Five New Genes Are Important for Common Polysaccharide Antigen Biosynthesis in *Pseudomonas aeruginosa*

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ABSTRACT Common polysaccharide antigen (CPA) is a conserved cell surface polysaccharide produced by *Pseudomonas aeruginosa*. It contains a rhamnan homopolymer and is one of the two forms of O polysaccharide attached to *P. aeruginosa* lipopolysaccharide (LPS). Our laboratory has previously characterized an eight-gene cluster (*pa5447-pa5454* in *P. aeruginosa* PAO1) required for biosynthesis of CPA. Here we demonstrate that an adjacent five-gene cluster *pa5455-pa5459* is also involved. Using reverse transcriptase PCR (RT-PCR), we showed that the original eight-gene cluster and the new five-gene cluster are both organized as operons. We have analyzed the LPS phenotypes of in-frame deletion mutants made in each of the five genes, and the results verified that these five genes are indeed required for CPA biosynthesis, extending the CPA biosynthesis locus to contain 13 contiguous genes. By performing overexpression experiments of different sets of these biosynthesis genes, we were able to obtain information about their possible functions in CPA biosynthesis.

IMPORTANCE Lipopolysaccharide (LPS) is an important cell surface structure of Gram-negative bacteria. The human opportunistic pathogen *Pseudomonas aeruginosa* simultaneously produces an O-antigen-specific (OSA) form and a common polysaccharide antigen (CPA) form of LPS. CPA, the focus of this study, is composed of α-1-2, α1-3-linked D-rhamnose sugars and has been shown to be important for attachment of the bacteria to human airway epithelial cells. Genome sequencing of this species revealed a new five-gene cluster that we predicted to be involved in CPA biosynthesis and modification. In this study, we have generated chromosomal knockouts by performing in-frame deletions and allelic replacements. Characterizing the function of each of the five genes is important for us to better understand CPA biosynthesis and the mechanisms of chain length termination and regulation of this unique D-rhamnan polysaccharide.

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Lipopolysaccharide (LPS) is an important cell surface structure of Gram-negative bacteria. It is the major component of the outer leaflet of the outer membrane, and it mediates direct interactions with the environment and host cell receptors. Due to its biological significance, it has been studied extensively. Our understanding of how these polysaccharides are assembled is based on detailed study of a number of model systems reviewed in reference 1. Currently, three mechanisms are known for LPS biosynthesis: the synthase-dependent pathway, the Wzy-dependent pathway, and the ABC (ATP binding cassette) transporter-dependent pathway (1). According to the current model, ABC transporter-dependent O polysaccharides are assembled on an undecaprenyl-pyrophosphate lipid carrier at the cytoplasmic face of the inner membrane. The chain grows by successive addition of monosaccharide units to the nonreducing terminus, catalyzed by a number of cytoplasmic glycosyltransferase enzymes which use sugar-nucleotides as activated precursors. The ABC transporter then exports the polysaccharide to the periplasmic face of the inner membrane where it is ligated to lipid A-core.

O polysaccharides (OPS) are found on the bacterial cell surface with a range of different polymer lengths, but they are not all equally common. A large proportion of chains usually have sizes close to one or more so-called modal chain lengths. Production of OPS chains with the correct size distribution can be important for the biological properties of this molecule, for instance, to confer resistance to complement-mediated killing (2). The means by which a modal chain length distribution is achieved has been a question of particular interest because of a desire to understand the quality control mechanisms that regulate the size of this biological polymer. Unlike DNA, RNA, and protein, polysaccharide chain lengths are not determined by the size of a nucleic acid template. Two models for ABC transporter-dependent OPS chain length control have been described. In *Klebsiella pneumoniae* O2a, the ABC transporter is critical for controlling chain length, and overexpression of the ABC transporter-encoding genes can dramatically shorten the OPS chains produced (3). In such a system, export functions as the termination step for chain synthesis by removing the OPS from the subcellular compartment where glycosyltransferases and sugar-nucleotides are located. In the model exemplified by the *Escherichia coli* O8 and O9a systems, chain
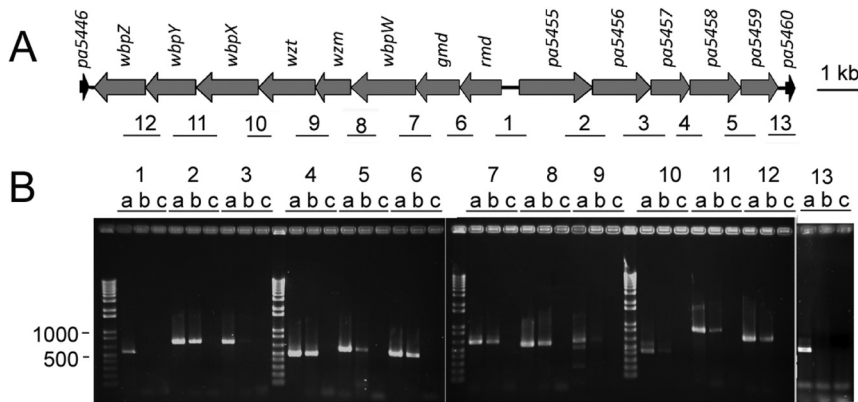
FIG 1 RT-PCR to examine cotranscription. (A) CPA (common polysaccharide antigen) regions amplified by the 13 primer pairs (see Table S1 in the supplemental material). (B) Agarose gel of the PCR amplification products using the 13 primer pairs. The molecular marker is the 1-kb+ DNA ladder standard from Invitrogen.

extension continues until a specific enzyme called WbdD catalyzes a covalent modification of the nonreducing terminal sugar. In the O8 serotype, the modification is a methylation reaction; in the O9a serotype, it is phosphorylation which is subsequently followed by methylation (4). Export can occur only after the terminal modification has been made, because the modified sugar is recognized by a specific domain of the transporter (5). In this case, overexpression of the ABC transporter genes does not substantially change the OPS chain lengths (3).

