MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 3, pp. 144-148, 1990

Isolation and Characterization of an *rcsA*-like Gene of *Erwinia amylovora* that Activates Extracellular Polysaccharide Production in *Erwinia* species, *Escherichia coli*, and *Salmonella typhimurium*

Asita Chatterjee, Wesley Chun, and Arun K. Chatterjee

Department of Plant Pathology, University of Missouri, Columbia 65211 U.S.A.
Received 30 August 1989. Accepted 14 November 1989.

Extracellular (= capsular) polysaccharide (EPS) production by *Erwinia amylovora* is required in the elicitation of the fire blight disease in apples and pears. To examine the regulation of EPS biosynthesis, we isolated from an *E. amylovora* cosmid library, *Escherichia coli* (HB101) clones that had a mucoid phenotype. The subcloning of a 2.2-kb DNA fragment of one of the cosmid clones resulted in the construct pAKC111, which conferred a mucoid phenotype in strains of *E. amylovora*, *E. stewartii*, *E. coli*, and *Salmonella typhimurium*. By analyzing a series of nested deletions, we localized the gene controlling EPS production within a 1-kb DNA segment. In *E. coli* and *S. typhimurium*, the *E. amylovora* gene activated the biosynthesis of structurally different polysaccharides as indicated by their differential susceptibility to a depolymerase specific for the *E. amylovora* EPS. The presence of an *rcsA*-like gene on pAKC111 was determined by its ability to complement *E. coli rcsA* mutations. However, with Southern hybridizations under high stringency conditions, homology between the cloned *E. amylovora* DNA and the *rcsA* genes of *E. coli*, *E. stewartii*, and *Klebsiella aerogenes* was not detected.

Erwinia amylovora (Burrill) Winslow *et al.* infects a wide variety of rosaceous plants and causes fire blight disease of apples and pears (Aldwinckle and Beer 1979). The bacterium also elicits a hypersensitive reaction in nonhost plants such as tobacco and cowpea. Several reports implicate extracellular (= capsular) polysaccharide (EPS) in the infection process (Ayers *et al.* 1979; Steinberger and Beer 1988). Despite the evidence for the involvement of EPS in pathogenicity of *E. amylovora* and other plant pathogenic bacteria (Billing 1984; Chatterjee and Vidaver 1986) as well as in *Rhizobium* symbiosis (Reed *et al.* 1988), little is known of the biosynthetic steps and their regulation.

In *Escherichia coli* (Migula) Castellani and Chalmers, capsule (= colanic acid) production is controlled by the positive regulators *rcsA* and *rcsB* and the negative regulators *rcsC* and *lon* (Brill *et al.* 1988; Gottesman *et al.* 1985; Torres-Cabassa *et al.* 1987). An *rcsA*-like gene of *Erwinia stewartii* (Smith) Dye was found to activate EPS (*cps*) genes of *E. stewartii* as well as *E. coli* (Torres-Cabassa *et al.* 1987). As a prelude to a detailed analysis of the EPS regulatory circuit, we report here the cloning of an *rcsA*-like gene and its expression in *E. amylovora* and in other enterobacteria. Our data show that although *E. amylovora rcsA* activates polysaccharide synthesis in various enterobacteria, the gene does not share extensive homology with the other *rcsA* genes. Preliminary reports of some of this work have been published (Chun *et al.* 1989; A. Chatterjee, W. Chun, and A. K. Chatterjee. Molecular cloning of an *rcsA*-like gene of *Erwinia amylovora* that stimulates extracellular polysaccharide synthesis in *Erwinia* species and other enterobacteria, (Abstr.) International Symposium of the Phytochemical Society of Europe, Biochemistry and Molecular Biology of Plant-Pathogen Interactions, University of East Anglia, Norwich, U.K., April 10-14, 1989).

