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Insights into the Extracytoplasmic Stress Response of *Xanthomonas campestris* pv. *campestris*: Role and Regulation of σ^E-Dependent Activity▽‡

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***Xanthomonas campestris* pv. *campestris* is an epiphytic bacterium that can become a vascular pathogen responsible for black rot disease of crucifers. To adapt gene expression in response to ever-changing habitats, phytopathogenic bacteria have evolved signal transduction regulatory pathways, such as extracytoplasmic function (ECF) σ factors. The alternative sigma factor σ^E, encoded by *rpoE*, is crucial for envelope stress response and plays a role in the pathogenicity of many bacterial species. Here, we combine different approaches to investigate the role and mechanism of σ^E-dependent activation in *X. campestris* pv. *campestris*. We show that the *rpoE* gene is organized as a single transcription unit with the anti-σ gene *rseA* and the protease gene *mucD* and that *rpoE* transcription is autoregulated. *rseA* and *mucD* transcription is also controlled by a highly conserved σ^E-dependent promoter within the σ^E gene sequence. The σ^E-mediated stress response is required for stationary-phase survival, resistance to cadmium, and adaptation to membrane-perturbing stresses (elevated temperature and ethanol). Using microarray technology, we started to define the σ^E regulon of *X. campestris* pv. *campestris*. These genes encode proteins belonging to different classes, including periplasmic or membrane proteins, biosynthetic enzymes, classical heat shock proteins, and the heat stress σ factor σ^H. The consensus sequence for the predicted σ^E-regulated promoter elements is $GGAACTN_{15\text{-}17}GTCNNA$. Determination of the *rpoH* transcription start site revealed that *rpoH* was directly regulated by σ^E under both normal and heat stress conditions. Finally, σ^E activity is regulated by the putative regulated intramembrane proteolysis (RIP) proteases RseP and DegS, as previously described in many other bacteria. However, our data suggest that RseP and DegS are not only dedicated to RseA cleavage and that the proteolytic cascade of RseA could involve other proteases.**

Bacteria often encounter diverse and rapidly changing environments. To overcome harmful situations, they must be capable of sensing external changes and transmitting this information across biological membranes into the cell, which results in the appropriate redirection of gene expression to prevent or repair cellular damages caused by stress. Extracytoplasmic function (ECF) σ factors provide one common means of bacterial signal transduction to regulate gene expression in response to various extracellular changes (65). ECF σ factors represent the largest and most diverse subfamily of σ^{70} proteins. They generally recognize a −35 box with a clear bias toward a GAAC in their target promoters, while the −10 region tends to be highly variable between ECF subfamily members (65). One of the best-studied ECF σ factors is the key regulator of the extracytoplasmic stress response factor σ^E from *Escherichia coli*, encoded by the *rpoE* gene (56). ECF proteins were recently divided into 43 major phylogenetically distinct groups named ECF01 to ECF43 (65). RpoE-like ECF σ factors are part of one predominant subgroup found in most bacterial phyla and comprise ECF01 to -04 proteins. RpoE-like ECF σ factors are autoregulated and are required for a wide range of functions. For instance, the *E. coli* σ^E factor is essential for growth and promotes the expression of factors that help to preserve and/or restore cell envelope integrity (2). *Salmonella enterica* serovar Typhimurium σ^E is required for protection against reactive oxygen species and antimicrobial peptides and for stationary-phase survival (20, 67). *Bacillus subtilis* σ^W seems to constitute an antibiosis regulon acting against cell envelope stress (34). *S.* Typhimurium σ^E, *Pseudomonas aeruginosa* AlgU, and *Vibrio cholerae* σ^E are required for virulence (5). ECFs can thus be considered models to understand how bacteria sense and respond to their environment both during their interaction with their host and in their free-living state.

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RpoE-like ECF σ factors are tightly regulated in order to coordinate their activation with the appropriate environmental cues. In most cases, the σ^E factor is cotranscribed with a cognate transmembrane anti-σ factor possessing an extracytoplasmic domain and an intracellular σ-binding domain. In the absence of stimulus, the membrane-bound anti-σ binds tightly to the σ factor, thereby keeping it inactive (33). Upon receiving a proper signal, the anti-σ factor is inactivated by regulated intramembrane proteolysis (RIP), resulting in the release and subsequent activation of the σ^E factor. This mechanism has been well studied for the anti-σ factors RseA, MucA, and RsiW, regulating the activity of *E. coli* σ^E, *P. aeruginosa* AlgU, or *B. subtilis* σ^W, respectively (1, 32, 75). In *E. coli*, the accumulation of C-terminal domains of unfolded porins is the activating signal of the RpoE response by triggering the activation of the inner-membrane-anchored protease, DegS (site 1 protease), and the subsequent cleavage of RseA within its periplasmic domain by DegS. The resulting truncated anti-σ factor is then a suitable substrate for a second inner-membrane protease, RseP/YaeL (site 2 protease), which cleaves RseA near the cytoplasmic face of the inner membrane, releasing an $RseA_{cyto}$-σ^E complex into the cytoplasm, where the remaining RseA fragment is degraded by cytoplasmic proteases, resulting in the active σ^E (1). Another important mediator of the extracytoplasmic stress response is the periplasmic protease DegP, also known as HtrA and DO in *E. coli* or MucD in *P. aeruginosa* (22, 55). DegP binds to and degrades misfolded proteins and acts as a chaperone to direct the proper folding of some envelope proteins (66). As such, this family of proteases regulates the σ^E stress response system by removing misfolded proteins in the periplasm that could activate the degradation pathway of the anti-σ^E factor, even in the absence of stress (27).

The Gram-negative phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris* is an epiphytic bacterium that can become a vascular pathogen, causing black rot disease of crucifers (52). The bacterium produces a large amount of extracellular polysaccharide (EPS) that plays an important role during bacterial infection, and *X. campestris* pv. *campestris* has been used as a model organism for investigating the mechanism of bacterial pathogenesis. *X. campestris* pv. *campestris* flourishes in and adapts to a wide range of habitats: during epiphytic life, *X. campestris* pv. *campestris* is exposed to harsh stresses, such as oligotrophic conditions, desiccation, or large changes in temperature. Upon entry into plant tissues, *X. campestris* pv. *campestris* cells must face defense reactions of the host, including oxidative conditions. Finally, the natural life cycle of *X. campestris* pv. *campestris* includes long periods of survival on seeds or plant scraps or in the soil, where again it must survive a variety of stressful conditions before it can infect a new host plant. Its ability to manage variable and often lethal external conditions can be partly attributed to its large repertoire of alternative σ factors. Of the 4,179 open reading frames (ORFs) comprising the large 5.1-Mb *X. campestris* pv. *campestris* strain ATCC 33913 genome, 15 ORFs encode characterized or putative σ factors, 10 of which belong to the ECF subfamily (23). Little is known about which σ factors are required for the survival of *X. campestris* pv. *campestris* under stress and the contribution of these factors to virulence. The classification of ECF σ factors strongly suggested that the *XCC1267* gene encodes the σ^E factor of *X. campestris* pv. *campestris* (65). Moreover, previous work by Cheng et al. (17) described the biochemical characterization of the σ^E factor of *X. campestris* pv. *campestris* strain 11 and suggested that it could have a role in the heat shock response. Therefore, we aimed at deciphering the roles and regulation mechanisms of the extracytoplasmic stress response regulator σ^E in *X. campestris* pv. *campestris*.

In the present work we characterized the *rpoE* operon genes, *rpoE*, *rseA*, and *mucD*. Using primer extension and *lacZ* transcriptional reporter fusions, we show that *rpoE* transcription is autoregulated and that RseA and MucD are negative regulators of σ^E activity. We identified 45 putative members of the σ^E regulon by a transcriptome analysis, including the heat stress σ factor σ^H and a number of periplasmic or membrane proteins. We provided evidence that σ^E is an important regulator of stress responses in *X. campestris* pv. *campestris*, since it has a role in heat adaptation, resistance to cadmium, and stationary-phase survival. Furthermore, our results strongly suggest that σ^E is regulated by a RIP mechanism involving RseP (XCC1366) and DegS (XCC3898) putative proteases, as in many other bacteria. However, our data suggest that the RIP proteases RseP and DegS are not only dedicated to RseA cleavage and that the proteolytic cascade of RseA could involve other proteases.

MATERIALS AND METHODS

Strains and growth conditions. The *X. campestris* pv. *campestris* strains, plasmids, and oligonucleotides used or generated in this study are listed in Table 1. *X. campestris* pv. *campestris* cells were grown at 30°C in MOKA (yeast extract, 4 g/liter; Casamino Acids, 8 g/liter; K_2HPO_4, 2 g/liter; $MgSO_4 \cdot 7H_2O$, 0.3 g/liter) (9) or in KADO (MOKA plus 1% sucrose) (39) medium. *E. coli* cells were grown at 37°C in LB medium (44). Antibiotics were used at the following concentrations for *X. campestris* pv. *campestris*: rifampin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 5 μg/ml. Antibiotics were used at the following concentrations for *E. coli*: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml. The following supplements were added when required: sucrose, 1%, and isopropyl-β-D-thiogalactopyranoside (IPTG), 0.5 mM.

Recombinant DNA procedures. Genomic DNA from *X. campestris* pv. *campestris* was extracted using the DNeasy Blood and Tissue kit according to the instructions of the manufacturer (Qiagen). Plasmid DNA and PCR products were purified with the Qiagen mini-plasmid purification kits and PCR purification kits, respectively. *E. coli* strain DH5α was used for cloning. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Phusion High-Fidelity DNA polymerase were used as specified by the manufacturer (New England Biolabs).

Construction of in-frame deletion mutant strains in *X. campestris* pv. *campestris*. The two-step recombination system (59), based on the inability of *X. campestris* pv. *campestris* carrying the *sacB* gene to grow in media with high sucrose concentrations, was used for the chromosomal inactivation of the *rpoE*, *rseA*, *mucD*, *rseP*, *degS*, and *XCC1664* genes of *X. campestris* pv. *campestris*. For each planned inactivation experiment, a mobilizable *X. campestris* pv. *campestris* integration vector was constructed, which contained two 1,000-bp fragments on each side of the gene to be deleted, comprising the first and the last 18 nucleotides of the selected gene, thus providing two homology regions for recombination.

Genomic DNA of *X. campestris* pv. *campestris* strain 568 and primer pairs rpoE F-Sma/rpoE R-Xba and rseA F-Xba/rpoE R-Hind were used to obtain two PCR products of ~1 kb from the region upstream of *rpoE* and downstream of *rpoE*, respectively. The products were sequentially cloned into the appropriate sites of the pK18*mobsacB* vector, starting with the upstream region, to finally obtain the mobilizable plasmid pK18-$rpoE_{U+D}$. The same cloning procedure was used for the other genes, using the primer pairs indicated in Table 1. The resulting plasmids were verified by sequencing from both ends with standard primers. The plasmids were transformed into *E. coli* DH5α and mobilized into *X. campestris* pv. *campestris* by conjugation, as described previously (68). Successful first re-

TABLE 1. *X. campestris* pv. *campestris* strains, plasmids, and oligonucleotides used or generated in this study

