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'*Candidatus* Phlomobacter fragariae' and the proteobacterium associated with the low sugar content syndrome of sugar beet are related to bacteria of the arsenophonus clade detected in hemipteran insects

Phylogeny and genetic diversity of plant pathogenic gamma-3 proteobacteria

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Abstract The phylogeny of '*Candidatus* Phlomobacter fragariae'(*Ca*. P. fragariae), the agent of the strawberry marginal chlorosis (SMC), and the proteobacterium associated with the low sugar content syndrome of sugar beet (SBRp) is not well understood. The *spo*T-*spo*U-*rec*G genetic locus initially characterised by genome walking from a '*Ca.* P. fragariae' partial *spo*T sequence was used to determine relatedness of '*Ca.* P. fragariae' and SBRp with bacteria detected in hemipteran insects. Both plant pathogenic bacteria belong to the same phylogenetic group as bacteria of the arsenophonus clade detected in hemipteran insects. The SBRp is closely related to arsenophonus-like proteobacteria from cixiids and more distantly related to psyllid and delphacid secondary endosymbionts, whereas the relatives of '*Ca.* P. fragariae' remain to be discovered. No genetic variability was found among isolates of '*Ca.* P. fragariae' or SBRp. Implications for explaining the emergence of both '*Ca*. P. fragariae' and SBRp as epidemic plant pathogens are discussed.

Keywords Phloem-restricted bacteria · Emerging plant disease · Strawberry · Sugar beet · Bacterium-like organism · Planthopper

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Phloem-restricted proteobacteria are plant pathogens that belong to the alpha or the gamma subdivision of *Proteobacteria* (Garnier et al. 2003). Two phylogenetically related proteobacteria of the gamma-3 subclade are causing emerging plant diseases in France. '*Candidatus* Phlomobacter fragariae' (*Ca*. P. fragariae) is the prevalent agent of strawberry marginal chlorosis (SMC) and is transmitted by the planthopper *Cixius wagneri* (Danet et al. 2003; Nourrisseau et al. 1993; Zreik et al. 1998). The proteobacterium SBRp associated with the low sugar content disease of sugar beet called 'basses richesses' syndrome (SBR) is transmitted by the planthopper *Pentastiridius leporinus* (Bressan et al. 2008; Gatineau et al. 2002; Sémétey et al. 2007) and belongs to the arsenophonus clade that contains secondary symbionts of insects (Sémétey et al. 2007).

These two bacterial plant pathogens cannot be cultured and genetic studies have therefore been limited. To identify possible original ecological reservoirs of these emerging pathogens, genetically related bacteria were looked for among hemipteran insects in which related bacteria had previously been

detected by 16SrDNA PCR (Foissac et al. 2000). For a better characterisation of these bacteria a less conserved genetic locus was preferred to the 16SrDNA. Genetic diversity of '*Ca.* P. fragariae' and SBRp was also assessed.

