AraC/XylS Family Stress Response Regulators Rob, SoxS, PliA, and OpiA in the Fire Blight Pathogen *Erwinia amylovora*

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Transcriptional regulators of the AraC/XylS family have been associated with multidrug resistance, organic solvent tolerance, oxidative stress, and virulence in clinically relevant enterobacteria. In the present study, we identified four homologous AraC/XylS regulators, Rob, SoxS, PliA, and OpiA, from the fire blight pathogen *Erwinia amylovora* Ea1189. Previous studies have shown that the regulators MarA, Rob, and SoxS from *Escherichia coli* mediate multiple-antibiotic resistance, primarily by up-regulating the AcrAB-TolC efflux system. However, none of the four AraC/XylS regulators from *E. amylovora* was able to induce a multidrug resistance phenotype in the plant pathogen. Overexpression of *rob* led to a 2-fold increased expression of the *acrA* gene. However, the *rob*-overexpressing strain showed increased resistance to only a limited number of antibiotics. Furthermore, Rob was able to induce tolerance to organic solvents in *E. amylovora* by mechanisms other than efflux. We demonstrated that SoxS from *E. amylovora* is involved in superoxide resistance. A *soxS*-deficient mutant of Ea1189 was not able to grow on agar plates supplemented with the superoxide-generating agent paraquat. Furthermore, expression of *soxS* was induced by redox cycling agents. We identified two novel members of the AraC/XylS family in *E. amylovora*. PliA was highly upregulated during the early infection phase in apple rootstock and immature pear fruits. Multiple compounds were able to induce the expression of *pliA*, including apple leaf extracts, phenolic compounds, redox cycling agents, heavy metals, and decanoate. OpiA was shown to play a role in the regulation of osmotic and alkaline pH stress responses.

*E*rwinia amylovora, a plant-pathogenic member of the family *Enterobacteriaceae*, causes the devastating disease fire blight on rosaceous plants, with economic importance for the disease on apple and pear plants (1). During pathogenesis in the plant, microbes are exposed to a variety of antimicrobial compounds produced by the host. Successful pathogens utilize a variety of different systems to circumvent the toxic effects of these substrates. Important among these mechanisms are multidrug efflux pumps that are able to recognize and efficiently expel a wide range of structurally diverse compounds from the cell (2). In Gram-negative bacteria, efflux pumps that form a large tripartite complex spanning both the inner and outer membranes play a key role in multidrug resistance (3–5). These pumps consist of an inner membrane transporter (ABC, MFS, or RND family transporter), a membrane fusion protein, and an outer membrane channel (6, 7). In *E. amylovora*, the RND-type efflux pump AcrAB-TolC has been demonstrated to play an important role in virulence (8, 9). Mutants defective in this efflux system were impaired in colonization of apple rootstock. Furthermore, the mutants were susceptible to apple leaf extracts as well as to the apple phytoalexins phloretin, naringenin, quercetin, and (+)-catechin (8).

Bacteria are able to utilize multidrug efflux pumps in a very efficient, precise, and complex way by specifically regulating the expression of the drug transporter genes. Many genes are under either local or global transcriptional control. For example, the multidrug transporter AcrAB is locally controlled by the transcriptional repressor AcrR in *Escherichia coli* (10, 11) and *Salmonella enterica* (12) and by global transcriptional activators of the AraC/XylS family, such as MarA, SoxS, and Rob (13–15).

Transcriptional regulators belonging to the AraC/XylS family are the most common positive regulators (16–18). The main characteristic of this family is a conserved 100-amino-acid sequence, constituting a helix-turn-helix (HTH) DNA-binding domain,

which is important for the activation of transcription upon binding to the target sequence of the respective promoter region. However, the DNA-binding domain is not involved in binding of effector molecules. Chemical signals may be recognized by additional, nonconserved domains located in the same polypeptide as the DNA-binding domain (e.g., AraC and XylS), or transcription of the AraC/XylS family member may be controlled by another regulator. Binding of effector molecules to this additional regulator induces transcription of the AraC/XylS protein gene and often leads to overexpression of the AraC/XylS regulator (e.g., MarA and SoxS) (reviewed in reference 17).

Most members of this family are involved in the control of carbon metabolism, pathogenicity, or stress responses (15, 17). In *E. coli*, the homologous regulators MarA, SoxS, and Rob control about 40 genes involved in resistance to antibiotics, heavy metals, organic solvents, and oxidative stress (19–22), and together they form the so-called mar/sox/rob regulon, whose function is associated with binding to the so-called marbox, a 20-bp asymmetric sequence (23). Due to the high levels of sequence similarity of the three regulators, an overlapping regulon is observed, albeit with different efficiencies for activation of particular promoter regions, depending on the transcriptional activator concentration (24),

TABLE 1 Bacterial strains used in this study

Strain	Relevant characteristics or genotype[a]	Reference or source
Escherichia coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tc^r)]	Stratagene
S17-1	Tc^r Sm^r *recA pro hsdR* (RP4-2-Tc::Mu-Km::Tn*7*)	90
S17-1 λ*pir*	λ*pir* phage lysogen of S17-1	90
DH5α λ*pir*	F$^-$ *supE44* Δ*lacU169* (φ*lacZ*Δ*M15*) *recA1 endA1 hsdR17*(r_K^- m_K^+) *thi-1 gyrA96 relA1* λ*pir* phage lysogen	D. Lies, Caltech
Erwinia amylovora strains		
Ea1189	Wild type	GSPB[b]
Ea1189-3	Ea1189 with Km^r cassette in *acrB*	8
Ea1189.opiA$^-$	Ea1189 with Cm^r cassette in *opiA*	This study
Ea1189.pliA$^-$	Ea1189 with Cm^r cassette in *pliA*	This study
Ea1189.rob$^-$	Ea1189 with Cm^r cassette in *rob*	This study
Ea1189.soxS$^-$	Ea1189 with Cm^r cassette in *soxS*	This study

[a] Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant.
[b] GSPB, Göttinger Sammlung Phytopathogener Bakterien, Göttingen, Germany.

DNA-binding affinity for the specific region (23–25), and RNA polymerase attraction (26, 27).

Expression of *marA*, *rob*, and *soxS* is increased upon exposure to a wide variety of signals. Transcription of MarA (multiple-antibiotic resistance) is controlled by the local repressor MarR and can be derepressed through various compounds, such as salicylate, benzoate, and plumbagin (28, 29). Expression of *soxS* (superoxide stress) is activated by the oxidative stress sensor SoxR. Upon exposure to superoxides, nitric oxide, or redox cycling compounds, such as paraquat and plumbagin, the [2Fe-2S] cluster of SoxR becomes oxidized, allowing it to activate the transcription of *soxS* (22, 30).

Rob (right *oriC* binding) is different from MarA and SoxS in that it is a larger protein consisting of two domains. The N-terminal, DNA-binding domain shows high degrees of sequence similarity to the small activators MarA and SoxS, whereas the C-terminal domain can bind low-molecular-weight effectors (31). Rob is constitutively present at high concentrations in cells and is post-translationally activated by a sequestering-dispersal mechanism (32–34). Under noninducing conditions, Rob is inactive because it is sequestered into aggregates. Several compounds, including decanoate, unconjugated bile salts, and dipyridyl, bind to the C-terminal domain of Rob and induce its dispersal from the sequestered state, thus enabling Rob to activate transcription of its target genes (14, 35).

MarA, SoxS, and Rob are known for their role in promoting antibiotic resistance due to the upregulation of the multidrug efflux pump AcrAB-TolC in *E. coli* (13, 36–38). Despite the fact that these activators are able to induce the expression of *acrAB* under stress conditions in *E. coli*, they appear to be less important in regulation of multidrug resistance in *S. enterica* (39). Interestingly, in *S. enterica*, RamA (resistance antibiotic multiple), another member of the AraC/XylS family, was found to be involved in conferring multidrug resistance by directly increasing the expression of *acrAB* (39). Furthermore, it has also been demonstrated that mutational inactivation of the AcrAB-TolC pump leads to increased expression of *ramA* in *S. enterica* (40).

