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RESEARCH ARTICLE

Analysis of biofilm formation in nosocomial *Stenotrophomonas maltophilia* isolates collected in Bulgaria: An 11-year study (2011–2022)

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ABSTRACT

The present study aimed to explore the genotypic and phenotypic characteristics of biofilm formation in Bulgarian nosocomial *Stenotrophomonas maltophilia* isolates ($n = 221$) during the period 2011–2022, by screening for the presence of biofilm-associated genes (BAG) (*spgM*, *rmlA* and *rpfF*), their mutational variability, and assessment of the adherent growth on a polystyrene surface. The methodology included: PCR amplification, whole-genome sequencing (WGS) and crystal violet microtiter plate assay for biofilm quantification. The overall incidence of BAG was: *spgM* 98.6%, *rmlA* 86%, and *rpfF* 66.5%. The most prevalent genotype was *spgM*+/*rmlA*+/*rpfF*+ (56.1%), followed by *spgM*+/*rmlA*+/*rpfF*- (28.5%), and *spgM*+/*rmlA*-/*rpfF*+ (9.5%), with their significant predominance in lower respiratory tract isolates compared to those with other origin ($P < 0.001$). All strains examined were characterized as strong biofilm producers (OD_{550} from 0.224 ± 0.049 to 2.065 ± 0.023) with a single exception that showed a weak biofilm-forming ability (0.177 ± 0.024). No significant differences were observed in the biofilm formation according to the isolation source, as well as among COVID-19 and non-COVID-19 isolates (1.256 ± 0.028 vs. 1.348 ± 0.128, respectively). Also, no correlation was found between the biofilm amounts and the corresponding genotypes. WGS showed that the *rmlA* accumulated a larger number of variants (0.0086 per base) compared to the other BAG, suggesting no critical role of its product to the biofilm formation. Additionally, two of the isolates were found to harbour class 1 integrons (7-kb and 2.6-kb sized, respectively) containing *sul1* in their 3′ conservative ends, which confers sulfonamide resistance. To the best of our knowledge, this is the first study on *S. maltophilia* biofilm formation in Bulgaria, which also identifies novel sequence types (ST819, ST820 and ST826). It demonstrates the complex nature of this adaptive mechanism in the multifactorial pathogenesis of biofilm-associated infections.

KEYWORDS

Stenotrophomonas maltophilia, biofilm formation, biofilm-associated genes, phenotypic characteristics, PCR screening, whole-genome sequencing

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INTRODUCTION

Stenotrophomonas maltophilia is an environmental, Gram-negative bacterium that has been recognized as an emerging multidrug-resistant opportunistic pathogen [1]. It is responsible

for a wide variety of clinical manifestations, predominantly healthcare-associated infections (HAIs) in intensive care unit patients, life-threatening diseases in immunocompromised patients with hematological malignancies and cancers and chronic pulmonary infections in patients with cystic fibrosis (CF) [2–5]. Since the beginning of the global pandemic, *S. maltophilia* has established itself as one of the most common pathogens causing respiratory co-infections and bacteremia in critically ill COVID-19 patients [6, 7]. The intrinsic resistance of the species to carbapenems and aminoglycosides, its ever-growing levels of acquired resistance towards key antimicrobials, such as trimethoprim-sulfamethoxazole and fluoroquinolones, and last, but not least the ability to form biofilms on various abiotic surfaces and tissues, like the bronchial tree, create significant difficulties with treating such infections [8–10].

The biofilm production by *Stenotrophomonas maltophila* is a prominent feature of its virulence [11]. The formed biofilm is composed of bacterial cells adherent to the surface and stick to each other via extracellular matrix, consisting of polysaccharides and proteins. It ensures higher resistance to antibiotics and antiseptic solutions, counteracting the immune defense mechanisms of the host, and contributes to the progression of CF lung disease and other chronic respiratory diseases [1, 9]. Biofilms have been estimated to be associated with 65% of HAIs caused by *S. maltophilia* [1].

The biofilm structure and levels of production vary between different *S. maltophilia* clinical isolates. The genetic mechanisms that determine biofilm formation include the participation of several biofilm-associated genes (BAG) with *spgM* (encoding a biofunctional enzyme with phosphoglucomutase and phosphomanomutase activity), *rmlA* (gluco-1-phosphate timidyltransferase) and *rpfF* (cis-11-methyl-2-dodecenoic acid, or synthase for the diffusible signal factor DSF) having the lead role [12].

The present study aimed to explore the main genotypic and phenotypic characteristics of biofilm formation in nosocomial *S. maltophilia* isolates obtained from six Bulgarian hospitals (2011–2022), via screening for the presence of BAG (*spgM*, *rmlA* and *rpfF*), their mutational variability, assessment of the adherent growth on a polystyrene surface, and searching for a correlation between these findings.

