Cross-Species GacA-Controlled Induction of Antibiosis in *Pseudomonads*[▽]

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Signal extracts prepared from culture supernatants of *Pseudomonas fluorescens* CHA0 and *Pseudomonas aeruginosa* PAO stimulated GacA-dependent expression of small RNAs and hence of antibiotic compounds in both hosts. *Pseudomonas corrugata* LMG2172 and *P. fluorescens* SBW25 also produced signal molecules stimulating GacA-controlled antibiotic synthesis in strain CHA0, illustrating a novel, *N*-acyl-homoserine lactone-independent type of interspecies communication.

In dense populations of many bacterial genera, cells communicate with one another by emitting and sensing chemical signals. These signal molecules regulate gene expression and enable the cells to engage collectively in various developmental processes and in the ability to colonize specific niches, in particular host organisms. As the extracellular signal concentrations increase with increasing cell population densities, it has been proposed that in this way the bacteria measure their population densities, and this phenomenon has been termed quorum sensing. Widely occurring and well-studied quorum-sensing signals include *N*-acyl-homoserine lactones (AHLs) in gram-negative bacteria, modified oligopeptides in gram-positive bacteria, and autoinducer 2 (AI-2) (a furanosyl diester) in both gram-negative and -positive bacteria (3, 10, 16, 40). Furthermore, some signal molecules having a relatively narrow host range have been characterized, for instance, the *Pseudomonas* quinolone signal (PQS) (2-heptyl-3-hydroxy-4-quinolone) in *Pseudomonas* and *Burkholderia* spp., *cis*-11-methyl-2-dodecenoate in *Xanthomonas* spp. and related genera, and 3-hydroxy-palmitate methyl ester in *Ralstonia* spp (5, 9, 41). When a signal molecule is shared by different species living in close association, interspecies communication can result (2, 30, 31).

The root-colonizing, plant-beneficial soil bacterium *Pseudomonas fluorescens* CHA0 produces several extracellular antibiotics and enzymes. Together, these exoproducts are important for the organism's ability to antagonize the development of fungal root pathogens and to suppress root diseases caused by these pathogens (12, 13). During in vitro cultivation, the exoproduct genes are expressed in a cell density-dependent manner, as is typical of a quorum-sensing response (1, 14, 20, 34). Both transcriptional and posttranscriptional regulatory mechanisms contribute to this expression pattern. However, global posttranscriptional control by the Gac/Rsm signal transduction pathway plays a decisive part. *P. fluorescens* CHA0 mutants that are defective in the GacS/GacA two-component system express exoproduct genes at a very low basal level throughout

growth (4, 21, 42). In strain CHA0, the GacS/GacA two-component system positively controls the expression of three small regulatory RNAs termed RsmX, RsmY, and RsmZ. Together, they sequester the translational repressors RsmA and RsmE and thereby relieve posttranscriptional repression of target genes, e.g., *phlA* (involved in the biosynthesis of the antibiotic 2,4-diacetylphloroglucinol) and *aprA* (encoding exoprotease AprA) (14, 18, 29, 39). The Gac/Rsm cascade also positively regulates the synthesis of extracellular signal molecules which act as autoinducers of the cascade (7, 14, 18, 39, 42). The chemical nature of these signals is not known. However, they are not related to AHLs, AI-2, or peptides (14). GacS- and GacA-negative mutants are signal blind (42).

Gac/Rsm signal transduction cascades occur in many different gram-negative bacteria (15). Here we investigated whether signal molecules activating the Gac/Rsm cascade are produced by *Pseudomonas* species other than *P. fluorescens* CHA0 and, if so, whether there is interspecies communication via this regulatory cascade. As a primary assay for GacA-dependent signal activity, we used reporter gene constructs which measure transcriptional activation of the small RNA genes *rsmY* and *rsmZ*; these genes are induced two- to fourfold by saturating signal concentrations in the presence of functional GacA (7, 18).

