Pigment and Virulence Deficiencies Associated with Mutations in the *aroE* Gene of *Xanthomonas oryzae* pv. *oryzae*

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Xanthomonadins are yellow, membrane-bound pigments produced by members of the genus *Xanthomonas*. We identified an ethyl methanesulfonate-induced *Xanthomonas oryzae* pv. *oryzae* mutant (BXO65) that is deficient for xanthomonadin production and virulence on rice, as well as auxotrophic for aromatic amino acids (Pig[−] Vir[−] Aro[−]). Reversion analysis indicated that these multiple phenotypes are due to a single mutation. A genomic library of the wild-type strain was used to isolate a 7.0-kb clone that complements BXO65. By transposon mutagenesis, marker exchange, sequence analysis, and subcloning, the complementing activity was localized to a 849-bp open reading frame (ORF). This ORF is homologous to the *aroE* gene, which encodes shikimate dehydrogenase in various bacterial species. Shikimate dehydrogenase activity was present in the wild-type strain and the mutant with the complementing clone, whereas no activity was found in BXO65. This clone also complemented an *Escherichia coli aroE* mutant for prototrophy, indicating that *aroE* is functionally conserved in *X. oryzae* pv. *oryzae* and *E. coli*. The nucleotide sequence of the 2.9-kb region containing *aroE* revealed that a putative DNA helicase gene is located adjacent to *aroE*. Our results indicate that *aroE* is required for normal levels of virulence and xanthomonadin production in *X. oryzae* pv. *oryzae*.

Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight, a serious disease of rice. Worldwide at least 350 different plant diseases are known to be caused by various xanthomonads (17). A characteristic feature of the genus *Xanthomonas* is the production of yellow, membrane-bound pigments called xanthomonadins (28). The xanthomonadins were initially thought to be carotenoids, but later they were characterized as a unique group of halogenated aryl polyene pigments (2, 3). The functional role of xanthomonadins is poorly understood. The vast majority of pigment-deficient mutants that have been isolated from several xanthomonads are prototrophs (20, 29) and virulence proficient upon wound inoculation (9, 20, 29). Pigment-deficient mutants of *Xanthomonas juglandis* and *X. oryzae* pv. *oryzae* have been reported to be more sensitive to photobiological damage than the wild-type strains are (13, 22), suggesting that the pigment may provide protection against photo-damage.

An 18.6-kb region containing seven transcriptional units required for xanthomonadin biosynthesis has been isolated from *Xanthomonas campestris* pv. *campestris* (20, 21). One of the transcriptional units, *pigB*, encodes a diffusible factor that is involved in both pigment and extracellular polysaccharide production (21). The *pigB* mutants have also been shown to be impaired for epiphytic survival and host infection (22).

We isolated an ethyl methanesulfonate (EMS)-induced pigment-deficient mutant of *X. oryzae* pv. *oryzae* that is also virulence deficient and auxotrophic for aromatic amino acids. A genomic clone that restores pigmentation, prototrophy, and virulence to this mutant was isolated by functional complementation. Characterization of this clone revealed that shikimate

dehydrogenase, an enzyme in the aromatic amino acid biosynthetic pathway, is required for normal levels of pigment production and virulence in *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Bacterial strains and media. All bacterial strains and plasmids used are listed in Table 1. *X. oryzae* pv. *oryzae* strains were grown at 28°C in either peptone sucrose (PS) medium (29) or modified Miller's minimal medium M4 (15). *Escherichia coli* strains were grown in Luria-Bertani medium (18) at 37°C. The following concentrations of antibiotics were used: rifampin, 50 µg ml^{−1}; spectinomycin, 50 µg ml^{−1}; ampicillin, 100 µg ml^{−1}; kanamycin, 50 µg ml^{−1}; chloramphenicol, 20 µg ml^{−1}; streptomycin, 50 µg ml^{−1}; and cycloheximide, 75 µg ml^{−1}.

