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Unifying bacteria from decaying wood with various ubiquitous *Gibbsiella* species as *G. acetica* sp. nov. based on nucleotide sequence similarities and their acetic acid secretion

Klaus Geider[a,*], Marina Gernold[a], Susanne Jock[a], Annette Wensing[a], Beate Völksch[b], Jürgen Gross[a], Dieter Spiteller[c]

[a] *Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim, Germany*
[b] *FSU Jena, Institute of Microbiology, Microbial Phytopathology, Neugasse 25, Jena, Germany*
[c] *Max-Planck-Institute for Chemical Ecology, Bioorganic Chemistry, Jena and Chemical Ecology/Biological Chemistry, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany*

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A B S T R A C T

Bacteria were isolated from necrotic apple and pear tree tissue and from dead wood in Germany and Austria as well as from pear tree exudate in China. They were selected for growth at 37 °C, screened for levan production and then characterized as Gram-negative, facultatively anaerobic rods. Nucleotide sequences from 16*S* rRNA genes, the housekeeping genes *dnaJ*, *gyrB*, *recA* and *rpoB* alignments, BLAST searches and phenotypic data confirmed by MALDI-TOF analysis showed that these bacteria belong to the genus *Gibbsiella* and resembled strains isolated from diseased oaks in Britain and Spain. *Gibbsiella*-specific PCR primers were designed from the proline isomerase and the levansucrase genes. Acid secretion was investigated by screening for halo formation on calcium carbonate agar and the compound identified by NMR as acetic acid. Its production by *Gibbsiella* spp. strains was also determined in culture supernatants by GC/MS analysis after derivatization with pentafluorobenzyl bromide. Some strains were differentiated by the PFGE patterns of *Spe*I digests and by sequence analyses of the *lsc* and the *ppiD* genes, and the Chinese *Gibbsiella* strain was most divergent. The newly investigated bacteria as well as *Gibbsiella querinecans*, *Gibbsiella dentisursi* and *Gibbsiella papilionis*, isolated in Britain, Spain, Korea and Japan, are taxonomically related *Enterobacteriaceae*, tolerate and secrete acetic acid. We therefore propose to unify them in the species *Gibbsiella acetica* sp. nov.

Introduction

Fire blight lesions on fruit trees are often populated with secondary invaders of many bacterial species. Some of those, such as the saprophyte *Erwinia billingiae* and the flower colonizing species *Erwinia tasmaniensis*, can be antagonistic to *Erwinia amylovora* (Jakovljevic et al. 2008). From tree lesions with fire blight we initially obtained two strains, BK1 and W4, with some phenotypic properties of *E. amylovora*, such as high levan production (Bereswill et al. 1998). We later isolated similar, taxonomically identical strains from stem sections of necrotic fire blight lesions collected in 2007 and 2010 in Carinthia, Austria. Other strains were isolated in Germany from plant tissue, such as dead wood samples of a compost pile as well as from sticky exudate of a pear tree in China. Similar bacteria were isolated from oozing oak trees in Britain, Spain and other countries, which were initially classified as *Brenneria quercina* (Biosca et al. 2003; Poza-Carrión et al. 2008), a species recently proposed to be named *Lonsdalea quercina* (Brady et al. 2012). *Gibbsiella quercinecans* has also been associated with Acute Oak Decline (AOD) (Brady et al. 2010). However, no experimental evidence has been provided to verify this species as the causative agent of the disease.

Several other species of bacteria have been associated with disease symptoms comprising bark discoloration and secretion of sticky tree exudates. *Pseudomonas syringae* pv. aesculi is involved in a disease of horse chestnuts (*Aesculus hippocastanum*). The

* Corresponding author. Tel.: +49 6221 86805 53; fax: +49 6221 86805 15.
E-mail addresses: Klaus.Geider@jki.bund.de (K. Geider), marina.gernold@yahoo.de (M. Gernold), Susanne.Epple@jki.bund.de (S. Jock), Annette.Wensing@jki.bund.de (A. Wensing), Beate.Voelksch@uni-jena.de (B. Völksch), Juergen.Gross@jki.bund.de (J. Gross), dieter.spiteller@uni-konstanz.de (D. Spiteller).

presumable causative bacterial agent has been isolated and could induce disease symptoms in plantlets (Schmidt et al. 2008; Green et al. 2009). Participation of a fungus in horse chestnut disease is still possible, but difficult to verify (Cerny et al. 2009). In the genus *Pantoea*, a type strain from pineapple, *Pantoea ananatis* was connected to disease symptoms on crops and trees and even human infections (Coutinho and Venter 2009). In an embryonated egg model this species was more virulent than *Pantoea agglomerans* strains (Völksch et al. 2009) isolated from different sources. Without the possibility to fulfill Koch's postulates, the differentiation between frequently occurring saprophytes and pathogenic bacteria is vague. Based on the isolation of *Gibbsiella* sp. our data suggests that the genus is not connected to oak decline but is rather a common ubiquitous bacterium. Here, we characterized *Gibbsiella* isolates of different origin. They tolerate and secrete acetic acid, which are typical features of the newly proposed genus *Gibbsiella acetica*.

Materials and methods

Bacteria used in the experiments and bacterial enrichments

Bacterial strains were isolated from pear and apple tissue with fire blight lesions or uprooted wood obtained in Germany and Austria. An additional isolate was obtained from exudate of a pear tree in China (Table 1). Small pieces of wood were rinsed with water in an Eppendorf tube and aliquots plated on Luria-Bertani (LB) agar with cycloheximide (50 μg/ml) and incubated at 37 °C for 1d. White colonies were transferred to LB agar with 5 g sucrose per liter (LBsuc). Levan-positive colonies were assayed for clearing zones on MM2 agar (Bereswill et al. 1998) with 1% glycerol and 0.05% $CaCO_3$, the addition originally designed for yeasts (Kurtzman and Fell 2001) indicating acid secretion. Copious levan-synthesis resembles *E. amylovora*, a species lacking growth at 37 °C. Suspicious strains were further characterized by molecular analysis, and two were kept from each isolation series.

Bioassays for inhibition of E. amylovora *and* Gibbsiella *sp.*

The basic minimal medium with streptomycin (500 μg/ml) used for testing growth inhibition was D1, which is similar to MM2 (Bereswill et al. 1998) and contains 5 mM $MgSO_4$, 2 g $(NH_4)_2SO_4$, 0.4 g asparagine, 0.2 g neutralized nicotinic acid, 0.2 g thiamine-HCl, 50 mM K_2HPO_4, 50 mM KH_2PO_4, and 1% glycerol (11.5 ml of 86%) per liter (Konecki et al. 2013). Medium D5 is medium D1 with 80 mM KH_2PO_4 and 20 mM K_2HPO_4. Over-night cultures of strain Ea1/79Sm in LB broth were diluted 10^3 fold in medium D1 or D5 and culture supernatants added, which were neutralized with NaOH or NH_3. The bacteria were grown in microtiter plates with 150 μl of the bacterial culture containing streptomycin and test samples which incubated on a rotary shaker at 240 rpm at 28 °C for 24 h or longer. Growth was measured with an ELISA reader (Titertec MCC340) at A620 as described previously (Konecki et al. 2013).

Tolerance of strain BK1 was assayed for growth with acetic or propionic acid in D1 and D5 medium.

Creation of a levansucrase mutant for sequencing of the lsc *gene*

Random Tn*5* mutants were created in BK1Sm by biparental mating using pRL27 (Larsen et al. 2002) as transposon donor in order to obtain sequence information of the *lsc* gene. Transconjugants were selected on Standard I agar with kanamycin (20 μg/ml) and transferred on LB agar with 5% sucrose to screen for a loss of levan formation. From a library of 1500 BK1-mutants a levan-deficient strain was selected and this phenotype was verified by restreaking several times on LBsuc. The transposon insertion site was identified by rescue cloning. Subcloning of the minitransposon was done with restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV and *Pst*I, respectively. The flanking sequences were analyzed with primers 13–2 and 17–1 (Larsen et al. 2002) and a consensus sequence created. BLAST analysis of the respective sequences showed similarities to various *lsc* genes. From the sequence of parts of the BK1-*lsc* gene primers #731/#732 and #806/#807 were created.

