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RESEARCH ARTICLE

Genotypic and phenotypic insights into virulence factors of nosocomial *Stenotrophomonas maltophilia* isolates collected in Bulgaria (2011–2022)

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ABSTRACT

The present study aimed to explore the virulence characteristics in 221 Bulgarian nosocomial *Stenotrophomonas maltophilia* isolates (2011–2022) via screening for the presence of virulence genes, their mutational variability, and the corresponding enzyme activity. PCR amplification, enzymatic assays, whole-genome sequencing (WGS), and biofilm quantification on a polystyrene plate were performed. The incidence of virulence determinants was as follows: *stmPr1* (encoding for the major extracellular protease StmPr1) 87.3%, *stmPr2* (minor extracellular protease StmPr2) 99.1%, Smlt3773 locus (outer membrane esterase) 98.2%, *plcN1* (non-hemolytic phospholipase C) 99.1%, and *smf-1* (type-1 fimbriae, biofilm-related gene) 96.4%. The 1621-bp allele of *stmPr1* was most frequently found (61.1%), followed by the combined allelic variant (17.6%), *stmPr1*-negative genotype (12.7%), and 868-bp allele (8.6%). Protease, esterase, and lecithinase activity was observed in 95%, 98.2%, and 17.2% of the isolates, respectively. The WGS-subjected isolates ($n = 9$) formed two groups. Five isolates possessed only the 1621-bp variant of *stmPr1*, higher biofilm formation ability (Optical Density at $\lambda = 550$ nm (OD_{550}): 1.253–1.789), as well as a low number of mutations in the protease genes and *smf-1*. Three other isolates had only the 868-bp variant, weaker biofilm production (OD_{550}: 0.788–1.108), and higher number of mutations within these genes. The only weak biofilm producer ($OD_{550} = 0.177$) had no *stmPr1* alleles. In conclusion, the similar PCR detection rates did not allow differentiation of the isolates. In contrast, WGS permitted *stmPr1* alleles-based differentiation. To the best of our knowledge, this is the first Bulgarian study presenting genotypic and phenotypic insights into virulence factors of *S. maltophilia* isolates.

KEYWORDS

Stenotrophomonas maltophilia, virulence genes, PCR screening, enzymatic assays, whole-genome sequencing, mutations

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INTRODUCTION

Stenotrophomonas maltophilia is a glucose non-fermenting, Gram-negative bacterium, widespread in many environmental sources. It is increasingly recognized as a multidrug-resistant

opportunistic pathogen responsible for a wide array of healthcare-associated infections in intensive care unit patients, life-threatening diseases in immunocompromised patients with malignancies, and chronic pulmonary infections in individuals with cystic fibrosis (CF) [1–4]. Moreover, *S. maltophilia* has established itself as one of the most common pathogens causing severe bacterial superinfections, including ventilator-associated pneumonia and bloodstream infections, in critically ill Coronavirus disease 2019 (COVID-19) patients [5, 6].

The *S. maltophilia* genome encodes various extracellular enzymes which have a key role in the pathogenesis of infections. Also, the biofilm formation on abiotic surfaces and host tissues is a prominent feature of the organism's virulence. This ability ensures higher resistance to many antibiotics and antiseptic solutions counteracting the host defence mechanisms, and contributes to the progression of CF lung disease and other chronic respiratory diseases [7, 8]. *S. maltophilia* virulence factors, as well as cellular and molecular pathogenic mechanisms have not yet been elucidated in detail, although they have been the subject of intensive research in recent years. Investigation of the virulence genes is of particular importance for expanding the fundamental knowledge about the pathogenesis of infections caused by *S. maltophilia*, as well as for development of new therapeutic approaches [7].

The aim of the present study was to explore the main virulence characteristics in Bulgarian nosocomial *S. maltophilia* isolates (2011–2022) via screening for the presence of virulence genes, determining their mutational variability, and the corresponding enzyme activity. The work follows on from a study recently published in this journal that focused on another important trait of the isolates' virulence, their biofilm-forming ability [9].

MATERIALS AND METHODS

Bacterial strains

A total of 221 non-duplicate nosocomial *S. maltophilia* isolates were included. The isolates were collected during an 11-year period (2011–2022) from inpatients aged 1–94 years in six university hospitals in Sofia, Bulgaria. They were obtained from various sources, including 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.

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 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 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 (Forward primer: 5′–GCTGGATTGGTTCTAGGAAAACGC–3′ and Reverse primer: 5′–ACGCAGTCACTCCTTGCG–3′) and amplification conditions described previously [10]. The identification of nine selected isolates was further confirmed by analyzing the assembled draft genome sequence using the Microbial Genomes Atlas (MiGA) Web server [11]. The included workflow for the NCBI Genome Database, Prokaryotic section was followed with default settings.

PCR-based screening for virulence determinants

PCR was performed to detect the presence of the following virulence determinants: *stmPr1* with two allelic variants (868-bp and 1621-bp sized, respectively) encoding for the major extracellular protease StmPr1, *stmPr2* (minor extracellular protease StmPr2), Smlt3773 locus (outer membrane esterase), *plcN1* (non-hemolytic phospholipase C), and *smf-1* (type-1 fimbriae, biofilm-related gene). Oligonucleotides used as primers for amplification [12] are listed in Table 1. DNA was amplified using the following protocol: initial denaturation at 95 °C for 5 min; followed by 28–30 cycles of denaturation at 95 °C for 35–45 s, annealing at 64–68.5 °C from 45 to 50 s and extension at 72 °C from 45 to 90 s; and a single final extension at 72 °C for 7 min. PCR products were separated in 1.5% agarose gel for 90 min at 80 V, stained with SimplySafe (0.05 µL mL^{-1}) (EURx, Gdansk, Poland) and detected by ultraviolet light (wavelength 312 nm). Amplified genes were identified on the basis of fragment size (Table 1).

