Stability of the GraA Antitoxin Depends on Growth Phase, ATP Level, and Global Regulator MexT

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ABSTRACT

Bacterial type II toxin-antitoxin systems consist of a potentially poisonous toxin and an antitoxin that inactivates the toxic protein by binding to it. Most of the toxins regulate stress survival, but their activation depends on the stability of the antitoxin that has to be degraded in order for the toxin to be able to attack its cellular targets. The degradation of antitoxins is usually rapid and carried out by ATP-dependent protease Lon or Clp, which is activated under stress conditions. The *graTA* system of *Pseudomonas putida* encodes the toxin GraT, which can affect the growth rate and stress tolerance of bacteria but is under most conditions inactivated by the unusually stable antitoxin GraA. Here, we aimed to describe the stability features of the antitoxin GraA by analyzing its degradation rate in total cell lysates of *P. putida*. We show that the degradation rate of GraA depends on the growth phase of bacteria being fastest in the transition from exponential to stationary phase. In accordance with this, higher ATP levels were shown to stabilize GraA. Differently from other antitoxins, the main cellular proteases Lon and Clp are not involved in GraA stability. Instead, GraA seems to be degraded through a unique pathway involving an endoprotease that cleaves the antitoxin into two unequal parts. We also identified the global transcriptional regulator MexT as a factor for destabilization of GraA, which indicates that the degradation of GraA may be induced by conditions similar to those that activate MexT.

IMPORTANCE

Toxin-antitoxin (TA) modules are widespread in bacterial chromosomes and have important roles in stress tolerance. As activation of a type II toxin is triggered by proteolytic degradation of the antitoxin, knowledge about the regulation of the antitoxin stability is critical for understanding the activation of a particular TA module. Here, we report on the unusual degradation pathway of the antitoxin GraA of the recently characterized GraTA system. While GraA is uncommonly stable in the exponential and late-stationary phases, its degradation increases in the transition phase. The degradation pathway of GraA involves neither Lon nor Clp, which usually targets antitoxins, but rather an unknown endoprotease and the global regulator MexT, suggesting a new type of regulation of antitoxin stability.

Bacterial toxin-antitoxin (TA) systems, consisting of a potentially poisonous toxin and an antitoxin that can efficiently inactivate the toxin, were first found in plasmids but are numerous in bacterial genomes as well (1–3). Plasmidial systems inhibit the proliferation of plasmid-free cells and, therefore, contribute to the maintenance of plasmids in bacterial populations (4). The function of chromosomal TA systems is not that straightforward, and several hypotheses about their roles have been proposed, the most plausible of which associates TA systems with stress response (5, 6). The activity of TA systems is often triggered by various stressful conditions, including different antibiotics, high temperatures, and glucose or amino acid starvation (7–9). Their expression is increased in biofilms (10) as well as in dormant, and therefore antibiotic-tolerant, persister cells (11–13). The role of TA systems in stress adaptation is also supported by the fact that some of them are integrated into the host's stress response network. For instance, the HipAB system is able to boost the (p)ppGpp-mediated stringent response and activation of the Lon protease, which degrades several antitoxins in *Escherichia coli* (14–17). Furthermore, MqsRA can modulate the RpoS-controlled stress response (18), and YafNO is upregulated during the DNA damage-induced SOS response (14, 19). Nevertheless, the real importance of genomic TA systems has remained ambiguous, as the deletion of a single system usually has no apparent effect on bacterial fitness (20, 21). Therefore, it is sometimes suggested that they may be just nonfunctional remnants of mobile genetic elements (22). How-

ever, the effect of TA systems on bacterial fitness can be seen when multiple TA loci have been deleted from the chromosome, indicating that chromosomal TA systems constitute a redundant network (21). As shown recently, the coordinated activation of the TA network in *E. coli* involves increased activity of the Lon protease that is triggered by the stringent response (15, 23). Transcriptional cross-activation between different TA systems has also been suggested as a mechanism for a synchronized response (23).

Five different types of TA systems have been described (24–29), but the most common and well-studied are type II TA systems, in which both the toxin and antitoxin are proteins. The activation of type II TA systems relies on the different levels of stability of the two proteins, with the toxin being more stable than the antitoxin (30, 31). For example, the half-lives ($t_{1/2}$ s) of the antitoxins RelB,

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HipB, and ϵ are estimated to be about 15 to 18 min, whereas their cognate toxins stay stable for much longer (24, 27, 32–34). Under normal growth conditions, the continuous synthesis of the antitoxin guarantees a sufficient amount of protein to bind and neutralize the toxin. However, under stress conditions, the rate of antitoxin proteolysis increases due to the activity of stress-induced ATP-dependent Lon and/or Clp protease (7, 35–37). The high levels of susceptibility of antitoxins to proteases are thought to be caused by unstructured and flexible parts of the proteins (31). For instance, the fast degradation of antitoxin RelB by Lon is supposed to be due to its unstable and flexible C-terminal tail (38). Unstructured C-terminal regions have also been reported for many other antitoxins, e.g., HipB and MazE (31, 39) from *E. coli*, as well as plasmidial CcdA and ParD (40, 41). However, there are also examples of antitoxins with structured C termini, like ParD of *Caulobacter crescentus* (31, 42, 43) and MqsA of *E. coli* (44). MqsA is still rapidly degraded by Lon but only under oxidative stress, when its half-life is just 1.25 min. Without stress, though, MqsA is stable for up to 60 min (45). Regardless of the exceptions, it is still commonly accepted that the unfolded state of specific regions of antitoxins is the reason for their instability and, therefore, an important determinant of the activation of TA systems (18).

