

ORIGINAL ARTICLE

Individual Nudix hydrolases affect diverse features of *Pseudomonas aeruginosa*

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UMO-2014/15/B/NZ6/02562; NIH Office
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Award Number: P40 OD010440**Abstract**

Nudix proteins catalyze the hydrolysis of pyrophosphate bonds in a variety of substrates and are ubiquitous in all domains of life. The genome of an important opportunistic human pathogen, *Pseudomonas aeruginosa*, encodes multiple Nudix proteins. To determine the role of nine Nudix hydrolases of the *P. aeruginosa* PAO1161 strain in its fitness, virulence or antibiotic resistance mutants devoid of individual enzymes were constructed and analyzed for growth rate, motility, biofilm formation, pyocyanin production, and susceptibility to oxidative stress and different antibiotics. The potential effect on bacterial virulence was studied using the *Caenorhabditis elegans*-*P. aeruginosa* infection model. Of the nine mutants tested, five had an altered phenotype in comparison with the wild-type strain. The $\Delta PA3470$, $\Delta PA3754$, and $\Delta PA4400$ mutants showed increased pyocyanin production, were more resistant to the β -lactam antibiotic piperacillin, and were more sensitive to killing by H_2O_2 . In addition, $\Delta PA4400$ and $\Delta PA5176$ had impaired swarming motility and were less virulent for *C. elegans*. The $\Delta PA4841$ had an increased sensitivity to oxidative stress. These changes were reversed by providing the respective *nudix* gene *in trans* indicating that the observed phenotype alterations were indeed due to the lack of the particular Nudix protein.

KEYWORDSmotility, Nudix mutants, oxidative stress, *Pseudomonas aeruginosa*, pyocyanin, virulence

1 | INTRODUCTION

Pseudomonas aeruginosa, a ubiquitous environmental bacterium capable of infecting a wide variety of organisms, has emerged as a leading source of nosocomial infections that displays not only an intrinsic resistance to many antibiotics but also a remarkable ability to adapt and develop novel mechanisms of resistance during treatment. Moreover, its pathogenicity is mediated by multiple cell-associated and excreted factors. Type III secretion, quorum sensing, biofilm formation, and motility are the most studied factors of *P. aeruginosa* that impact infections (Jimenez et al., 2012). However,

since almost half of the proteins encoded by the *P. aeruginosa* genome have not been assigned a function yet (Winsor et al., 2011), numerous additional determinants of pathogenesis, factors involved in resistance to antibacterial treatments and elements responsible for the high adaptive capacity are likely to exist. Among such putative unrecognized factors are members of the Nudix family which could have a significant impact on these processes. The *P. aeruginosa* genome encodes fourteen Nudix proteins: PA0336, PA0990, PA1823, PA2625, PA2769, PA3180, PA3470, PA3754, PA3755, PA4400, PA4841, PA4916, PA4971, and PA5176. Only for PA0336, PA1823, and PA4916 have the physiological consequences of their

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lack been described (Kujawa et al., 2017; Modzelan, Kujawa, Głąbski, Jagura-Burdzy, & Kraszewska, 2014; Okon et al., 2017).

Genes encoding Nudix hydrolases are present not only in all organisms but also found in viral genomes indicating that they are conserved evolutionarily. Nudix proteins are typically small (16–35 kDa) and have two domains. A highly conserved Nudix motif GX5EX7REUXEEXGU (where U is a hydrophobic amino acid, usually leucine, isoleucine, or valine, and X may be any amino acid) is located in the C-terminal domain and functions as the catalytic site. This motif is a part of the Nudix fold characteristic for all Nudix family members. Nudix proteins are enzymes catalyzing the hydrolysis of pyrophosphate bonds in a variety of substrates, mainly nucleoside diphosphate derivatives such as (d)NTPs and (r)NTPs (canonical and modified), nucleoside sugars, and coenzymes including NAD, NADH, and CoA (McLennan, 2006). Also, proteins from this family hydrolyze the m⁷GTP mRNA cap in eukaryotes and the 5'-triphosphorylated bacterial transcripts (Messing et al., 2009; Song, Bail, & Kiledjian, 2013). Apart from their catalytic activity, some Nudix proteins can act directly as transcription factors (Gao, Wei, Hassan, Li,

Deng, & Feng, 2019; Rodionov et al., 2008), and some play important regulatory functions in response to stress and in pathogenesis (Alva-Pérez, Arellano-Reynoso, Hernández-Castro, & Suárez-Güemes, 2014; Modzelan et al., 2014; Okon et al., 2017; Wagley et al., 2018; Zhang, Zborníková, Rejman, & Gerdes, 2018).

To address the question of the possible involvement of Nudix proteins in pathogenesis and stress response of *P. aeruginosa*, nine single *nudix* mutants were constructed and their phenotypic features determined.

2 | EXPERIMENTAL PROCEDURES

2.1 | Bacterial strains, plasmids, primers, and media

The *Escherichia coli* and *P. aeruginosa* strains used in this study are listed in Table 1. Plasmids and primers are listed in Tables A1 and A2, respectively. Primer synthesis and DNA sequencing were performed at the Institute of Biochemistry and Biophysics, PAS. Bacteria were

Strain	Description	Source
<i>Pseudomonas aeruginosa</i>		
PAO1161	<i>leu-r-Rif^R</i>	Lasocki, Bartosik, Mierzejewska, Thomas, and Jagura-Burdzy (2007)
PAO1161ΔPA0990	<i>leu,r, Rif^R, ΔPA0990</i>	This study
PAO1161ΔPA2769	<i>leu-,r-, Rif^R, ΔPA2769</i>	This study
PAO1161ΔPA3180	<i>leu-,r-, Rif^R, ΔPA3180</i>	This study
PAO1161ΔPA3470	<i>leu-,r-, Rif^R, ΔPA3470</i>	This study
PAO1161ΔPA3754	<i>leu-,r-, Rif^R, ΔPA3754</i>	This study
PAO1161ΔPA3755	<i>leu-,r-, Rif^R, ΔPA3755</i>	This study
PAO1161ΔPA4400	<i>leu-,r-, Rif^R, ΔPA4400</i>	This study
PAO1161ΔPA4841	<i>leu-,r-, Rif^R, ΔPA4841</i>	This study
PAO1161ΔPA5176	<i>leu-,r-, Rif^R, ΔPA5176</i>	This study
<i>Escherichia coli</i>		
S17-1	<i>recA pro hsdRhsdMTPRStrR ΩRPT-Tc::Mu-Km::TnT</i>	Simon, O'Connell, Labes, and Puhler (1986)
XL1-Blue MRF'	<i>Δ(mcrA)183 Δ(mcrCB- hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB</i>	Baba et al. (2006)
HB101	<i>supE44 hsdS20(rb-mB-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 (SmR) xyl-5 mtl-1</i>	Boyer and Roulland-Dussoix (1969)
DH5αλpir	<i>supE44, ΔlacU169 (φ80 lacZΔM15), hsdR17 (rk-mk+), recA1, endA1, thi1, gyrA, relA, λpirlysogen</i>	Martínez-García and de Lorenzo (2011)
OP50	Uracil auxotroph	Brenner (1974)

TABLE 1 Bacterial strains used in this study

grown in Luria-Bertani (L-broth) medium, on L-agar (L-broth with 1.5% w/v agar) at 37°C or in minimal media M9 (Sambrook, Fritsch, & Maniatis, 1989), supplemented with thiamine (1 µg/ml). If needed, appropriate antibiotics were added to the media as follows: ampicillin, 100 µg/ml for Ap^R in *E. coli*; chloramphenicol, 25 µg/ml for Cm^R in *E. coli* and 200 µg/ml in *P. aeruginosa*; kanamycin sulfate, 50 µg/ml for Km^R in *E. coli* and 500 µg/ml in *P. aeruginosa*; carbenicillin disodium salt, 300 µg/ml for Cb^R in *P. aeruginosa*; and rifampicin, 300 µg/ml in *P. aeruginosa*.

2.2 | Introduction of mutant nudix alleles into *Pseudomonas aeruginosa* PAO1161

Mutants were obtained with the use of two slightly different methods. Δ PA0990, Δ PA3180, Δ PA3755, Δ PA4841, and Δ PA5176 mutants were obtained as follows: for each gene, upstream and downstream DNA fragments of about 300–500 nucleotides were amplified using chromosomal DNA as a template. These fragments were subsequently ligated to the suicide pAKE600 vector and a gentamicin cassette was ligated in between. The pAKE600 vector encodes a pMB1 *ori* that enables it to replicate in *P. aeruginosa* (El-Sayed, Hothersall, & Thomas, 2001). *Escherichia coli* S17-1 was transformed with the obtained plasmids (pAKE Δ 0990, pAKE Δ 3180, pAKE Δ 3755, pAKE Δ 4841, or pAKE Δ 5176), and the transformants were conjugated with *P. aeruginosa* PAO1161 as the recipient strain using the procedure described by Bartosik, Mierzejewska, Thomas, and Jagura-Burdzy (2009). Following removal of the integrated suicide vector, *P. aeruginosa* colonies were analyzed by RT-PCR to determine whether the allele exchange was successful and the transcript of the particular *nudix* gene was absent (Figure A1).

The Δ PA3470, Δ PA3754, and Δ PA4400 mutants were obtained by the method developed by Martínez-García and de Lorenzo (2011). For each gene, upstream and downstream DNA fragments of 300–500 nucleotides in length were amplified as before and ligated to the suicide pEMG Km^R vector and a gentamicin cassette was ligated in between. The obtained plasmids (pEMG Δ 3470, pEMG Δ 3754, and pEMG Δ 4400) were introduced into *E. coli* DH5 α pir strain and transformants were conjugated by triparental mating with *P. aeruginosa* PAO1161 as the recipient strain with the assistance of the helper strain *E. coli* HB101 bearing the pRK2013 plasmid (de Lorenzo & Timmis, 1994). The pEMG-derived plasmids unable to replicate in *P. aeruginosa* integrated into the PAO1161 chromosome. The obtained transformants were selected and the pSW-I plasmid, carrying the *SceI* nuclease under the control of 3-methylbenzoate (3MB)-inducible promoter, was introduced to them by triparental mating. The excision of the integrated vector was achieved by adding 3MB (15 mM, final concentration) to bacterial cultures. Following the removal of the integrated vector, *P. aeruginosa* colonies were analyzed for the absence of the respective transcript (Figure A1).

