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Characterization of the *rcsB* Gene from *Erwinia amylovora* and Its Influence on Exopolysaccharide Synthesis and Virulence of the Fire Blight Pathogen

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RcsB belongs to a family of positive regulators of exopolysaccharide synthesis in various enterobacteria. The *rcsB* gene of the fire blight pathogen *Erwinia amylovora* was cloned by PCR amplification with consensus primers, and its role in exopolysaccharide (EPS) synthesis was investigated. Its overexpression from high-copy-number plasmids stimulated the synthesis of the acidic EPS amylovoran and suppressed expression of the levan-forming enzyme levansucrase. Inactivation of *rcsB* by site-directed mutagenesis created mutants that were deficient in amylovoran synthesis and avirulent on host plants. In addition, a cosmid which complemented *rcsB* mutants was selected from a genomic library. The spontaneous *E. amylovora* mutant E8 has a similar phenotype and was complemented by the cloned *rcsB* gene. The *rcsB* region of strain E8 was also amplified by PCR, and the mutation was characterized as a nine-nucleotide deletion at the start of the *rcsB* gene. Nucleotide sequence analysis of the *E. amylovora rcsB* region and the predicted amino acid sequence of RcsB revealed extensive homology to *rcsB* and the encoded protein of other bacteria such as *Escherichia coli* and *Erwinia stewartii*. In all three organisms, *rcsB* is localized adjacent to the *rcsC* gene, which is transcribed in the opposite direction of *rcsB*. The *E. amylovora rcsB* gene has now been shown to strongly affect the formation of disease symptoms of a plant pathogen.

The gram-negative bacterium *Erwinia amylovora* causes fire blight on rosaceous plants. Typical symptoms are wilting, necrotic lesions, and production of ooze which consists mainly of capsular exopolysaccharide (EPS) produced by the bacteria. Production of capsule polysaccharides is a common feature of many gram-negative bacteria (49), especially plant pathogens (32). The capsule creates a favorable microenvironment for the bacterial cells and protects them against water stress and plant defense mechanisms. *E. amylovora* produces two types of EPS, amylovoran and levan (2). Levan is a neutral homopolymer composed of β-2,6-linked fructose molecules, and its synthesis is mediated by the enzyme levansucrase. Levansucrase is secreted into the environment and uses sucrose as a substrate (29). The levansucrase gene (*lsc*) of *E. amylovora* was recently cloned and characterized and was found to be constitutively expressed by the pathogen (24). Amylovoran is an acidic heteropolymer composed of a pentasaccharide repeating unit that contains four galactose molecules and one glucuronic acid residue (35, 45). Its synthesis is encoded by the *ams* (for amylovoran synthesis) operon, which was also cloned and sequenced (13). The 12 genes *amsA* to *amsL* are organized in a large gene cluster on the bacterial chromosome. Transposon- and site-directed mutagenesis of the *lsc* gene and the *ams* operon revealed that both types of EPS are important for the virulence of *E. amylovora*. Mutants that lack the synthesis of levan were reduced in virulence (24), and mutants deficient in amylovoran synthesis were nonpathogenic (4, 9). The precise role of EPS in the infection process is still unknown, and genetic analysis revealed additional factors which are involved in virulence of the pathogen (40, 52). The capsule of amylovoran (5) may protect the bacteria from recognition by plant defense mechanisms after masking cell surface components (34). The regulation of EPS synthesis by the *rcs* (regulator of capsular synthesis) genes has been well studied in *Escherichia coli* (12, 27, 28, 46, 48) and in the corn pathogen *Erwinia stewartii* (16, 37), which produce colanic acid and stewartan, respectively. The genes *rcsA* and *rcsB* are conserved among enterobacteria and act as positive regulators of EPS synthesis (28, 46, 48). Inactivation of *rcsA* (37) or *rcsB* (16a) caused a strong reduction of EPS synthesis that was associated in *E. stewartii* with reduced virulence. In *E. coli*, and also in *E. amylovora*, the RcsA protein was shown to be inactivated by the Lon protease, which is a negative regulator of EPS synthesis (18, 46). The *rcsA* gene (10, 14, 16) and the *lon* gene (18) of *E. amylovora* were characterized with respect to nucleotide sequence and genetics of the mutated strains. These genes are involved in the regulation of amylovoran synthesis and have functions similar to those of the *E. coli* and *E. stewartii* genes. Mutants in *E. amylovora rcsA* showed a significant decrease in amylovoran production and were reduced in virulence. The inactivation of the *lon* gene resulted in overproduction of amylovoran. In *E. coli* and *E. stewartii*, the gene coding for the second positive regulator RcsB is localized adjacent to the *rcsC* gene (16, 31, 46). The two genes are transcribed in opposite directions, and the coded proteins are proposed to be part of a two-component regulatory system. A model for regulation based mainly on sequence homologies suggests that RcsB is a DNA binding effector protein that is negatively regulated by the membrane sensor RcsC on the level of phosphorylation (46). The spontaneous EPS-deficient mutant E8 has been suggested to carry a defect in *rcsB* (15). In this study, *rcsB* of *E. amylovora* was characterized by sequence analysis and shown to be strictly required for amylovoran synthesis. On the other hand, its overexpression diminished expression of levansucrase. Thus, the gene is unambiguously involved in development of fire blight symptoms.

