JOURNAL OF BACTERIOLOGY, Oct. 2000, p. 5351–5358 Vol. 182, No. 19
0021-9193/00/$04.00+0

Molecular Analysis of Sucrose Metabolism of *Erwinia amylovora* and Influence on Bacterial Virulence

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Received 18 February 2000/Accepted 26 June 2000

Sucrose is an important storage and transport sugar of plants and an energy source for many phytopathogenic bacteria. To analyze regulation and biochemistry of sucrose metabolism of the fire blight pathogen *Erwinia amylovora*, a chromosomal fragment which enabled *Escherichia coli* to utilize sucrose as sole carbon source was cloned. By transposon mutagenesis, the *scr* regulon of *E. amylovora* was tagged, and its nucleotide sequence was determined. Five open reading frames, with the genes *scrK*, *scrY*, *scrA*, *scrB*, and *scrR*, had high homology to genes of the *scr* regulons from *Klebsiella pneumoniae* and plasmid pUR400. *scrB* and *scrR* of *E. amylovora* were fused to a histidine tag and to the maltose-binding protein (MalE) of *E. coli*, respectively. ScrB (53 kDa) catalyzed the hydrolysis of sucrose with a K_m of 125 mM. Binding of a MalE-ScrR fusion protein to an *scrYAB* promoter fragment was shown by gel mobility shifts. This complex dissociated in the presence of fructose but not after addition of sucrose. Expression of the *scr* regulon was studied with an *scrYAB* promoter-green fluorescent protein gene fusion and measured by flow cytometry and spectrofluorometry. The operon was affected by catabolite repression and induced by sucrose or fructose. The level of gene induction correlated to the sucrose concentration in plant tissue, as shown by flow cytometry. Sucrose mutants created by site-directed mutagenesis did not produce significant fire blight symptoms on apple seedlings, indicating the importance of sucrose metabolism for colonization of host plants by *E. amylovora*.

The gram-negative bacterium *Erwinia amylovora* causes fire blight of apple, pear, and other rosaceous plants. Pathogenecity depends on the ability to produce the exopolysaccharide amylovoran (10, 13), to elicit a hypersensitive response on non-host plants (6, 8), and to metabolize sorbitol of the host plants (1). Rosaceous plants contain sorbitol and sucrose as storage and transport carbohydrates. The distribution of these carbohydrates is dependent on environmental conditions, species, and plant tissue (28, 52). The highest concentration of sucrose was found in the nectaries of host plants (14), which are assumed to be the main entry site for the pathogen when the pathogen is distributed by insects.

Sucrose is utilized by some but not all bacteria extracellularily or intracellularily. *E. amylovora* can metabolize sucrose via the secreted levansucrase, which polymerizes the homopolysaccharide levan and releases glucose from sucrose (27, 29), but also by uptake and intracellular metabolism.

The sucrose-utilizing system of enteric bacteria has been studied in *Klebsiella pneumoniae* and in some isolates of *Escherichia coli* and *Salmonella* spp. (45, 49). In *E. coli* and *Salmonella* spp., the conjugative plasmid pUR400 confers the ability to utilize sucrose (54), whereas the *scr* regulon of *K. pneumoniae* is located on the chromosome (42, 49). In these bacteria, the uptake of sucrose is mediated via the phosphoenolpyruvate-dependent carbohydrate:phosphotransferase system (PTS), yielding sucrose 6-phosphate, which is cleaved by an intracellular hydrolase into glucose 6-phosphate and fructose (45, 49). The *scr* regulons of *K. pneumoniae* and pUR400 consist of four structural genes: *scrK* codes for an ATP-dependent fructokinase (5), *scrY* codes for a sucrose-specific porin of the outer membrane (30), *scrA* codes for enzyme II^{scr} of the PTS, and *scrB* codes for an intracellular β-D-fructofuranoside fructohydrolase (EC 3.2.1.26), which cleaves sucrose 6-phosphate into β-D-fructose and α-D-glucose 6-phosphate (51). The regulon is controlled by the negative regulator ScrR (34) and is induced in medium containing sucrose, fructose, or raffinose (45, 46).

In this work, we cloned, sequenced, and characterized the *scr* regulon of *E. amylovora*. The regulation of sucrose metabolism was studied by gel shift assays and promoter-green fluorescent protein gene (*gfp*) fusions analyzed by flow cytometry also with bacteria extracted from plant tissue. Mutants carrying mutations in the *scr* genes were nonvirulent.

(A preliminary report has been published as a proceedings contribution [15].)

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. Table 1 lists the strains, plasmids, and oligonucleotides used in the experiments.

Mutagenesis and screening of *scr* mutants. Transposon insertions in *scr* genes were detected after infection of *Escherichia coli* S17-1(pSCR100) with phage λ::Tn*5*seq as white colonies on MacConkey agar (Gibco-BRL) with sucrose in the presence of kanamycin. Plasmids pSCR100-1 and pSCR100-3 were transferred into *E. amylovora* as described by Bernhard et al. (13). Plasmid pPH1JI, which is incompatible with pSCR100-1 and pSCR100-3, was then conjugated into a mutant, and cells were selected for resistance to chloramphenicol, kanamycin, and streptomycin to screen for bacteria with a transposon insertion in the chromosome.

Enzyme assays. Sucrose hydrolase activity was determined in 1 ml of 100 mM phosphate buffer (pH 7.0) with 200 mM sucrose incubated at 28°C for 30 min. The reaction was stopped by boiling for 1 min. After removal of denatured protein, the glucose was determined with 10-μl aliquots added to 1 ml of 10 mM phosphate buffer–0.05% sodium azide with peroxidase (1 U), glucose oxidase (10 U), and 1 mg of ABTS (2,2-azino-di-3-ethyl-benzthiazolinsulfonate) (Boehringer, Mannheim, Germany). The reaction was complete after 30 min at 28°C, and the absorption was determined at 436 nm.

