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Genus- and Isolate-Specific Real-Time PCR Quantification of *Erwinia* on Leaf Surfaces of English Oaks (*Quercus robur* L.)

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Abstract. In order to quantify pathogenic epiphytic bacteria on leaf surfaces of the important European forest tree *Quercus robur* without time-intensive cultivation and separation of microorganisms, methods were developed to selectively quantify DNA copy numbers of the genus *Erwinia* in DNA isolated from the leaf surface. By using the combination of the two different real-time PCR techniques SYBR®-Green and TaqMan®, methods were developed not only to allow quantification of the total DNA copy number of *Erwinia* on the oak leaf surface, but also to distinguish between two significantly different groups of *Erwinia* strains. In the present work, these techniques were successfully applied to quantify the copy number of the genus *Erwinia* and its subgroups compared with the total bacteria number in DNA samples extracted from the upper leaf surface of English oaks collected on the 4th of June 2001 (Julian day 155).

English oak (*Quercus robur* L.) is a widespread forest tree of central Europe. Oaks are often infected by numerous parasites. Especially on the leaf surface, a community of epiphytic bacteria and fungi can be found. These epiphytic microorganisms can cause serious damage to their host, lowering the yield of the total plant. Some information is available about the epiphytic bacteria on herbs [13, 10], but current knowledge about infection and the species spectrum of phytopathogenic bacteria invading oak leaves is poor. This is because phytopathogenic organisms can often not be grown separately from their host [3], and thus, species-specific cultivation and quantification is difficult and time consuming [1, 12].

Whereas in the case of crops and vegetables a series of specific pathogens are described, not much is known about phytopathogenic bacteria on trees [11], except for fruit-yielding species such as *Pyrus* [8]. No information is currently available about phytopathogenic bacteria on leaves of *Quercus robur*. Therefore, the scope of the present work was to develop a highly sensitive method, based on molecular techniques, allowing the detection of the phytopathogenic bacteria genus *Erwinia* and to quantify the degree of colonization of oak leaves by these bacteria. Detection as well as quantification was carried out using real-time PCR techniques and specific primers and probes for a segment of the internal transcribed spacer region (ITS region) of *Erwinia*.

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Materials and Methods

Isolation of DNA from the upper side of oak leaf surfaces. To be able to quantify the loss of DNA during the procedure, 10^8 copies of the plasmid pCR*myrS* carrying the myrcene synthase gene of *Quercus ilex* [4] were added as an internal standard to each sample before DNA isolation. After DNA isolation, the remaining copy number of plasmids in the samples was measured by using real-time PCR, and recovery rates were calculated. The quantitative removal of DNA from the upper surface of oak leaves, including a freezing and DNA extraction method for the real-time PCR quantification, has recently been described in detail [5].

Amplifying and cloning of *Erwinia* ITS segments. In order to amplify segments of the bacterial intragenic spacer region between the 16S and 23S rDNA, the oligonucleotide primers o-erw-for (5′ AAA GTC ACA CCA GAG TCC CCA TC 3′) and o-erw-rev (5′ GAA CAT ATC GTT A(AG)(CT) TTC ATT C 3′) were used. One microliter of the DNA solution isolated from the upper leaf surface of English oak was used for the PCR, which contained in a final volume of 50 μL 1 μM of the forward and reverse primer each, 2.0 mM $MgCl_2$, dNTPs each 0.2 mM, 5 μL 10 × PCR buffer (Invitrogen, Karlsruhe, Germany), 0.02% detergent W-1 (Invitrogen), and 1 U Taq DNA polymerase (Invitrogen). PCR was performed in 36 cycles (30 s 94°C, 60 s 53°C, and 60 s 72°C). After verification of the size of the amplification products by

