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Polyphenol Oxidase Activity Expression in *Ralstonia solanacearum*

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Sequencing of the genome of *Ralstonia solanacearum* revealed several genes that putatively code for polyphenol oxidases (PPOs). To study the actual expression of these genes, we looked for and detected all kinds of PPO activities, including laccase, cresolase, and catechol oxidase activities, in cellular extracts of this microorganism. The conditions for the PPO assays were optimized for the phenolic substrate, pH, and sodium dodecyl sulfate concentration used. It was demonstrated that three different PPOs are expressed. The genes coding for the enzymes were unambiguously correlated with the enzymatic activities detected by generation of null mutations in the genes by using insertional mutagenesis with a suicide plasmid and estimating the changes in the levels of enzymatic activities compared to the levels in the wild-type strain. The protein encoded by the RSp1530 locus is a multicopper protein with laccase activity. Two other genes, RSc0337 and RSc1501, code for nonblue copper proteins exhibiting homology to tyrosinases. The product of RSc0337 has strong tyrosine hydroxylase activity, and it has been shown that this enzyme is involved in melanin synthesis by *R. solanacearum*. The product of the RSc1501 gene is an enzyme that shows a clear preference for oxidation of *o*-diphenols. Preliminary characterization of the mutants obtained indicated that PPOs expressed by *R. solanacearum* may participate in resistance to phenolic compounds since the mutants exhibited higher sensitivity to L-tyrosine than the wild-type strain. These results suggest a possible role in the pathogenic process to avoid plant resistance mechanisms involving the participation of phenolic compounds.

Polyphenol oxidases (PPOs) are a group of copper enzymes that are able to catalyze the oxidation of aromatic compounds by oxygen (32). There are two main types of PPOs: laccases (EC 1.10.3.2) and tyrosinases (EC 1.14.18.1). Tyrosinases catalyze two kinds of reactions: *ortho* hydroxylation of monophenols (cresolase activity), such as L-tyrosine, yielding L-3,4-dihydroxyphenylalanine (L-DOPA); and the oxidation of this and other *o*-diphenols to *o*-quinones (catechol oxidase activity; EC 1.10.3.1). Laccases oxidize mainly *p*-diphenols and methoxy-substituted phenols, such as 2,6-dimethoxyphenol (DMP) (36).

In the bacterial kingdom there are few examples of PPOs that have been characterized at the molecular level, although genome sequencing has suggested that genes coding for these activities are widely distributed (1, 6). Laccase activity was described for the first time in *Azospirillum lipoferum* (13). Recently, laccase activity has been described in other microorganisms, such as *Marinomonas mediterranea* (34), *Bacillus subtilis* (23), and *Escherichia coli* (14). Bacterial tyrosinases were first described in *Streptomyces* in relation to melanin synthesis in this genus (16). Tyrosinase activity is also involved in melanization in *Sinorhizobium meliloti* (25) and *M. mediterranea* (19). At least in *Streptomyces* and *M. mediterranea*, the tyrosinase genes are present in an operon along with a second gene involved in copper transfer to the apotyrosinase (20, 37).

The melanogenic marine bacterium *M. mediterranea* was the first prokaryote that was found to express two different PPOs. One of these PPOs is a tyrosinase that is clearly involved in melanin synthesis, which is strongly activated in vitro by sodium dodecyl sulfate (SDS), and the other PPO is a membrane-bound laccase whose physiological role is still uncertain (30, 34). After this report, evidence was obtained for the coexistence of tyrosinases and laccases in some other genera. For instance, laccase activity has been described in *Streptomyces* (3, 11) and *S. meliloti* (5), whose tyrosinase activity has been described previously (16, 25). However, there are no data on the possible physiological advantage of expressing two PPOs that are so closely related in terms of substrate specificity.

In several microorganisms and particularly in fungi, the synthesis of melanins and the PPO activities have been related to pathogenesis (27). For bacterial pathogens of plants, a simple BLAST search revealed different genes that putatively encode PPOs. Moreover, many of the microorganisms described as microorganisms that actually express PPO activities are bacteria that interact with plants, such as *A. lipoferum*, *S. meliloti*, and the marine epiphyte *Microbulbifer degradans* (17, 33).

Plants use different pathways to defend against pathogens. The most powerful defense system for plants is the hypersensitive response. This is a highly concerted response that involves local cell death (programmed cell death), local accumulation of high levels of phenolic compounds, and cell wall reinforcement (35). In addition, plants produce a diverse array of secondary metabolites involved in resistance against microbial pathogens. Although there are defense compounds that are specific for selected plant families, some chemical compounds, such as phenylpropanoid derivatives, are often involved in these protective processes (10).

