MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 5, No. 1, pp. 81-88, 1992

Characterization of a Gene Cluster that Specifies Pathogenicity in *Erwinia stewartii*

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Received 8 August 1991. Accepted 24 October 1991.

A large cluster of pathogenicity genes from *Erwinia stewartii* DC283 was cloned in cosmid pES1044. These genes (*wts*) are required for both water-soaked lesion formation and wilting on corn seedlings. The *wts* region spanned 14 kb and was mapped by Tn*5* and Tn*3*HoHoI mutagenesis of pES1044 followed by introduction of selected mutations into the chromosome of DC283. Two chromosomal deletion mutants of the *wts* region, DM3001 and DM3020, were isolated. *Trans* complementation analysis identified three complementation groups in the map order *wtsA*, *wtsC*, and *wtsB*. *wtsA*, *wtsB*, and *wtsC* functions were not required for initial growth of the bacteria in corn seedlings; final populations of the *wts* mutants were only slightly lower than that of the parental strain. DM3001 Δ*wtsB* persisted in infected plants and was recovered from the leaves and stalks 6 wk after inoculation. *wtsA::lacZ* and *wtsB::lacZ* gene fusions were expressed *in planta* and in a minimal-salts glucose medium amended with casamino acids or yeast extract.

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

Erwinia stewartii (Smith) Dye causes both vascular wilt of sweet corn and leaf blight of field corn (*Zea mays* L.); it grows in the xylem vessels of the corn plant producing wilt and in the intercellular spaces of the leaves causing lesions. The primary mechanism of virulence for this pathogen is the production of extracellular polysaccharide (EPS) slime that occludes the xylem vessels and causes wilting. When *E. stewartii* grows in the intercellular spaces of young leaves it also produces "water-soaked" lesions. This water-soaking (Wts) symptom is due to cell membrane damage and accumulation of fluids in the leaf tissue. Although the cause of Wts is not known, we have proposed that EPS may function to prolong Wts symptoms and enhance bacterial growth by holding water and nutrients in the intercellular spaces (Coplin and Majerczak 1990; Coplin and Cook 1991).

In previous studies to identify and clone pathogenicity genes from *E. stewartii*, we used bacteriophage Mu *kan* pf7701 mutagenesis to obtain a number of nonpathogenic mutants (McCammon *et al.* 1985). These mutants had two basic phenotypes: EPS^- strains, which could not cause wilting but varied in their Wts ability, and EPS^+ strains, which could not cause either wilting or Wts. The latter were tentatively designated *wts* mutants. Pathogenicity was restored to many of these mutants by clones from a wild-type cosmid library (Coplin *et al.* 1986), and three major cosmid complementation groups were identified; two groups of mutants were defective in synthesis of EPS (Coplin and Majerczak 1990; Dolph *et al.* 1988; Torres-Cabassa *et al.* 1987) and the third group consisted of five out of six *wts* mutants that were complemented by cosmid pES1044. Hybridization of labeled pES1044 DNA with genomic Southern blots of the *wts* mutants revealed insertion mutations within the region represented by pES1044. Coplin *et al.* (1986) concluded that the *wts* region was chromosomal because indigenous plasmid DNA did not hybridize with pES1044 and none of the cryptic plasmids in the *wts* mutants contained Mu pf7701 insertions (McCammon *et al.* 1985).

In this study, we report the genetic and physical mapping of the *wts* gene cluster present in pES1044, the isolation of additional *wts* mutants by site-directed mutagenesis, the identification of genetic complementation groups, the expression of the *wts* genes *in vitro* and *in planta*, and the ability of *wts* mutants to grow in their host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacteria and plasmids used in this study are listed in Table 1. All *E. stewartii* strains were derived from DC283 or DC356, which are spontaneous nalidixic acid-resistant (Nal^r) and rifampicin-resistant (Rif^r) mutants of wild-type strain SS104 (Coplin *et al.* 1981), respectively. Capsular Wts^- mutants MU43, MU51, M136, and MU141, which cause very slight wilting but no Wts, were isolated by Mu *kan* pf7701 mutagenesis (McCammon *et al.* 1985). RDF6011 is a spontaneous Wts^- mutant, which has been cured of its 102-kb cryptic plasmid (Frederick and Coplin 1986). pES1044 is a pVK100 cosmid clone from a library of SS104 DNA partially digested with *Hin*dIII (Coplin *et al.* 1986). Additional subclones of pES1044 were constructed in pLAFR3 (Staskawicz *et al.* 1987) and pVK100 (Knauf and Nester 1982).

Culture media, growth of bacteria, and mating conditions for *E. stewartii* have been described previously (Coplin 1978; Coplin *et al.* 1986). Tetracycline (Tc, 20 μg/ml), kanamycin (Km, 20 μg/ml), ampicillin (Ap, 100 μg/ml), gentamicin (Gm, 10 μg/ml), nalidixic acid (Nal, 20 μg/ml), and rifampicin (Rif, 50 μg/ml) were added to selective media as appropriate. DBG minimal medium consisted of 0.1% (w/v) $NH_4H_2PO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, and 1.0% glucose, pH 7.0. Plasmids were mobilized from

E. coli HB101 into *E. stewartii* using pRK2013::Tn*7* as described previously (Coplin *et al.* 1986).

Recombinant DNA techniques. Procedures for plasmid DNA isolation, agarose gel electrophoresis, restriction analysis, transformation, ligation, Southern hybridization, and random primer labeling have been described previously (Coplin *et al.* 1981, 1986, 1990; Maniatis *et al.* 1982; Torres-Cabassa *et al.* 1987).

