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Identification of a Potential Antibiotic Agent Targeting Gram-Negative Bacteria From Urban Garden Soil

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Abstract

Antibiotic resistance is especially rampant among Gram-negative ESKAPE pathogens due to their structurally protective outer membrane, adaptive metabolic functions, and selective porins that make them more resistant to antibiotics than Gram-positive bacteria, which lack an outer membrane and instead possess a thick, exposed peptidoglycan layer that is more easily targeted by many antibiotics. This study investigated bacterial colonies in soil from an urban garden to identify bacteria capable of producing antibiotics effective against Gram-negative bacteria. One isolate, designated Isolate #9, demonstrated a clear zone of inhibition against *Escherichia coli*, a safe relative of *K. pneumoniae,* a Gram-negative bacterium. Morphological, biochemical, and metabolic characterization revealed that Isolate #9 is a Gram-negative bacillus with catalase activity, gelatin hydrolysis, glucose fermentation, nitrate reduction, and optimal growth at 30 °C, while lacking phospholipase, amylase, oxidase, and pigment production. Antibiotic susceptibility testing showed that the isolate is sensitive to trimethoprim and rifampin but resistant to tetracycline, penicillin, and gramicidin. Trimethoprim inhibits DNA synthesis by blocking dihydrofolate reductase, while rifampin targets RNA synthesis by binding to RNA polymerase. Although 16S rRNA sequencing produced inconclusive BLAST results, the biochemical profile suggests potential affiliation with the genus *Proteus*. These findings suggest that soils in high-traffic areas may harbor previously uncharacterized Gram-negative bacteria capable of producing antimicrobial properties that can combat clinically significant antibiotic-resistant Gram-negative pathogens.

Keywords: Gram-negative bacteria, Proteus, antibiotic resistance, antibiotic screening, urban soil microbiology, ESKAPE pathogens, antimicrobial discovery, 16S rRNA sequencing, Kirby-Bauer testing, biochemical assays, Klebsiella pneumoniae, multidrug resistance

Identification of a Potential Antibiotic Agent Targeting Gram-Negative Bacteria From Urban Garden Soil

Ever since the discovery of penicillin, antibiotics have been obtained from microbial cultures (Santajit & Indrawattana, 2016). However, multidrug or antibiotic resistance is one of the biggest threats to global public health, and is usually caused by the excessive or misuse of antibiotics (Mohammadi & Wc1e, n.d.). The ESKAPE pathogens—E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and Enterobacter species—especially E. faecium- are examples of this issue (Mulani et al., 2019). The majority of clinical antibiotics have been derived from Gram-positive bacteria, while Gram-negative bacteria make up most of the ESKAPE pathogens. K. pneumoniae, a Gram-negative bacterium, is the most common cause of nosocomial (hospital-acquired) pneumonia in the United States, accounting for 3% to 8% of all nosocomial bacterial infections (Breijyeh et al., 2020; O'Hara et al., 2000; Ashurst & Dawson, 2023).

Here, we show that we can sample soil from a garden with a high presence of cats to find antibiotic-producing bacteria against the safe relative of gram-negative bacteria, K. pneumoniae, which is E. coli. We found that a bacterial isolate (#9) exhibited a zone of inhibition when screened against the gram-negative ESKAPE pathogen relative, E. coli. Isolate #9 was characterized as a Gram-negative bacterium. Upon performing a Kirby-Bauer Disk Diffusion Susceptibility Test, we found that the isolate is susceptible to the antibiotics Trimethoprim and Rifampicin, and resistant to penicillin, trimethoprim, gramicidin, and tetracycline.

Molecular and biochemical assays revealed that Isolate #9 has no phospholipases, no pigment, is a mesophile as it grows well at 30 °C, no amylodextrin, secretes proteolytic enzymes, performs denitrification, contains catalase enzymes, and does not contain the cytochrome C oxidase system. Furthermore, the 16S rRNA gene was amplified from isolate #9, and sequencing analysis revealed inconclusive results. By utilizing sequencing and modern biochemical tests, we can significantly advance the medical field by discovering antibiotic-producing bacteria that may be effective against the ESKAPE pathogens (Mario Sergio Pino-Hurtado et al., 2023).

These antibiotic-producing bacteria can be found in the most common locations, such as gardens and fields (Mast & Stegmann, 2019). These findings can open new possibilities for innovative treatments against antibiotic-resistant bacteria, especially against K. pneumoniae.