The existence of genes that putatively encode PPOs in bacteria interacting with plants in general and in microbial pathogens in particular suggested that there is a link between the capacities of microorganisms to express PPO activities and infectivity and prompted us to study whether these activities

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TABLE 1. Strains, plasmids, and PCR primers used in this study

Strain, plasmid, or primer	Description and/or relevant genotype[a]	Reference or source
R. solanacearum strains		
GMI1000	Wild-type strain	29
R3	GMI1000, spontaneously Rif^r	This study
R3-1501$^-$	R3::pCN15, Km^r	This study
R3-337$^-$	R3::pCN337, Km^r	This study
R3-1530$^-$	R3::pCN1530, Km^r	This study
E. coli strains		
DH5α		Invitrogen
S17-1 (λ*pir*)	Tp^r Sm^r, *recA thi hsdRM*$^+$, λ*pir* phage lysogen RP4::Mu::Km Tn*7*	9
Plasmids		
pFSV	Cm^r Tc^r *ori* R6K *mob* RP4	4
pFSVIN3	Cm^r *ori* R6K *mob* RP4 *ppo*A::mini-Tn*10* Gm^r	21
pFSVCN	*ori* R6K *mob* RP4 *'ppo*A*'* Gm^r	21
pFSVK	*ori* R6K *mob* RP4 *'ppo*A*'* Km^r	21
pBRI15	pBKSII + 180-bp BamHI internal fragment from RSc1501 gene	This study
pCN1501	pFSVK + 235-bp EcoRI-SacI fragment from pBRI15	This study
pCN337	pFSVK + 300-bp NcoI-SacI fragment from RSc0337 amplified by PCR	This study
pCN1530	pFSVK + 800-bp SacI-EcoRI fragment from RSp1530 amplified by PCR	This study
Primers		
F1501	GTTTTCGAATTCTGCCTGACG (EcoRI)	
FI337	TCGCCATGCCGTACTGGAAC (NcoI)	
FI1530	ACGGCGAGCTCGACACCCTG (SacI)	
R1501	GGTGGTGAGCTCCCAATGAAG (SacI)	
RI337	GCGTGAGCTCCAGGATGC (SacI)	
RI1530	ACGGTGAATTCGAACGCGTCG (EcoRI)	

[a] Engineered restriction sites are underlined.

are really expressed in the microorganisms. We chose *Ralstonia solanacearum* as an appropriate model, since this organism is a pathogenic bacterium that causes wilting and death of solanaceous plants, like potato and tomato, and whose genome has been sequenced (29). In this work we demonstrated that at least three such genes are actually expressed and the proteins exhibit PPO activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *R. solanacearum* was usually grown in basal saline medium (BSM) medium containing 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgCl_2$, 2 μM $FeSO_4$, 0.2 mM $CaCl_2$, 8 μM Na_2MoO_4, 5 μM $MnCl_2$, 0.5% glycerol, and 0.01% yeast extract in 50 mM sodium-potassium phosphate buffer (pH 7.0). An alternative medium used in some experiments was PCG, which contained (per liter) 10 g peptone, 1 g casein hydrolysate, and 5 g glucose. *E. coli* was routinely grown in Luria-Bertani medium. When necessary, media were supplemented with kanamycin, ampicillin, or rifampin at a concentration of 50 μg/ml.

Construction of *R. solanacearum* mutants whose PPO activities are affected. Strains with mutations in several genes that putatively code for PPO activities were generated by homologous recombination. First, the RSc1501 gene was amplified by PCR using the appropriate forward and reverse primers (F1501 and R1501, respectively) (Table 1). Genomic DNA of the wild-type *R. solanacearum* strain, isolated with a Wizard kit from Promega, was used as the template. Twenty-five cycles of PCR consisting of 95°C for 1 min, 58°C for 1 min, and extension at 72°C for 2.25 min were performed. The reaction mixture contained 1.5 μM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 0.7 μg of each primer, 100 ng of template DNA, and EcoTaq polymerase (Ecogen). As expected, a PCR product that was ca. 1.6 kb long containing the complete RSc1501 gene was amplified. This product was cleaved with BamHI to obtain a fragment between the coding regions for the two copper-binding sites and was ligated to pBlueScript KSII(+) with T4 DNA ligase (Invitrogen). The ligation mixture was transformed into *E. coli* DH5α, and transformants were selected for ampicillin resistance. The plasmid obtained (pBRI15) was digested with EcoRI and SacI, and the internal RSc1501 gene fragment was subcloned in the pFSVK plasmid. The resulting plasmid (pCN1501) was transformed into *E. coli* S17-1 (λ*pir*), and transformants were selected for kanamycin resistance. After this, the plasmid was mobilized into spontaneous Rif^r *R. solanacearum* R3 by conjugation. Briefly, conjugation was performed on the surface of an agar plate in PCG. Forty microliters of the exponentially growing recipient cells was spotted on the surface of the plate and allowed to dry before 40 μl of the donor *E. coli* was added to the spot. The plate was incubated for 36 h, and then cells were collected by scraping and suspended in 1 ml of PCG. Appropriate dilutions were plated on PCG containing rifampin and kanamycin (50 μg/ml) to counterselect *E. coli*.

Plasmid pFSVCN contains the R6K origin of replication that requires the Pir protein, and hence it is suicidal in bacteria that do not express this protein (4), such as *R. solanacearum*. The only possible way to obtain kanamycin-resistant transconjugants is through homologous recombination between the RSc1501 gene and the fragment of this gene cloned in the plasmid. In order to confirm that there was RSc1501 gene disruption in transconjugants, a PCR using genomic DNA from several transconjugants and from the wild type and the F1501 and R1501 primers was performed as previously described. The absence of a 1.6-kb PCR product in the transconjugants and the presence of this DNA fragment in the control wild-type strain confirmed that there was gene disruption (data not shown). Strain R3-1501$^-$ was selected for further assays.

