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Control of Extracellular Polysaccharide Synthesis in *Erwinia stewartii* and *Escherichia coli* K-12: a Common Regulatory Function†

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A primary determinant of pathogenicity in *Erwinia stewartii* is the production of extracellular polysaccharide (EPS). A single mutation can abolish both EPS synthesis and pathogenicity; both properties are restored by a single cosmid clone. Subcloning and insertion analysis have defined a single positive regulatory function which shares a number of similarities with the *rcsA* function of *Escherichia coli* K-12, a positive regulator for capsular polysaccharide synthesis. In *E. stewartii*, the gene promotes the transcription of at least two operons (*cps*) involved in EPS synthesis; we have previously demonstrated a similar function for *rcsA* in *E. coli*. Both genes code for proteins of 25 to 27 kilodaltons; both proteins are unstable in *E. coli*. The *E. stewartii* RcsA protein was stabilized in *E. coli lon* mutants, as the RcsA product from *E. coli* is. The *E. stewartii* function complemented *E. coli rcsA* mutants, and the *E. coli* RcsA function increased *cps* expression and restored virulence in *E. stewartii* mutants. Therefore, these two gram-negative organisms share a similar component of their regulatory circuitry for the control of capsular polysaccharide synthesis.

Gram-negative bacteria produce a variety of extracellular polysaccharides (EPS) in the form of attached capsules or free slime (24). Bacterial capsules have been implicated in protection from toxic chemicals and dessication, in attachment to surfaces, and as virulence factors in plant and animal pathogens (8, 9, 10, 25, 28).

Erwinia stewartii causes both a leaf blight and vascular wilt of field and sweet corn. A primary determinant of pathogenicity of this bacterium is the production of EPS that occludes the xylem vessels, resulting in wilting of the plant (3, 4, 14). When the bacterium grows in the intercellular spaces of young leaves, it also causes a symptom called water-soaking (Wts), which is due to the loss of cell membrane semipermeability and to the accumulation of fluids in the leaf tissues. The bacterium produces EPS as a bound capsule under all conditions and as a copious slime when it is grown in the presence of a readily fermentable sugar. The heteropolysaccharide is composed of glucose, galactose, and glucuronic acid (13). One large gene cluster, cloned in plasmid pES2144, is needed for EPS synthesis (6; P. J. Dolph, D. R. Majerczak, and D. L. Coplin, submitted for publication). This cluster has been divided into five regions (*cpsA-E*) and represents at least three operons (*cpsA*, *cpsB-D*, and *cpsE*). The *galE* gene is also part of this cluster, which spans at least 19 kilobases (kb) of the chromosome. Mutants that cannot produce EPS are unable to cause wilting, and in addition *cpsB-E* mutants cannot elicit water-soaked lesions, suggesting that some common functions are required for both phases of symptom development.

In *Escherichia coli* K-12, which is normally nonmucoid, mutations at the *lon* locus result in the overproduction of the capsular polysaccharide colanic acid (18). At least six genes needed for the synthesis of this capsule (*cps*) are transcriptionally regulated by *lon* as well as by three other regulatory genes, *rcsA*, *rcsB*, and *rcsC* (12, 27). The product of the *rcsA* gene is an unstable protein that is degraded by the Lon protease; the accumulation of RcsA in *lon* mutants results in capsule overproduction (26). The availability of RcsA is the limiting factor for colanic acid synthesis, since increasing the gene dosage of $rcsA^+$ enhances the expression of the *cps* genes; plasmids containing $rcsA^+$ render *E. coli* K-12 hosts mucoid (26).

In a study done to clone virulence genes from *E. stewartii* that restored Wts ability to avirulent mutants (6, 20), plasmid pES4507 was obtained. This plasmid made *E. coli* strains mucoid and increased EPS synthesis fourfold in a wild-type *E. stewartii* strain. pES4507 concomitantly restored both Wts and EPS synthesis to MU14110, a Wts^- EPS^- mutant of *E. stewartii*. The pleiotrophic phenotype of MU14110 and the mucoidy of pES4507 transconjugants led us to suspect that MU14110 could be deficient in a positive regulator of EPS synthesis similar to $rcsA^+$ in *E. coli*. In this work, we present evidence that *E. stewartii* and *E. coli* share a common component for positive regulation of capsular polysaccharide synthesis.

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MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains used in this work and their sources or derivations are listed in Table 1. Plasmids are listed in Table 2. ΔTn*10* and Δ*kan* indicate

