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Aerobactin Mediates Virulence and Accounts for Increased Siderophore Production under Iron-Limiting Conditions by Hypervirulent (Hypermucoviscous) *Klebsiella pneumoniae*

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Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* (hvKP) strains are an emerging variant of "classical" *K. pneumoniae* (cKP) that cause organ and life-threatening infection in healthy individuals. An understanding of hvKP-specific virulence mechanisms that enabled evolution from cKP is limited. Observations by our group and previously published molecular epidemiologic data led us to hypothesize that hvKP strains produced more siderophores than cKP strains and that this trait enhanced hvKP virulence. Quantitative analysis of 12 hvKP strains in iron-poor minimal medium or human ascites fluid showed a significant and distinguishing 6- to 10-fold increase in siderophore production compared to that for 14 cKP strains. Surprisingly, high-pressure liquid chromatography (HPLC)-mass spectrometry and characterization of the hvKP strains hvKP1, A1142, and A1365 and their isogenic aerobactin-deficient (Δ*iucA*) derivatives established that aerobactin accounted for the overwhelming majority of increased siderophore production and that this was not due to gene copy number. Further, aerobactin was the primary factor in conditioned medium that enhanced the growth/survival of hvKP1 in human ascites fluid. Importantly the *ex vivo* growth/survival of hvKP1 Δ*iucA* was significantly less than that of hvKP1 in human ascites fluid, and the survival of outbred CD1 mice challenged subcutaneously or intraperitoneally with hvKP1 was significantly less than that of mice challenged with hvKP1 Δ*iucA*. The lowest subcutaneous and intraperitoneal challenge inocula of 3×10^2 and 3.2×10^1 CFU, respectively, resulted in 100% mortality, demonstrating the virulence of hvKP1 and its ability to cause infection at a low dose. These data strongly support that aerobactin accounts for increased siderophore production in hvKP compared to cKP (a potential defining trait) and is an important virulence factor.

The emergence of a "hypervirulent" (hypermucoviscous) variant of *Klebsiella pneumoniae* (hvKP) is a major challenge that needs to be confronted (1, 2). This pathogen is undergoing global dissemination from the Asian Pacific Rim, where it was first recognized in 1986 (3). In contrast to the usual health care-associated venue for "classical" *K. pneumoniae* (cKP) infections in the West, which have received increased notoriety due to their propensity for acquiring antimicrobial resistance determinants (4, 5), hvKP causes serious, life-threatening infections in younger, healthy individuals in the community setting (6–8). Due to a lack of awareness and of an objective diagnostic test to differentiate hvKP from cKP, the incidence and full spectrum of disease are still being defined. Nonetheless, it is clear that the initially described community-acquired pyogenic liver abscess (CA-PLA) in the absence of biliary tract disease represents just one of many possible primary infections (9), which also include endophthalmitis, meningitis, and necrotizing fasciitis (10–12). Another clinically defining feature is the ability to metastatically spread from primary sites of infection in noncompromised hosts, which further distinguishes hvKP from cKP and enteric Gram-negative bacilli (GNB) in general (3, 13, 14). Both clinically (2) and experimentally (7, 15), hvKP is more virulent than cKP. One of many knowledge gaps regarding hvKP is an understanding of hvKP-specific virulence mechanisms that distinguish these strains from cKP, from which they evolved.

The ability of bacterial pathogens to modify their inherent virulence most commonly occurs by horizontal gene transfer. Virulence plasmid acquisition may be an important mechanism for the increased virulence of hvKP. Genes that encode a number of virulence factors, including those that are responsible for the hypermucoviscous phenotype (RmpA) and the siderophores (SP) aerobactin and salmochelin, are located on a large, 200- to 220-kb virulence plasmid that is not present in most cKP strains (16–18). Since the hypermucoviscous phenotype (which is probably due to increased capsule production) (reviewed in reference 2) was the initial defining trait of hvKP strains, it has received significant attention as a virulence factor and, despite conflicting data (2, 19–21), likely contributes to the increased pathogenicity. Other studies have identified factors that contribute to the virulence of hvKP, but these factors or properties are often present in cKP strains. More experiments are needed to establish whether a virulence factor or property present in both cKP and hvKP is equally important for their pathogenesis or whether it accounts for the increased virulence of hvKP strains compared to cKP strains (22).

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TABLE 1 Bacterial strains used in this study

Strain	Siderophore genotype	Phenotype[a]	Site(s) of isolation/clinical syndrome(s)	Location (reference)
hvKP1	$entB^+$ $iroB^+$ $irp2^+$ $iucA^+$	ST^+, K2	Liver aspirate, blood/PLA	Buffalo, NY (7)
hvKP2	$entB^+$ $iroB^+$ $irp2^+$ $iucA^+$	ST^+, K1	Blood/endophthalmitis	Buffalo, NY (this study)
hvKP3	$entB^+$ $iroB^+$ $irp2^+$ $iucA^+$	ST^+, K1	Stool/colonization	Buffalo, NY (this study)
hvKP4	$entB^+$ $iroB^+$ *irp2* $iucA^+$	ST^+	Blood/PLA	Minneapolis, MN (this study)
NTUH-K2044	$entB^+$ $iroB^+$ $irp2^+$ *iucA*	ST^+, K1	Blood/PLA, meningitis	Taipei, Taiwan (31)
A1142	$entB^+$ $iroB^+$ *irp2* $iucA^+$	ST^+, K57	Blood/PLA	Taipei, Taiwan
A1365	$entB^+$ $iroB^+$ $irp2^+$ $iucA^+$	ST^+, K54	Blood/PLA	Taipei, Taiwan
A4528	$entB^+$ $iroB^+$ *irp2* $iucA^+$	ST^+, K2	Blood/PLA	Taipei, Taiwan
A9534	$entB^+$ $iroB^+$ *irp2* $iucA^+$	ST^+, K5	Blood/PLA	Taipei, Taiwan
N4252	$entB^+$ $iroB^+$ *irp2* $iucA^+$	ST^+, K57	Blood/cholangitis	Taipei, Taiwan
N6319	$entB^+$ $iroB^+$ $irp2^+$ $iucA^+$	ST^+, K2	Blood/sepsis	Taipei, Taiwan
N7205	$entB^+$ $iroB^+$ $irp2^+$ $iucA^+$	ST^+, K1	Blood/cholecystitis	Taipei, Taiwan
cKP1	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (7)
cKP2	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (7)
cKP3	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (7)
cKP4	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (7)
KP1	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP2	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP3	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP4	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP5	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP6	$entB^+$ *iroB* $irp2^+$ *iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP7	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP8	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP9	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)
KP24	$entB^+$ *iroB irp2 iucA*	ST^-	Blood/unknown	Buffalo, NY (this study)

[a] ST, string test; K, capsular serotype.

The ability to acquire iron (Fe) is essential for bacterial growth and replication. This trait has been shown to play a crucial role in the progression of infection, including cKP infection (23). The host has a number of Fe binding proteins (e.g., transferrin) that serve to withhold Fe from the invading pathogen. *K. pneumoniae*, like other *Enterobacteriaceae*, produces and secretes SP that acquire Fe from host binding proteins and then reenter the bacterial cell by SP-specific receptors (24). Aerobactin, enterobactin, salmochelin, and yersiniabactin are SP that have been described in *K. pneumoniae* (25). Gene clusters for the *Yersinia* high-pathogenicity island (encodes yersiniabactin) and *iucABCD-iutA* (encodes aerobactin and its cognate receptor) were more prevalent in hvKP (38/42 and 39/42 strains, respectively) than in cKP (7/32 and 6/32, respectively) (25). In addition, aerobactin genes and aerobactin production, as demonstrated by a cross-feeding assay, were more common in hvKP strains than in cKP strains (8, 15).

These bioinformatic-molecular epidemiologic analyses suggested that hvKP strains might have the capability to acquire Fe more readily than cKP strains. Hsieh et al. began to address this possibility by assessing the virulence of mutant derivatives of the hvKP strain NTUH-K2044 (25). However, a decrease in virulence after intraperitoneal (i.p.) challenge was seen only when the production of yersiniabactin, aerobactin, and salmochelin was disrupted together. Recent data from our group, however, have established that the hvKP strain hvKP1 produced more Fe acquisition factors than four cKP bacteremic isolates (22). Further, it was shown that this property enhanced the growth/survival of hvKP1 in human ascites fluid *ex vivo*. Although this study established that hvKP1 produced more Fe acquisition factors, it was not formally established whether SP were responsible or whether this observation was generally applicable for hvKP strains.

