Subinhibitory Concentrations of Antibiotics Alter the Response of *Klebsiella pneumoniae* to Components of Innate Host Defense

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ABSTRACT  Carbapenem-resistant *Klebsiella pneumoniae* isolates classified as multilocus sequence type 258 (ST258) are a problem in health care settings in many countries globally. ST258 isolates are resistant to multiple classes of antibiotics and can cause life-threatening infections, such as pneumonia and sepsis, in susceptible individuals. Treatment strategies for such infections are limited. Understanding the response of *K. pneumoniae* to host factors in the presence of antibiotics could reveal mechanisms employed by the pathogen to evade killing in the susceptible host, as well as inform treatment of infections. Here, we investigated the ability of antibiotics at subinhibitory concentrations to alter *K. pneumoniae* capsular polysaccharide (CPS) production and survival in normal human serum (NHS). Unexpectedly, pretreatment with some of the antibiotics tested enhanced ST258 survival in NHS. For example, a subinhibitory concentration of mupirocin increased survival for 7 of 10 clinical isolates evaluated and there was increased cell-associated CPS for 3 of these isolates compared with untreated controls. Additionally, mupirocin pretreatment caused concomitant reduction in the deposition of the serum complement protein C5b-9 on the surface of these three isolates. Transcriptome analyses with a selected ST258 isolate (34446) indicated that genes implicated in the stringent response and/or serum resistance were upregulated following mupirocin treatment and/or culture in NHS. In conclusion, mupirocin and/or human serum causes changes in the *K. pneumoniae* transcriptome that likely contribute to the observed decrease in serum susceptibility via a multifactorial process. Whether these responses can be extended more broadly and thus impact clinical outcome in the human host merits further investigation.

IMPORTANCE The extent to which commensal bacteria are altered by exposure to subinhibitory concentrations of antibiotics (outside resistance) remains incompletely determined. To gain a better understanding of this phenomenon, we tested the ability of selected antibiotics (at subinhibitory concentrations) to alter survival of ST258 clinical isolates in normal human serum. We found that exposure of ST258 to antibiotics at low concentrations differentially altered gene expression, capsule production, serum complement deposition, and bacterial survival. The findings were isolate and antibiotic dependent but provide insight into a potential confounding issue associated with ST258 infections.

KEYWORDS  *Klebsiella*, antibiotic resistance, capsular polysaccharide, serum resistance

*T. pneumoniae* is historically an opportunistic pathogen that can cause life-threatening pneumonia, bacteremia, and urinary tract infections (1). Carbapenem antibiotics have been used for decades to treat infections caused by β-lactam-resistant *K. pneumoniae* (2), and thus the emergence of carbapenem-resistant *K. pneumoniae* worldwide is a significant concern. *K. pneumoniae* strains that produce carbapenemase, such as *K. pneumoniae* carbapenemase (KPC), can inactivate carbapenems by hydrolysis and degrade β-lactam antibiotics and some β-lactamase inhibitors (3, 4). Resistance to fluoroquinolones and aminoglycosides in these organisms is often a confounding factor, and treatment options are limited (5–8).
The majority of KPC-producing clinical isolates in the United States are classified by multilocus sequence typing (MLST) as sequence type 258 (ST258) (9). The ST258 lineage is widely distributed and abundant in Europe, North America, and South America (4). Genetic characterization of ST258 isolates in the United States revealed two predominant capsular polysaccharide (CPS) types (CPS1 and CPS2, now known as KL106 and KL107, respectively), encoded by \( \text{cps1} \) and \( \text{cps2} \) loci (10). The basis for the success of ST258 and related clones outside antibiotic resistance remains incompletely determined, although CPS likely plays an important role. In general, \( K. \ pneumoniae \) CPS contributes to the often-observed resistance to serum bactericidal activity \( \text{in vitro} \) (11). Isogenic noncapsulated strains and capsule-defective strains are more susceptible to serum bactericidal activity than wild-type \( K. \ pneumoniae \) strains (12, 13), and increased serum killing of strains lacking CPS is in part attributed to increased complement deposition on the bacterial surface (12, 14, 15). Bacterial activity of serum is also enhanced by CPS-specific antibodies (13, 16), which may in part explain the varied survival of ST258 clinical isolates in human blood and serum \( \text{in vitro} \) (17).

