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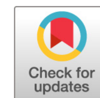

Pulmonary Surfactant Promotes Virulence Gene Expression and Biofilm Formation in *Klebsiella pneumoniae*

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ABSTRACT The interactions between *Klebsiella pneumoniae* and the host environment at the site of infection are largely unknown. Pulmonary surfactant serves as an initial point of contact for inhaled bacteria entering the lung and is thought to contain molecular cues that aid colonization and pathogenesis. To gain insight into this ecological transition, we characterized the transcriptional response of *K. pneumoniae* MGH 78578 to purified pulmonary surfactant. This work revealed changes within the *K. pneumoniae* transcriptome that likely contribute to host colonization, adaptation, and virulence *in vivo*. Notable transcripts expressed under these conditions include genes involved in capsule synthesis, lipopolysaccharide modification, antibiotic resistance, biofilm formation, and metabolism. In addition, we tested the contributions of other surfactant-induced transcripts to *K. pneumoniae* survival using engineered isogenic KPPR1 deletion strains in a murine model of acute pneumonia. In these infection studies, we identified the MdtJI polyamine efflux pump and the ProU glycine betaine ABC transporter to be significant mediators of *K. pneumoniae* survival within the lung and confirmed previous evidence for the importance of *de novo* leucine synthesis to bacterial survival during infection. Finally, we determined that pulmonary surfactant promoted type 3 fimbria-mediated biofilm formation in *K. pneumoniae* and identified two surfactant constituents, phosphatidylcholine and cholesterol, that drive this response. This study provides novel insight into the interactions occurring between *K. pneumoniae* and the host at an important infection site and demonstrates the utility of purified lung surfactant preparations for dissecting host-lung pathogen interactions *in vitro*.

KEYWORDS pneumonia, pulmonary surfactant, polyamines, putrescine, spermidine, colonization, type 3 fimbriae, metabolism

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen that causes an estimated 8 to 10% of nosocomial infections in the United States and Europe (1–3). *K. pneumoniae* is often found in the environment (4–6) and is also a frequent colonizer of the human gastrointestinal tract (7, 8). Infections by this bacterium occur in a range of tissues within immunocompromised individuals, with the tissues of the urinary and respiratory tracts being the most prevalent sites of infection (1, 2, 9). Pulmonary infections caused by *K. pneumoniae* are particularly concerning and are associated with high levels of morbidity and mortality. Unfortunately, treatment options for combating these infections are becoming increasingly limited due to the widespread development of drug resistance (10–12). The recent emergence of colistin resistance in *K. pneumoniae*, coupled with the increasing prevalence of extended-spectrum-beta-lactamase (ESBL)- and carbapenemase-producing strains, suggests that new therapeutics are urgently needed (13–15). Despite the clinical significance of *K. pneumoniae*, little is

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known about its interaction with the host lung environment during infection. *K. pneumoniae* transcriptional changes occurring following inhalation and deposition into the lung are likely associated with adaptation and niche colonization. Therefore, characterizing this ecological transition is critical to our understanding of the infection process.

One of the first aspects of the host lung environment encountered by inhaled bacteria is pulmonary surfactant. This phospholipid-rich mixture coats the alveolar surfaces at the air-liquid interface and serves to reduce surface tension within the lung to prevent collapse following expiration (16, 17). Aside from this mechanophysical role, lung surfactant also modulates the activity of inflammatory cells and directly participates in the innate immune response via two surfactant-associated collectins (SP-A and SP-D) (18–20). Lung surfactant contains roughly 100 unique components, including a minor proteinaceous fraction consisting of four surfactant-associated proteins (SP-A, SP-B, SP-C, SP-D), as well as a much larger lipid fraction comprising nearly 90% of the dry weight of this substance. Within the lipid fraction, dipalmitoylphosphatidylcholine and mixed-tail phosphatidylcholines are the major constituents, making up nearly 80% of the total lipid content, followed by phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin. Also present within the lipid fraction are fatty acids, free triglycerides, and neutral lipids, such as cholesterol (17, 21, 22).

Pathogenic bacteria entering the host lung must generate an appropriate transcriptional response to successfully transition to this environment and avoid clearance by the innate immune system. Recognition of components within lung surfactant has been associated with the survival and virulence of several other opportunistic pathogens, perhaps, unsurprisingly, given the locale of this substance at the respiratory surfaces of the alveoli and terminal bronchioles. Previous transcriptional profiling studies by our group with purified lung surfactant led to the determination that both the detection of sphingosine and the metabolism of the choline moiety of phosphatidylcholine by *Pseudomonas aeruginosa* are independently required for full virulence in a mouse model of acute pneumonia (23–25). Similarly, work by Ishii et al. concluded that fatty acids within lung surfactant invoked a membrane stress response in *Staphylococcus aureus* and identified a novel virulence determinant implicated in this process (26).

On the basis of these studies, purified lung surfactant represents a critical, yet experimentally tractable, aspect of the host lung environment that offers an attractive *in vitro* model to examine host-pathogen interactions occurring during the onset of infection. Here, we characterized the transcriptional response of *K. pneumoniae* MGH 78578, a multidrug-resistant clinical isolate (27), to purified bovine lung surfactant (Survanta). This transcriptome-based strategy allowed us to determine that numerous characterized virulence- and fitness-related genes of *K. pneumoniae* are expressed in response to lung surfactant, including those involved in capsule synthesis, biofilm formation, antibiotic resistance, lipopolysaccharide (LPS) modification, and metabolism (1, 2, 9). We also tested the contributions of some of the identified genes to survival in a mouse model of acute pneumonia. We identified the MdtJI polyamine efflux pump and the ProU glycine betaine ABC transporter to be significant mediators of *K. pneumoniae* survival within the lung and confirmed the importance of endogenous leucine synthesis for *K. pneumoniae* survival during infection. An additional goal of this study was to identify the constituents within lung surfactant that induced expression of *K. pneumoniae* virulence-associated transcripts. Here, we show that at least two components of lung surfactant, phosphatidylcholine and cholesterol, promote type 3 fimbria expression.

RESULTS

Lung surfactant alters expression of *K. pneumoniae* metabolic pathways and virulence factors. Our goal was to characterize the transcriptional changes occurring within *K. pneumoniae* as a result of exposure to purified lung surfactant. To accomplish this, we performed microarray analysis using a custom Affymetrix GeneChip designed

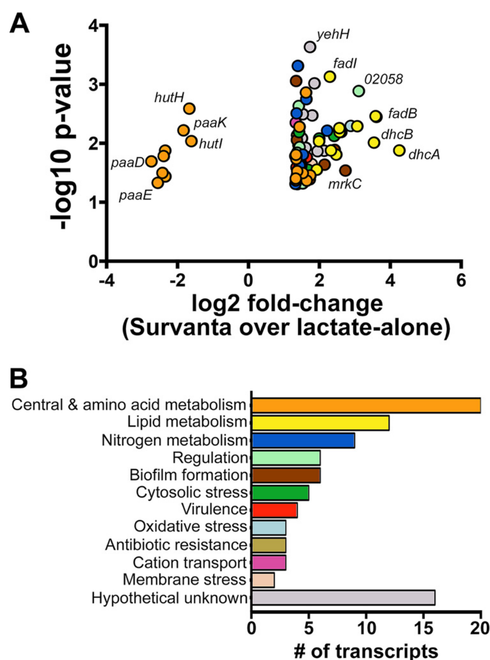

FIG 1 *K. pneumoniae* MGH 78578 transcriptome changes in response to lung surfactant. (A) Volcano plot of transcripts detected through the microarray as exhibiting more than a 2.5-fold change in expression ($P < 0.05$) following exposure to Survanta. (B) Survanta-regulated transcripts were categorized into groups reflecting their known or bioinformatically predicted functions. The color coding of the categories is the same for both panels.

for the *K. pneumoniae* MGH 78578 (ATCC 700721) genome and RNA collected from cells that were cultured in MOPS (morpholinepropanesulfonic acid) minimal medium containing lactate as a carbon source with or without purified bovine lung surfactant (Survanta). Under these conditions, 89 transcripts exhibited more than a 2.5-fold change in expression ($P < 0.05$) between the presence and absence of lung surfactant. Eighty of these genes increased in expression in response to surfactant, while nine genes were repressed. A summary of these changes is shown in Fig. 1A and B, with the transcripts being categorized into groups reflecting their known or bioinformatically predicted function (28–30). The 25 most highly expressed transcripts are also shown in Table 1, while a full list of the transcriptional changes occurring within *K. pneumoniae* in response to Survanta can be found in Table S1 in the supplemental material.

