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Research Note

Novel Genomic Locus with Atypical G+C Content that Is Required for Extracellular Polysaccharide Production and Virulence in *Xanthomonas oryzae* pv. *oryzae*

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Three exopolysaccharide (EPS)- and virulence-deficient mutants of *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight of rice, were isolated by Tn*5* mutagenesis. These insertions are not located within the *gum* gene cluster. A 40-kb cosmid clone that restored EPS production and virulence to all three mutants was isolated, and the three transposon insertions were localized to contiguous 4.3- and 3.5-kb *Eco*RI fragments that are included in this clone. Sequence data indicate that two of the transposon insertions are in genes that encode a putative sugar nucleotide epimerase and a putative glycosyl transferase, respectively; the third insertion is located between the glycosyl transferase gene and a novel open reading frame (ORF). A 5.5-kb genomic region in which these three ORFs are located has a G+C content of 51.7%, quite different from the G+C content of approximately 65.0% that is typical of *X. oryzae* pv. *oryzae*. Homologues of this locus have not yet been reported in any other xanthomonad.

The genus *Xanthomonas* comprises of a number of plant pathogenic bacteria (Starr 1981). One common feature of xanthomonads is the production of copious amounts of extracellular polysaccharide (EPS). The best-studied member of this genus, *Xanthomonas campestris* pv. *campestris*, produces an EPS called xanthan gum that is an industrially important product. The structure of this EPS has been shown to be composed of a repeating pentamer composed of two subunits of glucose, two subunits of mannose, and one of glucuronic acid, along with certain modifications like acetylation (Coplin and Cook 1990). Several gene clusters that are involved in EPS production have been identified (Harding et al. 1987; Hötte et al. 1990; Thorne et al. 1987). The 16-kb *gum* gene cluster has been shown to encode enzymes involved in EPS synthesis (Katzen et al. 1998). Several loci that are involved in synthesis of EPS precursors have been identified (Harding et al. 1993; Köplin et al. 1992). Genes that affect EPS production as well as lipopolysaccharide (LPS) production have also been characterized (Kingsley et al. 1993; Köplin et al. 1992), and it has been postulated that they might be involved in the synthesis of common precursors.

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Nucleotide and amino acid sequence data are available in the GenBank database under the accession number AF337647.

X. oryzae pv. *oryzae* causes a serious disease of rice called bacterial leaf blight. EPS appears to be an important virulence factor of *X. oryzae* pv. *oryzae* (Dharmapuri and Sonti 1999; Rajeshwari and Sonti 2000; Rajeshwari et al. 1997), and we are characterizing the genes required for EPS production in this bacterium. In an earlier work, a positive regulator of EPS production, *rpfC*, was cloned (Tang et al. 1996). Dharmapuri and Sonti (1999) had described an EPS and virulence-deficient mutant (BXO1002) of *X. oryzae* pv. *oryzae* that had a Tn*5* insertion in the *gumG* homologue of this bacterium. A 36-kb DNA clone containing the *X. oryzae* pv. *oryzae gum* cluster restored both EPS production and virulence to this mutant. This clone also restored EPS production and virulence to several spontaneous *X. oryzae* pv. *oryzae* mutants that carried insertion sequence (IS) element insertions in the *gumMXo* gene, which is a homologue of *gumM*, the last gene of the *gum* gene cluster of *X. campestris* pv. *campestris* (Rajeshwari and Sonti 2000). This article describes the identification of a novel genomic locus, distinct from the *gum* cluster, that is required for EPS production and virulence in *X. oryzae* pv. *oryzae*. Homologues of this gene cluster have not yet been reported from any other xanthomonad.

Tn*5*-induced EPS- and virulence-deficient mutants of *X. oryzae* pv. *oryzae*.