We recently identified the first TA system in *Pseudomonas putida*, a nonpathogenic soil bacterium which is able to colonize a wide variety of habitats and resist various stress conditions (46, 47). According to bioinformatic analysis, *P. putida* appears to have 16 genomic TA loci (48). We have shown that one of them represents a bona fide TA system of the HigBA family, named GraTA and encoding the growth rate-affecting toxin GraT and its antidote, GraA (49). GraT is a remarkably feeble toxin at optimal growth temperature, allowing the deletion of the antitoxin gene without drastic growth defects. However, GraT causes a severe growth defect at lower temperatures and total growth arrest below 20°C. The GraT-mediated growth inhibition is neutralized by GraA, and this involves complex formation between the two proteins (49). Akin to several other toxins, GraT can influence the bacterial stress survival. However, it seems to play a controversial role in the stress tolerance of *P. putida*: while increasing the tolerance to some antimicrobials, it sensitizes the bacteria to others (49). This indicates that GraT-mediated stress protection is costly and may result in harmful side effects if not properly controlled by GraA. Interestingly, GraA seems to be a very efficient antidote to GraT, as one chromosomal copy of *graA* can efficiently neutralize both the innate and the ectopically expressed GraT toxin (49). Such a high efficacy might be explained by the high level of stability of the protein, which is quite unusual among antitoxins. However, there are indications that GraA is relatively stable, as the GraA protein can be easily purified without any addition of protease inhibitors (49).

To get more insight into the stability of the antitoxin GraA, we aimed to determine the conditions and factors important for its degradation rate. We show that, compared to other antitoxins, GraA is uncommonly stable under most growth conditions, and neither the Lon nor the Clp protease that usually degrades antitoxins targets GraA. Moreover, our data suggest that the degradation pathway of GraA involves an endoproteolytic form of cleavage and depends on the growth phase, the ATP level, and the activity of the global transcriptional regulator MexT.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* strains are derivatives of PaW85 (52), which is isogenic to the fully sequenced strain KT2440 (58). Bacteria were grown in lysogeny broth (LB). When selection was needed, the growth medium was supplemented with ampicillin (100 $\mu\text{g} \cdot \text{ml}^{-1}$) or kanamycin (50 $\mu\text{g} \cdot \text{ml}^{-1}$) for *E. coli* and benzylpenicillin (1,500 $\mu\text{g} \cdot \text{ml}^{-1}$), kanamycin (50 $\mu\text{g} \cdot \text{ml}^{-1}$), or streptomycin (300 $\mu\text{g} \cdot \text{ml}^{-1}$) 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 (59).

Construction of plasmids and strains. The oligonucleotides used in PCR amplifications are listed in Table S1 in the supplemental material. The pEMG-based plasmids for the generation of deletion strains were constructed according to the protocol described previously (50). The upstream and downstream sequences (about 500 bp) of the gene to be deleted were amplified separately and joined into one fragment by overlap extension PCR. For deletion of the *lon1* (PP1443) and *lon2* (PP2302) genes, the obtained PCR fragments were cut with EcoRI and BamHI, for deletion of *clpP* (PP2300), with Acc65I and BamHI, and for deletion of *mexT* (PP2826), with SacI and XbaI and ligated into the corresponding sites in the pEMG plasmid, resulting in pEMG- Δ *lon1*, pEMG- Δ *lon2*, pEMG- Δ *clpP*, and pEMG- Δ *mexT*. The intactness of the DNA surrounding the gene deletion was controlled by sequencing. Plasmids were delivered to *P. putida* PaW85 (and, in the case of pEMG- Δ *lon2*, also to the Δ *lon1* strain to generate the double-deletion [Δ 2*lon*] derivative) by electroporation, and, after 3.5 h of growth in the LB medium, the bacteria were plated onto LB agar supplemented with kanamycin. The kanamycin-resistant cointegrates were selected and electrotransformed with the plasmid pSW(I-SceI). In order to resolve the cointegrate, the plasmid-encoded SceI enzyme (I-SceI) was induced overnight with 1.5 mM 3-methylbenzoate. Kanamycin-sensitive colonies were selected, and the deletion of the gene was verified by PCR. Plasmid pSW(I-SceI) was eliminated from the deletion strains by growing them overnight in LB medium without antibiotics.

For purification of the antitoxin, a hexahistidine tag was fused to the N terminus of GraA. The *graA*-containing fragment was amplified by PCR using oligonucleotides A-Nhis and 1585Bam. The NdeI-BamHI-treated PCR fragment was ligated into the corresponding sites of the plasmid pET11c, resulting in pET-hisA. For construction of pET-hisA_{E68A}, pET-hisA_{L79A}, pET-hisA_{R80A}, pET-hisA_{E83A}, pET-hisA_{Q84A}, and pET-hisA_{Q85A}, the site-directed mutagenesis of His₆-*graA* was performed using two sequential PCRs and the plasmid pET-hisA as a template. In the first PCR, one primer carried the substitution mutation (Table S1 in the supplemental material) and the other carried 1585Bam. The product of the first PCR served as a reverse primer for A-Nhis in the second PCR. The product of the second PCR was treated with DpnI, NdeI, and BamHI and ligated into the NdeI-BamHI-opened pET11c. All designed plasmids were sequenced in order to exclude PCR-generated errors in the cloned DNA fragments.