Fig. 1. Choice of genus-specific oligonucleotide primers for selective amplification and quantification of *Erwinia* in DNA samples isolated from leaf surfaces. The intragenic transcribed sequences between 16S and 23S rDNA (ITS1) of *Erwinia pyrifoliae* (Er.py), *Erwinia rhapontici* (Er.rh), and the next related enterobacteriacean species *Escherichia vulneris* (Es.vu) were aligned to design the primer pair o-erw-for/o-erw-rev and the real-time primer pair o-erw-rt-for/o-erw-rt-rev to selectively amplify *Erwinia* sequences in real-time PCR. Identical nucleotides in all three *Erwinia* sequences are marked in grey.

agarose gel electrophoresis, 1 μL of the PCR assay containing segments of the expected size was used for ligation with the vector pCRII-Topo (Invitrogen) and was cloned into *E. coli* 10F′ (Invitrogen).

DNA sequencing. After purification of the plasmids harboring the ITS segments, cycle sequencing dideoxy chain-termination reactions with Big Dye Terminators (Applied Biosystems, Weiterstadt, Germany) were performed for both DNA strands, by using vector-specific forward and backward primers (M13 primers, Invitrogen). The sequences were analyzed by using an ABI Prism 310 System (Applied Biosystems), and afterwards the isolated sequences 1–6 were submitted to the EMBL Nucleotide Sequence Database with the accession numbers AJ489444, AJ489441, AJ489442, AJ489443, AJ489445, and AJ489446, respectively.

Real-time PCR. Real-time PCR was performed by two methods. The first method used two primers and SYBR®-Green as fluorescent marker for the rising amount of double-stranded DNA (SYBR®-Green technique), a method already applied for the quantification of *Chlamydia* [6]. The second method used two primers and an additional TaqMan® probe labeled with a fluorescent marker at the 5′ end and a quencher at the 3′ end (TaqMan® technique), a method that was already successfully used, e.g., for *Salmonella* and *Pseudomonas* [7, 9]. The fluorescent marker is cut off from the annealed probe by the 5′ exonuclease activity of the Taq-polymerase, leading to an unquenched fluorescence signal for each new amplified copy during PCR. For the SYBR®-Green method, the assays contained 12.5 μL 2 × SYBR®-Green PCR Master Mix (Applied Biosystems), 300 nM of the primer o-erw-rt-for (5′ ACA CTG CCC TTT CAC GGC T 3′), the primer o-erw-rt-rev (5′ TTT CAA TTT TCA GCT TGT TCC G 3′), and 5 μL of the DNA solution isolated from the leaf surface in a final volume of 25 μL. For the TaqMan® method, the assays for the quantification of *Erwinia* contained 2.5 μL 10× TaqMan® buffer (Applied Biosystems), 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 300 nM of primers o-erw-rt-for and o-erw-rt-rev, 125 nM of one of the TaqMan® probes (erw-p1: 5′ ACC GGT AAC GAC GTG AAA GAC GTT GC 3′, erw-p2: 5′ ACG CCA TCT CCT GAT AAT GAG TGA AGG ACA 3′, erw-p3: 5′ ACG TGA CTC GTC CTT GCG GAG AGA ATC 3′), 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5 μL of the DNA solution isolated from the leaf surface in a final volume of 25 μL. After a hot start (10 min, 95°C) 45 cycles were performed with a 15 s melting step at 95°C and a 1-min annealing/extension step at 60°C on a GeneAmp® 5700 Sequence Detection System (Applied Biosystems). The quantification of copy numbers of total bacteria by real-time PCR was performed as described [2].

Results and Discussion

As there is almost no information available about the population of phytopathogenic microorganisms on the surface of leaves of the important forest tree *Quercus robur* and as these organisms can often not be grown separately from their host plants [3], the scope of this work was to develop a new, highly sensitive method based on molecular techniques that allows detection quantification of bacteria of the phytopathogenic genus *Erwinia* on oak leaves without cultivation and separation of the organisms.