Transposon mutagenesis and marker exchange. *E. coli* HB101 (pES1044) was mutagenized with Tn*3*HoHoI and λTn*5* as previously described (Dolph *et al.* 1988). Plasmids with transposon mutations in the pES1044 insert were mobilized from HB101 to RDF6011, MU141, and DM3001 in triparental matings and tested for pathogenicity as described below.

Selected transposon insertions were crossed into the chromosome of DC283. pES1044 plasmids containing Tn*5* and Tn*3*HoHoI insertions were mobilized into DC283 and then transferred three times on media selective only for the transposon marker (Km^r or Ap^r). pES1044 was then displaced by introduction of an incompatible plasmid, pPH1JI, and selection was maintained for pPH1JI Gm^r and the transposon marker. From this mating, 50 Tc^s transconjugants were tested for *wts* ability. Typically, from 10 to 50% had lost virulence. Marker exchange of the transposon mutation and loss of pES1044 was confirmed by Southern blotting.

Complementation tests. pES1044 derivative plasmids were mobilized from HB101 into strains with chromosomal *wts* mutations in triparental replica plate matings (Coplin and Majerczak 1990). Transconjugants were selected for Tc^r Nal^r and then inoculated on 8-day-old sweet corn (cv. Earliking) seedlings using a whorl assay for Wts ability (Coplin *et al.* 1986). Bacteria were suspended in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.2% Tween 40 at about 10^8 cells per milliliter. The inoculum was pipeted directly into the whorls of the seedlings. Plants were maintained in a controlled environment chamber at 29° C, 90% relative humidity, 16-hr light and 8-hr dark cycle, 355 $\mu E \cdot m^{-2} \cdot sec^{-1}$. Wts symptoms were rated at 3 days using the following continuous scale: 1 = no symptoms; 2 = a few restricted lesions; 3 = scattered water-soaked lesions; 5 = numerous water-soaked lesions; 6 = extensive lesions with prolonged water-soaking and ooze forming on leaf surfaces. Six to 10 plants were inoculated with each strain and pathogenicity tests were repeated two to four times.

Expression of β-galactosidase gene fusions. Bacteria containing *wts::lacZ* gene fusions were grown in liquid shake cultures at 28° C for 18 hr in various media. Assays were done according to Miller (1972).

To determine β-galactosidase production *in planta*, corn seedlings were inoculated with bacteria using the whorl assay as described above. The plants were kept at high

Table 1. Bacterial strains and plasmids used in this study

Strain, plasmid	Relevant properties[a]	Source or reference
Erwinia stewartii		
DC283	ICPPB SS104 Nal^r	Coplin *et al.* 1981
DC356	ICPPB SS104 Rif^r	Spontaneous, this study
DE0001	DC283 *wtsB70*::Tn*3*HoHoI	This study
DE0011	DC283 *wtsB236*::Tn*3*HoHoI	This study
DE0021	DC283 *wtsB1202*::Tn*5*	This study
DE0031	DC283 *wtsB1225*::Tn*5*	This study
DM061	DC283 *wtsC42*::Tn*3*HoHoI	This study
DM064	DC283 *wtsC1296*::Tn*5*	This study
DM067	DC283 *wtsB1243*::Tn*5*	This study
DM080	DC283 *wts1298*::Tn*5*	This study
DM092	DC283 *wtsB1216*::Tn*5*	This study
DM095	DC283 *wtsB1252*::Tn*5*	This study
DM3001	Δ*wtsB3001*	From MU51, this study
DM3020	Δ*wts3020*	From MU51, this study
MU43	*wtsB43*::Mu *kan* pf7701	McCammon *et al.* 1985
MU51	*wtsB51*::Mu *kan* pf7701	McCammon *et al.* 1985
MU136	*wtsB136*::Mu *kan* pf7701	McCammon *et al.* 1985
MU141	*wtsB141*::Mu *kan* pf7701	McCammon *et al.* 1985
RDF6011	DC283 *wtsA*	Frederick and Coplin 1986
Escherichia coli		
HB101	*thr leu thi recA hsdR hsdM pro* Sm^r	Boyer *et al.* 1969
Plasmids		
pVK100	Tc^r *cos* IncP	Knauf and Nester 1982
pLAFR3	Tc^r *cos* IncP	Staskawicz *et al.* 1987
pPH1JI	Gm^r Cm^r Sm^r Sp^r IncP	Hirsch *et al.* 1984
pRK2013::Tn*7*	ColE1 mob^+ Sm^r Sp^r Tp^r *kan*::Tn*7*	Dennis Dean[b]
pES1044	wts^+ clone in pVK100 from *E. stewartii* chromosome	Coplin *et al.* 1986
pRF201	3.6-kb *Hin*dIII-*Bam*H1 fragment from pES1044 in pLAFR3	This study
pRF203	9.2-kb *Bam*H1-*Hin*dIII fragment from pES1044 in pLAFR3	This study
pRF204, pRF205	1.8-kb *Hin*dIII fragment from pES1044 in pVK100	This study
pRF207	4.7-kb *Hin*dIII fragment from pES1044 in pVK100	This study

[a] Nal^r, Rif^r, Tc^r, Gm^r, Sm^r, Sp^r, Tp^r: resistant to naladixic acid, rifampicin, tetracycline, gentamycin, chloramphenicol, streptomycin, spectinomycin, and trimethoprim, respectively.