2.3 | Introduction of wild-type allele into mutant strain- trans-complementation

The PA3470, PA3754, PA4400, PA4841, or PA5176 genes were PCR-amplified from the *P. aeruginosa* genome and cloned into pQE80L vectors and subsequently subcloned into the pBBR plasmids (Table A1). The plasmid carrying the wild-type copy of a *nudix* gene was introduced by conjugation (see above) into the appropriate deletion mutant. Production of the protein was induced by 0.02% arabinose.

2.4 | Biofilm production assay

Overnight cultures of *P. aeruginosa* PAO1161 and mutant strains were diluted 1:100 in fresh L-broth medium in three replicates, and 100 µl of each diluted culture was transferred into 8 wells on a 96-well plate (wild-type *P. aeruginosa* PAO1161 and one mutant strain on each plate). The plates were incubated statically at 37°C for approximately 20 hr. OD₆₀₀ was measured with a plate reader. The medium with planktonic bacteria was removed, the wells were washed three times with PBS, 200 µl of 0.1% crystal violet solution was added to each well and incubated for 30 min at room temperature. The solution was removed, and the wells were washed three times with water and once with PBS. The plates were dried and 100 µl of 96% ethanol was added to dissolve the bound stain. After 10 min of incubation at room temperature, the solution was mixed by pipetting, OD₅₉₀ was determined, and the OD₅₉₀/OD₆₀₀ ratio was calculated.

2.5 | Motility assays

The assays were performed according to Rashid and Kornberg (2000). Swimming plates (1% tryptone, 0.5% NaCl, 0.3% agar), swarming plates (0.8% nutrient broth, 0.5% dextrose, 0.5% agar), and twitching plates (1% bactotryptone, 0.5% NaCl, 1.5% agar) were inoculated from fresh overnight cultures on L-agar plates with a sterile toothpick and observed after incubation at 37°C for 24 hr. Motility tests were repeated at least three times.

2.6 | Pyocyanin quantification

Overnight cultures of *P. aeruginosa* PAO1161 and mutant strains were inoculated 1:100 in 20 ml of L-broth and grown in triplicate at 37°C with aeration. After 12 hr of incubation, two 7.5-ml samples were withdrawn from each culture and extracted with 4.5 ml of chloroform and then 1.5 ml 0.2 M HCl was added to the extract causing the color change. OD₅₂₀ was determined and the obtained values were converted to pyocyanin content following Essar, Eberly, Hadero, and Crawford (1990). The experiment was repeated at least three times.

2.7 | Antibiotic sensitivity tests

Overnight cultures of *P. aeruginosa* PAO1161 and mutant strains were diluted 1:100 in 15 ml of fresh L-broth medium and incubated at 37°C to OD₆₀₀ equal to 0.1. Bacteria were spread evenly on Mueller–Hinton plates (Biomaxima S.A; 17.6 g/L casein hydrolase, 2.0 g/L beef extract, 1.5 g/L starch, 17 g/L agar) to give a homogeneous lawn, and then, antibiotic disks (Oxoid) were placed on the center of each plate. Disks with antibiotics from different groups such as cephalosporins: ceftazidime (10 µg) (CAZ); quinolones: ciprofloxacin (5 µg) (CIP); β-lactams: imipenem (10 µg) (IPM), meropenem (10 µg) (MEM), and piperacillin (100 µg) (PRL); polymyxins: polymyxin B (300 µg) (PB) and colistin (10 µg) (CT); and aminoglycosides: tobramycin (10 µg) (TOB) were used. The plates were incubated at 37°C for 20 hr, and the diameter of growth inhibition was measured. The tests were repeated several times.

2.8 | Determination of H₂O₂-induced killing

Overnight cultures of *P. aeruginosa* PAO1161 and mutant strains were diluted 1:100 in 15 ml of fresh L-broth medium and grown to an OD₆₀₀ of ~0.6. For each assay, 8 ml of culture was centrifuged and the pellet suspended in 8 ml of sterile 0.8% NaCl. A sample of the cell suspension was diluted and spread on L-agar plates and H₂O₂ (Sigma-Aldrich) was added to the remaining cells immediately to a final concentration of 200 mM. After 15 min of shaking at 37°C, the cells were transferred to the fresh L-broth medium to obtain appropriate dilutions. To determine survivability, the dilutions were spread on L-broth agar plates, incubated overnight at 37°C and colonies were counted. The survival rate was calculated as a ratio of the number of colonies formed by cells treated with H₂O₂ to that of colonies formed by untreated cells (taken as 100%).

2.9 | Mutation frequency

To determine the mutation frequency of PAO1161 and mutant strains in response to fosfomycin (Fos) or streptomycin (Str), overnight culture in 10 ml L-broth was centrifuged and the pellet was suspended in 1 ml of L-broth. Portions (100 µl) from this suspension and successive dilutions were plated onto L-agar plates as well as onto L-agar plates containing 128 µg/ml fosfomycin or 300 µg/ml streptomycin. The number of colonies (CFU) was counted after incubation at 37°C for 48 hr, and the ratio between colonies on L-agar agar and antibiotic plates refers to the mutation frequency. These experiments were reproduced at least three times.

2.10 | Nematode handling

Caenorhabditis elegans wild-type strain N2 (Brenner, 1974) used in this study was obtained from the *Caenorhabditis* Genetics Center

(GCG), University of Minnesota, USA. Worms were grown at 20°C on nematode growth medium (NGM) plates with *E. coli* HB101 as a food source. Gravid adults were synchronized by hypochloride treatment and eggs hatched in S-Basal buffer (Brenner, 1974) with vigorous shaking at 20°C. At approximately 18–20 hr, L1 larvae were harvested and grown to the L4 stage at 25°C for use in killing experiments.

2.11 | *Caenorhabditis elegans* killing assay

This method was first described by Tan et al. (1999). Overnight cultures of *P. aeruginosa* PAO1161 and mutant strains were diluted 1:100 in 15 ml of fresh L-broth medium, and 120 µl was spread on 35-mm NGM plates and incubated at 37°C for 24 hr and then for 24 hr at 25°C. Each plate was then seeded with 50 L4-stage hermaphrodite worms. Plates were incubated at 25°C and scored for live worms every 24 hr. Three or four replicates were carried out for each bacterial strain. *Escherichia coli* OP50 was used as a negative control. A worm was considered dead when it no longer responded to touch. Any worms that died as a result of getting stuck to the wall of the plate were excluded from the analysis. The experiment was repeated at least three times for each strain.

3 | RESULTS

Phenotypic characteristics of Δ PA0990, Δ PA2769, Δ PA3180, Δ PA3470, Δ PA3754, Δ PA3755, Δ PA4400, Δ PA4841, and Δ PA5176 deletion mutants.

3.1 | Growth

To examine the biological impact of Nudix hydrolases on *P. aeruginosa*, we tested the effects of individual chromosomal deletions of the PA0990, PA2769, PA3180, PA3470, PA3754, PA3755, PA4400, PA4841, or PA5176 genes on *P. aeruginosa* cells. The mutated allele in which the coding sequence was replaced with an antibiotic cassette was cloned into a suicide vector and introduced by mobilization into the *P. aeruginosa* PAO1161 strain and then incorporated into the chromosome by homologous allele exchange (Bartosik et al., 2009; Martínez-García & de Lorenzo, 2011). The effect of the insertion was verified by RT-PCR, and no wild-type transcripts of the respective genes were detected in any of the mutant strains (Figure A1).

To determine how the lack of the *nudix* genes affected growth, single bacterial colonies of each mutant were transferred into L-broth and growth was monitored. No major differences in the growth rate were observed between the mutants and the parental strain in either the exponential or stationary phase. (Figure 1a). Similarly, no growth alterations were found when the rich medium was replaced with the M9 minimal medium. The only mutant that showed slower growth was Δ PA4400 (Figure 1b). Interestingly, the amino acid sequence of the

FIGURE 1 Effect of *nudix* mutations on the growth of *Pseudomonas aeruginosa*. (a) Growth curve of wild-type *P. aeruginosa* PAO1161 and Δ PA0990, Δ PA2769, Δ PA3180, Δ PA3470, Δ PA3754, Δ PA3755, Δ PA4400, Δ PA4841, and Δ PA5176 mutants on L-broth. (b) Growth curve of wild-type *P. aeruginosa* PAO1161 and Δ PA0990, Δ PA2769, Δ PA3180, Δ PA3470, Δ PA3754, Δ PA3755, Δ PA4400, Δ PA4841, and Δ PA5176 mutants on M9 minimal medium. (c) Growth curve of wild-type *P. aeruginosa* PAO1161 and Δ PA4400 mutant on M9 minimal medium and M9 supplemented with thiamine (1 μ g/ml). (d) Alignment of *P. aeruginosa* PA4400 and *Burkholderia vietnamensis* thiamine synthase ThiE amino acid sequences. The alignment was performed using NCBI Protein BLAST[®]. Identical amino acids are in white-on-black, and similar ones are on a gray background. Nudix motif is boxed and the fragment used for alignment is underlined in red