Fifteen percent of the genes expressed by *K. pneumoniae* in response to Survanta are predicted to function in phospholipid and fatty acid metabolism. FadR regulon members are well represented among this group, with six β-oxidation-related genes (*fadBA*, *fadHIJ*, *fadE*) (31) exhibiting between 3.8- and 12.3-fold increases in transcript abundance in response to surfactant. In addition, the six genes within the *Kpn_02053-Kpn_02057* operon displayed between 5- and 19.2-fold increases in abundance under these conditions. Encoded within this operon are a predicted citrate permease-like transporter (which we designate *Kpn_02056.5*, as it was not annotated in the original genome numbering) and orthologs of genes found within the dehydroxycarnitine and 3-hydroxybutryate metabolism gene clusters of *P. aeruginosa* PAO1, reflecting their probable function in the uptake and metabolism of short-chain fatty acids (32, 33).

Other changes within the *K. pneumoniae* transcriptome reflect global alterations in nitrogen metabolism. The genes for glutamate synthase (*gltD*), the glutamine ABC transporter permease (*glnP*), and the nitrogen regulatory protein (*glnK*) exhibited between 2.6- and 3.1-fold increases in transcript abundance in response to lung surfactant, indicative of fluctuations in nitrogen pool homeostasis. Other transcriptional changes reflected the accumulation, metabolism, and excretion of polyamines during growth in lung surfactant. Notably, increases in the abundance of several putrescine-

TABLE 1 Summary of the 25 most highly induced transcripts expressed by *K. pneumoniae* MGH 78578 in response to lung surfactant

Fold increase in expression	Gene	Alternate name	Function
19.16	*Kpn_02053*	*dhcA*	Acetyl-CoA[a] transferase alpha subunit
12.25	*Kpn_04340*	*fadB*	3-Hydroxyacyl-CoA dehydrogenase
12.06	*Kpn_00235*	*fadE*	Acyl-CoA dehydrogenase
11.69	*Kpn_02054*	*dhcB*	Acetyl-CoA transferase beta subunit
8.68	*Kpn_02058*		LysR-family transcriptional regulator
8.41	*Kpn_02055*	*atoB*	Beta-ketothiolase
7.41	*Kpn_01989*	*nemA*	*N*-Ethylmaleimide reductase
6.67	*Kpn_03278*	*mrkC*	Type 3 fimbrial assembly chaperone
6.16	*Kpn_02505*	*Kp52D*	Glycosyltransferase: capsule synthesis
5.96	*Kpn_04339*	*fadA*	Acetyl-CoA acetyltransferase
5.94	*Kpn_02057*	*bdhA*	Short-chain dehydrogenase
5.58	*Kpn_01635*	*yneI*	Putative aldehyde dehydrogenase
5.40	*Kpn_01159*		Cyclic di-GMP phosphodiesterase
5.36	*Kpn_00406*	*queC*	7-Cyano-7-deazaguanine synthase
5.04	*Kpn_02056*	*bdhB*	3-Hydroxybutyryl-CoA dehydrogenase
4.93	*Kpn_02724*	*fadI*	Acetyl-CoA acetyltransferase
4.68	*Kpn_01565*	*mdtJ*	Polyamine efflux pump subunit
4.44	*Kpn_03277*	*mrkB*	Type 3 fimbrial usher protein
4.41	*Kpn_01316*		Hypothetical protein
4.00	*Kpn_03008*	*proV*	Glycine betaine ABC transporter subunit
3.99	*Kpn_02723*	*fadJ*	Enoyl-CoA hydratase
3.88	*Kpn_pKpn5p08207*		Hypothetical protein
3.82	*Kpn_03510*	*fadH*	2,4-Dieonyl-CoA reductase
3.65	*Kpn_01727*		Hypothetical protein
3.49	*Kpn_01676*		Hypothetical protein

[a]CoA, coenzyme A.

inducible transcripts (34, 35), including the *mdtJI* polyamine efflux pump and *yneI* succinate semialdehyde dehydrogenase (5.6- to 2.7-fold increases), were observed.

Exposure to lung surfactant also altered the expression of metabolic transcripts in *K. pneumoniae* in unexpected ways. Interestingly, Survanta stimulated the transcription of genes involved in the synthesis of branched-chain amino acids (BCAA), including the gene for valine-pyruvate transaminase (*avtA*; 2.6-fold increase) and the *leuABCD* leucine synthesis operon (2.9- to 2.3-fold increase). In addition, repression of the phenylacetic acid (*paaCDFEFIK*) and histidine (*hutUIH*) catabolism gene clusters was observed (6.7- to 3-fold decrease).

Numerous oxidative stress-related transcripts were also induced by *K. pneumoniae* in lung surfactant, potentially in response to the elevated amounts of reactive oxygen species generated through the β-oxidation of fatty acids. The levels of transcription of *nemAR*, encoding the oxidative stress-responsive regulator NemR and the reactive-electrophile neutralizing *N*-ethylmaleimide reductase NemA (36, 37) increased 2.8- and 7.4-fold, respectively, under these conditions. Other oxidative stress response genes were also expressed, including *ybbL* (2.5-fold increase) and a hydrogen peroxide-inducible gene of unknown function, *ybjM* (2.9-fold increase) (38, 39).

Other aspects of the *K. pneumoniae* transcriptional response to lung surfactant are reflective of metabolic/cytosolic stress. Notably, transcription of the multiple drug resistance and acid response regulator (40–42) gene *evgA* increased 3.5-fold in response to surfactant exposure. Transcription of the genes for the glycine betaine ABC transporter, *proVWX* (43), also increased 4-fold, suggesting that the lipid-rich environment of lung surfactant invokes osmotic stress in *K. pneumoniae*. Two tRNA nucleotide modification enzymes were also induced under these conditions, with *queC* and *gidA* exhibiting 5.4- and 2.8-fold increases in transcript abundance, respectively (44, 45). Finally, transcription of genes associated with antibiotic resistance were also upregulated, including the genes for the 23S rRNA methylation enzyme (*yfgB*) and aminoglycoside 3′-phosphotransferase (*strB*) (2.6- to 2.7-fold increase) (46, 47).

