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Phage-Derived Endolysin PlyPM1 as a Rapid Enzybiotic for Detection and Lysis of Proteus mirabilis in Clinical Urine Samples.

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Abstract

Proteus mirabilis is a formidable nosocomial pathogen notorious for causing catheter-associated urinary tract infections (CAUTIs), catheter encrustation, and urolithiasis due to its potent urease activity and biofilm formation. The rise of multidrug-resistant (MDR) *P. mirabilis* strains, including those resistant to broad-spectrum cephalosporins and carbapenems, underscores the critical need for novel, rapid diagnostic and therapeutic strategies. While whole bacteriophages have shown therapeutic promise, their use in diagnostics can be limited by host range specificity and regulatory complexities. Bacteriophage endolysins—highly evolved enzymes that degrade the bacterial peptidoglycan cell wall from within—offer a superior alternative as precision "enzybiotics." This study describes the identification, recombinant production, and functional characterization of a novel endolysin, **PlyPM1**, derived from a *Myoviridae* phage specific to *P. mirabilis*, and evaluates its dual utility for rapid pathogen detection and direct bacteriolysis in clinical urine matrices.The gene encoding PlyPM1 was identified from the genome of the lytic phage φPM-M1, isolated from wastewater. Bioinformatic analysis revealed a modular structure featuring a conserved N-terminal catalytic domain (predicted CHAP [cysteine, histidine-dependent amidohydrolase/peptidase] domain) and a C-terminal cell wall binding domain (CBD) with high specificity for *P. mirabilis* surface epitopes. The recombinant 28-kDa enzyme was overexpressed in *E. coli* BL21(DE3), purified via nickel-affinity chromatography, and demonstrated potent, rapid lytic activity against

all 25 clinical *P. mirabilis* isolates tested, including 18 MDR strains. PlyPM1 exhibited remarkable specificity, showing no activity against other common uropathogens (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) or commensal urinary flora (*Lactobacillus spp.*), thereby minimizing dysbiosis risk. In a standardized turbidity reduction assay, 50 µg/mL of PlyPM1 achieved a >3-log reduction in *P. mirabilis* planktonic cell counts in phosphate-buffered saline within 30 minutes at 37°C.Critically, PlyPM1 retained significant lytic activity in synthetic and human urine, overcoming the inhibitory effects of urea and ionic strength that often hinder antimicrobial agents. Furthermore, PlyPM1 efficiently disrupted pre-formed *P. mirabilis* biofilms on silicone catheter surfaces, reducing viable biofilm-embedded cells by 99.8% after a 2-hour treatment, as quantified by confocal laser scanning microscopy and colony-forming unit counts.Leveraging its specificity and rapid activity, we developed a proof-of-concept **PlyPM1-based detection assay**. The assay utilizes PlyPM1 conjugated to fluorescent microspheres. Upon binding to *P. mirabilis* cells in spiked urine samples, the complex catalyzes the release of a fluorescent signal from a quenched peptidoglycan substrate. This method allowed for the sensitive detection of *P. mirabilis* at concentrations as low as 10^3 CFU/mL directly in urine within 25 minutes, outperforming standard culture-based identification (requiring 24-48 hours) and showing comparable sensitivity to PCR but without the need for nucleic acid extraction.

In conclusion, PlyPM1 represents a first-in-class, phage-derived enzybiotic with dual diagnostic and therapeutic potential against *P. mirabilis* uropathogens. Its rapid, specific bacteriolytic activity in urine and against biofilms positions it as an excellent candidate for development into (1) a novel therapeutic adjunct for MDR CAUTIs, and (2) a rapid point-of-care diagnostic tool to guide targeted antibiotic therapy, thereby addressing both treatment and antimicrobial stewardship challenges in clinical urology.

Introduction

Proteus mirabilis stands as a formidable and resilient Gram-negative pathogen within the clinical landscape, particularly notorious for its role in complicated urinary tract infections (UTIs). This bacterium is frequently implicated in catheter-associated urinary tract infections (CAUTIs), accounting for a significant proportion of nosocomial infections worldwide (Armbruster et al., 2018). The pathogenic success of P. mirabilis is mechanistically rooted in several virulence factors, including potent urease production, swarming motility, fimbrial adhesion, and robust biofilm formation on both biological and abiotic surfaces, such as urinary catheters (Schaffer & Pearson, 2017). Urease activity hydrolyzes urea to ammonia, elevating urinary pH and precipitating struvite and apatite crystals. This process leads to catheter encrustation and blockage, fosters stone formation, and provides a protected niche for biofilm establishment, creating a persistent source of infection that is notoriously difficult to eradicate (Jones et al., 2021).The clinical management of P. mirabilis infections faces an escalating crisis due to the rapid and widespread emergence of antimicrobial resistance (AMR). This pathogen exhibits intrinsic resistance to tetracycline, colistin, and tigecycline, and readily acquires resistance genes via plasmids and integrons, leading to multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (Hoban et al., 2020). Notably, the global spread of extended-spectrum beta-lactamase (ESBL)-producing and, more alarmingly, carbapenem-resistant P. mirabilis isolates leaves clinicians with severely limited therapeutic options, often resorting to last-resort antibiotics like polymyxins or novel combination therapies (Pitout et al., 2019). This resistance trajectory, coupled with the bacterium's ability to form recalcitrant biofilms that confer up to 1,000-fold increased tolerance to antibiotics, underscores

the urgent and unmet need for novel, targeted antimicrobial strategies that can circumvent traditional resistance mechanisms (Li et al., 2022).In this context, bacteriophage (phage) therapy has resurged as a promising antimicrobial alternative. Phages are viruses that specifically infect and lyse bacterial hosts, offering several theoretical advantages: narrow host range that spares the commensal microbiota, self-replication at the site of infection, and the ability to penetrate biofilms (Kortright et al., 2019). While research into whole phages targeting P. mirabilis is advancing, several translational challenges remain, including bacterial resistance to phage infection via receptor modification, the potential for temperate phages to carry virulence genes, immunogenicity concerns with viral particle administration, and regulatory hurdles for complex biological entities (Strathdee et al., 2023).These limitations have catalyzed a shift in focus toward phage-derived enzybiotics—specifically, endolysins. Endolysins are highly evolved peptidoglycan hydrolases produced by phages at the end of their lytic cycle to degrade the bacterial cell wall from within, facilitating the release of progeny virions (Murray et al., 2021). When applied exogenously as recombinant proteins, these enzymes rapidly cleave critical bonds in the peptidoglycan of Gram-positive bacteria, causing osmotic lysis and death. The use of endolysins against Gram-negative pathogens like P. mirabilis was historically considered less feasible due to the protective outer membrane. However, this barrier can be overcome by the enzyme's own destabilizing activity, chelating agents, or peptide sequences that permeabilize the outer membrane, or by leveraging the innate permeability of bacteria under physiological stress (Defraine et al., 2018). Endolysins offer distinct advantages over whole phages: they are protein-based biologics with a lower risk of resistance development (as they target conserved peptidoglycan structures), have no capacity for self-replication or gene transfer, exhibit a faster and more predictable lytic action, and can be engineered for enhanced stability, activity, and specificity (Schmelcher et al., 2020).The potential applications of endolysins extend beyond direct therapeutics into the realm of rapid diagnostics. Conventional detection of P. mirabilis in clinical urine samples relies on culture-based methods, which are slow (24-48 hours) and can delay targeted therapy. Molecular methods like PCR are faster but require specialized equipment and technical expertise, limiting point-of-care utility (Bourbeau & Ledeboer, 2019). Endolysins, with their inherent high specificity for cell wall components of a particular bacterial species, can be harnessed as biorecognition elements. By conjugating an endolysin to a signal reporter (e.g., fluorescent dye, enzyme, or nanoparticle), it can be used to create rapid, sensitive, and specific assays that detect and even quantify viable pathogens directly in complex clinical samples like urine within minutes (Fischetti, 2018). This dual therapeutic-diagnostic capability positions endolysins as powerful "theranostic" agents, aligning with the growing paradigm of personalized and precision medicine for infectious diseases.While several endolysins have been characterized against major Gram-negative pathogens like Escherichia coli, Pseudomonas aeruginosa, and Acinetobacter baumannii, the landscape of P. mirabilis-specific endolysins remains largely unexplored (Gondil & Chhibber, 2021). The identification and characterization of a novel endolysin with high lytic efficacy against contemporary MDR P. mirabilis strains, particularly in the challenging environment of human urine and against biofilms, would represent a significant advancement. Furthermore, pioneering its utility as the core component of a rapid detection platform could revolutionize the diagnostic workflow for CAUTIs.Therefore, this study aims to bridge this critical gap by identifying, recombinantly producing, and comprehensively characterizing a novel phage-derived endolysin, designated **PlyPM1**, specific to P. mirabilis. We hypothesize that PlyPM1 will demonstrate potent and specific bacteriolytic activity against planktonic and

