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Genotypic and phenotypic characterization of a biofilm-forming *Serratia plymuthica* isolate from a raw vegetable processing line

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Abstract

Recently, we isolated from a raw vegetable processing line a *Serratia* strain with strong biofilm-forming capacity and which produced *N*-acyl-L-homoserine lactones (AHLs). Within the *Enterobacteriaceae*, strains of the genus *Serratia* are a frequent cause of human nosocomial infections; in addition, biofilm formation is often associated with persistent infections. In the current report, we describe the detailed characterization of the isolate using a variety of genotypic and phenotypic criteria. Although the strain was identified as *Serratia plymuthica* on the basis of its small subunit ribosomal RNA (16S rRNA) gene sequence, it differed from the *S. plymuthica* type strain in production of pigment and antibacterial compounds, and in AHL production profile. Nevertheless, the identification as *S. plymuthica* could be confirmed by *gyrB* phylogeny and DNA:DNA hybridization.

Keywords: *Serratia*; Identification natural isolate; *gyrB*; Phylogeny; Quorum sensing; *N*-Acyl-L-homoserine lactone

1. Introduction

The genus *Serratia*, named after the Italian physicist Serafino Serrati, belongs to the family *Enterobacteriaceae* and consists of the recognized species: *Serratia marcescens*, *S. liquefaciens*, *S. ficaria*, *S. rubidaea*, *S. fonticola*, *S. odorifera*, *S. plymuthica*, *S. grimesii*, *S. proteamaculans*, *S. quinivorans*, and *S. entomophila* [1]. All species except *S. entomophila* have been frequently isolated from clinical samples, and *S. marcescens* in particular is recognized as an important nosocomial pathogen capable of causing pneumonia, intravenous catheter-associated infections, urinary tract infections, osteomyelitis and endocarditis [2]. However, the recently described virulence-associated properties in *Serratia* strains other than *S. marcescens*, and the increasing number of documented infections caused by such strains, together with the difficult identification of these bacteria by commercial systems urges for a more detailed investigation of the physiology, virulence and taxonomy of this genus [3]. As ubiquitous inhabitants of soil, air and water, *Serratia* species are commonly associated with food raw materials and are implicated in the spoilage of various foods of plant and animal origin. In addition, as opportunistic pathogens, they may pose a foodborne health hazard. We have recently conducted an investigation on the biofilm-forming capacity and the production of quorum-sensing signalling molecules in Gram-negative bacteria isolated from a raw vegetable processing line [4]. Five out of 68 isolates produced *N*-acyl-L-homoserine lactones (AHLs), and two of these, one with strong and one with weak biofilm-forming capacity, were tentatively identified as *S. plymuthica*

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using Biolog carbon utilization patterns. *S. plymuthica* has been described as a non-motile, prodigiosin pigment-producing *Serratia* and is regarded as a significant pathogen [5] to which a variety of infections including peritonitis, pneumonia, sepsis and wound infections have been attributed [6–10]. The capacity to form biofilms often contributes to pathogen virulence because it provides protection against host defense and antibiotic therapy, it allows cells to survive in hostile environments and from there to disperse and colonize new niches, and may facilitate the spread of antibiotic resistance by horizontal gene transfer [reviewed in 11,12]. In the current report, we describe the detailed identification and characterization of the tentative *S. plymuthica* isolate with strong biofilm-forming capacity, using a variety of genotypic and phenotypic criteria.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Strains used in this study are listed in Table 1. All *Serratia* species type strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, Germany), except *S. liquefaciens* DSM 4487 (=LMG 7884), which was obtained from the Belgian Co-ordinated Collections of Micro-organisms (BCCM™/LMG). *Escherichia coli* ESS [13] and *Chromobacterium violaceum* CV026 [14] were obtained from Dr. Susan E. Jensen (University of Alberta) and Dr. René De Mot (Katholieke Universiteit Leuven), respectively. All strains were routinely grown in Luria–Bertani (LB) medium at 30 °C.

