Original Research Article

A Bioinformatic Comparative Analysis of TEM-1 and ROB-1 Ampicillin Resistance β-Lactamases in *Haemophilus influenzae*

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ABSTRACT

The growing issue of antibiotic resistance is causing an increasing crisis in bacterial infections. The discovery of new antibiotics is not aligned with the rapid increase in antibiotic resistance, resulting in significant challenges in treating antibiotic-resistant bacterial infections. One important bacterial pathogen with increased antibiotic resistance is *Haemophilus influenzae*, which was highlighted in the 2024 WHO bacterial priority pathogen list, especially in terms of the rise in resistance against ampicillin and other penicillin-based antibiotics. This study aimed to computationally characterize and compare the sequence, structure, and cellular localization of TEM-1 and ROB-1 β-lactamases in *H. influenzae*. In this study, multiple computational tools, including BLAST, Interpro, Phobius, Protein Data Bank, AlphaFold, and PyMOL, were used to investigate the prevalence of the most important genes and proteins associated with increased resistance to penicillin-based antibiotics in *Haemophilus influenzae*, specifically TEM-1 and ROB-1. BLASTn and BLASTp revealed a range of bacterial species, including those with intrinsic resistance, acquired resistance, and species with no documented ampicillin resistance, which were identified in the analysis as containing both genes and their associated proteins, thereby confirming the involvement of TEM-1 and ROB-1 in ampicillin resistance, as well as the widespread dissemination of TEM-1 and ROB-1-mediated resistance. A structural comparison of the two proteins revealed high similarity, and an Interpro analysis of protein domains revealed two distinct domains in both ROB-1 and TEM-1. Phobius analysis of protein cellular localization revealed that the two proteins are non-cytoplasmic. These findings were in line with the mechanism of action of ampicillin resistance.

Keywords: ampicillin resistance; beta-lactamase; TEM-1; ROB-1; Haemophilus influenzae; bioinformatics; antibiotic resistance

INTRODUCTION

Antibiotics are extremely important in that they have greatly increased the overall life expectancy. They started with the discovery of penicillin, which was used to treat bacterial infections (1). When bacterial infections caused by pathogens such as *Escherichia coli* or Staphylococcus occur, it is necessary to have the appropriate antibiotic to treat the infection. Improved research on bacteria also helps determine which antibiotics are needed to be used for different species of bacteria, including Gram-positive and Gram-negative.

However, there are issues with the continual usage of antibiotics. Eventually, antibiotics could stop working after repeated use during prolonged sickness. The issue presented is due to a variety of factors. One such factor is the rapid replication rate of bacteria, as

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bacterial generations, such as those of *E. coli,* can occur in as little as 20 minutes (2). This rapid multiplication allows for selection to take place, selecting for bacteria with genes that confer antibiotic resistance (3). The rapid selection of these genes then results in extreme resistance to these antibiotics, allowing for infection to occur at a faster rate (4).

This is noticeable in some well-known bacterial species. *Haemophilus influenzae* (*H. influenzae*), which commonly causes pneumonia and meningitis, has been showing increased antibiotic resistance. In the timeframe from 2007-2018, extreme drug-resistant *H. influenzae* was identified in Taiwan (5). In a sample of 2091 *H. influenzae* isolates, ≥41% of these isolates were resistant to ampicillin, chloramphenicol, levofloxacin, and trimethoprim-sulfamethoxazole and were isolated from patient wards (5). Another 50% of these strains were resistant to amoxicillin-clavulanate, cefotaxime, and cefuroxime and isolated from intensive care units (5). The study describes the need for managing the presence and growth of extreme drug-resistant *H. influenzae* to be acted upon (5).

There are two primary types of antibiotics: bactericidal and bacteriostatic, which target bacterial growth. Bactericidal antibiotics kill bacteria by lysing them, whereas bacteriostatic antibiotics primarily inhibit bacterial multiplication (6). Antibiotics are currently used all around the world and have saved millions of lives. The first antibiotic, known as penicillin, was derived from mold (Penicillium) that grew on an agar plate that seemed to have little to no bacterial growth around it (7). Other antibiotics were derived from penicillin to treat a broader spectrum of bacteria. One such example is ampicillin.

