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"ACQUISITION AND EXCLUSION OF PATHOGENICITY ISLAND 1 (PAPI-1) IN *PSEUDOMONAS AERUGINOSA*"

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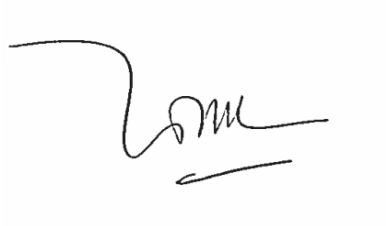
Declaration

I (Toan Phuoc Hong) confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.



ABBREVIATIONS

HGT	Horizontal gene transfer
CF	Cystic fibrosis
GIs	genomic islands
GST	glutathione S-tranferase
ICEs	Integrative and conjugative elements
IM	Inner membrane
IPTG	Isopropyl-D-thiopyranoside
kDa	Kilo Dalton
LB	Lubria-Bertani
LGT	Lateral gene transfer
MGEs	Mobile genetic elements
MW	Molecular weight
OD600	Optical density at 600nm
OM	outer membrane
OMPs	Outer membrane proteins
ORF	Open reading frame
PAPI-1	*Pseudomonas aeruginosa* pathogenicity island 1
PAPI-2	*Pseudomonas aeruginosa* pathogenicity island 2
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween 20
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate (SDS) polyacrylamide gel
T4SS	Type IV secretion system
TEM	Transmission electron microscopy

TABLE OF CONTENTS

FIGURE LEGENDS

LIST OF TABLES

ABSTRACT

Horizontal gene transfer (HGT) allows rapid exchanges of large genetic elements and is known to play an important role in bacterial evolution and adaptation. Conjugative transfer of genomic islands (GIs) has recently been reported in the opportunistic pathogen *Pseudomonas aeruginosa*. PAPI-1, one of the largest pathogenicity islands of *P. aeruginosa*, encodes several putative virulence genes and a major regulator of biofilm formation and antibiotic-resistant traits and was found to be horizontally transferable into strains lacking it. The conjugation of PAPI-1 island transfer is mediated by type IV pilus, which is encoded by ten genes located in PAPI-1. Nevertheless, the acquisition mechanism of PAPI-1 is currently not well understood.

The first part of this thesis was aimed at identifying the receptor for conjugative transfer on the bacterial cell surface. Based on previous knowledge on bacterial conjugation, we designed and performed a series of mating experiments and analyzed transfer efficiency between PAPI-1 donor and recipient strains. Our data showed that A-band lipopolysaccharide (LPS) is required to initiate PAPI-1 transfer, supporting the idea that this structure acts as a receptor for conjugative type IV pilus in recipient strains. These results were verified by PAPI-1 transfer inhibition experiments with outer membrane (OM) or LPS preparations. The addition of a low amount of OM or LPS derived from strains producing A-band decreased PAPI-1 transfer efficiency by 80% compared to controls.

In the second part, we demonstrated that *P. aeruginosa* strains which already acquired a copy of PAPI-1 almost completely lost the ability to receive additional copies of the island. Combination of strains with or without PAPI-1 were mated in-pair to investigate the redundancy in PAPI-1 transfer. The surface exclusion of PAPI-1 was characterized by investigating the effects of the addition of OM and LPS derived from strains with or without PAPI-1. In addition, LPS of the different strains were analyzed by western blot using antibodies directed against different parts of the molecule and by testing the in-vitro binding capacity of LPS to pilin protein. All experiments indicated that the strains carrying PAPI-1 produced much less A-band LPS compared to those lacking the island and lost the ability to bind to conjugative pilin. Finally, the screening of a series of mutants highlighted a role for two PAPI-1 genes in an entry exclusion activity, possibly through PAPI-1 island destabilization.

This study contributes with a step forward in the understanding of the acquisition of genomic islands in *P. aeruginosa,* which may be generalized to other gram-negative bacteria and may lead to the future development of new strategies to limit the spread of virulence or resistance functions in populations of pathogenic bacteria.

Keywords: *Pseudomonas aeruginosa*, PAPI-1 pathogenicity island, horizontal gene transfer.

INTRODUCTION

1. INTRODUCTION

1.1. Horizontal Gene Transfer (HGT) and bacterial evolution

Horizontal gene transfer (HGT), also termed lateral gene transfer (LGT), refers to the transfer of clusters of genes between organisms, in contrast to vertical transfer, the transmission of genes from the parental generation to their offspring. HGT has been shown to play an important role in bacterial evolution, adaptation and spread of antibiotic resistance or virulence.

1.1.1. Roles of HGT in bacterial evolution

Bacterial evolution is known to be driven by alterations of genome sequence and structure. Together with mutations created during DNA replication and reparation, HGT plays a major role in large-scale, rapid evolution, since hundreds of new genes can be acquired with a single genetic exchange event [1], [2]. HGT may result in a better adaptation of the recipient cell to new environments with advantageous functions encoded by the transferred genes. The first HGT event was reported in *Streptococcus pneumoniae* when virulence factors were observed to be transferred among them through transformation mechanism, which further explained in the next part 1.1.2 [3]. Later, a number of gene transfer processes mediated by mobile genetic elements (MGEs) including plasmids and viruses were subsequently described in bacteria [4]. Computational analysis has also revealed that a considerable proportion of most bacterial genomes consist of horizontally-acquired genes [4]. Therefore, genome analysis and comparisons suggested that HGT plays a key role in bacterial evolution and adaptation by sharing essential

metabolic functions, antibiotic resistances that might be beneficial under certain environmental conditions [5],[6],[7].

Acquisition of virulence or resistance genes may drastically alter the disease-causing potential or antibiotic resistance of a microorganism. In some instances, acquisition of a single gene or a small cluster of genes encoding critical virulence determinants was found to be the only genetic difference between an avirulent and virulent strain of the same species [8], [9]. Virulence genes are often organized in large blocks of DNA, also called genomic islands (GIs). GIs, accessory genomic regions, are present only in certain bacterial strains which are often flanked by direct repeats, inserted in the vicinity of tRNA genes and eventually excised out [10]. Those GIs enhancing the fitness in a host organism are named pathogenicity islands [6]. Horizontal gene transfer (HGT) spreading resistance against a wide range of antimicrobials has resulted in a worldwide impact of nosocomial and community infections caused by multidrug-resistant microorganisms, such as *Pseudomonas aeruginosa* or *Staphylococus aureus* [11], [12]. The figure 1 summarized the steps through which DNA must pass from donor to recipient.

1.1.2. Mechanisms of HGT in bacteria

Bacteria can implement HGT through three main mechanisms: uptaking free DNA (transformation) or encapsulated DNA (transduction), or cell-to-cell contact (conjugation) (Figure 2). Transformation is a common mode in which naked DNA, usually short fragments, is naturally uptaken by competent bacteria. Transduction is the transfer of DNA from one bacterium to another via bacteriophages.

Conjugation is the direct transfer of plasmids or other types of mobile genetic elements between two bacterial cells, requiring cell-to-cell contact via type IV pilus.

1.1.2.1. Transduction

Transduction is a process in which phages can pick up bacterial genes and carry them from one bacterial cell to another. Transduction was first described in studies on gene exchange between *Salmonella* bacteria [13]. There are two types of phage cycle: virulent phages immediately undergo their replicative cycle, lysing the host; temperate phages can remain integrated in the host cell genome for a period without killing it. A phage integrated into the bacterial genome is called a prophage and a bacterium harboring a quiescent phage is called lysogenic. There are two kinds of transduction: generalized and specialized. In the generalized transduction, phages can carry any fragment of the bacterial chromosome, whereas specialized transducing ones can pick up only certain specific parts. These strategies were identified to contribute to this horizontal transfer of genes between different host bacteria [14] .

Generalized transduction

Generalized transduction is a process in which any gene can be transferred from one bacterium to another by phages. The process includes two steps: the packaging of donor DNA into a phage particle and the stable introduction of this packaged DNA into the recipient cell, usually through genetic recombination with the recipient chromosome. The ability of a phage to perform generalized transduction thus depends on the mechanism of packaging DNA into phage

particles. There are numerous types of DNA metabolism that lead to generalized transduction. In each case, the capability for generalized transduction is a result of the mode of packaging phage DNA.

Specialized transduction

In this mode, the transduced genes can be covalently joined to the viral chromosome, then replicated, packaged, and introduced into a recipient with the rest of the viral chromosome. Besides, a specialized phage carries a specific chromosome segment, and consistently introduces it into the recipient [15].

The temperate phage λ is a classic example of this kind. This phage contains a linear double-stranded DNA molecule with complementary 12 nucleotide single-stranded ends [16]. When infecting to a cell, these ends are hybridized (cos sites); therefore, its chromosome is circularized, and the phage then chooses between two alternative life cycles. In the lytic cycle, the chromosome replicates to form concatemers to be packaged into particles with a limit of size about 35,000 bp to 50,000 bp. In this case, the phage DNA is integrated into the bacteria chromosome and a repressor for the lytic gene products is produced. Then, it is replicated and passively carried by the bacterium as a latent prophage. The integration occurs by breaking the phage chromosome at a specific site, *attP*, and joins it to the host at another specific one, *attB* [17]. When the prophage is induced, the phage repressor is inactivated and the reverse reaction occurs.

Phages contribute an extremely high impact on the bacterial evolution since the global rate of phage-mediated genetic modification in bacteria has been estimated

up to ~2×10^{16} events per second [18]. Phages are also indirectly responsible for horizontal gene transfer (HGT) by transformation. By inducing bacterial lysis, released bacterial DNA can then be acquired by neighboring competent cells. Many phage-mediated HGT occurs by generalized transduction mechanism, where bacterial DNA is accidentally packaged and delivered into neighboring cells [19]. Moreover, transduction can also facilitate the mobilization of antibiotic resistance and virulence genes [20] [21].

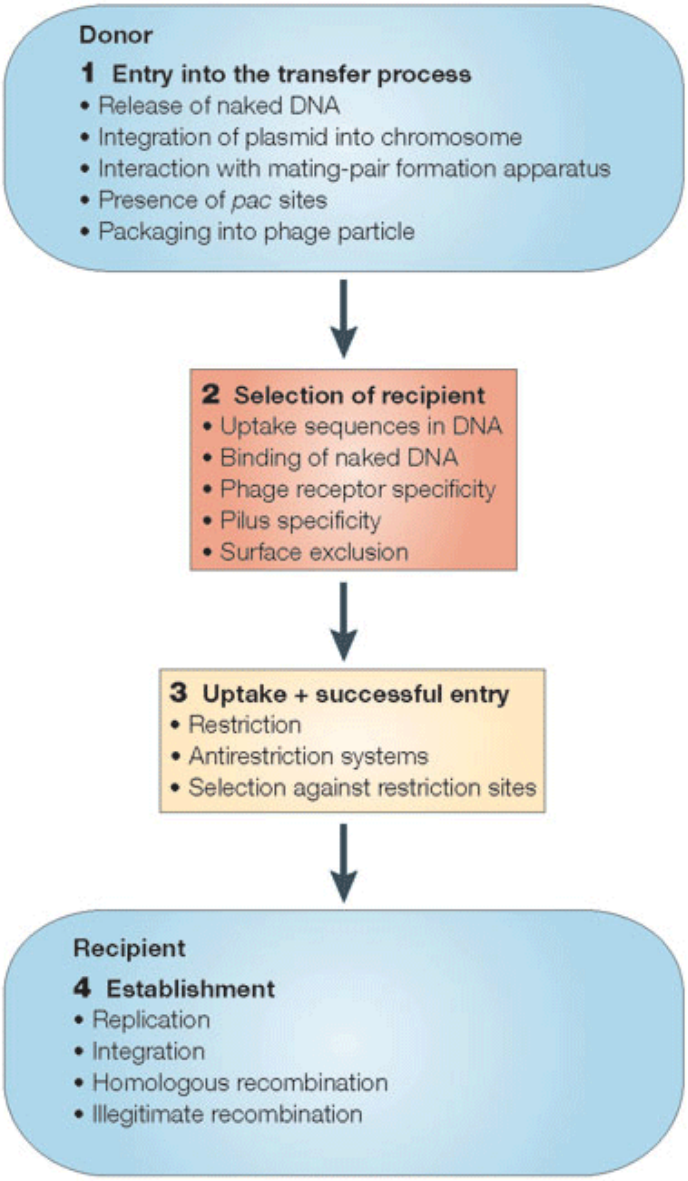


Figure 1. Horizontal gene transfer process. Summary of the steps through which DNA is transported from donor to recipient bacteria, starting from a potential DNA in donor cell becoming available for transfer and ending with a stable integration into a recipient's genome. This figure was reproduced by Thomas and Nielsen, 2005 [4].

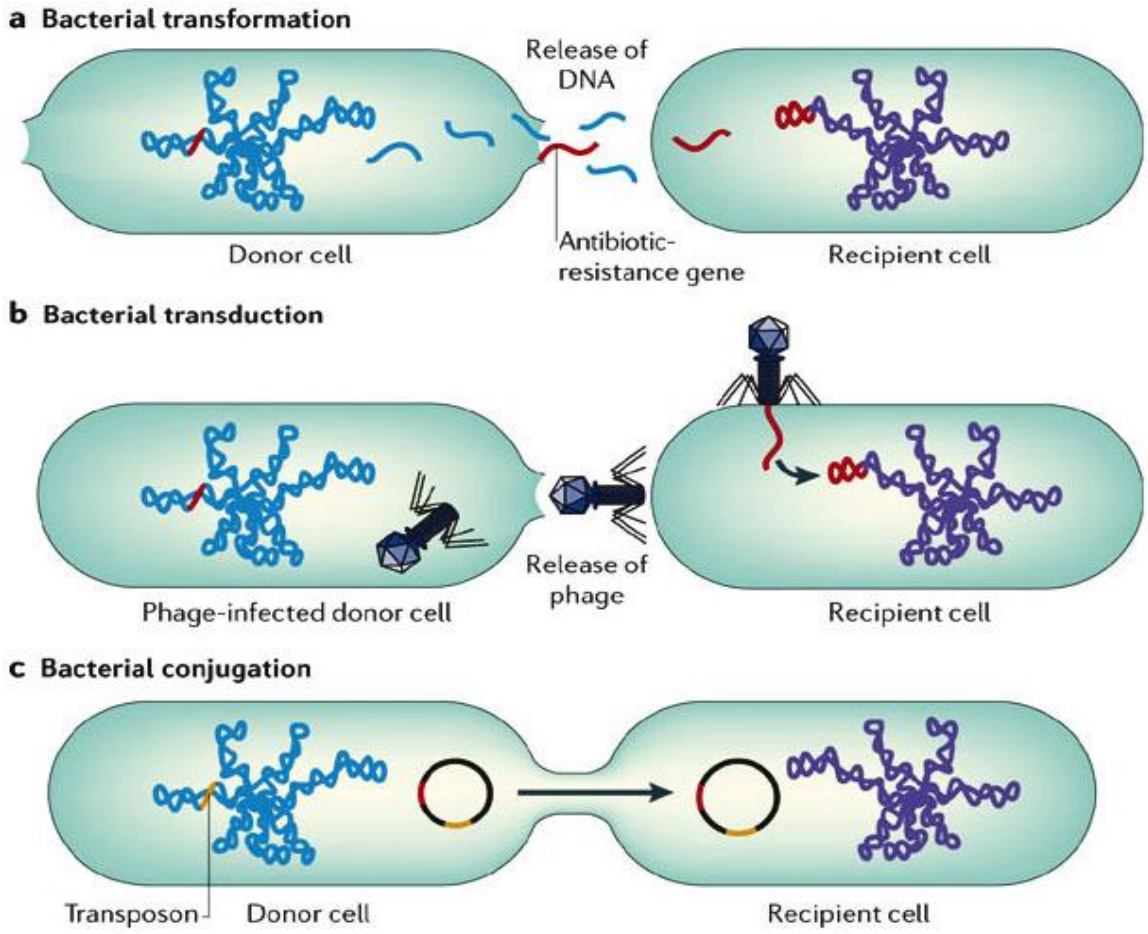


Figure 2. Mechanisms of HGT transfer. a. In transformation, the naked DNA released lysed bacteria can be taken up by another one. Then, the antibiotic-resistance genes can be incorporated to the recipient's chromosome. b. In transduction, bacteriophages transfer antibiotic-resistance genes from one bacterium to another and those can be integrated into the recipient's chromosome. c. Conjugation occurs by a direct contact between two bacteria, DNA is transferred to the recipient cells. This figure was reproduced by Furuya and Lowy, 2006 [22].

1.1.2.2. Natural Transformation

Natural transformation is the uptake of free DNA from the extracellular environment by bacteria, usually via a pore-like structure in the bacterial cell membrane [23]. This property is widely found among prokaryotes (including Archaea), inferring that natural competence has a long evolutionary history [24]. To initiate natural transformation, bacterial cells must first reach a regulated physiological state involving approximately 20 to 50 proteins. Remarkably, the proportion of bacteria that can develop competence ranges from near zero to almost 100% of the bacterial population [4]. The natural transformation has been detected in Archaea and different bacterial phyla, including Gram-positive bacteria and Cyanobacteria [24]. Importantly, many human pathogenic bacterial genera such as *Campylobacter*, *Haemophilus*, *Helicobacter*, *Neisseria*, *Pseudomonas*, *Staphylococcus* and *Streptococcus* are also naturally competent [24]. Natural transformation only occurs when there are free extracellular DNA, the presence of competent bacterial cells and the ability to stabilize the acquired DNA by integration into the bacterial genome or self-replicated in the recipient cells.

1.1.2.3. Conjugation

Conjugation is another HGT mechanism first described by Lederberg J and Tatum EL, 1953 [25] as bacterial sexual reproduction. Among different HGT mechanisms, conjugation has the most complex requirements. Known conjugative elements include plasmids, MGEs, or integrative and conjugative elements (ICEs) which are self-transmissible MGEs [26]. Plasmids or MGEs are generally transmitted by conjugation since they are usually too large to be transferred by transduction or

natural transformation [27], [28]. The schematic of the ICE transfer is well presented in the figure 3.

With conjugative apparatuses, large genetic elements, including chromosomes can be easily transmitted, providing a dynamic manner of bacterial evolution. Conjugation allows a rapid and efficient gene transfer in bacteria, mediated by factors which are usually encoded in plasmid or ICEs. Mating among *E. coli* cells mediated by IncF and IncI plasmids occurs in several steps: (i) pilus-to-wall contact formation, (ii) wall-to-wall contact, (iii) stabilization of the contacts, (iv) DNA transmission and (v) active disaggregation of the mating complexes [29], [30], [31], [32]. Remarkably, many studies, especially in gram-positive bacteria, suggested that before establishing stable mating pairs, the donors can recognize the recipients through signaling molecules such as pheromones [33], [34], [35]. [36, 37] or small peptides [38]. Recently, it has also been found in gram-negative bacteria that 3-oxo-C6- homoserine lactone (3-oxo-C6-HSL) which is a quorum sensing signal was able to control the gene transfer [39].

When the donors and recipients get closer and establish a physical contact sufficiently stable to allow transfer of DNA (Figure 4). During the transfer, metabolism of both cells has to be simultaneously active to allow DNA synthesis and other activities [24]. The cell-to-cell junctions allow DNA to pass, although the natures of these structures are not fully understood. The donor can contact

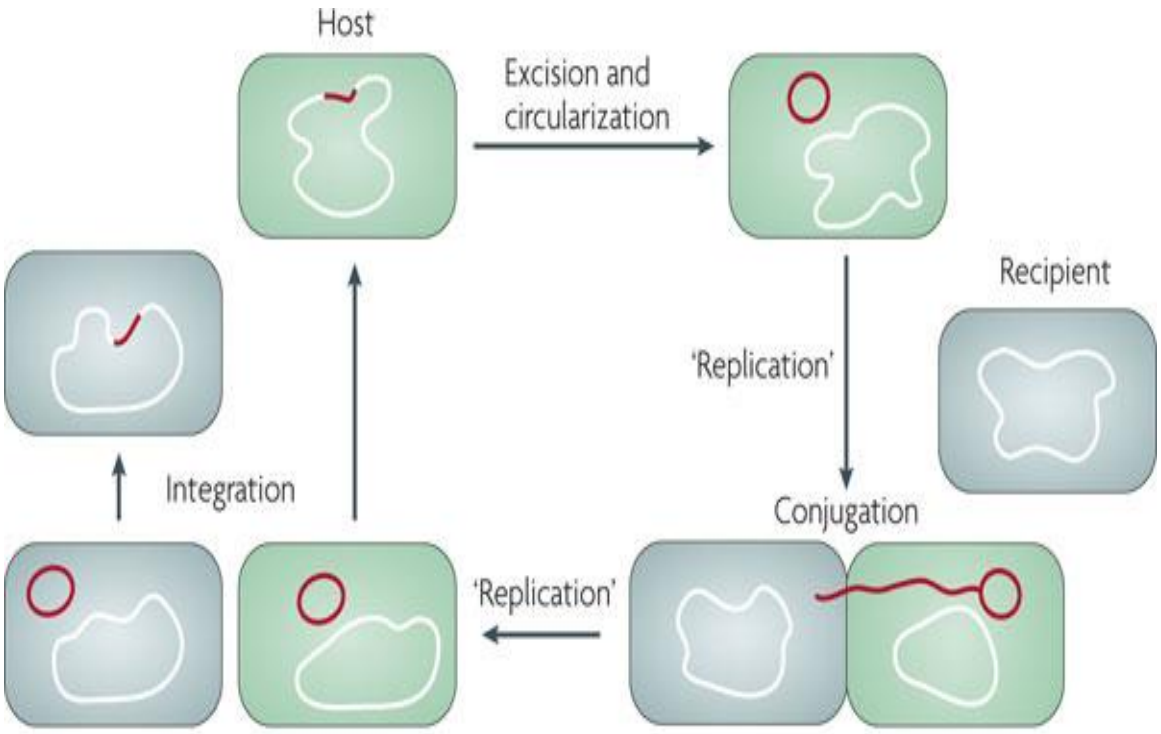


Figure 3. Life cycle of an integrative and conjugative element (ICE). An ICE can be integrated into the host chromosome at specific sites: the right (attR), and left (attL) ends. Excision of ICE by recombination between attL and attR to yield attP (in the ICE) and attB (in the host chromosome) can produce a circular molecule. During conjugation, the donor and potential recipient (ICE-free) establish a close contact, and a single DNA strand is transferred into the recipient through a rolling circle replication. Then, complementary strand is synthesized by DNA polymerase in the recipient regenerate the double-stranded in circular form. Finally, the ICE can be integrated into the host genome by a recombination event between attP and attB. This figure was reproduced by Wozniak and Waldor., 2010 [40].

recipients through a connecting tube (pilus) or in some cases through a pore [41]. *S. aureus,* a gram-positive bacterium, utilize transferring pores encoded by *tra* genes instead of pilus formation to allow DNA transfer [42].

In many cases, the donors bearing plasmids have been shown the ability to target different recipients through the specificity of the interaction between their pilus and LPS and/or outer membrane proteins on the recipient cell surface [43], [44]. For examples, the IncP and Ti plasmids, some mating-pair formation apparatuses can form productive junctions with different cell-type surfaces [45], [46], including not only gram-negative bacteria but also gram-positive bacteria, yeast, plant and animal cells.. However, the plasmid transfer efficiency among the same strains or different strains can be different. For instance, transfer efficiency of IncP-1 plasmids between *E. coli* strains or from *E. coli* to *Pseudomonas putida* is slower than transfer between *P. putida* strains [47].

In many gram-negative bacteria, type IV secretion systems (T4SSs) has been known to be responsible for conjugative transfer [48]. T4SS, one of six major classes of secretion system, is a large structure connecting both the inner and outer membranes of bacteria. Initially, the origin of transfer of plasmid (oriT) is processed in the cytoplasm leading to the formation of a multiprotein-DNA complex, so-called the relaxosome. The relaxosome contains relaxase enzyme as a crucial component and various accessory proteins which may be necessary for efficient recognition of oriT [49]. Subsequently, a coupling protein (T4CP) brings this complex to a T4SS and translocates the DNA from the donor to the recipient cell. Two types of T4SS pilus have been described, a long and flexible F-type

pilus, and a short and rigid P-type pilus [50]. Pilus contains a lumen, which is approximately 30A˚ in diameter, allowing passage of single-stranded DNA [51].

The conjugative pilus structure of *A. tumefaciens* was the most studied including the major and minor pilin proteins VirB2 and VirB5. It is known that the pilin proteins VirB2 of *A. tumefaciens* and TrbC encoded by the RP4 plasmid are post-translationally modified by cleaving the signal peptide followed by fusion of the N- and C-terminal ends to cyclize the peptide [52], [53], and then acetylated before being inserted into the inner membrane of the bacterium [54]. The T4SS possibly functions in two modes associated structural conformations, including pilus biogenesis and substrate transfer modes (Figure 5) [55].

