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Characterization of a Gene Cluster for Exopolysaccharide Biosynthesis and Virulence in *Erwinia stewartii*†

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We have previously cloned the genes for synthesis of capsular polysaccharide (*cps*) and slime from *Erwinia stewartii* in cosmid pES2144. In this study, pES2144 was shown to complement 14 spontaneous *cps* mutants. These mutants were characterized by probing Southern blots of mutant genomic DNA with pES2144; insertions were detected in four mutants and deletions in six mutants. Genetic and physical maps of the pES2144 *cps* region were constructed by subcloning, restriction analysis, and transposon mutagenesis with Tn*5*, Tn*5lac*, and Tn*3*HoHo1. Mutations affecting the ability of pES2144 to restore mucoidy to *cps* deletion mutants were located in five regions, designated *cpsA* to *cpsE*. None of the *cps* mutants were able to cause systemic wilting of corn plants, and mutations in *cps* regions B to E further abolished the ability of the bacterium to cause watersoaked lesions on seedlings. The gene for uridine-5′-diphosphogalactose 4-epimerase (*galE*) was linked to the *cps* genes on pES2144. In *E. stewartii*, *galE* was constitutively expressed, whereas the genes for galactokinase (*galK*) and galactose-1-phosphate uridyltransferase (*galT*) were inducible and not linked to *galE*. Thus, *galE* does not appear to be part of the *gal* operon in this species.

Most plant-pathogenic and -symbiotic bacteria are encapsulated and produce extracellular polysaccharides (EPSs). This material is in intimate contact with the plant cell surface and, in *Rhizobium* spp., is thought to play a crucial role in the signal exchange process that leads to infection and nodulation of the host (3, 21). EPS is probably just as important in plant-pathogenic interactions, either actively as a pathogenicity factor or passively as a barrier to host defenses and recognition events. *Erwinia stewartii* is a vascular pathogen of corn whose primary mechanism of virulence is production of EPS slime; this material occludes the xylem vessels and causes the plant to wilt (6, 18). The bacterium can also grow in the intercellular spaces of young leaves, where it produces a symptom called watersoaking (Wts), which is due to the loss of cell membrane semipermeability and the resulting accumulation of fluids in the tissue. The mechanism of Wts is not known, and no extracellular virulence factors other than EPS have been reported for this pathogen. If the bacterium loses the ability to produce EPS, it can no longer cause systemic wilting, and in some cases it also loses Wts ability (5, 8). On the other hand, Wts^- mutants include both mucoid and nonmucoid colony types. The Wts process appears to be prerequisite to the vascular wilt phase of the disease, since bacterial virulence correlates with the ability to induce Wts in seedlings (23), i.e., mutants that cannot cause Wts cannot cause systemic wilting either. In this paper, we consider the bacterium to be fully virulent if it can cause both Wts and wilting on susceptible corn plants; Wts^- mutants are therefore completely avirulent, whereas Wts^+ EPS^- mutants are considered partially virulent.

The EPS of *E. stewartii* is a very large (ca. 45 megadaltons) and viscous heteropolysaccharide composed of glucose, galactose, and glucuronic acid (16). Both bound capsule and loose slime are produced, and these two EPS fractions appear to have the same sugar composition and gel exclusion properties (A. Darus, Ph.D. thesis, University of Missouri, Columbia, 1980). The capsule layer is produced constitutively, whereas slime is produced only in the presence of readily fermentable carbohydrates. In addition to virulence, the EPS may also have a role in protecting the bacterium from phytoagglutinins (5) and in colonization of its insect vector.

In a previous study (8), we constructed a library of *E. stewartii* SS104 DNA and obtained a recombinant plasmid, pES2144, that restored EPS production and pathogenicity to a number of spontaneous EPS^- mutants. Among the mutants complemented by pES2144 were two putative *galE* mutants, GAL8 and GAL17, which are Gal^-, acapsular, completely avirulent, and sensitive to galactose (D. L. Coplin, C. Meaney, J. J. Bradshaw-Rouse, and S. L. McCammon, Phytopathology 72:1002, 1982). The purpose of this study was to locate the genes for EPS synthesis (designated *cps*), galactose utilization, and pathogenicity on this plasmid and to characterize several of the spontaneous acapsular mutants. A preliminary report of this work has appeared (10).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used in this study are listed in Table 1. All *E. stewartii* strains were derived from DC283, which is a spontaneous Nal^r mutant of wild-type strain SS104 (9). K9 series *E. stewartii* strains were derived from DC283 by selection for resistance to capsule-dependent bacteriophage K9 (5). Cosmid pLAFR3 contains the mp8 polylinker from pUC8 inserted into the *Eco*RI site of pLAFR1 (22; B. J. Staskawicz, University of California, Berkeley). SF800 (P1::Tn*5lac*) (20) was obtained from D. Kaiser (Stanford University, Stanford, Calif.). *Escherichia coli* HB101 (4) was used as a host for all cloning and transposon mutagenesis experiments.

