

Biosynthesis of the Antimetabolite 6-Thioguanine in *Erwinia amylovora* Plays a Key Role in Fire Blight Pathogenesis**

Sébastien Coyne, Cornelia Chizzali, Mohammed N. A. Khalil, Agnieszka Litomska, Klaus Richter, Ludger Beerhues, and Christian Hertweck*

Fire blight is one of the most devastating plant diseases that can eradicate entire orchards. It mainly affects apple and pear trees, among other members of the Rosaceae family. The causative agent of this rapidly spreading disease is the bacterium *Erwinia amylovora*, historically the first identified bacterial plant pathogen.^[1] Its high pathogenicity combined with an almost worldwide spread constitutes serious ecological and economic problems. Thus, deciphering the molecular mechanisms responsible for the success of this bacterium is of utmost importance. The typical symptom of the disease is necrosis of infected tissues with dissemination of the “scorched” phenotype from the infecting point, eventually involving the entire tree.^[2] The production of exudates, called ooze, on infected fruits is another characteristic of the disease. Several factors help this pathogen to establish its pathogenicity. An amylovoran exopolysaccharide, which participates in ooze formation, might confer resistance to plant-defensive reactive oxygen species.^[3] Effector proteins secreted through a type-three secretion system (T3SS) modulate the plant response such as induction of oxidative burst and manipulation of the jasmonic acid pathway.^[3] For iron acquisition the pathogen excretes the siderophore desferrioxamine E.^[4] However, despite considerable research, the mechanisms of fire blight development are still not fully understood.^[5] Since plant pathogenic microorganisms commonly produce secondary metabolites as virulence factors,^[6] it is likely that the aforementioned bacterial traits are only part of a wider

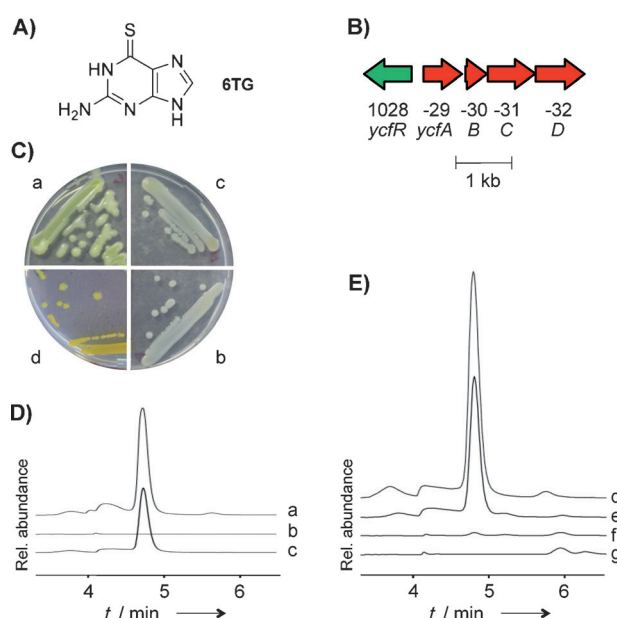


Figure 1. A) Structure of the antimetabolite 6-thioguanine (6TG). B) Organization of the *ycf* gene cluster (in red) in *E. amylovora*. Open arrows indicate coding sequences and direction of transcription. C) Phenotypic appearance of wild type and mutants on minimal medium 2 with Cu (MM2Cu) agar plate; a) *E. amylovora* wild type, b) $\Delta ycfA$ mutant, c) complemented mutant, d) *E. coli* heterologously expressing *ycfABCD*. D) HPLC profiles of extracts from liquid cultures of *E. amylovora*; a) wild type, b) $\Delta ycfA$ mutant and c) complemented mutant, $\Delta ycfA/p(ycfA)$. E) HPLC profiles of extracts from liquid cultures of *E. coli* heterologously expressing d) *ycfABCD*, e) *ycfABC*, f) *ycfAB*, and g) *ycfA*. Diode-array detection (DAD), $\lambda = 340$ nm.

