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Recycling of peptidyl-tRNAs by peptidyl-tRNA hydrolase counteracts Azithromycin-mediated effects on *Pseudomonas aeruginosa*

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ABSTRACT

Acute and chronic infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* pose a serious threat to human health care worldwide and the increasing resistance to antibiotics entails the requirement of more effective and alternative treatment strategies. Clinical studies clearly demonstrated that chronically *P. aeruginosa* infected cystic fibrosis (CF) patients benefit from long-term low-dose azythromycin (AZM) treatment. Thereby, an immunomodulating activity as well as an 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 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. Over-expression of the *P. aeruginosa* peptidyl-tRNA hydrolase, that recycles the tRNA from peptidyl-tRNA drop-off during translation, counteracted the AZM effects on stationary phase cell killing, cytotoxicity, the production of rhamnolipids, and partially restored swarming motility. Intriguingly, the exchange of a rare to frequent codon in *rhlR* also explicitly diminished the AZM-mediated decreased production of rhamnolipid. 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 greater extent and may explain the selective activity of AZM on the *P. aeruginosa* proteome and possibly also on the protein expression profile of other bacterial pathogens.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that causes both life-threatening acute and devastating chronic infections in the human host . 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 in these patients . Improved antimicrobial treatment strategies strongly increased the life-expectancy of CF patients in the last decades. Nevertheless, even aggressive antimicrobial therapy rarely eradicates established chronic *P. aeruginosa* infections . Hence, for the management of chronic infectious diseases, there is a strong need for alternative treatment strategies that amend classical antimicrobial therapy . Several clinical studies have now 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 growth of *P. aeruginosa* at concentration levels below the breakpoint concentration for susceptibility to the macrolides .

The nature of this beneficial effect of AZM is still unclear. Macrolides were shown to have an immunomodulatory activity, which results in a decreased inflammatory response to bacterial stimulations , and there are several studies demonstrating that macrolides inhibit virulence factor production in *P. aeruginosa in vitro* and *in vivo* and to interfere with biofilm formation .

Although macrolides are antibacterial agents that target the protein synthesis machinery, at subinhibitory concentrations AZM was demonstrated to both activate and repress the transcription of different subsets of genes in *P. aeruginosa* . It remains uncertain how these effects on transcriptions are mediated. Recently, it was clearly shown that bacterial stationary

phase cell killing and reduced expression of quorum sensing-dependent virulence factors require the interaction of AZM with the ribosome , indicating that there do not seem to be non-ribosomal targets of AZM which could 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, on the one hand, and depletion of the intracellular pools of aminoacyl-tRNAs by drop-off and incomplete peptidyl-tRNA hydrolase (Pth) activity, on the other hand . Pth is an essential enzyme and 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 .

In this study we aimed at analyzing to what extent a decreased intracellular tRNA pool also contributes to the observed AZM-mediated phenotype in respect to virulence factor production, motility and stationary phase cell killing in *P. aeruginosa*. Our results show that increasing the intracellular pools of tRNAs by over-expressing the peptidyl-tRNA hydrolase encoded by PA4672 clearly counteracted AZM induced stationary phase killing, reduced expression of rhamnolipids, pyocyanin, and swarming motility and increased cytotoxicity in *P. aeruginosa* cells. Furthermore, the AZM effect on rhamnolipid production could be explicitly diminished by the exchange of a rarely to a frequently used codon for arginine at the second position of *rhlR*. Our results suggest that AZM impacts on protein expression on a global scale and selectively impairs the expression of those proteins with an increased frequency of rare codons.

MATERIALS AND METHODS:

Strains and plasmids. Bacterial strains and plasmids used in this study are summarized in Table 1. *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 given concentrations. For solidification, agar was added to a final concentration of 1.5 % (w/v). 100 µg/ml ampicillin and 400 µg/ml carbenicillin were added to inhibit *E. coli* and PA14 growth, respectively.

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

For over-expression of PA4672 (*pth*) the plasmid pHERD-pth was constructed. The *pth* gene was PCR-amplified using PA14 genomic DNA as 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 . The resulting vector was introduced into PA14 by electroporation and pHERD20T empty plasmid was introduced into PA14 serving as wild-type control (wt control). The *pth* over-expression was induced with 0.2 % (w/v) arabinose at an OD_{600nm} of 0.3-0.5.

