Anaerobic survival of *Pseudomonas aeruginosa* by pyruvate fermentation requires an Usp-type stress protein

Running title: USP-TYPE STRESS PROTEIN IN *P. AERUGINOSA*

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ABSTRACT

Recently, we identified a pyruvate fermentation pathway in *Pseudomonas aeruginosa* sustaining anaerobic survival in the absence of alternative anaerobic respiratory and fermentative energy generation systems (M. Eschbach, K. Schreiber, K. Trunk, J. Buer, D. Jahn and M. Schobert, J. Bacteriol. 186:4596-4604, 2004). Anaerobic long-term survival of *P. aeruginosa* might be essential for survival in deeper layers of a biofilm and the persistent infection of anaerobic mucus plaques in the cystic fibrosis lung. Proteome analysis of *P. aeruginosa* cells during a seven-day period of pyruvate fermentation revealed the induced synthesis of three enzymes involved in arginine fermentation, ArcA, ArcB, ArcC and the outer-membrane protein OprL. Moreover, formation of two proteins of unknown function PA3309 and PA4352 increased by factors of 72- and 22-fold, respectively. Both belong to the group of universal stress proteins (Usp). Long-term survival of a PA3309 knock-out mutant by pyruvate fermentation was found drastically reduced. The oxygen-sensing regulator Anr controls expression of the P_{PA3309}-*lacZ* reporter gene fusion after a shift to anaerobic conditions and further pyruvate fermentation. PA3309 expression was also found induced during the anaerobic and aerobic stationary phase. This aerobic stationary phase induction is independent of the regulatory proteins Anr, RpoS, RelA, GacA, RhlR and LasR indicating a currently unknown mechanism of stationary phase dependent gene activation. PA3309 promoter activity was detected in the deeper layers of a *P. aeruginosa* biofilm using a P_{PA3309}-*gfp* fusion and confocal laser scanning microscopy. This is the first description of an Anr-dependent, anaerobically induced and functional Usp-like protein in bacteria.

INTRODUCTION

Pseudomonas aeruginosa is a highly adaptable bacterium that colonizes various environmental niches. It is also a leading opportunistic pathogen in human infections. *P. aeruginosa* is the dominant pathogen causing chronic respiratory infections of cystic fibrosis (CF) patients. This results in progressive lung damage and is the major cause of morbidity and mortality in CF patients (43). Recent data indicate that anaerobic conditions play an important role during persistent infection of the CF lung. *P. aeruginosa* forms biofilm-like microcolonies in the CF lung mucus embedded in an anaerobic environment (28, 47). Under these conditions, nitrate serves as an alternative electron acceptor sustaining growth under anaerobic conditions. Moreover, nitrate seems to favor the formation of more robust anaerobic biofilms (20, 50). Biofilm formation protects *P. aeruginosa* in hostile environments like the CF lung from the immune response of the host and leads to high antibiotic tolerance of the cells (7, 25, 41). A recent publication shows that oxygen restricted cells in deeper layers of a colony biofilm are highly resistant to antibiotics due to stationary phase conditions caused by oxygen depletion (4).

P. aeruginosa has a limited potential to survive in an anaerobic environment. Growth is supported by denitrification with nitrate or nitrite (5, 8, 51). Moreover, arginine sustains moderate anaerobic growth via fermentation (44). Recently, we have shown that pyruvate - one of the most abundant metabolites in all cells - allows anaerobic long-term survival of *P. aeruginosa* (13). However, in contrast to denitrification and arginine fermentation, pyruvate fermentation does not sustain anaerobic growth. We identified three enzymes essential for pyruvate fermentation: the phosphotransacetylase (Pta), acetate kinase (AckA) and a lactate dehydrogenase (LdhA). The operon encoding Pta and AckA is induced in response to oxygen limitation in dependence of the anaerobic regulatory protein Anr (13). The *ackA-pta* locus in *E. coli* is induced in response to starvation during aerobic conditions (31). Pyruvate

fermentation in *P. aeruginosa* might also play an important role as a general endogenous survival metabolism in response to energy starvation.

Here, we report the investigation of the physiological basis of pyruvate fermentation in *P. aeruginosa* starting with a proteome approach via two-dimensional (2D) gel electrophoresis. We identified two anaerobically induced Usp-type stress proteins (PA3309 and PA4352) and investigated the role of PA3309 during pyruvate fermentation and biofilm growth in more detail. In contrast to *E. coli* or Usp-type proteins investigated in *Mycobacteria* (33) our data indicate the presence of Usp-type proteins in *P. aeruginosa*, which are produced in response to oxygen limitation under the control of the oxygen sensing regulator Anr. We found evidence for an induction of the PA3309 gene in stationary phase by an Anr-independent unknown regulator.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are shown in Table 1. For standard molecular biology protocols, *E. coli* and *P. aeruginosa* strains were grown in LB medium as described before (13). Antibiotics were used at the following concentrations for *P. aeruginosa*: Carbenicillin at 500 µg/ml, gentamicin at 200 µg/ml and tetracycline at 100 µg/ml.

Pyruvate fermentation and anaerobic energy starvation experiments.

