Epidemiology and Virulence of Klebsiella pneumoniae

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ABSTRACT Strains of Klebsiella pneumoniae are frequently opportunistic pathogens implicated in urinary tract and catheter-associated urinary-tract infections of hospitalized patients and compromised individuals. Infections are particularly difficult to treat since most clinical isolates exhibit resistance to several antibiotics leading to treatment failure and the possibility of systemic dissemination. Infections of medical devices such as urinary catheters is a major site of K. pneumoniae infections and has been suggested to involve the formation of biofilms on these surfaces. Over the last decade there has been an increase in research activity designed to investigate the pathogenesis of K. pneumoniae in the urinary tract. These investigations have begun to define the bacterial factors that contribute to growth and biofilm formation. Several virulence factors have been demonstrated to mediate K. pneumoniae infectivity and include, but are most likely not limited to, adherence factors, capsule production, lipopolysaccharide presence, and siderophore activity. The development of both in vitro and in vivo models of infection will lead to further elucidation of the molecular pathogenesis of K. pneumoniae. As for most opportunistic infections, the role of host factors as well as bacterial traits are crucial in determining the outcome of infections. In addition, multidrug-resistant strains of these bacteria have become a serious problem in the treatment of K. pneumoniae urinary tract infections (UTIs) difficult (1–4). Although not frequently encountered as a cause of community-associated UTIs, K. pneumoniae is a leading cause of enterobacterial nosocomially acquired UTIs and is frequently prevalent as an infectious agent of patients with indwelling urinary catheters (5). These catheter-associated urinary-tract infections (CAUTIs) can be source of invading organisms into the bloodstream among these compromised individuals, leading to serious infections with high morbidity and mortality rates. In addition, the frequency of UTIs in individuals residing in long-term-care facilities is increasing (6, 7). Therefore, the epidemiology of Klebsiella UTIs and CAUTIs indicates that factors that result in a decrease in the efficiency of the host immune system, for example, the insertion of an indwelling device such as a catheter, represents a significant increase in susceptibility to infection by these bacteria (Table 1).

The presence of indwelling urinary devices leads to an accumulation in situ of host-derived material on the catheter surfaces (8, 9). This environment presents an excellent niche for the development of bacterial biofilms, particularly those of opportunistic pathogens such as...
Klebsiella pneumoniae. Biofilm formation plays a key role in many bacterial infections and contributes to the ability of bacteria to overcome host-defense mechanisms (8, 10, 11). CAUTIs are frequently associated with the development of K. pneumoniae biofilms and factors that contribute to this process are likely to play an important role in pathogenesis (8, 12, 13). The development of techniques to measure and quantitate bacterial biofilm formation has facilitated the analysis of K. pneumoniae biofilm formation on solid surfaces and this, in addition to historically older investigations into Klebsiella virulence, will hopefully lead to better management of UTIs by these bacteria. Clearly, the isolation of the so-called “superbugs” that exhibit resistance to many recently developed antibiotics necessitates consideration of non-antibiotic-associated treatments of both UTI- and non-UTI-associated K. pneumoniae infections. The major virulence factors and properties of K. pneumoniae are discussed below as well as biofilm formation and antibiotic resistance.

As for any opportunist, the outcome of infection in the urinary tract by K. pneumoniae is a function of the bacterial ability to resist clearance and/or killing by host-defense mechanisms. The bacteria causing these types of infection commonly do not produce single virulence factors that can be identified as a primary attribute but rely on a battery of gene products to facilitate successful colonization and growth in a host where the immune system may not be fully functional and damaged or impaired (Table 2). Colonization of the urinary tract may be asymptomatic or may progress to clinical disease. In contrast, relatively recent reports of K. pneumoniae isolates causing acute pyogenic and disseminated infections are associated with the production of a distinct hypermucoviscous phenotype and are most commonly associated with one capsular type of bacterium (14–16). The virulence factors of these strains will not be the focus of this chapter since they do not represent the typical K. pneumoniae isolates associated with UTIs. However, a comparison between the pyogenic strains and those causing UTIs will be made when the two groups share a common property. A summary of the virulence properties of K. pneumoniae associated with UTIs is shown in Table 3.

### Table 1: Incidence of UTIs caused by Klebsiella pneumoniae

<table>
<thead>
<tr>
<th>UTI type</th>
<th>Occurrence</th>
<th>Reference</th>
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<tbody>
<tr>
<td>ICU-acquired UTIs</td>
<td>4%</td>
<td>157</td>
</tr>
<tr>
<td>Community-acquired UTI w/ catheter</td>
<td>8%</td>
<td>158</td>
</tr>
<tr>
<td>Nosocomial catheter-associated UTI</td>
<td>10% to 11%</td>
<td>159</td>
</tr>
<tr>
<td>Community-acquired UTI</td>
<td>8.7% to 16.9%</td>
<td>52, 55, 160, 161</td>
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#### TABLE 2: Risk factors associated with Klebsiella pneumoniae UTI

<table>
<thead>
<tr>
<th>Risk Factor</th>
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<tbody>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Urinary-tract obstruction</td>
</tr>
<tr>
<td>Chronic renal insufficiency</td>
</tr>
<tr>
<td>Immunosuppression</td>
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<tr>
<td>Catheterization</td>
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#### TABLE 3: Summary of virulence factors involved in Klebsiella pneumoniae pathogenesis

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Role in pathogenesis</th>
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<tbody>
<tr>
<td>Capsule</td>
<td>Inhibit and evade phagocytosis by host cells, induces dendritic cell maturation, neutralizes antibacterial activity of host defense</td>
</tr>
<tr>
<td>LPS</td>
<td>O antigen provides serum resistance</td>
</tr>
<tr>
<td>Siderophore</td>
<td>Scavenge essential iron for survival, hypermucoviscous phenotypes have been linked to increased iron-binding activity</td>
</tr>
<tr>
<td>Urease</td>
<td>Limited role in precipitation of inorganic salts leading to catheter encrustation</td>
</tr>
<tr>
<td>Type 1 fimbriae</td>
<td>Involved in the formation of intracellular bacterial communities</td>
</tr>
<tr>
<td>Type 3 fimbriae</td>
<td>Important for biofilm formation on biotic and abiotic surfaces, role in biofilm formation on urinary catheters in vivo remains to be elucidated</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>Formation promotes resistance to host killing and antimicrobials, experimentally shown to be facilitated in part by fimbriae and capsule</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td>Carbapenem-resistance prevents many treatment options</td>
</tr>
</tbody>
</table>
but represented only approximately 13% of the isolates examined, with a broader range of serotypes being implicated in these types of infections (17). The relatively broad distribution of capsular types associated with K. pneumoniae UTIs was also reported in a study of 32 strains (18). Therefore, unlike the stains causing disseminated pyogenic infections and liver abscesses, the strains of K. pneumoniae implicated in UTIs represent a more diverse range of bacteria.

