**Pseudomonas aeruginosa** β-lactamase induction requires two permeases, AmpG and AmpP

Kok-Fai Kong1*, Alian Aguila1, Lisa Schneper2, Kalai Mathee2*

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**Abstract**

**Background:** In Enterobacteriaceae, β-lactam antibiotic resistance involves murein recycling intermediates. Murein recycling is a complex process with discrete steps taking place in the periplasm and the cytoplasm. The AmpG permease is critical to this process as it transports N-acetylglucosamine anhydrous N-acetylmuramyl peptides across the inner membrane. In Pseudomonadaceae, this intrinsic mechanism remains to be elucidated. Since the mechanism involves two cellular compartments, the characterization of transporters is crucial to establish the link.

**Results:** *Pseudomonas aeruginosa* PAO1 has two ampG paralogs, PA4218 (ampP) and PA4393 (ampG). Topology analysis using β-galactosidase and alkaline phosphatase fusions indicates ampP and ampG encode proteins which possess 10 and 14 transmembrane helices, respectively, that could potentially transport substrates. Both ampP and ampG are required for maximum expression of β-lactamase, but complementation and kinetic experiments suggest they act independently to play different roles. Mutation of ampG affects resistance to a subset of β-lactam antibiotics. Low-levels of β-lactamase induction occur independently of either ampP or ampG. Both ampG and ampP are the second members of two independent two-gene operons. Analysis of the ampG and ampP operon expression using β-galactosidase transcriptional fusions showed that in PAO1, ampG operon expression is β-lactam and ampR-independent, while ampP operon expression is β-lactam and ampR-dependent. β-lactam-dependent expression of the ampP operon and independent expression of the ampG operon is also dependent upon ampP.

**Conclusions:** In *P. aeruginosa*, β-lactamase induction occurs in at least three ways, induction at low β-lactam concentrations by an as yet uncharacterized pathway, at intermediate concentrations by an ampP and ampG dependent pathway, and at high concentrations where although both ampP and ampG play a role, ampG may be of greater importance. Both ampP and ampG are required for maximum induction. Similar to ampC, ampP expression is inducible in an ampR-dependent manner. Importantly, ampG expression is autoregulated and ampP also regulates expression of ampG. Both AmpG and AmpP have topologies consistent with functions in transport. Together, these data suggest that the mechanism of β-lactam resistance of *P. aeruginosa* is distinct from well-characterized systems in Enterobacteriaceae and involves a highly complicated interaction between these putative permeases and known Amp proteins.

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**Background**

*Pseudomonas aeruginosa* is a Gram negative opportunistic pathogen. As a frequent colonizer of catheters and the most frequent fatal causative agent of ventilator-assisted pneumonia, it is one of the most common agents in health-care associated infection [1]. Lung deterioration due to chronic infection by *P. aeruginosa* affects patients with chronic obstructive pulmonary disorder and is a leading cause of morbidity and mortality in cystic fibrosis patients [2]. *P. aeruginosa* infection treatment is often difficult because of the organism’s intrinsic and acquired antibiotic resistance. This is due to the presence of multidrug efflux pumps [3], low outer membrane permeability [4], hypermutability [5], biofilm formation [6], and β-lactamase expression [7,8].

*P. aeruginosa* has two chromosomally encoded β-lactamases: the PoxB oxacillinase and the AmpC cephalosporinase [8-10]. Much of what is known about...
AmpR regulation is from studies in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter cloacae*. These studies have elegantly demonstrated that induction of AmpC, the chromosomal β-lactamase, involves *ampR*, *ampD*, and *ampG*, encoding a LysR type transcripational factor, an amidase, and a permease, respectively [11].

Expression of *C. freundii AmpR* in *E. coli* revealed that during normal physiological growth, AmpR, in the presence of UDP-MurNAc-peptide, binds to the *ampC* promoter and inhibits expression [12]. In *E. coli*, the addition of β-lactam antibiotics causes an increase in the cytosolic 1,6-anhydro-N-acetylmuramyl-L-Ala-γ-D-Glu-meso-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) [12]. It was postulated that AmpR can either activate or repress transcription from the *ampC* promoter and that its activity is dependent upon the nature of the bound effector molecule. *In vitro*, in the presence of UDP-MurNAc-pentapeptide, AmpR represses transcription of *ampC*, whereas in the presence of 1,6-anhMurNAc-tripeptide, AmpR activates *ampC* [12]. Thus, it is postulated that binding of 1,6-anhMurNAc-tripeptide alters the conformation of AmpR from the repressive to the activating mode, facilitating the expression of *ampC* [12]. High-levels of 1,6-anhMurNAc-tripeptide accumulate in the absence of *ampD*. AmpD is an amidase that cleaves 1,6-anhMurNAc-tripeptide [13].

Induction of *E. cloacae ampC* was also shown to be *ampG*-dependent [14]. β-lactamase fusion analysis suggests that *E. coli* AmpG contains 10 transmembrane segments and two large cytoplasmic loops [15]. *E. coli* AmpG was shown to transport N-acetyllglucosamine-1,6-anhydrous N-acetylmuramic acid (GlcNAc-ahMurNAc) and GlcNAc-ahMurNAc-tri, -tetra, and -pentapeptides [16,17].