Lung surfactant also induced transcriptional changes within *K. pneumoniae* associated with colonization, virulence, and immune evasion. Exposure to Survanta induced

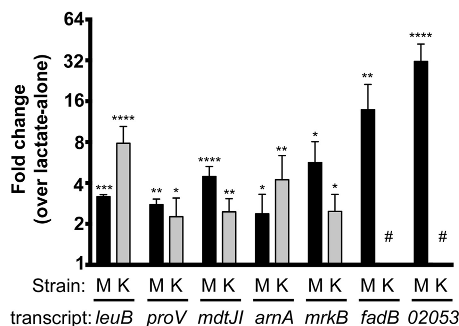

FIG 2 qRT-PCR validation of induced transcripts in *K. pneumoniae* following exposure to lung surfactant. The relative abundance of seven Survanta-induced transcripts detected through the microarray were reexamined using quantitative RT-PCR with RNA collected from three independent Survanta induction experiments as described in the Materials and Methods section. Genes for analysis were chosen to represent a range of cellular functions, including lipid metabolism (*fadB* and *Kpn_02053* [*dhcA*]), biofilm formation (*mrkA*), branched-chain amino acid synthesis (*leuA*), nutrient uptake (*proV*), polyamine efflux (*mdtJ*), and LPS modification (*arnA*). Raw transcript expression values were normalized to those for *Kpn_04184*, which exhibited no change in expression between conditions in our microarrays. We examined expression in both MGH 78578 (M) and KPPR1 (K), though our primers designed for *fadB* and *Kpn_02053* in MGH 78578 were not usable in KPPR1 due to multiple products (noted with the # symbol). Statistical analysis was conducted via two-way analysis of variance with a Sidak posttest analyzing in-strain changes comparing expression under conditions with Survanta additions to expression under the lactate-alone condition. The data shown summarize those from three independent Survanta induction experiments, and statistical significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

the expression of type 3 fimbriae encoded by the *mrkABCDF* gene cluster (6.7- to 2.6-fold increase) (48). Similarly, a 5.4-fold increase in transcript abundance was observed for a cyclic-di-GMP phosphodiesterase (KPN_01159) that has been implicated in promoting *mrK* operon expression *in vitro* (49). Increased transcription of genes encoded within the capsular polysaccharide synthesis (*cps*) region was also observed following exposure to surfactant, including *ugd*, *Kp52D*, and *Kpn_02483* (2.6- to 6.2-fold increase). Two other capsule synthesis genes, *Kpn_02503* and *Kpn_02506* (50), also exhibited statistically significant increases in transcript abundance but failed to surpass our 2.5-fold change cutoff for inclusion in this study (2.0- and 2.1-fold increases, respectively). Lung surfactant also invoked transcriptional changes within *K. pneumoniae* indicative of LPS modification. A 3.1-fold increase in transcript abundance was observed for *arnA*, whose product participates in conferring resistance to cationic peptides and polymyxin B through the addition of 4-amino-4-deoxy-L-arabinose (Ara4N) to lipid A (51–53).

Validation of microarray data. Quantitative reverse transcription-PCR (qRT-PCR) was used to confirm the Survanta-induced transcriptional changes within *K. pneumoniae* that we identified through the microarray and to determine the conservation of these responses in KPPR1. To accomplish this, reverse transcription-PCR (RT-PCR) was performed on *K. pneumoniae* MGH 78578 and KPPR1 RNA collected from three additional Survanta induction experiments, as described in the Materials and Methods section. The relative abundances of seven transcripts, representing nearly 10% of the genes identified through the microarray to be induced under these conditions, were examined. The genes for analysis were chosen to represent a range of cellular functions, including fatty acid and phospholipid metabolism (*fadB* and *Kpn_02053*), biofilm formation (*mrkA*), branched-chain amino acid synthesis (*leuA*), nutrient uptake (*proV*), polyamine efflux (*mdtJ*), and LPS modification (*arnA*). As shown in Fig. 2, all transcripts examined exhibited greater than a 2-fold increase in expression in response to Survanta, in close agreement with the microarray data. For the five primers that produced correct amplicons, all showed induction, with *leuB* and *arnA* showing higher relative induction in KPPR1.

Surfactant-induced transcripts contribute to *K. pneumoniae* fitness during lung infection. The contributions of other surfactant-induced transcripts to *K. pneumoniae*

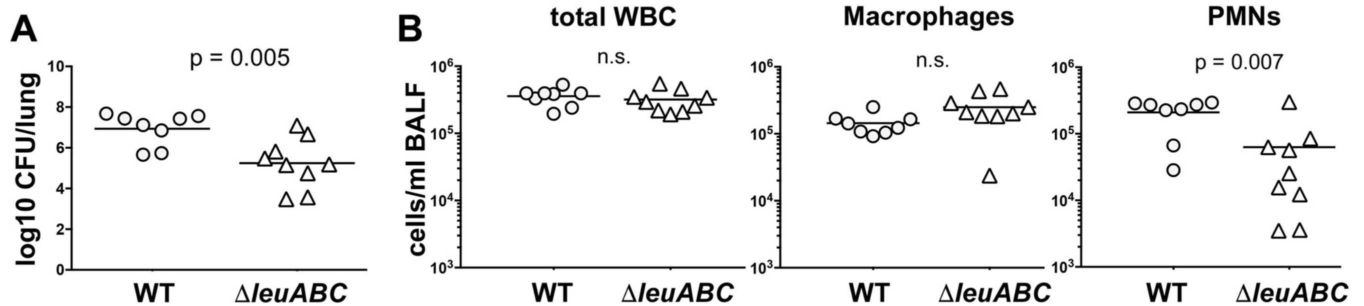

FIG 3 Leucine biosynthesis by *K. pneumoniae* is required for virulence during acute pneumonia. (A) Adult male C57BL/6J mice were infected via oropharyngeal aspiration with either *K. pneumoniae* KPPR1 WT or KPPR1 Δ*leuABCD*. The numbers of CFU per lung were measured at 24 h postinstillation. (B) White blood cells (WBC) within the bronchoalveolar lavage fluid collected from each infected mouse were enumerated. The total white blood cell, macrophage, and PMN counts are shown. The data shown summarize those from three independent experiments, with statistical significance being determined through an unpaired *t* test. The arithmetic mean is depicted for each strain, with the counts from individual mice being represented by individual points.

lung pathogenesis were explored using engineered *K. pneumoniae* gene deletion strains in a mouse oropharyngeal aspiration model of acute pneumonia. Due to the historic usage of KPPR1 as the model for *Klebsiella* lung infection, gene deletions were engineered into KPPR1 (ATCC 43816) (1, 54–58). KPPR1 and MGH 78578 share a high level of gene conservation, with 88% of their open reading frames being considered orthologous (59). More importantly, this genetic similarly is reflected within the surfactant microarray data, where 81% of transcripts expressed by MGH 78578 under these conditions are also encoded within the genome of KPPR1.

Exposure to lung surfactant induced the expression of the leucine synthesis gene cluster (*leuABCD*) in both strains of *K. pneumoniae* (Fig. 2). The importance of branched-chain amino acid synthesis to *K. pneumoniae* during pulmonary infection was recently demonstrated through an *in vivo* genetic screen that recognized that the *ilvADE* isoleucine and valine synthesis gene clusters are required for pathogenesis and that also noted that *leuABCD* disruption mutants display competitive fitness defects *in vivo* (60). Therefore, we generated a *leuABCD* deletion strain to determine if the defect was absolute or manifests only in competition with the wild type (WT). Deletion of *leuABCD* resulted in a nearly 50-fold decrease in the number of bacterial CFU in the lung compared to the number for the WT strain (a 48.98-fold decrease in the number of CFU; $P = 0.0048$) (Fig. 3A). Interestingly, although the total number of immune cells in bronchoalveolar lavage fluid (BALF) collected from mice infected with the Δ*leuABCD* strain was similar to those in BALF collected from mice infected with the KPPR1 WT, the composition of the infiltrating leukocytes differed. BALF collected from mice infected with the deletion strain demonstrated a reduction in the neutrophilic response to the mutant strain, likely as a consequence of the reduced number of bacterial CFU (Fig. 3B).

The *mdtJI* operon encodes a small multidrug resistance (SMR-family) efflux pump that was first implicated in resistance to deoxycholate and SDS in *Escherichia coli* (61). More recent reports have indicated that MdtJI primarily functions in the excretion of the polyamines spermidine and putrescine (35, 62). Polyamines have been recognized as important mediators of virulence in numerous bacterial genera, including *Shigella*, *Salmonella*, and *Staphylococcus* (63), leading to our interest in exploring the potential contribution of MdtJI to *K. pneumoniae* fitness during infection of the lung. As shown in Fig. 4A, deletion of *mdtJI* resulted in a more than 10-fold decrease in the bacterial lung burden relative to that of the WT strain at 24 h postinoculation (a 10.86-fold decrease in the number of CFU). Interestingly, BALF collected from mice infected with KPPR1 Δ*mdtJI* contained significantly fewer infiltrating leukocytes, neutrophils, and macrophages than BALF collected from mice infected with the WT strain (Fig. 4B).