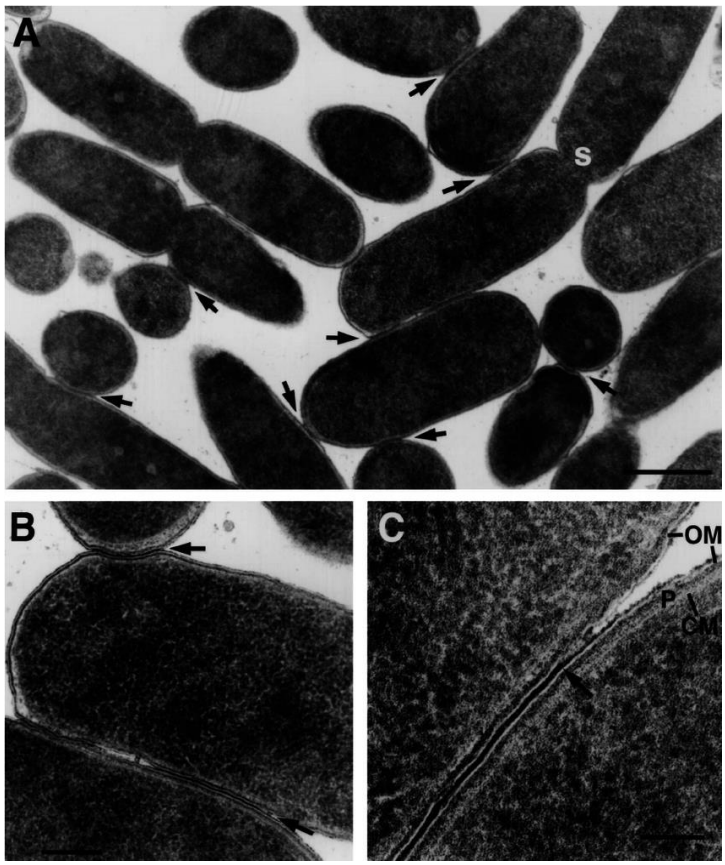


Figure 4. Junctions between *E. coli* strain during RP4-mediated conjugation on filter. (A) Mating cell aggregates at lower magnification. Arrows indicate junctions; s indicates septum. Bar, 0.5 μm. (B) Pairs of bacteria established conjugation junction (arrows). Bar, 0.25 μm. (C) Junction at high magnification showed a electron-dense area between outer membranes (arrow), lightly staining outer membrane (OM), dense periplasmic gel (P), and lightly staining inner cytoplasmic membrane (CM). Bar, 100 nm This figure was reproduced by Samuels *et al.,* 2000 [45].

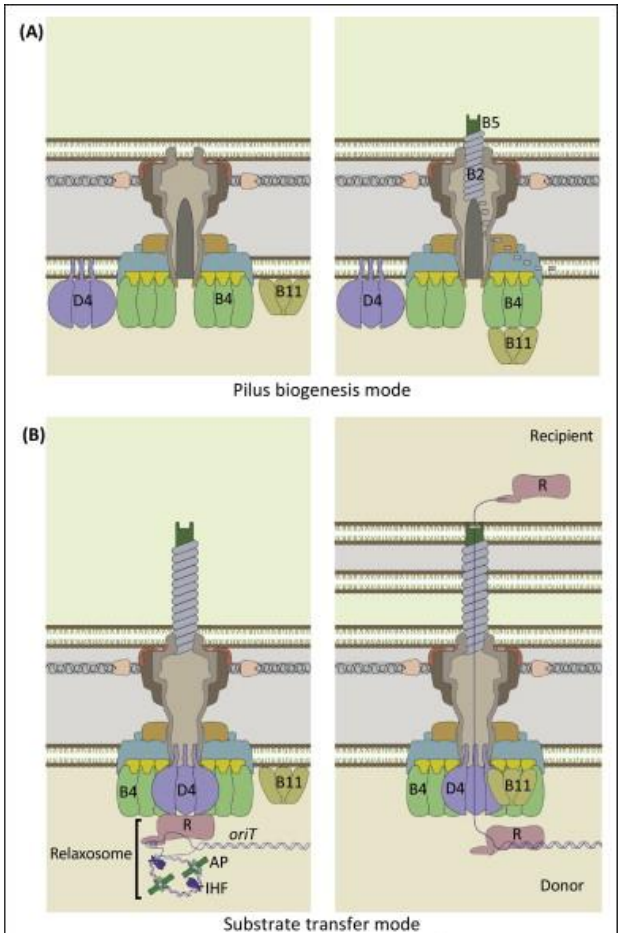


Figure 5. Type IV secretion system (T4SS) functioning modes. (A) In the biogenesis mode, the pilus is grown from a structure within the T4SS and at this point VirB11 (light brown) interacts with VirB4 (green) to activate this mode. (B) In the substrate translocation mode, VirB11 (light brown) interacts with VirD4 (purple) to assist the substrate transfer. The relaxosome [relaxase (R); accessory protein (AP); origin of transfer (oriT) DNA; and integration host factor (IHF)] processes the DNA and is recruited to the T4SS by the VirD4 coupling protein (CP; left panel). The DNA and relaxase are then transferred to the recipient cell (right panel). This figure was reproduced by Ilangovan *et al.*, 2015 [48].

1.1.3. HGT exclusion activity in bacteria

Back to the early history of conjugation, it has been known that recipients after they already acquired a plasmid can activate a exclusion system to avoid entry of another copy. This is usually a property of plasmids, encoded by at least one gene and essential for their stability. It has also been suggested that exclusion mechanism limits the damage of lethal zygosis in which bacterial death is induced by excessive rounds of conjugation. In addition, it may also avoid competition among identical plasmid backbones in a host. On the other hand, it could be understood that the lack of exclusion mechanism may generate a rapid evolutionary change [56]. There are at least two existing exclusion for the entry of plasmid or ICEs identified among bacteria : surface exclusion and entry exclusion.

1.1.3.1. Surface exclusion

This mechanism was originally described for the transfer of F plasmid in *E. coli*. The surface exclusion creates an effective barrier against conjugative transfer into bacterial cells already carrying specific genetic elements [57]. Surface exclusion has been found in conjugation systems of both gram-negative [52], [58] and gram-positive bacteria [59]. TraT is an outer membrane lipoprotein which can disturb the interaction between the pilus tip and OmpA receptor in *E.coli* [60]. In F plasmid, protein TraT changes the outer surface of the cell and reduces its binding capacity to the F pilus about 10-fold,. However, a large number of inter-plasmid recombination events were found among different F-like plasmids or IncP-1 plasmids [61].This indicates that despite surface exclusion, plasmids can still enter cells that carry a closely related element. In F plasmid transfer, the surface

exclusion activity is decreased in stationary phase in liquid culture, in non-growing populations on agar plates and under starvation of carbon source [62]. Studies on F plasmids revealed that surface exclusion might be relevant to the disruption of mating pairs after gene transfer and for release of the recipient [63].

1.1.3.2. Entry exclusion

Entry exclusion includes a wide-range of mechanisms inhibiting DNA entry after a mating pair established [30], [32]. For F plasmid, the entry exclusion showed a stronger barrier for gene transfer than surface exclusion mechanism, in which protein TraS, located in the inner membrane, prevents DNA entry by about 100-fold. An Eex system of the IncP plasmid RP4, including a single inner membrane lipoprotein (TrbK), further blocks acquisition of closely related plasmids by interacting and interfering with one or several proteins responsible for mating pair formation [52]. F plasmids utilize a similar mechanism where the lipoprotein TraS in the recipient cells blocks entry of the second copy of the F-plasmid by interacting with the TraG protein of the donor [64], [65]. An analogue exclusion system, consisting of two proteins, Eex and TraG, regulates entry exclusion of *Vibrio cholerae* ICE SXT [66]. A significant number of plasmids and ICEs that control entry exclusion mechanisms lack obvious homologues of the plasmid exclusion systems, but they could utilize analogues Eex systems that have significantly diverged in their primary sequence from their plasmid ancestors [56].

The entry of DNA can also lead to degradation by intracellular restriction endonucleases [67]. This exclusion activity depends on the plasmid size, and on whether it is single-stranded or double-stranded. Smaller plasmids contain fewer

restriction sequences and therefore are more likely to be protected from enzymatic cleavage [68].

The broad-host-range IncP-1 plasmid seems to have adapted to such barriers by losing most of its restriction sites [69]. Indeed, introduction of extra restriction sites into the plasmid increases the exclusion activity, resulting in a reduction of transfer efficiency [69], [70].

Besides, another barrier is to control plasmid replication and establishment in a heterologous host. This mechanism allows a plasmid having no orthologue in the recipient genome to self-replicate without the need for recombination into the chromosome. This could be explained by replication proteins encoded in the plasmid. The broad-host-range plasmids, like RSF1010 belonging to the IncQ plasmids, contain three replication proteins — an origin activation protein, RepA; a helicase, RepB; and aprimase, RepC. In contrast, studies on narrow-host-range plasmids showed various limitations to successful replication. The replication of plasmid F of *E. coli* in *Pseudomonas* species seems to be restricted due to the inability of its replication protein, RepE, to effectively recruit DnaB to complete the activation of the replication origin [71].

1.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacteria found in a wide range of enviroments, including soil, water, plants, and animals [72], [73]. It was first described in 1882 by Carle Gessard, a French chemist and bacteriologist, elucidating a mystery of more than 20 years about a blue-green pigment observed

in patients during the pre-antiseptic era of surgery. It was later found to be responsible for a broad range of infections, including endocarditis, corneal keratitis, meningitis and systemic infections of children [74], [75], [76]. Recently, it was reported that *P. aeruginosa* causes 8% of nosocomial infections and cost for nosocomial pathogen treatment in America was between $28.4 and $45 billion and cause an estimated 1.7 million infections and 99,000 deaths each year [77], [78]. In addition, *P. aeruginosa* is well-known as the main cause of morbidity and mortality in patients suffering cystic fibrosis (CF) [79] which is the most common autosomal recessive genetic disorder among Caucasians, with a high frequency of about 1 in 2,500 live births [80].

P. aeruginosa infections are often associated with compromised host defenses such as severe burns, urinary tract infections, AIDS [81] lung cancer, chronic obstructive pulmonary disease, and CF [82]. Most CF patients get infected and chronically colonized with multi-drug resistant (MDR) *P. aeruginosa,* becoming nearly impossible to be eliminated despite aggressive antibiotic therapy [83]. *P. aeruginosa* has become one of the most common MDR bacteria isolated from ventilator-associated pneumonia [84].

The broad environmental distribution of *P. aeruginosa* is reflected by its large genomic repertoire beyond the core genome. Indeed, the genome sequences of *P. aeruginosa* strains available to date show that a large core genome of about 5000 conserved genes is supplemented with an accessory gene pool of 1000-1500

additional genes, most of them being arranged in a limited number of genomic islands [85].

1.2.1. Pathogenicity islands

Integrative and conjugative elements (ICEs) are a large group of mobile genetic elements found in both gram-positive and gram-negative bacteria (Rachel, 2010), encoding various accessory activities such as symbiotic and pathogenesis functions. In many cases, ICEs have retained mobility [86, 87], while many others appear as ancient ICEs that became fixed in the bacterial chromosome due to degeneration of their conjugative elements [88]. The best-characterized ICEs to date contain specific features associated with conjugative plasmids and bacteriophages and are self-transmissible. ICEs, like all transmissible plasmids, are transferred following the recognition of the recipient cell by the donor utilizing a conjugative mechanism that, in many instances, is associated with the T4SS system [26].

In *P. aeruginosa*, two pathogenicity islands PAPI-1 (108 kb) and PAPI-2 (11 kb) has been recently identified and characterized as members of mobile ICE group [89]. It has been known that those islands can be integrated at into the chromosome at the *attB* site in the $tRNA^{Lys}$ gene PA4541.1 or PA0976.1 (Figure 7). Importantly, they were found in a highly virulent clinical strain, PA14, while absent the less virulent reference strain PAO1 (Figure 8). Many of the genes within these islands are homologous to genes from other human or plant pathogens. PAPI-1 carries several regulatory genes, such as the PvrSR/RcsCB two

components system, which controls biofilm formation and dispersal in *P. aeruginosa* strains causing chronic infections in individuals with cystic fibrosis [90], [91]. Significantly, many PAPI-1 ORFs are also present in several *P. aeruginosa* cystic fibrosis isolates [89]. However, more than 80% of the PAPI-1 DNA sequence is unique to strain PA14, and about 70% of predicted ORF products exhibit no homology with any known proteins or functional domains. 19 PAPI ORFs were found to be necessary for full plant or animal virulence, with 11 required for both [89], [92].

Conjugation has been known as the mechanism responsible for the transfer of PAPI-1 since this only occur when donor and recipient cells are co-cultured and not from a donor bacterium-free culture medium [93]. As mentioned above, a pilus is required to set-up mating pairs between donor and recipient cells. In PAPI-1, a 10-gene cluster encoding type IV pilus closely related to the homologous genes in the conjugative plasmid R64, was identified. However, only nine of PAPI-1 Pil proteins showed high similarities to those encoded in plasmid R64, as components of the type IV pilus system (PilL2, -N2, -O2, -P2, -Q2, -R2, -S2, -T2, -V2, and -M2). The PilS2 and PilV2 are homologous to the major and minor subunits of R64 thin pilus, PilS and PilV. Computational analyses predicted that the function of pilN2, PilO2, PilP2, PilQ2, pilR2, PilM2, are type IV lipoprotein, type IV pilus secretin protein, a pilus accessory protein, a pilus assembly protein, a pilus retraction ATPase, an integral membrane protein and an inner membrane protein, respectively [93].

Interestingly, the 10 PAPI-1 encoded pilus proteins are well conserved in several *P. aeruginosa* strains that carry this island, including PA2192, C3710, PACS2, PA7, and PSE9 (PAGI-5), and in *P. aeruginosa* clone C strain that carry a pKLC102-like element [78], [85], [94]. The PAPI-1 *pilS2* gene encodes for a major pilin subunit, which is a 176-amino-acid protein containing a conserved PilS superfamily domain. However, the PAPI-1 encoded conjugation system in *P. aeruginosa* is incomplete since it lacks prepillin peptidase, which is responsible for the cleavage of the PilS2 leader peptide and is located in the core genome. The PAPI-1 encoded pili was previously visualized under transmission electron microscopy (TEM) (Figure 6).

In plasmid-based conjugative transfer in *E. coli*, the F pilus is required for initiating the cell-to-cell contact and forming mating pairs during the conjugation process. R64 plasmid transfer is one of these well-studied systems. Plasmid R64 encodes type IV pilus with the PilV adhesins located at its tip [95], [96]. The C-terminal segments of the PilV adhesins are exchanged by multiple DNA inversions of the shufflon containing seven recombination sites, which flank and separate four DNA segments [97, 98] in order to determine the recipient specificity in R64 liquid matings [99, 100]. It was demonstrated that each adhesin can recognize a specific structure of LPS molecules of recipient cells [101].

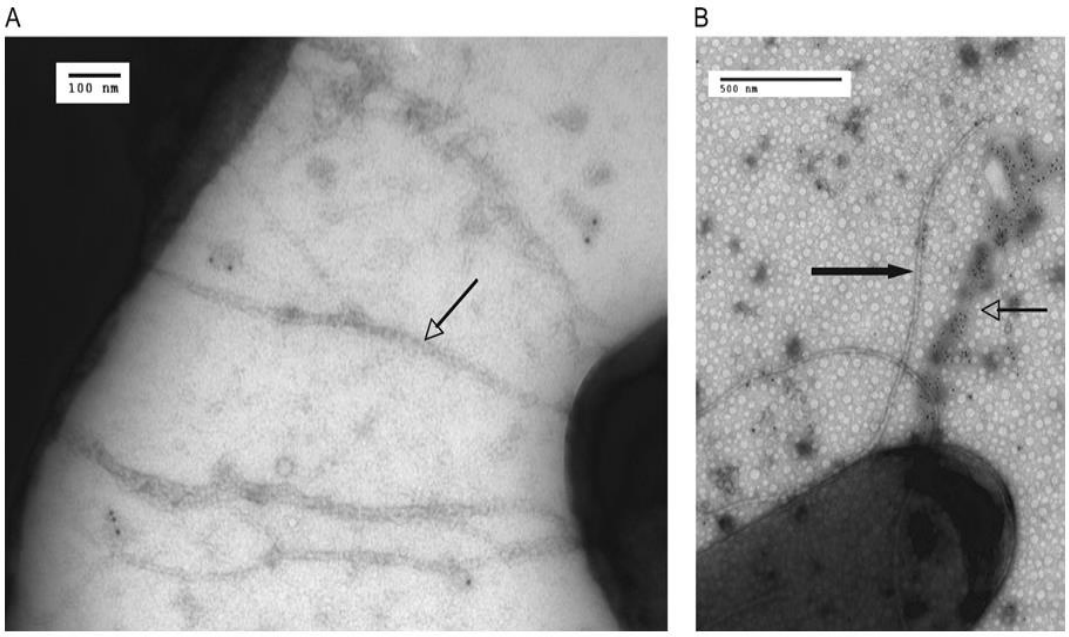


Figure 6. Visualization of PAPI-1-encoded pili under transmission electron microscopy (TEM). (A) The immunogold labelling is specific to the Flag tag, since there is little labelling of the native pili of the control cells without Flag-tagged PilS2 (PA14TnC2 ΔpilS2ΔpilA ppilS2). (B) gold-labelled PAPI-1 pili (arrow with open head) of Flag-tagged cells (PA14TnC2 ΔpilS2ΔpilA ppilS2-flag) next to the flagella (filled arrow) are well visualized. This figure was reproduced by Carter *et al.*, 2010 [93].

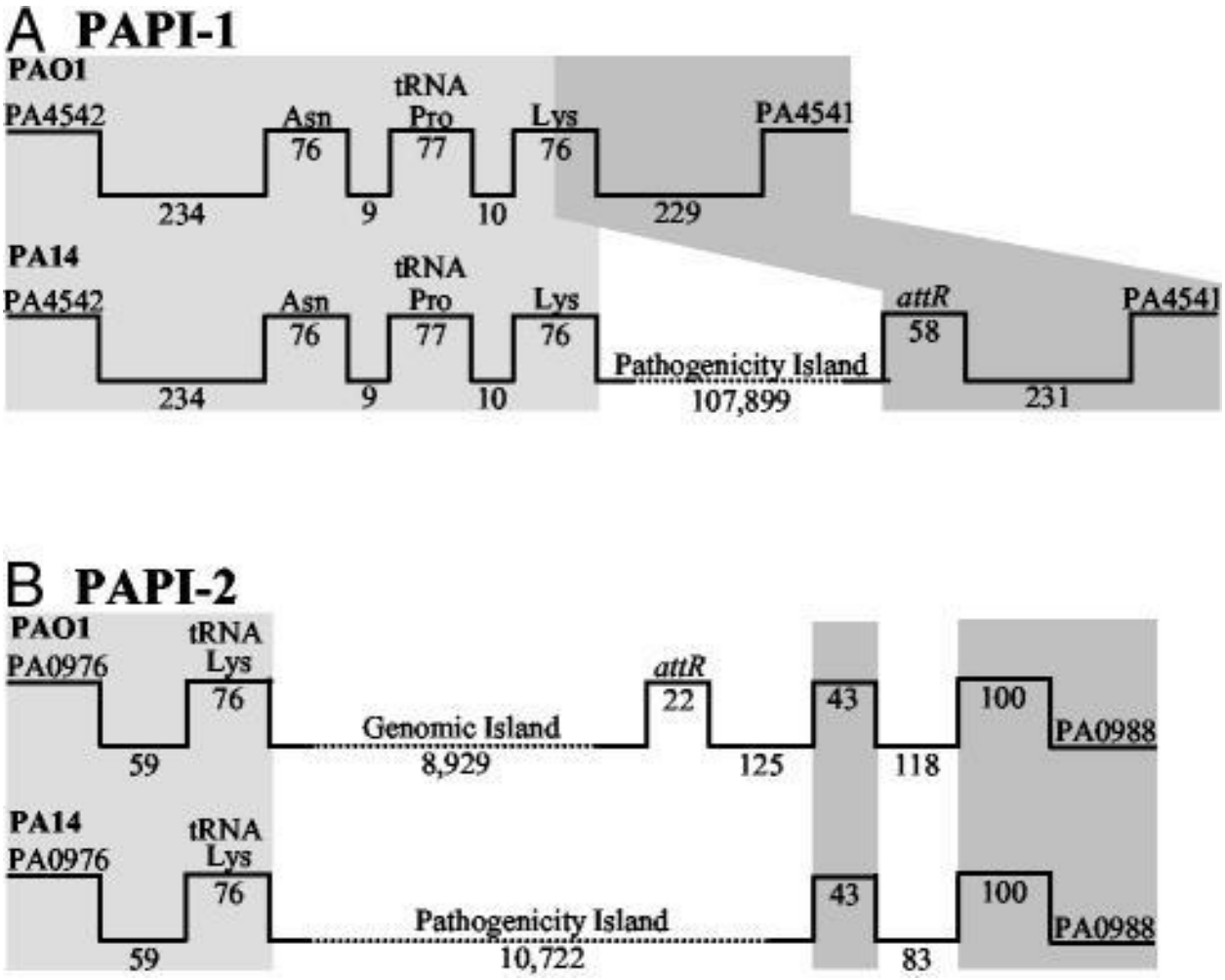


Figure 7. Genomic location of PAPI-1 (A) and PAPI-2 (B) in PA14 compared to PAO1 strain. Above and below the lines represent gene designations and length (bp), respectively. The left-boundary and right-boundary conserved regions are highlighted with light and dark gray shading represent, respectively. This figure was reproduced by He *et al.,* 2004 [89].

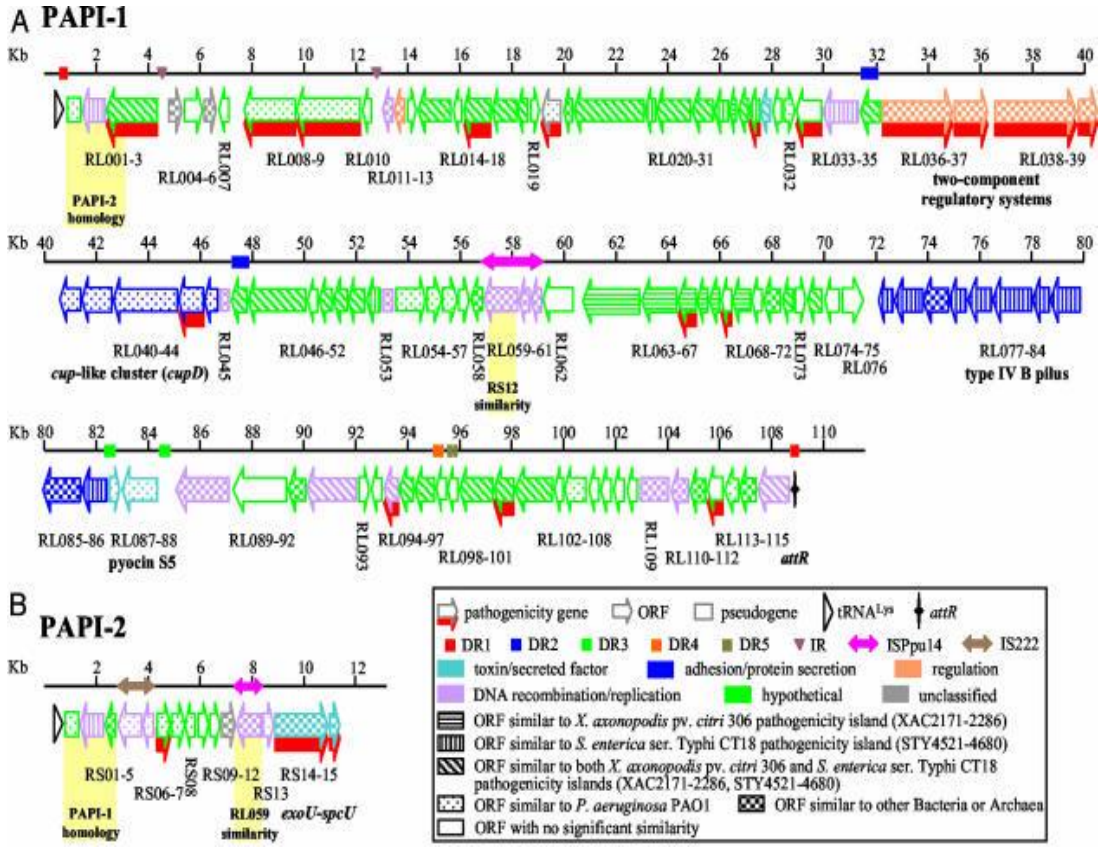


Figure 8. Genomic organization of PAPI-1 (A) and PAPI-2 (B). The individual ORFs and their transcriptional orientations are described by the boxes with arrows. Empty boxes indicate pseudogenes; triangles indicate tRNA genes; and the vertical black line indicates the attR "attachment" site. The numbered lines indicate size (kb). The direct repeats (DR1–5), inverted repeat (IR), and insertion sequences are marked by the coincident rectangles and single or double-headed arrows on the line, respectively. The color and pattern of ORF represent the putative protein function and the bacterial species it is most related to, respectively according to the key. Virulence-related ORFs are represented in red shading. Functions of gene clusters are correspondingly presented to the ORFs above the notations. The yellow shaded regions present the homology between PAPI-1 and PAPI-2. This figure was reproduced by He *et al.,* 2004 [89].

