Recycling of Peptidyl-tRNAs by Peptidyl-tRNA Hydrolase Counteracts Azithromycin-Mediated Effects on *Pseudomonas aeruginosa*

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Acute and chronic infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* pose a serious threat to human health worldwide, and its increasing resistance to antibiotics requires alternative treatments that are more effective than available strategies. Clinical studies have clearly demonstrated that cystic fibrosis (CF) patients with chronic *P. aeruginosa* infections benefit from long-term low-dose azithromycin (AZM) treatment. Immunomodulating activity, the impact of AZM on the expression of quorum-sensing-dependent virulence factors, type three secretion, and motility in *P. aeruginosa* seem to contribute to the therapeutic response. However, to date, the molecular mechanisms underlying these AZM effects have remained elusive. Our data indicate that the AZM-mediated phenotype is caused by a depletion of the intracellular pools of tRNAs available for protein synthesis. Overexpression of the *P. aeruginosa* peptidyl-tRNA hydrolase, which recycles the tRNA from peptidyl-tRNA drop-off during translation, counteracted the effects of AZM on stationary-phase cell killing, cytotoxicity, and the production of rhamnolipids and partially restored swarming motility. Intriguingly, the exchange of a rare for a frequent codon in *rhlR* also explicitly diminished the AZM-mediated decreased production of rhamnolipids. These results indicate that depletion of the tRNA pools by AZM seems to affect the translation of genes that use rare aminoacyl-tRNA isoacceptors to a great extent and might explain the selective activity of AZM on the *P. aeruginosa* proteome and possibly also on the protein expression profiles of other bacterial pathogens.

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that causes both life-threatening acute and devastating chronic infections in the human host (1). In cystic fibrosis (CF) patients, the respiratory tract is especially prone to chronic infections caused by the most dominant bacterial pathogen, *P. aeruginosa*, and these infections largely determine the fate and prognosis of these patients (2, 3). Improved antimicrobial treatment strategies have greatly increased the life expectancy of CF patients in the last decades. Nevertheless, even aggressive antimicrobial therapy rarely eradicates established chronic *P. aeruginosa* infections (4, 5, 6). Hence, for the management of chronic infectious diseases, there is a strong need for alternative treatment strategies that amend classical antimicrobial therapy (7, 8, 9). Several clinical studies have demonstrated that CF patients and patients suffering from diffuse panbronchiolitis (DPB) who are chronically infected with *P. aeruginosa* benefit from treatment with the macrolide azithromycin (AZM), although the 14- and 15-C macrolides (erythromycin, azithromycin, and clarithromycin) do not inhibit the growth of *P. aeruginosa* at concentration levels below the breakpoint concentration for susceptibility to the macrolides (10, 11, 12, 13, 14, 15).

The nature of this beneficial effect of AZM is still unclear. Macrolides were shown to have immunomodulatory activity, which results in a decreased inflammatory response to bacterial stimulation (16), and there have been several studies demonstrating that macrolides inhibit virulence factor production in *P. aeruginosa* *in vitro* and *in vivo* and interfere with biofilm formation (17, 18, 19, 20, 21, 22).

Although macrolides are antibacterial agents that target the protein synthesis machinery, AZM at subinhibitory concentrations was demonstrated to both activate and repress the transcription of different subsets of genes in *P. aeruginosa* (23, 24). It re-

mains uncertain how these effects on transcription are mediated. Recently, it was clearly shown that bacterial stationary-phase cell killing and reduced expression of quorum-sensing (QS)-dependent virulence factors require the interaction of AZM with the ribosome (25). This finding indicates that there are no nonribosomal targets of AZM, which might explain the AZM-mediated effects on *P. aeruginosa*. Furthermore, it was demonstrated that the bacteriotoxic activity of the macrolides is presumably caused by a combination of inhibition of protein elongation and depletion of the intracellular pools of aminoacyl-tRNAs by drop-off and incomplete peptidyl-tRNA hydrolase (Pth) activity (26, 27, 28). Pth is an essential enzyme, and it releases tRNA from the premature translation termination product peptidyl-tRNA by cleaving the ester bond between the peptide and the tRNA, thus allowing the tRNA species to return to the pool of accessible tRNAs available for protein synthesis (29).

