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Specific surface glycan decorations enable antimicrobial peptide resistance in plant-beneficial pseudomonads with insect-pathogenic properties

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Running Title: O-antigen function in biocontrol pseudomonads

Originality-Significance Statement: The study is the first to characterize the genetic basis of O-antigen biosynthesis in pseudomonads that can adopt both plant-beneficial and insect-pathogenic lifestyles. It reports on the remarkably high diversity of O-antigen gene clusters, some of which likely were acquired from other bacteria. The particular O-antigen decoration protects these pseudomonads against antimicrobial peptides (AMP), a hallmark of insect innate immunity, and significantly contributes to insect pathogenicity. This finding highlights for the first time the importance of O-antigen in the AMP resistance of pseudomonads.

SUMMARY

Some plant-beneficial pseudomonads can invade and kill pest insects in addition to their ability to protect plants from phytopathogens. We explored the genetic basis of O-polysaccharide (O-PS, O-antigen) biosynthesis in the representative insecticidal strains *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391 and investigated its role in insect pathogenicity. Both strains produce two distinct forms of O-PS, but differ in the organization of their O-PS biosynthesis clusters. Biosynthesis of the dominant O-PS in both strains depends on a gene cluster similar to the O-specific antigen (OSA) cluster of *Pseudomonas aeruginosa.* In CHA0 and other *P. protegens* strains, the OSA cluster is extensively reduced and new clusters were acquired, resulting in high diversity of O-PS structures, possibly reflecting adaptation to different hosts. CHA0 mutants lacking the short OSA form of O-PS were significantly impaired in insect virulence in *Galleria* injection and *Plutella* feeding assays. CHA0, PCL1391, and other insecticidal pseudomonads exhibited high resistance to antimicrobial peptides, including cecropins that are central to insect immune defense. Resistance of both model strains depended on the dominant OSA-type O-PS. Our results suggest that O-antigen is essential for successful insect infection and illustrate, for the first time, its importance in resistance of *Pseudomonas* to antimicrobial peptides.

INTRODUCTION

Root-associated pseudomonads with plant-beneficial and insect-pathogenic properties are promising biocontrol agents that may be exploited as an alternative to the chemical control of phytopathogenic fungi and pest insects in crops (Haas and Défago, 2005; Kupferschmied *et al.*, 2013). Their role in protection of plants against pathogenic fungi and the underlying mechanisms have been studied in some detail over the past three decades (Berendsen *et al.*, 2012; Mendes *et al.*, 2013), yet we still have only limited knowledge about the interaction of these microorganisms with insects. Plant-beneficial *Pseudomonas* strains with entomopathogenic properties belong to the *Pseudomonas fluorescens* group whose hallmark is its plant-protective activities including the production of various antifungal metabolites and induction of systemic resistance in the plant host (Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2015; Flury *et al.*, 2016). Strains of the *Pseudomonas fluorescens* group sub-clade encompassing the species *Pseudomonas chlororaphis* and *Pseudomonas protegens* efficiently infect and cause disease in certain, mainly Lepidopteran insect species upon oral or systemic infection (Péchy-Tarr et al., 2008; Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2013; Flury *et al.*, 2016). The host-specific production of an insecticidal protein, termed the Fit toxin, in the insect hemolymph contributes significantly to the pathogenicity in these bacteria (Péchy-Tarr et al., 2008; Ruffner *et al.*, 2013; Kupferschmied *et al.*, 2014; Ruffner *et al.*, 2015). However, we currently do not know the mechanisms that enable them to successfully invade and colonize insects and that make the immune system of the host organism fail to clear the infection.

Despite the central role it plays in host-microbe interactions, the cell surface structure of plant-beneficial pseudomonads has rarely been investigated. The surface of Gram-negative bacteria consists largely of lipopolysaccharide (LPS), exopolysaccharides and some proteins and is the site where these microorganisms directly interact with their host organisms (Raetz and Whitfield, 2002; Lebeer *et al.*, 2010; Silhavy *et al.*, 2010). LPS is the primary component of the outer membrane in Gram-negative bacteria and consists in general of three different regions: lipid A, core oligosaccharide and O-antigenic

polysaccharide (O-PS, also termed O-antigen) (Raetz and Whitfield, 2002; Trent *et al.*, 2006; Lam *et al.*, 2011). The conserved lipid A-core oligosaccharide (lipid A-core) serves as an attachment site for the highly variable O-PS, which consists of repeating units (O-units) of one to five sugar residues and is the exposed part of LPS. LPS is known as smooth when O-PS is attached to the lipid A-core (i.e., when capped) and rough when uncapped. There is a large variety of monosaccharides that can be incorporated into O-units with different linkages, different stoichiometry, in different orders, and with various modifications, which gives rise to a tremendous diversity of O-PS structures in Gram-negative bacteria (Lerouge and Vanderleyden, 2002). Since O-PS often constitutes the outermost part of a bacterial cell and thus is at the interface between the bacterium and its surrounding environment, LPS side chains play an important role in microbe-host interactions and are under high selective pressure (Raetz and Whitfield, 2002; Wildschutte *et al.*, 2004; Trent *et al.*, 2006; Lam *et al.*, 2011; Erbs and Newman, 2012). O-PS contributes to pathogenicity and is generally associated with the ability of a bacterium to colonize a particular host organism and to bypass or overcome host defense mechanisms (Moran, 2001; Lerouge and Vanderleyden, 2002; Skurnik and Bengoechea, 2003; Lam *et al.*, 2011).

The assembly and translocation of most O-PS occur via Wzx/Wzy-dependent and ATP-binding cassette (ABC) transporter-dependent pathways while a third pathway, which is dependent on a specific synthase, is only rarely found in bacteria (Raetz and Whitfield, 2002; Whitfield and Trent, 2014). The two prominent pathways have in common that undecaprenyl diphosphate (und-PP) is used as a lipid anchor for the synthesis of individual O-units or the entire O-PS at the cytoplasmic face of the inner membrane. Repeating units are assembled by glycosyltransferases, which use nucleotide-activated sugars, synthesized by specific enzymes, as precursors. Homopolymers (i.e., polysaccharides with a single monosaccharide component) are commonly synthesized via the ABC transporter-dependent pathway while heteropolymers are most often produced following the Wzx/Wzy-dependent pathway. In the latter pathway, individual und-PP-linked repeating units are transported across the inner membrane with the help of a flippase (Wzx) and are then assembled into O-PS by a polymerase

(Wzy) (Raetz and Whitfield, 2002; Kalynych *et al.*, 2014). The chain length of the O-PS can be controlled via one or several co-polymerases (Wzz). In the ABC transporter-pathway, the O-PS is completely synthesized in the cytoplasm and exported to the periplasm by an ABC transporter composed of the nucleotide-binding protein Wzt and the transmembrane protein Wzm (Raetz and Whitfield, 2002; Greenfield and Whitfield, 2012).

O-PS biosynthesis has been well studied in *Pseudomonas aeruginosa* (Lam *et al.*, 2011). This opportunistic pathogen produces two distinct forms of O-antigen simultaneously: a homopolymer composed of D-rhamnose and termed common polysaccharide antigen (CPA; formerly A band) and a heteropolymer known as O-specific antigen (OSA; formerly B band) (Rocchetta *et al.*, 1999; Lam *et al.*, 2011). CPA is assembled and transported in *P. aeruginosa* strains via an ABC transporter-dependent pathway, while OSA is synthesized via a Wzx/Wzy-dependent pathway. Most genes for the biosynthesis of these two O-PS types are located in two distinct gene clusters. Lack of OSA significantly reduces the virulence of *P. aeruginosa* in several model organisms (Lee *et al.*, 2006; Feinbaum *et al.*, 2012). Multiple studies suggested that O-PS are involved in immune evasion, resistance to complement-mediated killing, motility, and biofilm formation in this microorganism (Pier, 2007; Lau *et al.*, 2009; Lam *et al.*, 2011).

In the present study, we explored the genetic basis of O-PS biosynthesis in recently sequenced (Flury *et al.*, 2016), plant-beneficial *Pseudomonas* species displaying insecticidal activities. We found that *P. protegens* and closely related bacteria went through an extensive reduction of the dominant OSA gene cluster and in turn acquired novel O-antigen gene clusters via horizontal gene transfer. Furthermore, we discovered that many insecticidal pseudomonads with plant-beneficial properties are intrinsically resistant to cationic antimicrobial peptides (AMPs) and that this resistance is dependent on the presence of O-specific side chains on the surface of these bacteria. In *Pseudomonas*, a role of O-antigen in AMP resistance has not been demonstrated before. Protection of the cell envelope against attacks from AMPs might be critical for bacterial pathogenicity toward insects, as these do not possess the complement

system for clearing bacterial infections and thus massively produce such antimicrobial compounds for defense against invading microbes (Lemaitre and Hoffmann, 2007; Royet and Dziarski, 2007). Indeed, we found that loss of the ability to produce smooth LPS resulted in significantly attenuated virulence of a *P. protegens* strain in insect larvae, demonstrating the importance of O-PS biosynthesis for insect pathogenicity in these pseudomonads.

RESULTS

Identification of putative O-PS biosynthesis genes

O-PS biosynthesis genes are mostly organized in gene clusters. The search for them in a set of recently sequenced (Flury *et al.*, 2016) and publicly available genomes of plant-beneficial pseudomonads in this study was therefore focused on genomic regions where several characteristic O-PS genes (specifically genes encoding glycosyltransferases, O-antigen transporters, and nucleotide sugar biosynthesis proteins) were found located in close proximity to each other. The search for putative O-PS genes was initially focused on the two model strains *P. chlororaphis* subsp. *piscium* PCL1391 (Flury *et al.*, 2016) and *P. protegens* CHA0 (Jousset *et al.*, 2014). A gene cluster flanked by the *ihfB* and *comEA* genes and similar to the OSA cluster of *P. aeruginosa* strains (Fig. S1) was identified in both PCL1391 (Fig. 1) and CHA0 (Fig. 2). While in PCL1391 this gene cluster is similar to the one in *P. aeruginosa* PAO1 (Fig. 1, Fig. S1), it is reduced in CHA0 where only a few genes are present, a Wzy polymerase-encoding gene is absent and *wzz*, coding for a putative O-PS chain-length regulator, is a pseudogene (Fig. 2). In contrast to *P. protegens* CHA0, PCL1391 furthermore possesses a gene cluster orthologous to the CPA cluster in PAO1 (Fig. 1, Fig. S1). The three genes *rmd*, *gmd* and *wbpW* which encode enzymes required for the biosynthesis of GDP-D-rhamnose in PAO1 (Lam *et al.*, 2011) are, however, absent in PCL1391. The two newly identified gene clusters in PCL1391 and CHA0 are called OSA and CPA from here on, owing to their relatedness with the *P. aeruginosa* O-antigen clusters.

Three additional, so far uncharacterized gene clusters were found in *P. protegens* CHA0 and termed O-PS biosynthesis cluster (OBC) 1, 2 and 3 (Fig. 2). OBC1 and OBC3 contain genes that were predicted to code for ABC transporter proteins, suggesting an ABC-transporter-dependent biosynthesis of polysaccharides. In contrast, OBC2, which was also identified in the genome of PCL1391 (Fig. 1), harbors genes that code for proteins with predicted functions as for Wzx, Wzy and Wzz in PAO1, indicating the biosynthesis of polysaccharides via a Wzx/Wzy-dependent pathway (Fig. 2).

O-PS is covalently attached to lipid A-core in the periplasm by the O-antigen ligase WaaL (Raetz and Whitfield, 2002; Lam *et al.*, 2011). The WaaL-encoding gene (*waaL*) was identified by protein BLAST and protein sequence analysis in the LPS core biosynthesis loci of PCL1391 (29% amino acid sequence identity to PAO1 WaaL over 78% of the total protein length) and CHA0 (42% amino acid sequence identity to PAO1 WaaL over 61% of the total protein length) (Fig. S2). In CHA0, *waaL* seems to have undergone a fusion with a gene that is coding for a putative phosphotransferase and is orthologous to PA4998 in PAO1 (sharing 49% amino acid sequence identity over 83% of the total length of PA4998), and thus most likely encodes an O-PS ligase fused to a kinase domain at its C-terminus (Fig. S2B). This gene fusion was observed in all *P. protegens* strains examined in this study and in some additional *P. fluorescens* group lineages.

Mutational analysis of O-PS biosynthesis

Putative O-PS biosynthesis genes and gene clusters identified in PCL1391 and CHA0 were mutated individually as depicted in Fig. 1 and Fig. 2. The resulting LPS banding patterns were studied by extraction, separation and visualization of LPS on polyacrylamide gels (Fig. 3). *P. chlororaphis* subsp. *piscium* PCL1391 displayed a complex LPS banding pattern with a modular length distribution (Fig. 3A). The wild-type strain produced LPS with three distinct modules of bands with high to very high molecular weights (25 to over 100 kDa; indicated with “long” and “very long” in Fig. 3A) and additionally several low-molecular-weight bands (10 to approx. 20 kDa). Mutation of *waaL* (strain

PCL-1) resulted in the loss of most bands, except for the band with the lowest molecular weight which most probably corresponds to the uncapped lipid A-core moiety of LPS. Since WaaL is essential in most Gram-negative bacteria to produce smooth LPS (Raetz and Whitfield, 2002), this strongly indicated that bands visible above the lowest one on the gel corresponded to LPS molecules with covalently linked O-PS. Re-introduction of only *waaL* in strain PCL-1 restored all bands visible in the wild-type strain, indicating that there was no polar effect caused by disruption of *waaL* in PCL1391 (Fig. S3A). Disruption of *wzx* encoding the putative flippase, which translocates individual und-PP-linked repeating units across the inner membrane, in the OSA cluster resulted in a similar LPS phenotype as in the *waaL* mutant, with only weak bands remaining above the lipid A-core band on the gel (Fig. 3A).

The deletion of the CPA cluster in PCL1391 did not result in a significant change in the LPS banding pattern, except for a decreased intensity of some bands just below 25 kDa (Fig. 3A). Extraction and visualization of LPS from the wild type and isogenic mutants grown at 30°C suggested that the CPA cluster significantly contributed to O-PS production at elevated temperatures by encoding proteins that are responsible for the biosynthesis of a *wzx*-independent form of O-PS in this strain (Fig. S3B). LPS banding patterns of the *obc2* deletion mutant resembled those of the wild type, indicating that the *obc2* gene cluster is not involved in the biosynthesis of O-PS in PCL1391 (Fig. 3A). Mutagenesis of *waaL* and *wzx* in PCL1391 resulted in mutant strains with obvious growth defects, while disruption of *wzz1* or *wzz2* (Fig. 1), which were predicted to code for O-PS chain length regulators, did not alter the growth rate of the bacterium (Fig. S3C). Additionally, several attempts to delete genes in the OSA cluster failed.

All these findings suggest that, under the conditions tested, the OSA gene cluster is the dominant O-PS biosynthesis cluster in *P. chlororaphis* subsp. *piscium* PCL1391. Mutation of *wzz1* or *wzz2*, which code for putative O-PS chain length regulators, led to the disappearance of the dominant module with high-molecular-weight bands and the two modules with very-high-molecular-weight bands,

194 respectively (Fig. 3A). This result suggests that Wzz1 mediates the biosynthesis of long OSA-type O-
195 PS chains, while Wzz2 is essential for the production of very long O-PS chains.
196 *P. protegens* CHA0 displayed an LPS banding pattern distinct from PCL1391 (Fig. 3B). Separation of
197 the extracted LPS on polyacrylamide gels and subsequent silver staining revealed a dominant low-
198 molecular-weight band between 10 and 15 kDa and a smear comprising molecules with high molecular
199 weights of up to more than 100 kDa. The disruption of *waaL* and the deletion of *wbpL*, which encodes
200 the putative initial glycosyltransferase essential for both types of O-PS in PAO1 (Lam *et al.*, 2011),
201 both resulted in the loss of the high-molecular-weight bands and in a down-shift of the low-molecular-
202 weight band by several kDa. Complementation of the *waaL* and *wbpL* mutants rescued the production
203 of wild-type O-PS (Fig. S3D). This suggests that the smear with the high-molecular-weight bands and
204 the dominant low-molecular-weight band consisted of smooth LPS molecules with long and short O-
205 PS, respectively.
206 In-frame deletion of *wzx* encoding the putative flippase in CHA0 resulted in the shift of the low-
207 molecular-weight band observed before in the *waaL* and *wbpL* mutants, but not in the disappearance
208 of the LPS molecules with the long O-PS. In contrast, partial deletion of the OBC3 cluster and deletion
209 of the gene *fcl*, which is part of the OBC3 cluster (Fig. 2) and is predicted to encode a GDP-L-fucose
210 synthase (see following chapter), led to the loss of the LPS form with long O-PS without causing a
211 shift in the low-molecular-weight band (Fig. 3B; Fig. S3D). Complementation of the Δ*fcl* mutant
212 restored the production of the wild-type O-PS (Fig. S3D). Deletion of either OBC1 or OBC2 did not
213 alter the LPS banding pattern observed on the gels (Fig. 3B), nor did the double mutation in CHA5165
214 (data not shown). This indicates that, although they might somehow modify O-PS, the OBC1 and
215 OBC2 gene clusters are not essential for O-PS biosynthesis in strain CHA0.
216 Together, these observations suggest that genes in the OBC3 cluster are essential for the synthesis of
217 smooth LPS with long O-PS in *P. protegens* CHA0, while the remaining genes in the OSA cluster are
218 necessary for the production of LPS molecules capped with very short O-PS. WbpL, which is the

putative initial glycosyltransferase encoded in the OSA cluster, seems to be essential for the biosynthesis of both the short OSA form and the long OBC3 form of O-PS in CHA0. Since no gene coding for a Wzy polymerase was found in CHA0, it is likely that the short O-PS form consists of only one O-PS repeating unit as it was reported for a *wzy* mutant of *P. aeruginosa* PAO1 (Lam *et al.*, 2011). Although having different genetic bases for the biosynthesis of polysaccharides, both *P. chlororaphis* subsp. *piscium* PCL1391 and *P. protegens* CHA0 are capable of synthesizing two distinct types of O-PS simultaneously. The two bacterial strains may achieve this by employing two different biosynthesis pathways, i.e. a flippase-dependent (in the case of OSA) and an ABC-transporter-dependent (in case of CPA and OBC3), in parallel. In both strains, the OSA-type capped form of LPS dominated.

Reduction and acquisition of O-PS gene clusters

The available genome sequences (Flury *et al.*, 2016) and the knowledge gained about the genetic basis of O-PS biosynthesis in strains PCL1391 and CHA0 made it possible to analyze the diversity of genes and gene clusters predicted to be involved in the production of O-PS in diverse biocontrol pseudomonads. Analysis of banding patterns of LPS extracted from selected *Pseudomonas* strains indicated that they all produce smooth forms of LPS (Fig. S3E). The presence and genetic composition of predicted O-PS biosynthesis gene clusters varied extensively among the insect-pathogenic *Pseudomonas* strains analyzed in this study (Fig. 4).

All *P. protegens* strains in this study were found to possess an OSA gene cluster that is markedly reduced compared to the classical OSA cluster of *P. aeruginosa* and certain *P. chlororaphis* strains (Fig. 4, Table S1, Table S2). This genetic reduction was even more extensive in the *P. protegens* strains K94.41, PF and Pf-5 in which the OSA clusters consist of only *wbpL* and the two flanking genes (Fig. S4). A similarly reduced OSA cluster could also be detected in *P. chlororaphis* strains LMG1245, YL-1 and O6 as well as in *Pseudomonas* sp. CMR5c and Os17 (Fig. S4). The genome analysis also revealed that *P. protegens* strains not only have a reduced OSA cluster but also entirely lack the CPA cluster

(Fig. 4, Table S1). All strains with a reduced OSA gene cluster were found to possess additional gene clusters (OBC3-8) harboring genes that are predicted to contribute to O-PS biosynthesis (Fig. 4). Three genes in the OBC3 cluster (*gmd*, *fcl*, and PFLCHA0_c19690 (Fig. 2)) showed relatively high sequence identities (67-75%) to genes of the O-PS biosynthesis locus of *Rhizobium etli* CE3. These genes are sufficient for the synthesis of GDP-L-fucose from GDP-D-mannose and the covalent binding of the sugar to O-PS repeat units in CE3 (Ojeda *et al.*, 2013). Furthermore, three genes of the OBC5 cluster (PFL_5094, PFL_5095, and PFL_5096) were similar (66% nucleotide sequence identity) to genes of the O-PS biosynthesis gene cluster *rfb* of *Salmonella enterica* subsp. *enterica*. They were predicted to code for enzymes that catalyze the production of CDP-D-abequose, a nucleotide sugar used for the synthesis of O-PS in certain *Salmonella* serovars (Koropatkin *et al.*, 2005; Micoli *et al.*, 2014). These observations point to a possible acquisition of OBC clusters via horizontal gene transfer.

While certain *P. chlororaphis* strains have lost functionally important genes (i.e., *wzz*, *wzx* and *wzy*) of the OSA cluster (Fig. 4), independent events in other strains of the same species seem to have led to the loss of genes in the CPA gene cluster necessary for the synthesis of GDP-D-rhamnose. Strain PCL1391 does no longer carry the three genes *rmd*, *gmd* and *wbpW* that are predicted to encode enzymes (i.e., GDP-4-keto-6-deoxy-D-mannose reductase, GDP-D-mannose dehydratase, and phosphomannose isomerase/GDP-mannose pyrophosphorylase, respectively) required for the biosynthesis of GDP-D-rhamnose in PAO1 (Rocchetta *et al.*, 1999). Strain 30-84 only lost the gene *rmd*, but still possesses the *gmd* (Pchl3084_5934) and *wbpW* (Pchl3084_5935) genes, which points to a gradual loss of genes for GDP-D-rhamnose biosynthesis in *P. chlororaphis*.

