MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 5, pp. 286-292, 1990

Extracellular Polysaccharide Genes in *Erwinia stewartii*: Directed Mutagenesis and Complementation Analysis

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Received 10 October 1989. Accepted 20 March 1990.

A cosmid containing a cluster of *cps* genes required for extracellular polysaccharide synthesis in *Erwinia stewartii* was subjected to transposon mutagenesis, and the resulting *cps* mutations were crossed into the chromosome of *E. stewartii* SS104. *Trans* complementation tests between chromosomal mutants and mutant cosmids revealed that this cluster contains at least five complementation groups; these have been designated *cpsA* through *cpsE* and represent separate transcriptional units. The region from *cpsA* to *cpsD* is 10 kilobases long and appears to be contiguous. Strains of *E. stewartii* with mutations in *cpsA*, *cpsB*, and in some cases *cpsD* formed restricted, necrotic lesions on corn seedlings, whereas *cpsC* and *cpsE* mutants were completely avirulent. Strains with mutations in *cpsA* through *cpsD* grew normally *in planta* during the first 24 hr after infiltration into corn leaves, but a *cpsE* mutant failed to multiply.

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

Most plant pathogenic bacteria produce extracellular polysaccharides (EPSs) in the form of attached capsules and free slime. These polysaccharides may protect plant pathogens from phytoagglutinins, promote bacterial growth in intercellular spaces by retaining water, cause wilting by plugging xylem vessels, and aid in bacterial survival and insect transmission (Chatterjee and Vidaver 1986). *Erwinia stewartii* (Smith) Dye is a vascular pathogen of corn (*Zea mays* L.) whose only known virulence determinant is an exopolysaccharide. The EPS is produced as a bound capsule under all conditions and as copious slime when bacteria are grown in the presence of a readily fermentable sugar. It is an acidic heteropolysaccharide composed of glucose, galactose, and glucuronic acid (Huang 1980). The EPS occludes the xylem vessels of the corn plant and causes it to wilt (Bradshaw-Rouse *et al.* 1981; Braun 1982). In corn seedlings, bacteria also grow in the intercellular spaces of the leaves where they disrupt cell membrane function and cause the accumulation of fluids. This results in a "water-soaking" (Wts) symptom, which is typical of most bacterial leaf spot diseases. The mechanism of membrane damage by *E. stewartii* is not known; however, mutant analysis (Dolph *et al.* 1988) suggested that EPS has a role in enhancing Wts symptoms.

Dolph *et al.* (1988) characterized a 27-kilobase (kb) region of the *E. stewartii* chromosome containing the genes for EPS synthesis (*cps*). Genetic and physical maps of cosmid pES2144, which includes this region, were constructed by subcloning and transposon mutagenesis with Tn*5*, Tn*5lac*, and Tn*3*HoHo1. Transposon insertions that affected the ability of pES2144 to restore mucoidy to *cps* deletion mutants mapped in five clusters, tentatively designated *cps* regions A to E. The polarity of the *cps*::*lac* fusions constructed by Dolph *et al.* (1988) indicated that the genes in regions A through D are all transcribed in the same direction (from left to right as shown in Fig. 1). In addition, each is positively regulated by the product of the *rcsA* gene (Torres-Cabassa *et al.* 1987). A gene for uridine-5′-diphosphogalactose 4-epimerase (*galE*) is linked to the *cps* genes on pES2144; it is constitutively expressed and does not appear to be part of a *gal* operon (Dolph *et al.* 1988). Neutral (mucoid) transposon insertion mutations intervened between *cpsD*, *cpsE*, and *galE*, indicating that these regions are transcribed separately. However, we did not obtain any completely neutral insertions between the *cpsA*, *cpsB*, *cpsC*, and *cpsD* regions, so we could not determine if these clusters of mutations represent more than one operon.

The virulence of different *cps* mutants was tested by Dolph *et al.* (1988) using strains with large deletions of the chromosomal *cps* region that were carrying transposon-induced *cps* mutations in *trans* on pES2144. All of the *cps* mutants were uniformly unable to cause systemic wilting of corn seedlings, but varied in their ability to promote Wts. Strains with plasmid-borne *cpsA* and *galE* mutations produced small amounts of EPS and caused Wts, whereas those with *cpsD* and *cpsE* mutations were avirulent. We could not show that the *cpsB* and *cpsC* regions were necessary for Wts ability.

