Isolation, pure culture and characterization of *Serratia symbiotica* sp. nov., the R-type of secondary endosymbiont of the black bean aphid *Aphis fabae*

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An intracellular symbiotic bacterium was isolated from the flora of a natural clone of the black bean aphid *Aphis fabae*. The strain was able to grow freely in aerobic conditions on a rich medium containing 1 % of each of the following substrates: glucose, yeast extract and casein peptone. Pure culture was achieved through the use of solid-phase culture on the same medium and the strain was designated CWBI-2.3^T. 16S rRNA gene sequence analysis revealed that strain CWBI-2.3^T was a member of the class *Gammaproteobacteria*, having high sequence similarity (>99 %) with '*Candidatus Serratia symbiotica*', the R-type of secondary endosymbiont that is found in several aphid species. As strain CWBI-2.3^T (=LMG 25624^T=DSM 23270^T) was the first R-type symbiont to be isolated and characterized, it was designated as the type strain of *Serratia symbiotica* sp. nov.

Symbiosis is a common feature in insects, especially in insects feeding on exclusive and unbalanced diets (Buchner, 1965). For aphids, an association with an endosymbiotic bacterium *Buchnera aphidicola* combats the lack of essential amino acids in the phloem sap. In addition to this obligate symbiont, many aphids harbour facultative endosymbionts that enable them to expand their ecological niches and also help to provide protection against microbial and parasitoid attacks (Oliver *et al.*, 2003; Tsuchida *et al.*, 2004; Scarborough *et al.*, 2005). Both primary and secondary symbionts are maternally inherited over aphid generations and can reside intracellularly in specialized cells and organs (Buchner, 1965; Baumann *et al.*, 2000).

As they live in a nutrient-rich environment, many endosymbionts have relatively inactive biochemical profiles compared with those of their free-living relatives (Hypša & Dale, 1997; Dale & Maudlin, 1999; Pontes & Dale, 2006). Consequently, most of them are difficult to isolate using conventional *in vitro* culture conditions and their phenotypic and genetic characterization is mostly incomplete (Hypša & Dale, 1997; Darby *et al.*, 2005). To date, only a few of these symbionts have been successfully characterized and currently a trivial nomenclature system is in use to

allow their distinction and classification (Gherna *et al.*, 1991; Hypša & Dale, 1997; Dale & Maudlin, 1999).

'*Candidatus Serratia symbiotica*' is currently described as an endosymbiont from multiple aphid families. Recent studies have demonstrated that it plays different roles in the heat tolerance of the host and in their protection against parasitoids (Chen *et al.*, 2000; Montllor *et al.*, 2002; Oliver *et al.*, 2003; Burke *et al.*, 2010). In the aphid subgenus *Cinara*, '*Ca. S. symbiotica*' has a long-term relationship with its hosts and appears to be in a transition phase from a facultative to an obligate symbiont for host survival and reproduction (Gil *et al.*, 2002; Gómez-Valero *et al.*, 2004; Pérez-Brocá *et al.*, 2006; Lamelas *et al.*, 2008; Burke *et al.*, 2009).

In this study, the first isolation and *in vitro* culture of an endosymbiotic strain of '*Ca. S. symbiotica*' from the flora of a natural clone of the aphid *Aphis fabae* is described. This strain is proposed as the type strain of the secondary endosymbiont *Serratia symbiotica* sp. nov.

The novel strain, designated CWBI-2.3^T, was isolated during the characterization of the microbiota of several laboratory and natural aphids collected in Belgium. The strain was among the flora of the natural clone of the black bean aphid *Aphis fabae* collected over a period corresponding to the first parthenogenetic generations (May 2009).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CWBI-2.3^T is GU394001.

For strain isolation, 40 specimens were surface-sterilized and extracted with 863 medium containing (1^{-1} distilled water) 10 g each of glucose, yeast extract and casein peptone. The extract was then plated on 868 agar medium (corresponding to 863 medium with 1.7 % agar). Colonies of strain CWBI-2.3^T were visible after 2 weeks of incubation at 20 °C and the prevalence was about 10^3 c.f.u. mg^{-1} . The flora of the collected *A. fabae* clone contained two other bacteria identified as *Acinetobacter calcoaceticus* (1.3×10^3 c.f.u. mg^{-1}) and *Pseudomonas viridiflava* (0.7×10^3 c.f.u. mg^{-1}).

