Xanthomonas oryzae pv. *oryzae* XKK.12 Contains an AroQ_γ Chorismate Mutase That Is Involved in Rice Virulence

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ABSTRACT

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Chorismate mutase (CM) is a key enzyme in the shikimate pathway which is responsible for the synthesis of aromatic amino acids. There are two classes of CMs, AroQ and AroH, and several pathogenic bacteria have been reported to possess a subgroup of CMs designated AroQ_γ.

These CMs are usually exported to the periplasm or outside the cell; in a few cases, they have been reported to be involved in virulence and their precise role is currently unknown. Here, we report that the important rice pathogen *Xanthomonas oryzae* pv. *oryzae* XKK.12 produces an AroQ_γ CM which we have purified and characterized from spent supernatants. This enzyme is synthesized in planta and *X. oryzae* pv. *oryzae* knock-out mutants are hypervirulent to rice. The role of this enzyme in *X. oryzae* pv. *oryzae* rice virulence is discussed.

Xanthomonas oryzae pv. *oryzae* is a gram-negative bacteria and is the causal agent of rice bacterial leaf blight (BLB), a vascular disease in which *X. oryzae* pv. *oryzae* grows and colonizes the xylem vessels, eventually clogging them. BLB is the second most important rice disease and, consequently, is responsible for heavy loss in rice cultivation (1,18). Genetic and molecular studies have identified and elucidated the role of some determinants involved in plant-bacteria interaction, including type III effectors (34) and hypersensitivity response and pathogenicity (*hrp*) genes (7). In addition, a few cell-wall-degrading enzymes which are secreted have also been implicated in virulence (14). Importantly, several *X. oryzae* pv. *oryzae* genomes have recently been sequenced and annotated, providing insight into possible virulence mechanisms (19,20,25).

The sequence and annotation of the *X. oryzae* pv. *oryzae* genomes has revealed the presence of two chorismate mutase (CM) (EC 5.4.99.5) genes or enzymes. CM is a key enzyme in the shikimate pathway responsible for the synthesis of aromatic amino acids (13). Interestingly, the shikimate pathway is present in bacteria, fungi, algae, and plants but not in animals (13). There are two classes of CMs, AroQ and AroH, which are structurally different but possess a similar active site (for CM classification, see Calhoun et al. [6]). The nomenclature of the AroQ family was recently adjusted to four subclasses—AroQ_α, AroQ_β, AroQ_γ (previously designated *AroQ), and AroQ_δ—on the basis of their primary and other structural features (21). Members of the AroQ_γ subclass consist of two regions, each composed of three α-helices, and were initially found in some pathogenic bacteria such as *Erwinia herbicola* (36), *Salmonella typhimurium*, *Pseudomonas aeruginosa* (6), and *Mycobacterium tuberculosis* (17,27), and are either translocated to the periplasm or secreted (6,27,36).

In *Mycobacterium* spp., it has been proposed that the AroQ_γ CMs may correlate with pathogenicity because it is part of a three-gene operon where two genes have been shown to be implicated in virulence (27). In fact, AroQ_γ is also being considered as a target for new antimicrobial drugs because AroQ_γ is not present in mammals (13,24). The presence of AroQ_γ has also been reported in the root-knot nematodes obligate plant parasites *Meloidogyne javanica* (10), in the potato cyst nematode *Globodera pallida* (15), and in the beet cyst nematode *Heterodera schachtii* (32).

Two important questions which have arisen concerning the AroQ_γ CMs studied so far are (i) why locate a CM in the periplasm or outside the cell if the shikimate pathway is in the cytoplasm (13) and (ii) why is CM also present in nematodes if animals are believed to lack the shikimate pathway and to take up essential amino acids from their diet (24,28)? The role of the AroQ_γ CMs in pathogenic bacteria remains unclear, although current evidence has demonstrated that they strongly correlate with pathogenicity because (i) they are found almost only exclusively in pathogenic bacteria and nematodes (27), (ii) some AroQ_γ CMs are located in pathogenicity islands (31), and (iii) transgenic soybean expressing AroQ_γ in plant roots results in an altered cell development (10). Possible roles of AroQ_γ in pathogenicity and host-pathogen interaction have been suggested. In plant-parasitic nematodes, secreted CMs are responsible for remarkable changes in the hosts that could allow a continuous supply of nutrients for the invading pathogen (15). Moreover, it is known that salicylic acid is an important signal molecule in plant defense synthesized from chorismate via isochorismate through a pathway similar to that found in bacteria (30,35). The introduction of CM into plants may possibly interfere with this pathway and limit the availability of chorismate that can be converted to salicylic acid, altering the plant defense response during the early phases of infection. In addition to salicylic acid, the level of plant hormone indole-3-acetic acid or auxin also may be affected by the secretion of CM because chorismic acid is a precursor that can be modulated by the secreted CM, as proposed for nematodes (15).