1.2.3. Outer membrane and LPS structure

The cell envelope of Gram-negative bacteria consists of two membranes, the cytoplasmic membrane and the outer membrane, with periplasm containing peptidoglycan cell wall in between. The cytoplasmic membrane is a phospholipid bilayer constituted of glycerophospholipids mosaicked with proteins. The outer membrane is asymmetrical, containing glycerophospholipids in the inner leaflet, and lipopolysaccharides (LPSs) exposed to the cell surface, as well as integral outer-membrane proteins (OMPs) (Figure 9) [102]. The outer membrane protects the bacteria from harmful substances in the environment, such as antibiotics and bile salts, while it allows most nutrients to pass via a family of integral OMPs, so-called porins. Other OMPs are specified for transport functions, such as the secretion of proteins, or function as enzymes or structural composition of the outer membrane [103]. LPS is constituted of three parts: a proximal hydrophobic lipid A region, a core oligosaccharide region, and an O-antigen polysaccharide region.

In *Pseudomonas aeruginosa*, at least six conserved major proteins were identified in the outer membrane, including D (50 kDa), E (45 kDa), F (33 kDa), G and H (21 KDa), I (8 kDa) [104]. The cytoplasmic membrane consists of many protein species, and minimum fifty protein bands were detected by SDS-PAGE [104]. *Pseudomonas aeruginosa* shows a 10- to 100-fold lower outer membrane permeability, as compared to other gram-negative bacteria (e.g. *E. coli*). This unique feature slows down the trans-outer-membrane drug diffusion, aiding the

function of secondary resistance mechanisms (such as efflux systems and enzymatic modification or degradation). The main factor for *P. aeruginosa* outer membrane permeability to antibiotics is the major porin OprF [104]**,** and possibly other porins, such as OprB and OprD. More than 160 genes/ORFs encoding putative outer membrane proteins are found in the *P. aeruginosa* genome, implicating a much more complex system than in other gram-negative bacteria [105].

Lipopolysaccharide is a complex glycolipid structure, which is the main component of the outer membrane of gram-negative bacteria. LPS has an important role in antigenicity, inflammatory response, exclusion of external molecules, and also antibiotic interaction [106]. While the inner core is constituted of two D- *manno* -2-keto- octulosonic acid residues and two L- *glycero* -D- *manno* -heptose residues, the Lipid A moiety and the O-antigen are composed of various molecules. The lipid A contains diglucosamine biphosphate backbone with *O*- and *N*-linked primary and secondary fatty acids anchored to the LPS on the bacterial outer membrane. The lipid A structure differs in the number, the position, and the nature of acyl groups, as well as in the modification of the phosphate groups [107]. Lipid A is the domain of LPS that mediates inflammatory response-induced endotoxicity [108]. Many lipid A modifications are associated with environmental changes, including the presence of antibiotics or during CF infection [109], [110], [111].

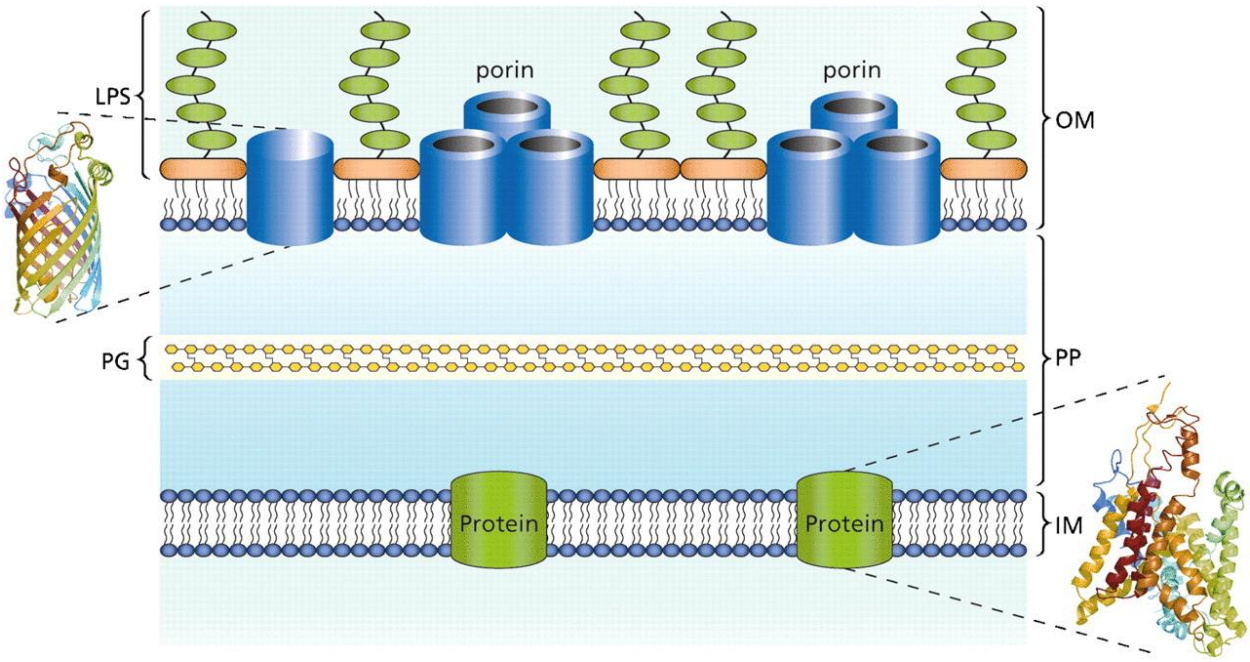


Figure 9. Structure of the gram-negative bacterial cell envelope. OM, Outer membrane containing glycerophospholipids in the inner leaflet, mosaicked with porins as the major protein components and LPS structure in the outer leaflet, exposed to the cell surface; PP, periplasm containing the peptidoglycan layer (PG); IM, inner membrane This figure was reproduced by Tommassen *et al*., 2010 [102].

The figure 10 presented the diversity of the LPS glycoforms on the surface of *P. aeruginosa.* Two different types of O-antigen have been characterized: A-band LPS is a homopolymer of D-rhamnose which elicits a weak antibody response; B-band LPS is a heteropolymer with three to five distinct sugars in its repeat units with a strong antibody response and is the chemical basis for serotyping [112]. Some isolates, lacking the O-antigen, have a “rough” colony morphology compared to the smooth isolates producing it, while others, identified as “semi-rough”, substitute the lipid A and core with only one O-saccharide unit [113].

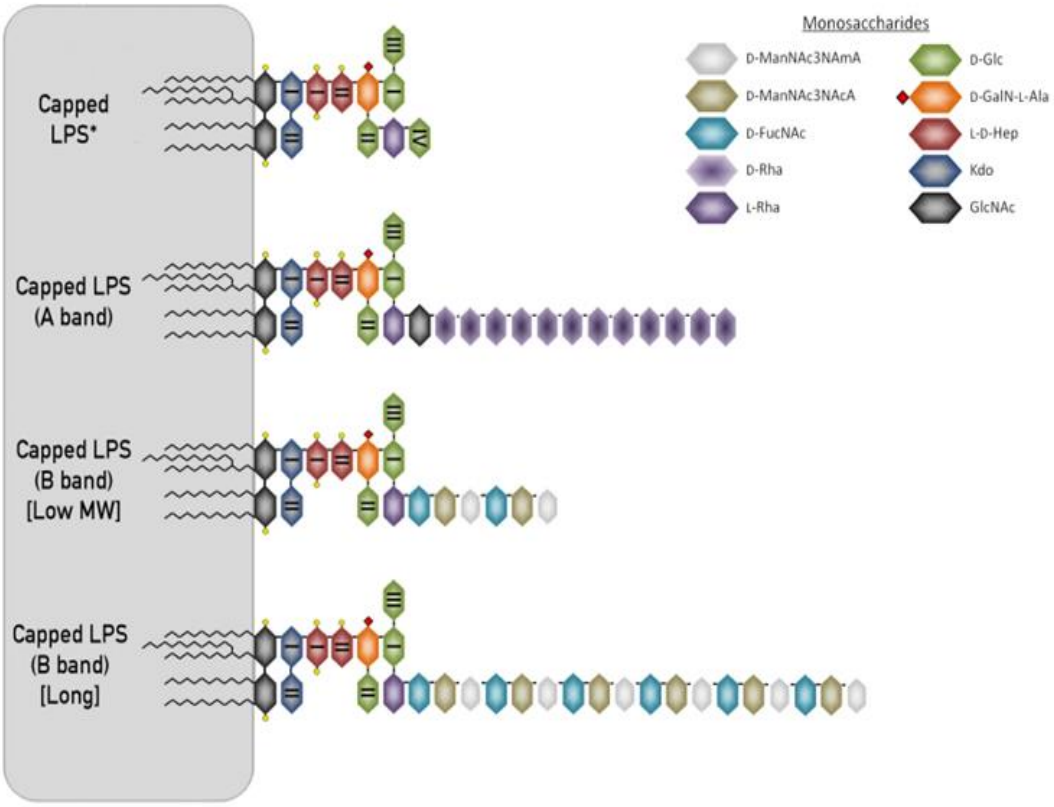


Figure 10. Heterogeneity of the LPS glycoforms on the surface of *P. aeruginosa* [107]. In *P. aeruginosa*, there are two types of O-antigen characterized. A-band LPS, or Common Polysaccharide Antigen, is a homopolymer of D-rhamnose with about 70 sugars long, elicits a weak antibody response. B-band LPS, or O-Specific Polysaccharide Antigen containing a heteropolymer of repeated units of three to five distinct sugars and constitutes the chemical basis for serotyping. B-band LPS is highly immunogenic and elicits a strong antibody response. This figure was reproduced by Knirel *et al.*, 2006 [112].

AIMS OF THE PROJECT

2. AIMS OF THE PROJECT

Horizontal gene transfer is known as a major evolutionary mechanism in prokaryotes and contributes to the virulence properties of many bacterial pathogens. *Pseudomonas aeruginosa* pathogenicity island 1 (PAPI-1) has previously been shown to be transferable from one strain to another through conjugation process mediated by type IV pilus. However, the detailed PAPI-1 transfer mechanism, especially acquisition and exclusion abilities have not been elucidated to date. To achieve this goal, this thesis project will address two specific aims.

First, we aim at demonstrating the existence of a physical contact between *P. aeruginosa* donors and recipients to initiate the PAPI-1 transfer. For this purpose we will perform a series of mating experiments and analyzed transfer efficiency between PA14ΔTnC2 donor carrying PAPI-1 and PAO1 recipients. The results will show us which components of the recipient's cell surface are involved in the transfer. Afterward, PAPI-1 transfer inhibition assays with the addition of OM or LPS derived from different strains will be carried out to verify the existence of a receptor on the recipient's outer membrane.

The second aim is to investigate the mechanism of PAPI-1 exclusion in *P. aeruginosa* strains that already acquired a copy of the island. For this purpose, we will test combinations of strains with or without PAPI-1 for in-pair mating to analyze the redundancy in PAPI-1 transfer. Experiments will consist in testing the effects of

OM and LPS derived from strains with or without the island, analyze LPS of the different strains by WB using antibodies directed against different parts of the molecule, and testing the in-vitro binding capacity of LPS to pilin protein. Last, a screening of mutants for PAPI-1 genes will be performed to identify any PAPI-1 genes involved in the exclusion system.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Strains and plasmids

All strains and plasmids used in this study are listed in Table 1, 2, 3, 4. *P. aeruginosa* strains and mutants were grown in Luria-Bertani (LB) broth (Sigma-Aldrich) supplemented with the appropriate antibiotics. For selection of *P. aeruginosa* mutants, the antibiotics used were gentamicin and tetracycline, both at a concentration of 75 µg/ml. For maintenance of plasmids in *E. coli*, the medium was supplemented with ampicillin at 100 µg/ml and chloramphenicol at 34 µg/ml. Isopropyl-D-thiopyranoside (IPTG) was added at a final concentration of 0.1 mM to induce GST-pilV2' expression in pGEX-2T plasmid.

Table 1. Strains and plasmids used in this study

Strains or plasmids	Antibiotic resistance[a]	Description	Source reference, or accession no.
E. coli strains			
E. coli SM10	None	Host strain for plasmids pEXG2, mini-CTX, and their derivatives	Lory's lab collection
E. coli λpir S17.1	None	Transfer pEXG2 plasmid into *P. aeruginosa* by conjugation	[113]
P. aeruginosa strains			
PA14	None	Burn isolate	[91]
PA14Δsoj (PA14 -)	Gm^R	Deletion mutant of PAPI-1 *soj* in strain PA14, which does not carry PAPI-1 island	[91]
PA14ΔTnC2::Gm^R (PA14+)	Gm^R	Strain PA14 with a transposon MAR2×T7 inserted at nucleotide 1634 of PAPI-1 gene RL090 (PA14_59200)	[114]
PA14ΔTnC2::Tc^R (PA14+)	Tc^R	Partially deletion of the PA14_59200 gene in strain PA14 by insertion of tetracycline resistant gene in the middle	This study
PAO1 (or PAO1 -)	Tc^R	PAO1 with Tet gene inserted at the CTX phage *att* site on the chromosome	Lory's lab collection
PAO1Bla6	Cb^R	PAO1 with genes *bla* and *lacZ* inserted at the CTX phage *att* site on the chromosome	[91]
PAO1Bla6TnC2::Gm^R (PAO1+)	Gm^R Cb^R	Transconjugant of the mating between PA14ΔTnC2 (Gm^R) and PAO1Bla6	This study
PAO1Bla6TnC2::Tc^R (PAO1+)	Gm^R Cb^R	Transconjugant of the mating between PA14ΔTnC2::Tc^R and PAO1Bla6	This study

PAO1Δwzx	Gm^R	In-frame deletion of the wzx gene in strain PAO1, deficient in B-band LPS synthesis	[114]Islam et al., 2012
Plasmids			
pEXG2	Gm^R	Gene replacement vector for constructing deletion or insertion mutants of *P. aeruginosa*	[115]
pJET1.2	Amp^R	Plasmid used for DNA blunt cloning	Thermo Scientific
pGEX-2T	Amp^R	Expression vector for GST-pilV2'	GE Healthcare
mini-CTX2	Tc^R	Gene delivery vector for inserting genes at the CTX phage *att* site on *P. aeruginosa* chromosome	AF140579
pFLP2	Amp^R	Plasmid used for resolving the backbone of mini-CTX	[115]
pEXG2	Gm^R	Gene replacement vector for constructing deletion or insertion mutants of *P. aeruginosa*	[115]
pJET1.2	Amp^R	Plasmid used for DNA blunt cloning	Thermo Scientific
pGEX-2T	Amp^R	Expression vector for GST-pilV2'	GE Healthcare

↵*a* Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance.

Table 2. List of PAO1 mutants for lipopolysaccharide biosynthesis used in the screening 3.2

Number	PA ORF	Gene Abbrev.	Putative ORF Function	Position in PAO1 transposon mutant library (source: [115])
1	PA0705	migA	alpha-1,6-rhamnosyltransferase MigA	phoAwp01q4A03
2	PA0936	lpxO2	lipopolysaccharide biosynthetic protein LpxO2	lacZwp03q3H09
3	PA3141	wbpM	nucleotide sugar epimerase/dehydratase	(*)
4	PA3157		probable acetyltransferase	phoAwp08q3G06
5	PA3160	wzz	O-antigen chain length regulator	phoAbp02q3G06
6	PA3193	glk	Glucokinase	phoAwp07q4C11
7	PA3337	rfaD	ADP-L-glycero-D-mannoheptose 6-epimerase	phoAwp05q3A01
8	PA3552	arnB	ArnB	phoAwp08q4G12
9	PA3554	arnA	ArnA	lacZwp07q3F04
10	PA3555	arnD	ArnD	phoAwp04q2C06
11	PA3556	arnT	inner membrane L-Ara4N transferase ArnT	lacZwp07q1F11
12	PA4458		conserved hypothetical protein	phoAwp07q2G01
13	PA4512	lpxO1	lipopolysaccharide biosynthetic protein LpxO1	phoAwp07q3E07
14	PA4661	pagL	Lipid A 3-O-deacylase	phoAbp02q4E08
15	PA5001		hypothetical protein	phoAwp01q3H11
16	PA5002		hypothetical protein	lacZbp03q3E06
17	PA5005		probable carbamoyl transferase	phoAwp09q3B06
18	PA5009	waaP	lipopolysaccharide kinase WaaP	phoAwp05q4G09
19	PA5011	waaC	heptosyltransferase I	lacZwp04q4G06
20	PA5012	waaF	heptosyltransferase II	lacZwp08q1C03
21	PA5447	wbpZ	glycosyltransferase WbpZ	lacZwp02q1H10
22	PA5448	wbpY	glycosyltransferase WbpY	phoAwp02q1F12
23	PA5449	wbpX	glycosyltransferase WbpX	lacZwp01q4A02
24	PA5450	wzt	ABC subunit of A-band LPS efflux transporter	phoAwp10q1E09
25	PA5452	wbpW	phosphomannose isomerase/GDP-mannose WbpW	lacZwp08q4H11
26	PA5453	gmd	GDP-mannose 4,6-dehydratase	lacZwp02q3E02
27	PA5454	rmd	oxidoreductase Rmd	lacZwp01q1B08
28	PA5455		hypothetical protein	phoAwp08q4H06
29	PA5456		hypothetical protein	lacZwp02q4C05
30	PA5457		hypothetical protein	lacZwp06q1F08
31	PA5458		hypothetical protein	phoAwp10q1C10
32	PA5459		hypothetical protein	phoAwp08q1B12
33	PA5322	algC	phosphomannomutase	phoAwp07q4D07

(*): Lory's lab collection

Table 3. List of PA14 mutants for PAPI-1 genes used in the screening 3.11

Number	PA ORF	Gene Abbrev.	Size(bp)	Gene function	Position in PA14 transposon mutant library (Source: [116])
1	PA14_60190	clpB	318	Hypothetical protein	PAMr_nr_mas_03_4:G11
2	PA14_60140	xerC	1284	Integrase	PAMr_nr_mas_11_2:F5
3	PA14_60130	RL003	1920	Hypothetical protein	PAMr_nr_mas_08_2:B6
4	PA14_60110	RL005	711	Hypothetical protein	PAMr_nr_mas_03_4:D3
5	PA14_60100	Dtd	540	Deoxycytidine triphosphate deaminase	PAMr_nr_mas_12_4:C1
6	PA14_60080	RL008	1932	Hypothetical protein	PAMr_nr_mas_10_3:E1
7	PA14_60070	RL009	2424	Hypothetical protein	PAMr_nr_mas_12_4:H8
8	PA14_60060	RL010	303	Hypothetical protein	PAMr_nr_mas_01_2:F9
9	PA14_60050	parE	351	Plasmid stabilization protein parE	PAMr_nr_mas_15_2:C5
10	PA14_60030	RL013	345	Hypothetical protein	PAMr_nr_mas_09_3:E5
11	PA14_60020	RL014	1512	Hypothetical protein	PAMr_nr_mas_06_4:D11
12	PA14_60010	RL015	342	Hypothetical protein	PAMr_nr_mas_13_3:D8
13	PA14_60000	RL016	1383	Hypothetical protein	PAMr_nr_mas_08_3:G8
14	PA14_59990	RL017	939	Hypothetical protein	PAMr_nr_mas_07_3:F5
15	PA14_59980	RL018	432	Hypothetical protein	PAMr_nr_mas_07_4:B10
16	PA14_59970	RL019	219	Hypothetical protein	PAMr_nr_mas_10_2:A3
17	PA14_59960	dsbG	660	Putative protein-disulfide isomerase	PAMr_nr_mas_10_2:D8
18	PA14_59950	RL021	285	Hypothetical protein	PAMr_nr_mas_10_3:B5
19	PA14_59940	RL022	2943	Hypothetical protein	PAMr_nr_mas_02_1:F1
20	PA14_59920	RL024	1506	Hypothetical protein	PAMr_nr_mas_09_1:G2
21	PA14_59910	RL025	885	Hypothetical protein	PAMr_nr_mas_07_3:F2
22	PA14_59900	RL026	660	Hypothetical protein	PAMr_nr_mas_03_4:F5
23	PA14_59890	RL027	387	Hypothetical protein	PAMr_nr_mas_10_1:D11
24	PA14_59870	RL029	240	Hypothetical protein	PAMr_nr_mas_13_2:H12
25	PA14_59850	RL031	306	Hypothetical protein	PAMr_nr_mas_04_1:E12
26	PA14_59840	RL033	1158	Hypothetical protein	PAMr_nr_mas_05_4:C10
27	PA14_59830	RL034	1482	Putative DNA helicase	PAMr_nr_mas_13_3:C1
28	PA14_59820	RL035	651	Hypothetical protein	PAMr_nr_mas_12_2:E10
29	PA14_59800	RL036	2796	sensor of two-component regulatory system	PAMr_nr_mas_01_4:A5
30	PA14_59790	pvrR	1200	regulator of two-component regulatory system; adhesion and antibiotic resistance	PAMr_nr_mas_15_2:A11
31	PA14_59780	rcsC	3255	sensor of two-	PAMr_nr_mas_05_3:D6

				component regulatory system	
32	PA14_59770	rcsB	696	regulator of two-component regulatory system	PAMr_nr_mas_10_2:E11
33	PA14_59760	cupD5	717	Probable pili assembly chaperone / adhesion and protein secretion	PAMr_nr_mas_11_3:G6
34	PA14_59750	cupD4	1347	adhesion and protein secretion	PAMr_nr_mas_03_2:A10
35	PA14_59720	cupD2	747	Probable fimbrial biogenesis usher / adhesion and protein secretion	PAMr_nr_mas_04_3:E2
36	PA14_59710	cupD1	549	Probable pili assembly chaperone / adhesion and protein secretion	PAMr_nr_mas_08_4:H11
37	PA14_59700	RL046	747	Probable fimbrial precursor / adhesion and protein secretion	PAMr_nr_mas_12_2:D7
38	PA14_59690	RL047	2235	Hypothetical protein	PAMr_nr_mas_05_4:A2
39	PA14_59680	RL048	258	Hypothetical protein	PAMr_nr_mas_05_4:E9
40	PA14_59670	RL049	501	Hypothetical protein	PAMr_nr_mas_03_4:A8
41	PA14_59660	RL050	582	Hypothetical protein	PAMr_nr_mas_04_4:A6
42	PA14_59640	RL052	690	Hypothetical protein	PAMr_nr_mas_13_2:B4
43	PA14_59630	RL054	1032	Hypothetical protein	PAMr_nr_mas_13_2:B3
44	PA14_59620	RL055	666	Hypothetical protein	PAMr_nr_mas_05_4:B8
45	PA14_59610	RL056	675	Hypothetical protein	PAMr_nr_mas_09_2:E8
46	PA14_59600	RL057	246	Hypothetical protein	PAMr_nr_mas_09_2:B10
47	PA14_59590	RL058	360	Hypothetical protein	PAMr_nr_mas_04_4:E5
48	PA14_59580	RL059	1536	Hypothetical protein	PAMr_nr_mas_12_1:C4
49	PA14_59570	RL060	336	Transposase	PAMr_nr_mas_08_2:A12
50	PA14_59550	RL062	1227	Hypothetical protein	PAMr_nr_mas_03_2:D8
51	PA14_59540	RL063	2250	Plasmid-related protein	PAMr_nr_mas_05_4:B6
52	PA14_59530	RL064	1452	Plasmid-related protein	PAMr_nr_mas_12_4:G9
53	PA14_59520	RL065	606	Hypothetical protein	PAMr_nr_mas_11_4:B6
54	PA14_59500	RL067	363	Hypothetical protein	PAMr_nr_mas_09_2:D8
55	PA14_59490	RL068	276	Hypothetical protein	PAMr_nr_mas_15_2:A1
56	PA14_59480	RL069	690	Hypothetical protein	PAMr_nr_mas_08_1:F2
57	PA14_59470	RL070	351	Hypothetical protein	PAMr_nr_mas_02_1:E1
58	PA14_59440	RL071	708	Hypothetical protein	PAMr_nr_mas_01_4:D4
59	PA14_59430	RL072	237	Hypothetical protein	PAMr_nr_mas_02_2:G6
60	PA14_59410	RL073	267	Hypothetical protein	PAMr_nr_mas_05_2:E4
61	PA14_59400	RL074	540	Hypothetical protein	PAMr_nr_mas_08_4:A3
62	PA14_59380	RL075	567	Hypothetical protein	PAMr_nr_mas_04_1:B3

63	PA14_59370	RL076	645	Hypothetical protein	PAMr_nr_mas_08_2:F11
64	PA14_59360	pilM2	438	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_14_1:F10
65	PA14_59350	pilV2	1329	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_13_1:H12
66	PA14_59320	pilS2	531	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_05_4:B5
67	PA14_59310	pilR2	1080	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_02_1:H1
68	PA14_59290	pilQ2	1581	ATPase / Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_08_2:G5
69	PA14_59280	pilP2	534	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_03_1:B3
70	PA14_59270	pilO2	1326	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_07_3:H2
71	PA14_59250	pilN2	1623	Secretin / Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_07_1:D10
72	PA14_59240	pilL2	1125	Type IV B pilus / adhesion and and protein secretion	PAMr_nr_mas_09_2:H8
73	PA14_59220	RL088	1497	Colicin-like toxin (pyocin S5)	PAMr_nr_mas_02_3:G7
74	PA14_59210	RL089	1974	DNA Helicase	PAMr_nr_mas_15_1:G8
75	PA14_59200	RL090	1890	Hypothetical protein	PAMr_nr_mas_09_3:A6
76	PA14_59190	RL091	471	Similar to luminal binding protein	PAMr_nr_mas_10_3:D5
77	PA14_59180	topA	1926	Topoisomerase I	PAMr_nr_mas_07_3:A4
78	PA14_59170	RL093	234	Hypothetical protein	PAMr_nr_mas_12_1:D9
79	PA14_59160	RL094	246	Hypothetical protein	PAMr_nr_mas_09_3:C10
80	PA14_59150	ssb	471	Single-stranded DNA binding protein	PAMr_nr_mas_06_4:H2
81	PA14_59140	RL096	534	Hypothetical protein	PAMr_nr_mas_06_4:C3
82	PA14_59130	RL097	729	Hypothetical protein	PAMr_nr_mas_06_3:D2
83	PA14_59120	RL098	240	Hypothetical protein	PAMr_nr_mas_13_2:B8
84	PA14_59100	RL100	1326	Hypothetical protein	PAMr_nr_mas_08_2:G6
85	PA14_59090	RL101	768	Hypothetical protein	PAMr_nr_mas_09_1:G8
86	PA14_59060	RL103	255	Hypothetical protein	PAMr_nr_mas_05_1:G3
87	PA14_59050	RL104	1017	Hypothetical protein	PAMr_nr_mas_04_3:F6
88	PA14_59010	RL107	258	Hypothetical protein	PAMr_nr_mas_05_1:A6
89	PA14_59000	RL108	528	Hypothetical protein	PAMr_nr_mas_12_1:E2