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source reference, or derivation
E. coli		
MC4100 background[a]		
SG1039	*proCYA221 zaj-403*::Tn*10 ilv*::Tn*5*	27
SG1041	*lon-100*	27
SB1087	*lon-100 rcsA40 zed-14*::Tn*10*	SG1041 + P1(SG12014)
JB3034	*cpsB10*::*lac* (immλ) *lon-100* Δ*rcsA26 recA*	J. Brill and S. Gottesman, manuscript in preparation
ATC5112	*cps-11*::Mu d1 *lon-100 rcsA72*::ΔTn*10*	26
ATC8109	*cpsB10*::*lac* (immλ) *lon-100 rcsA72*::ΔTn*10*	SG20581 + P1(ATC5112)
ATC8117	*cpsB10*::*lac* (immλ) *lon*$^+$ *rcsA72*::ΔTn*10*	SG20582 + P1(ATC5112)
SG20250	*lon*$^+$	12
SG20581	*cpsB10*::*lac* (immλ) *lon-100*	27
SG20582	*cpsB10*::*lac* (immλ) *lon*$^+$	27
SG20688	*cpsB10*::*lac* (immλ) *lon-100 rcsB15 ompC*::Tn*5*	J. Brill and S. Gottesman, manuscript in preparation
Other backgrounds		
ATC12017	F$^-$ *leu fhuA rpsL supE thr* Δ*lon-510*	C600 + P1(SG1039), + P1(SG4144)
C600	F$^-$ *leu fhuA rpsL supE thr*	NIH[b]
HB101	*thr leu thi recA hsdR hsdM pro rpsL*	2
SG4144	Δ*lon-510 galK2*	19
SG12014	*thr leu tonA zed-14*::Tn*10 rcsA40 lon-100*	12
E. stewartii		
DC283	Nal^r	7
MU14110	DC283 *rcsA14110*::Mu pf7701 *kan* EPS$^-$ Wts$^-$	20

[a] Δ(*lac*)*U169 araD flbB relA*.
[b] National Institutes of Health strain collection.

the use of deleted minitransposons derived from Tn*10* (26, 29).

Cosmid pLAFR3 contains the mp8 polylinker from pUC8 inserted into the *Eco*RI site of the broad-host-range plasmid pLAFR1 (B. Staskawicz, submitted for publication). *E. coli* HB101 was used as a host for cloning *E. stewartii* DNA.

pVK100 and pLAFR3 derivatives were mobilized in triparental matings with HB101(pRK2013) (6). Tn*3*HoHoI and Tn*5lac* insertions in pES2144 were previously constructed and failed to complement Δ*cps* mutants for colony type (Fig. 2; Dolph et al., submitted).

Media, enzyme assays, and pathogenicity assays. Media, growth conditions, and pathogenicity testing for *E. stewartii* strains have been previously described (5–7). *E. coli* strains were routinely grown in Luria broth (21). When required, ampicillin (75 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml), or trimethoprim (20 μg/ml) was added to the medium. Minimal broth and M56 minimal agar medium have been described previously (26). The colony type of *E. stewartii* was determined on CPG agar medium (3).

β-Galactosidase expression in the fusion strains was monitored on lactose-MacConkey agar indicator plates, and β-galactosidase assays were done as described by Miller (21).

Transposon mutagenesis. Insertional mutagenesis of the *E. stewartii rcsA* gene with Tn*5* (22) and Tn*3*HoHoI (23) was done in *E. coli* HB101 as described.

The Tn*5* insertions in pES4507 were crossed into the chromosome of wild-type *E. stewartii* DC283 by homogenotization. DC283 strains harboring different pES4507::Tn*5* derivatives were grown in Luria broth-kanamycin medium, and pR751, a plasmid that is incompatible with pES4507, was then conjugated into the strain. Transconjugants were selected for trimethoprim resistance (Tp^r) and kanamycin resistance (Kan^r) and screened for tetracycline sensitivity (Tet^s); loss of the pES4507 plasmid was confirmed by agarose gel electrophoresis.

TABLE 2. Plasmids

Plasmid	Relevant genotype or phenotype	Source or derivation
E. stewartii clones		
pES2144	Tet^r	pVK100 derivative from SS104 library; 6
pES4507	EPS$^+$ Wts^{+a} Tet^r	6
pRF101	EPS$^+$ Wts$^+$ Tet^r	pVK100 derivative
pRF111	EPS$^+$ Wts$^+$ Tet^r	pLAFR3 derivative
pRF121	EPS$^+$ Wts$^+$ Tet^r	pLAFR3 derivative
pRF1111	EPS$^+$ Wts$^+$ Amp^r	pUC8 derivative
pRF3011	EPS$^-$ Wts$^-$ *rcsA301*::Tn*5* Amp^r Kan^r	pUC8 derivative
pRF3021	EPS$^-$ Wts$^-$ *rcsA302*::Tn*5* Amp^r Kan^r	pUC8 derivative
E. coli clones		
pATC119	*rcsA*$^+$ Amp^r	26
pATC352	*rcsA*$^+$ Cm^r Amp^r	26
pATC400	*rcsA*$^+$ Amp^r	26
pATC401	*rcsA160*::Δ*kan* Amp^r	26
Other		
pLAFR3[b]	Tet^r *cos*	B. Staskawitz, submitted for publication
pR751	Tp^r IncP1	15
pRK2013	Kan^r ColE1 *mob*$^+$	11
pRK2013-Tn*7*	Str^r Spec^r Tp^r *kan*::Tn*7*	Dennis Dean

[a] Phenotype in MU14110.
[b] Polylinker from pUC8 cloned into *Eco*RI site of pLAFR1.

