RpoN (σ^{54}) Controls Production of Antifungal Compounds and Biocontrol Activity in *Pseudomonas fluorescens* CHA0

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Pseudomonas fluorescens CHA0 is an effective biocontrol agent of root diseases caused by fungal pathogens. The strain produces the antibiotics 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) that make essential contributions to pathogen suppression. This study focused on the role of the sigma factor RpoN (σ^{54}) in regulation of antibiotic production and biocontrol activity in *P. fluorescens*. An *rpoN* in-frame-deletion mutant of CHA0 had a delayed growth, was impaired in the utilization of several carbon and nitrogen sources, and was more sensitive to salt stress. The *rpoN* mutant was defective for flagella and displayed drastically reduced swimming and swarming motilities. Interestingly, the *rpoN* mutant showed a severalfold enhanced production of DAPG and expression of the biosynthetic gene *phlA* compared with the wild type and the mutant complemented with monocopy *rpoN*⁺. By contrast, loss of RpoN function resulted in markedly lowered PLT production and *plt* gene expression, suggesting that RpoN controls the balance of the two antibiotics in strain CHA0. In natural soil microcosms, the *rpoN* mutant was less effective in protecting cucumber from a root rot caused by *Pythium ultimum*. Remarkably, the mutant was not significantly impaired in its root colonization capacity, even at early stages of root infection by *Pythium* spp. Taken together, our results establish RpoN for the first time as a major regulator of biocontrol activity in *Pseudomonas fluorescens*.

Additional keyword: rhizosphere.

Certain strains of fluorescent *Pseudomonas* spp. that are closely associated with roots can protect crop plants from soilborne diseases caused by pathogenic fungi, oomycetes, and bacteria (Cook et al. 1995; Keel and Défago 1997). In addition to their capacity to efficiently colonize plant roots,

the production of metabolites that are inhibitory to plant-pathogenic rhizosphere inhabitants is considered to be one of the principal backbones of biocontrol activity in many of these bacteria (Haas and Keel 2003; Raaijmakers et al. 2002; Thomashow and Weller 1995). 2,4-Diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) are two metabolites that have received particular attention because of their broad-spectrum antifungal and antibacterial activity (Haas and Keel 2003; Keel et al. 1992; Maurhofer et al. 1992, 1995; Raaijmakers et al. 2002; Thomashow and Weller 1995). Some effective biocontrol pseudomonads produce both DAPG and PLT (Keel et al. 1996), among them the well-characterized *Pseudomonas fluorescens* strain CHA0 (Haas and Keel 2003; Keel and Défago 1997; Voisard et al. 1994) and the very closely related *P. fluorescens* strain Pf-5 (Howell and Stipanovic 1980; Kraus and Loper 1995; Sarniguet et al. 1995). DAPG and PLT make major contributions to biocontrol activity in these strains. DAPG has been shown to contribute to the protection of wheat from take-all disease (Keel et al. 1992) and of tobacco from black root rot (Keel et al. 1990), whereas PLT has been associated mainly with the suppression of root rot and damping-off diseases caused by *Pythium* spp. (Howell and Stipanovic 1980; Kraus and Loper 1995; Maurhofer et al. 1994).

The DAPG and PLT biosynthetic genes are organized as clusters, probably operons, and have been identified in several *Pseudomonas* strains (Haas et al. 2000; Haas and Keel 2003). The DAPG locus comprises the *phlACBD* biosynthetic genes and the divergently transcribed *phlF* gene located upstream of *phlA* and encoding a pathway-specific transcriptional repressor (Bangera and Thomashow 1999; Delany et al. 2000; Schnider-Keel et al. 2000). The PLT biosynthetic genes *pltLABCDEFG* are preceded by the divergently transcribed *pltR* gene, which encodes a pathway-specific transcriptional activator (Haas and Keel 2003; Nowak-Thompson et al. 1999). Remarkably, the biosyntheses of both compounds, DAPG and PLT, are controlled by positive autoregulatory circuits (Brodhagen et al. 2004; Haas and Keel 2003; Schnider-Keel et al. 2000). There is evidence for a molecular cross-talk between the DAPG and PLT biosynthetic pathways, inasmuch as the two metabolites were found to display an inverse relationship in which each metabolite, besides activating its own biosynthesis, represses the synthesis of the other metabolite (Brodhagen et al. 2004; Haas and Keel 2003; Schnider-Keel et al. 2000). In addition, levels of DAPG and PLT production may be modulated by other phenolic metabolites released by microorganisms and plant roots in the rhizosphere as well as in response to certain min-

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eral and carbon sources (Duffy and Défago 1999; Haas and Keel 2003; Maurhofer et al. 2004; Notz et al. 2001; Schnider-Keel et al. 2000).

In addition to the pathway-specific regulatory mechanisms, a series of global regulatory networks that respond to environmental and cell density-dependent signals and probably also to the physiological status of the bacterium contribute to the control of DAPG and PLT biosyntheses (Haas et al. 2000; Haas and Keel 2003; Thomashow and Weller 1995). They include a regulatory cascade controlled by a two-component system composed of the sensor kinase GacS and the response regulator GacA that positively controls antibiotic biosynthesis upon activation by a yet unidentified quorum sensing-signal (Haas and Keel 2003; Valverde et al. 2003; Zuber et al. 2003). An additional level of control may be provided by the relative concentrations of the housekeeping σ factor RpoD and the stress and stationary phase σ factor RpoS in the bacterial cell. In *P. fluorescens* CHA0, overexpression of the housekeeping σ factor RpoD enhances DAPG and PLT production in vitro and in the rhizosphere, resulting in improved biocontrol or phytotoxic effects depending on the plant species (Maurhofer et al. 1992, 1995; Schnider et al. 1995). Likewise, an *rpoS* mutant of the related strain Pf-5 shows strongly enhanced PLT and DAPG production (Sarniguet et al. 1995), whereas *rpoS* overexpression in strain CHA0 was found to shut off PLT production (Haas and Keel 2003).

In recent years, the alternative σ factor RpoN (σ^{54}) has evolved as an important regulator of virulence in pathogenic pseudomonads. RpoN works in concert with specialized transcriptional activators (enhancer-binding proteins) to control the expression of genes coding for very diverse functions in response to environmental stimuli (Buck et al. 2000; Merrick 1993). Typical features that depend on RpoN function in pseudomonads and in a broad range of other bacteria include motility and diverse metabolic functions such as transport and metabolism of various nitrogen and carbon sources (Cases et al. 2003; Köhler et al. 1989; Reitzer and Schneider 2001; Studholme and Buck 2000). In the plant-pathogenic *P. syringae* pvs. *maculicola* and *glycinea*, RpoN is required for the expression of the pathogenicity-related *hrp* genes, for the production of the phytotoxin coronatine, and for full virulence on host plants (Alarcón-Chaidez et al. 2003; Hendrickson et al. 2000a,b). Similarly, in the opportunistic human pathogen *P. aeruginosa*, RpoN regulates the expression of a series of virulence factors, including flagella, pili, alginate, rhamnolipid biosurfactants, exotoxins, cyanide, and pyocyanin, and contributes to virulence in certain animal models (Boucher et al. 2000; Hendrickson et al. 2001; Heurlier et al. 2003; Ishimoto and Lory 1989; Studholme and Buck 2000; Thompson et al. 2003; Totten et al. 1990). Recent evidence suggests that the RpoN regulon in pathogenic pseudomonads may be interlinked with other regulatory networks, in particular those controlled by the GacS/GacA two-component system, by quorum-sensing systems, and by other σ factors, but the exact mechanisms involved are not yet fully understood (Boucher et al. 2000; Chatterjee et al. 2003; Hendrickson et al. 2000a; Heurlier et al. 2003; Thompson et al. 2003).

To our best knowledge, a regulatory role for RpoN in plant-beneficial pseudomonads has not been described so far. To address this issue, we have cloned and sequenced the *rpoN* locus from *P. fluorescens* CHA0. In the present study, we show that an *rpoN* in-frame-deletion mutant of strain CHA0 displays several phenotypes that typically are associated with loss of RpoN function, including a drastically reduced motility and a defect in the utilization of several nitrogen and carbon sources. We report that *rpoN* mutation in strain CHA0 entails increased sensitivity to salt stress but does not affect tolerance to oxidative

stress. Finally, we demonstrate that deletion of *rpoN* in CHA0 results in an altered expression of the antifungal compounds DAPG and PLT and in a strongly reduced capacity to control *Pythium* root rot of cucumber, thus providing the first evidence that RpoN is a further key regulator of biocontrol activity in plant-beneficial pseudomonads.