Results

We performed an antibiotic screening on select colonies of bacteria from soil samples obtained from a garden with a high presence of cats. Through this process, we identified Isolate #9, which showed a zone of inhibition when screened against gram-negative ESKAPE relative, *E. coli.* This isolate had characteristics of a white colony that was circular, smooth, and raised (Figure 1B). The isolate was obtained when the soil sample was grown from an All Culture (AC) plate at a dilution 10^{-3} . A Gram stain was performed on the isolate, with *B. Subtillus* being the positive control and *E. coli* being the negative control. The results of the Gram stain for the isolate are Gram-negative (Figure 1). During the Gram stain process, the isolate absorbed the crystal violet dye strongly, which made it hard to wash off with ethanol. The isolate was lightly wiped with a paper towel to remove excess isolate, which allowed for the removal of the excess

crystal violet dye. When observed at 100x objective under a microscope, the isolate was identified as a bacillus, and some were observed as diplobacilli, with the individual bacillus being measured at around 2.5 µm (Figure 1).

Figure 1

Morphological Characterization of Isolate #9: Microscopy at 100X objective showing Gram stain of Isolate #9 is gram-negative; the positive control used was *S. Aureus*, and the negative control used was *E. coli*. Isolate #9 measured at around 2.5 µm in size, and some show to be a diplobacillus.

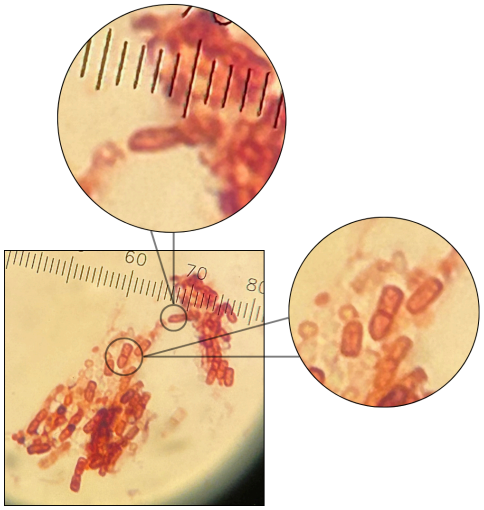


Table 1

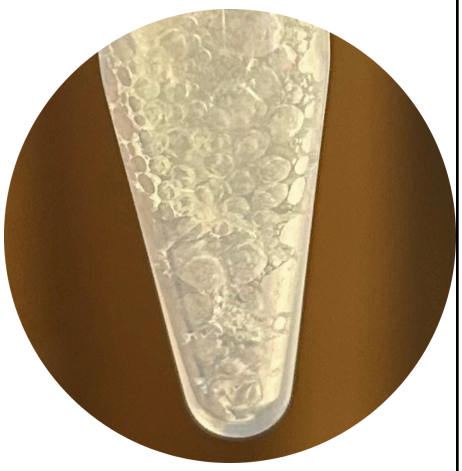
Morphological Characterization of Isolate #9 Colonies: The colonies of Isolate #9 are circular, raised, smooth, white, and opaque.

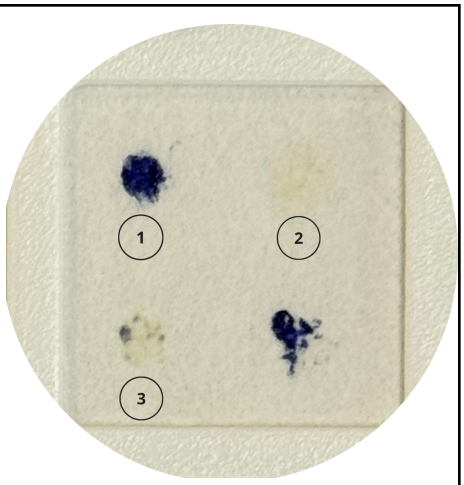
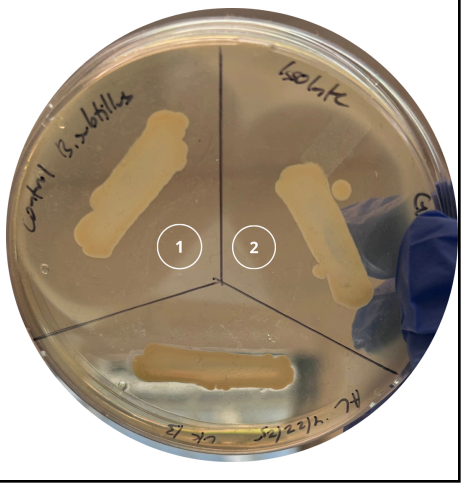
Colony Morphology Characterization of Isolate #9	
Shape	Circular
Elevation	Raised
Size	7 mm
Surface	Smooth
Pigmentation	White
Opacity	Opaque

