The rhamnosyltransferase genes migA and wapR are regulated in a differential manner to modulate the quantities of core oligosaccharide glycoforms produced by *P. aeruginosa*.

---

Dana Kocincová\(^1\)\(^\S\), Sarah L. Ostler\(^1\)\(^\S\), Erin M. Anderson\(^1\), and Joseph S. Lam\(^1\)*

\(^\S\) authors contributed to the work equally

Running title: *migA* and *wapR* modulate core glycoforms quantities

Keywords: *Pseudomonas aeruginosa*, core LPS, *migA*, *wapR*, quorum sensing

\(^1\)Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, University of Guelph, Ontario N1G 2W1, Canada; Tel +1 519 824 4120 extension 53823, Fax +1 519 837 1802; Email: jlam@uoguelph.ca
Abstract

*migA* and *wapR* are rhamnosyltransferase genes involved in the biosynthesis of *Pseudomonas aeruginosa* lipopolysaccharide core. Here we show that preferential expression of *migA* and *wapR* correlated with the levels of uncapped and O polysaccharide-capped core, respectively. *wapR* is negatively regulated, while *migA* is positively regulated by RhlR/RhlI quorum sensing.
Lipopolysaccharide (LPS) in Gram-negative bacteria is composed of three domains: lipid A, core oligosaccharide (OS) and O polysaccharides. *Pseudomonas aeruginosa* produces two core OS glycoforms, one is capped (linked to O polysaccharides) and the other is uncapped (devoid of O polysaccharides). The capped core can be substituted with various modal lengths of O polysaccharides, including high-molecular-weight O antigen or just a single O unit, which results in “core-plus-one LPS”. The two glycoforms differ in the position and linkage in which L-rhamnose (L-Rha) residue is bound to the backbone structure of the core OS (L-Rha^A or L-Rha^B, Fig. 1). Two rhamnosyltransferases encoded by homologous genes, *migA* and *wapR*, are responsible for the transfer of L-Rha to the uncapped and capped core, respectively (12).

Previously, Yang et al. (21) showed that *migA* is highly expressed, when *P. aeruginosa* is cultured in the presence of sputum samples collected from cystic fibrosis patients, and this expression was controlled by the RhlR/RhlI quorum sensing (QS) system. At present, the regulation of *wapR* is poorly understood. In this study, we test the hypothesis that *migA* and *wapR* are regulated in a differential manner to modulate the quantities of uncapped and capped core OS glycoforms being produced, respectively, by *P. aeruginosa*. When a *migA* mutant, affected in uncapped core, was complemented with *migA* carried on a multi-copy plasmid, the amount of core-plus-one LPS produced by the bacteria was significantly reduced (12). To test whether the observed phenotype of the complemented strain was due to a gene-dosage effect, we constructed vectors using the multi-copy pUCP26 vector carrying XbaI-HindIII fragments from pET30-*migA* or pET30-*wapR*; both constructs contain the ribosome binding site from pET30a and the lac promoter from pUCP26. We also constructed another set of vectors utilizing the integrative mini-CTX2 plasmid (9) carrying *migA* and *wapR* with their respective 400-bp upstream promoter regions that included the entire non-coding intergenic region and a part of the
upstream ORF. This is to ensure that complete regulatory region have been used for complementation. The gentamicin cassettes from migA::Gm and wapR::Gm mutants (12) were excised using the flippase-recombinase/target (FLP/FRT) plasmid system, pFLP2 (8). LPS from mutants and complemented strains were analyzed as described previously (11).