Address correspondence to A. K. Chatterjee, Department of Plant Pathology, 108 Waters Hall, University of Missouri, Columbia, MO 65211 U.S.A.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains and plasmids used in this study are listed in Table 1. Most of the *Erwinia* strains, *Klebsiella aerogenes* Trevisan, and *Salmonella typhimurium* (Loeffler) Castellani and Chalmers (LT2) have been described (Allen *et al.* 1987; Ayers *et al.* 1979; Chatterjee and Starr 1972; Steinberger and Beer 1988; Torres-Cabassa *et al.* 1987; Silhavy *et al.* 1984). *E. amylovora* wild-type strain E77 was from R. N. Goodman, Department of Plant Pathology, University of Missouri, Columbia; *Enterobacter aerogenes* Hormaeche and Edwards is from our laboratory collection. The nonmucoid *E. amylovora* strains EA225 Cap9 and EA225 Cap18A were previously derived from EA225 by nitrosoguanidine mutagenesis.

Erwinia strains were maintained on yeast extract-dextrose-$CaCl_2$ (YDC) agar, whereas *E. coli* and the other enterobacteria were kept on Luria-Bertani (LB) agar medium. The plasmid-carrying strains were kept on LB agar medium supplemented with the appropriate antibiotics.

Media and culture conditions. LB medium, minimal salts medium, tryptone broth, and YDC agar were described previously (Chatterjee 1980; Silhavy *et al.* 1984). For *E. amylovora* strains, the minimal medium was supplemented with 20 μg/ml of nicotinic acid (Chatterjee and Starr 1972).

Proline (50 μg/ml), leucine (50 μg/ml), and thiamine (20 μg/ml) were added to the minimal medium as necessary. When desired, antibiotics were added as follows: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml. The *E. amylovora* strains and other *Erwinia* spp. were grown at 28° C. *E. coli* and other enterobacterial strains were grown at 37° C unless otherwise indicated.

DNA techniques. Chromosomal DNA was extracted by the procedure of Staskawicz *et al.* (1984). Plasmid DNA was isolated by alkaline lysis (Maniatis *et al.* 1982). For cloning purposes and Southern analysis, chromosomal and plasmid DNAs were purified in cesium chloride density gradients (Maniatis *et al.* 1982). Standard procedures were used in gel electrophoresis, extraction of DNA fragments from low melting point (LMP) agarose gels, DNA ligations in liquid or in LMP agarose gels, and Southern hybridizations (de Bruijn and Lupski 1984; Maniatis *et al.* 1982; Whitford and DiCioccio 1988). A series of nested deletions were constructed using *Exo*III-*Exo*VII enzymes as described by Henikoff (1987) and according to the manufacturers' (Promega Biotechnology, Madison, WI, and Bethesda Research Laboratories, Gaithersburg, MD) instructions. Transformation of *E. coli* was conducted as described by Maniatis *et al.* (1982). Triparental matings were done according to Murata *et al.* (1988).

Table 1. *Escherichia coli* strains and recombinant plasmids used in this study

Designation	Relevant characteristics or genotype[b]	Source, reference, or derivation
E. coli		
DH5α	*ϕ80dlacZ*ΔM15, Δ(*lacZYA-argF*)U169, *thi, recA1, hsdR17*	Bethesda Research Laboratories, Gaithersburg, MD
HB101	*leu, thi, pro, recA, hsdR, hsdM, rpsL*	S. H. DeBoer, Agriculture Canada, Research Station, Vancouver, Canada
K-12	Prototrophic	Laboratory collection
SG1086	*lon-100, ompC*::Tn*5, rcsB42 arg, lac, str*$^+$	S. Gottesman, National Cancer Institute, Bethesda, MD
SG1087	*lon-100, rcsA40, zed-14*::Tn*10*	Torres-Cabassa *et al.* 1987
JB3030[a]	*lon-100, recA*	Brill *et al.* 1988
JB3032[a]	*lon-100, rcsB15, recA, ompC*::Tn*5*	Brill *et al.* 1988
JB3034[a]	*lon-100, rcsA26, recA*	Brill *et al.* 1988
Plasmids		
pAKC107	*rcsA-Ea*$^+$[c], Tc^r	pLAFR5 (Keen *et al.* 1988) cosmid clone containing *Erwinia amylovora* insert DNA
pAKC109	*rcsA-Ea*$^+$, Ap^r	pPR42 (Gill and Warren 1988) containing 2.2-kb insert DNA from pAKC107
pAKC111	*rcsA-Ea*$^+$, Tc^r	pRK415 (Keen *et al.* 1988) containing the 2.2-kb *Hin*dIII fragment (Fig. 1) from pAKC109
pAKC112	*rcsA-Ea*$^+$, Ap^r	pBluescript SK$^+$ (Stratagene, La Jolla, CA) containing the 2.2-kb *Hin*dIII fragment (Fig. 1) from pAKC109
pAKC113	Ap^r	pAKC112-DEL92 (Fig. 1)
pAKC114	*rcsA-Ea*, Ap^r	pAKC112-DEL111 (Fig. 1)
pAKC115	*rcsA-Ea*, Ap^r	pAKC112-DEL311 (Fig. 1)
pAKC116	Ap^r	pAKC112-DEL113 (Fig. 1)