RESULTS

Characterization of the chromosome region containing *rpoN* in *P. fluorescens* CHA0.

We cloned and sequenced a 2.2-kb chromosomal region in *P. fluorescens* strain CHA0 containing *rpoN* and adjacent genes (Fig. 1) in an arrangement identical to that described for other *Pseudomonas* spp. (Alarcón-Chaidez and Bender 2001; Härtig and Zumft 1998; Jin et al. 1994; Köhler et al. 1994; Stover et al. 2000). The deduced product (497 amino acids) of the *rpoN* gene of *P. fluorescens* CHA0 is very similar to σ factor RpoN (σ^{54}) of *P. syringae* pv. *maculicola* (accession no. AF199600; 95% identity), *P. syringae* pv. *glycinea* (AF283815; 93% identity), *P. syringae* pv. *tomato* (AE016872; 96% identity), *P. syringae* pv. *syringae* (ZP_00126321; 95% identity), *P. putida* (M24916 and X16474; 91% identity), *P. aeruginosa* (AE004860 and L26916; 86% identity), *P. stutzeri* MK21 (AJ223088; 85%), and *Azotobacter vinelandii* (X05888; 80% identity). The predicted protein sequence of RpoN of strain CHA0 is in accordance with the highly conserved domain structure identified in RpoN of other bacteria, in particular with respect to the helix-turn-helix motif (MKPLVLHDIAEAVGMHESITSRVTTQ) and the RpoN-box motif (ARRTVAKYRE) in the C-terminus which are identical to those in other pseudomonads and in *Azotobacter* sp. (Alarcón-Chaidez and Bender 2001; Buck et al. 2000; Jin et al. 1994; Merrick 1993). RpoN recognizes an YTGGCAC R-N4-TTG CW consensus sequence situated at positions -24 and -12 relative to the transcription start site (Barrios et al. 1999; Reitzer and Schneider 2001; Studholme and Buck 2000). A putative RpoN recognition site (CAGGCATATAAT TTGCT) was found 89 bp upstream of the translational start site of *rpoN* of strain CHA0. Remarkably, the sequence and location of the RpoN recognition site appears to be highly conserved in *P. fluorescens*, *P. syringae* (Alarcón-Chaidez and Bender 2001), *P. putida* (Köhler et al. 1994), and *P. aeruginosa* (Jin et al. 1994).

As in other pseudomonads, *rpoN* in *P. fluorescens* CHA0 is flanked by an upstream open reading frame (ORF) coding for the ATPase component of an ABC-type transport system (ORFA; Fig. 1) and by two downstream ORFs (ORFB and ORFC) encoding, respectively, a protein with putative σ^{54} -modulating function and the enzyme IIA (PtsN) of the phosphoenolpyruvate/sugar phosphotransferase system (Alarcón-Chaidez and Bender 2001; Cases et al. 1999; Härtig and Zumft 1998; Jin et al. 1994; Köhler et al. 1994; Stover et al. 2000). The amino acid sequences of the deduced products of ORFB and the partially sequenced ORFA and ORFC were found to be between 72 and 89% identical to the corresponding sequences of their counterparts in *P. syringae*, *P. aeruginosa*, *P. putida*, *P. stutzeri*, and *A. vinelandii*. Homologues of these ORFs also have been found in an identical arrangement in the *rpoN* regions of various other gram-negative bacterial species, but their precise function and their relation to RpoN function is not yet fully understood (Merrick 1993; Michiels et al. 1998). It has been suggested that the products of the downstream *rpoN*-linked genes might contribute to the modulation of RpoN activity or might be involved in the coregulation of some of the RpoN promoters (Cases et al. 1999; Jin et al. 1994; Merrick 1993; Michiels et al. 1998).

Growth characteristics of an *rpoN* mutant of *P. fluorescens*.

A chromosomal *rpoN* in-frame mutation was created in *P. fluorescens* CHA0 (Fig. 1) as described below. The *rpoN* mutant (CHA250) was complemented by a single copy of intact *rpoN*⁺ introduced into the chromosomal Tn7 attachment site, resulting in strain CHA251. The constructs then were compared with the wild type for changes in growth characteristics. In nutrient-rich media, the *rpoN* mutant CHA250 had a markedly longer doubling time (approximately 75 min in King's medium B [KMB] broth, 71 min in Luria-Bertani [LB] broth, and 133 min in glycerol-Casamino acids medium [GCM]) than the wild-type CHA0 and the complemented mutant CHA251 (approximately 44 min in KMB broth, 38 min in LB broth, and 72 min in GCM). However, in the stationary growth phase, all three strains had reached similar final cell densities in these media (data not shown). In the following, KMB broth was chosen as the standard culture medium for most of the experiments because growth kinetics of the *rpoN* mutant most resembled those of the wild type in this medium, except that the mutant had an extended lag phase and a slightly slower growth during the exponential phase.

Unlike wild-type CHA0 (doubling time of 99 min), the *rpoN* mutant CHA250 was unable to grow in the minimal glucose-ammonium medium (OSG). Therefore, the capacity of the mutant to utilize different nitrogen sources was tested in M9 minimal salt medium in which NH₄Cl was replaced or supplemented by an alternative nitrogen source. In contrast to the wild type or the complemented mutant, the *rpoN* mutant was unable to grow in M9 minimal medium when it contained NH₄Cl, histidine, or proline as the sole nitrogen source. The growth of the mutant in M9 minimal medium supplemented or not with NH₄Cl could be restored largely by addition of glutamine, glutamate, or aspartate to the medium (data not shown). In addition, carbon source utilization profiles were determined for wild-type CHA0 and the *rpoN* mutant using Biolog GN2 and GP2 plates. Of the 128 carbon sources tested, 53 were assimilated by strain CHA0 and, of these, the *rpoN* mutant could not utilize 18 (i.e., *N*-acetyl-D-glucosamine, *cis*-aconitate, 2-aminoethanol, D-arabitol, DL-

carnitine, citrate, L-histidine, γ -hydroxybutyrate, α -ketoglutarate, D-mannitol, monomethylsuccinate, L-proline, L-serine, succinamate, succinate, L-threonine, D-trehalose, and urocanate). Moreover, CHA250 displayed poor growth on 15 additional carbon sources (data not shown).

Overexpression of *rpoN* from the multicopy plasmids pME8013 (*P*_{rpoN}-*rpoN*⁺) and pME8014 (*P*_{lac}-*rpoN*⁺) (Fig. 1) in a CHA0 background did not significantly affect growth characteristics of the derivatives in rich and minimal media compared with the wild-type strain (data not shown). In conclusion, loss of RpoN function in *P. fluorescens*, as in other *Pseudomonas* spp. (Alarcón-Chaidez et al. 2003; Köhler et al. 1989; Totten et al. 1990), results in some growth defects, particularly with respect to the utilization of certain C and N sources.

RpoN is required for motility in *P. fluorescens*.

For *P. aeruginosa*, three types of surface motility have been described: i) swimming motility, which depends on the polar flagellum and can be observed on low-agar medium; ii) swarming motility, which occurs as a coordinate cell movement on semisolid agar surfaces and requires flagella, biosurfactants, and, in some cases, also type IV pili; and iii) twitching motility, which is mediated by type IV pili and takes place at surface interfaces (Köhler et al. 2000; Rashid and Kornberg 2000). Here, we report that these modes of surface translocation also can be observed in *P. fluorescens* (Table 1; Fig. 2). As in *P. aeruginosa*, swarming motility in *P. fluorescens* strain CHA0 was accompanied by a typical dendritic pattern of the swarm colony (Fig. 2A). Loss of RpoN function had a dramatic impact on the surface motility of *P. fluorescens*. Swimming and swarming motilities in the *rpoN* mutant CHA250 were drastically reduced, whereas twitching motility appeared to be less affected (Table 1; Fig. 2A and B). Motility could be restored to wild-type levels by complementation of the mutant with a single copy of intact *rpoN*⁺ (Table 1). Overexpression of *rpoN* from its natural promoter in CHA0/pME8013 and from the constitutive *lac* promoter in CHA0/pME8014 had no statistically significant effect on motility (Table 1). Transmission electron microscopy confirmed that cells of the *rpoN* mutant

Fig. 1. Organization of the *rpoN* region in *Pseudomonas fluorescens* strain CHA0; Δ = region deleted in strain CHA250 and in plasmid pME7066. The shaded arrows show the genes sequenced or partly sequenced on the 2209-bp *Bam*HI-*Xba*I fragment of pME7069. Open reading frame (ORF)A, ORFB, and ORFC designate ORFs that are highly similar to PA4461, PA4463, and PA4464 (*ptsN*), respectively, of *P. aeruginosa* PAO1 (Stover et al. 2000). The bars indicate the fragments cloned into the vectors pME6000, pME3087, pME6182, and pME6551 to give pME7069, pME7066, pME7070, and pME8014, respectively. The fragment inserted in pME7070 was used to complement the *rpoN* mutation in CHA250 with single-copy *rpoN*⁺ using the mini-Tn7 delivery system. Artificial restriction sites are marked with an asterisk.

were defective for flagella, whereas swarmer cells of the wild type, the complemented mutant, and the *rpoN*-overexpressing mutants possessed two to three, sometimes even more, polar flagella (Fig. 2C; data not shown). However, at least under the experimental conditions chosen here, we did not detect pili on swarmer cells. On swim plates, *P. fluorescens* cells with intact *rpoN* generally had one polar flagellum, although occasionally cells with two flagella also could be observed (data not shown). In summary, our results establish that RpoN, as in other bacterial species, is essential for flagella formation and thus for motility in *P. fluorescens*.

