Identification of *Klebsiella pneumoniae* by DNA Hybridization and Fatty Acid Analysis

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On the basis of the idea that DNA sequences encoding cell surface-exposed regions of outer membrane proteins are genus or species specific, two oligonucleotide probes which were based on the *PhoE* protein of *Klebsiella pneumoniae* were evaluated. In slot blot hybridizations and in polymerase chain reactions, no cross-hybridizations were observed with non-*Klebsiella* strains. When the probes were tested on 75 different K-antigen reference *Klebsiella* strains, 16 strains were not recognized although they did produce *PhoE* protein under phosphate starvation. To determine whether these 16 strains belong to (a) different species, the reference strains were also tested for the ability to produce indole and to grow at 10°C and their whole-cell fatty acid patterns were analyzed by gas chromatography. A strong correlation was observed among (i) reaction with the probes, (ii) the inability to produce indole, (iii) the inability to grow at 10°C, and (iv) the presence of the hydroxylated fatty acid C_{14:0-2OH}. From these results we conclude that the two oligonucleotides are specific for the species *K. pneumoniae*. Furthermore, analysis of fatty acid patterns appears to be a useful tool to distinguish *K. pneumoniae* from other *Klebsiella* species.

The genus *Klebsiella* consists of four species, i.e., *K. pneumoniae* (with subspecies *pneumoniae*, *ozaenae*, and *rhinoscleromatis*), *K. oxytoca*, *K. planticola*, and *K. terrigena* (12). Although the present definition of the genus is confined to nonmotile strains, proposals have been made to transfer *Enterobacter aerogenes* to the genus as *K. mobilis* (2, 5). The current classification of *Klebsiella* strains into the different species is based on their biochemical properties. The results of these assays are in many cases difficult to interpret. For instance, there is no key discriminatory test to differentiate the indole-negative *K. planticola* strains from *K. pneumoniae* (1). A genetically based procedure like the polymerase chain reaction (PCR) could potentially increase the precision and the rapidity and decrease the labor intensiveness of the identification.

The first step in the development of such an identification system is the selection of species-specific oligonucleotide probes. Recently, we have shown that DNA sequences encoding surface-exposed regions of outer membrane proteins can be used as specific probes (16). The *phoE* gene of members of the family *Enterobacteriaceae* encodes a pore-forming outer membrane protein which is induced when the bacteria are grown under phosphate limitation (14). According to the proposed topology model, the polypeptide traverses the outer membrane 16 times in an antiparallel β -sheet structure, thereby exposing eight areas at the cell surface (17, 22). Comparison of the primary structures of different *PhoE* proteins revealed four hypervariable regions, all of which were predicted to be cell surface exposed, whereas the membrane-spanning regions were found to be highly conserved (21).

In this study, we have evaluated the potential of two oligonucleotide probes, based on the *phoE* gene of *K. pneumoniae*, to discriminate between different *Klebsiella* species. The sensitivity of the probes was tested on the K antigen reference strains described by Ørskov and Ørskov

(13). The capsular polysaccharide, or K antigen, is most commonly used for serological typing of *Klebsiella* strains. The (lack of) reaction of the probes was correlated to species classification by testing the ability of the strains to produce indole and testing their growth at 10°C. *K. pneumoniae* strains are negative in the indole test and are the only *Klebsiella* strains unable to grow at 10°C (12). As an independent method, the fatty acid content of the strains was analyzed by gas-liquid chromatography, which is also considered a powerful approach to differentiate between bacterial species (6).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* K-12 strain CE1194, which carries a chromosomal *phoE* deletion, and its *phoE*⁺ derivative CE1195 (19) and strain K10 (CGSC4234) (18) have been described elsewhere. Other enterobacterial strains used were *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *K. pneumoniae*, *Providencia stuartii*, *Proteus mirabilis*, *Salmonella braenderup*, *Salmonella derby*, *Salmonella panama*, *Serratia marcescens*, *Shigella flexneri*, and *Shigella boydii*, all described by Hofstra and Dankert (4), and *Salmonella typhimurium* SJ2353, described by Sato and Yura (15). Of the 77 different K antigen reference strains described by Ørskov and Ørskov (13), 75 (missing K6 and K49) were kindly provided by W. Jansen at the National Institute for Public Health and Environmental Protection in Bilthoven, The Netherlands. The nonenterobacterial strains used, i.e., *Aeromonas hydrophila*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Sarcina flava*, and *Staphylococcus aureus*, were from our laboratory stocks.