Protein purification and analysis. $(His)_6$ tag fusions were expressed in *E. coli* strain GI698 after growth at 37°C to an optical density at 600 nm (OD_{600}) of 0.5. The gene was induced with 1 mM IPTG (isopropylthiogalactopyranoside) for 4 h at 28°C. The fused histidine residues of recombinant proteins were bound to an Ni-nitrilotriacetic acid matrix (Qiagen). The native protein (H-ScrB) was eluted with 250 mM imidazole, whereas the denaturated repressor (H-ScrR) was eluted with buffer D (8 M urea, 0.1 M NaH_2PO_4 [pH 5.9], 0.01 M Tris-HCl). The

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TABLE 1. Stains, plasmids, oligonucleotides, and phage used

Strain, plasmid, oligonucleotide, or phage	Properties	Source or reference
E. coli		
GI698	F$^-$ *lacI*^{q} *lacPL8 ampC*::P$_{trp}$ *cI*	Invitrogen
S17-1	*thi pro hsdR hsdM*$^+$ *recA tra*$^+$	47
E. amylovora		
Ea1/79	Wild-type strain isolated in 1979 in Germany	26
Ea7/74	Wild-type strain isolated in 1974 in Germany	26
Ea7/74-S1	Ea7/74 *scrY*::Tn*5*seq1, Km^r	This work
Ea1/79-S3	Ea7/74 *scrA*::Tn*5*seq1, Km^r	This work
PD494Sm	Levan-deficient strain	12
PD494-S1	PD494Sm *scrY*::Tn*5*seq1, Km^r	This work
Ea7/74-LS7	Ea7/74 with Tn*5* insertion in *lsc*, Km^r	27
Plasmids		
pSU18	P15A replicon, *lacZ′*, Cm^r, 2.3-kb polylinker pUC18	7
pGFPmut2	ColE1 *ori*, 3.9 kb, Ap^r, pKEN2 derivative	20
pFG30	0.7-kb *Bam*HI-*Pst*I fragment from pGFPmut2 in pBlueSK$^+$, 3.7 kb, Ap^r	Fangchery Gong[a]
pUC18	ColE1 replicon, *lacZ′*, Ap^r, 2.7 kb	55
pMAL-c2	Ap^r, expression vector	New England Biolabs
pQE31	ColE1 replicon, Ap^r, (His)$_6$ tag	Qiagen
pVK100	IncP, *mob*$^+$ *cos*, Tc^r, Km^r	35
pSU18-Y1	0.25-kb PCR product with primers 3342 and 3341 from pSCR109 in pSU18, *Xba*I and *Sal*I sites introduced, *scrYAB* promoter, Cm^r	This work
pSU18-Y1gfp	0.7-kb *Xba*I-*Asp*718 fragment (with *gfp*) from pFG30 in pSU18-Y1, Cm^r	This work
pSU18-Y1gfpR	1.2-kb PCR product with primers 3366 and 3367 from pSCR141 in pSU18-Y1gfp, *Eco*RV and *Asp*718 sites introduced, *scrR*$^+$, Cm^r	This work
pSCR100	Chromosomal 20-kb *Hin*dIII fragment from *E. amylovora* in pVK100, *scrK*$^+$*Y*$^+$*A*$^+$*B*$^+$*R*$^+$	This work
pSCR107	1.8-kb *Bam*HI fragment from pSCR100 in pUC18, *scrA′* scrB′, Ap^r	This work
pSCR109	3.9-kb *Bam*HI fragment from pSCR100 in pUC18, *scrK*$^+$ *scrY*$^+$ *scrA′*, Ap^r	This work
pSCR141	4.1-kb *Hin*dIII-*Eco*RI fragment from pSCR100 in pUC18, *scrB′ scrR*$^+$, Ap^r	This work
pQE31scrB	1.5-kb PCR product with primers 3584 and 3523 from pSCR100 in pQE31, *Sph*I and *Hin*dIII sites introduced, *scrB*$^+$, Ap^r	This work
pQE31scrR	1-kb PCR product with primers 3583 and 3506 from pSCR141 in pQE31, *Bam*HI and *Hin*dIII sites introduced, *scrR*$^+$, Ap^r	This work
pMalscrR	1-kb PCR product with primers 3583 and 3506 from pSCR141 in pMal-c2, *Bam*HI and *Hin*dIII sites introduced, *scrR*$^+$, Ap^r	This work
Oligonucleotides[b]		
3506	GCGAAGCTTTCACATGCCATTGCCGAAAGG (*Hin*dIII site)	This work
3523	GCGAAGCTTATTAGGCGCGATGGATCGTAG (*Hin*dIII site)	This work
3341	CGCGTCGACAGCCAGTAGCAGACATT (*Sal*I site)	This work
3342	CGCTCTAGAAACGCTTTCCCAGGTAATG (*Xba*I site)	This work
3366	CGCGATATCCTGTGACAGTTCCAGCATTGAGA (*Eco*RV site)	This work
3367	CGCGGTACCAGTCCTGCTTGTGTCGTTAATTG (*Asp*718 site)	This work
3583	GCGGGATCCACTAAAAACAAACGTATTACCATTAAC (*Bam*HI site)	This work
3584	CGCGCATGCGAGCGAAGCCCATTTGCTGAAAC (*Sph*I site)	This work
Bacteriophage		
λ::Tn*5*seq	Tn*5*, Km^r, λ b_{221} cI_{857}, Pam80, Km^r, promoter sequences from SP6 and T7	39

[a] Ohio State University, Columbus, Ohio.
[b] The site introduced by the underlined bases is indicated in parentheses.

purification of the proteins was done with the QIAexpress system according to the protocol of the manufacturer.

