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Research Notes

New Pathogenicity Loci in *Erwinia stewartii* Identified by Random Tn*5* Mutagenesis and Molecular Cloning

D. L. Coplin, R. D. Frederick, and D. R. Majerczak

Department of Plant Pathology, The Ohio State University, Columbus, OH 43210-1087.
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Pseudomonas aeruginosa **plasmid pMO75 was used as a delivery system to introduce random Tn*5* mutations into the genome of *Erwinia stewartii*. Nineteen avirulent mutants were identified by screening for loss of wilt induction and water-soaking (Wts) on corn seedlings. Five new *wts* mutations were mapped to the left of *wtsA*, thereby enlarging the *wts* gene cluster to approximately 28 kb. Four Wts$^-$ mutations appeared to be unlinked to the *wts* cluster and may represent new pathogenicity loci.**

Additional keywords: Stewart's wilt, *Zea mays*.

Erwinia stewartii (Smith) Dye causes Stewart's bacterial wilt and leaf blight of sweet corn and maize (*Zea mays* L.), which has been the most serious bacterial disease of corn in the north central and eastern United States in recent years. Synthesis of extracellular polysaccharide (EPS) is essential for the virulence of *E. stewartii* (Braun 1990; Dolph *et al.* 1988), but no other virulence factors have been reported, even though attempts have been made to find toxins and various degradative enzymes (Braun 1990) in culture and *in planta*. Work in our laboratory to genetically define virulence factors in *E. stewartii* has involved the isolation of transposon-induced mutants that cannot cause water-soaking (Wts) or produce EPS (McCammon *et al.* 1985), complementation of these mutants with wild-type library clones (Coplin *et al.* 1986), and the subsequent characterization of the genes of interest by site-directed mutagenesis and complementation analysis. This approach has identified: a 19-kb cluster of *cps* (*c*apsular *p*olysaccharide *s*ynthesis) genes, which was cloned in plasmid pES2144 and contains six complementation groups (*cpsA–E* and *galE*) (Dolph *et al.* 1988; Coplin and Majerczak 1990); *rcsA* (*r*egulation of *c*apsule *s*ynthesis), an activator of *cps* gene expression cloned in plasmid pES4507 (Torres-Cabassa *et al.* 1987; Poetter and Coplin 1991); and a 14-kb cluster of pathogenicity genes (designated *wts* for *w*ater-*s*oaking), which was cloned in plasmid pES1044 and contains three complementation groups (*wtsA*, *wtsC*, and *wtsB*) (Coplin *et al.* 1992).

Our previous search for avirulent mutants using bacteriophage Mu-pf7701 mutagenesis (McCammon *et al.* 1985) resulted in one *rcsA* and six *wtsB* mutants. The purpose of this study was to use a different transposon, Tn*5*, to create mutations in additional loci involved in pathogenicity.

Bacterial strains and general methods. *E. stewartii* DC356 was isolated as a spontaneous rifampin-resistant (Rif^r) mutant of wild-type strain ICPPB SS104 (Coplin *et al.* 1981). *Pseudomonas aeruginosa* (Schroeter) Migula PAO11 *trp-54 nal-19* carrying plasmid pMO75 (Whitta *et al.* 1985) was obtained from B. W. Holloway (Monash University, Clayton, Australia). pMO75 is a Tn*5*-containing derivative of IncP10 plasmid R91-5, which is derepressed for conjugal transfer and specifies resistance to carbenicillin (Cb^r) and kanamycin (Km^r). A cosmid library of partially *Sau*3a-digested, size-fractionated DNA from wild-type *E. stewartii* strain SS104 was prepared in vector pLAFR3 and maintained in *Escherichia coli* (Migula) Castellani and Chalmers JM109. Culture media, bacterial matings, and pathogenicity assays have been previously described (Coplin 1978; Coplin *et al.* 1986). Standard methods were used for DNA probe preparation, Southern hybridizations, and cosmid library construction (Maniatis *et al.* 1982).

Tn*5* mutagenesis. A major obstacle in *E. stewartii* genetics has been the lack of a suitable delivery system for transposon mutagenesis. Standard methods using suicide phages or plasmids do not work because *E. stewartii* is insensitive to phage λ infection, and the *E. coli lamB* receptor is not expressed in *E. stewartii*; because most plasmids from the Enterobacteriaceae and Pseudomonadaceae replicate well in this species; and because *E. stewartii* cannot grow above 37° C. In this study, we evaluated the use of plasmid pMO75 as a vector for transposon mutagenesis in *E. stewartii*. Although this plasmid will only replicate in certain strains of *P. aeruginosa*, it is derepressed for conjugation and will transfer into many gram-negative bacteria. Thus, Km^r *E. stewartii* transconjugants do not maintain pMO75, and only contain Tn*5* if it has transposed to a new site within the genome.

