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Whole-Genome Based Molecular Characterization of *Stenotrophomonas maltophilia*: An Emerging Human Pathogen

By

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I dedicate my work to my beloved sister Pascale.

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ABSTRACT

Stenotrophomonas maltophilia is a rod-shaped Gram-negative emerging opportunistic pathogen. It causes community and nosocomial infections and is most often recovered from respiratory infections. The organism infects severely ill or immunocompromised individuals and is intrinsically resistant to multiple antibiotics along with the development of acquired resistance through horizontal gene transfer. In this study, we used whole-genome sequencing (WGS), for the in depth molecular characterization of six clinical *S. maltophilia* isolates collected from hospitals in Lebanon during 2018-2019. Disk diffusion assay was used to determine resistance patterns which were confirmed through *in silico* detection of relevant genes. All the isolates were resistant to tetracycline, ticarcillin, and imipenem. The β-lactamase bla_{L1}, and the aminoglycoside *aac(6')-Iz* and *aph(3')-IIc* resistance determinants were detected in the studied isolates. Nucleotide sequences for virulence determinants were downloaded from NCBI and blasted against the isolates whole-genomes. Results showed the presence of a number of extracellular enzymes and other virulence factors (VFs) such as hemolysins and siderophores. The IslandViewer database was used to detect the number of genomic islands (GIs) and the gene pool. Isolates were found to harbor between 20 and 29 GIs each. The GIs were highly diverse and carried multiple VFs. The relatedness between

the isolates was investigated through multi-locus sequence typing (MLST), whole genome SNP-based analysis, and pulse field gel electrophoresis (PFGE). We reported three novel STs (ST639, ST640, and ST641). Two quorum sensing *rpfF* gene variants were detected and which were found to control biofilm formation. Our study is the first of its type in Lebanon and it helped in characterizing and identifying at the molecular level resistance and virulence patterns of an important globally emerging human pathogen. Prevention of related infections depends on establishing and developing infection-control strategies while controlling antibiotic use and environmental reservoirs.

Keywords: *Stenotrophomonas maltophilia,* WGS, PFGE, MLST, SNPs, MDR.

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LIST OF ABBREVIATIONS

AUBMC: American University of Beirut Medical Center

bp: Base Pairs

CF: Cystic Fibrosis

CGE: Center for Genomic Epidemiology

CLSI: Clinical and Laboratory Standards Institute

DC: dendritic cell

DNA: Deoxyribonucleic acid

DSF: Diffusible signal factor

DTA: deep tracheal aspirate

HGT: Horizontal Gene Transfer

HIV: Human Immunodeficiency Virus

LB: Luria-Burtani

LPS: Lipopolysaccharide

LRSM: Levofloxacin-resistant *S. maltophilia*

MBL: Metallo-β-Lactamase

MDR: Multidrug resistant

MGH: Makassed General Hospital

MLST: multi-locus sequence typing

NCBI: National Center for Biotechnology Information

OD: optical density

OMV: outer membrane vesicle

PCR: Polymerase Chain Reaction

PFGE: Pulse-field gel electrophoresis

RAST: Rapid Annotation using Subsystem Technology

SCV: small colony variant

SNP: Single Nucleotide Polymorphism

ST: Sequence Type

WGS: Whole Genome Sequencing

WHO: World Health Organization

Chapter One

Introduction

1.1 Nomenclature and Primary Characteristics

S. maltophilia is a rod-shaped Gram-negative bacterium that inhabits diverse niches like water and soil (Zheng et al; 2016). It's an environmental bacterium found in rhizospheres mainly as part of animals' microflora as well as in food (Adegoke et al; 2017). They are aerobic, glucose non-fermentative, Gram negative bacilli that are oxidase negative and catalase positive, motile due to their polar flagella, and react positively with extracellular DNase (Mukherjee & Roy, 2016). *S. maltophilia* was first isolated from pleural effusion and was designated as *Bacterium bookeri* in 1943 (Hugh & Leifson, 1963), and later as *Pseudomonas maltophilia* or *Xanthomonas maltophilia* (Senol, 2004). In the following years, other species appeared such as *S. rhizophila, S. terrae, S. humi,* and *S. pavanii*. In 1997 *S. africana* was identified as a new species, and later renamed to be *S. maltophilia* (Adamek et al, 2011). In 2008, the first two complete genome sequences of *S. maltophilia* were released and subsequently named as the "newly emerging superbug" due to the several chromosomal resistance determinants (Johnson & Duckworth, 2008). *S. maltophilia* has a large core genome, and the distribution of virulence genes is similar among clinical and environmental isolates (Lira et al; 2017).

Stenotrophomonas species share a common ancestor with *Xanthomonas citri* and has since then diverged to become more evolved from the progenitor of the *Xanthomonas* genus (Figure 1) (Pieretti et al., 2009).

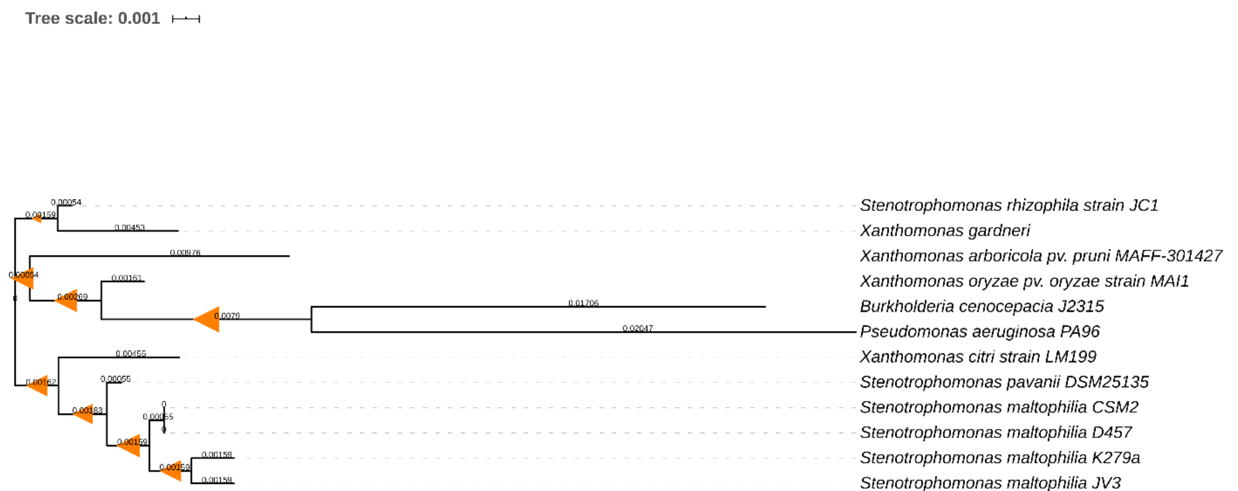


Figure 1. Phylogenetic tree describing the evolution of *S. maltophilia* from the *Xanthomonas* ancestor.