In this study, we analyzed to what extent a decreased intracellular tRNA pool in *P. aeruginosa* also contributes to the observed AZM-mediated phenotype in respect to virulence-factor production, motility, and stationary-phase cell killing. Our results show that increasing the intracellular pools of tRNAs by overexpressing the peptidyl-tRNA hydrolase encoded by PA4672 in *P. aeruginosa* cells clearly counteracted AZM-induced stationary-phase killing, reduced rhamnolipid and pyocyanin production and swarming

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TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain or plasmid	Relevant genotype or primer sequence ^a	Source or reference
Strains		
<i>E. coli</i> DH5α	F [−] <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> ϕ80 <i>dlacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169 hsdR17</i> (r _K [−] m _K ⁺) λ [−]	30
PA14	<i>Pseudomonas aeruginosa</i> PA14 wt	46
PA14 wt control	PA14 wt (pHERD20T), Cb ^r	This study
PA14- <i>pth</i>	PA14 (pHERD- <i>pth</i>), Cb ^r	This study
PA14 <i>rhlR</i>	<i>rhlR</i> transposon mutant from the NR PA14 transposon mutant library, ID 37943, Gm ^r	46
Plasmids		
pHERD20T	Shuttle vector, Cb ^r	31
pHERD- <i>pth</i>	pHERD20T carrying <i>pth</i> , Cb ^r	This study
pET21A	Expression vector, Ap ^r	Novagen
pUCP20	Shuttle vector, Ap ^r -Cb ^r	47
pUCP20:: <i>rhlR</i>	<i>rhlR</i> cloned into the EcoRI-XbaI site in MCS, Ap ^r -Cb ^r	This study
pUCP20:: <i>rhlR</i> -R2R	<i>rhlR</i> cloned into the EcoRI-XbaI site in MCS, Ap ^r -Cb ^r	This study
Primers		
<i>pth</i> -RBS-NcoI-fw	5′-GACCATGGAAAGAGGAGAAATACTAGGTGACTGCCGTACAACATGAT ^b	This study
<i>pth</i> -PstI-rev	5′-CGACTGCAGTCAGGCCTTCTGGCTGTG	This study
<i>rhlR</i> -NdeI-fw	5′-TATCATATGAGGAATGACGGAGGCTTT	This study
<i>rhlR</i> -(R ^{AGG} -2-R ^{CGC})-NdeI-fw	5′-TATCATATGCGCAATGACGGAGGCTTT	This study
<i>rhlR</i> -HindIII-rv	5′-TATAAGCTTTCAGATGAGACCCAGCG	This study
pET21A-pUCP20-fw	5′-GATCTCTAGATAGCAGCCGATCTCAGT	This study
pET21A-pUCP20-rv	5′-GATCGAATTCTTTTGTTAACTTAAAGAAGGAGATATAC	This study

^a Engineered restriction sites are underlined. Cb^r, carbenicillin resistance; Gm^r, gentamicin resistance; Ap^r, ampicillin resistance; MCS, multiple-cloning site.
^b Boldface indicates ribosomal binding site.

motility, and increased cytotoxicity. Furthermore, the AZM effect on rhamnolipid production might be explicitly diminished by the exchange of a rarely used for a frequently used codon for arginine at the second position of *rhlR*. Our results suggest that AZM has global impacts on the expression of proteins and selectively impairs the expression of those proteins with an increased frequency of rare codons.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are summarized in Table 1. The *Escherichia coli* DH5α and *Pseudomonas aeruginosa* PA14 (PA14) strains were routinely grown in lysogeny broth (LB) at 37°C with or without the addition of azithromycin (AZM) (Pfizer, Germany) at various concentrations. For solidification, agar was added to a final concentration of 1.5% (wt/vol). We added 100 μg/ml ampicillin and 400 μg/ml carbenicillin to inhibit the growth of *E. coli* DH5α and *P. aeruginosa* PA14, respectively.

