First Report of Serratia Species Isolated from Subterranean Cave Aquatic Environment

Michail G.[1], Reizopoulou A.[2], Vagelas I[3]

[1]Department of Ichthyology and Aquatic Environment, School of Agricultural Sciences, University of Thessaly, 38446 Fytoko Volos, Magnesia, Greece
[2]Natural History Museum, Volos, Greece
[3]Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Fytokou St., N. Ionia, GR38446 Magnesia, Greece

---

Abstract: During our studies in Greek caves with aquatic ecosystems we isolated from bats guano pile straight rod structure gram negative bacteria designated as strains Sl2, Sl3 and Sl4. Strains Sl2, Sl3 and Sl4 were able to ferment glucose (D-glucose) and other carbohydrates (i.e. D-mannitol, D-mannose) and Saccharose/Sucrose as a source of carbon and sugar. Strains have an optimal growth at 17-30° C whereas strain Sl4 were able to grow at 4° C. Strains Sl2, Sl3 and Sl4 were classified within the *Serratia liquefaciens* group by the VITEK® 2 system (bioMerieux) and were accurately identified at the species level by MALDI-TOF MS (bioMerieux). VITEK® 2 was able to identify bacteria isolates as *Serratia liquefaciens* group, whereas MALDI-TOF MS classified one as *S. proteamaculans* and the other as *S. liquefaciens*. This is the first report of *S. proteamaculans* isolation and identification from bat guano.

Keywords: *Serratia liquefaciens group, Serratia proteamaculans, subterranean aquatic environment, MALDI-TOF MS*

1. Introduction

The genus *Serratia*, which includes up to 18 species, can be found in many different habitats [1] (Garcia-Fraile et al., 2015). Strains of the genus *Serratia* have been isolated from water, soil, plants, and animals [2] (Grimont & Grimont, 2006). In mammals, *Serratia* strains have been associated with infections such as mastitis in cows, conjunctivitis in horses, septicaemia in foals, goats, and pigs, but have also been found associated with many clinically healthy individuals [2] (Grimont & Grimont 2006). *S. liquefaciens* and *S. marcescens* have been reported as opportunistic pathogens for the chiropteran species [3] (Muhldorfer et al., 2011).

Serratia liquefaciens group (Slg) consists of the species *S. liquefaciens*, *S. proteamaculans*, and *S. grimesii* [2] (Grimont & Grimont 2006). Strains of the *S. liquefaciens* group predominantly cause sepsis and bloodstream infections via contaminated clinical equipment and blood components [4] (Stock et al., 2003). In 1980 the "Approved list of Bacterial Names" listed *S. liquefaciens* and *S. proteamaculans* as separate species [5] (Skerman et al., 1980). Although probably rarely reported in clinical samples due to inability to easily discriminate between group species, *S. proteamaculans* has been shown to cause human disease [6] (Bollet et al., 1993).

The significant importance of *Serratia* species is also due to their potential antimicrobial resistance [7] (Mahlen, 2011). Specifically, β-lactam sensitivity patterns indicated that isolates of *S. liquefaciens, S. grimesii* and *S. proteamaculans* harbor chromosomal *ampC* genes. The sequence of *S. proteamaculans* strain 568 genome indicates the presence of a chromosomal *ampC* genes and several other β-lactamases [1] (Stock et al., 2003). Also, efflux pumps have not been well characterized for other *Serratia* species, but several are predicted from the genome sequence of *S. proteamaculans* strain 568 [7] (Mahlen, 2011).

Few studies have determined the actual mechanism of resistance to trimethoprim-sulfamethoxazole in *Serratia* species with one study from Greece to have found isolates with plasmid-mediated $dhfr_{II}$ genes [8] (Tsakris et al., 1992). Furthermore, quorum sensing, a cell-to-cell signaling mechanism employed by many bacteria for biofilm formation has been described for *S. proteamaculans* [9] (Van Houdt et al., 2007).

In this study, we sampled bat guano from several places in a subterranean aquatic environment which is the habitat of several bat species. From these samples, we recovered, cultured, isolated, and identified Serratia species of the *Serratia liquefaciens* group using VITEK® 2 identification system.

Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) typing we have been able to classify the isolates of *S. liquefaciens* group to *S. liquefaciens* and *S. proteamaculans*. This is the first bacterial identification from bat guano of *S. proteamaculans* strain.

2. Material and Methods

2.1 Description of site

The Malaki cave is located in central Greece (lat. 48°28′36″N, 20°29′09″E, alt. 339 m a.s.l.) is a small semi light limestones subterranean cave (Figure 1). Malaki cave environment is being separated into three zones i) a twilight zone near the entrance, ii) a middle zone with low light and variable temperature and iii) the deep zone of completely darkness and a constant temperature (17 ^{0}C), throughout the year. Cave's deep zone is associated with a small subterranean lake, and a large bat guano pile (Figure 2). In cave's deep zone the main species fauna found are the genus *Miniopterus*, *Myotis*, *Rhinolophus* [13] (Georgiakakis & Papadatou, 2015).

2.2 Bacterial Samples Collection

In this study we isolated gram negative bacteria from bats guano. For routine isolation of bacteria, NA (Nutrient Agar), Potato Dextrose Agar (PDA), and MacConkey agar (MCA, Oxoid) were used. We used quadrant steak method and agar plates were incubate at 22 ^{0}C for 2 days [1] (García-Fraile, et al., 2015).

2.3 Bacterial Identification

In this research we used VITEK® 2 and MALTI-TOF MS (bioMerieux) to identify all bacterial isolates at the species level [10] (Rodel et al., 2019).

Isolates were subjected to species identification using the VITEK® 2 system (bioMerieux) and MALDI-TOF MS (Biotyper 3.1 software) [10] (Rodel et al., 2019). A probability higher than 80 % with an acceptable profile was considered an acceptable identification within the possible identification spectrum of species or genera (taxa) by the VITEK® 2.

3. Results

3.1 Description of site and sampling points

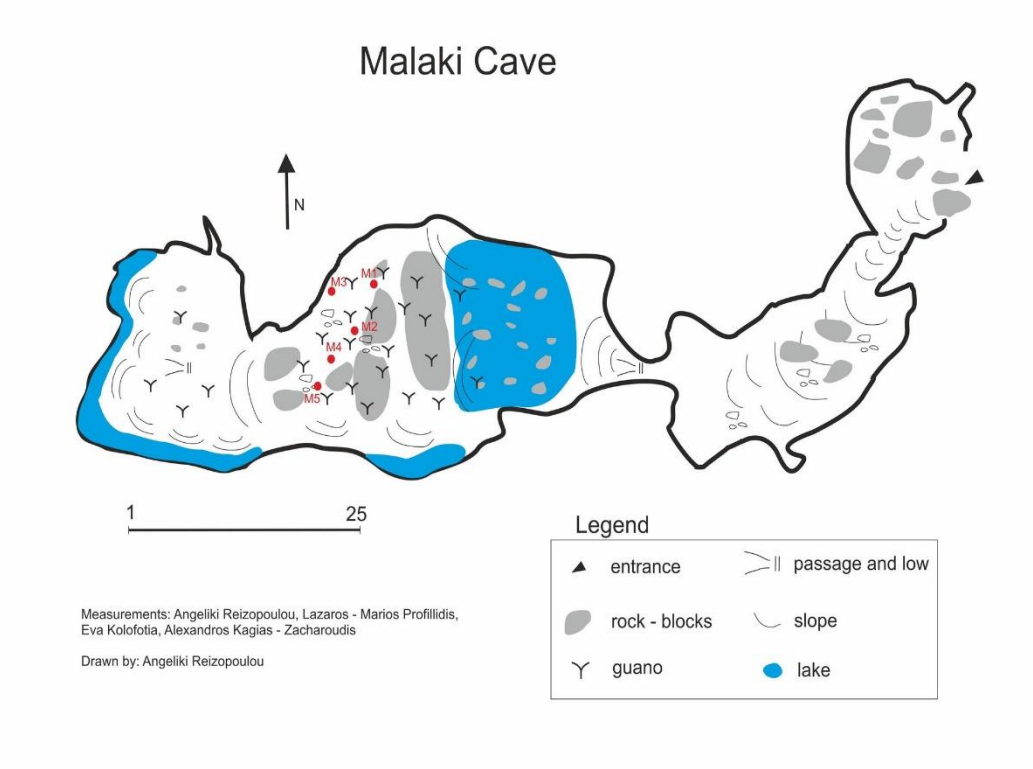

Figure 1: Cave mapping. Red dots indicate sampling sites close to indigenous bat nests. Drawn by Angeliki Reizopoulou