Multiple biochemical assays were performed on Isolate #9, which can test for the presence or activity of specific enzymes to aid in identifying the isolate. Several enzymatic tests were conducted, including a catalase test, which showed that Isolate #9 produces catalase to hydrolyze H_2O_2 (Table 2). Another test performed was starch hydrolysis, which shows that Isolate #9 does not produce amylases, as indicated by forming no clear halo or maroon color of the reagent, TMP (Table 2). An oxidase test was also performed and shows that Isolate #9 does not contain cytochrome C, as indicated by showing no blue pigment formation (Table 2). A lecithinase reaction test was performed, which identifies if the isolate secreted phospholipases. The isolate showed no indication of phospholipases (Table 2). Finally, a gelatin hydrolysis reaction was performed to visualize gelatin hydrolysis by adding 30% trichloroacetic acid (TCA). The isolate indicates that it can perform gelatin hydrolysis (Table 2). Several biochemical assays to test for metabolic patterns were performed. An Oxidative/Fermentation (O/F) test results show that Isolate #9 can metabolize glucose under aerobic and anaerobic conditions when comparing the change of color to the control (Table 2). A nitrate reduction test was also conducted, with results showing that Isolate #9 can perform denitrification, as indicated by the formation of the red color when exposed to nitrate (Table 2). A pigment production test was performed to determine if Isolate #9 can produce fluorescent pigments that are visible under UV light. Results show that Isolate #9 does not produce extracellular fluorescent pigments, as it did not show pigment under UV light compared to the control *P. aeruginosa* (Table 2). The last assay that was performed was the growth temperature test, and results show that Isolate #9 grows the best at 30 °C and grows substantially well at 42 °C (Table 2).

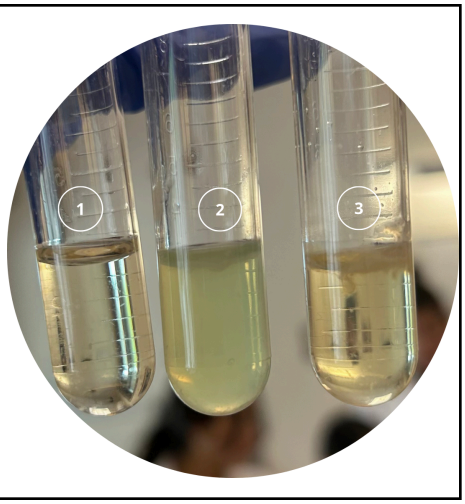
Table 2

***Biochemical Assays Performed on Isolate #9*:** Summary of all biochemical assays performed on Isolate #9 to determine biochemical properties and growth rates. The table includes controls used, images of the results, and a brief description of the conclusions made. In the images selected, numbers #1 and #2 refer to the separation on the agar and tubes between the control and isolate.

Biochemical Assay	**Control(s)**	**Results**	**Image**
Catalase	(+) Bubbles form	(+) Result Bubbles formed	
Starch Hydrolysis	(+) *B. subtilis*	(2) (-) Result No formation of a clear halo or maroon pigment production of the reagent	

Oxidase	(+) *P. fluorescence* (-) *S. epi*	(3) (-) No production of blue pigment	
Lecithin Hydrolysis	(+) *P. fluorescence*	(2) (-) No precipitation	
Gelatin Hydrolysis	(+) *B. subtilis*	(2) (+) Production of a clear halo	

Nitrate Reduction	(+) *P. fluorescence* (-) *P. acidovorans*	(+) Result Production of red pigment	
Oxidative/Fermentation (O/F)	(+) Without oil (aerobic condition)	(2) (+) Result with oil (3) (+) Result without oil	
Pigment Production	(+) *P. aeruginosa*	(2) (-) No pigment production	

Growth at Various Temperatures	(+) Growth at 30 °C	(1) (-) No growth at 4 °C (2) (+) Growth at 30 °C (3) (+) Growth at 42 °C	

As part of the biochemical characterization, the Kirby-Bauer Disk Diffusion Susceptibility Test was performed to test for sensitivity against antibiotics to analyze the potential antibiotic susceptibility of Isolate #9, and showed that it was sensitive to Trimethoprim and Rifampicin (Table 3). There was a zone of inhibition of 6mm radius against Trimethoprim (Figure 3A) and 3mm radius against Rifampicin (Figure 3B).

