Chilling and cultivar type affect the diversity of bacterial endophytes colonizing sweet pepper (*Capsicum anuum* L.)

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Abstract: A climate chamber experiment was conducted to assay the effect of low temperatures (chilling) on the diversity of bacteria colonizing the endospheres of two thermophilic sweet pepper (*Capsicum anuum* L.) cultivars, Milder Spiral and Ziegenhorn Bello. Structural diversity was analyzed by 16S rRNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis and by the generation of 16S rRNA gene libraries to determine dominant community members in T-RFLP profiles. Cultivable community members colonizing lines Milder Spiral and Ziegenhorn Bello were identified by 16S rRNA gene analysis. T-RFLP profiles and 16S rRNA gene libraries revealed a high heterogeneity of community composition due to chilling and suggested further the existence of cultivar-specific communities. The majority of isolates obtained from the cultivar Milder Spiral were assigned as high-G+C Gram-positive bacteria (*Microbacterium* sp., *Micrococcus* sp., *Rhodococcus* sp.) and *Firmicutes* (*Staphylococcus* sp.). Of the isolated endophytes obtained from cultivar Zeigenhorn Bello, 93% were affiliated with *Staphylococcus aureus* and *Bacillus* sp. (*Firmicutes*). The experimental set-up was suited to demonstrate that chilling and cultivar type can influence the diversity of bacterial endophytes colonizing sweet pepper. We propose additional chilling experiments to investigate the effect of chilling on functional, plant-beneficial abilities of bacterial endophytes associated with low-temperature-sensitive crops, such as sweet pepper.

Key words: chilling, thermophilic sweet pepper, bacterial endophyte diversity, 16S rRNA gene analysis.

Résumé : Nous avons effectué une expérience en chambre climatique afin d'évaluer l'impact des basses températures (refroidissement) sur la diversité des bactéries colonisant les endosphères de deux cultivars de piments doux (*Capsicum anuum* L.), Milder Spiral et Ziegenhorn Bello. La diversité structurelle fut analysée par les polymorphismes de la taille des fragments de restriction terminaux basées sur l'ARNr 16S (T-RFLP) et la génération de banques de gènes d'ARNr 16S afin de déterminer les membres dominants des communautés dans les profils de T-RFLP. Les membres cultivables des communautés colonisant les lignées Milder Spiral et Zeigenhorn Bello furent identifiés par analyse de l'ARNr 16S. Les profils de T-RFLP et les banques de gènes d'ARNr 16S ont révélé une hétérogénéité élevée dans la composition des communautés attribuable au refroidissement et a de nouveau suggéré l'existence de communautés spécifiques aux cultivars. La majorité des isolats obtenus du cultivar Milder Spiral furent classifiées en tant que bactéries Gram-positives à G+C élevé (*Microbacterium* sp., *Micrococcus* sp., *Rhodococcus* sp.) et *Firmicutes* (*Staphylococcus* sp.). Quatre-vingt-treize % des endophytes isolés du cultivar Zeigenhorn Bello étaient affiliées à *Staphylococcus aureus* et *Bacillus* sp. (*Firmicutes*). Le plan expérimental a pu démontrer adéquatement que le refroidissement et le type de cultivar pouvait influencer la diversité des endophytes bactéries colonisant le piment doux. Nous proposons davantage d'expériences de refroidissement afin d'étudier l'impact du refroidissement sur les attributs fonctionnels bénéfiques aux plantes des endophytes bactériens associés avec les cultures sensibles aux basses températures telles que le piment doux.

Mots clés : refroidissement, piments doux thermophiles, diversité des endophytes bactériens, analyse du gène de l'ARNr 16S.

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Introduction

Sweet pepper (*Capsicum anuum* L.) is nowadays frequently cultivated in temperate regions of Middle Europe. In Austria, temperate climate is characterized by nocturnal cold periods, which occur occasionally during the extensive cultivation period from March to October. Such short-term temperature descents within a temperature range of 0 °C (nonfreezing) to about 15 °C are called "chilling" and lead to derogations of various physiological processes within susceptible crops (Lyons 1973; Kratsch and Wise 2000). The severity of chilling injury tends to increase with decreasing temperatures and

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with length of low-temperature exposure (Lyons 1973). In particular, crops of subtropical and tropical origin, such as sweet pepper, are very sensitive to chilling. Their susceptibility is mainly manifested by restricted germination, seedling development, and growth, as well as by alterations of physiological conditions (Martin et al. 1981; Wise and Naylor 1987; Wang 1990; Kratsch and Wise 2000).

Bacteria that colonize the intercellular spaces and vascular tissues of plants are called endophytes, and numerous of these endophytic bacteria exhibit essential plant growth promoting activities (Sturz et al. 2000; Lodewyckx et al. 2002*a*; Sessitsch et al. 2004). The term endophyte is derived from the Greek "endon" (within) and "phyte" (plant), and until recently, this term had usually been applied to fungi (Caroll 1988), including the mycorrhizal fungi (O'Dell and Trappe 1992). To include bacteria in the group of endophytic microbes, Wilson (1995) defined endophytes as "bacteria and fungi, which for all part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease." It has been reported that plant genotype as well as cultivars of the same plant genotype strongly influence the individual community structure and functioning of the corresponding endophytic bacterial community (Siciliano et al. 1998; Sessitsch et al. 2002). In addition, recent studies by Sessitsch et al. (2002), Malinowski and Belesky (2000), and Dalmastri (1999) confirmed that abiotic factors, such as light deficiency, drought, and mineral stress, as well as soil type, can alter the diversity and functioning of bacterial endophyte populations.

No information is available on endophytes in sweet pepper and on how they are affected by chilling. Therefore, this study might be of high relevance, as chilling-related population shifts may lead to a modified functional potential of the endophytic community. Chilling-sensitive pepper varieties may particularly suffer from diminished beneficial interactions under low-temperature stress.