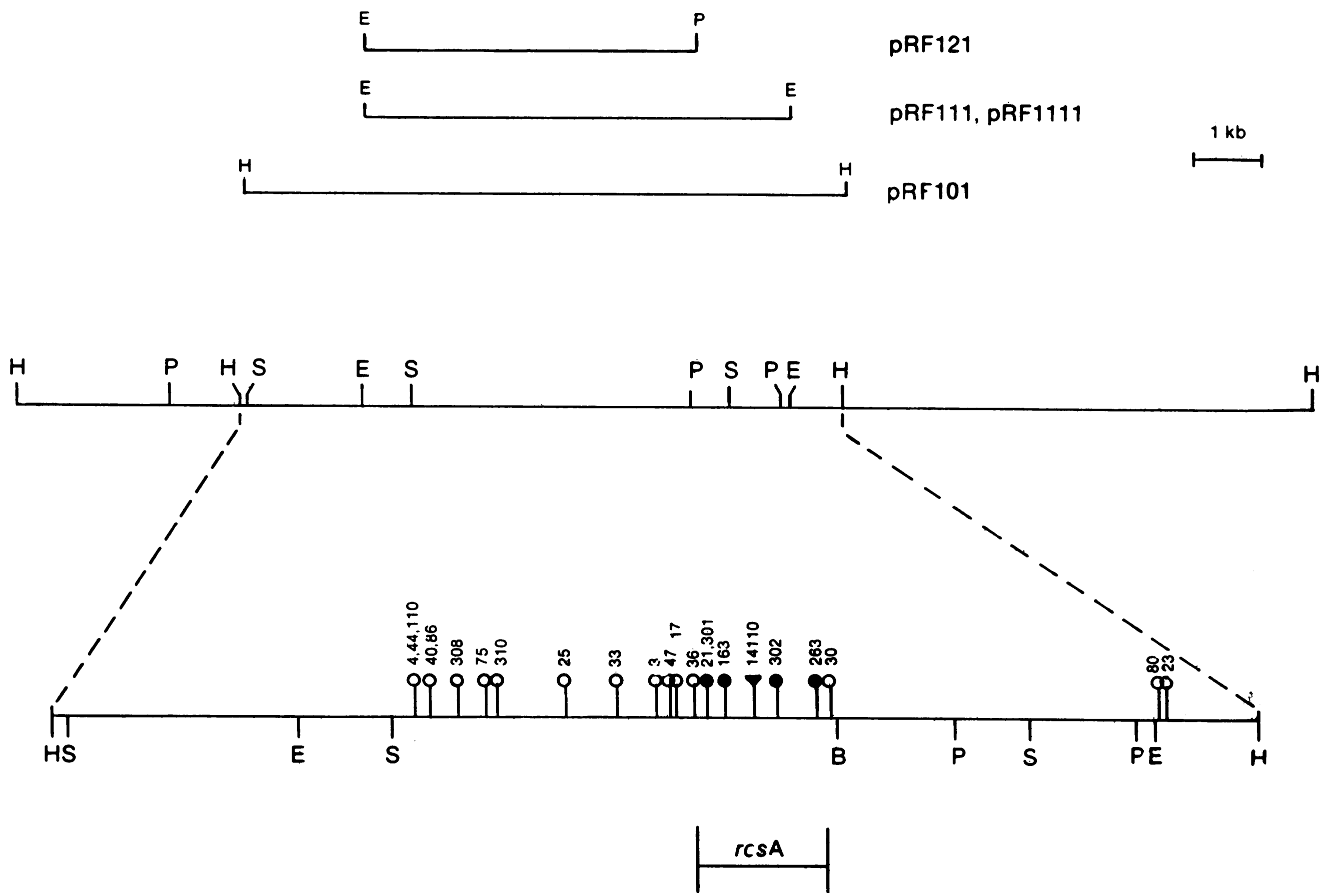

FIG. 1. Restriction map of the 19.5-kb *Hin*dIII insert from *E. stewartii* in pES4507 showing the corresponding sizes of the subclones which restored virulence to MU14110 and the location of Tn*5* insertions. pRF111 and pRF121 were derived from pLAFR3. pRF101 was derived from pVK100, and pRF1111 was derived from pUC8. Insertions which abolished (●) and did not abolish (○) the ability of the plasmid to complement EPS Wts mutations are indicated. Restriction sites: R, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; B, *Bgl*II. ▼, Site of Mu pf7701 insertion in MU14110.

Restrictions, ligations, and Southern blot analysis. Standard methods for plasmid DNA isolation, restriction analyses, ligations, and Southern blots were as described previously (6, 17). The blots (Zetabind; AMF Cumo) were hybridized at 52°C for 12 h and washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50°C.

Protein labeling. In vivo determination of proteins produced by the *rcsA* plasmids was done in maxicells as described previously (26). Cultures were labeled with [^{35}S]methionine (50 μCi/ml) for 20 min. The processed samples were suspended in 2% sodium dodecyl sulfate loading buffer and boiled for 5 min; 10-μl samples were electrophoresed in a 12% acrylamide gel. The buffers used were as described by Laemmli (16). The dried gels were autoradiographed at −70°C.

The in vivo stability of the *E. stewartii* RcsA protein was determined in growing cells as previously described for the *E. coli* RcsA protein (26).

RESULTS

Genetic and physical mapping of the EPS-Wts region on pES4507. An avirulent Mu pf7701 insertion mutant, MU14110, of *E. stewartii* has lost both the Wts ability and the ability to produce EPS (20). A cosmid library of *E. stewartii* DNA in the broad-host-range cosmid vector pVK100 yielded one plasmid, pES4507, which complemented both properties and restored virulence (6). From this plasmid, a 6.4-kb *Eco*RI fragment (plasmid pRF111) and a 4.9-kb *Eco*RI-*Pst*I fragment (plasmid pRF121) were subcloned into pLAFR3 (Fig. 1). Both subclones were able to restore the full wild-type phenotype to MU14110.

pES4507 and pRF101 were subjected to Tn*5* mutagenesis in *E. coli*, and the mutant plasmids were tested for the ability to restore colony type and Wts ability to MU14110. The location of Tn*5* insertions is indicated in Fig. 1. Insertions within a 1-kb region resulted in the loss of complementation for EPS and Wts. This same region, which was contained within the 4.9-kb fragment in pRF121, is the site of the Mu pf7701 insertion in MU14110 (Fig. 1; 6).