To generate the plasmids pUCP20::rhlR and pUCP20::rhlR-R2R (encoding 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 primers "*rhlR*-(R^{AGG}-2-R^{CGC})-*Nde*I-fw" or "*rhlR*-*Nde*I-fw" together with the reverse primer "*rhlR*-*Hind*III-rv." These PCR products were digested with *Nde*I/*Hind*III and in a first step cloned into similar digested pET21A, resulting in the plasmid 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 templates for a second PCR using the primers “pET21A-pUCP20-fw” and “pET21A-pUCP20-rv”. The PCR products were digested with *EcoR*I/*Xba*I and finally cloned into similar 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% (w/v) trichloroacetic acid. After centrifugation (3500 x g for 15 min at 4°C) the cell pellet was resuspended in ice cold 0.5 ml of lysis buffer [0.3M sodium acetate (pH 4.5) and 10 mM Na_2EDTA] and transfered to a fresh 1.5-ml Eppendorf tubes (on ice). The lysate was mixed with 0.5 ml acetate-saturated phenol/$CHCl_3$ (pH 4.5), vortexed three times for 15 s and placed 1 min on ice between each vortex mixing to ensure that the samples remain cold. After centrifugation at 20.817 x g for 12 min at 4°C, the aqueous layer was removed and subjected to another extraction with 1 volume acetate-saturated phenol/$CHCl_3$ (pH 4.5) solution. The mixture was vortexed for 15 s and centrifuged at 20.817 x g for 10 min at 4°C. The nucleic acids (essentially only tRNA) were recovered from the aqueous phase by precipitation with 1 ml EtOH (95-100%) for 15 min at -20°C followed by centrifugation at 20.817 x g for 30 min at 4°C. Finally the precipitated RNA was resuspended in 100 µl ice cold 10 mM NaOAc/HOAc (pH 4.5). Periodate oxidation of tRNA was adapted from a previous study by Dittmar and colleges . For the tRNA oxidation reaction tRNA with a concentration of 200 ng/µl was mixed with 25 µl $NaIO_4$ (50 mM) solved in KOAc/HOAc (200 mM; pH 4.8). The control reaction contained the same amount of tRNA and 25 µl NaCl (50 mM) solved in KOAc/HOAc (200 mM; pH 4.8). The mixture was incubated at 22°C for 30 min in the dark and 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 were subjected to a T4 Polynucleotide kinase (T4PNK) treatment. Both samples (control and oxidized tRNA) were ligated with the T4PNK treated Cy3-tagging oligonucleotide using following conditions: 66.6 ng/µl tRNA and 7.5 µM Cy3-tagging oligonucleotide in 50 mM Tris-HCL (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 13% DMSO and 1 U T4 DNA ligase for 15 h at 16°C. The ligation reaction was separated using a 2% agarose gel electrophoresis and fluorescence intensity was detected by a FLA-9000 FUJIFILM fluorescence scanner followed by a densiometric analysis using the software ImageJ vs. 1.43u . As sample handling control, the total tRNA amount was measured by post-staining 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 earlier . In brief, overnight cultures of plasmid harboring PA14 strains grown in LB supplemented with carbenicillin were diluted to an OD_{600nm} of 0.05 with antibiotic-free medium and incubated at 37°C. After reaching stationary phase (OD_{600nm} ~ 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 non-treated wild-type control.

Quantification of rhamnolipid production. Overnight cultures were freshly diluted to an OD_{600nm} 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 adopted from . 300 µl of culture supernatant were extracted twice with diethylether and the pooled ether fractions were evaporated to dryness. The remainders were dissolved in distilled water and incubated with 100 µl 1.6 % (w/v) 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 .

Assay for pyocyanin production. Pyocyanin production was determined as described previously . Briefly, a 5-ml aliquot of 24 h-old bacterial cultures grown in the absence or presence of AZM were extracted with chloroform and then re-extracted 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 .

Cytotoxicity assay. A549-Gluc cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, non-essential amino acids, 100U of penicillin per ml, 100 µg of streptomycin per ml, and 10 % fetal calf serum (DMEM complete). Cultures were grown at 37°C with 5% CO_2. A549-Gluc cells were generated from A549 by lentiviral gene transfer as previously described . Antibiotic selection was done by growing cultures in the presence of 10µg/ml blasticidin.

