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Research Article

NodC-based evaluation of the occurrence of bacteria in nodules of *Pisum sativum* isolated on YEM agar

NodC-basierte Bewertung des Auftretens von Bakterien in Wurzelknöllchen von *Pisum sativum*, isoliert auf YEM-Agar

Anna Lenart-Boroń[1]*, Tadeusz Zając[2], Piotr Mateusz Boroń[3], Agnieszka Klimek-Kopyra[2]

[1] Department of Microbiology, University of Agriculture in Cracow, Al. Mickiewicza 24/28, 30-059 Cracow, Poland
[2] Department of Crop Production, University of Agriculture in Cracow, Al. Mickiewicza 21, 31-120 Cracow, Poland
[3] Department of Forest Pathology, Mycology and Tree Physiology; University of Agriculture in Cracow, Al. 29 Listopada 46, 31-425 Cracow, Poland
* Corresponding author: anna.lenart-boron@urk.edu.pl

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Summary

The bacterial nodulation (*nod*) genes are essential in the formation process of root nodules. This study was aimed to verify the occurrence of nodule-associated bacteria in two pea varieties ("Tarchalska" and "Klif") inoculated with *Rhizobium* inoculants – Nitragine™ and a noncommercial one produced by the Polish Institute of Soil Science and Plant Cultivation (IUNG). The number of colonies isolated on yeast extract mannitol (YEM) agar from the nodules of "Klif" inoculated with IUNG inoculants was significantly higher than the number of colonies isolated from other variants. Species identification was based on sequencing of 16S rDNA, which revealed that despite careful sterilization of nodules, sequences of other bacterial species were detected. Among them, one sequence belonged to *Rhizobium leguminosarum* (isolated from IUNG inoculant). To assess the presence of nodulation-capable *Rhizobium*, amplification of the *nodC* gene was performed, which revealed that of 29 samples, 19 were positive. The remaining isolates, including reference strain and bacteria isolated from Nitragine™, lacked this gene. The results show that pea nodules harbor a very diverse community of bacteria. The lack of *nodC* gene in some strains isolated from plants inoculated with Nitragine™ and with IUNG inoculant proves that even if *R. leguminosarum* are abundant, they may not be efficient in nodulation.

Keywords: *Rhizobium leguminosarum* bv. *viceae*, *Pisum sativum* L., inoculant, *nod* genes, 16S rDNA sequencing

Zusammenfassung

Die bakteriellen Nodulationsgene (*nod*) sind für den Bildungsprozess der Wurzelknöllchen wesentlich. Diese Studie zielte darauf ab, das Vorkommen von knöllchenassoziierten Bakterien in zwei Erbsensorten zu überprüfen („Tarchalska" und „Klif"), die mit einem *Rhizobium*-Inokulat, Nitragine™, oder einem nicht kommerziellen Produkt, hergestellt vom Polnischen Institut für Bodenkunde und Pflanzenanbau (IUNG), beimpft wurden. Die Anzahl der auf YEM-Agar isolierten Kolonien, die aus mit IUNG geimpften Knöllchen von "Klif" isoliert wurden, war signifikant höher als die Anzahl der aus anderen Varianten isolierten Kolonien. Die Identifizierung der Spezies beruhte auf der Sequenzierung von 16S-rDNA, die ergab, dass trotz sorgfältiger Sterilisation der Knöllchen Sequenzen von anderen Bakterienarten nachgewiesen wurden. Unter ihnen gehörte nur eine Sequenz zu *R. leguminosarum* (Bakterien, die aus dem IUNG-Impfstoff isoliert wurden). Um das Vorhandensein von nodulationsfähigem *Rhizobium* zu bestimmen, wurde eine Amplifikation des *nodC*-Gens durchgeführt, die ergab, dass von 29 Proben nur 19 positiv waren. Bei den verbleibenden Isolaten, einschließlich des Referenzstamms und der aus Nitragine™ isolierten Bakterien, fehlte dieses Produkt. Die Studie zeigen, dass die Erbsenknöllchen eine sehr unterschiedliche Bakteriengemeinschaft beherbergen. Das Fehlen des *nodC*-Gens in einigen Stämmen, die sowohl aus mit Nitragine™ als auch mit IUNG beimpften Pflanzen isoliert wurden, beweist, dass, auch wenn *R. leguminosarum* reichlich vorhanden ist, diese bei der Pflanzennodulation nicht wirksam sein können.

