Biofilm formed by a hypervirulent (hypermucoviscous) variant of *Klebsiella pneumoniae* does not enhance serum resistance or survival in an in vivo abscess model

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**Abbreviations**: hvKP, hypervirulent *Klebsiella pneumoniae*; cKP, classic *Klebsiella pneumoniae*; CA-PLA, community-acquired pyogenic liver abscess; CNS, central nervous system; GNB, Gram-negative bacillus; LB, Luria-Bertani; cfu, colony forming units; PBS, phosphate-buffered saline; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; TE, Tris-ethylenediaminetetraacetic acid; bp, base pairs; delta 56°C, heated at 56°C for 30 minutes; rpm, rotations per minute; SEM, standard error of the mean; AUC, area under the curve; TCA, tricarboxylic acid; NHS, normal human serum; mL, milliliter; ORF, open reading frame

A new hypervirulent (hypermucoviscous) clinical variant of *Klebsiella pneumoniae* (hvKP) has emerged over the last decade. Our goal is to identify new mechanisms, which increase the virulence hvKP. It has been shown that hvKP strains produce more biofilm than “classical” stains of *K. pneumoniae*, therefore we hypothesized that biofilm formation may contribute to the pathogenesis of systemic infection. To test this hypothesis, transposon mutants of the model pathogen hvKP1 were generated and screened for decreased production of biofilm. Three mutant constructs with disruptions in *glnA* [putatively encodes glutamine synthetase, hvKP1 *glnA*::EZ::TN, KAN-2], *sucD* [putatively encodes succinyl-CoA synthase a subunit, hvKP1 *sucD*::EZ::TN, KAN-2], and *tag* [putatively encodes transcriptional antiterminator of glycerol uptake operon, hvKP1 *tag*::EZ::TN, KAN-2] were chosen for further characterization and use in biologic studies. Quantitative assays performed in rich laboratory medium and human ascites confirmed the phenotype and a hypermucoviscosity assay established that capsule production was not affected. However, compared with its wild-type parent, neither planktonic cells nor biofilms of *glnA*::Tn, *sucD*::Tn and *tag*::Tn displayed a change to the bactericidal activity of 90% human serum. Likewise, when assessed in a rat subcutaneous abscess model, the growth and survival of *glnA*:Tn, *sucD*:Tn and *tag*:Tn in abscess fluid was similar to hvKP1. In this report we identify three new genes that contribute to biofilm formation in hvKP1. However, decreased biofilm production due to disruption of these genes does not affect the sensitivity of these mutant constructs to 90% human serum when in planktonic form or within a biofilm. Further, their virulence in an in vivo abscess model was unaffected.

**Introduction**

A new, hypervirulent clinical variant of *Klebsiella pneumoniae* (hvKP) has emerged over the last two decades. Initial reports were from the Pacific Rim, but more recently hvKP has become an emerging pathogen in the United States, Canada, Europe, Israel, South Africa, Australia and elsewhere. At first, infection due to hvKP was characterized and distinguished from traditional infections due to “classical” *K. pneumoniae* (cKP) by: (1) presenting as community-acquired pyogenic liver abscess (CA-PLA), (2) affecting patients lacking a history of hepatobiliary disease and (3) a propensity for causing metastatic spread to distant sites in 11–80% of cases [e.g., lungs, pleura, prostate, bone, joints, kidneys, spleen, muscle/fascia, soft-tissue, skin, eyes and central nervous system (CNS)]. More recently, hvKP has also been described to cause a variety of serious extrahepatic...
not tested. Therefore, in order to test the hypothesis that biofilm formation by hvKP strains contributed to systemic infection a model hvKP strain (hvKP1) was randomly mutagenized and derivatives were screened for the production of biofilm. The primary goal of this report was not to perform an extensive screen for genes and their products that contribute to biofilm formation in hvKP, but rather to identify some mutants that produced less biofilm for use in biologic studies. Three mutant derivatives that produced less biofilm than hvKP1 were chosen for additional characterization and biologic studies to test our hypothesis. Although the results from this study did not support our hypothesis, a role for biofilm formation by hvKP during systemic infection cannot be excluded.

