Role of Gluconic Acid Production in the Regulation of Biocontrol Traits of *Pseudomonas fluorescens* CHA0

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The rhizobacterium *Pseudomonas fluorescens* CHA0 promotes the growth of various crop plants and protects them against root diseases caused by pathogenic fungi. The main mechanism of disease suppression by this strain is the production of the antifungal compounds 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT). Direct plant growth promotion can be achieved through solubilization of inorganic phosphates by the production of organic acids, mainly gluconic acid, which is one of the principal acids produced by *Pseudomonas* spp. The aim of this study was to elucidate the role of gluconic acid production in CHA0. Therefore, mutants were created with deletions in the genes encoding glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*), required for the conversion of glucose to gluconic acid and gluconic acid to 2-ketogluconate, respectively. These enzymes should be of predominant importance for rhizosphere-colonizing biocontrol bacteria, as major carbon sources provided by plant root exudates are made up of glucose. Our results show that the ability of strain CHA0 to acidify its environment and to solubilize mineral phosphate is strongly dependent on its ability to produce gluconic acid. Moreover, we provide evidence that the formation of gluconic acid by CHA0 completely inhibits the production of PLT and partially inhibits that of DAPG. In the *agcd* mutant, which does not produce gluconic acid, the enhanced production of antifungal compounds was associated with improved biocontrol activity against take-all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici*. This study provides new evidence for a close association of gluconic acid metabolism with antifungal compound production and biocontrol activity in *P. fluorescens* CHA0.

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Plant growth-promoting rhizobacteria (PGPR) (36) are root-colonizing bacteria that enhance the performance of crop plants by several mechanisms. First, they antagonize plant-pathogenic fungi, mainly by the production of antimicrobial metabolites, but also by competition for iron or rhizosphere niches (9, 23, 24, 59). The biocontrol activity of many disease-suppressive microorganisms is also attributed to stimulation of host defense (induced systemic resistance). Other mechanisms by which these rhizobacteria directly promote plant growth are the production of phytohormones and the increase of nutrient, in particular phosphate, availability to plants (18, 37). Certain rhizobacteria are able to solubilize insoluble or poorly soluble mineral phosphates by producing acid phosphatases and organic acids, mainly gluconic acid (2, 34, 60). Some PGPR combine these different plant-beneficial activities and are able to suppress soilborne plant diseases, as well as to increase phosphate availability for plants (72).

In fluorescent pseudomonads, gluconic acid production is catalyzed by periplasmic oxidation of glucose by membrane-bound glucose dehydrogenase (Gcd) (Fig. 1A) (16, 43). In many gram-negative bacteria, the synthesis of gluconic acid has been shown to be dependent on pyrroloquinoline quinone (PQQ) as an enzymatic cofactor of the Gcd (1, 14). A consecutive oxidation reaction is mediated by gluconate dehydrogenase (Gad), which converts gluconic acid to 2-ketogluconate (Fig. 1A) (11, 12, 44, 50). These enzymes should be of predominant importance for biocontrol soil pseudomonads, as major carbon sources provided by plant root exudates in the rhizosphere are made up of glucose (29, 69, 70). The two enzymes involved in glucose metabolism may have a substantial influence on general nutrient availability in the rhizosphere.

First, Gcd and Gad affect glucose levels, and second, they may modulate the availability of soluble phosphates by controlling the amount of gluconic acid released into the rhizosphere. Furthermore, the production of gluconic acid might substantially change the rhizosphere pH. Therefore, Gcd and Gad enzymes produced by fluorescent pseudomonads are likely to be important for soil fertility and to impact the activities of other organisms living in the rhizosphere, e.g., fungal pathogens attacking the roots. Indeed, gluconic acid metabolism has already been linked to antifungal activity. Recently, Kaur et al. (30) proposed that gluconic acid produced by a nonfluorescent *Pseudomonas* isolate may be important for the biological control of take-all disease.

*Pseudomonas fluorescens* CHA0 is a bacterial strain known to be able to suppress various soilborne plant diseases (24). Its biocontrol ability has been linked to the production of the antifungal compounds 2,4-diacetylphloroglucinol (DAPG) (31, 33) and pyoluteorin (PLT) (46, 47). The strain is also able to solubilize mineral phosphate and to improve plant growth under phosphate-limiting conditions (A. von Felten, personal communication). Gluconic acid is supposed to play a predominant role in the phosphate solubilization activity of *P. fluorescens*.
cens CHA0, and we hypothesize that the metabolite also has an impact on the biocontrol activity of this PGPR strain.

The aim of this study was to elucidate the role of gluconic acid production in *P. fluorescens* CHA0 with respect to its phosphate-solubilizing ability, antifungal metabolite production, and ability to suppress fungal root diseases. To this end, mutants of strain CHA0 carrying deletions in the *gcd* gene, encoding Gcd, and the *gad* gene, encoding Gad (Fig. 1), were created. The three in-frame deletion mutants, CHA1196 (Δ*gcd*), CHA1197 (Δ*gad*), and CHA1198 (Δ*gcd* Δ*gad*), were compared with their parental strain for the ability to produce organic acids, to solubilize inorganic phosphate, to produce the antifungal metabolites DAPG and PLT, to inhibit the growth of fungal pathogens, and to suppress different soilborne diseases. We provide evidence that in fact, gluconic acid production by *P. fluorescens* CHA0 is involved not only in the solubi-

![Figure 1](http://aem.asm.org/)

**FIG. 1.** (A) Periplasmic and intracellular glucose catabolism in pseudomonads based on studies with *P. aeruginosa* (10), *P. putida* (11, 12), and *P. fluorescens* (17, 28). Shown are membrane-bound enzymes involved in periplasmic glucose metabolism, Gcd (glucose dehydrogenase) and Gad (gluconate dehydrogenase), and enzymes involved in cytoplasmic glucose metabolism, Glk (glucokinase), Zwf (glucose-6-phosphate 1-dehydrogenase), GnuK (gluconokinase), KguK (2-ketogluconate kinase), and KguD (2-ketogluconate 6-phosphate reductase) (the names of the enzymes are derived from the nomenclature for *P. putida* KT2440 [12, 54]). (B and C) Physical locations of the *gcd* (B) and *gad* (C) genes in the genome of *P. fluorescens* strain CHA0. The shaded arrows show the sequenced or partly sequenced genes. The representation is based on the sequence data for strain CHA0 obtained by sequencing the chromosomal fragments inserted in the indicated vectors. The designations of the ORFs flanking the *gcd* and *gad* genes are based on the corresponding locus tags in the complete annotated sequence of the closely related *P. fluorescens* strain Pf-5 (56). Δ, region deleted in strains CHA1196 and CHA1197 and in plasmids pME3087::F34 and pME3087::F12. The bars designate the fragments cloned into the vector pME3087 to give pME3087::F34 and pME3087::F12 and into pColdI to give pColdI::gcd and pColdI::gad. Artificial restriction sites on the cloned fragments are marked with asterisks.
This study-D-galactoside (X-Gal) was incorpo-
ated, 5-bromo-4-chloro-3-indolyl-
chloride, 25/medium at the following concentrations: ampicillin sodium salt, 100 agar and in LB at 37°C. When required, antibiotics were added to the growth 
Escherichia coli
P. fluorescens

