The D3 bacteriophage α-polymerase-inhibitor (Iap) peptide disrupts O-antigen biosynthesis through mimicry of the chain length regulator Wzz in Pseudomonas aeruginosa.

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ABSTRACT

Lysogenic bacteriophage D3 causes seroconversion of *Pseudomonas aeruginosa* PAO1 from serotype O5 to O16 by inverting the linkage between O-specific antigen (OSA) repeat units from α to β. The OSA units are polymerized by Wzy to modal lengths regulated by Wzz1 and Wzz2. A key component of the D3 seroconversion machinery is the inhibitor of α-polymerase (Iap) peptide able to solely suppress α-linked long-chain OSA production in *P. aeruginosa* PAO1. To establish target specificity of Iap for Wzyα, changes in OSA phenotypes were examined via Western immunoblotting for single-knockout strains of *wzz1* and *wzz2*, as well as a *wzz1-wzz2* double knockout, following expression of iap from a tuneable vector. Increased induction of Iap expression completely abrogated OSA production in the *wzz1-wzz2* double mutant, while background levels of OSA production were still observed in either of the single mutants. Therefore, Iap inhibition of OSA biosynthesis was most effective in the absence of both Wzz proteins. Sequence alignment analyses revealed a high degree of similarity between Iap and the first transmembrane segment (TMS) of either Wzz1 or Wzz2. Subjecting the Iap sequence to various topology prediction analyses consistently predicted the presence of a single TMS, suggesting a propensity for Iap to insert into the IM. Taken together, the compromised ability of Iap to abrogate Wzyα function in the presence of Wzz1 or Wzz2 provides compelling evidence that inhibition occurs after Wzyα inserts into the IM and is achieved through mimicry of the first TMS from the Wzz proteins of *P. aeruginosa* PAO1.
Lipopolysaccharide (LPS) is an integral structural component of the outer membrane of Gram-negative bacteria and is important for the survival of these bacteria in the environment or in a host. In *Pseudomonas aeruginosa* and many other opportunistic pathogens, LPS is a major virulence factor and is composed of a lipid A membrane anchor, core oligosaccharide linker and a distal polysaccharide termed O antigen (O-Ag) (1). *P. aeruginosa* simultaneously produces two forms of O-Ag: a homopolymeric common antigen (CPA) and an immunodominant heteropolymeric O-specific antigen (OSA) composed of repeating trisaccharide units (2).

In *P. aeruginosa* PAO1, OSA is synthesized via the Wzx/Wzy-dependent pathway (3), which requires the activity of several integral inner membrane (IM) proteins (4). Synthesis begins at the cytoplasmic leaflet of the IM on the lipid carrier undecaprenyl pyrophosphate (UndPP), with the formation of an OSA trisaccharide repeat constructed from newly synthesized nucleotide sugar precursors (5). The UndPP-linked OSA repeat is then transported through the cationic interior of the OSA flippase Wzx to the periplasmic leaflet of the IM (6, 7) and polymerized at the reducing terminus of the growing chain (8) by Wzy, via a putative “catch-and-release” mechanism in an α-1-4 linkage (9, 10). The OSA chain length is regulated by the polysaccharide co-polymerase (PCP) proteins Wzz1 and Wzz2, which interact with the nascent polysaccharides and confer long (12-16 and 22-30 repeats) and very long (40-50 repeats) modal lengths, respectively (11, 12). Full-length OSA is then ligated to the lipid A-core moiety by the O-Ag ligase WaaL (13, 14) forming the mature LPS molecule.

