

A wide-ranging *Pseudomonas aeruginosa* PeptideAtlas build: A useful proteomic resource for a versatile pathogen

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ARTICLE INFO

Keywords:

Pseudomonas
Bacterial PeptideAtlas
Pathogen
Database
Proteomics
Tool for targeted proteomic studies

ABSTRACT

Pseudomonas aeruginosa is an important opportunistic human pathogen with high prevalence in nosocomial infections. This microorganism is a good model for understanding biological processes such as the quorum-sensing response, the metabolic integration of virulence, the mechanisms of global regulation of bacterial physiology, and the evolution of antibiotic resistance. Till now, *P. aeruginosa* proteomic data, although available in several on-line repositories, were dispersed and difficult to access. In the present work, proteomes of the PAO1 strain grown under different conditions and from diverse cellular compartments have been joined to build the *Pseudomonas* PeptideAtlas. This resource is a comprehensive mass spectrometry-derived peptide and inferred protein database with 71.3% coverage of the total predicted proteome of *P. aeruginosa* PAO1, the highest coverage among bacterial PeptideAtlas datasets. The proteins included cover 89% of metabolic proteins, 72% of proteins involved in genetic information processing, 83% of proteins responsible for environmental information processing, more than 88% of the ones related to quorum sensing and biofilm formation, and 89% of proteins responsible for antimicrobial resistance. It exemplifies a necessary tool for targeted proteomics studies, system-wide observations, and cross-species observational studies. The manuscript describes the building of the PeptideAtlas and the contribution of the different proteomic data used.

Significance: *Pseudomonas aeruginosa* is among the most versatile human bacterial pathogens. Studies of its proteome are very important as they can reveal virulence factors and mechanisms of antibiotic resistance. The construction of a proteomic resource such as the PeptideAtlas enables targeted proteomics studies, system-wide observations, and cross-species observational studies.

1. Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen with high prevalence in nosocomial infections [1,2]. In addition, this bacterial pathogen is the major cause of chronic infections in patients suffering obstructive pulmonary disease and cystic fibrosis (CF) [3–5]. It is able to survive, persist in, and colonize diverse environments [6], including soil and water. The phylogenetic analysis of clinical and environmental isolates of this organism show that the population is panmictic. There are no differentiated clinical and environmental lineages, and the success of infection relies more on the underlying situation of the patient than on the specific characteristics of a given clone that

may have evolved towards virulence [7–9]. Differing from other more specific pathogens that have become virulent through the acquisition of genetic material [10], the virulence determinants of *P. aeruginosa* belong to the core genome of this microorganism [11]: they are present in all members of the species and have not been acquired by a specific lineage evolved to be infectious. Using similar mechanisms, *P. aeruginosa* is able to infect a large variety of hosts, including amoebae, plants, worms, insects, and mammals [12]. As a highly versatile species, *P. aeruginosa* grows under aerobic and anaerobic conditions, over a wide temperature range, and can make use of several carbon sources [13]. Further, it presents a large repertoire of virulence determinants [13,14]. The expression of several of them is regulated through a complex quorum-

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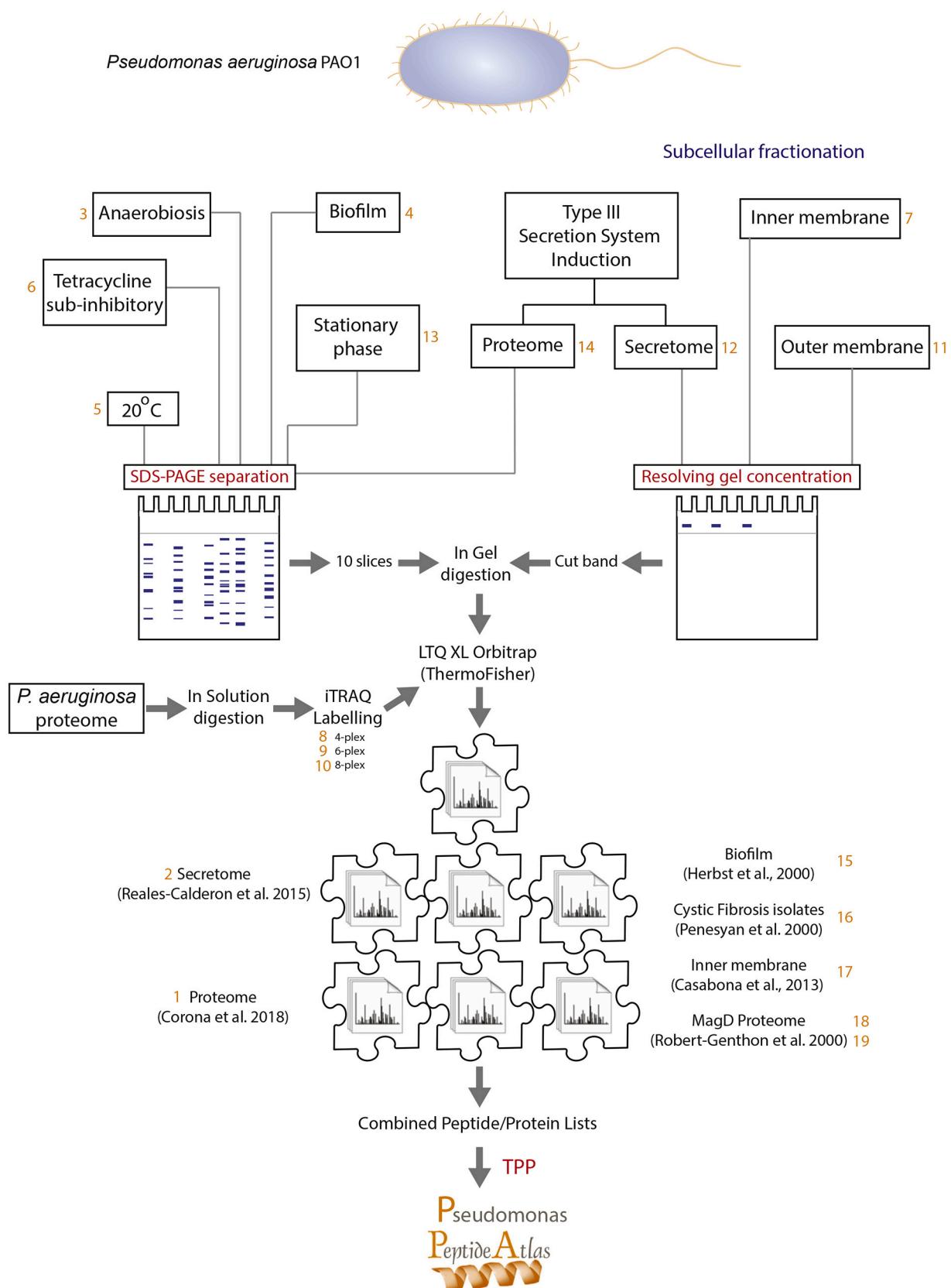


Fig. 1. Diagram showing the different samples obtained and analyzed to built-in the *Pseudomonas aeruginosa* PeptideAtlas. The different samples are identified by the number that PeptideAtlas has assigned (Table 1). Cytosolic, sub-cellular and secreted protein fractions were obtained for *P. aeruginosa* PAO1 grown under different conditions. Proteins were exhaustively separated on SDS-PAGE and peptides by OFFGEL separation. The MS output results, together with additional MS datasets from other authors were all processed with the TPP tool suite.

Table 1*Pseudomonas aeruginosa* proteomic samples included in this work.