Unless mentioned otherwise, strains were grown overnight at 37°C under aeration in L broth (20). When strains were grown at 10°C, incubation was continued for 48 h.

Isolation and characterization of cell envelopes. Strains were grown overnight at 37°C under aeration in a medium (7) in which the phosphate concentration is limiting for growth.

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FIG. 1. Comparison of the DNA sequences of the *Klebsiella* probes with the corresponding sequences of the *phoE* genes of *E. coli* and *E. cloacae*. KP5 is based on the DNA complementary to the sequence encoding the fifth surface-exposed region of the *phoE* gene, and KP8c is based on the DNA sequence encoding the eighth surface-exposed region of the *phoE* gene of *K. pneumoniae*.

Phosphate-replete conditions were obtained by supplementing the medium with 660 μ M K_2HPO_4 . Cell envelopes were isolated by differential centrifugation after ultrasonic disintegration of the cells (8). The protein patterns were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (8) using gels containing 6 M urea.

Indole reaction. Indole tests were performed as described by Frobisher (3).

Synthesis and labeling of the oligonucleotides. Oligonucleotides were automatically synthesized on a Bioscience 8600 DNA synthesizer. When used in hybridization assays, the oligomers were labeled by the enzymatically catalyzed transfer of ^{32}P from [γ - ^{32}P]ATP (3,000 Ci/mmol; Amersham International) with T4 polynucleotide kinase (Pharmacia, Uppsala, Sweden) according to the procedure described by Maniatis et al. (9).

DNA hybridizations and PCR assays. Slot blot hybridization assays were performed as described previously (16), except that the incubation temperature during (pre)hybridization and washings was 63°C instead of 60°C. DNA samples to be used in PCRs were isolated from 1-ml overnight cell cultures. Cells were harvested by centrifugation, resuspended in 100 μ l of demineralized water, and boiled for 5 min. Cell debris was removed by a 2-min centrifugation step at 14,000 $\times g$. PCR amplifications were performed on 5- μ l samples of the supernatant fraction by using a DNA thermal cycler (PHC-1; New Brunswick plc. Int.). One unit of *Taq* polymerase (Promega, Madison, Wis.) was used as described by the manufacturer except that RNase (Boehringer, Mannheim, Germany) was added to a final concentration of 20 μ g/ml and the total volume was adjusted to 25 μ l. A total of 25 PCR cycles was run under the following conditions: denaturation at 94°C for 1 min, primer annealing at 45°C for 2 min, and DNA extension at 72°C for 2 min but for 5 min in the last cycle. Of the PCR products, 10- μ l samples were analyzed by electrophoresis on 1% agarose gels.

Gas-liquid chromatographic analysis of fatty acids. For analysis of fatty acid patterns bacteria were harvested after growth for 24 h at 37°C on Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) solidified with 1.5% agar. Whole-cell fatty acids were analyzed as fatty acid methyl esters. Fatty acid methyl ester extracts were made by the techniques described by Miller and Berger (10). They were analyzed by high performance capillary gas-liquid chromatography with the MIDI Microbial Identification System (Microbial ID, Inc. Newark, Del.). The system configuration contained a Hewlett-Packard HP 5890 A gas chromatograph with autosampler and was equipped with a 25-m Ultra 2 column and a flame ionization detector. The system was completed with a HP9000 series 300 computer system for analysis control, data acquisition, and data evaluation, using the MIDI library-generating software.