O-PS contributes to insect pathogenicity of *Pseudomonas protegens* CHA0

O-PS plays an important role as virulence factors in many pathogenic bacteria (Moran, 2001; Lerouge and Vanderleyden, 2002; Skurnik and Bengoechea, 2003; Lam *et al.*, 2011). Therefore, the contribution of these glycan chains to insect pathogenicity in *P. protegens* CHA0 and *P. chlororaphis*

subsp. *piscium* PCL1391 was investigated. Mutant strains with no obvious growth defects (Fig. S3C) were selected for virulence and competition experiments. A mutant of *P. protegens* CHA0 lacking O-PS, i.e. both the short OSA form and the long OBC3 form (Δ*wbpL*, CHA5161; Fig. 3B), was strongly reduced in its ability to kill larvae of *Galleria mellonella* upon injection (Fig. 5A). Complementation of the mutant strain (CHA5169) restored its insecticidal activity to wild-type level. A lack of the short OSA-type O-PS (Δ*wzx*, CHA5206) resulted in similar strongly reduced injectable activity (Fig. 5A). Loss of the long OBC3-type O-PS (Δ*fcl*, CHA5205) resulted in a significant but only mild reduction of virulence (Fig. 5A). This indicates that OSA is the type of O-antigen that is essential for virulence in this infection model, while the OBC3 form is of minor importance. In contrast, mutation of *wzz1* and *wzz2* in strain PCL1391 did not reduce the virulence in *G. mellonella* (Fig. S5A), suggesting that regulation of O-PS chain lengths is not critical for virulence of PCL1391 during systemic infection of insects. As in the injection assay with *G. mellonella*, the Δ*wzx* and the Δ*wbpL* mutants of *P. protegens* CHA0 were also significantly less virulent than the wild type when orally administered to *Plutella xylostella* larvae (Fig. 5B). This indicates that in this bacterium the short O-antigen contributes to insect pathogenicity via the oral route of infection as well. Results from a competition assay (Fig. 5C) and a colonization assay (Fig. 5D) performed with fluorescently tagged strains suggest that loss of the OSA-type O-PS reduced the ability of strain CHA0 to colonize *G. mellonella* upon injection. Since O-antigen defective *Pseudomonas* strains previously were found to be impaired in root colonization ability under axenic conditions (de Weger *et al.*, 1989; Dekkers *et al.*, 1998), we tested O-PS mutants of *P. protegens* CHA0 also for their capacity to colonize root tips following inoculation of the stem base of axenically grown plants. However, lack of O-antigen did not change the ability of the *P. protegens* strain to colonize plant roots (Fig. S5B). Furthermore, isogenic mutants of PCL1391 lacking functional Wzz1 or Wzz2 were as competitive during insect infection and root colonization as the wild type (Fig. 5C; Fig. 5SB).

The dominant O-PS contributes to antimicrobial peptide resistance

Because O-PS was suggested to be important for resistance to cationic AMPs in bacteria other than pseudomonads (Nesper *et al.*, 2001; Skurnik and Bengoechea, 2003; West *et al.*, 2005; Nehme *et al.*, 2007), selected *Pseudomonas* strains were tested for their susceptibility to polymyxin B (PMB) and the two representative insect AMPs cecropin A and cecropin B (Yi *et al.*, 2014). PMB is a bacterial cationic AMP with a mode of action similar to that of AMPs of higher organisms and is therefore commonly used as a model AMP (Vaara, 1992; Anaya-López *et al.*, 2013; Cullen *et al.*, 2015).

All plant-beneficial *Pseudomonas* strains that were previously shown to possess insecticidal activities (Flury *et al.*, 2016) displayed resistance toward PMB (Fig. 6A). In contrast, with the exception of one strain, all non-insecticidal strains were susceptible to this model AMP. *P. protegens* and closely related strains were highly resistant to PMB, with a subpopulation growing at a concentration of 1024 µg/ml (Fig. 6A). Most *P. chlororaphis* strains only displayed PMB resistance when growing in liquid medium, but not on solid medium.

Isogenic mutants *wbpL* and *wzx* deletion mutant of *P. protegens* CHA0, incapable of synthesizing LPS capped with short O-PS, were significantly less resistant to PMB and the insect AMPs cecropin A and cecropin B than the wild type in a broth microdilution assay (Table 1). The presence of long O-PS, however, did not seem to be important for AMP resistance, since the Δ*fcl* mutant, which is defective for the predicted GDP-L-fucose synthase Fcl, was nearly as resistant to the three tested AMPs as the wild type (Table 1). In contrast to the insecticidal strains CHA0 and PCL1391, the non-insecticidal strain Q12-87 was highly susceptible to the AMPs. The importance of the short O-PS in AMP resistance was underscored by the results of a survival experiment in which *P. protegens* CHA0 and isogenic mutants were exposed to PMB (Fig. 6B). No survival at 100 µg/ml PMB was observed when *wbpL*, *waaL*, or *wzx* was mutated whereas complementation of these mutations restored wild-type resistance levels. By contrast, the Δ*fcl* mutant was as resistant to PMB as the wild type (Fig 6B). Modification of lipid A with 4-aminoarabinose by proteins encoded at the *arn* locus is well documented

to be important for antimicrobial peptide resistance in *P. aeruginosa* (Raetz *et al.*, 2007). Disruption of *arnA* (PFLCHA0_c30730) in CHA0 resulted in a decreased level of polymyxin resistance resembling that of the Δ*wzx* mutant CHA5206 (Fig. 6B). A *wbpL arnA* double mutant (CHA5214) was, as *P. aeruginosa* PAO1 and *P. fluorescens* Q12-87, completely sensitive to PMB. This suggests that lipid A modification with 4-aminoarabinose and capping LPS with short O-PS contributed to PMB resistance independently. Similar results were obtained with colistin (data not shown), another polymyxin AMP. Moreover, the presence of O-antigen seems to be essential for AMP resistance in *P. chlororaphis* subsp. *piscium* PCL1391 as well (Fig. S5C). The length of the O side chains thereby seems to shape the degree of resistance to these antimicrobial compounds.

DISCUSSION

Divergence in the genetic basis of O-PS biosynthesis in insecticidal pseudomonads

Although the composition of O-PS was determined in some plant-beneficial pseudomonads (de Weger *et al.*, 1989; Veremeichenko and Zdorovenko, 2004; Peretti *et al.*, 2011), this study is the first to address the genetic basis of O-PS biosynthesis in these particular microorganisms. Both model strains, *P. chlororaphis* subsp. *piscium* PCL1391 and *P. protegens* CHA0, produce two distinct forms of O-PS simultaneously, which seems to be a characteristic of pseudomonads (Cunneen and Reeves, 2011). However, the genetic basis of O-PS biosynthesis in the two bacteria differs remarkably, giving rise to entirely different O-antigen structures (Fig. 7). Our data indicate that, as in *P. aeruginosa* PAO1 (Lam *et al.*, 2011), the major O-PS type of PCL1391 is produced via the Wzx/Wzy-dependent pathway by proteins encoded in the OSA gene cluster and two chain length determination proteins (Wzz1 and Wzz2) regulate its length. Additionally, a slightly reduced but functional CPA gene cluster was found to be responsible for the biosynthesis of a minor form of O-PS in this strain when grown at elevated temperatures (Fig. S3B). It was observed in several bacterial species, including *P. aeruginosa*, that temperature and other environmental factors, in particular pH and osmotic conditions, can affect the

synthesis of O-PS (Rochetta *et al.*, 1999; Lerouge and Vanderleyden, 2002) and thus, we can only draw conclusions for the growth conditions that we tested in the present study. Future work should also address the possibility that O-PS genes mutated in the present study might have other functions besides being involved in the biosynthesis of O-PS.

While the regulation of OSA chain lengths by Wzz1 and Wzz2 was important for the pathogenesis of *P. aeruginosa* (Kintz *et al.*, 2008), it did not contribute to infection and killing of insect larvae by the *P. chlororaphis* strain in injection assays here, and needs to be addressed in future experiments, in particular by using an oral infection model. Because mutations in the OSA cluster of PCL1391 or the *waaL* gene negatively affected the growth rate of the bacterium, we were not able to investigate whether absence of O-antigen reduces the virulence of this particular pseudomonad.

Our data suggest that the genetic basis of O-PS biosynthesis in *P. protegens* strains, in contrast to *P. chlororaphis* subsp. *piscium* PCL1391 and *P. aeruginosa* PAO1, is characterized by reduction and acquisition of gene clusters during the evolution of the species. The OSA gene cluster in *P. protegens* strains has been reduced to various degrees by loss of genes, including the *wzy* gene coding for the O-antigen polymerase. Our findings indicate that the lack of this polymerase rendered *P. protegens* unable to synthesize OSA-type O-PS with more than one O-unit (Fig. 7B). *P. protegens* strains further lack the gene cluster for the biosynthesis of CPA, but possess additional gene clusters (termed OBC clusters) in return that enable them to synthesize O-PS with long sugar chains.

High O-PS diversity for better host or niche adaptation?

P. fluorescens group species either harbor OSA gene clusters with complex and diverse genetic compositions or, as in the case of many insecticidal strains, possess novel O-PS gene clusters outside of the OSA locus, which may have been acquired by horizontal gene transfer. High genetic diversity in O-PS gene clusters has been reported for other bacterial species such as *P. aeruginosa* and *Salmonella enterica* before (Xiang *et al.*, 1993; Popoff, 2001; Raymond *et al.*, 2002). Classically, this

glycan diversity has been explained with the selective pressure exerted by host immunity, i.e. bacteria producing rare or novel types of O-antigen are more likely to avoid detection by the host immune system (Reeves, 1995). Selection by the host immune system can however not explain why also non-pathogenic bacteria, such as plant-beneficial pseudomonads, display high O-antigen diversity. It has become quite evident that also other factors contribute to the diversity of glycan structures in microorganisms. O-PS may be involved in interbacterial competition: McCaughey *et al.* (2014) reported that rhamnose-containing LPS molecules are the target of lectin-like bacteriocins from pseudomonads. The reduction or loss of the CPA gene cluster thus could have rendered certain pseudomonads resistant to this kind of bacteriocins. It has further been suggested that infection by bacteriophages, which often use O-PS structures as receptors for cell entry, and predation by protozoa act as driving forces (Wildschutte *et al.*, 2004; Lam *et al.*, 2011; Chaturongakul and Ounjai, 2014). The diversity of O-PS structures in biocontrol pseudomonads thus might reflect the adaptation to specific niches and contrasting hosts (i.e., plants and insects), competition with related bacteria, or avoidance of infection or predation.

In the highly insecticidal sub-clade of the *P. fluorescens* group that comprises the species *P. protegens* and *P. chlororaphis*, reductions of the OSA cluster have taken place. The diversity of O-PS structures in many of these pseudomonads apparently has no longer been generated by the exchange of genes at the OSA locus, as it has been observed for other *Pseudomonas* bacteria (Raymond *et al.*, 2002; Cunneen and Reeves, 2011), but rather by acquisition of novel gene clusters at other genomic loci. *P. protegens* strains are all very similar in terms of genome sequence (Ruffner *et al.*, 2015; Flury *et al.*, 2016) and it is therefore remarkable that they display completely different surface glycan structures. Diversification of glycan structures might have allowed *P. protegens* and related insecticidal strains to successfully adapt to such contrasting hosts as plants and insects. The reduction of the OSA cluster and the acquisition of new O-PS gene clusters might have functionally disconnected the synthesis of

the dominant form of O-PS, which has protective functions and is now buried instead of exposed, from the production of O-PS with long and exposed sugar chains for long-term host or niche adaptation.

O-PS protect pseudomonads against attack by AMPs

O-antigen has been reported in several bacteria to be important for virulence in insects (Nehme *et al.*, 2007; Noonin *et al.*, 2010; Miyashita *et al.*, 2012; Bender *et al.*, 2013). We found that in particular the very short OSA is critical for *P. protegens* CHA0 to cause disease in insect larvae. The bacterium is less virulent when it lacks LPS molecules capped with O-antigen possibly because it might be less effective in bypassing or overcoming host defense mechanisms. The presence of O-PS on the cell surface likely renders the microorganism more resistant to antimicrobial factors produced by the insects during systemic infection. There is little but increasing evidence from several bacterial species outside of the *Pseudomonas* genus that O-PS is important for the resistance to AMPs (Nesper *et al.*, 2001; Skurnik and Bengoechea, 2003; West *et al.*, 2005; Nehme *et al.*, 2007), which play a central role in the immune system of insects (Lemaitre and Hoffmann, 2007; Royet and Dziarski, 2007). We present evidence in this study that plant-beneficial pseudomonads with insecticidal activities are naturally resistant against cecropin-type insect AMPs and the model AMP PMB and that OSA is crucial for this resistance in both CHA0 and PCL1391. In CHA0, it is possible that due to the uniform length of the OSA an effective surface barrier is formed that sterically hinders AMPs to bind to negatively charged lipid A moieties. Another explanation could be that OSA increases the resistance to AMPs indirectly, for example by acting as an anchor or stabilizer for proteases that neutralize AMPs such as reported for AprA in *Pseudomonas entomophila* (Liehl *et al.*, 2006). We currently have no explanation for the observation that most *P. chlororaphis* strains only displayed PMB resistance when growing in liquid medium, but not on solid medium. Possibly, the expression of various resistance genes (also of those involved in lipid A modification) is quite different when the bacteria are growing under the two contrasting conditions. This aspect needs further investigation in future work.

In general, resistance to PMB seems to correlate with the potential of bacteria of the *P. fluorescens* group to cause disease in lepidopteran insects (Fig. 6A). PMB resistance could therefore be used as a marker for insect pathogenicity in plant-beneficial pseudomonads. However, the genetic and molecular basis of the discovered PMB resistance in insecticidal pseudomonads needs further investigation in future work.

PMB and colistin are considered as last-resort antibiotics in clinics to treat patients with *Pseudomonas* infections (Landman *et al.*, 2008; Olaitan *et al.*, 2014). Our results suggest that development of high resistance to these polymyxins could take place in *P. aeruginosa* as well, for example by modification of the OSA gene cluster, which could render such strains resistant to AMPs. In the worst case, this event could also lead to cross-resistance to host AMPs (Anaya-López *et al.*, 2013).

It is possible that decreased AMP resistance is not the only reason why O-PS mutants of *P. protegens* CHA0 are significantly reduced in virulence towards insects. O-PS might contribute to equip CHA0 with an extremely tight and stable outer membrane, which would confer a general resistance to immune defenses beyond AMPs. Moreover, short smooth LPS could protect insect-pathogenic pseudomonads against phagocytosis by hemocytes, which are known to contribute to the clearance of bacterial infections in the insect hemolymph (Lemaitre and Hoffmann, 2007). That O-PS can protect against phagocytosis has been shown for other bacteria (Liang-Takasaki *et al.*, 1982; Lindell *et al.*, 2012; March *et al.*, 2013).

Deciphering the role of O-PS structures in direct host interactions

The present study suggests that O-antigens in plant-beneficial pseudomonads have evolved to high diversity, which might be linked to host or niche adaptation. Changes in LPS biosynthesis might have crucially contributed to the exploitation of insects as alternative hosts by insecticidal strains by creating a more protective cell envelope allowing the colonization and infection of these animals. Future studies should address the question whether high O-antigen diversity in these root-associated pseudomonads

is also advantageous for the interaction with plants. LPS plays an important role in interaction with plants as typical microbe-associated molecular pattern and elicitor of plant defenses (Zamioudis and Pieterse, 2012). Specific O-antigen structures may be significant for the molecular communication with the plant host, such as for the induction of systemic resistance. The knowledge gained in this study and the created mutant strains could now be used to investigate how these plant-beneficial microbes adapt to and communicate with their contrasting hosts.

EXPERIMENTAL PROCEDURES

Bacterial strains, O-PS mutant construction and complementation

All strains and plasmids constructed and used in this study are listed in Table S3. Detailed information on the growth conditions and on the construction and complementation of O-PS deletion and gene disruption mutants is provided in Text S1 in the supplemental material.

Bioinformatics

Genes and gene clusters putatively involved in the biosynthesis of O-PS in *P. protegens* CHA0 and *P. chlororaphis* subsp. *piscium* PCL1391 were identified by performing nucleotide (blastn) and protein BLAST on the NCBI website (http://blast.ncbi.nlm.nih.gov/) using sequences of genes and proteins (primarily glycosyltransferases, transporters, nucleotide sugar biosynthesis proteins, chain length regulators and the O-antigen ligase WaaL) reported to be important for the synthesis of O-PS in *P. aeruginosa* PAO1 (Lam *et al.*, 2011). Identification of such genes and gene clusters in a set of recently sequenced *Pseudomonas* strains (Flury *et al.*, 2016) and publicly available genome sequences was done similarly (with a minimum of 70% nucleotide sequence identity over 70% of the coding sequence (CDS)) by using the information from gene annotation and pan-genome calculations in EDGAR (Blom *et al.*, 2009), with standard settings as described in (Smits *et al.*, 2010), and from their localization on the chromosomes in GenDB (Meyer *et al.*, 2003). The functions encoded by the identified genes and

gene cluster were predicted using the NCBI Conserved Domain Database (CDD) Search (Marchler-Bauer *et al.*, 2011) and InterPro (Mitchell *et al.*, 2014) with default parameters and by finding orthologous genes in *P. aeruginosa* PAO1 by performing BLAST searches against this particular bacterial strain (Winsor *et al.*, 2011; http://www.pseudomonas.com/blast/set).

LPS extraction and visualization

Extraction of LPS was performed as previously described by Davis and Goldberg (2012) with the following modifications. If not otherwise stated, bacteria were sampled for LPS extraction after growing them for 16 h (i.e. to late exponential growth phase) in 10 ml of lysogeny broth (LB) at 25°C and 180 rpm as 1.5-ml suspensions with an optical density at 600 nm (OD_{600}) of 5.0. Because purification with phenol led to a loss of LPS with long O-PS in *P. protegens*, the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) directly after the proteinase K treatment. SDS-PAGE was performed with 15% acrylamide gels and the peqGOLD pre-stained Protein Marker V (PEQLAB Biotechnologie GmbH, Ehrlangen, Germany) was used as molecular mass standard. Silver staining of the gels was performed as described previously (Zhu *et al.*, 2012).

Insect assays

Injection assays for virulence determination using last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) were performed three times as described before (Péchy-Tarr *et al.*, 2013), with 18 larvae per treatment. Briefly, for infection, aliquots of 5 µl bacterial suspension containing approximately 50 washed cells were injected into the last proleg of each larva. The larvae were incubated at room temperature and their survival was monitored over 70 h. Feeding assays using larvae of the diamond back moth *P. xylostella* as an oral infection model were carried out two times as detailed previously (Flury *et al.*, 2016), with 64 larvae per treatment. Briefly, *P. xylostella* eggs (Syngenta Crop

Protection, Stein, Switzerland) were hatched in a growth chamber set at 26°C and 60% relative humidity. For oral infection, one-week-old larvae were placed individually into wells of Bio-Assay trays, each containing one pellet made of artificial insect diet and treated with 10 ul of a suspension of washed bacterial cells set to an OD of 0.5. Trays then were incubated in the 26°C growth chamber and larval survival was monitored during two days. Competition and colonization assays using fluorescently labelled strains and larvae of *G. mellonella* as an insect model were performed as described in Text S1 in the supplemental material.

Determination of minimum inhibitory concentrations (MIC) of AMPs

MIC determination in liquid medium was done by performing microdilution assays. Briefly, selected bacterial strains were grown overnight at 25°C and 180 rpm in LB and washed once in saline solution. The bacteria were inoculated into 96-well microtiter plates at a starting OD_{600} of 0.01 in a total volume of 200 µl LB supplemented with different concentrations of polymyxin B (Sigma, St. Louis, Missouri, USA) and in 150 µl LB supplemented with different concentrations of cecropin A and cecropin B (Bachem AG, Bubendorf, Switzerland). The MIC for a given strain was defined as the concentration at which no visible growth was observable after 24 h of incubation at 25°C and 300 rpm. The assay was carried out at least twice per strain at independent time points. MIC determination for polymyxin B on solid medium was performed using Etest strips (bioMérieux SA, Marcy l'Etoile, France) as described previously (Cullen *et al.*, 2015), with nutrient agar (NA) plates and incubation at 25°C, and interpreted according to manufacturer's instructions. The assay was performed twice per strain.

Polymyxin survival assay

To investigate the percentage of cells in a population that survive and grow in the presence of a certain AMP concentration, bacterial strains were grown on agar plates containing polymyxin B or colistin (Sigma, St. Louis, Missouri, USA) at two different concentrations. Therefore, bacterial suspensions

with an OD_{600} of 1.0 were prepared as described above and serially diluted to 10^{-4}. Ten microliters of each diluted bacterial suspension were spotted on NA plates supplemented with 10 or 100 µg/ml of polymyxin B or colistin sulfate. The same dilutions were also spotted on NA plates without polymyxins as a negative control. The plates were incubated for 24 h at 30°C and colony forming units (CFU) per 10 µl were calculated for each strain. The CFU counts were normalized with the numbers obtained from the negative controls to account for variations between bacterial samples. The experiment was performed three times per strain.

