

Control of Temperature-Responsive Synthesis of the Phytotoxin Coronatine in *Pseudomonas syringae* by the Unconventional Two-Component System CorRPS

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Abstract

The phytopathogen *Pseudomonas syringae* produces the phytotoxin coronatine (COR) as a major virulence factor. COR and its precursor, coronafacic acid, function as molecular mimics of the plant signaling molecule jasmonate. A 32.8-kb plasmid-borne gene cluster mediates COR biosynthesis, which is optimal at 18°C and non-detectable at 28°C, the optimal growth temperature for *P. syringae*. The thermoregulation is mediated at the transcriptional level by an unconventional two-component regulatory system consisting of a histidine protein kinase, CorS, and two transcriptional activators, CorR and CorP. Dissection of this regulatory triad revealed that CorR binds to its target sequences in a thermoresponsive manner and that its DNA-binding activity is controlled by CorS. A Preliminary model for thermo-sensing by CorS is proposed based on its membrane topology and the analysis of translational fusions of CorS to reporter enzymes at different temperatures. CorP lacks a typical helix-turn-helix motif but possibly functions as a modulator of CorR or CorS activity. The thermoregulation of COR biosynthetic genes is widespread among various COR-producing *P. syringae* strains. Post-translational processes also contribute to the thermo-responsiveness of COR production. Additionally, COR synthesis in *P. syringae* is influenced by nutrient availability, *rpoN* encoding the alternative sigma factor σ^{54} , and HrpV, a negative regulator of *hrp* gene expression, suggesting a complex regulatory network governing phytotoxin synthesis.

Introduction

Besides an intensively studied type III secretory system, the Hrp (*hypersensitive response and pathogenicity*)

system, which delivers so-called avirulence gene products to the host cell causing pathogenicity, virulence factors such as phytotoxins, exopolysaccharides, and extracellular enzymes significantly contribute to the disease development in pathogenic plant-microbe interactions (Alfano and Collmer, 1996). It remains to be determined how and under which environmental conditions most of these virulence factors impact the disease outcome. Opportunistic plant pathogens like our model organism, *Pseudomonas syringae*, preferably infect their host plants under conditions of high humidity and low temperature. Plants, as poikilothermic organisms do not maintain a given temperature. This complicates a direct comparison of thermoresponsive processes in plant pathogens with the well-studied thermoregulation of virulence factors in human and animal pathogens (Hurme and Rhen, 1998). While temperatures of 37–41°C signal to animal pathogens their arrival inside the warm-blooded host, numerous virulence factors of phytopathogens like *Erwinia amylovora*, *Agrobacterium tumefaciens*, *E. chrysanthemi*, and *P. syringae* are preferentially expressed, secreted, or assembled at lower temperatures, such as 18–22°C (Hugovieux-Cotte-Pattat *et al.*, 1992; Wei *et al.*, 1992; Rowley *et al.*, 2000; Banta *et al.*, 1998; Van Dijk *et al.*, 1999). In the current mini-review, we report on the dissection of one of these thermoresponsive systems, the regulation of biosynthesis of the phytotoxin coronatine in *P. syringae*.

The Phytotoxin Coronatine

Coronatine (COR) (Figure 1B) consists of a polyketide moiety, coronafacic acid (CFA), and a cyclized amino acid derivative, coronamic acid (CMA), which are fused to each other by an amide bond (Mitchell, 1982; Bender *et al.*, 1993). COR is synthesized by five different pathovars of *P. syringae* and functions in a non-host specific manner causing chlorosis, hypertrophy of storage tissue, compression of thylakoids, thickening of plant cell walls, and accumulation of plant-borne protease inhibitors (Sakai *et al.*, 1979; Mitchell, 1982; Palmer and Bender, 1995). COR and CFA structurally and functionally resemble jasmonic acid, a plant compound involved in signal transduction during plant defense mechanisms and global cell differentiation processes, and its precursor, 12-oxophytodienoic acid. COR and CFA are believed to mimic these compounds at the molecular level (Feys *et al.*, 1994; Weiler *et al.*, 1994; Krumm *et al.*, 1995). COR production enhances the virulence of *P. syringae* strains on tomato plants, *Arabidopsis thaliana*, and Chinese cabbage plants

