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The *rcsA* Gene from *Erwinia amylovora*: Identification, Nucleotide Sequence, and Regulation of Exopolysaccharide Biosynthesis

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RcsA is a positive activator of extracellular polysaccharide synthesis in the Enterobacteriaceae. A cosmid clone containing the *rcsA* gene from *Erwinia amylovora* was identified by its ability to restore mucoidy to an *E. stewartii rcsA* mutant. The *rcsA* gene was subcloned on a 2.2-kilobase *Hin*dIII-*Pst*I fragment that hybridized with an *E. stewartii rcsA* probe and complemented *E. stewartii* and *Escherichia coli rcsA* mutants. In addition, the cloned *E. amylovora rcsA* gene stimulated expression of *cps*::*lac* fusions in *E. coli* and *E. stewartii*. The *rcsA* region was sequenced, and one open reading frame of 211 amino acids was found. The predicted protein sequence specified by this open reading frame was 55% homologous with that of the *Klebsiella pneumoniae* RcsA protein. Highly conserved regions in the 3′ and 5′ ends of the two proteins were observed. An *E. amylovora rcsA* mutant was constructed by Tn*5* mutagenesis of the cloned gene followed by recombination of the mutation into the chromosome of wild-type strain Ea1/79. The synthesis of both amylovorin and levan was reduced by more than 90% in this mutant, indicating common regulation of the two polysaccharides by *rcsA*. Virulence of the *rcsA* mutant on immature pear fruit was diminished but not completely abolished.

Additional keywords: capsular polysaccharide, fire blight.

Erwinia amylovora (Burrill) Winslow *et al.* is the causal agent of fire blight on rosaceous plants. Typical disease symptoms are wilting, necrotic lesions, and droplets of bacterial ooze on plant surfaces. Production of extracellular polysaccharide (EPS) is an important factor in the virulence of *E. amylovora*. In all cases, EPS minus mutants have been avirulent (Bennett and Billing 1978; Ayers *et al.* 1979; Steinberger and Beer 1988). *E. amylovora* produces two different kinds of EPS, amylovorin and levan. Amylovorin is a complex acidic heteropolysaccharide that is produced both in culture and *in planta*. It is the main component of ooze from infected tissues. Synthesis of amylovorin seems to be influenced by a 29-kilobase (kb) plasmid, common to *E. amylovora* (Falkenstein *et al.* 1989). Levan is a fructan synthesized extracellularly on high-sucrose media by the enzyme levansucrase. So far, it has not been reported to be produced *in planta* by *E. amylovora*. Neither has it been observed to be produced *in planta* by other phytopathogenic bacteria (Fett and Dunn 1989).

The synthesis of capsules and slime is a common feature of gram-negative bacteria, especially plant pathogens. The regulation of EPS synthesis has been most extensively studied in *Escherichia coli*, which produces colanic acid. In *E. coli*, the genes for capsular polysaccharide synthesis (*cps*) are regulated positively by the product of the *rcsA* gene (Torres-Cabassa and Gottesman 1987). The RcsA protein, in turn, is rapidly degraded by the Lon protease (Gottesman 1984, 1989), which acts as a negative regulator. The availability of RcsA protein is a major limiting factor in colanic acid synthesis, and any increase in *rcsA* gene dosage results in increased EPS production (Torres-Cabassa and Gottesman 1987; Torres-Cabassa *et al.* 1987). RcsA-like proteins also regulate EPS synthesis in *E. stewartii* (Smith) Dye (Torres-Cabassa *et al.* 1987) and *Klebsiella pneumoniae* (Schroeter) Trevisan (syn. *K. aerogenes*) (Allen *et al.* 1987). In *E. coli*, additional control of capsule synthesis is provided by a two-component regulatory system consisting of a phosphorylated response regulator, RcsB, and a histidine protein kinase, RcsC (Brill *et al.* 1988; Gottesman *et al.* 1985; Stout and Gottesman 1990).

Our long-term goal is to determine the role of exopolysaccharides in pathogenicity of the fire blight pathogen *E. amylovora* and to study the regulation of EPS synthesis in response to the host environment. Since *E. stewartii* and *E. amylovora* are closely related pathogens and cause similar disease symptoms (Slade and Tiffin 1984), our approach has been to obtain *rcs* mutants of *E. amylovora* by first identifying cloned genes that complement characterized *E. stewartii* mutants and then using site-directed mutagenesis to construct the corresponding mutation in *E. amylovora*. In this study, we report the cloning and sequencing of the *E. amylovora rcsA* gene and show its function in other enterobacteria. By using Tn*5* mutagenesis and marker exchange techniques, an *rcsA* mutant of *E. amylovora* was constructed and tested for altered virulence. We also present evidence for RcsA activation of both amylovorin and levan synthesis in *E. amylovora*.

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While our work was in progress, Chatterjee *et al.* (1990) examined clones from *E. amylovora* EA246 that made *E. coli* HB101 mucoid, and they also found a gene that complements *rcsA* mutations in *E. stewartii* and *E. coli.* On this basis, they likewise proposed that EPS production in *E. amylovora* is regulated by *rcsA*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. All bacterial strains, plasmids, and phages used in this work are listed in Table 1. The S17-1 strain of *E. coli* was used as a host for a gene bank of chromosomal *E. amylovora* DNA in cosmid vector pVK100 (prepared by R. Theiler and K. Geider). The *E. stewartii cpsD*::*lacZ* fusion plasmid, pDM449, was derived from plasmid pES2144 (Dolph *et al.* 1988) by insertion of Tn*5lac* behind the promotor of *cpsD* (Coplin and Majerczak 1990). The DM3109 strain of *E. stewartii* was constructed by recombining the *cpsD*::Tn*5lac* insertion in plasmid pDM449 into the chromosome of Δ*rcsA* strain DM3032 (D. Coplin, unpublished).