Isolation and cloning of ITS sequences of *Erwinia* from English oak leaf surfaces. In order to obtain *Erwinia* sequences of strains host specific for leaves of *Quercus robur*, sequences covering the intragenic transcribed spacer region (ITS1) between the 16S rDNA and 23S rDNA of *Erwinia* and related enterobacteriacean

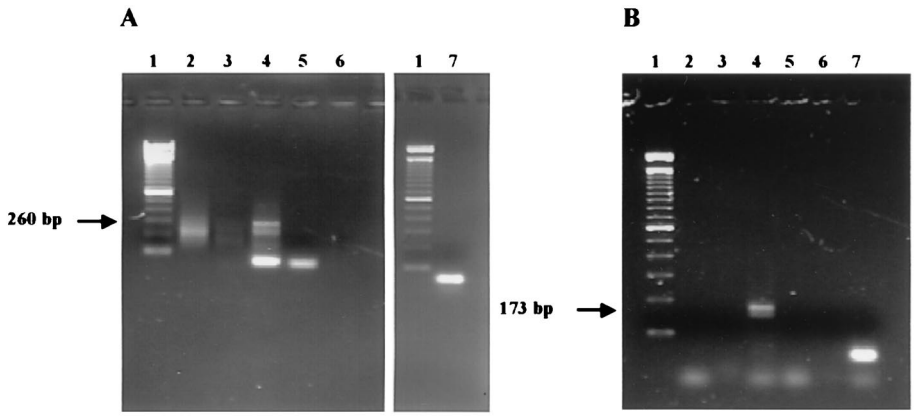

Fig. 2. Selective amplification of *Erwinia* ITS segments in DNA samples extracted from the upper leaf surface. The PCR assays were performed with the primer pair o-erw-for/o-erw-rev (A) and with the primer pair o-erw-rt-for/o-erw-rt-rev (B). Lanes: (1) 100 bp standard (Gibco BRL), (2–4 and 6) PCR assays with DNA from oak leaves and the following primer combinations: (2) forward primer only, (3) reverse primer only, (4) both primers, (5) control with both primers but without DNA, (6) no primer control, (7) DNA from *Escherichia coli* with both primers; 10 μL of the PCR assays was loaded on a 2% agarose gel.

species available in the gene libraries were aligned (as example, see Fig. 1). For *Erwinia*-specific PCR amplification of a segment of the ITS1 sequence, the primers o-erw-for and o-erw-rev were designed (Fig. 1). In an initial PCR with these primers and isolated DNA from the upper *Quercus robur* leaf surface, segments of the expected size of 252–262 bp could be amplified (Fig. 2A). However, with only the forward primer in PCR, a smaller segment of less than 200 bp was amplified, which occurred also in the assay with both primers and was not the expected one. Therefore, only the segments of 252–262 bp were cloned.

Sequence analysis. The inserts of the clones were sequenced on both strands. Four different ITS1 internal sequences were identified (Fig. 3A). Comparison with the gene libraries revealed that all of them were most closely related to *Erwinia rhapontici* (92.1–92.7% homology compared with AF232678), a species known to cause, e.g., the pink seed disease in wheat as well as a leaf disease in rhubarb. However, they are significantly different from known *Erwinia* species. As primer o-erw-for causes an additional segment not desired in PCR, and as the size of the amplicon obtained with o-erw-for and o-erw-rev is not suitable for an optimal real-time amplification, a new primer pair was designed. These primers were designed on the bases of the DNA sequences obtained from the oak leaf surface and were called o-erw-rt-for and o-erw-rt-rev (Fig. 1). The use of these primers in a PCR reaction with DNA isolated from the leaf surface led to the amplification of products of 163–173 bp, as expected (Fig. 2B). No amplification products of other sizes were obtained. After cloning and sequencing, the already known four *Erwinia* sequences were obtained, and in addition two new segments with slightly different sequences were identified (Fig. 3A) which also showed highest homology to *Erwinia rhapontici* (86.5 and 87.2% homology compared with AF232678). Calculating a phylogenetic tree (Fig. 3B) revealed that the isolated sequences from oak leaves indeed belong to the genus *Erwinia* but are well separated from *Erwinia pyrifoliae* (85.7–90.8% homology compared with AJ132969) and from *Erwinia rhapontici* (see above). In total, ten inserts of clones were sequenced, showing six different and four identical sequences, which fell into two significantly separated groups (Fig. 3B). Group A, with amplification products of 163 bp, consists of isolated sequences 3 and 4. Group B, with the larger amplification products of 173 bp, consists of isolated sequences 1, 2, 5, and 6. Group A is more closely related to *Erwinia rhapontici* than group B (Fig. 3B). To get more detailed information about the colonization of the two groups on the oak leaf, we aimed to distinguish between group A and group B.