EMS mutagenesis and isolation of pigment-deficient mutants. EMS (Sigma Chemical Co., St. Louis, Mo.) mutagenesis of *X. oryzae* pv. *oryzae* was done as described for *E. coli* by Miller (18). Twenty microliters of a cell suspension from a mutagenized and washed *X. oryzae* pv. *oryzae* cell preparation was inoculated into 2 ml of PS medium and incubated at 28°C for 24 h before the cells were dilution plated to obtain single colonies on PSagar (PSA) plates. Pigment-deficient mutants were obtained at a frequency of 1% of the surviving cells. One pigment-deficient mutant failed to grow on minimal medium, and the auxotrophic phenotype was diagnosed as a deficiency of all three aromatic amino acids by using the pool plate method (7). Spontaneous prototrophic revertants and rifampin-resistant (Rif^r) derivatives of *X. oryzae* pv. *oryzae* strains were obtained by plating saturated cultures (approximately 10⁸ CFU/plate) on plates containing minimal medium and plates containing PSA plus rifampin, respectively.

Virulence assays with rice plants. Forty-day-old greenhouse-grown rice plants of susceptible rice cultivar Taichung Native-1 were inoculated by clipping leaf tips with sterile scissors dipped in saturated cultures (10⁹ cells/ml) of *X. oryzae* pv. *oryzae* (14). When this method was used, approximately 10⁶ cells were deposited at the site of inoculation. The plants were incubated in a greenhouse with minimum and maximum temperatures of approximately 25 and 30°C, respectively, and a relative humidity of approximately 60%. Lesion lengths were measured at regular intervals. No lesions were observed in control experiments in which the leaves were inoculated with scissors dipped in water.

Extraction and quantification of pigment. The procedure described previously (2) for extraction of xanthomonadin from *X. juglandis* was used, with some modifications. *X. oryzae* pv. *oryzae* cultures were grown to the stationary phase, and xanthomonadin was extracted in chloroform-methanol (2:1) by shaking for 3 h at room temperature. The amount of pigment produced per 100 mg (dry weight) of bacterial cells was expressed as the absorbance (optical density at 445 nm) of the crude pigment extracts (21). We also quantified the pigment by

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>E. coli</i> strains		
DH5 α	F' <i>endA1 hsdR17</i> ($r_k^- m_k^+$) <i>supE44 thi-1 recA1 gyrA relA1 f80dlacZDM15 (lacZYA-argF)U169</i>	Lab collection
S17-1	RP4-2Tc::Mu-Kn::Tn7 <i>pro hsdR recA</i>	26
AB2835	<i>aroE353</i>	30
Plasmids		
pBluescript (KS)	Ap ^r	Stratagene, La Jolla, Calif.
pHM1	Sp ^r Sm ^r <i>cos parA</i> IncW derivative of pRI40	11
pUFR034	IncW Nm ^r Tra ⁻ Mob ⁺ <i>mob(P) LacZα⁺ Par⁺ cos</i>	8
pAG4	pUFR034 + 7.0-kb <i>EcoRI</i> fragment encoding <i>aroE</i>	This study
pAG5	pBluescript (KS) + 7.0-kb <i>EcoRI</i> fragment encoding <i>aroE</i>	This study
pAG7	pUFR034 + 1.2-kb <i>EcoRI-PstI</i> fragment encoding <i>aroE</i>	This study
pAG8	pAG4- <i>aroE1</i> ::Tn3-HoHo1	This study
pAG9	pAG5- <i>aroE2</i> ::mTn7	This study
pAG10	pAG5- <i>aroE3</i> ::mTn7	This study
pAG11	pHM1 + <i>aroE2</i> ::mTn7 (in the 7.0-kb <i>EcoRI</i> fragment)	This study
pAG12	pHM1 + <i>aroE3</i> ::mTn7 (in the 7.0-kb <i>EcoRI</i> fragment)	This study
<i>X. oryzae</i> pv. <i>oryzae</i> strains		
BXO1	Laboratory wild type, Indian isolate	Lab collection
BXO43	<i>rif-2</i> , derivative of BXO1	Lab collection
BXO62	<i>pig-10</i> , obtained by EMS mutagenesis of BXO1	This study
BXO65	<i>rif-5</i> , derivative of BXO62	This study
BXO1704	BXO65/pAG4	This study
BXO1706	<i>aroE1</i> ::Tn3-HoHo1 <i>rif-2</i>	This study
BXO1707	<i>aroE2</i> ::mTn7 <i>rif-2</i>	This study

^a *rif-2* and *rif-5* indicate mutations that confer resistance to rifampin; *pig-10* indicates pigment deficiency.

assuming that the structure and molar extinction coefficient of xanthomonadin from *X. oryzae* pv. *oryzae* are identical to those of a xanthomonadin from *X. juglandis* (3). Similar conclusions could be drawn by using either method for pigment estimation.