Pseudomonas aeruginosa is an important opportunistic human pathogen that can infect immunocompromised individuals such as patients with cystic fibrosis (CF), cancer, AIDS, or burn wounds. Among the virulence factors *P. aeruginosa* produces, LPS has been shown to be a major factor (6, 7). LPS can be conceptually divided into three structural domains: lipid A, which anchors the LPS in the outer membrane; a long-chain O polysaccharide (OPS); and a core oligosaccharide that links the lipid A and the OPS. Most *P. aeruginosa* strains simultaneously produce two distinct forms of OPS. One of these is a heteropolymer composed of repeating units of two to five distinct sugars and is termed O-specific antigen (OSA; formerly called B band). The other form of OPS contains a homopolymer of D-rhamnose (D-Rha). Since this structure is produced by the majority of *P. aeruginosa* strains, it has been named the common polysaccharide antigen (CPA; historically called A band) (8). Of the two O polysaccharides, CPA chains are shorter and less immunogenic than OSA, likely due to the neutral charge of the rhamnan. There is evidence that CPA is a virulence factor in the context of *P. aeruginosa* infections. During chronic lung infection in CF patients, there is a progressive decrease in the production of the OSA, while LPS-containing CPA is maintained on the cell surface of *P. aeruginosa* (9). It has also been demonstrated that CPA plays an important role in the attachment of *P. aeruginosa* to human airway epithelial cells *in vitro* (10).

The structure of CPA has been analyzed by various groups, including our laboratory, and the results showed that the central structural element of CPA is a polymer with a trisaccharide repeating unit: $[\rightarrow3)\text{-}\alpha\text{-}D\text{-Rha-}(1\rightarrow2)\text{-}\alpha\text{-}D\text{-Rha-}(1\rightarrow3)\text{-}\alpha\text{-}D\text{-Rha-}(1\rightarrow]_n$ (11–13). However, due to difficulties in isolating large quantities of CPA LPS, the structure of CPA has not been fully elucidated so that, for example, it is not known how the CPA is

attached to lipid A-core or whether there are any modifications or additional sugars on the distal terminus. In analyses of monosaccharide constituents of CPA preparations, small amounts of other components have been identified, albeit with some inconsistency between reports. These components include 3-*O*-methylrhamnose, 3-*O*-methylhexose, 3-*O*-methyl-6-deoxyhexose, ribose, mannose, glucose, xylose, alanine, galactosamine, and small amounts of *O*- and *N*-linked acetyl substitutions (11–13). The presence of these rare components suggests the possibility that consistent with what has been observed in the LPS of many other species, additional sugars may be present in the CPA domain either as linker/adapter moieties between the CPA OPS and the core, and/or as additional substituents to the nonreducing terminus of the OPS chain.

An eight-gene cluster (from *rmd* to *wbpZ*, *pa5447-pa5454* in *P. aeruginosa* PAO1 [Fig. 1]) important for the biosynthesis of CPA was previously identified and studied in our laboratory. Three of the proteins encoded by these genes, WbpW, Gmd, and Rmd are involved in the biosynthesis of the precursor nucleotide sugar, GDP−D-Rha. Three putative glycosyltransferases, WbpX, WbpY, and WbpZ, have been proposed to be involved in the assembly of the trisaccharide D-Rha repeating unit. Wzm and Wzt form the ABC transporter for the translocation of undecaprenol-linked CPA PS to the periplasmic side of the cytoplasmic membrane (14–16). The involvement of *wzm* and *wzt* imparts the biosynthesis of CPA into the particular category of ABC transporter-dependent pathways.

Most recently, our lab has presented bioinformatic evidence that a five-gene cluster (*pa5455-pa5459* [Fig. 1]) may also be involved in CPA biosynthesis and modification (7, 8). Gene sequence analysis indicated that *pa5455* and *pa5456* encode putative glycosyltransferases. The *pa5458* gene encodes a protein with a conserved acetyltransferase domain. PA5457 and PA5459 contain conserved methyltransferase domains and show sequence similarity to *E. coli* O8 O-antigen terminator protein WbdD. As mentioned earlier, it is not known how the rhamnan is linked to lipid A-core, or what the structure of the nonreducing terminus of the polysaccharide chain is. Given that methyl sugars and acetylation have been reported in CPA composition analyses and it is entirely plausible that previously unidentified sugars may be present at either end of the polymer, we hypothesized that the *pa5455-pa5459* gene cluster is part of the CPA biosynthesis locus encoding functions for biosynthesis and chain length regulation of the polymer.

To test the hypothesis, we prepared in-frame nonpolar deletion mutants, and the LPSs of these mutants and complemented strains were analyzed by SDS-PAGE and visualized via silver staining and Western immunoblotting techniques. Further information about the possible functions of these genes in CPA biosynthesis was obtained by analyzing the effects of overexpressing them in *P. aeruginosa*. Reverse transcriptase PCR (RT-PCR) was also performed to examine the cotranscription of the genes in the five-gene cluster as well as the previously identified eight-gene cluster.

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1 TGAGTCAAgga                    ttatcgcgctccaatctatgccaagaatgcgccgatttcaaccgcttattcgtgaccgg 160
  ACTCAGTTcct  (90 bp omitted)   aataGcgcgaggttagacacggttcttacgcggctaaagttggcgaataagcactggcc
      rmd                            ↑            <<<<<<                    <<<<<<
                                    TSS           -10 box                  -35 box

                -35 box                        -10 box          TSS
                >>>>>>                         >>>>>>             ↓                        → pa5455
(30 bp omitted) ctccgtcagccttcacgttttttgcgaaaggtatgtttagaatgcctcTcg (170 bp omitted) atgccATGAATTTC 424
                gaggcagtcggaagtgcaaaaacgctttccatacaaatcttacggagagc                      tacggTACTTAAAG
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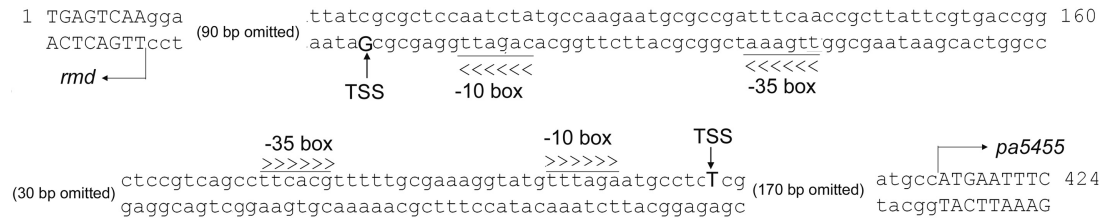
FIG 2 Identification of the promoter sequence upstream of *rmd* (8-gene cluster) and *pa5455* (5-gene cluster), respectively. The boldface capital letter letters with an arrow above or below it indicate the positions of the transcription start site (TSS) detected using 5′-RACE. The putative −10 and −35 boxes are indicated.

