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Multicopy suppression of a *gacA* mutation by the *infC* operon in *Pseudomonas fluorescens* CHA0: competition with the global translational regulator RsmA

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Abstract

The *gacA* gene of the biocontrol strain *Pseudomonas fluorescens* CHA0 codes for a response regulator which, together with the sensor kinase GacS (= LemA), is required for the production of exoenzymes and secondary metabolites involved in biocontrol, including hydrogen cyanide (HCN). A *gacA* multicopy suppressor was isolated from a cosmid library of strain CHA0 and identified as the *infC-rpmI-rplT* operon, which encodes the translation initiation factor IF3 and the ribosomal proteins L35 and L20. The efficiency of suppression was about 30%, as determined by the use of a GacA-controlled reporter construct, i.e. a translational *hcnA′–′lacZ* fusion. Overexpression of the *rsmA* gene (coding for a global translational repressor) reversed the suppressive effect of the amplified *infC* operon. This finding suggests that some product(s) of the *infC* operon can compete with RsmA at the level of translation in *P. fluorescens* CHA0 and that important biocontrol traits can be regulated at this level.

Keywords: GacA; RsmA; Global regulation; Secondary metabolism; Translational control; *Pseudomonas fluorescens*

1. Introduction

Pseudomonas fluorescens CHA0 is a root-colonizing biocontrol bacterium which suppresses root diseases caused by soil-borne fungi of various crop plants [1,2]. Extracellular antifungal metabolites, such as hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol, and pyoluteorin produced by strain CHA0, contribute to disease suppression. The global activator GacA is essential for antibiotic and HCN production in *P. fluorescens* [3]. Mutational inactivation of the *gacA* gene results in loss of virtually all secondary metabolites and exoenzymes in strain CHA0 and, concomitantly, impairs protection of dicotyledonous plants from a range of fungal root pathogens [3–5]. GacA is a response regulator belonging to a family of bacterial two-component regulatory systems [3,6]. Its cognate sensor kinase is encoded by the *gacS* gene, formerly designated *lemA* [7–9]. Homologs of the conserved *gacS*/*gacA* system have been identified as regulators of virulence in numerous animal- or plant-pathogenic species of *Pseudomonas* and enteric bacteria [8,10–12]. Our recent studies [13] have revealed that the structural genes for HCN biosynthesis (*hcnABC*) and extracellular protease (*aprA*) are regulated indirectly by GacA via a posttranscriptional mechanism involving RsmA, a translational repressor of secondary metabolism [14,15]. The GacA/RsmA regulatory cascade appears to act on specific mRNA recognition sites which can overlap with the ribosome binding sites of target genes [13]. GacA might stimulate the expression of a regulatory element that relieves RsmA-mediated translational repression. To identify such an additional component of the GacA/RsmA system, we searched for a suppressor of a *gacA* mutation in *P. fluorescens* CHA0. Here, we report the characterization of a multicopy suppressor that partially restores *hcn* gene expression to a *gacA* mutant of CHA0.

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2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

P. fluorescens CHA0 (wild-type), CHA89 (*gacA*::Km^R), and CHA500 (Δ*gacA*) [3] as well as the *Escherichia coli* cloning strains DH5α [16] and 3704 (*dam*) [17] have been described before. *P. fluorescens* strains CHA207 (chromosomal *hcnA′–′lacZ* fusion) and CHA89.207 (*gacA*, chromosomal *hcnA′–′lacZ* fusion) were constructed as previously reported [13]. Recombinant plasmids (Fig. 2) were constructed in the vectors pUK21 [18], pVK100 [19], pME6000 [20], pME6010 and pME6030 [21]. Derivatives of pVK100 and pME6000 were mobilized from *E. coli* to *P. fluorescens* with the helper plasmid pME497 [2]. A genomic library of strain CHA0 established in cosmid pVK100 and the recombinant plasmids pME6001 and pME6073 (overexpressing *rsmA*) have been described [13,22]. Bacterial strains were grown in nutrient yeast broth (NYB) or on nutrient agar plates [23] at 30°C (*P. fluorescens*) or at 37°C (*E. coli*). When required, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), gentamicin (10 μg ml^{-1}) or tetracycline (25 μg ml^{-1} for *E. coli* and 125 μg ml^{-1} for *P. fluorescens*) were added to the medium.