As a prerequisite for understanding potential chilling effects on endophyte functioning, the endophytic structural diversity associated with pepper plants under chilling stress needs to be adequately characterized. The present study, therefore, focused on diversity analyses of bacterial endophytic populations colonizing two different pepper cultivars exposed to chilling. We followed a cultivation-independent, community fingerprinting approach based on terminal-restriction fragment length polymorphisms (T-RFLP), using the 16S rRNA gene as a phylogenetic marker. Because of its sensitivity and high resolution, T-RFLP analysis has been proven to represent an ideal technique for the detection of the smallest genetic differences in complex bacterial populations in various ecosystems (Dunbar et al. 2001). T-RFLP analysis was supported by the establishment and sequence analysis of 16S rRNA gene libraries to determine dominant community members within T-RFLP fingerprint profiles of selected treatments. Further, the cultivable members of the endophyte community were isolated and identified by 16S rRNA gene analysis.

Materials and methods

Climate chamber experiment

Seeds of the sweet pepper (*Capsicum anuum* L.) cultivars Milder Spiral and Ziegenhorn Bello (Austrosaat, Vienna, Austria), which have been previously observed as susceptible and tolerant, respectively, cultivars against chilling (Christina Naglreiter, ARC Seibersdorf research GmbH, unpublished data), were sown in Frux ED 63 growing substrate (70% white peat, pH 5.5–6.5, 2.0 g L^{-1} sodium chloride, 200–350 mg L^{-1} N, 200–350 mg L^{-1} P_2O_2, 300–500 mg L^{-1} K_2O) (Patzer, Sinntal-Jossa, Germany) and were placed in a climate chamber (Vötsch Industrietechnik, Vienna, Austria) for 2 weeks. The climate within the chamber was adjusted to typical spring time temperate conditions in the area of Vienna: temperature (0900–1300 (i.e., time of day), 21 °C; 1300–1400, 23–25 °C; 1400–1900, 21 °C; 1900–0900, 18 °C), light (0800–1000, 400 μmol m^{-2} s^{-1} (proton flux density); 1000–1300, 800 μmol m^{-2} s^{-1}; 1300–1400, 1200 μmol m^{-2} s^{-1}; 1400–1800, 800 μmol m^{-2} s^{-1}; 1800–2000, 400 μmol m^{-2} s^{-1}; 2000–0800, darkness), and relative humidity (50%–60%). After germination, seedlings were planted in pots (10 cm by 10 cm by 10 cm) filled with Frux ED 63 substrate and were cultivated for 3 weeks under the same climatic conditions. After plantlets had reached the 7–8 leaf stage (principal growth stage 1 "Leaf development," as defined by Feller et al. (1995)), they were transplanted into 5 L pots with a 23 cm diameter containing Frux ED 63 substrate. Once a week, the plants were fertilized with 0.2% Wuxal® solution (Bayer, Langenfeld, Germany). When the plants reached the flowering stage (principal growth stage 6 "Flowering," as defined by Feller et al. (1995)), the chilling treatment was performed in two steps. For the first chilling treatment, half of the plants of both cultivars were transferred into a cold (6 °C), dark chamber to simulate a cool night, whereas the control plants remained in the chamber with a temperature of 18 °C. Chilled plants were kept in the cold room for 10 h (2200–0800). After this process, chilled plants were transferred back into the 18 °C chamber, where they were allowed to recover for 1 week. Then, the chilling treatment was repeated but with a chilling temperature of 4 °C. After the stressed plants recovered again for 1 week, sampling of control and chilled plants was performed.

Isolation and PCR–RFLP analysis of cultivable bacterial endophytes

Endophytic bacteria were isolated from three replicate plants from control, nonchilled plants. Isolation was performed from shoot segments (3 cm long) taken from the lower stem part. Each segment was surface sterilized with 5% sodium hyperchlorite for 2 min, rinsed in autoclaved water, dipped into 70% ethanol, and finally flamed. Sterilized segments were then aseptically peeled, cut into small pieces, and transferred into sterile plastic bags (30 mL, Whirl®-Pak, Nasco, Fort Atkinson, Wis., USA). Two millilitres of 0.85% sodium chloride solution was added, and samples were homogenized with a mortar and pestle. Tenfold dilution series were prepared with 0.85% sodium chloride solution, and 100 μL of each dilution was plated in triplicate onto King's B agar (King et al. 1954). Dishes were incubated for 7 days at 24 °C. After incubation, 30 colonies per line were randomly selected from both dilutions, were purified by restreaking on the same medium, and were incubated at 30 °C for 2 days. Isolates were propagated in 5 mL of King's B medium overnight at 30 °C. Cells were harvested

by centrifugation for 30 s at 10 000 r min^{-1}. The supernatant was decanted, and genomic DNA was isolated by using a bead beating protocol (Sessitsch et al. 2002). Endophytic DNA was stored in 60 μL of Tris–EDTA (TE) buffer (pH 8.0) containing 0.1 mg mL^{-1} RNase (Concert RNase A, Invitrogen, Carlsbad, Calif., USA) at –20 °C. RFLP analysis of the 16S rRNA gene was used to group isolates at the species level, whereas characterization of the 16S–23S rRNA intergenic spacer (IGS) region was used to distinguish different strains of the same species. The 16S rRNA genes of endophytic bacteria were amplified by PCR, using the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Weisburg et al. 1991) and 1520R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al. 1989). Primers pHr (5′-TGCGGCTGGATC ACCTCCTT-3′) and P23SR01 (5′-GGCTGCTTCTAAGCC AAC-3′) (Massol-Deya et al. 1995) were used for amplification of the 16S–23S rRNA IGS. PCR cocktails of 50 μL contained 0.5 μL of undiluted DNA, 1× PCR reaction buffer (Invitrogen), a 2.5 mmol L^{-1} concentration of $MgCl_2$, a 0.15 μmol L^{-1} concentration of each primer, a 0.2 mmol L^{-1} concentration of each deoxynucleoside triphosphate, and 2 U *Taq* DNA polymerase (Invitrogen). PCR amplifications were performed with an initial denaturation at 95 °C for 5 min and with 30 cycles each consisting of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 1 min, and polymerization at 72 °C for 2 min, and were completed with a final extension at 72 °C for 10 min. PCR products (5 μL) were checked by electrophoresis in 1% (*m*/*v*) agarose gels (Biozym, Vienna, Austria). Digestion of 10 μL of PCR products was performed with 5 U of restriction endonuclease *Alu*I (Invitrogen) at 37 °C for 4 h. The resulting DNA fragments were analyzed by gel electrophoresis in 3% (*m*/*v*) agarose gels (Biozym). A representative isolate of each IGS type was identified by partial 16S rRNA gene sequence analysis. Amplicons were purified with the NucleoTraPCR kit (Macherey-Nagel, Düren, Germany) and used as templates for sequence analysis. Partial sequencing of 16S rDNA was performed by applying the BigDye® Terminator v3.1 kit (Applied Biosystems, Warrington, UK) and the reverse primer 518r (5′-ATTACCGCGGCTGCTGG-3′) (Liu et al. 1997), resulting in sequence information of about 500 bp. Sequences were subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database.