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TABLE 1. Bacterial strains used in this study

Species	Strain	Properties (origin)	Reference or source
E. amylovora	Ea1/79	Wild type (Germany, 1979)	19
	Ea1/79-del100	Δ(*amsA-F amsJ*), avirulent, Cm^r	13
	Ea1/79-MG	Ea1/79, *rcsA*::Tn*5* Km^r	10
	Ea1/79-RB1	Ea1/79, *rcsB*-pfdA2, avirulent, Km^r	This work
	Ea1/79-RB3	Ea1/79, Ap^r cassette in *rcsB*	This work
	E8	E9, avirulent	38
	E9	Wild type (United States)	38
E. stewartii	DM4047	*rcsB*::Tn*5*, avirulent, Km^r	D. Coplin
E. coli	1100	K12, *sup*E *endA100*	Lab collection
	SG1086	DM2045, *rcsB42 lon100 ompC*::Tn*5* Km^r Nal^r	S. Gottesman via D. Coplin
	JM83-2	ϕ80 (Δ*lac-pro*) ΔM15 fd gene 2 in *gal*K (F′8 *galK*-fd gene 2)	22

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Bacterial strains and plasmids used are listed in Tables 1 and 2. *E. coli* strains containing recombinant plasmids were grown in the nutrient broth Standard I (Merck AG, Darmstadt, Germany) with appropriate antibiotics. For induction of amylovoran synthesis, *E. amylovora* was grown on agar with minimal medium MM2, described by Bennett and Billing (6), or in dialyzed LB medium with 1% sorbitol.

DNA procedures. Chromosomal DNA was isolated from 1-ml bacterial cultures by repeated treatment with phenol and chloroform. Plasmid DNA was isolated from 100-ml cultures after treatment with alkaline and purified with ion-exchange columns (AX100; Macherey und Nagel, Düren, Germany). Cell lysis and other methods were as described in reference 42.

Conditions for PCR amplification and cloning of the products. PCRs were carried out in a volume of 50 μl with 25 pmol of each primer, 10 ng of template DNA or 10,000 cells, and 1 U of *Tth* DNA polymerase in a buffer system described previously (8). Amplification was done in a thermocycler (Eppendorf) in 30 cycles of 15 s at 93°C for denaturation followed by 15 s at 42°C for annealing and 30 s at 72°C for polymerization. This time for polymerization was increased by 1 s per cycle. PCR products were separated on 1.5% agarose gels. For cloning, PCR products were recovered by ethanol precipitation and ligated into the thymidylated *Eco*RV site of vector pGEM5Z′f (pGEM-T; Promega) as described previously (7).

Selection of a cosmid clone with the *rcsB* gene. A cosmid library with DNA fragments from a partial *Sau*3A digest in vector pLA2917 (3), which was generously provided by A.-M. Barny and J. Laurent, Paris, France, was transferred by triparental mating in order to complement the *E. amylovora rcsB* mutant Ea1/79-RB1 to mucoid colonies. About 50 colonies of this morphology were obtained, and some were analyzed for their restriction patterns. Two clones carried plasmids which gave closely related patterns. One plasmid, named pEA-RB100, was further investigated. A strong hybridization signal was obtained with the *rcsB* fragment cloned by PCR amplification.

Nucleotide sequence analysis. PCR products cloned in plasmids pEA-RB1, pEA-RB2, pEA-RB3, and pEA-RB4 were sequenced by the dideoxy-chain termination method (43) with a Sequenase 2.0 kit (United States Biochemical).

Expression analysis of the *E. amylovora rcsB* gene. The *rcsB* gene was expressed with the T7 RNA polymerase/promoter system in *E. coli* K30(pGP1-2) (50). The *rcsB* gene cloned in plasmid pEA-RB2 was expressed by the T7 promoter of pGEM-T. The *E. coli* RNA polymerase was inhibited with rifampin, and the T7 RNA polymerase gene on plasmid pGP1-2 was heat induced (42°C). De novo-synthesized protein was labeled with [^{14}C]methionine and analyzed on a two-component denaturing sodium dodecyl sulfate-polyacrylamide gel. After separation, the protein band was visualized by exposure of the dried gel to X-ray film (Kodak X-Omat).

Polysaccharide assays. The amylovoran concentration in supernatants of bacterial cultures was quantitatively determined by a turbidity assay with cetylpyrimidinium chloride (CPC) as previously described (5).

Detection of levansucrase activity. The ability to produce levan was determined by the dome-shaped colony morphology on agar plates with the rich medium Standard II (Merck AG) containing 5% sucrose. The activity of secreted levansucrase was detected in supernatants of suspension cultures grown in Standard I medium. The bacteria from 1 ml of liquid cultures were removed by centrifugation, and the supernatant was diluted with the same volume of assay buffer containing 50 mM sodium phosphate, 2 M sucrose, and 0.05% sodium azide to prevent bacterial growth. The mixtures were incubated for 24 h at 28°C, and the turbidity caused by levan formation was estimated photometrically by measuring the absorbance at 580 nm.

Virulence assays. Virulence of *E. amylovora* strains was determined on apple seedlings (39) and immature pear fruits (11).

Nucleotide sequence accession number. The sequence of the *rcsB* gene has been assigned EMBL accession number Y09848.