S. maltophilia grows on numerous types of media, and appears as faint lavender colonies on blood agar, opaque gray/yellow colonies on nutrient agar, and being non-lactose fermenter appears as colorless colonies on MacConkey agar (Abbott et al., 2011). The transmission of *S. maltophilia* is through direct contact with the host (Schable et al., 1991), and it's the 6th most common cause of respiratory tract diseases, and the second most common cause of bacteremia (Chang et al, 2015). Other disorders include endocarditis, meningitis, and cutaneous infections (Adegoke et al; 2017). Malignancy, immunosuppressant therapy, cystic fibrosis (CF), and exposure to antibiotics are however, considered risk factors for *S. maltophilia* infections (Chang et al, 2015). Other predispositions include hemodialysis, HIV infection, prolonged hospital inpatient stay, indwelling devices such as catheters, pacemaker wires, and lenses (Abbott et al., 2011). In hospitalized patients, the most common isolation site is the respiratory

tract (Pathmanathan, 2005), recovered along with *P. aeruginosa* from CF patients' respiratory tract (Brooke, 2012). It was also linked to arthritis in dogs, mastitis in cows, lymphadenitis in goats, and recovered from trachea of horses with chronic coughs (Uçan et al., 2019).

1.2 Resistance Patterns

Antibiotic resistance is a global concern, it is acquired through horizontal gene transfer (transduction, transformation, and conjugation) of mobile genetic elements (plasmids, transposons, integrons, and insertion sequences), or it could be intrinsic (Sultan et al., 2018). Both acquired and inherent antibiotic resistant genes contributed to the high antibiotic resistance profile of *S. maltophilia* (Alonso, 2004). *S. maltophilia* can increase its resistance via horizontal gene transfer of plasmids or via recombinations with genomic islands, transposons, and integrons (Brooke et al., 2012). Plasmids capture and disseminate resistance genes and as a result act as resistance vehicles (Bennett, 2009).

Intrinsic factors are very common and are mediated by several different mechanisms such as low membrane permeability, multi-drug (MDR) efflux pumps, and antibiotic-modifying enzymes (Cho et al., 2012). *S. maltophilia* is intrinsically resistant to aminoglycosides, carbapenems, and β-lactams (Cho et al., 2012). Antibiotic resistance in *S. maltophilia* was mainly linked to quinolone resistance, and the presence of the class 1 integrons, efflux pumps, and β-lactamase production (Chang et al, 2015). The Sm*qnr* gene, which mediates resistance to quinolones, was detected in *S. maltophilia*, and is the homologue of the *qnr* gene (Shimizu et al., 2008; Sanchez and Martinez, 2008).

β-lactamase enzymes hydrolyze the amide bond of the four membered β-lactam rings (Tooke et al., 2019). These enzymes are divided into different classes, three of which (A, C, D) are the active serine β-lactamases, while class B includes the metallo β-lactamases (MBLs) or the zinc-dependent β-lactamases (Tooke et al., 2019). L1 and L2 are the main β-lactamases in *S. maltophilia* (Avison et al., 2001), along with TEM-2 which is carried on a Tn1-like transposon (Chang et al., 2015), and CTX-M (Peleg et al., 2015). L1 is a zinc-dependent metalloenzyme, it hydrolyzes penicillins, carbapenems, cephalosporins, and mainly all β-lactams, while L2 is a serine cephalosporinase (Avison et al., 2001). L1 and L2 are usually located on an approximately 200kb plasmid in clinical *S. maltophilia* (Avison et al., 2001).

S. maltophilia, like other non-fermentative bacteria, is intrinsically resistant to penicillin G, cefazolin, glycopeptides, fusidic acid, lincosamides, rifampicin, linezolid, cefamandole, macrolides, and daptomycin (Abbott et al., 2011). The intrinsic resistance of this bacterium is mediated by chromosomally encoded efflux pumps such as SmeYZ, SmeDEF, and SmeABC , with their overexpression leading to increased resistance (Garcia-Leon et al., 2015). Overexpression of the efflux pumps SmeVWX, SmeDEF, and SmQnr in *S. maltophilia* also led to fluoroquinolone resistance (Wu et al., 2019).

On the other hand, six resistance genes including the *floRv* genes which encodes phenicol resistance and other conferring resistance to tetracycline, streptomycin, and sulfonamides were found to be located in a novel genomic island in *S. maltophilia* (He et al., 2014). Furthermore, the *sul1*, *sul2* and *dfrA* genes which confer resistance to trimethoprim/sulfamethoxazole (SXT) were located on a 7.3kb plasmid-mediated class 1 integron in *S. maltophilia* (Hu et al., 2011). The acquisition of *sul1* or *sul2* can lead to

sulfonamide resistance in Gram-negative bacteria, whereas the resistance to trimethoprim is conferred via dihydrofolate reductase (*dfr*) genes (Gales et al., 2001).

1.3 Virulence and Pathogenesis

As *S. maltophilia* is considered a low-grade pathogen, its pathogenesis could involve a great deal of virulence factors besides the ability of these bacterial cells to form biofilms on host tissues and abiotic surfaces (Trifonova and Strateva, 2018). The virulence factors of *S. maltophilia* include small colony variant, pilli/fimbriae, quorum sensing, and extracellular enzymes (Adegoke et al; 2017). The host being immunocompromised or not is what determines the pathogenesis of this bacterium (Lira et al; 2017). *S. maltophilia* colonizes hospital settings fluids and invasive medical devices causing nosocomial diseases (de Oliveira-Garcia et al., 2003).

The small colony variant (SCV) phenotype is distinguished from the wild type by less growth on agar media, has a smaller colony size, and by being unable to give results for their susceptibility tests at standard conditions as described by the CLSI (Anderson et al., 2007).

Additionally, *S. maltophilia* are characterized by specific flagellar filaments which are made of a 38-kDa subunit known as *S. maltophilia* SM_{FLiC} having an amino acid N-terminus sequence similar to the flagellins of other organisms such as *Serratia marcescens, Escherichia coli*, *Proteus mirabilis*, *Shigella sonnei*, and *P. aeruginosa* (de Oliveira-Garcia et.al, 2002).

S. maltophilia uses quorum sensing and quorum quenching (the disruption of quorum sensing) to respond and sense its environment (Heudo et al, 2018). Quorum

sensing depends on the diffusible signal factor synthesis and sensing which varies between the *rpf1* and *rpf2* variants (Heudo et al, 2018). Moreover, the N-acyl homoserine lactone system and the Ax21 protein also have a role in quorum sensing in *S. maltophilia* (Heudo et al, 2018). The existence of two different alleles of the *rpfF* gene is vital for the synthesis of the diffusible signal factor (DSF) (Lira et al., 2017). The DSF of *S. maltophilia* can affect other bacterial species such as *Burkholderia cenocepacia* and *P. aeruginosa* (Ryan and Dow, 2011), which are usually found in the lungs of cystic fibrosis patients (Twomey et al., 2012). The *rpfF* gene through DSF signaling was found to regulate factors that contribute to the virulence of this organism via biofilm formation and motility (An & Tang, 2018). Besides the *rpfF* gene, *rmlA* and *spgM* are involved in biofilm formation (Zhuo et al, 2014).

