Component and protein domain exchange analysis of a thermoresponsive, two-component regulatory system of *Pseudomonas syringae*

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Two closely related phytopathogenic bacterial strains, *Pseudomonas syringae* pv. *glycinea* PG4180 and *P. syringae* pv. *tomato* DC3000, produce the chlorosis-inducing phytotoxin coronatine (COR) in a remarkably divergent manner. PG4180 produces COR at the virulence-promoting temperature of 18 °C, but not at 28 °C. In contrast, temperature has no effect on COR synthesis in DC3000. A modified two-component system consisting of the histidine protein kinase (HPK), CorS, the response regulator (RR), CorR, and a third component, CorP, governs COR biosynthesis in both strains. A plasmid-based component and domain swapping approach was used to introduce different combinations of RRs, HPKs and hybrid HPKs into *corS* mutants of both strains. Subsequently, expression levels of the COR biosynthetic *cma* operon were determined using RNA dot-blot analysis, suggesting that CorRSP of PG4180 mediates a thermoresponsive phenotype dependent on the genomic background of each strain. The reciprocal experiment demonstrated a loss of temperature dependence in the *corS* mutant of PG4180. The presence of *corR* from PG4180 led to more pronounced *cma* expression in DC3000 and was associated with thermoresponsiveness, while *corS* of PG4180 did not mediate a temperature-dependent phenotype in the DC3000 mutant containing native *corR* and *corP*. These findings were substantiated by RT-PCR experiments. The C-terminal domain of CorS of PG4180 mediated thermosensing, while the N terminus did not respond to temperature changes, suggesting cytosolic perception of the temperature signal.

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INTRODUCTION

The non-host-specific phytotoxin coronatine (COR) is a virulence factor produced by several pathovars of *Pseudomonas syringae* that significantly contributes to the development of disease symptoms on infected host plants. COR is produced by *P. syringae* pvs *alisalensis*, *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, *porri* and *tomato*, which infect broccoli, ryegrass, soybean, crucifers, *Prunus* spp., leek and tomato, respectively (Bender *et al.*, 1989; Cintas *et al.*, 2002; Koike *et al.*, 1999; Mitchell, 1982; Völksch & Weingart, 1998; Zhao *et al.*, 2002). COR consists of two distinct moieties, the polyketide coronafacic acid (CFA) and a cyclized amino acid, coronamic acid (CMA), which are synthesized independently and fused by amide bond linkage (Mitchell *et al.*, 1994; Parry *et al.*, 1994).

P. syringae pv. *glycinea* PG4180 produces COR in a strongly temperature-dependent manner, with maximum

synthesis at 18 °C and negligible synthesis at 28 °C, which is the optimal growth temperature of the bacterium (Budde *et al.*, 1998; Palmer & Bender, 1993). In PG4180, enzymes involved in COR biosynthesis are encoded on a plasmid-borne 32 kb DNA region which has been characterized extensively (Bender *et al.*, 1993; Liyanage *et al.*, 1995a, b; Ullrich & Bender, 1994; Ullrich *et al.*, 1995). Two biosynthetic operons, required for synthesis of CMA and CFA, respectively, and a 3.4 kb regulatory region encoding a modified two-component system (TCS) were identified (Couch *et al.*, 2004; Rangaswamy *et al.*, 1998; Ullrich & Bender, 1994; Bender *et al.*, 1996) (Fig. 1). Transcriptional fusions of a promoterless β -glucuronidase (*uidA*) gene to promoter regions of both operons showed maximal activity at 18 °C, indicating that COR biosynthesis in PG4180 is regulated by temperature at the transcriptional level (Budde *et al.*, 1998; Liyanage *et al.*, 1995b; Ullrich *et al.*, 1995).

The CorRSP regulatory system is composed of the histidine protein kinase (HPK), CorS, the response regulator (RR), CorR and a third component, termed CorP. Insertional

Abbreviations: CFA, coronafacic acid; CMA, coronamic acid; COR, coronatine; HPK, histidine protein kinase; RR, response regulator; TCS, two-component system.

Fig. 1. Functional map of the COR biosynthetic gene cluster of PG4180 showing DNA regions required for CFA and CMA biosynthesis, respectively, and for transcriptional regulation. Arrows indicate thermoresponsive *cfl* and *cma* promoter regions.

mutations in *corR*, *corS* and *corP*, respectively, completely abolished transcriptional activation of COR biosynthetic promoters (Bender *et al.*, 1993; Ullrich *et al.*, 1995). Moreover, a 3.4 kb DNA fragment containing *corRSP* restored temperature-regulated transcriptional activation of the COR biosynthetic promoters and COR production in the respective mutants (Ullrich *et al.*, 1995). CorS is believed to respond to a temperature change via autophosphorylation of a conserved histidine residue, followed by transduction of the signal to CorR via phosphorylation of its conserved aspartate residue (Rangaswamy & Bender, 2000). A conserved helix–turn–helix DNA-binding motif was identified in CorR, which binds to DNA upstream of both biosynthetic operons, while CorP lacks such a motif (Peñaloza-Vázquez & Bender, 1998; Wang *et al.*, 1999). Overproduction of CorR in a *corS* mutant background of PG4180 or at 28 °C resulted in an inactive protein in DNA-binding assays (Wang *et al.*, 1999), highlighting the importance of a functional CorS.

According to topology prediction and reporter enzyme fusion assays (Smirnova & Ullrich, 2004), the N terminus of CorS contains six transmembrane domains, whereas the C terminus is located in the cytosol. CorS contains typical histidine kinase domains: a dimerization and histidine phosphotransfer domain and a conserved catalytic and ATP-binding domain (CA). A conserved histidine residue (His-254) located in the H-box of CorS is the presumptive site of autophosphorylation. The CA domain of CorS consists of other conserved HPK motifs, such as the N-, D-, F- and G-boxes, which are involved in ATP-binding, catalysis and phosphotransfer (Grebe & Stock, 1999).