Enzymatic assays

All *S. maltophilia* isolates were grown in Luria Bertani broth (Liofilchem, Roseto degli Abruzzi, Italy) to OD_{600} = 1. Their extracellular enzyme activities were evaluated by plating 2 µL culture spots [12] onto specific agar plates.

Protease. Protease activity was determined using a casein hydrolysis on Mueller–Hinton agar containing 3% (w/v) skimmed milk (Oxoid, Hampshire, UK). The presence of a transparent zone around the inoculum spot after incubation at 37 °C for 24 h indicated a positive test [13].

Esterase. Esterase activity was assessed using Tributyrin Agar plates supplemented with tributyrin-Arabic gum (HiMedia, Thane, India) to a final concentration of 1%. The presence of a clear halo around the bacterial spot after overnight incubation at 37 °C was considered as a sign of esterase production [12].

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

Primer pair	Target[a]	Sequence (5′ – 3′)	Product size (bp)	Ta (°C)	Source
Pr1-F[b]	*stmPr1* (short allelic variant)	CGAGAACGACAACGAGTGCTACA	868	67	[6]
Pr1-R		CACGGCGGTCTTGTTGGTCA			
Pr1a-F[c]	*stmPr1* (long allelic variant)	GCCGCAGTGTTGTTCGATCCA	1621	68	[6]
Pr1a-R		CAGTTCTCGGTGCACGGCTCTT			
Pr2-F	*stmPr2*	GCCGATTCCGGCATTCACACC	1764	68.5	[6]
Pr2-R		GGTCAGGCCCGAGAAGGTGCT			
Lp1-F	Smlt3773	CGGTGCCGAACTCGTAACCGG	1342	68.5	[6]
Lp2-R		CTTCCGGCCATGGCAGGCGAA			
PlcN-F[d]	*plcN1*	GGCGATGATGAAGCTGACCT	174	59	This study
PlcN-R		GACAACTTCACCGACAACCC			
Fm3-F	*smf-1*	GGAAGGTATGTCCGAGTCCG	674	64	[6]
Fm2-R		GCGGGTACGGCTACGATCAGTT			

Ta, annealing temperature; F, forward primer; R, reverse primer.
[a]Genes and loci encoding for: *stmPr1*, major extracellular protease StmPr1; *stmPr2*, minor extracellular protease StmPr2; Smlt3773, outer membrane esterase; *plcN1*, non-hemolytic phospholipase C; *smf-1*, type-1 fimbriae.
[b]Primers designed on the basis of the *stmPr1* sequence, accession No. AJ291488.
[c]Primers designed on the basis of the *S. maltophilia* K279a genome sequence, accession No. AM743169.
[d]Primers designed using Primer-BLAST on the basis of the *S. maltophilia* K279a genome sequence, accession No. AM743169.

Lecithinase. Non-hemolytic phospholipase C hydrolyzes phosphatidylcholine [14], which is the main component of egg yolk lecithin [15]. Lecithinase activity of the studied *S. maltophilia* isolates was evaluated on Egg Yolk Agar plates supplemented with 10% Egg Yolk emulsion (HiMedia). The presence of a white precipitate around the inoculum spot after incubation at 37 °C for 72 h was taken as a positive test [16].

Microtiter plate assay for biofilm quantification

Microtiter plate crystal violet assay for biofilm quantification was performed as described previously [9]. Absorbance was measured at 550 nm wavelength (Optical density at $\lambda = 550$ nm – OD_{550}). The low cut-off (ODc) was calculated as the three standard deviations (3×SD) 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 ≤ 2×ODc), moderate biofilm producer (2×ODc < OD ≤ 4×ODc), and strong biofilm producer (4×ODc < OD) [9, 17].

Whole-genome sequencing (WGS)

Nine selected *S. maltophilia* isolates from various sources (LRT samples, wounds, medical devices, and hospital environment) differing in the amounts of biofilm formed on a polystyrene surface and demonstrating different *stmPr1*-genotypes, were subjected to WGS in order to investigate in detail their virulence genes. It was performed using DNA nanoball sequencing technology as previously described [9, 18]. In brief, genomic DNA was randomly fragmented using a Covaris g-TUBE device, and size-selected by magnetic beads to an average fragment length of 200–400 bp. The resulting fragments were end-repaired, 3′-adenylated, ligated to adapters, and PCR-amplified to generate sequencing libraries from each sample. These libraries were sequenced using an MGISEQ-2000 platform (BGI Group, Hong Kong, China), with 2 × 150 bp paired-end reads being generated.

Draft genome assembly

Quality control, raw read preprocessing and draft genome assembly were performed using tools integrated within the Galaxy online platform as follows: FastQC v0.11.9, Trimmomatic v0.38, and SPAdes v3.12.0 [19]. Default parameters were used for all programs.