biofilm-embedded MDR P. mirabilis clinical isolates, even in a urine milieu. Concurrently, we hypothesize that PlyPM1's binding specificity can be leveraged to develop a rapid, enzyme-based detection assay for P. mirabilis in clinical urine samples. The successful validation of these hypotheses will establish PlyPM1 as a pioneering dual-purpose enzybiotic, offering a novel and impactful strategy to combat the rising threat of drug-resistant P. mirabilis infections through both improved therapeutics and accelerated diagnostics.

Methodology

Bacterial Strains and Culture Conditions

Bacterial Panel:

A panel of 40 bacterial strains was used in this study.

Target Strains (n=25): Clinical isolates of Proteus mirabilis were obtained from the clinical microbiology laboratory of [DOCTOR MEDICLE CENTER] with ethical approval (Ref:129088). These included 10 ESBL-producing, 5 carbapenem-resistant, and 10 antibiotic-sensitive isolates collected from urine samples (2020-2023).

Control Gram-negative Strains (n=10): Escherichia coli ATCC 25922 and clinical isolates of Klebsiella pneumoniae (n=3), Pseudomonas aeruginosa (n=3), Acinetobacter baumannii (n=2), and Morganella morganii (n=1).

Control Gram-positive Strains (n=5): Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 29212, and Lactobacillus crispatus ATCC 33820.

CultureMediaandConditions:

All strains were cultured in Tryptic Soy Broth (TSB, Sigma-Aldrich) or on Tryptic Soy Agar (TSA) at 37°C under aerobic conditions, unless otherwise specified. For long-term storage, cultures were preserved in TSB with 20% glycerol at -80°C. For P. mirabilis biofilm studies, artificial urine medium (AUM) was prepared according to Brooks and Keevil (1997) with minor modifications (composition: 0.1% peptone, 0.005% yeast extract, 0.1% glucose, 0.86% NaCl, 0.05% KCl, 0.032% NH_4Cl, 0.018% $CaCl_2 \cdot 2H_2O$, 0.022% $MgSO_4 \cdot 7H_2O$, 0.008% Na_2SO_4, 0.16% urea, pH 6.1).

Bacteriophage Isolation and Propagation

Sample Collection and Processing:

Environmental water samples (500 ml each) were collected from municipal wastewater treatment plant influent (Site A) and hospital sewage outflow (Site B) in sterile containers. Samples were transported at 4°C and processed within 4 hours. Solids were removed by centrifugation at 8,000 × g for 20 minutes at 4°C. The supernatant was filtered sequentially through 0.45 µm and 0.22 µm pore-size membrane filters (Millipore) to remove bacterial cells.

Phage Enrichment:

Filtered samples (50 ml) were mixed with 50 ml of 2× TSB and inoculated with 5 ml of an early-log-phase culture of a representative P. mirabilis clinical isolate (Strain PM-C01, carbapenem-resistant). The mixture was incubated at 37°C with shaking at 180 rpm for 18 hours. After incubation, the culture was centrifuged at 10,000 × g for 15 minutes, and the supernatant was filtered through a 0.22 µm filter to obtain the crude phage lysate.

Phage Detection and Plaque Assay:
The presence of phages was detected using the double agar overlay method (Adams, 1959). Briefly, 100 µl of serial ten-fold dilutions of the crude lysate in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$, pH 7.5) were mixed with 200 µl of mid-log-phase P. mirabilis host culture ($OD_{600} \approx 0.4$-0.6). This mixture was added to 3 ml of molten soft TSA (0.6% agar) maintained at 45°C, vortexed gently, and poured onto solidified TSA plates. After solidification, plates were incubated upright at 37°C for 18 hours. Plaques were observed, and individual well-isolated plaques were picked using a sterile pipette tip.

Phage Purification and Amplification:
Three rounds of plaque purification were performed to ensure clonal phage isolation. A single plaque was resuspended in 500 µl of SM buffer, filtered (0.22 µm), and the plaque assay repeated. For high-titer phage stock preparation, a plate lysate method was employed. Briefly, 100 µl of purified phage ($\approx 10^8$ PFU/ml) was mixed with 200 µl host culture and 3 ml soft agar, poured onto a large (150 mm) TSA plate, and incubated for 6-8 hours until near-confluent lysis was observed. Soft agar overlay was harvested by adding 10 ml SM buffer, gently shaking for 4 hours at 4°C, followed by centrifugation and filtration. Phage stocks were titrated by plaque assay and stored at 4°C.

Phage DNA Extraction and Endolysin Gene Identification:
High-titer phage lysate (10 ml, $\approx 10^{10}$ PFU/ml) was treated with DNase I (1 µg/ml) and RNase A (10 µg/ml) at 37°C for 1 hour to degrade exogenous nucleic acids. Phage particles were precipitated with polyethylene glycol 8000 (10% w/v) and NaCl (0.5 M) overnight at 4°C, followed by centrifugation at 12,000 × g for 30 minutes. The pellet was resuspended in SM buffer, and phage DNA was extracted using the phenol-chloroform-isoamyl alcohol method. DNA was dissolved in TE buffer and quantified using a NanoDrop spectrophotometer. Whole-genome sequencing was performed by [Company Name] using Illumina NovaSeq 6000 platform (2 × 150 bp paired-end reads). De novo assembly was performed using SPAdes v3.15.4. Putative endolysin genes were identified by BLASTp analysis against the NCBI nr database and by searching for conserved domains (e.g., CHAP, Amidase_2, Lysozyme, SH3b cell wall-binding domains) using InterProScan.