Complementation of the migA mutant with the multi-copy (pUCP-migA) and integrative (CTX-migA) vectors restored the wildtype phenotype as analyzed by Western immunoblotting (Fig. 2A, panel II) using monoclonal antibody (MAb) 5c-101 specific against the uncapped core (4). Expression of migA carried on multi-copy pUCP-migA vector caused a great reduction in core-plus-one LPS (Fig. 2A, panel III), as analyzed by Western immunoblotting using core-plus-one-specific MAb 18-19 (4). In contrast, complementation with a single migA copy due to CTX-migA resulted in core-plus-one LPS production at a comparable level as that of the wildtype strain (Fig. 2A, panel I and III). Both pUCP-wapR and CTX-wapR constructs were able to complement the wapR mutant, and restored the production of LPS capped with O polysaccharides and core-plus-one O unit, as observed by SDS-PAGE silver staining and Western immunoblotting using MAb 18-19 and O-antigen-specific MAb MF15-4 (Fig. 2B, panel I, III and IV). When the wapR mutant was complemented with pUCP-wapR, the amount of uncapped core synthesized was greatly reduced, whereas in the strain complemented with the CTX-wapR construct, the intensity of the uncapped core OS band was similar to that in the wildtype (Fig. 2B, panel II). Together, these data showed that the aberrant phenotype of the recombinant strain of migA-FRT mutant complemented with pUCP-migA showing a great reduction in core-plus-one O unit was due to a gene-dosage effect. Overproduction of MigA favoured the production of uncapped core. In contrast, overproduction of WapR preferentially favoured the production of capped core, which became proportionally more abundant than the level of uncapped core. This mechanism of
regulating the quantity of either core OS glycoform is likely due to substrate competition between MigA and WapR glycosyltransferase reactions. Our data showed that changing the ratio of expression between MigA and WapR, simulated by using multi-copy versus single-copy expression constructs for complementation, influences the amounts of uncapped and capped core OS being produced by the bacteria. We further made hybrid constructs with swapped native promoters of migA and wapR by using overlap PCR, such that migA was regulated by the wapR promoter, while wapR expression was controlled by the migA promoter. The rhamnosyltransferase genes, migA and wapR, were separately cloned into intergrative mini-CTX2 plasmid as well as in a multicopy plasmid pTZ110, which contains a multi-cloning site downstream of a promoterless region (16). The pTZ and mini-CTX constructs containing migA and wapR with their native respective promoters were used as controls. The constructs were transformed into migA-FRT and wapR-FRT mutants. A single copy of migA controlled by the wapR promoter (in CTX-SWAPmigA) was able to fully complement the migA phenotype as verified by immunoblotting with MAb 5c-101 (Fig. 2A, panel II). Similarly, in the multicopy constructs, the migA gene expressed from its native promoter (in the pTZ-migA vector), and also from wapR promoter (in pTZ-SWAPmigA) was able to fully complement the migA mutant phenotype, as observed in Western immunoblotting using the uncapped core-specific MAb 5c-101 (Fig. 2A, panel II). Importantly, the amount of core-plus-one LPS was lower when migA was expressed from the wapR promoter than from the native migA promoter. These results suggest that the wapR promoter is stronger, leading to higher quantities of MigA, which outcompete for substrates of the WapR transferase reaction. This effect was obvious when the multicopy pTZ constructs were used, where a potential difference in promoters could be amplified by gene-dosage.
Surprisingly, the *wapR* gene regulated by the *migA* promoter encoded on the single-copy CTX-SWAP *wapR* construct was able to complement the *wapR* mutant phenotype only partially; a very small amount of LPS core capped with O antigen was detected by SDS-PAGE silver staining (Fig. 2B, panel I) and Western immunoblotting (Fig. 2B, panel IV). The use of the core-plus-one-specific MAb 18-19 did not detect a low-molecular-weight band representing the core-plus-one-LPS glycoform (Fig 2B, panel III). This phenotype can be attributed to the *migA* promoter being weaker compared to that of *wapR*. When expression of WapR was controlled by the *migA* promoter carried on the multi-copy plasmid, the amounts of high-molecular-weight O antigen-capped core was lower than in the wild-type, but higher than in the single copy swap experiment (Fig. 2B, panels I and IV). Therefore, it is very likely that a high level of WapR expression is required to assemble and produce the capped core oligosaccharide to the wildtype level. To date, nothing is known about regulation of LPS capped with only one O-antigen unit (semi-rough [SR] LPS) versus LPS capped with the polymer of O polysaccharide (wildtype LPS). However, based on genetic evidence through the generation of a *wzy* (formerly called *rfc*) knockout mutant (3) and immunochemical analysis using the core-plus-one-specific MAb 18-19 (4), both forms of LPS exists. When *wapR* was expressed from its native promoter (in pTZ-*wapR*), it was able to fully complement the *wapR* mutant phenotype (Fig. 2B, panel I and III), while the amount of uncapped core was not affected, as seen in the complementation experiment with pUCP-*wapR*, in which *wapR* expression is driven by the *lac* promoter (Fig. 2B, panel II).