T-RFLP analysis

For T-RFLP analysis, shoot segments of 10 cm in length were obtained from three replicate plants per treatment. Stem segments were surface sterilized and aseptically peeled, as described above, and transferred into sterile plastic bags (30 mL, Whirl®-Pak, Nasco). Samples were stored at –20 °C until DNA was isolated. For DNA isolation, 250–300 mg of frozen plant material was cut it into small pieces, transferred into a 2 mL bead beating tube, and 800 μL TN150 buffer (10 mmol L^{-1} Tris–HCl (pH 8.0), 150 mmol L^{-1} NaCl) was added. Samples were frozen in liquid nitrogen and then pulverized in a mixer mill (Retsch and Co., Haam, Germany) at full power (100%) twice for 90 s each in the presence of two sterile stainless steel beads (5 mm) at thawing. Three hundred milligrams of acid-washed glass beads (0.1 mm, Sigma-Aldrich Corp., St. Louis, Mo., USA) was added to aliquots, and bead beating was performed twice for 20 s at 4.0 m s^{-1} in a bead beater (FastPrep FP 120, Bio101, Savant Instruments, Inc., Holbrook, N.Y., USA). After extraction with phenol and chloroform, DNA was precipitated with a 0.1 volume of 3 mol L^{-1} sodium acetate solution (pH 5.2) and 0.7 volume of isopropanol at –20 °C for at least 30 min. DNA was centrifuged at 13 000 r min^{-1} for 20 min, washed with 100 μL 70% ethanol, and air-dried. DNA was dissolved in 30 μL TE buffer (pH 8.0) containing 0.1 mg mL^{-1} RNase (Concert RNase A, Invitrogen) and incubated at 37 °C for 15 min. DNA extracts were purified by passage through CL6B spin columns (filled with Sephadex™ G-50 (Amersham Biosciences, Piscataway, N.J., USA) in TE buffer (pH 8.0); 2800 r min^{-1} for 3 min). DNA extracts were stored at –20 °C. DNA isolations of one replicate control plant of both cultivars failed, therefore these samples had to be excluded from further analysis. Endophytic 16S rRNA genes were PCR amplified, using the primers 799F (5′-AAC(AC) GGATTAGATACCC(GT)-3′) (Chelius and Triplett 2001), which was labeled with 6-carboxyfluorescein at the 5′ end, and 1520R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al. 1989). PCR cocktails of 50 μL contained 1–4 μL of undiluted DNA, 1× PCR buffer (Invitrogen), a 2.5 mmol L^{-1} concentration of $MgCl_2$, a 0.15 μmol L^{-1} concentration of each primer, a 0.2 mmol L^{-1} concentration of each deoxynucleoside triphosphate, and 2 U *Taq* DNA polymerase (Invitrogen). PCR amplifications were performed with an initial denaturation at 95 °C for 5 min, 30 cycles each consisting of denaturation at 95 °C for 30 s, primer annealing at 53 °C for 1 min, and polymerization at 72 °C for 2 min, and were completed by a final extension at 72 °C for 10 min. PCR products (5 μL) were checked by electrophoresis in 1% (*m*/*v*) agarose gels (Biozym). Four PCR products of each sample were pooled and precipitated with a 0.1 volume of 3 mol L^{-1} sodium acetate solution (pH 5.2) and a 0.7 volume of isopropanol at –20 °C for at least 30 min. DNA was centrifuged at 10 000 r min^{-1} for 20 min and air-dried, and the pellet was dissolved in 40 μL of TE buffer (pH 8.0). PCR products were subjected to electrophoresis in 2% (*m*/*v*) agarose gels. The band of interest containing the PCR product of bacterial 16S rDNA (~720 bp) was excised and purified with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Digestion of 10 μL PCR product was performed with a combination of *Hae*III–*Hha*I (5 U each, Invitrogen) at 37 °C for 4 h. Prior to the T-RFLP analysis, digests were purified by passage through CL6B spin columns (filled with Sephadex™ G-50 in TE buffer (pH 8.0); 2800 r min^{-1} for 3 min). Labeled terminal restriction fragments (T-RFs) were detected by capillary electrophoresis with an ABI 3100 automatic DNA sequencer. Ten microlitres of digested PCR products was mixed with 15 μL HiDi formamide (Applied Biosystems) and a 0.3 μL internal size standard (500 ROX™ Size Standard, Applied Biosystems). Prior to analysis, samples were denatured at 92 °C for 2 min and immediately chilled on ice. The GeneScan® analysis software packet (version 3.7, Applied Biosystems) was used for data collection. Relative lengths of the T-RFs were determined by comparing them with the internal size standard. GenoTyper 3.7 NT software (Applied Biosystems) was used to transform the electropherograms of each sample into numeric data. Both fragment length and peak height were used as parameters for profile comparison. Normalization of T-RFLP fingerprint

Table 1. Sequence analysis of partial 16S rDNA (approximately 500 bp) of endophytic bacterial isolates obtained from flowering, nonchilled sweet pepper *Capsicum annum* L. 'Milder Spiral' and 'Ziegenhorn Bello'.