RESULTS

Cloning and analysis of the *E. amylovora rcsB* gene. The primers used for PCR amplification of the *rcsB* region of *E. amylovora* (RCSB/L2 [CGGCAAGCAGTTATGTG], RCSB/L3 [ATTGCCGATGACCATCC], RCSB/R1 [CTTCTGGCT ACTGATGG], and RCSB/R3 [CGTTGGCTGAAGAGAAG]; sequences are given 5′ to 3′) were chosen from DNA regions flanking the *rcsB* gene of *E. coli* (47). When DNA isolated from *E. amylovora* Ea1/79 was analyzed under conditions of low stringency (primer annealing at 42°C), the primer pairs RCSB/L2-RCSB/R3, RCSB/L2-RCSB/R1, and RCSB/L3-RCSB/R1 produced PCR products of 1.1, 0.6, and 0.5 kb, similar to PCR products obtained with *E. coli* DNA as the template. These products (Table 2) were cloned and subcloned (Fig. 1), and the nucleotide sequences of both strands were determined by the chain termination method. Analysis identified two open reading frames (ORFs) in opposite directions, which are highly homologous to the *rcsB* regions of *E. coli* and *E. stewartii*. The 1.1-kb PCR product cloned in plasmids pEA-RB1 and pEA-RB2 contained the complete reading frame for *rcsB* and the C-terminal part of *rcsC* (Fig. 1).

The coding nucleotide sequence of *E. amylovora rcsB* (Fig. 2) was found to be 82 and 85% identical to those of the *rcsB* genes of *E. coli* and *E. stewartii*, respectively. The 125 nucleotides upstream of the ATG start codon of the ORF also exhibit high degrees of identity (86% to *E. stewartii* and 75% to *E. coli*). This region contains a putative ribosome binding site (AAGG) at position −12 and a possible promoter structure with low homology to σ^{70}-dependent promoters of *E. coli*, including putative −35 and −10 regions for binding of RNA polymerase further upstream. The spacer region between *rcsB* and *rcsC* is not conserved, and the size is different in *E. coli*. The spacer region of *E. amylovora* contains neither direct repeats nor the REP consensus sequences that were described for the *E. coli* spacer region (46). The *E. amylovora rcsB* gene encoded a protein of 215 amino acids (Fig. 2) with a predicted molecular mass of 23,600 Da. This is in good agreement with the size of the encoded protein detected by expression analysis, where a single band was observed slightly below the marker protein bovine carbonic anhydrase, which has a molecular mass of 29 kDa (not shown). Due to base changes at the 5′ end of the ORF for the *rcsB* gene of *E. amylovora*, the predicted protein is one amino acid shorter than those of *E. coli* and *E. stewartii*. Comparison of the sequence of RcsB from *E. amylovora* to those of RcsB from *E. stewartii* and *E. coli* revealed 99% (97%) and 97% (93%) similarity (homology), respectively. The C-terminal amino acid sequence of RcsB also revealed significant homology to proteins of the LuxR family (20,

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pDM421	pKP2, *E. stewartii rcsB*::Tn*5*, Tc^r, Km^r	D. Coplin
pEA101	pSUP106 with a 3.6-kb *Hin*dIII fragment with the *rcsA* gene of *E. amylovora*, Ap^r	10
pEA-RB1	pGEM-T with a 1.1-kb PCR fragment (primers L2 and R3) with the *rcsB* gene of strain Ea1/79 in direction of P_{lac}, Ap^r	This work
pEA-RB2	pGEM-T with a 1.1-kb PCR fragment (primers L2 and R3) with the *rcsB* gene of strain Ea1/79 against P_{lac}, Ap^r	This work
pEA-RB3	*Eco*RV/*Sal*I deletion in pEA-RB2, Ap^r	This work
pEA-RB4	pGEM-T with a 0.7-kb PCR fragment (primers L2 and R1) with part of the *rcsB* gene of strain Ea1/79, Ap^r	This work
pEA-RB6	pEA-RB2 with pfdA2 inserted into the *Eco*RV site of *rcsB*, Ap^r, Km^r	This work
pEA-RB7	2.8-kb *Pst*I fragment from pEA-RB6 with pfdA2 but without pBR fragment, Km^r	This work
pEA-RB81	pGEM-T with a 1.1-kb PCR fragment with the *rcsB* gene of strain E8 in direction of P_{lac}, Ap^r	This work
pEA-RB82	pGEM-T with a 1.1-kb PCR fragment with the *rcsB* gene of *E. amylovora* E8 against P_{lac}, Ap^r	This work
pEA-RB100	Approx 20-kb fragment with *rcsB* gene of *E. amylovora* CFBP1430 in pLA2917, Tc^r	This work
pEC-RB1	pGEM-T with a 1.3-kb PCR fragment with the *rcsB* gene of *E. coli* 1100 against P_{lac}, Ap^r	This work
pEC-RB6	pEC-RB1 with insertion of pfdB14Z′ into the *Eco*RV site of *rcsB*, Ap^r, Cm^r	This work
pfdA2	fd ori, replication requires fd gene 2, 1.63 kb, Km^r	23
pfdA3	fd ori, replication requires fd gene 2, 3.3 kb, Ap^r, Km^r	23
pfdB14Z′	fd ori, replication requires fd gene 2, 3.0 kb, Cm^r, *lacZ′*	22
pGEM-T	pGEM5Z′f, thymidylated *Eco*RV site, 3.0 kb, Ap^r	Promega
pGP1-2	Km^r, vector with phage T7 polymerase gene	50
pKP2	pLAFR3 cosmid clone with the *rcsB* and *rcsC* genes from *E. stewartii*, Tc^r	D. Coplin

30), and a proposed DNA binding domain, called a helix-turn-helix motif (HTH-M) (36), was also detected for the *E. amylovora* protein (Fig. 3).