MATERIALS AND METHODS

Bacterial strains

A total of 221 non-duplicate nosocomial *S. maltophilia* isolates were studied. The isolates were collected during the period April 2011 to March 2022 from inpatients aged 1–94 years in six multiprofile university hospitals in Sofia, Bulgaria, namely: Military Medical Academy, University Hospital "Lozenetz", University Hospital "St. Ivan Rilski", University Hospital "Tokuda", University Hospital "Alexandrovska", and Medical Institute – Ministry of the Interior. They were obtained from lower respiratory tract (LRT) samples (n = 120), surgical wounds or abscesses (n = 27), upper respiratory tract (URT) samples (n = 26), blood (n = 17), urine (n = 11), medical devices (catheters and drainages) (n = 9), feces (n = 3), ascites (n = 2), bile (n = 1), cerebrospinal fluid (n = 1), and hospital environment (n = 4).

S. maltophilia ATCC 13637 was used as a control strain for species identification and was also subjected to tests to evaluate the produced biofilm.

All procedures involving patients were performed in accordance with the ethical standards of the Medical University of Sofia, Bulgaria and the Helsinki Declaration of 1964 and its later amendments. The current study was focused solely on bacterial isolates and no personal patient information or material was used; therefore, formal consent was not required.

Species identification of the isolates

Species identification was done using the VITEK 2 automated system (bioMérieux, Marcy-l'Ėtoile, France) and confirmed by a highly-specific polymerase chain reaction (PCR) targeting a 278-bp fragment of the 23S rRNA gene. Bacterial DNA was isolated by the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCR experiments were carried out with specific primers and amplification conditions described previously [13]. The identification of nine selected isolates was further confirmed by analyzing the assembled draft genome sequence using the Microbial Genomes Atlas (MiGA) Web server [14]. The included workflow for the NCBI Genome Database, Prokaryotic section was followed with default settings.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the isolates was performed by Minimum Inhibitory Concentration test strips (Liofilchem, Roseto degli Abruzzi, Italy) according to the EUCAST-2022 and CLSI-2020 guidelines (http://eucast.org; http://clsi.org).

PCR-based screening for BAG

PCR was performed to detect the presence of the main BAG (*spgM*, *rmlA* and *rpfF*). Oligonucleotides used as primers for PCR amplification were synthesized by Metabion (Planegg, Germany) and are listed in Table 1. Each 25-µL PCR mixture consisted of 2 µL of template DNA; a 0.1 µM of each primer; 12.5 µL of MyTaq PCR mix (Bioline, London, UK) and 8.5 µL of ultrapure 18.2 MΩ PCR water (Bioline).

DNA was amplified in a Gene ProThermal Cycler (Bioer Technology, Hangzhou, China) using the following protocol: initial denaturation at 95 °C for 5 min; followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 54.5–64 °C for 45 s and extension at 72 °C for 45 s; and a single final extension at 72 °C for 7 min. PCR products were separated in 1.5% agarose gel for 50 min at 130 V, stained with SimplySafe (0.05 µL mL^{-1}) (EURx, Gdansk, Poland) and

detected by ultraviolet light (wavelength 312 nm). Amplified gene fragments were identified on the basis of their size (Table 1).

Microtiter plate assay for biofilm quantification

The biofilm formation ability was evaluated by the crystal violet staining assay [15] among 92 selected *S. maltophilia* isolates received from: LRT (n = 41), URT (n = 20), wounds (n = 12), blood (n = 6), medical devices or hospital environment (n = 5), urine (n = 3), and feces (n = 3). Overnight Tryptic Soy Broth (Liofilchem, Roseto degli Abruzzi, Italy) cultures of the strains tested were diluted 1:100. One hundred and fifty microliters of the diluted inoculum were dispensed to each well of a sterile U-shaped polystyrene 96-wells microtiter plate and incubated at 37 °C for 24 h, with 6 repeats for every sample. The plates were shaken at several short intervals. Unattached bacteria were withdrawn, and the wells were washed with Phosphate-buffered saline. The wells were then coloured for 15 min with 0.1% crystal violet, washed extensively and solubilised in 70% ethanol. Absorbance was measured at 550 nm wavelength (Optical density at λ = 550 nm – OD_{550}).

The low cut-off (ODc) was calculated as the three standard deviations (3xSD) above the mean OD of control wells. Strains were classified according to the following criteria: no biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2xODc), moderate biofilm producer (2xODc < OD ≤ 4xODc), and strong biofilm producer (4xODc < OD) [16].

Whole-genome sequencing

Nine selected clinical *S. maltophilia* isolates from different sources (LRT, wounds, medical devices, and hospital environment), with a genetic profile of *spgM*+/*rmlA*+/*rpfF*+ and differing quantities of the biofilm formed on a polystyrene surface were subjected to whole-genome sequencing for the use of a detailed study of biofilm-associated genetic determinants. The WGS was performed using DNA nanoball sequencing technology as previously described [17]. Briefly, genomic DNA obtained from all selected strains was randomly fragmented using a Covaris g-TUBE device, and fragments were size selected by magnetic beads to an average size of 200–400 bp. The purified fragments from each sample were end repaired, 3′-adenylated, ligated to adapters, and, then, PCR amplified. All libraries generated in this way were then loaded onto an MGISEQ-2000 platform (BGI Group, Hong Kong, China). The following sequencing step was done generating 2 × 150-bp paired-end reads.

Draft genome assembly

All steps of quality control, raw reads preprocessing, and draft genome assembly were carried out through the Galaxy online platform as previously described [18, 19]. Default parameters were used for all following software tools unless otherwise specified.