For brevity, the *rcsA* genes from *E. amylovora, E. stewartii, E. coli,* and *K. pneumoniae* will be referred to as *rcsA-Ea, rcsA-Es, rcsA-Ec,* and *rcsA-Kp*, respectively.

Phage 4LM is a host range mutant of capsule-dependent *E. amylovora* phage 4L (Billing and Garrett 1980), which infects *E. stewartii.* It was isolated by plating approximately 6×10^8 plaque-forming units of 4L on *E. stewartii* wild-type strain DC283 on casein-peptone-glucose (CPG) agar (Bradshaw-Rouse *et al.* 1981). About 70 clear plaques were visible after 12 hr of incubation; one plaque was purified and designated 4LM.

Media, replica matings, and enzyme assays. Unless otherwise noted, cells were routinely grown in L broth or on L agar (Miller 1972). As appropriate, antibiotics were added to give the following final concentrations: ampicillin (200 μg/ml), carbenicillin (200 μg/ml), chloramphenicol (50 μg/ml), kanamycin (20 μg/ml), nalidixic acid (20 μg/ml), rifampicin (50 μg/ml), and tetracycline (20 μg/ml). Colony type of *E. stewartii* was evaluated on CPG agar. EPS production by *E. amylovora* was determined on asparagine minimal agar (ASP, Bennett and Billing 1980) supplemented with 1% sorbitol (ASP I) or 5% sucrose (ASP II).

β-Galactosidase was assayed as described by Miller (1972). Levansucrase was assayed according to Gross and Rudolph (1987). Bacteria were grown in ASP liquid medium supplemented with 1% fructose for 24 hr. The culture was centrifuged, and 1 ml of supernatant was used for the levansucrase assay. Sucrose was added to the supernatant to a final concentration of 5%, and either 20 μg/ml of kanamycin or 200 μg/ml of ampicillin was added to prevent further bacterial growth. The supernatant was incubated at 28° C for 3 hr, and the amount of glucose released from cleavage of sucrose was determined with glucose oxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The activity of one unit of levansucrase was defined as the production of 1×10^{-6} μg of glucose per cell in 3 hr.

Electroporation. Nonmobilizable plasmids were introduced into *E. amylovora* and *E. stewartii* by electroporation. Early exponential phase cells were washed twice in 1 mM HEPES buffer and once in 10% glycerol and finally resuspended in 10% glycerol. Forty microliters of the cell suspension was mixed with 4 μl of plasmid DNA (100 μg/ml) and electroporated using a Gene Pulser apparatus (Bio-Rad, Richmond, CA) with a pulse controller set at 200 Ω. Cells were pulsed in 0.2-cm cuvettes at 2,500 V and 25 μF capacitance. This produced a time constant of

Table 1. Bacterial strains, plasmids, and bacteriophages used in this study

Designation	Relevant characteristics[a]	Source or reference
Escherichia coli		
HB101	*recA hsdR hsdM rpsL leu thi pro*	Boyer and Roulland-Dussoix 1969
JB3034	Δ*rcsA26 lon-100 cpsB10*::*lac*(*imm*λ) *recA*	Brill *et al.* 1988
JM83	φ80 Δ(*lac-pro*) ΔM15	Messing *et al.* 1981
SG1087	*rcsA40 lon-100 zed-14*::Tn*10*	Trisler and Gottesman 1984
S17-1	*pro recA*, *tra*⁺ from RP4, Tp^r Sm^r	Simon *et al.* 1983
Erwinia stewartii		
DC283	SS104 Nal^r, EPS⁺	Coplin *et al.* 1981
MU14110	DC283 *rcsA14110*::Mu pf7701, Km^r	McCammon *et al.* 1985
DM3023	MU14110 Δ*rcsA*	D. Coplin, unpublished
DM3109	DM3023 *cpsD*::Tn*5lac*	D. Coplin, unpublished
E. amylovora		
Ea1/79	Wild-type strain	W. Zeller[b]
Ea1/79-MG	Ea1/79 *rcsA*::Tn*5*	This study
Plasmids		
pATC352	*E. coli rcsA* in pBR325, Cm^r Ap^r	Torres-Cabassa *et al.* 1987
pBluescript SK+	Ap^r	Stratagene[c]
pEA100	18.7-kb *Hin*dIII fragment in pVK100	This study
pEA101	3.6-kb *Hin*dIII subclone of pEA100 in pSUP106, Cm^r	This study
pEA102	2.2-kb *Hin*dIII-*Pst*I subclone of pEA101 in pBluescript SK+, Ap^r	This study
pEA108	pEA101 *rcsA*::Tn*5*, Cm^r Km^r	This study
pRK2013:Tn*7*	ColE1 *mob*⁺ *kan*::Tn*7*, Sm^r Sp^r Tp^r	Figurski and Helinski 1979
pDM449	*cpsD*::Tn*5lac*, Km^r Tc^r	Coplin and Majerczak 1990
pSB1	1.1-kb subclone of *E. stewartii rcsA* in pBluescript SK+, Ap^r	D. Coplin, unpublished
pSUP106	*cos mob*⁺, Cm^r Tc^r	Priefer *et al.* 1985
pVK100	*cos mob*⁺, Tc^r Km^r IncP	Knauf and Nester 1982
Phages		
4LM	Capsule-specific phage, host range mutant of phage 4L	Billing and Garrett 1980; this study
λ-Tn*5*	λ*cI857 rex*::Tn*5*	Ruvkin and Ausubel 1981

[a] Tp^r, Sm^r, Nal^r, Km^r, Cm^r, Ap^r, Sp^r, and Tc^r indicate resistance to trimethoprim, streptomycin, nalidixic acid, kanamycin, chloramphenicol, ampicillin, spectinomycin, and tetracycline, respectively; kb, kilobase.

[b] Biologische Bundesanstalt, Heidelberg-Dossenheim, Fed. Rep. of Germany.

[c] La Jolla, CA.

4.5 msec. Prior to pulsing, the cells were kept at 0° C. One milliliter of prewarmed SOC medium (Dower *et al.* 1988) was added immediately after pulsing. The cells were incubated for 2 hr at 28° C and then plated on L agar with appropriate antibiotics.