[b] Virginia Polytechnic Institute and State University, Blacksburg.

relative humidity for 3 days after inoculation, at which time droplets of ooze formed on the leaf surfaces. The ooze was rinsed from the leaves with 0.01 M potassium phosphate buffer (pH 7.0). Bacteria collected from the ooze were washed twice in buffer and then assayed for β-galactosidase. Ooze collected from plants inoculated with a wild-type strain, which is Lac⁻ and deficient in β-galactosidase, was used as a negative control.

Growth of bacteria *in planta*. A modification of the procedure of Lockwood and Williams (1957) was used to inoculate 7-day-old corn seedlings. Nal^r strains DC283, RDF6011, MU136, and DM064 were grown overnight in Luria broth and then adjusted to an A_{540nm} of 0.57 (about 1×10^9 cells per milliliter). The plants were cut off 2 cm above the soil line and a 5-μl drop of inoculum was placed on the cut surface. To ensure that the inoculum would be drawn into the cut xylem, rather than flushed out by bleeding, soil moisture was kept low until 1 hr after inoculation, at which time the plants were watered. Bacterial populations were determined at 2, 24, 48, 96, and 144 hr. At each time point, five seedlings were individually homogenized and plated for colony-forming units (cfu). An entire seedling was uprooted, rinsed, and homogenized in 5 ml of 0.01 M potassium phosphate buffer using a Tissumizer (Type SDT-1810, Tekmar, Cincinnati, OH). Serial dilutions of the suspension were plated on Luria agar plus Nal.

RESULTS

Physical mapping of pES1044 and chromosomal *wts* mutants. A restriction map of cosmid pES1044 was constructed and the region containing the *wts* gene cluster is shown in Figure 1. Genomic Southern blots probed with pES1044 revealed that pES1044 had been constructed by the ligation of two noncontiguous, partially digested *Hin*dIII fragments, which were 19.3 and 4.1 kb in size. Only the relevant, colinear 19.3-kb *wts* region is shown in Figure 1.

We previously reported that the group II *wts* mutants, MU43, MU51, MU141, and MU136, had Mu pf7701 insertions in a 12.8-kb *Hin*dIII fragment and the spontaneous *wts* mutant RDF6011 had a 1.3-kb insertion of unknown origin in a 1.8-kb *Hin*dIII fragment (Coplin *et al.* 1986). In this study, the locations of the insertions in MU43, MU51, MU136, MU141, and RDF6011 were determined more precisely and are shown in Figure 1. The spontaneous insertion in RDF6011 was mapped to the 1.2-kb *Hin*dIII-*Pst*I fragment from the right end of the 1.8-kb *Hin*dIII fragment.

Subcloning of pES1044. Subclones of pES1044 containing the 1.8-kb *Hin*dIII fragment (pRF204 and pRF205) and the 4.7-kb *Hin*dIII fragment (pRF207) were constructed in pVK100; the 3.6-kb *Hin*dIII-*Bam*HI (pRF201) and 9.2-kb *Bam*HI-*Hin*dIII (pRF203) fragments were subcloned in pLAFR3 (Fig. 1). Two complementation groups were identified based on the ability of the subclones to fully restore pathogenicity to chromosomal mutants. pRF205 complemented RDF6011, and pRF203 complemented MU43, MU51, MU136, and MU141. The mutation in RDF6011 was tentatively designated the *wtsA* region, and the Mu-induced mutations were designated the *wtsB* region. pRF207 did not complement any of the mutations.

In contrast to pRF205, pRF204, which contains the same 1.8-kb *Hin*dIII fragment in the opposite orientation, did not complement RDF6011. This result indicates that the *wtsA* region was transcribed from the vector P*lac* promoter in pRF205. *wtsA* is therefore transcribed from right to left, and its promoter lies to the right of the second *Hin*dIII site.