Strain, plasmid, or oligonucleotide	Sequence (5′ to 3′)	Features or purpose	Source or reference
Plasmids			
pVO155		pUC119 derivative containing the promoterless *gus* (*uidA*) reporter gene encoding β-glucuronidase; used for insertion mutagenesis; Km^r Amp^r	51
pFAJ1700		pTR102-derived expression vector containing a multiple-cloning site and transcriptional terminators in both orientations; Tet^r Amp^r	25
pCZ750		pFAJ1700 containing the KpnI-AscI *lacZ* gene from the pCZ367 plasmid; Tet^r Amp^r	9
p917		pFAJ1700 derivative containing 2,094 bp of pSC150 with *lacI*, *tacp* promoter, and T7 terminator; Tet^r Amp^r	11
p917-lacI		p917 derivative containing 1,526 bp of pMF533 with *lacI*, *tacp* promoter, ribosome binding site, and T7 terminator; used for protein overexpression; Tet^r Amp^r	This study
pCZ-rpoEp		pCZ750 derivative; *rpoEp-lacZ*; Tet^r Amp^r	This study
pCZ-rseAp		pCZ750 derivative; *rseAp-lacZ*; Tet^r Amp^r	This study
pCZ-rpoHp		pCZ750 derivative; *rpoHp-lacZ*; Tet^r Amp^r	This study
pCZ-prcp		pCZ750 derivative; *prcp-lacZ*; Tet^r Amp^r	This study
pCZ-ompWp		pCZ750 derivative; *ompWp-lacZ*; Tet^r Amp^r	This study
pCZ-hrpFp		pCZ750 derivative; *hrpFp-lacZ*; Tet^r Amp^r	This study
pCZ-xcc0401p		pCZ750 derivative; *xcc0401p-lacZ*; Tet^r Amp^r	This study
p917-rpoE		p917-lacI derivative; *tacp-rpoE*; Tet^r Amp^r	This study
pMF533		pMALc2E (New England Biolabs) containing *malE-gadE* fusion	16
pK18*mobsacB*		Mobilizable cloning vector containing a modified *sacB* gene from *B. subtilis*; used for gene disruption; Kan^r	61
pK18-$rpoE_U$		pK18 containing ~1 kb upstream of the *rpoE* gene of *X. campestris* pv. *campestris* (up to 15 nucleotides downstream of the ATG start codon)	This study
pK18-$rpoE_{U+D}$		pK18 containing ~1 kb upstream and ~1 kb downstream of the *rpoE* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$rseA_U$		pK18 containing ~1 kb upstream of the *rseA* gene of *X. campestris* pv. *campestris* (up to 15 nucleotides downstream of the ATG start codon)	This study
pK18-$rseA_{U+D}$		pK18 containing ~1 kb upstream and ~1 kb downstream of the *rseA* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$mucD_U$		pK18 containing ~1 kb upstream of the *mucD* gene of *X. campestris* pv. *campestris* (up to 15 nucleotides downstream of the ATG start codon)	This study
pK18-$mucD_{U+D}$		pK18 containing ~1 kb upstream and ~1 kb downstream of the *mucD* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$rpoE_U rseA_D$		pK18 containing ~1 kb upstream of the *rpoE* gene (up to 15 nucleotides downstream of the ATG start codon) and ~1 kb downstream of the *rseA* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$rpoE_U mucD_D$		pK18 containing ~1 kb upstream of the *rpoE* gene (up to 15 nucleotides downstream of the ATG start codon) and ~1 kb downstream of the *rseA* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$degS_U$		pK18 containing ~1 kb upstream of the *degS* gene of *X. campestris* pv. *campestris* (up to 15 nucleotides downstream of the ATG start codon)	This study
pK18-$degS_{U+D}$		pK18 containing ~1 kb upstream and ~1 kb downstream of of the *degS* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$rseP_U$		pK18 containing ~1 kb upstream of the *rseP* gene of *X. campestris* pv. *campestris* (up to 15 nucleotides downstream of the ATG start codon)	This study
pK18-$rseP_{U+D}$		pK18 containing ~1 kb upstream and ~1 kb downstream of the *rseP* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study
pK18-$xcc1664_U$		pK18 containing ~1 kb upstream of the *XCC1664* gene of *X. campestris* pv. *campestris* (up to 15 nucleotides downstream of the ATG start codon)	This study
pK18-$xcc1664_{U+D}$		pK18 containing ~1 kb upstream and ~1 kb downstream of the *XCC1664* gene of *X. campestris* pv. *campestris* (from 15 nucleotides upstream of the stop codon)	This study

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or oligonucleotide	Sequence (5′ to 3′)	Features or purpose	Source or reference
Strains			
E. coli			
DH5α		λ− ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Laboratory collection
X. campestris pv. *campestris*			
568		Wild-type strain; rifampin-resistant derivative of *X. campestris* pv. *campestris* LMG568/ATCC33913	9
XcPB1		Δ*rpoE*; Rif^r	This study
XcPB2		Δ*rseA*; Rif^r	This study
XcPB3		Δ*mucD*; Rif^r	This study
XcPB4		Δ*rpoE-rseA*; Rif^r	This study
XcPB5		Δ*rpoE-rseA-mucD*; Rif^r	This study
XcPB6		Δ*xcc1664*; Rif^r	This study
XcPB7		Δ*degS*; Rif^r	This study
XcPB8		Δ*rseP*; Rif^r	This study
rpoE::pVO		*XCC1267*::pVO155; Rif^r Km^r	This study
Oligonucleotides[a]			
rpoE F-Sma	TTTCCCGGGCATACGGCGCGGGATAGT GTTC	*rpoE* gene deletion	
rpoE R-Xba	TTTTCTAGAGACTTCGGCCATGTCGGG		
rpoE F-Xba	TTTTCTAGACACCGTGTATGACCAAT AACC		
rpoE R-Hind	TTTAAGCTTGGCCGAACATCTGGGTGCGG		
rseA F-Sma	TTTCCCGGGTGACATCGCCCAGTTCGAG	*rseA* gene deletion	
rseA R-Xba	TTTTCTAGAGTTATTGGTCATACACGGTG		
rseA F-Xba	TTTTCTAGACCGCAGGACTGATGTTCT CGCC		
rseA R-Hind	TTTAAGCTTGAAAGCCAGGTCGATCGG GATC		
mucD F-Eco (B)	TTTGAATTCGAAAGCGCTACCCGTGAG CGAC	*mucD* gene deletion	
mucD R-Xba	TTTTCTAGAGCGGGGATTCATCAGGTTG		
mucD F-Xba	TTTTCTAGAGCGGGCGGCTGAGACGC AGGG		
mucD R-Hind	TTTAAGCTTGGGTGTCGCCGACCGGCG CGCC		
rseP F-Sma	TTTCCCGGGACCCAGGCGCATGCCGGT GATC	*rseP* gene deletion	
rseP R-Xba	TTTTCTAGAGAAATCACCCATGGATG CAAC		
rseP F-Xba	TTTTCTAGAGTTCCACGATGAAGCTG CTCC		
rseP R-Hind	TTTAAGCTTGAAGATGTCGCCGGCCTT GGGG		
degS F-Eco	TTTGAATTCAACGCTGTTCTTGGCCA CCAC	*degS* gene deletion	
degS R-Xba	TTTTCTAGACAGCGGTCGCATGCAACGG ATTC		
degS F-Xba	TTTTCTAGACTCATGCGTTGATCCGG CGTG		
degS R-Hind	TTTAAGCTTCATGGCGCCGAATTTCA TGGG		
xcc1664 F-Eco	TTTGAATTCGCCGCCCAGATCGGGCGTG	*XCC1664* gene deletion	
xcc1664 R-Bam	TTTGGATCCCCCACCGGGCACTGCATGA TTTC		
xcc1664 F-Bam	TTTGGATCCGCGCCATGACCACGCCCTG		
xcc1664 R-Hind	TTTAAGCTTGGAAAGCCATCCAGGCGC		
rpoE RT-F (A)	GCGGCGTTCGATGTGTTGG		
rpoE-EXT (I)	CCAGCTCCTGAGGTGTATC	Mapping of *rpoEp*	
mucD RT-F (D)	CCATCACCCGCAAGGACGCC		
mucD-EXT (G)	GCCAACGGCAGGGTCATGG		
mucD(2)-EXT (K)	GAACATCTGGGTGCGGATG		
mucD(3)-EXT (H)	GGAAATGATGAAGCCCGAAC		
rseA-EXT (E)	CTTTCGGTCTCCAGCAGAGG		
rseA(2)-EXT (J)	GGACATGTCAGGGTTATTG	Mapping of *rseAp*	
rseA(3)-EXT (F)	GTTCGCGGGACACGAACAAG		
rseA RT-F (C)	GACGAAGAGTTGGCCGGCTG		
lacI-FseI	TTTGGCCGGCCATCGAATGGTGC		
PtaqNcoBam	TTGGATCCATGGGCTATGGTCCTTGTTG		
P1268-Hind	TTTAAGCTTCGGTGGCAGACAGG	Upstream region of *rseA* (fusion to *lacZ*)	
P1268-Xba	TTTCTAGATGGCCGACCGGAGTTC		
P1267-Hind	TTTAAGCTTGGGGCAGGGCAGCTCGG	Upstream region of *rpoE* (fusion to *lacZ*)	
P1267-Xba	TTTTCTAGAAGTCGGGCAATGAGACC		

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or oligonucleotide	Sequence (5′ to 3′)	Features or purpose	Source or reference
ORF1267-Nco	TTTCCATGGCCGAAGTCGATACACC	*rpoE* gene overexpression	
ORF1267-Hind	TTTAAGCTTCATACACGGTGTCGCTGAC		
xcc1535-EXT	GTTCACCACGTCCGGTGAGCTC	Mapping of *XCC1535p*	
xcc0964-EXT	CTCGGCCAGCATAATCTTGCC	Mapping of *XCC0964p*	
hrpF-EXT	CCTCGCAGTGACAGAGCAG	Mapping of *hrpFp*	
hpa1-EXT	CTGCAGGTTGATGAAGTTGG	Mapping of *hpaIp*	
ompW-EXT	GGAAATGGAACGCATGAGGG	Mapping of *ompWp*	
pqqB-EXT	CCGATCCCAAAACGATGATG	Mapping of *pqqAp*	
xcc0401-EXT	GTCGGTCTGGATCTGGATCTG	Mapping of *XCC0401p*	
prc-EXT	CCAGCAATGCCATCGGAGTG	Mapping of *prcp*	
xcc4186-EXT	GAAGCTGCGTACTGCGTTGCAG	Mapping of *XCC4186p*	
rpoH-EXT	GATTGTTTGCCACAAGGGCAGTC	Mapping of *rpoHp*	
xcc3227-EXT	GGAAGGAGCGGCGGTGCGTTC	Mapping of *XCC3227p*	
xcc1246-EXT	GGCAAGTCTGATCCTCTTGG	Mapping of *XCC1246p*	
P0539-Hind	TTTAAGCTTGGCCGGGCTGGTCGAGGG	Upstream region of *ompW* (fusion to *lacZ*)	
P0539-Xba	TTTTCTAGATTGTTAGGCCCTTGGAGTGG		
PhrpF-Hind	TTTAAGCTTCGGGGTCCAATCCAAGCC	Upstream region of *hrpF* (fusion to *lacZ*)	
PhrpF-Xba	TTTCTAGAGCAGTGGCGGAGCTG		
Pprc-Hind	TTTAAGCTTGTCACCCTGCGCGACCTG	Upstream region of *prc* (fusion to *lacZ*)	
Pprc-Xba	TTTTCTAGATGAAAGAAGGGCGGTGATC		
P0401-Hind	TTTAAGCTTCGTCCTGCGCCACCGCAG	Upstream region of *XCC0401* (fusion to *lacZ*)	
P0401-Xba	TTTTCTAGATTCGATTCTGCGACAGCC		
P3771-Hind	TTTAAGCTTCAATGCGGTGCTGACGGTGG	Upstream region of *rpoH* (fusion to *lacZ*)	
P3771-Xba	TTTTCTAGAACTATTGGACTGCTGGGTAC		
P1230-Hind	TTTAAGCTTCGGCATCGGCGTCCTCTTC	Upstream region of *XCC1230* (fusion to *lacZ*)	
P1230-Xba	TTTTCTAGATTGCTGCACCCCCATTCTG		

[a]Letters in parentheses after oligonucleotide designations indicate letter codes used for primers in Fig. 1 and 2 and Fig. S1 in the supplemental material.

combinants (chromosomal integration mutants) were selected by plating cells on MOKA containing kanamycin. For selection of the second recombination event, the integration mutants were plated on MOKA containing 5% (wt/vol) sucrose. Clones that had lost kanamycin resistance were screened by colony PCR using primers outside the cloned regions. Two of the confirmed transconjugants were randomly chosen for further study.

Construction of *rpoE*-overexpressing *X. campestris* pv. *campestris* strains for complementation. To construct the overexpression plasmid for the *rpoE* gene, an FseI-BamHI fragment encompassing the *lacI* gene and the *tacp* promoter was amplified by PCR using plasmid pMF533 (16) as a template, together with primers lacI-FseI and *tacp* NcoBam. The fragment generated was subsequently cloned into the pCZ917 plasmid (11) to yield p917-lacI. The resulting plasmid contains the *lacI* gene and the *tacp* promoter, followed by a ribosome binding site and the restriction sites for NcoI and BamHI. For complementation of deletion mutants of *X. campestris* pv. *campestris*, a 621-bp DNA fragment containing the entire *rpoE* gene was amplified by PCR using total DNA of *X. campestris* pv. *campestris* strain 568 as a template and primers ORF1267-Nco and ORF1267-Hind and cloned into the p917-lacI plasmid using the NcoI and HindIII enzymes. The resulting plasmid, p917-rpoE, was verified by sequencing and subsequently transferred to *X. campestris* pv. *campestris* by triparental conjugation. For the complementation experiments, different concentrations of IPTG were tested, and in order to avoid deleterious effects of σ^E overexpression, we chose not to add IPTG because the expression of σ^E from the leaky *tacp* promoter was sufficient.

Construction of promoter-reporting plasmids and β-galactosidase assays. The promoter regions (~500 bp upstream of the ATG) of genes *rpoE*, *rseA*, *prc*, *ompW*, *hrpF*, *XCC0401*, *rpoH*, and *XCC1230* were PCR amplified with primer sets shown in Table 1, using genomic DNA from *X. campestris* pv. *campestris* strain 568 as a template. These promoter regions were cloned as HindIII-XbaI fragments into the pCZ750 plasmid (9) upstream of a promoterless *lacZ* gene. The resulting plasmids were confirmed by sequencing and introduced into *X. campestris* pv. *campestris* strains by triparental conjugation. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and grown at 30°C for 9 h (~3 generations). β-Galactosidase activities were assayed as described previously (44). The data shown are the averages of at least two independent cultures, each measured in triplicate. Negligible β-galactosidase activity was derived from the control promoterless plasmid pCZ750 (less than 9 Miller units) (data not shown).

