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

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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 pACK111, 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 pACK111 was determined by its ability to complement E. coli rcsA mutations. However, with Southern hybridizations under high stringent 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 bacteria 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.


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 Hornbacek and Edwards is from our laboratory collection. The non-mucoid E. amylovora strains EA225 Cap9 and EA225 Cap18A were previously derived from EA225 by nitrosoquinidine mutagenesis. Erwinia strains were maintained on yeast extract-dextrose-CaCl2 (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 Sasaki 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 Luspi 1984; Maniatis et al. 1982; Whitford and DiCioccio 1988). A series of nested deletions were constructed using ExoIII-ExoVII 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).

**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 strains HB101 by ligating approximately 20- to 30-kilobase (kb) Sau3A fragments to the BamHI 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 Sau3A digest. The fragments were ligated to the BamHI 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 HindIII sites, with one site in the cloned DNA and the other in the multiple cloning site of the vector. The HindIII 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 HindIII fragment of pAKC112. By comparing the phenotype conferred by various deletions, we localized the gene within a 1-kb segment between the PstI site and a region near the BamHI 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).
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 I 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. stewardii (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

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Relevant genotype</th>
<th>β-Galactosidase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB3030</td>
<td>lon-100</td>
<td>80</td>
</tr>
<tr>
<td>JB3030(pAKC111)</td>
<td>lon-100/rcsA-Ea</td>
<td>431</td>
</tr>
<tr>
<td>JB3034</td>
<td>rcsA</td>
<td>ND</td>
</tr>
<tr>
<td>JB3034(pAKC111)</td>
<td>rcsA/rcsA-Ea</td>
<td>576</td>
</tr>
<tr>
<td>JB3032</td>
<td>rcsB</td>
<td>ND</td>
</tr>
<tr>
<td>JB3032(pAKC111)</td>
<td>rcsB/rcsA-Ea</td>
<td>ND</td>
</tr>
</tbody>
</table>

*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 37°C. The data are expressed as Miller units.

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 PstI-Scal fragment of pAKC109 (Fig. 1) as a probe against plasmids known to carry the rcsA genes and the EcoRI restricted genomic digests of the various enterobacteria. Under high stringency conditions (prehybridization, hybridization, and 0.1X SSC wash for 15 min at 65°C), strong signals were detected with an 8-kb chromosomal EcoRI 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* (Ec71), *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 (*eps*) genes, as postulated by Gottesman and associates for the *E. coli* system (Brill et al. 1988).

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**LITERATURE CITED**


stimulates extracellular polysaccharide (EPS) production in *Erwinia* spp. and other enterobacteria. (Abstr.) Phytopathology 79:1156.


