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Comparisons between Two *Klebsiella*: The Plant Endophyte *K. pneumoniae* 342 and a Clinical Isolate, *K. pneumoniae* MGH78578

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Abstract

Bacterial strains of potential value to agriculture that promote plant growth or control plant disease are often close relatives of isolates associated with human disease. *Klebsiella pneumoniae* endophytes isolated from maize, Kp342, Kp2028, and Kp zmvsy are examples of this dilemma. These strains enhance plant growth and are endophytes of a wide variety of plant species. This work represents a collection of experiments to define the taxonomic status of these endophytes and to compare

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several phenotypes of this strain to a clinical isolate, *K. pneumoniae* MGH78578 (KpMGH). Three genetic assays show that although the maize endophytes are members of *K. pneumoniae* they are more closely related to each other and to the type strain of the species (Kp13883) than they are to KpMGH. In addition, the endophytes lack the complete set of virulence factors assays that are present in KpMGH. Most physiological and metabolic characteristics were shared by all strains further confirming the identity of these endophytes as *K. pneumoniae*. However, the maize endophytes colonized the interior of wheat seedlings in much higher numbers than either KpMGH or Kp13883. These results show that these endophytes are strains of *K. pneumoniae* but they are distinct from a clinical isolate.

Keywords: Endophytic colonization, nitrogen fixation, enteric bacteria

1. Introduction

In medical microbiology, the genus *Klebsiella* includes many opportunistic human pathogens that are associated with alcoholics and immunocompromised individuals. Infections usually occur during hospitalization (Podschun and Ullmann, 1998). Several putative virulence factors including serum resistance, capsular polysaccharides, pili, and siderophores have been identified (Podschun et al., 2001). Brisse and Verhoef (2001) have shown the phylogenetic diversity of *K. pneumoniae* and *K. oxytoca* using several genetic markers. This has also been shown by a wide variety of phenotypes including capsular typing, O-antigen variation, biotyping, protein electrophoresis, pulsed field gel electrophoresis, automated ribotyping RFLP typing of *gyrA* and *parC*, and RAPD (Arlet et al., 1994; Arora et al., 1983; Brisse and Verhoef, 2001; Combe et al., 1994; Ferragut et al., 1989; Fournier et al., 1996; Guerin et al., 1998; Mizuta et al., 1983; Oerskov, 1984; Poh et al., 1993; Rennie and Duncan, 1974).

In environmental microbiology, strains of *Klebsiella pneumoniae* are perhaps best known as model organisms for the study of nitrogen fixation. Considerable effort since the 1970s has been done concerning the biochemistry and genetics of nitrogen fixation in *K. pneumoniae* (for review see Hoover, 2000). In recent years, strains of *Klebsiella* have been identified as endophytes of maize (Palus et al., 1996; Chelius and Triplett, 2000a,b; Chelius et al., 2001). That is, these bacteria reside within plants without causing any pathogenic symptoms. Genomic interspecies microarray analysis of one of these strains, *K. pneumoniae* 342 (Kp342) has identified 3000 genes in that strain, which are in common with *E. coli* K12 (Dong et al., 2001).

Two of these endophytic *Klebsiella* were found to express nitrogenase in planta provided that sucrose was added to the medium. The ability of these diazotrophic endophytes to contribute fixed nitrogen to plants is of interest for agricultural purposes. However, given that these strains are *Klebsiella* and are close relatives of human pathogens in the same genus, any agricultural use of these endophytes will require considerable assurance that these strains are not human or plant pathogens and are taxonomically distinct from clinical isolates. As a result, a comparison of clinical and endophytic *Klebsiella* is warranted and is the subject of this work.

2. Materials and Methods

Bacterial strains and culture conditions

In this work, the three *Klebsiella* endophytes used, Kp342, Kp2028, and Kp zmvsy, were previously isolated from maize (Palus et al., 1996; Chelius and Triplett, 2000a). The type strain of *K. pneumoniae*, ATCC 13883, and *K. oxytoca* ATCC 13183 were obtained from the American Type Culture Collection (Manassas, VA, USA, www.atcc.org). The clinical isolate, *K. pneumoniae* MGH78578, was obtained from Dr. Michael McClelland of the Sidney Kimmel Cancer Center, San Diego, CA, USA. A 7.9x shotgun sequence of MGH78578 is available at the Washington University Genome Center (http://www.genome.wustl.edu/).

