Characterization of *Serratia* isolates from soil, ecological implications and transfer of *Serratia proteamaculans* subsp. *quinovora* Grimont *et al.* 1983 to *Serratia quinivorans* corrig., sp. nov.

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Eleven strains of *Serratia* were isolated from different soils and the guts of invertebrates and characterized by their sensitivity to eight indigenous bacteriophages. They were also classified according to bacteriocin production and sensitivity, BiOLOG plate and API 20E strip profiles and 16S rRNA sequence information. One strain was thus identified as *Serratia plymuthica*, another as *Serratia fonticola*. The remaining strains were shown to be closely related to *Serratia proteamaculans* subsp. *quinovora* Grimont *et al.* 1983 after DNA–DNA cross-hybridization demonstrated relatedness greater than 70% with the type strain of this subspecies. From an ecological perspective, our results illustrated the wide variation in sensitivity that closely related *Serratia* strains have towards various indigenous soil phages and that these phages have broad host ranges within the genus. Furthermore, the phage and bacteriocin interactions within the *Serratia* strains examined were intricate and did not reflect phylogenetic relationships. These results together imply that complex interactions will occur in soil within the natural community of *Serratia* strains and their bacteriophages. DNA–DNA cross-hybridization and phenotypic characterization showed that *S. proteamaculans* subsp. *quinovora* strains formed a cohesive group at the species level. It is therefore concluded that these strains should be designated as *Serratia quinivorans* corrig., sp. nov.

Keywords: *Serratia*, DNA–DNA hybridization, 16S rRNA gene, bacteriophages, bacteriocins

INTRODUCTION

Serratia appears to be a ubiquitous genus in nature, and ten species are currently recognized (Grimont & Grimont, 1992). *Serratia* species have been isolated from water, soil, animals (including man) and from the surfaces of plants (Grimont & Grimont, 1992). Typical phytosphere isolates belong to the ‘*Serratia liquefaciens*–*proteamaculans*–*grimesii*’ complex (Grimont *et al.*, 1981), also called *S. liquefaciens*-like

species (Grimont *et al.*, 1982b). Strains of these species produce plant-growth-promoting chemicals, have anti-fungal properties, encourage the establishment of nitrogen-fixing symbionts and act as insect pathogens (Grimont & Grimont, 1992; Kalbe *et al.*, 1996; Zhang *et al.*, 1996, 1997). A greater understanding of the ecology of these bacteria is required if the impact of *Serratia* species on agriculture is to be adequately understood and, if necessary, manipulated. A potentially important controlling factor for bacterial populations is predation by bacteriophages (phages).

In previous studies, we characterized a population of phages that preyed on indigenous *Serratia* strains in the rhizosphere of sugar beet (Ashelford *et al.*, 1999a, b, 2000). This phage population was complex, both in terms of its temporal population dynamics and its diversity. The host species studied was a rhizosphere-derived strain named CP6 and originally

Full tables of results for bacteriocin production and DNA–DNA cross-reaction for the novel isolates are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org>).

Abbreviations: FAME, fatty acid methyl ester; SSM, *Serratia* selective medium; TSBA, tryptone soy broth agar.

The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA sequences analysed in this paper are AJ279044–AJ279055.

identified by fatty acid methyl ester (FAME) analysis as *S. liquefaciens*. In the current study, we aim to describe the isolation, enumeration, characterization and biodiversity of further examples of this bacterium from the same environment and from two other sites. Furthermore, we also investigate the identification and taxonomy of these isolates within the current understanding of the genus *Serratia* as a whole.

METHODS

Bacteria and bacteriophages. Bacteria used in this study (Table 1) were stored in 50% glycerol at -80°C and maintained on nutrient agar (Oxoid, CM3). The phages used were $\Phi\text{CP6-1}$ to $\Phi\text{CP6-6}$ (Ashelford *et al.*, 1999b) and two further phages, $\Phi\text{CP6-8}$ and $\Phi\text{CP6-9}$, isolated during this study. Fresh phage lysates were prepared by co-inoculating nutrient broth with phage and host bacterium and incubating overnight with agitation at 20°C . Cultures were centrifuged for 10 min at 4000 r.p.m. and their supernatants were filtered (0.22 μm pore size) to produce final lysates, which were stored at 4°C or under 50% glycerol at -80°C . CP6 (Ashelford *et al.*, 1999a) was used as the host bacterium for all phages except for $\Phi\text{CP6-8}$ and $\Phi\text{CP6-9}$, where strains CP6b and CP6d, respectively, were used. Phage lysates were titrated with the overlay-agar method of Adams (1959) using 1.3% (w/v) nutrient broth (Oxoid, CM1) solidified with 0.65% (w/v) agar (Oxoid, L11) as overlay on a nutrient agar base.

