Thiamine-Auxotrophic Mutants of Pseudomonas fluorescens CHA0 Are Defective in Cell-Cell Signaling and Biocontrol Factor Expression

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The root-colonizing strain Pseudomonas fluorescens CHA0 protects various crop plants from root-pathogenic fungi (15). This biocontrol ability of strain CHA0 depends on the production of secondary metabolites, such as 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide, as well as on exoenzymes (17, 18, 31, 34, 41). The GacS/GacA signal transduction cascade positively regulates the synthesis of these extracellular products and is activated by signal molecules which are produced by the bacterium at high cell population densities (3, 14). The chemical structure of the signal is not known, but it is not related to the structure of well-studied bacterial signals, such as N-acyl-homoserine lactones or autoinducer 2 (2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran) (13, 34). In strain CHA0, three small regulatory RNAs, designated RsmX, RsmY, and RsmZ, are expressed under positive GacS/GacA control and are induced by addition of the signal (13, 14, 38). These small RNAs together relieve posttranscriptional repression of target genes, including phlA involved in DAPG biosynthesis and aprA encoding exoprotease AprA, by titrating the translational repressors RsmA and RsmE (13, 29, 38).

The aim of the present study was to characterize mutations that affect the expression of the rsmZ gene in strain CHA0. The small RNA encoded by this gene is expressed late in growth and is induced severalfold by addition of a signal-containing culture extract (13). To find mutants with such mutations, we used an rsmZ-gfp reporter fusion and Tn5 mutagenesis. Apart from gacS or gacA mutants, we expected to isolate mutants affected in signal synthesis.

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In the biocontrol strain Pseudomonas fluorescens CHA0, the Gac/Rsm signal transduction pathway positively controls the synthesis of antifungal secondary metabolites and exoenzymes. In this way, the GacS/GacA two-component system determines the expression of three small regulatory RNAs (RsmX, RsmY, and RsmZ) in a process activated by the strain’s own signal molecules, which are not related to N-acyl-homoserine lactones. Transposon Tn5 was used to isolate P. fluorescens CHA0 insertion mutants that expressed an rsmZ-gfp fusion at reduced levels. Five of these mutants were gacS negative, and in them the gacS mutation could be complemented for exoprotein and signal synthesis by the gacS wild-type allele. Furthermore, two thiamine-auxotrophic (thiC) mutants that exhibited decreased signal synthesis in the presence of 5 × 10−8 M thiamine were found. Under these conditions, a thiC mutant grew normally but showed reduced expression of the three small RNAs, the exoprotease AprA, and the antibiotic 2,4-diacyethylphloroglucinol. In a gnotobiotic system, a thiC mutant was impaired for biological control of Pythium ultimum on cress. Addition of excess exogenous thiamine restored all deficiencies of the mutant. Thus, thiamine appears to be an important factor in the expression of biological control by P. fluorescens.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Strains of Erwinia chrysanthemi col and P. fluorescens were routinely grown in nutrient broth (NYB) (36) with shaking or on nutrient agar (36) plates amended with the following antibiotics, when required: ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 25 μg/ml (125 μg/ml for selection of P. fluorescens). Chloramphenicol was used at a concentration of 10 μg/ml to select P. fluorescens and to counterselect E. coli in mating experiments. When required, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to plates at a final concentration of 0.02%. For signal production, strains were cultured in glycerol-Casamino Acids medium (GCM) (33) with shaking or on GCM solidified with agar (Serva) (10 g/liter). Microcultures were grown in 200 μl of GCM with shaking in 96 well-microplates (Greiner Bio-One, Kremsmuenster, Austria). When required, thiamine (vitamin B12 hydrochloride) was added to the media at final limiting and excess concentrations (5 × 10−6 M and 10−3 M, respectively). The incubation temperatures were 30°C for P. fluorescens and 37°C for E. coli. P. fluorescens was grown at 35°C to improve its capacity to accept heterologous DNA (e.g., in electrotransformation or in biparental mating with E. coli).

DNA manipulation. Small-scale plasmid extraction was carried out by the cetyltrimethylammonium bromide method (7); large-scale preparations were obtained with a Jetstar kit (Genomed GmbH, Basel, Switzerland). Chromosomal DNA from P. fluorescens was prepared as previously described (11). DNA manipulations were carried out by standard techniques (32). DNA fragments were purified from agarose gels with a QIAquick gel extraction kit (QIAGEN).

Construction of an rsmZ-gfp fusion. The 340-bp BamHI fragment of pME7401 was inserted into pUK21 to obtain pME7401. Plasmids pME7401 and pPROBE-TT’ were restricted with BamHI and HindIII. The rsmZ fragment of pME7401 and the cleaved vector pPROBE-TT’ were ligated to obtain pME7402 containing the transcriptional rsmZ-gfp fusion (Fig. 1).