2.2. DNA manipulations and analysis

Standard recombinant techniques were used [16] or have been referenced elsewhere [20,24]. For subcloning experiments with cosmid pVK100 (23 kb), DNA fragments were ligated, after electrophoretic separation, in low melting point agarose gels. The complete nucleotide sequence of the *infC* operon was determined on both strands by Euro Sequence Gene Service (ESGS; Evry, France). For sequencing, fragments of the *infC* operon were cloned into pBluescript KS^+ (Stratagene), since it proved not possible to insert the entire *Kpn*I-*Eco*RV fragment carrying the operon into this vector. The nucleotide sequences of the *infC* in-frame deletion constructs were verified by using the Dye Terminator Kit (Perkin Elmer, #402080) and the ABI PRISM™ 373 sequencer. Nucleotide and deduced amino acid sequences were analyzed with the programs BLAST, GAP, BESTFIT and PILEUP using the Genetics Computer Group (GCG) package (University of Wisconsin).

2.3. GenBank accession number

The nucleotide sequence of the 2.4 kb *Kpn*I-*Eco*RV fragment containing the *infC-rpmI-rplT* operon of *P. fluorescens* CHA0 is reported in accession number AF136400.

2.4. Construction of mutations in the infC operon

For the construction of pME6534 (Fig. 1), the 2.4 kb *Kpn*I-*Eco*RV fragment from cosmid pME3020 was cloned into pVK100, after intermediate subcloning into the low copy number vector pME6030. The *Xho*I-*Stu*I fragment containing *infC* from pME6534 was first introduced into pME6010, excised together with the kanamycin resistance gene promoter (P_{kan}) on a *Stu*I fragment and cloned into pME6000, producing pME6527 (Fig. 1). To construct pME6525 (Fig. 1), a *Stu*I-*Eco*RV fragment carrying the *rpmI* and *rplT* genes was cloned into pVK100 using linkers from pME6010. In-frame deletions in *infC* were created by inserting the *Kpn*I-*Stu*I fragment containing *infC* into pUK21, from which the *Sfu*I (= *Bst*BI) site had been removed. Deletions of 327 bp and 99 bp were produced by digestion with *Tth*111I+*Bcl*I (using plasmid DNA extracted from *E. coli* 3704 to allow *Bcl*I cleavage) and with *Xmn*I+*Sfu*I, respectively, filling-in with T4 DNA polymerase and ligation. Deletions were verified by sequencing. The *Kpn*I-*Stu*I fragments from the resulting constructs were cloned into pME6534, creating pME6545 and pME6546, respectively (Fig. 1).

2.5. Biochemical assays

HCN production by *P. fluorescens* derivatives growing on nutrient agar was assessed by a qualitative test [25]. β-Galactosidase activities were determined in cells grown in NYB with aeration to an OD_{600} of 2.0–2.5.

3. Results

3.1. Isolation of a suppressor that partially restores HCN synthesis in a gacA mutant of strain CHA0

The *gacA* deletion mutant CHA500, which does not produce HCN, could be functionally complemented for HCN production by recombinant cosmids which contained either the $gacA^+$ gene [3], the $hcnABC^+$ structural genes [22,24], or a common 7.5 kb *Hin*dIII fragment unrelated to *gacA* or *hcnABC*. HCN production was assessed by a qualitative test [25]. A representative cosmid carrying the 7.5 kb insert, pME3020, was analyzed further in order to characterize the *gacA* suppressor.

Suppressor activity was monitored in the *gacA* mutant CHA89.207, which contains a chromosomal *hcnA′–′lacZ* fusion. Strain CHA89.207 formed light blue colonies on agar containing X-Gal and expressed β-galactosidase at a low level (56 ± 10 Miller units), whereas the $gacA^+$ parental strain CHA207 had a 40-fold higher β-galactosidase activity (2400 ± 400 Miller units). Upon introduction of pME3020 into strain CHA89.207, dark blue colonies and partial restoration of β-galactosidase activity (580 ± 30 Miller units) were found. The segment of pME3020 responsible for *gacA* suppression was located by deletion and subcloning experiments (not shown) to an internal 2.4 kb *Kpn*I-*Eco*RV fragment, which was inserted into cosmid pVK100, producing pME6534 (Fig. 1). Cosmid pVK100 was chosen as a vector because of its intermediate

