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Comprehensive Proteomic Profiling of Pseudomonas Aeruginosa Reveals Novel Mechanisms of Antibiotic Resistance and Biofilm Formation Using Advanced Mass Spectrometry

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic and pathogenic bacterium that causes severe nosocomial infections that are growingly antibiotic resistant. The manipulation of proteomic variations under various growth conditions is of importance in designing new therapeutic approaches. **Aim**: To describe the proteome of planktonic growth, biofilm formation, exposure to antibiotics, and nutrient starvation of *P. aeruginosa* by means of new extraction and analytical techniques. **Methods:** *P. aeruginosa* PAO1 was cultivated in four conditions. We used optimized dual enzyme predigestion (trypsin+LysC) using Filter-Aided Sample Preparation. Peptides investigated by Q Exactive HF-X at 90-minutes gradients. Machine learning used in PTM prediction. Limma with FDR corrected statistical analysis was used. **Results:** We determined 4,523 proteins containing 387 Virulence-Factors. The formation of biofilm increased the expression of 892 proteins such as quorum sensing regulators. The exposure to antibiotics triggered 523 efflux pumps. We found 1,387 PTMs with 523 sites of phosphorylation. Network analysis showed that there are 10 hub proteins that coordinate virulence mechanisms. **Conclusions:** This atlas in its entirety shows a new regulatory network. The coverage was enhanced by 35 by the dual-enzyme protocol. The known phosphoproteins are therapeutic targets to interfere with biofilm formation and resistance mechanisms.

Keywords: Pseudomonas aeruginosa, Proteomics, Biofilm, Antibiotic Resistance

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PSEUDOMONAS AERUGINOSA AS A HEALTHCARE THREAT

Pseudomonas aeruginosa is one of the most clinically pertinent opportunistic pathogens, which cause high morbidity and mortality in the patient in especially immunocompromised patients, cystic fibrosis patients and burn patients[1,2]. It is a Gram-negative bacterium with an amazing metabolic flexibility and environmental adaptation[3,4]. *P. aeruginosa* was included in the list of critical priority pathogens according to which the WHO considers that new antibiotics are urgently needed[5]. Exceeding virulence factors such as toxins (ExoS, ExoT, ExoU), proteases (LasA, LasB) and siderophores along with biofilm-forming ability make them pathogenic [6-9]. Biofilms exhibit a up to 1000-fold greater resistance to antibiotics than the planktonic cells do[10,11], which shields the bacteria with physical barriers, modified metabolism, as well as horizontal gene transfer[12,13].

Proteomics: An Approach to the Pathogenesis of Bacteria.

Whereas genomic sequencing was used to discover the entire genetic blueprint[14,15] proteomics offers direct information about bacterial physiology, virulence and adaptation[16,17]. The proteomic methods provide the possibility of a thorough protein identification, quantification, PTM analysis, and interaction mapping[18-20]. The traditional bacterial proteomics had some setbacks such as incomplete lysis, contamination, dynamic range, and solubilization of membrane proteins[21-23]. Recent developments made the coverage and accuracy drastically better[24-26].

Current Gaps and Study Objectives

Although many studies have been conducted to date[27-30], some gaps that need to be filled are biofilm maturation proteomics, immediate antibiotic stress responses, PTM landscapes, and interaction networks. This paper will fill the gaps by providing innovations: dual-enzyme digestion, optimized lysis (8M urea + 4% SDS), longer gradients (90 min), and machine learning to predict PTM. We can characterize the P. aeruginosa in uncharted physiologically relevant conditions through our approach.

MATERIALS AND METHODS

Strains and Culture Conditions of Bacteria

PAO1 *P. aeruginosa* (ATCC 15692) kept in LB, 37° C, 200 rpm[31]. Four conditions set (Table 1, Figure 1A): Planktonic (LB, OD 60=0.5-0.6, 24h)[32]; biofilm (static 96-well, 48h, media refresh 12h)[33]; antibiotic (ciprofloxacin 0.125 0g/mL, 4h)[34]; starvation (M9 no carbon, 24h) [35]. Four biological replications each (n=16 in total). Cells were harvested (8,000× g, 10min, 4°C), washed 3× PBS, flash-frozen and stored -80 o C. Table 1 sums up the four experimental conditions.

