

Potential Pathogens in the Environment: Cultural Reactions and Nucleic Acid Studies on *Klebsiella pneumoniae* from Clinical and Environmental Sources¹

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The phenotypic and nucleic acid properties of *Klebsiella pneumoniae* have been studied on cultures obtained from six different habitats (humans, vegetables, seeds, trees, rivers, and pulp mills). The 19 cultural reactions of 107 isolates varied significantly only in tryptophanase activity and dulcitol fermentation. The percentage of guanine plus cytosine base composition of 41 isolates varied from 53.9 to 59.2%. The range of percentage of guanine plus cytosine base composition for environmental klebsiellas was broader than that for the cultures of human origin. The range of deoxyribonucleic acid relative reassociation (homology) to the human *K. pneumoniae* reference strain extended from 5% to 100% and the chromosome molecular weights ranged from $2,200 \times 10^6$ to $3,000 \times 10^6$. The species of *K. pneumoniae* is thus molecularly more heterogeneous than previously thought and most isolates of human, pulp mill, and river origin are genetically indistinguishable. The presence of *K. pneumoniae* therefore represents a deterioration of the microbiological quality of the environment and should be considered of public health significance. At the present time the health significance of the molecularly more divergent strains, primarily of vegetable and seed origin, their relationship to klebsiellas of human origin, or to other genera of the *Enterobacteriaceae* is unclear.

Klebsiella pneumoniae, members of the family *Enterobacteriaceae*, comprise a group of nonmotile, nonsporeforming, lactose-fermenting, gram-negative rods. These bacteria are usually heavily encapsulated and are typically associated with nosocomially acquired infections of the genito-urinary, respiratory, and septicemic foci in debilitated, chronically ill, or postoperative situations (27). *K. pneumoniae* is also found as the primary agent of coliform mastitis in dairy herds (3). Classically, *Enterobacter* (formerly *Aerobacter*) and *Klebsiella* have been difficult to distinguish because of many similar cultural reactions and these two genera have not always been properly identified (20). As recommended by Hormaeche and Edwards (16), and subsequently demonstrated in other studies (3, 11), a few key cultural tests can be used to differentiate the two genera.

Several recent studies have demonstrated that bacteria which conform to the cultural definition of *K. pneumoniae* can be isolated from a variety of natural environments (1, 7, 10, 18, 19, 24). These observations coincide with the

increasing incidence of multiple antibiotic resistant nosocomially acquired *K. pneumoniae* infections in humans as well as infections in domestic and wild animals (5, 27, 28, 29). Because of the subtle phenotypic differences between *Enterobacter* and *Klebsiella* and the potential significance of high densities of *Klebsiella* in the natural environment, we have initiated several studies on the molecular taxonomy and ecology of the klebsiellas. In this report the molecular heterogeneity of the *K. pneumoniae* species is documented on the basis of nucleic acid studies.

MATERIALS AND METHODS

Bacterial cultures. *K. pneumoniae* cultures were selected from among lactose-positive and oxidase-negative colonies appearing on m-endo LES agar (Difco) that had been streaked with aliquots from samples of industrial waste waters, river water, vegetables and plant seeds (7). Several reference *K. pneumoniae* cultures isolated from human infections were obtained from the American Type Culture Collection, Rockville, Md., J. Matsen, University of Minnesota School of Medicine, Minneapolis, and Center for Disease Control, Atlanta, Ga. The cultures and their origin are listed in Table 1.

Identification of isolates and growth media.

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TABLE 1. *Origin of strains used in nucleic acid analyses*