Media, growth, and mating conditions. The culture media and growth and mating conditions for *E. stewartii* have been described previously (7). Colony type was evaluated on

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TABLE 1. Bacteria and plasmids

Strain or plasmid	Description[a]	Reference or source
Erwinia stewartii		
DC283	SS104 Nal^r	9
GAL8, GAL17	DC283 Δ(*cps-galE*)	5
PJD199	DC283 *galE199*::Tn*5lac*	This work
Escherichia coli		
C2110	Nal^r *polA1 rha his*	29
HB101	*thr leu thi recA hsdR hsdM pro* Str^r	4
SA6255	HfrH Δ*lac* Δ*gal*	S. Gottesman
W3107	*galT1*	ECGSC[b] (strain 4471)
W3102	*galK2*	ECGSC (strain 4468)
Plasmids		
pES2144	*cps*$^+$ *galE*$^+$ clone in pVK100 from *E. stewartii* chromosome	8
pHoHo1	Tn*3*::*lacZ* Cb^r *tnpA tnp*$^+$	29
pSShe	Cam^r *tnpR tnp*	29
pLAFR3	Tc^r IncP	B. J. Staskawicz
pRK2013	Km^r Tra$^+$ ColE1	19
pR751	Tp^r Sp/Sm^r IncP	26
pVK100	Tc^r Km^r IncP	19

[a] Cam^r, Chloramphenicol resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Str^r, streptomycin resistance; Tc^r, tetracycline resistance; *cps*, capsular polysaccharide synthesis; Sp/Sm^r, spectinomycin/streptomycin resistance; Tra, conjugative transfer.
[b] *Escherichia coli* Genetic Stock Center, Yale University, New Haven, Conn.

CPG agar (5). 5-Bromo-4-chloro-3-indolyl-D-galactopyranoside was added to minimal medium plates at 40 μg/ml to test for β-galactosidase activity. Cosmids were mobilized from *E. coli* into *E. stewartii* by using pRK2013 as described previously (8).

Genetic techniques. Techniques for Tn*5* (26), Tn*5lac* (20), and Tn*3*HoHo1 (29) mutagenesis were performed as described. A Tn*5lac* mutation in the *galE* gene of pES2144 was crossed into the chromosome by mobilizing the mutant plasmid into strain DC283 with pRK2013. A pES2144 transconjugant lacking pRK2013 was cultured repeatedly on kanamycin-containing medium, and then pR751, a Tp^r plasmid incompatible with pES2144, was transferred into this strain. Selection was maintained for Tp^r and Km^r, and transconjugants were screened for Tc^s, EPS$^-$, and Gal$^-$ phenotypes. The chromosomal location of the *galE*::Tn*5lac* mutation was verified by Southern blot analysis.

DNA manipulations. Procedures for plasmid DNA isolation, agarose gel electrophoresis, restriction analysis, transformation, ligation, and nick translation have been described previously (8). Southern blots were done in Hybrid-Ease chambers (Hoefer Scientific Instruments) on Zetabind filters (AMF Cuno) as specified by the manufacturer. Filters were hybridized at 65°C for 18 h and washed at 65°C in 2× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4) with 1% (wt/vol) sodium dodecyl sulfate.

Pathogenicity assays. Wts ability was assayed by a whorl inoculation technique (8) on 8-day-old sweet corn (cv. Earliking) seedlings in a controlled-environment chamber. Symptoms were rated on the following scale after 3 days: 0, no symptoms; 1, scattered small lesions; 2, numerous lesions; 3, extensive lesions that remained watersoaked, with ooze forming on leaf surfaces.

Enzyme assays. To measure the activity of enzymes involved in galactose catabolism, bacteria were grown overnight in DB or minimal A salts media (7) containing 1% sodium lactate (pH 7.0) or glucose at 30°C. Three hours prior to extraction, the cells were induced by addition of galactose or fucose (5 mM final concentration). Cells were washed and suspended in 50 mM Tris–1 mM EDTA–2 mM mercaptoethanol, pH 8.7, and then disrupted by sonication. Debris was removed by centrifugation, and the protein content of the supernatant was determined (28). Uridine-5′-diphosphogalactose 4-epimerase (epimerase; EC 5.1.3.2) activity was measured by the method of Moreno (24) and galactose-1-phosphate uridyltransferase (transferase; EC 2.7.7.12) was measured by the method of Isselbacher (17) as modified by Fietta et al. (12).