Sample number	Database name	Proteomic analysis	Runs	Number of identified proteins	Unique proteins	Reference	Repositories*
1	pao1_fcp001_proteome_4plex	iTRAQ/ LC-MS/MS	30	2762	51	[41]	PASS00809
2	pao1_vescl_secreteome_4plex	iTRAQ/ LC-MS/MS	12	1265	12	[24]	PASS00808
3	pao1_ananaerobiosis	LC-MS/MS	5	2252	20		PASS00804
4	pao1_biofilm	LC-MS/MS	5	1689	15		PASS00803
5	pao1_growth_20c	LC-MS/MS	5	2313	6		PASS00800
6	pao1_growth_TCN	LC-MS/MS	5	2465	13		PASS00801
7	pao1_inner_memb	LC-MS/MS	5	1158	3		PASS00805
8	pao1_iTRAQ_4plex	iTRAQ/ LC-MS/MS	2	1568	3		PASS00821
9	pao1_iTRAQ_6plex	iTRAQ/ LC-MS/MS	2	1582	2		PASS00822
10	pao1_iTRAQ_8plex	iTRAQ/ LC-MS/MS	2	1413	2		PASS00823
11	pao1_outer_memb	LC-MS/MS	1	1080	4		PASS00806
12	pao1_secreteome_T3SS	LC-MS/MS	1	473	4		PASS00807
13	pao1_stat_phase	LC-MS/MS	5	2504	18		PASS00799
14	pao1_T3SS_induction	LC-MS/MS	1	2486	11		PASS00802
15	PAO1_Fap	LC-MS/MS	288	3698	240	[43]	PXD001373
16	<i>Pseudomonas</i> _Cystic_Fibrosis	DIA LC-MS/MS	80	1787	223	[44]	PXD002865
17	PAO1_Inner_Membrane_Proteome	LC-MS/MS	6	1656	18	[45]	PXD000107
18	PAO1_MagD-WT	LC-MS/MS	2	1102	4	[46]	PXD000189
19	PAO1_delta-MagD	LC-MS/MS	2	446	0	[46]	PXD000189

* Datasets available in PeptideAtlas Database (accession code PASS_) and PRIDE Database (accession code PXD_).

sensing network [15,16], as well as a battery of secreted virulence factors (including toxins, siderophores and proteases) and vesicle-engulfed compounds [17–19]. One of the main problems in treating *P. aeruginosa* infections is the low susceptibility to antibiotics of this microorganism, being classified by WHO in the ESKAPE group of pathogens with higher risk of antibiotic resistance [14,20]. This is due to the presence in its genome of genes encoding beta-lactamases and several multidrug efflux pumps [21], among other elements [22]. In addition, *P. aeruginosa* can evolve to acquire increasing levels of resistance through mutations or through the acquisition of antibiotic resistance genes via horizontal gene transfer, allowing the dissemination of multidrug resistant strains causing nosocomial infections worldwide [23].

The versatility of *P. aeruginosa* is supported by its large genome, ranging between 5 and 7 Mbp in different strains [6], together with a large number of regulatory elements (both transcriptional and post-transcriptional) that allow the organism to adapt its physiology when confronted with different physicochemical conditions and cues specific to each of the habitats it can colonize. All these features make this microorganism, in addition to an important pathogen, a good model for understanding several biological processes, including the quorum-sensing response [15,16], the metabolic integration of virulence [24,25], the mechanisms of global regulation of bacterial physiology [26,27], and the evolution of antibiotic resistance [28–34], among others. For this reason, it has been proposed that *P. aeruginosa* should be considered a “lab rat”, similar to other model bacteria such as *Escherichia coli* and *Bacillus subtilis* [13].

The integrated analysis of *P. aeruginosa* under a systems biology umbrella requires the use of multi-omic techniques, and accurate databases are fundamental to this purpose. Among them, the on-line resource at the *Pseudomonas* Genomic Database (www.pseudomonas.com) [35] provides updated and well-curated information on the different sequenced strains of this microorganism at the genomic level, while PAMDB, <http://pseudomonas.umaryland.edu> [36] includes metabolomic information. Post-transcriptional regulation plays a major role in the adaptation of this microorganism to its different habitats [37]. Besides, the identification of factors that are secreted to the extracellular milieu or in extracellular vesicles (EVs) is fundamental to a full understanding of the virulence of this pathogen. In consequence, proteomic studies are needed for a comprehensive analysis of *P. aeruginosa* physiology. However, proteomic data, although available in several on-line repositories, are dispersed and difficult to access. Thus, resources like PeptideAtlas, which compiles the available LC-MS/MS-based shotgun proteomics data, are necessary for targeted proteomics

studies, system-wide observations, and cross-species observational studies [38]. To date, 43 PeptideAtlas builds are available, but only 8 of them are from bacteria (<http://www.peptideatlas.org/builds/>).

Here we present the *P. aeruginosa* PeptideAtlas. To build it, proteomes of *P. aeruginosa* strain PAO1 grown under very different conditions and from diverse cellular compartments have been obtained, analyzed, and joined with data from other authors, one of them including proteomes from clinical strains. The resulting on-line resource is a comprehensive mass spectrometry-derived peptide and inferred protein database with high coverage of the total predicted proteome from this bacterium.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *P. aeruginosa* strain used by our group to develop the *Pseudomonas* PeptideAtlas was the widely used wild-type PAO1. All the growth conditions employed to generate new proteomes used to build the PeptideAtlas are represented in Fig. 1 and Table 1, together with other additional datasets from our group and others deposited in PRIDE [39].

For most of the samples prepared for the present work, PAO1 was grown until exponential phase of growth (OD₆₀₀ of 0.6) in LB Luria Bertani (LB) broth (10 g Peptone, 5 g Yeast Extract, 5 g Sodium Chloride) at 37 °C. There were some exceptions: sample 4, biofilm formed in p24 plates at 37 °C during 48 h; sample 5, at 20 °C; sample 6, in LB agar; and sample 13, until OD₆₀₀ of 4 to reach stationary phase of growth. Also, additives were used for the following samples: for samples 12 and 14, 5 mM EGTA and 20 mM MgCl₂ for stimulating Type III secretion [40] during 4 h; and for sample 6, Tetracycline sub-inhibitory concentration (0.5 mg/l) during 4 h.

All the samples were grown under aerobic conditions except for sample 3, where anaerobiosis was achieved in LB agar plates placed in a generator for GENbox jar 2.5 l anaerobic environment (GENbox anaer, Biomerieux) during 48 h at 37 °C.

All the samples were total extracts (cytosolic proteins mainly) except for samples 7 and 11, where subcellular fractionations were needed to enrich in inner and outer membranes, and the secretome obtained for sample 12.

2.2. Protein extraction and digestion

Pellets from *P. aeruginosa* PAO1 cells grown under the different

conditions were washed and resuspended in 1 ml of PBS supplemented with 1/1000 Protease Inhibitor Cocktail (Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets). PAO1 biofilm was washed with PBS and cells were collected to protein extraction. Cells from the different samples were lysed by sonication (Labsonic U) for 3 times, 1 min each, and centrifuged for 20 min at 6000g at 4 °C. Cell debris was removed by centrifugation (14,000g, 10 min, 4 °C). Proteins were frozen at -80 °C until used. Protein concentration was measured using Bradford assay (Biorad).

The secretome from the T3SS-induction sample was collected by centrifugation at 7000g for 10 min at 4 °C and supernatants were filtered through a 0.2 µm pore size Nalgene Disposable Filter Unit (Thermo Fisher Scientific) to remove the remaining cells. Proteins were precipitated with methanol/chloroform. The pellet was washed 2 times with PBS and resuspended in 0.5 M Triethylammonium bicarbonate (TEAB) supplemented with 1/1000 Protease Inhibitor Cocktail.

In the case of the inner and outer membranes enriched fractions, cells were pelleted and washed with PBS and resuspended in 1.5 ml of 20 mM HEPES pH 8.0. Then, lysis was reached by sonication for 3 times, 1 min each, and centrifuged for 20 min at 6000g at 4 °C. After this, supernatant was collected and ultracentrifuged at 100,000g during 1 h at 4 °C. Then, supernatant was collected, and pellet was resuspended in 1.5 ml 20 mM HEPES pH 8.0 and the suspension was ultracentrifuged at 100,000g during 30 min at 4 °C. Total membranes were pelleted and resuspended in 1.5 ml of 20 mM HEPES pH 8.0 + 15 µl TRITON X-100, ultracentrifuged at 100,000g during 1 h at 4 °C. Outer membranes were in the pellet and inner membranes were enriched in the supernatant. Inner membranes were collected. The pellet was washed in 1.5 ml of 20 mM HEPES pH 8.0, ultracentrifuged at 100,000g during 1 h at 4 °C and the pellet, enriched in outer membranes, was resuspended in 300 µl of 20 mM HEPES pH 8.0.

