

Klebsiella pneumoniae Yersiniabactin Promotes Respiratory Tract Infection through Evasion of Lipocalin 2[∇]

Michael A. Bachman,¹ Jennifer E. Oyler,² Samuel H. Burns,² Mélissa Caza,^{3#}
François Lépine,³ Charles M. Dozois,³ and Jeffrey N. Weiser^{2,4*}

Departments of Pathology,¹ Microbiology,² and Pediatrics,⁴ University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, and INRS-Institut Armand-Frappier, Laval, Québec, Canada³

Received 23 March 2011/Returned for modification 19 April 2011/Accepted 5 May 2011

Klebsiella pneumoniae is a pathogen of increasing concern because of multidrug resistance, especially due to *K. pneumoniae* carbapenemases (KPCs). *K. pneumoniae* must acquire iron to replicate, and it utilizes iron-scavenging siderophores, such as enterobactin (Ent). The innate immune protein lipocalin 2 (Lcn2) is able to specifically bind Ent and disrupt iron acquisition. To determine whether *K. pneumoniae* must produce Lcn2-resistant siderophores to cause disease, we examined siderophore production by clinical isolates ($n = 129$) from respiratory, urine, blood, and stool samples and by defined siderophore mutants through genotyping and liquid chromatography-mass spectrometry. Three categories of *K. pneumoniae* isolates were identified: enterobactin positive (Ent⁺) (81%), enterobactin and yersiniabactin positive (Ent⁺ Ybt⁺) (17%), and enterobactin and salmochelin (glycosylated Ent) positive (Ent⁺ gly-Ent⁺) with or without Ybt (2%). Ent⁺ Ybt⁺ strains were significantly overrepresented among respiratory tract isolates ($P = 0.0068$) and β -lactam-resistant isolates ($P = 0.0019$), including the epidemic KPC-producing clone multilocus sequence type 258 (ST258). In *ex vivo* growth assays, gly-Ent but not Ybt allowed evasion of Lcn2 in human serum, whereas siderophores were dispensable for growth in human urine. In a murine pneumonia model, an Ent⁺ strain was an opportunistic pathogen that was completely inhibited by Lcn2 but caused severe, disseminated disease in *Lcn2*^{-/-} mice. In contrast, an Ent⁺ Ybt⁺ strain was a frank respiratory pathogen, causing pneumonia despite Lcn2. However, Lcn2 retained partial protection against dissemination. In summary, Ybt is a virulence factor that is prevalent among KPC-producing *K. pneumoniae* isolates and promotes respiratory tract infections through evasion of Lcn2.

Klebsiella pneumoniae colonizes >75% of hospitalized patients and causes an estimated 8% of all nosocomial infections in the United States (36). A nonmotile, encapsulated member of the *Enterobacteriaceae* family of Gram-negative bacteria, *K. pneumoniae* is a common cause of urinary tract infections and septicemia and the third-most-common bacterial cause of hospital-acquired pneumonia (23).

Antimicrobial resistance to fluoroquinolones, late-generation cephalosporins, and carbapenems among *K. pneumoniae* isolates is increasing rapidly, by >1% per year (23). Carbapenems have been the treatment of last resort against *K. pneumoniae* isolates with extended-spectrum β -lactamases (ESBLs), plasmid-encoded enzymes that inactivate penicillins and cephalosporins (36). However, strains encoding *Klebsiella pneumoniae* carbapenemases (KPCs) have spread throughout the United States and other regions worldwide and are associated with nearly complete antibiotic resistance, a 25 to 60% treatment failure rate, and fatal infections (21). One clone, multilocus sequence type 258 (ST258), accounts for over 70% of the KPC isolates that have been collected by the Centers for Disease Control (24).

Without effective antibiotics, a rapid immune response to *K.*

pneumoniae is critical for host defense. To acquire iron for DNA replication, amino acid synthesis, and electron transport (14), *K. pneumoniae* secretes the iron-scavenging molecule enterobactin (Ent), which has a higher affinity than lactoferrin or transferrin for iron (38). To counteract Ent, neutrophils (25) and mucosal surfaces (13, 34) produce the innate immune protein lipocalin 2 (Lcn2, or neutrophil gelatinase-associated lipocalin [NGAL], siderocalin, 24p3, or uterocalin). Lcn2 binds Ent in a cup-shaped ligand site (10), competes with the bacterial Ent receptor, and is bacteriostatic (20). Lcn2 also stimulates an acute inflammatory response when bound to afferic Ent, which induces the expression of the chemokine interleukin 8 (IL-8) from cultured respiratory cells (35) and promotes neutrophil influx in response to *K. pneumoniae* nasal colonization (2).

To evade Lcn2, some isolates of *K. pneumoniae* produce siderophores to which it cannot bind. Salmochelin is glycosylated Ent (gly-Ent), synthesized by genes encoded within the *iroA* locus and not bound by Lcn2 due to steric hindrance (16, 22). Alternative siderophores, such as yersiniabactin (Ybt) or aerobactin (Aer), are structurally distinct from Ent (28, 30). During nasal colonization, either gly-Ent or Ybt is sufficient to evade Lcn2 and support bacterial growth (2). In a pneumonia model, Ybt is required for maximal *K. pneumoniae* growth and lethality, although the reason for the contribution of Ybt has not been defined (30).

K. pneumoniae colonizes the colon, where Lcn2 is not normally expressed (18), but can cause disease in sites where Lcn2

* Corresponding author. Mailing address: 401A Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104-6076. Phone: (215) 573-3511. Fax: (215) 573-4856. E-mail: weiser@mail.med.upenn.edu.

Present address: Michael Smith Laboratories, 2185 East Mall, The University of British Columbia, Vancouver, BC, V6T 1Z4.

[∇] Published ahead of print on 16 May 2011.

is prevalent. In a human sepsis model, the Lcn2 levels correlate with the degranulation of circulating neutrophils (26). In the respiratory tract, Lcn2 is basally expressed (13, 34) and induced in response to *K. pneumoniae* infection (9). In the urinary tract, Lcn2 is basally produced in the renal tubules and induced by kidney injury (33). To determine whether Lcn2-resistant siderophores are required to cause disease, *K. pneumoniae* isolates from blood, the respiratory tract, urine, and stool were collected and characterized for siderophore genotype and phenotype and for their ability to evade Lcn2 *ex vivo* and *in vivo* in models of infection.