Tn5 mutagenesis. P. fluorescens CHA0/pME7402 was mutagenized by insertion of transposon Tn5 in a mating with E. coli W3110/ pLG022. The two strains, each from a 1.5-milliliter culture, were mixed, centrifuged, and plated on nutrient agar at 35°C. After 3 h of mating, the cells were resuspended in 1 ml of saline (0.9% NaCl) and incubated on selective plates (GCM containing tetracycline, kanamycin, and chloramphenicol) at 30°C. First, mutants exhibiting reduced green furescent protein (Gfp) expression on plates were screened. Each of the 2,400 candidates obtained was inoculated into a separate well of microplates containing 200 μl of GCM and incubated for 24 h. The fluorescence (excitation at 480 nm and emission at 520 nm) and optical density at 600 nm were
resulting sequences were compared to those in genomic banks using BLAST-N.

The reaction conditions were 5 min at 95°C, followed by 10 cycles of 45 s at 95°C, 30 s at 45°C, and 2.5 min at 72°C, and then 3 min at 72°C. The reaction products were purified on an agarose gel, and the purified products (one to four fragments for each product; ca. 10 ng) were amplified with a BigDye Terminator 1.1 kit (Perkin-Elmer). The reaction conditions were 35 cycles of 15 s at 96°C, 15 s at 45°C, and 3 min at 60°C. The products were sequenced with an ABI-PRISM 377 automatic sequencer, and the resulting sequences were compared to those in genomic banks using BLAST-N.

The method used for elimination of the plasmid from P. fluorescens CHA50 and pME7402 was based on the incompatibility between pME6001 and pME7402. The strain was transformed with pME6001, with selection for gentamicin resistance and screening for tetracycline sensitivity. Tetracycline-sensitive clones were cultivated in NYB without antibiotics for 40 generations to allow loss of pME6001, which is somewhat unstable without selection. This curing procedure resulted in 10% plasmid-free colonies. Sequence analysis of cured mutant CHA50 confirmed the presence of Tn5 in thIC. Signal extraction from cell-free culture supernatants. For signal production, strains were inoculated as single colonies and grown with shaking in 200 ml of GCM in 500-ml Erlenmeyer flasks. After 20 h of growth, cells were removed by centrifugation. The supernatant was passed through a 0.45-μm-pore-size filter (filter type HA; Millipore Corporation, Bedford, MA), the pH was adjusted to 5.0 with HCl, and the preparation was extracted three times with 1/3 volume of dichloromethane. The combined extracts were dried with anhydrous Na2SO4 and filtered through a...
Semiquantitative determination of signal activity. Strains CHA0, CHA19, and CHA50 were grown and signal molecules were extracted as described above. Various amounts of extracted culture supernatants (corresponding to 5 to 100 ml) were added to reporter strain CHA0/pME6091 grown in 20 ml NYB, and the equivalent of 50 ml extracted supernatant dissolved in 100 ml methanol were added. Control assays were done with 100 ml methanol. β-Galactosidase activities were quantified by the method of Miller (22), using cells permeabilized with 5% (vol/vol) toluene.

Detection of exoprotease activity and antibiotics. Exoprotease activity was qualitatively detected on skim milk nutrient agar (9). The antibiotic activity of P. fluorescens strains grown on GCM was determined with Bacillus subtilis or Pythium ultimum strain 67-1 (obtained from Allelix Agriculture, Mississauga, Canada) as the reporter. In the former assay, P. fluorescens was grown overnight and killed with UV. An overlay with B. subtilis revealed antibiotic production by growth inhibition zones. For the latter assay, bacteria were streaked around the edge of a plate, and an inoculum of P. ultimum was transferred to the center of the plate. The plate was incubated at room temperature until P. ultimum reached the edge of the plate. For quantitative measurement, exoprotease activity was detected by fluorimetry in microtiter plates containing liquid GCM, and 47 candidates exhibiting reduced Gfp expression were identified. Of these, 2,400 clones which appeared to have a less pronounced yellow color on plates were analyzed in more detail by fluorimetry in microtiter plates containing liquid GCM, and 47 candidates exhibiting reduced Gfp expression were kept. Among these, seven isolates whose growth was indistinguishable from that of the wild type received particular attention; five of these isolates did not produce Gfp, and two exhibited strongly reduced Gfp expression. Data for one mutant of each type are shown in Fig. 2. The 40 remaining candidates were not analyzed further because they had growth handicaps or did not show the altered exoproduct formation that is typical of gacS and gacA mutants (18, 44).

