AAC Journals.ASM.org

Genomic and Transcriptomic Analyses of Colistin-Resistant Clinical Isolates of *Klebsiella pneumoniae* Reveal Multiple Pathways of Resistance

Meredith S. Wright,[a] Yo Suzuki,[a] Marcus B. Jones,[a] Steven H. Marshall,[b] Susan D. Rudin,[b] David van Duin,[c] Keith Kaye,[d] Michael R. Jacobs,[e] Robert A. Bonomo,[b,f] Mark D. Adams[a]

J. Craig Venter Institute, La Jolla, California, USA[a]; Research Service, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, Ohio, USA[b]; Division of Infectious Diseases, University of North Carolina, Chapel Hill, North Carolina, USA[c]; Detroit Medical Center, Detroit, Michigan, USA[d]; Department of Pathology, Case Western Reserve University and University Hospitals Case Medical Center, Cleveland, Ohio, USA[e]; Departments of Medicine, Pharmacology, Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, Ohio, USA[f]

The emergence of multidrug-resistant (MDR) *Klebsiella pneumoniae* has resulted in a more frequent reliance on treatment using colistin. However, resistance to colistin (Col^r) is increasingly reported from clinical settings. The genetic mechanisms that lead to Col^r in *K. pneumoniae* are not fully characterized. Using a combination of genome sequencing and transcriptional profiling by RNA sequencing (RNA-Seq) analysis, distinct genetic mechanisms were found among nine Col^r clinical isolates. Col^r was related to mutations in three different genes in *K. pneumoniae* strains, with distinct impacts on gene expression. Upregulation of the *pmrH* operon encoding 4-amino-4-deoxy-L-arabinose (Ara4N) modification of lipid A was found in all Col^r strains. Alteration of the *mgrB* gene was observed in six strains. One strain had a mutation in *phoQ*. Common among these seven strains was elevated expression of *phoPQ* and unaltered expression of *pmrCAB*, which is involved in phosphoethanolamine addition to lipopolysaccharide (LPS). In two strains, separate mutations were found in a previously uncharacterized histidine kinase gene that is part of a two-component regulatory system (TCRS) now designated *crrAB*. In these strains, expression of *pmrCAB*, *crrAB*, and an adjacent glycosyltransferase gene, but not that of *phoPQ*, was elevated. Complementation with the wild-type allele restored colistin susceptibility in both strains. The *crrAB* genes are present in most *K. pneumoniae* genomes, but not in *Escherichia coli*. Additional upregulated genes in all strains include those involved in cation transport and maintenance of membrane integrity. Because the *crrAB* genes are present in only some strains, Col^r mechanisms may be dependent on the genetic background.

The increasing occurrence of multidrug-resistant (MDR) *Klebsiella pneumoniae* has expanded reliance on last-line therapies like colistin, a cationic antimicrobial peptide, for effective treatment (1). Of concern is the growing recovery of colistin-resistant (Col^r) strains from clinical settings (2–4). Colistin disrupts membrane integrity through displacement of cations like Mg^{2+} and Ca^{2+} in the outer membrane, leading to cell lysis (5). Resistance mechanisms described to date involve lipopolysaccharide (LPS) modification, particularly through derivatization of lipid A phosphate moieties with a sugar or ethanolamine. These modifications reduce the electrostatic affinity between the cationic colistin and anionic LPS. Mutations in the transcriptional regulatory systems controlling these LPS modifications are a common genetic mechanism leading to colistin resistance. For example, the PhoPQ and PmrAB two-component regulatory systems (TCRS) regulate expression of the gene (*pmrC*) that codes for the addition of phosphoethanolamine (pETN) and genes encoding biosynthesis and lipid A transfer of 4-amino-4-deoxy-L-arabinose (Ara4N) (*pmrHFIJKLM*) (Fig. 1A). Other regulatory components in this pathway include PmrD and MgrB, two connector proteins that convey feedback between the PmrAB and PhoPQ TCRS (6–9) (Fig. 1A). Mutations in *pmrAB*, *phoPQ*, and *mgrB* have been identified as mechanisms conferring Col^r in several Gram-negative pathogens, including *K. pneumoniae* (10–15).

