Stationary-Phase Variation Due to Transposition of Novel Insertion Elements in *Xanthomonas oryzae* pv. oryzae

R. RAJESHWARI AND R. V. SONTI*

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

Xanthomonas oryzae pv. oryzae causes bacterial leaf blight, a serious disease of rice. Spontaneous mutants which are deficient for virulence and extracellular polysaccharide (Eps) production accumulate in large numbers in stationary-phase cultures of this bacterium, a phenomenon which we have called stationary-phase variation. A clone (pSD1) carrying the Eps biosynthetic gene (*gum*) cluster of *X. oryzae* pv. oryzae restored Eps production and virulence to several *spv* (for stationary-phase variation) mutants. Data from localized recombination analysis, Southern hybridization, PCR amplification, and sequence analysis showed that the mutations are due to insertion of either one of two novel endogenous insertion sequence (IS) elements, namely, IS*Xo1* and IS*Xo2*, into *gumM*, the last gene of the *gum* gene cluster. The results of Southern analysis indicate the presence of multiple copies of both IS elements in the genome of *X. oryzae* pv. oryzae. These results demonstrate the role of IS elements in stationary-phase variation in *X. oryzae* pv. oryzae.

In their natural environment, bacteria often encounter nutritionally limited conditions which resemble the stationary-phase conditions of laboratory cultures. Gram-positive bacteria respond to the adverse conditions by forming resistant spores. Gram-negative bacteria exhibit reduced growth, and several genes that confer resistance to stress during starvation are transcriptionally induced in the stationary phase. Much of the transcriptional regulation in the stationary phase is mediated by *rpoS*, a gene that encodes the stationary-phase sigma factor (σ^S) of RNA polymerase (14, 21). Several *Escherichia coli* mutants that have a competitive advantage in stationary-phase cultures have been shown to carry mutations in the *rpoS* gene (38).

Spontaneous and reversible phenotypic variations mediated by various DNA rearrangements, such as insertions, DNA duplications, inversions, deletions, and frameshift mutations, are strategies adopted by bacteria to cope with adverse environmental conditions (7). A few examples are intragenic duplications in a regulatory gene in the mushroom pathogen *Pseudomonas tolaasii* (11), large chromosomal duplications that promote growth during carbon starvation in *Salmonella enterica* serovar Typhimurium (34), insertions in *Shigella flexneri* (24), frameshift mutations in *Bordetella pertussis* (35), and flagellar-phase variation mediated by DNA inversion in serovar Typhimurium (39). In the well-studied plant-pathogenic bacterium *Ralstonia solanacearum*, phenotypic changes were shown to be due to either mutations in the *phcA* regulatory gene (2), overproduction of a negative regulatory element (16), or excision of an episome from the bacterial chromosome (26).

Xanthomonas oryzae pv. oryzae causes bacterial leaf blight, a serious disease of rice. Like other xanthomonads, this bacterium produces copious amounts of an extracellular polysaccharide (Eps). The Eps produced by a related bacterium, *Xanthomonas campestris* pv. campestris, is the industrially important xanthan gum, which is comprised of a repeating pentamer unit made up of two subunits of glucose, two of mannose, and one of glucuronic acid, along with certain modifications like acetylation (4). A cluster of *X. campestris* pv. campestris genes (*gumA* through *gumM*) spanning 16 kb has been shown to encode enzymes involved in Eps biosynthesis (3, 17). A cosmid clone carrying the *X. oryzae* pv. oryzae homologue of the *X. campestris* pv. campestris *gum* cluster was isolated using transposon tagging methods (5).

The spontaneous loss of virulence associated with reduced levels of Eps production has been reported for long-term stabs and aging liquid cultures of *X. oryzae* pv. oryzae (9, 30). These mutants were shown to accumulate in large numbers in stationary-phase cultures but not in exponentially growing cultures (29). There was at least a 5,000-fold increase in the frequency of the mutants 10 days after day 1 in the stationary phase, with a large number of the cells comprised of these mutants. This phenomenon was referred to as stationary-phase variation. Independently isolated *spv* (for stationary-phase variation) mutants were reported to show phenotypic variation in the degree of loss of virulence, stability of the mutant phenotype, ability to outcompete the wild-type strain in stationary-phase cocultures, and sensitivity to hydrogen peroxide (29).

We are interested in determining the nature of mutations in the various *spv* strains and to understand why the mutants accumulate in stationary-phase cultures of *X. oryzae* pv. oryzae. In this study, we report that in some of the *spv* strains, the mutant phenotype is due to insertion of either one of two novel endogenous insertion sequence (IS) elements into the *gum* cluster of *X. oryzae* pv. oryzae.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics. The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type strain of *X. oryzae* pv. oryzae (BXO1) was obtained from the Directorate of Rice Research, Hyderabad, India, and the spontaneous rifampin-resistant strain BXO112 was derived from it in our laboratory. Several independent Eps- and virulence-deficient mutants (*spv* mutants) were isolated from stationary-phase cultures of the BXO112 strain (29). The Rif^r marker was useful for counterselection of *E. coli* donors in conjugal matings. All strains of *X. oryzae* pv. oryzae were grown at 28°C for 48 h in peptone-sucrose (PS) medium or PS agar (PSA) when maintained on plates (36). *E. coli* strains were grown in Luria broth medium at 37°C (23). The antibiotics kanamycin and rifampin were added wherever required (50 µg/ml).