EPS was isolated and estimated from different *X. oryzae* pv. *oryzae* strains by precipitation with acetone (Hancock and Poxton 1988) and quantified by the colorimetric method for estimation of pentoses and hexoses (DuBois et al. 1956). Three Tn*5*-induced EPS- and virulence-deficient mutants (BXO1001, BXO1003, and BXO1004) (Table 1) are severely deficient in EPS production (Table 2). The wild type (BXO151) typically produces glucose at 1,750 to 1,900 μg per 10^9 cells, whereas the mutants produce only 10 to 20 μg for the same number of cells. Inoculation of leaves of 40-day-old plants of the TN-1 rice cultivar (Dharmapuri and Sonti 1999) indicates that

BXO1001, BXO1003, and BXO1004 are severely virulence deficient (Table 2). Marker exchange of the Tn*5* insertions in BXO1001, BXO1003, and BXO1004 into the wild-type (BXO151) chromosomal background causes EPS and virulence deficiency (data not shown), indicating that the Tn*5* insertions were responsible for the mutant phenotypes of BXO1001, BXO1003, and BXO1004.

Functional complementation of BXO1001, BXO1003, and BXO1004.

Complementation analysis and Southern hybridization with a clone, pSD1 (Dharmapuri and Sonti 1999), that encodes the *gum* cluster of *X. oryzae* pv. *oryzae* indicated that the Tn*5* insertions in BXO1001, BXO1003, and BXO1004 are not located within this region (data not shown). Pools of genomic clones from a cosmid (pUFR034; DeFeyter et al. 1990) library of *X. oryzae* pv. *oryzae* maintained in *Escherichia coli* S17-1 (Rajagopal et al. 1999) were then conjugated with the EPS-deficient strains BXO1001, BXO1003, and BXO1004. One clone (designated as pSD5) from one pool restored wild-type levels of EPS production and virulence to all three strains (Table 2). The pSD5 plasmid was isolated and digested with *Eco*RI. The insert showed six fragments of sizes 12, 11, 6, 4.3, 3.5, and 2.9 kb and, therefore, represents a region of approximately 40 kb (data not shown). Southern analysis with pSD5 as a probe indicated that the Tn*5* insertions in BXO1001 and BXO1003 are located within the 3.5-kb *Eco*RI genomic fragment and that the Tn*5* insertion in BXO1004 is located in the 4.3-kb *Eco*RI genomic fragment (data not shown).

Analysis of a 6.149-kb genomic sequence in which *eps*::Tn*5* insertions are located.

A 6.149-kb region of genomic DNA flanking the three *eps*::Tn*5* insertions was sequenced using an ABI 3700 DNA sequencer (Perkin-Elmer, Foster City, CA, U.S.A.) and the sequence has been submitted to GenBank as accession no. AF337647. The location of four open reading frames (ORFs) (identified on-line by ORF Finder) within this sequence is indicated in Figure 1. The ORF1 encodes a predicted protein of 361 amino acids and a BLAST (Altschul et al. 1990) search reveals strong homology to nucleotide sugar epimerases from a number of microorganisms. The best matches were with *Gepi*A, a putative TDP-D-glucose-dehydratase from *Mycobacterium avium* (GenBank accession no. AF143772; 55% identity and 65% similarity at the amino acid level), and *WbiB*, a putative epimerase/dehydratase from *Burkholderia pseudomallei* (52% identity and 66% similarity at the amino acid level) which is encoded in the LPS biosynthetic gene cluster of this human pathogen (DeShazer et al. 1998). A Pfam search (available on-line) indicates that the *X. oryzae* pv. *oryzae* protein belongs to the family of NAD-dependent epimerases, which utilizes NAD as a cofactor and uses nucleo-

Table 2. Quantitation of extracellular polysaccharide (EPS) levels (glucose) and virulence (lesion lengths) of wild-type, EPS-deficient, and EPS-proficient (after complementation) strains of *Xanthomonas oryzae* pv. *oryzae*[a]