Variability in the OSA repeat sugar constituents, intra- and inter-glycosidic linkages of the OSA repeat residues, and the presence of side branch modifications classify *P. aeruginosa*...
into 20 distinct serotypes according to the International Antigenic Typing Scheme (1). Similar
OSA backbone sugar structures results in certain individual serotypes, for example O2, O5, O16, O18 and O20, being classified into a single serogroup (serogroup O2). Immunochemical cross-reactivity of LPS of these serotypes with specific typing antisera or monoclonal antibodies (MAb) substantiates their relatedness. In particular, the only difference between the OSA chemical structures of serotypes O5 and O16 is the α or β configuration of the inter-glycosidic bond at the reducing end, respectively (15, 16). Interestingly, the OSA biosynthesis cluster spanning \textit{pa3160-pa3145} (\textit{wzz1-wbpL}) genes of O5 is identical to that in serogroup O2. This suggests that genes located outside the \textit{wbp} cluster are responsible for the chemical differences in the OSA structures. As such, genes responsible for the chemical differences in OSA structures were likely to have come from external sources such as lysogenic bacteriophage (17) or other ancestral microbial species.

Serotype conversion following bacteriophage infection has long been observed in diverse Gram-negative bacteria (18-22). Upon infection of \textit{P. aeruginosa} PAO1 (serotype O5) by the bacteriophage D3, the bacteria became resistant to future infections, illustrating the lysogenic property of this lambdoid D3 phage (23). D3 phage utilizes the O5 OSA as a receptor, leading to downstream serotype conversion of the bacteria to O16 serotype. The conversion involved a switch from an α to a β bond linkage between OSA repeat units (24). Taking advantage of the annotated D3 phage genome (25), our group identified a 3.6-kb DNA fragment containing three open reading frames. Expression of the entire seroconverting unit in \textit{P. aeruginosa} PAO1 resulted in the same observed loss of reactivity to O5-specific MAb MF15-4 (26), and a conversion to serotype O16 as described above. Characterization of this 3.6-kb fragment
revealed a three-component system containing genes that encode a putative O-acetyl transferase (OAc), a putative β-polymerase Wzyβ, and an α-polymerase inhibitor (Iap) peptide (27).

The Iap peptide is encoded in a 0.9-kb fragment of the three-gene seroconverting unit, which when supplied in trans, inhibits production of α-linked long-chain OSA in serotypes O5, O18 and O20. Transformation of serotype O16 with iap did not show any inhibitory effect on OSA production, illustrating the specificity of Iap towards α-linked OSA biosynthesis in the O2 serogroup (27). Our group performed Southern blot analysis and demonstrated that iap is present in the genomes of serotypes O2 and O16, which would explain the lack of α-linked OSA in these strains. A subsequent study by our group showed that in addition to iap, a chromosomal copy of wzyβ (responsible for the β-linked OSA of these serotypes) is actively expressed in the O2 and O16 serotypes (28).

The mechanism of Wzyα suppression by iap is currently unknown. The translated product of iap is a 3.1-kDa protein (27). In this study, we further investigated Iap-mediated inhibition of OSA in the P. aeruginosa PAO1 background, utilizing previously generated chromosomal mutants deficient in the chain-length regulator(s) wzz1 and/or wzz2. The observed differences in Iap-mediated inhibition were dependent on the presence or absence of both Wzz proteins. These observations suggested that inhibition due to Iap occurred downstream of Wzyα insertion into the IM. Strong sequence homology was detected between a putative transmembrane segment (TMS) in the Iap and that of the first TMS of both PAO1 Wzz1 and Wzz2. Together, these findings indicate that the inhibitory activity of Iap is specifically targeted against Wzyα through mimicry of the Wzz TMS. Moreover, these data provide additional evidence to support direct interaction between Wzy and Wzz1/2 in the Wzx/Wzy-dependent LPS assembly pathway.
MATERIALS AND METHODS

DNA manipulations. Strains and plasmids used in this study are outlined in Table 1. Cloning iap into the arabinose-inducible expression vector pHERD20T (29) required amplification with iap-specific primers (Fig. S1), containing an EcoRI and PstI site, respectively from a previously-generated pET28-His6-Iap construct (not published). The His₆ coding sequence was retained for use in future experiments. Ligation products were introduced into E. coli DH10B by heat shock transformation. Plasmid DNA from positive clones was extracted using a Plasmid Purification kit (Life Technologies Inc, Burlington, ON) and identified by sequencing utilizing the pBAD forward primer. QuikChange (Agilent) site-directed mutagenesis was utilized to remove the leader sequence present in-frame at the 5’end of the pHERD20T vector (Fig. S1). Successful constructs were identified in the same manner as described above. Positive clones and previously generated plasmids were introduced into electrocoment P. aeruginosa PA01 wzz₁, wzz₂ and wzz₁-wzz₂ chromosomal mutants using a Gene Pulser instrument (Bio-Rad).