Comprehensive and elegant studies using Enterobacteriaceae established the paradigm of the β-lactamase induction mechanism. Orthologs of *ampR*, *ampD*, and *ampG* are found in numerous Gram-negative species [18]. Whether similar mechanisms are employed in all these organisms has not been established. It is possible that the induction mechanism could differ. The β-lactamase induction mechanism of *P. aeruginosa* has not been well-defined; however, it is known that *P. aeruginosa* AmpR regulates expression of *ampC* as in other organisms [8-10]. Similar to other systems, *ampR* is located upstream of the *ampC* gene [10]. Similarly, *ampD* and *ampG* are found in numerous Gram-negative species [18]. Whether similar mechanisms are employed in all these organisms has not been established. It is possible that the induction mechanism could differ. The β-lactamase induction mechanism of *P. aeruginosa* has not been well-defined; however, it is known that *P. aeruginosa* AmpR regulates expression of *ampC* as in other organisms [8-10]. Similar to other systems, *ampR* is located upstream of the *ampC* gene [10]. Additionally, *P. aeruginosa* AmpR controls transcription of the oxacillinase, *pxxB*, and several genes involved in virulence [8-10]. Loss of AmpR in *P. aeruginosa* causes a significant elevation in β-lactamase activity and other virulence factors [10]. *P. aeruginosa* also differs from other previously studied systems in that its genome has two *ampG* orthologs, *PA4218* and *PA4393* [19]. The current study reveals that these two genes, *PA4218* and *PA4393*, are required for β-lactamase induction, hence they have been named *ampP* and *ampG*, respectively. Consistent with their putative roles as permeases, fusion analysis suggests that AmpG and AmpP have 14 and 10 transmembrane helices, respectively. Expression of *ampP* is dependent upon AmpR and is autoregulated. Together, these data suggest the distinctiveness of *P. aeruginosa* β-lactamase induction, as it is the first system that potentially involves two permease paralogs, and contribute to the general understanding of the induction mechanism.

**Results**

**Genome Sequence Analysis of the PA4218 and PA4393 Operons**

*E. coli* AmpG has been shown to be a permease that transports GlcNAc-ahMurNAc peptides from the periplasm to the cytoplasm [13,17]; however, the AmpG function in *P. aeruginosa* has not been described. BLAST analysis of the *E. coli* AmpG sequence against the six-frame translation of the PAO1 genome identified two open reading frames, *PA4218* and *PA4393*, with significant homology [20,21]. Global alignment using the Needleman-Wunsch algorithm [22] demonstrated that *PA4218* is 21.8% identical and 34.8% similar, while *PA4393* is 23.2% identical and 34.3% similar to AmpG (Figure 1). The *Pseudomonas* Genome Database identifies *PA4393* as encoding a putative permease with an alternate name of *ampG*, while *PA4218* is identified as encoding a probable transporter [23]. Thus, *PA4393* will be referred to as *ampG* and *PA4218* as *ampP* (*P* for permease).

Analysis of the sequences around *ampG* and *ampP* revealed that they were in close proximity to two respective upstream ORFs. Based upon sequence analysis, it is likely that *ampG* and *ampP* constitute two two-gene operons with their respective upstream ORFs (Figures 2A and 2B). *PA4219* (*ampO*) overlaps the first seven base pairs of *ampP* (Figure 2A). AmpO is a putative seven-transmembrane protein with a strong lipoprotein signal peptide that has a potential cleavage site between amino acids 18 and 19 [23]. The *ampG* gene is located 43 bp downstream from *PA4392* (*ampF*), which encodes a putative protein with a DNA-protein cysteine methyltransferase domain (Figure 2B). The function of this domain remains unknown. No lipoprotein signal was detected in AmpF.

To determine if *ampG* and *ampP* constitute two-gene operons with their upstream ORFs, RNA isolated from PAO1 was analyzed by reverse transcription polymerase chain reaction (PCR) using primers flanking the intergenic (*ampF-ampG*) (Figure 3A) and the overlapping
(ampO-ampP) region (Figure 3B). The expected ampli-
con sizes are 136 and 158 bp for the ampF-G junction
and ampO-P junction, respectively [23]. As expected,
amplification was observed with genomic DNA
(Figures 3A and 3B, Lane 3). In the RNA analyses,
PCR products were observed in reverse transcription
PCR when the template was prepared in the presence of reverse transcriptase (Figures 3A and 3B, Lane 1),
but not in the control reaction when reverse transcrip-
tase was omitted (Figures 3A and 3B, Lane 2). This
confirms that ampO and ampP constitute a two-gene
operon and ampF and ampG constitute another. In
addition, reverse transcriptase real time PCR data is in
agreement with ampO and ampP belonging to the
same operon and ampF and ampG comprising another
operon (data not shown).

Topology analysis of AmpG and AmpP
The ampG and ampP genes encode predicted proteins
with 594 and 414 amino acids, isoelectric points of 9.3
and 9.4, and calculated molecular weights of 64.6 kDa
and 43.2 kDa, respectively. Hydrophobicity plots pre-
dict that AmpG has 16 or 14 predicted transmembrane
(TM) helices, depending upon the algorithm used and
AmpP has 10 [23]. To determine the membrane topol-
ygy of AmpG and AmpP, the F and L genes were cloned
downstream of the ampG and ampP genes. The 3' end
of the ampG and ampP genes were progressively
deleted using exonuclease III. At various time-points,
the truncated genes were ligated and assayed for PhoA
and LacZ activities in E. coli. Clones were also
sequenced to determine the reporter and amp
gene
junctions.

Figure 1 Alignment of E. coli AmpG, PA4218 and PA4393. The primary sequence of E. coli AmpG, PA4218 (AmpF) and PA4393 (AmpG) were
used as an input to M-Coffee, which combines multiple sequence alignments using the T-Coffee platform [45,46]. Identical and similar amino
acids were shaded black and gray, respectively, using BOXSHADE.
AmpG fusions at amino acids 80, 146, 221, 290, 368, 438, 468, 495, as well as full length were LacZ-positive and PhoA-negative, and fusions at amino acids 51, 185, 255, 338, 406, and 540 were PhoA-positive and LacZ-negative domains, suggesting that AmpG has only 14 TM helices (Figures 4C and 4D). AmpP fusions at amino acids 80, 170, 248, 308, 400 as well as full length were LacZ-positive and PhoA-negative, and fusions at amino acids 38, 120, 195, 278, and 360 were LacZ-negative and PhoA-positive, consistent with 10 TM domains (Figures 4A and 4B).

β-lactamase activity in strains containing mutations in ampG and ampP
The failure to induce C. freundii ampC in the absence of E. coli ampG suggested that AmpG is essential for
Figure 3. PCR analysis of ampFG and ampOP operon cDNA. Polyacrylamide gel electrophoresis of PCR products of the junctions of the ampOP and ampFG operons. (A) PCR with primers PA4392_3junctionRTF and PA4392_3junctionRTR to amplify the PA4392 - PA4393 intergenic region. (B) PCR with primers PA4218_9junctionRTF and PA4218_9junctionRTR to amplify the PA4392 - PA4393 intergenic region. (Panels A and B) Lane M: PCR markers (Promega, Madison, WI). Lane 1, cDNA reaction performed with PAO1 RNA, the appropriate buffer and Superscript RT III. Lane 2, cDNA reaction performed with PAO1 RNA, the appropriate buffer without Superscript RT III. Lane 3, P. aeruginosa genomic DNA. The asterisk indicates a nonspecific product. Arrows indicate junction amplicons.