Contribution of RpoN to stress tolerance in *P. fluorescens*.

We next tested whether lack of RpoN affects growth of *P. fluorescens* in response to osmotic stress. For this purpose, we determined the doubling times of strain CHA0 and its derivatives during exponential growth of the bacteria in KMB broth supplemented with NaCl or sorbitol. The *rpoN* mutant was significantly more sensitive to high salinity than the wild type. The doubling time of CHA250 cells quadrupled when they were exposed to 0.6 M NaCl, whereas it was only threefold longer in CHA0 and the complemented *rpoN* mutant CHA251 (Table 2). This effect also could be observed at higher salt concentrations (0.8 M NaCl) (data not shown). No such difference in stress sensitivity between the wild type and the *rpoN* mutant could be noted when bacteria were exposed to 1.2 M sorbitol (Table 2). The mutant complemented with monocopy *rpoN*⁺ and CHA0 derivatives overexpressing *rpoN* from plasmids pME8013 or pME8014 exhibited stress tolerances largely similar to that of the wild type (Table 2).

To test the contribution of RpoN to bacterial survival in response to osmotic stress, stationary-phase cells were exposed to 1.2 M NaCl or 2.4 M sorbitol (i.e., conditions which no longer allowed *P. fluorescens* to grow). In contrast to the wild-type CHA0 and the *rpoN*-overexpressing derivative CHA0/pME8014, culturability in the *rpoN* mutant CHA250 significantly dropped soon after exposure to salt stress (Fig. 3). By contrast, no such differences could be observed when bacteria were exposed to 2.4 M sorbitol (data not shown).

To determine whether inactivation of *rpoN* affects the susceptibility of *P. fluorescens* to agents generating reactive oxygen intermediates (ROIs), wild-type strain CHA0, the *rpoN* deletion mutant, and the *rpoN*-overexpressing derivatives were exposed to paraquat, hydrogen peroxide, and sodium hypochlorite. All mutant strains were found to display wild-type susceptibility to various concentrations of these agents (data not shown). In contrast, an *rpoS* mutant of strain CHA0 (Heeb et al. 2002) (Table 3) included as a control was significantly more sensitive to oxidative stress than the parental strain (data not shown), thus confirming previous findings of Sarniguet and associates (1995) obtained with an *rpoS* mutant of *P. fluorescens* Pf-5. In summary, these results suggest that RpoN contributes to tolerance of *P. fluorescens* to conditions of high salinity, but is not required for tolerance toward ROIs and osmotic stress induced by sorbitol.

RpoN controls the production of antifungal compounds in *P. fluorescens*.

We then evaluated the effect of the *rpoN* mutation on the production of the antifungal compounds DAPG and PLT, which make significant contributions to the biocontrol activity of *P. fluorescens* CHA0. For this purpose, we followed the expression of *lacZ* reporter fusions to the DAPG and PLT biosynthetic genes in bacterial cultures growing in KMB broth and we determined the production of the two antifungal compounds in parallel. Interestingly, the expression of a *phlA-lacZ* transcriptional fusion carried by pME6710 (Schnider-Keel et al. 2000) was enhanced severalfold in the *rpoN* mutant as of the end of the exponential growth phase compared with the wild-type CHA0 and the complemented mutant CHA251 (Fig.

Fig. 2. Swarming and swimming motilities of *Pseudomonas fluorescens* wild-type CHA0 (left) and its *rpoN* mutant CHA250 (right) on **A**, swarm plates (nutrient broth with 0.5% glucose and 0.5% agar) and **B**, swim plates (tryptone broth with 0.3% agar), respectively, after a 48-h incubation at room temperature. **C**, Electron micrographs showing flagellated cells of strain CHA0 (left) and nonflagellated cells of the *rpoN* mutant CHA250 (right) taken from the edge of swarm colonies.

Table 1. Contribution of RpoN to swimming, swarming, and twitching motilities of *Pseudomonas fluorescens* CHA0^z

Strain	Relevant genotype	Swim area, % wt	Swarm area, % wt	Twitch area, % wt
CHA0	Wild type	100 ± 12	100 ± 8	100 ± 6
CHA250	Δ <i>rpoN</i>	32 ± 4	4 ± 1	60 ± 12
CHA251	Δ <i>rpoN</i> attTn7:: <i>rpoN</i> ⁺	110 ± 4	118 ± 27	100 ± 20
CHA0/pME8013	<i>P</i> _{rpoN} - <i>rpoN</i> ⁺	122 ± 17	105 ± 12	88 ± 19
CHA0/pME8014	<i>P</i> _{lac} - <i>rpoN</i> ⁺	110 ± 8	79 ± 24	128 ± 21

^z Diameter of bacterial displacement on swim plates, swarm plates, and twitch plates after incubation for 24 h (swim and swarm plates) and 4 to 5 days (twitch plates) at room temperature, assessed as described in the Materials and Methods section. Data are expressed as percentage of the diameters obtained for the wild type which were arbitrarily set to 100%. Data represent the means ± standard deviation from three experiments with 12 replicate plates per treatment in each experiment.

4A). In contrast, expression levels of a *pltA-lacZ* transcriptional fusion carried by pME8007 (Table 3) were two- to threefold lower in the *rpoN* mutant than in the *rpoN*⁺ strains CHA0 and CHA251 (Fig. 4B). RpoN overexpression in strain CHA0 carrying the multicopy plasmids pME8013 or pME8014 (Table 3) did not significantly alter *phl* or *plt* gene expression (data not shown). The kinetics of DAPG and PLT production paralleled the expression of the *phlA-lacZ* and *pltA-lacZ* reporter constructs: DAPG levels were enhanced whereas PLT levels were reduced in cultures of the *rpoN* mutant (Fig. 4C and D). The *rpoN*-overexpressing strains CHA0/pME8013 and CHA0/pME8014 produced wild-type levels of DAPG and PLT (data not shown). The strong decrease of DAPG and PLT levels at the end of the experiments may be due to degradation of the two compounds by the producer strains (Schnider-Keel et al. 2000; *our unpublished observations*). Experiments also were carried out using a *phlA-lacZ* translational fusion on pME6259 (Schnider-Keel et al. 2000) and a *pltA-lacZ* translational fusion on pME6751 (Table 3), and kinetics of gene expression in the *rpoN* mutant, the wild type, and the complemented *rpoN* mutant were found to be almost identical to those obtained with the transcriptional reporter fusions described above (data not shown). Glycerol is known to favor PLT production in *P. fluorescens* (Duffy and Défago 1999; Schnider-Keel et al. 2000); therefore, the influence of RpoN on *phlA* expression also was tested in KMB broth in which glycerol was replaced by glucose (0.89%, wt/vol). A similar upregulation of *phlA* expression could be observed in the *rpoN* mutant (data not shown), thus excluding a potential effect of the carbon source. Taken together, these findings demonstrate that RpoN may control the balance of antibiotic production in a biocontrol strain of *P. fluorescens*.

RpoN is essential for biocontrol capacity of *P. fluorescens*.