Multilocus sequence typing (MLST) analysis

MLST analysis was done on the assembled draft genome sequences using the MLST tool (Galaxy Version 2.19.0, https://usegalaxy.eu/). Classification of the isolates into sequence types (STs) was done according to the MLST scheme of *S. maltophilia*. This scheme includes the identification of the conserved regions of seven housekeeping genes: *atpD* (H (+)-transporting two-sector ATPase), *gapA* (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase), *guaA* (GMP synthase [glutamine-hydrolyzing]), *mutM* (DNA-formamidopyrimidine glycosylase), *nuoD* (NADH dehydrogenase [ubiquinone]), *ppsA* (Pyruvate, water dikinase), and *recA* (RecA protein) [20].

Statistical analysis

Statistical analysis was done using Excel (Microsoft Office 365). The distribution of the major protease StmPr1 genotypes according to the isolates' origin as well as all comparisons between our results and recent ones reported by other authors were performed using the Student's *t*-test. For simple comparison test, a *P*-value below 0.05 was considered statistically significant. To counteract the problem of multiple comparisons, when used, a Bonferroni correction was applied.

RESULTS

Screening for virulence determinants

The overall incidence of virulence determinants was as follows: *stmPr1* 87.3%, *stmPr2* 99.1%, Smlt3773 98.2%, *plcN1* 99.1%, and *smf-1* 96.4%. The distribution of *stmPr1* allelic variants according to the isolates' origin is presented in Table 2. In general, the long allelic variant was most frequently found (61.1%), followed by the combined 868-bp+1621-bp allelic variant (17.6%) and the *stmPr1*-negative genotype (12.7%). The short variant was detected in a small number of isolates (8.6%). The 1621-bp allelic variant significantly predominated ($P < 0.001$) over all other *stmPr1* genotypes (868-bp variant, combined and gene absent) among the LRT and wound isolates. The combined allelic genotype outweighed ($P < 0.001$–0.01) the short as well as the *stmPr1*-negative genotype in the URT and blood isolates. Moreover, its prevalence (71.4%) was higher ($P < 0.001$) compared to all other genotypes in the COVID-19 group, where *stmPr1*-negative isolates were not found.

Table 2. Major protease StmPr1 genotype distribution according to the isolates' origin

Origin (N)	*stmPr1* (868 bp) Number (%)	*stmPr1* (1621 bp) Number (%)	Both variants Number (%)	No gene Number (%)
Clinical specimens				
LRT (120)	11 (9.2)	83 (69.2)	14 (11.7)	12 (10)
Wound (27)	5 (18.5)	15 (55.6)	2 (7.4)	5 (18.5)
URT (26)	1 (3.8)	9 (34.6)	14 (53.8)	2 (7.7)
Blood (17)	1 (5.9)	7 (41.2)	7 (41.2)	2 (11.8)
Urine (11)	1 (9.1)	5 (45.5)	1 (9.1)	4 (36.4)
Miscellaneous[a] (20)	0 (0)	16 (80)	1 (5)	3 (15)
COVID-19 infection				
COVID-19 (35)	1 (2.9)	9 (25.7)	25 (71.4)	0 (0)
Non-COVID-19 (186)	18 (9.7)	126 (67.7)	14 (7.5)	28 (15.1)
Total (221)	19 (8.6)	135 (61.1)	39 (17.6)	28 (12.7)

LRT, lower respiratory tract; URT, upper respiratory tract.
[a]Isolates obtained from: medical devices ($n = 9$), hospital environment ($n = 4$), feces ($n = 3$), ascites ($n = 2$), bile ($n = 1$), and cerebrospinal fluid ($n = 1$).

Phenotypic virulence features

Detection of extracellular enzyme activity. In accordance with the investigated virulence determinants encoding proteases, outer membrane esterase and non-hemolytic phospholipase C, the corresponding enzyme activities were determined. The distribution of extracellular enzyme-producing *S. maltophilia* isolates of various origins is showcased in Table 3. Protease and esterase activity was observed in 95% and 98.2% of the studied *S. maltophilia* isolates, respectively. In contrast, lecithinase production was detected in only 17.2% ($n = 38$) of them. The prevalence of enzyme production showed a slight variability according to the origin with exception of the urine isolates. They demonstrated the lowest frequencies (72.7% and 90.9% with protease and esterase activity, respectively, as well as an absolute absence of lecithinase activity).

Biofilm-forming ability on a polystyrene surface. Consistent with the observed high frequency of the biofilm-associated *smf-1* gene (96.4%), all *S. maltophilia* isolates were characterized as strong biofilm producers (OD_{550} from 0.224 ± 0.049 to 2.065 ± 0.023), excluding one from LRT (a weak producer with $OD_{550} = 0.177 \pm 0.024$). The phenotypic and genotypic characteristics of the biofilms formed by the investigated isolates were detailed in a recent study published in this journal [9].

Relations of the virulence genotypes and the corresponding enzyme activities

The relation of the protease (StmPr1 and StmPr2) genotype and the demonstrated protease activity is detailed in Fig. 1. The most common genotype among *S. maltophilia* isolates with protease activity (total 95%) was "*stmPr1* (l621 bp) + *stmPr2*", representing 59.7% of all isolates tested. The "*stmPr1* (868 bp) + *stmPr1* (1621 bp) + *stmPr2*" genotype was found in 39 of the protease-producing isolates (17.6% of all isolates). The predominant number of protease non-producers (6 out of 11) harbored only *stmPr2*.

Complete concordance between genotype and phenotype was detected regarding esterase: Smlt3773 locus (+)/enzyme activity (+) (98.2% of the isolates) and Smlt3773 locus (−)/no enzyme activity (1.8%).