Cytotoxicity of AZM-treated and non-treated *P. aeruginosa* strains were 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 2.5-5 x 10^4 cells per well and grown until ~ 90 % confluence. Cells were washed once with PBS and then inoculated with 6 h-old *P. aeruginosa* LB cultures adjusted to an MOI of 10 in cell culture medium with or without AZM. Plates were centrifuged for 5 min at 500 x g to increase chances of contact between the bacteria and epithelial cells. Cell culture supernatants were collected after 3 h of incubation at 37°C with 5 % CO_2 following a centrifugation step to pellet out remaining bacteria and cell debris. Gaussia luciferase activity was measured for 0.1 s using a 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 previously described . Briefly, swarming was evaluated on modified BM2 glucose plates containing 0.5 % (w/v) agar supplemented with 0.1 % (w/v) Casamino acids and AZM at indicated concentrations. Plates were incubated with 2 µl of a culture with OD_{600nm} of 1.0 and incubated at 37°C over night.

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

RESULTS AND DISCUSSION

Although macrolides do not exhibit an inhibitory activity against gram-negative bacteria at concentration below the breakpoint for susceptibility, it has been demonstrated in several clinical studies that patients, whose respiratory tract is chronically infected with *P. aeruginosa,* benefit from long-term low-dose AZM treatment . This effective treatment might be due to an immunomodulatory activity reducing bronchiolar inflammation and damage . However, AZM also significantly influences bacterial production of virulence factors and impacts on biofilm formation .

By applying a ribosome protection assay, Köhler *et al.* previously demonstrated that the AZM effect on 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 non-ribosomal 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, 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 . As a complement to the study of Köhler *et al.* , we aimed at analyzing 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*.

Over-expression of PA4672, encoding for a peptidy-tRNA hydrolase (Pth), increases the fraction of uncharged tRNAs in *Pseudomonas aeruginosa*. We over-expressed 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 . 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, over-expression of *pth* leads to an increased ligation efficiency of the tRNAs to the Cy3-tagging oligonucleotides of about two-fold. This is expected to 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* over-expressing 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* over-expressing *P. aeruginosa* cells. In previous studies it was shown that AZM mediates killing of stationary-phase cells of *P. aeruginosa* . Hence, we analyzed whether *pth* over-expression and the resulting increase of free available tRNAs counteracts the AZM-mediated killing effect. *P. aeruginosa* PA14 wild-type control and PA14 over-expressing *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 on cells that over-expressed the Pth enzyme was significantly compensated when treated with 2 µg/ml and 5 µg/ml AZM, respectively. This

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

Over-expressing *pth* counteracts the AZM-mediated effect on rhamnolipid and partially restores swarming activity. Subinhibitory concentrations of AZM have previously been shown to inhibit the production of mainly *rhl*-quorum sensing-dependent production of virulence factors, including pyocyanin and rhamnolipids, and inhibit swarming motility in *P. aeruginosa* . 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 / early stationary phase 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 over-expressing *pth* in AZM-treated cells (2 µg/ml), rhamnolipid production could be restored to wild-type levels, whereas pyocyanin production was only partially restored. Importantly, over-expression of *pth* in none-treated *P. aeruginosa* cells already 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* on BM2 agar plates containing different concentrations of AZM. Interestingly, over-expressing *pth* led to de-repression of swarming activity in cells treated with 2 µg/ml or 5 µg/ml AZM as compared to the wild-type control (Fig. 5). This suggests that the Pth-dependent recycling of peptidyl-tRNAs results in an enlarged pool of free tRNAs which counteracts the AZM-mediated inhibitory effect of *P. aeruginosa* swarming motility.

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 . We therefore tested the effect of *pth* over-expression 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 over-expression of *pth* compensated the effect of AZM, and cytotoxicity was not changed as compared to the untreated control. Higher dosages of AZM did not further enhance the cytotoxicity of PA14. However, this might be due to the AZM effect on the bacterial growth rate at the higher doses. Again, of note, over-expression of *pth* alone significantly reduced cytotoxicity in *P. aeruginosa* PA14 under all conditions tested.

Codon usage plays a role in *rhlR* translation efficiency of AZM treated cells. Obviously, upon treatment with subinhibitory AZM concentrations the translation of only some targets proceeds less efficiently, whereas others 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 *rhl*-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 *rhlR* 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; 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 *rhlR* transposon mutant and complemented the strain with the wild-type *rhlR* 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 *rhlR* transposon mutant carrying pUCP20 empty plasmid served as control.