Multilocus sequence typing (MLST) analysis

The MLST analysis was performed on the assembled draft genome sequences using the Multilocus sequence typing tool (Galaxy Version 2.19.0, https://usegalaxy.eu/).

Antibiotic resistance determinants (ARDs) screening

The draft genome contigs were screened for ARDs using the ABRicate tool (Galaxy Version 1.0.1) with the following settings: NCBI Bacterial Antimicrobial Resistance Reference Gene Database, Minimum DNA identity (70%) and Minimum DNA coverage (60%).

Statistical analysis

Statistical analysis was performed using Excel (Microsoft Office 365). The values of the data obtained were expressed as mean, standard deviation, number, and percentage frequencies.

The distribution of genetic determinants studied and the BAG genotypes according to the isolate origin as well as the comparison between our results and recent ones reported by other authors were performed using Student's t-test. For simple comparison tests, a P-value below 0.05 was considered statistically significant. To counteract the problem of multiple comparisons, when used, a Bonferroni correction was applied. The Spearman correlation coefficient was calculated using R 4.2.2.

RESULTS

Screening for BAG

The overall incidence of *spgM*, *rmlA* and *rpfF* among the full *S. maltophilia* collection including isolates from patients with HAIs and hospital environment (n = 221) was: 98.6%, 86.0% and 66.5%, respectively.

Table 1. Oligonucleotides used as primers for PCR amplification of biofilm-associated genes in 221 *S. maltophilia* isolates studied

Primer pair	Target	Sequence (5′–3′)	Product size (bp)	T_a (°C)	Source
spgM-F	*spgM*	AAGGCAGGATCATCTATGC	648	64	This study
spgM-R		TCAGAACCCCAGCGGGGTG			
rmlA-F	*rmlA*	CGGAAAAGCAGAACATCG	799	54.5	This study
rmlA-R		GCAACTTGGTTTCAATCAC			
rpfF-F	*rpfF*	ATGTCTGCAGTACGCCCCA	835	62	This study
rpfF-R		CGTACCAGCCGATCCATGG			

T_a, annealing temperature; F, forward primer; R, reverse primer.

The *S. maltophilia* strains used in this study were grouped in two categories: "Clinical isolates from secretion and bodily fluids" and "Clinical isolates from medical devices and hospital environment, therefore have been in contact with abiotic surfaces". This showed the following frequency of distribution of *spgM*, *rmlA* and *rpfF*: 98.6% vs. 100%, 86.5%/76.9% and 66.3%/69.2%, respectively. No statistically significant differences between the two groups were found.

Six genotypes of BAG were observed to have a wide prevalence range (from 0.5% to 56.1%). The predominant genotype was *spgM+/rmlA+/rpfF+* (56.1%), followed by *spgM+/rmlA+/rpfF-* (28.5%), *spgM+/rmlA-/rpfF+* (9.5%), *spgM+/rmlA-/rpfF-* (4.5%), *spgM-/rmlA+/rpfF+* (0.9%), and the lowest frequency was found for *spgM-/rmlA+/rpfF-* genotype (0.5%). The distribution of the studied *S. maltophilia* isolates with differing genetic BAG profiles based on their origin is showcased in Fig. 1. LRT isolates showed a significant dominance over other isolates in the group sharing the most frequent BAG gene profile – *spgM+/rmlA+/rpfF+* ($P < 0.001$). The distribution of clinical samples' findings within the groups with two BAG, *spgM+/rmlA+/rpfF-* and *spgM+/rmlA-/rpfF+* genotypes, revealed the same statistically significant values. Similar percentages of LRT isolates (30%) and wound isolates (20%), in comparison with the other clinical samples, are found to have a *spgM+/rmlA-/rpfF-* gene profile.

Biofilm-forming ability on a polystyrene surface and association between genotypic and phenotypic biofilm characteristics of *S. maltophilia* isolates tested ($n = 92$)

The measured biofilm production of all tested nosocomial *S. maltophilia* strains had optical density values (mean $OD_{550} \pm SD$) in a wide range (from 0.177 ± 0.024 to 2.065 ± 0.023) and mean $OD_{550} = 1.313 \pm 0.090$. All tested strains, excluding one from LRT (a weak producer), were determined to be strong biofilm producers (OD_{550} above 0.223).

The quantity of biofilm formed by the isolates from different sources, as well as its relation to the genotypes observed, are detailed in Table 2. In general, no significant differences were observed in the biofilm amounts concerning the source of strain isolation (almost identical mean OD_{550} values for biofilms formed by LRT, URT and wound isolates). Invasive bloodstream isolates demonstrated the highest ability of biofilm formation (1.596 ± 0.077), while those from feces, usually thought of as the colonists of the gastro-intestinal tract, showed the lowest biofilm formation capability (0.956 ± 0.034). What must be considered is the small isolate count, six and three, respectively.

Phenotype characteristics of the biofilm formed by *S. maltophilia* isolates from patients with COVID-19 ($n = 35$) and with non-COVID-19 infections ($n = 57$), included close mean values (1.256 ± 0.028 and 1.348 ± 0.128, respectively), but wider ranges within the non-COVID group of samples (0.177–2.065 vs. 0.362–1.698).