LPS preparation and visualization. Each of the P. aeruginosa strains were grown overnight from a single colony with shaking (200 rpm, 37°C) in the presence of 300 µg/ml of carbenicillin or 90 µg/ml of tetracycline to maintain the plasmids and L-arabinose concentrations to induce expression from pHERD. In the Wzz investigations using pHERD20T-His₆-Iap, 0, 0.1, 0.5, and 1% (w/v) L-arabinose concentrations were tested. The following morning, the cultures were equilibrated to the equivalent of 1 ml of culture at OD₆₀₀ of 0.45. LPS was then prepared as per the proteinase K digest method (12, 30). The LPS samples (5 µl) were analyzed by Western immunoblotting first probing with murine MAb MF15-4 (serotype O5 O-Ag specific) and MAb 5c-7-4 (inner-core specific). To quantify OSA levels in each of the wzz mutants, independently-isolated triplicate LPS samples were processed according to a previously described method (6).
Bioinformatic Analyses. The Iap sequence was analyzed by the following topology prediction algorithms to probe for the presence of a predicted transmembrane segment (TMS) (31): TopCons (http://topcons.cbr.su.se/) (32), HMMTOP (http://www.enzim.hu/hmmtop) (33), TMHMM (http://www.cbs.dtu.dk/services/TMHMM) (34), PHOBIUS (http://phobius.sbc.su.se) (35), TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) (36), Philius http://www.yeastrc.org/philius/pages/philius/runPhilius.jsp;jsessionid=6DD32D6307F337296FF (37), TOPPRED (http://www.sbc.su.se/~erikw/toppred2) (38) and PredictProtein (http://www.predictprotein.org) (39). Alignments of Iap to the PCP sequences were performed by ClustalW with a gap extension penalty of 1.0 (40, 41). The 3D model of Iap was generated using the I-TASSER de novo platform which produces a model structure based on sequence alignment to existing PDB files (42).
RESULTS

The OSA chain-length regulator proteins Wzz1 and Wzz2 are not the target of Iap. To determine whether Iap was inhibiting long-chain OSA biosynthesis through interference with either of the Wzz proteins in P. aeruginosa PAO1, the pD3 vector (i.e. pUCP26-Iap), was used to transform the WT strain as well as the wzz1, wzz2 and wzz1-wzz2 knockout strains.

Transformation of WT P. aeruginosa PAO1 with pD3 resulted in a reduction of OSA production (Fig. 1 lane 2) as detected through Western immunoblot analysis. Interestingly, OSA inhibition in either of the wzz1 or wzz2 single mutant backgrounds occurred at the same observed rate as WT PAO1. This indicates that neither of the two Wzz proteins is the specific target of inhibition by Iap, as there was no restoration of long-chain OSA. In contrast, when iap was expressed in the wzz1-wzz2 double mutant, total abrogation of OSA production was observed (Fig. 1 lane 8).

OSA inhibition by Iap is influenced by the presence of Wzz proteins. To ensure that the complete abrogation of OSA production observed in the wzz1-wzz2 double mutant transformed with pD3 was not simply a high-dosage artifact due to constitutive expression from the multi-copy pUCP26 backbone (43), iap was subcloned into the tuneable pHERD20T plasmid. Titratable iap expression was attained through addition of increasing amounts of L-arabinose. To rule out the possibility that any observed variations in OSA levels were simply due to differences in the amount of LPS being produced among the three Wzz mutant strains, the OSA levels from each were quantified via densitometry following Western immunoblotting analysis. The density of OSA was normalized by comparing the intensity of the OSA band to that of its respective inner-core oligosaccharide band, which is synthesized via an unrelated pathway and therefore levels would remain constant despite changes in OSA production. Thus
these lower-molecular-weight bands detected in the Western immunoblots were used as a loading control. Equivalent amounts of OSA were detected in all three \textit{wzz} mutants (Fig. S3).

