WHOP, a Genomic Region Associated With Woody Hosts in the *Pseudomonas syringae* Complex Contributes to the Virulence and Fitness of *Pseudomonas savastanoi* pv. *savastanoi* in Olive Plants

Eloy Caballo-Ponce,¹ Pieter van Dillewijn,² Regina Michaela Wittich,² and Cayo Ramos¹

¹Área de Genética, Facultad de Ciencias, Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora”, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Campus Teatinos s/n, E-29010 Málaga, Spain; and

²Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Profesor Albareda, 1. E-18008, Granada, Spain

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Bacteria from the *Pseudomonas syringae* complex belonging to phylogroups 1 and 3 (PG1 and PG3, respectively) isolated from woody hosts share a genomic region herein referred to as WHOP (from woody host and *Pseudomonas* spp.), which is absent in strains infecting herbaceous organs. In this work, we show that this region is also encoded in *P. syringae* pv. *actinidifoliorum* (PG1) and six additional members of PG3, namely, *Pseudomonas savastanoi* pv. *retacarpa*, three *P. syringae* pathovars, *Pseudomonas meliae*, and *Pseudomonas amygdali*. Partial conservation of the WHOP occurs in only a few PG2 strains. In *P. savastanoi* pv. *savastanoi* NCPPB 3335, the WHOP region is organized into four operons and three independently transcribed genes. While the *antABC* and *catBCA* operons mediate the catabolism of anthranilate and catechol, respectively, the *ipoABC* operon confers oxygenase activity to aromatic compounds. The deletion of *antABC*, *catBCA*, or *ipoABC* in NCPPB 3335 caused reduced virulence in woody olive plants without affecting knot formation in nonwoody plants; *catBCA*, *dhoAB*, and PSA3335_3206 (encoding a putative aerotaxis receptor) were also required for the full fitness of this strain exclusively in woody olive plants. Overall, this study sheds light on the evolution and adaptation of bacteria from the *P. syringae* complex to woody hosts and highlights the enzymatic activities encoded within the WHOP region that are essential for this process.

Bacteria belonging to the *Pseudomonas syringae* complex cause diseases in a wide variety of both herbaceous and woody plant species. In fact, *P. syringae* is one of the most important models for the molecular analysis of plant-pathogen interactions (Mansfield et al. 2012). Most research on the molecular interactions established by *P. syringae* has focused on herbaceous plant pathogens, since herbaceous plants are easier to handle than woody hosts. Moreover, the tomato plant pathogen *P. syringae* pv. *tomato* DC3000, whose genome sequence was published more than 10 years ago, infects the well-known model

plant *Arabidopsis thaliana* (Buell et al. 2003). However, diseases caused by *P. syringae* on woody hosts and, especially, on fruit trees are of extreme relevance. For example, the economic losses due to the bacterial canker of kiwifruit caused by *P. syringae* pv. *actinidiae* in New Zealand were estimated to cost over US\$885 million over a 15-year period (Lamichhane et al. 2014).

During the last decade, considerable efforts have been made to establish suitable models to study the interaction of bacteria from the *P. syringae* complex with woody plants. In this sense, three model systems have predominantly been used: *P. savastanoi* pv. *savastanoi*–olive (Matas et al. 2012; Ramos et al. 2012; Rodríguez-Palenzuela et al. 2010), *P. syringae* pv. *aesculi*–horse chestnut (Green et al. 2010), and *P. syringae* pv. *actinidiae*–kiwi (Bartoli et al. 2015b; Gao et al. 2016; McCann et al. 2013; Petriccione et al. 2014). Comparison of the draft genome sequence of *P. savastanoi* pv. *savastanoi* NCPPB 3335 with those of other *P. syringae* strains that infect herbaceous hosts (i.e., *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* B728a) revealed the presence of 12 regions longer than 10 kb unique to *P. savastanoi* pv. *savastanoi* NCPPB 3335. One of the most striking features found in one of these variable regions was a cluster of genes possibly related to the metabolism of phenolic compounds (Ramos et al. 2012; Rodríguez-Palenzuela et al. 2010). Similar genome comparisons carried out among four *P. syringae* pv. *aesculi* strains isolated from horse chestnut, i.e., the type strain NCPPB 3681 isolated from leaf spots on Indian horse chestnut and three strains isolated from bleeding stem cankers on European horse chestnut in Britain, revealed the presence of a similar variable genomic region in all strains but not in the sequenced genomes of other *P. syringae* pathovars (Green et al. 2010). Subsequent genome sequencing of *P. syringae* strains isolated from woody hosts revealed that this variable region, herein referred to as WHOP (from woody host and *Pseudomonas* spp.), was also present in *P. syringae* pv. *morsprunorum* MAFF 302280 and *P. syringae* pv. *actinidiae* MAFF 302091, which are pathogenic to apricot and kiwi trees, respectively (Ramos et al. 2012). Among other genes encoded in the WHOP region, the *antABC* and the *catBCA* operons were found, which are responsible for the transformation of anthranilate into catechol and the degradation of catechol into β -ketodipate enol-lactone via the *ortho* (β -ketoadipate) pathway, respectively, in other *Pseudomonas* spp. (Houghton et al. 1995; Li et al. 2010; Nojiri et al. 2002; Ornston 1966; Taniuchi et al. 1964; Urata et al. 2004). In

Corresponding author: C. Ramos; E-mail: crr@uma.es;
Telephone: +1 34-95213 1955; Fax: +1 34-95213 2001.

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addition to the *antABC* and *catBCA* operons, other genes potentially related to the metabolism of phenolic compounds were found in the *P. savastanoi* pv. *savastanoi* NCPPB 3335 WHOP region (Rodríguez-Palenzuela et al. 2010) and in the genomes of *P. syringae* pv. *aesculi* strains (Green et al. 2010).

One role of the WHOP region in bacterial adaptation to woody hosts and the catabolism of plant-derived aromatic compounds has been proposed by several authors (Green et al. 2010; O'Brien et al. 2011; Ramos et al. 2012; Rodríguez-

Palenzuela et al. 2010), and a recent comparative genomic analysis revealed that this region in *P. syringae* phylogroups (PG) 1 and 3 is significantly associated with woody hosts (Nowell et al. 2016). However, functional studies of the genes encoded in the WHOP region are still scarce. A recent study reported a correlation between the presence of a cluster of genes located in the WHOP region and the ability of several *P. syringae* and *P. savastanoi* pv. *savastanoi* strains, all belonging to PG1 and PG3, to grow endophytically in kiwifruit, i.e., *P. syringae* pv.

Table 1. Distribution of the WHOP region within the *Pseudomonas syringae* complex