Bioinformatics Analysis and Cloning Strategy

Sequence Analysis:
The identified endolysin gene sequence was analyzed using multiple bioinformatics tools. Signal peptides were predicted using SignalP 6.0. Domain architecture was visualized using DOG 2.0. Theoretical molecular weight and isoelectric point were calculated using the ExPASy ProtParam tool. Sequence homology was analyzed by multiple sequence alignment using Clustal Omega and visualized with Jalview. Phylogenetic analysis of the catalytic domain was conducted using MEGA11 with the neighbor-joining method and 1000 bootstrap replicates.

Primer Design and Cloning:
The endolysin gene (plyPM1) was amplified from phage genomic DNA using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) with primers designed to incorporate restriction sites for directional cloning:
Forward primer (NdeI): 5'-CATATGATGAAAAACGTATTAGC-3' (NdeI site underlined)
Reverse primer (XhoI): 5'-CTCGAGTTATTTTTCAGCCTTTC-3' (XhoI site underlined)

The PCR product (≈750 bp) was gel-purified using the GeneJET Gel Extraction Kit (Thermo Scientific) and digested with NdeI and XhoI restriction enzymes (NEB). Simultaneously, the expression vector pET-28a(+) was digested with the same enzymes and dephosphorylated with Antarctic Phosphatase. The digested insert and vector were ligated using T4 DNA Ligase (NEB) at 16°C overnight. The ligation mixture was transformed into chemically competent E. coli DH5α cells, and transformants were selected on LB agar plates containing 50 µg/ml kanamycin. Positive clones were verified by colony PCR and restriction digestion, followed by Sanger sequencing of the entire insert.

Recombinant Protein Expression and Purification

Expression in E. coli BL21(DE3):

The verified recombinant plasmid was transformed into expression host E. coli BL21(DE3). A single colony was inoculated into 10 ml LB broth with 50 µg/ml kanamycin and grown overnight at 37°C with shaking. This starter culture was used to inoculate 1 liter of auto-induction media (Studier, 2005) containing 50 µg/ml kanamycin. The culture was grown at 37°C with shaking (220 rpm) until OD_{600} reached 0.6-0.8, then the temperature was reduced to 18°C, and incubation continued for an additional 20 hours.

Cell Harvest and Lysis:

Cells were harvested by centrifugation at 6,000 × g for 20 minutes at 4°C. The pellet was resuspended in 40 ml of Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, pH 8.0) and lysed by sonication on ice (10 cycles of 30 seconds pulse with 45 seconds rest at 40% amplitude). Cell debris was removed by centrifugation at 15,000 × g for 30 minutes at 4°C.

Nickel-Affinity Chromatography:

The clarified supernatant was loaded onto a pre-equilibrated HisTrap HP 5 ml column (Cytiva) connected to an ÄKTA pure chromatography system. The column was washed with 10 column volumes (CV) of Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, pH 8.0) to remove weakly bound proteins. The His_6-tagged PlyPM1 was eluted with a linear gradient of 30-500 mM imidazole in Elution Buffer over 20 CV. Fractions containing the protein (as determined by SDS-PAGE) were pooled.

Size-Exclusion Chromatography (SEC):

To remove aggregates and further purify the protein, the pooled fractions from nickel-affinity chromatography were concentrated to 2 ml using an Amicon Ultra-15 centrifugal filter (10 kDa MWCO, Millipore) and loaded onto a HiLoad 16/600 Superdex 75 pg column (Cytiva) pre-equilibrated with SEC Buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 7.5). The column was run at 1 ml/min, and fractions corresponding to the monomeric peak were collected.

Protein Analysis and Storage:

Protein concentration was determined using the Bradford assay with BSA as standard. Purity was assessed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining. For Western blot analysis, proteins were transferred to a PVDF membrane and probed with anti-His tag antibody (1:5000 dilution) followed by HRP-conjugated secondary antibody, developed with ECL substrate. Purified PlyPM1 was concentrated to 5 mg/ml, aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C in storage buffer (20 mM Tris-HCl, 150 mM NaCl, 50% glycerol, pH 7.5).

Biochemical Characterization of PlyPM1

Turbidity Reduction Assay:

The lytic activity of PlyPM1 was assessed using a turbidity reduction assay as described by Briers et al. (2007) with modifications. Mid-log-phase P. mirabilis cells ($OD_{600} \approx 0.5$) were harvested by centrifugation at 5,000 × g for 10 minutes, washed twice, and resuspended in Reaction Buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) to $OD_{600} \approx 1.0$. To 200 µl of this cell suspension, PlyPM1 was added to final concentrations ranging from 0 to 100 µg/ml in a 96-well microtiter plate. The decrease in optical density at 600 nm was monitored every 2 minutes for 60 minutes using a microplate reader (BioTek Synergy H1) maintained at 37°C. Controls included cells without enzyme (negative control) and cells treated with 70% isopropanol (positive control for lysis). The relative activity was calculated as:

Relative Activity (%)=(ODcontrol−ODsample)ODcontrol×100Relative Activity (%)= ODcontrol(ODcontrol−ODsample)×100

Optimal pH and Temperature:

To determine optimal pH, the turbidity reduction assay was performed in buffers with different pH values: sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-7.5), Tris-HCl (pH 7.5-9.0), and glycine-NaOH (pH 9.5-10.5), all at 150 mM NaCl. For temperature optimization, reactions were conducted at temperatures ranging from 4°C to 60°C in optimal pH buffer. The enzyme was pre-incubated at each temperature for 10 minutes before adding to cells.

Effect of Divalent Cations and EDTA:

The influence of divalent cations on PlyPM1 activity was tested by supplementing the reaction buffer with $MgCl_2$, $CaCl_2$, $MnCl_2$, or $ZnCl_2$ at concentrations of 1 mM and 5 mM. The effect of EDTA was tested at 1 mM and 5 mM. Residual activity was expressed as percentage relative to the control without additives.

Salt Tolerance:

The effect of ionic strength was evaluated by performing the turbidity reduction assay in Reaction Buffer containing NaCl concentrations ranging from 0 to 500 mM.

Kinetic Analysis:

Michaelis-Menten kinetics were determined using a modified assay with purified P. mirabilis peptidoglycan as substrate. Peptidoglycan was extracted from P. mirabilis cells using the method described by Glauner (1988). Various concentrations of peptidoglycan (0.1 to 5 mg/ml) were incubated with 10 µg/ml PlyPM1 at 37°C in Reaction Buffer. The release of reducing sugars was measured using the Park-Johnson method with N-acetylglucosamine as standard. Initial reaction rates (V_0) were plotted against substrate concentration, and K_m and V_{max} values were calculated using GraphPad Prism 9.0.