In this study, we examined the contribution of each *cps* region to EPS production and virulence using strains with chromosomal rather than plasmid-borne mutations. We also determined that each of the *cps* regions corresponds to a separate complementation group and that most of the 10-kb region from *cpsA* to *cpsD* is involved in EPS production. A preliminary report of these findings has been published (Coplin and Majerczak 1988).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacteria and plasmids used in this study are listed in Table 1. All strains of *E. stewartii* were derived from DC283, which is a spontaneous nalidixic acid-resistant (Nal^r) mutant of wild-type strain SS104 (Coplin *et al.* 1981). Additional subclones

of pES2144 (pDM200 and pDM201; Table 1) were constructed in pLAFR3 (Staskawicz *et al.* 1987). The derivatives of pES2144 carrying transposon-induced *cps* mutations isolated by Dolph *et al.* (1988) are listed in Table 2. In this study, pDM453 *cps-453* and pDM449 *cps-449* were derived by mutagenesis of pES2144 with Tn*5lac* (Kroos and Kaiser 1984). Further restriction analysis of plasmid pPD2014 *cps-60* (Dolph *et al.* 1988) revealed that it lacks a 1.4-kb segment of DNA adjacent to the site of the Tn*3*HoHo1 insertion (Fig. 1).

Culture media and growth and mating conditions for *E. stewartii* have been described previously (Coplin 1978; Coplin *et al.* 1986). Colony type was evaluated on casamino acids-peptone-glucose (CPG) agar (Bradshaw-Rouse *et al.* 1981). Assays for β-galactosidase were done according to Miller (1972). Plasmids were mobilized from a rifampicin-resistant derivative of *Escherichia coli* HB101 into *E. stewartii* with pRK2013::Tn*7* as described previously (Coplin *et al.* 1986).

Replacement mutagenesis. Mutant pES2144 *cps*::Tn*3*HoHo1 or *cps*::Tn*5lac* plasmids were conjugated into parental strain DC283 with selection for tetracycline-resistant transconjugants. After several transfers on antibiotic-containing media (kanamycin for Tn*5lac* and ampicillin for Tn*3*HoHo1) to allow time for recombination between the plasmid and chromosome to occur, pES2144 was displaced by the introduction of an incompatible plasmid, pPH1JI. Transconjugants were selected for kanamycin and gentamycin resistance or ampicillin and gentamycin resistance and then screened for tetracycline-sensitivity, nonmucoid colonies. Exchange of the transposon insertions into the chromosome was verified by genomic Southern blots probed with appropriate subclones of pES2144 (that is pPD183, pDM201, or pPD012). Loss of the vector sequences and the wild-type chromosomal fragment, accompanied by the appearance of the predicted junction fragments, was observed in each case.

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

EPS determination. EPS was extracted from bacterial suspensions made from 48-hr-old CPG agar plate cultures, and total carbohydrate content (glucose equivalents) was determined by the anthrone reaction as previously described (Coplin *et al.* 1986). EPS production was normalized to viable cell counts of the suspensions made before extraction.

Virulence assays. Wts ability was assayed by a whorl inoculation technique (Coplin *et al.* 1986) on 8-day-old sweet corn (cv. Earliking) seedlings in two different controlled-environment chambers (29° C, 16-hr light and 8-hr dark cycle, 310–355 $\mu E \cdot m^{-2} \cdot sec^{-1}$, 90% relative humidity). Symptoms were rated on the following continuous scale at 3 days after inoculation: 0 = no symptoms; 1 = scattered small lesions; 2 = numerous lesions; and 3 = extensive lesions that remained water-soaked, with ooze forming on leaf surfaces. Wts assays were repeated from 4 to 12 times in each chamber. Means were compared using Fisher's LSD.

Wilting ability was assayed in 8-day-old seedlings inoculated by wounding the pseudostem 1 cm above the soil line. Symptoms were recorded at 10 days after inoculation.

Growth of bacteria *in planta*. To determine growth *in planta*, leaves of 7-day-old corn seedlings were vacuum infiltrated with a bacterial suspension (10^8 cells per milliliter in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.2% Tween 40). Infiltrated areas were sampled for viable cell counts at 6 and 24 hr after inoculation. Three leaf disks (1-cm diameter), each from a different seedling, were pooled to form a replicate. Population increases were expressed as log N/N_0, where N_0 was the population at 6 hr and N was the population at 24 hr. Means were taken