90	PA14_58990	pseudo	1363	DNA replication and recombination	PAMr_nr_mas_13_1:E5
91	PA14_58970	RL110	708	Putative phage protein	PAMr_nr_mas_01_4:H11
92	PA14_58950	RL112	687	Hypothetical protein	PAMr_nr_mas_05_1:C5
93	PA14_58930	RL113	498	Hypothetical protein	PAMr_nr_mas_08_3:H10
94	PA14_58920	RL114	681	Hypothetical protein	PAMr_nr_mas_06_1:B7
95	PA14_58910	soj	930	Chromosome partitioning	PAMr_nr_mas_15_1:F2

Table 4. Primers used in this study

Primer	Sequence (5'-3')	Description	Source
TnC2-US-F	GGTACCGGCAACACATTTCTCCCTCG	Amplify a fragment of 532 bp upstream of PA14_59200 gene	This study
TnC2-US-R	TCTAGATTGAGCCAGCCAGTTGTAGA		
TnC2-DS-F	TCTAGACGGCTGAGAGACATCAAGGA	Amplify a fragment of 594 bp downstream of PA14_59200 gene	This study
TnC2-DS-R	AAGCTTGTTCAGGTTCGTCGCTATGG		
Tc-F	TCTAGATCAGGTCGAGGTGGCCC	Amplify Tet gene from mini-CTX2 plasmid	This study
Tc-R	TCTAGAAGAGCGCTTTTGAAGCTAATTCGCTG		
TnC2-Li-F	CTTGACGAGTTTGCTGCACT	Check the insert of Tet gene on the left junction	This study
TnC2-Li-R	GAGAAGCAGGCCATTATCGC		
TnC2-Ri-F	GAACGGGTGCGCATAGAAAT	Check the insert of Tet gene on the right junction	This study
TnC2-Ri-R	TTCGACCAAGGAGCTGAACT		
pilV2-F	ATAGGATCCCTGTCCTGCCAAAACGGG	Amplify C-terminal region of pilV2 gene (97 amino acid)	This study
pilV2-R	ATATGAATTCCTAGTTCACGCAGGTAACGG		
intF	AGCTACATCGAGGCCGACTA	Check the insertion of PAPI-1 on the left junction of attL site	[91]
4542F	GTGGTGATGACCTCCAACCT		[91]
sojR	CGAGCACAGAAATGTCCTGA	Check the insertion of PAPI-1 on the right junction of attR site	[91]
4541F	GACAAGACCAGCCACAACCT		

3.2. Screening for PAO1 mutants deficient in PAPI-1 acquisition

A standard PAPI-1 transfer assay via liquid mating was carried out as previously described [93]. Mutant PA14ΔTnC2::Gm^R was used as donor and a series of PAO1 mutants (Tc^R) with altered LPS biosynthesis from PAO1 transposon mutant library [115] [106] were used as recipients. The list of PAO1 mutants for this experiment was presented in the table 2. After overnight growth at 37 ^oC and 200 rpm, the donor cells were harvested at an OD_{600} of 0.8 and were mixed with the recipient cells at an OD_{600} of 0.4, spun down and resuspended in 1 ml of fresh LB without antibiotics. The mating mixture was incubated in 15 ml culture tubes, statically at 37 °C for 24 h. The mating mixture was diluted to appropriate concentrations and plated on LB agar plates containing gentamicin and tetracycline at 75 µg/ml to select for transconjugants, and on LB agar plates containing only tetracycline at 75 µg/ml to select for recipients. The transfer efficiency was calculated as the transconjugants / recipients ratio of colonies in the mating mixture.

3.3. Outer membrane (OM) preparation

The outer membranes of *P. aeruginosa* were isolated by using sodium lauroylsarkosinate (sarkosyl) as described previously [117]. Briefly, cultures of *P. aeruginosa* were grown overnight at 37 °C, 200 rpm in LB broth. The pre-inoculum was then diluted 100-fold in fresh LB medium and grown at 37 °C and 200 rpm to an OD^{600} of 1.0. Cells were harvested and resuspended in 15 ml lysis buffer containing 20 mM Tris-Cl [pH 7.5], 100 mM NaCl, 1mM EDTA, lysozyme [0.5

mg/ml] and a complete protease inhibitor cocktail (Roche). The lysate was sonicated and spun down at 10,000 g, 10 min, 4 °C to remove cellular debris. The membrane fraction was isolated by ultracentrifugation (200,000 g at 4°C for 60 min). The pellets containing inner and outer membranes were further fractionated at 100,000 g for 30 min after incubation with sarkosyl 0.2%. OMs were finally resuspended in Tris-Cl buffer 20 mM [pH 7.5] and separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with Coomassie blue. The concentration of OMs was measured by protein assay kit (Biorad).

3.4. LPS preparation

LPS molecules from various *P. aeruginosa* strains were prepared by using the hot phenol-water extraction protocol from Westphal and Jann (1965) with minor modifications [118]. Briefly, cell suspensions in 100 mM NaCl were first heated to 68^{o}C before adding an equal volume of hot phenol and stir vigorously for 2 hours at 68°C. LPS was then fractionated by centrifugation at 12,000g for 15 min at 4°C. LPS in the upper phase was recovered and dialyzed against water to remove phenol residue. LPS extract was further treated with DNase, RNase and proteinase K to eliminate contaminations. LPS extract was finally lyophilized before use. The LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. LPS was then quantified with KDO assay [119].

3.5. LPS silver staining

LPS preparations were separated with 12% discontinuous PAGE gel and visualized by silver staining, previous described [120]. Shortly, LPS gel was incubated 22 °C for 20 min without prior fixation in oxidation solution containing 0.7% periodic acid in 40% ethanol and 5% acetic acid. The gel was washed with distilled water in 5 min for three times. The gel was stained for 10 min in a fresh staining solution, containing a 4-ml volume of concentrated ammonium hydroxide that was added to 56 ml of 0.1 M sodium hydroxide, 200 ml of water, 10 ml of 20% (wt/vol) silver nitrate (Sigma, Germany) was added in drops with stirring and 30 ml with water. The gel was washed again three times with distilled water for 5 min. The yellow color was rapidly developed in 200 ml solution of 10 mg of citric acid and 0.1 ml of 37% formaldehyde. The color exposure was stopped in 10% acetic acid for 1 min followed by washing in distilled water.

3.6. PAPI-1 transfer inhibition assay

OM and LPS preparations at various concentrations were added to a standard mating assay based on plasmid conjugal transfer [121], between the donor PA14ΔTnC2::Gm^R and the recipient PAO1::Tc^R. A mating mixture without addition of OM or LPS was also included as a negative control for this experiment. The transfer inhibition index was calculated by dividing the transfer efficiency observed with the addition of OM or LPS to that of the control.

3.7. Construction of PA14ΔTnC2::Tc^R mutant

The mutant PA14ΔTnC2 is proficient in transfer of PAPI-1 and has been used as a donor in PAPI-1 transfer assay [93]. In some experiments, a donor with TcR marker was required since the recipient containing GmR, and we expected to have an equivalent transfer efficiency to the PA14ΔTnC2::Gm^R obtained from PA14 transposon mutant library in which the transposon also inserted at nucleotide 1634 of PAPI-1 gene RL090 (PA14_59200). Therefore, the PA14_59200 gene of the wild-type PA14 was partially deleted and replaced by a tetracycline resistant marker. The mutant was constructed by using gene replacement vectors and methods, as previously described [122]. All primers used for generating the mutant PA14ΔTnC2::Tc^R are listed in Table 4. Briefly, DNA fragments of 500 bp, located at 5' and 3' end of the PA14_59200 gene and tetracycline resistance gene, were amplified and cloned in the vector pJET1.2 before subcloning into the vector pEXG2. The recombinant plasmid was conjugated from *E. coli* λpir S17.1 into *P. aeruginosa* [123]. The integrative plasmids were selected on LB plates supplemented with gentamicin, tetracycline or irgasan at 25μg/ml. To resolve merodiploids, which are cells possessing a partial duplication of their genetic material and potentially allows evolution of new genes [124], a second selection round on LB agar with 6% sucrose was performed. Transformants were screened by colony PCR and confirmed by DNA sequencing.

3.8. Expression of GST-pilV2 fusion protein

C-terminal region of pilV2 gene encoding 97 residues was amplified with the primers listed in Table 4 and cloned into pJet 1.2/Blunt before transformation into *E .coli* DH5α. The insert was then cloned into the expression vector pGEX-2T (Life Technologies) and transformed into *E. coli* BL21. *Escherichia coli* BL21 containing pGEX-2T-pilV2' was grown to an OD600 of 0.6 and IPTG at 0.1 mM was added to induce gene expression and translation of GST-pilV2' protein. The expression step was carried out at 37^{o}C, 200 rpm for 3 hours. The culture was centrifuged at 8000 rpm, 4^{o}C for 20 min to pellet the cells. GST-pilV2' fusion protein was then purified by using glutathione Sepharose 4B (GE Healthcares), as previously described [125]. The GST-pilV2' protein expression was also confirmed by anti-GST primary antibody (GE Healthcare) followed by anti-goat IgG Alkaline Phosphatase Conjugate and detected by using BCIP/NBT color development subtrate.

3.9. Western blotting for LPS samples

LPS samples prepared by Hitchcock and Brown method (1983) were used for western blotting analyses. The western blot protocol for LPS was described previously [118]. Briefly, 3 ul of LPS extract was loaded into a 12% discontinuous PAGE gel and run at 200 V for 50 min. LPS was electrophoretically transferred onto nitrocellulose membrane at 180 mA for 60 min. The membrane was blocked with 5 % skim milk for 20 min at room temperature, washed in PBS for 10 min before adding primary antibodies directed against different parts of LPS structure, and incubated overnight. Secondary antibodies were added after a 10-min wash

and followed by 1 hour of incubation. The membrane was washed again for 10 min and developed by using BCIP/NBT color development subtrate.

3.10. Microtiter plate binding assay

Binding of LPS to GST-pilV2' fusion was quantified by an enzyme-linked plate assay essentially as previously described [126] with slight modifications. Microtiter plates (Corning) were coated with 10 µg/ml LPS from PAO1 and PAO1 with PAPI-1 suspended in PBS (0.137 M NaCl, 0.005 M KCl, 0.009 M Na_2HPO_4, and 0.001 M KH_2PO_4 (pH 7.4)) containing 0.05% Tween 20 (v/v, PBST). The plates were then washed with PBST and blocked with 3% BSA. GST-pilV2' fusion was added to the wells coated with LPS and incubated for 2 h at room temperature. After three washes with PBST, mouse anti-GST antibody was added and incubated for 1.5 h. Then, HRP-labeled anti-mouse Ig (Sigma-Aldrich) was added after three other washes. TMB solution (Thermo Scientific) was used for color development and ODs were measured at 450 nm. LPS from *Salmonella enterica* was used as a negative control.

3.11. Screening for PAPI-1 genes involved in exclusion activity of *Pseudomonas aeruginosa*

The screening was followed the previous protocol (section 3.2). A standard PAPI-1 transfer assay via liquid mating was also carried out here between the mutant PA14ΔTnC2::Tc^R used as donor and a series of ninety-five PA14 mutants for PAPI-1 genes from PA14 transposon mutant library as recipients containing gentamicin resistant marker, listed in the table 3 [116]. The mating mixtures were

statically incubated at 37 °C for 24 h and then diluted to appropriate concentrations. Number of transconjugants was counted on LB agar plates with gentamicin and tetracycline at 75 µg/ml and that of recipients was selected on LB agar plates containing tetracycline at 75 µg/ml.

RESULTS

4. RESULTS

4.1. PAO1 mutants for A-band LPS biosynthesis are deficient in the acquisition of PAPI-1

Since in conjugal plasmid system the donor pilus is known to interact with specific components of LPS on the recipient membrane to initiate the transfer [101], we examined the effects of using various PAO1 mutants for LPS biosynthesis as recipients on PAPI-1 transfer assay (Figure 12). The mutant PA14ΔTnC2::Gm^R has previously been proposed as a proficient donor in PAPI-1 transfer [93], and was used as a donor in this study. We first checked the transfer efficiency of PAPI-1 into the mutant PAO1ΔalgC, in which algC gene was disrupted by transposon insertion at nucleotide 628 [115]. The gene algC encodes for a phosphoglucomutase, which is required for the synthesis of a complete LPS structure [127]. The mutant PAO1ΔalgC thus produces a truncated LPS core and is devoid of O-antigen. When the transfer assay was carried out between the donor PA14ΔTnC2::Gm^R and PAO1ΔalgC::Tc^R, the transfer efficiency was reduced by three orders of magnitude compared to that of wild-type PAO1 (Figure 12A). This suggests that the complete LPS structure plays an important role in PAPI-1 acquisition. We therefore decided to screen a series of 32 PAO1 mutants for LPS biosynthesis (Table 2) [106] using them as recipients in the PAPI-1 transfer assay. These mutants were obtained from PAO1 transposon mutant library, which contain Tc^R [115].

The results showed that transfer efficiency of PAPI-1 into 10 PAO1 mutants including PA5001; PA5447 (wbpZ); PA5448 (wbpY); PA5449 (wbpX); PA5450 (wzt); PA5453 (gmd); PA5454 (rmd); PA5455; PA5456 and PA5459 was significant decreased of two to three orders of magnitude compared to control. All these genes, except PA5001, were predicted as members of 2 operons [128]. One starts from 6135968 to 6144991 (8 constituent genes) and another is from 6145399 to 6151151 (5 genes). Interestingly, some of these genes are known to be involved in Common Polysaccharide Antigen (CPA) biosynthesis (Table 5). They encode enzymes responsible for the CPA biosynthesis pathway as shown in Figure 11. The CPA or A-band LPS has been shown to contain a tri-saccharide repeating unit: [→3)-α-D-Rha-(1→3)-α-DRha-(1→2)-α-D-Rha-(1→]$_n$, so-called D-rhamnose homopolymer or rhamnan structure. This structure has been characterised several times with good agreement between different studies [129], [130]. As a result, our data suggested that the A-band LPS could act as a receptor for conjugative type IV pilus as an initial step of PAPI-1 transfer.

Table 5. Genes involved or potentially involved in Common Polysaccharide Antigen (A-band LPS) biosynthesis. This table was reproduced by King *et al.*, 2009 [106].

Gene	Related proteins (% identity)	Proposed/demonstrated function	Key reference
wbpZ/PA5447	52% *E. coli* O9a WbdC	Glycosyltransferase (GT-4)	[131]
wbpY/PA5448	34% *E. coli* O9a WbdB	Glycosyltransferase (GT-4)	[131]
wbpX/PA5449	33% *E. coli* O9a WbdA C-terminal domain (over 301 amino acids)	Glycosyltransferase (GT-4)	[131]
	25% *E. coli* O9a WbdA N-terminal domain (over 262 amino acids)		
wzt/PA5450	61% *E. coli* O8 Wzt	ABC transporter	[132]
wzm/PA5451	56% *E. coli* O8 Wzm	ABC transporter	[132]
wbpW/PA5452	46% *P. aeruginosa* AlgA	D-Man-6-phosphate isomerase / GDP-D-Man pyrophosphorylase	[133]
	60% *P. aeruginosa* PslB		
gmd/PA5453	47% *E. coli* GMD	GDP-D-Man 4,6-dehydratase	[134]
rmd/PA5454	33% *Aneurinibacillus thermoaerophilus* RMD	GDP-D-Rha synthase	[134]
PA5455		Glycosyltransferase (GT-4)	
PA5456		Glycosyltransferase (GT-4)	
PA5457	20% *E. coli* O8 WbdD (over 149 amino acids)	Methyltransferase	
PA5458	24% *Staphylococcus aureus* OatA	Acetyltransferase	
PA5459	23% *E. coli* O8 WbdD (over 139 amino acids)	Methyltransferase	
algC/PA5322	31% *E. coli* ManB	Phosphomannomutase/phosphoglucomutase	[135]

The classification of Glycosyltransferase (GT) family is provided by the CAZy database [136]. * References including experimental study with *P. aeruginosa* genes are cited.

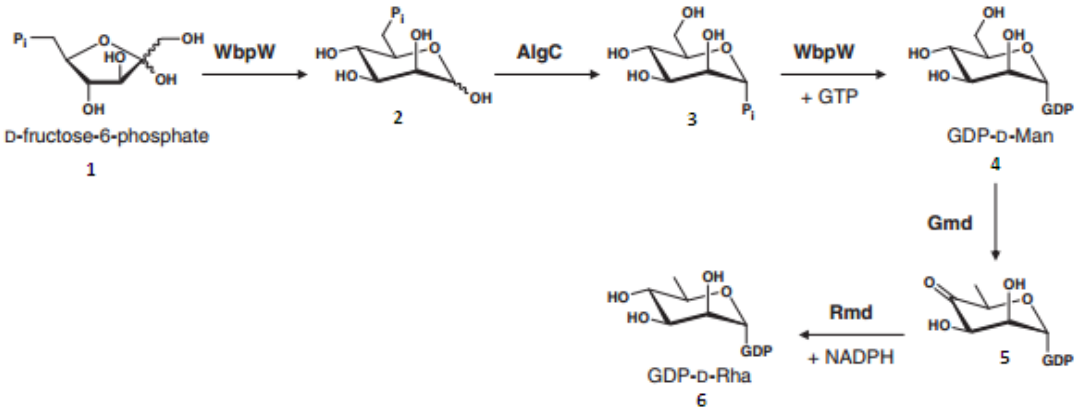


Figure 11. The GDP-D-rhamnose biosynthesis pathway. All the enzymes are encoded by a gene cluster, except the second enzyme phosphomannomutase encoded by algC gene in the alginate locus. D-fructose-6-phosphate; 1, Pi, phosphate; 2, mannose-6-Pi; 3, GDP-D-Man; 4, α-mannose-1-Pi; 5, GDP-4-keto-D-Rha; 6. This figure was modified from King *et al.*, 2009 [106].

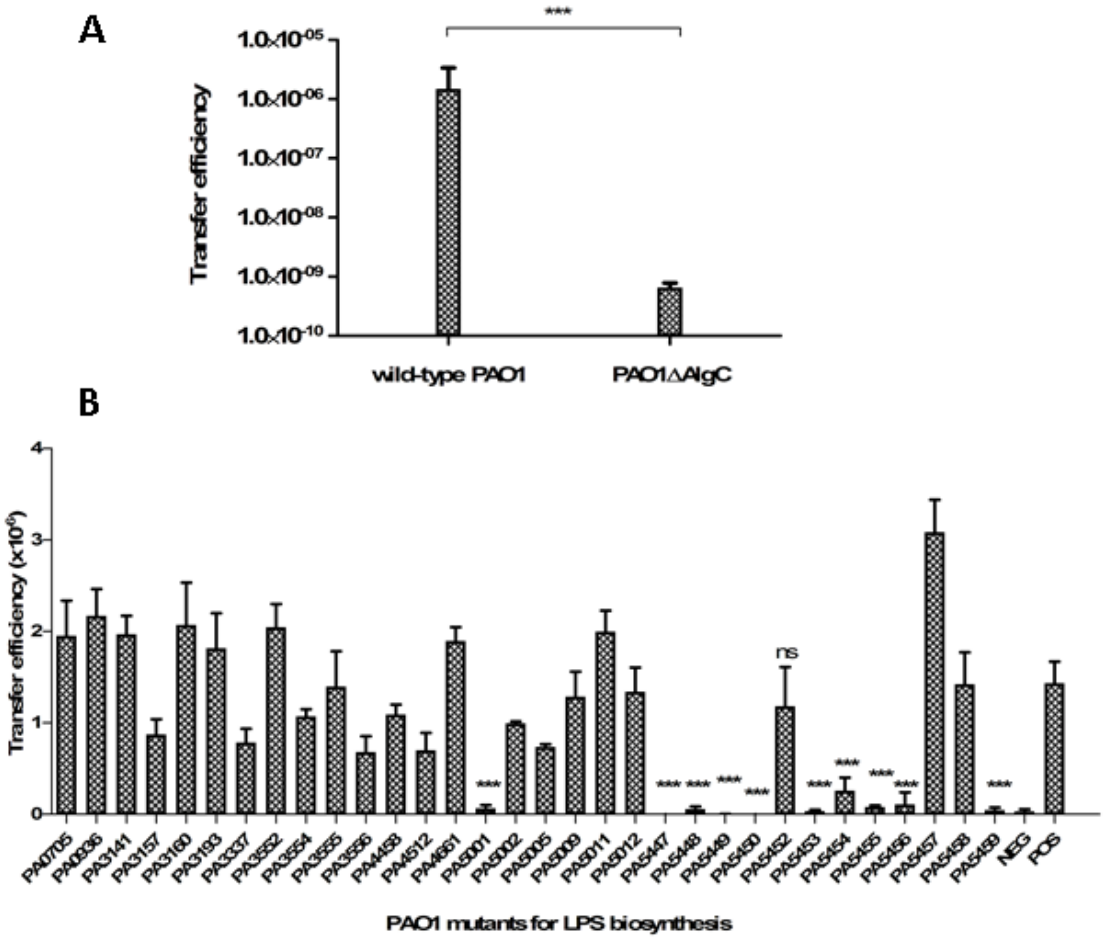


Figure 12. PAPI-1 transfer efficiency using PAO1 mutants for LPS biosynthesis genes as recipient strains. (A) Transfer efficiency into PAO1ΔalgC (B) Transfer efficiency into various PAO1 mutants for LPS biosynthesis. Positive control (POS): PAO1::Tc^R and Negative control (NEG): PAO1ΔalgC. Results were shown as mean ± SD for three independent replicates. Statistical significance was calculated by the unpaired t-test (A) and One-way ANOVA compared to the positive control (B) (ns: no significant; and *** $p < 0.001$).

4.2. Addition of OM and A-band LPS preparations inhibits PAPI-1 transfer

We hypothesized that OM fractions or A-band LPS preparations, the putative receptor for conjugative pilus, can compete with the recipients binding to the conjugative pilus and thus block the transfer of PAPI-1 to the recipient. We thus extracted OM and LPS from two PAO1 mutants producing only A-band LPS and two others producing only B-band LPS**.** The quality of OM and LPS preparations was controlled by SDS-PAGE (Figure 13) before adding them to the standard PAPI-1 transfer assay at different concentrations. As mentioned in the introduction section, the outer membrane fraction of *P. aeruginosa* contains at least six main proteins, including D (50 kDa), E (45 kDa), F (33 kDa), G and H (21 KDa), I (8 kDa) [104] (Figure 13A). The LPS preparations were processed to be free of protein, DNA and RNA contamination. In this experiment, OM and LPS samples were fractionated from two PAO1 mutants producing A-band LPS (PAO1ΔwbpM [137] and PAO1Δwzx [138]) and two others for B-band LPS (PAO1Δrmd [133] and PAO1ΔalgC).

Our results showed that the increased addition of OM and LPS amount from mutants lacking B-band LPS inhibited transfer efficiency, while the addition of OM and LPS from mutants lacking A-band LPS did not produce any significant effect on PAPI-1 transfer efficiency. The addition of OM and LPS from PAO1ΔwbpM and PAO1Δwzx strongly inhibited transfer even at low concentrations (< 5 μg) and reached the maximum index at 80% (Figure 14). These results strongly support the hypothesis that A-band LPS functions as a specific receptor for the IVb pilus in the recipient strain, and that it is required to initiate the transfer of PAPI-1.

Moreover, statistical analysis using two-way ANOVA for two factors (type and amount of OM or LPS) revealed that OM and LPS derived from A-band producing mutants have significant effects on PAPI-1 transfer efficiency compared to those from B-band producing ones with $P < 0.001$ (Table 6 and 7). The half maximal inhibitory concentration (IC_{50}), corresponding to the amount of preparations inhibiting 50% of the transfer efficiency, were calculated (Table 8). IC_{50} of OM from both PAO1Δwzx and PAO1ΔwbpM are 0.866 µg, whereas IC_{50} for LPS were of 1.184 µg for PAO1Δwzx and 2.114 µg for PAO1ΔwbpM.