Cross talk between *P. fluorescens* CHA0 and *P. aeruginosa* PAO. *P. fluorescens* CHA0 and the opportunistic human pathogen *Pseudomonas aeruginosa* PAO1 were grown in glycerol-Casamino Acids medium (34). Culture supernatants from these strains were acidified with HCl and extracted with dichloromethane, yielding crude signal preparations, as previously described (7, 18). The CHA0 signal markedly stimulated the expression of transcriptional *rsmY*_{CHA0}-*lacZ* and *rsmZ*_{CHA0}-*lacZ* fusions in the wild-type CHA0 (Fig. 1A and B), as reported before (14, 39). In this assay, addition of either PQS, *cis*-11-methyl-2-dodecenoate, or 3-hydroxypalmitate methyl ester, each at 10 μ M, had no effect (data not shown). Thus, none of these bacterial signals qualifies as a likely candidate for the CHA0 signal. However, a signal preparation from the wild-type *P. aeruginosa* PAO1 activated both fusions in strain CHA0, although signal activity appeared to be weaker than that of the CHA0 signal (Fig. 1A and B). An extract prepared from the *gacA* mutant PAO6281 provided very little signal activity (Fig. 1A and B), whereas an extract obtained from the

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FIG. 1. Cross talk between *P. aeruginosa* PAO1 and *P. fluorescens* CHA0. The signal activities of *P. aeruginosa* PAO1 (squares), *P. aeruginosa* PAO6281 (*gacA*) (circles), and *P. fluorescens* CHA0 (triangles) were tested in reporter strains grown in 20 ml nutrient yeast broth (36) in 50-ml Erlenmeyer flasks with shaking; the medium was amended with 0.05% (vol/vol) Triton X-100 to avoid cell clumping. An equivalent of 50 ml of extracted culture supernatant dissolved in 100 μ l methanol (7) was added as a standard signal preparation. Control mixtures (diamonds) contained 100 μ l methanol instead of extracted supernatant. Reporter strains were *P. fluorescens* CHA0/pME6916 ($rsmZ_{CHA0}$ - $lacZ$) (A), *P. fluorescens* CHA0/pME6091 ($rsmZ_{CHA0}$ - $lacZ$) (B), *P. aeruginosa* PAO6558 ($rsmY_{PAO}$ - $lacZ$) (C), and *P. aeruginosa* PAO6554 ($rsmZ_{PAO}$ - $lacZ$) (D). The incubation temperatures were 30°C for strain CHA0 and 37°C for strain PAO. β -Galactosidase measurements (24) were carried out in triplicate using cells permeabilized with 5% (vol/vol) toluene. Experiments were done three times. The symbols indicate averages, and the error bars show standard deviations. OD_{600} , optical density at 600 nm.

lasI rhII mutant PAO-JP2, which is devoid of AHLs (26), had wild-type activity (data not shown). In a reciprocal experiment, transcriptional $rsmY_{PAO}$ - $lacZ$ and $rsmZ_{PAO}$ - $lacZ$ fusions were used as chromosomal insertions in *P. aeruginosa* strain PAO1. (Note that the *P. aeruginosa* genome contains two GacA-regulated small RNA genes, *rsmY* and *rsmZ*, but no *rsmX* homolog [17, 19].) Both reporter fusions were induced by signal preparations of strains CHA0 and PAO, although the PAO signal did not fully activate $rsmY_{PAO}$ - $lacZ$. Again, the *gacA* mutant PAO6281 produced a very small amount of signal (Fig. 1C and D). These results show that *P. aeruginosa* PAO produces a GacA-dependent signal and that the PAO and CHA0 signals can cross-activate transcription of the *rsmY* and *rsmZ* genes in the heterologous background.

We confirmed these observations by assaying cellular activities that strongly depend on the functions of GacA and RsmY plus RsmZ. In *P. fluorescens* CHA0 we chose to monitor *phlA* expression by using a translational '*lacZ*' reporter in plasmid pME6702 (29). This construct specifically records posttranscriptional regulation; the *phlA* promoter, which responds to transcriptional effectors such as 2,4-diacetylphloroglucinol and other phenolic compounds (1, 34), was replaced by the constitutively expressed *tac* promoter. The expression of *phlA*'-'*lacZ*' was induced about fourfold by the CHA0 signal preparation and about threefold by the PAO signal extract, whereas the *gacA* mutant PAO6281 was almost devoid of signal activity (Fig. 2). In *P. aeruginosa* PAO, we chose to assay the formation of the antibiotic pyocyanin, whose synthesis is distinctly dependent on the

GacA system (19, 28). Ten milliliters of nutrient yeast broth (36) was inoculated with strain PAO1; after 16 h of incubation at 37°C, signal molecules extracted from 50-ml portions of various cultures were added and incubation was continued for 4 h. Pyocyanin concentrations in the cultures were then determined as previously described (8, 28). A signal