Isolating CP6-like bacteria from the environment. A crop of sugar beet (*Beta vulgaris* var. Amethyst) was grown at Wytham Farm, Oxford, from seeds (EB3 pellets; Germains UK, Kings Lynn, UK) sown on 16 May 1997. The resulting plants were sampled and homogenized as described previously (Ashelford *et al.*, 1999a). Fresh and freezer-stored homogenates were plated onto *Serratia* selective medium (SSM; Ashelford *et al.*, 2000) and incubated for 48 h at 30°C to select for bacteria that resembled CP6. Estimates of total viable heterotrophic bacteria were obtained by plating serial dilutions of the homogenates onto tryptone soya broth agar (TSBA) incubated for 7 days at 15°C . Three separate leaf-litter samples were collected from Oxleas wood, a

deciduous woodland in Kent, UK, and homogenates were plated onto SSM. Invertebrates in the samples, and the gut contents of earthworms from scrubland in Cornwall, were also homogenized and plated onto SSM.

Testing CP6-like colonies for phage sensitivity. Colonies on SSM plates with the typical CP6 morphology (1 mm orange colonies with yellow borders) were purified on TSBA and screened for phage sensitivity using overlay-agar plates. Phage lysates (5 μl ; $\Phi\text{CP6-1}$ to -9) were dropped onto each plate and examined for zones of lysis after overnight incubation at 15°C . CP6-like isolates were grouped by sensitivity to the phage lysates and representatives of these 'sensitivity-groups' were selected for analysis. In addition, two representative isolates that appeared CP6-like on SSM but not on TSBA were also studied further.

Phage sensitivity of CP6 isolates and various *Serratia* species representatives (Table 1) was quantified using fresh overlay-agar plates. Lysates were serially diluted and drop-plated (10 μl) in triplicate onto these plates and incubated overnight at 15°C and plaques were counted. All titrations were done in duplicate.

Assessing the host range of bacteriocins produced by the strains. All bacteria were checked for bacteriocin production and lysogeny. Nutrient-broth cultures were grown overnight under constant agitation at 20°C and 100 μl was used to inoculate overlay-agar plates. The remainder was centrifuged at 4000 r.p.m. for 10 min and the supernatant passed through a 0.22 μm -pore filter. Each filtered supernatant was then drop-plated (5 μl) onto the overlay plates. Plates were scored after overnight incubation at 15°C for the presence of zones of lysis. To confirm that lysis was not due to phage infection, the supernatants were serially diluted and plated to confirm that the zones would not resolve into individual plaques.

Probing bacterial DNA with DIG-labelled phage probes. DNA was extracted from phages $\Phi\text{CP6-1}$ to -6 by a proteinase-K method (Day & Marchesi, 1996) and resuspended in TE buffer (Sambrook *et al.*, 1989). DNA was digested with *EcoRI* according to the manufacturer's instructions (Promega), purified with buffered phenol/chloroform/isoamyl alcohol (25:24:1; Sambrook *et al.*, 1989) and randomly labelled with DIG using DIG-High Prime

Table 1. Bacterial strains used in this study

Culture collections are abbreviated as: ATCC, American Type Culture Collection; BCCM, Belgian Co-ordinated Collections of Microorganisms; CIP, Collection of bacterial strains of Institut Pasteur; NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures.

Strain(s)	Comments	Source
CP6 (= NCTC 13188)	Sugar-beet isolate from Wytham Farm, Oxford	Ashelford <i>et al.</i> (1999a)
CP6a, CP6b, CP6c, CP6d, CP6e (= NCTC 13190), CP6f, CP6g	Sugar-beet isolates from Wytham Farm, Oxford	This study
PC1 (= NCTC 13191)	Earthworm-gut isolate from scrubland, Cornwall	This study
RK (= NCTC 13192)	Sugar-beet isolate from Wytham Farm, Oxford	This study
Ox1	Leaf-litter isolate from Oxleas wood, Kent	This study
Ox2 (= NCTC 13194)	Invertebrate-gut isolate from Oxleas wood, Kent	This study
<i>Serratia marcescens</i> NCIB 1377		NCIB
<i>Serratia ficaria</i> NCTC 12148 ^T	Type strain	NCTC
<i>Serratia liquefaciens</i> CIP 103238 ^T	Type strain	NCTC
<i>Serratia proteamaculans</i> subsp. <i>proteamaculans</i> CIP 103236 ^T	Type strain	NCTC
<i>Serratia proteamaculans</i> subsp. <i>quinovora</i> LMG 7887 ^T	Type strain	BCCM
<i>Serratia grimesii</i> CIP 103361 ^T	Type strain	BCCM
<i>Serratia plymuthica</i> CIP 103239 ^T	Type strain	CIP
<i>Serratia fonticola</i> IB4r (= NCTC 13193)	Isolated from pilot-scale sewage bed	Ashelford <i>et al.</i> (2001)
<i>Serratia fonticola</i> LMG 7882 ^T	Type strain	BCCM
<i>Hafnia alvei</i> NCTC 8105 ^T	Type strain	NCTC

(Roche). Labelled DNA was then mixed together, in equal proportions, to produce a final DIG-labelled phage-DNA cocktail containing all six CP6 phages.