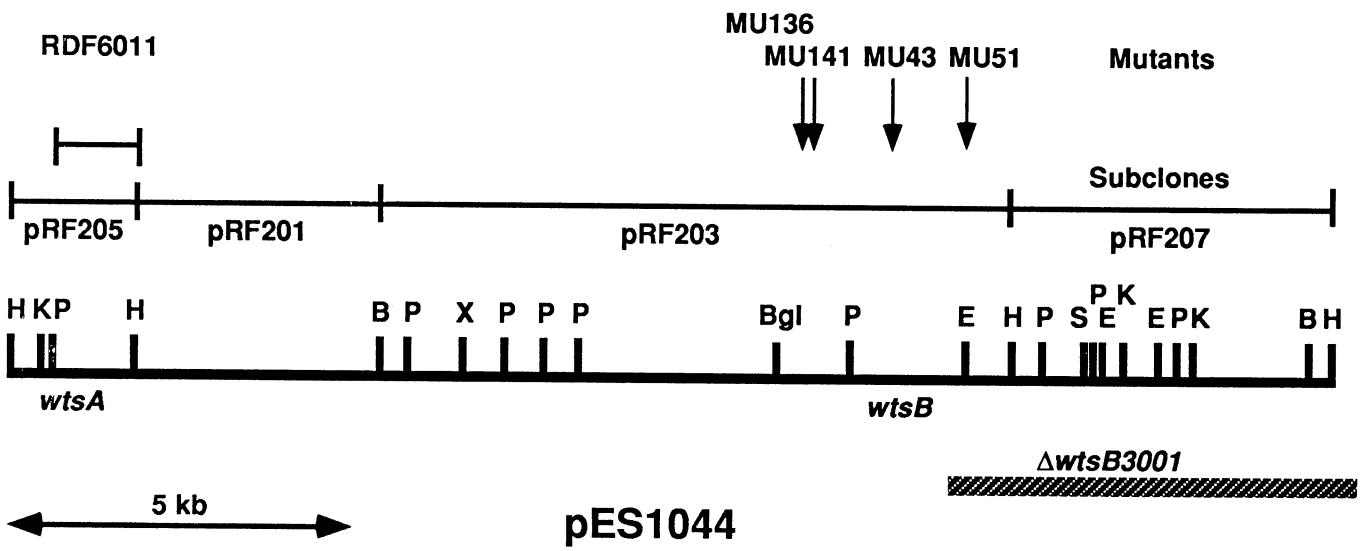

Fig. 1. Restriction map of the *wts* region cloned in pES1044. The inserts in subclones of pES1044 are indicated above the map. Arrows mark the sites of chromosomal Mu pf7701 insertions in *wtsB* and the bar shows the approximate location of a spontaneous insertion in the chromosome of RDF6011 affecting *wtsA*. The striped bar below the map shows the DNA deleted in strain DM3001; this deletion may extend beyond the right end of the pES1044 insert. Restriction sites are as follows: H = *Hin*dIII, E = *Eco*R1, Bgl = *Bgl*II, B = *Bam*H1, K = *Kpn*I, P = *Pst*I, S = *Sma*I, and X = *Xba*I. No *Xho*I sites were present.

Isolation of *wts* deletion mutants. *E. stewartii* is not sensitive to bacteriophage Mu infection but will support Mu replication if the prophage is introduced into the cell via plasmid transfer (Coplin 1979). Incubation of Mu *cts62* lysogens for several hours at 42° C followed by a shift down to 32° C is sufficient to induce Mu replication and cell lysis. MU51 *wtsB*::Mu pf7701 was cycled twice at 42° C to enrich for strains that had lost Mu pf7701 by deletion or imprecise excision. Two avirulent Km^s strains, DM3020 and DM3001, were obtained in which the prophage and adjacent DNA were deleted. Southern hybridizations, in which pES1044 was used as a probe, revealed that DM3020 had a large deletion that spanned the entire length of the pES1044 insert. DM3001 was missing the 2.7-kb *Pst*I fragment and all other DNA to its right. One strongly hybridizing junction fragment was present, indicating the deletion probably started in the right half of the 2.7-kb *Pst*I fragment and extended beyond the right end of pES1044 (Fig. 1). Because imprecise excision of Mu often creates deletions extending in one direction from the site in integration (Howe and Bade 1975), it is likely that the left end of the deletion in DM3001 corresponds to the site of Mu insertion in MU51, which is at the right end of the *wtsB* region. pES1044 fully restored the virulence of DM3001, but not DM3020. pRF203 did not complement DM3001.

Transposon mutagenesis and complementation analysis. To locate the *wts* genes on pES1044, this plasmid was mutagenized with transposons Tn*3*HoHoI and Tn*5*. Twenty-seven Tn*3*HoHoI and 41 Tn*5* insertions were mapped in pES1044 as shown in Figure 2. All of these insertions were independent and unique.

The transposon-mutagenized plasmids were mobilized to strains RDF6011, MU136, and DM3001 and tested for their ability to restore pathogenicity to the recipient strain (Fig. 2 and Table 2). The mutations that failed to com-