**Results**

**Generation and identification of hvKP1 mutant derivatives that produced less biofilm.** Two hundred thirty-two transposon mutant derivatives of hvKP1 were generated and screened for biofilm formation. The gene disrupted in mutant constructs that produced less biofilm was determined. Five unique mutants were identified that produced less biofilm; a rate of 2%. One of the five mutants had a disruption in the putative FimC chaperone protein. FimC is putatively part of a type 1, mannose-sensitive adhesin. A similar protein had already been identified as contributing to biofilm formation in hvKP ( locus tag K1_4245\(^\text{TM}\)). Therefore it was not further characterized; however, its identification validated our screen. Three mutant constructs (O10, 159 and H68) were chosen for further studies based on the identification of the gene disrupted. The mean ± SEM \( \Delta S_{505} \) (percentage decrease relative to wild-type) of this initial screen for biofilm formation was 0.77 ± 0.17 for hvKP1 and 0.21 ± 0.15 (73%), 0.51 ± 0.1 (34%) and 0.12 ± 0.018 (84%) for O10, 159 and H68 respectively.

The transposon insertions in O10, 159 and H68 were located in glnA (putatively encodes glutamine synthetase, locus tag KP1_0030 in the K. pneumoniae NTUH-K2044 chromosome), sucD (putatively encodes succinyl-CoA synthase \( \xi \) subunit, locus tag KP1_1690 in the K. pneumoniae NTUH-K2044 chromosome) and tag (putatively encodes transcriptional antiterminator of glyceral uptake operon, locus tag KP1_1112 in the K. pneumoniae NTUH-K2044 chromosome) respectively. These mutant constructs were respectively renamed hvKP1 glnA::EZ::TN < KAN-2 > (glnA::TN), hvKP1 sucD::EZ::TN < KAN-2 > (sucD::TN), and hvKP1 tag::EZ::TN < KAN-2 > (tag::TN). To confirm that the transposon insertion did not result in a polar effect, RT-PCR was performed on RNA purified from glnA::TN, sucD::TN, and tag::TN to determine if the transcript from the downstream gene was produced. This proved to be the case for all constructs thereby excluding potential polar effects.

**Characterization of the growth of hvKP1, glnA::TN, sucD::TN and tag::TN in Luria-Bertani (LB) medium and human ascites.** The first step in the characterization of the biofilm mutants was an assessment of growth in LB medium (rich laboratory medium) and in human ascites (a clinically relevant body fluid). The growth of glnA::TN, sucD::TN, and tag::TN was slightly less in LB medium compared with hvKP1 (Fig. 1A). The mean AUCs (\( \Delta A_{600} \) . hour)
+/− SEM were 68.7 ± 0.23, 59.1 ± 1.3, 62.2 ± 0.56 and 59.6 ± 2.7 for hvKP1, glnA::Tn, sucD::Tn and tag::Tn respectively. Although this difference was statistically significant for glnA::Tn and sucD::Tn and there was a trend for a difference for tag::Tn compared with hvKP1, the magnitudes of these differences were small (14% for glnA::Tn, 9.5% for sucD::Tn, 13.2% for tag::Tn).

Next the growth of glnA::Tn, sucD::Tn, and tag::Tn was assessed in human ascites (Fig. 1B). The mean AUCs (log10 cfu. hour) +/− SEM were 427.7 ± 1.7, 402.1 ± 3.2, 427.7 ± 1.7 and 383.7 ± 2.5 for hvKP1, glnA::Tn, sucD::Tn and tag::Tn respectively. Although the difference was statistically significant for glnA::Tn and tag::Tn compared with hvKP1, the magnitude of these differences were small (6.0% for glnA::Tn, 0.0% for sucD::Tn, 10% for tag::Tn).

Characterization of biofilm formation by hvKP1, glnA::Tn, sucD::Tn and tag::Tn in LB medium and human ascites. Next, quantitative biofilm assays were performed over time for hvKP1 and glnA::Tn, sucD::Tn, and tag::Tn in LB medium and human ascites. The assessment in ascites was important because it is clinically more relevant than LB; a rich laboratory medium. When performed in LB medium the mean AUCs (Å595 . hour) +/− SEM (percent decrease relative to wild-type) were 8.2 ± 0.96, 2.0 ± 0.3 (76%), 3.4 ± 0.89 (59%) and 3.1 ± 0.44 (62%) for hvKP1, glnA::Tn, sucD::Tn and tag::Tn respectively.

