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A Moderate Toxin, GraT, Modulates Growth Rate and Stress Tolerance of *Pseudomonas putida*

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Chromosomal toxin-antitoxin (TA) systems are widespread among free-living bacteria and are supposedly involved in stress tolerance. Here, we report the first TA system identified in the soil bacterium *Pseudomonas putida*. The system, encoded by the loci PP1586-PP1585, is conserved in pseudomonads and belongs to the HigBA family. The new TA pair was named GraTA for the growth rate-affecting ability of GraT and the antidote activity of GraA. The GraTA system shares many features common to previously described type II TA systems. The overexpression of GraT is toxic to the antitoxin deletion mutants, since the toxin's neutralization is achieved by binding of the antitoxin. Also, the *graTA* operon structure and autoregulation by antitoxin resemble those of other TA loci. However, we were able to delete the antitoxin gene from the chromosome, which shows the unusually mild toxicity of innate GraT compared to previously described toxins. Furthermore, GraT is a temperature-dependent toxin, as its growth-regulating effect becomes more evident at lower temperatures. Besides affecting the growth rate, GraT also increases membrane permeability, resulting in higher sensitivity to some chemicals, e.g., NaCl and paraquat. Nevertheless, the active toxin helps the bacteria survive under different stressful conditions and increases their tolerance to several antibiotics, including streptomycin, kanamycin, and ciprofloxacin. Therefore, our data suggest that GraT may represent a new class of mild chromosomal regulatory toxins that have evolved to be less harmful to their host bacterium. Their moderate toxicity might allow finer growth and metabolism regulation than is possible with strong growth-arresting or bactericidal toxins.

Toxin-antitoxin (TA) systems are widespread among bacteria. These gene cassettes consist of two small genes which code for a toxin that can influence bacterial life and an antitoxin that inhibits toxin action. Typically they have been divided into three types depending mainly on the nature and mode of action of the antitoxin. Type I and III antitoxins are RNAs that prevent toxin translation and inhibit the toxin protein by binding it, respectively (1, 2). In type II systems, which are the most abundant in prokaryotes (3), both the antitoxin and the toxin are small proteins, and toxin inactivation is achieved by complex formation between the two proteins (4). Recently, TA systems of new types have been reported, where the toxin neutralization mechanism involves stabilization of the toxin target (type IV) (5) or direct cleavage of the toxin mRNA by the antitoxin protein (type V) (6).

Toxins target various cellular processes, but the overwhelming majority of type II toxins impede translation (7). Inhibition of protein synthesis is achieved by different mechanisms, which include binding and inhibiting ribosomal subunits (8, 9) or elongation factor EF-Tu (10) and cleavage of rRNA, tRNA, and, most commonly, mRNA (11–13). Some toxins, such as RelE and HigB, associate with ribosomes and degrade only translated mRNAs (13, 14), while others, like MazF, cleave mRNA independently of ribosomes (12).

TA systems are coded on either plasmids or chromosomes. They were first identified on plasmids, where they contribute to plasmid maintenance in the bacterial population (15, 16). The functions of the chromosomal systems remain controversial—some seem to be just remnants of mobile elements (17), whereas others are involved in the regulation of crucial cellular functions (18). Still, accumulating evidence indicates that many TA systems are associated with a complex of functions that are important for survival under stressful conditions, including biofilm formation, bacterial persistence, growth regulation, protection against bacteriophages, and even programmed cell death (19–22). Activation of many TA systems in response to stresses, such as exposure to antibiotics or nutrient starvation (23–25), also indicates their role in stress survival. The hypothesis about TA systems as stress response elements is also supported by the finding that chromosomal TA systems are more abundant in free-living than in obligately intracellular bacteria (4, 26).

Members of the genus *Pseudomonas* are known for their versatility and adaptability, allowing their success in hostile and fluctuating habitats (27). *Pseudomonas putida*, for example, is common in polluted soil and aquatic environments and is thus extensively studied with respect to stress tolerance mechanisms (28). The high adaptability of *P. putida* relies on many features, including high genetic plasticity and broad metabolic, transport, signaling, and regulatory capabilities (29). Bioinformatic analyses suggest that the *P. putida* genome also codes for many TA systems. However, the number of predicted TA loci in the *P. putida* KT2440 chromosome differs between studies, varying from eight (26) to 12 (30), and only six of the TA systems are common to two predictions. To our knowledge, no information is available about the functionality of these putative TA systems. Still, we recently identified a transposon insertion in a putative antitoxin gene PP1585 of a predicted PP1586-PP1585 TA pair when we screened the suppressor mutants of *colR*-deficient *P. putida* (31).

ColR is a transcriptional regulator of the conserved ColRS two-

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component signal system, and it is conditionally essential for *P. putida*, as its deficiency results in a subpopulation lysis when bacteria grow on minimal glucose solid medium (32). Besides being important for cell survival on glucose medium, the ColRS system also contributes to the phenol and heavy metal tolerance of *P. putida* (33–35), the root colonization ability of *Pseudomonas fluorescens* (36, 37), and virulence of the human pathogen *Pseudomonas aeruginosa* and the plant pathogen *Xanthomonas* (38–40). Several lines of evidence suggest that all these phenotypes may be caused by impaired membrane homeostasis (31, 33, 37, 40) of *colRS*-deficient bacteria.

Recent selection of suppressors of lysis of the *colR*-deficient *P. putida* revealed among others an interruption of a predicted antitoxin gene PP1585 (31). Suppression of the lysis defect of a *colR* mutant by inactivation of an antitoxin gene seemed intriguing in several respects. The ability to disrupt the antitoxin gene is in itself uncommon among TA systems, as it results in the activity of the cognate toxin and normally leads to growth inability (25, 41). However, in the case of PP1586-PP1585, it was not only possible to disrupt the antitoxin gene of a putative TA system without significant growth impairment, but the antitoxin inactivation was even beneficial for the *colR* mutant, as it suppressed the lysis phenotype (31). Therefore, in this study, we addressed the question of whether the identified locus actually represents a bona fide TA system. We demonstrate that the PP1586-PP1585 locus of *P. putida* indeed codes for a functional TA system, which was named GraTA (growth rate-affecting TA system). GraT was shown to be an unusually mild toxin able to modulate the growth rate of bacteria. GraA is a very efficient antitoxin which neutralizes the innate GraT as well as overexpressed toxin. Interestingly, the growth-inhibitory effect of GraT increases significantly at lower temperatures, and its overexpression is toxic for *graA* deletion mutants. We present evidence that GraT can rescue the *colR* mutant from lysis and can also increase bacterial resistance to different antibiotics. This suggests that the GraTA system may be involved in stress adaptation of *P. putida*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. All *P. putida* strains are derivatives of PaW85 (42), which is isogenic to the fully sequenced strain KT2440 (43). Bacteria were grown in lysogeny broth (LB) or in M9 minimal medium (44) containing either 0.2% glucose, 0.2% gluconate, 0.2% fructose, or 0.2% sodium benzoate. When selection was necessary, the growth medium was supplemented with ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) for *E. coli* and benzylpenicillin (1,500 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) for *P. putida*. *E. coli* was incubated at 37°C and *P. putida* at 30°C if not specified otherwise. Bacteria were electrotransformed according to the protocol of Sharma and Schimke (45).

Construction of plasmids and strains. Oligonucleotides used in PCR amplifications are listed in Table S1 in the supplemental material. For generation of *graTA* deletion strains, the pEMG-based plasmids were constructed according to the protocol described elsewhere (46). The upstream and downstream regions (450 to 1,500 bp) of the gene(s) to be deleted were amplified separately and then joined into one fragment by overlap extension PCR. The obtained 1.05- to 1.5-kb DNA fragments were cut with EcoRI and BamHI and ligated into the corresponding sites of the plasmid pEMG, resulting in plasmids pEMG-ΔA, pEMG-Δ$A_{T(E80G)}$, pEMG-ΔT, and pEMG-ΔTA. Plasmid pEMG-Δ$A_{T(E80G)}$ with a mutant *graT* gene was obtained due to a PCR error. The pEMG plasmids were delivered to *P. putida* PaW85 by electroporation, and after 3 h of growth in LB medium, the bacteria were plated onto LB agar supplemented with kanamycin. Kanamycin-resistant cointegrates were selected and electrotransformed with the I-Sce-I expression plasmid pSW(I-SceI). In order to resolve the cointegrate, the plasmid-encoded I-SceI was induced with 1.5 mM 3-methylbenzoate overnight. Kanamycin-sensitive colonies were selected, and the deletion of *graA*, *graT*, or *graTA* was verified by PCR. Plasmid pSW(I-SceI) was eliminated from the deletion strains by growing them overnight in LB medium without antibiotics. Intactness of *graT* in the Δ*graA* strain was verified by sequencing. For the construction of double mutant strains, the disrupted *colR* gene was inserted into the chromosome of TA system deletion strains by homologous recombination. The suicide plasmid pGP704L/colR::Km was conjugatively transferred from *E. coli* CC118 λ*pir* into the Δ*graA*, Δ$graA_{T(E80G)}$, Δ*graT*, and Δ*graTA* strains using helper plasmid pRK2013. Kanamycin-resistant transconjugants were selected, and *colR* knockouts were verified by PCR analysis.