Subinhibitory concentrations of antibiotics can serve as signal molecules, directing changes in gene expression that result in altered virulence and physiology (18–22). Therefore, exposure of bacteria to subinhibitory concentrations of antibiotics has the potential to impact treatment negatively. To better understand the ability of ST258 to cause human infections, we tested the ability of antibiotics to alter bacterial susceptibility to normal human serum (NHS).

**RESULTS**

Subinhibitory concentrations of antibiotics alter ST258 survival in human serum.

We first evaluated the ability of subinhibitory concentrations of selected antibiotics to alter ST258 survival in NHS. Antibiotics were selected based on mechanism of action and/or use as a primary therapeutic agent for \( K. \ pneumoniae \) infections (see Tables S1 and S2 in the supplemental material). The 10 clinical isolates tested were recovered from patients with bacteremia and/or a wound infection and were assigned to KL106 (CPS1) and KL107 (CPS2) capsule subclades (Table 1). Pretreatment with five of the antibiotics tested, doxycycline, colistin, mupirocin, rifampin, and tigecycline, caused increased survival in NHS for one or more of the isolates evaluated in these assays (Table 2). Unexpectedly, subinhibitory concentrations of mupirocin caused increased survival in NHS for 7 of the 10 isolates (Table 2). Collectively, these data provide evidence that subinhibitory concentrations of antibiotics can enhance survival of ST258 in NHS.

Decreased surface deposition of serum complement following exposure to mupirocin. We next investigated the mechanism underlying increased survival of ST258 in NHS following exposure to mupirocin, the antibiotic that altered survival for the greatest number of isolates. We first measured the deposition of serum complement C5b-9 on the bacterial surface by using flow cytometry (Fig. 1). ST258 isolates 34446 (CPS1) and 35106 (CPS2) were selected for the initial experiments, but we ultimately evaluated complement deposition with all 10 ST258 clinical isolates. Doxycycline and vancomycin were used as controls for these assays because they had no significant impact on the survival of 34446 or 35106 in NHS (Table 2). There was a significant decrease in deposition of C5b-9 on the

| Isolate | CPS | Isolate source (U.S. state) | Culture site |
|---------|-----|-----------------------------|--------------|
| 34422   | CPS1/KL106 | PA                          | Blood        |
| 26598   | CPS1/KL106 | NJ                          | Blood        |
| 30678   | CPS1/KL106 | NJ                          | Blood        |
| 34576   | CPS1/KL106 | PA                          | Blood        |
| 34446   | CPS1/KL106 | PA                          | Deep wound   |
| 34473   | CPS2/KL107 | PA                          | Blood        |
| 34505   | CPS2/KL107 | PA                          | Blood        |
| 27386   | CPS2/KL107 | NY                          | Blood        |
| 27329   | CPS2/KL107 | NY                          | Blood        |
| 35106   | CPS2/KL107 | NY                          | Blood        |

*All isolates shown are ST258 by MLST.
surface of mupirocin- and/or doxycycline-treated 34422, 34446, and 35106 compared with untreated control bacteria (P, 0.05) (Fig. 1). In comparison, vancomycin failed to alter surface deposition of C5b-9 on these clinical isolates (Fig. 1). Overall, there was not necessarily a direct correlation between decreased C5b-9 surface deposition and survival in these assays, since only three of the seven ST258 clinical isolates whose survival was increased following pretreatment with mupirocin had corresponding decreased complement deposition.

Mupirocin treatment increases cell-associated CPS production. Inasmuch as CPS has been demonstrated previously to protect K. pneumoniae from killing by serum complement

### TABLE 2 Subinhibitory concentrations of antibiotics alter ST258 survival in NHS

| Antibiotic  | Change in survival of isolatea: | 34422 | 26598 | 30678 | 34576 | 34446 | 34473 | 34505 | 27386 | 27329 | 35106 |
|-------------|--------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Cefazidime  |                                |       |       |       |       |       |       |       |       |       |       |
| Ciprofloxacin |                               |       |       |       |       |       |       |       |       |       |       |
| Colistin    |                                |       |       |       |       |       |       |       |       |       |       |
| Doxycycline |                                |       |       |       |       |       |       |       |       |       |       |
| Erythromycin|                                |       |       |       |       |       |       |       |       |       |       |
| Linezolid   |                                |       |       |       |       |       |       |       |       |       |       |
| Meropenem   |                                |       |       |       |       |       |       |       |       |       |       |
| Mupirocin   | +                              | +     |       | +     | +     | +     | +     | +     |       |       |       |
| Rifampin    |                                |       |       |       |       |       |       |       |       |       |       |
| Gentamicin  |                                |       |       |       |       |       |       |       |       |       |       |
| Tigecycline |                                |       |       |       |       |       |       |       |       |       |       |
| Vancomycin  |                                |       |       |       |       |       |       |       |       |       |       |