As depicted in Fig. 7 the exchange of the rarely to 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 *rhlR* in trans, the exchange of the rarely used second codon in *rhlR* to the frequently used GCG did not make a difference even when higher AZM concentrations were used (Fig. 8). Thus, although 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

Over-expression 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 could be diminished by increasing the availability of uncharged tRNAs via over-expression of Pth. These results indicate that an AZM-mediated increased peptidyl-tRNA drop-off can be counteracted by an enhanced recycling of tRNAs. Similarly to Pth over-expression, the exchange of a rare to a frequent codon at the second position in *rhlR* counteracted the AZM-mediated inhibitory effect on RhlR-dependent phenotypes. Since rare codons within the first 2-6 codons have been implicated to enhance peptidyl-drop off , the exchange of a rare to a frequent codon might have the same effect as Pth over-expression: it diminishes the pool of peptidyl-tRNAs, and thus increases the

availability of uncharged tRNA important for protein translation. This latter finding suggests that a differential codon usage in *P. aeruginosa* may explain the selective activity of AZM on the translation of distinct proteins. The observations that AZM influences *P. aeruginosa* protein expression via a modulation of the availability of tRNAs for amino-acetylation might be of importance not only for long-term low-dose AZM therapy in CF patients, but subinhibitory concentrations of AZM might also affect the protein expression profile and possibly the virulence phenotype of other bacterial pathogens.

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493 sequence of the region required for their replication in *Pseudomonas aeruginosa*.
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495
496

497TABLE 1: Bacterial strains, plasmids and primers used in this study

Strains or plasmids	Relevant genotype	Source or reference
strains		
E. coli DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ (*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Woodcock
PA14	*Pseudomonas aeruginosa* PA14 wild type	
PA14 wt control	PA14 wt (pHERD20T), Cb^r	this study
PA14-*pth*	PA14 (pHERD-pth), Cb^r	this study
PA14 *rhlR*	*rhlR* transposon mutant from the NR PA14 transposon mutant library, ID 37943, Gm^r	
plasmids		
pHERD20T	shuttle vector, Cb^r	
pHERD-pth	pHERD20T carrying *pth*, Cb^r	this study
pET21A	expression vector, Ap^r	Novagen
pUCP20	shuttle vector, Ap^r /Cc^r	
pUCP20::rhlR	*rhlR* (promoter region and gene) cloned into *EcoR*I/*Xba*I site in MCS, Ap^r /Cb^r	this study
pUCP20::rhlR-R2R	*rhlR* (promoter region and gene) cloned into *EcoR*I/*Xba*I site in MCS, Ap^r /Cb^r	this study
primer		
pth-RBS-*Nco*I-fw	5`-GACCATGG**AAAGAGGAGAAATACTAG**GTGACTGCCGTACAACTGAT	this study
pth -*Pst*I-rev	5´-CGACTGCAGTCAGGCCTTCTGGCTGTG	this study
rhlR-*Nde*I-fw	5´- TATCATATGAGGAATGACGGAGGCTTT	this study
rhlR-(R^{AGG}-2-R^{CGC})-*Nde*I-fw	5`-TATCATATGCGCAATGACGGAGGCTTT	this study
rhlR-*Hind*III-rv	5`-TATAAGCTTTCAGATGAGACCCAGCG	this study
pET21A-pUCP20-fw	5`-GATCTCTAGATAGCAGCCGGATCTCAGT	this study
pET21A-pUCP20-rv	5`- GATCGAATTCTTTTGTTTAACTTTAAGAAGGAGATATAC	this study