One hundred µg of the different PAO1 cytoplasmic extracts were loaded onto conventional SDS-PAGE 10% Bis-Tris gels (mini-protean TGX Stain-free precast Gels, BioRad). The gel was stained with Coomassie blue and each lane was cut into 10 bands. Gel slices were cut into 1 mm³ cubes, washed twice with water and dehydrated with 100% ACN (v/v).

One hundred µg of protein of subproteomes (outer and inner membranes and T3SS secretome) were loaded in a conventional SDS-PAGE gel (1 mm-thick, 4% stacking, and 12% resolving). Then run was stopped as soon as the front entered the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (1 mm³), washed twice with water, and dehydrated with 100% ACN (v/v). All the gel slices were incubated with 10 mM DTT in 50 mM NH₄HCO₃ for 30 min at 56 °C for protein reduction; alkylation was carried out with 50 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate solution.

The gel pieces were washed with 50% ACN (v/v), and then washed again with 10 mM NH₄HCO₃, dehydrated with 100% ACN (v/v), and then dried in a vacuum concentrator. The gel pieces were rehydrated by adding sequence grade-modified trypsin (Roche) 1:20 in 50 mM NH₄HCO₃ and incubated overnight at room temperature in the dark for protein digestion. Supernatants were transferred to clean tubes, and gel pieces were incubated in 50 mM NH₄HCO₃ at 50 °C for 1 h. Then, remaining peptides were collected by incubation with 5% formic acid for 15 min and with 100% ACN for 15 min more. The extracts were combined, and the organic solvent was removed in a vacuum concentrator.

The tryptic eluted peptides were dried by speed-vacuum centrifugation and then desalted onto StageTip C18 Pipette tips (Thermo Scientific) until the mass spectrometric analysis. The 10 slices of the whole proteomes were combined, and 5 fractions were used for the mass spectrometry analysis.

2.3. LC-MS/MS analysis

The MS system used was an LTQ XL Orbitrap (ThermoFisher) equipped with a nanoESI ion source. A total amount of 5 µg from each sample (volume of 20 µl) was loaded into the chromatographic system consisting in a C18 pre-concentration cartridge (Agilent Technologies) connected to a 360 cm long, 100 µm i.d. C18 column (NanoSeparations). The separation was done at 0.25 µl/min in a 360 min acetonitrile gradient from 3 to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed of an Agilent 1200 capillary nano pump, a binary pump, a thermostated micro injector and a micro switch valve. The LTQ XL Orbitrap was operated in the positive ion mode with a spray voltage of 1.8 kV. The spectrometric analysis was performed in a data dependent mode, acquiring a full scan followed by 10 MS/MS scans of the 10 most intense signals detected in the MS scan from the global list. The full MS (range 400–1800) was acquired in the Orbitrap with a resolution of 60,000. The MS/MS spectra were done in the linear ion-trap.

2.4. Compiling of additional *P. aeruginosa* datasets

2.4.1. Datasets from our group

In addition to the current proteomes analyzed, three different *P. aeruginosa* MS/MS datasets from our lab were compiled to contribute to the *P. aeruginosa* PeptideAtlas, extending the coverage in two more specific conditions, the secretome of PAO1 compared with the mutant FCP001 (EVs and EV-free secretome) [24], and the comparison of the whole proteomes of PAO1 and mutant strain FCP001 grown until under exponential phase of growth [41].

Besides, the proteomic data from an experiment designed to compare the labelling by iTRAQ using the following kits: 4-plex, 6-plex (6 of the 8-plex kit markers) and 8-plex was added. For these experiments, 4, 6 or 8 biological replicates from the whole proteome of PAO1 grown until exponential phase growth were used. Briefly, 8 independent cultures of PAO1 (20 ml of each) were grown in LB medium to reach mid-exponential growth phase (OD₆₀₀ of 0.6), moment at which the cells were pelleted by centrifugation and proteins were obtained in 1 ml of PBS supplemented with 1/1000 Protease Inhibitor Cocktail (Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets) by sonication. Forty µg of protein from each condition were precipitated with methanol/chloroform and resuspended in 0.5 M Triethylammonium bicarbonate (TEAB) and after trypsin digestion, samples were labeled at room temperature for 2 h with a half unit of iTRAQ Reagent Multi-plex kit (AB SCIEX, Foster City, CA, USA). Finally, samples were combined and bRP-LC C18 fractionation of the iTRAQ labeled peptides was performed on the SmartLine (Knauer, Germany) HPLC system using the Waters, XBridge C18 column (100 × 2.1 mm, 5 µm particle). Thirty fractions were collected over the elution profile and pooled into 5 fractions. The peptide fractions were dried, desalting was performed using a SEP-PAK C18 Cartridge (Waters) and stored at -20 °C until the LC – MS analysis.

A 1.5 µg aliquot of each peptide fraction was subjected to 2D-nano LC ESI-MSMS analysis using a nano liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, AB SCIEX, Foster City, CA) coupled to high speed Triple TOF 5600 mass spectrometer (AB SCIEX, Foster City, CA) with a Nanospray III Source. The loading pump delivered a solution of 0.1% formic acid in water at 2 µl/min. The nano-pump provided a flow-rate of 300 nL/min and was operated under gradient elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Gradient elution was performed using a 120 min gradient ranging from 2% to 90% mobile phase B. Injection volume was 5 µl.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON). Data were acquired using an ionspray voltage floating (ISVF) 2800 V, curtain gas (CUR) 20, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 20, declustering potential (DP) 85 V. All data were acquired using information-dependent acquisition

(IDA) mode with Analyst TF 1.5 software (AB SCIEX, USA). For IDA parameters, 0.25 s MS survey scan in the mass range of 350–1250 Da were followed by 30 MS/MS scans of 150 ms in the mass range of 100–1800 (total cycle time: 4.04 s). Switching criteria were set to ions greater than mass to charge ratio (*m/z*) 350 and smaller than *m/z* 1250 with charge state of 2–5 and an abundance threshold of more than 90 counts (cps). Former target ions were excluded for 20 s. IDA rolling collision energy (CE) parameters script was used for automatically controlling the CE.

2.4.2. Other PRIDE *P. aeruginosa* datasets

Together with the experiments from our group, other published works that have been uploaded to the public repository PRIDE [42] have been included to increase the coverage of the *P. aeruginosa* PeptideAtlas. These are the datasets from Herbst et al. (PXD001373) [43], sample 15, Penesyan et al. (PXD002865) [44], sample 16, Casabona et al. (PXD000107) [45], sample 17, and Robert-Genthon et al. (PXD000189) [46], samples 18 and 19 (Table 1, Fig. 1).

2.5. Post spectra acquisition processing

LC-MS/MS spectra files resulting from the different proteomes (Fig. 1), in their native vendor-specific format, along with the meta data corresponding to each approach, were submitted to PeptideAtlas via the PeptideAtlas Submission System (PASS) on-line submission form with dataset identifications: PASS00799, PASS00800, PASS00801, PASS00802, PASS00803, PASS00804, PASS00805, PASS00806, PASS00807, PASS00808, PASS00809, PASS00821 and PASS00822, together with the already included in PRIDE previously cited. LC-MS/MS spectra files were converted to XML-based HUPO-PSI-adopted standard format for mass spectrometry output, mzML [47]. The protein sequence FASTA file was obtained from the *Pseudomonas* Genome Database (www.pseudomonas.com) [35]. Sequences were appended with a set of common contaminant proteins from the cRAP (common Repository of Adventitious Proteins) set from the GPM (<http://www.thegpm.org/crap/>) and decoy counterparts for every entry to add up a total of 11,376 entries.

Then database searches were performed using three different search engines: Comet [48], an open-source, freely available version of SEQUEST [49], X!Tandem [50] with the k-score algorithm plugin [51], and OMSSA [52]. The search parameters were established depending on the type of experiment and instrument (see supplementary table S1 'database search parameters' for a list of parameters).