The phenotype of the pES4507::Tn*5* mutants was verified by crossing them into the chromosome of strain DC283. Tn*5* insertions 21, 163, and 263 resulted in butyrous (EPS$^-$) Wts$^-$ strains, but homogenotization of adjacent insertions 40, 25, 36, 30, and 80 did not affect Wts or EPS production.

Expression of *cps*::*lac* fusions in *E. stewartii* MU14110 and *E. coli rcsA*. To test whether the complementing region in pES4507 regulated *cps* synthesis, we used 14 plasmids carrying independent Lac$^+$ Tn*3*HoHoI insertions in the *E. stewartii cpsA-D* regions and a Tn*5lac* mutation in *galE* (Fig.

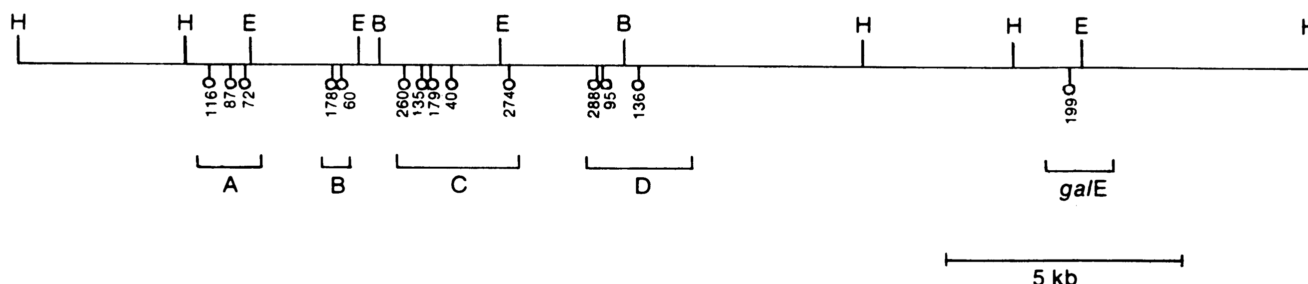

FIG. 2. Map positions of Lac$^+$ *cps*::*lacZ* gene fusions in pES2144 used in Table 3 (data from Dolph et al., submitted). Restriction sites: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. Insertion 199 is Tn*5lac*; all others are Tn*3*HoHoI.

2). Both Tn*3*HoHoI and Tn*5lac* produce transcriptional *lacZ* fusions. Therefore, insertions into the *cps* genes should result in *cps*::*lacZ* transcriptional fusions (Dolph et al., submitted).

E. stewartii is naturally Lac$^-$; therefore, we were able to use β-galactosidase production as a measure of *cps* expression when the Lac$^+$ plasmids were introduced into DC283 (EPS$^+$ Wts$^+$) and MU14110 (EPS$^-$ Wts$^-$). Transcription of *cpsA-B* was reduced about 20-fold and that of *cpsC-D* was reduced two- to sixfold in MU14110 as compared with the wild type (Table 3). These results confirmed our notion that MU14110 was deficient for a positive regulator of capsule synthesis. In two mutants, *cpsB*178::Tn*3*HoHoI (Table 3) and *cpsD*288::Tn*3*HoHoI (not shown), β-galactosidase activity was much higher than it was in adjacent fusions. This may be due to the formation of translational *lac* fusions, since this is also possible with the Tn*3*HoHoI transposon (23). The *galE* gene, which is not part of the *gal* operon in *E. stewartii* (Dolph et al., submitted), was expressed constitutively in both strains.

In *E. coli*, at least six structural genes involved in the synthesis of capsular polysaccharide (*cps*) are regulated in a positive fashion by the *rcsA* gene (12). Mutations in *rcsA* decrease *cps* transcription in a manner similar to the effect of the Wts$^-$ EPS$^-$ mutation in MU14110 on *E. stewartii cps* expression. We transferred the *E. stewartii cps* fusions into isogenic *rcsA*$^+$ and *rcsA E. coli* hosts and examined their expression (Table 3). Although the *E. stewartii cps*::*lac* fusions were expressed at significantly lower levels in *E. coli* than in *E. stewartii*, their expression was dependent on the *E. coli rcsA* function.

TABLE 3. β-Galactosidase synthesis of representative *E. stewartii cps*::*lacZ* fusions in *rcsA*$^+$ and *rcsA* strains of *E. stewartii* and *E. coli*

Region	Insertion	β-Galactosidase units[a]			
		E. stewartii[b]		*E. coli*[c]	
		rcsA$^+$	*rcsA*	*rcsA*$^+$	*rcsA*
cpsA	87	46.7	2.1	3.2	0.2
	72	48.2	1.7	1.5	0.4
cpsB	178	670.8	27.4	28	2.3
cpsC	260	29.9	5.8	3.4	0.1
	179	36.7	6.4	4.8	0.2
	274	10.8	2.1	1.9	0
cpsD	136	15.7	7.9	6.2	1.1
galE	199	7,386	9,430	1,703	1,658

[a] For β-galactosidase units, see Miller (21). Values are the averages of two or more experiments. All *cps*::*lacZ* fusions were constructed with Tn*3*HoHoI, except for 199, which is a Tn*5lac* fusion.
[b] The *rcsA*$^+$ strain was DC283; the *rcsA* host was MU14110.
[c] The *rcsA*$^+$ strain was SG1041; the *rcsA* host was SG1087. Both strains are *lon-100*.