The *P. syringae* pv. tomato DC3000 genome sequencing project (http://pseudomonas-syringae.org/pst_home.html) has allowed a comparative analysis of genes involved in regulation of COR biosynthesis. Genes encoding CorRSP are present in the DC3000 genome (Brooks *et al.*, 2004). However, in DC3000 the *corRSP* genes map with the CMA structural genes and are separated from the CFA structural genes by a 26 kb DNA region (Buell *et al.*, 2003; Sreedharan *et al.*, 2006). DC3000 produces significantly less COR than PG4180 *in vitro*, and temperature does not affect COR biosynthesis.

Recently, Smirnova *et al.* (2008) conducted site-directed mutagenesis of *corS* from PG4180 in order to convert its gene product into a non-temperature-responsive derivative, like CorS of DC3000. Either inactive enzyme variants or even more thermoresponsive ones were obtained, but no

clear conversion of phenotype could be accomplished. These results indicated that single or combined amino acid residue changes easily disturb the complex character of a membrane-bound HPK. Consequently, a novel strategy was approached in the present paper.

Herein, a *corRS* mutant of DC3000 was constructed to assess the role of HPK and RR in regulation of COR production in DC3000. By exchanging the respective TCS components between PG4180 and DC3000 in a plasmid-borne complementation assay with *corRS* mutants of either strain, differences in the regulatory function of CorS were investigated. Furthermore, CorS hybrids derived from PG4180 and DC3000 were generated to analyse the role of the membrane-embedded N terminus and the cytosolic C terminus of this HPK for thermosensing.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains used in this study are listed in Table 1. Plasmids are listed in Table 2. *Escherichia coli* cells were grown at 37 °C in Luria–Bertani (LB) medium. *P. syringae* cells were maintained at 28 °C on Mannitol–Glutamate (MG) medium (Keane *et al.*, 1970). For liquid cultures, *P. syringae* cells were grown at 18 and 28 °C at 280 r.p.m. in Hoitink–Sinden medium optimized for COR production (HSC) (Palmer & Bender, 1993). Antibiotics were used at the following concentrations: ampicillin (Ap, 100 µg ml^{−1}), kanamycin (Km, 25 µg ml^{−1}), tetracycline (25 µg ml^{−1}).

Construction of the *corRS* mutant DC3000.M1. Unless otherwise indicated, standard methods were performed according to Sambrook & Russell (2001). Marker exchange mutagenesis was used to generate a knockout mutant of *corS* in *P. syringae* DC3000. Two fragments, each of which contained sequences adjacent to *corS*, were PCR-amplified from the genomic DNA of DC3000. Using primers out_FcorS (5'-CTAG-TCTAGATGTAATGCGGAAAAACGCCT-3') and in_RcorS (5'-CCAGAATTCTGAGTTCGACCAGAAATGAGT-3'), a 1.4 kb *XbaI*–*EcoRI* fragment was amplified, and using primers in_FcorS (5'-CCGGAATTCCTGTTTATGCGCACCTCAAC-3') and out_RcorS (5'-CAAGGTACCGGTACAACGTTCTGTTGCCA-3') a 1.6 kb *EcoRI*–*KpnI* fragment was amplified, and subsequently ligated to pBluescript, yielding plasmid pBSXK30. A 1.7 kb Km cassette from pMKm (Murillo *et al.*, 1994) was inserted into a unique *EcoRI* site of pBSXK30, yielding the suicide vector pBSXB47, which was mobilized into DC3000 by electroporation followed by screening for Ap^r Km^r derivatives. Recombination of the Km cassette into genomic *corS* was verified by Southern blot analysis and PCR using primers corS_fwd_DC3000 (5'-GGAATTCGCGATAGACTCAATTAAT-3') and corS_rev_DC3000 (5'-GGAATTCAGGTATACGTAGTCCATG-3').

Construction of plasmid-borne TCS exchange systems. First, a plasmid-based system containing *corRSP* was created, which enabled

Table 1. Bacterial strains used in this study

Bacterial strain	Relevant characteristics*	Reference
<i>Escherichia coli</i>		
DH5 α	<i>F'</i> /endA1 <i>hsdR17</i> (τ_k^- m_k^+) <i>relA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA</i> (Nal ^r) Δ (<i>lacIZYA-argF</i>)U169 <i>deoR</i> ϕ 80 <i>dlacZ</i> Δ M15	Sambrook & Russell (2001)
HB101	<i>F'</i> Δ (<i>gpt-proA</i>)62 <i>leuB6</i> <i>supE44</i> <i>ara-14</i> <i>galK2</i> <i>lacY1</i> Δ (<i>mrcC-mrr</i>) <i>rpsL20</i> (Sm ^r) <i>xyl-5</i> <i>mtl-1</i> <i>recA13</i>	Sambrook & Russell (2001)
<i>P. syringae</i> pv. <i>glycinea</i>		
PG4180	COR ⁺ CFA ⁺ CMA ⁺	Mitchell (1982)
PG4180.D4	<i>corR::Tn5</i> ; Km ^r <i>corR</i> ⁻ <i>corS</i> ⁻	Bender <i>et al.</i> (1993)
<i>P. syringae</i> pv. <i>tomato</i>		
DC3000	Derivative of NCPPB1106, Rf ^r	Cuppels (1986)
DC3000.M1	Km ^r <i>corS</i> ⁻	This study

*Abbreviations: Km, kanamycin; Nal, nalidixic acid; Rf, rifampicin; Sm, streptomycin.