Biochemical analyses of K. pneumoniae capsules indicate that they are composed of complex polysaccharides consisting of repeating subunits. For example, the K21a, K36, and K50 serotypes possess di-mannose/rhamnose residues, whereas K2, K8, and K55 serotypes do not (19). The K53 capsule is comprised of D-glucuronic acid, D-galactose, D-mannose, and L-rhamnose and is similar to that found in the K74 serotypes that lack rhamnose (20, 21). The K60 capsule has been reported to be unusual in possessing three glucopyranosyl side chains, compared to one or two on most K-antigens, within a heptasaccharide-repeating subunit (22). The K1 capsule from isolates responsible for disseminated infections possess fucose and this is not found in non-K1 isolates (23). The association of the capsule with the lipopolysaccharide (LPS) has been investigated in a K2 serotype in which the capsule is bound by an ionic interaction to the LPS through a negative charge of carboxyl groups on GalA (24). Indeed, it has been reported that an enzyme playing an important role in LPS biosynthesis influences the amount of cell-bound capsule present on K. pneumoniae (25). These results indicate the interrelationship at the biochemical and synthetic level between these two structures in Klebsiella strains. It is clear that the K. pneumoniae LPS and capsule are not produced completely independently of each other and the formation of one of these structures can impact the amount and presence of the other.

Compared to E. coli and Salmonella enterica, the analysis of K. pneumoniae O-antigens associated with the LPS has been limited. Nine O-antigenic types have been described, with O1 being the most common serogroup associated with human infections (19, 26). The antigenic specificity may be a function of the modification of a single repeat subunit. For example, the O-antigenic side chain of serotype O2a is a polymer of the disaccharide D-galactan subunit. In other serotypes of O-antigens this subunit may be O-acetylated or capped by different forms of the repeat subunit.

Genetics of Capsule Production
The genes encoding the production of capsules by K. pneumoniae were originally designated the cps gene cluster and were mapped close to his on the bacterial chromosome (27). Cloning of the cps cluster indicated that the genes occupied approximately 15kb of DNA and possessed all the determinants necessary to impart the serotype of a strain (26, 28). Expression of the cps genes was originally described to be under the control of RcsB, a protein involved in colonic acid biosynthesis (29), and RcsB enhanced transcription of the cloned cps genes in an E. coli background. Subsequently, three alleles of a different gene encoding RmpA/A2 have been described that also positively activate cps-gene expression (30–32). The distribution of these alleles varies between serotypes and the geographic region of the isolates; two of the alleles are plasmid encoded and the third is a chromosomal gene. The presence of a DNA-binding domain in the C-terminal region of RmpA has led to the suggestion that it activates transcription of cps genes by binding to the promoter region of these genes. Excess RmpA production leads to a hypermucous phenotype in K. pneumoniae and transcriptional fusions of cps-promoter regions are increased in expression in the presence of RmpA (32).

K. pneumoniae grown under iron-replete conditions exhibit a decreased production of capsular polysaccharide. The Fur protein has been shown to inhibit transcription of rmpA as well as other genes associated with iron-acquisition systems in K. pneumoniae (33). Therefore, as in other enterobacterial systems, the concentration of available iron in the environment of the Klebsiella strains can play an important role in virulence-gene expression, including the amount of capsule produced by clinical isolates. The role of iron in affecting K. pneumoniae virulence is discussed below.

As indicated above, K1 serotypes of K. pneumoniae are associated with causing severe disseminated infections. The mucoviscosity-associated gene (magA) was identified to be part of the cps-gene cluster in these isolates and encodes a polymerase necessary for K1 biosynthesis (34). This gene has been used to identify, by polymerase chain reaction (PCR), K1-positive strains, but recent evidence indicates that a similar allele is present in other K-serotypes. The allele specific for the K1 serotype has recently been renamed wyz_K1 and the indication is that wyz alleles may be serotype-specific but functionally identical.

Role of Capsules in Virulence
The ability of specific K. pneumoniae capsules to inhibit or impair phagocytosis by host cells was reported over 20 years ago (35–39). Subsequently, it was demonstrated that capsules possessing the Man-\(\alpha\)-2-Man
sequence exhibited a greater binding and susceptibility to phagocytic cells, a phenomenon termed lectinophagocytosis (40). The K2 serotype does not possess the Man-α-2-Man disaccharide and is more frequently associated with human infections compared to serotypes that do possess this structure (e.g., K21a), indicating that, in part, the resistance to phagocytosis by some serotypes may be a function of decreased binding to these host cells. Simply exchanging the K21a cps genes for those from a K2 isolate, however, was not sufficient to restore full virulence to a strain and demonstrated that additional virulence factors must play a role during infection (26). Not only may the capsule influence susceptibility to phagocytosis by direct binding to these cells, but it has also been demonstrated that the composition of the capsule influences opsonophagocytosis. Serotypes or genetically engineered strains producing capsules possessing the di-Man/Rha epitope exhibited a decreased ability to stimulate polymorphonuclear leukocytes (PMNs). These epitopes were not recognized by many components of the innate immune system, such as lung-surfactant proteins, mannose-binding lectins, and alternative complement activation constituents, in addition to the mannose-receptor of phagocytic cells (19). This has led to the suggestion that virulent serotypes such as K2 escape eradication by producing a surface capsule comprised of glycopeptidomannans not recognized by these factors. Overall, strains of K. pneumoniae can be effectively killed by host phagocytic cells once internalized into these cells. Therefore, mechanisms by which the capsule can effectively inhibit interaction of the bacteria with these cells will facilitate survival.