Statistical analysis

Statistical analysis of experiments was performed in RStudio version 0.98.1091 (http://www.rstudio.com/). One-way or two-way analysis of variance (ANOVA) with Tukey's HSD test for post-hoc comparisons and Wilcoxon-Mann-Whitney test were performed when appropriate. The Log-Rank test of the *survival* package of RStudio was used to calculate significant differences in insect toxicity between the wild type and isogenic mutant strains in the *Galleria* and *Plutella* virulence assays. One-sample *t* tests were performed with data from competition assays.

Additional details on the materials and methods used in this study are provided in Text S1 in the supplemental material.

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730
731

732 **TABLES**

733 **Table 1.** Short O-PS contributes to resistance of *Pseudomonas protegens* CHA0 to cecropin-type
734 insect antimicrobial peptides.

		Minimal inhibitory concentration[1] (μg/ml) of		
Strain	Genotype	Polymyxin B	Cecropin A	Cecropin B
CHA0	Wild type	>512	256	128
CHA5161	Δ*wbpL*	64	64	32
CHA5205	Δ*fcl*	>512	128	128
CHA5206	Δ*wzx*	16	64	32
PCL1391	Wild type	64	64	32
Q12-87	Wild type	1	8	8

735 [1]The minimum inhibitory concentration (MIC) of the antimicrobial peptides was determined by
736 performing broth microdilution assays. The MIC was defined as the concentration at which no visible
737 growth of the bacteria was observed 24 h after inoculation. The entire experiment was repeated at an
738 independent time point and identical results were obtained.

739

740

FIGURE LEGENDS

Fig. 1. Gene clusters and genes predicted to be involved in the biosynthesis of O-PS in *Pseudomonas chlororaphis* subsp. *piscium* PCL1391. Putative functions of encoded proteins or gene names are indicated above the corresponding genes, information about the locus tags (PCL1391_...) are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for. Functional predictions for the displayed genes are listed in Table S2. Mutations that were introduced in specific genes and gene clusters in the present study are depicted below each cluster.

Fig. 2. Gene clusters and genes predicted to be involved in the biosynthesis of O-PS in *Pseudomonas protegens* CHA0. Putative functions of encoded proteins or gene names are indicated above the corresponding genes, information about the locus tags (PFLCHA0_c…) are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for. Functional predictions for the displayed genes are listed in Table S2. Mutations that were introduced in specific genes and gene clusters in the present study are depicted below each cluster.

Fig. 3. Mutation of O-PS biosynthesis genes in *Pseudomonas chlororaphis* subsp. *piscium* PCL1391 (A) and *Pseudomonas protegens* CHA0 (B). SDS-PAGE of LPS extracted from PCL1391, CHA0 and their isogenic mutant strains grown at 25°C to late exponential growth phase in Lysogeny Broth. LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of each gel and predicted compositions of LPS molecules on the right. The Δ*fcl* mutant CHA5205 shown in the inset in panel B was analyzed on a separate gel (see supplemental Fig. S3D).

Fig. 4. Genetic diversity of O-PS biosynthesis in selected pseudomonads. Phylogeny of selected pseudomonads, based on the full core genome, and presence of identified gene clusters predicted to contribute to the biosynthesis of O-PS in these strains. Strains that were previously shown to be able

to infect and kill lepidopteran insect larvae (Flury *et al.*, 2016) are marked in red and non-insecticidal strains in green. Gene clusters lacking functionally important genes (CPA: *rmd*, *gmd* and/or *wbpW*; OSA: *wzz1*, *wzy*) are depicted as "reduced" and the ones missing additional genes (OSA: *wzx*) are displayed as "strongly reduced". Gene clusters that comprise genes coding for both ABC transporters and flippases (Wzx/Wzy) are depicted as "extended". Locus tags for all genes and gene clusters are listed in Table S1.

Fig. 5. O-polysaccharides contribute to insect pathogenicity in *Pseudomonas protegens* CHA0. **(A)** Virulence of mutant strains of CHA0 was determined by injection of a low number of bacterial cells into larvae of *Galleria mellonella* and monitoring the survival of the insects over time. Deletion of *wbpL wzx* or *fcl* significantly reduced the mortality of the larvae (*, p-value < 0.001; Log-rank test), while the complemented Δ*wbpL* mutant was as virulent as the wild type. Saline solution served as a negative control (in gray). The experiment was performed with 18 larvae per treatment and was repeated twice with similar results (Fig. S6A). **(B)** The virulence for *wbpL wzx* or *fcl* mutants and the wild-type strain of *P. protegens* CHA0 upon oral infection was assessed using larvae of *Plutella xylostella*. The caterpillars were fed with bacteria-treated artificial diet and their survival was monitored over two days. Saline solution and a *gacA* mutant of CHA0 served as negative controls. Treatments that were significantly different from the one with wild-type CHA0 are indicated with * (p-value < 0.005; Log-rank test). This experiment was carried out with 64 larvae per treatment and was repeated once with similar results (Fig. S6B). **(C)** Competitiveness of mutant strains during insect infection was investigated by injecting equal numbers of fluorescently marked mutant and corresponding wild-type cells into larvae of *G. mellonella* and counting bacterial cells in hemolymph samples 20 to 24 h after the injection by epifluorescence microscopy. The competitive index indicates the ratio between the cell numbers of the mutant and the wild type, normalized by the ratio in the inoculum. Shown are means and standard deviations of three larvae per treatment. Significant

differences between the competing strains are indicated with * (p-value < 0.05; one-sample *t* test) and *** (p-value < 0.005; one-sample *t* test). The experiment was repeated once with similar results. NS, not significant. **(D)** Colonization efficiency of *P. protegens* CHA0 and its isogenic mutants in *G. mellonella*. Each bacterial strain (mCherry-tagged for selective plating) was injected into five individual larvae. Colony forming units (CFUs) were determined for each insect by extraction of hemolymph 24 h after injection and plating of serial dilutions on nutrient agar plates. Saline solution served as a negative control. Numbers indicate the average CFUs per strain and larva. Treatments labelled with different characters were significantly different (p-value < 0.05; One-way ANOVA with transformed data and post-hoc Tukey HSD).

Fig. 6. Occurrence of high antimicrobial peptide resistance in insect-pathogenic pseudomonads and underlying mechanisms in *Pseudomonas protegens* CHA0. (A) The minimum inhibitory concentration (MIC) of polymyxin B (PMB) was determined for selected *Pseudomonas* strains by Etest strips (orange circles) and broth microdilution assay (black diamonds). Shown are means of two independent experiments. Strains that were previously shown to be able to infect and kill lepidopteran insect larvae (Flury *et al.*, 2016) are marked in red and non-insecticidal strains in green. **(B)** Cell suspensions of *P. protegens* CHA0 and derivatives were serially diluted and aliquots were spotted on nutrient agar plates containing either 10 or 100 µg/ml PMB. Colonies were counted after incubating the plates for 24 h. Colony numbers were normalized by the number of colonies in the control plates without PMB, in order to compare them between strains. Shown are means and standard deviations from three independent assays with similar results. In *P. aeruginosa*, *arnA* is important for antimicrobial peptide resistance by being essential for lipid A modification with 4-aminoarabinose (Raetz *et al.*, 2007). Strains marked with different characters were significantly different from each other in respect to PMB resistance (p-value < 0.05; two-way ANOVA with transformed data).

Fig. 7. Models for the LPS structures in two plant-beneficial pseudomonads with insecticidal activity. *Pseudomonas chlororaphis* subsp. *piscium* PCL1391 **(A)** and *Pseudomonas protegens* CHA0 **(B)** both produce LPS with two distinct forms of O-polysaccharide (O-PS). The major type of O-PS is most probably a heteropolymer (in violet) and synthesized by proteins encoded in the OSA gene cluster via the Wzx/Wzy-dependent pathway. In PCL1391, the chain lengths of this particular O-PS vary and are regulated by the chain length determination proteins Wzz1 and Wzz2. In CHA0, the major form of O-PS consists of a single repeating unit due to the lack of a polymerase. Both strains produce a second, minor type of O-PS, which is synthesized via the ATP-binding cassette (ABC) transporter-dependent pathway by proteins encoded in the CPA gene cluster in PCL1391 (in red) and the OBC3 gene cluster in CHA0 (in green). The CPA in PCL1391 is mainly produced at temperatures above 25°C and likely a homopolymer. In CHA0, most of the lipid A-core molecules are capped with O-PS while in PCL1391 also rough LPS (i.e. lipid A-core without O-PS) is produced and presented on the bacterial surface.

SUPPORTING INFORMATION

Fig. S1. Gene clusters and genes involved in the biosynthesis of O-antigen in *Pseudomonas aeruginosa* PAO1. Gene names or predicted functions of encoded proteins are indicated above the corresponding genes, information about the locus tags are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for. The figure was drawn according to Lam *et al.* (2011).

Fig. S2. Identification of the *waaL* gene in *Pseudomonas chlororaphis* subsp. *piscium* PCL1391 and *Pseudomonas protegens* CHA0. (A) Candidate genes for *waaL* were identified in strains PCL1391 and CHA0 by protein BLAST searches with the corresponding amino acid sequence from *Pseudomonas aeruginosa* PAO1. Shown are the genetic loci of the putative *waaL* genes. The candidate gene in strain CHA0 (PFLCHA0_c05350) appears to be the result of a fusion between the *waaL* gene and a gene coding for a phosphatase. **(B)** The proteins encoded by *waaL* in CHA0 and PCL1391 were predicted by InterPro protein sequence analysis to possess 12 transmembrane domains and a periplasmic loop like it has been shown for PAO1 WaaL (Islam *et al.*, 2010). The WaaL protein in CHA0 was predicted to contain an additionally C-terminal kinase domain.

Fig. S3. Characterization of *Pseudomonas chlororaphis* subsp. *piscium* PCL1391, *Pseudomonas protegens* CHA0 and their isogenic O-polysaccharide (O-PS) mutants. (A) Complementation of the PCL1391 *waaL* mutant. SDS-PAGE of lipopolysaccharide (LPS) extracted from the wild type and selected isogenic mutants grown at 25°C to late exponential growth phase in Lysogeny Broth (LB). LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results. **(B)** Presence of CPA-type O-antigen at elevated temperatures in *P. chlororaphis* subsp. *piscium* PCL1391. SDS-PAGE of LPS extracted from PCL1391 and isogenic mutant strains grown at 30°C to late exponential growth phase in LB. LPS was

made visible by silver staining. The presence of CPA-type O-antigen bands in the *wzx* mutant and their absence in the Δ*cpa* mutant are highlighted with the red frame. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results. **(C)** Influence of mutation of selected O-PS genes on growth. Growth curves of *P. chlororaphis* subsp. *piscium* PCL1391 (left), *P. protegens* CHA0 (right), and their isogenic O-PS mutants in LB at 25°C. Shown are means and standard deviations of five independent cultures per bacterial strain. **(D)** Complementation of selected mutants of *P. protegens* CHA0 restores the expression of wild-type O-antigen. SDS-PAGE of LPS extracted from the wild type and selected isogenic mutants grown at 25°C to late exponential growth phase in LB. LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results. **(E)** SDS-PAGE of LPS extracted from diverse *Pseudomonas* strains grown until late exponential growth phase in LB at 25°C. Silver staining was used to visualize LPS on the gels. Molecular weights in kDa are indicated on the left of each gel.

Fig. S4. Reduction of the OSA locus in diverse *Pseudomonas* strains. Shown are the OSA loci of selected *Pseudomonas* strains. Gene names or predicted functions of encoded proteins are indicated above the corresponding genes, information about the locus tags are given below (locus prefixes are listed in Table S1). Genes are drawn to scale and are color-coded according to the function of the proteins they code for.

Fig. S5. Role of O-polysaccharide (O-PS) in virulence, root colonization, and resistance to antimicrobial peptides. (A) Regulation of O-PS chain length is not essential for virulence of *P. chlororaphis* subsp. *piscium* PCL1391 upon injection. Virulence of PCL1391 wild-type and mutant strains was determined by injection of low cell numbers into larvae of *Galleria mellonella* and monitoring the survival of the insects over time. Mutation of *wzz1* (in blue, PCL-11) and *wzz2* (in red,

PCL-13) did not significantly reduce the mortality of the larvae compared to the wild type (in black) (p-value > 0.05; Log-rank test). Saline solution served as a negative control (in gray). The experiment was performed with 18 larvae per treatment and repeated once with similar results. **(B)** Competitiveness of mutant strains during root colonization was studied by inoculation of cucumber plants with equal numbers of fluorescently marked mutant and corresponding wild-type cells and counting bacterial cells on root tips by epifluorescence microscopy 5 days after the inoculation. The competitive index indicates the ratio between the cell numbers of the mutant and the wild type, normalized by the ratio in the inoculum. Shown are means and standard deviations from three independent plants. No significant differences between the competing strains was observed (one-sample t test; $\alpha = 0.05$). The experiment was repeated once with similar results. NS, not significant. **(C)** Resistance of *P. chlororaphis* subsp. *piscium* PCL1391 to polymyxin B depends on the presence of the OSA-type O-antigen. The minimum inhibitory concentration (MIC) of polymyxin B was determined for *P. chlororaphis* subsp. *piscium* PCL1391 and isogenic mutant strains (PCL-1, *waaL*; PCL-3, Δ*obc2*; PCL-9, *wzx*; PCL-11, *wzz1*; PCL-12, Δ*cpa*; and PCL-13, *wzz2*) by performing a microdilution assay with LB. The MIC was defined as the concentration at which no visible growth of the bacteria was observed 24 h after inoculation. Shown are averages and standard deviations of three independent experiments. Different characters indicate statistically significant differences in MICs between the strains (p-value < 0.05; Wilcoxon-Mann-Whitney test).

Fig. S6. O-antigen contributes to insect pathogenicity in *Pseudomonas protegens* CHA0. (A) Repetitions of the *Galleria mellonella* virulence assay shown in Fig. 5 and performed with *P. protegens* CHA0 and isogenic mutant strains. Mutants displaying significantly reduced virulence upon injection into the hemocoel compared to the wild type are marked with asterisks (*, p-value < 0.005; Log-rank test). Saline solution served as a negative control (in gray). **(B)** Repetition of the oral virulence assay (shown in Fig. 5) performed with *P. protegens* CHA0 and isogenic mutant strains using larvae of

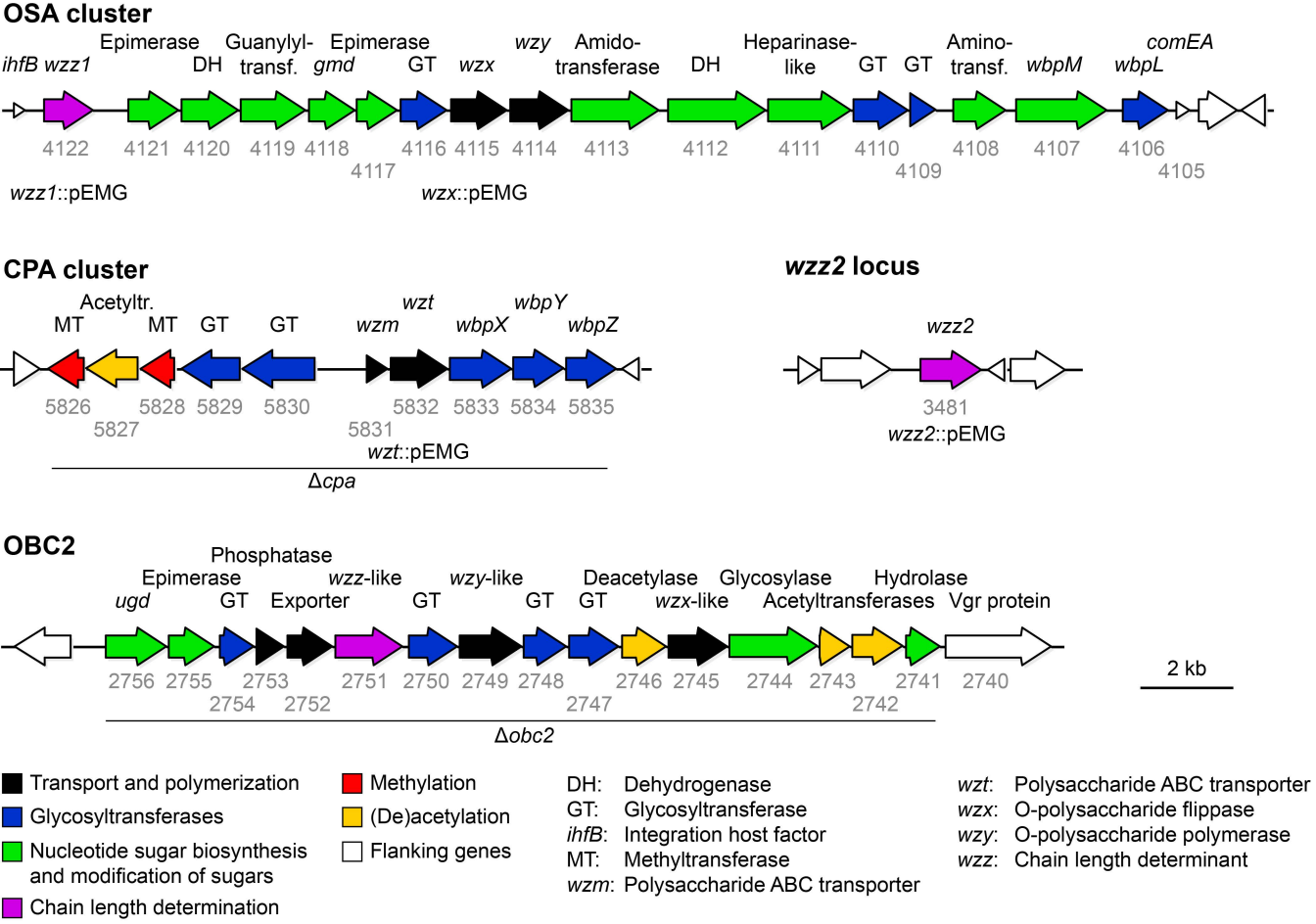
Plutella xylostella. Saline solution (in gray) and a *gacA* mutant of CHA0 (CHA89, in brown) served as negative controls. Treatments that were significantly different from the one with wild-type CHA0 are indicated with * (p-value < 0.005; Log-rank test).

Table S1. Polysaccharide biosynthesis gene clusters identified in selected *Pseudomonas* strains.

Table S2. Function predictions of putative O-polysaccharide biosynthesis genes in selected *Pseudomonas* strains.

Table S3. Bacterial strains, plasmids and primers used in this study.

