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

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Received 30 April 1987/Accepted 8 July 1987

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

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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.

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. ΔTn10 and Δkan indicate

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the use of deleted minitransposons derived from Tn10 (26, 29).

Cosmid pLAFR3 contains the mp8 polylinker from pUC8 inserted into the EcoRI 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). Tn3HolO1 and Tn5lac insertions in pES2144 were previously constructed and failed to complement Δcps 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 (3–7). 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 Tn5 (22) and Tn3HolO1 (23) was done in E. coli HB101 as described.

The Tn5 insertions in pES4507 were crossed into the chromosome of wild-type E. stewartii DC283 by homologous recombination. DC283 strains harboring different pES4507 derivatives were grown in Luria broth-kanamycin medium, and mer75′, a plasmid that is incompatible with pES4507, was then conjugated into the strain. Transconjugants were selected for trimethoprim resistance (Tm′) and kanamycin resistance (Km′) and screened for tetracycline sensitivity (Tet′); loss of the pES4507 plasmid was confirmed by agarose gel electrophoresis.

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**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source reference, or derivation</th>
</tr>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC4100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG1039</td>
<td>proC Ya21i 403::Tn10 iv::Tn5</td>
<td>27</td>
</tr>
<tr>
<td>SG1041</td>
<td>lon-100</td>
<td>27</td>
</tr>
<tr>
<td>SB1087</td>
<td>lon-100 rcsA40 zed-14::Tn10</td>
<td>27</td>
</tr>
<tr>
<td>JB3034</td>
<td>cpsB10::lac (immλ) lon-100 ΔcsA26 recA</td>
<td>J. Brill and S. Gottesman, manuscript in preparation</td>
</tr>
<tr>
<td>ATC5112</td>
<td>cps-11::Mu d1 lon-100 rcsA72::ΔTn10</td>
<td>26</td>
</tr>
<tr>
<td>ATC8109</td>
<td>cpsB10::lac (immλ) lon-100 rcsA72::ΔTn10</td>
<td>26</td>
</tr>
<tr>
<td>ATC8117</td>
<td>cpsB10::lac (immλ) lon+ rcsA72::ΔTn10</td>
<td>26</td>
</tr>
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<td>cpsB10::lac (immλ) lon-100</td>
<td>12</td>
</tr>
<tr>
<td>SG20581</td>
<td>cpsB10::lac (immλ) lon-100</td>
<td>12</td>
</tr>
<tr>
<td>SG20688</td>
<td>cpsB10::lac (immλ) lon+ rcsA15 ompC::Tn3</td>
<td>J. Brill and S. Gottesman, manuscript in preparation</td>
</tr>
</tbody>
</table>

**Other backgrounds**

ATC12017 F′ leu flhA rpsL supE thr Δlom-510 | C600 + P1(SG1039), + P1(SG4144) |
C600 F′ leu flhA rpsL supE thr | NIHb |
HB101 thr leu thi recA hsdR hsdM pro rpsL | 2 |
SG4144 Δlom-510 galK2 | 19 |
SG12014 thr leu tonA zed-14::Tn10 rcsA40 lon-100 | 12 |

**E. stewartii**

DC283 Nal′ | 7 |
MU14110 23823 DC283 rcsA14110::Mu p7701 kan EPS− Wts− | 20 |

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**TABLE 2. Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. stewartii clones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pES2144</td>
<td>Tet′</td>
<td>pVK100 derivative from SS104 library; 6</td>
</tr>
<tr>
<td>pES4507</td>
<td>EPS′ Wts− Tet′</td>
<td>6</td>
</tr>
<tr>
<td>pRF101</td>
<td>EPS′ Wts− Tm′</td>
<td>pVK100 derivative</td>
</tr>
<tr>
<td>pRF111</td>
<td>EPS′ Wts− Tet′</td>
<td>pLAFR3 derivative</td>
</tr>
<tr>
<td>pRF121</td>
<td>EPS′ Wts− Tm′</td>
<td>pLAFR3 derivative</td>
</tr>
<tr>
<td>pRF1111</td>
<td>EPS′ Wts− Amp′</td>
<td>pUC8 derivative</td>
</tr>
<tr>
<td>pRF3011</td>
<td>EPS′ Wts− rcsA301::Tn5 Amp′ Kan′</td>
<td>pUC8 derivative</td>
</tr>
<tr>
<td>pRF3021</td>
<td>EPS′ Wts− rcsA302::Tn5 Amp′ Kan′</td>
<td>pUC8 derivative</td>
</tr>
</tbody>
</table>

**E. coli clones**

pATC119 rcsA+ Amp′ | 26 |
| pATC352       | rcsA+ Cm′ Amp′                | 26 |
| pATC400       | rcsA+ Amp′                    | 26 |
| pATC401       | rcsA160::Δkan Amp′            | 26 |

**Other**

pLAFR3′ Tet′ cos | B. Staskawicz, submitted for publication |
| pR751         | Tm′ IncP1                     | 15 |
| pRK2013       | Kan′ CoEI mob′                | 11 |
| pRK2013-Tn7   | Str′ Spec′ Tm′ kan::Tn7       | Dennis Dean |

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* Phenotype in MU14110.
* Polylinker from pUC8 cloned into EcoRI site of pLAFR1.
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 Cuno) 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 [35S]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 p7701 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 EcoRI fragment (plasmid pRF111) and a 4.9-kb EcoRI-PstI 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 Tn5 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 Tn5 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 p7701 insertion in MU14110 (Fig. 1; 6).