The aim of this study was to identify members of the AraC/XylS family involved in regulation of multidrug efflux in *E. amylovora*. We identified genes for four transcriptional regulators of the AraC/XylS family with homology to MarA, SoxS, and Rob of *E. coli* in the genome sequence of *E. amylovora*. Two regulators were homologous to either Rob or SoxS, whereas the other two proteins, PliA and OpiA, did not show significant similarity to characterized members of the AraC/XylS family. We characterized the roles of these regulators in antibiotic resistance, organic solvent tolerance, and oxidative stress. Moreover, their contributions to virulence in apple rootstock and immature pear fruits were investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1 and in Table S1 in the supplemental material, respectively. *E. amylovora* strains were cultured at 28°C in lysogeny broth (LB), double yeast tryptone (dYT), or asparagine minimal medium 2 (AMM2; 10 g fructose, 4 g L-asparagine, 12.8 g Na$_2$HPO$_4$ · 7H$_2$O, 3 g K$_2$HPO$_4$, 3 g NaCl, 0.2 g MgSO$_4$ · 7H$_2$O, 0.25 g nicotinic acid, 0.2 g thiamine per liter) (8). *E. coli* XL-1 Blue was used as the cloning host. *E. coli* DH5α λ*pir* was used as the host for the replacement vectors. *E. coli* cells were routinely maintained at 37°C in dYT medium. Cultures harboring individual vectors were supplemented with 50 μg/ml ampicillin (Ap) for *E. coli* or 250 μg/ml Ap for *E. amylovora*, 25 μg/ml chloramphenicol (Cm), 2 μg/ml gentamicin (Gm), or 25 μg/ml kanamycin (Km), where necessary. Bacterial growth was monitored using a spectrophotometer to measure the optical density at 600 nm (OD$_{600}$).

PCR amplifications, modifications, and protein purification. PCR primers are listed in Table S2 in the supplemental material. Primers were designed based on the genome sequence of *E. amylovora* CFBP1430 available from NCBI (accession no. NC_013961.1). Screening PCRs were carried out using DreamTaq DNA polymerase (Thermo Scientific) in accordance with the manufacturer's instructions. For high-fidelity PCRs, Phusion DNA polymerase (Thermo Scientific) was used.

Restriction enzyme (Thermo Scientific) and T4 DNA ligase (Thermo Scientific) reactions were performed following the manufacturer's instructions, with restriction enzyme reactions performed at the appropriate temperature and all ligation reaction mixtures incubated at room temperature. DNA purifications were performed using either a GeneJET PCR purification kit (Thermo Scientific) or a GeneJET gel extraction kit (Thermo Scientific) according to the manufacturer's instructions.

Protein purification was carried out using a Ni-nitrilotriacetic acid (Ni-NTA) spin kit (Qiagen) following the manufacturer's instructions.

Construction of *pliA*- and *rob*-deficient mutants of *E. amylovora*. For mutagenesis of the *pliA* gene, a 1,013-bp fragment was amplified using

the primer pair pliA_ko_fwd and pliA_ko_rev. For deletion of the *rob* gene, a 1,087-bp fragment was amplified using the primer pair rob-_ko_fwd and rob_ko_rev. Obtained PCR products were verified by sequencing. Next, a chloramphenicol cassette flanked by Flp-*FRT* sites was cut from plasmid pFCm1 (96) and inserted into the PstI-digested *pliA* fragment as well as into the AatII-digested, blunt-ended *rob* fragment. The deletion alleles were further cloned into the final replacement vector pCAM-Km by use of SpeI and EcoRI, yielding plasmids pCAM-Km.pliA-ko.Cm and pCAM-Km.rob-ko.Cm. The plasmids were transformed into electrocompetent cells of *E. amylovora* Ea1189, which subsequently were grown for 3 h at 28°C in dYT broth. Putative mutants were screened for homologous recombination events by testing their antibiotic resistance. Mutants that resulted from single-crossover events were identified by the ability to grow on plates containing Km. In order to confirm gene deletion through a double-crossover event in Cm-resistant and Km-sensitive colonies, primers binding up- and downstream of the knockout fragments were designed (pliA_out and rob_out primers). PCRs were done using these locus-specific primers in combination with outward-facing primers binding within the Cm cassette (cat_out primers). PCR products were verified by sequencing.

Construction of *soxS*- and *opiA*-deficient mutants of *E. amylovora*. For generation of the *soxS* knockout vector, a 1,268-bp fragment was amplified using the primer pair soxS_ko_fwd and soxS_ko_rev. A chloramphenicol cassette flanked by Flp-*FRT* sites was cut from plasmid pFCm1 (96) and inserted into the EcoRV-digested *soxS* fragment. The deletion allele was further cloned into the final replacement vector pCAM-Km via BglII, yielding plasmid pCAM-Km.soxS-ko.Cm.

The construction of the *opiA* knockout vector was based on the protocol described by Zumaquero et al. (41). Briefly, a 1,220-bp fragment flanking the 5′ region and a 777-bp fragment flanking the 3′ region of the *opiA* gene were PCR amplified using primer pairs opiA-A5-1– opiA-A6 and opiA-B3– opiA-B4, respectively. Primers opiA-A6 and opiA-B3 share a 20-nucleotide homologous sequence at their 5′ ends, consisting of the T7 primer sequence and a KpnI restriction site (41). The obtained fragments were gel purified, and approximately 40 ng (each) of A and B fragments was used for a fusion PCR with primers opiA-A5 and opiA-B4. The resulting fusion product was gel purified and confirmed by sequencing. Next, a chloramphenicol cassette flanked by Flp-*FRT* sites was cut from plasmid pFCm1 and subsequently inserted into the KpnI-digested *opiA* fragment. The deletion allele was cut with BglII and further ligated into BglII-digested pCAM-Km, yielding the final replacement plasmid pCAM-Km.opiA-ko.Cm.

Next, knockout constructs were transformed into electrocompetent cells of *E. amylovora* Ea1189, and double-crossover mutants were selected as described above.

Cloning of the *pliA*, *rob*, *soxS*, and *opiA* regions of *E. amylovora*. The *pliA*, *rob*, *soxS*, and *opiA* genes, including the respective promoter regions, were PCR amplified using the primer pairs pliA-P-fwd-KpnI–pliA_rev_BamHI (594 bp), rob-P-fwd-ApaI–rob_rev_SacII (1,160 bp), soxS-P-fwd-KpnI–soxS_rev_BamHI (606 bp), and opiA-P-fwd-ApaI–opiA_rev_BamHI (720 bp), respectively. The obtained PCR products were sequenced and subsequently cloned into pBlueScript II SK(+) in the opposite orientation with respect to the *lac* promoter to obtain expression from the native promoters, yielding plasmids pBlueSK.pliA-Pro, pBlueSK.rob-Pro, pBlueSK.soxS-Pro, and pBlueSK.opiA-Pro. In order to obtain expression from the *lac* promoter, the respective genes were amplified using the forward primers pliA_fwd_KpnI, rob_fwd_ApaI, soxS_fwd_KpnI, and opiA_fwd_KpnI in combination with the above-mentioned reverse primers. The PCR products were cloned into pBlueScript II KS(+), yielding plasmids pBlueKS.pliA, pBlueKS.rob, pBlueKS.soxS, and pBlueKS.opiA. *E. amylovora* does not carry a homologue of the *lac* repressor. Therefore, the *lac* promoter shows full activity in *E. amylovora* without the addition of inducers.

Drug susceptibility tests. The MICs of antimicrobial compounds on *E. amylovora* strains were determined by a 2-fold dilution assay using 96-well plates and Mueller-Hinton broth (MHB). All tests were performed at least in triplicate following the recommendations of the Clinical and Laboratory Standards Institute (42). Growth of bacteria at 28°C was examined by visual inspection after 48 h of incubation. The MIC was defined as the lowest concentration of an antibiotic that completely prevented visible cell growth.

Solvent tolerance assay. Organic solvent tolerance levels of bacteria were visually determined by the appearance of colony formation on agar plates overlaid with *n*-hexane and cyclohexane. Therefore, an overnight culture was diluted to an OD_{600} of 0.1, and 20 µl of the diluted cell suspension was spotted onto an agar plate and kept until the spot was completely dried. Next, plates were overlaid with 2 ml *n*-hexane or 1 ml cyclohexane, sealed, and incubated at 28°C for 48 to 60 h. Growth was visually determined by means of colony formation.

Paraquat-induced superoxide resistance. Superoxide resistance was tested on MHB II agar plates supplemented with different concentrations of paraquat (10, 20, 30, 40, and 50 µg/ml) as previously described (43). Briefly, overnight cultures grown in MHB broth were diluted to an OD_{600} of 0.1 and further cultivated until an OD_{600} of approximately 1.0. Next, 10-µl aliquots of the bacterial cultures were spread on agar plates supplemented with paraquat. After 48 to 60 h of incubation at 28°C, the plates were visually monitored for bacterial growth by means of colony formation.