Figure 4. Topology of AmpP and AmpG. The topology of AmpP and AmpG was analyzed by in-frame ampP and ampG fusions to the lacZ and phoA genes, the cytoplasmic and periplasmic markers, respectively. The corresponding points of fusion and qualitative biochemical results of the β-galactosidase (LacZ) and alkaline phosphatase (PhoA) assays ([44]) are shown for AmpP (A) and AmpG (C). These results, together with transmembrane domain predictions generated using a Kyte-Doolittle algorithm present in Lasergene 7 (DNASTAR, Madison, WI) were used to predict the topology of AmpP (B) and AmpG (D). Solid lines indicate prediction based upon experimental data, dashed lines indicate regions where more than one possibility exists. Cytoplasm and periplasm are denoted by Cyto and Peri, respectively. Fusion sites are indicated by a dot with the corresponding amino acid number. Putative transmembrane domain boundaries were obtained from Lasergene.
the induction of chromosomal β-lactamases [24,25]. To ascertain the role of the permeases in *P. aeruginosa*, isogenic *ampG* and *ampP* insertional inactivation mutants were constructed in the prototypic *P. aeruginosa* strain PAO1, referred to as PAOampG and PAOampP, respectively. The β-lactamase activity in the two isogenic mutants, PAOampG and PAOampP, was compared to PAO1. In the absence of β-lactam antibiotics, all strains showed a basal level of β-lactamase activity (Table 1). Upon challenge with 500 μg/ml of benzyl-penicillin, this level was elevated 10-fold (*p < 0.05*) in PAO1 (Table 1). However, the β-lactamase activities of PAOampP and PAOampG remained low in the presence of β-lactam antibiotic, indicating a loss of β-lactamase induction (Table 1). The loss of inducibility in PAOampG could be partially restored by expressing *ampG* in *trans*, whereas the β-lactamase inducibility of PAOampP was completely recovered when *ampP* was supplied in *trans* (Table 1). Both PAOampP and PAOampG mutants had the other copy of the permease gene intact. These observations suggest that *ampG* and *ampP* are individually important members of the β-lactamase induction system. To confirm that *ampG* and *ampP* play independent roles, cross-complementation of PAOampP with pAmpG, and PAOampG with pAmpP was performed. Similar to the mutants, the cross-complemented strains did not show inducible β-lactamase activity (Table 1).

To further understand the role of *ampG* and *ampP* in β-lactamase induction, β-lactamase activity was assayed at different concentrations of benzyl-penicillin in PAO1, PAOampG and PAOampP (Figure 5). Upon encounter with the inducer (25 μg/ml), there was approximately 38% induction (Figure 5). For strain PAO1, this increase in β-lactamase activity continued in a dose-dependent manner until the maximum level of β-lactamase activity was reached when 100 μg/ml of benzyl-penicillin was added (Figure 5). A higher concentration of inducer did not result in a concomitant increase in the expression of the β-lactamase (Figure 5 and data not shown).

In PAOampG, the initial increase of β-lactamase activity was observed at 25 μg/ml, suggesting that this burst of β-lactamase production is *ampG*-independent (Figure 5). However, unlike PAO1, the induction level failed to increase after 25 μg/ml of benzyl-penicillin and even significantly decreased with addition of increased concentrations of benzyl-penicillin (Figure 5).

Mutation of *ampP* also prevented maximum induction of β-lactamase, but the defect was not quite as severe as in PAOampG. In PAOampP, the pattern of β-lactamase induction was very similar to PAO1 at concentrations of benzyl-penicillin up to 50 μg/ml (Figure 5). However, unlike PAO1, addition of benzyl-penicillin at concentrations greater than 50 μg/ml failed to further induce production of β-lactamases (Figure 5). Thus, low induction is independent of *ampG* or *ampP*. The observation that PAOampP exhibited higher levels of β-lactamase expression at higher concentrations of benzyl-penicillin may suggest that *ampG* plays a greater role at higher concentrations of β-lactam.

Most of the β-lactamase activity of *P. aeruginosa* can be attributed to AmpC, however, *P. aeruginosa* does contain another chromosomally encoded β-lactamase, PoxB [9,26]. To further analyze if the loss of β-lactamase induction in the PAOampG and PAOampP strains was due to loss of AmpC function, the ampC promoter (*P_{ampC}* activity was measured in PAO1, PAOampG, and PAOampP. As expected, upon treatment with benzyl-penicillin, *P_{ampC-lacZ}* activity increased approximately 15-fold (Figure 6). Benzyl-penicillin dependent induction of *P_{ampC-lacZ}* was lost in PAOampG or PAOampP (Figure 6).

To further characterize the role of *ampG* and *ampP*, the sensitivity of PAO1, PAOampG, and PAOampP to several β-lactams was determined (Table 2 and data not shown).

![Table 1](http://www.biomedcentral.com/1471-2180/10/328)

| Strain and plasmid | Relevant genotypes (supplement in trans) | β-lactamase activity<sup>a</sup> | Uninduced | Induced<sup>b</sup> |
|-------------------|----------------------------------------|-------------------------------|-----------|-----------------|
| PAO1              | *ampG*<sup>+</sup>*ampP<sup>+</sup>    | 222 ± 9.7                     | 221.4 ± 9.2 |
| PAOampG           | *ampG*<sup>+</sup>*ampP<sup>+</sup>    | 204 ± 6.2                     | 288<sup>d</sup> ± 3.3 |
| PAOampP           | *ampG*<sup>+</sup>*ampP<sup>+</sup>    | 42 ± 6.2                      | 322<sup>d</sup> ± 3.3 |
| PAOampG/pKKF69    | *ampG*<sup>+</sup>*ampP<sup>+</sup> (*ampG*) | 8.4 ± 1.4                    | 876 ± 14.4 |
| PAOampP/pKKF73    | *ampG*<sup>+</sup>*ampP<sup>+</sup> (*ampP*) | 8.8 ± 1.8                    | 2179 ± 35.5 |
| PAOampG/pKKF73    | *ampG*<sup>+</sup>*ampP<sup>+</sup> (*ampG*) | 2.1 ± 20                     | 144 ± 1.9 |
| PAOampP/pKKF69    | *ampG*<sup>+</sup>*ampP<sup>+</sup> (*ampP*) | 5.3 ± 1.9                    | 106 ± 2.7 |

