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## Yersiniabactin Is a Virulence Factor for *Klebsiella pneumoniae* during Pulmonary Infection<sup>∇</sup>

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**Iron acquisition systems are essential for the in vivo growth of bacterial pathogens. Despite the epidemiological importance of *Klebsiella pneumoniae*, few experiments have examined the importance of siderophores in the pathogenesis of this species. A previously reported signature-tagged mutagenesis screen identified an attenuated strain that featured an insertional disruption in *ybtQ*, which encodes a transporter for the siderophore yersiniabactin. We used this finding as a starting point to evaluate the importance of siderophores in the physiology and pathogenesis of *K. pneumoniae*. Isogenic strains carrying in-frame deletions in genes required for the synthesis of either enterobactin or yersiniabactin were constructed, and the growth of these mutants was examined both in vitro and in vivo using an intranasal infection model. The results suggest divergent functions for each siderophore in different environments, with enterobactin being more important for growth in vitro under iron limitation than in vivo and the reverse being true for the yersiniabactin locus. These observations represent the first examination of isogenic mutants in iron acquisition systems for *K. pneumoniae* and may indicate that the acquisition of nonenterobactin siderophores is an important step in the evolution of virulent enterobacterial strains.**

Iron is an essential nutrient for the majority of bacterial species. It plays a key role as a cofactor for the electron transport chain and for various other enzymes (34). In anaerobic environments, Fe<sup>2+</sup> iron is prevalent and imported into the bacterial cytoplasm via porins and an ABC transport system (23). However, in aerobic conditions and mammalian tissues (in vivo), the majority of iron is found as Fe<sup>3+</sup>; iron in vivo is almost entirely sequestered by transferrin and lactoferrin (49). To grow successfully in host tissues, bacteria must be able to obtain iron from these host transport proteins. Different species have evolved a variety of secreted factors in order to obtain iron. Among the bacteria in the *Enterobacteriaceae* family, a number of different siderophore systems have developed to fill this role. After being synthesized and secreted by bacteria, these siderophores compete iron off of iron-loaded host proteins; they bind Fe<sup>3+</sup> iron with an affinity that is nearly 10 orders of magnitude higher than that of either transferrin or lactoferrin (49). In gram-negative bacteria, the iron-bound siderophores are then taken up via bacterial outer membrane receptors and are imported into the bacterium, where the iron is released by various mechanisms (10).

Among the *Enterobacteriaceae*, three siderophore systems are most prevalent: enterobactin, aerobactin, and yersiniabactin. Enterobactin (also known as enterochelin) is a catecholate siderophore that was among the first characterized bacterial siderophores and is produced by more than 90% of examined enterobacterial isolates (12, 32, 38, 47). Aerobactin is a hydroxamate siderophore which is produced by a smaller fraction of enterobacterial strains and has a lower affinity for free Fe<sup>3+</sup>

than either enterobactin or yersiniabactin (8, 36). Found among even fewer isolates is the phenolate siderophore yersiniabactin. This siderophore system was first described and characterized for *Yersinia* species, but it can be found among some isolates of other enterobacterial species and is believed to be acquired via horizontal gene transfer (2). In *Yersinia* isolates, the yersiniabactin locus is located within the high pathogenicity island, which is responsible for much of the disseminated disease and mortality caused by pathogenic yersiniae (9). The loss of the yersiniabactin system (Ybt) within this island has been shown to attenuate both *Yersinia enterocolitica* and *Yersinia pestis* strains in vivo (4, 6). However, in *Y. pestis*, this attenuation is specific for the subcutaneous (peripheral) route, as Ybt<sup>-</sup> mutants administered by the intravenous route are fully virulent (4, 46).

Extensive research has been performed on each of these siderophore systems to evaluate their genetic regulation, the functions of the components of each locus, and the relative iron binding ability of the purified siderophores (10, 14, 16, 36). Strains deficient in one or more siderophore systems have been examined for their growth defects in iron-restricted medium and for their abilities to acquire iron in vitro from transferrin and lactoferrin (8). These three systems have also been studied for their importance during mammalian infection. *Escherichia coli* strains deficient in both aerobactin and enterobactin show defects in kidney dissemination using a murine urinary tract infection model; however, mutants deficient in either aerobactin or enterobactin alone showed no colonization or dissemination defects (45). Aerobactin production was more prevalent among extraintestinal pathogenic *E. coli* strains than among intestinal strains, and the production of aerobactin was found to impact virulence in a subcutaneous infection model (11). *Y. pestis* bacteria that were deficient in yersiniabactin production caused systemic disease when inoculated intravenously but failed to disseminate following subcutaneous inoculation (4,

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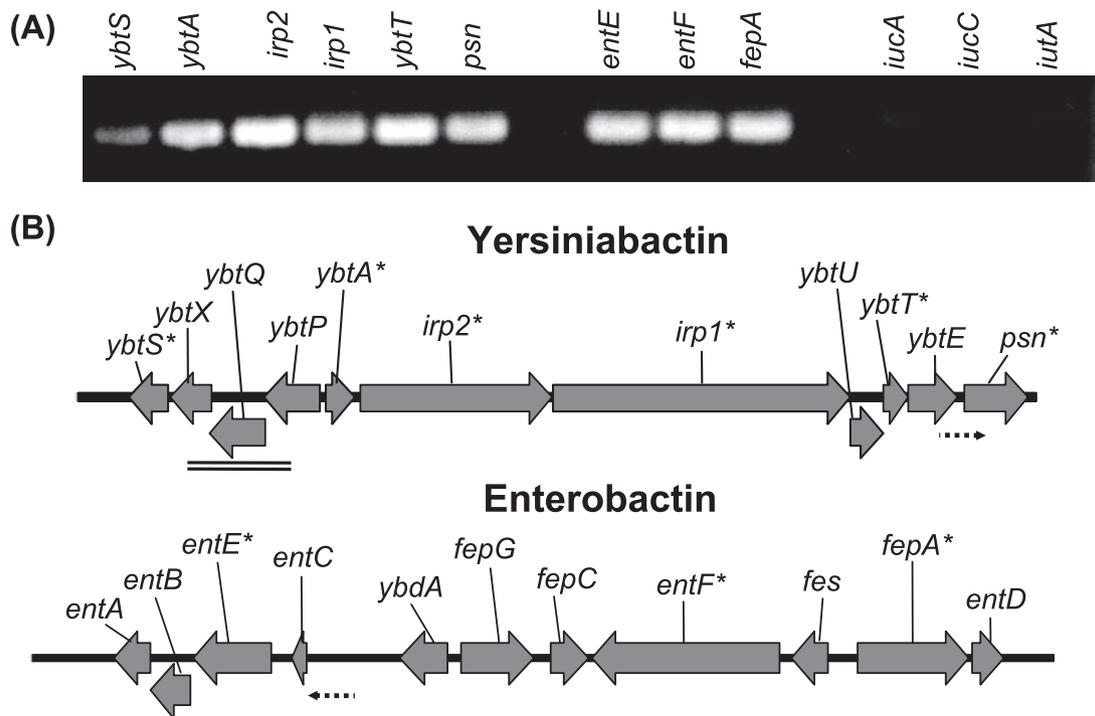