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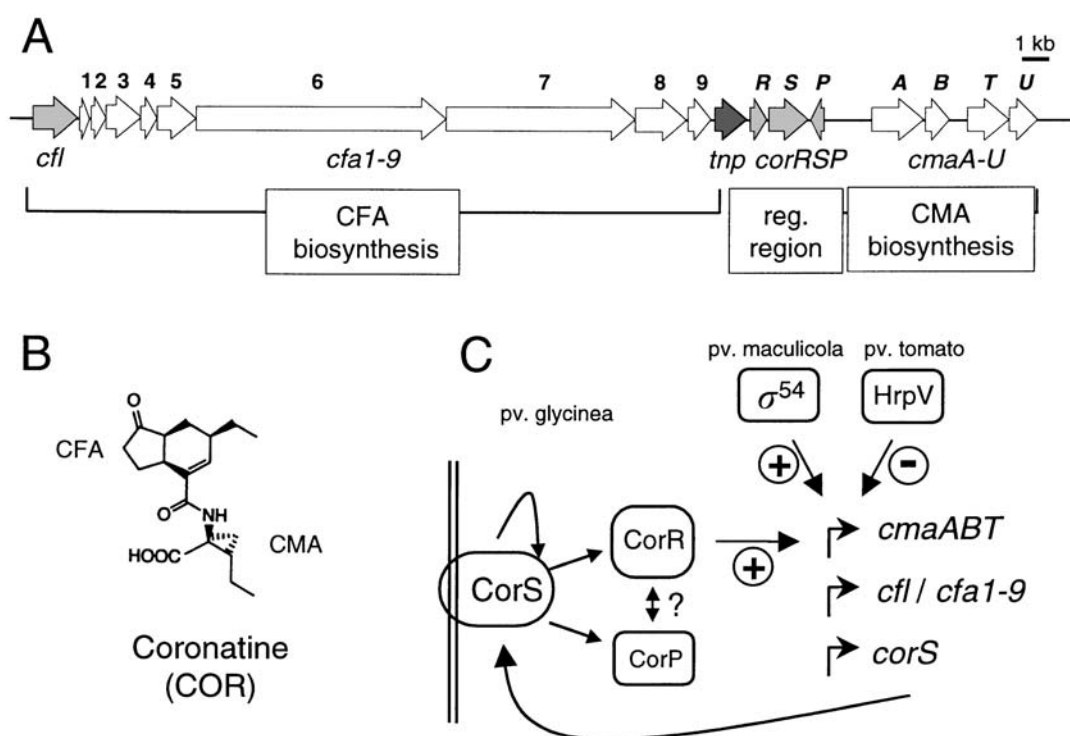


Figure 1. (A) Functional map of the COR biosynthetic gene cluster of PG4180 indicating the DNA regions required for CFA and CMA biosynthesis, respectively, and for transcriptional regulation. (B) Structure of COR which consists of CFA and CMA fused by amide bond. (C) Hypothetical scheme for transcriptional regulation of COR genes. A modified two-component regulatory system consisting of CorS, CorR, and CorP was characterized in *P. syringae* pv. *glycinea*. Additional regulatory factors found in *P. syringae* pvs. *maculicola* and tomato are indicated. It remains to be tested whether the symbolized regulatory proteins play a role in all three pathogens studied.

(Bender *et al.*, 1987; Mittal and Davis, 1995; Tamura *et al.*, 1998). Analysis of the accumulation of mRNAs encoded by defense-related genes in *Arabidopsis* demonstrated that COR is required for successful infection under natural conditions and that COR plays a critical role during the early stages of infection by suppressing the activation of defense-related plant genes (Mittal and Davis, 1995). These findings were confirmed by Budde and Ullrich (2000), who investigated the impact of COR production by *P. syringae* PG4180 during compatible and incompatible interactions with soybean and tobacco plants, respectively. After spray inoculation, PG4180 caused typical bacterial blight symptoms on soybean plants only when the bacteria produced COR at 18°C. This effect was not observed when PG4180 was infiltrated into soybean leaves, indicating that COR synthesis is important for bacterial invasion via natural plant openings. In the incompatible interaction, PG4180 elicited a rapid localized cell death termed the hypersensitive response (HR) on tobacco plants regardless of COR synthesis. However, the HR was significantly delayed when tobacco plants were treated with cells of a CFA-overproducing derivative of PG4180. Results of Budde and Ullrich (2000) indicated that the amount of synthesized CFA but not that of COR influenced the outcome of the HR, shedding further light on CFA's putative role as a molecular mimic of jasmonic acid.