RESULTS

Physical map and subcloning of pES2144. A restriction map was constructed of the 27-kilobase (kb) insert of pES2144 (Fig. 1). The order of restriction sites was deduced from single and double digestions with *Eco*RI, *Bam*HI, and *Hin*dIII and partial digestions with *Eco*RI and *Hin*dIII. Restriction sites for *Pst*I, *Bgl*I, *Kpn*I, *Sal*I, *Sma*I, and *Pvu*II were individually mapped in reference to the *Hin*dIII, *Bam*HI, and *Eco*RI sites. Restriction mapping of the subclones described below verified the map. The insert had no sites for *Xho*I, *Xba*I, or *Sst*I.

Since pES2144 was obtained from a *Hin*dIII library and the insert contained three *Hin*dIII sites, it could have been formed by the ligation of several noncontiguous *Hin*dIII fragments. To determine whether the insert was colinear with the chromosome, Southern blots of *Eco*RI- and *Pst*I-digested wild-type SS104 genomic DNA were probed with nick-translated pES2144 DNA (data not shown). The 7.2-, 3.4-, and 1.6-kb *Pst*I fragments of pES2144 spanned the three internal *Hin*dIII sites of the insert, and the 12.1-kb *Eco*RI fragment spanned two internal *Hin*dIII sites. The predicted fragments were present in blots of both *Eco*RI- and *Pst*I-digested genomic DNA, indicating that the four *Hin*dIII fragments cloned into pES2144 were contiguous on the *E. stewartii* chromosome.

Subclones of pES2144 were constructed; their dimensions are shown in Fig. 1. *Hin*dIII fragments were recloned in vector pVK100, and other types of fragments were recloned in vector pLAFR3. The 5-kb *Bam*HI fragment was unstable when inserted in pLAFR3, so it was recloned in pBR322 to give a stable plasmid, pPD098. A subclone of the 14-kb *Hin*dIII fragment was not obtained.

Characterization of spontaneous acapsular mutants. We previously reported that pES2144 restored full virulence and

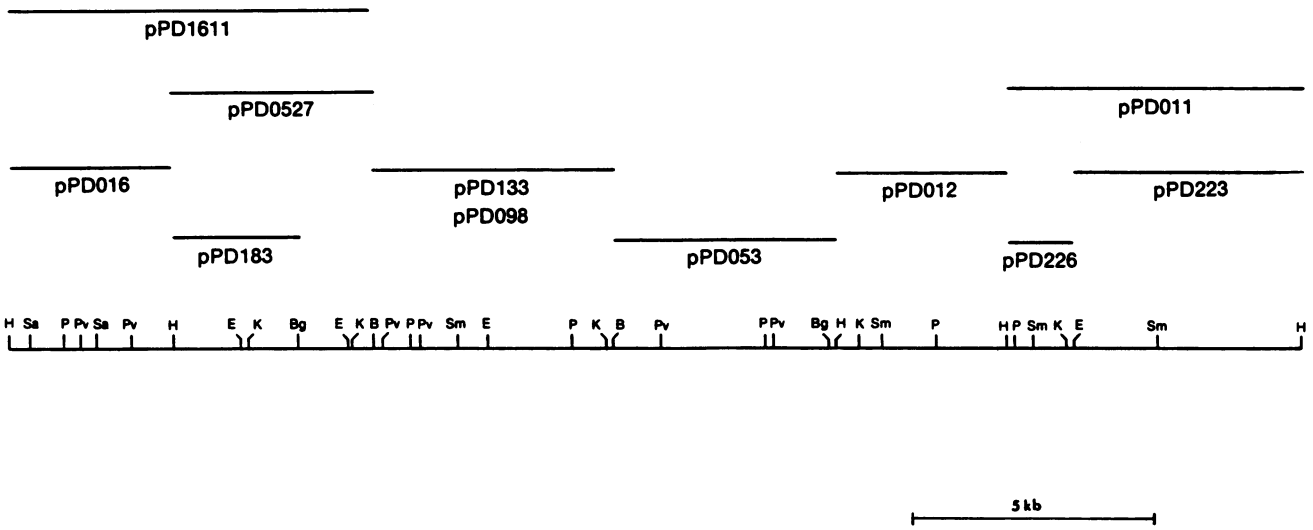

FIG. 1. Restriction map of the 27.0-kb insert in pES2144. Horizontal lines above the map delineate subclones of pES2144. Restriction sites are shown for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Pvu*II (Pv), *Sal*I (Sa), and *Sma*I (Sm).

EPS production to four spontaneous phage K9-resistant, nonmucoid mutants, GAL8, K91J, K91K, and K91R (8). In this study, we examined 13 additional EPS$^-$ K9-resistant mutants and found that all but 3 were complemented by pES2144. The mutants have been grouped into seven classes based on their colony type, type of mutation, and ability to be corrected by pES2144 and subclone pPD0527 (Table 2). Except for classes 1 and 7, the mutants were able to cause moderate Wts symptoms (disease ratings from 1.1 to 2.2) but were unable to wilt plants. pES2144 restored EPS production, as well as full wilting and Wts ability, to these strains. Wts$^+$ EPS$^-$ mutants caused as many lesions as the wild-type strain but received lower disease ratings because lesion size was restricted and bacterial exudate did not form on the surface of the leaves. Presumably, the slime produced by mucoid strains prolonged the period of Wts and allowed more bacterial growth to occur before the lesion became necrotic.