MATERIALS AND METHODS

Bacterial strains and media. KPPR1, a rifampin-resistant derivative of *K. pneumoniae* subsp. *pneumoniae* (ATCC 43816), was used as the wild-type (WT) strain in these studies. The construction of isogenic siderophore mutants with *entB* (strain VK087, referred to hereinafter as KP5), *ybtS* (VK088, referred to hereinafter as KP6), *entB ybtS* (VK089, referred to hereinafter as KP7) (30), *iroA* (KP25), and *iroA ybtS* (KP20) (2) has been previously described. *K. pneumoniae* isolates were prospectively collected without patient identifiers at the Hospital of the University of Pennsylvania (HUP) clinical microbiology laboratory. Isolates from respiratory, urine, and blood samples were identified using a Vitek-2 system (bioMérieux, Durham, NC) and tested for antimicrobial susceptibility using standard methods (11). Screening for ESBLs and KPCs was based on Vitek susceptibility patterns. ESBL carriage was confirmed with double-disk diffusion testing (12); KPC carriage was confirmed with either the modified Hodge test or PCR (31). Stool isolates were identified by conventional microbiological and biochemical methods (37). For comparison of siderophore prevalence among β -lactamase-positive isolates, a curated collection of antibiotic-resistant *K. pneumoniae* isolates from 2007 was examined. All strains were cultivated overnight in Luria-Bertani (LB) medium either at 30°C on agar or at 37°C with shaking in broth. For liquid chromatography coupled to mass spectrometry (LC-MS) analyses, strains were cultivated in M63-glycerol medium at 37°C for 17 h (8). KPPR1 and its derived mutants were grown with rifampin (30 μ g/ml); kanamycin (50 μ g/ml) was added for *iroA* and *iroA ybtS* mutants.

Genotyping of clinical isolates. To detect the presence of siderophore loci among hospital clinical isolates, genotyping was performed by PCR using primers targeting conserved gene sequences as follows: *entB* (forward, 5'-TGAAGACG ATACCGTGCTGGTGGGA, and reverse, 5'-GTCGGCGACAAAGAACGGTT TGAT), *entE* (forward, 5'-GCTGGTGGTTGAACAAAGC, and reverse, 5'-C AATGTCGCGGAGTTTTACA) (30), *ybtS* (forward, 5'-CAAAAATGGGC GGTGGATTC, and reverse, 5'-CCTGACGGAACATAAACGAGCG), *iroN* (forward, 5'-GCATTGGTATTCCAGTTCAGACG, and reverse, 5'-GAAAGG CAACGGTTGTCCAAA), and *iucA* (forward, 5'-GTACATCCGTGGCAGTG GCAG, and reverse, 5'-CAAGCGCGGCATAGCCTTCAT). The cycling parameters for *entB*, *ybtS*, and *iroN* were 94°C for 2 min, 25 cycles of 94°C for 15 s, 59°C for 15 s, and 72°C for 15 s, and one cycle of 72°C for 2 min. The cycling conditions for *entE* and *iucA* included an annealing temperature step-down of 1°C per cycle for the first seven cycles as follows: 94°C for 2 min, 30 cycles of 94°C for 15 s, 63°C to 56°C (*entE*) or 69.6°C to 62.6°C (*iucA*) for 15 s, and 72°C for 15 s, and one cycle of 72°C for 2 min. *Escherichia coli* CFT073 was used as a positive control for *iucA*; KPPR1 was used for all other reactions.

Liquid chromatography-mass spectrometry of siderophores. Strains were grown in M63-glycerol (0.6%) without the addition of iron at 37°C for 17 h. Each strain was cultured in triplicate. Multiple-reaction-monitoring (MRM) analyses were performed using a Waters 2795 Alliance HT high-performance liquid chromatography (HPLC) system coupled to a Micromass Quattro Premier XE mass spectrometer (Micromass MS Technologies). Samples were injected onto a Zorbax Eclipse XDB-C8 4.6- by 150-mm column at a flow rate of 400 μ l/min and with a linear gradient of water-acetonitrile with 1% acetic acid. Aliquots of 1 ml of supernatant were prepared, and 0.12 ng/ml of 5,6,7,8-tetradeuterio-3,4-dihydroxy-2-heptylquinoline was added as an internal control. The specific transitions monitored, from pseudomolecular to daughter ions of salmochelins, enterobactins, and aerobactin, are described elsewhere (8). The transition for yersiniabactin was *m/z* 482 > 295. These transitions were used for relative quantification of the siderophores.

MLST. Multilocus sequence typing (MLST) was performed to determine the diversity of a selection of clinical isolates. Genomic DNA was isolated from bacteria using a Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA). Seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) were

amplified by PCR, using conditions and primers designated by the Pasteur Institute *Klebsiella pneumoniae* MLST Database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>), with 50 ng of genomic DNA as template. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequencing was performed at the Nucleic Acid and Protein Research Core within the Children's Hospital of Philadelphia (Philadelphia, PA). Each primer set included an identical 5' sequence such that universal sequencing primers were used. Sequence alignment was performed using MacVector editing software, and the resulting contig was queried against the Pasteur Institute MLST database to determine the designated allele. To identify the sequence type, allelic profiles were generated for each clinical isolate and compared to the MLST database.

Preparation of recombinant human lipocalin 2. Recombinant human lipocalin 2 expressed in *E. coli* strain BL21 as a glutathione *S*-transferase fusion protein (a gift from J. Cowland) was purified and cleaved with human thrombin as previously described (2, 7, 39). Purified Lcn2 was quantified using a Micro BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL). Siderophore-binding activity was confirmed by incubation with Fe-Ent followed by measurement of absorbance at 340 nm.

Serum growth assay. RPMI with 10% (vol/vol) heat-inactivated human serum from two sources and with or without 1.6 μ M recombinant human Lcn2 was inoculated with 1×10^3 CFU/ml of an overnight culture of *K. pneumoniae* and incubated overnight in a final volume of 100 μ l in 96-well plates at 37°C with 5% CO₂ (17). To determine bacterial density, samples were serially diluted and plated on LB agar.

Urine growth assay. Pooled human urine, sterilized by passage through a 0.22- μ m sterile filter (Millipore, Billerica, MA), was inoculated with 1×10^3 CFU/ml of an overnight culture of *K. pneumoniae*, with or without 1.6 μ M recombinant human Lcn2, and incubated overnight in a final volume of 100 μ l in 96-well plates at 37°C with 5% CO₂. To determine bacterial density, samples were serially diluted and plated on LB agar.