FIG. 1. Siderophore loci of *K. pneumoniae*. (A) PCR products obtained from each siderophore locus using *K. pneumoniae* DNA as a template. Primers were directed against the yersiniabactin locus sequence from *Y. pestis* and the enterobactin locus sequence from *E. coli*. Primers for the aerobactin genes *iucA*, *iucC*, and *iutA* were also tested and produced negative results. (B) Genetic analysis of *K. pneumoniae* siderophore loci. ORFs marked with asterisks were detected in the *K. pneumoniae* genome by PCR as shown in panel A. The solid double line indicates the segment of the yersiniabactin locus that was sequenced from the wild-type *K. pneumoniae* strain. Promoter regions that were used to generate transcriptional fusions are marked with dashed arrows.

15). It is believed that the more distal inoculation (i.e., subcutaneous) introduces *Y. pestis* into a local environment that features different sources of iron, and other siderophore systems cannot compensate for the loss of yersiniabactin.

Among the *Enterobacteriaceae*, *Klebsiella pneumoniae* is one of the most epidemiologically important species. It is a non-motile, gram-negative bacterium that is typically found as a commensal resident of the mammalian gastrointestinal tract (41). *K. pneumoniae* is also a substantial cause of human disease, particularly among immunocompromised individuals (39). The increasing acquisition of extended-spectrum beta-lactamase plasmids among *K. pneumoniae* isolates has led to a dramatic increase in antibiotic-resistant outbreaks (44).

A number of studies have assayed the distribution of siderophores among *K. pneumoniae* clinical isolates and have found that nearly all of them produce enterobactin, while a much smaller percentage produce either aerobactin or yersiniabactin (25, 40). In addition, aerobactin has been shown to play a role in enhancing the virulence of *K. pneumoniae* by the intraperitoneal (i.p.) route (7, 33). Another study using in vivo expression technology to examine genes upregulated during i.p. infection found that both an aerobactin synthesis gene and an enterobactin receptor gene were upregulated (26). However, no *K. pneumoniae* studies to date have evaluated isogenic mutant strains in different siderophore systems or examined the importance of siderophores for pathogenesis using a natural route of infection.

In an intranasal genetic screen to identify virulence factors

of *K. pneumoniae*, we identified an attenuated mutant featuring a disruption in *ybtQ*, a gene that encodes an ATPase necessary for the proper transport of yersiniabactin (28). Using this finding as a starting point to evaluate the importance of siderophores in the physiology and pathogenesis of *K. pneumoniae*, we have examined the expression of siderophore loci both in vitro and in vivo, the impact of these systems on growth in iron-restricted environments, and their relative significance on the ability of *K. pneumoniae* to grow in vivo.

#### MATERIALS AND METHODS

**Bacterial strains and media.** The wild-type strain used in this study is KPPR1, a rifampin-resistant derivative of a *K. pneumoniae* subsp. *pneumoniae* clinical pneumonia isolate featuring a type 1 O antigen and a type 2 polysaccharide capsule (ATCC 43816) (28). Unless otherwise noted, bacteria were grown overnight at 37°C, either on Luria-Bertani (LB) agar or by shaking in LB broth. Media were supplemented with 30  $\mu\text{g ml}^{-1}$  rifampin, 50  $\mu\text{g ml}^{-1}$  kanamycin, and/or 25  $\mu\text{g ml}^{-1}$  chloramphenicol as needed.

**Yersiniabactin locus sequencing.** By using the chromosome capture sequencing method, approximately 500 bp of *K. pneumoniae* sequence flanking the transposon insertion in mutant strain 7-13 was obtained (28). Two hundred nanograms of chromosomal DNA from the wild-type strain was then digested overnight at 37°C with equal units of Sall and XhoI restriction enzymes (New England Biolabs, Ipswich, MA). Following heat inactivation at 80°C for 20 min, the reaction was diluted to a final volume of 300  $\mu\text{l}$  and ligated for 3 h at 25°C with 2 units of T4 DNA ligase (Invitrogen, Carlsbad, CA). The ligation reaction was purified and concentrated by ethanol precipitation, and 1  $\mu\text{l}$  of the resuspension was used as a template for the PCR using standard *Taq* polymerase and dimethyl sulfoxide. Nested primers were used for both the PCR and subsequent sequencing of the resulting 5-kb amplicon (see Table 2).

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference
<i>K. pneumoniae</i> strains		
KPPR1	Wild type	22
VK087	KPPR1 $\Delta$ entB	This study
VK088	KPPR1 $\Delta$ ybtS	This study
VK089	KPPR1 $\Delta$ entB $\Delta$ ybtS	This study
VK096	KPPR1 pE2	This study
VK097	KPPR1 pY4	This study
VK099	VK087 pY4	This study
VK146	KPPR1 pPsn	This study
VK147	VK087 pPsn	This study
VK123	VK088 pE2	This study
VK124	VK087 pMLentB	This study
VK125	VK088 pMLybtS	This study
Plasmids		
pPROBE GFP	Km <sup>r</sup> , GFP	25
pE2	entC promoter-GFP in pPROBE	This study
pY4	ybtA promoter-GFP in pPROBE	This study
pPsn	psn promoter-GFP in pPROBE	This study
pJB1806	Cm <sup>r</sup> , low-copy-number plasmid, RSF1010ori	3
pMLentB	entB in pJB1806	This study
pMLybtS	ybtS in pJB1806	This study

**Qualitative reverse transcriptase PCR (RT-PCR).** Primers were designed to amplify internal regions of each gene, with melting temperature values between 55 and 60°C and with resulting products of approximately 150 bp (see Table 2). PCRs were carried out for 30 cycles at an annealing temperature of 50°C using standard reagents and protocols. Ten microliters of each PCR mixture was examined by using a 1% agarose gel run at approximately 75 mV for 45 min.