Pyruvate fermentation experiments with *P. aeruginosa* were performed with a slightly modified protocol. Instead of OS medium, we used a potassium-phosphate (100 mM) buffered LB medium at a pH value of 7.4. *P. aeruginosa* was grown aerobically up to an OD_{578} = 0.3. Cells were shifted to anaerobic conditions by transferring the culture to rubber-stoppered bottles and immediate addition of 40 mM pyruvate. Incubation was carried out at 37 °C without shaking. Viable cell counts were determined as described before (13). Formation of fermentation products and consumption of pyruvate were analyzed using HPLC analysis as outlined previously (13). Control experiments revealed no consumption of pyruvate during aerobic growth in LB up to an OD_{578} = 0.3.

For anaerobic long-term energy starvation experiments, 40 ml LB was inoculated with 10^4 *P. aeruginosa* cells/ml and incubated under anaerobic conditions for 20 days. Cell numbers increased up to $4x10^8$ cells/ml within 24 h. After 24 hours cells faced a severe energy starvation, resulting in a dramatic reduction of cells/ml.

Construction and testing of the promoter-*lacZ* reporter gene fusions.

Chromosomal promoter-*lacZ* reporter gene fusions were constructed using the mini-CTX-*lacZ* vector. A 492 bp PCR product, covering the region from 450 bp upstream and 26 bp

downstream of the translational start of the PA3309 gene, was generated using primer Pa-3309-for (5'-CGGAATTCGCCATGGACGAGGAACTG-3') and Pa-3309-rev (5'-CGGGATCCTCCACGGCTACCAGAATG-3'). Pa-3309-for contained an *Eco*RI restriction site at the 5'-end, and Pa-3309-rev a restriction site for *Bam*HI also at its 5'-end. The *Eco*RI and *Bam*HI digested PCR product was cloned into the *Eco*RI and *Bam*HI site of mini-CTX-*lacZ* to generate pKS15. Transfer of pKS15 in *P. aeruginosa* was carried out by a diparental mating using *E. coli* S17 λ-pir as donor. The CTX integrase of pKS15 promoted integration of the vector into the *attB* site of the *P. aeruginosa* genome. The vector was transferred into PAO1 and the *anr, rpoS, rhlR, lasR, gacA* and *relA* mutant strains to generate the *P. aeruginosa* strains KS06, KS08, KS10, KS31, KS32, KS33 and KS37, respectively (see also Table 1). In these mutant strains parts of the mini-CTX-*lacZ* vector containing the tetracycline resistance cassette were deleted using a FLP-recombinase encoded on the pFLP2 plasmid. Reporter gene fusion assays were performed as outlined before in detail (13, 37). Obtained activities were given in Miller units (29).

Construction of a P_{PA3309}-*gfp* fusion.

We used a transcriptional P_{PA3309}-*gfp* reporter gene fusion to follow promoter activity of PA3309 in biofilms. A 526 bp fragment of the PA3309 promoter region without the translational start codon was amplified by PCR with primers oKS16 (5'-GGAATTCGCCATGGACGAGGAACTG-3') containing an *Eco*RI restriction site at the 5'-end and oKS17 (5'-CGGGATCCAAGGTGTCCCTCCAGAGTG-3') with a *Bam*HI site. The PCR product was cloned into pMH305 upstream of *gfp* to generate pKS08. The P_{PA3309}-*gfp* reporter gene fusion was liberated via *Not*I digestion and ligated into mini-CTX2 to generate pKS09. This vector was transferred and integrated into the *P. aeruginosa* genome using the procedure described above. The resulting strain was named KS15.

Construction of *P. aeruginosa* ΔPA3309, ΔPA4352, Δ*relA*, *rhlR*::Tc^R and *lasR*::Gm^R mutants.

Unmarked gene deletion mutants were obtained using the well-established strategies based on *sacB* counter selection and FLP recombinase excision (23). First a suicide vector pKS10 was constructed to replace the PA3309 gene with a gentamicin cassette. After transfer of this vector into *P. aeruginosa*, a double crossover mutant was obtained by *sacB*-based counter selection. The resulting mutant, KS16, was verified by Southern blot analysis. Finally, FLP recombinase encoded on the pFLP2 plasmid removed the FRT-flanked gentamicin cassette to generate KS17. To construct the suicide vector pKS10, the *Bam*HI digested gentamicin resistance cassette of pPS858 was cloned between two PCR fragments of the PA3309 gene in the multiple cloning site of pEX18Ap. The two PCR fragments contained DNA homologous to the upstream and downstream areas of the PA3309 gene. A 723 bp fragment containing the upstream promoter region of the PA3309 gene was amplified using primer oKS10 (5'-CGGAATTCGAACAAGGCGCTGAAG-3') with an *Eco*RI restriction site at the 5'-end and oKS11 (5'-CGCGGATCCAACTTCAAGGACACTGTA-3') with a *Bam*HI site. The primers oKS12 (5'-CGCGGATCCTCCGGTCCTGCTGGT-3') with a *Bam*HI restriction site and oKS13 (5'-ACGCGTCGACACGCCATCATCGTCCT-3') with a *Sal*I restriction site were used for the amplification of 625 bp of the PA3309 downstream region.