[a] Strains containing *cpsB10-lacZ* chromosomal fusions.
[b] Tc = tetracycline, and Ap = ampicillin.
[c] *rcsA-Ea* = *rcsA* gene isolated from *E. amylovora*.

Preparation of an EPS depolymerase. *E. coli* HB101(pJH94) (Hartung *et al.* 1988), which produces a polysaccharide depolymerase specific for the *E. amylovora* EPS, was grown for 18 hr at 37° C in LB medium plus ampicillin. Cells were collected by centrifugation (12,000 × *g*) at 4° C, resuspended in 1/20th volume of 10 mM Tris-HCl (pH 7.5), and disrupted by sonicating with a Braunsonic 1510 sonicator (Thurn and Chatterjee 1985). The sonicated sample was centrifuged (12,000 × *g*) at 4° C for 10 min, and the supernatant was then used as the source of the EPS depolymerase.

RESULTS

Identification of an *E. amylovora* DNA segment that regulates EPS production. A genomic library of *E. amylovora* strain EA246 was constructed in *E. coli* strain HB101 by ligating approximately 20- to 30-kilobase (kb) *Sau*3A fragments to the *Bam*HI site of the cosmid vector, pLAFR5. Among the 2,000 tetracycline-resistant colonies, five appeared mucoid. The insert DNA (approximately 25 kb) of one such plasmid (pAKC107) was subjected to a partial *Sau*3A digest. The fragments were ligated to the *Bam*HI site of the mobilizable vector, pPR42, and transformed into DH5α. pAKC109, derived in this manner, carried 2.2 kb of insert DNA and conferred a mucoid phenotype in HB101.

A partial restriction map (Fig. 1) shows the insert DNA residing within two *Hin*dIII sites, with one site in the cloned DNA and the other in the multiple cloning site of the vector. The *Hin*dIII fragment was subsequently recloned into pRK415 (for example, pAKC111) and pBluescript SK$^+$ (for example, pAKC112). Cloning of this fragment in either orientation in pRK415 had no effect on the expression of the mucoid phenotype in *E. coli* transformants.

To localize the gene controlling EPS production on the 2.2-kb DNA segment, we created nested unidirectional deletions into the *Hin*dIII fragment of pAKC112. By comparing the phenotype conferred by various deletions, we localized the gene within a 1-kb segment between the *Pst*I site and a region near the *Bam*HI site (Fig. 1).

The mucoid phenotype conferred by pAKC109 suggested that the gene was functionally similar to the *rcsA* gene of *E. coli* (Gottesman *et al.* 1985), *E. stewartii* (Torres-Cabassa *et al.* 1987), and *K. aerogenes* (Allen *et al.* 1987).