Determination of intracellular ROS levels. In order to measure intracellular peroxide levels, we utilized the reactive oxygen species (ROS)-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCF; Sigma-Aldrich) as previously described (44). Briefly, overnight cultures grown in LB broth were diluted to an OD_{600} of 0.1 and further incubated at 28°C until an OD_{600} of 0.5 was obtained. Cells were harvested by centrifugation and washed with phosphate-buffered saline (PBS). Next, the cell suspension was incubated with 10 µM DCF at 28°C with shaking at 220 rpm for 30 min. Fluorescence intensity was measured using an Infinite M1000 Pro microplate reader (Tecan, Crailsheim, Germany), with an excitation wavelength of 485 nm and emission detection at 528 nm.

Effects of pH and osmotic stress on growth of *Erwinia amylovora*. Overnight cultures grown in AMM2 medium were diluted to an OD_{600} of 0.1 and further cultured in AMM2 medium at pH 5.5, 7.0, or 8.5 or in AMM2 medium supplemented with 300 mM NaCl. Cultures were incubated at 28°C for 24 h in a shaking incubator at 220 rpm. Growth of the bacteria was determined using a spectrophotometer to measure the OD_{600}.

Promoter activity of the RND efflux pump AcrAB *in vitro*. A transcriptional fusion between the promoter region of *acrAB* and the reporter gene *egfp* was employed to study the impact of overexpression of *pliA*, *rob*, *soxS*, and *opiA* on expression of the multidrug efflux pump AcrAB in *E. amylovora*. The overexpression plasmids carrying the AraC/XylS regulator genes under the control of the *lac* promoter were transformed into Ea1189 harboring plasmid pBBR.acrA-Pro.egfp (45, 46). Transformed cells of Ea1189 were grown at 28°C in LB broth until stationary phase, harvested by centrifugation, resuspended in phosphate-buffered saline, and adjusted to an OD_{600} of 0.5. Next, enhanced green fluorescent protein (EGFP) fluorescence was measured using an Infinite M1000 Pro microplate reader (Tecan, Crailsheim, Germany) set to an excitation wavelength of 470 nm, with emission detection at 516 nm.

Virulence assay on apple rootstock. Apple plants (rootstock Malus MM106) were grown in a greenhouse at 20 to 25°C with 60% humidity and a 12-h photoperiod (15,000 lx). *E. amylovora* strains were grown on LB agar for 24 h and then resuspended and diluted to an OD_{600} of 1.0 in sterile demineralized water. Apple plants were inoculated by the prick technique (47). Each bacterial strain was inoculated into one shoot each of a minimum of five single plants. A bacterial suspension (5 µl) was placed onto each wound on the shoot tip. Plants were monitored for symptom development daily. Survival of bacteria in plant tissue was examined by reisolation of bacterial cells 1, 3, and 7 days after inoculation, from 1 cm of the shoot tip around the inoculation area.

In order to analyze mRNA transcript abundances of *pliA*, *rob*, *soxS*, and *opiA* during growth of *E. amylovora* in apple rootstock MM106, total RNAs were isolated from infected shoots at 1 and 7 days postinoculation. Five individual wound regions were pooled, homogenized in sterile water (1 ml/wound), and centrifuged for 2 min at 4,000 rpm. The supernatant was transferred to 15 ml killing buffer (20 mM Tris-HCl, pH 7.5; 20 mM NaN$_3$) (48) and centrifuged for 20 min at 4,000 rpm. The supernatant was decanted and the pellet frozen at −80°C for further RNA extraction. Total RNA was isolated by acid phenol-chloroform extraction (48).

Virulence assay on immature pears. Virulence of *E. amylovora* Ea1189 and *pliA*, *rob*, *soxS*, and *opiA* mutants was determined on immature pears (*Pyrus communis* L. cv. Bartlett). Bacteria grown on LB agar plates at 28°C for 24 h were resuspended and adjusted to an OD$_{600}$ of 1.0 in sterile demineralized water for inoculation. Immature pear fruits were surface sterilized and pricked with a sterile needle (49). Wounds were inoculated with 5 × 10^6 CFU/ml, and fruits were incubated in a humidified chamber at 18°C for 14 days. Disease symptoms were visually recorded as detection of necrosis surrounding the infection site.

To analyze gene expression of *E. amylovora* during growth on pear fruits, immature fruits were cut in slices (approximately 0.5 cm), and five slices were inoculated with 100 μl of a bacterial suspension adjusted to an OD$_{600}$ of 1.0 in sterile demineralized water. The suspension was evenly distributed on the slice. After 12 h of incubation in a humidified chamber at room temperature, the upper layer of the surface was scratched from the five slices, resuspended in 25 ml of PBS, and centrifuged for 2 min at 4,000 rpm (45). The supernatant was transferred to 15 ml killing buffer and further processed as described above.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR). Cell cultures were grown in LB broth until the desired optical densities were reached. An aliquot containing 15 × 10^9 CFU (equivalent of 15 ml at an OD$_{600}$ of 1.0) was transferred to 15 ml killing buffer (48) and centrifuged for 20 min at 4,000 rpm. The supernatant was decanted and the pellet frozen at −80°C for further RNA extraction.

Total RNA was isolated using a GeneJET RNA purification kit (Thermo Scientific) following the manufacturer's instructions. The obtained RNA was treated with DNase (Ambion/Life Technologies) and subsequently checked for purity by gel electrophoresis and determination of the A_{260}/A_{280} and A_{260}/A_{230} ratios, using a Nanodrop ND-2000 spectrophotometer (Thermo Fischer Scientific). High-quality RNA was reverse transcribed and amplified with a OneStep RT-PCR kit according to the manufacturer's protocol (Qiagen). Template RNA (5 ng) was used in a standard 25-μl qRT-PCR mixture with specific primers (see Table S2 in the supplemental material). As a control, RNA samples without reverse transcriptase were included to detect possible DNA contamination.

For analysis, a Mastercycler ep realplex^2 gradient S machine (Eppendorf, Hamburg, Germany) was used. Cycling parameters included a 15-min initial denaturation at 95°C to activate the DNA polymerase followed by 40 cycles consisting of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The final step consisted of 1 min at 95°C and 30 s at 55°C. A melting curve analysis with a temperature ramp from 25°C to 95°C in 20 min was performed at the end of each run to determine the specificity of amplified qPCR products.

Each sample was analyzed for gene expression in triplicate. Quantification of mRNA transcripts was performed by the comparative threshold cycle (C_T) method. Briefly, the C_T values of the samples of interest were compared with that of a nontreated sample. All C_T values were normalized to the housekeeping gene *recA*, which shows constant expression at different ODs and with different medium compositions, as well as an amplification efficiency similar to that of the target gene (45, 50). The comparative C_T method was calculated as $2^{-(\Delta C T,\text{sample} - \Delta C T,\text{reference})}$, where the ΔC_T value was normalized to that for the endogenous housekeeping gene *recA*. Subsequently, fold changes between the samples were determined based on the calculated C_T method.

RESULTS

Bioinformatic analysis of MarA/Rob/Sox homologues in *E. amylovora*. Searches with the BLASTP program (NCBI), using the amino acid sequences of MarA, Rob, and SoxS from *E. coli* K-12 as queries, identified four homologous sequences in the genome of *E. amylovora* CFBP1430 (see Table S3 in the supplemental material). One homologue (EAMY_2956) shared 72% amino acid sequence identity with Rob from *E. coli*. A sequence alignment between Rob from *E. amylovora* and Rob from *E. coli* showed that the N-terminal, DNA-binding HTH domains share 77% identity with each other, whereas the C-terminal, effector-binding GyrI-like domains share only 59% identity. Analysis of the up- and downstream regions flanking the *rob* homologues from *E. amylovora* and *E. coli* using the Artemis comparison tool (91) revealed several differences. The upstream region of *rob* from *E. coli* contains the *creABCD* locus, including the two-component system *creBC*, involved in catabolic regulation (51). In *E. amylovora*, only *creA* is present upstream of *rob* (see Fig. S1). The downstream regions are highly similar. However, an additional small hypothetical protein gene is carried downstream of *rob* in *E. amylovora*.