The Tn5 insertions in the seven isolates kept were mapped by DNA sequencing. The five clones that did not produce Gfp (CHA52 to CHA56) were all gacS negative. The sequence data indicated that each Tn5 insertion had occurred at a different location in gacS (Table 1). The remaining two clones (CHA50 and CHA51), both of which showed reduced Gfp expression, were identified as thiC-negative mutants, as the sequences flanking the Tn5 insertion exhibited >98% nucleotide se-
implemented by a single distinguishable from the gacS (data not shown). The was not sensitive to addition of signal in strain CHA52/ (14, 18, 44). The these mutants do not respond to the CHA0 signal molecules when it was tested qualitatively for antibiotic and protease production. Furthermore, expression of the gacS gene of the closely related strain P. fluorescens Pf-5 (25).

Complementation of gacS mutant CHA52. Previous research has shown that gacS-negative mutants produce very low levels of secondary metabolites and signal molecules; moreover, these mutants do not respond to the CHA0 signal molecules (14, 18, 44). The gacS::Tn5 mutant CHA52/pME7402 was indistinguishable from the gacS deletion mutant CHA19 (44) when it was tested qualitatively for antibiotic and protease production. Furthermore, expression of the rsmZ-gfp fusion was not sensitive to addition of signal in strain CHA52/ pME7402 (data not shown). The gacS::Tn5 mutation was complemented by a single gacS copy in CHA52.1/pME7402, which resulted in restored production of secondary metabolites (data not shown) and Gfp expression (Fig. 2). A gacA mutant (CHA89) carrying rsmZ-gfp was defective for signal production and perception, like a gacA mutant (data not shown).

Growth properties of thiC mutant CHA50. The thiC mutant P. fluorescens CHA50/pME7402 was cured of its plasmid by introduction of the incompatible, somewhat unstable plasmid pME6001, which was lost during subsequent growth without antibiotic selection. When strain CHA50 was grown in 200-μl cultures in microtiter wells containing GCM not supplemented with thiamine, it did not appear to have a growth handicap compared with wild-type strain CHA0. However, when strain CHA50/pME7402 was grown in 20-ml GCM cultures in Erlenmeyer flasks, it had to be supplemented with 5 x 10^{-8} M thiamine in order to obtain wild-type growth rates and yields. Under these conditions, the pME7402-driven Gfp expression was significantly lower in CHA50 than in CHA0. With thiamine at a concentration of 10^{-6} M or higher, Gfp expression was the same in both strains (data not shown).

Signal production by P. fluorescens CHA50. As decreased Gfp production by P. fluorescens CHA50/pME7402 may result from diminished signal molecule synthesis, the signal production in strain CHA50 was assessed by a semiquantitative method and compared to that in strains CHA19 (gacS) and CHA0. When strains CHA50 and CHA19 were grown with 5 x 10^{-8} M thiamine, they produced comparable, small amounts of signal (Table 2). When the thiC mutant was grown with excess thiamine (10^{-3} M), signal production was stimulated about 25-fold and was similar to that in the wild type. Signal production in CHA0 and CHA19 was not influenced by the thiamine concentration (5 x 10^{-8} M or 10^{-3} M) (Table 2).

Expression of the Gac/Rsm regulatory cascade in P. fluorescens CHA50. The thiC mutant CHA50 produced a substantially reduced amount of signal under thiamine limitation conditions, we hypothesized that key elements in the Gac/Rsm cascade would be expressed at below those levels expressed in the wild type. We first compared the levels of expression of RsmX, RsmY, and RsmZ in strains CHA0 and CHA50 grown in GCM containing 5 x 10^{-8} M thiamine, using transcriptional rsmX-lacZ, rsmY-lacZ, and rsmZ-lacZ fusions. In each case, a decrease in expression was observed in the mutant (Fig. 3). Moreover, the fact that rsmZ was expressed later in growth than rsmX and rsmY was confirmed.

We then compared the expression of AprA protease and DAPG in the two strains grown with a limiting thiamine concentration, using translational aprA’-lacZ and phlA’-lacZ fusions, respectively (Fig. 4). In both cases, the expression of the reporter plasmids was clearly reduced in the thiC mutant compared to the expression in the wild type. To confirm the results obtained with the phlA’-lacZ fusion, the DAPG production of strains CHA0 and CHA50 was measured after growth in GCM with 5 x 10^{-8} M thiamine for 24 h. The thiC mutant produced 11.5 ± 2.0 nmol DAPG/10^6 cells, which was about threefold less than the amount produced by the wild type (33.2 ± 2.9 nmol DAPG/10^6 cells).