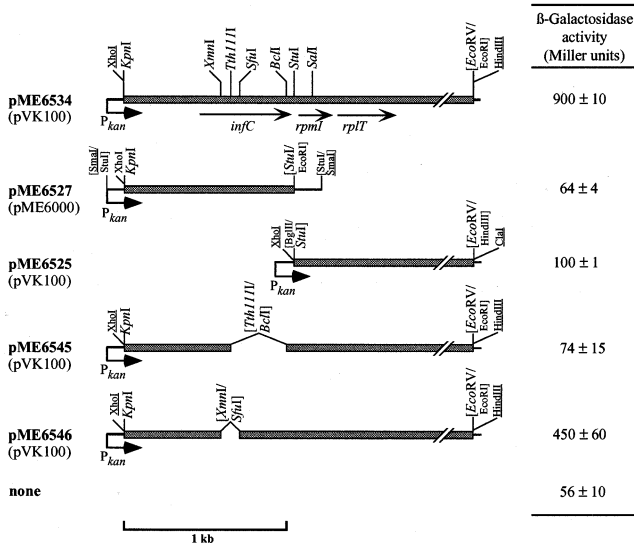

Fig. 1. Deletion constructs of the *infC-rpmI-rplT* operon and their ability to restore *hcn* expression to a *gacA* mutant. Strain CHA89.207 (*gacA*, *hcnA′–′lacZ*) was transformed with the constructs shown; the vectors used are indicated in parentheses and the constructions are described in Section 2. Restriction sites derived from genomic DNA are indicated in italics. Vector restriction sites are in roman, those used for cloning are underlined, and those lost are shown in parentheses. P_{kan}, kanamycin resistance gene promoter. β-Galactosidase expression (Miller units) was determined when cells reached an OD_{600} of about 2.5 in NYB. Activities are mean values of triplicate experiments ± standard deviation.

(though unknown) copy number, a property which appeared to favor *gacA* suppression without interfering with cell growth. Vector pME6000, which has about 18 copies in strain CHA0 [20], was used for some constructs, but pME6000 derivatives carrying the 2.4 kb segment mentioned above caused marked growth inhibition in *P. fluorescens*.

3.2. The gacA suppressor consists of the infC-rpmI-rplT operon

The 2.4 kb *Kpn*I-*Eco*RV fragment was sequenced and found to contain three open reading frames forming the *infC-rpmI-rplT* operon (Fig. 2), which encodes the translation initiation factor IF3 and the ribosomal proteins L35 and L20 present in the 50S subunit. The deduced amino acid sequences show identities of 66% for IF3, 53% for L35 and 82% for L20 of *E. coli*, and 98% for IF3, 100% for L35 and 98% for L20 of *Pseudomonas syringae* [26,27]. Conserved amino acid residues in these proteins are highlighted in Fig. 2. Interestingly, the *infC* operon has previously been identified as a multicopy suppressor of a *gacS* mutation in *P. syringae* [27]. The *infC-rpmI-rplT* operon of strain CHA0 is flanked upstream by *thrS*, coding for threonyl-tRNA synthetase, and downstream by *pheS*, the gene for phenylalanyl-tRNA synthetase (Fig. 2). The organization of these five genes is the same in *E. coli* and in *P. syringae*. In *E. coli* the genes belonging to the *infC* operon are expressed from four different promoters, one of which is located at the 3′ end of the *thrS* gene and probably accounts for most of the *infC-rpmI-rplT* expression [26]. The same promoter appears to be present also in *P. fluorescens* (Fig. 2) and in *P. syringae* [27], since the promoter sequence and location are identical in the three organisms.

Deletions were created in the *infC* operon of *P. fluorescens* CHA0 to determine the suppressor locus more precisely. A large in-frame deletion in *infC*, which removed 109 of the 183 codons of *infC* on plasmid pME6545, abolished suppression, whereas a 33 codon deletion in *infC* (on pME6546) did not (Fig. 1). Plasmid pME6527 carrying the *infC* gene alone had no suppressor activity, whereas pME6525 carrying *rpmI-rplT* behind the kanamycin resistance gene promoter had low activity (Fig. 1). These results indicate that optimal suppression is brought about by the intact *infC* operon carried by pME6534; the efficiency of suppression was 25–40% (Fig. 1), by comparison with the *hcnA′–′lacZ* activity in a $gacA^+$ background.

3.3. Overexpression of the rsmA gene reverses the suppressor effect of the infC operon

In *P. fluorescens*, the GacS/GacA system regulates genes of secondary metabolism including the *hcn* genes, via a cascade in which the translational repressor RsmA is involved, apparently at the level of translation initiation [13]. Overexpression of *rsmA* caused a 7-fold reduced expression of a *hcnA′–′lacZ* translational fusion in strain CHA207 (Table 1). We tested whether multiple copies of the *infC* operon could out-compete RsmA. This was done

Table 1
Antagonistic effects of *infC-rpmI-rplT* and *rsmA* overexpression on *hcnA′–′lacZ* expression in *P. fluorescens*

Strain/plasmid	β-Galactosidase activity[a]		RsmA repression factor
	+pME6001[b] (vector control)	+pME6073[b] ($rsmA^{++}$)	
CHA207	2500 ± 500	350 ± 40	7
CHA89.207	70 ± 6	< 5	> 14
CHA89.207/pME6534	800 ± 100	110 ± 20	7
CHA89.207/pME6546	670 ± 30	130 ± 10	5

[a] β-Galactosidase activities (Miller units) were determined in triplicate; mean values ± standard deviation are given.
[b] The *hcn* expression was tested in the presence (pME6073) or absence (pME6001) of overexpressed *rsmA* (designated by ($rsmA^{++}$). Cells were grown in 20 ml NYB with gentamicin (10 μg ml^{-1}) to an OD_{600} of 2.0–2.5.