When performed in ascites the mean AUCs (Å595 . hour) +/− SEM (percent decrease relative to wild-type) were 6.2 ± 0.97, 2.3 ± 0.78 (63%), 2.2 ± 0.70 (65%) and 1.2 ± 0.07 (81%) for hvKP1, glnA::Tn, sucD::Tn and tag::Tn respectively. There was a trend for glnA::Tn, sucD::Tn and tag::Tn to produce significantly less biofilm in 100% human ascites medium compared with its wild-type parent hvKP1 (Fig. 2B). These data confirm that glnA::Tn, sucD::Tn and tag::Tn produce a decreased amount of biofilm.
compared with their wild-type parent hvKP1 in both a rich laboratory medium and an ex vivo human body fluid.

Characterization of the hypermucoviscosity of hvKP1, glnA::Tn, sucD::Tn and tag::Tn in LB medium. Capsular polysaccharide production has been shown to contribute to biofilm formation, including hvKP strains.\textsuperscript{28,32} The hypermucoviscosity assay was used to assess the amount of capsule since this assay has been shown to reflect the degree of capsule production.\textsuperscript{22,28} Sedimentation of bacterial cells after low speed centrifugation is inversely proportional to the quantity of capsule. Therefore, the $\Delta A_{600}$ of the supernatant reflects the degree of capsule production. Each of the mutant derivatives produced a higher mean $\Delta A_{600}$ than their wild-type parent (0.88 ± 0.002, 0.75 ± 0.007, 0.78 ± 0.008 and 0.71 ± 0.001 for glnA::Tn, sucD::Tn, tag::Tn and hvKP1 respectively; $n = 5$). These data support the concept that these biofilm mutants do not produce less capsule than their wild-type parent.

The ability to produce less biofilm did not affect resistance to complement-mediated bactericidal activity in vitro. Next, the effect of producing a decreased amount of biofilm on a biologically relevant host defense system was assessed in vitro, namely complement-mediated bactericidal activity. As a first step, the effect of complement mediated bactericidal activity was tested against planktonic cells of hvKP1, glnA::Tn, sucD::Tn and tag::Tn. No significant decrease in growth and/or survival was observed for all strains exposed to 90% normal human serum over 24 h compared with strains exposed to 90% normal human serum in which complement was inactivated (data not shown). The effect of complement mediated bactericidal activity against hvKP1, glnA::Tn, sucD::Tn and tag::Tn contained within a biofilm was assessed next (Fig. 3). Note that, as expected, the starting inoculum of cfu within the biofilm was greater for hvKP1 compared with glnA::Tn, sucD::Tn and tag::Tn. Despite that, the effect of complement-mediated bactericidal activity was similar for all strains. The AUC was used to compare growth and/or survival of strains when exposed to 90% serum in which complement was inactivated (delta 56°C) or remained active. The growth and/or survival of hvKP1, glnA::Tn, sucD::Tn and tag::Tn when exposed to active complement vs. inactive complement was $-6.8\%$, $+1\%$, $-8.7\%$ and $-8.8\%$ respectively. Taken together these data

![Figure 3](image_url)

**Figure 3.** The growth and/or survival of glnA::Tn, sucD::Tn and tag::Tn within a biofilm is similar to hvKP1 when exposed to 90% human serum. The effect of 90% normal human serum on the growth and/or survival of hvKP1, glnA::Tn, sucD::Tn, and tag::Tn contained with a biofilm was assessed (see Materials and Methods for experimental details). (A) hvKP1, (B) glnA::Tn, (C) sucD::Tn and (D) tag::Tn. Data are mean ± SEM. $n = 3$ for each strain, condition and time point.
support that decreased biofilm production does not result in increased sensitivity to complement-mediated bactericidal activity and therefore biofilm formation may not be an important bacterial defense mechanism against this innate host defense factor for hypervirulent \textit{K. pneumoniae}.