In addition to the role of capsules in the interaction between K. pneumoniae and macrophages or PMNs, recent evidence indicates that the capsules affect interaction with dendritic cells (41). In these studies, capsular material was shown to induce dendritic-cell maturation with increased expression of the markers CD83, CD86, and TLR4 but decreased production of CD14. The results indicate that K. pneumoniae capsular material induces a defective immunological response characterized by dendritic-cell maturation with increased pro-Th1 cytokine production. In these studies it could also be demonstrated that although LPS played no role in dendritic cell maturation, the LPS played a role in dendritic-cell activation. Very little is known about the interaction of K. pneumoniae with these important antigen-presenting cells and further studies may shed light on possible mechanisms that enable the bacteria to avoid recognition and processing by dendritic cells.

The role of K. pneumoniae capsule in experimental UTIs indicates that this structure plays a significant role in this type of infection (42). Using competitive-infection studies and non-capsulate variants in addition to the parental strains, it was possible to demonstrate that the capsulate strains outgrew the variants in vivo. However, no such advantage was observed when colonization of the murine gastrointestinal tract was monitored. In contrast, the non-capsulate variants were able to adhere in vitro to epithelial-cell lines better than capsulate strains, but this increase in adherence did not extrapolate to increased infectivity in vivo. Increased adherence in vitro by non-capsulate strains may be due to unmasking of surface-associated adherence factors that are covered by capsule in wild-type strains during growth under these conditions.

Antimicrobial peptides are predicted to play an important role against infection, particularly against mucosal pathogens, such as K. pneumoniae. Most of these peptides are cationic and the role of anionic capsules in neutralizing their affect has been investigated (43). One host defensin has been shown to stimulate the release of K2-capsular material from bacterial cells. The release of this anionic capsule from the bacterial cell in vivo has been postulated to neutralize the anti-bacterial activity of a host defensin by preventing the peptide from localizing to the bacterial membrane. Considering that K. pneumoniae is an opportunistic that can colonize mucosal surfaces of a variety of organ systems, the ability of these bacteria to evade innate defense mechanisms represents one of the primary and early mechanisms in the establishment of infection.

Overall, the most virulent strains of K. pneumoniae produce a capsule composed of saccharides that do not facilitate binding to phagocytic cells and therefore are more resistant to phagocytosis. In addition, these capsules also present as poor opsonins and evade phagocytosis by complement activation. Since there are a greater variety of capsular serotypes implicated in UTIs compared to disseminating K. pneumoniae infections, the role of capsules in mucosal infections is less clear and a few studies of UTI-causing strains have indicated that serotypes from a wide variety of capsular types are found. The role of these capsules in protecting against innate defense mechanisms in the urinary tract has not been investigated in detail, but these structures could play a role in neutralizing host factors in this environment.

**SIDEROPHORES**

**Siderophore Production**

K. pneumoniae must compete with host cells in vivo for essential iron that is a necessary cofactor for bacterial
metabolism. In a study of more than thirty strains representing 23 different serotypes of \textit{K. pneumoniae} implicated in urinary tract infections, the siderophore enterochelin was the predominant iron-salvaging-system compound produced \cite{18}. This is in contrast to bacteremic strains, primarily K1 and K2 serotypes, in which aerobactin was correlated with virulence in an experimental murine model of infection \cite{44}. Enterochelin is a catechol-type of molecule comprised of three phenolic rings that are involved in the uptake of ferric ions \cite{45}. The ferrienterochelin complex is bound to an 81-kDa outer-membrane receptor that is found in most strains of both \textit{K. pneumoniae} and \textit{E. coli}. Aerobactin is a hydroxamate compound comprised of a citrate molecule with N6-hydroxyacetyl lysine and is derived by the oxidation of lysine.

Transport of ferric iron into the bacterial cells is dependent upon a family of proteins that includes TonB, Exb, and ATP-binding cassette (ABC) transporters \cite{46}. The iron-siderophore complex is recognized by a TonB-dependent outer-membrane receptor that mediates transport into the periplasm. Subsequently, the siderophore binds a periplasmic protein that facilitates transport to an ABC-transporter that mediates passage of iron through the inner membrane prior to reduction from ferric to ferrous iron. Although the role of iron-chelating systems in uropathogenic \textit{K. pneumoniae} has not been experimentally examined in detail, both airway-infection models and bacteremic studies have suggested that mutants lacking the ability to take up iron are attenuated.

In addition to the two described siderophores aerobactin and enterochelin, it is possible that highly invasive strains of \textit{K. pneumoniae} possess multiple iron-acquisition systems. Using experimental infection models it has been demonstrated that invasive strains express genes that encode proteins related to iron-binding molecules from other bacterial species and the relationship between these systems has yet to be examined \cite{47, 48}. It appears that the more invasive that some strains of \textit{K. pneumoniae} have evolved to become, the greater the necessity to efficiently compete with the host for iron. This may have led to the evolution of a complexity of iron acquisition systems by these bacteria.

**Genetics of Iron-Acquisition Systems**

The ferric-uptake regulator (Fur) is a principal regulatory protein controlling gene expression in response to iron concentrations. In the presence of excess iron, i.e., iron-replete conditions, this protein can bind to specific DNA sequences (the Fur box) in promoter regions of iron-regulated genes. This binding results in repression of target-gene transcription and derepression under iron-depleted conditions. Fur is, therefore, a global regulator of iron-regulated genes in enterobacteria. For \textit{K. pneumoniae} Fur has been shown to affect the expression of genes encoding capsule biosynthesis and iron-acquisition systems. For example the regulatory genes, \textit{rpmA2} and \textit{rcsA}, are themselves regulated by Fur in addition to at least six of eight genes involved in iron uptake \cite{49}. Fur-deletion mutants of \textit{K. pneumoniae} exhibit reduced siderophore production as well as changes in adhesin production \cite{50}. Consequently, Fur can have multiple effects on gene expression in \textit{K. pneumoniae} and many of these genes encode products involved in colonization and growth in the host.

Genome analysis of available \textit{K. pneumoniae} sequences indicates the presence of 10 putative iron-uptake systems in some strains. Many (at least seven) of these systems exhibit properties associated with a dependency on the TonB machinery for their complete function. Although the precise function of each of these systems has yet to be elucidated, the presence of multiple systems in \textit{K. pneumoniae} indicates that the uptake of iron from a limiting environment is an important and essential function to maintain the viability of the bacteria.