In either of the \textit{wzz}_1 or \textit{wzz}_2 single knockout strains, OSA production was inhibited to levels similar to WT, retaining background levels equivalent to each other when induced with up to 1\% L-arabinose (Fig. 2A and B). In contrast, un-induced expression of \textit{iap} in the \textit{wzz}_1-\textit{wzz}_2 double mutant was sufficient to inhibit OSA production. Total abrogation was observed at 0.1\% L-arabinose induction, a ten-fold lower concentration than that required to observe changes in OSA production in either of the single \textit{wzz} mutants (Fig. 2C).

**Sequence conservation between \textit{iap} and the predicted N-terminal TMS of \textit{Wzz}\textsubscript{1/2}**

suggests a mechanism of specificity. Upon aligning the \textit{iap} amino acid sequence to that of either full-length \textit{Wzz}\textsubscript{1} or \textit{Wzz}\textsubscript{2} sequences, a high degree of sequence conservation was observed between \textit{iap} and the N termini of both \textit{Wzz} proteins in a region corresponding to the predicted first TMS of either \textit{Wzz} (Fig. 3 and Fig. S4). Conversely, alignments of \textit{iap} to annotated full-length \textit{Wzz} protein sequences from a heterologous strain, \textit{P. aeruginosa} PA7 (serotype O12), and from other bacteria (\textit{Escherichia coli} K12, \textit{Salmonella enterica} sv. \textit{typhimurium}, and \textit{Vibrio cholerae}) did not display homology to their respective TMS regions; instead, the \textit{iap} sequence was more favourably aligned to tracts present in the soluble periplasmic domains of these heterologous \textit{Wzz} proteins. This difference in alignment regardless of amino acid characteristics demonstrates an association with the OSA biosynthesis machinery of the O5 serotype of \textit{iap} to \textit{Wzz}\textsubscript{05}, (Fig. S4).

**In silico prediction of \textit{iap} topology suggests a single TM spanning domain.** The \textit{iap} sequence is composed of 31 amino acids, wherein 52\% of residues are hydrophobic, supporting
the notion of membrane localization. The Iap amino acid sequence was subjected to in silico topology prediction analyses using nine distinct algorithms (31, 44), which consistently predicted a single TMS spanning domain. Based on these outputs, Iap was predicted to contain a TMS between residue 6 and residue 28. In addition, the N and the C termini were predicted to reside in the cytoplasm and periplasm, respectively (Fig. 4).

To further support these findings, a de novo 3D structure of Iap was generated by the alignment modeling platform I-TASSER. Upon superimposition of the three highest-scoring models, a conserved α-helical region between residues 12 and 29 was displayed. This corresponds to the putative TMS of Iap (Fig. 5). Surface electrostatics analysis of the Iap structure revealed hydrophobic properties for the α-helical segment, while the N-terminal tail was shown to be cationic. These data fit with the proposed orientation of Iap in the IM (Fig. 4) and are consistent with the demonstrated “positive-inside rule”, which states that the cytoplasmic domains of TMS-containing proteins or peptides retain a net-positive charge (45, 46) (Fig. S6).