RNA purification. Overnight cultures were diluted to an OD_{600} of 0.05 and grown at 30°C for 9 h (~3 generations) to reach an OD_{600} of 0.4. If the cells required a temperature upshift, it was done for 60 min at 35°C. For all experiments, 10-ml culture samples were harvested by immediately adding 1.25 ml of ice-cold 5% water-saturated phenol in ethanol and centrifugation at 5,000 rpm. The cell pellets were flash frozen in liquid N_2 and stored at −80°C. Total RNA was extracted from the cell pellets by use of TRIzol reagent following the manufacturer's specifications (Invitrogen). A further treatment with 0.03 U RQ1 DNase I (Promega) per μg of RNA for 30 min at 37°C, followed by phenol extraction and ethanol precipitation, was carried out. RNA was evaluated for quantity and quality using the NanoDrop1000 spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis. The absence of DNA contamination was confirmed by PCR.

RT-PCR. One microgram of total RNA was reverse transcribed into cDNA by using PrimeScript reverse transcriptase (RT) (Takara Bio) with random hexamers according to the manufacturer's instructions, and 0.5 μl of each retrotranscription reaction mixture was subjected to PCR using GoTaq DNA polymerase (Promega). Positive controls were performed with genomic DNA, and negative controls were performed with RNA that had not been subjected to retrotranscription (data not shown). The cycling conditions used were 95°C for 2 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min 30 s, followed by incubation at 72°C for 5 min.

Microarray data collection and statistical analyses. Briefly, total RNA was extracted from four sets of identically treated batch cultures of Δ*rpoE* (no σ^E) and Δ*rseA* (overactivation of σ^E) mutant *X. campestris* pv. *campestris* strains grown up to mid-exponential phase in MOKA medium at 30°C. Total RNA (10 μg) was reverse transcribed in the presence of aminoallyl dUTP using Superscript II (Invitrogen) and random hexamers for priming, according to the manufacturer's instructions. The resulting amine-modified cDNA was then chemically labeled at the aminoallyl group using Alexa Fluor 555 and 647 reactive dyes (Invitrogen). Hybridization took place underneath a coverslip in 60 μl of warmed DIG Easy Hybridization buffer (Roche) at 42°C for 16 h in a sealed humidified chamber.

Xcc5kOLI microarrays (62) based on the genome sequence of *X. campestris* pv. *campestris* strain B100 (70) were used for hybridizations. The array contains 4,441 50-mer to 70-mer oligonucleotides representing the predicted protein-encoding genes. In addition, it contains 15 stringency controls of the genes *gapA*, *rpsA*, *rpsB*, *rpsL*, and *rpmI* (70%, 80%, and 90% identity to the native sequence), 12 alien DNA oligonucleotides, and 5 spiking control oligonucleotides. Each probe was spotted in three replicates.

After being washed, the hybridized microarray slides were scanned using an Axon GenePix 4100A scanner (Molecular Devices). The acquired microarray images were analyzed with GenePix Pro (version 3.0.6.90). Preprocessing of raw data and statistical analyses were performed using the Bioconductor and

LIMMA packages in the R programming environment (64). Spots marked as "bad" (flags ≤ −49) by GenePix were excluded from further analysis. Intensity normalization was performed using the robustspline method (within arrays) and the aquantile method (between arrays). Within array replicates, correlation was estimated and incorporated in the linear model was inferred by LIMMA, which was further used to build an empirical Bayes moderated *t* test statistic to assess differential expression. *P* values were adjusted for multiple testing using the method of Benjamini and Hochberg (8) to control the false-discovery rate at a level of 0.05, and the statistical significance threshold to decide differential expression was set to 0.05.

Primer extension analyses. For these experiments, we used one of the four RNA sample sets that were used for the microarray experiments. Primer extension experiments were performed at 50°C using PrimeScript Reverse Transcriptase (Takara Bio) and primers hybridizing in the 5′ region of the coding sequence of the respective genes (Table 1). Ten micrograms of total RNA and 1 pmol of ^{32}P end-labeled primers were used per reaction. The extension products were loaded on a 6% denaturing polyacrylamide gel adjacent to a sequencing ladder obtained with the ^{32}P-labeled universal cycle primer and pUC18 plasmid provided in the Thermo Sequenase cycle-sequencing kit (USB).

Microarray data accession numbers. Fully annotated microarray data from this study have been deposited in ArrayExpress under accession no. E-MEXP-2935. The array design is available in ArrayExpress under accession no. A-MEXP-1909.

RESULTS

***rpoE* genomic organization.** The *rpoE* (*XCC1267*) genomic organization found in the *X. campestris* pv. *campestris* genome is similar to that of other gammaproteobacteria, such as *E. coli*, *P. aeruginosa*, and *Xanthomonas fastidiosa* (Fig. 1A). The gene immediately downstream of *rpoE* is predicted to encode an alanine-rich protein of 286 amino acids that contains an N-terminal anti-σ^E protein RseA domain (pfam03872; RseA_N). Taken together with the fact that the genes encoding the σ^E factors are contiguous to a coding region specifying an anti-σ factor, this strongly suggested that XCC1268 was the putative anti-σ^E factor, and *XCC1268* has been renamed *rseA*. The third gene of the *rpoE* cluster is the *mucD* orthologue *XCC1269*. MucD is a periplasmic serine protease of *P. aeruginosa* that belongs to the HtrA protein family and alleviates periplasmic stress by degrading misfolded outer membrane proteins (OMPs) selectively (75). Hence, MucD is an indirect negative regulator of σ^E activity. Interestingly, *P. aeruginosa* and *X. fastidiosa* carry a *mucD* gene in the *rpoE* operon, but *E. coli* does not (Fig. 1A). In other bacteria, there may be one or two genes downstream of *rseA* (namely, *rseB-mucB* and *rseC-mucC*), which encode accessory σ^E regulatory proteins (Fig. 1A). RseB is a negative regulator of σ^E that binds to the periplasmic domain of RseA, probably in order to block the access of DegS to the cleavage site of RseA (40). The function of RseC is generally unknown, but it has been reported to be both a positive and a negative regulator (10, 46). Strikingly, there are no orthologues of *rseB* and *rseC* in the *X. campestris* pv. *campestris* genome (Fig. 1A), suggesting that σ^E activity might be regulated only by RseA binding in this bacterium.

To investigate whether *rpoE-rseA-mucD* constituted a single transcription unit, RT-PCR experiments were carried out using appropriate primers located within the *rpoE-rseA-mucD* region. As shown in Fig. 1B, we could identify specific transcripts encompassing *rpoE* and *rseA* (Fig. 1B, primer pairs AF and BF), *rseA* and *mucD* (Fig. 1B, primer pair CG), and *rpoE* and *mucD* (Fig. 1B, primer pairs AG and BG). These data strongly suggest that *rpoE-rseA-mucD* form a single transcription unit.

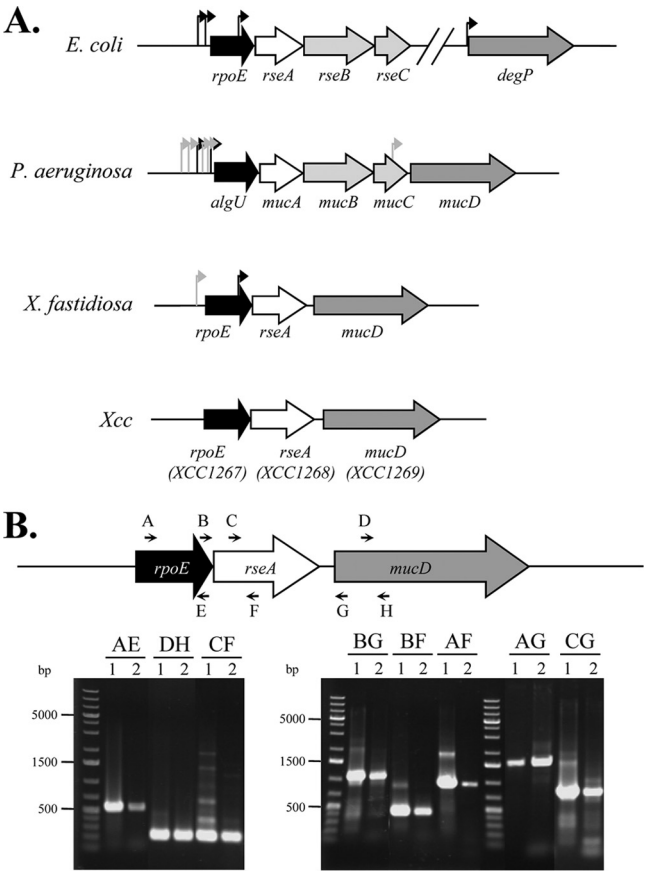


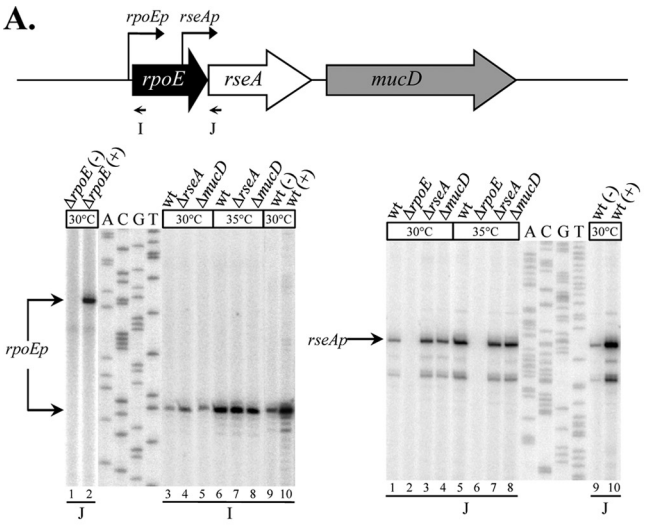
FIG. 1. Transcriptional organization of the *X. campestris* pv. *campestris rpoE* region. (A) Schematic (to scale) showing the organization of the *rpoE* region in *E. coli*, *P. aeruginosa*, *X. fastidiosa*, and *X. campestris* pv. *campestris*. The arrows indicate experimentally demonstrated promoter start sites (black, σ^E-dependent promoter; gray, σ^E-independent promoter). (B) Organization of the *rpoE* region showing the primer pairs used for the amplifications (Table 1 shows primer sequences) and agarose gel of the RT-PCR amplification products. For each primer pair (named according to the letter code of each primer), two lanes are shown (lane 1, positive control using genomic DNA templates; lane 2, RT-PCR using RNA extracted from cells in exponential phase). The molecular size marker is the O'GeneRuler 1-kb Plus DNA Ladder (Fermentas). In each case, the main extension product migrated at the expected size: AE, 524 bp; DF, 228 bp; CF, 235 bp; BG, 1,058 bp; BF, 393 bp; AF, 912 bp; CG, 900 bp; and AG, 1,577 bp.

Transcriptional regulation of the *rpoE* operon. A typical σ^E-dependent promoter has been identified upstream of the *rpoE* gene in *X. campestris* pv. *campestris* strain 11 (Fig. 2B) (17). Since this promoter sequence is conserved in *X. campestris* pv. *campestris*, we postulated that *rpoE* was autoregulated in *X. campestris* pv. *campestris*. A single transcription start site upstream of the *rpoE* gene (*rpoEp*) was identified by primer extension (Fig. 2A, left, lane 3), and it matched P1 of *X. campestris* pv. *campestris* strain 11 (Fig. 2B). To test the transcriptional regulation of *rpoEp*, we conducted primer extension experiments using RNAs purified from strains with *rpoE*, *rseA*, or *mucD* deleted (chromosomal unmarked in-frame deletions; see Materials and Methods for details). The growth of the mutants was comparable to that of the parental strain in rich

medium (MOKA) at 30°C, evidencing a doubling time of ~3 h (data not shown), suggesting that *rpoE* plays a nonessential role under ordinary growth conditions. As expected, *rpoEp* expression was strongly reduced when *rpoE* was inactivated (Fig. 2A, left, lane 1), and conversely *rpoEp* expression was increased when *rseA* or *mucD* was deleted (Fig. 2A, left, compare lanes 4 and 5 with lane 3). Note that the effect of *mucD* deletion is minor but reproducible. Further, the wild-type (WT) or Δ*rpoE* mutant strain was complemented with plasmid p917-rpoE, carrying the *rpoE* gene under the control of the IPTG-inducible promoter *tacp*. Overexpression of σ^E from plasmid p917-rpoE significantly increased the amount of *rpoEp* expression in the wild-type strain (Fig. 2A, left, lanes 9 and 10) and restored *rpoEp* activity, while no activity was seen when the control plasmid p917 was introduced into the Δ*rpoE* mutant strain (Fig. 2A, left, lanes 1 and 2).

These results were confirmed by the analysis of the β-galactosidase activity expressed from the reporter plasmid pCZ-rpoEp carrying a fusion between a DNA fragment containing *rpoEp* and the *lacZ* gene (see Materials and Methods). This plasmid was introduced into the *X. campestris* pv. *campestris* wild-type strain and the Δ*rpoE*, Δ*rseA*, Δ*mucD*, Δ*rpoE-rseA*, and Δ*rpoE-mucD* mutants. We observed that deletion of *rpoE* caused an ~4-fold reduction in *rpoEp*-driven β-galactosidase activity (Fig. 2C), showing that the expression of σ^E is autoregulated in *X. campestris* pv. *campestris*, as in many other bacteria and as previously reported in *X. campestris* pv. *campestris* strain 11 (17). However, significant β-galactosidase activity remained when *rpoE* was deleted (~256 Miller units) (Fig. 2C), suggesting that the *rpoE* operon could also be controlled by a σ^E-independent promoter. This is consistent with the regulation of *rpoE* transcription by a combination of σ^E-dependent and σ^E-independent promoters in other bacteria (Fig. 1). Nevertheless, we were unable to map such a promoter using primer extension, possibly because its expression was too low under our experimental conditions. The deletion of *rseA* or *mucD* caused ~5-fold and ~2-fold activation, respectively, of *rpoEp* expression (Fig. 2C), indicating that RseA and MucD are negative regulators of σ^E-dependent activity. The modest effect of *mucD* deletion on the transcriptional activation of *rpoEp* compared with the impact of *rseA* deletion (Fig. 2A, left, and C) was in agreement with the predicted role of MucD as an indirect negative regulator of σ^E that acts by removing misfolded proteins that activate proteases for degradation of anti-σ^E.