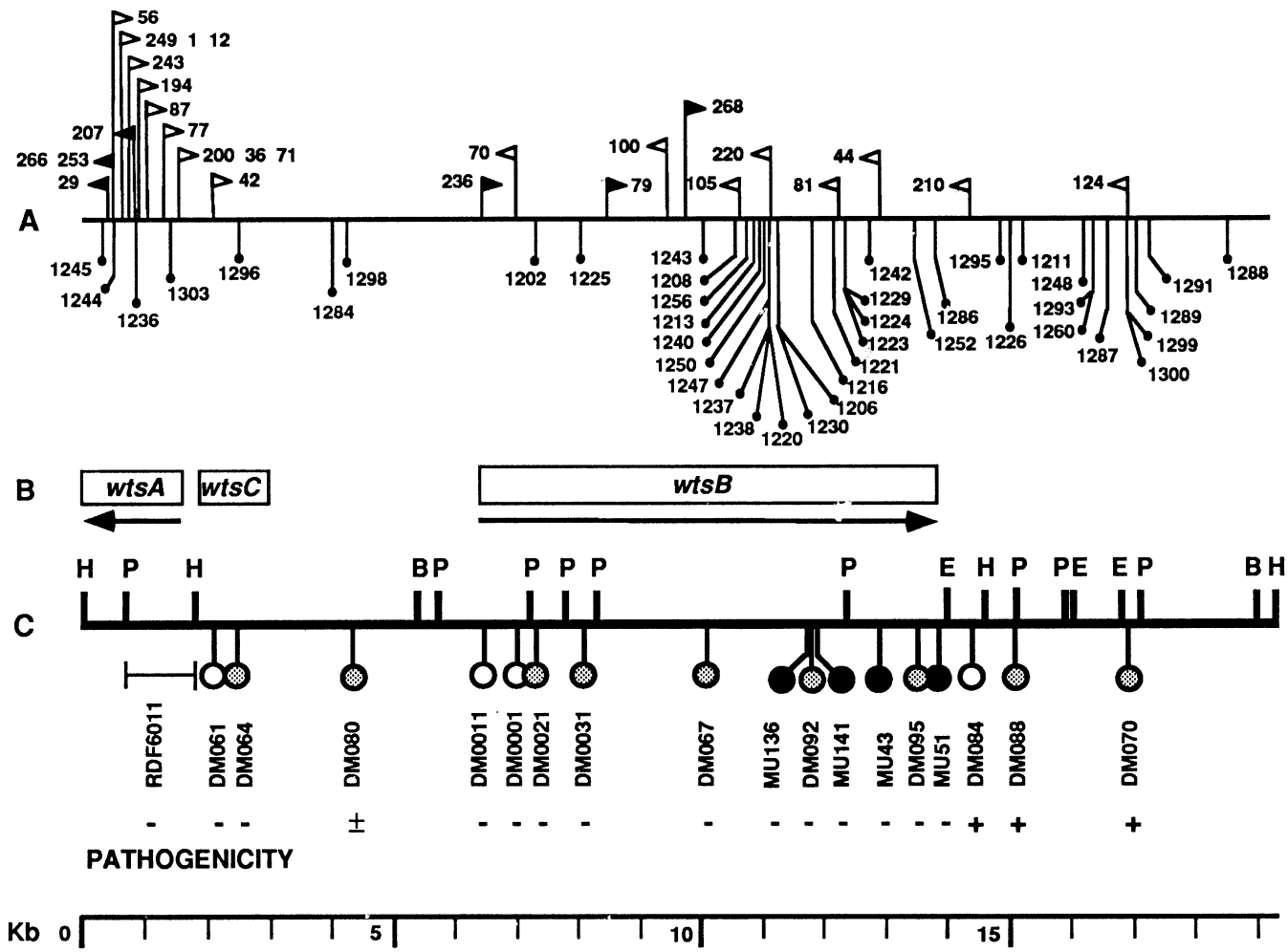

Fig. 2. Transposon mutagenesis, marker-exchange mutagenesis, and complementation analysis of the *wts* region. **A,** Plasmid mutations. Flags above the line show positions of Tn*3*HoHoI insertions and point in the direction of *lacZ* transcription. Solid flags denote Lac^+ gene fusions. Positions of Tn*5* insertions are shown below the line. Numbers designate *wts* mutant alleles. **B,** Complementation groups. Plasmid mutations above a box did not complement chromosomal mutations below the box. **C,** Chromosomal mutations. Tn*3*HoHoI (open symbol) and Tn*5* (shaded symbol) insertions that were marker exchanged into the chromosome are shown below the restriction map. The bar shows the approximate location of a spontaneous insertion in RDF6011 *wtsA* and the filled insertion symbols denote Mu pf7701 insertions. Pathogenicity in whorl assays (+ or −) is given below each strain number.

plement RDF6011 were located from map positions 0.3 to 1.6 kb, and the mutations failing to fully complement MU136 and DM3001 were located between 7.3 and 13.8 kb. These two complementation groups corresponded to those defined by complementation with pRF205 and pRF203, which have been designated *wtsA* and *wtsB*, respectively. They are shown by the boxes in Figure 2. Two insertions adjacent to the left end of *wtsB* (insertions 236 and 70) gave variable results between pathogenicity tests with certain crosses, suggesting they may have caused mutations in *wtsB*. The 15 pES1044 plasmids with insertions to the right of *wtsB* restored pathogenicity to DM3001. Therefore, these insertions probably did not inactivate any pathogenicity genes because DM3001 was missing this region of the chromosome.

To better define the borders of *wtsB* and to test for additional *wts* genes, selected mutations in pES1044 were introduced into the chromosome of DC283 by marker exchange; the homogenotes were verified by Southern hybridizations and tested for pathogenicity. Five mutations between *wtsA* and *wtsB* (insertions 42, 1296, 1298, 236, and 70) and four mutations within *wtsB* (wtsB1202, wtsB1225, wtsB1243, and wtsB1216) had an avirulent phenotype (Fig. 2). As reported previously for the Mu-induced *wts* mutants (McCammon *et al.* 1985), the homogenotes were prototrophic and grew normally on minimal media. Their pathogenicity was completely restored by introduction of pES1044. In addition, *wts-42* and *wts-1296* were fully complemented by pRF201 and *wts-236*, *wts-70*, *wtsB1202*, *wtsB1225*, *wtsB1243*, and *wtsB1216* were fully complemented by pRF203. Three insertions to the right of *wtsB* (insertions 210, 1226, and 124) remained fully virulent after marker exchange (Fig. 2). We were not successful in marker exchanging insertion 1284 or any of the wtsA mutant alleles.

Plasmid-borne and chromosomal *wts* mutants were used in more extensive complementation analyses, the results of which are shown in Table 2. These experiments confirmed the above assignment of mutants to the *wtsA* and *wtsB* complementation groups. The two mutations adjacent to *wtsB* (*wts-236* and *wts-70*) were found to be part of *wtsB*, thereby extending this region leftward to map position 6.5 kb. Two mutations near the right end of *wtsA* (*wts-42* and *wts-1296*) formed a new complementation group that we have designated *wtsC*. The status of the two mutations between *wtsC* and *wtsB* (insertions 1284 and 1298) is still uncertain. Although strain DM080, resulting from marker exchange of *wts-1298*, was avirulent, the homologous complementation test between pDM1298 and DM080 was positive. This result could be due to leakiness

Table 2. *Trans* complementation analysis of pES1044 and chromosomal *wts* mutants