In this study, we tested the hypotheses that hvKP strains produce more SP than do cKP strains and that this trait is an important mechanism contributing to the hypervirulence of hvKP. We demonstrate that increased SP production in hvKP compared to cKP strains was a distinguishing trait that enhanced the growth of hvKP strains in human ascites fluid. Surprisingly, physical and genetic evidence illustrated that nearly all of the increased SP production by hvKP strains was due to increased aerobactin production and that this increased production appears to be mediated by a novel mechanism. Lastly, aerobactin was shown to increase growth/survival *ex vivo* in human ascites fluid and in mice challenged subcutaneously (s.c.) or intraperitoneally, enabling infection to occur at a low challenge inoculum, a critical feature for a pathogen. These data establish aerobactin as an important virulence factor for hvKP.

MATERIALS AND METHODS

Strain description. hvKP1 (ST86, K2 serotype, ampicillin resistant) was isolated from blood and liver abscess aspirate from a previously healthy 24-year-old male from Buffalo, NY, USA, with CA-PLA and metastatic spread to the spleen (7). Additional wild-type hvKP and cKP clinical isolates used in this study are described in Table 1. Although the definition is imperfect, since an unequivocal test that distinguishes between hvKP and cKP strains is presently lacking, hvKP strains were defined as having a positive string test and/or being associated with community-acquired *K. pneumoniae* infections with clinical features characteristic of hvKP, such as metastatic spread (2). Genotypes for SP biosynthetic genes were designated by the presence or absence of PCR-generated amplicons: en-

terobactin (*entB* plus, 5′-GATGAAGACGATACCGTGC-3′; *entB* minus, 5′-ACCGAATCCAGACCGTAGTC-3′), salmochelin (*iroB* plus, 5′-ATCTCATCATCTACCCTCCGCTC-3′; *iroB* minus, 5′-GGTTCG CCGTCGTTTTCAA-3′), yersiniabactin (*irp2* plus, 5′-GCTACAATG GGACAGCAACGAC-3′; *irp2* minus, 5′-GCAGAGCGATACGGAAA ATGC-3′), and aerobactin (*iucA* plus, 5′-AATCAATGGCTATTCCC GCTG-3′; *iucA* minus, 5′-CGCTTCACTTCTTTCACTGACAGG-3′). Aerobactin-deficient derivatives of hvKP1 (hvKP1 Δ*iucA*), A1142 (A1142 Δ*iucA*), and A1365 (A1365 Δ*iucA*) were generated by allelic exchange as described previously (26). Constructs were confirmed by sequence analysis of PCR-generated amplicons using primers outside *iucA* (plus, 5′-ATAAGGCAGGCAATCCAG-3′; minus, 5′-TAACGGC GATAAACCTCG-3′). Polar effects were excluded by reverse transcription-PCR (RT-PCR) identification of the expected transcript for *iucB* (plus, 5′-GCAGTTACCGCTATGGCTGAGTTC-3′; minus, 5′-C CGCTTTGCTCCCAGAAATACTC-3′), which is immediately downstream from *iucA* (data not shown). Further, the Δ*iucA* derivatives remained *rmpA*, *iroN*, and string test positive. For *trans* complementation of hvKP1 Δ*iucA*, the plasmid pFUS2[*iucA-D*] was constructed by ligating the *iucA-D* PCR-generated amplicon (plus, 5′-ATGGATC CGAACAGCATTGAGCCAAGC-3′ [261bp 5′ to the *iucA* open reading frame {ORF}); minus, 5′-GTAGGTACCAGAAGCAGTGGGTTG AGAG-3′ [131bp 3′ to the *iucD* ORF]), which contains its native promoter and upstream regulatory sequence, into pFUS2 (confers gentamicin resistance) (27). pFUS2[*iucA-D*] was introduced by electroporation into hvKP1 Δ*iucA*, resulting in hvKP1 Δ*iucA*/pFUS2[*iucA-D*]. A quantitative SP assay performed on conditioned medium, generated by the growth of hvKP1 Δ*iucA*/pFUS2[*iucA-D*] grown in Fe-poor minimal medium (MM) plus gentamicin, demonstrated that SP production was 81% of that observed for its parent hvKP1. pFUS2[*iucA-D*] was poorly retained by hvKP1 Δ*iucA* in the absence of positive selection by the addition of gentamicin (2.5 μg/ml). Initially, a plasmid construct that contained only *iucA* (pFUS2[*iucA*]) was generated, but it did not result in SP production when used for *trans* complementation of hvKP1 Δ*iucA*. In addition, pFUS2 was introduced into hvKP1 (resulting in hvKP1/pFUS2) and hvKP1 Δ*iucA* (resulting in hvKP1 Δ*iucA*/pFUS2) for use in complementation experiments. All strains were maintained at −80°C in 50% Luria-Bertani (LB) broth and 50% glycerol prior to use.

Media. The procedures for obtaining human ascites fluid were reviewed and approved by the Western New York Veterans Administration Institutional Review Board. The Western New York Veterans Administration Institutional Review Board waived the need for informed consent for the process of obtaining ascites fluid. An expedited review was performed because the ascites fluid was collected from deidentified patients who were undergoing therapeutic paracentesis for symptoms due to abdominal distension. These individuals were not being treated with antimicrobials and were not infected with human immunodeficiency, hepatitis B, or hepatitis C virus. The ascites fluid was cultured to confirm sterility, divided into aliquots, and stored at −80°C. Each batch was obtained from a different patient and was designated by the date of removal. Ascites fluid batch 8/27/2009 was used for the growth studies reported in Fig. 1, and batch 10/18/2012 was used for all other studies in this report. For various *in vitro* growth studies, 100% ascites fluid, LB medium, or M9 MM was used.

Development of conditioned medium. Conditioned medium was generated as described previously (22).

***In vitro* growth in ascites fluid, LB, and M9 MM.** Growth in ascites fluid, LB, and M9 MM was determined as described previously with aliquots removed for bacterial enumeration at various times (28). The growth rates and plateau densities achieved by wild-type cKP and hvKP strains were similar in ascites fluid treated at 56°C for 30 min to inactivate complement activity, LB, and M9 MM. In some experiments, conditioned medium generated from various strains or high-pressure liquid chromatography (HPLC)-generated fractions of conditioned medium were added.

Quantitative SP assay. The quantitative SP concentration in conditioned medium and in HPLC fractions of conditioned medium generated by cKP and hvKP strains was determined using a modified chrome azurol S (CAS) assay (29). In brief, standards containing 0, 1.5, 3.1, 6.25, 12.5, 25, 50, and 100 μg/ml of SP were prepared. The SP assay solution consisted of 50 ml of 1.2 mM hexadecyltrimethylammonium bromide, 7.5 ml of 2 mM CAS, 1.5 ml of 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl, and 1.37 M piperazine (the pH was adjusted to 5.6 with HCl). In a flat-bottom 96-well plate, 100 μl of each standard or sample was added to wells, followed by the addition of 100 μl of 98% SP assay solution and 2% 0.2 M 5-sulfosalicyclic acid solution in duplicate. The reaction mixture was incubated for 30 min, and results were read at 630 nm. For quantitative interpretation, a reference curve was calculated as follows: (optical density [OD] standard/OD zero standard) × 100. A curve was generated using cubic spline analysis in Prism software. The SP concentration in each sample was extrapolated from the linear portion of the reference curve. SP concentrations were reported in μg/ml/1×10^9 bacterial CFU. SP assays performed on HPLC-generated fractions of unconditioned MM and human ascites fluid demonstrated background detection levels of 10.8 μg/ml and 2.4 μg/ml, respectively, in fraction 1. These amounts were subtracted from the appropriate conditioned-medium SP values.

Analysis of conditioned medium via HPLC. Conditioned medium was subjected to fractionation utilizing HPLC on an Agilent 1260 system in line with a C_{18} column (Eclipse; 4.6 by 100 mm, 3.5 μm) running an aqueous mobile phase containing 0.1% (vol/vol) formic acid at a flow rate of 1 ml/min. All runs were monitored at 230 nm with the samples reconstituted in the initial HPLC gradient buffer. Exploratory runs were conducted with an initial linear gradient from 5% methanol (MeOH) to 40% MeOH over 40 min at a column temperature of 40°C. The 20 largest peaks were collected, spotted on chrome azurol agar, and monitored qualitatively for SP activity. Of the 20 peaks, only one peak, subsequently designated fraction 5 (see Fig. 3A), displayed substantial Fe acquisition capability (data not shown). The HPLC gradient profile was subsequently modified to a 5% to 23% linear MeOH gradient over 20 min, and 5 fractions from the conditioned-medium sample, designated fractions 1 to 5, were collected.

Further purification of fraction 5 was undertaken by evaporating the fraction to dryness and reconstituting in 5% acetonitrile (ACN). This sample was run using a linear gradient from 5 to 40% ACN over 20 min at a column temperature of 40°C. Fractions collected from this run were designated 5a to 5e.