DNA manipulations were performed according to standard protocols or following the manufacturers’ instructions. Kits for the isolation of chromosomal DNA, isolation of plasmids, and purification of PCR products were purchased from Qiagen GmbH (Hilden, Germany). Enzymes were purchased from Roche Diagnostics Deutschland GmbH (Mannheim, Germany) and Fermentas (St Leon-Rot, Germany).

For overexpression of PA4672 (*pth*), the plasmid pHERD-*pth* was constructed. The *pth* gene was PCR amplified using PA14 genomic DNA as the template. The primers used are listed in Table 1. The resulting PCR product, including an artificial ribosomal binding site, was ligated in frame into the NcoI-PstI site of pHERD20T (31). The resulting vector was introduced into PA14 by electroporation, and a pHERD20T empty plasmid was introduced into PA14 to serve as a wild-type (wt) control. We induced *pth* overexpression with 0.2% (wt/vol) arabinose at an optical density at 600 nm (OD_{600 nm}) of 0.3 to 0.5.

To generate the plasmids pUCP20::*rhlR* and pUCP20::*rhlR*-R2R (carrying the *rhlR* gene with the codon change from AGG to CGC at the

second position), the *rhlR* gene was amplified from *P. aeruginosa* PA14 chromosomal DNA using the forward primer *rhlR*-(R^{AGG}-2-R^{CGC})-NdeI-fw or *rhlR*-NdeI-fw together with the reverse primer *rhlR*-HindIII-rv. These PCR products were digested with NdeI-HindIII and in a first step were cloned into similarly digested pET21A, resulting in the plasmids pET21A::*rhlR*-(R^{AGG}-2-R^{CGC}) and pET21A::*rhlR*, which were verified by sequence analysis (data not shown). Both plasmids were separately used as the templates for a second PCR using the primers pET21A-pUCP20-fw and pET21A-pUCP20-rv. The PCR products were digested with EcoRI-XbaI and finally cloned into similarly digested pUCP20, resulting in the plasmids pUCP20::*rhlR*-R2R and pUCP20::*rhlR*, which were verified by sequence analysis (data not shown).

Total tRNA isolation and hybridization with a Cy3-tagging oligonucleotide. Total tRNA was isolated by mixing 10 ml cell culture with 10 ml cold 10% (wt/vol) trichloroacetic acid. After centrifugation (3,500 × g for 15 min at 4°C), the cell pellet was resuspended in 0.5 ml of ice-cold lysis buffer (0.3 M sodium acetate [pH 4.5] and 10 mM Na₂EDTA) and transferred to a fresh 1.5-ml Eppendorf tube (on ice). The lysate was mixed with 0.5 ml acetate-saturated phenol-CHCl₃ (pH 4.5), vortexed three times for 15 s, and placed on ice for 1 min between each vortex mixing to ensure that the samples remained cold. After centrifugation at 20,817 × g for 2 min at 4°C, the aqueous layer was removed and subjected to another extraction with 1 volume acetate-saturated phenol-CHCl₃ (pH 4.5) solution. The mixture was vortexed for 15 s and centrifuged at 20,817 × g for 10 min at 4°C. The nucleic acids (essentially only tRNA) were recovered from the aqueous phase by precipitation with 1 ml ethyl alcohol (EtOH) (95 to 100%) for 15 min at −20°C followed by centrifugation at 20,817 × g for 30 min at 4°C. Finally, the precipitated RNA was resuspended in 100 μl ice-cold 10 mM sodium acetate (NaOAc)/HOAc (pH 4.5). Periodate oxidation of tRNA was adapted from a previous study by Dittmar and colleagues (32). For the tRNA oxidation reaction, tRNA with a concentration of 200 ng/μl was mixed with 25 μl NaIO₄ (50 mM) dissolved in KOAc/HOAc (200 mM [pH 4.8]). The control reaction mixture contained the same amount of tRNA and 25 μl NaCl (50 mM) dissolved in

FIG 1 Overexpression of peptidyl-tRNA hydrolase (Pth) leads to an elevated level of uncharged tRNAs. (A) Agarose gel (2%) with fluorescent Cy3-labeled tRNA after a ligation reaction and (B) densitometric analysis of each fluorescent band. Cy3 tagging oligonucleotide-labeled tRNA from the PA14 wild-type (wt) control pretreated with (1) NaCl or (2) NaIO₄ and from the *pth*-overexpressing PA14 (PA14-*pth*) pretreated with (3) NaCl or (4) NaIO₄. Shown are representative results from three independent experiments.