DNA was extracted from all bacteria using the cetyltrimethylammonium bromide (CTAB) method of Powell *et al.* (1993). DNA from four CP6 lysogens (CP6-ly-Φ1, CP6-ly-Φ2, CP6-ly-Φ3, CP6-ly-Φ5; Ashelford *et al.*, 1999b) was extracted as positive controls. DNA extracts were transferred onto positively charged nylon membrane (Roche) using a slot-blotter and the membrane was probed with the DIG-labelled phage DNA at 68 °C using standard hybridization buffer [5 × SSC; 1% (w/v) blocking reagent; 0.1% (w/v) *N*-lauroylsarcosine, sodium salt; 0.02% SDS] following the manufacturer's methodology.

Phenotype-based identification of representative isolates. All strains (Table 1) were characterized phenotypically using the API 20E (bioMérieux) and BiOLOG GN microplate (Biolog) identification systems. The sugar-beet isolates CP6 and CP6a–CP6g were also characterized in triplicate by FAME and cluster analysis. Isolates CP6 and CP6e were additionally characterized using the Biotype 100 system (bioMérieux). This was undertaken for the authors by the BCCM/LMG (Belgian Co-ordinated Collections of Micro-organisms; Laboratorium voor Microbiologie, Universiteit Gent, Belgium) in conjunction with Professor P. A. D. Grimont. Biotype growth profiles were compared to the 'Enterobacteriaceae '94' database and strain identities with Recognizer (Institut Pasteur Taxolab). CP6-like isolates and some type strains of *Serratia* species were also tested for growth on M70 minimal agar with 0.1% (w/v) quinic acid (Sigma) at 30 °C for 14 days (Grimont & Grimont, 1984).

16S rRNA amplification and sequencing. Genomic DNA was extracted using the CTAB method and amplified by PCR with primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al.*, 1998). The PCR products (approx. 1300 bp) were cloned using the pGEM-T Easy vector system (Promega) and sequenced from each end with M13 primers on a LI-COR DNA4000L sequencer (MWG-Biotech), using fluorescent-primer labelling with near-infrared IRD800, according to the manufacturer's instructions.

Phylogenetic analysis of 16S rRNA sequences. Sequences were aligned with those of *Serratia* and other members of the *Enterobacteriaceae* from the DDBJ/EMBL/GenBank databases with CLUSTAL W (Thompson *et al.*, 1994). Only sequences > 1300 bp were used and we ensured that a representative of each available enterobacterial genus was included in our analysis. After editing the aligned dataset to remove gaps, unknown bases and obvious mismatches, evolutionary distances were calculated with the Jukes and Cantor algorithm. Phylogenetic trees were determined by the neighbour-joining method using TREECON for Windows (Van de Peer & De Wachter, 1994). For comparison, additional phylogenetic trees were constructed using other tree-estimation methods. The Fitch–Margoliash method was applied to Jukes and Cantor-derived distance matrices using the programs FITCH and KITSCH of the PHYLIP computer package (Felsenstein, 1993). From the same package, the programs DNAML and DNAMLK were also used to calculate tree topology by maximum-likelihood, whilst DNAPARS was used for parsimony analysis.

DNA–DNA cross-hybridization. This was undertaken on the authors' behalf by BCCM/LMG. DNA was prepared from strains CP6 and CP6e, *Serratia plymuthica* CIP 103239^T, *Serratia grimesii* CIP 103361^T, *S. liquefaciens* CIP 103238^T,

Serratia proteamaculans subsp. *proteamaculans* CIP 103236^T, *S. proteamaculans* subsp. *quinovora* LMG 7887^T and *Serratia fonticola* LMG 8772^T using the methods of Pitcher *et al.* (1989) and Wilson (1987).

Two DNA–DNA cross-hybridization methods were used. Strains CP6 and CP6e, *S. plymuthica*, *S. grimesii*, *S. liquefaciens*, *S. proteamaculans* subsp. *proteamaculans* and *S. fonticola* were cross-hybridized with each other using the fluorimetric method of Ezaki *et al.* (1989). Hybridizations were performed under high-stringency conditions in 50% formamide at 48 °C. Strains CP6 and CP6e, *S. proteamaculans* subsp. *proteamaculans* and *S. proteamaculans* subsp. *quinovora* were cross-hybridized with each other using the initial renaturation method of De Ley *et al.* (1970), undertaken at 75 °C. All hybridizations were performed in both directions and in duplicate and the mean percentage DNA relatedness values were determined.

Statistical analysis of data. Statistics were performed with the MINITAB version 12 computer package. Mean phage titres were compared by analysis of variance after log₁₀ transformation and the minimum significant difference (MSD) between means was calculated (Fry, 1993). Bacteriocin profiles of the various bacteria were investigated by cluster analysis. Jaccard's coefficients (*S_j*) were calculated by hand. Average linkage clustering (UPGMA) was then applied to the resulting distance matrix using the PHYLIP package.