Overexpression of Wzz in P. aeruginosa serotype O16 results in recovery of α-linked OSA. As mentioned in the introduction, serotype O16 has previously been shown to be unable to produce detectable α-linked OSA. If Iap in this background is indeed mimicking a Wzz TMS, then overexpression of Wzz should be able to out-compete Iap for access to Wzyα and restore synthesis of α-linked OSA. To test this hypothesis, Western immunoblotting of triplicate LPS samples obtained from the previously generated P. aeruginosa serotype O16 wzyβ::Gm8 strain transformed with the pUCP26-wzz1 plasmid showed LPS bands that reacted with O5-specific antibodies (Fig. 6). This indicates that α-linked OSA similar to that seen in strain PAO1 was produced by this transformant.
Seroconversion in *P. aeruginosa* PAO1 caused by bacteriophage D3 was observed earlier by our group after transformation of the bacterium with the aforementioned ‘seroconverting unit’ (consisting of three-genes from the D3 chromosome), resulting in abrogation of long-chain OSA production. One of these three genes, *iap*, was identified to be the cause of inhibition of long and very-long chain OSA biosynthesis (Newton et al. 2001).

Results from the current study indicate that the specificity of the Iap inhibitory activity is against the native Wzy\(_{α}\) of serotype O5. We initially attempted co-localization studies to directly examine Iap-Wzy\(_{α}\) interaction; however, due to the hydrophobic nature of the Iap peptide, such evidence could not be obtained. Subsequently, we decided to take advantage of the well-defined *wzz*\(_1\), *wzz*\(_2\), *wzz*\(_1\)-*wzz*\(_2\*) chromosomal mutants and the unique panel of MAbs from our laboratory to elucidate the target of Iap inhibition. Our results show that the target of the Iap-mediated OSA inhibition is Wzy\(_{α}\), a key component of the putative membrane complex involved in the Wzx/Wzy-dependent LPS assembly pathway.

The phenotype resulting from Iap expression was the loss of long- and very-long-chain OSA in *P. aeruginosa* PAO1, especially when the peptide was expressed in the multi-copy plasmid pUCP26. Iap expressed in the *wzz*\(_1\) and *wzz*\(_2\*) single PCP mutants did not display observable changes in OSA production when compared to the effect of Iap on the WT PAO1 background; therefore, the chain-length regulators are not the target of Iap function. This is consistent with previous observations made by our group in which expression from the pD3 plasmid (consisting of the 3-gene seroconverting segment of D3 bacteriophage) in *P. aeruginosa* PAO1 resulted in β-linked OSA subunits being polymerized by the phage-encoded Wzy\(_β\) to form
LPS of defined modal lengths (despite the presence of Iap) that PCP proteins are not the target of Iap inhibition (27, 28).

In order to be able to draw conclusions of the effect of Iap activity in the different wzz mutant backgrounds, it is essential to determine whether equivalent levels of OSA are being produced. To our knowledge, this is the first study to investigate whether the absence of either or both Wzz1 or Wzz2, would affect the total amount of OSA on the cell surface (Fig. S3). With equivalent levels of OSA being synthesized between the three wzz mutant strains, the low Iap levels required for suppression of LPS production in the wzz1-wzz2 double mutant suggest that in the absence of Wzz proteins, Iap has unencumbered access to Wzyα. This is in contrast to either of the single wzz mutants, where a higher amount of Iap would be required to out-compete Wzz for Wzyα by the presence of the remaining Wzz protein.

In addition, this suggests that Iap-mediated inhibition of Wzyα function occurs after the α-polymerase has inserted into the IM. If the Iap were to interfere with the wzyα RNA or exert its effect to disrupt transport or insertion of Wzyα into the cell membrane, then utilizing the pHERD system to express iap in P. aeruginosa would have resulted in a consistent inhibition phenotype regardless of WT or co-polymerase mutant background strain. This is regardless of whether wild-type or co-polymerase mutant backgrounds were used to test the effect of iap.