IGS type[a]	16S rDNA type	Closest NCBI match (acc. No.); % homology	Phylogenetic group
Milder Spiral			
iMSN51 (3)	iMSN51	*Staphylococcus aureus* (BX571857); 100	*Firmicutes*
iMSN50 (1)	IMSN50	*Staphylococcus aureus* (DQ193528); 99	*Firmicutes*
iMSN20 (1)	iMSN20	*Staphylococcus* sp. MSB2032 (AY275505); 99	*Firmicutes*
iMSN37 (5)	iMSN04	*Microbacterium* sp. GWS-SE-H100 (AY332149); 98	High-G+C Gram-positive bacteria
iMSN23 (2)	iMSN23	*Micrococcus luteus* (AJ409096); 100	High-G+C Gram-positive bacteria
iMSN49 (1)	iMSN49	*Micrococcus luteus* (AJ516052); 99	High-G+C Gram-positive bacteria
iMSN17 (1)	iMSN17	*Rhodococcus erythropolis* (AY357220); 99	High-G+C Gram-positive bacteria
iMSN24 (6)	iMSN17	*Rhodococcus* sp. djl-6 (DQ090961); 100	High-G+C Gram-positive bacteria
iMSN27 (1)	iMSN27	*Rhodococcus* sp. iRV10 (AY358017); 98	High-G+C Gram-positive bacteria
iMSN04 (3)	iMSN04	*Streptomyces* sp. YIM26 (AF389343); 99	High-G+C Gram-positive bacteria
iMSN10 (1)	iMSN10	*Rhizobium* sp. Phr-9 (AF510387); 99	α-Proteobacteria
iMSN36 (5)	iMSN36	*Frateuria* sp. A12 (AF406661); 99	γ-Proteobacteria
Ziegenhorn Bello			
iZBN10 (1)	iZBN10	*Bacillus* sp. BacB1 (AF497247); 100	*Firmicutes*
iZBN13 (3)	iZBN13	*Bacillus* sp. Mali 47 (AY211141); 98	*Firmicutes*
iZBN11 (24)	iZBN11	*Staphylococcus aureus* (BX571857); 99	*Firmicutes*
iZBN12 (1)	iZBN12	*Rhodococcus* sp. djl-6 (DQ090961); 100	High-G+C Gram-positive bacteria
iZBN22 (1)	iZBN22	*Paracoccus yeeii* (AY014173); 99	α-Proteobacteria

[a]Numbers in parentheses indicate the number of isolates showing the intergenic spacer (IGS) restriction fragment length polymorphism type.

profiles was performed according to Dunbar et al. (2001). Finally, the values of peak heights of ≥30 fluorescence units of 55 normalized T-RFs with different fragment lengths were used for analysis of community patterns. Analysis of variance combined with post hoc Bonferroni tests (SPSS for Windows, version 11.7, SPSS Inc., Chicago, Ill., USA) was used to determine significant treatments effects on the T-RFLP data. The values of peak height of T-RFs were examined for significant differences in relation to cultivar and chilling. The T-RFLP data set was further subjected to discriminant analysis to investigate differences between treatments and to identify important discriminating variables.

Cloning and sequence analysis

The 16S rRNA gene libraries were generated from both nonchilled control lines and chilled Milder Spiral. PCR products of the corresponding treatment replicates were pooled and purified with the QIAquick Gel Extraction kit (Qiagen). Purified amplicons were ligated into the TpCR 4-TOPO vector (Invitrogen). *Escherichia coli* DH5α-T1^R (Invitrogen) was then transformed with the ligation products. Fifty colonies of each treatment were randomly picked and transferred to a new medium for a 24 h incubation period. Colonies were suspended in a reaction tube containing 50 μL TE buffer (pH 8.0), boiled for 10 min, chilled on ice, and centrifuged for 10 min at 13 000 r min^{-1} and the supernatant was used for PCR. Clones were PCR amplified with the primers M13f and M13r. Amplicons were purified by passage through the CL6B spin columns (filled with Sephadex™ G-50 (Amersham) in TE buffer (pH 8.0); 2800 r min^{-1} for 3 min) and used as templates for sequencing analysis. Partial sequencing of 16S rDNA was performed by applying the BigDye® Terminator v3.1 kit (Applied Biosystems) and the reverse primer 1385r (5′-CGGTGTGTACAAGACCC-3′) (Lane 1991), resulting in sequence information of about 500 bp. Clones were finally checked for chimaeric artifacts by using CHECK_CHIMERA of the Ribosomal Database Project, and chimaeric sequences were discarded. Sequences were subjected to BLAST analysis with the NCBI database. To identify the 16S rRNA clones with the T-RFs in the corresponding T-RFLP fingerprint, clones were subjected to T-RFLP analysis as described above.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession Nos. DQ401242–DQ401258 (bacterial isolates) and DQ401196–DQ401241 (16S rRNA gene clones).

Results

Characterization of isolated bacterial endophytes

Colony forming units (cfu) of isolated endophytes obtained from the nonchilled plants Milder Spiral and Ziegenhorn Bello revealed no significant difference between the two cultivars ($P < 0.05$). On average, $1.24 \times 10^3 \pm 5.23 \times 10^2$ SE (standard error) cfu (g fresh plant material)$^{-1}$ were isolated from each shoot segment. A total of 60 bacterial isolates were characterized by 16S rRNA gene analysis. Endophytes of the nonchilled Milder Spiral cultivar could be grouped into 12 16S–23S rRNA intergenic spacer (IGS) region types and into 10 16S rDNA types, whereas for the nonchilled Ziegenhorn Bello cultivar, five IGS and five 16S rRNA types were obtained (Table 1). Phylogenetic affiliation of endophytic isolates with different IGS patterns was done by partial sequence analysis. Sequences covered approximately 500 bp each, and most sequences showed at least 98% similarity to known partial 16S rRNA gene sequences in the NCBI database (Table 1). The majority of isolates obtained from nonchilled Milder Spiral line belonged to the high-G+C