FIG. 2. Autoradiogram of a slot blot hybridization using probe KP5. The K strains are different *Klebsiella* K antigen reference strains. The *E. coli* strain used was CE1195. *Kpn* is an unserotyped *K. pneumoniae* strain, the *phoE* gene of which has been sequenced previously (21). The probes were based on this sequence.

RESULTS

The *phoE* gene of *K. pneumoniae* has been cloned and sequenced previously (21). Comparison of the DNA sequences of the *phoE* genes of *K. pneumoniae*, *E. cloacae*, and *E. coli* revealed four hypervariable regions, corresponding to cell surface-exposed segments of the PhoE proteins (21). The hypervariable regions where the homology between the proteins was the lowest were chosen for two oligonucleotide probes. Probes KP5 and KP8c were based on DNA sequences encoding the fifth and the eighth surface-exposed regions of PhoE, respectively (Fig. 1). They correspond to segments of the noncoding and coding DNA strands, respectively, so that they can be used in PCRs. An ideal DNA probe has to be both sensitive and selective; i.e., it has to recognize all strains and serotypes of a certain taxon, but it should not cross-react with other bacteria. These conditions were tested in hybridization and PCR assays.

Sensitivity assays. The capacity of the probes to recognize different *Klebsiella* strains was tested on 75 of the 77 K-antigen reference strains (13). The strains are named K1 to K82; K73 and K75 to K78 have been eliminated because of misdiagnosis and overlapping (13). Strains K6 and K49 were lost from our collection. The probes were tested on these strains in slot blot hybridization assays. An example of an autoradiogram is shown in Fig. 2. Both probes reacted

FIG. 3. Analysis of PCR products on a 1% agarose gel. The K strains are different *Klebsiella* K antigen reference strains. The *E. coli* strain used was CE1195. *Kpn* is an unserotyped *K. pneumoniae* strain, the *phoE* gene of which has been sequenced previously (21). The probes were based on this sequence. demi means that demineralized water was used as the substrate.

TABLE 1. Characteristics of the *Klebsiella* strains

Strain	PCR results	Growth at 10°C ^a	Indole production	Presence of fatty acid C _{14:0-2OH}
<i>K. pneumoniae</i> ^b	+	—	—	+
K1	+	—	—	+
K2	+	—	—	+
K3	+	—	—	+
K4	+	±	—	+
K5	—	—	—	+
K7	+	—	—	+
K8	+	±	—	+
K9	+	—	—	+
K10	+	±	—	+
K11	+	—	—	+
K12	+	±	—	+
K13	+	±	—	+
K14	+	±	—	+
K15	+	—	—	+
K16	+	—	—	+
K17	+	—	—	+
K18	+	—	—	+
K19	+	±	—	+
K20	+	—	—	+
K21	+	—	—	+
K22	+	±	—	+
K23	+	—	—	+
K24	+	±	—	+
K25	+	—	—	+
K26	—	++	+	—
K27	+	±	—	+
K28	+	—	—	+
K29	—	++	+	—
K30	+	±	—	+
K31	+	±	—	+
K32	+	±	—	+
K33	+	—	—	+
K34	+	±	—	+
K35	—	++	—	—
K36	+	±	—	+
K37	+	—	—	+
K38	+	—	—	+
K39	+	—	—	+
K40	+	—	—	+
K41	—	++	+	—
K42	+	±	—	+
K43	+	±	—	+
K44	—	++	+	—
K45	+	±	—	+
K46	+	±	—	+
K47	+	±	—	+
K48	+	±	—	+
K50	+	—	—	+
K51	+	—	—	+
K52	+	—	—	+
K53	+	±	—	+
K54	+	±	—	+
K55	+	±	—	+
K56	+	±	—	+
K57	+	—	—	+
K58	+	±	—	+
K59	—	++	—	—
K60	+	—	—	+
K61	+	—	—	+
K62	+	—	—	+
K63	+	—	—	+
K64	+	—	—	+
K65	—	++	—	—
K66	—	++	+	—
K67	—	++	—	—
K68	—	++	—	—

Continued

TABLE 1—Continued

Strain	PCR results	Growth at 10°C ^a	Indole production	Presence of fatty acid C _{14:0-2OH}
K69	—	++	—	—
K70	—	±	+	—
K71	+	—	—	+
K72	—	++	+	—
K74	—	++	+	—
K79	—	++	—	—
K80	+	±	—	+
K81	+	—	—	+
K82	+	±	—	+

^a Symbols for optical density (OD) values at $\lambda = 660$ nm after 48 h: —, OD < 0.1; ±, $0.1 \leq \text{OD} < 0.5$; ++, OD ≥ 1 .