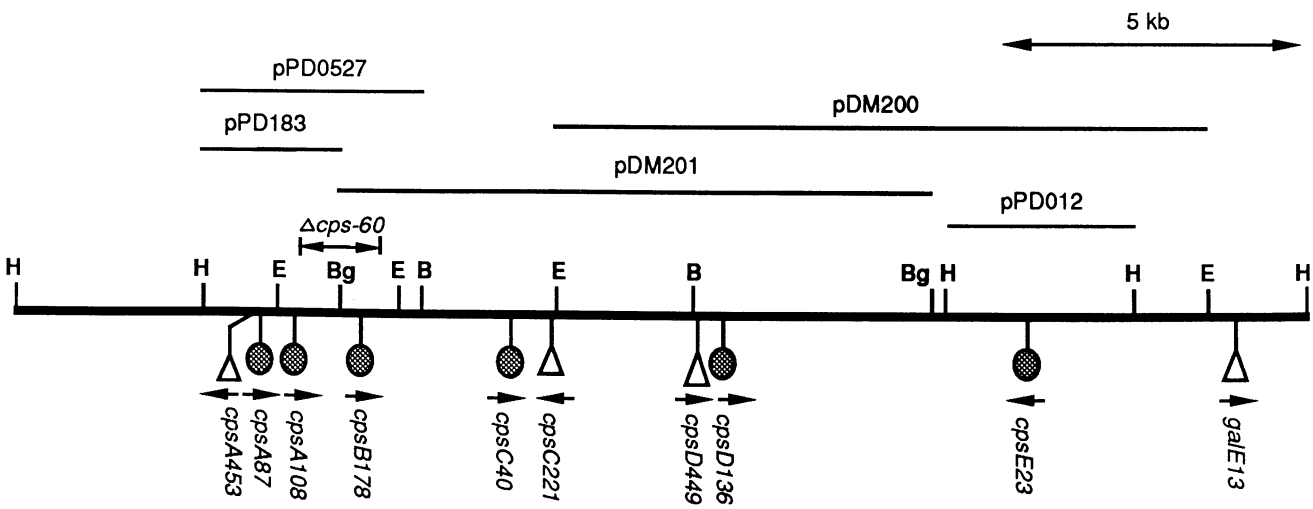

Fig. 1. Restriction map of pES2144 showing Tn*3*HoHo1 (●) and Tn*5lac* (△) insertions that were introduced into the chromosome. The Tn*3*HoHo1 insertion in *cps-60* has an adjacent deletion, which is shown above the map. Arrows indicate the orientation of *lacZ* in the transposon. Insertions oriented from left to right are Lac^+; those in the opposite orientation are Lac^- (Dolph *et al.* 1988). Bars above the map delineate subclones of pES2144 that were used in complementation tests. Restriction sites are as follows: H = *Hin*dIII, E = *Eco*RI, Bg = *Bgl*II, and B = *Bam*HI.

over three replicates and compared using Fisher's LSD.

Complementation tests. Plasmids derived from pES2144 were transferred into *E. stewartii* recipients from *E. coli* HB101 in triparental matings by mobilization with the helper plasmid pRK2013::Tn*7*. Overnight, L broth cultures (5×10^9 cells per milliliter) of the plasmid donor, the helper-plasmid donor, and the *E. stewartii* Nal^r recipient were mixed 1:1:2, respectively, in the wells of a microtiter plate and then transferred with a multipoint inoculator to an L agar plate. The bacteria were allowed to conjugate for 6 hr at 28° C and then transferred with a velveteen replicator pad to L agar and CPG agar plates containing tetracycline and nalidixic acid. Bacterial growth from the L agar plates was purified by streaking onto CPG agar to check for mixed colony types. Transconjugants were rated for mucoidy on CPG agar and for growth on L agar. A scale from 1 to 5 was used to rate the predominate colony type for EPS production: 1 = butyrous (nonmucoid), 3 = intermediate (partially mucoid), and 5 = very fluidal (mucoid).

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant properties[a]	Source or reference
Erwinia stewartii		
DC283	ICPPB SS104 Nal^r	Coplin *et al.* 1981
GAL8	Δ*cpsB-galE*, from DC283	Dolph *et al.* 1988
K91J	Δ*cpsA-cpsC*, from DC283	Dolph *et al.* 1988
Escherichia coli		
HB101	*thr leu thi recA hsdR hsdM pro* Sm^r	Boyer and Roulland-Dussoix 1969
Plasmids		
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]
pES2144	*cps*$^+$ pVK100 cosmid	Coplin *et al.* 1986
pPD012	3.5-kb *Hin*dIII fragment from pES2144 in pVK100	Dolph *et al.* 1988
pPD183	2.6-kb *Hin*dIII-*Bgl*II fragment from pES2144 in pLAFR3	Dolph *et al.* 1988
pPD0527	4.1-kb *Hin*dIII-*Bam*HI fragment from pES2144 in pLAFR3	Dolph *et al.* 1988
pDM200	12.2-kb *Eco*RI fragment from pES2144 in pLAFR3	This study
pDM201	11.1-kb *Bgl*II fragment from pES2144 in pLAFR3	This study
pPD1210	pPD0527 *cps-28*::Tn*5*	Dolph *et al.* 1988
pPD1213	pPD0527 *cps-60*::Tn*5*	Dolph *et al.* 1988
pPD1217	pPD0527 *cps-90*::Tn*5*	Dolph *et al.* 1988
pPD2017	pES2144 *cps-72*::Tn*3*HoHo1	Dolph *et al.* 1988
pPD2024	pES2144 *cps-108*::Tn*3*HoHo1	Dolph *et al.* 1988
pPD2031	pES2144 *cps-121*::Tn*3*HoHo1	Dolph *et al.* 1988

[a] Nal^r, Tc^r, Gm^r, Cm^r, Sm^r, Sp^r, and Tp^r indicate resistance to nalidixic acid, tetracycline, gentamycin, chloramphenicol, streptomycin, spectinomycin, and trimethoprim, respectively; kb, kilobase.
[b] Virginia Polytechnic Institute and State University, Blacksburg.