498Cb^r, carbenicillin resistance; Engineered restriction sites are underlined,

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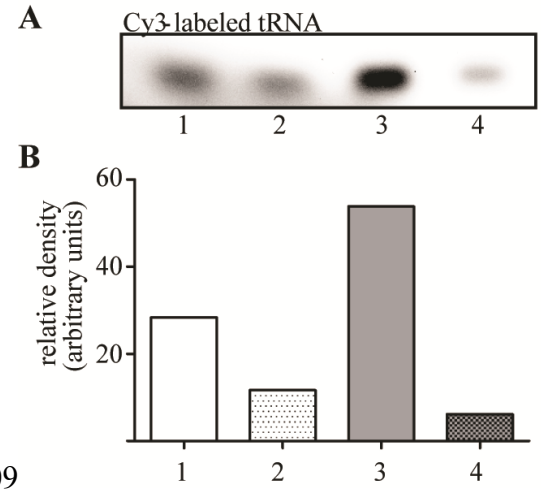
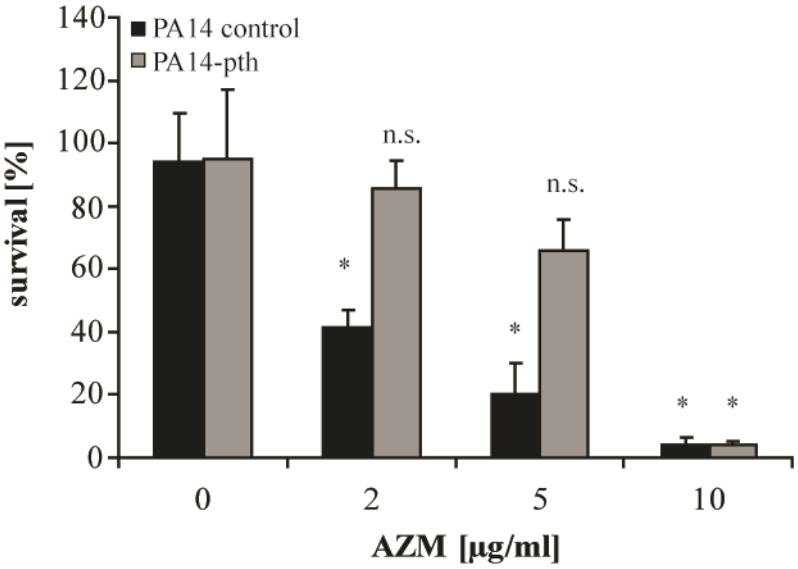


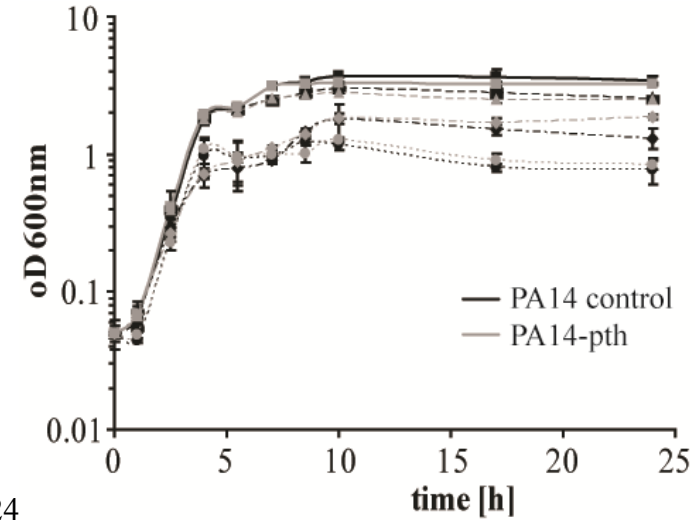
509

510FIG 1. Over-expression of peptidyl-tRNA hydrolase (Pth) leads to an elevated level of 511uncharged tRNAs. A) Agarose gel (2%) with fluorescent Cy3-labeled tRNA after ligation 512reaction and (B) densiometric analysis of each fluorescent band. Cy3-tagging oligonucleotide 513labeled tRNA from PA14 wt control pretreated with (1) NaCl or (2) $NaIO_4$ and from *pth* over-514expressing PA14 (PA14-*pth*) pretreated with (3) NaCl or (4) $NaIO_4$. The figure shows a 515representative result from three independent experiments.