Strain	Source or reference
<i>Klebsiella pneumoniae</i>	
13882	ATCC; human serotype 64
13883	ATCC; neotype strain, human serotype 3
8047	ATCC; human serotype 1
15574	ATCC; leaf nodule endosymbiont, serotype 24
141	J. Matsen, human
143	J. Matsen, human
UOMS-1	Univ. Oregon Medical School, human
UOMS-5	Univ. Oregon Medical School, human
MUSC-2	Medical University South Carolina, human
004	Pulp mill; ammonium base sulfite
012	Pulp mill; ammonium base sulfite
037	Pulp mill; ammonium base sulfite
045	Pulp mill; ammonium base sulfite
050	Pulp mill; ammonium base sulfite
084	Pulp mill; Kraft
089	Pulp mill; Kraft
093	Pulp mill; Kraft
094	Pulp mill; Kraft
102	Pulp mill; Kraft
116	Pulp mill; defiberization
118	Pulp mill; defiberization
131	
4064-19	Living fir tree (1)
4082-20A	Living fir tree (1)
006	River
051	River
002	River
R301	River downstream from pulp mill, serotype 64 (7)
R302	River downstream from pulp mill, serotype 15 (7)
R303	River downstream from pulp mill
R304	River downstream from pulp mill, serotype 47 (7)
R306	River downstream from pulp mill
R307	River downstream from pulp mill
V-101b	Potato
V-104a	Mushroom, serotype related to 6 and 7 (7)
V-112	Potato, serotype 26 (7)
V-233	Radish, serotype 11 (7)
V-236	Radish, serotype 21 (7)
V-244	Green onion, serotype 47 (7)
V-171	Turnip seeds, serotype 23 (7)
V-151	Carrot seeds, serotype 23 (7)
Ka-1	Soil (19)
M5A1	2,3 butylene glycol fermentation, serotype related to 8, 11, 21, 26, and 69 (20)
<i>Enterobacter aerogenes</i>	
13048	ATCC; neotype strain, human
<i>E. cloacae</i>	
13047	ATCC; neotype strain, human

Biochemical examination of all isolates was accomplished according to the recommendations of Edwards and Ewing (11) and the *Manual of Clinical Microbiology* (3).

Cultures for deoxyribonucleic acid (DNA) extraction were propagated in 2 to 4 liters of nutrient broth (BBL) with the addition of 1% glucose or in a broth consisting of 1% tryptone (Difco) plus 0.3% yeast extract (Difco).

DNA preparation. Saline-ethylenediaminetetraacetic acid washed and frozen cells were thawed and suspended in saline-ethylenediaminetetraacetic acid and lysed by the addition of 1 to 2% sodium lauryl sulfate (22). DNA purification involved a modified Marmur technique by using neutralized phenol deproteinization immediately after cell lysis and ribonuclease treatment (22).

The capsular polysaccharide was removed from the

DNA solution after the last ethanol precipitation. The DNA was dissolved in 0.1 strength standard saline citrate ($0.1 \times$ SSC) and an equal volume of 2.5 M potassium phosphate, pH 7.5, was added. An equal volume of 2 methoxy-ethanol was added, dropwise, until the initial precipitate could no longer be dissolved by gentle swirling. The slurry was centrifuged at 10,000 rpm, and then the clear supernatant was decanted. The DNA was precipitated with *N*-propanol and collected on a glass rod. The above is a modification of the procedure of Bellamy and Ralph (4).

Determination of percentage guanine plus cytosine (% G+C) base composition. The % G+C base composition of purified DNA was determined by the thermal denaturation procedure as described by Marmur and Doty (23). The % G+C content was calculated from the midpoint of the thermal melting curve (T_m) by using equation 3 of DeLey (9).

Quantitative measurements of DNA reassociation. Two procedures were used in determining DNA homology between environmental and human *K. pneumoniae* strains. In one procedure, the DNA membrane filter technique was used and hybridization was measured by the competition assay (13, 17). About 20% of the input homologous radioactive DNA renatured to the membrane-bound DNA. This resulted in some 10,000 counts/min of radioactivity. Approximately 400 counts/min of DNA was retained on blank filters. The second technique utilized measurement of $C_0t_{0.5}$ values of renaturing DNA mixtures (26). Membrane filter competition assays

were performed in $2 \times$ SSC, whereas optical measurement of renaturing DNA mixtures were in $3 \times$ SSC plus 20% Me_2SO . DNA duplex formation occurred at $T_m - 25$ C.

Determination of genome size. Genome sizes were estimated by the technique of DNA renaturation kinetics (26). Renaturation occurred in $3 \times$ SSC plus 20% Me_2SO at $T_m - 25$ C. *Escherichia coli* WP2 was used as the molecular weight standard.