Gram-positive bacteria (63%), with *Firmicutes* and γ-Proteobacteria following at 17% each. The high-G+C Gram positives were dominated by *Rhodococcus* sp., *Micrococcus luteus*, and *Streptomyces* sp., whereas the *Firmicutes* and the γ-Proteobacteria were mainly represented by *Staphylococcus* sp. and *Microbacterium* sp., respectively. The α-Proteobacteria were represented by one isolate (3% *Rhizobium* sp.). Ninety-three percent of the endophytes isolated from nonchilled Zeigenhorn Bello were identified as *Staphylococcus aureus* and *Bacillus* sp. (*Firmicutes*). The remaining two isolates belonged to the high-G+C Gram-positive bacteria (3% *Rhodococcus* sp.) and the α-Proteobacteria (3% *Paracoccus yeeii*), respectively (Table 1).

T-RFLP fingerprint profiles and gene libraries

After normalization of T-RFLP fingerprint profiles, a total of 55 terminal restriction fragments (T-RFs) with different fragment lengths were identified. In individual profiles, between 13 and 37 T-RFs were identified, which had peak heights of at least 30 fluorescence units. The endosphere bacterial communities showed different population structures in relation to the two cultivars, Milder Spiral and Ziegenhorn Bello, and were affected by chilling. In particular, analysis of variance determined 13 T-RFs (71, 72, 78, 158, 175, 176, 189, 207, 241, 268, 272, 287, and 289 bp), which were significantly affected by the two cultivars, whereas chilling affected nine T-RFs (71, 78, 79, 111, 158, 189, 207, 241, and 289 bp) and 11 T-RFs (71, 78, 79, 111, 175, 176, 247, 268, 272, 287, and 289 bp) within Ziegenhorn Bello and Milder Spiral, respectively ($P < 0.05$) (Table 2). A comparison of T-RFLP fingerprint profiles was based on discriminant analysis, which confirmed the results determined by analysis of variance. Discriminant analysis revealed diversity differences in relation to cultivar and chilling. In detail, the first two discriminating functions were able to explain 99.9% of the total variance. The discriminating power of the functions was confirmed by their high canonical correlations ($r = 0.999$) with the two treatments, cultivar and chilling. The capability of the discriminating functions to significantly discriminate the treatments was further confirmed by a Wilks' lambda of $P = 0.004$. In detail, the nonchilled Milder Spiral and Ziegenhorn Bello exposed the greatest effect on the bacterial endophytic community. When analyzing the chilling effect, differences between the chilled and nonchilled plants of Milder Spiral were greater than the differences between the chilled and nonchilled Ziegenhorn Bello, which showed only a slight chilling-related diversity difference. In total, 16 significant T-RFs (71, 72, 78, 79, 111, 158, 175, 176, 189, 207, 241, 247, 268, 272, 287, and 289 bp) confirmed these treatment separations ($P < 0.05$). To identify the dominant community members within the T-RFLP fingerprint profiles, 16S rRNA gene libraries were constructed and clones were partially sequenced. These gene libraries consisted of sequences derived from the nonchilled, control plants of Milder Spiral and Ziegenhorn Bello and from the chilled plants of the Milder Spiral line. In total, 150 clones were sequenced; however, for 34 sequences, the presence of chimaeric sequences could not unambiguously be excluded. Most of the clearly nonchimaeric sequences showed at least 97% similarity to known sequences in the NCBI database, whereas 11 clones were only distantly (94%–96%) related to known 16S rRNA genes (Table 3). The majority of the sequences obtained from all treatments used for cloning were assigned to chloroplasts (nonchilled Milder Spiral, 53%; nonchilled Ziegenhorn Bello, 65%; chilled Milder Spiral, 63%). The remaining clones belonged to the divisions *Firmicutes* (nonchilled Milder Spiral, 12%; nonchilled Ziegenhorn Bello, 17%; chilled Milder Spiral, 2%), high-G+C Gram-positive bacteria (nonchilled Milder Spiral, 3%; nonchilled Ziegenhorn Bello, 3%), α-Proteobacteria (nonchilled Milder Spiral, 9%; nonchilled Ziegenhorn Bello, 3%; chilled Milder Spiral, 25%), β-Proteobacteria (nonchilled Milder Spiral, 6%; nonchilled Ziegenhorn Bello, 6%; chilled Milder Spiral, 6%), and γ-Proteobacteria (nonchilled Milder Spiral, 17%; nonchilled Ziegenhorn Bello, 6%; chilled Milder Spiral, 4%). To identify clones corresponding to dominant T-RFs in the community profiles, clones were subjected to T-RFLP analysis. This was necessary, since the actual T-RF lengths may differ from the theoretical, sequence-determined T-RF lengths. The drift ranged from 0 to 5 bases (Table 3). In general, the results obtained by sequence analysis and by T-RFLP analysis were in good agreement. Bacteria belonging to the divisions of *Firmicutes*, high-G+C Gram-positive bacteria, α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria were highly represented in the T-RFLP patterns of all analyzed treatments by the 79 bp T-RF, indicating high abundance (Fig. 1). The 16S rRNA gene libraries indicated differences between both nonchilled cultivars as well as a chilling effect for Milder Spiral, although to a certain extent highly similar sequences were

Table 2. Terminal-restriction fragments (T-RFs) that were significantly affected by cultivar and chilling, as determined by analysis of variance.

