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.

**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, 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.
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 (Penaloza-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 derivate, 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 acyl 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-TCTAGATTGGATCCGCGGAAACACGCCT-3’) and in_FcorS (5’-CCAGAGTTCGAGTGGCAGGCAGAATGAGT-3’), a 1.4 kb Xbal–EcoRI fragment was amplified, and using primers in_FcorS (5’-CCCGAATTCCCGTTTTAGCGCACCTCAAC-3’) and out_RcorS (5’-CAAGGTACCCGTACAAAGTGGTTCGAC-3’), a 1.6 kb EcoRI–KpnI fragment was amplified, and subsequently ligated to pBluescript, yielding plasmid pBSXX30. A 1.7 kb Km cassette from pMKm (Murillo et al., 1994) was inserted into a unique EcoRI site of pBSXX30, yielding the suicide vector pBSX847, which was mobilized into DC3000 by electroporation followed by screening for Ap² Km¹ derivatives. Recombination of the Km cassette into genomic corS was verified by Southern blot analysis and PCR using primers corS_fwd_DC3000 (5’-GGATTCCCGGATGACTCAATTAAAT-3’) and corS_rev_DC3000 (5’-GGATTCCGTTGATCTAGTCCATG-3’).

Construction of plasmid-borne TCS exchange systems. First, a plasmid-based system containing corRSP was created, which enabled
To generate a CorRSP exchange system which contains corRSP from DC3000 with unique restriction sites, plasmid pYBH34 was constructed. To use colony PCR, corR 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'-GAACATGCTGGGCAGCAGTTATTTTGGCAG-3’) and corR_Spe_rev (5'-TGCTGGCTCAGAGCTAGTCTCTGAGGCGACG-3’) and subsequently digested with 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 corS, T7_HindIII (5’-GACAAGCTTAAATACGACTCACTATAG-3’) and corS_Spe_rev (5’-TGCAAGCCGAGGCCAGGCGCAG-3’); for corS, corS_Spe_fwd (5’-ATGCCGCGCGCCGCTCTAGACTGCTCTGTGAGGCCAGC-3’) and corS_Xba_rev (5’-TGAAAGTACTACGGTCAGAGGCGCTAGTCTTGCTG-3’); for corP, corP_Spe_fwd (5’-CAGCAAGGCGATGCTCTCCATAGGCCTAGGTATAGTCTC-3’) and corP_HindIII (5’-GGCAAGCTTATTAACCCTCACTAAAG-3’). The thereby generated plasmid, pASE34, was sequenced 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).

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 Schenck 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 cma-fw

<table>
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<tr>
<th>Bacterial strain</th>
<th>Relevant characteristics*</th>
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<tr>
<td><em>Escherichia coli</em></td>
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<tr>
<td>DH5*&lt;sup&gt;z&lt;/sup&gt;</td>
<td>F’/endA1 hsdR17( rˌm˼&lt;sup&gt;_&lt;2&gt;&lt;/sup&gt;) relA1 supE44 thi-1 recA1 gyrA (Na&lt;sup&gt;+&lt;/sup&gt;) Δ(lacZΔM15[U169 deor]) K12 Δ(lacZYA-argF)U169 deor 800lacZΔM15</td>
<td>Sambrook &amp; Russell (2001)</td>
</tr>
<tr>
<td>HB101</td>
<td>F’ Δ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1 Δ(mrcC-mrr) rpsL20 (Sm&lt;sup&gt;+&lt;/sup&gt;) xyl-5 mit-1 recA13</td>
<td>Sambrook &amp; Russell (2001)</td>
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<td><em>P. syringae pv. glycinea</em></td>
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<tr>
<td>PG4180</td>
<td>COR&lt;sup&gt;+&lt;/sup&gt; CFA&lt;sup&gt;+&lt;/sup&gt; CMA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mitchell (1982)</td>
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<tr>
<td>PG4180.D4</td>
<td>corR: T7:5 Km&lt;sup&gt;+&lt;/sup&gt; corR&lt;sup&gt;-&lt;/sup&gt; corS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Bender et al. (1993)</td>
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### Table 1. Bacterial strains used in this study

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<td><strong>P. syringae pv. glycinea</strong></td>
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<tr>
<td>PG4180</td>
<td>COR&lt;sup&gt;+&lt;/sup&gt; CFA&lt;sup&gt;+&lt;/sup&gt; CMA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mitchell (1982)</td>
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<td>corR: T7:5 Km&lt;sup&gt;+&lt;/sup&gt; corR&lt;sup&gt;-&lt;/sup&gt; corS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Bender et al. (1993)</td>
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*Abbreviations: Km, kanamycin; NaI, nalidixic acid; Rif, rifampicin; Sm, streptomycin.*
**Table 2.** Plasmids used in this study