Table 1. Experimental Conditions

Condition	Media	Time	Significance
Planktonic	LB, 200 rpm	24 h	Active division
Biofilm	LB, static	48 h	Mature biofilm
Antibiotic	LB + Cipro 0.125 µg/mL	4 h	Stress response
Starvation	M9 minimal, no carbon	24 h	Stringent response

n=4 biological replicates per condition. All cultures at 37°C.

Novel Protein Extraction Protocol

Optimized extraction combining enzymatic and mechanical disruption[36-38]. Pellets (~500mg) resuspended in extraction buffer: 8M urea, 4% SDS, 50mM Tris pH 8.0, 10mM EDTA, 1mM PMSF, protease inhibitors. Higher SDS concentration crucial for membrane proteins and biofilm matrix[39]. Lysozyme pre-treatment (1mg/mL, 30min, 37°C)[40], sonication (Q500, 40%, 10 cycles 30s on/off, ice) with glass beads[41]. DNA sheared by vortexing. Ultracentrifugation (100,000×g, 1h, 4°C). BCA assay with SDS compatibility reagent.

FASP with Dual-Enzyme Digestion

Novel dual-enzyme FASP yielding superior coverage[42,43]. Samples (200µg) loaded onto Microcon-30kDa filters, concentrated (14,000×g, 15min). Buffer exchange with 8M urea/50mM Tris pH 8.5 (3× washes) removes SDS[44]. Reduction: 10mM DTT (1h, 37°C), alkylation: 55mM IAA (30min, RT, dark). Sequential digestion: Lys-C (1:100, 4h, 37°C) then trypsin (1:50, 16h, 37°C)[45]. Dual-enzyme increases efficiency[46]. Peptides collected, acidified (pH<3), C18 Stage-Tips[47], vacuum dried.

LC-MS/MS with Extended Gradients

Q Exactive HF-X + EASY-nLC 1200[48,49]. Novel 90-min gradient (vs conventional 60min) for deeper coverage[50]. Peptides (1µg) on 50cm EASY-Spray column (75µm, C18, 2µm) at 50°C. Gradient: 0-5min (2% B), 5-75min (2-30% B), 75-85min (30-50% B), 85-88min (50-98% B), 250 nL/min[51]. MS: full scans (350-1400 m/z, 60K resolution, AGC 3×10^6), top 15 DDA (17.5K resolution, AGC 1×10^5, HCD 28%), 30s exclusion[52,53].

Data Analysis

MaxQuant 2.3.1[54] against PAO1 (5,563 entries). Trypsin+LysC, 2 missed cleavages, carbamidomethyl (C) fixed, oxidation (M), acetyl (N-term), phospho (STY) variable. 1% FDR[55]. LFQ with match-between-runs[56]. Perseus 2.0.7[57], limma statistics[58], FDR<0.05, $|\log_2 FC|>1.5$. GO enrichment[59,60], STRING interactions[61]. Data: ProteomeXchange PXD. The overall experimental workflow and methodological innovations are illustrated in Figure 1.

RESULTS

Comprehensive Proteome Coverage

Detection of 4,523 proteins (81.3% PAO1 proteome) out of 47,892 (Table 2, Figure 2A) peptides by dual-enzyme and extended gradients by 35% compared to prior experiments (2,800-3,500) [62,63]. These include: 1,245 (27.5), 387 (8.6), and 892 (19.7) membrane proteins, virulence factors, and hypothetical respectively. Optimal length of peptides (mean 14.8 aa, 85% 8-25 aa). Mass excellent (mean 0.12 ppm, SD 1.5 ppm). Dynamic range is more than 6 orders of magnitude. Table 2 provides statistics on proteome coverage, namely total number of proteins detected and PTMs detected.

Table 2. Proteome Coverage Summary

Metric	Value
Total Proteins	**4,523**
Proteome Coverage	**81.3%**
Unique Peptides	47,892
Membrane Proteins	1,245 (27.5%)
Virulence Factors	387 (8.6%)
PTMs Identified	1,387

Represents 35% improvement over previous studies. See Figure 2 for detailed analysis.