*aST258 clinical isolates were exposed to subinhibitory concentrations of the indicated antibiotics and then incubated in 90% NHS for 30 min as described in Materials and Methods. **, survival increased significantly compared to untreated control; *, survival decreased significantly compared to untreated control. Unmarked table cells indicate no significant change in survival compared to bacteria not pretreated with antibiotics (untreated control).

FIG 1 Antibiotics alter deposition of complement onto the bacterial surface. ST258 clinical isolates were treated with subinhibitory concentrations of the indicated antibiotics for 2 h and then incubated in 90% NHS for 30 min. The level of surface-associated C5b-9 was determined by flow cytometry as described in Materials and Methods. Results are presented as the mean ± standard deviation of the indicated number of experiments (where each symbol represents a unique experiment). *, P < 0.05 versus LB control assays as determined by using a repeated-measures one-way ANOVA and Dunnett’s posttest to correct for multiple comparisons. LB, Luria-Bertani broth; Mup, mupirocin; Dox, doxycycline; Van, vancomycin.

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We tested the ability of mupirocin to alter CPS production by ST258. To determine if CPS contributes to survival of the mupirocin-treated bacteria, we isolated cell-associated CPS and measured uronic acid content following exposure to subinhibitory concentrations of antibiotic (Fig. 2). Compared with untreated bacteria, CPS production was increased significantly in the three clinical isolates that had corresponding decreases in surface complement deposition (i.e., isolates 34422, 34446, and 35106) after pretreatment with mupirocin ($P < 0.01$) (Fig. 2A). On the other hand, increased cell-associated CPS failed to correlate with decreased C5b-9 surface deposition for 34446 and 35106 following exposure to doxycycline (Fig. 1 and Fig. 2A). Consistent with increased CPS production, transmission electron microscopy (TEM) analysis with isolate 34446 indicated that mupirocin pretreatment increased thickness of the CPS layer compared with that of untreated control bacteria or bacteria pretreated with doxycycline or vancomycin (Fig. 2B and C). Collectively, these data support the idea that mupirocin enhances survival of some ST258 clinical isolates (i.e., 34422, 34446, and 35106) in NHS in part by eliciting increased CPS production. These findings are consistent with previous studies showing that mupirocin enhances survival of ST258 in NHS by altering complement deposition and CPS production.

**FIG 2** Antibiotics and CPS production. (A) Cell-associated CPS recovered from ST258 clinical isolates treated with the indicated antibiotic was quantified based on uronic acid content. (B) Representative TEM images showing the effect of antibiotic treatment on cell-associated CPS production with isolate 34446. Bar = 500 nm. (C) Capsule thickness of 34446 following antibiotic treatment was measured using TEM images and ImageJ software. *, $P < 0.01$ versus LB control as determined with a repeated-measures (A) or ordinary (C) one-way ANOVA and Dunnett’s posttest to correct for multiple comparisons.
with those of Álvarez et al., who reported that the amount of CPS rather than K-type is important for *K. pneumoniae* resistance to complement-mediated killing (24).

