JOURNAL OF PROTEOMICS 96 (2014) 328–342

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LTQ-XL mass spectrometry proteome analysis expands the *Pseudomonas aeruginosa* AmpR regulon to include cyclic di-GMP phosphodiesterases and phosphoproteins, and identifies novel open reading frames

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ARTICLE INFO

Article history:
Received 8 October 2013
Accepted 18 November 2013
Available online 28 November 2013

Keywords:
AmpR
Core Proteome
Antibiotic resistance
Transcriptional regulators
Phosphoproteome
Cyclic di-GMP

ABSTRACT

Pseudomonas aeruginosa is well known for its antibiotic resistance and intricate regulatory network, contributing to its success as an opportunistic pathogen. This study is an extension of our transcriptomic analyses (microarray and RNA-Seq) to understand the global changes in PAO1 upon deleting a gene encoding a transcriptional regulator AmpR, in the presence and absence of β-lactam antibiotic. This study was performed under identical conditions to explore the proteome profile of the *ampR* deletion mutant (PAOΔ*ampR*) using LTQ-XL mass spectrometry. The proteomic data identified ~53% of total PAO1 proteins and expanded the master regulatory role of AmpR in determining antibiotic resistance and multiple virulence phenotypes in *P. aeruginosa*. AmpR proteome analysis identified 853 AmpR-dependent proteins, which include 102 transcriptional regulators and 21 two-component system proteins. AmpR also regulates cyclic di-GMP phosphodiesterases (PA4367, PA4969, PA4781) possibly affecting major virulence systems. Phosphoproteome analysis also suggests a significant role for AmpR in Ser, Thr and Tyr phosphorylation. These novel mechanisms of gene regulation were previously not associated with AmpR. The proteome analysis also identified many unannotated and

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1874-3919/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jprot.2013.11.018

misannotated ORFs in the *P. aeruginosa* genome. Thus, our data sheds light on important virulence regulatory pathways that can potentially be exploited to deal with *P. aeruginosa* infections.

Biological significance

The AmpR proteome data not only confirmed the role of AmpR in virulence and resistance to multiple antibiotics, but also expanded the perimeter of AmpR regulon. The data presented here points to the role of AmpR in regulating cyclic di-GMP levels and phosphorylation of Ser, Thr and Tyr, adding another dimension to the regulatory functions of AmpR. We also identify some previously unannotated/misannotated ORFs in the *P. aeruginosa* genome, indicating the limitations of existing ORF analyses software. This study will contribute towards understanding complex genetic organization of *P. aeruginosa*. Whole genome proteomic picture of regulators at higher nodal positions in the regulatory network will not only help us link various virulence phenotypes but also design novel therapeutic strategies.

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1. Introduction

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, frequently causes life-threatening infections in cystic fibrosis (CF) patients, and several other hospitalized immunocompromised individuals such as those with burn wounds, medical implants, cancer and AIDS [1–4]. The extensive range of infections caused by *P. aeruginosa* is due, in part, to the multitude of virulence factors coded for by its genome. The genome of *P. aeruginosa* strain PAO1 encodes a predicted 5569 open reading frames (ORFs) and remains one of the largest sequenced bacterial genomes on a single chromosome with 36% of hypothetical proteins [5]. While most bacterial pathogens adopt a strategy of genome reduction [6,7], the plasticity of the *P. aeruginosa* genome that allows incorporation of acquired DNA, has enabled the bacterium to thrive in a diverse range of habitats [8]. The genome also aids in clinical setting by encoding numerous virulence factors to establish and maintain an infection, as well as for different antibiotic resistance mechanisms [9,10].

P. aeruginosa is intrinsically primed to evade antibiotics [11]. The membrane impermeability [12,13] and ability to modify drug targets [14], compounded by the expression of multiple efflux pumps [15] and ß-lactamases (AmpC and PoxB) [16,17] makes it a formidable pathogen. The major β-lactamase, AmpC, is a clinically-important, chromosomally-encoded enzyme that mediates resistance to most cephalosporins [16]. In *P. aeruginosa* and Enterobacteriaceae members, AmpR, a LysR-type transcriptional regulator (LTTR) [18,19], induces the expression of *ampC* in the presence of β-lactams [20–23]. In addition to *ampC*, AmpR was previously shown to regulate genes involved in *P. aeruginosa* virulence [24,25]. Recent transcriptome analyses have shown that the *P. aeruginosa* AmpR regulon is quite extensive [26,27]. AmpR microarray studies showed AmpR-regulation of non-β-lactam resistance through MexEF-OprN efflux system, as well as several virulence determinants under quorum sensing control, secretion systems and biofilm formation [26]. Deep sequencing of RNA further revealed the role of AmpR in other processes such as oxidative stress, iron acquisition and heat shock, most of which are mediated by regulation of small RNAs [27]. Importantly, the transcriptome studies reveal that AmpR activates expression of genes associated with acute infection and represses those that control chronic infection phenotypes [26,27]. Thus, it is important to know whether AmpR-mediated differential regulation of genes is also evident at the protein level. The dynamics of the *P. aeruginosa* proteome during exposure to β-lactams and the role of AmpR in the process are yet to be elucidated.

This study describes the shotgun proteomic analysis of the wild type *P. aeruginosa* PAO1 and its isogenic *ampR* deletion mutant, PAOΔ*ampR* in the presence and absence of β-lactam stress, similar to previous transcriptome studies [26,27]. Proteins were identified using two proteomic database search tools namely, Crux [28] and InsPecT [29]. The proteome data further expanded the AmpR regulon to include novel virulence mechanisms. The data also revealed a role for AmpR in protein phosphorylation, and identified several previously unannotated or misannotated ORFs in *P. aeruginosa* genome.

2. Materials and methods

2.1. Bacterial cell culture and primers

P. aeruginosa strains PAO1 [5] and PAOΔ*ampR* [26] were grown and harvested essentially identical to the two transcriptome (array and deep-sequencing of RNA) studies [26,27]. Briefly, the cells were grown to an OD_{600} of 0.8 in LB medium and divided into two pools. To one pool, added 100 μg/mL of benzyl penicillin (induced sample) and a second pool was kept without antibiotic as control (uninduced). The cells were further grown for two hours before harvesting. The culture OD_{600} at that point was ~4.0. The numbers of replicates for each of the conditions were: two each for uninduced and induced PAO1 and three each for uninduced and induced PAOΔ*ampR*, making a total of 10 samples. The sub-inhibitory ß-lactam exposure did not have a significant effect on the growth of PAO1 and PAOΔ*ampR*.

2.2. GeLC-MS analysis of PAO1 and PAOΔampR samples

For each samples, 1 g of cell pellet was resuspended in 5 mL of guanidinium chloride solution (8 M guanidinium chloride, 5% n-propanol, 10 mM NH_4HCO_3 pH 8.6 and 10 mM DTT added fresh) and sonicated at room temperature. The sonicated sample was aliquoted into four Lysing Matrix B tubes (MP

Biomedical; 1 mL per tube) and vortexed for 30 s. The extracts were processed and analyzed as described [30] at the Proteomics facility at the Harvard-Partners Center for Genetics and Genomics. Briefly, each sample was run on a NuPAGE and 10 slices were obtained from each. The protein fragments from each gel slice were characterized using nanoflow high-pressure liquid chromatography (HPLC) in conjunction with microelectrospray ionization on a LTQ XL mass spectrometer (Thermo Fisher Scientific Inc., USA). Henceforth, this whole process is referred to as GeLC-MS analysis.

2.3. Data analysis

The binary RAW files were converted to the generic mzXML format [31] using MakeMS2 (http://proteome.gs.washington.edu/software/makems2/). *P. aeruginosa* PAO1 protein sequences were obtained from the *Pseudomonas* Community Annotation Project (PseudoCAP) [32] and combined with the commonly encountered contaminant protein list available from ftp://ftp.thegpm.org/fasta/cRAP. All pre-and post-processing of text files were carried out using sed, awk and perl scripts [33].