Pathovar	Strain	PG ^a	Host ^b	Symptoms ^c	WHOP ^d	Reference
<i>P. syringae</i> pv. <i>spinaceae</i>	ICMP 16929	2	Spinach		C	Thakur et al. 2016
<i>P. syringae</i> pv. <i>coryli</i>	ICMP 17001	2	European hazelnut	Twig and branch dieback	C	Thakur et al. 2016
<i>P. syringae</i> pv. <i>japonica</i>	MAFF 301072	2	Barley		AC	Baltrus et al. 2011
<i>P. syringae</i> pv. <i>actinidiae</i>	ICMP 9617 MAFF 302091 NCPPB 3871	1	Kiwi tree	Cankers on stems	+	McCann et al. 2013 Baltrus et al. 2011 Marcelletti et al. 2011
<i>P. syringae</i> pv. <i>actinidifoliorum</i>	CFBP 8161 CFBP 8180 CFBP 8043	1	Kiwi tree	Leaf spots	+	Cunty et al. 2016 Cunty et al. 2016 Cunty et al. 2016
<i>P. syringae</i> pv. <i>aesculi</i>	NCPPB 3681 2250	3	Horse chestnut	Cankers on stems	+	Green et al. 2010 Green et al. 2010
<i>P. syringae</i> pv. <i>castaneae</i>	ICMP 9419	3	Japanese chestnut	Galls and cankers on twigs	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>cerasicola</i>	ICMP 17524	3	Cherry	Galls on trunks and twigs	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>ciccaronei</i>	ICMP 5710	3	Carob	Leaf spots	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>cunninghamiae</i>	ICMP 1894	3	Chinese fir	Needle blight	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>daphniphylli</i>	CFBP 4219	3	Himeyuzuriha	Galls on stems and twigs	+	Bartoli et al. 2015a
<i>P. syringae</i> pv. <i>dendropanacis</i>	CFBP 3226	3	Kakuremino	Galls on trunks and twigs	+	Bartoli et al. 2015a
<i>P. syringae</i> pv. <i>eriobotryae</i>	ICMP 4455	3	Loquat	Cankers on stems	+	Thakur et al. 2016
<i>P. savastanoi</i> pv. <i>fraxini</i>	ICMP 7711	3	European ash	Cankers and blisters on stems	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>myricae</i>	ICMP 7118	3	Chinese bayberry	Galls on trunks and twigs	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>morsprunorum</i>	MAFF 302280	1	Apricot	Leaf spots, cankers on branches and trunks	+	Baltrus et al. 2011
<i>P. savastanoi</i> pv. <i>nerii</i>	ICMP 16943	3	Oleander	Knots on trunks, twigs and leaves	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>photiniae</i>	ICMP 7840	3	Japanese photinia	Leaf spots, shoot blight	+	Thakur et al. 2016
<i>P. savastanoi</i> pv. <i>retacarpa</i>	ICMP 16945	3	Broom bush	Knots on stems	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>rhapiolepidis</i>	ICMP 9756	3	Sharinbai	Galls on trunks and twigs	+	Thakur et al. 2016
<i>P. savastanoi</i> pv. <i>savastanoi</i>	NCPPB 3335 DAPP-PG 722 ICMP 4352 PseNe107	3	Olive tree	Knots on stems	+	Rodríguez-Palenzuela et al. 2010 Moretti et al. 2014 Thakur et al. 2016 Bartoli et al. 2015a
<i>P. syringae</i> pv. <i>theae</i>	ICMP 3923	1	Tea	Shoot blight	+	Thakur et al. 2016
<i>P. syringae</i> pv. <i>ulmi</i>	ICMP 3962	3	Elm	Bacterial spots, shoot blight	+	Thakur et al. 2016
<i>P. amygdali</i>	CFBP 3205	3	Almond	Cankers on stems and branches	+	Bartoli et al. 2015a
<i>P. meliae</i>	CFBP 3225	3	Chinaberry	Galls on twigs and shoots	+	Bartoli et al. 2015a
<i>P. syringae</i> pv. <i>aceris</i>	ICMP 2802 MAFF 302273	2	Maple	Leaf spots	–	Thakur et al. 2016 Baltrus et al. 2011
<i>P. syringae</i> pv. <i>alisalensis</i>	ICMP 15200	5	Broccoli		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>antirrhini</i>	ICMP 4303	1	Snapdragon		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>apii</i>	ICMP 2814	1	Celery		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>aptata</i>	ICMP 459 DMS 50252	2	Sugar beet		–	Thakur et al. 2016 Baltrus et al. 2011
<i>P. syringae</i> pv. <i>atrofaciens</i>	ICMP 4394	2	Wheat		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>atropurpurea</i>	ICMP 4457	4	Italian ryegrass		–	Thakur et al. 2016

(continued on next page)

^a PG = phylogroup.

^b Woody hosts are shown in bold.

^c Only for woody host pathogens.

^d C = presence of the *catBCA* operon; AC = presence of the *antABC* and *catBCA* operons; + = presence of the WHOP region; – = absence of the WHOP region.

^e Not assigned.

^f *P. congelans* is not a confirmed plant pathogen.

actinidia strains of biovars 1, 3, and 4, *P. syringae* pv. *mor-sprunorum* MAFF 302280, *P. syringae* pv. *aesculi* 0893_23, *P. syringae* pv. *theae* NCPPB 2598, and *P. savastanoi* pv. *savastanoi* strains NCPPB 3335 and PseNe107. In contrast, other *P. syringae* strains isolated from water or herbaceous hosts lacking this cluster did not colonize kiwifruit plants, suggesting a role for these genes in the colonization of the plant vascular system, in which lignin is an important compound (Bartoli et al.

2015b). A role in knot development on olive plants has been reported for the *P. savastanoi* pv. *savastanoi* NCPPB 3335 *catJ* gene (Matas et al. 2012). This gene, which is not encoded in the WHOP region but is present in the genomes of *P. syringae* strains isolated from nonwoody hosts, encodes the subunit B of a putative β -ketoadipate CoA-transferase, an enzyme of the β -ketoadipate pathway involved in the conversion of β -ketoadipate enol-lactone into succinyl Co-A (Matas et al. 2012). Conversely,

Table 1. (continued from previous page)

Pathovar	Strain	PG ^a	Host ^b	Symptoms ^c	WHOP ^d	Reference
<i>P. syringae</i> pv. <i>avellanae</i>	ICMP 9746 PaVt10	1	Hazelnut	Twig dieback and cankers	–	Scortichini et al. 2013 Bartoli et al. 2015a
<i>P. syringae</i> pv. <i>avii</i>	CFBP 3846	1	Cherry	Cankers	–	Nowell et al. 2016
<i>P. syringae</i> pv. <i>berberidis</i>	ICMP 4116	1	Barberry	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>broussonetiae</i>	ICMP 13650	3	Paper mulberry	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>coriandricola</i>	ICMP 12471	5	Cilantro		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>coronafaciens</i>	ICMP 3113		Oat		–	An et al. 2015
<i>P. syringae</i> pv. <i>delphinii</i>	ICMP 529	1	Delphinium		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>garcae</i>	ICMP 4323	4	Coffee	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>glycinea</i>	B706 race 4 ICMP 2189	3	Soybean		–	Qi et al. 2011 Qi et al. 2011 Thakur et al. 2016
<i>P. syringae</i> pv. <i>helianthi</i>	ICMP 4531	6	Sunflower		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>hibisci</i>	ICMP 9623	3	Chinese hibiscus	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>lachrymans</i>	ICMP 3507 MAFF 302278	3	Cucumber		–	Thakur et al. 2016 Baltrus et al. 2011
<i>P. syringae</i> pv. <i>lapsa</i>	ICMP 3947	3	Corn hybrid		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>maculicola</i>	CFBP 1657 ES4326	1	Cauliflower		–	Bartoli et al. 2015a Baltrus et al. 2011
<i>P. syringae</i> pv. <i>mellea</i>	ICMP 5711	3	Tobacco		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>mori</i>	ICMP 4331 MAFF 301020	3	White mulberry	Leaf spots	–	Thakur et al. 2016 Baltrus et al. 2011
<i>P. syringae</i> pv. <i>oryzae</i>	ICMP 9088 1_6	4	Rice		–	Thakur et al. 2016 Reinhardt et al. 2009
<i>P. syringae</i> pv. <i>panici</i>	LMG 2387	NA ^e	Millet		–	Liu et al. 2012
<i>P. syringae</i> pv. <i>papulans</i>	CFBP 1754	2	Apple	Leaf and fruit spots	–	Bartoli et al. 2015a
<i>P. syringae</i> pv. <i>phaseolicola</i>	ICMP 2740 1448A	3	Bean		–	Thakur et al. 2016 Joardar et al. 2005
<i>P. syringae</i> pv. <i>philadelphi</i>	ICMP 8903	1	Mock orange	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>pisi</i>	1704B	2	Pea		–	Baltrus et al. 2011
<i>P. syringae</i> pv. <i>porri</i>	ICMP 8961	4	Leek		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>primulae</i>	ICMP 3956	7	Polyanthus		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>ribicola</i>	ICMP 3882	7	Golden currant	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>sesame</i>	ICMP 763	3	Sesame		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>solidagae</i>	ICMP 16925	1	Tall golden rod		–	Thakur et al. 2016 Thakur et al. 2016
<i>P. syringae</i> pv. <i>syringae</i>	B728a ICMP 3023	2	Bean, lilac		–	Feil et al. 2005 Thakur et al. 2016
<i>P. syringae</i> pv. <i>tabaci</i>	ICMP 2835 ATCC 11528	3	Tobacco		–	Thakur et al. 2016 Baltrus et al. 2011
<i>P. syringae</i> pv. <i>tagetis</i>	ICMP 4091	6	African marigold		–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>tomato</i>	DC3000 ICMP 2844 NCPPB 1108 Max13 T1 K40	1	Tomato		–	Buell et al. 2003 Thakur et al. 2016 Cai et al. 2011 Cai et al. 2011 Almeida et al. 2009 Cai et al. 2011
<i>P. syringae</i> pv. <i>tremae</i>	ICMP 9151	3	Urajiroenoki	Galls on trunks and twigs	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>viburni</i>	ICMP 3963	1	Viburnum	Leaf spots	–	Thakur et al. 2016
<i>P. syringae</i> pv. <i>zizaniae</i>	ICMP 8921	4	Rice		–	Thakur et al. 2016
<i>P. cannabina</i>	ICMP 2823	5	Indian hemp		–	Thakur et al. 2016
<i>P. caricapapayae</i>	ICMP 2855	6	Papaya	Leaf spots	–	Thakur et al. 2016
<i>P. congelans</i> ^f	ICMP 19117	2	Grass phyllosphere		–	Thakur et al. 2016
<i>P. ficuserectae</i>	ICMP 7848	3	Inubiwa	Leaf spots	–	Thakur et al. 2016
<i>P. viridiflava</i>	ICMP 2848	7	Bean		–	Thakur et al. 2016

epidemic strains of *P. syringae* pv. *actinidiae* have been reported to possess a plasmid encoding two gene clusters also related to the metabolism of aromatic compounds that are shared with the xylem pathogen *Xylella fastidiosa*. Interestingly, one of these clusters is reportedly related to the biosynthesis of anthranilate. In addition, a putative phenylacetate CoA ligase encoded in the second cluster has also been proposed to use anthranilate as a substrate (McCann et al. 2013).