For expression and purification of maltose-binding protein (MBP)-ScrR, *E. coli* strain DH5α(pMalscrR) was grown in Luria-Bertani (LB) medium with 0.2% glucose to an OD_{600} of 0.5. Expression of the *malE-scrR* fusion was induced with 0.5 mM IPTG for 3 h at 37°C. Cells were harvested, washed, and resuspended in column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and sonicated. The cell debris was pelleted by centrifugation (13,000 × *g*), and the supernatant was mixed with the amylose resin (1:1) and incubated at 4°C for 30 min. After extensive washing with column buffer, the bound protein was eluted with column buffer supplemented with 10 mM maltose.

Protein concentrations were determined by the method of Lowry (37) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels (36).

Sequencing. Sequencing was done with the automated laser fluorescent DNA sequencer (A.L.F. Express; Pharmacia Biotec). Initial sequences were obtained with fluorescent universal primers. Nucleotide sequences were determined by primer walking using unlabeled primers synthesized with an oligonucleotide synthesizer (Beckman), after brief incorporation of fluorescein-labeled dATP followed by a chase with unlabeled dATP. Nucleotide sequences were determined with plasmids pSCR107, pSCR109, and pSCR141 in both strands of the inserts. Computer analysis was done with databases for similarities to DNA and protein sequences using the programs BLAST (3; http://www3.ncbi.nlm.nih.gov/Entrez/), IDENTIFY (41; http://dna.stanford.edu/identify/), and additional programs from the BCM-Launcher (47; http://www.hgsc.bcm.tmc.edu/SearchLauncher/).

Media and virulence assays. MM2 medium has been described (9, 11, 18). Sorbitol (1%) was replaced with other carbohydrates when indicated. DNA manipulations followed standard procedures (44).

Leaf tips of young apple seedlings (cv. Golden Delicious) were cut with scissors and inoculated with a toothpick dipped into an overnight culture of a *gfp*-labeled *E. amylovora* strain. Migration of bacteria was evaluated in a fluorescence microscope (Zeiss Axiovert, type 135) under fluorescein isothiocyanate conditions after 5 days of incubation in a growth chamber. Virulence on pears was visually determined from ooze formation at 1 week after inoculation of 0.5-cm-thick slices in a petri dish. The assays were done with at least three plant specimens for each strain.

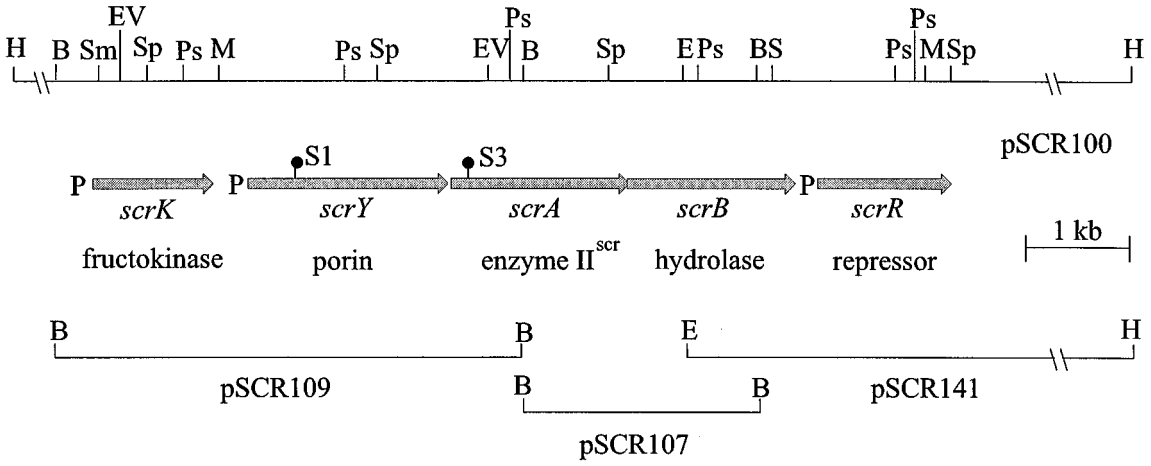

FIG. 1. Schematic map of the *scr* regulon of *E. amylovora* and the plasmids constructed for sequencing. The insertions S1 and S3 of Tn*5*seq are marked by ●. P, putative promoter; B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; M, *Mlu*I; Ps, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I.

Cytometric and fluorometric analyses. For analysis by flow cytometry and spectrofluorometry, bacteria were grown for 16 h in LB medium with 0.5% carbohydrate, centrifuged, and washed in phosphate-buffered saline (PBS) (44). The bacterial pellet was suspended in 0.5 ml of PBS for determination of fluorescence. Flow cytometry was carried out in a FACScan system (Becton Dickinson). Illumination was done with a 15-mW, 488-nm argon laser, and emission light was detected by a 530 ± 30-nm band pass filter. Photomultiplier voltages were kept constant in a given series of experiments. The fluorescence detector was set at a photomultiplier tube voltage of 546 V. Forward scatter was collected by a photomultiplier tube set at 100, and sideward scatter was collected at a photomultiplier tube set at 400 V. Data were collected for 10^4 particles per sample and analyzed with the program WinMDI 2.7 (http://facs.scripps.edu/software.html). Bacteria and other particles were separated by their different light-scattering properties (32), which are a complex function of their size, shape, and refractive indices (2, 40, 43). The region R1 (see Fig. 4) was defined to measure bacterial fluorescence on the light-scattering plots. LB cultures of Ea1/79(pSU18-Y1gfpR), LB medium, and extracts from uninfected plant tissue were compared to define an area where more than 95% of the particles were recovered as bacterial signals. For evaluation of R1, at least 1,000 particles were measured. An SPF-500 spectrofluorometer (American Instrument Company) was used to measure bacterial fluorescence at an excitation wavelength of 488 nm and an emission wavelength of 510 nm.