Schlagworte: *Rhizobium leguminosarum* bv. *viceae*, *Pisum sativum* L., Beimpfung, nod Gene, 16S-rDNA-Sequenzierung

1. Introduction

Among legumes, pea (*Pisum sativum* L.) is the second most important grain legume crop in the world, which is widely used as green vegetable, seed, pasture, silage, hay, and green manure (Cristou, 1997; Wadhwa et al., 2011). The global area of green peas cultivation is 2.3 million ha (FAO, 2013). The general awareness of increasing soil and water pollution caused by the use of chemical fertilizers has also increased the importance of legumes (Tas et al., 1995). However, the pea yield can vary strongly depending on various factors, resulting in lower yields of pea plants in Eastern Europe than in Western part of the continent (Doré et al., 1998). According to Klimek-Kopyra et al. (2017), the seed yield of pea can vary in different years of cultivation, which might be associated with agro-climatic conditions, and also can be affected by the genotype variety and—to some extent—by the applied inoculant. At present, rhizobial inoculation of pea seeds is one of agronomic solutions for sustainable agriculture development. Commercial bacterial inoculants are produced in many countries. Their quality depends on both the abundance of *Rhizobium* spp. bacteria and their effectiveness in fixing nitrogen with the target host (Amarger et al., 1997). Symbiotic effectiveness is one of the most important factors when selecting an inoculant strain (Stephens and Rask, 2000). On the other hand, *Rhizobium* populations present in the soil will affect the outcome of the inoculation (Thies et al., 1991), because indigenous inefficient strains are often better adapted to the prevailing soil and climatic conditions (Wadhwa et al., 2011). Therefore, searching for best strains and evaluation of the existing inoculants is crucial both for bacterial improvement and for pea cultivation. The process of interaction between leguminous plants and *Rhizobium* bacteria starts when root hairs—the target for rhizobia—start to deform and curl to form nitrogen-fixing nodules. The bacterial nodulation (nod) genes are essential for interactions with root hairs. The common group of nod genes (nodABC), which are located on a Sym plasmid, is required in all phases of root hair interaction (Debellé et al., 1986). Gloudemans et al. (1989) demonstrated that the *nodC* gene is required and essential for the induction and enhancement of expression of genes essential in the nodule formation process.

Another important aspect in terms of legume root nodules is the fact that they are hosts to several eubacterial genera other than *Rhizobium*, the number of which can reach even 10^4 colony forming units (CFUs) of viable bacteria per gram of fresh nodule tissue. The non-rhizobial occupants of root nodules of leguminous plants may include the representatives of *Bacillus*, *Streptomyces*, *Herbaspirillum*, Arbuscular Mycorrhizal Fungi, *Agrobacterium*, and many others (Selvakumar et al. 2013). Some of these bacteria, such as *Pantoea agglomerans*, *Enterobacter kobei*, *Enterobacter cloacae*, *Leclercia adecarboxylata*, *Escherichia vulneris*, or *Pseudomonas* spp. were implicated in the nodulation process (Selvakumar et al., 2013). For a number of reasons, the use of 16S rRNA gene sequencing has been the most common method to study bacterial phylogeny and taxonomy. These reasons include the presence of the 16S rRNA gene in almost all bacteria, the fact that its function has not changed over time and its size (i.e. 1,500 bp), which is large enough for bioinformatics purposes (Patel, 2001).

Having the above factors in mind, this study was aimed to assess the effect of pea plants' inoculation with two preparations containing symbiotic N_2-fixing bacteria (*Rhizobium* spp.)—a commercial product (Nitragine™) and a noncommercial one produced by the Polish Institute of Soil Science and Plant Cultivation (IUNG)—in terms of the abundance and identification of nodule-associated bacteria. Particular emphasis was on the verification of the presence and molecular diversity of the *NodC* gene, which is essential for the effective process of nodule formation in leguminous plants.