Plasmids

pColdI Cold shock-based expression vector; Ap
pColdI:ged pColdI with the ged gene flanked by an N-terminal His6 tag under the control of the cspA promoter; Ap
This study
This study

pColdI:ged Suicide vector; ColEl replicon; RK2-Mob; Te
pME3087 carrying the 1,127-bp KpnI-XbaI fragment from pUKF12; Te
This study
This study
This study

pME7100 phrA-gfp transcriptional fusion; Te
This study

pME7109 pchrA-gfp transcriptional fusion; Te
This study

pME497 Mobilizing plasmid; IncP-1 Tra RepA(Ts) Ap
This study

pUK21 Cloning vector; lacZa Km
This study

pUKF12 pUK21 carrying a 1,127-bp KpnI-XbaI insert with a deletion in ged; Km
This study

pUKF34 pUK21 carrying a 1,247-bp KpnI-XbaI insert with a deletion in ged; Km
This study

Oligonucleotides

Gad1 GGGGTACCGAATCATCGACCGGAGATGA; KpnI
This study

Gad2 GGAATTCCGCTGACACTTCTATCATCTAG; EcoRI
This study

Gad3 GGAATTCCGTACAGGATTTGCATCGATCC; EcoRI
This study

Gad4 GCTCTAGACTTCTCCAGCACTGGTGCTTC; NdeI
This study

Gcd1 GGGGTACCGAATACATCGACCGGCAATG; KpnI
This study

Gcd2 GGAATTCCGCTGACACTTCTATCATCTAG; EcoRI
This study

Gcd3 GGAATTCCGTACAGGATTTGCATCGATCC; EcoRI
This study

Gcd4 GCTCTAGACTTCTCCAGCACTGGTGCTTC; NdeI
This study

PCold gad1 CGGGATCCTTATGCCTGAACCAGCG; BamHI
This study

PCold gad2 CGGGATCCTTATTCCGACAGTTTGTAGGCA; BamHI
This study

PCold gad3 CGGGATCCTTATTCCGACAGTTTGTAGGCA; BamHI
This study

PCold gad4 CGGGATCCTTATTCCGACAGTTTGTAGGCA; BamHI
This study

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. P. fluorescens strains were cultivated on King’s medium B agar (KMB) (35) at 27°C and in Luria broth (LB) (62) at 27°C, unless otherwise specified. Escherichia coli strains were grown on nutrient agar and LB at 37°C. When required, antibiotics were added to the growth medium at the following concentrations: ampicillin sodium salt, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin sulfate, 50 μg/ml; and tetracycline hydrochloride, 25 μg/ml for E. coli and 125 μg/ml for P. fluorescens strains. When appropriate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was incorporated into solid media to monitor β-galactosidase expression (62). For monitoring antifungal gene expression, derivatives of P. fluorescens strain CHA0 carrying rhizosphere-stable plasmids with transcriptional fusions of a stable variant of the gfp gene to the DAPG biosynthetic gene phlA (pME7100) or the PLT biosynthetic gene phlA (pME7109) (3) were used.

Fungal strains and culture conditions. Gaumannomyces graminis var. tritici strain ETH1000, the causal agent of take-all disease of wheat, and Magnaporthe grisea no. 283 (obtained from Syngenta, Stein, Switzerland), the causal agent of rice blast, were grown and maintained on potato dextrose agar (PDA) (24 g/liter of Difco potato dextrose broth and 12 g/liter of Oxoid technical agar, pH 7.0) at 24°C in the dark. Rhizoctonia solani strain 160 (obtained from Ciba-Geigy SA, Basel, Switzerland), the causal agent of root rot of cucumber in this study, was grown and maintained under the same conditions on malt extract agar (15 g/liter of Difco malt extract and 12 g/liter of Oxoid technical agar, pH 7.0).

DNA manipulations and sequence analyses. Chromosomal DNA of P. fluorescens was isolated as described previously (66). Small- and large-scale plasmid preparations were performed by the cetyltrimethylammonium bromide method (62), with the GenElute HP Plasmid Miniprep kit (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), or with the Jetstar 2.0 kit (Genomed, Basel, Switzerland). Standard techniques were used for restriction, agarose gel electrophoresis, dephosphorylation, generation of blunt ends with T4 DNA polymerase (Boehringer, Ingelheim, Germany), isolation of DNA fragments from low-melt- ing-point agarose gels, and ligation (62, 65, 66). Restriction fragments were purified from agarose gels using the MinElute gel extraction or Qiaquick gel extraction kit (Qiagen, Basel, Switzerland), depending on the size of the fragment. Electroporation of bacterial cells with plasmid DNA was done as described previously (66). Nucleotide primers were designed with the online software Primer3 (v.0.4.0) (61) and purchased from Microsynth (Balgach, Switzerland). PCR products were carried out using GoTaq DNA polymerase (Promega, WI) and PrimeStar HS DNA Polymerase (Takara Bio Inc., Shiga, Japan), following procedures detailed elsewhere (3, 57). Nucleotide sequences were determined on
both strands with the Big Dye terminator cycle-sequencing kit 3.1 (Applied Biosystems, Foster City, CA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Nucleotide and deduced amino acid sequences were analyzed with the programs Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI) and the ExPaSy Proteomics Server (http://www.expasy.org). Sequence alignments were performed using Clustal W software (39).