Colonies of strain CWBI-2.3^T were circular, uniform in size with entire edges, not pigmented and appeared off-white, smooth and shiny. Morphological features were examined by light microscopy and transmission electron microscopy (100-SX; JEOL) (Fig. 1). The strain was motile, rod-like ($0.5 \times 0.8\text{--}1.3$ μm) and appeared mainly as single cells or in pairs.

The novel strain was maintained in the laboratory for several months and used for all of the subsequent experiments described in this study. Long-term preservation was possible by freezing at -80 °C in culture medium with 20 % (w/v) glycerol.

For strain identification, genomic DNA was extracted from cells grown at 20 °C for 120 h in 868 medium and purified by using a Wizard Genomic DNA purification kit (Promega). The primers used for PCR amplification of the 16S rRNA gene were the universal primers 16SP0

GAAGAGTTTGATCCTGGCTCAG and 16SP6 CTACGG-CTACCTTGTTACGA (Ventura *et al.*, 2001). The PCR product was purified using GFX PCR DNA and a Gel Band kit (GE Healthcare). Sequencing was achieved using a Big Dye v.3.1 kit and a 3730 DNA Analyzer (Applied Biosystems). The primers used for sequencing were F1 (CTGGCTCAGGAYGAACG), F2 (GAGGCAGCAGTRG-GGAAT), F3 (ACACCARTGGCGAAGGC) and F4 (GCA-CAAGCGGYGGAGCAT) for the coding DNA segment, and R1 (CTGCTGGCACGTTAGT), R2 (AATCTGT-TTGCTMCCCCA), R3 (CCAACATCTCAGACACG) and R4 (TGTGTAGCCCWGGTCRTAAG) for the non-coding DNA segment. The sequences obtained (400–600 bp) were then assembled by using the BioEdit program. The resulting sequence (1478 bp) of strain CWBI-2.3^T was deposited in GenBank database with the accession number GU394001. The sequence of strain CWBI-2.3^T was compared with other sequences in GenBank by using the BLASTN program (Altschul *et al.*, 1997).

The 16S rRNA gene sequence of the novel strain shared 99.9 % similarity with that of '*Ca. Serratia symbiotica*' strains found in *Aphis craccivora* (1463/1464 bp) (Tsuchida *et al.*, 2006). It was also closely related to '*Ca. Serratia symbiotica*' strains found in *Acyrtosiphon pisum* (99.7 %) and *Cinara cupressi* (99.6 %) (Unterman *et al.*, 1989; Fukatsu *et al.*, 2000; Tsuchida *et al.*, 2006; Lamelas *et al.*, 2008; Burke *et al.*, 2009).

A comparison with 16S rRNA gene sequences of the type strains of free-living members of the genus *Serratia* showed that strain CWBI-2.3^T shared 97.7 % similarity with *Serratia ficaria*, 97.5 % with *Serratia entomophila*, 97.4 % with *Serratia plymuthica*, 97.1 % with *Serratia proteamaculans*, 97.0 % with *Serratia grimesii*, 96.9 % with *Serratia nematodiphila*, 96.8 % with *Serratia odorifera*, 96.7 % with *Serratia marcescens*, 96.4 % with *Serratia fonticola*, 96 % with *Serratia ureilytica*, 96 % with *Serratia glossinae* and 95.6 % with *Serratia rubidaea*.

Phylogenetic analysis was conducted by using MEGA 4.0 software (Tamura *et al.*, 2007). Sequence similarity was estimated using the likelihood method and phylogenetic trees were constructed with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (1000 resamplings) was used to evaluate the topology of the trees (Felsenstein, 1985).

Recent phylogenetic reconstructions of lineages of symbiotic members of the genus *Serratia* have revealed the existence of two groups of '*Ca. S. symbiotica*' in aphids (Lamelas *et al.*, 2008; Burke *et al.*, 2009). The first (cluster A) encompasses S-symbionts belonging to different subfamilies of the family Aphididae; Aphidinae, Chaitophorinae, Eriomatinae and some members of the subfamily Lachninae. The second (cluster B) comprises only endosymbionts of the genus *Serratia* from the subfamily Lachninae which are considered to be primary-type endosymbionts (Lamelas *et al.*, 2008). The phylogenetic tree (Fig. 2) showed that

Fig. 1. (a) Transmission electron micrograph (TEM) of cells of strain CWBI-2.3^T after incubation for 7 days on 868 agar medium. (b) TEM of a thin section of *Aphis fabae* showing the secondary symbiont (arrows) located in the bacteriosome in the cytoplasm of sheath cell (shc), (ss) secondary symbionts, (ps) primary symbionts. Bars, 1 μm (a); 5 μm (b).