Therefore, the amount of uncapped core produced by the bacteria varies depending on which complementation constructs (differing by the promoter of *migA*, *wapR*, or *lac*) was used to drive the expression of WapR and vice versa for the expression of MigA. Our findings showed that the amounts of core-plus-one capped core produced was influenced by gene dosage and promoter,
which controlled migA expression. The data from the “promoter-swap” experiment supports our hypothesis and also indicates that the wapR and migA promoters possess different strengths. To provide quantitative evidence that wapR and migA promoters have differential strengths, we cloned the 400-bp promoter regions of migA and wapR upstream of a plasmid-borne β-galactosidase gene. We transformed PAO1 with the constructs and measured β-galactosidase activity in three replicates as described previously (19). Indeed, based on the calculated Miller units, PAO1 with the migA construct showed 8.2X lower β-galactosidase activity (P<0.05) than that with the wapR construct (data not shown). The migA promoter region has been studied previously (21), but nothing is known about the regulation of wapR expression. To investigate the wapR promoter region, we mapped the transcription start site (TSS) of wapR using a technique called 5’ Random Amplification of cDNA Ends (5’ RACE) following the protocol recommended by the manufacturer (Roche) (data not shown). Two TSS sites (TSS¹ and TSS²) separated by 165 bp were identified. TSS¹ was the A base of the annotated initiation ATG codon (Fig. 3). This wapR transcript can be considered an example of a leaderless mRNA found in various bacterial species. Leaderless mRNAs do not require RBS to bind to ribosomes and the interaction with ribosomes is mediated directly through AUG of mRNA (2). Importantly, the same TSS¹ site of the wapR transcript has been identified recently by RNA sequencing in a study where genome-wide TSS sites of P. aeruginosa PA14 were determined (5). Such findings strongly support our data. In the upstream region of the wapR gene, potential -10 (TAAGGT) and -35 (TTGAAT) promoter hexamers were identified. The sequences of the hexamers display similarities to -10 (TAtAAT) and -35 (TTGaCc/a) boxes recognized by P. aeruginosa RpoD (13), a principal sigma factor. Note that the degree of conservation in the consensus sequences is not absolute; and among the 149 RpoD-dependent promoters published in the literature, they are
designated as the following: nucleotides in underlined and uppercase letters are those with 50% 
conservation, in plain uppercase letters for those with 40% conservation and in lower case letters 
for those with 30% conservation. The sequence identity between the putative wapR promoter 
elements and consensus nucleotides is high in the most conserved nucleotides. The distances 
identified between TSS¹ and -10 sites (8 bp) as well as between -10 and -35 sites (18 bp) (6, 18) 
have been observed in functional promoters, which support correct identification of TSS¹ and the 
promoter hexamers. We did not discern any consensus sequences for the promoter used for 
initiation of the longer wapR transcript.