Figure 2: Overview of the subterranean cave lake and bat guano pile in Malaki Cave (Central, Greece). Photo Angeliki Reizopoulou

3.2 Bacterial Identification

All bacterial strains with negative straight rod morphology, capable of growth into MacConkey Agar were correctly identified to the species level (99,9% probability) by the VITEK® 2 system (bioMerieux) using VITEK 2 GN ID card (Table 1). VITEK 2 GN ID card recognised all strains (Sl2 & Sl3) as *Serratia liquefaciens* group. The direct identification reporting time of VITEK® 2 was from 4.17 h and 4.65 h for both isolates.

Further, all strains identified in VITEK 2 were identified to the species level (99,9% Confidence level) with MALDI-TOF MS system (bioMerieux) as *S. proteamaculans* and *S. liquefaciens*.

Table 1. Evaluation of VITEK® 2 GN ID Card for Rapid Identification of Gram Negative Bacterial Isolated from bats guano

Test	Mnemonic	Amount	Sl2	Sl3
Ala-Phe-Pro-ARYLAMIDASE	APPA	0.0384 mg	-	-
ADONITOL	ADO	0.1875 mg	-	-
L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018 mg	+	+
L-ARABITOL	1ARL	0.3 mg	-	-
D-CELLOBIOSE	dCEL	0.3 mg	-	-
BETA-GALACTOSI DAS E	BGAL	0.036 mg	+	+
H2S PRODUCTION	H2S	0.0024 mg	-	-
BETA-N-ACETYL-	BNAG	0.0408 mg	+	+
Glutamyl Arylamidase pNA	AG LTp	0.0324 mg	-	-
D-GLUCOSE	dGLU	0.3 mg	+	+
GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0228 mg	+	-
FERMENTATION/ GLUCOSE	OFF	0.45 mg	+	+
BETA-GLUCOSIDASE	BGLU	0.036 mg	+	+
D-MALTOSE	dMAL	0.3 mg	-	-
D-MANNITOL	dMAN	0.1875 mg	+	+
D-MANNOSE	dMNE	0.3 mg	+	+
BETA-XYLOSIDASE	BXYL	0.0324 mg	-	-
BETA-Alanine arylamidase pNA	BAlap	0.0174 mg	-	-

L-Proline ARYLAMIDASE	ProA	0.0234 mg	+	+
LIPASE	LIP	0.0192 mg	-	-
PALATINOSE	PLE	0.3 mg	+	-
Tyrosine ARYLAMIDASE	TyrA	0.0276 mg	+	+
UREASE	URE	0.15 mg	-	-
D-SORBITOL	dSOR	0.1875 mg	+	+
SACCHAROSE/SUCROSE	SAC	0.3 mg	+	+
D-TAGATOSE	dTAG	0.3 mg	-	-
D-TREHALOSE	dTRE	0.3 mg	+	+
CITRATE (SODIUM)	CIT	0.054 mg	+	+
MALONATE	MNT	0.15 mg	-	-
5-KETO-D-GLUCONATE	5KG	0.3 mg	+	+
[-LACTATE alkalinisation	1LATk	0.15 mg	+	+
ALPHA-GLUCOSIDASE	AGLU	0.036 mg	+	-
SUCCINATE alkalinisation	SUCT	0.15 mg	+	+
Beta-N-ACETYL-	NAGA	0.0306 mg	+	+
ALPHA-GALACTOSIDASE	AGAL	0.036 mg	-	+
PHOSPHATASE	PHOS	0.0504 mg	+	-
Glycine ARYLAMIDASE	GIyA	0.012 mg	-	
ORNITHINE DECARBOXYLASE	ODC	0.3 mg	+	+
LYSINE DECARBOXYLASE	LDC	0.15 mg	+	+
DECARBOXYLASE BASE	ODEC	N/A		
L-HISTIDINE assimilation	1H1Sa	0.087 mg	-	-
COUMARATE	CMT	0.126 mg	+	+
BETA-GLUCORONIDASE	BGUR	0.0378 mg	-	-
0/129 RESISTANCE (comp.vibrio.)	0129R	0.0105 mg	+	+
Glu-Gly-Arg-ARYLAMIDASE	GGAA	0.0576 mg	+	+
L-MALATE assimilation	1MLTa	0.042 mg	-	-
ELLMAN	ELLM	0.03 mg	-	-
[-LACTATE assimilation	1LATa	0.186 mg	-	-