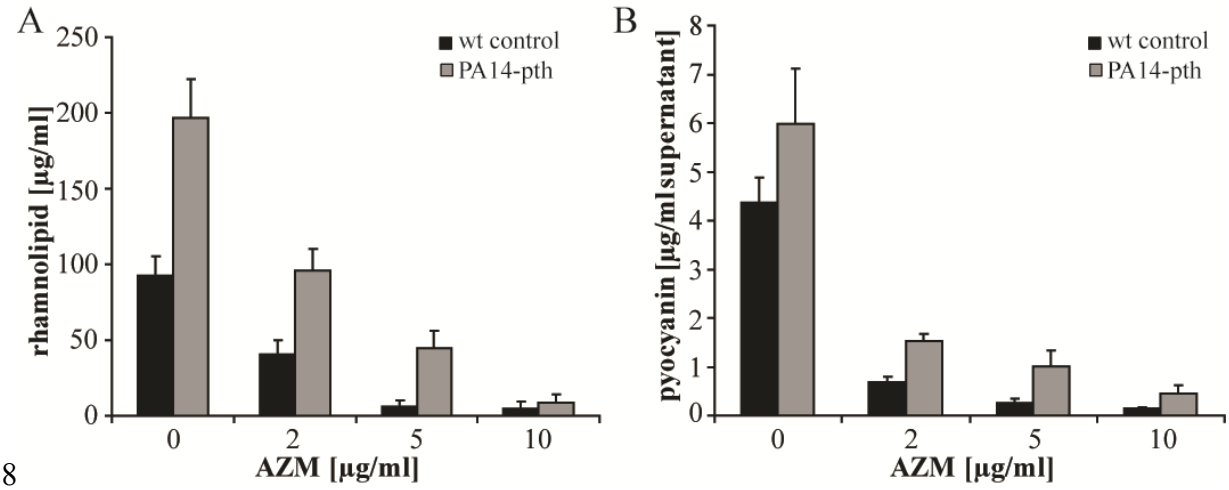
516

517FIG 2. AZM-mediated killing of PA14 wild-type control and the mutant over-expressing *pth*
518(PA14-*pth*). Stationary-phase cells were treated with 2 µg/ml, 5 µg/ml and 10 µg/ml AZM,
519respectively, and incubated for 20 h at 37°C. The viable counts were determined by plating
520serial dilution aliquots onto LB agar plates. The averages and associated standard deviations
521of three replicates are shown. The asterisks (*, $p < 0.005$; n.s., not significant) indicate the
522statistically significant differences of AZM-treated cells compared to the corresponding non-
523treated strains.



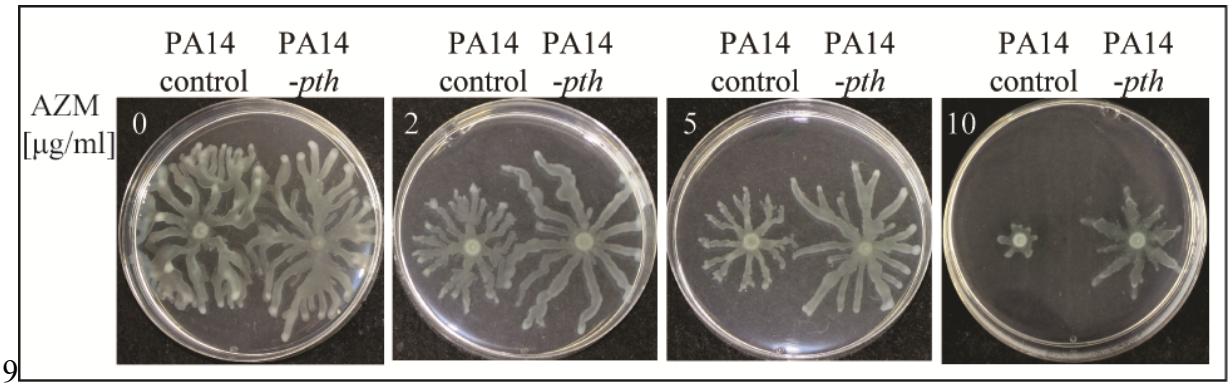
524

525FIG. 3. Growth of PA14 wild-type control (black) and the mutant over-expressing *pth* (grey)
526in the absence (squares) or presence of 2 µg/ml (triangles), 5 µg/ml (diamonds) or 10 µg/ml
527(circles) AZM (dashed line).