Another potential virulence determinant is the O-specific polysaccharide, which is a part of the lipopolysaccharide (LPS), this factor aids the bacterium in adapting to a specific niche (Steinmann et al., 2018). *rmlA, rmlC*, and *xanB* are coupled to the biosynthesis of LPS in *S. maltophilia* (Huang et al, 2006). Additionally, outer membrane vesicles (OMVs) secreted by *S. maltophilia* have a role in virulence through quorum sensing (Devos et al, 2015). OMVs contain the chromosomally encoded L1 metallo and L2 serine β-lactamases in addition to the Ax21 homologs which are associated with biofilm formation. The secretion levels of OMVs and the Ax21 are controlled by the DSF system (Devos et al, 2015).

The pathogenesis of *S. maltophilia* is also facilitated through *Stenotrophomonas maltophilia* serine protease (StmPr1) cytotoxic activities. This serine protease along with others, such as StmPr2 and StmPr3 were found to be the substrates for the Xps-type II

secretion system in *S. maltophilia* K279a strain and hence contributed to the virulence of *S. maltophilia*. StmPr1 and the other proteases could activate the human lung epithelial cells to produce IL-8 via the protease activated receptor 2, causing inflammation. They could also cause tissue damage in the lungs (DuMont & Cianciotto, 2017).

The positively charged surface of *S. maltophilia* also functions as a virulence factor as it mediates resistance to antiseptics and disinfectants, which bind to the negatively charged membranes and cell walls of bacteria. In addition, the melanin-like pigment (encoded by gene *mel*) protects the cell from environmental insult (Abbott et al., 2011).

Most of the bacteria acquire all nutrients required for its survival such as nitrogen, phosphates, and amino acids, except for iron since it's not freely available in the host (Ratledge and Dover, 2000). Iron is well known for its role in cell growth, proliferation and development, and pathogenicity (Kalidasan et al., 2018). Iron is a signal that has an effect on extracellular enzymes production, DSF and siderophore production, and oxidative stress response in *S. maltophilia* (Kalidasan et al., 2018). Iron was also found to have a role in the virulence of *S. maltophilia* strains. When it infects an immunocompromised host it will be deprived of iron, and iron limitation was found to improve biofilm formation (Garcia et al., 2015). Biofilm formation is a virulence factor embedded within extracellular polymeric substances whose production is in turn regulated by iron limitation (Garcia et al., 2015). The expression of two iron acquisition systems in *S. maltophilia* enables this bacterium to acquire iron for its cellular process. *S. maltophilia* has two different iron uptake systems: a siderophore and heme-mediated system. When iron is limited, the regulators of *S. maltophilia* are the fur and sigma factors (Kalidasan et al., 2018).

1.4 *S. maltophilia* and the environment

S. maltophilia could be isolated from the environment and from other sources as well. It was recovered from leafy green vegetables such as kale, parsley, and sweet basil in Singapore (Li et al,. 2019). Several studies have shown the isolation of this bacterium from tap water (Amoli et al., 2017), soil (Bollet et al., 1995), and even from dairy milk (El-Prince et al., 2019), but most importantly from plant rhizosphere (Messiha et al., 2007).

S. maltophilia plays a significant role in plant fungal disease control, in bioremediation, and in plant growth promotion (Suckstorff et al, 2003). It acquires DNA from environmental bacteria, which has severe implications on the transfer of genes within microbial environmental communities such as wastewater (Brooke et al., 2012). *S. maltophilia* has as well been reported to transfer resistance genes to other MDR bacteria with which it was found in association, such as *P. aeruginosa* and members of *Enterobacteriaceae* (Brooke et al., 2012). Interestingly, the efflux pump SmeDEF was found to mediate plant colonization of *S. maltophilia,* with the plants producing flavonoids that induce SmeT, which regulates the efflux pump (Garcia-Leon et al., 2014). On the other hand, *S. maltophilia, B. cepacia,* and *A. xylosoxidans* share significant environmental benefits, and are used in agriculture and bioremediation, due to their ability to degrade pollutants and protect plants through promoting the growth and health of these plants (An & Berg, 2018).

The *rpf*/DSF system controls several genes that are responsible for *S. maltophilia* plant growth promotion and plant colonization via biofilm formation, chemotaxis, cell motility, and efflux pumps (Alavi et al, 2013).

1.5 *S. maltophilia* and the immune system

S. maltophilia plays distinct roles in the host immune response. In the case of CF patients for example, it contributes to an inflammatory process that compromises the respiratory function (Di Bonaventura et al., 2010), and induces the production of proinflammatory cytokines such as IL-6 and IL-8 and tumor necrosis factor alpha (Härtel et al., 2013; Waters et al., 2007). Moreover, it enhances the expression of the latter cytokines in macrophages (Di Bonaventura et al., 2010). This bacterium also induces dendritic cell (DC) maturation and the expression of DC surface maturation markers (Roscetto et al.; 2015). Furthermore, *S. maltophilia* fimbriae (SMF) has a role in stimulating immune response in the bladder via pro-inflammatory cytokine production and neutrophil infiltration (Zgair and Al-Adressi, 2012). Similarly, flagellin stimulates the innate immunity as detected in the lungs of mice (Zgair and Chhibber, 2010).

1.6 Treatment

The first line of treatment is trimethoprim-sulfamethoxazole (SXT), even though resistance to this antibiotic was reported (Chang et al, 2015). SXT resistance rates are generally less than 10% (Gales et al., 2001), and it is recommended to be given at high doses because of its bacteriostatic action (Zelenitsky et al., 2005). Along with SXT, ticarcillin and clavulanic acid are also being considered as primary drugs of choice against *S. maltophilia* since clavulanic acid inhibits the L2 β-lactamase (Kataoka et al., 2004). Similarly, aztreonam was also reported to inhibit L2 β-lactamase (Sanchez et al., 1997), and it is used in combination with clavulanic acid (Kataoka et al, 2004).

Moreover, the newest fluoroquinolones, such as moxifloxcin, act against biofilms (Korakianitis et al., 2010), and are preferred due to their in vitro activity (Sobottka et al., 2002). Other alternatives include colistin, chloramphenicol, and derivatives of tetracycline (minocycline, tigecycline) (Abbott et al., 2011).

In case of severe sepsis, polymicrobial infections, or neutropenia, combination therapy is the best to be employed. Due to the bacteriostatic action of most drugs, combination therapy diminishes the risk of forming antibiotic resistance during the treatment (Nicodemo et al., 2007). The most commonly used combinations include ceftazidime, ciprofloxacin, and ticarcillin-clavulanic acid, and these are for infections that can't be treated by SXT (Falagas et al., 2008).