A partial sequence of the '*Ca.* P. fragariae' *spo*T gene had been previously cloned by comparative random amplification (Foissac et al. 2000). The genome walking strategy was used to enlarge the *spo*T sequence using genome walking primer pairs directed toward the regions neighbouring the known sequence (Table 1). Two rounds of genome walking PCR were performed using ten genome walking libraries prepared according to GenomewalkerTM kit (BD Bioscience, San Jose, CA, USA). In this procedure total DNA (4 µg) from '*Ca.* P. fragariae'-infected periwinkle (isolate LG2001) (Danet et al. 2004) was digested by *Eco*RV, *Pvu*II, *Hinc*II, *Sca*I, *Stu*I, *Sna*I, *Eco*105I, *Ksp*AI, *Pme*I and *Swa*I and ligated to the genome walking adaptor designed to allow specific amplification of the DNA region between the adaptor and the genome walking primers. Genome walking PCR and nested-PCR were carried out according to the manufacturer's instructions. The PCR products were either directly sequenced on ABI-prism or cloned into pGEMt-easy (Promega Corp) prior to sequencing. Sequences were assembled and edited with the Phred-Phrap-Consed package (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998). The 3364 bp consensus sequence was annotated using ORF finder (http://www.ncbi.nlm.nih.gov/gorf) and blastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). It encoded from positions 44 to 2161 a 705 amino acid protein 84% identical to the SpoT p-ppGpp synthetase II of *Escherichia coli*, from positions 2165 to 2821 a 218 amino acid protein 59% identical to the spoU tRNA (Guanosine-2′-O-)-methyltransferase of *E. coli* and from positions 2883 to 3364 the 218 N-terminal amino acids of a protein 61% identical to the RecG ATP-dependent DNA helicase of *E. coli* (Fig. 1). Several oligonucleotide primers were designed to amplify and sequence a 2.4 kbp genetic locus corresponding to the end of the *spo*T gene, the *spo*U gene and the beginning of *rec*G gene. The sequence was determined for eight '*Ca.* P. fragariae' isolates detected in six strawberry plants and two *C. wagneri*, for six SBRp isolates detected in two sugar beets and four *P. leporinus* and for related proteobacteria detected in *Hyalesthes scotti, Pentastiridius* sp., *Delphacodes* sp., *Delphacidae* sp., *Conomelus anceps* planthoppers, in *Glycaspis brimbelcombei* and *Diaphorina citri* psyllids, in *Trialeurodes vaporariorum* whiteflies and in three different batches of *Mocydia crocea* leafhoppers (Table 2).

All *spo*T-*spo*U-*rec*G sequences obtained from strawberry plants affected by SMC and from *C. wagneri* insect vectors were 100% identical to the '*Ca.* P. fragariae' LG2001 *spo*T-*spo*U-*rec*G sequence, revealing the absence of genetic diversity among isolates of '*Ca.* P. fragariae'. The *spo*T-*spo*U-*rec*G sequence determined for a SBRp infected sugar beet was 100% identical to that of two other sugar beets affected by SBR and four *P. leporinus* insect vectors showing that no genetic diversity could be detected among SBRp isolates.

The SBRp *spo*T-*spo*U-*rec*G sequence presented only seven single nucleotide polymorphisms (SNP)

Table 1 Primers used for genome walking, PCR and sequencing of *spo*T-*spo*U-*rec*G locus

Primers	*Nucleotide sequence (5'-3')*
Genome walking	
phloD1	ACCATGAGTGTCGTCGTAGCGCATA
phloD1N	AATTACCTTTGCCAAATGTTGTCGC
phloG1	GGGCTCCAACACAGGCATGACCTAT
phloG1N	CGGGGGTAAAAACATAGATTTCGTC
phloG2	GGAAGCGCTGCTCTTTCAGGTGCAT
phloG2N	CCTGGCTTCCAATAATCTACCTTCA
PCR and sequencing	
SpoTf2	GATCGAACGCACAATATGCG
SpoTr1	ACATCAGGCATGATACGGAT
SpoTf3	GGCCTTGGCAATACCATGAG
SpoUr1	TGTCTTTGCCGTTGCGCTTC
SpoUf2	GCGGGCAGTAATCATTGGGT
RecGr2	CTAATTTGGCTGGTCATCAT
SBRTF1	CAAAACTGAATTGGAAGAAC
SBRTR1	AAACGGCGGCCTAAACTAAT
SBRTF5	GAGAAACATTAGAAATTTATAGCCC
SBRTR2	TTTTTGCTCTGGCTTTGGAG
SBRCOM7	AGGAGAATCTAGCACAACCG
SBRUR3	CATGGCGATCACTTGACG
SBRCOM3	CGTAATATTCGAGGCTATCAAAAAG
SBRCOM4	GTAAAGGTTTAGTCATACACC
SBRGR1	CATTCGCTTACGTCCAAATG
SBRGR2	CCGCTTACAGTCATAGAGCT