To check the effect of unfolding stress on *rpoEp* transcription, we tested the effect of temperature stress, since σ^E has been shown to be involved in cell survival after a heat shock stress in *X. campestris* pv. *campestris* strain 11 and the closely related bacterium *X. fastidiosa* (17, 24). More generally, σ^E is involved in the transcription of a set of heat shock response genes and the heat shock sigma factor σ^H in several bacterial species (3, 28, 69). We conducted primer extension experiments using RNAs extracted from *X. campestris* pv. *campestris* strains that had been shifted from 30°C to 35°C. As shown in Fig. 2A (left), there was a strong increase in *rpoEp* transcription upon heat treatment (compare lane 6 with lane 3). Similar transcription levels were obtained in WT, Δ*rseA*, and Δ*mucD* strains (compare lanes 7 and 8 with lane 6). These results showed that *rpoE* expression was induced by temperature



B.

	-35		-10	
rpoEp Xcc	GAACTTT	TCTTTTCAATGGCG	GTCTCATT	GCCCC**G** - 32 nt- ATG$_{rpoE}$
P1 Xc11	GAACTTT	TCTTTTCAATGGCG	GTCTCATT	GCCCC**G** - 32nt - ATG$_{rpoE}$
rseAp Xcc	GAACAAA	CGGTGATGCGTGCG	GTCCAAGC	CT**T** - 189 nt - ATG$_{rseA}$
P2 Xf	GAACAAA	TCGTGATGAGCGCG	GTCCAAGC	AC**T** -225 nt -ATG$_{rseA}$
Consensus	GAACTTT	- 16 nt -	$_G$TCTA$_{AA}$	-5/6 nt- **+1**

C.

Strain	β-galactosidase activity (Miller Units)	
	rpoEp	*rseAp*
WT	981±176	3082±992
Δ*rpoE*	256±75	36±6
Δ*rpoE-rseA*	244±95	33±9
Δ*rpoE-rseA-mucD*	225±17	29±4
Δ*rseA*	5193±303	16075±5952
Δ*mucD*	2236±584	5914±1317

FIG. 2. Expression of the *rpoE* operon genes. (A) Determination of the transcription start site of the *rpoE* operon genes by primer extension. The schematic (to scale) shows the primers used for transcriptional start site mapping experiments, and the black arrows indicate the positions of the two identified σ^E-dependent promoters. Total RNAs from the WT, Δ*rpoE*, Δ*rseA*, Δ*mucD*, and WT or Δ*rpoE* strains containing the control plasmid p917 (−) or the σ^E-overexpressing plasmid p917-rpoE (+) were used as templates in primer extension experiments when they were suited. Total RNAs were obtained from cells incubated at 30°C or after a 60-min shift at 35°C. The arrows indicate the bands corresponding to the observed start site. (B) Sequence alignment depicting the relevant features of *rpoEp* and *rseAp* compared to σ^E-binding sites of P1 of *X. campestris* pv. *campestris* strain 11 (Xc11) and P2 of *X. fastidiosa* (Xf) (17, 24). The transcription start sites identified by primer extension are indicated in boldface, and the putative −10 and −35 regions are boxed. The consensus ECF02 group of ECF σ factor sites is indicated below (65). (C) Determination of *rpoEp* and *rseAp* activities in different strains. Plasmids containing a transcriptional fusion of the upstream region of *rpoEp* or *rseAp* to the *lacZ* gene were transferred into *X. campestris* pv. *campestris* strains. Overnight cultures of these strains grown in MOKA medium were diluted in the same medium and grown for 9 h before determination of β-galactosidase activity. The results represent the mean values of at least two independent experiments, each performed in triplicate, with the standard errors.

stress. Moreover, these strongly suggest that RseA and MucD are negative regulators of σ^E in the absence of activating signal, which could be the presence of nonfolded proteins in the periplasm.

To go further into the regulation of the *rpoE* operon, we tested for the presence of other promoters using primer extension experiments with primers located at the 3′ end of *rpoE* and the 5′ end of *rseA* or *mucD*. As for *X. fastidiosa* and *E. coli*, a typical σ^E-dependent promoter was found within the *rpoE* coding sequence and was named *rseAp* (Fig. 2A, right, and B). Note that we used a primer further upstream to show that the upper band corresponded to the transcription start site of *rseAp* (data not shown). The *rseAp* regulation pattern followed that of *rpoEp*, being activated by σ^E (Fig. 2A, right, compare lane 10 to lane 9), repressed by RseA and MucD (Fig. 2A, right, compare lanes 3 and 4 to lane 1), and induced by heat stress (Fig. 2A, right, compare lane 5 to lane 1). As a negative control, we checked that there was no transcript corresponding to *rseAp* when RNAs purified from the Δ*rpoE* strain were used (Fig. 2A, right, lanes 2 and 6). Moreover, we could not detect any independent transcription start site for *mucD* if we used a primer located in the *mucD* gene, while we could still see a band corresponding to the *rseAp* start site (see Fig. S1 in the supplemental material). Taken together with the RT-PCR results (Fig. 1B), this strongly suggested that the *mucD* gene was transcribed from both *rpoEp* and *rseAp*. Moreover, as shown in Fig. 2C, the β-galactosidase activity driven from a plasmidic *rseAp-lacZ* fusion was higher than that of *rpoEp* (~3 fold) and was strictly dependent on σ^E, since almost no activity was detected in the absence of *rpoE*. Our results suggest that σ^E positively autoregulates itself and upregulates RseA and MucD. Since RseA and MucD are negative regulators of σ^E activity, this could set up a negative feedback loop to ensure rapid downregulation of the σ^E-dependent response for a return to the baseline level after a protein-folding stress condition or during normal growth.

σ^E is involved in stationary-phase survival and in response to diamide and cadmium. Several reports have indicated that *rpoE* mutants of Gram-negative bacteria are more susceptible to environmental stresses (60). To evaluate the role of σ^E in the physiology and environmental stress response of *X. campestris* pv. *campestris*, bacterial growth and the resistance levels of isogenic WT, Δ*rpoE*, Δ*rpoE-rseA*, Δ*rpoE-rseA-mucD*, Δ*rseA*, and Δ*mucD* strains against different stresses were determined. All of the strains exhibited mucoid phenotypes when grown on KADO plates, suggesting that the production of exopolysaccharides was not affected by the absence or the overactivation of σ^E (data not shown), as previously described in *X. campestris* pv. *campestris* strain 11 (17). Moreover, the morphology of the mutant cells (determined by phase-contrast microscopy) grown on MOKA medium was unaffected in stationary phase, but Δ*rseA* mutant cells were significantly smaller than WT cells in exponential phase (data not shown). This change in cell shape could indicate a link between σ^E and peptidoglycan assembly, as suggested for *E. coli* (31).

Bacteria were cultivated aerobically in MOKA medium and exposed to different treatments during mid-exponential growth for several periods of time, and viable-cell counts were determined. Resistance to acidic pH, 0.1% SDS, alkaline stress caused by 40 mM NaOH, oxidative stress caused by 5 mM H_2O_2, and hyperosmotic stress caused by 1.5 M NaCl was assayed. No statistically significant differences between the WT and Δ*rpoE* mutant strains were observed (data not shown). In addition, the different strains were tested for survival upon oxidative stress (1 M diamide and 200 mM paraquat) and against cell wall-active antibiotics (50 mg/ml vancomycin, 10 mg/ml polymyxin B sulfate, and 100 mM chlorpromazine) using a disk diffusion assay. Briefly, *X. campestris* pv. *campestris* cultures in mid-exponential phase were plated on MOKA agar, and sterile paper disks saturated with 10 μl of chemicals were layered on top prior to incubation at 30°C. Strains with *rpoE* deleted showed no significant differences (data not shown) apart from increased sensitivity to diamide: the diameter of growth inhibition for Δ*rpoE*, Δ*rpoE-rseA*, and Δ*rpoE-rseA-mucD* mutants was 2.85 cm (±0.20 cm) compared with 1.9 cm (±0.02 cm) for the WT strain. This suggests that σ^E is involved in the oxidative-stress response via a thiol oxidation pathway in *X. campestris* pv. *campestris*.

Further, σ^E factors have been reported to be required for metal resistance in *E. coli* (26). We submitted *X. campestris* pv. *campestris* cells to cadmium stress by spotting serial dilutions of cultures of WT and mutant strains in the late exponential phase of growth on MOKA agar plates containing cadmium (40 μM) and incubating them at 30°C for 72 h. The results of a representative spot dilution experiment are shown in Fig. 3A.

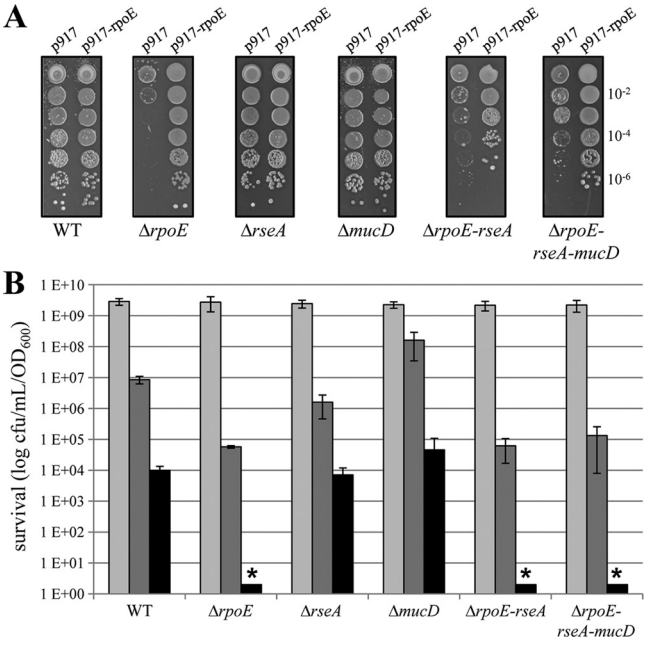


FIG. 3. Stress sensitivity of Δ*rpoE* mutants. (A) Influence of cadmium on growth of *X. campestris* pv. *campestris* WT and mutant strains containing control plasmid p917 or σ^E-overexpressing plasmid p917-rpoE. Serial 10-fold dilutions of late-exponential-phase bacteria were spotted on MOKA plates containing 40 μM cadmium and incubated at 30°C for 72 h. Each experiment was repeated three times. (B) Effect of *rpoE* deletion on stationary-phase survival of *X. campestris* pv. *campestris*. Overnight cultures in MOKA medium were diluted to an OD_{600} of 0.05 (time zero), and survival was monitored by viable-cell counting 24 h (light-gray bars), 48 h (dark-gray bars), and 96 h (black bars) postinoculation. The data are the mean values from four experiments, with the error bars representing standard deviations. The asterisks indicate no detectable cells.

We checked that control plasmid p917 had no impact on growth in the different *X. campestris* pv. *campestris* strains (data not shown). The deletion of *rpoE* was detrimental for resistance to cadmium, since there was a clear growth defect compared to the WT strain. Normal growth was restored when σ^E was overexpressed from plasmid p917-rpoE. These results indicate that σ^E is required for full adaptation to cadmium stress in *X. campestris* pv. *campestris*, as in *E. coli*.

It has been shown that σ^E activity increases upon entry into stationary phase in *E. coli* (19) and that *S.* Typhimurium σ^E is required for stationary-phase survival (67). To monitor the stationary-phase survival of *X. campestris* pv. *campestris* WT and mutant strains, the cells were grown at 30°C on a continuously shaking platform, and when the cells reached stationary phase, viable-cell counts were determined periodically by plating cells onto MOKA plates. As shown in Fig. 3B, the survival of Δ*rpoE* mutants was severely impaired, since the mutants displayed a 100-fold reduction in viability with respect to the WT after 48 h in stationary phase and there was no survival after 72 h, whereas the Δ*rseA* mutant did not show any appreciable phenotype under the conditions tested. These results strongly suggest that σ^E is required for stationary-phase survival of *X. campestris* pv. *campestris*.