KOAc/HOAc (200 mM [pH 4.8]). The mixture was incubated at 22°C for 30 min in the dark and the oxidation reaction was quenched for 5 min with glucose (100 mM). The tRNAs were then purified by the use of G25 spin columns and ethanol precipitation. Prior to the ligation reaction, the Cy3-tagging oligonucleotide was subjected to a T4 polynucleotide kinase (T4PNK) treatment. Both samples (control and oxidized tRNA) were ligated with the T4PNK-treated Cy3-tagging oligonucleotide under the following conditions: 66.6 ng/μl tRNA and 7.5 μM Cy3-tagging oligonucleotide in 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 10 mM dithiothreitol (DTT), 1 mM ATP, 13% dimethyl sulfoxide (DMSO), and 1 U T4 DNA ligase for 15 h at 16°C. The ligation reaction was separated using 2% agarose gel electrophoresis, and fluorescence intensity was detected by an FLA-9000 FujiFilm fluorescence scanner followed by a densitometric analysis using the software ImageJ version 1.43u (33). As a sample handling control, the total tRNA amount was measured by poststaining of the agarose gel with GelStar nucleic acid gel stain (Lonza) and a standard UV transilluminator (312 nm).

Stationary-phase killing assay. The killing assay was performed essentially as described previously (34). In brief, overnight cultures of the plasmid harboring PA14 strains grown in LB supplemented with carbenicillin were diluted to an OD_{600 nm} of 0.05 with antibiotic-free medium and incubated at 37°C. After reaching stationary phase (OD_{600 nm} ~3.0), AZM (2, 5, or 10 μg/ml) was added to 2-ml aliquots of the cultures and the incubation was continued for 20 h at 37°C. The viable counts were determined by plating serial-dilution aliquots onto LB agar plates. The relative survival of each strain was normalized to that of the nontreated wild-type control.

Quantification of rhamnolipid production. Overnight cultures were freshly diluted to an OD_{600 nm} of 0.05 and incubated in LB supplemented with or without AZM at 37°C for 48 h. The colorimetric analysis of the orcinol reaction was adapted from the method described in reference 35. Three hundred microliters of culture supernatant was extracted twice with diethyl ether and the pooled ether fractions were evaporated to dryness. The remainders were dissolved in distilled water and incubated with 100 μl 1.6% (wt/vol) orcinol and 800 μl 60% sulfuric acid at 80°C for 30 min. The adsorption at 421 nm was determined. In parallel, rhamnose at defined concentrations was also assayed as described above and used as a standard for determining the rhamnose in the culture samples. Rhamnolipid concentrations were then calculated based on the assumption that 1 μg of rhamnose corresponds to 2.5 μg of rhamnolipid (36).

Assay for pyocyanin production. Pyocyanin production was determined as described previously (37). Briefly, 5-ml aliquots of 24-h-old

FIG 2 AZM-mediated killing of PA14 wild-type control and *pth*-overexpressing (PA14-*pth*) cells. Stationary-phase cells were treated with 2 μg/ml, 5 μg/ml, or 10 μg/ml AZM and incubated for 20 h at 37°C. The viable counts were determined by plating serial-dilution aliquots onto LB agar plates. The averages and associated standard deviations of three replicates are shown. The asterisks (*, $P < 0.005$; n.s., not significant) indicate the statistically significant differences of AZM-treated cells compared to the corresponding nontreated cells.

bacterial cultures grown in the absence or presence of AZM were extracted with chloroform and then reextracted into 0.2 N HCl to give a pink solution. The absorbance was measured at 520 nm, and the pyocyanin produced per milliliter of culture supernatant was calculated as described elsewhere (37).