Table 3. Enzyme activity of the studied *S. maltophilia* isolates from different sources

Number of positive isolates (Percentages)							
Enzyme activity	LRT ($n = 120$)	Wound ($n = 27$)	URT ($n = 26$)	Blood ($n = 17$)	Urine ($n = 11$)	Miscellaneous[a] ($n = 20$)	Total ($n = 221$)
Protease	117 (97.5)	25 (92.6)	25 (96.2)	16 (94.1)	8 (72.7)	19 (95)	210 (95.1)
Esterase	120 (100)	27 (100)	26 (100)	17 (100)	10 (90.9)	19 (95)	219 (99.1)
Lecithinase	22 (18.3)	6 (22.2)	5 (19.2)	3 (17.6)	0	2 (10)	38 (17.2)

LRT, lower respiratory tract; URT, upper respiratory tract.
[a]Isolates obtained from: medical devices ($n = 9$), hospital environment ($n = 4$), feces ($n = 3$), ascites ($n = 2$), bile ($n = 1$), and cerebrospinal fluid ($n = 1$).

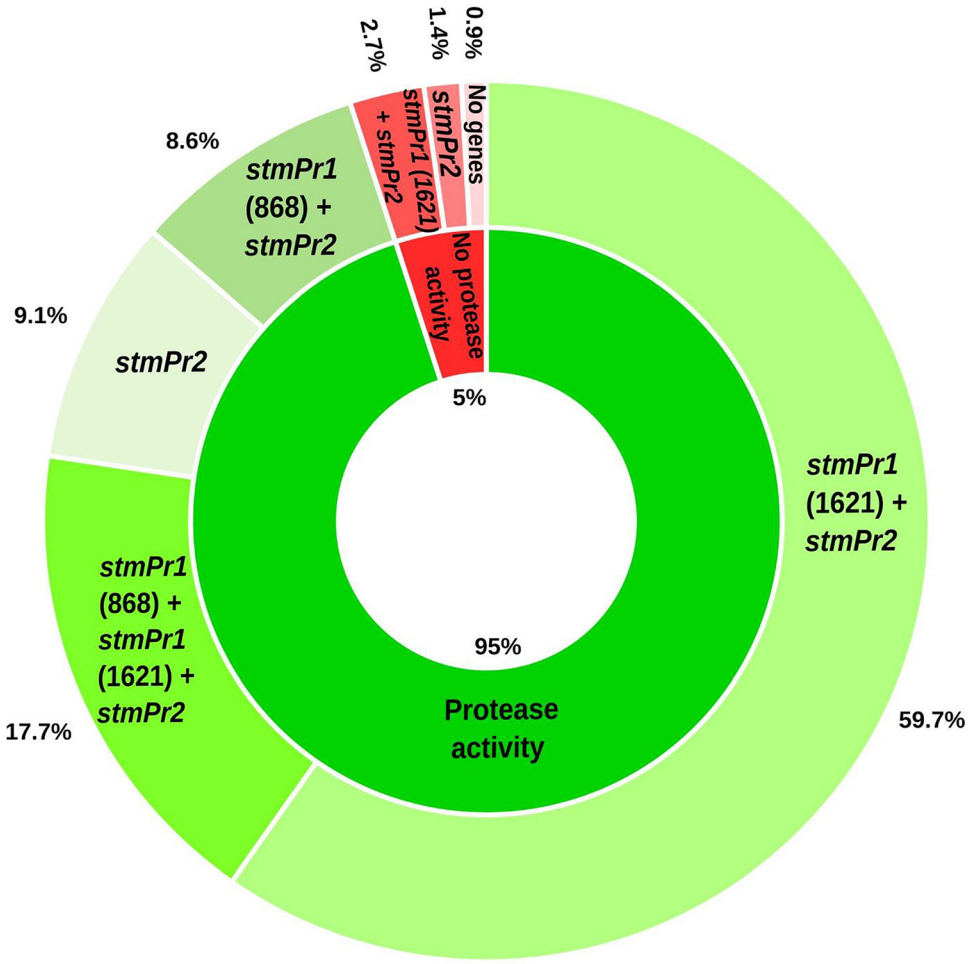

Fig. 1. Relation of the SmltPr1/2 genotypes and the protease activity in 221 nosocomial *S. maltophilia* isolates

All *S. maltophilia* isolates that demonstrated lecithinase activity (17.2%) were *plcN1*-positive. Also, almost all isolates without lecithinase activity contained the gene (Fig. 2).

Draft genome assemblies: evaluation and comparison

The nine assembled draft genomes varied in size between 4.38 and 4.99 Mbps and had an estimated GC% content between 65.80% and 66.78%. The isolates were found to belong to seven STs, as follows: ST27, ST119, ST139, ST172 (two *S. maltophilia* isolates), ST819, ST820 (two isolates), and ST826.

Identifying missense mutations in the studied virulence genes

The genomes were screened for mutations affecting the virulence genes selected. All identified amino acid (AA) exchanges were divided as conservative and non-conservative substitutions. Their numbers are shown in Table 4, and the complete list is presented in Table 5. The lowest variation was observed within the *smf-1* gene. In contrast, the 868-bp allelic variant of *stmPr1* showed the highest diversity. As can be seen from the table, the isolates formed two groups regarding the *stmPr1* gene. Five of them (SM49, SM64, SM79, SM105, and SM130) possessed only the 1621-bp allele. They demonstrated higher biofilm formation ability (OD_{550}: 1.253–1.789) and had a low number of mutations in the protease genes and *smf-1*. In contrast, three other isolates (SM8, SM62, and SM148) had only the 868-bp variant, weaker biofilm production (OD_{550}: 0.788–1.108), as well as higher number of mutations within *stmPr1*, *stmPr2* and *smf-1*. The only weak biofilm producer (SM135, OD_{550} = 0.177) had no *stmPr1* alleles. The mutations in Smlt3773 and *plcN1* were evenly distributed among the groups of isolates. As shown in Table 4, all WGS-subjected *S. maltophilia* isolates could produce detectable protease regardless of the genotype present. Also, no association was found between virulome and phenotypic expression of esterase and lecithinase.