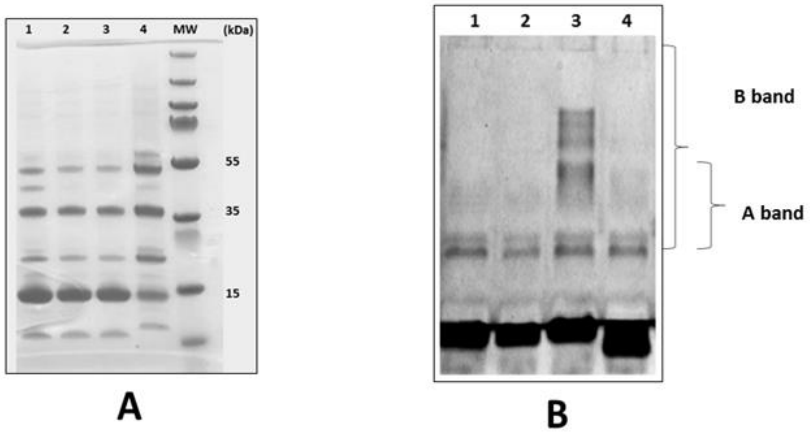


Figure 13. OM (A) and LPS (B) preparations from PAO1 mutants lacking either A-band or B-band LPS. Well 1, PAO1ΔwbpM (A+, B-); Well 2, PAO1Δwzx (A+,B-); Well 3, PAO1Δrmd (A-,B+); Well 4, PAO1ΔalgC (A-, B+).

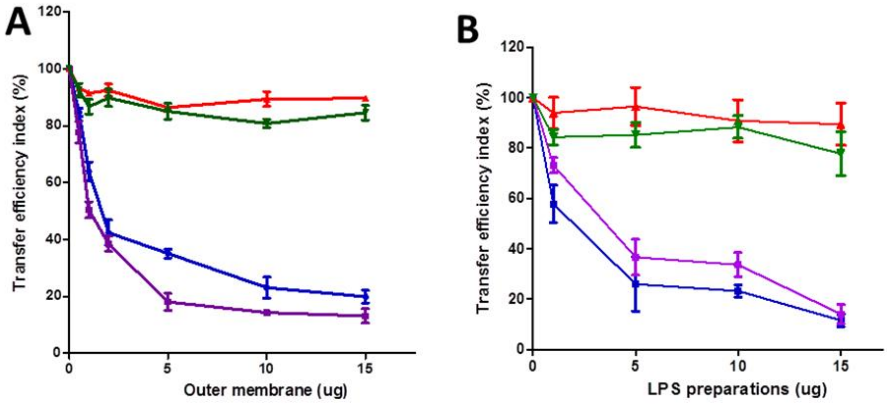


Figure 14. Effect on PAPI-1 transfer efficiency of the addition of OM (A) and LPS (B) preparations from PAO1 mutants lacking either A-band or B-band LPS. Red, PAO1Δrmd (A-,B+); green, PAO1ΔalgC (A-, B+); blue, PAO1ΔwbpM (A+, B-); purple, PAO1Δwzx (A+,B-).

Table 6. Statistical analysis for the effects of OM from PAO1 mutants lacking either A-band or B-band LPS on PAPI-1 transfer efficiency. Significant differences were analyzed by two-way ANOVA for two factors (type and amount of OM)

PAO1ΔwbpM (+A -B) vs PAO1Δrmd (-A +B)				
Amount of OM (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
0.5	8.482	4.67	P<0.001	***
1	27.57	15.18	P<0.001	***
2	50.1	27.58	P<0.001	***
5	51.36	28.27	P<0.001	***
10	66.31	36.51	P<0.001	***
15	69.84	38.45	P<0.001	***
PAO1ΔwbpM (+A -B) vs PAO1ΔalgC (-A +B)				
Amount of OM (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
0.5	7.742	3.679	P<0.01	**
1	22.81	10.84	P<0.001	***
2	47.44	22.55	P<0.001	***
5	50.05	23.79	P<0.001	***
10	57.74	27.44	P<0.001	***
15	64.72	30.76	P<0.001	***
PAO1Δwzx (+A -B) vs PAO1Δrmd (-A +B)				
Amount of OM (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
0.5	15.49	9.14	P<0.001	***
1	41.05	24.22	P<0.001	***
2	54.08	31.91	P<0.001	***
5	68.34	40.32	P<0.001	***
10	75.07	44.29	P<0.001	***
15	76.67	45.23	P<0.001	***
PAO1Δwzx (+A -B) vs PAO1ΔalgC (-A +B)				
Amount of OM (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
0.5	14.75	7.375	P<0.001	***
1	36.29	18.14	P<0.001	***
2	51.42	25.71	P<0.001	***
5	67.03	33.51	P<0.001	***
10	66.5	33.24	P<0.001	***
15	71.55	35.77	P<0.001	***

Table 7. Statistical analysis for the effects of LPS from PAO1 mutants lacking either A-band or B-band LPS on PAPI-1 transfer efficiency. The significant differences were analyzed by two-way ANOVA for two factors (type and amount of LPS)

PAO1∆wbpM (+A -B) vs PAO1∆rmd (-A +B)				
Amount of LPS (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
1	36.21	6.104	P<0.001	***
5	70.02	11.8	P<0.001	***
10	63.77	10.75	P<0.001	***
15	78.19	13.18	P<0.001	***
PAO1∆wbpM (+A -B) vs PAO1∆algC (-A +B)				
Amount of LPS (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
1	25.65	4.372	P<0.01	**
5	58.77	10.02	P<0.001	***
10	62.57	10.66	P<0.001	***
15	66.01	11.25	P<0.001	***
PAO1∆wzx (+A -B) vs PAO1∆rmd (-A +B)				
Amount of LPS (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
1	20.44	4.214	P<0.01	**
5	59.51	12.27	P<0.001	***
10	56.82	11.71	P<0.001	***
15	76.02	15.67	P<0.001	***
PAO1∆wzx (+A -B) vs PAO1∆algC (-A +B)				
Amount of LPS (ug)	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
1	9.89	2.072	P > 0.05	ns
5	48.26	10.11	P<0.001	***
10	55.62	11.65	P<0.001	***
15	63.84	13.38	P<0.001	***

Table 8. Analysis of half maximal inhibitory concentration (IC_{50}) for the addition of OM and LPS from the mutants producing only A-band into the PAPI-1 transfer assay. IC50 was calculated by an equation "log(inhibitor) vs. normalized response -- Variable slope" with software GraphPad Prism 5.

Additives	OM		LPS	
	PAO1∆wzx (+A, -B)	PAO1∆wbpM (+A, -B)	PAO1∆wbpM (+A -B)	PAO1∆wzx (+A -B)
Best-fit values				
LogIC50	-0.06247	-0.06247	0.0735	0.325
IC50	0.866	0.866	1.184	2.114
P value	0.6114		0.0084	
Preferred model	LogIC50 SAME for all data sets		LogIC50 DIFFERENT for each data set	

4.3. Construction of the PA14∆TnC2::TcR mutant

Since some experiments required a donor with tetracycline resistant marker, we constructed the mutant PA14∆TnC2 by inserting tetracycline gene within PA14_59200 (or TnC2 gene), and named it PA14∆TnC2::Tc^R. We expected to obtain a donor providing an equivalent transfer efficiency as PA14∆TnC2 which was throughout used in this study. We constructed a pJet 1.2/blunt plasmid containing an fragment ranging from 500-600 bp at 5' and 3' end of the TnC2 gene with Tet resistance gene inserted in the middle. We obtained specific PCR amplicons in a range of annealing temperatures (60 to 65^{o}C) (Figure 15). All fragments were successfully ligated in a correct order into pJet 1.2/blunt, subcloned in pEXG2, and used to transform into wild-type *P. aeruginosa* PA14. Eventually, the correct mutation in TnC2 gene was confirmed by PCR assay for left and right junctions (Figure 16). The transfer efficiency of PAPI-1 into PAO1

recipient for PA14ΔTnC2::Tc^R was comparable to that of PA14ΔTnC2::Gm^R (data not shown).

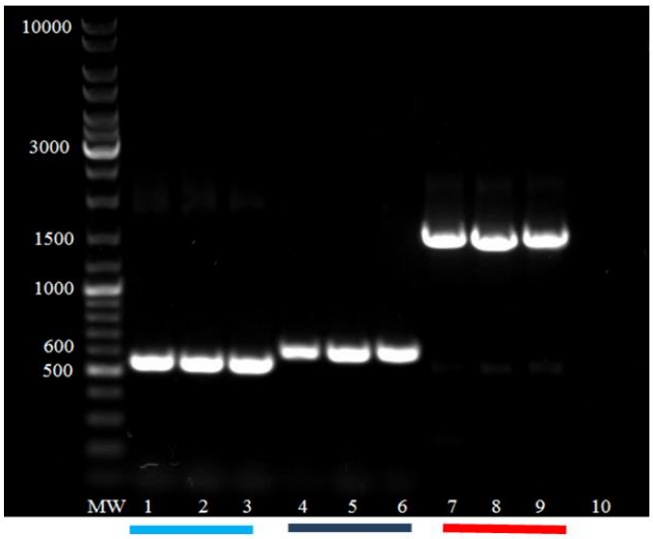


Figure 15. PCR amplification of upstream and downstream fragments and PA14_59200 gene with gradient annealling temperature (60^{o}C; 62,5^{o}C; 65°C). Well 1-3: amplicon of US fragment; Well 4-6: amplicon of DS fragment; Well 7-9: amplicons of tetracycline resistant gene. Well 10: negative control.

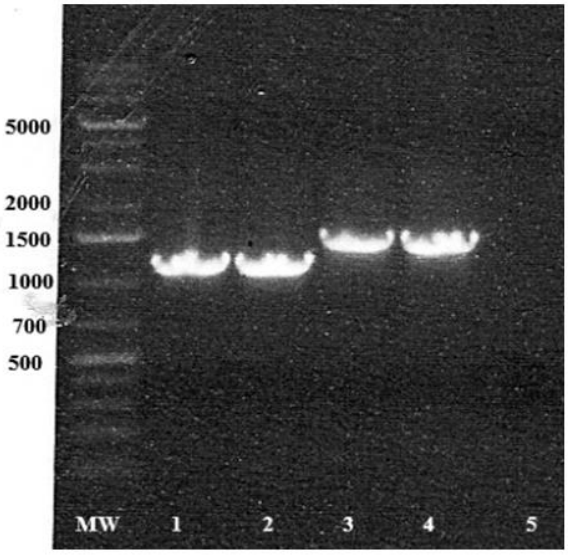


Figure 16. PCR amplification confirming the insertion of tetracycline resistance cassette in PA14_5900 gene. Well 1-2: Right insertion of two tested colonies (Primers TnC2-Ri-F and TnC2-Ri-R); Well 3-4: Left insertion of two tested colonies (Primers TnC2-Li-F e TnC2-Li-R).

4.4. Recipient strains carrying PAPI-1 can acquire additional copies of the island at a low frequency

After having demonstrated that the conjugative transfer is mediated by contact with A-band LPS, we asked if the recipients already carrying PAPI-1 can acquire additional copies of this island. In this experiment, we carried out a combination of mating assays between the donors PA14+ or PAO1+ and the recipients also with PAPI-1 (PA14+ or PAO1+) or without PAPI-1 (PA14 – or PAO1 –).

As shown in Figure 17, the transfer efficiency of PA14+ to PA14- is significant lower than the control PAO1-. This would suggest that the transfer efficiency of PAPI-1 is also influenced by the identity of the recipient strain. Remarkably, it can be noted that the strain PAO1 carrying PAPI-1 can act as a donor of the island, transferring it to the control recipient (PAO1-) at a similar efficiency as that of PA14+. These results also demonstrate the ability of recipient strains to acquire more than one PAPI-1 copy, even if this occurs at much lower efficiency compared to controls, with a decrease of one and three orders of magnitude and three orders of magnitude for PA14+/PA14+ and PAO1+/PAO1+, respectively. Our data thus indicates that *P. aeruginosa* which already acquired PAPI-1 strongly and significantly decreased their ability of receiving additional copies. This implies that *P. aeruginosa* strains carrying PAPI-1 specify a mechanism to exclude the acquisition of additional copies of the island.

4.5. The acquisition of PAPI-1 specifies a surface exclusion mechanism

We hypothesized that after acquisition of PAPI-1, recipient *P. aeruginosa* strains modify their membrane structure to avoid further contact and transfer with the donor cells. To infer if the presence of PAPI-1 in the cell genome can affect the structure of its OM and LPS, we performed again the standard transfer assay with the addition of OM and LPS from strains with and without PAPI-1. Similar to section 4.2, the quality of OM and LPS was controlled before added to the transfer assay (Figure 18). As shown in Figure 19, the addition of OM preparations derived from strains with PAPI-1 do not impact on transfer efficiency, compared to the ones with OM of the strains without the island. The effect of LPS preparations (Figure 19B) is similar for PAO1 strain but not for PA14, since the addition of LPS from PA14- did not produce significant variation in transfer efficiency. This data suggests that *P. aeruginosa* specify mechanisms to exclude the acquisition of additional copies of PAPI-1 via OM and/or LPS modification. However, the mechanism seems to be different for PAO1 and PA14. The statistical analysis also showed that the effect of OM from PAO1 and PA14 strains with or without PAPI-1 are significant different ($P<0.001$) (Table 9). However, only the effect of LPS from PAO1+ and PAO1- is significant; but not for PA14 (Table 10). The half maximal inhibitory concentration (IC_{50}) values of OM and LPS from strains without PAPI-1 were also analyzed (Table 11). For OM, the IC_{50} of PAO1- (0.9726 µg) was lower than that of PA14- (1.453 µg). For the LPS of PAO1-, the IC_{50} was 1.232 µg.

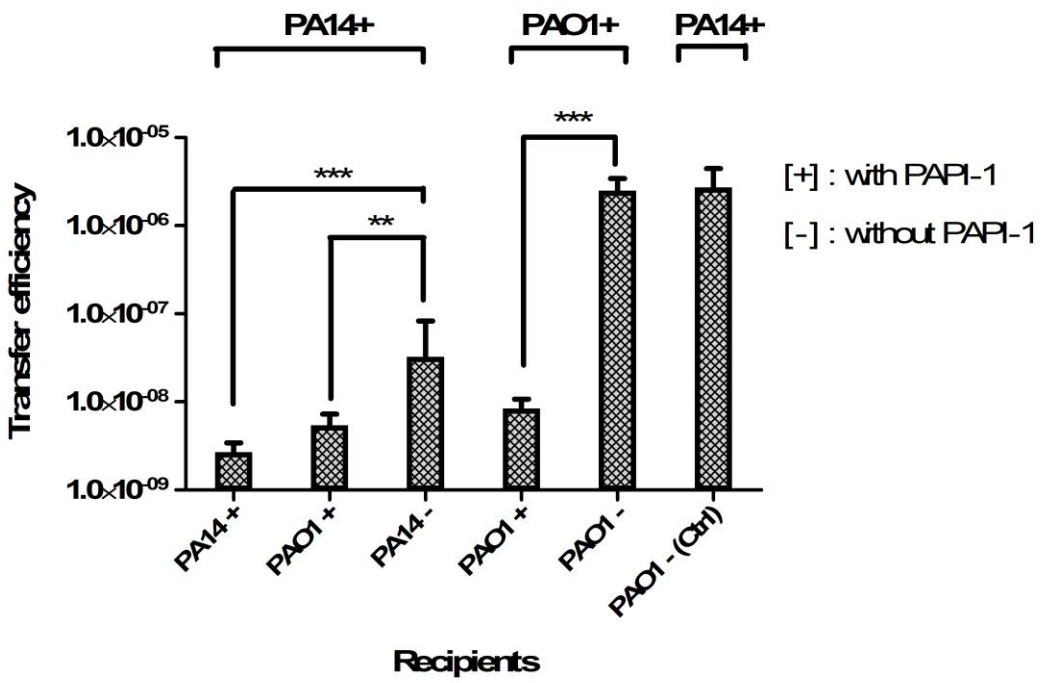


Figure 17. Transfer efficiency of multiple PAPI-1 copies into recipient strains. Marks [+] or [-] stand for strains with or without PAPI-1, respectively. After acquisition of PAPI-1, PAO1 becomes a stable donor which can transfer PAPI-1 to another recipient and decreases its ability of receiving additional copies of PAPI-1. Results are presented as mean ± SD for three independent experiments. Statistical significance was calculated by the unpaired t-test (A) (** $p<0.01$, and *** $p<0.001$).

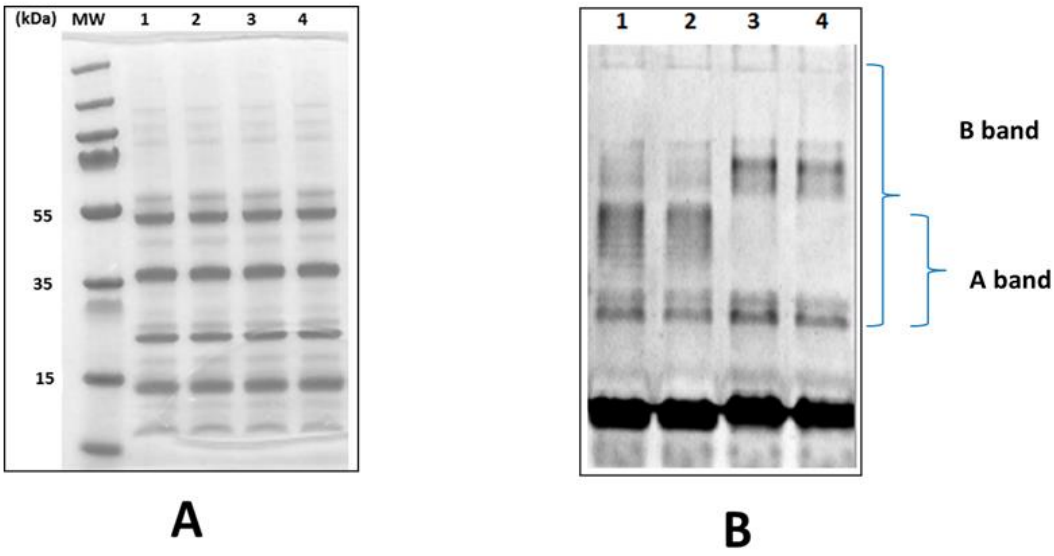


Figure 18. OM (A) and LPS (B) preparations from strains with or without PAPI-1 island. OM preparations were stained with Coomassie 1%. LPS samples were visualized by silver staining. Well 1, PAO1+; Well 2, PAO1-; Well 3, PA14+; Well 4, PA14-.

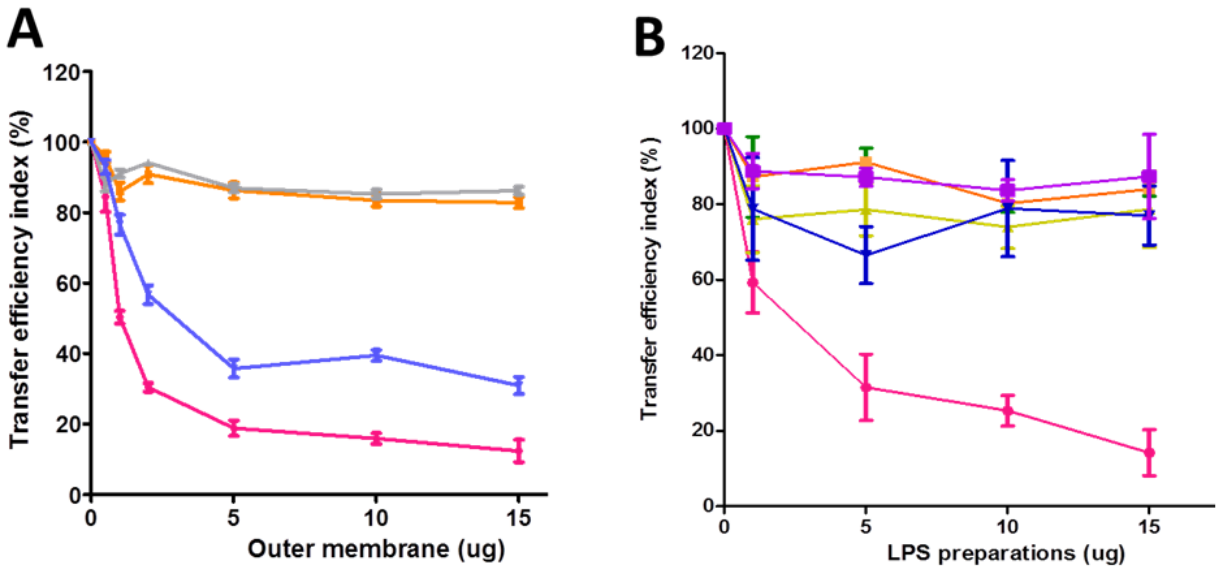


Figure 19. Effect on PAPI-1 transfer of the addition of OM (A) and LPS (B) preparations derived from strains with (+) or without (-) PAPI-1 island. Pink, PAO1-; orange, PAO1+; gray, PA14+; blue, PA14-. Purple data point on (B) are LPS from *Salmonella enterica* used as negative control.

Table 9. Statistical analysis for the effects of OM preparations from the strains with and without PAPI-1. The significant differences were analyzed by two-way ANOVA for two factors (type and amount of OM).

PAO1- vs PAO1+				
Amount of OM	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
0.5	11.27	6.377	P<0.001	***
1	35.65	20.17	P<0.001	***
2	60.47	34.22	P<0.001	***
5	67.47	38.18	P<0.001	***
10	67.54	38.22	P<0.001	***
15	70.47	39.88	P<0.001	***
PA14- vs PA14+				
Amount of OM	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
0.5	4.863	3.353	P < 0.05	*
1	-14.5	9.995	P<0.001	***
2	-37.4	25.79	P<0.001	***
5	-51.22	35.31	P<0.001	***
10	-45.74	31.54	P<0.001	***
15	-55.29	38.12	P<0.001	***

Table 10. Statistical analysis for the effects of LPS preparations from the strains with and without PAPI-1. The significant differences were analyzed by two-way ANOVA for two factors (type and amount of LPS).

PAO1- vs PAO1+				
Amount of LPS	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
1	27.93	5.937	P<0.001	***
5	59.62	12.67	P<0.001	***
10	54.93	11.68	P<0.001	***
15	69.82	14.84	P<0.001	***
PA14- vs PA14+				
Amount of LPS	Difference	t	P value	Summary
0	0	0	P > 0.05	ns
1	2.71	0.3892	P > 0.05	ns
5	-12.11	1.739	P > 0.05	ns
10	4.92	0.7066	P > 0.05	ns
15	-1.65	0.237	P > 0.05	ns

Table 11. Analysis of half maximal inhibitory concentration (IC_{50}) for the addition of OM and LPS from the mutants without PAPI-1 into the PAPI-1 transfer assay. IC50 was calculated by an equation "log(inhibitor) vs. normalized response -- Variable slope" with software GraphPad Prism 5.

Additives	**OM**		**LPS**
	PA01 -	**PA14 -**	**PAO1-**
Best-fit values			
LogIC50	-0.01205	0.1622	0.0906
HillSlope	-1.897	-2.565	-1.119
IC50	0.9726	1.453	1.232
P value	P<0.0001		
Preferred model	LogIC50 DIFFERENT for each data set		

4.6. Reduction of A-band LPS production plays a role in the surface exclusion mechanism of PAPI-1

We wanted to better understand the putative mechanism that *P. aeruginosa* uses to exclude additional copies of PAPI-1. We therefore asked if the strains containing PAPI-1 could modify their LPS structure, which would result in the reduction of LPS binding capacity to the donor's pilus. With silver staining of LPS, no differences between the same strain with or without PAPI-1 could be observed (Figure 20A). Then, Western blotting using a combination of antibodies (MF15-4 [139], [140]; N1F10 [139], [141]; 5c7-4 [139], [142]; 5c101 [139], [142] recognizing different parts of LPS from PAO1+ and PAO1- was carried out. This experiment could not be performed for PA14 strains because the corresponding antibodies are not available.

We observed that there were no differences between different parts of LPS, with the notable exception of the A-band receptor, since PAO1+ seemed to produce significant less amount of A-band LPS compared to PAO1- (Figure 20B). An possible explanation for this is that after acquiring PAPI-1 island, PAO1 recipients represses the expression of genes involved in the biosynthesis of A-band LPS, leading to a reduced ability to bind the conjugative pilus and thus to act as a recipient for PAPI-1.

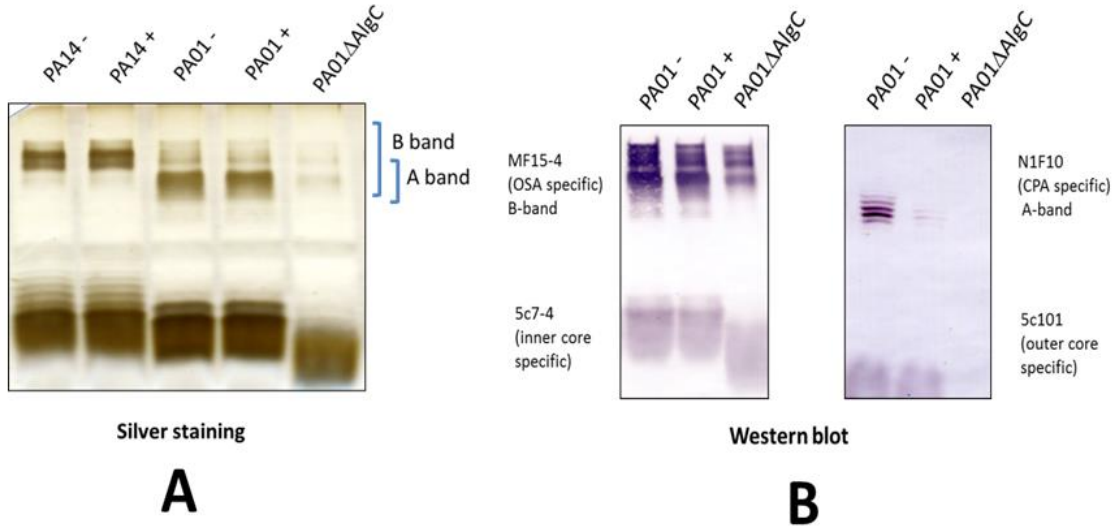


Figure 20. Analysis of LPS preparations from PAO1 and PA14 strains with (+) or without (-) PAPI-1. (A) LPS silver staining. (B) Western_blot with a combination of antibodies specifically recognizing A-band (N1F10), B-band (MF15-4), outer core (5c101) and inner core (5c7-4).