2.2. Phenotypic analysis

2.2.1. Swimming and swarming motility

Motility was tested by stab inoculating the strain to be tested in both LB and minimal AB [15] medium solidified with either 0.3% agar to examine swimming through the water-filled channels in the agar, or 0.7% agar to examine swarming over the agar surface [16].

2.2.2. Proteolytic activity

Production of extracellular proteolytic enzymes was evaluated by observation of clearing zones around stab inoculated bacteria on LB agar supplemented with 10% skimmed milk after 24 h of incubation at 30 °C.

2.2.3. Production of antibacterial factors

Bacteria were checked for the production of antibacterial compounds active against various target strains by scoring inhibition or lysis zones. Briefly, 100 μl of an overnight LB broth culture of the target strain was mixed with liquid 0.7% LB agar at 50 °C and poured into a petri dish. After solidification, potential antibacterial producer strains were stab inoculated onto this lawn, and plates were scored after overnight incubation at 30 °C for the presence of inhibition or lysis zones.

2.2.4. Analysis of the N-acyl-L-homoserine lactone production pattern

Analysis of the AHL production pattern was performed by thin-layer chromatography (TLC) on C18 reversed-phase plates (VWR International, Leuven, Belgium) using a methanol/water (60:40 v/v) solvent system essentially as described by Shaw et al. [17]. Briefly, cell-free culture supernatants from 21 h LB broth

Table 1
Strains used in this study

Species	Strain[a]	GenBank Accession No.	
		16S rDNA	*gyrB*
Chromobacterium violaceum	CV026 (*cviI*::mini-Tn5 derivative of ATCC 31532, Km^r, AHL^-)		
Escherichia coli	ESS		
Plesiomonas shigelloides	DSM 8224^T = ATCC 14029^T	M59159	AJ300545
Serratia entomophila	DSM 12358^T = ATCC 43705^T	AJ233427	AJ300543
Serratia ficaria	DSM 4569^T = ATCC 33105^T	AJ233428	AJ300541
Serratia fonticola	DSM 4576^T = ATCC 29844^T	AJ233429	AJ300539
Serratia grimesii	DSM 30063^T = ATCC 14460^T	AJ233430	AJ300538
Serratia liquefaciens	DSM 4487^T = ATCC 27592^T	AJ306725	AJ300537
Serratia marcescens	DSM 30121^T = ATCC 13880^T	AJ233431	AJ300536
Serratia odorifera	DSM 4582^T = ATCC 33077^T	AJ233432	AJ300533
Serratia plymuthica	DSM 4540^T = ATCC 183^T	AJ233433	AJ300532
Serratia proteamaculans	DSM 4543^T = ATCC 19323^T	AJ233434	AJ300531
Serratia quinivorans[b]	DSM 4597^T = ATCC 33765^T	AJ233435	
Serratia rubidaea	DSM 4480^T = ATCC 27593^T	AJ233436	AJ300530
Serratia sp.	RVH1	AY394724	AY787168

[T]Type strain.
[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ATCC, American Type Culture Collection, Manassas, VA, USA.
[b] Originally classified as *S. proteamaculans* subsp. *quinovora* the transfer to *Serratia quinivorans* [30] reduces *Serratia proteamaculans* subsp. *proteamaculans* to *Serratia proteamaculans*.

stationary-phase cultures (500 ml) of the *Serratia* spp. were extracted twice with the same volume of ethyl acetate, dried over anhydrous $MgSO_4$, evaporated to dryness, and the residue was dissolved in a small volume of ethyl acetate and loaded onto the TLC-plates. After chromatographic separation, the presence of AHLs was detected by overlaying the dried TLC-plates with a thin film of AHL sensor strain *C. violaceum* CV026 in 1.4% LB agar, and looking for the appearance of purple spots indicative of induction of violacein production after incubation at 30 °C for 24 h.