DISCUSSION

A high prevalence (87.3%–99.1%) of all virulence determinants was found among our isolates. These data were close to recent results for clinical *S. maltophilia* isolates from Europe and South Korea [21, 22]. On the other hand, the spread rates of protease genes (*stmPr1* 87.3% and *stmPr2*

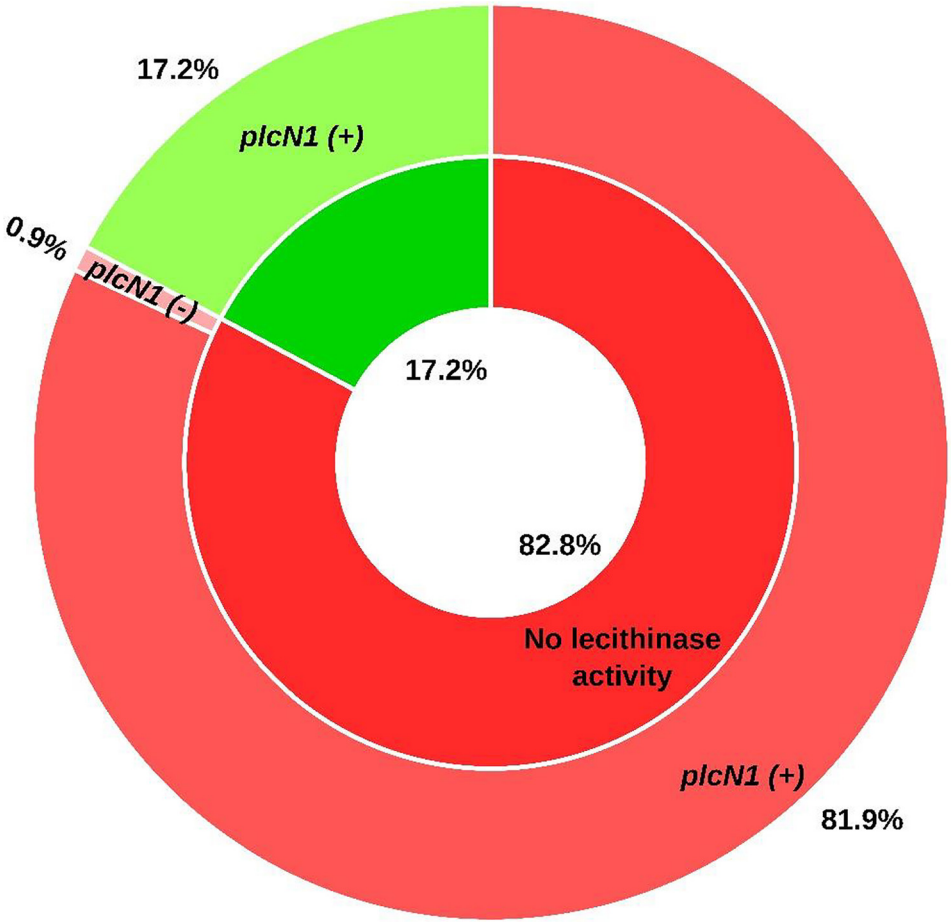

Fig. 2. Relation of the *plcN1* genotypes and the lecithinase activity in 221 nosocomial *S. maltophilia* isolates

Table 4. Number of amino acid substitutions found in the coding sequences of virulence genes in selected *S. maltophilia* isolates, biofilm production on a polystyrene surface and enzymatic activities

Isolate No (source)	ST	*stmPr1* (868 bp) 617 AA	*stmPr1* (1621 bp) 630 AA	*stmPr2* 580 AA	Smlt3773 611 AA	*plcN1* 707 AA	*smf-1* 169 AA	Biofilm OD_{550} ± SD	Enzymatic activity		
									Protease	Esterase	Lecithinase
SM8 (sputum)	820	32/26[a]	No allele	1/0	0/0	0/1	3/1	1.108 ± 0.143	+	+	–
SM49 (wound)	172	No allele	3/0	0/1	1/0	11/16	0/4	1.253 ± 0.191	+	+	–
SM62 (wound)	826	8/5	No allele	16/10	15/32	18/24	2/2	0.788 ± 0.098	+	+	+
SM64 (wound)	27	No allele	0/0	0/0	0/2	4/5	0/0	1.576 ± 0.150	+	+	–
SM79 (gastroscope)	820	No allele	2/0	1/0	0/0	0/1	0/0	1.391 ± 0.116	+	+	–
SM105 (drainage)	139	No allele	1/0	0/0	0/0	5/3	0/0	1.789 ± 0.171	+	+	+
SM130 (TBA)	119	No allele	2/0	1/0	10/25	5/9	0/4	1.430 ± 0.125	+	+	–
SM135 (BAL)	172	No allele	No allele	0/1	1/2	9/14	4/4	0.177 ± 0.024	+	+	–
SM148 (TBA)	819	28/25	No allele	11/14	32/48	14/28	5/6	0.989 ± 0.033	+	+	–

ST, sequence type determined by the MLST tool (Galaxy Version 2.19.0); AA, amino acid; OD_{550}, optical density read at $\lambda = 550$ nm (microtiter plate assay); SD, standard deviation; TBA, tracheobronchial aspirate; BAL, bronchoalveolar lavage.
[a]Number of conservative (left)/non-conservative (right) amino acid substitutions calculated by BLASTP algorithm against the equivalent sequences of the *S. maltophilia* K279a strain.