In this work, we analyze the organization, distribution, function, and role of the WHOP region in virulence and fitness in planta, using the well-established model pathogen of woody hosts *P. savastanoi* pv. *savastanoi*. The genetic organization of the WHOP region has been determined as well as the function of several genes located within this chromosomal region. Our results broaden the distribution of the WHOP region to other PG1 and PG3 members infecting woody hosts and show its partial conservation in only three PG2 pathovars. Furthermore, we demonstrate that several of the genes located within this region contribute to the fitness and virulence of this bacterium in woody olive plants but not in nonwoody olive plants.

RESULTS

Distribution of the WHOP region in the *Pseudomonas syringae* complex.

Due to the development of next-generation sequencing technologies, the genome sequences of almost every single pathovar (type and pathotype strains) of *P. syringae* and related species are currently available (Bartoli et al. 2015a; Moretti et al. 2014; Nowell et al. 2016; Thakur et al. 2016). To broaden the analysis of the distribution of the WHOP region to all phylogroups of the *P. syringae* complex including at least one sequenced strain, the genomes of 81 *P. syringae* strains (59 pathovars), seven *P. savastanoi* strains (four pathovars), and one strain of the remaining eight species of this complex were downloaded from GenBank and the presence of the WHOP region was analyzed. Of the 96 genomes examined, 49 (51%) corresponded to strains isolated from woody hosts. As shown in Table 1, a complete WHOP region is conserved in four PG1 pathovars (*P. syringae* pvs. *actinidiae*, *morsprunorum*, *actinidifoliorum*, *theae*) of the 14 analyzed and several PG3 strains, i.e., 12 *P. syringae* pathovars (pvs. *aesculi*, *castaneae*, *cerasicola*, *ciccaronei*, *cunninghamiae*, *daphniphylli*, *dendropanacis*, *erobotryae*, *myricae*, *photiniae*, *rhapiolepidis*, and *ulmi*), all four *P. savastanoi* pathovars (pvs. *fraxini*, *nerii*, *savastanoi*, and *retacarpa*), as well as *Pseudomonas amygdali* CFBP 3205, and *Pseudomonas meliae* CFBP 3225. All of these strains were isolated from woody hosts, causing most of the symptoms on the woody organs of their respective plant hosts. In contrast, the WHOP region is absent in the genomes of all sequenced *P. syringae* pathovars infecting herbaceous hosts or causing symptoms on the nonwoody organs of woody plants, with the exception of *P. syringae* pvs. *actinidifoliorum* and *ciccaronei*, which cause leaf spot lesions on kiwifruit trees (Chapman et al. 2012; Cuntly et al. 2015) and carob (Ercolani and Caldarella 1972), respectively. These results, which are in agreement with those previously reported for a smaller number of PG1 and PG3 strains (Bartoli et al. 2015b; Nowell et al. 2016), broaden the distribution of the WHOP region to *P. syringae* pv. *actinidifoliorum* (PG1) and six additional members of PG3, *P. syringae* pvs. *ciccaronei*, *cunninghamiae*, and *photiniae*, *P. savastanoi* pv. *retacarpa*, *P. amygdali*, and *P. meliae*.

Partial conservation of the WHOP region was observed in three of the 12 PG2 *P. syringae* pathovars analyzed, i.e., *P. syringae* pv. *spinaceae* ICMP 16929 and *P. syringae* pv. *coryli* ICMP 17001, which harbor only the *catABC* operon, and *P. syringae* pv. *japonica*

MAFF 301072, which carries both the *catBCA* and *antABC* operons (Table 1). However, in these three strains, the *catBCA* operon was found in close proximity to the *catBCA* transcriptional activator gene *catR*, whereas *catR* was not present in the genomes of strains encoding a complete WHOP region (Fig. 1A to C). Furthermore, a gene encoding a transposase was found immediately upstream of the *catA* gene in the *P. syringae* pv. *japonica* MAFF 301072 genome (Fig. 1C), which encodes four additional open reading frames (ORFs) unrelated to the WHOP region between *antR* and *catA*. Conversely, the *catBCA* and *antABC* operons are located in close proximity in all strains carrying a complete WHOP region (Fig. 1A). These results suggest that horizontal transfer might have occurred in the evolution of the *catBCA* operon.

A phylogenetic tree based on the *catBCA* operon was constructed using maximum likelihood and showed that PG1 and PG3 strains clustered monophyletically to the exclusion of PG2. In addition *P. syringae* pv. *japonica* clustered separately from the other two PG2 strains encoding a partial WHOP region (Supplementary Fig. S1). These results are discordant with the phylogeny of the *P. syringae* complex based on house-keeping genes (Berge et al. 2014), reinforcing the hypothesis of horizontal transfer in the evolutionary history of this operon across the *P. syringae* complex.

The WHOP region is organized into four operons and three independently transcribed genes.

Fourteen putative ORFs are encoded in the WHOP region (Fig. 1A; Table 2). PSA3335_3199 to PSA3335_3201 and PSA3335_3202 to PSA3335_3204 encode homologs of the *catBCA* and *antABC* operons, respectively, found in other pseudomonads (Houghton et al. 1995; Li et al. 2010; Maeda et al. 2003; Nojiri et al. 2002; Urata et al. 2004). The *antABC* operon participates in the conversion of anthranilate into catechol, which is further transformed into β -ketoadipate enol-lactone by the *catBCA*-encoded activity (Supplementary Fig. S2). PSA3335_3205 (*antR*) and PSA3335_3213 (*benR*), which are the only genes oriented in the opposite direction to all others (Fig. 1A), encode positive transcriptional regulators of the *antABC* and *benABCD* operons, respectively. However, the *benABCD* operon, which is involved in the degradation of benzoate to catechol in *Pseudomonas putida* (Jeffrey et al. 1992), is not present in the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome.

Annotation of the PSA3335_3207 to PSA3335_3211 genes also yielded functions likely to be involved in the metabolism of phenolic compounds (Rodríguez-Palenzuela et al. 2010). A search for protein domains using the MyHits web tool revealed that PSA3335_3206 (annotated as an aerotaxis receptor) may, in fact, play a role in chemotaxis, since it encodes a protein containing a methyl-accepting domain and a bacterial chemotaxis sensory transducer domain. No specific domain was obtained for PSA3335_3207, whose product was annotated as a protein involved in the *meta*-pathway of phenol degradation. The products of PSA3335_3208 and PSA3335_3209 contain two structural motifs characteristic of iron-sulfur proteins (2Fe-2S and 4Fe-4S centers) and an NADP-dependent oxidoreductase domain, respectively. The product of PSA3335_3211 contains a dienelactone hydrolase family domain. This family of enzymes is involved in the metabolism of halogenated catechols in *Pseudomonas knackmussii*, previously known as *Pseudomonas* sp. strain B13 (Schlömman et al. 1990). PSA3335_3210, annotated as a short chain alcohol dehydrogenase gene, encodes a protein containing a KR domain, which is characteristic of bacterial polyketide synthases involved in the reductive modification of the β -carbonyl centers in the growing polyketide chain (Caffrey 2012).