On GCM agar plates (not supplemented with thiamine because agar contains traces of thiamine), growth of P. ultimum was inhibited by wild-type strain CHA0 but not by the thiC mutant CHA50 or by the gacS mutant CHA19 (Fig. 5A), confirming that the production of antibiotics (i.e., essentially DAPG) was greatly reduced in the mutants.

In control experiments, the wild type and the thiC mutant were grown in medium supplemented with excess thiamine (10^{-3} M). The two strains exhibited similar expression of rsmX,

TABLE 2. Signal production by P. fluorescens CHA0, thiC mutant CHA50, and gacS mutant CHA19

<table>
<thead>
<tr>
<th>Strain</th>
<th>Thiamine concn (M)</th>
<th>Culture vol (ml) required to give half-maximal induction of rsmZ-lacZ fusion</th>
<th>Relative amt of signal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHA0</td>
<td>5 x 10^{-8}</td>
<td>9 ± 2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>11 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>CHA19</td>
<td>5 x 10^{-8}</td>
<td>271 ± 36</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>238 ± 67</td>
<td>4</td>
</tr>
<tr>
<td>CHA50</td>
<td>5 x 10^{-8}</td>
<td>276 ± 19</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>11 ± 4</td>
<td>100</td>
</tr>
</tbody>
</table>

a Different volumes of extracted supernatant (corresponding to 5 to 100 ml) of P. fluorescens CHA0, CHA19 (gacS), and CHA50 (thiC) were added to 20-ml cultures of P. fluorescens CHA0/pME6001 (rsmZ-lacZ) and assayed for β-galactosidase activity. The cultures to be extracted were grown in GCM with thiamine at a concentration of 5 x 10^{-8} M or 10^{-3} M. The values are average ± standard deviations from three different experiments. Culture volumes that gave half-maximal induction of the reporter fusion were calculated using the equation of Hanes: $[S] \cdot v = V_{max} \cdot [S] + K_m \cdot V_{max}$. 

FIG. 2. Gfp expression in P. fluorescens CHA0/pME7402 (triangles), CHA50/pME7402 (thiC) (circles), and CHA52/pME7402 (gacS) (diamonds) and complemented mutant CHA52.1/pME7402 (gacS') (squares). The strains were grown in microplate wells containing GCM without added thiamine. P. fluorescens CHA0/pME7402 and CHA50/ pME7402 were cultivated in the presence (solid symbols) and absence (open symbols) of 1 mM thiamine. Gfp expression and cell densities were measured with a FluoStar fluorimeter. Each value is the average from eight different cultures; the error bars indicate standard deviations. OD_{600}, optical density at 600 nm.
**Suppression of disease caused by P. ultimum in a gnotobiotic system.** To assess the importance of thiamine in disease suppression, we grew cress on agar plates containing dilute GCM (see Materials and Methods). *P. fluorescens* CHA0, CHA19, and CHA50 were assessed to determine their abilities to protect the plant from *P. ultimum* during 5 days of incubation. The *gacS* mutant was unable to protect cress, whereas the wild type provided partial protection (as determined by stem length). With both strains, addition of thiamine had no effect (Fig. 6). The *thiC* mutant CHA50 provided intermediate protection, which could be restored to the wild-type level by addition of thiamine (Fig. 6). These experiments show that thiamine plays a role in plant protection, probably via antibiotic production.

**DISCUSSION**

In this search for mutants with reduced expression of *rsmZ*, we obtained mutants which were defective for both signal production and perception. All five Tn5 insertion mutants in this group were found to be *gacS* negative. The pleiotropic phenotypes of *gacS::Tn5* mutant CHA52 and restoration of these phenotypes by complementation with *gacS*+ confirmed the results of previous studies with *gacS* mutants (6, 44). Mutations in either *gacS* or *gacA* cause equally strong reductions in the expression of secondary metabolism in strain CHA0 (6, 44). In the present study, no *gacA* mutants were obtained, perhaps because the probability of a Tn5 insertion in the 2.7-kb *gacS* sequence (44) is greater than the probability of a Tn5 insertion in the 0.6-kb *gacA* sequence (18).