RESULTS

The two gene clusters (*pa5447-pa5454* and *pa5455-pa5459*) are organized as two operons. Reverse transcriptase PCR was performed using forward and reverse primer pairs designed from neighboring genes in each of the gene clusters (Fig. 1A; see Table S1 in the supplemental material). For each pair of primers, three types of template were used: *P. aeruginosa* PAO1 genomic DNA (positive control), reverse-transcribed first strand cDNA (for testing cotranscription), and total RNA (negative control to ensure that there is no genomic DNA contamination in the RNA samples). As expected, all positive controls gave PCR products of expected sizes (Fig. 1B, lanes a) and all negative controls did not give any PCR product (Fig. 1B, lanes c). Fragment 1 which covers the intergenic region between the two clusters that are known not to be cotranscribed did not give any PCR product after the RT-PCR procedure (Fig. 1B, lane 1b). RT-PCR products were observed for fragments 2, 3, 4, and 5, covering *pa5455-pa5459*, suggesting that these genes are cotranscribed, while fragment 13 did not yield any RT-PCR product (Fig. 1), indicating that *pa5460* is not part of the operon. Fragments 6 to 12, which cover the genes within the *rmd-wbpZ* cluster, all yielded positive RT-PCR results (Fig. 1B), confirming that these eight genes are also transcriptionally linked.

Identification of promoter elements. By using 5′ rapid amplification of cDNA ends (5′-RACE), a putative transcription start site (TSS) was identified for the *rmd-wbpZ* operon 98 bp upstream of the *rmd* start codon (Fig. 2). By manually examining the sequence upstream of the detected TSS, putative −10 (CAGATT) and −35 (TTGAAA) boxes were identified (Fig. 2). A putative TSS was also identified for the *pa5455-pa5459* cluster at 178 bp upstream of the start codon of *pa5455* using 5′-RACE. Putative −10 box (TTTAGA) and −35 box (TTCACG) regions were present as indicated in Fig. 2.

The presence of the −10 and −35 boxes upstream of the detected transcription start sites indicates that these promoters are likely recognized by the sigma 70 factor RpoD. Electrophoretic mobility shift assay (EMSA) was then performed to detect the binding of RpoD (from *E. coli*) to the predicted promoter region. It was found that similar to the control promoter P-*lac*UV5 (17), the *E. coli* holoenzyme containing RpoD was able to bind the wild-type probe for both P-*rmd* and P-*pa5455*, but not to the mutated versions where the −10 boxes were either deleted (P-*lac*UV5-M and P-*rmd*) or replaced with a GC-rich sequence (P-*pa5455*) (Fig. 3).

To test the promoter activity, a promoter trap vector, pTZ110, was used. Based on the in vitro β-galactosidase activity assay carried out with the various pTZ110 constructs, P-*rmd* showed an activity of 834.6 ± 17.1 Miller units and P-*pa5455* showed an activity of 474.6 ± 51.6 Miller units, and both activities were significantly higher than that of the empty vector (which only showed a background level of activity of 11.9 ± 1.2 Miller units), while the positive control P-*rpoD* showed an activity of more than 19,000 Miller units.

Mutational analysis of *pa5455-pa5459*. To characterize the function of each of the individual genes, we made a set of isogenic mutants in the PAO1 background in which each of the *pa5455-pa5459* genes was knocked out by a nonpolar in-frame deletion within its coding sequence. The LPSs produced by these mutants were analyzed by SDS-PAGE with silver staining and Western immunoblotting. Production of OSA LPS or the uncapped core structure was not affected by mutation of any of the genes in this cluster because the LPS preparations from each of the Δ*pa5455-5459* mutants were reactive to monoclonal antibody (MAb) MF15-4 (OSA specific), and 5c-101 (core specific) in Western blots (see the bottom panel of Fig. S1 in the supplemental material). Western immunoblotting using the CPA-specific MAb N1F10 showed that most of these mutations caused obvious changes to CPA biosynthesis and that complementation was successful in restoring the wild-type phenotype, indicating that this operon is required for CPA biosynthesis (Fig. S1, middle panel).

It is valuable to know that knocking out these genes does not affect OSA biosynthesis. However, the presence of OSA in the LPS samples from the PAO1 mutants made it difficult to observe the CPA phenotypes on silver-stained SDS-polyacrylamide gels. This is because the resolved OSA and CPA bands occupy the same region on the gel. Therefore, to observe more clearly the effect of each deletion mutation on the CPA phenotype, another set of
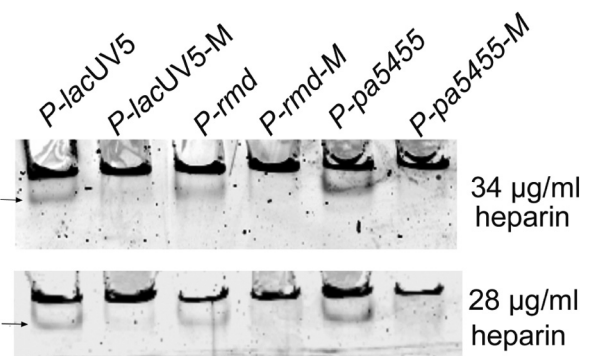

FIG 3 EMSA for detection of binding of *E. coli* RpoD saturated holoenzyme to the predicted promoter sequences. Heparin was supplied to a final concentration of 34 μg/ml (top panel) or 28 μg/ml (bottom panel). The small black arrows to the left of the gels indicate the positions of the bound oligonucleotides on the gels.
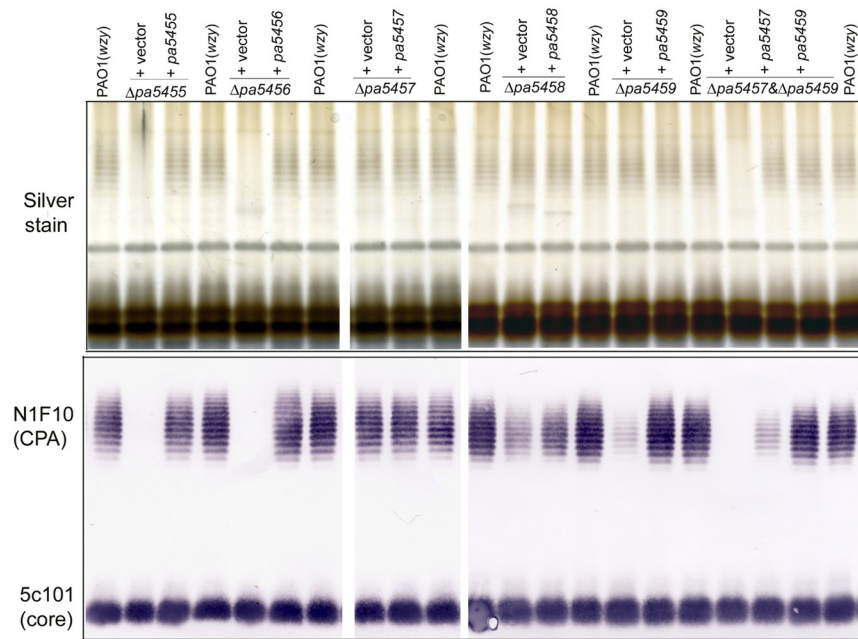
FIG 4 Lipopolysaccharides (LPSs) of in-frame deletion mutants in *P. aeruginosa* PAO1(*wzy*) background. (Top) Silver-stained SDS-polyacrylamide gel; (bottom) Western blot obtained with MAb N1F10 (CPA-specific) and MAb 5c101 (core O-antigen-specific) antibodies (in a 30:1 ratio). Complementation by a plasmid-borne gene in *trans* is indicated by a plus sign and the name of the gene (e.g., + *pa5456*). "+ vector" indicates the presence of the empty vector pHERD20T.