DNA mobility shift assay. The *scrYAB* promoter fragment was amplified by PCR using primers 3341 and 3342. About 0.5 pmol of the promoter fragment was incubated with protein MBP-ScrR (37 to 50 pmol) for 30 min at 30°C in 20 μl of DNA-protein binding buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM dithiothreitol, 0.1 mM EDTA, 0.05 μg of λ DNA per μl, and 0.5 μg of bovine serum albumin per μl). Before incubation, 10 mM sugar was added if indicated. Protein binding to DNA was assayed by electrophoresis in a native 5% polyacrylamide gel. After electrophoresis, the gel was stained with a $1{:}10^4$ dilution of SYBR Green I (Biozym) in Tris-Borate-EDTA buffer for 30 min. The gel was analyzed with a Fluor-S Multi-Imager from Bio-Rad.

RESULTS

Identification and analysis of the *scr* regulon from *E. amylovora*. Cosmid pSCR100 (Fig. 1) was isolated from a genomic library of *E. amylovora* (10) obtained after *Hin*dIII digestion. Cells of *E. coli* strain S17-1 with the library were screened for the ability to utilize sucrose as a carbon source on MacConkey agar plates with sucrose and tetracycline and to grow on minimal medium with 1% sucrose. Cosmid pSCR100 contained a 20-kb *Hin*dIII fragment, and the *scr* regulon of *E. amylovora* was localized by transposon mutagenesis of S17-1(pSCR100) using phage λ::Tn*5*seq and screening on 1% sucrose, tetracycline, and kanamycin. From 1,100 transposon mutants, 2 colonies with an *scr*-negative phenotype were found, and cosmids pSCR100-1 and pSCR100-3 were isolated from white colonies with different insertions of Tn*5*seq in the *scr* regulon of pSCR100. By subcloning after digestion with various restriction endonucleases, the transposon insertions were localized in a 10-kb region of pSCR100.

Restriction fragments from the *scr* regulon of *E. amylovora*, which was located by transposon mutagenesis, were subcloned from pSCR100 into pUC18 and sequenced in both strands by primer walking. The cloned fragments and a summary of the mapping data are shown in Fig. 1. By computer analysis of the obtained sequence of 6.9 kb (accession number AJ250722), five open reading frames (ORFs) (encoding proteins of 308, 514, 457, 469, and 342 amino acids) with putative ribosome-binding sites were found. The sequenced ORFs showed 67 to 88% similarity to ORFs of the *scr* regulon from *K. pneumoniae* and the mobilizable plasmid pUR400. The corresponding genes were named *scrK*, *scrY*, *scrA*, *scrB*, and *scrR* in analogy to other *scr* regulons. In the region preceding *scrK*, a 6-bp palindrome (TAAACC/CGTTTA) was detected, similar to the palindrome TAAACC/CCTTTA identified as the binding site of the repressor ScrR in pUR400 and *K. pneumoniae* (5). The genes *scrY*, *scrA*, and *scrB* are organized in an operon structure with an intergenic space of 18 nucleotides between *scrY* and *scrA* and an overlap of *scrA* and *scrB* (ATGA), which has also been described for related genes and may indicate translational coupling (42). The nucleotide sequences in front of *scrK*, *scrY*, and *scrR* are less conserved than the structural genes. The 266-bp intergenic region between *scrK* and *scrY* showed the palindrome AACC/GGTT. As for pUR400 (34), the possible promoter region for *scrR* overlaps the end of *scrB*.

The insertion sites of Tn*5*seq in pSCR100-1 and pSCR100-3 were also determined by sequence reactions with T7 and SP6 primers, which bind to the asymmetric ends of Tn*5*seq. In pSCR100-1, the transposon was inserted into *scrY*, whereas in pSCR100-3 the insertion was in *scrA* (Fig. 1).

The deduced amino acid sequence of ScrK showed 67% similarity to that of the ATP-dependent fructokinase of pUR400 (5), with a typical kinase consensus sequence motif ([AG]-G-x(0,1)-[GAP]-x-N-x-[STA]-x(6)-[GS]-x(9)-G) in the N terminus of the protein. The motif is defined by the distance of specified amino acids, one amino acid of a list [in brackets] or the indicated number of any amino acid (x).

ScrY showed 76% similarity to ScrY of pUR400, a sucrose-specific porin of the outer membrane (30, 46). ScrA shows high similarity (88%) to enzyme II^{scr} (EII^{scr}) of the PTS system from *K. pneumoniae* and pUR400 (51). A consensus sequence

of the EIIB cysteine phosphorylation region (N-[LIVMFY]-x(5)-C-x-T-R-[LIVMF]-x-[LIVMF]-x-[LIVM]-x-[DQ]) was identified in the N terminus of ScrA. ScrB of *E. amylovora* has 66% similarity with the β-D-fructofuranoside fructohydrolase (EC 3.2.1.26) encoded by pUR400 (46, 51). A consensus sequence of the catalytic active domain (H-x(2)-P-x(4)-[LIVM]-N-D-P-N-G) of invertases was found in the N terminus of the protein. ScrR of *E. amylovora* shows 83% similarity to the regulator protein of the *scr* regulon of pUR400. The proteins share a well defined N-terminal helix-turn-helix DNA-binding motif, characteristic of many repressors of the LacI/GalR family (53).