The ability to produce less biofilm did not affect in vivo survival in a rat abscess model. Since biofilm formation has been implicated as being important for human infection and in closed space infection such as abscesses, the ability of hvKP1, \textit{glnA::Tn, sucD::Tn} and \textit{tag::Tn} to grow and survive in vivo was assessed in a rat subcutaneous abscess model. Further, this model was deemed appropriate since hypervirulent \textit{K. pneumoniae} strains cause hepatic and extra-hepatic abscesses. In the initial experiment the bacterial strains were individually inoculated into the abscess. The growth and survival of hvKP1, \textit{glnA::Tn, sucD::Tn} and \textit{tag::Tn} in the abscess fluid, as measured by bacterial enumeration, was similar (Fig. 4).

A competition experiment has an increased sensitivity for detecting differences between strains. Therefore this experimental design was used next to assess the growth and survival of hvKP1, \textit{glnA::Tn, sucD::Tn} and \textit{tag::Tn} within the abscess fluid. A small but insignificant 1.5% difference in the growth and survival was observed for \textit{glnA::Tn} when in competition with hvKP1 in the abscess fluid, but not for \textit{sucD::Tn} nor \textit{tag::Tn} (Fig. 5).

In both the single bacterial strain challenge and competition design, no significant differences were noted. Therefore, these results do not support an important role in the ability to produce more biofilm as being important for the growth and/or survival of hvKP1 within abscess fluid.

**Discussion**

In order to test the hypothesis that hvKP biofilm formation contributes to systemic infection, three mutant derivatives of hvKP1 were generated that produced a decreased amount of biofilm when grown in either rich laboratory medium or human ascites compared with their wild-type parent (Fig. 2). The genes disrupted in these constructs included \textit{glnA} (putatively encodes glutamine synthetase), \textit{sucD} (putatively encodes succinyl-CoA synthase α subunit) and \textit{tag} (putatively encodes transcriptional antiterminator of glycerol uptake operon). Next, we used hvKP1 and these mutant derivatives to assess the role of biofilm in contributing to resistance of complement-mediated bactericidal activity in vitro and growth and/or survival in a rat abscess model. Although planktonic hvKP1 were resistant to complement-mediated bactericidal activity, the effect of complement activity against bacteria within a biofilm has been minimally studied.
overall (in contrast to phagocytosis and resistance to antimicrobials) and not studied, as far as we are aware of, against *K. pneumoniae* including hvKP. Further, biofilm-associated bacteria are phenotypically and metabolically different from planktonic bacteria, biofilm formation has been shown to promote intestinal colonization of hvKP, and complement is central to the innate host defense. Therefore, an experiment that assessed the role of biofilm protecting against complement-mediated bactericidal activity would generate new and potentially important data. However, in the assessment of three independent mutants that produced a decreased amount of biofilm compared with its wild-type parent hvKP1, we were unable to demonstrate any biologically significant differences in sensitivity to complement-mediated bactericidal activity against either planktonic cells or cells within a biofilm (Figs. 4 and 5). Further, when tested in an in vivo abscess model using both single infection and competition experimental designs, we were also unable to demonstrate any biologically meaningful differences in growth and/or survival of these mutants compared with their wild-type parent hvKP1 (Figs. 4 and 5). Although we did not assess the role of biofilm protecting against neutrophil-mediated bactericidal activity in vitro, this critical host defense factor is indirectly assessed in the rat abscess model since neutrophils migrate into the abscess in response to bacterial challenge. Taken together, these experiments were unable to demonstrate a direct role for hvKP biofilm formation in systemic infection.

However, these studies by no means exclude a role of biofilm in the pathogenesis of hvKP extraintestinal infection. Recently published work has demonstrated that mutants that produce less biofilm have diminished intestinal colonization in mice. Although it is unclear whether intestinal colonization leads to infection in humans, it is certainly clinically plausible. Likewise, although we were unable to demonstrate that biofilm conferred resistance to complement-mediated bactericidal activity in vitro or growth and/or survival within an abscess, it remains possible that biofilm still contributes to infection within an abscess or other aspects of hvKP infection. In our abscess model we measured planktonic, but not bacteria that were tissue bound. Although the planktonic population is likely seeded in part from the tissue bound cells it remains possible that biofilm mutants associate with tissue less well than their wild-type parent. Another consideration is that biofilm production could contribute to metastatic spread. We are in the process of testing this possibility.