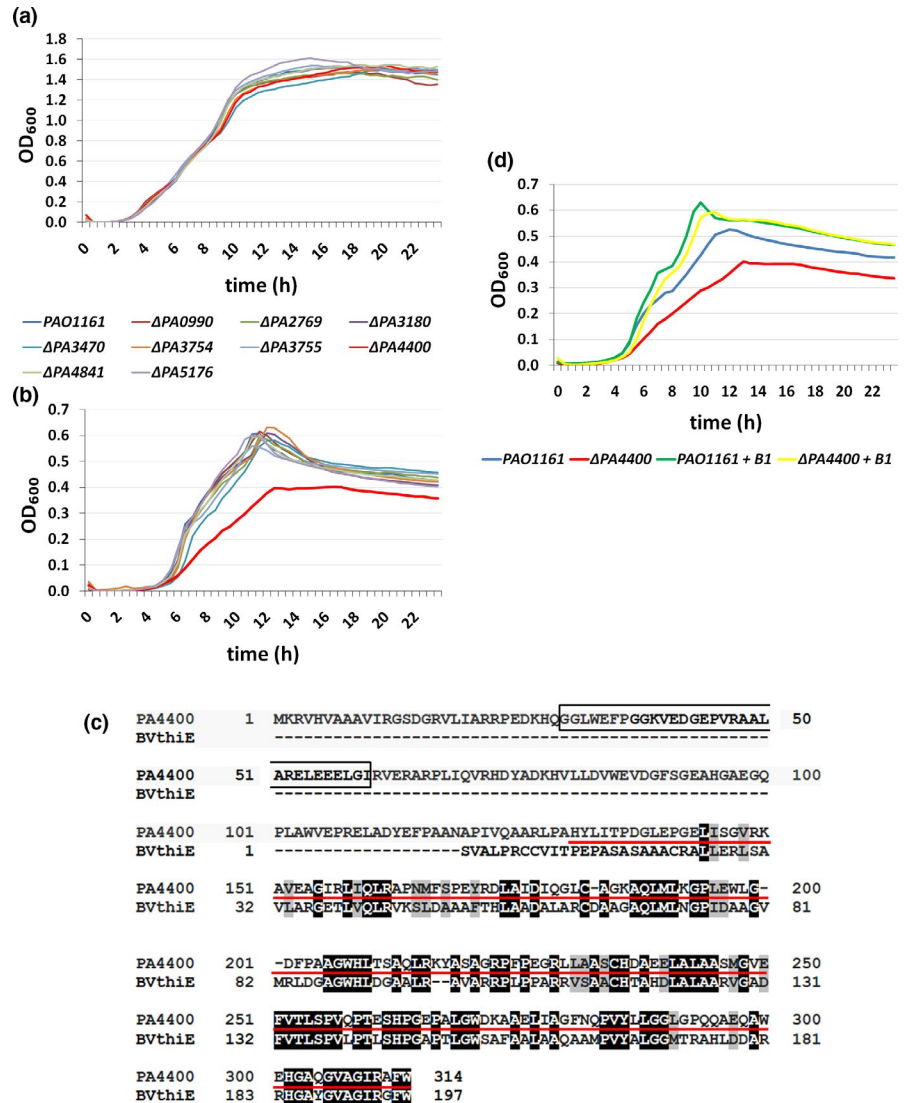
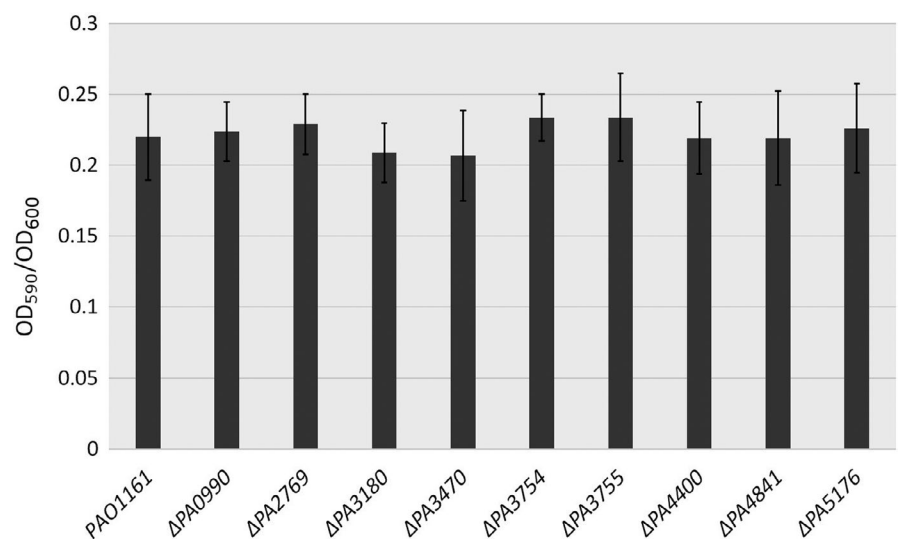


FIGURE 2 Effect of *nudix* mutations on biofilm formation. Biofilm production by wild-type *Pseudomonas aeruginosa* PAO1161 and *nudix* mutants was determined as in *Experimental Procedures*. The experiment was repeated at least three times with similar results



C-terminal part of PA4400 protein is 47% identical to the consensus sequence of thiamine monophosphate synthase (Figure 1c), suggesting that PA4400 is bifunctional and apart from its nudix function plays a

role in thiamine biosynthesis. Indeed, when the M9 medium was supplemented with thiamine no differences in growth were observed between the mutant and the parental strain (Figure 1d).

3.2 | Biofilm formation

Recently, it was shown that the RenU Nudix hydrolase of *Mycobacterium smegmatis* influences biofilm formation (Wolff et al., 2015). To check whether any of the *P. aeruginosa* Nudix enzymes tested had a similar effect, biofilm production by the mutants was determined. No significant differences between the mutants and the parental strain were observed indicating that none of these Nudix proteins participated in this process (Figure 2).

3.3 | Motility

It is well recognized that motility is strongly associated with bacterial adaptation and virulence. To establish whether the Nudix proteins influenced motility, each mutated strain was tested for their swimming, swarming, and twitching ability. While the swimming and twitching motility was not disturbed in the mutants (Figures A2 and A4), the lack of either PA4400 or PA5176 protein severely impaired the type IV pili-flagella-rhamnolipids-dependent swarming as compared to the wild-type cells (Figures 3 and A3).

3.4 | Pyocyanin production and antibiotic susceptibility

We have earlier shown that Nudix hydrolase PA0336 (RppH) influences pyocyanin production in *P. aeruginosa* (Kujawa et al., 2017). To check whether any other Nudix protein could be important for this process, the level of pyocyanin was determined in the *nudix* mutants. Pyocyanin content was increased in the Δ PA3470, Δ PA3754, and Δ PA4400 mutants by 43%, 58%, and 33%, respectively (Figure 4a). Interestingly, the same mutants were also more resistant to piperacillin than was the parental strain or the other *nudix* mutants.

(Figure 5a). Notably, both differences were nullified following trans-complementation with the respective *nudix* gene (Figures 4b and 5b). Except for piperacillin, no significant differences in the response to other antibiotics were observed between the strains assayed (Figure A5).

3.5 | Oxidative stress and mutation frequency

As a consequence of inflammation, bacteria colonizing human airways are exposed to massive oxidative stress caused by the reactive oxygen species released by leukocytes as the first line of defense (Ciofu, Riis, Pressler, Poulsen, & Høiby, 2005). To infect humans effectively *P. aeruginosa* responds with adaptive and protective strategies against these toxic molecules. To examine whether Nudix hydrolases participate in the response to such stress, the mutated *P. aeruginosa* strains were exposed to hydrogen peroxide. The Δ PA3470, Δ PA3754, Δ PA4400, and Δ PA4841 mutants were sensitized to killing by H₂O₂ (Figure 6a), indicating that these Nudix proteins may play a protective role, for example, by hydrolyzing the oxidized dNTPs which appear in the nucleotide pool following oxidative stress. Oxidized dNTPs are highly mutagenic (Pericone et al., 2002). To establish whether indeed these Nudix hydrolyze mutagenic dNTP derivatives mutation frequency was determined for the *nudix* mutants by measuring the frequency of appearance of antibiotic-resistant colonies for two types of antibiotics. Apart from Δ PA4400, no mutant tested displayed an increased spontaneous mutation frequency (Table 2), suggesting that except PA4400, the other Nudix enzymes have no antimutator activity. Interestingly, the lack of PA5176 hydrolase increases bacterial resistance to killing by H₂O₂. Comparing to the parental strain, the Δ PA3470, Δ PA3754, Δ PA4400, and Δ PA4841 mutants complemented with a wild-type copy of the respective gene were no longer sensitized to killing by H₂O₂ (Figure 6b).

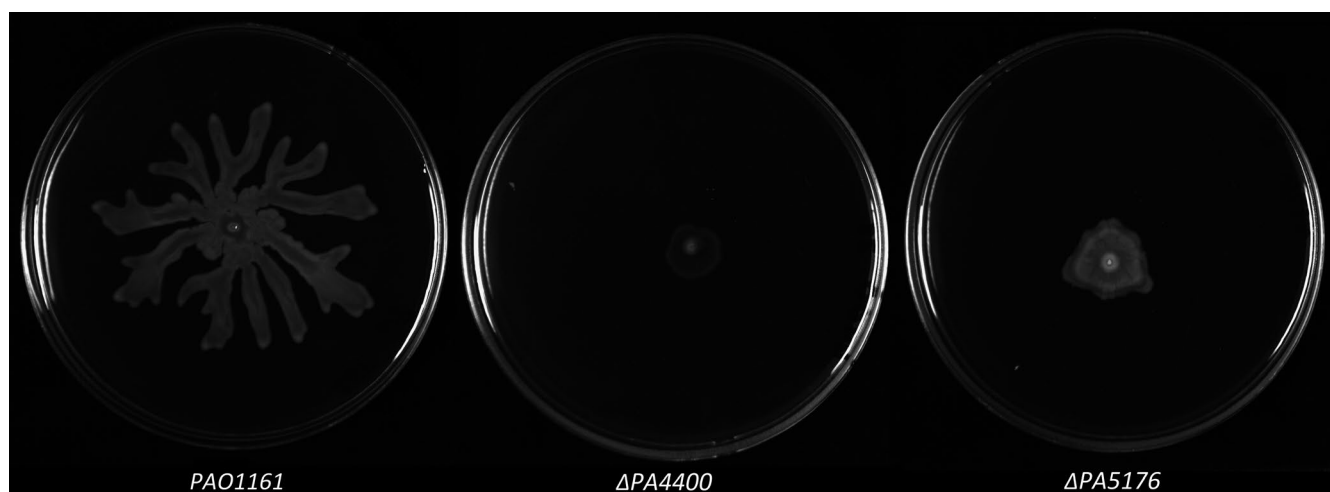


FIGURE 3 Effect of Δ PA4400 and Δ PA5176 mutation on swarming. Swarming motility of wild-type *Pseudomonas aeruginosa* PAO1161 and Δ PA4400 or Δ PA5176 mutants was examined as described in *Experimental Procedures*. Representative results obtained from three independent experiments are shown