Sequence analysis of 16S rRNA and housekeeping genes and phylogenic trees

A small shotgun library of strain BK1 DNA was created by M. Kube (MPI of Molecular Genetics, Berlin) and a fragment was identified that encoded a peptidyl-prolyl cis-trans isomerase (*ppiD*) and the adjacent gene for a ComEA protein. Part of the fragment was sequenced with primers #556 and #557. Then part of the *lsc* gene was amplified with primers #731 and #732 and sequenced with primer #731. Parts of the 16*S* rRNA, *dnaJ*, *gyrB*, *rpo*B, and *recA* genes were also amplified and sequenced with primers listed in Table 2. The Genbank/ENA accession numbers are provided in the dendrograms and with sequence alignments.

Phylogenic trees were constructed using MEGA v. 5 (Tamura et al. 2011) software and minimal evolution analysis with bootstrapping (1000 replicates).

Identification of Gibbsiella *sp. strains by PCR*

Conventional PCR (cPCR) and quantitative PCR (qPCR) assays were done as described previously (Wensing et al. 2012). Bacterial cells were lysed in 0.1% Tween 20 at 65 °C for 15 min. cPCR analysis was performed with *Gibbsiella* sp. BK1 primers #726/#728 deduced from *ppiD* or #731/#732 and #806/#807 from *lsc* (Table 2). For identification by qPCR for mass screening and confirmation of cPCR the primers #726/#727 from the *ppiD* gene were applied with EvaGreen for signal detection and carried out in the CFX96 cycler (Bio-Rad, Hercules, CA, USA).

Phenotypic characterization isolates from decaying wood

Strain morphology and motility were investigated by microscopic examination. Gram reaction, oxidative/fermentative glucose utilization, the presence of catalase and oxidase, growth on Standard I-agar (Merck, Darmstadt, Germany) at 28 °C, 37 °C, and 45 °C, and with 5% NaCl were studied. In addition, each strain was biochemically characterized using API 20E and API 50CHB/E test strips (bioMérieux, Nürtingen, Germany). The tests were carried out according to the manufacturer's instructions. The API-strips were incubated at 28 °C, and the results were read after 24 and 48 h.

Levan synthesis was visually determined on LB agar with 5% sucrose. For quantitative assays, turbidity of the formed levan was measured (Bereswill et al. 1997) and the more sensitive method with dinitrosalicylic acid (DNS) was applied to determine the amount of reducing sugar (glucose) released from sucrose (Miller 1959). Supernatants of cultures in LB or D1 medium were added to levansucrase buffer (2 M sucrose, 100 mM Tris, pH 7.5; 0.1% azide) (1:1) and incubated overnight at 37 °C. Levan was measured as turbidity at 600 nm or as the amount of glucose in cleared aliquots with the DNS assay at 575 nm.

The hypersensitive response (HR) was determined by infiltration of watery cell suspensions (10^8 CFU/ml) into tobacco leaves (*Nicotiana tabacum*). A formation of gray spots was determined after 2d.

Table 1
Strains investigated in the experiments.

Strain	Source, origin	Reference; sample
Isolates from decaying wood		
BK1	Necrotic apple wood, Darmstadt, Germany, 1991	W. Zeller
BK1Sm	Spontaneously Sm-resistant mutant of strain BK1	This study
W4	Necrotic apple wood, Darmstadt, Germany, 1991	W. Zeller
BK2	Necrotic pear wood, Carinthia (Austria), 2010	M. Bartosik; this study
BK3	Necrotic pear wood, Carinthia (Austria), 2010	M. Bartosik; this study
BK23a	Necrotic pear wood, Carinthia (Austria), 2007	M. Bartosik; this study
BK23b	Necrotic pear wood, Carinthia (Austria), 2007	M. Bartosik; this study
BK30	Necrotic wood, Dossenheim, Germany, 2011	This study
BK31	Necrotic wood, Dossenheim, Germany, 2011	This study
C22	Exudate of pear tree, China, 2012	B. Hu, Nanjing, China
Gibbsiella quercinecans		
NCPPB 4470^T	FRB 97^T, AOD, Hoddesdon Park (Britain)	Brady et al. (2010)
NCPPB 4471	FRB 92, AOD, Hoddesdon Park (Britain)	Brady et al. (2010)
NCPPB 4472	FRB 185, AOD, Gorse Covert (Britain)	Brady et al. (2010)
N78	from *Q. pyrenaica*, Spain	Poza-Carrión et al. (2008), Brady et al. (2010)
Gibbsiella dentisursi		
NUM1720^T	DSM 23818^T, oral cavity of bear, Japan	Saito et al. (2012)
Gibbsiella papilionis		
DSM 27944^T	LEN33^T, KACC 16707^T, butterfly intestine, Korea	Kim et al. (2013)
Other strains with similarity to *Gibbsiella* sp. and Genbank nucleotide sequences (ac)		
Ag03	16S rRNA (ac EU554429)	Zhongkang et al. (2008)
Ag10	16S rRNA (ac EU554436)	Zhongkang et al. (2008)
Ag11	16S rRNA (ac EU554437)	Zhongkang et al. (2008)
Bacterium 2134	*gyrB* (ac FJ268865)	Green et al. (2009)
Other bacteria isolated from wood		
Isolate 10–1	From dead wood	This work
Isolate 12–2	From dead wood	This work
Erwinia amylovora		
Ea1/79	Apple, Germany	Falkenstein et al. (1988)
Ea1/79Sm	Sm-resistant strain Ea1/79	
Erwinia uzenensis		
NCPPB 4475^T	YPPS951^T, LMG 25843^T, Western pear, Japan	Matsuura et al. (2012)
Brenneria quercina		
(syn. *Lonsdalea quercina* subsp. *quercina*)	Brady et al. (2012)	
DSM 4561^T	USA	
IVIA-1251-3	From *Q. ilex*, Spain	Poza-Carrión et al. (2008)
IVIA-1940-1	From *Q. ilex*	Poza-Carrión et al. (2008)
Brenneria salicis		
DSM 30166^T	United Kingdom	

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; NCPPB, National Collection of Plant Pathogenic Bacteria (UK). AOD, acute oak decline. In case of multiple names of a strain, the name in the text refers to the collection from where the strain was obtained.

MALDI-TOF MS and PFGE analysis of Gibbsiella *sp. strains*

Sample preparation was performed as described previously (Sauer et al. 2008). Briefly, bacteria were grown for 24 h at 28 °C in 1 ml of LB broth with 1% glycerol in 2 ml reaction tubes. Cells were harvested by centrifugation, the pellet washed with 1 ml water to remove residual components of the growth medium, cells were pelleted again, and then they were resuspended in 0.3 ml water with 0.8 ml ethanol. For lysis, cells were pelleted, air dried to remove ethanol, and resuspended thoroughly in 40 µl 70% formic acid and 40 µl acetonitrile. The cell debris was removed by centrifugation and clear lysates were stored at −20 °C. 1 or 2 µl of the extracts were placed on a MSP 96 polished steel target and co-crystallized with the same amount of matrix solution (saturated α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid/47.5% water) for several minutes and analyzed with a Bruker Microflex mass spectrometer. For fast identification of bacteria, direct analysis of fresh single colonies was performed. Cell material was transferred to the target grid with a sterile tip, overlaid with 1 µl of matrix solution and analyzed by MALDI-MS. The spectra were compared by Bruker biotyper software 2.0 against reference spectra created for strain BK1 and *G. quercinecans* strain NCPPB 4470^T and evaluated in a score system (Sauer et al. 2008) defining values above 2 for high relatedness.