**Siderophore mutant strains.** Deletion mutants of *K. pneumoniae* were constructed with the pKO3 vector as described previously (29). In-frame deletions were made with pKO3 constructs that included approximately 1,000 bp of flanking sequence on each side of the gene to be targeted, with sequences that were amplified using primers indicated (see Table 2). For each deleted gene, the coding regions were removed, with the exception of three codons at the 5' and 3' ends of each open reading frame (ORF). Biosynthetic genes for both enterobactin (*entB*, encoding 2,3-dihydro-2,3-dihydroxybenzoate synthase) and yersiniabactin (*ybtS*, encoding salicylate synthase) were targeted, and mutant *K. pneumoniae* strains that were deficient in one or both genes were constructed (Fig. 1; Table 1). Both *entB* and *ybtS* encode enzymes responsible for early steps in the biosynthesis of enterobactin and yersiniabactin, respectively (19, 38).

**Siderophore transcriptional fusions.** To study the expression of siderophore loci in vitro, transcriptional fusions were made to approximately 500-bp fragments of the promoters for each locus (Fig. 1; Table 2). The *entC* promoter was used for enterobactin, and the *ybtA* and *psn* promoters were used for yersiniabactin (1, 14, 30). The amplified promoter sequences were moved into the low-copy-number pPROBE green fluorescent protein (GFP) reporter plasmid, and constructs were then transformed into either wild-type or siderophore mutant *K. pneumoniae* strains (31).

**Complementation studies.** Complementation clones were made for *entB* and *ybtS* using primers outside of each end of each gene (Table 2). These fragments were ligated into the low-copy-number plasmid pJB1806, and these constructs were moved into the *entB* and *ybtS* mutant strains of *K. pneumoniae* (Table 1) (3).

**In vitro growth curves.** In order to examine bacterial growth in vitro, bacterial strains were grown overnight in LB broth, diluted 1:1,000 into fresh LB broth, placed into 48-well plates, and shaken for 7 h at 37°C (Synergy HT multidetection microplate reader; BioTek Instruments, Winooski, VT). Values for the optical density at 600 nm (OD<sub>600</sub>) of each sample were recorded at regular intervals, and all strains were assayed in quintuplicate. Medium was supplemented with 2,2'-dipyridyl (Sigma Chemical, St. Louis, MO) at different concentrations to examine bacterial growth under iron-limiting conditions.

To examine siderophore expression, strains containing promoter-GFP fusion plasmids were used for growth curves as described above and, at each time point, GFP fluorescence was monitored using wavelengths of 485 and 528 nm.

**Uronic acid quantitation.** To determine the quantity of capsular polysaccharide produced by *K. pneumoniae*, cultures were grown at 37°C in LB broth and, at various time points, 500  $\mu$ l of culture was removed and stored at 4°C. The extraction and measurement of uronic acids were carried out as described previously (13). A standard curve was calculated using known concentrations of glucuronolactone (Sigma Chemical, St. Louis, MO).

**Lipopolysaccharide purification.** By using lysozyme and ultracentrifugation modifications as previously described (22), we performed lipopolysaccharide purification using a protocol of hot phenol and water developed by Westphal and Jann (51). Samples were fractionated using 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels and stained using the Pro-Q Emerald 300 lipopolysaccharide gel stain kit (Molecular Probes, Eugene, OR).

**Intranasal infection model.** Five- to 7-week-old female C57BL/6j mice (Jackson Labs, Jackson, ME) were anesthetized by i.p. injection with a mixture containing ketamine (8 mg ml<sup>-1</sup>) and xylazine (1.6 mg ml<sup>-1</sup>). Overnight bacterial cultures were diluted in phosphate-buffered saline, and 20  $\mu$ l of the bacterial suspension was inoculated intranasally in four 5- $\mu$ l aliquots. To facilitate consistent inoculations, mice were held vertically during inoculation and placed on a 45° incline while recovering from anesthesia.

In order to examine bacterial growth in vivo, mice were inoculated intranasally and, at various time points after infection, sacrificed by a lethal injection of 300  $\mu$ l of sodium pentobarbital (20 mg ml<sup>-1</sup>). Lungs and spleens were dissected, weighed, and homogenized in 500  $\mu$ l of phosphate-buffered saline. Homogenates were plated on LB agar with rifampin to determine the CFU per gram of tissue.

To compare the lethalities of various *K. pneumoniae* strains, groups of 20 mice were intranasally inoculated with  $2 \times 10^5$  CFU of bacteria. Surviving mice were counted twice each day for 7 days, and survival curves were compared using log rank statistical tests.

All animal experiments were performed under the guidance of the Department of Comparative Medicine at Washington University, and protocols were approved by the Animal Studies Committee.

**In vivo gene expression.** Three groups of four mice each were inoculated intranasally with approximately  $1 \times 10^4$  CFU of wild-type *K. pneumoniae*. After 72 h, whole lungs and spleens were recovered, tissues were disrupted into a single-cell suspension, mammalian cells were lysed using saponin, and RNA was extracted using a previously established protocol (27). Due to the low quantity of bacterial RNA extracted, qualitative RT-PCR was used to assess transcript levels of each gene by using cDNA synthesis protocols as described previously (27).

**Statistical analysis.** All figures were prepared and subsequent statistical analysis was performed using Prism 3 for Macintosh, version 3.0cx (GraphPad Software, San Diego, CA).

## RESULTS

**Identification of siderophore systems.** We previously reported the development of an intranasal mouse model of *K. pneumoniae* infection and the use of this model in a signature-tagged mutagenesis screen (28). One insertional mutant that was not recovered from either lung or spleen tissues in this screen contained a transposon insertion in *ybtQ*, a gene in the yersiniabactin synthesis locus (strain 7-13). YbtQ is a member of the traffic ATPase/ABC transporter family of proteins and, along with its homolog YbtP, is required for proper yersiniabactin transport (15). An approximately 5-kb region of *K. pneumoniae* chromosomal DNA flanking the transposon insertion was amplified and sequenced. This segment included the *ybtX*, *ybtQ*, and *ybtP* genes, each with greater than 90% nucleotide identity to the corresponding genes from *Yersinia pestis*. In addition, these genes were in the same orientation in the *K. pneumoniae* and *Y. pestis* sequences. Other genes present in the *Y. pestis* yersiniabactin locus and the *E. coli* enterobactin locus were also positively identified in this wild-type *K. pneumoniae* isolate by PCR (Fig. 1). By contrast, none of the three genes examined from the aerobactin synthesis locus of *E. coli* were detected (Fig. 1). The primers for the aerobactin genes were able to amplify the expected products from an *E. coli* strain producing aerobactin, and the primers were designed to