**Mupirocin and/or human serum alters *K. pneumoniae* gene expression.** To gain insight into the molecular mechanisms used by ST258 to survive in NHS, we used transcriptome sequencing (RNA-Seq) to measure changes in the 34446 transcriptome during culture in NHS pretreatment with doxycycline or mupirocin (Fig. 3). We selected 34446 as a representative ST258 clinical isolate for RNA-Seq experiments because it had a strong survival phenotype following exposure to mupirocin and it was also not feasible to conduct these experiments with multiple clinical isolates. Principal-component analysis (PCA) was used as a first step to evaluate RNA-Seq data based on sources of experimental variance (Fig. 3A). Data obtained from bacteria exposed to subinhibitory concentrations of doxycycline clustered with control bacteria not exposed to antibiotic (LB and LB plus NHS) (Fig. 3A). On the other hand, bacteria cultured in NHS were separated clearly by PCA from those not cultured in NHS, regardless of the antibiotic pretreatment (Fig. 3A). In addition, data from bacteria treated with mupirocin clustered in groups separate from control bacteria or those treated with doxycycline (Fig. 3A). Collectively, these results suggest that NHS and/or subinhibitory concentrations of mupirocin but not doxycycline elicited significant changes in 34446 gene expression. Therefore, we analyzed data obtained from mupirocin-treated bacteria in more detail (Fig. 3B).

First, culture in NHS alone caused significant changes in the ST258 transcriptome, including upregulation of genes involved in CPS biosynthesis and serum fitness (Fig. 3B). The finding that *arnDEF, amtI, wcaJ, wecB*, and *wzc* were upregulated by ST258 during culture in NHS is

![FIG 3](https://example.com/figure3.png)

**FIG 3** Treatment with mupirocin alters ST258 gene expression. Gene expression was measured in ST258 isolate 34446 pretreated with the indicated antibiotics and/or cultured in NHS as described in Materials and Methods. (A) Data were visualized by using a PCA plot. (B) Selected genes whose expression was increased or decreased by mupirocin pretreatment alone (Mup versus LB), culture in NHS alone (NHS versus LB), or mupirocin pretreatment followed by culture in (Mup-NHS versus LB).
consistent with a recent study by Short et al, who used a transposon library to identify ST258 genes involved in serum resistance (25). Although mupirocin alone (no NHS) elicited changes in gene expression that were more limited in scope than those elicited by NHS (Fig. 3B, second column), the data are consistent with the increased CPS production (Fig. 2B). That is, treatment with mupirocin alone caused upregulation of transcripts involved in CPS biosynthesis (wzy, wcaJ, and ugd) and those encoding glycosyl transferases (Fig. 2B). In addition, glpD, pyrB, and pyrC, genes involved in \textit{K. pneumoniae} capsule regulation and serum fitness, were upregulated after pretreatment with mupirocin alone (Fig. 3B) (26–28). \textit{csrD} was downregulated following exposure to mupirocin, and mutation of \textit{csrD} increases \textit{K. pneumoniae} survival in serum (Fig. 3B) (25, 28). Collectively, these data provide support to the idea that the ability of subinhibitory concentrations of mupirocin to alter bacterial gene expression underlies the observed enhanced survival of 34446 in NHS.

**DISCUSSION**

Subinhibitory concentrations of antibiotics are known to elicit multiple responses in bacteria, including global changes in gene expression that can facilitate persistence in the host (18). Previous studies have reported synergy or antagonism between antibiotics and serum bactericidal activity, depending on the bacterium (29, 30). For example, Davis et al. showed that a subinhibitory concentration of colistin synergized with components of serum to kill \textit{Escherichia coli} (29). A similar observation was made by Loose et al. with colistin-resistant \textit{E. coli} (30). In contrast, the bactericidal activity of polymyxin B toward \textit{Pseudomonas aeruginosa} was inhibited completely by 20% human serum (29). The extent to which these phenomena impact treatment of human infections, including those caused by carbapenem-resistant \textit{K. pneumoniae}, remains incompletely determined. As a first step toward gaining a better understanding of this phenomenon, we tested the ability of antibiotics at subinhibitory concentrations to alter ST258 survival in NHS.

Although we tested a limited selection of antibiotics at subinhibitory concentrations, 5 of the 10 antibiotics evaluated increased survival in NHS for one or more ST258 clinical isolates. Our finding that mupirocin enhanced survival of the majority (7/10) of ST258 isolates in NHS is unrelated to clinical use of mupirocin, since it is used externally (e.g., on skin as a topical ointment or intranasally to eliminate \textit{Staphylococcus aureus} from the nose). Mupirocin inhibits bacterial iso-leucyl-tRNA and thereby inhibits protein synthesis (31–33), which in turn triggers the stringent response in some bacteria (34–37). The gene encoding SpoT, an enzyme that contributes to regulation of stress responses (including the stringent response) in \textit{K. pneumoniae}, was upregulated during culture in NHS, but it was not changed significantly by exposure to mupirocin under our assay conditions (Fig. 3B) (38). It is possible that the stringent response was induced by mupirocin in these ST258 isolates, but our ability to detect changes in gene expression was limited technically. There was increased C5b-9 surface deposition and concomitant increased cell-associated CPS on only 3 of the 10 ST258 isolates tested (Fig. 1 and 2). Inasmuch as we measured cell-associated CPS only, any CPS shed from the surface would have gone undetected in our uronic acid assays—and shed CPS has potential to alter serum bactericidal activity.