An important aspect of evaluating biofilm formation was our assessment in ascites in addition to LB broth, a rich laboratory medium. The mutants were initially identified via a screen that used LB medium. However, since biofilm formation is dependent on the growth environment and LB medium is not reflective of growth conditions within the human host, confirmation that the biofilm production was decreased in the mutants compared with their wild-type parent in human ascites was a critical validation. Although the growth of *glnA*:Tn, *sucD*:Tn and *tag*:Tn in LB and *glnA*:Tn and *tag*:Tn in ascites was slightly less compared with their parent hvKP1, the magnitude of these differences were small compared with the much larger differences in biofilm formation observed in these media (Figs. 1 and 2). Interestingly the growth of *glnA*:Tn, *sucD*:Tn and *tag*:Tn was similar to their wild-type parent hvKP1 when single strain challenge was used in the in vivo abscess model (Fig. 4). We appreciate that other methodologies could have been used to assess biofilm formation. Confocal or scanning microscopies are additional experimental tools that may lend insight into the gene-specific mechanisms responsible for biofilm formation. However, this was not the goal of this study. The primary goal of this report was to identify some mutants that produced less biofilm for use in biologic studies.

A number of studies have focused on the identification and characterization of genes and/or their products that contribute to biofilm formation in *K. pneumoniae*. In cKP strains type 3 fimbriae, capsular and lipopolysaccharide, amino acid synthesis genes, L-arabinose metabolism, sugar phosphotransferase systems, the type 2 quorum sensing regulatory system, the Lys-R-type regulator oxyR and a putative cell surface protein are among the factors that have been identified as being involved. The body of work that includes studies on biofilm formation in hvKP is limited. The most comprehensive study screened transposon mutants generated from the model pathogen NTUH-K2044 for biofilm formation. Twenty-three mutants were identified that exhibited reduced biofilm formation and four that displayed increased biofilm formation. A number of genes were identified from this hvKP strain, which shared similarities with factors identified in cKP. These included capsular polysaccharide, lipopolysaccharide, pilin, carbohydrate transport and metabolism and type 2 quorum sensing genes. Further, a role for capsule in biofilm formation in hvKP variants was also confirmed in another study of the hvKP strain KpL1. Unique genes identified by Wu et al. encoded for a putative exonuclease, a peptidase, a helicase, a multidrug resistance protein, a cold-shock protein, and eight genes of unknown function.

The three genes we identified that contribute to biofilm formation have not yet been identified as contributing to biofilm formation in *K. pneumoniae* in general or in hvKP strains. *glnA* putatively encodes glutamine synthetase. Although amino acid synthesis genes have been identified for contributing to biofilm formation in cKP, the details of these genes were not reported. The mechanism by which *glnA* contributes to biofilm formation is speculative at this point. *sucD* putatively encodes the succinyl-CoA synthase α subunit. Although this gene has not been described as contributing to biofilm formation in *K. pneumoniae*, it has been implicated in *Mycobacterium avium* and *E. coli*. Succinyl-CoA is a part of the tricarboxylic acid (TCA) cycle. Some of the metabolic products of this cycle are various carbohydrates, which could contribute to capsular polysaccharide synthesis. However, a hypermucoviscosity assay established that hvKP1 *sucD*:Tn does not produce less capsule than hvKP1. Alternatively, the TCA cycle is an important contributor to energy production and conservation and perhaps it is within this context that *sucD* affects biofilm production. *tag* putatively encodes transcriptional antiterminator of glycerol uptake operon. Glycerol phosphate is a component of t-caseic acids, which are obtained from biofilm extracts from *Staphylococcus epidermidis*. Perhaps of greater relevance for hvKP, cyclic β-linked glucans substituted with
phosphoglycerol are present in the biofilm matrix of *Pseudomonas aeruginosa*.