Functional complementation. A partial *EcoRI*-digested genomic library of our laboratory wild-type *X. oryzae* pv. *oryzae* strain, having an average insert size of 30 kb, was prepared (L. Rajgopal, Y. Jameer, S. Dharmapuri, M. Karunakaran, R. Ramanan, A. P. K. Reddy, and R. V. Sonti, Abstr. Rice Genet. III Symp., p. 939–944, 1995) in the broad-host-range Km^r cosmid cloning vector pUFR034 (8). A total of 960 clones from this library were transferred from *E. coli* DH5 α to S17-1 by using pRK600 as a helper (24). Genomic clones from the library in S17-1 were mobilized in pools of 12 clones each into the pigment-deficient mutant by performing biparental mating. Transconjugants that appeared on PSA-rifampin-kanamycin selection plates were replica plated onto minimal medium plates containing kanamycin to identify prototrophic and pigment-proficient colonies.

Transposon mutagenesis and marker exchange. Transposon 3 insertions were obtained with the pAG4 clone by using Tn3-HoHo1 as described by Stachel et al. (27), with some modifications. Plasmids were isolated, and the insertions were localized on either the insert or the vector DNA by restriction analysis.

The 7.0-kb *EcoRI* fragment in pAG4 was also cloned into the *EcoRI* site of pBluescript(KS) to obtain pAG5 and was mutagenized by mini-transposon 7 (mTn7) derivatives by using an in vitro transposition kit (Genome Priming System; New England Biolabs [NEB], Beverly, Mass.). The mTn7 element encodes kanamycin resistance. Two different mTn7 insertions on the cloned DNA in plasmids pAG9 and pAG10 (Table 1) were found to be in the *aroE* gene (see below). These two insertions were cloned into the shuttle vector pHM1 (Sp^r) (Table 1) by using *EcoRI* restriction sites (*EcoRI* does not cut within the mTn7 element) to obtain plasmids pAG11 and pAG12. Plasmids pAG11 and pAG12 and the derivatives of pAG4 containing Tn3-HoHo1 insertions were mobilized individually into BXO43 for marker exchange. Marker exchange was done by growing the cells in either PS-ampicillin medium (for Tn3-HoHo1) or PS-kanamycin medium (for mTn7) for more than 30 generations by serial passage. Colonies that were either Ap^r Km^r (for Tn3-HoHo1) or Km^r Sp^r (for mTn7) were analyzed by Southern hybridization to confirm that marker exchange had occurred as expected.

Protein extraction, quantification, and enzyme assay. Cell lysates were prepared as described by Chaudhuri and Coggins (6), and protein contents were estimated by the Bradford method (5). Shikimate dehydrogenase activities in cell lysates of *X. oryzae* pv. *oryzae* strains were assayed as described previously for *E. coli* (32). Shikimic acid and NADP were used as substrates, and reduction of NADP to NADPH was assayed by monitoring increase in absorbance at 340 nm. One unit of enzyme activity was defined as 1 μ mol of NADPH produced per min.

Plasmid preparation and DNA sequencing. Plasmid DNA was isolated by the alkaline lysis method (25). Restriction digestions were performed by using enzymes obtained from NEB as recommended by the supplier. Primers that are outwardly directed from Tn3-HoHo1 were used to obtain the sequence of DNA flanking the Tn3-HoHo1 insertions. mTn7-specific primers provided in the Genome Priming System kit from NEB were used to sequence the mTn7 insertion sites. M13 forward and reverse primers were used to sequence the ends of a 7.0-kb insert in clone pAG5. The sequencing reactions, electrophoresis, and sequence data analysis were performed with an ABI Prism 377 automated DNA sequencer (Perkin-Elmer, Foster City, Calif.). A homology search in the database was performed through the National Center for Biotechnology Information by using the BLAST algorithm (1). Promoter prediction was performed by using software at the Baylor College of Medicine search launcher (www.hgsc.bcm.tmc.edu).