Estimation of Eps. Eps was isolated from colonies grown on PSA for 8 to 10 days at 28°C by precipitation with acetone, as described by Hancock and Poxton (12). It was quantitated by colorimetric estimation of pentoses and hexoses (6) using D-glucose as the standard; the amount of Eps was calculated in milligrams per 10^{10} cells of *X. oryzae* pv. oryzae.

* Corresponding author. Mailing address: Centre for Cellular and Molecular Biology, Uppal Rd., Hyderabad 500 007, India. Phone: 91-40-7172241. Fax: 91-40-7171195. E-mail: sonti@ccmb.ap.nic.in.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
X. oryzae pv. oryzae		
BXO1	Natural isolate (Chinsuria, West Bengal, India)	Lab collection
BXO112	*rif-1*; Rif^r derived from BXO1	29
BXO113	*spv-2 rif-1*	29
BXO114	*spv-3 rif-1*	29
BXO115	*spv-4 rif-1*	29
BXO117	*spv-5 rif-1*	29
BXO119	*spv-6 rif-1*	29
BXO121	*spv-7 rif-1*	29
BXO123	*spv-8 rif-1*	29
BXO125	*spv-9 rif-1*	29
BXO127	*spv-10 rif-1*	29
BXO148	*rif-1*; Eps$^+$ Vir$^+$ recombinant of BXO125	This study
BXO149	*rif-1*; Eps$^+$ Vir$^+$ recombinant of BXO125	This study
BXO150	*rif-1*; Eps$^+$ Vir$^+$ recombinant of BXO125	This study
BXO151	BXO125/pSD2	This study
BXO152	BXO125/pSD1	This study
E. coli		
DH5α	F′ *end A1 hsdR17* (r$_K$$^-$ m$_K$$^+$) *supE44 thi-1 recA1 gyrA relA1* ɸ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)U169	Lab collection
S17-1	RP4-2-TC::Mu-Km::Tn7 *pro hsdR recA*	33
Plasmids		
pSD1	pUFR034 + 36 kb of BXO1 DNA containing the *gum* gene cluster	5
pSD2	pUFR034 + 14.2 kb of BXO1 DNA containing part of the *gum* gene cluster	5
pRR7	pBKS + the 4.5-kb *Bam*HI fragment from pSD2	This study

Virulence assays. Cultures of *X. oryzae* pv. oryzae that were grown to saturation, pelleted, and resuspended in sterile water to about 10^9 cells/ml were used as the inoculum. Forty-day-old greenhouse-grown plants of the susceptible rice cultivar Taichung Native-1 (TN-1) were inoculated by the leaf clip method of inoculation (18). Symptoms were measured as lesion lengths at regular intervals.

Conjugation and complementation of *spv* mutants. The cosmid clones containing the *gum* genes of *X. oryzae* pv. oryzae were transferred from *E. coli* to BXO112 using biparental mating procedures (5, 15). Cultures of BXO112 were concentrated to 10^{12} cells/ml, and 200 μl was spotted onto Hybond N$^+$ membranes placed on nutrient agar plates and then mixed with 10 μl of the donor *E. coli* strain, S17-1 (10^9 cells/ml), carrying either pSD1 or pSD2. The plates were incubated for 48 h at 28°C before the cells were washed into sterile distilled water and plated on selection medium containing PSA with rifampin and kanamycin.

Isolation of genomic DNA and Southern blot analysis. Genomic DNA was isolated according to the protocol of Leach et al. (20). Restriction digestions were done using enzymes obtained from New England Biolabs (Beverly, Mass.), as per the manufacturer's instructions. The digested DNA was separated on agarose gels, denatured, neutralized, and vacuum transferred to a Hybond N$^+$ membrane as described by Sambrook et al. (32). Probes were labeled with [α-^{32}P]dATP using a random prime labeling kit (BRIT, Mumbai, India). Prehybridization, hybridization, and autoradiography were performed as described by Yashitola et al. (37).

PCR assays. Six forward (F1 to F6) and six reverse (R1 to R6) primers with sequences specific to *gum* genes were used in various combinations to PCR amplify regions from genomic DNA of the wild-type and *spv* strains of *X. oryzae* pv. oryzae. The sequences of the primers can be obtained from us on request. Genomic DNA (100 ng) was used as the template in 25-μl reaction volumes containing 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl$_2$, and 0.01% gelatin) from Perkin-Elmer (Foster City, Calif.); dATP, dGTP, dTTP, and dCTP (all 0.2 mM; Pharmacia Biotech, Uppsala, Sweden); 10 pmol of each primer; and 2 U of *Taq* polymerase. PCRs were carried out in a PTC-200 Peltier thermal cycler (MJ Research Inc., Watertown, Mass.), with denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 5 min, and a final extension at 72°C for 5 min. PCR-amplified products were analyzed in 0.7% agarose gels stained with ethidium bromide (10 mg/ml) and visualized under UV.