The genome sequence of *E. amylovora* also contains genes for a homologue of the oxidative stress regulator system SoxRS from *E. coli*, which consists of the oxidative stress sensor SoxR, a member of the MerR family, and the AraC/XylS family member SoxS. SoxS from *E. amylovora* (EAMY_3287) shares 64% identity with SoxS from *E. coli*, and SoxR from *E. amylovora* (EAMY_3286) shares 72% identity with SoxR from *E. coli* (see Table S3 in the supplemental material). The up- and downstream regions flanking the *soxRS* locus in *E. amylovora* CFBP1430 and *E. coli* K-12 are similar (see Fig. S1). However, both regions of *E. amylovora* contain insertions. The *gstA* gene, encoding a glutathione *S*-transferase, is located upstream of *soxR* in *E. amylovora*. The downstream region of *soxS* from *E. amylovora* contains an insertion of about 5.8 kb, encoding several small hypothetical proteins, and *hecB*, encoding a member of the two-partner secretion family involved in protein export.

BLASTP searches identified genes for two additional members of the AraC/XylS family in the genome of *E. amylovora*, with the proteins sharing high levels of amino acid sequence identity with MarA, Rob, and SoxS from *E. coli* K-12. The proteins EAMY_1795 (annotated Rob1) and EAMY_3560 (annotated SoxS3) were found to share about 50% identity to MarA, Rob, and SoxS from *E. coli* (see Table S3 in the supplemental material). Based on our findings, we propose renaming EAMY_1795 "PliA" (plant-inducible activator) and renaming EAMY_3560 "OpiA" (osmotically and pH-inducible activator).

Phylogenetic analysis of all 13 AraC/XylS family members from *E. amylovora* showed that Rob, SoxS, PliA, and OpiA are closely related (92, 93) (see Fig. S2 in the supplemental material). The close relationship of the four proteins is based on the conserved HTH domain responsible for specific DNA binding (23, 92, 94, 95) (see Fig. S3).

Inactivation of the transcriptional regulators PliA, Rob, SoxS, and OpiA has no effect on antibiotic resistance of *E. amylovora*. Transcriptional regulators of the AraC/XylS family are well known for their role in coordinating the responses of enterobacteria to various chemical stresses and antimicrobial compounds. In order to investigate the impact of the transcriptional

regulators PliA, Rob, SoxS, and OpiA of *E. amylovora* Ea1189 on antibiotic resistance, the respective genes were disrupted by insertion of a chloramphenicol cassette. Antibiotic susceptibility tests against a variety of antimicrobial agents were performed. However, disruption of the transcriptional regulators resulted in no change in sensitivity to any tested compound (see Table S4 in the supplemental material).

Overexpression of transcriptional regulators increases resistance to fusidic acid, nalidixic acid, tetracycline, and novobiocin. Previous studies have shown that high-level expression of AraC/XylS regulators is required to induce antibiotic resistance in enteric bacteria (52–54). Therefore, we generated high-copy-number plasmids overexpressing the *pliA*, *rob*, *soxS*, and *opiA* genes from their native promoters. Levels of overexpression determined by qRT-PCR were 69-fold for *pliA*, 34-fold for *rob*, 48-fold for *soxS*, and 56-fold for *opiA* in cells grown in LB broth to an OD_{600} of 0.5.

The overexpression plasmids were mobilized into the *E. amylovora* Ea1189 wild type as well as into the *acrB*-deficient mutant Ea1189-3, which is hypersensitive to many drugs (8), and the sensitivities of the transformants to various substrates were determined (Table 2). Overexpression of *pliA* caused elevated resistance of the wild type to fusidic acid (4-fold), nalidixic acid (4-fold), and tetracycline (4-fold), whereas overexpression of *rob* increased the resistance of the wild type to fusidic acid (4-fold) and tetracycline (4-fold). The resistance profiles of transformants overexpressing *soxS* and *opiA* in the wild-type background were not altered. In the *acrB*-deficient mutant, overexpression of *rob* and *opiA* caused a 4-fold increase in resistance to novobiocin.

Organic solvent tolerance. Because overexpression of *soxS* or *robA* causes increased organic solvent tolerance in *E. coli* (53, 55), we were prompted to investigate solvent tolerance levels of *E. amylovora* mutants deficient in PliA, Rob, SoxS, and OpiA, as well as those of transformants overexpressing these regulators.

Our data show that *E. amylovora* Ea1189 is able to grow in the presence of *n*-hexane but not in the presence of cyclohexane. Interestingly, the *pliA*- and *rob*-deficient mutants showed decreased growth in the presence of *n*-hexane compared to the wild-type strain (Table 3), indicating a possible involvement of PliA and Rob in regulation of solvent detoxification mechanisms. In agreement with this observation, we found that overexpression of *pliA* and *rob* increased the resistance of *E. amylovora* Ea1189 to *n*-hexane. Furthermore, we demonstrated that overexpression of *pliA* and *soxS* increased tolerance toward cyclohexane.

It was previously reported that deletion of the multidrug efflux pump AcrAB from *E. coli* resulted in hypersusceptibility to both *n*-hexane and cyclohexane (21). Our data revealed that the *acrB*-deficient mutant of *E. amylovora* was able to survive on *n*-hexane but not cyclohexane (Table 3), indicating that resistance mechanisms other than efflux are involved in organic solvent tolerance in the plant pathogen *E. amylovora*.

Rob affects expression of the RND-type efflux pump AcrAB in *E. amylovora*. Since the expression of the multidrug efflux pump AcrAB is modulated by the AraC/XylS family members MarA, SoxS, and Rob in *E. coli* (13, 14), as well as by RamA in *S. enterica* (56), we investigated whether the expression of *acrAB* is regulated by the homologous regulators PliA, Rob, SoxS, and OpiA in *E. amylovora* Ea1189. Therefore, we used a transcriptional fusion between the promoter region of *acrAB* and the reporter gene *egfp*, as previously described (45). Our results showed a

2-fold induction of *acrAB* in cells harboring the *rob* overexpression plasmid. However, overexpression of *pliA*, *soxS*, or *opiA* did not affect the promoter activity of *acrAB* (Table 4).

In addition, we used qRT-PCR analysis to elucidate whether overexpression of the transcriptional activators PliA, Rob, SoxS, and OpiA causes induction of the AcrAB efflux pump in *E. amylovora*. Therefore, overexpression of the transcriptional regulators was achieved from high-copy-number plasmids carrying the genes under the control of the *lac* promoter (163-fold for *pliA*, 332-fold for *rob*, 287-fold for *soxS*, and 407-fold for *opiA*). Fold changes in mRNA transcript levels were determined for cells grown in LB broth to an OD_{600} of 0.5.

In agreement with the fluorescence data, we found a 2.3-fold induction of the *acrAB* efflux pump in cells overexpressing *rob*, while the overexpression of the other regulators did not affect the expression of *acrAB* in *E. amylovora* (Table 4).

In light of the above findings, we investigated whether Rob is able to bind to the *acrAB* promoter region under *in vitro* conditions. Therefore, an electrophoretic mobility shift assay using Cy5-labeled DNA fragments of the *acrAB* promoter region and His-tag-purified Rob protein was performed. However, no interaction between Rob and the *acrAB* promoter region was detected (data not shown), indicating that the observed induction of *acrAB* is not directly mediated by Rob in *E. amylovora* Ea1189.

Transcriptional analysis of *pliA*, *rob*, *soxS*, and *opiA* in *acrB*- and *tolC*-deficient mutants. In *E. coli*, the mar/sox/rob regulon includes the major multidrug efflux pump AcrAB and the outer membrane channel TolC. It has been found that transcription of *marA*, *soxS*, and *rob* is increased in *tolC* mutants (57), and this prompted us to investigate whether the expression of the regulators *pliA*, *rob*, *soxS*, and *opiA* is also influenced in an *acrB* or *tolC* mutant of *E. amylovora* Ea1189. However, our analysis revealed that the expression of *pliA*, *rob*, *soxS*, and *opiA* was not significantly changed in mutants defective in AcrB or TolC (data not shown).

Induction of *opiA* by osmotic and pH stress. We analyzed the involvement of the AraC/XylS regulators in response to pH and osmotic stress. First, we investigated the effects of pH and salt concentration on the growth of *E. amylovora* Ea1189. We found that the growth of Ea1189 was significantly reduced at pH 5.5 and 8.5. The cultures grown in LB broth adjusted to these pH values reached an OD_{600} of about 1.0 after 24 h, whereas cultures grown at pH 7.0 reached an OD_{600} of 2.6 (see Fig. S4A in the supplemental material). A similar growth reduction was found in cultures of Ea1189 supplemented with 300 mM NaCl (see Fig. S4B).