Text S1. Supplemental experimental procedures.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Epimerase DH
Guanylyl-transf.
Epimerase gmd
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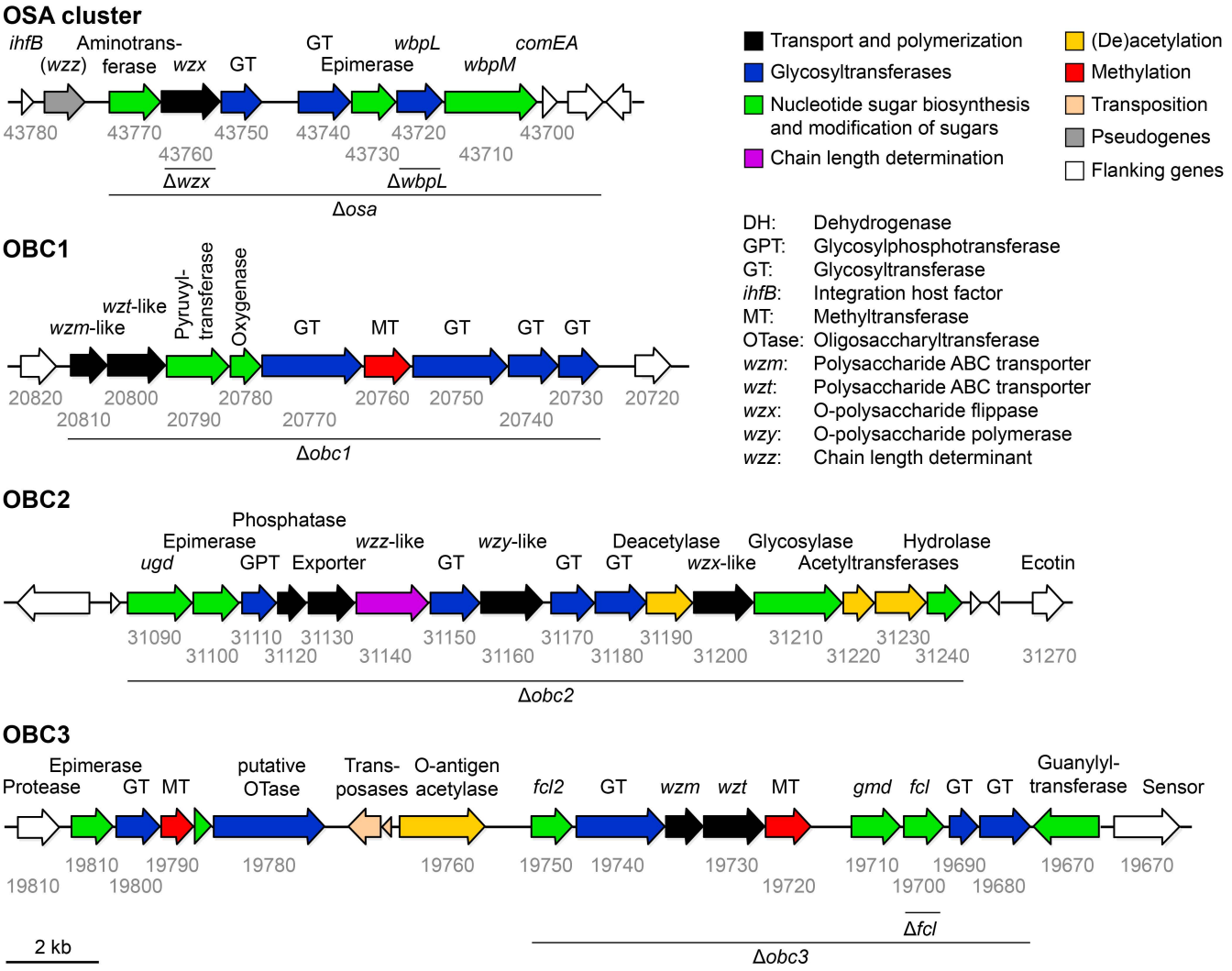
wzx
wzy
Amido-transferase
DH
Heparinase-like
GT
GT
Amino-transf.
wbpM
wbpL
comEA
4122 4121 4120 4119 4118 4117 4116 4115 4114 4113 4112 4111 4110 4109 4108 4107 4106 4105
wzz1::pEMG
wzx::pEMG
CPA cluster
Acetyltr. MT
MT
GT
GT
wzm
wzt
wbpX
wbpY
wbpZ
5826 5827 5828 5829 5830 5831 5832 5833 5834 5835
wzt::pEMG
Δcpa
wzz2 locus
wzz2
3481
wzz2::pEMG
OBC2
ugd
Phosphatase
Epimerase GT
wzz-like Exporter
GT
wzy-like
GT
GT
Deacetylase
wzx-like
Glycosylase Acetyltransferases
Hydrolase
Vgr protein
2756 2755 2754 2753 2752 2751 2750 2749 2748 2747 2746 2745 2744 2743 2742 2741 2740
Δobc2
2 kb
Transport and polymerization
Glycosyltransferases
Nucleotide sugar biosynthesis and modification of sugars
Chain length determination
Methylation
(De)acetylation
Flanking genes
DH: Dehydrogenase
GT: Glycosyltransferase
ihfB: Integration host factor
MT: Methyltransferase
wzm: Polysaccharide ABC transporter
wzt: Polysaccharide ABC transporter
wzx: O-polysaccharide flippase
wzy: O-polysaccharide polymerase
wzz: Chain length determinant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Aminotrans-ferase
wzx
GT
GT Epimerase
wbpL
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43750
43740
43730
43720
43710
43700
Δwzx
ΔwbpL
Δosa
Transport and polymerization
Glycosyltransferases
Nucleotide sugar biosynthesis and modification of sugars
Chain length determination
(De)acetylation
Methylation
Transposition
Pseudogenes
Flanking genes
DH: Dehydrogenase
GPT: Glycosylphosphotransferase
GT: Glycosyltransferase
ihfB: Integration host factor
MT: Methyltransferase
OTase: Oligosaccharyltransferase
wzm: Polysaccharide ABC transporter
wzt: Polysaccharide ABC transporter
wzx: O-polysaccharide flippase
wzy: O-polysaccharide polymerase
wzz: Chain length determinant
OBC1
wzm-like
wzt-like
Pyruvyl-transferase
Oxygenase
GT
MT
GT
GT
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20780
20770
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20730
20720
Δobc1
OBC2
Phosphatase
Epimerase
ugd
GPT
Exporter
wzz-like
GT
wzy-like
GT
Deacetylase
GT
wzx-like
Glycosylase
Acetyltransferases
Hydrolase
Ecotin
31090
31100
31110
31120
31130
31140
31150
31160
31170
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31190
31200
31210
31220
31230
31240
31270
Δobc2
OBC3
Epimerase
Protease
GT
MT
putative
OTase
Trans-posases
O-antigen acetylase
fcl2
GT
wzm
wzt
MT
gmd
fcl
GT
GT
Guanylyl-transferase
Sensor
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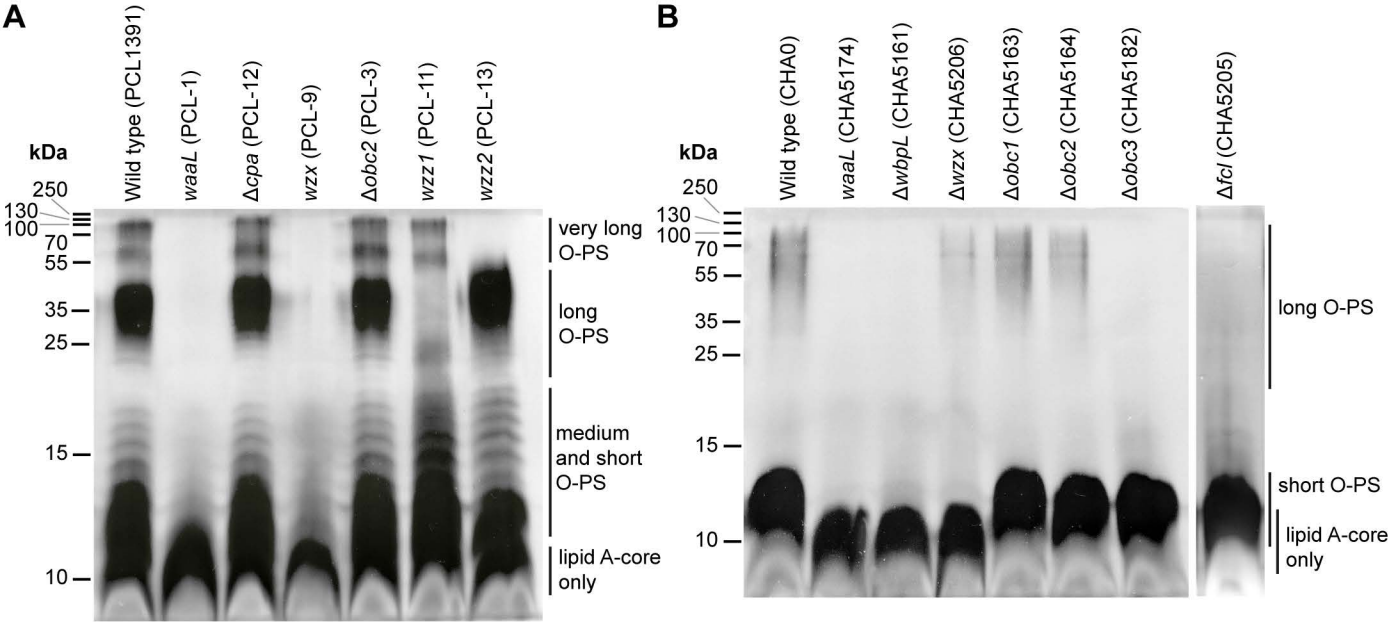
A
kDa
250
130
100
70
55
35
25
15
10
Wild type (PCL1391)
waaL (PCL-1)
Δcpa (PCL-12)
wzx (PCL-9)
Δobc2 (PCL-3)
wzz1 (PCL-11)
wzz2 (PCL-13)
very long O-PS
long O-PS
medium and short O-PS
lipid A-core only
B
kDa
250
130
100
70
55
35
25
15
10
Wild type (CHA0)
waaL (CHA5174)
ΔwbpL (CHA5161)
Δwzx (CHA5206)
Δobc1 (CHA5163)
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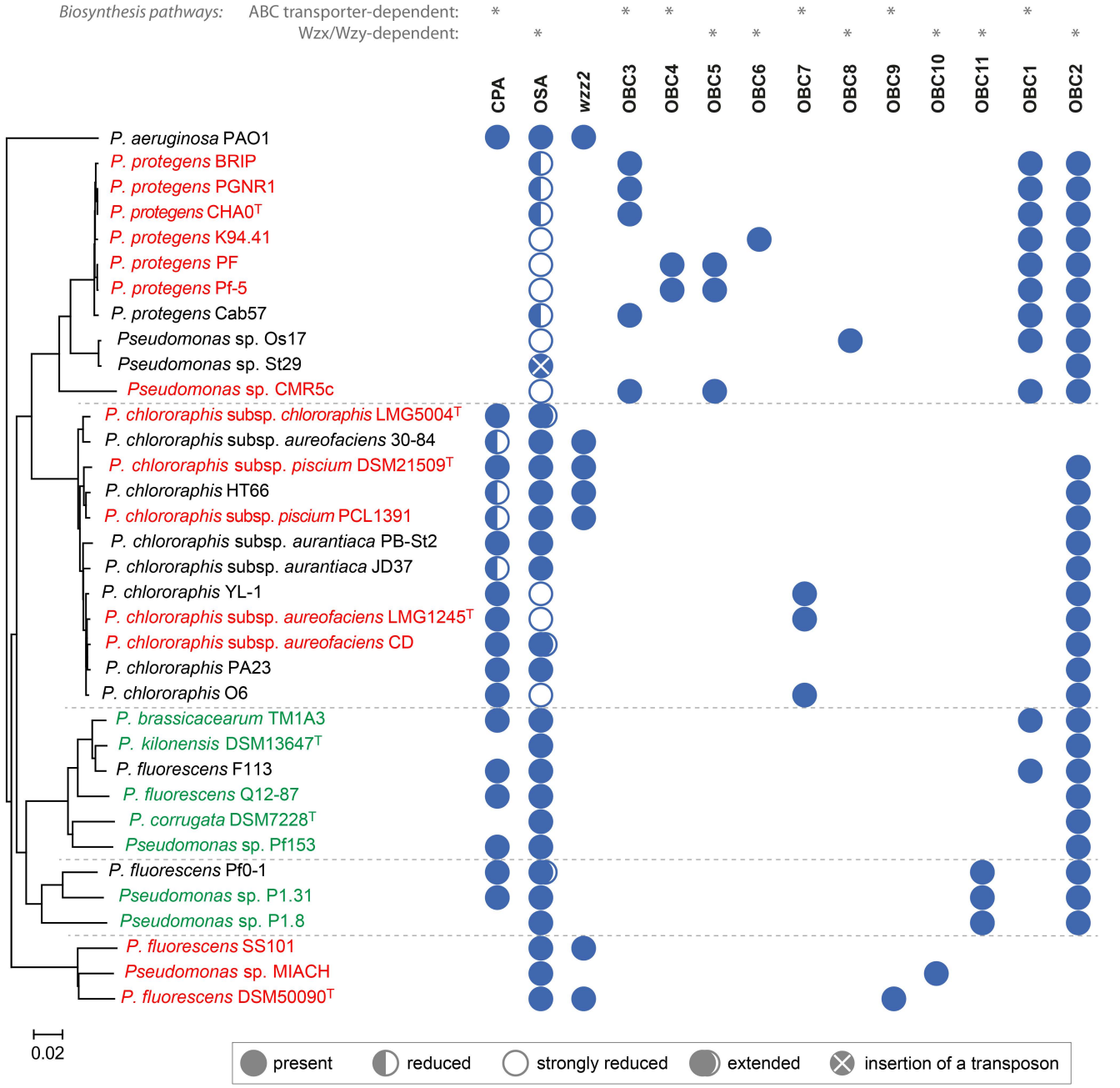
Δobc3 (CHA5182)
Δfcl (CHA5205)
long O-PS
short O-PS
lipid A-core only


Biosynthesis pathways:
ABC transporter-dependent:
Wzx/Wzy-dependent:
CPA
OSA
wzz2
OBC3
OBC4
OBC5
OBC6
OBC7
OBC8
OBC9
OBC10
OBC11
OBC1
OBC2
P. aeruginosa PAO1
P. protegens BRIP
P. protegens PGNR1
P. protegens CHA0T
P. protegens K94.41
P. protegens PF
P. protegens Pf-5
P. protegens Cab57
Pseudomonas sp. Os17
Pseudomonas sp. St29
Pseudomonas sp. CMR5c
P. chlororaphis subsp. chlororaphis LMG5004T
P. chlororaphis subsp. aureofaciens 30-84
P. chlororaphis subsp. piscium DSM21509T
P. chlororaphis HT66
P. chlororaphis subsp. piscium PCL1391
P. chlororaphis subsp. aurantiaca PB-St2
P. chlororaphis subsp. aurantiaca JD37
P. chlororaphis YL-1
P. chlororaphis subsp. aureofaciens LMG1245T
P. chlororaphis subsp. aureofaciens CD
P. chlororaphis PA23
P. chlororaphis O6
P. brassicacearum TM1A3
P. kilonensis DSM13647T
P. fluorescens F113
P. fluorescens Q12-87
P. corrugata DSM7228T
Pseudomonas sp. Pf153
P. fluorescens Pf0-1
Pseudomonas sp. P1.31
Pseudomonas sp. P1.8
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Pseudomonas sp. MIACH
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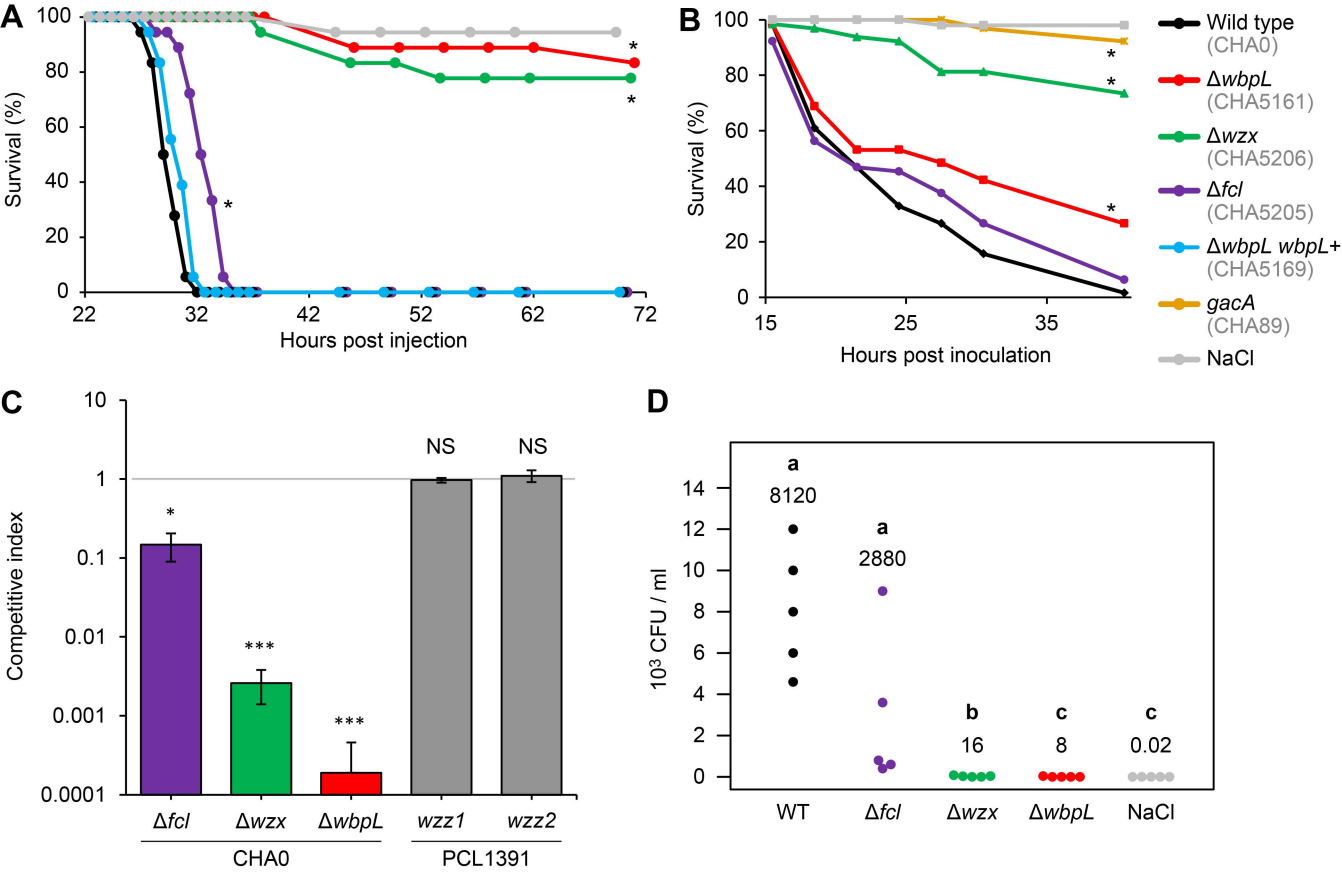
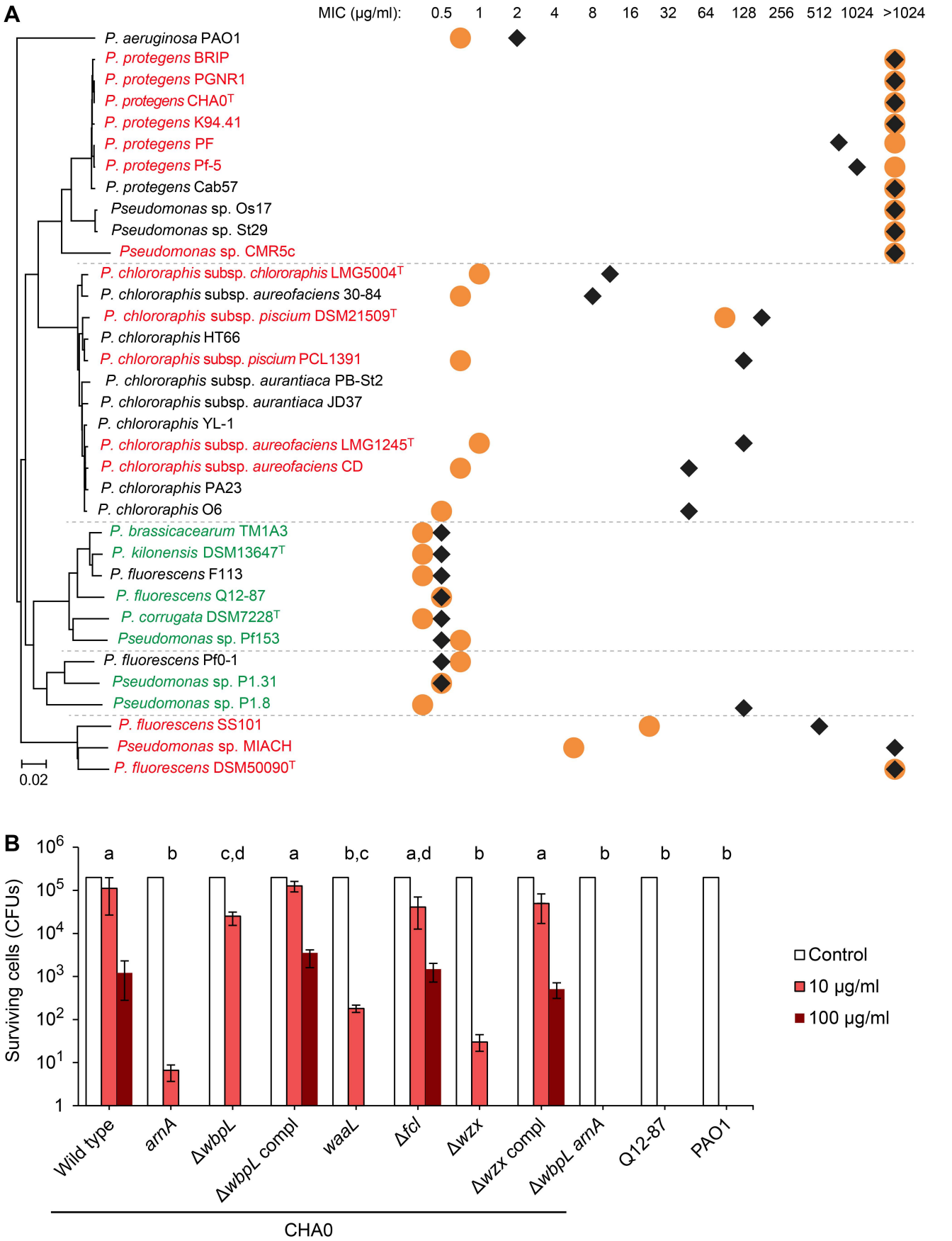
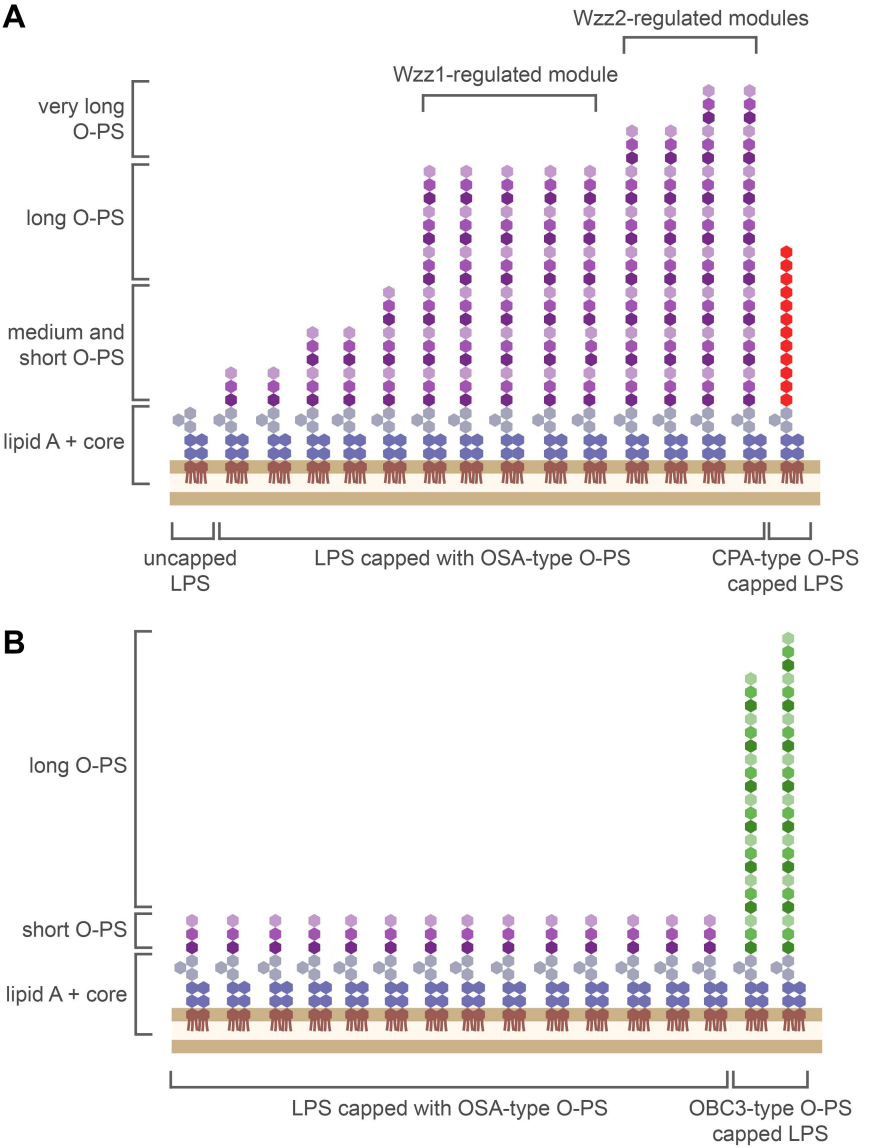
present
reduced
strongly reduced
extended
insertion of a transposon