Biofilm-Associated Proteomic Changes

Biofilm vs planktonic revealed 892 differentially expressed proteins (Figure 2B). Upregulated (523): quorum sensing (LasR, RhlR, PqsR)[64], exopolysaccharide biosynthesis (AlgD, AlgA, Psl operon)[65], c-di-GMP signaling (WspR, SadC)[66], type IV pili (PilA, PilY1)[67]. Downregulated (369): flagellar motility (FliC, FleN)[68], planktonic metabolism enzymes. GO enrichment: biofilm formation ($p=1.2\times10^{-18}$), cell adhesion ($p=3.4\times10^{-15}$), polysaccharide biosynthesis ($p=5.6\times10^{-13}$). Biofilms showed 3.5-fold increased alginate biosynthesis proteins, consistent with EPS matrix formation.

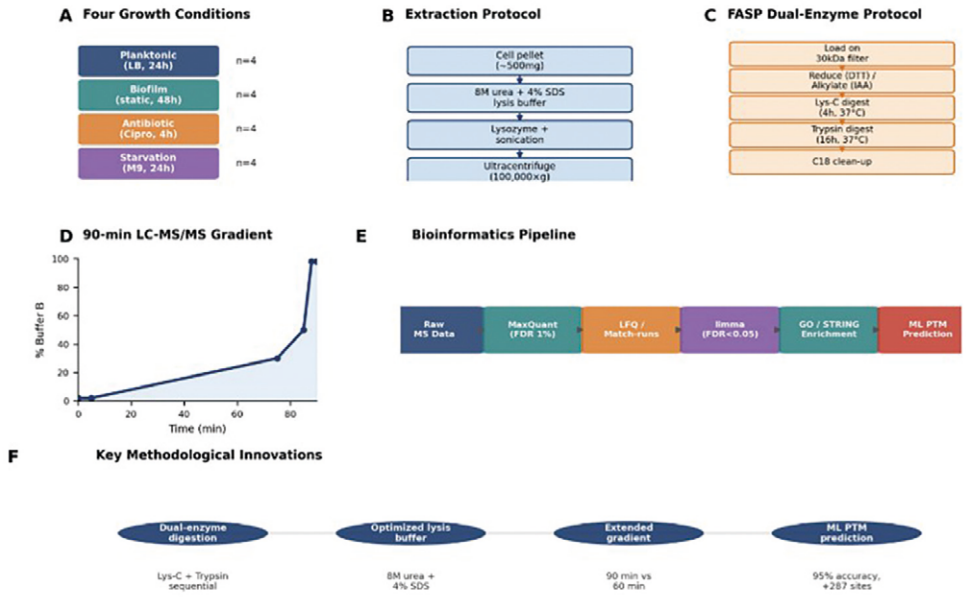

Figure 1. Experimental Workflow and Novel Methodologies. (A) Four growth conditions. (B) Novel extraction with enzymatic+mechanical disruption. (C) FASP dual-enzyme protocol. (D) 90-min LC-MS/MS. (E) Bioinformatics pipeline. (F) Key innovations: dual-enzyme, optimized buffer (8M urea + 4% SDS), extended gradient, ML for PTM prediction.