Recombinant DNA and hybridization techniques. Standard methods for preparation of small- and large-scale plasmid DNA, isolation of chromosomal DNA, restriction analysis, ligation, transformation, and Southern blotting were done as described previously (Maniatis *et al.* 1982; Torres-Cabassa *et al.* 1987). Blots were hybridized in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 50 mM Tris, 1% sodium dodecyl sulfate (SDS), 0.5% dried milk at 68° C for 12 hr and subsequently washed in 2× SSC plus 0.1% SDS and in 0.1× SSC plus 0.1% SDS, both at 68° C. The DNA probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim). Labeling and signal detection were conducted according to the supplier's specifications.

DNA sequencing and construction of nested deletions. Sequencing was done using the dideoxy chain termination technique of Sanger *et al.* (1977). DNA was cloned into the polylinker of phagemid vector pBluescript SK+ (Stratagene, La Jolla, CA). For the sequencing reactions, the Sequenase kit (U.S. Biochemical Corporation, Cleveland, OH) was used. Nested deletions in the 2.2-kb insert of pEA102 were made from both directions with an ExoIII/Mung Bean nuclease kit (Stratagene). After exonuclease digestion, plasmids were religated and transformed into *E. coli* JM83. Since plasmid pEA102 DNA made JM83 mucoid, transformants harboring clones with an inactive *rcsA* gene were identified by their nonmucoid phenotype after incubation at 28° C. Plasmids from both nonmucoid and mucoid transformants were isolated, linearized, and sized by electrophoresis in a 0.8% agarose gel. Plasmids containing inserts with suitable deletions adjacent to or within *rcsA-Ea* were chosen for sequencing. Data was verified by sequencing from both strands.

Transposon and replacement mutagenesis. Insertional mutagenesis of the *rcsA-Ea* gene in pEA101 was done in *E. coli* HB101 by infection with phage λ-Tn*5* as described by Ruvkin and Ausubel (1981). Plasmids from nonmucoid, kanamycin-resistant transductants were mobilized from HB101 into a nalidixic acid-resistant derivative of JM83 in triparental matings with intermediate donor HB101 (pRK2013::Tn*7*). The transconjugants were again screened for nonmucoidy and one plasmid, designated pEA108, was chosen for further study. The Tn*5* insertion in this plasmid mapped within the *rcsA-Ea* gene (Fig. 1).

The *rcsA*::Tn*5* mutation in pEA108 was introduced into the *E. amylovora* chromosome by marker exchange. Plasmid pEA108 was conjugated into wild-type strain Ea1/79 with selection for chloramphenicol-resistant transconjugants. After several transfers on kanamycin-selective medium to allow time for recombination between the plasmid and chromosome to occur, pEA108 was displaced by introduction of an incompatible plasmid, pSUP106 (Priefer *et al.* 1985); transconjugants were selected for kanamycin and tetracycline resistance and then screened for nonmucoid colonies on L agar plus 2% sucrose.

Polysaccharide extraction and quantification. Bacteria were grown on ASP I and ASP II agar, supplemented with antibiotics, for 3 days and harvested by suspending the bacterial growth in 3.0 ml of 0.85% NaCl. Numbers of cells were determined by dilution plating. Bacteria were removed by centrifugation for 1 hr at 15,000 × *g*, and then the supernatant was dialyzed against distilled water. The concentration of nondialyzable polysaccharides in the supernatant was determined by the anthrone reaction (Dubois *et al.* 1956) using galactose as a standard.

Virulence assays. Immature pear fruit (cv. Packham) were generously supplied by J. Young, Auckland, New Zealand, and stored at 4° C. Fruit were surface-sterilized with 10% sodium hypochlorite and cut into slices approximately 5 mm thick. Inoculations were made by stabbing the pear slices with toothpicks dipped into inocula or by pipetting 10 μl of an *E. amylovora* suspension (10^9 cells per milliliter) onto the surface of the freshly cut slices. Two inoculations were made in each pear slice, one in the center and another halfway between the center and edge of the slice. Inoculated slices were incubated in petri dishes sealed with Parafilm at 28° C in the dark. Each strain was replicated on at least four pear slices. Inoculated pear slices were rated for water-soaked lesions and ooze droplets at 2- to 3-day intervals until 2 wk after inoculation according to the following scale: 0 = no symptoms; 1 = symptoms at the inoculation site; 2 = symptoms over one third of the surface; 3 = symptoms over two thirds of the surface; and 4 = whole surface of the pear slice affected. Ratings were the average of at least four replicates.

RESULTS

Identification of an *E. amylovora rcsA* clone. Our approach to cloning the *rcsA* gene from *E. amylovora* was to screen clones of *E. amylovora* DNA for their ability

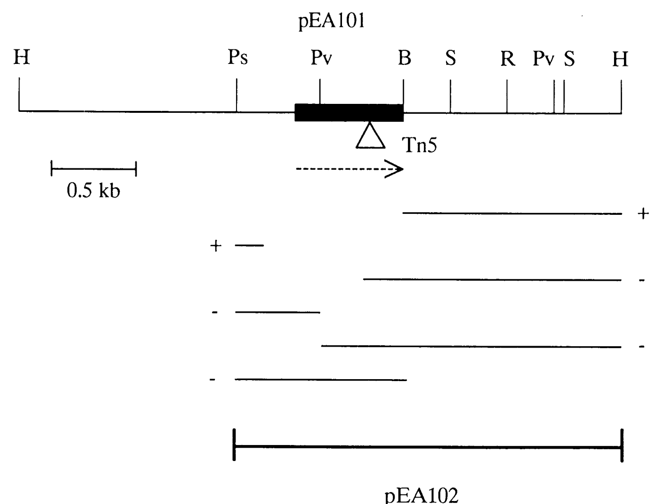

Fig. 1. Restriction map and deletion analysis of plasmid pEA101. The solid bar defines the open reading frame of the *rcsA* gene, and the arrow indicates the direction of transcription. The insertion site of Tn*5* in plasmid pEA108 is marked (Δ). The exonuclease III deletions of pEA102, used for locating the *rcsA* gene, are indicated below the map; the lines indicate DNA deleted from pEA102. (+) denotes inserts with extracellular polysaccharide-inducing activity. (−) denotes inserts inactive in extracellular polysaccharide induction. B, *Bam*HI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*I; R, *Rsa*I; and S, *Sal*I.

