American Journal of Biology and Natural Sciences
https://biojournals.us/index.php/AJBNS
ISSN: 2997-7185

Evolutionary and Virulence Signatures of *S. Maltophilia* and *S. Paucimobilis* Uncovered Through Genomic and Proteomic Profiling

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Received: 2025, 28, Nov
Accepted: 2025, 29, Dec
Published: 2026, 30, Jan

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Abstract: This paper is a comparative study of Stenotrophomonas maltophilia and Sphingomonas paucimobilis using phenotypic, biochemical, genomic and proteomic characterization. There were 32 isolates that were obtained in the clinical and environmental sources (18 S. maltophilia, 14 S. paucimobilis). The growth (2436 h) and biofilm formation (83.3%, OD595: 0.28116) of S. maltophilia were higher and the maximum environmental tolerance was high (survivance in 8178.NaCl). Antibiotic susceptibility profiles indicated that S. maltophilia was largely multidrug resistant, with a resistance rate of 88.9-100 per cent. to β-lactam, up to 94.4 per cent. to carbapenem and 83.3 per cent. to aminoglycoses whilst S. paucimobilis only exhibited moderate resistance to β-lactam (4271) and high susceptibility to fluoroquinolones and tetracyclines. These results were supported by genomic analysis in which percentages of resistance and virulence determinants were found to be high in S. maltophilia at; smeDEF (100%),

rmlA-D (94.4%), rpfF (88.9%), and fliC (83.3%), in comparison with the percentages at 73.5-35% in S. paucimobilis. The addition of MALDI-TOF MS greatly improved accuracy of identifications that produced 100 percent concordance with 16S rRNA sequencing and correct two mis-identifications created by VITEK 2. All these findings highlight the strong pathogenic potential and evolutionary specialization of S. maltophilia, and place S. paucimobilis as a low-virulence, but environment-adapted species.

Keywords: S. maltophilia, S. paucimobilis, VITEK 2, mini-genomics, antibiotic resistance, phylogeny.

Introduction:

Gram-negative, non-fermenting bacterium S. maltophilia and S. paucimobilis have become increasingly important as the opportunistic pathogens over the last several years(1). Once taken as innocuous environmental agents that relate to mold, they have now become key contributors of hospital-acquired infections, especially in immunocompromised or medically dependent patients(2). These bacteria are exceptionally adaptable, able to survive in nutrient-depleted conditions, they are highly resistant to disinfectants and their tolerability to antibiotic pressure is extremely high which has enhanced their clinical importance(3, 4).

They live under similar environments e.g. soil, water and hospital environments though they vary in classification and morphological features. *S. maltophilia* is a subfamily Gammaproteobacteria of the family Xanthomonadaceae and *S. paucimobilis* is a subfamily Alphaproteobacteria of the family Sphingomonadaceae(5). *S. maltophilia* has been described as a rod shaped, obligate aerobe which forms smooth yellow green colonies. It is also hyperenzymatic against protein and lipids, as well as develops thick biofilms on medical surfaces, which increases its stability and resistance to antibiotics(6) (7). Conversely, S. paucimobilis colonies are yellow in color as a result of carotenoid like molecules and grow slowly in aerobic conditions, which is in contact with a single polar flagellum. It is not as virulent as it is but can nonetheless lead to outbreaks of both blood-stream and catheter-based infections and is thus able to resist being in sterile hospital conditions(8).

Biochemically, the two exhibit a variety of enzymatic activities, which have been attributed to the variation in the metabolic activities of these organisms. *S. maltophilia* is neither oxidase-negative nor oxidase-positive but has the ability to produce proteases, lipases and DNases. Conversely, *S. paucimobilis* is oxidase positive and catalase positive(9). Computerized identification systems, e.g. VITEK 2, are based on these biochemical interactions to quickly determine the non-fermenting Gram-negative bacteria. The misidentification may however occur because of the similarity that prevails in appearance of the species thus bringing out the significance of

combining biochemical and genomic data to ascertain the correct diagnosis(10, 11). Because the automated system (VITEK 2) occasionally has false identifications due to rare non-fermenting bacteria, including a false positive distribution of *S. paucimobilis*, the introduction of more high-resolution instruments like MALDI-TOF MS has become even more significant in modern microbiological studies. This proteomic method is used to generate species-specific mass spectral fingerprints, which can be used to make a rigorous distinction between closely related organisms and forms a powerful complementary method to 16S rRNA gene sequencing.

Genomically, *S. maltophilia* possesses large and complex genome with the high level of mobile genetic components which favour horizontal gene transfer (HGT), (12) that will contribute to its future development of multidrug resistance. Other beta-lactamases (L1 and L2) along with overexpression of multidrug efflux pumps and new biofilm-related strategies are its mechanisms of resistance to beta-lactams, carbapenems, aminoglycosides, and macrolides(13). This bacterium is frequently not treated effectively because of trimethoprim-sulfamethoxazole or recently developed drugs such as minocycline and cefiderocol(14). By contrast, S. paucimobilis has a small genome, about 4.2 Mb, with distinct cell membrane characteristics(15). It is a stable cell membrane with sphingoglycolipids rather than lipopolysaccharides that increase its stability in the environment and its capacity to resist against oxidative stress(16). It is generally sensitive to fluoroquinolones, aminoglycosides and tetracyclines, but resistant to beta-lactams as it has the beta-lactamase systems genetically coded in its DNA, and flow proteins coded in its chromosomes. Recent genomic research has also found plasmid-mediated resistance genes and integrons, which have led to an augmentation of the capacity of the bacteria to accept the challenging hospital environments(17).