Second, to obtain mutants affected in the RSc0337 gene, an internal fragment between the two copper-binding sites of this gene was amplified using the appropriate forward and reverse primers, primers FI337 and RI337 (Table 1). PCR was performed under the conditions described above for the RSc1501 gene, except that the reaction mixture contained 2 μM $MgCl_2$ and 1 μg of each primer. Twenty-five PCR cycles consisting of 95°C for 1 min, 56°C for 1 min, and 72°C for 30 s were performed. A 300-bp PCR product was amplified. Then it was cloned in the pFSVK plasmid using the NcoI and SacI restriction sites. The resulting plasmid, pCN337, was transformed into *E. coli* and mobilized into spontaneous rifampin-resistant *R. solanacearum* strain R3 as described above for the RSc1501 gene. To confirm that there was RSc0337 disruption, a PCR was performed using primers FI337 and RevKm. This last primer hybridized to the kanamycin resistance gene present in the pFSVK plasmid. In transconjugants with mutations in the RSc0337 gene, an 800-bp fragment that was not present in the control wild-type strain was detected. Strain R3-337$^-$ was selected.

Finally, to obtain mutants affected in the RSp1530 gene, a PCR using primers FI1530 and RI1530 (Table 1) was performed to amplify an approximately 800-bp internal fragment of the RSp1530 gene between copper-binding sites 2 and 3. Twenty-five PCR cycles consisting of 95°C for 1 min, 54°C for 1 min, and extension at 72°C for 3 min were performed using Pyrobest polymerase from Takara. The reaction mixture contained 5% dimethyl sulfoxide, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 μg of each primer, and 100 ng of template DNA. The amplified internal fragment of RSp1530 was cloned in plasmid pFSVK as previously described, and the resulting plasmid, designated pCN1530, was mobilized into *R. solanacearum* R3 as described above for creation of the R3-1501$^-$ and R3-337$^-$ mutants. To confirm that there was disruption of the RSp1530 gene, a PCR with FI1530 and RevKm was carried out, which resulted in a 1.2-kb PCR product in positive transconjugants that was not detected in the control wild-type strain (data not shown). Strain R3-1530$^-$ was selected to complete the set of mutants with mutations in different putative PPO genes.

Enzymatic determinations. To obtain bacterial cellular extracts, cells were grown in BSM for 48 h and centrifuged at 5,000 × *g* for 10 min. The pellet was washed with a 0.9% NaCl solution, resuspended in 1 ml of 0.1 M sodium phosphate (pH 7.0) plus 0.1 mM phenylmethylsulfonyl fluoride and a 1/500 dilution of the Protease inhibitor cocktail (Sigma Chemicals), and disrupted by sonication with a Braun Labsonic U sonicator (4-min treatments at a relative output power of 0.5 with 0.7 duty period). The homogenate was centrifuged at 12,000 × *g* for 4 min, and the supernatant was used for enzymatic activity determinations as previously described (32). Tyrosine hydroxylase (TH) and DOPA oxidase (DO) activities were determined by monitoring the oxidation of 2 mM L-tyrosine and L-DOPA, respectively, to dopachrome at 475 nm (ε = 3,700 M^{-1} cm^{-1}) in 0.1 M phosphate buffer adjusted to pH 5.0 for the TH determination and to pH 7.0 for the DO determination. For the TH activity standard assay 0.05% SDS was added, while for the DO activity assay 0.02% SDS was added. Dimethoxyphenol oxidase (DMPO) activity was determined by monitoring the oxidation of 2 mM DMP to 3,3′,5,5′-tetramethoxydiphenylquinone at 468 nm (ε = 14,800 M^{-1} cm^{-1}) in 0.1 M phosphate buffer (pH 5.0). In this assay 0.05% SDS was also added. In all cases, 1 U was defined as the amount of enzyme that catalyzed the appearance of 1 μmol of product per min at 37°C. Specific activities were normalized to milligrams of protein, measured using the bicinchoninic acid assay.

Ascorbate oxidase activity was determined by the decrease in absorbance at

TABLE 2. Comparison of *R. solanacearum* PPOs with representative proteins with the highest levels of identity

Similar protein			*R. solanacearum* protein[a]	
Organism	Accession no.	No. of amino acids	Score	E value
			RSp1530	
Xylella fastidiosa	NP_299954	721	636	0.0
Agrobacterium vitis	AAQ08598	716	582	1×10^{-164}
Rhizobium etli	AAM55015	669	553	1×10^{-156}
Burkholderia fungorum	ZP_00281779	749	320	1×10^{-85}
Microbulbifer degradans	ZP_00318139	569	266	1×10^{-69}
Marinomonas mediterranea	AAF75831	675	235	1×10^{-60}
			RSc0337	
Rhizobium etli	NP_659960	609	306	1×10^{-81}
Chromobacterium violaceum	NP_902932	296	128	4×10^{-28}
Marinomonas mediterranea	AAV49996	488	115	3×10^{-24}
Lycopersium esculentum	CAA78296	596	116	1×10^{-24}
Solanum tuberosum	T07097	599	115	3×10^{-24}
Vicia faba	CAA7764	606	114	8×10^{-24}
			RSc1501	
Rudrobacter xylanophilus	ZP_00186219.2	279	108	3×10^{-22}
Streptomyces avermitilis	NP_822312	274	105	2×10^{-21}
Streptomyces antibioticus	B23971	273	103	1×10^{-20}
Streptomyces glaucescens	A24089	274	103	1×10^{-20}
Nostoc punctiforme	ZP_B23971	406	104	4×10^{-21}
Caenorhabditis elegans	AAB52481	601	98.6	3×10^{-19}