RESULTS

Over 100 presumptive *K. pneumoniae* strains from nine different ecological habitats were examined for their reactions in 19 diagnostic media (Table 2). In general, isolates from all groups conformed to the acceptable pattern(s) exhibited by the species, *K. pneumoniae* (4, 11). Perhaps the most atypical group of strains comes from white fir trees (1). Although some of these isolates exhibited a weak production of H_2S and nearly two-thirds did not grow in KCN, all were confirmed as *K. pneumoniae* serotype 68 by the Center for Disease Control.

The single most variable cultural reaction was the ability to produce indole. In general, strains of plant-associated *K. pneumoniae* had a somewhat higher incidence for indole production than would be expected from the published key (11). Nearly one-third of the vegetable,

TABLE 2. Percentage of positive cultural reactions of *K. pneumoniae* from various environments

Test or substrate	Key ^b	Origin ^a								
		Vegetables	Seeds	Trees	Pulp mill	River	Human clinical	CDC	ATCC	Other
Indole	6	20.0	25	100	0	0	17	0	33	50
Methyl Red	13.3	12.0	5	38	0	0	0	0	0	0
Voges-Proskauer	91	92.0	100	100	100	100	100	100	100	100
Citrate	98	88	100	100	100	100	100	100	100	100
H_2S	0	12	0	63	0	0	0	0	0	0
Urease	95	80	80	100	95	100	92	100	100	100
KCN	98	ND ^c	38	100	ND	ND	ND	100	100	100
Motility	0	0	0	0	0	0	0	0	0	0
Gelatin	3.3	0	0	0	0	ND	0	0	0	0
Lysine	100	100	85	100	100	100	100	100	100	100
Arginine	1	0	0	0	0	0	0	0	0	0
Ornithine	0	0	0	0	0	0	0	0	0	0
Phenylalanine	0	ND	ND	0	0	ND	ND	0	0	0
Malonate	100	96	88	100	100	100	100	100	100	100
Glucose gas	97	84	100	100	100	100	100	100	100	100
Lactose	100	100	100	100	100	100	100	100	100	100
Sucrose	99	ND	ND	ND	100	ND	ND	100	67	100
Mannitol	100	100	100	100	100	100	100	100	100	100
Dulcitol	32	32	70	ND	47	67	17	43	33	50

^a Number of cultures included in each group: vegetables, 25; seeds, 20; trees, 8; pulp mills, 19; river, 9; human clinical, 12; CDC, 7; ATCC, 3; other, 4.

^b Key of expected cultural reactions taken from Edwards and Ewing (11).

^c ND, Not done.

seed, and tree strains were positive for this trait (18/53).

The % G+C of 41 strains of *K. pneumoniae* from various habitats is summarized in Table 3. The % G+C content for all groups ranged from 53.9% to 59.2%. There was no significant difference in the average G+C contents among any of the groups. However, the groups represented by pulp mill and environmental klebsiellas contained isolates with more divergent base composition than the human group (54.7 to 59.2% G+C, 53.9 to 58.2% G+C and 56.2 to 57.8% G+C, respectively).

One of the main goals of the present study was to determine the degree of genetic relatedness of the environmental *K. pneumoniae* strains with the human reference strain. The DNA polynucleotide diversity was ascertained by DNA hybridization experiments (Table 4). The strains of *K. pneumoniae* were divided into four groups which reflect their origin in nature. A broad range of relative reassociation (homology) values were observed for these phenotypically indistinguishable *K. pneumoniae* strains. Most of the human isolates were homogeneous illustrating some 75% or higher relatedness to the neotype reference strain DNA, 13883. Comparable high values were also obtained with five out of seven pulp mill isolates and all five isolates from rivers. However, only the leaf nodule symbiont (8) exhibited a high homology with the human reference DNA in the plant-vegetable-seed group of strains. The average homology value for the remaining eight out of nine vegetable isolates is 35%. This low average value is comparable to that observed for one human strain UOMS-1 and for two strains from pulp mills, 094 and 012.