TABLE 2. Primers used in this study<sup>a</sup>

Primer function	Primers	Sequence <sup>b</sup>
<i>K. pneumoniae</i> sequencing	Ybtq5'/out1 Ybtq5'/out2 Ybtq3'/out1 Ybtq3'/out2	GTCAAAGCGGATATCGTAAC AGGTGTAGCTGATAAACATCG TAATCATGTTTCTTTGACGTTC CTACGGTCACCAAACCTGTTAAT
RT-PCR	<i>gyrB</i> gy5 gy3 <i>ybtS</i> ys5 ys3 <i>ybtA</i> ya5 ya3 <i>irp2</i> y25 y23 <i>irp1</i> y15 y13 <i>ybtT</i> yt5 yt3 <i>psn</i> yps5 Yps3 <i>iucA</i> ia5 ia3 <i>iucC</i> ic5 ic3 <i>iutA</i> it5 it3 <i>entA</i> ea5 ea3 <i>entE</i> ee5 ee3 <i>fepG</i> fg5 fg3 <i>fepE</i> ef5 ef3 <i>fepA</i> fa5 fa3	TGGCGGTAATTCGATGATA GGATAACCAGCTCCAGCTTC CCGGCATCATCAATAAATGT AGCGTCAACCCTGTTGGTAT CTGTTTCGTTGCAGGAAGGTA CCAGGATGCACGAGAAGTTA GAAAGTACCCCTTCCCATT ATGGCCTTCAAACCTCTGAT TTCCCGGATGAGTACCTACC GCGGGTGGCAATATAGTCTT AGCTGAGCAACTGACTGATTG CGCCAGTTGTGTAATGCTTC CAAACCTCCCAGAGTCTTGC TAGAAGTCTGCGCTGAAGA TTTCTGCTCATCTGGTCAC CTGGCAGAAAAAGTTGATGC TGGATTGATGCTCAAACCTCTG TGCATCGCTCATTGACAGTA GATGAAACGTTCTGTTGTC GAATCTGCTGTTCCAGTTCG AAGGTTTGCTGCCAGTCTTC GCTGCTCAACAACATCGAAC GCTGGTGGTTGAACAAAGC CAATGTCGCCGAGTTTTACA CGCTGTTTGGGATCACTTC GCTGCCGGTAAAGGCTAC CCCTCCTCTTCTCAAAGC GGCCCACTATGTTGAACTACG CGACGTCTCGGAGATCATT GATATCAATCTGGCGGTTGTT
<i>entB</i> mutant construction	E1 E2 E3 E4	TCTCGTCATACACCACTATCTC GGTGAAATAATGGCGGCGCTGGATTTC GCCATTCTCTGTTCTCCTTCAGCC CTGTTTGCTGACGATAGCTT
<i>ybtS</i> mutant construction	S1 S2 S3 S4	ACCGTATTAGCGGTAAGAAT GTGTAGGAGTAACTGTTTTCTAACCTC CATCCCTTACCTCTCTGTGTT TGTTCTATGCAGTCCTTACCC
<i>entB</i> -complementing clone construct	CCE1 CCE4	TCTCGTCATACACCACTATCTC CTGTTTGCTGACGATAGCTT
<i>ybtS</i> -complementing clone construct	CCS1 CCS4	ACCGTATTAGCGGTAAGAAT TGTTCTATGCAGTCCTTACCC
<i>entC</i> promoter fusion construct	Ecp5'in Ecp3'in	GATGGTTTTAAGCTGATCGT CATCTGCTGCTGGAACCTC
<i>ybtA</i> promoter fusion construct	yap5'in yap3'in	AGTCCAGCCAGAACAAGATA GACCACCAACTGGTGAATAG
<i>psn</i> promoter fusion construct	Ppsn5' Ppsn3'	CCCCCGGATCCGATGAGGTGGGGAACCTGC CCCCCGAATTCTCATTTGTTGAGTCCC

<sup>a</sup> *K. pneumoniae* sequencing primers were designed from sequences obtained via chromosome capture with strain 7-13 as performed as previously described (21). *gyrB* primers for *K. pneumoniae* were designed using sequences from the *K. pneumoniae* genome sequencing project at Washington University in St. Louis (<http://genome.wustl.edu>). Primers for the yersiniabactin and aerobactin loci were designed from sequences in the NCBI nucleotide database, using an *E. coli* locus for aerobactin (AY553855) and a *Y. pestis* locus for yersiniabactin (AF091251). The enterobactin sequence was taken from the *K. pneumoniae* genome sequencing project.

<sup>b</sup> Underlining indicates restriction enzymes.

TABLE 3. Doubling times of *K. pneumoniae* strains in LB broth with various concentrations of 2,2'-dipyridyl<sup>a</sup>

Concentrations of 2,2'-dipyridyl in LB ( $\mu$ M)	WT	$\Delta$ entB	$\Delta$ ybtS	$\Delta$ entB $\Delta$ ybtS
0	13 $\pm$ 0.37	15 $\pm$ 0.11	14 $\pm$ 0.34	15 $\pm$ 0.27
200	13 $\pm$ 0.51	17 $\pm$ 1.06	14 $\pm$ 0.13	25 $\pm$ 0.91*
400	39 $\pm$ 0.90	170 $\pm$ 59.68*	31 $\pm$ 1.31	163 $\pm$ 21.84*

<sup>a</sup> Growth curves were performed in quintuplicate by measuring OD<sub>600</sub> values every 20 min for 7 h. Unless otherwise indicated, mean doubling times with standard deviations are shown. \*, *P* value was  $\leq$ 0.01 compared to that of the wild-type (WT) strain under the same growth conditions (Mann-Whitney *t* test).

amplify a region that is identical among the *E. coli* aerobactin genes and aerobactin genes found in some *K. pneumoniae* strains (data not shown). Thus, we concluded that the wild-type strain harbors the enterobactin and yersiniabactin loci but not the aerobactin locus.

In order to evaluate the contributions of each siderophore system to the pathogenesis of this *K. pneumoniae* strain, in-frame deletions were constructed in synthetic enzymes for enterobactin and yersiniabactin. Mutant strains with deletions in *entB* and *ybtS* were generated, along with a double-mutant strain (Table 1; Fig. 1). To insure that these mutations did not impact the production of known *K. pneumoniae* virulence factors, the double-mutant strain was assayed for its polysaccharide production. This strain produced normal levels of capsular polysaccharide, along with lipopolysaccharide that appeared identical to that of wild-type samples by SDS-polyacrylamide gel electrophoresis analysis (data not shown).