us to remove the *corS* gene of PG4180 and substitute it with *corS* from DC3000 or hybrid-*corS* (Fig. 2). *corRSP* from PG4180 were amplified as a 3.4 kb PCR product using primers T3*Hind*III (5'-GGCAAGCTTATTAACCCCTCACTAAAG-3') and T7*Hind*III (5'-GACAAGCTTAATACGACTCACTATAG-3'), and subsequently ligated into pRK415, resulting in plasmid pASH34. To generate a construct with unique restriction sites upstream and downstream of *corS* and within non-coding regions, three PCR fragments were amplified using pASH34 as template. The following primers were used: for *corR*, T7*Hind*III (5'-GACAAGCTTAATACGACTCACTATAG-3') and *corR*SpeI_{rev} (5'-GTCGTGCCTCACAGGACTAGT-CCTAGGCGGCGCGCGGCA-3'); for *corS*, *corS*SpeI_{fwd} (5'-ATGCCGCGCGCGCTAGGACTAGTCTGTGAGGCACG-3') and *corS*XbaI_{rev} (5'-TGAAAGGTACTAACGGTCTAGAAGGCATTGCTTGTCTG-3'); for *corP*, *corP*XbaI_{fwd} (5'-CAGCAAGGCGATGCCTTC-TAGACCGTTAGTACCTTTCA-3') and T3*Hind*III (5'-GGCAAGCTTATTAACCCCTCACTAAAG-3'). The 1.4 kb amplification product containing *corR* was digested with SpeI and HindIII and ligated to similarly treated pBluescript II SK, yielding pHS14. A 1.5 kb PCR product containing *corS* was treated with SpeI and XbaI and ligated into similarly treated pHS14 to give pHX29. A 0.5 kb PCR product containing *corP* was treated with XbaI and ligated into XbaI-treated pHX29 to give pHX34. The 3.4 kb fragment containing *corRSP* was subcloned into pRK415 using EcoRI. The thereby generated plasmid, pASE34 (Fig. 2), was analogous to pMUH34 (Ullrich *et al.*, 1995), but contained SpeI and XbaI restriction sites upstream and downstream of *corS*, respectively. The PCR products used to construct pASE34 were verified by nucleotide sequencing. Colony PCR was used to amplify a 1.5 kb SpeI-XbaI fragment comprising *corS* from DC3000. The PG4180 *corS* was excised as a 1.5 kb SpeI-XbaI fragment from pASE34 and was replaced by the DC3000 *corS* to yield pASE34D.

Hybrid *corS* genes were constructed by fusion of the N and C termini of *corS* from PG4180 and DC3000, respectively. For the N terminus, the sequence encoding all transmembrane domains was PCR-amplified (0.6 kb) with primers *corS*SpeI_{fwd} and SRN-terminus (5'-CAATCGGCAGAGCCCAAGCCGTAAAGCAGCAC-3'). The C terminus, consisting of the cytosol-located catalytic domains (1.2 kb), was amplified using primer SFC-terminus (5'-GTGCTGCTTTACGG-CTTGGGGCTCTGCCGATTG-3') and *corS*XbaI_{rev}. Subsequently, one N terminus and one C terminus were used as templates in a PCR with primers *corS*SpeI_{fwd} and *corS*XbaI_{rev}. Primers SRN-terminus and SFC-terminus have homologous sequences, therefore PCR products of the N and C terminus will align, resulting in *corS* gene hybrids when amplified with the outer primers *corS*SpeI_{fwd} and *corS*XbaI_{rev}. PCR products were ligated into pGEM-T Easy and

confirmed by nucleotide sequencing. The *corS* gene hybrids were cloned instead of the native *corS* genes into the pRK415-based exchange constructs pASE34 and pASE34:D respectively. Resulting plasmids were then mobilized into *P. syringae* by triparental mating with the helper plasmid pRK2013 (Figurski & Helinski, 1979).

To generate a CorRSP exchange system which contains *corRSP* from DC3000 with unique restriction sites, plasmid pYBH34 was constructed. Using colony PCR, *corR*, *corS* and *corP* from DC3000 were amplified. Restriction sites upstream and downstream of the respective genes were introduced via primers. For *corR*, *corR*HindIII_{fwd} (5'-GAACATGCTGGGCGCAAGCTTATTTGGC-AG-3') and *corR*SpeI_{rev} (5'-GTCGTGCCTCACAGGACTAGT-CCTAGGCGGCGCGCGGCA-3'); for *corS*, *corS*SpeI_{fwd} (5'-AT-GCCGCGCGCGCGCTAGGACTAGTCTGTGAGGCACG-3') and *corS*XbaI_{rev} (5'-TGAAAGGTACTAACGGTCTAGAAGGCATTGCTTGTCTG-3'); and for *corP*, *corP*XbaI_{fwd} (5'-TTGCAAGGCTA-TGCCTTCTAGACCGTGAGTACC-3') and *corP*SacII_{rev} (5'-CCG-CGGGCTCTAGGTCCAGGTTTGTGTGAACA-3') were used. PCR products of *corR* (1.0 kb) and *corS* (1.8 kb) were used as templates in a subsequent PCR with primers *corR*HindIII_{fwd} and *corS*XbaI_{rev}. The 2.8 kb *corRS* PCR product as well as the *corP* PCR product (0.7 kb) were ligated into pGEM-T Easy, yielding plasmids pGEM:*corRS* and pGEM:*corP*, which were confirmed by sequencing. Subsequently, *corP* was transferred into pGEM:*corRS* using XbaI and SacII. The resulting construct (3.5 kb), designated pGEM:*corRSP*, contained all three genes of the TCS from DC3000 which were subsequently transferred to vector pBluescript II SK, yielding pBcorRSP-DC. In this vector, exchange of *corS* from DC3000 against recombinant *corS* from PG4180 and hybrid-*corS* sequences was carried out. Plasmid pBcorRSP-DC was digested with SpeI and XbaI to remove *corS*. The remaining pBcorRP-DC was purified. Subsequently, SpeI/XbaI-treated recombinant or hybrid-*corS* was ligated with pBcorRP-DC. The resulting constructs (Table 2) were mobilized into *P. syringae* by triparental mating.