The C-terminal domain of the secreted CM from the nematode *H. schachtii* is similar to secreted CMs of pathogenic bacteria

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possessing a single catalytic site (32). This suggests a possible similar mechanism of action of CMs from plant-pathogenic nematodes and bacteria in the plant–pathogen interaction. This hypothesis is also supported by the presence in both plant-pathogenic bacteria and the subventral gland of nematodes of cell-wall-degrading enzymes such as cellulases and pectinases that assist nematodes and bacteria in penetrating the host (10,19).

In the present study, we investigated the production and secretion of an *X. oryzae* pv. *oryzae* AroQ_γ and its role in planta on the assumption that it would lead to a better understanding of the role played by the AroQ_γ enzyme in virulence of bacterial leaf blight on rice.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strain used in this study is *X. oryzae* pv. *oryzae* XKK.12, isolated in Kerala State (India) and previously reported as being highly virulent to rice (11). Plasmids used in this study are listed in Table 1. The *Xanthomonas* sp. was grown at 28°C on peptone-yeast extract (PY) liquid medium or on peptone-sucrose agar (PSA) plates as described previously (11). Antibiotics were used at the following concentrations: kanamycin at 50 µg/ml, gentamycin at 10 µg/ml, tetracycline at 5 µg/ml, and ampicillin at 100 µg/ml.

Analysis of *X. oryzae* pv. *oryzae* secreted proteins. *X. oryzae* pv. *oryzae* was grown in PY medium with (PY + R) and without (PY) the addition of 0.5% (wt/vol) macerated rice. Rice was the Italian cv. Baldo, 6 weeks old, grown in sterilized peat substrate (Klassmann substrate 4; Geeste, Germany) under greenhouse conditions (28 ± 2°C, 70% humidity) with a photoperiod of 14 h of light and 10 h of darkness. Macerated rice was prepared as follows. Fresh rice leaves and stems were frozen using liquid nitrogen, then ground with a pestle in a mortar. The fine powder obtained was suspended in warm water and the solid fraction was recovered and dried in an oven at 80°C for 24 h prior to the addition to the liquid medium to support *Xanthomonas* sp. growth. *X. oryzae* pv. *oryzae* was grown at 28°C for 2 days, and the cultures were then centrifuged at 10,000 × g for 20 min to remove bacterial cells and other solid material. The supernatant from each culture was precipitated by adding 10% (wt/vol) trichloroacetic acid (TCA). After 1 h on ice, the samples were centrifuged in Corex tubes at 15,000 × g for 1 h. The resulting pellets were washed with acetone, dried, and resuspended in 1× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (26) before loading onto a 12% SDS-PAGE gel. After the run, the gel was stained with Coomassie Brilliant Blue R-250 and protein bands independently analyzed by using a

matrix-assisted laser desorption ionization tandem time-of-flight (MALDI TOF/TOF) mass spectrometer (Model 4800; Applied Biosystem) after trypsin digestion. Each band was processed as follows. Gel bands were individually cut out from the gel and cut in small pieces of ≈1 mm. Protein bands were destained by adding 100 µl of 50% (vol/vol) 100 mM ammonium bicarbonate in acetonitrile and shaking for 20 min. This step was repeated after decanting the liquid. Then, 100 µl of distilled water was added to the decanted tube and shaken for 15 min. After decanting, another 100 µl of water was added and the gel was allowed to stand for 5 min. The water was decanted, and the spots were soaked in 100 µl of acetonitrile for 5 min. The destained gel spots were dried in a speed-vac. To each dried gel band, 15 µl of sequencing grade trypsin in 50 mM ammonium bicarbonate at 50 ng/µl was added. After 20 min, additional buffer was added until the gel pieces were covered and incubated for 12 to 16 h at 37°C. The digestion was quenched by adding 50 µl of 0.1% (vol/vol) TFA. The solution was sonicated for 20 min. Liquid was separated from the intact piece of gel using a micropipette and then saved in a separate tube. Then 100 µl of 50% (vol/vol) acetonitrile were added to the gel spot and the solution was sonicated for 20 min. The liquid was extracted once again. Finally, this was repeated with 100 µl of 60% (vol/vol) acetonitrile. The liquid obtained from these three steps was combined and centrifuged to dryness. Finally, peptides were resuspended in 0.1% TFA and cleaned by ZipTip C18. The protein of interest was analyzed by both mass spectrometry (MS) and MS/MS mode.