Table 3

Kirby-Bauer Disk Diffusion Susceptibility Test: Shows what antibiotics Isolate #9 is sensitive to when the Kirby-Bauer Disk Diffusion Susceptibility Test was performed. The table also shows the size of the zone of inhibition against the antibiotics.

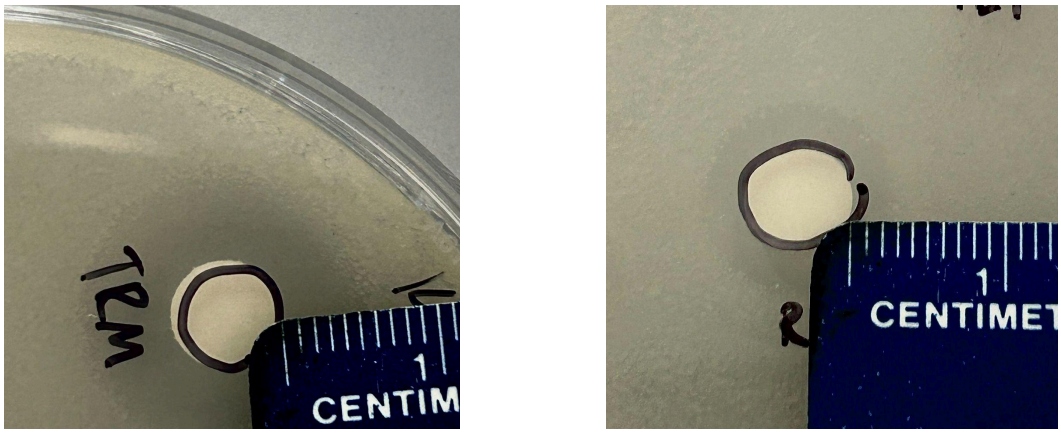
Antibiotic	**Acronym**	**Zone of Inhibition**	**Size of Zone of Inhibition**
Trimethoprim	TRM	Yes	6 mm
Gramicidin	GRA	No	-
Tetracycline	TET	No	-

Rifampin	RIF	Yes	3 mm
Penicillin	PEN	No	-

Figure 3

Kirby-Bauer Disk Diffusion Susceptibility Test Against Trimethoprim and Rifampin: **A)** Zone of inhibition of Isolate #9 against Trimethoprim (TRM) measured at 6mm. **B)** Zone of inhibition of Isolate #9 against Rifampin (RIF) measured at 3mm.

A. **B.**



A gel electrophoresis was performed to amplify the 16S rRNA gene, which is highly conserved among different species of bacteria. This allows for the use of universal primers for amplification and sequencing to identify unknown bacteria. Gel electrophoresis shows a band amplification of this DNA product around the size of 1.5 kb. Because the amplification was successful, the product was sent for sequencing. The genomic sequence obtained was sent to the Basic Local Alignment Search Tool (BLAST), a bioinformatics tool used to compare amino-acid sequences of proteins or the nucleotides of DNA and/or RNA sequences. BLAST could not find highly similar nucleotide sequences (>93%) in its database when compared to the obtained sequence from Isolate #9.

Figure 4

PCR & BLAST Results: Gel electrophoresis was performed to identify the 16S rRNA of Isolate #9. The control used was *B. subtillus*. Both bands show a visible band at 1.5kb long.