The partially cloned second ORF encodes 49 amino acids and showed significant homology to the C-terminal part of *rcsC* of *E. coli* and *E. stewartii*. The nucleotide sequence is more than 60% homologous to the corresponding parts of *E. coli* and *E. stewartii rcsC*, and the predicted amino acid sequence is also highly homologous to RcsC of both enterobacteria. The ORF of *E. amylovora* RcsC is terminated by an ochre stop codon, and the predicted sequence is six amino acids longer than reported for RcsC of *E. coli* and *E. stewartii* due to a frameshift at the 3′ end of the genes.

Site-directed mutagenesis of the chromosomal *rcsB* gene of *E. amylovora*. The suicide vector pfdA2 (1.6 kb) with the kanamycin resistance gene and the origin of replication of bacteriophage fd was used to inactivate the *E. amylovora rcsB* gene. Plasmid pEA-RB2 was cleaved with restriction enzyme *Eco*RV and ligated with *Eco*RI-cleaved and blunt-ended pfdA2 DNA to give plasmid pEA-RB6. The resulting plasmid was digested with restriction enzyme *Pst*I, and religation of the 2.7-kb fragment created plasmid pEA-RB7, where the pBR part of pEA-RB6 and the promoter region of *rcsB* were removed. This plasmid was propagated in *E. coli* JM83-2 expressing fd gene 2 and transformed into *E. amylovora* Ea1/79 by electroporation. Kanamycin-resistant transformants resulted from a single crossover between pEA-RB7 and the chromosomal *rcsB* gene, which caused an interruption of the *rcsB* gene. The mutant showed a drastic decrease in the production of the EPS amylovoran on plates with MM2 agar. Four of the mutants were analyzed by PCR with primers RCSB/L2 and RCSB/R3, and one of them was designated Ea1/79-RB1. Due to the deletion of the promoter region of *rcsB* in the suicide plasmid pEA-RB7, the primer RCSB/L2 could bind only to its chromosomal target. The 2.8-kb PCR product obtained from the mutants indicated correct insertion of pEA-RB7 into the *rcsB* gene. The disruption of the gene was further analyzed by recloning of the insertion and restriction analysis. A second mutant was created after the ampicillin resistance cassette (1.6 kb) from plasmid pfdA3 (22) was inserted as blunt-ended *Eco*RI/*Hae*II fragment into *rcsB*. The resulting mutant, Ea1/79-RB3, had the same properties as the gene disruption mutant Ea1/79-RB1.

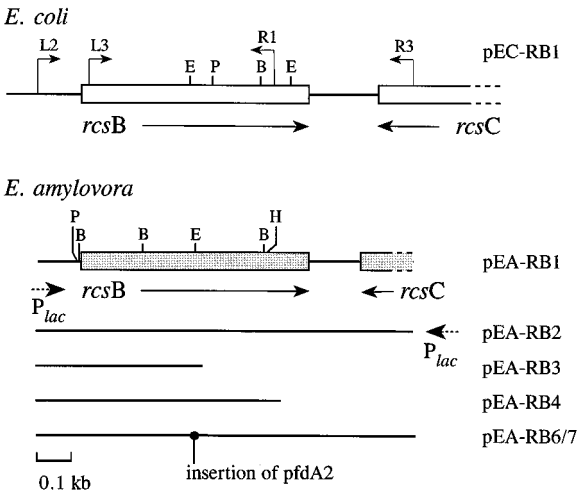

FIG. 1. Genetic organization of the *rcsB* regions of *E. coli* and *E. amylovora*. The genes *rcsB* and *rcsC* are transcribed in opposite directions (arrows). The positions of the primers (suffixes "L" and "R") used for PCR amplification and sequence analysis and the direction of the *lac* promoter (P_{lac}) are indicated by arrows. B, *Bst*EII; E, *Eco*RV; P, *Pst*I; H, *Hin*dIII; X, *Xho*I. The lines at the bottom indicate various subclones with the *rcsB* gene.

Influence of *rcsB* on amylovoran synthesis and complementation of *rcsB* mutants. To measure the influence of *rcsB* on amylovoran synthesis of *E. amylovora*, various plasmids with an intact *rcsB* gene were introduced into *E. amylovora* wild-type and mutant strains. Plasmid pEA-RB6 was used as a negative control. EPS synthesis was measured in the culture supernatant by the CPC turbidity assay (Fig. 4) and estimated from the mucoid phenotype of colonies on minimal agar MM2 (Table 3). Ea1/79-RB1 did not produce amylovoran in liquid cultures and showed a nonmucoid colony morphology on minimal agar. This phenotype could be converted to mucoid appearance by complementation of Ea1/79-RB1 with plasmid pEA-RB2 and also with pEA-RB100, which was derived from a cosmid library by complementation of the *rcsB* mutant. In addition, the *rcsB*