mentation with the wild-type gene reversed the band shift and increased the Western blot staining of the CPA banding pattern, although not back to the intensity of wild-type bands (Fig. 4 and 5A). Because this is an in-frame deletion mutant, it is unlikely that the apparent incomplete restoration of CPA production level is due in this case to a polar effect of the mutation. Subsequent experiments (see below) showed that overexpression of this gene from a plasmid causes a reduction in Western blotting band intensity, so that even with the low arabinose concentrations used in induction of gene expression in the complementation experiment, there may be an effect of the increased copy number of the plasmid-borne gene compared with expression from a single chromosomal copy.

The knockout mutants of the putative methyltransferase genes (*pa5457* and *pa5459*) exhibited different phenotypes. LPS from the *pa5457* mutant was indistinguishable from LPS from the wild type by SDS-PAGE and silver staining analysis and by Western blotting (Fig. 4). Analysis of LPS from the other putative methyltransferase gene mutant, the *pa5459* mutant, showed that the amount of polysaccharide produced by this mutant was similar to that produced by the wild type as judged by silver staining intensity (Fig. 4), but the bands were shifted (Fig. 5B). Furthermore, the Western blotting signals were less in-
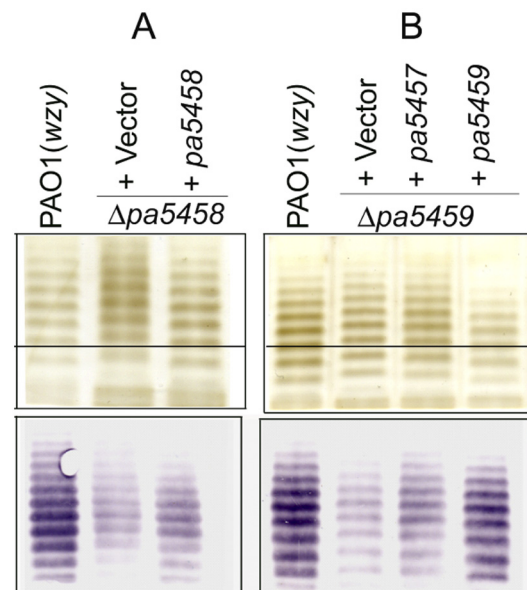
mutants was constructed in an OSA-minus background, PAO1(*wzy*) (6). The phenotypes of these mutants (Fig. 4) were all consistent with those made in the PAO1 strain (see Fig. S2 in the supplemental material), but with clear CPA banding by silver-stained SDS-PAGE analysis.

Mutation of either one of the two putative glycosyltransferase genes, *pa5455* and *pa5456*, completely abrogated CPA biosynthesis as shown by the absence of any detectable CPA banding patterns on either silver-stained gels or Western blots. In both cases, supplying the corresponding gene in *trans* restored production of CPA which was indistinguishable from production by the wild type (Fig. 4). Mutation of the putative acetyltransferase-encoding gene *pa5458* did not abrogate polysaccharide production, and bands were detected on both silver-stained SDS-polyacrylamide gels and Western blots. However, the polymer produced by this mutant has different properties compared to the polymer produced by the wild type. When the run time of SDS-PAGE was increased, it was clear that the bands produced by the mutant were shifted relative to the positions of bands produced by the wild type, and the bands also appeared to be more diffuse (Fig. 5A). In the Western blot, the intensity of staining for the mutant was lower than that for the wild type, and comparison with the silver stain indicates that this was not due to a smaller amount of polysaccharide present (Fig. 4). Note that the differences in LPS reactivity with MAb N1F10 were not due to sample loading variations. The sample loads were monitored on the Western blots by reacting the blots simultaneously with MAb N1F10 and MAb 5c101 (outer core specific) to ensure that the level of reactivity of LPS with the latter antibody remains constant among the different LPS samples. Therefore, the fainter bands on the Western blot indicate weaker binding of MAb N1F10 to the mutant polymer. Comple-

FIG 5 Analysis of LPS from *pa5458* and *pa5459* mutants. These results depict the effects of deletion of *pa5458* (A) or *pa5459* (B) on CPA biosynthesis in the *P. aeruginosa* PAO1(*wzy*) strain. (Top) Silver-stained SDS-polyacrylamide gels; (bottom) Western blots obtained with MAb N1F10.
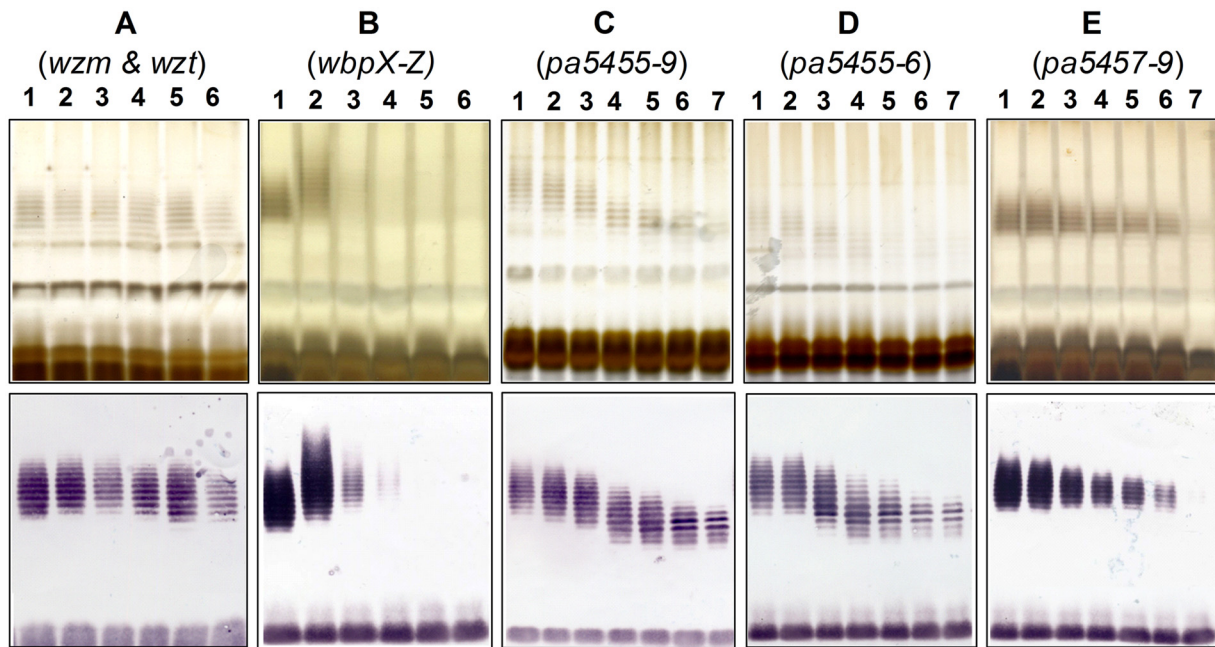
FIG 6 Overexpression of different CPA biosynthesis genes in *P. aeruginosa* PAO1(*wzy*) background. Overexpression of *wzm* and *wzt* genes (A), *wbpX-Z* operon (*wbpX* to *wbpZ*) (B), *pa5455-pa5459* operon (*pa5455* to *pa5459*) (C), *pa5455-pa5456* operon (D), and *pa5457-pa5459* operon (E). Lanes 1, PAO1(*wzy*) control; lanes 2 to 7, induction with increasing concentrations of arabinose, i.e., 0%, 0.01%, 0.05%, 0.1%, 0.2%, and 0.5% arabinose. (Top) Silver-stained SDS-polyacrylamide gels; (bottom) Western blots obtained with MAb N1F10 and MAb 5c101 (uncapped core-specific antibody). The core serves as a loading control to make sure equal amounts of samples were loaded into the different lanes.