To validate the results obtained by the RACE experiment, we mutated the putative -10 hexamer 
and cloned the constructs into a β-galactosidase reporter vector. Since the putative hexamer 
TAAGGT is present on the 3’ end of the promoter, we introduced mutations by PCR using 
reverse primers containing nucleotide substitutions. These primers mutated either all six 
nucleotides to GCCAAC (in the “1-6” constructs) or four conserved A/T nucleotides (in general 
required for DNA unwinding during transcription activation), which changed the hexamer to 
GCCGCG (in the “1/2/3/6” constructs). Two sets of plasmids were constructed; the short (S) set 
contained 136-bp wapR upstream sequence, while the long (L) set contained 400-bp upstream 
promoter regions. The short version therefore contained only the promoter, which drives 
expression of the transcript with TSS¹, whereas the long version contained both potential 
promoters. The constructs were electroporated in PAO1, and β-galactosidase assay was 
performed using 6 replicates. These results have been summarized in Table 2. β-Galactosidase 
activity of cells with constructs containing the S and L wild type promoter sequences differed, 
being more than 5X lower in the S version, which indicates that the promoter driving expression 
of the longer transcript with TSS² have a higher strength. Both mutations (1-6, 1/2/3/6) in the
hexamer caused significant decrease in \( \beta \)-galactosidase activity (\( P<0.05 \)) in both S and L constructs, and in the S constructs, the enzyme activity dropped to the level <0, when the activity in the empty plasmid control was subtracted from these values. Together, these data revealed the existence of more than one promoter in the \( \text{wapR} \) upstream region as well as existence of the putative -10 hexamer.

A previous study by our group showed that expression of \( \text{migA} \) in the PAK strain (serotype O6) is positively regulated by the RhlR/RhlI quorum sensing (QS) pathway (21). To determine if and how \( \text{wapR} \) expression might be regulated by QS either by RhlR/RhlI or by the other system, LasR/LasI, we used a real-time quantitative PCR (qPCR) approach. The bacterial strains used for this part of the study have been listed in Table 1. Data were obtained using three technical replicates of three biological replicates. RNA was purified from cultures grown in Tryptone broth that reached the cell density measured by optical density at 600 nm (\( \text{OD}_{600} \)) of 0.9-1.1, in which QS is active. Cell activation of QS in Tryptone broth is immediately apparent by visual inspection, since \( \text{P. aeruginosa} \) produces a QS regulated blue-green secondary metabolite. cDNA was produced from RNA transcripts using reverse transcriptase. qPCR was performed with QuantiTect SYBR Green I PCR Master Mix (QIAGEN) and analyzed with the Rotor-Gene 6000 (Corbett Robotics Inc) following the protocols recommended by the manufacturers (sequences of primers used will be available upon request). The expression level of \( \text{rpoD} \), a non-QS-regulated reference gene (15) used as a control, was monitored to normalize for differences in total RNA concentration between samples. An additional control used was \( \text{lasB} \), which encodes elastase and is predominantly regulated by LasR/LasRI (14). The expression level of \( \text{migA} \) in the \( \text{rhl}^- \) mutant of PAO1 background was reduced by 2.8 folds (\( P<0.05 \)) as compared to expression in the wildtype PAO1 (data not shown), indicating that \( \text{migA} \) in wildtype PAO1 is positively regulated
by RhlR/RhlI QS. This is similar to observations made previously in the P. aeruginosa PAK (21). In contrast, expression of wapR increased by 2.0 folds (P<0.05) in the rhl\textsuperscript{I} mutant (data not shown), which indicates that wapR is negatively regulated by RhlR/RhlI QS. This negative regulation is likely indirect, since we have not identified potential RhlR binding sites within the wapR promoter region. We did not observe any significant differences in expression of migA, and wapR in the lasI strain. In the control experiment, we found that lasB expression was 357X lower (P<0.05) in the lasI- mutant than in the wild-type, whereas in the rhl\textsuperscript{I} strain the expression level was not significantly changed (data not shown). The observation of differential regulation between migA and wapR gene expression by the RhlR/RhlI QS system supports our hypothesis that the levels of migA and wapR expression influence the amount of uncapped- and capped-core OS that were synthesized, respectively. Based on our data and previously published results (21), it is evident that regulation of migA and wapR expression is very complex and involves the action of multiple transcription regulators. Further experiments are required to investigate how RhlR regulation of wapR expression occurs, and what role RpoD plays in the wapR expression. In addition, development of a competition rhamnosyltransferase assay would clarify if the difference in the strengths of migA and wapR promoters can be compensated by diverse transferase efficiency.