Cloning of the *gcd* and *gad* genes from *P. fluorescens*. The *gcd* gene was amplified from chromosomal DNA of strain CHA0 using primers PColgd1 and PColgd2 (Table 1). To amplify the *gad* gene from CHA0, primers PColgd1-1 and PColgd-2 (Table 1) were used. Primers were designed based on genomic data available for the very closely related *P. fluorescens* strain Pf-5 (http://www.pseudomonas.com) (56). For *gcd*, the PCR amplification yielded a 2.4-kb band. The PCR product was digested with NdeI and BamHI and was cloned into the vector pColdI to give pCold::gcd (Table 1). For *gad*, the PCR amplification produced a 1.7-kb fragment that was digested with NdeI and BamHI and was cloned into pColdI to give pCold::gad. For both constructs, the inserts of two of the obtained clones were sequenced to confirm the identities of the cloned fragments.

Construction of *gcd* and *gad* in-frame deletion mutants of *P. fluorescens*. To generate the *gcd* mutant strain CHA1196, a 2,328-bp fragment was deleted in frame in the *gcd* gene. For this purpose, a 666-bp KpnI-EcoRI fragment including the first nine codons of *gcd* and the adjacent upstream chromosomal region was amplified, by PCR from chromosomal DNA of strain CHA0, with primers Gcd1 and Gcd2. A 575-bp EcoRI-XbaI fragment including the last 16 codons of *gcd* and the adjacent downstream region was amplified by PCR with primers Gcd3 and Gcd4. The resulting upstream and downstream fragments were digested with KpnI and EcoRI and with EcoRI and XbaI, respectively, and were cloned by a triple ligation into pKU21 digested with KpnI and XbaI, giving plasmid pUKF34. The 1.24-kb KpnI-XbaI insert in pME34 was checked by sequencing. The insert was excised from pME34 with EcoRI and XbaI, respectively, and were cloned by a triple ligation into the plasmid, pUKF12, giving plasmid pME3087 digested with KpnI-XbaI, producing pME3087::F34. To obtain the *gcd* mutant strain CHA1196, the suicide plasmid pME3087::F34 was then integrated into the chromosome of strain CHA0 by triparental mating, with *E. coli* HB101/pME947 as the mobilizing strain, with selection for tetracycline- and chloramphenicol-resistant recombinants (65, 66). Excision of the vector by a second crossing over occurred after enrichment for tetracycline-sensitive cells.

An analogous gene replacement strategy was followed to obtain the 1,731-bp *gad* deletion mutant strain CHA1197. Using CHA0 DNA as a template, two fragments including the first nine and the last three codons of the *gad* gene and the respective up- and downstream flanking regions were amplified by PCR with primers Gad1 plus Gad2 and Gad3 plus Gad4, respectively. The 494-bp upstream and 627-bp downstream fragments were digested with KpnI and EcoRI and with EcoRI and XbaI, respectively, and were cloned by a triple ligation into pKU21 digested with KpnI and XbaI, giving plasmid pUKF34. The 1.24-kb KpnI-XbaI insert in pME34 was checked by sequencing. The insert was excised from pME34 with EcoRI and XbaI, respectively, and were cloned by a triple ligation into the plasmid, pUKF12, giving plasmid pME3087 digested with KpnI-XbaI, producing pME3087::F34. To obtain the *gad* mutant strain CHA1197, the suicide plasmid pME3087::F34 was then integrated into the chromosome of strain CHA0 by triparental mating, with *E. coli* HB101/pME947 as the mobilizing strain, with selection for tetracycline- and chloramphenicol-resistant recombinants (65, 66). Excision of the vector by a second crossing over occurred after enrichment for tetracycline-sensitive cells.

Quantification of antifungal compound production. Strain CHA0 and its mutants were grown in 300-ml Erlenmeyer flasks in NBRIP medium (3, 4) with 25 mM glucose (5% glucose) (for growth and gene expression rates for *P. fluorescens*) and 0.01% yeast extract (OS yeast extract) (for growth and gene expression). The OD600 of 0.1 was inoculated into overnight LB cultures diluted to an OD600 of 0.05. For each treatment, six wells of the microtiter plate were then partially filled with aliquots of 200 μl of the respective bacterial culture. The cultures were incubated at 30°C with orbital shaking at 500 rpm in a Thermotost incubator (BMG Labtechnologies, Offenburg, Germany). The OD600 was a parameter of growth and green fluorescence (excitation at 480 nm and emission at 520 nm) as a parameter of antifungal gene expression were measured with a SpectraFluor الأسبوع (Tecan Group Ltd., Männedorf, Switzerland) throughout the exponential and stationary growth phases (3). For each individual measurement, the green fluorescence value was divided by the corresponding OD600 value, giving the specific fluorescence of the cells expressed as relative fluorescence units (3). The green fluorescence emitted by cells of wild-type strain CHA0 without the gfp reporter fusion was determined for background correction.

Fungal inhibition assays. The inhibitory effects of CHA0 and its *gcd* and *gad* mutants on the growth of fungal pathogens were monitored on one-fifth-strength PDA plates. For *G. graminis var. tritici*, 0.6-cm plugs from a 1-week-old growing agar culture were placed in the centers of one-fifth-strength PDA plates and incubated at 24°C for 2 days in the dark. Samples (OD600 = 0.1) of washed bacterial cells grown overnight in LB were then applied to the plate in a square of 5.5-cm side length centered on the fungal plug. After an additional 4 days of incubation at 24°C, the radial growth of the fungi was measured. Bioassays with *R. solani* and *M. grisea* were performed in the same manner, except that the fungal agar plug and the bacterial suspensions were placed at the same time. The experiment consisted of six plates per treatment and was repeated twice.

To test the sensitivity of *G. graminis var. tritici* to gluconic acid, the fungus was grown for 5 days at 24°C on one-fifth-strength PDA plates with 10 μl drops of gluconic acid solutions of different concentrations (10 to 20 mg/ml distilled water) added. Drops were spotted in the corners of a square of 5.5-cm side length centered on the fungal plug.

Plant disease suppression and root colonization assays. The effects of *gcd* and *gad* gene deletions in *P. fluorescens* CHA0 on its biocontrol ability were tested in two different plant-pathogen systems: wheat-*G. graminis var. tritici* and cucumber-*R. solanum*. Seeds of cucumber (*Cucumis sativus* cv. Chinesische Schlange) and seeds of winter wheat (*Triticum aestivum* cv. Arina) were surface sterilized for 10
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RESULTS

Identification and characterization of the chromosome regions containing gad and gad in P. fluorescens CHA0 and construction of deletion mutants. Prior to this study, we had undertaken an approach to identify novel regulators of antibiotic production in P. fluorescens. To this end, a derivative of CHA0 carrying a phlA-gfp fusion on pME7100 (3) had been subjected to random Tn5 insertion mutagenesis. From this collection of Tn5 mutants (4), those with altered antifungal gene expression were characterized and Tn5-flanking regions were amplified by arbitrary PCR, cloned, and sequenced. Several mutants were identified for which the Tn5 insert was located in genes involved in glucose metabolism (L. Rochat and P. de Werra, unpublished data).