2.4. Database searching and protein identification

An overview of the work-flow is shown in Fig. 1. FASTA files containing the PAO1 protein sequences (5569 entries) and common contaminant sequences (112 entries) were used to generate the forward and shuffled database using *PrepDB.py* and *ShuffleDB.py* for InsPecT (Version 20100804) [34] and using *create-index* command for Crux (Version 1.31) [28]. Iodoacetic acid derivative of cysteine was specified as a fixed modification, while oxidation of methionine, phosphorylation of serine, threonine and tyrosine were specified as variable modifications in both Crux and InsPecT searches.

For Crux, the maximum number of modified methionine was set to two and phosphorylated serine, threonine and tyrosine residues were set to three. For InsPecT, the maximum number of variable modifications was set to three. The instrument type was set to *ESI-ION-TRAP* for InsPecT, all other parameters were default for both Crux and InsPecT database searches. The output files from Crux *search-for-matches* were filtered using Percolator [35] to determine the peptide spectrum matches (PSM) having minimal false discovery rate. Only those PSM that fulfilled the criteria of $q < 0.01$ [35] were chosen. Search results from InsPecT were filtered using *PValue.py* and only PSM with $p < 0.01$ were chosen. In InsPecT, *Summary.py* was used to generate a subset of identified protein database (1574 entries) to search against, for identifying phosphorylation modifications. The results were further analyzed using *PhosphateLocalization.py* script to filter post-translational modifications (PTM) based on phosphate localization score (PLS) and only those PTM having PLS > 20 were considered.

For determining novel proteins, the PseudoCAP [32] annotated intergenic regions of the PAO1 genome were translated in all six frames using the EMBOSS [36] command transeq, combined with the common contaminants FASTA file and was used for searching the tandem mass spectra using Crux and InsPecT as described above. Data analysis using a third

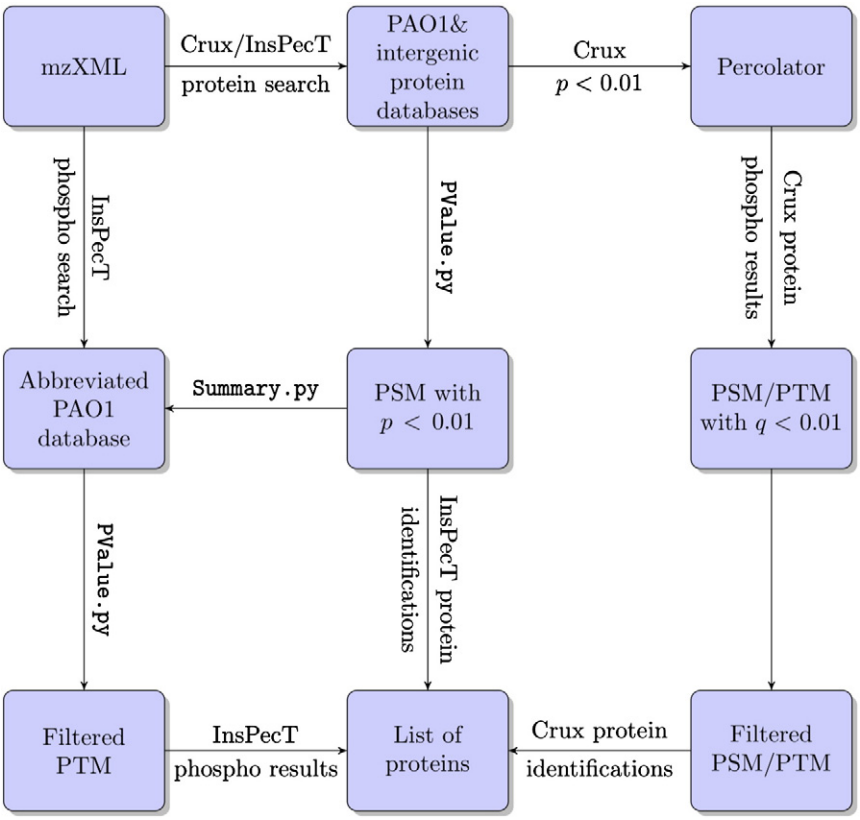

Fig. 1 – Overview of the work-flow for proteome analysis using Crux and InsPecT. Details of the analysis are given in material and methods section 2.4. PSM, Peptide Spectrum Matches; PTM, Post-Translational Modifications.

program, BioWorks (Ver 3.3.2) generated data similar to Crux (data not shown) and so was not used further.

2.5. Distribution and functional enrichment analysis

Functional categorization of all the identified proteins followed that of the PseudoCAP [32]. Gene distribution under individual functional categories was plotted as percentage of query genes vs. percentage of PAO1 genes in each category.

Enrichment analysis: Functional enrichment analysis was performed for both the total proteome dataset and AmpR-regulated proteins. Enrichment of a particular functional category in the total proteome data was assessed by comparing the percentage of proteins in that category in the proteome dataset with its percentage distribution in PAO1. A higher or lower percentage compared to PAO1 signified enrichment (E) or underrepresentation (U), respectively. The significance of E or U was determined by *p*-values, computed by hypergeometric distribution. A conservative-threshold of 0.05/N (N = 26, number of functional categories) was chosen to account for multiple hypotheses setting. Thus, a p-value ≤ 0.002 was considered significant.

Similarly, the protein datasets regulated by AmpR in the absence and presence of ß-lactam were also tested for E/U of specific functional categories by comparing percentage of proteins in a particular functional category in each AmpR-dependent dataset against percentage of that category in the total proteome dataset.

2.6. RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

Total RNA was isolated from PAO1 and PAOΔ*ampR* following the same culture condition as for the proteome assay. The antibiotic was added to the cells grown to mid-log phase, and the cells with or without antibiotic exposure were further incubated for one hour before harvesting in the stationary phase. We had previously demonstrated that the gene expression trend is the same, whether we exposed the cells for 40 min or for two hours [26]. RNA isolation, cDNA synthesis and qPCR assays were performed as described earlier [26]. Ten nanograms of cDNA were used per reaction well in the qPCR assays. The *clpX* gene (*PA1802*) was used as an internal control to ensure equal amounts of RNA were used in all samples. Assays were performed at least in biological triplicates, each with technical triplicates, for every gene analyzed. Melt curves were determined to ensure primer specificity. Gene expression in PAOΔ*ampR* was normalized to the corresponding PAO1 values, for both the uninduced and ß lactam induced conditions, and is presented as fold-expression in PAOΔ*ampR*. All data were analyzed for statistical significance using *t*-test on GraphPad statistical analysis software. Primers used for the qPCR analysis are listed in Supplementary Table 1.

2.7. Verification of unannotated ORFs

The basic alignment search tool [37,38] was used to map identified peptides to ORFs not previously annotated in the PseudoCAP [32]. To verify that the putative ORF was expressed, reverse transcriptase PCR (RT-PCR) was performed on RNA isolated from PAO1. Briefly, PAO1 RNA was isolated and cDNA was synthesized as described earlier [26]. As a control, the cDNA synthesis was also performed in the absence of reverse transcriptase enzyme and the sample was processed along with the cDNA. Using cDNA as template, putative ORFs were amplified using primers listed in Supplementary Table 1. The amplification products were analyzed using standard DNA gel electrophoresis.

3. Results and discussion

3.1. GeLC-MS analysis of *P. aeruginosa* proteome

It has been well-established that the β-lactam antibiotic exposure results in the AmpR-induced expression of *ampC* encoding a β-lactamase in *P. aeruginosa* and many *Enterobacteriaceae* members [23,24]. Further, transcriptome studies and complementary assays demonstrated that the AmpR regulon is extensive and includes additional genes involved in antibiotic resistance, virulence and metabolism [24–27]. However, the transcriptional profile does not necessarily reflect proteomic profile due to post-transcriptional regulation. In addition, transcriptome analyses [26,27] could have missed key AmpR-regulated targets. In order to complement the previous transcriptomic studies [26,27] to determine the global regulatory role of AmpR, proteomic analyses of PAO1 and PAOΔ*ampR* were performed using GeLC-MS.