pMO75 (Cb^r, Km^r) was transferred from *P. aeruginosa* PAO11 to *E. stewartii* DC356 (Rif^r); 4×10^9 donor cells were mixed with 8×10^9 recipient cells, collected on filters,

Present address of R. D. Frederick: Department of Biological Sciences, Purdue University, W. Lafayette, IN 47907.
Address correspondence to D. L. Coplin.
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and incubated over semisolid L-agar for 5 hr at 28° C. Transconjugants were selected for Km^r and Rif^r on casamino acids-peptone-glucose (CPG) agar. The transfer of Km^r occurred at $4 \pm 2 \times 10^{-5}$ per donor cell, and none of the transconjugants were Cb^r. PAO11 (pMO75) had a high rate of spontaneous mutation to Rif^r, but these mutants were easily distinguished from *E. stewartii* on the basis of colony morphology and pigmentation. Two percent of the *E. stewartii* transconjugants were auxotrophic mutants that failed to grow on DB-glucose minimal medium. Southern hybridizations of *Eco*RI-digested genomic DNA from the mutants in Table 1 with a probe made from the internal 3.3-kb *Hin*dIII fragment of Tn*5* revealed single Tn*5* insertions in all but one mutant (DM4030). (Secondary transposition of IS50 could have occurred and would not be detected by this probe.) These results demonstrate that pMO75 is a good delivery system for Tn*5* mutagenesis in *E. stewartii*; pMO75 produces mostly single transposition events with a high frequency of mutations to auxotrophy.

Table 1. Characterization of avirulent Tn*5* mutants

Mutants	Phenotype (genotype)	Number of mutants	Complemented by plasmids
DM4019, DM4021	EPS^-Wts^-(*cpsA–E*)	2	pES2144
DM4007	$EPS^-Wts^{\pm}$(*rcsA*)	1	pES4507
DM4025, DM4061	$EPS^{\pm}Wts^-$	2	Not done
DM4002, DM4003, DM4008, DM4012, DM4013, DM4027, DM4029, DM4062	EPS^+Wts^-(*wtsA–C*)	8	pES1044
DM4005, DM4028, DM4031, DM4057, DM4059	EPS^+Wts^-(*wts*)	5	pES411
DM4001, DM4006, DM4011, DM4030	EPS^+Wts^-	4	Not done

A bank of Tn*5* mutants was screened for colony type and prototrophy and tested for virulence on 8-day-old sweet corn seedlings (cv. Earliking). Plant inoculations were done in two stages: first, all of the Tn*5* mutants were inoculated by wounding plants near the soil line, and, second, any avirulent and weakly virulent strains were retested by using the whorl assay for Wts ability (Coplin *et al.* 1986). Although many mutants exhibited decreased disease severity in wound inoculations, only mutants that were prototrophic and completely avirulent in the whorl assay were selected for further study. Nineteen mucoid avirulent mutants were obtained from a total of 2,521 colonies derived from nine separate matings (Table 1). In addition, the virulence screen also yielded three EPS^- mutants; mucoidy was restored to two mutants by pES2144 and to the third by pES4507, suggesting that they have mutations in previously characterized *cpsA–E* and *rcsA* genes, respectively (Table 1).

Characterization of Wts^- mutants. In a previous study (Coplin *et al.* 1992), we suggested that *wtsA–C* was part of a larger pathogenicity region extending to the left of *wtsA*. Pathogenicity was completely restored to eight of the Wts^- Tn*5* mutants by pES1044 (Table 1), indicating that they probably have mutations in either *wtsA*, *wtsB*, or *wtsC*. The leftmost 1.8-kb *Hin*dIII fragment from pES1044, containing *wtsA*, was used as a hybridization probe to screen a wild-type *E. stewartii* cosmid library for adjacent DNA, and cosmid pES411 was obtained. This plasmid overlapped 6 kb of the pES1044 insert and extended another 19.6 kb farther to the left (Fig. 1). It complemented known *wtsA* and *wtsC* mutants (RDF6011 and DM061, respectively; Coplin *et al.* 1992) and completely restored pathogenicity to five of the Tn*5* mutants (DM4005, DM4028, DM4031, DM4057, and DM4059; Table 1). Genomic Southern blots of these mutants probed with pES411 revealed that they all had independent Tn*5*

