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Characterization of *Serratia* species isolated from fresh vegetables using genomics

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Background

Bacteria of the genus *Serratia* (*S.*) are motile, facultatively-anaerobic and non-endospore forming, gram-negative rods. They have been isolated from various sources, including soil, water, plants, animals and even air. Some species, i.e. *S. marcescens*, *S. liquefaciens* and *S. odorifera* are considered to be opportunistic pathogens and have been associated with various human infections. Some plant-associated species of the genus *Serratia,* i.e. *S. ficaria and S. rubidaea* have been isolated from coconuts, figs, or leafy vegetables. Some diseased root isolates of *S. marcescens* were shown to be resistant to a wide variety of antimicrobial agents, including ampicillin and both second and third generation cephalosporins (1). These were also found to carry resistance genes.

Produce has recently been recognized as a potential reservoir of pathogens and of antibiotic-resistant bacteria, which can harbor transferable resistance genes. In this study, we isolated 17 *Serratia* strains from fresh produce including herbs, salads, cucumbers, carrots and mixed salads. All isolates were initially characterized using 16S rRNA gene sequencing, multi locus sequence analysis (MLSA) and rep-PCR for genomic fingerprinting. In addition, antibiotic resistance testing was done by the Kirby-Bauer disc diffusion method, and the complete genome of 7 of the 17 strains was sequenced.

Phenotypic and genotypic characteristics of *Serratia spp*.

Seventeen *Serratia* spp. strains were isolated on violet red bile dextrose agar plates from fresh produce. They are oxidase-negative, catalase-positive and grew at 10 °C. Total genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrepTM (Zymo Research) kit. Rep (repetitive extragenic palindromic) PCR was used to identify all isolates with GTG_5-primer and band profiles were analyzed using the Bionumerics (v. 7.5) software (Applied Maths). Clustering analysis was carried out with means of the Dice correlation coefficient (S_D) and UPGMA. The S_D of rep-genotyping analysis clustered at >66.7%, except for strain S16 (Fig. 1).

Strain	Oxidase	Catalase	Growth		Indole from tryptophan
			10°C	45°C	
S1	-	+	+	-	-
S2	-	+	+	-	-
S3	-	+	+	-	-
S5	-	+	+	-	-
S6	-	+	+	-	-
S7.1	-	+	+	-	-
S8	-	+	+	-	-
S9	-	+	+	-	-
S10	-	+	+	-	-
S11	-	+	+	-	-
S12	-	+	+	-	-
S13	-	+	+	-	-
S14	-	+	+	-	-
S15	-	+	+	-	-
S16	-	+	+	-	-
S17	-	+	+	-	-
S18	-	+	+	-	-

Table 1. Phenotypic characteristics of *Serratia* strains. Growth temperature determination was performed using Luria Bertani broth under aerobic conditions.

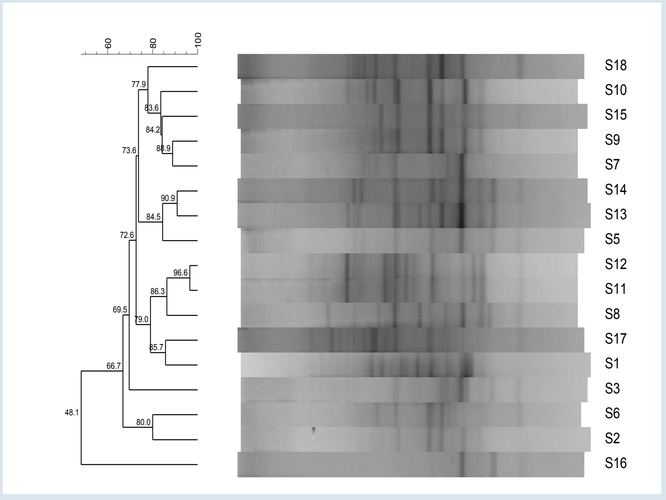


Figure 1. Dendrogram obtained by UPGMA using Dice correlation coefficient (S_D) of digitized $(GTG)_5$-PCR fingerprints with *Serratia* strains isolated from fresh produce.