RNA dot-blot analysis. Bacteria were grown in HSC medium at 18 and 28 °C until an OD₆₀₀ of 1.3–1.5 was reached. Total RNA was isolated by acid phenol/chloroform extraction as described by Schenk *et al.* (2008). Aliquots of total RNA (200 ng per dot) were transferred to positively charged nylon membranes (Pall) using the Minifold I Spot-Blot System (Schleicher & Schuell), according to the manufacturer's recommendations. The digoxigenin-labelled specific RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase and specific PCR products as templates. PCR amplification of the *cma* template was performed using primers *cmaA*-fwd

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics*	Reference/source
pBluescript II SK	Ap ^r ; ColE1 origin, high-copy-number cloning vector	Stratagene
pGEM-T Easy	Ap ^r ; TA cloning vector	Promega
pRK415	Tc ^r ; <i>mob</i> ⁺ ; low-copy-number cloning vector, RK2-derivative	Keen <i>et al.</i> (1988)
pRK2013	Km ^r ; <i>mob</i> ⁺ <i>tra</i> ⁺ ; helper plasmid for triparental mating	Figurski & Helinski (1979)
pMUH34	Tc ^r ; contains a 3.4 kb <i>Hind</i> III fragment with <i>corRSP</i> genes from PG4180 in pRK415	Ullrich <i>et al.</i> (1995)
pBSXK47	Ap ^r Km ^r ; suicide vector for <i>corS</i> mutagenesis	This study
pASH34	Tc ^r ; contains a 3.4 kb <i>Hind</i> III fragment with <i>corRSP</i> genes from DC3000 in pRK415	This study
pHS14	Ap ^r ; contains a 1.4 kb <i>Hind</i> III– <i>Spe</i> I PCR fragment of <i>corR</i> from PG4180 in pBluescript II SK	This study
pHX29	Ap ^r ; contains a 2.9 kb <i>Hind</i> III– <i>Xba</i> I fragment with <i>corRS</i> from PG4180 in pBluescript II SK	This study
pHX34	Ap ^r ; contains a 3.4 kb <i>Hind</i> III– <i>Xba</i> I fragment with <i>corRSP</i> from PG4180 in pBluescript II SK	This study
pASE34	Tc ^r ; contains a 3.4 kb <i>Eco</i> RI fragment with <i>corRSP</i> from PG4180, <i>Spe</i> I and <i>Xba</i> I sites upstream and downstream of <i>corS</i> , in pRK415	This study
pASE34:D	Tc ^r ; contains a 3.4 kb <i>Eco</i> RI fragment with <i>corRP</i> genes from PG4180 and <i>corS</i> from DC3000 in pRK415	This study
pASE34:PG-DC	Tc ^r ; contains a 3.4 kb <i>Eco</i> RI fragment with <i>corRP</i> from PG4180, hybrid <i>corS</i> N terminus from PG4180, C terminus from DC3000, in pRK415	This study
pASE34:DC-PC	Tc ^r ; contains a 3.4 kb <i>Eco</i> RI fragment with <i>corRP</i> from PG4180, hybrid <i>corS</i> N terminus from DC3000, C terminus from PG4180, in pRK415	This study
pGEM: <i>corRS</i>	Ap ^r ; contains a 2.8 kb <i>Hind</i> III– <i>Xba</i> I PCR fragment with <i>corRS</i> from DC3000 introduced in pGEM-T Easy	This study
pGEM: <i>corP</i>	Ap ^r ; contains a 0.7 kb <i>Hind</i> III– <i>Xba</i> I PCR fragment with <i>corP</i> from DC3000 introduced in pGEM-T Easy	This study
pGEM: <i>corRSP</i>	Ap ^r ; contains a 3.5 kb <i>Hind</i> III– <i>Sac</i> I PCR fragment with <i>corRSP</i> from DC3000 introduced in pGEM-T Easy	This study
pB <i>corRSP</i> -DC	Ap ^r ; contains a 3.5 kb <i>Hind</i> III– <i>Sac</i> I fragment with <i>corRSP</i> from DC3000 with introduced <i>Spe</i> I and <i>Xba</i> I sites upstream and downstream of <i>corS</i> in pBluescript II SK	This study
pYBH34	Tc ^r ; contains <i>corRSP</i> from DC3000, <i>Spe</i> I and <i>Xba</i> I sites upstream and downstream of <i>corS</i> , in pRK415	This study
pYBH34:P	Tc ^r ; contains <i>corRP</i> from DC3000, <i>corS</i> from PG4180, in pRK415	This study
pYBH34:PG-DC	Tc ^r ; contains <i>corRP</i> from DC3000, hybrid <i>corS</i> N terminus from PG4180, C terminus from DC3000, in pRK415	This study
pYBH34:DC-PG	Tc ^r ; contains <i>corRP</i> from DC3000, hybrid <i>corS</i> N terminus from DC3000, C terminus from PG4180, in pRK415	This study

*Abbreviations: Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; *mob*, mobilization function; *tra*, transfer function.

(5'-TTTGAGTCGGTCTGCACGCA-3') and *cmaA*-revT7 (5'-TAATACGACTCACTATAGGGAGGGCTGTACGTTGTCTACTAG-3'). For the synthesis of DIG-labelled RNA probes, the Strip-EZ RNA Probe Synthesis and Removal kit (Ambion Europe) and digoxigenin-11-UTP (Roche Diagnostics) were used. The probe was hybridized to the membrane in hybridization solution (50% formamide, 7% SDS, 2% blocking reagent, 0.1% *N*-laurylsarcosine, 5×SSC) at 68 °C for 16 h. After hybridization, the membrane was washed twice for 5 min at room temperature in 2×SSC containing 0.1% SDS, followed by two washes for 15 min at 68 °C in 0.2×SSC containing 0.1% SDS. Hybridization signals were detected by incubation with anti-digoxigenin-AP Fab fragments (Roche Diagnostics) and a fluorescence substrate for alkaline phosphatase (ECF) (Amersham-Pharmacia Biotech) using an FLA-3000 phosphorimager (Raytest). Signals were quantified using the AIDA Image Analyser software package (Raytest).