The contribution of RpoN to the biocontrol ability of *P. fluorescens* strain CHA0 against a root rot and damping-off disease of cucumber caused by the pathogenic oomycete *Pythium ultimum* was tested in natural soil microcosms. *P. ultimum* severely reduced growth and survival of cucumber plants when no biocontrol bacteria were applied to soil (Table 4). Addition of strain CHA0 to pathogen-infested soil enhanced plant survival by 29% and plant fresh weights were increased threefold (Table 4). The *rpoN* mutant CHA250 was severely impaired in its capacity to protect cucumber from the root disease; plant survival and plant fresh weights were only marginally higher than those of plants treated with the pathogen alone (Table 4). Biocontrol activity of the *rpoN* mutant could be restored by complementing the strain with monocopy *rpoN*⁺ (Table 4). Overexpression of *rpoN* from the natural promoter or from the constitutive *lac* promoter in strains CHA0/pME8013 and CHA0/pME8014, respectively, did not result in a significantly altered biocontrol activity (Table 4). In the absence of the patho-

gen, plant growth was not significantly affected by addition of bacteria (Table 4). Remarkably, root colonization levels at the end of the experiment were similar for all bacterial strains and varied between 0.4 and 1.7 × 10⁷ CFU/g of roots in the absence of *Pythium* spp. and between 2.1 and 4.9 × 10⁷ CFU/g of roots in pathogen-infested soil. To check whether differences in bacterial root colonization levels occurred eventually at earlier stages of *Pythium* spp. infection, we determined population densities of the wild-type CHA0 and the *rpoN* mutant CHA250 on cucumber roots also at 2 and 3 days after bacterial application to soil. However, in no case were population densities of the two strains on cucumber roots significantly different in either the presence or absence of the pathogen (data not shown).

DISCUSSION

In this study, we identified the *rpoN* locus in the plant-beneficial *Pseudomonas fluorescens* strain CHA0. The arrangement of *rpoN* and adjacent genes in *P. fluorescens* (Fig. 1) is identical to that described for *P. putida* (Köhler et al. 1994), *P. stutzeri* (Härtig and Zumft 1998), *P. aeruginosa* (Jin et al. 1994; Stover et al. 2000), and *P. syringae* (Alarcón-Chaidez and Bender 2001), thus confirming that the gene alignment in this chromosomal region is conserved in different *Pseudomonas* spp. Our results demonstrate that RpoN of *P. fluorescens* is

Fig. 3. Survival of *Pseudomonas fluorescens* wild-type CHA0 (black circle), the *rpoN* mutant CHA250 (open circle), and the *rpoN*-overexpressing derivative CHA0/pME8014 (gray-shaded circle) during exposure to 1.2 M NaCl. Survival was expressed as the log₁₀ of the ratio of CFU in the exposed suspension to the CFU in the initial suspension (Sarniguet et al. 1995). Means ± standard deviation from three replicate cultures are shown. The experiment was repeated, with similar results. Some of the error bars were too small to be included.

Table 2. Effect of an *rpoN* mutation on tolerance of *Pseudomonas fluorescens* to osmotic stress

Strain ^z	Relevant genotype	Without stress	Doubling time (h) ^x		Ratio ^y	
			0.6 M NaCl	1.2 M Sorbitol	0.6 M NaCl	1.2 M Sorbitol
CHA0	Wild type	0.74 c	2.28 b	1.51 c	3.1	2.0
CHA250	$\Delta rpoN$	1.09 a	4.52 a	2.42 a	4.2	2.2
CHA251	$\Delta rpoN$ attTn7::rpoN ⁺	0.75 c	2.02 b	1.62 bc	2.7	2.2
CHA0/pME8013	<i>P</i> _{rpoN} -rpoN ⁺	0.82 bc	1.92 b	2.06 b	2.3	2.5
CHA0/pME8014	<i>P</i> _{lac} -rpoN ⁺	0.86 b	1.69 b	2.07 b	2.0	2.4

^x Doubling time was calculated from optical density (OD) at 600 nm values determined during exponential growth phase (eight measurements). During this, the relation of OD at 600 nm values to CFU counts was linear (data not shown). Data are means from two independent experiments, with three replicate cultures per experiment. Values in the same column with different letters are significantly different at *P* = 0.05 (Fisher's least significant difference test).

^y Ratio denotes the *x*-fold increase in doubling time of stressed (0.6 M NaCl or 1.2 M sorbitol) versus nonstressed cultures for each bacterial strain.

^z Bacteria were grown at 30°C in King's medium B broth with (and without) the addition of NaCl or sorbitol.

functionally equivalent to its counterparts in other *Pseudomonas* spp. with respect to its importance for motility and utilization of various carbon and nitrogen sources (Alarcón-Chaidez et al. 2003; Hendrickson et al. 2000b, 2001; Ishimoto and Lory 1989; Köhler et al. 1989; Merrick 1993; Totten et al. 1990).

We also tested whether RpoN, as well as RpoS (Sarniguet et al. 1995) and AlgU (Schnider-Keel et al. 2001), may be an additional σ factor required for tolerance of *P. fluorescens* to environmental stress. Indeed, the *rpoN* mutant of strain CHA0 was significantly more sensitive to hyperosmotic conditions induced by NaCl (Table 2; Fig. 3), confirming previous results obtained with an *rpoN* mutant of *P. aeruginosa* PAO1 (Sage et al. 1997). By contrast, the *rpoN* mutant of strain CHA0 displayed wild-type sensitivities to agents that generate ROIs. Similarly, an *rpoN* mutant of *P. putida* KT2440 was reported not to be impaired in its tolerance toward oxidative stress; however, this mutant was not affected in its sensitivity to either salt stress or high temperature (Cases and de Lorenzo 2001).

More intriguingly, our findings provide evidence for novel roles of RpoN as a key regulator of antibiotic production and biocontrol activity in *P. fluorescens*. On the one hand, RpoN was found to positively control the expression of *plt* biosynthetic genes (Fig. 4B) and PLT production (Fig. 4D) in strain CHA0. Interestingly, RpoN has been reported previously to be essential for the expression of the phytotoxin coronatine in the plant-pathogenic bacteria *P. syringae* pv. *maculicola* (Hendrickson et al. 2000b) and *P. syringae* pv. *glycinea* (Alarcón-Chaidez et al.

2003). In the same vein, RpoN was required for the production of the antibiotic and virulence factor pyocyanin in *P. aeruginosa* (Hendrickson et al. 2001; Thompson et al. 2003). On the other hand, RpoN exerted a strong negative effect on the expression of *phl* biosynthetic genes (Fig. 4A) and DAPG production (Fig. 4C) in *P. fluorescens* CHA0. Recently, a negative effect of RpoN has been reported by Heurlier and associates (2003), who found that mutation of *rpoN* in *P. aeruginosa* leads to overexpression of *N*-acyl homoserine lactones and some quorum-sensing-regulated virulence factors such as the biocide hydrogen cyanide, but the precise mechanisms involved remain unknown.

At the current stage, we can only speculate on how RpoN might affect the balance of antibiotic production in *P. fluorescens* CHA0. We could not detect typical motifs recognized by σ^{54} (i.e. YTGGCACR-N4-TTGCW) (Barrios et al. 1999; Reitzer and Schneider 2001; Studholme and Buck 2000) in the *phlA* and *pltA* leader regions, suggesting that RpoN does not directly interact with the promoters of the DAPG and PLT biosynthetic genes. However, we could find a potential σ^{54} -binding motif (ATGGCCCCAGCACTGCT) approximately 100 bp upstream of the translational start of *pltR* (M. Bottiglieri and C. Keel, unpublished data). Because PltR functions as a pathway-specific activator of PLT gene expression (Haas and Keel 2003; Nowak-Thompson et al. 1999), it seems possible that the positive effect of RpoN on *pltA* expression is mediated by σ^{54} -dependent activation of *pltR* transcription. Using a transcriptional *pltR-lacZ* fusion, we have obtained preliminary evi-