Functional complementation shown by the Wts-EPS locus of *E. stewartii* and the *rcsA* locus of *E. coli*. To determine if the two regulatory genes are complementary, plasmid pRF1111, a pUC8 derivative carrying a 6.8-kb subinsert which includes the *E. stewartii* Wts-EPS region (Fig. 1), was introduced into *E. coli* C600 (*cps*$^+$ *lon*$^+$). A dramatic increase in capsule synthesis was observed. In addition, mobilization of the *E. stewartii* plasmids which carry this region, pRF121 and pES4507, into *E. coli* SG1087 (*rcsA lon*) also resulted in a mucoid phenotype. In the reciprocal experiment, plasmid pATC352, which carries the *rcsA*$^+$ gene from *E. coli*, was mobilized into *E. stewartii* MU14110 (EPS$^-$ Wts$^-$); full virulence in wilting and Wts was restored concomitantly with mucoidy to these transconjugants.

If plasmid pRF1111 carries a positive regulator of capsule that acts in a similar fashion to the *E. coli rcsA*$^+$ gene product, then it would be expected to increase β-galactosidase expression from *E. coli cps*::*lacZ* transcriptional fusions. To test this, β-galactosidase expression was compared in a set of *E. coli cps*::*lac* fusion hosts harboring either the *E. stewartii* or the *E. coli* positive regulator (Table 4).

E. coli cps::*lac* strains in which the chromosomal copy of *rcsA* has been inactivated by an insertion of the mini-transposon Δ*kan* (26) synthesize β-galactosidase at very low levels in both *lon*$^+$ and *lon* cells and form white (Lac$^-$) colonies on lactose-MacConkey agar indicator plates. When these strains were transformed with pRF1111 (Wts$^+$ EPS$^+$) or pATC400 (*rcsA*$^+$ of *E. coli*), increases in the expression of the fusions occurred in *lon*$^+$ *rcsA* and *lon rcsA* cells (Table 4). This enhanced expression was not observed in transformants of the same *cps*::*lac* fusion strains with either plasmid pRF3021 or plasmid pATC401 (Table 4). pRF3021 carries a Tn*5* insertion which abolishes the ability of the plasmid to confer the EPS$^+$ Wts$^+$ phenotype. pATC401 carries the *rcsA 160*::Δ*kan* mutation from *E. coli* (Table 4). These results suggest that the Wts-EPS locus on the *E. stewartii* plasmids is able to complement the *E. coli rcsA* mutation fully and that their gene products are functionally equivalent. For this reason, the region defined by the Tn*5* insertions in the *E. stewartii* clone has been designated *rcsA*.

We have shown previously that in *E. coli* strains transformed with a plasmid carrying a defective copy of *rcsA*, titration of a negative effector occurs, resulting in the expression of the chromosomal *rcsA*$^+$ copy and escape synthesis of *cps*::*lac* (26); these results are confirmed in Table 4. In contrast, such titration did not occur when the *E. stewartii rcsA* plasmid pRF3021 (*rcsA302*::Tn*5*) was used to transform a *lon*$^+$ *cps*::*lac* strain (Table 4), suggesting that in *E. coli* the

TABLE 4. Effect of cloned *E. stewartii* and *E. coli rcsA* genes on *cps*::*lac* expression in *E. coli rcsA* hosts

Genotype of *cps*::*lac* strain[a]	β-Galactosidase units[b]				
	No plasmid	Plasmid			
		pRF1111[c] (*rcsA*$^+$)	pRF3021[c] (*rcsA302*::Tn*5*)	pATC400[d] (*rcsA*$^+$)	pATC401[d] (*rcsA160*::Δ*kan*)
lon$^+$	0.7	178	1.1	750	91
lon$^+$ *rcsA72*::ΔTn*10*	0.9	442	0.8	122	14
lon	246	480	60.0	721	386
lon rcsA72::ΔTn*10*	1.4	377	2.2	554	13
lon rcsB15	0.7	1.1	0.9	1.5	0.8

[a] All strains are *E. coli* K-12. Strain designations: SG20582, *lon*$^+$; ATC8117, *lon*$^+$ *rcsA72*::ΔTn*10*; SG20581, *lon-100*; ATC8109, *lon-100 rcsA72*::ΔTn*10*; SG20688, *lon-100 rcsB15*.
[b] For units, see Miller (21).
[c] The plasmid carries *rcsA* from *E. stewartii* in the pUC8 vector.
[d] The plasmid carries *rcsA* from *E. coli* in the pBR322 vector.

rcsA gene from *E. stewartii* may not be under the same negative regulation or may not be as efficient a titrator.