RT-PCR analysis of *cma* and *corR* expression. Bacterial cells were grown and total RNA was extracted as described above. RNA samples were treated with an excess of RNase-free DNase (Ambion). The oligonucleotides used to amplify *cma* and *corR* signals were: *cmaA*_RT_fw (5'-CCTACCGCCGATTGAGT-3'), *cmaA*_RT-rev (5'-CCAGGCCTTGACGTATCAGT-3'), *corR*_RT_fw (5'-ATGCCGAGCTCTTCGATCT-3'), *corR*_PG_rev (5'-AGACGCCTCCTCGA-TCCT-3') and *corR*_DC_rev (5'-AAGCGCCTGCTCGATCCT-3'). QuantiTect SYBR Green one-step RT-PCRs were performed in triplicate, following the manufacturer's instructions (Qiagen), and using an Mx3000P Real-time cycler instrument (Stratagene). Reactions in which reverse transcriptase was omitted served as negative controls and demonstrated the lack of DNA contamination.

Extraction and quantification of COR. Organic acids were extracted from cell-free bacterial supernatants (1.5 ml) and analysed

Fig. 2. Construction of the TCS-Exchange system illustrated for PG4180. (a) Primer-binding sites (thin arrows) with introduced restriction sites for the generation of three PCR products which were fused to construct the system. Genes encoding CorRSP are represented by large arrows. (b) Primer-binding sites for amplification of the N and C termini of CorS which were fused to generate CorS hybrids. (c) Broad-host-range plasmid containing the final recombinant TCS for introduction into *P. syringae*.

for the presence of COR by high-pressure liquid chromatography (HPLC) as described by Budde *et al.* (1998). Bacterial cell pellets were used to determine total protein content according to Bradford (1976).

RESULTS

Expression levels of *cma* and complementation of PG4180, DC3000 and respective *corRS* mutants

PG4180 had previously been characterized as temperature-dependent with regard to COR biosynthesis and promoter activity of COR biosynthetic genes (Ullrich *et al.*, 1995). In contrast, temperature had little or no effect on DC3000 *in vitro*. In this study, *cmaA* mRNA levels at 18 and 28 °C were quantified in PG4180, DC3000 and *corRS* mutants of both strains (Fig. 3). In PG4180, wild-type *cma* expression was 20-fold higher at 18 compared to 28 °C. The respective *corRS* mutant, PG4180.D4, displayed only basal low levels of

Fig. 3. Expression of *cma* at 18 (hatched bars) and 28 °C (white bars) in *P. syringae* strains PG4180, DC3000, PG4180.D4 and DC3000.M1, and complemented mutants. Quantities represent means of two experiments with six replicates. The *cma* expression at 18 °C in PG4180 represents 100 %. Error bars represent the SD from the means ($n=12$, $P<0.005$).

cma expression at both temperatures. Plasmid pMUH34, containing *corRSP* from PG4180, restored temperature-dependent *cma* expression to this mutant, demonstrating that PG4180.D4 could be fully complemented. In contrast, temperature had virtually no effect on *cma* expression in DC3000. Moreover, DC3000 displayed much lower *cma* expression at 18 °C compared to PG4180. At 28 °C, both strains showed comparable *cma* expression levels. Subsequently, *cma* mRNA abundance was quantified in the *corRS* mutant DC3000.M1. Transcription of *cma* was completely abolished at both temperatures (Fig. 3), suggesting that transcriptional activation of COR biosynthetic genes was *corRS*-dependent in DC3000. Not surprisingly, neither mutant DC3000.M1 nor mutant PG4180.D4 produced any detectable amounts of COR, regardless of temperature, in contrast to the wild-type strains (data not shown; Ullrich *et al.*, 1995; Smirnova *et al.*, 2008).

Previous results revealed that a 3.4 kb fragment containing *corRSP* from PG4180 is sufficient to complement mutations in these regulatory genes with respect to COR production and activity of COR biosynthetic promoters (Ullrich *et al.*, 1995). To achieve complementation of DC3000.M1, we constructed plasmid pASH34 carrying a 3.4 kb fragment containing the three regulatory genes of DC3000. In DC3000.M1(pASH34), equal levels of COR and *cma* mRNA were detected at 18 and 28 °C, comparable to those of the wild-type (Fig. 3 and data not shown), suggesting that recombinant *corRSP* successfully complemented the mutant and were required and sufficient for *cma* expression in DC3000.

Analysis of TCS exchange systems

CorRSP regulate COR production in strains PG4180 and DC3000, despite the obvious differences in thermorespon-

siveness (Weingart *et al.*, 2004). The specific role(s) of individual CorRSP components and CorS domains in thermosensing for COR gene expression were investigated using recombinant CorRSP exchange systems. Constructs with different combinations of CorRSP and CorS hybrids from PG4180 and DC3000 were analysed with respect to *cma* mRNA synthesis in *corRS* mutants of either strain at 18 and 28 °C, respectively (Fig. 4). Three major results obtained from these analyses allowed us to draw the following conclusions.

First, regardless of the mutant background, any combination with *corR* and *corP* from DC3000 yielded low or almost negligible *cma* expression, suggesting that CorR of DC3000 is either not properly expressed or barely active under the tested conditions. In contrast, any combination with *corR* and *corP* from PG4180 gave rise to significant *cma* expression, indicating that CorR of PG4180 was functional.

Second, a clear thermoresponsive activity of CorRSP associated with significantly high *cma* expression was only obtained when CorR of PG4180 was combined with its cognate CorS or the CorS hybrid containing the C terminus derived from PG4180, suggesting that the molecular interplay of CorR and the C terminus of CorS might be essential for temperature-mediated *cma* expression. However, this interaction additionally seemed to depend on the cellular background, since a combination of PG4180-derived CorR with its cognate CorS or a CorS hybrid containing a PG4180-based C terminus remained non-thermoresponsive in the DC3000 mutant.

Third, very puzzling results were obtained when *corR* and *corP* from PG4180 were combined with *corS* of DC3000: while this construct yielded moderate *cma* expression in the PG4180 mutant regardless of temperature, it showed

moderate but thermoresponsive *cma* expression in the DC3000 background. Consequently, the construct was generated *de novo* and the analyses were repeated several times, each time giving the same inconclusive combination of results. Similarly unclear was the finding that the C terminus of CorS of DC3000 in combination with the N terminus of CorS of PG4180 and CorRP from PG4180 yielded low but still thermoresponsive *cma* expression. After generating this construct again, similar results were obtained. These inconsistencies prompted us to conduct confirmatory RT-PCR experiments with some of the exchange constructs.