Murine pneumonia model. All animal work was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (assurance number A3079-01). Six- to 8-week-old C57BL/6 mice (Jackson Laboratory, Jackson, ME) or isogenic *Lcn2*^{-/-} mice (provided by Shizuo Akira via Alan Aderem and backcrossed for ≥ 8 generations) were anesthetized with isoflurane and inoculated in the pharynx with 1×10^4 CFU of *K. pneumoniae*. LB broth-grown cultures were centrifuged, resuspended, and diluted in phosphate-buffered saline (PBS), and 50 μ l of the suspension was administered. Mice were monitored daily for signs of illness (hunched posture, ruffled fur, and decreased activity); any mice displaying these signs were euthanized. To determine bacterial density, mice were sacrificed by CO₂ asphyxiation and lungs and spleens were removed, homogenized in 400 μ l PBS, and plated on LB agar with appropriate antibiotics.

RESULTS

Clinical isolates of *K. pneumoniae* encoding yersiniabactin are more prevalent in respiratory samples than other sites. In total, 129 clinical isolates (from 33 respiratory, 46 urine, 14 blood, and 36 stool samples) were screened for siderophore gene loci by PCR. As expected, all strains contained Ent synthesis genes (Table 1). Twenty-two strains (17%) also contained Ybt synthesis genes, and three strains (2%) had Aer and gly-Ent synthesis genes. Ybt was significantly more prevalent among isolates from the respiratory tract than in other sites (33% versus 11%, respectively; $P = 0.0068$; relative risk (RR), 2.4; 95% confidence interval (CI), 1.39 to 4.26).

To correlate the detection of siderophore genes with siderophore production, a subset of clinical isolates was tested for siderophore production by liquid chromatography-mass spectrometry (Table 2). To validate the mass spectrometry method, a wild-type strain and five defined siderophore synthesis gene mutants were assayed. These strains (KP4, KP5, KP6, KP7, KP20, and KP25) had patterns of detectable siderophores that correlated with their genotypes. Based on mass spectrometry, genotyping correlated with the production of each siderophore in iron-limited minimal medium for the majority of isolates (Ent 12/12; gly-Ent 11/12; Ybt 11/12; and Aer 12/12). Import-

TABLE 1. Comparison of siderophore prevalence by site of infection

Siderophore (PCR target)	No. (%) of PCR-positive isolates from indicated infection site						P value ^a	Relative risk (95% CI)
	All sites (n = 129)	Respiratory (n = 33)	Nonrespiratory					
			Urine (n = 46)	Blood (n = 14)	Stool (n = 36)	Combined (n = 96)		
Ybt (<i>ybtS</i>)	22 (17)	11 (33)	4 (9)	1 (7)	6 (17)	11 (11)	0.0068	2.43 (1.39-4.26)
Aer (<i>iucA</i>)	3 (2)	1 (3)	0 (0)	0 (0)	2 (6)	2 (2)	1.0	1.3 (0.26-6.89)
gly-Ent (<i>iroN</i>)	3 (2)	1 (3)	0 (0)	0 (0)	2 (6)	2 (2)	1.0	1.3 (0.26-6.89)
Ent (<i>entB</i> or <i>entE</i>)	129 (100)	33 (100)	46 (100)	14 (100)	36 (100)	96 (100)	ND	ND

^a Calculated using Fisher's exact test to compare respiratory and combined nonrespiratory prevalence. ND, not determined.

tantly, the amount and proportion of each siderophore produced is likely to vary by growth conditions and site of infection (8). Together, these data indicate that the majority (105/129, 81%) of *K. pneumoniae* clinical isolates produce only Ent. However, the subset of strains encoding Ybt was significantly more common in the respiratory tract than in blood, urine, or stool.

The yersiniabactin locus is often coincident with β-lactam resistance. The spread of antibiotic-resistant strains, particularly KPC clone ST258, could be aided by additional fitness advantages. To determine whether carriage of the Ybt locus is associated with antibiotic resistance, available antimicrobial susceptibility data were compared to Ybt prevalence (Table 3). Based on phenotype, 21 of 89 strains analyzed had enhanced β-lactam resistance attributable to a KPC (11), ESBL (6), or other mechanism (4). Of these strains, 43% had the Ybt locus, compared to only 10% of nonresistant strains (*P* = 0.0019; RR, 3.4; CI, 1.74 to 6.72). Strikingly, 64% of KPC-encoding strains also had the Ybt locus.

Because of the coincidence of Ybt occurrence among KPC strains, it is possible that the high prevalence of Ybt-positive

(Ybt⁺) isolates in our collection is due to overrepresentation of a single clone. To test this hypothesis, we performed MLST on the 11 respiratory isolates encoding Ybt (Table 4). Eight of these isolates were ST258, two isolates were ST353, and one was ST234. However, when combined with the observed resistance patterns, there was significant heterogeneity, such that 5 unique patterns were observed. Among ST258 isolates, three resistance patterns were observed: KPC, ESBL, and levofloxacin/tobramycin resistance. These data indicated that although ST258 was common, there was both intra- and interclone variability in antibiotic resistance mechanisms among Ybt⁺ respiratory isolates.

If Ybt improves fitness in the lung independently from antibiotic resistance, then it should be more prevalent in β-lactam-resistant isolates from the respiratory tract than in resistant isolates from other sites. To test this hypothesis, a collection containing only β-lactam-resistant isolates was genotyped for siderophores. Ybt was significantly more prevalent among respiratory isolates (13/16) than among isolates from other sites of infection (6/16; *P* = 0.0290; RR, 2.97; CI, 1.05 to 8.4). These data suggest that Ybt is often produced by β-lactam-

TABLE 2. Relative distribution of siderophores detected by LC-MS in culture supernatants of strains grown in M63-glycerol