PAO1 and PAOΔ*ampR* cells were grown under identical conditions as in our previous transcriptome (microarray and RNA-Seq) studies in the presence or absence of β-lactam [26,27]. The total cell lysate was separated by SDS-PAGE and the MS–MS spectra for each gel slice were obtained as described under materials and methods. Analysis of the GeLC-MS data was performed using two different tools, Crux [28] and InsPect [29]. Crux uses a modified SEQUEST algorithm [39], whereas InsPecT uses a combination of database searching and de novo peptide tag-based filtering algorithms [29]. It should also be noted that the mere absence of a protein in a group does not mean that the protein is not expressed, but that it was not detectable in that condition using our proteomic methodology.

The list of genes encoding the non-redundant peptides found in the GeLC-MS analysis by Crux and InsPecT is given in Supplementary Table 2. These analyses led to the identification of 2965 non-redundant proteins in all of the samples analyzed (Supplementary Table 2). A total of 1302 proteins were detected by both the analytic tools, whereas 15 and 1648 proteins were exclusively identified in Crux and InsPecT, respectively.

Our proteome data thus identified 53% of the total PAO1 encoded proteins. Previous proteome studies in *P. aeruginosa* identified between 1% and 30% of total proteins [40–44]. The number of proteins identified in the current analysis is significantly higher than the previous studies. The widely varying results between the studies can potentially be explained by the differential sensitivities of the various analytical techniques (iTraq, MudPit, 2-DE), the strains (PAO1 and isogenic mutants, PA14, AES-1R) and the media (LB, PIA, CF media) used.

3.2. *Overall classification of identified proteins based on their functional categories*

In order to find out if the proteins identified by GeLC-MS analysis are uniformly distributed or skewed in some specific categories, functional categorization of the 2965 non-redundant proteins was followed as that of PseudoCAP [32].

For each functional category (Categories a to z), the percentage of ORFs in the PAO1 database was compared to the percentage of gene products in that category identified in the proteome data (Fig. 2; Supplementary Table 3). There was an overall good representation of the genes under various functional categories in our proteome data, compared to the distribution in PAO1 (Fig. 2). The largest number of proteins identified in our analysis (31%) belonged to the hypothetical, unclassified or unknown class (Category c), which makes up 36% of the PAO1 genome. Thus, the proteins identified in Category c in our proteome data are no longer hypothetical. The next highest number of proteins identified belonged to putative enzymes (Category o), membrane proteins (Category d) and transcriptional regulators (Category l) in both PAO1 (8.1, 11.5 and 7.8%, respectively) and proteome data (8.4, 7.0 and 6.8%, respectively; Fig. 2; Supplementary Table 3).

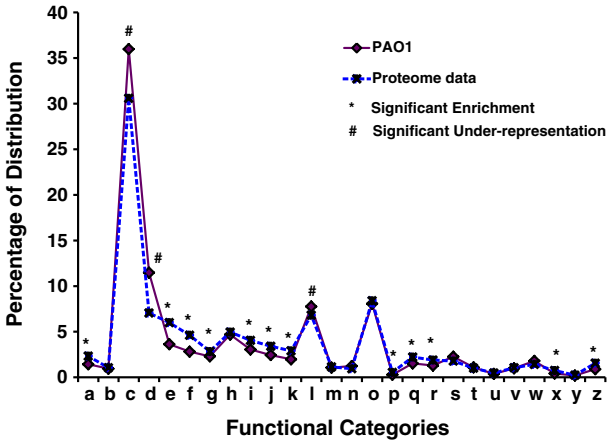

Fig. 2 – Functional categorization of the total proteins identified in GeLC-MS analysis by PseudoCAP [32]. All the non-redundant proteins (2965) identified by Crux and InsPecT were functionally categorized and plotted as percentage distribution of each category in PAO1 (purple) vs. proteome data (blue). Hypergeometric analysis was performed to determine significantly enriched (*) or under-represented (#) categories (*p*-values of ≤0.002). The functional categories are (a) DNA replication, recombination, modification and repair; (b) fatty acid and phospholipid metabolism; (c) hypothetical; (d) membrane proteins; (e) amino acid biosynthesis, metabolism; (f) translation, post-translational modification, degradation; (g) cell wall/LPS/capsule; (h) transport of small molecules; (i) energy metabolism; (j) biosynthesis of cofactors, prosthetic groups, carriers; (k) adaptation, protection; (l) transcriptional regulators; (m) two-component regulatory systems; (n) secreted factors — toxins, enzymes, alginate; (o) putative enzymes; (p) chaperones, heat-shock proteins; (q) central intermediary metabolism; (r) nucleotide biosynthesis and metabolism; (s) carbon compound catabolism; (t) motility and attachment; (u) chemotaxis; (v) related to phage, transposon, plasmid; (w) protein secretion, export apparatus; (x) cell division; (z) transcription, RNA processing, degradation.

The percentage of ORFs in each functional category in proteome data and PAO1 genome was compared to determine if there is under/over representation of a particular category. The significance of enrichment (E) or under-representation (U) was determined using hypergeometric distribution *p*-value (Supplementary Table 3). Significant enrichment was seen for proteins involved in vital cellular functions (* in Fig. 2; Supplementary Table 3) such as DNA replication, recombination, modification and repair (Category a), cell division (Category x), transcription, RNA processing and degradation (Category z), amino acid biosynthesis and metabolism (Category e), translation (Category f), nucleotide biosynthesis and metabolism (Category r), central intermediary metabolism (Category q), biosynthesis of cofactors (Category j), energy metabolism (Category i) and cell-wall/LPS (Category g). In addition, it was not surprising that the proteins in the categories of adaptation and protection (Category k) and chaperones and heat shock proteins (Category p) were over-represented because exposure of cells to ß-lactam antibiotic results in stress [45].

The significantly under-represented proteins (# in Fig. 2; Supplementary Table 3) belonged to the functional classes of hypothetical (Category c), membrane proteins (Category d) and transcriptional regulators (Category l). Although not statistically significant, substantially reduced number of proteins involved in protein secretion apparatus (Category w), and carbon compound metabolism (Category s) were detected (Supplementary Table 3). Expression of many of these proteins is condition-specific, thus low representation of these functional groups is expected as they may not be expressed under the experimental condition used in the study.

3.3. *Identification of AmpR- and AmpR-ß-lactam-dependent proteins*

The GeLC-MS data identified numerous proteins expressed under individual conditions. In the absence of ß-lactam exposure, the analyses identified 2139 and 2267 proteins in PAO1 and PAOΔ*ampR*, respectively. In the presence of ß-lactam, the PAO1 and PAOΔ*ampR* expressed 2052 and 2150 proteins, respectively (Table 1). However, there can be potential overlaps in the proteins that are expressed under different conditions. Hence, these numbers do not reflect the true difference between

Table 1 – Summary of proteins identified in each sample condition by GeLC-MS analysis.

Sample	β-lactam	Crux	InsPecT	Combined
PAO1	–	848	2109	2139
	+	990	2029	2052
PAOΔ*ampR*	–	1003	2246	2267
	+	552	2011	2150
Total		1317	2950	2965

The number of proteins present in each condition tested identified by either the Crux or InsPecT algorithms is indicated. The number of unique proteins identified with either algorithm is summarized in the combined column.

β-lactam or AmpR-dependent proteins. In order to determine the exclusively AmpR-dependent proteins in the presence and absence of β-lactam antibiotics, a four-way Venn diagram was plotted (Fig. 3). The complete list of identified proteins in each of the Venn groups is given in Supplementary Table 4.