Pulsed-field gel electrophoresis (PFGE) analysis of Gibbsiella *sp. strains*

The bacterial cells were lysed, embedded in agar and their DNA digested with restriction enzyme *Spe*I as described previously (Jock et al. 2002). Pulsed field gel electrophoresis (PFGE) was performed with a CHEFDRIII apparatus (Bio-Rad) in a 1% agarose gel with a linear ramping of 5 V/cm for 1–25 s for 22 h at 14 °C.

Enrichment of an acidic E. amylovora *growth inhibiting compound from a culture supernatant of strain BK1*

The culture supernatant from 1 L of MMA medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.2 g nicotinic acid, 0.2 g thiamin) was alkalinized by adding NaOH to 50 mM and then evaporated to near dryness in a rotary evaporator. The residue was acidified with 55 ml 1 M H_2SO_4 and again evaporated to dryness. The residue was then extracted again with 50 ml H_2O. The distillates were combined, evaporated to dryness,

Table 2
Primers for sequencing and detection by PCR.

Primer number	Other name	Sequence	Fragment length
cPCR for detection of *Gibbsiella* sp. and sequencing			
#556	BK1PPID140	GAAGACAGCCAACTGGTTGA	
#557	BK1PPID943[a]	CAAGGCAGCTTGCCATACAG	0.8 kb
#726	BK1PPID216	AGGATCGCCAGGACAATGTG	
#728	BK1PPID813[a]	TCACGGTAACGCACAATGGT	0.6 kb
Sequencing of *lsc* genes and detection			
#731	BK1LSC313	GTTGACGGCTGGTCAGTGAT	
#732	BK1LSC1119[a]	GAACAGTGAATCCGCGACAA	0.8 kb
#806	BK1LSC285	GCGTGATATCAACGGTAACG	
#807	BK1LSC944[a]	CAGTCATCGCCGTCTTCATC	0.66 kb
qPCR (from BK1 *ppiD-comEA* region)			
#594	BK1PPID75	GGCGATGAGGCAATGAAAG	
#595	BK1PPID218[a]	CCTGCGACATACCATAAACC	
#727	BK1PPID355Q[a]	GTTAGCCAGCAGCGAGTCAA with #726	
16S rRNA Weisburg et al. (1991)			
#63	fd2	AGAGTTTGATCATGGCTCAG	
#64	rP1	ACGGTTACCTTGTTACGACTT	
rpoB Brady et al. (2010)			
#710	CM7-F	AACCAGTTCCGCGTTGGCCTG and	
#711	CM31b-R	CCTGAACAACACGCTCGGA (PCR)	
#712	CM81-Fsq	CAGTTCCGCGTTGGCCTG (sequencing)	
#714	CM32b-Rsq	CGGACCGGCCTGACGTTGCAT (sequencing)	
recA (from *E. amylovora* gene)			
#93	RECA81	CATGCGCCTGGGTGAAGACC	
#94	RECA779[a]	TCAGCCTGCTTGAACGGCGC	
dnaJ Pham et al. (2007)			
#583	DN1-1F	GATYTRCGHTAYAACATGGA with	
#584	DN1-2R	TTCACRCCRTYDAAGAARC or	
#725	DN1-2REp	TTCACRCCRTYGAAGAARG (modified from Ep1/96 *dnaJ* gene (ac FP236842)	
gyrB (from BK1 gene)			
#789	BK1GYRB58	TCGGTTGTTAACGCCTTGTC with	
#790	BK1GYRB773[a]	CAGCCAGCAGTTCGTTCATC or	
#791	BK1GYRB872[a]	TCATTTCACGGGCCTTACG	

[a]Primer from complementary strand.

and again extracted with 50 ml H_2O. For further purification by ion exchange chromatography, 10 ml of the distillate was applied to a 1 ml Dowex-1 column (OH-form), which was washed with water (5 ml) and eluted with 8 ml 0.5 M H_2SO_4. The fractions in the elution zone with a pH decrease were neutralized and also inhibited growth of *E. amylovora*. The residual distillate was alkalized with NH_3 and concentrated to 5 ml, when salt crystallization started.

NMR analysis

For NMR, 10 ml distillate of a culture supernatant was applied to a 1 ml Dowex-1 column (OH-form) and the column washed with 5 ml D_2O. In order to avoid the background from protons of the solvent, 5 ml 2 N DCl in D_2O were used for the ion exchange chromatography. The fractions with a pH decrease were subjected to ^1H NMR analysis using a Bruker Avance 500 MHz NMR spectrometer. The ^{13}C NMR-signals were measured at 125 MHz in D_2O/DCl. The identity of the isolated compound was proven by comparison with commercial acetic acid.

Determination of acetic acid by GC/MS after alkylation with pentafluorobenzylbromide (PFBBr)

Bacteria were grown in MMA for 2 d at 28 °C. The concentration of acetic acid in culture supernatants was determined according to a described procedure (Kage et al. 2004). Briefly, *ca.* 30 μl of a bacterial culture supernatant was added to 100 μl 0.5 M sodium phosphate buffer (pH 6.8) and mixed with 1 ml 100 mM pentafluorobenzyl bromide (PFBBr, Sigma–Aldrich) in acetone in 2 ml Eppendorf tubes. The mixture was incubated for 60 min at 60 °C. One ml of n-hexane with 0.1 mM 1,3,5-tribromobenzene (TBB) was added at room temperature. The mixture was vortexed and then centrifuged for 10 min. A part of the large hexane/acetone phase was transferred to an injection vial.

For analysis by coupled gas chromatography-mass spectrometry (GC/MS), 1 μl of a sample was injected using the splitless mode by an autosampler into a Clarus 680 gas chromatograph (PerkinElmer, Waltham, MA, USA). The injector temperature was set to 280 °C. Separation was performed on an Elite-5 capillary column (30 m × 0.25 mm, 0.25 μm) using helium as carrier gas at a flow rate of 2.5 ml/min and a solvent delay of 3 min. The initial oven temperature was held at 50 °C for 3 min, and raised at a rate of 10 °C/min to 220 °C, held for 9 min, followed by an increase at 10 °C/min to 280 °C, held for 5 min. The gas chromatograph was coupled to a quadrupole mass spectrometer (Clarus 600, PerkinElmer), which was operated in the SCAN mode. The electron ionization (EI) mass spectra were recorded at 70 eV, scanning from 35 to 350 *m/z*.

The MS spectra were analyzed according to Kage et al. (2004) for the molecular mass of PFB-acetate by ion trace search for 240 *m/z*. The peak area of the compound in the chromatogram was correlated with the acetic acid standards (0.15, 0.5 and 1.5 μmol in 100 μl sodium phosphate). The ion at 314 *m/z* was used for the detection of TBB to confirm the correct processing after extraction with n-hexane.

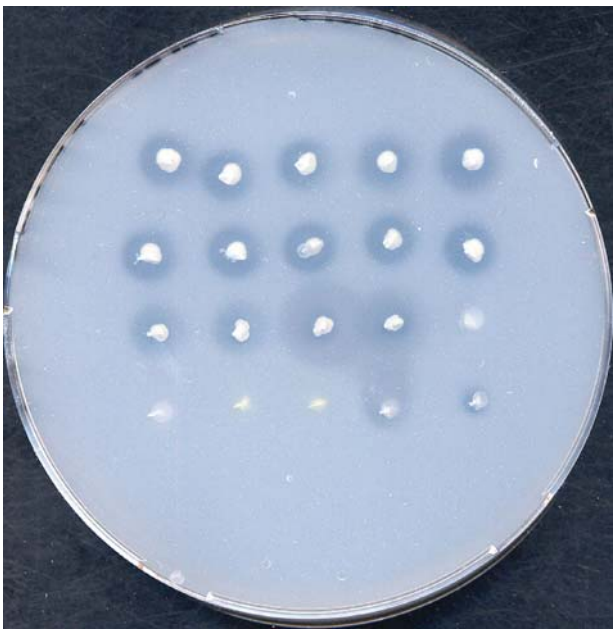

Fig. 1. Halo formation from acid secretion on MM2 agar with $CaCO_3$. Two colonies of BK1, BK2, BK3, BK30, BK31, C22, NCPPB4470^T, Ea1/79, EhNZ, Eb661^T were transferred to the agar (from left to right) and incubated for 18 h at 28 °C.