[a] The RSp1530 protein contains 717 amino acids, and its accession number is NP_523089. The RSc0337 protein contains 496 amino acids, and its accession number is NP_518458. The RSc1501 protein contains 412 amino acids, and its accession number is NP_519622.

245 nm by measuring the oxidation of 0.1 mM L-ascorbate to dehydroascorbate (8). A 10 mM L-ascorbic acid stock solution was prepared in 100 ml of 1 mM HCl supplemented with 1 mM EDTA. The 10 mM L-ascorbic acid stock solution was diluted to obtain 0.1 mM L-ascorbic acid with dilution buffer consisting of 0.1 M NaH_2PO_4 and 1 mM EDTA. Then 0.1 ml of *R. solanacearum* cell extract was added to a cuvette containing 0.9 ml of 0.1 mM L-ascorbic acid. The reference cuvettes contained only 1 ml of dilution buffer without ascorbic acid.

Specific activity staining of the gels. Nondissociating SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Hames (15), using acrylamide concentrations of 9% for the separating gel and 3% for the stacking gel. The resolving buffer was Tris-HCl (pH 8.8), and the reservoir buffer was Tris-glycine (pH 8.3); both of these buffers contained 0.1% SDS. Samples were mixed at a 2:1 (vol/vol) ratio with sample buffer (0.18 M Tris-HCl [pH 6.8], 15% glycerol, 0.075% bromophenol blue, 9% SDS). Electrophoresis was performed at 20°C and at constant currents of 15 mA for 20 min and 30 mA for about 90 min.

To perform specific activity staining of the gels after electrophoresis, the slabs were equilibrated by immersion for 5 min in 0.1 M phosphate buffer at pH 5 (TH and DMPO) or pH 7 (DO). TH and DO specific activity staining was performed by incubating the gels at 37°C in 2.5 mM L-tyrosine or L-DOPA plus 2.5 mM 3-methyl-2-benzothizolinone hydrazone in 0.1 M phosphate buffer (pH 5 for TH and pH 7 for DO) for about 1 h (32); 0.05% SDS and 0.02% SDS were also added to the staining mixtures for TH and DO activities, respectively. The DMPO activity staining procedure was identical to the TH staining procedure except that the substrate was 2.5 mM 2,6-dimethoxyphenol instead of L-tyrosine and 3-methyl-2-benzothizolinone hydrazone was not added. After staining, the gels were dried at 60°C in a gel dryer.

Melanin synthesis assay. *R. solanacearum* strain R3 and PPO-disrupted mutants of this strain (R3-1501$^-$, R3-337$^-$, and R3-1530$^-$) were grown for 48 h in BSM. Eight milliliters of a stationary-phase culture was centrifuged at $5,000 \times g$ for 10 min, and the cells were suspended in 10 ml of 0.9 M NaCl and incubated overnight at 30°C. Tyrosine (5 mM) was added to the cell suspensions, which were incubated further, as described above, until pigmentation was observed.

RESULTS

Detection of genes coding for putative PPOs in *R. solanacearum*. A preliminary BLAST search (2) detected up to four genes that putatively code for PPOs in the genome of *R. solanacearum*. Two genes, RSp1530 and RSp0656, putatively code for multicopper oxidases. The RSp1530 product was in fact annotated as ascorbate oxidase (29). A BLAST search using the hypothetical protein encoded by this gene detected several proteins with scores higher than 200 (Table 2). Interestingly, some of these proteins are from other plant pathogens, such as *Xylella fastidiosa*, *Agrobacterium vitis*, and *Burkholderia fungorum*, or from bacteria that interact with plants, such as the epiphyte *Microbulbifer degradans* and the symbiont *Rhizobium etli*. Another protein with a high score is PpoA from the marine bacterium *M. mediterranea* (31). All these proteins were aligned (7), and it was observed that except for the hypothetical protein from *A. vitis*, all of them showed good conservation in the characteristic four copper-binding domains of multicopper proteins (data not shown). Among these microorganisms, laccase activity has previously been described for *M. mediterranea* (32) and *M. degradans* (33). The scores obtained for other bacterial multicopper proteins with demonstrated laccase activity were very low; for instance, the score was 53 and the E value was 10^{-5} for *E. coli* CueO (accession number NP_752102), and the score was 44.3 and the E value was 0.011 for *B. subtilis* CotA (accession number NP_388511).

The second gene putatively coding for a multicopper protein, RSp0656, is present in a typical operon containing genes involved in copper resistance, and accordingly it was annotated as CopA (29). This copper resistance operon is well known in a variety of bacteria, and it is strongly induced by copper (24). However, in our preliminary experiments we did not detect any induction of laccase activity by copper addition to the culture medium (data not shown). This result indicates that either the *R. solanacearum* RSp0656 gene is not expressed or the protein does not show laccase activity with the phenolic substrates and

under the conditions used in this work; hence, RSp0656 was not considered further in this study, which focused on PPO activities.