The organization of all genes encoded in the WHOP region into different operons was analyzed in *P. savastanoi* pv. *savastanoi* NCPPB 3335 by reverse transcription-polymerase chain

reaction (RT-PCR). Primers targeting sequential ORFs were used to amplify intergenic regions, except for those of the *catBCA* operon, in which a region spanning from PSA3335_3199 (*catA*) to PSA3335_3201 (*catB*) was amplified (Fig. 1A). Positive controls using genomic DNA as a template were used in all PCR analyses. As shown in Figure 1D, RT-PCR amplification of the *antC-antB* and *antB-antC* regions yielded DNA fragments of identical sizes to those obtained for their respective positive controls, confirming that *antA*, *antB*, and *antC* are also organized as an operon in NCPPB 3335. A similar result was obtained for the region spanning from *catB* to *catA*, revealing the operon organization of *catB*, *catC*, and *catA*. No amplification product was obtained for the intergenic region located between the *catBCA* and *antABC* operons, showing that *antABC* and *catBCA* are transcribed independently. Regarding the intergenic regions of PSA3335_3207 to PSA3335_3208 and PSA3335_3208 to

PSA3335_3209, amplification products of the expected sizes were observed in both cases, while DNA fragments were not obtained for PSA3335_3206 to PSA3335_3207 and PSA3335_3209 to PSA3335_3210. These results indicate that transcription of the PSA3335_3206 gene is independent. However, PSA3335_3207 to PSA3335_3209 cotranscribe in an operon, herein referred to as the *ipoABC* operon (from indigo-producing operon), in relation to the ability of these genes to transform indole into the blue pigment indigo (discussed below). The intergenic region between PSA3335_3211 (*dhoA*) and PSA3335_3210 (*dhoB*) was also amplified, suggesting their organization into an operon, hereafter referred to as the *dhoAB* operon (from diene lactone hydrolase operon). No amplification products corresponding to the intergenic regions located immediately upstream or downstream from this operon were obtained. In summary, the genes encoded in the WHOP regions are organized into four operons

Fig. 1. Schematic map of the WHOP region and related genes in the genomes of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and other *P. syringae* strains. **A**, *P. syringae* pathovars *aesculi*, *actinidiae* (ICMP 9617, MAFF 302091, and NCPPB 3871), *morsprunorum* MAFF 302280, *actinidifoliorum* (CFBP 8161, CFBP 8180, and CFBP 8043), *castaneae* ICMP 9419, *cerasicola* ICMP 17524, *ciccaronei* ICMP 5710, *cunninghamiae* ICMP 1894, *daphniphylli* CFBP 4219, *dendropanacis* CFBP 3226, *erobotryae* ICMP 4455, *myricae* ICMP 7118, *photinieae* ICMP 7840, *rhapiolepidis* ICMP 9756, *theae* ICMP 3923 and *ulmi* ICMP 3962; *P. savastanoi* pathovars *savastanoi* (NCPPB 3335, DAPP-PG 722, PseNe107 and ICMP 4352), *fraxini* ICMP 7711, *nerii* ICMP 16943, and *retacarpa* ICMP 16945; *P. amygdali* CFBP 3205 and *P. meliae* CFBP 3225. **B**, *P. syringae* pv. *coryli* ICMP 17001 and *P. syringae* pv. *spinaciae* ICMP 16929. **C**, *P. syringae* pv. *japonica* MAFF 301072; T = IS222 transposase protein *orfB*. **D**, Gel electrophoresis (1% agarose) of reverse transcription-polymerase chain reaction amplicons obtained from *P. savastanoi* pv. *savastanoi* NCPPB 3335. Black bars indicate the hybridization regions of the primers used (A). Genes in the same color are transcribed as an operon. M = 1-kb Plus DNA ladder (Life Technologies).

Table 2. Genes located within the WHOP region

Demonstrated function	Accession number ^a	Gene ^b	Annotated gene product
Catechol catabolism	PSA3335_3199	<i>catA</i>	Catechol 1,2-dioxygenase
	PSA3335_3200	<i>catC</i>	Muconolactone isomerase
	PSA3335_3201	<i>catB</i>	Muconate cycloisomerase
Anthranilate catabolism	PSA3335_3202	<i>antC</i>	Anthranilate dioxygenase reductase component
	PSA3335_3203	<i>antB</i>	Anthranilate dioxygenase beta subunit
	PSA3335_3204	<i>antA</i>	Anthranilate dioxygenase alpha subunit
	PSA3335_3205	<i>antR</i>	<i>antABC</i> regulatory protein
	PSA3335_3207	<i>ipoC</i>	Involved in <i>meta</i> pathway of phenol degradation
Indigo-producing oxygenase	PSA3335_3208	<i>ipoB</i>	Nitrilotriacetate monooxygenase component B
	PSA3335_3209	<i>ipoA</i>	Putative oxygenase subunit
Not determined	PSA3335_3206	UN	Aerotaxis receptor
	PSA3335_3210	<i>dhoB</i>	Short chain alcohol dehydrogenase
	PSA3335_3211	<i>dhoA</i>	Dienelactone hydrolase
	PSA3335_3213	<i>benR</i>	Positive regulator of the <i>benABCD</i> operon

^a Accession number corresponding to the genome of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335.

^b UN = unnamed.

(*catBCA*, *antABC*, *ipoABC*, and *dhoAB*) and three independently transcribed genes (*antR*, PSA3335_3206, and *benR*).

Involvement of the *antABC* operon in the degradation of anthranilate.

To confirm the activity of the *antABC* operon in *P. savastanoi* pv. *savastanoi* NCPPB 3335, a $\Delta antA$ mutant and its complemented strain harboring the *antABC* operon in the plasmid pBRR:*antABC* were constructed. *P. savastanoi* pv. *savastanoi* cells grown overnight in lysogeny broth (LB) medium were transferred to M9 minimal medium containing 500 μ M anthranilate (M9A) and, after 24 h, the filtered supernatants were analyzed by high-performance liquid chromatography (HPLC). A single peak corresponding to anthranilate was observed at a retention time (Rt) of 9.72 min in the chromatogram of the noninoculated M9A (Fig. 2A), whereas this peak was not present in the supernatant of M9A inoculated with the wild-type strain *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Fig. 2B), suggesting the total disappearance of the compound under the conditions tested. In contrast, a concentration of 472 μ M anthranilate was detected in the supernatant of M9A inoculated with the $\Delta antA$ mutant (Fig. 2C). Two peaks (Rt = 7.8 and 9.9 min) corresponding to 229 μ M catechol and 66 μ M anthranilate, respectively, were detected in the M9A inoculated with the complemented strain (Fig. 2D). Thus, total

degradation of anthranilate did not occur in the culture supernatant of the complemented strain. These results confirm the implication of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 *antABC* operon in the conversion of anthranilate into catechol.

Involvement of the *catBCA* operon in the degradation of catechol and halogenated catechols.

In *P. putida*, the transcription of the *catBCA* operon is activated by CatR (Rothmel et al. 1990). However, a homolog of this activator protein is not found in the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome. The expression of the *catBCA* operon in *P. savastanoi* pv. *savastanoi* NCPPB 3335 and its $\Delta antA$ mutant was analyzed by quantitative RT-PCR (RT-qPCR) of the *catB* gene. For this purpose, *P. savastanoi* pv. *savastanoi* cells were inoculated in LB medium and LB medium amended with 7.3 mM anthranilate (LBA). In the wild-type strain, expression of the operon was 232-fold higher in LBA than in the non-amended medium. However, a similar expression level was obtained for the $\Delta antA$ mutant in both media (Supplementary Fig. S3), indicating that the activity of the *antABC* operon is essential for the activation of *catBCA* mediated by anthranilate. Together, these results suggest that the transcription of *catBCA* may be activated by the product of the *antABC* operon (catechol), by other catechol-related compounds, or both in a CatR-independent manner.

Fig. 2. Functional analysis of the *antABC* and *catBCA* operons. **A to D**, High-performance liquid chromatograms of filtered supernatants after 24-h exposure to M9 minimal media supplemented with 500 μ M anthranilate or **E to H**, 300 μ M catechol. Simultaneous detection at 210 (blue lines) and 230 nm (red lines) was performed. **A**, Control cell-free medium supplemented with anthranilate, **B**, anthranilate-amended medium (M9A) inoculated with *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335, **C**, M9A with the $\Delta antA$ mutant, **D**, M9A with the $\Delta antA$ mutant complemented with pBRR:*antABC*, **E**, control cell-free medium supplemented with catechol, **F**, catechol-amended medium with *P. savastanoi* pv. *savastanoi* NCPPB 3335, **G**, catechol-amended medium with the $\Delta catB$ mutant, or **H**, catechol-amended medium with the $\Delta catB$ mutant complemented with pBRR:*catBCA*. The concentrations of the compounds were determined using a standard curve of anthranilate or catechol (5 to 500 μ M). The intensity of the signal is indicated in millivolts (mV).