In order to construct the broad-host-range expression vector pKTlacItac, the 1.9-kb BamHI-HindIII fragment containing the *lacI tac* cassette from pBRlacItac was inserted into the corresponding sites of pKT240. The resulting plasmid was modified by replacing its HindIII-SacI region with a HindIII-SacI fragment containing a multicloning site of pSEVA-Km(RK2). The plasmid obtained, pKTlacItac, was used to construct plasmids pKTlacItac-TA, pKTlacItac-T, pKTlacItac-T_{E80G}, and pKTlacItac-A. First, the whole *graTA* operon was amplified from the *P. putida* PaW85 chromosome with the primers 1585Acc and 1586Sal. The obtained DNA fragment was cut with Acc65I and SalI and cloned into Acc65I-SalI-opened pKTlacItac. The resulting plasmid, pKTlacItac-TA, was treated with SalI and XhoI and religated to obtain pKTlacItac-A. pKTlacItac-T was constructed by deleting *graA* from pKTlacItac-TA by the use of SacI and SacII. To construct pKTlacItac-T_{E80G}, the mutant *graT* gene was amplified from the chromosome of the Δ$graA_{T(E80G)}$ strain using primers 1586Sal and 1586Bam. The resulting PCR fragment was treated with BamHI, Klenow fragment, and XhoI and inserted into Ecl136II-XhoI-opened pKTlacItac-TA.

To construct the transcriptional fusion of the *graTA* promoter with *lacZ*, the *graT* gene and its upstream region was amplified from the *P. putida* PaW85 chromosome with primers 1585ATG and 1585Sac. The resulting PCR fragment was treated with Eco47III and Ecl136II and inserted into SmaI-opened p9T$T_B$lacZ.

For purification of the antitoxin or the toxin-antitoxin complex, a hexahistidine tag was fused to the C terminus of GraA or to the N terminus of GraT, respectively. The *graA*- and *graTA*-containing fragments were amplified by using the oligonucleotide pair A-XhoNde and A-his and the pair T-Nhis and 1585Bam (see Table S1 in the supplemental material), respectively. For expression of untagged GraA, the *graA* gene was amplified by using oligonucleotides A-XhoNde and 1585Bam. All PCR fragments were treated with NdeI and BamHI and ligated into the corresponding sites of the plasmid pET11c, resulting in pET-Ahis, pET-hisTA and pET-A. All plasmids designed were sequenced in order to exclude PCR-generated errors in the cloned DNA fragments.

Determination of the generation time of bacteria. Generation time was calculated for bacteria growing on microtiter plates. LB-grown overnight cultures were diluted 100-fold into 100 μl LB medium in microtiter plate wells. Microtiter plates were incubated with shaking at 37°C, 30°C, 25°C, or 20°C, and the optical density at 580 nm (OD_{580}) was measured every 30 min to obtain the growth curve of bacteria. Generation time was calculated from the slope of the exponential growth curve according to the equation $G = [t/3.3\log(b/B)]$, where G is the generation time, t is the time interval (in minutes), and B and b are the numbers of bacteria (OD_{580}) at the beginning and the end of the time interval, respectively.

Ectopic overexpression of GraTA proteins in *P. putida*. In order to evaluate the effect of the overexpression of GraT, GraT(E80G), and GraA, the *P. putida* PaW85 and its *graTA* deletion derivatives were electrotransformed with plasmids pKTlacItac-T, pKTlacItac-T_{E80G}, and pKTlacItac-A. After 1 h of growth in 1 ml LB medium, the bacterial cultures were serially diluted into LB, and 5 μl of diluted cultures was spotted

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics	Source or reference
E. coli strains		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	74
DH5α λ*pir*	λ*pir* lysogen of DH5α	46
CC118 λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir* lysogen	75
BL21(DE3)	*hsdS gal* (λcI *ts*857 *ind-1 S*am7 *nin-5 lacUV5*-T7 gene *1*)	76
P. putida strain or genotype		
PaW85	Wild type, isogenic to KT2440	42
Δ*graA*	PaW85 Δ*graA*	This study
$\Delta graA_{T(E80G)}$	PaW85 Δ*graA*; *graT* contains an E80G substitution mutation	This study
Δ*graT*	PaW85 Δ*graT*	This study
Δ*graTA*	PaW85 Δ*graTA*	This study
colR	PaW85 *colR*::Km^r (Km^r)	77
colR Δ*graA*	*colR* Δ*graA* (Km^r)	This study
colR $\Delta graA_{T(E80G)}$	*colR* Δ*graA*; *graT* contains an E80G substitution mutation (Km^r)	This study
colR Δ*graT*	*colR* Δ*graT* (Km^r)	This study
colR Δ*graTA*	*colR* Δ*graTA* (Km^r)	This study
Plasmids		
pEMG	Suicide plasmid containing *lacZ*α with two flanking I-SceI sites (Km^r)	46
pSW(I-SceI)	Plasmid for I-SceI expression (Ap^r)	78
pEMG-ΔA	pEMG containing a PCR-designed 1.5-kb EcoRI-BamHI insert for deleting *graA* (Km^r)	This study
$pEMG\text{-}\Delta A_{T(E80G)}$	pEMG-ΔA; *graT* contains an E80G substitution mutation (Km^r)	This study
pEMG-ΔT	pEMG containing a PCR-designed 1.05-kb EcoRI-BamHI insert for deleting *graT* (Km^r)	This study
pEMG-ΔTA	pEMG containing a PCR-designed 1.2-kb EcoRI-BamHI insert for deleting *graTA* (Km^r)	This study
pGP704L/colR::Km	Suicide plasmid containing *colR* disrupted by Km (Ap^r Km^r)	77
pRK2013	Helper plasmid for conjugal transfer of pGP704L derivatives (Km^r)	79
pKT240	Cloning vector (Ap^r Km^r)	80
pBRlacItac	Expression vector containing P_{tac} promoter and *lacI*^{q} repressor in pBR322 (Ap^r)	81
pSEVA-Km (RK2)	Cloning vector (Km^r)	82
pKTlacItac	Expression vector containing P_{tac} promoter and *lacI*^{q} repressor from pBRlacItac and MCS from pSEVA-Km (RK2) (Ap^r)	This study
pKTlacItac-TA	pKTlacItac containing *graTA* operon under the control of *lacI* and P_{tac} promoter (Ap^r)	This study
pKTlacItac-T	pKTlacItac containing *graT* under the control of *lacI* and P_{tac} promoter (Ap^r)	This study
$pKTlacItac\text{-}T_{E80G}$	pKTlacItac containing mutant $graT_{E80G}$ under the control of *lacI* and P_{tac} promoter (Ap^r)	This study
pKTlacItac-A	pKTlacItac containing *graA* under the control of *lacI* and P_{tac} promoter (Ap^r)	This study
$p9TT_BlacZ$	Promoter probe plasmid (Cm^r Ap^r)	33
p9TT1586	$p9TT_BlacZ$ containing *graTA* promoter fused with *lacZ* (Cm^r Ap^r)	This study
pET11c	Protein expression vector (Ap^r)	Stratagene
pET-Ahis	pET11c containing *graA* with C-terminal His_6 tag (Ap^r)	This study
pET-hisTA	pET11c containing *graT* with N-terminal His_6 tag and untagged *graA* (Ap^r)	This study
pET-A	pET11c containing *graA* (Ap^r)	This study

onto LB agar plates supplemented with benzylpenicillin for plasmid selection. For the overexpression of TA proteins, the bacteria were also spotted onto LB medium containing 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Plates were incubated up to 48 h at 30°C or 25°C.