Where: Sl2 *S. proteamaculans* and Sl3 *S. liquefaciens* strain

3.3 MALDI-TOF MS results

It is clear from the results from MALDI-TOF MS (Figure 3) that the Confidence value of 99.9% is beyond doubt evident of the presence of *S. proteamaculans* strain.

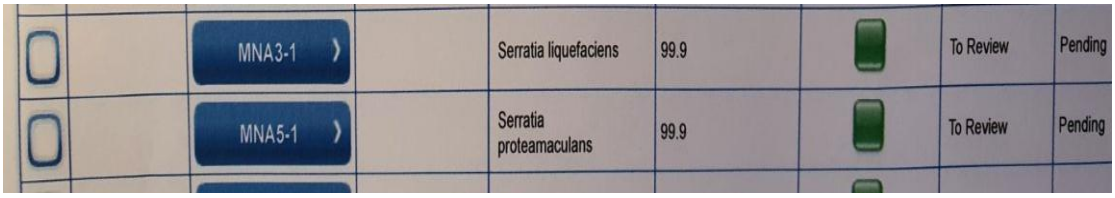

Figure 3. MALDI-TOF MS results

4. Discussion - Conclusions

It is well known that the genus Serratia can be found in many different habitats such as water, soil, plants, and animals [2] (Grimont & Grimont, 2006, [1] García-Fraile, et al., 2015). In our research, we isolated form bat guano piles similar bacterial species matched as *Serratia liquefaciens* group. *S. liquefaciens* and *S. marcescens* have been reported as opportunistic pathogens for many European bat species [3] (Muhldorfer et al., 2011) but there has been till now no evidence of *S. proteamaculans* presence in either bat guano or as a member of their bacterial microbiome.

In another recent study by Newman et al., 2018, in caves of New Mexico, USA, there was no evidence of isolation nor identification of any *Serratia* species either by culturing in TSA, blood agar, and bat guano medium (BGM) or by PCR amplification of 16s rRNA gene. Their study examined fresh and decaying bat guano even though they found different

bacterial taxa between fresh and decaying guano [11] (Newman et al., 2018). According to our results, this may represent different bacterial strains in bat microbiome and/or in guano between European [3] (Muhldorfer et al., 2011) and American [11] (Newman et al., 2018) continent indigenous bats.

The *Serratia* strains cultured and then identified by Vitek® 2 identification system, were identified as *Serratia liquefaciens* group with two phenotypic characteristics different between them as you can see in Table 1. These characteristics (Table 1) that were positive is the Gamma-Glutamyl, Palatinose, Alpha-Glucosidase and the Phosphatase biochemical reaction proved after the use of MALDI-TOF MS to be the *S. proteamaculans* strain. The *S. liquefaciens* had negative reactions to these characteristics. The use of MALDI-TOF MS can be used to identify the species between the *Serratia liquefaciens* group if necessary as it has been used before with other *Serratia* species nosocomial outbreaks [12] (Batah et al., 2015, [10] Rodel et al. 2019).

Based on the above we consider that there is a possible strong association between *Serratia* species and European continent bats as identified with VITEK 2 and MALDI-TOF MS. Furthermore, according to our data, we provide evidence of the first-time isolation of *S. proteamaculans* in bat guano.