**Role of Siderophore and Iron-Uptake Systems in Virulence**

Growth of \textit{K. pneumoniae} \textit{in vivo} requires the capture and utilization of iron for essential metabolic processes. Since iron is also required by host cells, in order to survive the bacteria must compete with the host for available iron that is frequently in limiting supplies. Host iron-binding compounds, such as transferrin and lactoferrin, are strong chelators of elemental iron and \textit{K. pneumoniae} has evolved to produce its own iron-binding systems to scavenge the necessary iron required for its survival. These iron-acquisition systems are described above. The importance of iron availability and \textit{K. pneumoniae} pathogenesis was suggested by the early studies of Khimji and Miles \cite{51} who demonstrated that excess iron in hosts’ tissues increased bacterial pathogenicity.

The \textit{K. pneumoniae} TonB system has been shown to play an important contribution in bacterial virulence. Using murine experimental models of infection, it has been demonstrated that a TonB mutant is significantly attenuated \cite{47}. In addition, this mutant could be used...
as a vaccine strain to prevent experimental infections by the parental virulent strain due to stimulation of a protective immune response in vaccinated animals. In contrast, individual mutations in genes involved in the iron-transport systems and siderophore production did not appear to have any effect on K. pneumoniae pathogenesis. However, a triple mutant, deleted in the irp2, iuc, and iroA genes exhibited decreased virulence (47). No studies have yet examined the role of these systems in UTIs caused by K. pneumoniae.

Recently, the isolation of hypervirulent strains of K. pneumoniae exhibiting a hypermucoviscous phenotype has been described (53). Investigations of virulence factors of these strains indicated that a small molecule (approximately 3kD) was produced that enabled the strains to grow and survive in human ascites. In addition, this molecule increased the growth of K. pneumoniae in vivo following subcutaneous inoculation of mice. Preliminary evidence indicates that this molecule is involved in iron uptake and is a siderophore. It is speculated that the enhanced virulence of these strains may, in part, be a function of increased siderophore production as well as an increased iron-binding activity compared to less virulent K. pneumoniae isolates (53).

**UREASE**

**Genetics and Structure**

Many strains of Klebsiella produce the extracellular enzyme urease that is a nickel-containing enzyme responsible for hydrolyzing urea to ammonia and carbamoyl (54). The molecular genetics of urease production and the structure of the enzyme have been described (54, 56). The enzyme is assembled by the interaction of four accessory proteins (UreD, UreE, UreF, and UreG) involved in delivering nickel to the urease apoprotein and one of these components (UreG) serves as a GTPase for activation. The molecular biology of urease production and activity have been described in a series of studies by Hausinger and colleagues (54, 56–60).

**Role of Urease in Virulence**

The significance of urease as a virulence factor has been suggested for bacteria that grow in the urinary tract and contribute to the formation of infection stones. Urea hydrolysis is proposed to lead to an increase in the localized pH resulting in precipitation of inorganic salts that are insoluble at a relatively high pH (pH 9.0). This precipitation leads to encrustation, particularly on indwelling urinary catheters that may influence the course of infection. First, urine flow is impaired under these conditions, leading to a deficiency in bacterial clearance from the site of infection. Second, the encrustations can facilitate the formation of biofilms on biotic or abiotic surfaces that themselves may inhibit effective treatment by antibiotics.

The role of urease production by K. pneumoniae in virulence and mediating the pathogenesis of the organisms, however, has yet to be demonstrated. Compared to species of Proteus, K. pneumoniae produces significantly less urease in vitro. In addition, it has been demonstrated that bacterially induced precipitation of salts onto abiotic surfaces in vitro by K. pneumoniae is less than that observed by urease-producing strains of Proteus (61, 62). More recently, these results were confirmed by Broomfield and coworkers (63) who demonstrated that stains of Klebsiella were less efficient at forming encrustations and subsequently blocking catheters in a laboratory model of infection, compared to strains of Proteus and Providencia. Direct experiments to investigate the role of urease production by K. pneumoniae in vivo using an animal model of infection have not yet been performed. However, the evidence to date indicates that this enzyme may only have a limited role in virulence by these bacteria.

**COLONIZATION AND ADHERENCE**

As for many of members of the Enterobacteriaceae, a review of the available on-line genome sequences for strains of K. pneumoniae indicates the presence of multiple gene clusters that potentially encode fimbrial- and non-fimbrial-adherence antigens. The presence of these multiple-gene clusters appears to be the norm for most enteric bacteria, although investigations into the function of most of these genes and their products have been restricted to a small number of factors. For example, there are up to eleven putative fimbrial-gene clusters located on the K. pneumoniae genome, but only a few of these have been associated with specific adherence phenotypes (Fig. 1). Many of the gene clusters are not readily expressed under laboratory conditions of growth. However, it is likely that all of these systems encode functional adherence factors since their presence and expression represents a significant metabolic load to the bacterial cell. Most of the investigations into the role of K. pneumoniae adhesins that play a role in colonization of the urinary tract have focused upon two fimbrial types, type 1 and type 3 fimbriae, which are produced by many urinary isolates of K. pneumoniae (Fig. 1).
Structure and Genetics of Fimbriae

All of the fimbriae that are produced by strains of *K. pneumoniae* are assembled by the chaperone/usher-assembled pathway and no adhesins similar to type IV pili of other bacteria have been described in *Klebsiella* (64–69). The fimbriae assembled by the chaperone/usher pathway are characterized by the presence of at least two genes that encode a periplasmic chaperone and a scaffolding protein necessary for ordered fimbrial assembly. Some gene clusters possess more than one gene encoding a periplasmic chaperone, but all the clusters are characterized by having a single gene encoding the scaffolding or usher protein. These proteins facilitate assembly of a functional fimbrial structure by the processes of donor-strand complementation and donor-strand exchange (64, 66). The structural subunits possess an incomplete immunoglobulin-like groove that is completed by a strand of the chaperone protein (donor-strand complementation). At the usher protein in the outer membrane, that strand is exchanged by a region from the most recently incorporated subunit (donor-strand exchange). The fimbrial appendages are composed of a major fimbrial subunit, adaptor molecules, and a specific adhesin, and the number of adaptor molecules associated with any particular fimbrial type is variable. In *K. pneumoniae*, each of the gene clusters encoding these components of the fimbrial-adherence factors are unlinked, but each cluster exists as a contiguous set of genes. There is no evidence to indicate that the individual genes, and their products, are interchangeable, and so the chaperone and usher molecules of one system are not functional in assembling the appendage of a heterologous fimbrial system. Similarly, the adhesin protein of one type of fimbria cannot be assembled onto the shaft of a different fimbrial type.