to restore EPS production to MU14110, an *rcsA* mutant of *E. stewartii.* Five hundred clones from a genomic library in cosmid vector pVK100 were individually transferred from *E. coli* S17-1 into MU14110 by conjugation. Transconjugants were selected on CPG agar supplemented with tetracycline and nalidixic acid. Three plasmids converted MU14110 from the nonmucoid to mucoid colony type. Restriction analysis revealed that these plasmids had identical 19.6-kb inserts, each containing 3.6-, 7.5-, and 8.5-kb *Hin*dIII fragments. One of the cosmids was designated pEA100 and used for further study.

The portion of pEA100 that contained the *rcsA-Ea* gene was determined by hybridization with a 1.1-kb *Sst*I-*Hin*dIII DNA fragment from pSB1, containing the *E. stewartii rcsA* gene. A 3.6-kb *Hin*dIII fragment, which hybridized with the *rcsA-Es* probe, was subcloned into vector pSUP106, and the resulting plasmid, pEA101, was mapped with various restriction enzymes (Fig. 1). No recognition sites were found for *Acc*I, *Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Kpn*I, *Sal*I, *Sma*I, *Sst*I, and *Xho*I. The 2.2-kb *Hin*dIII-*Pst*I fragment from pEA101 was further subcloned into vector pBluescript SK+, and the new plasmid was designated pEA102. Both subclones were able to restore EPS production to MU14110, indicating that they contained an intact *rcsA-Ea* gene.

Complementation of *E. stewartii* and *E. coli rcsA* mutants by *E. amylovora rcsA*$^+$ clones. Introduction of plasmid pEA101 into *E. stewartii* DM3023 Δ*rcsA* and *E. coli* SG1087 *rcsA40 lon-100* resulted in a mucoid colony type indicative of increased EPS production. Complementation of the *rcsA* deletion mutant DM3023 further showed that *rcsA-Ea* acted in *trans* rather than by recombination between homologous regions within the *rcsA* gene. EPS was isolated from the complemented strains and quantitated (Table 2). Plasmid pEA101 increased EPS production in both *E. stewartii* and *E. coli rcsA* mutants by more than 100-fold. For transconjugants of strains MU14110 and DM3023 of *E. stewartii*, this represented an increase of 10- to 20-fold over the wild-type strain DC283. A similar increase in EPS production over wild-type levels has been reported for the same strains containing *rcsA-Es* plasmids and is due to the increased gene dosage of *rcsA* (Torres-Cabassa *et al.* 1987).

A host range mutant of *E. amylovora* phage 4L that could infect wild-type *E. stewartii* DC283 was isolated. Sensitivity of *E. stewartii* to this phage, 4LM, was dependent on EPS production and an active *rcsA* gene since no plaques were formed by 4LM on MU14110 *rcsA* on CPG agar. Phage sensitivity and plaque morphology were fully restored to MU14110 by pEA101 (data not shown).

To demonstrate transcriptional activation of *cps* genes in *E. stewartii* and *E. coli* by RcsA-*Ea*, we examined β-galactosidase production from chromosomal *cps*::*lacZ* fusions in each species. pEA102 was introduced into *E. stewartii* DM3109 Δ*rcsA cpsD*::*lacZ* and *E. coli* JB3034 Δ*rcsA26 lon-100 cpsB*::*lac* by electroporation, and β-galactosidase production by the transformants was determined (Table 3). Expression of the *cps* genes was stimulated sevenfold in *E. stewartii* and 20-fold in *E. coli* by pEA102.

Nucleotide sequence of the *E. amylovora rcsA* gene. The identity of the *rcsA-Ea* gene was determined by sequencing pEA102 and locating regions of homology with previously sequenced *rcsA* genes. To prepare the 2.2-kb insert of pEA102 for sequencing, unidirectional nested deletions were made from both ends using exonuclease III and mung bean exonuclease. The digested plasmids were religated and transformed into *E. coli* JM83. Due to the high copy number of pEA102, most transformants had a smooth, slightly mucoid colony type on L agar at 28° C as compared to the normally rough, nonmucoid phenotype of JM83. This enabled us to use colony type to identify plasmids with deletions extending into the *rcsA* gene. Deletions extending 0.5 kb or more right of the *Pst*I site or more than 1.3 kb left of the right *Hin*dIII site abolished RcsA function. In this manner, we located the active element within the 1-kb *Pst*I-*Bam*HI fragment. This fragment was sequenced, and only one open reading frame (ORF) of 633 base pairs with recognizable translation initiation and termination sites was found (Fig. 2). This ORF could encode a 211 amino acid (24 kDa) protein, which corresponds in size to those of RcsA-*Ec* and RcsA-*Es* (Torres-Cabassa *et al.* 1987). A potential ribosome binding site and a possible −10 promoter sequence were detected upstream of the putative start codon of the protein. However, an element homologous to the *E. coli* −35 consensus promoter

Table 2. Complementation of *Erwinia stewartii* and *Escherichia coli rcsA* mutants with plasmid pEA101 containing the *E. amylovora rcsA* gene

Strain	Genotype	Presence of pEA101 *rcsA-Ea*$^+$	EPS[a] production (10^{-8} μg/cell)	Colony type[b]
E. stewartii				
DC283	*rcsA*$^+$	−	13.3	F
MU14110	*rcsA*	−	0.3	B
		+	150.3	F
DM3023	Δ*rcsA*	−	0.1	B
		+	380.0	F
E. coli				
SG1087	*rcsA*	−	0.1	B
		+	11.1	F

[a] EPS, extracellular polysaccharide.
[b] F = mucoid colony type (fluidal); B = nonmucoid colony type (butyrous). *E. stewartii* and *E. coli* colony types were evaluated on casein-peptone-glucose and L agars, respectively.