No significant differences were found within the mean amount of biofilm formed among the *S. maltophilia* isolates with three (*spgM+/rmlA+/rpfF+*) and two BAG (*spgM+/rmlA-/rpfF+* and *spgM+/rmlA+/rpfF-*) as indicated by the calculated Spearman correlation coefficient of 0.133 ($P = 0.214$) (Table 2). The three studied isolates with a *spgM+/rmlA-/rpfF-* genotype were characterized by the weakest ability to form a biofilm on a polystyrene surface (0.888 ± 0.031), but, taking the small count of samples into account, the assumption that there is a direct correlation

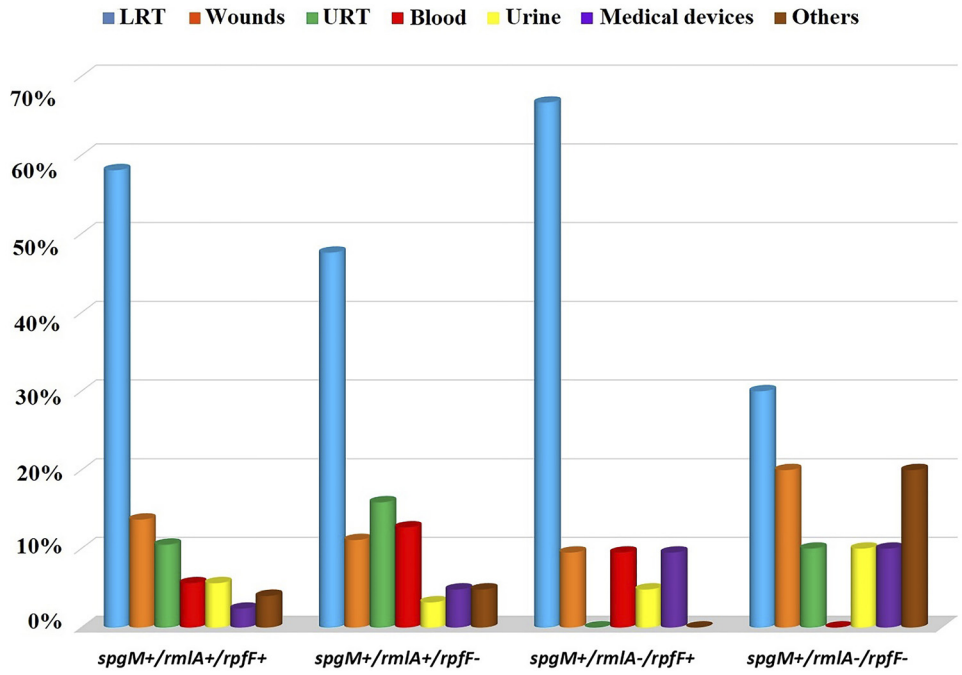

Fig. 1. **Distribution of the *S. maltophilia* isolates of different genotypes according to their origin**. Both genotypes *spgM-/rmlA+/rpfF+* and *spgM-/rmlA+/rpfF-* are excluded because they consist of single isolates 2 and 1, respectively

Table 2. Relation of the genotypes and the biofilm formation ability in 92 selected *S. maltophilia* isolates of various origins

Genotype	N	Mean OD_{550} ± SD	LRT N	LRT Mean OD_{550} ± SD	Wounds N	Wounds Mean OD_{550} ± SD	URT N	URT Mean OD_{550} ± SD	Blood N	Blood Mean OD_{550} ± SD	Urine N	Urine Mean OD_{550} ± SD	Feces N	Feces Mean OD_{550} ± SD	M.Dev/H.Env N	M.Dev/H.Env Mean OD_{550} ± SD
spgM+/rmlA+/rpfF+	50	1.278 ± 0.098	22	1.255 ± 0.116	11	1.340 ± 0.106	11	1.199 ± 0.028	2	1.630 ± 0.031	–	–	1	0.847 ± 0.031	3	1.42 ± 0.265
spgM+/rmlA-/rpfF+ [a]	12	1.42 ± 0.136	8	1.385 ± 0.117	–	–	–	–	1	1.847 ± 0.160	1	1.303 ± 0.121	–	–	1	1.347 ± 0.195
spgM+/rmlA+/rpfF- [b]	27	1.377 ± 0.06	11	1.377 ± 0.075	1	1.276 ± 0.037	8	1.446 ± 0.050	3	1.489 ± 0.080	1	1.349 ± 0.039	1	1.058 ± 0.041	1	0.772 ± 0.024
spgM+/rmlA-/rpfF-	3	0.888 ± 0.031	–	–	–	–	1	1.356 ± 0.034	–	–	1	0.345 ± 0.029	1	0.962 ± 0.031	–	–
Total	**92**	**1.313 ± 0.090**	**41**	**1.313 ± 0.105**	**12**	**1.334 ± 0.100**	**20**	**1.305 ± 0.037**	**6**	**1.596 ± 0.077**	**3**	**0.999 ± 0.063**	**3**	**0.956 ± 0.034**	**5**	**1.276 ± 0.203**

N, number of isolates; OD_{550}, optical density read at λ = 550 nm; SD, standard deviation; LRT, lower respiratory tract; URT, upper respiratory tract; M.Dev, medical devices; H.Env, hospital environment.
[a] one ascites isolate is not presented; [b] one cerebrospinal fluid is not presented.

between the number of BAG and the quantity of biofilm produced could be thought of as speculative.