The unmarked ΔPA4352 mutant (NB015) was generated with the same strategy described above using the suicide vector pNB007. In this suicide vector, the following PCR fragments flanked the gentamicin resistance cassette: The 596 bp fragment of the upstream region of the PA4352 gene was amplified using primers oNB01 (5'-CGAGCTCTACGGCGACTTCGTCAAGG-3') with a *Sac*I restriction site and oNB02 (5'-CGGGATCCAAGCGGATGCTTCGGACT-3') with a *Bam*HI site. The primers oNB03 (5'-CGCGGATCCCTTCCGCCGCGCGCTGA-3') with a *Bam*HI site and oNB04 (5'-

CCCAAGCTTCCCTGGCGCCGCTGACC-3') with a *Hind*III site amplified 617 bp of the corresponding downstream region of PA4352.

The Δ*relA* mutant KS35 was constructed with the same strategy as described above. For the construction of the suicide vector pKS18 the primers oKS40 (5'-GGAATTCGGCCAGTGCATTGCTGTTG-3') with an *Eco*RI restriction site at the 5'-end and oKS41 (5'-CGGGATCCTTACCACGGTGCGCGTAG-3') with a *Bam*HI site amplified 796 bp of the putative promoter region of the *relA* gene. The primers oKS42 (5'-CGCGGATCCCGAGCAGGTCGAGATCA-3') with a *Bam*HI site and oKS43 (5'-CCCAAGCTTTGGGCAGTTGCGAGACG-3') with a *Hind*III site were used to amplify 824 bp of the 3'-end of *relA*.

The two quorum sensing mutants *rhlR*::Tc^R (KS31) and *lasR*::Gm^R (KS32) were constructed as described (1) using KS06 as the parent strain.

Plasmid construction for the complementation of the PA3309 knockout mutant.

For the construction of the complementation plasmid for the chromosomal PA3309 knockout mutant, a 1200 bp PCR product covering 455 bp of the PA3309 promoter region, the PA3309 gene and 289 bp downstream of PA3309 was amplified using primers oKS14 (5'-CGGAATTCGCCATGGACGAGGAACTG-3') with an *Eco*RI restriction site at the 5'-end and oKS15 (5'-CCAAGCTTATCCACGTGCCGATGGTC-3') with a *Hind*III site. The product was digested with *Eco*RI and *Hind*III and ligated into mini-CTX2 to generate pKS13. The vector was transferred into *P. aeruginosa* KS17 to generate KS29 by a diparental mating with *E. coli* S17 λ-pir and integrated at the *att*B site in the genome as described above. As a control, the empty mini-CTX2 vector was integrated into the genome of PAO1 and KS17 to generate KS27 and KS28, respectively.

Proteomic analysis.

To prevent changes of the cellular protein pattern during cell harvesting and cell free extract preparation, the culture was mixed with the double volume of ice cold potassium-phosphate-buffer (0.1 M; pH 7.4) and allowed to cool for 20 min. Cells were centrifuged at 8000 x g for 30 minutes at 4 °C and washed twice with potassium-phosphate buffer. Cells were resuspended in a small volume of potassium-phosphate buffer. Protein concentration in whole cell suspensions was determined using the BCA protein assay (Sigma, Taufkirchen, Germany). Cells were disrupted by incubation of a 360 µl culture aliquot with 150 µl NaOH for 1 h at 70 °C. For protein isolation we modified a protocol described previously (19) and extracted proteins directly from whole cells with phenol and a subsequent acetone precipitation. The precipitated proteins were solubilized in sample buffer consisting of 7 M urea, 2 M thiourea, 4 % 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS), 50 mM dithioerythritol (DTT) and 2 % ampholytes (Bio-Lyte; BioRad, Munich, Germany). Protein concentration was determined in the sample buffer using the PlusOne 2D Quant Kit (Amersham Biosciences, Freiburg, Germany). 2D gel electrophoresis was performed using immobilized pH gradient (IPG) strips of 11 cm or 17 cm length covering two different pH ranges (pH 4.7-5.9 or 5-8) (IPG Ready Strips; BioRad, Munich, Germany). For the narrow pH range, the IPG strips (11 cm) were rehydrated overnight in rehydration buffer containing 50 µg of protein. Isoelectric focusing (IEF) was carried out at 20 °C under mineral oil in the PROTEAN IEF Cell (BioRad, Munich, Germany) for a total of 35 000 Vh. The focused IPG strips were reduced for 15 min in a SDS equilibration solution containing 15 mM DTT and afterwards alkylated twice for 15 min in the same buffer containing 150 mM iodacetamide prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The IPG strips were transferred to 10.5-14 % SDS-PAGE (Criterion Tris-HCl Gel; BioRad, Munich, Germany) gels and electrophoresis was performed at constant 200 V for 55 min. Large IPG strips (17 cm, pH 5-8) were loaded with 700 µg of protein and IEF was conducted for a total