The phenotypic profiles of bacteria from API strips and BiOLOG plates were also analysed by cluster analysis. The positive/negative scorings from both tests were pooled for each bacterium tested, a simple matching coefficient (*S_{sm}*) matrix was constructed from the resulting binary dataset and a cluster analysis was performed with UPGMA.

RESULTS AND DISCUSSION

Initial screening for CP6-like bacteria

Sugar beets were sampled 25, 52 and 82 days after sowing. Overall, 0.045% of the viable bacteria counted were CP6-like and 78.6% of these putative CP6-like bacteria (*n* = 262) possessed the correct morphology when grown on TSBA and were therefore investigated further. Of these, 84.0% had varying degrees of sensitivity to the CP6-phage lysates. Seven patterns of phage sensitivity were identified, ranging from successful infection by most of the phages to insensitivity to all phages. A representative of each of these seven groups (CP6a–CP6g) was selected for subsequent analysis.

CP6-like bacteria were also isolated from samples taken from Oxleas wood in Kent and from scrubland in Cornwall. Although morphologically identical, these had phage-sensitivity patterns different from any of those identified from the Wytham Farm isolates. Isolates Ox2 (from Oxleas wood) and PC1 (from the earthworm) were chosen to represent these bacterial strains.

Finally, two further isolates, named RK and Ox1, were also selected for further investigation. Isolate RK was one of several isolates from stored 1996 sugar-beet samples from Wytham Farm which appeared CP6-like in all respects except for its red colony pigmentation on

Table 2. Sensitivity of environmental isolates and selected type and reference strains to various CP6 bacteriophages

Values are means of two replicate titrations. The MSD (Fry, 1993) between $\log_{10}$ means at $P = 0.05$ was 0.521. L, Lysis characteristic of bacteriocins. Not all of the lysis observed was due to successful phage infection. Frequently, neat lysates would lyse bacterial lawns but, when diluted, their lytic effect would disappear completely rather than resolve into individual plaques, indicating bacteriocin, rather than phage, activity. —, No lysis observed. Isolate Ox1, *S. fonticola* IB4r and *H. alvei* NCTC 8105^T were insensitive to all the phages tested. Furthermore, *S. proteamaculans* subsp. *proteamaculans* CIP 103236^T and *S. grimesii* CIP 103361^T had the same sensitivity as *S. liquefaciens* CIP 103238^T.

Strain	Mean titre [$\log_{10}$ (p.f.u. ml ⁻¹)]							
	ΦCP6-1	ΦCP6-2	ΦCP6-3	ΦCP6-4	ΦCP6-5	ΦCP6-6	ΦCP6-8	ΦCP6-9
Environmental isolates								
CP6	9.256	9.528	9.580	9.996	9.153	7.971	L	—
CP6a	8.860	9.054	L	9.946	8.942	8.023	L	—
CP6b	L	L	L	9.317	L	L	9.662	—
CP6c	L	L	L	L	L	6.384	—	—
CP6d	L	L	L	L	L	L	—	10.042
CP6e	—	—	—	—	—	—	L	10.190
CP6f	—	—	L	8.314	—	L	—	—
CP6g	—	—	L	—	—	—	L	—
PC1	L	L	L	L	L	L	—	—
Ox2	L	L	L	L	L	9.150	6.688	—
RK	L	L	L	L	L	L	—	9.872
Type and reference strains								
<i>S. marcescens</i> NCIB 1377	—	—	—	—	—	L	—	—
<i>S. ficaria</i> NCTC 12148 ^T	—	—	—	—	—	L	L	8.302
<i>S. liquefaciens</i> CIP 103238 ^T	L	L	L	L	L	L	—	—
<i>S. proteamaculans</i> subsp. <i>quinovora</i> LMG 7887 ^T	L	L	L	4.477	L	L	L	—
<i>S. plymuthica</i> CIP 103239 ^T	L	L	L	L	L	9.444	—	8.710

TSBA when incubated at less than 30 °C. Isolate RK was notable in showing definite sensitivity to the lysate of phage ΦCP6-9. In contrast, Ox1, an isolate from Oxleas wood, was typical of isolates that appeared CP6-like on SSM but did not show CP6-like colony morphology when grown on TSBA and were insensitive to all of the phages.

Quantifying phage sensitivity of environmental isolates and type and reference strains

All 11 isolates described above, the original CP6 strain and various type and reference strains were titrated against all the phages in our collection. The resulting mean titres (Table 2) showed that, not only were the various CP6-like isolates sensitive to different combinations of phages, but there was significant variation in sensitivity. For example, isolates CP6, CP6a, CP6b and CP6f were all sensitive to phage ΦCP6-4, yet CP6f was significantly less sensitive than CP6b, which in turn was significantly less sensitive than CP6 and CP6a (Table 2). This variation in sensitivity frequently coincided with variations in plaque morphology. Thus, ΦCP6-4 produced large, clear plaques surrounded by several concentric rings on CP6 (Ashelford *et al.*, 1999b), smaller plaques without rings on CP6a and tiny, barely discernible plaques on CP6b and CP6f.