Bacterial growth is assisted by penicillin-binding proteins. These penicillin-binding proteins catalyze the formation of a bacterium's peptidoglycan cell wall (8). The catalysis of the cell wall allows for bacterial growth to propagate and help bacteria thrive (Figure 1A). The peptidoglycan cell wall resists a large amount of internal cellular pressure in bacteria. Ampicillin prevents bacterial growth by acting as a competitive inhibitor. By binding to the active site of the penicillin-binding proteins, ampicillin prevents the binding of peptidoglycan to the active site (9). Peptidoglycan cannot be catalyzed into the cell wall needed to maintain a hypotonic environment inside the cell (9). Due to bacterial pressure not being maintained, the bacterium will lyse and die (Figure 1B).

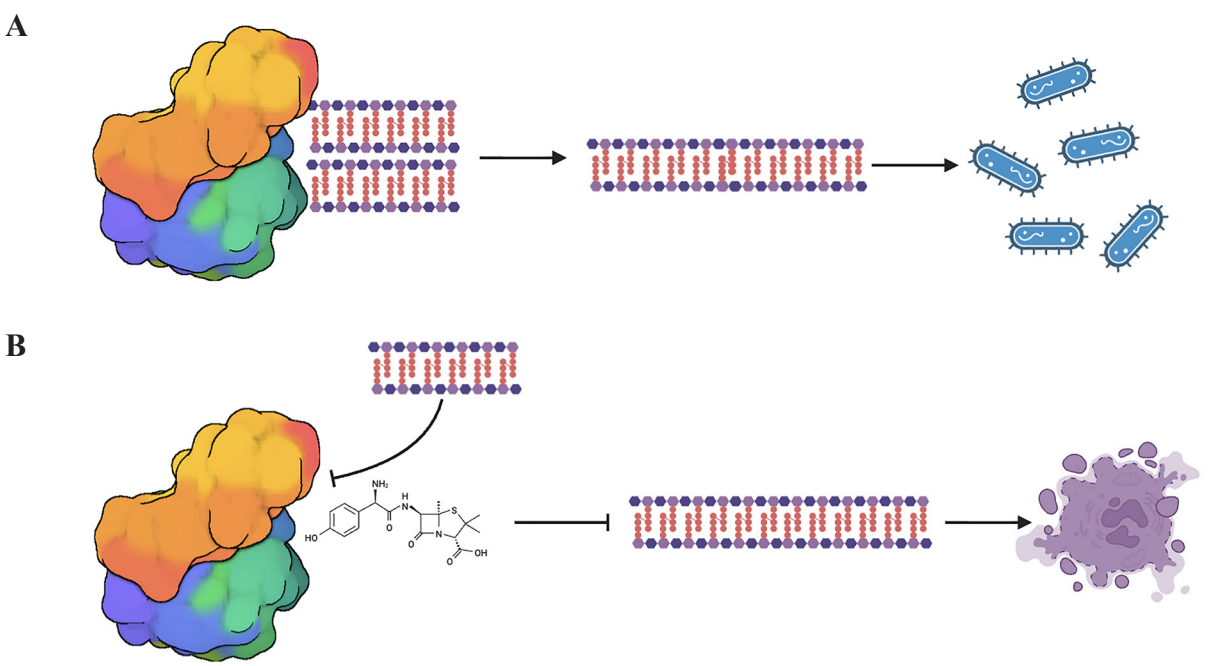


Figure 1. *Catalysis of the bacterial cell wall mediated by penicillin-binding proteins [A], and inhibition of this process by ampicillin [B]. Figure made using BioRender.*

H. influenzae has the genes *TEM-1* and *ROB-1,* providing resistance to many penicillin-based antibiotics such as ampicillin. The growing antibiotic resistance in *H. influenzae* and in many other bacteria warrants research into the prevalence and molecular mechanisms of TEM-1/ROB-1-mediated antibiotic resistance. *TEM-1* and *ROB-1* produce enzymes called Class A Serine β-lactamases. β-lactamases are hydrolases, which are enzymes that use water molecules to inactivate compounds. β-lactamases use strategically placed water molecules to inactivate these penicillin-based antibiotics before they reach the penicillin-binding proteins, resulting in minimal bacterial death (Figure 2).