RESULTS

Chromosomal *cps* mutants. To examine the phenotype of single-copy mutations in *cps* genes, 11 transposon insertions (Fig. 1) were introduced into the chromosome of parental strain DC283 from derivatives of pES2144. Nonmucoid chromosomal mutants for each *cps* region and *galE* were obtained (Table 2). The constructions were verified by blot hybridization. Mucoidy could be restored to each mutant by reintroduction of pES2144. EPS production was measured and is given in Table 2. All of the mutants produced small amounts of EPS ($1–2 \times 10^{-8}$ μg per cell) compared to the wild-type strain (18×10^{-8} μg per cell). Unlike other *cps* mutations, those in *cpsA* had an intermediate colony type when present on a plasmid in K91J Δ*cpsA—C*, but were nonmucoid when introduced into the chromosome (Tables 2 and 3). Some strains with insertions in *cpsB* (DM138), *cpsC* (DM101), *cpsD* (DM113), and *cpsE* (DM145) grew very poorly; colonies were small and slow to develop, indicating that these mutations were detrimental to the bacterium.

Some of the Tn*3*HoHo1 insertions (*cps-87*, *cps-178*, *cps-60*, *cps-40*, and *cps-136*) and one of the Tn*5lac* insertions (*cps-449*) created *cps*::*lac* transcriptional fusions (Fig. 1, Dolph *et al.* 1988). In pES2144, the Tn*3*HoHo1 fusions expressed 11–48 units of β-galactosidase; however, this decreased to 2–10 units after the mutations were crossed into the chromosome. In contrast, the *cpsD449*::Tn*5lac* fusion was expressed much better in both cases. It produced

Table 2. Extracellular polysaccharide (EPS) production by chromosomal *cps* mutants derived by marker exchange of pES2144 plasmids containing *cps* insertion mutations

Region	Chromosomal mutant	Plasmid mutant	*cps* allele	Lac phenotype[a]	EPS production (μg/cell × 10^8)
A	DM223	pDM453	*cps-453*::Tn*5lac*	−	1
	DM136	pPD2019[a]	*cps-87*::Tn*3*HoHo1	+	1
	DM149	pPD2024[a]	*cps-108*::Tn*3*HoHo1	+	1
B	DM138	pPD2041[a]	*cps-178*::Tn*3*HoHo1	+	2
ΔA—B	DM108	pPD2014[a]	Δ*cps-60*::Tn*3*HoHo1	+	1
C	DM101	pPD2013[a]	*cps-40*::Tn*3*HoHo1	+	1
	PJD1910	pPD1910[a]	*cps-221*::Tn*5lac*	−	2
D	DM220	pDM449	*cps-449*::Tn*5lac*	+	1
	DM113	pPD2037[a]	*cps-136*::Tn*3*HoHo1	+	1
E	DM145	pPD208[a]	*cps-23*::Tn*3*HoHo1	−	1
galE	DM201	pPD195[a]	*galE130*::Tn*5lac*	+	1
	DC283	None	*cps*$^+$	−	18

[a] Dolph *et al.* 1988.

2,000–2,500 units on pES2144 and 300–500 units after introduction into the chromosome.

Virulence tests. In wilt assays, none of the chromosomal *cps* mutants were able to cause any systemic wilting of corn seedlings (data not shown). However, they varied in their ability to cause water-soaked lesions in the whorl assay. The virulence tests were done in two plant growth chambers at Wooster and Columbus, OH, and four strains (DM223 *cpsA453*, DM138 *cpsB178*, DM220 *cpsD449*, and DM201 *galE130*) differed in virulence depending on the chamber used. Data obtained in both chambers are given in Figure 2. All of the *cps* mutants had significantly decreased Wts ratings (5% level) compared to parental strain DC283, except for DM223 in Columbus. The *cpsA* and *cpsB* mutants, the *cpsD449* mutant, and the *galE* mutant caused water-soaked lesions that were smaller, were fewer in number, and turned necrotic sooner than those produced by DC283. Both *cpsC* mutants, the *cpsD136* mutant, and the *cpsE* mutant were almost completely avirulent and formed only a few restricted lesions.