Sequence analysis and homology search. The PCR-amplified products were purified from agarose gels using the QIAEX kit (Qiagen Inc., Chatsworth, Calif.) according to the supplier's instructions. The purified DNA samples were sequenced using the same set of primers as were used for their amplification. The 4.5-kb *Bam*HI DNA fragment cloned into pBluescript (pRR7) was sequenced using vector-specific primers. The internal sequences of the IS elements and the pRR7 clone were obtained by primer walking. Sequencing reactions, polyacrylamide gel electrophoresis, and sequence output processing were performed on an automated sequencing unit (ABI Prism 377; Perkin-Elmer) according to the

manufacturer's instructions. Computer-based sequence homology searches were performed using the BLAST algorithm (1), available on the World Wide Web.

Isolation of *spv* mutants. A culture of BXO112 was grown by inoculating a single colony in 20 ml of PS medium for 48 h at 28°C. The culture was then placed on top of the laboratory bench, and bacterial cells were plated at appropriate dilutions at various time intervals to yield single colonies on PSA plates. The mutants were scored visually for altered colony morphology (i.e., Eps deficiency).

Nucleotide sequence accession numbers. The nucleotide sequences of the *gumI* and *gumJ* genes and the *gumK*, *gumL*, and *gumM* genes have been deposited in GenBank under accession no. AF231923 and AF231924, respectively. The nucleotide sequences of IS*Xo1* and IS*Xo2* have been deposited under accession no. AF225214 and AF225215, respectively.

RESULTS

The phenotype of several *spv* strains is due to mutations in the *gum* locus. In a previous study, inactivation of *gum* genes by transposon insertion was shown to affect Eps production and virulence in *X. oryzae* pv. oryzae (5) and the transposon tag was used to isolate cosmid clones carrying the *X. oryzae* pv. oryzae homologue of the *X. campestris* pv. campestris *gum* cluster. One clone, pSD1, carried a 36-kb insert containing six *Eco*RI fragments of 1.2, 5.2, 5.5, 6.5, 7.8, and 10 kb, whereas a second clone, pSD2, had three fragments of 1.2, 5.2, and 7.8 kb. Only the pSD1 clone complemented the Eps and virulence deficiency of a *gumGXo*::Tn5 mutant. To determine whether the functional *gum* gene cluster could restore a similar phenotype to the *spv* mutants, these two clones were transferred in biparental matings to nine independently isolated *spv* mutants. The Eps-deficient phenotype of four *spv* mutants (BXO119, -121, -123, and -125) was complemented by pSD1 as determined by the appearance of mucoid colonies and quantification of Eps. The wild-type strain produced ~110 mg of glucose/10^{10} cells, whereas the mutants produced only ~10 mg for the same number of cells. The pSD1 clone restored wild-type levels of Eps production to these four *spv* mutants. The mucoid phenotype depended on the continued presence of the plasmid, since the exconjugants grown in the absence of the drug marker, viz.,
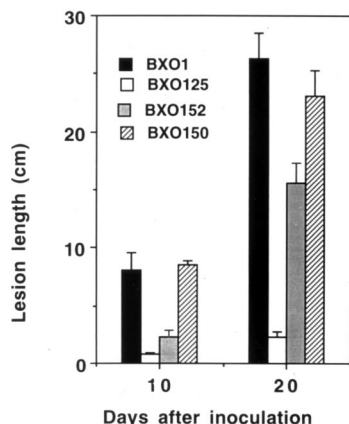
FIG. 1. Virulence of *X. oryzae* pv. oryzae strains on rice. Inoculations were performed on 40 one-day-old plants of rice cultivar TN-1 as described in Materials and Methods. The data are the averages of measurements from 10 leaves that had been inoculated with particular strains: BXO1 (wild type), BXO125 (*spv* mutant), BXO152 (BXO125/pSD1), and BXO150 (an Eps⁺ Vir⁺ recombinant obtained in a BXO125/pSD2 background). Error bars indicate standard deviations. Similar results were obtained in independent experiments.

kanamycin, showed Eps⁻ phenotypes and kanamycin sensitivity due to the loss of the transforming plasmid. Introduction of the pSD2 clone did not restore Eps production to the four mutants, as judged by colony morphology and further confirmed by quantification of Eps production for two of the strains (data not shown). Neither pSD1 nor pSD2 restored Eps production to the remaining five *spv* mutants (BXO113, -114, -115, -117, and -127), as judged by colony morphology.

Pathogenicity tests on rice cultivar TN-1 revealed that the *spv* mutants which were restored for mucoidy by complementation with pSD1 also regained virulence, though the lesion lengths were not as extensive as those caused by the wild-type strain (Fig. 1). Although only the data for BXO125 are presented, similar results were obtained with BXO119, -121, and -123. While the lesions caused by the wild-type strain covered the entire length of the leaf (~30 cm) within 20 days of inoculation and the mutant strain was restricted to 4 cm, the exconjugants showed lesion lengths of ~15 cm (about 40 to 50% of that caused by the wild type), possibly due to the progressive loss of recombinant pSD1. Introduction of the pSD2 clone did not restore virulence to these *spv* mutants (data not shown). The virulence-deficient phenotype of the remaining five *spv* mutants (BXO113, -114, -115, -117, and -127) was not complemented by either the pSD1 or pSD2 clone (data not shown).