Results

Isolation of novel saprophytic bacteria from trees with fire blight symptoms and from dead wood

In the 1990s we obtained two strains, which were isolated from necrotic apple tissue with fire blight symptoms that resembled *E. amylovora* (BK1 and W4). After selection for growth at 37 °C and levan formation, additional strains were subsequently isolated from extracts of pear bark with fire blight obtained from Carinthia (Austria). These were named BK2, BK3, BK23a, and BK23b. We also extracted pieces of dead wood from a compost pile in Germany and isolated strains BK30 and BK31. Strain C22 was isolated in China from a diseased pear tree with stem oozing.

Acid production and hypersensitive response assays

BK1 and similar strains, as well as *G. quercinecans* strains, were assayed for acid secretion on MM2 agar with $CaCO_3$ (Fig. 1). The colonies of our strains formed halos indicating a reaction with carbonate (Table 1). These halos were not observed upon growth on carbonate agar buffered with 100 mM potassium phosphate because it neutralized the secreted acid.

The tested strains BK1, BK2, BK30 and NCPPB 4470^T did not produce a hypersensitive response (HR) on tobacco leaves in contrast to *E. amylovora* Ea1/79 and *E. uzenensis* NCPPB 4475^T as positive controls.

Growth inhibition of E. amylovora *by an acetic compound in culture supernatants of* Gibbsiella *sp. strains*

Culture supernatants of BK1 and W4 inhibited the growth of *E. amylovora* in liquid medium (Fig. 2a). As described previously (Konecki et al. 2013), short-chain fatty acids can cause a similar significant growth retardation of *E. amylovora* that is dependent on the pH of the medium.

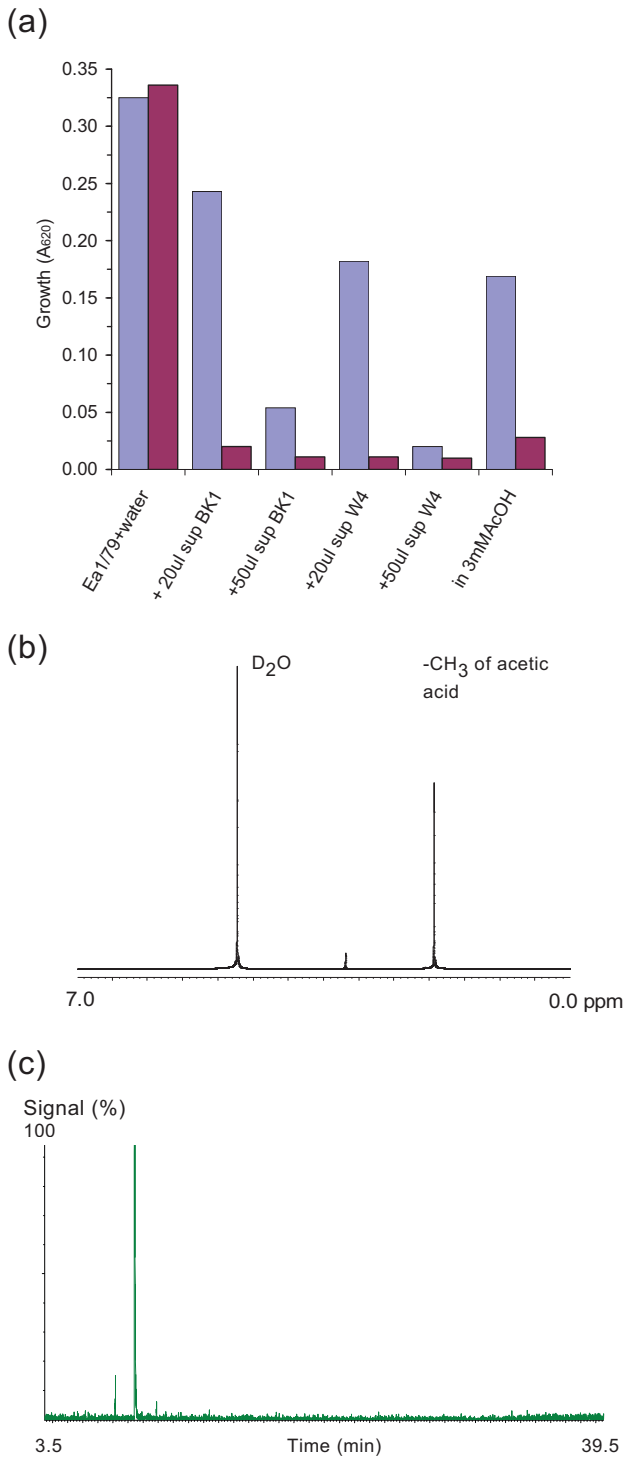

Fig. 2. Growth inhibition of *E. amylovora* and identification of acetate. (a) Growth inhibition of strain Ea1/79Sm by culture supernatants of strains BK1 and W4 in D1 and D5 medium as compared to the addition of neutralized acetic acid. Left bars are growth of Ea1/79Sm in D1 medium (pH 6.5); the right (dark) bars growth in D5medium. (b), ^{1}H NMR-spectrum of the fraction from DCl elution in D_2O (500 MHz). The CH_3-signal at 1.95 ppm was identical with commercial acetic acid as reference. (c) Selective ion chromatogram of acetic acid after derivatization with pentafluorobenzyl bromide and separation by GC/MS.

Purification of acetic acid from culture supernatants and its identification by NMR

Culture supernatants of BK1 in MMA medium were alkalinized in order to fully convert acid compounds to their salts and then

evaporated in a rotary evaporator to dryness. The remaining residue was acidified in order to protonate any organic acid compounds and then distilled. Growth inhibiting activity was found in neutralized samples of the distillates. The distillates were subjected to ion exchange chromatography using Dowex-1 resin. Bioactive fractions were obtained. For identification of the acid compound by NMR. The distillate was applied to a Dowex-1 column and eluted with DCl in D_2O in order to avoid any background of solvent protons in ^1H NMR analysis.

A clean spectrum of acetic acid was observed for the elution fraction. The ^1H NMR signal at 1.94 ppm (CH_3) (Fig. 2b) and the ^{13}C signals at 20.20 ppm (CH_3) and 176.50 ppm (COOH) (not shown) matched those of commercial acetic acid that was used as reference.

Determination of acetic acid in culture supernatants of bacteria from apple and pear trees

A reliable, fast and accurate method for determination of acetic acid in culture supernatants was found to be GC/MS (Fig. 2c). All of our putative *Gibbsiella* sp. strains produced acetic acid at the level of 25 mM during cultivation in MMA medium (Table 3). The advantage of the GC/MS measurement was the use of the 240 *m/z* selective ion mass identifying the pentafluorobenzyl ester of acetic acid. From its peak area in the chromatograms, the amount of acetic acid in culture supernatants was calculated. For comparison, *E. coli* and *B. salicis* supernatants produced more than 25 mM acetic acid, whereas *E. billingiae* and *B. quercina* produced very little, and *E. amylovora*, *E. tasmaniensis*, or *P. agglomerans* did not synthesize any detectable amounts of acetic acid (Table 3).