In previous investigations, neither the WT O16 nor O16 wzyβ::GmR was able to produce α-linked OSA due to the presence of the seroconverting unit, particularly iap, within the genome. Previous work in our lab demonstrated that Wzyα from serotype O16 is indeed functional; however, it is being actively inhibited. To further investigate the Iap-Wzy-Wzz interaction, Wzz was overexpressed in the O16 wzyβ::GmR background to determine whether any amount of α-
linked OSA can be restored. Several advantages were exploited through the use of this particular strain. Firstly, the lack of CPA allowed for a more clear observation of changes in the OSA. In addition, iap expressed from the chromosome precluded the use of two separate plasmids. Finally, with the absence of the β-polymerase any restoration of α-linked OSA was more easily detected. As reported previously, (28), there is no background O5 OSA being polymerized in the absence of Wzyβ. When Wzz was overexpressed, partial restoration of the α-linked OSA phenotype was restored indicating that Wzz was able to out-compete Iap to interact with Wzyα. Future characterization of the binding kinetics and affinity of Iap for Wzyα will help to better understand events at the molecular level leading to the abrogation of OSA biosynthesis.

Wzz proteins exist throughout all Gram-negative organisms and are required not only for the biosynthesis of LPS but also for the biosynthesis of Enterobacterial Common Antigen (ECA) (47) and capsule polysaccharide (48). Wzz proteins are characterized by two TMS, a large periplasmic domain and the preference to organize in a higher ordered homo-oligomeric state (11, 12, 49-53). The sequence similarity between PCP proteins is limited to approximately 19-20%; however, the secondary structures of these proteins are quite conserved (12). The sequence similarity between Iap and the proposed N-terminal TMS of either P. aeruginosa PAO1 Wzz suggests a mechanism of specificity to the O5 OSA machinery. Based simply on hydrophobicity, it would not be unexpected for a hydrophobic IM-spanning peptide to align with other TMS domains. However, alignment of Iap to heterologous Wzz protein sequences does not yield the same alignment positioning as that detected for the P. aeruginosa PAO1 Wzz1 or 2 templates. This similarity might provide a clue to substrate specificity explaining the targeted inhibition of OSA production in serotype O5 strain. Recently, high-resolution X-ray crystal structures of the periplasmic domain of Wzz from Shigella flexneri and from other bacterial species have been...
obtained (53, 54). These structures revealed a conserved structural motif, and functional
investigation through site-directed mutagenesis of residues proposed to be involved in substrate
binding and oligomerization (49, 50, 55).

A leading hypothesis to describe the interplay between proteins in the Wzx/Wzy-
dependent pathway, including Wzy and Wzz, is based through loose proximity to one another
and the resulting interaction is mediated through mutually-shared OSA substrate bridging the
two proteins (11, 56, 57). However, genetic data indirectly support interaction of the Wzx
flippase with its corresponding Wzy and Wzz proteins (58). In addition, a new investigation
using heterologous bacterial 2-hybrid screens have provided evidence to support interactions
between Wzy proteins and their corresponding PCP (59).

Although considerable efforts have been made to investigate the periplasmic domain of
Wzz proteins, the importance of the two TMS regions has been largely overlooked. Site-directed
mutagenesis of conserved motifs spanning both the N- and C-terminal TMS have been
investigated in *S. flexneri* (60). Of particular relevance to this study are the residues substituted
in the N-terminal TMS contained within the conserved “KTMII” motif. Complete loss of high-
molecular-weight O-Ag was observed in the K31A mutant whereas an M32T mutation resulted
in altered O-Ag modal lengths approximately 10-15 residues shorter than WT, thus
demonstrating the importance of the N-terminal TMS regions in O-Ag chain length regulation
(60). In a recent study by our group, (S. T. Islam, S. M. Huszczynksi, T. Nugent, A. C. Gold, and
J. S. Lam, submitted), substitution of cytoplasmic amino acid residues in Wzyα from *P.
aeruginosa* PAO1 were found to eliminate OSA bands at modal lengths corresponding to those
regulated by Wzz1 (but not those regulated by Wzz2); these cytoplasmic amino acid substitutions
in Wzyα have led to altered predicted packing of the C-terminal TMS of the polymerase. These
observations suggest that a specific interaction between Wzy and Wzz\(_1\) had been disrupted, as OSA bands corresponding to Wzz\(_2\)-mediated very-long chain regulation were not affected. If Wzy\(_\alpha\) and Wzz\(_{1/2}\) were simply localized in close proximity, the site-directed mutations introduced to the cytoplasmic amino acid residues of Wzy\(_\alpha\) should not have exerted an effect on the downstream process of OSA chain-length regulation. These observations are consistent with the result shown in the present study, providing the first evidence for a direct interaction between homologously-expressed Wzy and Wzz proteins, with the TMS of Wzz proteins likely playing an integral role in this process.