For generation of the *graA*-deficient strain with an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible copy of *graA*_{L79A} (designated the Δ A-*tac*-A_{L79A} strain), the mutant *graA*_{L79A} gene was first amplified from pET-hisA_{L79A} with primers A-Nhis and 1585Acc. The PCR fragment was cut with SacII and Acc65I and cloned into SacII-Acc65I-opened pKtlacItac-A, resulting in replacement of the 3' end of *graA*. The *lacI*^q-P_{tac}-*graA*_{L79A} cassette was excised from the plasmid pKtlacItac-A_{L79A} with BamHI and SacI and ligated into the corresponding sites of the plasmid pUC18NotKm to obtain pUCNotKmlacItac-A_{L79A}. Finally, the *graA*_{L79A} expression cassette was subcloned as a NotI fragment into the miniTn7 delivery plasmid pBK-miniTn7- Ω Gm. The pminiTn7-lacItac-A_{L79A} plasmid was introduced into a *P. putida* *graA*-deficient strain by coelectroporation together with the helper plasmid pUXBF13. The presence of the *graA*_{L79A} expression cassette in the attTn7 site of the Δ A-*tac*-A_{L79A} strain chromosome was verified by PCR.

TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or characteristic(s)	Source or reference
<i>E. coli</i> strains		
DH5 α λ pir	λ pir lysogen of DH5 α	50
BL21(DE3)	<i>hsdS gal</i> (λ cl <i>ts857 ind-1 Sam7 nin-5 lacUV5-T7</i> gene 1)	51
<i>P. putida</i> strains		
PaW85	Wild type, isogenic to KT2440	52
Δ graA strain	PaW85 Δ graA	49
Δ lon1 strain	PaW85 Δ lon1	This study
Δ lon2 strain	PaW85 Δ lon2	This study
Δ 2lon strain	PaW85 Δ lon1 Δ lon2	This study
Δ clpP strain	PaW85 Δ clpP	This study
Δ mexT strain	PaW85 Δ mexT	This study
Δ A-tac-A strain	PaW85 Δ graA containing genomic <i>lacI</i> ^q - <i>P</i> _{tac} - <i>graA</i> expression cassette (Gm ^r)	This study
Δ A-tac-A _{L79A} strain	PaW85 Δ graA containing genomic <i>lacI</i> ^q - <i>P</i> _{tac} - <i>graA</i> _{L79A} expression cassette (Gm ^r)	This study
Plasmids		
pEMG	Suicide plasmid containing <i>lacZ</i> α with two flanking I-SceI sites (Km ^r)	50
pSW(I-SceI)	Plasmid for I-SceI expression (Ap ^r)	53
pEMG- Δ lon1	pEMG plasmid containing a PCR-designed 1.05-kb EcoRI-BamHI insert for deleting <i>lon1</i> (Km ^r)	This study
pEMG- Δ lon2	pEMG plasmid containing a PCR-designed 1.01-kb EcoRI-BamHI insert for deleting <i>lon2</i> (Km ^r)	This study
pEMG- Δ clpP	pEMG plasmid containing a PCR-designed 0.95-kb Acc65I-BamHI insert for deleting <i>clpP</i> (Km ^r)	This study
pEMG- Δ mexT	pEMG plasmid containing a PCR-designed 0.9-kb SacI-XbaI insert for deleting <i>mexT</i> (Km ^r)	This study
pET11c	Protein expression vector (Ap ^r)	Stratagene
pET-hisA	pET11c containing <i>graA</i> with N-terminal His ₆ tag (Ap ^r)	This study
pET-hisA _{E68A}	pET11c containing <i>graA</i> _{E68A} with N-terminal His ₆ tag (Ap ^r)	This study
pET-hisA _{L79A}	pET11c containing <i>graA</i> _{L79A} with N-terminal His ₆ tag (Ap ^r)	This study
pET-hisA _{R80A}	pET11c containing <i>graA</i> _{R80A} with N-terminal His ₆ tag (Ap ^r)	This study
pET-hisA _{E83A}	pET11c containing <i>graA</i> _{E83A} with N-terminal His ₆ tag (Ap ^r)	This study
pET-hisA _{Q84A}	pET11c containing <i>graA</i> _{Q84A} with N-terminal His ₆ tag (Ap ^r)	This study
pET-hisA _{Q85A}	pET11c containing <i>graA</i> _{Q85A} with N-terminal His ₆ tag (Ap ^r)	This study
pKTLacItac-A	Expression plasmid containing <i>graA</i> under the control of <i>lacI</i> and the <i>P</i> _{tac} promoter (Ap ^r)	49
pKTLacItac-A _{L79A}	Expression plasmid containing <i>graA</i> _{L79A} under the control of <i>lacI</i> and the <i>P</i> _{tac} promoter (Ap ^r)	This study
pUC18NotKm	Cloning vector (Km ^r)	54
pUCNotKmlacItac-A _{L79A}	pUC18NotKm containing <i>graA</i> _{L79A} under the control of <i>lacI</i> and the <i>P</i> _{tac} promoter (Km ^r)	This study
pBK-miniTn7- Ω Gm	pUC19-based delivery plasmid for miniTn7- Ω Gm (Ap ^r Gm ^r)	55
pminiTn7-lacItac-A _{L79A}	pBK-miniTn7- Ω Gm containing the <i>lacI</i> ^q - <i>P</i> _{tac} - <i>graA</i> _{L79A} expression cassette (Ap ^r Gm ^r)	This study
pUXBF13	Helper plasmid coding for the Tn7 transposition proteins (Ap ^r <i>mob</i> ⁺)	56
pUTmini-Tn5Sm/Sp	Delivery plasmid for mini-Tn5Sm/Sp (Ap ^r Sm ^r)	57