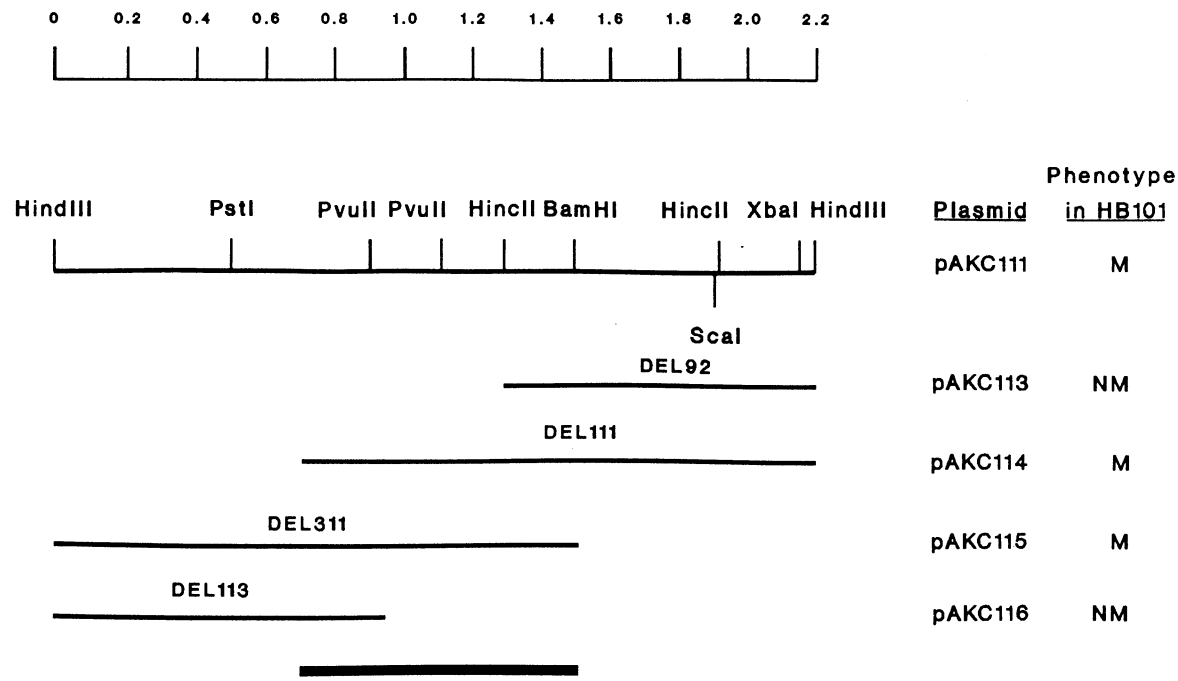

Fig. 1. Partial restriction map and deletion analysis of the *Erwinia amylovora rcsA*$^+$ DNA segment. The left *Hin*dIII restriction endonuclease site is in the *E. amylovora* insert DNA. The right *Hin*dIII site is in the multiple cloning site present in pPR42. Sites for *Sal*I and *Pst*I also present in the multiple cloning site were not mapped. The 2.2-kilobase (kb) insert was not cleaved by *Acc*I, *Ava*I, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Eco*RV, *Kpn*I, *Mbo*I, *Mlu*I, *Nar*I, *Nde*I, *Not*I, *Nru*I, *Sac*I, and *Sma*I. Unidirectional deletions in the *Hin*dIII fragment cloned into pBluescript SK$^+$ were made by using the exonucleases *Exo*III and *Exo*VII. The solid lines represent the DNA segment retained after the deletions. The plasmids carrying the deletions were introduced into *Escherichia coli* strain HB101, and the appearance of the mucoid phenotype was noted as mucoid (M) or nonmucoid (NM) on Luria-Bertani agar medium supplemented with ampicillin. The thick line spanning about 1 kb indicates the limits of the *rcsA* gene.

To test if indeed the cloned gene behaved like *rcsA*, pAKC109 or pPR42 was transferred to an *rcsA* strain (SG1087) and an *rcsB* strain (SG1086) of *E. coli*. SG1087(pAKC109) became mucoid whereas SG1087 (pPR42) remained nonmucoid. SG1086 colonies containing pAKC109 or pPR42 were not mucoid. Thus, the cloned *E. amylovora* gene is functionally similar to the *E. coli rcsA*. We designated the gene as *rcsA-Ea* to distinguish it from that of *E. coli*.

As a further test of complementation of the *rcsA* mutation, we transferred pAKC111 to *rcsA* and *rcsB* derivatives of *E. coli* carrying a *cpsB-lacZ* transcriptional fusion (see Table 1 for the genotype). The plasmid-carrying strains were grown for 18 hr in tryptone broth supplemented with the appropriate drugs, and β-galactosidase activity in the cells was determined (Miller 1972). The data (Table 2) show activation of a *cpsB-lacZ* fusion by the *E. amylovora* gene in an *rcsA* strain but not in the *rcsB* strain. The level of β-galactosidase was 26-fold higher in the *rcsA*-deficient strain (JB3034) carrying pAKC111 compared to the level in JB3034 alone.