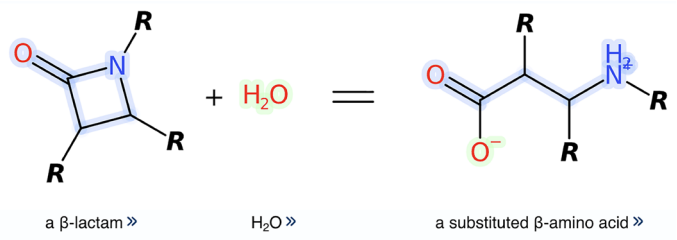


Figure 2. *Diagram of TEM-1 or ROB-1 hydrolysis of a β-lactam. Figure adapted from UNIPROT.*

There is a pressing need to understand bacteria across numerous species. In the WHO 2024 bacterial priority list, there are three groups: the critical group, the high group, and the medium group (10). The medium group, in comparison to the other two groups, is relatively understudied in terms of how to treat these infections (10). *H. influenzae* is a medium group bacterium that causes pneumonia, meningitis, and an illness known as the Hib infection. The Hib infection has similarities to many other bacterial infections, with symptoms such as high temperature, rashes, sore throats, etc. However, Hib infections can lead to serious complications, such as meningitis or sepsis (11).

Overall, the paper will conduct an in-depth bioinformatic analysis of the mechanisms of ampicillin resistance in *H. influenzae*. The study will target the notable *TEM-1* and *ROB-1* genes for ampicillin resistance analysis using multiple bioinformatic methods. The study will use BLAST to review how ampicillin resistance mechanisms are conserved in a variety of species through protein and nucleotide sequences. The TEM-1 and ROB-1 proteins will be extensively analyzed through their properties, such as their domains, localization, and structure. Considering that both TEM-1 and ROB-1 are involved in ampicillin resistance in *H. influenzae*, we hypothesize that the prevalence of their associated genes, as well as their structure and localization within the bacterial cell, are likely to be rather similar.

METHODS AND MATERIALS

This study uses a variety of bioinformatic tools. The nucleotide and protein sequences were retrieved from the GenBank database using the protein and nucleotide filters. The NCBI BLAST search was used to find matches for the input sequences. The entries were then run through 4 BLAST programs: BLASTp (protein BLAST), tBLASTn (protein-to-nucleotide BLAST), BLASTn (nucleotide BLAST), and BLASTx (nucleotide-to-protein BLAST). Protein BLAST used the default scoring matrix of BLOSUM62. The BLAST data consists of the number of matches between bacterial species and the input sequence, along with the first hit values for each species: query coverage, E-value, and percentage identity. BLAST hits were rerun on June 15th, 2026. BLAST hits that detect protists were included to keep full transparency of the BLAST hits.

The TEM-1 nucleotide sequence (accession number EU531509) and protein sequence (accession number AFM77583) were obtained from NCBI GenBank. The ROB-1 nucleotide sequence (accession number HM236409) and protein sequence (accession number ADK23949) were also obtained. Significant hits were defined as those with at least 70% query coverage and 70% percent identity. The same significance thresholds were applied for tBLASTn and BLASTx analyses to enable direct comparison across BLAST programs.

After obtaining the BLAST data, the protein structures were identified. Experimentally derived proteins were found using the PDB database, from which the TEM-1 protein structure was retrieved. ROB-1 was not experimentally determined, so the AlphaFold protein predictor was used to retrieve the predicted protein structure of ROB-1. The protein was retrieved from the Alphafold Protein Structure Database (accession AF-P67918-F1, model_v4). InterPro was used to analyze these protein sequences to find any specific domains that are similar across the two proteins. Phobius was used to predict the probability of protein localization within the cell.

RESULTS

The prevalence of TEM-1 protein across bacterial species

Using BLASTp, the TEM-1 protein sequence from *H. influenzae* was analyzed for similarity across other bacterial species. Examples of bacterial species with TEM-1, including the number of hits emerging for each species and the significance of the top hit from each species (as evaluated based on query cover, E-value, and percent identity), are summarized in Table 1. Overall, bacterial species that harbor TEM-1 express the protein, conferring ampicillin resistance via beta-lactamases encoded by the *TEM-1* gene. Note that the same analysis was performed with tBLASTn, and the only result was a couple of *E. coli hits*.

The prevalence of *TEM-1* gene across bacterial species

Using BLASTn, the input nucleotide sequences matched those from other species. Examples of bacterial species containing the *TEM-1* gene are presented in Table 2A. The same analysis was performed with BLASTx, and the top-identified species are summarized in Table 2B.