Strain and siderophore genotype or site of infection	Synthesis gene detected (+/-) and % (±SD) total siderophores produced ^a			
	Enterobactin (Ent)	Salmochelin (gly-Ent)	Yersiniabactin (Ybt)	Aerobactin (Aer)
Isogenic mutants				
KP4 (KPPR1) WT	+ 39.3 ± 3.6	+ 40.8 ± 3.8	+ 20.0 ± 7.3	- 0
KP5 (VK087) <i>entB</i>	- 0	- 0	+ 100	- 0
KP6 (VK088) <i>ybtS</i>	+ 49.9 ± 0.4	+ 50.1 ± 0.4	- 0	- 0
KP7 (VK089) <i>entB ybtS</i>	- 0	- 0	- 0	- 0
KP20 <i>iroA ybtS</i>	+ 100	- 0	- 0	- 0
KP25 <i>iroA</i>	+ 85.5 ± 4.3	- 0	+ 14.5 ± 4.3	- 0
Clinical isolates				
KP30, respiratory	+ 100	- 0	- 0	- 0
KP33, urine	+ 58.8 ± 5.3	- 0	+ 41.2 ± 5.3	- 0
KP34, urine	+ 74.4 ± 4.5	- 0.2 ± 0.02	+ 25.4 ± 4.5	- 0
KP35, urine	+ 100	- 0	- 0	- 0
KP36, urine	+ 100	- 0	- 0	- 0
KP56, respiratory	+ 100	- 0	+ 0	- 0
KP57, respiratory	+ 92.4 ± 2.3	- 0	+ 7.6 ± 2.3	- 0
KP58, respiratory	+ 100	- 0	- 0	- 0
KP76, stool	+ 100	- 0	- 0	- 0
KP77, stool	+ 100	- 0	- 0	- 0
KP78, stool	+ 26.4 ± 1.4	+ 9.6 ± 0.4	+ 1.9 ± 0.2	+ 62.1 ± 1.3
KP102, stool	+ 1.0 ± 0.2	+ 0.2 ± 0.02	+ 12.5 ± 3.4	+ 86.3 ± 3.6

^a Results for siderophores detected by both genotype and LC-MS are in boldface.

TABLE 3. Comparison of yersiniabactin prevalence among invasive isolates according to β -lactam resistance pattern

Antibiotic resistance pattern	No. (%) of isolates PCR positive for Ybt
Enhanced β -lactam resistance	
KPC ($n = 11$)	7 (64)
ESBL ($n = 6$)	2 (33)
Other ($n = 4$)	0 (0)
Combined ($n = 21$)	9 (43)
Classical ($n = 68$)	7 (10)
Total ($n = 89$)	16 (18)
P value ^a	0.0019
Relative risk (95% CI)	3.4 (1.74–6.72)

^a Calculated using Fisher's exact test to compare prevalence among resistant (combined) and nonresistant (classical) isolates.

producing strains but that among resistant strains, Ybt is still more prevalent in the respiratory tract.

Glycosylated enterobactin but not yersiniabactin promotes growth in human serum. To determine which siderophores could evade Lcn2 in the bloodstream, bacterial growth was measured in human serum with or without recombinant human Lcn2 (Fig. 1). As controls, isogenic siderophore-producing strains were used. The wild-type strain (KP4) showed robust growth with or without added Lcn2. The siderophore-negative mutant KP7 had a >3 -log growth defect under either condition, as did the Ybt-dependent mutant KP5. Ent⁺ (KP20) and Ent⁺ Ybt⁺ (KP25) strains were severely inhibited by Lcn2. Conversely, a gly-Ent⁺ strain (KP6) was resistant to Lcn2. In this strain background, gly-Ent is both necessary and sufficient to evade Lcn2 in serum.

For the clinical isolates, a subset was selected to represent each site of isolation and to include strains with LC-MS data. Two clinical isolates encoding gly-Ent (KP78 and KP237) were Lcn2 resistant. KP102, which produces Ybt and Aer but only trace amounts of gly-Ent, was also Lcn2 resistant. In contrast, five of six strains with only Ent synthesis genes were significantly inhibited by Lcn2. One exception, KP225, showed a nonsignificant trend toward Lcn2-dependent inhibition. Yersiniabactin-positive strains displayed two phenotypes: three strains were defective for serum growth, and four strains grew well in serum but were significantly inhibited by Lcn2. The serum growth defect for KP29, KP48, and KP57 was not at-

tributable to a lack of functional siderophore receptors, since the addition of purified Ent or Ybt restored growth (data not shown). Furthermore, KP57 is capable of producing both Ent and Ybt as measured by LC-MS (Table 2). Despite functional synthesis pathways and receptors, these strains do not appear to produce siderophores at sufficient concentrations to allow replication. Together, these data suggest that gly-Ent and perhaps Aer, but not Ybt, allow the evasion of Lcn2 in human serum.

Ex vivo growth in human urine does not require siderophores. To determine whether the production of alternative siderophores allows *K. pneumoniae* to evade Lcn2 in urine, bacterial growth was measured in pooled human samples with or without added recombinant human Lcn2 (Fig. 2). All control strains, including the siderophore-negative mutant KP7, showed robust growth in urine. Curiously, Lcn2 had a small but statistically significant inhibitory effect on KP7. This defect may be attributable to bacterial utilization of endogenous catechol compounds (3, 32) that transport iron, ligands that Lcn2 also binds. The majority of clinical isolates tested had maximal growth regardless of Lcn2. This growth pattern is similar to that seen for *E. coli*, where uropathogenic, commensal, and laboratory strains grew to equivalent final densities in urine (1). Only the Ybt-positive strain KP48 showed a significant defect in urine growth and was further inhibited by Lcn2. This reason for this growth defect is unclear. Together, these data indicate that *K. pneumoniae* does not require siderophores for urine growth and that most clinical isolates are not inhibited by Lcn2 in urine.

Yersiniabactin is sufficient to evade Lcn2 and cause pneumonia, but Lcn2 protects against disseminated disease. Because of the high prevalence of Ybt⁺ Ent⁺ clinical isolates in the respiratory tract and their inability to grow in serum, we asked whether Ybt is able to support growth in the respiratory tract and evade Lcn2. Attempts to grow *K. pneumoniae* in human bronchoalveolar lavage (BAL) fluid were unsuccessful: some BAL fluid samples had antimicrobial activity that could not be inactivated, while others allowed siderophore-independent growth (data not shown). Instead, a model of murine pneumonia induced by *K. pneumoniae* was employed. To control for strain-to-strain differences that may affect *in vivo* fitness, isogenic mutant strains were used. At day 3 postinfection, the siderophore-negative strain (KP7) had replicated poorly in the lung, with a median CFU count of 4×10^4 (Fig. 3A). The