Following sequence database searching, we used the TPP (Trans-Proteomic Pipeline) tool suite [53,54] to validate the results. First, PeptideProphet [55] creates a discriminant search engine-independent score, models distributions of correctly and incorrectly assigned peptide spectrum matches (PSMs) and computes PSM posterior probabilities. Next, iProphet [56], was used to combine three search results, further refine the PSM-level probabilities and calculate distinct peptide-level probabilities using corroborating information from other PSMs in the dataset. ProteinProphet [57] then was used to further refine peptide probabilities based on the Number of Sibling Peptides (NSP) that each peptide shares within a protein; it also groups and reports proteins with a protein-level probability estimated from peptide-level probabilities. To assemble the *P. aeruginosa* PeptideAtlas, all individual iProphet files from the 13 compiled datasets were filtered at a variable probability threshold to reach a constant PSM-level FDR threshold of 0.0002 across all datasets.

The *Pseudomonas aeruginosa* PeptideAtlas is made available at https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/buildDetails?atlas_build_id=455.

Mayu [58] software, designed for large-scale protein FDR estimation, was used to report FDR values at different levels (PSM, unique peptides and protein-level) for the whole build based on a strategy that estimates the number of false positive protein identifications from the number of

proteins containing false positive PSMs, including a correction for high proteome coverage.

2.6. In silico functional analyses

Gene Ontology classifications, and subcellular protein localization studies were performed using the *Pseudomonas* Genome Database (www.pseudomonas.com) [35] and KEGG PATHWAY Database (<https://www.genome.jp/kegg/>) was used for pathway analysis [59,60].

3. Results and discussion

3.1. Construction of the *P. aeruginosa* PeptideAtlas and strategies for exhaustive proteome characterization

The *Pseudomonas* PeptideAtlas has been built using data mainly obtained from the wild-type strain PAO1, one of the most widely used model strains, whose genome sequence is available [61]. We used a wide range of experimental growth conditions and different subcellular fractions to increase the proteome coverage as much as possible (Fig. 1 and Table 1). Table 1 and Supplemental Table S2 show the number of proteins identified in the different samples. In Table S2, proteins are ordered by number in the *Pseudomonas* Genome Database (www.pseudomonas.com) [35], facilitating the visualization of the identified proteins in the different conditions to observe commonalities and differences between conditions. Uniquely identified proteins in each dataset are highlighted. Of the total 649 uniquely identified proteins, 164 belong to the datasets generated by our group. This shows the necessity of analyzing of proteomes produced under different environmental conditions.

Samples 1 and 2 were obtained from PAO1 and the mutant strain Fcp001 (*Δcrc*) grown until they reached the exponential phase of growth at 37 °C. In the work by Corona et al. [41], the whole proteomes were studied (Sample 1), and in the case of Reales-Calderon et al. [24], the fraction used was the secretome (Sample 2). In both cases, proteins were labeled with iTRAQ and analyzed by LC-MS/MS. In sample 1, 2762 proteins were identified in a total of 30 runs, 51 of them unique to sample 1 and in sample 2, a total of 1265 proteins in the secretome and EVs were identified in 12 runs, 12 of them only detected in this sample. The same growth conditions were used for samples 8, 9, and 10, allowing the identification of around 1550 proteins in each experiment. The difference between these samples was the iTRAQ labeling strategy, with 4, 6, or 8 plex used. Only two runs were done for each sample. As expected, the three samples differed only in a few proteins (Table S2), allowing the addition of peptides from 7 more proteins to PeptideAtlas. All of them were grown until the exponential phase of growth, a condition widely used for microbiological studies, with the expectation that many proteins should be abundant because the microorganism is actively growing. However, the stationary phase of growth can unveil proteins that are important for the maintenance of the microorganism once the carbon source has been exhausted and secreted components are accumulating in the culture. This condition is represented by sample 13, which comprises 2504 proteins, including 18 not detected in the other samples.

For sample 3, anaerobic conditions were used because, during chronic infection of the CF lung, *P. aeruginosa* is able to grow and persist in a microaerobic to anaerobic environment [62]. This analysis rendered 2252 proteins, 20 of them uniquely identified in this sample. Growth as a biofilm is very important in *P. aeruginosa* virulence and antibiotic resistance [14], in its persistence in environments such as water supplies [63], and in CF patients [3]. Samples 4, 15, and 16 represent this kind of growth. In sample 4, 1689 proteins were identified, including 15 unique for this sample.

As *P. aeruginosa* is present in natural environments, we grew PAO1 at 20 °C (sample 5). In 5 runs, 2313 proteins were identified, of which only 6 were uniquely detected in this condition. Because *P. aeruginosa* is quite

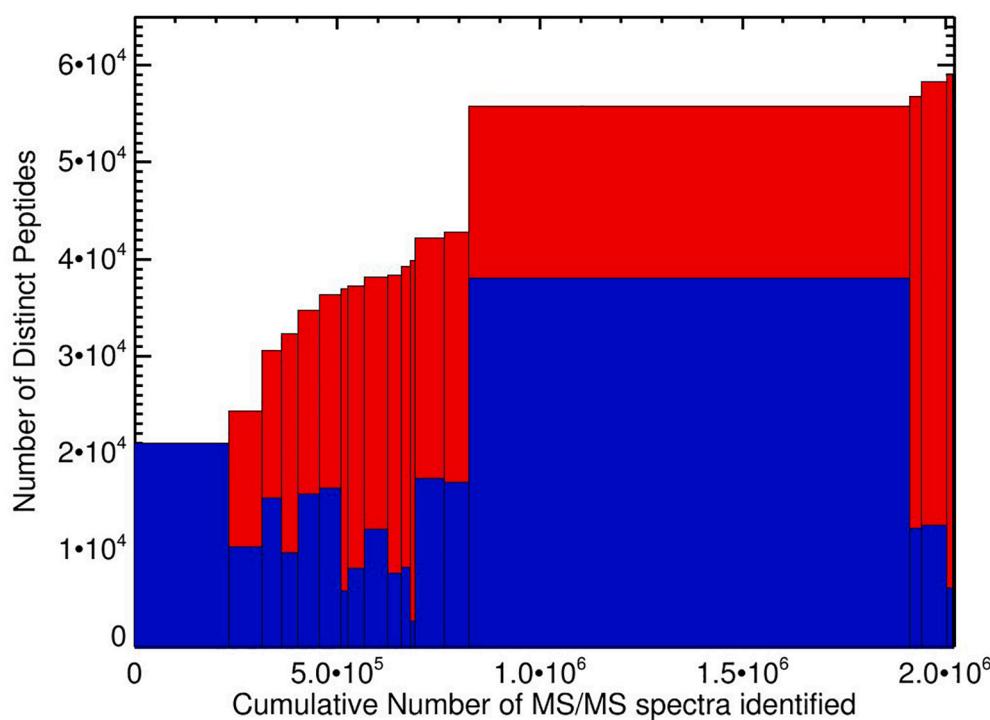


Fig. 2. Contribution of the different constituent datasets of the *Pseudomonas* PeptideAtlas. Plot shows the number of peptides contributed by each experiment, and the cumulative number of distinct peptides for the build as of that experiment. For each dataset, the height of the blue bar represents the number of distinct peptides and the total height of bar represents the cumulative number of distinct peptides identified. The width of bars represents the contributions in terms of spectra identified. Numbers designate the samples as ordered in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resistant to antibiotics [14,20] and it has been reported that subinhibitory antibiotic concentrations may modify *P. aeruginosa* physiology [64], sample 6, grown in tetracycline (TCN) was included, with 2465 proteins identified, including 13 more unique to this condition. In addition, PAO1 was cultured in the medium that induces the type III secretion system (T3SS), as T3SS is a very important virulence factor, and the proteome and secretome were analyzed (samples 14 and 12, respectively). This condition yielded a total of more than 2800 identified proteins from the two samples, including 15 that were unique to them.