To confirm the involvement of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 *catBCA* operon in catechol metabolism, a $\Delta catB$ mutant and its complemented strain carrying the complete *catBCA* operon in the plasmid pBBR:*catBCA* were constructed. *P. savastanoi* pv. *savastanoi* cells grown overnight in LBA medium were collected by centrifugation and were transferred to M9 media containing 300 μ M catechol (M9C). After 24 h, the filtered supernatant was analyzed by HPLC. As shown in Figure 2E, the chromatogram corresponding to the cell-free medium showed a peak at $R_t = 7.61$ min, corresponding to nonmodified catechol. Conversely, the chromatogram of the wild-type *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Fig. 2F) did not show a peak at this R_t , indicating that catechol was completely removed from the medium. However, several other peaks corresponding to the accumulation of unidentified compounds were observed in this chromatogram, some of which showed an R_t between 1 and 4 min and a single additional peak at 12 min. Two large peaks ($R_t = 8.01$ and 8.66 min) showing UV absorption spectra resembling that of catechol were detected in the chromatogram of the $\Delta catB$ strain (Fig. 2G) grown in M9C, indicating that several catechol-related compounds may be present in the culture supernatant of this strain. This hypothesis was reinforced by the fact that the chromatogram of the complemented $\Delta catB$ strain ($\Delta catB$ -pBBR:*catBCA*) did not display these peaks (Fig. 2H). Since the *catBCA* operon is unique in the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome and modification of the amended catechol was not observed in the cell-free M9C, we propose that one or more other enzymes encoded by this strain may be able to transform catechol under the conditions tested.

Dienelactone hydrolases are reportedly involved in the metabolism of halogenated catechols in *Pseudomonas* sp. strain B13 (Schlmann et al. 1990). In this pathway, halogenated catechols are initially attacked by catechol 1,2-dioxygenase (CatA), followed by muconate cycloisomerase (CatB) activities. To address the possible involvement of the *catBCA* and *dhoAB* operons in the catabolism of 4-chlorocatechol in *P. savastanoi* pv. *savastanoi* NCPPB 3335, the wild-type strain and the $\Delta catB$ and $\Delta dhoAB$ mutants were cultured in M9 media supplemented with 4-chlorocatechol. A peak corresponding to 4-chlorocatechol ($R_t = 5.05$ min) was observed in the cell-free culture (Supplementary Fig. S4). However, this compound was not detected in the chromatograms of the wild-type strain or its $\Delta dhoAB$ mutant, indicating the ability of *P. savastanoi* pv. *savastanoi* NCPPB 3335 to degrade 4-chlorocatechol through a pathway independent of *dhoAB*. In addition, other peaks corresponding to unidentified compounds ($R_t = 4.45$ and 4.85 min) but resembling that of 4-chlorocatechol were observed, suggesting the accumulation of 4-chlorocatechol-related compounds in the culture medium of these two strains. In contrast, although 4-chlorocatechol was detected in the supernatant of the $\Delta catB$ mutant, the calculated area under the peak for this compound was approximately fourfold lower than that of the cell-free medium, indicating that the observed partial degradation of 4-chlorocatechol is independent of the *catBCA* operon in this strain. Thus, as mentioned before for nonmodified catechol, one or more other *P. savastanoi* pv. *savastanoi* NCPPB 3335 proteins could enzymatically attack 4-chlorocatechol under the conditions tested.

Involvement of the *ipoABC* operon in the oxidation of indole into indigo.

To analyze the function of the *ipoABC* operon, genes of which were annotated as putative components of an oxygenase involved in the metabolism of aromatic compounds (Table 2), we constructed a plasmid harboring the whole operon under the control of its own promoter region (plasmid pBBR:*ipoABC*).

Several authors have reported that heterologous expression of certain oxygenases in *Escherichia coli* leads to the production of the blue pigment indigo (Choi et al. 2003; Doukyu et al. 2003; Ensley et al. 1983; van Hellemond et al. 2007; Woo et al. 2000), which is synthesized via the oxidation of indole (Fig. 3A). *E. coli* DH5 α cells harboring pBBR:*ipoABC* were grown in M9 medium (colorless) amended with 0.5 mM indole. After 24 h of incubation at 37°C, the culture turned blue, whereas the culture of the untransformed strain remained colorless. Furthermore, a *P. savastanoi* pv. *savastanoi* NCPPB 3335 $\Delta ipoABC$ mutant and its complemented strain harboring the plasmid pBBR:*ipoABC* (strain $\Delta ipoABC$ -pBBR:*ipoABC*) were constructed and were incubated in M9 minimal medium amended with 0.5 mM indole. While cultures of this medium inoculated with the wild-type strain *P. savastanoi* pv. *savastanoi* NCPPB 3335 or its $\Delta ipoABC$ mutant remained colorless, the culture of the complemented strain turned blue and accumulated insoluble blue precipitates (Fig. 3B). HPLC analysis of these blue precipitates showed a single peak at $R_t = 1.7$ min (Fig. 3C) with a UV spectrum identical to that previously reported for indigo (Olvera Vargas et al. 2010). These results confirm the involvement of the *ipoABC* operon in the transformation of indole into indigo,

Fig. 3. Functional analysis of the *ipoABC* operon. **A**, Proposed pathway for the biosynthesis of indigo by toluene dioxygenase (Ensley et al. 1983). **B**, Appearance of M9 minimal media supplemented with 0.5 mM indole after the growth of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335, its $\Delta ipoABC$ mutant, and the complemented $\Delta ipoABC$ strain expressing the whole *ipoABC* operon (strain $\Delta ipoABC$ -pBBR:*ipoABC*). **C**, High-performance liquid chromatogram of the blue compound partially purified from the supernatant of the $\Delta ipoABC$ -pBBR:*ipoABC* culture.

suggesting the codification of an oxygenase able to use aromatic compounds as substrates.

Role of the WHOP region in the virulence and fitness of *P. savastanoi* pv. *savastanoi* NCPPB 3335 in olive plants.

Due to the exclusivity of the WHOP region to strains of *P. syringae* complex—infecting woody organs of woody hosts (Table 1), it was of particular interest to analyze its role in the interaction between *P. savastanoi* pv. *savastanoi* NCPPB 3335 and olive plants. Two different model systems were used for this purpose, micropropagated olive explants (nonwoody plants) (Fig. 4A) and one-year-old woody olive plants (Fig. 4B). Disease severity was evaluated after 30 or 90 days, respectively. Plants were inoculated with either the wild-type strain, its mutant derivatives affected in the WHOP region ($\Delta antA$, $\Delta catB$, $\Delta 06$, $\Delta ipoABC$ and $\Delta dhoAB$), or each of the complemented strains. No significant differences in the volume of the knots induced in micropropagated olive plants were found among the strains tested (Fig. 4A and C; not shown for the complemented $\Delta 06$ and $\Delta dhoAB$ strains). However, significant differences in the knot sizes generated on woody olive plants were observed among the strains tested (Fig. 4B and D). The largest reduction in knot volume (3.1-fold smaller than those generated by the wild-type strain) was observed in plants inoculated with the $\Delta antA$ mutant. In addition, plants inoculated with the $\Delta catB$ and $\Delta ipoABC$ mutants induced knots whose volumes were 2.4- and 1.6-fold smaller, respectively (Fig. 4D). Moreover, while the complemented $\Delta catB$ -pBBR:*catBCA* and $\Delta ipoABC$ -pBBR:

ipoABC strains developed knots similar to those observed in plants infected with the wild-type strain, the knots induced by the complemented strain $\Delta antA$ -pBBR:*antABC* were slightly larger. No differences in the volumes of the knots developed on woody olive plants by the $\Delta 06$ and $\Delta dhoAB$ mutants (Fig. 4B and D) or their respective complemented strains (not shown) relative to the wild-type strain were observed. Despite the observed differences in strain virulence, all strains reached equal final populations within the developed knots, both on micropropagated (equivalent to 10^7 CFU per knot) and woody olive plants (equivalent to 10^6 CFU per knot). In summary, the *antABC*, *catBCA*, and *ipoABC* operons play a role in the virulence of *P. savastanoi* pv. *savastanoi* NCPPB 3335 in woody olive plants but not in micropropagated (nonwoody) olive plants.

Mixed suspensions of the wild-type strain *P. savastanoi* pv. *savastanoi* NCPPB 3335 and each of the mutants affecting the WHOP region were prepared to perform competitive assays on both micropropagated and woody olive plants (Fig. 5). On nonwoody olive plants, the resulting competitive index (CI) values were not significantly different from 1.0, except for that of the $\Delta ipoABC$ mutant (CI = 0.39 ± 0.16) (Fig. 5A). However, when mixed inoculations were performed on woody olive trees, the obtained CI values were statistically lower than unity, except for the $\Delta antA$ mutant (Fig. 5B). The lowest value was observed for the $\Delta catB$ mutant (CI = 0.11 ± 0.04). Similar to the results described above in relation to the virulence of the mutant strains, the $\Delta catB$, $\Delta 06$, and $\Delta dhoAB$ mutants were impaired in their competitive growth exclusively in woody olive plants,

Fig. 4. Role of the genetic clusters located within the WHOP region in the virulence of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 in olive plants. **A**, Knots developed by the indicated strains on nonwoody plants and **B**, woody olive plants at 30 and 90 days postinoculation, respectively. **C**, Quantification of knot volume generated by the indicated strains on nonwoody plants and **D**, woody olive plants. Error bars represent the standard deviation from the average volume of a minimum of three knots. The same letters indicate differences that were not significant ($\alpha = 0.05$), using the one-way analysis of variance statistical test.

whereas their fitness and survival in nonwoody olive plants was similar to that of the wild-type strain.