Fig. 2. Phylogenetic position of strain CWBI-2.3^T among 'Ca. S. symbiotica' strains of aphids. The evolutionary history was inferred using the neighbour-joining tree based on analysis of 16S rRNA gene sequence data. Endosymbionts are referenced by their aphid host taxa and by the GenBank accession numbers for their 16S rRNA gene sequences. Bootstrap values >50% (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

strain CWBI-2.3^T belonged to cluster A of the S-symbiont lineages.

For free-living species of the genus *Serratia*, phylogenetic affinities revealed two clusters. The first cluster (cluster II) included *S. marcescens*, *S. rubidaea*, *S. liquefaciens*, *S. ureilytica*, *S. nematodiphila* and *S. odorifera*. The second cluster (cluster I) included *S. proteamaculans*, *S. grimesii*, *S. plymuthica*, *S. fonticola*, *S. entomophila* and *S. ficaria*. Strain CWBI-2.3^T clearly belonged to the second cluster (cluster I, Fig. 3).

DNA–DNA hybridizations were performed with strain CWBI-2.3^T and the three most closely related species in the second cluster (cluster I); *S. ficaria*, *S. entomophila* and *S. plymuthica*. The analysis was performed fluorometrically according to the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Strain CWBI-2.3^T showed DNA–DNA relatedness values of 23%, 22% and 21% with *S. ficaria*, *S. entomophila* and *S. plymuthica*, respectively. This indicated that the secondary symbiont of the aphid, strain CWBI-2.3^T, represented a distinct species of the genus *Serratia* according to the criteria used for the delineation of bacterial species (Wayne *et al.*, 1987).

For DNA G+C analysis, genomic DNA was extracted and purified according to Gevers *et al.* (2001) and the G+C content was determined by HPLC (Mesbah *et al.*, 1989). The DNA G+C content of CWBI-2.3^T strain was 52.7 mol% and was similar to that found for *S. liquefaciens* (51.9 mol%) and *S. fonticola* (51.5 mol%), but was less than that determined for most free-living species of the genus *Serratia* (Grimont *et al.*, 1982; Gavini *et al.*, 1979).

Genome size reduction and a low G+C content of the genomic DNA are common characteristics of endosymbiotic bacteria (Moran *et al.*, 2008). The G+C content of the strain CWBI-2.3^T was relatively low when compared with that of many free-living species of the genus *Serratia*; however, it was much higher than that reported for several other insect endosymbionts, in which the DNA G+C content is often below 33 mol% (Moran *et al.*, 2008).

The novel strain was able to grow aerobically and microaerobically at 0–4% (w/v) NaCl and at 10–30 °C, with an optimum growth temperature of 25 °C. In an attempt to define a minimal medium for the growth of the novel strain, it was found that the strain was unable to grow on a mineral medium (MM) [0.2% (NH₄)₂SO₄; 0.2% Na₂SO₄; 1.5% K₂HPO₄; 0.05% KH₂PO₄; 0.01% MgSO₄·7H₂O and 0.003% CaCl₂] containing glucose (1%) and the 20 amino acids (0.01% each) (OD₆₀₀>0.1). However, it was able to grow on MM containing 1% glucose and 1% casein peptone instead of pure amino acids (OD₆₀₀=0.6). Weak growth was obtained on MM containing only casein peptone (1%) (OD₆₀₀=0.2). On 863 medium (containing 1% each of glucose, casein peptone and yeast extract), growth reached an OD₆₀₀ of 1.4.