Genetics and Biosynthesis of COR

Naturally COR⁻ strains of *P. syringae* acquired the ability to produce COR when transformed with p4180A, a 90-kb indigenous plasmid from PG4180 (Bender *et al.*, 1993). Tn5 mutagenesis indicated that the COR biosynthetic genes are clustered within a 32.8-kb region on p4180A (Figure 1A). The phenotype of COR-defective mutants was determined by supplying them with CFA and CMA and by complementation studies with cloned DNA from the COR biosynthetic cluster. The regions encoding CFA and CMA synthesis and coupling activity were localized to 18.8-, 6.9- and 2.3-kb regions of the cluster, respectively. Mutants in a 3.4-kb region required the addition of both CFA and CMA for COR synthesis indicating a regulatory role for this part of the cluster (Bender *et al.*, 1993). The COR gene cluster in *P. syringae* is normally plasmid-encoded in pvs. *atropurpurea*, *glycinea*, *morsprunorum*, and tomato but chromosomally encoded in pv. *maculicola* (Bender *et al.*, 1991; Ullrich *et al.*, 1993). Alarcón-Chaidez *et al.* (1999) demonstrated that some COR plasmids were self-transmissible, and that all COR plasmids shared a related *oriV/par* region. Sequence analysis of the region adjacent to the COR gene cluster in p4180A indicated the presence of IS elements which may have contributed to the horizontal transfer of the COR gene cluster.

The biosynthetic regions within the COR gene cluster have been analyzed in detail. The 6.9-kb DNA region required for CMA biosynthesis was sequenced, revealing four distinct open reading frames, which shared a common orientation for transcription (Ullrich *et al.*, 1994; Ullrich and Bender, 1994; Budde *et al.*, 1998) (Figure 1A). The deduced amino acid sequence of *cmaA* contained six core sequences, which are present in amino acid-activating enzymes, including nonribosomal peptide synthetases. The *cmaB* gene showed high similarity to a gene required for syringomycin production in *P. syringae*. The deduced amino acid sequence of *cmaT* was related to thioesterases. These data suggest that CMA assembly is similar to the thiotemplate mechanism of nonribosomal peptide synthesis. No significant similarities were found between *cmaU* and other database entries (Ullrich and Bender, 1994). Furthermore, we localized and studied the promoter region upstream of *cmaABT*. Data of transcriptional fusions with a promoterless β -glucuronidase gene (*uidA*) indicated that CMA biosynthesis is regulated by temperature at the transcriptional level (see below).

Liyanage *et al.* (1995b) analyzed the nucleotide sequence of the coronafacate ligase-encoding gene (*cfl*), which is required for the amide linkage of CFA and CMA. The deduced amino acid sequence of *cfl* showed homology to a variety of adenylate-forming enzymes, which bind and hydrolyze ATP in order to activate their substrates for further ligation. Liyanage *et al.*, (1995a) showed that *cfl* and the CFA biosynthetic genes are located on a single transcript and that the *cfl* promoter directs its transcription. The *cfl* promoter was shown to be activated in a thermoresponsive manner.

Subsequently, nine genes (*cfa1* to *cfa9*) were identified that are required for CFA biosynthesis (Figure 1A). While no meaningful sequence similarity was observed for *cfa4*, the predicted translation products of *cfa1*, *cfa2*, *cfa3*, and *cfa5* were related to acyl carrier proteins, fatty acid dehydrases, β -ketoacyl synthases, and adenylating enzymes, respectively (Penfold *et al.*, 1996). Two large genes, *cfa6* and *cfa7*, encode polyketide synthases (PKS) that are structurally and functionally similar to multifunctional modular PKS (Rangaswamy *et al.*, 1998a). Additionally, the predicted translation products of *cfa8*, and *cfa9* showed relatedness to oxidoreductases and thioesterases, respectively (Rangaswamy *et al.*, 1998b). Mutagenesis indicated that *cfa8* is required for the production of CFA whereas *cfa9* is dispensable for CFA synthesis but may accelerate the release of enzyme-bound products. Rangaswamy *et al.*, (1998a) presented a scheme for CFA biosynthesis that incorporates the activities of all proteins encoded by the *cfa1–9* region.