The ProU (*proVWX*) ABC transporter was the most highly induced metabolite acquisition system expressed by *K. pneumoniae* following exposure to Survanta. The role of this transporter has been extensively studied in *Escherichia coli* and *Salmonella eneterica* serovar Typhimurium and participates in the uptake of glycine betaine from the

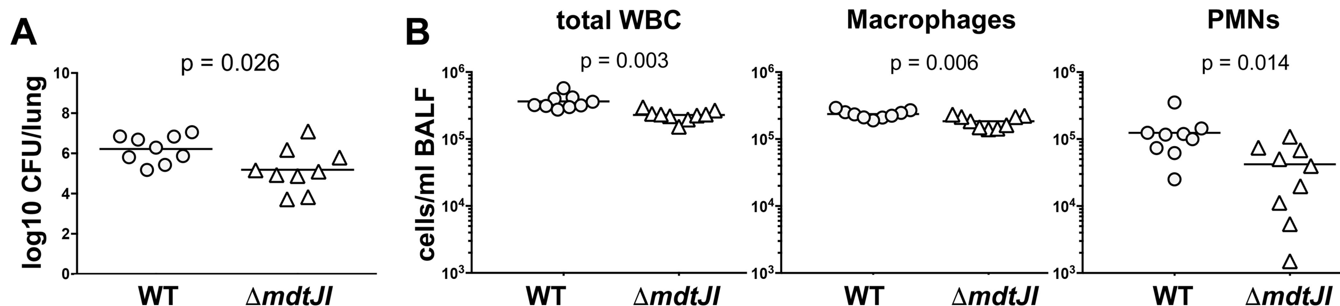

FIG 4 The MdtJI polyamine efflux pump contributes to *K. pneumoniae* fitness during acute pneumonia. (A) Adult male C57BL/6J mice were infected via oropharyngeal aspiration with either *K. pneumoniae* KPPR1 WT or KPPR1 Δ*mdJI*. The numbers of CFU per lung were measured at 24 h postinstillation. (B) The white blood cells within the bronchoalveolar lavage fluid collected from each infected mouse were enumerated. The total white blood cell, macrophage, and PMN counts are shown. The data shown summarize those from three independent experiments, with statistical significance being determined through an unpaired *t* test. The arithmetic mean is depicted for each strain, with the counts from individual mice being represented by individual points.

environment during periods of osmotic stress (43, 64). Phosphatidylcholine is the most abundant phospholipid in lung surfactant and has previously been shown to serve as an important source of the osmoprotectant glycine betaine, which is required for *Pseudomonas aeruginosa* fitness within the lung (24). We were therefore curious to determine if ProU-mediated glycine betaine uptake also contributed to *K. pneumoniae* fitness during lung infection. As shown in Fig. 5A, deletion of *proV* resulted in a significant decrease in the bacterial lung burden compared to that of the WT KPPR1 strain at 24 h postinoculation (a 6.99-fold decrease in the number of CFU). Examination of immune cells in BALF collected from these mice indicated that deletion of *proV* altered polymorphonuclear leukocyte (PMN) recruitment (Fig. 5B). It is important to note for the changes in the number of CFU reported for these three strains that although we suggest a survival difference, we did not enumerate the CFU in the BALF or other body compartments, and it therefore remains a formal possibility that localization is affected instead of or in addition to survival.

KPPR1 and isogeneic deletion strains exhibit wild-type growth kinetics in TSB. Our *K. pneumoniae* gene deletion strains were assessed for general growth defects in nutrient-rich media to ensure that the decreased CFU counts that we observed *in vivo* were not a consequence of generalized growth defects. In order to address this question, the growth of the KPPR1 WT and engineered gene deletion strains were measured in tryptic soy broth (TSB), the medium used to culture the bacteria prior to inoculation into mice. As shown in Fig. 6, the growth kinetics of all deletion strains closely mirrored those of the WT strain. We were also interested in exploring the impact that deletion of these genes had on the ability of *K. pneumoniae* to grow in the

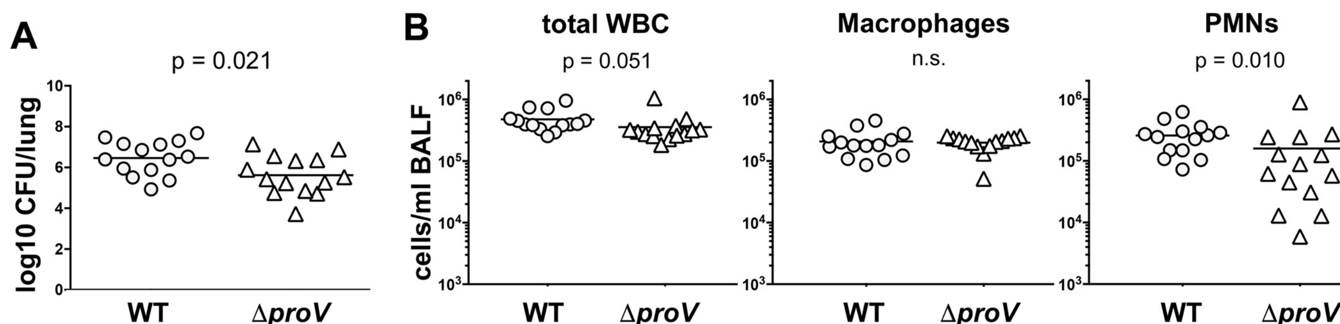

FIG 5 The ProU glycine betaine ABC transporter (*proVWX*) contributes to *K. pneumoniae* fitness during acute pneumonia. (A) Adult male C57BL/6J mice were infected via oropharyngeal aspiration with either *K. pneumoniae* KPPR1 WT or KPPR1 Δ*proV*. The numbers of CFU per lung were measured at 24 h following instillation. (B) The white blood cells within the bronchoalveolar lavage fluid collected from each infected mouse were enumerated. The total white blood cell, macrophage, and PMN counts are shown. The data shown summarize those from four independent experiments, with statistical significance being determined through an unpaired *t* test. In each panel, the arithmetic mean is depicted for each strain, with the counts from individual mice being represented by individual points.

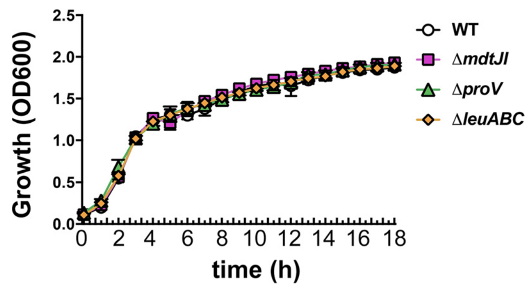

FIG 6 KPPR1 isogenic deletion strains exhibit wild-type growth kinetics in TSB. The growth of the KPPR1 WT and gene deletion strains in TSB was measured via measurement of the OD_{600} over an 18-h period. The growth curves shown are representative of those from three independent experiments, with error bars indicating standard deviation.

presence of Survanta. However, both *K. pneumoniae* MGH 78578 and KPPR1 failed to effectively utilize Survanta as a nutrient source in a range of media, including lysogeny broth (LB), TSB, R2A, MOPS, and M63 (data not shown). Specifically, addition of Survanta to either these rich or minimal medium formulations did not result in an increase or a decrease of the CFU counts compared to those in the media without Survanta.