	Significance level		
T-RF size[a] (bp)	Cultivar effect on MS vs ZB	Chilling effect on MS	Chilling effect on ZB
71	***	***	*
72	*	–	–
78	*	***	*
79	–	*	**
111	–	***	***
158	***	–	***
175	**	**	–
176	***	***	–
189	***	–	***
207	*	–	*
241	**	–	***
247	–	**	–
268	***	***	–
272	***	***	–
287	*	**	–
289	**	***	*

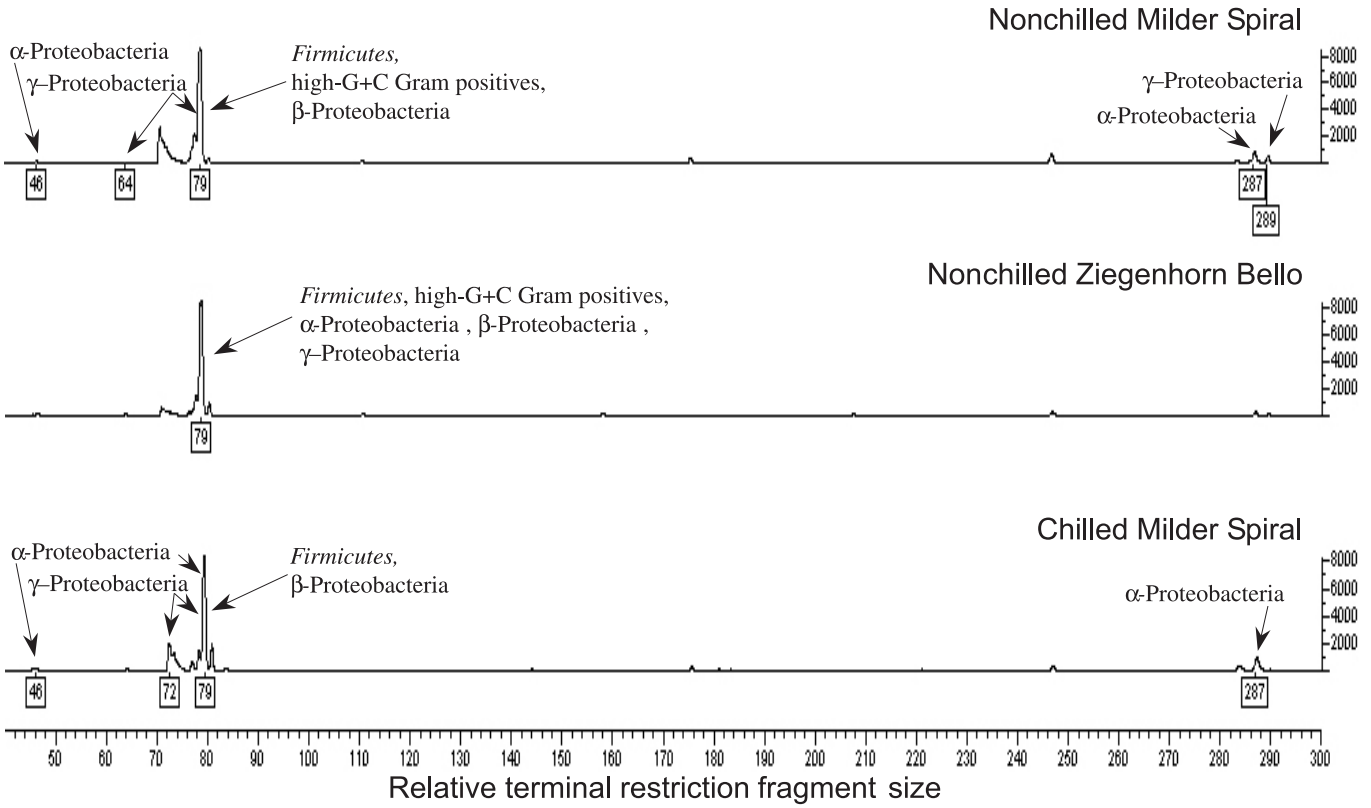
Note: Sweet pepper cultivars: MS, Milder Spiral; ZB, Ziegenhorn Bello. Significance levels: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; –, $P > 0.05$.

[a]T-RFs that were significantly influenced by cultivar or (and) chilling treatment.

Table 3. Phylogenetic assignment of gene libraries of amplified bacterial 16S rRNA genes derived from the endosphere of nonchilled *Capsicum annum* L. 'Milder Spiral' and 'Ziegenhorn Bello' and chilled 'Milder Spiral'.