2.3. 16S rDNA analysis

Analysis of 16S rDNA of *S. plymuthica* RVH1 was performed by BCCM™/LMG (Gent, Belgium). Briefly, genomic DNA was extracted following the protocol of Pitcher et al. [18] and the part of the 16S rRNA gene corresponding to positions 28–1521 of the *E. coli* 16S rRNA gene was PCR amplified with the primers 16F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16R1522 (5′-AAGGAGGTGATCCAGCCGCA-3′). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced using five forward primers and three reverse primers annealing to universally conserved regions, with the ABI PRISM TM BigDye TM Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Applied Biosystems Div., Foster City, CA, USA) and an Applied Biosystems 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The sequence assembly was performed using the program AutoAssembler (Perkin–Elmer).

2.4. DNA:DNA hybridizations

DNA:DNA hybridizations were performed by BCCM™/LMG (Gent, Belgium). Briefly, DNA was prepared according to a slightly modified procedure of Wilson [19] and hybridizations were performed at 46 °C using the method described by Ezaki et al. [20] with some modifications.

2.5. gyrB *gene amplification and sequencing*

The *gyrB* gene of *S. plymuthica* RVH1 was PCR amplified as described by Dauga [21]. Briefly, 50 pmol of each primer gyr-320 (5′-TAARTTYGAYGAYAACTCYTAYAAAGT-3′) and rgyr-1260 (5′-CMCCYTCCACCARGTAMAGTTC-3′) were used in a reaction mixture (100 μl) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$. PCR amplification was carried out as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final incubation at 72 °C for 10 min. The amplification product was purified using the High Pure PCR Purification Kit (Roche Diagnostics, Vilvoorde, Belgium) and sequenced in both directions using the same primers as used for amplification at a commercial sequencing facility (MWG-Biotech AG, Ebersberg, Germany).

2.6. Phylogenetic data analysis

Multiple-sequence alignments were performed using the CLUSTAL W algorithm from the European Bioinformatics Institute (EBI) toolbox (http://www.ebi.ac.uk/clustalw/) and were further refined by eye, introducing gaps to improve overall alignment. Sequence distance matrices were established in pairwise comparisons by use of the Kimura algorithm [22]. Phylogenetic trees were constructed by the neighbour-joining method [23] using the PHYLIP version 3.5 software package [24]. Statistical significance was evaluated by bootstrap analysis [25] with 100 repeats of bootstrap samplings.

3. Results and discussion

3.1. Phenotyping of strain RVH1

In a screening of 68 biofilm-forming Gram-negative bacteria from a raw vegetable processing line, one of the strongest biofilm-forming isolates that also produced different AHLs and AI-2 as quorum signalling compounds, designated RVH1, was a catalase negative and oxidase positive rod-shaped organism (1.0 μm width; 1.2–1.5 μm length), and was tentatively identified as *S. plymuthica* based on phenotypic analysis with the Biolog GN2 Microplate System [4]. The results of additional phenotypic analysis of this strain, in comparison to type strains of the nine *Serratia* species most closely related to *S. plymuthica*, are described below and summarized in Table 2 and Fig. 1.

3.1.1. Proteolytic activity and swimming and swarming motility

All bacteria, including RVH1, showed proteolytic activity on skimmed milk plates except *S. proteamaculans* and *S. grimesii*. Since all strains except *S. fonticola* score positive in gelatin hydrolysis assays [26], it is possible that the proteases produced by *S. proteamaculans* and *S. grimesii* are not able to hydrolyse caseins.

All strains showed swimming motility, as expected for the genus *Serratia*, but only *S. ficaria* showed swarming motility on both LB and AB medium with 0.7% agar. The *S. liquefaciens* type strain showed no swarming motility; contrary to *S. liquefaciens* strain MG1, which we used as an internal control for our swarming motility assay because it is a model organism in many studies of swarming motility [16].

Table 2
Summary of phenotypic tests

Strain	Pigment production	Swimming motility	Swarming motility	Proteolytic activity	ESS[a]	RVH1[b]
S. entomophila	–	+	–	+	–	+
S. ficaria	–	+	+	+	–	+
S. fonticola	–	+	–	+	–	–
S. grimesii	–	+	–	–	–	+
S. liquefaciens	–	+	–	+	–	–
S. odorifera	–	+	–	+	–	+
S. plymuthica	Red	+	–	+	–	+
S. proteamaculans	–	+	–	–	–	+
S. quinivorans	–	+	–	+	–	–
RVH1	–	+	–	+	+	–

[a] ESS: Production of antibacterial factor scored on *E. coli* ESS overlay plates.
[b] RVH1: Production of antibacterial factor by RVH1 scored on overlay plates of listed strains.