Comparative evolutionary studies have revealed that the two species (although belonging to different taxonomic groups) have evolved separate processes that allow antibiotic tolerance, stability, and formation of biofilms(18). Stress response genes, multiple drugs transporters, and quorum sensing are among the genes that are critical in improving their survival in adverse conditions. It is believed that these convergent evolutionary characteristics came about due to the similar environmental stress factors, including disinfectants and non-lethal amounts of antibiotics(19).

The paper intends to examine the evolutionary diversity and determine the virulence capacity of an isolate of *S. maltophilia* and *S. paucimobilis* through a combination of comprehensive genomic work and biochemical work with the VITEK 2 system(20). A high throughput phenotyping method is presented, which allows the simultaneous identification of rapid phenotyping with in-depth genomic understanding, facilitating the relationship of identified biochemical elements to genetic elements including beta-lactamases, efflux pumps and biofilm-related genes. Results of the study associate VITEK 2 response patterns with genetically encoded resistance and virulence markers, improving the knowledge of the genotype-phenotype association and aiding further enhancement of the accuracy of species identification and clinical interpretative meaning.

The need to determine the accuracy of the VITEK 2 system towards identifying these organisms is also discussed by the fact that an error in identification may result in a misplaced treatment decisions. Thus, the validity of the VITEK 2 data is confirmed by its comparison with more advanced techniques like the 16S ribosomal RNA sequencing and whole-genome analysis that are used in establishing the quality of automated systems in case of rare non-fermenting bacteria. Also, the study compares the most important genes on virulence to emphasize on the pathway of adhesion, motility, biofilm formation, and immune evasion. In the case of *S. maltophilia*, it will be genes like smeABC, rmlA-D, and fliC and quorum-sensing factors like rpfF and rpfC. In the case of *S. paucimobilis*, the analysis will look at those genes used to defend against oxidative stress and those genes used to defend against the synthesis of the sphingolipids. This comparative study will eventually offer a holistic perspective of evolution and function as a means of comprehending how the two bacteria survive and evolve in clinical environments. The combination of phenotypic tests, the application of the VITEK 2 automated identification system, the use of advanced techniques, i.

e., MALDI-TOF MS and the bioinformatics platforms allowed the study to contribute to the further understanding of the mechanisms of bacterial adaptation, the development of their resistance, and the increased accuracy of diagnosis in the context of the challenges of multidrug-resistant infections.

Methodology

2.1. Study Design and Location

The research was done in diwaniyah in Iraq, and involved a partnership between the Ministry of Health (Diwaniyah Teaching Hospital) and the College of Biotechnology at Al-Qadisiyah University. The timeline of the study was Jul- 2023 to Jun- 2024, where phenotypic, biochemical and genomic data were combined to evaluate the evolutionary diversity and virulence potential of *S. maltophilia* and *S. paucimobilis*. The two supervising institutions gave all the regulatory and ethical approvals.

2.2. Sample Collection

S. maltophilia and *S. paucimobilis* clinical as well as environmental isolates had been sourced through accredited microbial collections and healthcare facilities. Bronchoalveolar lavage, respiratory aspirates, nasal swabs, and sputum were used as clinical samples, whereas hospital water and equipment and staff surfaces were used as environmental samples. Purified isolates were streaked on nutrient agar, and colony morphology and Gram staining were used to identify them.

2.3. Culture Maintenance and Growth Conditions

Isolates were incubated on Mueller-Hinton agar and broth (Sigma-Aldrich) as per Bilinskaya et al. (2020) (21), and on chromogenic agar (Conda Laboratory) as per Akter, L., et al. (2014) (22). Further growth was done on blood agar and MacConkey agar (HIMEDIA) at aerobic conditions at 30 37 o C within 24-72 h. The hanging-drop method and semi-solid motility agar (0.3% agar) were used to determine motility. Morphology, pigmentation, and hemolytic activity were noted, which was in a colonial form.

Isolates were cultured on Mueller-Hinton agar and broth (Sigma-Aldrich) following Bilinskaya *et al*. (2020) and on chromogenic agar (Conda Laboratory) according to Akter, L., *et al.* (2014) Additional growth was performed on blood agar and MacConkey agar (HIMEDIA) at 30–37°C for 24–72 h under aerobic conditions. Motility was evaluated using the hanging-drop technique and semi-solid motility agar (0.3% agar). Colonial morphology, pigmentation, and hemolytic activity were recorded.

2.4. Biochemical and Metabolic Characterization

Manual assays and VITEK 2 GN card were used to study the biochemical properties. A test of the activity of catalase, oxidase, lipase, protease, and DNase enzymes was done with the help of standard methods. Qualitative assessment was done with the help of the crystal violet stain in a microliter assay, whereas qualitative analysis was through the use of the Congo red agar. Also, it was found that growth tolerance was in different conditions of sodium chloride concentration (0-10%), pH (4-10), and temperatures of 20-45 C.**(6)**

2.5. Antibiotic Susceptibility Testing

Kirby Bauer disk diffusion and MIC were used to identify the susceptibility to antibiotics. Antibiotics that were tested were β-lactams, aminoglycosides, macrolides, fluoroquinolones, and tetracyclines. The VITEK 2 Compact System (BioMerieux, France) was used to analyze all the isolates using the AST-P577 and AST-N117 cards. There were 64 micro-wells of antimicrobial agents or substrates in each card. Advanced Expert System (AES) deciphered resistance schemes and patterns.