Amplification and sequencing of 16S rDNA genes

Nearly full-length sequences of the 16S rDNA gene of Kp342 were determined as described previously (Chelius et al., 2000a). The GenBank accession numbers for this sequence is AF394537.

Spectroscopic DNA-DNA hybridization

DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al. (1977). DNA-DNA hybridization was carried out as described by De Ley et al. (1970), with the modification described by Huss et al. (1983) and Escara and Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program described by Jahnke (1992). These hybridizations were done by the DSMZ-Identification Service (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (http://www.dsmz.de).

Automated ribotyping

Strains Kp342, Kp zmvsy, Kp2028, KpMGH were grown overnight on Columbia agar with sheep blood (Oxoid). Automated ribotyping was performed under the conditions described by Brisse and Verhoef (2001). The digests were probed with the small and large subunit rRNA genes amplified from *E. coli*.

Presence of pectinase genes

A PCR method was used to detect the conserved pectinase gene, *pehX*, in these strains. Two forward primers 5'-ggactacgccgtctatcgtcaag-3' and 5'-gatacggagtatgccttta cggtg-3' and reverse primer 5'-tagcctttatcaagcggatactgg-3' were used in reactions to generate 344 and 513 bp fragments. Templates included Kp342, Kp2028, Kp zmvsy, KpMGH, Kp13883, and *K. oxytoca* ATCC 13183 as a positive control. The PCR conditions were 94°C denaturation for 20 sec, 58°C annealing for 30 sec, and 72°C elongation for 30 sec. PCR products were separated by electrophoresis. PCR reactions included 1U TAQ polymerase, 2.5 mM $MgCl_2$, 1 ng template DNA, 25 pmol of each primer, 0.2 mM dNTP, and a buffer recommended by Fermentas, Inc. (www.fermentas.com). The primer sequences were compared with the available sequences with the Blast N program of the Blast service at the NCBI and the FASTA one of the EMBO network services. Priming oligonucleotides were synthesized in the Institute of Bioorganic Chemistry of RAN (Russian Federation).

Determination of the expression of virulence factors

Capsule typing, determination of siderophore production, serum bactericidal assays and hemagglutination assays were done as described by Podschun et al. (2001).

Plant growth conditions and bacterial inoculations

Plant growth conditions were as described by Chelius and Triplett (2000a). Wheat seeds were surface sterilized using the method of Broek et al. (1993). Seeds were immersed in 70% ethanol for 2 minutes followed by 3 washes with sterile water and immersion in 12% bleach, 0.1% SDS for 5 min. Seeds were then rinsed four times with sterile water and allowed to soak in the last water rinse for 1 hour. The surface sterilization procedure was repeated. Seeds were allowed to pre-germinate in the dark in TSA medium with until the roots were approximately 1 cm in length. Uncontaminated seedlings were transferred to glass tubes (20 mm × 200 mm) containing 20 ml solid, N-free MPCL medium

(Broek et al., 1993) and inoculated immediately. All plants were placed in the growth chamber of a 15 h day cycle at 22°C. Bacteria were scraped from the surface of Luria-Bertani agar and diluted in PBS to provide an inoculum containing 4 $\log_{10}$ CFU per seed.

Physiological characteristics of the Klebsiella isolates

Many physiological characteristics of the *Klebsiella* strains were determined using the 20E and 50CH API kits from BioMerieux Vitek (Hazelwood, MO, USA) according to the manufacturer's instructions. API 20E tests were performed on Kp342, Kp2028, Kp zmvsy, KpMGH, and Kp13883. API 50CH tests were performed on Kp342, KpMGH, and Kp13883.

Antibody generation, immunostaining, confocal microscopy

Antibody was raised against heat killed Kp342 cells in a rabbit provided by the Animal Care Unit, University of Wisconsin Medical School. One ml cell suspensions with 5×10^9 CFU were used for the first injection followed by a second injection of the same dosage two weeks later. The titers of the antibody were determined by double diffusion.

From 3, 6, 9 to 12 days after inoculation of plants, duplicate samples of plant roots were removed, surface sterilized as described by Chelius and Triplett (2000a), and fixed for 1 h in 4% paraformaldehyde. The whole immunostaining procedure was followed as described by Chelius et al. (2000a) with some modifications. Protein blocking was done with 20% goat serum for 90 min, followed by primary antibody staining (1:250) for 4 h. When necessary, the blocking was extended overnight if background was high. The secondary antibody was a rhodamine red-X conjugate (Jackson Immuno Research) used at a 1:250 dilution. Roots were stained with the secondary antibody for 2h at room temperature and mounted using an antifade kit (Prolong, Molecular Probes). Confocal microscopy was done as described by Chelius et al. (2000a).