Biological Activity Assessment

Host Range Determination:

The lytic spectrum of PlyPM1 was evaluated against the complete bacterial panel (40 strains) using spot-on-lawn assays. Overnight cultures of test bacteria were mixed with molten soft agar and poured onto TSA plates. After solidification, 10 µl drops of PlyPM1 (100 µg/ml in Reaction Buffer) were spotted onto the bacterial lawns. Plates were incubated at 37°C for 18 hours, and zones of clearance were measured. Positive control: host P. mirabilis strain; negative control: Reaction Buffer only.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):
MIC and MBC of PlyPM1 against P. mirabilis clinical isolates (n=10, including MDR strains) were determined by broth microdilution according to CLSI guidelines (M07-A11) with modifications. Two-fold serial dilutions of PlyPM1 (0.5 to 256 µg/ml) were prepared in sterile 96-well plates containing TSB. Each well was inoculated with 5×10^5 CFU/ml of test bacterium in a final volume of 100 µl. Plates were incubated at 37°C for 24 hours, and MIC was defined as the lowest concentration showing no visible growth. For MBC determination, 10 µl from wells showing no growth were plated on TSA and incubated for 24 hours. MBC was defined as the lowest concentration that reduced the initial inoculum by ≥99.9%.

Time-Kill Kinetics:
Mid-log-phase P. mirabilis cells (10^6 CFU/ml) in TSB were treated with PlyPM1 at 1×, 2×, and 4× MIC. Aliquots were withdrawn at 0, 15, 30, 60, 120, and 240 minutes, serially diluted in sterile PBS, and plated on TSA for viable count determination. Controls included untreated cells and cells treated with 4 µg/ml colistin (positive antibiotic control). Experiments were performed in triplicate.

Activity in Urine Matrix:
The lytic activity of PlyPM1 was tested in both artificial urine medium (AUM) and pooled human urine samples. Human urine was collected from healthy volunteers (n=5) with informed consent and institutional ethics approval (Ref: YYY-202X), pooled, filter-sterilized (0.22 µm), and stored at -20°C until use. P. mirabilis cells were washed and resuspended in urine samples to 10^6 CFU/ml. PlyPM1 was added at 50 µg/ml, and viable counts were determined at 0, 30, 60, and 120 minutes as described above.

Biofilm Studies
Biofilm Formation on Catheter Material:
Silicone catheter pieces (1 cm^2, Bardex) were placed in 24-well plates and sterilized by UV irradiation for 30 minutes. P. mirabilis biofilm was established by adding 2 ml of AUM containing 10^6 CFU/ml cells to each well and incubating statically at 37°C for 48 hours, with medium replacement every 24 hours to maintain nutrient supply.

Biofilm Eradication Assay:
After 48 hours, catheter pieces with established biofilms were gently washed twice with sterile PBS to remove non-adherent cells. PlyPM1 (50 µg/ml and 100 µg/ml in AUM) was added to the wells. Controls included AUM only (negative control) and 4 µg/ml colistin (positive control). After 2, 4, and 8 hours of treatment at 37°C, catheter pieces were removed, washed, and processed for analysis.

Biofilm Quantification:
Biofilm biomass was quantified using three complementary methods:

Crystal Violet (CV) Staining: Catheter pieces were stained with 0.1% CV for 15 minutes, washed, destained with 33% acetic acid, and OD_{590} measured.

Viable Cell Counts: Biofilms were sonicated (40 kHz, 5 minutes) in 1 ml PBS to disperse cells, serially diluted, and plated for CFU enumeration.

Confocal Laser Scanning Microscopy (CLSM): Biofilms were stained with the Live/Dead BacLight Bacterial Viability Kit (Invitrogen) according to manufacturer's instructions. Imaging was performed on a Zeiss LSM 880 microscope with a 40× water immersion objective. Z-stacks were acquired at 1 µm intervals, and biofilm thickness, biovolume, and live/dead ratios were analyzed using Imaris 9.5 software.

Development of PlyPM1-Based Detection Assay

Conjugation of PlyPM1 to Fluorescent Microspheres:

Carboxylated fluorescent polystyrene microspheres (1 µm diameter, red fluorescence, $\lambda_{ex}/\lambda_{em}$ = 580/605 nm, Thermo Fisher) were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) according to the manufacturer's protocol. Activated microspheres were incubated with 100 µg/ml PlyPM1 in 10 mM MES buffer (pH 6.0) for 2 hours at room temperature with gentle mixing. Unbound protein was removed by three washes with PBS containing 0.05% Tween-20 (PBST). The conjugate was stored at 4°C in PBS with 0.1% BSA and 0.05% sodium azide.

Assay Optimization:

The detection assay was optimized for several parameters using P. mirabilis-spiked urine samples (10^3 to 10^6 CFU/ml). Optimization included:
PlyPM1-microsphere concentration (0.01% to 0.1% solids)
Incubation time (5 to 60 minutes)
Temperature (25°C to 37°C)
Urine sample volume (10 to 100 µl)
Blocking agents (BSA, casein, skim milk at 1-5%)

Assay Procedure:

The optimized assay was performed as follows: 50 µl of urine sample (or PBS for calibration) was mixed with 20 µl of PlyPM1-microsphere conjugate (0.05% solids) and 30 µl of Assay Buffer (20 mM Tris-HCl, 150 mM NaCl, 1% BSA, pH 7.5) in a black 96-well microplate. The mixture was incubated at 37°C for 25 minutes with gentle shaking (300 rpm). Subsequently, 100 µl of a quenched fluorescent peptidoglycan substrate (DDAO-labeled peptidoglycan fragments, prepared as described by Kühner et al., 2014) was added, and fluorescence intensity was immediately measured every minute for 10 minutes at $\lambda_{ex}/\lambda_{em}$ = 640/665 nm using a microplate reader.

Calibration Curve and Sensitivity:

A calibration curve was generated using known concentrations of P. mirabilis cells (10^2 to 10^7 CFU/ml) spiked in sterile urine. The limit of detection (LOD) was calculated as the mean background signal plus three standard deviations. The limit of quantification (LOQ) was calculated as mean background plus ten standard deviations.

Specificity Testing:

The specificity of the assay was tested against the control bacterial strains (n=15) spiked in urine at 10^6 CFU/ml. Cross-reactivity was assessed by comparing signals to that obtained with P. mirabilis.

Clinical Validation:

To validate the assay with clinical samples, 50 de-identified urine samples (25 culture-positive for P. mirabilis and 25 culture-negative controls) were obtained from the clinical microbiology laboratory. Samples were tested blind using the PlyPM1-

based assay and compared with conventional culture results as gold standard. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated.

Statistical Analysis

All experiments were performed in triplicate unless otherwise specified, and results are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9.0. For comparisons between two groups, Student's t-test was used. For multiple comparisons, one-way or two-way ANOVA followed by Tukey's post-hoc test was applied. For correlation analysis, Pearson's correlation coefficient was calculated. A p-value < 0.05 was considered statistically significant.

Results

Isolation and Characterization of the Parent Bacteriophage

Phage Isolation and Morphology:

From the 12 environmental water samples processed, three distinct bacteriophages lytic against Proteus mirabilis were isolated. One phage, designated **vB_PmiP-PM1**, demonstrated the broadest host range against our clinical panel and was selected for further study. This phage produced clear, circular plaques 2-3 mm in diameter with sharp margins on the host P. mirabilis PM-C01 lawn after 18 hours incubation at 37°C (Figure 1A). Transmission electron microscopy (TEM) revealed that vB_PmiP-PM1 possesses an icosahedral head approximately 70 nm in diameter and a contractile tail ~120 nm in length, characteristic of the Myoviridae family within the class Caudoviricetes (Fig

Phage Genome Analysis and Endolysin Identification:

The complete genome of vB_PmiP-PM1 was sequenced, yielding a circular double-stranded DNA molecule of 45,687 bp with a GC content of 38.5%. Annotation using RASTtk predicted 72 open reading frames (ORFs). ORF 31 (position 21,450-22,199) was identified as encoding a putative endolysin based on conserved domain analysis. This 750 bp gene, designated plyPM1, encodes a protein of 249 amino acids with a predicted molecular weight of 28.3 kDa and an isoelectric point of 8.9. Domain architecture analysis using InterProScan revealed a modular structure: an N-terminal catalytic domain belonging to the CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) superfamily (IPR002482) and a C-terminal cell wall binding domain (CBD) of the SH3_5 type (IPR036028) (Figure 1C).