TABLE 4. MLST results for yersiniabactin-positive respiratory isolates

Strain	Allelic profile							Sequence type	Antibiotic resistance pattern	Phenotype pattern
	<i>tonB</i>	<i>gapA</i>	<i>pgi</i>	<i>infB</i>	<i>mdh</i>	<i>phoE</i>	<i>rpoB</i>			
KP156	79	3	1	3	1	1	1	ST258	KPC	1
KP223	79	3	1	3	1	1	1	ST258	KPC	1
KP228	79	3	1	3	1	1	1	ST258	KPC	1
KP248	79	3	1	3	1	1	1	ST258	KPC	1
KP250	79	3	1	3	1	1	1	ST258	KPC	1
KP216	79	3	1	3	1	1	1	ST258	ESBL	2
KP235	79	3	1	3	1	1	1	ST258	ESBL	2
KP246	79	3	1	3	1	1	1	ST258	Levofloxacin/tobramycin	3
KP217	16	3	1	9	47	13	1	ST353	None	4
KP151	16	3	1	9	47	13	1	ST353	None	4
KP152	24	2	1	1	2	7	1	ST234	None	5

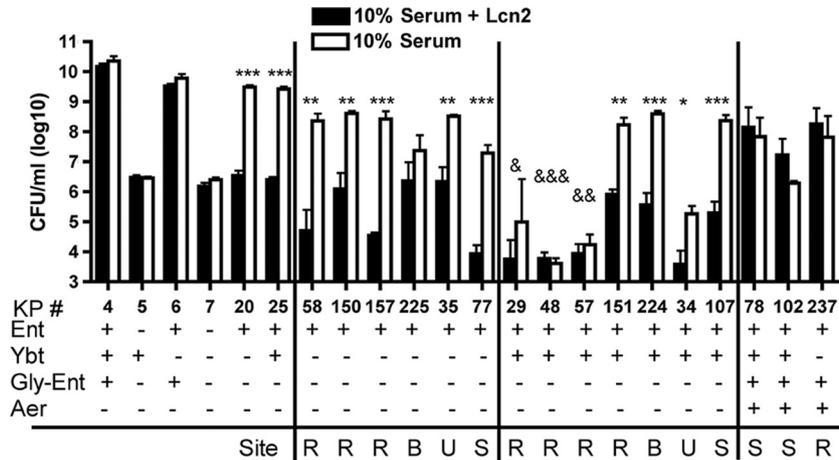


FIG. 1. Glycosylated enterobactin but not yersiniabactin promotes growth in human serum. Overnight growth in 10% heat-inactivated human serum with or without 1.6 μ M recombinant human Lcn2 was determined for the *K. pneumoniae* strains indicated (KP #). The means \pm standard errors of the means for at least three independent experiments are shown as log₁₀ CFU/ml. The siderophore genotype of each strain is indicated by plus signs (+). For clinical isolates, the site of infection is noted as R (respiratory), B (blood), U (urine), or S (stool). *, growth is inhibited by rLcn2 as measured by unpaired *t* test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001). &, growth is inhibited in serum alone compared to growth of KP7 by one-way analysis of variance (ANOVA) (&, *P* < 0.05; &&, *P* < 0.01; &&&, *P* < 0.001).

Ent⁺ strain (KP20) also replicated poorly. The Ybt⁺ Ent⁺ strain (KP25) had significantly enhanced growth in the lungs compared to the growth of both KP20 and KP7.

To determine whether Ybt is sufficient to evade Lcn2, strains expressing combinations of Ybt, Ent, and gly-Ent were used to infect wild-type (WT) and *Lcn2*^{-/-} mice. The Ent⁺ strain (KP20) was severely inhibited for growth in WT mice (Fig. 3B). This growth inhibition was Lcn2 dependent, since KP20 replicated ~10,000 times more in *Lcn2*^{-/-} mice. Ybt was sufficient to evade Lcn2; the Ybt⁺ strain (KP5) grew robustly in both WT and *Lcn2*^{-/-} mice. The Ybt⁺ Ent⁺ strain (KP25) grew robustly in WT mice, but in *Lcn2*^{-/-} mice, it replicated even

further. The addition of gly-Ent in strain KP4 did not promote evasion of Lcn2.

To measure the progression of pneumonia to disseminated disease, spleens were sampled for bacterial CFU counts, and the health of the mice was monitored daily. The Ent⁺ strain (KP20) was at or below the limit of detection in 8 of 9 spleens from WT mice (Fig. 3C). Strikingly, by day 3 postinfection, the majority of *Lcn2*^{-/-} mice were moribund or dead (Fig. 3D), correlating with high bacterial burdens in the spleen. The Ybt⁺ strain (KP5) was recovered in moderate numbers from the spleen in both WT and *Lcn2*^{-/-} mice. However, the majority of these mice appeared healthy throughout the experiment

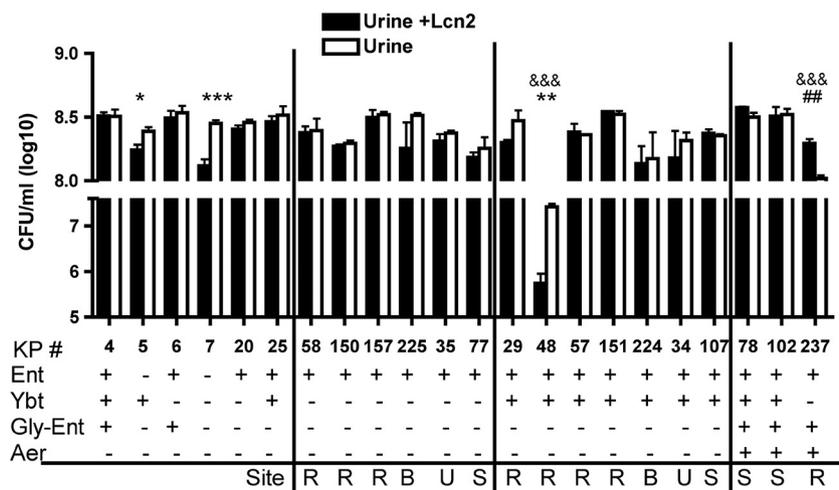


FIG. 2. Siderophores are not required for growth in human urine. Overnight growth in pooled human urine with or without 1.6 μ M recombinant human Lcn2 was determined for the *K. pneumoniae* strains indicated (KP #). The means \pm standard errors of the means for at least three independent experiments are shown as log₁₀ CFU/ml. The siderophore genotype of each strain is indicated by plus signs (+). For clinical isolates, the site of infection is noted as R (respiratory), B (blood), U (urine), or S (stool). *, growth is inhibited by Lcn2 as measured by unpaired *t* test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001). &&&, growth is inhibited in urine compared to growth of KP4 by one-way ANOVA (*P* < 0.001). ##, growth is stimulated by Lcn2 as measured by unpaired *t* test (*P* < 0.01).