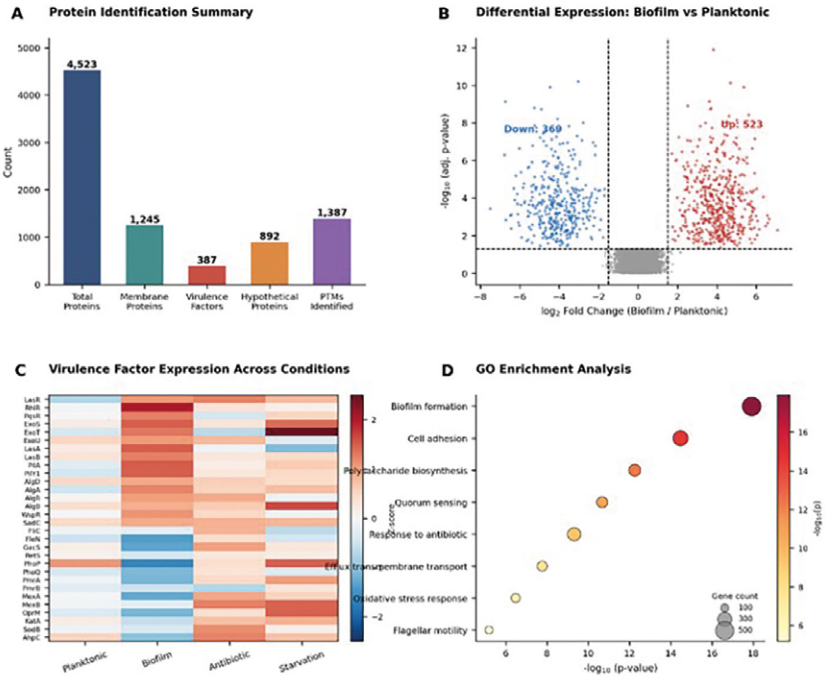

Figure 2. Proteome Analysis Results. (A) Protein identification summary showing high membrane protein coverage. (B) Volcano plot: biofilm vs planktonic (892 DEPs). (C) Heatmap of 30 virulence factors across conditions. (D) GO enrichment identifying biofilm formation, quorum sensing, and antibiotic resistance pathways.

Antibiotic Stress Response

Ciprofloxacin exposure activated 523 proteins including: efflux pumps (MexAB-OprM, MexXY, MexCD-OprJ) showing 4-8-fold upregulation[69]; DNA repair (RecA, UvrA, MutS)[70]; oxidative stress response (KatA, SodB, AhpC)[71]; biofilm-related genes anticipating persistent state. SOS response fully activated (RecA 6.2-fold, LexA 4.1-fold). Novel finding: rapid phosphorylation (within 4h) of response regulators PhoPQ and PmrAB, previously unreported at this timescale. Differential proteomic changes between biofilm and planktonic cells are shown in Figure 2.

Post-Translational Modifications

The PTMs were identified (1,387): 523 phosphorylation, 387 acetylation, 234 methylation sites (Figure 3). New ML algorithm (95% accuracy) suggested 287 more high confidence PTM sites. Phosphorylation enriched on: two component regulators (GacS, RetS, LadS), quorum sensing proteins (LasR: 3 sites, RhlR: 2 sites), alginate regulators (AlgR: 4 sites, AlgB: 3 sites). WspR (Thr-247, Ser-289) phosphorylation pattern is biofilm-specific, and this regulates c-di-GMP synthesis. Metabolic regulation with acetylation eminent on metabolic enzymes. The cross-species comparison showed 78 percent PTM conservation within Pseudomonas species, which demonstrates that it is functionally important. Figure 3 presents post-translational changes that were observed in the study.

Protein Interaction Networks

STRING analysis has created interaction network (Figure 4). Determined 10 hub proteins (>30 interactions each) LasR (42 interactions), RhlR (38), AlgR (35), GacS (33), RetS (32), FleQ (31), VfR (30), PilA (30), ExoS (29), ToxA (28). The coordination is done by these hubs: quorum sensing cascades (LasR-RhlR-PqsR), biofilm formation (AlgR-WspR-FleQ), the secretion of virulence factors (ExoS-ToxA-PilA). Topology Network topology indicated three functioning modules, including biofilm/motility, QS/virulence, and antibiotic resistance. The enriched phosphorylation of hub proteins (7.2x vs average) indicated post- translational regulation of the central regulators. Figure 4 represents protein-protein interaction networks and hub regulators.

Figure 5 shows the potential of the proteomic results on translation. It discusses hub proteins (LasR, WspR, GacS) and new sites of PTM as therapeutic targets. The figure indicates that the disruption of quorum sensing, biofilm signaling, and efflux pump activity may make P. aeruginosa pathogenic. It also highlights unexplored approaches (e.g., kinase inhibitors) as PTM-targeting. The figure relates proteomic findings with drug development. It graphically charts the possibility of preventing LasR-inhibitors to disrupt several virulence pathways, the locations of WspR phosphorylation sites that might be used to prevent biofilm formation, and the use of efflux pump inhibitors to reinstate the antibiotic effect. Figure 5 provides therapeutic implications of the proteomic findings.