FIG. 2. Stimulation of *phlA* gene expression in *P. fluorescens* CHA0 by signal preparations. Signal activities in extracted supernatants from *P. aeruginosa* PAO1 (squares), *P. aeruginosa* PAO6281 (*gacA*) (circles), and *P. fluorescens* CHA0 (triangles) were tested in the reporter strain *P. fluorescens* CHA0/pME6702 (*phlA*'-'*lacZ*) as described in the legend to Fig. 1. Control mixtures (diamonds) contained 100 μ l methanol instead of extracted supernatant. The incubation temperature was 30°C. β -Galactosidase measurements were carried out in triplicate using cells permeabilized with 5% (vol/vol) toluene. Experiments were done three times. The symbols indicate averages, and the error bars show standard deviations. OD_{600} , optical density at 600 nm.

FIG. 3. Effects of strains *P. fluorescens* SBW25, *P. corrugata* LMG 2172, and *P. alcaligenes* Ps93 on the Gac/Rsm cascade and antibiotic production in strain CHA0. (A to C) Signal activities in extracted supernatants from *P. fluorescens* CHA0 (triangles), *P. fluorescens* SBW25 (empty circles), *P. corrugata* LMG 2172 (filled circles), and *P. alcaligenes* Ps93 (filled diamonds) were tested in CHA0 reporter strains as described in the legend to Fig. 1. Reporter strains were *P. fluorescens* CHA0/pME6916 (*rsmY*_{CHA0}-*lacZ*) (A), *P. fluorescens* CHA0/pME6091 (*rsmZ*_{CHA0}-*lacZ*) (B), and *P. fluorescens* CHA0/pME6702 (*ptac-phlA'*-*lacZ*) (C). Control mixtures (empty diamonds) contained 100 μ l methanol instead of extracted supernatant. β -Galactosidase measurements were carried out in triplicate. Experiments were done three times. The symbols indicate averages, and the error bars show standard deviations. OD₆₀₀, optical density at 600 nm. (D) Antibiotic production by *P. fluorescens* CHA0 in the presence *P. fluorescens* SBW25, *P. corrugata* LMG 2172, or *P. alcaligenes* Ps93. Strains SBW25, LMG 2172 and Ps93 were inoculated by placing 5- μ l drops of overnight cultures onto nutrient agar (36). After incubation at 30°C for 24 h, 5- μ l drops of a CHA0 culture were added and incubation continued for 24 h. An overlay with *Bacillus subtilis* revealed antibiotic production by growth inhibition zones.

preparation from strain CHA0 increased pyocyanin production by $15.9\% \pm 1.4\%$, whereas a signal extract from the AHL-deficient mutant PAO-JP2 enhanced pyocyanin production by $10.6\% \pm 0.7\%$, relative to the control without addition ($2.7 \mu\text{g/ml} = 100\%$). Strain PAO-JP2 was used in order to avoid any effect of AHLs on pyocyanin production. A signal extract of the *gacA* mutant PAO6281 had no significant influence ($\leq 1.5\%$) on pyocyanin production.

Stimulation of antibiotic production in strain CHA0 by antibiotic-negative *Pseudomonas* spp. We wondered whether environmental isolates of *Pseudomonas* spp. might produce signals that activate the Gac/Rsm cascade in strain CHA0. In a collection of some 30 strains, the entire spectrum ranging from high to low or undetectable signal activity was found (6). We selected three strains that did not produce antibiotics under our experimental conditions: the biocontrol strain *P. fluorescens* SBW25 (25); the plant pathogen *Pseudomonas corrugata* LMG2172, which causes tomato pith necrosis (33, 38); and the industrial lipase producer *Pseudomonas alcaligenes* Ps93

(11). Signal preparations from strains SBW25 and LMG2172 stimulated the expression of *rsmY-lacZ*, *rsmZ-lacZ*, and *ptac-phlA'*-*lacZ* fusions in strain CHA0, albeit less effectively than did the homologous CHA0 signal. No activity was detected in a culture supernatant of strain Ps93 (Fig. 3A, B, and C). On nutrient agar, colonies of SBW25 and LMG2172 markedly stimulated antibiotic production in a neighboring colony of strain CHA0, as detected by a *Bacillus subtilis* overlay (Fig. 3D). We presume that the CHA0 antibiotic that is produced predominantly under these conditions is 2,4-diacetylphloroglucinol. The significance of the biphasic antibiotic halos seen in the CHA0-SBW25 and CHA0-LMG2172 interactions is not clear. As expected from the results of Fig. 3A, B, and C, strain Ps93 had no significant effect on antibiotic production in strain CHA0 (Fig. 3D). These experiments demonstrate that both plant-beneficial and -pathogenic strains have the potential to cross-induce antibiotic production in strain CHA0 and that the signal molecules produced by strains SBW25 and LMG2172 are diffusible in agar. Furthermore, the data in Fig. 3C suggest that