99.1%) were significantly higher than those found among nosocomial *S. maltophila* isolates in Mexico (*stmPr1* 50%; $P < 0.001$) [23] and Egypt (*stmPr2* 75%; $P < 0.05$) [24]. The dominant frequency of the long allele of *stmPr1* reported in earlier investigations [12, 24] was confirmed by us. StmPr1 genotype distribution in our LRT isolates was very similar to that found in CF-LRT isolates in Italy (2003–2005): 69.8% (long allelic variant), 11.6% (both variants), 11.6% (no

Table 5. List of all amino acid (AA) substitutions found in the coding sequences of virulence genes in the *S. maltophilia* isolates that were subjected to whole-genome sequencing

Isolate No	Gene (Coding sequence)	
	Conservative substitutions	Non-conservative substitutions
stmPr1 – short allelic variant (617 AA)		
SM8	pD47E, pS57A, pA80S, pA84S, pS87N, pV103L, pL106V, pK123Q, pA127S, pS140T, pY157F, pS176A, pV186I, pL191I, pV248L, pH278Y, pI302V, pA311S, pV313I, pV322I, pS332A, pD371E, pS393T, pV414I, pS417T, pD420N, pS429T, pS483A, pV495L, pI564L, pS573T, pK578R	pA41T, pT46A, pG61S, pG98A, pV99T, pA152V, pY161N, pT169V, pA222P, pT245N, pT303V, pA317T, pD334G, pG335S, pY430P, pS431G, pA465T, pT486A, pT524A, pT532A, pG534A, pA544V, pG577A, pN591T, pK592A, pV594A
SM62	pQ43E, pS57T, pA80S, pV103L, pA127S, pS140T, pD249E, pS393T	pA41T, pD47Q, pA84T, pT88A, pT524A
SM148	pD47E, pS57A, pA80S, pA84S, pR96K, pV103L, pL106V, pA127S, pS140T, pD146N, pY157F, pS176A, pV186I, pL191I, pV248L, pH278Y, pI302V, pV313I, pV322I, pS332A, pD371E, pS393T, pV414I, pS417T, pD420N, pS429T, pS483A, pV495L	pA41T, pG61T, pG98S, pV99T, pA152V, pY161N, pT169V, pA222P, pT245D, pT303V, pA317T, pS330G, pD334A, pG335S, pT359A, pR366Q, pY430P, pS431G, pA465T, pT486A, pT524A, pT532A, pG534A, pA544V, pG577A
stmPr1 – long allelic variant (630 AA)		
SM49	pK96R, pI136V, pV195I	No substitutions
SM64	pS35N	No substitutions
SM79	pE207Q, pV195I	No substitutions
SM105	pV67L	No substitutions
SM130	pK96R, pV195I	No substitutions
stmPr2 (580 AA)		
SM8	pQ89E	No substitutions
SM49	No substitutions	pS293G
SM62	pK101R, pI159V, pR160Q, pA166S, pT401S, pA449S, pA466S, pA494S, pT495S, pA497S, pN500S, pT510S, pS520T, pV527I, pI553V, pL574V	pA59P, pT72N, pG156S, pA184P, pA188G, pN474T, pG492S, pE498S, pA504N, pT537V
SM64	No substitutions	No substitutions
SM79	pQ89E	No substitutions
SM105	No substitutions	No substitutions
SM130	pS227A	No substitutions
SM135	No substitutions	pS293G
SM148	pT72S, pS227A, pT291S, pT392S, pT401S, pN462D, pT495S, pN500S, pV527L, pS548A,pI553V	pP44Q, pA59P, pL113Q, pG207N, pA222D, pG226Q, pI229Y, pA231G, pT375N, pS385G, pN474_, pE498S, pA504N, pT537V
Smlt3773 locus (611 AA)		
SM8	No substitutions	No substitutions
SM49	pN89S	No substitutions
SM62	pT10S, pR55Q, pS70A, pN89S, pM108L, pN187D, pL190I, pT231S, pV232I, pK254R, pL265I, pL527V, pL533V, pD568N, pT610S	pL2Q, pR6H, pP51D, pA52P, pD53G, pA82G, pG101S, pV104E, pP105A, pS106G, pA107G, pT110V, pA119T, pV133A, pA148T, pA149R, pA150AAAGGAS, pP151Q, pA154V, pA156G, pA193Q, pT201A, pG234R, pA237T, pA241G, pA258N, pQ262A, pT266G, pA307G, pV380F, pG545A, pK564N
SM64	No substitutions	pA152T, pT462A
SM79	No substitutions	No substitutions
SM105	No substitutions	No substitutions
SM130	pR55Q, pM108L, pN187D, pL190I, pT231S, pV232I, pN239S, pL265I, pV420I, pT610S	pP51D, pA52P, pD53G, pA82G, pN89K, pG101S, pV104E, pP105A, pS106G, pA107G, pT110A, pV133A, pA148T, pA149R, pA150AAAGGAS, pP151Q, pA154V, pA156G, pA193Q, pT201A, pG234R, pA237T, pA258N, pQ262A, pT266G
SM135	pN89S	pP51D, pT110A
SM148	pT10S, pR55Q, pV57L, pT58S, pD103E, pM108L, pN187D, pL190I, pL205M, pT231S, pV232I, pD256N, pL265I, pS271T, pI321V, pL363V, pI371V, pD376N, pM378V, pL389M, pN390S, pI418V, pL498M, pI516V,	pA52E, pD53A, pP56G, pG81K, pA82D, pA83P, pG101S, pV104G, pP105G, pS106G, pT110A, pG131R, pV133A, pA149G, pA150AGAGGAS, pA154V, pA156G, pA193Q, pT201A, pA207V, pG234R, pA237T,