2.6. Genomic DNA Extraction and 16S rRNA Amplification

A commercial kit (Promega) was used to extract genomic DNA according to the instructions provided by the manufacturer. PCR was done to amplify the 16S rRNA gene and used the universal primers 27F, 907R, and 1525R to amplify 1500 bp. On 1% agarose gel, the visualization of products and sequencing on an ABI 3100 analyzer Table 1 were performed. Moreover, potential target genes (smeDEF, rpfF, rpfC, fliC, rmlA-D) related to antibiotic resistance and biofilm formation were obtained and extracted by the Prokka software and analyzed by the Roary software (presence/absence) (23) . BLASTn software was used to verify the functions of the gene by comparing it with the VFDB, CARD, and KEGG databases with an identity threshold of 95%(24).

Table 1. General primers applied in the amplification of the 16S rRNA gene.

Primer Name	Sequence (5′ → 3′)	Target Region	Product Size (bp)	Reference
27F	AGAGTTTGATCCTGGCTCAG	5′ conserved region	~1500	Weisburg et al., 1991(25)
907R	CCGTCAATTCMTTTRAGTTT	Mid-region of 16S rRNA		Lane, 1991(26)
1525R	AAGGAGGTGATCCAGCCGCA	3′ conserved region	~1500	Lane, 1991(26)

2.7. MALDI-TOF MS Identification and Validation

Pick isolates were further analyzed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics) to increase the accuracy of the identification of bacterial isolates and also to confirm the results of automated biochemical analysis. The pure colonies were placed on a stainless-steel target plate and covered with α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution. Mass spectral profiles were obtained in a linear positive mode that produced protein fingerprint spectra in the normal m/z range of bacteria identification. The results of identifying were interpreted as good matches of the species with the results of 2.0 and above as good matches of the species and 1.7-1.99 as good matches of the genus. The obtained spectra were matched to Bruker Biotyper reference database and cross-validated with VITEK 2 GN identification results and 16S rRNA sequencing to identify concordance and possible misidentification events.

2.8. Mini-Genomic and Phylogenetic Analyses

Sequences were modified using FinchTV, and related to GenBank by BLASTn. Clustal Omega and WebPRANK generated alignments. Mega X and IQ-TREE were used to construct phylogenetic trees through Maximum Likelihood and Neighbor-Joining (500 bootstraps). Prokka and Roary were used in core and accessory genome analysis. Annotations were done based on CARD, VFDB and KEGG databases (27).

2.9. Statistical and Bioinformatic Analyses

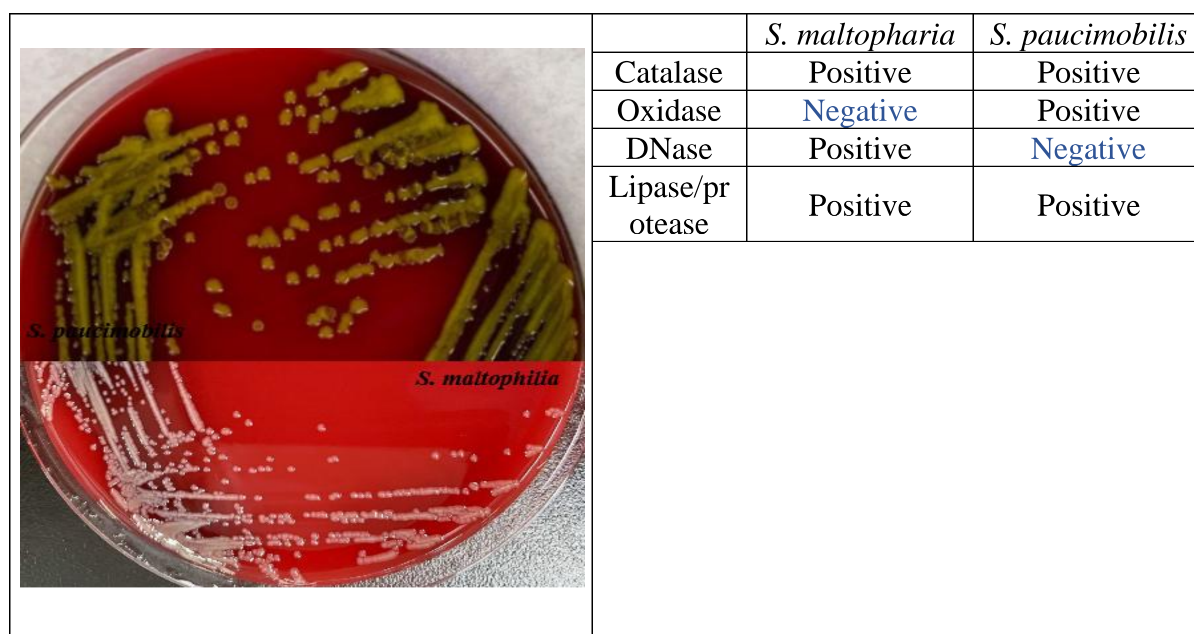
Statistical analysis was done using SPSS v26 and R v4.3. Pearson correlation and Fisher exact test were used to explore the correlations between VITEK 2 biochemical profiles and genomic characteristics. ggplot2 and factoextra were used to visualize PCA and clustering analyses and iTOL v6 was used to visualize phylogenies.(28).

Results

Both clinical and environmental samples of Diwaniyah Teaching Hospital were collected between July 2023 and June 2024 in order to recover a total of 32 bacterial isolates. Among them, 18 out of these isolates were *S. maltophilia* and 14 out of them were *S. paucimobilis*. Initial morphological

and Gram staining data were performed, which proved that all of the isolates were Gram-negative, rod-shaped, and non-fermentative. *S. maltophilia* colonies were smooth, yellow-green, circular, and smooth on nutrient agar, with colonies of *S. paucimobilis* being yellow-pigmented, opaque, and slightly mucoid. Both species were non-lactose fermenting as confirmed by the chromogenic and MacConkey agar tests.