2. Material and methods

2.1 Collection site and pea plants

Bacterial strains were isolated from nodules of two varieties of field pea (*Pisum sativum* L.)—"Tarchalska" and "Klif"—cultivated in the experimental field of Bayer® company located in Modzurów, Silesia, Poland (N50°9'24" E18°7'55"). A randomized system was adopted in the conducted field experiment, and it consisted of four replicates; each plot had a size of 8.4 m^2. The experimental field soil was an Umbrisol, a slightly degraded chernozem, formed from loess, classified as good wheat complex and the second bonitation class; therefore, the soil conditions were sufficient for the pea needs. The topsoil had a neutral pH (pH in 1 mol/dm^3 KCl: 6.28) and a high nutrient content: 191 ppm P_2O_5, 217 ppm K_2O, and 101 ppm Mg. The following pre-sowing doses were applied: 48 kg ha^{-1} of phosphorus (P_2O_5) and 72 kg ha^{-1} potassium (K_2O). Ammonium nitrate was applied as a "starting dose" with 20 kg N ha^{-1}. Both varieties were inoculated before sowing

with either of the two inoculants containing *Rhizobium leguminosarum* bv. *viceae*: Nitragine™ produced by the BIOFOOD company (Poland) and noncommercial gel inoculant produced by the Polish IUNG. Non-inoculated plants were used as control. The plant seeds were sown in the first week of April 2011 and in the fourth week of March 2012. One hundred and twenty germinable seeds of "Tarchalska" and of 100 germinable seeds of "Klif" variety were sown per square meter at a row distance of 15 cm and sowing depth of 6 cm. Four types of nodules (large red, large cream, small red, and small cream) were selected for the study and were collected in July 2011 and 2012.

2.2 Bacterial strains

From each plant sampled, six pea nodules of each type (large red, large cream, small red, and small cream) were randomly excised. In total, 24 samples were used for further analyzes in each year. The collected nodules were washed with sterile water and surface sterilized using 1% NaOCl for 2 min and then with 70% ethanol for 1 min and washed with sterile water for four times. After sterilization, each nodule (one nodule per each type in six replicates) was crushed with a sterile glass rod in 1 cm^3 of sterile 0.85% NaCl solution and shaked for 15 min in a Petri dish. Afterwards, the bacteria were isolated on yeast extract mannitol (YEM) agar plates. The isolates were grown at 28°C for 5 days and purified by repeated streaking on YEM (Vincent, 1970). Following incubation, the numbers of *Rhizobium*-like bacteria CFUs per one nodule of each type and each plant–inoculant combination were determined.

Moreover, bacterial isolates were derived from the two *Rhizobium*-based inoculants used in this study by streaking serial dilutions onto YEM agar plates. A reference strain of *R. leguminosarum* bv. *viceae* (DSM 30143) was also used in this study.

2.3 Isolation of genomic DNA

The bacterial isolates were cultured on tryptone yeast agar plates at 28°C and genomic DNA was extracted by following the protocol of Genomic Mini kit for genomic bacterial DNA isolation (A&A Biotechnology, Poland).

2.4 Amplification and sequencing of 16S rRNA gene

The species identification of bacterial isolates was based on the sequencing of the 16S rRNA gene. The partial fragment of 16S rRNA gene was amplified using the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg et al., 1991). Polymerase chain reaction (PCR) solution contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1X PCR buffer, and 2.5 U Taq DNA polymerase in a total volume of 25 µl. The following temperature profile was used for DNA amplification: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62.5°C for 30 s, and elongation at 72°C for 1 min; 15 cycles of denaturation at 94°C, annealing at 48°C, and elongation at 72°C for 1 min; and final elongation at 72°C for 10 min. PCR amplifications were performed using Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). The PCR products were visualized by 1×TBE electrophoresis in ethidium-bromide-stained, 1% agarose gel and purified using the Gel Out purification kit (A&A Biotechnology, Poland) before sequencing. Sequencing reactions were performed on 24 samples of 16S rRNA amplicons—one sample per each of the combination. The obtained nucleotide sequences were queried against the NCBI GenBank database using BLAST search to determine the most closely related bacterial species.