Selection of group-specific *Erwinia* probes for TaqMan® PCR. Whereas the use of primer o-erw-rt-for and o-erw-rt-rev allowed a genus-specific amplification of *Erwinia* in DNA samples extracted from the leaf surface in a SYBR®-Green method, it was not sufficient to differentiate between the identified group A and group B inside the genus. In order to improve the specificity, we applied the TaqMan® technique. This uses, in addition to the two primers, a selective probe localized inside the amplicon. On the basis of the alignment of the six sequenced *Erwinia* segments, probes were chosen that allowed a more specific identification and quantification of the different *Erwinia* groups in DNA samples isolated

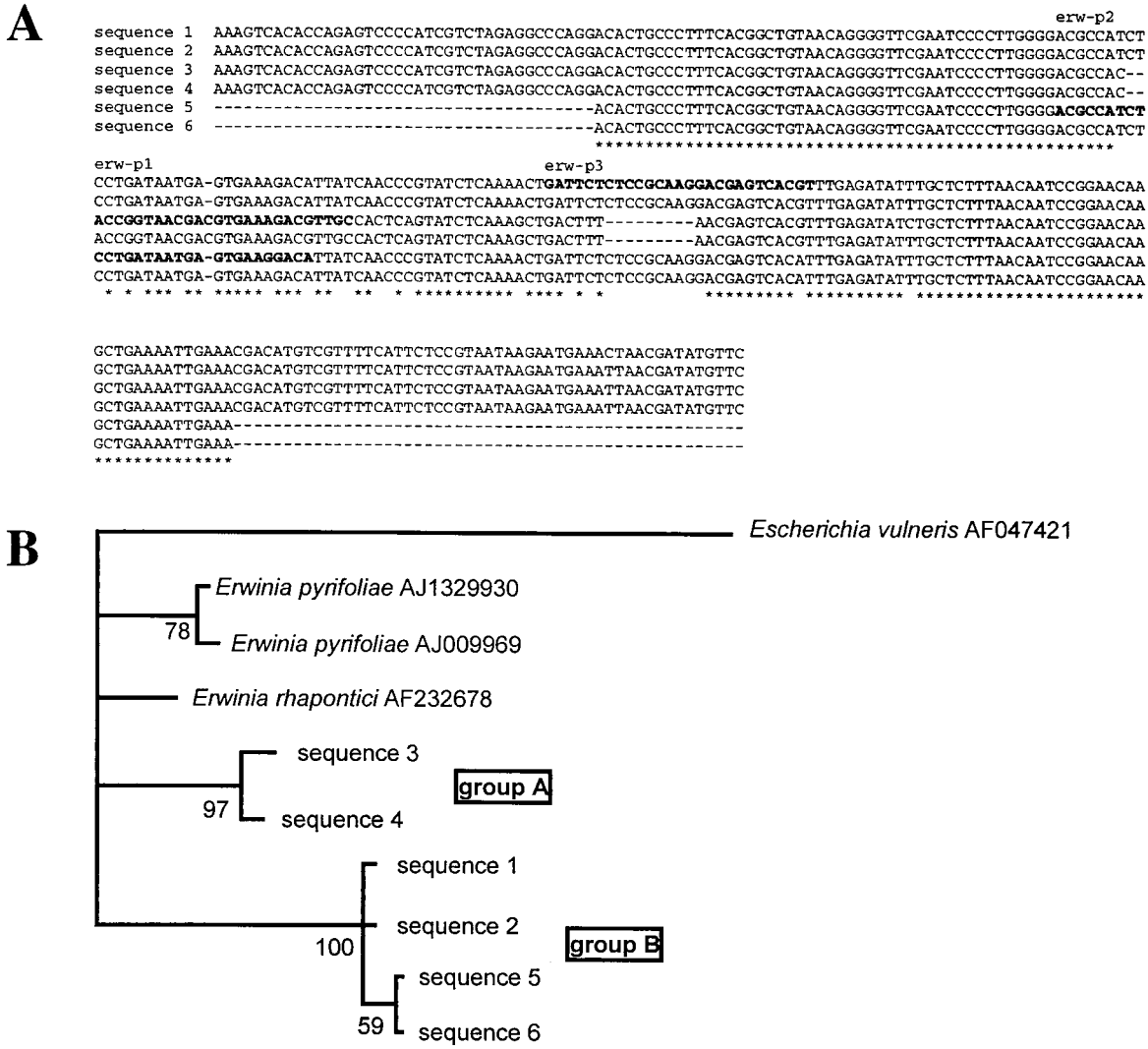

Fig. 3. Alignment of the cloned *Erwinia* sequences. (A) Isolated sequences 1–4 were amplified by using the primer pair o-erw-for/o-erw-rev. Isolated sequences 5 and 6 were amplified by using the primer pair o-erw-rt-for/o-erw-rt-rev. The start of the designed TaqMan® probes erw-p1–erw-p3 is given above the alignment. The alignment was performed by the program CLUSTALW 1.8. (B) The tree was calculated by the program PAUP 3.0 on the basis of a CLUSTALW 1.8 alignment. The lengths of the horizontal lines represent the relative distances. The values below the lines are the bootstrap values from 100 replications for the given branches.

from the leaf surface. For group A (sequences 3 and 4), probe erw-pl was designed (Fig. 3A). For group B (sequences 1, 2, 5, and 6), probe erw-p2 (highly specific for sequence 5) and probe erw-p3 (highly specific for sequences 1 and 2) were designed (see Fig. 3A).