The more conventional DNA membrane filter hybridization technique was used in early studies. Experiments involving hybridization trials with 141 as the reference included strains 131, 116, and 094. Comparable relative reassociation values were obtained by both optical and membrane filter hybridization procedures (agreement within 10%).

The question now arises as to the significance of the *K. pneumoniae* strains which exhibit less than 50% homology with the human neotype reference strain, 13883. It therefore was relevant to examine further DNA reassociation levels among these low homology strains in an attempt to clarify their taxonomic status and possible health significance. Additional experiments were studied by using neotype strains of *Enterobacter* spp. as reference DNA (Table 5). *E. aerogenes* and *E. cloacae* share similarity in polynucleotide sequences over one-third of their genome, whereas *E. aerogenes* and *K.*

TABLE 3. Midpoint of thermal melting curve and % G + C mean base composition of DNA from *K. pneumoniae* from various habitats

Origin	T_m	% G + C base composition ^{a, b}
Pulp mills		
004	93.5 ± 0.5	57.8
012	93.8	58.4
037	94.1 ± 0.4	59.2
045	93.5 ± 0.4	57.8
050	93.0	56.6
084	93.5	57.7
089	92.6 ± 0.3	55.6
093	93.6 ± 0.3	58.0
094	92.9 ± 0.2	56.3
102	93.2	57.0
116	93.2	57.0
118	92.2 ± 0.1	54.7
Average	93.3	57.2
Environmental isolates		
M5A1	92.6	55.6
UW-1	93.6	58.0
V-101b	92.0	54.2
V-104a	92.1	54.4
V-112	93.7	58.2
V-151	93.4	57.4
V-171	93.7	58.2
V-233	93.6	58.0
V-236	91.9	53.9
V-244	92.5	55.4
4069-19	92.4	55.2 ^c
4082-20A	93.2	57.0 ^c
006	93.2	57.0
002	93.5	57.8
051	92.9	56.3
R-302	93.2	57.1
R-304	93.6	58.0
R-306	93.6	58.0
R-307	93.7	58.2
Ka-1	93.5	57.8
ATCC 15574	93.5	57.8
Average	93.1	56.8
Human isolates		
UOMS-1	93.1	56.8
UOMS-5	93.5	57.8
MUSC-1	92.8	56.2
13882	93.3	57.2
141	92.9 ± 0.5	57.7
143	92.9 ± 0.2	57.7
ATCC 13883	93.5	57.8
ATCC 8047	93.1	56.9
Average	93.1	56.9

^a % G + C base composition was calculated using equation 3 of DeLey (9).

^b Results of two or more determinations.

^c Reported in reference 2.

pneumoniae 13883 exhibit a slightly greater extent of relative reassociation. These data are in agreement with other published studies (6).

TABLE 4. Relative reassociation and % G + C of human and environmental *K. pneumoniae* DNA

Origin	Strain	% Relative reassociation	% G+C base composition
Human	ATCC 13883 ^a	100	57.8
Human	13882 ^b	100	57.2
Human	MUSC-2	74	56.2
Human	141 ^b	91	56.3
Human	UOMS-5	98	57.7
Human	UOMS-1	40	56.8
River	006	91	57.0
River	002	81	57.8
River	051	85	56.3
River	R-302	97	57.1
River	R-307	92	58.2
Mushroom	V-104a	23	54.4
Radish	V-236	13	53.9
Green onion	V-244	5	55.4
Potato	V-112	70	58.2
Radish leaves	V-233	55	55.2
Carrot seeds	V-151	26	57.4
Turnip seeds	V-171	48	58.2
<i>Psychotria</i> leaf nodule symbiont	ATCC 15574	93	57.8
Pulp mill:			
Raw mill effluent	116 ^b	91	57.0
Primary pond	102	90	57.0
Primary pond	045	90	57.8
Aeration basin	037	87	59.2
Secondary pond	084	73	57.7
Primary pond	094 ^b	41	56.3
Primary pond	012	32	58.4

^a Reference DNA from ATCC 13883 *K. pneumoniae* neotype strain.