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^z	Reference or source
<i>Pseudomonas fluorescens</i>		
CHA0	Wild type	Voisard et al. 1994
CHA250	$\Delta rpoN$	This study
CHA251	CHA250:: <i>atfTn7-rpoN</i> ⁺ ; Gm ^r	This study
CHA815	$\Delta rpoS$	Heeb et al. 2002
<i>Escherichia coli</i>		
DH5 α , HB101	Laboratory strains	Sambrook and Russell 2001
Plasmids		
pBluescript II KS+	Cloning vector; ColE1 replicon; Ap ^r	Stratagene
pME497	Mobilizing plasmid; IncP-1, Tra; RepA(Ts); Ap ^r	Voisard et al. 1994
pME3087	Suicide vector; ColE1 replicon; RK2-Mob; Tc ^r	Voisard et al. 1994
pME3280a	Carrier plasmid for Tn7; containing the mini Tn7-Gm transposon with translational and transcriptional signals; pUC19ori; Ap ^r Gm ^r	Schnider-Keel et al. 2000; Zuber et al. 2003
pME6000	Cloning vector, pBBR1MCS derivative; Tc ^r	Schnider-Keel et al. 2001
pME6001	Cloning vector, pBBR1MCS derivative; Gm ^r	Valverde et al. 2003
pME6010	Cloning vector; pACYC177-pVS1 shuttle vector; Tc ^r	Heeb et al. 2000
pME6014, pME6015	Cloning vectors for construction of translational <i>lacZ</i> -fusions; derived from pME6010; Tc ^r	Schnider-Keel et al. 2000; this study
pME6016	Cloning vector for construction of transcriptional <i>lacZ</i> fusions; derived from pME6010; Tc ^r	Schnider-Keel et al. 2000
pME6182	Carrier plasmid for Tn7 containing the mini Tn7-Gm transposon; derived from pME3280a; multiple cloning site flanked by transcription terminators; Ap ^r Gm ^r	C. Reimmann
pME6259	pME6014 carrying a translational <i>phlA</i> '-' <i>lacZ</i> fusion; Tc ^r	Schnider-Keel et al. 2000
pME6551	Cloning vector; derivative of pME6000; Gm ^r	Schnider-Keel et al. 2001
pME6710	pME6016 carrying a transcriptional <i>phlA-lacZ</i> fusion; Tc ^r	Schnider-Keel et al. 2000
pME6751	pME6015 with a 864-bp <i>pltA</i> upstream fragment and a translational <i>pltA</i> '-' <i>lacZ</i> fusion containing the first 43 <i>pltA</i> codons; Tc ^r	This study
pME7066	pME3087 carrying a 867-bp <i>Bam</i> HI- <i>Hind</i> III insert with a deletion in <i>rpoN</i> ; Tc ^r	This study
pME7069	pME6000 with a 2,199-bp <i>Bam</i> HI- <i>Xba</i> I fragment containing <i>rpoN</i> and its flanking regions of CHA0; Tc ^r	This study
pME7070	pME6182 with the 1,952-bp <i>Bam</i> HI- <i>Nco</i> I fragment of pME7069 containing <i>rpoN</i> ; Ap ^r Gm ^r	This study
pME8007	pME6016 with a 798-bp <i>pltA</i> upstream fragment and a transcriptional <i>pltA-lacZ</i> fusion containing the first 9 <i>pltA</i> codons; Tc ^r	This study
pME8013	pME6551 with a 2.2-kb <i>Hind</i> III- <i>Eco</i> RI fragment of pME7070 containing <i>rpoN</i> ; Gm ^r	This study
pME8014	pME6551 with a 1,515-bp fragment derived from pME7070 containing <i>rpoN</i> and a 21-bp upstream region placed under the control of the <i>lac</i> promoter; Gm ^r	This study
pNAB1	pBluescript II KS+ with a 0.86-kb <i>Pst</i> I- <i>Bam</i> HI fragment encompassing the <i>pltR-pltLA</i> intergenic region	This study
pUK21	Cloning vector; <i>lacZ</i> α ; Km ^r	Vieira and Messing 1991
pUX-BF13	Helper plasmid for Tn7-based transposon mutagenesis containing the transposition functions; R6K-replicon; Ap ^r	Bao et al. 1991

^z Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

dence that RpoN may function as an activator of *pltR* transcription (M. Bottiglieri and C. Keel, *unpublished data*). Analogously, in *P. syringae*, putative σ^{54} recognition motifs were localized upstream of the start codon of *corR* encoding a transcriptional activator of coronatine gene expression, but were not found in the promoter regions of the coronatine biosynthetic genes (Alarcón-Chaidez et al. 2003; Hendrickson et al. 2000b). Surprisingly, a similar plausible σ^{54} recognition motif appears to lack in the leader region of *phlF* (M. Bottiglieri and C. Keel, *unpublished findings*), which encodes a pathway-specific transcriptional repressor of DAPG gene expression in *P. fluorescens* (Bangera and Thomashow 1999; Delany et al. 2000; Schnider-Keel et al. 2000). PLT is known to strongly repress DAPG gene expression in *P. fluorescens* CHA0 (Schnider-Keel et al. 2000); therefore, it is tempting to speculate that the negative effect of RpoN on *phl* gene expression may take place indirectly through enhanced production of PLT following σ^{54} -dependent activation of *pltR* expression.

However, other regulatory elements might interfere with RpoN-mediated control of antibiotic production as well. A po-

tential candidate is the alternative σ factor RpoS, which is known to exert an effect on PLT expression that is opposite to that of RpoN. In *rpoS* mutants of *P. fluorescens* strains CHA0 and Pf-5, expression of *plt* biosynthetic genes and PLT production is enhanced severalfold (Haas and Keel 2003; Sarniguet et al. 1995), pointing to the possibility that cellular levels of RpoN and RpoS could influence the relative amounts of this antifungal compound produced by the bacterium. Future work also may include investigations of the potential interactions of RpoN with components of the GacS/GacA signal transduction cascade (Haas and Keel 2003; Valverde et al. 2003; Zuber et al. 2003).

The *rpoN* mutant of *P. fluorescens* CHA0 had a drastically reduced capacity to protect cucumber plants from *Pythium* root rot and damping-off disease in natural soil (Table 4), thus establishing RpoN, in addition to RpoD and RpoS, as a further σ factor involved in the regulation of biocontrol activity in plant-beneficial pseudomonads. Previous studies have shown that RpoD overexpression or loss of RpoS function enhances the disease suppressive effect of *P. fluorescens* against *Pythium* spp. on cucumber (Maurhofer et al. 1992; Sarniguet et al. 1995;

Fig. 4. RpoN control of the biosynthesis of antifungal compounds in *Pseudomonas fluorescens* strain CHA0. Expression of the plasmid-borne transcriptional reporter fusions **A**, *phlA-lacZ* (pME6710) and **B**, *pltA'-lacZ* (pME8007) and production of **C**, 2,4-diacetylphloroglucinol and **D**, pyoluteorin in growing cultures of wild-type CHA0 (black circle), the *rpoN* in-frame-deletion mutant CHA250 (open circle), and the *rpoN* mutant complemented with monocopy *rpoN*⁺ CHA251 (gray-shaded circle). Specific β -galactosidase activities and antibiotic production were determined for bacteria cultivated in King's medium B broth at 30°C. Growth (optical density [OD] at 600 nm) of strains CHA0, CHA250, and CHA251 are indicated by black, open, and gray-shaded triangles, respectively. Means $\pm$ standard deviation from three replicate cultures are shown. The experiment was repeated, with similar results. Some of the error bars were too small to be included.

Schnider et al. 1995), suggesting that a balance between the three σ factors may be important for calibrating levels of biocontrol activity. Due to the pleiotropic effects of the *rpoN* mutation in *Pseudomonas fluorescens* CHA0, it is difficult to assign the defect in biocontrol efficacy to a certain phenotype of the *rpoN* mutant. Reduced production of the antifungal compound PLT (Fig. 4D), which has been associated with the suppression of Pythium diseases by *P. fluorescens* (Howell and Stipanovic 1980; Kraus and Loper 1995; Maurhofer et al. 1994), may have accounted, at least partly, for this effect. However, there is the conflicting finding that the *rpoN* mutant excretes enhanced levels of DAPG (Fig. 4C) and there is some evidence that DAPG also may contribute to *Pythium* spp. suppression (Maurhofer et al. 1992, 1995; Schnider et al. 1995; M. Péchy-Tarr and C. Keel, unpublished data). However, the actual kinetics of DAPG and PLT expression on plant roots might be significantly different from those observed in vitro. Moreover, because the *rpoN* mutant shows several defects in nitrogen and carbon source metabolism, it also may be impaired in the utilization of certain root exudates that may be required for effective production of the two antifungal compounds. In pathogenic pseudomonads, deletion of *rpoN* results in significantly reduced virulence in some animal and plant models, but decreased pathogenicity also could be only partly explained by lowered expression of virulence factors (i.e., certain toxins) by the *rpoN* mutants (Alarcón-Chaidez et al. 2003; Hendrickson et al. 2000a,b, 2001). In some cases, decreased virulence of these mutants was associated with defects in their host colonization ability. For instance, *rpoN* mutants of *Pseudomonas aeruginosa* and *P. syringae* showed strongly reduced proliferation in planta (Alarcón-Chaidez et al. 2003; Hendrickson et al. 2000b, 2001) and a *P. aeruginosa rpoN* mutant was impaired in its capacity to form biofilms on the fungal pathogen *Candida albicans* (Hogan and Kolter 2002). In contrast, root colonization levels of the *P. fluorescens rpoN* mutant used in the present study were not significantly different from those of the wild type at different plant growth stages (Table 4; data not shown). Nevertheless, it still is possible that the *rpoN* mutation could have resulted in altered root colonization patterns at a microniche level, eventually leading to reduced biocontrol efficacy.