^b *K. pneumoniae* is an unserotyped strain, the *phoE* gene of which has been sequenced previously (19). The probes were based on this sequence.

with 59 of the 75 strains, and each of them failed to recognize 16 strains, which were the same strains in both cases. The probes were designed in such a way that they could also be used as primers in PCRs. When the primers recognize their complementary sequences, there will be amplified a 368-bp DNA fragment which can be detected when the PCR products are analyzed by electrophoresis on agarose gels. An example of such an analysis is shown in Fig. 3. The expected amplified fragment was observed in the same 59 strains that reacted positively in the slot blot hybridization assays, whereas the remaining strains again were negative (Table 1).

Selectivity assays. To test the selectivity of the probes, the 14 different non-*Klebsiella* strains belonging to the *Enterobacteriaceae* and 5 non-*Enterobacteriaceae* strains mentioned in Material and Methods were tested in slot blot hybridizations. The results of probe KP8c have been published earlier (16) and showed that only the *K. pneumoniae* strain from which the *phoE* nucleotide sequence was established was recognized. Similar results were found when KP5 was used as the probe or when KP5 and KP8c were used together in PCRs (data not shown).

Expression of the *phoE* genes. The two probes turned out to be selective, since they did not recognize any other strains except *Klebsiella* strains, but when tested for their sensitivity, they failed to recognize 16 of 75 *Klebsiella* strains, i.e., K5, K26, K29, K35, K41, K44, K59, K65 to K70, K72, K74, and K79. To test whether these strains contain a *phoE* gene, a number of them were grown under high- and low-phosphate conditions and their cell envelope protein patterns were analyzed. Figure 4 shows that all tested *Klebsiella* strains induced a membrane protein of the expected apparent molecular weight when grown under low-phosphate conditions. Of the strains tested, K26, K29, K44, K59, and K65 did not react with the probes. The DNA sequence of their *phoE* genes must therefore differ from those of strains recognized by the probes.

Species classification reactions. To determine whether the different reactions with the DNA probes can be correlated to differences at the species level, more information about the 75 *Klebsiella* strains was necessary. Therefore, the strains were tested for the ability to produce indole and to grow at 10°C (Table 1). Strains that are positive in the indole test belong to either the species *K. oxytoca* or *K. planticola*, and of the different *Klebsiella* species, only *K. pneumoniae* is not able to grow at 10°C (12). All strains found to produce indole, i.e., K26, K29, K41, K44, K66, K70, K72, and K74, were not recognized by the probes. Furthermore, we found a

FIG. 4. SDS-polyacrylamide gel electrophoresis patterns of cell envelope proteins of *Klebsiella* strains either grown under high-phosphate (H) or low-phosphate (L) conditions. The PhoE proteins are indicated with asterisks. Only the relevant part of the gel is shown.

strong correlation between positive results in the probe assays and no or poor growth at 10°C (Table 1). The strains that gave negative results in the probe assays, on the other hand, could all grow at 10°C, except for strains K5 and K70, which showed no and poor growth, respectively.

Gas-liquid chromatographic analysis of fatty acids. The analysis of fatty acid content has been propagated for the identification of bacterial species (6) but has, to our knowledge, never been used to differentiate between different *Klebsiella* species. Therefore, as a totally independent determination method, the fatty acid methyl esters of the isolated phospholipids of the 75 *Klebsiella* strains were analyzed. The most common fatty acids found in all the *Klebsiella* strains were C_{16:0} (34%), C_{17:0 cyclo} (14%), and C_{14:0} (9%). The major hydroxylated fatty acid found was C_{14:0-2OH} (2%). Interestingly, only those *Klebsiella* strains that were recognized by the probes contained this hydroxylated fatty acid, whereas the strains which were not recognized by the probes did not (Table 1). The only exception is strain K5, which, although negative in the probe assays, contains fatty acid C_{14:0-2OH}. Strain K60, which is positive in the probe assays, does contain fatty acid C_{14:0-2OH}, albeit at a lower level (1%).