Thermoregulation of COR Biosynthesis

Transcription of fusions of the *cmaABT* and *cfl/cfa1–9* operons with *uidA* were influenced by temperature and showed maximal reporter gene activity at 18°C (Ullrich

and Bender, 1994; Liyanage *et al.*, 1995a; Budde *et al.*, 1998). Furthermore, transcription of both biosynthetic operons was dependent on a modified two-component regulatory system located within the COR biosynthetic gene cluster (Ullrich *et al.*, 1995). A 3.4-kb DNA fragment from the COR biosynthetic gene cluster restored temperature-regulated phytotoxin production to Tn5 mutants that were defective in production of both CFA and CMA. Nucleotide sequence analysis of this fragment revealed three genes, *corS*, *corP*, and *corR*, which encode an unconventional two-component regulatory system consisting of one sensor protein, CorS, and two response regulator proteins, CorP and CorR (Figure 1C). Although only one response regulator, CorR, has a DNA-binding domain, the phosphate-receiving domains of both response regulator proteins are highly conserved.

Transcriptional fusions of *corP* and *corR* to *uidA* indicated that these two genes are expressed constitutively. In contrast, a *corS::uidA* fusion exhibited the temperature dependence previously observed for COR biosynthetic promoters and exhibited maximal transcriptional activity at 18°C and low activity at 28°C suggesting a positive auto-regulation of the *corS* gene (Ullrich *et al.*, 1995). Furthermore, *uidA* activity for *corS::uidA*, *cmaABT::uidA*, and *cfl::uidA* was decreased in *corP*, *corR*, and *corS* mutants as compared to wild type levels. This difference was not found for *corP::uidA* and *corR::uidA* transcriptional fusions. The three regulatory genes functioned in a *P. syringae* strain lacking the COR gene cluster to achieve thermoresponsive activation of an introduced COR biosynthetic promoter, indicating that this triad of genes has the primary control over COR biosynthesis and is responsible for thermoregulation.

Later, we furthered our understanding of the transcriptional and post-translational effects of temperature on *cmaB*, which encodes an enzyme involved in CMA biosynthesis (Budde *et al.*, 1998). Transcriptional *cmaABT::uidA* fusions and Northern blot analyses were used to monitor promoter activities and transcript abundance, respectively, during bacterial growth at 18 and 28°C. Promoter activity and transcript abundance were maximal when cells were incubated at 18°C and sampled at mid-exponential phase. Western blot analysis indicated that CmaB accumulated in *P. syringae* cultures grown at 18°C but not in cultures incubated at 28°C. Temperature shift experiments indicated that CmaB was more stable at 18°C than at 28°C (Budde *et al.*, 1998). Rangaswamy *et al.*, (1997) used Anti-Cfl antibodies and a transcriptional *cfl::uidA* fusion to find that transcription of *cfl* is thermoresponsive. In summary, we propose that thermoregulation of both, transcription and protein stability, might control COR synthesis.

Using phenotypic determination of COR synthesis, a transcriptional *cmaABT::uidA* fusion, and Western blot analysis, we screened a representative number of natural isolates of *P. syringae* for effects of temperature on expression of *cmaABT* (Rohde *et al.*, 1998). Thermoregulation of *cmaABT* expression was frequent among the tested strains.

One important question currently addressed in our laboratory is whether the *in vitro* thermoregulation of COR gene expression also plays a role *in planta*. Transcriptional fusions of *cmaABT* with a *gfp* derivative encoding the enhanced green fluorescent protein were generated, introduced to PG4180, and shown to be thermoresponsive *in vitro* as well as *in planta* (Weingart and Ullrich, unpublished).

It was investigated whether CorR binds specifically to the DNA region upstream of *cfl* (Peñaloza-Vázquez and Bender, 1998). Complementation analysis with a *corR* mutant and transcriptional fusions indicated that CorR functions as a positive regulator of COR gene expression. Deletion analysis of the 5'-end of the *cfl* upstream region was used to define the minimal region required for COR gene expression. An area extending from -704 to -650 with respect to the *cfl* transcriptional start site was protected from DNase I in footprinting experiments, indicating a rather large binding area. This area was also conserved in the promoter region for *cmaABT*.

To substantiate this, fusions of CorR and CorP to the maltose-binding protein (MBP) were overproduced and tested for functionality by complementation of *corR* and *corP* mutants of PG4180 (Wang *et al.*, 1999). The *cmaABT* promoter region was defined by deletion mapping and the DNA-binding capability of CorR and CorP was examined by gel retardation assays. Once overproduced in *P. syringae* at 18°C and purified, MBP-CorR was shown to bind specifically to a DNA fragment corresponding to positions -841 to -623 bp upstream of the transcriptional start of *cmaABT*. In contrast, MBP-CorP and MBP did not bind to this or to any other DNA fragment analyzed. The MBP-CorR activity was completely abolished when the fusion protein was overproduced at 28°C or in a *corS* mutant, indicating that the DNA binding of CorR depended on the growth temperature at which it was produced, and was controlled by CorS. DNA-binding assays with CorR were also conducted at different temperatures. However, the actual temperature at which DNA-binding assays were carried out had no effect suggesting that thermally-induced changes in the DNA structure might not play a role in this system (Wang *et al.*, 1999). In addition, overproduction of MBP-CorR in a *corP* mutant of PG4180 also yielded inactive protein, highlighting the importance of CorP for CorR activation. Rangaswamy and Bender (2000) demonstrated the *in vitro* phosphorylation of both, CorR and an N-terminally truncated form of CorS, thereby biochemically proving their function in phospho-transfer. We propose that CorR is activated by CorS at low temperature and that CorP is required for this activation before CorR can bind to its target DNA sequences (Figure 1C).