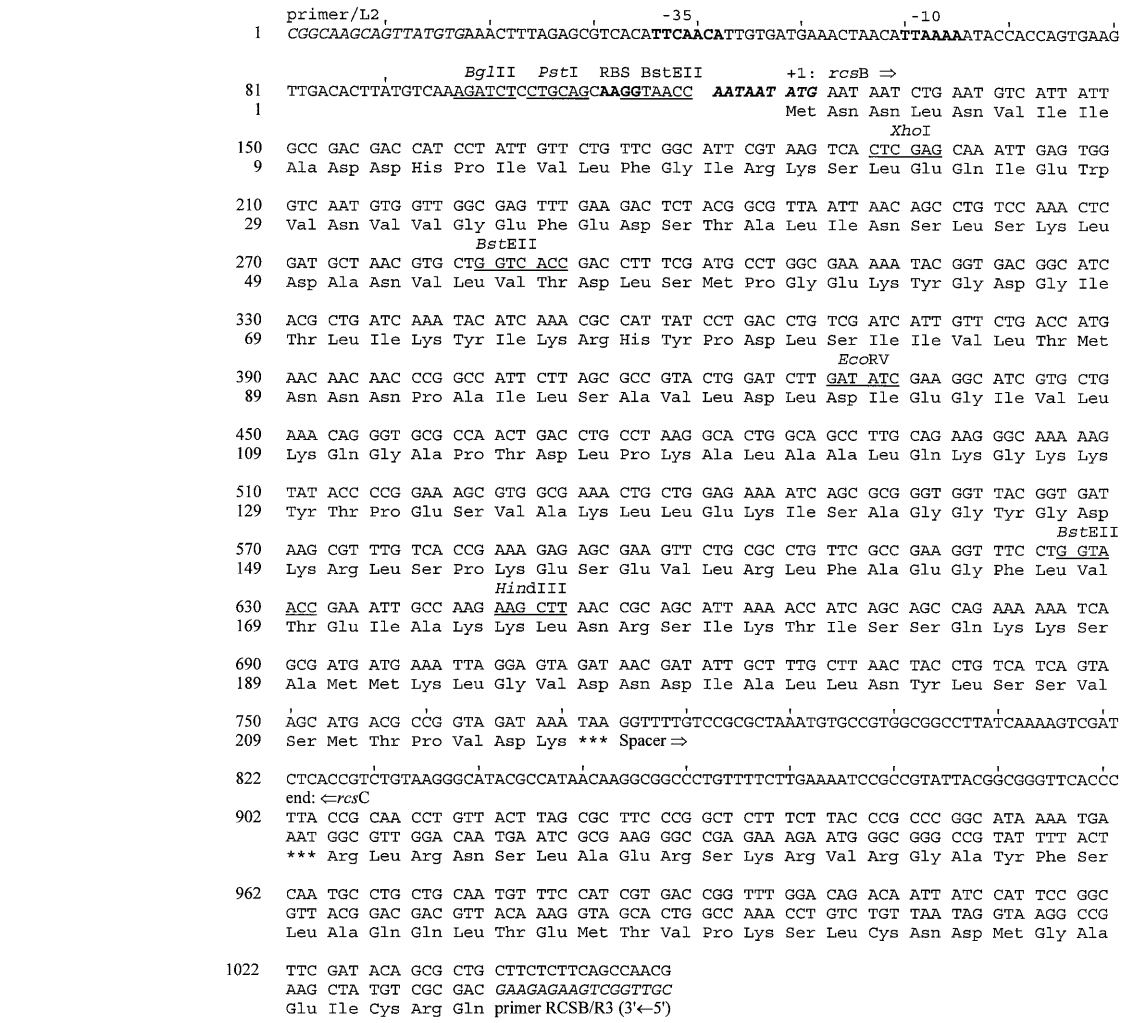

FIG. 2. Nucleotide sequence of the *rcsB* region of *E. amylovora* Ea1/79. The 1,053 nucleotides comprise the *rcsB* gene and parts of the adjacent *rcsC* gene. The sequences of the primers used for amplification are italicized. The 9-bp deletion at the start of the *rcsB* gene of strain E8 is in boldface italics. Restriction sites for enzymes *Bst*EII, *Eco*RV, *Hin*dIII, *Pst*I, and *Xho*I are underlined. Possible consensus structures with homologies to σ^{70}-dependent promoters of *E. coli* (−10 and −35) and a putative ribosome binding site (RBS) are in boldface.

genes of *E. coli* and *E. stewartii* on plasmids pEC-RB1 and pKP2 could also restore mucoidy of Ea1/79-RB1. Conversely, the *rcsB* mutants SG1086 of *E. coli* and DM4047 of *E. stewartii* were complemented by the *E. amylovora* gene on plasmid pEA-RB2. Since the gene in pEA-RB2 was inserted in the direction opposite that of the *lac* promoter, a promoter existed in front of the gene, which was active in all three organisms. The overexpression of *rcsB* by pEA-RB2 increased amylovoran synthesis of the wild-type strain Ea1/79 but did not affect the *ams* mutant Ea1/79-del100, where the nonmucoid appearance of colonies was not changed.

Genetic analysis of strain E8. Plasmids pEA-RB1, pEA-RB2, and pEA-RB100 could restore amylovoran synthesis of mutant E8 (Table 3; Fig. 4), which was originally selected by its nonmucoid phenotype. The mutant displayed a deficiency for amylovoran and for levan synthesis (2). To test a defect of the *rcsB* gene in E8, the chromosomal region with *rcsB* was amplified by PCR with primers RCSB/L2 and RCSB/R3 and cloned as described before for pEA-RB1 and pEA-RB2 in both directions relative to the *lac* promoter. Both plasmids, designated pEA-RB81 and pRB-RB82, did not complement the amylovoran-negative phenotype of strains Ea1/79-RB1 and E8. Restriction analysis revealed that in the coding sequence of the E8 *rcsB* gene, at least five nucleotides were deleted. The deletion located in the shortened 79-bp *Bst*EII-*Xho*I fragment at the 5′ end of the gene (Fig. 2) was also detected when PCR products obtained from the *rcsB* region of E8 were analyzed without cloning. This fragment was the same size as found for strain Ea1/79 when amplified from E9, the parent strain of E8. Sequence analysis of this region in strain E8 revealed a 9-bp deletion after nucleotide 119, removing the start codon of *rcsB* and adjacent nucleotides upstream. Although the deletion does not alter the reading frame, the next possible ATG start site is as far as 60 amino acids downstream of the deletion (Fig. 2). For E8 *rcsB*, the triplet adjacent to the wild-type ATG start site is changed from AAT to AAC, both coding for Asn. No additional base changes were detected in the DNA fragment with *rcsB* of strain E8. This analysis confirmed the sequence for Ea1/79 presented in Fig. 2.