Based on our current observations and the documented relationship between Wzy and Wzz, a possible mechanism of Iap-mediated inhibition of OSA polymerization would involve Iap outcompeting Wzz for interaction with Wzy. Hence, the bacteria would exhibit a phenotype lacking long-chain polysaccharide with \(\alpha\)-linked O-units. Herein, we have exploited Iap as a unique tool with which to examine the biosynthesis of O-Ag via the Wzx/Wzy-dependent pathway. Further investigations on the molecular mechanism of Iap-mediated O-Ag inhibition would help to shed more light on the assembly of various virulence-associated cell-surface polysaccharides via this assembly scheme.
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FIG. 1. Effect of iap expression in *P. aeruginosa* PAO1 wzz chromosomal mutant strains. Western immunoblot probed with OSA-specific MAb MF15-4. The control lanes include: *P. aeruginosa* PAO1, PAO1 transformed with pD3 and each wzz mutant construct (*wzz*1, *wzz*2, and *wzz*1-*wzz*2). The wzz mutants are then transformed with pD3.

FIG. 2. Effects of iap titration on OSA levels in various wzz knockout strains. Western immunoblot of LPS prepared from *P. aeruginosa* PAO1 wzz chromosomal mutants transformed with pHERD20T-His6-Iap. Expression levels were titrated via increasing concentrations of L-arabinose (0, 0.1, 0.5 and 1.0%). Immunoblots were subsequently probed with MAb specific to O5 OSA (MAb MF15-4) and inner-core oligosaccharide (MAb 5c-7-4) (denoted by *). WT *P. aeruginosa* PAO1 and PAO1 transformed with pUCP26-Iap were used as wild type (WT) and OSA− controls in each set of blots. (A) LPS harvested from the *wzz*1 chromosomal mutant background. (B) LPS harvested from *wzz*2 chromosomal mutant background. (C) LPS harvested from the *wzz*1-*wzz*2 double chromosomal mutant.
FIG. 3. Sequence alignment of Iap to (A) Wzz₁ and (B) Wzz₂ of *P. aeruginosa* PAO1. The alignment was produced in ClustalW and colored according to conservation score (out of 10) as represented by Jalview: red = 10, orange = 9, yellow = 8 and blue = 7. The black line denotes the predicted transmembrane region of either Wzz. Both alignments demonstrate strong similarities between the Iap sequence and Wzz from *P. aeruginosa* strain PAO1, alluding to substrate specificity.

FIG. 4. Topology prediction algorithm analysis of Iap. The amino acid sequence of Iap was analyzed using nine different algorithms to determine whether a conserved single TMS spanning domain was predicted for a putative TMS. The Iap amino acid sequence is depicted from 1 – 31. Color key: blue line, cytoplasmic localization; black box, range of amino acids predicted to form a TMS; red line, periplasmic localization.

FIG. 5. I-TASSER three-dimensional modeling of Iap. The three highest-quality models for the Iap structure were superimposed using PyMOL. Residues from 1-13 are predicted to form a disordered coil, while residues 14-31 are predicted to form an α-helix.

FIG. 6. Effect of *wzz₁* overexpression in *P. aeruginosa* serotype O16 *wzy::Gm*<sup>R</sup>. Western immunoblot probed with OSA-specific MAb MF15-4 (denoted by the *) and MAb 5c7-4 (denoted by *). The control lanes include: *P. aeruginosa* PAO1, O16 *wzy::Gm*<sup>R</sup> and PAO1 *wzz₁*-wzz₂ double mutant transformed with pUCP26-*wzz₁*. The last 3 lanes contain O16 *wzy::Gm*<sup>R</sup> transformed with pUCP26-*wzz₁*.