(continued)

Table 5. Continued

Isolate No	Gene (Coding sequence)	
	Conservative substitutions	Non-conservative substitutions
	pL527V, pL533V, pD568N, pE579D, pS592T, pQ599R, pL603I, pT610S	pA241G, pS250G, pQ262G, pT266G, pA278Y, pN279Q, pT291V, pA292T, pM331N, pG335A, pG412A, pL429S, pA494T, pA495V, pA531V, pG545A, pK564D, pV572A, pT578M, pS585G, pF587V, pA591T, pG594A, pN606T, pF607V, pS608G
plcN1 (707 AA)		
SM8	No substitutions	pS378I
SM49	pA23S, pS83A, pY229H, pR263Q, pY265H, pL293I, pV367L, pS450T, pT461S, pM526I, pA539S	pA90T, pT102A, pA129P, pR199H, pH217Q, pA260V, pS266R, pP286A, pA294G, pD368G, pG369T, pA396L, pG462N, pV510S, pA569P, pD580G
SM62	pA23S, pS83A, pM160L, pR199Q, pE337D, pD368N, pT461S, pV481M, pA539S, pQ597K, pL605V, pD643E, pE653Q, pA655S, pQ670R, pI671V, pM677V, pS698T	pQ40L, pA90T, pA129P, pK234N, pG279A, pA294D, pG462N, pV510S, pA537T, pM568R, pA569P, pD580G, pK601T, pD602G, pT612V, pG614D, pG626S, pQ633H, pP634G, pA645T, pV647A, pG672S, pE675G, pQ706H
SM64	pR199Q, pT461S, pV512I, pA539S	pA294D, pA445P, pG462N, pA490G, pD580G
SM79	No substitutions	pS378I
SM105	pS83A, pR199Q, pE509D, pV512I, pA539S	pA129P, pA294D, pD580G
SM130	pS83A, pR199Q, pT461S, pV512I, pA539S	pA39T, pA129P, pP286S, pA294D, pG462N, pG493R, pG567R, pA569P, pD580G
SM135	pS83A, pY229H, pR263Q, pY265H, pV367L, pS450T, pT461S, pM526I, pA539S	pA90T, pT102A, pR199H, pH217Q, pA260V, pS266R, pP286A, pA294G, pD368G, pG369T, pA396L, pG462N, pV510S, pA569P
SM148	pA23S, pS83A, pM160L, pR263Q, pY265H, pL293I, pV367L, pT461S, pM526I, pA539S, pL605V, pD643E, pI671V, pE675D	pA82P, pA90T, pT102A, pA129P, pH142Q, pR199H, pH217Q, pA260V, pS266R, pP286A, pA294G, pA308T, pD368G, pG369T, pA396L, pG462N, pV510S, pA569P, pD580G, pK601T, pD602G, pI609T, pT612A, pG626S, pA645T, pQ670L, pM677A, pQ706H
smf-1 (169 AA)		
SM8	pT87S, pL91V, pQ112E	pV113F
SM49	No substitutions	pN19T, pT51A, pA54N, pL123Q
SM62	pT87S, pL91V	pN40T, pV113F
SM64	No substitutions	No substitutions
SM79	No substitutions	No substitutions
SM105	No substitutions	No substitutions
SM130	No substitutions	pN19T, pT51A, pA54N, pL123Q
SM135	pT66S, pT87S, pL91V, pQ112E	pN19T, pT51A, pA54N, pL123Q
SM148	pT66S, pT87S, pL91V, pS138T, pD140N	pQ112T, pV113F, pA121T, pT126D, pG134A, pT135G

Amino acid substitutions were calculated by BLASTP algorithm against the equivalent sequences of the *S. maltophilia* K279a strain.

stmPr1 gene), and 7% (short variant) [12]. A recent study on bloodstream *S. maltophilia* isolates in South Korea reported similar values (48.38% and 45.16% for the combined *stmPr1* genotype and the 1621-bp variant, respectively) to ours regarding the blood isolates. Both 868-bp allele and gene absent were found in 3.22% [22].

The *plcN1* frequency established by us was higher than that detected among *S. maltophilia* isolates from patients with respiratory and septicemia infections in Iraq (2016–2020) – 99.1% vs. 84%; $P < 0.001$ [25]. Similar results were also found for the *smf-1* gene in our isolates (96.4%) compared to its prevalence among older clinical isolates from Brazil (23%; $P < 0.001$) [26]. Also, in contrast to ours, most Brazilian isolates were weak and moderate biofilm producers. A significant association was recently reported between *smf-1* and biofilm-forming ability of *S. maltophilia* [27].

In the study of extracellular enzyme activity, most of the isolates were able to produce protease (95%) and esterase (98.2%), whereas lecithinase activity was found in a lower proportion of our *S. maltophilia* collection (17.2%).