For the assimilation and acidification tests, API 20E, API 20NE and API 50CH strips (bioMérieux) were used and the incubation times were about 2 weeks for strain CWBI-2.3^T and 24 h for the free-living *Serratia* strains. For the assimilation tests (API 20NE strips), the novel strain gave a positive result only for the assimilation of glucose and *N*-acetylglucosamine as a carbon source. However, the novel strain was unable to grow on arabinose, mannose,

Fig. 3. Phylogenetic position of strain CWBI-2.3^T among related free-living species of the genus. The tree was generated using the neighbour-joining method and based on 16S rRNA gene sequence data analysis. Species are referenced by strain numbers and GenBank accession numbers for their 16S rRNA sequences. Bootstrap values are expressed as percentages of 1000 replications and are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

mannitol, maltose, gluconate, caprate, adipate, malate, citrate or phenylacetate. In acidification tests (API 50CH strips), strain CWBI-2.3^T was weakly positive for acid production from glucose, sucrose and aesculin, but was unable to produce acid from the 46 remaining carbohydrates present on the strips (Table 1). With the API 20E strips, strain CWBI-2.3^T was positive for the Voges–Proskauer reaction but gave a negative result for β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase. The novel strain was also unable to reduce nitrate or produce H₂S or indole. The strain was also negative in tests for DNase, lipase (Tween 80), lecithinase, chitinase and in the methyl red test.

Phenotypic characterization revealed that strain CWBI-2.3^T was characterized by a highly restricted metabolic profile in comparison with the free-living members of the genus *Serratia* (Grimont *et al.*, 1988; Ajithkumar *et al.*, 2003; Bhadra *et al.*, 2005; Zhang *et al.*, 2009; Geiger *et al.*, 2010). Indeed, in general, members of the genus *Serratia* are able to produce a wide range of hydrolases and to develop on many carbon sources. They are usually distinguished from other members of the family *Enterobacteriaceae* by their growth on mineral medium without growth factors, as well as by their acid production from maltose, mannitol and trehalose and by their ability to produce DNase, lipase, and gelatinase (Farmer *et al.*, 1985). It appears that strain CWBI-2.3^T has lost all these characteristics during its adaptation to its symbiotic life. In the genus *Serratia*, this kind of adaptation, with loss of metabolic diversity, has been previously reported for the

biogroup 1 of *S. marcescens*. The latter has lost some of its metabolic activities through the natural selection of strains adapted to the human urinary tract (Farmer *et al.*, 1985). For mutualistic intracellular bacteria in general, the loss of metabolic and genetic diversity is usually a consequence of evolution in a closed environment in which many molecules can be obtained from the host (Andersson & Kurland, 1998; Dale & Maudlin, 1999; Moran & Wernegreen, 2000; Dale & Moran, 2006). This loss of metabolic and genetic diversity is probably the main reason why many insect endosymbionts are difficult to isolate and characterize.

For quantitative analysis of the cellular fatty acid composition, cells were grown aerobically for 24 h at 28 °C on TSA medium (BBL 11768). Inoculation and harvesting of cells were performed as described by the Microbial Identification System, Inc. (MIDI). The extraction and analysis were performed as prescribed by the MIDI system and the whole-cell fatty acid composition was determined using GC.

The fatty acid profile of strain CWBI-2.3^T was compared with those of some related free-living species of the genus *Serratia* (Table 2). Summed features 2 and 3 (comprising C_{12:0} alde, an unknown fatty acid with equivalent length 10.928 and C_{14:0} 3-OH/C_{16:1} iso I, and C_{16:1} ω 7c iso C_{15:0} 2-OH, respectively) were also among the predominant fatty acids of most of the strains, except for *S. nematodiphila*, in which summed feature 3 was present only in trace amounts but the amount of C_{19:0} cyclo ω 7c was particularly high. The most marked differences between strain CWBI-2.3^T

Table 1. Biochemical and physiological characteristics of strain CWBI-2.3^T compared with the most closely related type strains of free-living taxa

Taxa: 1, strain CWBI-2.3^T; 2, *S. ficaria* LMG 7881^T; 3, *S. entomophila* LMG 8456^T; 4, *S. plymuthica* LMG 7886^T; 5, *S. grimesii* LMG 7883^T; 6, *S. proteomaculans* LMG 7887^T. All strains were rod-shaped, motile and non-spore-forming. All strains were negative for indole and H₂S production and for tryptophan deaminase, oxidase and urease. All strains assimilated *N*-acetylglucosamine. All strains were unable to grow on adipate and phenylacetate. All strains were able to produce acid from *N*-acetylglucosamine, aesculin, D-fructose, D-glucose and sucrose. All strains were unable to produce acid from erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose or L-arabitol.