There was growth pattern that *S. maltophilia* was the faster growing organism as it normally needed 24 to 36 hours to develop a visible colony, whereas *S. paucimobilis* needed about 48 to 72 hours to reach the same growth rate. It was established that the best temperature to grow both species was between 35 and 37 o C and under aerobic conditions. *S. maltophilia* was actively mobile on semi-solid agar with characteristic green pigment formation, with *S. paucimobilis* less mobile and remaining at a stable yellow color because of the carotenoid pigment formation. The hemolytic activity of *S. maltopharia* was also significant in the blood agar culture, which was more enzyme active and showed the presence of a high degree of virulence. Figure 1.



	S. maltopharia	*S. paucimobilis*
Catalase	Positive	Positive
Oxidase	Negative	Positive
DNase	Positive	Negative
Lipase/protease	Positive	Positive

Figure 1: Phenotypic and biochemical characteristics *S. maltophilia*; and S. *S. paucimobilis* on blood agar.

Although biochemical characterization identified *S. maltophilia* to be catalase-positive and oxidase-negative with major DNase activity, *S. paucimobilis* was catalase-positive and oxidase-negative, but DNase-negative. In terms of the enzyme production, both species were active in terms of lipase and protease, although *S. maltophilia* presented a higher level of enzyme secretion, as evidenced by larger hydrolytic zones on tripoterine agar and skimmed milk. They were all correctly identified based on VITEK 2 GN card with confidence limits ranging between 95% and 99, which supports the accuracy of the automated biochemical assays. There are however minor differences in the environmental *S. paucimobilis* isolates which are at times confused with Burkholderia cepacia complex and this illustrates the need to validate the findings genetically.

In order to enhance the quality of identification and eliminate the differences related to automated biochemical methods, all isolates were analyzed using MALDI-TOF MS analysis. The method was able to produce species-specific spectral fingerprints with unique high-intensity protein mass spectra of *S. maltophilia* and *S. paucimobilis*. The MALDI-TOF MS outcome showed 100 per cent concord with 16S rRNA sequencing and thus the taxonomic identity of all the isolates was confirmed. Notably, MALDI-TOF MS was able to resolve two incorrect classifications yields by the VITEK 2 system, where *S. paucimobilis* isolates have been mistakenly classified as members of the Burkholderia cepacia complex. These results emphasize the excellent discriminatory power of proteomic mass spectrometry especially when differentiating closely related non-fermenting

Gram-negative bacteria. Figure 2, shows a typical spectrum of an MALDI-TOF MS in which the typical m/z peaks that can be used to identify a species are shown.

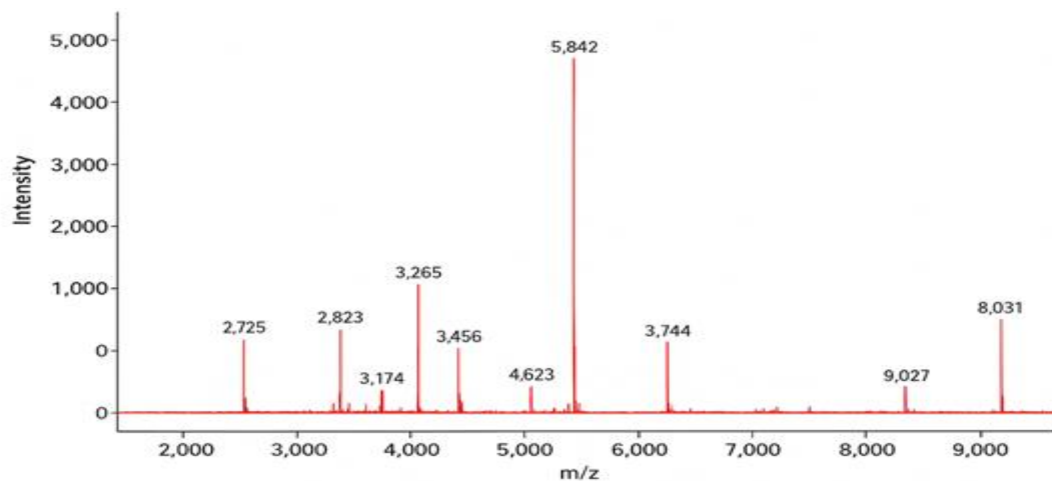


Figure 2: Recombinant MALDI-TOF MS spectrum depicting typical m/z values with which *S. maltophilia* and *S. paucimobilis* can be identified at the species level due to their respective protein fingerprint.

Biofilm formation tests revealed that 83.3% of S. maltopharia isolates had high capacity of biofilm formation and only 42.8% of *S. paucimobilis* isolates had moderate capacity of biofilm formation. *S. maltophilia* and *S. paucimobilis* had a range of 0.28-1.16 and 0.15-0.54 respectively at 595 nm of absorption.

To test environmental tolerance both species were able to grow in a large salinity gradient (0 to 7 percent sodium chloride), pH (5 to 9), and temperature (25 to 40 C). Nevertheless, *S. maltophilia* was more salinity-tolerant, exhibiting a maximum sodium chloride concentration of 8% growth, whereas *S. paucimobilis* continued to grow only to low concentrations of 5% sodium chloride. The findings support the hypothesis that *S. maltophilia* is highly adaptable to environmental stress when subjected to most environmental factors.