Antibiotic resistance testing

The antibiotic discs used in this study included ampicillin (AMP, 10 µg), cefepime (FEP, 30 µg), cefotaxime (CTX, 5 µg), cefoxitin (30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), meropenem (MEM, 10 µg), oxacillin (OX, 5 µg), streptomycin (S, 10 µg), tetracycline (TET 30 µg) and tobramycin (TOB, 10 µg). The inhibition zones were measured and the isolates were grouped into the categories susceptible, intermediate or resistant according to CLSI criteria (Fig 3).

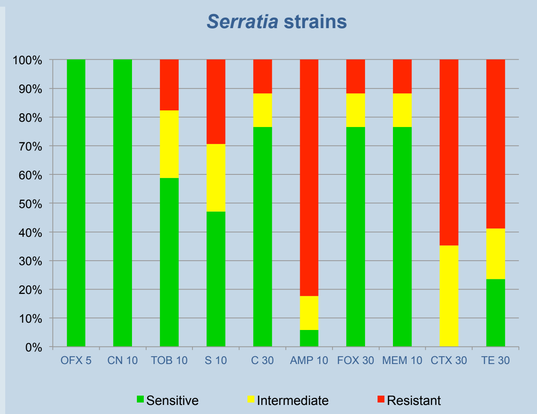
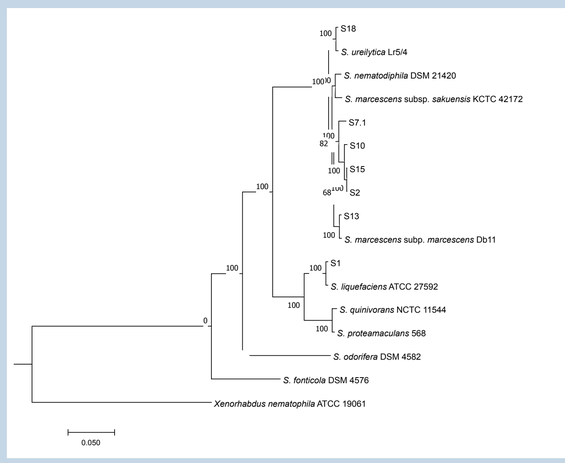


Figure 3. Antibiotic resistance profile of *Serratia* strains based on inhibition zone diameters (mm) according to CLSI (2018)

Whole genome sequencing

The genomic DNA of 7 selected strains were sequenced. The library was prepared with an Illumina Nextera XT library prep kit (Illumina) and genome sequencing was done with an Illumina MiSeq (paired-end, 2 x 250 bp reads). Raw sequence data which contained adapters were trimmed using Trimmomatic (v. 0.32) and *de-novo* genome assembly was performed using SPAdes 3.10.1. The quality of the obtained genome contigs was evaluated with the QUAST tool and all contigs that were longer than 500 bp were used for genome annotation using the PATRIC genome annotation server (4).



The phylogenomic tree showed that strains S1 and S18 could be unequivocally identified as *S. liquefaciens* and *S.ureilytica*, respectively, while strain S13 could be identified as *S. marcescens* subsp. *marcescens*. As in MLSA, the strains S2, S7.1, S10 and S15 grouped closely together, but apart from the *S. marcescens* or *S. nematodiphila* reference genomes, indicating that these may constitute a new species (Fig. 4).

Figure 4. Phylogenetic tree based on whole genome sequences of selected *Serratia* isolates and reference strains. Using the PATRIC Fasttree pipeline.

Identification of *Serratia spp.* Isolates

Total genomic DNA of *Serratia* isolates was used to amplify the 16S rRNA gene (ca. 1450 bp) using the primers 27F and 1540R. All PCR products were purified and commercially sequenced. For MLSA, four housekeeping genes were PCR amplified and sequenced (2). The different primers and housekeeping genes analyzed for *Serratia* spp. are shown in Table 2. The homology of concatenated housekeeping gene sequences were clustered using MEGA 7 with the maximum likelihood method, 1 000 x bootstrap resampling and the kimura-2 parameter for nucleotide substitutions (Fig. 2). All strains were also identified using the EzTaxon 16S rRNA gene database (3). The S1 strain was identified as *S. quinivorans* based on 16S rRNA gene sequence similarity, but clustered closely together with *S. liquefaciens* strain in MLSA. Three strains (S11, S12, and S17) were identified with the highest 16S rRNA gene sequence similarity with *S. nematodiphila* KCTC 22130, while thirteen isolates (S2, S3, S5, S6, S7.1, S8, S9, S10, S13, S14, S15, S16 and S18) were identified as *S. marcescens* by this method. The MLSA cluster, however, showed that strains S2, S7.1, S9, S10 and S15 were potentially novel species.