tense than those of wild-type LPS (Fig. 4). In all these respects, the wild-type phenotype was restored by complementation (Fig. 4 and 5B). The changes in electrophoretic mobility and antibody binding suggest that the polysaccharide produced by the *pa5459* mutant has a different chemical structure than that of wild-type CPA.

Because the *pa5457* and *pa5459* genes encode proteins with the same class of enzyme activity, we were interested in investigating the phenotype of the double mutant in case there was any redundancy between these two functions. When both of the putative methyltransferase-encoding genes *pa5457* and *pa5459* were deleted, CPA biosynthesis was completely abolished (Fig. 4). When the *pa5457 pa5459* double mutant was provided with *pa5457* or *pa5459* on a plasmid, the resulting LPS resembled that of the *pa5459* mutant or *pa5457* mutant (Fig. 4). Although mutating *pa5457* alone did not result in an altered CPA phenotype, the fact that the phenotype of the *pa5457 pa5459* double mutant is different from that of the *pa5459* single-gene mutant confirms that *pa5457* has a function in CPA biosynthesis.

Overexpression of the CPA biosynthesis cluster. As described in the introduction, overexpression of the ABC transporter components can help to distinguish between different mechanisms for control of the OPS chain length. When we expressed the CPA biosynthesis locus Wzm and Wzt from a plasmid with an arabinose-controlled promoter, we observed a subtle shift in CPA chain length distribution with increasing concentration of the inducer (Fig. 6A). High arabinose concentrations produced a chain length distribution shifted slightly to shorter chains compared to the distribution at low arabinose concentrations. At low arabinose concentrations, the chain length distribution shifted slightly toward longer chains compared to the wild type. It is notable, how-

ever, that all of the CPAs observed in any of the overexpression samples had sizes within the normal range for wild-type CPA. To verify that Wzm and Wzt are expressed in an active form from plasmid pYHJL18, we used the plasmid to perform complementation assays of a *P. aeruginosa wzm wzt* double-knockout mutant and observed restoration of CPA production (see Fig. S2 in the supplemental material).

All three genes, *wbpX, wbpY* and *wbpZ*, have been reported to encode rhamnosyltransferases responsible for assembly of the CPA rhamnan polymer (14). If one or more of these genes encodes the processive transferase(s) responsible for chain elongation, then it would be anticipated that overexpression of these genes would result in production of longer CPA chains. This is exactly what was observed when *wbpX, wbpY*, and *wbpZ*, carried as contiguous genes on a plasmid, were expressed from an arabinose-inducible promoter (Fig. 6B). With increasing concentrations of arabinose, average chain lengths increased, and the intensity of CPA signals on both silver-stained SDS-polyacrylamide gels and Western blots progressively diminished. At the highest arabinose concentration (0.5%), no CPA production was discerned (Fig. 6B).

Overexpression of the five-gene cluster *pa5455-pa5459* resulted in a definite shortening of CPA chains (Fig. 6C). In response to increasing arabinose concentrations, the chain length distribution shifted to shorter lengths until at the highest arabinose concentration (0.5%) most of the chains were shorter than the shortest chains visible in the CPA banding pattern of the wild-type LPS samples. At the highest levels of expression, there was also a reduction in the total amount of CPA detected by both silver-stained SDS-PAGE and Western immunoblotting. This dramatic change in chain length suggested that one or more genes within
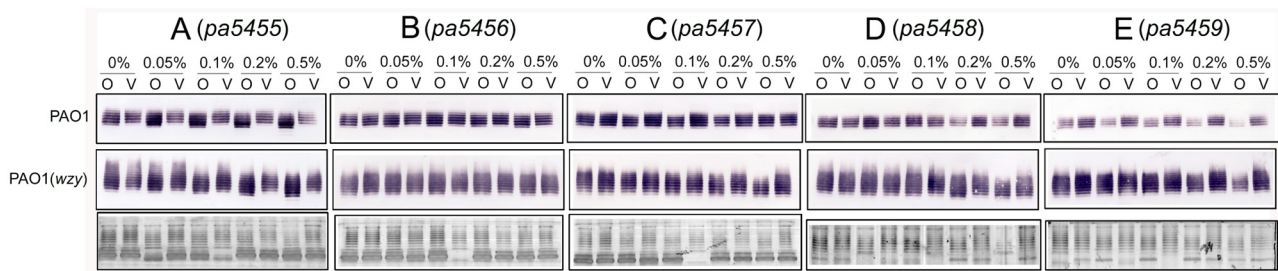
FIG 7 Effects of overexpression of *pa5455* (A), *pa5456* (B), *pa5457* (C), *pa5458* (D), or *pa5459* (E) in *P. aeruginosa* PAO1 or in PAO1(*wzy*) background (middle and bottom panels). (Top and middle) Western blots obtained with MAb N1F10; (bottom) silver-stained SDS-polyacrylamide gels. The percentages (0% to 0.5%) above the lanes are the inducer arabinose concentrations used, and the letters V and O above the lanes indicate whether the *P. aeruginosa* PAO1 or PAO1(*wzy*) strain contains the empty vector pHERD20T (V) or the corresponding overexpression vector (O).

this cluster may play a direct role in a chain length-regulating process.