A
Survival (%)
Hours post injection
B
Survival (%)
Hours post inoculation
Wild type (CHA0)
ΔwbpL (CHA5161)
Δwzx (CHA5206)
Δfcl (CHA5205)
ΔwbpL wbpL+ (CHA5169)
gacA (CHA89)
NaCl
C
Competitive index
NS
Δfcl
Δwzx
ΔwbpL
wzz1
wzz2
CHA0
PCL1391
D
10³ CFU / ml
a
8120
a
2880
b
16
c
8
c
0.02
WT
Δfcl
Δwzx
ΔwbpL
NaCl


A
MIC (µg/ml):
0.5
1
2
4
8
16
32
64
128
256
512
1024
>1024
P. aeruginosa PAO1
P. protegens BRIP
P. protegens PGNR1
P. protegens CHA0T
P. protegens K94.41
P. protegens PF
P. protegens Pf-5
P. protegens Cab57
Pseudomonas sp. Os17
Pseudomonas sp. St29
Pseudomonas sp. CMR5c
P. chlororaphis subsp. chlororaphis LMG5004T
P. chlororaphis subsp. aureofaciens 30-84
P. chlororaphis subsp. piscium DSM21509T
P. chlororaphis HT66
P. chlororaphis subsp. piscium PCL1391
P. chlororaphis subsp. aurantiaca PB-St2
P. chlororaphis subsp. aurantiaca JD37
P. chlororaphis YL-1
P. chlororaphis subsp. aureofaciens LMG1245T
P. chlororaphis subsp. aureofaciens CD
P. chlororaphis PA23
P. chlororaphis O6
P. brassicacearum TM1A3
P. kilonensis DSM13647T
P. fluorescens F113
P. fluorescens Q12-87
P. corrugata DSM7228T
Pseudomonas sp. Pf153
P. fluorescens Pf0-1
Pseudomonas sp. P1.31
Pseudomonas sp. P1.8
P. fluorescens SS101
Pseudomonas sp. MIACH
P. fluorescens DSM50090T
0.02
B
Surviving cells (CFUs)
10^6
10^5
10^4
10^3
10^2
10^1
1
a
b
c,d
a
b,c
a,d
b
a
b
b
b
Control
10 µg/ml
100 µg/ml
Wild type
arnA
ΔwbpL
ΔwbpL compl
waaL
Δfcl
Δwzx
Δwzx compl
ΔwbpL arnA
Q12-87
PAO1
CHA0


A
Wzz2-regulated modules
Wzz1-regulated module
very long O-PS
long O-PS
medium and short O-PS
lipid A + core
uncapped LPS
LPS capped with OSA-type O-PS
CPA-type O-PS capped LPS
B
long O-PS
short O-PS
lipid A + core
LPS capped with OSA-type O-PS
OBC3-type O-PS capped LPS

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SUPPORTING INFORMATION: Supplemental Figures S1, S2, S3, S4, S5 and S6

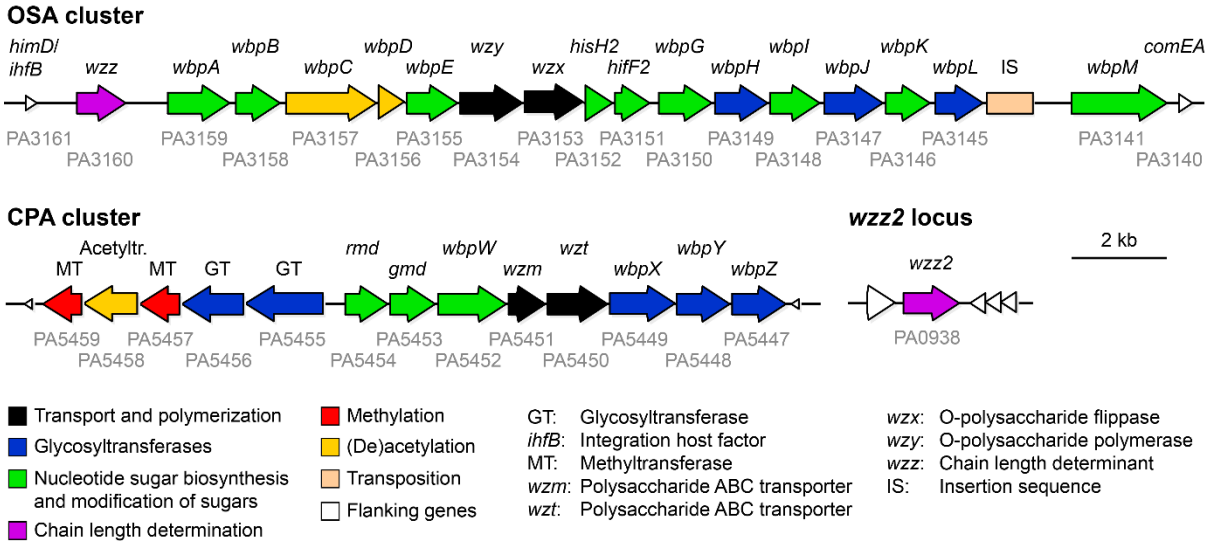


Fig. S1. Gene clusters and genes involved in the biosynthesis of O-antigen in *Pseudomonas aeruginosa* PAO1. Gene names or predicted functions of encoded proteins are indicated above the corresponding genes, information about the locus tags are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for. The figure was drawn according to Lam *et al.* (2011).

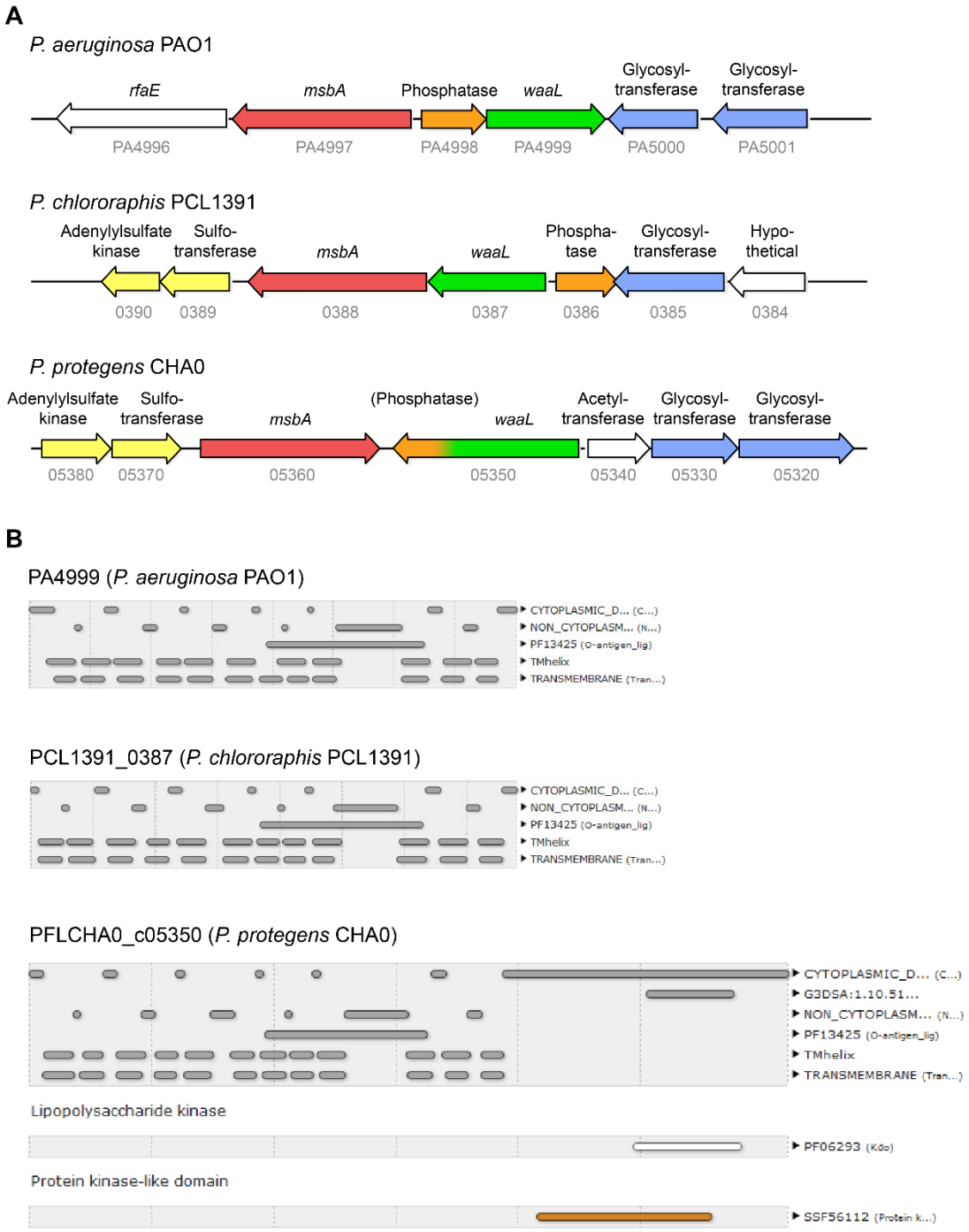
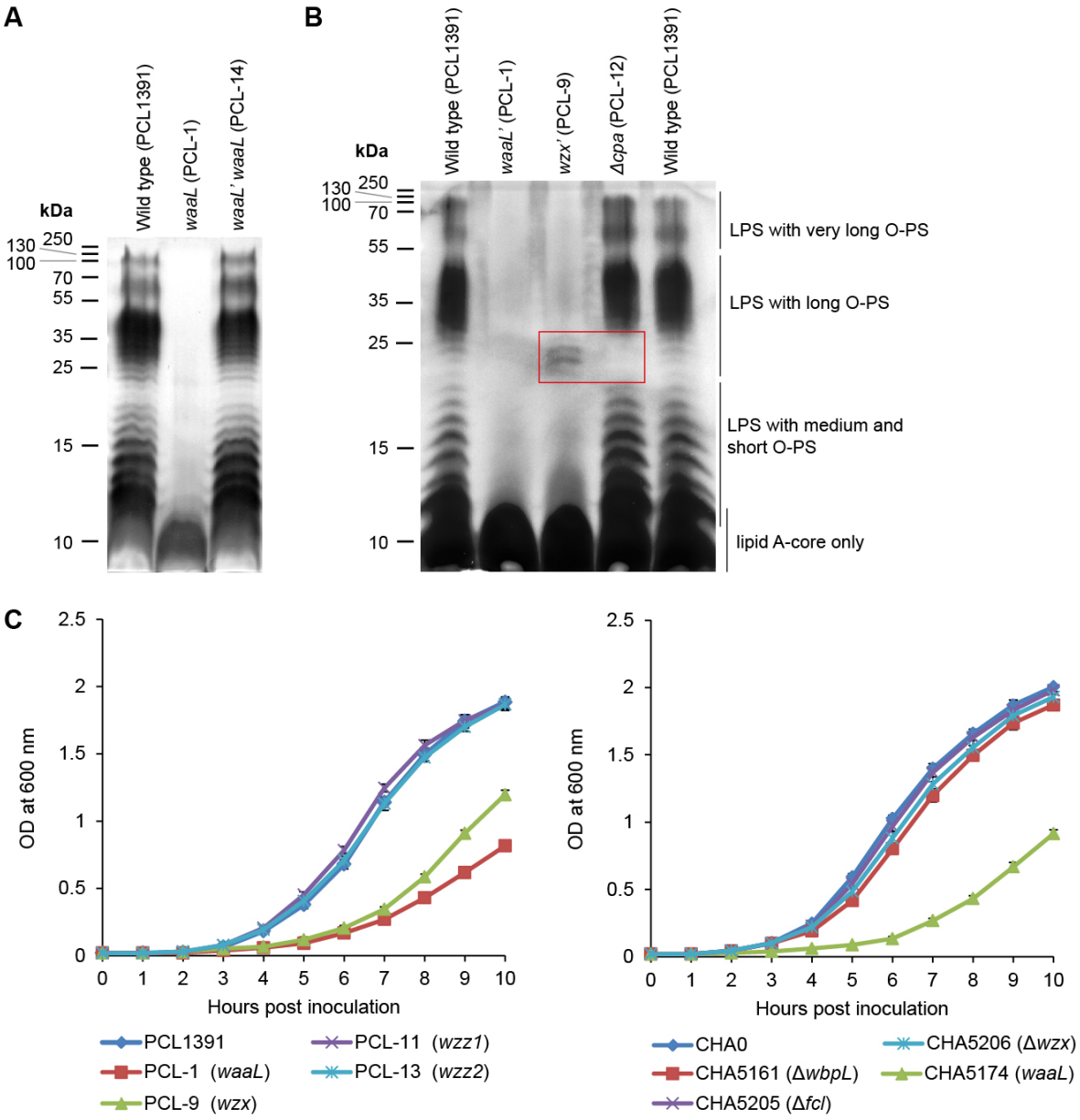


Fig. S2. Identification of the *waaL* gene in *Pseudomonas chlororaphis* subsp. *piscium* PCL1391 and *Pseudomonas protegens* CHA0. (A) Candidate genes for *waaL* were identified in strains PCL1391 and CHA0 by protein BLAST searches with the corresponding amino acid sequence from *Pseudomonas aeruginosa* PAO1. Shown are the genetic loci of the putative *waaL* genes. The candidate gene in strain CHA0 (PFLCHA0_c05350) appears to be the result of a fusion between the *waaL* gene and a gene coding for a phosphatase. **(B)** The proteins encoded by *waaL* in CHA0 and PCL1391 were predicted by InterPro protein sequence analysis to possess 12 transmembrane domains and a periplasmic loop like it has been shown for PAO1 WaaL (Islam *et al.*, 2010). The WaaL protein in CHA0 was predicted to contain an additionally C-terminal kinase domain.


A
kDa
Wild type (PCL1391)
waaL (PCL-1)
waaL' waaL (PCL-14)
130
250
100
70
55
35
25
15
10
B
kDa
Wild type (PCL1391)
waaL' (PCL-1)
wzx' (PCL-9)
Δcpa (PCL-12)
Wild type (PCL1391)
LPS with very long O-PS
LPS with long O-PS
LPS with medium and short O-PS
lipid A-core only
C
OD at 600 nm
Hours post inoculation
PCL1391
PCL-1 (waaL)
PCL-9 (wzx)
PCL-11 (wzz1)
PCL-13 (wzz2)
CHA0
CHA5161 (ΔwbpL)
CHA5205 (Δfcl)
CHA5206 (Δwzx)
CHA5174 (waaL)

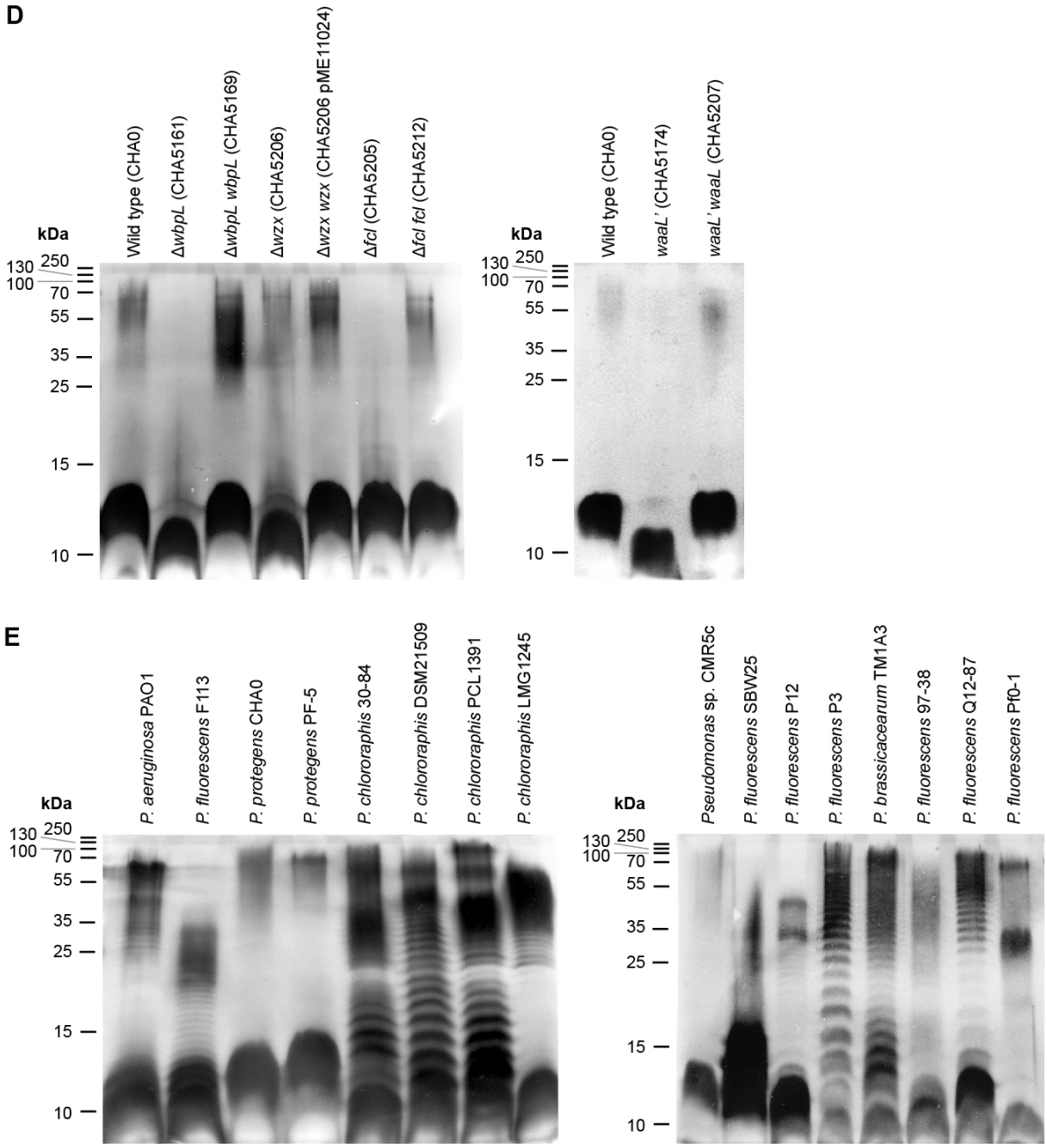


Fig. S3. Characterization of *Pseudomonas chlororaphis* subsp. *piscium* PCL1391, *Pseudomonas protegens* CHA0 and their isogenic O-polysaccharide (O-PS) mutants. (A) Complementation of the PCL1391 *waaL* mutant. SDS-PAGE of lipopolysaccharide (LPS) extracted from the wild type and selected isogenic mutants grown at 25°C to late exponential growth phase in Lysogeny Broth (LB). LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results. **(B)** Presence of CPA-type O-antigen at elevated temperatures in *P. chlororaphis* subsp. *piscium* PCL1391. SDS-PAGE of LPS extracted from PCL1391 and isogenic mutant strains grown at 30°C to late exponential growth phase in LB. LPS was made visible by silver staining. The presence of CPA-type O-antigen bands in the *wzx* mutant and their absence in the Δ*cpa* mutant are highlighted with the red frame. Molecular weights in kDa are depicted on the left

of the gel. The experiment was repeated once with similar results. **(C)** Influence of mutation of selected O-PS genes on growth. Growth curves of *P. chlororaphis* subsp. *piscium* PCL1391 (left), *P. protegens* CHA0 (right), and their isogenic O-PS mutants in LB at 25°C. Shown are means and standard deviations of five independent cultures per bacterial strain. **(D)** Complementation of selected mutants of *P. protegens* CHA0 restores the expression of wild-type O-antigen. SDS-PAGE of LPS extracted from the wild type and selected isogenic mutants grown at 25°C to late exponential growth phase in LB. LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results. **(E)** SDS-PAGE of LPS extracted from diverse *Pseudomonas* strains grown until late exponential growth phase in LB at 25°C. Silver staining was used to visualize LPS on the gels. Molecular weights in kDa are indicated on the left of each gel.