Table 3. Induction of *cps*::*lacZ* fusions in *Erwinia stewartii* and *Escherichia coli* by the *E. amylovora rcsA* gene and regulation of an *E. stewartii cpsD*::*lacZ* fusion by *rcsA* in *E. amylovora*

Strain	Chromosomal genotype	Plasmid genotype	β-Galactosidase assay[a]
E. stewartii			
DM3109	Δ*rcsA cpsD*::*lacZ*	−	176.9
DM3109 (pEA102)	Δ*rcsA cpsD*::*lacZ*	*rcsA*$^+$	1,225.5
DM3109 (pEA108)	Δ*rcsA cpsD*::*lacZ*	*rcsA*::Tn*5*	140.0
E. coli			
JB3034	Δ*rcsA cpsB*::*lacZ*	−	12.9
JB3034 (pEA102)	Δ*rcsA cpsB*::*lacZ*	*rcsA*$^+$	246.4
JB3034 (pEA108)	Δ*rcsA cpsB*::*lacZ*	*rcsA*::Tn*5*	10.4
E. amylovora			
Ea1/79	*rcsA*$^+$	−	3.3
Ea1/79 (pDM449)	*rcsA*$^+$	*cpsD*::*lacZ*	1,743.1
Ea1/79-MG (pDM449)	*rcsA*::Tn*5*	*cpsD*::*lacZ*	6.0

[a] β-Galactosidase units are as defined by Miller (1972). Data are averages of three or more determinations.

sequence was not found. The largest 5′ deletion of pEA102 that did not affect *rcsA* function in MU14110 terminated 218 nucleotides upstream of the ORF.

The nucleotide sequence of the *E. amylovora* ORF had 58% homology to the sequence of the *K. pneumoniae rcsA* gene reported by Allen *et al.* (1987). The predicted protein sequence of RcsA-*Ea* was compared with that of RcsA-*Kp* (Fig. 2). The overall homology of identical amino acids amounted to 55% and that of conserved amino acid changes was 65%. Four regions with identical homology of 64%

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-268  TGGATAACTGCATAAAATTCCGTGTTAAGTCAGATAGTTACGTATTTTGACTGCTAAATTACACAAA

-201  CAGGTGTTACGGCGTTTCTTGCCATATCAAATCTCAAACTGTCAGTTGGCAAAAAGCGCATTGTTCA

-134  CCTCGCTGATGATGACAGCGTTTTGCTAACCTTGGGTAATTATTACCAGCATGAAAACGGAATTTTT

 -67  GCTCGTAGCGCAAATGCGTAATTCATCGTTGAATTTAACCTATAACAAATTGGTGAGGGTCGCTGTT

   1  ATG CCG ACG ATT ATT ATG GAT TCA TGC AAT TAT ACA CGG CTG GGT TTA ACT
      Met Pro Thr Ile Ile Met Asp Ser Cys Asn Tyr Thr Arg Leu Gly Leu Thr

  51  GAA TAT ATG ACT GTC AAG GGA GTT AAA AAG AAA AAT ATA TCC CTG ATC AAT
      Glu Tyr Met Thr Val Lys Gly Val Lys Lys Lys Asn Ile Ser Leu Ile Asn

 102  GAT ATT GCA CAG TTA CAG AAT AAA TGC CAG CAG CTG AAA CCC GGC GTC GTG
      Asp Ile Ala Gln Leu Gln Asn Lys Cys Gln Gln Leu Lys Pro Gly Val Val

 153  CTT ATT AAC GAA GAC TGC TTT ATC CAT GAA TCT GAC GCC AGT GAG CGC ATC
      Leu Ile Asn Glu Asp Cys Phe Ile His Glu Ser Asp Ala Ser Glu Arg Ile

 204  AGG AAA ATT ATT CTT CAG CAT CCT GAT ACA CTA TTT TTC ATC TTT ATG GCC
      Arg Lys Ile Ile Leu Gln His Pro Asp Thr Leu Phe Phe Ile Phe Met Ala

 255  ATC TCT AAT ATT CAC TTT GAA GAA TAC CTG TAT GTG CGT AAC AAC CTG ATT
      Ile Ser Asn Ile His Phe Glu Glu Tyr Leu Tyr Val Arg Asn Asn Leu Ile

 306  ATT ACC TCA AAA GCG ATC AAA ATT TCT ACG CTG GAC TCC CTG CTC AAC GGC
      Ile Thr Ser Lys Ala Ile Lys Ile Ser Thr Leu Asp Ser Leu Leu Asn Gly

 357  TAT TTT CAG AAG AAA TTA AAC CTG TCT GTG CGT CAT GGC ACA CAT TCA GAA
      Tyr Phe Gln Lys Lys Leu Asn Leu Ser Val Arg His Gly Thr His Ser Glu
                                  *** *** *** ***

 408  GTC CAT CCG TTG ACG CTT AGC CAG ACC GAG TCA AAC ATG CTG AAA ATC TCC
      Val His Pro Leu Thr Leu Ser Gln Thr Glu Ser Asn Met Leu Lys Ile Ser

 459  ATG TCT GGC CAT GAT ACC ATT CAA ATA TCA GAC AAA ATG CAA ATC AAA GCC
      Met Ser Gly His Asp Thr Ile Gln Ile Ser Asp Lys Met Gln Ile Lys Ala

 510  AAA ACG GTT TCT TCG CAT AAA GGC AAT ATC AAA CGC AAG ATC AAA ACC CAT
      Lys Thr Val Ser Ser His Lys Gly Asn Ile Lys Arg Lys Ile Lys Thr His