However, it was observed that approximately 3 out of every 10 leaves inoculated with transconjugants of BXO119, -121, -123, and -125 carrying pSD2 showed extended lesions. Bacteria isolated from these leaves were mucoid and kanamycin sensitive and appeared to be recombinants that had regained wild-type characteristics. In an attempt to isolate the putative recombinants from laboratory cultures, the exconjugants were also dilution plated in the laboratory on PSA. Mucoid revertants were obtained at a frequency of 1 in 1,000 for *spv* mutants BXO119 and -125 carrying pSD2 as the noncomplementing clone. These mucoid colonies were patched simultaneously on plates containing PSA and PSA with kanamycin. The majority of the colonies were found to be kanamycin sensitive, indicating the loss of the transforming plasmid. No such revertants were obtained when the *spv* strains alone or containing only the vector were used to inoculate rice plants (none in 30 inoculated leaves) or plated on PSA (none out of ~10,000 colo-

nies), indicating a reversion frequency of less than 1 in 10⁴. These observations indicate that the wild-type revertants were obtained by homologous recombination of DNA between the plasmid and the bacterial chromosome, accompanied by the subsequent loss of plasmid in the kanamycin-sensitive colonies. Pathogenicity tests revealed that virulence was restored to these recombinants, as the lesion lengths on rice cultivar TN-1 were comparable to those caused by the wild-type strain (represented by BXO150 in Fig. 1). The complementation and localized recombination data suggest that mutations in the *gum* locus are the cause of the mutant phenotype in four out of nine *spv* strains.

RFLP indicates a DNA rearrangement at the *gum* locus. In order to determine the nature of mutations in the *gum* region of the *spv* strains which are complemented by the pSD1 clone, the total genomic DNA from the wild-type and mutant strains was each digested with *Eco*RI and analyzed by Southern hybridization with pSD2 (Fig. 2A) and pSD1 (Fig. 2B) clones. The results revealed the presence of a restriction fragment length polymorphism (RFLP) in the four *spv* strains (BXO119, -121, -123, and -125) in comparison to the wild-type strain. The *spv* strains were characterized by a 9-kb *Eco*RI fragment instead of the 7.8-kb band observed in the wild-type strain, while the other bands hybridizing to the two clones were unaltered in all the strains (representative data for BXO125 are shown in Fig. 2). Southern analysis of three independently isolated recombinants from each of the exconjugants between BXO123/pSD2 and BXO125/pSD2 showed that the altered 9-kb fragment characteristic of the mutant strains was restored to the original 7.8-kb fragment in all the recombinant strains that were examined (data not shown). The data therefore indicate that a DNA rearrangement of the *gum* cluster occurred in several *spv* mutants of *X. oryzae* pv. oryzae. This DNA rear-
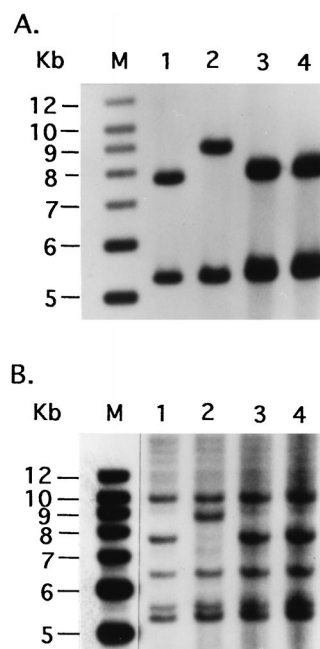

FIG. 2. DNA rearrangement at the *gum* locus in several *spv* strains. Southern analyses of *Eco*RI-digested genomic DNA were performed using α-³²P-labeled probes as described in Materials and Methods. pSD2 (A) and pSD1 (B) were used as probes. Lanes: M, ladder marker DNA from Stratagene; 1, BXO1 (wild type); 2, BXO125 (*spv* mutant); 3 and 4, BXO113 and BXO114 (both are *spv* mutants which cannot be complemented by pSD1). The 7.8-kb fragment is present as a 9-kb fragment in BXO125.
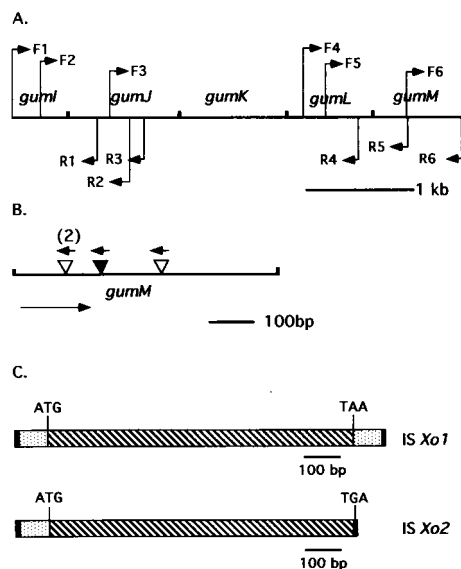
FIG. 3. (A) The *gumI*-to-*gumM* region of the *gum* locus, included in the pRR7 clone. The locations where PCR primers were designed are indicated. F1 to F6 are forward primers, whereas R1 to R6 are reverse primers. (B) The *gumM* region of the *gum* locus. Sites of insertions of the IS elements are shown. ▽, sites of IS*Xo1* insertion in BXO121, -123, and -125. IS*Xo1* is inserted in the same site in BXO121 and -125. ▼, site of IS*Xo2* insertion in BXO119. Note that the putative transposase genes of IS*Xo1* and IS*Xo2* insertions are of a transcriptional orientation (←) opposite to that of the *gumM* gene (——). (C) Schematic representation of the organization of IS*Xo1* and IS*Xo2* elements. ▨, the ORF encoding putative transposase; ▦, transposon-specific flanking sequences; ■, terminal inverted repeats. ATG is the start codon; TAA and TGA are the stop codons.