Cloning of *scrB* and purification and enzymatic activity of the sucrose hydrolase from *E. amylovora*. The gene *scrB* of *E. amylovora* was amplified from plasmid pSCR100 by PCR with primers 3584 and 3523, creating *Sph*I and *Hin*dIII sites at the ends of the 1.5-kb PCR fragment. The amplified fragment with *scrB* was digested and inserted into pQE31 to create pQE31scrB by fusion of *scrB* with the $(His)_6$ tag sequence of the vector. The plasmid was transformed into *E. coli* strain GI698. The protein was expressed and purified as described in Materials and Methods. After elution of the native gene product from an Ni column, a major band of 53 kDa was detected by SDS-PAGE, corresponding in size to ScrB as deduced from the encoding ORF.

The purified sucrose hydrolase cleaved sucrose with an optimal enzymatic activity of 18,300 U/mg of homogeneous enzyme in 100 mM phosphate buffer (pH 7.0) and 200 mM sucrose at 28°C. The Michaelis-Menten constant of the sucrose hydrolase for cleavage of sucrose was 125 mM (data not shown). After freezing the enzyme in 10% glycerol at −80°C, no significant (<1%) loss of enzymatic activity was detected after 1 month. Enzymatic activity decreased at ionic strengths higher than 200 mM or lower than 50 mM sodium phosphate. The temperature optimum of the hydrolase was between 18 and 28°C. The maximum enzymatic activity was recovered at pH 7.0, whereas a pH shift to 5.6 or 8.6 caused loss of the activity.

Gene cloning, purification, and characterization of the repressor ScrR from *E. amylovora*. The gene *scrR* of *E. amylovora* was amplified by PCR with primers 3583 and 3506 as a 1-kb fragment. The amplified *scrR* was digested with *Bam*HI and *Hin*dIII and inserted into pQE31 to create pQE31scrR, expressed in strain GI698. Purification of the gene product was done under denaturing conditions due to low solubility of the fusion protein. The purified probe was analyzed by SDS-PAGE and showed a dominant band of 38 kDa, corresponding to the size deduced from the ORF. Several attempts to renature the protein after purification were unsuccessful.

Insolubility after overexpression in *E. coli* has been described for other ScrR repressors (33). However, a fusion of the repressor to *E. coli* MBP could yield a soluble protein. Therefore, the 1-kb *Bam*HI-*Hin*dIII fragment from pQE31scrR was cloned into pMa1-c2 to create pMalscrR. After expression in *E. coli*, the MBP-ScrR protein was purified by its affinity to an amylose resin. In order to show that *scrR* codes for a regulatory protein which binds to the *scrYAB* promoter region, a gel mobility shift assay with the 278-bp promoter fragment (P_{scrYAB}) of pSU18-Y1 was carried out. The promoter fragment was shifted after addition of MBP-ScrR but was not affected by the presence of λ DNA (Fig. 2). To identify the intracellular inducer, fructose, sucrose, and glucose were tested for their ability to release bound ScrR from the promoter fragment. Fructose but not sucrose or glucose dissociated the repressor-promoter complex (Fig. 2), suggesting that fructose is the intracellular inducer of the *scr* operon.

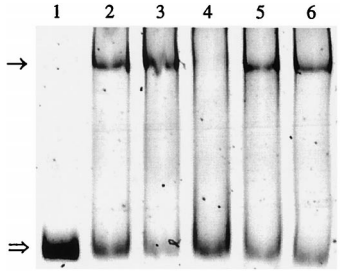

FIG. 2. DNA mobility shift assay with promoter fragment P_{scrYAB} and protein MBP-ScrR. Lane 1, no added protein; 2, 30 pmol of MBP-ScrR; 3, 50 pmol of MBP-ScrR; 4, 50 pmol of MBP-ScrR and 10 mM fructose; 5, 50 pmol of MBP-ScrR and 10 mM sucrose; 6, 50 pmol of MBP-ScrR and 10 mM glucose. The arrow indicates the promoter fragment with the bound 81-kDa protein MBP-ScrR. The double arrow indicates the unbound fragment.

Regulation of *scrYAB* in *E. amylovora*. To study transcriptional regulation of the *scrYAB* operon of *E. amylovora*, the putative promoter-operator region preceding the operon was amplified as a 278-bp PCR fragment with primers 3342 and 3341. The fragment was inserted into vector pSU18 via the restriction sites *Sal*I and *Xba*I. In the resulting plasmid (pSU18-Y1), the orientation of the putative promoter P_{scrYAB} is directed against the *lac* promoter of the vector. To fuse the promoterless *gfp* gene with P_{scrYAB}, a 700-bp *Xba*I-*Sal*I fragment from pFG30 was subcloned into pSU18-Y1, yielding pSU18-Y1gfp. Constitutive expression of the reporter gene in Ea1/79(pSU18-Y1gfp) without the repressor gene cloned on the plasmid was assayed by fluorometry and flow cytometry in LB medium with and without sucrose. In order to increase the intracellular level of the repressor, a 1.2-kb fragment containing *scrR* was amplified with primers 3366 and 3367 by PCR. The primers introduced *Eco*RV and *Asp*718 restriction sites into pSU18-Y1gfp. The plasmid created, pSU18-Y1gfpR, was transferred into Ea1/79, and expression of the *gfp* gene in LB cultures with various carbohydrates was determined (Fig. 3). A high induction of the transcriptional fusion was found for 0.1% sucrose, with less effect of lower or higher concentrations (Fig. 3A). Fructose also induced the reporter fusion to some extent (Fig. 3B). Catabolite repression was observed for growth with glucose in addition to sucrose. For the levan-deficient mutant Ea7/74-LS7(pSU18-Y1gfpR), expression of the reporter gene was increased twofold compared to expression in strain Ea1/79(pSU18-Y1gfpR). Crude protein extracts of Ea7/74-LS7 cultures grown in LB with 0.5% sucrose, fructose, glucose, glycerol, or sucrose plus glucose were tested for sucrose hydrolase activity. The induction of enzyme activities by the different carbohydrates correlated with the induction of the *gfp* gene measured by flow cytometry and spectrofluorometry with Ea7/74-LS7(pSU18-Y1gfpR) or Ea1/79(pSU18-Y1gfpR) (Fig. 3).