Growth tolerance of Gibbsiella *sp. to acetic acid*

Bacteria of the species *Gibbsiella* grow at low pH in the presence of neutralized acetic acid, when *E. amylovora* and *E. tasmaniensis* are significantly retarded. In D1 medium with almost neutral pH growth of *E. amylovora* was slightly inhibited by moderate concentrations of acetic and propionic acid, at concentrations well inhibiting growth of *E. tasmaniensis* (Fig. 3), whereas *Gibbsiella* strain BK1 was unaffected. Growth retardation was slightly visible in D1 for propionic acid but strongly pronounced at the low pH of medium D5 even for strain BK1. On the other hand, acetic acid did not affect growth *Gibbsiella* sp. BK1 at both pHs. In conclusion, strain BK1 is selectively tolerant for growth in the presence of acetic acid.

Nucleotide sequences from 16S rRNA genes for identification of bacteria from decaying wood

In order to initially classify the bacteria that we isolated from decaying wood parts, their 16*S* rRNA sequences were determined. The sequences of BK1, W4, BK2, BK3, BK23a, BK3 and C22 were largely identical. In a dendrogram (sup. Fig. 1), they clustered with the 16*S* rRNA of *G. quercinecans*, a newly described species isolated from exudates of diseased oak trees in Britain and Spain (Brady et al. 2010). Strain NUM 1720^T, isolated in Japan from a bear tooth cavity, was classified as *Gibbsiella dentisursi* (Saito et al. 2012) is also closely related to *G. quercinecans*. Strain LEN33^T, isolated in Korea from intestinal tracts of a butterfly, was classified as *Gibbsiella papilionis* (Kim et al. 2013), and is another close neighbor. In addition, three strains, AG 03, 10 and 11, isolated from insect intestines (*Apriona germari*) in China, were characterized by their 16*S* rRNA sequences (Zhongkang et al. 2008) and are also related to *G. quercinecans*.

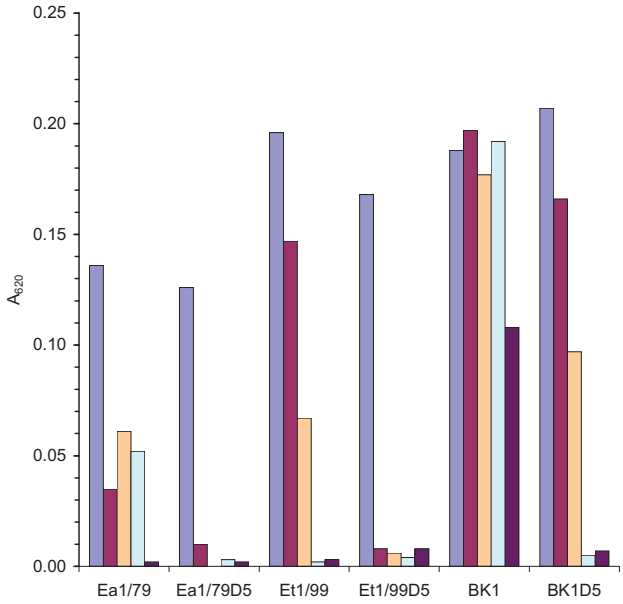

Fig. 3. Growth tolerance of *Gibbsiella* sp. strain BK1 to the presence of acetic acid. An ON culture of the strain was inoculated into medium D1 (pH 6.8) or D5 (pH 6.2) and neutralized acetic acid was added to 2 mM and 5 mM as well as propionic acid to 1 mM and 2.5 mM. Similarily, growth on *E. amylovora* strain Ea1/79 and *E. tasmaniensis* strain Et1/99 were assayed. Order of bars: first bar control, then addition of acids.

Classification of putative Gibbsiella *strains using nucleotide sequences from housekeeping genes*

In addition to the 16*S* rRNA genes, we analyzed several housekeeping genes, *dnaJ*, *gyrB*, *recA*, and *rpoB*. The dendrograms are provided in the supplementary material. In the cases of *dnaJ* and *recA*, nucleotide sequence information was not available for *G. quercinecans*, *B. quercina* and *B. salicis*, so we sequenced the corresponding genes.

For the taxonomic description of *G. quercinecans*, sequence information for *gyrB* and *rpoB* was used (Brady et al. 2010). A part of the *gyrB* gene of BK1 was amplified with primers #789 and #790 or #791 (sup. Fig. 2). The *gyrB* sequence from strain BK1 was compared against the NCBI nucleotide database using BLAST searches. Forty-two hits were obtained for strains of the species *G. quercinecans* with 98% to 99% identity, separated from the genus *Serratia*. This confirms that BK1 belongs to the genus *Gibbsiella*. A phylogenetic tree revealed (sup. Fig. 2) that all previously described *G. quercinecans* strains clustered with *G. dentisursi* (NUM1720^T) and *G. papilionis* as well as our isolate BK1 and bacterium 2134 (Table 1). Other *Gibbsiella*-like strains in this cluster were Ag03 and Ag10.

Using *rpoB* sequences, all our isolates from decaying wood clustered with *G. quercinecans* (sup. Fig. 3). Although part of the same *rpoB*-cluster, the Chinese strain C22 was slightly separated mapping with *G. dendisursi* and *G. papilionis*. Neighboring branches included the genera *Edwardsiella*, *Rahnella* and *Serratia*.

We also investigated nucleotide sequences from *dnaJ* and *recA*. The *dnaJ* gene is quite versatile and is therefore well suited to distinguish species (Pham et al. 2007). The nucleotide sequences from *dnaJ* of the wood isolates, including the Chinese strain C22, clustered with *G. quercinecans* strains NCPPB 4470^T, NCPPB 4472 and N78 (sup. Fig. 4).

The *recA* gene is also highly conserved in bacterial species. Deviations from the consensus sequence are typical and are used for species differentiation (Wensing et al. 2010). The *recA* sequences of the wood isolates and the Chinese strain C22 also showed that they cluster with the *G. quercinecans* strains NCPPB 4472 and N78

Table 3
Analyses of epiphytic bacteria for acid production and hypersensitive response as well as with MALDI-TOF MS and PCR assays.

Strain	Acid[a]	GC/MS	MT score	cPCR	qPCR (Ct)	Levan	HR
B. quercina							
DSM 4561^T	–	7	2.51 Bq	–	28.9	+/++	
IVIA-1940-1	–	0	2.28 Bq	–	28.7		
B. salicis							
DSM 30166	±	28	2.45 Bs	–	29.4	+/–+	
Gibbsiella sp.							
BK1	+	32	2.51 Gq	+	15.2	+/++	–
BK2	+	22	2.59 Gq	+	17.9	+/++	–
BK3	+	20	2.58 Gq	+	18.4	+/++	
BK23a	+	15	2.55 Gq	+	19.3	+/++	
BK23b	+	28	2.34 Gq	+	18.9	+/++	
BK30	+	25	2.50 Gq	+	18.3	+/++	–
BK31	+	33	2.50 Gq	+	17.9	+/++	
C22	+	33	2.34 Gq	+	19.7	+/++	
W4	+	29	2.49 Gq	+	19.2		
G. quercinecans							
NCPPB4470^T	+	20	2.44 Gq	+	21.8	+/++	–
NCPPB4471	+	28	2.56 Gq	+	22.1	+/++	
NCPPB4472	+	30	2.48 Gq	+	21.7	+/–+	
N78	+	24	2.48 Gq	+	20.0	+/++	
G. dentisursi							
DSM 23818^T	+	12	2.1 Gq	+	21.1	+/++	
G. papilionis							
DSM 27944^T	+	25	2.0 Gq	+	21.7	+/++	
E.amylovora							
Ea1/79	–	0			33.8	+/++	+
Ea273	–	0					
E. uzenensis							
NCPPB 4475^T	–				29.2	–/–±	+
E. tasmaniensis							
Et1/99^T	–	0			30.1	+/++	
Et2/99	–					+/±+	
E. billingiae							
Eb661^T	±	5	2.37 Eb	–	30.1		
P. agglomerans							
EhNZ	–	0					
E. coli							
1100	+	108				–/––	

GC, GC/MS assays for acetate, calculated concentration (mM) in culture supernatants; MT score, MALDI-TOF MS values; cPCR, with *ppiD* (#726#728)-*lsc*-primers (731#732, #806#807); qPCR, with primers #726#727; Ct, cycle threshold; HR, hypersensitive response on tobacco leaves; MALDI-TOF MS references: Gq, *G. quercinecans*: NCPPB4470^T; Bq, *B. quercina*: DSM 4561^T; Bs, *B. salicis*: DSM 30166; Eb, *E. billingiae:* Eb661^T.