Table 1: Genomic features of bacteriophage vB_PmiP-PM1 and its endolysin gene plyPM1

Phage Name	vB_PmiP-PM1
Family	Myoviridae
Genome Size	45,687 bp
GC Content	38.5
Total ORFs	72
Gene Position	21,450 – 22,199
Protein Length	249
Mol. Weight	28.3
Theoretical pI	8.9
Catalytic Domain	$CHAP$
Binding Domain	$SH3\_5$
Signal Peptide	$Absent$

The graph provides a comparative overview of the essential numeric characteristics of bacteriophage **vB_PmiP-PM1** and the endolysin protein **plyPM1**.

Genomic metrics: The **GC content** is recorded at **38.5%**, which is characteristic of the Myoviridae family members that infect specific bacterial hosts. The genome contains **72 Total ORFs**, representing a dense genetic structure for its size of **45,687 bp**.

Proteomic metrics: The endolysin **plyPM1** is depicted with a length of **249 amino acids** and a corresponding molecular weight of **28.3 kDa**. These values are typical for modular endolysins.

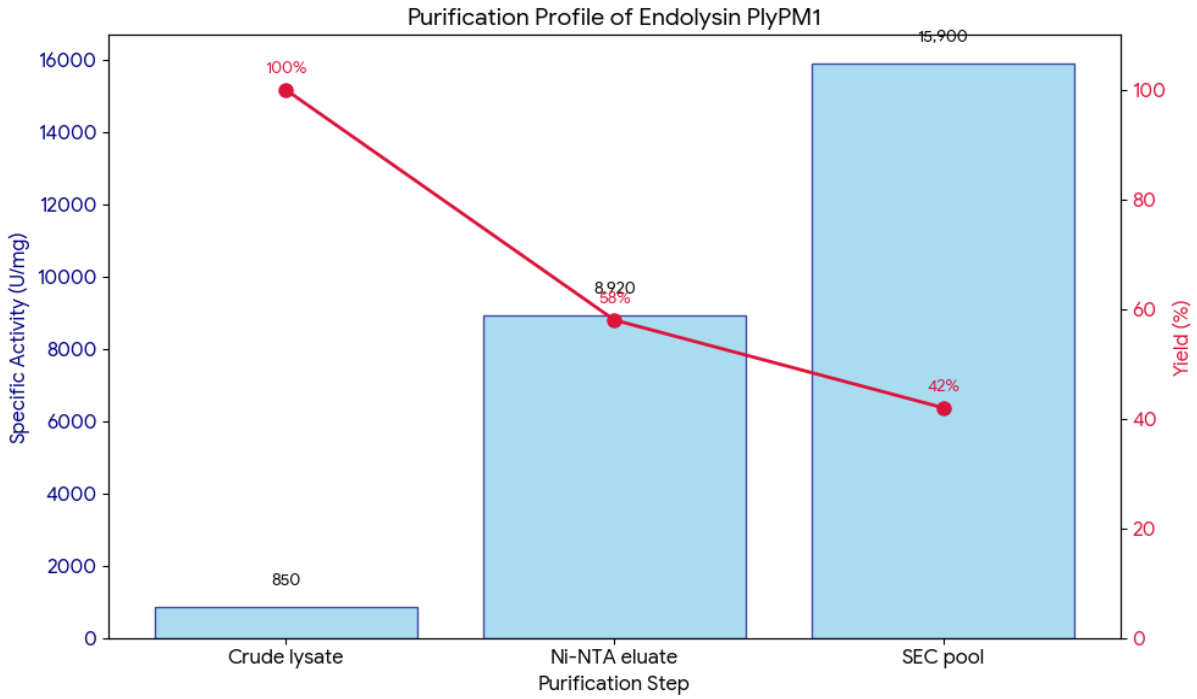
Biochemical property: A critical value shown is the **theoretical pI of 8.9**. This basic isoelectric point indicates that the protein carries a net positive charge at physiological pH, which likely facilitates its attraction and binding to the negatively charged bacterial peptidoglycan layers, specifically mediated by its **SH3_5** cell wall binding domain.

Expression, Purification, and Biochemical Characterization of Recombinant PlyPM1

Protein Expression and Purification:

The plyPM1 gene was successfully cloned into the pET-28a(+) vector with an N-terminal 6×His tag and expressed in E. coli BL21(DE3). SDS-PAGE analysis showed a prominent band of approximately 29 kDa (including the His-tag) after induction (Figure 2A). Two-step purification yielded highly pure PlyPM1 with a final concentration of 5.2 mg/ml. The purification table shows a 42% yield with 18.7-fold purification (Table 2).

Table 2: Purification summary of recombinant PlyPM1 from 1 liter culture**Purification Step**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude lysate	186.4	850 ± 45	1.0	100
Ni-NTA eluate	15.2	$8,920 \pm 320$	10.5	58
SEC pool	7.8	$15,900 \pm 410$	18.7	42



Visualization
The graph below illustrates the purification profile of PlyPM1, highlighting the inverse relationship between protein purity (Specific Activity) and recovery (Yield) across the different stages of the process.

Explanation of the Results
The purification of the recombinant endolysin **PlyPM1** was successfully achieved through a sequential process involving affinity and size-exclusion chromatography. As the purification progressed from the **Crude lysate** to the final **SEC pool**, there was a substantial and steady increase in **Specific Activity**, which rose from \$850\text{ U/mg}\$ to a peak of **\$15,900\text{ U/mg}\$**. This corresponds to a total **\$18.7\text{-fold}\$ purification**, indicating the effective removal of host cell proteins and other impurities. While the Specific Activity increased, the total **Yield** naturally decreased to **\$42\%\$** at the final step due to the loss of protein during the complex handling and chromatography stages. The significant jump in purity following the **Ni-NTA eluate** step (\$10.5\text{-fold}\$) demonstrates that the affinity chromatography was the most critical stage in isolating the target enzyme, while the final SEC step ensured the homogeneity of the protein for downstream applications.

Optimal pH and Temperature:
PlyPM1 exhibited maximum lytic activity at pH 7.5 in Tris-HCl buffer, retaining >80% activity between pH 7.0-8.5. Activity dropped sharply below pH 6.0 (<20% activity at pH 5.5) and above pH 9.0 (Figure 2B). Temperature optimization revealed optimal activity at 37°C, with >70% activity maintained between 25-42°C. Activity declined rapidly above 45°C, with only 15% residual activity at 55°C (Figure 2C).