RT-PCR analysis with selected TCS exchange constructs

To substantiate the main results of the RNA dot-blot analyses, RT-PCR experiments were performed with six of the TCS exchange constructs harbouring different combinations of CorRSP and CorS hybrids from PG4180 or DC3000. Samples were analysed with respect to *cma* and *corR* mRNA synthesis in *corRS* mutants of either strain at 18 and 28 °C, respectively (Fig. 5). As expected, the presence of *corRSP* from PG4180 and from DC3000 resulted in strong thermoresponsive *cma* expression versus low level temperature-independent *cma* expression, respectively. Whenever *corRP* were derived from DC3000, a low level of expression of *cma* was detected, while *corRP* from PG4180 resulted in higher levels of expression, thus confirming the RNA dot-blot results. The analysis of the construct, in which CorRP and the N terminus of CorS from PG4180 were combined with the C terminus of CorS derived from DC3000 and expressed in PG4180.D4, did not confirm the low-level but thermoresponsive result of

Fig. 4. Expression of *cma* at 18 (hatched bars) and 28 °C (white bars) in *P. syringae* strains PG4180.D4 and DC3000.M1 harbouring CorRSP exchange plasmids. The table summarizes the recombinant systems: grey boxes represent non-native genes or non-native parts of genes. PG, PG4180-derived components or domains; DC, DC3000-derived components or domains. Quantities represent means of two experiments with six replicates. The relative mRNA level is relative to the level of *cma* mRNA synthesized at 18 °C in PG4180, which was defined as 100 %. Error bars represent the SD from the means ($n=12$, $P<0.005$).

Fig. 5. RT-PCR analyses for the expression of *cma* and *corR* with selected TCS exchange constructs at 18 (hatched bars) and 28 °C (white bars). The table summarizes the recombinant systems: grey boxes represent non-native genes or non-native parts of genes. PG, PG4180-derived components or domains; DC, DC3000-derived components or domains. Quantities represent means of three experiments with four replicates. Relative expression levels are related to the level of the highest signals, which were defined as 100%. Error bars represent the SD from the means ($n=12$, $P<0.005$).

this construct derived from RNA dot-blot analysis (Fig. 5). Also, in contrast to the above results, thermoresponsive *cma* expression of the construct with CorRP from PG4180 and CorS from DC3000 present in DC3000.M1 was moderate but temperature-independent.

The analysis of *corR* expression using RT-PCR revealed that *corR* of PG4180 is generally more highly expressed compared to its homologue from DC3000, regardless of the genomic background of the mutants (Fig. 5). No statistically significant temperature dependence of *corR* expression was observed except in one case, where *corRP* from PG4180 were combined with *corS* from DC3000 and tested in mutant DC3000.M1.

DISCUSSION

Results of this study regarding strain-specific differences in temperature-dependent *cma* expression in *P. syringae* substantiated our earlier findings (Weingart *et al.*, 2004) and demonstrated that the frequently used *P. syringae* strain DC3000 does not produce significant amounts of the phytotoxin *in vitro*. Previously, transcriptional fusions of the *cma* promoter to an *egfp* reporter gene were monitored *in vitro* and *in planta* (Weingart *et al.*, 2004), suggesting that *cma* promoter activity in DC3000 was plant-inducible, while in PG4180 it was temperature-dependent regardless of any plant-borne signals.

COR production and *cma* expression were completely abolished in strain DC3000 when *corRS* were knocked out, indicating a vital role of these genes for phytotoxin regulation. Unexpectedly, cross-complementation of the *corRS* mutant of DC3000 with *corRSP* derived from PG4180 did not result in thermoresponsiveness of *cma* expression, suggesting that the genomic background of DC3000 dictates either *corR* expression or CorR activity and, consequently, transcription of the *cma* operon. In contrast, the genetic background of PG4180 did not

render the regulatory triad of DC3000 thermoresponsive, indicating that thermosensing requires both indigenous CorRSP and the genetic background of PG4180. In this respect, RNA dot-blot results were confirmed by RT-PCR analysis and may reflect evolutionary adaptations of either pathovar, i.e. pv. *glycinea* (PG4180) on soybeans and pv. *tomato* (DC3000) on tomato and *Arabidopsis*, to their host plants. The cold-weather pathogen, PG4180, might sense abiotic parameters such as temperature (Budde *et al.*, 1998) while strain DC3000 might respond directly to plant signals (Weingart *et al.*, 2004).

The expression of *corR* was investigated in selected TCS exchange constructs using RT-PCR. The results suggest that *corR* expression in all but one case is not thermoresponsive, and indicate that temperature sensing is restricted to CorS activity. This result is not surprising, since regulatory genes are not usually expressed at a high level. Why *corR* expression was thermoresponsive and unusually high in DC3000.M1 harbouring CorRP from PG4180 and CorS from DC3000 remains to be investigated in future studies.

Interestingly, upstream of the DC3000 *corR* gene, a binding site for the alternative sigma factor, HrpL, responsible for expression of the *hrp* operon encoding a type III secretion system, was found (Fouts *et al.*, 2002). An *hrpL* mutant of DC3000 was significantly impaired in expression of *corR* (Sreedharan *et al.*, 2006). Additionally, a putative *corR* binding site was identified upstream of *hrpL*, demonstrating an interconnection of the expression of these two regulatory proteins in DC3000 (Sreedharan *et al.*, 2006). In this context it is noteworthy that PG4180 does not possess a recognizable *hrp*-box upstream of its *corR* gene (data not shown).

Besides the activity of CorRSP, COR synthesis in PG4180 and DC3000 seems to be governed by the alternative sigma factor RpoN (σ^{54}) (Alarcon-Chaidez *et al.*, 2003). σ^{54} is required for a number of metabolic functions, including

utilization of alternative carbon and nitrogen sources, nitrogen fixation, and expression of virulence genes (Kustu *et al.*, 1989; Studholme & Buck, 2000; Wösten, 1998). Thus, a combination of regulatory pathways governed by σ^{54} and CorRSP may lead to responsiveness of COR production to various abiotic environmental parameters in PG4180.