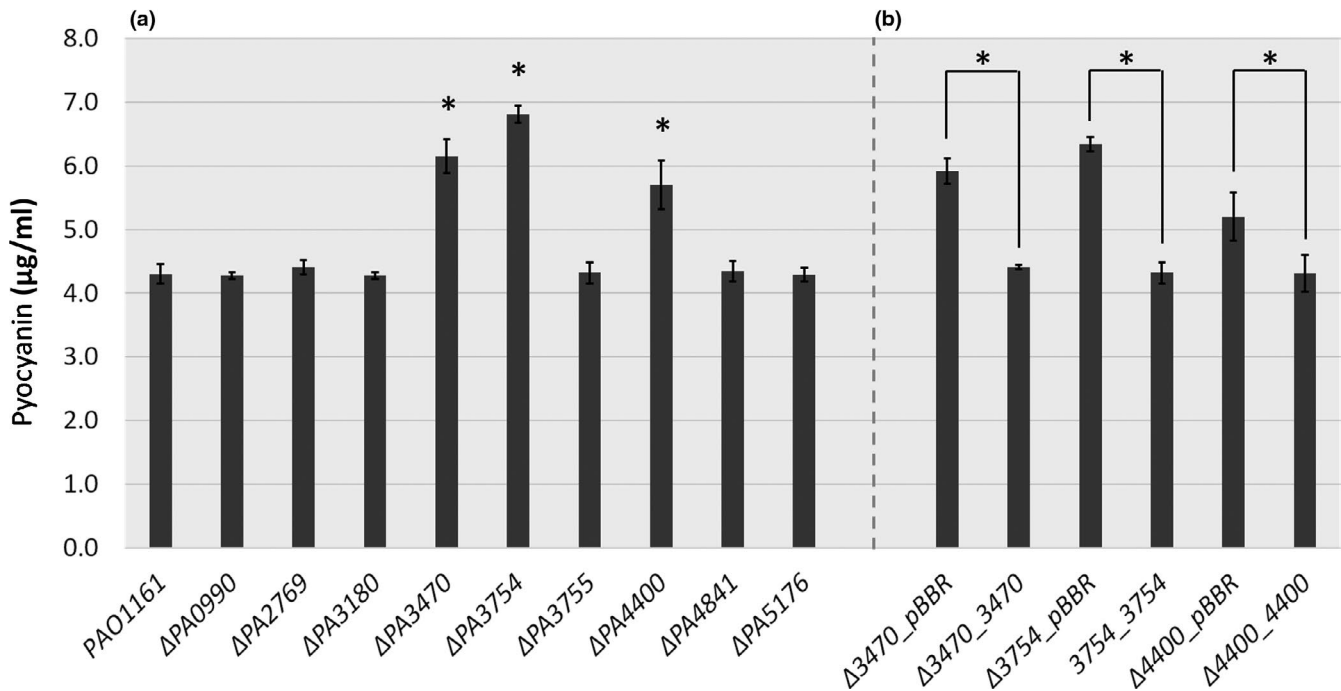


FIGURE 4 Effect of *nudix* mutation on pyocyanin production. (a) Production of pyocyanin by wild-type *Pseudomonas aeruginosa* PAO1161 and *nudix* mutants. (b) Production of pyocyanin by *nudix* mutants transformed either with empty pBBR vector or pBBR carrying wild-type copy of the respective *nudix* gene. Pyocyanin level (µg/ml) was determined at the stationary phase of growth. The mean value of at least three independent replicates \pm SD is shown. Significant differences were indicated as * $p \leq .05$

3.6 | Virulence

To determine whether the lack of Nudix hydrolase affected bacterial virulence, we employed *C. elegans*, a widely used model eukaryotic organism to study various aspects of host-pathogen interactions (Tan, Mahajan-Miklos, & Ausubel, 1999). Only the Δ PA4400 and Δ PA5176 mutants displayed an attenuated virulence compared to the wild type, indicating that these Nudix proteins participate in *P. aeruginosa* pathogenicity (Figure 7a and Appendix Figure A6). As before, the differences in virulence between the mutants and the parental strain were lost when the mutants were trans-complemented with a wild-type copy of the PA4400 or PA5176 gene (Figure 7b).

4 | DISCUSSION

Here, we present characteristics of the Δ PA0990, Δ PA2769, Δ PA3180, Δ PA3470, Δ PA3754, Δ PA3755, Δ PA4400, Δ PA4841, and Δ PA5176 *nudix* mutants of *P. aeruginosa*, focusing on pathogenesis and stress-related features.

The first characterized Nudix family member was the MutT protein from *E. coli*. A *mutT1* mutant strain displayed a significantly higher spontaneous mutation frequency than the wild type (Treffers, Spinelli, & Belser, 1954). Expression, purification, and characterization of the gene product led to the identification of a new enzyme, a nucleoside triphosphatase with a preference for dGTP and its mutagenic oxidized derivative 8-oxo dGTP (Yanofsky, Cox, & Horn, 1966).

Functional homologues of the *E. coli* antimutator MutT protein were identified in other bacteria including *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Bdellovibrio bacteriovorus*, *Vibrio parahaemolyticus*, and *Streptococcus oligofermentans* (Castellanos-Juárez et al., 2006; Patil, Sang, Govindan, & Varshney, 2013; Steyert, Messing, Amzel, Gabelli, & Piñeiro, 2008; Wagley et al., 2018; Zhou, Liu, Tong, & Dong, 2012). It was also noticed that the PA4400 gene of *P. aeruginosa* can complement *mutT* deficient strain of *E. coli* (Oliver, Sánchez, & Blázquez, 2002). Inactivation of the *P. aeruginosa* PA4400 gene increased spontaneous mutation frequency indicating that the encoded protein has an antimutator effect (Sanders, Sudhakara, & Sutton, 2009). In addition to these observations, we found here that the Δ PA4400 mutation stimulates pyocyanin production, severely impairs swarming motility, decreases virulence, and increases resistance to piperacillin. Moreover, we found possible participation of PA4400 protein in thiamine biosynthesis. This hypothesis is under investigation.

A vast majority of Nudix enzymes are not highly specific and exhibit considerable substrate ambiguity, which makes it difficult to assess their biological functions based solely on the substrate preferences established in vitro. Up to now, more than one hundred chemical compounds that are hydrolyzed by Nudix enzymes have been identified (Srouji, Xu, Park, Kirsch, & Brenner, 2017). Despite this, in numerous cases, the identity of the true physiological substrate of many of these hydrolases is uncertain (McLennan, 2013; Nguyen et al., 2016). It appears therefore that the best way to assess the biological role of a Nudix hydrolase is to study the cellular effects of its deficiency.

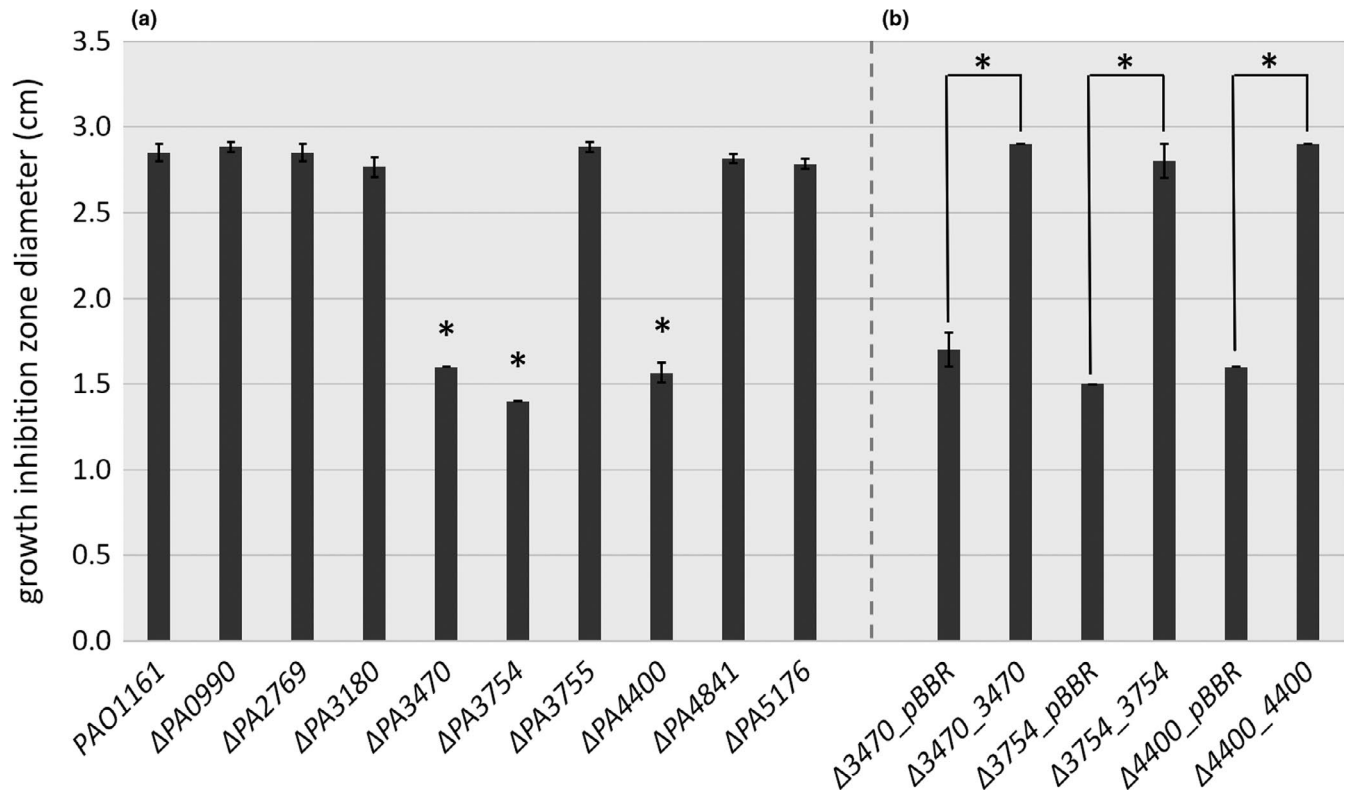


FIGURE 5 Effect of *nudix* mutation on antibiotic susceptibility. (a) Susceptibility to piperacillin of wild-type *Pseudomonas aeruginosa* PAO1161 and *nudix* mutants. (b) Susceptibility to piperacillin of Δ PA3470, Δ PA3754, and Δ PA4400 transformed either with empty pBBR vector or pBBR carrying wild-type copy of the respective *nudix* gene. Susceptibility to the antibiotic was determined by disk diffusion assay as described in *Experimental Procedures*. The mean value of at least three independent replicates \pm SD is shown. Significant differences were indicated as * $p \leq .05$