Analysis of HPLC fraction by MS. Mass spectrometry (MS) analysis was carried out by injecting 250 ng of purified fraction 5c onto a ThermoFinnigan LCQ Advantage equipped with an electrospray ionization source. Conditions were evaluated in 60% MeOH with a source voltage of 4.5 kV, a capillary voltage of 23.5 V, and a transfer tube temperature of 250°C. Spectra were scanned in negative-ion mode over the range of *m/z* 150 to 1000.

Mouse s.c. challenge infection model. The mouse s.c. challenge infection models have been previously described (22, 30). These animal studies were reviewed and approved by the University at Buffalo-SUNY and Veterans Administration Institutional Animal Care Committee. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all efforts were made to minimize suffering. In brief, outbred male CD1 mice (18 to 22 g) were challenged subcutaneously (s.c.) with either 3.0×10^2 ($n = 5$), 3.5×10^3 ($n = 10$), 3.5×10^4 ($n = 10$), or 3.5×10^5 ($n = 10$) CFU of hvKP1 or 2.8×10^2 ($n = 5$), 3.6×10^3 ($n = 10$), 3.6×10^4 ($n = 10$), or 3.6×10^5 ($n = 10$) CFU of hvKP1 Δ*iucA* (results for groups with $n = 10$ represent average titers from 2 experiments). In another experiment, CD1 mice were challenged i.p. with either 3.2×10^1 ($n = 5$), 3.2×10^2 ($n = 5$), 3.2×10^3 ($n = 5$), or 3.2×10^4 ($n = 5$) CFU of hvKP1 or 2.4×10^1 ($n = 5$), 2.4×10^2 ($n = 5$), 2.4×10^3 ($n = 5$), or

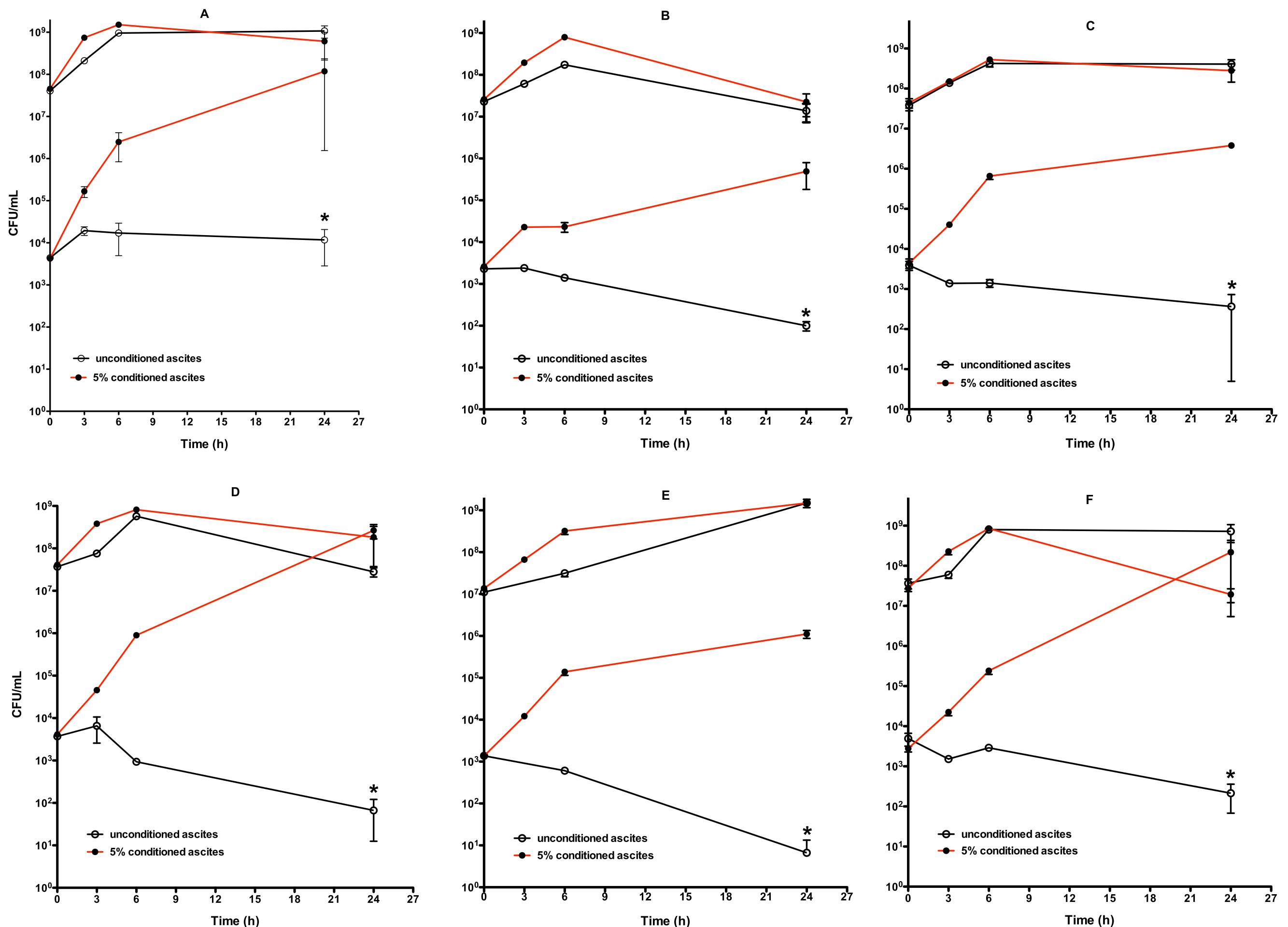

FIG 1 Growth/survival of hypervirulent *Klebsiella pneumoniae* (hvKP) strains grown in human ascites fluid supplemented with 0% and 5% homologous, conditioned ascites fluid. The growth/survival of six hvKP strains was assessed at 0, 3, 6, and 24 h in 100% human ascites fluid supplemented with 0% or 5% homologous conditioned ascites fluid. Both low (between 10^3 and 10^4 CFU) and high (between 10^7 and 10^8 CFU) starting titers were evaluated. (A) NTUH-K2044. (B) N7205. (C) A4528. (D) A1365. (E) A9534. (F) A1142. Supplementation with conditioned, homologous ascites fluid (5% final concentration) resulted in a significant increase in growth/survival for all strains at the low starting titer compared to 0% supplementation (*, $P < 0.05/2$). Data are means ± SEM; n = 3 or 4.

2.4×10^4 (n = 5) CFU of hvKP1 Δ*iucA*. Animals were followed for 14 days, with an *in extremis* state or death used as the study endpoint.

Statistical analyses. Data are presented as means ± standard errors of the means (SEM). P values of $0.05/n$ (n = the number of comparisons) are considered statistically significant based on the Bonferroni correction for multiple comparisons, and P values of $>0.05/n$ but <0.05 are considered as representing a trend. To normalize *in vitro* and *ex vivo* growth/survival data (see Fig. 1, 6, and 7), $\log_{10}$-transformed values were utilized, the area under each curve was calculated, and these areas were compared using two-tailed unpaired t tests (Prism 4 for MacIntosh; GraphPad Software Inc.). Two-tailed unpaired t tests were used for comparison of quantitative SP data (see Fig. 2 and 4). A log rank (Mantel-Cox) test was used for the analysis of the Kaplan-Meier plot (see Fig. 8) (Prism 4 for MacIntosh; GraphPad Software Inc.).

RESULTS

The growth/survival of hvKP strains in human ascites fluid is enhanced by the addition of conditioned medium. It was initially observed that the addition of conditioned medium enhanced the growth/survival of the hvKP strain hvKP1 in human ascites fluid at a low ($<10^5$ CFU/ml), but not at a high ($>10^7$CFU/ml), starting titer. An Fe acquisition factor(s) was established to be responsible (22). To extend these observations for additional hvKP strains, 6 hvKP clinical isolates were assessed for growth in human ascites fluid with and without the addition of 5% homologous conditioned medium. The addition of conditioned medium significantly increased the growth of all hvKP strains tested for low, but not high, starting inocula (Fig. 1).

hvKP strains secrete 6- to 9.6-fold more SP than cKP strains when grown under Fe-poor conditions. Our collection of 12 hvKP strains and 14 cKP strains were grown overnight in human ascites fluid (with complement inactivated to enable comparable growth of all strains) or Fe-poor minimal medium (MM) (Table 1). Total SP were measured in the supernatants using a quantitative chrome azurol S (CAS) assay and normalized to 1×10^9 CFU (Fig. 2A and B). The median SP concentration in ascites fluid was 6-fold greater for hvKP strains than for cKP strains ($P < 0.0001$) (median [range] of 138 [33.2 to 186] versus 23.1 [9.7 to 39.6] μg/ml) and 9.6-fold greater for hvKP strains grown in MM than for cKP strains (190 [55.6 to 624] versus 19.8 [13 to 74.7] μg/ml)