To measure regulation of P_{scrYAB} in plant tissue, immature pears were used directly or soaked with 200 μl of 0.2% sucrose solution and inoculated with Ea1/79(pSU18-Y1gfpR). Four-week-old apple seedlings and cotoneaster flowers were also inoculated with Ea1/79(pSU18-Y1gfpR). For the isolation of bacteria, a crude extract from infected plant tissue was filtered and analyzed by flow cytometry. The mean fluorescence of bacteria inoculated into untreated immature pear slices was 6.55 after 2 days, while the fluorescence was increased to 8.55 after soaking the pears with 0.2% sucrose solution 1 day before inoculation (Fig. 4). Ea1/79(pSU18-Y1gfpR) isolated from the first two leaves of apple seedlings 5 days after inoculation produced a fluorescence value of 2.7; conversely, the fluores-

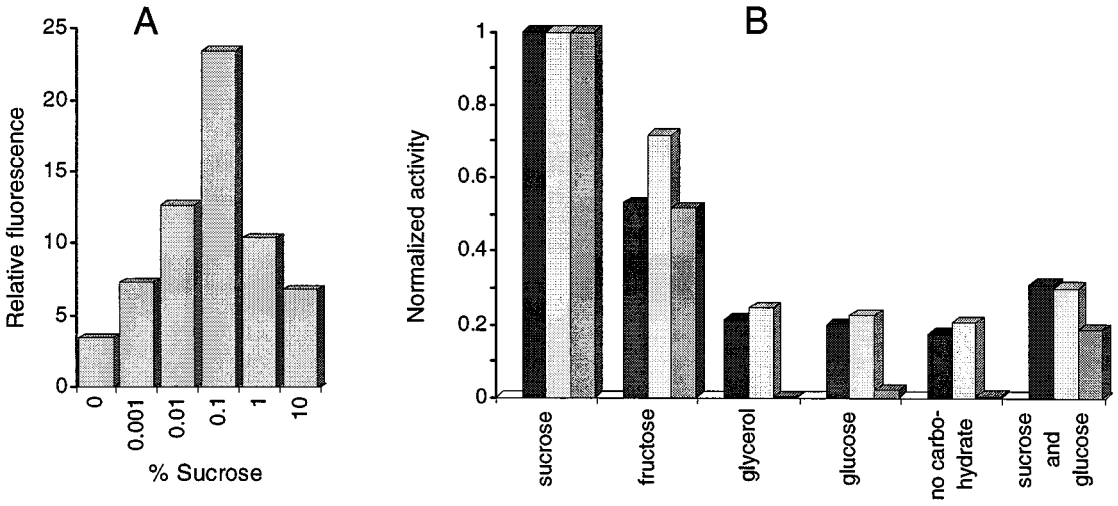

FIG. 3. Induction of the *scrYAB* promoter fragment in Ea1/79(pSU18-Y1gfpR). (A) Expression dependent on sucrose concentration. Fluorescence measurements were done by flow cytometry. (B) Expression of the reporter gene fusion in the presence of various sugars and expression of the sucrose hydrolase activity of Ea7/74-LS7 grown in LB medium with 0.5% of each carbohydrate. The expression of the *gfp* reporter gene was determined by flow cytometry (dark bars) and spectrofluorometry (light bars). The sucrose hydrolase activity is shown in an intermediate bar color. Values for sucrose were set at 1.

cence of the bacteria increased to a value of 6.9 when they were isolated from stem tissue.

Site-directed mutagenesis of the *scr* regulon in *E. amylovora*. Plasmid pSCR100-1 was transferred into *E. amylovora* strain Ea7/74 and the levan-deficient strain PD494, and pSCR100-3 was transformed into strain Ea1/79. Homolog recombination events were screened after conjugation of plasmid pPH1JI, which is incompatible with RP4-derived plasmids. Mutants with site-specific recombination of the transposon were white on MacConkey plates with sucrose and unable to grow on solidified MM2 medium with sucrose. The mutants with transposon insertions in *scrY* (pSCR100-1) and *scrA* (pSCR100-3) were named Ea7/74-S1, PD494-S1, and Ea1/79-S3, accordingly. Complementation of the mutants was verified with pSCR100 on MacConkey agar plates with sucrose. Their level of levan synthesis was the same as for the parent strains.