Nucleotide sequence accession number. The sequence of a 2.953-kb region containing *aroE* was determined. This sequence has been submitted to GenBank with accession no. AF258797.

RESULTS

Isolation of a pigment- and virulence-deficient aromatic amino acid auxotroph of *X. oryzae* pv. *oryzae*. A pigment-deficient mutant (BXO62) that is auxotrophic for all three aromatic amino acids was isolated after EMS mutagenesis of the BXO1 strain (see Materials and Methods). To aid in the subsequent analysis, spontaneous Rif^r derivatives of BXO1 and BXO62 designated BXO43 and BXO65, respectively, were obtained as described in Materials and Methods. The pigment production, prototrophic-auxotrophic, and virulence properties of the Rif^r derivatives were similar to those of their respective parent strains (data not shown). Pigment was extracted from strains BXO43 and BXO65 and quantified as described in Materials and Methods. BXO65 produces approximately 23% of the pigment produced by BXO43 (Table 2). An absorption spectrum of the pigment extracted from the BXO43 strain showed a characteristic structure with peak at 445 nm and shoulders at 425 and 470 nm (Fig. 1). These features were missing from the absorption spectrum of the BXO65 strain

TABLE 2. Pigment production and shikimate dehydrogenase activity in various *X. oryzae* pv. *oryzae* strains

Strain ^a	Pigment production (optical density at 445 nm) ^b	Shikimate dehydrogenase activity (mU/mg of protein) ^c
BXO43	0.632 ± 0.006 ^d	5.35 ± 0.57
BXO65	0.15 ± 0.018	NDA ^e
BXO1704	0.816 ± 0.03	65.3 ± 6.07

^a BXO43 is the wild-type strain, BXO65 is an EMS-induced Pig⁻ Aro⁻ Vir⁻ mutant, and BXO1704 is BXO65 with pAG4 (a complementing plasmid).

^b Pigment production was measured by determining absorbance at 445 nm as described in Materials and Methods.

^c Protein was extracted and shikimate dehydrogenase activity was assayed as described in Materials and Methods.

^d Mean ± standard error based on three independent experiments.

^e NDA, no detectable activity.

(Fig. 1). The virulence characteristics of these strains were assayed with rice leaves as described in Materials and Methods. Lengths of lesions caused by BXO43 and BXO65 were measured 10 and 15 days after inoculation (Fig. 2). It is apparent that BXO65 is severely virulence deficient (Vir⁻). Prototrophic revertants of BXO65 were isolated and were found to have regained pigment and virulence proficiency (data not shown). Simultaneous reversion suggests that a single mutation is responsible for the pleiotropic phenotype of BXO65.

Isolation of a genomic clone that restores prototrophy, pigmentation, and virulence to BXO65. Clones from a cosmid genomic library of BXO1 were mobilized into BXO65 in 55 pools containing 12 clones each (see Materials and Methods). We identified three donor pools which yielded Aro⁺ Pig⁺ exconjugants. Individual clones from the three pools were mobilized into BXO65, which led to identification of three inde-

FIG. 1. Structure of xanthomonadin I and absorption spectra of crude pigment extracts from *X. oryzae* pv. *oryzae* cultures. (A) Structure of isobutyl derivative of xanthomonadin isolated from *X. juglandis*, a walnut pathogen (3). (B) Absorption spectra of crude pigment extracts from BXO431 (wild-type strain), BXO65 (an EMS-induced Pig⁻ Aro⁻ Vir⁻ mutant), BXO1706 an *aroE1*::Tn3-HoHo1 marker exchange mutant, and BXO1704 (BXO65 with a complementing plasmid pAG4). The dashed vertical lines indicate the wavelengths corresponding to the characteristic peak and shoulders in the absorption spectrum of xanthomonadin.