Table 1. *Examples of BLASTp hits for bacterial species containing similar protein to H. influenzae TEM-1.*

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Acinetobacter baumannii	100%	0.0	100.00%	9
Escherichia coli	100%	0.0	99.65%	17
Klebsiella pneumoniae	100%	0.0	99.65%	14
Eimeria maxima	100%	0.0	99.30%	1
Providencia stuartii	100%	0.0	98.95%	1
Enterobacter asburiae	100%	0.0	99.65%	1
Salmonella enterica	90%	0.0	99.22%	4
Pectobacterium brasiliense	100%	2e-175	82.17%	1
Acinetobacter seifertii	100%	6e-172	85.66%	1
Morganella morganii	99%	0.0	100%	1
Chromatium okenii	88%	0.0	100%	1
Serratia marcescens	82%	3e-172	99.57%	1
Staphylococcus haemolyticus	98%	0.0	98.93%	1
Pseudomonas aeruginosa	99%	0.0	95.41%	1

Table 2. *Examples of BLASTn [A] and BLASTx [B] hits for bacterial species containing similar gene to H. influenzae TEM-1.*

[A]

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Haemophilus influenzae	100%	0.0	100%	8
Haemophilus parainfluenzae	95%	0.0	99.70%	5
Acinetobacter baumannii	100%	0.0	99.56%	3
Klebsiella pneumoniae	100%	0.0	99.79%	27
Escherichia coli	89%	0.0	99.79%	33
Escherichia fergusonii	100%	0.0	99.68%	2
Salmonella enterica	100%	0.0	99.68%	10

Continued Table 2. *Examples of BLASTn [A] and BLASTx [B] hits for bacterial species containing similar gene to H. influenzae TEM-1.*

[A]

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Phytobacter diazotrophicus	100%	0.0	99.68%	1
Enterobacter hormaechei	99%	0.0	99.68%	4
Enterobacter asburiae	100%	0.0	99.68%	1
Citrobacter freundii	100%	0.0	99.68%	1
Enterobacter roggenkampii	99%	0.0	99.68%	1
Neisseria gonorrhoeae	100%	0.0	99.68%	1
Providencia hangzhouensis	100%	0.0	99.68%	1
Shigella sonnei	100%	0.0	99.68%	1
Klebsiella planticola	100%	0.0	99.68%	1
Enterobacter hormaechei	100%	0.0	99.68%	1
Proteus mirabilis	100%	0.0	99.68%	1
Citrobacter braakii	100%	0.0	99.68%	1
Shigella flexneri	94%	0.0	99.68%	2

[B]

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Acinetobacter baumannii	93%	0.0	86.81%	7
Klebsiella pneumoniae	73%	0.0	99.65%	14
Escherichia coli	73%	0.0	99.65%	25
Eimeria maxima	73%	0.0	99.30%	1
Enterobacter asburiae	73%	0.0	99.65%	1
Providencia stuartii	73%	0.0	98.95%	1
Morganella morganii	73%	0.0	98.25%	1
Chromatium okenii	64%	5e-172	100.00%	1
Pectobacterium brasiliense	73%	5e-165	82.17%	1
Serratia marcescens	60%	2e-158	99.57%	1
Acinetobacter seifertii	73%	6e-158	85.66%	1
Enterobacter hormaechei	72%	2e-157	81.69%	2

The prevalence of ROB-1 protein across bacterial species

Using BLASTp, the ROB-1 protein sequence from *H. influenzae* was analyzed for similarity across other bacterial species. Examples of bacterial species with ROB-1, including the number of hits emerging for each species and the significance of the top hit from each species (as evaluated based on query cover, E-value, and percent identity), are summarized in Table 3A. Overall, bacterial species containing ROB-1 indicate that they actively express the protein for antibiotic resistance against ampicillin using beta-lactamases derived from the *ROB-1* gene. Note that the same analysis was performed with tBLASTn, and examples of the top hits are summarized in Table 3B.

Table 3. *Examples of BLASTp [A] and tBLASTn [B] hits for bacterial species containing similar protein to H. influenzae ROB-1.*

[A]