At present, it is difficult to explain our finding that overexpression of RpoN has no major effects on the expression of the RpoN-dependent phenotypes described for *P. fluorescens* CHA0 in this study. One possibility is that higher intracellular

levels of σ^{54} also require higher levels of the different enhancer-binding proteins that are necessary for activation of the σ^{54} -RNAP complex and, thus, for efficient expression of RpoN-dependent functions (Buck et al. 2000). Alternatively, it is possible that wild-type levels of σ^{54} in the cells are already sufficient for effective expression of the RpoN-dependent phenotypes. In *P. putida* and *Escherichia coli*, intracellular levels of σ^{54} remain constant at relatively low levels (compared with the housekeeping σ factor σ^{70}) throughout the different growth stages (Jishage et al. 1996; Jurado et al. 2003). This is in agreement with our recent finding that, in *P. fluorescens* wild-type CHA0, an *rpoN-lacZ* reporter fusion was expressed at a very low and constant level throughout bacterial growth (S. Mathys and C. Keel, unpublished data). Remarkably, *rpoN* expression was enhanced approximately fivefold in the *rpoN* mutant CHA250, pointing to a mechanism of negative autoregulation of *rpoN* expression. By contrast, in CHA0 derivatives overexpressing RpoN from the vectors pME8013 and pME8014, *rpoN* expression was reduced to levels below that of the wild type (S. Mathys and C. Keel, unpublished findings), thus providing a possible explanation for the lack of a significant effect of RpoN overexpression in *P. fluorescens*. Negative autoregulation of *rpoN* expression has been reported previously in *P. putida* (Köhler et al. 1994) and several other bacterial species (Buck et al. 2000).

In conclusion, our results confirm the important role of RpoN in bacterial motility and nitrogen and carbon metabolism and establish this σ factor for the first time as a major regulator of antibiotic production and biocontrol activity in *P. fluorescens*. However, due to the complex consequences of the *rpoN* mutation, the exact mechanism of loss of biocontrol activity awaits further elucidation.

MATERIALS AND METHODS

Microorganisms, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are described in Table 3. *P. fluorescens* strains were cultivated on nutrient agar (NA) (Stanisich and Holloway 1972), on KMB agar (King et al. 1954), in nutrient yeast broth (NYB) (Stanisich and Holloway 1972), in KMB broth, and in LB broth (Sambrook and Russell 2001) at 30°C with aeration. *E. coli* strains were grown on NA and in NYB at 37°C. Antimicrobial compounds, when required, were added to the growth

Table 4. Contribution of RpoN to the suppression of Pythium damping-off and root rot of cucumber by *Pseudomonas fluorescens* CHA0 in natural soil^x

Bacterial strain added ^y	Pythium added	Surviving plants per flask (%)	Shoot fresh weight per flask (g)	Root fresh weight per flask (g)	Colonization by <i>P. fluorescens</i> (log ₁₀ CFU/g of roots) ^z
None	–	100 a	2.09 a	0.46 a	ND
CHA0 (wild type)	–	99 a	2.12 a	0.45 a	6.67 ± 0.37
CHA250 ($\Delta rpoN$)	–	100 a	1.92 a	0.40 b	6.55 ± 0.15
CHA251 ($\Delta rpoN$ attTn7::rpoN ⁺)	–	98 ab	1.96 a	0.42 ab	7.19 ± 0.24
CHA0/pME8013 (<i>P</i> _{rpoN} -rpoN ⁺)	–	97 ab	1.92 a	0.41 ab	6.62 ± 0.10
CHA0/pME8014 (<i>P</i> _{lac} -rpoN ⁺)	–	100 a	2.16 a	0.49 a	6.53 ± 0.02
None	+	63 c	0.52 d	0.13 d	ND
CHA0	+	92 ab	1.54 b	0.33 c	7.56 ± 0.43
CHA250	+	73 c	0.75 c	0.18 d	7.20 ± 0.73
CHA251	+	90 b	1.43 b	0.31 c	7.30 ± 0.15
CHA0/pME8013	+	95 ab	1.61 b	0.38 bc	7.44 ± 0.16
CHA0/pME8014	+	93 ab	1.65 b	0.33 c	7.57 ± 0.48

^x Data represent the means from six individual repetitions of the same experimental setup, with 5 to 10 replicates (flasks containing three cucumber plants) per treatment in each experiment. Means within the same column followed by different letters differ significantly at *P* = 0.05, according to Fisher's least significant difference test; ND = not detected.

^y *P. fluorescens* strains were added at 10⁷ CFU/g of natural soil contained within 200-ml flasks (60 g of soil per flask), after planting three 72-h-old, sterile-grown cucumber seedlings per flask. *Pythium ultimum* was added as a millet-seed inoculum at 2.5 g/kg of soil before planting. Plants were harvested after 7 days.

^z A derivative of the rhizosphere-stable plasmid pME6010 (Heeb et al. 2000) was introduced into *Pseudomonas fluorescens* strains to determine their root colonization capacity.

media at the following concentrations: ampicillin sodium salt, 100 µg/ml; chloramphenicol, 25 µg/ml; gentamicin sulphate, 10 µg/ml; kanamycin sulphate, 25 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 was incorporated into solid media to monitor β-galactosidase expression (Sambrook and Russell 2001). *P. ultimum* strain 67-1 was cultured on malt agar and, for use in plant disease suppression assays, on autoclaved millet seed as described previously (Maurhofer et al. 1992).

DNA manipulations and sequencing.

Chromosomal DNA of *P. fluorescens* was prepared as described elsewhere (Schnider-Keel et al. 2000). Isolation of pME3087-based plasmids from *E. coli* was done by alkaline lysis (Sambrook and Russell 2001). All other small- and large-scale plasmid preparations were performed with the cetyltrimethylammonium bromide method (Sambrook and Russell 2001) and the Jetstar 2.0 kit (Genomed, Basel, Switzerland), respectively. Standard techniques were used for restriction, agarose gel electrophoresis, generation of blunt ends with T4 DNA polymerase (Roche Diagnostics, Rotkreuz, Switzerland), isolation of DNA fragments from low-melting-point agarose gels, and ligation (Sambrook and Russell 2001; Schnider-Keel et al. 2000, 2001). Restriction fragments were purified from agarose gels using the Qiagen Gel extraction kit (Qiagen, Hombrechtikon, Switzerland). Bacterial cells were transformed with plasmid DNA by electroporation (Schnider-Keel et al. 2000). Southern blotting with Hybond N membranes (Amersham Biosciences, Otelfingen, Switzerland), random-primed DNA labeling with digoxigenin-11-dUTP, hybridization, and detection (Roche Diagnostics) were performed according to the protocols of the suppliers.

Polymerase chain reactions (PCRs) typically were carried out with 1 to 2 U of thermostable DNA polymerase (Expand High Fidelity PCR System, Roche Diagnostics) in a reaction mixture containing 100 to 200 ng of target DNA, 200 µM each of the four dNTPs (Roche Diagnostics), 0.5 µM each primer, 1 to 3 mM MgCl₂, and 1× PCR buffer (Roche Diagnostics) in a final volume of 20 µl. For the amplification reaction, an initial denaturation step of 5 min at 95°C was followed by 25 to 30 thermal cycles (1 min at 95°C, 30 s at 55 to 60°C, and 1 to 2 min at 72°C) and a final elongation step of 5 min at 72°C.

Nucleotide sequences of PCR-derived constructs were determined on both strands with the Big Dye Terminator Cycle sequencing kit 1.1 (Perkin Elmer, Fremont, CA, U.S.A.) and an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.), and, in part, by Microsynth (Balgach, Switzerland). Nucleotide and deduced amino acid sequences were analyzed with programs of the European Molecular Biology Open Software Suite.

Cloning of the *rpoN* gene from *P. fluorescens*.