[a] halos on MM2 agar + 0.5% $CaCO_3$; Levan: on plates/levansucrase by turbidity and DNS assay.

and *G. dendisursi* and *G. papilionis*. The cluster was neighbored by *Serratia* and *Dickeya* species (sup. Fig. 5).

Microbiological properties compared to G. quercinecans

The strains that we isolated from diseased and decaying wood exhibited typical morphological and microbiological characteristics of the newly described genus *Gibbsiella* (Brady et al. 2010) within the family *Enterobacteriaceae*. They were Gram-negative short rods, facultatively anaerobic, oxidase negative, and catalase positive. They were also arginine dihydroxylase, lysine decarboxylase, ornithine decarboxylase, and tryptophane deaminase negative, but positive for β-galactosidase. Gelatine was not degraded and H_2S was not produced, but citrate was utilized and nitrate was reduced to nitrite. The strains metabolized a broad spectrum of carbon sources with the exception of erythritol, L-xylose, D-adonitol, β-methyl-D-xyloside, dulcitol, inulin, D-melezitose, starch, xylitol, D-rhamnose, D-tagatose, D-fucose, and 5-keto-gluconate. Unlike in the description of *G. quercinecans* (Brady et al. 2010) our strains did not significantly utilize D-lactose and glycogen. The indole test with Kovacs reagent (Wensing et al. 2010) was negative.

In addition to the strains obtained by us from diseased and decaying wood, the strains NCPPB 4470^T from Britain, N78 from Spain, and C22 from China produced levan on LB sucrose agar plates as well as levansucrase shown in the turbidity and in the DNS assays (Table 3).

Biotyping by MALDI-TOF MS

The reference strains described as *G. quercinecans*, BK1 and the other isolates from wood identified as BK1-like by PCR and phenotypic analysis were in addition compared by MALDI-TOF MS analysis. Protein spectra of fresh cell extracts (from pure cultures) were compared using the Bruker biotyper software. As *Gibbsiella* species are not yet included in the commercial biotyper database, reference spectra were created for *G. quercinecans* NCPPB 4470^T, strain BK1 and *Brenneria* species. All *Gibbsiella* isolates showed almost identical protein patterns including *G. dentisursi* NUM1720^T (DSM 23818^T), *G. papilionis* LEN33^T (DSM 27944^T). The ID score values for these strains were above 2 against the NCPPB 4470^T reference. These score values are considered sufficient for species identification (Wensing et al. 2010).

Detection of Gibbsiella *strains in PCR assays*

We have designed several primer pairs, which specifically amplify *Gibbsiella* spp. Two pairs for cPCR were derived from the *ppiD* gene and two from the *lsc* gene (Table 2). Since the nucleotide sequences of these genes can vary (Fig. 4), we designed two pairs from each gene, #556/#557 (Fig. 5), #726/#728 for *ppiD* and #731/#732 and #806/#807 for *lsc*. These combinations identified *Gibbsiella* strains (Table 3) and for other bacteria produced no or only faint bands at an incorrect position. This technique also could detect *Gibbsiella* sp. strains in a mixture of bacteria. No signals were obtained with cell lysates of other levan producing bacteria isolated from wood, *E. amylovora* strain Ea1/79 (Fig. 5) or *B. quercina* strains from Spain.

For qPCR analysis, primers pairs from the *lsc* gene were used. The combination #726/#727 produced threshold values for *Gibsiella* sp. strains that differed by 10 and more from those obtained with other bacteria and the water control (Table 3). DNA samples from lysates of fully grown cultures were applied to qPCR. The

(a)

```
BK1            1 AAACGGCAACTATGACATCAACCTCGACTGGAACGATCGTCATGGCCGGGCAAAAATGTGCTACTGGTTCTCCCGCACCA
BK2            1 ..............................................................................T....
BK23a          1 ................................................................................
BK30           1 ................................................................................
NCPPB4470      1 ................................................................................
N78            1 ................................................................................
C22            1 ......................G.........................................................A.......

BK1           81 GCAAAGACTGGGTATATGGCGGCCGTGTGATGGCGGACGGTGTGTCACCGACCACCCGCGAATGGGCCGGCTCGCCGATT
BK2           81 ....................................C...........................................
BK23a         81 ....................................C...........................................
BK30          81 ................................................................................
NCPPB4470     81 ................................................................................
N78           81 ..............G.............................C.....................................
C22           81 ..............G...........C..A...........C..T.....A.................A............

BK1          161 TTGCTCAACGACCAGGGCGATGTGGATCTGTACTACACTGCCGTTACCCCTGGCGCGACCATTGTGAAAGTGCGTGGCCG
BK2          161 ....................C..C.....................................................
BK23a        161 ................................................................................
BK30         161 ................................................................................
NCPPB4470    161 ................................................................................
N78          161 ................................................................................
C22          161 ....................C..C........T.....C..G.....G.....T.........................

BK1          241 TGTGGTGACTACTGAACACGGCGTTGACATGGTTGGCTTCAAAAAAGTGTCTTCGCTGTTCGAAGCCGACGGCAAAATGT
BK2          241 C.................T..............................................................
BK23a        241 C..A.....C........T..............................................................
BK30         241 ................................................................................
NCPPB4470    241 C..............................................................................
N78          241 C.................T.............................................................
C22          241 C........C........T.....................................A.........................

BK1          321 ATCAAACCGAAGCGCAAAACCCGTACTGGGGCTTCCGCGACCCATGGCCGTTCCGCGATCCGGAGAGCGGCAAGCTGTAC
BK2          321 ................................................................................
BK23a        321 ................................................................................
BK30         321 ................................................................................
NCPPB4470    321 ....................................................................T...........T
N78          321 ................................................................................
C22          321 ..............................................................T........T........T

BK1          401 ATGCTGTTTGAAGGCAATGTGGCCGGGGAACGAGGTTCGCACAAAGTGGGTGAAGCGGAAATCGGCGACGTGCCACCGGG
BK2          401 ................................................................................
BK23a        401 ................................................................................
BK30         401 ................................................................................
NCPPB4470    401 ................................................................................
N78          401 ................................................................................
C22          401 ...T....C.................T..G...........T........C...................A..G.....

BK1          481 CTACGAAGATGTAGGCAACTCGCGTTACCAGACCGCCTGCGTCGGTATTGCGGTTGCGCGTGATGAAGACGGCGATGACT
BK2          481 ................................................................................
BK23a        481 ................................................................................
BK30         481 ................................................................................
NCPPB4470    481 ................................................................................
N78          481 ......................A.........................................................
C22          481 T..T.....C..G........A..C..............A.T...........G........C..............T.

BK1          561 GGGAACTGCTGCCGCCATTGATTACAGCGGTTGGCGTTAATGACCAGACGGAACGCCCACACCTGTTGTTCCAGGACGGC
BK2          561 ....................................................................T.............
BK23a        561 ................................................................................
BK30         561 ................................................................................
NCPPB4470    561 ................................................................................
N78          561 ................................................................................
C22          561 ............T...C..T.G..G.......................C..G............C...........T..G

BK1          641 AAATATTACCTGTTCACCATCAGCCATCAGTACACTTATGCCGATGGCCTGACCGGCCCGGATGGTGTGTACGGTTTTGT
BK2          641 ................................................................................
BK23a        641 ................................................................................
BK30         641 ................................................................................
NCPPB4470    641 ................................................................................
N78          641 ..................................................................T.............
C22          641 ................T........C....T...C.....T...................C.................
```

Fig. 4. Differentiation of *Gibbsiella* strains with nucleotide sequences from the *lsc* and *ppiD* genes. (a) Part of the *lsc* gene was amplified with primers #731 and #732 and sequenced with both primers. Accession numbers: BK1, HG932523; BK2, HG932524; BK23a, HG932525; BK30, HG932526; C22, HG932527; N78, HG932528; NCPPB 4470^T, HG932529. (b) Part of the *ppiD* gene was amplified with primers #556 and #557 and sequenced with both primers. Genbank/ENA accession numbers: BK1, HG932530; BK2, HG932531; BK3, HG932532; BK23a, HG932533; BK23b, HG932534; BK30, HG932535; BK31, HG932536; C22, HG932537; W4, HG932538; N78, HG932539; NCPPB 4470^T, HG932540; NCPPB 4471, HG932541; NCPPB 4472, HG932542.