Cytotoxicity assay. A549-Gluc cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal calf serum (DMEM complete). Cultures were grown at 37°C with 5% CO₂. A549-Gluc cells were generated from A549 by lentiviral gene transfer as described previously (38, 39). Antibiotic selection was done by growing cultures in the presence of 10 μg/ml blasticidin.

Cytotoxicity of AZM-treated and nontreated *P. aeruginosa* strains was assessed by infecting A549-Gluc cells, which secrete Gaussia luciferase as a measure of cell integrity. A549-Gluc cells were seeded in 96-well plates at a density of 2.5×10^4 to 5×10^4 cells per well and grown to ~90% confluence. Cells were washed once with phosphate-buffered saline (PBS) and then inoculated with 6-h-old *P. aeruginosa* LB cultures adjusted to a multiplicity of infection (MOI) of 10 in cell culture medium with or without AZM. Plates were centrifuged for 5 min at $500 \times g$ to increase the chances of contact between the bacteria and the epithelial cells. Cell culture supernatants were collected after 3 h of incubation at 37°C with 5% CO₂ following a centrifugation step to pellet out remaining bacteria and cell debris. Gaussia luciferase activity was measured for 0.1 s using an LB 960 Centro XS3 plate luminometer (Berthold Technologies) after the addition of 60 μl of 10 μM coelenterazine (P.J.K.).

Motility assay. Swarming assays were performed as described previously (40). Briefly, swarming was evaluated on modified BM2-glucose plates containing 0.5% (wt/vol) agar supplemented with 0.1% (wt/vol) Casamino Acids and AZM at indicated concentrations. Plates were incubated with 2 μl of a culture with an OD_{600 nm} of 1.0 and incubated at 37°C overnight.

Statistical analysis. When indicated, Student's *t* test (two-tailed) was used to determine whether the presence of AZM and the overexpression of *pth* resulted in any significant differences compared to the nontreated wild-type cells.

RESULTS AND DISCUSSION

Although macrolides do not exhibit an inhibitory activity against Gram-negative bacteria at concentrations below the breakpoint for susceptibility, it has been demonstrated in several clinical studies that patients whose respiratory tract is chronically infected

FIG 3 Growth of PA14 wild-type control (black) and *pth*-overexpressing mutant (gray) in the absence (squares) or presence of 2 µg/ml (triangles), 5 µg/ml (diamonds), or 10 µg/ml (circles) AZM (dashed line).

with *P. aeruginosa* benefit from long-term low-dose AZM treatment (10, 11, 12, 13, 14, 15). This effective treatment might be due to an immunomodulatory activity that reduces bronchiolar inflammation and damage (41). However, AZM also significantly influences bacterial production of virulence factors and has an impact on biofilm formation (18, 42).

By applying a ribosome protection assay, Köhler et al. (34) previously demonstrated that the AZM effect on quorum-sensing (QS)-dependent virulence factor production and cell killing in *P. aeruginosa* requires AZM interaction with the ribosome. These results clearly show that there does not seem to be a second, so far uncharacterized, nonribosomal target which explains the effect of subinhibitory AZM concentrations on *P. aeruginosa* protein expression. Macrolides are inhibitors of protein biosynthesis. They seem to block the peptide exit channel of the 50S ribosomal subunit through interaction with the 23S rRNA and promote dissociation of the peptidyl-tRNA and, thereby, increase the rate of peptidyl-tRNA drop-off. It has been suggested that the inhibitory activity of macrolides is at least partially mediated via a depletion of the intracellular pool of aminoacyl-tRNAs as the result of the increased peptidyl-tRNA drop-off (26, 27, 28). As a complement to the study of Köhler et al. (34), we analyzed whether the depletion of the tRNA pools as a result of the enhanced peptidyl-tRNA drop-off contributes to the observed AZM-mediated modulation

of the expression of QS-dependent virulence factors, motility, and cytotoxicity in *P. aeruginosa*.