**In vitro growth and expression.** In order to compare the roles of these iron acquisition systems in *K. pneumoniae* biology, we began by observing the abilities of the siderophore mutant strains to grow under iron-limiting conditions in vitro. *K. pneumoniae* cultures were grown in LB broth overnight and then subcultured into medium containing increasing concentrations of the iron chelator 2,2'-dipyridyl for each growth curve experiment. Similar rates of growth were observed when all strains were grown in rich medium. However, these doubling times slowed when the strains were placed in iron-limited medium (Table 3). With the addition of a moderate amount of the iron chelator 2,2'-dipyridyl (200  $\mu$ M), the wild-type and *ybtS* mutant strains showed no defects in their rates of growth. By comparison, the *entB* mutant grew slower, decreasing from a doubling time of 15 min in LB to 17 min in the presence of 200  $\mu$ M chelator (Table 3). The double mutant showed an even greater attenuation in growth, slowing to a doubling time of 25 min.

In the presence of a higher concentration of chelator (400  $\mu$ M), the wild-type and *ybtS* mutant strains grew at substantially different rates compared to those of the *entB* and double-mutant strains. Both wild-type and *ybtS* mutant strains increased their doubling times by more than twofold to more than 30 min. By contrast, the *entB* and double-mutant strains increased their doubling times more than 10-fold under these conditions to more than 160 min for each strain (Table 3). The growth defects of the single-mutant strains were complemented by providing either *ybtS* or *entB* on a low-copy-number plasmid (data not shown).

These data suggest that for iron acquisition in vitro, enterobactin plays a greater role than yersiniabactin does. In an effort to understand the reason for this difference, we constructed GFP fusions to the promoter regions of each siderophore locus by using the *psn* and *ybtA* promoters for yersiniabactin and the *entC* promoter for enterobactin. After moving these plasmids into wild-type *K. pneumoniae*, strains were grown in both rich and iron-limited media. None of the constructs were upregulated at any time point during growth in LB broth (data not shown). However, under iron-limited conditions, these promoters were expressed, with the *entC* promoter transcriptionally upregulated to a level approximately sevenfold higher than that for the *psn* promoter and approximately fourfold higher than that for the *ybtA* promoter (Fig. 2B).

In order to determine the potential cross talk between these iron acquisition systems, we examined the expression of each

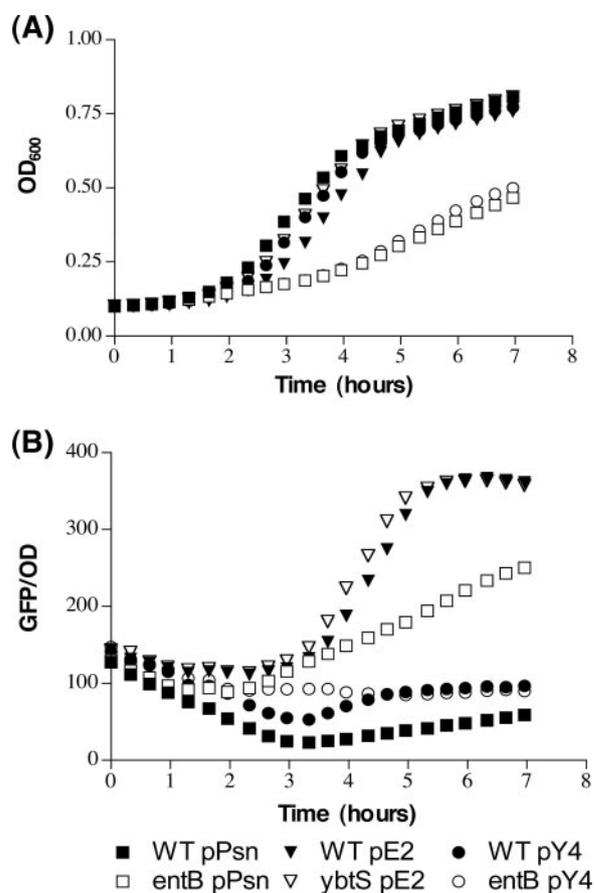


FIG. 2. Expression of siderophore loci in vitro. Bacteria were grown in LB broth supplemented with 200  $\mu$ M 2,2'-dipyridyl, and OD<sub>600</sub> values and GFP fluorescence were measured at each point along the growth curve. Each point on the OD<sub>600</sub> curves represents the means of five replicates  $\pm$  standard deviations. (A) Growth curve of either wild-type (WT) *K. pneumoniae*, the *entB* mutants or the *ybtS* mutant carrying the indicated reporter constructs. (B) Comparison of siderophore expression levels in wild-type and mutant strains. In order to correct for the growth deficiency of the *entB* strain under these medium conditions, GFP values are normalized to OD<sub>600</sub> measurement at each time point. Also assayed but not shown were the same strains carrying the pPROBE GFP vector; background fluorescence readings throughout the course of the experiment were never more than 2 relative light units.