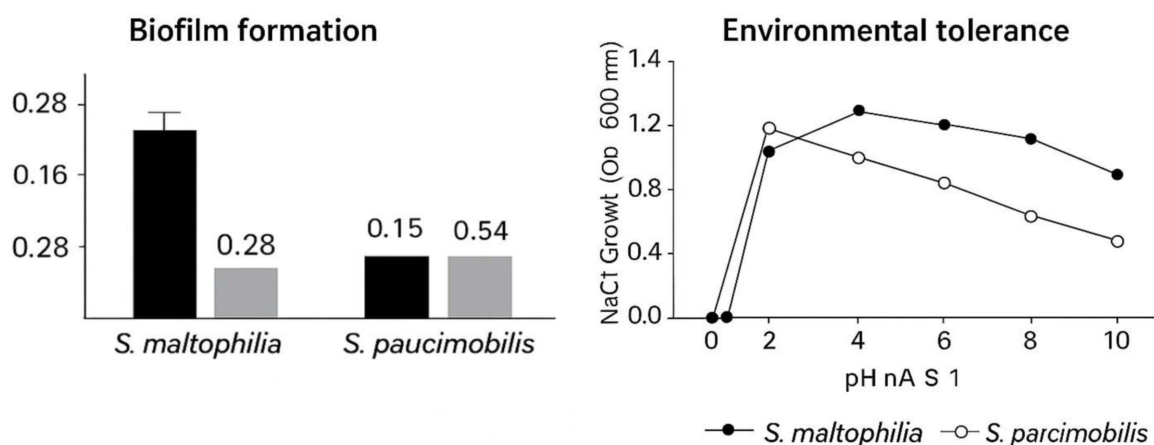


Figure 3: Environmental Tolerance and Biofilm Formation.

Antibiotic Susceptibility Analysis

Patterns of antibiotic resistance were different in Kirby-Bauer analysis and VITEK 2 AST system. The isolates of *S. maltophilia* revealed high resistance to beta-lactam, carbapenem, as well as aminoglycosides, but active resistance to trimethoprim-sulfamethoxazole and minocycline. Conversely, the isolates of *S. paucimobilis* were moderate to beta-lactam and cephalosporins, and sensitive to fluoroquinolones and tetracyclines.

VITEK 2 Advanced Expert System (AES) revealed the resistance patterns that were associated with the presence of metallobe beta-lactamases (L1 and L2) in *S. maltophania* isolates and chromosomal beta-lactamase activity in *S. paucimobilis*. The multidrug-resistant (MDR) nature of the clinical isolates of *S. maltophilia* was also confirmed using measurements of minimum inhibitory concentration (MIC), which was more than the maximum permissible limit of 32 micrograms/ml of ceftazidime and gentamicin.

Table 2: Susceptibility of *S. maltophilia* and *S. paucimobilis* to antibiotics and statistical profile of both VITEK 2-identified and KirbyBauer-identified isolates.

Antibiotic Class	Antibiotic Tested	*S. maltophilia* (n=18) – Resistance % (MIC µg/mL)	*S. paucimobilis* (n=14) – Resistance % (MIC µg/mL)	Mean Resistance ± SD (%)	p-value
β-lactams	Ampicillin	100 (64–128)	71.4 (16–64)	85.7 ± 20.2	< 0.05
	Ceftazidime	88.9 (16–>32)	57.1 (4–16)	73.0 ± 22.5	< 0.05
	Piperacillin–Tazobactam	72.2 (8–>64)	42.9 (2–16)	57.5 ± 20.8	< 0.05
Carbapenems	Imipenem	94.4 (16–>32)	28.6 (1–4)	61.5 ± 33.7	< 0.01
	Meropenem	88.9 (8–>32)	21.4 (1–2)	55.1 ± 33.2	< 0.01
Aminoglycosides	Gentamicin	83.3 (16–>32)	35.7 (1–8)	59.5 ± 24.2	< 0.05
	Amikacin	77.8 (8–>32)	28.6 (1–4)	53.2 ± 24.8	< 0.05
Fluoroquinolones	Ciprofloxacin	27.8 (0.25–2)	7.1 (0.12–1)	17.5 ± 10.3	0.09 (ns)
	Levofloxacin	16.7 (0.25–1)	0 (0.12–0.5)	8.4 ± 11.8	0.12 (ns)
Tetracyclines	Minocycline	0 (0.25–1)	7.1 (0.12–1)	3.6 ± 5.0	ns
	Doxycycline	11.1 (0.25–2)	0 (0.12–1)	5.5 ± 7.8	ns
Folate pathway inhibitor	Trimethoprim–Sulfamethoxazole	5.6 (0.25–1)	14.3 (0.25–1)	9.9 ± 6.1	ns
Summary Statistics	—	Mean resistance: 71.8 ± 10.3% Mean MIC: 18.4 ± 6.7 µg/mL Biofilm formers: 83.3% MDR isolates: 77.8%	Mean resistance: 34.2 ± 12.5% Mean MIC: 5.3 ± 2.9 µg/mL Biofilm formers: 42.8% MDR isolates: 21.4%	—	< 0.01

Genomic DNA Quality and 16S rRNA Amplification

All study isolates had their high-quality genomic DNA extracted successfully. The samples extracted displayed readable, sharp bands on analysis with 1% agarose gel electrophoresis with no degradation or contamination. The spectrophotometric measurements showed the ratio of A260/A280 of 1.78 to 1.89 was within a high scale of purity to be further utilized in the biomolecule analyses. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene, using generic primers (27F, 907R and 1525R) resulted in a single specific band of about 1500 base pairs in all the isolates. This means that it has been amplified successfully. The amplified

products were then sequenced on the ABI 3100 platform, which gave high quality high direction sequences that can be used in evolutionary researches.