Overexpression of the two glycosyltransferase-encoding genes, *pa5455* and *pa5456*, closely mimicked the effects of overexpressing the entire operon (*pa5455-pa5459*) (cf. Fig. 6D and C), suggesting that these two genes alone may account for the chain length phenotype observed from overexpression of the whole cluster. We also examined the effect of overexpressing these glycosyltransferase genes individually. When *pa5455* was overexpressed in either the PAO1 or PAO1(*wzy*) background, there was a correlation between the induction strength (arabinose concentration) and shortening of the CPA chain length (Fig. 7A). Overexpression of this gene did result in the production of CPA chains shorter than those observed in the controls, but the effect was not as dramatic as that observed when *pa5455* and *pa5456* were overexpressed together (Fig. 6D). Overexpression of *pa5456* alone had no discernible effect on CPA phenotype. With arabinose-induced *pa5456* expression in the PAO1 background, a subtle shift toward shorter CPA chains was observed, albeit the effect was very slight (Fig. 7B).

Overexpression of the three genes (*pa5457-pa5459*) caused a progressive reduction in the quantity of CPA produced that is proportional to the concentration of the arabinose inducer. With 0.5% arabinose, CPA LPS was barely detectable (Fig. 6E). There was no dramatic shift in the chain length distribution, but as the total amount of polymer diminished, it was mainly the longer chains that were lost. When the putative methyltransferase-encoding gene, either *pa5457* or *pa5459*, was overexpressed, it caused a shortening of the average CPA chain lengths, mainly due to loss of production of the longer chains (Fig. 7C and E). At the highest arabinose concentration (0.5%), the high level of *pa5459* overexpression caused the CPA production level to decrease to <50% compared to that produced by the wild-type controls (as judged by Western immunoblotting) (Fig. 7E). On the silver-stained SDS-polyacrylamide gel of samples from bacteria overexpressing *pa5459*, a band shift was evident, indicating a change in chemical structure of the polymer (Fig. 7E, bottom panel). Overexpression of the putative acetyltransferase resulted in a modest reduction in the amount of CPA produced and a subtle shift in the chain length distribution which was mainly due to loss of longer chains (Fig. 7D).

DISCUSSION

We have presented evidence from genetic studies that the genes in the *pa5455-pa5459* cluster are involved in CPA biosynthesis in *P. aeruginosa*. To our knowledge, this is the first experimental investigation of this gene cluster. This is an important development for understanding the biosynthesis, structure, and regulation of this cell surface polysaccharide.

Results from RT-PCR experiments demonstrated that the five genes in the *pa5455-pa5459* gene cluster are organized in an operon. We also showed that the previously identified eight genes in the *rmd-wbpZ* cluster are cotranscribed and organized in an operon. These two operons are appropriately juxtaposed, with each being transcribed from a different strand, hence taking on opposite orientations for gene transcription. This arrangement is conserved among most of the *P. aeruginosa* genomes that have been sequenced (including strains PAO1, PA14, and LESB58), as well as in *Pseudomonas fluorescens* pfO-1 (7), with the exception that in the genome of *P. aeruginosa* strain PA7, the five-gene cluster is localized approximately 10 kb upstream of the other CPA eight-gene biosynthesis cluster (7). We also identified a promoter for each of the two operons using the 5′-RACE, EMSA, and promoter trap methods. Based on the EMSA data, binding between these promoters and the *E. coli* holoenzyme containing the sigma 70 factor RpoD was evident. The *rpoD* gene of *P. aeruginosa* shares high sequence homology with *E. coli rpoD* and was able to complement a temperature-sensitive mutant of *E. coli rpoD* (18); hence, the two are orthologs and would presumably perform similar functions (19).

Each of the two putative glycosyltransferase-encoding genes *pa5455* and *pa5456* is essential for CPA biosynthesis, as mutants with either of these genes deleted are devoid of CPA. The other three novel genes described in this study are not essential for biosynthesis of a CPA-like polymer. In the case of the putative acetyltransferase-encoding gene, *pa5458*, its function is clearly not required for assembly and processing of the polysaccharide, although the mutant produces CPA with a different structure, which is consistent with previous reports of the presence of acetylation substitutions in the sugars of CPA (12). The observation that overexpression of this gene also resulted in a reduction in binding of the LPS from the recombinant bacteria to the CPA-specific MAb suggests that the epitope in the wild-type CPA that is recognized by this antibody may depend on the level of acetylation. The two putative methyltransferase-encoding genes fulfill partially redundant functions; however, when both putative methyltransferase-encoding genes are knocked out, no CPA production could be discerned.

Because of sequence similarities between the putative methyl-

transferases encoded by genes in this cluster with the chain length-regulating methyltransferase WbdD from *E. coli* O8 (4), we were particularly interested in determining whether any of these genes might participate in chain length-regulating activities. This would include chain extension and chain termination activities and any mechanism that coordinates these two processes. In general, polymer chain lengths are determined by the balance and/or coordination between chain-extending and chain-terminating activities, and it can be informative to examine the influence of the level of gene expression on the length of the chain. Shifts in the CPA chain length distribution may indicate that the overexpressed genes participate directly in chain length-regulating processes. For example, overexpression of an enzyme that catalyzes chain-terminating activity results in a shortening of polysaccharide chains (4). Therefore, to investigate the functions of CPA biosynthesis genes, we examined the phenotypes that resulted from overexpression of genes and combinations of genes of the CPA biosynthesis locus. It must be borne in mind, however, that gene overexpression could influence chain length in a number of other ways. For example, overexpression of an enzyme that decreases the concentration of GDP−D-Rha, which is a precursor needed for repeat unit biosynthesis, may reduce the observed chain lengths; overexpression of any member of a putative multienzyme complex could affect the composition, localization, and efficiency of biosynthesis in unpredictable ways; and overexpression of an integral membrane protein could affect the stability or structure of the cell wall with unpredictable consequences for the biosynthesis of CPA, which is a membrane-associated cell surface macromolecule. There is also the possibility that overexpression of the enzymes may alter the biochemistry of the enzymatic reactions, hence affecting the overall CPA length. It is also possible that these products may be used for other purposes by *P. aeruginosa* and that therefore, the whole biosynthesis process is out of balance when the product is overexpressed. We therefore focused on results where gene overexpression produced a dramatic shift in CPA chain length—where bands were observed that are out of the normal size range.