CM bioassay. CM activities were determined spectrophotometrically at λ = 320 nm based on the formation of phenylpyruvate by treatment with HCl, as previously described (8). One unit (U) of enzyme is defined as the formation of 1 µmol of prephenate (assayed as phenylpyruvate after acidification) per minute at 37°C.

CM purification and analysis. The supernatant from 800 ml of *X. oryzae* pv. *oryzae* culture grown in PY with the addition of 0.5% macerated rice was used for purification of CM. Prior to loading onto the chromatographic column, the supernatant was dialyzed against 50 mM Tris-Cl, pH 8.0, and concentrated using an Amicon ultrafiltration device equipped with a YM10 filter (Millipore). The sample was directly loaded onto a Q Sepharose FF column (Amersham Bioscience) previously equilibrated with 50 mM Tris-Cl, pH 8.0. After washing with 40 ml of the same buffer, proteins were eluted using a 160-ml linear gradient of 0 to 0.4 M NaCl, followed by 40 ml at 0.4 to 1 M NaCl. Flow rate was 3 ml min⁻¹ and fractions of 5 ml each. Positive fractions for the CM activity were pooled, dialyzed against 50 mM Tris-Cl, pH 8.0, and loaded again onto the same column but eluted with a

TABLE 1. Plasmids used

Plasmids, strains	Description ^a	Reference
Plasmids		
pGEM2T	Cloning vector, Amp ^r	Promega
pBlueScriptKS	Cloning vector, Amp ^r	Stratagene
PQE30	Expression vector	Qiagen
pKNOCK-Km	Conjugative suicide vector, Km ^r	3
pKNOCK-Tc	Conjugative suicide vector, Tc ^r	3
pUFR047	Wide host range vector, Gm ^r , Amp ^r	9
pUFRCM	pUFR047 containing <i>aroQ_γ</i> gene and its promoter	This study
pUC19	Cloning vector, Amp ^r	37
pQEAROQ	pQE30 containing <i>aroQ_γ</i> gene from <i>Xanthomonas oryzae</i> pv. <i>oryzae</i> XKK.12	This study
pKmCHORI	Internal PCR fragment of <i>aroQ_γ</i> gene cloned in pKNOCK-Km	This study
pTcCM	pKNOCK-Tc containing <i>aroQ_γ</i> gene and its promoter	This study
Strains		
<i>Escherichia coli</i> M15(pREP4) (pQEAROQ)	<i>E. coli</i> expressing 6xHis-tagged chorismate mutase (CM)	Qiagen
XKK.12	<i>X. oryzae</i> pv. <i>oryzae</i> wild type	11
XKK.12CHORI	AroQ _γ ::Km of XKK.12; Km ^r	This study
XKK.12CHORIpUFRCM	AroQ _γ ::Km of XKK.12 complemented by plasmid pUFRCM; Km ^r Amp ^r	This study
XKK.12CHORIpTcCM	AroQ _γ ::Km of XKK.12 complemented by integration of pTcCM; Km ^r Tc ^r	This study

^a Amp^r, Km^r, Tc^r, and Gm^r indicated resistance to ampicillin, kanamycin, tetracycline, and gentamycin, respectively; PCR = polymerase chain reaction.

160-ml gradient of 0.15 to 0.4 M NaCl. Fractions with CM activity were pooled and concentrated to 2 ml before loading onto a Sephacryl HR 200-gel filtration column XK16 (Amersham Bioscience). Flow rate was 0.5 ml min⁻¹ and fractions of 2.5 ml each. The column was calibrated with an MW-GF-200 kit (Sigma Chemical Co.) for molecular mass estimation.

CM molecular mass and N-terminal amino acid sequence were determined as follows. SDS-PAGE was performed according to Sambrook et al. (26) using 15% acrylamide and the broad-range protein marker (New England Biolabs) as molecular mass reference. N-terminal amino acid sequence was determined by automated Edman degradation on a pulsed liquid-phase protein sequencer (Model 470A; Applied Biosystem) equipped with an online phenylthiohydantoin amino acid analyzer (Model 120A; Applied Biosystem). Prior to the analysis, the purified protein was resolved by 15% SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane.