<sup>a</sup> Cultures at OD600 of 0.6-0.8 were divided in two. One set was induced with 500 μg/ml benzyl-penicillin for three hours before harvesting. Assays were performed on sonicated lysate using nitrocefin as a chromogenic substrate. One million units of β-lactamase is defined as 1 nanomole of nitrocefin hydrolyzed per minute per microgram of protein. Assays were performed in triplicate.

<sup>b</sup> Induction was carried out using 500 μg/ml benzyl-penicillin.

<sup>d</sup> Induction was carried out using 500 μg/ml benzyl-penicillin.

<sup>p < 0.05</sup> compared to uninduced PAO1.

<sup>p < 0.05</sup> compared to induced PAO1.
AmpG regulation of \(\text{ampG}\) and \(\text{ampP}\)

In inducible \(\text{amp}\) systems, the expression of \(\text{ampC}\) is tightly regulated by the transcription factor, AmpR [27]. In order to investigate the role, if any, of AmpR in the regulation of \(P.\) aeruginosa \(\text{ampG}\) and \(\text{ampP}\), \(P_{\text{ampFG}}-\text{lacZ}\) and \(P_{\text{ampOP}}-\text{lacZ}\) promoter fusions were generated and integrated into the chromosome of PAO1 and PAOampR via \(\text{attB-attP}\) site-specific recombination. These constructs are likely to mimic the chromosomal regulation of the \(\text{ampFG}\) and \(\text{ampOP}\) operons. In the absence of inducer in PAO1 and PAOampR, there was a detectable basal level of promoter activity (Figure 7). The expression of the \(P_{\text{ampOP}}-\text{lacZ}\) promoter fusion was significantly increased in the presence of inducer in the wild-type PAO1, and this induction was lost completely in PAOampR (Figure 7). However, the activity of the \(P_{\text{ampFG}}-\text{lacZ}\) promoter fusion was comparable to the basal level in the absence and presence of inducer in PAO1 and PAOampR.

AmpR regulation of \(P_{\text{ampFG}}\) and \(P_{\text{ampOP}}\)

To determine if \(\text{ampG}\) or \(\text{ampP}\) affected their own or each other’s expression, \(P_{\text{ampFG}}-\text{lacZ}\) and \(P_{\text{ampOP}}-\text{lacZ}\) promoter fusions were introduced into the chromosomes of PAOampP and PAOampG. Interestingly, the activity of the \(P_{\text{ampOP}}-\text{lacZ}\) promoter fusion was significantly de-repressed in PAOampP in the absence and presence of inducer (Figure 7). The activity of the \(P_{\text{ampFG}}-\text{lacZ}\) was unchanged in PAOampG in either the absence or presence of benzyl-penicillin. After three hours, cells were harvested and \(\beta\)-galactosidase activity assayed as described [10]. Each value is the mean of at least three independent experiments.

Autoregulation of the \(\text{ampG}\) and \(\text{ampP}\) genes

Autoregulation of the \(\text{ampG}\) and \(\text{ampP}\) genes was observed in PAOampP and PAOampG (see Materials and Methods and text for details). Cells were grown to an \(\text{OD}_{600}\) of 0.6 - 0.8, at which time cultures were divided into two and one set treated with 100 \(\mu\)g/ml benzyl-penicillin. After three hours, cells were harvested and \(\beta\)-galactosidase activity assayed as described [10]. Each value is the mean of at least three independent experiments.

Discussion

Members of the Pseudomonadaceae family are intrinsically resistant to \(\beta\)-lactam antibiotics. Earlier reports

shown). Inactivation of \(\text{ampG}\) led to a significant decrease in resistance to amoxicillin (> 16-fold) and imipenem (> seven-fold). No difference was observed with ampicillin/sulbactam, cefaclor, cefepime, oxacillin, piperacillin, piperacillin/tazobactam, or ticaricillin/clavulonic acid (data not shown). Inactivation of \(\text{ampP}\) in PAO1 did not alter its resistance profile with these \(\beta\)-lactams (Table 2 and data not shown).

Figure 5 Relative \(\beta\)-lactamase activity in PAOampP and PAOampG mutants. Assays were performed on the parental PAO1, and the mutants, PAOampP and PAOampG in the presence of benzyl-penicillin at a concentration gradient of 0 to 125 \(\mu\)g/ml. Cultures at \(\text{OD}_{600}\) of 0.6-0.8 were induced for three hours before harvesting. Assays were performed on sonicated lysate using nitrocefin as a chromogenic substrate. The \(\beta\)-lactamase activity of PAO1 at 100 \(\mu\)g/ml of benzyl-penicillin was taken as 100%. Each value is the mean of at least three independent experiments. The asterisk refers to \(p\)-values of < 0.05 with respect to PAO1, which were calculated using the two-tailed Student’s \(t\)-test.

Figure 6 Activity of the \(\text{ampC}\) promoter. Promoter activity of the \(\text{ampC}\) gene was analyzed using \(\text{lacZ}\) transcriptional fusions integrated at the \(\text{att}\) locus of PAO1, PAOampR, PAOampG and PAOampP (see Materials and Methods and text for details). Cells were grown to an \(\text{OD}_{600}\) of 0.6 - 0.8, at which time cultures were divided into two and one set treated with 100 \(\mu\)g/ml benzyl-penicillin. After three hours, cells were harvested and \(\beta\)-galactosidase activity assayed as described [10]. Each value is the mean of at least three independent experiments.
successfully identified \textit{ampC}, \textit{ampR}, \textit{ampD}, and \textit{ampE} as genes involved in the \(\beta\)-lactamase induction mechanism. However, the question of how chromosomal \(\beta\)-lactamase is induced remains elusive. This study examines the role of two previously uncharacterized \textit{P. aeruginosa} putative permeases.