DISCUSSION

The WHOP region of the *P. syringae* complex, first described in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Rodríguez-Palenzuela et al. 2010) and *P. syringae* pv. *aesculi* (Green et al. 2010), is currently viewed as a relevant genomic feature for the evolution of *P. syringae* PG1 and PG3 strains associated with woody hosts (Bartoli et al. 2015b; Nowell et al. 2016). In this study, we extended the analysis of the WHOP region distribution to 40 additional members of the *P. syringae* species complex, 32 strains of *P. syringae* pathovars included in PG1 to PG7, *P. savastanoi* pv. *retarcarpa*, *P. amygdali*, *Pseudomonas cannabina*, *Pseudomonas caricapapayae*, *Pseudomonas congelans*, *Pseudomonas fuscuserectae*, *P. meliae*, and *Pseudomonas viridiflava*. Besides other PG3 strains also infecting woody organs of woody hosts (*P. savastanoi* pv. *retarcarpa*, *P. syringae* pvs. *cunninghamiae* and *photiniae*, *P. meliae*, and *P. amygdali*), a complete WHOP region was also found in *P. syringae* pv. *actinidifoliorum* (PG1) and *P. syringae* pv. *ciccaronei* (PG3), both inducing leaf spots in woody hosts (Table 1). It could be possible that these two pathovars represent a transition point from woody to herbaceous organs that maintain the WHOP region. In fact, strains of *P. syringae* pv. *actinidifoliorum*, which has been proposed to be a new and less-virulent pathovar emerging from *P. syringae* pv. *actinidiae*, are unable to cause symptoms on woody organs of *Actinidia deliciosa* and *Actinidia chinensis* (Cunty et al. 2015). *P. syringae* pv. *avellanae* and *P. syringae* pv. *avii*, both included in PG1 and pathogenic to woody hosts, have been shown not to encode the WHOP region (Nowell et al. 2016). Here, we show that this cluster is neither found in *Pseudomonas tremae* (PG3), causing galls on trunks and twigs of urajiroenoki (*Trema orientalis*). The genetic traits governing adaptation of these strains to woody niches remain unknown.

Interestingly, we also found that some *P. syringae* pathovars of PG2 conserve a partial sequence of the WHOP region, the *catBCA* operon (Table 1). Similarly to the phylogeny of the genes flanking the WHOP region (Nowell et al. 2016), the evolutionary history of the *catBCA* operon reveals phylogenetic discordance with that of the *P. syringae* complex based on housekeeping genes (Berge et al. 2014), suggesting the existence of horizontal transfer. Nowell et al. (2016) explained the presence of the WHOP region in both PG1 and PG3 by a recombination event between an ancestral PG1 lineage and an ancestral PG3 lineage, resulting in the horizontal transfer of the WHOP genes across these phylogroups. This hypothesis is further supported by the topology of the *catBCA* operon. In relation to the exclusion of PG2 in the *catBCA* phylogeny, distinct recombination events between ancestral lineages of both PG2 and other *Pseudomonas* sp. might explain the acquisition of the *catBCA/catR* cluster in *P. syringae* pvs. *coryli*, *spinaceae*, and *japonica*. The *catBCA* operon, which is widely distributed within the genus *Pseudomonas*, has been proposed to play an important role in the degradation of catechol and catechol-derived compounds in the plant-associated bacterium *P. putida* KT2440 (Jiménez et al. 2002). Adaptation of *P. syringae* pathovars *coryli*, *spinaceae*, and *japonica* to their respective hosts may have required the incorporation of the *catBCA* operon into their genomes, whereas the other WHOP-encoded pathways (except for the *antABC* operon in *P. syringae* pv. *japonica*) are dispensable in these niches. In agreement with this hypothesis, a transposase was found immediately downstream of the *cataA* gene in *P. syringae* pv. *japonica* (Fig. 1C), suggesting that the *antABC* and *catBCA* operons, which are encoded in close proximity, may have been subjected to genetic instability in this

pathovar. Interestingly, *P. resinovorans* NBRC 106553, isolated from activated sludge, carries the *antABC* operon in the plasmid pCAR1, a 200-kb plasmid of the incompatibility group P-7 essential for the degradation of carbazole (Urata et al. 2004), further supporting the hypothesis that these operons are subjected to horizontal transfer within the *Pseudomonas* genus.

The transcription of the *catBCA* operon is activated by CatR in *P. putida* (Rothmel et al. 1990). Since *P. savastanoi* pv. *savastanoi* NCPPB 3335 does not encode a *catR* homolog in its genome, transcriptional regulation of *catBCA* in this strain is intriguing. Although expression of the *catB* gene in this strain was higher when anthranilate, the substrate of the upper *antABC* operon, was present in the media, this increase was not observed in a mutant unable to convert anthranilate into catechol. Thus, anthranilate or catechol-related compounds may also act as inducers of the *catBCA* operon. Moreover, induction of the *catBCA* operon in *P. putida* by *cis,cis*-muconate, the product generated by the oxidation of catechol via CatA, has been shown to be mediated by CatR (McFall et al. 1998). Although both CatR and AntR belong to the LysR/XylS family of transcriptional regulators, their divergent sequences do not suggest that AntR may also be in charge of *catBCA* regulation. Codification of an orphan *benR* gene in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Fig. 1; Table 2) prompted us to analyze the possibility that BenR is involved in the activation of *catBCA*. However, BenR boxes (Silva-Rocha and de Lorenzo 2012) were not found upstream of this operon in *P. savastanoi* pv. *savastanoi* NCPPB 3335. Thus, the transcriptional activator of *catBCA* in *P. savastanoi* pv. *savastanoi* NCPPB 3335 and

Fig. 5. Fitness of *Pseudomonas savastanoi* NCPPB 3335 mutants affected in the WHOP region in planta. **A**, Competitive index (CI) assays between *P. savastanoi* pv. *savastanoi* NCPPB 3335 and each of the indicated mutants on nonwoody olive plants at 30 days postinoculation (dpi) and **B**, woody olive plants at 90 dpi. Error bars represent the standard deviation from the mean of three independent replicates. Asterisks represent CI values that were significantly different from 1.0, according to Student's *t* test ($P < 0.05$).

other *P. syringae* strains encoding the WHOP region remains unknown.

We confirmed the involvement of *antABC* and *catBCA* in the transformation of anthranilate and catechol, respectively, and detected oxygenase activity for the *ipoABC* operon. Exposure of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 Δ *catB* mutant to a medium containing catechol resulted in the accumulation of several unidentified compounds that were not detected in the supernatants of the Δ *catB* complemented strain. Since catechol is highly toxic to *P. savastanoi* (Capasso et al. 1995), this result suggests that *P. savastanoi* pv. *savastanoi* is able to detoxify catechol in the absence of the *catBCA* operon using alternative pathways. Catechol 2,3-dioxygenase, an enzyme that modifies catechol by opening the aromatic ring in the *meta* position (Parales et al. 1997), is widespread among plant-associated bacteria (Olapade and Ronk 2015), including an endophytic *Pseudomonas* strain (Pawlik and Piotrowska-Seget 2015). However, the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome does not encode this enzyme.

The contribution of *P. savastanoi* pv. *savastanoi* NCPPB 3335 to the virulence and fitness in planta was analyzed for each of the individual clusters encoded within the WHOP region, with the exception of the orphan *benR* gene. Regarding the virulence of the bacteria, the Δ *antA*, Δ *catB*, and Δ *ipoABC* mutants generated olive knots that were significantly smaller than those induced by the wild-type strain on woody plants but not on nonwoody plants. With respect to the Δ *antA* and Δ *catB* strains, several pathways for bacterial mineralization of the auxin indole 3-acetic acid (IAA) involving anthranilate and catechol have been proposed (Leveau and Gerards 2008). Additionally, IAA levels have been demonstrated to positively regulate knot size induced by *P. savastanoi* pv. *savastanoi* NCPPB 3335 on olive plants (Aragón et al. 2014). Although *P. savastanoi* synthesizes IAA from tryptophan by the action of the *iaaMH* operon, which follows the indol-3-acetamide pathway (Comai and Kosuge 1982; Kosuge et al. 1966), a secondary pathway for the biosynthesis of this phytohormone remains to be elucidated (Aragón et al. 2014). Conversely, anthranilate is an intermediate metabolite in the biosynthesis of tryptophan in bacteria (Radwanski and Last 1995). Considering that the *P. savastanoi* pv. *savastanoi* NCPPB 3335 Δ *antA* mutant is affected by its capacity to eliminate anthranilate (Fig. 2C), a disequilibrium of the tryptophan pool and, therefore, of IAA biosynthesis could occur in this strain. Furthermore, several *iac* locus genes, which are involved in the catabolism of IAA in *P. putida* 1290 (Leveau and Gerards 2008), showed relative similarity (approximately 30% amino acid identity) to genes located within the WHOP region, i.e., *antA* (*iacC*), *antB* (*iacD*), PSA3335_3210 (*iacE*), *antC* (*iacF*), and PSA3335_3208 (*iacG*).