Acknowledgement

This work is funded by an operating grant from Cystic Fibrosis Canada (CFC) D.K is a recipient of a CFC fellowship, S.L.O. was a recipient of a Canadian Institutes of Health Research Canada Graduate Scholarship Master`s Award, and J.S.L. holds a Canada Research Chair in Cystic Fibrosis and Microbial Glycobiology. We thank Dr. Herbert Schweizer and Dr. Tung Hoang for providing all of the molecular biology vectors including pUCP26, pFLP2, pTZ110 and mini-
CTX systems, and Dr. Peter Krell for the use of the Rotor-Gene instrument to perform real-time PCR analyses.

This paper is dedicated to the memory of Dr. Bill Costerton who was a tremendous mentor, a pioneer, and a distinguished microbiologist. For almost four decades, through numerous publications and public lectures, Bill tirelessly educated scientists, medical personnel, and the general public about the importance of biofilms. Full validation of his concepts became apparent when the US National Institutes of Health publicly announced “80% of all microbial infections in the body are caused by biofilms” (e.g. PA-03-047, PA-06-537).
References


<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype, phenotype, or properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>cloning strain, F−φ80lacΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rlK− mK+) supE44 Δ thr-1 gyrA96 relA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SM10</td>
<td>strain for bi-parental mating thi-1 thr leu tonA lacY supE recA</td>
<td>(17)</td>
</tr>
<tr>
<td><strong>P. aeruginosa strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>IATS serotype O5; wild-type type strain</td>
<td>(7)</td>
</tr>
<tr>
<td>migA-FRT</td>
<td>gentamicin cassette excised from migA::Gm (12), leaving behind FRT sequence to disrupt migA</td>
<td>this study</td>
</tr>
<tr>
<td>wapR-FRT</td>
<td>gentamicin cassette excised from wapR::Gm (12)</td>
<td>this study</td>
</tr>
<tr>
<td>migA-FRT/pUCP-migA</td>
<td>migA::FRT complemented with pUCP-migA</td>
<td>this study</td>
</tr>
<tr>
<td>migA-FRT/CTX-migA</td>
<td>migA::FRT complemented with CTX-migA</td>
<td>this study</td>
</tr>
<tr>
<td>wapR-FRT/pUCP-wapR</td>
<td>wapR::FRT complemented with pUCP-wapR</td>
<td>this study</td>
</tr>
<tr>
<td>wapR-FRT/CTX-wapR</td>
<td>wapR::FRT complemented with CTX2-wapR</td>
<td>this study</td>
</tr>
<tr>
<td>migA-FRT/pTZ-migA</td>
<td>migA::FRT complemented with pTZ-migA</td>
<td>this study</td>
</tr>
<tr>
<td>migA-FRT/CTX2-wapR</td>
<td>migA::FRT complemented with pTZ-wapR</td>
<td>this study</td>
</tr>
<tr>
<td>migA-FRT/pTZ-SwapR</td>
<td>wapR::FRT complemented with pTZ-SwapR</td>
<td>this study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET30a</td>
<td>expression vector, KmR</td>
<td>Novagen</td>
</tr>
<tr>
<td>pUCP26</td>