Specificity of the different TaqMan® methods to the isolated sequences from oak leaf surfaces. In order to verify the specificity of the three different TaqMan® methods, we added 100,000 plasmid copies of sequence 3 (group A), sequence 2 (group B), or sequence 5 (group B) to the TaqMan® assay already containing the primers o-erw-rt-for and o-erw-rt-rev. The quantitative PCR reaction was performed for each of these three assays with either probe erw-p1, erw-p2, or erw-p3. Each assay had three replicates, allowing us to calculate a mean value and standard deviation (Table 1). Probe erw-p1, which was designed for group A, indeed was specific for group A represented by sequence 3 and did not cross-react with the other isolated sequences (Table 1). Probe erw-p3 resulted in a quantitative recovery in the case of sequence 2 (group B), but was also sensitive (40%) for sequence 5 (group B). Probe erw-p2 quantitatively detected sequence 5 (group B) but also cross-reacted with sequence 2. In the case of the probes erw-p2 and erw-p3,

Table 1. Cross-sensitivity of the TaqMan® probes to the isolate-specific DNA[a]

Probe	Detected copies		
	Isolated sequence 3 (group A)	Isolated sequence 2 (group B)	Isolated sequence 5 (group B)
erw-p1	$(1.0 \pm 0.1) \times 10^5$	0	0
erw-p2	0	$(0.9 \pm 0.2) \times 10^5$	$(1.0 \pm 0.3) \times 10^5$
erw-p3	0	$(1.0 \pm 0.3) \times 10^5$	$(0.4 \pm 0.2) \times 10^5$

[a] 100,000 plasmids with the cloned isolated DNA of either isolated sequence 2, 3, or 5 were added to the real-time PCR assays. The TaqMan®-PCR reactions were performed with the primers o-erw-rt-for and o-erw-rt-rev and one of the given probes (erw-p1, erw-p2 or erw-p3) in each sample. All assays were performed in three replicates.