Type strains of *S. proteamaculans* subsp. *quinovora*, *Serratia ficaria* and *S. plymuthica* were also susceptible to some of the phages tested, as was the red-pigmented isolate, RK (Table 2). All other strains were insensitive to successful phage infection, although considerable amounts of non-specific cell lysis were observed.

Bacteriocin production and sensitivity of isolates and type and reference strains

Most of the environmental isolates produced detectable bacteriocins, as did many of the type and reference strains tested (a full table of data is available as supplementary material in IJSEM Online at <http://ijs.sgmjournals.org/>). Bacteriocin production was most noticeable when supernatants were harvested from bacteria grown at 20 °C rather than 30 °C. There was a large variation in the degree of lysis observed, ranging from total clearing of the bacterial lawn to turbid zones that were only just discernible. On only two occasions was this lysis the result of phage infection. This was when CP6g was inoculated with neat supernatant from CP6d and when CP6d was inoculated with neat supernatant from the *S. proteamaculans* subsp. *proteamaculans* type strain. On both of these occasions, a faint scattering of tiny plaques was seen. These plaques were few in number

Fig. 1. Cluster analysis of bacteria according to bacteriocin sensitivity. A distance matrix of Jaccard's coefficients (S_j) was clustered according to the UPGMA algorithm. Other tree-construction methods gave similar tree topologies.

and were barely discernible. In all other cases, serial dilution of the lytic supernatants failed to resolve the observed zones of lysis into individual plaques; rather, the lysis effect disappeared between successive dilutions, a phenomenon characteristic of bacteriocin activity (Day, 1997).

All the bacteria, with the exception of *Hafnia alvei*, were sensitive to at least some of the bacteriocins produced, and each bacterium tested had a different sensitivity profile. A cluster analysis of these data is presented in Fig. 1.

Probing whole-genome extracts with DIG-labelled phage DNA

To assay prophages, total genomic bacterial DNA was probed with DIG-labelled DNA from phages Φ CP6-1 to -6. Positive cross-hybridizations were only observed for the four lysogen controls, the CP6-like isolate Ox2, *S. fonticola* IB4r and the *S. liquefaciens* and *S. proteamaculans* subsp. *quinovora* type strains. A very weak hybridization signal was also obtained with the type strain of *S. grimesii*.

16S rRNA gene sequence analysis

The cloned 16S rRNA gene from strain CP6 was sequenced three times in both directions and the resulting sequences were compared to assess sequencing error. Sequences varied by 9 bases at most prior to editing and this resulted in an uncorrected evolutionary distance of 0.2% in the edited dataset. A consensus sequence of 1360 bp was constructed (ac-

cession no. AJ279044) and compared with sequences generated (sequenced once in both directions) from the other isolates along with those from the DDBJ/EMBL/GenBank databases.

Sequences from isolates CP6a–CP6d, CP6g and PC1 showed similarities of 99.7–99.9% towards CP6 and were thus unambiguously confirmed to be members of the same species. The CP6f sequence was 99.5% similar, showing that it too was most likely to belong to the same species as CP6. Isolate CP6e showed the greatest divergence, at 99.2% similarity (14 bases different before editing). The non-CP6-like isolates RK and Ox1 were respectively 1.2 and 2.1% distant, corresponding to 21 and 33 unedited base differences from CP6.

Comparisons with sequences from the public databases revealed the CP6 sequence to be almost identical to that of the *S. proteamaculans* subsp. *quinovora* type strain (99.9% similarity). The next closest sequence was from *S. proteamaculans* subsp. *proteamaculans* DSM 4543^T (99.5%) followed by *S. grimesii* DSM 30063^T (99.3%). This order of similarity was repeated for all the CP6-like bacterial sequences with the exception of CP6e, which was most similar to *S. proteamaculans* subsp. *proteamaculans* DSM 4543^T (99.7%), then *S. grimesii* DSM 30063^T (99.5%) and only then *S. proteamaculans* subsp. *quinovora* DSM 4597^T (99.2%). Of the non-CP6-like bacteria, the red-pigmented isolate RK was closest to *S. plymuthica* DSM 4540^T (99.8%) and, equally, isolate Ox1 was closer to *S. fonticola* DSM 4576^T (99.5%) than any other sequence.

All but one of the CP6-like bacteria clustered unambiguously with *S. proteamaculans* subsp. *quinovora* DSM 4597^T. The exception, CP6e, clustered with *S. proteamaculans* subsp. *proteamaculans* DSM 4543^T and *S. grimesii* DSM 30063^T (Fig. 2). Isolate RK grouped closely with *S. plymuthica* DSM 4540^T, whilst Ox1 clustered unequivocally with *S. fonticola* DSM 4576^T. Other tree-constructing methods were employed to test the rigour of these groupings (data not shown). Although the overall topology of the genus *Serratia* sometimes varied in these trees, all but the unrooted parsimony method of Felsenstein (1993) were consistent in clustering the environmental isolates with the same type strains as seen in Fig. 2.