Purification of proteins. To purify the N-terminally histidine-tagged GraA proteins, nickel affinity chromatography was used. For the overexpression of proteins, the *E. coli* strain BL21(DE3) containing different expression plasmids was first grown in LB medium at 30°C up to an optical density at 580 nm (OD₅₈₀) of ~0.1. Then, the temperature was shifted to 20°C, and protein expression was induced with 0.5 mM IPTG at an OD₅₈₀ of ~0.5. After 4 to 5 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 4°C for 20 min at 16,000 $\times$ g. The supernatant was filtered through a 0.22- μ m filter before it was loaded 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 Akta 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 a 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; and (iii) 10 mM Tris-HCl (pH 7.5), 200 mM KCl, 50% glycerol. The protein purity was estimated to be >95% by silver staining of proteins in a Tricine-SDS gel (see Fig. S1 in the supplemental material). Proteins were stored at -20°C.

For the purification of the N-terminal degradation intermediate of His₆-GraA, the degradation mixture was loaded on a Ni-nitrilotriacetic acid (Ni-NTA) column. The column was washed with buffer A, and proteins were eluted with buffer A containing 500 mM imidazole. The proteins in the eluate were separated on a 10% Tricine-SDS-PAGE gel and stained with Coomassie blue G250. The band corresponding to the degradation fragment of His₆-GraA was excised from the gel, in-gel digested with trypsin, and subjected to analysis by mass spectrometry. As a control, full-length His₆-GraA was analyzed as well.

Protein degradation assay. The cell lysates were prepared from *P. putida* grown in LB media at 30°C to an OD₅₈₀ of ~0.5, 1.5, 2.5, or 3.5 or overnight (OD₅₈₀ of ~4.5). The cells were pelleted, flash frozen, and stored at -80°C. For the degradation experiment, the cells were thawed on ice, washed with ice-cold 1 $\times$ phosphate-buffered saline (PBS) buffer, and resuspended in the same buffer. Bacteria were sonicated, and cellular debris was removed by centrifugation at 16,000 $\times$ g for 12 min at 4°C. The protein concentrations were measured using the Bradford reagent and bovine serum albumin as a standard.

The degradation mixture (80 to 120 μ l) contained 30 ng (1.5 pmol) of His₆-GraA or its mutant derivative per 50 μ g of the cell lysate protein. In some experiments, ATP, ADP, or GTP at a final concentration of 4 mM and MgCl₂ at a final concentration of 5 mM was added to the mixture. The degradation mixture was incubated at 30°C, and samples of 10 μ l were removed at the time points indicated (see Fig. 1). Proteins were denatured at 96°C, separated on a 10% Tricine-SDS-PAGE gel, and transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes. His₆-GraA was detected either with HisProbe-horseradish peroxidase (HRP) (Pierce) or by probing membranes with anti-GraA antibodies, followed by treatment with alkaline phosphatase-conjugated anti-mouse immunoglobulin G. The blots with antibodies against GraA were developed using bromochloroindolyl phosphate-nitroblue tetrazolium (BCIP-NBT). Blots with HisProbe-HRP were developed using the Pierce enhanced-chemiluminescence (ECL) substrate. Band intensities were quantified with ImageQuant TL software.

Protein-ATP binding assay. The differential radial capillary action of ligand assay (DRaCALA) was carried out as described elsewhere (60). Mixtures containing 20 μ M His₆-GraA or, as a control, ColS-His₆ (61), 4 nM [γ -³²P]ATP, and 4 μ M unlabeled ATP in binding buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol) were incubated for 10 min at room temperature. Then, 5 μ l of mixture was pipetted onto a dry untreated nitrocellulose membrane (GE Healthcare) in triplicate and allowed to dry completely before a 5-min exposure on a PhosphorImager screen. Images were scanned with a Typhoon Trio imager (GE Healthcare).

Transposon mutagenesis screen of the Δ A-tac-A_{L79A} strain. For the identification of factors implicated in the degradation of GraA, the *graA*-deficient strain complemented with the *graA*_{L79A} gene (Δ A-tac-A_{L79A}) was subjected to mutagenesis using a Tn5-based mini-transposon that contains a streptomycin resistance marker. Plasmid pUTmini-Tn5Sm/Sp was conjugatively transferred from *E. coli* CC118 λ pir into the *P. putida* Δ A-tac-A_{L79A} strain with the aid of the helper plasmid pRK2013. Transconjugants with random chromosomal insertions of the minitransposon were selected on minimal 0.2% gluconate plates supplemented with streptomycin and 0.3 mM IPTG at 20°C. Primary screening of about 250,000 transconjugants yielded 460 suppressor clones that had lost the GraA-caused growth arrest at 20°C. To find the suppressor mutants that depend on expression of GraA_{L79A}, the secondary screen on gluconate plates with or without 0.3 mM IPTG was performed at 20°C. Five clones that grew in the presence of 0.3 mM IPTG but not in the medium without IPTG were subjected to arbitrary PCR and sequencing as described previously (62).

Statistical analyses. For single pairwise comparisons, Student's *t* test was applied. For multiple comparisons, analysis of variance (ANOVA) was performed. If several groups were compared with one control group, the two-sided Dunnett *post hoc* test was performed, and, if all groups were compared with all others, Tukey's *post hoc* test was used instead. The analyses were carried out using the Statistica 10 software.