Apart from the multicopper proteins mentioned above, in the preliminary BLAST search for PPOs two other genes in the bacterial chromosome whose products show homology to tyrosinases were detected. These gene products were annotated as catechol oxidase (encoded by RSc0337) and tyrosinase (encoded by RSc1501) (29). The typical signatures of six histidine residues involved in the binding of a pair of copper ions to tyrosinases (12) are present in both predicted proteins. When a BLAST search was performed using both sequences, two different sets of proteins exhibiting the highest levels of similarity were detected.

For the protein encoded by RSc0337, the highest scores were obtained with three bacterial proteins from *R. etli*, *M. mediterranea*, and *Chromobacterium violaceum* and several plant catechol oxidases (Table 2). The protein from *C. violaceum* appeared to be divided and to be encoded by two different, consecutive open reading frames (CV3262 and CV3263). In order to construct the alignment, we considered this protein a single protein with 464 amino acids. All the sequences contained the two copper-binding sites with absolute conservation of the six histidines directly involved in copper binding (data not shown). The first *Streptomyces* tyrosinase detected in this BLAST search was from *Streptomyces tanashiensis* (accession number BAB20029); its score was lower (84), and the E value was 10^{-14}.

A BLAST search using the RSc1501 protein showed that the closest homologues of this protein were several tyrosinases from actinobacteria, particularly members of the genus *Streptomyces*, and a hypothetical protein from the cyanobacterium *Nostoc punctiforme*. A similar score was obtained for a tyrosinase present in *Caenorhabditis elegans* (Table 2). All of these proteins also contained the two copper-binding sites with conservation of the six histidines (data not shown). In some *Streptomyces* species (for example, *Streptomyces avermitilis*), two genes coding for tyrosinases have been detected (28); the score obtained for the second tyrosinase in this microorganism (accession number NP_826539) was 84.7, and the E value was 4×10^{-15}. The highest-scoring plant tyrosinase detected was the tyrosinase from *Vicia faba* (accession number CAA7764), with a score of 68.9 and an E value of 2×10^{-10}; these values are much lower than the values obtained in a comparison with the RSc0337 protein (Table 2). Finally, a comparison of the two *R. solanacearum* proteins resulted in a score of 56.6 and an E value of 2×10^{-6}, showing that these two proteins from the same microorganism are less similar to each other that to other proteins of plants and animals.

Generation of mutants with mutations in genes putatively coding for PPO and enzymatic determination. Preliminary experiments revealed that *R. solanacearum* showed all types of PPO activities, namely, laccase, cresolase, and catechol oxidase activities, represented by DMPO, TH, and DO (32). In order to correlate these activities with the products of the genes detected, we constructed strains with mutations in the three genes of interest using the strategy described in Materials and Methods. The PPO activities in cellular extracts of the strains generated were determined and compared to those of the wild-type strain. The conditions for the PPO enzymatic assay were optimized. In short, two parameters were found to be very important: pH and SDS concentration. Accordingly, all the PPO activities were measured at pH 5 and 7 and at different SDS concentrations.

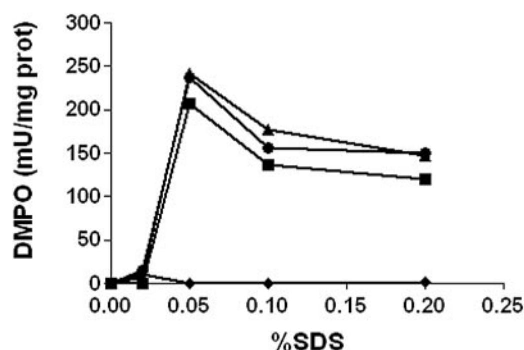

FIG. 1. DMPO activity in extracts of different *R. solanacearum* strains, determined at pH 5 and with different SDS concentrations. ●, R3; ▲, R3-337⁻; ■, R3-1501⁻; ◆, R3-1530⁻.

In the assay for DMPO activity the optimum pH was 5. This activity was strongly activated by SDS, and a peak was observed at an SDS concentration of 0.05% (Fig. 1). Mutant R3-1530⁻ exhibited no DMPO activity in these conditions, indicating that the protein encoded by the RSp1530 locus is the only protein responsible for the activity observed under optimal standard assay conditions (pH 5 and 0.05% SDS). However, there was some residual activity at an SDS concentration of 0.02% (Fig. 1). SDS gels were stained for DMPO activity, which revealed a band with strong activity for DMP that was not present in mutant strain R3-1530⁻ (Fig. 2). This result confirmed that the DMPO activity of the protein is encoded by the RSp1530 gene. Since this protein was annotated as ascorbate oxidase, we also tried to detect this enzymatic activity. However, in spite of numerous efforts, we were unable to observe it in any of the *R. solanacearum* strains used in this study (data not shown).