arsenal developed by the bacterium to harm the plant hosts. Interestingly, *E. amylovora* is capable of producing 6-thioguanine (6TG, Figure 1 A).^[7] Thioguanine is a highly potent cytotoxin discovered and synthesized in the 1950s by Elion and Hitchings, who received a Nobel Prize in Medicine or Physiology for their discovery of antimetabolite drugs.^[8] The high cytotoxicity of 6TG results from its *in situ* transformation into 6-thioguanine nucleotides (6TGN),^[9] which are incorporated into DNA. As a result, a process that involves the mismatch repair pathway triggers cell-cycle arrest and apoptosis.^[10] Owing to its cytotoxic properties, 6TG is currently being used in clinics for the treatment of leukemia.^[11] The natural role of 6TG and its biosynthesis in *E. amylovora*, however, have remained enigmatic until now. In this study, we unveil the molecular basis of 6TG biosynthesis, provide first insights into the formation of a peculiar

[*] Dr. S. Coyne, A. Litomska, Prof. Dr. C. Hertweck
Dept. of Biomolecular Chemistry, Leibniz Institute for Natural Product Research and Infection Biology, HKI
Beutenbergstrasse 11a, 07745 Jena (Germany)
E-mail: christian.hertweck@hki-jena.de
Homepage: <http://www.hki-jena.de>

Dr. C. Chizzali, M. N. A. Khalil, Prof. Dr. L. Beerhues
Institute for Pharmaceutical Biology
Technische Universität Braunschweig (Germany)

Dr. K. Richter
Julius Kühn Institute, Federal Research Centre for Cultivated Plants
Institute for Resistance Research and Stress Tolerance
Quedlinburg (Germany)

Prof. Dr. C. Hertweck
Chair of Natural Product Chemistry
Friedrich Schiller University Jena (Germany)

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enzymatic carbon–sulfur bond formation, and demonstrate the crucial role of 6TG in fire blight pathogenesis.

To select candidate 6TG biosynthetic genes we mined the full genome sequence of *E. amylovora*.^[12] Whereas no promising candidate genes for potential secondary metabolite sulfur transferases^[13] were found, we noted that a small gene cluster consisting of five genes (EAM_1028 to 1033, Figure 1B) is conserved in *Erwinia* species. Two genes of this cluster, *ycfA* and *ycfB*, have been implicated in “yellow compound formation” (*ycf*) of *E. amylovora*.^[14] Surprisingly, no biochemical functions have been assigned to these genes, and apart from its absorption spectrum and its ability to bind Cu^{2+} , the nature of the pigment has remained fully elusive. In an independent study using a random mutagenesis strategy, it was found that a transposon insertion in the vicinity of *ycfB* led to the loss of yellow pigmentation, too.^[15] Notably, in none of these studies the phenotypes have been correlated to the formation of 6TG, which shares the physicochemical properties with the pigment. Thus, we analyzed cultures of *E. amylovora* by HPLC–UV/MS and MSⁿ using synthetic 6TG as a reference and found that the yellow compound is identical with the antimetabolite (the Supporting Information). Next, we analyzed the cryptic *ycf* gene locus consisting of a LysR-type transcriptional regulator gene (*ycfR*) and four structural genes (*ycfABCD*), which clustered on a chromosomal region encoding several conserved proteins related to nucleotide metabolism.