4.7. Expression and purification of pilV2' pilin

PilV2' is a small pillin protein constituting the type IVb pilus [93] showing significant similarity to adhesions PilVB and PilVA' of plasmid R64, with 40% of identity (data not shown). C-terminal variable segments of R64 pilV adhesins were previously shown to interact with LPS of recipient in vitro [44]. Therefore, we engineered a glutathione transferase (GST) fusion protein carrying at its C-terminus a 97 amino acid C-terminal segment of PilV2', so-called GST-pilV2' fusion, containing analogue constructs of R64 PilV adhesin.

Two *E.coli BL21* colonies containing pGEX with GST- pilV2' construct were tested the expression of the target protein. As shown in Figure 21, a band appears at the expected size of 36 kDa for GST-pilV2' with increased concentrations after induction. This suggests the target protein was well expressed in the studied condition. Furthermore, the small-scale purification process was also established for GST-pilV2'. The result showed that GST-pilV2' protein was mostly expressed in the insoluble fraction (lysis pellet), while there was very low concentration of the target protein recovered in the soluble phase (Figure 22). N-laurylsarkosine (sarkosyl) and Triton X-100 was added to lysis buffer to aid in solubilization of the fusion protein. It was observed that GST-pilV2' protein was well solubilized with this protocol (Figure 23).

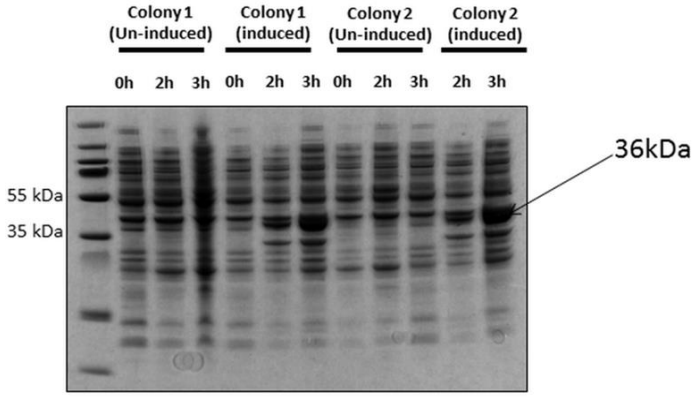


Figure 21. Expression of GST-pilV2' fusion protein in two tested colonies. Samples were taken at 0h, 2h, 3h of induction of IPTG 0.1mM at 37 ^{o}C and 200 rpm.

Figure 22. Western-blotting on different purification steps for GST-pilV2' fusion protein using anti-GST antibody. Well 1: 0h of induction (as a negative control); Well 2-5: samples from the culture of colony 1 (3h induction; insoluble fraction; soluble fraction; purifed fraction after reduced from GST resin binding respectively). Similarly, well 6-9: samples from the culture of colony 2 with the same order.

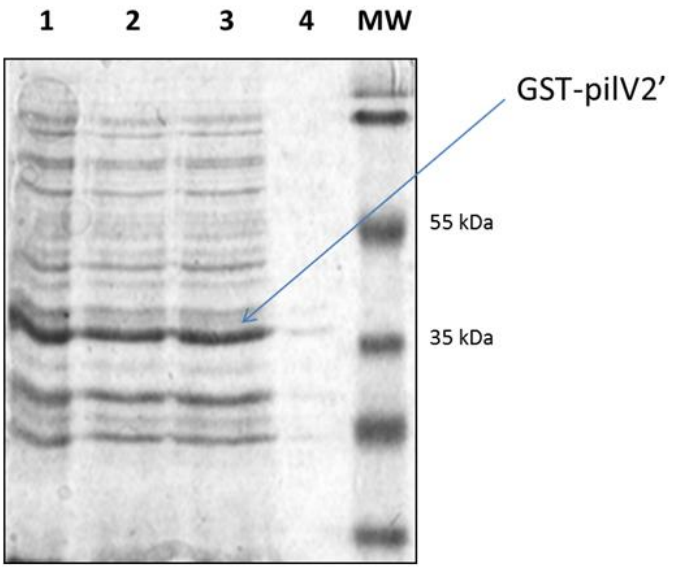


Figure 23. Solubilization of GST-pilV2' fusion protein with Sarkosyl. Well 1: 3h induction; well 2: Crude lysate; Well 3: Soluble fraction with Sarkosyl; Well 4: Insoluble fraction.

4.8. LPS derived from PAO1 strain carrying PAPI-1 strongly reduces the binding capacity to conjugative pilus

To confirm that PAO1+ strain lost the recognition of donor's conjugative pilus, we over-expressed and purified a GST-pilV2' fusion protein and performed enzyme-linked immunosorbent assay (ELISA) to compare the in vitro binding capacity of pilV2' to LPS derived from PAO1+ and PAO1- strains.

The purified GST-PilV2' fusion was then tested for binding to various LPSs. As shown in Figure 24, the binding capacity of GST-PilV2' to LPS from PAO1+ was significantly decreased, compared to LPS from PAO1-, supporting the idea that the

acquisition of PAPI-1 results in the reduction of A-band LPS of PAO1, which in turn causes the loss of interaction with the pilus from donor strains.

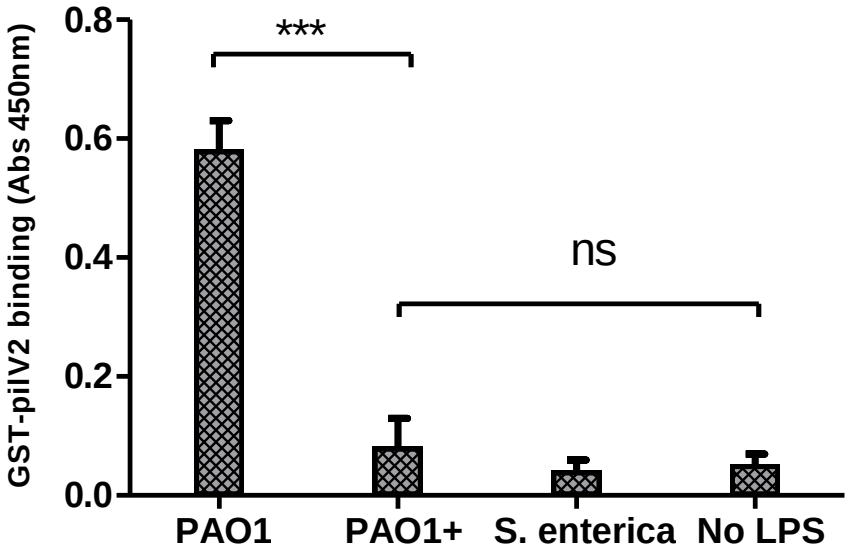


Figure 24. Binding of GST-pilV2' to various LPS preparations in an enzyme-linked immunosorbent *assay*. LPS from *Salmonella enterica* and sample without LPS used as negative control. LPS derived from PAO1 carrying PAPI-1 showed the loss of binding capacity to GST-pilV2'. Results were presented as mean ± SD for three independent experiments. Statistical significance was assessed by One-way ANOVA (*** $p<0.001$; ns: no significance, $P>0.05$).

4.9. Disruption of RLO68 and parE in PA14 partially restores transfer efficiency

In plasmid transfer, bacteria have been known that they may perform different barriers to exclude extra copies of the conjugative elements. Considering that PAPI-1 lacks any identifiable homologues of genes involved in LPS biosynthesis and modification (data not shown), the factors associated with the reduced amount of A-band LPS are more likely to be located in the core genome of *P. aeruginosa*. However, some other factors controlling LPS biosynthesis or responsible for other exclusion mechanisms may still be present in PAPI-1. To test this hypothesis, we screened a series of PA14 mutants for PAPI-1 genes for their eventual ability to restore the acquisition of PAPI-1. Two of ninety-five PA14 mutants for PAPI-1 genes, PA14_59490 (RL068) and PA14_60050 (parE), were shown possibility to receive additional copies of PAPI-1 after mated to PA14ΔTnC2::Tc^R (Figure 25). Compared to the control, RL068 and parE mutants were able to restore 16.6% and 55% transfer efficiency. The result will be further discussed in the discussion section.

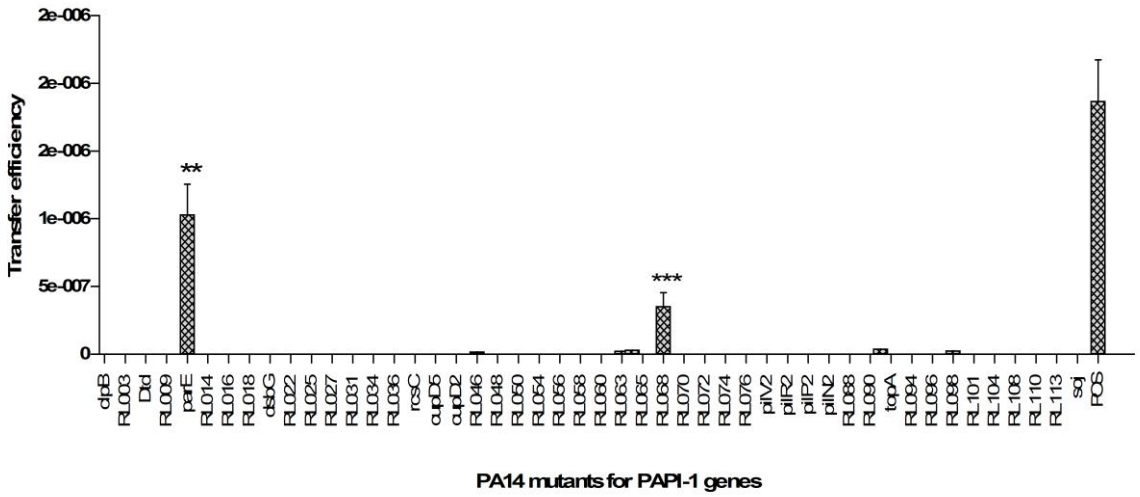


Figure 25. PAPI-1 transfer efficiency using PA14 mutants for PAPI-1 genes as recipient strains. Disruption of RL068 and parE genes partially restores 16.6% and 55% of transfer efficiency of PAPI-1 compared to the positive control, respectively. Results are shown as mean ± SD for three independent experiments. Statistical significance was analyzed by the unpaired t-test (A) (** $p<0.01$, and *** $p<0.001$).

DISCUSSION

5. DISCUSSION

Conjugation is a complex process which plays an important role in bacterial evolution and adaptation. Conjugation is usually started by the contact between donors and recipients, which is diverse among species. In Gram-negative bacteria, complex transfer systems are mostly encoded by large self-transmissible plasmids by using a type IV secretion apparatus to produce a pilus, so-called the mating-pair apparatus. This structure generates a junction between the mating bacterial cells, forming a pore through which the plasmid DNA and some donor-encoded proteins can be transported to the recipient [143]. In particular, for some plasmid groups, the pilus has also been shown to determine the specificity of the interaction with the recipient cell surface, in particular with LPS and OM proteins [43] [101]. Interestingly, pili encoded by IncP and Ti plasmid groups are able to generate junctions with a range of cell types, not only Gram-negative bacteria but also Gram-positive bacteria, yeast, plant and animal cells [45] [46]. The general process seems to be the same but the transfer can be dramatically affected by the identity of the donor [144] [47]. The cell-to-cell contact modalities studied in Gram positive bacteria are more diverse than those of Gram-negative bacteria [41]. The transfer apparatuses encoded by plasmids in the Gram-positive enterococci is only switched on in response to an appropriate recipient through production of pheromones, usually hydrophobic peptides [33, 34]. The activation results in the production of a membrane protein that promotes aggregation of the donors and recipients [35]. Another case of pJI101 plasmid in *Streptomyces* seems to rely on

natural bridges between host hyphae, using a single plasmid-encoded transfer protein to implement the movement from donor to recipient [145] [146].

Little is known about the conjugative systems in the ICEs. However, bioinformatic analyses suggest that in Gram-negative bacteria, ICE DNA can mostly be transferred through systems similar to type IV secretion systems (T4SSs) [40]. This system consists of a membrane-spanning secretion channel and often includes an extracellular pilus [147]. Whereas nearly all ICEs discovered in Gram-negative bacteria possess at least one gene homologous to T4SS [40], several ICEs encode almost entire transfer systems that are similar to plasmid-encoded T4SSs. The proteins mediating conjugation of *Vibrio cholerae* ICE SXT, have 34–78% identity with those in IncA–IncC plasmids [148, 149]. The PAPI-1 conjugation system of *Pseudomonas aeruginosa* also showed a close relationship to plasmid transfer apparatus. Homologs of nine PAPI-1 proteins encoding for structural and assembly components of a thin conjugative pilus were found in plasmid R64, except PilD peptidase, responsible for the cleavage of the PilS2 leader peptide, located in the chromosome [93].

It is known that PAPI-1 can be mobilized from the donor to a recipient lacking this island through conjugation. A number of plasmid-based conjugative transfer systems, exemplified by the F pilus encoded by the F sex factor of *E. coli*, are required for the initiation of cell-to-cell contact during conjugation and formation of mating pairs. Since PAPI-1 conjugation system shares similarity to other conjugative plasmids, Herein, we demonstrated that the contact between donor and recipient was also established when PAPI-1 is transferred. Our screening on

the 32 PAO1 mutants for LPS biosynthesis showed that eleven of them significantly reduced the transfer efficiency. Among them, only the PA5001 mutant is not clear about its function in LPS biosynthesis, though it was predicted to be involved in the core oligosaccharide biosynthesis [106]. The genes *algC, wbpZ, wbpY, wbpX, wzt, wbpM, gmd, rmd* encode enzymes participating in common polysaccharide antigen biosynthesis, while the genes *PA5455, PA5456, PA5459* potentially play a role in this pathway [106]. They have been computationally predicted to be in 2 operons, except *algC* which belong to another locus. Our data showed that the mutants for those genes significantly decrease the transfer efficiency of PAPI-1, compared to control. Noteworthy, the rmd mutant showed a slightly higher efficiency compared to negative control which can be explained by the cross effect of gmd gene. RMD enzyme (encoded by rmd gene) catalyses the final step in the GDP-D-Rha biosynthesis pathway. The disruption of the rmd gene on *P. aeruginosa* chromosome impairs A-band synthesis [133], while in vitro gmd is partially capable of catalyzing the same reaction as RMD enzyme [134]. Therefore, it could somehow contribute to the A-band biosynthesis in the *rmd* mutant which can help the recipient to receive PAPI-1. Moreover, we also observed that the transfer efficiency of the *wbpW* mutant was comparable to the positive control. This is not surprising since it has been recently found that *PslB* gene is able to substitute wbpW gene to promote A-band LPS production in a *wbpW* mutant [150] despite of a low level of this structure [133]. Compared to *algC* mutant, the transfer efficiency of these mutants was a bit higher. As mentioned earlier, algC gene is required for the synthesis of a complete LPS core and A-band

LPS. Therefore, a complete LPS core would also contribute to the low transfer efficiency of algC mutant. However, other mutants such as *waaC, waaF and waaP*, which are involved in core LPS synthesis, did not show a significant decrease in PAPI-1 transfer. This would suggest that the A-band is the main LPS structure driving the contact and interaction between donor and recipient in *P. aeruginosa*. Moreover, the competition assays with the addition of outer membrane and LPS preparations have provided a strong evidence that A-band LPS is involved in the recognition of recipient cells in the mating mixture since only LPS preparations derived from wbpM and wzx mutants producing only A-band could significantly inhibit the transfer of PAPI-1.

Interestingly, LPS structure has also been found as a receptor for conjugative transfer and bacteriophages [101] [151]. A detailed study on plasmid R64, the most related conjugative system to PAPI-1, showed that LPS core structure, such as GlcNAc(α1-2)Glc or Glc(α1-2)Gal structures, plays an important role in establishing contact between donor and recipient by specifically binding to PilVB′ and PilVC′ adhesins in a liquid medium, respectively [101]. In particular, D-rhamnose common lipopolysaccharide antigen was also characterized as a receptor for A7 bacteriophage. The molecule is hydrolyzed by rhamnanase enzymes contained in the phages particles to expose core-lipid A containing only two or three rhamnose repeats [152]. This would suggest that the PAPI-1 reception might be inherited from bacteriophage transduction in the course of evolution. Our findings support the idea that the transfer of PAPI-1 is derived from the plasmid

conjugation mechanism in which the conjugative pilus interacts with components of the recipient membrane in order to initiate the transfer.

The second main argument in this thesis deals with the behavior of recipients after acquisition of PAPI-1 island. ICE acquisition has been shown many benefits for the host cell such as the devlopment of resistance to antimicrobial [149],acquisition of virulence factors [153] [154], or establishment of symbiotic association with plants [155]. However, transfer of *ICEclcB13* has been shown to result in a strong fitness cost to the host since it was observed that transfer-competent cells are sacrificed after the transfer of ICEs. This could be a consequence of specific high energy demand to produce the conjugative system, or excessive oxidative damage; the transconjugants might profit from released nutrients from lysed cells [156]. Therefore, it is likely that bacteria probably gain profits when they acquire one or few ICEs and avoid many others to reduce the fitness costs. In our study, we aimed at better understanding how *P. aeruginosa* behaves after acquisition of PAPI-1. We demonstrated that after receiving the PAPI-1 island, the recipients can act as stable donors to transfer the island to other recipient cells. This ability is well-known for conjugative plasmid transfer (Griffith, 1999) and was recently also observed in ICEBs1 from *Bacillus subtilis*. However, it has not been reported for gram-negative bacteria, in particular *P. aeruginosa*. In this work, we demonstrated that the recipient was able to act as a donor for PAPI-1 after it acquired it, with a transfer efficiency comparable to that of PA14 strain with original PAPI-1. It still remains unclear how much time or generations the recipient needs to act as a

donor in *P. aeruginosa*, though in the *B. subtilis* cell chains, the recipient can rapidly behave as a donor and spread ICEBs1 to the neighboring cells. Remarkably, PAPI-1 acquisition was stable after several generations and in fact during evolution PAPI-1 is partially or entirely retained in some clinical isolates [89]. This would suggest that the acquisition of PAPI-1 would probably benefit to the host though it is not well understood in which manner. However, since the size of PAPI-1 is pretty large, about 100 kb, it is likely to cause inconvenience to the cells due to metabolic cost and genome expansion. Similarly to conjugative plasmid, the recipients may respond to a number of incoming ICEs.

Herein, we demonstrated that the recipient which already acquired PAPI-1 island were able to receive more copies though transfer efficiency was significantly decreased. In spite we have not defined how many copies of PAPI-1 the recipient might carry and how long it might maintain it, the occurrence of multiple copies of PAPI-1 at the attB site as a tandem array was previously reported [92]. The pathogenicity islands in *P.aeruginosa*, PAPI-1 and PAPI-2, are known to be inserted and excised at the specific att sites located in the two $tRNA^{Lys}$ genes, which were identified as “hot spots” for insertion and excision of large genetic elements in several *P. aeruginosa* strains [92]. For instance, the large plasmid pKLK106 in *P. aeruginosa* clone K was able to recombine sequentially with either of the two $tRNA^{Lys}$ genes PA4541.1 and PA0976.1 to rearrange the genomes of sequential K isolates from the airway of a CF patient [157]. In *P. aeruginosa* clone C, the plasmid pKLC102 reversibly integrated only into PA4541.1 but not into PA0976.1, which was occupied by a 23-kb PAGI-4 island [94]. In *Vibrio cholerae*, it

was reported that tandem arrays of SXT and R391 elements occurred after their transfer, and this arrangement was observed to be stably maintained for many generations [58]. These suggested that the *attB* site in a recipient's genome can be used as a platform to build composite GIs by sequentially acquired independent genetic elements to form a superintegron [158]. Finally, the integration of PAPI-1 into the site for PAPI-2 means that this *attB* site is conserved and remains intact at the borders of the composite element. This feature could be used for acquiring multiple ICEs in *P. aeruginosa*. Harboring at least two ''hot spots'' for integration of genetic elements, this bacterium is likely to employ an exclusion system to avoid the expansion of its genome and metabolism. This activity has been well documented for conjugative plasmids, but there is not much evidence of this for ICEs. After acquiring a genetic element, the bacteria can modify their cell surfaces or express specific factors to ignore or cleave incoming elements, which are classified in different barrier levels. The bacteria might possess and activate one or some of them to maintain a stable state, and this would affect the efficiency of the transfer after acquiring these elements. In this study, the exclusion activity of PA14 and PAO1 with PAPI-1 were successfully addressed. Exclusion Index (EI) was calculated as the transfer efficiency of PAPI-1 to the recipient lacking this element divided by that to a recipient already carrying the same element [56]. The EI for mating between PA14 donor and PA14+ recipient was 12, while the EI for mating between PAO1+ donor and PAO1+ recipient was about 298, indicating that the exclusion activity of PAO1 is stronger than PA14. The observed EI were comparable with those of the SXT family of ICEs [66] but about two times lower

than that of virulence plasmid pVAPA1037 [159], six times lower than that of plasmid R27-mediated entry exclusion [160] and twenty times lower than exclusion mediated by highly promiscuous plasmid RP4, where the EI ranged from 10^3 to 10^4 [52]. This suggested that the exclusion activity for ICEs are less stringent than that for conjugative plasmids. In this study, we provided strong evidence that the recipient PAO1+ reduced transfer efficiency by three order of magnitudes compared to PAO1-. This implies that the PAO1+ can activate a system for excluding the acquisition of additional copies of PAPI-1. The exclusion activity is well documented for plasmid transfer; but not for ICEs systems, though the underlying mechanism seems to be very diverse among bacteria and ICE types. Some mechanisms for inhibiting redundant ICE transfer have been reported. For example, in the SXT/R391 ICE family, the entry exclusion is mediated by the interaction between the Eex inner membrane protein with the TraG protein, a component of the mating pore [161, 162]. For the pSAM2 ICE, when it is present in the recipient cells, Pif (pSAM2 immunity factor) is expressed to abolish the additional transfer from the donor; in consequence, the transfer rate was 2000 decreased times [59]. A third example deals with an ImmR repressor encoded by a recipient bacterium to reduce the integration efficiency of an incoming ICEBs1 by 1000-fold [163]. However, some other ICEs do not prevent redundant conjugative transfer. The presence of Tn916 ICE family in a recipient cell showed a low integration specificity, but does not impede transfer of a related element [164]. These mechanisms have been grouped as an entry exclusion. In this study, we demonstrated that the presence of PAPI-1 in the recipient may detract the

interaction between the donor pilus and recipient's receptor by inhibiting A-band LPS biosynthesis. This result was also confirmed by the demonstration of the loss of *in vitro* binding capacity of the pilus tip PilV2' and LPS preparations and *in vivo* transfer inhibition assays. Noticeably, both LPS preparations from PA14 and PA14- did not show any altering effects on transfer efficiency, whereas OM extract of PA14– partially inhibited conjugation. This would suggest that A-band LPS of PA14 may not be involved in PAPI-1 exclusion activity. Not surprisingly, the PA14 strain which originally carries PAPI-1 was recently found lacking A-band LPS structure [165]. In addition, PA14 and PAO1 genomes (6.5 and 6.3 Mb respectively) are remarkably similar, except for differences on their genomic islands [166]. One might speculate that during the bacterial evolution, PA14 could have evolved from PAO1 after acquiring PAPI-1 from other strains. The different behaviors of PA14 and PAO1 strains suggested that more than one exclusion mechanisms could be activated in response to the presence of PAPI-1. As mentioned earlier, PAPI-1 island belongs to proteobacterial ICEs, such as pKLC102 and ICE*Hin1056*, which were described as plasmids and can be present as multiple circular copies per cell [157] [167]. Recently, the maintenance of this ICE family was also found to be ensured by theta-replication, similar to Tn*GBS1* and Tn*GBS2* from the firmicute *S. agalactiae* [92, 168, 169]. Therefore, once additional PAPI-1 copies succeed to enter into the recipient cell, they may activate exclusion mechanisms to avoid the extensive integration of the element. Restriction enzymes [170] or clustered regularly interspaced short palindromic

repeats (CRISPR) systems [171, 172] are common used by the recipient to recognize and cleave incoming ICEs.