Determination of the TH activity revealed that it was higher at pH 5 and SDS concentrations of at least 0.05%. In these conditions the activity is due to the protein encoded by the RSc0337 locus, since the strain with a mutation in this gene

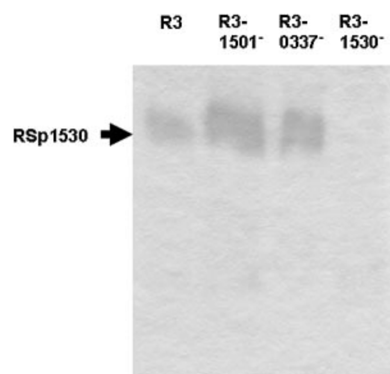

FIG. 2. Electrophoretic analysis of the DMPO activity in different *R. solanacearum* strains. PAGE was performed under nondissociating conditions, and the gel was stained for laccase activity. The arrow indicates the activity due to the protein encoded by the RSp1530 gene.

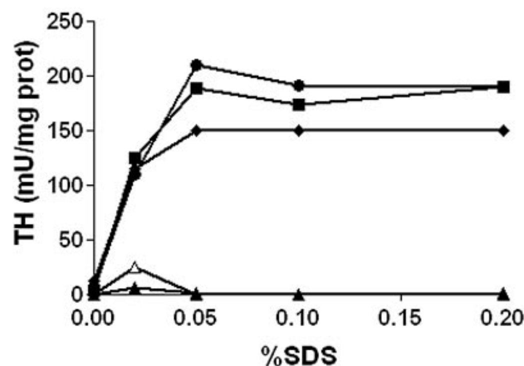

FIG. 3. TH activity in extracts of different *R. solanacearum* strains, determined at different SDS concentrations. ●, R3; ▲ and △, R3-337⁻; ■, R3-1501⁻; ◆, R3-1530⁻. The solid symbols indicate activities measured at pH 5; the open symbols indicate activities measured at pH 7.

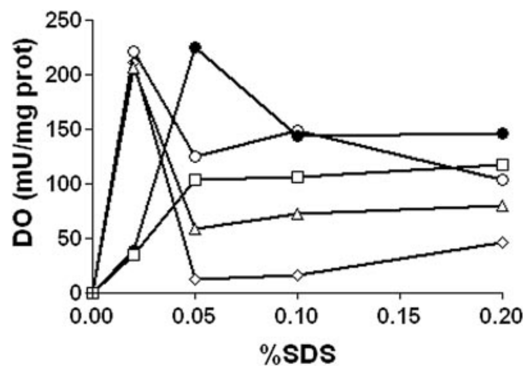

FIG. 5. DO activity in extracts of different *R. solanacearum* strains, determined at different SDS concentrations. ● and ○, R3; △, R3-337⁻; □, R3-1501⁻; ◇, R3-1530⁻. The solid symbols indicate activities measured at pH 5; the open symbols indicate activities measured at pH 7.

completely lacked the activity (Fig. 3). However, it was clearly observed that in this mutant there was some residual activity on this substrate and that the optimum activity occurred at pH 7 and 0.02% SDS. SDS-PAGE and specific staining with L-tyrosine allowed us to correlate the band due to the protein encoded by RSc0337 with TH activity. As expected, this band was not observed for the mutant with a mutation in this gene (Fig. 4).

For the oxidation of L-DOPA the optimal SDS concentrations were different depending on the pH of the assay mixture. On the one hand, at pH 7 oxidation showed a very abrupt peak at 0.02% SDS (Fig. 5). This peak was not observed in mutant strain R3-1501⁻, suggesting that the protein encoded by the RSc1501 locus is responsible for the activity in these conditions. On the other hand, at pH 5 the optimal SDS concentration for L-DOPA oxidation was 0.05% or higher. Under these conditions, the oxidation seemed to be the result of the residual DO activities encoded by both RSp1530 and RSc0337, since strains with mutations in any of these genes exhibited significant decreases in activity (data not shown). This result was confirmed by SDS-PAGE and staining for L-DOPA activity; in these experiments the two DOPA-staining bands corresponding to these enzymes were observed (Fig. 6). In spite of numerous efforts involving different substrates, pH conditions, and SDS concentrations, we were unable to detect in these gels the band corresponding to the RSc1501 locus. This surely reflected the instability of the protein encoded by this locus under the electrophoretic conditions used. This protein was strongly activated by 0.02% SDS, but exposure to this detergent for the periods needed to perform the SDS-PAGE seemed to inactivate it.

Preliminary characterization of the PPO mutants. The growth of strain R3 and mutants of this strain and their PPO activities were studied in different conditions. It was observed that the growth rate of none of these strains in standard BSM or PCG was affected (data not shown). Later, the effects of L-tyrosine supplementation on the growth and pigmentation of the mutant strains were studied. It was observed that in PCG the addition of 5 mM L-tyrosine did not affect the growth of strain R3, but it resulted in significant inhibition of the growth of the different mutants, especially the mutants affected in tyrosinase-like activities (Fig. 7). Under these conditions all the cultures acquired a reddish pigmentation, suggesting that there was degradation of L-tyrosine through the *p*-hydroxyphenylpyruvate catabolic pathway. In fact, in the *R. solanacearum* genome several genes putatively coding for *p*-hydroxyphenylpyruvate dioxygenase have been detected (29).