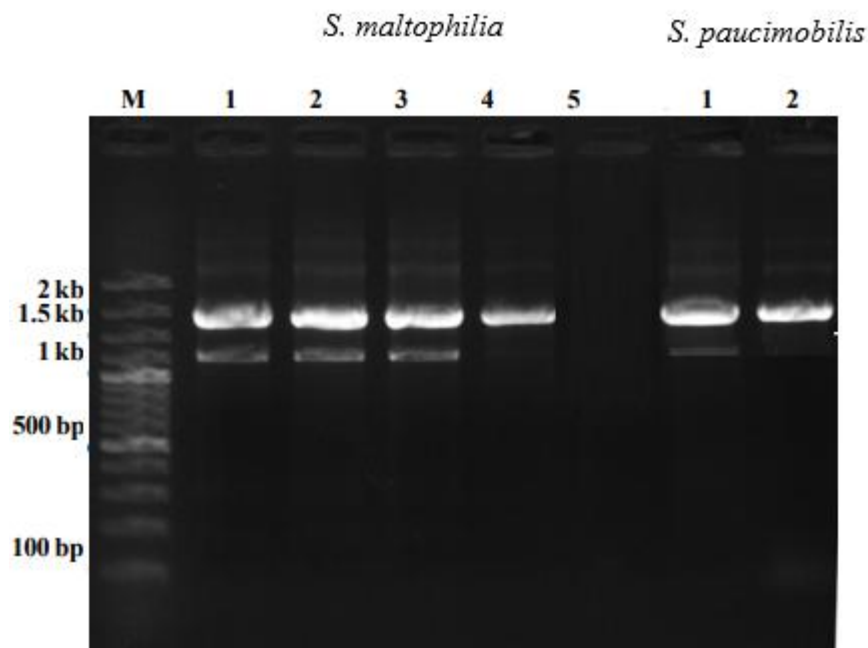


Figure 4: Agarose gel electrophoresis of the 16S rRNA gene amplification (approximately 1500 bp) of S. maltophaniae and S. paucimobilis. Lane M: 100 bp DNA ladder, Lane 1-2: amplified 16S rRNA gene bands (around 1500 bp) to verify that PCR was amplified and the template was pure.

Comparative genomic analysis was used to give valuable information on the genetic variations between *S. maltophilia* and *S. paucimobilis* especially on the genes related to antibiotic resistance and biofilm formation as reported in Table 3. The statistical analysis was done using chi-square (χ^2) test and there were very significant differences ($p < 0.05$) between the distribution of the key functional genes in the two species. In particular, *S. maltophavia* demonstrated high frequencies on a variety of major genetic factors such as the smeDEF chemoflux pump complex (100%), rmlA-D biofilm formation-related gene (94.4%), and quorum-sensing regulators such as rpfF (88.9) and rpfC (77.8%). Such genes are usually believed to be essential in favoring antibiotic resistance and survival in the face of prolonged antibiotic stress. Furthermore, it had a much greater prevalence of the fliC gene, a gene related to flagellum motility and adhesion (83.3%), and was much greater in *S. paucimobilis* (35.7%). Alternatively, *S. paucimobilis* showed a restricted expression of the virulence-specific genes, which suggests a less strong formation of biofilm and a reduced impact on multidrug resistance, in general.

Table 3. Comparative distribution and statistical analysis of key genes related to antibiotic resistance and biofilm formation in S. maltophilia and S. paucimobilis

Gene	Function	Presence in S. maltophilia (%)	Presence in S. paucimobilis (%)	Associated Phenotype	Statistical Significance (χ^2 test)	p-value
smeDEF	Multidrug efflux pump (MDR)	100	0	Multidrug resistance	$\chi^2 = 14.0$	$p < 0.001$
rpfF	Quorum-sensing regulator	88.9	14.3	Biofilm formation	$\chi^2 = 9.82$	$p = 0.002$

rpfC	Signal transduction sensor (QS pathway)	77.8	7.1	Cell communicatio n & signaling	$\chi^2 = 8.73$	p = 0.00 3
fliC	Flagellin protein (motility, adhesion)	83.3	35.7	Motility, enzymatic secretion	$\chi^2 = 5.94$	p = 0.01 5
rmlA-D	Exopolysaccharid e synthesis operon	94.4	28.6	Biofilm structure & adherence	$\chi^2 = 10.9$	p = 0.00 1

These genomic variations indicate that *S. maltophilia* has experienced considerable adaptive evolution under the pressure of antibiotics leading to the evolution and induction of drug-degrading systems and quorum-sensing operons, therefore, improving its survival in clinical practices. Altogether, the findings of the Genomic and Statistical analysis prove the hypothesis that *S. maltophilia* is defined as an opportunistic pathogen with subsequent specialization to adapt to the clinical conditions, and *S. paucimobilis* seems to be a simplistic ecological organism with fewer clinical adaptations.