Recombinant DNA techniques. Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA polymerase, Southern hybridization, and transformation of *Escherichia coli* were performed as described (26). Plasmids were purified using the JetStar plasmid purification mini or midi kit (Genomed, GmbH); genomic DNA was isolated by sarcosyl-pronase lysis as described previously (4). Nucleotide sequencing was performed by MacroGen (www.macrogen.com).

aroQ_γ overexpression, purification, and CM antibodies. The CM *aroQ_γ* gene from *X. oryzae* pv. *oryzae* XKK.12 was amplified by PCR using GoTaq DNA polymerase (Promega), using genomic DNA as template and with the oligonucleotide primers Bamchori Fw 5'-CCAGGATCCCAGCCGCCGCTGGATCTGT-3' and Hindchori Rv 5'-TCTAAGCTTTCAGTGGCAAAATCGCCC-3'. Primers were designed according to the sequence from the *X. oryzae* pv. *oryzae* KACC10331 annotated genome, which has a CM encoding gene 100% homologue to the CM gene of *X. oryzae* pv. *oryzae* XKK.12. The resulting 480-bp PCR product was cloned in the pGEM2T easy vector, sequenced, excised from pGEM as a *Bam*HI-*Hind*III fragment, and cloned in the corresponding sites of the 6xHis-tag expression vector pQE30 yielding pQEPHEA. Expression and purification of 6xHis-tagged AroQ_γ were carried out in *E. coli* M15 (pREP4) (pQEAROQ) according to the instruction of the supplier (Qiagen). Polyclonal antibodies against 6xHis-tagged CM were generated by injecting purified protein into rabbits.

Inactivation of *aroQ_γ* gene in *X. oryzae* pv. *oryzae*. The *X. oryzae* pv. *oryzae* *aroQ_γ* gene encoding CM was amplified, in part, by PCR as a 270-bp fragment using the primers 5'-TGCAGTGGCGCTGAGCAAG-3' and 5'-CAGTTCGCCCTGCAGTTGA-3'. The PCR product was cloned in pBlueScriptIIKS, sequenced, and cloned as a *Kpn*I and *Xba*I fragment in the corresponding sites in pKNOCK-Km, generating pKmCHORI. This latter plasmid was delivered to *X. oryzae* pv. *oryzae* XKK.12 by electroporation and, after appropriate selection of a recombination event, a CM knock-out mutant was generated and designated XKK.12CHORI. The fidelity of the marker exchange event

was confirmed by Southern blot analysis (data not shown). The *aroQ_γ* gene of *X. oryzae* pv. *oryzae* KACC10331 (YP_199373) has been annotated as a single transcription unit (19) (Fig. 1); therefore, it is postulated that the XKK.12CHORI mutant was inactivated only in the *aroQ_γ* gene.

Complementation of XKK.12CHORI. In order to complement the CM knock-out mutation present in strain XKK.12CHORI, the pUFRCM and pTcCM plasmids were constructed as follows: the complete *aroQ_γ* gene, including the promoter region, was amplified using genomic DNA as template and the oligonucleotides BamchoriPROM Fw 5'-CCAGGATCCGATCACCGCC AATGGCGA-3' and Hindchori Rv. The 1,072-bp PCR product obtained, that comprises 500 bp upstream of the ATG start codon, was cloned in pUC19, sequenced, and cloned as a *Bam*HI-*Hind*III fragment in the corresponding sites in the pUFRO47 vector and in the pKNOCK-Tc vector, generating pUFRCM and pTcCM, respectively. These latter plasmids were delivered to *X. oryzae* pv. *oryzae* XKK.12 by electroporation and transformants were selected after appropriate selection. Plasmid pUFRCM can replicate in *Xanthomonas* spp., whereas pTcCM integrated in the genome via homologous recombination (3).

Bacterial leaf blight virulence assay on rice plants. *X. oryzae* pv. *oryzae* strains were grown in PY medium at 28°C for 2 days before being used for infection of rice plants. The bacterial concentration was adjusted to 1 × 10⁹ CFU/ml with demineralized water prior to inoculation of rice plants. Pathogenicity tests on *X. oryzae* pv. *oryzae* strains were carried out on the Italian rice cv. Baldo, which showed high sensitivity to *X. oryzae* pv. *oryzae* XKK.12 infection, and rice plants were inoculated by the clipping method (16). Three to four leaves were inoculated and water was used as negative control. Five to six plants were inoculated per strain and were kept for 24 h in humid chambers (>92% relative humidity) at 30 ± 4°C, and were then transferred to greenhouse conditions for disease development. Fourteen days after inoculation, symptoms were evaluated by measuring the lesion length of the infected leaves. Experiments were performed in triplicate. Lesion-length data not fulfilling the requirements for analysis of variance were statistically analyzed by the program SPSS (SPSS Inc., version 15.0) using the nonparametric Kruskal-Wallis multiple comparison test completed by the Mann-Whitney test.

The CFU per gram of infected leaves were also calculated for *X. oryzae* pv. *oryzae* XKK.12 and mutant derivatives. One gram of infected leaves was taken, cutting 3 cm above and 1 cm below the end of the lesion. The infected leaves were ground in a mortar, resuspended in phosphate-buffered saline and serial dilution plated on petri dishes of PSA with ampicillin.