 561  AAC AAG CAG GTT ATC TAT CAC GTT GTC CGC CTG ACC GCT AAT GTG ACC AGC
      Asn Lys Gln Val Ile Tyr His Val Val Arg Leu Thr Ala Asn Val Thr Ser

 612  GGT AGG GAT GTT AAC GAA AGA TAG GATCCCCGCCATGGCTGGCAGCGCGGTTTTAATGT
      Gly Arg Asp Val Asn Glu Arg STOP
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Fig. 2. Nucleotide sequence of a DNA fragment containing the *Erwinia amylovora rcsA* gene and the derived amino acid sequence of the RcsA protein. A potential ribosomal binding site (GAGG) and a possible *Escherichia coli* −10 consensus promoter sequence (TATAA) are marked with double underlines. Identical amino acids in the RcsA proteins of *E. amylovora* and *Klebsiella pneumoniae* are underlined. An insertion of 12 nucleotides, not present in the *K. pneumoniae rcsA* gene, is marked with asterisks.

or more were found (amino acids 1–27, 50–60, 68–106, and 140–211), indicating highly conserved domains of the two proteins. In the carboxy-terminus of the two RcsA proteins, beginning at amino acid position 168, the homology was 84%. RcsA-*Ea* had four additional amino acids resulting from an insertion at amino acid position 127. Only 18% homology was present in the vicinity of this insertion from amino acid position 107 to 139. Hydrophilicity plots (Hopp and Woods 1981) of the two proteins also revealed a very conserved pattern (data not shown).

Site-directed mutagenesis of the *E. amylovora rcsA* gene and phenotype of an *rcsA* mutant. Tn*5* mutagenesis of pEA101 resulted in an *rcsA*::Tn*5* insertion mutation that mapped close to the 3′ end of the gene (Fig. 1). The mutant plasmid, designated pEA108, no longer complemented MU14110 and SG1087 and failed to induce *cps*::*lacZ* fusions in DM3109 and JB3034 (Table 3). An *rcsA* mutant of *E. amylovora* was constructed by crossing the *rcsA*::Tn*5* mutation from pEA108 into the chromosome of wild-type strain Ea1/79. pEA108 was introduced into Ea1/79, allowed to recombine with the chromosome, and then displaced with incompatible plasmid pSUP106. About 0.7% of the transconjugants had a nonmucoid colony type, which was different from the mucoid appearance of wild-type colonies. Genomic blots of one nonmucoid mutant, Ea1/79-MG, were probed with labeled *rcsA-Ea* and Tn*5* DNA (Fig. 3). A single 12-kb *Eco*RI fragment hybridized with the Tn*5* probe. This indicated that only one Tn*5* insertion was present. We could not predict the size of the genomic *Eco*RI fragment because pEA100 lacks sites for this enzyme. In the *Hin*dIII digest probed with *rcsA-Ea* DNA, the wild-type 3.6-kb fragment was missing and two new fragments (3.2 and 2.6 kb) hybridized with the probe. These fragments were the correct size for the junction fragments created by insertion of Tn*5* into the 3.6-kb *Hin*dIII fragment of pEA101 and indicated that the mutation had been successfully exchanged into the chromosome of Ea1/79-MG.

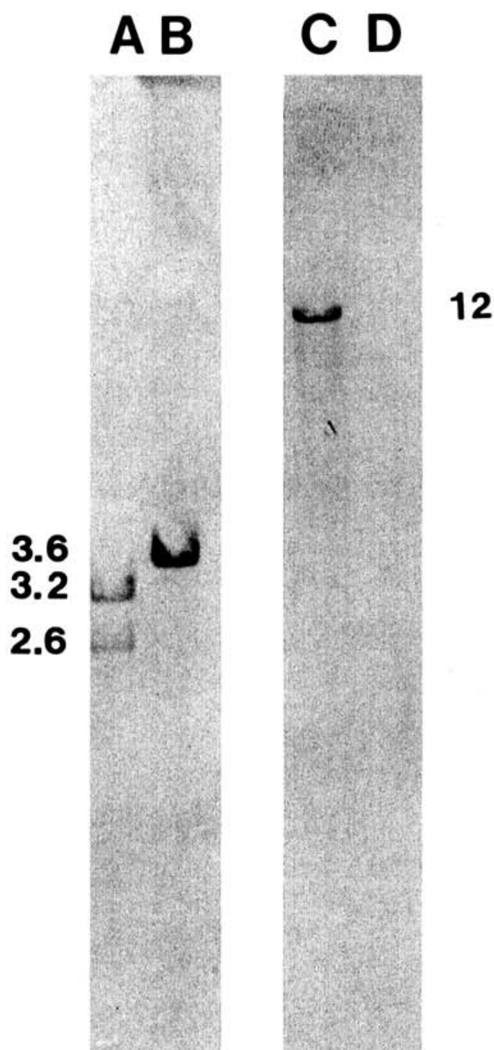

Fig. 3. Verification of the Tn*5* insertion in the chromosomal *rcsA* gene of strain Ea1/79-MG. Restricted chromosomal DNA was separated on a 0.8% agarose gel and transferred to a nylon membrane. (**A**) Ea1/79-MG and (**B**) Ea1/79 genomic DNA restricted with *Hin*dIII and hybridized with the 1.0-kilobase *Pst*I-*Bam*HI fragment of pEA102 containing the entire *Erwinia amylovora rcsA* gene. (**C**) Ea1/79-MG and (**D**) Ea1/79 genomic DNA restricted with *Eco*RI and hybridized with the 3.3-kilobase *Hin*dIII fragment of Tn*5*.