of 110 000 Vh. SDS-PAGE was performed at a constant temperature of 20 °C with 1 W per gel for approximately 20 h using 10 % polyacrylamide gels (25.5 x 20.5 cm). All gels were stained with Ruthenium-(II)-tris-(bathophenanthroline disulfonate) (RuBPS) as described before (35). Gels were documented with a FX-Scanner (BioRad, Munich, Germany). Analysis and quantification of differential protein spot patterns was performed by using the Software Z3 (Compugen, Tel Aviv, Israel). Gel spots were excised and treated using a method described before introducing minor modifications (40). Briefly, the gel pieces were washed with water, dehydrated with acetonitrile (ACN) and digested with trypsin (sequencing grade, Promega). Peptides were extracted and collected in four elution steps (each 15 min, 37 °C) using 25 mM NH_4HCO_3, ACN, 5 % formic acid and again ACN. Extracted peptides were purified using ZipTip C18-microcolumns (Millipore), following the manufacturer's instructions. Proteins were identified by peptide-mass fingerprint (PMF) as well as post-source decay fragmentation data recorded on a Bruker Ultraflex MALDI-TOF mass spectrometer. PMF-data were analyzed using an internal MASCOT-server at the GBF (version 1.9; Matrix Science) (34) and the NCBI database (restricted to the taxon *Pseudomonas aeruginosa*). Only peptides with a MASCOT rank of 1 were considered significant and used for the combined peptide score. The criteria used to accept protein identifications based on PMF-data included the extent of sequence coverage (minimum of 30 %), the number of peptides matched (minimum of 5) and the score of probability (minimum of 70 for the Mowse score). Lower-scoring proteins were either verified manually or rejected.

Biofilm experiments.

Biofilm studies were performed in three-channel flow cells with individual channel dimensions of 1 x 4 x 40 mm supplied with a flow of 3 ml h^{-1} of AB-medium supplemented with 300 µM glucose and where indicated with 50 mM KNO_3 (6). The system was assembled and cultures for inoculation were prepared as described before (6, 21). Biofilms were stained

with Syto62 (Molecular Probes) to visualize the biofilm matrix. Dead cells in the biofilm were visualized with propidium iodide (Sigma). Microscopy and image acquisition were done as outlined (22) with a Zeiss LSM 510 confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany). Quantitative image analysis was done using the COMSTAT software (22). Images were processed using the Imaris software (Bitplane AG, Zurich, Switzerland). For protein analysis biofilms were grown in silicone tubes (30 cm, inner square 0.5 cm), in AB medium as described above and supplied with a flow rate of 20 ml h^{-1}.

RESULTS

Proteome analysis of *P. aeruginosa* under pyruvate fermentation conditions.

Recently, we showed that *P. aeruginosa* can survive anaerobically for several weeks by pyruvate fermentation in the absence of nitrate, nitrite or arginine (13). Pyruvate fermentation allows survival but not growth of *P. aeruginosa* cells during severe anaerobic energy starvation conditions. We used a proteomics approach to identify proteins which are preferentially synthesized during pyruvate fermentation. Long-term survival pyruvate fermentation experiments using cultures grown in LB medium were set up as described previously (13). For this purpose, cells were grown aerobically to an OD_{578} = 0.3, immediately shifted to anaerobic flasks containing pyruvate at a final concentration of 40 mM and further incubated for seven days at 37 °C. Viable cell counts of three independent cultures were determined and remained almost constant during the whole experiment ($2.8x10^8$ ± $1.3x10^8$ cells per ml medium at day 0 and $1.6x10^8$ ± $1.0x10^7$ at day 7).

We compared the protein pattern of the aerobically grown culture in phosphate buffered LB medium (OD_{578} = 0.3) with that of the culture incubated for seven days under pyruvate fermentation conditions (phosphate buffered LB plus 40 mM pyruvate, Fig. 1A and B). The concentration of eleven proteins was found highly increased at least nine-fold under anaerobic pyruvate fermentation conditions (Table 2). MALDI-TOF analysis identified the eleven separated proteins to represent 6 different proteins. Three proteins involved in arginine fermentation (ArcA, ArcB and ArcC) were found highly upregulated as well as the outer membrane protein OprL and two proteins, PA3309 and PA4352, that are annotated as conserved hypothetical proteins, but that each have universal stress protein motifs (Table 2 and Fig. 1B). None of these proteins was expected to be involved or contribute to pyruvate fermentation. The results of the proteome analysis raised the question if proteins involved in

arginine fermentation or the two hypothetical proteins contribute to survival during pyruvate fermentation.

Contribution of *arcDABC* and PA3309 to anaerobic long-term survival of *P. aeruginosa.*

Since we used a complex medium containing arginine, we investigated if arginine fermentation contributes to survival under anaerobic pyruvate fermentation conditions. The Δ*arcDABC* mutant PAO6251 cannot grow anaerobically by arginine fermentation. Viable cell counts of the Δ*arcDABC* mutant decreased by a factor of 30 compared to wild type during 20 days of pyruvate fermentation. In contrast the viable cell counts of the wild type control, which was incubated under anaerobic conditions without pyruvate, decreased by a factor of 500 (Fig. 2). These results demonstrated that arginine fermentation contributes to anaerobic long-term survival, however to a lesser extent than the PA3309 protein.