2.5 Amplification and sequencing of *nodC* gene

Five primer pairs were tested for amplification of approximately 930 bp of the 1,300 bp *nodC* gene: forward primers used were nodCF, nodCFu, nodCF2, nodCF4, or nodCFn, and reverse primer used were nodCI (Laguerre et al, 2001) (Table 1).

PCR solution contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1X PCR buffer, and 2.5 U Taq DNA polymerase in a total volume of 25 µl. The following temperature profile was used for DNA amplification: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62.5°C for 30 s, and elongation at 72°C for 1 min; 15 cycles of denaturation at 94°C, annealing at 48°C, and elongation at 72°C for 1 min; and final elongation at 72°C for 10 min. PCR amplifications were performed using Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). The PCR products were visualized by 1×TBE electrophoresis in ethidium-bromide-stained, 1% agarose gel and purified using the Gel Out purification kit (A&A Biotechnology, Poland) before sequencing. The part of *nodC* gene of the selected isolates was sequenced and queried against NCBI GenBank database using BLAST search to confirm their species affiliation.

Table 1. Oligonucleotides used for *nodC* amplification
Tabelle 1. Für die *nodC*-Amplifikation verwendete Oligonukleotide

Primer	5′–3′ nucleotide sequence
nodCF	AYGTHGTYGAYGACGGTTC
nodCFu	AYGTHGTYGAYGACGGITC
nodCF2	AYGTHGTYGAYGACGGCTC
nodCF4	AYGTHGTYGAYGACGGATC
nodCFn	AGGTGGTYGAYGACGGTTC
nodCI	CGYGACAGCCANTCKCTATTG

Y = C or T; H = A, C or T; R = A or G; S = C or G; K = G or T; N = A, C, G or T; I = inosine

2.6 PCR-RFLP of *nodC* gene

The PCR amplified, 930-bp long, *nodC* products were subjected to the restriction fragment length polymorphism (RFLP) analysis by digesting with five restriction endonucleases *AluI*, *BamHI*, *BsuRI*, *EcoRI*, and *MspI* following the manufacturer's instructions (Thermo Fisher Scientific-Fermentas, Canada). This procedure was aimed to determine the molecular diversity of rhizobial strains isolated from the analyzed pea plants. Each digestion reaction was conducted individually with one restriction enzyme at a time. After digestion, resulting fragments were visualized by 1×TBE electrophoresis in ethidium-bromide-stained, 1% agarose gel. The GeneRuler™ 1kb DNA Ladder (Thermo Scientific) DNA size marker was used to determine the size of the bands obtained as a result of the restriction digestion. Similarly, as in the case of the restriction reactions, the results of restriction reactions were assessed in the form of one electrophoresis per one enzyme. Therefore, five different electrophoreses were prepared to analyze the RFLP results.

2.7 Statistical analysis

Comparison of means and least significant difference (LSD) for the CFUs of bacteria colonizing pea nodules was performed using STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA).

3. Results

3.1 Bacterial isolation

The numbers of CFUs per one nodule varied significantly (Table 2). The isolates formed colonies that were visible after 5–7 days. All isolates produced colorless to whitish convex colonies with abundant extracellular gum on the surface. Of all examined variants, the highest number of colonies of nodule-colonizing bacteria was observed for the large red nodules of the IUNG-inoculated "Klif" variety of pea (310 CFU/nodule). In general, this variant showed the highest mean number of bacterial colonies per nodule (175.9 CFU/nodule). On the other hand, the smallest number of CFUs per nodule (CFU/nodule) was observed for the uninoculated "Tarchalska" pea plants. The smallest mean number per nodule was observed for the combination of "Tarchalska" plants inoculated with Nitragine™ (46.0 CFU/nodule). When considering the differences in the numbers of CFUs per nodules, the highest mean abundance was observed for the large red nodules (132.7 CFU/nodule), as it was nearly twice higher than that in the case of small red and nearly three times higher than in large cream nodules, where the mean number of CFUs per nodule was the smallest (53.2 CFU/nodule).