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Pasteurella multocida	89%	0.0	100%	1
Actinobacillus pleuropneumoniae	78%	6e-174	99.58%	1
Haemophilus influenzae	79%	7e-180	100.00%	1
Escherichia coli	68%	7e-149	99.52%	1
Actinobacillus minor	61%	2e-132	100.00%	1
Mannheimia haemolytica	54%	3e-113	98.17%	2
Glaesserella parasuis	46%	2e-96	100.00%	2
Neisseria meningitidis	61%	8e-136	100.00%	2
Paenibacillus faecalis	97%	8e-100	47.39%	1
Marinisporobacter balticus	99%	1e-98	48.36%	1
Scopulibacillus cellulosilyticus	97%	4e-97	50.00%	1
Sporosarcina globispora	97%	6e-97	47.00%	1
Sediminibacillus massiliensis	96%	1e-95	50.34%	1
Brevibacillus reuszeri	87%	1e-102	55.68%	1
Virgibacillus ihumii	98%	2e-95	47.18%	1
Clostridium kluyveri	98%	2e-95	47.67%	1
Bacillus manliponensis	98%	3e-95	46.69%	1
Paenibacillus spongiae	98%	4e-95	48.03%	1
Paenibacillus pasadenensis	95%	9e-95	49.66%	1
Clostridium senegalense	83%	1e-94	52.76%	1
Bacillus pacificus	98%	2e-94	47.68%	1
Brevibacillus panacihumi	94%	2e-94	49.31%	1

[B]

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Actinobacillus pleuropneumoniae	100%	0.0	92.79%	16
Actinobacillus porcitonsillarum	100%	0.0	92.79%	4
Actinobacillus indolicus	100%	0.0	92.79%	1
Mannheimia haemolytica	100%	0.0	92.79%	7
Glaesserella parasuis	100%	0.0	92.79%	24
Pasteurella haemolytica	100%	0.0	92.79%	1
Pasteurella multocida	100%	0.0	92.79%	21
Pasteurella aerogenes	100%	0.0	92.79%	4
Bibersteinia trehalosi	100%	0.0	92.79%	4
Moraxella bovoculi	100%	0.0	92.79%	1
Wielerella bovis	100%	0.0	92.79%	2
Bergeyella porcorum	100%	0.0	92.79%	1
Haemophilus influenzae	100%	0.0	92.79%	9

The prevalence of *ROB-1* gene across bacterial species

Through the BLASTn analysis, the inputted nucleotide sequences matched those present in other species. Examples of bacterial species containing the *ROB-1* gene are presented in Table 4. The same analysis was done with BLASTx, with no significant hits, as all hits have query coverages well below 70%.

Structural and functional insights into TEM-1 and ROB-1 proteins in *H. influenzae*

UniProt described both proteins as hydrolases capable of cleaving the ring of the beta-lactam subunit in beta-lactam antibiotics, resulting in new substituted beta-amino acids. Structural similarities between TEM-1 (experimentally solved, hence retrieved from PDB) and ROB-1 (not experimentally solved, hence predicted via AlphaFold) were performed in PyMol protein modeling software, where superimposing the structures of the two proteins revealed high similarity with an RMSD value of 0.754 (Figure 3A). Structural domain analysis using Interpro revealed two beta-lactamase/transpeptidase-like domains, identical in both proteins, thereby confirming the structural homology (Figure 3B). Cellular localization analysis using Phobius indicated that both proteins were primarily non-cytoplasmic, as shown in the graph for TEM-1 and ROB-1. From the visualization, the Phobius output values hover around 1, indicating a signal peptide from amino acids 1–24, with a high probability for this section of the proteins. For the probability of being non-cytoplasmic and beyond for TEM-1, the probabilities remain around 0.95–0.90, while for ROB-1, the probabilities hover around 1–0.98 (Figure 3C).

DISCUSSION

The TEM-1 and ROB-1 proteins have similar mechanisms of action to confer ampicillin resistance. Protein and gene similarity analysis using BLAST has demonstrated that the proteins and their associated genes are present in multiple species of bacteria known to be either intrinsically resistant to ampicillin or to carry ampicillin resistance genes, thereby confirming the accuracy of our analysis. Also, some of these species appeared in the BLAST analysis for both TEM-1 and ROB-1, indicating that the two resistance proteins are sometimes coexpressed. This aligns with our initial hypothesis that, given that both proteins are involved in ampicillin resistance in *H. influenzae,* their prevalence, as well as that of their associated genes, is likely to be rather similar. Examples of significant ampicillin-resistant species (≥70% query coverage, ≥80% identity) identified in our analysis are summarized in Table 5.

It is interesting to note that we obtained far more hits with BLASTn/BLASTx analysis (i.e., species with the *TEM-1* and *ROB-1* genes) compared to BLASTp/tBLASTn analysis (i.e., species with the TEM-1 and ROB-1 proteins). This could be associated with differences in protein expression among bacterial species or differences in exposure to ampicillin, as the expression of resistance genes (i.e., the presence of the proteins) typically occurs only when the bacterium is in ampicillin-containing environments.