The growth of the *pliA*, *rob*, *soxS*, and *opiA* mutants was not altered compared to the growth of the wild type in response to pH changes or increasing salt concentrations (Fig. 1A). However, overexpression of *opiA* increased growth of *E. amylovora* >2-fold at alkaline pH and a high salt concentration (300 mM) (Fig. 1B).

pliA and soxS are induced by phenolic acids and ROS. We conducted qRT-PCR analysis to explore whether the expression of *pliA*, *rob*, *soxS*, or *opiA* is induced by phytochemicals, phenolic acids, ROS producers, or heavy metals (Table 5). Expression of *pliA* was increased upon exposure to a wide variety of signals. Transcript levels of *pliA* were increased in response to apple leaf extracts (4.4-fold), the phenolic acids gallic acid (6.2-fold) and salicylic acid (3.7-fold), the ROS inducers indole (10-fold), paraquat (3.9-fold), plumbagin (67.8-fold), and phenazine methosulfate (29.9-fold), the fatty acid sodium decanoate (109.6-fold), and

TABLE 2 Antimicrobial susceptibility profiles of *E. amylovora* Ea1189 and the *acrB*-deficient mutant Ea1189-3, harboring the plasmids pBlueScript II SK, pBlueSK.pliA, pBlueSK.rob, pBlueSK.soxS, and pBlueSK.opiA[a]

Drug or compound	MIC (μg/ml)[b]									
	Ea1189					Ea1189-3				
	pBlueScript II SK	pBlueSK.pliA	pBlueSK.rob	pBlueSK.soxS	pBlueSK.opiA	pBlueScript II SK	pBlueSK.pliA	pBlueSK.rob	pBlueSK.soxS	pBlueSK.opiA
Flavones										
Daidzein	>5,000	>5,000	>5,000	>5,000	125	125	125	125	125	125
Genistein	>1,000	>1,000	>1,000	>1,000	62.5	62.5	62.5	62.5	62.5	62.5
Naringenin	1,000	1,000	1,000	1,000	250	250	250	250	250	250
Phloretin	5,000	5,000	5,000	5,000	312	312	312	312	312	312
Antibiotics										
Amikacin	6.25	6.25	6.25	6.25	6.25	6.25	12.5	12.5	6.25	6.25
Azithromycin	0.63	0.63	0.63	0.63	0.63	0.31	0.31	0.31	0.31	0.31
Cefepime	12.5	12.5	12.5	12.5	12.5	5	10	10	5	5
Chloramphenicol	3.13	6.25	6.25	3.13	3.13	1.56	1.56	1.56	1.56	1.56
Ciprofloxacin	0.03	0.06	0.03	0.03	0.03	0.006	0.006	0.006	0.006	0.006
Erythromycin	1.25	1.25	1.25	1.25	1.25	1.25	1.25	2.50	1.25	1.25
Fusidic acid	250	**1,000**	**1,000**	250	250	3.13	6.25	3.13	3.13	3.13
Nalidixic acid	2.5	**10**	2.5	2.5	2.5	0.63	0.63	0.63	0.63	0.63
Norfloxacin	0.16	0.16	0.16	0.16	0.16	0.02	0.02	0.02	0.02	0.02
Novobiocin	250	500	250	250	250	6.25	**25**	6.25	**25**	6.25
Tetracycline	1.25	**5**	**5**	1.25	1.25	0.63	0.63	0.63	0.63	0.63
Heavy metals										
Cadmium acetate	50	50	100	50	50	100	100	100	100	100
Copper sulfate	1,250	1,250	1,250	1,250	1,250	1,250	1,250	1,250	1,250	1,250
Silver nitrate	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Zinc sulfate	500	500	500	500	500	500	500	500	500	500
Antimicrobials										
Bile	5,000	5,000	5,000	5,000	5,000	2,500	2,500	2,500	2,500	2,500
Phenazine	50	50	50	50	50	12.5	12.5	12.5	12.5	12.5
Plumbagin	50	100	100	100	100	12.5	12.5	12.5	12.5	12.5
Tannin	2,500	2,500	2,500	2,500	2,500	1,250	1,250	1,250	1,250	1,250
Dyes										
Acriflavine	125	125	125	125	125	12.5	12.5	12.5	12.5	12.5
Crystal violet	12.5	12.5	12.5	12.5	12.5	1.25	1.25	1.25	1.25	1.25
Ethidium bromide	500	500	500	500	500	6.25	6.25	6.25	6.25	6.25

[a] The *pliA*, *rob*, *soxS*, and *opiA* genes, including the respective promoter regions, were cloned into pBlueScript II SK (+) in the opposite orientation with respect to the *lac* promoter to obtain expression from the native promoters.

[b] MICs were determined by 2-fold dilution assay in three or more independent experiments, with similar results. Numbers in bold indicate increases of >2-fold.

TABLE 3 Organic solvent tolerance of *E. amylovora* Ea1189 and *pliA*-, *rob*-, *soxS*-, and *opiA*-deficient mutants, as well as of the wild-type strain and the *acrB*-deficient mutant Ea1189-3 harboring the plasmids pBlueScript II SK, pBlueSK.pliA, pBlueSK.rob, pBlueSK.soxS, and pBlueSK.opiA[a]

| Strain | Mutation or plasmid | Organic solvent tolerance[b] | |
		n-Hexane	Cyclohexane
Ea1189	Wild type	+	−
	pliA	−	−
	rob	+/−	−
	soxS	+	−
	opiA	+	−
	pBlueScript II SK	+	−
	pBlueSK.pliA	++	+
	pBlueSK.rob	++	−
	pBlueSK.soxS	+	+
	pBlueSK.opiA	+	−
Ea1189-3	pBlueScript II SK	+	−
	pBlueSK.pliA	+	−
	pBlueSK.rob	++	−
	pBlueSK.soxS	+	−
	pBlueSK.opiA	+	−

[a] The *pliA*, *rob*, *soxS*, and *opiA* genes, including the respective promoter regions, were cloned into pBlueScript II SK(+) in the opposite orientation with respect to the *lac* promoter to obtain expression from the native promoters.
[b] ++, growth; +, single colonies; −, no growth. Data presented are observations from at least three replicates.

TABLE 4 Effects of overexpression of the transcriptional regulators PliA, Rob, SoxS, and OpiA on expression of the multidrug efflux pump AcrAB[a]

Plasmid	Fold change in *acrAB* promoter activity[b]	Fold change in mRNA transcript level[c]
pBlueKS.pliA	1	1.1
pBlueKS.rob	2	2.3
pBlueKS.sox	0.9	1
pBlueKS.opiA	0.9	1

[a] The *pliA*, *rob*, *soxS*, and *opiA* genes were cloned into pBlueScript II KS(+) to obtain expression from the *lac* promoter.
[b] Fold changes in the *acrAB* promoter activity were determined by measuring the fluorescence of Ea1189 cells cotransformed with regulator overexpression plasmids and pBBR.acrA-Pro.egfp, harboring a transcriptional fusion of the *acrAB* promoter to a promoterless *egfp* gene. Cells were grown in LB until stationary phase.
[c] Total RNA was isolated from cells grown in LB broth to an OD_{600} of 0.5. Transcript abundances in cells overexpressing the regulators were determined by quantitative RT-PCR and compared to the RT-PCR signal from cells transformed with an empty expression plasmid.

the heavy metals copper (2.7-fold) and zinc (2.6-fold). Expression of *soxS* was induced by gallic acid (3.3-fold) and after treatment with the ROS producers indole (8.8-fold), paraquat (192.2-fold), plumbagin (16.2-fold), and phenazine methosulfate (18-fold). Expression of *rob* and *opiA* was not induced by any of the tested compounds (Table 5). It is worth mentioning that expression of *rob* was downregulated in response to most of the tested compounds. Additionally, a >3-fold downregulation of *soxS* and *opiA* was detected upon exposure of *E. amylovora* Ea1189 to zinc sulfate for 10 min.

Paraquat inhibits growth of a *soxS*-deficient mutant. Members of the AraC/XylS family have been found to coordinate the induction of genes for resistance to oxidative stress (e.g., SoxS of *E. coli*). To investigate a possible involvement of PliA, Rob, SoxS, and OpiA in oxidative stress resistance, we tested the respective mutants for growth on agar plates supplemented with increasing concentrations of the superoxide-generating agent paraquat. Our results showed that the *soxS*-deficient mutant was not able to grow on MHB II agar plates containing 10 μg/ml paraquat (see Fig. S5 in the supplemental material), indicating that SoxS is involved in superoxide resistance.

Deletion of Rob increases intracellular ROS levels. To elucidate the effect of disruption or overexpression of the transcriptional regulator genes *pliA*, *rob*, *soxS*, and *opiA* on the intracellular redox status of *E. amylovora*, we determined intracellular ROS levels by measuring the oxidation of the nonfluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate to its highly fluorescent derivative 2′,7′-dichlorofluorescein. Our data revealed that cells of the *rob* mutant possessed an approximately 2-fold higher intracellular ROS level than that in cells of the wild type (Fig. 2).