1.7 Objectives of this study

In this study, we sought to conduct an in-depth whole-genome molecular characterization and comparative genome analysis of six clinical *S. maltophilia* isolates recovered from patients hospitalized in two different hospitals in Lebanon in 2018 and 2019. We used whole-genome sequencing to elucidate their clonal relatedness, virulence and antimicrobial resistance profiles. Genomes of the isolates were used to build a genomic landscape and were put into a comprehensive context via comparative analysis with global isolates from various clinical source.

Chapter Two

Materials and Methods

2.1 Bacterial Isolate Collection

A total of six *S. maltophilia* isolates were collected between 2018 and 2019 from the American University of Beirut Medical Center (AUBMC) and Makassed General Hospital (MGH) and were designated as AST1-3 and AST6-8. The isolates were recovered from various body parts including: diagnostic tracheal aspirate (DTA), throat, and ear. Patients' age range was 23-87, with a 1:1 male to female ratio (Table 1).

Table 1. Isolate metadata and accession numbers.

#	Isolate	Date	Age	Specimen	Sex	Accession Number
1	AST 1	2018	23	DTA	M	GCA_004522355.1
2	AST 2	2018	84	DTA	F	GCA_004522395.1
3	AST 3	2018	87	DTA	F	GCA_004522405.1
4	AST 6	2018	39	DTA	M	GCA_008080405.1
5	AST 7	2019	70	Throat	M	WKLG00000000
6	AST 8	2019	71	Ear Cult	F	WLYO00000000

2.2 DNA Extraction

DNA was extracted using the NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The extracted DNA was stored at -20 ℃ after being quantified using the NanoDrop spectrophotometer.

2.3 Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disk diffusion technique on Mueller-Hinton agar and it included a panel of 8 antibiotic disks (Trimethoprim/Sulfamethoxazole, Tetracycline, Amikacin, Ticarcillin, Imipenem, Ceftazidime, Levofloxacin, and Minocycline) belonging to 7 classes (sulfonamides, tetracyclines, penicillins, cephalosporins, quinolones, aminoglycosides and carbapenems). The obtained data was interpreted according to the CLSI guidelines (CLSI, 2017)

2.4 Biofilm assay

A freshly prepared bacterial culture was used to inoculate LB broth and was incubated at 37°C overnight. After incubation, the optical density was set to 2 using a wavelength of 600 nm. The cultures were diluted 1:10 and 1:100 folds using LB broth. 100 µL of the diluted bacteria were added to a 96-well plate (each dilution in a separate plate) and incubated 18 h at 37°C. Growth was first determined via measuring the OD at 600 nm, followed by 2-3 washes with water then the addition of 95% ethanol. The resulting aliquot was loaded into a new fresh 96-well plate, and the absorbance was measured at 540 nm. All tests were done in triplicates. A negative control was used which was fresh LB broth.

2.5 Pulse Field Gel Electrophoresis

PFGE fingerprinting was performed using the XbaI restriction enzyme (ThermoScientific, Waltham, MA, USA), 1% SeaKem agarose gel, and the universal

laboratory standard *Salmonella enterica subsp. enterica serovar Braenderup* (ATCC® BAA664™) according to the standard PulseNet protocol (http://www.pulsenetinternational.org). Electrophoresis was performed using the Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., Hercules, CA, USA) with a run time of 21 h and the first-block switch time was 5 to 15 s for 10 h, and the second-block switch time was 15 to 60 s for 11 h (Shueh et al., 2013). Gels were stained with ethidium bromide. PFGE profiles were analyzed with the BioNumerics software version 7.6.1 (Applied Maths, Sint-Martens-Latem, Belgium), with profiles assigned as different pulsotypes if three or more bands were different (Tenover et al., 1995). Pulsotypes were clustered based on the BioNumerics software analysis through dice correlation coefficients with an optimization of 1.5% and tolerance of 1.5%.

2.6 Multi-Locus Sequence Typing

MLST 2.0 database (MLSTfinder) available on the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org) was used for the *in silico* determination of the sequence types (STs) for the isolates (Larsen et al, 2012). Novel STs were submitted to the pubMLST database for new ST designations (https://pubmlst.org/), and included AST1, AST3, and AST8.

2.7 Whole Genome based virulence and resistance profiling

FastQC was used to check the quality of the raw reads (Andrews, 2010). The presence of different virulence determinants was revealed using ncbiBLAST, by blasting

the gene sequences against the isolates' whole-genomes (https://blast.ncbi.nlm.nih.gov/Blast.cgi). PHASTER (a phage search tool) was used to identify intact phages (Arndt et al, 2016). BioNumerics software version 7.6.1 was used for genome comparisons and alignments. IslandViewer 4 was used to detect genomic islands (Bertelli et al., 2017). Uniprot database was used to download protein sequences (https://www.uniprot.org/).

2.8 Genome assembly and annotation

Genomes were assembled then annotated using the RAST online server (http://rast.nmpdr.org) (Aziz et al, 2008). Resistance genes were detected *in silico* using the ResFinder 3.0 (Zankari et al., 2012) available on the Center for Genomic Epidemiology website (www.genomicepidemiology.org).

2.9 Recombination and pangenome analysis

The annotation of *S. maltophilia* genomes was performed using Prokka version 1.13 (Seemann, 2014). The resulting annotated .gff files were then submitted to Roary through the terminal (Page et al, 2015). Snippy pipeline was used to generate a SNP tree which classifies the isolates based on SNP similarities (Seeman, 2015). Snippy tree was generated using the interactive tree of life (iTOL) https://itol.embl.de/ (Letunic & Bork, 2016). Gubbins was used to detect loci with base substitutions that are indicative of horizontal gene transfer (Croucher et al., 2015). The Gubbins output was submitted to Phandango (Hadfield et al., 2018).

Chapter Three

Results

3.1 Antibiotic Susceptibility Test

The disk diffusion assay conducted showed that all *S. maltophilia* isolates were susceptible to trimethoprim/sulfamethoxazole and minocycline. All, however, were resistant to tetracycline, ticarcillin, and imipenem, 66.7% to amikacin, 83.3% to ceftazidime, and 33.3% to levofloxacin.

The β-lactamase resistance gene *bla*$_{L1}$ was detected in 66.7% of the isolates, the aminoglycoside resistance genes *aac(6')-Iz* and *aph(3')-IIc* were detected in 50% and 83.3% of the isolates, respectively.

The disk diffusion assay results and the resistance genes detected using CGE's Resfinder are summarized in Table 2.

Table 2. Antibiotic susceptibilities, *in silico* detected resistance genes using Resfinder and isolates' metadata. Light blue: sensitive; Dark Blue resistant; DTA: deep tracheal aspirate; F: female; M: male; SXT: Trimethoprim/Sulfamethoxazole; TE: Tetracycline; AKN: Amikacin; TIC: Ticarcillin; IPM: Imipenem; CAZ: Ceftazidime; LVX: Levofloxacin; MIN: Minocycline.