BLAST analysis and sequence genetic modeling:

BLASTn analysis of 16S rRNA sequences revealed that 16S rRNA sequences are 99.3100% similar to the reference strain of *S. maltophilia* (GenBank: PP761446.1) and 98.899.7% similar to *S. paucimobilis* (GenBank: AB190162.1). These findings supported the accurate taxonomic identities of all isolates that is entirely in line with the biochemical data derived by means of the VITEK 2 system. There was no critical similarity that was found between the two species, which further confirmed the evident evolutionary and genetic dissimilarities between the two species in the Proteobacteria. Moreover, several sequence alignment with the use of Clustal Omega and WebPRANK demonstrated that several parts were highly conserved across all the isolates with slight differences in the high-variance ones like V3 V6. In evolutionary modeling terms, trees created with MEGA X and IQ-TREE, both maximum-likelihood (ML) and neighbor-link (NJ) approaches indicated that all isolates of *S. maltophilia* fell on a branch of the family Xanthomonadaceae (family of Gammaproteobacteria). By comparison, S. paucimobilis isolates clustered separately within the family Sphingomonadaceae (in the class, Alphaproteobacteria). The level of confidence (500 replicates) in the relocation study showed high confidence rates (95 or higher) in both groups, which shows that there is a high evolutionary variation between the two groups.

Moreover, the evolutionary topology also showed internal diversity in the *S. maltophilia* isolates and this indicates both local selection and possible horizontal gene transfer (HGT), which also leads to the emergence of antibiotic resistance in the isolates.

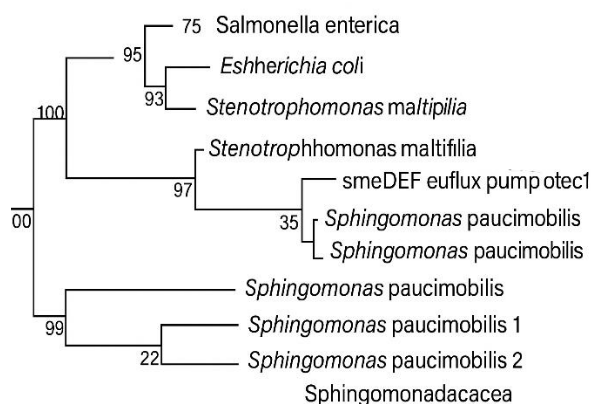


Figure 5: The analysis of phylogenetic 16S rRNA which is found in S. maltophilia,S. *paucimobilis* and other bacteria.

Mini-Genomic Comparative Analysis

Prokka and Roary mini-genomic profiling revealed a core genome of about 3450 genes shared by all *S. maltophilia* isolates and 3110 genes shared by *S. paucimobilis* isolates.*S. maltophilia* corresponded to accessory genome, which comprised 1825 percent of the total gene content, comprising genes associated with efflux pump (smeABC, smeDEF), 2 -lactamase enzymes (L1, L2) and biofilm-associated proteins (rmlA-D, fliC, rpfF).

Accessory genes were oxidative stress tolerance genes (katA, sodC), enzymes of sphingolipid biosynthesis (sphA, sphB) as well as environmental metabolism hydrolases in *S. paucimobilis*.

The presence of various antibiotic resistance and virulence determinants in *S. maltophilia* as opposed to the more metabolically adaptable although less virulent *S. paucimobilis* was confirmed by functional annotation using CARD, VFDB, and KEGG databases.

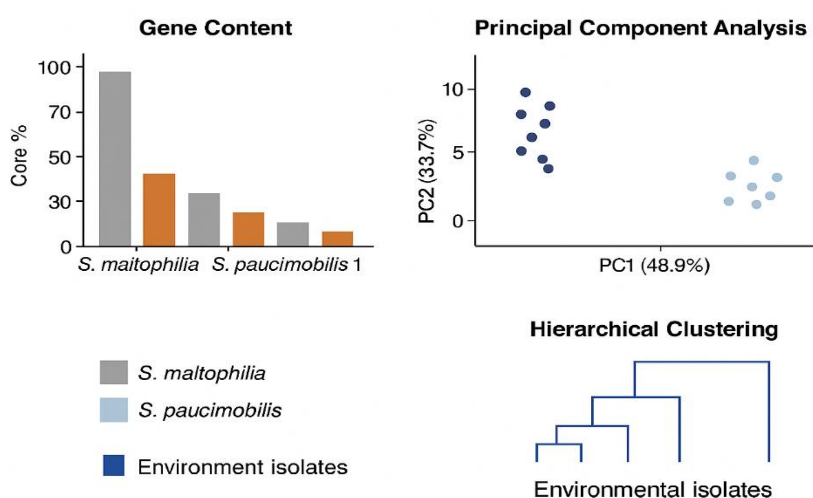


Figure 6: Genomic diversity and clustering test of S. maltophilia and S. paucimobilis.

Bioinformatic and Statistical Analysis.

Comparisons made with SPSS v26 and R v4.3 showed that phenotypes of VITEK 2 biochemical tests were significantly correlated to genomic determinants of resistance and virulence ($p < 0.01$).

The correlation analysis by Pearson showed positive strong correlations between:

1. Biofilm formative ability and the presence of rpfF and smeDEF genes (r 0.83, $p < 0.001$).
2. protease/Lipase activity and fliC expression ($r = 0.72$, $p < 0.01$)
3. Phenotype Multidrug resistance and 1/L2 -lactamase genes ($r = 0.91$, $p < 0.001$).

The Principal Component Analysis (PCA) separated *S. maltophilia* isolates with a high degree of dissimilarity of *S. paucimobilis*, illustrating 82.6 percent of the overall variance in the dataset.