We used an RNA-Seq approach to gain insight into the mechanism underlying increased ST258 survival in human serum following pretreatment with mupirocin. We selected a single isolate (34446) for the RNA-Seq studies because it was one of three isolates that had concomitant increased C5b-9 surface deposition and decreased cell-associated CPS. Our finding that wzy, wcaJ, and ugd as well as genes encoding glycosyltransferases were upregulated following pretreatment with mupirocin (Fig. 3B) is consistent with the observed increase in CPS thickness in isolate 34446 (Fig. 2B). In addition, \textit{pyrB} and \textit{pyrC} were upregulated in ST258 isolate 34446 following exposure to mupirocin (Fig. 3B). Weber et al. reported \textit{pyrB} and \textit{pyrC} are important for \textit{K. pneumoniae} growth in serum (26), although specific roles for the encoded proteins in the survival in serum are not known. The ST258 genes reported here as induced by mupirocin and/or NHS may contribute to the observed phenotypes, but it remains incompletely determined how this antibiotic affected the changes that led to increased ST258 survival in serum.

Unintended effects of subinhibitory concentrations of antibiotics, such as increasing the ability of bacteria to survive in human serum, may confound treatment of infections.
This phenomenon and others related to antibiotics and altered bacterial responses merit further investigation.

**MATERIALS AND METHODS**

**Antibiotics, bacterial strains, and culture.** Linezolid and muipirocin were purchased from ChemPacific Corp. (Baltimore, MD) and AppliChem GmbH (Darmstadt, Germany), respectively. All other antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). The *K. pneumoniae* isolates used in this study are clinical isolates classified as multilocus sequence type 258 (ST258) (Table 1). All strains were routinely cultured in Luria-Bertani (LB) broth. For treatment/pretreatment of bacteria with antibiotics, overnight cultures were diluted 1:1,000 in fresh LB and then cultured at 37°C with shaking to an optical density at 600 nm (OD600) of 0.2 to 0.3. Antibiotics were added to bacterial culture aliquots to attain subinhibitory concentrations based on the MIC of each as determined below.

**Antibiotic susceptibility assay.** The MICs of antibiotics were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute. Briefly, all antibiotics were diluted into cation-adjusted Mueller-Hinton broth (CA-MHB) in wells of sterile 96-well microtiter plates. The concentrations tested ranged from 1 μg/mL to 512 μg/mL. A McFarland standard inoculum of 0.5 was prepared from bacterial colonies cultured on tryptic soy agar plates. The standardized cultures were diluted 1:100 in CA-MHB, and aliquots were added to wells containing antibiotic. Control wells lacking antibiotic or bacteria (medium only) were prepared accordingly. The microplates were incubated for 20 h at 37°C and inspected for turbidity. The MIC represented the lowest concentration of antibiotic that yielded no growth (see Table S1 in the supplemental material). To determine subinhibitory concentrations, bacteria were treated with dilutions of antibiotics starting from one-fourth the MIC in LB at 37°C with shaking at 220 rpm. To determine CFU per milliliter, 100-μL aliquots of culture were plated on LB agar and enumerated after overnight incubation at 37°C. We selected the lowest concentration of antibiotic that did not significantly decrease bacterial viability in these assays (Table S2).

**Isolation of blood and preparation of normal human serum.** Venous blood was obtained from healthy volunteers in accordance with a protocol (01IN055) approved by the Institutional Review Board for Human Subjects at the National Institutes of Health. All subjects gave written informed consent to participate in the study. NHS was prepared by using a standard method (coagulation at 37°C for 30 min followed by centrifugation to pellet cells and coagulated material) and frozen and thawed one time before use. Heat-inactivated serum was prepared by incubating normal human serum at 56°C for 30 min.