For strain R3 the addition of L-tyrosine to PCG did not

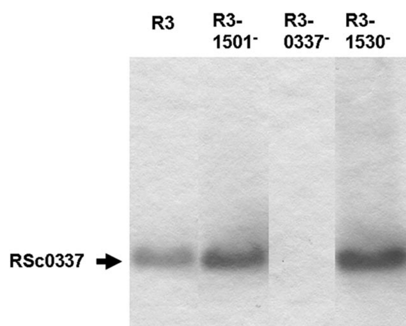

FIG. 4. Electrophoretic analysis of the TH activity in different *R. solanacearum* strains. PAGE was performed under nondissociating conditions, and the gel was stained for TH activity. The arrow indicates the activity due to the protein encoded by the RSc0337 gene.

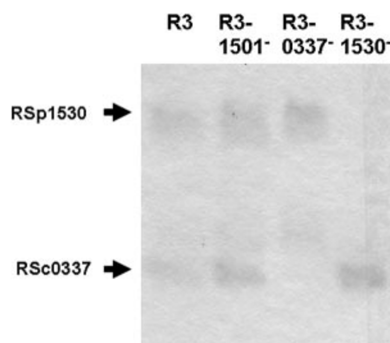

FIG. 6. Electrophoretic analysis of the DO activity in different *R. solanacearum* strains. PAGE was performed under nondissociating conditions, and the gel was stained for DO activity. The lower arrow indicates the activity due to the protein encoded by the RSc0337 gene. The upper arrow indicates the activity due to the protein encoded by the RSp1530 gene.

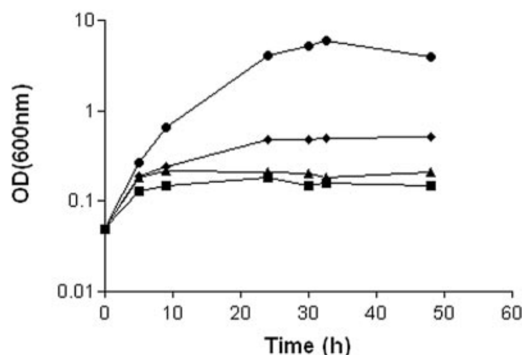

FIG. 7. Growth curves for different *R. solanacearum* strains in PCG supplemented with 5 mM L-tyrosine. ●, R3; ▲, R3-337$^-$; ■, R3-1501$^-$; ◆, R3-1530$^-$. OD (600 nm), optical density at 600 nm.

significantly affect the level of PPO activities (data not shown). However, even in these conditions, in which there was high precursor availability, melanins were not visibly generated. This could have been the result of the competition for L-tyrosine between use in the catabolic pathway and hydroxylation by the TH activity leading to melanin. To further explore the capacity to synthesize melanins, the different strains were incubated overnight in media lacking any other nutrient and then supplemented with L-tyrosine. Under these conditions it was observed that the wild type and most of the mutant strains were able to synthesize melanins; the only exception was mutant R3-337$^-$ (Fig. 8), whose TH activity was affected, which is the key activity for oxidizing L-tyrosine to melanin.

DISCUSSION

Until now, there have been few examples of bacteria that express more than one PPO activity. In this work we observed that in *R. solanacearum* three different genes actually code for PPO activities. Laccase activity, detected as DMPO activity, was detected in cellular extracts of *R. solanacearum*. This activity is maximal at pH 5 and 0.05% SDS. It is catalyzed by the multicopper protein encoded by the RSp1530 locus, as revealed by the fact that strains with mutations in this gene completely lack the laccase activity (Fig. 1 and 2). Although this protein was annotated preliminarily as ascorbate oxidase because of its similarity to other ascorbate oxidases, we were unable to detect the activity in *R. solanacearum*, in spite of the fact that we tried different conditions. Thus, we concluded that the multicopper protein encoded by the RSp1530 gene exhibits PPO activity rather than ascorbate oxidase activity. The proteins exhibiting the highest levels of similarity to the RSp1530 product are hypothetical proteins, with the exception of the protein expressed by *M. mediterranea*, whose laccase activity has been described previously (31). A laccase activity very similar to that of *M. mediterranea* was also detected in extracts of *M. degradans* (33), and it seems reasonable to assume that it is due to the protein detected in this microorganism by a BLAST search (Table 2). The capacity to exhibit laccase activity is also present in other prokaryotic proteins that have a low score compared to the *R. solanacearum* RSp1530 protein, such as CueO from *E. coli* or CotA from *B. subtilis*, whose physiological roles are known (14, 23). Apart from its enzymatic activity, the results obtained in this study suggest that the RSp1530 protein may be involved in resistance to phenolic compounds. Complete characterization of the natural substrate and the physiological role of the RSp1530 protein and the proteins similar to it would require further studies to determine if all of them may play similar roles in microorganisms.