Expression of *rcsA-Ea* in various enterobacteria. To test the expression of the regulatory function of *rcsA-Ea* in various enterobacteria, pAKC109 was transferred by triparental matings. The plasmid conferred a mucoid phenotype in strains of *E. amylovora* (EA178, EA321, and EA101B), *E. stewartii* (Mu14110), *E. coli* (DH5α and HB101), and *S. typhimurium* (LT2). pAKC109-mediated mucoid phenotype was not detected in *E. amylovora* strains

Table 2. Activation of a *cpsB-lacZ* fusion in *Escherichia coli* strains by the *Erwinia amylovora rcsA*$^+$ gene

Strain (plasmid)[a]	Relevant genotype	β-Galactosidase units
JB3030	*lon-100*	80
JB3030(pAKC111)	*lon-100/rcsA-Ea*$^+$	431
JB3034	*rcsA*	ND[b]
JB3034(pAKC111)	*rcsA/rcsA-Ea*$^+$	576
JB3032	*rcsB*	ND
JB3032(pAKC111)	*rcsB/rcsA-Ea*$^+$	ND

[a] *E. coli* mutants carrying a chromosomal copy of the *cpsB10-lacZ* fusion were transformed with pACK111 (see Table 1 for further details). The transformants were purified by two successive single colony isolations on Luria-Bertani agar medium containing tetracycline. Bacteria were grown in tryptone broth supplemented with appropriate drugs and assayed for β-galactosidase activity (Miller 1972) after 12 hr of incubation at 32° C. The data are expressed as Miller units.

[b] ND = Not detectable, that is, absorbance of the reaction mixture at 420 nm was < 0.05.

EA225 Cap9 and EA225 Cap18A; *E. carotovora* subsp. *atroseptica* (van Hall) Dye strain Eca12; *E. c.* subsp. *carotovora* (Jones) Bergey *et al.* strain Ecc71; *E. chrysanthemi* Burkholder *et al.* strain EC16; and *E. herbicola* (Lohnis) Dye strain EH106. A mucoid phenotype did not result from the transfer of pPR42 in these strains.

The quantity of EPS produced in plasmid-carrying strains was determined by an anthrone assay (Disch 1962) using galactose as a standard. Compared to pRK415, with pAKC111, the level of EPS was 20-fold higher in *E.*

amylovora strain EA178, sixfold higher in *E. coli* strain HB101, and twofold higher in *S. typhimurium* strain LT2.

The viscosity of the *E. amylovora* (EA178/pAKC111) EPS rapidly decreased in the presence of a depolymerase known to degrade the *E. amylovora* EPS (Hartung *et al.* 1988). The enzyme did not cause a change in the viscosity of the polysaccharides obtained from *E. coli* (HB101/pAKC111) and *S. typhimurium* (LT2/pAKC111) (Fig. 2).

Occurrence of *rcsA* homologues in enterobacteria. To test if the nucleotide sequence of the *rcsA* genes was conserved in these bacteria, Southern hybridizations were conducted using the 1.5-kb *Pst*I-*Sca*I fragment of pAKC109 (Fig. 1) as a probe against plasmids known to carry the *rcsA* genes and the *Eco*RI restricted genomic digests of the various enterobacteria. Under high stringency conditions (prehybridization, hybridization, and 0.1× SSC wash for 15 min at 65° C), strong signals were detected with an 8-kb chromosomal *Eco*RI fragment of the *E. amylovora* strains E9, E77, EA101B, EA178, EA225, EA246, and EA321. In contrast, under similar stringency conditions, we did not detect signals with *rcsA* genes cloned from *E. coli* (Gottesman *et al.* 1985), *E. stewartii* (Torres-Cabassa *et al.* 1987), and *K. aerogenes* (Allen *et al.* 1987) or with resident plasmids of *E. amylovora*. No signals were observed with chromosomal DNA from *E. c.* subsp. *carotovora* (Ecc71), *E. c.* subsp. *atroseptica* (Eca12), *E. chrysanthemi* (EC16), *E. herbicola* (EH106), *E. stewartii* (Mu14110), *E. coli* (HB101, K-12, and SG1087), *S. typhimurium* (LT2), and *Enterobacter aerogenes*.