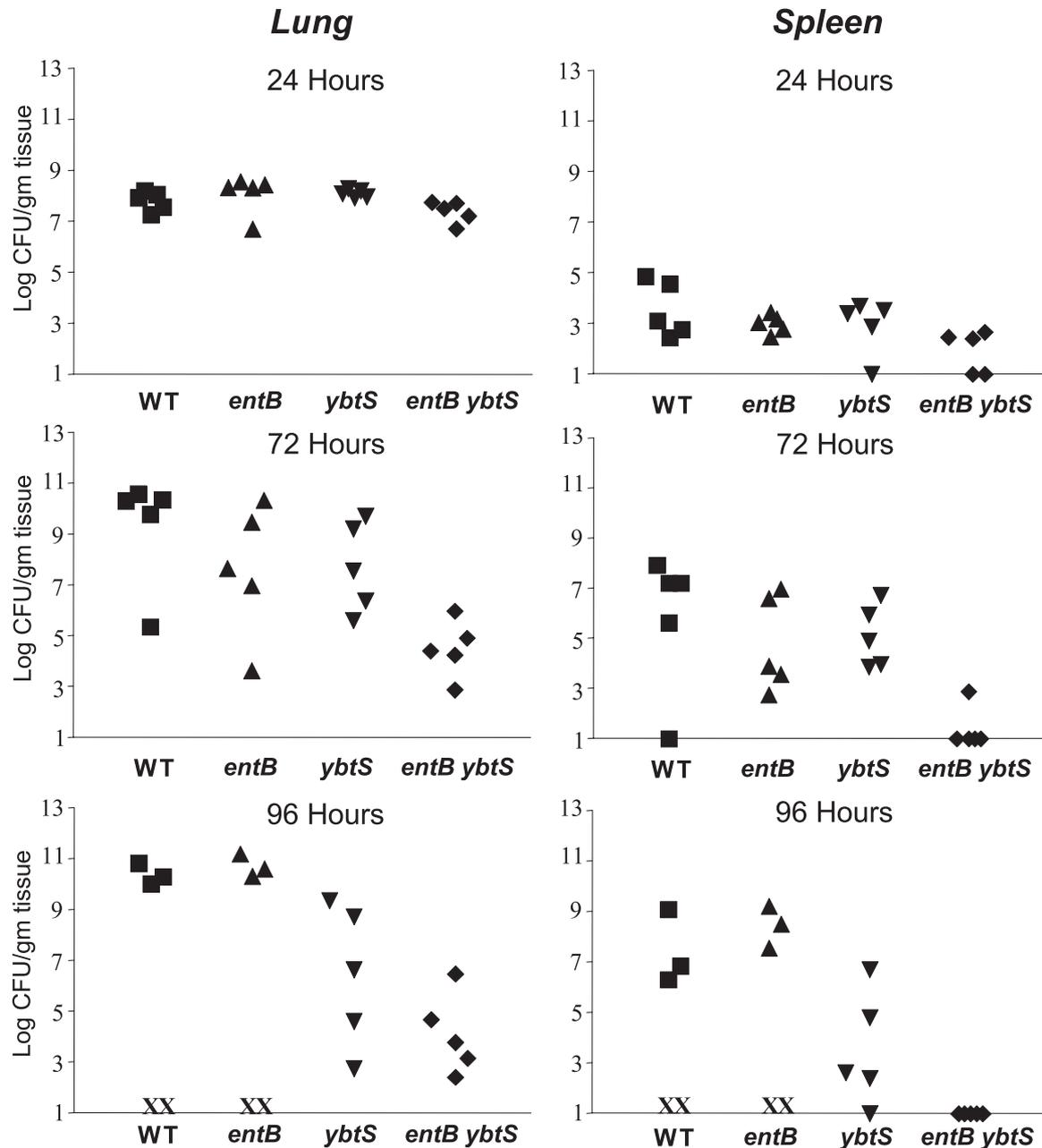


FIG. 3. Growth of *K. pneumoniae* siderophore mutants in vivo. Mice were intranasally inoculated with  $1 \times 10^4$  CFU of either the wild-type (WT) or a mutant strain, and at each time point, lungs and spleens were taken from five mice in each group. The limit of detection for this assay is approximately 10 CFU. Results are representative of three independent experiments.

of these systems in the absence of the other siderophore. By moving the promoter-GFP fusion plasmids into each single-mutant strain, we could determine whether compensatory siderophore expression was occurring. In the wild-type strain, the *psn* promoter is expressed at a low level across the growth curve. However, in an *entB* mutant background, the expression of *psn* increases approximately fivefold (Fig. 2B). In contrast, the expression of the yersiniabactin regulatory gene *ybtA* did not show differential expression in the wild-type background versus that in the *entB* mutant background (Fig. 2B). While expressed at a much higher level than either *ybtA* or *psn*, the

transcription of the *entC* promoter was identical regardless of the production of yersiniabactin (Fig. 2B).

**Intranasal comparison.** We next evaluated the three siderophore deletion strains by using our intranasal mouse model and compared bacterial growth in both lung and spleen tissues (Fig. 3). In the lungs, all strains showed nearly identical concentrations of bacteria at the 24-h time point, indicating that these mutations in iron acquisition systems do not lead to dramatic defects in overall bacterial fitness or in susceptibilities to the early innate immune response (Fig. 3).

As expected, mice infected by our wild-type strain showed

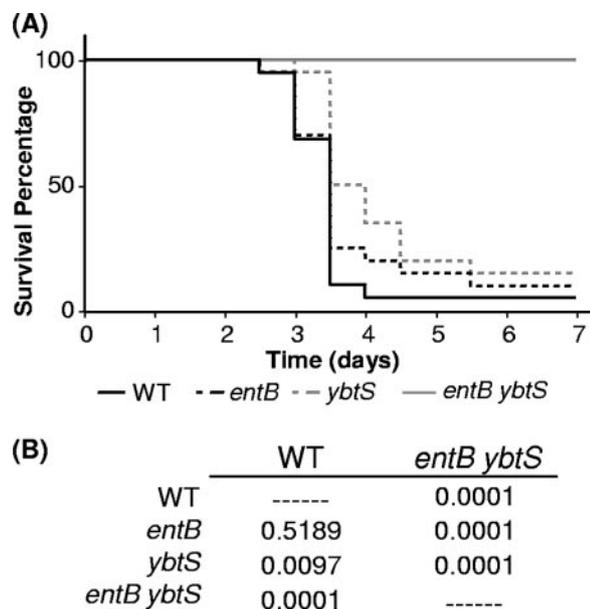


FIG. 4. Lethality of siderophore mutant strains. Intranasal inoculations were performed with  $2 \times 10^5$  CFU of *K. pneumoniae*. (A) Survival curves. Mouse survival was monitored twice daily for 7 days. (B) Statistical analysis. Log rank survival curve comparisons were made with each strain against the survival curves of the wild type (WT) and *entB ybtS*, and the resulting *P* values are shown.

significant amounts of growth in the lungs by 72 h postinfection (Fig. 3). By 96 h, two of five mice from this group succumbed to the infection and the remaining three mice had lung bacterial counts of nearly  $10^{11}$  CFU/g of tissue. The growth of the *entB* mutant strain in the lungs was almost identical to that of the wild-type strain, and two of five mice in this group also died by the 96-h time point (Fig. 3). By comparison, the *ybtS* mutant showed a reduced ability to grow in the lungs. At 96 h, there were relatively low levels of *ybtS* bacteria, with a mean concentration near  $10^6$  CFU/g, almost 5 orders of magnitude lower than that of the wild-type strain. The enterobactin and yersiniabactin double mutant showed the most dramatic defect for growth in vivo, with consistently lower bacterial concentrations at each time point after 24 h.

Following intranasal infection, *K. pneumoniae* disseminates beyond the respiratory tract and causes a systemic infection, which was monitored in this model by quantifying bacterial concentrations in the spleen. As expected from the lung data, there were steady increases in bacterial concentrations in the spleens of mice infected with both wild-type and *entB* mutant strains (Fig. 3). The *ybtS* mutant demonstrated an increase in splenic bacterial growth between 24 and 72 h but then decreased by the 96-h time point. Only a few mice became infected systemically with the double-mutant strain at any time point, and none of these mice had detectable bacteria in the spleen at 96 h (Fig. 3).