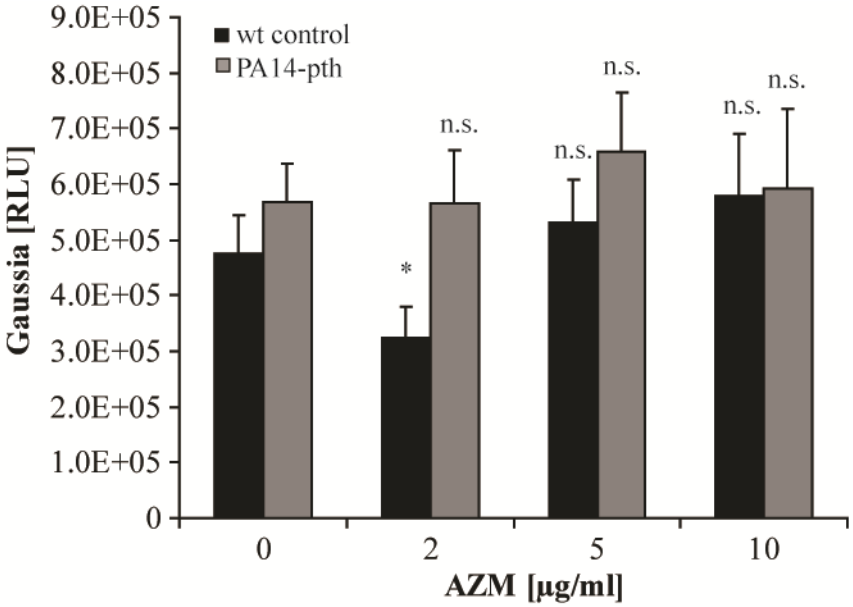
528

529FIG. 4. Effects of AZM on the production of rhamnolipid (A) and pyocyanin (B). PA14 wild-
530type control and the *pth*-over-expressing mutant were incubated at 37°C in the absence or
531presence of 2 µg/ml, 5 µg/ml and 10 µg/ml AZM, respectively. (A) The amounts of
532rhamnolipids in 48 h-old cultures were determined by an indirect colorimetric assay (orcinol
533test). There was no statistical significant difference in rhanmolipid production between non-
534AZM-treated wild type and *pth* over-expressing mutant treated with AZM ($p > 0.05$). (B)
535Pyocyanin production was assayed in 24 h-old cultures. Although *pth* over-expression
536increased pyocyanin production in the wild-type strain, Pth activity could not restore
537pyocyanin to wild-type levels in AZM treated cultures. The values are means of three
538replicates and the error bars display the standard deviations.