Taxon	Theoretical T-RF size (bp)	Actual T-RF size (bp)	Corresponding clone	Closest NCBI match (acc. No.); % homology
Nonchilled Milder Spiral				
Firmicutes	81	79	cloMSN45	*Bacillus* sp. R002A (AY188839); 98
	82	79	cloMSN22	*Exiguobacterium* sp. HHS31 (AJ846291); 99
	82	79	cloMSN01/91	*Staphylococcus aureus* subsp. *aureus* (CP000046); 99
High G+C Gram-positive bacteria	82	79	cloMSN48	*Rhodococcus fascians* (AY730713); 99
α-Proteobacteria	46	46	cloMSN30	*Methylobacterium* sp. SKJH-1 (AY741724); 98
	49	46	cloMSN93	*Methylobacterium* sp. SKJH-1 (AY741724); 100
	287	287	cloMSN07	*Methylobacterium* sp. iRIV1 (AY358005); 99
β-Proteobacteria	80	79	cloMSN05	Uncultured *Duganella* sp. CTBH-18 (AF067655); 99
	80	79	cloMSN31	Uncultured β-proteobacterium CLi96 (AF529344); 99
γ-Proteobacteria	66	64	cloMSN85	*Pseudomonas mucidolens* (D84017); 100
	81	79	cloMSN100	*Acinetobacter lwoffii* (DQ144736); 99
	81	79	cloMSN28	*Pseudomonas* sp. GP30 (DQ003219); 98
	83	79	cloMSN47	*Stenotrophomonas maltophilia* (AY841799); 99
	288	289	cloMSN64	*Enhydrobacter aerosaccus* (AJ550856); 99
	288	289	cloMSN63	*Pseudomonas* sp. T213BY5 (AY741257); 99
Nonchilled Ziegenhorn Bello				
Firmicutes	81	79	cloZBN24	*Staphylococcus epidermis* (AY730712); 99
	82	79	cloZBN09/13/60	*Staphylococcus aureus* subsp. *aureus* (CP000046); 100
	82	79	cloZBN47	*Staphylococcus aureus* subsp. *aureus* (CP000046); 99
	84	79	cloZBN100	*Streptococcus* sp. 106-03c (AJ871182); 98
High G+C Gram-positive bacteria	83	79	cloZBN10	*Arthrobacter* sp. Fa21 (AY131225); 99
α-Proteobacteria	78	79	cloZBN101	*Brevundimonas* sp. FWC30 (AJ227796); 98
β-Proteobacteria	80	79	cloZBN57	*Burkholderia stabiliz* (AY616143); 99
	81	79	cloZBN23	Uncultured *Paucimonas* sp. CLOENT34 (DQ124793); 99
γ-Proteobacteria	81	79	cloZBN35	*Acinetobacter* sp. DG880 (AY258108); 100
	81	79	cloZBN50	*Serratia marcescens* (DQ112331); 99
Chilled Milder Spiral				
Firmicutes	79	79	cloMSC31	*Staphylococcus epidermidis* (D83362); 99
α-Proteobacteria	46	46	cloMSC09	*Methylobacterium* sp. iRIV1 (AY358005); 99
	79 or 80	79	cloMSC24/38	*Brevundimonas mediterranea* (AJ244706); 99
	79	79	cloMSC68	*Bosea* sp. 7F (AF531764); 99
	283	287	cloMSC34	*Methylobacterium* sp. RKT-5 (AY770515); 95
	284 or 286	287	cloMSC11/19/63	*Methylobacterium extorquens* (AF531770); 98
	284	287	cloMSC57	*Methylobacterium* sp. RKT-5 (AY770515); 99
	286	287	cloMSC36	*Methylobacterium* sp. RKT-5 (AY770515); 99
	287 or 288	287	cloMSC22/40	*Methylobacterium extorquens* (AF531770); 99
β-Proteobacteria	79	79	cloMSC07	*Burkholderia stabiliz* (AY616143); 99
	80	79	cloMSC35	*Janthinobacterium* sp. PI12 (DQ202711); 99
	80	79	cloMSC30	*Massilia* sp. Tibet-IIU65 (DQ177481); 99
γ-Proteobacteria	75	72	cloMSC05	*Klebsiella* sp. TNT1 (DQ229100); 98
	81	79	cloMSC17	*Serratia marcescens* (DQ226210); 99

Fig. 1. Representative terminal restriction fragment length polymorphism electropherograms of endophytic bacterial communities derived from the nonchilled sweet pepper Milder Spiral and Ziegenhorn Bello as well as from the chilled Milder Spiral. Fragments corresponding to dominant phylogenetic groups represented by 16S rRNA gene libraries from the same endosphere samples of assayed cultivars are indicated and labeled with the respective fragment size.



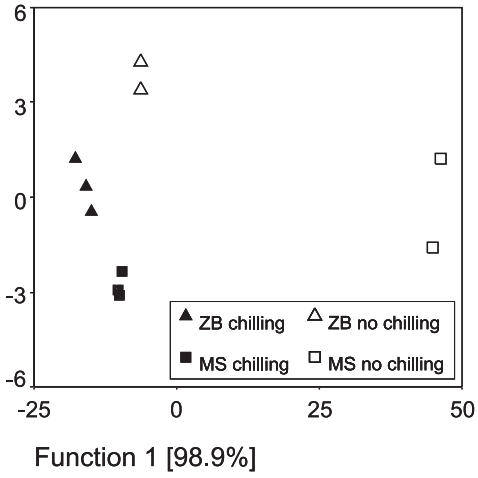
found in different treatments. Nevertheless, the number of clones analyzed was far too small to allow any statistical analysis.

Discussion

Chilling is characterized by low, nonfreezing temperatures that limit the productivity and quality of chilling-sensitive crops (e.g., sweet pepper (*Capsicum anuum* L.)) (Li et al. 2003). Such thermophilic crops, originating from tropical and subtropical areas, are now frequently cultivated in temperate regions. Thus, they are endangered to be easily affected by short chilling periods, which occur occasionally in temperate regions (e.g., in Austria) (Allen et al. 2001). It has been previously confirmed that particularly biochemical processes (e.g., photosynthesis activity) in sweet pepper are impaired by chilling events (Li et al. 2001, 2004). Apart from biochemical and physiological measurements, there is presently no report available that focuses on the potential response of the endosphere bacterial diversity associated with thermophilic sweet pepper under low-temperature stress. This investigation of chilling effects on endophyte community structure is of high relevance as chilling-related diversity changes can probably affect the functional potential of the endophytic community. These modifications consequently might have adverse effects on the host development, as numerous bacterial endophytes exhibit essential plant-beneficial functions, such as growth promotion and biocontrol activities (Sturz et al. 2000; Lodewyckx et al. 2002*a*; Sessitsch et al. 2004). This study was therefore dedicated to the analysis of structural characteristics of bacterial endophytic populations colonizing two different sweet pepper cultivars exposed to chilling events. In particular, bacterial endosphere communities were characterized by molecular 16S rRNA gene based cultivation-dependent and cultivation-independent techniques.

Genetic differences between plant genotypes or cultivars of a certain plant species influence the endophytic bacterial community composition (Elvira-Recuenco and van Vuurde 2000; Adams and Kloepper 2002). The two sweet pepper cultivars Milder Spiral and Ziegenhorn Bello analyzed in this study harbored unique endophytic communities. In detail, T-RFLP analysis yielded 11 out of 55 detected T-RFs that were significantly influenced by the plant genotype (Table 2). 16S rDNA gene library analysis revealed dominant bacteria related to significantly affected T-RFs within the corresponding T-RFLP community patterns. In particular, several clones with respective T-RF lengths of 46, 64, and 289 bp identified as *Methylobacterium* sp., *Pseudomonas* sp., and *Enhydrobacter* sp. were determined in the gene library of nonchilled Milder Spiral but not in that of Ziegenhorn Bello. All T-RFLP fingerprint profiles were dominated by a T-RF of 79 bp length, which corresponded to clones of various bacterial divisions that could be referred to this T-RF (Table 3). In detail, *Micrococcus* sp., *Paracoccus* sp., and *Rhodococcus* sp. represent potential human or plant pathogens (Verville et al. 1994; Goethals et al. 2001; Daneshvar et al.