Next, we investigated if the hypothetical protein PA3309 promotes survival during pyruvate fermentation. First, a PA3309 chromosomal knockout mutant strain was constructed as described in Materials and Methods. The PA3309 mutant strain exhibited a clear 400-fold reduction of anaerobic long-term survival over a 20 day period (Fig. 2). We were able to complement the PA3309 mutant by chromosomal integration of pKS13 which contains the cloned PA3309 gene and the putative promoter region (Table 1). Survival rates of the complemented mutant strain (KS29) do not completely reach wild type level, but were found significantly increased (100-fold) compared to the PA3309 mutant (KS17) and the PA3309 mutant containing the empty mini-CTX2 vector integrated in the *attB* locus (KS28, data for this mutant not shown). A PA4352 knockout mutant (NB015) without the second Usp-type stress protein showed no reduced survival during pyruvate fermentation (Fig. 2).

Our studies revealed an essential role of PA3309 for anaerobic long-term survival during pyruvate fermentation.

The conserved hypothetical protein PA3309 belongs to the group of anaerobically induced universal stress proteins.

The conserved hypothetical protein PA3309 consists of 151 amino acids with a calculated mass of 16496 Da and a theoretical pI of 5.31. The Pfam database indicates the presence of a single Usp-domain (Pfam accession number PF00582) which originates from the universal stress protein A (UspA) of *E. coli*. PA3309 shares the single conserved Usp domain, a similar molecular mass and a moderate amino acid sequence identity of 37 % with *E. coli* UspA. Six Usp-type stress protein paralogues have been identified in *E. coli* which are produced in response to a variety of different stress conditions, most of them leading to growth arrest (27). We tested if the PA3309 knockout mutant showed similar phenotypes as the *E. coli uspA* mutant in response to UV-stress and stationary phase survival. However, no similar behavior was observed (data not shown). Despite the observed phenotype of the PA3309 knockout mutant during pyruvate fermentation, we did not observe a defect in anaerobic denitrifying growth using different media supplemented with 50 mM nitrate. However, PA3309 contributes to survival of severe anaerobic energy stress conditions. We incubated wild type *P. aeruginosa* and the PA3309 mutant anaerobically in LB medium without nitrate, nitrite or pyruvate. Within 20 days cell numbers of the wild type strain decreased by a factor of 9.0×10^2 while cell numbers of the mutant strain decreased by a factor of 9.7×10^4.

PA3309 is induced upon a shift to anaerobic conditions by the oxygen regulator Anr.

We studied the regulatory behavior of the PA3309 promoter towards various environmental stimuli and corresponding metabolic conditions with a chromosomal transcriptional promoter-*lacZ* reporter gene fusion. We monitored β-galactosidase activities of the P_{PA3309}-*lacZ* reporter gene fusion (KS06) during pyruvate fermentation for the first four days. The β-galactosidase activities increased linear up to 1594 ± 20 MU during the first four days (Fig. 3A) indicating strong anaerobic induction. Anr is a global transcriptional regulatory protein of the Crp-Fnr

family which activates gene expression in *P. aeruginosa* in response to oxygen limitation (16, 39). We detected a putative Anr-Box 86 bp upstream of the translational start codon in the presumed promoter region of PA3309 using tools of the PRODORIC database (30). We also checked dependence of the PA3309 promoter on Anr. No significant increase was detected when the P_{PA3309}-*lacZ* reporter gene fusion was monitored in the *anr* mutant strain PAO6261 (Fig. 3A). We also measured a strong increase in β-galactosidase activity of the P_{PA3309}-*lacZ* reporter gene fusion in wild type *P. aeruginosa* upon a shift to anaerobic conditions in the absence of pyruvate. Again, under these conditions the *anr* mutant failed to induce the PA3309 promoter activity (Fig. 3B). Clearly, Anr induces an anaerobic expression of PA3309 independent of the presence of pyruvate.

PA3309 is induced in stationary phase.

Further examination of the P_{PA3309}-*lacZ* behavior during various anaerobic growth phases revealed a 5.3-fold induction during anaerobic exponential growth compared to aerobic exponential growth. Interestingly, the PA3309 promoter was further 1.8-fold induced in the anaerobic stationary phase (Table 3). Next, we investigated the behavior of the PA3309 promoter during aerobic stationary phase. To our surprise, a 5.4-fold induction was observed. Gene expression of PA3309 in the aerobic stationary phase was further confirmed by 2D gel electrophoresis (Fig. 4B and 4C). In contrast to pyruvate fermentation, where we identified only one protein spot representing PA3309 (Fig. 4D), two spots representing PA3309 were identified in the protein pattern of aerobic stationary phase cells (Fig. 4C). This might indicate a phosphorylation of PA3309 as reported for *E. coli* UspA (15). The Anr regulator was the candidate responsible for the aerobic induction of the P_{PA3309}-*lacZ* reporter gene fusion upon entry into the stationary phase, since the strong respiration of a high cell density culture leads to oxygen limitation. However, expression of the P_{PA3309}-*lacZ* reporter gene fusion in the *anr* mutant still increased 4.2-fold upon entry in the aerobic stationary phase (Table 3). Therefore,

promoter activity in the aerobic stationary phase is independent of Anr. Expression of the *uspA, uspC, uspD* and *uspE* genes in *E. coli* upon entry in the stationary phase requires stringent control via the nucleotide guanosine 3',5'- bisdiphosphate (ppGpp) and the RelA protein (14, 18, 26). In *P. aeruginosa* the RelA protein (PA0934) synthesizes ppGpp in response to amino acid starvation conditions or in the stationary phase (12, 42). Deletion of the *P. aeruginosa relA* gene abolishes the production of ppGpp and also decreases production of RpoS (12, 42). Expression of the *P. aeruginosa* PA3309 promoter in a *P. aeruginosa relA* mutant remained unchanged compared to wild type conditions (Table 3). Furthermore, we did not detect increased PA3309 promoter activity when wild type cells faced carbon-starvation during growth in 1/20 LB medium (data not shown). Therefore, the stringent response system does not contribute to PA3309 regulation under the tested conditions.