Some Survanta-induced transcripts are expressed in response to specific lung surfactant components. An additional goal of this study was to identify the molecules within lung surfactant that promote *K. pneumoniae* virulence gene expression. The ability of individual constituents of lung surfactant to stimulate *mrkA*, *proV*, and *mdtJ* transcription was examined through quantitative RT-PCR. For these experiments, RNA was collected from *K. pneumoniae* MGH 78578 cells grown in MOPS minimal medium and subsequently exposed to the individual components found within lung surfactant or lactate as a control. The compounds tested included phosphatidylcholine, diacylglycerol, palmitate, sphingosine, and cholesterol, in addition to choline and ethanolamine, which have previously been shown to induce fimbria expression in enterohemorrhagic *E. coli* (65). Exposure to cholesterol and phosphatidylcholine stimulated the transcription of *mrkA*, but none of the individual compounds tested significantly induced the transcription of *mdtJ* or *proV* (Fig. 7).

Surfactant-induced biofilm formation is mediated by type 3 fimbriae. Both our microarrays and subsequent qRT-PCR revealed that the type 3 fimbria gene cluster (*mrkABCDF*) is expressed by *K. pneumoniae* MGH 78578 and KPPR1 following exposure to lung surfactant (Fig. 1 and 2 and Table 1). In addition, our gene induction experiments indicated that two constituents of lung surfactant, phosphatidylcholine and cholesterol, induced transcription from the *mrkA* promoter (Fig. 7). We were therefore curious to determine if these observations were reflected through increased biofilm production. To address this question, we cultured *K. pneumoniae* MGH 78578, the KPPR1 WT, and KPPR1 Δ*mrkABC* in minimal medium in the presence and absence of Survanta, phosphatidylcholine, or cholesterol and then on the following day quantified the resultant biofilm material that adhered to the plastic culture wells through a crystal violet staining assay.

As shown in Fig. 8A, exposure to Survanta, phosphatidylcholine, and cholesterol resulted in significant increases in biofilm production in *K. pneumoniae* MGH 78578 and KPPR1. The biofilms generated by KPPR1 under these conditions were notably less robust than those produced by MGH 78578. These observations can be explained in part by the hypermucoid phenotype of KPPR1 (cps_{K2} serotype [56]) relative to the phenotype of MGH 78578 (cps_{K52} serotype [66]), since capsule production is known to negatively impact biofilm formation in *K. pneumoniae* (67, 68). Furthermore, deletion of the *mrkABC* fimbria genes in KPPR1 disrupted biofilm formation at the air-liquid interface in the presence of lung surfactant and resulted in a substantial reduction in adhered biofilm material compared to that for the WT strain under every condition tested (Fig. 8A). These data indicate that lung surfactant-induced biofilm formation is primarily mediated by type 3 fimbriae.

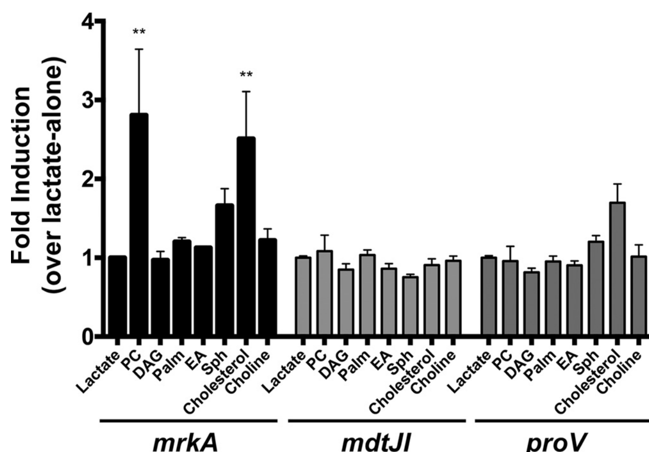

FIG 7 Constituents of lung surfactant stimulate *K. pneumoniae* gene expression. Gene induction assays were performed for 4 h with *K. pneumoniae* MGH 78578 in MOPS minimal medium containing lactate and individual compounds found within PS. RNA collected from these inductions was then used for quantitative RT-PCR, with the raw transcript values being normalized to those for *Kpn_04184*. The data shown encompass those from three separate experiments. Statistical analysis was performed via two-way analysis of variance and Dunnett's multiple-comparison test, using the uninduced (lactate-alone) condition as the comparator. For this analysis, the *Kpn_04184*-adjusted transcript values under the uninduced (lactate-alone) condition were first set equal to 1 for comparison. Statistical significance is depicted as follows: **, $P < 0.01$. PC, phosphatidylcholine; DAG, diacylglycerol; Palm, palmitate; EA, ethanolamine; Sph, sphingosine.

DISCUSSION

Our understanding of the genetic factors influencing *Klebsiella pneumoniae* pathogenesis has significantly improved in recent years (1, 9). *In vivo* genetic screens and deep sequencing have been particularly effective in identifying genes associated with *K. pneumoniae* fitness during infection in a range of tissue types (42, 69–72). Bachman and colleagues recently applied this methodology to uncover numerous *K. pneumoniae* genes that contribute to pathogenesis within the lung (60). Despite these advances, there is still much that we do not understand regarding the role that *K. pneumoniae*'s response to the host environment plays in shaping colonization and pathogenesis.

Lung surfactant serves as an initial point of contact for inhaled bacteria entering the lung, particularly those in small aerosol droplets, and likely contains molecular cues that influence colonization and pathogenesis. Our group has demonstrated the utility of the lung surfactant preparation Survanta for dissecting host-lung pathogen interactions in *P. aeruginosa*. We previously showed that lung surfactant leads to induction of transcripts involved in the detection of sphingosine and the metabolism of choline and that both of these pathways are required for *P. aeruginosa* survival in a mouse model of acute pneumonia (23, 24). We also showed that the utilization of phosphatidylcholine metabolites in lung surfactant by *P. aeruginosa* promoted virulence factor expression (25) and directly contributed to the loss of surfactant function during murine infection (73).

In this study, we expanded on our previous efforts with *P. aeruginosa* and characterized the transcriptional changes within *Klebsiella pneumoniae* MGH 78578 resulting from exposure to Survanta. We observed numerous alterations within the *K. pneumoniae* transcriptome that likely promote colonization, adaptation to the host, and virulence *in vivo*. Notable transcripts expressed by *K. pneumoniae* under these conditions included genes involved in capsule synthesis, LPS modification, antibiotic resistance, and biofilm formation (Fig. 1 and 2). Furthermore, a sizeable fraction of the transcripts identified through this work indicates that the lipid-rich environment of lung surfactant invokes significant membrane, cytosolic, and oxidative stress in *K. pneumoniae* (Fig. 1B and Table 1). These results parallel our earlier findings in *P. aeruginosa* (23, 25) and support similar observations in *Staphylococcus aureus* (26) suggesting that