The production of proteases plays a key role in bacterial pathogenesis, participating of invasion, tissue damage and host-defence evasion [7, 28]. A high incidence of protease-producing clinical isolates of *S. maltophilia* has been reported in previous studies in Italy (90.7%) [12], Thailand (91.5%) [29], Brazil (100%) [30], and Malaysia (100%) [31]. The small differences observed are probably dependent on variations in media and methods (qualitative or quantitative) used, or are related to the exoenzyme potential of the tested *S. maltophilia* isolates.

The *stmPr2* gene was found in nearly all our isolates (219/221) regardless of their protease production. In contrast,

the *stmPr1* alleles were predominantly detected in isolates with enzyme activity (90.5%) compared to those without such activity (27.3%). These results support the StmPr1 role as a major extracellular protease which was previously described in a toxicity model in *Galleria mellonella* larvae [12]. It is worth mentioning that a small number of protease non-producers were characterized to be *stmPr1* (1621 bp)-positive. A plausible explanation for this outcome could be the existence of inactivating frameshift mutations within the gene as described previously [12].

Esterases are a group of hydrolytic enzymes that may be associated with the virulence of *S. maltophilia* isolates [7]. The frequency of isolates demonstrating esterase activity in our study was significantly higher than that found among clinical *S. maltophilia* isolates obtained primarily from CF patients in Italy (98.2% vs. 81.4%, $P < 0.01$) [12]. In contrast to our esterase non-producers (1.8%), which did not harbor Smlt3773 locus, the authors found isolates without esterase activity despite the presence of an encoding gene (18.6%) and explained that with detected frameshift mutations forming premature stop codons. Their analysis of amplicons of esterase-producing strains confirmed the presence of wild-type Smlt3773 locus. The results reported by *Nicoletti* et al. indicate that the outer membrane esterase-encoding locus is highly conserved and there are highly prevalent, non-functional gene variants among clinical CF *S. maltophilia* isolates.

Genes encoding for non-hemolytic phospholipase C and class D phospholipases were identified in the *S. maltophilia* K279a genome by *Crossman* et al. [32]. Bacterial phospholipases, including lecithinase, are a heterogeneous group of metalloenzymes produced by many organisms, some of which play roles in the infectious pathogenesis. As virulence factors, they are involved in destruction of protective molecules or structures such as mucin, lipoprotein membranes and immunoglobulins [7, 13]. The prevalence of lecithinase-producing clinical *S. maltophilia* isolates varies widely according to earlier studies [13, 30, 31]. *Figueirêdo* et al. established lecithinase activity only in isolates originated from trachea and liver [13]. *Thomas* et al. reported 69.4% isolates with lecithinase activity among a collection of nosocomial *S. maltophilia* isolates of various origins from Malaysia [31], a significantly higher frequency than ours (17.2%, $P < 0.001$). The authors did not detect lecithinase producers in urine isolates, which was also confirmed by us. An older Brazilian study found phospholipolytic activity in all clinical strains with variable but low amounts of enzyme (from 0.0011 to 0.49 PU) [30]. It should be mentioned that enzyme activity testing was performed with a more precise quantitative method.

Despite all the available data about the distribution rates of lecithinase activity in nosocomial *S. maltophilia* isolates of various origins and geographic locations, very little is known about the underlying genetic basics of this virulence-related feature. To provide some new insights regarding this matter, a *plcN1* genotyping was performed. The gene encodes for a non-hemolytic phospholipase C, which was found to hydrolyze the main component of egg yolk lecithin, making it a strong candidate for relatedness with lecithinase production. Our results revealed that 99.1% of the analyzed isolates were *plcN1*-positive, suggesting that this gene is ubiquitously distributed among them and does not correlate with the phenotypic expression of lecithinase activity. Two of the nine WGS-subjected isolates formed white precipitate around the inoculum spot after incubation on Egg Yolk Agar. The corresponding promoter regions of their *plcN1* genes were analyzed for the presence of common regulatory mutations and/or insertion sequences. Nothing was found, ruling out the hypothesis that a mutational upregulation of this locus can lead to development of lecithinase activity.

Overall, despite the large number of WGS-identified mutations found in the present study, no frame shifts or premature stop codons were detected within the selected virulence genes. This finding supported their role in the pathogenicity of *S. maltophilia*. Moreover, a trend was observed that strains with the short *stmPr1* allele possessed higher number of mutations in their protease-encoding genes and lower ability to form biofilms. It can be concluded that determining the protease determinants of *S. maltophilia* has a potential to also evaluate other aspects of its virulence like biofilms formation.

In conclusion, to our knowledge, this is the first Bulgarian study presenting phenotypic, genotypic, and genomic insights into virulence factors of nosocomial *S. maltophilia* isolates. The similar PCR detection rates of genes encoding for extracellular proteases, outer membrane esterase, non-hemolytic phospholipase C, and type-1 fimbriae did not allow differentiation of the isolates. In contrast, WGS permitted differentiation according to their *stmPr1* alleles. Strains harboring the long allelic variant also possessed the lowest number of mutations within both proteases and type-1 fimbriae genes, and showed the most pronounced biofilm formation. Furthermore, the phenotypic expression of enzyme activity demonstrated that the isolates irrespective of their origin, are capable of producing virulence-associated extracellular enzymes with contribution to the *S. maltophilia* infectious pathogenesis.

Conflict of interest: None to declare.

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