Table 3. *Trans* complementation of chromosomal *cpsA* and *cpsB* mutants with pES2144 *cps*::Tn*3*HoHo1 and pPD0527 *cps*::Tn*5* plasmids

Group	Plasmid allele	EPS rating[a] K91J Δ*cpsABC*	DM136 *cpsA87*	DM223 *cpsA453*	DM108 Δ*cps-60*	DM138 *cpsB178*
A	*cps-87*[b]	3	3	2	2	5
A	*cps-453*[c]	3	2	2	2	5
A	*cps-72*[b]	3	3	3	3	5
A	*cps-108*[b]	3	3	3	3	5
A	*cps-121*[b]	3	3	3	3	5
A, B	*cps-32*[d]	ND[e]	1	1	1	1
B	*cps-90*[d]	ND	4	5	1	1
B	*cps-28*[d]	ND	5	5	1	1
B	*cps-178*[b]	1	3	4	1	1
B	*cps-61*[d]	ND	5	5	1	1
	cps^{+}[f]	5	4	5	5	5
	None	1	1	1	1	1

[a] Extracellular polysaccharide (EPS) rating: 1 = butyrous colony type to 5 = fluidal colony type.
[b] Tn*3*HoHo1 insertion in pES2144.
[c] Tn*5lac* insertion in pES2144.
[d] Tn*5* insertion in pPD0527.
[e] ND, not done; pPD0527 does not complement K91J.
[f] pES2144.

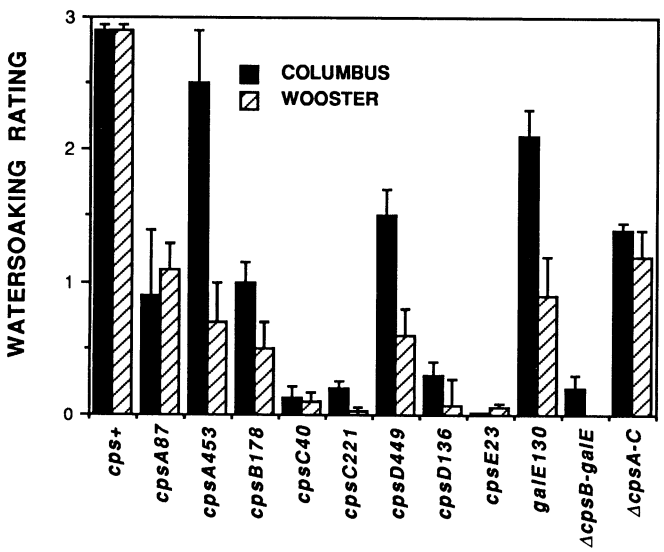

Fig. 2. Water-soaking caused by chromosomal *cps* mutants on sweet corn seedlings in growth chambers at Columbus and Wooster, OH. Symptoms were rated on the following scale after 3 days: 0 = no symptoms; 1 = scattered small lesions; 2 = numerous lesions; and 3 = extensive lesions that remained water-soaked, with ooze forming on leaf surfaces. DC283 is the parental strain; GAL8 and K91J are spontaneous deletion mutants (see Tables 1 and 2); and DM201 is *galE*. Complementation groups are indicated below the strain numbers.

The possibility that the decrease in virulence of *cps* mutants could be due to a detrimental effect of these mutations on the growth rate of the mutants *in planta* rather than the loss of EPS was investigated. Bacteria were vacuum infiltrated into leaves of corn seedlings, and the changes in populations between 6 and 24 hr after infiltration were measured (Fig. 3). Initial studies showed that the wild-type parental strain, DC283, grew exponentially during this time period (data not shown). All of the *cps* insertion mutants grew as well as or better than DC283, and their populations increased more than 47-fold, except for DM145 *cpsE*, which failed to multiply in the plant. By comparison, the population of GAL8, a Δ*cpsB-galE* deletion mutant, increased only sevenfold.

Complementation analysis of *cps* mutants. To determine if the previously defined *cps* regions represented different transcription units, we conducted *trans* complementation tests between different chromosomal and plasmid-borne *cps* mutations. The merodiploids were scored for colony type, and the results are shown in Tables 3 and 4. With the exception of *cps-60*, the *cps* mutants could be placed into five complementation groups corresponding to the original five *cps* regions; crosses involving mutations in different *cps* regions produced more mucoid merodiploids, whereas crosses between mutants in the same *cps* region did not increase EPS production. *cps-60*, which is a deletion mutation (Fig. 4), appeared to be in both *cpsA* and *cpsB* complementation groups. In most cases where complementation occurred, the plasmid converted the recipient strain

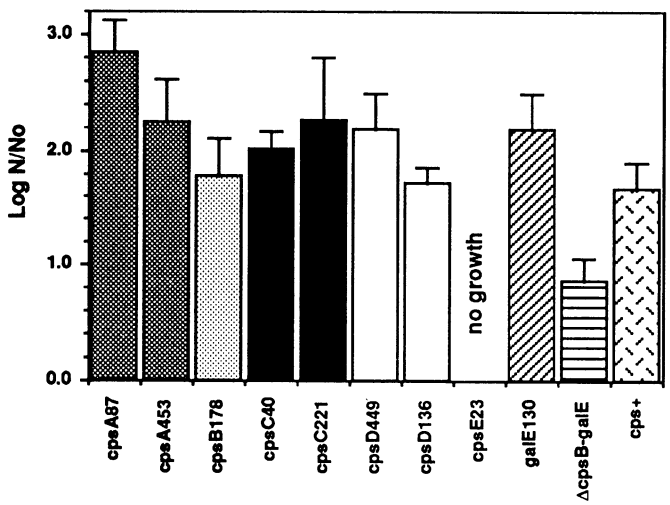

Fig. 3. Growth of chromosomal *cps* mutants in corn leaves. N_0 and N are the respective bacterial populations at 6 and 24 hr after vacuum infiltration. The experiment was done in Columbus, OH. Error bars indicate the standard error of the mean.