<td>pUC18-based broad-host-range vector, TcR</td>
<td>(20)</td>
</tr>
<tr>
<td>mini-CTX2</td>
<td>Int-mediated Integrative vector TcR</td>
<td>(9)</td>
</tr>
<tr>
<td>pFLP2</td>
<td>Contains Flp-recombinase gene, AmpR</td>
<td>(8)</td>
</tr>
</tbody>
</table>
pTZ110 broad-host-range promoterless lacZ vector, AmpR (16)
pET30-migA migA cloned into NdeI-HindIII of pET30a this study
pET30-wapR wapR cloned into NdeI-HindIII of pET30a this study
pUCP-migA XbaI-HindIII fragment from pET30-migA cloned into XbaI-HindIII of pUCP26 this study
pUCP-wapR XbaI-HindIII fragment from pET30-wapR cloned into XbaI-HindIII of pUCP26 this study
CTX-migA migA + upstream 400 bp cloned into mini-CTX2 (PstI-XhoI) this study
CTX-wapR wapR + upstream 400 bp cloned into mini-CTX2 (PstI-XhoI) this study
CTX-SWAPmigA wapR promoter (400 bp)+migA gene cloned into mini-CTX2 (HindIII-BamHI) this study
CTX-SWAPwapR migA promoter (400 bp)+wapR gene cloned into mini-CTX2 (HindIII-BamHI) this study
pTZ-migA migA + upstream 400 bp cloned into pTZ110 (HindIII-BamHI) this study
pTZ-wapR wapR + upstream 400 bp cloned into pTZ110 (HindIII-BamHI) this study
pTZ-SWAPwapR migA promoter (400 bp)+wapR genecloned into pTZ110 (HindIII-BamHI) this study
pTZ-SWAPmigA wapR promoter (400 bp)+migA gene cloned into pTZ110 (HindIII-BamHI) this study
pPmigA migA promoter (400 bp) cloned into pTZ110 (HindIII-BamHI) this study
pPwapR_L wapR promoter (400 bp) cloned into pTZ110 (HindIII-BamHI) this study
pPwapR_L (1/2/3/6) wapR promoter (400 bp) with mutated -10 hexamer cloned into pTZ110 (HindIII-BamHI) this study
pPwapR_L (1-6) wapR promoter (400 bp) with mutated -10 hexamer cloned into pTZ110 (HindIII-BamHI) this study
pPwapR_S wapR promoter (136 bp) cloned into pTZ110 (HindIII-BamHI) this study
pPwapR_S (1/2/3/6) wapR promoter (136 bp) with mutated -10 hexamer cloned into pTZ110 (HindIII-BamHI) this study
pPwapR_S (1-6) wapR promoter (136 bp) with mutated -10 hexamer cloned into pTZ110 (HindIII-BamHI) this study
Table 2. β-galactosidase assay on mutated putative -10 hexamer of the wapR promoter.

<table>
<thead>
<tr>
<th>Construct</th>
<th>MU (wapR construct) - MU (empty plasmid)* ± St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPwapR_S wt</td>
<td>145.8 ± 87.0</td>
</tr>
<tr>
<td>pPwapR_S (1-6)</td>
<td>&lt;0 ± 44.6</td>
</tr>
<tr>
<td>pPwapR_S (1/2/3/6)</td>
<td>&lt;0 ± 39.8</td>
</tr>
<tr>
<td>pPwapR_L wt</td>
<td>1231.7 ± 125.6</td>
</tr>
<tr>
<td>pPwapR_L (1-6)</td>
<td>402.7 ± 51.5</td>
</tr>
<tr>
<td>pPwapR_L (1/2/3/6)</td>
<td>579.8 ± 75.9</td>
</tr>
</tbody>
</table>

*: Miller units (MU) obtained by subtraction of the MU value calculated in the empty plasmid control

S and L: 136-bp and 400-bp wapR upstream region, respectively

1-6 and 1/2/3/6: mutations of -10 hexamer (TAAGGT) to GCCAAC and GCCGGC, respectively
Legend to figures