Cytosolic extracts are the most commonly studied fractions in proteomic analysis, but some proteins are under-represented in or missing from them, for instance membrane proteins [65]. With the goal of maximum coverage of the proteome, subcellular fractions enriched in membrane proteins (samples 7 and 11) were also analyzed, and 7 more unique proteins were identified. According to *Pseudomonas* Genome Database, in sample 7, from the total of 1158 proteins, 199 are membrane proteins, while in sample 11, of the total 1080 proteins identified, there are 290 membrane proteins. As part of the datasets from other authors included in the PeptideAtlas, sample 17 [45], is described as an inner membrane enriched proteome, with 670 membrane proteins and 18 proteins uniquely identified in this sample.

Besides, proteins identified in other published works [24,41,43,44,46] were also included, adding 529 more uniquely identified proteins to the PeptideAtlas (Samples 15, 16, 18 and 19). Penesyan et al. [44] (Sample 16) included proteins from four clinical isolates. As expected, the proteomes of these freshly isolated strains differ from that of the PAO1 strain, as only 29.4% of the identified proteins were common to all 5 strains used in this work. Although this does not mean that the proteins are not present in all these strains, it is a sign of differences in the most abundant proteins between samples showing, as the authors highlight, the need for analyses of strains isolated from clinical or environmental samples [44]. Sample 16 contributed with 1787 proteins, of which 223 were uniquely identified. The dataset with the highest number of proteins identified is sample 15 [43] with 3698, and it also comprises the highest number of unique proteins, 240.

A total of 5,078,535 tandem mass (MS/MS) spectra from the 19 different proteomic experiments and 459 individual runs using a wide

variety of mass spectrometers (TripleTOF 5600, LTQ Orbitrap XL, LTQ-Orbitrap Velos, LTQ Orbitrap Velos pro and Q Exactive) were used to generate the *P. aeruginosa* PeptideAtlas. Almost 40% (2,021,521) of the generated spectra were matched to peptides with probability high enough to pass our threshold. From these, 13,059 peptides were identified in only one dataset. Sample 15, with the highest number of runs (288), contained 2,516,514 spectra. Overviews of the contributions of each dataset to the entirety of the PeptideAtlas build are depicted in Table 1 and in Fig. 2.

As above stated, *P. aeruginosa* is one of the most prevalent opportunistic human pathogens. It is able to grow as biofilm, under aerobic and anaerobic conditions and in diverse host environments. Besides, it harbours several antibiotic resistance determinants, the expression of which is a relevant problem for the treatment of the infections it produces. Consequently, the exploration of growth conditions such as biofilm formation, anaerobic growth, in the presence of antibiotics, or situations that trigger T3SS, is crucial for the analysis of proteins involved in virulence and antibiotic resistance. Particularly relevant is the study of the exoproteome and the EVs-associated proteome since several *P. aeruginosa* virulence determinants are extracellular. Thus, the total of peptides and proteins included in the *Pseudomonas* PeptideAtlas Database, together with the information on the conditions used to trigger them, make the database a powerful tool for implementing targeted proteomic approaches aiming to track proteomic changes associated with infecting conditions as well as to analyze the expression of proteins involved in *P. aeruginosa* antibiotic resistance.

3.2. Functional and gene ontology enrichment analyses of the proteins included in the *P. aeruginosa* PeptideAtlas

The proteomic analyses identified 4043 proteins from a total of 5668 proteins predicted to be encoded by the *P. aeruginosa* PAO1 genome, achieving 71.3% coverage of the predicted *Pseudomonas* proteome. Table 2 lists the more represented KEGG pathways, and those with coverage superior to 90% are highlighted. The proteins in the *Pseudomonas* PeptideAtlas cover 89% of metabolic proteins, 72% of proteins involved in genetic information processing, 83% of proteins responsible

Table 2
KEGG pathway enrichment analysis.

KEGG identifiers and KEGG pathway maps	Identified proteins	Total protein in KEGG pathway maps	Percent of protein in Peptide Atlas*
1. Metabolism			
Global and overview maps			
01200 Carbon metabolism	120	124	96.77
01210 2-Oxocarboxylic acid metabolism	28	30	93.33
01212 Fatty acid metabolism	42	49	85.71
01230 Biosynthesis of amino acids	136	146	93.15
01240 Biosynthesis of cofactors	166	185	89.73
01220 Degradation of aromatic compounds	10	28	35.71
Carbohydrate metabolism			
00010 Glycolysis / Gluconeogenesis	34	37	91.89
00020 Citrate cycle (TCA cycle)	31	31	100.00
00030 Pentose phosphate pathway	24	27	88.89
00040 Pentose and glucuronate interconversions	4	7	57.14
00051 Fructose and mannose metabolism	17	19	89.47
00052 Galactose metabolism	4	5	80.00
00053 Ascorbate and aldarate metabolism	4	6	66.67
00500 Starch and sucrose metabolism	14	16	87.50
00520 Amino sugar and nucleotide sugar metabolism	36	37	97.30
00620 Pyruvate metabolism	58	62	93.55
00630 Glyoxylate and dicarboxylate metabolism	54	59	91.53
00640 Propanoate metabolism	41	43	95.35
00650 Butanoate metabolism	34	42	80.95
00660 C5-Branched dibasic acid metabolism	12	14	85.71
00562 Inositol phosphate metabolism	6	7	85.71
Energy metabolism			
00190 Oxidative phosphorylation	51	57	89.47
00680 Methane metabolism	25	26	96.15
00910 Nitrogen metabolism	30	34	88.24
00920 Sulfur metabolism	35	49	71.43
Lipid metabolism			
00061 Fatty acid biosynthesis	24	26	92.30
00071 Fatty acid degradation	26	31	83.87
00072 Synthesis and degradation of ketone bodies	9	10	90.00
00561 Glycerolipid metabolism	12	15	80.00
00564 Glycerophospholipid metabolism	23	26	88.46
00565 Ether lipid metabolism	2	3	66.67
00590 Arachidonic acid metabolism	3	4	75.00
00592 alpha-Linolenic acid metabolism	2	3	66.67
01040 Biosynthesis of unsaturated fatty acids	5	5	100.00
Nucleotide metabolism			
00230 Purine metabolism	61	68	89.70
00240 Pyrimidine metabolism	28	33	84.85
Amino acid metabolism			
00250 Alanine, aspartate and glutamate metabolism	34	37	91.89
00260 Glycine, serine and threonine metabolism	46	49	93.88
00270 Cysteine and methionine metabolism	40	43	93.02
00280 Valine, leucine and isoleucine	41	46	89.13

KEGG identifiers and KEGG pathway maps	Identified proteins	Total protein in KEGG pathway maps	Percent of protein in Peptide Atlas*
degradation			
00290 Valine, leucine and isoleucine biosynthesis	18	19	94.74
00300 Lysine biosynthesis	14	15	93.33
00310 Lysine degradation	19	22	86.36
00220 Arginine biosynthesis	28	29	96.55
00330 Arginine and proline metabolism	39	49	79.59
00340 Histidine metabolism	23	24	95.83
00350 Tyrosine metabolism	15	27	55.56
00360 Phenylalanine metabolism	22	27	81.48
00380 Tryptophan metabolism	28	31	90.32
00400 Phenylalanine, tyrosine and tryptophan biosynthesis	27	30	90.00
Metabolism of other amino acids			
00410 beta-Alanine metabolism	18	20	80.00
00430 Taurine and hypotaurine metabolism	7	8	87.50
00440 Phosphonate and phosphinate metabolism	2	9	22.22
00450 Selenocompound metabolism	9	9	100.00
00460 Cyanoamino acid metabolism	11	11	100.00
00471 D-Glutamine and D-glutamate metabolism	5	5	100.00
00472 D-Arginine and D-ornithine metabolism	2	2	100.00
00473 D-Alanine metabolism	4	4	100.00
00480 Glutathione metabolism	28	29	96.55
Glycan biosynthesis and metabolism			
00540 Lipopolysaccharide biosynthesis	27	28	96.43
00541 O-Antigen nucleotide sugar biosynthesis	24	25	96.00
00550 Peptidoglycan biosynthesis	19	20	95.00
Metabolism of cofactors and vitamins			
00730 Thiamine metabolism	12	13	92.31
00740 Riboflavin metabolism	9	12	75.00
00750 Vitamin B6 metabolism	8	9	88.89
00760 Nicotinate and nicotinamide metabolism	23	24	95.83
00770 Pantothenate and CoA biosynthesis	21	26	80.77
00780 Biotin metabolism	19	21	90.48
00785 Lipoic acid metabolism	2	2	100.00
00790 Folate biosynthesis	29	32	90.63
00670 One carbon pool by folate	17	18	94.44
00860 Porphyrin and chlorophyll metabolism	40	47	85.11
00130 Ubiquinone and other terpenoid-quinone biosynthesis	17	18	94.44
Metabolism of terpenoids and polyketides			
00900 Terpenoid backbone biosynthesis	15	16	93.75
00903 Limonene and pinene degradation	7	8	87.50
00281 Geraniol degradation	14	17	82.35
00523 Polyketide sugar unit biosynthesis	5	5	100.00
01053 Biosynthesis of siderophore group nonribosomal peptides	7	7	100.00
Biosynthesis of other secondary metabolites			
00332 Carbapenem biosynthesis	2	2	100.00
00261 Monobactam biosynthesis	8	9	88.89
00521 Streptomycin biosynthesis	8	8	100.00
00525 Acarbose and validamycin biosynthesis	2	2	100.00
00401 Novobiocin biosynthesis	5	5	100.00