The experiments in which we overexpressed the Wzm/Wzt ABC transporter help to set CPA biosynthesis into context with respect to the *E. coli* and *K. pneumoniae* model systems which have been described for ABC transporter-dependent polysaccharide biosynthesis and chain length regulation. Because overexpression of the CPA ABC transporter genes resulted in only very minor shifts in the chain length distribution, it most closely resembles the results of overexpressing the ABC transporter in the *E. coli* O9a model system (3). In contrast, overexpression of the ABC transporter in the *K. pneumoniae* O2a model system completely shifted the size distribution out of the normal range (3). We conclude from this that export of the nascent polysaccharide chain is not the key event that terminates chain elongation. Therefore, by analogy with the *E. coli* O8 and O9a systems, there is probably an enzyme-catalyzed modification of the nascent CPA chain that takes place before the ABC transporter recognizes it as a substrate for export. It would then be the timing and/or chain length specificity of this modification reaction that is the key step in regulating chain length. In the *E. coli* O9a model system, the modification takes place on the nonreducing terminal sugar, and it prevents further elongation (20). In CPA biosynthesis, however, there is as yet no evidence that the modification that is recognized by the ABC transporter takes place at the nonreducing terminus or that it

prevents further elongation. Full structural elucidation of the wild-type CPA would greatly help to clarify these questions.

Overexpression of the glycosyltransferase-encoding genes *wbpX*, *wbpY*, and *wbpZ* resulted in increased chain lengths and decreases in the intensities of CPA signals. This result is consistent with one or more of these genes encoding the chain-extending glycosyltransferase functions required for CPA biosynthesis as has previously been suggested. When these genes are overexpressed, we would anticipate that available sugar precursors may be incorporated into a smaller number of much longer polysaccharide chains. It is possible that this accounts for the loss of intensity in CPA banding patterns observed on the silver-stained SDS-polyacrylamide gels and Western blots.

Because of the sequence similarities between the two putative methyltransferase-encoding genes *pa5457* and *pa5459*, with *wbdD* from *E. coli* O8, we had hypothesized that one or both of these genes might catalyze an analogous polysaccharide methylation reaction and that this methylation would be the signal recognized by the ABC transporter. Our data do not rule out this possibility, but it is clear that these *P. aeruginosa* methyltransferases do not have the same kind of chain length-regulating function as WbdD from *E. coli* O8 (WbdD$_{O8}$). Overexpression of WbdD results in dramatic shortening of OPS chains, whereas overexpression of the CPA locus methyltransferase genes does not. The *E. coli* WbdD proteins are larger because they contain domains for interacting with the membrane and glycosyltransferase enzymes (21), whereas these domains are not present in PA5457 and PA5459. The precise mechanism for coordination of elongation and termination of OPS synthesis in the *E. coli* model systems is still unknown, but it is clear that these interacting domains are important. Of the two putative methyltransferase-encoding genes in the CPA cluster, *pa5459* seems to be more critical for biosynthesis of the CPA structure. Knocking out this gene, or overexpressing it, resulted in observable changes in CPA phenotypes, indicating an alteration in the chemical structure of the molecule. Although the phenotype of the *pa5457 pa5459* double mutant confirms that both genes participate in CPA biosynthesis, mutation of *pa5457* alone did not confer detectable phenotype change, and overexpression of this gene had only a very subtle effect on the chain length distribution. Possibly the SDS-PAGE−silver staining and Western immunoblotting methods we used to characterize LPS were not sensitive enough to detect changes caused by genetic experiments with *pa5457*, and it may yet be the case that the function of this gene is important biologically. The requirement for at least one of these putative methyltransferase-encoding genes for CPA production substantiates previous findings that methyl sugars are present in the structure of the CPA polysaccharide, although there is not perfect agreement about which methyl sugars are present (11–13). Future structural study of the wild-type and mutant forms of the CPA LPS is warranted and has the potential to illuminate the specific functions encoded by these genes. Since methylation appears to be important for completion of CPA biosynthesis, it seems probable that at least one methyl sugar fulfills a critical signal function to permit export and processing of the polymer. It is therefore possible that methylation is one of the features recognized by the ABC transporter before export is triggered as we had hypothesized.

Overexpression of the two putative glycosyltransferase-encoding genes *pa5455* and *pa5456* produced a dramatic, inducer concentration-dependent shortening of the length of the chain.

One or both of the proteins encoded by these genes is therefore the best candidate for the catalyst of the chain length-determining reaction. However, neither gene when overexpressed individually could reproduce this dramatic change. It is tempting to speculate that since overexpression of *pa5455* was able to induce a modest, but genuine shortening of CPA chain length, then PA5455 catalyzes the critical chain-terminating event. We must then account for the less dramatic phenotype when this protein is overexpressed by itself. One possibility is that these two proteins interact in a complex and that without coexpression with PA5456, PA5455 is either unstable or is inappropriately localized within the cell. However, at present, there is no data to specifically support this hypothesis.

In conclusion, we have presented data to describe the arrangement and functions of five new genes involved in biosynthesis of CPA in *P. aeruginosa*. The effects of overexpression experiments with these and other CPA biosynthesis genes have enabled us to define some of the features of chain length control in the biosynthesis of this polysaccharide. On the basis of these data, we hypothesize that the key step in controlling the length of the CPA polymer is not export by the Wzm-Wzt ABC transporter but a covalent modification of the nascent polysaccharide chain. This is consistent with the bioinformatic analyses (8). In this respect, the mechanism of chain length regulation resembles that of the *E. coli* O9a model system. However, we have presented data that lead us to tentatively suggest that the key modification step in CPA biosynthesis is probably catalyzed by a glycosyltransferase encoded by *pa5455*, whereas in the *E. coli* O9a system, the key step is phosphorylation by WbdD (20). We are at an early stage of fully understanding CPA biosynthesis, and our ability to assign specific gene functions is hampered to a large degree by the lack of a full structure of the CPA-containing LPS molecule, including information about how the rhamnan is attached to lipid A-core and its structure at the nonreducing terminus. We are currently undertaking detailed study of the wild-type and mutant CPA structures by high-field nuclear magnetic resonance (NMR) and mass spectrometry analyses in order to fill these gaps in our knowledge.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Tables S2 and S3 in the supplemental material. *P. aeruginosa* strains were grown in lysogeny broth (also commonly known as Luria-Bertani [LB] medium) or on *Pseudomonas* isolation agar (PIA) at 37°C with appropriate antibiotics at the following concentrations: carbenicillin (Cb) at 300 μg ml^{-1} (150 μg ml^{-1} for strains containing pTZ110-derived promoter trap vectors) and gentamicin (Gm) at 200 μg ml^{-1}. *E. coli* strains were grown in LB medium at 37°C, and when required, antibiotics were added at the following concentrations: ampicillin (Ap) at 100 μg ml^{-1}, Gm at 50 μg ml^{-1}, and chloramphenicol (Cm) at 17 μg ml^{-1}.