^b Percentage of relative DNA reassociation also determined by the membrane filter competition procedure (17).

K. pneumoniae R-307, which shows high relatedness to the reference strain 13883, also exhibits the expected 50% reassociation with *E. aerogenes*. That is, R-307 is just as different from *E. aerogenes* as is 13883. It is more significant that two lower homology vegetable isolates of *K. pneumoniae* (V-104a and V-233) are also less related to *E. aerogenes* and *E. cloacae* than to the 13883 neotype strain. It is clear that such genetically divergent strains are not *Enterobacter* spp. which are masquerading as *K. pneumoniae*.

The low relative reassociation exhibited by the human strain UOMS-1 prompted experiments with environmental isolates also showing low relatedness values to 13883. Three strains of environmental *K. pneumoniae* exhibiting high, intermediate, and low levels of relative reas-

sociation with 13883 were chosen for such a study. Strain V-233 was found to be less related to UOMS-1 than to the neotype *K. pneumoniae* reference strain. Strain R-302 which has 97% relative reassociation with 13883, exhibited the expected 40% value range with UOMS-1. Thus, R-302 and 13883 share a common 40% of the genome with human strain UOMS-1. On the other hand, mushroom isolate V-104a shared more than twice the level of polynucleotide similarity with UOMS-1 than with 13883.

Genome sizes of selected strains were estimated from the renaturation kinetics of DNA (Table 6). The genome sizes of all strains examined were in the range of $2,200 \times 10^6$ to $3,000 \times 10^6$ daltons. This range is typical for species of the *Enterobacteriaceae* (2, 12, 14). In general, *K. pneumoniae* strains with genome sizes similar to 13883, showed the highest relative reassociation values to this human strain. Thus, six out of seven *K. pneumoniae* isolates with $2,200 \times 10^6$ to $2,600 \times 10^6$ daltons genome size exhibited 55% or greater relative reassociation values with 13883. With one exception, strains with larger genomes ($2,700 \times 10^6$ to $3,000 \times 10^6$) and the *Enterobacter* species showed less than 50% relatedness.

DISCUSSION

The biochemical testing of cultural reactions on over 100 *K. pneumoniae* strains illustrated some phenotypic variability existed among the isolates. A variety of indole, methyl red, Voges-Proskauer, and citrate patterns was observed and this variability appears comparable to other studies on *K. pneumoniae* from the environ-

TABLE 5. Relative reassociation values for additional reference strains of DNA

DNA species	Strain	% Relative reassociation
<i>Enterobacter aerogenes</i>	ATCC 13048 ^a	100
<i>E. cloacae</i>	ATCC 13047	33
<i>Klebsiella pneumoniae</i>	ATCC 13883	49
<i>K. pneumoniae</i>	river R-307	52
<i>K. pneumoniae</i>	V-233	18
<i>K. pneumoniae</i>	V-104a	11
<i>E. cloacae</i>	ATCC 13047 ^a	100
<i>K. pneumoniae</i>	V-104a	33
<i>K. pneumoniae</i>	V-233	45
<i>K. pneumoniae</i>	UOMS-1 ^a	100
<i>K. pneumoniae</i>	V-233	29
<i>K. pneumoniae</i>	R-302	43
<i>K. pneumoniae</i>	V-104a	57

^a Reference DNA.

TABLE 6. Genome sizes calculated from renaturation rates

Source of DNA	$C_{0t_{0.5}}$ (mol s per liter)	Calculated genome size ^a ($\times 10^6$ daltons)
<i>Escherichia coli</i>	1.08 ± 0.06 (4)	2,200 ^b
<i>Klebsiella pneumoniae</i> :		
13883	1.11 ± 0.06 (7)	2,300
15574	1.08	2,200
R-301	1.12	2,300
MUSC-2	1.18	2,400
UOMS-5	1.19	2,400
UOMS-1	1.23	2,500
V-233	1.28	2,600
R-302	1.29	2,600
R-303	1.31	2,700
V-104a	1.32	2,700
V-101b	1.36	2,800
V-112	1.39	2,800
V-171	1.47	3,000
<i>E. cloacae</i>		
13047	1.25	2,600
<i>E. aerogenes</i>		
13048	1.28	2,600

^a Corrected for effect of G+C content on renaturation rate constant (26).