Purification of proteins. Nickel affinity chromatography was used to purify the C-terminally histidine-tagged GraA and the GraT-GraA complex consisting of the N-terminally tagged GraT and the untagged GraA. The untagged GraA was also overexpressed and used as a negative control in protein purification. For the overexpression of proteins, the *E. coli* strain BL21(DE3) containing different expression plasmids was grown in LB medium at 30°C up to an OD_{580} of ~0.5. Protein expression was induced with 0.5 mM IPTG. After 4 h of induction, cells were pelleted and sonicated in buffer A (50 mM phosphate buffer [pH 7.6], 1 M NaCl). Cellular debris was removed by centrifugation at 16,000 × *g* for 20 min at 4°C. The supernatant was filtered through a 0.22-μm filter before loading it on a 1-ml HisTrap HP (GE Healthcare Life Sciences) column equilibrated with buffer A. Protein purification was performed by fast protein liquid chromatography (FPLC) using an Äkta Prime chromatography system (GE Healthcare Life Sciences). The column was washed with buffer B (50 mM phosphate buffer [pH 7.6], 0.5 M NaCl, 50 mM imidazole, 10% glycerol) until the absorbance of the flowthrough at 280 nm approached baseline. Proteins were eluted from the column with linear imidazole gradient by using buffer C (50 mM phosphate buffer [pH 7.6], 0.5 M NaCl, 600 mM imidazole, 10% glycerol). Elution fractions with peak absorbance at 280 nm were collected and the purified proteins were dialyzed stepwise against the following buffers: (i) 10 mM Tris-HCl (pH 7.5), 300 mM KCl, 20% glycerol; (ii) 10 mM Tris-HCl (pH 7.5), 250 mM KCl, 35% glycerol; (iii) 10 mM Tris-HCl (pH 7.5), 200 mM KCl, 50% glycerol. Proteins were stored at −20°C.

Enzyme assay. β-Galactosidase activities were measured from overnight cultures of LB-grown bacteria carrying plasmid p9TT1586 according to a previously described protocol (47).

5′ RACE analysis. To determine the transcriptional start site of the *graTA* operon by 5′ RACE (random amplification of cDNA ends), the total RNA was isolated from exponential-phase cells of *P. putida* PaW85 using the NucleoSpin RNA II kit (Macherey-Nagel). RNA samples were additionally treated with DNase I (Thermo Scientific) to remove contaminating DNA. The first-strand cDNA was synthesized from total RNA using the primer graAstop and Maxima reverse transcriptase (Thermo Scientific). cDNA was purified with DNA Clean & Concentrator-5 kit (Zymo Research) and tailed with poly(dG) sequence by terminal deoxynucleotidyl transferase (Thermo Scientific). The second strand of the poly(dG)-tailed single-stranded cDNA was made using the primer C-ankur and *Taq* polymerase. Purified double-stranded cDNA was amplified by PCR using the primers graAsees and ARB2. The obtained PCR product was sequenced.

qRT-PCR. Total RNA for *graT* mRNA quantification was isolated as described above for 5′ RACE. The quantitative reverse transcription-PCR (qRT-PCR) assay was performed on the Rotor-Gene Q system (Qiagen) using the SuperScript III Platinum SYBR green one-step qRT-PCR kit (Invitrogen) according to the manufacturer's protocol, except with double primer concentrations and a total reaction volume of 10 μl. A 10-ng portion of total RNA was used for each reaction. The *graT* gene was amplified using the primers graTqFw and graTqRev, and the *rpoD* gene was amplified using the primers rpoDqFw and rpoDqRev. Raw data were analyzed with the Rotor-Gene Q software version 2.02 (Qiagen), and mRNA amounts were calculated using the LinRegPCR software version 2013.0 (48). Data from three separate qRT-PCR experiments performed on two independently extracted RNAs were averaged and normalized against *rpoD* levels.

DNase I footprinting. DNA fragments for DNase I footprinting assay were amplified from the plasmid p9TT1586 by PCR. Oligonucleotides used in PCRs are listed in Table S1 in the supplemental material. Prior to the PCR, one oligonucleotide was end labeled by phosphorylation with [γ-^{32}P]ATP. The labeled DNA fragments were purified by native 5% polyacrylamide gel electrophoresis, eluted with buffer (0.5 M ammonium acetate [NH_4Ac], 10 mM MgAc, 1 mM EDTA, 0.1% SDS) and resuspended in water. For the binding reaction, the purified His-tagged GraA or GraT-GraA complex (0.45, 0.9, 4.5, 9, and 45 pmol) was combined with 30,000 cpm of labeled DNA fragment, 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 10 mM KCl, 5 μg of bovine serum albumin (BSA), 1 μg of salmon sperm DNA, and 5% glycerol in a final volume of 50 μl. Reactions with different volume of proteins were equalized with the addition of appropriate amount of GraA storage buffer. Proteins were allowed to bind to DNA during 30 min at room temperature before the start of digestion by DNase I (0.06 U; Thermo Scientific) for 3 min. Reactions were stopped by the addition of 50 μl of stop solution (20 mM Tris-HCl [pH 8.0], 20 mM EDTA, 0.8% sodium dodecyl sulfate, 100 μg of salmon sperm DNA per ml). The footprinting reaction mixtures were subsequently extracted once with phenol and chloroform (1:1, vol/vol) and once with chloroform, and finally, the DNA was precipitated with ethanol and NaCl. The DNA fragments were resuspended in 7 μl of sequence loading buffer (50% formamide, 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol) and loaded onto a 6.5% polyacrylamide gel that contained 8 M urea. DNA sequencing reactions were performed with a Sequenase kit, version 2.0 (USB Corporation), and the products were loaded on a sequencing gel as size markers. After the run, the gels were dried, exposed to a PhosphorImager screen, scanned using a Typhoon Trio imager, and analyzed by ImageQuant TL v2005 software (GE Healthcare).

Flow cytometry analysis. Different bacterial strains were grown on LB agar or on glucose or gluconate minimal plates as thin radial stripes. In order to enhance the glucose-dependent cell lysis of the *colR* mutant, the glucose plates were supplemented with 1 mM phenol. After 48 h of growth, the cells were scraped off the plates and suspended in M9 buffer. The cell suspension was diluted to an OD_{580} of 0.015. The two components of the LIVE/DEAD BacLight kit (Invitrogen), the red fluorescent dye propidium iodide (PI) and the green fluorescent dye SYTO9, were mixed in a 1:1 ratio and then diluted 17.6-fold into filter-sterilized M9 buffer. For staining of bacteria, the diluted cell suspension was mixed with the freshly prepared reagent mixture in a 20:1 ratio. Samples were incubated at 30°C in the dark for 30 min, and approximately 10,000 events from every sample were analyzed with the FACSAria flow cytometer (BD Biosciences). Fluorescent dyes were excited at 488 nm. Forward and side scatter (FSC and SSC) of the light and fluorescence emission at 530 and 616 nm were acquired for every event. Populations of intact, PI-permeable, and dead cells were defined as previously described (34). While PI-permeable cells differ from the intact subpopulation only by their PI-permeable membranes, the dead cells are more compromised, containing less DNA than other cells (34).

Stress tolerance assays. Phenol sensitivity was evaluated on agar plates containing 0.2% glucose as a carbon source and 7 mM phenol. The cultures that had been grown overnight in LB were 10-fold serially diluted, spotted onto plates as 5-μl drops, and incubated at 30°C for up to 7 days.

In order to evaluate the stress tolerance of bacteria to different compounds, the serially diluted bacteria were spotted onto LB agar plates supplemented with different antibiotics and chemicals (specified in Results). Plates were incubated at 30°C for 24 or 48 h.

Streptomycin-mediated killing assay. Bacterial cultures pregrown overnight in liquid LB medium at 37°C were diluted about 55-fold into fresh LB and grown at 25°C or 37°C until the optical density (580 nm) reached about 0.5. A 1.4-ml portion of each culture was transferred to an Eppendorf tube and treated with 300 μg/ml streptomycin with shaking at 25°C or 37°C. The number of CFU was determined before streptomycin addition and at various time points during treatment. For that, 200 μl of bacterial culture was centrifuged and resuspended in M9 buffer, and 10-fold serial dilutions were spotted onto solid LB medium as 5- to 10-μl spots. Plates were incubated at 30°C overnight.