Sequence analysis of 16S rRNA genes was very consistent in identifying CP6 and all but one (CP6e) isolate as *S. proteamaculans* subsp. *quinovora*, despite its frequent unreliability for distinguishing bacteria beyond the genus level (Fox *et al.*, 1992; Wayne *et al.*, 1987). However, this was only achieved once reliable sequences for all the known *Serratia* species had become available from the DDBJ/EMBL/GenBank databases (after Spröer *et al.*, 1999), highlighting that this form of identification can only be as good as the public database upon which it relies.

Considering the genus *Serratia* as a whole, our 16S rRNA gene sequence phylogenetic tree (Fig. 2) shows

Fig. 2. Phylogenetic reconstruction of the genus *Serratia* based on edited 16S rRNA sequence data (1312 bp) illustrating the phylogeny of the environmental strains isolated during this study, highlighted in bold. Sequences for type strains were from Spröer *et al.* (1999). The tree was constructed from a Jukes and Cantor distance matrix using the neighbour-joining method and was rooted with *Buchnera aphidicola* (accession no. L18927; not shown). The dataset was bootstrapped 100 times; only bootstrap values 50% or greater are shown. Accession numbers for sequences are shown in parentheses. Bar, 2 substitutions per 100 nucleotides.

H. alvei splitting the genus *Serratia*; other enterobacterial genera (*Obesumbacterium*, *Rahnella*, *Yersinia*, *Ewingella*, *Budvicia*, *Pragia* and *Leminorella*) also split the genus *Serratia* in this manner. This genus-splitting phenomenon is consistent with the 16S rRNA gene sequence trees published by Spröer *et al.* (1999) and Dauga (2002). However, using *gyrB* sequences, Dauga (2002) found that all the *Serratia* species formed a monophyletic group supported by high bootstrap values and phenotypic information. So, 16S rRNA gene sequences do not give an accurate picture of the more deeply branching phylogenies within *Enterobacteriaceae*.

Whole-genome DNA–DNA cross-hybridization

Isolate CP6 shared 100% DNA relatedness with *S. proteamaculans* subsp. *quinovora*, conclusively identifying CP6. Consistent with this identification, isolate CP6 exhibited DNA relatedness values with the other type strains at values well below the value of 70% required to signify species identity (Wayne *et al.*, 1987). A table with full results for all the DNA–DNA cross-hybridizations carried out is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Isolate CP6e was the only CP6-like bacterium that failed to cluster closely with CP6 during 16S rRNA

Fig. 3. Cluster analysis of bacteria according to API 20E and BiOLOG profiles. A distance matrix of simple similarity coefficients (S_{sm}) was clustered with the UPGMA algorithm. Other tree-construction methods gave similar tree topologies.

sequence analysis. The mean DNA–DNA relatedness between isolates CP6 and CP6e was 88%, so they are members of the same species, in spite of the 16S rDNA sequence analysis. Moreover, CP6e showed greater than 70% relatedness with *S. proteamaculans* subsp. *quinovora* (85%) and less than 70% relatedness with *S. proteamaculans* subsp. *proteamaculans* (63–68%). Cross-hybridizations amongst the type strains were consistently less than 70% similar (19–57%); this included the two *S. proteamaculans* subspecies (46–68%).

Identification methods based on phenotype

API 20E strips failed to identify many of the isolates, and those for which an identification was obtained could only be identified unambiguously at the genus level. Isolates CP6d, CP6e, PC1 and Ox2 were tentatively identified as *S. liquefaciens*, with the percentage identities ranging from 73 to 76.2%. Isolate RK was identified as either *Serratia rubidaea* or *S. plymuthica* at 46 and 42.6%, respectively. Isolate Ox1 was identified as *S. fonticola* at 31.6%. Using BiOLOG plates, all but one of the CP6-like bacteria were identified as *S. liquefaciens*/*S. grimesii*, with similarity values ranging from 0.548 to 0.970. The exception was CP6b, being identified as *Serratia marcescens* (0.743). Isolate RK was identified as *S. plymuthica* at 0.610, whilst Ox1 was identified as *S. fonticola* at 0.468. A cluster analysis of the combined API 20E and BiOLOG profiles was attempted to determine whether identities could be further clarified (Fig. 3). All but one of the CP6-like isolates clustered closely with *S. liquefaciens*

CIP 103238^T and *S. proteamaculans* subsp. *quinovora* LMG 7887^T, the exception being isolate Ox2. A cluster analysis of the FAME profiles of CP6a–CP6g with CP6 indicated they were the same (data not shown). Like CP6 (similarity coefficient, 0.774), they were identified by FAME analysis as *S. liquefaciens*/*S. proteamaculans* (Ashelford *et al.*, 1999a).

Overall, these three phenotypic identification methods were unreliable for natural isolates of *Serratia* spp. API performed least well and BiOLOG proved more effective, with positive identifications obtained regularly. Furthermore, the dendrogram of the BiOLOG and API data was not consistent with the 16S rDNA trees and DNA–DNA hybridization results.