rangement was not observed in two *spv* strains (BXO113 and -114) that were not complemented by pSD1 (Fig. 2).

PCR amplification of mutant alleles from *spv* strains. Southern analysis of *Bam*HI-digested genomic DNA from the wild type and the four *spv* strains (BXO119, -121, -123, and -125) probed with pSD2 revealed a 4.5-kb fragment in the wild-type strain which was altered by 1.2 kb to yield a 5.7-kb fragment in the four mutants (data not shown). The 4.5-kb *Bam*HI fragment was subcloned from pSD2 into pBluescript (pRR7), and sequence analysis using vector-specific primers indicated homology to the *gumI* gene of *X. campestris* pv. campestris at one end and to the *gumM* gene at the other end (Fig. 3A). The sequence of most of the pRR7 clone was then obtained by primer walking. A sequence of 1,637 bp from the *gumI* end showed homology to the *gumI* and *gumJ* genes of *X. campestris* pv. campestris (85 and 82% identity to *gumI* and *gumJ*, respectively). A 2,181-bp sequence from the *gumM* end showed homology to the *gumK*, *gumL*, and *gumM* genes from *X. campestris* pv. campestris (~83% identity at the nucleotide level). A contiguous stretch of sequence spanning an ~700-bp interval between the 3' end of *gumJ* and the start of the *gumK* gene of *X. oryzae* pv. oryzae remains to be sequenced. The gene order in the *gum* locus is *gumA* through *gumM* in *X. campestris* pv. campestris (3) and appears to be so also in *X. oryzae* pv. oryzae (at least from *gumG* through *gumM* based on earlier [5] and our present work).

For use in PCRs, six pairs of forward as well as reverse primers were then designed, the locations of which are shown in Fig. 3A. PCRs were then performed to identify the region which shows polymorphism in the *spv* strains. One set of primers (F6 and R6) amplified a 300-bp fragment in BXO112, -121, and -125 and a 1.5-kb fragment in two of the *spv* strains, BXO119 and -123 (Fig. 4, lanes 1 through 5). Another set of

primers (F5 and R5) amplified a 500-bp fragment in BXO112, -119, and -123 compared to a 1.7-kb fragment in the other two *spv* strains, BXO121 and -125 (Fig. 4, lanes 6 through 10). In both cases the altered fragment showed an increase of ~1.2 kb over the normal allele, suggesting a DNA rearrangement in that region.

The DNA rearrangement is due to IS element insertion into the *gumM* gene of *X. oryzae* pv. oryzae. The 300-bp fragment amplified in the wild-type strain (BXO112) by the F6-R6 primer combination showed homology to the *gumM* gene, whereas the 500-bp fragment amplified by the F5 and R5 primers showed homology to the *gumL* and *gumM* genes of *X. campestris* pv. campestris. The fragments amplified from the *spv* strains were sequenced with the aid of the primers used for PCR as well as with internal primers. Two distinct and novel IS elements, designated IS*Xo1* and IS*Xo2*, were found to be inserted in different regions of the *gumM* gene (Fig. 3B) in the four *spv* strains. IS*Xo1* was inserted at an identical site in *gumM* in the independently isolated mutants BXO121 and BXO125. The BXO123 strain had an IS*Xo1* insertion at another location in the *gumM* gene. The BXO119 strain had an insertion of IS*Xo2* at a third site within the *gumM* gene. The IS*Xo1* sequence was 1,156 bp long, with a 16-bp inverted repeat (Fig. 3C), and caused a 4-bp target site duplication. There was one open reading frame (ORF) with the potential to encode a 322-amino-acid-long polypeptide, which showed over 88% identity with the gene for the putative transposase of IS*1646*, an IS element from *X. campestris* pv. vesicatoria (19). The ~150-bp flanking region on either side of the ORF, however, did not show homology to any known sequence and was unique to IS*Xo1*. The IS*Xo2* element was 1,070 bp long, with a 24-bp terminal inverted repeat (Fig. 3C), and caused an 8-bp target site duplication. The element has the potential to encode a polypeptide of 320 amino acids which exhibits ~46% similarity with the putative transposase of IH*4*, an IS element from *Halobacterium halobium* (28). The termination codon of the IS*Xo2* ORF extended into the right inverted terminal repeat. The putative transposase genes of IS*Xo1* and IS*Xo2* insertions in *gumM* were of a transcriptional orientation opposite to that of the *gumM* gene (Fig. 3B).