Draft genome assemblies: evaluation and comparison

The nine assembled draft genomes varied in size between 4.38 and 4.99 Mbp, and their GC% content was between 65.80% and 66.78% (Table 3). These values are comparable with the accessible data from sequenced *S. maltophilia* genomes.

Successful multilocus sequence typing of the selected isolates was performed using the available sequences. They were found to belong to seven different sequence types (STs) and none of them included more than two species. Three of those STs (ST819, ST820 and ST826) were detected for the first time within the frame of our study and next deposited in the PubMLST database (https://pubmlst.org/bigsdb?db=pubmlst_smaltophilia_seqdef&page=query&scheme_id=1). Two of the novel STs (ST820 and ST826) contained four new alleles in total, while ST819 was a new combination of alleles that were already known (Table 3).

Calculating the average nucleotide identity (ANI) of the sequenced genomes against the *S. maltophilia* K279a (Accession No NC_010943) one showed significantly lower values for the SM62 and SM148 isolates compared to the others.

Identifying missense mutations in BAG

All mutations, leading to amino acid exchanges in the coding sequences of *spgM*, *rmlA* and *rpfF* were identified and divided into two groups: conservative amino acid substitutions and non-conservative substitutions (Table 4). The corresponding sequences in *S. maltophilia* ATCC 13637 strain (classified as a strong biofilm producer – OD_{550} = 1.374 ± 0.169) were used as references. The lowest amount of variation from both types was found in the *rpfF*, while the *rmlA* gene was found to be the most variable one among all BAG analysed. The average frequency of conservative substitutions per base pair in the coding sequences for BAG was 0.0016 for *spgM*, 0.0014 for *rpfF* and 0.0041 for *rmlA*. The corresponding values for the non-conservative variants were calculated to be 0.0011, 0.0003 and 0.0045, respectively.

ARDs screening

The ARDs detected in the nine WGS-subjected *S. maltophilia* isolates are summarized in Table 5. All genomes possessed typical ARDs conferring intrinsic resistance to β-lactams, including carbapenems, and aminoglycosides. Two of the isolates (SM130 and SM148) were found to harbour class 1 integrons (7-kb and 2.6-kb sized, respectively) containing *sul1* (encoding dihydropteroate synthase type-1) in their 3′ conservative ends. The SM130 integron also included a resistance gene cassette embedded into the variable region. It consisted of bla_{OXA-74} (encoding an OXA-10 family class D β-lactamase OXA-74), *aac(6′)-Ib-cr* (fluoroquinolone-acetylating aminoglycoside acetyltransferase) and *cmlA7* (chloramphenicol acetyltransferase) as described

Table 3. Whole genome-based characterization of 9 selected *S. maltophilia* isolates producing different amounts of biofilm

Isolate No	Genome size (Mbp)	GC%	ANI (%)	ST	Alleles							OD_{550}
					atpD	*gapA*	*guaA*	*mutM*	*nuoD*	*ppsA*	*recA*	
SM8	4.62	66.62	98.17	820	154	104	7	235	7	108	1	1.108
SM49	4.92	65.80	97.26	172	5	94	122	79	70	105	85	1.253
SM62	4.47	65.93	92.30	826	81	236	494	70	190	201	135	0.788
SM64	4.68	66.50	98.70	27	3	1	1	3	6	4	1	1.576
SM79	4.99	65.83	98.22	820	154	104	7	235	7	108	1	1.391
SM105	4.50	66.71	98.33	139	3	4	110	46	6	38	58	1.789
SM130	4.47	66.67	97.50	119	4	76	92	5	70	84	9	1.430
SM135	4.53	66.57	98.10	172	5	94	122	79	70	105	85	0.177
SM148	4.38	66.78	92.58	819	116	165	124	176	4	163	201	0.989

ANI, average nucleotide identity; ST, sequence type; OD_{550}, optical density read at $\lambda = 550$ nm. Isolates obtained from: SM8, sputum; SM49, wound; SM62, wound; SM64, wound; SM79, gastroscope; SM105, drainage; SM130, tracheobronchial aspirate; SM135, bronchoalveolar lavage; SM148, tracheobronchial aspirate.

Note: Underlined STs and alleles were detected for the first time in the present study.

in our previous study [10]. These findings were in accordance with the antibiotic resistance profiles of both isolates (SM130 was characterized as extensively drug-resistant, including to trimethoprim-sulfamethoxazole, levofloxacin, ceftazidime, and chloramphenicol; SM148 showed resistance to trimethoprim-sulfamethoxazole and levofloxacin).

DISCUSSION

Biofilms produced by *S. maltophilia* play a pivotal role in HAIs, especially those related to the use of medical devices, such as ventilator-associated pneumonia, central venous catheter-related bloodstream infections, catheter-associated urinary tract infection and surgical site infections [1]. However, it is currently not fully understood whether there is any variation in biofilm formation among clonally diverse clinical isolates of *S. maltophilia* and whether there are any relationships between the biofilm-forming ability and the mutation or expression of main BAG such as *spgM*, *rmlA* and *rpfF* [12].