Although the *rcsA* genes from *E. coli* and *E. stewartii* appear to substitute for each other in the regulation of capsule synthesis in either host, they showed little homology in Southern blot hybridizations at low stringency. pRF1111 (*E. stewartii rcsA*$^+$) failed to hybridize to genomic DNA blots from *E. coli* HB101 and *Erwinia amylovora* E8. Likewise, pATC352 (*E. coli rcsA*$^+$) DNA did not hybridize to genomic blots of *E. stewartii* DC283 DNA. Furthermore, *rcsA*-containing restriction fragments from pRF1111 and pATC352 showed only very weak hybridization signals in reciprocal hybridizations to each other.

Nonetheless, some sequence homology probably exists, since the *rcsA72*::ΔTn*10* insertion from *E. coli* was successfully crossed from a λ transducing bacteriophage carrying the *E. coli rcsA* gene into plasmid pRF1111, abolishing its ability to complement a chromosomal *rcsA* mutation in *E. coli* (data not shown). In contrast, attempts to cross *rcsA*::Δ*kan* from λ*rcsA* clones into pRF1111 failed.

pRF1111 did not complement an *rcsB* mutation in *E. coli*. An alternative explanation for the ability of pRF1111 to complement *E. coli rcsA* mutants is that the *E. stewartii* function bypasses the normal *E. coli* regulatory circuit completely. If so, we might expect the plasmid to allow capsule expression in *rcsB* mutants, an additional positive regulatory function essential for colanic acid synthesis in *E. coli* (12). We assayed β-galactosidase expression from the same *cps*::*lac* fusion in strain SG20688 (*lon rcsB15*) transformed with pRF1111 and pRF3021, as well as with pATC400 and pATC401 (Table 4). Neither the *E. coli rcsA*$^+$ plasmid (pATC400) nor the *E. stewartii rcsA*$^+$ plasmid (pRF1111) increased *cps*::*lac* transcription in this strain. It is unlikely either that the *rcsA* gene product from *E. stewartii* is functionally homologous to the *rcsB* gene product of *E. coli* or that it completely bypasses the *E. coli* regulatory pathway.

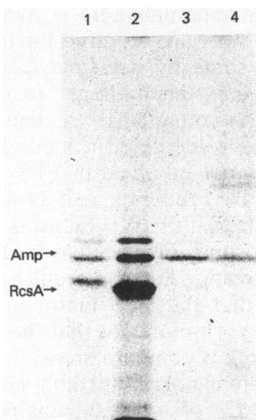

FIG. 3. In vivo labeling of proteins expressed in JB3034 by recombinant plasmids. Proteins were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, pATC119 (*rcsA*$^+$ *E. coli*); 2 to 4, *E. stewartii* plasmids pRF1111 (*rcsA*$^+$) (2), pRF3011 (*rcsA301*::Tn*5*) (3), and pRF3021 (*rcsA302*::Tn*5*) (4). Amp, β-Lactamase.

Identification of the *E. stewartii* RcsA protein and comparison with the *E. coli* RcsA protein. The maxicell strain JB3034 (*lon rcsA recA*) was transformed with plasmids pATC119, a pUC19 derivative carrying the *rcsA*$^+$ gene from *E. coli*; pRF1111; and its derivative plasmids pRF3011 (*rcsA301*::Tn*5*) and pRF3021 (*rcsA302*::Tn*5*), in which the *rcsA* gene has been inactivated by Tn*5* insertions. To identify the specific polypeptides synthesized by each plasmid, a maxicell experiment was performed. One major protein of about 25 kilodaltons (kDa) was made by pRF1111 (Fig. 3, lane 2) and not by the *rcsA*::Tn*5* derivatives (lanes 3 and 4). The protein was slightly smaller than the *E. coli* RcsA protein (27 kDa) (lane 1). Although the two Tn*5* insertions mapped at different positions on the clone, no truncated polypeptides were detected. These results suggest that the 25-kDa product is the RcsA protein from *E. stewartii*.

Sensitivity of the RcsA protein from *E. stewartii* to *lon* proteolysis in *E. coli*. It has been shown previously that the RcsA protein from *E. coli* is unstable and subject to proteolysis by the Lon protease (26). To determine if the RcsA protein from *E. stewartii* is also unstable and affected by Lon proteolysis, a pulse-chase experiment was carried out. *E. coli* C600 (*cps*$^+$ *lon*$^+$) and ATC12017 (*cps*$^+$ Δ*lon-510*) were transformed with pRF1111. The *E. stewartii* RcsA protein had a half-life of about 5 (±1) min in *lon*$^+$ cells; it was far more stable in the *lon* mutant, with a half-life of greater than 30 min (Fig. 4). In both *lon*$^+$ and *lon* hosts, β-lactamase made from the same plasmids was properly processed and was stable. These results are similar to those seen with the *E. coli* RcsA protein (5 min in *lon*$^+$ cells and 20 min in *lon* hosts [26]). The data indicate that at least in *E. coli*, the stability of the *E. stewartii* RcsA protein is affected by Lon proteolysis. The *lon* gene has not yet been identified in *E. stewartii*.