Transcriptional analysis of *pliA*, *rob*, *soxS*, and *opiA in planta*. In order to analyze the expression of *pliA*, *rob*, *soxS*, and

opiA transcripts *in planta*, *E. amylovora* was inoculated onto immature pear fruit slices or injected into shoot tips of apple rootstock MM106. Bacteria were reisolated from immature pear fruit slices after 12 h or from apple plants after 1 and 7 days (Table 6). Transcript abundances were determined by qRT-PCR and compared to qRT-PCR signals from cells grown in LB broth to an OD_{600} of 0.5. Analysis of fold changes in mRNA transcript levels showed 15.3-, 5.5-, and 5.0-fold increases in expression of *pliA*, *soxS*, and *opiA*, respectively, on immature pear fruits. Expression of *rob* was similar in cells grown in LB broth and cells recovered from infected pear fruits (Table 6).

Furthermore, our results demonstrated that expression of *pliA* was 97.3-fold, expression of *soxS* 11.5-fold, and expression of *opiA* 2.5-fold higher during infection of apple rootstock 1 day after inoculation than during growth in LB medium. The expression of the three genes declined to basal levels after 7 days of infection. Like the case for pear fruit infection, expression of *rob* was not induced during infection of apple rootstock (Table 6).

Contributions of PliA, Rob, SoxS, and OpiA to virulence of *E. amylovora* on apple rootstock and immature pear fruits. The development of disease symptoms on apple rootstock MM106 was analyzed to study the impact of the transcriptional regulators PliA, Rob, SoxS, and OpiA on virulence of *E. amylovora* Ea1189. After 7 days, all shoots showed typical disease symptoms, including shepherd's crook-like bending, tissue necrosis, and ooze formation, regardless of whether the shoots were infected with the wild type or with one of the regulatory mutants. In order to study the establishment of bacterial populations within the tissue, samples were taken at 1, 3, and 7 days postinoculation, and numbers of CFU per stem were determined. The wild type and all regulatory mutants showed no significant differences in growth 3 days after infection. However, the population size of the *soxS* mutant was approximately 10 times lower than the population size of the wild type after 7 days (Fig. 3; see Table S5 in the supplemental material). This result indicates that *soxS* contributes to the ability of *E. amylovora* to colonize apple rootstock.

Furthermore, since *E. amylovora* is also able to infect pears, immature pear fruits were used to study the impact of the transcriptional regulator mutants on virulence in this host plant. Fruits were infected with the wild-type strain and the corresponding regulatory mutants and incubated at 18°C for 14 days. How-
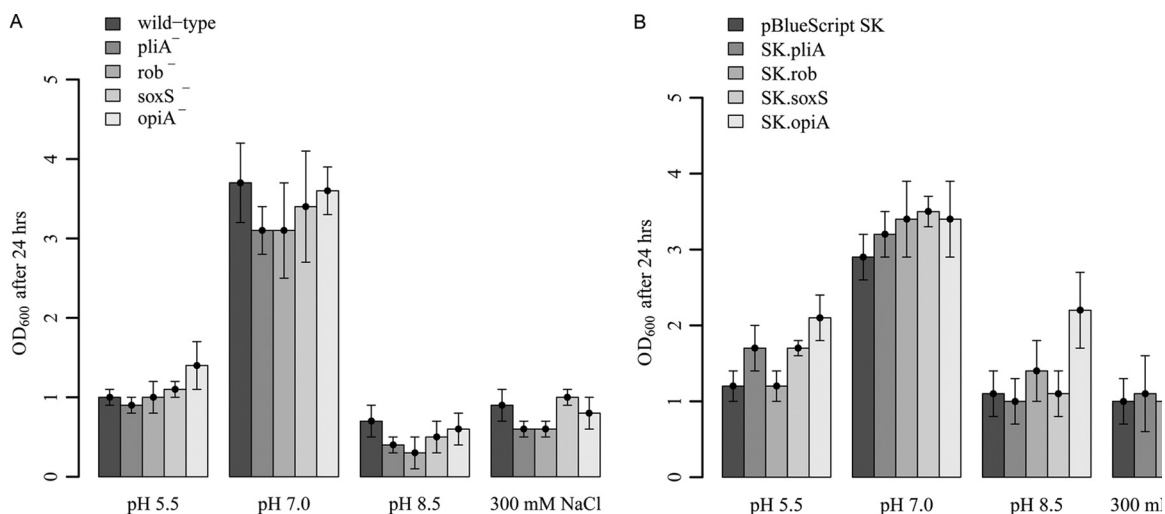
FIG 1 pH and osmolarity sensitivity assays. (A) *E. amylovora* Ea1189 wild type and *pliA*-, *rob*-, *soxS*-, and *opiA*-deficient mutants. (B) *E. amylovora* Ea1189 harboring overexpression plasmids. Tests were performed at pH 5.5, 7.0, and 8.5 and with 300 mM NaCl (stress concentrations were determined prior to assays [see Fig. S4 in the supplemental material]). Optical densities were determined after 24 h of growth in AMM2 medium.

ever, no significant difference between the wild type and the mutants was observed (see Fig. S6 in the supplemental material).

DISCUSSION

Multidrug efflux systems, such as the AcrAB-TolC complex, play an important role in the intrinsic resistance of bacteria against

TABLE 5 Fold changes of *pliA*, *rob*, *soxS*, and *opiA* mRNA transcript levels in *E. amylovora* Ea1189 after 2 h of incubation (or 10 min, where indicated) with various substrates, as determined by qRT-PCR[a]

Substrate (concn)	Fold change in mRNA transcript level[b]			
	pliA	*rob*	*soxS*	*opiA*
Phytochemicals				
Apple leaf extracts (10 μl/ml)	**4.4**	0.7	1.7	1.3
Indole-3-acetic acid (2 mM)	1.4	0.7	1.6	1.2
Phloretin (4 μg/ml)	1.3	0.6	1.4	0.5
Sodium decanoate (5 mM)	**109.6**	0.7	1.5	0.5
Tannin (0.5 mg/ml)	1.0	**0.4**	1.2	0.5
Phenolic acids				
Gallic acid (1 mg/ml)	**6.2**	0.7	**3.3**	1.4
Salicylic acid (5 mM)	**3.7**	0.5	1.0	0.9
ROS producers				
Indole (2 mM)	**10.0**	0.8	**8.8**	1.1
Paraquat (0.2 mM)	**3.9**	0.7	**192.2**	0.7
Plumbagin (0.1 mM)	**67.8**	**0.4**	**16.2**	1.0
Phenazine methosulfate (0.1 mM)	**29.9**	**0.2**	**18.0**	0.6
Heavy metals				
Copper sulfate (1 mM)	1.3	0.6	1.3	0.6
Copper sulfate (1 mM, 10 min)	**2.7**	1.2	1.5	1.8
Zinc sulfate (1 mM)	1.4	0.7	1.5	0.5
Zinc sulfate (1 mM, 10 min)	**2.6**	0.9	**0.2**	**0.3**

[a] Total RNA was isolated from bacterial cells incubated with the indicated substrates in LB broth. Transcript abundances were determined by quantitative RT-PCR and compared to the RT-PCR signal from cells grown in LB broth containing only the solvent of the respective substrate.
[b] Values in bold indicate an increase or decrease of >2-fold. The presented data values are the means of results from at least three replicates.

antimicrobial compounds. The regulation of the *acrAB* genes has been studied extensively in human-pathogenic enterobacteria. Several members of the AraC/XylS family, including MarA, RamA, Rob, and SoxS, have been identified as positive transcriptional regulators of this efflux system (10, 13, 14, 21, 54, 58).