**Fig. 1. Structures of the two outer-core oligosaccharide glycoforms.** Uncapped-core OS is devoid of O polysaccharides and contains an \( \alpha-1,6 \)-linked L-Rha\(^A\) transferred by MigA. The migA mutant LPS is lacking L-Rha\(^A\) and Glc\(^{IV}\). Star depicts the epitope (Glc\(^{IV}\)) for MAb 5c-101. Capped core OS is substituted with O polysaccharides through an \( \alpha-1,3 \)-linked L-Rha\(^B\), which is transferred by WapR. The capped core can be substituted with various modal lengths of O antigen, including one single repeat of O unit leading to core-plus-one LPS or multiple repeats of O unit. Circle and square depict the epitope for MAb 18-19 (one O antigen unit) and MF 15-4 MAb (O antigen polysaccharide). Abbreviations: GalN, galactosamine; Ala, alanine; Rha, rhamnose; Glc, glucose. The complete core oligosaccharide structures consisting of both inner and outer core sugars have been reported in reference (10).

**Fig. 2. SDS-PAGE and immunoblotting analysis of LPS from PAO1, migA-FRT (Fig. 2A), wapR-FRT (Fig. 2B) and strains complemented with mini-CTX (CTX), pUCP26 (pUCP), pTZ110 (pTZ) constructs.** In the constructs CTX-SWAPmigA, pTZ-SWAPmigA, migA is controlled by the wapR promoter and vice versa, in the CTX-SWAPwapR, pTZ-SWAPwapR, wapR is regulated by the migA promoter. Lines 1-7 of panels I-III are identical and for Fig. 2A are as follows: 1, PAO1 wild type; 2, migA-flp mutant; 3, migA-flp/pUCPmigA; 4, migA-flp/CTXmigA; 5, migA-flp/pTZ-SWAPmigA; 6, migA-flp/pTZ-migA; 7, migA-flp/CTX-SWAPmigA. For Fig. 2B, lines 1-7 of panels I-IV are as follows: 1, PAO1 wild type; 2, wapR-flp mutant; 3, wapR-flp/pUCPwapR; 4, wapR-flp/CTXwapR; 5, wapR-flp/pTZ-SWAPwapR; 6, wapR-flp/pTZ-wapR; 7, wapR-flp/CTX-SWAPwapR. LPS separated on SDS-PAGE was silver stained (panel I) and immunoblots were probed with outer-core-specific MAb 5c-101 (panel II),
core-plus-one-specific MAb 18-19 (panel III) and O antigen specific MAb MF 15-4 (panel IV).

Note that MAb 18-19 also recognizes O antigen repeats, which is caused by a very close chemical composition of core-plus-one LPS and complete O antigen polysaccharide. In the \textit{wapR}-\textit{FRT} mutant (Fig. 2B, panel IV), detection of O antigens by MAb 15-4 is caused by a presence LPS precursors, i.e. O antigen linked to a lipid carrier and not by O antigen linked to lipidA-core, as previously demonstrated by our group (12).

\textbf{Fig. 3. Analysis of the \textit{wapR} promoter region.} 5' RACE was performed to determine the transcription start sites (TSS) sites of \textit{wapR}. The TSS$^1$ site overlaps with the annotated initiation ATG codon, TSS$^2$ is located 165 bp from TSS$^1$. Grey boxes depict potential -10 and -35 hexamers.
Uncapped core

MigA

\[ \beta-D-Glc^{IV} \rightarrow 6-L-Rha^{II} \rightarrow 6-D-Glc^{II} \]

1

\[ \downarrow \]

\[ D-Glc^{III} \rightarrow 6 \beta-D-Glc^{I} \rightarrow 3-D-GalN-Ala \]

Capped core

WapR

\[ 3-L-Rha^{II} \rightarrow 6-D-Glc^{II} \]

1

\[ \downarrow \]

\[ \downarrow \]

\[ 3 \beta-D-Glc^{I} \rightarrow 3-D-GalN-Ala \]

MAb

5c-101

18-19

MF15-4

one O-antigen unit or O-antigen polymer