FIG. 2. Virulence of *X. oryzae* pv. *oryzae* strains for rice. Inoculations were performed with greenhouse-grown plants of susceptible rice cultivar Taichung Native-1 as described in Materials and Methods. Lesion lengths were measured 10 and 15 days after inoculation. The values are means and standard deviations based on three independent experiments. BXO43, wild-type strain; BXO65, EMS-induced Pig⁻ Aro⁻ Vir⁻ mutant; BXO1704, BXO65 with complementing plasmid pAG4. Similar results were obtained following inoculation of growth chamber-grown rice plants (data not shown).

pendent complementing clones, pAG1, pAG2, and pAG3. The *EcoRI* restriction patterns of the three plasmids revealed that there were three common *EcoRI* fragments (7.0, 4.0, and 1.5 kb). When the 7.0-kb fragment was subcloned (pAG4) and mobilized into BXO65, it was found to contain the complementing activity. The absorption spectrum of the pigment extracted from BXO1704 (BXO65/pAG4) was identical to that of BXO43 (Fig. 1), and the amount of pigment produced was estimated to be 30% more than the amount produced in the wild type (Table 2). This could have been due to the presence of the *aroE* gene on a multicopy plasmid in BXO1704. The pAG4 plasmid also complemented BXO65 partially (~60%) for virulence on rice (Fig. 2). This partial complementation could have been a result of instability of the clone because of the absence of antibiotic selection inside the plant.

Transposon mutagenesis of pAG4 and marker exchange. The 7.0-kb *EcoRI* fragment in the pAG4 clone was subjected to mutagenesis by using Tn3-HoHo1 and mTn7 (see Materials and Methods). Seven independent Tn3-HoHo1 insertions were obtained on pAG4, and one of them, *aroE1*::Tn3-HoHo1, affected the ability to complement BXO65 (data not shown). This insertion was marker exchanged in the BXO43 background. The marker exchange mutant BXO1706 was found to be Pig⁻ (Fig. 1), Vir⁻ (data not shown), and auxotrophic for aromatic amino acids. Introduction of pAG4 into BXO1706 restored the Pig⁺ Vir⁺ Aro⁺ phenotype (data not shown). This indicated that *aroE1*::Tn3-HoHo1 disrupted a transcriptional unit that is required for pigmentation, virulence, and prototrophy. Marker exchange mutants obtained with six other Tn3-HoHo1 insertions were Pig⁺ Vir⁺ Aro⁺. Twenty-nine mTn7 insertions were obtained for the 7.0-kb DNA cloned in pAG5 (see Materials and Methods). Two of these insertions, *aroE2*::mTn7 and *aroE3*::mTn7 in plasmids pAG9 and pAG10, respectively, were found to be in the *aroE* open reading frame (ORF) (see below). These two insertions along with flanking 7.0-kb sequences were cloned in the *EcoRI* site of the plasmid

FIG. 3. Nucleotide sequence of *X. oryzae* pv. *oryzae* *aroE* gene and deduced amino acid sequence. The solid and shaded triangles indicate mTn7 and Tn3-HOHO1 insertions, respectively. The *aroE3::mTn7* insertion could not be marker exchanged. The predicted ribosome binding site (S/D) and -35 and -10 promoter regions are indicated. Restriction sites for *EcoRI* and *HindIII* are also indicated.

shuttle vector pHM1 to give plasmids pAG11 and pAG12 (see Materials and Methods). Both pAG11 and pAG12 failed to complement BXO65, indicating that *aroE2::mTn7* and *aroE3::mTn7* abolished the complementing ability of the 7.0-kb genomic fragment. When the *aroE2::mTn7* insertion was marker exchanged in the BXO43 background, it produced the *Pig⁻ Vir⁻ Aro⁻* mutant strain BXO1707 (data not shown). The *aroE3::mTn7* insertion could not be marker exchanged, most likely because of limited homology on one side of the insertion (Fig. 3).