Growth features of *scr* mutants in culture. The growth properties of the *scr* mutants on various carbon sources were compared with those of their parent strains. No difference in the growth kinetics was observed in LB medium or minimal me-

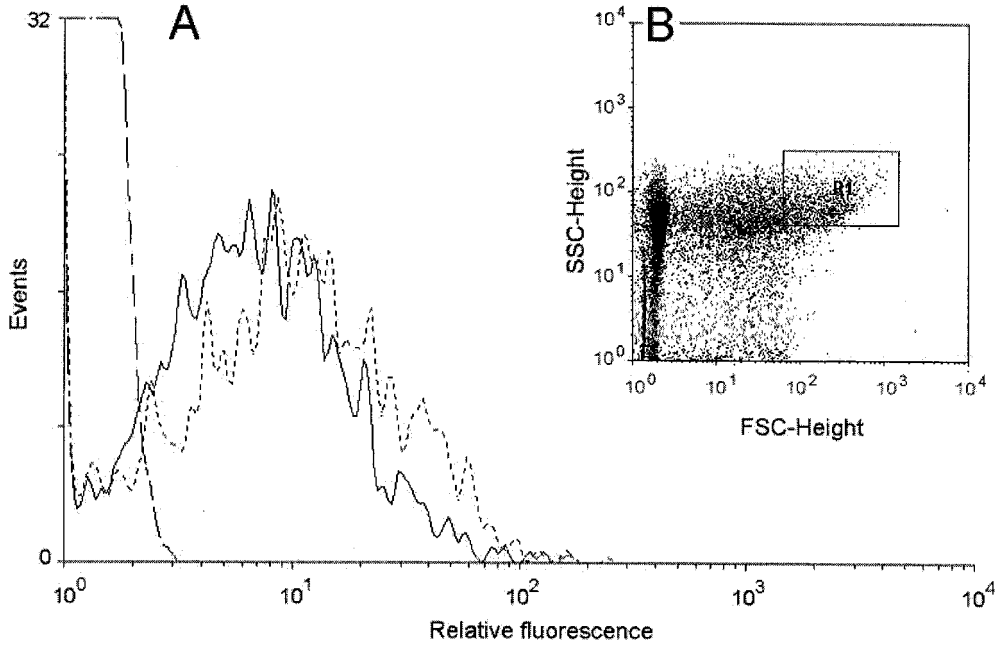

FIG. 4. Flow cytometry of Ea1/79(pSU18-Y1gfpR) isolated from immature pears. (A) Comparison of the relative fluorescence of Ea1/79 (—·—) (background signals), Ea1/79(pSU18-Y1gfpR) isolated from immature pears (——), and Ea1/79(pSU18-Y1gfpR) isolated from immature pears which were soaked in 0.2% sucrose solution before inoculation (– – –). Mean fluorescence values: Ea1/79(pSU18-Y1gfpR), 6.55; Ea1/79(pSU18-Y1gfpR) plus 0.2% sucrose, 8.87; Ea1/79, 1.26 (background). (B) Dot plot of forward (FSC) (*x* axis) and side (SSC) (*y* axis) scatter of the particles isolated from immature pears infected with *E. amylovora*. For measurement of bacterial fluorescence, region R1 was defined for optimal recovery of bacteria (95%).

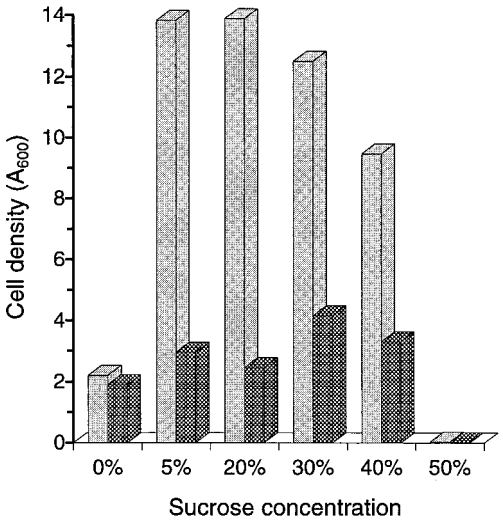

FIG. 5. Growth of PD494Sm (light bars) and the sucrose mutant PD494-S1 (dark bars) in LB medium. The sucrose concentrations in the medium are indicated. Strain PD494Sm was used to avoid a slight interference by levan in determination of the cell density.

dium with glucose or sorbitol. In contrast to the parent strains, there was no growth of the *scr* mutants in minimal medium with 1% sucrose after 24 h. In LB medium with sucrose (5 to 40%, wt/wt), the growth of the mutants was reduced fourfold compared to the wild type, which is able to use sucrose as a carbon source, whereas the mutants must rely on the ingredients of the LB medium. For sucrose concentrations higher than 40%, the mutants and the wild-type strains were unable to grow, as shown for the levan-deficient strain PD494Sm and corresponding sucrose mutant PD494-S1 (Fig. 5), limiting *E. amylovora* to propagation in extreme sugar concentrations independent of their genetic features.

Virulence of *E. amylovora scr* mutants. In virulence assays on slices of immature pears, the *E. amylovora* wild-type strains and the *scr* mutants produced similar amounts of ooze. The low content of sucrose in those pears (G. Geier and K. Geider, unpublished) is probably compensated for by other carbon sources, such as sorbitol, fructose, and glucose, or organic acids.

When inoculated into leaves of apple seedlings, wild-type strains of *E. amylovora* caused necrosis and sometimes even wilt. In contrast, the sucrose mutants Ea7/74-S1, PD494-S1, and Ea1/79-S3 showed strongly reduced symptoms in these assays. To confirm the inability of the mutants to colonize leaf tissue, the strains were labeled with plasmid pfdC1Z′-gfp in order to trace them via the fluorescence of the GFP (16). The parent strains labeled with *gfp* showed the expected migration in the veins of apple leaves at day 5 after inoculation. Conversely, the labeled mutant strains did not move from the narrow zone of inoculation at the leaf tip. Sucrose, which cannot be utilized by *scr* mutants, is thus an important energy source for *E. amylovora* to colonize the tissue of the fire blight host plants.