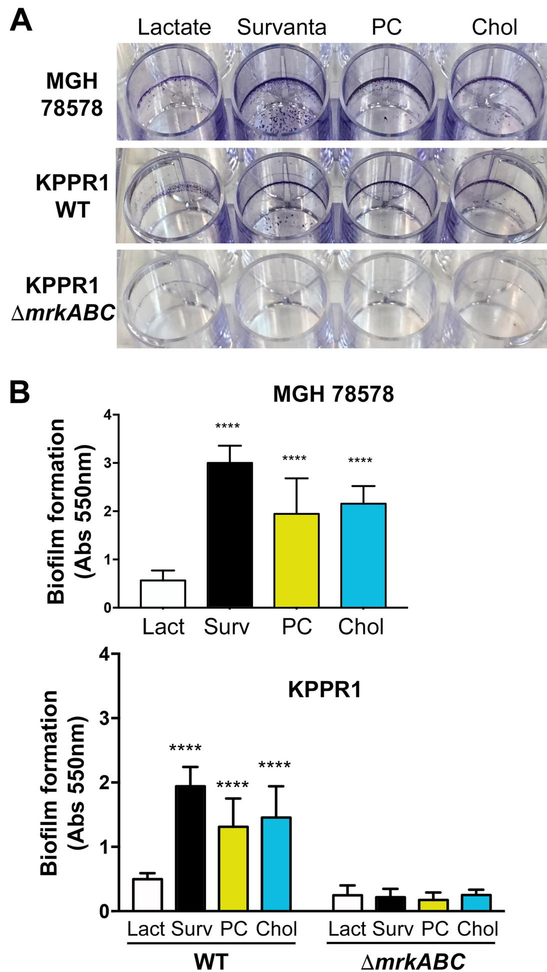

FIG 8 Type 3 fimbriae mediate biofilm formation in response to lung surfactant. *K. pneumoniae* MGH 78578, KPPR1, and KPPR1 Δ*mrkABC* were cultured in MOPS minimal medium containing 20 mM lactate in the presence and absence of Survanta, phosphatidylcholine (PC), and cholesterol (Chol). After 18 h, the extracellular material remaining adhered to the culture dish was stained with 0.1% crystal violet. (A) Representative crystal violet-stained biofilms generated by each strain under the culturing conditions described above. (B) Biofilm-adhered crystal violet was solubilized with 30% glacial acetic acid and quantified through measuring the absorbance at 550 nm. The data shown are the summary of those from four individual experiments that were performed in technical triplicate, with error bars representing standard deviations. Lact, lactate; Surv, Survanta. Statistical analysis was performed using the MOPS-lactate condition of each strain as the comparator via one-way analysis of variance and Dunnett's multiple-comparison test for *K. pneumoniae* MGH 78578 and two-way analysis of variance with Sidak's multiple-comparison test for the KPPR1 WT and KPPR1 Δ*mrkABC*. Statistical significance is specified as follows: ****, $P < 0.0001$.

lung surfactant likely promotes the expression of virulence and stress-related genes in a range of lung pathogens.

Lung surfactant-induced transcripts contribute to *K. pneumoniae* survival during acute murine pneumonia. We also demonstrated that lung surfactant-induced transcripts contribute to *K. pneumoniae* survival and the resulting inflammation during acute pneumonia. For these experiments, we focused on metabolism-related genes induced by surfactant.

Our interest in BCAA synthesis and the role that these genes play in bacterial fitness during infection stems from our observation that lung surfactant specifically stimulates transcription of the leucine synthetic operon (*leuABCD*) in *K. pneumoniae*. In contrast, the expression of genes for other amino acid anabolic pathways was not altered by lung surfactant under these conditions. The mechanism driving this induction is unclear, but lung surfactant metabolism by *K. pneumoniae* could invoke a specific,

previously unknown need for increased leucine synthesis. The ability to synthesize BCAAs during infection is known be critical for the survival and virulence of several bacterial lung pathogens, given its scarcity in the lung environment (60, 74–76). The necessity of BCAA synthesis for *K. pneumoniae* during pulmonary infection was recently highlighted through an *in vivo* transposon mutant screen that recognized that *ilvADE* and *leuABCD* gene disruption mutants displayed competitive fitness defects in the murine lung (60). Results from our mouse infections with an engineered leucine auxotroph of *K. pneumoniae* support these earlier findings and confirm that BCAA biosynthesis is required for both fitness and survival in the absence of competition during lung infection (Fig. 3), suggesting that, like in other bacterial lung pathogens, the loss of leucine synthesis is deleterious to survival in the lungs.

Polyamines have been recognized to be significant mediators of bacterial virulence and often have pleiotropic effects on pathogenesis (63, 77). Within enteric species, the accumulation of putrescine and spermidine has been shown to promote biofilm formation in *Yersinia pestis*, type 3 secretion system expression in *Salmonella* Typhimurium, and increased resistance to reactive oxygen species in *Shigella flexneri* during macrophage infection (78–80). Surprisingly, however, the influence of polyamines on *K. pneumoniae* survival had not previously been explored. Here, we have shown that deletion of the genes encoding the spermidine and putrescine efflux pump *mdtJI* (35, 62) resulted in a significant defect in *K. pneumoniae* survival relative to that of the parental WT strain in our murine model of pneumonia (Fig. 4). We propose two potential explanations for these observations. First, putrescine and spermidine are present on the outer membranes of enteric species and have been shown to alter membrane permeability through modifying the charge and shape of porins in *E. coli* (81, 82). Likewise, the presence of these polyamines on the outer membrane surface of *P. aeruginosa* has been shown to protect against oxidative stress and antibiotic-mediated killing (83). Therefore, MdtJI-mediated polyamine efflux could similarly facilitate resistance against oxidative killing in *K. pneumoniae*. Second, the polyamines secreted by bacteria and fungi have been shown to interfere with the innate immune response by disrupting polymorphonuclear leukocyte (PMN) function (84–88).

Our lung surfactant lipid induction experiments failed to reveal any individual components within surfactant that stimulated transcription of *mdtJI* (Fig. 7). The expression of this pump is primarily regulated by the intracellular concentration of putrescine (35). However, transcription of *mdtJI* has also been shown to be stimulated by deoxycholate and bile salts in *S. flexneri* (35), suggesting that this efflux pump could also be induced by membrane stress or other environmental cues. We predict that the expression of *mdtJI* in *K. pneumoniae* under these conditions could be a consequence of either membrane stress or the metabolism of multiple components within lung surfactant.

The ProU (*proVWX*) ABC transporter has been well characterized in *E. coli* and *S.* Typhimurium and functions in the uptake of the osmoprotectant glycine betaine under periods of osmotic stress (43, 64). Phosphatidylcholine is the most abundant phospholipid within lung surfactant and serves as a vital precursor of glycine betaine for *P. aeruginosa*, the accumulation of which is required for bacterial survival in the lung (24). The survival defect that we observed in the *K. pneumoniae* Δ*proV* strain in our acute murine pneumonia model is in close agreement with these earlier findings and suggests that the ability to obtain glycine betaine from phosphatidylcholine is likely important for other Gram-negative respiratory pathogens as well (Fig. 5). Host-derived glycine betaine has additionally been shown to promote *K. pneumoniae* success at other sites of infection. An *in vivo* screen previously revealed that *proV* gene disruption mutants displayed a competitive fitness defect in the colon and liver (42), indicating that glycine betaine likely serves as a preferred osmoprotectant for *K. pneumoniae* during infection.

It is important to note that not all surfactant-induced transcripts expressed by *K. pneumoniae* contribute to bacterial fitness during lung infection. The products of the six-gene *Kpn_02053-Kpn_02057* operon are predicted to function in the uptake and

metabolism of short-chain fatty acids (28, 29), and these genes represent the most highly induced transcripts expressed by *K. pneumoniae* in response to lung surfactant (Fig. 1 and Table 1). Despite the dramatic increase in transcription of this operon in response to lung surfactant, the deletion strain exhibited no defect in bacterial lung burden compared to the WT strain at 24 h postinoculation. Similar results were also observed in a Δ*fadBA* strain, indicating that the metabolism of fatty acids within lung surfactant does not directly contribute to *K. pneumoniae* fitness during acute pneumonia (data not shown). The lack of a phenotype for these highly expressed transcripts is not unexpected, as there is no evidence of a direct relationship between gene expression and fitness phenotype in bacterial lung infections to date (89).

Lung surfactant promotes type 3 fimbria expression and biofilm formation in *K. pneumoniae*. Exposure to lung surfactant induced type 3 fimbria-mediated biofilm formation in *K. pneumoniae* MGH 78578 and KPPR1 (Fig. 8). Type 3 fimbriae (Mrk fimbriae) have been extensively studied in *K. pneumoniae* and facilitate cell adhesion to a range of biotic and abiotic substrates, including type IV and type V collagen, silicone, and hard plastics (90–93). Although type 3 fimbriae are not directly involved in *K. pneumoniae* virulence, their requirement for colonization and persistence in catheter-associated urinary tract infections (CAUTI) has been demonstrated by multiple groups (94, 95). The transcriptional regulation of type 3 fimbria expression in *K. pneumoniae* is complex and governed by multiple integrated regulatory networks, including being dependent on the coordinated activities of MrkH and MrkI in response to the intracellular accumulation of the secondary messenger cyclic-di-GMP (96, 97). Surprisingly, the environmental signals and regulatory networks acting upstream of MrkHI that drive type 3 fimbria expression are largely unknown, particularly in the context of infection. Recent reports have identified iron- and oxidative stress-responsive transcription regulators that modulate *mrk* fimbria expression (98–100), and Chen et al. also identified bile salts to be stimulators of type 3 fimbria-mediated biofilm formation (101). Here, we expand on these previous findings and report that at least two components of lung surfactant, phosphatidylcholine and cholesterol, promote type 3 frimbria transcription and biofilm formation in *K. pneumoniae* (Fig. 7 and 8).