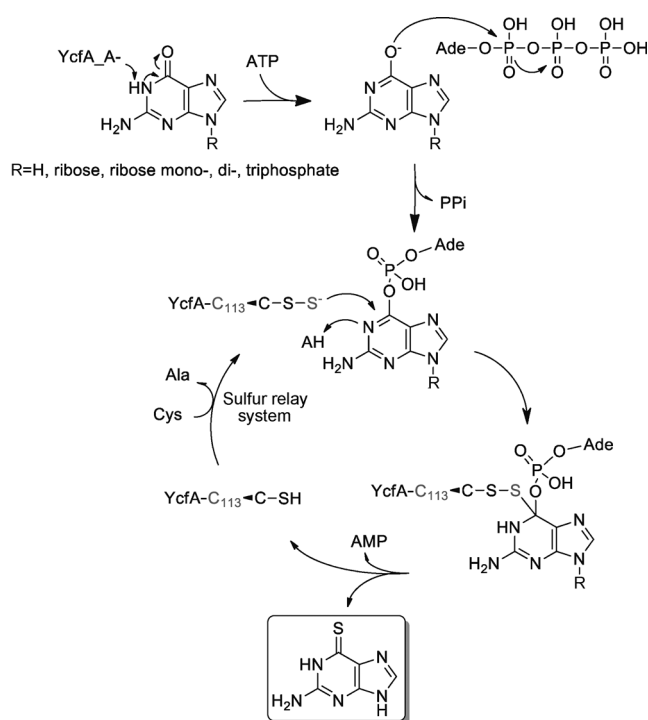
The deduced amino acid sequence of YcfA points to the presence of an adenylation domain, yet the overall sequence does not share any homology with known proteins. However, by protein modeling (Swiss Model)^[16] we found that YcfA shares structural homologies with GMP synthetase from *Thermus thermophilus* and MnmA, a 2-thiouridylase from *E. coli*.^[17] MnmA is a conserved enzyme responsible for the thiolation of tRNA–uridine, a modification involved in tRNA maturation.^[17] YcfB possesses the characteristic motif of NUDIX (nucleotide diphosphate linked to X) hydrolases, which typically catalyze the hydrolysis of NMP–X into NMP + X.^[18] YcfC does not exhibit any homology with known proteins, and YcfD likely belongs to the drug/metabolite transporter (DMT) family.

To evaluate the identity of the *ycf* gene cluster we deleted *ycfA* in the *E. amylovora* genome by PCR targeting.^[19] HPLC–MS analysis showed that inactivation of the *ycfA* gene totally abolished 6TG production in *E. amylovora* (Figure 1C/D). Trans-complementation by a plasmidic *ycfA* copy restored 6TG production in *E. amylovora* Δ *ycfA* (Figure 1D, lane c). This result unequivocally showed that YcfA is crucial for 6TG biosynthesis.

To further corroborate this finding we reconstituted the entire pathway in *E. coli*. Therefore, by using PCR techniques we amplified *ycfABCD* together with the putative *ycfA* promoter region and cloned the cassette into an expression plasmid. After introduction of p(*ycfABCD*) into *E. coli* BL21 (DE3), the metabolic profile of the transformant was monitored by HPLC–UV and HRESI-MS. Heterologous expression of *ycfABCD* conferred the ability to produce 6TG to *E. coli* (Figure 1C/E). The level of 6TG production was even five times higher than the production observed in

the natural producer. This result unequivocally proved that the *ycfABCD* gene cluster is responsible and sufficient for 6TG biosynthesis. To define the minimal set of genes required for 6TG biosynthesis, we constructed plasmids harboring reduced *ycf* gene cassettes and introduced these into *E. coli* for heterologous expression. The metabolic profile of *E. coli* carrying *ycfABC* showed that the strain still produced substantial amounts of 6TG. Although the production level was slightly decreased in comparison to the *ycfABCD* construct (Figure 1E), it indicates that the predicted transporter YcfD is not essential for 6TG production in the heterologous host. In an *E. coli* strain carrying the *ycfAB* genes, however, the antimetabolite was formed only in trace amounts. Finally, expression of *ycfA* alone was not sufficient to heterologously produce 6TG (Figure 1E).