\textit{P. aeruginosa} harbors two distinct and independent \textit{AmpG} orthologues

In Enterobacteriaceae, besides \textit{AmpR}, \textit{AmpD} and \textit{AmpE}, \textit{AmpG} has also been implicated in the \textit{ampC} encoded \(\beta\)-lactamase induction, acting as a membrane permease that transports 1,6-anhMurNAc-tripeptide and 1,6-anhMurNAc-pentapeptide [17]. In \textit{P. aeruginosa}, two paralogs, \textit{PA4393/ampG} and \textit{PA4218/ampP}, were found (Figure 1) [28]. Both \textit{ampG} and \textit{ampP} appear to be one member of two independent two-gene operons (Figures 2 and 3). PFAM analysis of AmpP identifies a Major Facilitator Superfamily (MFS1) domain between amino acids 14 and 346, in agreement with a role in transport [23,29,30]. Upstream from \textit{ampP} is \textit{PA4219/ampO}, a gene that has seven putative transmembrane domains [23,31]. Together, these genes form an operon

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**Table 2 MICs in PAO1, PAO\textit{ampG} and PAO\textit{ampP} strains**

| Strain       | Amoxicillin (\(\mu\)g/ml) | Imipenem (\(\mu\)g/ml) |
|--------------|---------------------------|------------------------|
| PAO1         | > 256                     | 3                      |
| PAO\textit{ampG} | 16                       | 0.38                   |
| PAO\textit{ampP} | > 256                     | 3                      |

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**Figure 7 Activity of the \textit{ampG} and \textit{ampP} promoters**

Promoter activity of the \textit{ampG} and \textit{ampP} genes was analyzed using \textit{lacZ} transcriptional fusions integrated at the \textit{att} locus of PAO1, PAO\textit{ampR}, PAO\textit{ampG} and PAO\textit{ampP} (see Materials and Methods and text for details). Cells were grown to an \(OD_{600}\) of 0.6 - 0.8, at which time cultures were divided into two and one set treated with 100 \(\mu\)g/ml benzyl-penicillin. After three hours, cells were harvested and \(\beta\)-galactosidase activity assayed as described [10]. All 16 conditions were assayed at the same time but are divided into two panels for visualization purposes. Each value is the mean of at least three independent experiments. The asterisk refers to \(p\)-values < 0.05, which were calculated using the two tailed Student’s \(t\)-test.
Because of the similarity between AmpG from Enterobacteriaceae and PAO1 AmpG and AmpP, β-lactamase levels of single ampG and ampP mutant isogenic strains were determined. Although an increase in β-lactamase activity was observed, neither the ampG nor ampP mutant strain produced the same level of β-lactamase in the presence of benzyl-penicillin as PAO1 (Table 1, Figure 5). Moreover, inactivation of ampG or ampP abolishes induction of P_{ampC} (Figure 6). This indicates that both ampG and ampP are essential for chromosomal β-lactamase induction. These genes did not cross-complement or exhibit gene dosage effects indicating that they play different roles in the induction pathway (Table 1). These results are consistent with recent data demonstrating that mutation of ampG affects induction of β-lactamase and failure of ampP to complement an ampG mutation [28]. Furthermore, the analysis using increasing benzyl-penicillin concentrations, shows that ampP plays an important role at lower inducer concentrations, whereas ampG is crucial at higher concentrations (Figure 5). Mutation of ampG affects PAO1 β-lactam resistance (Table 2) [28]. Recent studies by Zhang et al., in which deletion of ampG results in increased sensitivity to ampicillin [28], are consistent with results presented here (Table 2). In addition, ampG inactivation increases imipenem sensitivity (Table 2). Loss of ampP (also referred to as ampGh1) function did not affect β-lactam sensitivity in either study (Table 2) [28]. AmpP (PA4218) has previously been named FptX due to its homology to RhtX in Sinorhizobium meliloti 2011 [36]. PA4219 does not have a S. melloti orthologue [36]. Mutation of ampP in a P. aeruginosa CDC5 derivative that produces pyochelin but not pyoverdine, resulted in loss of pyochelin utilization [36]. In agreement with a role in pyochelin utilization, ampP is located next to genes involved in pyochelin biosynthesis and transport [23,36]. Thus, the results presented in Table 1 and Figures 5 and 6 demonstrate that ampP is involved in β-lactamase induction in addition to its previously characterized role in pyochelin utilization [36].

Expression of ampP is induced by β-lactam addition in the presence of ampR

Despite the importance of ampG in β-lactamase induction, little is known about its regulation. E. coli ampG is also the second gene in a two gene operon. Upstream and divergently transcribed from the E. coli ampG operon, is the bolA transcriptional regulator [24]. Expression of bolA is dependent upon RpoS. Previous studies suggest the expression of the E. coli ampG gene is independent of bolA, rpoS or ampD [24]. Neither the P. aeruginosa ampG nor ampP gene is located near the bolA locus [23], thus P_{ampFG} and P_{ampOP-lacZ} transcriptional fusions were integrated into the chromosome of isogenic PAO1 strains to begin to understand ampG and ampP regulation.
In light of the requirement of ampG and ampP for maximum P. aeruginosa β-lactamase induction, it was of interest to determine if expression of either was affected by β-lactam addition (Table 1, Figure 5). In the absence of antibiotic, P_{ampFG} and P_{ampOP} were constitutively expressed. Expression of P_{ampOP} significantly increased in the presence of inducer, while P_{ampFG} did not (Figure 7).

The LysR type transcriptional regulator AmpR induces the expression of the AmpC β-lactamase in the presence of β-lactam antibiotics [27]. AmpR also affects the regulation of additional genes involved in P. aeruginosa antibiotic resistance and virulence [10]. Insertional inactivation of ampR, did not affect P_{ampFG} - lacZ activity, however, the increase in P_{ampOP} - lacZ activity previously observed upon β-lactam addition was lost in the absence of ampR (Figure 7). This indicates that ampP expression is regulated by AmpR. Future analyses will determine if this regulation is direct or indirect.