Isolates CP6 and CP6e were identified using Biotype 100 strips. CP6 was identified as *S. proteamaculans* subsp. *quinovora*, whilst CP6e was identified as *S. proteamaculans* subsp. *proteamaculans* biotype C1c. Hence, based on the two results obtained, this proved the most accurate phenotypic method.

Ecological implications

Our CP6 isolates were distinguishable from one another in two ecologically significant ways. They varied both in their sensitivity to indigenous phages and in their sensitivity to, and production of, bacteriocins. These differences are not remarkable, as medical strains can be classified by their bacteriocin profiles (Farmer, 1972a, b) and ‘phage-typed’ with suites of characterized phages. However, our bacteriocin and phage-sensitivity results do not correlate with the phylogeny of the *Serratia* species and so cannot be used for identifying these bacteria. Almost all these isolates came from the same environment as the original phage collection. Our previous investigations have demonstrated that these phages show distinct, different temporal population fluctuations and competition within the rhizosphere (Ashelford *et al.*, 1999a, 2000). Now we have shown that the bacterial species on which they predate is diverse. Collectively, our results imply a complex underlying interaction amongst bacterial species and with predatory phages *in situ*.

The bacteriocin diversity profiles observed demonstrate that these CP6 strains and related species have the potential to interact antagonistically amongst themselves *in situ*. It is probable that the bacteriocin activity derives from previous phage infections, either through the creation of a viable lysogenic relationship with the host or by leaving a disabled prophage within the genome. Some *Serratia* bacteriocins are prophage derived (Prinsloo, 1966), and we have observed phage tail-like structures within CP6 supernatant (data not shown) that cannot be accounted for by known phage infections and could explain the lytic effects observed. Similar phage-tail-like pyocins in *Pseudomonas aeruginosa* have been shown recently (Nakayama *et al.*, 2000) to be phage tails that have been evolutionarily specialized as bacteriocins and not

simply defective phages. The absence of prophages from all our Wytham Farm isolates indicates that the same might be true for these bacteria.

Our results also show that the host ranges of the CP6 phages were not restricted to a single species. Titration experiments showed that some of these phages could also infect *S. plymuthica* (both the indigenous isolate RK and the type strain) and the *S. ficaria* type strain successfully (Table 2). We have no evidence that these phages are capable of infecting bacteria beyond the genus *Serratia*. However, other work has shown that *Serratia*-infecting phages can infect non-*Serratia* members of the *Enterobacteriaceae* (Prinsloo, 1966). There are also some descriptions in the literature of broad-host-range phages that infect other genera and that have been isolated from barley rhizosphere (Campbell *et al.*, 1995) and aquatic environments (Matsuzaki *et al.*, 1992; Jensen *et al.*, 1998).

One way in which the complex interaction between phage and bacteria may manifest itself is through horizontal gene transfer, leading to a mixing of the genetic pool that makes phylogenetic determination more difficult. The cluster analysis of our bacteriocin data (Fig. 1) showed that bacteriocin interactions do not follow phylogeny. If the observed bacteriocin activity was the consequence of earlier phage infections, especially as the phages have broad host ranges, this should not be surprising. Horizontal gene transfer may go some way to explain the difficulties in resolving the phylogeny of genera such as *Serratia*.

Taxonomy of the *S. proteamaculans* subspecies

A combination of 16S rRNA sequence analysis and DNA–DNA cross-hybridization clearly demonstrated that all our CP6-like isolates and PC1 were members of the same species as *S. proteamaculans* subsp. *quinovora*. Strain Ox2 is most likely to be a member of *S. proteamaculans* subsp. *quinovora*, based on its bacteriophage sensitivity (Table 2), although not all the key BiOLOG test results were consistent with those from the other strains of this subspecies (Table 3). However, isolates RK and Ox1 were respectively readily identified as *S. plymuthica* and *S. fonticola* for the following reasons, and so were not true CP6-like isolates. The high (> 99.5%) 16S rDNA sequence similarity between these isolates and the closest type strains indicate that the DNA–DNA genomic hybridization frequencies between these bacteria are highly likely to be > 70% (Keswani & Whitman, 2001). Isolate RK had red colonies on TSBA and both isolates had BiOLOG substrate utilization profiles similar to those expected for their identities (Fig. 3).

Our DNA–DNA hybridization results are consistent with those of Grimont *et al.* (1982a). Taking both these sets of results together, it is clear that all strains of *S. proteamaculans* subsp. *quinovora* show > 80% relatedness ($n = 16$) and so form a more coherent species by DNA–DNA hybridization than any of the other *S. liquefaciens*-like species investigated by

Table 3. Characteristics useful in differentiating *S. quinivorans* sp. nov. isolates from *S. proteamaculans*

Strains are designated as: 1, *S. quinivorans* strains CP6, CP6a, CP6b, CP6c, CP6e, CP6f, CP6g, PC1 and LMG 7887^T; 2, *S. quinivorans* CP6d; 3, *S. quinivorans* Ox2; 4, *S. proteamaculans* subsp. *proteamaculans* CIP 103236^T.