These intranasal infections demonstrated clear differences in bacterial survival among the siderophore mutant strains. During these experiments, we also observed differences in host survival during infection. Both wild-type and *entB* infections caused a mortality rate of 40% by the 96-h time point, while all mice infected with the *ybtS* or double-mutant bacteria survived

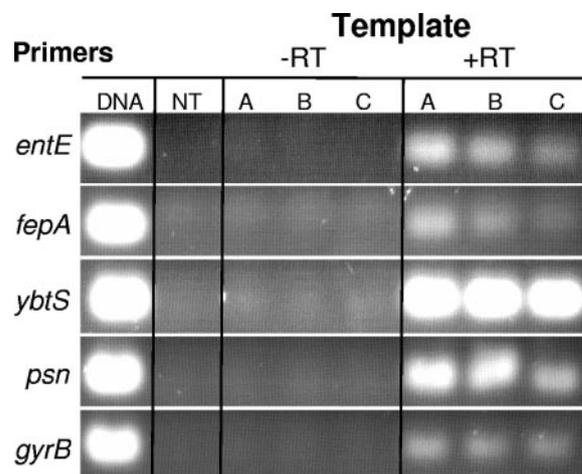


FIG. 5. Expression of siderophore systems during *K. pneumoniae* intranasal infection. Mice were infected with  $1 \times 10^4$  CFU of wild-type *K. pneumoniae*, whole lungs were harvested at 72 h postinfection, and RNA extractions were performed. For each cDNA synthesis reaction, amounts of RNA were used that provided roughly equivalent levels of *gyrB* signal. PCR was performed using primers for enterobactin synthesis (*entE*), enterobactin receptor (*fepA*), yersiniabactin synthesis (*ybtS*), and yersiniabactin receptor (*psn*) genes. Gyrase (*gyrB*) was used as a positive control. No template (NT) controls and RT reactions without reverse transcriptase (-RT) were used as controls for bacterial DNA contamination. Each group (A, B, and C) consisted of tissues from four infected mice. Ten microliters of each PCR product was run on the same 1% agarose gel and photographed at the same time with identical exposure settings.

(Fig. 3A). We followed up on this observation by conducting lethality experiments with these strains by using intranasal inoculations with a dose approximately 40-fold higher than the 50% lethal dose ( $LD_{50}$ ) for the wild type (28). As expected, nearly all of the wild-type-infected mice succumbed by 96 h postinfection (Fig. 4A). The *entB* and *ybtS* mutant infections exhibited slightly decreased rates of lethality. As expected by the lack of systemic bacterial dissemination at later time points, the double-mutant strain did not cause any host lethality by this route of infection. When these data were compared using a log rank test, the survival curves for both *ybtS* and *entB ybtS* were significantly different from the curve for the wild type, whereas the curve for the *entB* mutant was not significantly different (Fig. 4B). The above data strongly suggest that yersiniabactin is more important than enterobactin for iron acquisition in vivo following intranasal inoculation. While yersiniabactin is sufficient to allow growth and dissemination to wild-type levels in the absence of enterobactin, the reverse is not true. Nevertheless, the results from the double-mutant ( $\Delta entB \Delta ybtS$ ) infection suggest that enterobactin can partially compensate for the lack of yersiniabactin.

**Siderophore gene expression in vivo.** The *ybtS* mutant strain demonstrated a greater deficiency for growth in vivo than did the *entB* mutant strain; this contrasts with the greater in vitro growth deficiency of the *entB* mutant strain. As a complementary experiment, we wanted to examine the expression of these systems in vivo. Unfortunately, the yield of bacterial RNA from infected tissues at the 24-h time point was too low to provide a comparison by RT-PCR, presumably due to the relatively low concentration of bacteria present. Spleen tissues

at the 72-h time point were also examined for bacterial gene expression, but consistent amounts of control transcript were not obtained. This difficulty may be a reflection of the dense cellularity of the spleen and, consequentially, the large amount of mammalian RNA that may be diluting the amount of bacterial RNA in these samples.

Following intranasal inoculation with our wild-type strain, we were able to extract sufficient bacterial RNA from infected lung tissues at the 72-h time point using previously established protocols (27). These samples were used to compare the production of enterobactin and yersiniabactin genes in the lungs at 72 h postinfection by using a qualitative RT-PCR method (Fig. 5). Our control primers for bacterial gyrase (*gyrB*) show a substantial level of transcript present that is roughly equivalent across the three separate groups of infected mice. Using these levels as sources of comparison, we can see the expression of all four iron acquisition ORFs that were examined: two enterobactin genes and two yersiniabactin genes. These results suggest that both enterobactin and yersiniabactin loci are expressed by *K. pneumoniae* during an infection.

## DISCUSSION

Siderophores have long been implicated as bacterial virulence factors (52). The role of iron acquisition systems is especially important in light of new findings that siderophores may represent a key front in the interplay between host and pathogen. Recent work using an intraperitoneal infection model of *E. coli* has demonstrated that the mammalian protein lipochalin 2 may serve to sequester enterobactin, therefore inhibiting bacterial replication in vivo (18). Lipochalin 2 production is stimulated by the recognition of bacteria by Toll-like receptors and may be one of the earliest steps in antibacterial host defense. Lipochalin 2 production is also strongly upregulated by bacterial colonization of nasal mucosa (35). Thus, successful colonization of the nasal mucosa may require the expression of iron acquisition systems other than enterobactin or a modified form of enterobactin (17).

Only a few in vivo studies have evaluated the relative importance of different iron acquisition systems in bacterial growth and virulence. One study examined the importance of three iron-regulated outer membrane proteins in the pathogenesis of *Vibrio cholerae* and concluded that the enterobactin receptor IrgA played a greater role in vivo than either vibriobactin or heme binding proteins did (42). A chronic respiratory infection model was used to evaluate the iron acquisition systems of *Burkholderia cepacia*, and strains with mutations in two previously characterized siderophore systems were compared for their levels of virulence (48). A strain with a mutation in the ornibactin system was substantially reduced in its ability to persist in the rat lung, while the pyochelin system was apparently dispensable under these circumstances. The relative importance of aerobactin and enterobactin for *E. coli* also was examined in a urinary tract infection model (45). Strains with mutations in either system alone displayed no defect relative to the wild type when assayed as a pure culture; thus, a differential phenotype was not detected in this infection model for *E. coli*.