Characteristic	1	2	3	4	5	6
Pigment production	–	–	–	+	–	–
β-Galactosidase	–	+	+	+	+	+
Arginine dihydrolase	–	+	–	+	+	–
Lysine decarboxylase	–	–	–	–	+	+
Ornithine decarboxylase	–	–	–	–	+	+
Gelatinase	–	+	+	+	+	+
Voges-Proskauer reaction	+	+	+	+	+	+
Methyl red test	–	–	–	+	+	+
DNase	–	+	+	+	+	+
Lipase (Tween 80)	–	+	+	+	+	+
Lecithinase	–	+	+	+	+	+
Chitinase	–	+	+	+	+	+
Caseinase	–	–	+	+	–	–
Catalase	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+
Growth temperature (°C)	10–30	5–37	5–40	5–37	5–37	5–37
NaCl (% w/v)	0–4	0–8	0–8	0–8	0–8	0–8
DNA G + C content (mol%)	52.7	59.6	58	ND	ND	ND
Carbohydrate assimilation						
Caprate	–	+	–	–	+	+
Citrate	–	+	+	+	–	+
Gluconate	–	+	+	+	+	+
Glycerol	–	–	+	–	+	+
Malate	–	+	+	+	+	+
Maltose	–	+	+	+	+	+
Mannitol	–	+	+	+	+	+
Mannose	–	+	+	+	+	+
Acid production						
Amygdalin	–	+	+	+	+	+
L-Arabinose	–	+	–	+	+	+
D-Arabitol	–	+	+	–	–	–
Arbutin	–	–	+	+	+	+
D-Cellobiose	–	+	–	+	+	–
D-Galactose	–	+	+	+	+	+
Gentiobiose	–	+	+	–	+	+
Inositol	–	+	+	+	+	+
Lactose	–	–	–	+	–	–
Maltose	–	+	+	+	+	+
D-Mannitol	–	+	+	+	+	+

Table 1. cont.

Characteristic	1	2	3	4	5	6
D-Mannose	–	+	+	+	+	+
Melezitose	–	+	–	+	+	–
Melibiose	–	+	+	+	+	+
Potassium 2-ketogluconate	–	+	+	+	+	+
Potassium 5-ketogluconate	–	+	+	+	+	+
Potassium gluconate	–	+	+	+	+	+
Raffinose	–	+	–	+	–	–
Ribose	–	+	+	+	+	+
Salicin	–	+	+	+	+	+
Sorbitol	–	+	–	+	+	–
D-Sorbitol	–	+	–	+	+	–
Starch	–	–	–	–	+	–
Trehalose	–	+	+	+	+	+
Turanose	–	+	–	+	+	+
D-Xylose	–	+	–	+	+	+

and the free-living strains tested concerned the fatty acid C_{17:0} cyclo, which was absent from strain CWBI-2.3^T and present in large amounts in most tested strains. The content of summed feature 3 was particularly high in strain CWBI-2.3^T when compared with the level in the other species tested. Such differences confirmed that strain CWBI-2.3^T represented a distinct and separate species within the genus *Serratia*.

Strain CWBI-2.3^T was highly sensitive to rifampicin, tetracycline and chloramphenicol (<10 µg ml^{–1}). It was sensitive to streptomycin (10 µg ml^{–1}), kanamycin (20 µg ml^{–1}), ampicillin (20 µg ml^{–1}), erythromycin (30 µg ml^{–1}) and gentamicin (40 µg ml^{–1}), but resistant to vancomycin.

Due to their inability to grow axenically, no strain of '*Ca. S. symbiotica*' has been isolated and characterized previously, as required for formal description of the species. Consequently, the taxon has previously been named under the *Candidatus* provision for the informal naming of uncultured representatives of new genera and species (Moran *et al.*, 2005). As strain CWBI-2.3^T is the first strain of '*Ca. S. symbiotica*' to be purified and characterized, it is designated as the type strain of *Serratia symbiotica* sp. nov.

The availability of a pure culture of *S. symbiotica* sp. nov. will provide valuable opportunities to promote the position of the species in the genus *Serratia*. The provisional name '*Candidatus Serratia symbiotica*' has been assigned to many strains residing in several aphid species mainly on the basis of morphological observations and 16S rRNA gene sequence similarities. Even though the 16S rRNA gene sequences of strains of '*Ca. S. symbiotica*' have shown very high similarities, they may actually belong to separate species and so warrant further investigation. The isolation of strain CWBI-2.3^T should also stimulate further studies on the role of this species in aphids.