			Water-soaking rating[a,b,c]										
Plasmid	**Allele**	**Map Position (kb)[d]**	**RDF6011 *wtsA6011***	**DM061 *wtsC42* (2.09)**	**DM064 *wtsC1296* (2.47)**	**DE0011 *wtsB236* (6.46)**	**DE0001 *wtsB70* (7.00)**	**DE0031 *wtsB1225* (8.04)**	**MU136 *wtsB* (11.8)**	**MU141 *wtsB* (11.9)**	**MU43 *wtsB* (12.9)**	**MU51 *wtsB* (14.0)**	**DM3001 Δ*wtsB***
pDM1029	*wtsA243*::Tn*3*HoHoI	0.76	**2**	6	6	6	5	6	6	5	6	4	6
pDM1236	*wtsA1236*::Tn*5*	0.87	**2**	4	6	6	6	6	6	NT	NT	NT	6
pDM1016	*wtsA87*::Tn*3*HoHoI	1.06	**1**	5	3	NT	NT	NT	6	6	5	5	5
pDM1004	*wtsA36*::Tn*3*HoHoI	1.56	**2**	6	6	6	6	6	5	6	6	6	5
pDM1005	*wtsC42*::Tn*3*HoHoI	2.09	3	**3**	**2**	5	5	5	6	3v	4	3v	4
pDM1296	*wtsC1296*::Tn*5*	2.47	6	**3**	**2**	3	6	6	6	3	6	4	6
pDM1284	*1284*::Tn*5*	4.02	6	6	6	4	4	6	6	6	6	3	6
pDM1298	*wts-1298*::Tn*5*	4.23	6	6	6	4	6	6	6	6	6	4	6
pDM1028	*wtsB236*::Tn*3*HoHoI	6.46	6	6	6	**1**	**1**	**1**	**2v**	**2v**	**2**	**1**	**2**
pDM1009	*wtsB70*::Tn*3*HoHoI	7.00	6	5	3	**1**	**1**	**1**	**1**	**2v**	**2**	**1**	**1v**
pDM1202	*wtsB1202*::Tn*5*	7.29	6	6	4	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1v**
pDM1225	*wtsB1225*::Tn*5*	8.04	6	4	4	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1v**
pDM1013	*wtsB79*::Tn*3*HoHoI	8.42	6	NT	NT	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1v**
pDM1017	*wtsB100*::Tn*3*HoHoI	9.44	4	NT	NT	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
pDM1034	*wtsB268*::Tn*3*HoHoI	9.73	4	6	6	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**
pDM1018	*wtsB105*::Tn*3*HoHoI	10.6	6	NT	NT	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
pDM1027	*wtsB220*::Tn*3*HoHoI	11.2	6	NT	NT	**1**	**3**	**2**	**1**	**1**	**1**	**1**	**1**
pDM1216	*wtsB1216*::Tn*5*	11.8	6	NT	NT	**2**	**2**	**3**	**1**	**1**	**1**	**1**	**1**
pDM1014	*wtsB81*::Tn*3*HoHoI	12.2	5	NT	NT	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1v**
pDM1242	*wtsB1242*::Tn*5*	12.7	6	NT	NT	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**
pDM1006	*wtsB44*::Tn*3*HoHoI	12.9	6	NT	NT	**2**	**2**	**2**	**1**	**1**	**1**	**1**	**1**
pDM1252	*wtsB1252*::Tn*5*	13.5	6	NT	NT	**1**	**1**	**2**	**1**	**1**	**1**	**2**	**1**
pDM1025	*210*::Tn*3*HoHoI	14.4	3	NT	NT	5	6	5	5	5	3	4	4
pDM1019	*124*::Tn*3*HoHoI	16.9	6	NT	NT	4	5	6	5	5	6	5	6
pES1044	*wts*$^+$	...	6	6	6	6	6	6	6	6	6	6	6
None	...	...	1	1	1	1	1	1	1	1	1	1	1

[a] Water-soaking rating: 1 = no symptoms; 3 = scattered lesions turning necrotic early; 5 = numerous water-soaked lesions; 6 = numerous water-soaked lesions and ooze; v = variable results between experiments; NT = not tested. Ratings within a complementation group are shown in boldface.

[b] *wtsA*::Tn*5* alleles 1245 and 1244 and *wtsA*::Tn*3*HoHoI alleles 29, 56, 266, 253, 249, 1, 2, 207, 194, 87, 77, and 200 (Fig. 2) had ratings of 1 to 2 in RDF6011 and 5 to 6 in MU136.

[c] *wtsB*::Tn*5* alleles 1243, 1208, 1256, 1213, 1240, 1250, 1247, 1238, 1236, 1220, 1230, 1206, 1216, 1221, 1223, 1224, and 1229 (Fig. 2) had ratings of 1 to 2 in MU136 and 5 to 6 in RDF6011.

[d] Distance in kb from left end of pES1044 insert. Also given for chromosomal mutants (below column headings).

of the *wts-1298* mutation when present in multicopy or to suppression of its phenotype by increasing the copy number of other *wts* genes in pES1044.

Expression of *wts::lac* fusions. Tn*3*HoHoI can create transcriptional β-galactosidase gene fusions if it is inserted in the correct orientation downstream of an indigenous promoter and translational fusions if it is located in the correct reading frame (Stachel *et al.* 1985). *lacZ* is a convenient reporter gene to use in *E. stewartii* because wild-type strains cannot utilize lactose unless a functional β-galactosidase gene is introduced into them. Insertions *wtsA266, wtsA207, wtsB79,* and *wtsB268* created Lac^+ gene fusions (Fig. 2). The orientation of the transposons in these mutants indicates that *wtsA* is transcribed from left to right and *wtsB* from right to left as shown in Figure 2. Because only a single Lac^- Tn*3*HoHoI insertion was obtained in *wtsC,* we could not determine the direction of transcription of this cistron.