The nature of the mutation in each EPS$^-$ strain was shown by probing Southern blots of *Hin*dIII-digested wild-type and mutant genomic DNAs with nick-translated pES2144 and selected subclones; insertion mutations were found in four mutants (class 4), and deletions were found in six mutants (classes 1, 2, and 3). When the entire pES2144 plasmid was used as a probe, it hybridized to many bands in the genomic digests, indicating that it contained repeated DNA sequences, possibly insertion elements, present in over 25 copies on the chromosome and plasmids. In some experiments this limited our ability to use pES2144 and certain subclones as probes. The multicopy sequences were present in pPD0527 (Fig. 2, lanes K to N) and pPD011 probes but not in pPD098 (Fig. 2, lanes A to J), pPD016, pPD012, or pPD053 probes. The pES2144 probe revealed insertion mutations in the 14-kb *Hin*dIII fragment of class 4 strains (data not shown). When K91K was examined in more detail with the pPD098 and pPD0527 probes, a 1.2-kb insertion was found in the 7.2-kb *Pst*I fragment (Fig. 2, lane L) and its internal 1.3-kb *Hin*dIII-*Eco*RI fragment (Fig. 2, lane L). Both class 1 mutants had deletions spanning three adjacent *Hin*dIII fragments (14, 3.5, and 6.2 kb). The deletion in GAL8 must start within the region shown by the slashed lines in Fig. 3 because the pPD0527 probe revealed that the 2.3-kb *Eco*RI fragment was missing, whereas the 1.3-kb *Hin*dIII-*Eco*RI fragment was still present (Fig. 2, lane N). Class 2 and 3 mutants had deletions within the 14-kb *Hin*dIII fragment, and in each case a new 9-kb fusion fragment was formed. The right end of the deletion in K91J involved both the 2.9-kb *Eco*RI and 3.4-kb *Pst*I fragments (Fig. 2, lanes C and

TABLE 2. Classes of *E. stewartii* EPS$^-$ mutants grouped by colony type, type of mutation, and ability to be complemented by pES2144 and pPDO527

Class	Type of mutation	Strain(s)	No plasmid		pES2144		pPD0527	
			Colony type[a]	Avg Wts[b] ± SD	Colony type	Avg Wts ± SD	Colony type	Avg Wts ± SD
1	Deletion	GAL8, GAL17	B	0.1 ± 0.2	F	2.7 ± 0.4	B	0
2	Deletion	K91C, K91J, K91R	B	1.4 ± 0.6	F	2.7 ± 0.4	B	0.8 ± 0.6
3	Deletion	K91M	I	1.6 ± 0.5	F	2.9 ± 0.2	F	2.3 ± 0.4
4	Insertion	K91G, K91H, K91K, K91L	I	2.2 ± 0.3	F	2.9 ± 0.2	F	2.8 ± 0.3
5	Point	K91I, K91O, K91P, K91Q	I	1.9 ± 0.3	F	2.4 ± 0.4	F	2.7 ± 0.4
6	Unknown	K91N, K91T	I	1.1 ± 0.2	I	1.2 ± 0.2	I	0.9 ± 0.4
7	Unknown	K91S	B	0	B	0	B	0
—	Wild type	DC283	F	2.9 ± 0.2				

[a] F, B, and I indicate fluidal (mucoid), butryrous (nonmucoid), and intermediate colony types, respectively.
[b] Values indicate the amount of Wts at 3 days after inoculation scored from 0 (no symptoms) to 3 (severe Wts with exudate) and are combined averages of duplicate assays for each strain.