Strain	EPS (μg 10^{-9} cells)	Lesion lengths (cm)[b]	
		7 days	14 days
BXO151	1,866.5 ± 100.3	7.0 ± 2.2	16.5 ± 3.0
BXO1001	11.1 ± 2.7	0.66 ± 0.11	1.1 ± 0.11
BXO1003	13.7 ± 3.2	0.71 ± 0.11	1.0 ± 0.15
BXO1004	12.3 ± 2.0	0.5 ± 0.33	1.2 ± 0.22
BXO1023	1,765.3 ± 116.6	6.5 ± 2.2	15.5 ± 3.5
BXO1024	1,835.6 ± 133.3	6.5 ± 1.5	15.25 ± 2.5
BXO1025	1,750.5 ± 83.6	7.0 ± 2.5	15.5 ± 3.5

[a] EPS was isolated and estimated as described in text. BXO151 is the wild-type strain of *X. oryzae* pv. *oryzae.* BXO1001, BXO1003, and BXO1004 are EPS-deficient mutants, while BXO1023, BXO1024, and BXO1025 are the mutants with the pSD5 clone that restores EPS production. Each data point is the mean and standard deviation of three independent experiments.

[b] Lesion lengths on rice leaves. Inoculation of 40-day-old rice plants of TN-1 cultivar was performed as described in text. The data at each time point represents the average and standard deviation of lesion lengths obtained from 12 inoculated leaves. Similar results were obtained in independent experiments.

Table 1. Strain list

Strain	Relevant characteristics[a]	Reference/Source
Plasmids		
PBluescript (KS)	Ap^r	Stratagene, La Jolla, CA, U.S.A.
pUFR034	IncW Km^r Mob^+ *mob* (P) lacZα^+ Par^+ *cos* (8.7 kb)	DeFeyter et al. 1990
pRK600	pRK2013 npt::Tn*9*, Cm^r	Laboratory collection
pSD1	pUFR034 + 36-kb *Xanthomonas oryzae* pv. *oryzae* genomic DNA insert that encodes the *gum* gene cluster	Dharmapuri and Sonti 1999
pSD5	pUFR034 + 40 kb *X. oryzae* pv. *oryzae* genomic DNA insert that encodes certain genes required for EPS production and virulence	This study
Escherichia coli		
DH5α	F', *endA*1 *hsdR*17 (r_k^- m_k^+) *supE*44 *thi*-1 *recA*1 *gyrA relA*1 ϕ80d*lac*M15 (*lacZYA-argF*) U169	Laboratory collection
S17-1	RP4-2-Tc::Mu-Km::Tn*7 pro hsdR recA*	Simon et al. 1983
X. oryzae pv. *oryzae*		
BXO1	Natural isolate; Chinsuria, West Bengal	Laboratory collection
BXO151	*rif-10*; derivative of BXO1	This study
BXO1001[b]	*eps-1*::Tn*5gusA40 rif-10* (derived from BXO151)	This study
BXO1003[b]	*eps-3*::Tn*5gusA40 rif-10* (derived from BXO151)	This study
BXO1004[b]	*eps-4*::Tn*5gusA40 rif-10* (derived from BXO151)	This study
BXO1023	*eps-1*::Tn*5gusA40 rif-10* /pSD5	This study
BXO1024	*eps-3*::Tn*5gusA40 rif-10* /pSD5	This study
BXO1025	*eps-4*::Tn*5gusA40 rif-10* /pSD5	This study
BXO1002[b]	*gumG*::Tn*5gusA40 rif-10*	Dharmapuri and Sonti 1999

[a] EPS = extracellular polysaccharide; *eps* indicates a mutation that confers extracellular polysaccharide deficiency; *rif* indicates a mutation that confers resistance to rifampicin; and Ap^r, Cm^r, and Km^r indicate resistance to ampicillin, chloramphenicol, and kanamycin, respectively.

[b] Isolated following random Tn*5gusA40* (Wilson et al. 1995) mutagenesis of BXO151.

tide sugar substrates for a variety of chemical reactions. The Tn*5* insertion in BXO1004 is inserted within ORF1.