Structural analyses of hvKP1 biofilm will assist in determining if this is the mechanism by which *tag* contributes to biofilm formation.

In summary, we were unable to support the hypothesis that hvKP biofilm formation plays a role in systemic infection; the primary goal of this report. However, three new genes that contribute to biofilm formation in hvKP1 were identified; a secondary goal.

**Materials and Methods**

**Bacterial strains and media.** hvKP1 was isolated from the blood and liver abscess of a previously healthy 24 y old male from Buffalo, NY with CA-PLA and metastatic spread to the spleen. It possesses a K2 serotype and is hypermucoviscous. hvKP1 and mutant derivatives were maintained at -80°C in 50% LB broth and 50% glycerol. Human ascites was collected from a de-identified patient who was undergoing therapeutic paracentesis for symptoms due to abdominal distension. This individual was not being treated with antimicrobials and was not infected with human immunodeficiency, hepatitis B and hepatitis C viruses. The ascites was cultured to confirm sterility, divided into aliquots, and stored at -80°C. For various in vitro growth studies 100% ascites or LB medium was used.

**Transposon mutagenesis.** hvKP1 transposon mutant derivatives were generated using the EZ::TN < KAN-2 > Transposome system (Epigenic, TSM99K2) as described previously. Mutant derivatives of hvKP1 were generated and screened qualitatively for biofilm formation.

**Screen for biofilm deficient mutants.** On day 1 a single colony from a given mutant construct was inoculated into LB medium plus 40 μg/mL of kanamycin and grown overnight at 37°C. On day 2 the bacterial concentration was adjusted in triplicate to an absorbance (A) of 0.1 in 1 mL of LB medium in a 5 mL polystyrene tube and subsequently grown at 37°C in a shaking water bath overnight (Precision Scientific, Model 25, 110 rpm). Pilot studies establish that biofilm formation for hvKP1 was greater with shaking vs. static incubation. On day 3 tubes were gently washed twice with 3 mL 1X phosphate-buffered saline (PBS) (pH 7.4) and then dried overnight at 37°C. On day 4 the biofilm was stained with freshly diluted 0.1% Gram Crystal Violet for 15 min. The dye was carefully removed with a glass pipette and then tubes were gently washed with 5 mL of distilled water three times. The tubes were dried overnight. On day 5, to maximize the recovery of biofilm-bound crystal violet, 0.5 g of borosilicate spheres (1 mm, VWR, 80066-214) and 2.5 mL 95% ethanol were added to each of the dried tubes, which were subsequently vortexed at the maximum setting for 30 sec. The resulting solution was read at A595, after subtracting the absorbance of the 1X PBS solvent, and values recorded. A given set of mutant constructs were screened in parallel with their wild-type parent hvKP1.

**Quantitation of biofilm in vitro.** This assay was performed in an identical fashion to the screen for biofilm deficient mutants except that biofilm was quantitated at 1, 24 and 48 h.

**Identification of gene disrupted in mutants with decreased biofilm formation.** The gene disrupted by transposon mutagenesis in the mutant derivatives of hvKP1 was initially established by chromosomal sequencing off of the end of the transposon for two mutants (*ghnA:*Tn, *tag:*Tn) as described.

For the third mutant (*sucD:*Tn) chromosomal sequencing was unsuccessful, therefore a portion of the transposon and the gene into which it was inserted was subcloned into pBluescript as described, except the restriction enzymes EcoRV and SacI were used. Gene disruptions were confirmed by sequencing of PCR-generated amplicons derived from chromosomal DNA from the mutant in question. Primers sequences were: (1) for *ghnA:*Tn 5’-TGGTCCATCAATGACGCTATC-3’, plus and 5’-TAATGGGGTGCCAAACAG-3’, minus (product 454 bp, annealing temperature 55.7°C); (2) for *sucD:*Tn 5’-ATGTCAAGTTTTAATTAAATA-3’ plus and 5’-TTACTTGATAATAGCTTTCTC-3’, minus (product 870 bp, annealing temperature 53.2°C); (3) for *tag:*Tn 5’-ATGATTCTCAATAAACCAT-3’, plus and 5’-TACCATAAGCGGGG-3’, minus (product 552 bp, annealing temperature 51.2°C).