DISCUSSION

For colonization of plants by *E. amylovora*, the causative agent of fire blight, not only the sorbitol (1) but also the sucrose metabolism of the bacteria is important. These carbohydrates vary among species and within parts of a plant (14, 28, 50). Sucrose is high in the nectaries of flowers (14), a main entry site for the pathogen. High sucrose concentrations usually inhibit bacterial growth, but *E. amylovora* can grow even in 40% sucrose (38). Its inability to grow in 50% sucrose restricts propagation of the pathogen in an environment with extreme sugar concentrations.

The genes involved in sucrose metabolism of *E. amylovora* showed significant homology to the *scr* genes of *Salmonella enterica* serovar Typhimurium with pUR400 and of *K. pneumoniae* (45, 49). Conserved amino acid motifs in the N terminus of *scr* proteins of *E. amylovora* indicated biological function related to the enzymes from pUR400, *K. pneumoniae*, and other proteins in the family. The overlapping stop codon of *scrA* and start codon of *scrB* (ATGA) support transcriptional and translational coupling of the two genes, as described for the *scr* genes of other species (42, 51).

A transcriptional fusion of the promoter P_{scrYAB} with *gfp* in the presence of *scrR* (pSU18-Y1gfpR) and expression studies of ScrB indicated regulation of the promoter activity from a region preceding *scrYAB*. Computer analysis of P_{scrYAB} predicted binding domains for the regulator ScrR and a cyclic AMP-cyclic AMP receptor protein complex. The promoter was not only regulated by ScrR, but also induced by sucrose and fructose and suppressed by glucose. Gel mobility shift assays with the 300-bp DNA fragment preceding *scrY* and the repressor showed that ScrR binds to this region and that fructose and not sucrose is the molecular inducer of *scrYAB* transcription, as in pUR400 and *K. pneumoniae*. The *scr* repressors of pUR400 and *K. pneumoniae* interact with a helix-turn-helix with the operator palindrome TAAACC/GGTTTA preceding *scrY* and *scrK* and bind to fructose or fructose 1-phosphate but not to sucrose (34). A part of this sequence (AACC/GGTT) was found in front of *scrY* and is possibly the operator in the *scr* regulon of *E. amylovora*. Based on a possible leucine zipper motif at the C terminus of ScrR and the observation that the palindrome is present twice in the P_{scrYAB} region of *K. pneumoniae*, Jahreis and Lengeler (34) proposed binding of ScrR as a tetramer to the operator. This is not supported by *E. amylovora*, with one palindrome in this region and without a leucine zipper motif in ScrR.

An influence of host cells on the promoter activity of a pathogen has been measured in *Mycobacterium smegmatis* (23), *Bartonella henselae* (22), *Bacillus cereus* (24), and *Listeria monocytogenes* (17) by the combination of flow cytometry and the *gfp* reporter gene as a marker. *gfp* expression in *E. amylovora* was also analyzed by flow cytometry. Fluorescence of bacteria from stem sections was increased twofold compared to bacteria from young leaves of apple seedlings. This increased fluorescence can be explained by the relatively low sucrose concentration in young leaves and the high concentration in stem tissue (28). The highest in vitro induction was found for sucrose concentrations between 0.01 and 0.8%, comparable to concentrations in the xylem of apple plants. Other parts of the plants can contain much higher concentrations of the sugar (14, 25, 31). In LB medium with sucrose concentrations higher than 0.8%, the activity of P_{scrYAB} was reduced. Low fluorescence of the bacteria from cotoneaster flowers (unpublished data) can be explained by the high sucrose concentration in the nectaries, which also caused reduced fluorescence in LB medium.

Reduced virulence of sorbitol (1) and sucrose mutants could be due to a low level of nutrients in xylem vessels, requiring access to sucrose and sorbitol for colonization of the host plant by *E. amylovora*. The *scr* mutants Ea7/74-S1, PD494-S1, and Ea1/79-S3 did not grow in minimal medium with sucrose as the sole carbon source, independent of levan synthesis.

Levan may provide fast protection of *E. amylovora* against plant defense mechanisms (27, 29). Levansucrase could reduce the induction of the *scr* regulon by decreasing the sucrose concentration and providing glucose for catabolite repression shown for the levan mutant Ea7/74-LS7(pSU18-Y1gfpR) with increased fluorescence in the presence of sucrose compared with the wild type. Intracellular sucrose metabolism and extracellular levan formation can depend on a single enzyme, as in SacB of *Bacillus subtilis* (19). At high sucrose concentrations (>30 mM), sucrose is cleaved by extracellular SacB to form levan, and at low sucrose concentrations (≤1 mM) it is hydrolyzed in the cells. The *sacB* gene is regulated by sucrose via an antitermination process (21). Expression of the levansucrase gene of *E. amylovora* is not induced by sucrose (27), but is regulated by LsrA, encoded in the *hrp* region (56). Unlike the nitrogen-fixing bacterium *Acetobacter diazotrophicus*, which depends on an extracellular levansucrase to use sucrose as a carbon source (4), *E. amylovora* thus encodes two enzymes to metabolize sucrose (ScrB and Lsc). The external release of glucose did not substitute for a deficiency in sucrose metabolism, since the defect in *scr* mutants of *E. amylovora* cannot be suppressed by secreted levansucrase. Sucrose metabolism via the *scr* regulon of *E. amylovora* is thus strictly required for pathogenicity.

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