**Serum bactericidal activity assay.** To test survival of ST258 clinical isolates in normal human serum (NHS), ± antibiotic pretreatment, bacteria were cultured in LB to an OD600 of ~0.2 and treated with antibiotics (Table 1) for 2 h. Antibiotic-free LB cultures were used as controls. After antibiotic pretreatment, bacteria were washed with Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO), and 40 μL of washed bacteria was mixed with 360 μL of NHS to yield ~10^7 bacteria in 90% NHS. Cultures were incubated at 37°C for 30 min with continuous shaking (1,200 rpm). Culture aliquots were serially diluted and plated on LB agar plates. Colonies were enumerated the following day and used to determine CFU per milliliter. The percentage of survival ± antibiotic at 30 min was determined relative to CFU per milliliter at 0 min (start of the assay) by using the following equation: % survival = CFU/mL at 30 min/CFU/mL at 0 min × 100.

**Complement deposition assay.** Bacteria were treated ± antibiotics as described above for 2 h prior to culture in 90% NHS for 30 min at 37°C. Deposition of serum complement component C5b-9 on *K. pneumoniae* was measured by flow cytometry as described previously (17). Bacteria were pelleted by centrifugation at 1,000 × g for 10 min at 4°C. Washed LB cultures were used as controls. After antibiotic pretreatment, bacteria were washed with Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO), and 40 μL of washed bacteria was mixed with 360 μL of NHS to yield ~10^7 bacteria in 90% NHS. Cultures were incubated at 37°C for 30 min with continuous shaking (1,200 rpm). Culture aliquots were serially diluted and plated on LB agar plates. Colonies were enumerated the following day and used to determine CFU per milliliter. The percentage of survival ± antibiotic at 30 min was determined relative to CFU per milliliter at 0 min (start of the assay) by using the following equation: % survival = CFU/mL at 30 min/CFU/mL at 0 min × 100.

**Capsule isolation and quantification.** Bacteria were treated ± antibiotics as described above for 2 h prior to culture and then centrifuged at 3,300 × g for 10 min at room temperature. Bacterial pellets were washed with 1 mL DPBS in 1.5-mL microcentrifuge tubes, and the supernatant was discarded. The pellets were suspended in 500 μL DPBS plus 100 μL of 1% zwittergent 3-14-[3-(N,N-dimethyl-myristylammonio)propanesulfonate] (Sigma-Aldrich, St. Louis, MO) in 100 mM citric acid at pH 2.0. The tubes were incubated for 30 min at 50°C with occasional shaking (10 s of mixing at 700 rpm and 20 s at rest). The samples were centrifuged at 14,100 × g for 2 min at room temperature. CPS was precipitated from 300 μL of the supernatants in new 1.5-mL tubes by adding 1,200 μL of 100% ethanol (to yield a final concentration of 80% ethanol) and incubating tubes on ice. After 30 min, samples were centrifuged at 16,100 × g for 10 min at 4°C. The supernatants were discarded, and pellets were air dried for 30 min at room temperature. The CPS pellets were resuspended in 100 μL of distilled water (dH2O) overnight. The CPS concentration was determined by quantifying the amount of uronic acid residues in the samples. Briefly, 120 μL of 12.5 mM sodium tetraborate (Sigma-Aldrich) in concentrated sulfuric acid was added to wells of 96-well plates containing 20 μL of CPS samples. Wells containing serial dilutions of galacturonic acid (0 to 100 μg/mL) were included to generate a linear standard curve. The plate was then incubated at 100°C for 5 min with shaking (500 rpm). After allowing the plates to sit at room temperature for 15 min, 2 μL of 0.15% 3-phenylpheno/ (Sigma-Aldrich) in 0.5% NaOH was added to each well and allowed to cool to room temperature for 15 min. Control wells received only 0.5% NaOH. The plate was incubated at room temperature for 5 min with shaking (500 rpm), and then 20 μL of 1% 3-phenylphenol was added to each well. After 10 min, the absorbance at 520 nm of each well was measured.
absorbance at 520 nm was measured on a Synergy MX plate reader (Bio-Rad Laboratories). The absorbance of the blank sample (0 μg/mL in the well) was subtracted from sample absorbance readings, and the CPS concentration (μg/mL) was calculated for each sample using a linear standard curve.