Effect of *rcsB* on the *rcsA* mutation and on levan synthesis. The network of regulatory genes for EPS synthesis of *E. amylovora* includes *rcsA*. We therefore overexpressed the *E. amy-*

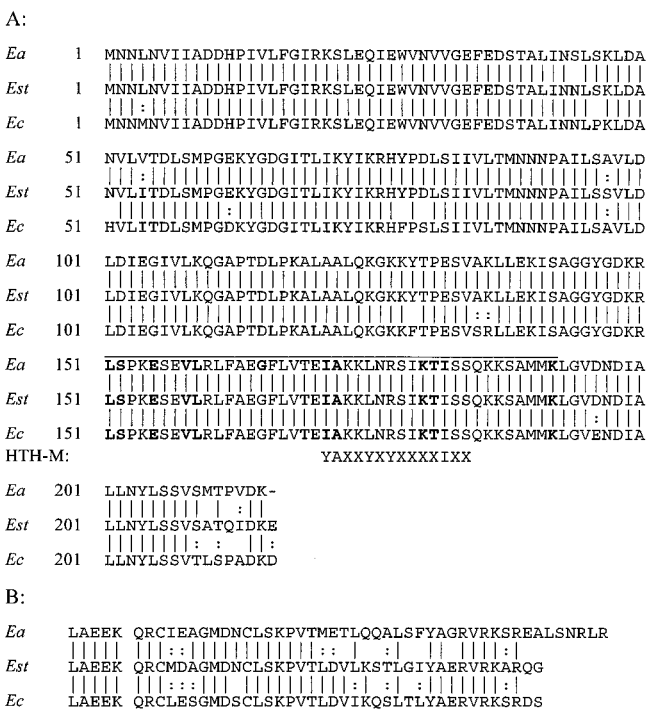

FIG. 3. Alignment of RcsB (A) and the C-terminal part of RcsC (B) from *E. amylovora* (*Ea*), *E. stewartii* (*Est*), and *E. coli* (*Ec*). The amino acid sequences of RcsB (A) and RcsC (B) were deduced from DNA sequences. The sequence derived from the primer region is listed in a separate block for the RcsC proteins. The region with homologies to the two-component regulators of the LuxR family that contains a putative DNA binding domain is underlined. |, identical amino acids; :, conservative exchange of amino acids. The putative DNA binding site of the HTH-M is indicated: Y, hydrophobic amino acid Ile, Val, or Met; X, any amino acid. In this region, the LuxR consensus sequence is indicated by a line beginning at amino acid 151, and conserved amino acids of the Lux family (20) are in boldface.

lovora rcsB gene in the *rcsA* mutant Ea1/79-MG and found significant restoration of amylovoran production (Fig. 4). This is in agreement with the role of RcsA to support RcsB in activation of genes involved in EPS synthesis. The *rcsB* mutant Ea1/79-RB1 did not show a change in levansucrase activity compared to the wild type, Ea1/79 (Fig. 5). On the other hand, overexpression of both *rcs* genes strongly reduced the expression of levansucrase. Ea1/79(pEA101) with the *rcsA* gene on the plasmid and Ea1/79(pEA-RB2) with the *rcsB* gene on the plasmid had a more than 10-fold reduction of levansucrase activity. When Ea1/79(pEA-RB100) was assayed for levansucrase, no significant change of levan synthesis was observed compared to the wild type.

Influence of *rcsB* on the virulence of *E. amylovora*. Amylovoran is an important virulence factor for *E. amylovora*, and mutants which are deficient in its synthesis are avirulent. Virulence of strains Ea1/79-RB1 and E8 with and without pEA-RB2 was assayed on slices of immature pears and on apple seedlings. The results indicated that strains Ea1/79-RB1 and E8 were avirulent on apple seedlings and caused no fire blight symptoms. Complementation of an *rcsB* mutant by plasmid pEA-RB2 restored virulence. Both strains carrying the plasmid also produced the typical fire blight symptoms of mucoid colonies on slices of immature pear fruits, in contrast to the mutant strains (Fig. 6). Mutant Ea1/79-RB3 was also strongly reduced in symptom formation on immature pears. Due to the gene disruption by cointegrate formation and its occasional reversion into wild-type cells in the case of Ea1/79-RB1, droplets of ooze became visible after prolonged incubation, whereas these weak symptoms were not observed for Ea1/79-RB3.

DISCUSSION

Capsular EPSs have multiple roles in the life of bacteria (32). Their syntheses are therefore regulated by a network of factors, which are affected by environmental conditions and the cellular growth stage. For pathogenesis, the fire blight pathogen *E. amylovora* requires the formation of amylovoran (4, 6), an acidic polysaccharide of four differently linked galactose residues and a glucuronic acid residue (35). Its synthesis is encoded by a large operon with 12 genes, which is expressed from an RcsA-regulated promoter in front of *ams*G (13).