IS*Xo1* and IS*Xo2* are present in multiple copies within the genome of *X. oryzae* pv. oryzae. Genomic DNA from each of the strains BXO112, -119, -121, -123, and -125 that had been digested with *Bam*HI was probed after Southern blotting, with the PCR-amplified product containing either IS*Xo1* or IS*Xo2*. Sequence analysis had indicated that neither of these elements is cleaved by *Bam*HI. Southern analysis showed the presence of multiple copies of each of the elements in the genome of *X. oryzae* pv. oryzae (Fig. 5). The number of copies of IS*Xo1* was
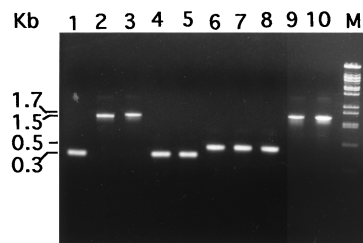

FIG. 4. PCR amplification of mutant alleles from *spv* strains. Agarose gel electrophoresis of PCR products was performed by using the F6-R6 (lanes 1 to 5) and F5-R5 (lanes 6 to 10) sets of *gum*-specific primers with genomic DNA from *X. oryzae* pv. oryzae strains. Lanes: 1 and 6, BXO112; 2 and 7, BXO119; 3 and 8, BXO123; 4 and 9, BXO121; 5 and 10, BXO125. The molecular size marker (lane M) is a *Bst*EII digest of lambda DNA.
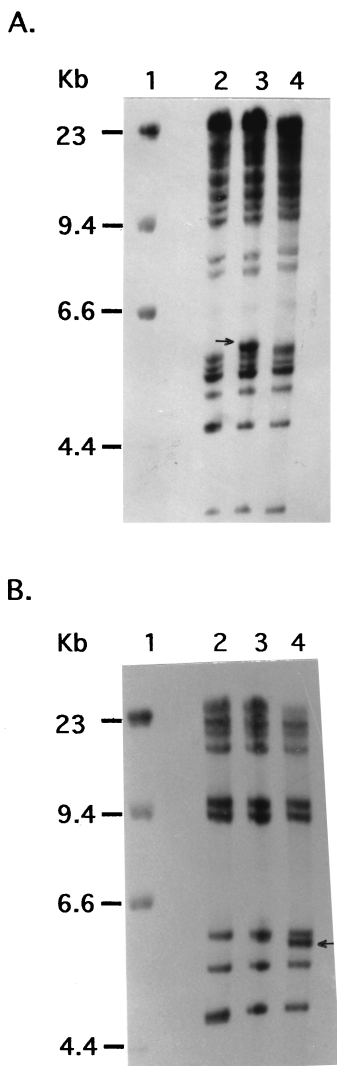
A.

B.

FIG. 5. Distribution of IS*Xo1* and IS*Xo2* in the *X. oryzae* pv. oryzae genome. Southern analyses of *Bam*HI-digested genomic DNA were performed using α-^{32}P-labeled probes IS*Xo1* (A) and IS*Xo2* (B). Lanes: 1, *Hin*dIII-digested lambda DNA marker; 2, BXO1; 3, BXO125; 4, BXO119. An additional 5.7-kb band (indicated by arrows) was detected in BXO125 with the IS*Xo1* probe and in BXO119 with the IS*Xo2* probe.

>20, whereas the number of copies of IS*Xo2* was 9 in BXO112. As expected, an additional 5.7-kb fragment hybridized to IS*Xo1* in BXO121, -123, and -125 (representative data shown for BXO125 in Fig. 5A) and to IS*Xo2* in BXO119 (Fig. 5B).

***spv* mutants from a single stationary-phase culture of *X. oryzae* pv. oryzae are genetically heterogeneous.** The data presented so far indicate that independently isolated *spv* mutants are heterogeneous in nature. To determine whether *spv* mutants from a single stationary-phase culture show similar variation, several mutants were isolated from a single colony of BXO112 as described in Materials and Methods. As observed earlier (29), the frequency of mutants in the culture increased from undetectable levels (<1 in 10^5 cells) after 1 day to ~5 × 10^8 cells after 10 days in the stationary phase. The pSD1 clone was transferred in biparental matings from *E. coli* strain S17-1 to 13 such *spv* mutants, and only 5 of them were complemented by pSD1 for Eps production and virulence (data not shown).

This suggests that a single stationary-phase culture of *X. oryzae* pv. oryzae can have genetically heterogeneous *spv* mutants.

DISCUSSION

In prolonged stationary-phase cultures of *X. oryzae* pv. oryzae grown in the laboratory, spontaneous Eps- and virulence-deficient mutants (referred to as *spv* strains) accumulated in large numbers (29). This study shows that in several of these *spv* strains, the mutant phenotype is due to transposition of either one of two endogenous IS elements into *gumM*, the last gene of the Eps biosynthetic gene cluster. Four different IS elements (IS*1112*, IS*1113*, TNX6, and TNX7) have been previously reported for *X. oryzae* pv. oryzae (20, 27). All four elements are present in multiple copies within the *X. oryzae* pv. oryzae genome. However, when used as probes, they give DNA fingerprinting patterns in the BXO1 strain (37; J. Yashitola and R. V. Sonti, unpublished data) distinctly different from those of either IS*Xo1* or IS*Xo2*, indicating that the latter two elements are different from the previously described IS elements. The sequences of IS*1112* and IS*1113* are also different from those of IS*Xo1* and IS*Xo2* (M. Ryba-White and J. E. Leach, unpublished results). TNX6 and TNX7 have not yet been sequenced.