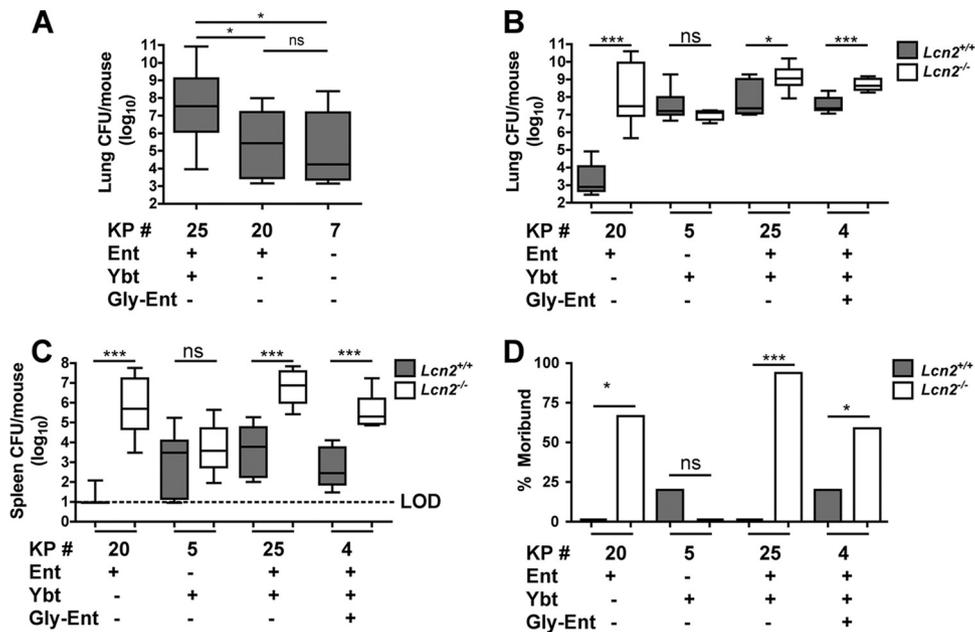


FIG. 3. Yersiniabactin is sufficient to evade Lcn2 and cause pneumonia but not sepsis. (A) Lung bacterial burden (\log_{10} CFU) at day 3 after retropharyngeal inoculation of 1×10^4 CFU of the *K. pneumoniae* mutants indicated (KP #) was determined in C57BL/6 mice ($n \geq 10$ mice per group). Box-and-whisker graph shows the median and interquartile ranges. *, $P < 0.05$ as determined by one-way ANOVA with Tukey's post test. ns, not significant. (B, C) Lung (B) and spleen (C) bacterial burdens at day 3 after retropharyngeal inoculation of 1×10^4 CFU of the *K. pneumoniae* mutants indicated were compared between C57BL/6 (*Lcn2*^{+/+}) and isogenic *Lcn2*^{-/-} mice (≥ 5 mice per group). *, $P < 0.05$, and ***, $P < 0.001$, as determined by the Mann-Whitney test. Box-and-whisker graphs show the median and interquartile ranges. LOD, limit of detection. (D) The percentages of dead or moribund mice were determined at day 3 after inoculation. *, $P < 0.05$, and ***, $P < 0.001$, as determined by the log rank test. The siderophore genotype of each strain is indicated by plus signs (+). ns, not significant.

regardless of Lcn2. Similar to Ybt⁺ strain KP5, both the Ent⁺ Ybt⁺ (KP25) and Ent⁺ Ybt⁺ gly-Ent⁺ (KP4) strains were recovered from the spleen in moderate numbers in WT mice, and the majority of mice were healthy. However, in *Lcn2*^{-/-} mice, these strains disseminated to reach high numbers in the spleen, and the majority of mice were moribund or dead by day 3 postinfection. Together, these data indicate that Ybt allows evasion of Lcn2 in the lung but confers limited potential to disseminate and cause sepsis. Conversely, Ent confers the potential to cause severe pneumonia and fatal sepsis, but only in the absence of Lcn2.

DISCUSSION

The purpose of this study was to determine whether Lcn2-resistant siderophores are required for *K. pneumoniae* to cause disease. Our results demonstrate by two independent measures, prevalence among sites of clinical infection and a murine pneumonia model, that the Lcn2-resistant siderophore Ybt is a significant risk factor for *K. pneumoniae* respiratory infection. Furthermore, our data indicate that the requirement for Ybt is attributable to its ability to evade Lcn2. The ability of Ent⁺ Ybt⁺ strains to evade Lcn2 is likely to have a significant impact on human disease, since these strains are a substantial subset of clinical isolates and are overrepresented among β -lactam-resistant isolates, including the epidemic KPC clone ST258.

Ybt is sufficient to allow colonization of the respiratory tract (2) and to cause pneumonia through evasion of Lcn2. Therefore, strains expressing this siderophore have simultaneously

expanded their tissue tropism and their ability to cause disease. Colonization of the upper respiratory tract is likely to enhance the fitness of strains by providing an additional site of replication and the potential to spread through oral and nasal secretions. Therefore, Ybt likely improves the fitness of epidemic KPC clone ST258 independently of antibiotic resistance. In fact, our limited MLST analysis suggests that Ybt is more consistent in this strain than KPC carriage itself. In a larger analysis, ST258 strains also demonstrated significant diversity in the KPC plasmids superimposed on this genetic background (24). By allowing access to the respiratory tract, carriage of Ybt likely enhances the ability of clone ST258 to colonize, infect, and spread among hospitalized patients independent of its antibiotic resistance.

Integrating the prevalence of Ybt by site of infection with its ability to support growth in blood, urine, and a murine model of pneumonia highlights its specialized function. Indeed, Ybt prevalence correlates with Lcn2 levels. The high prevalence of Ybt⁺ isolates in the respiratory tract compared to their levels in blood, urine, and stool is consistent with the high expression of Lcn2 by the respiratory mucosa. The trachea and lung have the highest levels of mRNA expression among 50 human tissue types examined (13). This explains why Ybt has been described as a virulence factor in the lung (30); it significantly increases the ability to cause respiratory tract infection despite Lcn2.