Characteristic	1	2	3	4
Metabolism of:*				
Inositol	+	+	+	—
Sucrose	+	+	+	—
Growth on:†				
Cellobiose	+	—	+	—
L-Rhamnose	+	+	—	—
D-Galactonic acid lactone	+	+	—	—
D-Gluosaminic acid	+	+	+	—
D-Glucuronic acid	+	+	—	—
Hydroxy-L-proline	+	+	+	—

* From API 20E strips incubated at 30 °C for 24 h.

† From BiOLOG GN microplates incubated at 30 °C for 24 h.

Grimont *et al.* (1982a). *S. proteamaculans* subsp. *quinovora* is also distinct from subsp. *proteamaculans*, as these two studies show that 90% ($n = 42$) of the DNA–DNA hybridizations between these two sub-species showed < 70% DNA relatedness. *S. proteamaculans* subsp. *proteamaculans* is also not as coherent as subsp. *quinovora*, because 16% ($n = 38$) of the hybridizations of strains within subsp. *proteamaculans* showed < 70% but > 60% DNA–DNA relatedness. In addition, Grimont *et al.* (1982b) were able to distinguish the two *S. proteamaculans* sub-species phenotypically with biochemical tests. So, we propose that *S. proteamaculans* subsp. *quinovora* be transferred to a novel species, *Serratia quinivorans* corrig., sp. nov. The type strain for *S. quinivorans* sp. nov. should remain the same as previously designated for *S. proteamaculans* subsp. *quinovora* (CIP 8195^T = ATCC 33765^T = LMG 7887^T).

Distinguishing *S. quinivorans* sp. nov. from *S. proteamaculans* and the other *Serratia* species

A combination of 16S rRNA gene sequence analysis and DNA–DNA hybridization is the best way to distinguish members of this species group. BiOLOG GN microplate and Biotype 100 identification can provide a useful first step, although, as discussed previously, neither system gives definitive results for all *Serratia* species. Table 3 gives the BiOLOG tests, after incubation at 30 °C for 24 h, that provided definitive separation between all the strains of *S. quinivorans* sp. nov. that we tested and the *S. proteamaculans* type strain. The lack of both aesculin hydrolysis (API CH) after 2 days and acid production from salicin in 5 days also distinguishes *S. quinivorans* sp. nov. from all the other *S. liquefaciens*-like species (Grimont *et al.*,

1982b). Further tests for distinguishing *Serratia* species are provided elsewhere (Grimont *et al.*, 1982b; Grimont & Grimont, 1984, 1992).

Grimont *et al.* (1982b) gave growth on quinic acid as the sole carbon source at 30 °C in 14 days as a distinguishing test between *S. quinivorans* sp. nov. and other *S. liquefaciens*-like species, except for *S. plymuthica*. However, although all our strains of *S. quinivorans* sp. nov. grew on M70 quinic acid plates in 14 days (colony size 0.4–0.8 mm), so did the type strains of *S. liquefaciens* and *S. proteamaculans*, with the same colony size. The type strain of *S. plymuthica* and isolate RK grew more strongly (colony size after 14 days 1.2–1.7 mm), but the type strain of *S. grimesii* did not grow at all. This test is therefore only useful for distinguishing *S. quinivorans* sp. nov. from *S. grimesii*. In all cases, growth was apparent after 48 h and relative colony sizes distinguishable by 7 days.

Description of *Serratia quinivorans* corrig., sp. nov.

Serratia quinivorans (qui.ni.vor'ans. N.L. n. *acidum* quinicum quinic acid; L. v. *vorare* to devour; N.L. fem. part. adj. *quinivorans* devouring quinic acid).

Originally classified as *S. proteamaculans* subsp. *quinovora*. Cells are straight, Gram-negative, non-spore-forming rods, 0.5–0.8 µm wide by 0.9–2.0 µm long with rounded ends, that are motile by means of peritrichous flagella. Facultatively anaerobic, growing aerobically on nutrient agar in 24 h at 30 °C as colonies that are 1–3 mm in diameter, circular, shiny, domed, opaque, cream-white and smooth with an entire margin, having a distinctive musty odour like other *Serratia* species. Does not require growth factors. Grows on a wide range of carbon sources including glucose, sucrose, quinic acid and, for most strains, *trans*-aconitate, cellobiose, malate and L-rhamnose, but not adonitol or benzoate. Most strains produce lysine decarboxylase, ornithine decarboxylase, chitinase and gelatinase, but not arginine decarboxylase. Aesculin is not hydrolysed within 2 days but is within 7 days. Acid is produced from glucose and triacetin but not from lactose or salicin. Full details of other phenotypic tests are available elsewhere (Grimont *et al.*, 1982b; Grimont & Grimont, 1984, 1992). Occurs in soil, in association with plants and in the digestive tracts of soil invertebrates. The G + C content of the DNA is 53–54 mol % and the type strain is strain 4123^T (= CIP 8195^T = ATCC 33765^T = LMG 7887^T) (Grimont *et al.*, 1982a, b).

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