The *spgM* gene plays a significant role in virulence and biofilm formation of *S. maltophilia* (Pompilio A. et al., 2011). Earlier studies reported the following prevalence of the gene's distribution: 88.8% among clinical and environmental *S. maltophilia* isolates from Italy and the Czech Republic [20], 83.3% among respiratory isolates from China (2010–2011) [12] and 71.6% among Serbian CF and non-CF *S. maltophilia* strains, mostly isolated from children between 2013 and 2015 [21]. Three recent studies on biofilm production of clinical isolates from several University hospitals in Iran [22–24] showed a *spgM* frequency ranging from 97.3% to 100% between 2016 and 2019, which corresponds to our values (98.6%).

The lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes *rmlA*, *rmlC*, and *xanB* are necessary for biofilm formation and twitching motility of *S. maltophilia* [25]. The overall frequency of *rmlA* found by us (86.0%) corresponded to that in the aforementioned earlier studies – 87.5% established by *Zhuo* et al. in China [12], and 88.41%–89.41% reported by *Bostanghadiri* et al. in Iran [22, 24]. A significantly lower distribution of the genetic determinant was recorded by *Pompilio* et al. (65.2%, $P < 0.001$) [20], and higher – among clinical *S. maltophilia* isolates from a pediatric hospital in Belgrade, Serbia (97.7%, $P < 0.001$) [21].

The *rpfF* gene is part of the *rpf* (regulation of pathogenicity factors) cluster and the synthesis of the diffusible signal factor (DSF) is completely dependent on it [26]. The *rpf*/DSF signalling system of *S. maltophilia* positively regulates biofilm formation, production of extracellular virulence factors and L1/L2 β-lactamase induction [27]. The accounted frequency of *rpfF* among our nosocomial *S. maltophilia* isolates (66.5%) was higher than the one stated in the Chinese study [12], very close to that registered among the Italian (65.2%) and Serbian (70.4%) clinical isolates [20, 21] and significantly lower than the one among the *S. maltophilia* strains from Iran (83.53%–84.71%, $P < 0.001$) [22, 24]. We need more data from different regions as well as a higher count of strains to be studied, to determine a possible geographical distribution of the gene.

In the predominant part of the studied nosocomial *S. maltophilia* isolates from Bulgarian hospitals was proven the presence of all three BAG. The *spgM+/rmlA+/rpfF+* genotype was the most reported by other authors as well, and the frequency we found was higher than the incidence found for the genotype among *S. maltophilia* isolates from CF patients in Italy (56.1% vs. 34.8%, $P < 0.001$) [20] and lower than that in clinical strains from university hospitals in Iran, for the period 2016–2017 (68.7%, $P < 0.02$) [23]. Our study included three LRT isolates obtained from CF patients, in which the three BAG were detected. The second most common BAG profile among our isolates had a missing *rpfF* (28.5%), and its frequency was very close to the one stated by *Pompilio* et al. (21.3%) among the studied clinical and environmental *S. maltophilia* isolates from Italy [20]. Only one of the clinical isolates we used demonstrated a gene profile of *spgM-/rmlA+/rpfF-* and none – *spgM-/rmlA-/rpfF-*, which was reported earlier and by other authors [23].

Table 4. Missense mutations found in biofilm-associated genes of the *S. maltophilia* isolates that were subject to whole genome sequencing

Isolate No	Gene (CDS)		Identities (%)	Positives (%)
	Similar mutations	Dissimilar mutations		
		***rmlA* (888 bp)**		
SM8	pQ79R, pE98D, pD131E, pS154T, pI158V, pN182D, pA188S, pK205R, pS211N	pV130E, pN166I, pG209A, pN212A, pA217P, pC252S, pQ261N, pT265N	94	97
SM49	pE98D, pD131E, pI158V, pN182D, pK205R, pS211N	pV130K, pN166I, pG209A, pN212T, pA217P, pC252S, pQ261N, pT265N	95	97
SM62	pK97R, pE98D, pE189D	pV130T, pG209A, pS211G, pT265N	98	98
SM64			100	100
SM79			100	100
SM105			100	100
SM130	pE98D, pD131E, pI158V, pN182D, pK205R, pS211N	pV130K, pN166I, pG209A, pN212T, pA217P, pC252S, pQ261N, pT265N	95	97
SM135	pS154T		99	100
SM148	pK97R, pE98D, pK124R, pE128Q, pH186Y, pK205Q, pN212H, pQ261K	pR125N, pV130E, pD131Q, pS154D, pA185D, pG209R, pS211G, pT229N	95	97
		***rpfF* (870 bp)**		
SM8	pS35N		99	100
SM49	pS35N		99	100
SM62	pS35N		99	100
SM64	pS35N	pA69T	99	99
SM79	pS35N		99	100
SM105	pS35N		99	100
SM130	pS35N		99	100
SM135	pS35N		99	100
SM148	pS35N, pE59D	pV213A	99	99
		***spgM* (2343 bp)**		
SM8	pM703L		99	100
SM49	pA43S, pA457S	pE10G	99	99
SM62	pD106E, pV220I, pV234I, pS270T, pD306E, pT307S, pE336Q, pV429I, pT564S, pE566D, pR568K, pE694D	pE10G, pE50A, pQ104H, pT114N, pQ132G, pG137A, pK138G, pG140S, pA147G, pQ159P, pT189A, pT198A, pQ204L, pG208S, pA215V, pA236P, pA301G, pA304T, pA305T, pG317E, pG322D, pT385A, pQ446A, pA454V, pG565A, pA677G, pIns314P	95	96
SM64		pT671A	99	99
SM79	pM703L		99	100
SM105	pM703L	pP313del	99	99
SM130	pA43S, pI392V, pD712E		99	100
SM135	pQ293H, pD306N, pE318K, pD328N, pV333I	pT671A	98	99
SM148	pD106E, pV220I, pS270T, pD306E, pT307S, pE336Q, pV449I, pE694D, pD775E	pE10G, pE50A, pQ104H, pT114N, pQ132A, pG137T, pK138G, pG140S, pA147G, pQ159P, pT189A, pT198A, pG208S, pA236P, pA300V, pA301E, pA304T, pA305T, pG317E, pT385A, pA454V, pT722I	96	96