Ea1/79-MG was tested for production of amylovorin and levan on ASP I (1% sorbitol) or ASP II (5% sucrose) media, respectively. The synthesis of both polysaccharides was reduced more than 90% in Ea1/79-MG as compared to Ea1/79 (Table 4). In addition, activity of the enzyme levansucrase was decreased from 440 units for wild-type Ea1/79 to 50 units for mutant Ea1/79-MG. Sensitivity to EPS-dependent phage 4LM was also diminished for strain Ea1/79-MG; very turbid plaques were visible on ASP I agar in contrast to clear plaques on strain Ea1/79. EPS synthesis on ASP I and ASP II media was restored to Ea1/79-MG by *rcsA-Ea*$^{+}$ and *rcsA-Es*$^{+}$ plasmids (Table 4). However, the cloned *rcsA* gene from *E. coli* only partially complemented Ea1/79-MG. An *E. stewartii cpsD*::*lacZ* fusion, carried in plasmid pDM449, was expressed well in wild-type strain Ea1/79 but not in Ea1/79-MG (Table 3).

Table 4. Regulation of the synthesis of amylovorin and levan by the *rcsA* gene in *Erwinia amylovora* and complementation of an *E. amylovora rcsA* mutant by the *E. stewartii* and *Escherichia coli rcsA* genes[a]

Strain	Genotype	EPS production on ASP I[b] (10^{-8} μg/cell)	EPS production on ASP II[c] (10^{-8} μg/cell)
Ea1/79	*rcsA*$^{+}$	3.23	24.07
Ea1/79-MG	*rcsA*::Tn*5*	0.23	2.13
Ea1/79-MG (pEA102)	*rcsA*::Tn*5*/*rcsA-Ea*$^{+}$	2.87	23.77
Ea1/79-MG (pSB1)	*rcsA*::Tn*5*/*rcsA-Es*$^{+}$	3.20	24.31
Ea1/79-MG (pATC352)	*rcsA*::Tn*5*/*rcsA-Ec*$^{+}$	1.09	9.12

[a] Extracellular polysaccharide (EPS) was quantitated after bacterial growth on agar medium for 3 days. Results of at least three determinations were averaged.

[b] Asparagine minimal medium with 1% sorbitol; cultivation of wild-type *E. amylovora* on this medium results in synthesis of amylovorin (Bennett and Billing 1980).

[c] Asparagine minimal medium with 5% sucrose; cultivation of wild-type *E. amylovora* on this medium results in synthesis of levan (Bennett and Billing 1980).

The virulence of the *rcsA* mutant Ea1/79-MG was decreased on immature pear slices (Fig. 4) when compared to the wild-type strain Ea1/79. Small droplets of bacterial ooze were produced by Ea1/79-MG on the slices, but the amount was much less than that produced by Ea1/79. Virulence was restored to Ea1/79-MG by complementation with *rcsA-Ea*$^+$, but not by *rcsA-Es*$^+$ or *rcsA-Ec*$^+$ plasmids. Stability of plasmid pEA102 *rcsA-Ea*$^+$ in Ea1/79-MG was positively correlated with virulence, whereas pSB1 *rcsA-Es*$^+$ and pATC352 *rcsA-Ec*$^+$ were much less stable in Ea1/79-MG *in planta* (Table 5) and failed to completely restore virulence (Fig. 4). Segregation and loss of pATC352 were greater in pear slices that supported limited bacterial growth than in slices with no symptoms and little bacterial growth. In L broth, retention of the *rcsA*$^+$ plasmids in Ea1/79-MG was 100% for pEA102, 34% for pSB1, and 86% for pATC352 after growth for 10 generations without selection. The growth rate of Ea1/79-MG in L broth was not altered by any of the plasmids (data not shown).

DISCUSSION

In this study, we report the cloning of a gene from *E. amylovora* that complements *rcsA* mutants of *E. stewartii* and *E. coli* and activates transcription from *cps::lac* fusions in these species. When present in multiple copies, this gene also stimulates overproduction of EPS. It appears to be the same *rcsA*-like gene that was described by Chatterjee *et al.* (1990) in a different strain of *E. amylovora.* We have found that Chatterjee's clone and ours hybridize in Southern blots (data not shown), and except for an extra *Pvu*II site in the middle of their *rcsA* region, the restriction maps of the two clones are identical. We have extended the findings of Chatterjee *et al.* (1990) by constructing an *rcsA* mutation in *E. amylovora*, demonstrating that RcsA is required for synthesis of amylovorin and levan and virulence in this pathogen, and sequencing the *rcsA* gene. On the basis of phenotype, interspecific complementation, and nucleotide sequence, we conclude that this is the same *rcsA* gene which is present in other enteric bacteria. It remains to be shown, however, that RcsA is a transcriptional activator of the exopolysaccharide genes in *E. amylovora*, because cloned genes for amylovorin and levan biosynthesis are not yet available for regulation studies.

RcsA regulation of capsule synthesis has now been described in four species of the Enterobacteriaceae, and in all cases the RcsA proteins appear to be functionally equivalent in activation of *cps* genes. Allen *et al.* (1987) reported that *rcsA-Kp* functions in *E. coli*; Torres-Cabassa *et al.* (1987) that *rcsA-Es* and *rcsA-Ec* cross-complement; and Chatterjee *et al.* (1990) that multicopy *rcsA-Ea* plasmids confer a mucoid phenotype on *E. coli, E. stewartii*, and *Salmonella typhimurium* (Loeffler) Castellani and Chalmers. We likewise found that *rcsA-Ea* is interchangeable with *rcsA-Es* and *rcsA-Ec*, with the exception that *rcsA-Ec* did not fully complement an *E. amylovora rcsA* mutant. In addition, *cps::lac* fusions from *E. stewartii* and *E. coli* were specifically induced by RcsA-*Ea*, even in heterologous backgrounds. However, complementation data are based on phenotypic characterization (that is colony type) and quantitative determination of EPS. The failure of *rcsA-Ec* and *rcsA-Es* to restore virulence to an *E. amylovora rcsA* mutant, despite partial restoration of EPS synthesis, could be due to differences in the activation of individual *cps* operons by the heterologous RcsA and production of an altered EPS. Furthermore, some divergence in structure and function of the heterologous *rcsA* gene itself, its interaction with RcsB, or regulation of other genes required for pathogenicity could also influence the restoration of virulence in an *rcsA* mutant. Alternatively, instability of the recombinant plasmids combined with poor expression of the *rcsA-Es* and *rcsA-Ec* promoters in *E. amylovora* may have contributed to reduced EPS synthesis and virulence *in planta*.