The ORF2 is 618 bp long and, in a BLAST search, exhibits moderate homology to glycosyl/mannosyl transferases from a number of microorganisms. The best matches were with a putative glycosyl transferase from *Streptomyces coelicolor* A3(2) (GenBank accession no. AL121855; Redenbach et al. 1996) and a Mannosyltransferase B from a *Synechocystis* sp. (Kaneko et al. 1996). The ORF2-encoded protein was assigned by a Pfam search to group 1 of the family of glycosyl transferases that transfers activated (UDP-, ADP-, GDP-, or CMP-linked) sugars to a variety of substrates like LPS, glycogen, and fructose-6-phosphate. The Tn*5* insertion in BXO1003 is in ORF2. The ORF3 is a novel ORF that exhibits weak homology to a putative glycosyl transferase encoded in the *Actinobacillus actinomycetemcomitans* gene cluster responsible for synthesis of serotype e-specific polysaccharide antigen (Yoshida et al. 1999). Promoter search (available on-line) revealed a strong promoter 432 bp upstream of ORF3. The third Tn*5* insertion (BXO1001) is inserted between this promoter and ORF3 and is in a short ORF (129 bp) that does not exhibit significant homology to any sequence in the database. It is possible that this insertion may be polar on ORF3. The ORF4 (for which only partial sequence is available) exhibits very strong homology to cystathionine gamma-lyase-like protein from *Stenotrophomonas maltophilia* (accession no. AF031709; 90% identity and 95% similarity at amino acid level) and a number of other microorganisms.

A 5.51-kb region starting at the end of ORF4 and including the rest of the sequence reported here has a G+C content of 51.7%. Within this region, the G+C content of ORF1 (1,083 bp) is 54.5%; ORF2 (618 bp) is 56.5%, and ORF3 (609 bp) is 51.7%. The pattern of codon usage in these three ORFs is quite different from that of other *X. oryzae* pv. *oryzae* genes (data not shown). The G+C content of the 882-bp sequence between ORF4 and ORF1 is 49.7%, the 1,335 bp between ORF1 and ORF2 is 51.9%, the 873 bp between ORF2 and ORF3 is 48.2%, and the available 110 bp following ORF3 is 47.3%. The partial sequence (693 bp) of ORF4 obtained in this work has a G+C content of 64.5%, which is typical of DNA sequences from a number of different genomic regions of *X. oryzae* pv. *oryzae* that are currently available in the DNA databases. This figure is also similar to the average G+C content of 63 to 71% that has been reported for xanthomonads (Starr 1981).

The atypical G+C content and codon usage suggests that the genomic region containing ORF1, ORF2, and ORF3 may be similar to the genomic islands described in a number of bacteria (Hentschel and Hacker 2001; Ochman et al. 2000). These genomic islands are postulated to have been acquired by horizontal gene transfer. To date, there are very few, if any, reported examples of genomic islands in xanthomonads. It is interesting that ORF1 and ORF2 have a slightly higher G+C content than the rest of this "putative genomic island," suggesting that selection may be operating on these two ORFs for promoting optimal expression in the host bacterium. The observation that the ORFs described here are separated from each other by DNA regions of 500 to 1,000 bp is consistent with the possibility that this represents a genomic island as a surprising amount of noncoding sequence has been reported in other genomic islands (Hentschel and Hacker 2001). However, it is also possible that some of the small ORFs that are located in this region are functional.

LPS analysis.

LPS was isolated by the hot phenol method (as described for *X. campestris* pv. *campestris* by Dow et al. 1995) from BXO151 (wild type); EPS- and virulence-deficient mutants BXO1001, BXO1003, and BXO1004; the three mutant strains complemented with pSD5 clone (BXO1023, BXO1024, and BXO1025); as well as an EPS- and virulence-deficient mutant (BXO1002) that has a *gumG*::Tn*5* insertion (Dharmapuri and Sonti 1999). LPS was purified, separated by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lesse et al. 1990), and detected by the silver staining method (Tsai and Frasch 1982). The results (Fig. 2) indicate that, as in *X. campestris* pv. *campestris* (Dow et al. 1995), two LPS bands of high mobility are detected in BXO151. In *X. campestris* pv.