DISCUSSION

A large variety of capsular polysaccharides are synthesized by gram-negative bacteria. While some have been

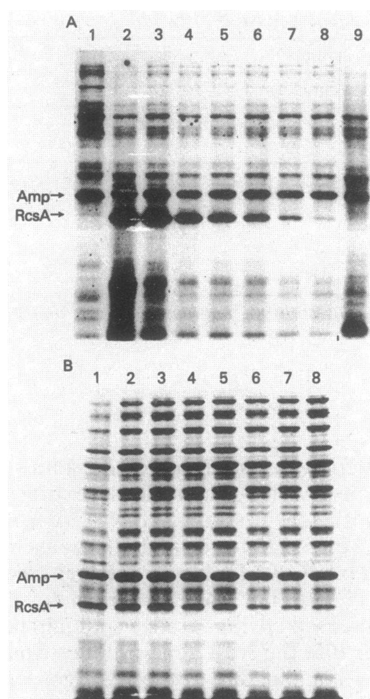

FIG. 4. Pulse-chase labeling of RcsA from *E. stewartii* in *E. coli* lon^+ and *lon* strains. Cells were pulse-labeled with [^{35}S]methionine for 2 min and chased with excess unlabeled methionine (2 mM). (A) lon^+ (C600). Lanes: 1, pBR322; 2, pRF1111 (2-min pulse); 3 to 8, chases of 2, 4, 6, 10, 15, and 30 min; 9, pRF3021 (2-min pulse). (B) *lon* (ATC12017). Lanes: 1, pRF1111 (2-min pulse); 2 to 8, chases of 2, 4, 6, 8, 10, 15, and 30 min. Amp, β-Lactamase.

implicated in the pathogenicity of plant and mammalian hosts, others have not yet been assigned functions but may serve as adhesion factors or to protect the bacterium from the environment (8, 10, 24, 25). The data presented here suggest a common component for the regulation of the synthesis of EPS of *E. stewartii* and *E. coli*. The synthesis of both capsules was dependent on an unstable positive regulator, RcsA, and the activator from one host functioned in the other. In both cases, the positive regulator was limiting for capsule synthesis; increasing the gene dosage led to the increased synthesis of EPS.

Wild-type *E. coli* K-12 synthesizes only low levels of capsule under all conditions thus far tested. *lon* mutants, which synthesize capsule at high rates, are more mucoid and express *cps::lac* better at low temperatures (less than 30°C) than at temperatures above 37°C. This temperature regulation is dependent on the negative regulator *rcsC* (T. Klopotowski and S. Gottesman, unpublished results). In addition to the *rcsA* function, a second positive regulator of *cps* expression, *rcsB*, has been identified for *E. coli* (12).

E. stewartii makes a bound capsule under all growth conditions but increases the synthesis of unbound slime dramatically when cells are grown on readily fermentable sugars. Since *E. stewartii* does not grow above 37°C, it is not clear if there is an effect of temperature on EPS synthesis. Additional regulatory genes have not yet been identified in *E. stewartii*, and clones from an *E. stewartii* library failed to complement *E. coli rcsB* or *rcsC* mutants (D. L. Coplin, unpublished results). We have observed that *E. stewartii cps* genes are poorly expressed in *E. coli*, which suggests that the *rcsB* and *rcsC* components of the system, if present in *E. stewartii*, may differ sufficiently so as not to function with the heterologous *cps* functions. Given that the pattern and pathogenicity function of capsule synthesis in the two organisms appear to be rather different, it may not be surprising if much of the regulatory circuitry has diverged. It is possible that different regulatory networks, using common factors such as RcsA, have been adopted for the control of synthesis of different capsules.

The product of the *rcsA* gene of *E. stewartii* was identified as a 25-kDa protein, which is slightly smaller than the estimated size of the *E. coli* RcsA product (26). As for the RcsA protein from *E. coli*, *E. stewartii* RcsA was unstable in *E. coli* lon^+ cells, with a half-life of 5 min. The half-life was longer than 30 min in *lon* cells. Therefore, at least in *E. coli*, the RcsA protein from *E. stewartii* was unstable and was affected by Lon proteolysis. The possible presence of the Lon protease has not been examined in *E. stewartii*.

The similarity observed here between a positive regulator of capsule function in *E. stewartii* and *E. coli* can probably be extended to many other gram-negative organisms. Allen et al. (1) have recently reported the isolation and sequencing of a regulatory gene, which they called *rcsA*, from *Klebsiella aerogenes*. This gene also increases colanic acid synthesis when present in multicopy plasmids in *E. coli*. However, functional complementarity with *E. coli rcsA* has not been demonstrated. The *Klebsiella rcsA* gene makes a 23-kDa protein, which is slightly smaller than those produced by *E. coli* and *E. stewartii*. Allen and co-workers were unable to detect homology between their plasmid and the *E. coli* K-12 chromosome (presumably in an $rcsA^+$ host) in Southern blots; their results are similar to the results indicating a lack of hybridization between *E. stewartii* and *E. coli* clones reported here. However, we have observed that their sequence shares extensive DNA and protein homology with the sequence we have determined for *E. coli rcsA* (A. Torres-Cabassa and S. Gottesman, manuscript in preparation). Presumably the *E. stewartii rcsA* gene would show the same sorts of extensive but interrupted homologies to the *E. coli* gene.