Heat sensitivity of the *X. campestris* pv. *campestris* ΔrpoE mutant. Since we had shown that exposure to heat (35°C) activated σ^E, we tested the effect of temperature stress in isogenic WT, Δ*rpoE*, Δ*rpoE-rseA*, Δ*rpoE-rseA-mucD*, Δ*rseA*, and Δ*mucD* strains. Heat shocks from 42°C to 50°C were applied to exponentially growing cultures for several time points up to 30 min. Viable-cell counts were determined for all strains, and all the mutants gave similar responses to heat-killing treatment compared to the WT (data not shown), indicating that σ^E is not essential for survival after a heat shock in *X. campestris* pv. *campestris*. To check if σ^E could be involved in adaptation to heat stress, the growth of *rpoE* mutants was compared to that of the WT strain by spotting serial dilutions of bacterial cultures in the late exponential phase of growth on MOKA agar plates and incubating them at 30°C and 35°C. The results of a representative experiment are shown in Fig. 4A. Growth at 35°C was strongly impaired for the Δ*rpoE* mutants (Δ*rpoE*, Δ*rpoE-rseA*, and Δ*rpoE-rseA-mucD*), while there was no or little effect on growth for mutants that overactivate σ^E (Δ*rseA*) or for mutants of other ECF σ factors (Δ*XCC1664*). Conversely, there was no difference between the WT and mutant strains in plating efficiency when the strains were incubated for 72 h at 30°C. However, it must be noted that the colonies formed at 35°C with the Δ*mucD* mutant are reproducibly smaller than those formed at 30°C. This could be due to its putative protective role during protein folding stress by degrading misfolded proteins in the periplasm.

Complementation of the Δ*rpoE* mutants with plasmid p917-rpoE partially restored growth at 35°C (Fig. 4A). When we introduced the control plasmid p917 into the WT strain, we observed a small decrease in growth at 35°C compared to 30°C that could be the result of the combination of heat and antibiotic stresses (since we added tetracycline in the plates to maintain the plasmid). This inhibitory effect was even stronger when plasmid p917-rpoE was introduced into the WT strain, probably due to the toxicity of σ^E overexpression, as has been described for *E. coli* (58). This could explain why complementation of Δ*rpoE-rseA* and Δ*rpoE-rseA-mucD* mutants is less efficient than that of the Δ*rpoE* mutant, since the lack of appropriate posttranslational regulation of σ^E could be detrimental to bacterial fitness. We also tested the effect of σ^E on adaptation to cold stress at 14°C, and there was no difference between the WT and mutant strains (data not shown). Our results show that deletion of *rpoE* makes *X. campestris* pv. *campestris* cells extremely vulnerable to temperature adaptation at 35°C.

Given that ethanol is an amphiphilic compound that mimics the effects of high-temperature stress and that σ^E is required for growth in the presence of ethanol in several bacteria, such as *X. fastidiosa* or *V. cholerae* (24, 42), we tested the growth of WT and mutant *X. campestris* pv. *campestris* strains in the presence of 1.5% ethanol. As shown in Fig. 4B, the deletion of the *rpoE* gene strongly impaired growth, while deletion of *rseA* or *mucD* had no significant effect. Overall, these results indicate that σ^E contributes to cell envelope stress adaptation of *X. campestris* pv. *campestris*.

Identification of genes regulated by the σ^E factor. To gain insight into the functions regulated by σ^E in *X. campestris* pv. *campestris*, a global approach was chosen by comparing transcriptomes of the Δ*rpoE* strain (no σ^E activity) to that of the Δ*rseA* strain (overactivation of σ^E) using Xcc5kOLI microarrays (62). Given that there are nine ECFs in *X. campestris* pv. *campestris* and that they recognize close promoter sequences (65), we chose these setups in order to mimic a physiological activation of σ^E and to minimize nonspecific promoter recognition by σ^E. In the microarray analyses, any gene with a *P* value of ≤0.05 showing an increase of expression of 1.3-fold or more and a reduction in expression of 1.5-fold or more in the Δ*rseA* strain in comparison to the Δ*rpoE* strain was defined as being regulated by σ^E, either directly or indirectly (Tables 2 and 3). We have chosen relatively low thresholds to identify the significantly σ^E-regulated genes, since we have done four independent biological replicates, including a dye swap experiment. A total of 45 genes comprising 37 putative transcription units (TUs) were induced (Table 2), and 20 genes were repressed (Table 3). As expected, the *rpoE* and *mucD* transcripts were included in the group of upregulated genes. Nevertheless, the signal for the *rseA* transcript was in the range of the background under the conditions employed (data not shown), suggesting that there could be a problem with the detection of the oligonucleotide probe.

Of the upregulated genes, σ^E transcribes an array of biosynthetic enzymes that are involved in fatty acid metabolism (*fadE2* and *XCC3937*), redox metabolic functions (*XCC1588*), and electron transport systems (*bioI* and *XCC3906*). This raised electron-transport system activity could possibly compensate for proton leakage across the membrane when its integrity is compromised or could be a response to the formation of reactive oxygen species generated by a perturbation of the electron transport chain. This is further supported by the induction of *pqqA*, since the redox cofactor PQQ can act as an antioxidant metabolite to detoxify reactive oxygen species (45).

A large number of σ^E-upregulated genes bear signal sequences or transmembrane domains, which is consistent with the σ^E response having a role in monitoring and preserving the membrane during stress. In addition, three genes encode cell envelope proteins. Among them, *XCC0539* encodes a pre-

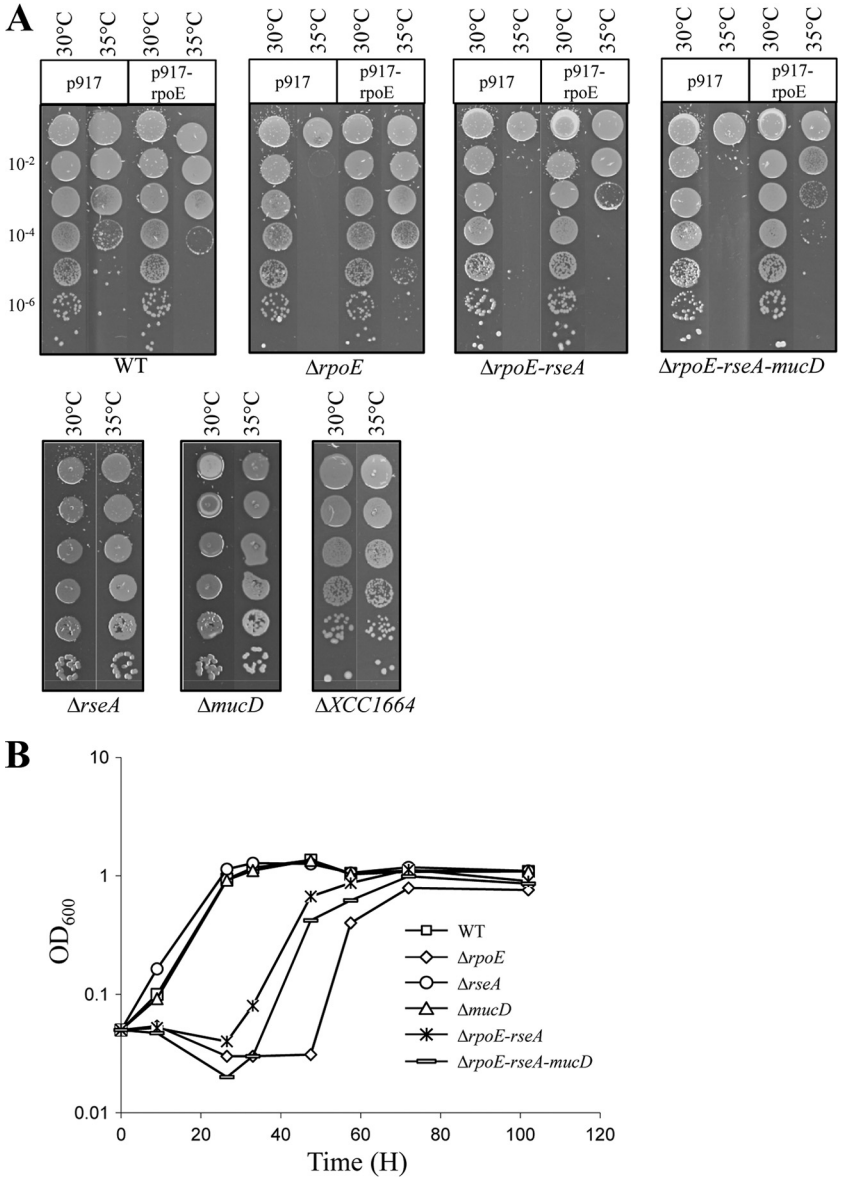


FIG. 4. The Δ*rpoE* mutant of *X. campestris* pv. *campestris* is sensitive to heat and ethanol. (A) Heat sensitivities of *X. campestris* pv. *campestris* WT and mutant strains containing no plasmid, control plasmid p917, or σ^E-overexpressing plasmid p917-rpoE. Serial 10-fold dilutions of late-exponential-phase bacteria were spotted on plates and incubated at 35°C for 72 h. Each experiment was repeated three times. (B) Growth curve of *X. campestris* pv. *campestris* WT and mutant strains in the presence of 1.5% ethanol. Cells were precultured in MOKA medium overnight and then diluted to an OD_{600} of 0.05 in MOKA medium containing 1.5% ethanol. One representative experiment out of three independent replicates is shown.

dicted member of the OmpW/AlkL family that is found in all Gram-negative bacteria and is involved in the protection of bacteria against various forms of environmental stress (36). *pglA* encodes a predicted polygalacturonase (PG) to selectively degrade the pectic polymers of the plant cell walls, and PGs are virulence factors in closely related bacterial species (73). Interestingly there is also an overrepresentation of type III secretion (T3S)-related genes within the σ^E regulon of *X. campestris* pv. *campestris*. In plant-pathogenic bacteria, the T3S system (T3SS) is one of the key pathogenicity factors and is encoded by the chromosomal *hrp* gene cluster. Here, we identified 12 genes whose products are involved in all aspects of T3S machinery and effector proteins that are under the control (at least partial) of σ^E. In xanthomonads, the *hrp* cluster is organized into at least six transcriptional units and is under the positive control of HrpG and HrpX (30). In our experiments, only two *hrp* operons (*hrpE* and *hrpF*) were upregulated, suggesting that σ^E-dependent regulation of T3S genes did not occur via HrpG or HrpX. To check this, we tested the effect of *rpoE* inactivation on plasmid-driven *hrpXp*- or *hrpGp-lacZ* transcriptional fusion reporters, and there was no significant change in their activity (data not shown).

As expected from the known σ^E-dependent stress responses, a heat shock response was induced in *X. campestris* pv. *campes-*

TABLE 2. Genes with increased expression in the Δ*rseA* strain compared with the Δ*rpoE* strain

ID	Gene	Ratio[a]	Description of gene product	Putative TU[b]	Characteristics
Regulatory function					
XCC1267	*rpoE2*	4.28	RNA polymerase σ factor σ^E	XCC1267, -68, -69	Transcription initiation
XCC3771	*rpoH*	1.52	Heat shock RNA polymerase factor σ^H		Response to heat; transcription initiation
XCC3348		1.48	Putative sensor-response regulator hybrid	XCC3347, -48	Two-component signal transduction system (phosphorelay)
Metabolism					
XCC2432	*fadE2*	3.42	Putative acyl-coenzyme A dehydrogenase	XCC2430, -31, -32	Oxidation reduction
XCC3937	*baf*	3.14	Putative type III pantothenate kinase	XCC3938, -37, -36	Positive regulation of transcription
XCC3047	*bioI*	1.45	Putative cytochrome P450 hydroxylase		Heme b metabolic process
(NC_003902: 3492579-3492659, plus strand[c])	*pqqA*	1.41	Putative coenzyme PQQ biosynthesis protein A	XCC2937, -38, -39, -40, -41	Pyrroloquinoline quinone biosynthetic process
XCC1588		1.32	Putative sulfite oxidase subunit YedY		Electron carrier activity
XCC3906		1.31	Putative cytochrome B561	XCC3905, -06	Respiratory electron transport chain
Protein synthesis and fate					
XCC1269	*mucD*	2.91	Putative periplasmic protease	XCC1267, -68, -69	Proteolysis
XCC1047	*hspA*	2.02	Low-molecular-wt heat shock protein		Response to stress
XCC3493-like degradation complex	*hslU*	1.83	Chaperone subunit of a proteasome	XCC3493, -94	Response to stress
XCC3227	*moxR*	1.67	MoxR-like AAA^+ ATPase chaperone	XCC3227, -26, -25, -, 24, -23, -22	ATPase activity
XCC2393	*htpG*	1.53	Molecular chaperone Hsp90 family		Protein folding
XCC1535	–[d]	1.41	FKPB-type peptidyl-prolyl *cis-trans* isomerase		Protein folding
XCC1475	*dnaJ*	1.36	Molecular chaperone Hsp40 family	XCC1474, -75	Response to heat; protein folding
XCC3450	*prc*	1.34	Putative carboxyl-terminal processing protease		Proteolysis
XCC1474	*dnaK*	1.31	Molecular chaperone Hsp70 family	XCC1474, -75	Response to heat; protein folding
T3S, translocation, and regulation machinery					
XCC1240	*hpa1*	2.15	Harpin-like elicitor protein (T3SS-dependent secreted protein)		
XCC1217	*hrpF*	2.04	Type III translocon protein		Interaction with host via protein secreted by T3SS
XCC1246		1.99	Type III effector protein (XopAL class)		Pathogenesis
XCC1241	*hpa2*[d]	1.78	Lytic transglycosylase-like		
XCC1222	*hrpD6*	1.64	Type III secretion system component	XCC1222, -21, -20, -19	Protein secretion by the T3SS
XCC1220	*hpaB*	1.54	Global T3S chaperone	XCC1222, -21, -20, -19	T3SS
XCC2565		1.50	Leucin-rich-repeat-containing protein/type III effector protein (XopAC class)		Pathogenesis
XCC4186		1.49	Leucin-rich-repeat-containing protein/type III effector (XopL class)		Pathogenesis
XCC1221	*hrpE*	1.47	Type III secretion system pilus protein	XCC1222, -21, -20, -19	T3SS complex
XCC2896	*psvA*	1.42	Peptidase C48 family/type III effector protein (XopD class)		Proteolysis
XCC1224	*hpaA*	1.34	Type III secreted virulence factor	XCC1222, -21, -20, -19	Interaction with host via protein secreted by T3SS
XCC1219	*hrpW*	1.33	Harpin pectate lyase	XCC1222, -21, -20, -19	Interaction with host via protein secreted by T3SS
Cell envelope					
XCC0539	*ompW3*	1.39	Putative outer membrane protein		Cell outer membrane
XCC2266	*pglA*[d]	1.31	Putative polygalacturonase		Carbohydrate metabolic process
XCC3925	*ecnA*[d,e]	1.31	Putative entericine A		Response to toxin
Unknown function					
XCC1308	–[d]	2.22	Hypothetical protein	XCC1306, -07, -08	
XCC0401		2.06	Hypothetical protein/ribosomal protein S30Ae/σ^{54} modulation protein		Primary metabolic process
(NC_003902: 3290964-3290833, minus strand[c])		2.05	Small putative membrane protein		
XCC3224		2.03	Hypothetical membrane protein	XCC3227, -26, -25, -24, -23, -22	
XCC1244		1.95	Hypothetical protein		
XCC0944	–[d]	1.83	Conserved hypothetical protein		
XCC3226		1.54	Hypothetical protein	XCC3227, -26, -25, -24, -23, -22	
XCC3798	–[d]	1.47	Calcium-binding EF hand motif		Calcium ion binding
XCC3887		1.35	Hypothetical protein		
XCC2566		1.35	Putative carboxymethylenebutenolidase		Hydrolase activity
XCC0863	–[d,e]	1.33	Putative membrane protein		
XCC1736	–[d]	1.33	Putative secreted protein	XCC1737, -36, -35, -34	Catalytic activity