References

[1] García-Fraile, P., Chudíčková, M., Benada, O., Pikula, J., & Kolařík, M. (2015). Serratia myotis sp. nov. and Serratia vespertilionis sp. nov., isolated from bats hibernating in caves. International Journal of Systematic and Evolutionary Microbiology, 65(Pt_1), 90–94. doi:10.1099/ijs.0.066407-0

[2] Grimont F., Grimont P. A. D. (2006). The genus Serratia. . In The Prokaryotes: a Handbook on the Biology of Bacteria, 3rd edn., vol. 6, pp. 219–244. Edited by Dworkin M., Falkow S., Rosenberg E., Schleifer K. H., Stackebrandt E. New York: Springer

[3] Mühldorfer, K., S. Speck, A. Kurth, R. Lesnik, C. Freuling, T. Müller, S. Kramer-Schadt, and G. Wibbelt, (2011): Diseases and causes of death in European bats: dynamics in disease susceptibility and infection rates. PLoS ONE **6**, e29773

[4] Stock I, Grueger T, Wiedemann B. (2003). Natural antibiotic susceptibility of strains of Serratia marcescens and the S. liquefaciens complex: S. liquefaciens sensu stricto, S. proteamaculans and S. grimesii. Int J Antimicrob Agents **22**: 35–47

[5] Skerman, V. B. D., McGowan, V. & Sneath, P. H. A. (editors) (1980). Approved lists of bacterial names. Int J Syst Bacteriol 30, 225–420

[6] Bollet C., et al. (1993). Fatal pneumonia due to Serratia proteamaculans subsp. quinovora. J. Clin. Microbiol. 31:444–445

[7] Mahlen SD. (2011). Serratia infections: from military experiments to current practice. Clin Microbiol Rev **24**: 755–791.

[8] Tsakris A., A C Vatopoulos, A P Johnson, T L Pitt, N J Legakis, L S Tzouvelekis (1992). Prevalence of a plasmid-mediated type II dihydrofolate reductase gene among trimethoprim-resistant urinary pathogens in Greek hospitals. J. Antimicrob. Chemother. 29:405–413

[9] Van Houdt R., Givskov M., Michiels C. W. (2007). Quorum sensing in Serratia. FEMS Microbiol. Rev. 31:407–424

[10] Rodel J., Mellmann A., Stein C., Alexi M., Kipp F., Edel B., et al. . (2019). Use of MALDI-TOF mass spectrometry to detect nosocomial outbreaks of Serratia marcescens and Citrobacter freundii. Eur. J. Clin. Microbiol. Infect. Dis. 38, 581–591. 10.1007/s10096-018-03462-2

[11] Newman, M. M., Kloepper, L. N., Duncan, M., McInroy, J. A., & Kloepper, J. W. (2018). Variation in Bat Guano Bacterial Community Composition With Depth. Frontiers in Microbiology, 9. doi:10.3389/fmicb.2018.00914

[12] Batah R., Loucif R., Olaitan A. O., Boutefnouchet N., Allag H., and Rolain J., Microbial Drug Resistance VOL. 21, NO. 4 | Epidemiology normal Outbreak of Serratia marcescens Coproducing ArmA and CTX-M-15 Mediated High Levels of Resistance to Aminoglycoside and Extended-Spectrum Beta-Lactamases, Algeria Published Online: 22 Jul 2015 https://doi.org/10.1089/mdr.2014.0240

[13] Georgiakakis, P. & Papadatou, E., 2015. Chiroptera, In Papamichael G., Arapis T., Petkidi K., Fytou I & Chatirvasanis V. 2015. Deliverable 7, C – Phase of Study 7: "Monitoring and evaluation of conservation status mammal species of Community interest in Greece.". Ministry of Environment, Energy and Climatic Change, Athens, scholars Partnership and consultancy firms “ARAPIS THOMAS EYAGGELOY, GEOANALYSI S.A. and PAPACHARISI ALEXANDRA THEODORA ", Athens. pp. 59 (in Greek)