The predilection of Ybt⁺ *K. pneumoniae* for the respiratory tract is further supported by Ybt's low prevalence and lack of function in other sites of infection. In blood isolates, Ybt carriage was rare. Accordingly, Ybt could not evade Lcn2 in

serum, and some strains could not replicate in serum regardless of Lcn2 levels. The ability of Ybt to evade Lcn2 in the lung but not the blood may explain the limited dissemination of Ybt⁺ *K. pneumoniae* bacteria in our murine model. These bacteria were recoverable from the spleen only in moderate numbers, which could represent decreased capacity to grow in extrapulmonary sites. In urine isolates, Ybt was also rare, and *K. pneumoniae* did not require Ybt or, indeed, any siderophore for growth in urine. Together, these data suggest that Ybt has a specialized function suited to Lcn2 evasion in the lungs. This site specificity could be due to enhanced activity of Ybt, increased production of Ybt, or preferential utilization of an iron source. This is similar to *Yersinia pestis*, in which Ybt is required for intranasal and intradermal but not intravenous routes of infection (5, 6, 15).

Whereas *K. pneumoniae* expressing Ybt can efficiently infect the lungs regardless of Lcn2, Ent⁺ strains represent opportunistic pathogens that cause severe disease in an immunocompromised (*Lcn2*^{-/-}) host. In the absence of Lcn2, Ent was sufficient to cause robust replication in the lung, high-level dissemination to the spleen, and sepsis. The fact that Ent⁺ strains were common among clinical isolates (81%) and can cause severe disease when iron acquisition is unfettered suggests two possibilities. The first is that Lcn2 is produced at normal levels in affected patients but that certain infections do not require siderophore-dependent growth. For example, bacteremia from *K. pneumoniae* is likely caused by seeding from other, more permissive sites (19), such as the urinary tract. The second possibility is that Ent⁺ isolates cause opportunistic infections under conditions of Lcn2 deficiency. *K. pneumoniae* is a common cause of nosocomial infections, affecting patients with underlying disease (23). Diseases affecting myelopoiesis, renal tubule function, and mucosal integrity would be predicted to disrupt Lcn2 production. Intriguingly, HIV⁺ patients have decreased serum Lcn2 levels that are restored with antiretroviral therapy (29). Conversely, Lcn2-resistant siderophores are associated with community-acquired disease. For example, community-acquired pyogenic liver abscesses reported in Southeast Asia (27) are caused by *K. pneumoniae* strains expressing multiple Lcn2-resistant siderophores, including gly-Ent and Ybt (22). Furthermore, a defining feature of *Salmonella enterica*, a leading cause of community-acquired gastroenteritis, is production of the Lcn2-resistant siderophore gly-Ent (*iroA*) (4). A large correlation study of Lcn2 levels and siderophore genotypes of bacterial infections would be needed to determine protective concentrations.

Utilizing isogenic *K. pneumoniae* siderophore mutants reveals that the degree of protection by Lcn2 against *K. pneumoniae* is significantly greater than previously appreciated based on studies using the Ent⁺, Ybt⁺, and gly-Ent⁺ strain ATCC 43816 (9). In fact, Lcn2 confers almost complete protection against an Ent⁺ strain, representing the majority of isolates. Since Lcn2 specifically binds Ent, it is remarkable that it also confers significant protection against strains encoding resistant siderophores. During infection with strains producing Ent and Ybt, sequestration of Ent by Lcn2 could provide protection since Ybt is inefficient at promoting systemic disease. By the same reasoning, gly-Ent may also have limited virulence potential compared to that of Ent, since strains encoding this siderophore are partially inhibited by Lcn2. Alter-

natively, Lcn2-dependent acute inflammation, similar to what is observed during nasal colonization (2), could explain this partial protection. The mechanism by which Lcn2 retains protection against strains encoding multiple siderophores is currently under investigation. Nevertheless, Lcn2 is partially protective against all combinations of Ent, gly-Ent, and Ybt represented in our collection of clinical isolates, including strains producing multiple Lcn2-resistant siderophores.

In summary, this study adds to our understanding of the importance of siderophores in *K. pneumoniae* pathogenesis by demonstrating the following. First, Ybt-encoding strains are associated with an increased risk of respiratory infection. Second, Ybt confers the ability to evade Lcn2 in the lungs and efficiently cause pneumonia. Third, Ybt function is specialized such that it promotes growth in the lungs but not in serum. Fourth, Ent-expressing strains are opportunistic lung pathogens, causing severe disease in the absence of Lcn2. Understanding the function of each siderophore in *K. pneumoniae* disease may allow for improved detection of virulent strains and provide targets for antimicrobial therapy against this increasingly deadly pathogen.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant K08GM085612 (M.A.B.) from the National Institute for General Medical Sciences, by grants R01AI038446 and R01AI078538 (J.N.W.) from the National Institute of Allergy and Infectious Diseases, and by the Natural Sciences and Engineering Research Council (C.M.D.).

M.A.B. would like to thank Paul Edelstein for access to the collection of *K. pneumoniae* β-lactam-resistant isolates and, along with Irving Nachamkin, for guidance with biochemical identification of *K. pneumoniae* from stool specimens.