#	Isolate	Date	Age	Specimen	Sex	Antibiotics								Resistance Genes		
						SXT	TE	AKN	TIC	IPM	CAZ	LVX	MIN	bla_{L1}	*aph(3')-IIc*	*aac(6')-Iz*
1	AST 1	2018	23	DTA	M											
2	AST 2	2018	84	DTA	F											
3	AST 3	2018	87	DTA	F											
4	AST 6	2018	39	DTA	M											
5	AST 7	2019	70	Throat	M											
6	AST 8	2019	71	Ear Cult	F											

3.2 Biofilm assay

The results were interpreted by comparing the cut-off value *ODc* with the average of the triplicates of every sample. The *ODc* is defined as three standard deviations above the mean of the negative control:

$$OD_c = \overline{OD}_{negative\ control} + (3 \times \sigma_{negative\ control})$$

The strains were classified as follows:

- $OD_{strain} \leq OD_c$ = no biofilm producer (0)
- $OD_c \leq OD_{strain} \leq 2 \times OD_c$ = weak biofilm producer (+ or 1)
- $2 \times OD_c \leq OD_{strain} \leq 4 \times OD_c$ = moderate biofilm producer (++ or 2)
- $4 \times OD_c \leq OD_{strain}$ = strong biofilm producer (+++or 3)

Accordingly, all the tested isolates could be classified as being strong biofilm producers.

3.3 SNP Analysis

A neighbor-joining (NJ) phylogenetic tree was constructed between our isolates and the clinical (pink) as well as the environmental (green) reference genomes uploaded from NCBI (Figure 1). Two of our isolates AST2 (from DTA) and AST8 (from ear cult) were found to cluster together along with the two environmental reference genomes, which were namely: *S. maltophilia* CSM2 (from the laboratory sink) and *S. pavanii* DSM (from stems of sugar cane).

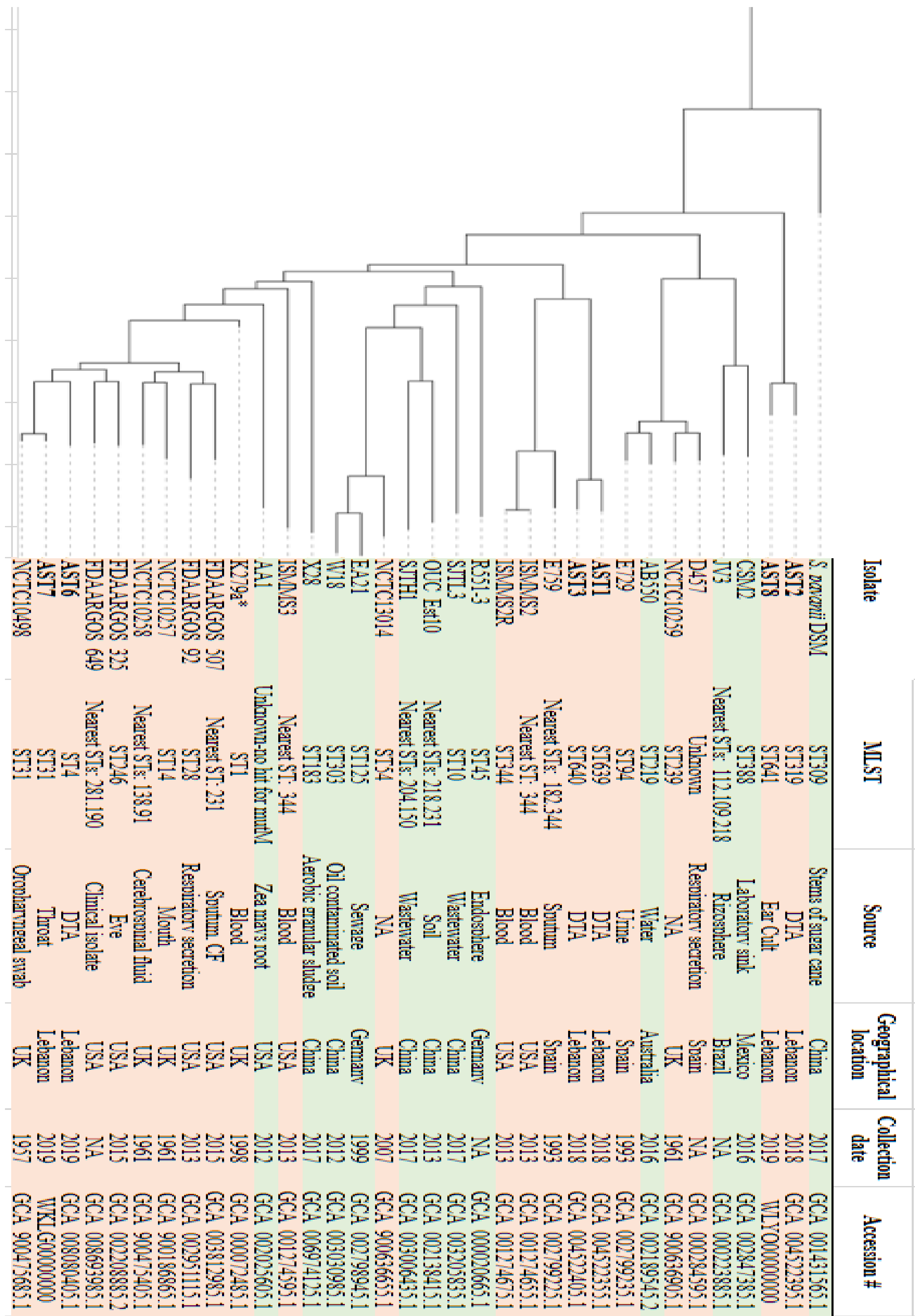


Figure 2. SNPs based neighbor-joining (NJ) phylogenetic tree. 1000 bootstrap values were used. Pink: clinical isolates; green: environmental isolates; bold: isolates sequenced

in this study; * reference genome. NA: not available; CF: cystic fibrosis; DTA: deep tracheal aspirate.