The proteins expressed under all conditions are found in Group O (1433) and, most likely, are part of the core proteome (discussed in the following section). The 585 proteins in Groups G, H, K, L, M and N were eliminated from further analysis, as they could not be assigned to any one class unequivocally. Thus, of the remaining 947 proteins, we identified a total of 491 AmpR-dependent proteins (Groups A, E, C and J, Fig. 3), 362 proteins that are AmpR-dependent in the presence of ß lactam (Groups B and D, Fig. 3), and 94 β-lactam dependent proteins (Groups F and I, Fig. 3).

The 207 proteins found in Groups A and E are positively regulated by AmpR, since they are produced only in the presence of *ampR* and not in any other condition. Similarly, AmpR negatively regulates the proteins present in Groups C and J (284 proteins), since they are produced only in the absence of *ampR*. A similar logic was applied to identify 106 positively regulated and 256 negatively-regulated proteins that are AmpR-dependent only under ß-lactam stress (Groups B and D, Fig. 3).

3.4. *Proteins expressed under all conditions: core proteome*

Of the 2965 proteins identified, 1433 proteins (26% of PAO1 genome) were detected in all four conditions irrespective of the presence of AmpR or antibiotic (Group O; Fig. 3). Many of the previously identified AmpR-regulated ORFs such as AmpC, LasR, RhlR, the MexAB-OprM efflux pump, and some Psl proteins were present in Group O. This is probably because in addition to the housekeeping proteins that are expected to be expressed under all test conditions, Group O is also likely to contain AmpR-regulated proteins that have a basal-level of expression (higher or lower expression in *ampR* mutants compared to PAO1). The reason could be that our current proteome analysis, unlike the previous transcriptome analyses, is not quantitative and will

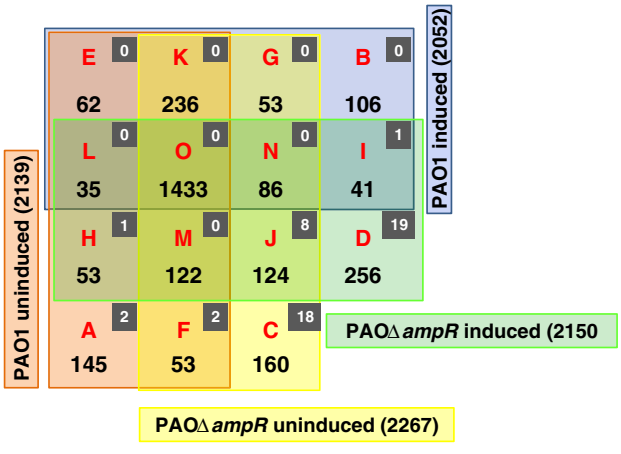

Fig. 3 – Venn diagram of proteins identified in different samples. Distribution of non-redundant proteins identified in PAO1 and PAOΔ*ampR* without (uninduced) and with (induced) sub-MIC ß-lactam. Distribution of phosphoproteins identified in each group is shown in the inset (gray squares).

not detect changes in protein levels between the conditions. Thus, AmpR potentially regulates subsets of the proteins that are detected in Group O. Another possibility is that the basal level expression of the proteins ensures a rapid response to the different stress conditions.

As one would expect, many genes of Group O are involved in cellular metabolism and ribosome biosynthesis (Supplementary Table 4). Accordingly, functional categorization and hypergeometric distribution analysis (Supplementary Table 5) revealed a significant enrichment in categories e (amino acid biosynthesis and metabolism), f (translation, post translation and modification), k (adaptation, protection), q (central intermediary metabolism), r (nucleotide biosynthesis and metabolism), x (cell division) and z (transcription, RNA processing and degradation). As expected many of the proteins involved in murein biosynthesis (MurA, MurC, MurD, MurE, MurF, MurG, MurI and MraW), the peptidoglycan formation (PBP1A, PBP1B, PBP2, PBP3, PBP5 and PBP6), the cell wall shape (EnvA/LpxC, EnvB, and LpxD) and others (AmpDh3, Mp1, MltA, AmiB and LdcA) were detected in Group O (Supplementary Table 4).

The characterized proteins in the adaptation and protection category (k), which was significantly enriched, include OstA, Ohr, AmpC, HtpX, SodB, KatA, LasR, RhlR, Lon, PpkA, CheZ, and several chemotactic transducers. In addition, proteins of the major RND efflux pumps MexAB-OprM, MexEF-OprN and its regulator MexT, and TriABC were also detected under all conditions (Supplementary Table 4).

Among the functional categories significantly under-represented in Group O were categories c (hypothetical), d (membrane proteins), l (transcriptional regulators) and m (two-component regulatory systems). A low representation of these in the core set is probably due to their condition-specific expression. The outer membrane protein category is under-represented and the proteome analysis detected the following characterized members: OprD, OprH, OprF, OprQ, OprI, Opr86, OprC, OprG, OpdO, OprL, OstA and IcmP. The analysis also detected the sigma factors, RpoS, RpoD, RpoN, AlgU/T and FliA and global regulators, Anr, Vfr, Dnr, Crc, GacA and Hfq (Supplementary Table 4).

A major adaptive phenotype of *P. aeruginosa* during chronic infection is the production of alginate. This study detected the following alginate-specific regulatory proteins: AlgU, MucA, MucB, MucC, MucD, AlgO/Prc, AlgW, AlgP, AlgR, AlgC, AlgB, and Ndk [46–49]. However, none of the proteins from *algD* operon involved in alginate biosynthesis [50] were detected in this category (Supplementary Table 4). Previous studies have demonstrated that genes of the AlgT/U regulon are expressed to deal with cell envelope homeostasis [51–53]. However, our proteome data suggests that the regulators of this system may have additional roles, since the proteins are detected in all four conditions.

3.5. *Analysis of AmpR-dependent proteins (independent of ß-lactam)*

The functional categorization and enrichment analysis of AmpR-dependent proteins in the absence of β-lactam antibiotics was done as described earlier. In the protein set positively regulated by AmpR (Groups A and E, Fig. 3; Supplementary Table 6), significant enrichment was seen only in Category c

(the hypothetical, unclassified, unknown), which accounts for 46% of the proteins in this group. Only Category f proteins (translation, post-translational modification and degradation) were found to be significantly under-represented (Supplementary Table 6).

The proteins that are positively regulated by AmpR (Supplementary Table 7) include major virulence determinants, such as the LasA protease (PA1871), the alkaline protease secretion protein AprD (PA1246), and the phospholipase PlcB (PA0026). The proteins LasA, PlcB and AprD are under QS regulation [54,55]. The QS process is positively regulated by AmpR [26,27], supporting the proteome findings. The proteome data also identified modulators of cyclic di-GMP (c-di-GMP) levels such as BifA (PA4367), CdpA (PA4969), TpbB (PA1120) to be under AmpR-regulation (Supplementary Table 7). The role of AmpR in c-di-GMP signaling is discussed in a later section. Two other proteins in the AmpR-dependent group are the sensor kinases of two TCSs, RoxS (PA4494) and RocS2 (PA3044). The RoxSR TCS plays a crucial role in attachment of *P. aeruginosa* to the epithelial cell surface to initiate the infection process [56]. The RocS2A2 TCS is a major regulator of fimbrial gene expression, affecting the attachment process to host cell surfaces [57]. AmpR-dependent expression of these two proteins suggests a role for AmpR in the establishment of infection and warrants further investigation.

Among the proteins negatively regulated by AmpR (detected in the absence of *ampR*; Groups C and J, Fig. 3; Supplementary Table 6), proteins in Categories m and n corresponding to two-component regulatory systems and secreted factors, respectively, were significantly enriched. Proteins in Categories e (amino acid biosynthesis and metabolism) and f (translation) were under-represented.