<table>
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<th>Plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference/source</th>
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<tr>
<td>pBluescript II SK</td>
<td>Ap⁺; ColE1 origin, high-copy-number cloning vector</td>
<td>Stratagene</td>
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<tr>
<td>pGEM-T Easy</td>
<td>Ap⁺; TA cloning vector</td>
<td>Promega</td>
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<tr>
<td>pRK415</td>
<td>Tc⁺; mob⁺; low-copy-number cloning vector, RK2-derivative</td>
<td>Keen et al. (1988)</td>
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<tr>
<td>pRK2013</td>
<td>Km⁺; mob⁺ tra⁺; helper plasmid for triparental mating</td>
<td>Figurski &amp; Helinski (1979)</td>
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<td>pMUH34</td>
<td>Tc⁺; contains a 3.4 kb HindIII fragment with corRSP genes from PG4180 in pRK415</td>
<td>Ullrich et al. (1995)</td>
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<td>pBSXX47</td>
<td>Ap⁺ Km⁺ suicide vector for corS mutagenesis</td>
<td>This study</td>
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<tr>
<td>pASH34</td>
<td>Tc⁺; contains a 3.4 kb HindIII fragment with corRSP genes from DC3000 in pRK415</td>
<td>This study</td>
</tr>
<tr>
<td>pH14</td>
<td>Ap⁺; contains a 1.4 kb HindIII–SpeI PCR fragment of corR from PG4180 in pBluescript II SK</td>
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<td>pHX29</td>
<td>Ap⁺; contains a 2.9 kb HindIII–XbaI fragment with corRS from PG4180 in pBluescript II SK</td>
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<td>pHX34</td>
<td>Ap⁺; contains a 3.4 kb HindIII–XbaI fragment with corRSP from PG4180 in pBluescript II SK</td>
<td>This study</td>
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<td>pASE34</td>
<td>Tc⁺; contains a 3.4 kb EcoRI fragment with corRSP from PG4180, SpeI and XbaI sites upstream and downstream of corS, in pRK415</td>
<td>This study</td>
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<td>pASE34:D</td>
<td>Tc⁺; contains a 3.4 kb EcoRI fragment with corRP genes from PG4180 and corS from DC3000 in pRK415</td>
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<td>pASE34:PG-DC</td>
<td>Tc⁺; contains a 3.4 kb EcoRI fragment with corRP from PG4180, hybrid corS N terminus from PG4180, C terminus from DC3000, in pRK415</td>
<td>This study</td>
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<tr>
<td>pASE34:DC-PC</td>
<td>Tc⁺; contains a 3.4 kb EcoRI fragment with corRP from PG4180, hybrid corS N terminus from DC3000, C terminus from PG4180, in pRK415</td>
<td>This study</td>
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<td>pGEM:corRS</td>
<td>Ap⁺; contains a 2.8 kb HindIII–XbaI PCR fragment with corRS from DC3000 introduced in pGEM-T Easy</td>
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<td>Ap⁺; contains a 0.7 kb HindIII–XbaI PCR fragment with corP from DC3000 introduced in pGEM-T Easy</td>
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<td>pGEM:corRSP</td>
<td>Ap⁺; contains a 3.5 kb HindIII–SacI PCR fragment with corRSP from DC3000 introduced in pGEM-T Easy</td>
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<td>pBorRSP-DC</td>
<td>Ap⁺; contains a 3.5 kb HindIII–SacI fragment with corRSP from DC3000 with introduced SpeI and XbaI sites upstream and downstream of corS in pBluescript II SK</td>
<td>This study</td>
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<td>pYBH34</td>
<td>Tc⁺; contains corRSP from DC3000, SpeI and XbaI sites upstream and downstream of corS, in pRK415</td>
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<td>pYBH34:P</td>
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<td>This study</td>
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*Abbreviations: Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; mob, mobilization function; tra, transfer function.

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’- CCTACCGCCGATTTGAGT-3’), cmaA_RT-rev (5’- CGAGCCCTTGACGTATCAGT-3’), corR_RT-fw (5’- ATGCCGAAGCCTTCCTCCTGATCTT-3’), corR_RT-rev (5’- AGACGCCTCCTGATCTT-3’) and corR_DC_rev (5’- AAGCGCCCTGCTGATCTT-3’). QuantifTect 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

(5’-TTTGAATGCCTGCTGACCGA-3’) and cmaA-rev17 (5’-TAATTACTGCTACATATTGCCGGAGGTAGTTGCTATTG-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 x SSC) at 68 °C for 16 h. After hybridization, the membrane was washed twice for 5 min at room temperature in 2 x SSC containing 0.1 % SDS, followed by two washes for 15 min at 68 °C in 0.2 x 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 phosphoimager (Raytest). Signals were quantified using the AIDA Image Analyser software package (Raytest).
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 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 thermodrespons-
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-thermosensitive 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).](http://mic.sgmjournals.org)
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 genomic 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 (σ54) (Alarcon-Chaidex et al., 2003). σ54 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; Wöstten, 1998). Thus, a combination of regulatory pathways governed by $\sigma_{34}$ 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 thermostressive 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 thermostasis, 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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