Sequence analysis. A 2.953-kb region cloned in pAG5 was sequenced by using transposon-specific primers, as well as primer walking (see Materials and Methods). The insertions *aroE1::Tn3-HoHo1*, *aroE2::mTn7*, and *aroE3::mTn7* were found to be present in a 849-bp ORF with the potential to encode a 283-amino-acid protein (Fig. 3). A homology search using the BLAST algorithm revealed that the ORF is homologous to *aroE*, which encodes shikimate dehydrogenase, an enzyme in the aromatic amino acid biosynthetic pathway of various bacterial species. Maximum homologies were found with *aroE* from *Pseudomonas aeruginosa* (EMBL/GenBank/DBJ database accession no. X85015), *Neisseria meningitidis* (33), *E. coli* (4), and *Haemophilus influenzae* (10), as shown in Table 3. Promotor prediction, as described in Materials and Methods, indicated the presence of -35 and -10 promoter regions 100 and 75 nucleotides upstream of the putative ATG start codon, respectively. Within the ORF the *aroE1::Tn3-HoHo1*, *aroE2::mTn7*, and *aroE3::mTn7* insertions are located

at the 634th, 350th, and 15th nucleotides, respectively (Fig. 3). A *PstI* restriction site was identified 200 bp downstream of the *aroE* stop codon. This site was used to clone a 1.2-kb *EcoRI-PstI* fragment that included the *aroE* ORF to obtain plasmid pAG7. When pAG7 was introduced into BXO65, this minimal fragment was sufficient to confer the *Pig⁺ Vir⁺ Aro⁺* phenotype (data not shown).

In the 2.953-kb sequence, another ORF downstream of *aroE* was identified by using the BLAST algorithm. This incomplete ORF started at 1,546th nucleotide and showed very strong homology in the 1,407 bp sequenced (55% similarity and 36% identity at the amino acid level) to *dinG* (DNA damage-inducible gene G), which encodes an ATP-dependent helicase in *E. coli* (16).

Complementation of *E. coli* *aroE* mutant by pAG4. *E. coli* *aroE* mutant strain AB2835 (30) was transformed individually

TABLE 3. Homology of the *X. oryzae* pv. *oryzae* *aroE* gene with the *aroE* genes of other bacterial species

Organism	Length of gene product (amino acids)	% Similarity (% identity)	E value ^a	Accession no.
<i>P. aeruginosa</i>	274	58 (45)	e-54	X85015
<i>N. meningitidis</i>	269	57 (41)	e-50	U82840
<i>E. coli</i>	272	58 (44)	e-50	U18997
<i>H. influenzae</i>	272	53 (38)	e-35	U32748

^a E values were obtained by using the BLAST algorithm.

with pAG4 and pAG5 and with plasmids pAG8, pAG9, and pAG10 having transposon insertions in the *aroE* ORF (Table 1). Plasmids pAG4 and pAG5 complemented AB2835 for growth on minimal medium, whereas the other plasmids having an insertion in the *aroE* ORF were not able to do so. All strains grew on minimal medium supplemented with the three aromatic amino acids and the aromatic vitamins *p*-aminobenzoic acid and *p*-hydroxybenzoic acid. Plasmid curing from strain AB2835/pAG4 resulted in a loss of prototrophy (data not shown).

Assay of shikimate dehydrogenase activity. Protein extracts were prepared from the wild-type and *aroE* mutant strains of *X. oryzae* pv. *oryzae*, and shikimate dehydrogenase activity was measured (see Materials and Methods). The Michaelis constant of this enzyme for shikimic acid was calculated to be 5×10^{-5} M, a value very similar to that in *E. coli* (32). The enzyme activity was found to be 5.4 mU/mg of protein in wild-type strain BXO43 (Table 2). No activity was detected in *aroE* mutant-strain BXO65 or in BXO1706 (*aroE1::Tn3-HoHo1*) or BXO1707 (*aroE2::mTn7*). The BXO1704 strain (BXO65/pAG4) had an activity of 65 mU/mg of protein, approximately 12 times that of BXO43 (Table 2).

***X. oryzae* pv. *oryzae* *aroE* mutants are unable to synthesize a diffusible factor involved in xanthomonadin biosynthesis.** Cross-feeding of BXO65 with 15 independently isolated EMS-induced prototrophic pigment-deficient mutants was tested by streaking the mutant strains adjacent to BXO65 in pairwise combinations on PSA plates. Development of a yellow color in BXO65 at the adjacent colony boundary was considered to indicate cross-feeding for pigmentation. BXO65 could not be cross-fed for pigmentation by wild-type strain BXO43, whereas 8 of 15 EMS-induced prototrophic pigment-deficient mutants could be. None of the 15 prototrophic pigment-deficient mutants could be cross-fed by each other, BXO43, or BXO65 for pigmentation.