[a] Ratio, averaged expression ratio of σ^E induced (Δ*rseA*)/no σ^E (Δ*rpoE*).
[b] TUs are listed in chromosomal order.
[c] Nonannotated gene in the genome of *X. campestris* pv. *campestris* ATCC 33913. Genomic location contains *X. campestris* pv. *campestris* ATCC 33913 chromosome accession number followed by the start coordinate, end coordinate, and strand.
[d] A predicted cleavable signal sequence.
[e] Predicted transmembrane helices.

TABLE 3. Genes with decreased expression in Δ*rseA* strain, as compared with Δ*rpoE*

ID	Gene	Ratio[a]	Description of gene product	Characteristic
Transporter activity				
XCC2867	*btuB*[b]	−1.97	TonB-dependent transporter	Transport; membrane
XCC3316	–[b]	−1.73	TonB-dependent transporter	Transport; membrane
XCC2497	–[b]	−1.61	Pseudo-TonB-dependent transporter/Oar like	Transport; membrane
XCC3271	–[b]	−1.54	Pseudo-TonB-Dependent Transporter/Oar like	Transport; membrane
XCC1892	*cirA*[b]	−1.39	TonB-dependent transporter	Transport; membrane
Regulatory function				
XCC3677		−9.64	Putative two-component system sensor kinase	Two-component signal transduction system (phosphorelay)
XCC1935	*rpoN2*	−1.54	RNA polymerase σ^{54} factor	Transcription initiation
XCC1276		−1.50	Putative sensor/response regulator hybrid	Two-component signal transduction system (phosphorelay)
Metabolism				
XCC2410		−23.07	Ketoglutarate semialdehyde dehydrogenase	Metabolic process
XCC2295		−1.68	Putative polyhydroxyalkanoate synthesis repressor	
XCC3324	*ilvB*	−1.57	Acetolactate synthase	Branched-chain family amino acid biosynthetic process
XCC0550	*atpF*	−1.55	F0F1 ATP synthase subunit B	ATP synthesis-coupled proton transport
Nucleic acid metabolic process				
XCC2904	*hsdS*	−7.86	Type I restriction enzyme (specificity chain) homologue	DNA modification
XCC0377	*mnmG*	−1.38	Pyridine nucleotide-disulfide oxidoreductase, class II	tRNA processing
Catalytic activity				
XCC0700	–[b]	−1.39	Putative peptidase S15	Proteolysis
Motility and attachment				
XCC3232	*pilM*	−1.35	Type IV pilus assembly protein PilM	Pilus assembly
Cell envelope				
XCC3017	*ompP6*[b]	−.53	Outer membrane protein P6 precursor (OmpA family)	Cell outer membrane
Oxidative stress function				
XCC1109	*katE*	−5.64	Catalase	Response to oxidative stress
Unknown function				
XCC1080		−5.74	Hypothetical protein	
XCC2823		−1.37	Hypothetical protein	

[a] Ratio, averaged expression ratio of σ^E induced (Δ*rseA*)/no σ^E (Δ*rpoE*).
[b] A predicted cleavable signal sequence.

tris, since several upregulated gene products are highly conserved heat shock proteins (HSPs), such as *dnaK-dnaJ*, *hspA*, *htpG*, and *hslU*, and the heat stress σ^H factor encoded by *rpoH*. Not only important during heat stress, many HSPs assist protein folding and homeostasis and are general stress proteins (49). Two other heat shock protease genes, *hslV* (forming a putative TU, *hslU-hslV*) and *lon*, showed a small but statistically significant 1.23-fold induction (data not shown). In many bacteria, most of these HSP genes are transcribed by the σ^H factor and *rpoH* expression is regulated by σ^E (50), and our data strongly suggest that heat stress regulation is the same in *X. campestris* pv. *campestris*. This is further supported by the presence of σ^H promoter elements (CTTGAAN$_{13\text{-}14}$CCCCA TNT) (41) within the 300 nucleotides upstream of the start codons of *dnaK*, *hspA*, *htpG*, *lon*, and *hslV* (data not shown). Nine σ^E-regulated genes encode proteins involved in protein quality control in the cytoplasm (e.g., DnaK and HslU) and in the periplasm (e.g., Prc and XCC1535), possibly to cope with the defects in membrane protein insertion, folding, and assembly resulting from σ^E-activating stress signals. Interestingly, the predicted periplasmic protease Prc has been reported to be implicated in the proteolysis of the anti-σ^E factors MucA in *P. aeruginosa* and RsiW in *B. subtilis* (32, 57). It is also worth noting that we expected to find *XCC1535* in our analysis, since

it shares the same promoter elements with its orthologue in *X. fastidiosa* (*xf0644*), which has been shown to be a direct target of σ^E (24).

Among the functions encoded by 20 σ^E-downregulated genes, there was an overrepresentation of proton motive force (PMF) consumers that could compete with putative proton leakage in response to localized disturbances in membrane integrity: (i) TonB-dependent transporters (TBDTs), which are in the outer membrane and are mainly involved in iron, vitamin B_{12}, or plant-derived carbohydrate uptake (9); (ii) ATP synthase; and (iii) flagella, since the transcript for the alternative σ factor RpoN2, which is responsible for the transcription of the flagellar T3S system (76), was downregulated upon σ^E overactivation. Thus, σ^E could function to maintain the membrane potential component of the PMF in *X. campestris* pv. *campestris* as in *S.* Typhimurium (7). In addition, the *pilM* gene was repressed by σ^E, and type IV pili could also represent an energy burden for the cell under stressful conditions.

One of the most downregulated genes was *hsdS*, possibly to provide restriction alleviation in response to stress stimuli, in agreement with work showing that the signal of chromosomal DNA damage might be transmitted to the cell surface via activation of the σ^E regulon (4). Among genes negatively regulated by σ^E, there was only one OMP. This minor inhibitory effect on OMP biosynthesis contrasts with other bacteria in which the σ^E response limits the expression of a number of OMPs via small RNAs (54) to prevent the accumulation of misfolded intermediates. This discrepancy may be due to the setup of our experiments, since we did not overexpress σ^E (instead, σ^E was constantly activated), but it could also suggest that the σ^E signaling system is regulated differently in *X. campestris* pv. *campestris*.

Validation of microarray experiments and determination of a σ^E-binding consensus motif. In order to validate the results of the microarray data, a subset of five genes from the σ^E regulon was randomly chosen for verification of σ^E dependence *in vivo*, using *lacZ* transcriptional reporter fusion assays. The upstream regions (~500 bp) of the chosen genes were fused to a promoterless *lacZ* reporter gene in a broad-host-range vector as described in Materials and Methods. We also included in this study a non-σ^E-dependent promoter region as a negative control. To test the expression of these promoters with different levels of σ^E activity, the plasmids were transferred to the isogenic WT, Δ*rpoE*, and Δ*rseA* strains. As expected, the gene fusions showed dramatically increased expression in the Δ*rseA* mutant strain containing constitutively high σ^E activity (Fig. 5). However, there was only a small decrease of β-galactosidase activity upon loss of σ^E, probably because the cells were grown under nonactivating conditions. As expected, the expression levels of the control gene *XCC1230* were similar in all strains.

To confirm our transcriptome analysis, we also checked some phenotypes associated with predicted σ^E-regulated functions. In *X. campestris* pv. *campestris*, the alternative σ^{54} factor RpoN2 is responsible for the transcription of the flagellar genes (76). Since RpoN2 was negatively regulated by σ^E, it was possible that *rseA* mutants possessed reduced motility. To test this, we measured the motility of the WT, Δ*rpoN2*, and Δ*rseA* strains (data not shown). The Δ*rpoN2* mutant cells had impaired motility, and Δ*rseA* mutant motility was delayed, since its swimming zone was reproducibly smaller than that of the WT after 2 days at 30°C. These results are consistent with the microarray analyses. In addition, we tested the Δ*rpoE* mutants for pathogenicity on a host plant, since σ^E controlled virulence-associated genes. The *X. campestris* pv. *campestris* WT and mutant strains were inoculated into *Arabidopsis thaliana Sf-2* ecotype leaves by using the leaf-clipping method. The deletion of *rpoE* did not reproducibly alter symptom development (data not shown), suggesting that σ^E is not required for the virulence of *X. campestris* pv. *campestris* under these conditions.

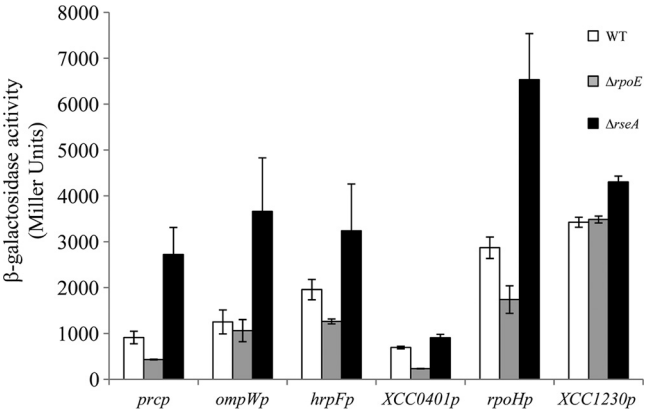


FIG. 5. Expression of σ^E-regulated genes. Reporter plasmids carrying the 5′ ends of selected genes and their upstream promoter regions fused to *lacZ* were transferred to three *X. campestris* pv. *campestris* strains with different σ^E activities: WT, Δ*rpoE*, and Δ*rseA*. Overnight cultures of these strains grown in MOKA medium were diluted in the same medium and grown for 9 h before determination of β-galactosidase activity. The results represent the mean values of at least two independent experiments, each performed in triplicate, and the error bars indicate the standard errors.