Given these observations and also the presence of multiple fimbrial-gene clusters in *K. pneumoniae*, the control of fimbrial production, localization, and assembly must represent a complex regulatory pathway. It has been suggested that the ability of enteric pathogens to colonize different ecological niches within the urinary tract, for example, may be a function of temporal expression of gene clusters encoding adherence factors facilitating attachment to different types of cells encountered throughout the organism system (70–73).

The three *K. pneumoniae* fimbrial adhesins that have been described in any detail are types 1 and 3 fimbriae and the Kpc fimbriae (65, 74) (Fig. 1). Type 1 fimbriae, or mannose-sensitive fimbriae, so-called because of their ability to bind soluble mannose as a competitive inhibitor to binding, are encoded by the fim gene cluster. This fimbrial type is produced by many members of the *Enterobacteriaceae* and yet, although characterized by the presence of functionally related proteins (e.g., periplasmic chaperone, scaffolding protein, adhesin, major structural protein, etc.), there is little amino acid-sequence relatedness between species (75–79). For example, the genes encoding the type 1 fimbriae of *Salmonella* cannot complement those encoding the same fimbrial type from *K. pneumoniae* (79). The regulation of the *K. pneumoniae* fim gene expression is controlled using an invertible DNA element (fimS) that facilitates transcription of the fim operon by a mechanism similar to that described in *E. coli* (65, 80, 81). In one orientation (phase “ON”) the promoter enabling fim-gene expression allows transcription of the operon, whereas in the opposite orientation (phase “OFF”), transcription of the fim genes cannot occur. However, in addition the *K. pneumoniae* fim-gene cluster possesses a gene, fimK, not present in the *E. coli* cluster (82). This gene encodes...
a putative phosphodiesterase that may modulate the intracellular levels of cyclic-dimeric-guanosine monophosphate (c-di-GMP) that is an important bacterial second messenger (83–85). As indicated below, the intracellular concentrations of c-di-GMP has been shown to effect many bacterial attributes including virulence-factor production.

Type 3 fimbriae are encoded by the *mrk*-gene cluster and may be present in *K. pneumoniae* as a plasmid-borne and/or a chromosomally borne gene cluster (86–88). MrkA is the major structural component of the fimbrial shaft and MrkD facilitates binding to extracellular-matrix proteins and functions as the fimbrial adhesin. Structural stability of the fimbriae is associated with β strands in the C-terminal region of MrkA (89). Regulation of *mrk*-gene expression is achieved using a combination of a phosphodiesterase (MrkJ) and a c-di-GMP-binding protein (MrkH) in conjunction with a DNA-binding protein (MrkI). These gene products are suggested to influence and respond to the intracellular concentration of c-di-GMP that modulates the level of *mrk* gene expression (90–93). The level of c-di-GMP within bacterial cells has been demonstrated to influence the expression of numerous genes and plays an important role in virulence-factor production (84, 85, 94).

The Kpc fimbriae are most frequently associated with K1-positive strains of *K. pneumoniae* causing disseminated pyogenic infections (95). The production of these fimbriae is mediated by the *kpcABCD* gene cluster, although the conditions for optimal expression of these genes has yet to be elucidated. Preliminary investigations indicate that *kpc*-gene expression is mediated, in part, by the orientation of an invertible DNA element within the gene cluster. Like the *fimS* switch of *E. coli*, it is possible that this mechanism is responsible for a phase-variable phenotype of Kpc fimbriae in *K. pneumoniae*.

**Role of Fimbriae in Colonization and Virulence**

The role of type 1 and type 3 fimbriae in colonization and pathogenesis of urinary isolates of *K. pneumoniae* has been investigated. Since early studies had indicated that *E. coli* type 1 fimbriae facilitated colonization and intracellular survival within the urinary tract of experimentally infected animals, the role of *K. pneumoniae* type 1 fimbria in uropathogenesis was examined (82, 96–98). The ability of type 1 fimbriae to mediate *in vivo* the formation of *K. pneumoniae* intracellular-bacterial communities (IBCs) within bladder-epithelial cells was compared to that of *E. coli* fimbriae. *E. coli* IBC formation is proposed to be a strategy whereby uropathogenic *E. coli* evade host innate immune mechanisms and subsequently migrate through the urinary tract (99, 100). Bacteria growing as IBCs exhibit properties similar to those growing as a biofilm community. Although *K. pneumoniae* did form IBCs, their frequency compared to those produced by *E. coli* was considerably less and this correlated with a decreased production of fimbriae *in vivo* (82). The reason for a decreased production of type 1 fimbriae in the murine model of infection by *K. pneumoniae* is unclear. *E. coli* type 1 fimbriae are less frequently produced in the kidney compared to the bladder and it has been suggested that *Klebsiella* has evolved to more efficiently colonize different regions of the urinary tract. However, the results investigating *K. pneumoniae* pathogenesis do indicate that IBC formation may be a general strategy among enteric pathogens implicated in UTIs.

Type 3 fimbriae have been shown to play a major role in the formation of *K. pneumoniae* biofilms *in vitro* on both abiotic and biotic surfaces (101–104). The MrkD adhesin facilitates binding to extracellular-matrix proteins, such as collagen, and also mediates binding to cells derived for the urinary tract (105, 106). Biofilm formation on abiotic surfaces such as plastic and silicon is mediated by the major structural fimbrial protein MrkA and can occur independently of MrkD (102). However, indwelling devices, such as urinary catheters, are coated *in situ* over time with host-derived factors and thus are more likely to represent a substrate for bacterial-biofilm formation that is mediated by MrkD binding (107). Examination of multiple clinical isolates of *K. pneumoniae* have demonstrated that the surface production of type 3 fimbriae was a significant indicator of a strain’s ability to adhere to and form a biofilm on solid surfaces (108). Type 3 fimbriae are produced by several different genera of enterobacteria (109). Given the role of type 3 fimbriae in adherence to host-derived matrices, it has been speculated that type 3 fimbria-producing bacteria may be selected during growth on indwelling urinary catheters (110).