**RT-PCR analysis of hvKP1 mutant constructs.** RT-PCR analysis was deemed sufficient to exclude polar effects in *ghnA:*Tn, *sucD:*Tn and *tag:*Tn for several reasons. Terminator sequence is present in the intergenic region between *ghnA*, *sucD* and their respective downstream genes. Although a terminator sequence is not present in the intergenic region downstream of *tag*, the orientation of *EZ::TN* < KAN-2 in *tag:*Tn is in the opposite transcriptional direction. This is also the case for *sucD:*Tn. Therefore, we are confident that the demonstration of RT-PCR-generated amplicons from the respective ORFs downstream of *ghnA:*Tn, *sucD:*Tn and *tag:*Tn are not from *EZ::TN* < KAN-2-mediated read-through transcription, thereby excluding a transposon-mediated polar effect. Approximately 1 × 10⁸ cfu were resuspended in 500 μl of RNAlater (Ambion, 7020) and incubated at room temperature for 10 min. Next, the bacteria were maintained at 4°C while they were concentrated and resuspended in 100 μl of TE buffer containing 0.5% SDS and 60 μg of Proteinase K (Amresco, 0706). The cells were incubated at 37°C for 40 min, resulting in lysis. At this point the Qiagen RNeasy Protect Kit (Qiagen, 74124) was used to clean the sample, including on-column DNase I digestion (Qiagen RNase-Free DNase, 79254). The samples were eluted in 50 μl of RNase free water and digested a second time with DNase I to ensure complete digestion of chromosomal DNA. Lastly, the samples underwent a final column wash (Qiagen RNeasy Protect Kit) and were eluted in 50 μl of RNase free water. The RNA concentration was determined using the Nanodrop analyzer and the samples were diluted to 50 ng/μl for further analysis. The absence of chromosomal DNA was subsequently confirmed by testing all RNA samples prior to RT-PCR analysis using the appropriate primer pair and GoTaq Green Master Mix (Promega, M712B). The Qiagen OneStep RT-PCR kit (Qiagen, 210210) was used for subsequent RT-PCR analysis of all samples.

Primers were designed to amplify 100–500 bp of the RNA transcript downstream from the gene disruption, based on
sequence generated from hvKP1 (unpublished), to exclude a polar effect. The primer sequences used were: (1) 5'–GGCTTTATT CTGCTGAAATGG–3', plus and 5'–TGGTAGACCTGGG GTGCTAATCG–3', minus (product 384 bp, annealing temperature 59.4°C); amplifies transcript for nitrogen regulation protein, immediately downstream from glnA::Tn; (2) 5’–CGACTTTCG CTTTGGCAAGC–3' plus and 5’–GATACGCTCCTTGT GCTGAAC–3', minus (product 323 bp, annealing temperature 58.6°C); amplifies transcript for cytochrome d ubiquinol oxidase subunit I, downstream from sucD::Tn; (3) 5’–GTGTTGGGG ACTGTGATAACTG–3', plus and 5’–TTGTTTATGCT CCAGGTGCCG–3', minus (product 220 bp, annealing temperature 57.2°C); amplifies transcript for d-xylulose/3-keto-d-gulonate kinase, immediately downstream from tag::Tn. Of note, immediately downstream from sucD is a predicted open reading frame (ORF) that could encode 48 amino acids of a hypothetical protein. However, we were unable to generate a RNA transcript from this predicted ORF using RNA from hvKP1, thereby supporting the concept that this predicted ORF is not transcribed.

**Hypermucoviscosity assay**. This assay was performed as described. In brief, 1.2 mL of $\bar{A}_{600}$ normalized bacteria growing in LB medium overnight was centrifuged in microcentrifuge tubes at 2,000 g for 5 min. The absorbance of the supernatant was measured at $\bar{A}_{600}$.