In vitro and in planta assay for CM production by *X. oryzae* pv. *oryzae*. Strain XKK.12 and mutant derivatives were grown for 2 days in PY liquid medium, with and without the addition of macerated rice at 28°C on a rotary shaker. The CM activity in the cell-free supernatant was measured in triplicate as described above. The CM production in planta was evaluated by Western blot analysis using the antibodies raised against CM. Four infected leaves for each *X. oryzae* pv. *oryzae* bacterial strain were taken and cut 3 cm above and 1 cm below the end of the lesion,

Fig. 1. Physical map of the region surrounding the chorismate mutase gene (*pheA*) in *Xanthomonas oryzae* pv. *oryzae* KACC10331. *atpD* and *atpC* belong to the ATP synthase operon. XOO0735: hypothetical protein. *glmU*: UDP-N-acetylglucosamine pyrophosphorilase. *pheA* is 100% identical to *aroQ_γ*.

giving ≈ 0.5 g of infected material. Each sample was ground with a pestle in a mortar, and proteins were extracted using 2 ml of 50 mM Tris-Cl, pH 6.8. Then, 100 μ l were mixed with the same volume of SDS-PAGE loading buffer (26) and loaded onto SDS-PAGE gel. After electrophoresis, resolved proteins were Western blotted onto a PVDF membrane and analyzed using polyclonal antibodies against CM. After incubation with the secondary horseradish peroxidase-labeled antibody, the protein was detected with 3,3-diaminobenzidine tetrahydrochloride tablets (Sigma Chemical Co.), according to the instructions of the supplier.

RESULTS

Identification of secreted proteins from *X. oryzae* pv. *oryzae* cultures. In order to obtain some insight into proteins produced in planta, total proteins from the spent culture supernatant of *X. oryzae* pv. *oryzae* strain XKK.12 grown in the presence of macerated rice were compared with those expressed in the same medium without macerated rice. The proteins showing different levels of production are shown in Figure 2 and were individually analyzed by MALDI MS. The following proteins were identified by comparing MS data with the sequenced and annotated genome of *X. oryzae* pv. *oryzae* KACC10331 (19): a chaperonin (YP_202927, nominal mass 57121, score 53, sequence coverage 33%), a cellulase (YP_202658, nominal mass 58293, score 75, sequence coverage 30%), a cellobiohydrolase (YP_202674, nominal mass 72474, score 37, sequence coverage 15%), a putative outer membrane protein (YP_202335, nominal mass 20653, score 56, sequence coverage 41%), and a CM (YP_199373, nominal mass 20672, score 60, sequence coverage 52%). The chaperonin and cellulase were found to have a molecular mass (MM) determined by SDS-PAGE similar to the theoretical one whereas cellobiohydrolase showed an MM lower than the predicted one. A possible explanation for this lower MM is degradation at the C-

terminus because no peptides were identified in the cellobiohydrolase C-terminal region (data not shown). Cellulase displayed lower levels when macerated rice was provided in the growth medium, whereas the chaperonin and CM were present in apparently higher amounts upon addition of macerated rice (Fig. 2A). The CM YP_199373 identified here belongs to the AroQ₇ subclass and is one of the two CM proteins present in the annotated *X. oryzae* pv. *oryzae* KACC10331 genome (19). The band corresponding to the CM (Fig. 2A, e) was first identified by peptide mass fingerprinting (PMF) and then analyzed by MS/MS sequencing generating the following selected precursors: WDSGKPVLDQAR (ions score 17), SVQYALLNR (ions score 51), and QWQLDALHHAALVR (ions score 17). The YP_199373 protein was predicted to belong to the AroQ₇ subfamily and contains a putative signal sequence indicating that it was most likely transported outside the cytoplasm. The fact that this protein was found in the spent supernatant indicates that it was likely to be secreted by *X. oryzae* pv. *oryzae* although, in gram-negative bacteria, AroQ₇ are believed to be usually exported to the periplasm.

Purification and characterization of secreted *X. oryzae* pv. *oryzae* CM. In order to characterize the AroQ₇ CM, the protein was purified by liquid chromatography. The AroQ₇ CM of *X. oryzae* pv. *oryzae* XKK.12 was purified from a cell-free culture supernatant of PY culture grown in the presence of macerated rice. When loaded onto an anion exchange column previously equilibrated at pH 8.0 and eluted with NaCl gradient, the peak of activity was typically at 360 mM NaCl. Ammonium sulfate precipitation and hydrophobic interaction chromatography were not used in the purification process due to the interference of ammonium sulfate with the enzyme assay. An ultrafiltration step and two consecutive steps of ion exchange chromatography with broader and narrower NaCl gradients, respectively, followed by a gel filtration chromatography step were effective in partially resolving the enzymatic activity. After the gel filtration step, one