Comparison of sequence data with the complete genomic sequence available for the closely related P. fluorescens strain Pf-5 (56) revealed that in one CHA0 mutant the transposon had been inserted into an open reading frame (ORF) that was highly similar to the Pf-5 locus PFL_4916 (http://www.pseudomonas.com), encoding a putative Gcd. The CHA0 locus was termed gcd by analogy with closely related sequences in other pseudomonads (see below). Pf-5 sequence information was used to PCR amplify, clone, and sequence a 3,581-bp chromosomal region in strain CHA0 containing gcd and its surroundings (Fig. 1B). The deduced product of the 2,421-bp gcd of strain CHA0 (806 amino acids; 86.4 kDa) is 99% identical to the putative Gcd of P. fluorescens Pf-5 encoded by PFL_4916 (GenBank accession no. AAY94145.1). Gcd of CHA0 was also found to be 80% identical to the well-characterized Gcd of Pseudomonas putida KT2440 (PP_1444 [12, 15, 54]) and 83% identical to the Gcd of Pseudomonas aeruginosa PA01 (PA2290 [67]) and to show similarity (46% identity) to the Gcd of E. coli K-12 (GenBank accession no. D12651 [74, 75]). The gcd gene of P. fluorescens CHA0 is located between two ORFs (Fig. 1B) and are also present at the corresponding loci of P. fluorescens Pf-5. The partially sequenced upstream ORF (ORFA) encodes a putative porin B (with a C terminus 100% identical to that of the PFL_4915 product of P. fluorescens Pf-5 and also 85% identical to that of the PP_1445 product of P. putida KT2440). The sequenced part of the downstream ORF (ORFB) is 100% identical to PFL_4917, encoding a hypothetical protein of P. fluorescens Pf-5.

A second CHA0 mutant had the Tn5 transposon inserted in an ORF, which we termed gad by analogy with published sequences (see below), encoding a Gad. We amplified and sequenced a 2,864-bp chromosomal region in strain CHA0 containing gad and its surroundings (Fig. 1C). The deduced product (594 amino acids; 65.2 kDa) of the 1,785-bp gad gene of strain CHA0 is 99% identical to the corresponding ORF encoding the putative Gad in P. fluorescens Pf-5 ( locus tag PFL_0054; GenBank accession no. AAY95472.1). Gad of CHA0 was also found to share 89% amino acid similarity with the Gad of P. putida KT2440 encoded by the gadB (PP_3383) (11) and 77% with the Gad of P. aeruginosa PA01 (PA2265 [67]). Moreover, the Gad amino acid sequence of CHA0 is still highly similar (77% identity) to that of the well-described membrane-bound Gad (GADH) of Erwinia cyrippe- dii ATCC 29267 (GenBank accession no. U97665 [76]). The deduced product of the only partially sequenced ORFC (Fig. 1C) upstream of gad shows similarities to the C terminus of a conserved hypothetical protein of P. fluorescens Pf-5 (PFL_0053; 99% identity) and P. putida KT2440 (PP_3384; 76% identity). The adjacent downstream gene (ORFD) was partially sequenced in its 5' region. Its deduced N terminus amino acid sequence displays similarity to the Gad cytochrome c subunit.
of *P. fluorescens* Pf-5 (PFL_0055; 99% identity) and *P. putida* KT2440 (PP_3382; 74% identity).

In-frame deletion mutations in the *gcd* and *gad* genes of *P. fluorescens* CHA0 (Table 1) were constructed as described in Materials and Methods, and the resulting *gcd* mutant CHA1196, *gad* mutant CHA1197, and *gcd* *gad* double mutant CHA1198 were compared with the parental strain for growth characteristics, acid production, phosphate solubilization, and antifungal and biocontrol activities.

**Growth characteristics of **<sup>**gcd**</sup>** and **<sup>**gad**</sup>** mutants of *P. fluorescens* CHA0.** The *gcd*, *gad*, and *gcd* *gad* mutants were indistinguishable from the wild type with respect to their growth characteristics and morphologies in liquid or solid nutrient-rich media, such as KMB and LB. However, in liquid OS minimal medium amended with glucose as the sole carbon source, the loss of Gcd in strain CHA1196 markedly slowed its initial growth phase compared to that of the wild-type strain (Fig. 2A). The lack of Gad in strain CHA1197 also resulted in a slight delay in initial growth. However, the exponential growth curves of both mutants were similar to that of the wild type (Fig. 2A). All strains attained the same final cell density at the stationary growth phase (Fig. 2A). When the strains were grown in OS minimal medium amended with glycerol, no difference in growth was observed between the two mutants and wild-type CHA0 (Fig. 2B).

A difference in colony morphology was observed for mutants grown on NBRIP agar plates containing glucose as the sole carbon source (Fig. 3B). Colonies of wild-type CHA0 and the *gcd* mutant were less than 1 mm in diameter, whereas the two other mutants, *gad* and *gcd* *gad*, formed colonies 2 to 2.5 mm in diameter. A second morphological difference was observed on one-fifth-strength PDA. On this medium, the *gcd* mutant had a strong yellow coloration that also diffused into the agar zone surrounding the colony.