Based on these genetic results and the in silico prediction, we concluded that YcfA is the key enzyme that catalyzes the sulfur transfer onto a guanine backbone. For the homologous MnmA 2-thiouridylase, it has been shown that a catalytic cysteine thiol is transformed into a persulfidic sulfur donor.^[20] YcfA harbors three cysteine residues (at positions 25, 43, and 113), which could be involved in the thiolation of the guanine heterocycle. By site-directed mutagenesis, we exchanged each of these cysteines for alanine in the *E. coli* construct carrying *ycfABCD*. While the substitutions of Cys25 and Cys43 did not alter the metabolic profiles of the mutants, the Cys113-to-Ala mutation completely abolished 6TG production (the Supporting Information). The YcfA Cys113 residue thus likely represents the persulfide-activatable residue. Altogether, these results indicate that YcfA-mediated 6TG biosynthesis is analogous to thiouridine formation (Scheme 1). By adeny-



Scheme 1. Model of YcfA-mediated 6-thioguanine biosynthesis. Ade = adenosine, A = base sulfur relay system.

lation, YcfA would activate the guanine backbone. In analogy to the catalytic cycle of MmA, the highly reactive persulfide sulfur of YcfA is likely provided by a sulfur relay system that likely includes a cysteine desulfurylase and sulfur carrier proteins. Transfer of the sulfur on the guanine backbone would form 6TG with concomitant release of AMP and restoration of the native YcfA enzyme.

The deletion of the *ycfA* gene in *E. amylovora* has provided a 6TG-negative mutant that appeared to be ideally suited for investigating the biological role of this cytotoxin in *E. amylovora* pathogenicity. For this purpose we inoculated shoots of the apple (*Malus × domestica*) cultivar “Holsteiner Cox” with the wild type and the $\Delta ycfA$ mutant and monitored morphological changes. Seven days after inoculation with the *E. amylovora* wild-type strain, the veins of the two cut leaves became necrotic (Figure 2A). After 14 days, necrosis in the wild-type-treated plants embraced the first three leaves and a 5 cm segment of the shoot tip, and four weeks after inoculation, the transition zone, that is, the interface between necrotic and healthy stem segments, had extended downwards by another 3 cm. The two attached leaves had turned necrotic after 42 days when the transition zone stopped migrating. In stark contrast to the infection with the wild type, plants inoculated with the $\Delta ycfA$ mutant lacked any disease symptoms (Figure 2B). In fact, they looked identical to mock-inoculated plants.

In addition to the macrovisual effect of the infection, we monitored the diversity and quantity of phytoalexins formed in the apple plants. The production of biphenyl and dibenzofuran phytoalexins by apple plants has been shown to occur in response to an infection by *E. amylovora* and has been implicated as an induced defense mechanism.^[21] Biphenyl and dibenzofuran phytoalexins were detected in *E. amylovora*-inoculated Holsteiner Cox plants in the stem segment between the necrotic and the healthy tissue after this transition zone had stopped migrating (Figure 2A). The four biphenyls, 3-hydroxy-5-methoxybiphenyl (**1**, $(15.43 \pm 9.86) \mu\text{g g}^{-1}$ DW; DW = dry weight), aucuparin (**2**, $(36.86 \pm 8.29) \mu\text{g g}^{-1}$ DW, LOD: $1.9 \mu\text{g g}^{-1}$ DW; LOD = limit of detection), noraucuparin (**3**, $(121.43 \pm 26.26) \mu\text{g g}^{-1}$, LOD: $1.59 \mu\text{g g}^{-1}$ DW), and 2'-hydroxyaucuparin (**4**, $(30.71 \pm 15.93) \mu\text{g g}^{-1}$ DW), were detected by GC–MS

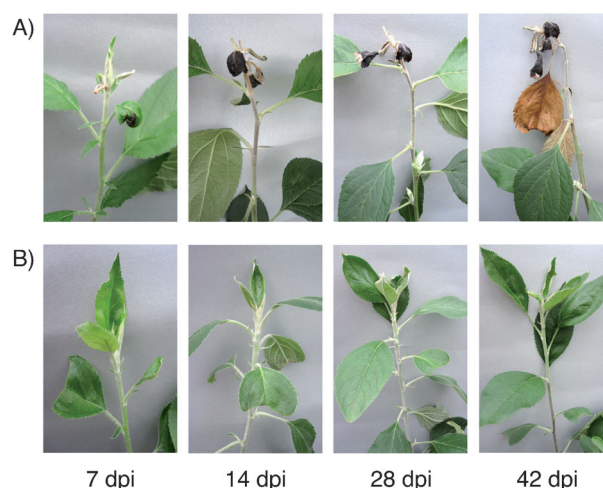


Figure 2. Morphological changes observed in Holsteiner Cox plants at various times after inoculation with A) the *E. amylovora* wild type and B) the 6TG-deficient $\Delta ycfA$ mutant. dpi = days after inoculation.