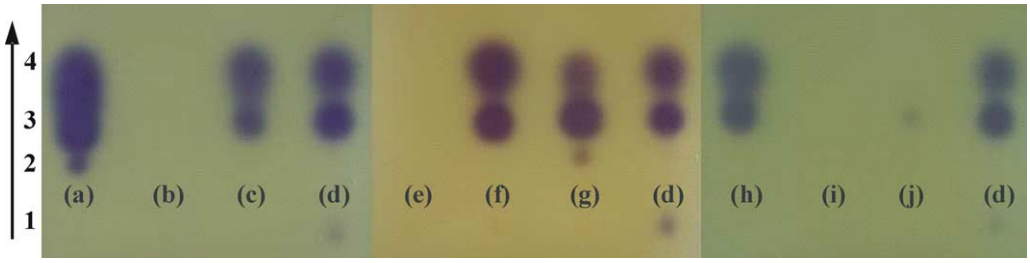

Fig. 1. *N*-Acyl-L-homoserine production profile as indicated by biosensor strain *Chromobacterium violaceum* CV026. (a) *S. ficaria* DSM 4569; (b) *S. liquefaciens* DSM 4487; (c) *S. quinivorans* DSM 4597; (d) strain RHV1; (e) *S. plymuthica* DSM 4540; (f) *S. entomophila* DSM 12358; (g) *S. odorifera* DSM 4582; (h) *S. proteamaculans* DSM 4543; (i) *S. grimesii* DSM 30063; and (j) *S. fonticola* DSM 4576.

3.1.2. Production of antibacterial factor

Serratia strains have been reported to produce certain compounds with antibacterial activity, such as the simple carbapenem, 1-carbapen-2-em-3-carboxylic acid, identified in *Serratia* sp. strain ATCC 39006 [27]; Serracin P, a phage-tail-like bacteriocin, produced by *S. plymuthica* J7 [28]; and bacteriocin 28b produced by most *S. marcescens* biotypes [29]. Therefore, the production and activity spectrum of possible antibacterial factors produced by RVH1 and the type strains was analyzed by stab inoculating each strain onto a series of plates each containing a lawn of one of the other *Serratia* strains or of *E. coli* ESS, a β-lactam supersensitive strain used in carbapenem production analysis. None of the *Serratia* spp. type strains produced a halo in this test except for *S. proteamaculans*, which caused a weak inhibition of *S. grimesii*. However, strain RVH1 caused complete inhibition (clear halo) of *S. entomophila*, *S. ficaria*, *S. grimesii*, *S. odorifera*, *S. plymuthica*, *S. proteamaculans*, and *E. coli* ESS, but not of *S. fonticola*, *S. liquefaciens*, and *S. quinivorans*. The activity spectrum of the antibacterial factor produced by RVH1 differs from Serracin P, which shows no activity towards the *E. coli* strains tested [28]. Preliminary tests suggest that the antibacterial activity can be ascribed to a protein, but the presence of other compounds cannot be excluded at this stage (data not shown). The antibacterial spectra of the type strains in this study differ from those reported by Ashelford et al. [30], possibly due to differences in growth temperature and other experimental parameters.

3.1.3. N-Acyl-L-homoserine lactones

N-Acyl-L-homoserine lactone mediated quorum-sensing is a widespread communication system in Gram-negative bacteria, in which small diffusible AHL signalling molecules, synthesized by a LuxI homologue, interact with a LuxR homologue and activate or repress the target genes when their concentration reaches a certain threshold, related to population density [31]. Since quorum sensing regulates a range of important biological functions, such as antibiotic production, plasmid transfer, motility, virulence and biofilm formation [reviewed in 31], it is considered as a possible target for antibacterial treatment, and several studies have demonstrated the feasibility of interfering with quorum sensing by the use of specific antagonists of the signalling molecules, an approach known as 'quorum quenching' [32,33].