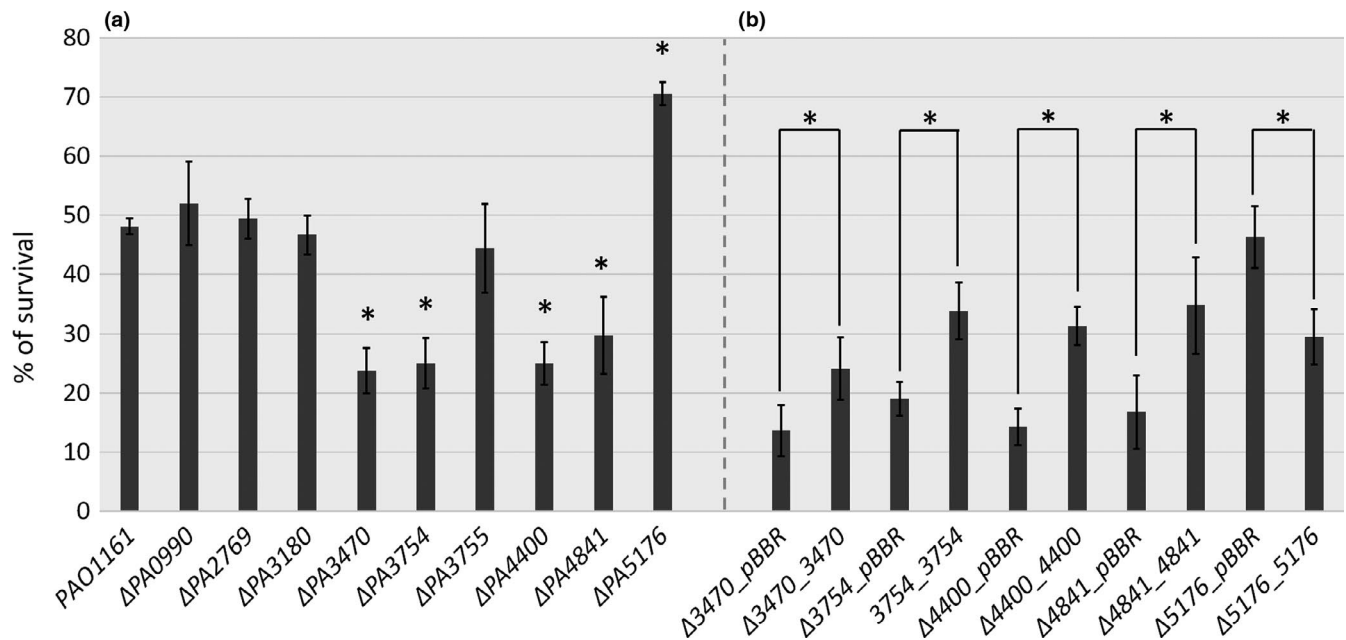


FIGURE 6 Effect of *nudix* mutation on susceptibility to killing by H_2O_2 . (a) Susceptibility to H_2O_2 of *Pseudomonas aeruginosa* wild-type PAO1161 and *nudix* mutant mutants. (b) Susceptibility to H_2O_2 of Δ PA3470, Δ PA3754, Δ PA4400, Δ PA4841, or Δ PA5176 transformed either with empty pBBR vector or pBBR carrying wild-type copy of the gene indicated. To determine survivability, the bacteria were treated with 200 mM H_2O_2 as described in *Experimental Procedures*. The mean value of at least three independent replicates \pm SD is shown. Significant differences were indicated as * $p \leq .05$

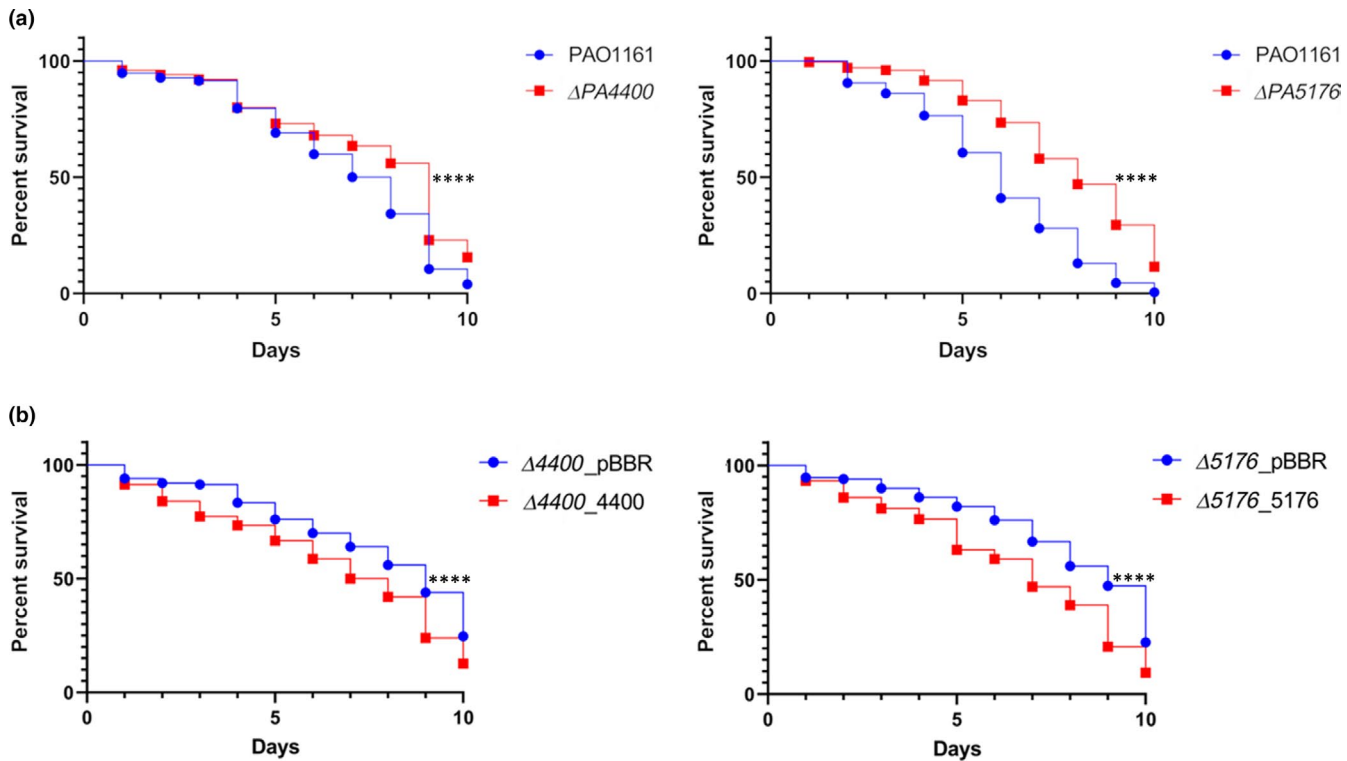


FIGURE 7 Effect of *nudix* mutation on virulence. (a) Effect of $\Delta PA4400$ and $\Delta PA5176$ mutation on *Pseudomonas aeruginosa* virulence. (b) Effect of $\Delta PA4400$ and $\Delta PA5176$ transformed either with empty pBBR vector or pBBR, carrying wild-type copy of the gene indicated, on *P. aeruginosa* virulence. *Pseudomonas aeruginosa*-*Caenorhabditis elegans* infection model was used to determine the pathogenicity of *Pseudomonas* strains as described in *Experimental Procedures*. The experiment was repeated at least three times. Shown are results of the representative experiment presented as the Kaplan-Meier survival curves of *C. elegans* fed wild-type PAO1161, *nudix* mutants or mutants complemented by the wild-type copy of the *PA4400* or *PA5176* gene. The significant differences between wild type and mutant were determined by the log-rank test (**** $p < .0001$)

TABLE 2 Str^R and Fos^R mutation frequency of PAO1161 and *nudix* mutants. Mutation frequency was measured as described in *Experimental Procedures*

Strain	Str ^R	Fold change	Fos ^R	Fold change
PAO1161 Rif ^R	6.26×10^{-10}	-	2.30×10^{-6}	-
PAO1161 Rif ^R $\Delta 0990$	6.22×10^{-10}	-	2.00×10^{-6}	-
PAO1161 Rif ^R $\Delta 2769$	5.80×10^{-10}	-	2.52×10^{-6}	-
PAO1161 Rif ^R $\Delta 3180$	6.20×10^{-10}	-	1.92×10^{-6}	-
PAO1161 Rif ^R $\Delta 3470$	5.95×10^{-10}	-	2.33×10^{-6}	-
PAO1161 Rif ^R $\Delta 3754$	6.00×10^{-10}	-	2.30×10^{-6}	-
PAO1161 Rif ^R $\Delta 3755$	5.50×10^{-10}	-	1.88×10^{-6}	-
PAO1161 Rif ^R $\Delta 4400$	2.33×10^{-7}	372	0.80×10^{-4}	35
PAO1161 Rif ^R $\Delta 4841$	6.18×10^{-10}	-	2.20×10^{-6}	-
PAO1161 Rif ^R $\Delta 5176$	5.89×10^{-10}	-	2.41×10^{-6}	-

Of the nine *nudix* mutants tested, four, $\Delta PA0990$, $\Delta PA2769$, $\Delta PA3180$, and $\Delta PA3755$, did not display any significant phenotypic changes compared to the parental strain. This observation suggests that either these proteins are not essential for the bacteria under the experimental conditions used or that they can be functionally substituted by other Nudix enzymes. Despite the effort, we were unable to construct the $\Delta PA2625$ and $\Delta PA4971$ mutants. Whether it was due to the physiological significance of these genes or to the recombination difficulties in the respective loci remains to be recognized.

Similarly to the $\Delta PA4400$, disabling of the *PA3470*, *PA3754*, or *PA4841* genes sensitized *P. aeruginosa* to killing by H₂O₂, suggesting that the gene products could participate in the repair of the cytotoxic lesions caused by oxidative stress as has been shown for the *P. aeruginosa* PA4400 protein (Oliver et al., 2002 and Table 2 here). It has also been observed that in addition to their preferred substrates some other non-MutT Nudix hydrolases often display a residual antimutator activity (Arczewska et al., 2011; Dos Vultos, Blazquez, Rauzier, Matic, & Gicquel, 2006). However, no increase in the mutation frequency was observed in these *nudix* mutants indicating that the *PA3470*, *PA3754* and *PA4841* proteins have no such activity.

It is well known that cell motility and pyocyanin production are among the factors modulating *P. aeruginosa* virulence (Hall et al., 2016; Kazmierczak, Schniederberend, & Jain, 2015). However, the

increased pyocyanin level in $\Delta PA3470$, $\Delta PA3754$, and $\Delta PA4400$ was not accompanied by an increased virulence. Most probably, this was due to the mutant's increased sensitivity to H_2O_2 . It is well recognized that H_2O_2 production by macrophages is often the host's first line of defense against a pathogen.

Of the mutants tested only $\Delta PA4400$ and $\Delta PA5176$ with severely affected swarming motility also displayed lower virulence as compared to the parental strain, which suggests that in the experimental conditions used swarming motility is the most significant determinant of *P. aeruginosa* pathogenicity.