CDS, coding sequence. Positions of the mutations are given according to the corresponding sequences of *S. maltophilia* ATCC 13637. Similar (conservative substitutions) and dissimilar (non-conservative) substitutions are determined by BLASTP alignment/BLOSUM62 matrix. Identities and Positives are calculated by BLASTP comparisons against the equivalent sequences of *S. maltophilia* ATCC 13637 (CP008838.1)

All *S. maltophilia* isolates tested ($n = 92$) in this study were able to produce bioflms, and 98.9% were characterized as strong producers. Previous studies also showed biofilm production by all or most of the tested isolates, but demonstrated a higher relative proportion of weak and moderate biofilm producers in contrast to our findings [12, 20, 22, 28, 29]. In a study by *Flores-Treviño* et al. covering clinical *S. maltophilia* isolates from Mexico (2006–2013), isolates were categorized as: weak (47.9%), moderate (38.7%) and strong (13.4%) biofilm producers [28]. In a recent study, conducted in Iran, the predominant part of the tested *S. maltophilia* isolates (2018–2019) were also weak and moderate biofilm producers, 28.23% and 37.65% respectively [24]. In contrast, *Gallo* et al. showed that Brazilian clinical isolates were weak (3%), moderate (45%), or strong (48%) biofilm producers [30]. The relative percentage of strong

Table 5. Antibiotic resistance determinants found in the WGS-subjected *S. maltophilia* isolates

Isolate No	Intrinsic ARDs	Intrinsic resistance	Acquired ARDs	Acquired resistance
SM8	bla_{L1}, bla_{L2} *aph(3′)-IIc*, *aac(6′)-Iz*	β-lactams, incl. carbapenems Aminoglycosides		
SM49	bla_{L1}, bla_{L2} *aph(3′)-IIc*, *aac(6′)-Iz*	β-lactams, incl. carbapenems Aminoglycosides		
SM62	bla_{L1}, bla_{L2} *aph(3′)-IIc*	β-lactams, incl. carbapenems Aminoglycosides		
SM64	bla_{L1}, bla_{L2} *aph(3′)-IIc*	β-lactams, incl. carbapenems Aminoglycosides		
SM79	bla_{L1}, bla_{L2} *aph(3′)-IIc*, *aac(6′)-Iz*	β-lactams, incl. carbapenems Aminoglycosides	*aph(3′)-IIb*, *aph(3″)-Ib*, *aph(6)-Id*	Aminoglycosides
SM105	bla_{L1}, bla_{L2} *aph(3′)-IIc*, *aac(6′)-Iz*	β-lactams, incl. carbapenems Aminoglycosides		
SM130	bla_{L1}, bla_{L2} *aph(3′)-IIc*, *aac(6′)-Iz*	β-lactams, incl. carbapenems Aminoglycosides	*sul1* [a] bla_{OXA-74} [b] *aac(6′)-Ib-cr5* [b] *cmlA7* [b] Sm*qnr11*	Sulfonamides Cephalosporins, carbapenems Fluoroquinolones, aminoglycosides Chloramphenicol Quinolones
SM135	bla_{L1}, bla_{L2} *aph(3′)-IIc*, *aac(6′)-Iz*	β-lactams, incl. carbapenems Aminoglycosides		
SM148	bla_{L1}, bla_{L2} *aph(3′)-IIc*	β-lactams, incl. carbapenems Aminoglycosides	*sul1* [a] *qnrE1* [c]	Sulfonamides Quinolones

WGS, whole-genome sequencing; ARDs, antibiotic resistance determinants.
[a] part of the 3′ conservative end of a class 1 integron; [b] part of the gene cassette (variable region) of a class 1 integron; [c] partial sequence (60% coverage).

biofilm producers we found was almost identical to that (98.41%) published by a recent study of clinical *S. maltophilia* isolates from patients treated at a university hospital in Argentina over an 8-year period (2004–2012) [31]. The authors related the high percentage of such strains to their isolation from infections associated with invasive medical devices, such as bronchoscopes, intubation tubes, catheters and drains (76.92%).