RESULTS

GraA is least stable at the transition to stationary phase. To evaluate the degradation rate of GraA, we used Western blot analysis with an anti-GraA polyclonal antibody. Since endogenous GraA was not detectable, we introduced an extra copy of *graA* under the control of an IPTG-inducible promoter into the chromosome of wild-type *P. putida*. Unfortunately, as a large portion of the overexpressed GraA was produced as insoluble protein (data not shown), *in vivo* characterization of GraA stability was impossible. Therefore, we used an *in vitro* approach, and the half-life of purified His-tagged GraA was assessed in the cell lysates of *P. putida*. To investigate if His₆-GraA degradation depends on the growth phase of bacteria, the lysates were prepared from bacteria taken from different time points over the growth curve (Fig. 1A). His₆-GraA turned out to be extremely stable in cell extracts prepared

from logarithmic (OD₅₈₀ of ~0.5)- and late-stationary (OD₅₈₀ of ~4.5)-phase bacteria, with a half-life of more than 4 to 5 h (Fig. 1B and C; see also Table S2 in the supplemental material). Yet, at the transition from logarithmic to stationary phase (OD₅₈₀ of ~2.5), the degradation of antitoxin was considerably faster, with a *t*_{1/2} of about 1 h (Fig. 1B and C; see also Table S2 in the supplemental material).

During the degradation of His₆-GraA (11.7 kDa), a smaller band of about 10 kDa sometimes appeared below the full-length antitoxin band on the Western blot (Fig. 1B, gel IV). As this small protein was detected sporadically in some experiments, we assumed that it may represent an unstable intermediate of GraA degradation. To find out if this protein originates from His₆-GraA, we probed the degradation mixtures with His-Probe-HRP. This revealed that the small product was a His-tagged peptide that probably comprised the N terminus of the antitoxin His₆-GraA (Fig. 1D). To further analyze the origin of the ~10-kDa peptide, the degradation intermediate was purified and identified by mass spectrometry. Comparison of the trypsin-generated peptides of full-length His₆-GraA with that of the ~10-kDa protein revealed that while the degradation intermediate possesses the N-terminal part of His₆-GraA, it lacks the C-terminal tryptic peptide TAEQQ HGDEIIGSVQR (amino acids 81 to 96 in GraA). Thus, the ~10-kDa degradation intermediate of His₆-GraA is truncated from its C terminus.

These data suggest that the degradation of GraA is most rapid at the transition from the logarithmic to stationary phase. The appearance of a discrete His-tagged degradation intermediate suggests that GraA degradation may involve cleavage by an unknown endoprotease.

GraA is not degraded by the Lon or ClpP protease and is instead stabilized by ATP. Most TA system antitoxins are degraded by the ATP-dependent proteases Lon or Clp (31), which processively degrade their substrates to oligopeptides (63). Although our results imply that GraA degradation involves endoproteolytic cleavage, we decided to test whether Lon or ClpP can also influence the stability of GraA. There are two Lon protease-encoding genes in the chromosome of *P. putida* PaW85, PP1443 (*lon1*) and PP2303 (*lon2*); the ClpP protease is encoded by PP2300. To determine the impact of Lon or ClpP on the stability of GraA, we constructed the single and double *lon* deletion strains and the *clpP* deletion strain. Construction of a protease triple-deletion strain was, unfortunately, impossible. Comparison of the degradation rates of the purified His₆-GraA added to cell lysates of the wild type as well as of protease-deficient bacteria showed that the antitoxin was equally stable in all lysates (Fig. 2 [only data for the Δ 2lon strain are shown]; see also Table S2 in the supplemental material).

To rule out the possibility that low ATP or Mg²⁺ levels limit the activity of Lon or Clp proteins, the degradation mixtures were supplied with 4 mM ATP and 5 mM MgCl₂. Contrarily to our expectations, addition of ATP remarkably increased the stability of His₆-GraA in both wild-type and protease-deficient lysates (Fig. 2 [only data for the Δ 2lon strain are shown]; see also Table S2 in the supplemental material). To test whether the stabilization of GraA is specific to ATP, we measured the His₆-GraA degradation dynamics also in the presence of ADP and GTP. However, neither of these nucleotides influenced the antitoxin stability in cell lysate (see Fig. S2 and Table S2 in the supplemental material).

To test whether the antitoxin stabilization could be the result of direct binding of ATP to GraA, the ligand-binding DRaCALA as-

FIG 1 The stability of His₆-GraA depends on growth phase. (A) Growth curve of *P. putida* in LB broth at 30°C. Dots represent the time points when the cells for lysates were collected (I, OD₅₈₀ ~0.5; II, OD₅₈₀ ~1.5; III, OD₅₈₀ ~2.5; IV, OD₅₈₀ ~3.5; V, OD₅₈₀ ~4.5). (B) Immunoblot analysis (using anti-GraA antibodies) of His₆-GraA incubated in cell lysates of bacteria at different stages of growth (gels I through V). Numbers above and to the right of the images indicate reaction times and protein half-lives ($t_{1/2}$), respectively. GraA is indicated with > signs, and the + sign shows the unstable degradation intermediate of GraA sometimes detected on the Western blot. L, lysate; A, antitoxin; L + A, lanes of degradation assay. Representative images from at least three independent experiments are shown. The data from all experiments were analyzed with ANOVA using Tukey's *post hoc* test. Differences were confirmed between all time points ($P < 0.005$) except those for IV versus II and III ($P = 0.2$ and 0.6 , respectively) and I versus V ($P = 0.1$). ND, not determined. (C) Quantification of GraA bands from immunoblots in panel B. (D) Immunoblot analysis of His₆-GraA using HisProbe-HRP. The lower picture is an overexposed (OE) version of the upper picture. +, ~10-kDa histidine-rich peptide that appears during the degradation of His₆-GraA. The 7-h and 23-h samples were loaded in duplicate.

say was performed. Data in Fig. 2B show that there is no direct binding between ATP and His₆-GraA.