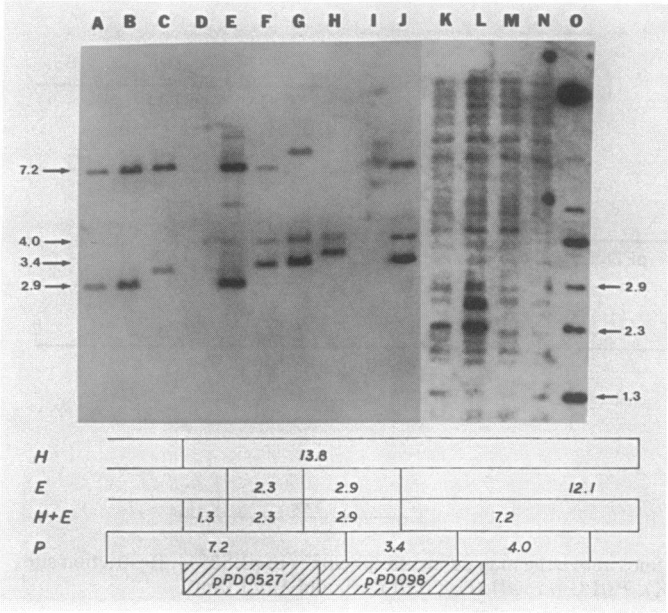

FIG. 2. (Top) Southern blots of genomic DNA of spontaneous EPS$^-$ mutants probed with the 5-kb *Bam*HI fragment from pPD098 (lanes A to J) and pPD0527 (lanes K to O). DNA was digested with *Hin*dIII and *Eco*RI (lanes A to E and K to O) or *Pst*I (lanes F to J). Lanes A, F, and K, DC283; lanes B, G, and L, K91K; lanes C, H, and M, K91J; lanes D, I, and N, GAL8; lanes E, J, and O, pES2144. (Bottom) Maps of pES2144 *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P) restriction fragments that hybridized with pPD0527 and pPD098 probes (slashed area). Fragment sizes are given in kilobases.

H) and therefore must lie within the overlap between these two fragments (Fig. 3). Using pPD016 as a probe revealed that the other end of the deletion in K91J originated within the left-hand 3.5-kb *Hin*dIII fragment. In contrast, the latter fragment was present in K91M, so the deletion in this strain was probably internal to the 14-kb *Hin*dIII fragment. No rearrangements were apparent in Southern blots of the class 5 mutants, so these strains may have point mutations in the *cps* region, since they were corrected by pES2144. These mutants were not studied further.

Location of *cps* and *gal* genes on pES2144. All of the subclones shown in Fig. 1 were tested for their ability to restore colony type and Wts ability to the EPS$^-$ mutants listed in Table 2. Plasmids containing a common 2.6-kb *Hin*dIII-*Bgl*I fragment (pPD1611, pPD0527, and pPD183) restored EPS production and full virulence to class 4 insertion mutants, and K91M (Table 2) and pPD011, containing the right-end *Hin*dIII fragment, restored the Gal$^+$ phenotype to class 1 mutants. Although pPD011 complemented GAL8 and GAL17 for galactose utilization and restored Wts ability (disease rating of 1.0), it did not restore the fluidal colony type. None of the other subclones complemented any of the mutants.

pES2144 was mutagenized with transposons Tn*3*HoHo1 and Tn*5lac*. Since the deletions in GAL8 and K91J were overlapping and together spanned the entire length of the insert DNA (Fig. 3), we were able to use these two strains as recipients in complementation tests and determine the effect of each transposon-induced mutation in pES2144 on EPS synthesis. This approach eliminated the need to cross every mutation into the chromosome in order to determine its phenotype. Mutations in six regions affected colony type in one or both of the recipients. These have been designated *cps* regions A to E as shown in Fig. 3; the sixth region was identified as the *galE* gene (see below). Insertions in region A resulted in an intermediate level of EPS production in K91J rather than a nonmucoid colony type. In similar experiments, plasmids with insertions in *cpsA* also failed to complement class 4 mutants, indicating that the chromosomal insertions in these strains were in region A. Mutations to the left of *cps-12* in region C failed to complement K91J, and mutations within and to the right of region B failed to complement GAL8. Although possible polar effects prevented us from using this method to determine the endpoints of the deletions in K91J and GAL8, these results are consistent with the physical mapping described above.

When inserted in the proper orientation, both Tn*3*HoHo1 and Tn*5lac* could create transcriptional *lacZ* gene fusions. The Lac$^+$ insertions in all of the *cps* regions and *galE* were oriented so that transcription of *lacZ* proceeded in the direction from region A to *galE* (Table 3).

To characterize *cpsA* further, the 4-kb insert in pPD0527 was mutagenized with Tn*5* and the plasmids were tested for complementation of EPS production in K91K (class 4) (Fig. 4). We mapped primarily insertions affecting colony type, and these were located within a 0.75-kb region. Insertions near the promoter resulted in an intermediate level of EPS production, whereas insertions at the end of the region