The obscure finding that a combination of *corRP* from PG4180 and *corS* from DC3000 gave rise to moderate and temperature-independent *cma* expression in PG4180, but thermoresponsive expression in DC3000 cannot be explained. Interestingly, the latter result could not be confirmed by respective RT-PCR experiments, suggesting that it might reflect some kind of technical artefact. A similar explanation might be appropriate for the construct in which CorRP and the N-terminal portion of CorS from PG4180 were brought together with the C terminus of CorS from DC3000 and expressed in PG4180.D4.

The most promising result of this study came from the exchange analysis of the N- and C-terminal domains of CorS in PG4180. Our data suggested a particular role of the C terminus of this HPK in thermosensing, and furthermore indicated that temperature-mediated *cma* expression was most pronounced when the C-terminal part of CorS interacted with CorR of PG4180. Thus, it is tempting to speculate that CorS dimerization or phosphotransfer to CorR via its C terminus, but not the membrane-embedded N-terminal part of CorS, is involved in the actual temperature-sensing process. However, CorS activity also requires its N terminus as reported in a previous study (Smirnova & Ullrich, 2004). To our knowledge, this is the first report of a potential role of the C terminus in temperature sensing for one of the three well-studied bacterial, low-temperature-sensing HPKs, CorS of *P. syringae*, DesK of *Bacillus subtilis* (Aguilar *et al.*, 2001), and DspA (Hik33) of *Synechocystis* sp. PCC 6803 (Suzuki *et al.*, 2000; Inaba *et al.*, 2003). All three kinases differ significantly in their primary amino acid sequences and secondary structures. Hunger *et al.* (2004) and Albanesi *et al.* (2004) demonstrated an involvement of the N-terminal membrane-embedded domain of DesK in thermosensing, but no such studies have thus far been conducted with DspA. However, the divergence of these findings from ours could be explained by the fact that DesK controls the saturation level of phospholipids in the membrane of *B. subtilis* and thus is likely to sense changes in the membrane, while CorS controls synthesis of a virulence-associated secondary metabolite in *P. syringae*, which in fact might be embedded in other regulatory circuits.

Future studies will focus on the in-depth analysis of the molecular interactions taking place in the CorRSP system, i.e. CorS dimerization and phosphotransfer reactions, as well as *in planta* COR gene expression analysis in both PG4180 and DC3000, with variable CorRSP combinations, using a novel *in planta* bacterial mRNA quantification technique recently developed in our laboratory (Schenk *et al.*, 2008).

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REFERENCES

- Aguilar, P. S., Hernandez-Arriaga, A. M., Cybulski, L. E., Erazo, A. C. & de Mendoza, D. (2001). Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. *EMBO J* **20**, 1681–1691.
- Alarcon-Chaidez, F. J., Keith, L., Zhao, Y. & Bender, C. L. (2003). RpoN (σ^{54}) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid* **49**, 106–117.
- Albanesi, D., Mansilla, M. C. & de Mendoza, D. (2004). The membrane fluidity sensor DesK of *Bacillus subtilis* controls the signal decay of its cognate response regulator. *J Bacteriol* **186**, 2655–2663.
- Bender, C. L., Malvick, D. K. & Mitchell, R. E. (1989). Plasmid-mediated production of the phytotoxin coronatine in *Pseudomonas syringae* pv. tomato. *J Bacteriol* **171**, 807–812.
- Bender, C. L., Liyanage, H., Palmer, D., Ullrich, M., Young, S. & Mitchell, R. (1993). Characterization of the genes controlling the biosynthesis of the polyketide phytotoxin coronatine including conjugation between coronafacic and coronamic acid. *Gene* **133**, 31–38.
- Bender, C. L., Palmer, D., Peñaloza-Vázquez, A., Rangaswamy, V. & Ullrich, M. (1996). Biosynthesis of coronatine, a thermoregulated phytotoxin produced by the phytopathogen *Pseudomonas syringae*. *Arch Microbiol* **166**, 71–75.
- Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brooks, D. M., Hernández-Guzmán, G., Kloeck, A. P., Alarcón-Chaidez, F., Sreedharan, A., Rangaswamy, V., Peñaloza-Vázquez, A., Bender, C. L. & Kunkel, B. N. (2004). Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant Microbe Interact* **17**, 162–174.
- Budde, I. P., Rohde, B. H., Bender, C. L. & Ullrich, M. S. (1998). Growth phase and temperature influence promoter activity, transcript abundance, and protein stability during biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *J Bacteriol* **180**, 1360–1367.
- Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Scott Durkin, A. & other authors (2003). The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc Natl Acad Sci U S A* **100**, 10181–10186.
- Cintas, N. A., Koike, S. T. & Bull, C. T. (2002). A new pathovar, *Pseudomonas syringae* pv. alisalensis pv. nov., proposed for the causal agent of bacterial blight of broccoli and broccoli raab. *Plant Dis* **86**, 992–998.
- Couch, R., O'Connor, S. E., Seidle, H., Walsh, C. T. & Parry, R. (2004). Characterization of CmaA, an adenylation-thiolation didomain enzyme involved in the biosynthesis of coronatine. *J Bacteriol* **186**, 35–42.
- Cuppels, D. A. (1986). Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. tomato. *Appl Environ Microbiol* **51**, 323–327.