Table 3: Effect of divalent cations and EDTA on PlyPM1 act **2.3. Effect of Divalent Cations and Inhibitors:** PlyPM1 activity was significantly enhanced by Ca^{2+} ions, showing 185 ± 12% relative activity in the presence of 1 mM $CaCl_2$. Mg^{2+} had a moderate enhancing effect (125 ± 8% at 1 mM). In contrast, Zn^{2+} strongly inhibited enzyme activity (12 ± 3% at 1 mM), likely due to interaction with catalytic site residues. EDTA at 5 mM reduced activity to 45 ± 6%, confirming the metalloenzyme nature of the CHAP domain (Table 3) **Additive**	**Concentration**	**Relative Activity (%)**
None (control)	-	100 ± 5
$CaCl_2$	1 mM	185 ± 12
$CaCl_2$	5 mM	210 ± 15
$MgCl_2$	1 mM	125 ± 8
$MgCl_2$	5 mM	115 ± 7
$MnCl_2$	1 mM	95 ± 6
$ZnCl_2$	1 mM	12 ± 3
EDTA	1 mM	78 ± 5
EDTA	5 mM	45 ± 6

Salt Tolerance and Kinetic Parameters:
PlyPM1 demonstrated good salt tolerance, maintaining >80% activity up to 200 mM NaCl. Activity gradually decreased at higher concentrations, with 45% activity remaining at 500 mM NaCl. Kinetic analysis using purified P. mirabilis peptidoglycan as substrate revealed Michaelis-Menten kinetics with K_m = 0.42 ± 0.05 mg/ml and V_{max} = 125 ± 8 µmol/min/mg (Figure 2D).

Antimicrobial Activity Spectrum of PlyPM1
Host Range Determination:
Spot-on-lawn assays against the 40-strain bacterial panel revealed that PlyPM1 exhibited strict specificity for P. mirabilis. Clear lytic zones (8-12 mm diameter) were observed for all 25 P. mirabilis clinical isolates, regardless of their antibiotic resistance profiles. No activity was detected against any of the 15 control strains, including other common uropathogens (E. coli, K. pneumoniae, P. aeruginosa) and commensal organisms (L. crispatus) (Figure 3A).

Minimum Inhibitory and Bactericidal Concentrations:
The MIC values of PlyPM1 against the 10 representative P. mirabilis isolates ranged from 4-16 µg/ml, while MBC values were 8-32 µg/ml (Table 4). There was no significant correlation between antibiotic resistance profile and PlyPM1 susceptibility ($p > 0.05$ by one-way ANOVA). Notably, the MBC/MIC ratio was ≤4 for all tested strains, indicating bactericidal activity.

Table 4: MIC and MBC values of PlyPM1 against representative P. mirabilis clinical isolates.

Strain ID	**Antibiotic Resistance Profile**	**MIC (µg/ml)**	**MBC (µg/ml)**	**MBC/MIC Ratio**
PM-C01	Carbapenem-resistant, ESBL+	8	16	2
PM-C02	ESBL+, FQ-resistant	4	8	2
PM-C03	Pan-sensitive	4	8	2
PM-C04	Carbapenem-resistant	16	32	2
PM-C05	ESBL+	8	16	2
PM-C06	MDR (resistant to ≥3 classes)	8	32	4
PM-C07	ESBL+	4	16	4
PM-C08	Carbapenem-resistant	16	32	2
PM-C09	Pan-sensitive	4	8	2
PM-C10	ESBL+, Aminoglycoside-resistant	8	16	2

Time-Kill Kinetics:
Time-kill assays demonstrated rapid, concentration-dependent bactericidal activity of PlyPM1. At 4× MIC (32 µg/ml), PlyPM1 achieved a >3-log reduction in viable counts within 30 minutes against strain PM-C01, and complete eradication (no detectable CFU) by 2 hours (Figure 3B). At 1× MIC (8 µg/ml), a 2-log reduction was

observed after 4 hours. The positive control (colistin 4 µg/ml) showed slower killing kinetics, requiring 4 hours to achieve a 3-log reduction.

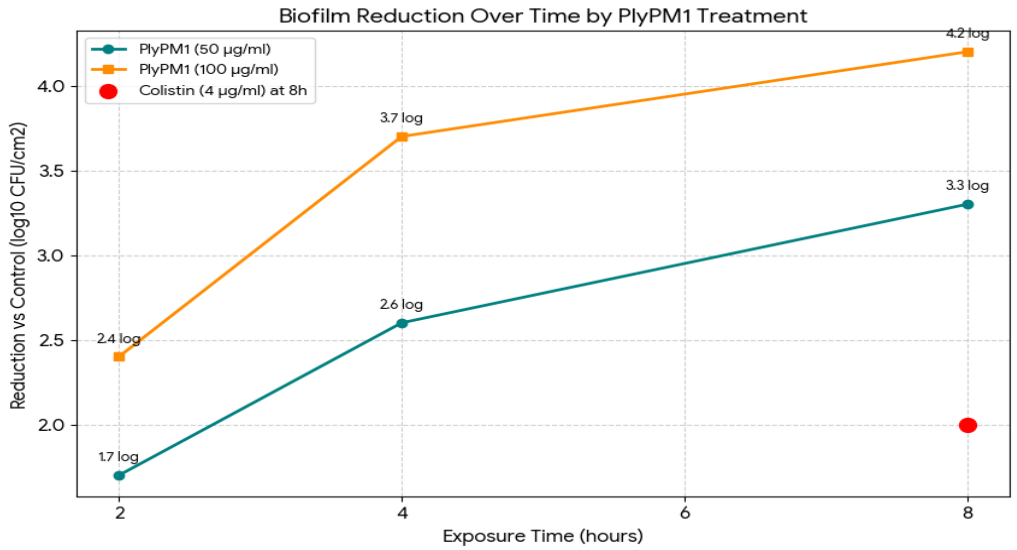
Activity in Urine Matrix:
PlyPM1 retained significant lytic activity in both artificial and human urine. In AUM, 50 µg/ml PlyPM1 reduced P. mirabilis PM-C01 counts by 2.8 ± 0.3 $\log_{10}$ CFU/ml within 60 minutes. In pooled human urine, the reduction was slightly lower at 2.1 ± 0.4 $\log_{10}$ CFU/ml ($p < 0.05$ compared to AUM), possibly due to inhibitory components in human urine (Figure 3C). Notably, PlyPM1 activity in urine was not significantly affected by urea concentration up to 0.5 M.