**Complement-mediated bactericidal activity against planktonic cells and cells within a biofilm in vitro**. Complement-mediated bactericidal activity against planktonic cells. This assay was performed as described except the starting inoculum was approximately $1 \times 10^8$ cfu/mL to approximate the number of cfu/mL in a biofilm. Ninety percent human serum was buffered with 1X PBS (pH 7.4). Pilot experiments, done previously in our laboratory, have established that the magnitude of complement-mediated bactericidal activity in serum is at least as great when 1X PBS is used alone instead of in combination with isotonic gelatin-veronal buffer with Ca$^{2+}$ and Mg$^{2+}$ (Isogever). Further, bacterial enumeration was measured at 6 and 24 h. Growth in heat-inactivated serum served as a positive control, which established that decreased survival, if present, was due to complement-mediated bactericidal activity.

**Complement-mediated bactericidal activity against cells within a biofilm**. A complement-mediated bactericidal assay was designed to assess the bactericidal activity of 90% human serum against hvKP1, glnA::Tn, sucD::Tn and tag::Tn contained within a biofilm. The first step was biofilm development. This was accomplished by growing hvKP1, glnA::Tn, sucD::Tn or tag::Tn in 1 mL of LB medium in 5 mL polypropylene tubes (starting inoculum of $\bar{A}_{600}$ of 0.1) followed by incubation for 48 h at 37°C in a shaking water bath (Precision Scientific, Model 25, 110 rpm). Next, planktonic bacteria were removed by gently washing and the remaining bacteria within the biofilm were exposed to either 1 mL of 90% human serum:10% 1X PBS or 90% human serum in which complement-mediated bactericidal activity was inactivated:10% 1X PBS [heated at 56°C for 30 min (delta 56°C)] at 37°C. After 0, 6 and 24 h of serum exposure, bacterial counts within the biofilm were measured by gentle removal of the measured supernatant was measured at $\bar{A}_{600}$.

**Growth and survival in a rat abscess model**. The rat subcutaneous abscess model was approved by the University at Buffalo and Veterans Administration Institutional Animal Care Committees. This study was performed 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. Dalhoff originally described this model, which was modified by our group. In brief, a space is created by subcutaneous injection of 30 mL of air into the back of anesthetized Long-Evans rats (200–225 g), which is injected with 1 mL of 1% croton oil in a filter-sterilized vegetable oil vehicle. This space matures into an encapsulated, fluid-filled (8–12 mL) “abscess” over 6–8 d. Neutrophils will migrate into the abscess in response to appropriate chemotactic signals. The abscess’ subcutaneous location enables multiple injections and samplings to be performed over time. In the first set of experiments, hvKP1, glnA::Tn, sucD::Tn and tag::Tn were injected alone into the abscess of an anesthetized animal, resulting in an estimated starting abscess concentration of $1 \times 10^5$ cfu/mL. Within one minute after the bacteria were injected into the abscess 0.5 mL of abscess fluid was removed to measure the actual starting bacterial titer. Fluid aliquots (0.5 mL) were subsequently obtained from anesthetized animals 3, 6, 24 and 48 h after the initial bacterial challenge, and bacterial titers were enumerated. The next set of experiments used a competition format where both hvKP1 and a given mutant construct were inoculated into the pouch together; each at a starting inoculum of approximately $1 \times 10^5$ cfu/mL. The harvest time points were identical to the initial experiment.

**Statistical analyses**. Data are presented as mean ± 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 < 0.05/n but > 0.05/n and < 0.1 are considered as representing a trend. To normalize in vitro and in vivo data log$_{10}$ transformed values were utilized. When multiple measurements were performed over time, the area under each curve (AUC) was calculated. AUCs, which reflect the data in its entirety, were compared using two-tailed unpaired t-tests (Prism 4 for MacIntosh, GraphPad Software Inc.).

**Ethics statement**. The procedures for obtaining human serum and ascites were reviewed and approved by the Western New York Veterans Administration Institutional Review Board. Informed consent was used to obtain human blood for the preparation of serum (approval ID # 00063). The Western New York Veterans Administration Institutional Review Board for the process of obtaining ascites waived informed consent (approval ID # 00098). An expedited review was performed because the ascites was collected from de-identified patients who were undergoing therapeutic paracentesis for symptoms due to abdominal distension.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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