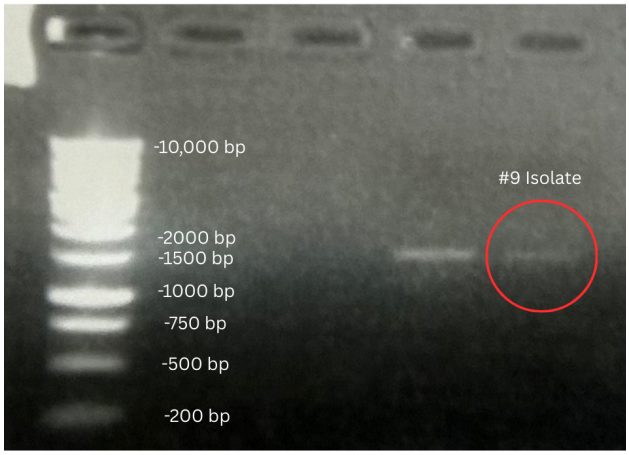


Table 4 PCR & BLAST Results Legend: Legend indicating identity of band numbers.

Band Number	Identity
1	Ladder
4	Control
5	Isolate #9

Discussion

The majority of clinical antibiotics are derived from Gram-positive Actinobacteria, especially from the genus *Streptomyces*, which has produced important antibiotics such as β-lactams and tetracyclines *(Esselmann & Liu, 1961; Gupta et al., 2014)*. The antibiotic screening results showed that the Gram-negative Isolate #9 bacterium inhibited the growth of *E. coli*, a safe relative of the Gram-negative ESKAPE pathogen *K. pneumoniae*, suggesting that it produces natural antibiotics that may inhibit the growth of *K. pneumoniae* itself.

The outer membrane of Gram-negative bacteria is the main reason for resistance to a wide range of antibiotics, including β-lactams and tetracyclines *(Breijyeh et al., 2020)*. Most antibiotics must pass the outer membrane to enter the cell, although hydrophilic antibiotics like β-lactams pass through porins *(Breijyeh et al., 2020)*. Alterations made in the outer membrane by Gram-negative bacteria, such as changing the hydrophobic properties or mutations in porins and other factors, can create resistance to these antibiotics. Gram-positive bacteria lack this important layer, which makes Gram-negative bacteria more resistant to antibiotics than Gram-positive ones *(Breijyeh et al., 2020)*.

The results of the Kirby-Bauer antibiotic susceptibility screening on Isolate #9 revealed that it is susceptible to Trimethoprim and Rifampin and resistant to Gramicidin, Tetracycline, and Penicillin. Both Trimethoprim and Rifampin target DNA or RNA synthesis; Trimethoprim inhibits bacterial dihydrofolate reductase (DHFR), an enzyme involved in synthesizing folic acid, which is essential for synthesizing purine and pyrimidine needed to build DNA and RNA *(Sikora & Zahra, 2023)*. Rifampin, on the other hand, directly inhibits DNA-dependent RNA polymerase (RNAP), halting the activity of the enzyme responsible for RNA synthesis *(Belas et al., 1998)*.

Due to the failed sequencing of Isolate #9, BLAST results could not be obtained to identify the species. Instead, we will dissect the biochemical assay results and cross-reference the morphology of Isolate #9 with peer-reviewed research articles and textbooks to identify a possible genus.

Seven out of the nine biochemical assays performed are consistent with the biochemical characteristics of the genus *Proteus*, a Gram-negative bacillus known for its swarming motility *(Beloor Suresh & Wadhwa, 2023)*.

From one research article, three strains of a *Proteus* sp. produce the same results for nitrate reduction, catalase, and oxidase tests as Isolate #9 *(Drzewiecka, 2016)*. The positive nitrate test result, indicated by the formation of a red color, shows that both the *Proteus* sp. strain and Isolate #9 perform denitrification in the presence of nitrate, reducing nitrate (NO_3^-) to nitrite (NO_2^-) *(Drzewiecka, 2016)*. The positive catalase test result, shown by bubble formation, indicates that both the *Proteus* strain and Isolate #9 produce the enzyme catalase and can catalyze hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) *(Drzewiecka, 2016)*. The negative oxidase test result, indicated by no blue color formation when exposed to TMP, shows that neither contains the cytochrome c oxidase system *(Drzewiecka, 2016)*.

According to the *Encyclopedia of Food Safety*, *Proteus* strains can grow between 10 and 43 °C, with an optimal temperature of 25 °C *(Batt & Tortorello, 2014)*. This is consistent with Isolate #9, which showed the best growth at 30 °C, indicative of a mesophilic bacterium.