from a butyrous (EPS rating = 1) to fluidal (EPS rating = 4 or 5) colony type; however, two exceptions were observed. Crosses involving two *cpsA* mutations resulted in partially mucoid (EPS rating = 2 or 3), instead of butyrous, merodiploids. This was not considered to be complementation because the same plasmid-borne *cpsA* mutations were also partially mucoid in a Δ*cpsA—C* chromosomal background. In crosses where the trans-conjugants grew poorly (Table 4, footnote b), partial restoration of EPS production (EPS rating = 3) was considered to be complementation.

Dolph *et al.* (1988) reported several pES2144::Tn*3*HoHo1 insertions distal to region A that did not affect colony type (*cps-72*, *cps-108*, and *cps-121*; Fig. 4). We reexamined these plasmids in the *cpsA* chromosomal mutants and found that they had the partially mucoid phenotype typical of plasmid-borne *cpsA* mutations (Table 3). Furthermore, introduction of *cps-108* into the chromosome of DC283 resulted in loss of EPS production (Table 2). This suggests that these mutations are in the *cpsA* complementation group. Dolph *et al.* (1988) also isolated four pPD0527::Tn*5* insertions in the vicinity of *cpsB* (Fig. 4), but the phenotype of these mutations could not be determined for lack of chromosomal *cps* mutants that could be complemented by pPD0527. In the present study, three of these mutations (*cps-90*, *cps-28*, and *cps-61*) were placed in the *cpsB* complementation group (Table 3). However, *cps-32*, the leftmost insertion (Fig. 4), appears to be in both *cpsA* and *cpsB* complementation groups.

The ability of the subclones of pES2144 shown in Figure 1 to complement the chromosomal *cps* mutants was also tested. pPD183 fully complemented *cpsA* mutants; pPD0527, *cpsA* and *cpsB* mutants; pDM201, *cpsC* and *cpsD* mutants; and pPD012 and pDM200, the *cpsE* mutant. pDM201 did not complement *cpsB* mutants, and pDM200 did not complement *cpsD* mutants.

DISCUSSION

The genetics of EPS synthesis has been examined in very few bacteria despite the importance of capsules to the ecological fitness and pathogenicity of many species. In those systems that have been examined, clustering of genes for heteropolysaccharide synthesis has been reported. Large clusters of EPS genes occur in *E. coli* (Trisler and Gottesman 1984), *Xanthomonas campestris* (Pammel) Dowson (Harding *et al.* 1987; Thorne *et al.* 1987; Vanderslice *et al.* 1989), *Rhizobium* sp. strain NGR234 (Chen *et al.* 1988), *R. meliloti* Dangeard (Leigh *et al.* 1985), and *Zoogloea ramigera* Itzigsohn (Easson *et al.* 1987). In this study, we report that EPS genes in *E. stewartii* form a 10-kb cluster (*cpsA* through *cpsD*) with other EPS genes (*cpsE* and *galE*) nearby. This cluster is about the size needed to encode the enzymes in the EPS biosynthetic pathway. *E. stewartii* EPS has a heptasaccharide repeating unit with two side-chains (J. Costa, D. Horton, and D. Coplin, unpublished data), so at least seven specific transferases, one or more polymerases, and an export system would be necessary to produce it.

The construction of chromosomal *cps* mutations in *E. stewartii* permitted complementation analysis of the major *cps* gene cluster. Five complementation groups were identified (Table 4). Groups A through D are coordinately regulated by *rcsA* (Torres-Cabassa *et al.* 1987) and probably represent four operons, each containing one or more genes. Group E is represented by a single Tn*3*HoHo1 mutation, which is Lac$^-$, so we cannot tell how large this transcription unit is or how it is regulated. The complementation groups have been designated *cpsA* through *cpsE* to correspond to the clusters of transposon mutations identified by Dolph *et al.* (1988).