**FIG. 2. Growth curve of *P. fluorescens* wild-type CHA0 (**■**); its *gcd* mutant, CHA1196 (**○**); and its *gad* mutant, CHA1197 (**△**) in OS glucose (**A**) and OS glycerol (**B**) media at 30°C. The data represent the means (± standard errors) of six replicate cultures. The experiment was repeated twice with similar results.**

**FIG. 3. In vitro acid production (**A** and **C**) and phosphate solubilization (**B**) by *P. fluorescens* CHA0 and its *gcd* (CHA1196), *gad* (CHA1197), and *gcd* *gad* (CHA1198) mutants. The bacterial strains were grown on NBRIP agar plates with (**A**) or without (**B**) a pH indicator. (A) Acidification of the medium resulted in the formation of a blurred red halo. (B) The solubilization of tricalcium phosphate \([\text{Ca}_3(\text{PO}_4)_2]\) resulted in the formation of cleared zones. (A and B) The experiments were repeated twice with eight replicates per treatment. (C) Acidification of a glucose solution by addition of washed cells of wild-type CHA0 (**■**), the *gad* mutant (**○**), the *gcd* mutant (**△**), and the *gcd* *gad* double mutant (**×**). The pHs of the solutions were measured at different time intervals. The experiment was repeated twice with similar results.**

**FIG. 3. In vitro acid production (**A** and **C**) and phosphate solubilization (**B**) by *P. fluorescens* CHA0 and its *gcd* (CHA1196), *gad* (CHA1197), and *gcd* *gad* (CHA1198) mutants. The bacterial strains were grown on NBRIP agar plates with (**A**) or without (**B**) a pH indicator. (A) Acidification of the medium resulted in the formation of a blurred red halo. (B) The solubilization of tricalcium phosphate \([\text{Ca}_3(\text{PO}_4)_2]\) resulted in the formation of cleared zones. (A and B) The experiments were repeated twice with eight replicates per treatment. (C) Acidification of a glucose solution by addition of washed cells of wild-type CHA0 (**■**), the *gad* mutant (**○**), the *gcd* mutant (**△**), and the *gcd* *gad* double mutant (**×**). The pHs of the solutions were measured at different time intervals. The experiment was repeated twice with similar results.**

**Roles of *gcd* and *gad* in acid production.** A simple agar plate experiment was performed to visualize the acidification of NBRIP medium amended with a pH indicator by the wild-type CHA0 and its derivatives affected in glucose metabolism. The wild-type strain CHA0 produced a blurred red halo around the colony (Fig. 3B). A stronger and larger red halo was observed for the *gad* mutant, probably due to an accumulation of gluconic acid. In contrast, no red coloration was observed for the *gcd* mutant and the *gcd* *gad* double mutant (Fig. 3A), reflecting the defect in gluconic acid production in these strains.
In order to quantify the amounts of acid produced by the mutants, washed pellets of bacteria were inoculated in a glucose solution as described in Materials and Methods. Wild-type CHA0 and the \( \Delta \text{gcd} \) mutant converted the glucose into gluconic acid, which strongly acidified the solution. After 5 days, the solution reached a minimal \( \text{pH} \) of 4.0 and did not change during the following days (Fig. 3C). Using the titration method of Schleissner et al. (63), the wild-type strain and the \( \Delta \text{gad} \) mutant were estimated to produce acid at a concentration of about 1.3 mM. As expected, the absence of Gcd in the \( \Delta \text{gcd} \) mutant, CHA1196, showed a remarkably strong dependence on its ability to produce gluconic acid. As a consequence, gluconic acid accumulated in larger amounts than in the wild type (Fig. 3A), which resulted in the formation of a clear halo (4.2 \( \pm \) 0.8 mm in diameter) on the agar plate (Fig. 3B). The Gcd defect in strain CHA1196 and the \( \Delta \text{gcd} \) \( \Delta \text{gad} \) double mutant, CHA1198, resulted in a loss of phosphate-solubilizing ability on NBRIP agar medium. On the other hand, the absence of Gad in strain CHA1197 impaired the transformation of gluconic acid into 2-ketogluconate. As a consequence, gluconic acid accumulated in larger amounts than in the wild type (Fig. 3A), which resulted in significantly greater phosphate solubilization (halo diameter, 5.6 \( \pm \) 0.9 mm) on the NBRIP agar plates (Fig. 3B). These results indicate that phosphate solubilization by CHA0 is strongly dependent on its ability to produce gluconic acid.

Effects of \( \text{gcd} \) and \( \text{gad} \) deletions on the production of antifungal compounds and on the expression of antifungal genes.

The production of the two antifungal compounds DAPG and PLT by wild-type CHA0 and its \( \Delta \text{gcd} \) and \( \Delta \text{gad} \) mutants was investigated in YM medium containing glucose or gluconate and GCM containing glycerol as carbon sources. DAPG levels obtained in YM glucose medium for the \( \Delta \text{gcd} \) mutant CHA1196 reached 118 \( \mu \)M after 1 day of incubation and were 22-fold higher than those for the wild-type CHA0 (Table 2). After 3 days, the concentration of DAPG in YM glucose medium was still 50% higher in cultures of the \( \Delta \text{gcd} \) mutant than in those of the wild type. DAPG production by the \( \Delta \text{gad} \) mutant did not differ significantly from that of the wild-type strain. Neither the wild-type CHA0 nor the \( \Delta \text{gad} \) mutant CHA1197 produced any PLT, the second antifungal compound studied, in YM glucose medium. The \( \Delta \text{gcd} \) mutant, however, was able to form PLT at concentrations of 7 to 12 \( \mu \)M, which did not substantially change over time (Table 2).

### TABLE 2. Production of the antifungal compounds DAPG and PLT by \( P. \text{fluorescens} \) CHA0 and its \( \Delta \text{gcd} \) and \( \Delta \text{gad} \) mutants

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Incubation time (days)</th>
<th>DAPG production (( \mu )M)*</th>
<th>PLT production (( \mu )M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CHA0 (wild type)</td>
<td>CHA1196 (( \Delta \text{gcd} ))</td>
</tr>
<tr>
<td>YM glucose</td>
<td>1</td>
<td>5.3 ± 1.2</td>
<td>118.1 ± 47.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>51.7 ± 2.7</td>
<td>94.7 ± 28.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>73.7 ± 9.8</td>
<td>113.3 ± 35.7</td>
</tr>
<tr>
<td>YM gluconate</td>
<td>1</td>
<td>ND</td>
<td>40.9 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>66.9 ± 13.5</td>
<td>62.1 ± 15.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18.2 ± 3.6</td>
<td>15.5 ± 1.6</td>
</tr>
</tbody>
</table>

* \( P. \text{fluorescens} \) wild-type CHA0 and its derivatives, CHA1196 (\( \Delta \text{gcd} \)) and CHA1197 (\( \Delta \text{gad} \)), were cultivated in 100 ml YM medium amended with 25 mM glucose or 25 mM gluconate and extracted with ethyl acetate. DAPG and PLT contents in extracts were quantified by high-performance liquid chromatography. The data represent the means (± standard errors) of three replicate cultures per treatment.