after derivatization (Figure 3A). In addition to these biphenyls, the transition zone accumulated the two dibenzofurans eriobofuran (**5**; $(19.43 \pm 6.44) \mu\text{g g}^{-1}$ DW) and noreriobofuran (**6**; $(31.14 \pm 12.13) \mu\text{g g}^{-1}$ DW). The total concentration of all biphenyl and dibenzofuran phytoalexins in the transition zone of apple plants treated with wild-type *E. amylovora* was $(255.0 \pm 78.91) \mu\text{g g}^{-1}$ DW. In contrast, no phytoalexins could

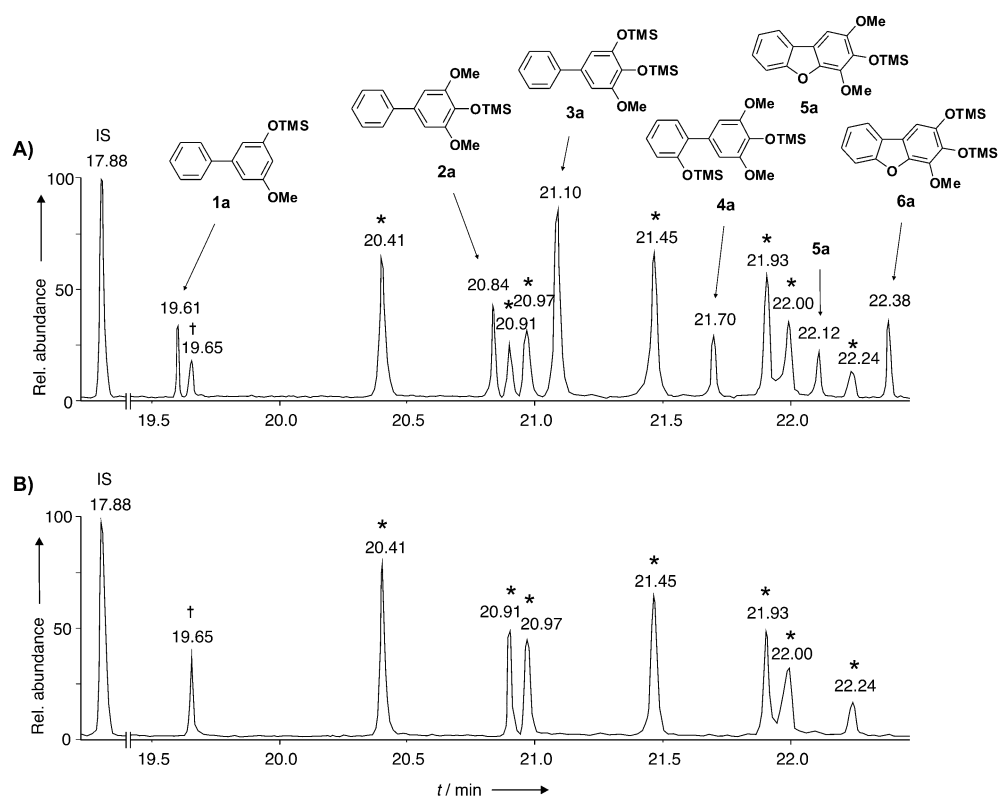


Figure 3. GC–MS analysis of biphenyls and dibenzofurans present in methanolic extracts from Holsteiner Cox shoots 42 days after inoculation with A) the *E. amylovora* wild type and B) the 6TG-deficient $\Delta ycfA$ mutant. Compounds were separated as trimethylsilyl (TMS) derivatives named **1a–6a**. IS = internal standard (4-phenylphenol), †sugar derivatives, *fatty acid derivatives.

be detected in the Holsteiner Cox plants inoculated with the $\Delta ycfA$ mutant (Figure 3B). This result is again identical to the one obtained with the mock-inoculated plants. The absence of symptoms and of phytoalexin production from apple plants inoculated with the $\Delta ycfA$ mutant corroborated the crucial role of 6TG in the development of fire blight disease.