DISCUSSION

The two *Klebsiella* probes evaluated in this study were found to be selective; i.e., no cross-reactivity occurred with tested *Enterobacteriaceae* strains other than *Klebsiella* strains and with non-*Enterobacteriaceae* strains. When the probes were tested on 75 of the 77 K antigen reference strains, 16 strains were not recognized. Among these strains are K26, K29, K44, K59, and K65, which normally induced a PhoE protein when grown under phosphate limitation (Fig. 4). Therefore, the *phoE* gene is probably commonly present in *Klebsiella* strains. An explanation for the fact that 16 of the *Klebsiella* strains were not recognized by the probes might be that the probes are specific for the species *K. pneumoniae*. It has been known for some time that a number of the K antigen reference strains are *K. oxytoca* strains (12), and more recent research has indicated that *K. planticola* and *K. terrigena* strains might also be present in this collection (11). To obtain more information on the species classification of the *Klebsiella* strains, their ability to produce indole and to grow at 10°C and their whole-cell fatty acid patterns were analyzed. A good correlation was observed among (i) reaction with the probes, (ii) the inability to produce indole, (iii) the inability to grow at 10°C, and (iv) the

presence of the hydroxylated fatty acid C_{14:0-2OH}. Only two exceptions were found. Strain K5, despite its inability to grow at 10°C and the presence of C_{14:0-2OH} in its fatty acid profile, was not recognized by the probes. This strain, like K4, which normally reacted with the probes, has been classified as a *K. ozaenae* strain (12a). Strain K70, although positive in the indole test, negative in the probe assays, and missing fatty acid C_{14:0-2OH}, showed only poor growth at 10°C.

From the results, we conclude that recognition by the probes is probably restricted to the species *K. pneumoniae* and that therefore, with the exception of strain K5, all strains which are not recognized by the probes belong to other *Klebsiella* species. The finding that K26, K29, K35, K41, K44, K59, K65, K66, K70, K72, K74, and K79 do not belong to the species *K. pneumoniae* is in agreement with the results of Mori et al. (11). However strains K67, K68, and K69, which, according to our tests, did not behave like *K. pneumoniae* strains, were classified as such by Mori et al. On the other hand, strains K8, K14, K32, K39, K48, K49, K56, K57, K58, and K71, classified by Mori et al. as *K. planticola* strains, behaved in our tests as normal *K. pneumoniae* strains. Most differences occur in differentiating *K. pneumoniae* strains from the indole-negative *K. planticola* strains. Since there is no key discriminatory test in the biochemical classification, Mori et al. used, as recommended by Bagley et al. (1), a combination of an L-sorbose fermentation test and a hydroxy-L-proline utilization test to distinguish between the two species. Also, two other tests, namely, the fecal coliform reaction at 44.5°C and growth at 10°C, were used. Mori et al. found only 65% of the strains which were fecal coliform negative and had growth at 10°C to be positive in the tests recommended by Bagley et al., whereas Bagley et al. found a correlation of more than 90%. According to Ørskov, some *Klebsiella* strains are biochemically hard to classify (12a).

The above-mentioned results demonstrate the difficulty in the biochemical classification assays to distinguish *K. pneumoniae* from indole-negative *K. planticola*. In our opinion, the use of the DNA probes as described in this article and the analysis of fatty acid content will contribute to a more precise classification of *Klebsiella* strains. Moreover, the use of these probes will lead to more rapid detection and classification assays. Presently, we are developing similar DNA probes for the other *Klebsiella* species to enable the specific detection and identification of these species as well.

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