RESULTS

Orthologs of PP1585 and PP1586 are present in the genomes of most pseudomonads. It has been predicted that the *Pseudomonas putida* genes PP1586 and PP1585 code for a toxin and an antitoxin, respectively (26, 30). To determine whether this putative TA pair is conserved in 48 other completely sequenced pseudomonads, we performed a search in the Pseudomonas Genome Database (49). According to this analysis, the antitoxin protein has 30 and the toxin 17 annotated orthologs in other *Pseudomonas* species (see Table S2 in the supplemental material). Importantly, all 17 PP1586 orthologs constitute a pair with a putative antitoxin orthologous to PP1585. Moreover, the uncommon gene order, the toxin gene preceding the antitoxin, is conserved in these putative TA loci. The remaining 13 PP1585 orthologs were annotated as single gene loci, but careful sequence analysis revealed that nine of them possess a putative PP1586 toxin ortholog upstream of the antitoxin gene (see Table S2). Only four PP1585 orthologs in other pseudomonads did not have an obvious toxin partner. We also searched the genomes of the *Pseudomonas* species that at first glance did not contain any PP1585 and PP1586 orthologs and found three more TA loci orthologous to PP1586-PP1585. The loci most similar to PP1586-PP1585 were in the genomes of *P. putida* F1 and *P. putida* NBRC 14164 (97% and 92% identity of the toxin gene and 99% and 97% identity of the antitoxin gene, respectively), but well conserved TA loci were also present in all 13 *P. aeruginosa* strains, in two *P. fluorescens* strains, in one *Pseudomonas stutzeri* strain, in one *Pseudomonas syringae* strain, and in *Pseudomonas denitrificans* strains (64 to 72% identity of the toxin gene and 48 to 62% identity of the antitoxin gene) (see Table S2). Conclusively, of 48 completely sequenced pseudomonads, 30 contain a putative TA pair orthologous to the PP1586-PP1585 operon.

Using the NCBI BLAST tool, we searched for putative PP1586-

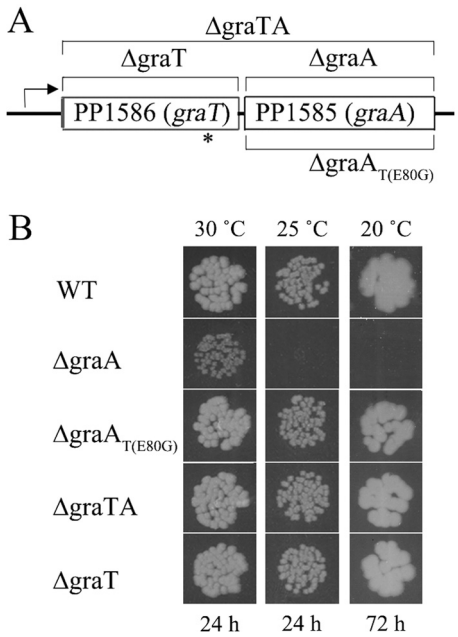

FIG 1 GraT-mediated growth inhibition of the Δ*graA* strain. (A) Organization of genes in the *P. putida graTA* locus. DNA regions which are absent from the chromosomes of the deletion strains are indicated by brackets. The approximate location of the E80G substitution mutation in *graT* of the $\Delta graA_{T(E80G)}$ strain is indicated by an asterisk. (B) Growth of *P. putida* wild-type strain PaW85 (WT) and its *graTA* deletion derivatives on solid LB medium. Approximately 50 cells of each strain were inoculated onto LB agar plates. Incubation temperatures and growth times are indicated.

PP1585 homologs in other bacteria and found that the protein most similar to PP1585 was a HigA family antitoxin protein from *Nitrosococcus halophilus* Nc4 (57% identity), and the one most similar to PP1586 was a putative killer protein of the plasmid maintenance system from *Delftia* sp. Cs1-4 (71% identity). The proteins most similar to PP1585 and PP1586 that have already been experimentally described are HigBA system proteins from *Vibrio cholerae* (41), with sequence identities of 38% for the antitoxin and 37% for the toxin (see Fig. S1 in the supplemental material). Given that the structure of the PP1586-PP1585 operon also resembles that of HigBA systems, where the toxin precedes the antitoxin gene and the antitoxin is larger than the toxin, we deduce that PP1585 and PP1586 belong to the HigBA family of TA systems.

PP1586 and PP1585 genes code for the growth rate-modulating TA system GraTA. In order to examine whether PP1585 and PP1586 code for a functional TA system, we constructed three derivatives of *P. putida* PaW85 which had deletions of either PP1585 (Δ*graA*), PP1586 (Δ*graT*), or both genes (Δ*graTA*) (Fig. 1A). Accidentally, we also obtained an antitoxin (PP1585) deletion strain [$\Delta graA_{T(E80G)}$] which had a PCR-generated point mutation in the toxin (PP1586) coding region, resulting in the replacement of glutamic acid with glycine in the 80th position of the toxin (E80G). To investigate the effect of this substitution on the toxin activity, we included the antitoxin deletion strain with the mutant toxin [$\Delta graA_{T(E80G)}$] in the experiments presented below.

It is quite unusual that we could easily construct a stable antitoxin deletion strain. As sequencing evidenced that toxin gene was intact in the Δ*graA* strain, we considered that the toxin is either very weak or entirely inactive. However, while constructing the deletion strains, we noticed that the PP1585 (antitoxin) deletion mutant had a somewhat lower growth rate than the parent strain and other deletion strains. This was the first hint of the functionality of the toxin in the absence of the antitoxin, and therefore we named the system GraTA, for growth rate-affecting toxin-antitoxin system.

We first noticed the GraT-mediated inhibition of growth on agar plates, where the colony formation of the Δ*graA* strain was significantly delayed compared to that of the parent strain (Fig. 1B). This growth defect was obviously caused by GraT, because the colony formation rates of the Δ*graTA* and Δ*graT* mutants were equal to that of the wild-type strain. Moreover, the fact that the $\Delta graA_{T(E80G)}$ strain grows similarly to the parent strain clearly showed that GraT is responsible for the growth inhibition of the Δ*graA* strain and that the mutation E80G in GraT reduces its activity. The slower growth of the GraA deletion strain was observed on different carbon sources, including glucose, gluconate, fructose, and sodium benzoate (data not shown). Interestingly, the GraT-mediated growth inhibition was stronger when the bacteria were grown at lower temperatures (Fig. 1B). While the outgrowth of the Δ*graA* strain on LB solid medium was delayed for only a few hours at 30°C, this strain could not form colonies on LB plates at 20°C within 3 days. The temperature-sensitive growth defect of the Δ*graA* strain is clearly caused by the activity of GraT, because the $\Delta graA_{T(E80G)}$ strain behaved similarly to the wild type (Fig. 1B), with only a slight hint of slower growth at lower temperatures (not observable in Fig. 1B). To investigate the effect of the toxin on growth rate more specifically, we determined the generation time of *P. putida* PaW85 and its *graTA* deletion derivatives grown in liquid LB medium. The generation times of wild-type and Δ*graA* bacteria differed markedly at 30°C (64 min for the wild type versus 82 min for the mutant) but even more at 20°C (151 min for the wild type versus 804 min for the mutant) (Table 2). Surprisingly, the growth rate suppression effect of GraT was not observable at 37°C. Other deletion strains, including the $\Delta graA_{T(E80G)}$ mutant, revealed growth rates comparable to that of the wild-type strain at all temperatures. These results show that

TABLE 2 Generation times of *P. putida* strains grown in LB medium

Strain genotype	Generation time (*P*) at[a]:			
	20°C	25°C	30°C	37°C
WT	151.0 ± 9.3	120.2 ± 2.8	64.7 ± 3.4	44.7 ± 1.6
Δ*graA*	804.2 ± 77.7 (1.9×10^{-11})	247.7 ± 9.6 (5.2×10^{-14})	81.9 ± 3.1 (2.4×10^{-7})	45.5 ± 2.7 (0.34)
$\Delta graA_{T(E80G)}$	153.4 ± 11.55 (0.52)	122.8 ± 2.9 (0.02)	61.8 ± 5.8 (0.53)	43.8 ± 1.2 (0.31)
Δ*graTA*	149.7 ± 4.8 (0.63)	117.6 ± 2.7 (0.03)	62.7 ± 8.5 (0.81)	45.3 ± 2.5 (0.43)
Δ*graT*	149.2 ± 9.3 (0.53)	120.6 ± 4.1 (0.76)	64.8 ± 1.4 (0.42)	45.7 ± 1.6 (0.12)

[a] Values are means ± standard deviations. *P* values (in parentheses) are for comparison between each strain and the wild type (WT) at a given temperature.