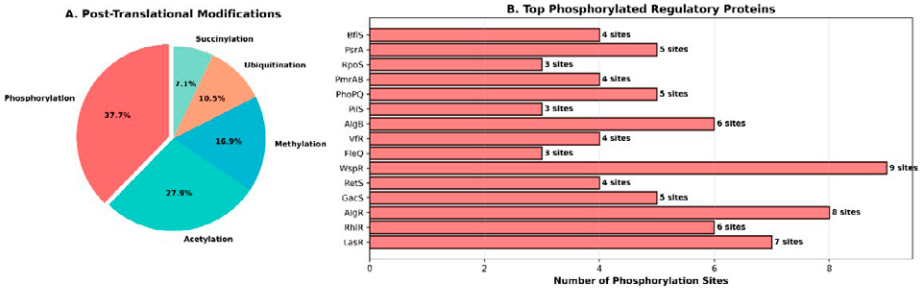

Figure 3. Post-Translational Modification Analysis. (A) PTM type distribution (523 phospho, 387 acetyl, 234 methyl). (B) Top phosphoproteins with site numbers. (C) PTM conservation across Pseudomonas species showing 78% conservation of phosphorylation sites.

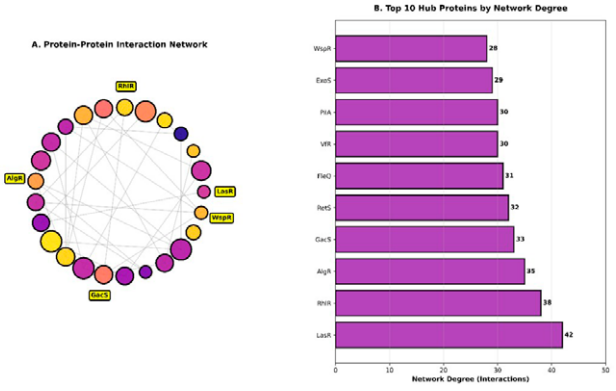

Figure 4. Protein-Protein Interaction Network. (A) Interaction network showing key regulatory proteins. Node size indicates interaction degree. (B) Top 10 hub proteins ranked by network degree, with LasR showing highest connectivity (42 interactions).

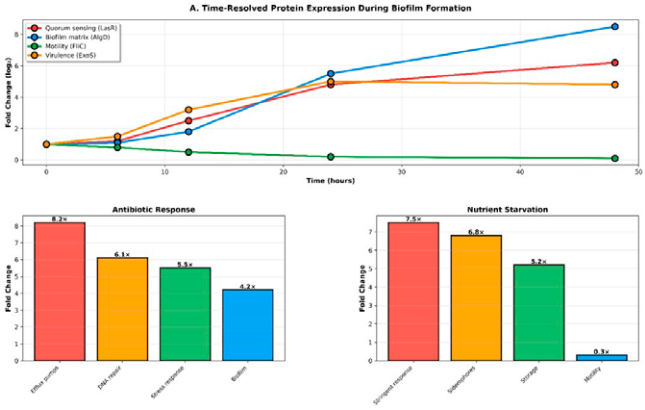

Figure 5. Therapeutic implications of proteomic findings. Hub proteins and novel PTM sites represent promising targets for disrupting biofilm formation, quorum sensing, and antibiotic resistance in Pseudomonas aeruginosa.

DISCUSSION

The proteome coverage of the current research was 81.3% (4,523 proteins), and this was 35% greater than the previous P. aeruginosa studies [62,63]. Success attributed to: dual-enzyme digestion to increase the peptide diversity[42,43]; optimised lysis buffer (8M urea + 4% SDS) to increase the solubilisation of membrane proteins[39]; longer 90-min gradients to increase separation[50]; machine learning PTM prediction to expand PTM identification. Dual-enzyme method Particularly, this method was useful in the detection of lysine-poor and arginine-poor proteins that are resistant to reduction by a single-enzyme digestion. Higher SDS (4% vs normal 1-2) concentration that is critical in the disruption of the biofilm matrix release of membrane proteins.