The expression of heme and yersiniabactin receptors by *Y. enterocolitica* was examined in vitro and in vivo through the

construction of translational fusions (21). Both receptors were strongly expressed under iron starvation conditions in vitro. When comparing different inoculation methods, the highest level of expression for both heme and yersiniabactin receptors in vivo was found in the peritoneal cavity following intraperitoneal inoculation. The natural oral route of infection resulted in lower levels of expression of both receptors in the intestinal lumen and the Peyer's patches. However, this study found no conditions that led to differential expression for these iron acquisition receptors. In addition, while the virulence level of a yersiniabactin receptor mutant strain had been studied previously, the relative virulence levels of strains with mutations in each or both of these receptors were not investigated (37). However, the effect on virulence of mutations in other iron uptake systems in *Y. pestis* has been examined. For *Y. pestis*, yersiniabactin is the dominant iron acquisition system in vitro and is required for virulence via subcutaneous inoculation (4, 24). The Yfe system transports iron and manganese and is required for *Y. pestis* virulence during the systemic phase of infection (5). Several other iron acquisition systems have been described in *Y. pestis*, including the Yfu, Hmu, and Yiu systems, but a role in virulence for these systems has not been detected (20, 24, 43).

Compared to those for other bacterial pathogens, there are very few studies that examine the importance of iron acquisition systems during *K. pneumoniae* infection. If excess iron is provided to the host by injection of ferric ammonium citrate, the LD<sub>50</sub> value of *K. pneumoniae* by intramuscular injection is reduced 100-fold (7). When the aerobactin locus was provided in *trans* to a weakly virulent *K. pneumoniae* strain, the intraperitoneal LD<sub>50</sub> value also decreased 100-fold (33). However, no published experiments have evaluated isogenic *K. pneumoniae* mutants for their abilities to grow in vitro or in vivo.

The results presented in the current study represent the first attempt to examine the importance of iron acquisition systems for *K. pneumoniae* via a natural route of infection. These data suggest divergent roles for enterobactin and yersiniabactin under different environmental conditions. Under iron-limited conditions in vitro, the enterobactin locus is more highly expressed than is the yersiniabactin locus and an isogenic enterobactin mutant shows slower growth than does a yersiniabactin mutant. Interestingly, the double-mutant strain grows significantly slower than the enterobactin mutant does in a low concentration of iron chelator, suggesting that yersiniabactin may be a minor but important player in iron acquisition under these conditions. However, in a higher concentration of iron chelator, the enterobactin mutant strain grows just as poorly as does the double mutant. Therefore, enterobactin appears to play a dominant role in iron acquisition under the more iron-restrictive conditions. The gene encoding the yersiniabactin receptor, *psn*, was more highly expressed in an enterobactin mutant than in the wild-type strain. This is consistent with observations of *Yersinia* that show that the regulation of the Ybt locus by YbtA requires yersiniabactin itself (14). Perhaps a threshold level of iron-loaded yersiniabactin in the cell available for interaction with YbtA occurred under these iron-restricted growth conditions only when enterobactin was absent.

Studies of mammalian iron binding proteins have shown that transferrin is expressed abundantly throughout the body, while lactoferrin is produced mostly in mucosal secretions and the

intestinal tract (49). In particular, lactoferrin is produced most abundantly in the airways and in the secondary granules of neutrophils, where it is believed to serve as an iron donor to catalyze the oxidative burst (50). Mucosal infections and neutrophil recruitment are characteristic of *K. pneumoniae* infections, and it is possible that *K. pneumoniae* has acquired virulence traits to take advantage of the high concentrations of lactoferrin that may be present in vivo. Yersiniabactin and enterobactin may respond differently in the presence of transferrin or lactoferrin, and this may help explain the divergent results from our single-mutant infections. Similarly, lipocalin 2 is present in neutrophil granules and is secreted in response to the activation of innate immune receptors, including Toll-like receptor 4 (18). Lipocalin 2 differentially inhibits iron acquisition by enterobactin, suggesting that a pathogen expressing an alternative siderophore (e.g., yersiniabactin) will be at an advantage. Together, these results raise the intriguing possibility that nonenterobactin siderophore systems are selected for during infection and this selective pressure is due to a preference for the source of iron acquisition and the local innate immune responses present.

The double-mutant strain provides another interesting perspective on the in vivo results. While the *ybtS* mutant strain shows a more significant defect than does the *entB* mutant in both bacterial growth and host lethality after intranasal inoculation, the double-mutant strain is even more attenuated. This suggests that enterobactin must be playing some role in iron acquisition in vivo, even if the role is relatively minor compared to that of the yersiniabactin system. One advantage of the intranasal model is that it provides highly reproducible results, and typically, the concentrations of bacteria are within a fivefold range among all infected mice at a given time point. A unique aspect of the yersiniabactin mutant infection is the substantial difference in bacterial counts in the lungs among infected mice, with this variance as high as 5 orders of magnitude. We surmise that this range of concentrations represents a variation in the efficiency of iron acquisition by the bacterium, which may be acquiring iron from other sources with various degrees of success, and that as a result, the *ybtS* mutant strains are more susceptible to the immune response overall.

The technical difficulties of obtaining reliable concentrations of bacterial RNA from infected tissues have impacted the strength of our conclusions from these data. We would prefer to be able to compare different time points or different tissues for the expression of iron loci. Quantitative RT-PCR would provide a better direct comparison, but the quantity of RNA we recovered from tissues was not sufficient for this use. Our attempts at qualitative RT-PCR resulted in cycle threshold values that were too high to allow for a comparison between the control *gyrB* locus and the different iron acquisition genes. While our in vivo expression data provide a qualitative comparison between these siderophore systems, further experiments are required to determine whether the yersiniabactin genes are expressed at substantially higher levels than are the enterobactin genes, as they appear to be in these samples. Nevertheless, these data clearly demonstrate that both enterobactin and yersiniabactin genes are expressed during infection.

Taken together, these data show an inverse relationship between yersiniabactin and enterobactin, depending on the growth conditions. In vitro, enterobactin is more important

than yersiniabactin under iron-limited conditions, while in vivo, yersiniabactin is the dominant player. Despite substantial previous research on siderophores, we believe that this study represents the first observation of such a differential relationship between these two iron acquisition systems and clearly establishes yersiniabactin as important for the ability of *K. pneumoniae* to maintain infection in a mammalian host. The results presented in this study also suggest that yersiniabactin contributes to a more virulent phenotype of *K. pneumoniae*, and an earlier study (33) suggests that aerobactin can enhance the virulence levels of less virulent strains. Together, these results suggest that the acquisition of a siderophore system in addition to the ubiquitous enterobactin system, whether it is yersiniabactin or aerobactin, may be a key factor in enhancing the virulence of *K. pneumoniae*.

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