RcsA belongs to a group of proteins regulating bacterial polysaccharide synthesis, which include RcsB and RcsC (27, 46, 48). RcsC is suggested to be a sensor in the membrane which modifies RcsB (47). RcsB is enhanced in the presence of RcsA for full activity, but it can also act alone. The protein can therefore suppress *rcsA* mutants by overexpression on high-copy-number plasmids, but RcsA cannot suppress *rcsB* mutants. The *rcs* genes are well conserved for various bacteria, demonstrated by homology of RcsA for *E. amylovora*, *E. coli*, *E. stewartii*, and *Klebsiella pneumoniae* (10). RcsB also seems to be conserved in these bacteria (17). The amplified DNA fragment from *E. amylovora* carried promoter activity for expression of *rcsB*. Further upstream of the cloned region of *E. amylovora*, a consensus sequence for σ^{54}- and σ^{70}-dependent promoters and a LexA binding site were detected for *E. coli* and *E. stewartii* (17, 21, 26, 47), which could be present also for *E. amylovora*. It can be assumed that modulation by σ factors and DNA binding proteins can affect expression of the *rcsB* gene in these bacteria. In *E. coli*, silencing by H-NS can be suppressed by a high level of a short RNA from the *dsrA* gene, which is located just upstream of *rcsA* (44). Chaperons DnaJ and DnaK may stabilize RcsA itself or its complex with RcsB (27). RcsF is another protein affecting colanic acid synthesis, presumably by activation of RcsB (25). In *E. amylovora*, the *rcsB* mutant lost its ability to synthesize amylovoran, whereas

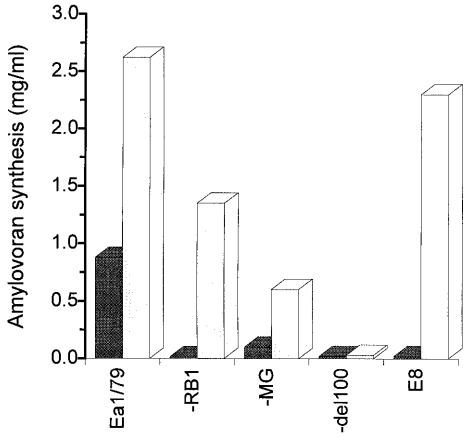

FIG. 4. Influence of *rcsB* overexpression on amylovoran synthesis in various *E. amylovora* strains. Strains transformed with plasmids pEA-RB6 (dark bars) and pEA-RB2 (light bars) were grown in dialyzed LB liquid medium with 1% sorbitol. The amylovoran concentration in the supernatant was determined by using the CPC turbidity assay for the wild-type strain Ea1/79, for Ea1/79 with mutations in *rcsB* (-RB1) and in *rcsA* (-MG) and with a deletion in the *ams* region (-del100), and for the spontaneous *rcsB* mutant E8. Strain Ea1/79 produced similar amounts of amylovoran with and without pEA-RB6.

TABLE 3. Complementation analysis of mutants from various species

Strain	Chromosomal genotype	Plasmid	Plasmid genotype	Source of *rcs* gene[a]	Mucoid colonies[b]
E. amylovora					
Ea1/79-RB1	*rcsB*-pfdA2	None			−
		pEA101	*rcsA*$^+$	Ea1/79	−
		pEA-RB2	*rcsB*$^+$	Ea1/79	+
		pEA-RB6	*rcsB*-pfdA2	Ea1/79	−
		pEC-RB1	*rcsB*$^+$	Ec 1100	+
		pKP2	*rcsB*$^+$ *rcsC*$^+$	Est DC283	+
		pDM421	*rcsB*::Tn*5* *rcsC*$^+$		−
		pEA-RB81	*rcsB*	E8	−
		pEA-RB82	*rcsB*	E8	−
		pEA-RB100	*rcsB*$^+$	See reference 3	+
E8	Δ*rcsB*	None			−
		pEA-RB2	*rcsB*$^+$	Ea1/79	+
		pEC-RB1	*rcsB*$^+$	Ea1/79	+
		pKP2	*rcsB*$^+$	Est DC283	+
		pEA101	*rcsA*$^+$	Ea1/79	−
		pEA-RB81	*rcsB*$^+$	E8	−
		pEA-RB82	*rcsB*$^+$	E8	−
		pEA-RB100	*rcsB*$^+$	See reference 3	+
Ea1/79-MG	*rcsA*::Tn*5*	None			−
		pEA-RB2	*rcsB*$^+$	Ea1/79	+
Ea1/79-RB3		None			−
		pEA-RB2	*rcsB*$^+$	Ea1/79	+
Ea1/79-del100	Δ*amsA-F,J*	None			−
		pEA-RB2	*rcsB*$^+$	Ea1/79	−
E. stewartii DM4047	*rcsB*::Tn*5*	None			−
		pEA-RB2	*rcsB*$^+$	Ea1/79	+
		pEC-RB1	*rcsB*$^+$	Ec 1100	+
E. coli					
1100		None			−
		pEA-RB2			+
SG1086	*rcsB42*	None			−
		pEA-RB2	*rcsB*$^+$	Ea1/79	+
		pKP2	*rcsB*$^+$	Est DC283	+

[a] Ec, *E. coli*; Est, *E. stewartii.*
[b] On MM2 agar (*E. amylovora*) and Standard I agar (*E. stewartii* and *E. coli*).