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	AHL production ^b	Reference or source
Strains			
<i>Escherichia coli</i> DH5 α	Laboratory strain	–/–	32
<i>Pseudomonas aeruginosa</i>			
PAO1	Wild type	+/+	36
PAO-JP2	$\Delta lasI::Tc^r \Delta rhlI::Tn501; Tc^r Hg^r$	–/–	26
PAO6281	$gacA::\Omega-Sp^r/Sm^r; Sm^r Sp^r$	+/±	28
PAO6554	$rsmZ_{PAO-lacZ}; Gm^r$	+/+	19
PAO6558	$rsmY_{PAO-lacZ}; Gm^r$	+/+	19
<i>Pseudomonas alcaligenes</i> Ps93	Res [–] Mod ⁺	–/–	11
<i>Pseudomonas corrugata</i> LMG 2172 (= CFBP 2431)	Wild type	+/+	33
<i>Pseudomonas fluorescens</i>			
CHA0	Wild type	–/–	37
CHA89	$gacA::Km; Km^r$	–/–	21
SBW25	Wild type	–/–	25
<i>Vibrio harveyi</i> BB120	Wild type	ND ^c	2
<i>Vibrio natriegens</i>	Wild type	ND	A. Kukangara
Plasmids			
pME6091	Transcriptional $rsmZ_{CHA0-lacZ}$ fusion; Tc^r		14
pME6702	Translational $ptac-phlA'-lacZ$ fusion; Tc^r		29
pME6916	Transcriptional $rsmY_{CHA0-lacZ}$ fusion; Tc^r		39

^a Gm^r, gentamicin resistant; Hg^r, mercuric chloride resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant.

^b The first symbol indicates the result of a qualitative assay for long-chain AHLs using *Agrobacterium tumefaciens* NTL4/pZLR4 (35) as an indicator; the second symbol indicates the result of a qualitative assay for short-chain AHLs using *Chromobacterium violaceum* CV026 (23) an indicator. +, presence of AHL; ± reduced AHL concentration; –, absence of AHL.

^c ND, not determined. *N*-3-Hydroxybutanoyl-L-homoserine lactone (AI-1) is known to be produced by *V. harveyi* (2).

this effect occurs at a posttranscriptional level. Qualitative assays for AHLs were positive for strain LMG2172 but negative for strains SBW25 and Ps93 (Table 1); thus, at least in the case of SBW25, signal activity was not correlated with AHL production.

Further investigation to find signal-producing bacteria that do not belong to the *Pseudomonas* spp. revealed that culture extracts from *Vibrio harveyi* BB120 and *Vibrio natriegens* had high signal activities in assays with *P. fluorescens* reporter strains. By contrast, an *Escherichia coli* DH5 α extract had no detectable activity (data not shown).

Concluding remarks. In *P. aeruginosa*, the GacS/GacA system modulates the quorum-sensing response by regulating AHL synthesis (19, 28). In *Vibrio cholerae*, the GacS/GacA (= VarS/VarA) system acts on the expression of the central virulence regulator LuxO, in parallel with two other quorum-sensing pathways involving AI-2 and cholera autoinducer 1, respectively (22). In *P. fluorescens* CHA0, the absence of quorum-sensing mechanisms controlled by AHLs and AI-2 (14, 27) has facilitated the detection of signals that activate the Gac/Rsm cascade. As we have shown here, such signals are not confined to strain CHA0. Their existence in plant-beneficial and -pathogenic pseudomonads suggests considerable scope for cross talk in ecosystems. Moreover, as both *V. harveyi* and *V. natriegens* appear to produce signal molecules that induce the Gac/Rsm pathway in *P. fluorescens*, it is conceivable that

V. cholerae might also use similar signals in the VarS/VarA branch of quorum sensing.

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