Table 2. The mean numbers of colony forming units (CFUs) of bacteria colonizing pea nodules per one nodule depending on variants (pea varieties and inoculants).
Tabelle 2. Die Durchschnittswerte koloniebildender Einheiten (KBE) von Bakterien, die Erbsenknollen besiedeln, pro Knöllchen in Abhängigkeit der Varianten (Erbsensorten und Inokulen).

Variant	Nodules				
	Large cream	Small cream	Large red	Small red	**Mean**
Klif (uninoculated)	20.5	87.0	128.5	52.0	**72.0^{b}**
Tarchalska (uninoculated)	6.0	110.0	157.5	36.0	**77.4^{b}**
Klif + IUNG	84.0	97.5	310.0	212.0	**175.9^{a}**
Tarchalska + IUNG	110.0	45.0	101.0	49.0	**76.3^{b}**
Klif + Nitragine™	54.0	79.5	67.0	39.0	**59.9^{b}**
Tarchalska + Nitragine™	45.0	54.0	32.0	53.0	**46.0^{b}**
Mean	**53.2^{b}**	**78.8^{ab}**	**132.7^{a}**	**73.5^{ab}**	

Pairs of treatments that are not significantly different according to the LSD test ($p<0.05$) share the same letter.

Considering the plant cultivar–inoculant combination, the LSD test showed that the number of bacterial CFUs isolated from nodules of "Klif" variety combined with IUNG inoculant was significantly higher than the number of colonies isolated from other variants ($p<0.05$) (Table 2).

3.2 Molecular characterization of isolates

In order to verify the identity of isolated strains, the 16S rRNA gene was amplified and sequenced. The experiment involved DNA samples extracted from bacterial isolates derived from all 24 experimental variants and isolated from inoculants as well as from a *R. leguminosarum* reference strain (DSM 30143). Amplification of the 16S rRNA region produced a single sharp band of the expected size (1,500 bp).

Sequencing of the product revealed, however, that despite careful sterilization of nodules and using a *Rhizobium*-selective medium sequences of other bacterial species were mostly detected. Sequences of seven samples (apart from *R. leguminosarum* bv. *viceae* DSM 30143) could only be analyzed, as the rest of sequencing patterns presumably resulted from multiple templates that were amplified. Among them only one sequence belonged to *R. leguminosarum* bv. *viceae* (Table 3). Such result may indicate that the pea nodules as well as the Nitragine™ inoculant were dominated by bacterial species other than *R. leguminosarum*.

To eliminate the *Rhizobium*-negative samples, the isolates were tested for the presence of *nodC* gene. This test also confirmed the theoretical ability of strains to form nodules with roots. This experiment was conducted on 46 bacterial strains isolated from the YEM agar plates. These were from one to four isolates per one experimental variant and one strain from each inoculant (IUNG and Nitragine) along with the reference strain of *R. leguminosarum*. The expected 930-bp region of 1,300-bp long *nodC* gene was observed only in the case of 22 of 46 samples (21 bacterial isolates from nodules and 1 from the IUNG inoculant; Table 4). The remaining isolates, including the reference strain and the bacteria isolated from a commercial Nitragine™ inoculant, lacked the expected product. The comparison of the obtained *nodC* sequences with the NCBI showed that all samples resulted in positive identification of *R. leguminosarum* bv. *viceae*.

The RFLP analysis using five restriction enzymes (*AluI*, *BamHI*, *BsuRI*, *EcoRI*, and *MspI*) provided conclusive results only in the case of *BamHI* digestion; therefore, the results obtained only for this enzyme are shown (Figure 1). This reaction resulted in the digestion of a 930-bp long PCR product (*nodC* gene) into two or three fragments of various sizes, revealing two patterns (A and B). In the pattern A, the digestion process gave two fragments, 780 and 150 bp, whereas in the pattern B, the digestion resulted in three fragments, 560, 220, and 150 bp. In some cases, the digestion revealed a mixed profile (A + B), that is, four bands were observed 780, 560, 220, and 150 bp, indicating that in those cases two profiles were combined in the same sample.