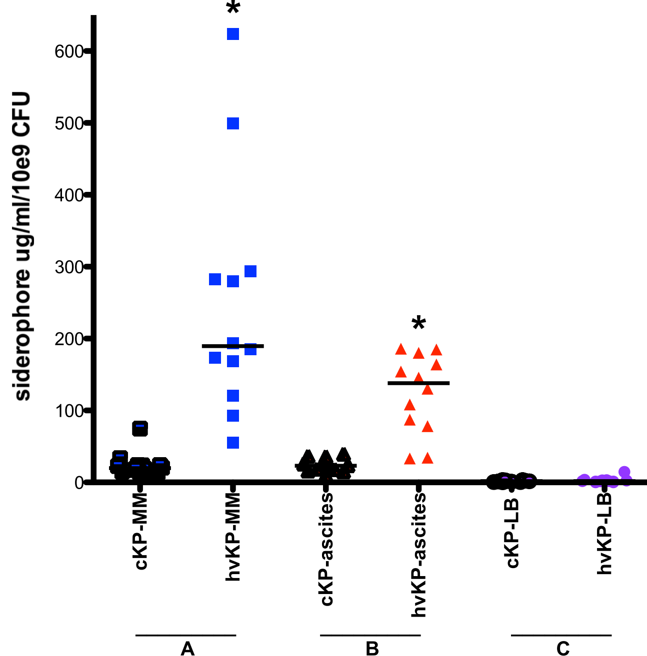

FIG 2 Quantitative measurement of siderophores (SP) in 12 hvKP and 14 cKP strains grown in iron-poor minimal medium (MM), human ascites fluid, and Luria-Bertani broth (LB). Quantitative SP measurements were performed on bacterium-free supernatants harvested after overnight growth. A, MM. B, human ascites fluid. C, LB. The median SP concentration for hvKP strains grown in MM and human ascites fluid, but not LB, was significantly greater than that for cKP strains (*, $P < 0.05/3$). Each symbol represents the mean concentration from 3 independent conditioned media measured in duplicate for a single strain.

($P < 0.0001$). These data extended our published observations and strongly supported the hypothesis that increased SP production was a defining trait for hvKP strains.

SP production is appropriately low when hvKP and cKP strains are grown under Fe-replete conditions. The same hvKP and cKP strains were grown overnight in Luria-Bertani (LB) medium (Fe replete), and SP production was measured as described above. Both hvKP and cKP strains produced similarly low levels of SP ($P = 0.3$) (median [range] of 1.9 [0.08 to 14.7] versus 1.8 [0.92 to 3.1] μg/ml, respectively) (Fig. 2C). SP production normally is low when *Klebsiella* is grown under Fe-replete conditions. Therefore, these data indicated that Fe concentration-mediated regulation of SP production was appropriate in these strains.

HPLC-MS analysis of conditioned medium from hvKP1 demonstrates that aerobactin is the major SP secreted into human ascites fluid and MM. High-pressure liquid chromatography (HPLC) was performed on conditioned media generated from the growth of hvKP1 in human ascites fluid and Fe-poor MM. The observed HPLC profiles were similar, demonstrating 5 major peaks, which were separated and collected as five fractions (1 to 5) (Fig. 3A). Quantitative SP analyses performed on these fractions established that 74.8% and 96.4% of SP from growth in MM and ascites fluid, respectively, were located in fraction 5 (Table 2). Next, fraction 5 was separated into an additional 5 fractions (5a to 5e) (Fig. 3B). One hundred percent of the SP were present in fraction 5c and 5d, with nearly 90% present in 5c. To confirm these quantitative assays, biologic growth/survival assays were performed in which human ascites fluid was supplemented with fractions 5a to 5e. As expected, growth/survival of hvKP1 was optimized by supplementation with fractions 5c and 5d (Fig. 3D). As 5c and 5d elute in neighboring fractions, the activity in fraction 5d probably represents carryover from fraction 5c. Fraction 5c underwent mass spectrometric (MS) analysis, which revealed that fraction 5c contained the SP aerobactin (Fig. 3C). These data demonstrated that for hvKP1, aerobactin was the dominant SP produced, a surprising result considering that all SP are believed to be regulated in a similar manner.

Quantitation of SP from hvKP1 and hvKP1 Δ*iucA* further substantiates that aerobactin is the major SP secreted into human ascites fluid and MM. To further substantiate that aerobactin was the major SP produced by hvKP1 in Fe-poor MM and human ascites fluid, an isogenic derivative of hvKP1 with a disruption in the aerobactin biosynthetic gene *iucA* was generated (hvKP1 Δ*iucA*). hvKP1 and hvKP1 Δ*iucA* were grown overnight in Fe-poor MM, human ascites fluid, or LB medium, and total SP were measured in the supernatants and normalized to 1×10^9 CFU (Fig. 4A). hvKP1 produced a median (range) of 581 (318 to 676) μg/ml in MM, 183 (73 to 214) μg/ml in ascites fluid, and 2.0 (1.1 to 7.8) μg/ml in LB. In contrast, hvKP1 Δ*iucA* produced 21.9 (8.3 to 53.9) μg/ml in MM, 14.8 (8.8 to 17.5) μg/ml in ascites fluid, and no detectable SP in LB, which represent 96.2% and 92% decreases, respectively, in SP production in MM and ascites fluid. These data further demonstrated that aerobactin was the dominant SP produced by hvKP1 when grown under Fe-poor conditions.

Aerobactin is also the major SP secreted by the hvKP strains A1142 and A1365. To assess whether aerobactin was also the major SP secreted by other hvKP strains, conditioned medium generated by the growth of A1142 and A1365 in ascites fluid was fractionated by HPLC and assayed for SP production. HPLC analysis for both strains demonstrated a fraction 5 peak (data not shown) similar to that observed for hvKP1 and shown to contain aerobactin (Fig. 3A and C). Quantitative SP analysis of the HPLC fractions demonstrated that 96% and 95.9% of the total SP activity resided in fraction 5 for A1142 and A1365, respectively (Table 2). To further substantiate these findings, aerobactin-deficient isogenic derivatives of A1142 and A1365 were constructed, and quantitative SP production was measured in conditioned medium and normalized to 1×10^9 CFU (Fig. 4B and C). A1142 produced a median (range) of 177 (109 to 394) μg/ml in MM, 57.5 (30.8 to 196) μg/ml in ascites fluid, and undetectable (undetectable to 3.1 μg/ml) SP in LB. In contrast, A1142 Δ*iucA* produced 45.3 (31.9 to 78.7) μg/ml in MM, 16.1 (13.0 to 17.4) μg/ml in ascites fluid, and undetectable SP in LB, which represented 79.6% and 78.1% decreases, respectively, in SP production in MM and ascites fluid. A1365 produced 219 (146 to 356) μg/ml in MM, 67.1 (42.5 to 125) μg/ml in ascites fluid, and undetectable (undetectable to 1.5 μg/ml) SP in LB. In contrast, A1365 Δ*iucA* produced 10.3 (4.3 to 12.2) μg/ml in MM, 10.2 (4.7 to 11.4) μg/ml in ascites fluid, and undetectable SP in LB, which represented 95.5% and 86.8% decreases, respectively, in SP production in MM and ascites fluid. These data demonstrated that aerobactin was the dominant SP produced by other hvKP strains when grown under Fe-poor conditions.

The gene copy number for *iucA* (aerobactin) is similar to those for *entH* (enterobactin), *iroB* (salmochelin), and *irp2* (yersiniabactin). The aerobactin and salmochelin biosynthetic genes are present on large, 200- to 224-kDa plasmids in CG43, NTUH-