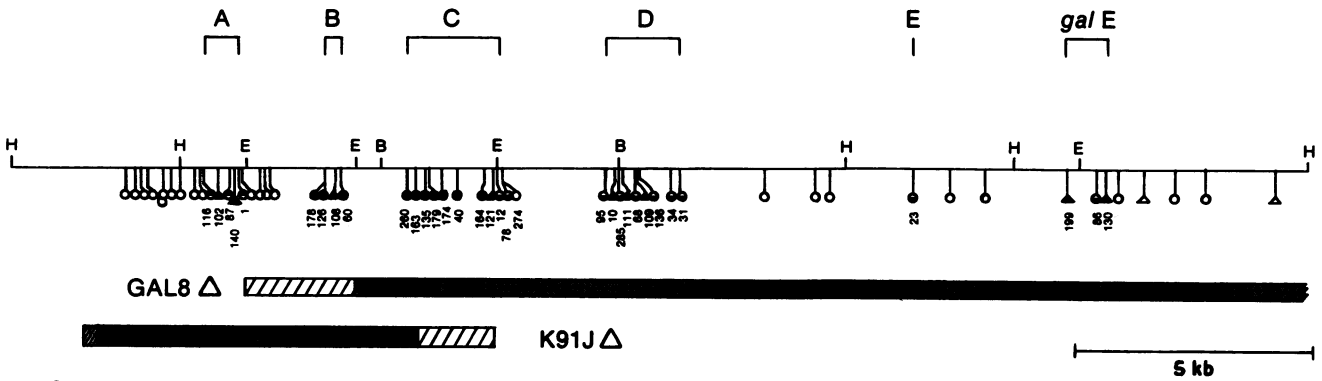

FIG. 3. Transposon mutagenesis of pES2144 showing Tn*5lac* (triangles) and Tn*3*HoHo1 (circles) insertions that affect the ability of the plasmid to restore mucoidy to GAL8 and K91J. Bars indicate deletions in GAL8 and K91J; deletions begin within hatched areas, and in GAL8 one end is external to the insert. Symbols: ●, ▲, mutation did not complement either GAL8 or K91J; ◒, ◭, mutation failed to complement K91J; and ◒, ◭, mutation failed to complement GAL8.

TABLE 3. Restoration of EPS production and Wts ability to K91J and GAL8 by pES2144 plasmids carrying *cps*::Tn*3*HoHo1 mutations

pES2144 mutation	*cps* region	Tn*3*HoHo1 orientation[a]	Lac phenotype	K91J		GAL8	
				Colony type[b]	Wts[c]	Colony type	Wts
No plasmid				B	2.5	B	0
cps-87	A	R	+	I	2.5	F	3.0
cps-72	A	R	+	I	2.5	F	2.9
cps-178	B	R	+	B	1.7	B	0
cps-126	B	L	−	B	2.5	B	0
cps-260	C	R	+	B	1.5	B	0
cps-40	C	R	+	B	1.4	B	0
cps-164	C	L	−	I	1.5	B	0
cps-12	C	L	−	F	2.5	I	0
cps-274	C	R	+	F	2.5	F	0
cps-285	D	L	−	F	2.8	B	0
cps-136	D	R	+	F	3.0	B	0
cps-23	E	L	−	F	3.0	B	0.5
galE86		R	±	F	2.5	B	2.5
cps$^+$				F	3.0	F	3.0

[a] R, Transcription of *lacZ* is from left to right, as shown in Fig. 3; L, opposite orientation.
[b] See Table 2, footnote *a*.
[c] Values indicate the amount of Wts scored from 0 (no symptoms) to 3 (severe Wts with exudate) and are averages of two to five experiments.

resulted in butyrous transconjugants. Leakiness of promoter-proximal Tn*5* insertions has been observed in other systems (2) and may be the reason that mutations in the first part of region A resulted in an intermediate colony type.

Effect of *cps* and *galE* mutations on pathogenicity. The Wts ability of GAL8 and K91J strains containing each of the mutant pES2144 plasmids shown in Fig. 3 was tested on corn seedlings. Representative data are given in Table 3. Mutations in *cps* regions B through E failed to complement GAL8, and the resulting transconjugants were completely avirulent and butyrous. An exception was *cps-274*:: Tn*3*HoHo1 at the end of region C, which was fluidal yet avirulent. Since K91J was initially Wts$^+$, it was therefore not possible to determine the effect of *cpsBCD* mutations on the ability of pES2144 to restore Wts ability to this strain. On the other hand, some plasmid mutations in *cps* regions B to D appeared to decrease Wts in K91J (e.g., *cps-164* in Table 4). However, these plasmids also decreased the growth rate and viability of K91J and GAL8 transconjugants, which may account for this effect. Inactivation of the *galE* gene on pES2144 eliminated visible EPS production in GAL8 but did not affect its Wts ability.

Identification of *galE* on pES2144. Because the extent of the deletion in GAL8 was unknown, we crossed the *galE199*::Tn*5lac* insertion from pES2144 into the chromosome and examined the properties of this mutation in the resulting strain, PJD199. This strain was identical to GAL8 containing the mutant plasmid in lack of EPS production, inability to utilize galactose, sensitivity to galactose, and ability to cause watersoaked lesions. Epimerase activity was present in a wild-type *E. stewartii* strain but was not detected in sonicates of either GAL8 or PJD199 (Table 4). When pES2144 was introduced into an *E. coli* strain (SA2655) carrying a deletion of the *gal* operon, epimerase activity was expressed (Table 4).