The following observations are evidence that *cpsA* through *cpsD* represent a contiguous cluster of *cps* genes. The *cpsA* operon is contained within the pPD183 subclone, and mutations in this region are not polar on *cpsB* mutants. Reevaluation of neutral transposon insertions distal to *cpsA* (Dolph *et al.* 1988; Fig. 4) revealed that they also have reduced EPS production and are probably within *cpsA*. This was confirmed by the loss of EPS production when *cps-108* was introduced into the chromosome. The *cps-90*::Tn*5* mutation extends *cpsB* to within 0.5 kb of *cpsA* (Fig. 4), so these two operons may be directly adjacent to each other. The failure of subclone pPD201 to complement *cpsB* is further evidence that the *cpsB* promoter lies to the left of the *Bgl*II site in Figure 4. Complementation of *cpsB* mutants by pPD0527 shows that this operon ends before the *Bam*HI site in Figure 4 and that mutations in this region are not polar on *cpsC*. In addition, *cps-61*

Table 4. *Trans* complementation analysis of *cps* mutants

Group	Chromosome *cps* allele or strain	Complementation by plasmid mutation[a]								
		Δ*cps-60*	*cps-178*	*cps-40*	*cps-221*	*cps-136*	*cps-449*	*cps-23*	*cps*$^+$	None
ΔA—B	Δ*cps-60*	−	−[b]	±[b,c]	+	±	±	ND[d]	+	−
B	*cps-178*	−[b]	−[b]	±[b,c]	+	±	+	ND	+	−[b]
C	*cps-40*	+	+[b,c]	−[b]	−[b,c]	+	+	ND	+	−[b]
	cps-221	+	±	−[b,c]	−	±	±	ND	+	−
D	*cps-136*	±	+[b]	+[b]	+	−[b]	−[b]	−[b]	±[b,c]	−[b]
	cps-449	+	±	±	+	−	−	±	+	−
E	*cps-23*	ND	ND	ND	ND	+[b]	+	−[b]	+	−[b]
ΔA—C	K91J	−	−[b]	−	−	+	+	+	+	−
ΔB—E	GAL8	−	−[b]	−	−	−	−	−	+	−

[a] Plasmids are pES2144 derivatives. Extracellular polysaccharide (EPS) rating: − = butyrous to + = fluidal colony type.
[b] Poor growing.
[c] Some fluidal recombinants present.
[d] ND, not done.

extends *cpsB* to within 1 kb of the first *cpsC* insertion (Fig. 4). Failure of pDM200 to complement *cpsD* mutants suggests that *cpsC* and *cpsD* are also contiguous and that the *cpsD* promoter is located within the small region between *cpsC221* and the *Eco*RI site at the beginning of pDM200.

The *cps-32* mutation, which maps between *cpsA* and *cpsB*, is interesting because it appears to be in both complementation groups A and B and, unlike upstream *cpsA* mutations, completely eliminates EPS production when located on a plasmid. The deletion associated with *cps-60*::Tn*3*HoHo1 also includes the *cps-32* insertion site and has the same phenotype. The basis for this finding is unknown, but a possible explanation is that these mutants lack a *cis*-acting control element which is necessary for expression of both *cpsA* and *cpsB*.

In most complementation tests, all of the purified transconjugants from a cross had the same colony type, so that recombination events, which rescued the wild-type *cps* allele, did not interfere with the interpretation of our results. However, some crosses between poor-growing mutants within the same complementation group produced about 1 to 10% stable fluidal transconjugants (Table 4, footnote c). The lack of a recombination deficient host for these studies, the presence of an incompatible plasmid, pPH1JI, in the recipients, and selection pressure from poor-growth effects probably contributed to the high apparent frequency of recombination and *cis* complementation in these cases. The poor growth noted for some *cps* mutants and merodiploids (Table 4) may have been caused by the accumulation of intermediates of EPS synthesis within the cell due to blockage of the pathway at a late step or a defect in export of the repeating unit. A similar lethal effect was observed for a subunit export mutant (*gumJ*) of *X. campestris* (Vanderslice *et al.* 1989). Alternatively, poor growth of *cps*::Tn*3*HoHo1 mutants may have been a consequence of fusing exported Cps proteins to β-galactosidase.

In *X. campestris*, it is possible to isolate mutants that cannot add glucuronic acid and mannose to the side-chain of xanthan gum (Betlach *et al.* 1987; Tait and Sutherland 1989). These mutants make a new polytrimeric EPS, but produce 100-fold less gum because the truncated subunit is not readily polymerized. The unusual phenotype of *cpsA* mutants of *E. stewartii* might be explained by a similar defect. Chromosomal *cpsA* mutants appeared nonmucoid, but they may still have made small amounts of an altered EPS lacking a side-chain. Increasing the copy number of the *cps* cluster in pES2144 *cpsA*/Δ*cpsA—C* merodiploids could have increased EPS production by boosting polymerase activity. It will be interesting to examine the sugar composition of EPS from *cpsA* strains to test this hypothesis.

A secondary purpose of this study was to construct *cps*::*lac* gene fusions to be used in studies on the expression of each operon in response to environmental signals and different regulatory genes. The *cpsD449*::Tn*5lac* fusion has already been very useful for this purpose. It expressed fairly high levels of β-galactosidase after introduction into the chromosome and continued to respond to *rcsA* and culture conditions (D. Coplin and D. Majerczak, unpublished data). However, the other *cps*::*lac* fusions, described in this study, produced low levels of β-galactosidase after they were introduced into the chromosome. This decrease was probably due to the lowered copy number of the genes.