Taken together, we have demonstrated that most of the Nudix hydrolases present in *P. aeruginosa* are important in the response to genotoxic stress and only a few play a role in bacterial pathogenicity.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Elzbieta Kraszewska: Conceptualization (lead); Funding acquisition (lead); Supervision (lead); Writing – review and editing (lead). **Joanna Drabinska:** Formal analysis (equal); Investigation (equal); Methodology (equal). **Mateusz Ziecina:** Data curation (equal); Investigation (equal); Methodology (equal). **Marta Modzelan:** Investigation (supporting); Methodology (supporting). **Grazyna Jagura-Burdzy:** Funding acquisition (supporting); Supervision (supporting); Writing – review and editing (supporting).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data are provided in full in the results section of this paper.

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APPENDIX 1

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Bacterial transformation

Competent *Escherichia coli* were prepared by the standard CaCl₂ method, and plasmid DNA was introduced by the heat shock method (Sambrook et al., 1989).

DNA manipulations

Purification of plasmids and *Pseudomonas* genomic DNA was conducted using Plasmid MiniKit and Genomic Mini Kit, respectively

(A&A Biotechnology), according to the manufacturer's instructions. To purify DNA from agarose, gel slices were cut out and processed using the Gel-Out system (A&A Biotechnology) according to the manufacturer's instructions. PCR amplification was performed using high fidelity Pfu DNA polymerase (Promega). Usually, 30 cycles of amplification were performed, at reaction conditions depending on the pairs of primers and the size of the final amplification product. The PCR reactions were conducted on a PTC-200 EngineCycler (MJ Research) and the PCR products

TABLE A1 Plasmids used in this study

Name	Relevant feature	Source
pQE-80L	<i>oriColE1 ApR T5p lacOlaclq His6 tag, expression vector</i>	Qiagen
pBBR	<i>araBADp, araC, Cm^R broad-host-range expression vector</i>	Bartosik et al., (2014)
pAKE600	<i>oriMB1 oriRK2 Ap^RsacB</i>	El-Sayed et al. (2001)
pEMG	<i>KmR, oriR6K, lacZα with two flanking I-SceI sites</i>	Martínez-García and de Lorenzo (2011)
pRK2013	<i>ori (ColE1) tra⁺ (RK2) Km^R helper plasmid for conjugation</i>	Martínez-García and de Lorenzo (2011)
pSW-I	<i>ApR, oriRK2, xylS, Pm → I-sceI (transcriptional fusion of I-sceI to Pm)</i>	Wong and Mekalanos (2000)
pAKEΔ0990	pAKE600 derivative with 297 upstream bp (incl. first 15 bp of the gene) and 300bp downstream incl. 3 bp of PA0990 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into PstI site	This study
pAKEΔ2769	pAKE600 derivative with 368 bp upstream (incl. first 6 bp of the gene) and 249 bp downstream incl. 7 bp of PA2769 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into PstI site	This study
pAKEΔ3180	pAKE600 derivative with 300 bp upstream (incl. first 15 bp of the gene) and 351 bp downstream incl. last 3 bp of PA3180 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into PstI site	This study
pEMGΔ3470	pEMG derivative with 320 bp upstream (incl. first 15 bp of the gene) and the last 435 bp downstream incl. 7 bp of PA3470 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into PstI site	This study
pEMGΔ3754	pEMG derivative with 446 bp upstream (incl. first 9 bp of the gene.) and the 369 bp downstream incl. 9 bp of PA3754 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into XbaI site	This study
pAKEΔ3755	pAKE600 derivative with 305 bp upstream (incl. 6 first bp of the gene) and 297 bp downstream incl. 3 bp of PA3755 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into PstI site	This study
pEMGΔ4400	pEMG derivative with 302 bp upstream (incl. first 3 bp of the gene) and the 317 bp downstream incl. 9 bp of PA4400 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into XbaI site	This study
pEMGΔ4841	pEMG derivative with 301 bp upstream (first 3 bp of the gene) and 301 bp incl. 12 bp of PA4841 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into KpnI site	This study
pAKEΔ5176	pAKE600 derivative with 327 bp upstream (incl. first 22 bp of the gene) and 300 bp downstream incl. 50 bp of PA5176 gene cloned as EcoRI-BamHI fragment separated by Gm ^R gene cloned into PstI site	This study
pQE3470	pQE-80L derivative with PA3470 cloned into BamHI-PstI site of the vector	This study
pQE3754	pQE-80L derivative with PA3754 cloned into BamHI-PstI site of the vector	This study
pQE3755	pQE-80L derivative with PA3755 cloned into BamHI-PstI site of the vector	This study
pQE4400	pQE-80L derivative with PA4400 cloned into BamHI-PstI site of the vector	This study
pQE4841	pQE-80L derivative with PA4841 cloned into BamHI-PstI site of the vector	This study
pQE5176	pQE-80L derivative with PA5176 cloned into BamHI-PstI site of the vector	This study
pBBR3470	pBBR derivative with His ₆ -tag-PA3470 cloned into BamHI-PstI site of the vector	This study
pBBR3754	pBBR derivative with His ₆ -tag-PA3754 cloned into EcoRI-SacI site of the vector	This study
pBBR3755	pBBR derivative with His ₆ -tag-PA3755 cloned into EcoRI-SacI site of the vector	This study
pBBR4400	pBBR derivative with His ₆ -tag-PA4400 cloned into EcoRI-SacI site of the vector	This study
pBBR4841	pBBR derivative with His ₆ -tag-PA4841 cloned into EcoRI-SacI site of the vector	This study
pBBR5176	pBBR derivative with His ₆ -tag-PA5176 cloned into EcoRI-SacI site of the vector	This study

TABLE A2 Primers used in this study

Name	Sequence 5' → 3'	Restriction enzyme if used (underlined)	Usage
RT0990F	CCGTCTTCTGGAATTGATACT		RT-PCR analysis
RT0990R	CCAGAGCGATCCAGTTCTCG		RT-PCR analysis
RT2625F	CTGAGCGCCGTCACCGGCAT		RT-PCR analysis
RT2625R	AGTTCGTCGCGGGTCAGCCA		RT-PCR analysis
RT2769F	TGATCTTGCGGGACGGCAAG		RT-PCR analysis
RT2769R	CGCCCTCGAAGACGTCATT		RT-PCR analysis
RT3180F	AACCTCGTATCGAATGGCAG		RT-PCR analysis
RT3180R	ATCGCCAGTATTGCCTTCG		RT-PCR analysis
RT3470F	GCACCTCGGCAGCTTCCAG		RT-PCR analysis
RT3470R	GAGATTGTCCGGCTGCGCTTG		RT-PCR analysis
RT3754F	GCCTTCATCCCGACTTCGT		RT-PCR analysis
RT3754R	TTGACCAACTCCACCACCATGA		RT-PCR analysis
RT3755F	ATGGAGAACGCGGAGACCCT		RT-PCR analysis
RT3755R	GGGAATCTCCGCTTCGTCGA		RT-PCR analysis
RT4400F	AGTGGAGTTCGTCACCCTTT		RT-PCR analysis
RT4400R	GCCAGAACGCACGGATAC		RT-PCR analysis
RT4841F	GCCTCCGACGCCGAACATCAT		RT-PCR analysis
RT4841R	GATAGACCGCCTTGCTCAGGG		RT-PCR analysis
RT4971F	GCTCCGTGGCTTCTATCGT		RT-PCR analysis
RT4971R	GGTTGGCGAGCTTCTGCATC		RT-PCR analysis
RT5176F	GAGTTGCAACTGCGCTTCAG		RT-PCR analysis
RT5176R	CTTGGGCAACGACAACCTGGT		RT-PCR analysis
Δ0990-F1	CC <u>GG</u> AATTCTCTCTACGGTAAGTC	EcoRI	Mutant construction
Δ0990-R1	A <u>ACTGC</u> AGGCTGACCGATGGCAT	PstI	Mutant construction
Δ0990-F2	A <u>ACTGC</u> AGTAGCCCCCGACCTC	PstI	Mutant construction
Δ0990-R2	CGGGAT <u>CC</u> CTAGCACGGATGAGC	BamHI	Mutant construction
Δ2625-F1	CGGAAT <u>TC</u> CGAAGACGCTGAGC	EcoRI	Mutant construction
Δ2625-R1	A <u>ACTGC</u> AGCCAGCTCATGGATGTC	PstI	Mutant construction
Δ2625-F2	A <u>ACTGC</u> AGGCCTGATAGAATCCGCG	PstI	Mutant construction
Δ2625-R2	CGGGAT <u>CC</u> AAGTGCTCGAACACG	BamHI	Mutant construction
Δ2769-F1	CGGAAT <u>TC</u> ATTACCTCGTGGTGGTCGA	EcoRI	Mutant construction
Δ2769-R1	A <u>ACTGC</u> AGCGGCATCGTCTACTCCTG	PstI	Mutant construction
Δ2769-F2	A <u>ACTGC</u> AGGCGCTGAGCGACAGGC	PstI	Mutant construction
Δ2769-R2	CGGGAT <u>CC</u> ATGTCCTGCTGGGCTG	BamHI	Mutant construction
Δ3180-F1	CGGAAT <u>TC</u> CTGGCCGAGGCTG	EcoRI	Mutant construction
Δ3180-R1	A <u>ACTGC</u> AGAGGGACATCGTGCACC	PstI	Mutant construction
Δ3180-F2	A <u>ACTGC</u> AGTAACGCCGATGACGTTAG	PstI	Mutant construction
Δ3180-R2	CGGGAT <u>CC</u> CTTCAAGCTGTTTCC	BamHI	Mutant construction
Δ3470-F1	CGGAAT <u>TC</u> GTGCTCACCCGCAATC	EcoRI	Mutant construction
Δ3470-R1	A <u>ACTGC</u> AGCAGGTTGTCGGTCATTGC	PstI	Mutant construction
Δ3470-F2	A <u>ACTGC</u> AGCATGTGCGCTGAGCG	PstI	Mutant construction
Δ3470-R2	CGGGAT <u>CC</u> ACAAACGCCTATATCTTCC	BamHI	Mutant construction
Δ3754-F1	CGGAAT <u>TC</u> AAGGGTATAGAGCTGCAG	EcoRI	Mutant construction
Δ3754-R1	T <u>ATCT</u> AGAGCAGTTCATACTGCCGGG	XbaI	Mutant construction

(Continues)

TABLE A2 (Continued)