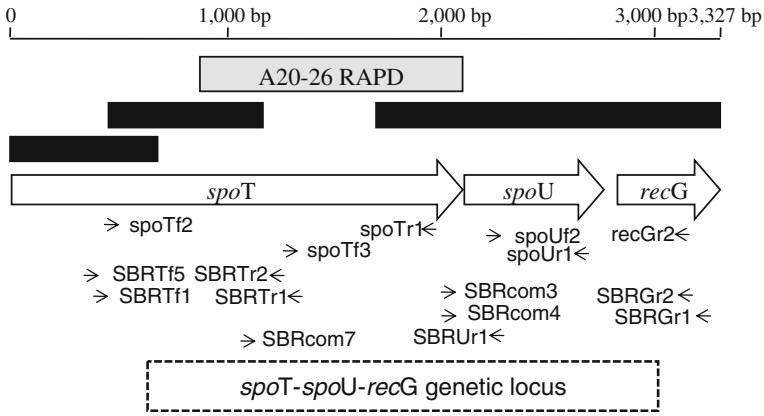

Fig. 1 Genome walking assembly and gene content of the *spo*T-*spo*U-*rec*G sequence of '*Ca.* P. fragariae'. Grey box represents the original partial *spot* sequence obtained by RAPD, black boxes indicate the genome walking PCR fragments. White arrows correspond to coding sequences and short arrows identicate the positions of primers used for PCR and sequencing

Table 2 Insect and plant samples

Insects and Plants	Origin, date of collection	*spo*T-*spo*U-*rec*G accession number
Periwinkle LG2001	Lot et Garonne, France, 2001	FM992113
Strawberry plant #31	Dordogne, France, 1996	FM992113
Strawberry plant #99	Lot et Garonne, France, 1997	FM992113
Strawberry plant #116	Lot et Garonne, France, 1997	FM992113
Strawberry plant #433	Dordogne, France, 1999	FM992113
Strawberry plant #490	Vaucluse, France, 2000	FM992113
Strawberry plant #503	Loir et Cher, France, 2000	FM992113
Cixius wagneri	Dordogne, France, 2001	FM992113
Cixius wagneri	Lot et Garonne, France, 2001	FM992113
Sugar Beet #P421	Côte d'Or, France, 1997	FM992680
Sugar Beet #P3844	Côte d'Or, France, 2005	FM992680
Sugar Beet #SB6	Hungary, 2004	FM992680
Pentastiridius beiri #X858	Côte d'Or, France, 1998	FM992680
Pentastiridius beiri # X4315	Côte d'Or, France, 1999	FM992680
Pentastiridius beiri # X6056	Côte d'Or, France, 2002	FM992680
Pentastiridius beiri #X7247	Côte d'Or, France, 2005	FM992680
Hyalesthes scotti #X7261	Jura, France, 2006	FM992686
Pentastiridius sp.#X7278	Gard, France, 2004	FM992685
Delphacodes sp.#X2588	Côte d'Or, France, 1999	FM992684
Delphacidae sp. #X2575	Côte d'Or, France, 1999	FM992683
Diaphorina citri	Florida, USA, 2003	FM992682
Glycaspis brimblecombei	California, USA, 2003	FM992681
Mocydia crocea #112	Dordogne, France, 1995	FM992679
Mocydia crocea #X2334	Côte d'Or, France, 1999	FM992679
Mocydia crocea #X2518	Côte d'Or, France, 1999	FM992679
Mocydia crocea #X2634	Côte d'Or, France, 1999	FM992679
Conomelus anceps #182	Dordogne, France, 1995	FM992678
Trialeurodes vaporariorum #18	Gironde, France, 1996	FM992677