The stationary phase sigma factor RpoS activates gene expression upon entry into the stationary phase. Anr-dependent genes like *azu* encoding the blue copper protein azurin have been reported to be RpoS-dependent (45). However, an *rpoS* mutant showed no influence on the PA3309 promoter activity under aerobic or anaerobic stationary phase conditions (Table 3).

In *Mycobacterium smegmatis*, a two-component regulator similar to the *Mycobacterium tuberculosis* DevR protein was shown to induce expression of three genes encoding Usp-type proteins (3, 32). DevR shares domain organization and a 37 % identity on the amino acid sequence level to the *P. aeruginosa* GacA regulator of the global GacA/GacS system (global antibiotics and cyanide control). This system regulates the expression of multiple phenotypes in *Pseudomonads.* Again, no change in PA3309 promoter activity was found in a *gacA* mutant strain (Table 3). Similar observations were made using *lasR, rhlR* mutants carrying defects in both quorum-sensing systems of *P. aeruginosa* (Table 3).

So far, we did not check if PA3309 expression in the stationary phase is RecA/FtsK dependent as shown for *E. coli uspA* (10). However, since the PA3309 deletion mutant is not sensitive to UV exposure (data not shown) a RecA/FtsK dependent regulation seems unlikely.

Expression of PA3309 in biofilms.

We checked the spatial distribution of PA3309 promoter induction within biofilms, its dependence on the Anr regulatory protein and the effect of nitrate. To visualize promoter activity of PA3309 in biofilms, a P_{PA3309}-*gfp* reporter gene fusion was constructed and transferred to the *P. aeruginosa* chromosome (see Materials and Methods). Biofilms were grown for six days in flow cells and inspected by scanning confocal laser microscopy (SCLM). As depicted in Fig. 5A, PA3309 promoter activity visualized by GFP fluorescence, represented as yellow areas in the red colored biofilm matrix, was only detectable in the deeper layers of the biofilm. Quantitative analysis of the biofilms using COMSTAT, revealed a two-fold increase of GFP fluorescence when biofilms grew in the presence of nitrate (Fig. 5B). Since GFP requires small amounts of oxygen for activity, the deeper layers of the inspected biofilms were not strictly anaerobic but oxygen limited. In control experiments biofilms were stained with propidium iodide to visualize dead cells. Only a small portion of the biofilm cells (1-2 %) were not alive (data not shown).

To examine if the expression of PA3309 in biofilms is Anr-dependent, a proteome approach was applied. Biofilms of *P. aeruginosa* PAO1 and the *anr* mutant strain PAO6261 were grown for six days under aerobic conditions, since the *anr* mutant is unable to grow and, consequently, cannot form biofilms under strict anaerobic conditions. Fig. 4E and 4F show the partial enlargements of a 2D gel image containing two PA3309 protein spots. Both strains contain similar amounts of PA3309. Therefore, we conclude, that under these microaerobic conditions induction of the PA3309 promoter is independent of Anr comparable to the Anr-independent induction in the aerobic stationary phase.

DISCUSSION

During pyruvate fermentation, the survival of *P. aeruginosa* depends on PA3309, a protein with the signature domain of Universal stress proteins (Usp). The PA3309 protein contains a single Usp domain and shares 37 % amino acid sequence identity to UspA of *E. coli*. Universal stress proteins have been identified in bacteria, archaea, plants and fungi. Six different Usp proteins (UspA, UspC, UspD, UspE, UspF and UspG) are present in *E. coli* (27). Although much is known about the regulation, the function of Usp-type proteins still remains unknown (27). The *E. coli* proteins are produced in response to a large number of different stresses, including DNA damaging, stationary phase and growth arrest. In some cases, Usp-type proteins have been linked to the resistance to DNA-damaging agents and respiratory uncouplers (18). However, a *P. aeruginosa* PA3309 deletion mutant shows no comparable phenotype to an *E. coli uspA* mutant strain, regarding UV-resistance and survival in the stationary phase (data not shown). The phenotype of the PA3309 mutant seems to be restricted to anaerobic conditions. Besides the essential role of PA3309 during pyruvate fermentation, PA3309 also contributes to survival during long-term anaerobic energy starvation in the absence of pyruvate. During these stress conditions, cell numbers of the wild type decrease dramatically by a factor of 9.0×10^2 while cell numbers of the PA3309 mutant decrease by a factor of 9.7×10^4. Consistent with the anaerobic phenotypes, regulation of the PA3309 gene was found to be dependent on the oxygen sensing regulatory protein Anr. The β-galactosidase activities of a P_{PA3309}-*lacZ* reporter gene fusion remained unchanged in an *anr* mutant strain upon a shift to anaerobic conditions while they increased 4- to 8-fold (Fig. 3) in wild type *P. aeruginosa*. As shown in Fig. 3, this anaerobic induction of the PA3309 promoter is independent of the presence of pyruvate. The regulatory behavior is consistent with the observed anaerobic phenotype of the PA3309 mutant. However, we also observed a production of the PA3309 protein in the aerobic stationary phase and monitored a 5-fold increased β-galactosidase activity of the P_{PA3309}-*lacZ* reporter gene fusion. We showed that this induction in the stationary phase is independent of Anr. It is also independent of other