Different AHL production profiles and target genes have been described in a number of *Serratia* spp., showing the specificity and diversity of quorum sensing signal molecules and regulation in this genus. For example, *Serratia proteamaculans* strain B5a produces

3-oxo-*N*-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) and *N*-hexanoyl-L-homoserine lactone (C6-HSL) [34], *S. liquefaciens* strain MG1 produces primarily *N*-butanoyl-L-homoserine lactone (C4-HSL) and also C6-HSL [35], while *S. marcescens* strain SS-1 produces at least four AHLs, namely 3-oxo-C6-HSL, C6-HSL, *N*-heptanoyl-L-homoserine lactone (C7-HSL) and *N*-octanoyl-L-homoserine lactone (C8-HSL) [36]. Recently, three AHLs produced by *S. plymuthica* IC1270 were tentatively identified as 3-hydroxy-*N*-hexanoyl-L-homoserine lactone (3-hydroxy-C6-HSL), 3-hydroxy-*N*-octanoyl-L-homoserine lactone (3-hydroxy-C8-HSL) and an unidentified compound by comigration with synthetic compounds in thin layer chromatography [37]. These AHL molecules are produced by the AHL synthase from the substrates *S*-adenosyl-L-methionine (SAM) and acylated acyl carrier protein (acyl-ACP) [38] and can vary in acyl chain length (from C4 to C14), oxidation at the C3 position and saturation [39,40] due to the enzyme acyl chain specificity and the available cellular pool of acyl-ACPs [39,41].

As additional phenotype, we examined the AHL production profile of strain RVH1 and the *Serratia* sp. type strains by TLC analysis in combination with *C. violaceum* CV026 biosensor overlay, and compared the AHL profiles to those already described in *Serratia* spp. In *C. violaceum* CV026, the proper production of AHL molecules has been blocked by mutation of the AHL synthase but the gene encoding the production of the purple pigment violacein remains AHL-responsive. In the presence of specific AHLs with acyl chain lengths shorter than C_{10}, this strain will therefore produce purple pigment due to violacein production [14]. The results of the AHL profile analysis are shown in Fig. 1, and reveal at least four different AHLs with a different TLC migration. The two farthest migrating spots (spots 3 and 4 in Fig. 1) are the most common AHLs, being present in *S. ficaria*, *S. quinovorans*, *S. entomophila*, *S. odorifera*, *S. proteamaculans*, and RVH1. Since another strain of *S. proteamaculans* (B5a) was previously reported to produce 3-oxo-C6-HSL and C6-HSL [34], these two spots most likely correspond to these two AHL molecules, although the existence of other AHLs with the same migration cannot be excluded at this stage. Strain RVH1 shows a third spot (spot 1 in Fig. 1) which did not migrate from the point of application and which was not seen in any of the other *Serratia* species. The slow migration could indicate the presence of an AHL with a long-chain hydrophobic fatty acid residue that binds strongly to the C-18 solid phase, but such an AHL should not be able to elicit violacein production. Even so, when a lot of material is loaded onto a TLC-plate, molecules with shorter acyl chains sometimes get blocked, but on the other hand preliminary mass spectrometry analysis suggests indeed the presence of 3-oxo-C12-HSL in the ethyl acetate extracts of RVH1 (data not shown). Finally, one TLC spot (spot 2 in Fig. 1) was observed only in *S. ficaria* and *S. odorifera*, but its nature remains unknown. No AHLs capable to induce violacein production were found for the *S. grimesii*, *S. plymuthica*, and *S. liquefaciens* type strains, although AHL production has been described for *S. plymuthica* IC1270 [37] and *S. liquefaciens* MG1 [35], indicating that the AHL synthases in *S. plymuthica* and *S. liquefaciens* type strains are absent or mutated, resulting in the loss of AHL production.