4. Discussion

The first part of our experiment showed that the abundance of nodule-dwelling bacteria can be associated with the variety of the pea plants combined with the inoculant used. In our study, the combination of the noncommercial IUNG inoculant coupled with the "Klif" variety of pea was characterized by a significantly higher number of CFU per nodule of bacteria grown on *Rhizobium*-selective medium than the one observed for all other variants. Also Klimek-Kopyra et al. (2017) observed that the application of IUNG inoculant resulted in the formation of a higher

Table 3. Identification of bacteria colonizing pea nodules
Tabelle 3. Identifizierung der Bakterien, welche die Erbsenknöllchen besiedelten

Variant	Bacteria species	Maximum identity (%)
IUNG	*Rhizobium leguminosarum* bv. *viciae* USDA 2370 strain USDA 2370; ATCC 10004	99
Nitragine™	*Achromobacter insolitus* strain LMG 6003	99
Klif (uninoculated)	*Bacillus megaterium* strain IAM 13418	99
Tarchalska (uninoculated)	*Stenotrophomonas maltophilia* strain IAM 12423	99
Tarchalska + IUNG	*Stenotrophomonas maltophilia* strain IAM 12423	99
Klif + Nitragine™	*Stenotrophomonas maltophilia* strain IAM 12423	98
Tarchalska + Nitragine™	*Stenotrophomonas maltophilia* strain IAM 12423	99