Despite the virulence reduction observed in woody olive plants for the Δ *antA* mutant, its competitiveness in this tissue was similar to that of the wild-type strain (Fig. 5). These results suggest that degradation of anthranilate-related compounds is not required for growth and survival of *P. savastanoi* pv. *savastanoi* in woody olive tissues. A recently published study showed that *P. syringae* strains lacking the “catechol operon” were unable to grow endophytically in kiwifruit trees, whereas strains carrying this region could do so (Bartoli et al. 2015b). Similarly, the *P. savastanoi* pv. *savastanoi* NCPPB 3335 Δ *catBCA*, Δ *dhxAB*, and Δ 06 mutants were less competent than the wild-type strain on woody olive plants but not on micropropagated plants (Fig. 5), a finding that reinforces the hypothesis that the WHOP region contributes to the adaptation of bacteria from the *P. syringae* complex to woody niches. Wood is composed mainly of the polysaccharides cellulose and hemicellulose (65 to 75%) and lignin (18 to 35%) (Pettersen 1984). Lignin is a complex organic network synthesized from

three hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohols) (Boerjan et al. 2003; Ralph et al. 2004). It is well-known that lignin-related monomers are funneled into protocatechuate and catechol prior to degradation through the β -ketoadipate pathway, which is widely distributed in soil bacteria and fungi (Harwood and Parales 1996). While the catechol degradation machinery is restricted to several pathovars within the *P. syringae* complex (Table 1), the degradation of protocatechuate into Krebs cycle intermediaries is a feature that is widely distributed among *P. syringae* pathovars. Thus, WHOP-encoded activities may complement the protocatechuate catabolic pathway to allow for the degradation of additional lignin-related compounds in bacteria that colonize plant woody organs. For example, cinnamate, an unsubstituted lignin-related compound, is metabolized via catechol (Andreoni et al. 1991). Thus, the WHOP region could help bacteria utilize lignin precursor compounds as carbon and nitrogen sources. Another putative advantage conferred to bacteria by the WHOP-encoded activities could be the modification of these compounds to decrease their toxicity. In fact, catechol and guaiacol, the latter being a type of lignin subunit derived from coniferyl alcohol, are highly toxic to *P. savastanoi* (Capasso et al. 1995). Other published works support an association between lignin and catechol-degrading activities. For example, the amendment of a soil with wood sawdust favored the development of bacteria carrying the *catA* gene (catechol 1,2-dioxygenase) compared with a nonamended soil (Cébron et al. 2015). In addition, strains possessing high lignocellulose-degrading activity were able to partially mineralize catechol (Větrovský et al. 2014). Unlike the Δ *catBCA*, Δ *dhxAB*, and Δ 06 mutants, which are impaired in their fitness exclusively on woody olive plants, the *P. savastanoi* pv. *savastanoi* NCPPB 3335 Δ *ipoABC* operon mutant displayed a slower growth rate in both woody and nonwoody plants compared with the wild-type strain. This finding suggests that this impairment may not be directly related to lignin-related compounds. Phenolic compounds are secondary metabolites produced by plants upon pathogen attack (Poiatti et al. 2009). Therefore, perhaps the *ipoABC* operon is involved in the degradation of such defense compounds, which are produced by both nonwoody and woody plants, to decrease their toxicity. However, further work is needed to confirm this hypothesis.

In summary, we show that besides PG1 and PG3 strains infecting woody organs of woody hosts, a complete WHOP region is also encoded in *P. syringae* pv. *ciccaronei* and *P. syringae* pv. *actinidifoliorum*, which cause leaf spots in woody hosts. Partial conservation of the WHOP occurs in only a few PG2 strains. Furthermore, several clusters located within the WHOP region play a role in the adaptation of *P. savastanoi* pv. *savastanoi* NCPPB 3335 to woody hosts but not to nonwoody plants, supporting the role of the WHOP region in the adaptation of other *P. syringae* pathovars to fruit trees and other woody hosts. We confirmed the function of the *antABC* and the *catBCA* operons and identified an oxygenase activity for the *ipoABC* operon. However, further work is necessary to determine one or more environmental or plant-derived substrates used by the enzymes encoded in the WHOP region as well as to elucidate the activity of the *dhxAB* operon and the PSA3335_3206 gene.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions.

The bacterial strains used in this study are listed in Supplementary Table S1. *P. savastanoi* pv. *savastanoi* and *Escherichia coli* were grown at 28 and 37°C, respectively, in LB (Bertani 1951), super optimal broth (SOB) (Hanahan 1983), or M9 minimal salts medium with an additional carbon source (Sambrook and

Russell 2001). Solid and liquid media were amended with the appropriate antibiotic when required. The concentrations of antibiotics used were kanamycin (Km) at $10 \mu\text{g ml}^{-1}$ for *P. savastanoi* pv. *savastanoi* and $50 \mu\text{g ml}^{-1}$ for *E. coli*, gentamycin at $10 \mu\text{g ml}^{-1}$, ampicillin (Ap) at $400 \mu\text{g ml}^{-1}$ for *P. savastanoi* pv. *savastanoi* and $100 \mu\text{g ml}^{-1}$ for *E. coli*, and tetracycline at $10 \mu\text{g ml}^{-1}$.

Distribution of the WHOP region across the *Pseudomonas syringae* complex.

Genome sequences of 96 strains belonging to the *P. syringae* complex, 49 (51%) of which correspond to strains isolated from woody hosts, were downloaded from GenBank, and the presence of the WHOP region was analyzed by blastn, using Geneious software v7.1.13 and the nucleotide sequence of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 WHOP regions as template. Positive hits were determined using the “Grade” value, a percentage calculated by Geneious, combining the query coverage, e-value, and identity values for each hit with weights 0.5, 0.25, and 0.25, respectively. Genomes yielding a Grade value $\geq 90\%$ were considered to encode a complete WHOP region. Genomes yielding pairwise alignments with $>70\%$ identity to specific clusters were considered to encode a partial WHOP region.

Construction of bacterial strains and plasmids.

The plasmids and oligonucleotides used in this study are listed in Supplementary Tables S2 and S3. For the generation of *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutants, DNA fragments of approximately 1 kb, corresponding to the upstream and downstream flanking regions of the gene or operon to be deleted, were amplified in three independent rounds of PCR, using genomic DNA from NCPPB 3335 as a template and Expand high fidelity polymerase (Roche Applied Science, Mannheim, Germany). Restriction sites for *EcoRI* (for the plasmid pECP1), *BamHI* (for the plasmid pIMC), or *HindIII* (for plasmids pECP2, pECP3, and pECP4) were included in the primers as previously described (Matas et al. 2014). The resulting products, consisting of upstream and downstream flanking regions separated by one of the mentioned restriction sites, were cloned into pGEMT (Promega, Madison, WI, U.S.A.) and were sequenced to discard mutations. Next, the Km-resistance gene *npII* was extracted by enzyme restriction from pGEM-T-KmFRT-*EcoRI*, pGEM-T-KmFRT-*HindIII*, or pGEM-T-KmFRT-*BamHI* and was cloned into the vectors described above to yield pECP1-Km, pIMC-Km, pECP2-Km, pECP3-Km, and pECP4-Km, respectively. The Km-resistance cassette was then cloned in an inverted position with respect to the transcription orientation of each cluster. All plasmids generated for the construction of *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutants were suicide vectors in *P. savastanoi* pv. *savastanoi*. Plasmids were transferred to NCPPB 3335 by electroporation (Pérez-Martínez et al. 2007) and transformants were selected in LB-Km plates. Plasmids pECP1-Km, pIMC-Km, pECP2-Km, pECP3-Km, and pECP4-Km were used for the construction of $\Delta catB$, $\Delta antA$, $\Delta 06$, $\Delta ipoABC$, and $\Delta dhoAB$, respectively. To select the allelic interchange (a double recombination event) and to discard plasmid integration (a single recombination event), individual colonies were replicated onto LB-Ap plates and Ap-resistant colonies were discarded. Finally, Southern blot analyses were performed to confirm integration in the correct position in the genome.