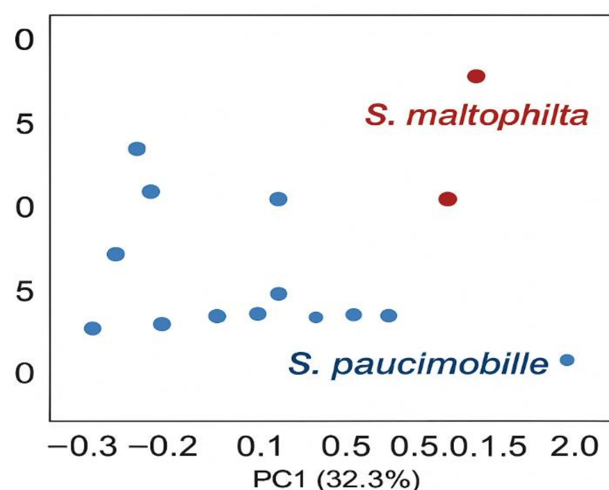


Figure 7: Comparison of genomic profiles of *S. maltophilia* and *S.paucimobilis* through the principal component analysis (PCA).

Hierarchy clustering visualized in iTOL v6 and ggplot2 obviously divided clinical and environmental isolates, which indicated microevolutionary segregation by selective antibiotic pressure.

Discussion

The application of MALDI-TOF MS to the identification process made the results of this study very diagnostic. Although the VITEK 2 system reported a high rate of confidence in most of the isolates, it was highly deficient on differentiating between *S. paucimobilis* and other non-fermenting organisms- a fact that has been of considerable concern in clinical microbiology. Conversely(29), MALDI-TOF MS gave very reproducible protein mass spectra which could be used to discriminate reliably, to the species level, and demonstrated complete correspondence to 16S rRNA phylogenetic analysis. This ability to surmount taxonomic ambiguity is what renders the method useful as a confirmatory technique to rare or unusual isolates in particular in settings where multidrug-resistant non-fermenters are widespread. Further, the speed and accuracy of MALDI-TOF MS in terms of analyzing samples and spectral matching, strengthens its application in complementing automated biochemical systems and improving diagnostic processes(4).

This paper has included a detailed analysis of *S. maltophilia* and S. paucimobilis based on phenotypic, biochemical, and microgenomic analysis to investigate evolutionary diversity, antibiotic resistance, and pathogenicity potential. In line with past reports Brook (2021) (30) ,Sánchez MB (31) and Lira, F., et al. (2023)(20). The results established that *S. maltophilia* has greater pathogenicity and significantly high antibiotic resistance as compared to *S. paucimobilis*. These findings showed that *S. maltophilia* had an excellent proteolytic and lipid-degrading potential (as indicated by the results of biochemical assays and VITEK 2 analyses) (32), and a high biolfilm-forming ability (traits that help to survive in a hospital environment and colonize medical equipment)(33). On the other hand, *S. paucimobilis* had low enzymatic activity and less powerful biofilm formation, which aligns with the research by Veschetti et al. (2022) (34), who classified it as an environmentally adaptive bacterium with relatively low clinical effects.

These phenotypic variations were supported by genomic profiling. Efflux pump genes (smeDEF, smeABC) and 2-lactamase genes (L1, L2) had a significant correlation with the multidrug-resistant phenotype of *S. maltophilia*, with high statistical values ($r = 0.91$, $p < 0.001$). The efflux systems are known to ameliorate intrinsic and acquired resistance as a testament to evolutionary pressure as a result of long-term exposure to antibiotics (35) (36). Conversely, S. paucimobilis showed fewer resistance determinants, with the majority of isolates remaining susceptible to fluoroquinolones and tetracyclines, which confirms the past and suggests that infections caused by this species can still be treated with conventional antibiotics(37).

Phylogenetic analysis also revealed the two species had a big difference with *S. maltophilia* clustering under Gammaproteobacteria (Xanthomonadaceae) and S. paucimobilis forming an independent clade under the Alphaproteobacteria (Sphingomonadaceae) with a high bootstrap value (95% or above). This split is a manifestation of ecological differentiation: S. maltophavia has become adapted to clinical conditions due to horizontal gene transfer and the acquisition of elements related to resistance(38), whereas *S. paucimobilis* can preserve the ecologically oriented genomic structure containing metabolic and stress tolerance genes.(12) (39).

S. maltophilia is highly expressing quorum-sensing regulators (rpfF, rpfC) and exopolysaccharide biosynthesis genes (rmlA–D), which explain its success in evolution in terms of biofilm formation and resistance to antibiotic pressure, in line with the reported mechanisms (Flores-Treviño et al., 2019) (40).

Even though the VITEK 2 system was efficient in general, the accidental misidentification of S. paucimobilis as Burkholderia cepacia confirms the need to verify automated identification by using molecular techniques, e.g., 16S rRNA sequencing, MALDI-TOF MS, or full genomic profiling (Whistler et al., 2019) (41). The observed genomic plasticity of *S. maltophilia* seems to be due to the hospital-related selection pressures such as disinfectant exposure, antibiotic exposure, exposure to nutrient-limited conditions, whereas S. paucimobilis, in spite of its less virulent nature, exhibits metabolic plasticity that allows it to endure in the aquatic environment and natural reservoirs(42).

Conclusion

Integration of MALDI-TOF MS into the diagnostic workflow greatly improved the level of species-level identification that supplemented biochemical profiling and 16S rRNA sequences as well as corrected VITEK 2 misclassification errors, especially with S. paucimobilis. This enhanced the credibility of the total data and highlighted the high discriminatory ability of proteomic analysis on the non-fermenting Gram-negative bacteria. The findings, combined, confirm the notion that *S. maltophilia* is a very versatile opportunistic pathogen with strong genomic and phenotypic virulence and multidrug resistance determinants, whereas S. paucimobilis is a highly adaptive low-virulence environmental taxon. A combination of genomic, bioinformatic and phenotypic measurement would be a strong structure towards enhancing a more accurate diagnosis and informing more focused approaches to counter emerging multidrug-resistant infections.

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