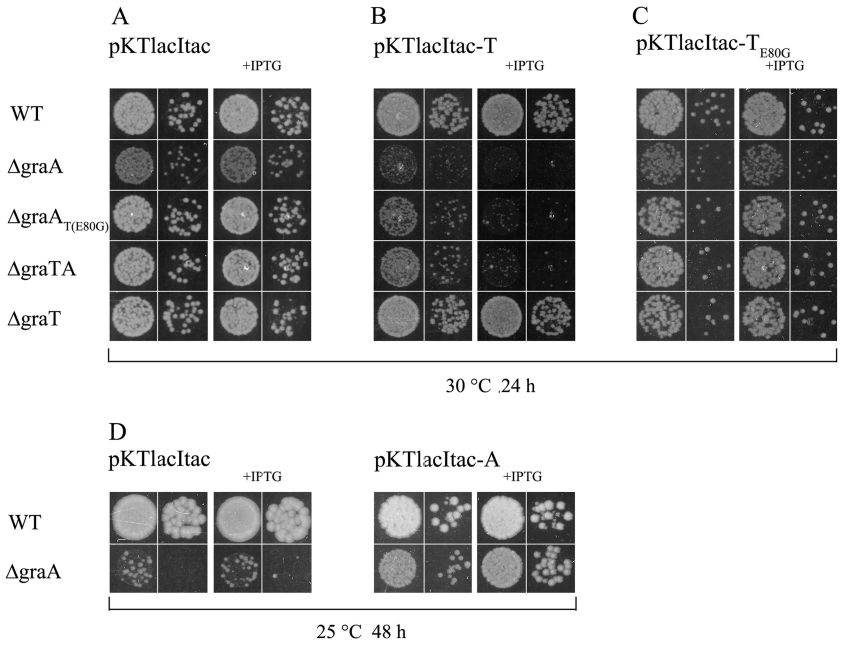

FIG 2 Ectopic overexpression of GraT but not GraT(E80G) is toxic to strains devoid of *graA*. *P. putida* wild-type strain PaW85 (WT) and its *graTA* deletion derivatives were transformed with plasmids pKTlacItac (A and D), pKTlacItac-T (B), pKTlacItac-T_{E80G} (C), and pKTlacItac-A (D), and series of 10-fold dilutions were spotted onto LB agar or onto LB with 0.5 mM IPTG (+IPTG). Plates were incubated either at 30°C for 24 h (A, B, and C) or at 25°C for 48 h (D).

GraTA forms a functional TA system consisting of the temperature-dependent toxin GraT, which is able to inhibit the growth rate of bacteria, and the antitoxin GraA, capable of neutralizing the toxin's effect. Moreover, we show that the single E80G amino acid substitution suppresses the activity of GraT toxin.

Overexpression of GraT is toxic to *graA* deletion mutants. The deletion of an antitoxin gene has been impossible for several other TA systems (41, 50), but we gained the *graA* deletion strain quite easily. This indicated that either the expression of *graT* is weak, the stability of the *graT* mRNA or of the GraT protein is low, or the activity of GraT is somehow hindered. To find out if the overexpression of GraT can be highly toxic to bacteria, plasmids pKTlacItac-T and pKTlacItac-T_{E80G}, enabling overproduction of GraT and GraT(E80G), respectively, were constructed. The empty vector pKTlacItac was used as control plasmid (Fig. 2A). Data in Fig. 2B show that at 30°C, the ectopic expression of GraT was most toxic for the Δ*graA* strain, which lacked the GraA antitoxin but retained the native *graT* gene in the genome. Notably, comparison of pKTlacItac-T-carrying bacteria with an empty vector control shows that the *tac* promoter used to overexpress the GraT is leaky, because the growth of the Δ*graA*, Δ$graA_{T(E80G)}$, and Δ*graTA* strains was already retarded without induction with IPTG (Fig. 2A and B). Although the Δ*graTA* and Δ$graA_{T(E80G)}$ strains are devoid of *graA* like the Δ*graA* strain, they were less affected by ectopic GraT, and the total growth arrest was observed only under IPTG-induced conditions. Interestingly, neither the wild-type nor the Δ*graT* strain showed significant sensitivity to GraT overexpression at 30°C (Fig. 2A and B). This shows that overexpression of GraT can be toxic to bacteria but the native antitoxin can effectively inactivate the artificially overproduced toxin. As was expected, the overexpression of GraT(E80G) at 30°C was not toxic to bacteria (Fig. 2C), confirming the ability of the E80G substitution to reduce the GraT activity.

Complementation of the Δ*graA* strain with the GraA overexpression plasmid pKTlacItac-A eliminated the growth defect of the strain even at lower temperatures (Fig. 2D), indicating that ectopic expression of GraA can neutralize the effect of GraT toxin. Thus, these overexpression experiments confirmed that GraT is a toxin which is inactivated by GraA antitoxin, whether artificial or innate. Unfortunately, all plasmids containing the *lacI tac* cassette are unstable in *P. putida*. Therefore, the effect of the ectopic expression of GraT and GraA can be tested only immediately after transformation, and no long-term experiments are possible.

GraA and GraT form a complex. In type II TA systems, the toxin is normally inactivated by a protein complex formation between the toxin and the antitoxin (51). To clarify if GraA and GraT form a complex, a pulldown assay was used. GraT with an N-terminal hexahistidine tag was overexpressed together with untagged GraA. As a negative control, the untagged GraA alone was also overexpressed. Affinity purification using Ni^{2+}-coated agarose beads and subsequent SDS-PAGE revealed that while His_6-GraT and GraA copurify in a 1:1 ratio, the untagged GraA alone cannot bind to the Ni^{2+} column (Fig. 3). This suggests specific binding between GraA and GraT, which can be considered the mechanism of toxin inactivation. Binding of GraT and GraA, despite the use of N-terminally His-tagged GraT, suggests that the N terminus of the toxin is not specifically important for antitoxin binding.

GraA represses the *graTA* promoter and binds just upstream of the *graT* start codon. Commonly, the transcription of a type II TA operon is autorepressed by the antitoxin or toxin-antitoxin complex (52). Computational analysis of GraTA proteins predicted that GraA can putatively bind DNA, as it possesses a helix-turn-helix motif of XRE-family proteins. In order to test whether the expression of the *graTA* operon depends on the presence of the antitoxin, the *graTA* transcriptional fusion with the β-galactosidase gene was analyzed in wild-type and TA deletion mutants. The

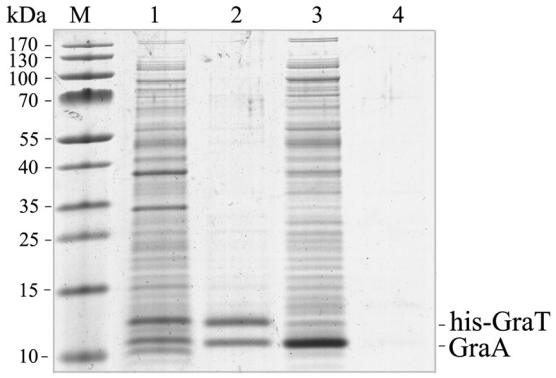

FIG 3 GraT and GraA form a complex. His-tagged GraT and untagged GraA were coexpressed (lane 1) and copurified by nickel affinity chromatography (lane 2). The untagged GraA alone was also overexpressed (lane 3) and passed through the nickel affinity column (lane 4) as a negative control.

transcription from the *graTA* promoter was 5-fold upregulated in the Δ*graA* and Δ*graTA* strains compared to the parent strain, which indicated antitoxin-mediated autorepression (Fig. 4A). Promoter activity in the Δ*graT* strain was even lower than in the wild-type strain. We also quantified the *graT*-specific mRNA in the wild type and the Δ*graA* strain by qRT-PCR. As the Δ*graA* strain displayed an approximately 85-fold-higher level of toxin mRNA than the wild type, GraA indeed acts as an autorepressor of *graTA* expression. Notably, this also indicates that the mild effect of the toxin cannot be attributed to the low expression level of the *graT* gene in the Δ*graA* strain.

To determine the transcription start site of *graTA* genes, we carried out 5′ RACE analysis with a primer complementary to the end of the operon. We detected a single transcription start site located 26 nt upstream of *graT* ATG codon (Fig. 4B), indicating that *graTA* genes are transcribed polycistronically. Consensus −10 and −35 hexamers of the sigma 70-dependent promoter were identified upstream of the transcriptional start site (Fig. 4B).

To confirm that the autorepression is caused by direct binding of GraA to the promoter region, a DNase I footprint analysis was performed with both the antitoxin and the toxin-antitoxin complex (Fig. 4C). The results show that both the GraA antitoxin alone and the GraTA complex bind a 35-nucleotide-long sequence directly in front of the toxin gene (Fig. 4B and C). The area protected from DNase I digestion is exactly the same for both GraA and the GraTA complex. The recognition site contains a perfect palindromic sequence (TTAACGAATAACGTTAA; underlining indicates the palindrome) and partially overlaps the −10 region of the *graTA* promoter. Such a location of the GraA binding site indicates that antitoxin binding may directly hinder the binding of RNA polymerase to the promoter.