Thus, ATP-dependent Lon and ClpP proteases are not responsible for the degradation of GraA. Intriguingly, our data show that

GraA is stabilized by higher levels of ATP, indicating that antitoxin degradation may be controlled by the energy status of the cell.

Positions L79 and R80 are important for the degradation rate of GraA. Our data suggest that degradation of GraA may be initi-

FIG 2 The degradation rate of His₆-GraA is not affected by Lon1 and Lon2 proteases. (A) Immunoblots of GraA (detected by anti-GraA antibodies) degradation in cell lysates of the wild-type (wt) and doubly Lon protease deficient *P. putida* (Δ lon) strain prepared at an OD₅₈₀ of ~2.5. Numbers above and to the right of the images indicate reaction times and protein half-lives, respectively. Representative images from at least three independent experiments are shown. The data from all experiments were analyzed with ANOVA using Tukey's *post hoc* test. *, $P < 0.05$; ***, $P < 0.0005$. (B) DRACALA experiment testing ATP binding to a protein. The ColS histidine sensor kinase of the ColRS signal system was used as a positive control.

FIG 3 Effects of substitution mutations on the degradation rate of GraA. (A) Immunoblots of His₆-GraA (detected by anti-GraA antibodies) variants that possess the E68A, L79A, R80A, E83A, Q84A, or Q85A substitution mutation. Each protein was mixed with cell lysate prepared from wild-type *P. putida* at an OD₅₈₀ of ~2.5. Numbers above and to the right of the images indicate reaction times and protein half-lives, respectively. (B) Immunoblot of degradation of His-GraA(L79A) (indicated with a > sign) in a shorter time frame than in panel A. L, lysate; L+A_{L79A}, lanes of degradation assay. Representative images from at least three experiments are shown. The data from all experiments were analyzed with ANOVA using the two-sided Dunnett's *post hoc* test, comparing each mutant protein to His₆-GraA. *, *P* < 0.05; ***, *P* < 0.0005.

ated by endoproteolytic cleavage. The occasionally detected degradation intermediate of His₆-GraA was about 10 kDa (about four-fifths of the whole 105-amino-acid His₆-GraA protein), and mass spectrometry analysis confirmed that it is indeed the N-terminal portion of His₆-GraA. Although the mass spectroscopy analysis could not determine the exact cleavage site, it indicated that the degradation intermediate lacks the C-terminal trypsin-generated peptide TAEQQHGDEIIGSVQR, which comprises the GraA sequence from amino acid residues 81 to 96. Thus, we estimated that the cleavage site is somewhere around the 80th amino acid of the antitoxin and constructed His₆-GraA variants with amino acid replacements near the 80th position, i.e., E68A, L79A, R80A, E83A, Q84A, and Q85A. The mutant proteins were purified and analyzed in the degradation experiments with wild-type cell lysate prepared from the transition phase (OD₅₈₀ of ~2.5). The results clearly show that the mutations at positions L79 and R80 change the stability of GraA. While GraA_{R80A} is significantly more stable than the wild-type

FIG 4 Transpositional inactivation of *mexT* suppresses GraT-caused growth arrest. *P. putida* wild-type (wt) strain PaW85, the Δ*graA* strain complemented with the *lacI*-*P*_{tac}-*graA*_{L79A} expression cassette (Δ*AtacA*_{L79A}), and the transposon insertion derivative of the Δ*tac*-*A*_{L79A} strain (Δ*AtacA*_{L79A} mexT::TnSm) were serially diluted and spotted onto LB agar or LB with 0.3 or 0.5 mM IPTG. Plates were incubated at 20°C for 24 h.

protein, GraA_{L79A} is degraded very rapidly, with a *t*_{1/2} of 12 min (Fig. 3; see also Table S2 in the supplemental material). Other mutations did not change the degradation rate of GraA significantly (Fig. 3A; see also Table S2). These data suggest that the position of the endoproteolytic cleavage of GraA is near the 80th amino acid of the antitoxin.

The disruption of MexT rescues bacteria from the GraT-caused growth arrest. In order to search for factors that might be involved in the regulation of the GraA stability, we took advantage of the rapid degradation rate of GraA_{L79A}. We hypothesized that if we inserted the mutant *graA* gene into a Δ*graA* strain and induced GraA_{L79A} expression at a low level, it should not complement the lack of GraA, because the degradation speed of GraA_{L79A} would exceed its synthesis rate. These cells would effectively be GraA deficient and would not form colonies on a plate at 20°C due to the GraT-mediated growth defect. A transposon insertion into the putative GraA-degrading protease gene would increase the half-life and, thus, the cellular concentration of GraA_{L79A}, which would then suppress GraT and allow growth at 20°C.