AmpR was previously shown to negatively regulate chronic infection phenotypes, such as biofilm formation [26]. The type VI secretion system (T6SS) is one of the attributes of chronic infection [58]. In accordance with this data, the proteome analysis shows negative regulation of proteins of the T6SS Tse3 (PA3484), TssJ1 (PA0080) and TssG1 (PA0089; Supplementary Table 7). Similarly, AmpR negatively regulates BfiS (PA4197) and BfiR (PA4196), which play critical roles in biofilm formation [59], and PelG (PA3058), a biofilm matrix protein involved in pellicle formation [60]. These findings add further support to our previous data [26,27].

3.6. AmpR regulates some proteins only under ß-lactam exposure

The proteins that were expressed only in the presence of AmpR and β-lactam (Group B, Fig 3; Supplementary Table 6) were not enriched for any functional category. However, there was a significant under-representation of proteins involved amino acid biosynthesis and metabolism (Category e). Although not statistically significant (p-value ≤ 0.002), this group had a large number of transcriptional regulators (13%; p-value of 0.007). This group included an ECF sigma factor VreR (PA0676), a positive regulator of *P. aeruginosa* virulence [61], and KynR (PA2082), an activator of the kynurenine pathway for anthralinate (a PQS precursor) biosynthesis [62]. These findings agree with previous studies demonstrating that AmpR is a positive regulator of many acute virulence factors, including those regulated by the PQS system [26,27]. AmpR also positively regulates two metabolic regulators — PcaQ (PA0152), a homolog of a phenolic compound catabolism regulator in *Agrobacterium tumefaciens* [63], and BkdR (PA2246) that is involved in amino acid metabolism in *P. putida* [64]. BkdR is also regulated by Crc (PA5332) [65]. Since both AmpR and Crc have previously been demonstrated to regulate metabolism [26,66], there is a potential interplay between these regulators and needs further investigation.

The proteins that are expressed only in the absence of AmpR and ß-lactam (Group D, Fig. 3) were significantly enriched in Categories d (membrane proteins), and l (transcriptional regulators), whereas under-represented in Categories e (amino acid biosynthesis and metabolism) and q (central intermediary metabolism; Supplementary Table 6). Proteins whose synthesis was negatively regulated by AmpR in the presence of ß-lactam (Supplementary Table 7) encompassed those involved in cofactor biosynthesis (CobC/PA1276, CobD/PA1275 and CobV/PA1281) and proteins involved in antibiotic resistance (MexXY/PA2018-19, ArnT/PA3556, Aph/PA4119 and MexD/PA4598). The *cob* genes are part of an 11-gene operon that is involved in biosynthesis of the cofactor cobalamin [32]. Cobalamin has been demonstrated to enhance growth under anaerobic conditions and suppress biofilm formation in *P. aeruginosa* [67]. The MexXY RND efflux system confers resistance to aminoglycosides and macrolides [68,69]. Aph (PA4119) is an aminoglycoside phosphotransferase [70], which is encoded as part of a two-gene operon along with a transcriptional regulator PA4120 [32]. It is interesting to note that AmpR negatively regulates expression of aminoglycoside resistance proteins in response to ß-lactam stress. This is not very surprising since one would expect up-regulation of resistance to ß-lactam and not to aminoglycoside in response to ß-lactam exposure. However, there was no differential regulation of the *mexXY* or *aph* in our transcriptome data [26,27]. The current proteome analysis suggests potential AmpR-mediated regulation of aminoglycoside resistance, further expanding the AmpR regulon. This was further confirmed by a differential susceptibility profile for aminoglycosides, amikacin and tobramycin, observed between PAO1 and PAOΔ*ampR* (data not shown).

Furthermore, upon ß-lactam exposure, AmpR negatively regulates additional proteins involved in chronic infection phenotype such as those involved in chaperone–usher pathway, CupB2 (PA4085) and CupB3(PA4084); alginate regulation, AlgZ/FimS (PA5262); biofilm formation, MifS (PA5512), PslG (PA2237) and PslJ (PA2240); and the T6SS proteins, TssE1 (PA0087) and Tse2 (PA2702; Supplementary Table 7). The negative regulation of *cupB2* by AmpR in the presence of β-lactam was confirmed by qPCR (2 fold, p-value 0.004). The proteome data thus, supports the role of AmpR as a positive and negative regulator of acute and chronic infection phenotypes, respectively.

An interesting observation is downregulation of HacB (PA0305), an acyl homoserine lactone acylase by AmpR. HacB degrades AHL molecules and serves to quench QS signaling [71]. Thus, AmpR not only activates QS genes by controlling expression of major QS regulators [27] but also downregulates QS quenchers.

3.7. AmpR regulates c-di-GMP signaling

C-di-GMP-mediated signaling plays a critical role in determining *P. aeruginosa* pathogenesis [72,73]. Intracellular c-di-GMP

levels are modulated by diguanylate cyclases (DGCs) containing GGDEF domains, and phosphodiesterases (PDEs) containing EAL domains [54] that synthesize and degrade c-di-GMP, respectively. The *P. aeruginosa* genome encodes 39 proteins that are capable of modulating c-di-GMP levels [32,74]. The proteome data suggests that two of these PDEs, BifA (PA4367) and CpdA (PA4969) were positively regulated by AmpR. qPCR analyses concurred with the proteome data: *bifA* (−2.7 fold, *p*-value 0.0003), *cpdA* (−2.5 fold, *p*-value 0.0001). Another PDE, PA4781 was determined to be under negative AmpR-regulation in the proteome data. However, *PA4781* expression was positively regulated by AmpR, as seen in the qPCR analysis (−2.0 fold, *p*-value 0.0003). Elevated c-di-GMP levels in the cell positively regulate chronic infection phenotypes, such as biofilm formation [75]. *P. aeruginosa* AmpR negatively regulates biofilm formation [26] and one possible mode is by regulating PDE gene expression.

3.8. AmpR regulon includes transcriptional regulators

P. aeruginosa AmpR has an extensive regulon. We hypothesized that gene regulation by AmpR may, in part, be mediated by intermediate transcriptional regulators. Accordingly, previous transcriptome analyses identified 22 [26] and 66 [27] transcriptional regulators under AmpR regulation. The proteome data identified 102 putative transcriptional regulators (~24% of transcriptional regulators encoded by PAO1) to be AmpR-dependent under different categories (Supplementary Tables 6 and 7). However, the specific role for most of these is yet to be elucidated [32,76]. Eighteen of the AmpR-regulated transcriptional regulators identified by proteome analysis overlapped with either of the transcriptome studies (Table 2). Many of these regulators have no assigned role. The known regulators include PrtN (PA0610) and PrtR (PA0611), which are part of the regions of genome plasticity in *P. aeruginosa* [8] and regulate pyocin production to confer a survival advantage [77]. ParR (PA1799), a transcriptional regulator of the ParRS TCS, contributes to adaptive resistance to polymyxin B and colistin by activating the *arn* operon involved in LPS modification [78]. AmpR-mediated regulation of ParR, identified in the RNA-Seq and proteome studies, expands the role of AmpR in non-β-lactam resistance [26].

One transcriptional regulator that was found to be AmpR-regulated in all three studies is PA2588 (Table 2). PA2588 is flanked by PqsH (PA2587), which is involved in synthesis of the PQS signaling molecule [79], and a two-gene operon (PA2589–PA2590) that is potentially involved in iron uptake [32]. AmpR is known to positively regulate both the iron uptake and PQS-mediated QS, affecting diverse virulence phenotypes [27]. The role of PA2588 in *P. aeruginosa* is not yet known, but given the genomic location and the potential role of AmpR in its regulation, it warrants further study.

3.9. Phosphoproteome analysis

Post translational modifications (PTM) are beginning to be widely accepted in microbial systems not only as means of regulation [80] but also in the control of protein–protein interactions [81]. Recent evidence suggests that apart from the previously known histidine/aspartate phosphorylation, the phosphorylation of serine, threonine and tyrosine residues is common in bacteria and is no longer exclusive to eukaryotes [82,83]. In *P. aeruginosa*, Ser/Thr/Tyr phophorylations have been shown to regulate virulence factor production [84,85]. Analysis of bacterial phosphoproteomes has traditionally been less common than proteome analysis since the identification of PTM tremendously increases the time required for database searching.