GraT increases membrane permeability but nevertheless prevents the lysis of the *colR* mutant. The data presented above show that GraTA is a functional TA system and that the toxin GraT is able to reduce bacterial growth rate. Slower growth has been shown to be beneficial for bacteria under stressful conditions (53). Considering that the disruption of *graA* can eliminate the glucose-specific subpopulation lysis phenotype of *colR*-deficient strain (31), we hypothesized that this may occur due to GraT-caused growth inhibition, which results in lower membrane stress of the *colR* mutant. To test this possibility, we decided to analyze the effects of GraT and GraT(E80G) on phenotypes of the *colR*-

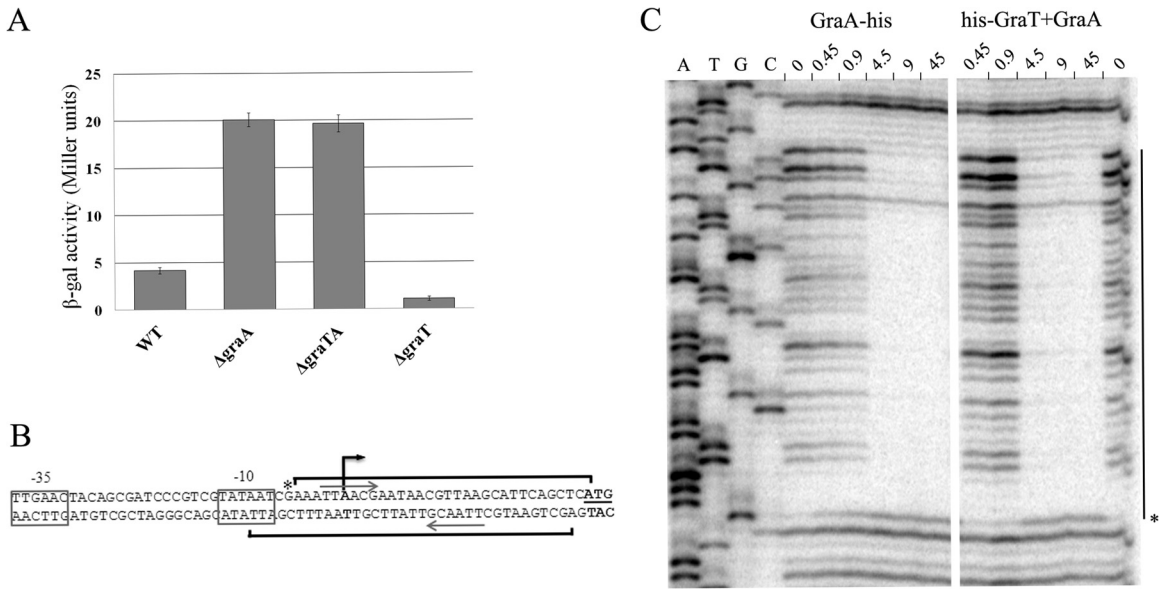

FIG 4 Autorepression of *graTA* promoter by GraA. (A) β-Galactosidase activities measured in *P. putida* wild-type (WT) and *graTA* deletion strains carrying the transcriptional fusion of the *graTA* promoter with *lacZ* in plasmid p9TT1586. Bacteria were grown overnight in LB medium at 30°C. Data (means with 95% confidence intervals) of at least three independent experiments are presented. (B) Promoter region of *graTA*. The −10 and −35 elements of the promoter are boxed, the transcriptional start site is indicated by a black arrow, and the ATG of *graT* is underlined. Brackets indicate the regions protected by DNase I cleavage by binding of GraA, and the asterisk marks a hypersensitive cleavage site. The palindromic sequences of GraA binding site are designated by gray arrows. (C) DNase I footprint analysis for determining GraA and GraT-GraA binding sites in the *graTA* promoter region. Footprints on the upper strand of the promoter region are presented. Lane 0 represents the DNase I reaction carried out in the absence of TA proteins. Other lanes represent reactions which contain increasing amounts (in pmol) of either the GraA-His or the His-GraT+GraA complex. The line on the right designates the region of protection against DNase I cleavage, and the asterisk signifies a base which becomes hypersensitive to DNase I cleavage upon the formation of the DNA complex with TA protein.

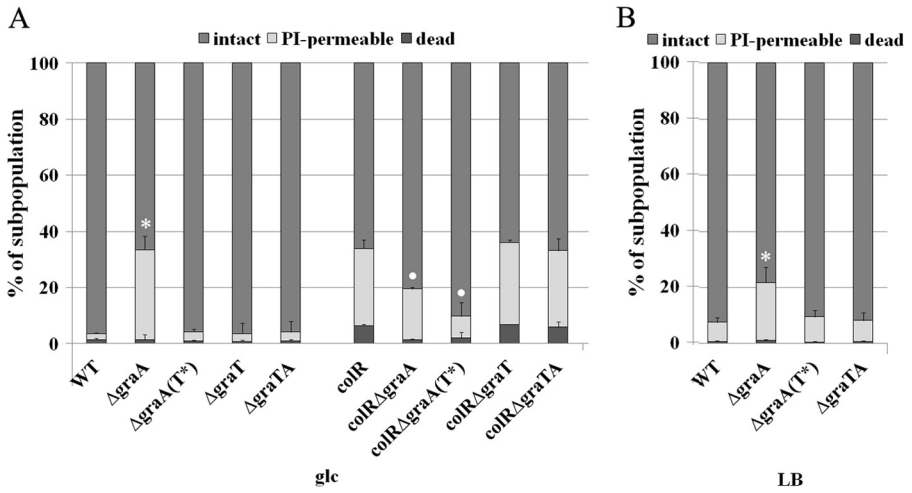

FIG 5 Cell population structure by flow cytometry analysis. (A) The *P. putida* wild type (WT), the *colR* mutant (colR) and their *graTA* deletion derivatives were grown for 48 h on solid glucose minimal medium supplemented with 1 mM phenol at 30°C. Inactive GraT(E80G) is designated T*. (B) The *P. putida* wild type and its *graTA* deletion derivatives were grown on LB agar at 30°C for 48 h. Cells were stained with PI and SYTO9 and analyzed by flow cytometry. Relative proportions of intact, PI-permeable, and dead subpopulations (specified in Fig. S1 in the supplemental material) are shown. Data (mean with 95% confidence intervals) of at least four independent determinations are presented. Asterisks indicate statistically significant differences ($P < 0.001$, Student's *t* test) between the PI-permeable populations of a particular strain and the wild type. Dots indicate that both the PI-permeable and the dead subpopulations of a particular strain differ significantly ($P < 0.01$) from that of the *colR* mutant.

deficient strain. We presumed that only GraT could suppress the lysis defect of *colR* mutant, as only GraT and not GraT(E80G) can significantly affect the growth rate of bacteria.

The lysis-susceptible population of *colR*-deficient *P. putida* grown on glucose solid medium is highly heterogeneous, as judged by SYTO9 and propidium iodide (PI) staining and single-cell analysis by flow cytometry (32, 34). Besides a subpopulation of PI-impermeable cells similar to the wild type, the *colR* mutant culture also contains a large amount of PI-permeable cells and a characteristic subpopulation of dead cells (see Fig. S2 in the supplemental material) (34). In order to examine how GraT and GraT(E80G) can influence the population structure as well as membrane permeability of glucose-grown bacteria, we stained the wild type, *colR*-deficient strain, and TA deletion mutants with the LIVE/DEAD kit and assessed the populations at the single-cell level. Flow cytometry analysis revealed that the characteristic population structure with a dead subpopulation was present in *colR*-deficient bacteria and also in *colR* derivatives lacking either *graT* or the whole *graTA* operon (Fig. 5A). However, the subpopulation of dead cells had almost disappeared in the *colR* Δ*graA* double mutant ($P = 4.1e-04$, Student's *t* test) and, contrary to our expectations, had also significantly decreased in the *colR* $\Delta graA_{T(E80G)}$ mutant (Fig. 5A) ($P = 6.4e-04$). The latter finding indicates that mutant GraT(E80G) retained part of its activity but also suggests that the growth rate reduction by GraT is not the only reason why the lysis of the *colR* mutant is suppressed.

Population analysis also revealed that deletion of *graA* essentially influenced the membrane permeability, because a large PI-permeable subpopulation was observed in Δ*graA* bacteria (Fig. 5A; also, see Fig. S2 in the supplemental material). As this PI-permeable population was absent in the $\Delta graA_{T(E80G)}$ strain, we conclude that the toxin GraT is responsible for the membrane permeabilization. To test whether the higher membrane permeability of Δ*graA* cells is specific to bacteria grown on glucose medium or whether this effect is also detectable on other carbon sources, we performed flow cytometry analyses with LB- and gluconate-grown bacteria. The membrane-permeabilizing effect of GraT also appeared on solid gluconate (data not shown) and LB media (Fig. 5B) ($P = 1.6e-04$). As bioinformatic analysis does not give any clue that GraT could be a membrane protein (data not shown), we conclude that the increased membrane permeability of the Δ*graA* strain should be an indirect effect of the GraT toxin. Taken together, these data show that the toxin GraT increases membrane permeability to PI but can nevertheless eliminate the membrane stress and cell death of the *colR* mutant.