Plasmids for the complementation of the mutants were generated as follows. The complete open reading frames of *catBCA*, *antABC*, *ipoABC*, and *dhoAB* operons and the PSA3335_3206 gene, together with their corresponding promoters and transcriptional terminator regions, were amplified by PCR using NCPPB 3335 genomic DNA as a template and Phusion DNA polymerase (Thermo Scientific, Waltham, MA, U.S.A.). Fragments

were then cloned into pBluescript SK II⁺ or pENTR/SD/D-TOPO (only for the *antABC* operon) (Invitrogen, Carlsbad, CA, U.S.A.). After sequencing to discard mutations, the fragments were subcloned into pBRR1MCS-5 and the correct orientation was selected by PCR, using primers hybridizing to the plasmid and the cloned fragment. Plasmids pBRR:*catBCA*, pBRR:*antABC*, pBRR:PSA3335_3206, pBRR:*ipoABC*, and pBRR:*dhoAB* were used for the complementation of $\Delta catB$, $\Delta antA$, $\Delta 06$, $\Delta ipoABC$, and $\Delta dhoAB$, respectively.

RNA extraction, RT-PCR and qRT-PCR.

For RT-PCR, *P. savastanoi* pv. *savastanoi* NCPPB 3335 cells were grown in LB to an optical density at 600 nm (OD_{600}) = 2, were pelleted, and were stored at -80°C . Total RNA was extracted using the TriPure isolation reagent (Roche Applied Science), according to the manufacturer's instructions, with the following exceptions: TriPure was preheated to 65°C , the lysis step was performed at 65°C and 1-bromo-3-chloropropane (Molecular Research Center, Cincinnati, OH, U.S.A.) was used instead of chloroform, as previously described (Matas et al. 2014). RNA concentration was determined spectrophotometrically, and its integrity was assessed by agarose gel electrophoresis. Total RNA was treated with a TURBO DNA-free kit (Applied Biosystems, Foster City, CA, U.S.A.), as detailed by the manufacturer's instructions. The samples were later tested for genomic contamination by PCR. DNA-free RNA was reverse transcribed using random hexamers included in the iScript cDNA synthesis kit (BioRad, Hercules, CA, U.S.A.). cDNA (50 ng) was used as a template to amplify intergenic regions by PCR, using *GoTaq* polymerase (Promega). PCR products were analyzed by 1% agarose gel electrophoresis.

For RT-qPCR assays, *P. savastanoi* pv. *savastanoi* and the $\Delta antA$ mutant were grown in LB to an OD_{600} = 0.5 and anthranilate was then added to a final concentration of 7.3 mM. After 4 h, bacteria were pelleted and were frozen in liquid nitrogen, and RNA was subsequently extracted. RNA extraction and cDNA synthesis were performed as described above. Bacteria grown in nonamended LB were used as a control. The primer efficiency tests, RT-qPCR, and confirmation of the specificity of the amplification reactions were performed as previously described (Vargas et al. 2011). The relative transcript abundance was calculated using the cycle threshold ($\Delta\Delta Ct$) method (Livak and Schmittgen 2001). Transcriptional data were normalized to the housekeeping gene *gyrA* and are presented as the fold change of expression in LB amended with anthranilate relative to expression in nonamended LB. The relative expression ratio was calculated as the difference in qPCR threshold cycles ($\Delta Ct = Ct \text{ gene of interest} - Ct_{gyrA}$). One PCR cycle represents a twofold difference in template abundance; therefore, fold-change values were calculated as $2^{-\Delta\Delta Ct}$, as previously described (Pfaffl 2001; Rotenberg et al. 2006).

Identification and quantification of aromatic compounds by HPLC.

The separation and identification of aromatic compounds of interest and related compounds were performed by HPLC, using an HP series 1050 chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array UV-Vis detector and a 5- μm C18RP column (Novapak C18, $150 \times 3.9 \text{ mm}$, Waters S.A., Barcelona, Spain). Compounds were identified by comparison of their respective UV absorption spectra and Rts with commercial pure standards (Sigma Aldrich, Saint Louis).

For the detection of anthranilate and catechol, samples were run for 20 min at a flow rate of 1 ml/min with the detector set at 210 and 230 nm. The eluents used were 0.1% *ortho*-phosphoric acid in deionized water and methanol in a gradient of concentrations, as follows: 5 to 25% methanol for 15 min, 25 to 5% methanol for 1 min, and a final step for column re-equilibration at 5% methanol for 5 min. Under these conditions, the Rt for

anthranilate and catechol were 9.72 and 7.61 min, respectively. For the functional analysis of the *antABC* operon, bacteria were grown overnight in LB, were washed twice in M9 medium, and were resuspended in M9 minimal medium amended with 5 mM succinate and 500 μ M M9A to an approximate final concentration of 10^9 CFU/ml. After 24 h of incubation at 28°C, cells were removed by centrifugation and the supernatant was filtered (pore diameter 0.22 μ m) and was analyzed by HPLC. For the functional analysis of the *catBCA* operon, the same procedure was followed, with the exception that 7.3 mM anthranilate was added to the overnight culture of LB (LBA) and 300 μ M catechol was added to the M9 minimal media (M9C) instead of anthranilate. As a control, cell-free media was subjected to the same procedure. For the quantification of anthranilate and catechol in the media, calibration curves with pure standards were performed, in which the concentration was compared with the area of each peak.

For the detection of 4-chlorocatechol, an isocratic protocol consisting of 30% methanol and 70% deionized water with 0.1% *ortho*-phosphoric acid was used. The flow rate was 1 ml/min for 15 min and the detector was set at 210 and 230 nm. The *Rt* of 4-chlorocatechol was 5.05 min. Samples were prepared as described above for the analysis of the *catBCA* function, with the exception that 300 μ M 4-chlorocatechol was added to the M9 minimal media instead of catechol.

For the detection of indigo, the HPLC protocol consisted of an isocratic method with a mixture of 90% acetonitrile and 10% deionized water and a flow rate of 1 ml/min. The running time was 5 min, and the detector was set to 290 nm. Cells were grown overnight in LB, were washed twice, were resuspended in M9 minimal media amended with 5 mM succinate and 500 μ M indole, and were incubated at 28°C until the color of the culture turned blue. When formed, the blue compound was partially purified by centrifugation of 2 ml of the culture. A blue precipitate formed along the inner side of the tube and was then grazed with a scraper, was solubilized in acetonitrile, and was transferred to a new tube without disrupting the cell pellet. The partially purified blue precipitate was filtered and was analyzed by HPLC.

Plant infection and isolation of bacteria from knots.

Olive plants were micropropagated, were rooted, and were maintained as previously described (Rodríguez-Moreno et al. 2008). Micropropagated (nonwoody) plants were infected in a stem wound with a bacterial suspension (equivalent to 5.10^3 CFU) and were incubated in a growth chamber for 30 days, as previously described (Rodríguez-Moreno et al. 2008). The morphology of the knots was observed with a stereoscopic microscope (Leica MZ FLIII; Leica Microsystems, Wetzlar, Germany). Bacteria were recovered from the knots, using a mortar and pestle containing sterile 10 mM $MgCl_2$. Serial dilutions were plated onto LB plates or LB supplemented with the corresponding antibiotic, and population sizes were determined from three different knots. A minimum of three representative knots were three-dimensionally scanned and knot size was determined using Neftabb Basic 5.2 software. Statistical analyses were performed using analysis of variance (ANOVA) tests ($\alpha = 0.05$).

The pathogenicity of *P. savastanoi* pv. *savastanoi* on one-year-old (woody) olive explants was also analyzed as previously described (Matas et al. 2012; Penyalver et al. 2006; Pérez-Martínez et al. 2007). Morphological changes scored at 90 days post-inoculation were captured on a high-resolution Canon D6200 camera (Canon Corporation, Tokyo). Knot volume was calculated from a minimum of three representative knots as previously described (Hosni et al. 2011; Moretti et al. 2008). Statistical analyses were performed by ANOVA using SigmaPlot 12.0.

Competitive assays between the wild-type and mutant strains were performed in both woody and nonwoody plants as

previously described (Matas et al. 2012; Rodríguez-Moreno et al. 2008). Bacteria were mixed at a 1:1 ratio and 5×10^3 cells were placed on each inoculation point.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Geneious software platform: <http://www.geneious.com>
MyHits web tool: <http://myhits.isb-sib.ch>