Evaluation of the contribution of both type 1 and type 3 fimbriae in biofilm formation has suggested differing results. In some studies, it has been demonstrated that strains unable to produce type 1 fimbriae are as proficient at forming biofilm as bacteria that can produce this type of fimbria, indicating that type 1 fimbriae play a minimal role. Also, it has been suggested that type 1-fimbrial expression is decreased during biofilm formation. In addition, several investigations have shown that type 3 fimbriae are essential for typical biofilm...
formation (91, 103). However, more recent investigations have demonstrated that both fimbral types play a role in biofilm formation (104). One probable reason for the observed differences is the method of assessing biofilm formation in vitro among the investigations and also the nature of the substrate being used to examine biofilm formation. *K. pneumoniae* biofilm formation in vivo on urinary catheters is most likely to be an important stage in the pathogenesis of these organisms. However, there is currently no available animal infection-model system to mimic these conditions. Currently, we are developing a murine-infection model with silicone tubing implanted in the bladder to investigate the role of fimbral adhesins in vivo.

As indicated above, urinary isolates of *K. pneumoniae* have the genetic capability of producing an array of adherence factors. The role of these factors in colonization and virulence has not been investigated and the difficulty of expressing these genes in vitro has made assessment of their function difficult. It is possible that many of these genes clusters are expressed only under conditions that are found in vivo. Therefore, the development of in vivo infection models along with increased assays to measure gene transcription under these conditions may provide mechanisms to examine the kinetics of fimbral-gene expression in vivo. Examination of the transcriptome of *E. coli* during infection in vivo has recently been described and the application of these techniques to growth of *K. pneumoniae* in experimentally infected animals should be informative, and may address the question of multiple fimbral-gene expression (111, 112).

**BIOFILM FORMATION**

*K. pneumoniae*-biofilm formation has been postulated to be an important stage in the pathogenesis of these bacteria, particularly in the case of CAUTIs. Investigations into bacterial-biofilm formation on solid surfaces has been a major focus of research into bacteria pathogens over the last decade (111, 112, 113–115). Biofilm formation is a complex process that has been divided into a number of stages involving attachment, microcolony production, mature-biofilm formation, and release of free-living planktonic bacteria from the biofilm. The formation of biofilms is believed to be a mechanism of promoting persistence since bacteria within a biofilm are proposed to be less susceptible to killing by host-defense mechanisms. Also, there is evidence that the biofilm community is more resistant to the action of many antibiotics and exhibits an increased resistance to antimicrobials. The mechanisms by which growth as a biofilm enables increased resistance can be attributed to several factors. The biofilm matrix that frequently consists of a dense matrix of proteins, polysaccharides, and DNA prevents the efficient diffusion of antibiotics resulting in significantly decreased exposure of bacteria. However, for some antibiotics, the rate of diffusion through a biofilm may not be impaired but the breakdown of these antimicrobials may be enhanced within the biofilm (116). Bacteria growing deep within biofilms grow at a much slower rate than planktonic bacteria or those close to the surface of the biofilm, again making the biofilm bacteria less susceptible to killing by antibiotics that act upon actively dividing bacteria. Also, for these slow-growing bacteria, specific antimicrobial-binding proteins are poorly expressed. Changes in gene expression by bacteria growing as a biofilm compared to free-living organisms may also lead to resistance to killing. Also, transfer of genetic material between bacterial cells can be enhanced in a biofilm, thus facilitating transfer of material conferring antibiotic resistance (10, 117–119). For all these reasons, the benefit of bacterial-biofilm formation provides an important attribute for increasing the persistence and establishment of chronic infections in the urinary tract.

The role of *K. pneumoniae* fimbral adhesins in the initial stages of biofilm formation has been discussed in the preceding section and will not be considered here. However, as mentioned above, biofilm formation involves a complex series of stages and developmental processes that require differential gene expression and the production of specific gene products at defined stages. For this reason, many gene products are likely to be involved in biofilm formation and their production may also be transitory during the process. For *K. pneumoniae*, the important contributing factors to biofilm formation have only recently been explored and relatively little is known about the array of gene products that are required for this process. Most of this early work has focused upon constructing libraries of defined insertion mutants of *K. pneumoniae* and identifying from these libraries specific genes that play a role in facilitating biofilm formation. Alternatively, recombinant-plasmid libraries comprised of *K. pneumoniae* DNA segments have been used to transform *E. coli* strains to detect genes that increase biofilm formation in specific transformants.

Signature-tagged mutagenesis (STM) has been used to identify genes that influence biofilm formation in vitro on extracellular-matrix material using a continuous-flow biofilm chamber (120). Using this procedure,
mutations in three different groups of genes that resulted in decreased biofilm formation were identified. These groups were genes encoding transcriptional regulators (e.g., LuxR-, LysR-, and CRP-related genes), sugar phosphotransferases, and genes involved in the synthesis of extracellular structures such as fimbriae and capsule. LysR-related transcriptional regulators affect a diverse group of genes and functions and have been reported to influence biofilm formation in other pathogenic bacteria. More recently, OxyR, a LysR-related regulator, has been shown to influence K. pneumoniae colonization of both mucosal and abiotic surfaces (121). Often the genes encoding LysR regulators are located adjacent to and inversely transcribed from the target genes that they regulate. However, further analyses of the genes surrounding this group of mutants were not performed.