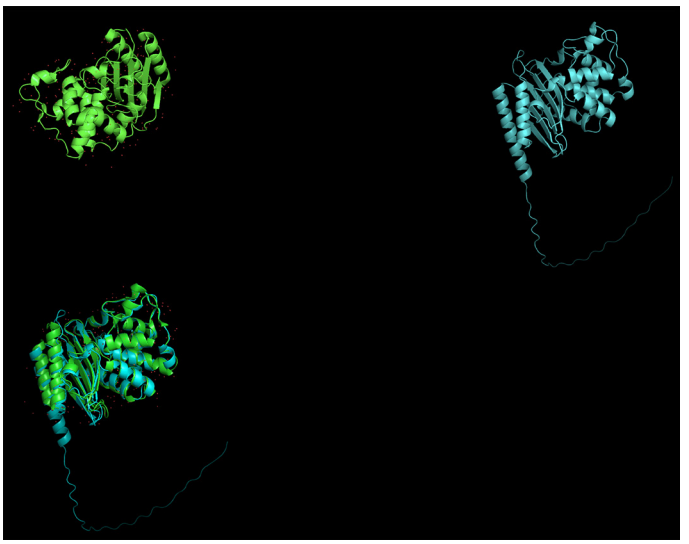
Considering that both TEM-1 and ROB-1 are involved in ampicillin resistance in *H. influenzae*, it can also be hypothesized that the structure and localization within the bacterial cell of the two proteins are likely to be similar. Our hypothesis was confirmed through the structural comparison of TEM-1 and ROB-1. Using PyMol, we were able to overlay the two structures, highlighting that they are very similar.

This structural similarity was further highlighted through the domain analysis in Interpro, which highlighted two beta-lactamase/transpeptidase-like domains in each protein, and the two domains were identical for

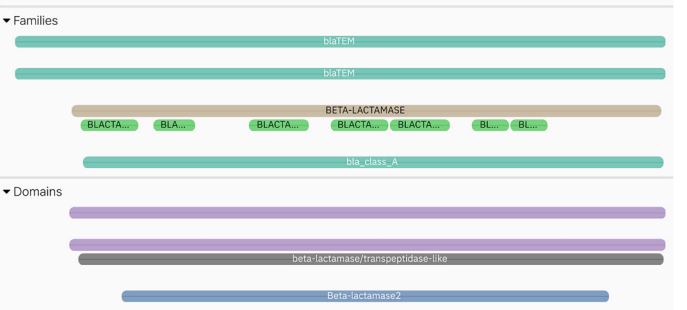
Table 4. Examples of BLASTn hits for bacterial species containing similar gene to *H. influenzae* ROB-1.

Bacterial species	Query Cover	E-value	Percent Identity	Number of Hits
Haemophilus influenzae	100%	0.0	100.00%	9
Glaesserella parasuis	100%	0.0	99.89%	22
Pasteurella multocida	100%	0.0	99.89%	22
Pasteurella aerogenes	100%	0.0	99.87%	4
Actinobacillus pleuropneumoniae	100%	0.0	99.85%	34
Mannheimia haemolytica	100%	0.0	98.85%	9
Mannheimia pernigra	61%	0.0	98.17%	2
Actinobacillus porcitonsillarum	52%	0.0	97.97%	1

A



B

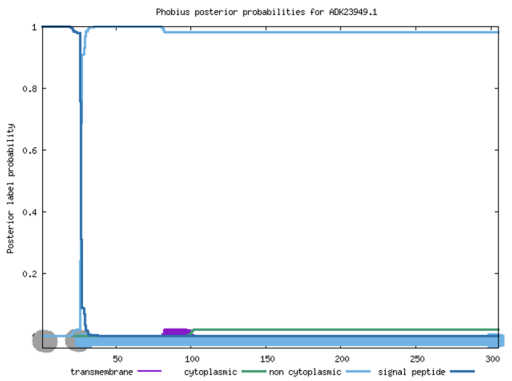


C

Phobius prediction

Prediction of ADK23949.1

```
ID   ADK23949.1
FT   SIGNAL       1     25
FT   REGION       1      6       N-REGION.
FT   REGION       7     18       H-REGION.
FT   REGION      19     25       C-REGION.
FT   TOPO_DOM    26    305       NON CYTOPLASMIC.
//
```

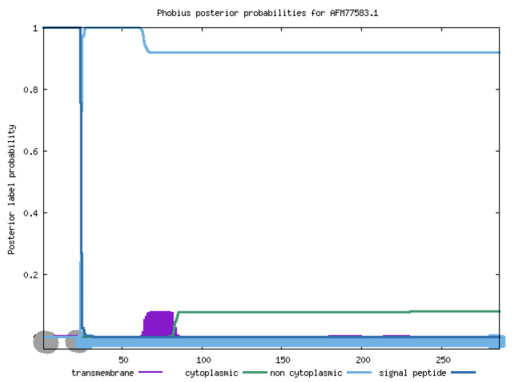


The probability data used in the plot is found here, and the gnuplot script is here.