Fig. 2. Discriminant analysis of the terminal restriction fragment length polymorphism data derived from the differently treated pepper endospheres. Data points represent the pepper lines, Milder Spiral (MS) and Ziegenhorn Bello (ZB), and the chilling treatment, no chilling or chilling. One replicate of each of the nonchilled control lines was missed due to failed DNA isolations.



2003). Interestingly, numerous isolates and clones have been identified as the pathogenic bacterium *Staphylococcus aureus* causing serious infections in humans, including endocarditis, deep-seated abscesses, and osteomyelitis (Brakstad et al. 1992). In contrast to the high number of potential pathogens, there was only a small number of nonpathogenic endophytes, such as *Bacillus* sp. and *Arthrobacter* sp., obtained from both cultivars that have been previously found in other crops (Reiter et al. 2002; Sessitsch et al. 2002; Kwon et al. 2005). A possible explanation for the high abundance of potential human pathogens, above all *Staphylococcus aureus*, and nonpathogenic bacteria in the endospheres might be their origin from the non-heat-treated white peat growth substrate that was used for the climate chamber experiment. Earlier studies have demonstrated that soils are a potential source of bacterial endophytes entering the plant through natural openings or wounds of plant roots (Hallmann et al. 1997; Dalmastri 1999; Girvan et al. 2003). However, the fact that the plant genotype may drastically influence the internal colonization by human pathogens merits further investigation.

Abiotic factors affect the bacterial community composition of plant tissue colonizing bacterial endophytes (Dalmastri 1999; Kinkel et al. 2000; Malinowski and Belesky 2000; Sessitsch et al. 2002). It is therefore very likely that chilling directly affects the bacterial endophyte community composition. This argumentation finds support by Cambours et al. (2005) who showed that endophytic populations associated with *Salix* plants are affected by frost. In addition, Pesaro et al. (2003) confirmed that freeze–thaw processes in soils can lead to significant changes in microbial community characteristics. In the present study, a low-temperature-related diversity difference was found for both cultivars (Fig. 2). In particular, the endophyte population associated with Milder Spiral was more affected than the bacterial community of Ziegenhorn Bello. Because of the major chilling effect on Milder Spiral plants, 16S rRNA gene libraries were generated of the nonchilled and chilled plants to determine dominant, chilling-affected community members. In detail, *Methylobacterium* sp. was more abundant in chilled plants than in control plants. Former studies showed that methylobacteria commonly occur in the endosphere and rhizosphere of stressed plants, such as highly nickel-accumulating *Thlaspi goesingense* (Idris et al. 2004) or the zinc hyperaccumulator *Thlaspi caerulescens* (Lodewyckx et al. 2002*b*). These methylobacteria might be generally important for plants suffering from abiotic stress, since Idris et al. (2004), Ivanova et al. (2001), Holland (1997), as well as Holland and Polacco (1994) have shown that these bacteria are able to produce plant growth promoting enzymes, like 1-aminocyclopropane-1-carboxylic acid deaminase, and siderophores or hormones, such as cytokinins and indole acetic acid. Apart from the direct effect of low temperature on bacterial endophytes, it is also possible that the population shifts have been a consequence of chilling-related plant physiological modifications within the sweet pepper cultivars. In a previous experiment in which the same Milder Spiral and Ziegenhorn Bello cultivars were used, it was shown that plant physiological parameters (e.g., net rate of CO_2 assimilation and actual photochemical capacity) of sweet pepper were strongly affected by chilling (Christina Naglreiter, ARC Seibersdorf research GmbH, unpublished data). In detail, Milder Spiral showed a decrease in the net rate of CO_2 assimilation and actual photochemical capacity, indicating a higher chilling susceptibility than Ziegenhorn Bello, for which smaller decreases were determined. This might thus be a conceivable explanation for the greater diversity differences between the chilled and nonchilled Milder Spiral plants compared with those of Ziegenhorn Bello.

In all three 16S rRNA gene libraries that have been generated from the nonchilled cultivars and the chilled Milder Spiral, there were large numbers of clone sequences obtained, which could be clearly assigned as chloroplast sequences (or in some cases chloroplast sequences wrongly designated as uncultured bacterial 16S rRNA gene sequences) after comparison with known sequences of the NCBI database. This was very surprising, since for the cultivation-independent analyses (e.g., establishment of 16S rRNA gene libraries), the 799F forward primer was used, which amplifies only bacterial 16S rRNA genes and no plant-derived small-subunit rRNA genes (Chelius and Triplett 2001). However, this is the first assay in which this forward primer was applied to characterize bacterial communities associated with sweet pepper shoots. Chelius and Triplett (2001) used the 799F primer for the detection of endophytic bacteria colonizing maize roots. In addition, this forward primer was successfully used by Rasche et al. (2006) and Idris et al. (2004) to characterize bacterial communities colonizing shoots of potatoes and *Thlaspi goesingense*, respectively.

In conclusion, it was shown that the genotype of thermophilic sweet pepper plants strongly influences its associated endophytic bacterial community. It was further shown that the strength of chilling impact on endophytic bacterial diversity was dependent on the cultivar-specific chilling tolerance, which correlated with measurements of physiological changes. This study therefore contains valuable baseline information for prospective assays focusing on chilling-related diversity alterations of bacterial endophytes associated with low-temperature-sensitive crops, such as sweet pepper. Structural

community changes may translate into functional changes, such as plant beneficial effects. We therefore point out that additional chilling experiments are required to confirm obtained results and, particularly, to gain insight into the potential effects on functioning of active but also dormant bacterial endophytes associated with chilling-sensitive crops.

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