LuxR-like transcriptional regulators are frequently involved in affecting the genes involved in quorum sensing. They have been implicated in regulation of biofilm formation for a variety of organisms and members of this family usually respond to cell density by binding to autoinducers (122). The LuxR-like homolog identified in the STM studies did possess a putative DNA-binding domain, but lacked a conserved autoinducer-binding region. Therefore, its role in quorum sensing and biofilm formation has not been established. The role of quorum sensing in K. pneumoniae was further investigated by Balestrino and coworkers (123) who demonstrated that autoinducer-1 (AI-1), an acylhomoserine lactone, was not produced by K. pneumoniae, but autoinducers of the class AI-2 were detected in culture supernatants. The gene, luxS, encoding the synthesis of this molecule was able to complement a LuxS-negative strain of E. coli. Comparison of a LuxS K. pneumoniae mutant with its wild-type parent indicated that the mutant was impaired in its initial stages of biofilm formation in a flow chamber. In a second series of studies, it could be shown that LuxS was involved in altering the expression of two genes involved in LPS biosynthesis by K. pneumoniae and this phenotype may be responsible for the observed changes in biofilm formation (124). However, the mutant did not demonstrate any significant differences in its ability to colonize the murine intestine following oral inoculation and the role of quorum sensing in colonization and growth in the urinary tract has not been examined.

As indicated above, the third type of mutation identified using the STM screen was an insertion into a K. pneumoniae open-reading frame that contains a CRP-activation domain. CRP mediates catabolite repression and the role of this specific K. pneumoniae gene in affecting biofilm formation has not been determined. However, the cAMP-CRP system has been shown to influence biofilm formation in other enteric bacteria. In E. coli, cAMP-CRP activates genes that have been shown to mediate biofilm formation (125). In Serratia marcescens, mutations in crp result in a large increase in biofilm formation and this was, in part, due to an increased expression of fimA and production of type 1 fimbriae in this strain (126). The influence of sugars on biofilm formation has also been demonstrated in Vibrio cholerae (127). Therefore, it is likely that the carbohydrate concentration of the environment that K. pneumoniae is growing in will have a significant effect on its ability to form biofilms and persist in the urinary tract. Increase in K. pneumoniae biofilm formation as a response to carbohydrate presence is not necessarily associated with virulence in acute infections. Mutations in K. pneumoniae genes that convert mannose to fucose have been reported to result in increased biofilm formation but decreased virulence in a murine model of sepsis (23). The observation has been made that increased gene expression resulting in efficient production of biofilms by pathogens is often associated with the establishment of chronic infections, whereas it has been reported that these genes are down regulated in acute infections (128).

More recently, a K. pneumoniae-derived clone library was constructed in E. coli to identify genes that played a role in biofilm formation on abiotic surfaces and might be implicated in the colonization of devices such as urinary catheters (129). These results confirmed that type 3 fimbriae production was an important property in mediating biofilm formation by K. pneumoniae. Three clones possessed genes implicated in arabinose or amino-acid biosynthesis and one additional gene encoding a large surface protein that had been implicated in collagen binding was also identified. However, only the genes encoding type 3 fimbriae were subsequently shown to increase biofilm formation on catheters.

With the exception of types 1 and 3 fimbriae, the only other well-defined phenotype that has been examined in relationship to K. pneumoniae biofilm formation is capsule production. For example, mutations within the genes wza and wzc that are involved in capsule biosynthesis resulted in a decreased ability of K. pneumoniae strains to form biofilms. In addition, mutations in the genes treC and sugE that alter both capsule production and affect the mucoviscosity of the bacteria, altered biofilm-forming properties of K. pneumoniae. Although it is unclear if capsule production influences biofilm formation in the urinary tract, these studies clearly
indicated that *K. pneumoniae* biofilm formation is an important bacterial virulence factor in colonization of the gastrointestinal tract (130). In an interesting study, Dzul and colleagues (131) examined the role of *K. pneumoniae*-capsule production in bacterial spacing and interactions during biofilm formation. Their results indicate that the capsular material enables the bacteria to maintain shorter spacing between cells and thus tighter packing within the biofilm. The reason for this phenotype is unclear, but the authors indicate that greater capsule production could inhibit the separation of daughter cells during bacterial division or that the capsule could interfere with other factors, such as fimbriae, that might affect space-filling between cells. Indeed, the interaction of the capsule expression and fimbrial production has been examined in *K. pneumoniae* (132, 133). In these studies it was noted that the production of capsules interfered with adhesin function.

Another factor that influences *K. pneumoniae*-biofilm formation is cellobiose metabolism (134). Using a *K. pneumoniae* isolate causing pyogenic liver abscesses, it was demonstrated that a mutation in *celB* resulting in cellobiose deficiency was significantly reduced in biofilm formation and this phenotype could be restored by complementation using the cloned *celB* gene. In addition, the *celB* mutant was reduced in its ability to colonize the gastrointestinal tract of experimentally infected mice. These experiments provided another indication that a reduced-biofilm phenotype of *K. pneumoniae* correlates with decreased colonization activity in vivo.

It is now becoming apparent that colonization of and subsequent biofilm formation on indwelling urinary catheters by *K. pneumoniae* are important stages in the pathogenesis of the bacteria during CAUTIs. Also, the complexity of biofilm formation and the multiple genes involved in this process makes investigations into the molecular pathogenesis of this process very intriguing. The absence of a good in vivo model system to mimic CAUTI has limited the extrapolations that can be made from studying biofilms in vitro. However, recent developments have led to investigations of bacterial biofilm formation on silicone tubing that has been inserted into the bladders of mice (135). This technique is now being adapted to investigate *K. pneumoniae*-biofilm formation in the urinary tract and may provide validation of many of the results observed using in vitro techniques.

**ANTIBIOTIC RESISTANCE**

The emergence of multidrug-resistant (MDR) *K. pneumoniae* isolates has presented a significant problem in the management and treatment of infection by these bacteria. *K. pneumoniae* infections of the urinary tract are a leading cause of Gram-negative CAUTIs in the hospital environment and long-term healthcare facilities. The carriage of resistance determinants on promiscuous plasmids has enabled the spread of resistance between strains. As an example of this rapid spread, it can be noted that *K. pneumoniae* resistance to the carbapenem family of antibiotics was initially described in the United States in 1997 and periodically thereafter for the next three years (136–138). However, over the last decade carbapenem-resistant strains of *K. pneumoniae* have emerged globally and this resistance has now been reported to be present in more than 33% of the isolates from medical centers in one major population area (139). Horizontal transfer of the plasmid carrying the resistance determinant between *E. coli* and *K. pneumoniae* residing in the gastrointestinal tract has been suggested (140). This transfer represents a major concern since the GI tract of hospitalized patients is frequently colonized with *K. pneumoniae* and represents an important reservoir of infection in this environment. Establishment of this plasmid in the normal flora will facilitate its transfer to strains causing extraintestinal infections.