(b)

```
BK1          1 AAACGAAATGAAAACCTTTGCTGAGAAAATGCAAGAAAGTGCCACTGGCGTGACCTTTGACTCGCTGCTGGCTAACTTGC
W4           1 ...............................................................................
BK2          1 ...T.....................G...........CA.....................................
BK3          1 ...T.....................G...........CA.....................................
BK23a        1 ..............................................A................................
BK23b        1 ..............................................A................................
BK30         1 ...............................................................................
BK31         1 ...............................................................................
NCPPB4470    1 ...............................................................................
NCPPB4471    1 ...............................................................................
NCPPB4472    1 ..............................................................................T.
N78          1 ...............................................................................
C22          1 ............G.......G.................C.....C.....................................

BK1         81 GCACCGAGGCCAAGATTAAGATGGGCTCTGCGGAACAATCGCCGCAATAATCCCCGCATTTCTTTGCAACGCAATGTAAT
W4          81 ...............................................................................
BK2         81 ...............................................................................
BK3         81 ...............................................................................
BK23a       81 ...............................................................................
BK23b       81 ...............................................................................
BK30        81 ...............................................................................
BK31        81 ...............................................................................
NCPPB4470   81 ...............................................................................
NCPPB4471   81 ...............................................................................
NCPPB4472   81 ...............................................................................
N78         81 ...............................................................................
C22         81 .......A.................T........G.....................................................

BK1        161 AACCAAAGGCCGCTTTCGCGGCCTTTTCCACATCTGGCGTCTGCCACTTGTTGCCCATCTGCCGCTGCGGCAAGGTAGCC
W4         161 ...............................................................................
BK2        161 ............................G..................................TG...T.............
BK3        161 ............................G..................................TG...T.............
BK23a      161 ...............................................................................
BK23b      161 ...............................................................................
BK30       161 ...............................................................................
BK31       161 ...............................................................................
NCPPB4470  161 ..........................................................TG...T.............
NCPPB4471  161 ..........................................................TG...T.............
NCPPB4472  161 ..........................................A....................................
N78        161 ...............................................................................
C22        161 ..........................................T...............G...T.............

BK1        241 GTGCTGTTAAACACAAGGAGGATACAGCATGCAACCGAATGAAAGAAAAGCGGCGCACTGCCCGCGTATTAATCTGAAAG
W4         241 ...............................................................................
BK2        241 ..............................................................C............C.......
BK3        241 ..............................................................C............C.......
BK23a      241 ........................................G......................................
BK23b      241 ........................................G......................................
BK30       241 ...............................................................................
BK31       241 ...............................................................................
NCPPB4470  241 ..........................................................A...C............C.......
NCPPB4471  241 ..........................................................A...C............C.......
NCPPB4472  241 ..............................................................C............C.......
N78        241 ..................................................T.........C............C.......
C22        241 ..............................................................C............C.......
```

Fig. 4. (*Continued*).

primer pair caused an elevated background and was well suited for qualitative analysis.

PFGE analysis of Gibbsiella *strains and differentiation with nucleotide sequences of the levansucrase and phenyl-proline isomerase genes*

Chromosomal DNA of the *G. quercinecans* strains was digested with restriction enzyme *Spe*I. After separation of the genomic digests by PFGE, some patterns were divergent and some had minor differences (Fig. 6). The strains BK1 and BK30 produced identical patterns (lanes 1 and 3). These strains originated from necrotic wood with fire blight and dead wood without disease symptoms. Both strains were isolated from German orchards *ca.* 50 km apart from each other. The *Spe*I genomic digests of *Gibbsiella* sp. strains from Carinthia (Austria), and the *G. quercinecans* type strain largely differed from the other strains from Britain (Fig. 6). Nevertheless, a

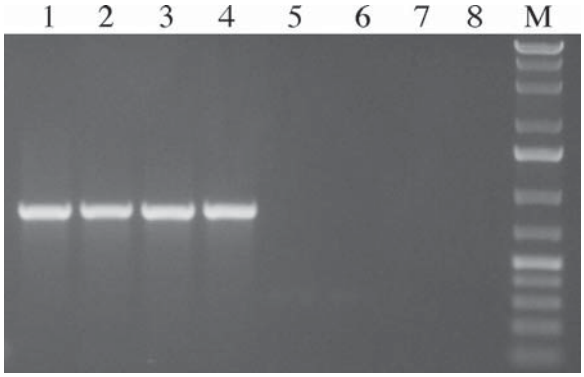

Fig. 5. Detection of *Gibbsiella* strains with PCR assays using primers #556 and #557 for cPCR. Lane 1, BK1; 2, BK2; 3, NCPPB 4470^T; 4, NCPPB 4472; 5. wood sample 10–1; 6, wood sample 12–2; 7, *E. amylovora* Ea1/79; 8, water control.

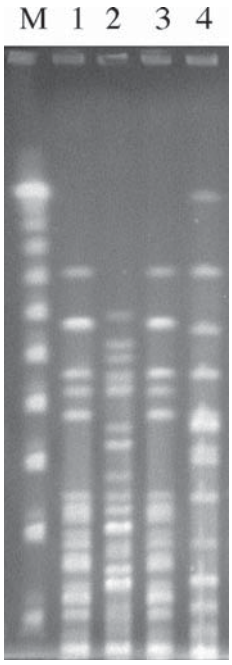

Fig. 6. PFGE profiles of *Spe*I digests of genomic DNA from *Gibbsiella* strains. Lane 1, *Gibbsiella* sp. BK1 (matches 3); 2, *Gibbsiella* sp. BK2; 3, *Gibbsiella* sp., BK30 (matches 1); 4, *G. quercinecans* NCPPB 4470^T. M, marker of phage lambda concatemers.

partial consensus of bands was observed in the patterns in strains BK1, BK30 and NCPPB 4470^T (lanes 1, 3, and 4). However, the divergence of BK2 (lane 2) suggests that PFGE analysis does not always create patterns typical of all *Gibbsiella* sp. strains.

Differentiation was extended by sequence analysis of two partial genes, *lsc* (with primers #731/#732) and *ppiD* (with primers #556/#557) (Fig. 4). The single nucleotide polymorphisms in the sequences agreed with deviations in PFGE analysis. In both cases, the sequences of BK1 and BK30 from Germany were identical. NCPPB 4470^T from Britain showed a different pattern and N78 from Spain was more divergent than the type strain. BK2 and BK23a from Carinthia also differed from BK1 and are not identical to each other. The sequence comparison of the partial *ppiD* gene was in accordance to the results of the sequence analysis of the *lsc* gene. The data showed low genetic diversity for strains isolated in a narrow geographic region and divergence for strains from distant locations.

The *Gibbsiella* strain NCPPB 4472 from Britain, was different from the other English strains, NCPPB 4470^T and NCPPB 4471, which were identical. Nucleotide sequence identity was found for BK1, W4, BK30 and BK31 from Germany as well as for BK2/BK3 from Carinthia isolated in 2007 and BK23a/BK23b isolated in 2010.