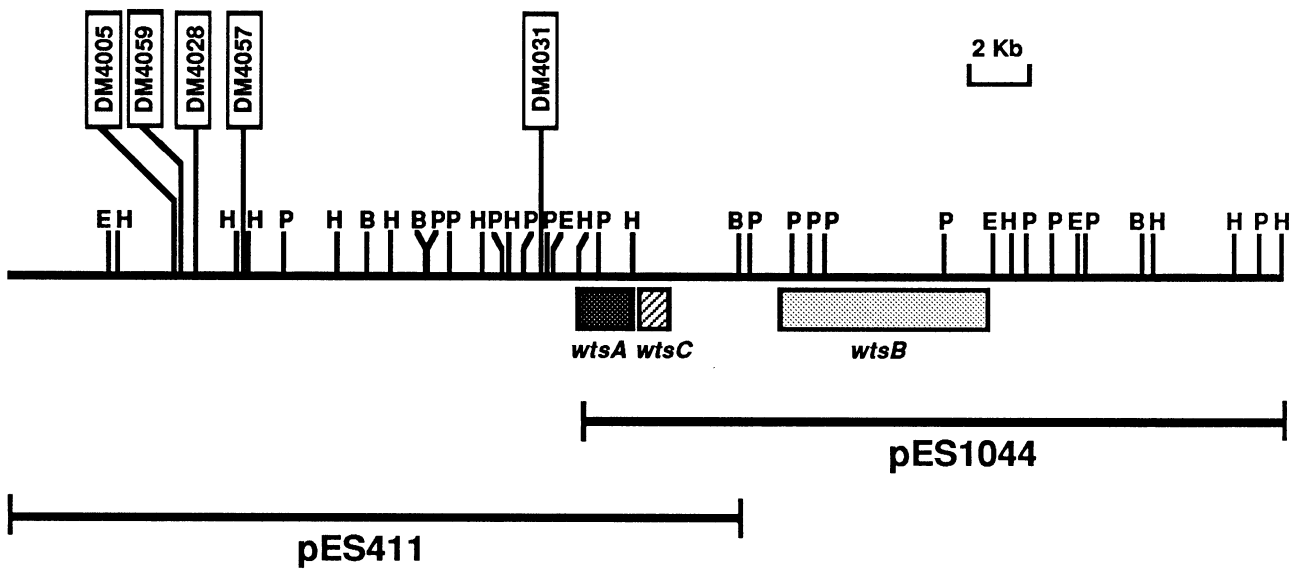

Fig. 1. The *wts* region of *Erwinia stewartii* cloned into plasmids pES411 and pES1044. Locations of chromosomal Tn*5* mutations are shown by the flagged boxes above the map, and the locations of the *wts* complementation groups determined by Coplin *et al.* (1992) are shown by the horizontal boxes below the map. Restriction endonucleases: B = *Bam*HI, Bg = *Bgl*II, E = *Eco*RI, H = *Hin*dIII, and P = *Pst*I.

insertions within the same 7.6-kb *Eco*RI fragment. These insertions were mapped with respect to the *Pst*I and *Hin*dIII sites in pES411 and are shown in Figure 1.

To determine if the mutations in the remaining four mucoid, uncomplemented Wts$^-$ mutants (DM4001, DM4006, DM4011, and DM4030) were also part of the same *wts* gene cluster, we used the 3.5-kb *Bgl*II fragment from pES411 to probe the *E. stewartii* library for another overlapping insert. Cosmid pES873 was found to overlap pES411 by 8 kb and extend an additional 22 kb to the left; however, pES873 failed to restore pathogenicity to these mutants. Hybridization of mutant genomic DNA with a Tn*5* probe, as described above, revealed at least two sites for Tn*5* insertions; DM4006 and DM4001 had insertions in 20-kb *Eco*RI fragments, and DM4011 and DM4030 had insertions in 30-kb *Eco*RI fragments.

Physiological studies on *E. stewartii* (Braun 1990) have only implicated EPS as a virulence factor. Thus, the simplest model for pathogenicity of *E. stewartii* invokes EPS and a postulated cell-leakage factor (Coplin *et al.* 1992). It is interesting that genetic studies have not yet added to the complexity of this model. The majority of the mutants isolated by McCammon *et al.* (1985) and in this study are either defective in EPS synthesis or have *wts* mutations. The function of the *wts* genes is not known, and it is possible that they are directly involved in the water-soaking process, because they are not required for growth in the plant (Coplin *et al.* 1992). Recently, the *wtsA–C* region has been shown to hybridize with *hrp* genes from *E. amylovora* (Burrill) Winslow *et al.* and *P. syringae* pv. *phaseolicola* (Burkholder) Young *et al.* (Beer *et al.* 1990; Frederick *et al.* 1991), confirming the importance of these genes to pathogenicity. The expansion of the *wts* cluster in *E. stewartii* to about 28 kb shows that the *wts* cluster is similar in size to the large *hrp* clusters of other bacteria. The four uncomplemented Wts$^-$ mutations discovered in this study are not closely linked to the main *wts* cluster and could represent one or more new pathogenicity factors.

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