The deletion of two PAPI-1 genes (RL068 and parE) was found here to partially restore PAPI-1 transfer efficiency. According to the *Pseudomonas* database, RL068 is a short hypothetical protein, a 91-aa peptide, which does not show any conserved domains [128]. Whereas, the parE is a 13.2 kDa protein, belonging to the plasmid stabilization protein family. Its sequence shows about 30%-45% identity with parE protein encoded in broad-host-range plasmid RK2, and with plasmid stabilization protein in *Agrobacterium tumefaciens*, respectively (data not shown). It is also highly conserved among *Pseudomonas* species. In plasmid RK2 system, parE was identified as toxic for cell growth by inactivating DNA gyrase; however, this activity is prevented by anti-toxin parD protein by forming a complex with the parE toxin protein to protect the bacterial cell [173]. This finding supports the hypothesis that the presence of PAPI-1 might activate the parE gene to avoid its replication and integration into the bacteria chromosome.

In summary, these results suggest that the *P. aeruginosa* would activate a complex exclusion system after PAPI-1 is acquired. It would include both surface exclusion by reducing the amount of A-band LPS and entry exclusion by inhibiting the replication and integration of PAPI-1. However, there was no factors encoded in PAPI-1 found to be responsible for the reduction of A-band LPS production. It remains interesting to study about any factors in the core genome which are involved in this exclusion activity.

CONCLUSION AND PERSPECTIVES

6. CONCLUSION AND PERSPECTIVES

In this thesis work, we demonstrated for the first time that the horizontal transfer of PAPI-1 island is mediated by the interaction between conjugative type IVb pilus of the donor cell and A-band LPS on the recipient cell membrane. PAO1 mutants not producing A-band LPS showed a significantly decreased efficiency in acquiring the island. This statement was confirmed by experiments investigating the effects of the addition of outer membrane and LPS preparations from various strains in a competitive transfer assay. In the second part of the project, we provided evidence for an exclusion mechanism allowing *P. aeruginosa* to restrict the acquisition and integration of additional copies of PAPI-1. Our data suggests that even among *P. aeruginosa* strains PAO1 and PA14 different barriers for PAPI-1 reception may occur. We showed that the modified recipient strain PAO1 carrying PAPI-1 reduces the production of A-band LPS as a probable surface exclusion mechanism, by decreasing the binding capacity to the conjugative pilus tip (pilin protein pilV2). On the other hand, we showed that strain PA14, that naturally carries PAPI-1 island in its genome, tends to exclude the acquisition of extra copies. The putative role of PA14 LPS in this exclusion mechanism has still to be demonstrated. We demonstrated that the disruption of two genes located in PAPI-1 partially restore PAPI-1 transfer efficiency although their function has not been experimentally demonstrated to date. While PA14_59490 gene has not been assigned any function, PA14_60050 encoded for parE functionally predicted as a DNA stabilization protein, which may play a role in inhibiting processing and integration of further copies of the island.

As perspectives, it can be noted that the detailed molecular basis of PAPI-1 exclusion mechanism has still to be elucidated. A transcriptome analysis of strains with or without PAPI-1 should provide important information about regulatory aspects of this process. Moreover, a genome-wide screening for factors involving in LPS biosynthesis would also help a better understanding on how *P. aeruginosa* controls the transfer and exclusion of PAPI-1. Taken altogether, such approaches should assist to get a more complete insight on the horizontal acquisition and exclusion of genomic islands, which may result in future development of new strategies to limit the spread of virulence or resistance functions in *P. aeruginosa* populations, and potentially in other pathogenic bacteria.

7. CONTRIBUTIONS

***Contributions on the topic of the doctoral thesis**:* My PhD project was focused on the PAPI-1 acquisition mechanism in *Pseudomonas aeruginosa*. The first aim was to identify the specific receptor of the recipient which can be recognized by the conjugative pilus of the donor. The second aim was to study the exclusion mechanisms can be activated when the recipient already acquired PAPI-1 island. This project was carried out in collaboration with Prof. Stephen Lory (Department of Microbiology and Immunobiology, Harvard Medical School, USA). Moreover, the Figure 20 was done with a strongly support from Prof. Joseph Lam and Dr. Youai Hao (University of Guelph, Canada).

Publications

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9. APPENDICES

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Title: Conjugative type IVb pilus recognizes lipopolysaccharide of recipient cells to initiate PAPI-1 pathogenicity island transfer in Pseudomonas aeruginosa

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Running Head: Horizontal transfer of PAPI-1 in Pseudomonas aeruginosa

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ABSTRACT

Pseudomonas aeruginosa pathogenicity island 1 (PAPI-1) is one of the largest genomic islands of this important opportunistic human pathogen. Previous studies have shown that PAPI-1 encodes several putative virulence factors, a major regulator of biofilm formation, antibiotic-resistance traits, and that it is horizontally transferable into recipient strains lacking this island. PAPI-1 island is transferred by conjugation mediated by specialized type IV pili encoded by a cluster of ten genes located in PAPI-1. However, the PAPI-1 acquisition mechanism is currently not well understood. In this study, we performed a series of conjugation experiments and identified determinants of PAPI-1 acquisition by analyzing transfer efficiency between the donor and a series of mutant recipient strains. Our data shows that A-band lipopolysaccharide (LPS) is required to initiate PAPI-1 transfer, supporting the idea that this structure acts as a receptor for conjugative type IV pilus in recipient strains. These results were verified by PAPI-1 transfer assay experiments supplemented with outer membrane or LPS preparations, and by the binding of pilin fusion protein GST-pilV2' to immobilized LPS molecules in vitro. We also demonstrated that *P. aeruginosa* strains that already acquired a copy of PAPI-1 almost completely loss the ability to import additional copies of the island, and that such strains produced much less A-band LPS compared to the strains lacking PAPI-1. This may specify a PAPI-1 exclusion mechanism in *P. aeruginosa* to avoid uncontrolled expansion of the bacterial genome.

IMPORTANCE

Horizontal gene transfer (HGT) represents a major evolutionary mechanism for the acquisition of new phenotypes by microorganisms. HGT allows rapid evolution on a large scale, since hundreds of new genes can be acquired during a single genetic exchange event. HGT plays a particularly important role in the evolution of virulence and antibiotic resistance as it allows acquisition of genes that can alter the pathogenic potential of a bacterial strain.

The significance of this work is in our ability to experimentally test the molecular mechanism of acquisition of genomic islands by HGT. This is not possible in the majority of cases as the mobility of these elements is frequently lost because of evolutionary decay. New insights about PAPI-1 mobility and its dissemination by HGT could be applicable to other systems where experimental validation of transmission models is not possible. To our knowledge, this mechanism has never been investigated in *P. aeruginosa*.

INTRODUCTION

Horizontal gene transfer (HGT) mediated by microorganisms is a major evolutionary mechanism for the acquisition of new functionalities. HGT allows rapid and drastic changes in bacterial genomes, since many, even hundreds of new genes can be acquired during a single genetic exchange event, and is recognized to play an important role in the evolution of virulence, antibiotic resistance and adaptation to the new environments (1), (2). The acquisition of virulence genes may radically alter the disease-causing potential of a microorganism. In some instances, acquisition of a single gene or a small cluster of genes encoding critical virulence determinants may be the only genetic difference between an avirulent and virulent strain of the same species (3), (4). Virulence genes are often part of large blocks of DNA referred to as genomic islands (GIs). GIs are accessory genomic segments present only in certain bacterial strains; they are often flanked by direct repeats and inserted in the vicinity of tRNA genes. Reversible excision and integration further implicate their potential for inter-bacterial transfer (5) Those genomic islands that lead to an enhancement of fitness in a host organism are called pathogenicity islands (6). Conjugative and integrative elements (ICEs), or conjugative transposons, are well-characterized genomic islands that in many cases have retained mobility (7, 8). In contrast, a number of genomic islands appear to be ancient ICEs that became fixed in the bacterial chromosome due to degeneration of their conjugative elements by deletion of integration sites or mutations in genes encoding transfer functions (9). The best-characterized ICEs to date contain specific features associated with conjugative plasmids and

bacteriophages; can be transferred horizontally following recognition of the recipient cell by the donor utilizing a conjugative mechanism that, in many instances, is associated to the type IV protein secretion pathway (10). The recipient cell is recognized by the pilus structure that is part of the type IV secretion apparatus of the donor (11).

Pseudomonas aeruginosa has a broad environmental distribution that is reflected by its large genomic repertoire. Indeed, the genome sequences of *P. aeruginosa* strains available to date show that a large core genome of about 5000 conserved genes is supplemented with an accessory gene pool of 1000-1500 additional genes, most of them being arranged in a limited number of genomic islands (12). PAPI-1 is one of the largest island characterized in *P. aeruginosa* PA14 (13) a highly virulent strain which can infect a broad range of plants, insects, and animals. It is integrated at the attB site, located in tRNA-lys genes (14) and consists of a cluster of 108 genes that encode a number of virulence determinants, whose disruption resulted in the attenuation of the virulence phenotype in several infection models (13). In addition, PAPI-1 carries several regulatory genes, such as the PvrSR/RcsCB two components system, which controls biofilm formation and dispersal in *P. aeruginosa* strains causing chronic infections in individuals with cystic fibrosis [15]. PAPI-1 island is naturally presented in wild-type PA14 strain whereas it is missing in PAO1, but it can easily be transferred from PA14 to PAO1. PAPI-1 transfer has previously been described as a conjugation process mediated by type IVb pilus in co-culture experiments with donor and recipient cells (14, 15). Type IVb pilus is encoded by a 10-gene cluster in PAPI-1 (15) and is closely

related to the genes found in the enterobacterial plasmid R64. Previous studies on conjugal plasmid R64 suggested that the thin pilus PilV adhesins, formed by a recombinant mechanism between various cassettes, a shufflon (16) recognize a specific structure of the lipopolysaccharide molecules of recipient cells, therefore determining the transfer specificity of the plasmid R64 (17).

The aim of this study was to investigate the mechanism of acquisition of PAPI-1 island in *P. aeruginosa*. We performed a series of conjugation experiments on wild-type or mutant donor and recipient strains, and analyzed the determinants of PAPI-1 transfer efficiency. We demonstrated for the first time that the conjugative type IVb pilus of the donor can recognize A-band LPS on the recipient outer membrane, and that this structure is required to initiate the transfer of PAPI-1. Our data also indicates that *P. aeruginosa* strains containing PAPI-1 specify a mechanism to exclude additional copies of PAPI-1 by producing less A-band LPS.

RESULTS

PAO1 mutants for A-band LPS biosynthesis are deficient in the acquisition of PAPI-1

Since in plasmid conjugal transfer the donor pilus is known to recognize specific components of LPS on the recipient membrane (18), we decided to examine the impact on transfer efficiency by using various mutants for LPS biosynthesis as recipients in PAPI-1 transfer assay (Figure 1). The gene AlgC encodes for a phosphoglucomutase, which is required for the synthesis of a complete LPS structure (19). The mutant PAO1ΔalgC thus produces a truncated LPS core and is

devoid of O-antigen. When the transfer assay was carried out between the donor PA14ΔTnC2 and PAO1ΔalgC, the transfer efficiency was reduced by three orders of magnitude compared to that of wild-type PAO1 (Figure 1A). This suggests that the complete LPS structure plays an important role in PAPI-1 transfer. We therefore decided to screen a series of 32 PAO1 mutants for LPS biosynthesis (20), using them as recipients in the PAPI-1 transfer assay. The results showed that 10 mutants were deficient in PAPI-1 transfer (Figure 1B). Interestingly, these mutated genes are located in 2 operons encoding enzymes involved in D-Rhamnose biosynthesis pathway. This would suggest that the D-rhamnose homopolymer, also known as A-band polysaccharide, may act as a receptor for conjugative type IV pilus as an initial step of PAPI-1 transfer.

Addition of OM and A-band LPS preparations inhibits PAPI-1 transfer

We postulated that OM fractions or A-band LPS preparations, the putative receptor for conjugative pilus, can compete with recipient cells binding to the conjugative pilus and thus blocking the transfer of PAPI-1 to the recipient. We extracted OM and LPS from two PAO1 mutants producing only A-band LPS and two mutants producing only B-band LPS (Figure 2). These preparations were added to the standard PAPI-1 transfer assay at different concentrations. The increased addition of OM and LPS amount from mutants lacking B-band LPS (PAO1ΔwbpM and PAO1Δwzx) strongly inhibited transfer efficiency, while the addition of OM and LPS from mutants lacking A-band LPS (PAO1Δrmd and PAO1ΔalgC) did not significantly affect the efficiency of PAPI-1 transfer. The addition of OM and LPS from PAO1ΔwbpM and PAO1Δwzx strongly inhibited transfer even at low

concentrations (< 5 µg) and reduced transfer efficiency to 20%. These results strongly support the hypothesis that A-band LPS of the recipient strain acts as a specific receptor for the IVb pilus, and is required to initiate the transfer of PAPI-1.

Recipient strains carrying PAPI-1 show a strongly reduced ability to acquire additional copies of the island

We then asked if the *P. aeruginosa* strains already carrying PAPI-1 can acquire additional copies of this island. In this experiment, we carried out transfer assays using the donor PA14ΔTnC2 or PAO1Bla6TnC2 and the recipients with PAPI-1 (PA14ΔTnC2 or PAO1Bla6TnC2) or without PAPI-1 (PA14ΔsojR and PAO1). Herein, we indicate [+] or [-] for strains with or without PAPI-1, respectively. As shown in Figure 3, the transfer efficiency of PA14+ to PA14- is significant lower than the control PAO1-. This would suggest that the transfer efficiency of PAPI-1 may be also influenced by the identity of the recipient strain. Interestingly, the data shows that PAO1 strain carrying PAPI-1 can act as a donor of the island, transferring it to the control recipient (PAO1-) at an efficiency comparable to the PA14+ donor. These results also demonstrate the ability of recipient strains to acquire more than one copy of PAPI-1, even if this occurs at much lower efficiency compared to controls, with a decrease of one and three orders of magnitude and three orders of magnitude for PA14+ to PA14+ and PAO1+ to PAO1+ transfers, respectively. Our data thus indicates that *P. aeruginosa* which already acquired PAPI-1 strongly and significantly decreased their ability to receive additional copies of the island. This implies that *P. aeruginosa* strains carrying PAPI-1 specify a mechanism to exclude the acquisition of additional copies of the island.

The acquisition of PAPI-1 activates a surface exclusion mechanism

We hypothesized that after acquisition of PAPI-1, recipient *P. aeruginosa* strains modify their surface to avoid further contact and subsequent transfer from the donor cells. To infer if the presence of PAPI-1 in the cell genome can affect the structure of its OM and LPS, we performed the standard transfer assay with the addition of OM and LPS, prepared from strains with and without PAPI-1. As shown in Figure 4, the addition of OM preparations derived from strains with PAPI-1 does not impact on transfer efficiency, compared to the ones with OM of the strains without the island. The effect of LPS preparations (Figure 4B) is similar for PAO1 strain but not for PA14, since the addition of LPS from PA14- did not produce significant variation in transfer efficiency. This data suggests that *P. aeruginosa* specify mechanisms to exclude the acquisition of additional copies of PAPI-1 via OM and/or LPS modification. However, the mechanism seems to be different for PAO1 and PA14.

Reduction of A-band LPS production plays a role in the surface exclusion mechanism of PAPI-1

In order to better understand the putative mechanism utilized by *P. aeruginosa* to exclude additional copies of PAPI-1 we first codiered potential alterations in surface exclusion. We therefore asked whether the strains containing PAPI-1 could modify their LPS structure, which would result in the reduction of the donor's pilus to bind to its receptor. With silver staining of LPS fractionated by SDS-PAGE, no differences between the amount of this molecule in a given strain with or without

PAPI-1 could be observed (Figure 5A). We then subjected gels to Western blotting using a combination of antibodies: MF15-4 (21, 22); N1F10 (21, 23); 5c7-4 (21, 24); 5c101 (21, 24) recognizing different parts of LPS from PAO1+ and PAO1-. This could not be done for PA14 strains because the corresponding antibodies are not available. We observed that there were no differences between different part of LPS, with the notable exception of the A-band component, showing that PAO1+ produces significantly lower amount of A-band LPS compared to PAO1- (Figure 5B). An possible explanation for this is that after acquiring PAPI-1 island, PAO1 represses the expression of genes involved in the biosynthesis of A-band LPS, leading to a reduced ability to bind the conjugative pilus and thus to act as a recipient for PAPI-1 transfer. To confirm that PAO1+ strain lost the recognition of donor's conjugative pilus, we over-expressed and purified a GST-pilV2' fusion protein and performed enzyme-linked immunosorbent assay (ELISA) to compare the in vitro binding capacity of pilV2' to LPS derived from PAO1+ and PAO1- strains. PilV2' is a small pillin protein constituting the type IVb pilus (15) showing significant similarity to adhesins PilVB and PilVA' of plasmid R64, with 40 % of identity (data not shown). C-terminal variable segments of R64 pilV adhesins were previously shown to interact with LPS of recipient in vitro (17). Therefore, we engineered a glutathione transferase (GST) fusion protein carrying at its C-terminus a 97 amino acid C-terminal segment of PilV2, so-called GST-pilV2' fusion, analogous to the construct used to analyze the R64 pilin interactions with its receptor. This GST-PilV2' fusion was then tested for binding to various LPS preparations. As shown in Figure 6, the binding capacity of GST-PilV2' to LPS

from PAO1+ was significantly decreased compare to LPS from PAO1-, supporting the idea that the acquisition of PAPI-1 results in the modification of PAO1 LPS, which in turn causes the loss of interaction with the pilus from donor strains.

DISCUSSION

It is known that horizontal gene transfer plays an important role in driving the bacterial evolution and adaptation to various environments. Pseudomonas aeruginosa genome has a mosaic structure composed of a variable number of horizontally-acquired accessory regions containing up to hundreds of genes (12). The largest genomic island in Pseudomonas aeruginosa, PAPI-1, was previously shown to be transferable to recipients lacking it through direct cell-to-cell interaction and by a conjugation mechanism (15).

Here, we demonstrate, for the first time, that during conjugation and transfer of PAPI-1, the conjugative pilus of the donor recognizes a specific structure, A-band LPS, on the recipient membrane. The R64 plasmid transfer also requires the recognition by the pilus of specific parts of LPS core. Specifically, the GlcNAc(α1-2)Glc or Glc(α1-2)Gal structures, are recognized by PilVB′ and PilVC′ adhesins, respectively (18). Moreover, LPS molecules have been also found as receptors for many bacteriophages (25). In bacteriophage A7, D-rhamnose common lipopolysaccharide antigen was also characterized as a receptor which is hydrolyzed to expose core-lipid A containing only two or three rhamnose repeats by rhamnanase from the phages (26). Our data strongly suggests that the conjugative pilus interacts with A-band LPS on the surface of the recipients in

order to initiate the transfer of PAPI-1. We also demonstrated that following acquisition of the PAPI-1 island, the recipients can become stable donors for transfer of the island to other recipient cells. On the other hand, the recipients which already acquired PAPI-1 island specify an exclusion mechanism precluding the acquisition of additional copies. In conjugal plasmid transfer, a number of studies have shown that after acquired plasmids, the recipient bacteria prevent entry of additional plasmid copies (27). Exclusion index (EI), which was calculated as the transfer efficiency of PAPI-1 to the recipient lacking this element divided by that to a recipient already carrying the same element has been used to evaluate the exclusion activity (27). The EI for mating between PA14 donor and PA14+ recipient was 12, while the EI for mating between PAO1+ donor and PAO1+ recipient was about 298, indicating that the exclusion activity of PAO1 is stronger than PA14. The observed EI were comparable with those of the SXT family of ICEs (28) but about two times lower than virulence plasmid pVAPA1037 (29), six times lower than plasmid R27-mediated entry exclusion (30), and twenty times lower than exclusion mediated by highly promiscuous plasmid RP4, where the EI ranged from 103 to 104 (31).

At least two exclusion mechanisms for plasmid or ICEs acquisition are known. One is entry exclusion (Eex) mediated by inner membrane proteins. This mechanism is able to inhibit DNA entry after a stable mating pair has been established (28, 31, 32). Another mechanism occurs via surface exclusion, inhibiting formation of a stable mating pair. TraT, encoded by the F plasmid, is an outer membrane lipoprotein which can disturb the interaction between the pilus tip and OmpA

receptor in *E. coli* (32-34). In this study, we provide evidence for another surface-associated mechanism that Pseudomonas aeruginosa utilizes for excluding acquisition of multiple copies of the PAPI-1 island. Preliminary data supporting this mechanism is that the strains carrying PAPI-1 produce reduced amounts of the A-band LPS compared to strains lacking the island and this presumably adversely effects the efficiency of the contact between donor and recipient cells.

The molecular basis of PAPI-1 exclusion mechanism has still to be elucidated. Considering that PAPI-1 lacks any identifiable homologues of genes involved in LPS biosynthesis and modification (data not shown), the factors causing a reduction in the amount of A-band LPS may be conceivably located in the core genome of *P. aeruginosa*. PAPI-1 could therefore specify regulatory functions controlling the expression of the enzymes for LPS biosynthesis. We are currently screening a series of PA14 mutants for PAPI-1 genes for their eventual ability to restore the acquisition of PAPI-1. Our study provides new insights on the horizontal acquisition and exclusion of genomic islands which may lead in the future development of new strategies to limit the spread of virulence or resistance functions in *P. aeruginosa* populations.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions

All strains and plasmids used in this study are listed in supplementary table S1. *P. aeruginosa* strains and mutants were grown in Luria-Bertani (LB) broth (Sigma-Aldrich) supplemented with the appropriate antibiotics. For selection of *P. aeruginosa* mutants, the antibiotics used were gentamicin and tetracycline, both at a concentration of 75 µg/ml. For maintenance of plasmids in *Escherichia coli*, the medium was supplemented with ampicillin at 100 µg/ml and chloramphenicol at 34 µg/ml. Isopropyl-D-thiopyranoside (IPTG) was added at a final concentration of 0.5 mM to induce GST-pilV2' expression in pGEX-2T plasmid.

Construction of PA14ΔTnC2::TcR mutant

The deletion mutant was constructed by using gene replacement vectors as previously described (35). All primers used for generating the mutant PA14ΔTnC2::TcR are listed in supplementary table S3. Briefly, a cassette conferring Tet resistance flanked by two DNA fragments of about 500 bp flanking the PA14_59200 gene was cloned in the vector pJET1.2 before subcloning into the vector pEXG2. The recombinant plasmid was conjugated from E. coli λpir S17.1 into *P. aeruginosa* (36). The integrative plasmids were selected on LB plates supplemented with gentamicin, tetracycline or irgasan at 25 µg/ml. To resolve merodiploids a second selection round on LB agar with 6% sucrose was performed. Transformants were screened by colony PCR and confirmed by DNA sequencing.

Screening for PAO1 mutants deficient in PAPI-1 acquisition

A standard PAPI-1 transfer assay via liquid mating was carried out as previously described (15). Mutant PA14ΔTnC2 (GmR) was used as donor and a series of PAO1 mutants, with altered LPS biosynthesis, obtained from a PAO1 transposon mutant library were used as recipients (supplementary table S2). After overnight growth at 37 ^{o}C and 200 rpm, the donor cells were harvested at an OD 600 of 0.8 and were mixed with the recipient cells at an OD 600 of 0.4, spun down and resuspended in 1 ml of fresh LB without antibiotics. The mating mixture was incubated in 15-ml culture tubes, statically at 37 °C for 24 h. The mating mixture was diluted to appropriate dilutions and plated on LB agar plates containing gentamicin and tetracycline at 75 µg/ml to select transconjugants, and on LB agar plates containing tetracycline at 75 µg/ml to select recipients. The transfer efficiency was calculated as the ratio of transconjugants and recipients colonies in the mating mixture.

Outer membrane (OM) preparation

The outer membranes of *P. aeruginosa* were isolated by using sodium lauroylsarkosinate (sarkosyl) as previously described (37). Briefly, cultures of *P. aeruginosa* were grown overnight at 37 °C, 200 rpm in LB broth. The pre-inoculum was then diluted 100-fold in fresh LB medium and grown at 37 °C and 200 rpm to an OD 600 of 1.0. Cells were harvested and resuspended in 15 ml lysis buffer containing 20 mM Tris-Cl [pH 7.5], 100 mM NaCl, 1 mM EDTA, lysozyme [0.5 mg/ml] and a complete protease inhibitor cocktail (Roche). The lysate was

sonicated and spun down at 10,000 g, 10 min, 4 °C to remove cellular debris. The membrane fraction was isolated by ultracentrifugation (200,000 g at 4 °C for 60 min). The pellets containing inner and outer membranes were further fractionated at 100,000 g for 30 min after incubation with sarkosyl 0.2 %. Outer membranes were finally resuspended in Tris-Cl buffer 20 mM [pH 7.5] and separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with Coomassie blue. The concentration of OM was measured by Lowry method.

LPS preparation

LPS molecules from various *P. aeruginosa* strains and mutants were prepared by using the hot phenol-water extraction protocol from Westphal and Jann (1965) with minor modifications (38). Briefly, cell suspensions in 100 mM NaCl were first heated to 68 ^{o}C before adding an equal volume of hot phenol and stirring vigorously for 2 hours at 68 °C. LPS was then fractionated by centrifugation at 12,000 g for 15 min at 4 °C. LPS in the upper phase was recovered and dialyzed against water to remove residual phenol. LPS extract was further treated with DNase, RNase and proteinase K to eliminate contaminations. LPS extract was finally lyophilized before use. The LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (39). LPS was then quantified with KDO assay (40).