To amplify the *rpoN* gene and its surroundings from *P. fluorescens* CHA0, the degenerate primers CK-P1 (5'-CGGGATCCCGGAAGTBTAYCTGGGBCACGARTTCCG-3') and CK-P2 (5'-GCTCTAGAGCTTCACVAGGGAACGGCCGGGGT CAGG-3') containing artificial restriction sites (underlined) for *Bam*HI and *Xba*I, respectively, were used. Primers were designed based on the alignment of genomic sequences of strains *P. fluorescens* Pf0-1, *P. syringae* pv. *glycinea* PG4180 (accession number AF283815), *P. syringae* pv. *maculicola* ES4326 (accession number AF199600), *P. putida* KT2440 (accession number X16474), *P. putida* TN2100 (accession number M24916), *P. aeruginosa* PAO1 (accession number AE004860), *P. stutzeri* MK21 (accession number AJ223088), and *A. vinelandii* OP (accession number X05888). The PCR amplification yielded a 2.2-

kb band with an annealing temperature of 59°C. The PCR product was digested with *Bam*HI and *Xba*I and cloned into vector pME6000 to give pME7069 (Fig. 1; Table 3). The inserts of three of the clones obtained were sequenced to confirm the identity of the cloned fragment.

Construction of an *rpoN* in-frame-deletion mutant of *P. fluorescens*.

For the construction of the *rpoN* mutant CHA250, a 1,212-bp fragment, including the XLINK motif, the helix-turn-helix motif, the RpoN box, and associated motifs (Buck et al. 2000; Merrick 1993) was deleted in-frame in the *rpoN* gene (Fig. 1) as follows. A 487-bp *Bam*HI-*Xho*I fragment, including the first 88 codons of *rpoN*, was isolated from pME7069. A 510-bp fragment from pME7069, including the last six codons of *rpoN*, ORF1, and the start of *ptsN* (Fig. 1), then was amplified by PCR with primers CK-P3 (5'-GCGCCTTCCCTCGAGCGTAAGCGG-3'), which created an artificial *Xho*I site (underlined), and CK-P2 (discussed above). The PCR product was trimmed to 380 bp with *Xho*I-*Hind*III (Fig. 1). The 487-bp *Bam*HI-*Xho*I and 380-bp *Xho*I-*Hind*III fragments were cloned by a triple ligation into the suicide plasmid pME3087 digested with *Bam*HI-*Hind*III, giving plasmid pME7066 (Fig. 1). In parallel, the two fragments also were cloned into pUK21 and the insert was checked by sequencing. Plasmid pME6077 then was integrated into the chromosome of strain CHA0 by triparental mating using *E. coli* HB101/pME497 as the mobilizing strain (Schnider-Keel et al. 2000, 2001), with selection for tetracycline- and chloramphenicol-resistant recombinants. Excision of the vector by a second crossing-over occurred after enrichment for tetracycline-sensitive cells (Schnider-Keel et al. 2000, 2001). The *rpoN* mutation was verified by PCR and Southern blotting (data not shown).

Complementation of the *rpoN* mutation by monocopy *rpoN*⁺.

To complement strain CHA250 (Δ *rpoN*), the *rpoN* gene was introduced as a single copy into the chromosome using a mini-Tn7 delivery system described previously (Bao et al. 1991; Schnider-Keel et al. 2000; Zuber et al. 2003). For this purpose, the 1,952-bp *Bam*HI-*Nco*I fragment of pME7069 (Fig. 1) was cloned into the mini-Tn7-Gm carrier plasmid pME6182, a derivative of pME3280a (Schnider-Keel et al. 2000; Zuber et al. 2003) in which the multiple cloning site is flanked on both sides by transcriptional termination signals. The construct obtained, pME7070 (Fig. 1), and the Tn7 transposition helper plasmid pUX-BF13 (Bao et al. 1991) were co-electroporated into the recipient strain CHA250. The single Tn7-*rpoN*⁺ insertion in strain CHA251 was checked by PCR using primers PTn7Gm (5'-GGCGGTAGTTGGGTCGATAT-3') and PTn7glmS (5'-AATCATGGCCAAGTCGGTCACC-3') and by Southern blotting (data not shown).

Overexpression of *rpoN* in *P. fluorescens*.

For *rpoN* overexpression, the CHA0 *rpoN* gene was cloned into the multicopy vector pME6551 (18 copies per chromosome equivalent in *P. fluorescens*) (Schnider-Keel et al. 2001) and expressed from its natural promoter or the constitutive *lac* promoter. Plasmid pME8013 (Table 3) was constructed by cloning a 2.2-kb *Hind*III-*Eco*RI fragment of pME7070 (Fig. 1) containing *rpoN* under the control of its own promoter into pME6551. For the construction of pME8014 (Fig. 1), a 1,515-bp fragment derived from pME7070 containing the entire *rpoN* gene and a 21-bp region upstream of its start codon was PCR amplified with primers SM-P1 (5'-CCGGAATTCATAAGGTGTTAAGCCCCTGCC-3') and SM-P2 (5'-CGCGGATCCCTACATCAACCGCTTACGTTTC-3') containing artificial restriction sites (underlined) for *Eco*RI and *Bam*HI, respect-

tively. The PCR product was digested with *Eco*RI and *Bam*HI, ligated in pME6001 cut with the same enzymes, and verified by sequencing. A 1.6-kb *Kpn*I-*Bam*HI fragment from the resulting plasmid then was placed under the control of the *lac* promoter in pME6551 to give pME8014 (Fig. 1).

Construction of transcriptional and translational *lacZ* fusions to the *pltA* gene.

A *pltA*'-'*lacZ* translational fusion was constructed as follows. Sequence information available from the very closely related strain *P. fluorescens* Pf-5 (GenBank accession number AF081920) was used to PCR amplify the *pltR*'-'*pltL*-'*pltA*' chromosomal region of CHA0 encompassing the promoter region of the pyoluteorin biosynthetic genes, which is located in the intergenic region between *pltR* and *pltL* (Nowak-Thompson et al. 1999). Primers CGB-P1 (5'-AACTGCAGAGGTGGGATGCCAAGTAGTC-3') and CGB-P2 (5'-CGGGATCCCATGCTCGCGCTCGAACAGTT-3'), containing artificial restriction sites (underlined) for *Pst*I and *Bam*HI, respectively, served to amplify an 864-bp upstream region and the first 43 codons of the *pltA* gene. The PCR product was cloned into pBluescript II KS+ giving pNAB1 and the insert was sequenced on both strands. The nucleotide sequence (accession number AY459536) showed 99.8% identity to the corresponding sequence of *P. fluorescens* Pf-5. A 994-bp *Eco*RI-*Bam*HI fragment from pNAB1 then was fused in-frame with the '*lacZ*' gene in vector pME6015 (Table 3), to give the *pltA*'-'*lacZ* reporter pME6751 (Table 3). The fusion was checked by sequencing.

For the construction of the *pltA*-*lacZ* transcriptional fusion, a fragment encompassing a 798-bp upstream region and the first nine codons of the *pltA* gene was amplified by PCR with primers MB-P14 (5'-GGAATTCGCTCTATGTCATTGACAACGCCCTAGCGCCTTCATTC-3'; artificial *Eco*RI site underlined) and MB-P15 (5'-CGGGATCCTTACACTACATCATCATAATCATGATCGCTCATTGCC-3'; artificial *Bam*HI site underlined). The PCR product was cloned into the vector pUK21 and the construct was sequenced on both strands. The 825-bp *Eco*RI-*Bam*HI fragment from the pUK21 derivative carrying *pltR*'-'*pltL*-'*pltA*' then was fused to *lacZ* in plasmid pME6016 (Schnider-Keel et al. 2000), producing the *pltA*-*lacZ* reporter pME8007 (Table 3).

Assessment of growth characteristics of the *P. fluorescens* *rpoN* mutant.

Growth rates for *P. fluorescens* CHA0 and its mutant derivatives were assessed by growing the strains at 30°C in different rich media, including NYB, LB broth, KMB broth, and GCM (Schnider-Keel et al. 2000), as well as in OSG (Schnider-Keel et al. 2000). Nitrogen source utilization tests were performed in tubes containing 2 ml of M9 salts minimal medium (Sambrook and Russell 2001) with 0.4% glucose (wt/vol) by replacing or complementing NH_4Cl with an alternative nitrogen source at 10 mM when required. After inoculation of the media with aliquots of 20 μl of a suspension of washed bacterial cells adjusted to an optical density (OD) of 1.0, cultures were incubated for 24 h at 30°C with shaking at 180 rpm and growth was determined by recording OD at 600 nm values. In addition, carbon source utilization profiles of wild-type CHA0 and its *rpoN* mutant were compared by growing the strains in Biolog GN2 and Biolog GP2 microplates (Biolog Inc., Hayward, CA) containing 128 different carbon sources, including sugars, amino acids, and organic acids (Wang et al. 2001). Bacterial cell suspensions were prepared from LB overnight cultures grown at 30°C. Cells were washed twice in sterile distilled water and cell density was adjusted to an OD at 600 nm of 0.25. The Biolog GN2 and GP2 plates were inoculated with aliquots of 150 μl of the bacterial suspension per well and were incubated at 30°C for 24 h with

shaking (500 rpm) in a Thermostar incubator (BMG Labtechnologies, Offenburg, Germany). Bacterial growth was determined by measuring OD at 595 nm with a Fluostar Galaxy microplate reader (BMG Labtechnologies) and wells were scored positive for growth when OD readings exceeded those of control wells that did not contain a carbon source by at least 0.35. The experiment was repeated twice.