To further verify the transcriptome data and to identify σ^E promoter elements, we selected 11 σ^E-upregulated genes from nearly all the functional categories identified, and their promoters were mapped by primer extension (Fig. 6; see Fig. S1 in the supplemental material). We added to our study the *XCC0964* gene (encoding a putative membrane metalloprotease), since it shares promoter elements with its *X. fastidiosa* orthologue (*xf2594*), which has been shown to be a direct target of σ^E (24). We could not detect any reverse transcription product for *XCC1246* unless σ^E was overexpressed from plasmid p917-rpoE, suggesting that the amount of *XCC1246* transcripts was below the detection threshold. For *rpoH*, two primer extension products (*rpoHp1* and *rpoHp2*) were detected. All primer extension experiments confirmed the trend of changes in transcript levels following σ^E activation observed in microarray experiments (Fig. 6, compare lanes Δ*rseA* and Δ*rpoE*), with the exception of the distal promoter of *rpoH* (*rpoHp2*) (see below). Moreover, all promoters except *rpoHp2* were induced when σ^E was overexpressed from the p917-rpoE plasmid [Fig. 6, compare lanes wt(+) and wt(−)]. The proximal promoter *rpoHp1* was controlled by σ^E, since its activity increased when *rseA* or *mucD* was deleted and decreased when *rpoE* was deleted. In addition, the loss of *rpoHp1* activity was complemented by the overexpression of σ^E from plasmid p917-

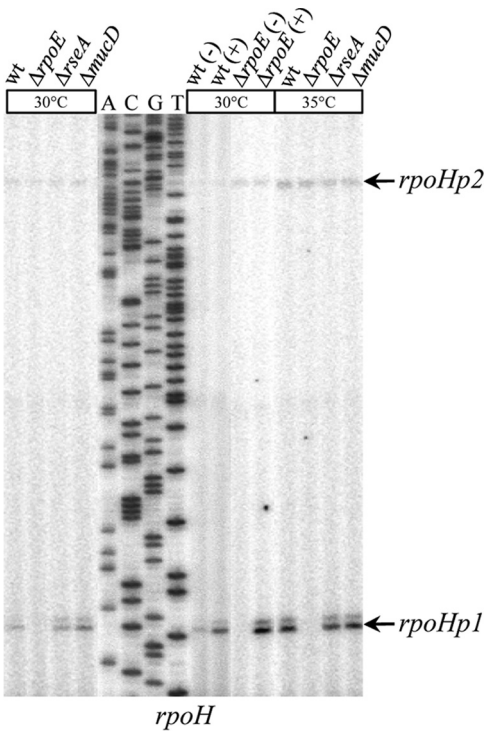


FIG. 6. Determination of the transcription start sites of *rpoH*. Total RNAs from WT, Δ*rpoE*, Δ*rseA*, Δ*mucD*, and WT or Δ*rpoE* strains containing control plasmid p917 (−) or σ^E-overexpressing plasmid p917-rpoE (+) were used as templates in primer extension experiments. Total RNAs were obtained from cells incubated at 30°C or after a 60-min shift at 35°C. Primer rpoH-EXT was 5′ end labeled with ^{32}P and extended with reverse transcriptase to map its corresponding gene promoter sequences. The arrows indicate the bands corresponding to the main start sites observed.

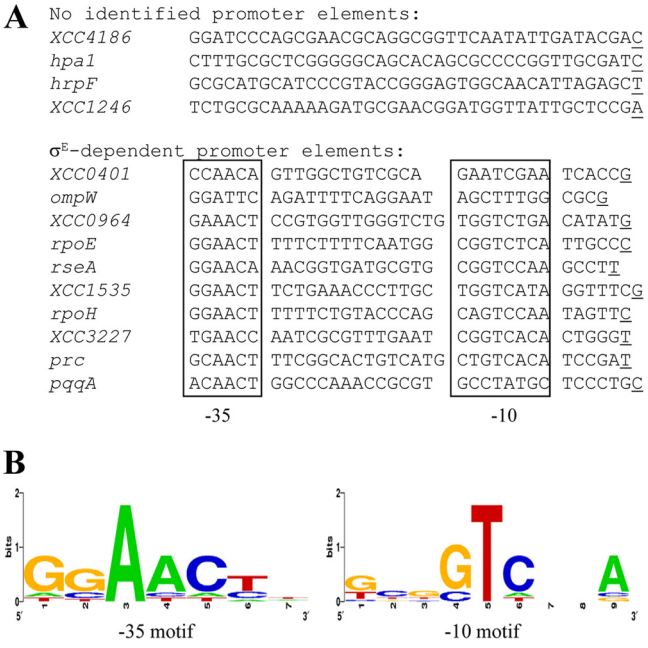


FIG. 7. Identification of the *X. campestris* pv. *campestris* σ^E promoter recognition sequence. (A) Alignment of σ^E-dependent promoters identified by primer extension assays. The transcription start site is underlined, and the −35 and −10 motifs are boxed. (B) The aligned promoter sequences were analyzed using the WebLogo program (http://weblogo.berkeley.edu/). The height of a stack indicates sequence conservation (2 = 100% conservation), and the height of each individual nucleotide within the stack indicates its relative frequency at that position.

rpoE [Fig. 6, compare lanes Δ*rpoE*(+) and Δ*rpoE*(−)]. The distal *rpoHp2* was σ^E independent, since its activity did not vary in mutants of the *rpoE* operon. Overall, these results indicated good verification of the microarray data.

A consensus for the σ^E-binding motif was obtained by alignment of the 40 nucleotides upstream of the experimentally determined start sites and a search for conserved promoter elements identified from other bacterial σ^E regulons and for *rpoEp* and *rseAp* (65) (Fig. 2B). As shown in Fig. 7A, 8 out of the 12 selected genes had these conserved promoter elements, indicating that they could be directly regulated by σ^E. The proposed consensus for *X. campestris* pv. *campestris* σ^E target promoter motifs ($GGAACTN_{15\text{-}17}GTCNNA$) is very similar to the σ^E-binding sequence of homologous ECF σ factors from the ECF02 group (65) (Fig. 2B and 7B). It harbors the hallmark feature of an ECF-type promoter, the AAC motif in the −35 region, and the −10 region contains highly conserved TC and A residues. Interestingly, only half of the 14 mapped promoters depended solely on σ^E for their transcription: *rpoEp*, *rseAp*, *XCC1535p*, *rpoHp2*, *XCC3227p*, *XCC0964p*, and *prcp* (Fig. 2A and 6; see Fig. S1 in the supplemental material). This suggests that partially σ^E-dependent genes could be controlled by other σ factors that overlap functionally in *X. campestris* pv. *campestris*. We were unable to identify σ^E conserved promoter elements for the four T3S-related genes *XCC1246*, *XCC4186*, *hpaI*, and *hrpF*, suggesting that the effect of σ^E on their expression is indirect.

To gain further insight into σ^E activity under heat stress conditions and to further validate our microarray data, we extracted total RNAs from bacterial cultures after a shift to 35°C. We included only the genes whose expression was heat inducible (Fig. 6 and data not shown; see Fig. S1 in the supplemental material). The relative levels of *rpoH*, *ompW*, *XCC3227*, *hspA*, *XCC1535*, and *prc* transcripts dramatically increased upon heat treatment, indicating that a subset of the σ^E regulon was heat responsive. Both promoters of *rpoH* were induced by a temperature upshift, but only *rpoHp1* was upregulated in a σ^E-dependent manner. We failed to identify conserved promoter elements upstream of the *rpoHp2* start site, so we could not predict which σ factor could be in charge of its transcription. The heat inducibility of *XCC3227p*, *XCC1535p*, and *prcp* expression was dependent on σ^E (see Fig. S1 in the supplemental material), as for *rpoEp* and *rseAp* (Fig. 2A). Hence, this suggests that their induction is likely to be accomplished by activation of σ^E upon heat exposure, probably mediated by anti-σ RseA cleavage. In contrast, *ompWp* heat induction was σ^E independent, and this will need further investigation. Interestingly, we could not directly test the impact of σ^H on gene expression because, despite numerous attempts, we could not inactivate the *rpoH* gene. It is thus likely (although not formally proven) that *rpoH* is essential in *X. campestris* pv. *campestris*, as it is thought to be in other bacteria (29, 63).

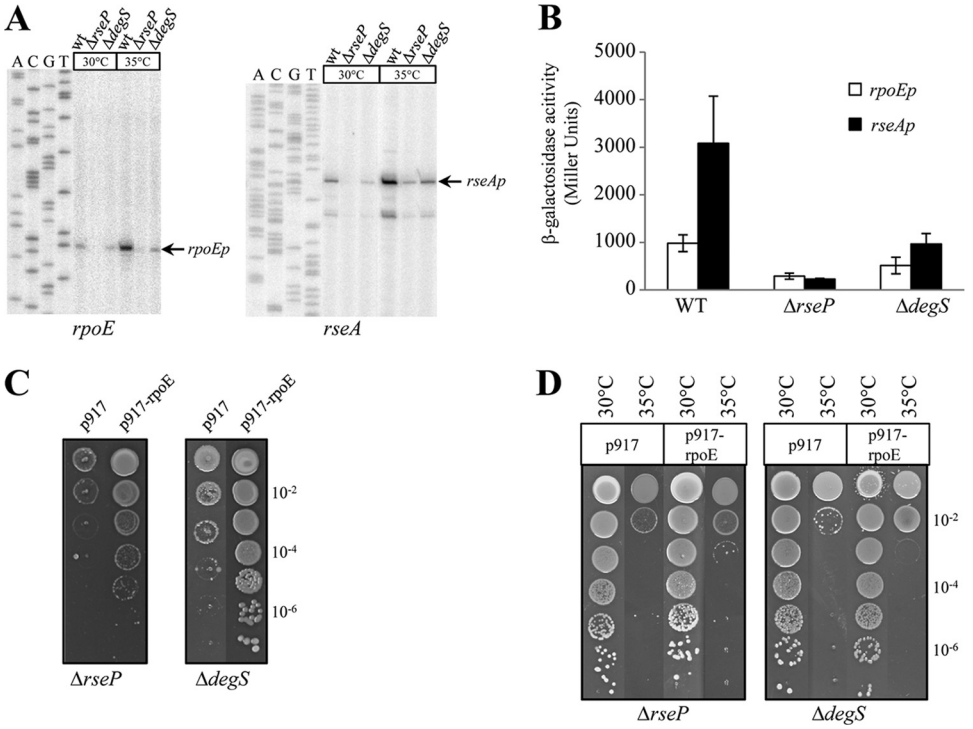


FIG. 8. The deletion of *rseP* or *degS* impairs σ^E activation. (A) Expression of σ^E-dependent promoters using primer extension assays. Total RNA was prepared from the WT, Δ*rseP*, and Δ*degS* mutant strains grown at 30°C or exposed to a temperature upshift at 35°C for 60 min. Primers rpoE-EXT and rseA(2)-EXT were used. (B) Determination of *rpoEp* and *rseAp* activities in WT, Δ*degS*, and Δ*rseP* strains. Plasmids containing a transcriptional fusion of the upstream region of *rpoEp* or *rseAp* to the *lacZ* gene were transferred into *X. campestris* pv. *campestris* strains. Overnight cultures of these strains grown in MOKA medium were diluted in the same medium and grown for 9 h before determination of β-galactosidase activity. The results represent the mean values of at least two independent experiments, each performed in triplicate, and the error bars indicate the standard errors. (C) Influence of 40 μM cadmium on growth of *X. campestris* pv. *campestris* WT, Δ*degS*, and Δ*rseP* strains containing control plasmid p917 or σ^E-overexpressing plasmid p917-rpoE. Serial 10-fold dilutions of late-exponential-phase bacteria were spotted on MOKA plates containing cadmium and incubated at 30°C for 72 h. (D) Heat sensitivities of *X. campestris* pv. *campestris* WT, Δ*degS*, and Δ*rseP* strains containing control plasmid p917 or σ^E-overexpressing plasmid p917-rpoE. Serial 10-fold dilutions of late-exponential-phase bacteria were spotted on plates and incubated at 35°C for 72 h. For panels C and D, each experiment was repeated three times.

Regulation of σ^E-mediated response by RseP (XCC1366) and DegS (XCC3898). Upon cell envelope stress in *E. coli*, RseA is sequentially cleaved by the RIP proteases DegS and YaeL (1). Specifically, the recognition of the C termini of unfolded OMPs allows DegS to cleave the periplasmic C terminus (site 1) of RseA. This converts RseA into a substrate for RseP, which cleaves the transmembrane segment (site 2) of RseA. To gain insights into the σ^E activation pathway, we asked whether the σ^E-activating signal generated in *X. campestris* pv. *campestris* also requires DegS and RseP. To find the *X. campestris* pv. *campestris* homologues of DegS and RseP, BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches were performed using DegS and RseP homologues from *E. coli* and *P. aeruginosa*. The *X. campestris* pv. *campestris* genome contains a homologue of RseP (XCC1366, with its predicted amino acid sequence sharing 56% and 62% similarity with *E. coli* RseP and *P. aeruginosa* MucP, respectively; XCC1366 will be referred to as RseP protease), and XCC3898, annotated as protease DO, had the highest level of homology to DegS proteins (XCC3898, with its predicted amino acid sequence sharing 61% similarity with *E. coli* DegS and *P. aeruginosa* AlgW; XCC3898 will be referred to as DegS protease). In *X. campestris* pv. *campestris*, there was also significant similarity (62%) between DegS and MucD, since they belong to the widely conserved family of HtrA proteins (18).

To probe the role of the RseP and DegS proteases in σ^E-dependent transcription in *X. campestris* pv. *campestris*, we constructed chromosomal in-frame deletions of *rseP* or *degS*. As shown in Fig. 8A, upon deletion of *degS* or *rseP*, there was a strong decrease in the expression of the σ^E-dependent promoters *rpoEp* and *rseAp* at both 30°C and 35°C compared to the WT levels. Moreover, σ^E-dependent transcriptional activity from *rpoEp-lacZ* or *rseAp-lacZ* in the Δ*rseP* and Δ*degS* mutants was significantly reduced during normal growth (Fig. 8B). These results indicated that *degS* and *rseP* are required for the activation of σ^E, thus suggesting a role for DegS and RseP proteases in cleaving RseA in *X. campestris* pv. *campestris*. Interestingly, the two proteases do not have the same impact on σ^E-dependent activity, since the Δ*rseP* mutant was the most affected. If the RIP protease cascade is conserved in *X. campestris* pv. *campestris*, this raises the possibility that, at least under certain conditions, site 1 proteases other than DegS could initiate the cleavage of RseA or that the second-site cleavage of RseA by RseP could occur independently of a site 1 protease. However, σ^E-dependent activity was still induced in Δ*rseP* and Δ*degS* mutants following a temperature upshift to

35°C (Fig. 8A), indicating that RseP and DegS had little or no impact on the heat stress response of σ^E activation.