KEGG identifiers and KEGG pathway maps	Identified proteins	Total protein in KEGG pathway maps	Percent of protein in Peptide Atlas*
00405 Phenazine biosynthesis	24	24	100.00
Xenobiotics biodegradation and metabolism			
00362 Benzoate degradation	22	35	62.86
00625 Chloroalkane and chloroalkene degradation	6	8	75.00
00361 Chlorocyclohexane and chlorobenzene degradation	1	6	16.67
00633 Nitrotoluene degradation	2	2	100.00
00643 Styrene degradation	8	12	66.67
00930 Caprolactam degradation	8	9	88.89
00626 Naphthalene degradation	2	2	100.00
2. Genetic Information Processing			
Transcription			
03020 RNA polymerase	4	4	100.00
Translation			
03010 Ribosome	55	68	80.88
00970 Aminoacyl-tRNA biosynthesis	27	90	30.00
Folding, sorting and degradation			
03060 Protein export	17	18	94.44
04122 Sulfur relay system	20	21	95.24
03018 RNA degradation	16	17	94.12
Replication and repair			
03030 DNA replication	14	15	93.33
03410 Base excision repair	12	13	92.31
03420 Nucleotide excision repair	8	8	100.00
03430 Mismatch repair	16	17	94.12
03440 Homologous recombination	22	23	95.65
3. Environmental Information Processing			
Membrane transport			
02010 ABC transporters	151	193	78.24
02060 Phosphotransferase system (PTS)	8	8	100.00
03070 Bacterial secretion system	64	92	69.57
Signal transduction			
02020 Two-component system	194	212	91.51
4. Cellular Processes			
Cellular community - prokaryotes			
02024 Quorum sensing	84	95	88.42
02025 Biofilm formation - <i>Pseudomonas aeruginosa</i>	102	113	90.27
Cell motility			
02030 Bacterial chemotaxis	46	46	100.00
02040 Flagellar assembly	41	46	89.13
5. Human Diseases			
Drug resistance: Antimicrobial			
01501 beta-Lactam resistance	24	27	88.89
01502 Vancomycin resistance	8	8	100.00
01503 Cationic antimicrobial peptide (CAMP) resistance	24	28	85.71

*KEGG pathways represented in a percentage higher than 90% are highlighted.

for environmental information processing (91% of two component system proteins), more than 88% of the ones related to quorum sensing and biofilm formation, and 89% of proteins responsible for antimicrobial resistance.

The contribution of each sample and the role of some of the proteins included in the PeptideAtlas in processes important for *P. aeruginosa* virulence or adaptation to the conditions studied are discussed below. Processes like iron acquisition, antibiotic resistance, quorum sensing response or response to external inputs, as the two component signal transduction systems, have been included in the analysis.

3.2.1. Proteins relevant to biofilm formation and maintenance

As previously mentioned, samples 4, 15, and 16 were grown as biofilms. When comparing the proteins identified in strains from CF patients and the proteome of PAO1 biofilm (sample 4), we found 1154 proteins common to both proteomes. Of them, 23 are related to biofilm formation, 33 to the quorum-sensing response, and 19 to antibiotic resistance. In sample 4, of the 15 unique proteins, we found ErcS (PA1976), ErbR (PA1978), EraR (PA1980), and PqqC (PA1987), proteins involved in ethanol oxidation, to be highly expressed in biofilm cells and probably involved in the ciprofloxacin resistance of biofilms [66]. TspR (PA4857), required for T3SS expression, biofilm formation,

and bacterial motility [67,68], was also found.

In sample 15, in which functional amyloid was induced, 4 of the amyloids, FapE (PA1952), FapD (PA1953), FapC (PA1954), and FapB (PA1955) were uniquely identified [43]. It is also noteworthy, but not surprising, that the genes involved in alginate biosynthesis (PA3540 to PA3551) were identified only in samples 15 and 16, as alginate is the main component of mucoid biofilms and of the *P. aeruginosa* biofilms encountered in CF patients [43,69]. Another group of proteins involved in biofilm formation, the Pel extracellular polysaccharide biosynthetic proteins PelD (PA3061), PelC (PA3062), and PelB (PA3063), were identified only in sample 15. These proteins are the back-up system for the extracellular polysaccharide biosynthetic process for single-species biofilm formation [70] when Psl polysaccharide biosynthesis fails. The proteins for Psl biosynthesis are present in almost all the samples (PA2231–PA2245). We found other proteins related to biofilm formation only in sample 15. These included IcmF3 (PA2361); Arr (PA2818), which is essential for biofilm induction and aminoglycoside resistance [71]; and CupE3 (PA4650) and CupE5 (PA4652), part of the cupE gene cluster that encodes a chaperone usher pathway that is homologous to the *Acinetobacter baumanii* Csu pili gene cluster and involved in the early and late stages of biofilm formation [72].

CdrA is a cyclic-di-GMP-regulated adhesin that reinforces the extracellular matrix of *P. aeruginosa* biofilm [73]. In accord with its function, it was identified in both biofilm proteomes (samples 4 and 15) and is also common to both secretomes (samples 2 and 12) and is thus determined to be secreted. Mep72 is a metalloendopeptidase [74] common to secretomes and to samples 18 and 19, and it has been described as secreted in biofilms [75].

As previously mentioned, the study of the proteome under anaerobic conditions (sample 3) is also interesting because during chronic infection of the CF lung, *P. aeruginosa* is able to grow and persist in a microaerobic to anaerobic environment [62], thus suffering drastic physiological changes. Among the 20 unique proteins in this sample, we highlight PscU (PA1690), PopN (PA1698), ExsB (PA1712), and PscC (PA1716), related to T3SS, which is induced in these conditions [76]. AtvR (PA2899), identified only in sample 15, is an important regulator of the *Pseudomonas* adaptation to hypoxia and crucial for survival in the host [77].

3.2.2. Proteins involved in secretion systems

Microorganisms often relate to their environments by secreting proteins, either by using classic secretion mechanisms, or through extracellular vesicles. Two secretomes with a large proportion of their proteins in common have been included in this study: a secretome obtained during exponential growth, with 12 unique proteins (sample 2), and a secretome sampled under the conditions for T3SS induction (sample 12), with 4 more. With respect to the cytoplasmic proteome under T3SS induction conditions (sample 14), proteins from glycine betaine catabolism [Cdhr (PA5389), DgcA (PA5398), GbcB (PA5411), SoxD (PA5417), and Soxa (PA5418)] were detected only in this sample. The generation of glycine betaine via carnitine catabolism allows carnitine to induce the expression of the virulence factor hemolytic phospholipase C, PlcH [78], which induces a proinflammatory response, suppresses oxidative burst in neutrophils, degrades pulmonary surfactant, and increases endothelial cell death [79] during infection. Phospholipase C precursor (PlcH, PA1249) was detected as a unique protein in the secretome under T3SS induction conditions, which included other virulence factors, such as AprA (PA1249) [80] and the lipase LipA (PA2862) [81]. Thus, the analysis of the proteome under T3SS induction conditions has provided important contributions to the *P. aeruginosa* PeptideAtlas. Proteins involved in T3SS regulation, such as MexT (PA2492) [82], also involved in the regulation of the expression of the multidrug efflux pump MexEF-OprN [83] were identified only in sample 15.