3.4 Roary

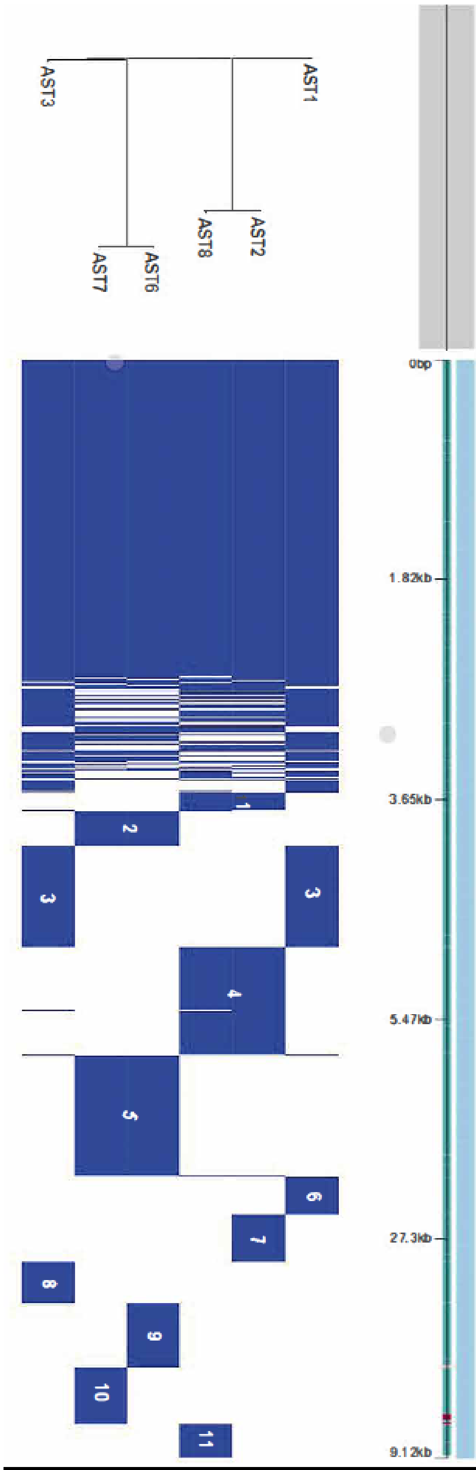


Figure 3. Roary pan-genome similarity matrix. Maximum likelihood phylogenetic tree, based on the alignment of accessory genomes, was generated using FastTree 2 (Price et al, 2010). Pan-genome was constructed using Roary based on the core and accessory genes showing phylogenetic relatedness of the isolates. Blue: present; White: absent fragments (Page et al, 2015). Fragments distinguishing the isolates were numbered as 1 to 11.

Figure 2 is divided into two parts, the core genome that represents the genes common to all *S. maltophilia* isolates, depicted as a dark blue chunk on the left side of Figure 2. The right part represents the accessory genome which are the genes that are characteristic to each isolate and are correlated with virulence and other functions. The isolates were grouped based on the shared genes in the phylogenetic tree on the very left side of Figure 2. The main virulence genes found in each numbered fragment were summarized in Table 5.

Table 3. Features related to virulence/resistance in each fragment.

Fragment	Important Genes Involved	References
1	Fe(3+) ions import ATP-binding protein FbpC, Sensor histidine kinase RcsC	Dhungana et al., 2005; Wang et al., 2011
2	Sensor histidine kinase DesK, Cobalt-zinc-cadmium resistance protein CzcA, Stress response kinase A, Porin B	Hunger et al., 2004; Nies, 1995; Lee & Young, 1996; Lewis et al., 2003
3	Putative multidrug resistance protein MdtD, Beta-lactamase Toho-1, Type IV secretion system protein virB2, Metallo-beta-lactamase L1 type 3	Nagakubo et al., 2002; Yagi et al., 1997; Kerr et al., 2010; Avison et al., 2000
4	Sensory/regulatory protein RpfC, Virulence sensor protein BvgS, Biofilm growth-associated repressor BigR, Purine ribonucleoside efflux pump NepI	Huedo et al., 2018; Beier et al., 1995; Guimarães et al., 2011; Gronskiy et al., 2005
5	Fimbrial protein fimA, Multidrug resistance protein Stp, Type II secretion system protein F	Klemm et al., 1984; Chen et al., 2004; Cianciotto, 2005
6	Type IV secretion system protein virB10, Type II secretion system protein F, Fimbrial protein pilE	Kerr et al., 2010; Cianciotto, 2005; Alm & Mattick, 1997
7	Type II secretion system protein F, Fimbrial protein pilA, Type IV secretion system protein virB10, CFA/I fimbrial subunit E	Cianciotto, 2005; Alm & Mattick, 1997; Kerr et al., 2010; Jordi et al., 1992
8	Type II secretion system protein F	Cianciotto, 2005
9	Multidrug resistance protein MdtA, Cobalt-zinc-cadmium resistance protein CzcB, A-type flagellin fliC, Multidrug export protein MepA	Nagakubo et al., 2002; Nies, 1995; Brimer & Montie, 1998; Kaatz et al., 2006
10	Chromosomal, Multidrug resistance protein MdtC, Efflux pump periplasmic linker BepF, Multidrug export protein MepA	Nagakubo et al., 2002; Truttman et al., 2010; Kaatz et al., 2006
11	Type IV secretion system protein virB2 and b10, Type II secretion system protein F, Fimbrial protein pilA	Yagi et al., 1997; Cianciotto, 2005; Schmoll et al., 1987

3.5 Phages

The PHASTER database was used to detect the phages present in our isolates. Only the intact phages were reported, these include *S. maltophilia* phages in addition to phages from other organisms such as *Burkholderia* and *Pseudomonas*, all summarized in Table 4 below.

Table 4. List of the intact phages found in some *S. maltophilia* isolates along with their GC% using the PHASTER database.

Isolate	Intact Phage		GC% of Phage
AST2	Burkholderia phage KS9	PHAGE_Burkho_KS9_NC_013055(5)	64.45%
AST3	Mesorhizobium phagevB_MloP_Lo5R7ANS	PHAGE_Mesorph_phagevB_MloP_Lo5R7ANS_NC_025431(12)	64.67%
AST6	Stenotrophomonas phage Smp131	PHAGE_Stenot_Smp131_NC_023588(38)	64.67%
	Stenotrophomonas phage phiSHP2	PHAGE_Stenot_phiSHP2_NC_015586(9)	61.18%
	Pseudomonas phage vB_PaeS_PM105	PHAGE_Pseudo_vB_PaeS_PM105_NC_028667(20)	64.74%

3.6 SNP-based phylogenetic analysis

The isolates were studied through core genome SNP based phylogenetic analysis using the reference strain *S. maltophilia* K279a (accession number: GCF_000072485.1). Figure 3 demonstrates the clustering of the isolates into two main clades. AST6 and AST7 that clustered together, were more closely related to the clinical reference genome (*S. maltophilia* K279a) than the other ones. In the second clade, we had isolates AST1 AST2, AST3, and AST8 all clustering together. The core genome consisted of 4851126 bp. The percentage covered by the core genome for each isolate was: AST1: 75%, AST2: 73.5%, AST3: 74%, AST6: 87%, AST7: 88.4%, and AST8: 69.5%.