3. Results

Spectroscopic DNA-DNA hybridization

One of the most important criteria for the species identification of bacteria is DNA:DNA hybridization. Where the DNA:DNA hybridization level between two strains exceeds 70%, the strains are considered to be in the same species. Two of *Klebsiella* endophytes, Kp342 and Kp zmvsy exceeded this standard when compared with the type strain of *K. pneumoniae*, Kp13883

Table 1. Percent genomic DNA:DNA hybridization between pairs of *Klebsiella* strains

	K. pneumoniae 342	*K. pneumoniae* zmvsy
K. pneumoniae ATCC13883	78.4	72.1
K. oxytoca ATCC 13182	42.9	42.2
K. pneumoniae MGH78578	63.5	63.0
K. pneumoniae ZMVSY	96.4	

(Table 1). And the two endophytic strains were much more similar to each other than they were to Kp13883. Both of the endophytes were very distantly related to the type strain of *K. oxytoca*, ATCC 13182. The similarity between the two endophytes and the clinical isolate, KpMGH, fell below the standard required for two strains of the same species.

Automated ribotyping and pectinase gene

Previous ribotyping results showed three specific groups of *K. pneumoniae*: KpI, KpII, and KpIII (Brisse and Verhoef, 2001). A 3.8 kb *Mlu*I fragment specific for Kp1 strains was not found in the endophytes but was present in KpMGH and Kp13883 (data not shown). Overall, the patterns showed that the endophytes were members of KpIII whereas KpMGH and Kp13883 were members of KpI (data not shown). Kp13883, KpMGH, and Kp342 lacked a pectinase gene which is present in strains of *K. oxytoca* (see Fig. 1).

Expression of virulence factors

Kp13883, Kp342, Kp2028, and Kp zmvsy did not produce aerobactin, which was produced by the clinical isolate, KpMGH. Only KpMGH and Kp zmvsy produced the ferric aerobactin receptor. None of the *K. pneumoniae* tests were sensitive to anti-capsule serum. Kp13883 and KpMGH strongly expressed type 1 pili (mannose-sensitive hemaglutination [MSHA]). Type 3 pili (mannose-resistant *Klebsiella*-like hemagglutination [MR/K-HA]) were expressed strongly by KpMGH, weakly by Kp2028 and Kp342, and not at all by Kp zmvsy and Kp13883.

Physiological distinctions between the Klebsiella strains

Using the 50CH API kit, very few metabolic differences were observed

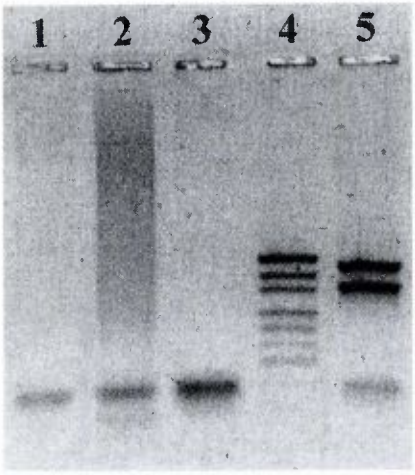

Figure 1. 1: Kp342; 2: Kp2028; 3: Kp MGH78578; 4: marker-pUC19/HpaII; 5: *K. oxytoca* ATCC13183. Two pairs of primers, specific to polygalacturonase-encoding gene, have been used to do PCR. These primers generated two DNA fragments (344- and 513-bp) as seen in lane 5.

between Kp13883, Kp342, and KpMGH. Of the three strains, Kp342 was the only strain incapable of utilizing adonitol and amidon. It was also the only strain of the three to use 5-ceto-gluconate as a substrate. KpMGH was the only strain incapable of using methyl-D-glucoside. Kp13883 was the only strain incapable of using D-tagatose, L-sorbose, and dulcitol as substrate. Kp13883 was the only strain to produce acetoin from pyruvate. These results are strongly supportive of all three endophytes being strains of *K. pneumoniae*. None of the *Klebsiella* strains utilize pectate or possess a pectinase gene as assessed by PCR (Fig. 1).