The protease mutants were then tested for σ^E-dependent stress responses, such as resistance to cadmium (Fig. 8C) and adaptation to increased temperature (Fig. 8D). As expected, deletion of *rseP* or *degS* resulted in a strong growth defect in the presence of cadmium, similar to the results obtained with a Δ*rpoE* mutant (Fig. 3A). The overexpression of σ^E from plasmid p917-rpoE restored resistance to cadmium, indicating that overexpression of σ^E bypasses the need for RseP and DegS for metal resistance. These data point to a role of proteolytically active DegS and RseP in the activation of σ^E in *X. campestris* pv. *campestris* during cadmium stress. At 35°C, growth of the Δ*degS* and the Δ*rseP* mutants was strongly impaired, similar to the results obtained with a Δ*rpoE* mutant (Fig. 4A). However, the overexpression of σ^E from plasmid p917-rpoE did not fully restore the growth defect phenotype of the protease mutant strains, probably because DegS and RseP are essential for growth at 35°C. These results strongly suggest that DegS and RseP degrade cytoplasmic-membrane-localized substrates (other than RseA) that could be involved in the heat stress response. Furthermore, it must be noted that, as for *X. fastidiosa*, there are no σ^E-dependent promoter elements upstream of the *degS* gene, but instead, its promoter presents a putative σ^H consensus sequence (data not shown). Unfortunately, we have been unable to experimentally identify *degS* or *rseP* promoters, so that the regulation of these genes under stress conditions is still under investigation. All these data show that DegS and RseP are required to activate σ^E, and this is probably achieved by two-step proteolysis to liberate σ^E from RseA. However, a residual induction of σ^E activity could still occur without these proteases, depending on growth conditions.

DISCUSSION

Here, we have combined different approaches to investigate the role and mechanism of σ^E-dependent activation in *X. campestris* pv. *campestris*. We demonstrated that σ^E is an important regulator of the stress response of *X. campestris* pv. *campestris*, since it is involved in stationary-phase survival and resistance to membrane-perturbing stresses. However, we were unable to find any increased sensitivity of the *rpoE* mutant to a variety of chemical stresses (data not shown). This limited response was not surprising, since there are 9 other ECF σ factors in *X. campestris* pv. *campestris*. Each one could respond to a specific stress stimulus and could substitute functionally for the others, since ECFs tend to share overlapping promoter specificity, as has been well described in *B. subtilis* for σ^M, σ^W, and σ^X (43). Knowledge of the remaining ECF σ factors and their inducing signals is sparse, and it is crucial to address these issues in future studies.

As in *X. campestris* pv. *campestris* strain 11 and *X. fastidiosa*, the *rpoE* gene of *X. campestris* pv. *campestris* is organized as a single transcription unit with the anti-σ gene *rseA* and the protease gene *mucD*. However, the transcriptional control of *rpoE* expression is different in these bacterial species, since it is controlled by the housekeeping σ factor in *X. fastidiosa* (24), while transcription of *rpoE* is autoregulated in *X. campestris* pv. *campestris*, like most of its known orthologues (our results and reference 17). This indicates that regulatory pathways may have evolved differently in these closely related bacterial species in order to be able to process different environmental parameters, since *X. fastidiosa* appears to have an *in planta*-restricted lifestyle while *X. campestris* pv. *campestris* is able to survive in the environment between infections. Our results strongly suggest that RseA is the anti-σ^E factor and that MucD is a negative regulator of σ^E-dependent activity. The transcription of *rseA* and *mucD* is controlled by a highly conserved σ^E-dependent promoter within the σ^E gene (24, 59). This negative feedback loop would ensure the tight control of σ^E-dependent activity in *X. campestris* pv. *campestris* in order to avoid the energy cost of an inappropriate σ^E response, as we observed that the overexpression of σ^E was deleterious to *X. campestris* pv. *campestris* fitness.

We used microarrays to define the *X. campestris* pv. *campestris* σ^E regulon by comparing the levels of the transcripts of all annotated ORFs in the *rpoE* deletion mutant with those in the *rseA* deletion mutant in order to be closer to physiological levels of induction of the σ^E response. σ^E upregulates at least 45 genes, including *rpoE* regulon members themselves, and 20 genes are downregulated upon σ^E overactivation. We identified the *X. campestris* pv. *campestris* σ^E promoter consensus motif (GGAACTN$_{15\text{-}17}$GTCNNA), which has the conserved characteristics of promoter elements of ECF σ factors from the ECF02 group (65). Most σ^E-dependent genes fall broadly into the same categories previously described for the core and extended σ^E regulon members (59) with structural components of the cell envelope, OMP assembly, the periplasmic chaperone, fatty acid biosynthesis, and two-component systems. The higher electron transport system activity could possibly compensate for proton leakage across the membrane when its integrity is compromised in order to maintain the PMF. In agreement with this notion, a functional σ^E regulon was essential for the maintenance of PMF in *S.* Typhimurium (7), and we observed that genes encoding functions that are PMF-energized processes (ATP synthesis, motility, and active transport) were downregulated when σ^E was overactive.

In contrast to core σ^E regulons of enterobacteria, we did not find genes involved in cell envelope biogenesis (59). Variation in growth conditions, treatment conditions, and microarray platforms could be the reasons for this discrepancy. A subset of σ^E-dependent genes in *X. campestris* pv. *campestris* belongs to the highly conserved heat shock regulon involved in aiding protein folding, protein disaggregation, and proteolysis and comprising the heat shock sigma factor (*rpoH*). We also showed that *rpoH* transcription was directly regulated by σ^E under both normal and heat stress conditions, so that σ^H could in turn direct transcription of the heat shock regulon. The ability of σ^E to mediate the expression of *rpoH* has been reported in several other bacteria, particularly in gammaproteobacteria (3, 69). It is noteworthy that the *rpoH* gene of *X. campestris* pv. *campestris* strain 11 has σ^E promoter elements identical to those in *X. campestris* pv. *campestris*, but previous work did not correctly predict the σ^E-dependent promoter of *rpoH* in *X. campestris* pv. *campestris* strain 11 (37). We have also identified a distal σ^E-independent promoter for *rpoH*, and its expression was slightly induced by temperature. These data underline the complex regulation of *rpoH*, as in many other bacteria, e.g., in *E. coli* there are five promoters upstream of

the *rpoH* gene, recognized by σ^{70}, σ^{S}, σ^{E}, and σ^{54} (38). This ensures that the protective heat shock response can be triggered by several environmental cues or during the cell cycle. In *X. campestris* pv. *campestris* it seems particularly relevant, since we could not inactivate the *rpoH* gene under normal growth conditions (data not shown). Moreover, we observed that *rpoEp*, *rseAp*, *XCC1535p*, *XCC3227p*, and *prcp* were directly activated by σ^{E} following heat treatment at 35°C. XCC1535 and Prc are predicted to be periplasmic proteins and to have roles, respectively, in protein folding and proteolysis. This suggests that they could prevent aggregation of misfolded periplasmic protein derivatives under increased export stress, which could occur when cellular envelope integrity is compromised. Taken together, our results support the essential role of *rpoE* in heat stress protection of *X. campestris* pv. *campestris* and suggest that the heat shock response in *X. campestris* pv. *campestris* is similar to that of *E. coli* and other gammaproteobacteria.

We also found that σ^{E} is involved in the transcriptional activity of a subset of genes encoding proteins involved in the T3S translocation and regulation machinery. Only two of the six *hrp* operons of *X. campestris* pv. *campestris*, *hrpE* and *hrpF*, were induced. This is reminiscent of the regulation of the *hrpC* and *hrpE* operons by the two-component regulatory system ColR/ColS in *X. campestris* pv. *campestris* 8004 (77), suggesting that individual *hrp* operons might be targeted by alternative regulatory networks integrating diverse environmental signals. Taken together with the contribution of σ^{E} to the functionality of T3S systems of the enteropathogens *Yersinia pseudotuberculosis* and *S.* Typhimurium (15, 53), this regulation suggests a link between cell envelope perturbation and T3S induction in *X. campestris* pv. *campestris*. This could occur during the pathogenesis process in response to host factors (oxidative antimicrobial systems and membrane-targeted host defense peptides) that compromise bacterial membrane integrity. We tested the hypothesis that σ^{E} could be involved in *X. campestris* pv. *campestris* pathogenesis, but we were unable to see significant differences between the WT and the Δ*rpoE* mutant when we inoculated them into *A. thaliana* (data not shown). These results are in agreement with previous studies based on *X. campestris* pv. *campestris* strain 11 (17). This lack of a phenotype does not exclude the possibility that σ^{E} has a role under natural infection conditions of *X. campestris* pv. *campestris* that may be discovered using a different plant model or route of infection. Moreover, T3S is involved not only in pathogenesis, but also in pathogen dissemination. Mutation of genes encoding structural components and regulatory genes of the T3SS of *Xanthomonas fuscans* subsp. *fuscans* altered the ability of the bacterium to transmit to bean seeds through both vascular and floral pathways (21). The importance of *rpoE* in resisting external stresses suggests that σ^{E} may be involved in survival in the environment, which constitutes a prerequisite to plant infection and disease development (35). Bacterial epiphytic fitness and seed transmission experiments will be crucial to decipher the role of σ^{E} in the dispersion of plant-pathogenic bacteria, colonization, and survival on their hosts.

In *E. coli*, the σ^{E} response is triggered when periplasmic protein folding and assembly are compromised. The accumulation of unassembled OMPs is the activation signal for the initiation of a sequential proteolytic cascade of the membrane-spanning anti-σ RseA by DegS and RseP (72). DegS and RseP homologues have been identified in *X. campestris* pv. *campestris* based on sequence homologies. The deletion of their corresponding genes led to a decrease in σ^{E}-dependent activity at both 30°C and 35°C. Our results strongly suggest that the predicted proteases DegS and RseP regulate σ^{E} by the mechanism of RIP as described in *E. coli*. However, there were differences in the proteolytic process: when proteases were inactivated, residual σ^{E}-dependent transcription under normal growth conditions remained, and there was still induction of the σ^{E} response by a temperature upshift. This was rather unexpected, because it would be the first example of σ^{E} activation by a temperature upshift that did not require DegS and RseP proteases. Consequently, it must be assumed that RseA/σ^{E} cleavage can be performed by other proteases, depending on stress stimuli, in *X. campestris* pv. *campestris*. This is supported by the recent findings that acid stress activation of σ^{E}-dependent gene expression in *S.* Typhimurium is independent of the unfolded OMP signal or the DegS protease (48). These observations unravel a new activation pathway for the σ^{E} response. In addition, in *E. coli*, it has been shown that the HtrA family members DegQ and DegS can complement the loss of the periplasmic protease Prc (6). Interestingly, *prc* was one of the σ^{E} target genes identified in our microarray analysis, and we also showed that its expression was heat inducible. Tail-specific proteases like Prc have been reported to be implicated in RIP: CtpB of *B. subtilis* triggers activation of σ^{K} indirectly by cleaving the SpoIVFA protein (14), and Prc of *P. aeruginosa* is implicated in the AlgU/MucA σ/anti-σ system (57). Hence, it is conceivable that Prc is involved in RseA/σ^{E} cleavage in *X. campestris* pv. *campestris* under some conditions, and it will be the aim of future work to address these hypotheses. These unusual cleavage features of the RseA/σ^{E} system in *X. campestris* pv. *campestris* could also be due to the absence of the *rseB* gene in the *rpoE* operon. RseB is a negative regulator of the σ^{E}-dependent envelope stress response that binds to the periplasmic domain of RseA (1). RseB prevents DegS and RseP from cleaving RseA, suggesting that interaction of RseB with RseA must be altered before the signal transduction cascade is activated. There is no homologue of RseB in the *X. campestris* pv. *campestris* genome, and it is unknown if another protein could fulfill its protective function for inappropriate cleavage of RseA in the absence of activating stress.

Other observations were that *degS* and *rseP* mutants were strongly affected for growth at 35°C and in the presence of cadmium but that σ^{E} overexpression in these mutant strains restored growth only in the presence of heavy metals. These data implied that both DegS and RseP are required for growth at 35°C, possibly because they could have other substrates. It is the case for *E. coli* RseP, which could use the signal-transducing protein FecR as a substrate (13), and the *B. subtilis* RseP orthologue, RasP, which can attack the cell division protein FtsL (12). In addition, DegS and MucD belong to the HtrA protein family. In *E. coli*, the HtrA family member DegQ can rescue the conditional lethality of *degP* deletion (6, 71). It is tempting to speculate that since there is no homologue of DegQ in *X. campestris* pv. *campestris*, DegS could also substitute for the *X. campestris* pv. *campestris* DegP orthologue, MucD. This could explain why the *mucD* mutant is not affected by temperature stress in *X. campestris* pv. *campestris*, whereas

in most bacterial species *degP* mutants show a thermosensitive phenotype *in vitro* (47, 74).

Taken together, the data show that while the general function of σ^E is regulation of the extracellular stress response in *X. campestris* pv. *campestris*, its specific functions and regulation pathways are complex and multifaceted. A lot of questions remain to be answered, and continued work on the role and RIP mechanism of the σ^E response will be of major importance in ultimately understanding the nature and complexity of the envelope stress response, as these regulatory proteins can be found in a great variety of prokaryotes.

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