IS elements have been shown to be ubiquitously distributed within bacterial genomes (22). The data presented in this paper demonstrate that mutations with clearly defined phenotypes caused by IS element transposition can accumulate in large numbers in stationary-phase cultures. IS element transposition has been demonstrated within bacterial cells recovered from stabs that have been stored for several decades (25). These elements have also been shown to play a role in adaptive mutation of the *bgl* operon of *E. coli* (10). These results suggest that IS elements may play prominent roles in the adaptation of *X. oryzae* pv. oryzae and possibly other bacteria to life in the stationary phase.

The results of this study indicate that the *spv* mutants of *X. oryzae* pv. oryzae are heterogeneous in nature, and even in a single stationary-phase culture, at least two different classes of *spv* mutants are found to coexist. Finkle and Kolter (8) have also found different morphotypes coexisting in populations of surviving cells in prolonged stationary-phase cultures of *E. coli*. An intriguing feature in the case of *X. oryzae* pv. oryzae is that the vast majority of *spv* mutants (including the four characterized in this study) do not appear to have a discernible growth advantage in stationary-phase cocultures with the wild-type strain (29). Yet in these same cocultures, new *spv* mutants that are derived from the wild-type strain were found to accumulate in large numbers. Further experimentation is required to determine if the increase in frequency of the *spv* strains is due either to a selection for preexisting mutants or to the generation of new mutants.

The spontaneous insertion of IS*1*-like elements into the *virF* gene of an invasion plasmid in *S. flexneri* has been found to be associated with the stability of the plasmid outside the host, whereas its precise excision allowed the pathogen to revert to the invasive form inside the host (24). Under natural conditions, *X. oryzae* pv. oryzae has been reported to survive on dried plant parts and seeds for extended periods of likely starvation (31). Mutants that are similar to the *spv* strains might accumulate under such conditions. This variability may not be a dead end for the pathogen, if the IS elements can be excised at reasonable frequencies and permit selection for the virulent form under conditions favorable for disease development.