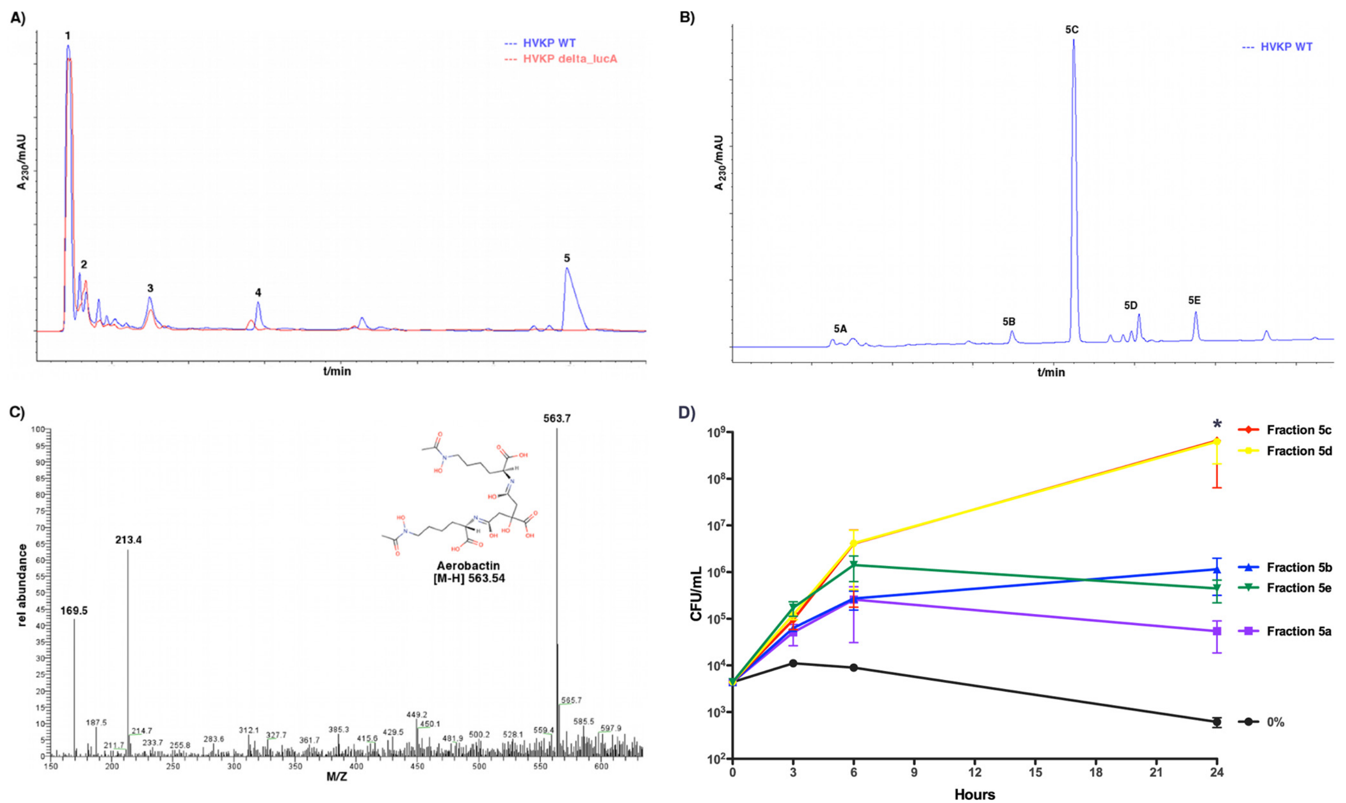

FIG 3 HPLC-mass spectrometry analysis of hvKP1-generated conditioned medium. (A) A representative HPLC profile of conditioned medium generated from the growth of hvKP1 in human ascites fluid. The fractions were designated 1 to 5. (B) Fraction 5 was further separated by HPLC into fractions 5a to 5e. (C) Mass spectrometric analysis was performed on fraction 5c and established that the species present was aerobactin. (D) The biological activities of fractions 5a to 5e were assessed by assessing the growth of hvKP1 in human ascites fluid supplemented with each fraction (5% final concentration). Supplementation with fractions 5c and 5d resulted in a significant increase in growth and/or survival for hvKP1 compared to 0% supplementation (*, $P < 0.05/5$). Data are means ± SEM; $n = 3$.

K2044, and hvKP1, the hvKP strains assessed to date (16, 31, 32). In contrast, enterobactin and yersiniabactin biosynthetic genes are chromosomally located. Quantitative PCR was performed to assess gene copy number. As expected due to the presence of 8 RNA gene clusters in *K. pneumoniae*, the quantitative PCR-calculated threshold, an inverse correlate for gene copy number, was significantly less for genes encoding 23S rRNA than for *iucA*, *entH*, *iroB*, and *irp2* under all conditions tested ($P < 0.05/4$) (Fig. 5). In contrast, the calculated threshold was similar for *iucA*, *entH*, *iroB*, and *irp2* under nearly all conditions tested (Fig. 5). There was a trend for an increase in the calculated threshold for *entH* compared to *iucA*, *iroB*, and *irp2* when 5,000 picograms of DNA was used ($P > 0.05/4$ but $P < 0.05$). However, since both *entH* and *irp2* were chromosomally located, this difference did not appear to be biologically significant (33). Likewise, the calculated threshold for *iucA* was statistically less than those for *entH* and *irp2* when 50 picograms of DNA was used. However, this difference was not observed when *iroB*, which like *iucA* is plasmid located, was compared to *entH* and *irp2* at this DNA concentration. Therefore, again these small differences did not appear to be biologically significant. Taken together, these data do not support increased copy number of the aerobactin biosynthetic genes as being responsible for its increased production.

Aerobactin enables the growth of hvKP1 and hvKP1 Δ*iucA* in human ascites fluid. To confirm that aerobactin was the primary factor in conditioned medium that enhanced the growth/survival of hvKP1 in human ascites fluid, the growth/survival of hvKP1 was assessed in human ascites fluid in the presence or absence of various growth supplements. With a starting inoculum of $<1 \times 10^5$ CFU/ml, the growth of hvKP1 in ascites fluid was limited (Fig. 6A). Likewise, the growth of hvKP1 was similarly limited when the ascites fluid was supplemented with conditioned medium deficient in aerobactin (conditioned medium generated by hvKP1 Δ*iucA*) (Fig. 6A). However, growth of hvKP1 was significantly greater ($P < 0.05/3$) in ascites fluid supplemented with purified aerobactin (460 nM; 260 μg/ml) or conditioned medium containing aerobactin (conditioned medium generated by hvKP1) (Fig. 6A). Similar results were obtained with hvKP1 Δ*iucA* and the same supplements (Fig. 6B). Importantly, hvKP1 Δ*iucA* was chemically complemented with purified aerobactin. These data confirm that aerobactin is the primary factor responsible for the phenotype of increased growth/survival of hvKP1 in human ascites fluid supplemented with conditioned medium.

The growth/survival of the wild-type strain hvKP1 and hvKP1/pFUS2 is significantly greater than that of their isogenic aerobactin-deficient derivatives hvKP1 Δ*iucA* and hvKP1 Δ*iucA*/pFUS2, respectively, in human ascites fluid *ex vivo*. To determine whether aerobactin enhanced growth/survival under clinically relevant conditions, hvKP1, hvKP1/pFUS2, hvKP1 Δ*iucA*, and hvKP1 Δ*iucA*/pFUS2 were grown in human ascites fluid *ex vivo* at starting titers of approximately 3×10^4 and 1×10^6 CFU/ml. The growth/survival of hvKP1 and hvKP1/pFUS2 was

TABLE 2 Siderophore concentrations in the different HPLC fractions

Strain/medium	Mean siderophore concn (μg/ml) ± SEM (% of total) in HPLC fraction[a]:									
	1	2	3	4	5	5a	5b	5c	5d	5e
hvKP1/MM[b]	69.1 ± 11.6 (24.9)	0.18 ± 0.18 (<0.1)	0.18 ± 0.18 (<0.1)	0.25 ± 0.25 (<0.1)	207.2 ± 22.7 (74.8)					
hvKP1 Δ*iucA*/MM	39.6 ± 0.84 (100)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)					
hvKP1/ascites fluid	13.1 ± 0.40 (3.6)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	350.8 ± 13.3 (96.4)					
A1142/ascites fluid	14.1 ± 0.40 (4.0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	343.3 ± 11.8 (96.0)					
A1365/ascites fluid	13.9 ± 0.50 (4.1)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	327.6 ± 21.8 (95.9)					
hvKP1/MM						0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	210.9 ± 24.1 (89.3)	25.3 ± 4.3 (10.7)	0.0 ± 0.0 (0)

[a] $n = 4$ to 6 for all measurements.
[b] MM, iron-poor M9 minimal medium.

significantly greater than that of hvKP1 Δ*iucA* and hvKP1 Δ*iucA*/pFUS2, respectively ($P < 0.05/2$) (Fig. 7A and B). The diminished growth of hvKP1 Δ*iucA* was complemented in *trans* by the plasmid pFUS2[*iucA-D*], which expressed IucA (Fig. 7A). Interestingly, when the higher starting inoculum of 1×10^6 CFU/ml was used, the difference in growth/survival between hvKP1 and hvKP1 Δ*iucA* was observed at 24 h. However, when the lower inoculum of 3×10^4 CFU/ml was used, this difference was not discerned over the first 24 h. These data demonstrated that aerobactin was needed for optimal growth/survival in a time- and inoculum-dependent fashion.