**Transmission electron microscopy.** Antibiotic-treated bacteria (as described above) and LB control samples were fixed for 30 min in 2% paraformaldehyde plus 2.5% glutaraldehyde in 0.1 Sorenson’s phosphate buffer (PB) supplemented with 0.05% alcan blue to enhance the capsule structure. Subsequent steps were performed using a microwave processor (see reference 39). Briefly, samples were rinsed in buffer and then fixed in 0.5% OsO4 plus 0.8% K2Fe(CN)6, in 0.1 M Sorenson’s PB, rinsed in buffer and stained with 1% aqueous tannic acid. Samples were rinsed in dH2O and en bloc stained with 1% aqueous samarium acetate. Samples had a final rinse with dH2O and were dehydrated in ethanol into Epon Araldite resin. Seventy-nanometer sections were imaged in a Hitachi HT7800 transmission electron microscope operating at 80 kV with an XR-818 detector (AMT). Images were taken at the same nominal magnification of ×25,000 with a pixel size of 0.62 nm. Emphasis was given to cross sections of cells where the membrane was crisp, indicating that it was a true cross section. Capsule thickness was measured from at least 3 sides of 20 images from each treatment using ImageJ software v.2.0.0 (https://imagej.nih.gov/ij/). The program was used to segment for the outer membrane and then for the outer boundary of the capsule. The shortest distance between those two segments was used to calculate capsule thickness. The output measurement unit was pixels. Conversion from pixels to nanometers was done using a 500-nm scale bar (with 50-nm calibrations) on the images. Two independent experiments were performed.

**RNA isolation, RNA-seq and data processing.** Total RNA was isolated from the samples before and after treatment with mupirocin, doxycycline, and/or NHS using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions. Residual genomic DNA was removed from RNA samples using the Baseline-ZERO DNase (Lucigen Corporation). RNA integrity and quality were evaluated on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano assay kit (Agilent Technologies, Santa Clara, CA). Samples with an RNA integrity number (RIN) score of greater than 8.0 were used for further analysis.

Two hundred nanograms of RNA was prepared for next-generation sequencing (NGS) using the Illumina Stranded Total RNA Prep Kit and Ribo-Zero Plus (Illumina, Inc., San Diego, CA) workflow. In lieu of bacterial rRNA depletion with Ribo-Zero Plus, the Qiagen FastSelect—55/165/235 kit (Qiagen Sciences, Germantown, MD) was used with the modification of reducing the fragmentation step to 7 min to account for slight degradation of the RNA. After this initial RNA treatment step, the samples were prepared for cDNA synthesis and library preparation following the Illumina protocol. The libraries were amplified for 14 cycles based on a 200-ng input. Final libraries were assessed on BioAnalyzer DNA 1000 chips (Agilent Technologies, Santa Clara, CA) and quantified using a Kapa SYBR FAST Universal qPCR kit for Illumina sequencing (Roche, Basel, Switzerland) on the CFX384 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Libraries were normalized to 4 nM, pooled, denatured and further diluted to a 1.5 pM stock for clustering and paired-end 2 × 75 cycle sequencing on a NextSeq with a Mid Output flow cell (Illumina, Inc., San Diego, CA).

Raw reads were trimmed of adapter sequence using cutadapt (https://cutadapcutreadthedocs.io/en/stable/). The remaining reads were then filtered for low-quality bases and low-quality reads using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). The remaining reads were mapped, using Bowtie2 (40), to the genome of Klebsiella pneumoniae Kp 32192. Finally, read counts were tabulated using HTseq-count (41) using genes from the gff modified to include the additional plasmids (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000597905.1_ASM59790v1/GCA_000597905.1_ASM59790v1.gff). Genes were defined as significantly altered when the P value is <0.05 and the absolute fold change (FC) is ≥2.

**Statistical analyses.** Statistical analyses were performed using Prism 9.1 software (GraphPad Software, La Jolla, CA). With the exception of gene expression data, all other data were analyzed by using a repeated-measures one-way analysis of variance (ANOVA) and Dunnnett’s posttest.

**Data availability.** All next-generation sequence data are available on GEO (GSE201383).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.**

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We declare no conflict of interest.

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