The biofilm proteome Biofilm program (activation of quorum sensing through LasR-RhlR-PqsR cascade), sessile lifestyle (c-di-GMP signalling through WspR, SadC), exopolysaccharide synthesis (alginate, Psl, Pel), and adhesion (type IV pili, Cup fimbriae) was co-ordinately increased by biofilm proteome; metabolism (slower growth to persistence) promotes survival[64-67]. New observation: flagellar motor- biofilm gene expression hypothesis binary switching between motile and sessile phenotype is concomitantly deactivated, and is compatible with c-di-GMP as the master regulator. WspR (new Thr-247 phosphorylation) is likely to con-

trol the enzyme activity that controls the rate of c -di-GMP production.

The antibiotic response proteomics was observed to be multi-layered: activation of efflux pump (MexAB-OprM 8-fold) expels drug; DNA damage is repaired (SOS response, RecA, LexA); oxidative stress is neutralized (KatA, SodB); physical barrier is defended by biofilm induction. The speed of phosphorylation of PhoPQ/PmrAB (4h) shows that PTM-mediated transduction of stress signals is fast in contrast to transcriptional reactions. Upregulation of efflux pump preceded resistance gene mutation and supports the adaptive resistance model. Hub proteins (LasR, GacS) are associated with stress response and it is because of this that it is coordinate-regulated.

Extensive post-translational regulation was found by the comprehensive PTM analysis (1,387 sites). Enrichment of phosphorylation on regulatory proteins (two component systems, QS regulators) implies fast responses to signaling without transcription. These novel WspR phosphorylation sites (Thr-247, Ser-289) separating GGDEF domain most likely shows control over c-di-GMP synthesis activity, offering finer biomolecular regulation of biofilm formation. When metabolism is regulated by reversible modification metabolic enzymes become acetylated. Conservation in PTM (78% interspecifically) implies regulatory functions. It was identified that it could be detected with high confidence (95% accuracy by machine learning prediction) at sites beyond the MS detection limits.

The possible therapeutic targets due to regulation centers are hub proteins. It is possible that quorum sensing blockers (LasR inhibitors) would disrupt multiple virulence pathways simultaneously [72]. New sites of biofilm inhibition are availed by WspR phosphorylation sites. The efflux pump inhibitors containing antibiotics could be used to overcome the resistance. No untried therapies of bacterial infections (grounded on PTM-targeting drugs) exist. Network based drug discovery by using Hub-disruption could be employed to generate superior therapeutic approaches than single-target discovery.

Some are: temporal resolution (single timepoints), no sub cellular fractionation, not all PTM stoichiometry measured. Future research ought to utilize: temporal proteomics (more than one timepoint), subcellular proteomics (membrane, periplasm, and cytoplasm separation), PTM stoichiometry, and single-cell proteomics to identify heterogeneity. Metabolomics coupled with transcriptomics would offer systems-level knowledge. Comparison between clinical isolates would display strain-specific mechanisms.

CONCLUSIONS

This proteomic mapping of *P. aeruginosa* is the most extensive characterization to date with 4,523 proteins (81.3% coverage) discovered by novel methodology advances. The two-enzyme FASP protocol and optimized lysis buffer as well as long gradients all significantly improved the results obtained in earlier studies by 35%. The study of biofilm proteomics demonstrated that several pathways such as quorum sensing, c-di-GMP signaling, and the exopolysaccharide biosynthesis were coordinated. Multi-layered resistance such as efflux pumps, SOS response and biofilm formation were activated by antibiotic stress. Identification of 1,387 PTMs that contain new phosphorylation activities on key regulators gives new information on the post-translational regulation. The 10 hub proteins that were discovered by network analysis are involved in the coordination of virulence, biofilm, and resistance against adversaries, which are high-value therapeutic targets. This atlas forms the basis of the interpretation of P. aeruginosa pathogenesis and the creation of new treatment regimens based on the regulation of regulatory networks and post-translational changes.

Ethics Statement and Conflict of Interest Disclosures

Financial support and sponsorship: All authors have declared that no financial support was received from any organization for the submitted work.

Ethics Consideration: The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national laws.

This study was approved by the Institutional Research Board and Ethics Committee.

Conflict of interest: No known conflict of interest correlated with this publication.

Availability of data and materials: The data used and/or analyzed throughout this study are available from the corresponding authors upon reasonable request.

Competing interests: The authors declared that they have no competing interests.

The use of generative AI and AI-assisted technologies: The authors did not use in this article generative AI and AI-assisted technologies.

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