Phobius prediction

Prediction of AFM77583.1

```
ID   AFM77583.1
FT   SIGNAL       1     23
FT   REGION       1      7       N-REGION.
FT   REGION       8     19       H-REGION.
FT   REGION      20     23       C-REGION.
FT   TOPO_DOM    24    286       NON CYTOPLASMIC.
//
```



The probability data used in the plot is found here, and the gnuplot script is here.

Figure 3. *Structural and functional analysis of TEM-1 and ROB-1 proteins in H. influenzae. [A] The structures of H. influenzae TEM-1 (retrieved from PDB) (top left, green) and ROB-1 (predicted by AlphaFold) (top right, cyan), as well as the two structures superimposed on each other (bottom) using PyMOL. The analysis highlights high structural conservation between the two proteins. [B] The two beta-lactamase/transpeptidase-like domains, which were identical for both proteins, as predicted by Interpro. [C] The cellular localization for the two proteins as predicted by Phobius with numerical data regarding where sections of the protein remain.*

Table 5. *Examples of species identified in our BLAST analysis as resistant to ampicillin through the presence of TEM-1, ROB-1 or both.*

Bacterial species	Ampicillin Resistance	Importance	Reference
Escherichia coli	Gained	Infections in the gut and urinary tract. Often treated with ampicillin	(20)
Acinetobacter baumannii	Intrinsic	Multi-drug-resistant infections especially in hospitals. Not typically treated with ampicillin	(21)
Klebsiella pneumoniae	Intrinsic	Pneumonia and respiratory infections	(16)
Eimeria maxima	Intrinsic	A protozoan that causes coccidiosis in poultry that isn't treated with antibiotics. ampicillin is used as a supportive treatment of bacterial complications associated with E. maxima infections.	(22) (23)
Providencia stuartii	Intrinsic	Catheter-associated UTIs especially in elderly patients	(24)
Enterobacter asburiae	Intrinsic	Pneumonia and UTIs	(25)
Salmonella enterica	Gained	A foodborne pathogen transmitted mainly through contaminated or undercooked food	(26) (27)
Pectobacterium brasiliense	Gained	A bacterial plant pathogen causing lot of damage to horticulture plants	(28)
Acinetobacter seifertii	Gained	Associated with intensive care unit infections as a part of Acinetobacter genus	(18) (14)
Morganella morganii	Intrinsic	Causes septic arthritis and sepsis	(29)
Chromatium okenii	No reports of resistance	A water bacterium capable of photosynthesis	(30)
Pseudomonas aeruginosa	Intrinsic	Blood, lung, and urinary infections occur after resistance.	(31) (19)
Staphylococcus haemolyticus	Gained	Healthcare associated pathogen usually found in intensive care units. Also found in bacterial meningitis. Not universal resistance.	(32) (33) (34)
Actinobacillus porcitonsillarum	Gained	Associated with tonsils in pigs and are non-pathogenic with minimal clinical relevance. Susceptible to ampicillin	(13)
Actinobacillus indolicus	No Data	Ampicillin generally used on Actinobacillus	
Mannheimia haemolytica	Gained	Primary pathogen associated with bovine respiratory disease. Near 100% resistance to ampicillin	(35) (36)
Glaesserella parasuis	Gained	Main pathogen associated with Glässer disease in pigs.	(37)
Pasteurella multocida	Gained	After close contact with an animal such as a scratch or bite from dog or cat Pasteurella multocida is the most common cause of infection	(38) (39)
Pasteurella aerogenes	No Data Available	Usually found in pig respiratory and digestive tracts but could eventually cause harm and become pathogenic.	(17)
Bibersteinia trehalosi	Gained	Commonly affects ruminants (hoofed animals such as sheep and cattle).	(40)
Moraxella bovoculi	No resistance	Common bacterial cause of infectious bovine keratoconjunctivitis. Common ocular disease caused by corneal infection.	(41) (42)
Wielerella bovis	Potential resistance but minimal	Very new bacterial species found in bovine endocarditis.	(15)
Bergeyella porcorum	Potentially Intrinsic	Unsure relationship with pneumonia in pigs	(43)

both proteins. This also aligns with the function of the two proteins as beta-lactamases.