The above reporter gene fusions were used to investigate *wts* gene expression in common culture media and *in planta.* The expression of the *wtsA* and *wtsB* gene fusions in pDM1024 *wtsA207::lacZ*, pDM1013 *wtsB79::lacZ*, and pDM1034 *wtsB268::lacZ* was tested in wild-type strain DC283 to ensure proper gene regulation. Controls consisted of oppositely oriented (Lac^-) insertions in *wtsA* and *wtsB.* These strains were grown in Luria broth, casamino acids-peptone-glucose broth (CPG), DBG minimal liquid medium, and DBG medium amended with 0.1% yeast extract, casamino acids, or tryptone. β-Galactosidase activity was measured and the results are shown in Table 3. Both *wtsA* and *wtsB* were expressed coordinately in the amended DBG media and CPG broth. However, β-galactosidase levels were not above background in unamended DBG medium and were depressed 9- to 15-fold in Luria broth. Increasing the concentrations of yeast extract, casamino acids, and tryptone from 0.1 to 1.0% in DBG did not alter *lacZ* expression (data not shown).

Table 3. Expression of plasmid-borne *wts::lacZ* Tn*3*HoHoI gene fusions in bacteria grown in different culture media and in bacteria collected from ooze on lesions

	β-Galactosidase[a]		
Medium[b]	pDM1024[c] *wtsA207::lacZ*	pDM1013[c] *wtsB79::lacZ*	pDM1034[c] *wtsB268::lacZ*
LB	117	121	93
CPG	744	833	582
DBG	22	9	20
DBG + CA	1,829	1,063	1,072
DBG + YE	1,555	1,184	1,335
DBG + TRYP	1,890	1,102	1,046
Ooze	462	192	180

[a] β-Galactosidase units according to Miller (1972). Insertions of Tn*3*HoHoI in the Lac^- orientation, *wtsA56* (pDM1007) and *wtsB100* (pDM1017), were used as controls. β-Galactosidase levels for the controls were ≤30 units in liquid medium and ≤12 units in ooze. Values are the average of two experiments.

[b] LB, Luria broth; CPG, casamino acids-peptone-glucose broth; DBG, DBG minimal medium with 1% glucose; CA, 0.1% casamino acids; YE, 0.1% yeast extract; TRYP, 0.1% tryptone. Ooze was collected from the surface of water-soaked lesions at 3 days after inoculation.

[c] Plasmids were tested in wild-type *E. stewartii* strain DC283.

β-Galactosidase was also detected in washed bacteria recovered from ooze that formed on the surface of water-soaked lesions on whorl-inoculated corn seedlings. Both the *wtsA* and *wtsB* gene fusions were expressed at moderate levels (180 to 462 units) *in planta.* Ooze from control plants inoculated with the β-galactosidase deficient, parental strain (DC283) had negligible enzyme activity (<12 units) indicating that all plant β-galactosidase was removed from the bacteria by washing.

Growth of *wts* mutants *in planta.* To determine the growth of *wts* mutants *in planta* we used a procedure by which a uniform quantity of inoculum was added to the cut surface of a decapitated corn seedling. At specific time intervals, the bacterial population in the entire seedling was determined. The growth of DC283 wts^+, RDF6011 *wtsA*, MU136 *wtsB*, and DM064 *wtsC* was determined from 0 to 6 days and is shown in Figure 3. The parental strain, DC283, produced water-soaked lesions at 48 hr and severe wilting and necrosis by 144 hr. Populations of DC283 reached a maximum of 1×10^9 cfu/plant at 48 hr and then declined to 2×10^8 cfu/plant as the tissue wilted and dried up. In contrast, plants inoculated with the *wts* mutants remained symptom free, although the bacteria grew well during the first 48 hr. An estimate of the population doubling times was obtained by simple linear regression of log cfu/plant vs. time for the period from 2 to 48 hr. For DC283, RDF6011, MU136, and DM064 the doubling times (± SE) were 5.7 ± 0.2, 6.8 ± 0.8, 6.3 ± 0.8, and 7.0 ± 0.5 hr, respectively. When the doubling

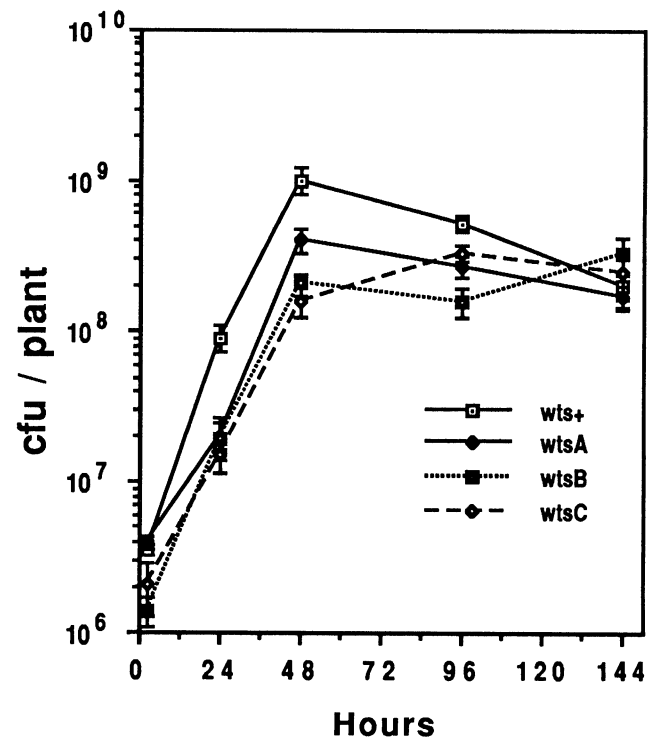

Fig. 3. Growth of *wts* mutants *in planta.* Corn seedlings were inoculated with DC283 wts^+, RDF6011 *wtsA*, MU136 *wtsB*, and DM064 *wtsC* as described in the text. At intervals entire plants were sampled. Bacterial populations are given as cfu/plant and error bars represent the standard error of the mean.

times for the mutants were compared to that of the wild type using Student's *t* tests, RDF6011 and MU136 were not significantly different from DC283 at the 5% level; however, DM064 differed from DC283 at the 5% level but not at 1% level of significance. After 48 hr, the mutant populations remained fairly constant at 1–3 $\times$ 10^8 cfu/plant. Comparable results were obtained using either stem injection or the whorl assay to inoculate plants, but the above inoculation technique produced less variability because sampling the entire plant avoided problems with nonuniform distribution of the bacteria throughout the vascular system.