Triticum asetivum L. cv. Trenton root colonization

An antibody specific for *K. pneumoniae* was used to detect strains of *K. pneumoniae* within plant tissues by scanning confocal laser microscopy. This antibody cross-reacted with Kp342, Kp2028, Kp zmvsy, Kp13883, and KpMGH but did not cross-react with *E. coli* K12. Strong cross-reactivity was observed in roots inoculated with Kp342 but not KpMGH or Kp13883 (Fig. 2). Very little non-specific binding was observed in the uninoculated control (Fig. 2B). Kp342 colonized roots in much higher numbers than did KpMGH, Kp13883, or *E. coli* K12 (Fig. 2). Kp13883 and KpMGH colonized wheat roots in similar numbers (Fig. 2).

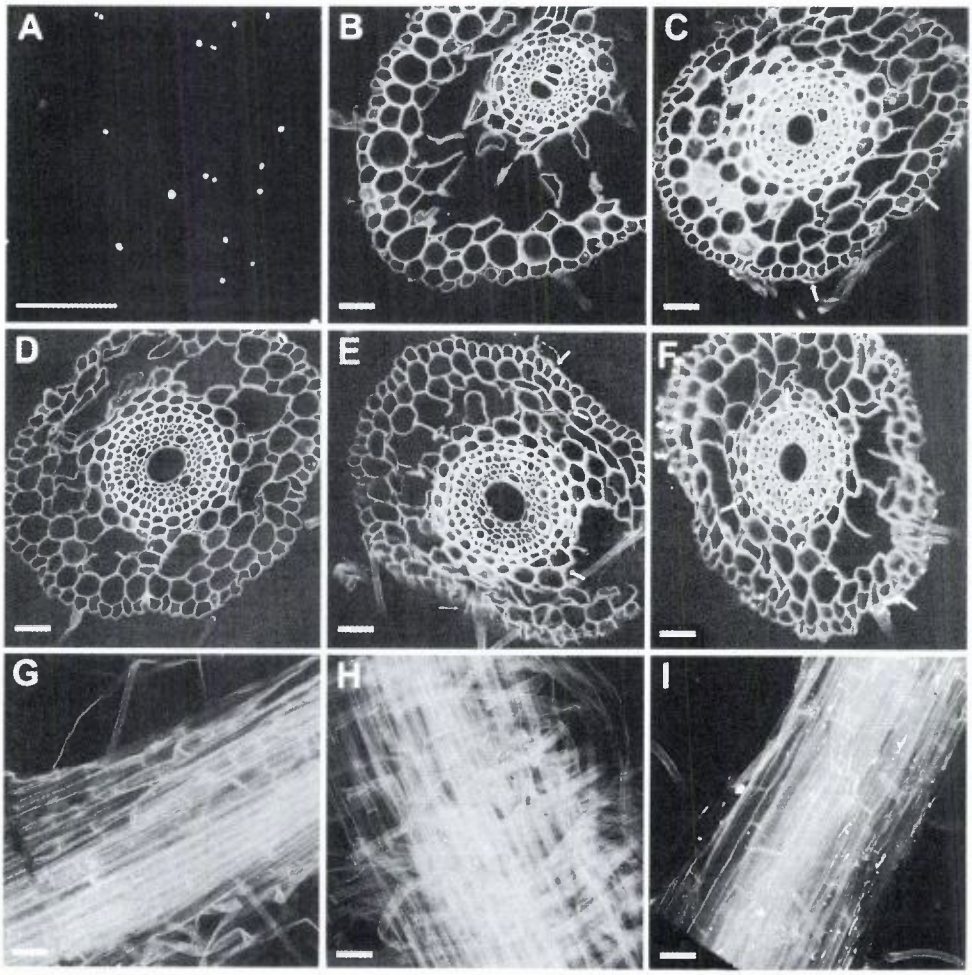

Figure 2. Immunolocalization of *K. pneumoniae* cells in wheat roots. (A) Immunostaining of *K. pneumoniae* MGH78578 cells (red) conjugated with rhodamine red-X. Transverse sections of immunostained wheat roots (green) control without inoculation (B), wheat roots inoculated with *K. pneumoniae* type strain ATCC 13883 (C), *E. coli* K12 (D), *K. pneumoniae* MGH78578 (E) and *K. pneumoniae* 342 (F) after 9 days with the inoculation level of 10^4 CFU/seed. Longitudinal sections of wheat roots inoculated with *E. coli* K12 (G), *K. pneumoniae* MGH78578 (H) and *K. pneumoniae* 342 (I). Arrows point to rhodamine red-X stained bacterial cells, and they are in red. The auto-fluorescence from wheat roots are shown in green for viewing the structure of the wheat roots. Bars: 50 μm.