We used InsPecT to determine if there are phosphorylation modifications in any of the identified proteins from *P. aeruginosa* (Table 3). InsPecT identified a total of 51 proteins with 52 unique phosphorylation modifications (PLS > 20, *p*-value < 0.05) (Table 3). The cell division protein ZipA (PA1528) was phosphorylated on serine at two different positions (Table 3). Out of the 52 phosphorylation sites, 24 (46.2%) were on the Ser, 17 (32.7%) were on the Thr and 11 (21.2%) were on Tyr.

Table 2 – AmpR-regulated transcriptional regulators overlapping with Array and RNA-Seq analysis.

PA #	Gene name	Product name	Array	RNA-Seq	Proteome
PA0601		Probable two-component response regulator	–	+	+
PA0610	*prtN*	Transcriptional regulator PrtN	+	–	+
PA0611	*prtR*	Transcriptional regulator PrtR	+	–	+
PA1142		Probable transcriptional regulator	–	+	+
PA1359		Probable transcriptional regulator	–	+	+
PA1799	*parR*	Two-component response regulator, ParR	–	+	+
PA1961		Probable transcriptional regulator	–	+	+
PA2588		Probable transcriptional regulator	+	+	+
PA2718		Probable transcriptional regulator	–	+	+
PA2877		Probable transcriptional regulator	–	+	+
PA3027		Probable transcriptional regulator	–	+	+
PA3034		Probable transcriptional regulator	–	+	+
PA3077		Probable two-component response regulator	–	+	+
PA3630		Probable transcriptional regulator	–	+	+
PA4145		Probable transcriptional regulator	–	+	+
PA4781		Cyclic di-GMP phosphodiesterase	+	–	+
PA4983	*dmsR*	Probable two-component response regulator	–	+	+
PA4987		Probable transcriptional regulator	–	+	+

Presence and absence of a AmpR dependent transcriptional regulator is depicted by (+) and (–), respectively.

Interestingly the distribution of phosphorylated proteins varied greatly in PAOΔ*ampR* samples. While PAO1 samples showed only five (uninduced) and one (induced) phosphorylated peptides, 28 (uninduced) and 29 (induced) phosphopeptides were detected in PAOΔ*ampR* samples. Owing to the potential overlap between the peptides detected in the four conditions, a four way Venn-diagram was constructed to identify AmpR-dependent phosphorylations (inset, Fig. 3). Using the same logic as for the AmpR-dependent proteins (Fig. 3), phosphorylation of two proteins were positively regulated (Fig. 3, A and E), and 26 were negatively regulated by AmpR (Fig. 3, C and J). AmpR negatively regulated phosphorylation of another 19 in the presence of β-lactam (Fig. 3, D). The list of AmpR-dependent phosphorylated proteins is given in Supplementary Table 8. Phosphorylations under negative AmpR-regulation included proteins involved in energy utilization such as SucC (PA1588),

Table 3 – List of identified phosphopeptides from PAO1 and PAOΔ*ampR* samples in the presence and absence of β-lactam antibiotic.

PA #	Protein description	Phosphopeptide
PA0389	Hypothetical protein	R.HGWASphosRLWPNLLGEIGIYR.V
PA0427	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor	R.AAFFPSISLTANAGTMSphosR.Q
PA0437	Cytosine deaminase	K.ALLSphosHEDVKQRAWQTLK.W
PA0620	Probable bacteriophage protein	K.GRVTAGMALAATDIPGLDASphosK.L
PA0943	Hypothetical protein	K.QMPISphosGNASR.S
PA1174	Periplasmic nitrate reductase protein NapA	K.GKTLYphosDVLFRNGQVDR.F
PA1206	Hypothetical protein	K.QAYIAMDVETphosIATIR.D
PA1528	Cell division protein ZipA	R.DESphosGFKGPALLQNILESGLR.F
PA1528	Cell division protein ZipA	K.LKFKLDRSFANLPDDDGDSphosAELLGPAR.V
PA1544	Transcriptional regulator Anr	R.FRARGFSAQQFRLAMSphosR.N
PA1588	Succinyl-CoA synthetase beta chain	K.ILVESCTDIDKELYphosLGAVVDRSSRR.I
PA1589	Succinyl-CoA synthetase alpha chain	R.SLADIGKALAELTphosGWEVK.K
PA1805	Peptidyl-prolyl cis-trans isomerase D	K.GEDFAALAKEFSphosQDIGSAATGGDLGYAGR.G
PA2015	Putative isovaleryl-CoA dehydrogenase	R.AYLYphosAVAAACDRGETTRK.D
PA2229	Conserved hypothetical protein	R.CHPDWSLLRLSphosEVLFDR.R
PA2291	Probable glucose-sensitive porin	K.MSphosGSGTKGALLPVELIWQPK.V
PA2304	AmbC	R.NYRAGLGLSphosWREAFQTDSR.A
PA2462	Hypothetical protein	K.GQTDETphosVRQSQIVAQGNLAIK.A
PA2492	Transcriptional regulator MexT	R.TphosLFDDPLFVRTGR.S
PA2735	Probable restriction–modification system protein	K.YphosRDVILPFTVLR.R
PA2744	Threonyl-tRNA synthetase	K.KEAADFIKLTLQVYphosR.D
PA2950	Hypothetical protein	R.ADYphosKELQPEVQSRVEELWDK.V
PA3040	Conserved hypothetical protein	R.GKIHDSLKRARDTphosLR.D
PA3168	DNA gyrase subunit A	K.GQQLISphosMLIPESGAQILTASER.G
PA3227	Peptidyl-prolyl cis-trans isomerase A	R.NGFADVPSphosDDVVILSAKR.L
PA3313	Hypothetical protein	K.ELKVSphosAIPDEAPTELLR.K
PA3552	ArnB	K.NLTphosCAEGAMFVSDDSALAERVR.R
PA3567	Probable oxidoreductase	R.DLLVEVRAISVNPVDTphosK.V
PA3659	Probable aminotransferase	R.CQILFLCSphosPGNPTGALVPLETLK.K
PA3700	Lysyl-tRNA synthetase	R.YphosPFEVSPLARR.N
PA3728	Hypothetical protein	R.HRFSVNTQELDLTphosLMPR.G
PA3796	Hypothetical protein	K.LTphosPDGQAPQGDLDIGSLLAR.F
PA3817	Probable methyltransferase	R.LYGRSAISphosKLEMNILR.G
PA4251	50S ribosomal protein L5	K.ITphosGQKPVVTYARK.S
PA4256	50S ribosomal protein L16	R.GSphosKVSFGEYALKATSRGR.L
PA4414	UDP-N-acetylmuramoylalanine-D-glutamate ligase	R.YphosLARRGLPFAVVDTR.E
PA4448	Histidinol dehydrogenase	R.SYHEKQKQGSphosWR.Y
PA4496	Probable binding protein component of ABC transporter	K.AKIVTYEWGEYphosIKR.A
PA4576	Probable ATP-dependent protease	R.AFNQRYDRALDSphosVERR.A
PA4700	Penicillin-binding protein 1B	R.SRNSKARPAPGLNKWLSphosWALK.L
PA5022	Conserved hypothetical protein	K.MTphosPTLLKNQLTEIPWGSGVR.E
PA5044	Type 4 fimbrial biogenesis protein PilM	R.RYphosGLSVEEAGLAKK.Q
PA5050	Primosomal protein N′	R.LALPSPLRRLFDYphosRAPR.G
PA5171	Arginine deiminase	R.KAGVEVITphosISASELGR.G
PA5194	Hypothetical protein	K.ASGWLVQVTphosEPLFR.L
PA5232	Conserved hypothetical protein	K.TVETphosANEREKLMFR.V
PA5240	Thioredoxin	K.DGNVEATKVGALSphosK.S
PA5345	ATP-dependent DNA helicase RecG	R.RRSphosLLVRLQDGSGTLSLR.F
PA5372	Choline dehydrogenase	R.GRPNLTIVTphosHALSDR.I
PA5492	Conserved hypothetical protein	R.HPLTphosDFDRLMLDWAQASQLPIHVLMTK.A
PA5497	Class II (cobalamin-dependent) ribonucleotide-diphosphate reductase subunit, NrdJa	R.IRGSphosVLHAKYSRYMQR.V
PA5556	ATP synthase alpha chain	R.NEGTphosIVSVSDGIVR.I