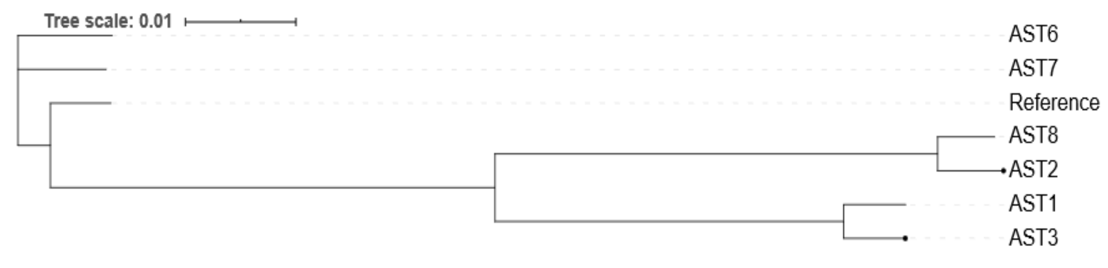


Figure 4: Snippy tree generated from iTOL. The reference genome used is *Stenotrophomonas maltophilia* K279a (accession number: GCF_000072485.1).

3.7 Virulence factors

Nucleotide sequences for virulence determinants were downloaded from NCBI and blasted against the isolates whole-genomes. Results showed the presence of a number of extracellular enzymes such as: esterases, lipases/hydrolases, phospholipases and nucleases (DNase and RNase) and heparinases. Moreover, other virulence factors such as haemolysins and siderophores were detected in all of the isolates. The virulence genes/proteins are summarized in Table 5 below.

Table 5. Virulence determinants shared by all the *S. maltophilia* isolates.

Virulence Factors Present in all isolates	
Gene/Protein	Gene/Protein Name
Proteases	StmPr1, StmPr2, and StmPr3
Lipopolysaccharide (LPS) genes	*xanB, xanA, etfA, etfB, rmlB, rmlA, rmlC, rmlD, lpsI, lpsJ, and waaE*
Type IV pili virulence genes	*pilA, pilC, pilE, pilN, pilM, pilO, pilP, and pilQ*
Fimbriae gene	*smf-1*
Afimbrial adhesion genes	*bmaC, upaG, ehaG*
Flagella genes	*flhA, flhB, flhF, flhQ*

Virulence genes associated with quorum sensing/biofilm formation were also detected in the studied isolates and included: *spgM* 83.3%, *rpfF-1* 66.7%, *rpfF-2* 33.3%, *bsmR* 66.7%, and *Ax21* 100%.

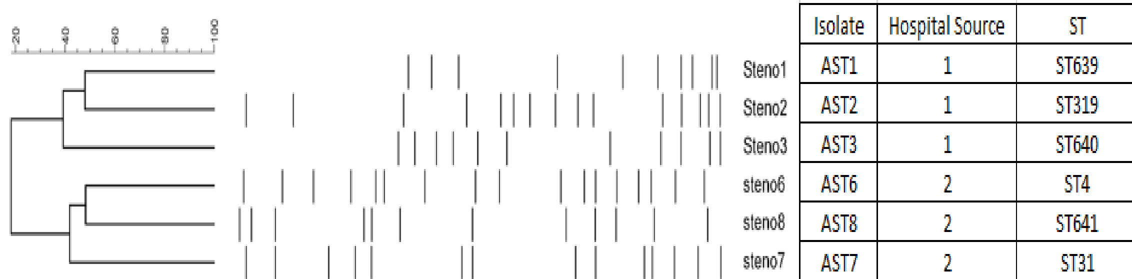
3.8 Genomic Islands

The IslandViewer database was used to detect the number of genomic islands and the gene pool. Isolates were found to harbor between 20 and 29 GIs each. The GIs were highly diverse and carried multiple VFs. Tetracycline resistance encoded by *tetA* was also detected on a common GI found in AST2 (GI2.13) and in AST6 (GI6.29). Isolates AST1

and AST3 shared two conserved GIs, 10,513 bp and 5,797 bp in size, while 3 GIs were shared between AST6 and AST7 (19,778 bp, 13,352 bp and 11,674 bp).

3.9 PFGE

Pulse field gel electrophoresis was performed to study the isolates' genetic relatedness (Figure 4). All the isolates had different STs and pulsotypes. However, it is noteworthy that isolates recovered from the same hospital clustered together; AST1, AST2, and AST3 (AUBMC) versus AST6, AST7, and AST8 (Makassed).



Isolate	Hospital Source	ST
AST1	1	ST639
AST2	1	ST319
AST3	1	ST640
AST6	2	ST4
AST8	2	ST641
AST7	2	ST31

Figure 5. Pulse Field Gel Electrophoresis (PFGE) results. 1: AUBMC, 2: Al Makassed Hospital.

Chapter Four

Discussion

Antimicrobial resistance contributes to elevated mortality and morbidity (Osman et al., 2019). The faulty diagnosis, the irrational consumption and misuse of antibiotics, and improper prescription are all reasons behind the elevated resistance of bacteria nowadays (Chamoun et al., 2016). Chamoun et al (2016) showed that AMR increased the costs of healthcare in Lebanon. The world health organization (WHO) has classified *S. maltophilia* as one of the leading MDR organisms in clinical settings (Brooke et al., 2013). *S. maltophilia* was previously detected in Lebanon in 2015 in a water canal and among 20 household water tap samples (Hamieh et al., 2015). With time, it became a major opportunistic nosocomial pathogen associated with high rates of mortality, especially in people who are immunocompromised or debilitated (Liu et al., 2017).

This study aimed at the molecular characterization of six clinical *S. maltophilia* isolates collected between 2018 and 2019 from two hospitals. As the sequencing technologies are advancing, a shift to WGS-based phylogenetic core single nucleotide polymorphism approaches is needed especially when it involves closely related emerging opportunistic pathogens such as *S. maltophilia* (Steinmann et al., 2018). A SNP based approach was used and it showed the relatedness of the clinical and environmental *Stenotrophomonas* sp. isolates. It is assumed that antibiotic resistance originated in the environment through gene exchange between clinical and environmental isolates (Perry & Wright, 2013). The

environmental resistome undergoes selective pressure linked to human activities, which in turn impacts resistance patterns and gene transfer events (Perry & Wright, 2013).

S. maltophilia is intrinsically resistant to aminoglycosides and β-lactams. *aac(6')-Iz* and *aph(3')-IIc* were the only two detected aminoglycoside resistance genes detected genes. Li (2003), previously reported that *aac(6')-Iz* gene was found in 57% of clinical isolates and contributed to the resistance to tobramycin (an aminoglycoside) .The *aac(6')-Iz* codes for aminoglycoside acetyltransferase, whereas the chromosomal intrinsic gene *aph(3')-IIc* encodes for an aminoglycoside phosphotransferase. *aph(3')-IIc* was also detected in the reference *S. maltophilia* K279a genome (Okazaki & Avison, 2006), which we used as a reference genome. *aph(3')-IIc* contributed to the increase in the minimum inhibitory concentrations (MICs) of many aminoglycosides when expressed in other bacteria (Okazaki & Avison, 2006).