GAL8 retained the other enzymes in the LeLoir pathway for galactose utilization. Since *galE*, *galT*, and *galK* are arranged in an operon in *E. coli*, we expected all of these genes would be missing in GAL8 due to the size and location of the deletion in this strain. However, in nonquantitative assays, galactokinase (*galK*), transferase (*galT*), and UDP glucose pyrophosphorylase (*galU*) activities were demonstrated (data not shown). This finding suggested that *galE* might not be part of a classical *gal* operon in *E. stewartii*. Further tests revealed that pES2144 was not able to complement *galT* (W3107), *galK* (W3102), or Δ*galETK* (SA2655) mutants of *E. coli* for growth on minimal galactose medium. In addition, *galE* was not coordinately regulated with *galT* in *E. stewartii* (Table 4). Transferase activity in wild-type *E. coli* and *E. stewartii* strains and epimerase in *E. coli* were induced by galactose and fucose, but epimerase activity was constitutive in *E. stewartii*. Addition of glucose to the medium depressed transferase activity in both species and epimerase in *E. coli* but did not affect epimerase levels in *E. stewartii*.

DISCUSSION

In this study, we characterized a large region of the *E. stewartii* chromosome that is involved in synthesis of extracellular polysaccharide. A similar cluster of function has been described for *E. coli* (31); six *cps* genes are located near *his* and are positively regulated by *rcsA* (14). We have shown that the *cps-galE* region in *E. stewartii* is also linked to *his* (S. L. McCammon and D. L. Coplin, Phytopathology

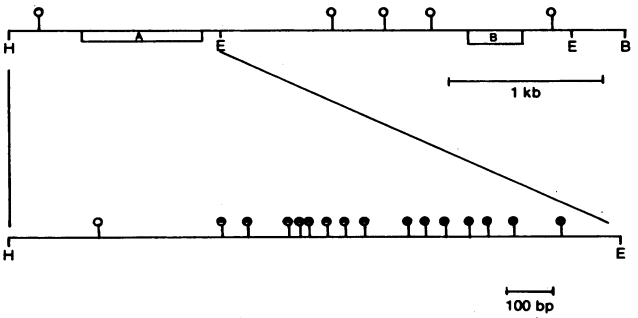

FIG. 4. Tn*5* mutagenesis of pPD0527. Shaded symbols indicate that the mutation did not complement K91K. Symbols: ○, mucoid colonies (complemented K91K); ◓, intermediate colony type; ●, nonmucoid colony type. Boxes A and B designate *cps* regions A and B, respectively. Restriction sites: H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI. bp, Base pairs.

TABLE 4. Expression of UDP-galactose 4-epimerase and galactose-1-phosphate uridyltransferase activity in wild-type and Gal$^-$ strains of *E. stewartii* and *E. coli*

Strain[a]	Carbon source	Inducer	Activity (mU/mg of protein)[b]	
			UDP-galactose 4-epimerase	Galactose-1-phosphate uridyltransferase
Erwinia stewartii				
DC283	Lactate	None	34	3
	Lactate	Galactose	27	13
	Lactate	Fucose	16	22
	Glucose	Fucose	31	5
GAL8	Lactate	Fucose	1	15
	Glucose	Fucose	2	ND[c]
PJD199	Glucose	Fucose	1	ND
Escherichia coli				
C600	Lactate	None	5	2
	Lactate	Galactose	40	19
	Lactate	Fucose	48	22
	Glucose	Fucose	11	3
SA2655	Glucose	Fucose	2	ND
SA2655(pES2144)	Glucose	Fucose	66	ND
SA2655(pJD199)	Glucose	Fucose	9	ND

[a] DC283 and C600 are Gal$^+$; GAL8 is Δ(*galE-cps*); PJD199 and pJD199 carry *galE199*::Tn*5lac* on the chromosome and pES2144, respectively; and SA2655 is Δ*gal*.
[b] One unit = 1 μmol of substrate converted per min.
[c] ND, Not done.

72:1001, 1982) and is likewise regulated by *rcsA* (30). In view of the similarities reported for the linkage maps of other *Erwinia* species and *E. coli* (13, 25, 27), these findings suggest that the *cps* regions in *E. stewartii* and *E. coli* may be analogous and code for similar enzymatic functions. We have not, however, been able to complement *E. coli cpsBCDEF* mutants with pES2144, so either the *E. stewartii cps* genes are not expressed well in *E. coli* or their products are not equivalent. Since the capsular polysaccharides made by these bacteria are somewhat different (colanic acid contains fucose, which is missing in *E. stewartii* EPS), this result is not unexpected. Both species could use similar pathways for synthesis of EPS, but the glycosyltransferases would have different substrate specificities and might therefore not be able to complement one another.