Conclusions. In summary, we characterized the transcriptional response of *K. pneumoniae* MGH 78578 to the lung surfactant preparation Survanta. This work revealed numerous transcripts expressed by *K. pneumoniae* in response to lung surfactant that reflect metabolic adaptation, stress resistance, virulence, and host colonization. We also demonstrated that some surfactant-induced transcripts contribute to bacterial survival *in vivo* in a mouse model of acute pneumonia. Through this effort we confirmed the necessity of BCAA synthesis to *K. pneumoniae* success during infection and provided novel evidence suggesting that glycine betaine uptake and polyamine efflux also contribute to *Klebsiella* survival during respiratory tract infection. Finally, we identified multiple components within lung surfactant that stimulate type 3 fimbria-mediated biofilm formation. This study provides novel insight into the interactions occurring between *K. pneumoniae* and the host at an important infection site. This work, together with our previous studies in *P. aeruginosa*, highlights the utility of using lung surfactant to uncover important aspects of host-lung pathogen interactions *in vitro*.

MATERIALS AND METHODS

Bacterial strains and compounds. *K. pneumoniae* KPPR1 (ATCC 43816) and *K. pneumoniae* MGH 78578 (ATCC 700721) were maintained on lysogeny broth (LB), Lennox formulation, supplemented with 200 μg/ml of hygromycin B when appropriate. All cloning steps were performed with *E. coli* DH5α λ*pir*, while *E. coli* S17-1 λ*pir* was used for conjugation with *K. pneumoniae*. Both *E. coli* strains were maintained in LB, supplemented with 150 μg/ml of hygromycin B when appropriate. The strains and plasmids used in this study are described in Table 2. The purified bovine pulmonary surfactant preparation Survanta (Beractant; AbbVie, Lake Bluff, IL) was utilized for our surfactant-response microarrays and biofilm experiments. Survanta is an organic extraction of lung surfactant from cows and as such is missing the polar surfactant proteins involved in pulmonary defense (SP-A and SP-D) as well as most antimicrobial peptides (including defensins) and antimicrobial proteins present in the lung lining fluid (e.g., lysozyme); thus, it is composed of the lipids naturally present in lung surfactant along with the hydrophobic proteins SP-B and SP-C. Because it is an organic extraction product, physiological concentrations of salts and dissolved polar compounds are added back by dilution of this product into minimal medium. The lung

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
K. pneumoniae strains		
GGW112	MGH 78578	ATCC 700721
GGW231	KPPR1	ATCC 43816
GGW178	Δ*proV* in GGW231	This study
GGW180	Δ*leuABCD* in GGW231	This study
GGW192	Δ*mrkABC* in GGW231	This study
GGW194	Δ*mdtJI* in GGW231	This study
E. coli strains		
DH5α λ*pir*	*sup E44* Δ*lacU169* ϕ80Δ(*lacZ*)M15 *recA1 endA1 hsdR17 thi-1 gyrA96 relA* λ*pir*	Bio-Rad
NEB5α	*fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44* ϕ80Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
S17-1 λ*pir*	*thi pro hsdR* negative *hsdM* positive Δ*recA RP4-2*::TcMu-Km::Tn*7* λ*pir*	112
GGW166	pGW74 in S17-1 λ*pir*	This study
GGW168	pGW76 in S17-1 λ*pir*	This study
GGW172	pGW78 in S17-1 λ*pir*	This study
GGW186	pGW79 in S17-1 λ*pir*	This study
Plasmids		
pGW65	Suicide vector, R6Kγ ori Hm^r *sacB*	This study
pGW74	*proV*-SOE in pGW65	This study
pGW76	*leuABCD*-SOE in pGW65	This study
pGW78	*mdtJI*-SOE in pGW65	This study
pGW79	*mrkABC*-SOE in pGW65	This study

[a]SOE, splice overlap extension products used for gene deletion.

surfactant constituents used in our gene induction assays were purchased from Avanti Polar Lipids (Alabaster, AL) and Sigma-Aldrich (St. Louis, MO).

Construction of *K. pneumoniae* gene deletion strains. Gene deletion strains in *K. pneumoniae* KPPR1 were generated through allelic exchange facilitated by the suicide vector pGW65. To create pGW65, pMQ310 and pMQ30 (102, 103) were first digested with NcoI and KpnI (New England BioLabs, Ipswich, MA). The 3.9-kbp fragment of pMQ310 carrying the hygromycin B resistance cassette and R6Kγ origin and the 4.6-kbp fragment of pMQ30 carrying the *sacB* counterselectable marker were gel extracted using Thermo Fisher's GeneJET kit (Waltham, MA) and subsequently ligated together before transformation into chemically competent DH5α λ*pir* cells. Gene deletion constructs were engineered into this vector using the molecular cloning methodology previously described with pMQ30 (103, 104). Briefly, ~1-kbp fragments immediately upstream and downstream of the gene (or genes) targeted for deletion were amplified using the primers listed in Table S2 in the supplemental material. For each deletion construct, tailed primers were used to facilitate the fusion of each fragment via overlap extension PCR as well as ligation into pGW65 through incorporated flanking restriction sites. The ligation reaction mixtures were then chemically transformed into DH5α λ*pir* cells, and transformants were selected for on LB supplemented with 150 μg/ml of hygromycin B. Plasmid DNA was harvested from these colonies by use of a miniprep kit (Qiagen) and verified by restriction digestion.

Deletion constructs were subsequently transformed into chemically competent *E. coli* S17-1 λ*pir* cells and mobilized into *K. pneumoniae* KPPR1 via conjugation (105). Following overnight incubation at 37°C, merodiploids were selected by plating on MOPS (morpholinepropanesulfonic acid) minimal agar medium supplemented with 200 μg/ml of hygromycin B and 25 mM sodium pyruvate. To select for the Δ*leuABCD* strain, 0.5% Casamino Acids was added to this medium. KPPR1 merodiploids that arose the next day were then restreaked onto this medium to ensure that *E. coli* S17-1 λ*pir* cells were not carried over. A second round of recombination was then permitted by first growing hygromycin B-resistant colonies overnight in LB containing 200 μg/ml of the antibiotic, diluting the overnight culture 1:500, and growing the culture to mid-log phase in LB in the absence of hygromycin B. Dilutions of this culture were then plated on low-salt LB agar containing 6% sucrose and incubated overnight at 25°C, as suggested previously (105). Sucrose-resistant colonies arising 24 h later were screened for deletion of the gene(s) of interest via PCR with the primers listed in Table S2.

Growth conditions and RNA purification for microarrays/qRT-PCR. *K. pneumoniae* MGH 78578 was grown overnight at 37°C in modified MOPS minimal medium (106, 107) supplemented with 25 mM lactate and 5 mM D-glucose. On the following day, cells were collected by centrifugation, washed with 1 ml of MOPS medium, and resuspended in MOPS medium containing 4 mM lactate to achieve an optical density at 600 nm (OD_{600}) of 0.6. These cultures were then mixed 1:1 with MOPS medium containing 4 mM lactate or the same medium supplemented with Survanta (AbbVie, Lake Bluff, IL) at a dilution of 1:50 to reflect the physiological concentration of pulmonary surfactant in the airway surface liquid (15 mg/ml). Cultures were incubated at 37°C with shaking at 170 rpm for 4 h, at which point the cells

were harvested via centrifugation, immediately lysed in ~85°C RNAzol reverse transcriptase (Sigma-Aldrich, St. Louis, MO), and frozen at −80°C. RNA extractions were first performed using Zymo Research's RNA miniprep kit (Irvine, CA) following the manufacturer's provided protocol. The resulting RNA was then incubated for 1 h with DNase I (NEB) before being repurified using RNeasy columns (Qiagen) to remove small RNAs in preparation for their use in the microarrays, as we have done previously (23, 108). The quality of each RNA sample was then assessed via an Agilent BioAnalyzer and quantified through a Qubit fluorometer.