**ND**, not detected.
strain, CHA0 (Fig. 4C). The expression of the PLT biosynthetic gene \( \text{pltA} \) did not differ between the \( /H9004 \text{gcd} \) mutant and the wild type and was very poor in both strains (Fig. 4D).

Together, these results indicate that the presence of gluconic acid, either produced by CHA0 or added to the medium, completely prevents the biosynthesis of PLT and partially prevents that of DAPG.

Biocontrol activities of \( P. \text{fluorescens} \) CHA0 and its \( /H9004 \text{gcd} \) and \( /H9004 \text{gad} \) mutants. In order to determine the relative contributions of Gcd and Gad to the biocontrol ability of \( P. \text{fluorescens} \) CHA0, we investigated the abilities of the \( /H9004 \text{gcd} \) and \( /H9004 \text{gad} \) mutants to inhibit the growth of fungal pathogens in vitro (Table 3) and to suppress take-all of wheat (Table 4), as well as \( Rhizoctonia \) root rot of cucumber, under gnotobiotic conditions. The radial growth of \( G. \text{graminis var. tritici} \), the causal agent of take-all, on one-fifth-strength PDA was strongly inhibited (by 72%) by strain CHA0 after 5 days (Table 3). The \( /H9004 \text{gcd} \) mutant reduced the growth of \( G. \text{graminis var. tritici} \) significantly more strongly (by 83%) than wild-type CHA0. No difference in fungal growth reduction was observed between the \( /H9004 \text{gad} \) mutant and the parental strain (Table 3). To test the inhibitory activity of gluconic acid on the growth of \( G. \text{graminis var. tritici} \), we confronted the fungus growing on one-fifth-strength PDA with 10-\( \mu \)l drops of gluconic acid solution at different concentrations. Only the highest tested concentrations of 100 and 200 mg/ml inhibited fungal growth (data not shown).

**TABLE 3. Growth inhibition of different plant-pathogenic fungi by \( P. \text{fluorescens} \) CHA0 and its \( /H9004 \text{gcd} \) and \( /H9004 \text{gad} \) mutants.**

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Radial growth of fungal colony (radius in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No bacteria added</td>
</tr>
<tr>
<td>( G. \text{graminis var. tritici} )</td>
<td>29a</td>
</tr>
<tr>
<td>( M. \text{grisea} )</td>
<td>17a</td>
</tr>
<tr>
<td>( R. \text{solani} )</td>
<td>43a</td>
</tr>
</tbody>
</table>

\( ^\text{a} \) \( G. \text{graminis var. tritici}, M. \text{grisea}, \) and \( R. \text{solani} \) were inoculated in the center of a one-fifth-strength PDA plate. \( P. \text{fluorescens} \) wild-type CHA0 and its derivatives, CHA1196 (\( /H9004 \text{gcd} \)) and CHA1197 (\( /H9004 \text{gad} \)), were streaked out in a square of 5.5-cm lateral length around the fungal plugs and grown for 5 days at 24°C.

\( ^\text{b} \) The data are means of eight replicates. Means for the same fungus within the same row followed by different letters are significantly different at a \( P \) value of \( \leq 0.05 \) according to Fisher’s least significant difference test. The experiment was repeated twice with similar results.
TABLE 4. Suppression of take-all of wheat by *P. fluorescens*/H9004\(gcd\) and \(gcd\) mutants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity</th>
<th>Root fresh CFU/g of root (ratio to wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81.3b</td>
<td>12.5c</td>
</tr>
<tr>
<td><em>G. graminis</em> var. <em>tritici</em></td>
<td>7.84a</td>
<td>79.0c</td>
</tr>
<tr>
<td><em>G. graminis</em> var. <em>tritici</em> + CHA0</td>
<td>7.48a</td>
<td>84.3a</td>
</tr>
<tr>
<td><em>G. graminis</em> var. <em>tritici</em> + (gcd) mutant</td>
<td>7.77a</td>
<td>8.31a</td>
</tr>
</tbody>
</table>

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A similar effect of the \(gcd\) deletion was observed on the inhibition of *M. grisea*. The \(\Delta gcd\) mutant, but not the \(\Delta gad\) mutant, reduced the growth of *M. grisea* significantly more (47% growth reduction) than the wild type (29% growth reduction) (Table 3). Interestingly, no such difference in fungal growth inhibition between the wild type and the \(\Delta gcd\) mutant was observed in the assay with *R. solani* (Table 3).

In summary, the loss of Gcd activity in CHA0 resulted in increased inhibition of two out of three tested fungal plant pathogens on agar plates.

The results for the three repetitions of the take-all biocontrol assay are presented individually in Table 4. Due to differences in general take-all severity levels among the three experiments, the protection provided by CHA0 was also subject to high variation and ranged from 15% (experiment 2, with the highest take-all incidence in the *G. graminis* var. *tritici* control) to 85% (experiment 1, with the lowest take-all incidence in the *G. graminis* var. *tritici* control) reduction of disease severity (Table 4). Root weights were increased by 27% to 41% when plants were protected against *G. graminis* var. *tritici* by the \(\Delta gcd\) mutant, the disease severity index was even lower compared to wild-type treatments in all three independent experiments (the \(\Delta gcd/CHA0\) ratio ranged from 0.61 to 0.74). This effect was statistically significant in only one (experiment 2) out of three experiments. However, when the ratios of disease severities between \(\Delta gcd\) and CHA0 treatments in all three experiments were analyzed together (average \(\Delta gcd/CHA0\) ratio, 0.69), the decrease in biocontrol ability due to lack of Gcd was significant (Table 4). Since after 2 weeks *G. graminis* var. *tritici* infected only the uppermost 2 cm of the root system, better disease protection did not necessarily result in much higher root weights. Therefore, roots protected by the \(\Delta gcd\) mutant had only slightly higher fresh weights than roots protected by CHA0 (Table 4). In all three experiments, take-all disease severity was significantly higher in the \(\Delta gad\) mutant treatments than in the \(\Delta gcd\) treatments. In the absence of *G. graminis* var. *tritici*, plant growth was not significantly affected by the addition of any of the *P. fluorescens* strains (data not shown).

Root colonization by the tested bacterial strains ranged between 7.48 and 7.84 log\(_{10}\) CFU/g of root in experiment 1 and between 8.31 and 8.58 log\(_{10}\) CFU/g of root in experiments 2 and 3. No significant difference between the individual strains was observed, with the exception of experiment 2, where root colonization by the \(\Delta gcd\) mutant was about two times lower than that of the wild-type strain (Table 4). Likewise, there was also no difference in root colonization levels between plants with or without pathogen infection (data not shown).