Opposite effects of GraT on survival under different stress conditions. In addition to glucose-specific lysis phenotype, the *colR*-deficient *P. putida* also displays decreased phenol tolerance (33, 34). As phenol mostly affects the cell membrane and GraT increases membrane permeability (Fig. 5), it was interesting to find out how GraT affects the phenol tolerance of both wild-type and *colR*-deficient bacteria. Thus, we compared the growth of different strains on glucose solid medium supplemented with phenol. Bacteria with a disrupted *colR* gene tolerated 7.5 mM phenol only when *graA* was absent (Fig. 6), indicating that GraT can alleviate the phenol sensitivity of the *colR*-deficient strain. Also, GraT(E80G) can increase the phenol tolerance of the *colR* mutant, although to a lesser extent than wild-type GraT (Fig. 6). The tolerance had not changed for any of the deletion mutants in the wild-type background. Thus, despite the fact that GraT increases membrane permeability, it restores the phenol tolerance of the *colR*-deficient strain without affecting the tolerance of wild-type bacteria.

To analyze whether GraT can increase the overall stress tolerance of *P. putida*, we tested the growth of the wild type and the *graTA* deletion strains on media with different compounds (Fig. 7) which inhibit transcription (rifampin), translation (streptomycin, kanamycin, and tetracycline), replication (mitomycin C and ciprofloxacin), or cell wall synthesis (ceftazidime and benzylpenicillin) or generate oxidative (paraquat, nitroquinoline, and

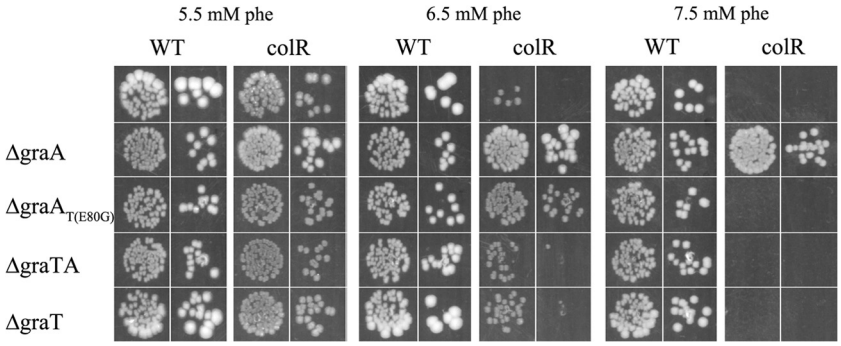

FIG 6 Plate assay of phenol tolerance. The *P. putida* wild-type strain PaW85 (WT), the *colR*-deficient strain (colR), and their *graTA* deletion derivatives were grown on glucose solid medium supplemented with 5.5, 6.5, or 7.5 mM phenol for 7 days at 30°C. In each pair, the panels show serial dilutions of approximately 50 and 5 cells per spot.

H_2O_2) or osmotic stress (NaCl, LiCl, and urea). We observed that the Δ*graA* strain had remarkably increased tolerance for the translation inhibitors streptomycin and kanamycin, the replication inhibitors mitomycin C and ciprofloxacin, and the cell wall inhibitor ceftazidime, whereas the Δ$graA_{T(E80G)}$, Δ*graTA*, and Δ*graT* strains were as sensitive to these chemicals as the wild type (Fig. 7). However, the Δ*graA* mutant showed an increased sensitivity to H_2O_2 (data not shown), paraquat, and tetracycline and particularly to NaCl (Fig. 7). The tolerance of the Δ*graA* strain to other tested chemicals was not significantly changed. We also tested the effect of these chemicals on the *graTA* promoter, but no remarkable response of the *graTA* promoter was observed (data not shown). Therefore, GraT helps bacteria survive some stresses and increases the sensitivity to others, but the expression of the *graTA* operon is not responsive to these stress situations.

GraT increases the tolerance of bacteria to the killing activity of streptomycin. TA loci are known to increase the tolerance of bacteria to the killing activity of antibiotics (54, 55). To test whether GraT can increase the survival of bacteria after antibiotic treatment, we exposed the exponentially growing cells of *P. putida* wild-type, Δ*graA*, and Δ$graA_{T(E80G)}$ strains to a lethal concentration of streptomycin. If the strains were pregrown at 25°C, i.e., under conditions in which the growth rate of the Δ*graA* strain is decreased, clearly higher persistence of the Δ*graA* strain was observed compared to the wild-type and Δ$graA_{T(E80G)}$ strains (Fig. 8A). Bacteria growing at 37°C were killed much more rapidly, and no significant differences between killing curves of analyzed strains were recorded (Fig. 8B). These data suggest that a GraT-caused growth rate reduction is important for the increased streptomycin tolerance of the Δ*graA* strain.

DISCUSSION

In this work, we identified the first TA system in *P. putida*, which was named GraTA, for the growth rate-affecting effect of the toxin GraT. The *graTA* system is homologous to the *higBA* system of *Vibrio cholerae* (41), and we show that it possesses several characteristic features of type II TA loci. First, GraT overexpression was toxic to cells in the absence of antitoxin GraA and resulted in severe growth inhibition (Fig. 2B). As the toxicity of GraT was inhibited by the substitution mutation E80G (Fig. 2C), the growth inhibition is clearly caused by the GraT protein. Second, chromosomally encoded GraA is an efficient antitoxin which can counteract the GraT-mediated growth inhibition even if GraT is ectopically overexpressed (Fig. 2B). Third, GraA directly associates with GraT (Fig. 3), suggesting that protein binding is the mechanism for toxin neutralization. Fourth, *graT* and *graA* form an operon and are cotranscribed in a single bicistronic mRNA. Fifth, *graTA* operon transcription is autorepressed by the antitoxin GraA (Fig. 4A).

Although the GraTA system possesses many features common to other TA loci, it is still unusual among TA pairs. While the antitoxin deletion is generally lethal for bacteria (41, 50), the deletion of the antitoxin gene *graA* not only was possible but was only modestly influential on the growth rate of *P. putida*. This shows that GraT is an unusually moderate growth inhibitor and even raises the question of whether it can actually be classified as a

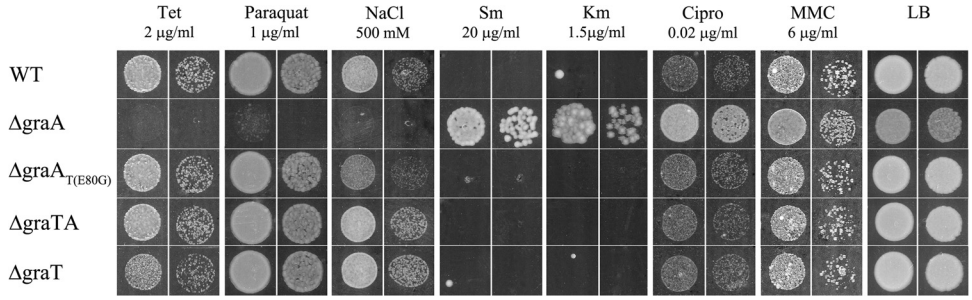

FIG 7 Plate assays of stress tolerance. The *P. putida* wild-type strain PaW85 (WT) and its *graTA* deletion derivatives were grown on LB solid medium containing different chemicals at 30°C for 24 h (Sm and Km, 48 h). The final concentrations of tetracycline (Tet), paraquat, NaCl, streptomycin (Sm), kanamycin (Km), ciprofloxacin (Cipro), and mitomycin C (MMC) are indicated. In each pair, the panels show serial dilutions of approximately 5,000 and 500 cells per spot.