In a similar study, DM3001 Δ*wtsB* was found to persist up to 6 wk after inoculation and was distributed throughout the plants. Populations in stalks ranged from 4 $\times$ 10^6 to 3 $\times$ 10^7 cfu/g tissue (fresh weight) and were about 3 $\times$ 10^3 cfu/g in leaves. About half of the plants appeared healthy, but the remainder exhibited slight stunting and chlorosis and had several elongated lesions on the leaves.

DISCUSSION

The only presently known virulence factor in *E. stewartii* is EPS (Coplin and Majerczak 1990). Other classical virulence factors, such as degradative enzymes and toxins, are notably absent (Braun 1990). The simplest model for pathogenicity in this bacterium therefore involves the EPS, an unknown cell-leakage factor to cause Wts symptoms, and maybe a set of underlying basic pathogenicity genes similar to *hrp* genes (Willis *et al.* 1991). We describe here a large cluster of pathogenicity genes (*wts*) from *E. stewartii* that are required for both lesion formation and wilting. The finding that *wts* mutants grow well *in planta* during the first few days after infection suggests that these mutants are affected only in their ability to directly damage host tissue rather than in their ability to grow in the plant environment and avoid host defenses.

The *wts* gene cluster cloned in cosmid pES1044 spans 14 kb and includes three complementation groups, *wtsA, wtsC,* and *wtsB.* Analysis of deletion mutants obtained by excision of the Mu prophage from MU51 indicates that the complete *wts* region may be even larger. The deletion in DM3001 could be complemented by pES1044 even though it started at the right end of *wtsB* and extended beyond the right end of the pES1044 insert. On the other hand, the deletion in DM3020 encompassed all of the pES1044 insert and could not be complemented by this plasmid. These results suggest that additional *wts* genes may lie to the left of *wtsA*.

Complementation analysis using plasmid-borne *wts* mutations, subclones of pES1044, and chromosomal mutants revealed three *wts* complementation groups. *wtsA* mutants were defined by complementation of mutant RDF6011. The functional *wtsA* gene(s) is contained in the 1.8-kb *Hin*dIII fragment cloned in pRF205. This construct, however, appears to be transcribed from the vector P*lac* promoter, indicating that the *wtsA* promoter is located to the right of the *Hin*dIII site at 1.8 kb. Two mutations adjacent to *wtsA* defined a second complementation group, *wtsC.* Chromosomal *wtsC* mutations were complemented by pRF201, indicating that they do not have a polar effect on *wtsA* and are located in a different transcriptional unit. It is not known why *wtsC42* only partially complemented RDF6011, but this may reflect complex interactions between *wtsA* and other *wts* genes. The largest complementation group is *wtsB.* Mutations in this region span 7.5 kb and were complemented by pRF203. The size of the *wtsB* region suggests that it may be an operon comprised of many genes.

Some pathogenicity genes, such as the *Agrobacterium tumefaciens vir* genes, are induced by specific plant products (Peters and Verma 1990), whereas others are induced or repressed by a variety of nutritional and environmental signals. For example, the *hrp* genes of *Pseudomonas syringae* pathovars and *E. amylovora* (Willis *et al.* 1991) are repressed by organic nitrogen, pH, and osmolarity. The construction of *wts::lacZ* reporter gene fusions in this study allowed us to determine that *wtsA* and *wtsB* are expressed in culture and in ooze collected from lesions. The *wts* genes were not expressed in a minimal salts-glucose medium until it was amended with other nutrients. The ability of either yeast extract, casamino acids, or tryptone to increase β-galactosidase activity suggests that simple nutrients, rather than a specific plant product, are required for induction. The *wts* genes were also expressed in a rich medium, CPG broth, indicating that repression by organic nitrogen sources may not occur. However, the latter observations will need to be tested with chromosomally located gene fusions in order to avoid gene dosage effects that might bypass normal regulation.

It was recently discovered that pES1044 hybridizes with the right half of the *hrp* gene cluster from *E. amylovora* (Beer *et al.* 1990), suggesting that *wts* and *hrp* genes might have a common function in pathogenicity. The results of this study show that the *wts* genes are similar to some *hrp* genes in that they comprise a very large region of the chromosome and are expressed in nutritionally limiting media; however, *E. stewartii* is not known to cause a hypersensitive reaction in any host or nonhost. For this reason, we have chosen to retain the designation *wts*, rather than *hrp* or *dsp*, for the genes described in this paper. It will be interesting to learn if the *wts* genes encode the synthesis of the postulated cell-leakage factor or determine a more basic pathogenicity function.

ACKNOWLEDGMENTS

We thank D. Estes and Scot Olah for technical assistance.

Salaries and research support were provided by the U.S.Department of Agriculture under grant 83-CRCR-1-1205 from the Competitive Research Grants Office and grant 593 0009-13 from the Midwest Plant Biotechnology Consortium and by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

This is journal article 192-91 of the Ohio Agricultural Research and Development Center.

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