In this study, we identified four regulators from the plant pathogen *E. amylovora* that are closely related to the MarA/Rob/SoxS group of AraC/XylS transcriptional activators from *E. coli* (see Fig. S3 in the supplemental material). Due to the high level of sequence similarity, it was tempting to speculate that the four AraC/XylS regulators from *E. amylovora* might have a similar function in the regulation of the multidrug efflux pump AcrAB.
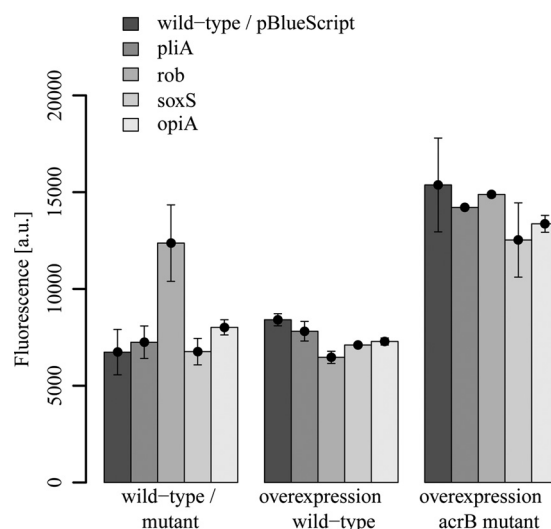

FIG 2 Intracellular ROS levels in the *E. amylovora* Ea1189 wild type and its *pliA*-, *rob*-, *soxS*-, and *opiA*-deficient mutants and in strains harboring overexpression plasmids in wild-type and *acrB*-deficient backgrounds. Cells were grown to an OD_{600} of 0.5 and incubated with DCF diacetate. Whole-cell fluorescence was determined after 30 min. Fluorescence intensity (absorbance units [a.u.]) was normalized to an OD_{600} value of 0.1. Experiments were performed in triplicate, with similar results.

TABLE 6 Fold changes of *pliA*, *rob*, *soxS*, and *opiA* mRNA transcript levels after inoculation of *E. amylovora* Ea1189 on apple rootstock MM106 and immature pear fruit slices[a]

| | Fold change in mRNA transcript level | | |
| | Apple rootstock[b] | | Immature pear[c] |
Gene	1 dpi	7 dpi	12 hpi
pliA	97.3	1.9	15.3
rob	1.4	0.9	1.3
soxS	11.5	1.1	5.5
opiA	2.5	0.5	5.0

[a] Total RNAs were isolated from bacterial cells recovered from infected plant tissues. Transcript abundances were determined by quantitative RT-PCR and compared to RT-PCR signals from cells grown in LB broth to an OD_{600} of 0.5.
[b] Bacteria were reisolated from infected shoots of apple rootstock at 1 and 7 days postinoculation (dpi).
[c] Bacteria were reisolated from infected immature pears at 12 h postinoculation (hpi).

However, a key finding of this work was that none of the identified regulators is able to induce a multidrug resistance phenotype in *E. amylovora*. *E. amylovora* strains overexpressing the regulatory proteins from a high-copy-number vector showed only minor changes in the substrate resistance profiles compared to the wild type (Table 2). Overexpression of *rob* led to an about 2-fold increase in expression of the *acrA* gene. However, the strain overexpressing *rob* showed increased resistance to only a limited number of antibiotics, not to a wide range of antimicrobials as usually associated with induction of the AcrAB-TolC multidrug efflux pump.

Overexpression of *rob* and of *pliA* increased the resistance of *E. amylovora* Ea1189 to fusidic acid and tetracycline and, in the case of *pliA*, also toward nalidixic acid. The overlap in the resistance patterns of the strains overexpressing Rob and PliA suggests that these regulators induce similar drug resistance mechanisms. However, overexpression of *rob* and of *pliA* in the *acrB*-deficient mutant Ea1189-3 did not increase the resistance to fusidic acid, tetracycline, and nalidixic acid, suggesting that the AcrAB pump is involved in the resistance mechanism promoted by Rob and PliA.

Rob (right origin-binding protein). There is plenty of evidence that Rob is involved in the regulation of genes conferring a multidrug resistance phenotype in various species of the *Enterobacteriaceae* family, and the reduced susceptibility to antibiotics has often been linked to the AcrAB-TolC efflux system (21, 59, 60). We also observed a 2-fold increase in expression of *acrA* upon overexpression of Rob from a high-copy-number vector in *E. amylovora* (Table 4). However, the increased expression of the *acrA* gene was not associated with a multidrug resistance phenotype. A similar effect was found in clinical isolates of *Klebsiella pneumoniae* when the mean expression levels of *acrA* were correlated with the MIC values for the antibiotic tigecycline. The *acrA* expression levels differed over the range of MICs for tigecycline (61).

In addition to the role of AcrA as a periplasmic fusion protein in the AcrAB-TolC system, it was shown that AcrA is required for the function of the AcrD efflux pump (62, 63). Thus, the Rob-mediated 2-fold increase in expression of *acrA* may lead to a higher level of activity of the AcrD pump, which could partially explain the observed resistance phenotype. We recently reported that AcrD of *E. amylovora* contributes to resistance against fusidic

acid, for which the Rob-overexpressing strain had a 4-fold higher MIC value (45). Another possible explanation for why the increased expression of *acrA* did not lead to an elevated resistance of *E. amylovora* toward multiple antibiotics may be that the constitutive level of AcrAB is so high that additional expression shows no effect.

Organic solvent tolerance has not been investigated in *E. amylovora*. Therefore, we assessed the sensitivity of the plant pathogen to *n*-hexane and cyclohexane and investigated the role of the AraC/XylS regulators in organic solvent tolerance. Disruption of *rob* caused increased susceptibility to *n*-hexane, whereas its overexpression resulted in an increased tolerance toward *n*-hexane (Table 3). Similar phenotypes have been described for *E. coli* (21). However, overexpression of *rob* also increased tolerance of *E. coli* to cyclohexane, a phenotype which was not observed in *E. amylovora* (21, 53). Furthermore, organic solvent tolerance of *E. coli* requires a functional AcrAB pump. Deletion of *acrAB* from the *E. coli* wild type resulted in a loss of tolerance to *n*-hexane, and it abolished the induced resistance to cyclohexane in *E. coli* strains overexpressing *rob*, *marA*, and *soxS* (21). In contrast, overexpression of *rob* in an *acrB*-deficient mutant of *E. amylovora* resulted in an increased *n*-hexane tolerance. Although organic solvent tolerance mediated by AraC/XylS transcriptional regulators has been associated with the expression of the RND-type efflux pump AcrAB in *E. coli* and *S. enterica* (21, 64), our data suggest that Rob of *E. amylovora* induces mechanisms other than efflux to overcome organic solvent toxicity. Bacteria use a wide variety of mechanisms to increase their organic solvent tolerance (65). Bacteria prevent entry of organic solvents into the cell by modification of lipopolysaccharides (66). To compensate for the damaging membrane fluidity changes imposed by solvents, bacteria allow a denser packing of their membranes by *cis/trans*-isomerization of unsaturated fatty acids (67). Moreover, activation of general stress responses, including the induction of chaperones that refold proteins denatured by the solvent, and activation of the oxidative stress response have been reported to contribute to bacterial solvent resistance (55, 68). However, the nature of the mechanism(s) of organic solvent tolerance induced by Rob in *E. amylovora* remains to be identified in future studies.
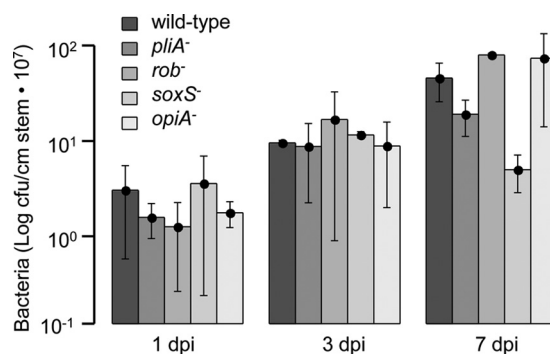

FIG 3 Virulence assay on apple rootstock MM106. Bacteria were inoculated into the shoot tips by the prick technique, with an inoculum of 5×10^6 CFU/ shoot. Establishment of populations of the *E. amylovora* Ea1189 wild type and its *pliA*-, *rob*-, *soxS*-, and *opiA*-deficient mutants was determined at 1, 3, and 7 days postinoculation (dpi). A minimum of five shoot tips were inoculated for each strain. Data represent the numbers of CFU/cm of stem/shoot tip times 10^7. Average values and standard deviations are listed in Table S5 in the supplemental material.

Our data revealed a general downregulation of *rob* transcription after addition of various compounds to the growth medium (Table 5). It is known that the transcriptional regulators MarA, SoxS, and Rob regulate each other's expression in *E. coli*. Both MarA and SoxS were shown to repress the *rob* promoter (58, 69, 70). Thus, the downregulation of *rob* might indicate a possible transcriptional cross talk between the homologous regulators of the AraC/XylS family to coordinate the cellular responses to environmental stresses.