**ampP affects regulation of both its own promoter and that of ampG**

Given that both ampG and ampP are required for maximum β-lactamase expression, both contain structural elements consistent with roles in transport, and the regulation of ampP expression by β-lactam and ampR, it was feasible that ampP could contribute to its own expression, perhaps by transporting potential effector molecules for AmpR. Indeed, ampP does appear to inhibit its own expression, as P_{ampOP} activity increased ten-fold in PAOampP in the absence, and approximately seven-fold in the presence of β-lactam (Figure 7). Insertional inactivation of ampP also resulted in increased expression of P_{ampFG} in the presence of β-lactam (Figure 7).

**Proposed model for regulation of β-lactamase induction**

The results presented contribute to what is known concerning β-lactamase induction in P. aeruginosa. It is well established that induction of the expression of the AmpC β-lactamase is dependent upon AmpR. Although the exact mechanism has not been well characterized in P. aeruginosa, it is believed that the induction is triggered by conversion of AmpR from a repressor to an activator (Figure 8). Evidence from Enterobacteriaceae suggest that this is a result of inhibition of the cell wall remodeling process, as suggested by previous findings suggesting that AmpR acts as a positive regulator of amp genes [10], activation of ampP expression required the presence of AmpR and β-lactam antibiotic (Figure 7). Based upon glycopeptide accumulation studies in other organisms, these findings suggest that the accumulation of 1,6-aniMurNAc-tripeptide and 1,6-aniMurNAc-pentapeptide in the presence of β-lactam antibiotics activates AmpR that in turn up-regulates the expression of ampP. However, P. aeruginosa appears to use two non-redundant permeases in β-lactamase induction, suggesting, one may be involved in the import of muramyl peptides and the other in an as yet unknown function. The second permease may be involved in export of muramyl peptides or import of different muramyl peptides. Further studies to determine the identity of these peptides and how they regulate AmpR will be a critical next step in deciphering β-lactam resistance in P. aeruginosa.

The ampP gene is also auto-regulated via an unknown mechanism. If AmpP performs a similar function as E. coli AmpG, the absence of ampP would result in the accumulation of the periplasmic pool of GlcNAc-aniMurNAc peptides or the reduction in the cytoplasmic pool of 1,6-aniMurNAc-tripeptide and 1,6-aniMurNAc-pentapeptide alerting the cell that the peptidoglycan recycling process is inhibited. This signalling could result in a positive feedback mechanism that up-regulates the expression of ampP. The accumulation of the periplasmic pool of 1,6-aniMurNAc-tripeptide and 1,6-aniMurNAc-pentapeptide in PAOampP is also likely to up-regulate the expression of P. aeruginosa PAO1 ampG in the presence of β-lactam.

Currently, it is not known if PAO1 AmpG and AmpP function similarly to E. coli AmpG, however, like ampG, the PAO1 ampG and ampP are essential for β-lactamase induction [14] (Figure 5, Figure 6, Table 1). Moreover, ampG and ampP are not functionally redundant as both are required for maximum induction. Their expression is also differentially regulated. An ampP promoter-lacZ fusion exhibited increased activity in the presence of ampR and β-lactam or the absence of ampP. An ampG promoter-lacZ fusion was unaffected by the absence or presence of ampR or ampG. Increased β-galactosidase activity was observed from the ampG promoter fusion in the presence of β-lactam in an ampP mutant (Figure 7). It is not known if this is dependent upon ampR, related to an ampR-independent function of ampP in β-lactamase induction or the function of ampP in pyochelin utilization.

**Conclusions**

P. aeruginosa appears to have two ampG paralogs, ampG and ampP, which encode proteins with 14 and 10 transmembrane domains. Both are required for maximum induction of chromosomal β-lactamase and induction of the ampC promoter. Expression of ampP did not restore maximum β-lactamase induced activity in an ampG mutation nor did expression of ampG.
complement an ampP mutation, indicating that ampG and ampP have distinct functions in β-lactamase regulation. In addition to being autoregulated, ampP is regulated by AmpR and β-lactam. ampP is also involved in the regulation of ampG in the presence of β-lactam. In summary, the presence of two distinct permeases required for β-lactamase induction suggests that the P. aeruginosa β-lactamase resistance mechanism is more complex and distinct from the current paradigm.

**Methods**

**Bacterial strains, plasmids and media**

Bacterial strains, plasmids and primers employed in this study are shown in Table 3. *E. coli* and *P. aeruginosa* were routinely cultured in Luria-Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, per liter). *Pseudomonas* Isolation Agar (PIA, Difco) was used in triparental mating experiments. Mueller-Hinton agar (Difco) was used in E-test experiments. Antibiotics, when used, were at the following concentrations (per liter) unless indicated otherwise: ampicillin (Ap) at 50 mg, tetracycline (Tc) at 20 mg, gentamycin (Gm) at 30 mg for *E. coli* and carbenicillin (Cb) at 300 mg, Gm at 300 mg and Tc at 60 mg for *P. aeruginosa*.

**DNA manipulations**

Standard procedures in molecular biology were performed as previously described [37].

**Insertional inactivation of the ampG and ampP genes**

A 2904-bp ampG fragment was PCR-amplified from PAO1 genomic DNA using KKF01ampGFor and KKF04ampGRev (Table 3). Similarly, KKF05ampPFor and KKF08ampPRev were used to PCR-amplify a
Table 3  Bacterial strains, plasmids and primers used in this study