In spite of the tentative biochemical identification of strain RVH1 as *S. plymuthica*, the difficulties in precise phylogenetic positioning of *Serratia* strains combined with the phenotypic differences between strain RVH1 and the *S. plymuthica* type strain (see Table 2) motivated us to perform a detailed phylogenetic study based on 16S rDNA and *gyrB* sequence comparison, and on DNA:DNA hybridization.

3.2. 16S rRNA-based phylogeny

Part of the 16S rDNA gene sequence of strain RVH1 was amplified and analysed (GenBank Accession No. AY394724). Fig. 2(a) shows a neighbour-joining phylogenetic tree based on the alignment of the nearly complete 16S rDNA gene sequence of strain RVH1 with 16S rDNA sequences of the 11 described *Serratia* type strains available in GenBank and EMBL databases (see Table 1 for corresponding accession numbers), and rooted by using *Plesiomonas shigelloides*, which is the most closely related species to the *Enterobacteriaceae* family [42]. The 16S rDNA sequence similarity between strain RVH1 and the 11 described *Serratia* species ranged between 99.3% and 96.3%, with the highest similarity to *S. plymuthica* (99.3%) and *S. ficaria* (99.2%) and the lowest to *S. rubidaea* (96.4%) and *S. marcescens* (96.3%). Two separate clusters were obtained as described by Sproër et al. [43]. One cluster comprised *S. rubidaea*, *S. marcescens*, *S. odorifera*, *S. entemophila*, and *S. ficaria* and the second cluster comprised *S. plymuthica*, *S. fonticola*, *S. liquefaciens*, *S. quinivorans*, *S. grimesii*, and *S. proteamaculans*. The phylogenetic information obtained from these sequences is poor due to the low rate of variation of 16S rDNA sequences. Therefore, we also determined a phylogeny based on the *gyrB* sequence.

3.3. gyrB-based phylogeny

Dauga [21] described the use of the *gyrB* sequence for determining relationships among *Serratia* species. In general, phylogenetic trees based on *gyrB* sequences appear to be more reliable for closely related bacterial species than trees based on 16S rDNA. The *gyrB* nucleotide sequence from strain RVH1 was determined from the PCR-amplified *gyrB* gene, revealing a 910 bp open reading frame (GenBank Accession No. AY787168). The