The cellular localization analysis with Phobius yielded identical predictions for both proteins, indicating that they are predominantly non-cytoplasmic. This not only aligns with our initial hypothesis but also with the function of the proteins as beta-lactamases, which act on the bacterial cell wall that is outside the cell.

In this study, the structural and localization components of the hypothesis were supported; however, prevalence differed substantially between the two proteins, ROB-1 and TEM-1. BLAST results indicate that ROB-1 is largely confined to animal-associated bacterial species, whereas TEM-1 is more widely distributed among human-associated pathogens. This distinction is biologically significant: it suggests that the two resistance genes reflect the environments in which they evolved, ROB-1 in animal agriculture and TEM-1 in human medicine.

The functions of TEM-1 and ROB-1 beta-lactamases are tied to their localization as "non-cytoplasmic". The periplasmic space is a defining feature of Gram-negative bacteria, located between the inner cytoplasmic membrane and the outer membrane. Gram-positive bacteria, which lack an outer membrane, do not possess a true periplasmic space in this structural sense. As H. influenzae is a Gram-negative bacterium, the non-cytoplasmic localization of both TEM-1 and ROB-1 is consistent with periplasmic positioning (12). PBPs reside in the periplasmic space, where penicillin-based antibiotics bind, leading to bacterial cell death. Based on the data, researchers can infer that TEM-1 and ROB-1 can hydrolyze ampicillin when it is present in the periplasmic space, allowing PBPs to continue building the cell wall without interference. This is specific to gram-negative bacteria in such as *Haemophilus influenzae*.

This study has limitations that should be acknowledged. The analysis is computational, and additional experimental research is needed to confirm the functional expression of TEM-1 and ROB-1 in all species identified through BLAST. The presence of a homologous sequence does not necessarily confirm active ampicillin resistance, as epigenetic regulatory factors, such as methylation, can affect ampicillin resistance expression. Additionally, the structural comparison of TEM-1 relied on an experimentally solved PDB structure, whereas ROB-1 was modeled using AlphaFold (predicted structure), which introduces inconsistency and uncertainty in the final comparison of both genes. Future studies should incorporate antimicrobial susceptibility testing across the various species detected by BLAST, as well as other methods to experimentally determine the ROB-1 structure.

To summarize, the study conducted an in-depth computational bioinformatic analysis focusing on the two most important ampicillin resistance proteins in *H. influenzae*, namely TEM-1 and ROB-1, hypothesizing that their involvement in the same ampicillin resistance pathway might mean that they have similar characteristics in terms of structure, cellular localization, and prevalence across bacterial species. The similarity of the two proteins was confirmed by superimposing their structures and analyzing their domain characteristics and predicted cellular localization. Also, the similarity in the prevalence of the two proteins and their associated genes across bacterial species was demonstrated via multiple BLAST analyses. This study therefore highlights the importance of monitoring TEM-1- and ROB-1-mediated ampicillin resistance in bacteria and demonstrates the similar characteristics of these two proteins.

CONCLUSION

The hypothesis states that both TEM-1 and ROB-1 have similar structures, localization, and prevalence. However, analysis of the study indicates that the structure and localization of the two proteins are extremely similar (supporting similar mechanisms of action); however, the hypothesis is challenged by the differing prevalence between the two species. In the ROB-1-associated species, using BLAST, these resistance proteins are commonly found in bacteria related to animals (pigs, cows, etc.) rather than in bacteria commonly found in humans, which contain the TEM-1 protein. The differences in prevalence show which proteins commonly indicate antibiotic resistance in humans and which mark clinically relevant bacteria. However, understanding which antibiotic resistance proteins are present in animals can be crucial to understanding how ampicillin resistance poses a pressing issue in the meat industry. Currently, as antibiotic resistance is a pressing issue, a leading cause of hospital deaths worldwide, it is imperative to understand bacterial mechanisms of action across multiple protein families to develop effective strategies for neutralizing these proteins through methods such as antagonist drug discovery. This study overall shows that studying medium-group bacteria, such as *H. influenzae*, can reveal surprising similarities and differences between two proteins that are presumed to

act similarly, suggesting potential for further study in fields beyond human clinical settings.

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interest related to this work.

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