Most divergent was strain C22 from China when compared to the other isolates from decaying wood by sequence analysis using 16*S* rRNA and housekeeping genes. Its *lsc* and *ppiD* sequences differed the most from other strains (Fig. 4).

Discussion

Gibbsiella is a widely distributed bacterial genus. *G. quercinecans* strains were originally isolated from trees in Britain with Acute Oak Decline (AOD) (Brady et al. 2010). We have isolated similar bacteria from apple or pear trees with fire blight symptoms in Germany and Austria and from asymptomatic decaying wood of a compost pile. Other strains were isolated from pear trees with a sticky exudate in China, from intestine of insects or from a bear tooth.

An array of tools is available to group bacterial strains into a genus or species. The approaches are finally equivalent in the taxonomic conclusions such as DNA/DNA hybridization or nucleotide sequence alignments. The tedious kinetics applying liquid reassociation have been replaced by membrane filter hybridization (Ezaki et al. 1989) producing a much lower quality of data for species relations. A relatedness of 64% and 41% to *G. quercinecans* has been claimed for *G. dentisursi* and *G. papilionis*, respectively (Saito et al. 2012; Kim et al. 2013). We have preferred alignments of 16S rRNA and nucleotide sequences of four house keeping genes (*dnaJ*, *gyrB*, *recA*, *rpoB*). These genes are not equally represented in our data collection of the investigated strains. Consensus PCR primers for housekeeping genes do not produce signals in all cases. This may be due slight nucleotide sequence in homogeneities for a worldwide distributed genus. Nevertheless, Fig. 4 shows in part a clonal sequence conservation in particular for the *ppiD*-gene. Phylogenetic trees with concatenates could not be created due to a small subset of nucleotide sequences for many strains. Average Nucleotide Identity (ANI) cannot be given due to missing whole-genomic sequence informations. AFLP has been substituted by PFGE, MALDI-TOF analysis and gene sequence comparisons (Table 3). Fatty acid profiles can fluctuate, although data for five *G. quercinecans* strains were typical for *Enterobacteriaceae* (Brady et al. 2010).

Housekeeping genes have very conserved sequences, which are due to their important enzymatic functions. For instance the *recA* recombinase is a multifunctional enzyme (Kowalczykowski et al. 1994) and cannot tolerate many amino acid substitutions. On the other hand, the DnaJ protein supports chaperones in protein folding and may be evolutionarily adjusted to the demands of individual species (Pham et al. 2007). Out of 376 amino acids of DnaJ, positions 2–108, retain most of the entire protein's biological function and capabilities (Szyperski et al. 1994). *gyrB*, which encodes a subunit of gyrase (topoisomerase II), and *rpoB*, which encodes the β-subunit of RNA polymerase, are widely used for taxonomic identification. The variations observed in divergent PFGE patterns and in SNPs in the *recA* and *ppiD* genes of *Gibbsiella* sp. strains, may represent their need to adapt to a wide range of conditions and competitors that they face during saprophytic growth. This would be in contrast to the narrow host environment encountered by pathogens that interact with a limited range of host plants. The divergence is most pronounced for strains that were collected from geographically far distant sites, such as Europe and China.

An oozing pear tree in China showed symptoms described for horse chestnuts in Germany (Schmidt et al. 2008) and Britain (Green et al. 2009). In the latter case the bacteria isolated were mostly classified as *P. syringae* pv. *aesculi* based on nucleotide sequences from *gyrB*. However, in an English collection of isolates from oozing chestnut trees, one strain, 2134 (Green et al. 2009) most likely belonged to *Gibbsiella* sp. according to the high

similarity of its *gyrB* sequence (FJ268865). Other *Gibbsiella* strains isolated from the oral cavity of a bear (strain NUM 1720^T) were named *G. dentisursi* (Saito et al. 2012) and from a butterfly (strain LEN33^T) *G. papilionis* (Kim et al. 2013). The fact that they belong to this genus was supported by 16*S* rRNA and *gyrB* sequence data. However, doubts can be raised about their differentiation as separate species. Three other strains (AG3, AG10, AG11) were isolated in China from intestine of the Mulberry longhorn beetle (*Apriona germari*) (Zhongkang et al. 2008) and 16*S* rRNA sequences (EU554429, EU554436, EU554437) indicate a close relationship to *G. quercinecans* (sup. Fig. 1).

Species of the *Enterobacteriaceae* are known to occur in many different environments, including both animals and plants. A prominent member is *P. agglomerans*. This species was originally isolated from both plant samples as *Erwinia herbicola* and from human specimens as *Enterobacter agglomerans*. Since strains from both sources are taxonomically closely related. They were placed into a single species as *P. agglomerans* (Gavini et al. 1989) and later disassembled (Brady et al. 2008). Their occurrence on plant surfaces and growth at 37 °C made them ubiquitous for diverse environments. Their isolation from wounds or blood bottles does not prove a clinical impact, it rather indicates a broad distribution. Since strains from apple and pear orchards are also applied as control agents against fire blight, differentiation of within species could avoid application of clinical strains in the field (Rezzonico et al. 2009). One such attempt was the embryonated egg test, but no differences in symptom development were found between plant and clinical strains (Völksch et al. 2009).

Bacteria of the genus *Gibbsiella* belong to a group of bacteria that can secrete acid. Acetic acid bacteria are widespread in nature and commercially used in vinegar and cheese production. Six genera of acetic acid bacteria were proposed, with *Gluconobacter* and *Acetobacter* being most prominent (Gonzalez et al. 2006; Cleenwerck and De Vos 2008). They are characterized by the ability to convert ethanol to acetic acid. Like other bacteria, they can be distinguished by their rRNA sequences. Similar approaches with *G. quercinecans* strains suggest a close relationship to the genus *Serratia*. The phenyl proline isomerase gene used to design primers for detection of *Gibbsiella* strains is also present in the genome of *Serratia* species. The secretion of acetic acid may provide a growth advantage in the presence of other microorganisms. Some but not all *Gibbsiella* strains also produce another compound that inhibits the growth of *E. amylovora* (unpublished observation). An *in vitro* test for this compound and its identification is still unavailable. L-2,5-dihydroxyphenylalanine is another example of a growth inhibitor, which is synthesized by several American *E. amylovora* strains (Gehring and Geider 2012).

Similar to other ubiquitous bacteria, *Gibbsiella* sp. strains can grow at 37 °C and thus can survive conditions not tolerated by many plant-associated bacteria. The close resemblance of nucleotide sequences of 16*S* rRNA genes and several housekeeping genes justifies grouping the newly described strains isolated from decaying wood, *G. quercinecans*, *G. dentisursi* and *G. papilionis* into a single species. The ability of the strains that we characterized in this study and the previously described *G. quercinecans* strains to secrete acetic acid suggests combining these highly related bacteria into one species with the name *G. acetica*. The origins of their isolation indicate a worldwide distribution (Fig. 7).

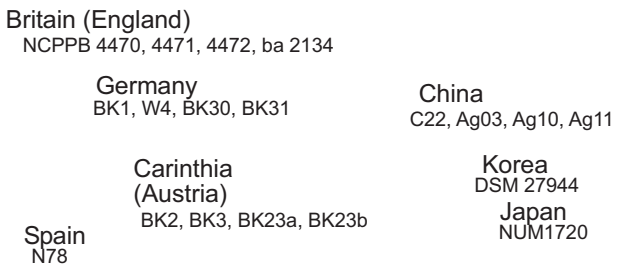

Fig. 7. Worldwide distribution of *G. acetica* strains described in Table 1. The countries of their isolation are indicated.

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Description of Gibbsiella acetica sp. nov.

L. n. *acetum*, vinegar; N.L. fem. adj. a.ce.'tica, related to acetic acid. The novel suggested species produces acetic acid and essentially overlaps with the microbiological description of *G. quercinecans* (Brady et al. 2010). NCPPB 4470^T is the type strain of *G. acetica*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2015.05.003

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