Enterobacteria utilize galactose by the LeLoir pathway. The enzymes in this pathway are galactokinase (*galK*), galactose-1-phosphate uridyltransferase (*galT*), UDP galactose 4-epimerase (*galE*), and UDP glucose pyrophosphorylase (*galU*). In *E. coli* and in *Klebsiella* and *Salmonella* spp., genes for the first three enzymes are arranged in an operon which is induced by galactose, whereas *galU* is unlinked and constitutively expressed (1). Both the epimerase and pyrophosphorylase are key enzymes in the synthesis of nucleotide phosphate sugar precursors for polysaccharide biosynthesis, so it is understandable that *galU* is constitutive. On the other hand, why *galE* is regulated as a catabolic enzyme is puzzling. In *E. stewartii* SS104, we found that *galE* was linked to *cps* rather than *galTK* and was constitutively expressed. This arrangement is different from the *E. coli* model but makes sense in view of the epimerase's important biosynthetic role and the large amount of EPS produced by *E. stewartii*. A similar observation has been made for *Vibrio cholerae*, in which *galE* is not linked to *galTK* (15). The repeated sequences found near *galE* in pES2144 suggest that during the evolution of *E. stewartii* a transposition event may have moved this gene to its present location adjacent to the *cps* locus. It will be interesting to determine the location of *galE* in other *E. stewartii* strains.

The *cps* gene cluster is very large, extending from region A to *galE*, but we need to analyze mutants for structural changes in EPS before we can tell how much of this region is actually involved in EPS synthesis. So far, transposon mutagenesis has identified only about 7 kb that affects colony type and/or virulence. Since these phenotypes do not reflect subtle changes in the pattern of EPS synthesis or structural changes in the polysaccharides, it is possible that more *cps* genes may be located in this region. In addition, some *cps* mutations may be lethal, leading us to underestimate the number of *cps* genes. This is suggested by the poor growth of many *cpsBCD* mutants. Moreover, we have not been able to obtain certain subclones from the *cpsBCDE* region, and repeated attempts to fill in the gaps in our genetic map by using Tn*3*HoHo1, Tn*5lac*, and Tn*5* mutagenesis have failed. If *cps* mutations that block the final stages of EPS synthesis are potentially lethal because they allow toxic intermediates to accumulate, then mutations which also remove the early portion of the pathway should permit normal growth. This possibility may explain the high frequency of deletions and insertions that we observed among spontaneous EPS$^-$ mutants, since these types of mutations could affect many genes.

Mutations in *cps* regions B to E affected the ability of *E. stewartii* to cause Wts. Knowledge of the operon structure of the *cps* region is needed, however, before we can determine whether all of these regions are really required for Wts. At least three operons, *cpsA*, *cpsBCD*, and *cpsE*, are present, and these have been separated by intervening neutral insertion mutations; further research may reveal more *cps* operons. The avirulent phenotype of the *cps-274*::Tn*3*HoHo1 mutation, along with the finding that it is regulated by *rcsA* (30), indicates that this insertion is probably within a *cps* gene, but this mutant is still mucoid. Thus, the apparent lack of polarity of *cps-274* on *cpsD* suggests that *cpsC* and *cpsD* are separate operons. On the other hand, Tn*3*HoHo1 has been reported to cause nonpolar mutations in *Agrobacterium tumefaciens* (11), so more work is needed to determine if this interpretation is correct. Without knowing whether mutations in *cpsB* and *cpsC* are polar on *cpsD*, we cannot say whether all of the *cpsBCD* region is required for Wts.

Assuming that *cpsBCD* is one operon and that the deletion removing regions A to C in K91J has fused region D to a new promoter, then regions B and C may not be required for Wts, since K91J is still Wts^+.

The remaining genes in the *cps* cluster do not appear to be necessary for Wts, and this may be because EPS synthesis in *cpsA* and *galE* mutants is not completely blocked. *cpsA* mutants have an intermediate colony type, and *galE* mutants can still produce a small amount of slime if grown on both glucose and a subinhibitory concentration of galactose.

Our previous studies have shown that normal EPS synthesis is necessary for *E. stewartii* to cause wilt symptoms and move systemically through the vascular system of the corn plant (5). In this study, we have further implicated the EPS-biosynthetic pathway in the Wts process. However, the cause and mechanism of Wts are still unknown, and the fact that we have identified a second chromosomal locus that also affects Wts but does not alter colony type (8) suggests that the Wts factor is probably not the major capsular polysaccharide. Moreover, it would be difficult for a molecule as large as the capsular polysaccharide to act on the plant cell membrane through the plant cell wall. Therefore, at least two possibilities exist: either EPS aids the Wts factor in producing symptoms, or synthesis of the two molecules involves common steps in the EPS pathway.

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