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Variation of the *Pseudomonas* community structure on oak leaf lettuce during storage detected by culture-dependent and -independent methods

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ABSTRACT

The genus *Pseudomonas* plays an important role in the lettuce leaf microbiota and certain species can induce spoilage. The aim of this study was to investigate the occurrence and diversity of *Pseudomonas* spp. on oak leaf lettuce and to follow their community shift during a six day cold storage with culture-dependent and culture-independent methods. In total, 21 analysed partial *Pseudomonas* 16S rRNA gene sequences matched closely (> 98.3%) to the different reference strain sequences, which were distributed among 13 different phylogenetic groups or subgroups within the genus *Pseudomonas*. It could be shown that all detected *Pseudomonas* species belonged to the *P. fluorescens* lineage. In the culture-dependent analysis, 73% of the isolates at day 0 and 79% of the isolates at day 6 belonged to the *P. fluorescens* subgroup. The second most frequent group, with 12% of the isolates, was the *P. koreensis* subgroup. This subgroup was only detected at day 0. In the culture-independent analysis the *P. fluorescens* subgroup and *P. extremaustralis* could not be differentiated by RFLP. Both groups were most abundant and amounted to approximately 46% at day 0 and 79% at day 6. The phytopathogenic species *P. salmonii*, *P. viridiflava* and *P. marginalis* increased during storage. Both approaches identified the *P. fluorescens* group as the main phylogenetic group. The results of the present study suggest that pseudomonads found by plating methods indeed represent the most abundant part of the *Pseudomonas* community on oak leaf lettuce.

1. Introduction

The bacterial microbiota of lettuce is dominated by Gram-negative microorganisms (97.3%) and the dominant genus is *Pseudomonas* (King et al., 1991). In diverse lettuce cultivars, 43.4–48.2% of the overall microbiota was assigned to this genus (Hunter et al., 2010), while Rastogi et al. (2012) reported 17% *Pseudomonas* on Romaine lettuce. Pseudomonads are highly versatile organisms and can adapt to a wide range of habitats. This adaptability accounts for their constant presence in the environment, and also on plants (Mena and Gerba, 2009). Some members of the genus *Pseudomonas* are known for their beneficial role to plants, while others are used for bioremediation and as biocontrol agents (Ghyselinck et al., 2013; Haas and Défago, 2005; Scherwinski et al., 2008). In addition, some members play an important role as plant pathogens (Mansfield et al., 2012) or opportunistic animal and human pathogens (Gershman et al., 2008; Mena and Gerba, 2009). Thus, pseudomonads have an extensive impact on ecology, agriculture, and commerce. Approximately 30% of fresh vegetables, such as lettuce, are lost on account of food spoilage due to colonization of bacteria (Lee et al., 2013). Commonly detected species on minimally processed vegetables are *P. fluorescens*, *P. putida*, *P. chicorii* and *P. maltophila* (Franzetti and Scarpellini, 2007). They are able to produce enzymes that catalyse proteolytic and lipolytic reactions that contribute to spoilage of refrigerated fresh produce, as well as pectolytic enzymes to degrade pectic substances of plant cell walls (Barth et al., 2009; Wulfkuehler et al., 2013).

Certain *Pseudomonas* species are easy to isolate on standard growth media (Sánchez et al., 2014). However, it is still not known if and how well these bacteria represent the total environmental population of *Pseudomonas* spp. Li et al. (2013) estimated that less than 1% of bacterial population in environmental samples is cultivable, and that cultivation will inevitably lead to an underestimation of total cell counts and total diversity. Plate counts and molecular biological techniques, such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) (Johnson et al., 2007) and denaturing gradient gel electrophoresis (DGGE) (Evans et al., 2004) have been used to study the diversity of *Pseudomonas* spp. However, these methods lack the ability to distinguish species in complex bacterial communities such as lettuce communities. The taxonomy of the genus *Pseudomonas* has undergone many changes since the initial description published by Palleroni (1984). Phylogenetic trees, based on partial sequences of four 'housekeeping' genes, enabled the classification of the pseudomonads into two lineages, called *P. aeruginosa* lineage and *P. fluorescens* lineage (Mulet et al., 2010). The latter lineage was divided into six groups.