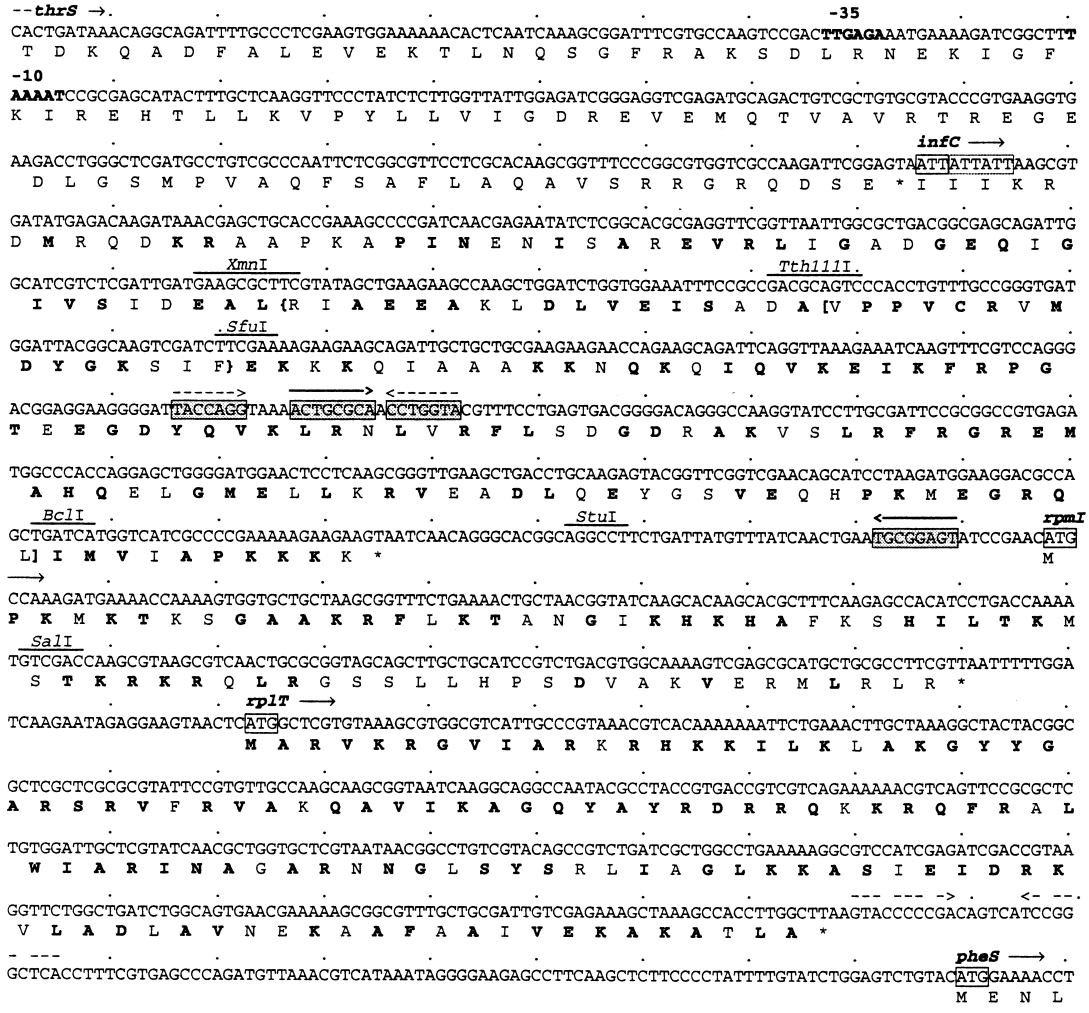

Fig. 2. Nucleotide sequence of the *infC-rpmI-rplT* operon of *P. fluorescens* CHA0 and deduced amino acid sequences of its protein products. The putative start codons are boxed. Restriction sites are indicated by lines above the nucleotide sequence. { }, deletion in pME6546; [ ], deletion in pME6545. The −35 and −10 elements of a potential internal promoter in *thrS* are shown in boldface. Amino acid residues in *infC-rpmI-rplT* which are conserved in *P. fluorescens*, *P. syringae* and *E. coli* are also indicated in boldface. Sequences with the potential to form a pseudoknot are shaded and boxed. Facing arrows indicate inverted repeats; this includes a putative ρ-independent terminator downstream of *rplT*. Nucleotide numbering starts at the unique *Kpn*I site of pME6534.