The virulence of the wild-type strain hvKP1 is significantly greater than that of its isogenic aerobactin-deficient derivative hvKP1 Δ*iucA* in both mouse s.c. and i.p. challenge models. To determine whether aerobactin contributed to the hypervirulent phenotype of hvKP1 *in vivo*, first an outbred mouse s.c. challenge model was used. Four challenge inocula were used, ranging from 3.0×10^2 to 3.5×10^5 CFU and from 2.8×10^2 to 3.6×10^5 CFU in approximately $\log_{10}$ intervals for hvKP1 and hvKP1 Δ*iucA*, respectively. The mortality of mice challenged with hvKP1 was significantly greater than that of mice challenged with hvKP1 Δ*iucA* ($P < 0.05$) (Fig. 8A and B). Generally, the timing of mortality was inversely proportional to the challenge titer. Remarkably, animals challenged with 3.0×10^2 CFU of hvKP1 experienced 100% morality, with deaths observed from 6 to 9 days postchallenge. Next, an i.p. challenge model was employed. Four challenge inocula were used, ranging from 3.2×10^1 to 3.2×10^4 CFU and from 2.4×10^1 to 2.4×10^4 CFU in $\log_{10}$ intervals for hvKP1 and hvKP1 Δ*iucA*, respectively. The mortality of mice challenged with hvKP1 was significantly greater than that of mice challenged with hvKP1 Δ*iucA* ($P < 0.05$) (Fig. 8C and D). These data demonstrated the hypervirulence of hvKP and strongly supported the importance of aerobactin in contributing to this hypervirulent phenotype.

DISCUSSION

In this study, we supported our hypotheses that hvKP strains produce more SP than cKP strains and that this trait is an important mechanism contributing to their hypervirulence. The data presented demonstrate that all 12 hvKP strains tested produced more SP than 14 cKP blood isolates under Fe-poor conditions, such as occur in the human host (Fig. 2). Further, we showed that the SP aerobactin primarily accounted for the significant increase in SP production observed with the hvKP strains hvKP1, A1142, and A1365 (Table 2; Fig. 4). In addition, we demonstrated that aerobactin was the primary factor in conditioned medium that enhanced the growth/survival of hvKP1 in human ascites fluid (Fig. 6). More importantly, hvKP1 Δi*ucA* grew/survived less well than its wild-type parent *ex vivo* in human ascites fluid (Fig. 7), and this growth defect was complemented in *trans* by the aerobactin biosynthetic genes (Fig. 7A). Lastly, hvKP1 Δi*ucA* was significantly less virulent in mouse s.c. and i.p. challenge models than its wild-type parent hvKP1 (Fig. 8). Taken together, these data directly established that under Fe-poor conditions, hvKP strains produce increased levels of SP compared to cKP strains, that aerobactin is the dominant SP produced by hvKP strains, and that aerobactin contributes to the virulence of hvKP1 *ex vivo* and *in vivo*.

The mechanism responsible for the increased aerobactin production in hvKP remains unclear. The ferric uptake regulator (Fur) has been established as an important negative regulator of Fe acquisition systems in *K. pneumoniae*, including hvKP strains

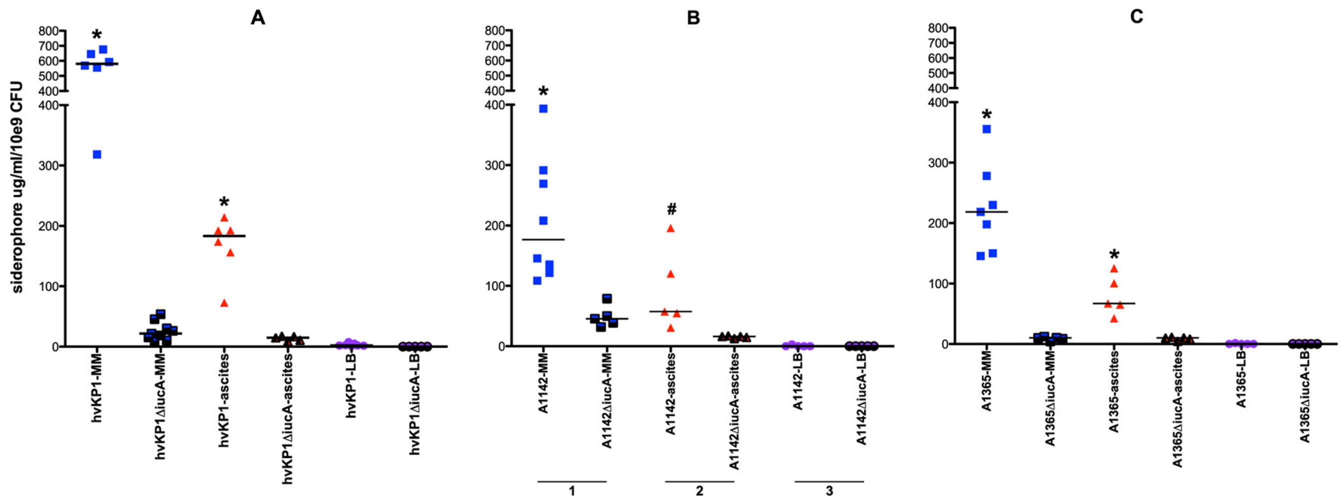

FIG 4 Quantitative measurement of siderophores (SP) in hvKP1, hvKP1 Δ*iucA*, A1142, and A1142 Δ*iucA* grown in iron-poor MM, human ascites fluid, and LB broth. Quantitative SP measurements were performed on bacterium-free supernatants harvested after overnight growth. 1, MM; 2, human ascites fluid; 3, LB. (A) hvKP1 and hvKP1 Δ*iucA*. (B) A1142 and A1142 Δ*iucA*. (C) A1365 and A1365 Δ*iucA*. The median SP concentrations for hvKP1, A1142, and A1365 compared to hvKP1 Δ*iucA*, A1142 Δ*iuc*, and A1365 Δ*iucA* when grown in MM and for hvKP1 and A1142 compared to hvKP1 Δ*iucA* and A1142 Δ*iuc* when grown in ascites fluid were significantly greater (*, $P < 0.05/3$). There was a trend for the median SP concentration of A1365 compared to A1365 Δ*iucA* when grown in ascites fluid (#, $P < 0.05$ but $>0.05/3$). Each symbol represents the mean concentration from an independent conditioned medium measured at least in duplicate for each strain.

(34). The fact that SP production was appropriately decreased in Fe-replete LB medium (Fig. 2C) suggests that regulation is appropriate under conditions where a high concentration of Fe is present. Presumably this is mediated, at least in part, by Fur, but this has not yet been formally established for hvKP1. Since the aerobactin genes were present on a large plasmid, increased gene copy number was a consideration. However, quantitative PCR data that examined this possibility demonstrated that this was not the case. The copy numbers of the yersiniabactin and enterobactin genes (*irp2* and *entH*), which are located on the chromosome, are similar to those of the aerobactin and salmochelin genes (*iucA* and *iroB*), which are located on the plasmid (Fig. 5). Published data have shown that environmental conditions can significantly affect SP production. pH and carbon sources have been shown to modulate SP production in *Escherichia coli* (35). Optimal aerobactin production occurred at a pH of 5.6 when glycerol was used as the carbon source (as opposed to glucose). In an avian extraintestinal pathogenic *E. coli* strain (36) grown in MM, aerobactin comprised 1.9% of the total SP produced. In contrast, in a chicken infection model this proportion increased to 48% and 58.9% in pericardia and air sacs, respectively. We did not observe this discordance between MM and a more clinically relevant setting (ascites fluid in this study). Recent work on the small RNA RyhB has established that regulators beyond Fur are important for bacterial Fe homeostasis and SP production (37). Although, the mechanism(s) responsible for increased aerobactin production in hvKP1 is presently unclear and requires further study, its delineation would be important and may lend insight into potential therapeutic interventions.

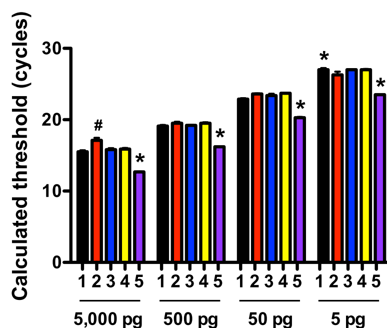

FIG 5 The gene copy numbers for *iucA*, *entH*, *iroB*, and *irp2* are similar. Quantitative PCR was performed on 4 independent concentrations of chromosomal DNA. The calculated threshold (cycles) correlates inversely with gene copy number. Black, *iucA*; red, *entH*; blue, *iroB*; yellow, *irp2*; purple, 23S RNA. *, $P < 0.05/4$ for 23S RNA compared to *iucA*, *entH*, *iroB*, and *irp2* for all DNA concentrations. #, $P > 0.05/4$ but $P < 0.1$ for *entH* compared to *iucA*, *iroB*, and *irp2* for 5,000 pg. *, $P < 0.05/4$ for *iucA* compared to *entH* and *irp2* for 50 pg. $n = 3$ for all measurements.