Analysis of the inner and outer membranes of *P. aeruginosa* PAO1 yielded 25 more unique proteins in datasets 7, 11, and 17 (Table 1). Ten

of them are related to transport through membranes or secretion systems. As an example, FleS (PA1098), identified in the outer-membrane proteome, is involved in the regulation of both mucin adhesion and motility [84]. The Tad proteins, involved in the Type II secretion system (PA2494–PA4303), have mostly been identified only in sample 15, and thus might be important for amyloid-induced biofilm growth.

The Type VI secretion system proteins and the proteins secreted are relevant to bacterial pathogenesis and cell survival in the host [85]. All these proteins (PA0070–PA0091) were identified in the amyloid-induced biofilm (sample 15) and many of them in the rest of the samples, together with several VgrG proteins, including VgrG4a (PA3294), VgrG4b (PA3486), VgrG5 (PA5090), and VgrG6 (PA5266), that are also secreted by this system [86].

3.2.3. Proteins involved in bacterial response to antibiotics

The study of antibiotic resistance suggests new methods to fight it [87]. Besides, antibiotics can challenge bacterial physiology in some aspects going beyond resistance to these compounds [22].

Thus, the analysis of the proteome under a sub-inhibitory concentration of tetracycline was also included and yielded 2465 proteins. Thirteen were unique, including GltR (PA3192), which is required for glucose transport and involved in the regulation of the expression of exotoxin A, a primary *P. aeruginosa* virulence factor [88]. Biofilms are more resistant to antibiotics, and proteins related to antibiotic resistance were uniquely identified in sample 15. These include PA5514, a probable beta lactamase; PoxB, regulated by AmpR, together with AmpC (PA4110) [89], identified in samples 15 and 16 and in the inner membrane proteome (sample 7); and CzcR (PA2523), which modulates antibiotic, Zn, Cd, and Co resistance [90].

3.2.4. Proteins involved in iron acquisition

Iron is a key nutrient that is often in limiting concentrations [91], a situation of particular relevance in the case of infection because bacteria must compete with the host's cells to acquire the iron required for growth. Consequently, siderophores and proteins involved in Fe transport are fundamental to bacterial survival.

FepB (PA4159), an iron-enterobactin transporter, and PfeA (PA2688), a ferric enterobactin receptor, were identified in the secretome of PAO1 (sample 2) [24]. As previously mentioned, PvdS (PA2426) has been identified in cells in stationary phase. PvdS is an RNA polymerase sigma-70 factor from the extracytoplasmic function sigma factor subfamily [92] that controls the synthesis of the siderophore pyoverdine. Its expression is regulated by FpvR (PA2388), a protein present in 6 datasets, and FpvA (PA2398), identified in PAO1 cytosolic extract (sample 9). An increased abundance of the pyoverdine synthesis machinery was observed in sample 15 [43]. In this sample there are several uniquely identified proteins related to iron, such as Pvdr (PA2389) and PvdT (PA2390), which are involved in pyoverdine biosynthesis and secretion together with OmpQ (PA2391) [93]; FemA (PA1910), a ferric-mycobactin receptor; FvbA (PA4156), a receptor homologous to the one from *Vibrio cholerae* and able to use vibriobactin as a source of iron in the absence of siderophores [94]; FiuR (PA0471), involved in the uptake of ferrioxamine B [95] with FiuI (PA0472), in sample 16; FvpG (PA2403) and the rest of the proteins of a putative ABC transporter complex (PA2404–PA2410); and FoxR (PA2467), part of the Fox cell-surface signaling (CSS) pathway employed by *P. aeruginosa* to sense and respond to the presence of ferrioxamine in the environment. FoxA (PA2466) is the receptor controlled by this system and was identified only in samples 15 and 13 (a stationary phase sample) [96]. These results demonstrate the importance of iron capture and transport for *P. aeruginosa* biofilm growth.

Iron acquisition is also important for survival outside of host. The heme oxygenase BphO (PA4116), necessary to yield iron from heme [97], was identified in the sample grown at 20 °C.

Table 3

Proteins in *Pseudomonas* PeptideAtlas involved in two component signal transduction systems.

Locus	Protein name
Tag	
PA0001	chromosomal replication initiator protein DnaA
PA0112	hypothetical protein
PA0173	probable methylesterase
PA0175	CheR2
PA0176	erotaxis transducer Aer2
PA0177	probable purine-binding chemotaxis protein
PA0178	probable two-component sensor
PA0179	probable two-component response regulator
PA0180	chemotactic transducer for trichloroethylene [positive chemotaxis], CttP
PA0296	Glutamylpolyamine synthetase
PA0298	Glutamylpolyamine synthetase
PA0408	twitching motility protein PilG
PA0409	twitching motility protein PilH
PA0410	twitching motility protein PilI
PA0411	twitching motility protein PilJ
PA0412	methyltransferase PilK
PA0413	component of chemotactic signal transduction system
PA0414	probable methylesterase
PA0415	probable chemotaxis protein
PA0463	two-component response regulator CreB
PA0464	two-component sensor CreC
PA0472	FluI
PA0518	cytochrome c-551 precursor
PA0652	transcriptional regulator Vfr
PA0749	hypothetical protein
PA0753	hypothetical protein
PA0754	hypothetical protein
PA0756	probable two-component response regulator
PA0757	probable two-component sensor
PA0766	serine protease MucD precursor
PA0884	C4-dicarboxylate-binding periplasmic protein
PA0886	probable C4-dicarboxylate transporter
PA0905	RsmA
PA0920	alanyl-phosphatidylglycerol synthase
PA0928	sensor/response regulator hybrid
PA0929	two-component response regulator
PA0930	two-component sensor
PA0931	ferric enterobactin receptor PirA
PA0958	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor
PA1092	flagellin type B
PA1097	transcriptional regulator FleQ
PA1098	two-component sensor
PA1099	two-component response regulator
PA1157	probable two-component response regulator
PA1158	probable two-component sensor
PA1179	two-component response regulator PhoP
PA1180	two-component sensor PhoQ
PA1183	C4-dicarboxylate transport protein
PA1251	chemotaxis transducer
PA1335	AauR
PA1336	AauS
PA1337	glutaminase-asparaginase
PA1339	AatP
PA1340	AatM
PA1341	AatQ
PA1342	putative acidic amino acid ABC transporter substrate-binding protein
PA1423	BdlA
PA1437	probable two-component response regulator
PA1438	probable two-component sensor
PA1455	sigma factor Flia
PA1456	two-component response regulator CheY
PA1458	probable two-component sensor
PA1459	probable methyltransferase
PA1460	MotC
PA1463	hypothetical protein
PA1464	probable purine-binding chemotaxis protein
PA1552	Cytochrome c oxidase, cbb3-type, CcoP subunit
PA1552	Cytochrome c oxidase, cbb3-type, CcoQ subunit
PA1553	Cytochrome c oxidase, cbb3-type, CcoO subunit
PA1554	Cytochrome c oxidase, cbb3-type, CcoN subunit

Table 3 (continued)