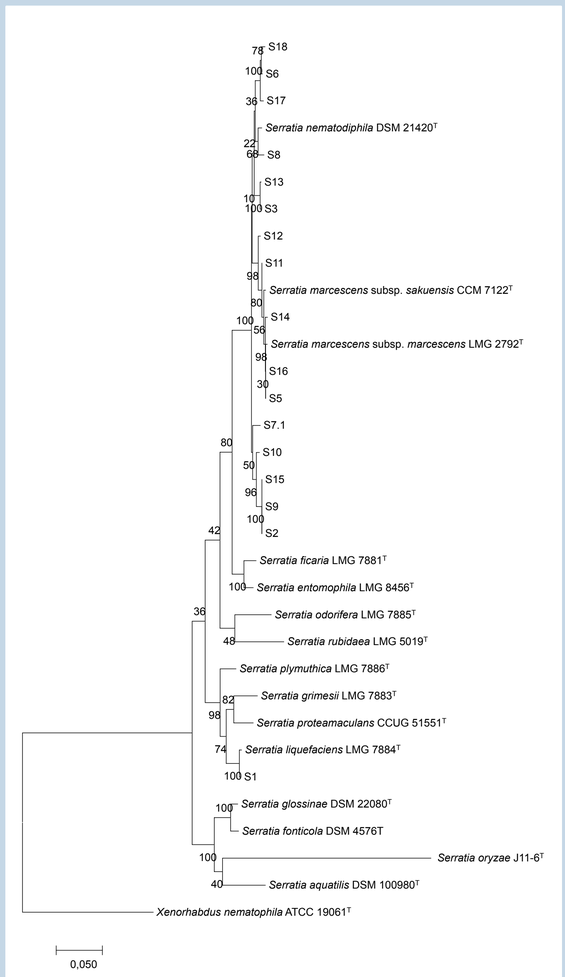


Figure 2. Phylogenetic tree based on 4 house keeping gene sequences of *Serratia* type strains.

Digital DNA-DNA hybridization (dDDH) (Table 3) (5) showed that the strains S2, S7.1, S10 and S15 hybridized at values below 70% when hybridized to the *S. marcescens* or *S. nematodiphila* reference strains, confirming that these are probably novel species. The strain S13 could also be confirmed as a *S. marcesens* strain (Table 3).

Table 3: Digital DNA-DNA hybridization of *Serratia* isolates and referrence strain genome sequences.

dDDH	S18	S13	S7.1	S10	S15	S2	*S. nematodiphila*	*S. marcescens* ssp. *sakuensis*
S. marcescens* ssp. *marcescens	62.01	96.56	62.83	60.29	59.97	60.22	64.8	70.8
S. marcescens* ssp. *sakuensis	61.37	70.99	61.09	59.23	59.66	59.6	84.7	
S. nematodiphila	59.99	63.78	58.91	56.44	55.95	55.92		
S2	44.0	59.9	80.0	96.3	98.3			
S15	44.2	59.7	79.5	96.4				
S10	46.5	58.0	79.9					
S7.1	44.2	63.5						
S13	63.0							

Table 2. Multi-locus sequence analysis primers and their sequences and temperatures used for annealing in PCR reactions

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing temperature (°C) and PCR product length (bp)	Length of the concatenated sequence (bp)
DNA gyrase β-subunit (gyrB)	TAA RTT YGA YGA YAA CTC YTA YAA AGT	CMC CYT CCA CCA RGT AMA GTT	55°C / 742 bp	2651 bp
RNA polymerase β-subunit (rpoB)	AAC CAG TTC CGC GTT GGC CTG	CCT GAA CAA CAC GCT CGG A	55°C / 637 bp	
ATP-sythase β-subunit (atpD)	RTA ATY GGM GCS GTR GTN GAY GT	TCA TCC GCM GGW ACR TAW AYN GCC TG	55°C / 657 bp	
Translation initiation factor IF-2 (infB)	ATY ATG GGH CAY GTH GAY CA	ACK GAG TAR TAA CGC AGA TCC A	55°C / 615 bp	

R: A or G, Y: C or T, S: G or C, W: A or T, K: G or T, M: A or C, B: C or G or T, D: A or G or T, H: A or C or T, V: A or C or G, N: any base

References

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