Table 2. Quantification of copy numbers of total bacteria and *Erwinia* in DNA samples isolated from oak leaf surfaces[a]

Target	Detection system	Primers and probe	Log_{10}(copy number/m^2 leaf surface)
Total bacteria	TaqMan®	primers and probe are from [2]	9.1 ± 0.5
Erwinia	SYBR®-Green	o-erw-rt-for, o-erw-rt-rev	6.2 ± 0.6
Isolated sequences 3 and 4, group A	TaqMan®	o-erw-rt-for, o-erw-rt-rev, erw-p1	4.5 ± 0.4
Isolated sequence 5, group B	TaqMan®	o-erw-rt-for, o-erw-rt-rev, erw-p2	4.6 ± 0.5
Isolated sequences 1 and 2, group B	TaqMan®	o-erw-rt-for, o-erw-rt-rev, erw-p3	5.3 ± 0.6

[a] DNA was extracted from the upper side of leaves harvested on the Julian day 155 (4th June 2001) of *Quercus robur* grown in the valley of Garmisch-Partenkirchen, Germany at 700 m a.s.l. Values and standard deviations are calculated for 19 independent leaves.

there were no detectable cross-reactions to sequence 3 representing group A (Table 1). The cross-reactions inside group B are due to the fact that only one base (G to A) differs in the target region of erw-p3 to sequence 5 and in erw-p2 to sequence 2. Obviously, this little difference is not sufficient under the given conditions to get a clear-cut separation of the two isolated sequences inside group B and highlights the limit of the resolution of the TaqMan® method.

Quantification of copy numbers of total bacteria, the genus *Erwinia*, and groups of *Erwinia* in DNA samples extracted from the upper leaf surface of English oaks. After the development of the *Erwinia*-specific SYBR®-Green and the group-specific TaqMan® methods, they were used to quantify the amount of *Erwinia* in DNA extracted from the upper leaf surface. Therefore, DNA of 19 leaf samples collected on 4th June 2001 (Julian day 155) was isolated by the described freezing and extraction technique [5]. Copy numbers of eubacteria were quantified by a well-established TaqMan® method [2]. The total number of DNA copies of *Erwinia* was determined with SYBR®-Green technique based on the primer pair o-erw-rt-for/o-erw-rt-rev, and the separation into the *Erwinia* group A and group B was performed by using in addition the probes erw-p1, erw-p2, and erw-p3 in a TaqMan® method. Among the more than 10^9 bacterial DNA copies per m^2 on the leaf, about 10^6 were identified to belong to the genus *Erwinia* by the use of the SYBR®-Green technique (Table 2). From these about 10^4 belong to group A and 10^4–10^5 to group B (Table 2). Inside group B the probes erw-p2 and erw-p3 resulted in similar results because of the cross-sensitivity mentioned above. A comparison of the sum of the copy numbers of the *Erwinia* groups A and B obtained by the TaqMan® technique (7.1×10^4 copies m^{-2} leaf area for the sum of group A and sequence 5 of group B, or 2.3×10^5 copies m^{-2} leaf area for the sum of group A and sequences 1 and 2 of group B) to the copy numbers of the total *Erwinia* amount, determined by the SYBR®-Green technique (1.6×10^6 copies m^{-2} leaf area), suggests the existence of more *Erwinia* groups that are not recognized by the developed TaqMan® probes so far (Table 2).

Concluding Remarks

In the present work, powerful real-time PCR methods were developed and successfully applied, allowing the direct quantification of genus-specific DNA copy numbers of *Erwinia* on leaves of English oaks. Because this technique is not based on isolation and growth of the epiphytic bacteria, the procedure can be done in less than 1 day and will detect culturable as well as non-culturable microorganisms. On the basis of this technique, it will be possible to determine the degree of invasion of pathogenic bacteria during the vegetative period in dependence on external factors such as humidity, light, and

temperature, which is important for the prediction of tree development and health, e.g., during global warming.

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