regulators which induce promoter activity in the stationary phase, during starvation or at high cell densities as RpoS, RelA, GacA and the quorum sensing regulators RhlR and LasR. We also did not find a phenotype of the PA3309 mutant during aerobic stationary phase in LB medium with or without supplemented pyruvate as reported for the *E. coli uspA* mutant. These experiments clearly indicate a different role and regulation of the Usp-type protein PA3309 in *P. aeruginosa* compared to the Usp proteins in *E. coli*. Stationary phase expression of the *P. aeruginosa* PA3309 promoter is independent of RelA, which induce expression of *usp* genes in *E. coli* and GacA, which shows similarities to DevR of *M. smegmatis*.

To identify the role of PA3309 during aerobic stationary phase, we currently investigate the PA3309 promoter in more detail and aim to identify the involved regulator.

We also investigated the spatial distribution of PA3309 promoter induction within biofilms. Biofilms are dense bacterial communities attached to a surface and surrounded by an exopolysaccharide matrix. Differences in density and architecture determine the access to nutrients and oxygen within the biofilm. Oxygen limitation can start within the first 30 µm below the surface of an aerobically grown *P. aeruginosa* biofilm (48). Proteome studies of *P. aeruginosa* biofilms indicate that a large portion of a mature biofilm population is under oxygen limitation (38). Moreover, *P. aeruginosa* forms even more robust biofilms when grown anaerobically in the presence of nitrate (50). Since the CF airway mucus is anaerobic (47), anaerobic biofilms might mirror a persistent infection situation in the CF lung. Oxygen limitation contributes to antibiotic resistance of the oxygen-restricted layers of aerobically grown biofilms (4). Previously, increased expression of PA3309 in aerobic and anaerobic biofilms was described (50). Our biofilm experiments clearly showed an induction of the PA3309 promoter in the deeper layers of biofilms and an increased promoter activity when biofilms were grown in the presence of nitrate. However, 2D gel electrophoresis revealed that PA3309 production in biofilms is independent of the oxygen sensing regulator Anr. Currently, we investigate if PA3309 also contributes to survival of cells in deeper layers of a biofilm.

2D gel analysis of *P. aeruginosa* cells during pyruvate fermentation revealed production of a second Usp-type stress protein, PA4352, as well as proteins involved in arginine fermentation. While a PA4352 mutant had no phenotype during pyruvate fermentation, a mutant defect in arginine fermentation, Δ*arcDABC*, showed decreased survival. Since we used a complex medium and shifted the culture to anaerobic conditions during the early exponential phase, minor amounts of arginine could support survival. We determined arginine levels in LB to be approximately 1.6 mM (data not shown), but these amounts do not allow survival in the absence of pyruvate (see wild type control in Fig. 2). However, the mutant defect in arginine fermentation also grows poorly even in aerobiosis (17). This suggests that the arginine deiminase operon in general supports growth and survival of *P. aeruginosa* including pyruvate fermentation.

This is the first report of an Usp-type stress protein in *P. aeruginosa* which contributes to anaerobic survival and pyruvate fermentation and which is produced in response to anaerobiosis in an Anr-dependent manner. Usp-type stress proteins recently gained attention from investigations using *Mycobacteria.* Data of proteome and transcriptome analysis showed that Usp-type proteins of *Mycobacteria* were produced upon oxygen limitation and phagocytosis (33). The stationary phase regulator DevR, which is required for oxygen starvation, was identified to control expression of *usp*-gene expression in *Mycobacterium smegmatis* (3, 32). Oxygen limitation has been shown to induce a transition from active growth to a non-replicative persistent stage important for *Mycobacterium tuberculosis* latency and infection (33). Our results indicate that an Usp-type protein in *P. aeruginosa* also contributes to anaerobic survival and may play a role for survival in anaerobic mucus plaques in CF lungs.

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TABLE 1. Strains of *P. aeruginosa* and *E. coli* and plasmids used.