As discussed in the introduction, molecular epidemiologic studies were suggestive that aerobactin may be a factor that enhanced the virulence of hvKP due to its increased prevalence in hvKP strains compared to cKP strains (8, 15, 25). Older studies had demonstrated that the virulence of *E. coli* and *K. pneumoniae* was enhanced with the presence of a plasmid containing genes that encoded aerobactin (17, 38, 39). However, the presence of other virulence factors encoded on these plasmids, such as RmpA, which increased capsule production and mediated the hypermucoviscosity phenotype, was a confounding variable. More recent studies using isogenic derivatives have shown that aerobactin was the only SP needed to mediate virulence for an avian extraintestinal pathogenic *Escherichia coli* strain in a chicken infection model (40) and that in a murine urinary tract infection model the absence of the aerobactin receptor decreased fitness (41). Therefore, despite having a lower Fe association constant ($K_f = 10^{22.9}$) (42) than enterobactin ($K_f = 10^{52}$) (42), yersiniabactin ($K_f = 10^{36}$) (43), and transferrin ($K_f \sim 10^{30}$) (44), aerobactin appears to overcome this perceived disadvantage under clinically relevant conditions. Potential mechanisms for these observations include the facts that aerobactin is recycled (45) and transfers Fe from trans-

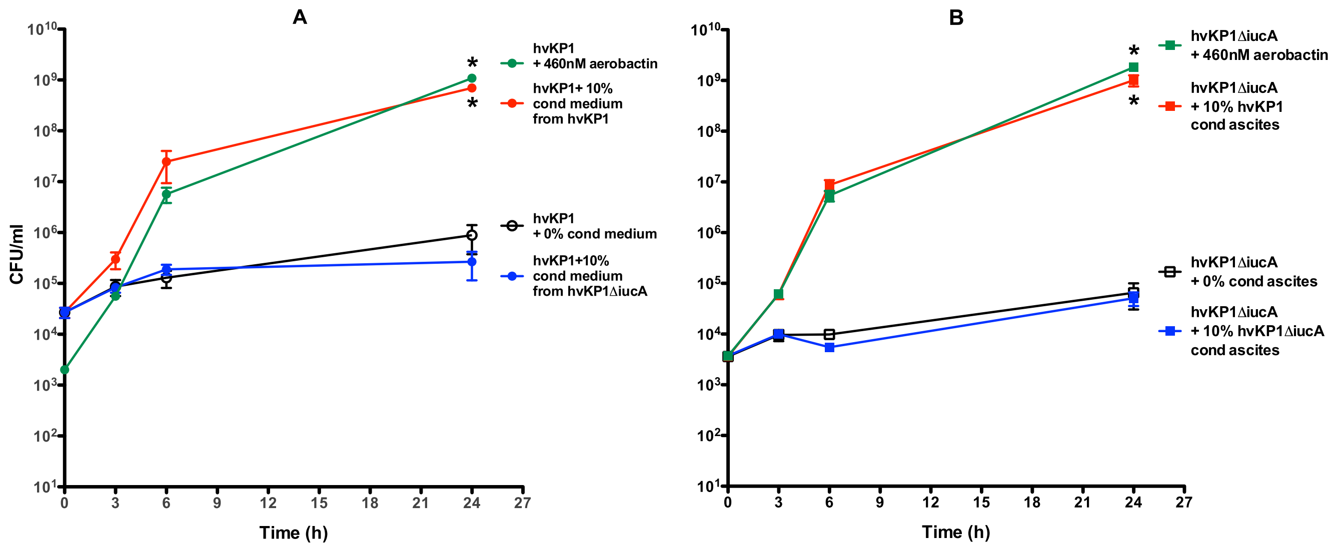

FIG 6 Growth/survival of hvKP1 and hvKP1 Δ*iucA* in human ascites fluid supplemented with purified aerobactin or conditioned medium generated by hvKP1 or hvKP1 Δ*iucA*. The growth/survival of hvKP1 and hvKP1 Δ*iucA* was assessed at 0, 3, 6, and 24 h in 100% human ascites fluid. (A) Growth/survival of hvKP1 in ascites fluid that was not supplemented (0%) or was supplemented with 10% conditioned ascites fluid generated by hvKP1 (aerobactin replete) or hvKP1 Δ*iucA* (aerobactin deficient) or purified aerobactin (460 nM; 260 μg/ml). *, $P < 0.05/3$. (B) Growth/survival of hvKP1 Δ*iucA* in ascites fluid that was not supplemented (0%) or was supplemented with 10% of conditioned ascites fluid generated by hvKP1 (aerobactin replete) or hvKP1 Δ*iucA* (aerobactin deficient) or purified aerobactin (460 nM; 260 μg/ml). *, $P < 0.05/3$. Data are means ± SEM; $n = 4$ to 6.

ferrin more efficiently than enterobactin (46) and that in contrast to the case for enterobactin, this transfer is not impeded by albumin or immunoglobulins (47). Further, aerobactin is resistant to the inhibitory effects of lipocalin 2 (48).

An increasing body of data has begun to address whether certain SP may be more efficacious in certain settings (41, 49, 50). Our *ex vivo* and *in vivo* data with human ascites fluid and mouse infection models determined that the beneficial effect of aerobactin at lower challenge titers was delayed. In contrast to enterobactin, salmochelin, and yersiniabactin, which are produced during logarithmic phase (51), aerobactin is maximally produced in late logarithmic-secondary metabolism (stationary) phase (52). Therefore, it is possible that aerobactin may contribute to the phenotype of hvKP under conditions when the host and bacterium are at a "stalemate," such as might occur with a lower challenge inoculum. The possession of a factor that enables infection

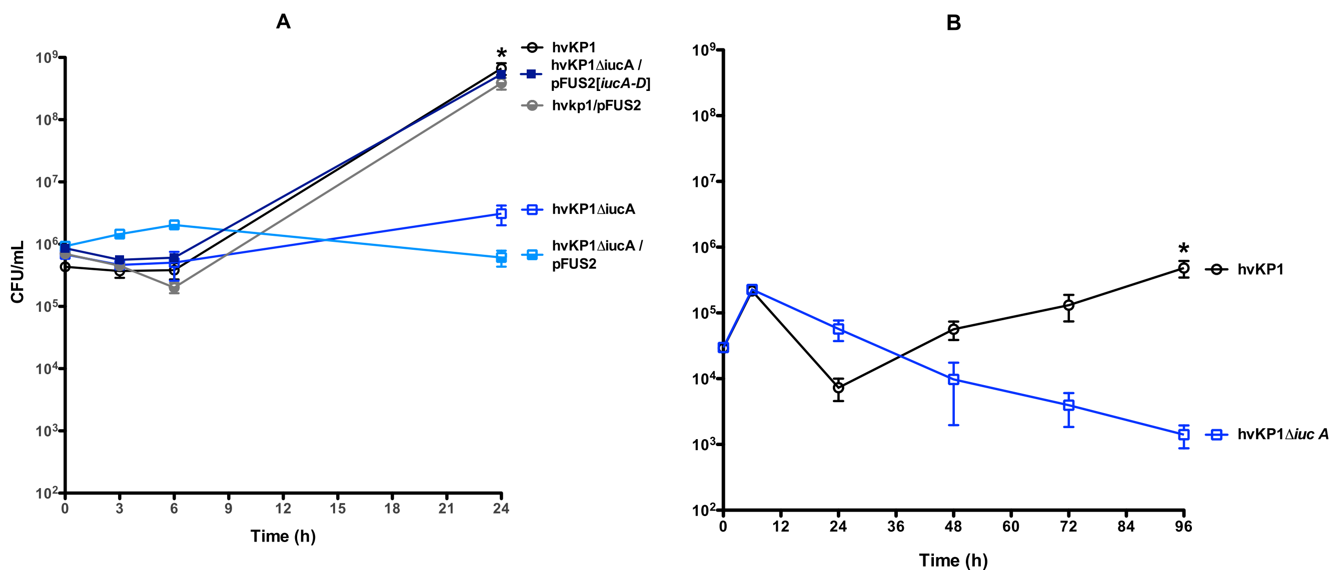

FIG 7 Growth/survival of hvKP1 and hvKP1 Δ*iucA* in human ascites fluid. The growth/survival of hvKP1, hvKP1/pFUS2, hvKP1 Δ*iucA*, hvKP1 Δ*iucA*/pFUS2, and hvKP1 Δ*iucA*/pFUS2[*iucA-D*] was assessed in 100% human ascites fluid. (A) A midlevel starting inoculum of approximately 1×10^6 CFU/ml was used, and growth/survival was assessed at 0, 3, 6, and 24 h. The growth/survival of hvKP1 and hvKP1/pFUS2 was significantly increased compared to that of hvKP1 Δ*iucA* and hvKP1 Δ*iucA*/pFUS2, respectively. *, $P < 0.05/2$. Data are means ± SEM ($n = 5$ to 8). (B) A low starting inoculum of $<1 \times 10^5$ CFU/ml was used, and growth/survival was assessed at 0, 12, 24, 48, 72, and 96 h. The growth/survival of hvKP1 was significantly increased compared to that of hvKP1 Δ*iucA*. *, $P < 0.05$. Data are means ± SEM from 3 independent experiments with $n = 3$ for each experiment.