Overexpression of PA4672, encoding a peptidyl-tRNA hydrolase, increases the fraction of uncharged tRNAs in *Pseudomonas aeruginosa*. We overexpressed the peptidyl-tRNA hydrolase (Pth) encoded by PA4672 by introducing the gene on the plasmid pHERD-*pth* into the wild-type strain PA14 (PA14-*pth*). To monitor the functionality of the Pth, the total tRNA pool of PA14 and PA14-*pth* was extracted under mild acidic conditions to retain the amino acid charging, adjusted in respect to total tRNA concentration, and fluorescently labeled as described by Dittmar et al. (32). If Pth recycles the tRNAs from the peptidyl-tRNA pool, we would expect that in the *pth*-overexpressing strain PA14-*pth* the relative abundance of uncharged versus amino-acetylated tRNAs should be increased. Indeed, as depicted in Fig. 1, overexpression of *pth* leads to an increased ligation efficiency of about 2-fold of the tRNAs to the Cy3-tagging oligonucleotides. This increased efficiency might be due to the absence of the peptidyl moiety, which, if present, presumably leads to a hindrance of the ligation process. Vice versa, if the total tRNA pool in the *pth*-overexpressing strain was pretreated with periodate, which oxidizes uncharged tRNAs and thus hinders the ligation process, the efficiency of ligation was reduced. By a permanent delivery of Pth-recycled tRNAs, the intracellular pool of tRNAs is constantly filled up with uncharged tRNA, which presumably also increases the overall amount of amino-acetylated tRNAs as more freely accessible tRNAs are present in the cell.

AZM stationary-phase killing is decreased in *pth*-overexpressing *P. aeruginosa* cells. In previous studies it was shown that AZM mediates the killing of stationary-phase cells of *P. aeruginosa* (34). Hence, we analyzed whether *pth* overexpression and the resulting increase of free available tRNAs counteract the AZM-mediated killing effect. *P. aeruginosa* PA14 wild-type control and PA14 overexpressing *pth* (PA14-*pth*) were grown to stationary phase and subjected to AZM concentrations of 2 µg/ml, 5 µg/ml, or 10 µg/ml (Fig. 2). Whereas supplementation with 10 µg/ml AZM had a strong bactericidal effect on both PA14 and PA14-*pth* cells, the AZM-mediated killing of cells that overexpressed the Pth enzyme was significantly compensated for when the PA14 and PA14-*pth* cells were treated with 2 µg/ml and 5 µg/ml AZM, re-

FIG 4 Effects of AZM on the production of rhamnolipid (A) and pyocyanin (B). PA14 wild-type control and *pth*-overexpressing mutant cells were incubated at 37°C in the absence or presence of 2 µg/ml, 5 µg/ml, and 10 µg/ml AZM. (A) The amounts of rhamnolipids in 48-h-old cultures were determined by an indirect colorimetric assay (ornic test). There was no statistically significant difference in rhamnolipid production between non-AZM-treated wild-type and *pth*-overexpressing mutant cells treated with AZM ($P > 0.05$). (B) Pyocyanin production was assayed in 24-h-old cultures. Although *pth* overexpression increased pyocyanin production in the wild-type strain, Pth activity could not restore pyocyanin to wild-type levels in AZM-treated cultures. The values are the means of three replicates and the error bars display the standard deviations.

FIG 5 AZM-mediated inhibition of swarming motility. PA14 wild-type control and *pth*-overexpressing cells were analyzed on BM2 plates containing 0.5% agar and AZM at the indicated concentrations. The plates were incubated overnight at 37°C.

spectively. This result indicates that depletion of the intracellular tRNA pool is critical for the AZM inhibitory activity against stationary *P. aeruginosa* cells.