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of **A**, culture supernatants precipitated by trichloroacetic acid; **B**, partially purified chorismate mutase (CM); and **C**, 6xHis-tagged CM. Lane 1, molecular weight markers; lane 2, peptone-yeast extract (PY); lane 3, supernatant of *Xanthomonas oryzae* pv. *oryzae* culture grown in PY; lane 4, PY medium with macerated rice (PY + R); lane 5, supernatant of *X. oryzae* pv. *oryzae* culture grown in PY + R; lane 6, partially purified CM; lane 7, 6xHis-tagged CM. The bands indicated by letters are putative proteins: cellulase (a), chaperonin (b), cellobiohydrolase (c), outer membrane protein (d), and chorismate mutase (e).

predominant band was found when the peak of CM activity was analyzed by SDS-PAGE (Fig. 2B). This protein showed a molecular mass slightly lower than the marker of 16.5 kDa and similar to the MM of the band corresponding to the CM of sequenced *X. oryzae* pv. *oryzae* strain KACC10331 (Fig. 2A). In order to confirm that the predominant band of the partially purified protein was the CM, the band was resolved by SDS-PAGE and subjected to N-terminal amino acid sequence analysis by Edman degradation. This revealed the amino acid sequence GLLLG, which indicated the precise cleavage site of the predicted signal peptide of 19 residues, based on the amino acid sequence of the *X. oryzae* pv. *oryzae* KACC10331 CM. This suggests that it is probably a type II secreted protein. This data confirmed that the protein analyzed is the secreted CM found in the culture supernatant. Based on the elution volume in gel filtration column, the MM of the purified enzyme was estimated to be ≈ 40 kDa, suggesting a possible homodimeric structure because the theoretical MM of the monomeric secreted peptide is 18,539.93 Da. There is discrepancy between the theoretically and the experimentally determined MM of the partially purified CM. Either a partial degradation at the C-terminus or differential migration in the lower part of the SDS-PAGE could be responsible for this observed difference. However, the specific activity of the protein seems not to be affected because it constantly increases along the purification steps (Table 2) and is only slightly lower than the specific activity of 63.8 U/mg reported for the CM of *Erwinia herbicola* (36). Due to the low yield of enzyme obtained from the spent culture supernatant (0.2 mg/liter), we overexpressed the protein in *Escherichia coli* as a 6xHis-tagged protein (as explained in Materials and Methods).

The His₆-AroQ₇ CM protein devoid of its signal sequence was purified in a single step and, when analyzed by SDS-PAGE, showed the expected MM of ≈ 19 kDa (Fig. 2C).

***X. oryzae* pv. *oryzae* CM knock-out mutants are more virulent toward rice.** It was of interest to determine whether the AroQ₇ CM played a role in *X. oryzae* pv. *oryzae* rice virulence. Rice leaves were infected by the clipping method (16) with *X. oryzae* pv. *oryzae* XKK.12 wild type and the *aroQ₇* CM knock-out mutant derivative; the results of this experiment are shown in Figure 3. Importantly, the difference between the wild type and the CM-deficient mutant was statistically significant and suggests that the CM plays a role in virulence; surprisingly, the *X. oryzae* pv. *oryzae* CM mutants are considerably more pathogenic than the parent wild-type strain. Not only were the lesion lengths different when infected with the two strains but the leaves also appeared to be more yellowish when infected with the CM knock-out mutant with more evident disease symptoms (Fig. 4). Leaves infected with the complemented CM knock-out mutant harboring the wild type *aroQ₇* gene in a plasmid showed lesion lengths similar to those caused by the mutant, with a statistically insignificant reduction (data not shown). However, by sampling the recovered bacteria from infected leaves and plating on selective PSA solid media, we estimated that only $\approx 20\%$ of the *X. oryzae* pv. *oryzae* cells retained the plasmid. This was an indication that, in planta, this plasmid was lost by *X. oryzae* pv. *oryzae* strains, most probably due to the absence of the antibiotic selection. However, when the complementation was achieved by integration into the genome of the CM gene by using pTcCM, the phenotype was restored and the virulence was reduced to levels similar to the parent wild-type strain (Fig. 3).

TABLE 2. Purification of chorismate mutase from 800 ml of *Xanthomonas oryzae* pv. *oryzae* culture

Purification step ^a	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (% of activity)
Cell-free supernatant	126	56	0.44	1	100
Concentration and dialysis	14	44	3.14	7.1	78
Q Sepharose FF 1	4.5	30	6.6	15	53
Q Sepharose FF 2	0.44	16	36.3	82.5	28
Sephacryl HR200	0.16	7.7	48.6	110.4	13

^a Details are provided in Materials and Methods.