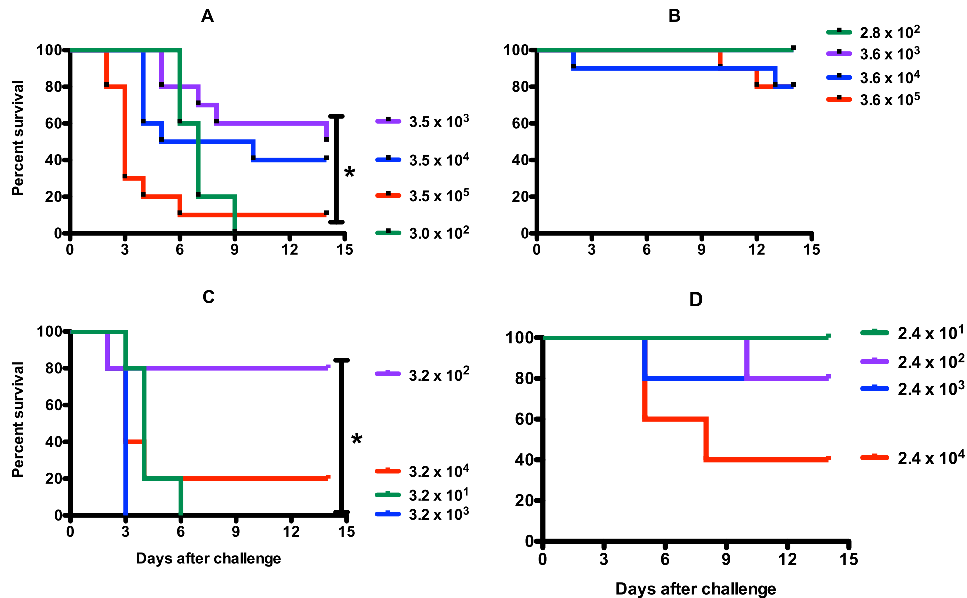

FIG 8 Survival of outbred CD1 mice after subcutaneous (s.c.) or intraperitoneal (i.p.) challenge with hvKP1 and hvKP1 Δ*iucA*. (A and B) Animals were challenged s.c. with 3.0×10^2 ($n = 5$), 3.5×10^3 ($n = 10$), 3.5×10^4 ($n = 10$), or 3.5×10^5 ($n = 10$) CFU of hvKP1 (A) or with 2.8×10^2 ($n = 5$), 3.6×10^3 ($n = 10$), 3.6×10^4 ($n = 10$), or 3.6×10^5 ($n = 10$) CFU of hvKP1 Δ*iucA* (B). (C and D) Animals were challenged i.p. with 3.2×10^1 ($n = 5$), 3.2×10^2 ($n = 5$), 3.2×10^3 ($n = 5$), or 3.5×10^4 ($n = 5$) CFU of hvKP1 (C) or with 2.4×10^1 ($n = 5$), 2.4×10^2 ($n = 5$), 2.4×10^3 ($n = 5$), or 2.4×10^4 ($n = 5$) CFU of hvKP1 Δ*iucA* (D). Strains were grown overnight in LB medium. An *in extremis* state or death was scored as nonsurvival. *, $P < 0.05$ for hvKP1 compared to hvKP1 Δ*iucA*.

with a lower inoculum would significantly enhance virulence. This characteristic may be critical for a pathogen, such as hvKP, which infects healthy ambulatory hosts who do not have an overt portal of entry (2). This is in contrast to the case for cKP, where infections are usually health care associated and often in the setting of intravascular or urinary catheters, endotracheal tubes, or open wounds.

Two challenge routes were employed to assess the role of aerobactin *in vivo*. Initially s.c. challenge was used, since hvKP strains may gain entry into the human host via this route, thereby potentially mimicking human infection (2). Further, it had been previously established that s.c. challenge with hvKP1 resulted in subsequent systemic dissemination over the next 24 to 72 h (22), making this model clinically relevant since the ability to disseminate is a defining characteristic of hvKP strains. Further and importantly, this route of infection will result in bacteria entering the bloodstream at a physiologically appropriate titer, which in turn reflects what happens during human infection and avoids a "cytokine storm" that may occur soon after bacterial challenge by other routes, such as intravenous or intraperitoneal challenge. Lastly, since aerobactin is produced during the late logarithmic-secondary metabolism phase (52), models that use metrics generated over the first 24 h may not be able to define a role for aerobactin. Further, the ability to produce aerobactin during nonlogarithmic growth may be important for growth/survival within abscesses. In contrast to our results, an isogenic aerobactin-deficient derivative of the hvKP strain NTUH-K2044 had a 50% lethal dose (LD_{50}) similar to that of its wild-type parent after i.p. and intragastric challenge in BALB/cByl mice (25). Therefore, to determine whether this contrasting observation was related to bacterial strain, challenge route, or animal breed differences, we challenged mice i.p. with hvKP1 and hvKP1 Δ*iucA*. Similar to our findings after s.c. challenge, hvKP1 was significantly more virulent than hvKP1 Δ*iucA* (Fig. 8C and D). Since our studies used outbred CD1 mice and the studies with NTUH-K2044 used an inbred BALB/c derivative, we believe that the observed difference is most likely related to the mouse strains used; however, we cannot exclude a role for differences in the bacterial strains.

The observation that hvKP strains produce more SP than cKP strains has potential implications beyond pathogenesis. Presently, since we are still establishing which factors confer hvKP strains with their unique virulence capabilities, reliable markers for differentiating cKP and hvKP pathotypes are lacking. The hypermucoviscous phenotype (mediated by RmpA/A2) as manifested by a positive string test, in combination with the clinical syndrome, are perceived to be the best markers for hvKP strains (2). However, even if the clinical microbiology laboratory considered this test, its performance can be challenging and its interpretation subjective. Further, the sensitivity and specificity of the string test for hvKP strains have not been defined, and cKP strains may also be string test positive (2). The development of a more objective diagnostic test(s) that can be employed by the clinical microbiology laboratory to reliably identify hvKP strains is requisite. An assessment of total SP production, which could be measured by a plate assay that incorporates chrome azurol S dye, may represent the solution or at least an ancillary test that could be used in conjunction with the string test and clinical features. The ability to objectively identify hvKP strains will enhance our ability to perform more comprehensive epidemiologic studies and to define the full spectrum of infectious syndromes caused by hvKP. It will also enable the incidence of infection, especially outside the Asian Pacific Rim, to be defined. Perhaps most importantly, the identification of a *K. pneumoniae* isolate as being an hvKP strain will assist the clinician in disease management. The knowledge that an hvKP strain is causing infection should prompt a search for concomitant or subsequent metastatic sites of infection, which may require drainage or

a site-driven modification of the antimicrobial regimen. It is critical to be particularly vigilant for endophthalmitis and central nervous system infection.

K. pneumoniae strains variably possess genes encoding enterobactin, salmochelin, yersiniabactin, and aerobactin. However, in contrast to hvKP strains, where most if not all strains are aerobactin positive, only 7 to 18% of putative cKP strains are aerobactin-positive (8, 15, 25). The data presented here support the concept that in an hvKP background, aerobactin is an important virulence factor and is the major contributor of high SP levels observed under Fe-poor conditions. However, due to the lack of an unequivocal definition of the hvKP pathotype, it is unclear what proportion of aerobactin-positive putative cKP strains are truly cKP, unrecognized hvKP, or of an intermediate phenotype. Further, for true cKP aerobactin-positive strains (if they exist), the contribution of aerobactin to total SP production and virulence requires assessment. In one study, for 2 stool isolates of *K. pneumoniae* grown in M63-glycerol that produced enterobactin, salmochelin, yersiniabactin, and aerobactin, aerobactin production accounted for 62% and 86% of SP production (53). However, total SP levels were not reported, and it was unclear whether these stains were cKP or hvKP isolates. Interestingly, genes that encode yersiniabactin were more prevalent in hvKP (91%) than in cKP (22%) (25), but data from this report do not support yersiniabactin as a major contributor to the high SP levels observed in hvKP strains under Fe-poor conditions.

In summary, the data presented in this report strongly support that hvKP strains produce significantly higher levels of SP than cKP strains. In the hvKP strains tested to date (hvKP1, A1141, and A1365) increased aerobactin production accounts for most if not all of this increased SP production. Further, we demonstrated that aerobactin is a major virulence determinant for hvKP1. These data have potential implications for the development of novel diagnostic tests and preventative and therapeutic strategies in the management of hvKP infection. This may be particularly important since recent data support that hvKP has the potential to be acquire significant antimicrobial resistance (54), similar to what is now occurring with cKP (55).

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