For tyrosinase-like activities in *R. solanacearum*, we found that two different genes coding for proteins that have the typical copper-binding sites of this kind of PPO are actually expressed. The RSc0337 locus codes for an enzyme exhibiting high TH activity under optimum assay conditions (pH 5 and 0.05% SDS). The RSc1501 locus codes for a PPO that exhibits optimal activity at pH 7 and 0.02% SDS. It oxidizes L-DOPA, although it also exhibits low activities with DMP and L-tyrosine, as revealed by the residual DMPO and TH activities detected in mutants R3-1530$^-$ and R3-337$^-$, respectively. Of the three PPO activities detected and described here, this is the only activity that we were unable to detect after SDS-PAGE. This enzyme produces a strong activation peak with 0.02% SDS, but it is very sensitive to concentrations above this value (Fig. 6), suggesting that the failure to stain the enzyme after electrophoresis is due to inactivation caused by the high level of sensitivity of the RSc1501 protein to SDS exposure.

Interestingly, a BLAST search performed with both tyrosinase-like proteins showed that the closest homologues are members of a different group of proteins. Proteins similar to the RSc0337 protein are detected in some gram-negative bacteria that are able to synthesize melanins, such as *R. etli* (26) and *M. mediterranea* (32). At least in *M. mediterranea* the participation of the tyrosinase in melanin synthesis has been clearly demonstrated (20). In this study it was found that although *R. solanacearum* is not a melanogenic bacterium in standard growth conditions, it is able to synthesize melanins in the presence of L-tyrosine when it is deprived of other nutrients, such as phosphorus and a sulfur source, and that the protein encoded by the RSc0337 locus is the enzyme that is most directly involved in melanin synthesis since mutants with mutations in this gene are amelanogenic even in the conditions described above (Fig. 8). For the genes similar to RSc1501, the closest homologues include the genes coding for tyrosinase in different *Streptomyces* species. Although in *Streptomyces* the tyrosinases encoded by these genes are involved in melanin synthesis, we have not found any evidence of the participation of the product of the *R. solanacearum* RSc1501 gene in melanin synthesis. Genome sequencing of *S. avermitilis* has revealed

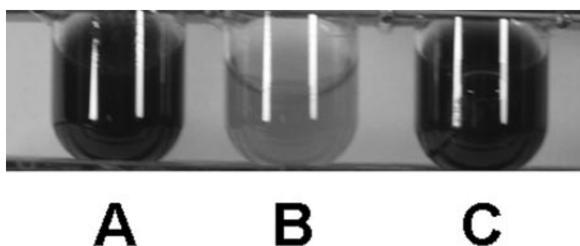

FIG. 8. Melanin synthesis by strains R3 (A), R3-337$^-$ (B), and R3-1501$^-$ (C) incubated in a saline solution with 5 mM L-tyrosine. The result for strain R3-1530$^-$ was identical to the results shown for strains R3 and R3-1501$^-$.

the presence of two operons with genes coding for tyrosinases, but it was suggested that one of the tyrosinase genes is not expressed or exhibits a very low level of transcription (28). In contrast, we found that in *R. solanacearum* both genes are expressed, resulting in active enzymes. In principle, it is unclear what kind of physiological advantage the expression of two proteins that are so similar provides to the bacteria. One attractive possibility is that the two proteins interact during the process of melanin synthesis. The RSc0337 gene product could catalyze the rate-limiting step, monophenol hydroxylation, and the RSc1501 product could catalyze the following step, oxidation of the *o*-diphenol to *o*-quinone. A similar complex has been described in mammals for tyrosinase and tyrosinase-related protein 1, a catechol oxidase-like protein (18). On the other hand, the possibility that RSc1501 plays a role that is not related to melanin synthesis cannot be ruled out.

From an evolutionary perspective it is interesting to speculate that if the presence of two genes coding for tyrosinases in *R. solanacearum* is the result of duplication, the duplication could have occurred very early. The two genes present in this microorganism are less similar to each other that they are to plant homologues (in the case of the protein encoded by the RSc0337 locus) or to animal homologues (for the RSc1501 locus). The last tyrosinase is more similar to both copies of the enzyme present in *Streptomyces*. This observation indicates that there is similarity between *R. solanacearum* and some *Streptomyces* strains with regard to the presence of two tyrosinases, but that the evolutionary origins of the two copies may be different in these bacteria. Establishment of a relationship of the genes in terms of orthology and paralogy must await a more comprehensive analysis.

The strains with mutations in the different genes coding for the PPOs are not affected in terms of growth in the standard media assayed, suggesting that these activities could be related to secondary metabolism, as they are in other bacteria that have been studied (22). The results obtained after L-tyrosine addition to the culture media revealed that in contrast to the wild-type strain, the addition of large amounts of this amino acid resulted in inhibition of growth in all the mutants assayed. These results suggest that, at least in vitro, the expression of PPOs in *R. solanacearum* plays a role in the defense against phenolic compounds, with a detoxification mechanism involving all three enzymes. In future experiments we will examine whether the pathogenicity in plants of these mutants is also affected and the possible role of the expression of PPO activities and melanin synthesis in this process.

Bacterial PPOs, particularly laccases, have received a lot of attention lately because of their possible biotechnological applications (3, 6). Until now, in spite of the fact that genes coding for PPOs have been detected in the genomes of several microorganisms (1), there have been few examples of bacteria in which the activity has actually been demonstrated. In this regard, this study showed for the first time that *R. solanacearum* expresses three different PPOs, and experiments are under way to purify and fully characterize these enzymes in order to evaluate their possible biotechnological applications when they are either isolated or mixed to amplify the range of phenol oxidation.

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