SucD (PA1589), a transcription regulator Anr (PA1544), outer membrane protein OprM (PA0427) involved in the antibiotic efflux, proteins involved in translation including PpiA (PA3227), RplE (PA4251), and RplP (PA4256). Anr is an important regulator of anaerobic growth and biofilm formation in *P. aeruginosa* [86,87]. AmpR-mediated negative phosphorylation in the presence of β-lactam stress included proteins involved in antibiotic resistance such as a transcriptional regulator MexT (PA2492), and proteins involved in cell wall synthesis such as a penicillin binding protein MrcB (PA4700) and MurD (PA4414; Supplementary Table 8). AmpR has been previously demonstrated to negatively regulate expression of *mexT* using qPCR, and consequently, the functioning of the MexEF-OprN efflux pump [26]. This could potentially be mediated by differential phosphorylation of MexT. However, at this point, it is not clear how phosphorylation affects the activity of these proteins.

3.10. *Unannotated gene analysis*

High-throughput proteomic data have been successfully employed to aid microbial genome annotation [88–91]. Having identified proteins that are expressed under different conditions such as antibiotic stress and the presence or absence of AmpR, we then asked the question if our tandem mass spectra could provide clues to correct potentially misannotated ORFs in the PAO1 genome. The tandem mass spectra were searched against a database containing protein sequences resulting from all possible six-frame translations of all the intergenic regions in the PAO1 genome as described in materials and methods. The hypothesis is that if the so-called intergenic regions contain protein-encoding genes, peptide sequences corresponding to these proteins should be identified in the database search. Crux identified 30 peptides belonging to 13 intergenic regions, while InsPecT identified 218 peptides belonging to 57 intergenic regions.

Based on the sequence analysis with PAO1 and other sequenced *P. aeruginosa* strains (PA7, PA14, LESB58), primers were designed to test nine intergenic regions by reverse transcriptase polymerase chain reaction (RT-PCR; Supplementary Table 1). Our results suggest that at least 9 novel, unannotated conserved ORFs exist in the PAO1 genome that are misannotated as intergenic regions (Table 4, Fig. 4). All of previously unannotated novel ORFs were tested by RT-PCR (Supplementary Fig. 1). Additionally, 11 peptides were mapped to intergenic regions that were in-frame with the downstream annotated ORF (Table 5), suggesting that the putative start codon was erroneously annotated in the Pseudomonas database. One of these, PA3732, was tested by RT-PCR (Supplementary Fig. 1). The new proposed coordinates for these misannotated ORFs based upon the peptides are listed in Table 5. All the proposed new ORFs in PAO1 genome that were confirmed by RT-PCR are depicted on PAO1 co-ordinates in Fig. 4. The confirmation of new ORFs reflects the limitations of existing software to predict all the ORFs in the genome accurately.

3.11. *Comparison of AmpR transcriptome and proteome*

We had previously analyzed the transcriptome of AmpR using both DNA microarrays [26] and RNA deep sequencing [27]. Comparing the normalized data of all four conditions from the two-transcriptome studies with the proteome data revealed an extensive overlap (Fig 5A). As expected, the transcriptome data had much more reads that were not identified in the proteome studies, and this can be attributed to short half-lives of the mRNA, tRNA reads and various kinds of post-transcriptional regulation. Moreover, almost all the proteins detected in the proteome analysis were found in one of the two-transcriptome studies (Fig 5A). Various technical (sample preparation, instrumentation etc.), functional (posttranscriptional or post-translational modifications) or analysis software variations can account for the differences observed in these three approaches. The findings also highlight the fact that in order to obtain a comprehensive picture, studying gene expression on a global scale should involve a combination of approaches.

The transcriptome data and complementary assays established the global regulatory nature of AmpR [26], which includes regulatory RNAs [27]. Comparison of the transcriptome (Array and RNA-Seq) and proteome datasets revealed that 27 ORFs are AmpR-regulated in all the three experiments (Fig. 5B; Supplementary Table 9). As expected, many of the genes/proteins that were detected in all three assays contribute to *P. aeruginosa* virulence. These include the Psl exopolysaccharide biosynthetic proteins, PslF, PslG and PslH (PA2236–PA2238) that play a role in biofilm formation [60], the PQS response protein PqsE (PA1000) [92], a component of the MexGHI-OpmD efflux pump MexI [93], and the phenazine-modifying enzyme PhzS (PA4217) ([94]; Supplementary Table 9). These findings support previous phenotypic assays and qPCR studies that determined a role for AmpR in regulating biofilm formation, QS and antibiotic resistance [26,27].

Even though we found 2121 non-redundant AmpR-dependent genes combining all the three analysis, 363 genes

Table 4 – List of unannotated ORFs in PAO1 genome verified by reverse transcription PCR.

Proposed ORF	Proposed PAO1 coordinates	Orthologs
PA0306.1	348473–347835	PSPA7 0398, PA14 04010, PALES 31051, PA14 36010, PACG_03535
PA0708.1	782205–782113	None
PA0852.1	932105–931395	Unannotated orthologs in PA14, PA7 and LESB58
PA2566.1	2902048–2902155	Unannotated orthologs in LESB58 and PA14
PA3259.1	3647746–3647399	PSPA7 1865, unannotated orthologs in PA14 and LESB58
PA3440.1	3847538–3847200	PALES 16201, PA14 19600, PSPA7 1687
PA3574.1	4007324–4007130	PA14 18070, PSPA7 1570, unannotated ortholog in LESB58
PA3865.1	4327697–4327362	PA14 13950
PA5183.1	5836685–5836470	PALES 55771, PA14 68470, PSPA7 5926