Fig. 3. Results of lesion length caused by *Xanthomonas oryzae* pv. *oryzae* strains: (i) XKK.12 wild type, (ii) XKK.12CHORI, and (iii and iv) two independent XKK.12CHORI complemented with pTcCM. Bars with different letters are significantly different using the Mann-Whitney comparison test performed on lesion length data. *X. oryzae* pv. *oryzae* inoculum at 10^9 CFU/ml was used for the infection of rice leaves.

The number of *X. oryzae* pv. *oryzae* cells in infected leaves was estimated for the wild-type strain, the CM knock-out mutant, and the mutant complemented by chromosomal integration and found to be $1.49 \pm 0.26 \times 10^{13}$, $2.42 \pm 0.22 \times 10^{13}$, and $2.06 \pm 0.33 \times 10^{13}$ per gram of infected leaves, respectively. Consequently, no significant difference between in planta growth and colonization was observed; similar results were obtained when the

three strains were grown under laboratory conditions, as shown in Figure 5.

CM activity in *X. oryzae* pv. *oryzae* strains. It was of interest to determine CM activities of *X. oryzae* pv. *oryzae* strains grown under different conditions. When grown in flasks, *X. oryzae* pv. *oryzae* CM knock-out mutants showed no significant CM activity in the culture supernatant whereas the mutants complemented by

Xoo XKK.12

Xoo XKK.12CHORI

Fig. 4. Leaves of rice infected with *Xanthomonas oryzae* pv. *oryzae* XKK.12 and XKK.12CHORI. Arrows indicate the end of the lesion along the central vein. The bar represents a 1-cm scale.

Fig. 5. Growth curve of *Xanthomonas oryzae* pv. *oryzae* XKK.12 strains XKK.12, XKK.12CHORI, and XKK.12CHORIpTcCM grown in peptone-yeast extract (PY) and PY medium with macerated rice (PY + R).

Fig. 6. A, Chorismate mutase (CM) activity measured in the supernatant of *Xanthomonas oryzae* pv. *oryzae* cultures grown in peptone-yeast extract (PY) and PY medium with macerated rice (PY + R). For strains, see Table 1. **B,** Western blotting analysis of *X. oryzae* pv. *oryzae* culture supernatants. Lane 1, partially purified CM; lane 2, *X. oryzae* pv. *oryzae* XKK.12 grown in PY; lane 3, *X. oryzae* pv. *oryzae* XKK.12CHORI grown in PY; lane 4, XKK.12CHORIpTcCM grown in PY; lane 5, PY + R; lane 6, *X. oryzae* pv. *oryzae* XKK.12 grown in PY + R; lane 7, *X. oryzae* pv. *oryzae* XKK.12CHORI grown in PY + R; lane 8, *X. oryzae* pv. *oryzae* XKK.12CHORIpTcCM grown in PY + R.

integration had activity similar to the wild type (Fig. 6A). Surprisingly, the addition of macerated rice to the growth medium did not significantly affect the CM activity (Fig. 6A). This is in contrast to what was previously observed in Figure 2, where apparently more CM protein was produced when macerated rice was added. The reason for this is currently unknown; however, enzyme quantity and activity are not necessarily related. The mutant complemented by plasmid pUFRM showed two times higher activity than the wild type in the same conditions (data not shown), probably due to overexpression of the gene carried in a plasmid. The His₆-AroQ_γ CM polyclonal antibody raised (see Materials and Methods) was able to detect CM protein only in the spent supernatants of the wild-type and complemented mutant strains, both grown in PY medium with and without the addition of macerated rice (Fig. 6B). The CM protein secreted by *X. oryzae* pv. *oryzae* strains was also detected in planta by Western blot analysis of extracts from infected leaves (Fig. 7). The CM protein was detected in leaves infected with the wild-type *X. oryzae* pv. *oryzae* and in leaves infected with the CM knock-out complemented by integration of the *X. oryzae* pv. *oryzae* wild-type *aroQ_γ* in the chromosome via the utilization of pTcCM (see Materials and Methods). From this data, we concluded that the CM is expressed in planta. No CM was detected in leaves infected with either the CM knock-out mutant or the mutant complemented by plasmid pUFRM, indicating that the plasmid was lost when *X. oryzae* pv. *oryzae* grew in planta, as previously suggested.