Overexpressing *pth* counteracts the AZM-mediated effect on rhamnolipid production and partially restores swarming activity. Subinhibitory concentrations of AZM have previously been shown to inhibit the production of mainly *rhl*-quorum-sensing-dependent virulence factors, including pyocyanin and rhamnolipids, and inhibit swarming motility in *P. aeruginosa* (18, 34). We cultured PA14 wild type and PA14-*pth* in LB with and without adding 2 µg/ml, 5 µg/ml, or 10 µg/ml AZM. Whereas higher concentrations of AZM (5 µg/ml or 10 µg/ml) led to a reduced growth rate in the late exponential and early stationary phases in both strains, lower concentrations of AZM (2 µg/ml) only slightly impacted bacterial growth (Fig. 3). Interestingly, under low AZM concentrations rhamnolipid and pyocyanin production were significantly reduced (Fig. 4). When *pth* was overexpressed in AZM-treated cells (2 µg/ml), rhamnolipid production was restored to wild-type levels, whereas pyocyanin production was only partially restored. Importantly, overexpression of *pth* in nontreated *P. aeruginosa* cells revealed an enhanced production of both rhamnolipid and pyocyanin, indicating that the availability of tRNAs

for amino-acetylation is critical for the expression of *rhl*-dependent virulence factors.

To further analyze whether changes in the tRNA pools also have an influence on the swarming motility, we cultured PA14 wild-type and PA14-*pth* cells on BM2 agar plates containing different concentrations of AZM. Interestingly, overexpression of *pth* led to derepression of swarming activity in cells treated with 2 µg/ml or 5 µg/ml AZM compared to the swarming activity in the wild-type control cells (Fig. 5). This suggests that the Pth-dependent recycling of peptidyl-tRNAs results in an enlarged pool of free tRNAs which counteract the AZM-mediated inhibitory effect of *P. aeruginosa* swarming motility.

FIG 6 Effects of AZM on the cytotoxicity of PA14 strains as determined by a Gaussia luciferase assay of A549 cells. The Gaussia assay is used to monitor cell viability. PA14 wild-type control and *pth*-overexpressing mutant cells were treated with AZM at indicated concentrations and incubated for 6 h prior to infecting eukaryotic A549-Gluc cells (MOI 10). After cocultivation for 3 h at 37°C and 5% CO₂, the activity of the secreted Gaussia luciferase, given in relative light units (RLU), was determined using a luminometer. The results represent the means $\pm$ standard deviations of eight independent replicates. The asterisks indicate the statistically significant differences ($P < 0.002$; n.s., not significant) of AZM-treated cells compared to the corresponding nontreated cells.

FIG 7 Relation between *rhlR* codon usage and the AZM-mediated reduction of rhamnolipid (A) and pyocyanin (B) production. PA14 strains carrying either pUCP20 empty plasmid or *rhlR* complementation constructs were incubated at 37°C in the absence or presence of AZM (2 µg/ml). (A) The amounts of rhamnolipids in 48-h-old cultures were determined by an indirect colorimetric assay. (B) Pyocyanin production was assayed in 24-h-old cultures. The values are the means of three replicates and the error bars display the standard deviations of the mean. The asterisks indicate the statistically significant differences ($P < 0.005$; n.s., not significant) of the strains carrying either the native *rhlR* or the codon-exchanged *rhlR* gene in the presence or absence of AZM.

FIG 8 Effects of codon exchange in *rhIR* on AZM-mediated inhibition of swarming motility. The PA14 *rhIR* mutant (3) and the complementation mutant containing either the *rhIR* wild-type gene (1) or the *rhIR* gene with the rare-to-frequent codon exchange (2) were analyzed on BM2 plates containing 0.5% agar $\pm$ AZM at the indicated concentrations. The plates were incubated overnight at 37°C.