SoxS (superoxide stress protein). Expression of the AraC/XylS family member SoxS has been described to be activated in response to oxidative stress in *E. coli* and other enterobacteria (15). In the present study, we demonstrated that SoxS from *E. amylovora* Ea1189 is also involved in superoxide resistance. A *soxS*-deficient mutant of Ea1189 was not able to grow on agar plates supplemented with the superoxide-generating agent paraquat (see Fig. S5 in the supplemental material). Furthermore, expression of *soxS* was induced by treatment of the cultures with redox cycling agents, such as phenazine methosulfate, paraquat, and plumbagin. Additionally, *soxS* expression was increased in the presence of indole, a biological oxidant. Indole is proposed to dissolve in membrane lipids, enabling direct interaction between the redox cycling agents isoprenoid quinones and dioxygen and resulting in the generation of superoxide (71). In the SoxRS regulatory cascade, SoxR, a regulator of the MerR family, senses oxidative stress via the oxidation state of its [2Fe-2S] cluster (72). Upon oxidation, SoxR becomes an activator of *soxS* transcription, and the resulting increased level of SoxS activates a variety of cellular defense mechanisms against oxidative stress (73).

Our data demonstrated that the expression of *soxS* was significantly downregulated after exposure of *E. amylovora* to zinc (Table 5), a finding that has been reported before for *E. coli*, by Graham et al. (74). Zn(II) is an essential micronutrient for bacteria, although it has significant toxicity at high concentrations (75). Previous results showed that SoxS is a major regulator responsible for growth under Zn-depleted conditions. SoxS increases the expression of the zinc uptake system ZnuACB, and deletion of *soxS* led to reduced growth of *E. coli* in Zn-depleted medium (76). We observed a downregulation of *soxS* in *E. amylovora* after exposure to 1 mM zinc sulfate, suggesting that the cells of the plant pathogen avoid zinc toxicity by reduction of its import via the ABC transporter ZnuACB. Recently, McDevitt et al. (77) identified a molecular mechanism linking the bacterial susceptibility to zinc with oxidative stress. Zinc interferes with acquisition of manganese by bacterial cells. Mn(II) plays an important role in oxidative stress management, e.g., as a cofactor of the manganese superoxide dismutase or as a substitute for ferrous iron in the enzyme ribulose-5-phosphate 3-epimerase under oxidative stress (78, 79).

The oxidative burst, a rapid production of ROS, is one of the earliest responses of plants to microbial infection (80). The predominant ROS detected in plant-pathogen interactions are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) (81). The oxidative burst is usually correlated with incompatible plant-pathogen interactions leading to a rapid and localized programmed cell death known as the hypersensitive response (82). However, it was previously reported that *E. amylovora* is able to generate an oxidative stress response even in a compatible situation with pear (83). It is suggested that *E. amylovora* uses the production of ROS as a tool to provoke host cell death during pathogenesis to invade plant tissues.

Our results showed that the promoter activity of *soxS* was induced during growth of *E. amylovora* in apple rootstock and immature pear fruits (Table 6). We identified gallic acid, a phenolic compound found in a wide variety of plants, including apple and pear (84, 85), as a plant-derived signal which increased *soxS* expression 3.3-fold. Furthermore, we found that the *soxS*-deficient mutant of *E. amylovora* reached an ~10-fold-lower population density than that of the wild type on apple rootstock 7 days after infection (Fig. 3). Our results show that SoxS can be induced in response to phenolic acids and the oxidative stress generated by the host during pathogenic interaction and that it is required for successful colonization of a host plant.

PliA (plant-inducible activator protein). In this work, we characterized a novel regulator of the AraC/XylS family, PliA, in *E. amylovora* Ea1189. The amino acid sequence of PliA shares about 50% identity to MarA, Rob, and SoxS from *E. coli* as well as to Rob, SoxS, and OpiA from *E. amylovora* (see Table S3 in the supplemental material). We demonstrated that plasmid-mediated overexpression of *pliA* produced resistance to fusidic acid, nalidixic acid, and tetracycline, as well as increased tolerance to *n*-hexane and cyclohexane, in *E. amylovora*. The resistance phenotypes required the presence of the functional RND-type efflux pump AcrAB. However, we did not observe an increased expression of *acrA* upon overexpression of PliA, suggesting that resistance mechanisms other than efflux are induced by the transcriptional activator PliA.

The most striking finding was the high level of induction of *pliA* expression during the early infection phase in apple rootstock and immature pear fruits (Table 6). Transcription of *pliA* was induced almost 100-fold 1 day after inoculation of *E. amylovora* Ea1189 into apple shoot tips. On day 7, the expression level came back to the low expression level of *pliA* in *E. amylovora* cells grown in LB broth.

We identified gallic acid and salicylic acid, as well as ROS, as inducers of *pliA* expression *in vitro* that may also be responsible for the increased *pliA* expression *in planta*. Both gallic and salicylic acids are hydroxybenzoic acids and, as major plant phenolics, play an important role in plant-pathogen interactions (86). The rapid production of ROS is described as one of the earliest responses to pathogen infection (80). Our results strongly suggest that PliA is involved in the immediate response of *E. amylovora* to contact with its host and that plant phenolics or ROS induce stress responses in *E. amylovora* via PliA during the early plant infection phase.

Despite the fact that we observed a significant induction of *pliA* expression *in planta*, deletion of the *pliA* gene had no effect on the ability of *E. amylovora* to colonize its host plants, apple and pear. This inconsistency can be explained by the fact that MarA-like regulators often show redundancy among their regulons. We observed, for instance, that oxidative stress induced SoxS as well as PliA in *E. amylovora*. Casaz et al. (87) investigated the functions of MarA, SoxS, and Rob from *E. coli* as virulence factors in a murine model of urinary tract infection. Only an *E. coli* mutant lacking all three transcriptional regulators was significantly less virulent than the parental strain. Furthermore, complementation of the triple-knockout mutant with *marA*, *soxS*, and *rob* individually restored wild-type virulence.

Multiple compounds were able to induce the expression of *pliA*, including plant extracts, phenolic compounds, redox cycling agents, heavy metals, and the saturated fatty acid decanoate. How-

ever, PliA is a small protein, consisting of 113 amino acids, that contains only a DNA-binding domain. For this protein to exert its regulatory action, the intracellular concentration of PliA must increase. The transcription of other AraC/XylS regulators (e.g., MarA and SoxS) that contain only a DNA-binding motif is controlled by another regulator (17). We identified a gene for a transcriptional regulator of the Rrf2 repressor family upstream of the *pliA* gene in *E. amylovora*. One of the best-studied Rrf2 proteins, IscR, represses the *iscRSUA* operon, encoding Fe-S cluster biosynthesis proteins (88). Several regulators of the widespread but poorly characterized Rrf2 family coordinate an Fe-S cluster that participates in signal transduction (89). The oxidation state of the Fe-S cluster may modulate the function of these regulators, as found with the [2Fe-2S]-containing transcription factor SoxR (72). Future work will examine the role of the Rrf2-type regulator located upstream of *pliA* in the induction of the AraC/XylS regulator by different environmental signals.

Furthermore, our induction experiments point to a possible transcriptional cross talk between Rob and PliA. The expression of *pliA* was induced >100-fold by decanoate. Sodium decanoate was originally found to posttranslationally activate Rob in *E. coli* (14). Whether the observed induction of *pliA* expression by decanoate is due to binding and activation of Rob in *E. amylovora* will be a subject of our future studies.

OpiA (osmotically and pH-inducible activator). OpiA, the fourth MarA-like transcriptional regulator of the AraC/XylS family from *E. amylovora*, did not show significant sequence similarity to characterized members of this family. The first experiments to characterize this protein did not yield any obvious phenotype, except for a downregulation of *opiA* transcripts upon exposure to zinc. However, we made an interesting observation during the generation of the *opiA* mutant. We isolated *opiA*-deficient mutants only on LB agar without salt. No mutants were found on high-salt LB agar containing 10 g/liter sodium chloride. This finding suggested that OpiA might play a role in the regulation of osmotic stress responses. In order to prove this hypothesis, we tested the *opiA* mutant for growth at various pHs and salt concentrations. However, we were not able to detect a significant difference in the growth of the mutant and the wild type. Next, we investigated whether the overexpression of *opiA* might increase the tolerance to pH or osmotic stress, and indeed, we found that *opiA* overexpression increased tolerance to alkaline pH and high salt concentrations (Fig. 1). Therefore, we concluded that OpiA is involved in the adaptation of *E. amylovora* to alkaline pH and high salinity. Further experiments need to be conducted to identify which genes are regulated by OpiA in *E. amylovora*.

ACKNOWLEDGMENTS

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