Two species of *Proteus*, *P. vulgaris* and *P. mirabilis*, were tested for phospholipase secretion via the lecithinase test, and both produced negative results consistent with Isolate #9 *(Juarez et al., 2020; Kang et al., 2018)*. Phospholipases hydrolyze lecithin, a group of phospholipids found naturally in animal and plant tissues *(Juarez et al., 2020)*.

A research article that performed gelatin hydrolysis on five *Proteus* species showed that *P. mirabilis*, *P. myxofaciens*, and Biogroup 3 of *P. vulgaris* produced 90–100% positive

hydrolysis results *(Kiani et al., 2021)*. These species likely secrete proteolytic enzymes that hydrolyze gelatin *(Kiani et al., 2021)*.

One research article examining starch hydrolysis in *P. vulgaris* revealed that the strain did not produce amylase to digest starch, consistent with Isolate #9 *(Augustine, 2024)*.

Although no research articles performed an oxidative/fermentation test on *Proteus* strains, one study performed a glucose fermentation test and showed that *Proteus* can ferment glucose *(Augustine, 2024)*. In addition, according to the *Encyclopedia of Food Safety*, *Proteus* is a facultative anaerobic bacterium *(Batt & Tortorello, 2014)*.

No studies were found on pigment production in *Proteus*, although *Proteus* does not produce pyoverdin or pyocyanin—pigments characteristic of *Pseudomonas (Abdelaziz et al., 2023; Masschelein et al., 2017)*.

Future directions to further identify Isolate #9 include performing additional biochemical assays and repeating PCR and sequencing. Repeating PCR and sequencing would allow BLAST analysis to identify the species of Isolate #9. One biochemical assay that may help confirm whether Isolate #9 belongs to the *Proteus* genus is the urease test, which identifies bacteria capable of hydrolyzing urea into ammonia and carbon dioxide *(Brink, 2010)*. *Proteus* is known to produce ammonia, and a positive result would support this identification *(Drzewiecka, 2016; Kiani et al., 2021)*. If confirmed to belong to the genus *Proteus*, performing an indole test can help distinguish between species. One study showed that 95.7% of *P. mirabilis* strains tested negative, while 88.9% of *P. vulgaris* strains tested positive *(Bale et al., 1985)*. According to

Feigin and Cherry's Textbook of Pediatric Infectious Diseases, *P. mirabilis* is differentiated from other *Proteus* species by its inability to produce indole from tryptophan *(Kang et al., 2018)*.

Three *Proteus* species—*P. vulgaris*, *P. mirabilis*, and *P. penneri* are known opportunistic pathogens that can cause UTIs *(Juarez et al., 2020)*. In both case studies and artificial urine media, *P. mirabilis* has been documented to produce antimicrobial compounds, specifically ammonia, that inhibit the growth of Gram-negative bacteria such as *E. coli* and *K. pneumoniae (Kiani et al., 2021; Salam et al., 2023)*. The possibility that Isolate #9 is a Gram-negative antibiotic-producing *Proteus* strain capable of inhibiting Gram-negative ESKAPE pathogens is significant, as it suggests the potential for discovering antibiotic-producing bacteria beyond traditional Gram-positive producers.

Limitations

Although this study provides valuable insight into the potential of soil-derived Gram-negative bacteria as antibiotic producers that can target other resistant Gram-negative bacteria, several limitations must be acknowledged. The full identification of Isolate #9 remains incomplete due to the failure of 16S rRNA sequencing to produce a high-confidence BLAST match. Without successful molecular identification, the taxonomic placement of the isolate heavily relies on the results of the biochemical assays, which can be difficult to interpret due to overlapping traits among closely related bacterial genera. Environmental factors, including soil composition, moisture, and microbial diversity, were not measured, which limits the reproducibility of the environment from which the isolate was obtained.

Conclusion

There is potential for urban garden soils as valuable sources of novel antibiotic-producing bacteria. Isolate #9, a Gram-negative bacillus with biochemical characteristics consistent with the *Proteus* genus, demonstrated inhibitory activity against *E. coli*, a safe relative of *K. pneumoniae,* suggesting the ability to produce antibiotics effective against Gram-negative pathogens. These findings show the importance of continued exploration of common environments for microbial diversity and antibiotic discovery among bacteria. Further molecular identification and expanded biochemical testing, including urease and indole assays, can fully characterize this isolate and evaluate its potential as a potential source of discovering new antibiotics against resistant Gram-negative bacteria.

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