Survanta microarray methodology. Microarray analyses were performed by the UVM Advanced Genome Technology Core using a custom Affymetrix chip containing probes specific to the genomes of *Klebsiella pneumoniae* MGH 78578, *Stenotrophomonas maltophilia* K279A, *Burkholderia thailandensis* E264, and *Pseudomonas aeruginosa* PA14 (109). Analyses with the arrays were performed in biological duplicate, with RNA being collected from two independent Survanta induction experiments that were performed on separate days. *K. pneumoniae* cDNA hybridization was performed simultaneously with a 1:1 mixture of Survanta-induced *Stenotrophomonas maltophilia* K279A cDNA (cultured under the same conditions) per the manufacturer's recommendation. Each condition was analyzed in duplicate, with the intensity of the probes for each gene being averaged into the intensity of one probe using the Affymetrix Expression Console and Transcriptome Analysis Console software packages (version 3.0). Surfactant-altered transcripts were identified as those exhibiting at least a 2.5-fold change in signal between the two conditions, as determined using robust multiarray average (RMA) analysis, and a P value of <0.05.

Quantitative RT-PCR. Total RNA was prepared from three additional Survanta inductions with *K. pneumoniae* MGH 78578 as described above. Twenty nanograms of RNA from each sample was then utilized as the template for cDNA synthesis using SuperScript IV reverse transcriptase and random hexamers (Thermo Fisher) per the manufacturer's instruction. Quantitative PCR was performed using the resulting cDNA in technical duplicate with the primers listed in Table S1 and NEB's Q5 2× master mix supplemented with SYBR green I nucleic acid gel stain (Thermo Fisher) at a concentration of 0.2×, as we have done previously (108). A standard curve dilution series was generated for each primer set to determine transcript abundance (110). The values for each reaction were normalized to those for *Kpn_04184*, which exhibited no change in expression between conditions in the Survanta microarrays. The fold change in expression for each transcript was determined by dividing the normalized surfactant-exposed values by their corresponding control condition values. The absence of reverse transcriptase during cDNA synthesis resulted in no product from any primer set when the isolated RNA was used.

Mouse infections. Mouse infections were performed as previously described (57, 73). Briefly, *K. pneumoniae* KPPR1 WT and isogenic deletion strains were grown in TSB overnight, and the growth was normalized by the OD_{600}, harvested via centrifugation, washed in 2 ml of phosphate-buffered saline (PBS), and finally, resuspended in PBS to achieve 2×10^3 CFU per 50 μl. For each strain, the actual input inoculum was determined by serial dilution plating on LB agar. Eight- to 10-week-old adult male C57BL/6J mice (The Jackson Laboratory, Detroit, MI) were briefly anesthetized with isoflurane and inoculated with 2×10^3 CFU of either the KPPR1 WT or isogenic deletion strains through oropharyngeal aspiration. Twenty-four hours later, the mice were euthanized with sodium pentobarbital, delivered through intraperitoneal injection. Bronchoalveolar lavage fluid was then collected, and the lungs were then quickly removed, placed into 1 ml of cold PBS, and immediately homogenized.

Serial dilutions of the resulting lung homogenates were plated on LB agar to determine the bacterial burden by counting the number of CFU. The white blood cell content within the bronchoalveolar lavage fluid was enumerated manually. Infections were performed at least three times with 3 to 4 mice per strain per experiment. In each case, paired infections were performed with one gene deletion strain and the parental WT strain for comparison of the number of lung CFU. The protocol for animal infection was approved by the University of Vermont Institutional Animal Care and Use Committee, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines (Animal Welfare Assurance A3301-01).

Growth assays. Growth assays were conducted with *K. pneumoniae* KPPR1 WT and isogenic deletion strains as we have done previously with *P. aeruginosa* (104). Briefly, the KPPR1 WT and our isogenic deletion strains were grown overnight at 37°C on a roller drum in MOPS minimal medium supplemented with 20 mM lactate and 5 mM D-glucose. In the case of KPPR1 Δ*leuABC*, 0.5% Casamino Acids was added to this medium to permit growth. On the following day, cells were collected via centrifugation, washed with 1 ml MOPS medium, and resuspended in TSB at a final optical density of 0.05 OD_{600} unit. Growth assays were performed three times, each with technical triplicates, in a 48-well tissue culture plate, and growth was determined by measurement of the OD_{600} using a Synergy 2 plate reader (Biotek). Growth assays in Survanta were conducted as described above, except that growth was quantified by serial dilution plating, as Survanta is a colloidal suspension and prevents growth assessment by measurement of the OD_{600}.

Gene induction assays with components of lung surfactant. To identify the transcript-inducing molecules within lung surfactant, quantitative RT-PCR was performed on *K. pneumoniae* MGH 78578 RNA collected from cells exposed to 1 mM phosphatidylcholine, sphingosine, cholesterol, diacylglycerol, palmitate, choline, or ethanolamine or no compound as a control. For these experiments, *K. pneumoniae* was first grown overnight in MOPS minimal medium as described above. On the following day, cells were collected by centrifugation, washed in 1 ml MOPS, and resuspended in MOPS–20 mM lactate to achieve a final OD_{600} of 0.3. One-milliliter aliquots of this culture were then added to a plastic culture dish with wells containing these compounds, deposited via the evaporation of ethanol, and incubated for 4 h at 37°C and 170 rpm. Following the induction period, RNA was purified from these cells, cDNA was synthesized, and quantitative PCR was performed as described above.

Biofilm assay. *K. pneumoniae* MGH 78578, the KPPR1 WT, and KPPR1 Δ*mrkABC* were grown overnight at 37°C on a roller drum in MOPS minimal medium supplemented with 20 mM sodium pyruvate and 5 mM glucose. On the following day, cells were collected by centrifugation, washed in 1 ml of MOPS medium, and adjusted to an OD_{600} of 0.1. Each strain was then added 1:1 to MOPS medium containing 20 mM sodium lactate, in addition to the same medium supplemented with Survanta, to achieve a final surfactant dilution of 1:50. The OD_{600}-adjusted cultures were also diluted 1:1 in the same medium (MOPS, 20 mM sodium lactate) and added to the wells of a 48-well dish containing phosphatidylcholine or cholesterol that had been deposited the night prior through ethanol evaporation. These cultures were incubated for 18 h at 37°C and agitated at 170 rpm to loosely reflect the continuous aeration and mixing of surfactant that occurs within the lung. Following the incubation, the cell suspension was removed from the wells and the remaining biofilm material was stained using 0.1% crystal violet, followed by a water rinse and solubilization of the remaining crystal violet in 30% acetic acid (111). Biofilm was quantified by measuring the A_{550} using a Biotek Synergy 2 plate reader. This experiment was performed four times with technical triplicates of each experiment.

Statistical analysis and data visualization. All statistical analyses and figure generation were performed using GraphPad Prism software (version 7.0), unless otherwise noted. Microarray analysis and statistical assessment were performed through RMA using Affymetrix's Expression Console and Transcriptome Analysis Console software packages (version 3.0) as described above. Gene functional classification was done by manually combining related Gene Ontology, Clusters of Orthologous Groups, and KEGG predictions into more general functions.

Accession number(s). The array data have been submitted to the GEO database under accession number GSE110628.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00135-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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