The interchangeability of *rcsA* genes among enteric bacteria in induction of *cps* genes implies that they are highly conserved and have a common function and mode of action in each species. This was apparent in our comparison of

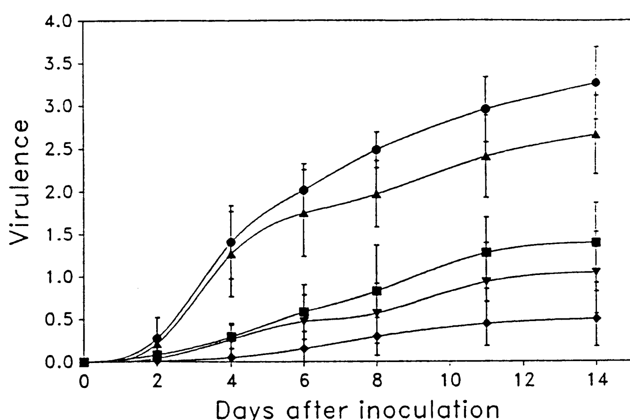

Fig. 4. Virulence of an *Erwinia amylovora rcsA* mutant. Slices of immature pears were inoculated with wild-type strain Ea1/79 (●), *rcsA* mutant Ea1/79-MG (■), and Ea1/79-MG with plasmids pEA102/*rcsA-Ea*$^+$ (▲), pSB1/*rcsA-Es*$^+$ (▼), and pATC352/*rcsA-Ec*$^+$ (◆), respectively. Virulence was rated on a scale from 1 to 4. Results are means of at least four determinations; error bars indicate the standard error.

Table 5. Stability of *rcsA*$^+$ plasmids in strain Ea1/79-MG growing in immature pear fruits[a]

Plasmid	Genotype	Virulence[b]	Plasmid retention[c] (%)
pEA102	*rcsA-Ea*$^+$	1.5–2.0	52.5 ± 26.3
		3.0–4.0	91.8 ± 9.0
pSB1	*rcsA-Es*$^+$	0.5–1.0	55.1 ± 35.1
		1.5–2.0	60.6 ± 40.7
pATC352	*rcsA-Ec*$^+$	0.1–0.2	64.3 ± 12.7
		1.5–1.8	8.8 ± 5.7

[a] Bacteria were reisolated from infected pear slices 10 days after inoculation. Bacteria were recovered by washing the inoculation site with 10 μl of water or from ooze droplets on the surface of an infected pear slice. Samples were dilution-plated on L agar supplemented with kanamycin.

[b] Bacteria were reisolated from pear slices showing the minimum and maximum levels of symptom expression for each strain. The two ranges in this column represent the extremes of virulence for each strain. Virulence was rated on a scale from 0 (no visible symptoms) to 4 (pear slice completely covered with ooze and/or water-soaked lesions).

[c] From each isolation, 100 colonies were checked for retention of the plasmid antibiotic resistance marker. Results are means (± SE) of at least four determinations.

the *rcsA* sequences from *E. amylovora* and *K. pneumoniae*. The overall similarity of the amino acid sequences was 55%, and highly conserved regions in the 3′ and 5′ ends of the protein were found; more variability occurred in the center of the gene. The conserved regions may be the active sites of the protein. The *rcsA-Ea* sequence was also compared with the unpublished sequences of *rcsA-Ec* (S. Gottesman, personal communication) and *rcsA-Es* (K. Poetter and D. Coplin, unpublished), and the amino acid homologies were 59% and 82%, respectively. In each case, the conserved regions were apparent.

Amylovorin and levan, the two exopolysaccharides produced by *E. amylovora*, are quite different in structure and mode of synthesis. Amylovorin is a high molecular weight, acidic heteropolymer, which is the primary capsular polysaccharide. The site of amylovorin synthesis is probably the periplasm, as has been shown for EPS synthesis in *Klebsiella, Xanthomonas*, and other gram-negative bacteria (Sutherland 1979). Nothing, however, is known about the biosynthetic pathway or the many enzymes that must be involved. In contrast, levan is a homopolymer of fructose, which is produced extracellularly by the enzyme levansucrase (Gross *et al.*, in press). Levansucrase is secreted constitutively in *Pseudomonas syringae* pv. *phaseolicola* (Burkholder) Young *et al.* (Gross and Rudolph 1987), and we found a similar pattern of synthesis in *E. amylovora* (Gross *et al.*, in press). The possibility that two very different polysaccharides respond to the same mechanism of regulation is very interesting.

Loss of amylovorin production in the *rcsA* mutant Ea1/79-MG resulted in reduced virulence on pear slices. Complementation of Ea1/79-MG with a plasmid carrying an *rcsA-Ea*$^+$ gene resulted in both increased virulence and EPS production, indicating that this mutant does not have a second-site mutation responsible for its phenotype. Since Ea1/79-MG is a regulatory mutant, we are assuming that the residual EPS it produced (approximately 10% of the wild-type amount) was unaltered amylovorin and that this may have been enough EPS to account for the water-soaking symptoms and ooze shown by this strain on pear slices. Since multiple regulatory controls operate on EPS synthesis in *E. coli* (Gottesman *et al.* 1985; Stout and Gottesman 1990), it is likely that *E. amylovora* may have other regulatory genes which can turn on amylovorin synthesis *in planta* and bypass the requirement for RcsA. It is unlikely that the residual virulence was due to levan, since G. Geier and K. Geider (unpublished results) have recently found that Tn*5* mutants deficient in levan production are not affected in virulence on pear slices. Our results are in agreement with previous studies on the pathogenicity of EPS minus mutants (Bennett and Billing 1978; Ayers *et al.* 1979; Steinberger and Beer 1988) and support the importance of amylovorin in pathogenesis.

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