PAPI-1 transfer inhibition assay

OM and LPS preparations at various concentrations were added to a standard mating assay based on plasmid conjugal transfer (41), between the donor PA14ΔTnC2::GmR and the recipient PAO1::TcR. A mating mixture without the addition of OM or LPS was also included as a negative control for this experiment. The transfer inhibition index was calculated by dividing the transfer efficiency observed with the addition of OM or LPS to that of the control.

Western blotting for LPS samples

LPS samples prepared, by the Hitchcock and Brown method (42), were used for western blotting analyses. The western blot protocol for LPS was previously explained (38). Briefly, 3 µl of LPS samples was loaded into the 12 % discontinuous PAGE gel electrophoresis and run at 200 V for 50 min. LPS was electrophoretically transferred onto nitrocellulose membrane at 180 mA for 60 min. The membrane was then blocked with 5 % skim milk for 20 min at room temperature. The membranes were washed in PBS for 10 min; the primary antibodies against to different parts of LPS structure,were added and incubation continued overnight. Following a 10 min PBS wash, secondary antibodies were added for an additional hour. The membrane was washed for 10 min in PBS and developed by BCIP/NBT detection kit.

Expression and purification of GST-pilV2' fusion protein

The C-terminal region of pilV2 gene encoding 97 residues was amplified with the primers listed in supplementary table S3 and cloned into pJET 1.2/Blunt. The insert was then subcloned into the expression vector pGEX-2T (Life Technologies) and

transformed into E. coli BL21. E. coli BL21 containing pGEX-2T-pilV2' was grown to an OD of 0.6 at 37oC, at 200 rpm, before adding IPTG to induce expression of GST-pilV2' protein. The culture was incubated for additional 3 hours. The cells were collected by centrifugation at 8000 rpm, 4 ^{o}C for 20 min. The GST-pilV2' fusion protein was then purified by using glutathione Sepharose 4B (GE Healthcare), as previously described (43).

Microtiter plate binding assay

Binding of LPS to GST-pilV2' fusion was quantified by a modified enzyme-linked plate assay essentially as previously described (44). Microtiter plates (Corning) were coated with 10 µg/ml LPS from PAO1 and PAO1 with PAPI-1 suspended in PBS (0.137 M NaCl, 0.005 M KCl, 0.009 M Na2HPO4, and 0.001 M KH2PO4 (pH 7.4)) containing 0.05% Tween 20 (v/v, PBST). The plates were then washed with PBST and blocked with 3% BSA. GST-pilV2' fusion was added to the wells coated with LPS and incubated for 2 h at room temperature. After three washes with PBST, mouse anti-GST antibody was added and incubated for 1.5 h following three washes with PBST, HRP-labeled anti-mouse Ig (Sigma-Aldrich) was added for one hour, follwed by three aditional washes. A solution of 3,3′,5,5′-tetramethylbenzidine (Thermo Scientific) was used for color development and ODs were measured at 450 nm. LPS from *Salmonella enterica* was used as a negative control.

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FIGURES

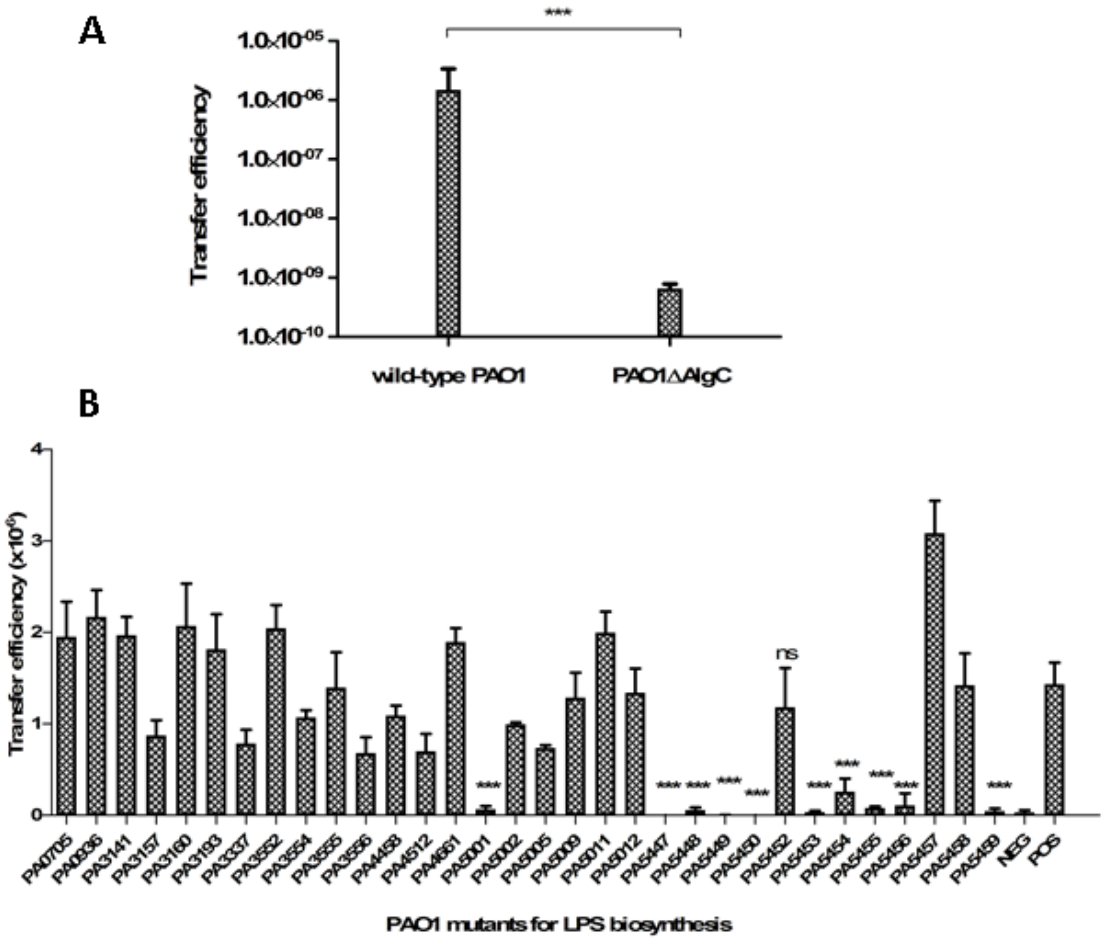

Figure 1. PAPI-1 transfer efficiency using PAO1 mutants for LPS biosynthesis genes as recipient strains. (A) Transfer efficiency into PAO1ΔalgC (B) Transfer efficiency into various PAO1 mutants for LPS biosynthesis. Positive control (POS): PAO1::Tc^R and Negative control (NEG): PAO1ΔalgC. Results were shown as mean ± SD for three independent replicates. Statistical significance was calculated by the unpaired t-test (A) and One-way ANOVA compared to the positive control (B) (ns: no significant; and *** $p < 0.001$).

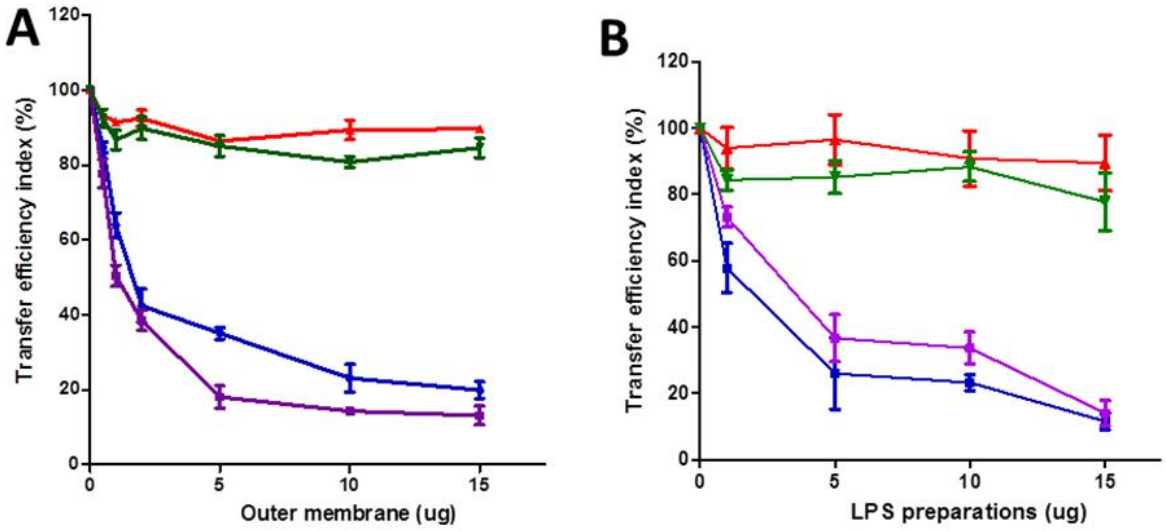

Figure 26. Effect on PAPI-1 transfer efficiency of the addition of OM (A) and LPS (B) preparations from PAO1 mutants lacking either A-band or B-band LPS. Red, PAO1Δrmd (A-,B+); green, PAO1ΔalgC (A-, B+); blue, PAO1ΔwbpM (A+, B-); purple, PAO1Δwzx (A+,B-).

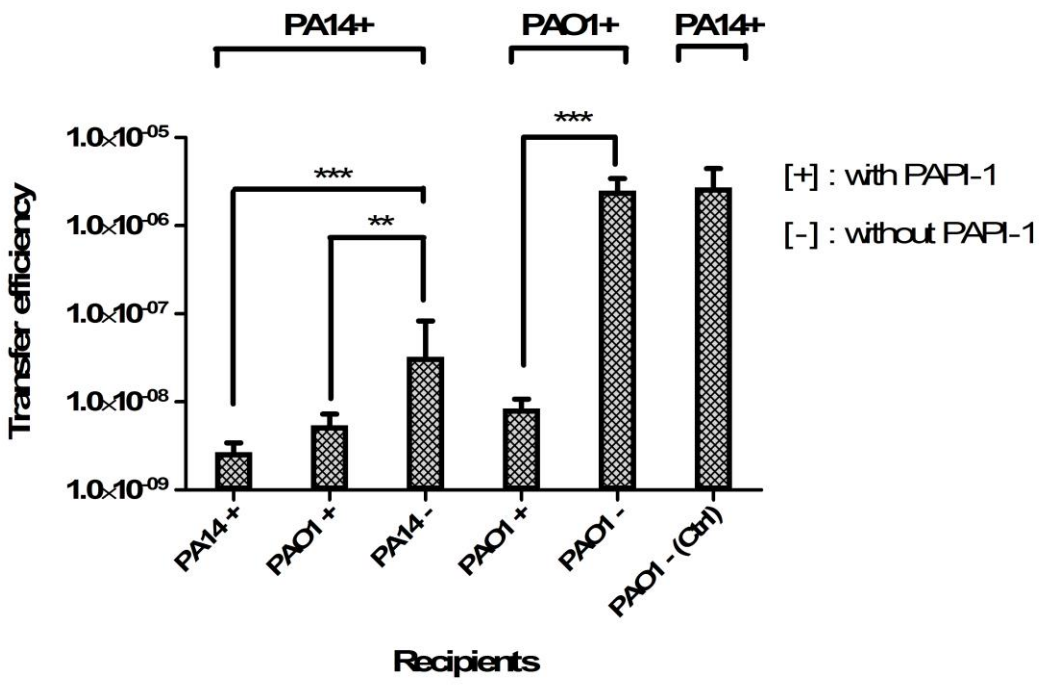

Figure 3. Transfer efficiency of multiple PAPI-1 copies into recipient strains. Marks [+] or [-] stand for strains with or without PAPI-1, respectively. After acquisition of PAPI-1, PAO1 becomes a stable donor which can transfer PAPI-1 to another recipient and decreases its ability of receiving additional copies of PAPI-1. Results are presented as mean ± SD for three independent experiments. Statistical significance was calculated by the unpaired t-test (A) (** $p<0.01$, and *** $p<0.001$).

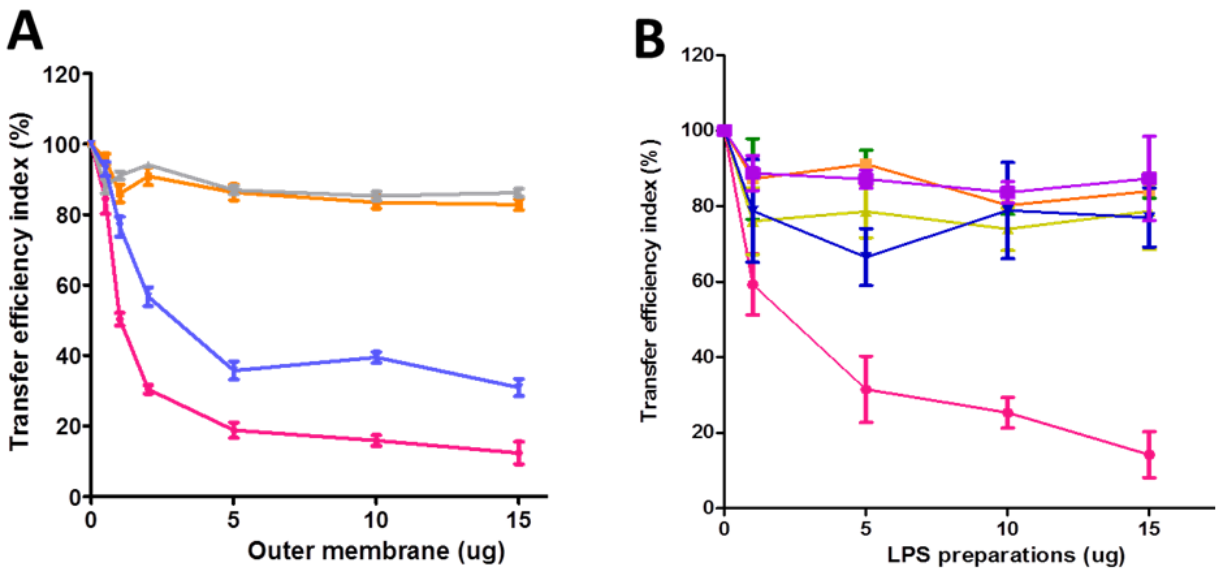

Figure 4. Effect on PAPI-1 transfer of the addition of OM (A) and LPS (B) preparations derived from strains with (+) or without (-) PAPI-1 island. Pink, PAO1-; orange, PAO1+; gray, PA14+; blue, PA14-. Purple data point on (B) are LPS from *Salmonella enterica* used as negative control.

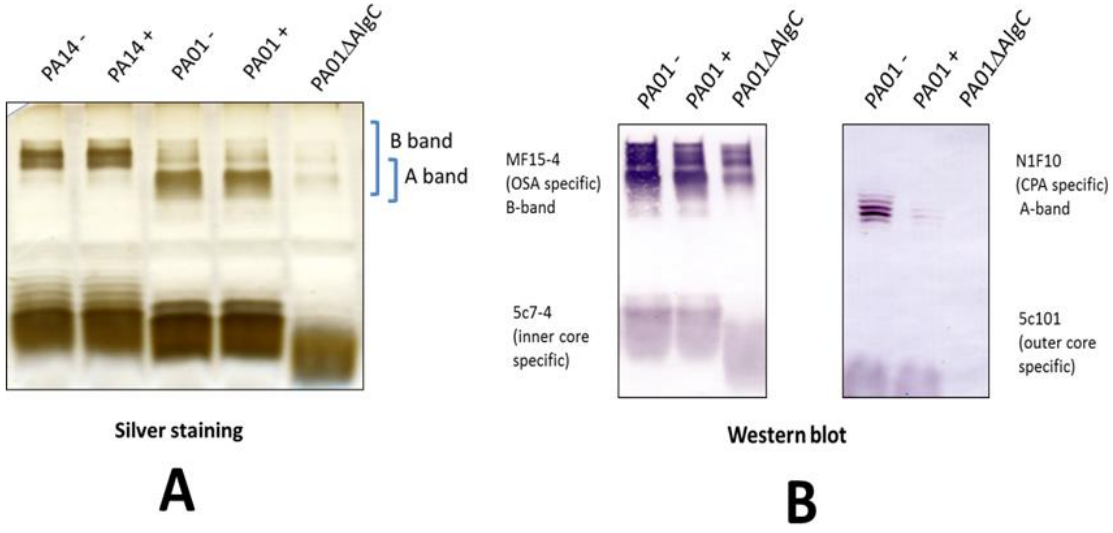

Figure 5. Analysis of LPS preparations from PAO1 and PA14 strains with (+) or without (-) PAPI-1. (A) LPS silver staining. (B) Western blot with a combination of antibodies specifically recognizing A-band (N1F10), B-band (MF15-4), outer core (5c101) and inner core (5c7-4).

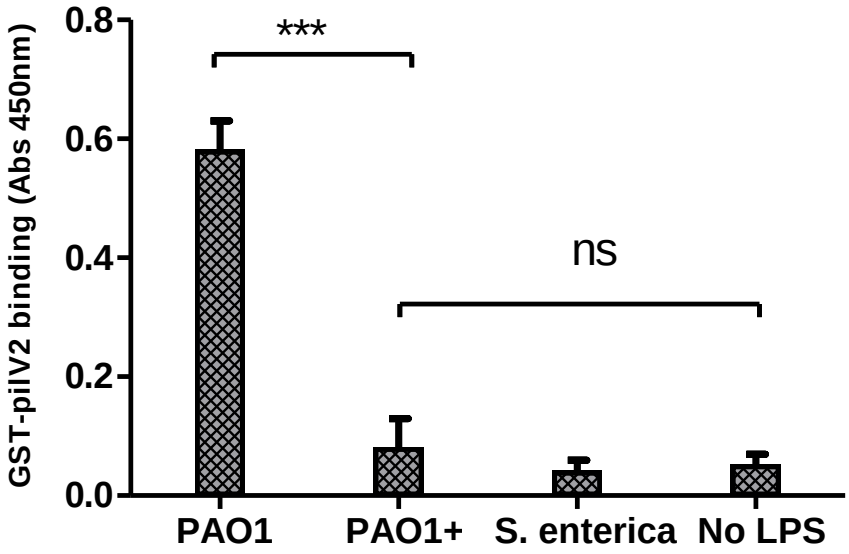

Figure 6. Binding of GST-pilV2' to various LPS preparations in an enzyme-linked immunosorbent *assay*. LPS from *Salmonella enterica* and sample without LPS used as negative control. LPS derived from PAO1 carrying PAPI-1 showed the loss of binding capacity to GST-pilV2'. Results were presented as mean ± SD for three independent experiments. Statistical significance was assessed by One-way ANOVA (*** $p<0.001$; ns: no significance, $P>0.05$).

Table 1. Strains and plasmids used in this study

Strains or plasmids	Antibiotic resistance[a]	Description	Source reference, or accession no.
E. coli strains			
E. coli SM10	None	Host strain for plasmids pEXG2, mini-CTX, and their derivatives	Lory's lab collection
E. coli λpir S17.1	None	Transfer pEXG2 plasmid into *P. aeruginosa* by conjugation	(1)
P. aeruginosa strains			
PA14	None	Burn isolate	(2)
PA14Δsoj (PA14 -)	Gm^R	Deletion mutant of PAPI-1 *soj* in strain PA14, which does not carry PAPI-1 island	(2)
PA14ΔTnC2::Gm^R (PA14+)	Gm^R	Strain PA14 with a transposon MAR2×T7 inserted at nucleotide 1634 of PAPI-1 gene RL090 (PA14_59200)	(3)
PA14ΔTnC2::Tc^R (PA14+)	Tc^R	Partially deletion of the PA14_59200 gene in strain PA14 by insertion of tetracycline resistant gene in the middle	This study
PAO1 (or PAO1 -)	Tc^R	PAO1 with Tet gene inserted at the CTX phage *att* site on the chromosome	Lory's lab collection
PAO1Bla6	Cb^R	PAO1 with genes *bla* and *lacZ* inserted at the CTX phage *att* site on the chromosome	(2)

PAO1Bla6TnC2::Gm^R (PAO1+)	Gm^R Cb^R	Transconjugant of the mating between PA14ΔTnC2 (Gm^R) and PAO1Bla6	This study
PAO1Bla6TnC2::Tc^R (PAO1+)	Gm^R Cb^R	Transconjugant of the mating between PA14ΔTnC2::Tc^R and PAO1Bla6	This study
Plasmids			
pEXG2	Gm^R	Gene replacement vector for constructing deletion or insertion mutants of *P. aeruginosa*	(4)
pJET1.2	Amp^R	Plasmid used for DNA blunt cloning	Thermo Scientific
pGEX-2T	Amp^R	Expression vector for GST-pilV2'	GE Healthcare
pJET1.2	Amp^R	Plasmid used for DNA blunt cloning	Thermo Scientific
pGEX-2T	Amp^R	Expression vector for GST-pilV2'	GE Healthcare

a Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance.

Supplementary table SA1. List of PAO1 mutants for lipopolysaccharide biosynthesis

Number	PA ORF	Gene Abbrev.	Putative ORF Function	Position in PAO1 transposon mutant library (source:[5])
1	PA0705	migA	alpha-1,6-rhamnosyltransferase MigA	phoAwp01q4A03
2	PA0936	lpxO2	lipopolysaccharide biosynthetic protein LpxO2	lacZwp03q3H09
3	PA3141	wbpM	nucleotide sugar epimerase/dehydratase	(*)
4	PA3157		probable acetyltransferase	phoAwp08q3G06
5	PA3160	wzz	O-antigen chain length regulator	phoAbp02q3G06
6	PA3193	glk	Glucokinase	phoAwp07q4C11
7	PA3337	rfaD	ADP-L-glycero-D-mannoheptose 6-epimerase	phoAwp05q3A01
8	PA3552	arnB	ArnB	phoAwp08q4G12
9	PA3554	arnA	ArnA	lacZwp07q3F04
10	PA3555	arnD	ArnD	phoAwp04q2C06
11	PA3556	arnT	inner membrane L-Ara4N transferase ArnT	lacZwp07q1F11
12	PA4458		conserved hypothetical protein	phoAwp07q2G01
13	PA4512	lpxO1	lipopolysaccharide biosynthetic protein LpxO1	phoAwp07q3E07
14	PA4661	pagL	Lipid A 3-O-deacylase	phoAbp02q4E08
15	PA5001		hypothetical protein	phoAwp01q3H11
16	PA5002		hypothetical protein	lacZbp03q3E06
17	PA5005		probable carbamoyl transferase	phoAwp09q3B06
18	PA5009	waaP	lipopolysaccharide kinase WaaP	phoAwp05q4G09
19	PA5011	waaC	heptosyltransferase I	lacZwp04q4G06
20	PA5012	waaF	heptosyltransferase II	lacZwp08q1C03
21	PA5447	wbpZ	glycosyltransferase WbpZ	lacZwp02q1H10
22	PA5448	wbpY	glycosyltransferase WbpY	phoAwp02q1F12
23	PA5449	wbpX	glycosyltransferase WbpX	lacZwp01q4A02
24	PA5450	wzt	ABC subunit of A-band LPS efflux transporter	phoAwp10q1E09
25	PA5452	wbpW	phosphomannose isomerase/GDP-mannose WbpW	lacZwp08q4H11
26	PA5453	gmd	GDP-mannose 4,6-dehydratase	lacZwp02q3E02
27	PA5454	rmd	oxidoreductase Rmd	lacZwp01q1B08
28	PA5455		hypothetical protein	phoAwp08q4H06
29	PA5456		hypothetical protein	lacZwp02q4C05
30	PA5457		hypothetical protein	lacZwp06q1F08
31	PA5458		hypothetical protein	phoAwp10q1C10
32	PA5459		hypothetical protein	phoAwp08q1B12
33	PA5322	algC	phosphomannomutase	phoAwp07q4D07

(*): Lory's lab collection

Supplementary table SA2. Primers used in this study

Primer	Sequence (5'-3')	Description	Source
TnC2-US-F	GGTACCGGCAACACATTTCTCCCTCG	Amplify a fragment of 532 bp upstream of PA14_59200 gene	This study
TnC2-US-R	TCTAGATTGAGCCAGCCAGTTGTAGA		
TnC2-DS-F	TCTAGACGGCTGAGAGACATCAAGGA	Amplify a fragment of 594 bp downstream of PA14_59200 gene	This study
TnC2-DS-R	AAGCTTGTTCAGGTTCGTCGCTATGG		
Tc-F	TCTAGATCAGGTCGAGGTGGCCC	Amplify Tet gene from mini-CTX2 plasmid	This study
Tc-R	TCTAGAAGAGCGCTTTTGAAGCTAATTCGCTG		
TnC2-Li-F	CTTGACGAGTTTGCTGCACT	Check the insert of Tet gene on the left junction	This study
TnC2-Li-R	GAGAAGCAGGCCATTATCGC		
TnC2-Ri-F	GAACGGGTGCGCATAGAAAT	Check the insert of Tet gene on the right junction	This study
TnC2-Ri-R	TTCGACCAAGGAGCTGAACT		
pilV2-F	ATAGGATCCCTGTCCTGCCAAAACGGG	Amplify C-terminal region of pilV2 gene (97 amino acid)	This study
pilV2-R	ATATGAATTCCTAGTTCACGCAGGTAACGG		
intF	AGCTACATCGAGGCCGACTA	Check the insertion of PAPI-1 on the left junction of attL site	(2)
4542F	GTGGTGATGACCTCCAACCT		(2)
sojR	CGAGCACAGAAATGTCCTGA	Check the insertion of PAPI-1 on the right junction of attR site	(2)
4541F	GACAAGACCAGCCACAACCT		

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