With the establishment of rapid whole-genome-sequencing technologies it is now possible to compare the genomes of individual bacterial isolates. For *K. pneumoniae*, there are currently available six genome sequences that are accessible on-line, representing at least three different clinical isolates with differing antibiotic susceptibilities (141). A comparison of the genome sequences in these strains indicates that approximately 70% of the proteins that could be encoded in these strains are conserved. This indicates a large degree of plasticity in the *K. pneumoniae* genome, a condition that would be conducive to allowing the spread of antibiotic resistance. Indeed, a comparison of these strains suggested that the acquisition of an MDR phenotype was not clonal but due to the horizontal gene transfer between strains with quite different genetic backgrounds (141). In fact, the presence of plasmids can only partially account for observed differences in antibiotic sensitivities. In *K. pneumoniae*, there are many examples of chromosomally borne two-component regulatory systems that appear to control the production and efficiency of efflux pumps (142–144). The activity of these pumps plays a vital role in determining resistance to a wide range of antimicrobials.

Bioinformatic analysis has also been used to identify possible regulators that control the expression of genes
involved in antibiotic resistance. This approach has been used to characterize an AraC-type regulator, termed RarA, that controls the expression of an operon (oapRxAB) encoding an efflux pump in *K. pneumoniae* (145). Overexpression of RarA results in a MDR phenotype that requires a functional efflux pump. RarA is also predicted to be encoded by the genomes of *Enterobacter* and *Serratia* species. This regulator is another protein that can be added to the family of transcriptional regulators that have been shown to control AcrAB efflux pump activity and include SoxS, RamA, MarA, and Rob.

**DISCUSSION AND FUTURE PERSPECTIVES**

*K. pneumoniae* has become of increasing concern in the clinical environment over the last two decades. The isolation of MDR strains causing nosocomially acquired infections and the relatively rapid spread of this resistance phenotype among strains has made the treatment and management of these types of infections difficult. The most common type of *K. pneumoniae* infection associated with hospitalized patients is CAUTIs. In most cases, removal of the infected device can lead to elimination of the infection, but while the device remains indwelling, there is a risk of more severe and disseminated infections. Many of these patients are immunologically compromised and are, therefore, at an increased risk of morbidity and mortality. If the bacteria migrate from the bladder to deeper tissues and the bloodstream it is possible that antibiotic therapy will not be successful in eradicating the organisms. For this reason, the development of alternative, non-antibiotic-based strategies to prevent urinary infections has been sought. For example, the use of probiotics to prevent *E. coli* UTIs has been investigated (146, 147). In addition, small molecule inhibitors and competitors of essential bacterial metabolic processes are beginning to be evaluated as methods of therapy (148–155). However, to date, no studies have been reported for the use of these molecules to prevent *K. pneumoniae* infections.

In order to fully understand the uropathogenesis of *K. pneumoniae* in CAUTIs it will be necessary to define those bacterial attributes that mediate adherence, colonization, and growth on both uroepithelial cells and catheters residing in the urinary tract. Murine models of experimental enterobacterial UTI have been developed and are widely used to investigate this type of infection, but do not involve the use of implanted devices. To date, there is no reported *in vivo* model to mimic urinary catheterization. Most models have used *in vitro* techniques employing silicone catheters and urine. The development of an animal model with silicone tubing implanted into the bladder has been developed to investigate growth on the tubing by Gram-positive bacteria (135). The adaptation of this model to investigate growth of *K. pneumoniae* on foreign bodies in the urinary tract *in vivo* is currently being examined by our group.

Bacterial growth on urinary catheters has been associated with biofilm formation in this environment. Research into this form of growth by bacterial pathogens has significantly increased over the last decade, and results have indicated that specific gene products can be produced only under these conditions. This is also likely to be the case for growth of *K. pneumoniae* in the urinary tract. Consequently, a more complete understanding of *K. pneumoniae* pathogenesis in CAUTIs will require investigations into this phase of growth. In addition, the insertion of medical devices into humans and animals rapidly results in the coating of these devices by host-derived material. Therefore, the biofilm growth on both abiotic and biotic surfaces should be examined. This is important since it is clear that that bacterial-gene expression during biofilm formation can be influenced by the substrate on which the bacteria are growing (156).

Another factor regarding growth of *K. pneumoniae* in the urinary tract is the ability of the bacteria to internalize within epithelial cells and avoid host defense mechanisms. Originally observed in *E. coli* and referred to as intracellular bacterial communities (IBCs), the bacteria exhibiting this phenotype were observed to demonstrate properties similar to organisms growing as biofilms (99). Uropathogenic *K. pneumoniae* isolates were subsequently shown to form IBCs in experimentally infected animals (82). Consequently, efficient elimination of bacteria from the urinary tract may have to employ strategies that take into consideration this phenomenon.

One troubling aspect of *K. pneumoniae* infections is the emergence of strains causing disseminated pyogenic infections. These isolates were originally described in patients from Asia and associated with diabetic individuals. However, there are increasing reports of such isolates from non-diabetic individuals and a more widespread geographic occurrence. Although these strains are not generally associated with UTIs, they are clearly genetically related bacteria and present the potential to transfer, either directly or indirectly, genetic information into urinary isolates. The evolution of *K. pneumoniae* strains producing factors that mediate rapid dissemina-
tion from the urinary tract and subsequent tissue injury and destruction may be a possibility.

In conclusion, *K. pneumoniae* has become an important pathogen of immunocompromised individuals and presents significant potential problems with respect to treatment and elimination for the host. This is certainly the case for UTIs caused by these bacteria. Challenges that face the future management of these infections include the development of non-antibiotic based therapies since the ability of *K. pneumoniae* to rapidly evolve to antibiotic-resistant strains is alarming. The increased quality of healthcare has resulted in a greater population of susceptible hosts for *K. pneumoniae* infection. The prevention of infection and management of patients with infections will provide enormous challenges in the future.

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REFERENCES


Epidemiology and Virulence of *Klebsiella pneumoniae*