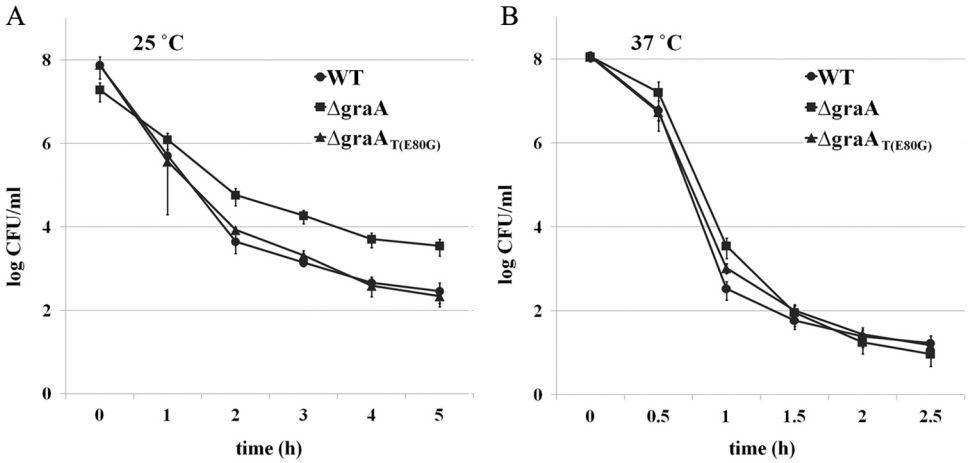

FIG 8 GraT increases bacterial tolerance to streptomycin-mediated killing. Exponentially growing cultures of *P. putida* PaW85 (WT) and its Δ*graA* and Δ$graA_{T(E80G)}$ derivatives were exposed to 300 μg/ml streptomycin either at 25°C for 5 h (A) or at 37°C for 2.5 h (B). The surviving cells were determined by plating and counting the CFU. Data (means with 95% confidence intervals) of at least four independent experiments are presented.

toxin. However, one should consider that, as with other TA toxins, the overexpression of GraT had a strong toxic effect on *P. putida* with *graA* deleted. At the same time, GraT seems not to be toxic to *E. coli*, as we never observed growth inhibition by different *graT*-containing plasmids in this bacterium (data not shown). This indicates that the activity of GraT might be host specific. Another interesting feature of GraT is that its effect depends on the growth conditions, as we observed significantly greater GraT-mediated growth rate suppression at 25°C and 20°C than at 30°C. Moreover, at 37°C, we did not detect a decrease in growth rate for *P. putida* with *graA* deleted. The temperature sensitivity of GraT raises the intriguing possibility that bacteria may benefit from GraT-mediated growth regulation during adaptation to lower temperatures. We tested this hypothesis in cocultivation experiments over 2 weeks, but as wild-type *P. putida* could not outcompete the Δ*graTA* double mutant at either high or low temperatures (data not shown), the results did not support the possibility of the GraTA system influencing the fitness of bacteria at lower temperatures. Nevertheless, the temperature sensitivity of GraT deserves further study, especially considering that the temperature dependence of TA systems is not well documented. To our knowledge, there is only one recent study which reports a temperature-sensitive toxin of a type I system (56). The authors showed that the toxin's mRNA is less stable at 48°C, but the biological significance of the phenotype remained unclear.

Our data show that while ectopic overexpression of GraT is toxic to the Δ*graA* and Δ*graTA* strains, it is tolerated by wild-type *P. putida* (Fig. 2A), indicating that chromosomally encoded GraA can efficiently neutralize both the innate and the additionally expressed GraT. Such a high efficacy of the antitoxin would be difficult to achieve in the case of an unstable antitoxin. And indeed, we have seen indications of GraA's unusual stability, as His-tagged GraA was stable during the overexpression, lysate preparation, and protein purification processes, even though we did not use any protease inhibitors. Hence, the efficacy of GraA in counteracting artificially overproduced GraT possibly relies on its high stability.

It is common in type II TA systems that their operons are autorepressed by either the antitoxin or the toxin-antitoxin complex (52, 57, 58). In the latter case, the antitoxin is the DNA-binding moiety and the toxin acts as a corepressor if the ratio of toxin to antitoxin is low or as a derepressor if toxin is in excess (59, 60). Transcriptional coregulation of TA operons by toxin abundance is common among TA systems and is known as conditional cooperativity (61–63). However, TA operons can also be regulated by other mechanisms involving promoter repression by antitoxin alone or even activation by antitoxin (18, 57). Similarly to most TA systems, the *graTA* operon is regulated by autorepression (Fig. 4). We detected significantly higher repression of the *graTA* promoter in Δ*graT* bacteria than in the wild type, which indicates that GraA antitoxin alone is an efficient repressor and the presence of the GraT causes partial derepression of the promoter activity. However, *in vitro* data show that both GraA alone and the GraA-GraT complex can bind to the promoter DNA, suggesting that the toxin GraT may also affect the promoter regulation. Still, we never observed that the protein complex could bind DNA more avidly than GraA alone, which shows that GraT cannot essentially influence the DNA-binding properties of GraA. Thus, our data do not support GraT-mediated corepression, and we conclude that the *graTA* operon is autorepressed by GraA only. Therefore, *graTA* regulation seems to resemble that of the *mqsRA* operon, as recent data demonstrate that the MqsR toxin is not a corepressor but rather alleviates MqsA-mediated repression (57).

The importance of chromosomal TA operons in bacterial physiology is highly debated, and different functions have been proposed for genomic TA systems, ranging from their participation in programmed cell death, persister formation, and stress tolerance to even considering them junk DNA and selfish modules (20, 22, 64). Still, accumulating evidence supports the idea that chromosomal TA systems are stress response loci which contribute to bacterial maintenance in unfavorable situations (4, 54, 65). Activation of the toxin leads to growth arrest, which facilitates bacterial survival under different stress conditions (66, 67). Our data also show that the GraT toxin can act as a stress-relieving factor, as it increased bacterial tolerance to several antibiotics. Given that antibiotics are more efficient against rapidly growing bacteria (53), the increased resistance of the Δ*graA* strain is most obviously caused by its slower growth. This is supported by the

finding that GraT(E80G), which is not able to decrease the growth rate of bacteria, is not able to increase antibiotic tolerance (Fig. 7 and 8). However, the observation that both GraT and GraT(E80G) can rescue the *colR* mutant from lysis indicates that the stress-relieving activity of GraT is more complicated and does not rely only on its ability to reduce growth rate. Furthermore, we also showed that GraT is not a universal stress-relieving factor, and its activity results in trade-offs between different types of resistance. While it increased resistance to streptomycin, kanamycin, ciprofloxacin, and mitomycin C, GraT decreased resistance to compounds such as tetracycline, paraquat, and NaCl (Fig. 7). Here, it is important to note that besides inhibiting the growth rate, GraT also compromised the barrier functions of membrane, as judged by the increased permeability of Δ*graA* cells to propidium iodide (Fig. 5). This membrane defect is possibly responsible for the increased sensitivity of Δ*graA* bacteria to tetracycline, paraquat, and NaCl. Thus, these data demonstrate that although GraT can in principle increase the tolerance of *P. putida* to particular stress factors, such stress protection is highly costly and results in harmful side effects. However, it should be emphasized that these data were obtained with the Δ*graA* strain, and GraT most likely cannot exert such a severe effect in wild-type bacteria, as it is normally neutralized by GraA. Nevertheless, it would be worthwhile to find out if some stress conditions can lead to the destabilization of GraA and if the released GraT can have an impact on the survival of *P. putida*.

Bioinformatic analysis of the genomes in the Pseudomonas Genome Database (49) revealed that 30 out of 48 different *Pseudomonas* strains possess orthologs of *graTA* genes. Such a high occurrence of the GraTA system in the highly heterogeneous *Pseudomonas* genus indicates that this TA system should be beneficial for both human and plant pathogens like *P. aeruginosa* and *P. syringae* as well as plant-beneficial soil bacteria like *P. fluorescens* and *P. putida*. Interestingly, all *P. aeruginosa* genomes annotated in the Pseudomonas Genome Database possessed highly conserved *graTA* orthologs. In accordance with these findings, recent analysis of 46 clinical isolates of *P. aeruginosa* revealed that all of them contain *graTA* homologs and, furthermore, that all these operons are actively transcribed (68). Considering the rapid evolution of microbial pathogens (69), the maintenance of active GraTA modules indicates that they might contribute to the virulence of *P. aeruginosa*.

The activation of a TA system may be bactericidal (70, 71), but more commonly, it results in rapid growth arrest until favorable conditions return (41, 72, 73). Hence, in spite of the fact that TA systems were discovered as killing modules on plasmids, most chromosomal systems actually operate as components of a cellular regulatory network dedicated to optimizing the bacterial growth rate. Still, compared to other growth modulators, the participation of toxins in growth regulation seems to be rather strange, especially considering that most of the well-studied TA systems code for quite harmful proteins. Contrary to most toxins, the effect of GraT on bacterial growth is moderate, resembling that of the general cellular regulators. We suggest that GraT represents a new class of chromosomal toxins which have evolved to less toxic variants either to better suit the needs of bacterial regulatory network or just to lessen the harm they can cause to the host bacterium. We believe that further research on TA systems will discover more GraTA-like TA pairs in different bacteria.

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