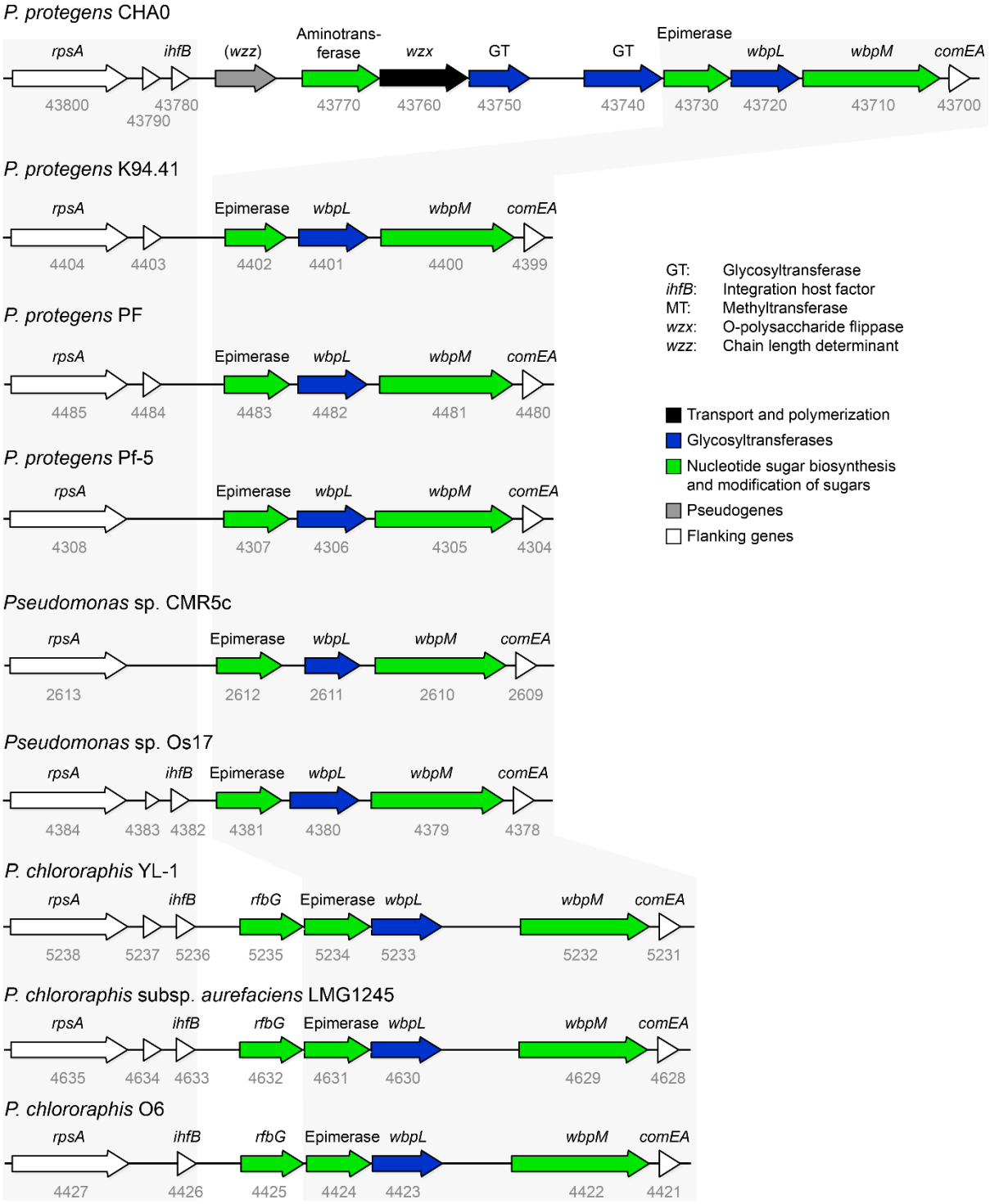


Fig. S4. Reduction of the OSA locus in diverse *Pseudomonas* strains. Shown are the OSA loci of selected *Pseudomonas* strains. Gene names or predicted functions of encoded proteins are indicated above the corresponding genes, information about the locus tags are given below (locus prefixes are listed in Table S1). Genes are drawn to scale and are color-coded according to the function of the proteins they code for.

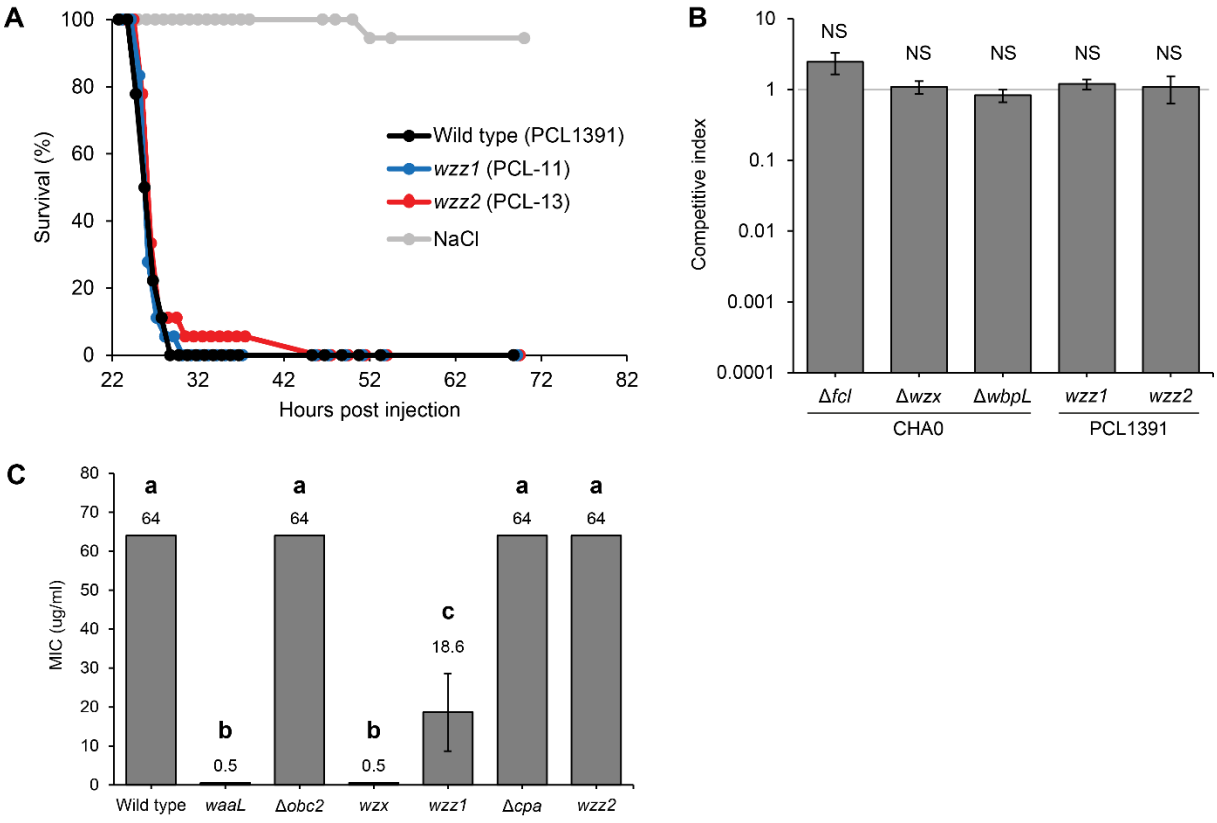


Fig. S5. Role of O-polysaccharide (O-PS) in virulence, root colonization, and resistance to antimicrobial peptides. (A) Regulation of O-PS chain length is not essential for virulence of *P. chlororaphis* subsp. *piscium* PCL1391 upon injection. Virulence of PCL1391 wild-type and mutant strains was determined by injection of low cell numbers into larvae of *Galleria mellonella* and monitoring the survival of the insects over time. Mutation of *wzz1* (in blue, PCL-11) and *wzz2* (in red, PCL-13) did not significantly reduce the mortality of the larvae compared to the wild type (in black) (p-value > 0.05; Log-rank test). Saline solution served as a negative control (in gray). The experiment was performed with 18 larvae per treatment and repeated once with similar results. **(B)** Competitiveness of mutant strains during root colonization was studied by inoculation of cucumber plants with equal numbers of fluorescently marked mutant and corresponding wild-type cells and counting bacterial cells on root tips by epifluorescence microscopy 5 days after the inoculation. The competitive index indicates the ratio between the cell numbers of the mutant and the wild type, normalized by the ratio in the inoculum. Shown are means and standard deviations from three independent plants. No significant differences between the competing strains was observed (one-sample t test; $\alpha = 0.05$). The experiment was repeated once with similar results. NS, not significant. **(C)** Resistance of *P. chlororaphis* subsp. *piscium* PCL1391 to polymyxin B depends on the presence of the OSA-type O-antigen. The minimum inhibitory concentration (MIC) of polymyxin B was determined for *P. chlororaphis* subsp. *piscium* PCL1391 and isogenic mutant strains (PCL-1, *waaL*; PCL-3, Δ*obc2*; PCL-9, *wzx*; PCL-11, *wzz1*; PCL-12, Δ*cpa*; and PCL-13, *wzz2*) by performing a microdilution assay with LB. The MIC was defined as the concentration at which no visible growth of the bacteria was observed 24 h after inoculation. Shown are averages and standard deviations of three independent experiments. Different characters indicate statistically significant differences in MICs between the strains (p-value < 0.05; Wilcoxon-Mann-Whitney test).

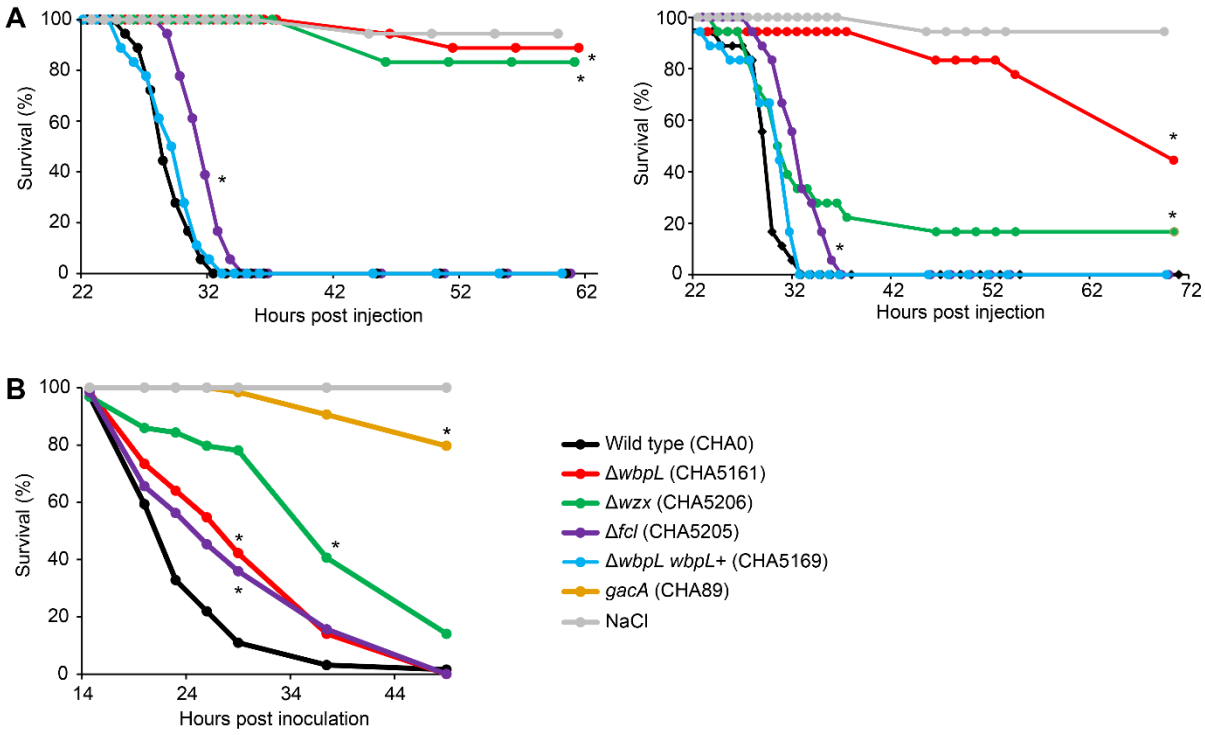


Fig. S6. O-antigen contributes to insect pathogenicity in *Pseudomonas protegens* CHA0. (A) Repetitions of the *Galleria mellonella* virulence assay shown in Fig. 5 and performed with *P. protegens* CHA0 and isogenic mutant strains. Mutants displaying significantly reduced virulence upon injection into the hemocoel compared to the wild type are marked with asterisks (*, p-value < 0.005; Log-rank test). Saline solution served as a negative control (in gray). **(B)** Repetition of the oral virulence assay (shown in Fig. 5) performed with *P. protegens* CHA0 and isogenic mutant strains using larvae of *Plutella xylostella*. Saline solution (in gray) and a *gacA* mutant of CHA0 (CHA89, in brown) served as negative controls. Treatments that were significantly different from the one with wild-type CHA0 are indicated with * (p-value < 0.005; Log-rank test).

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SUPPORTING INFORMATION: Supplemental Tables S1, S2 and S3

Table S1. Polysaccharide biosynthesis gene clusters identified in selected *Pseudomonas* strains.

Strain	Gene/gene cluster	Locus tag(s)		Function (prediction) / remarks[1]
CHA0	OSA	PFLCHA0_c…	43710-43770	Synthesis of very short O-antigen
	OBC1		20730-20810	Unknown function
	OBC2		31090-31240	Unknown function
	OBC3		19670-19810	Synthesis of long O-antigen
	psl		42710-42820	Psl biosynthesis
	pel		30140-30210	Pel biosynthesis
	alg		10330-10440	Alginate biosynthesis
	pga		01630-01660	PNAG biosynthesis
Cab57	OSA	PPC_...	4407-4413	O-antigen synthesis
	OBC1		2048-2056	Unknown function
	OBC2		3106-3121	Unknown function
	OBC3		1981-1998	O-antigen synthesis
	psl		4306-4317	Psl biosynthesis
	pel		3008-3015	Pel biosynthesis
	alg		1051-1062	Alginate biosynthesis
	pga		0165-0168	PNAG biosynthesis
PGNR1	OSA	PGNR1_...	4406-4409	O-antigen synthesis
	OBC1		2119-2127	Unknown function
	OBC2		3145-3160	Unknown function
	OBC3		2007-2023	O-antigen synthesis
	psl		4305-4316	Psl biosynthesis
	pel		3052-3059	Pel biosynthesis
	alg		1079-1090	Alginate biosynthesis
	pga		222-225	PNAG biosynthesis
BRIP	OSA	BRIP_...	4339-4342	O-antigen synthesis
	OBC1		2039-2047	Unknown function
	OBC2		3076-3091	Unknown function
	OBC3		1926-1942	O-antigen synthesis
	psl		4238-4249	Psl biosynthesis
	pel		2984-2991	Pel biosynthesis

	alg		970-981	Alginate biosynthesis
	pga		113-116	PNAG biosynthesis
Pf-5	OSA	PFL_…	4305-4307	O-antigen synthesis
	OBC1		2024-2032	Unknown function
	OBC2		3078-3093	Unknown function
	OBC4		5480-5496	O-antigen synthesis
	OBC5		5092-5107	O-antigen synthesis
	psl		4208-4219	Psl biosynthesis
	pel		2971-2978	Pel biosynthesis
	alg		1013-1024	Alginate biosynthesis
	pga		0161-0164	PNAG biosynthesis
PF	OSA	PF_...	4481-4483	O-antigen synthesis
	OBC1		2168-2176	Unknown function
	OBC2		3230-3245	Unknown function
	OBC4		5663-5680	O-antigen synthesis
	OBC5		5290-5303	O-antigen synthesis
	psl		4381-4392	Psl biosynthesis
	pel		3124-3131	Pel biosynthesis
	alg		1161-1172	Alginate biosynthesis
	pga		312-315	PNAG biosynthesis
K94.41	OSA	K94_41 _...	4400-4402	O-antigen synthesis
	OBC1		2013-2021	Unknown function
	OBC2		3089-3104	Unknown function
	OBC6		4616-4627	O-antigen synthesis
	psl		4299-4310	Psl biosynthesis
	pel		3002-3009	Pel biosynthesis
	alg		973-984	Alginate biosynthesis
	pga		113-116	PNAG biosynthesis
Os17	OSA	POS17_…	4379-4381	O-antigen synthesis
	OBC1		1999-2005	Unknown function
	OBC2		3083-3097	Unknown function
	OBC8		4982-4995	O-antigen synthesis
	pel		2950-2957	Pel biosynthesis
	alg		1019-1030	Alginate biosynthesis
	pga		0166-0169	PNAG biosynthesis
St29	OSA	PST29_...	4351-4369	O-antigen synthesis
	OBC2		3178-3193	Unknown function
	pel		3076-3083	Pel biosynthesis
	alg		1036-1048	Alginate biosynthesis
	pga		0161-0164	PNAG biosynthesis

CMR5c	OSA	CMR5c_...	2610-2612	O-antigen synthesis
	OBC1		6059-6065	Unknown function
	OBC2		0652-0667	Unknown function
	OBC3-A		3747-3756	O-antigen synthesis
	OBC3-B		5999-6004	O-antigen synthesis
	OBC5		1744-1759	O-antigen synthesis
	psl		2510-2521	Psl biosynthesis
	alg		3365-3376	Alginate biosynthesis
	pga		2427-2430	PNAG biosynthesis
PCL1391	CPA	PCL1391_...	5826-5835	Synthesis of O-antigen of medium length
	OSA		4106-4122	Synthesis of O-antigen of various lengths
	wzz2		3481	Chain length determination
	OBC2		2741-2756	Unknown function
	psl		4983-4994	Psl biosynthesis
	alg		0858-0869	Alginate biosynthesis
	pga		0057-0060	PNAG biosynthesis
LMG1245	CPA	LMG1245_...	0135-0147	O-antigen synthesis
	OSA		4629-4632	O-antigen synthesis
	OBC2		3112-3127	Unknown function
	OBC7		0185-0190	O-antigen synthesis
	psl		5501-5512	Psl biosynthesis
	alg		1184-1195	Alginate biosynthesis
	pga		0359-0362	PNAG biosynthesis
30-84	CPA	PCHL3084_...	5929-5941	O-antigen synthesis
	OSA		4169-4186	O-antigen synthesis
	wzz2		3541	Chain length regulation
	psl		5061-5072	Psl biosynthesis
	alg		1006-1017	Alginate biosynthesis
	pga		0182-0185	PNAG biosynthesis
LMG5004	CPA	LMG5004_...	5952-5964	O-antigen synthesis
	OSA		4268-4276	O-antigen synthesis
	psl		5111-5122	Psl biosynthesis
	pel		3097-3104	Pel biosynthesis
	alg		922-933	Alginate biosynthesis
	pga		125-128	PNAG biosynthesis
DSM21509	CPA	PisciumT_...	180-192	O-antigen synthesis
	OSA		4713-4733	O-antigen synthesis
	wzz2		4044	Chain length regulation
	OBC2		3184-3199	Unknown function
	psl		5584-5595	Psl biosynthesis
	alg		1229-1240	Alginate biosynthesis
	pga		407-410	PNAG biosynthesis
HT66	CPA	HT66_...	3323-3332	O-antigen synthesis
	OSA		1779-1795	O-antigen synthesis

	wzz2		696	Chain length regulation
	OBC2		5341-5356	Unknown function
	psl		3965-3976	Psl biosynthesis
	alg		2808-2819	Alginate biosynthesis
	pga		4843-4846	PNAG biosynthesis
CD	CPA	CD_...	139-151	O-antigen synthesis
	OSA		4388-4402	O-antigen synthesis
	OBC2		3059-3074	Unknown function
	psl		5266-5277	Psl biosynthesis
	alg		1162-1173	Alginate biosynthesis
	pga		358-361	PNAG biosynthesis
YL-1	CPA	YL-1_...	6922-6934	O-antigen synthesis
	OSA		5232-5235	O-antigen synthesis
	OBC2		3883-3898	Unknown function
	OBC7		6880-6884	O-antigen synthesis
	psl		6197-6186	Psl biosynthesis
	alg		1987-1998	Alginate biosynthesis
	pga		1149-1152	PNAG biosynthesis
O6	CPA	PchlO6_...	6277-6289	O-antigen synthesis
	OSA		4422- 4425	O-antigen synthesis
	OBC2		3083-3098	Unknown function
	OBC7		0005-0009	O-antigen synthesis
	psl		5328-5339	Psl biosynthesis
	alg		1018-1029	Alginate biosynthesis
	pga		0181-0184	PNAG biosynthesis
JD37	CPA	JM49_...	00300-00350	O-antigen synthesis
	OSA		09345-09395	O-antigen synthesis
	OBC2		15745-15820	Unknown function
	psl		04850-04905	Psl biosynthesis
	alg		25125-25180	Alginate biosynthesis
	pga		29630-29645	PNAG biosynthesis
PA23	CPA	EY04_...	29610-29670	O-antigen synthesis
	OSA		21770-21845	O-antigen synthesis
	OBC2		14180-14255	Unknown function
	psl		26830-26885	Psl biosynthesis
	alg		04465-04520	Alginate biosynthesis
	pga		00305-00320	PNAG biosynthesis
PB-St2	CPA	U724_...	12200-12260	O-antigen synthesis
	OSA		06825-06920	O-antigen synthesis
	OBC2		02620-02690	Unknown function
	psl		23050-23105	Psl biosynthesis
	alg		24885-24940	Alginate biosynthesis
	pga		26515-26530	PNAG biosynthesis
F113	CPA	PSF113_...	5880-5892	O-antigen synthesis
	OSA		1644-1659	O-antigen synthesis

	OBC1-like		2067-2081	Unknown function
	OBC2		1955-1970	Unknown function
	alg		4752-4763	Alginate biosynthesis
	pga		0161-0164	PNAG biosynthesis
TM1A3	CPA	TM1A3_...	104-117	O-antigen synthesis
	OSA		1777-1788	O-antigen synthesis
	OBC1-like		3905-3920	Unknown function
	OBC2		4015-4030	Unknown function
	alg		4851-4862	Alginate biosynthesis
	pga		329-332	PNAG biosynthesis
DSM7228	OSA	corrugata_T_...	4355-4372	O-antigen synthesis
	OBC2		5172-5187	Unknown function
	alg		1708-1719	Alginate biosynthesis
	pga		74-77	PNAG biosynthesis
Pf153	CPA	Pf153_...	1397-1407	O-antigen synthesis
	OSA		3766-3783	O-antigen synthesis
	OBC2		5054-5070	Unknown function
	alg		266-277	Alginate biosynthesis
	pga		3121-3124	PNAG biosynthesis
Q12-87	CPA	Q12_87_...	5612-5624	O-antigen synthesis
	OSA		3823-3846	O-antigen synthesis
	OBC2		1782-1797	Unknown function
	alg		980-991	Alginate biosynthesis
	pga		5514-5517	PNAG biosynthesis
DSM13647	OSA	kilonensis_T_...	1804-1821	O-antigen synthesis
	OBC2		3860-3875	Unknown function
	alg		4708-4719	Alginate biosynthesis
	pga		221-224	PNAG biosynthesis
Pf0-1	CPA	Pfl01_...	5676-5688	O-antigen synthesis
	OSA		4042-4071	O-antigen synthesis
	OBC2		2010-2024	Unknown function
	OBC11		2812-2826	Unknown function
	alg		0949-0960	Alginate biosynthesis
	pga		0177-0180	PNAG biosynthesis
P1.31	CPA	P1_31_...	5492-5504	O-antigen synthesis
	OSA		3819-3834	O-antigen synthesis
	OBC2		2021-2037	Unknown function
	OBC11		2594-2608	Unknown function
	alg		1010-1021	Alginate biosynthesis
	pga		167-170	PNAG biosynthesis
P1.8	OSA	P1_8_...	4237-4256	O-antigen synthesis
	OBC2		2221-2236	Unknown function
	OBC11		3136-3149	Unknown function
	alg		906-917	Alginate biosynthesis
	alg2		5421-5432	Alginate biosynthesis?