DISCUSSION

In this paper we present evidence that mutations in the *aroE* gene of *X. oryzae* pv. *oryzae* result in reduced levels of xanthomonadin production and virulence. To the best of our knowledge, this is the first report of a mutation affecting a specific step in a general metabolic pathway that also affects xanthomonadin biosynthesis. Insertional inactivation of any of the seven transcriptional units previously shown to be required for xanthomonadin biosynthesis in *X. campestris* pv. *campestris* has not been reported to cause any nutrient deficiencies (20, 21). Also, clones containing the *aroE* gene of *X. oryzae* pv. *oryzae* do not show any homology with the *pig* gene cluster of either *X. campestris* pv. *campestris* or *X. oryzae* pv. *oryzae* (Goel, Rajagopal, and Sonti, unpublished results). This indicates that the *aroE* gene is from a different genomic locus than the gene cluster that was previously shown to be required for xanthomonadin biosynthesis.

Shikimate dehydrogenase catalyzes the conversion of dehydroshikimate to shikimic acid. How might shikimate production be related to xanthomonadin biosynthesis? One possibility, as previously suggested (12), is that the aromatic ring in xanthomonadin may be derived from the shikimate pathway. This would be interesting because the aromatic ring in aro-

matic carotenoid pigments is derived from cyclization of the polyene chain and not from the shikimate pathway. However, we have observed that shikimic acid facilitates neither growth on minimal medium nor pigmentation in *aroE* mutants (Goel and Sonti, unpublished results). This might be because *aroE* mutants of *X. oryzae* pv. *oryzae* are deficient in shikimic acid uptake, as reported previously for *E. coli* *aroE* mutants (19).

The residual amount of pigment made in the *aroE* mutants may be derived either from a very small amount of shikimic acid produced by spontaneous conversion of dehydroshikimate to shikimic acid or through a second minor pathway. It is also possible that the pigment produced by the *aroE* mutants is devoid of the aromatic ring. Detailed structural analysis and biochemical experiments, such as feeding wild-type cells with labelled shikimic acid and monitoring the incorporation of the label, are required to address these issues.

Several EMS-induced pigment-deficient mutants are able to cross-feed *aroE* mutant strains for pigment production. This suggests that a diffusible compound that can enter the pigment biosynthetic pathway accumulates in these strains. As xanthomonadins are membrane-bound pigments, the compound or intermediate must act prior to commitment to the membrane-bound state. The inability of the wild-type strain and some of the EMS-induced mutants to cross-feed *aroE* strains suggests that the compound does not accumulate in these strains. The inability of the prototrophic EMS-induced pigment-deficient mutants to cross-feed each other suggests that they are defective in reactions that involve commitment to either the membrane-bound state or subsequent steps in xanthomonadin production and transport.

The virulence deficiency of the *aroE* mutants is most likely due to a growth defect. The in planta doubling times for the wild type, *aroE* mutant BXO65, and a mutant with a complementing clone are 8.6, 25.7, and 10.5 h, respectively (data not shown). This is the first report which suggests that one or more aromatic amino acids may be limiting for growth of *X. oryzae* pv. *oryzae* in rice plants. A virulence deficiency has previously been reported to be associated with either arginine or leucine auxotrophy in *X. oryzae* pv. *oryzae* (31). The results also suggest that shikimate dehydrogenase can be used as a target to develop novel bactericides against *X. oryzae* pv. *oryzae*. These bactericides can be compounds that affect the shikimate dehydrogenase of the pathogen without affecting the host. The shikimate pathway may be a good target for this purpose as it is absent in mammals. It is pertinent to note that effective bactericides are not available for use against *X. oryzae* pv. *oryzae* and that judicious application of such compounds could help control yield losses due to this devastating rice pathogen.

ACKNOWLEDGMENTS

We thank Mehar Sultana and N. Nagesh for primer synthesis and DNA sequencing and Jan E. Leach, K. Veluthambi, Jim Pittard, J. Gowrishankar, and A. R. Poplawsky for providing bacterial strains.

A.K.G. and L.R. were supported by fellowships from the Council of Scientific and Industrial Research (CSIR), Government of India.

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