Table 2. Cellular fatty acid compositions of strain CWBI-2.3^T and the type strains of some related free-living members of the genus *Serratia*

Taxa: 1, strain CWBI-2.3^T; 2, *S. ficaria* LMG 7881^T; 3, *S. entomophila* LMG 8456^T; 4, *S. plymuthica* LMG 7886^T; 5, *S. nematophilus* DZ0503SBS1^T (data from Zhang *et al.*, 2009); 6, *S. odorifera* LMG 7885^T (data from the Sherlock MIS TSBA50 identification library); 7, *S. marcescens* DSM 30121^T. tr, Trace amount (<1.0%); –, not detected; ND, not determined.

Fatty acid	1	2	3	4	5	6	7
C _{10:0}	tr	tr	–	–	–	–	–
C _{10:0} 3-OH	–	–	–	–	tr	–	–
C _{11:0}	–	tr	–	–	–	–	–
C _{12:0}	3.74	5.80	4.73	4.93	2.47	2.95	1.46
C _{13:0}	–	tr	tr	–	–	–	–
C _{12:0} 2-OH	–	1.51	tr	–	tr	tr	tr
C _{12:0} 3-OH	–	–	–	–	tr	–	–
C _{14:0}	8.87	8.14	6.44	7.60	8.40	7.40	6.94
C _{14:0} 2-OH	–	–	2.38	–	1.02	–	2.05
C _{15:0}	–	–	–	–	tr	–	–
C _{15:0} anteiso	tr	–	–	–	–	–	–
C _{16:0}	29.86	24.51	24.47	31.48	34.76	30.74	31.86
C _{16:1} ω5c	tr	–	–	–	–	–	–
C _{17:0} cyclo	–	12.45	10.86	8.71	20.03	4.96	11.68
C _{17:0}	–	2.26	1.85	–	–	–	tr
C _{18:0}	–	tr	tr	1.13	tr	tr	tr
C _{18:1} ω7c	4.22	9.56	10.51	7.69	1.67	14.40	16.88
C _{19:0} iso	–	–	–	–	tr	–	–
C _{19:0} cyclo ω7c	–	–	–	–	17.24	tr	1.24
C _{19:0} cyclo ω8c	–	1.68	tr	–	–	–	–
Unknown ECL* 14.502	tr	–	–	tr	tr	tr	tr
Summed features†							
1	–	tr	tr	–	–	–	–
2	12.20	14.53	15.14	13.94	7.56	8.98	8.44
3	40.11	13.71	19.53	23.84	tr	28.04	16.51

*ECL, Equivalent chain-length.

†Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 comprises C_{13:0} 3-OH and/or iso-C_{15:1} I/H; summed feature 2 comprises C_{12:0} alde, unknown ECL 10.928, iso-C_{16:1} and C_{14:0} 3-OH; summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1} ω7c.

The inactive biochemical profile of the novel species suggests that the loss of metabolic diversity could be gradual and it may be that phenotypic diversity may exist between strains of *S. symbiotica*. This diversity is probably a result of an overall evolution within the species which forms a continuum from partially free-living strains, such as strain CWBI-2.3^T, to the highly host-dependent strain recently described in *Cinara* aphids (Pérez-Brocail *et al.*, 2006).

Description of *Serratia symbiotica* sp. nov.

Serratia symbiotica [sym.bi.o'ti.ca. N.L. fem. adj. *symbiotica* (from Gr. n. *symbios* a companion, partner) living together].

Cells are motile, Gram-negative-staining rods (0.5 × 0.8–1.3 µm). Grows axenically on a rich medium containing glucose, casein peptone and yeast extract. Growth is optimal in aerobic conditions but growth also occurs in micro-aerobic conditions. On nutrient agar, colonies are circular, non-pigmented, off-white and smooth and shiny with entire margins. Cells grow at 10–30 °C (optimum, 25 °C), pH 6–8 (optimum, 7) and with 0–4% (w/v) NaCl. Positive in tests for catalase and the Voges–Proskauer reaction. Negative result in tests for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, gelatinase, DNase, lipase (Tween 80), lecithinase, chitinase, caseinase and for the methyl red test. Assimilation and acidification tests, performed on API 20NE and API 50CH strips, give a positive result for the assimilation of glucose and *N*-acetylglucosamine. Weakly positive for acid production from glucose, sucrose and aesculin. Negative result for all remaining tests present on API 20NE and API 50CH strips. Whole-cell fatty acids are C_{10:0}, C_{12:0}, C_{14:0}, an unknown fatty acid with equivalent length 14.502, C_{15:0} anteiso, C_{16:1} ω5c, C_{16:0}, C_{18:1} ω7c, summed feature 2 (comprising C_{12:0} alde, unknown fatty acid with equivalent length 10.928, and C_{14:0} 3-OH/C_{16:1} iso I) and summed feature 3 (comprising C_{16:1} ω7c/C₁₅ iso 2-OH).