DISCUSSION

When the culture supernatant of *X. oryzae* pv. *oryzae* was analyzed for the presence of secreted proteins, a chaperonin, cellulase, cellobiohydrolase, putative outer membrane protein, and CM were identified. Interestingly, the cellulase and cellobiohydrolase have been recently reported in *X. oryzae* pv. *oryzae* and shown to be virulence-associated factors (14). The role of the chaperonin in folding secreted proteins involved in virulence has also been demonstrated (2). However, there are no reports on the presence and function of an AroQ_γ CM of *X. oryzae* pv. *oryzae*. Our data demonstrate that *X. oryzae* pv. *oryzae* produces and most probably secretes a CM belonging to the AroQ_γ subfamily; this enzyme is most probably secreted via a type II secretion system, as postulated by the presence of a signal peptide. The enzyme was partially purified and showed a homodimeric structure, as observed for the CMs of *Mycobacterium tuberculosis* (22), *E. coli*, and yeast (23). *X. oryzae* pv. *oryzae* CM knock-out mutants were significantly more pathogenic on rice than the *X. oryzae* pv. *oryzae* wild type. Based on this observation, we hypothesize that the AroQ_γ CM could have a role in the interaction between the host and the pathogen, possibly

attenuating some of the adverse effects of *X. oryzae* pv. *oryzae* infection; this could have the evolutionary advantage for the pathogen of conferring longer life in the host.

The AroQ_γ CM of *Meloidogyne javanica* has been shown to reduce lateral root formation and to alter plant cell growth through the formation of giant cells (10). An alteration of plant development has been also reported in transgenic soybean expressing the AroQ_γ CM from *M. javanica*, suggesting that this enzyme from nematodes can be involved in interference with the plants' shikimate pathway or, possibly, by suppression of auxin formation. Several reports have evidenced the possible role of AroQ_γ from plant-pathogenic nematodes (10,15,32); on the other hand, very little is known of the role of CMs of plant-pathogenic bacteria. The bacterial CMs are similar to those secreted by nematodes with a similar catalytic site at the C-terminus, as previously presented (32). Importantly, in the human pathogen *Mycobacterium tuberculosis*, AroQ_γ is secreted and seems to be involved in pathogenesis (17,27,29). *M. tuberculosis* is known to have evolved sophisticated mechanisms in order to evade the host immune system; thus, it cannot be excluded that a secreted CM could play a role in this process. Unlike plants, however, mammals do not possess a shikimate pathway; thus, the role of AroQ_γ secreted by *M. tuberculosis* is even more intriguing. Interestingly, the *aroQ_γ* gene in *M. tuberculosis* is part of a transcriptional unit encoding for three proteins, of which the other two are also virulence-associated factors (27).

At present, we do not have any evidence of what the role of the AroQ_γ CM is in the infected rice plants; however, the symptoms are clearly attenuated when the *X. oryzae* pv. *oryzae* pathogen produces the enzyme. We can speculate that it could be involved in the activation of a defense response mechanism in the rice plant; for example, interfering with the salicylic acid biosynthetic pathway in which CM is involved and, possibly, altering the salicylate-dependent defense response mechanism and the resistance to abiotic stress (38). In addition, it cannot be excluded that the plant shikimate pathway could be affected by the AroQ_γ CM converts chorismate into prephenate and could be involved in the shift of the pathway toward tyrosine and phenylalanine instead of tryptophan. This could possibly result in an increased concentration of intermediates for the synthesis of phenolic compounds used in the synthesis of lignin (33) such as ferulic acid and related compounds that are also known for their antioxidant properties (12). Moreover, salicylate is involved in induction of allelopathy in rice and influences the concentration of phenolic acid compounds that could also be affecting the induction of defense mechanisms (5). Alternatively, this shift of the shikimate pathway resulting in a reduced tryptophan synthesis could result in a reduced synthesis of auxin, as previously proposed (10).

In summary, we report the presence of AroQ_γ in *X. oryzae* pv. *oryzae* XKK.12 and its importance in the infection process. The absence of AroQ_γ results in hypervirulence by *X. oryzae* pv. *oryzae*, indicating that it is involved in timing and regulating the virulence potential in planta. Future studies will need to focus on the role of AroQ_γ in the *X. oryzae* pv. *oryzae*-rice interaction.

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Fig. 7. In planta analysis of chorismate mutase (CM) production in *Xanthomonas oryzae* pv. *oryzae*-infected leaves. For experiment details, see Materials and Methods. Lane 1, noninfected rice leaves; lane 2, rice leaves infected with *X. oryzae* pv. *oryzae* XKK.12CHORI; lane 3, rice leaves infected with *X. oryzae* pv. *oryzae* XKK.12pUFRM; lane 4, rice leaves infected with *X. oryzae* pv. *oryzae* XKK.12pTcCM; lane 5, rice leaves infected with *X. oryzae* pv. *oryzae* XKK.12pTcCM; lane 6, partially purified CM.

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