^b Value based on two literature reports (12, 26).

ment (1, 7, 10, 24). A few strains representing most ecogroups produced tryptophanase, a trait not classically recognized as being associated with *K. pneumoniae* but acknowledged to be present in some 6% of the more recent clinical isolates (11). As is typical, a variable number of strains fermented dulcitol. Careful analyses of the cultural reactions tested indicated that variable properties in tryptophanase, dulcitol, and indole, methyl red, Voges-Proskauer, citrate patterns were not correlated with habitat of origin nor with DNA reassociation levels. The authors of a recent study on 339 strains of *K. pneumoniae* of clinical origin also recognized cultural variability and subdivided their isolates into eight biotypes on patterns of dulcitol, sorbitol, and tartrate fermentation (25). The most common patterns of dulcitol, sorbose, and d-tartrate fermentations were --- (109 strains), --+ (70 strains) and -+- (52 strains). Unfortunately, the phenotypic biotypes did not correlate with capsule serotyping and no nucleic acid studies were performed.

The DNA thermal melting curves indicated that the average G+C base composition for all *K. pneumoniae* isolates was 57%. This overall average is indistinguishable from the average obtained for each of the four ecogroups. These values are similar to most literature reports

which indicate a G+C range of about 53 to 57% (9, 21, 23). The present overall range of 54 to 59% may be more broad simply because more isolates were examined from diverse origins. The 5 to 6% spread is much broader than the usual 2% range typical of other bacterial species (15). In fact, the G+C base composition spread for each genus of the enteric bacteria is typically about 2% (21). This larger variation in base composition is indicative of a higher degree of molecular heterogeneity among strains of the *K. pneumoniae* species.

The 5 to 100% range observed in the relative DNA reassociation levels among *Klebsiella* clearly confirms the molecular heterogeneity of this group. However, it is most significant that the strains of *K. pneumoniae* examined from rivers, most pulp mill isolates and strain V-112 from potato are only as different from the human reference DNA as are most of the human cultures themselves.

We might speculate that strains having similar genetic composition presumably have the same potential to induce infections as do strains of known human origin. However, we recognize the limitations in the DNA hybridization technique and do not claim the procedure is specifically capable of detecting differences in levels of virulence. We are stating that, in terms of the tests we do comprehend, the cultural reactions, serotyping, and genetic analyses, most environmental isolates are indistinguishable from strains of human origin.

Although the number of strains are limited, the evidence indicates a good correlation exists between the % G+C base composition, DNA homology, and genome size. Cultures with genome sizes and G+C contents most divergent from the reference DNA, also have the lowest homology. This is typified by isolates V-101b and V-104a. Isolates with a base composition similar to the reference strain, but having larger genome sizes (V-112, V-171) also exhibit depressed homology levels. Additional studies are necessary before this apparent anomaly of phenotypic uniformity and genetic heterogeneity can be fully understood. This apparent anomaly is theoretically explainable since the 19 cultural reactions examined represent only 2% or less of the total genes present in a bacterium. In addition, we do not know whether there is amino acid sequence homology in the enzymatic reactions the isolates have in common.

The inference here is that one must consider the possibility it is merely fortuitous that the human and vegetable klebsiellas have many phenotypic properties in common. *Enterobacter* and *Klebsiella* are specific examples of bacteria

which share numerous phenotypic properties but nevertheless, differ in some 60% of the polynucleotide sequences in the genome (6). Unfortunately, many relevant questions still remain concerning the evolutionary relationships of the low homology level klebsiellas. Are they ancestors of human *Klebsiella*? Are the low homology strains closely related to each other or to other already described genera/species of the enteric group? Finally, what are the public health implications of bacteria which are of environmental origin, conform to the cultural definition of *K. pneumoniae*, but are genetically distantly related to the human pathogenic ecotypes? The answers to these and other important questions are currently under study.

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