The proportion of unsaturated fatty acids in bacterial membranes is increased when temperature decreases to maintain membrane fluidity. This common feature was also observed for *P. syringae* grown at 18 or 28°C using gas chromatography analysis of membrane fatty acid methyl esters (Smirnova *et al.*, unpublished). Since CorS is a membrane-bound

sensor kinase, it was tempting to speculate that CorS might sense changes in the fatty acid composition of the inner membrane. Respective experiments to test this are currently underway in our laboratory. Translational fusions of CorS to alkaline phosphatase (AP) and β -galactosidase were generated to biochemically confirm the computer prediction of membrane topology for this enzyme. Six potential membrane-spanning helices could be confirmed by this approach (Smirnova *et al.*, unpublished). Interestingly, CorS-AP fusions downstream of one of these helices had either a cytoplasmic or a periplasmic location depending on the bacterial incubation temperature, suggesting that CorS might modify its conformation in a thermoresponsive manner.

Additional Regulatory Factors Influencing COR Synthesis

Palmer *et al.*, (1997) demonstrated that selected carbon and amino acid sources significantly decreased COR biosynthesis of PG4180 at the transcriptional level. Interestingly, changes in osmolarity and the addition of complex carbon and nitrogen sources to the growth medium did not significantly affect COR transcription. These results indicate that multiple factors impact COR production. The influence of plant-borne signals on COR gene expression in *P. syringae* pv. tomato DC3000 was studied by Li *et al.*, (1998). Crude leaf extracts and plant intercellular fluids strongly induced expression of COR genes in this organism.

The co-ordinated regulation of different systems required for pathogenicity and virulence was recently addressed by analyzing COR production in defined *hrp* mutants of *P. syringae* pv. tomato DC3000 (Peñaloza-Vázquez *et al.*, 2000). COR was produced *in vitro* by mutants of DC3000 defective in *hrcC*, which encodes an outer-membrane protein required for type III-mediated secretion. Furthermore, a DC3000 mutant containing a polar mutation in *hrcC*, which inactivates *hrcC*, *hrpT*, and *hrpV*, produced significantly higher amounts of COR than the wild type suggesting that the regulatory protein HrpV might negatively control COR synthesis (Figure 1C). The results indicated that the *hrp* secretion system is not required for COR production, but mutations in this system may have regulatory effects on COR production.

Hendrickson *et al.*, (2000) constructed a mutant defective in the *rpoN* gene encoding σ^{54} from *P. syringae* pv. maculicola and showed that *rpoN* was essential for production of COR and for expression of the *cmaABT* operon (Figure 1C). In summary, it remains to be tested whether the additional regulatory principles play a role in all COR-producing pathovars of *P. syringae*.

Perspectives

Several intriguing questions regarding the CorRPS triad remain to be answered in future studies. Most importantly, the molecular mechanism by which CorS senses temperature changes needs to be addressed.

The postulated role of CorP as a modulator of CorR/CorS activity will be studied using phosphorylation assays and two-hybrid protein-protein interactions systems. Another important task will be to dissect the regulatory cascades or networks governing the concerted regulation of different pathogenicity and virulence determinants, with special emphasis on COR, *hrpV*, and *rpoN*. Ultimately, our studies aim at a better understanding of the molecular processes taking place in *P. syringae* during the onset of virulence in response to micro-climatic temperature changes in *planta*.

Acknowledgements

We thank Carol L. Bender and her coworkers for continuing support and fruitful cooperation. Critical comments by Alexander Schenk, Hongqiao Li, Hilary King, Erhard Bremer, and Uwe Völker are gratefully acknowledged. This work was supported by grants from the Deutscher Akademischer Austauschdienst, Deutsche Forschungsgemeinschaft, and the Max-Planck-Gesellschaft.

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