The results of this study show that *E. stewartii* EPS is involved not only in causing wilt of corn seedlings but in the Wts process as well. Since the EPS is highly hygroscopic, its function in lesion formation may be to retain water released by damaged host cells and thereby enhance bacterial growth in the intercellular spaces. If this

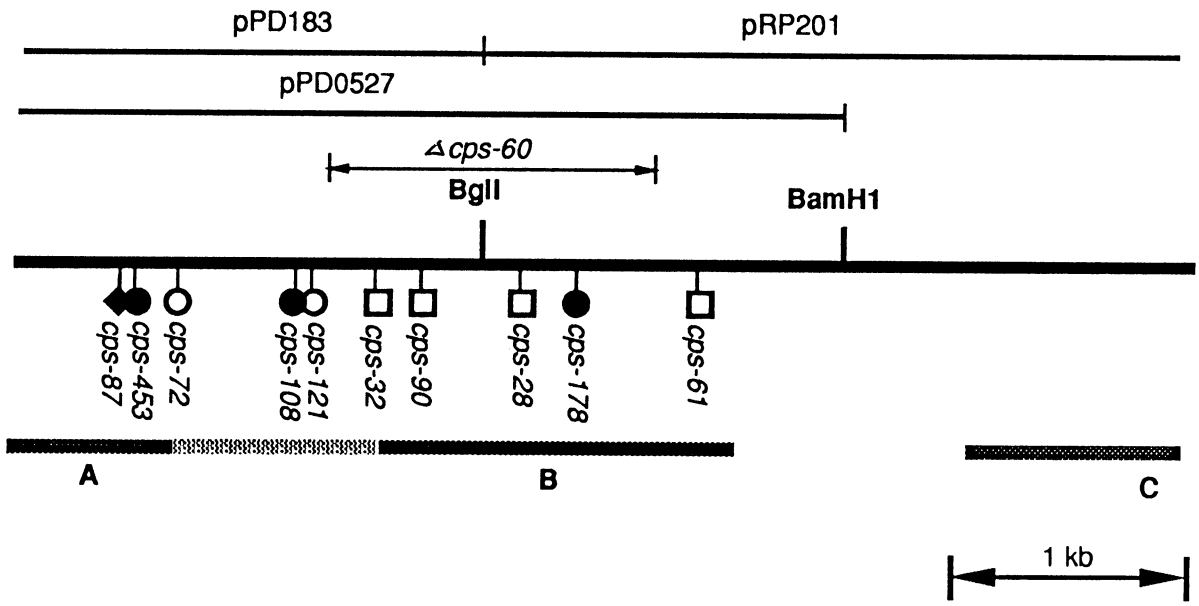

Fig. 4. Detailed map of the *cpsA-cpsB* region of pES2144 showing locations of Tn*3*HoHo1 (○), Tn*5lac* (◇), and Tn*5* (□) insertions used in complementation tests. Solid symbols are insertions that were introduced into the chromosome. Portions of subclones pPD183, pDM201, and pPD0527 as well as the deletion associated with *cps-60*::Tn*3*HoHo1 are shown above the map. *cps* regions A, B, and C are indicated by the more solid bars below the map. The lightly shaded bar indicates mutations that result in a partially mucoid colony type when plasmid-borne and that are probably in *cpsA*. *cps-32* had a nonmucoid colony type in both *cpsA* and *cpsB* hosts.

hypothesis is true, then bacteria without EPS would be expected to produce restricted lesions that turn necrotic much sooner than normal. The symptoms produced by *cpsA*, *cpsB*, and some *cpsD* mutants are consistent with this hypothesis. The variability observed in the Wts assays with some of these mutants (*cpsA453*, *cpsD449*, and *galE130*) between growth chambers suggests that the requirement for EPS synthesis in these strains further depends upon environmental conditions. Other *cps* mutants (*cpsC40*, *cpsC221*, *cpsD136*, and *cpsE23*) could not cause Wts even though all but *cpsE23* initially grew well *in planta*. To explain the differences in pathogenicity between *cps* mutants, we will need to determine if any of these mutants are still producing small amounts of EPS *in planta* or are blocked in the synthesis of other cell surface polysaccharides.

ACKNOWLEDGMENTS

Salaries and research support were provided by the U.S. Department of Agriculture under grant 85-CRCR-1-1781 from the Competitive Research Grants Office, the National Science Foundation under grant DMB-8703722, and by state and federal funds to the Ohio Agricultural Research and Development Center, The Ohio State University.

This is journal article 333-89 of the Ohio Agricultural Research and Development Center.

We thank Scot Olah and Russell Pastian for technical assistance and T. Graham, R. Garber, and K. Poetter for critical reading of the manuscript.

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