Bacterial strain or plasmid	Genotype or phenotype	Reference
P. aeruginosa		
PAO1	Wild type	(11)
PAO6261	PAO1 Δ*anr*	(49)
PAO-MW20	PAO1 *rpoS::aacC1*, Gm^R	(46)
PAO6251	PAO1 Δ*arcDABC*	(17)
PAO6281	PAO1 *gacA::*Ω-Sp/Sm	(36)
KS06	PAO1 *attB*::(P_{PA3309}-*lacZ*)	This study
KS08	PAO6261 *attB*::(P_{PA3309}-*lacZ*)	This study
KS10	PAO-MW20 *attB*::(P_{PA3309}-*lacZ*)	This study
KS11	PAO1 *attB*::(mini-CTX-*lacZ*)	This study
KS15	PAO1 *attB*:: (P_{PA3309}-*gfp*)	This study
KS16	PAO1 ΔPA3309*::aacC1*, Gm^R	This study
KS17	PAO1 ΔPA3309	This study
KS27	PAO1 *attB*::(mini-CTX2)	This study
KS28	KS17 *attB*::(mini-CTX2)	This study
KS29	KS17 *attB*::(pKS13)	This study
KS31	PAO1*rhlR attB*::(P_{PA3309}-*lacZ*)	This study
KS32	PAO1*lasR attB*::(P_{PA3309}-*lacZ*)	This study
KS33	PAO6281 *attB*::(P_{PA3309}-*lacZ*)	This study
KS35	PAO1 Δ*relA*	This study
KS37	PAO1 Δ*relA attB*::(P_{PA3309}-*lacZ*)	This study
NB015	PAO1 ΔPA4352	This study

E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX*74 *deoR recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*) 7697 *galU galK* λ$^-$ *rpsL nupG*	GibcoBRL (Invitrogen)
S17 λ-pir	*pro thi hsdR*$^+$ Tp^r Sm^r; chromosome::RP4-2 Tc::Mu-Km::Tn7/ λpir	(9)
SM10	*thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^R)	(9)
Plasmids		
pEX18Ap	Ap^R; *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS from pUC18	(23)
mini-CTX-*lacZ*	Tc^R; promoterless *lacZ* gene	(2)
mini-CTX2	Tc^R; integration vector	(24)
pSB219.9A	Gm^R; *lasR*::Gm suicide vector	(1)
pSB224.10A	Tc^R; *rhlR*::Tc suicide vector	(1)
pPS858	Ap^R; Gm^R; source of gentamicin cassette	(23)
pFLP2	Ap^R; source of FLP recombinase	(23)
pMH305	Ap^R; Gm^R; Source of *gfp* cassette	Morten Hentzer
pKS08	Ap^R; Gm^R; pMH305 with 526 bp of the PA3309 promoter between *Eco*RI and *Bam*HI	This study
pKS09	Tc^R; mini-CTX2 with a *Not*I fragment liberated from pKS08 containing the PA3309 promoter fused to *gfp*	This study
pKS10	Ap^R; Gm^R; pEX18Ap with 723 bp promoter of PA3309, Gm^R-*gfp* fragment from pPS858 and 625 bp downstream of the coding region of PA3309 between *Eco*RI and *Sal*I	This study
pKS13	Tc^R; mini-CTX2 with a 1200 bp PCR fragment covering the entire PA3309 gene and 455 bp of the putative promoter region and 289 bp of the downstream region between *Eco*RI and *Hind*III	This study
pKS15	Tc^R; mini-CTX-*lacZ* containing a 492 bp fragment of the putative promoter region of the PA3309 gene between *Eco*RI and *Bam*HI	This study
pKS18	Ap^R; Gm^R; pEX18Ap with 796 bp promoter of *relA* (PA0934), Gm^R-*gfp* fragment from pPS858 and 824 bp of the 3' coding region of *relA* between *Eco*RI and *Hind*III	This study
pNB007	Ap^R; Gm^R; pEX18Ap with 596 bp promoter PA4352, Gm^R-*gfp* fragment from pPS858 and 617 bp downstream of the coding region of PA4352 between *Sac*I and *Hind*III	This study

TABLE 2. Identified proteins under pyruvate fermentation in LB-medium.

Spot number	PA number[a]	Protein[a]	Description[a]	Regulation[b] pyr. vs aerob
1	PA5171	ArcA	Arginine deiminase	132 fold
2	PA5171	ArcA	Arginine deiminase	9 fold
3	PA5171	ArcA	Arginine deiminase	unmatched
4	PA5172	ArcB	Catabolic ornithine carbamoyltransferase	29 fold
5	PA5172	ArcB	Catabolic ornithine carbamoyltransferase	14 fold
6	PA5173	ArcC	Carbamate kinase	unmatched
7	PA5173	ArcC	Carbamate kinase	60 fold
8	PA4352		Conserved hypothetical protein	22 fold
9	PA0973	OprL	Outer membrane protein	99 fold
10	PA0973	OprL	Outer membrane protein	unmatched
11	PA3309		Conserved hypothetical protein	72 fold

[a] PA number, protein short name and description according to the Pseudomonas database (www.pseudomonas.com).

[b] Ratios of amount of proteins from a *P. aeruginosa* PAO1 culture incubated for seven days under pyruvate fermentation conditions at 37 °C in phosphate buffered LB to the aerobic grown culture (see Materials and Methods).

Proteins were separated by 2D gel electrophoresis and stained with RuBPS (see Materials and Methods for details). Protein ratios of 2D gel images were determined by the Z3 software (Compugen, Tel Aviv, Israel). Unmatched, describes a protein spot which is detected only in the protein pattern of the pyruvate fermentation culture.