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One of these groups, the *P. fluorescens* group, was subdivided further into nine subgroups (Mulet et al., 2010). In case of the genus *Pseudomonas*, the analysis of 16S rRNA gene sequences alone does not always differentiate sufficiently to allow the resolution of intrageneric relationships (Mulet et al., 2009). Other approaches for the discrimination of *Pseudomonas* spp. were the sequence analysis of genes such as *gyrB*, which encodes the subunit B of DNA gyrase, *rpoD*, which encodes the sigma subunit of the RNA polymerase, *rpoB*, which encodes the beta subunit of bacterial RNA polymerase, and the outer membrane lipoprotein I gene *oprI* (Ait Tayeb et al., 2005; De Vos et al., 1998; Yamamoto et al., 2000). The *rpoB* gene is a highly conserved 'housekeeping' gene and one copy is present in all bacteria because of its essential role in cellular metabolism. Therefore it has been used as a signature for bacterial identification, as well as locus for phylogenetic analysis (Ait Tayeb et al., 2005; Mollet et al., 1997).

The present study aimed at assessing the diversity of *Pseudomonas* species on oak leaf lettuce applying culture-dependent and -independent methods on the same sample, and at comparing the results concerning species that are known to affect human health and to induce food spoilage. Secondly, to investigate changes within the *Pseudomonas* community composition during six day storage at 4 °C, again using culture-dependent as well as -independent methods. In the culture-dependent approach the *rpoB* gene was analysed additionally to the 16S rRNA gene to get more detail information in *Pseudomonas* species assignment.

2. Material and methods

2.1. Sample collection and processing

Ten heads of green oak leaf lettuce (*Lactuca sativa* var. *crispa*) were purchased from a local supermarket. Five of these heads were analysed on the day of purchase (day 0). To mimic commercial lettuce storage, the remaining five heads were packaged loosely in a plastic box covered with aluminium foil and stored in a dark cooling chamber (4 °C) until analysis at day 6, which was the shelf life of oak leaf lettuce under the given storage conditions. In Fig. 1, an overview of the experimental design of the study is given. Sample preparation on each day was carried out as follows:

The outer leaves of each lettuce head were removed by cutting with a sterile scalpel. Ten leaves with a weight of approximately 10 g per leaf were used to prepare biomass. The cut edges of the leaves were closed with commercial nail polish to reduce chloroplast effusion. The leaves were transferred into sterile blender bags (Rotilabo®-Homogenizing bags, PE; Carl Roth GmbH, Germany) and the nine-fold volume of sterile buffer (1% (*w*/*v*) TWEEN® 80 (Sigma-Aldrich Co., U.S.A.), 1% (*w*/*v*) buffered peptone water (Merck KGaA, Germany), 10 mM EDTA (Biomol GmbH, Germany); pH 6.7) was added. The leaves were completely covered by the buffer. The bags were then heat-sealed and placed into a Pulsifier® PUL 100E device (Microgen Bioproducts Ltd., UK), and treated for 45 s to detach microorganisms from the leaf surface. Afterwards, the buffer was transferred from each of the bags into two sterile 50 mL centrifuge tubes. The tubes were centrifuged at 4000 × *g* for 20 min at 4 °C. Supernatants were discarded, and all 20 cell pellets of the ten leaves per lettuce head were pooled into one sterile 50 mL centrifuge tube by suspending each pellet in 1 mL buffered peptone water (Merck KGaA) and quantitatively transferring this solution to the 50 mL tube. The volume was filled up to 50 mL with buffered peptone water. Out of this solution a decimal dilution was carried out as described in 2.2.1. The remaining bacterial suspension was centrifuged at 8000 × *g* for 20 min at 4 °C. The supernatant was discarded and the cell pellet was stored at −20 °C until the DNA isolation as described in 2.3.1.