The type strain is CWBI-2.3^T (=LMG 25624^T=DSM 23270^T). The type strain is the R-type of the secondary endosymbiont associated symbiotically with a natural clone of *Aphis fabae* collected in Belgium. The bacterium lives in the host body and can be found in some host tissues and fluids, mostly in the bacteriosome in the cytoplasm of the sheath cells. The DNA G + C content of the type strain is 52.7 mol%.

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References

- Ajithkumar, B., Ajithkumar, V. P., Iriye, R., Doi, Y. & Sakai, T. (2003). Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank. *Int J Syst Evol Microbiol* **53**, 253–258.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Andersson, S. G. E. & Kurland, C. G. (1998). Reductive evolution of resident genomes. *Trends Microbiol* **6**, 263–268.
- Baumann, P., Moran, N. & Baumann, L. (2000). Bacteriocyte-associated endosymbionts of insects. In *The Prokaryotes, a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*. Edited by M. Dworkin. New York: Springer-Verlag.

- Bhadra, B., Roy, P. & Chakraborty, R. (2005). *Serratia ureilytica* sp. nov., a novel urea-utilizing species. *Int J Syst Evol Microbiol* **55**, 2155–2158.
- Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. Edited by J. W. Sons. New York: Interscience Publishers.
- Burke, G. R., Normark, B. B., Favret, C. & Moran, N. A. (2009). Evolution and diversity of facultative symbionts from the aphid subfamily Lachninae. *Appl Environ Microbiol* **75**, 5328–5335.
- Burke, G., Fiehn, O. & Moran, N. (2010). Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *ISME J* **4**, 242–252.
- Chen, D. Q., Montllor, C. B. & Purcell, A. H. (2000). Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrtosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. *Entomol Exp Appl* **95**, 315–323.
- Dale, C. & Maudlin, I. (1999). *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *Int J Syst Bacteriol* **49**, 267–275.
- Dale, C. & Moran, N. A. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell* **126**, 453–465.
- Darby, A. C., Chandler, S. M., Welburn, S. C. & Douglas, A. E. (2005). Aphid-symbiotic bacteria cultured in insect cell lines. *Appl Environ Microbiol* **71**, 4833–4839.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Farmer, J. J., III, Davis, B. R., Hickman-Brenner, F. W., McWhorter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, C., Wathen-Grady, H. G., Elias, C. & other authors (1985). Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J Clin Microbiol* **21**, 46–76.
- Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–791.
- Fukatsu, T., Nikoh, N., Kawai, R. & Koga, R. (2000). The secondary endosymbiotic bacterium of the pea aphid *Acyrtosiphon pisum* (Insecta: homoptera). *Appl Environ Microbiol* **66**, 2748–2758.
- Gavini, F., Ferragut, C., Izard, D., Trinel, P. A., Leclerc, H., Lefebvre, B. & Mossel, D. A. A. (1979). *Serratia fonticola*, a new species from water. *Int J Syst Bacteriol* **29**, 92–101.
- Geiger, A., Fardeau, M.-L., Falsen, E., Ollivier, B. & Cuny, G. (2010). *Serratia glossinae* sp. nov., isolated from the midgut of the tsetse fly *Glossina palpalis gambiensis*. *Int J Syst Evol Microbiol* **60**, 1261–1265.
- Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31–36.
- Gherna, R. L., Werren, J. H., Weisburg, W., Cote, R., Woese, C. R., Mandelco, L. & Brenner, D. J. (1991). *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. *Int J Syst Bacteriol* **41**, 563–565.
- Gil, R., Sabater-Muñoz, B., Latorre, A., Silva, F. J. & Moya, A. (2002). Extreme genome reduction in *Buchnera* spp.: toward the minimal genome needed for symbiotic life. *Proc Natl Acad Sci U S A* **99**, 4454–4458.