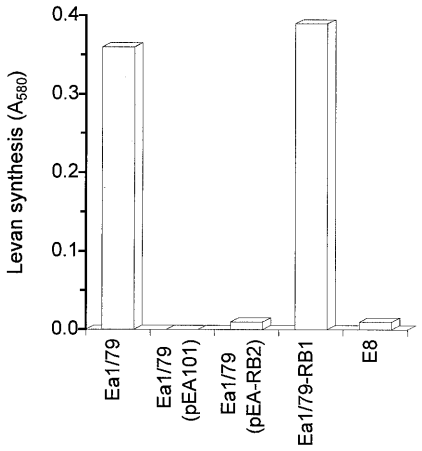

FIG. 5. Suppression of levansucrase by overexpression of *rcs* genes in *E. amylovora*. Strains Ea1/79-RB1 and Ea1/79 carrying plasmid pEA101 or pEA-RB2 with the *rcsA* or *rcsB* gene, respectively, of *E. amylovora* were grown overnight in liquid medium (Standard I). The levansucrase activity in the supernatant of bacterial cultures was estimated by the turbidity assay. The values for relative activity of the enzyme are means from three measurements. The vectors of the recombinant plasmids were found to have no effect on levansucrase activity.

amylovoran production was stimulated by RcsB overexpression in a wild-type strain. Similar to the case for *E. coli* (1b), a high level of RcsB resulted in growth inhibition of *E. amylovora.*

RcsB of *E. amylovora* belongs to proteins of the LuxR family (20, 30), which carry a consensus sequence of 12 amino acids. The sequence occurs at the C termini of LuxR of *Vibrio fischeri* and other regulatory proteins from various bacteria. Typical is the HTH-M domain, thought to act in protein binding to DNA (36). The RcsA proteins of *E. coli*, *E. stewartii*, *K. pneumoniae*, and *E. amylovora* also show homology to RcsB at the C terminus. A novel activator protein from *E. amylovora*, RcsV, has been recently characterized for activation of EPS synthesis in *E. stewartii* and *E. amylovora* (1), and may also belong to a group of several other proteins involved in regulation of polysaccharide production.

Overexpression of the *rcsA* and the *rcsB* genes of *E. amylovora* strongly interfered with levan synthesis. The *lsc* gene was found to be expressed constitutively in *E. amylovora* (24), whereas the analogous *sacB* gene of *Bacillus subtilis* is induced by sucrose, the substrate of the levan-forming enzyme levansucrase (33). Although an *rscA* mutation was found to be reduced for levan synthesis (10), another *rcsA* mutant had a normal level of levansucrase (1a). The spontaneous mutant E8 (38) is deficient for amylovoran and for levan synthesis. Analysis of the E8 *rcsB* gene and complementation with the cloned

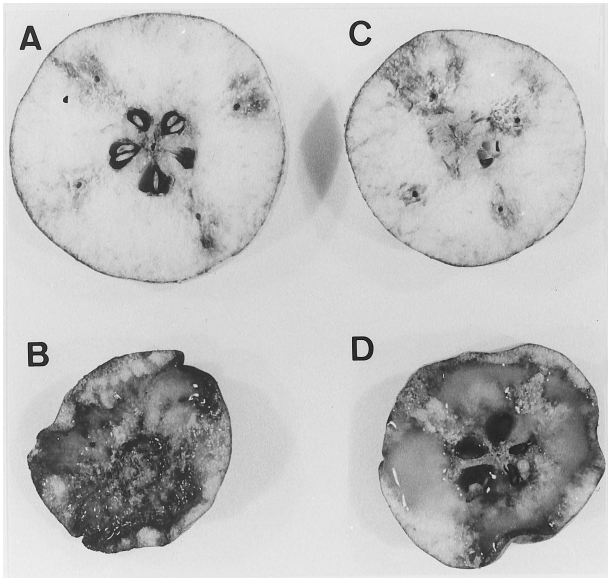

FIG. 6. Complementation of an *rcsB* mutant and of strain E8 to virulence by the cloned *rcsB* gene. Slices from immature pears were inoculated with cells from colonies of *E. amylovora* mutants without and with an *rcsB* plasmid: Ea1/79-RB1 (A), Ea1/79-RB1(pEA-RB2) (B), E8 (C), and E8(pEA-RB2) (D).

rcsB gene confirmed the suggestion that E8 carries a mutation in *rcsB* (15). Since the *rcsB* mutants were not altered for expression of levansucrase, strain E8 should carry a second mutation in a regulatory gene(s) in addition to the deletion in *rcsB*. The *lsc* gene was cloned from strain E8 by PCR amplification and was found intact as in several natural levan-deficient strains (6a). Considering the influence of *rcsA* or *rcsB* on EPS synthesis of *E. amylovora*, a deficiency of the regulatory proteins reduces amylovoran synthesis without an effect on levan synthesis, and their overexpression increases amylovoran production and diminishes levan formation. Thus, the cells attempt to keep at least one EPS species available. A similar observation has been made for *Salmonella typhi*, where RcsA interfered with capsular polysaccharide synthesis of the group 2 K54 antigen (41). For *S. typhi*, *rcsA*, *rcsB*, and *rcsC* have been cloned. RcsA did not affect Vi antigen synthesis (51). In this case, another regulator, TviA, was assumed to activate transcription in concert with RcsB. *E. amylovora* seems to regulate synthesis of EPS in a way such that the pathogen can adjust amylovoran synthesis and secretion of levansucrase according to its environment and life cycle during spread and persistence in host plants, and RcsB is an important component in this complex regulation of EPS synthesis.

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