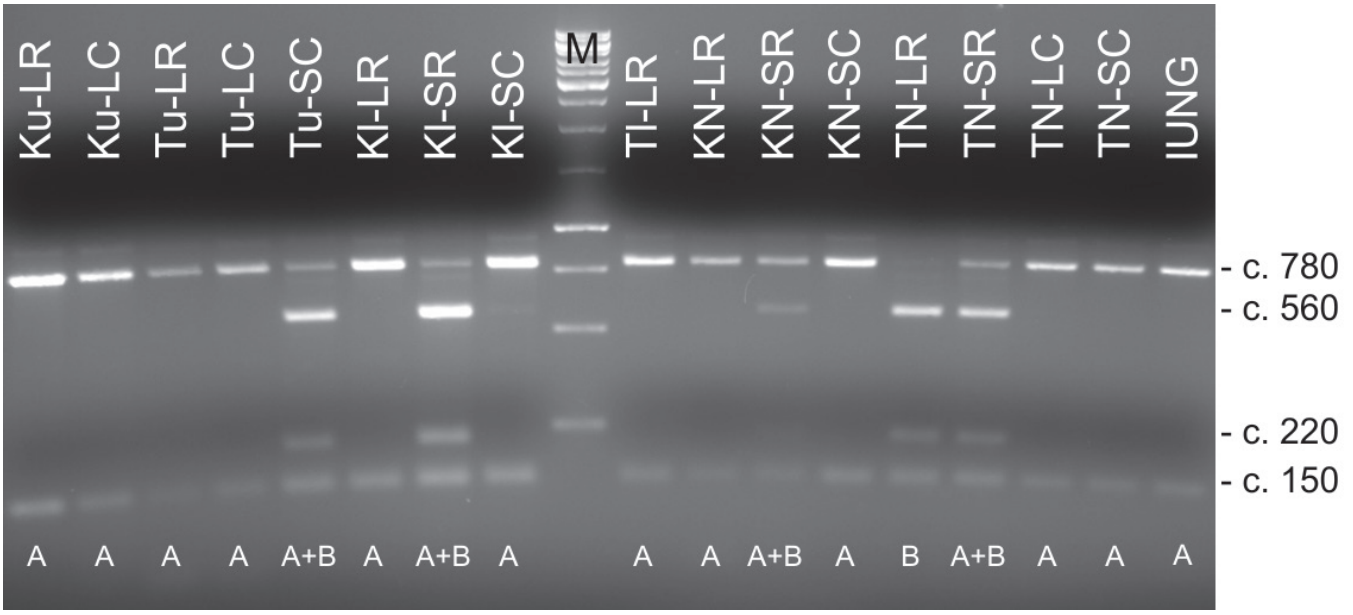

Figure 1. BamHI digestion of *nodC* gene of 17 selected *Rhizobium leguminosarum* samples. Letters at the top of the figure show experimental variants, whereas letters at the bottom of the figure show the digestion patterns.
Legend for the top of the figure: Ku, uninoculated "Klif" pea plants; KI, "Klif" plants inoculated with IUNG; KN, "Klif" plants inoculated with Nitragine™; Tu, uninoculated "Tarchalska" pea plants; TI, "Tarchalska" plants inoculated with IUNG; TN, "Tarchalska" plants inoculated with Nitragine™; IUNG, bacterial isolates derived from the IUNG inoculant; LR, LC, SR, and SC refer to the types of nodules: LR, large red; LC, large cream; SR, small red; SC, small cream.
Legend for the bottom of the figure: M, GeneRuler™ 1kb DNA Ladder (Thermo Scientific); A, B, and C, clear digestion profiles; A + B, mixed digestion profiles.
Abbildung 1. BamHI-Verdauung des *nodC*-Gens von 17 ausgewählten Proben von *Rhizobium leguminosarum*. Buchstaben oben in der Abbildung zeigen experimentelle Varianten, während Buchstaben unten in der Abbildung die Verdauungsmuster zeigen.

number of seeds per pod, pods per plant, and seeds per plant. Even though the presence of small, cream-colored nodules is considered as evidence of ineffective nitrogen fixation (Carr et al., 2000), no significant differences were observed in this analysis in the number of bacteria isolated from nodules of various size and color. Similarly, the size and color of nodules did not have an impact on the detection of *R. leguminosarum* based on the *nodC* gene.

Sequencing of 16S rRNA gene revealed the presence of bacterial species, other than *R. leguminosarum*, including *Stenotrophomonas maltophilia*, *Bacillus megaterium*, and *Achromobacter insolitus*. *B. megaterium*, as well as many other bacteria, was reported to be one of endophytic bacteria in pea cultivars (Elvira-Recuanco and van Vuurde, 2000; Sturz et al., 1997). Similarly, species of *Stenotrophomonas* spp. were detected as bacterial isolates derived from nodules of several leguminous plants (Dudeja et al., 2011). Sharikov and Khakimov (2010) using the 16S rRNA gene study revealed that the leguminous plant nodule-isolated bacteria were related to *Rhizobium*, *Burkholderia*, and *Achromobacter* genera.

The detection of *nodC* gene fragment revealed that not all nodule-derived strains possessed the expected *nodC* fragment. Because nodulation (*nod*) genes are essential for symbiosis and *nodC* is a key symbiotic gene that, together with *nodA* and *nodB*, is responsible for the synthesis of the core structure of Nod factors (Bontemps et al., 2005), the lack of this particular gene in bacteria isolated from pea nodules as well as from isolates derived from the Nitragine™ could indicate that nodules of the six plants were dominated by bacteria that belonged to other nitrogen-fixing species. However, in order to certainly confirm whether the *Rhizobium* strains contained in Nitragine™ possess or lacks the *nodC* gene, that is, whether they are capable of initiating the effective nodulation process, the PCR detection of this gene should be conducted on the DNA samples extracted not from individual isolates growing on agar media but from the representative sample of the inoculant itself.

For a number of samples, RFLP analysis revealed the presence of multiple *nodC* variants within a single isolate; this was indicated by the mixed digestion patterns in *BamHI* reaction. Such result indicates either the occurrence of mul-

Table 4. Detection of *nodC* gene in the studied bacterial isolates
Tabelle 4. Nachweis des *nodC*-Gens in den untersuchten Bakterienisolaten