DSM50090	OSA	fluorescens_T_...	1707-1718	O-antigen synthesis
	wzz2		2899	Chain length regulation
	OBC9		4168-4186	O-antigen synthesis
	psl		1984-1994	Psl biosynthesis
	alg		1012-1023	Alginate biosynthesis
	pga		171-174	PNAG biosynthesis
SS101	OSA	PflSS101_...	1657-1676	O-antigen synthesis
	wzz2		2494	Chain length regulation
	psl		1926-1937	Psl biosynthesis
	alg		0998-1009	Alginate biosynthesis
	pga		0155-0158	PNAG biosynthesis
MIACH	OSA	Miach_...	2608-2630	O-antigen synthesis
	OBC10		671-688	unknown function
	psl		2530-2540	Psl biosynthesis
	alg		2118-2129	Alginate biosynthesis
	pga		4575-4578	PNAG biosynthesis

[1] Putative O-PS biosynthesis clusters (OBC) were identified using bioinformatic tools as gene clusters that contain several O-antigen-associated genes, such as glycosyltransferases and O-antigen transporters. Clusters identified in strains other than CHA0 and PCL1391 are predicted to contribute to the biosynthesis of O-antigen if not otherwise stated.

Abbreviations: Pel, exopolysaccharide contributing to pellicle formation; PNAG, poly-N-acetylglucosamine; Psl, exopolysaccharide synthesized by proteins encoded at the polysaccharide synthesis locus.

Table S2. Function predictions of putative O-polysaccharide biosynthesis genes in selected *Pseudomonas* strains.

Strain	Gene cluster	Locus tag (gene description)	NCBI Conserved Domain Database or EMBL-EBI Interpro search (E-values of the top hits)
CHA0	OSA	PFLCHA0_c43780 (*ihfB*)	Beta subunit of integration host factor (IHFB) (1.06e-43)
		PFLCHA0_c43770	TDP-4-keto-6-deoxy-D-glucose transaminase, WecE (0e+00)
		PFLCHA0_c43760 (*wzx*)	Uncharacterized subfamily of the multidrug and toxic compound extrusion (MATE) proteins (5.67e-47); O-antigen translocase (1.51e-34)
		PFLCHA0_c43750	Glycosyltransferase involved in cell wall biosynthesis, WcaA (5.55e-17)
		PFLCHA0_c43740	Glycosyltransferase involved in cell wall biosynthesis, RfaB (5.46e-43)
		PFLCHA0_c43730 (*wbpK*)	UDP-glucose 4 epimerase (1.12e-120); nucleoside-diphosphate-sugar epimerase, WcaG (5.87e-47)
		PFLCHA0_c43720 (*wbpL*)	WbpL-like glycosyltransferase (7.76e-71)
		PFLCHA0_c43710 (*wbpM*)	NDP-sugar epimerase (0e+00)
		PFLCHA0_c43700 (*comEA*)	DNA uptake protein ComE and related DNA-binding proteins (1.29e-11)
	OBC1	PFLCHA0_c20810 (*wzm*-like)	ABC-type polysaccharide/polyol phosphate export permease (4.60e-43)
		PFLCHA0_c20800 (*wzt*-like)	ATP-binding cassette component of polysaccharide transport system, Wzt-like (9.78e-110)
		PFLCHA0_c20790	Polysaccharide pyruvyl transferase (8.14e-24)
		PFLCHA0_c20780	2-oxoglutarate-Fe(II) oxygenase superfamily (2.18e-14)
		PFLCHA0_c20770	Glycosyltransferase (2.41e-119)
		PFLCHA0_c20760	SAM-dependent methyltransferase (1.37e-06)
		PFLCHA0_c20750	Glycosyltransferase (3.89e-117)
		PFLCHA0_c20740	Glycosyltransferase (6.93e-46)
		PFLCHA0_c20730	Glycosyltransferase (1.21e-57)
	OBC2	PFLCHA0_c31090 (*ugd*)	UDP-glucose 6-dehydrogenase, Ugd (0e+00)
		PFLCHA0_c31100	UDP glucuronic acid epimerase (0e+00)
		PFLCHA0_c31110	Bacterial sugar transferase (8.86e-69); WcaJ-like exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase (1.11e-68)
		PFLCHA0_c31120	Chromosome partitioning ATPase, Mrp family (4.45e-18); exopolysaccharide/PEP-CTERM locus tyrosine autokinase (1.52e-17)
		PFLCHA0_c31130	Periplasmic protein involved in polysaccharide export Wza (5.24e-22)

		PFLCHA0_c31140 (*wzz*-like)	GumC, Uncharacterized protein involved in exopolysaccharide biosynthesis (2.07e-25); exopolysaccharide transport protein family (2.96e-08)
		PFLCHA0_c31150	Glycosyltransferase (2.55e-58)
		PFLCHA0_c31160 (*wzy*-like)	Wzy-like O-Antigen ligase (2.50e-12)
		PFLCHA0_c31170	Glycosyltransferase (2.81e-27)
		PFLCHA0_c31180	Glycosyltransferase (8.42e-80)
		PFLCHA0_c31190	Carbohydrate esterase 4 superfamily, NodB (4.88e-46); polysaccharide deacetylase (7.88e-15)
		PFLCHA0_c31200 (*wzx*-like)	Wzx, a subfamily of the multidrug and toxic compound extrusion (MATE)-like proteins (1.93e-22)
		PFLCHA0_c31210	Glycoside hydrolase superfamily (Interpro, IPR017853)
		PFLCHA0_c31220	N-Acyltransferase superfamily (2.98e-03)
		PFLCHA0_c31230	Acetyltransferase (7.84e-11)
		PFLCHA0_c31240	YdjC-like protein, involved in cleavage of cellobiose-phosphate (9.95e-38)
	OBC3	PFLCHA0_c19810	UDP-glucose 4-epimerase, GalE (3.46e-157)
		PFLCHA0_c19800	Bacterial DPM1_like enzymes are related to eukaryotic DPM1 (6.60e-89); glycosyltransferase 2 superfamily (5.30e-63)
		PFLCHA0_c19790	S-adenosylmethionine-dependent methyltransferases (4.69e-08)
		PFLCHA0_c19780	Mannosyltransferase (0.04)
		PFLCHA0_c19780*	GtrA-like protein (2.13e-08)
		PFLCHA0_c19760**	Integrase core domain (1.95e-14)
		PFLCHA0_c19760*	Helix-turn-helix domains (1.96e-07); transposase and inactivated derivatives (1.57e-04)
		PFLCHA0_c19750 (*fcl2*)	GDP-fucose synthetase (1.85e-145)
		PFLCHA0_c19740	Glycosyltransferase (5.79e-03)
		PFLCHA0_c19730* (*wzm*)	ABC-type polysaccharide/polyol phosphate export permease (2.23e-47)
		PFLCHA0_c19730 (*wzt*)	ABC-type polysaccharide/polyol phosphate transport system, ATPase component (8.90e-104)
		PFLCHA0_c19720	S-adenosylmethionine-dependent methyltransferases (0.04)
		PFLCHA0_c19710 (*gmd*)	Gmd, GDP-D-mannose dehydratase (0e+00)
		PFLCHA0_c19700 (*fcl*)	GDP-fucose synthetase (4.82e-159)
		PFLCHA0_c19690	Glycosyltransferase (1.41e-54)
		PFLCHA0_c19680	Glycosyltransferase (3.60e-169)
		PFLCHA0_c19670	Mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase (0e+00)
PCL1391	OSA	PCL1391_4123 (*ihfB*)	Beta subunit of integration host factor (IHFB) (7.93e-44)
		PCL1391_4122 (*wzz1*)	Chain length determinant protein WzzB (2.47e-20)

	PCL1391_4121	UDP-N-acetylglucosamine 2-epimerase, WecB (1.06e-171)
	PCL1391_4120	UDP-N-acetyl-D-mannosamine dehydrogenase, WecC (0e+00)
	PCL1391_4119	Mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase (0e+00)
	PCL1391_4118 (*gmd*)	Gmd, GDP-D-mannose dehydratase (0e+00)
	PCL1391_4117	GDP-mannose 4,6 dehydratase (8.13e-67); nucleoside-diphosphate-sugar epimerase (2.38e-46)
	PCL1391_4116	Glycosyltransferase (2.12e-41)
	PCL1391_4115 (*wzx*)	Wzx, a subfamily of the multidrug and toxic compound extrusion (MATE)-like proteins (1.87e-05)
	PCL1391_4114 (*wzy*)	Ten transmembrane domains (Interpro); Von Willebrand factor type A (vWA) domain (0.36)
	PCL1391_4113	Exosortase A system-associated amidotransferase 1 (1.29e-109)
	PCL1391_4112	2-Desacetyl-2-hydroxyethyl bacteriochlorophyllide and other MDR family members (1.65e-93); predicted dehydrogenase (3.79e-46)
	PCL1391_4111	Heparinase II/III-like protein (8.86e-23)
	PCL1391_4110	Glycosyltransferase (9.20e-134)
	PCL1391_4109	Bacterial sugar transferase (3.69e-88)
	PCL1391_4108	3-Amino-5-hydroxybenzoic acid synthase family, aspartate aminotransferase superfamily (9.16e-129)
	PCL1391_4107 (*wbpM*)	NDP-sugar epimerase (0e+00)
	PCL1391_4106 (*wbpL*)	WbpL/WbcO-like (5.60e-77)
	PCL1391_4105 (*comEA*)	Competence protein ComEA helix-hairpin-helix repeat region (5.76e-14)
wzz2 locus	PCL1391_3481 (*wzz2*)	LPS O-antigen chain length determinant protein, WzzB/FepE family (1.17e-20)
CPA	PCL1391_5826	Methyltransferase domain (8.50e-14)
	PCL1391_5827	Peptidoglycan/LPS O-acetylase OafA/YrhL (6.72e-16)
	PCL1391_5828	Methyltransferase domain (7.25e-13)
	PCL1391_5829	Glycosyltransferase (1.77e-11)
	PCL1391_5830	Glycosyltransferase (9.41e-16)
	PCL1391_5831 (*wzm*)	ABC-type polysaccharide/polyol phosphate export permease (8.27e-25)
	PCL1391_5832 (*wzt*)	ABC-type polysaccharide/polyol phosphate transport system, ATPase component (5.26e-100)
	PCL1391_5833 (*wbpX*)	Glycosyltransferase (3.54e-71)
	PCL1391_5834 (*wbpY*)	Glycosyltransferase (8.01e-111)
	PCL1391_5835 (*wbpZ*)	Glycosyltransferase (7.37e-172)
OBC2	PCL1391_2756 (*ugd*)	UDP-glucose 6-dehydrogenase, Ugd (0e+00)
	PCL1391_2755	UDP glucuronic acid epimerase (0e+00)
	PCL1391_2754	Bacterial sugar transferase (3.91e-68); exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase (4.42e-66)

PCL1391_2753	Chromosome partitioning ATPase, Mrp family (1.04e-18); P-loop containing nucleoside triphosphate hydrolases (2.56e-13)
PCL1391_2752	Polysaccharide biosynthesis/export protein (1.59e-20); polysaccharide export protein EpsE (3.29e-12)
PCL1391_2751 (*wzz*-like)	Uncharacterized protein involved in exopolysaccharide biosynthesis, GumC (1.28e-24); chain length determinant protein, Wzz (1.20e-03)
PCL1391_2750	Glycosyltransferase (5.85e-55)
PCL1391_2749 (*wzy*-like)	O-Antigen ligase (9.40e-14)
PCL1391_2748	ExoA is involved in the biosynthesis of succinoglycan (8.81e-26); glycosyltransferase (2.64e-09)
PCL1391_2747	Glycosyltransferase (1.12e-48)
PCL1391_2746	Carbohydrate esterase 4 superfamily, deacetylase (1.15e-45)
PCL1391_2745 (*wzx*-like)	Wzx, a subfamily of the multidrug and toxic compound extrusion (MATE)-like proteins (9.76e-20)
PCL1391_2744	Glycoside hydrolase superfamily (Interpro, IPR017853)
PCL1391_2743	N-Acyltransferase superfamily (5.74e-04)
PCL1391_2742	Acetyltransferase (GNAT) domain (8.98e-15)
PCL1391_2741	Catalytic NodB homology domain of the carbohydrate esterase 4 superfamily (1.24e-49)
PCL1391_2731	Type VI secretion system Vgr family protein (0e+00)

Table S3. Bacterial strains, plasmids and primers used in this study.

Strain	Genotype or phenotype	Reference or source
Pseudomonas protegens		
BRIP	Wild type	Ruffner et al., 2015
Cab57	Wild type	Takeuchi et al., 2014; NIAS
CHA0	Wild type	Stutz et al., 1986
CHA0-*gfp2*	CHA0::attTn*7*-*gfp2*; Gm^r	Péchy-Tarr et al., 2013
CHA89	*gacA*::ΩKm^r	Laville et al., 1992
CHA5129	*arnA*::pEMG (PFLCHA0_c30730); Km^r	This study
CHA5161	Δ*wbpL* (PFLCHA0_c43720)	This study
CHA5161-*mChe*	CHA5161::attTn*7*-*mcherry*; Gm^r	This study
CHA5163	Δ*obc1*	This study
CHA5164	Δ*obc2*	This study
CHA5165	Δ*obc1* Δ*obc2*	This study
CHA5169	CHA5161::attTn*7*-$P_{tac/lacIq}$-*wbpL*(CHA0); Gm^r	This study
CHA5174	*waaL*::pEMG (PFLCHA0_c05350); Km^r	This study
CHA5182	Δ*obc3*	This study
CHA5205	Δ*fcl* (PFLCHA0_19700)	This study
CHA5205-*mChe*	CHA5205::attTn*7*-*mcherry*; Gm^r	This study
CHA5206	Δ*wzx* (PFLCHA0_43760)	This study
CHA5206-*mChe*	CHA5206::attTn*7*-*mcherry*; Gm^r	This study
CHA5207	CHA5174::attTn*7*-$P_{tac/lacIq}$-*waaL*(CHA0); Km^r, Gm^r	This study
CHA5212	CHA5205::attTn*7*-$P_{tac/lacIq}$-*fcl*(CHA0); Gm^r	This study
CHA5214	CHA5161 *arnA*::pEMG (PFLCHA0_c30730); Km^r	This study
K94.41	Wild type	Wang et al., 2001
PF	Wild type	Keel et al., 1996
Pf-5	Wild type	Howell and Stipanovic, 1979
PGNR1	Wild type	Keel et al., 1996
Pseudomonas chlororaphis		
30-84	Wild type	Pierson and Thomashow, 1992
CD	Wild type	Ruffner et al., 2015
DSM21509	Wild type	Gobeli et al., 2009
HT66	Wild type	Chen et al., 2015
JD37	Wild type	Fang et al., 2015
LMG1245	Wild type	Kluyver, 1956; Peix et al., 2007
LMG5004	Wild type	Peix et al., 2007
O6	Wild type	Tucker et al., 1995
PA23	Wild type	Savchuk et al., 2004

PB-St2	Wild type	Mehnaz et al., 2009; Peix et al., 2007
PCL1391	Wild type	Chin-A-Woeng et al., 1998
PCL1391-*gfp1*	PCL1391::attTn*7*-*gfp1*; Km^r	This study
PCL-1	*waaL*::pEMG (PCL1391_0387); Km^r	This study
PCL-3	Δ*obc2*	This study
PCL-9	*wzx*::pEMG (PCL1391_4115); Km^r	This study
PCL-11	*wzz1*::pEMG (PCL1391_4122); Km^r	This study
PCL-11-*mChe*	PCL-11::attTn*7*-*mcherry*; Gm^r	This study
PCL-12	Δ*cpa*	This study
PCL-13	*wzz2*::pEMG (PCL1391_3481); Km^r	This study
PCL-13-*mChe*	PCL-13::attTn*7*-*mcherry*; Gm^r	This study
PCL-14	PCL-1::attTn*7*-$P_{tac/lacIq}$-*waaL*(PCL1391); Km^r Gm^r	This study
YL-1	Wild type	Liu et al., 2014
Pseudomonas spp.		
CMR5c	Wild type	Perneel et al., 2007
P. aeruginosa PAO1	Wild type	Holloway et al., 1979
P. brassicacearum TM1A3	Wild type	Frapolli et al., 2007
P. corrugata DSM7228	Wild type	Scarlett et al., 1978
P. fluorescens 97-38	Wild type	Frapolli et al., 2007
P. fluorescens DSM50090	Wild type	Rhodes, 1959
P. fluorescens F113	Wild type	Fenton et al., 1992
P. fluorescens Pf0-1	Wild type	Compeau et al., 1988
P. fluorescens Q12-87	Wild type	Vincent et al., 1991
P. fluorescens SBW25	Wild type	Silby et al., 2009
P. fluorescens SS101	Wild type	De Souza et al., 2003
P. fluorescens P3	Wild type	Meyer et al., 2011
P. kilonensis DSM13647	Wild type	Sikorski et al., 2001
P. kilonensis P12	Wild type	Keel et al., 1996
Pseudomonas sp. MIACH	Wild type	Meyer et al., 2011
Pseudomonas sp. Os17	Wild type	Takeuchi et al., 2015; NIAS
Pseudomonas sp. P1.31	Wild type	Ruffner et al., 2015
Pseudomonas sp. P1.8	Wild type	Ruffner et al., 2015
Pseudomonas sp. Pfl153	Wild type	Fuchs et al., 2000
Pseudomonas sp. St29	Wild type	Takeuchi et al., 2015; NIAS
Escherichia coli		
DH5α, DH5α λpir, HB101	Laboratory strains	Sambrook and Russel, 2001