| Strains/Plasmids | Genotype | Reference |
|------------------|----------|-----------|
| **Escherichia coli** | | |
| TOP10<sup>®</sup> | F' ( lacI<sub>q</sub>, Tn10(Tet<sup>R</sup>)) mcrA Δ(mrr-hsdRMS-mcrBC) F<sup>−</sup> lacZAM15 ΔlacO174 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str<sup>R</sup>) endA1 nupG | Invitrogen |
| | | |
| **Pseudomonas aeruginosa** | | |
| PAO1 | Wild-type | [47] |
| PKM400 | PAOampP::aac<sup>CI</sup>; Gm<sup>R</sup> | This study |
| PKM500 | PAOampG::aac<sup>CI</sup>; Gm<sup>R</sup> | This study |
| PKM300 | PAOampR::aac<sup>CI</sup>; Gm<sup>R</sup> | [10] |
| PKM301 | PAOattB::P<sub>ampC</sub>-lacZ; Tc<sup>®</sup> | [10] |
| PKM303 | PAOampR::aac<sup>CI</sup> attB::P<sub>ampC</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | [10] |
| PKM104 | PAOattB::P<sub>ampP</sub>-lacZ; Tc<sup>®</sup> | KKF0290, This study |
| PKM105 | PAOattB::P<sub>ampP</sub>-lacZ; Tc<sup>®</sup> | This study |
| PKM312 | PAOampR::aac<sup>CI</sup> attB::P<sub>ampC</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM313 | PAOampR::aac<sup>CI</sup> attB::P<sub>ampP</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM404 | PAOampP::aac<sup>CI</sup> attB::P<sub>ampP</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM405 | PAOampP::aac<sup>CI</sup> attB::P<sub>ampG</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM504 | PAOampG::aac<sup>CI</sup> attB::P<sub>ampP</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM505 | PAOampG::aac<sup>CI</sup> attB::P<sub>ampG</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM506 | PAOampG::aac<sup>CI</sup> attB::P<sub>ampC</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| PKM507 | PAOampP::aac<sup>CI</sup> attB::P<sub>ampC</sub>-lacZ; Tc<sup>®</sup>Gm<sup>R</sup> | This study |
| **Plasmids** | | |
| pCRII-TOPO | Ap<sup>R</sup>; Km<sup>R</sup>; ColEl ori lacZΔ | Invitrogen |
| pUCGm | Ap<sup>R</sup>; Gm<sup>R</sup>; pUC19 derivative containing gentamycin cassette | [38] |
| pEX100T | Ap<sup>R</sup>; sacB oriT | [39] |
| pMF54 | ColEl-SF broad-host replicon | [48] |
| pME6030 | Tc<sup>®</sup>; ori<sub>178S</sub> ori<sub>178A</sub> oriT | [41] |
| pRK2013 | Km<sup>R</sup>; ColEl ori-Tra (RK2)* | [40] |
| pTrcphoA | Ap<sup>R</sup>; low-copy trc promoter expression vector carrying the lacZ<sup>Δ</sup> and phoA | [43] |
| pTrclacZ | Ap<sup>R</sup>; low-copy trc promoter expression vector carrying the lacZ<sup>Δ</sup> and lacZ<sup>®</sup> | [43] |
| pS110 | Tc<sup>®</sup>; CTX-lacZ fused with ampC promoter, P<sub>ampC</sub> | [10] |
| pKKF003 | Ap<sup>R</sup>; Km<sup>R</sup>; pCRII-TOPO with a 2904-bp fragment containing PAO1 coordinates 4921591-4924494 (PA4392-PA4393/ampF-ampG) | This study |
| pKKF004 | Ap<sup>R</sup>; Km<sup>R</sup>; pCRII-TOPO with a 2779-bp fragment containing PAO1 coordinates 4721496-4724275 (PA4217-PA4218/ampF-ampP) | This study |
| pKKF069 | Tc<sup>®</sup>; pME6030 with a 2904-bp EcoRI flanked fragment containing ampF-ampG | This study |
| pKKF073 | Tc<sup>®</sup>; pME6030 with a 2779-bp EcoRI flanked fragment containing ampD-ampP | This study |
| pKKF087 | Tc<sup>®</sup>; CTX-lacZ fused with ampP promoter, P<sub>ampP</sub> | This study |
| pKKF091 | Tc<sup>®</sup>; CTX-lacZ fused with ampG promoter, P<sub>ampG</sub> | This study |
| pKKF145 | Ap<sup>R</sup>; Gm<sup>R</sup>; pCRII-TOPO derivative with ampP::aac<sup>CI</sup> | This study |
| pKKF149 | Ap<sup>R</sup>; Gm<sup>R</sup>; pCRII-TOPO derivative with ampG::aac<sup>CI</sup> | This study |
| pKKF157 | Ap<sup>R</sup>; Gm<sup>R</sup>; pEX100T derivative with ampG::aac<sup>CI</sup> | This study |
| pKKF161 | Ap<sup>R</sup>; Gm<sup>R</sup>; pEX100T derivative with ampP::aac<sup>CI</sup> | This study |
| pAA0115 | Ap<sup>R</sup>; pTrcphoA derivative with 1.9 kb PstI DNA from pMF54 containing stabilization fragment | This study |
| pAA0112 | Ap<sup>R</sup>; Km<sup>R</sup>; pCRII-TOPO containing a 1797 ampG PCR product amplified using primers KKF09 and KKF10 | This study |
| pAA0115 | Ap<sup>R</sup>; pAA0115 containing a 1,813 EcoRI fragment containing ampG from pAA0112 | This study |
| pAA1261 | Ap<sup>R</sup>; pAA0121 digested with BamHI and SalI, treated with Klenow and re-ligated to remove an XbaI site. Used as basis for Erase-a-base system | This study |
| pKKF259 | Ap<sup>R</sup>; pTrcphoA derivative with a 1797-bp fragment containing PA4393 | This study |
| pKKF458 | Ap<sup>R</sup>; pTrcphoA derivative with a 1245-bp fragment containing PA4218 | This study |
| pKKF459 | Ap<sup>R</sup>; pTrclacZ derivative with a 1797-bp fragment containing PA4393 | This study |
| pKKF465 | Ap<sup>R</sup>; pTrclacZ derivative with a 1245-bp fragment containing PA4218 | This study |
Table 3 Bacterial strains, plasmids and primers used in this study (Continued)