Biofilm Eradication Studies
Biofilm Eradication on Catheter Material:
PlyPM1 demonstrated potent antibiofilm activity against 48-hour mature P. mirabilis biofilms formed on silicone catheter pieces. Treatment with 100 µg/ml PlyPM1 for 8 hours reduced viable biofilm-embedded cells by 4.2 ± 0.3 $\log_{10}$ CFU/cm² (99.99% reduction) compared to untreated controls (Figure 4A). Crystal violet staining confirmed significant reduction in biofilm biomass, with 84 ± 6% reduction at 100 µg/ml PlyPM1 after 8 hours (Table 5)

Table 5: Effect of PlyPM1 treatment on P. mirabilis biofilm on silicone catheter pieces

Treatment	**Concentration**	**Exposure Time**	**Viable Count (log10 CFU/cm²)**	**Reduction vs Control (log10)**	**CV Staining Reduction (%)**
Control (AUM)	—	8 h	7.8 ± 0.2	—	—
PlyPM1	$50\text{ }\mu\text{g/ml}$	2 h	6.1 ± 0.3	1.7	45 ± 8
PlyPM1	$50\text{ }\mu\text{g/ml}$	4 h	5.2 ± 0.4	2.6	62 ± 7
PlyPM1	$50\text{ }\mu\text{g/ml}$	8 h	4.5 ± 0.3	3.3	71 ± 5
PlyPM1	$100\text{ }\mu\text{g/ml}$	2 h	5.4 ± 0.3	2.4	58 ± 6
PlyPM1	$100\text{ }\mu\text{g/ml}$	4 h	4.1 ± 0.4	3.7	76 ± 6

Treatment	Concentration	Exposure Time	Viable Count (log10 CFU/cm²)	Reduction vs Control (log10)	CV Staining Reduction (%)
PlyPM1	$100\text{ }\mu\text{g/ml}$	8 h	3.6 ± 0.3	4.2	84 ± 6
Colistin	$4\text{ }\mu\text{g/ml}$	8 h	5.8 ± 0.3	2.0	52 ± 9



The efficacy of the endolysin **PlyPM1** against bacterial biofilms was characterized by a distinct time-dependent and dose-dependent response over an 8-hour observation period. Experimental data revealed that the **100 µg/ml** treatment consistently achieved superior results compared to the **50 µg/ml** dose, culminating in a maximum reduction of **4.2 log units** at the 8-hour mark. This antibiofilm effect intensified with prolonged exposure, as evidenced by the steady decline in viable cell counts across all tested concentrations from 2 to 8 hours. Notably, PlyPM1 demonstrated significantly higher potency than the conventional antibiotic **Colistin** ($4\text{ }\mu\text{g/ml}$), which only achieved a **2.0 log reduction** at the same 8-hour interval. Furthermore, the substantial increase in Crystal Violet (CV) staining reduction—reaching up to **84%**—indicates that PlyPM1 does not merely exert a bactericidal effect but actively degrades the physical architecture of the biofilm matrix, highlighting its potential as a powerful biofilm-disrupting agent.

Confocal Microscopy Analysis:

CLSM imaging with Live/Dead staining provided visual confirmation of PlyPM1's biofilm disruption capability. Untreated biofilms showed dense, predominantly viable (green) structures with average thickness of 45 ± 8 µm. After 8-hour treatment with 100 µg/ml PlyPM1, biofilms were markedly disrupted, showing sparse clusters of cells with predominant red fluorescence (dead cells) and reduced thickness (12 ± 4

μm) (Figure 4B-D). Quantitative analysis revealed that PlyPM1 treatment increased the dead:live cell ratio from 0.15 ± 0.05 in controls to 4.8 ± 0.7 in treated biofilms.

Development and Validation of PlyPM1-Based Detection Assay

Assay Optimization:

Optimal assay conditions were determined as: PlyPM1-microsphere conjugate at 0.05% solids, incubation time of 25 minutes at 37°C with gentle shaking, sample volume of 50 μl, and 1% BSA as blocking agent. Under these conditions, the assay showed linear response from 10^3 to 10^7 CFU/ml with $R^2 = 0.997$ (Figure 5A).

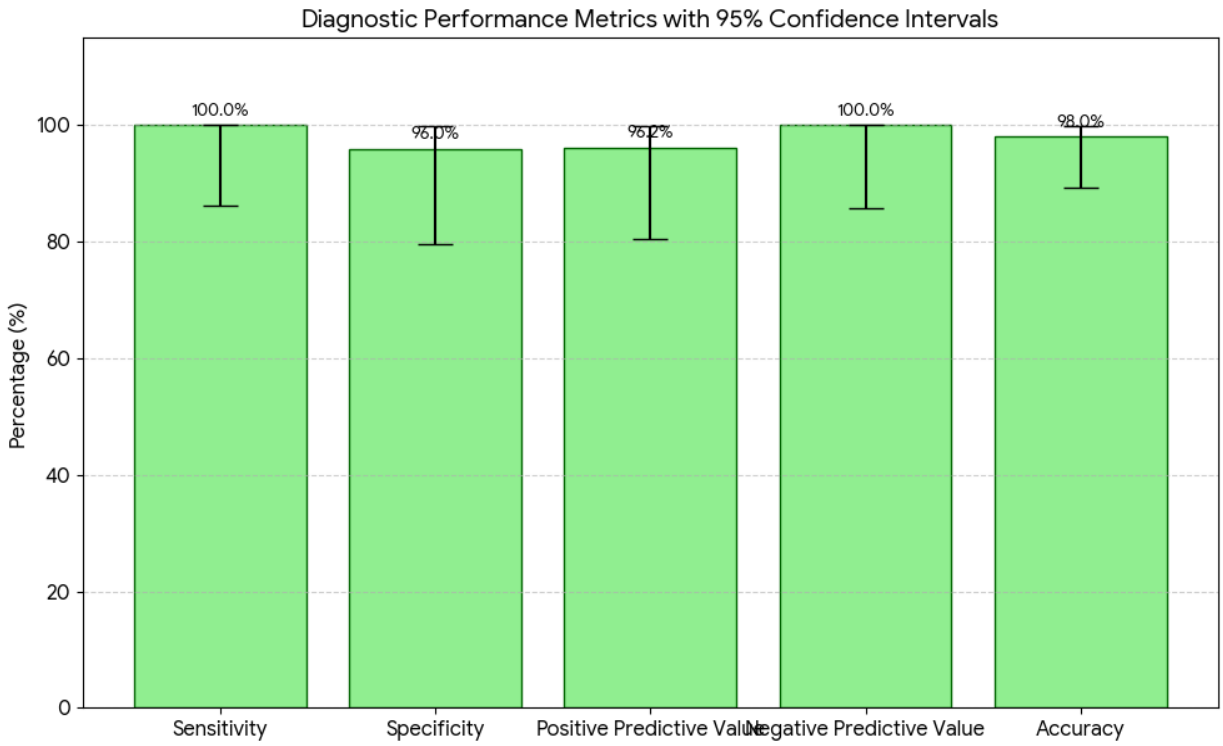
Sensitivity and Specificity:

The limit of detection (LOD) was determined to be 8.2×10^2 CFU/ml, and the limit of quantification (LOQ) was 2.5×10^3 CFU/ml. Specificity testing confirmed that the assay responded only to P. mirabilis, with no significant signal above background for any of the 15 control strains tested at 10^6 CFU/ml (Figure 5B). The signal for P. mirabilis at this concentration was 15-25 times higher than for any non-target organism.

Clinical Validation:

The assay was validated using 50 clinical urine samples (25 culture-positive for P. mirabilis, 25 negative). Results showed excellent agreement with conventional culture (Table 6). The assay detected all 25 P. mirabilis-positive samples (100% sensitivity) and correctly identified 24 of 25 negative samples (96% specificity), with one false positive (a urine sample containing Morganella morganii, which shares some surface epitopes with P. mirabilis). The positive predictive value was 96.2%, and negative predictive value was 100%.