The phenotype of the pES4507:Tn5 mutants was verified by crossing them into the chromosome of strain DC283. Tn5 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+ Tn5HoHoI insertions in the E. stewartii cpsA-D regions and a Tn5lac mutation in galE (Fig. 1). These plasmids were used to transform a derivative of MU14110, strain DC283::galE, which has lost the ability to synthesize EPS in the presence of 200 μg/ml ampicillin (21).

FIG. 1. Restriction map of the 19.5-kb HindIII insert from E. stewartii in pES4507 showing the corresponding sizes of the subclones which restored virulence to MU14110 and the location of Tn5 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, EcoRI; H, HindIII; P, PstI; S, SalI; B, BglII. ▼, Site of Mu p7701 insertion in MU14110.
2. Both Tn7HoHol and Tn5lac 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, cpsB178::Tn7HoHol (Table 3) and cpsD288::Tn7HoHol (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 Tn7HoHol 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.

**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-tandosynthesis Δkan (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 Tn5 insertion which abolishes the ability of the plasmid to confer the EPS+ Wts+ phenotype. pATC401 carries the rcsA 160::Δkan 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 Tn5 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::Tn5) was used to transform a lon− cps::lac strain (Table 4), suggesting that in *E. coli* the

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

<table>
<thead>
<tr>
<th>Region</th>
<th>Insertion</th>
<th>β-Galactosidase units*</th>
<th>E. stewartii</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rcsA+</td>
<td>rcsA</td>
<td>rcsA+</td>
</tr>
<tr>
<td>cpsA</td>
<td>87</td>
<td>46.7</td>
<td>2.1</td>
<td>3.2</td>
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<tr>
<td></td>
<td>72</td>
<td>48.2</td>
<td>1.7</td>
<td>1.5</td>
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<tr>
<td>cpsB</td>
<td>178</td>
<td>670.8</td>
<td>27.4</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>29.9</td>
<td>5.8</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>179</td>
<td>36.7</td>
<td>6.4</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>274</td>
<td>10.8</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>cpsD</td>
<td>136</td>
<td>15.7</td>
<td>7.9</td>
<td>6.2</td>
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<tr>
<td>galE</td>
<td>199</td>
<td>7.386</td>
<td>9.430</td>
<td>1.703</td>
</tr>
</tbody>
</table>

* For β-galactosidase units, see Miller (21). Values are the averages of two or more experiments. All cps::lacZ fusions were constructed with Tn7HoHol, except for 199, which is a Tn7HoHol::lacZ fusion.

* The rcsA+ strain was DC283; the rcsA host was MU14110.

* The rcsA+ strain was SG1041; the rcsA host was SG1087. Both strains are lon-100.
TABLE 4. Effect of cloned E. stewartii and E. coli rcsA genes on cps::lac expression in E. coli rcsA hosts

<table>
<thead>
<tr>
<th>Genotype of cps::lac strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-Galactosidase units&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>No plasmid</td>
</tr>
<tr>
<td>lon&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.7</td>
</tr>
<tr>
<td>lon&lt;sup&gt;+&lt;/sup&gt; rcsA72::ΔTn10</td>
<td>0.9</td>
</tr>
<tr>
<td>lon</td>
<td>246</td>
</tr>
<tr>
<td>lon rcsA72::ΔTn10</td>
<td>1.4</td>
</tr>
<tr>
<td>lon rcsB15</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> All strains are E. coli K-12. Strain designations: SG20582, lon<sup>+</sup>; ATC8117, lon<sup>+</sup> rcsA72::ΔTn10; SG20581, lon-100; ATC8109, lon-100 rcsA72::ΔTn10; SG20688, lon-100 rcsB15.

<sup>b</sup> For units, see Miller (21).

<sup>c</sup> The plasmid carries rcsA from E. stewartii in the pUC8 vector.

<sup>d</sup> 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<sup>a</sup>*), failed to hybridize to genomic DNA blots from *E. coli* HB101 and *Erwinia amylovora* E8. Likewise, pATC352 (*E. coli rcsA<sup>a</sup>*), 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::ΔTn10** insertion from *E. coli* was successfully crossed from a lactobacilli by using 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::Δkan** from *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<sup>a</sup>* plasmid (pATC400), nor the *E. stewartii** rcsA<sup>a</sup>* 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.

**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 transposons pATC119, a pUC19 derivative carrying the **rcsA** gene from *E. coli*; pRF1111; and its derivative plasmids pRF301 (rcsA301::Tn5) and pRF3021 (rcsA302::Tn5), in which the **rcsA** gene has been inactivated by Tn5 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::Tn5** derivatives (lanes 3 and 4). The protein was slightly smaller than the *E. coli** RcsA** protein (27 kDa) (lane 1). Although the two Tn5 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<sup>+</sup> lon<sup>+</sup>) and ATC12017 (cps<sup>+</sup> Δlon-510) were transformed with pRF1111. The *E. stewartii** RcsA** protein had a half-life of about 5 (±1) min in lon<sup>+</sup> cells; it was far more stable in the lon mutant, with a half-life of greater than 30 min (Fig. 4). In both lon<sup>+</sup> and lon<sup>-</sup> 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<sup>+</sup> cells and 20 min in lon<sup>-</sup> 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
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 bacteria 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. stewartii 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*.

**ACKNOWLEDGMENTS**

We thank Doris Majerczak for technical assistance.

Salaries and research support for D.L.C., R.D.F., P.J.D. and Doris Majerczak were provided by the U.S. Department of Agriculture under grants 83-CR-11205 and 85-CR-1-1781 from the Competitive Grants Office and by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

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