Isolate No.	Variant	Nodule	Number of isolates per variant	*nodC* presence
1	Klif (uninoculated)	Large red	1	Yes
2	Klif (uninoculated)	Large red	2	No
3	Klif (uninoculated)	Small red	1	No
4	Klif (uninoculated)	Small red	2	No
5	Klif (uninoculated)	Large cream	1	Yes
6	Klif (uninoculated)	Small cream	1	Yes
7	Klif (uninoculated)	Small cream	2	No
8	Tarchalska (uninoculated)	Large red	1	Yes
9	Tarchalska (uninoculated)	Small red	1	Yes
10	Tarchalska (uninoculated)	Small red	2	No
11	Tarchalska (uninoculated)	Large cream	1	Yes
12	Tarchalska (uninoculated)	Small cream	2	Yes
13	Klif + IUNG	Large red	1	Yes
14	Klif + IUNG	Small red	1	Yes
15	Klif + IUNG	Large cream	1	Yes
16	Klif + IUNG	Small cream	1	No
17	Tarchalska + IUNG	Large red	1	Yes
18	Tarchalska + IUNG	Large red	2	No
19	Tarchalska + IUNG	Large red	3	No
20	Tarchalska + IUNG	Large red	4	No
21	Tarchalska + IUNG	Small red	1	No
22	Tarchalska + IUNG	Small red	2	No
23	Tarchalska + IUNG	Small red	3	No
24	Tarchalska/IUNG	Large cream	1	No
25	Tarchalska + IUNG	Large cream	2	No
26	Tarchalska + IUNG	Large cream	3	No
27	Tarchalska + IUNG	Small cream	1	No
28	Klif + Nitragine™	Large red	1	No
29	Klif + Nitragine™	Large red	2	No
30	Klif + Nitragine™	Large red	3	No
31	Klif + Nitragine™	Large red	4	No
32	Klif + Nitragine™	Small red	1	Yes
33	Klif + Nitragine™	Small red	2	No
34	Klif + Nitragine™	Large cream	1	Yes
35	Klif + Nitragine™	Small cream	1	Yes
36	Tarchalska/Nitragine™	Large red	1	Yes
37	Tarchalska + Nitragine™	Large red	2	No
38	Tarchalska + Nitragine™	Small red	1	Yes
39	Tarchalska + Nitragine™	Large cream	1	Yes
40	Tarchalska + Nitragine™	Small cream	1	Yes
41	Tarchalska + Nitragine™	Small cream	2	Yes
42	Tarchalska/Nitragine™	Small cream	3	Yes
43	Tarchalska + Nitragine™	Small cream	4	Yes
44	Nitragine™	Nonapplicable	1	No
45	IUNG	Nonapplicable	1	Yes
46	*R. leguminosarum* bv. *viceae* DSM 30143	Nonapplicable	1	No

tiple *Rhizobium* strains in a single nodule or the presence of rhizobial strains carrying multiple variants of *nodC* gene in a Sym plasmid. The restriction digestion of *nodC* gene indicated the presence of more than one rhizobial strain in one nodule, as RFLP analysis resulted in mixed digestion patterns (at least two different restriction patterns could be detected within a single sample). It is not unusual, as numerous studies confirm that nodules of leguminous plants may often harbor more than one *Rhizobium* spp. strain (Gross and Vidaver, 1978; Simms and Taylor, 2002).

5. Conclusions

The performed study showed that the abundance of nodule-dwelling bacteria is affected by a few factors, among which the variety of pea plants coupled with the type of inoculant seem to have the most significant impact. Most abundant nodule colonization was observed in the case of the "Klif" variety combined with a noncommercial inoculant. We also observed that the pea nodules may harbor a very diverse community of bacteria, and this diversity was also observed within the *nodC*-carrying strains of *Rhizobium* isolated from the pea nodules. Moreover, even though the *nodC* gene is required for establishing a symbiosis with plants, it was not detected in the majority of nodule-derived bacterial isolates. This could have been caused by the fact that the *nod* genes are located on a plasmid; therefore, after the completion of nodulation, this plasmid could have been lost because it constitutes a genetic burden for the bacteria. However, because of the fact that the *nod* genes are essential for establishing a legume–bacterium symbiosis, in order for an inoculant to be effective, it needs not only to carry viable *Rhizobium* strains but it also requires the presence of a Sym plasmid that, as we demonstrated, can be lost in many generations of bacteria. Thus, the lack of *nodC* gene in strains isolated from a commercial Nitragine™ inoculant proves that even if *R. leguminosarum* are abundant, they might not be efficient in nodulation. However, the results do not disqualify the examined inoculant, as we are not fully convinced that the bacterial strains that were able to grow on the YEM medium are in fact the representative group. In order to conduct a more certain assessment of the nodulation efficiency of an inoculant, the PCR tests aimed at detection of the *nodC* gene should be carried out on the DNA extracts derived from the inoculant itself.

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