Motility assays.

Swimming, swarming, and twitching motility of *P. fluorescens* CHA0 and its derivatives was tested following the procedures described by Rashid and Kornberg (2000). Flagellar swimming was tested on plates containing 0.3% agar (Serva, Heidelberg, Germany), yeast extract at 10 g/liter (Difco, Detroit), tryptone at 10 g/liter (Difco), and NaCl at 12.5 g/liter. Swarming motility was examined on plates containing 0.5% agar, nutrient broth at 8 g/liter (Difco), and glucose at 5 g/liter. Twitching motility was assayed on LB broth solidified with 1% agar. Cells from bacterial overnight cultures on NA were inoculated into the center of the swim and swarm plates using a sterile toothpick. Plates were incubated for 24 h at room temperature prior to the assessment of the diameter of bacterial displacement. For twitch plates, cells were stab inoculated with a toothpick to the bottom of the petri dish. After incubation for 4 to 5 days at room temperature, the agar was carefully removed and the bacterial cells attached to the bottom of the petri dish were stained with crystal violet (1%, wt/vol). In all assays, motility was scored as the diameter of bacterial displacement from the point of inoculation.

Transmission electron microscopy.

Cells from the edge of a colony grown on swarm plates were deposited with a toothpick on a drop of water. Carbon-film-coated transmission electron microscopy grids were placed on top of the drop for 15 to 20 s to allow for adhesion of bacterial cells. Grids then were stained with 2% (wt/vol) potassium phosphotungstic acid (pH 3.0) for 20 to 30 s and washed for 20 s in a drop of water. The grids were allowed to air dry and then were examined with a Philips CM12 transmission electron microscope (Heindoven, The Netherlands) at 60 to 80 keV. Electron micrographs were taken at a magnification of $\times 10,000$. At least 15 fields of view were analyzed for each sample.

Assays for sensitivity

of *P. fluorescens* to osmotic and oxidative stress.

P. fluorescens CHA0 and its mutant derivatives were grown in 20 ml of KMB broth without selective antibiotics in 100-ml Erlenmeyer flasks plugged with cotton stoppers. To induce conditions of osmotic stress which still allow growth of *P. fluorescens*, KMB was supplemented with 0.6 M NaCl, 0.8 M NaCl, or 1.2 M sorbitol, a nonionic solute which cannot be metabolized by strain CHA0. For inoculation, aliquots of exponential-growth-phase LB cultures of the bacterial strains were used to adjust the cell density to an OD at 600 nm of 0.05. Cultures were incubated with rotational shaking (180 rpm) at 30°C. Doubling times of strain CHA0 and its derivatives were calculated from OD at 600 nm values between 0.05 and 1.5 (i.e. during exponential growth) (Schnider-Keel et al. 2001).

Susceptibility to oxidative stress was tested by exposing bacterial cells to paraquat, hydrogen peroxide (H_2O_2), or sodium hypochlorite (NaOCl) as described previously (Schnider-Keel et al. 2001). Sterile filter disks (6-mm diameter; Millipore, Volketswil, Switzerland) were soaked with 10 μl of paraquat (1.9 or 3.8%; wt/vol), H_2O_2 (3 or 12%; vol/vol), or NaOCl (5 or 10%; vol/vol) and placed on a layer of soft agar (2 ml of NYB with 0.8% agar) containing 100 μl of an LB overnight culture covering NA. The diameter of the inhibition zone sur-

rounding the impregnated disks was measured after overnight incubation at 30°C.

To assess survival in response to osmotic or oxidative stress, stationary-phase cells were washed and suspended in 10 ml of distilled H₂O to obtain an OD at 600 nm of 1.0. Suspended cells were exposed to 1.2 M NaCl, 2.4 M sorbitol, or 30 mM H₂O₂ (i.e., conditions which no longer allowed growth of *P. fluorescens*) and incubated at 30°C with shaking. Cell culturability was determined at different intervals by plating serial dilutions of the cultures on NA.

Assay for expression of DAPG and PLT biosynthetic genes.

P. fluorescens CHA0 and its mutant derivatives carrying a transcriptional *phlA-lacZ* fusion on plasmid pME6710, a translational *phlA'-lacZ* fusion on pME6259, a transcriptional *pltA-lacZ* fusion on pME8007, or a translational *pltA'-lacZ* fusion on pME6715 (Table 3) were grown in 20 ml of KMB broth without selective antibiotics using 100-ml Erlenmeyer flasks sealed with cellulose stoppers. For inoculation, 200 µl of a suspension of washed cells (OD at 600 nm adjusted to 2) from overnight KMB broth cultures of the bacterial strains were used. Cultures were incubated at 30°C with rotational shaking at 180 rpm, and β -galactosidase-specific activities of at least three independent cultures were determined by the method of Miller (Sambrook and Russell 2001). The experiment was done twice.

Quantification of DAPG and PLT production.

Production of DAPG and PLT was assessed for bacteria grown in 500-ml Erlenmeyer flasks with 100 ml of KMB broth. For inoculation, 1-ml aliquots of overnight cultures of the bacterial strains grown in KMB broth at 30°C and adjusted to an OD at 600 nm of 2.0 were used. Cultures were incubated at 30°C with rotational shaking at 180 rpm. DAPG and PLT were extracted with ethyl acetate from acidified culture supernatants and quantified by established high-performance liquid chromatography procedures as described before (Keel et al. 1992; Maurhofer et al. 1992, 1994). The experiment was repeated twice.

Plant disease suppression and root colonization assays.

For the plant assays, aliquots of 60 g of natural sandy loam soil from Eschikon, Switzerland (Keel et al. 2002) were distributed into 200-ml Erlenmeyer flasks with wide openings. When appropriate, the soil was artificially infested with the pathogenic oomycete *P. ultimum* 67-1 by adding 2.5 g of a 5-day-old millet-seed inoculum (Maurhofer et al. 1992; Keel et al. 2002) of the pathogen per kilogram of soil. Three sterile-grown, 72-h-old cucumber seedlings (*Cucumis sativus* cv. Chinese Snake) then were placed in each flask as previously described (Keel et al. 2002). *P. fluorescens* strains were added to soil as a suspension (5 ml per flask) of cells washed twice in sterile distilled water to give 1×10^7 CFU/g of soil. Control flasks received the same amount of sterile water. Seedlings were covered with 10 g of nontreated soil and flasks were sealed with cotton stoppers. The microcosms were incubated in a randomized block design in a growth chamber containing 80% relative humidity and 22°C with light (200 µmol sec⁻¹ m⁻²; ratio of 1.37 of light at 660 to 730 nm) for 16 h, followed by an 8-h dark period at 15°C. No watering was necessary. At 6 days after inoculation, the percentage of surviving plants was determined. Plants were removed from the flasks, washed, briefly dried with paper towels, and weighed. Roots from each flask were pooled and transferred into 50-ml plastic tubes containing 20 ml of sterile distilled water. Tubes were vigorously shaken at 240 rpm for 30 min and the resulting suspensions were used to determine CFU numbers. In a subset of experiments, root colonization also was determined at 2 and 3 days after bacterial inoculation. Data represent the means from six

individual repetitions of the same experiment with 5 to 10 replicates per treatment in each experiment (1 replicate corresponds to one flask containing three cucumber plants). Data were analyzed for significance with variance analysis, followed by Fisher's least significant difference test. Data for CFU counts were log₁₀-transformed prior to statistical analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The EMBnet EMBOSS database: www.ch.embnet.org/EMBOSS/index.html
 United States Department of Energy *Pseudomonas fluorescens* genome website: genome.jgi-psf.org/draft_microbes/psefl/psefl.home.html
 The Pseudomonas Genome Project *Pseudomonas aeruginosa* database: www.pseudomonas.com