REFERENCES

1. Alteri, C. J., and H. L. Mobley. 2007. Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. *Infect. Immun.* **75**:2679–2688.
2. Bachman, M. A., V. L. Miller, and J. N. Weiser. 2009. Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin. *PLoS Pathog.* **5**:e1000622.
3. Bao, G., et al. 2010. Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex. *Nat. Chem. Biol.* **6**:602–609.
4. Bäumler, A. J., F. Heffron, and R. Reissbrodt. 1997. Rapid detection of *Salmonella enterica* with primers specific for *iroB*. *J. Clin. Microbiol.* **35**:1224–1230.
5. Bearden, S. W., J. D. Fetherston, and R. D. Perry. 1997. Genetic organization of the yersiniabactin biosynthetic region and construction of avirulent mutants in *Yersinia pestis*. *Infect. Immun.* **65**:1659–1668.
6. Bearden, S. W., and R. D. Perry. 1999. The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. *Mol. Microbiol.* **32**:403–414.
7. Bundgaard, J. R., H. Sengelov, N. Borregaard, and L. Kjeldsen. 1994. Molecular cloning and expression of a cDNA encoding NGAL: a lipocalin expressed in human neutrophils. *Biochem. Biophys. Res. Commun.* **202**:1468–1475.
8. Caza, M., F. Lepine, S. Milot, and C. M. Dozois. 2008. Specific roles of the *iroBCDEN* genes in virulence of an avian pathogenic *Escherichia coli* O78 strain and in production of salmochelins. *Infect. Immun.* **76**:3539–3549.
9. Chan, Y. R., et al. 2009. Lipocalin 2 is required for pulmonary host defense against *Klebsiella* infection. *J. Immunol.* **182**:4947–4956.
10. Clifton, M. C., C. Corrent, and R. K. Strong. 2009. Siderocalins: siderophore-binding proteins of the innate immune system. *Biomaterials* **22**:557–564.
11. CLSI. 2008. Performance standards for antimicrobial susceptibility testing, 18th informational supplement. CLSI document M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
12. CLSI/NCCLS. 2005. Screening and confirmatory tests for ESBLs in *K. pneumoniae*, *K. oxytoca*, *E. coli* and *P. mirabilis*. CLSI document M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA.
13. Cowland, J. B., and N. Borregaard. 1997. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. *Genomics* **45**:17–23.

14. Earhart, C. F. 1996. Uptake and metabolism of iron and molybdenum, p. 1075–1090. In F. Neidhart et al. (ed.), *Escherichia coli* and *Salmonella*, 2nd ed. ASM Press, Washington, DC.
15. Fetherston, J. D., O. Kirillina, A. G. Bobrov, J. T. Paulley, and R. D. Perry. 2010. The yersiniabactin transport system is critical for the pathogenesis of bubonic and pneumonic plague. *Infect. Immun.* **78**:2045–2052.
16. Fischbach, M. A., et al. 2006. The pathogen-associated *iroA* gene cluster mediates bacterial evasion of lipocalin 2. *Proc. Nat. Acad. Sci. U. S. A.* **103**:16502–16507.
17. Flo, T. H., et al. 2004. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* **432**:917–921.
18. Friedl, A., S. P. Stoesz, P. Buckley, and M. N. Gould. 1999. Neutrophil gelatinase-associated lipocalin in normal and neoplastic human tissues. Cell type-specific pattern of expression. *Histochem. J.* **31**:433–441.
19. Garcia de la Torre, M., et al. 1985. *Klebsiella* bacteremia: an analysis of 100 episodes. *Rev. Infect. Dis.* **7**:143–150.
20. Goetz, D. H., et al. 2002. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol. Cell* **10**:1033–1043.
21. Hirsch, E. B., and V. H. Tam. 2010. Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *J. Antimicrob. Chemother.* **65**:1119–1125.
22. Hsieh, P. F., T. L. Lin, C. Z. Lee, S. F. Tsai, and J. T. Wang. 2008. Serum-induced iron-acquisition systems and TonB contribute to virulence in *Klebsiella pneumoniae* causing primary pyogenic liver abscess. *J. Infect. Dis.* **197**:1717–1727.
23. Jones, R. N. 2010. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. *Clin. Infect. Dis.* **51**(Suppl. 1):S81–S87.
24. Kitchel, B., et al. 2009. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: clonal expansion of multilocus sequence type 258. *Antimicrob. Agents Chemother.* **53**:3365–3370.
25. Kjeldsen, L., A. H. Johnsen, H. Sengelov, and N. Borregaard. 1993. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J. Biol. Chem.* **268**:10425–10432.
26. Klausen, P., C. U. Niemann, J. B. Cowland, K. Krabbe, and N. Borregaard. 2005. On mouse and man: neutrophil gelatinase associated lipocalin is not involved in apoptosis or acute response. *Eur. J. Haematol.* **75**:332–340.
27. Ko, W. C., et al. 2002. Community-acquired *Klebsiella pneumoniae* bacteremia: global differences in clinical patterns. *Emerg. Infect. Dis.* **8**:160–166.
28. Koczura, R., and A. Kaznowski. 2003. Occurrence of the Yersinia high-pathogenicity island and iron uptake systems in clinical isolates of *Klebsiella pneumoniae*. *Microb. Pathog.* **35**:197–202.
29. Landro, L., et al. 2008. Decreased serum lipocalin-2 levels in human immunodeficiency virus-infected patients: increase during highly active anti-retroviral therapy. *Clin. Exp. Immunol.* **152**:57–63.
30. Lawlor, M. S., C. O'Connor, and V. L. Miller. 2007. Yersiniabactin is a virulence factor for *Klebsiella pneumoniae* during pulmonary infection. *Infect. Immun.* **75**:1463–1472.
31. McGettigan, S. E., K. Andreadchio, and P. H. Edelstein. 2009. Specificity of ertapenem susceptibility screening for detection of *Klebsiella pneumoniae* carbapenemases. *J. Clin. Microbiol.* **47**:785–786.
32. Miethke, M., and A. Skerra. 2010. Neutrophil gelatinase-associated lipocalin expresses antimicrobial activity by interfering with L-norepinephrine-mediated bacterial iron acquisition. *Antimicrob. Agents Chemother.* **54**:1580–1589.
33. Mori, K., et al. 2005. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J. Clin. Invest.* **115**:610–621.
34. Nelson, A. L., J. M. Barasch, R. M. Bunte, and J. N. Weiser. 2005. Bacterial colonization of nasal mucosa induces expression of siderocalin, an iron-sequestering component of innate immunity. *Cell. Microbiol.* **7**:1404–1417.
35. Nelson, A. L., A. J. Ratner, J. Barasch, and J. N. Weiser. 2007. Interleukin-8 secretion in response to aferric enterobactin is potentiated by siderocalin. *Infect. Immun.* **75**:3160–3168.
36. Podschun, R., and U. Ullmann. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin. Microbiol. Rev.* **11**:589–603.
37. Winn, W. C., et al. 2006. The Enterobacteriaceae, p. 259–264. Koneman's color atlas and textbook of diagnostic microbiology, 6th ed. Lippincott Williams and Wilkins, Baltimore, MD.
38. Wooldridge, K. G., and P. H. Williams. 1993. Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol. Rev.* **12**:325–348.
39. Yang, J., et al. 2002. An iron delivery pathway mediated by a lipocalin. *Mol. Cell* **10**:1045–1056.

Editor: A. J. Bäumlner