On the other hand, *S. maltophilia* are intrinsically resistant to imipenem. The intrinsic resistance to imipenem is due to the production of L1 zinc-metalloenzyme with carbapenemase activity (Howe et al, 1997), encoded by the β-lactamase L1 (bla_{L1}) gene, and which was detected in this study. Similarly, Yang et al (2014) showed that all *S. maltophilia* isolates recovered in China harbored the bla_{L1} gene (Yang et al., 2014). The expression of this β-lactamase L1 is linked or triggered by the use of β-lactam antibiotics (Abbott et al., 2011).

All the studied isolates were susceptible to trimethoprim/sulfamethoxazole (SXT). SXT inhibits folate synthesis and is as a result bacteriostatic (Denton & Kerr, 1998). SXT is not always well tolerated due to hyperkalemia, lactic acidosis, myelosuppression, and bone marrow suppression (Abbott et al., 2011). Moreover, the resistance of *S. maltophilia*

to SXT is increasing as well as the diseases linked to nosocomial infections (Li et al., 2019; Hu et al., 2016). The *sul* genes (*sul1* and *sul2*) encoding for dihydropteroate synthases were linked to SXT resistance (sulfonamide) in isolates recovered in Korea (Radstrom et al., 1988; Chung et al., 2015), United Kingdom (Toleman et al., 2007), and Mexico (Herrera-Heredia et al., 2017). SXT resistance could also be through dihydrofolate reductase encoded for by *dfrA* gene (Hue et al., 2011). *dfr* and *sul* genes could be both present in isolates showing high level SXT resistance (Hu et al., 2016). No SXT resistance related genetic determinants was detected in our study isolates. Alternative treatment options however, are needed as strains with low or full resistance to SXT were detected in other studies in Korea (Chung et al., 2013). One such alternative could be levofloxacin (Falagas et al., 2008). But, even levofloxacin resistant *S. maltophilia* (LRSM) are emerging worldwide (Wang et al., 2020), and three of our isolates (AST1, AST7, and AST8) showed resistance to it. Another alternative is minocycline, which is characterized by its rapid penetration into the lungs and improved tolerability (Hand et al., 2016). Recently, it was shown that all *S. maltophilia* were susceptible to minocycline and the majority (80%) to SXT, which was in accordance to our results (Gallo et al., 2016; Magrum et al., 2020).

The LPS synthesis associated gene *spgM* interferes with the bacterial envelope structure and alters the susceptibility to β–lactam antibiotics (Liaw et al., 2010). Yet, *spgM*, was previously shown to be correlated with resistance to ceftazidime (Liaw et al., 2010), which was detected in all of our isolates (except AST8) which were also phenotypically resistant to ceftazidime.

Three novel STs, namely ST639, ST640, and ST641, were detected in this study. AST1, AST3, and AST8 had an altered loci patterns for the seven housekeeping genes used in determining the MLST type, and consequently were tagged as being new. The other STs in this study were ST319 (AST2), ST31 (AST7), and ST4 (AST6). ST4 and ST31 were previously detected in China, France, and Serbia (Yang et al., 2014; Madi et al., 2016; Corlouer et al., 2017).

Extracellular enzymes such as lipases, gelatinases, proteinases, lecithinases, DNases, and haemolysins, which were detected in all the study isolates, contributed to *S. maltophilia* pathogenicity and helped the bacterium to evade the innate immune response of the host (Thomas et al., 2014). These enzymes cause damage to the host tissues rendering it permissive to infection through mediating cell to cell spread and adhesion (Thomas et al., 2014).

In this study we also tested our isolates' ability to form biofilms, and all were positive. Biofilm production helps avoid antimicrobial agents, and promotes bacterial attachment to surfaces (Abbott et al., 2011). Different factors play a role in the formation of biofilms including the flagella, pili, afimbrial adhesion, and the outer membrane LPS layer (Abbott et al., 2011). Some of the genetic determinants linked to biofilm formation are the *spgM, rmlA*, and the *rpf* genes (Zhuo et al., 2014). Having detected all the three genes in all our isolates, supports our observation of all being strong biofilm formers, which is an important element enhancing virulence capabilities and resistance towards the used antimicrobial treatment options.

The quorum sensing system (QS) in *S. maltophilia* is controlled by the synthesis of DSF. This synthesis functions via the *rpfF-1* and *rpfF-2* variants. *rpfF-1* positive strains produce detectable diffusible signal factor (DSF) (Huedo et al., 2015). Unlike the *rpfF-1*

strains, the *rpfF-2* strains have a permanently repressed DSF synthesis under wildtype conditions, and gets activated only upon having exogenous DSF (Heudo et al, 2018). AST2 and AST8 harbored *rpfF-2* while the other isolates were *rpfF-1* positive. A similar finding was reported in a *S. maltophilia* study by Huedo et al (2014) where the *rpfF-1* variant was found in 60.26% of the isolates and the *rpfF-2* variant in the rest (Huedo et al., 2014).

The uptake of external DNA by horizontal gene transfer (HGT) as well as gene loss are considered main factors in bacterial evolution along with phage excision and integration (Lee et al, 2014). We detected several different bacteriophages in this study. One intact phage was the *Stenotrophomonas* phage Smp131 (detected in AST6) with a length of 33,525-bp and 47 open reading frames (Lee et al, 2014). Smp131 was only found to infect *S. maltophilia,* although it has sequence homology with *Xanthomonas* prophages, and with other phages, such as phiSMA7, phiSHP1, and phiSMA (Lee et al, 2014).

To our knowledge, this is the first comprehensive study of an important emerging human pathogen linked to nosocomial infections in Lebanon. The abundance of virulence factors, especially those associated with quorum sensing and biofilm formation, and the resistance determinants to β–lactams and aminoglycosides highlights the important health impacts of *S. maltophilia.* As future plans, we are planning to study in depth the quorum sensing mechanism in our isolates, and to dig into the molecular characteristics of the *rpf* genes. Our plans include checking for lost transmembrane proteins when we have the *rpfF-2* variant, checking if we also have two *rpfC* variants, inducing mutations/deletions/knockouts in the *rpf* genes and studying their effects, and studying the 3D structures of these proteins to detect any difference that renders one more specific than the other. Additionally, we also need to study quorum sensing via co-culturing our *S.*

maltophilia isolates with other organisms such as *P. aeruginosa* and study experimentally the *rpf* variant system and other proteins of the quorum sensing system (Ax21, AHL, *luxR*).

Conclusion:

This study aimed at the molecular characterization of *S. maltophilia* which has a low abundance in Lebanon. The organism is a global emerging opportunistic and multi-drug resistant human pathogen. *S. maltophilia* has emerged as an important opportunistic pathogen in immunosuppressed individuals. *S. maltophilia* has emerged as an important opportunistic pathogen. Prevention depends upon establishing and developing infection-control strategies while emphasizing the need to control antibiotic use and environmental reservoirs.

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