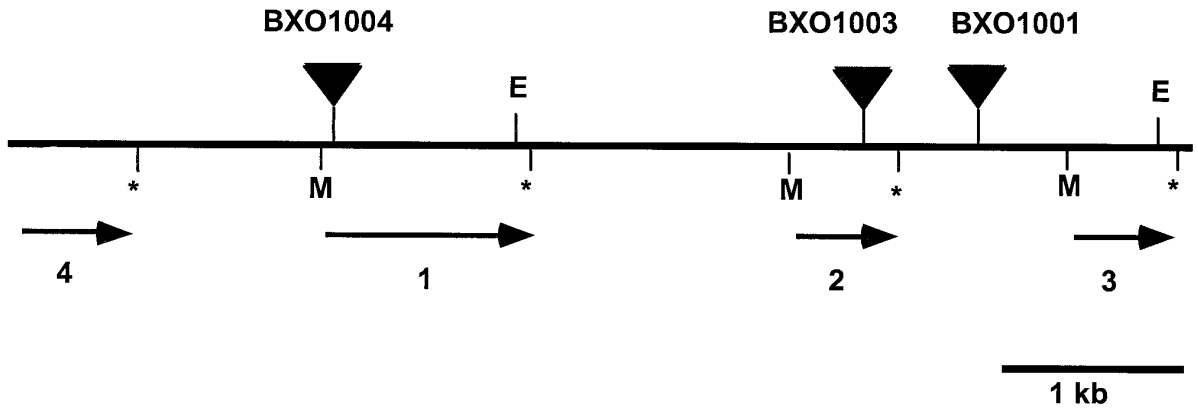

Fig. 1. Schematic map of a 6.149-kb region of *Xanthomonas oryzae* pv. *oryzae* genome showing Tn*5* insertions that cause loss of extracellular polysaccharide production and virulence. The location of Tn5 insertions in BXO1004, BXO1003, and BXO1001 is indicated by the filled triangular flags. 1 = open reading frame (ORF) 1, 2 = ORF2, 3 = ORF3, and 4 = ORF4. E = *Eco*RI sites, M = methionine start codon, and * = stop codon. ORFs were identified as described in text. Arrows indicate the transcriptional orientation of the ORFs. ORF1 encodes a putative epimerase/dehydratase, ORF2 encodes a putative glycosyl transferase, ORF3 is a novel ORF, and ORF4 encodes a cystathionine gamma-lyase-like protein.

campestris, these bands have been interpreted to represent core oligosaccharide and lipid A (Dow et al. 1995). Compared with the three Tn*5*-induced mutants (BXO1001, BXO1003, and BXO1004), the lower mobility band is strikingly more intense in BXO151. Introduction of the complementing clone (pSD5) restores wild-type staining pattern to all three Tn*5*-induced mutants. The *gumG*::Tn*5* insertion mutant (BXO1002) exhibits a wild-type LPS pattern, indicating that the LPS phenotype of the three mutants (BXO1001, BXO1003, and BXO1004) is not merely due to their EPS deficiency. Although it is difficult to specify the exact nature of the LPS deficiency, this suggests that these three mutants might be exhibiting an alteration in the synthesis of core oligosaccharides and lipid A. An altered LPS phenotype was also detected in the three mutants (data not shown) by the rapid LPS isolation protocol of Yi and Hackett (2000) which involves the use of phenol and guanidium thiocyanate in aqueous solutions. However, unlike previously described EPS and LPS deficient mutants of *X. campestris pv. campestris* (Köplin et al. 1993), the three mutants did not exhibit a rough colony morphology, autoagglutination, or motility defects.