Plasmid	Relevant characteristics	Reference
pBK-miniTn7-*gfp1*	pUC19-based delivery plasmid for miniTn7-*gfp1*; *mob*$^+$; Km^r, Cm^r, Ap^r	Koch et al., 2001
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r, Ap^r	Martínez-García and de Lorenzo, 2011
pME497	Mobilizing plasmid; Ap^r	Voisard et al., 1994
pME8300	Carrier plasmid for Tn*7* for $P_{tac/lacIq}$ controlled target gene expression; Gm^r, Ap^r	Kupferschmied et al., 2014
pME4510	Broad host range promoter-probe plasmid vector for Gram-negative bacteria; Gm^r	Rist and Kertesz, 1998
pME8362	pEMG-Δ*wbpL*(CHA0); suicide plasmid for the in-frame deletion of PFLCHA0_c43720 (*wbpL*) in CHA0; Km^r	This study
pME8366	pEMG-Δ*obc1*(CHA0); suicide plasmid for the deletion of the OBC1 cluster in CHA0; Km^r	This study
pME8367	pEMG-Δ*obc2*(CHA0); suicide plasmid for the deletion of the OBC2 cluster in CHA0; Km^r	This study
pME8368	pME8300-$P_{tac/lacIq}$-*waaL*(CHA0); IPTG-inducible expression of CHA0 *waaL* (PFLCHA0_c05350); Gm^r, Ap^r	This study
pME8378	pME8300-$P_{tac/lacIq}$-*wbpL*(CHA0); IPTG-inducible expression of CHA0 *wbpL* (PFLCHA0_ c43720); Gm^r, Ap^r	This study
pME8379	pEMG-*waaL*(PCL1391); suicide plasmid for the disruption of PCL1391 *waaL* (PCL1391_0387); Km^r	This study
pME8382	pME8300-$P_{tac/lacIq}$-*waaL*(PCL1391); IPTG-inducible expression of PCL1391 *waaL* (PCL1391_456); Gm^r, Ap^r	This study
pME8383	pEMG-*waaL*(CHA0); suicide plasmid for the disruption of CHA0 *waaL* (PFLCHA0_c05350); Km^r	This study
pME8388	pEMG-Δ*obc2*(PCL1391); suicide plasmid for the deletion of the OBC2 cluster in PCL1391; Km^r	This study
pME8393	pEMG-Δ*cpa*(PCL1391); suicide plasmid for the deletion of the CPA cluster in PCL1391; Km^r	This study
pME8394	pEMG-Δ*obc3*(CHA0); suicide plasmid for the deletion of the OBC3 cluster in CHA0; Km^r	This study
pME8399	pEMG-*wzx*(PCL1391); suicide plasmid for the disruption of PCL1391 *wzx* (PCL1391_4115); Km^r	This study

pME11001	pEMG-*wzz1*(PCL1391); suicide plasmid for the disruption of PCL1391 *wzz1* (PCL1391_4122); Km^r	This study
pME11007	pEMG-Δ*fcl*(CHA0); suicide plasmid for the in-frame deletion of PFLCHA0_c19700 (*fcl*) in CHA0; Km^r	This study
pME11009	pEMG-Δ*wzx*(CHA0); suicide plasmid for the in-frame deletion of PFLCHA0_c43760 (*wzx*) in CHA0; Km^r	This study
pME11012	pEMG-*wzz2*(PCL1391); suicide plasmid for the disruption of PCL1391 *wzz2* (PCL1391_3550); Km^r	This study
pME11022	pME8300-$P_{tac/lacIq}$-*fcl*(CHA0); IPTG-inducible expression of CHA0 *fcl* (PFLCHA0_19700); Gm^r, Ap^r	This study
pME11024	pME4510-*wzx*-locus; expression of PFLCHA0_c43760 (*wzx*) under the control of its native promoter; Gm^r	This study
pME9407	pUC19-based delivery plasmid for miniTn7-*mcherry*; *mob*$^+$; Gm^r, Cm^r, Ap^r	Rochat et al., 2010
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	Martínez-García and de Lorenzo, 2011
pUX-BF13	Helper plasmid encoding Tn*7* transposition functions; R6K-replicon; Ap^r	Bao et al., 1991

Primer	Sequence 5' → 3', restriction enzyme[1]	Purpose
arnA-im-1	CGGGATCCAACTGGGTGCTGGTCAAG, BamHI	Disruption of CHA0 *arnA*
arnA-im-2	GGAATTCGTCCACCAGGCCCAGTTC, EcoRI	Disruption of CHA0 *arnA*
fcl-del-1	GGAATTCCCTTCGTCACTCGCAAGATTAC, EcoRI	Deletion of CHA0 *fcl*
fcl-del-2	CCCAAGCTTGATCGCAGAGCCAGCCATAC, HindIII	Deletion of CHA0 *fcl*
fcl-del-3	CCCAAGCTTACGCAACTGCGTGAAGGTATAG, HindIII	Deletion of CHA0 *fcl*
fcl-del-4	CGGGATCCAGTCGCCACCAAAGATCGTAG, BamHI	Deletion of CHA0 *fcl*
fcl-expr-F	GACTAGTGTGGATAAACGCGCCAAAATATAC, SpeI	Expression of CHA0 *fcl*
fcl-expr-R	CCCAAGCTTCATTCAGTGGCGATCCAGAC, HindIII	Expression of CHA0 *fcl*
obc1-del-1	CGGGATCCGCATCCTCTTCAGCTTCGAC, BamHI	Deletion of CHA0 *obc1*
obc1-del-2	CCCAAGCTTGGTACTTCCGGTCAACTCC, HindIII	Deletion of CHA0 *obc1*
obc1-del-3	CCCAAGCTTGTCCTCTCCTGGAGCATCT, HindIII	Deletion of CHA0 *obc1*
obc1-del-4	GGAATTCCCCAATCATAGCCACTCAC, EcoRI	Deletion of CHA0 *obc1*

obc2-del-1	CGGAATTCATGGGCACGGATTGAGTAAGG, EcoRI	Deletion of CHA0 *obc2*
obc2-del-2	CCCAAGCTTGAGGGTGGTTCTCACCTTCT, HindIII	Deletion of CHA0 *obc2*
obc2-del-3	CCCAAGCTTATTGCCTACAGCGAGCTGTAA, HindIII	Deletion of CHA0 *obc2*
obc2-del-4	GGGATCCAACGAGCTGATTGCCGAATTG, BamHI	Deletion of CHA0 *obc2*
obc3-del-1	GGAATTCTGTTACCCTTCCCCCATACAG, EcoRI	Deletion of CHA0 *obc3*
obc3-del-2	GACTAGTACAGCAGCCTGGTTGGTAAG, SpeI	Deletion of CHA0 *obc3*
obc3-del-3	GACTAGTCCGCAAGAAGCATTGGTAATGG, SpeI	Deletion of CHA0 *obc3*
obc3-del-4	CGGGATCCGACCATGCCCAAGACATCAAG, BamHI	Deletion of CHA0 *obc3*
PCL.cpa-del-1	CGGAATTCGGTGATCAGGGTTGCTGAAG, EcoRI	Deletion of PCL1391 *cpa*
PCL.cpa-del-2	CCCAAGCTTCGAGTTGTTCACCGGTTTTCC, HindIII	Deletion of PCL1391 *cpa*
PCL.cpa-del-5	CCCAAGCTTCCACCGGTTTCTCCTACAG, HindIII	Deletion of PCL1391 *cpa*
PCL.cpa-del-6	GGGATCCAGACGATGGCGTAGCTCAGT, BamHI	Deletion of PCL1391 *cpa*
PCL.obc2-del-1	CGGAATTCCTTGGATGTCAGCGTTTTTGG, EcoRI	Deletion of PCL1391 *obc2*
PCL.obc2-del-2	CCCAAGCTTACAGCTCGCTCAACTGCTG, HindIII	Deletion of PCL1391 *obc2*
PCL.obc2-del-3	CCCAAGCTTCCATATCGACTCCCATCAGC, HindIII	Deletion of PCL1391 *obc2*
PCL.obc2-del-4	GGGATCCGATCGTCAGGGAAATATGGG, BamHI	Deletion of PCL1391 *obc2*
PCL.waaL-check-F	GGGCCTGATCAGTCTGTTGT	Disruption of PCL1391 *waaL*
PCL.waaL-check-R	TGATCGAACGAAAACCCTTCAG	Disruption of PCL1391 *waaL*
PCL.waaL-expr-F	GACTAGTATGCAGGCCACACGTTGGG, SpeI	Expression of PCL1391 *waaL*
PCL.waaL-expr-R	CCCAAGCTTGTCGAGCTCACTGTTTAGGAG, HindIII	Expression of PCL1391 *waaL*
PCL.waaL-im-1	GGAATTCCTGTTGTTTTTCCCGGTTTTCG, EcoRI	Disruption of PCL1391 *waaL*
PCL.waaL-im-2	CGGGATCCAACATCGCCAGTAGCAATG, BamHI	Disruption of PCL1391 *waaL*
PCL.wzx-check-1	CGGGTAGTCTAGGGATGCAA	Disruption of PCL1391 *wzx*
PCL.wzx-check-2	CAACATTGTTCCGCAAGAGAGC	Disruption of PCL1391 *wzx*
PCL.wzx-im-1	CGGGATCCTGGTCGTGCTCACCAACTTAC, BamHI	Disruption of PCL1391 *wzx*
PCL.wzx-im-2	CGGGATCCCAAGAACGAGGCTCAATATG, BamHI	Disruption of PCL1391 *wzx*
PCL.wzz2-check-1	GGTACGGCATGACAGGGTTA	Disruption of PCL1391 *wzz2*
PCL.wzz2-check-2	CTGCAGCTCCTTACCGATTTC	Disruption of PCL1391 *wzz2*

PCL.wzz2-im-1	GGAATTCGCTCGGAAGTCTACAAACTG, EcoRI	Disruption of PCL1391 *wzz2*
PCL.wzz2-im-2	CGGGATCCTTGCGCTGTTCTTTCAACTG, BamHI	Disruption of PCL1391 *wzz2*
PCL.wzz-check-1	AGTGGATCTGCAGGAGCTTG	Disruption of PCL1391 *wzz1*
PCL.wzz-check-2	GTAGCTCACGCAAACGATCAG	Disruption of PCL1391 *wzz1*
PCL.wzz-im-1	GGAATTCGTAGTGCCTCCGACACAAAAAG, EcoRI	Disruption of PCL1391 *wzz1*
PCL.wzz-im-2	CGGGATCCAATTTGCTGCTCGACGTTTCG, BamHI	Disruption of PCL1391 *wzz1*
waaL-expr-F	GACTAGTATGCAACCCAATGCCCTTCAC, SpeI	Expression of CHA0 *waaL*
waaL-expr-R	CCCAAGCTTCCCTGTGTAGCTGTCATGGA, HindIII	Expression of CHA0 *waaL*
waaL-fs-1	GGAATTCTGCCGATCGGTTATCTACTG, EcoRI	Disruption of CHA0 *waaL*
waaL-fs-2	CCCAAGCTTGAGAGCAACGATCGCAGCAC, HindIII	Disruption of CHA0 *waaL*
waaL-fs-3	CCCAAGCTTTCGCCTCGATCTTCAACCTG, HindIII	Disruption of CHA0 *waaL*
waaL-fs-4	CGGGATCCAGAAGCATCTGCGAGAAC, BamHI	Disruption of CHA0 *waaL*
wbpL-del-1	CGGGATCCGCCTTGCACGACCTTATTGTG, BamHI	Deletion of CHA0 *wbpL*
wbpL-del-2	GACTAGTCCAGTACACATGGCTCATACG, SpeI	Deletion of CHA0 *wbpL*
wbpL-del-3	GACTAGTGCAAGAGAGAACTGAGATGCG, SpeI	Deletion of CHA0 *wbpL*
wbpL-del-4	GGAATTCAGCGCCTGCTCCATAAATAG, EcoRI	Deletion of CHA0 *wbpL*
wbpL-expr-F	GACTAGTATGAGCCATGTGTACTGGG, SpeI	Expression of CHA0 *wbpL*
wbpL-expr-R	CCCAAGCTTCGCATCTCAGTTCTCTCTTGC, HindIII	Expression of CHA0 *wbpL*
wzx-del-1	GGAATTCGTCATTCGAGAGAAAGGGACGA, EcoRI	Deletion of CHA0 *wzx*
wzx-del-2	GACTAGTAGAGGCGACAGTTAAAGCAGC, SpeI	Deletion of CHA0 *wzx*
wzx-del-3	GACTAGTGTGGTTGCCGTATCAGGTTTT, SpeI	Deletion of CHA0 *wzx*
wzx-del-4	CGGGATCCACAGCCTTTCCTTCGGGAAT, BamHI	Deletion of CHA0 *wzx*
wzx-expr-F2	GGAATTCATCCTGATTGAGGTTCTCGTAG, EcoRI	Expression of CHA0 *wzx*
wzx-expr-R2	CGGGATCCCTCATGCAAACGAGAGTAC, BamHI	Expression of CHA0 *wzx*

Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; NIAS, NIAS Genebank Project (http://www.nias.affrc.go.jp/); Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

[1] Restriction sites are underlined.

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SUPPORTING INFORMATION: Supplemental experimental procedures (Text S1)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids, media, and culture conditions

Bacterial strains were routinely maintained on nutrient agar (NA) plates or cultured in lysogeny broth (LB) (BD Difco, Franklin Lakes, New Jersey, United States) supplemented with appropriate antibiotics as needed. *Pseudomonas* strains were grown at 25°C while *E. coli* was cultured at 37°C. The following antibiotic concentrations were used: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; kanamycin, 25 µg/ml for *E. coli* and *P. protegens*, 50 µg/ml for *P. chlororaphis*; gentamicin, 10 µg/ml for *E. coli* and *P. protegens*, 20 µg/ml for *P. chlororaphis*; and tetracycline, 25 µg/ml or 125 µg/ml for *E. coli* and *Pseudomonas*, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM, if required.

Recombinant DNA techniques

DNA manipulations and PCRs were performed according to standard protocols (Sambrook and Russel, 2001). Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit from Promega (Fitchburg, Wisconsin, United States). Plasmid DNA preparations were done using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands) and the Genomed JETStar Plasmid Purification Midi Kit for small and large scale purifications, respectively. DNA gel extractions were conducted using the MinElute Gel Extraction Kit and the QIAquick Gel Extraction Kit (Qiagen). DNA restriction and modification enzymes were purchased from Promega and were used according to the manufacturer's recommendations. DNA enzyme reaction cleanups were conducted using the QIAquick PCR Purification Kit (Qiagen). PCR for molecular cloning was conducted according to the recommendations of the manufacturer with the PrimeSTAR HS high-fidelity DNA polymerase kit (Takara Bio Inc., Shiga, Japan), while the DNA Polymerase kit (Promega) was used to perform PCRs for analytic purposes. The primers used in this study were synthesized by Microsynth AG (Balgach, Switzerland) and are listed in Table S3. DNA sequencing was conducted at GATC Biotech (Konstanz, Germany) and obtained sequences were analyzed using the DNASTAR Lasergene software suite (version 12).

Construction of in-frame deletion mutants

In-frame deletions of selected genes in *P. protegens* CHA0 and *P. chlororaphis* subsp. *piscium* PCL1391 were performed based on homologous recombinations using the suicide vector pEMG and the I-SceI system (Martínez-García and de Lorenzo, 2011). For the construction of the suicide vectors (Table S3), upstream and downstream regions of 500–600 bp length flanking the region to be deleted were amplified by PCR using the primer pairs listed in Table S3 and chromosomal DNA from strains CHA0 or PCL1391 as DNA templates. Purified PCR products were digested using the corresponding restriction enzymes (indicated in Table S3) and subsequently cloned into the suicide vector pEMG via triple ligation. Correct insert sequences of the resulting plasmids were confirmed via DNA sequencing. The obtained suicide vectors then served to construct strains CHA5161, CHA5163, CHA5164, CHA5165, CHA5182, CHA5205, CHA5206, PCL-3, and PCL-12 (Table S3), respectively, using the I-SceI system as described previously (Kupferschmied *et al.*, 2014).

Construction of gene disruption mutants

In cases where genes or gene clusters could not be deleted using the method described above (*waaL*, *P. chlororaphis* subsp. *piscium* PCL1391 *wzz1*, *wzz2*, *wzx*, and *wzt*), selected genes were mutated by sequence-specific insertion of pEMG-based suicide vectors via homologous recombination, which led to the disruption of the coding sequences (CDSs). Suicide vectors were constructed by PCR amplification of approximately 500-bp regions in the middle of the genes of interest, using the primer pairs listed in Table S3 and chromosomal DNA from the respective bacterial strain. The resulting fragments were digested with BamHI and EcoRI and cloned into pEMG opened with the same restriction enzymes. The insert sequences of the constructed vectors were verified by DNA sequencing and the plasmids were integrated into the CHA0 or PCL1391 chromosome by transformation of the bacteria via electroporation or via triparental mating using *E. coli* HB101 possessing the plasmid pME497 (Voisard *et al.*, 1994). Correct insertion of the suicide vectors in the chromosomes was confirmed by conducting specific PCR using the "check" primers specified in Table S3.

In order to introduce a frame shift in CHA0 *waaL*, two 350-bp fragments of the gene were amplified by PCR using primer pairs waaL-fs-1/waaL-fs-2 and waaL-fs-3/waaL-fs-4 (Table S3) and chromosomal DNA from CHA0. The obtained DNA fragments were digested with the corresponding restriction enzymes and cloned into pEMG opened with EcoRI and BamHI. The insert sequence of the resulting plasmid pME8383 was verified by DNA sequencing. The created vector was integrated into the chromosome of CHA0 by electroporation of electrocompetent cells, resulting in strain CHA5174 (Table S3). Since several attempts to get frame shift mutants by selection for bacteria that underwent second homologous recombination failed, strain CHA5174 served as a gene disruption mutant for this study.

Complementation of mutant strains

For complementation of selected gene deletion and disruption mutants of CHA0 and PCL1391, the respective genes were cloned under the control of the $P_{tac/lacIq}$ promoter and introduced into the unique chromosomal Tn*7* attachment site of corresponding mutant strains using the mini-Tn*7* delivery vector pME8300 (Kupferschmied *et al.*, 2014) as follows. Primer pairs listed in Table S3 were used to amplify the CDS of selected genes of *P. protegens* CHA0 and *P. chlororaphis* subsp. *piscium* PCL1391 by PCR using chromosomal DNA from the respective bacterial strains. Resulting PCR products were digested with the restriction enzymes indicated in Table S3 and consequently cloned individually into pME8300 opened with the same enzymes. The sequences of the inserts in the resulting plasmids were verified by DNA sequencing. The pME8300 derivatives and the Tn*7* transposition helper plasmid pUX-BF13 were co-electroporated into competent cells of the respective mutant strains to create strains CHA5169, CHA5207, CHA5208, CHA5211, CHA5212, PCL-8, and PCL-14 (Table S3).

Because the above described cloning strategy did not work for CHA0 *wzx*, CHA5206 was complemented as follows. A 3-kb region, comprising the CDS of *wzx*, the upstream-flanking gene and its native promoter, was amplified by PCR using the primer pair wzx-expr-F2/wzx-expr-R2 and chromosomal DNA from CHA0. The purified PCR product was digested with EcoRI and BamHI and subsequently ligated into pME4510 which was opened with the same restriction enzymes. The sequence of the insert of the resulting vector pME11024 (Table S3) was verified by DNA sequencing and the plasmid was used to transform CHA5206 by electroporation.

Growth curves

Growth defects in constructed mutant strains were assessed by performing growth curve assays. Therefore, five glass test tubes per strain containing 3 ml of LB each were inoculated with aliquots of 60 µl of a bacterial suspension at an optical density at 600 nm (OD_{600}) of 1. The OD_{600} of each culture incubated at 25°C and 180 rpm was monitored over time by spectrometry.

Competition and colonization assays with insects

For the competition assay, O-PS mutant strains of CHA0 and PCL1391 were marked with a constitutively expressed mCherry tag using the Tn*7* delivery vector pME9407 as described previously (Rochat *et al.*, 2010) (Table S3). The PCL1391 wild-type strain was marked analogously with a constitutively expressed GFP tag using the plasmid pBK-miniTn7-*gfp1* (Table S3). The resulting strains and the GFP-tagged CHA0 wild-type strain (Péchy-Tarr *et al.*, 2013) were grown overnight in 10 ml of LB at 25°C and 180 rpm. The cells were washed once in 0.9% NaCl solution and the OD_{600} of the bacterial suspension was adjusted to 1.0. Each mCherry-tagged mutant strain was mixed 1:1 with the corresponding GFP-marked wild-type strain and the resulting suspension was diluted 200-times in saline solution. Aliquots of 5 µl were injected into the last left pro-leg of *G. mellonella* larvae. After incubation at room temperature for approximately 22 hours, hemolymph of the larvae was collected and visualized by fluorescence microscopy as described before (Péchy-Tarr *et al.*, 2013). Mutant and wild-type cells were counted using the DsRed and GFP channels, respectively. The competitive index was calculated by first normalizing the counts for the wild type and the mutant with the ratio observed in the undiluted inoculum (to correct for variation within the inocula) and then dividing the number of mutant cells by the number of wild-type cells. Three larvae per treatment were analyzed and the experiment was performed three times independently.

For the colonization assay, mCherry-tagged strains were injected into larvae of *G. mellonella* (with 5 larvae per treatment) as described before (Péchy-Tarr *et al.*, 2013). After incubation at room temperature for 24 h, the insects were killed in liquid nitrogen, cut into two pieces and the hemolymph was collected in Eppendorf tubes placed on ice. The hemolymph samples were serially diluted in saline solution and 10 µl of each dilution and the undiluted sample were spotted on nutrient agar plates (containing 10 µg/ml gentamicin) for determination of colony forming units per larva. The experiment was performed twice.

Competitive root colonization

The assay to assess the competitiveness of mutant strains on cucumber roots was performed as described previously (Kupferschmied *et al.*, 2014) with some modifications. Briefly, three-day-old cucumber (*Cucumis sativus* cv. Chinese Snake) seedlings were grown axenically in 50-ml tubes (three plants per tube) containing 35-ml of 0.35% (w/v) water agar. Tubes with seedlings were incubated for three days in a growth chamber set to 80% relative humidity and 16 h with light (160 $mE/m^2/s$) at 22°C, followed by an 8-h dark period at 18°C. Fluorescently marked wild-type and mutant strains constructed for the competition assay with *G. mellonella* were grown as mentioned above, washed in saline solution and their OD_{600} was adjusted to 1. Each mutant strain was mixed 1:1 with its corresponding wild-type strain and each plant was inoculated at the stem base with 30 µl of the bacterial mixture. After another 5 days of incubation in the growth chamber, bacteria were isolated from root tips and counted by fluorescence microscopy as described before (Kupferschmied *et al.*, 2014). The competitive index was calculated as mentioned above. Three plants from three different tubes were analyzed per treatment and the experiment was performed twice.

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