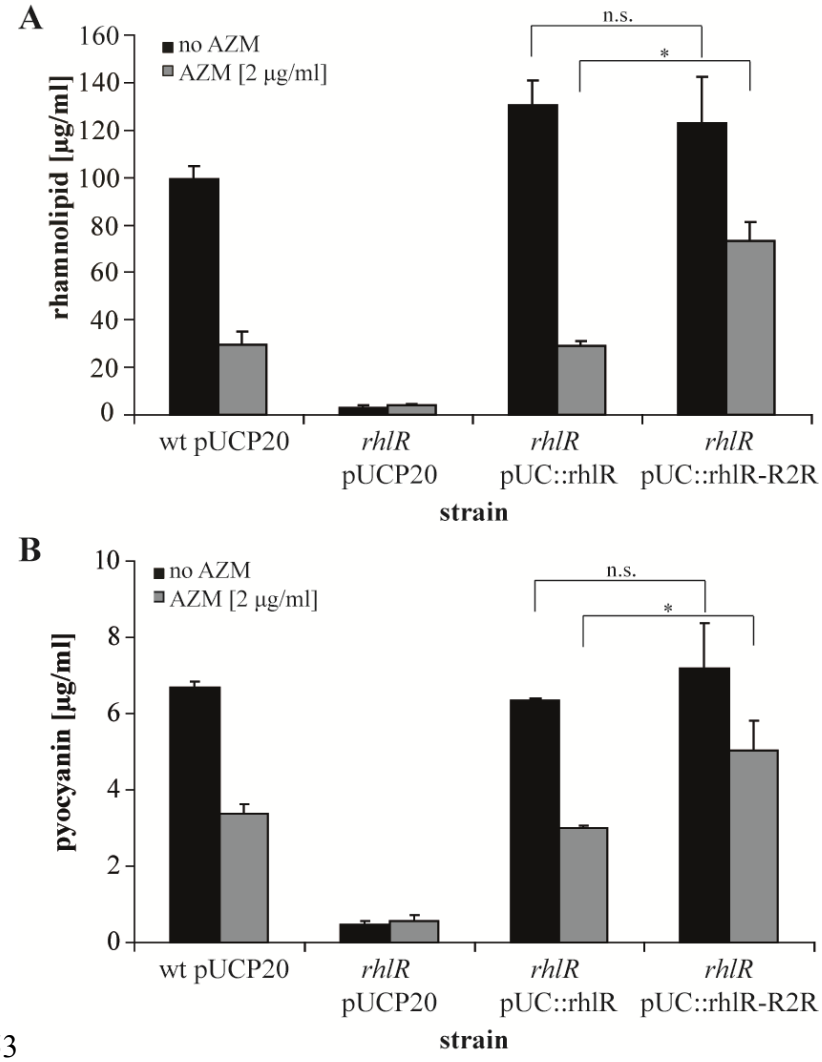
539

540FIG. 5. AZM-mediated inhibition of swarming motility. PA14 wild-type control and the
541mutant over-expressing *pth* were analyzed on BM2 plates containing 0.5 % agar and AZM at
542indicated concentrations. The plates were incubated overnight at 37°C.



543

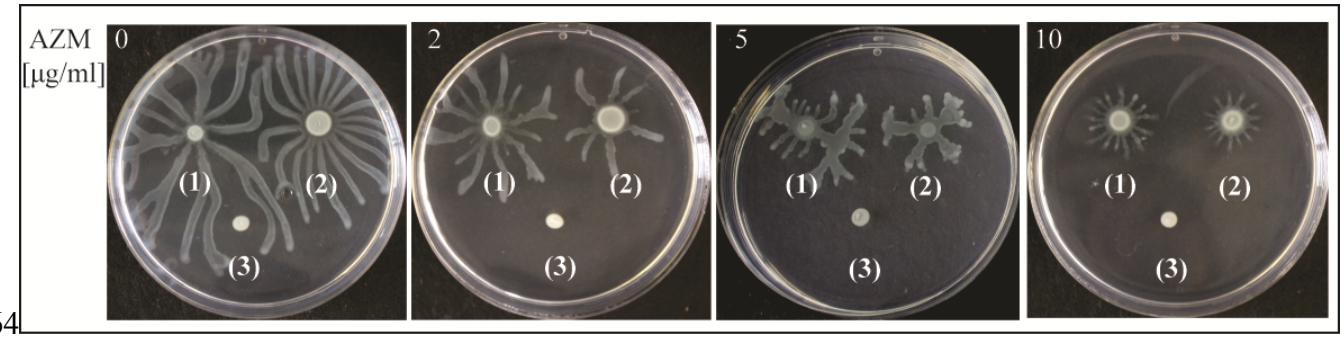
544FIG. 6. Effects of AZM on the cytotoxicity of PA14 strains as determined by Gaussia 545luciferase assay of A549 cells. The Gaussia assay is used to monitor cell viability. PA14 wild-546type control and *pth*-over-expressing mutant were treated with AZM at indicated 547concentrations and incubated for 6 h prior to infecting eukaryotic A549-Gluc cells (MOI 10). 548After co-cultivation for 3 h at 37°C and 5 % CO_2 the activity of the secreted Gaussia 549luciferase given in relative light units (RLU) was determined using a luminometer. The results 550represent the means ± standard deviations of eight independent replicates. The asterisks (*, p 551< 0.002; n.s., not significant) indicate the statistically significant differences of AZM-treated 552cells compared to the corresponding non-treated strains.



553

554FIG. 7. Relation between *rhlR* codon usage and the AZM-mediated reduction of rhamnolipid
555(A) and pyocyanin (B) production. PA14 strains either carrying pUCP20 empty plasmid or
556*rhlR* complementation constructs were incubated at 37°C in the absence or presence of AZM
557(2 µg/ml). (A) The amounts of rhamnolipids in 48 h-old cultures were determined by an
558indirect colorimetric assay. (B) Pyocyanin production was assayed in 24 h-old cultures. The
559values are means of three replicates and the error bars display the standard deviations of the
560mean. The asterisks (*, $p < 0.005$; n.s., not significant) indicate the statistically significant
561differences of the strains carrying either the native *rhlR* or the codon-exchanged *rhlR* in the
562precence or absence of AZM.

563



564

565FIG. 8. Effects of codon exchange in *rhlR* on AZM-mediated inhibition on swarming motility. 566PA14 *rhlR* mutant (3) and the complementation mutant containing either the *rhlR* wild-type 567gene (1) or *rhlR* with the rare-to-frequent codon exchange (2) were analyzed on BM2 plates 568containing 0.5 % agar ± AZM at indicated concentrations. The plates were incubated 569overnight at 37°C.