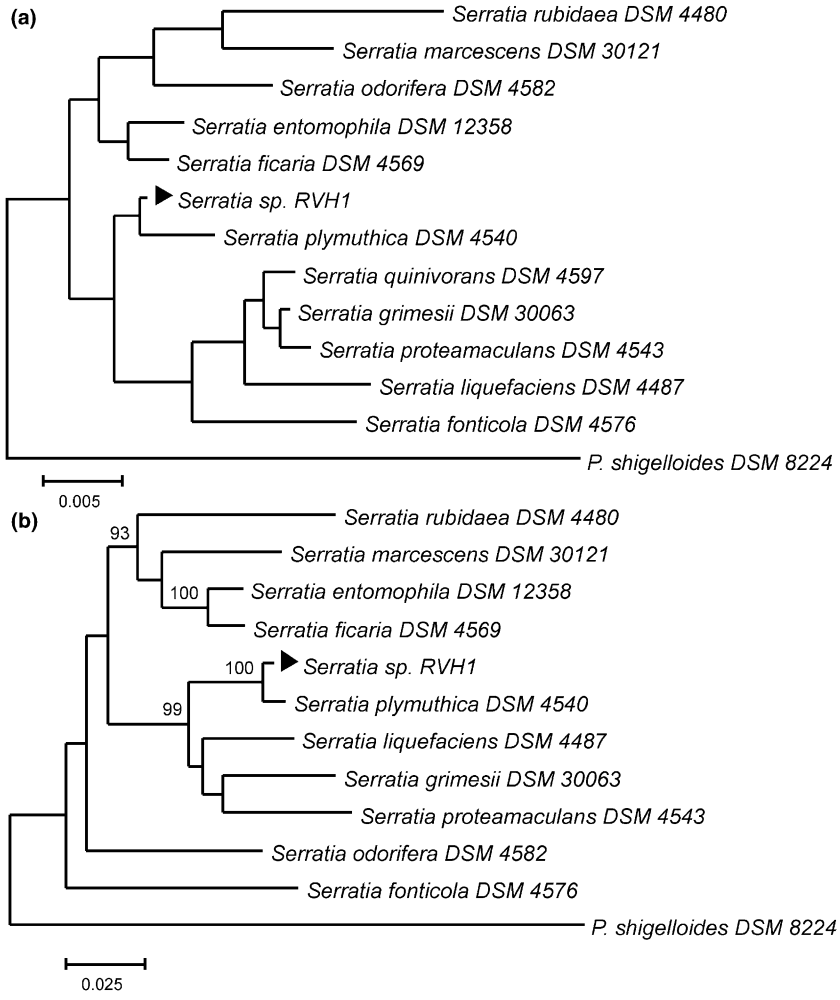

Fig. 2. Neighbour-joining phylogenetic tree obtained from (a) 16S rRNA gene sequences, with the scale bar representing an estimated five base substitutions per 1000 nt positions and (b) *gyrB* sequences, with the scale bar representing an estimated 25 substitutions per 1000 nt positions. Numbers refer to significant bootstrap values of 100 calculated trees.

translated amino acid sequence had a lysine (K) at codon 206 (*E. coli* amino acid numbering system, Accession No. X04341) in a β-sheet-shaped region of the ATP binding site, which is a *Serratia* signature sequence [21]. The sequence similarity of strain RVH1 to the 10 *Serratia* species examined (the *gyrB* sequence of *S. quinovorans* was not available in a database) ranged between 98.9% and 86.5%, with the highest similarity to *S. plymuthica* (98.9%) and *S. liquefaciens* (94.2%) and the lowest to *S. rubidaea* (87.8%) and *S. fonticola* (86.5%). Fig. 2(b) represents a phylogenetic tree based on the alignment of the *gyrB* gene sequence of strain RVH1 and the *Serratia* sp. type strain *gyrB* sequences available in GenBank and EMBL databases (see Table 1 for corresponding accession numbers) and rooted by using *P. shigelloides*. Two phylogenetic clusters with significant bootstrap values were again found. The first cluster (bootstrap value 93%) contained *S. rubidaea*, *S. marcescens*, *S. entomophila*, and *S. ficaria*. The second cluster (bootstrap value 99%) contained strain RVH1, *S. grimesii*, *S. proteamaculans*, *S. liquefaciens*, and *S. plymuthica*. Within this cluster strain RVH1 and *S. plymuthica* formed a coherent group validated by a significant bootstrap value of 100%.

3.4. DNA:DNA hybridization

Bacterial strains are generally considered to belong to the same species if they share a 16S rDNA sequence identity of >97% and/or 70% or greater DNA–DNA relatedness with 5 °C or less difference of melting temperature (ΔT_m), with the latter criterion being decisive [44]. Therefore, to conclusively confirm the identity of RVH1, we performed DNA:DNA hybridization between RVH1 and the two most closely related type strains based on 16S rDNA sequence identity, i.e., *S. plymuthica* and *S. ficaria*. We found 100% DNA–DNA hybridization with the *S. plymuthica* type strain and 46% with the *S. ficaria* type strain, confirming the identity of RVH1 as *S. plymuthica*.

4. Conclusions

We have performed a comparative characterization of *Serratia* sp. RVH1, which was previously isolated as a biofilm-forming strain from a raw vegetable processing line [4], with the type strains of the 9 or 10 most closely related *Serratia* species. Phenotypically, the isolate could not be clearly assigned to any of the described *Serratia* species, but 16S rRNA and *gyrB* sequence comparison and DNA:DNA hybridization unequivocally identified the strain as *S. plymuthica*. Furthermore, these observations add new phenotypic and genotypic information to the *Serratia* genus, thus contributing to a more precise phylogenetic positioning of *Serratia* strains.

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