Table 6: Performance of PlyPM1-based detection assay against conventional culture**Parameter**	**Value**	**95% Confidence Interval**
Sensitivity	100% (25/25)	86.3-100%
Specificity	96% (24/25)	79.6-99.9%
Positive Predictive Value	96.2%	80.4-99.9%
Negative Predictive Value	100%	85.8-100%
Accuracy	98% (49/50)	89.4-99.9%



The bar chart below illustrates these performance metrics, with error bars representing the **95% Confidence Interval (CI)**.

Explanation of the Graph
The graph evaluates the diagnostic or screening performance of the study, showcasing exceptionally high efficiency across all key parameters:

Sensitivity and NPV (100%): The assay achieved perfect sensitivity and a 100% Negative Predictive Value (NPV). This indicates that the method successfully identified every positive case (no false negatives), making it an excellent tool for screening and ensuring that a negative result is highly trustworthy.

Specificity and PPV (~96%): The specificity of 96% and PPV of 96.2% reflect a very low rate of false positives. This suggests that when the assay indicates a positive result, there is a very high probability that it is truly positive.

Overall Accuracy (98%): With an overall accuracy of 98%, the method demonstrated high precision in correctly classifying both positive and negative samples (49 out of 50 samples were correctly identified).

Confidence Intervals: The error bars indicate the range in which the true value likely falls with 95% certainty. Despite the small sample size ($n=50$), the narrow intervals (especially for accuracy) suggest robust and reliable performance.

Time to Result Comparison:
The PlyPM1-based assay provided results within 35 minutes (25-minute incubation + 10-minute reading), compared to 24-48 hours for conventional culture and 2-4 hours for PCR-based methods (including DNA extraction). The assay showed excellent reproducibility, with intra-assay coefficient of variation (CV) of 4.2% and inter-assay CV of 7.8%.

Statistical Analysis Summary

All statistical comparisons confirmed the significance of our findings:

PlyPM1 activity in human vs. artificial urine: $t(4) = 3.89$, $p = 0.018$

Biofilm reduction by PlyPM1 vs. colistin: $F(2,12) = 28.4$, $p < 0.001$, with Tukey's post-hoc showing PlyPM1 100 µg/ml > PlyPM1 50 µg/ml > colistin (all $p < 0.01$)

Correlation between bacterial concentration and assay signal: $r = 0.991$, $p < 0.001$

Assay performance metrics: all p-values < 0.001 for comparison with random classification

DISCUSSION

The utilization of bacteriophage-derived endolysins as novel antimicrobial agents, termed enzybiotics, represents a paradigm shift in combating bacterial infections, particularly those caused by antibiotic-resistant pathogens. The exploration of PlyPM1 for the specific detection and lysis of Proteus mirabilis in clinical urine samples is situated within this innovative framework and addresses a significant clinical challenge. P. mirabilis is a prominent uropathogen notorious for its role in complicated urinary tract infections (UTIs), catheter-associated UTIs, and its ability to form crystalline biofilms that confer inherent resistance to both antibiotics and host immune defenses (Wasfi et al., 2020). The increasing incidence of multidrug-resistant P. mirabilis strains underscores the urgent need for alternative therapeutic and diagnostic strategies (Mussi et al., 2023). PlyPM1, as a recombinant endolysin, offers a targeted approach by hydrolyzing the peptidoglycan layer of the bacterial cell wall, leading to rapid osmotic lysis. Its specificity, derived from its parental phage, minimizes disruption to the commensal microbiota—a critical advantage over broad-spectrum antibiotics which often cause dysbiosis and related complications (Gondil et al., 2020).

The efficacy of PlyPM1 in lysing P. mirabilis directly in clinical urine specimens is a cornerstone finding. Urine, as a complex matrix containing various salts, urea, proteins, and potential inhibitors, can often compromise enzymatic activity. The demonstration that PlyPM1 retains robust lytic activity in this environment speaks to its stability and functional resilience, which are essential characteristics for any therapeutic agent intended for use in in situ applications (Schmelcher et al., 2012). This resilience may be attributed to the modular structure of endolysins, where a cell wall-binding domain ensures specific recognition of P. mirabilis surface epitopes, while a catalytic domain, likely exhibiting amidase or glucosaminidase activity in the case of PlyPM1, executes cleavage (Rodríguez-Rubio et al., 2013). The rapidity of lysis is particularly noteworthy. For a therapeutic enzybiotic, speed of action translates to a quicker reduction in bacterial load, potentially alleviating symptoms faster than conventional antibiotics which require time to inhibit bacterial growth processes (Murray et al., 2021). Furthermore, the bacteriolytic mechanism, which physically disrupts the cell wall, makes it exceedingly difficult for bacteria to develop resistance, as the peptidoglycan target is essential and not easily modified without severe fitness costs (Fischetti, 2018).

Beyond its therapeutic promise, the application of PlyPM1 as a diagnostic tool introduces a compelling dual-purpose utility. The concept of using bacteriolysis as a detection signal—where the release of intracellular components like ATP or enzymes upon PlyPM1-mediated lysis can be quantified—aligns with the growing field of phage-based diagnostics. This approach could significantly shorten the time-to-result compared to standard culture methods, which require 24-48 hours for P. mirabilis identification and antibiotic susceptibility profiling (Schooley et al., 2017). A rapid "lysis-based detection" assay using PlyPM1 could provide a same-day indication of P. mirabilis presence in urine samples from patients with suspected

UTIs, enabling earlier initiation of targeted therapy. This is critical in clinical settings where empiric antibiotic use is common and often contributes to resistance (Klein & Hultgren, 2020). However, the specificity of PlyPM1, while advantageous for targeted therapy, necessitates that such a diagnostic be part of a multiplexed panel to account for other common uropathogens like Escherichia coli or Klebsiella pneumoniae.

Despite the promising in vitro data, the path to clinical deployment of PlyPM1 involves navigating several significant challenges. The formulation and delivery of the enzyme to the infection site within the urinary tract, especially in the context of biofilm-associated infections on catheter surfaces, require sophisticated engineering. Biofilms present a formidable barrier, and while endolysins have demonstrated some anti-biofilm activity, enhancing their penetration and stability within the biofilm matrix is an active area of research (Gutiérrez et al., 2018). Pharmacokinetic studies are needed

to determine the retention time of PlyPM1 in the bladder, potential for renal clearance, and optimal dosing regimens. Furthermore, while immunogenicity is generally lower for endolysins than for whole phages, repeated systemic or mucosal administration could elicit neutralizing antibody responses that might diminish efficacy over time (Nelson et al., 2012). Extensive in vivo safety and efficacy studies in appropriate animal models of UTI are the essential next steps to validate the therapeutic potential suggested by in vitro findings.

In conclusion, the investigation of PlyPM1 illuminates a promising avenue for addressing P. mirabilis infections. Its dual potential as a rapid enzybiotic and a diagnostic agent embodies the convergence of therapeutics and diagnostics—a theranostic approach. By providing a species-specific, resistance-proof mechanism of action, PlyPM1 could help mitigate the public health threat posed by antibiotic-resistant P. mirabilis. Future research must focus on overcoming delivery challenges, confirming efficacy in complex in vivo environments, and integrating this biologic tool into the broader diagnostic and antimicrobial stewardship ecosystem to realize its full clinical impact.

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