In summary, this study shows that in four of the nine *spv*

mutants tested, the spontaneous loss of virulence and Eps production was due to transposition of one of the two endogenous IS elements, IS*Xo1* or IS*Xo2*, in the *gum* locus. The other *spv* mutants do not show any RFLP in the *gum* locus containing the biosynthetic genes, nor are they complemented by the pSD1 clone. It would be interesting to see if these strains show an RFLP when probed with the IS elements. At least three different non-*gum* loci have been shown to be involved in the production of Eps precursor molecules (UDP-glucose, UDP-mannose, UDP-glucosamine, and GDP-mannose) in *X. campestris* pv. campestris (13). It is possible that either the *X. oryzae* pv. oryzae homologues of these genes or putative Eps regulatory loci are affected in some of the *spv* mutants. The nature of the mutations in these mutants is being investigated.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Brumbley, S. M., and T. P. Denny.** 1990. Cloning of wild-type *Pseudomonas solanacearum phcA*, a gene that when mutated alters expression of multiple traits that contribute to virulence. J. Bacteriol. **172:**5677–5685.
3. **Capage, M. A., D. H. Doherty, M. Betlach, and R. W. Vanderslice.** September 1996. Recombinant DNA mediated production of xanthan gum. U.S. patent 5,559,015.
4. **Coplin, D. L., and D. Cook.** 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. **3:**271–279.
5. **Dharmapuri, S., and R. V. Sonti.** 1999. A transposon insertion in the *gumG* homologue of *Xanthomonas oryzae* pv. oryzae causes loss of extracellular polysaccharide production and virulence. FEMS Microbiol. Lett. **179:**53–59.
6. **DuBois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
7. **Dybvig, K.** 1993. DNA rearrangements and phenotypic switching in prokaryotes. Mol. Microbiol. **10:**465–471.
8. **Finkle, S. E., and R. Kolter.** 1999. Evolution of microbial diversity during prolonged starvation. Proc. Natl. Acad. Sci. USA **96:**4023–4027.
9. **Goto, M.** 1972. Interrelationship between colony type, phage susceptibility and virulence in *Xanthomonas oryzae*. J. Appl. Bacteriol. **35:**505–515.
10. **Hall, B. G.** 1998. Activation of the *bgl* operon by adaptive mutation. Mol. Biol. Evol. **15:**1–5.
11. **Han, B., A. Pain, and K. Johnstone.** 1997. Spontaneous duplication of a 661 bp element within a two-component sensor regulator gene causes phenotypic switching in colonies of *Pseudomonas tolaasii*, cause of brown blotch disease of mushrooms. Mol. Microbiol. **25:**211–218.
12. **Hancock, I., and I. Poxton.** 1988. Isolation of exopolysaccharides, p. 121–125. *In* I. Hancock and I. Poxton (ed.), Bacterial cell surface techniques. Wiley Interscience, Chichester, United Kingdom.
13. **Harding, N. E., S. Raffo, A. Raimondi, J. M. Cleary, and L. Ielpi.** 1993. Identification, genetic and biochemical analysis of genes involved in sugar nucleotide precursors of xanthan gum. J. Gen. Microbiol. **139:**447–457.
14. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell **72:**165–168.
15. **Hopkins, C. M., F. F. White, S. H. Choi, A. Guo, and J. E. Leach.** 1992. Identification of a family of avirulence genes from *Xanthomonas oryzae*. Mol. Plant-Microbe Interact. **5:**451–459.
16. **Huang, Y., and L. Sequeira.** 1990. Identification of a locus that regulates multiple functions in *Pseudomonas solanacearum*. J. Bacteriol. **172:**4728–4731.
17. **Katzen, F., D. U. Ferreiro, C. G. Oddo, M. V. Ielmini, A. Becker, A. Pühler, and L. Ielpi.** 1998. *Xanthomonas campestris* pv. campestris *gum* mutants: effects on xanthan biosynthesis and plant virulence. J. Bacteriol. **180:**1607–1617.
18. **Kauffman, H. E., A. P. K. Reddy, S. P. Y. Hsieh, and S. D. Merca.** 1973. An improved technique for evaluation of resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis. Rep. **57:**537–541.
19. **Kousik, C. S., and D. F. Ritchie.** 1998. Identification of an insertion sequence (IS1646) from *Xanthomonas campestris* pv. *vesicatoria* and its distribution among Xanthomonads. Phytopathology **88:**S49.
20. **Leach, J. E., F. F. White, M. L. Rhoads, and H. Leung.** 1990. A repetitive DNA sequence differentiates *Xanthomonas campestris* pv. *oryzae* from other pathovars of *X. campestris*. Mol. Plant-Microbe Interact. **3:**238–246.
21. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^S (*katF*) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
22. **Mahillon, J., and M. Chandler.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. **62:**725–774.
23. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Mills, J. A., M. M. Venkatesan, L. S. Baron, and M. Buysse.** 1992. Spontaneous insertion of an IS1-like element into the *virF* gene is responsible for avirulence in opaque colonial variants of *Shigella flexneri* 2a. Infect. Immun. **60:**175–182.
25. **Naas, T., M. Blot, W. M. Fitch, and W. Arber.** 1994. Insertion sequence-related genetic variation in resting *Escherichia coli* K-12. Genetics **136:**721–730.
26. **Negishi, J., T. Yamada, T. Shiraishi, H. Oku, and H. Tanaka.** 1993. *Pseudomonas solanacearum*: plasmid pJTPS1 mediates a shift from the pathogenic to nonpathogenic phenotype. Mol. Plant-Microbe Interact. **6:**203–209.
27. **Nelson, R. J., M. R. Baraoidan, C. M. Vera Cruz, I. V. Yap, J. E. Leach, T. W. Mew, and H. Leung.** 1994. Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. Appl. Environ. Microbiol. **60:**3275–3283.
28. **Ng, W.-L., S. Kothakota, and S. DasSarma.** 1991. Structure of the gas vesicle plasmid in *Halobacterium halobium*: inversion isomers, inverted repeats, and insertion sequences. J. Bacteriol. **173:**1958–1964. (Erratum, **173:**3933.)
29. **Rajeshwari, R., J. Yashitola, A. P. K. Reddy, and R. V. Sonti.** 1997. Characteristics of stationary-phase variation affecting virulence in *Xanthomonas oryzae* pv. oryzae. Can. J. Microbiol. **43:**862–867.
30. **Reddy, A. P. K., and H. E. Kauffman.** 1977. Loss of virulence associated with aging of *Xanthomonas oryzae* cultures. Indian Phytopathol. **30:**106–111.
31. **Reddy, R., and Y. Shang-zhi.** 1988. Survival of *Xanthomonas campestris* pv. *oryzae*, the causal organism of bacterial blight of rice, p. 65–77. *In* Proceedings of the International Workshop on Bacterial Blight of Rice. International Rice Research Institute, Manila, the Philippines.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
34. **Sonti, R. V., and J. R. Roth.** 1989. Role of gene duplications in the adaptation of *Salmonella typhimurium* to growth on limiting carbon sources. Genetics **123:**19–28.
35. **Stibitz, S., W. Aaronson, D. Monack, and S. Falkow.** 1989. Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. Nature **338:**266–269.
36. **Tsuchiya, K., T. W. Mew, and S. Wakimoto.** 1982. Bacteriological and pathological characteristics of wild types and induced mutants of *Xanthomonas campestris* pv. oryzae. Phytopathology **72:**43–46.
37. **Yashitola, J., D. Krishnaveni, A. P. K. Reddy, and R. V. Sonti.** 1997. Genetic diversity within the population of *Xanthomonas oryzae* pv. oryzae in India. Phytopathology **87:**760–765.
38. **Zambrano, M. M., D. A. Siegele, M. Almiron, A. Tormo, and R. Kolter.** 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science **259:**1757–1760.
39. **Zieg, J., M. Silverman, M. Hilman, and M. Simon.** 1977. Recombinational switch for gene expression. Science **196:**170–172.