Locus	Protein name
Tag	
PA1555	Cytochrome c oxidase, cbb3-type, CcoP subunit
PA1556	Cytochrome c oxidase, cbb3-type, CcoO subunit
PA1557	Cytochrome c oxidase, cbb3-type, CcoN subunit
PA1561	erotaxis receptor Aer
PA1566	Glutamylpolyamine synthetase
PA1608	probable chemotaxis transducer
PA1611	hybrid sensor kinase
PA1633	potassium-transporting ATPase, A chain
PA1634	potassium-transporting ATPase, B chain
PA1635	potassium-transporting ATPase, C chain
PA1636	two-component sensor KdpD
PA1637	two-component response regulator KdpE
PA1638	glutaminase
PA1646	probable chemotaxis transducer
PA1736	probable acyl-CoA thiolase
PA1798	two-component sensor, ParS
PA1799	two-component response regulator, ParR
PA1856	probable cytochrome oxidase subunit
PA1930	probable chemotaxis transducer
PA1976	ErcS'
PA1980	response regulator EraR
PA2001	acetyl-CoA acetyltransferase
PA2002	conserved hypothetical protein
PA2018	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexY
PA2019	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein MexX precursor
PA2040	Glutamylpolyamine synthetase
PA2051	transmembrane sensor
PA2523	CzcR
PA2526	MuxC
PA2527	MuxB
PA2528	MuxA
PA2553	probable acyl-CoA thiolase
PA2561	CtpH
PA2573	probable chemotaxis transducer
PA2583	probable sensor/response regulator hybrid
PA2586	response regulator GacA
PA2652	methyl-accepting chemotaxis protein
PA2654	TlpQ
PA2688	Ferric enterobactin receptor, outer membrane protein PfeA precursor
PA2809	two-component response regulator, CopR
PA2810	two-component sensor, CopS
PA2824	surface attachment and growth sensor hybrid, SagS
PA2920	probable chemotaxis transducer
PA2978	ptpA; phosphotyrosine protein phosphatase
PA3045	Two-component response regulator, RocA2
PA3345	Histidine phosphotransfer protein HptB
PA3346	HptB-dependent secretion and biofilm regulator HsbR
PA3347	HptB-dependent secretion and biofilm anti anti-sigma factor HsbA
PA3348	CheR1
PA3349	probable chemotaxis protein
PA3351	FlgM
PA3356	Glutamylpolyamine synthetase
PA3409	transmembrane sensor
PA3471	probable malic enzyme
PA3476	autoinducer synthesis protein RhlI
PA3540	GDP-mannose 6-dehydrogenase AlgD
PA3552	ArnB
PA3658	protein-PII uridylyltransferase
PA3702	WspR
PA3703	probable methylesterase
PA3704	probable chemotaxis sensor/effector fusion protein
PA3705	hypothetical protein
PA3706	probable protein methyltransferase
PA3707	hypothetical protein
PA3708	probable chemotaxis transducer
PA3714	probable two-component response regulator
PA3872	respiratory nitrate reductase gamma chain
PA3873	respiratory nitrate reductase delta chain
PA3874	respiratory nitrate reductase beta chain
PA3875	respiratory nitrate reductase alpha chain
PA3878	two-component sensor NarX
PA3879	two-component response regulator NarL
PA3900	transmembrane sensor

(continued on next page)

Table 3 (continued)

Locus	Protein name
PA3925	probable acyl-CoA thiolase
PA3929	cyanide insensitive terminal oxidase
PA3930	cyanide insensitive terminal oxidase
PA3946	two-component sensor RocS1
PA3948	Two-component response regulator RocA1
PA4080	probable response regulator
PA4133	cytochrome c oxidase subunit (cbb3-type)
PA4290	probable chemotaxis transducer
PA4307	chemotactic transducer PctC
PA4309	chemotactic transducer PctA
PA4310	chemotactic transducer PctB
PA4429	probable cytochrome c1 precursor
PA4430	probable cytochrome b
PA4431	probable iron-sulfur protein
PA4462	RNA polymerase sigma-54 factor
PA4493	RoxR
PA4494	RoxS
PA4520	probable chemotaxis transducer
PA4525	type 4 fimbrial precursor PilA
PA4546	two-component sensor PilS
PA4547	two-component response regulator PilR
PA4616	C4-dicarboxylate-binding protein
PA4633	probable chemotaxis transducer
PA4776	PmrA: two-component regulator system response regulator PmrA
PA4777	PmrB: two-component regulator system signal sensor kinase PmrB
PA4785	probable acyl-CoA thiolase
PA4886	probable two-component sensor
PA4915	probable chemotaxis transducer
PA4954	chemotaxis protein MotA
PA4974	probable outer membrane protein precursor
PA5072	McpK
PA5119	glutamine synthetase
PA5124	two-component sensor NtrB
PA5125	two-component response regulator NtrC
PA5165	DctB
PA5166	DctD
PA5167	DctP
PA5168	DctQ
PA5199	AmgS
PA5200	AmgR
PA5261	alginate biosynthesis regulatory protein AlgR
PA5262	FimS
PA5360	two-component response regulator PhoB
PA5361	two-component sensor PhoR
PA5369	phosphate ABC transporter, periplasmic phosphate-binding protein, PstS
PA5483	two-component response regulator AlgB
PA5484	KinB
PA5508	Glutamylpolyamine synthetase homologue
PA5511	MifR
PA5512	MifS
PA5522	Glutamylpolyamine synthetase

3.2.5. Proteins in two-component signal transduction systems

Two-component signal transduction systems in *P. aeruginosa* enable the bacteria to sense the environment and respond and adapt its behaviour to better suit prevailing conditions. *P. aeruginosa* encodes genes for over 60 two-component regulatory systems, being around 10% of the *P. aeruginosa* genome dedicated to these regulatory networks [61]. These genes encode sensor kinases (SKs) and response regulators (RRs). A total of 212 proteins are annotated in the Two-component System pathway in the KEGG database, being 194 of them (91.5%) included in *Pseudomonas* PeptideAtlas. These proteins are listed in Table 3.

3.2.6. Other proteins with relevance for *P. aeruginosa* infection

Another group of proteins important for *P. aeruginosa* virulence are the Alp proteins, which constitute a self-lysis pathway that enhances virulence in the host [98]. Only three Alp proteins were identified, two of them unique to samples 15 (AlpA, PA0907) and sample 2 (vesicle and exoproteome; AlpE [PA0911]).

4. Conclusions

This work describes the results of analyses of widely diverse *P. aeruginosa* proteomes. The datasets used to build the *P. aeruginosa* PeptideAtlas include the proteomes obtained from strain PAO1 grown under various conditions and from different subcellular fractions (cytosol, membranes, and secretomes). They were analyzed using different proteomic techniques of fractionation, labeling, and MS analysis. It also incorporates datasets from other authors, including one comprising proteins from clinical *P. aeruginosa* strains. All this has allowed the widest coverage of *P. aeruginosa* PAO1 proteins and the highest published coverage by a bacterial PeptideAtlas, higher than those of *Leptospira interrogans* (65.9%) [99], *Halobacterium salinarum* (62.7%) [100], and *Streptococcus pyogenes* (55.7%) [101]. Only 8 PeptideAtlas builds are from bacteria, including 6 derived from pathogens, highlighting the importance of the present work. Quantitative comparative analysis of the level of expression of specific proteins traditionally relies in immunodetection techniques as Western blot or ELISA, amenable to any molecular biology laboratory. However, this is feasible just when the number of proteins to analyze is low and the required antibodies are available. Targeted proteomics is a useful approach when the number of proteins to analyze is large or when the needed antibodies are not available. However, targeted proteomics requires comprehensive information on informative peptides of bacterial proteomes as that provided in the current work. The Atlas here described is hence also a valuable resource for the selection of candidate proteotypic peptides for targeted proteomics experiments via selected reaction monitoring (SRM) or parallel reaction monitoring (PRM).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2021.104192>.

Credit author statement

All the authors have contributed significantly to the manuscript.

Declaration of Competing Interest

None.

Acknowledgements

This study was supported by RTI2018-094004-B-100 from the Spanish Ministry of Science and Innovation, InGEMICS-CM B2017/BMD3691 from the Comunidad de Madrid, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0011), and PRB3 (PT17/0019/0012) from the ISCIII. InGEMICS-CM, REIPI, and PRB3 are co-financed by the European Development Regional Fund (ERDF) "A way to achieve Europe". These results are lined up with the Human Infectious Diseases HPP initiative from the Human Proteome Project (HID-HPP). The proteomics analyses were performed in the Proteomics facilities of Centro Nacional de Biotecnología (CNB) and Complutense University of Madrid (UCM), both members of the ProteoRed-ISCIII network. This work was funded in part by the National Institutes of Health grants R01GM087221 (EWD/RLM) and R24GM127667(EWD), and by the National Science Foundation grant DBI-1933311 (EWD) and Award Number 1920268 (RLM).

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