Hötte and colleagues (1990) have described a 35.3-kb region of the *X. campestris* pv. *campestris* genome that contains several genes that are involved in production of both LPS and EPS. Two genes (*xanA* and *xanB*) from this genomic region have been shown to be required for EPS and LPS production and are involved in the synthesis of UDP-glucose and GDP-mannose (Köplin et al. 1992). Both of these nucleotide sugars are considered to be precursors for synthesis of EPS and LPS in *X. campestris* pv. *campestris*. A homology search reveals no relatedness between *xanA, xanB*, and the *X. oryzae* pv. *oryzae* genes that are described here. Also, no homology was detected between these *X. oryzae* pv. *oryzae* genes and the *opsX* locus that had been previously shown to affect EPS and LPS production in *X. campestris* pv. *citrumelo* (Kingsley et al. 1993).

A recently submitted sequence (accession no. AF204145) indicates that the LPS biosynthetic gene cluster (which includes the *xanA* and *xanB* genes) of *X. campestris* pv. *campestris* is located next to the cystathionine gamma lyase gene, in almost exactly the same position as the 5.51-kb region localized in *X. oryzae* pv. *oryzae*. However, there is no homology between the sequence of the 5.51-kb region and the *X. campestris* pv. *campestris* LPS biosynthetic gene cluster. Further studies are required to determine if the *X. oryzae* pv. *oryzae* homologue of the LPS biosynthetic gene cluster of *X. campestris* pv. *campestris* can be located in the genomic region flanking the 5.51-kb region.

In addition to the ORFs described in this article, partial sequencing and homology search reveals that a putative glucose-1-phosphate thymidyltransferase, a putative O-antigen acetylase, and a putative ATP-binding component of an ABC transporter involved in LPS transport are encoded in the genomic region cloned in pSD5 (M. R. Vishnupriya and R. V. Sonti, *unpublished data*). These sequences also have an atypical G+C content that is similar to that of the 5.51-kb region. Detailed sequence characterization and functional analysis of the entire 40-kb clone in pSD5 are currently in progress. These studies should provide insights into the evolutionary history of this genomic region and its role in the production of EPS and LPS in *X. oryzae* pv. *oryzae*.

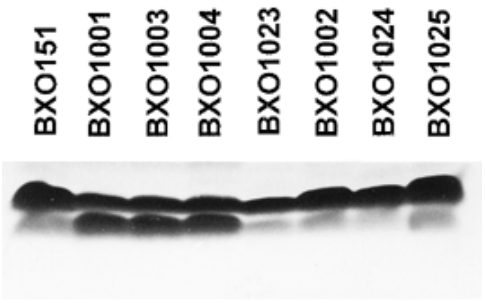

Fig. 2. Lipopolysaccharide (LPS) production by wild-type and extracellular polysaccharide (EPS)-deficient mutants of *Xanthomonas oryzae* pv. *oryzae*. BXO151 = wild-type strain of *X. oryzae* pv. *oryzae*; BXO1001, BXO1003, and BXO1004 are Tn*5*-induced EPS- and virulence-deficient mutants; BXO1023 = BXO1001/pSD5, BXO1024 = BXO1003/pSD5, and BXO1025 = BXO1004/pSD5. The pSD5 clone restores EPS production and virulence to all three mutants. BXO1002 = a *gumG*::Tn*5* mutant that is EPS and virulence deficient. Note that LPS production is not affected in BXO1002. The procedure for LPS isolation, tricine-sodium dodecyl sulfate-golyacrylamide gel electrophoresis, and silver staining is as described in text.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ORF Finder at the National Center for Biotechnology Information: www.ncbi.nlm.nih.gov/gorf

Pfam Homepage of Washington University in St. Louis: pfam.wustl.edu

Promoter search through Search Launcher at Baylor College of Medicine: searchlauncher.bcm.tmc.edu/seq-search/gene-search.html