Like we stated above, no relation between BAG genotypes (including the number of detected genes) and the quantity of biofilm produced on a polystyrene surface was determined. In earlier studies, the simultaneous presence of *spgM* and *rpfF* was shown to significantly affect the biofilm-forming ability of the strains, whereas such correlation was not found with other gene combinations (*spgM* + *rmlA* and *rmlA* + *rpfF*) [12, 21]. It was also reported that the presence of all three genes improved the biofilm-forming capacity, but did not significantly affect its quantity [12, 21, 22].

The lack of correlation between the amounts of biofilm produced by the isolates and their origin found by us was also established by *Madi* et al. [21] in a studied population of Serbian *S. maltophilia*, including isolates from the three categories – weak, moderate, and strong biofilm producers. The authors reported that all strong producers were respiratory isolates, except for one from blood. Among our strains tested for biofilm production (98.9% strong producers), isolates from respiratory secretions predominated (as in the whole study).

A recent prospective multicenter study of clinical *S. maltophilia* isolates from five European countries (Italy, Serbia, Czech Republic, Germany, and Spain) revealed that the isolates from blood showed the highest capacity for biofilm formation compared to those from other clinical sources [29], which was also confirmed in our work. The authors reported that the "strong-producer" phenotype was most prevalent among strains isolated from blood (78.3%; $P < 0.0001$ vs. other groups). Also, they found that the strains able to form a higher biofilm amount were significantly more prevalent among hospital-acquired infection than community-acquired strains (60.6% vs. 33.3%, respectively; $P < 0.05$). In the present study, all isolates tested were nosocomial, including isolates from patients with COVID-19 (predominantly recovered from respiratory specimens), and as already reported, we found no significant differences in biofilm-forming capacity between COVID-19 and non-COVID-19 *S. maltophilia* isolates. No similar comparative analyses currently exist in the literature. The wider OD_{550} range found within the "non-COVID-19 isolates" group could be accounted to the greater variety of isolation sites and the related strain-to-strain differences in the biofilm formation efficiency [29].

The MLST analysis performed on a WGS subset revealed that all the STs were represented by one or two isolates, or a high genetic diversity. These findings confirm those from previous studies [4, 29, 32, 33], where *S. maltophilia* strains also showed a high heterogeneity.

Analysis of the coding sequences of *rmlA* revealed over 2 times more conservative and over 4 times more non-conservative amino acid substitutions compared to the other two genes. A similar high mutation load in *rmlA* has been reported in other studies [12], suggesting a secondary role of the gene product in biofilm production. *Brooke* also reported increased biofilm production by a *rmlA- S. maltophilia* mutant on glass and polyvinylchloride surfaces as well as decreased production over polystyrene surface [1]. This data can explain the decreased biofilm production by SM62 and SM148 isolates (Table 3), as they both possessed a common unique non-conservative amino acid substitution pS211G in the *rmlA* (Table 4). This finding can be considered as a promising candidate for further investigations. It is worth mentioning the lack of isolates with a *spgM+/rmlA-/rpfF+* genotype from URT and wound samples. A possible explanation of this phenomenon is that the enzyme encoded by *rmlA* is required for the formation of the O-antigen that benefits the colonization of such habitats.

The *rpfF* gene accumulated disproportionally lower amounts of both amino acid substitution types than the other two BAG we studied. This finding, combined with its frequency of detection during the conducted PCR screening, suggested that the encoded product is important for biofilm formation in *rpfF*-positive isolates. Additional data supporting this hypothesis also came from recently published comparative analyses between *S. maltophilia* K279a and K279a*rpfF* strains that demonstrated the importance of this gene in biofilm formation and virulence [27].

The *spgM* gene was the most frequently identified BAG during our PCR screening. Even higher detection rates were reported in a recent study, stating the presence of *spgM* in 100% of the tested isolates and that all of them had the ability to form biofilms [24]. Analysis of the nine assembled genomes showed that *spgM* is the only gene with non-conservative amino acid substitutions in the three weakest producers among the studied isolates (SM62, SM135 and SM148). All this suggested that its product plays an important role in biofilm formation and the accumulation of possible inactivating mutations within it can serve as an indicator for decreased biofilm formation on a polystyrene surface.

CONCLUSION

To the best of our knowledge, this is the first Bulgarian study on biofilm formation by *S. maltophilia*, including PCR detection and WGS analysis of the main BAG, as well as measuring the biofilm biomass on an abiotic surface. Established are wide distribution of the *spgM*, *rmlA* and *rpfF* genes, strong biofilm production among nosocomial strains studied (98.9%) and lack of correlation between the quantity of biofilm formed and the isolates' genotype (number and combination of BAG) and origin. The obtained results emphasize the complex nature of this adaptive mechanism of *S. maltophilia* in the multifactorial pathogenesis of biofilm-associated infections.

The still limited therapeutic approaches for the prevention of biofilm formation and the antibiotic tolerance of bacteria within the biofilm, pose as a serious threat to public health. Future studies are needed to better understand the genetic mechanisms of biofilm formation and dynamics in *S. maltophilia* in order to develop successful strategies for its control.

NUCLEOTIDE SEQUENCING

Whole-genome shotgun sequencing project of the *S. maltophilia* SM130 isolate has been deposited in GenBank under Accession no. JABVAZ000000000. The three novel sequence types were submitted to the PubMLST database.

Conflict of interest: The authors report no conflicts of interest.

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