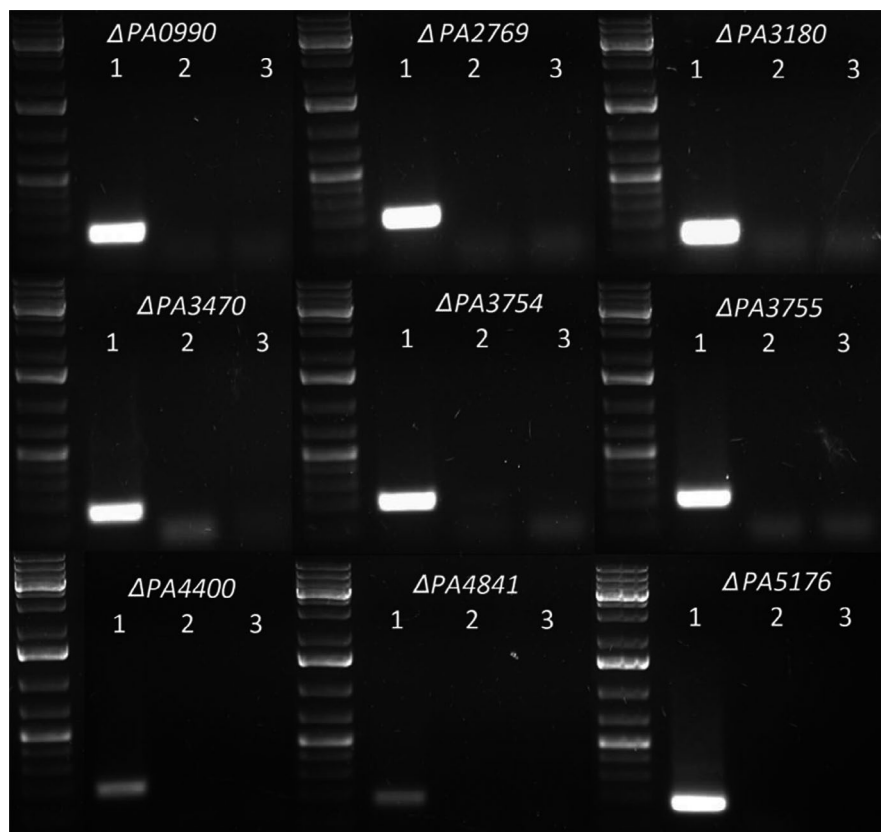
Name	Sequence 5' → 3'	Restriction enzyme if used (underlined)	Usage
Δ3754-F2	TCTCTAGACTGAGTTGACCGACGG	XbaI	Mutant construction
Δ3754-R2	AGGGATCCGTA ^T CTTGCCGATCTTC	BamHI	Mutant construction
Δ3755-F1	CGGAATTCATGGGTGGACAAACCG	EcoRI	Mutant construction
Δ3755-R1	AACTGCAGTTTCATGCCAGGTTCTTC	PstI	Mutant construction
Δ3755-F2	AACTGCAGTGAACGCCAAACACTCG	PstI	Mutant construction
Δ3755-R2	CGGGATCCTCCAGTAGAAGCCTTC	BamHI	Mutant construction
Δ4400-F1	CGGAATTCGCGACACGACAGGATA	EcoRI	Mutant construction
Δ4400-R1	CGTCTAGACACGGATCATCTC	XbaI	Mutant construction
Δ4400-F2	GCTCTAGAGGCC ^T TTGACGG	XbaI	Mutant construction
Δ4400-R2	ATGGATCCGGGCCCGCTGAA	BamHI	Mutant construction
Δ4841-F1	CGGAATTCCTGGCTGCAGATCGAC	EcoRI	Mutant construction
Δ4841 R1	GGGGTACCCATGGGTCAGCC	KpnI	Mutant construction
Δ4841-F2	GGGGTACCTGGACGCCTGAG	KpnI	Mutant construction
Δ4841-R2	CGGGATCCGCGTCTTTGGGTTGC	BamHI	Mutant construction
Δ4971-F1	CGGAATTCCTTGAAGAGGCT	EcoRI	Mutant construction
Δ4971-R1	TATCTAGAGGTTTCGGACATC	XbaI	Mutant construction
Δ4971-F2	TATCTAGAGCCTGAACCTGCT	XbaI	Mutant construction
Δ4971-R2	CGGGATCCTCATTCAACTGG	BamHI	Mutant construction
Δ5176-F1	CGGAATTCAGTACCAGTTGAGCTGGC	EcoRI	Mutant construction
Δ5176-R1	AACTGCAGGTACGGTGGGTTTCTGAC	PstI	Mutant construction
Δ5176-F2	AACTGCAGTGTACCTGGTGCGCG	PstI	Mutant construction
Δ5176-R2	CGGGATCCTGAACTCCTCGCTAC	BamHI	Mutant construction
gentxbaF	GCTCTAGAATGTTACGCAGCA	XbaI	Mutant construction
gentxbaR	GCTCTAGATTAGGTGGCGGT	XbaI	Mutant construction
gentpstF	ATCTGCAGATGTTACGCAGCA	PstI	Mutant construction
gentpstR	ATCTGCAGTTAGGTGGCGGT	PstI	Mutant construction
gentkpnF	ATGGTACCATGTTACGCAGCA	KpnI	Mutant construction
gentkpnR	ATGGTACCTTAGGTGGCGGT	KpnI	Mutant construction
QE3470F	TAGGATCCACCGACAACCTGCTG	BamHI	Mutation complementation
QE3470R	GCCTGCAGTCAGCGCACATGAT	PstI	Mutation complementation
QE3754F	TAGGATCCA ^A ACTGCACGCTCGA	BamHI	Mutation complementation
QE3754R	GCCTGCAGTCAACTCAGTTTG	PstI	Mutation complementation
QE3755F	CGGGATCCAAATCTGCAGCCTG	BamHI	Mutation complementation
QE3755R	ACAAGCTTTCAGTCTTTCTTATAGGAAGCC	HindIII	Mutation complementation
QE4400F	CGGGATCCAAACGAGTACATGTCTG	BamHI	Mutation complementation
QE4400R	ACAAGCTTTCAAAGGCCGCCCG	HindIII	Mutation complementation
QE4841F	ATGGATCCATGGCCGCGCCGAT	BamHI	Mutation complementation
QE4841R	CGCAAGCTTTCAGGCGTCCAGGT	HindIII	Mutation complementation
QE5176F	ATGGATCCCGTCAGAAACCCAC	BamHI	Mutation complementation
QE5176R	AGCTGCAGTCATGCCTGGTACTC	PstI	Mutation complementation
QEecoF	CCGGAATTCATGAGAGGATCGCATCACCATC	EcoRI	Mutation complementation
BBR3470R	GCGAGCTCTCAGCGCACATGATC	SacI	Mutation complementation
BBR3754R	GCGAGCTCTCAACTCAGTTTGATG	SacI	Mutation complementation
BBR3755R	GCGAGCTCTCAGTCTTTCTTATAGGAA	SacI	Mutation complementation

(Continues)

TABLE A2 (Continued)

Name	Sequence 5' → 3'	Restriction enzyme if used (underlined)	Usage
BBR4400R	TT <u>GAGCTCT</u> CAAAGGCCGCCCGG	SacI	Mutation complementation
BBR4841F	GCGCTAGCATGAGAGGATCGCA	NheI	Mutation complementation
BBR4841R	AT <u>GAGCTCT</u> CAGGCGTCCAGGT	SacI	Mutation complementation
BBR5176R	CGAGCTCTCATGCCTGGTACTC	SacI	Mutation complementation

FIGURE A1 Expression of nudix genes in *Pseudomonas aeruginosa* wild-type PAO1161 and mutant strains analyzed by RT-PCR. Lane 1: positive control, RT-PCR product from wild-type *P. aeruginosa* genomic DNA; Lane 2: negative control; Lane 3 RT-PCR product from mutant strain genomic DNA



were identified by agarose gel electrophoresis. The primers are listed in Table A2.

RNA isolation and RT-PCR

RNA was isolated with the use of Total RNA Zol-Out™ Kit (A&A Biotechnology) and genomic DNA was removed with the use of the Clean-UP RNA Concentrator kit (A&A Biotechnology), according

to the manufacturer's instructions. The RT-PCR reaction was performed with the use of the QuantiTect® Reverse transcription Kit (Qiagen) according to the manufacturer's instructions. A reaction without the addition of reverse transcriptase was prepared for negative control. RNA isolated from wild-type *P. aeruginosa* PAO1161Rif^R was used as a positive control.

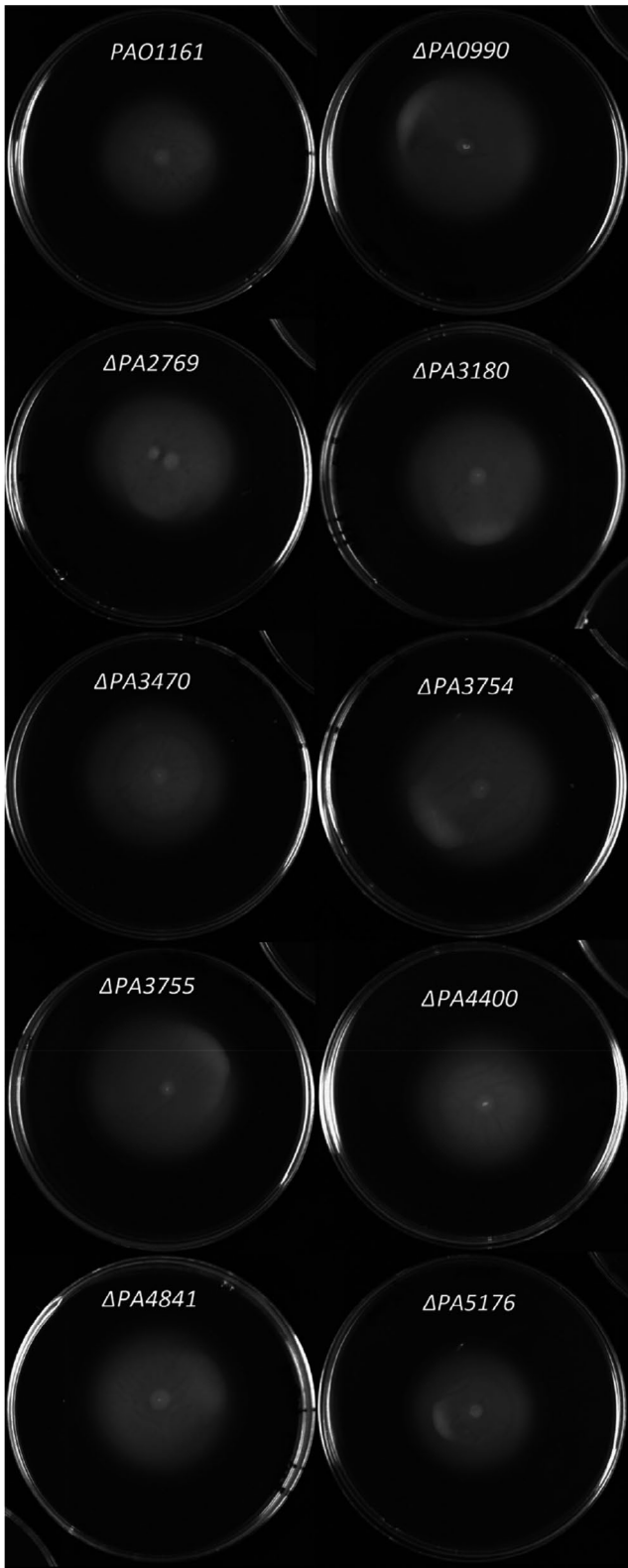


FIGURE A2 Swimming motility of *Pseudomonas aeruginosa* PAO1161 wild type and mutant strains, tested as described under Experimental Procedures