Tn5 mutagenesis also resulted in two mutants in which signal synthesis was conditionally reduced. These mutants have a Tn5 insertion in the *thiC* gene, which takes part in thiamine pyrophosphate (TPP) biosynthesis. Most studies of
TPP synthesis have been performed with *B. subtilis*, *E. coli*, and *Salmonella enterica*, and about 12 genes are involved in TPP synthesis (2, 39). TPP synthesis starts with two separate pathways, which generate the thiamine precursors 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate and 4-methyl-5-(β-hydroxyethyl)thiazole phosphate (10, 21). The ThiC enzyme converts 5-aminimidazole ribonucleotide to 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (19, 39), probably with the help of another unknown component, and ThiD converts 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate to 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (2, 19). Mutation in thiC completely blocks 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate synthesis and hence the formation of TPP (2). In a thiC mutant, TPP production can be restored by supplementation with 4-amino-2-methyl-5-hydroxymethylpyrimidine or with thiamine (42), which is transformed into TPP by ThiK and ThiL (19).

TPP is an essential cofactor of several enzymes, such as transketolase, pyruvate dehydrogenase, and 2-oxoglutarate dehydrogenase (12). A lack of TPP disturbs the tricarboxylic acid (TCA) cycle, which produces precursors for the biosynthesis of amino acids, purines, pyrimidines, and vitamins (8); TPP is needed for pyruvate dehydrogenase, which supplies acetyl coenzyme A to the TCA cycle, as well as for the transformation of 2-oxoglutarate to succinyl coenzyme A by 2-oxoglutarate dehydrogenase in the TCA cycle itself (8). These essential functions explain why mutants such as CHA50 do not grow in media that completely lack thiamine. Growth of CHA50 in GCM with thiamine at a concentration of $5 \times 10^{-8}$ M was restored to the wild-type level. However, the synthesis of signals, DAPG and AprA protease, as well as the expression of RsmX, RsmY, and RsmZ, remained low. Thus, thiamine at a concentration of $5 \times 10^{-6}$ M restored essential functions in the primary metabolism of the bacteria but not several key functions of GacS/GacA-dependent secondary metabolism.

The pronounced decrease in signal synthesis in CHA50 (Table 2) and CHA51 (data not shown) suggests a role for TPP in signal synthesis. TPP could be an essential cofactor for enzymes involved in signal synthesis or could indirectly affect signal production. Thiamine itself is unlikely to be a component of the Gac/Rsm cascade. First, addition of thiamine at a concentration of $10^{-3}$ M did not enhance expression of reporter plasmids in strain CHA0 (data not shown), whereas addition of the CHA0 signal does (13, 14, 44). Second, strain CHA19 (gacS), which produced very little signal (Table 2), is not thiamine auxotrophic, and its growth was not affected by thiamine. We suspect that the reduced signal levels caused by thiamine limitation cause decreased expression of the RsmXYZ RNAs and, therefore, reduced secondary metabolite synthesis, resulting in a partial loss of inhibition of *P. ulitum* (Fig. 5 and 6). The mechanism by which thiamine causes reduced signal concentrations is not known.

Early studies suggested that some plants use thiamine as a growth factor (4), whereas other plants are able to secrete thiamine into the rhizosphere (43). Thiamine can also be a product of soil microorganisms (30). A thiamine auxotroph of *P. fluorescens* strain WCS365 has been found to be unable to colonize the roots of tomato plants under axenic conditions (35). By contrast, our mutant *P. fluorescens* CHA50 colonized cucumber roots with wild-type efficiency in a standard gnotobiotic system (16) consisting of quartz sand, clay minerals, and Knop solution (data not shown). This suggests that under these conditions thiamine is readily available to rhizosphere microorganisms, either because thiamine is a contaminant of the artificial soil used or because it is present in the root exudates. For these reasons, we chose a gnotobiotic system in which the thiamine contents could be controlled (i.e., a system with an agar support instead of soil).

The thiC mutants produced a residual amount of signal molecules. No mutants that were totally defective in signal synthesis were obtained. The reasons for this are not entirely clear as our screening (Fig. 2) should have picked up such mutants. It is possible that in strain CHA0 signal synthesis involves more than one pathway. This possibility is supported by the observation that two peaks of rsmZ-stimulating activity were obtained when a crude signal preparation was fraction-
ated on a silica gel column (Dubuis and Haas, unpublished data).

The Gac/Rsm system controls quorum sensing in several gram-negative bacteria. In *P. aeruginosa*, GacA function favors the production of *N*-butyl-homoserine lactone, which is one of the quorum-sensing signals in this organism (20–28). In *Vibrio cholerae*, the function of the VarA (\( = \) GacA) response regulator antagonizes the expression or the activity of the central quorum-sensing regulator LuxO via a cascade that involves three small VarA-dependent RNAs (20). In *P. fluorescens* CHA0, the function of the GacS/GacA system is positively autoregulated by CHA0 signals in densely growing cultures (14), reflecting a quorum-sensing behavior. An important conclusion of the present study is that the function of this quorum-sensing circuitry requires thiamine in *P. fluorescens*.

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