in the *gacA* mutant CHA89.207 whose chromosomal *hcnA'–'lacZ* fusion was repressed to an undetectable low level by the *rsmA* overexpressing plasmid pME6073 (Table 1). In the suppressed *gacA* mutant CHA89.207 carrying the *infC* construct pME6534 or pME6546 (Fig. 1), *rsmA* overexpression no longer resulted in complete repression of *hcnA'–'lacZ*, but a basal level of 110 to 130 Miller units was detected (Table 1). The data of Table 1 also show that *rsmA* overexpression strongly reduced the suppressive effect of pME6534 and pME6546, suggesting that multiple copies of the *infC* operon and *rsmA* have antagonistic effects on *hcn* expression.

4. Discussion

In this study, we have identified the *infC-rpmI-rplT* operon as a multicopy suppressor of a *gacA* mutation in *P. fluorescens* CHA0. Suppression was detected as restoration of HCN production and quantified by measuring *hcnA'–'lacZ* expression. In an entirely independent approach, the same operon had previously been isolated as a *gacS* (= *lemA*) suppressor in *P. syringae*, with restoration of protease production [27]. Kitten and Willis [27] carried out an extensive deletion analysis of the *infC* operon and concluded that overproduction of the strongly basic ribosomal proteins L20 (p*I* 11.6) and/or L35 (p*I* 12.2) was responsible for *gacS* suppression. Our data (Fig. 1) agree with this conclusion, except that the construct carrying only the *rpmI* and *rplT* genes, pME6525, had low suppressor activity. However, we are uncertain whether the ribosomal proteins L35 and L20 were overproduced from this construct. One major reason for our difficulty to pinpoint the suppressor locus more precisely

might be the complex regulation of the *infC* operon. In *E. coli*, this operon is subject to transcriptional regulation involving four different promoters and two different translational control circuits. IF3 represses the translation of its own gene by a regulation mechanism that involves the unusual AUU initiation codon [28], which also occurs in the *infC* gene of *P. fluorescens* (Fig. 2). Furthermore, in *E. coli*, L20 translationally represses the expression of *rpmI* and its own gene, *rplT* [29]. L20-mediated repression depends on a pseudoknot formed between the loop of a hairpin structure within *infC* and a region within the *rpmI* translation initiation site [30]. The *infC-rpmI* sequence of *P. fluorescens* (Fig. 2) has the same potential to form such a pseudoknot. Finally, the expression of all three genes *infC*, *rpmI*, and *rplT* is translationally coupled in *E. coli* [30]. Such intricate regulation might also apply to the *infC* operon of *P. fluorescens*, making predictions about the expression of subfragments difficult. Nevertheless, we deduce from our results that translation initiation factor IF3 is not, or not solely, responsible for *gacA* suppression, since a deletion of about 20% of the *infC* gene, which presumably inactivates IF3, still gave *gacA* suppression in strain CHA89.207/pME6546 (Fig. 1). In addition, overexpression of *infC* driven by P_{kan} on the high copy number plasmid pME6527 did not restore *hcnA′–′lacZ* expression of strain CHA89.207 (Fig. 1). A similar picture concerning the role of IF3 has also emerged from the analysis of the *infC* operon acting as a *gacS* suppressor in *P. syringae* [27].

Our current understanding of the GacS/GacA regulatory cascade implies that RsmA is one of several components mediating translational repression of target genes. This is indicated by the finding that mutational inactivation of the *rsmA* gene suppresses a *gacS* mutation with an efficiency of about 30% [13]. As we have shown here, multicopy suppression of a *gacA* mutation by the *infC* operon has a similar efficiency. The fact that overexpressions of *rsmA* and *infC-rpmI-rplT* have opposite effects on the expression of a translational *hcnA′–′lacZ* fusion (Table 1), supports our model according to which genes of exoproducts and secondary metabolism such as *hcnA* are controlled at the level of translation initiation. This RsmA-dependent control might also affect mRNA stability [14,15]. Since the GacA/RsmA signal transduction pathway may well be conserved in many beneficial biocontrol strains as well as in pathogenic bacteria, a range of important biocontrol and virulence traits may be regulated, in part, at a posttranscriptional level.

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