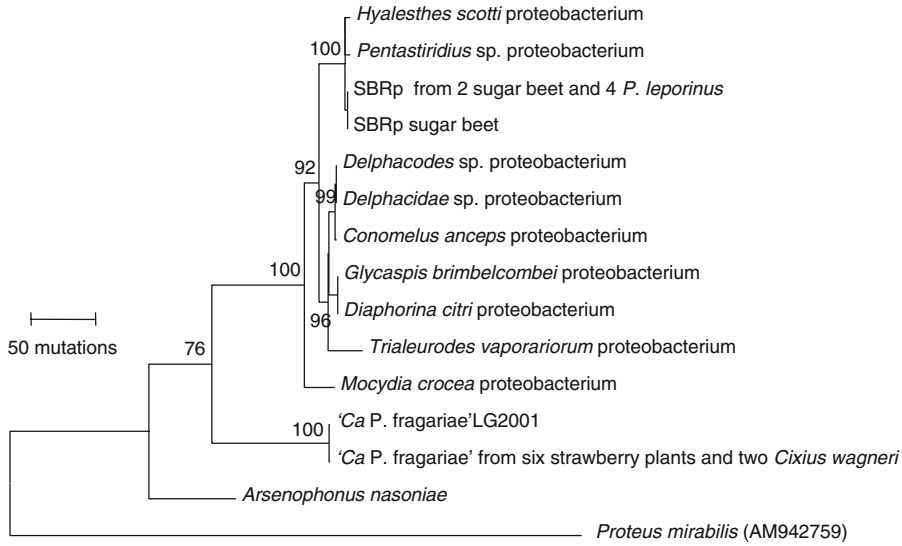

Fig. 2 Phylogenetic analysis of the *spo*T-*spo*U-*rec*G genetic locus of '*Ca.* P. fragariae', SBRp and related proteobacteria of the arsenophonus clade detected in hemipteran insects. The evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 2370 positions in the final dataset, out of which 242 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

(99.7% similarity) and a deletion of seven nucleotides by comparison to the *spo*T-*spo*U-*rec*G sequence detected in *H. scotti*, and five SNP (99.8% similarity) by comparison to the *spo*T-*spo*U-*rec*G sequence detected in a *Pentastiridius* sp. insect. Phylogenetic analysis of the *spo*T-*spo*U-*rec*G sequences showed that all sequences obtained from hemipteran insects except *C. wagneri* clustered together on a statistically robust branch with a bootstrap value of 100% (Fig. 2). Sequences differed by 34 and 35 nucleotides between the SBRp and the *Delphacodes* sp. proteobacterium (98.6% similarity) and the *Glycaspis brimbelcombei* proteobacterium (98.5% similarity) respectively. The sequence of the *Delphacodes* sp. proteobacterium had a 10 nucleotide deletion at position 1718. The *spo*T-*spo*U-*rec*G sequence of '*Ca.* P. fragariae' significantly differed from the SBRp *spo*T-*spo*U-*rec*G genetic locus by 200 nucleotides (92.8% similarity).

Up to now both '*Ca.* P. fragariae' and SBRp have limited geographical distribution, restricted to France and Japan for '*Ca.* P. fragariae' (Danet et al. 2003; Tanaka et al. 2006) and to the Burgundy region of France for the SBRp (Sémétey et al. 2007). The absence of genetic diversity in both '*Ca.* P. fragariae' and the SBRp is consistent with recent emergence of these two pathogens in strawberry and sugar beet crop in France. Cases of SMC have been recently reported in Italy, but amplicon sequences covering 2.4 kbp of the *spo*T-*spo*U-*rec*G genetic locus of the Italian strawberry proteobacterium showed it was 100% identical to the *spo*T-*spo*U-*rec*G genetic locus of the SBRp, demonstrating that the Italian SMC was not associated to '*Ca.* P. fragariae' (Terlizzi et al. 2007). In the absence of plant hosts other than strawberry and sugar beet, the existence of an original reservoir for '*Ca.* P. fragariae' and SBRp from which epidemics could have emerged is an important issue that remains to be established. In the present paper we show that the proteobacterium of *Cixiidae, Delphacidae* and *Psyllidae* are related to SBRp. These proteobacteria belong to the clade of *Arsenophonus* facultative secondary endosymbionts of hemipteran insects (Hansen et al. 2007; Thao and Baumann 2004). The occasional transmission of these members of the *Arsenophonus* clade to the sap of plants by their hemipteran hosts could lead to erratic emergence of bacteria such as '*Ca.* P. fragariae' and SBRp. Their subsequent acquisition and transmission to plants by *Cixiidae* growing on the infected crop may explain the emerging epidemics.

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