In the cucumber-*R. solani* disease suppression assay, both the \(\Delta gcd\) and the \(\Delta gad\) mutants provided plant protection similar to that of wild-type CHA0. The average root fresh weight of healthy control plants was 3.1 ± 0.2 g, and that of plants attacked with *R. solani* was 0.6 ± 0.4 g. When protected by either the wild type or one of the two mutants, root fresh weights of cucumber plants ranged between 2.9 and 3.1 g (data not shown).

In summary, the deletion of the *gcd* gene in CHA0 resulted in improved protection of wheat against take-all, but not of cucumber against *R. solani*.
ROLE OF GLUCONIC ACID IN CHA0

DISCUSSION

In the present study, we constructed in-frame deletions in the gcd (CHA1196) and gad (CHA1197) genes in P. fluorescens CHA0 in order to study the influence of gluconic acid production on different characteristics of the strain. Our results show that alteration of gluconic acid production in the two mutants has an impact not only on the metabolism of the bacterium, but also on its interaction with fungal pathogens.

First, the loss of Gcd activity strongly impaired strain CHA0 in its ability to produce acids. Mutants impaired in Gcd activity (CHA1196 and CHA1198) were unable to acidify the surrounding solid medium (Fig. 3A) or showed a strongly reduced ability to produce acids in glucose solution (Fig. 3C). We suggest that these effects were due to the loss of Gcd activity, which resulted in an inability to transform glucose into gluconic acid. Interestingly, the Δgcd mutant and the Δgcd Δgad double mutant, both impaired in acid production, grew faster and formed bigger colonies on NBRIP agar plates than the wild-type strain CHA0 and the Δgad mutant. We suppose that the difference in colony development between the strains is due to the lack of gluconic acid production. On a solid medium containing only glucose as the carbon source (NBRIP medium) (Fig. 3A and B), gluconic acid formation by the wild-type strain and the Δgad mutant resulted in strong acidification of the medium, which may have hampered colony development.

It has been established that in pseudomonads gluconate can enter the cell either by a direct oxidative or by a phosphorylative pathway (Fig. 1A) (10, 12, 40). Along the direct oxidative route, glucose is transformed to gluconic acid and 2-ketogluconate in the periplasmic space by Gcd and Gad, which are located in the inner membrane. Both products can be transported through the inner membrane and be then transported to 6-phosphogluconate (6-PGA) in the cytoplasm. Along the phosphorylative route, glucose is directly transported into the cell and converted to 6-PGA via glucokinase (Glk) and glucose-6-phosphate dehydrogenase (Zwf) in the cytoplasm (Fig. 1A). Berka et al. (6) showed for Burkholderia cepacia that Gcd-deficient strains were blocked in the direct oxidative pathway and glucose was forced to be catabolized entirely by the phosphorylative route. In the same manner, a gcd isogenic mutant of P. putida KT2440 did not produce any gluconate (12), and this indicated that 6-PGA was produced only from glucose-6-phosphate. The phosphorylative pathway is also the only route available in cells during growth under anaerobic (denitrifying) conditions because Gcd activity is not expressed (25, 26, 51).

Interestingly, Fuhrer et al. (17) showed that for P. fluorescens 52-1C, extracellular glucose was mainly converted to gluconate and 2-ketogluconate, suggesting that the oxidative pathway is preferred to the phosphorylative pathway for this strain. Since we have identified gcd and gad genes in P. fluorescens CHA0, it is likely that in our strain also, glucose can pass through the inner membrane in three different forms: as glucose itself or as its oxidized forms gluconic acid and 2-ketogluconate (Fig. 1A). It is evident that the proportion of each form varies between the wild type and the different mutants investigated in this study. In a medium with glucose as the sole carbon source, the Δgcd mutant can take up only the glucose form because the oxidative pathway, and thus production of gluconic acid and 2-ketogluconate, is blocked. This may slow down growth and can explain the delayed initial growth observed for the Δgcd mutant in OS glucose medium (Fig. 2A). Such a reduced growth rate of a gcd mutant was also observed for P. putida KT2440 by del Castillo and coworkers (12). Even a partial inhibition of the oxidative pathway slightly delayed growth, as observed for the Δgad mutant, which is blocked in 2-ketogluconate formation. When mutants were grown in OS glycerol medium (Fig. 2B), no difference in growth was observed between any of the mutants and the wild type. As in pseudomonads glycerol enters the cell by a different route (10, 40), mutations in the gcd and gad genes did not affect bacterial growth in this medium.

Several bacterial genera are known to hydrolyze mineral phosphates to organic phosphates and thus increase phosphate availability for plants (60). The bacterial production of organic acids as the main mechanism of phosphate solubilization has been well documented. Goldstein (19, 20) has proposed that direct periplasmic oxidation of glucose to gluconic acid forms the metabolic basis of the mineral phosphate solubilization in some gram-negative bacteria. Gluconic acid seems to be the most frequent organic acid involved in mineral phosphate solubilization by microorganisms such as Pseudomonas spp. and Penicillium spp. (27, 71), Pantoea agglomerans (41), B. cepacia (21), and P. aeruginosa (7). In this study, we provide evidence that gluconic acid is responsible for phosphate solubilization by P. fluorescens wild-type CHA0, as well as by the Δgad mutant (Fig. 3). The degree of phosphate solubilization is correlated with the degree of acid production. The Δgad mutant, which produced larger amounts of acid (Fig. 3A) than the wild type, also showed better ability to solubilize phosphate (Fig. 3B). In contrast, the Δgcd mutant blocked in gluconic acid production was impaired in phosphate solubilization on NBRIP agar plates (Fig. 3B).