DISCUSSION

Several lines of evidence show that the gene cloned from *E. amylovora* is indeed a functional homologue of *rcsA*, a positive activator of the *E. coli* genes for polysaccharide biosynthesis. The *E. amylovora* gene restored EPS production and also activated the transcription of a *cpsB-lacZ* operon fusion in the *rcsA* but not in the *rcsB* strains of *E. coli* (Table 2). The polysaccharides produced by *E. coli* and *S. typhimurium* harboring *rcsA-Ea* were structurally different from those produced by *E. amylovora*. These findings taken together with those previously reported for *E. stewartii* (Torres-Cabassa *et al.* 1987), *E. coli* (Gottesman *et al.* 1985), and *K. aerogenes* (Allen *et al.* 1987) show a functional homology in *rcsA* of enterobacteria.

In light of the expression of *rcsA-Ea* in various enterobacteria, the lack of homology in Southern hybridizations between this and *rcsA* genes of other species was somewhat unexpected. We should, however, note that the *rcsA* gene of *K. aerogenes* was found not to share extensive homology with the *E. coli* gene (Allen *et al.* 1987). Likewise, Torres-Cabassa *et al.* (1987) did not detect in Southern hybridizations significant homology between the *rcsA* genes of *E. stewartii* and *E. coli* or between the *rcsA* genes of *E. stewartii* and *E. amylovora*.

The findings presented here reveal that some aspects of the regulation of EPS production in *E. amylovora* and *E. coli* are similar. Moreover, the lack of restoration of EPS production or the absence of the activation of the *cpsB-lacZ* fusions in the *rcsB* strain of *E. coli* implies a cooperative interaction between the *rcsB* and *rcsA* products for the activation of the EPS (*cps*) genes, as postulated by Gottesman and associates for the *E. coli* system (Brill *et al.* 1988).

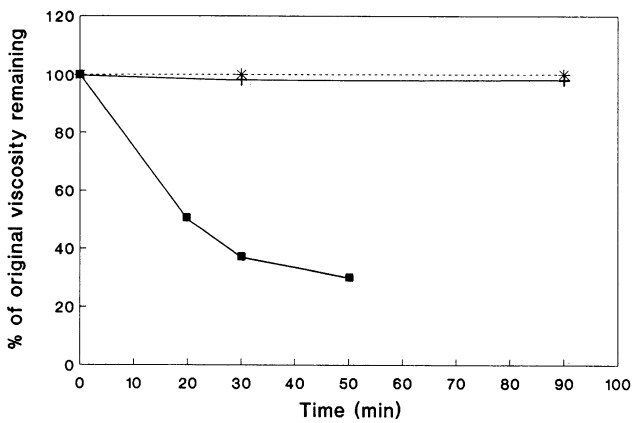

Fig. 2. Effect of *Erwinia amylovora* extracellular polysaccharide (EPS) specific depolymerase (DP) on EPS produced by *Escherichia coli*, HB101(pAKC111), *; *Salmonella typhimurium*, LT2(pAKC111), +; and *E. amylovora*, EA178(pAKC111), ■. Polysaccharides, extracted by the method of Hollingsworth *et al.* (1984), were dissolved in water to yield the following concentrations: HB101(pAKC111), 3.5 mg/ml; LT2(pAKC111), 3.5 mg/ml; and EA178(pAKC111), 5 mg/ml. Five microliters of cell extract (approximately 4 mg total protein per milliliter) from HB101(pJH94) containing EPS DP was added to 10 ml of EPS preparations. Viscosity was recorded at various time intervals using a Fisher Brand viscometer (ASTM size 50). Changes in viscosity were calculated as: (efflux time of EPS + DP)/(efflux time of EPS alone) × 100.

ACKNOWLEDGMENTS

This research was supported by a grant from the Food for the 21st Century program of the University of Missouri, Columbia.

We wish to thank S. V. Beer, D. Coplin, R. N. Goodman, S. Gottesman, N. T. Keen, and G. Warren for the bacterial strains and plasmids.

This work is journal series 10,928 of the Missouri Agricultural Experiment Station.

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