To this end, we constructed a Δ*graA*-derivative strain, the Δ*tac*-*A*_{L79A} strain, that enabled the IPTG-inducible expression of GraA_{L79A}. To test whether the expression of GraA_{L79A} could save the bacteria from the GraT-caused growth defect, bacteria were grown in the presence of different concentrations of IPTG. Figure 4 shows that overexpression of GraA_{L79A} with 0.5 mM IPTG can suppress the growth arrest at 20°C, indicating that mutated GraA is able to neutralize GraT. However, the effect of overexpressed GraA_{L79A} is partial, which is in accordance with its high instability. As a slightly lower induction level of GraA_{L79A} with 0.3 mM IPTG was insufficient for growth recovery, we concluded that these conditions would be suitable for the screening of suppressor mutants. Screening of about 250,000 transposon insertion derivatives of the Δ*tac*-*A*_{L79A} strain disclosed 460 mutants able to grow at 20°C on plates supplemented with 0.3 mM IPTG. These mutants were subjected to a secondary screen by analyzing them on plates with or without 0.3 mM IPTG. This screen enabled us to select the mutants that depended on the production of GraA_{L79A} and exclude those that grew due to mutations that inactivated the toxin gene. Five GraA_{L79A}-dependent mutants, i.e., those that grew only on the IPTG-containing plates, were identified (Fig. 4). Sequencing of the transposon insertion sites revealed that in all five mutants, the *mexT* gene (PP2826) was inactivated. One of the five insertions was directly in front of the gene, most probably disrupting the expression of *mexT*. Four mutants were probably siblings, as they contained identical transposon insertions in the middle of the gene.

FIG 5 The deletion of *mexT* stabilizes GraA. (A) Immunoblots of His₆-GraA (indicated with a > sign and detected by anti-GraA antibodies) in cell lysates of the wild-type (wt) and *MexT*-deficient *P. putida* (*ΔmexT*) prepared at an OD₅₈₀ of ~2.5. Numbers above and to the right of the images indicate reaction times and protein half-lives (*t*_{1/2}), respectively. L, lysate; L+A, lanes of degradation assay. Representative images from at least three independent experiments are shown. The data were analyzed with Student's *t* test. ***, *P* < 0.0005. (B) Quantification of GraA bands from immunoblots on panel A.

The degradation of GraA is slower in the absence of the regulator *MexT*. The *mexT* (PP2826) gene codes for a transcriptional regulator and is annotated to be the first gene of an operon also encoding a putative phage integrase (PP2825) and a TetR family transcriptional regulator (PP2824). To eliminate the possibility that transpositional inactivation of *mexT* has a polar effect on expression of downstream genes and to provide more evidence of *MexT* influence on the stability of GraA, we constructed a *ΔmexT* strain by using a markerless gene deletion procedure. Notably, deletion of *mexT* did not affect bacterial growth, as the *ΔmexT* strain was identical to the wild-type *P. putida* strain in growth rate and overall growth curve (data not shown). The *in vitro* degradation experiment with both His₆-GraA_{L79A} and His₆-GraA demonstrated that both proteins were significantly more stable in the cell lysate of *ΔmexT* bacteria; the half-life increased about 3.5 times for both proteins (Fig. 5A and B; see also Table S2 in the supplemental material). These results show that *MexT* is indeed involved in regulating the degradation of GraA. Furthermore, given that the *in vitro* degradation experiments verified the *in vivo* transposon mutagenesis results, the *in vitro* approach seems to be relevant in studying GraA stability.

DISCUSSION

The activation of a type II TA system toxin depends on the proteolytic degradation of the antitoxin (31, 64, 65). Therefore, the identification of cellular and environmental factors that influence the stability of the antitoxin is crucial to understand the regulation and functioning of a particular TA system. In this study, we provide evidence that the stability of the antitoxin GraA of the GraTA system is influenced by several factors, including growth phase, ATP level, and *MexT*.

TA systems are important in stress response (32, 66), and in accordance with this, the degradation rate of the antitoxin also depends on growth conditions and is increased upon stress. For

instance, carbon or amino acid deficiency or the presence of different antibiotics increases (p)ppGpp levels that lead to Lon protease activation and the degradation of antitoxins in *E. coli* (14). This results in the liberation of cognate toxins and the inhibition of growth, which help bacteria to survive unfavorable conditions (14, 32, 67, 68). Our data suggest that, as with other TA systems, the activation of the GraTA system may also be triggered by stressful conditions, as we show that the stability of GraA is responsive to growth phase. While GraA is unusually stable in rapidly growing exponential-phase cells and also in late-stationary-phase cells, its degradation rate increases at the onset of stationary phase (Fig. 1B and C). This suggests that some transition phase-specific factor(s) is involved in destabilizing GraA. Interestingly, it has been shown that the intracellular level of ATP decreases drastically at the transition from logarithmic to stationary phase, after which the ATP pool slightly increases again in stationary phase (69). Considering that GraA is stabilized by exogenous ATP (Fig. 2; see also Fig. S1 in the supplemental material), it is possible that the decreased ATP level in the transition phase is one reason for the increased degradation rate of GraA in this phase of growth. The physiological meaning of the faster degradation of GraA in the transition phase may be that the GraT-mediated growth rate inhibition helps bacteria adapt to changing conditions. Our data suggest that the protective effect of ATP on antitoxin stability is indirect and does not involve the direct binding of ATP to GraA. Therefore, we hypothesize that the activation of the degradation machinery that influences GraA stability depends on the level of ATP.

Most TA system antitoxins characterized so far are targeted by the Lon or Clp protease, which processively degrades the whole protein (31, 63), yet the degradation of GraA seems to differ from that of the previously described antitoxins, because neither Lon nor ClpP degraded GraA (Fig. 2). Rather, our data indicate that an unknown endoprotease is involved in the proteolysis of GraA, because a discrete degradation intermediate was sometimes observed on Western blots. Moreover, as the degradation intermediate was a histidine-rich peptide lacking the C-terminal end of GraA, and as the mutations in positions L79 and R80 drastically changed the stability of GraA (Fig. 3), we have reason to suspect that the cleavage site of the endoprotease is located near the 79th and 80th amino acids of the antitoxin. Given that the GraA degradation intermediate was detected only occasionally, the C-terminally truncated protein is probably highly unstable, which suggests that endoprotease-mediated cleavage is meant to trigger or accelerate the degradation of GraA.