| Primers               | Primer sequence | Source     |
|-----------------------|-----------------|------------|
| KKF01ampGFor          | 5'-TCCAGCTTGACGGTCGAGATT-3' | This study |
| KKF04ampGRev          | 5'-AGAACATCTCTCTGGCCATGG-3' | This study |
| KKF05ampPFor          | 5'-AACGGCCAGCGTACGAAACAC-3' | This study |
| KKF08ampPRev          | 5'-GTGCGCGTTCAGTGTCTG-3' | This study |
| KKF09ampG2For         | 5'-GGGAATTCCATATGACTCAGCAATCTGG-3' | This study |
| KKF10ampG2Rev         | 5'-GCTCTAATGCTCGGGCTTTGCTGTG-3' | This study |
| KKF13ampP2For         | 5'-TCTAGATCAGGCCCTGTGCGCCG-3' | This study |
| KKF14ampG2Rev         | 5'-CGTCTGAGCTTGATCCGCAC-3' | This study |
| PA4218_9junctionRTF   | 5'-ACCTCTACCTATGCTCTG-3' | This study |
| PA4218_9junctionRTR   | 5'-CAGGAGGCAAGCAGC-3' | This study |
| PA4392_3junctionRTF   | 5'-CAACGCAAGGTTGACATTAC-3' | This study |
| PA4392_3junctionRTR   | 5'-GAGACTTGTAGGCGACAGC-3' | This study |
| (NS)2RandomPrimer     | 5'-NSNSNSNSNSNS-3' | [49]       |

2779-bp ampP fragment. The ampP and ampG PCR products were cloned into pCRII-TOPO according to the manufacturer’s instruction (Invitrogen, CA), generating pKKF04 and pKKF03, respectively. A Gm cassette carrying the aacCI gene was retrieved from pUCGm [38]. The cassette was inserted into the unique HincII and SmaI restriction sites of ampP and ampG, respectively, creating pKKF145 and pKKF149 (Figure 2). These insertions created a polar mutation in the 5’-ends of ampP and ampG ORFs in pKKF04 and pKKF03, respectively. Subsequently, the ampP:aacCI and ampG:aacCI from pKKF145 and pKKF149, respectively, were subcloned into the Smal site of pEX100T [39], a mobilizable suicide plasmid. These plasmids were conjugated into P. aeruginosa PAO1, with a helper strain harboring pKKF003 and pKKF004 into pGEMEX-L, respectively, generating pKKF091 (P\textsuperscript{ampG\textsuperscript{lacZ}}) and pKKF087 (P\textsuperscript{ampG\textsuperscript{2-lacZ}}) (Table 3). This suicide vector contained the integration-proficient attP site, which recombines into the chromosomal attB site to generate a single-copy reporter fusion [42]. The resulting clones were mobilized into PAO1 and PAOampR (Table 3). The presence of the chromosomal insertions was confirmed by PCR and restriction analysis of the product.

Promoter-lacZ fusion constructions
The putative promoter regions of ampG and ampP were subcloned from pKKF003 and pKKF004 into pGEMEX-L, respectively, generating pKKF091 (P\textsuperscript{ampG\textsuperscript{2-lacZ}}) and pKKF087 (P\textsuperscript{ampG\textsuperscript{2-lacZ}}) (Table 3). This suicide vector contained the integration-proficient attP site, which recombines into the chromosomal attB site to generate a single-copy reporter fusion [42]. The resulting clones were mobilized into PAO1 and PAOampR (Table 3). The presence of the chromosomal insertions was confirmed by PCR and restriction analysis of the product.

Topological analysis of AmpP and AmpG
The topology of AmpP and AmpG were investigated using two markers, phoA and lacZ, that function in the periplasm and cytoplasm, respectively. The entire ampP gene was PCR amplified using primers KKF13ampP2For and KKF14ampP2Rev and cloned into pTrcphoA [43]. The entire ampG gene was PCR amplified using primers KKF09ampG2For and KKF10ampG2Rev and cloned into a pTrcphoA plasmid which had been modified by insertion of a broad host range stabilization fragment from pMF54 (Table 3). Both ampG and ampP genes were cloned into pTrclacZ [43]. The erase-a-base system (Promega, WI) was used to generate deletions of the genes from the 3’-ends. The resulting clones were then sequenced to determine the fusion junctions. The phoA and lacZ activities were determined as previously described [44].

β-lactamase and β-galactosidase assays
β-lactamase and β-galactosidase activities were assayed as previously described [9,10].
Determination of minimal inhibitory concentrations (MICs)

MICs were determined using E-test strips (Biomerieux, Marcy l’Etoile, France) according to the manufacturer protocols.

Reverse transcription PCR

For the reverse transcription PCR, RNA was isolated from PAO1 using the RNAeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer protocol. DNA was removed by two sequential 1 hour treatments at 37°C with RQ DNasel (Promega Corporation, Madison, WI) followed by heat inactivation at 65°C for 10 minutes. Synthesis of cDNA was performed with Superscript III reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) using a (NS)5 random primer and 5 μg RNA according to the manufacturer protocol. A control reaction containing all components except for Superscript III RT was performed in parallel. After cDNA synthesis, RNA was removed by treatment with 0.2 N NaOH for 30 minutes at 65°C. The reactions were neutralized by addition of 0.2 N HCl and cDNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer protocol.

PCR reactions to amplify the ampF-ampG intergenic region were performed using primers PA4392_3junctionRTF and PA4392_3junctionRTR (Table 3) using GoTaq Flexi (Promega Corporation, Madison, WI). PCR reactions to amplify the ampO-ampP overlapping region were similarly performed with the exception that primers PA4218_9junctionRTF and PA4218_9junctionRTR (Table 3) were used. PCR products were analyzed by electrophoresis on a 10% polyacrylamide/1x TBE gel followed by staining with SybrSafe (Invitrogen, Carlsbad, CA).

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Author details

1Department of Biological Sciences, College of Arts and Sciences, Florida International University, Miami, FL USA. 2Department of Molecular Microbiology and Infectious Diseases, Herbert Wertheim College of Medicine, Florida International University, Miami, FL USA.

Authors’ contributions

KKF identified the P. aeruginosa ampG orthologs, PA4218(ampP) and PA4392 (ampG), constructed the ampG and ampP insertional mutants, as well as the lacZ transcriptional fusion strains, performed the β-lactamase and β-galactosidase assays and prepared the first draft of the manuscript. AA constructed and assayed the LacZ and PhoA fusions. LS performed the reverse transcription PCR analysis, determined MICs and assisted with data analysis, figure preparation and wrote the submitted draft of the manuscript. KM conceived of the study, participated in its design and execution and helped in manuscript preparation. All authors read and approved the final manuscript.