Colanic acid, a capsular polysaccharide of *E. coli* K-12, *Salmonella* spp., and other enteric bacteria, has not been implicated in pathogenesis. Given the poor synthesis of this capsule at 37°C, it seems unlikely that this particular capsule is even produced in mammalian hosts. On the other hand, a capsule and slime layer are required for full virulence of *E. stewartii*, and the slime by itself can account for the wilt symptoms produced by this pathogen on corn. The cause of the Wts observed on young corn seedlings is unknown, but the fact that we have identified a locus for Wts cloned in pES1044 (6) that is not involved in EPS synthesis or regulated by *rcsA* (R. D. Frederick and D. L. Coplin, unpublished data) suggests that the Wts factor is probably separate from the major capsular polysaccharide. Because *cps* functions and RcsA activation are also required for Wts ability, it is possible either that the EPS factor and Wts factor act together to produce symptoms or that the synthesis of these two molecules involves common steps.

What is the advantage of an unstable positive regulator in these systems? For *E. coli*, one can imagine a mechanism for the rapid turn-on of capsule in response to environmental stimuli by increasing both the synthesis and stability of the protein. Similarly, the turn-off of capsule synthesis will also be rapid if the stability of the regulatory protein is low. Since *E. stewarti* seems to have set its capsule synthesis high when carbohydrates are available, either it synthesizes more RcsA

than *E. coli* does or the protein is more stable in the *E. stewartii* host. It will be interesting to see if Lon plays a role in regulation in *E. stewartii* as it does in *E. coli*.

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LITERATURE CITED

1. **Allen, P., C. A. Hart, and J. R. Saunders.** 1987. Isolation from *Klebsiella* and characterization of two *rcs* genes that activate colanic acid capsular biosynthesis in *Escherichia coli*. J. Gen. Microbiol. **133**:331–340.
2. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. **41**:459–472.
3. **Bradshaw-Rouse, J. J., M. A. Whatley, D. L. Coplin, A. Woods, L. Sequeira, and A. Kelman.** 1981. Agglutination of strains of *Erwinia stewartii* with a corn agglutinin: correlation with extracellular polysaccharide production and pathogenicity. Appl. Environ. Microbiol. **42**:344–350.
4. **Braun, E. J.** 1982. Ultrastructural investigation of resistant and susceptible maize inbreds infected with *Erwinia stewartii*. Phytopathology **72**:159–166.
5. **Coplin, D. L.** 1978. Properties of F and P group plasmids in *Erwinia stewartii*. Phytopathology **68**:1637–1643.
6. **Coplin, D. L., R. D. Frederick, D. R. Majerczak, and E. S. Haas.** 1986. Molecular cloning of virulence genes from *Erwinia stewartii*. J. Bacteriol. **168**:619–623.
7. **Coplin, D. L., R. G. Rowan, D. A. Chisholm, and R. E. Whitmoyer.** 1981. Characterization of plasmids in *Erwinia stewartii*. Appl. Environ. Microbiol. **42**:599–604.
8. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The role of bacterial surface structures in pathogenesis. Crit. Rev. Microbiol. **8**:303–338.
9. **Dudman, W. F.** 1977. The role of surface polysaccharides in natural environments, p. 357–414. *In* I. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, Inc. (London), Ltd., London.
10. **Ferris, F. G., and T. J. Beveridge.** 1985. Functions of bacterial cell surface structures. BioScience **35**:172–177.
11. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
12. **Gottesman, S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. **162**:1111–1119.
13. **Huang, J.** 1980. Galactosyltransferase activity in *Erwinia stewartii* and its role in biosynthesis of extracellular polysaccharide. Physiol. Plant Pathol. **17**:73–80.
14. **Ivanoff, S. S., A. J. Riker, and H. A. Dettwiler.** 1938. Studies on cultural characteristics, physiology, and pathogenicity of strain types of *Phytomonas stewartii*. J. Bacteriol. **35**:235–253.
15. **Jobanputra, R. S., and N. Datta.** 1974. Trimethoprim R factors in enterobacteria from clinical specimens. J. Med. Microbiol. **7**: 169–177.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Markovitz, A.** 1977. Genetics and regulation of bacterial capsular polysaccharide synthesis and radiation sensitivity, p. 415–462. *In* I. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, Inc. (London), Ltd., London.
19. **Maurizi, M. R., P. Trisler, and S. Gottesman.** 1985. Insertional mutagenesis of the *lon* gene in *Escherichia coli*: *lon* is dispensable. J. Bacteriol. **164**:1124–1135.
20. **McCammon, S., D. L. Coplin, and R. Rowan.** 1985. Isolation of avirulent mutants of *Erwinia stewartii* using bacteriophage Mu pf7701. J. Gen. Microbiol. **131**:2993–3000.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Ruvkin, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) **289**: 85–88.
23. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn*3* *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4**:891–898.
24. **Sutherland, I. W.** 1979. Microbial exopolysaccharides: control of synthesis and acylation, p. 1–34. *In* R. C. W. Berkeley, G. W. Gooday, and D. C. Ellwood (ed.), Microbial polysaccharides and polysaccharases. Academic Press, Inc. (London), Ltd., London.
25. **Sutherland, I. W.** 1983. Microbial exopolysaccharides: their role in microbial adhesion in aqueous systems. Crit. Rev. Microbiol. **10**:173–201.
26. **Torres-Cabassa, A., and S. Gottesman.** 1987. Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. J. Bacteriol. **169**:981–989.
27. **Trisler, P., and S. Gottesman.** 1984. *lon* transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. J. Bacteriol. **160**:184–191.
28. **Van Alfen, N. K.** 1982. Wilts: concepts and mechanisms, p. 459–474. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 1. Academic Press, Inc. (London), Ltd., London.
29. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32**:369–379.