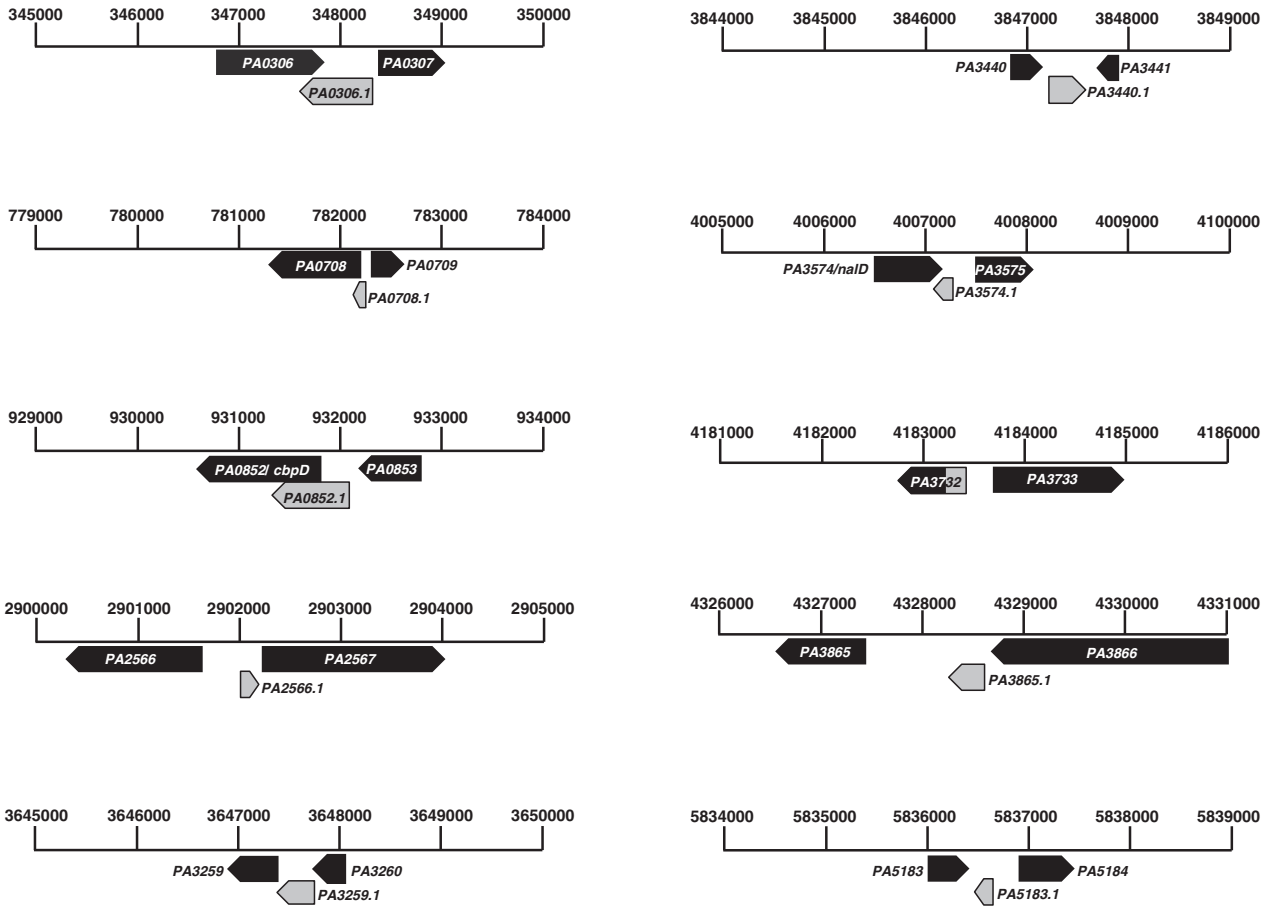

Fig. 4 – Proposed new ORFs shown on the PAO1 genome co-ordinates.

overlapped between any two analyses (Fig. 5B). Thus, conservatively speaking, actual AmpR regulon can be anywhere between >363 < 2121 genes. The 363 AmpR-dependent genes identified in any two analyses included a long list of genes involved in ß-lactam and non-ß-lactam resistance, QS-regulation, phenazine biosynthesis, hydrogen cyanide biosynthesis, T6SS, biofilm and alginate production, etc. (Supplementary Table 9). These sufficiently paint the global picture of AmpR regulation in *P. aeruginosa*.

Further, there were many AmpR-regulated proteins that were unique to any one of the analysis (Fig. 5; Supplementary Table 9). Proteome, array and RNA-Seq identified 640, 291, 827 AmpR-dependent genes, respectively, that did not overlap with any other lists. The RNA-Seq gene set also included the AmpR regulated small RNAs [27].

The AmpR-dependent proteins detected uniquely in the proteome analysis further identified many other virulence proteins such as the alkaline protease AprD (PA1246), the BfiSR (PA4196-4197) TCS and proteins of the chaperone–usher pathway (CupB2, B3 and C3) (Supplementary Table 9). In addition, regulation of 3 of the c-di-GMP PDEs by AmpR was identified by proteomic data. While one (PA4781) overlapped with array, two unique PDEs CpdA (PA4969) and BifA (PA4367) were identified in proteome analysis (Supplementary Table 9). The role of AmpR in modulating c-di-GMP levels needs further investigation. Thus, both the previous transcriptome and the current proteome data

Table 5 – List of misannotated ORFs in PAO1 genome.

Proposed ORF	Proposed PAO1 coordinates	Orthologs
PA0369	413840–413364	PA14, C3719, PACS2, 39016, LESB58, 2192, PA7
PA0459	517837–520635	PA14, 39016, 2192, PACS2, LESB58, C3719, PA7
PA0799	878818–876617	39016, PA14, LESB58, C3719, PACS2, 2192, PA7
PA1926	2104177–2106447	2192, LESB58, PACS2, 39016, PA14, C3719
PA2731	3090231–3089644	None
PA3248	3635589–3634924	C3719, PACS2, LESB58, 2192, 39016, PA14, PA7
PA3732	4183405–4182785	LESB58, PACS2, C3719, 39016, 2192, PA14, PA7
PA3861	4321104–4322627	C3719, 39016, LESB58, PA14, 2192, PACS2, PA7
PA4985	5601131–5599884	PACS2, C3719, 39016, PA14, LESB58, 2192, PA7
PA5117	5764611–5762659	LESB58,PACS2, C3719, 39016, 2192, PA14
PA5322	5991168–5993774	PACS2, C3719, LESB58, 2192, PA14, PA7

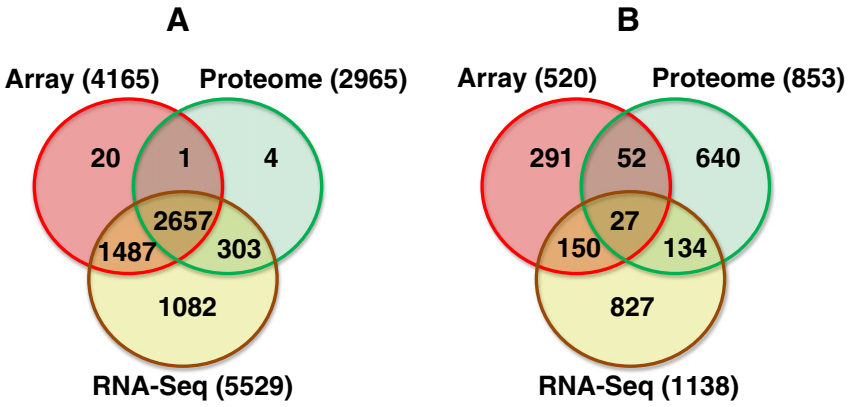

Fig. 5 – Comparative analyses of AmpR microarray, RNA-Seq and proteome datasets. Venn diagram was constructed using the datasets from the microarray, RNA-Seq and Proteome analysis; (A) Comparison of total identified datasets and, (B) Comparison of the AmpR-dependent datasets.

not only attest to its role as global regulator of virulence in *P. aeruginosa*, but also revealed new modes of gene regulation by AmpR.

4. Conclusion

Our study is the first report to look at the proteomic response of *ampR* mutant of *P. aeruginosa* in the presence and absence of ß-lactam antibiotic. The data presented here not only supports the previous transcriptomic studies [26,27], but also strengthens the master regulatory role of *P. aeruginosa* AmpR in regulating antibiotic resistance, virulence factors as well as protein phosphorylation. In addition, we also describe the Ser/Thr/Tyr phosphorylations in PAO1 and PAOΔ*ampR* in the presence and absence of ß-lactam antibiotics that were not previously reported. The huge difference between the phosphoproteomes of PAO1 and PAOΔ*ampR* under the same conditions is interesting and needs to be explored further. Finally, we identified and confirmed several unannotated and misannotated ORFs in the *P. aeruginosa* genome. Since the function of over ~36% of the ORFs in the PAO1 genome is yet to be determined, it will take a considerable amount of research to decipher the specific role of these newly identified proteins. Thus, the information provided in this analysis opens up new areas of research in understanding complex strategies that contribute to the success of *P. aeruginosa* as a pathogen. Global approaches like the current study will not only aid in connecting previously isolated areas of research but also in finding therapeutic strategies to combat this formidable pathogen.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.11.018.

Acknowledgments

This work was supported by National Institutes of Health-Minority Biomedical Research Support SCORE grants (grant numbers S06 GM08205, 5SC1AI081376 to K.M.) and Florida International University (FIU) Research Assistantship (Herbert Wertheim College of Medicine, to D.B.).We also thank Soumya Vemuri for her assistance with the data clarification.

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