To validate the results obtained for apple plants, we investigated the effects of an infection by the $\Delta ycfA$ mutant on *Sorbus aucuparia* cell cultures. *S. aucuparia* (mountain ash) belongs to the Rosaceae family and its cell cultures are an established model to study aspects of fire blight disease.^[22] Using a trypan blue assay, we compared the plant cell viability after infection by wild-type and $\Delta ycfA$ strains. After 72 h infection, the *E. amylovora* wild type caused 36% cell death, in contrast to only 9% observed with the $\Delta ycfA$ mutant (see the Supporting Information). Since *S. aucuparia* cells infected by wild-type *E. amylovora* resemble apple plants in synthesizing a cocktail of phytoalexins,^[22] we also analyzed the metabolites released as an indicator of virulence. In contrast to the wild-type *E. amylovora*, which stimulated the production of biphenyls and dibenzofurans, the $\Delta ycfA$ mutant did not induce the biosynthesis of any diagnostic phytoalexins in the *S. aucuparia* cell suspension cultures (the Supporting Information). Infection of *S. aucuparia* cell cultures confirmed the results obtained in planta and unambiguously demonstrated a major role of *ycfA*, and thus of 6TG production, in the development of the fire blight disease.

These results are particularly intriguing in light of earlier studies, which raised considerable ambiguity with regard to the biological function of 6TG. Addition of purified 6TG to a Bartlett pear cell suspension culture (BASC) led to neither growth inhibition nor necrosis.^[7] In addition, an apparently 6TG-negative strain was described to be able to induce fire blight disease, albeit only in the presence of a 6TG producer.^[7] Our data, in contrast, clearly correlate the symptoms of fire blight with 6TG biosynthesis. Apart from being a cytotoxic antimetabolite, it is also well conceivable that 6TG affects the plant response to the infection and for example, favors the survival and/or dissemination of the bacterium in its hosts. Its remarkable structural similarity with the cytokinins hints to a dysregulation of the phytohormone balance. Alternatively, a role of this sulfur compound in oxidative burst resistance is plausible.

In summary, we have unveiled the genetic basis for the biosynthesis of 6-thioguanine, an antimetabolite in clinical use. By taking advantage of published but thus far overlooked information, a unique five-gene cluster was identified in *E. amylovora*. Through computational studies and mutational analyses, we have revealed that *YcfA* represents the key enzyme for the biosynthesis of the sulfur compound. Both *YcfA* and *MnmA* appear to possess an adenylation domain and form an active persulfide-bound cysteine to transfer sulfur on a nucleobase (in a tRNA complex for *MnmA*). Notably, 6TG contains a thioamide moiety, which is scarce in nature,^[23] and to date no mechanism for the formation of this peculiar moiety has been described. This work reports a new avenue to thioamides that is analogous to thiourea formation by *MnmA*.^[20] From a biotechnological point of view it is noteworthy that heterologous gene expression production

allows the high-titer production of the valuable chemotherapeutic in a nonpathogenic, readily culturable bacterium. The most important result to emerge from this study is that 6TG plays a key role in the pathogenicity of *E. amylovora*, which ranks among the top ten plant pathogens.^[24] For the first time, we have correlated the biosynthesis of 6TG with necrosis and phytoalexin formation in intact apple plants and cultured *S. aucuparia* cells. Our findings are an important addition to the knowledge on fire blight pathogenesis, since they may point to novel prophylactic and/or therapeutic treatments of fire blight disease. Such measures are urgently needed, because fire blight rapidly spreads worldwide—and antibiotic-resistant *E. amylovora* strains are on the rise.^[25]

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