Our results show that the absence of Gcd in the Δgcd mutant enhances the production of both DAPG and PLT in a glucose-based medium (Table 2 and Fig. 4). Similar observations were made previously with a PQO mutant of P. fluorescens CHA0. PQO is a cofactor of Gcd (14), and a lack of PQO in CHA0 leads to inactivation of Gcd and to overproduction of PLT (64). Because the Δgcd mutant is not able to produce gluconic acid, we attributed the change in the strain’s antifungal compound production to the absence of this metabolite. So far, little is known about the specific role of gluconic acid in the regulation of antifungal compounds. Several groups have shown that glucose metabolism and carbon sources in general play a role in the regulation of antifungal metabolites. James and Gutterson (28), for example, have postulated that glucose and gluconate might have important roles in the regulation of antibiotic biosynthesis in P. fluorescens strain HV37a, which shows biocontrol activity against Pythium ultimum. Gutterson (22) proposed that glucose may block antibiotic production through repression of dehydrogenases that catalyze glucose oxidation, a reaction that transfers electrons from the enzyme cofactor PQO to electron transport. Duffy and Défago (13) and other authors showed that carbon sources found in root exudates have different influences on the production of various antifungal compounds in P. fluorescens. In strain CHA0, production of PLT is stimulated by glycerol and the production of DAPG by glucose (13, 46, 66). Glucose, however, represses.
PLT production (13, 66). The stimulation of PLT production by glycerol and its inhibition by glucose were also described for _P. fluorescens_ Pf-5 by Kraus and Loper and Nowak-Thompson et al. (38, 55). All these studies suggest that glucose interferes with PLT production in CHA0 and the closely related strain Pf-5. However, we postulate here that it is the presence of gluconic acid rather than that of glucose that inhibits PLT production in CHA0 for several reasons. First, the lack of Gcd activity and subsequently the lack of gluconic acid production enable a Δgcd mutant to synthesize PLT in a medium containing glucose (Table 2), in which the wild type cannot produce the antifungal compound. Moreover, in a medium with glucose as the sole carbon source, a PLT biosynthetic gene was expressed at a much higher level in the Δgcd mutant than in the wild type (Fig. 4B). Finally, in OS glucan medium, the expression of PLT is not only completely repressed in the wild type, but also in the Δgcd mutant (Fig. 4D). How exactly gluconic acid interacts with the PLT biosynthetic pathway still remains unclear; however, the interaction seems to occur at the transcriptional level. It is also unclear why the absence of Gcd results in enhanced DAPG production and DAPG biosynthetic gene expression (Table 2 and Fig. 4A). The lack of gluconic acid formation cannot explain this observation because gluconic acid inhibits neither DAPG production (Table 2) nor the expression of the DAPG biosynthetic gene _phiA_ (Fig. 4C). The only slight difference we have observed in the gluconate-based medium in comparison to the glucose-based medium is a delay of DAPG production in the wild-type strain. Further studies are needed to assess the exact role of Gcd for the regulation of antifungal metabolite production in _P. fluorescens_ CHA0. In the Δgcd mutant, the oxidative pathway is thought to be completely inhibited, and we suppose that all the glucose is forced to enter the cell via the phosphorolytic pathway. It is likely that this leads to many modifications within the cell and to the accumulation of different compounds, which might impact the regulation of secondary-metabolite production.

The lack of Gcd in strain CHA1196 resulted in 30% better protection (in terms of disease severity) of wheat against take-all disease than the wild type (Table 4). We suggest that the improved ability to suppress the disease is due to enhanced production of antifungal compounds by the Δgcd mutant, which results in enhanced antibiotic. This suggestion is supported by the fact that the Δgcd mutant produces larger amounts of DAPG and PLT in vitro and indeed shows an enhanced ability to inhibit the growth of _G. graminis var. tritici_ on agar plates (Tables 2 and 3). As a major part of root exudates are composed of glucose (29, 42, 69, 70), it is tempting to assume that more DAPG and PLT is produced in the rhizospheres of plants inoculated with the Δgcd mutant. Since no significant difference in root colonization was observed for the tested bacterial strains, the improved plant protection could not be attributed to higher rhizosphere colonization by the Δgcd mutant. However, the increased ability of the Δgcd mutant to suppress disease seems to be pathogen specific. In the cucumber- _R. solani_ system, the lack of Gcd did not result in improved plant protection. Also, on agar plates, increased inhibition by the Δgcd mutant was observed only for _G. graminis var. tritici_ and _M. grisea_ and not for _R. solani_. The reasons for such specificity remain unclear; however, different sensitivities of individual plant-pathogenic fungi to DAPG and PLT have been observed. Maurhofer et al. (45) showed that _G. graminis var. tritici_ is more sensitive to DAPG and PLT than _R. solani_. Even different isolates of _G. graminis_ vary in sensitivity to DAPG (49). Moreover, biocontrol activity is not only defined by antibiosis (24), and for the protection of cucumber against _R. solani_, other mechanisms might also be important.

The direct role of gluconic acid production in the biocontrol activity of CHA0 remains unclear at present. Gluconic acid itself has been proposed to be an antifungal agent and to be involved in the biocontrol ability of an Australian nonfluorescent, non-DAPG-producing pseudomonad (30). A transposition insertion mutant of strain AN5 which had lost its ability to produce gluconic acid was partially impaired in its ability to inhibit _G. graminis var. tritici_ in vitro (30, 53), yet the genetic basis of the mutation was not described. The authors suggested that gluconic acid production is involved in the suppression of take-all disease by the bacterium, probably by lowering the pH in the wheat rhizosphere. In our study, we could not demonstrate that gluconic acid produced by CHA0 is involved in inhibition of _G. graminis var. tritici_ or suppression of take-all. First, the lack of gluconic acid production in the Δgcd mutant did not result in reduced inhibition of _G. graminis var. tritici_ (Table 3). Still, it is possible that the effect of loss of gluconic acid production was masked by the effect of increased antifungal compound production in the Δgcd mutant. Second, the Δgcd mutant, which produces the same DAPG and PLT levels as the wild type (Table 2) but increased amounts of gluconic acid (Fig. 3A), did not either better inhibit _G. graminis var. tritici_ in vitro (Table 3) or better suppress take-all of wheat (Table 4). However, we do not know whether the Δgcd mutant in fact produces higher levels of gluconic acid in the rhizosphere. Moreover, the _G. graminis var. tritici_ isolate used in this study appears not to be as sensitive to gluconic acid as the isolate used by Kaur et al. (30). The growth of the isolate used by Kaur et al. was already inhibited at a concentration of 6 mg/ml gluconic acid, whereas growth of our _G. graminis var. tritici_ isolate was inhibited only at concentrations of 100 mg/ml and higher. Therefore, we suggest that the direct role of gluconic acid produced by a biocontrol bacterium in the suppression of take-all may also be dependent on the sensitivity of the fungus to the acid.

Taken together, our results demonstrate that gluconic acid production is involved in the regulation of antimicrobial compound production in a rhizosphere-associated pseudomonad and as a consequence indirectly modulates the bacterium’s biocontrol activity.

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