- Figurski, D. H. & Helinski, D. R. (1979).** Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci U S A* **76**, 1648–1652.
- Fouts, D. E., Abramovitch, R. B., Alfano, J. R., Baldo, A. M., Buell, C. R., Cartinhour, S., Chatterjee, A. K., D'Ascenzo, M., Gwinn, M. L. & other authors (2002).** Genomewide identification of *Pseudomonas syringae* pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc Natl Acad Sci U S A* **99**, 2275–2280.
- Grebe, T. W. & Stock, J. B. (1999).** The histidine protein kinase superfamily. *Adv Microb Physiol* **41**, 139–227.
- Hunger, K., Beckering, C. L. & Marahiel, M. A. (2004).** Genetic evidence for the temperature-sensing ability of the membrane domain of the *Bacillus subtilis* histidine kinase DesK. *FEMS Microbiol Lett* **230**, 41–46.
- Inaba, M., Suzuki, I., Szalontai, B., Kanesaki, Y., Los, D. A., Hayashi, H. & Murata, N. (2003).** Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. *J Biol Chem* **278**, 12191–12198.
- Keane, P. J., Kerr, A. & New, P. B. (1970).** Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Aust J Biol Sci* **23**, 585–595.
- Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988).** Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**, 191–197.
- Koike, S. T., Barak, J. D., Henderson, D. M. & Gilbertson, R. L. (1999).** Bacterial blight of leek: a new disease in California caused by *Pseudomonas syringae*. *Plant Dis* **83**, 165–170.
- Kustu, S., Santero, E., Keener, J., Popham, D. & Weiss, D. (1989).** Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiol Rev* **53**, 367–376.
- Liyanage, H., Palmer, D. A., Ullrich, M. & Bender, C. L. (1995a).** Characterization and transcriptional analysis of the gene cluster for coronafacic acid, the polyketide component of the phytotoxin coronatine. *Appl Environ Microbiol* **61**, 3843–3848.
- Liyanage, H., Penfold, C., Turner, J. & Bender, C. L. (1995b).** Sequence, expression and transcriptional analysis of the coronafatec ligase-encoding gene required for coronatine biosynthesis by *Pseudomonas syringae*. *Gene* **153**, 17–23.
- Mitchell, R. E. (1982).** Coronatine production by some phytopathogenic pseudomonads. *Physiol Plant Pathol* **20**, 83–89.
- Mitchell, R. E., Young, S. A. & Bender, C. L. (1994).** Coronamic acid, an intermediate in coronatine biosynthesis by *Pseudomonas syringae*. *Phytochemistry* **35**, 343–348.
- Murillo, J., Shen, H., Gerhold, D., Sharma, A., Cooksey, D. A. & Keen, N. T. (1994).** Characterization of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. tomato PT23. *Plasmid* **31**, 275–287.
- Palmer, D. A. & Bender, C. L. (1993).** Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. glycinea. *Appl Environ Microbiol* **59**, 1619–1626.
- Parry, R. J., Mhaskar, S. V., Lin, M. T., Walker, A. E. & Mafoti, R. (1994).** Investigations of the biosynthesis of the phytotoxin coronatine. *Can J Chem* **72**, 86–99.
- Peñaloza-Vázquez, A. & Bender, C. L. (1998).** Characterization of CorR, a transcriptional activator which is required for biosynthesis of the phytotoxin coronatine. *J Bacteriol* **180**, 6252–6259.
- Rangaswamy, V. & Bender, C. L. (2000).** Phosphorylation of CorS and CorR, regulatory proteins that modulate production of the phytotoxin coronatine in *Pseudomonas syringae*. *FEMS Microbiol Lett* **193**, 13–18.
- Rangaswamy, V., Jiralerspong, S., Parry, R. & Bender, C. L. (1998).** Biosynthesis of the *Pseudomonas* polyketide coronafacic acid requires monofunctional and multifunctional polyketide synthase proteins. *Proc Natl Acad Sci U S A* **95**, 15469–15474.
- Sambrook, J. & Russell, D. W. (2001).** *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schenk, A., Weingart, H. & Ullrich, M. S. (2008).** Extraction of high-quality bacterial RNA from infected leaf tissue for bacterial *in planta* gene expression analysis by multiplexed fluorescent Northern hybridization. *Mol Plant Pathol* **9**, 227–235.
- Smirnova, A. V. & Ullrich, M. S. (2004).** Topological and deletion analysis of CorS, a *Pseudomonas syringae* sensor kinase. *Microbiology* **150**, 2715–2726.
- Smirnova, A. V., Braun, Y. & Ullrich, M. S. (2008).** Site-directed mutagenesis of the temperature-sensing histidine protein kinase CorS from *Pseudomonas syringae*. *FEMS Microbiol Lett* **283**, 231–238.
- Sreedharan, A., Peñaloza-Vázquez, A., Kunkel, B. N. & Bender, C. L. (2006).** CorR regulates multiple components of virulence in *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant Microbe Interact* **19**, 768–779.
- Studholme, D. J. & Buck, M. (2000).** The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS Microbiol Lett* **186**, 1–9.
- Suzuki, I., Los, A. D., Kanesaki, Y., Mikami, K. & Murata, N. (2000).** The pathway for perception and transduction of low-temperature signals in *Synechocystis*. *EMBO J* **19**, 1327–1334.
- Ullrich, M. & Bender, C. L. (1994).** The biosynthetic gene cluster for coronamic acid, an ethylcyclopropyl amino acid, contains genes homologous to amino acid-activating enzymes and thioesterases. *J Bacteriol* **176**, 7574–7586.
- Ullrich, M., Peñaloza-Vázquez, A., Bailey, A. M. & Bender, C. L. (1995).** A modified two-component regulatory system is involved in temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *J Bacteriol* **177**, 6160–6169.
- Völksch, B. & Weingart, H. (1998).** Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. *J Basic Microbiol* **38**, 135–145.
- Wang, L., Bender, C. L. & Ullrich, M. S. (1999).** The transcriptional activator CorR is involved in biosynthesis of the phytotoxin coronatine and binds to the *cmaABT* promoter region in a temperature-dependent manner. *Mol Gen Genet* **262**, 250–260.
- Weingart, H., Stubner, S., Schenk, A. & Ullrich, M. S. (2004).** Impact of temperature on *in planta* expression of genes involved in synthesis of the *Pseudomonas syringae* phytotoxin coronatine. *Mol Plant Microbe Interact* **17**, 1095–1102.
- Wösten, M. M. S. M. (1998).** Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**, 127–150.
- Zhao, Y. F., Damicone, J. P. & Bender, C. L. (2002).** Detection, survival, and sources of inoculum for bacterial diseases of leafy crucifers in Oklahoma. *Plant Dis* **86**, 883–888.

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