- Gómez-Valero, L., Soriano-Navarro, M., Pérez-Brocal, V., Heddi, A., Moya, A., García-Verdugo, J. M. & Latorre, A. (2004). Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *J Bacteriol* **186**, 6626–6633.
- Grimont, P. A. D., Grimont, F. & Irino, K. (1982). Biochemical characterization of *Serratia liquefaciens sensu stricto*, *Serratia proteamaculans*, and *Serratia grimesii* sp. nov. *Curr Microbiol* **7**, 69–74.
- Grimont, P. A. D., Jackson, T. A., Ageron, E. & Noonan, J. M. (1988). *Serratia entomophila* sp. nov. associated with amber disease in the New Zealand grass grub *Costelytra zealandica*. *Int J Syst Bacteriol* **38**, 1–6.
- Hypša, V. & Dale, C. (1997). *In vitro* culture and phylogenetic analysis of “*Candidatus Arsenophonus triatominarum*”, an intracellular bacterium from the triatomine bug, *Triatoma infestans*. *Int J Syst Bacteriol* **47**, 1140–1144.
- Lamelas, A., Pérez-Brocal, V., Gómez-Valero, L., Gosalbes, M. J., Moya, A. & Latorre, A. (2008). Evolution of the secondary symbiont “*Candidatus Serratia symbiotica*” in aphid species of the subfamily Lachninae. *Appl Environ Microbiol* **74**, 4236–4240.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Montllor, C. B., Maxmen, A. & Purcell, A. H. (2002). Facultative bacterial endosymbionts benefit pea aphids *Acyrtosiphon pisum* under heat stress. *Ecol Entomol* **27**, 189–195.
- Moran, N. A. & Wernegreen, J. J. (2000). Lifestyle evolution in symbiotic bacteria: insights from genomics. *Trends Ecol Evol* **15**, 321–326.
- Moran, N. A., Russell, J. A., Koga, R. & Fukatsu, T. (2005). Evolutionary relationships of three new species of *Enterobacteriaceae* living as symbionts of aphids and other insects. *Appl Environ Microbiol* **71**, 3302–3310.
- Moran, N. A., McCutcheon, J. P. & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet* **42**, 165–190.
- Oliver, K. M., Russell, J. A., Moran, N. A. & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci U S A* **100**, 1803–1807.
- Pérez-Brocal, V., Gil, R., Ramos, S., Lamelas, A., Postigo, M., Michelena, J. M., Silva, F. J., Moya, A. & Latorre, A. (2006). A small microbial genome: the end of a long symbiotic relationship? *Science* **314**, 312–313.
- Pontes, M. H. & Dale, C. (2006). Culture and manipulation of insect facultative symbionts. *Trends Microbiol* **14**, 406–412.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Scarborough, C. L., Ferrari, J. & Godfray, H. C. J. (2005). Aphid protected from pathogen by endosymbiont. *Science* **310**, 1781.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Tsuchida, T., Koga, R. & Fukatsu, T. (2004). Host plant specialization governed by facultative symbiont. *Science* **303**, 1989.
- Tsuchida, T., Koga, R., Sakurai, M. & Fukatsu, T. (2006). Facultative bacterial endosymbionts of three aphid species, *Aphis craccivora*, *Megoura crassicauda* and *Acyrtosiphon pisum*, sympatrically found on the same host plants. *Appl Entomol Zool (Jpn)* **41**, 129–137.
- Unterman, B. M., Baumann, P. & McLean, D. L. (1989). Pea aphid symbiont relationships established by analysis of 16S rRNAs. *J Bacteriol* **171**, 2970–2974.
- Ventura, M., Elli, M., Reniero, R. & Zink, R. (2001). Molecular microbial analysis of *Bifidobacterium* isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Microbiol Ecol* **36**, 113–121.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Zhang, C. X., Yang, S. Y., Xu, M. X., Sun, J., Liu, H., Liu, J. R., Liu, H., Kan, F., Sun, J. & other authors (2009). *Serratia nematodiphila* sp. nov., associated symbiotically with the entomopathogenic nematode *Heterorhabditoides chongmingensis* (Rhabditida: Rhabditidae). *Int J Syst Evol Microbiol* **59**, 1603–1608.