2.2. Culture-dependent microbiological approach

2.2.1. Bacterial viable counts

Cultural analyses were carried out directly after pooling the cell pellets as described in 2.1 (Fig. 1). Appropriate dilutions were made in sterile physiological sodium chloride solution and surface spread plated in triplicate. Total aerobic mesophilic bacterial cell counts were determined on Standard I nutrient agar (Merck KGaA). *Pseudomonas* colonies were enumerated on *Pseudomonas* selective agar consisting of *Pseudomonas* agar base (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with cephaloridine, fucidin and cetrimide (CFC; Oxoid Ltd., UK) as well as on cetrimide agar (Merck KGaA). Plates were incubated aerobically at 30 °C for 48 h before colonies were counted. Viable counts were performed in duplicate with determination of the arithmetic means and standard deviations. Of the viable counts of each sampling day, 100 colonies were randomly selected from the *Pseudomonas* CFC-agar and transferred onto Standard I nutrient agar. The plates were incubated at 30 °C for 24 h and checked for uniform colony morphology. The isolates were subcultured two times to ensure purity.

2.2.2. Biochemical characterization of the isolates

Pigment production on King's B medium, oxidase and catalase reaction, and the oxidative/fermentative metabolism of glucose were tested for all isolates. The fluorescent and phenazine pigment production was tested with the King's B medium (King et al., 1954). Samples were incubated for 48 h at 30 °C. Colonies with fluorescence under UV light (302 nm) indicate a positive reaction. The cytochrome c oxidase reaction was performed with cytochrome c oxidase test strips (Kallies Feinchemie AG, Germany) according to manufacturer's instructions. The presence of the catalase enzyme in the isolates was detected using 3% hydrogen peroxide (Carl Roth GmbH, Germany). If the bacteria possessed catalase, bubbles of oxygen were observed when a small amount of bacterial isolate was added into a drop of hydrogen peroxide. The oxidative/fermentative metabolism of glucose was tested with the OF basal medium (Merck KGaA) according to Hugh and Leifson (1953), and the samples were incubated at 28 °C for 48 h. A colour change from green to yellow indicates a positive result. *P. fluorescens* strain DSM 50091^T was used as a control for all four tests. The control strain was characteristically fluorescent, oxidase and catalase positive and able to oxidize glucose in the OF test.

2.2.3. PCR analysis of the isolates

The genus affiliation of presumptive *Pseudomonas* isolates were confirmed using genus-specific primers Ps-for (5′-GGT CTG AGA GGA TGA TCA GT-3′) and Ps-rev (5′-TTA GCT CCA CCT CGC GG C-3′) (Widmer et al., 1998) targeting a 990 bp fragment of the 16S rRNA gene. One colony of each isolate was suspended in 50 μL DNase- and RNase-free sterile water (Gibco®, Thermo Fisher Scientific Inc., Germany). Each 25 μL PCR reaction contained: 2.5 μL 10 × ThermoPol™ reaction buffer, 0.5 μL dNTPs (10 mM each), 1 μL of each primer (10 pmol/μL), 0.125 μL Taq polymerase (5 U/μL; New England BioLabs GmbH, Germany) and 1 μL of the bacterial suspensions. PCR cycle parameters consisted of an initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min and extension at 68 °C for 1 min, followed by a final extension step at 68 °C for 10 min. PCR reactions were performed in a Biometra Thermocycler T1 (Biometra GmbH, Germany).

The *rpoB* gene was amplified with the primers LAPS (5′-TGG CCG AGA ACC AGT ACC GCG T-3′) and LAPS27 (5′-CGG CTT CGT CCA GCT TGT TCA G-3′) (Ait Tayeb et al., 2005), yielding a 1247 bp fragment. The PCR reaction was performed as described above, under following conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 68 °C for 1.5 min, followed by a final extension step at 68 °C for 10 min.

The PCR products were separated by electrophoresis in 2% (*w*/*v*) agarose gels (LE agarose, Biozym Scientific GmbH, Germany) at 100 V