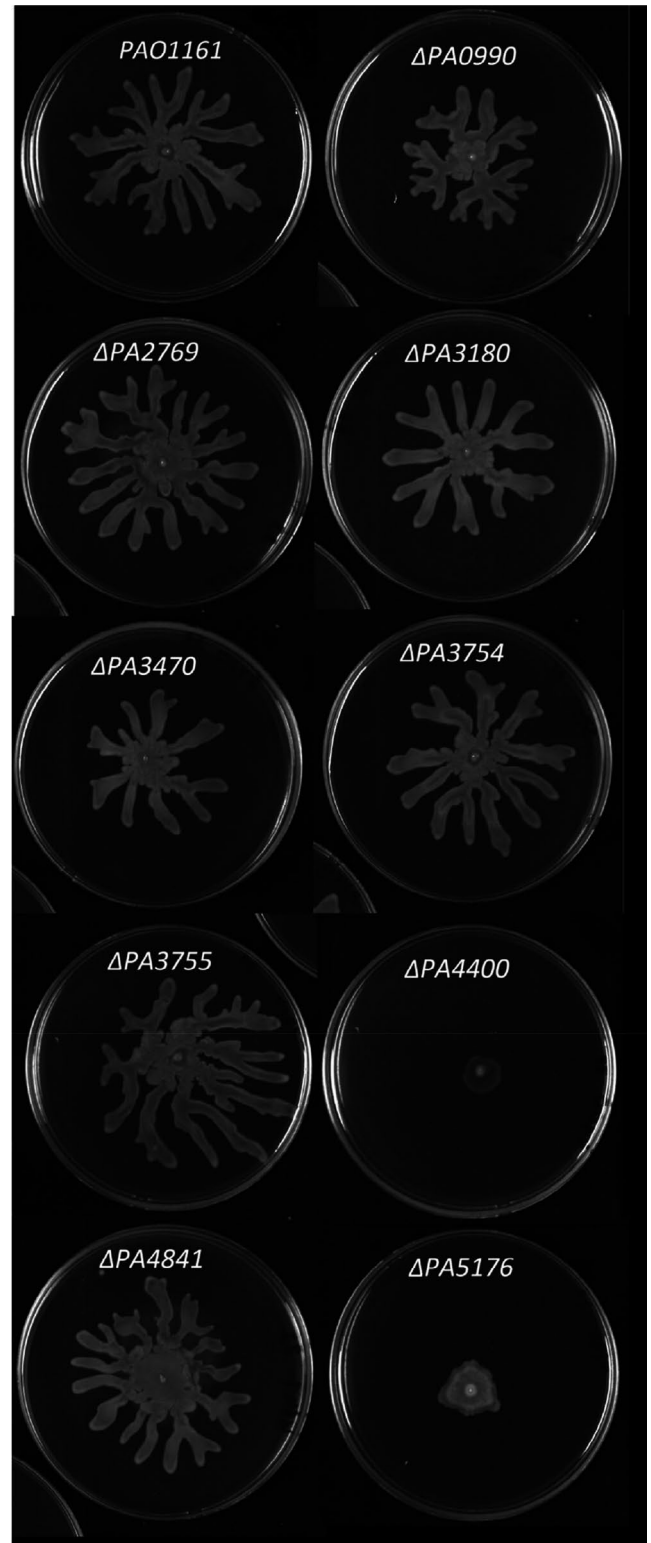


FIGURE A3 Swarming motility of *Pseudomonas aeruginosa* PAO1161 wild type and mutant strains. Tested as described under Experimental Procedures



FIGURE A4 Twitching motility of *Pseudomonas aeruginosa* PAO1161 wild type and mutant strains. Tested as described under Experimental Procedures

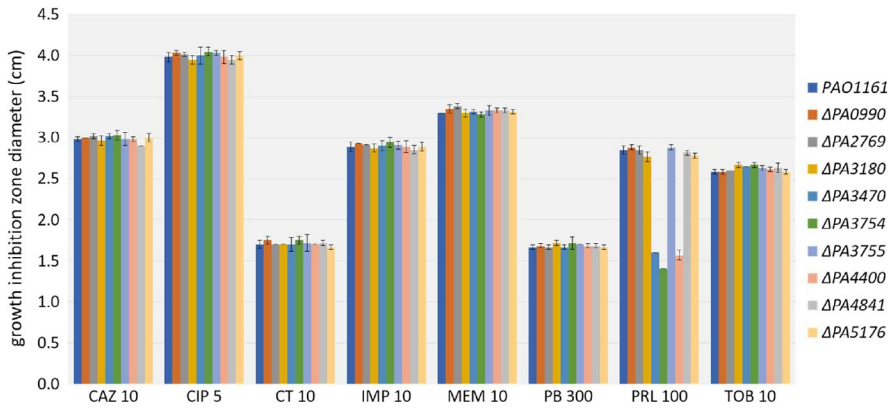


FIGURE A5 Antibiotics susceptibility of wild type *Pseudomonas aeruginosa* PAO1161 and nudix mutants tested with the use of disc diffusion assay as described in Experimental Procedures. CAZ10—ceftazidime (10 μ g), CIP—ciprofloxacin (5 μ g) CT—colistin (10 μ g), IMP—imipenem (10 μ g), MEM—meropenem (10 μ g), PB—polymyxin B (300 μ g), PRL—piperacillin (100 μ g), TOB—tobramycin (10 μ g)

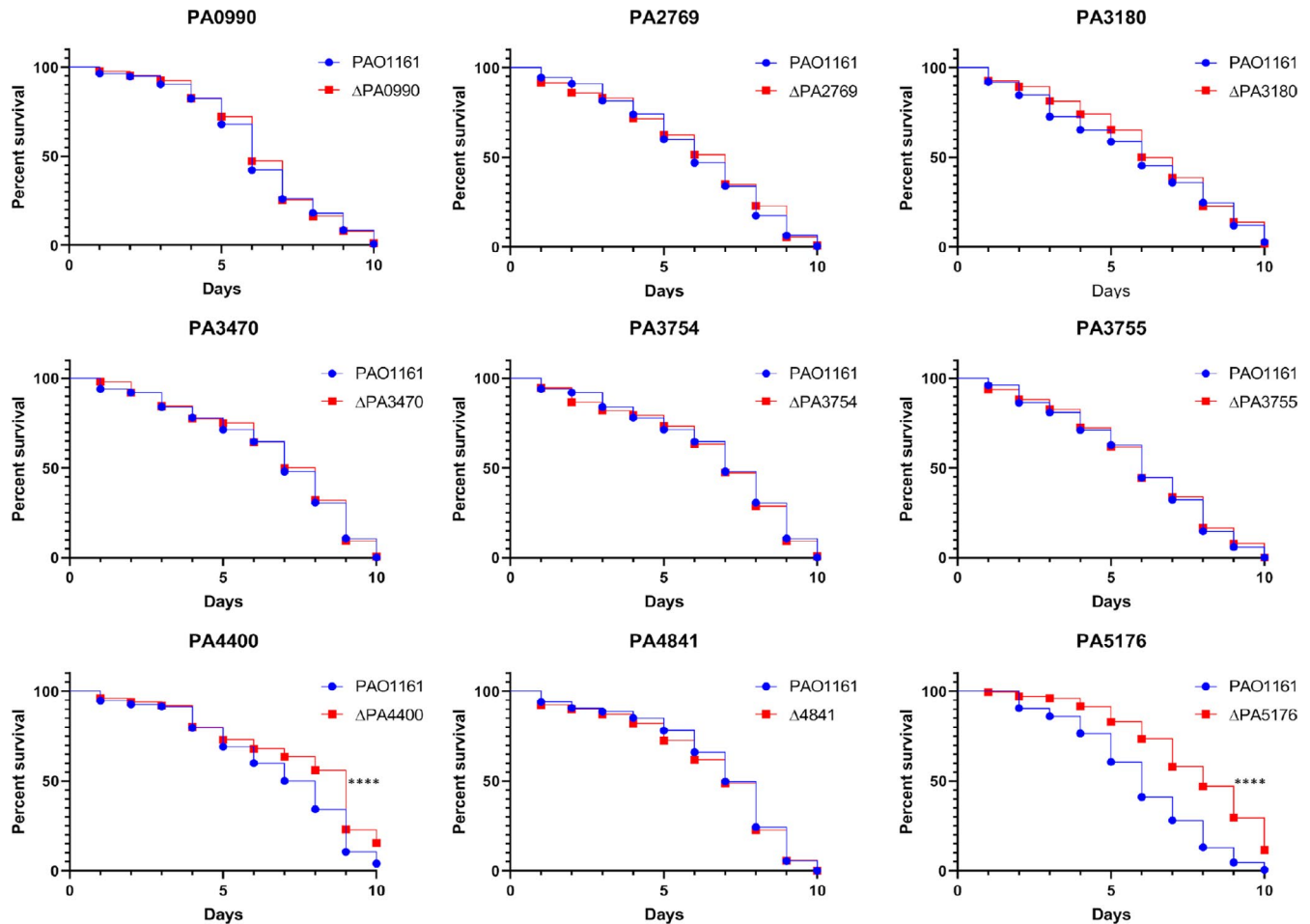


FIGURE A6 Effect of nudix mutations on *Pseudomonas aeruginosa* virulence. *Pseudomonas aeruginosa*–*Caenorhabditis elegans* infection model was used to determine pathogenicity of *Pseudomonas* strains as described under Experimental Procedures. The experiment was repeated at least three times. Shown are results of the representative experiments presented as the Kaplan–Meier survival curves of *C. elegans* fed wild type PAO1161 and nudix mutant strains. The significant differences between wild type and mutant were determined by the log-rank test (**** $p < .0001$)