Recycling of the peptidyl-tRNAs diminished AZM-mediated cytotoxicity. One of the most striking AZM-mediated phenotypes in *P. aeruginosa* is the enhanced cytotoxicity upon treatment with subinhibitory concentrations of AZM (43). We therefore tested the effect of *pth* overexpression on the virulence of *P. aeruginosa* cells that have been cultured with and without the addition of subinhibitory concentrations of AZM. As depicted in Fig. 6, the cultivation of *P. aeruginosa* PA14 in medium containing 2 µg/ml AZM significantly enhanced cytotoxicity as lysis of A549 cells was clearly increased. Strikingly, the overexpression of *pth* compensated for the effect of AZM, and cytotoxicity was not changed compared to the untreated control. Higher dosages of AZM did not further enhance the cytotoxicity of PA14. However, this might have been due to the effect on the bacterial growth rate of AZM at the higher doses. Again, of note, overexpression of *pth* alone significantly reduced cytotoxicity in *P. aeruginosa* PA14 under all conditions tested.

Codon usage plays a role in *rhIR* translation efficiency of AZM treated cells. Obviously, upon treatment with subinhibitory AZM concentrations, the translation of only some targets proceeds less efficiently, whereas other targets seem to be unaffected and growth is hardly inhibited in *P. aeruginosa* upon the addition of 2 µg/ml AZM. Since we found that the accumulation of peptidyl-tRNAs and, therefore, the decreased availability of tRNAs is critical for the effect of AZM on the expression of *rhIR*-mediated phenotypes, we wondered whether the deprivation of distinct tRNA isoacceptors that bind to different codons for the same amino acid might affect the translation efficiency of the *RhlR*-encoding mRNA. We found that the second codon of the *rhIR* gene was AGG, which is read by an arginine tRNA isoacceptor that is very rarely used during *P. aeruginosa* protein synthesis (2.1 per thousand) (see www.kazusa.or.jp). In order to determine whether codon usage plays a role in translation efficiency in AZM-treated cells, we used a PA14 *rhIR* transposon mutant and complemented the strain with the wild-type *rhIR* gene in *trans* and the wild-type gene where the second codon AGG was exchanged to the most frequently used codon CGC (48.8 per thousand). The *rhIR* transposon mutant carrying pUCP20 empty plasmid served as a control.

As depicted in Fig. 7, the exchange of the rarely for the frequently used codon significantly reduced the AZM-mediated inhibitory effect on the production of rhamnolipids and pyocyanin. Of note, albeit the AZM-mediated repression of the swarming motility was partially restored in the PA14 strain complemented with *rhIR* in *trans*, the exchange of the rarely used second codon in *rhIR* for the frequently used GCG did not make a difference even when higher AZM concentrations were used (Fig. 8). Thus, al-

though many of the AZM-mediated effects on *P. aeruginosa* are at least partially due to a reduced expression of *RhlR*, these results indicate that other factors besides the AZM-mediated effect on rhamnolipid production play a role in the AZM-mediated repression of swarming motility and there seem to be other yet to be identified targets of AZM.

Conclusion. Overexpression of *Pth* in PA14 wild-type cells revealed a diametrically opposed phenotype to that seen in PA14 exposed to subinhibitory AZM concentrations, and the AZM-mediated effects on the *RhlR*-dependent phenotypes were diminished by increasing the availability of uncharged tRNAs via overexpression of *Pth*. These results indicate that an AZM-mediated increase in peptidyl-tRNA drop-off can be counteracted by an enhanced recycling of tRNAs. Similarly to *Pth* overexpression, the exchange of a rare for a frequent codon at the second position in *rhIR* counteracted the AZM-mediated inhibitory effect on *RhlR*-dependent phenotypes. Since rare codons within the first 2 to 6 codons have been implicated in the enhancement of peptidyl-tRNA drop-off (44, 45), the exchange of a rare for a frequent codon might have the same effect as *Pth* overexpression: it diminishes the pool of peptidyl-tRNAs and thus increases the availability of uncharged tRNA that is important for protein translation. The latter finding suggests that a differential codon usage in *P. aeruginosa* might explain the selective activity of AZM in the translation of distinct proteins. The observation that AZM influences *P. aeruginosa* protein expression via a modulation of the availability of tRNAs for amino acetylation might be important in long-term low-dose AZM therapy in CF patients, and subinhibitory concentrations of AZM might also affect the protein expression profile and possibly the virulence phenotype of other bacterial pathogens.

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