6-thioguanine biosynthesis in *Erwinia* species

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Abstract

Erwinia amylovora **produces a compound with an absorbance maximum at 340 nm that forms a yellow colored complex with copper. This compound was identified as 6-thioguanine, a guanine analogue that is used in chemotherapy and the treatment of inflammatory bowel disease. Synthesis of 6-thioguanine could be linked to five genes common in *Erwinia* genomes, but missing in related genera. Expression of *Erwinia tasmaniensis* genes *tgsA-D* in *Escherichia coli* was sufficient for heterologous production of 6-thioguanine. Transfer of the four biosynthetic *E. tasmaniensis* genes did not enhance resistance of *E. coli* against 6-thioguanine. Bacterial and synthetic 6-thioguanine have a strong growth inhibitory effect on many bacteria, such as *E. coli* or a number of *Pantoea agglomerans* isolates. While this inhibition of competing species might provide an advantage to *Erwinia*, further biological functions of 6-thioguanine cannot be excluded. A direct link between 6-thioguanine synthesis of *E. amylovora* and its pathogenicity was not observed, as two 6-thioguanine negative mutants produced as severe symptoms on apple and pear shoots as did producing strains.**

INTRODUCTION

On minimal medium supplemented with copper sulfate, *Erwinia amylovora* colonies show a yellow coloration (Bereswill, Jock et al. 1998). This phenotype can be used as selection criterion for isolation from infected plant material. The yellow color formation has been linked to a substance with an absorption maximum of 340 nm (CP340) at neutral pH that can be detected in culture supernatants (Zhang et al. 2000). An open reading frame (ORF) tentatively annotated as *ycfA* (yellow color formation) could be linked to biosynthesis of this compound. Notwithstanding the growing amount of sequence information, BLAST comparison of the *E. amylovora ycfA* against the databases showed no similarities to genes with known function.

In 1986, Feistner and Staub investigated potential phytotoxin production in *E. amylovora* and therefore analyzed secondary metabolites produced by the pathogen (Feistner and Staub 1986). They demonstrated the production of 6-thioguanine (6-TG) by various *E. amylovora* isolates, but could exclude a function as phytotoxin for this compound. 6-thioguanine is a

guanine analogue with various medical applications. The synthetic compound among other thiopurines has been applied in treatment of acute lymphoblastic leukemia and ulcerative colitis (Hedeland et al. 2010, Bar et al. 2013). It acts as an antimetabolite, inhibits purine *de-novo* synthesis and can be incorporated into DNA and RNA (McCollister et al. 1964, Nelson et al. 1975, Somerville et al. 2003). Less is known on potential influences on regulatory pathways as for example methylation patterns are also affected by 6-TG content of the cells (Wang and Wang 2009). An inhibitory effect of 6-TG on several bacterial species has been described (Mandel et al. 1965, Hill and Pittillo 1973).

For *E. amylovora*, function and biosynthetic basis of 6-TG production remained unclear. In this current study we investigated distribution of 6-TG biosynthesis among *Erwinia* species and related genera. We identified a five open-reading-frame gene cluster involved in 6-TG biosynthesis and analyzed its function in heterologous expression.

RESULTS AND DISCUSSION

Comparison of *Erwinia* spp. showed a high conservation of CP340 within this genus. Supernatants from closely related pathogens like *E. pyrifoliae* as well as epiphytic species like *Erwinia billingiae* and *Erwinia tasmaniensis* showed an absorption peak at 340nm. While absolute concentrations varied, *E. tasmaniensis* Et1/99 repeatedly produced high amounts of this compound, so purification by ion exchange chromatography was performed starting with Et1/99 supernatants. CP340 was confirmed as 6-TG by HPLC/MS and NMR analysis. To identify potential 6-TG biosynthetic genes, transposon mutagenesis with a mini-Tn*5* derivative was performed in Et1/99. Six mutants with reduced 6-TG production and three mutants that did not produce any 6-TG were selected for further analysis. Insertion sites could be located in genes from purine as well as pyrimidine metabolism or from other pathways like a putative sulfate/thiosulfate transporter. No potential dedicated 6-TG biosynthetic region was identified in the screening.

The genetic region flanking the previously annotated *ycfA* is highly conserved among *Erwinia*. Comparison to other *Enterobacteriaceae* like *Escherichia coli* revealed a possible insertion of five 6-TG related ORFs between *htpG* and *adk*. A putative transcriptional regulator and four adjacent genes from *E. tasmaniensis* Et1/99 where subcloned into the expression vector p3T for further analysis. Heterologous expression in *E. coli* XL1blue showed, that the four genes *tgsA* (formerly *ycfA*), *tgsB*, *tgsC*, and *tgsD* where sufficient for 6-TG production. Smaller fragments missing one or more genes did not result in 6-TG formation. A deletion mutagenesis eliminating individual *tgs* genes from expression plasmid p3T-tgsA-D showed that deletion of any of the four genes resulted in loss of heterologous 6-TG production.

Neither gene from the *tgs* region enhanced resistance of *E. coli* against 6-TG. The heterologous expression strains could only grow in minimal medium if supplemented with either adenosine or guanosine. We could show a diverse degree of sensitivity towards 6-TG among plant pathogenic bacteria, saprophytes and common epiphytes. While no strain from the genus *Pectobacterium* and *Dickeya* was inhibited by 6-TG, sensitivity among *Pantoea agglomerans* isolates varied. Given the low minimal inhibitory concentration of 6-TG necessary to inhibit sensitive bacteria, 6-TG production might enable *E. amylovora* to antagonize competing bacteria in its host environment.

Few strains of *E. amylovora* lacking 6-TG production are found among isolates from plant material. One such example is Ea25/82. This strain and an artificial insertion mutant of *tgsA*, Ea1/79-YA, form white colonies on MM2Cu and show no 6-TG in culture supernatants. A complementation of both strains was possible by plasmid expression of *tgsA* (Zhang et al. 2000). In plant assays on detached apple flowers, pear slice assays and shoot infection on apple and pear seadlings (MM106 and Kirchensaller Mostbirne) both 6-TG mutants still induced symptom formation. No necrosis was observed when bathing pear slices in a solution

of synthetic 6-TG, confirming the findings of Feistner (1986) that it is unlikely to act as phytotoxin. No direct correlation of 6-TG synthesis to pathogenicity was observed.

CONCLUSIONS

Synthesis of 6-TG is highly conserved among *Erwinia* spp., while rarely found in other genera. A chromosomal region, consisting of five genes flanked by the *htpG* and *adk* could be identified in the genomes of *E. amylovora*, *E. pyrifoliae* and *E. billingiae* and *E. tasmaniensis*. One putative transcriptional regulator, *tgsR*, and four adjacent genes *tgsA-D* could be linked to 6-TG synthesis. The genes *tgsA-D* were sufficient and necessary for heterologous production of 6-TG by *E. coli* XL1blue. The mechanism that protects 6-TG-producing *Erwinia* against the compound remains unclear. 6-TG is highly active against various bacteria like *E. coli* and some isolates of *P. agglomerans*, yet this inhibitory effect is abolished in the presence of adenosine or guanosine. It is possible that 6-TG production supresses growth of competitors of *Erwinia* on the plant surface. Many modified nucleobases produced by bacteria are incorporated into their RNA. For example, in tRNA such incorporation at specific positions is supposed to have a regulatory effect. It remains to be investigated if 6-TG is incorporated into DNA or RNA of the producing *Erwinia* species and if its synthesis has an effect on gene regulation. No direct correlation between 6-TG production and pathogenicity of *E. amylovora* on apple and pear was observed.

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