We have previously shown that GraA is very efficient at inactivating GraT, because one chromosomal copy of *graA* was able to neutralize both the innate and the ectopically expressed GraT (49). This suggested that GraA is a highly stable protein and even led us to wonder whether the GraTA system could be activated at all. Indeed, for activation of a TA system, the antitoxin should be degraded quite rapidly to counter its continuous synthesis; otherwise, the toxin would always be in an inactive form. Our current finding that unstable GraA_{L79A} was not as successful at inactivating the toxin as wild-type GraA (Fig. 4), however, suggests that, as with other type II TA systems (8, 31), the stability features of GraA are important for the regulation of GraT toxin activity. Nevertheless, the rate of degradation of GraA differs markedly from that of previously studied antitoxins. While the half-lives of most antitoxins are usually measured in minutes (for HipB and RelB in *E.*

coli, 17 and 15 min, respectively; for MazE in *Staphylococcus aureus*, 18 min) (7, 35, 70), GraA is very stable under most conditions, with a half-life greater than 4 h. Even at the transition phase, where the measured half-life is about 1 h, the degradation of GraA is still quite slow compared to those of most other antitoxins. Yet, we have to consider that our results were obtained from *in vitro* experiments, so it is possible, therefore, that the degradation of GraA *in vivo* would be faster. This has previously been observed for HipB and RelB antitoxins, which are about four times more stable *in vitro* than *in vivo* (7, 35, 39). Still, the *in vitro* results for HipB and RelB have been obtained by mixing together purified protease with the antitoxin and may lack various cellular factors important for degradation (7, 35, 39). The approach that we used includes whole-cell lysate, so all cellular factors that might affect the degradation characteristics are present, and the *in vitro* measured degradation rate should be quite similar to the *in vivo* degradation rate.

The relatively high stability of GraA is comparable to that of the antitoxin MqsA from the MqsRA system of *E. coli*, which is also a quite stable protein ($t_{1/2}$ of about 1 h). Interestingly, the degradation rate of MqsA increases enormously during oxidative stress ($t_{1/2}$ of only 1.25 min) (18, 45). Thus, we cannot rule out the possibility that GraA also is degraded in response to a specific, so-far-undiscovered stress.

The screen for the suppressor mutants that stabilize GraA gave an unexpected result. Instead of discovering any GraA-degrading proteases, we identified the global transcription factor MexT as a stability factor of GraA. The reason for not identifying the protease gene in our suppressor screen may be that this putative protease is essential or that its locus contains other essential genes, the expression of which is disturbed due to a polar effect of the transposon insertion. It is also possible that the screen was not saturated, although this seems unlikely, because suppressor mutants with a disrupted *mexT* gene were identified repeatedly.

MexT, a LysR-type transcription regulator, has been a subject of intense investigation in *Pseudomonas aeruginosa*, where it modulates antibiotic tolerance and virulence (71, 72). MexT in *P. putida* PaW85 is about 84% to 87% identical to MexTs in different *P. aeruginosa* strains (73), which reflects its similar functions in these bacteria. Indeed, in both of the species, MexT has been shown to positively regulate the MexEF-OprN efflux pump and to have many other common target genes (74, 75), some of which have unknown functions, so their cellular importance still needs investigation (74). MexT is known to be quiescent in *P. aeruginosa* under standard laboratory growth conditions and somehow inactivated by MexS, which is encoded by an adjacent gene that is transcribed divergently from *mexT* (76, 77). We have studied the antitoxin stability in rich medium at the optimal growth temperature of 30°C, where the MexT-activated genes probably have a low expression level. Interestingly, it has recently been shown that MexT functions as a redox-responsive regulator, which is activated under oxidative conditions and which modulates disulfide stress resistance (71). Considering that MexT is a negative regulator of GraA stability (Fig. 5), it is tempting to speculate that the quiescence of the MexT regulator is one of the reasons for the low degradation rate of GraA under unstressed conditions. Furthermore, one may hypothesize that alterations in the cellular redox state may lead to activation of MexT and, therefore, to the MexT-controlled factors that are responsible for the degradation of GraA.

FIG 6 Hypothetical model for regulation of GraA degradation. Solid lines represent links with experimental proofs. Dashed lines show possible links, which may include intermediate steps or some additional factors.

As the disruption of the *mexT* gene stabilized GraA and allowed the GraT-affected $\Delta A\text{-}tac\text{-}A_{L79A}$ cells to grow at low temperature, the degradation of GraA indeed is a crucial part of the regulation of the activity of the GraTA system. Our future studies will be directed at understanding the connections between MexT and GraA stability, and we will try to determine the conditions under which the antitoxin is degraded faster and the GraTA system is activated so that we can start to understand the physiological importance of the GraTA system. Nevertheless, considering the factors that we have identified so far to be important in the stability of GraA, we propose a possible model for the regulation of GraA stability that includes activation of MexT, possibly by alterations in the cellular redox state, and a decrease in ATP levels (Fig. 6). We hypothesize that these stress conditions will ultimately lead to activation of an unknown endoprotease that triggers the degradation of GraA by cleavage of about 20 amino acids from its C terminus. After the degradation of the antitoxin GraA, the released toxin GraT can affect its cellular target and regulate the growth of *P. putida*.

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