Extraction of RNA and reverse transcriptase PCR (RT-PCR). Total RNA was extracted from *P. aeruginosa* PAO1 using the RNeasy minikit (Qiagen). DNA was digested from the total RNA samples by treatment with amplification-grade DNase I (Invitrogen). An aliquot of 2 μg of the total RNA was reverse transcribed into first strand cDNA using Superscript II reverse transcriptase (Invitrogen) with random primers (Invitrogen). Finally, 1.5-μl portions of the transcription reaction products were then used as the template to perform PCR with primers listed in Table S1 in the supplemental material using *Taq* DNA polymerase in a 25-μl reaction mixture.

5′-RACE. 5′ rapid amplifications of cDNA ends (5′-RACEs) were performed with the primers listed in Table S4 in the supplemental material

using the second generation 5′/3′ RACE kit and following the protocols from the manufacturer (Roche Applied Science).

EMSA. Oligonucleotide sequences used in the electrophoretic mobility shift assay (EMSA) are listed in Table S5 in the supplemental material. The *E. coli* RNA polymerase sigma-saturated holoenzyme was obtained from Epicentre. EMSA experiment was performed by the method of Fenton et al. (17) with slight modifications; heparin was added to a concentration of either 28 μg/ml or 34 μg/ml to inhibit nonspecific binding of the enzyme to the oligonucleotides, and glycerol was added to 5% to the acrylamide gel and to 10% in the 0.5× Tris-borate-EDTA (TBE) running butter. The gels were dyed with Sybr gold (Invitrogen) and scanned using a Typhoon scanner (GE Healthcare Life Sciences).

Promoter trap vector construction and β-galactosidase activity assay. To test the promoter activity for the *rmd-wbpZ* operon, a region from 46 to 151 bp upstream of the *rmd* start codon was amplified and inserted into the HindIII and BamHI sites of pTZ110 to yield pTZ-P-*rmd*. To test the promoter activity for the *pa5455-pa5459* operon, a region from 10 to 228 bp upstream of *pa5455* was amplified and inserted into pTZ110 to construct pTZ-P-*pa5455*. Plasmid pTZ-P-*rpoD* (which is pTZ110 containing the *rpoD* promoter) (22) was used as a positive control. These plasmids were transformed into *P. aeruginosa* PAO1, and the strains were incubated overnight at 37°C before the β-galactosidase activity was analyzed by the method of Miller (23).

Construction of in-frame nonpolar deletion mutants. To construct the gene replacement vector for *pa5458*, a fragment containing *pa5458* was amplified with primers 58MF1 and 58MR1 (see Table S6 in the supplemental material) and inserted into the SmaI site of pEX18AP to yield pEX*pa5458*. Primers 58MF2 and 58MR2 were then used to amplify a fragment from pEX18*pa5458* containing the whole vector backbone and the N- and C-terminal ends of *pa5458*. The amplified fragment was self-ligated to yield the replacement vector pEXΔ*pa5458* which contains a deletion of 822 bp in the *pa5458* gene. To construct gene replacement vectors for the other genes, DNA fragments containing each gene and flanking regions were amplified with primers listed in Table S6. The PCR products were first inserted into the HincII site of pUC18, and restriction digestions were then performed on the resulting plasmids to generate in-frame deletions in the corresponding genes. AgeI digestion cut out a 123-bp fragment from *pa5455*. HincII digestion deleted 645-bp and 330-bp fragments from *pa5456* and *pa5459*, respectively. NgoMIV digestion removed a 528-bp fragment from *pa5457*. The linearized vectors were then self-ligated and used as the templates for amplification of the truncated genes by PCR using the same primers as mentioned above. The amplified, truncated genes were then cloned into the SmaI-cut suicide vector pEX18Ap to yield the replacement vectors pEXΔ*pa5455*, pEXΔ*pa5456*, pEXΔ*pa5457*, and pEXΔ*pa5459*.

The allelic replacement vectors were conjugated to *P. aeruginosa* PAO1 or PAO1(*wzy*), and chromosome replacement mutants were selected and screened essentially by the methods of Choi et al. and Hoang et al. (24, 25). A double mutant strain with deletions of both *pa5457* and *pa5459* was constructed by conjugal transfer of the gene replacement vector pEXΔ*pa5459* into a *P. aeruginosa pa5457* mutant.

Construction of vectors for complementation or overexpression experiments. *P. aeruginosa* PAO1 genes or groups of genes were amplified using primers listed in Table S7 in the supplemental material and inserted into the broad-host-range vector pHERD20T (26). Complementation and overexpression vectors constructed in this manner are listed in Table S3. Plasmids were transferred into *P. aeruginosa* by electroporation by the method of Choi et al. (27). In complementation experiments, the strains were grown in liquid medium containing 0.001% arabinose overnight before LPS was extracted and analyzed. In overexpression experiments, the strains were cultured overnight in liquid medium containing arabinose at 0%, 0.01%, 0.05%, 0.1%, 0.2%, 0.5%, or 1% (wt/vol) to induce different levels of expression of the genes or gene combinations.

LPS extraction, SDS-PAGE, silver staining, and Western immunoblotting. LPS was prepared using the proteinase K method of Hitchcock

and Brown (28). The LPS samples were resolved by electrophoresis on 12% SDS-polyacrylamide gels, and the banding patterns were visualized by the ultrafast silver staining method (29). LPS was also transferred onto BioTraceNT nitrocellulose membranes (Pall), and Western immunoblotting was performed using monoclonal antibody (MAb) N1F10 (CPA specific), a mixture of MAb MF15-4 (OSA specific) and MAb 5c101 (core specific) (in a 1:1 ratio), or a mixture of MAb N1F10 and 5c101 (in a 20:1 ratio). The secondary antibody used was alkaline phosphatase-conjugated goat anti-mouse Fab$_2$ (Jackson ImmunoResearch), and the blots were developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) as described previously (30, 31).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00631-12/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.2 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.1 MB.
Table S5, PDF file, 0.1 MB.
Table S6, PDF file, 0.1 MB.
Table S7, PDF file, 0.1 MB.

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