The number of cells within surface sterilized wheat roots were enumerated from the homogenized roots. Nine days after inoculation, Kp342 colonized

inside wheat roots up to 5.9±0.1 $\log_{10}$ CFU per gram fresh root weight while KpMGH levels reached only 2.9±0.2 $\log_{10}$ CFU per gram fresh root weight. This is done with three replicates in each treatment. A t-test showed that, at a 95% confidence level, Kp342 inhabits the interior of wheat roots at a significantly higher level than does KpMGH.

4. Discussion

All of the physiological and genetic evidence presented here confirm the identification of Kp342, Kp2028, and Kp zmvsy as strains of *K. pneumoniae*. These data include the 16S rDNA sequences, the DNA:DNA hybridization results, and the physiological tests. Despite this identification, other results show that the *Klebsiella* endophytes are distinct from the clinical isolate KpMGH genetically and with respect to host-microbe interactions. Similar results were found in *Serratia marcescens* where an endophytic strain isolated from rice (Gyaneshwar et al., 2001) was found to be taxonomically distinct from clinical isolates (Tan et al., 2001).

The most striking difference between Kp342 and KpMGH is the ability of Kp342 to enter wheat in numbers 1000-fold higher than does KpMGH. This was shown by counting of cells extracted from the interior of surface sterilized roots and was confirmed by direct observation using confocal microscopy. The mechanism by which the maize endophyte enters wheat in such high numbers is unknown. However, as two strains of *K. pneumoniae* differ greatly in their ability to enter wheat roots, it is reasonable to assume that bacterial genetic determinants are involved in the process.

With respect to virulence factors involved in human-microbe interactions, only KpMGH expressed the full complement of virulence factors with Kp342 lacking more than any other strain of *K. pneumoniae* tested here. Future work needs to address the question of whether the absence of some of these factors in Kp342 is related to the ability of this strain to colonize animals. Bach et al. (2000) reported that of three *K. penumoniae* strains tested for the presence of the *Yersinia* high pathogenicity island, only one strain contained this set of genes coding for the siderophore yersiniabactin. The presence of this island in maize endophytes was not tested here but Kp342 was found to lack production of the siderophore, aerobactin.

Pectate or pectin lyases play a very important role in the bacterial penetration of plant surfaces. Phytopathogens need pectin lyases to attack plants. However, little is known about their role in nonpathogenic bacteria. For example, among species of *Azospirillum* only *A. irakense* possesses pectate lyase (Bekri et al., 1999), but another species of *Azospirillum*, *A. brasilense*, is able to enter plant tissues (Schloter and Hartmann, 1998). Though *K. oxytoca*

possesses pectate lyases, it showed levels of colonization on different plants. Induction of pectate lyase activity led to an increased number *K. oxytoca* within wheat roots (Kovtunovych et al., 1999). However, the *K. penumoniae* endophytes used in this work lack pectate lyase activity and pectate lyase genes so that pectate degradation in these strains is not important for plant entry by these strains unlike endophytes of *K. oxytoca*.

By DNA:DNA hybridization, the maize endophytes are very closely related to each other but are sufficiently related to the type strain of *K. pneumoniae* to be within the same species. However, the endophytes are below this threshold when compared to the clinical isolate, KpMGH. Similarly, ribotyping of the rrn operons shows that the maize endophytes are closely related to each other within group KpIII while KpMGH and Kp13883 are both in another group, KpI.

The criteria used to distinguish species from each other in bacterial taxonomy fail to capture distinctions in host-microbe interactions that may exist between strains of a given species. The dilemma associated with this problem is that often strains that may have important applications in plant-microbe interactions cannot be exploited commercially because of their taxonomic relatedness to clinical isolates. This has been a significant problem in *Burkholderia* and *Bacillus* where strains have been identified as useful agents for the control of plant disease (Emmert and Handelsman, 1999; Helgason et al., 2000; Parke and Gurian-Sherman, 2001; Pruss et al., 1999). The genetic change required to convert a harmless or beneficial bacterium to one that can cause human disease can be the simple lateral transfer of a plasmid carrying virulence determinants to an avirulent cell (Stephens and Murray, 2001). As a result, the demonstration that a particular strain is not a pathogen in humans may not be sufficient to permit widespread use of a beneficial strain in agriculture. With an increased understanding of lateral transfer mechanisms among bacteria and of the phenotypes related to host-microbe interactions, new approaches may be developed that will reduce safety concerns related to the agricultural use of bacteria that are close relatives of human pathogens.

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