How colistin resistance mechanisms alter the global transcription profile and how transcriptome profiles vary in the genetic mechanism(s) that confers colistin resistance remain to be examined. By coupling complete genome sequence data and whole-genome transcriptional characterization of colistin-susceptible (Col^s) and Col^r *K. pneumoniae*, we were able to relate genetic mechanisms of colistin resistance to gene expression changes.

MATERIALS AND METHODS

Strains and genomic analyses. Strains originated from a collection of KPC-producing *K. pneumoniae* isolates obtained from a consortium of tertiary-care hospitals previously described (16, 17). Colistin MICs were determined with a Sensititre system (17) and confirmed by Etest strips (bioMérieux). Genome sequencing was previously described (17) and consisted of Illumina HiSeq reads assembled with Newbler and annotated using the Comprehensive Microbial Resource annotation pipeline (18). Genome sequences were compared using Mauve (19), and gene content

Received 6 August 2014 **Returned for modification** 1 September 2014
Accepted 2 November 2014

Accepted manuscript posted online 10 November 2014

Citation Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. Antimicrob Agents Chemother 59:536–543. doi:10.1128/AAC.04037-14.

Address correspondence to Robert A. Bonomo, Robert.Bonomo@va.gov, or Mark D. Adams, madams@jcvi.org.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.04037-14.

doi:10.1128/AAC.04037-14

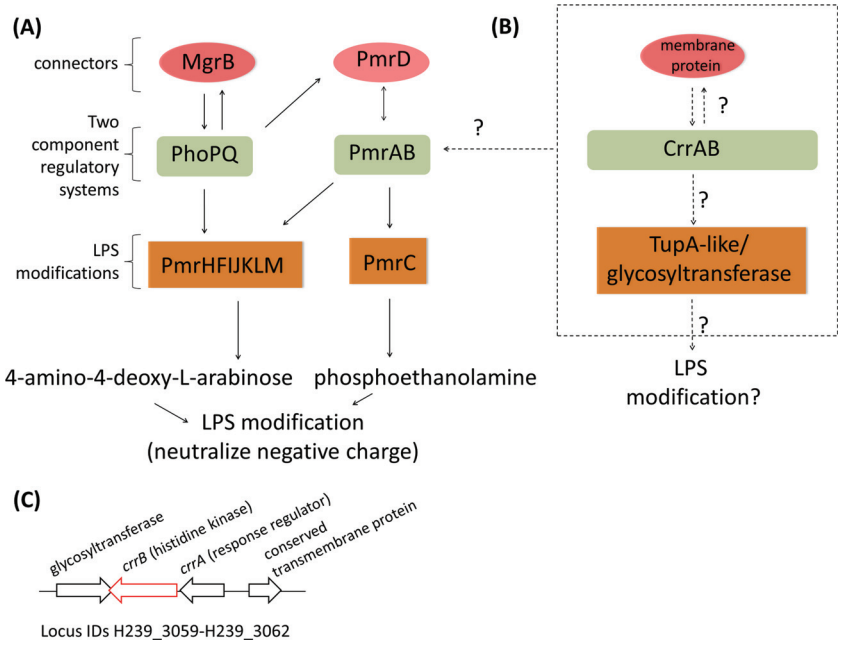

FIG 1 Model of Col^r mechanisms. (A) Diagram of genes involved in colistin resistance based on published summaries from Gram-negative bacteria. (B) Model of newly identified proteins and potential interactions with previously described pathways of Col^r. (C) Genome segment comprising differentially expressed genes, including the mutated histidine kinase gene in UHKPC26 and UHKPC28.

analysis used PanOCT (20). Single-nucleotide variants (SNVs) were determined using BWA for sequence read mapping (21) and Mpileup (22) for SNV detection in paired and closely related strains. A SNV-based phylogeny was constructed from kSNP software (23) output, as described in reference 17, with the inclusion of additional non-ST258 reference genomes. Two strains had paired isogenic Col^s isolates (UHKPC57 with UHKPC179, and UHKPC27 with UHKPC52). SNVs were associated with Col^r based on their presence in one of the Col^r strains (Table 1) and the absence of that allele in all 48 Col^s strains from reference 17 and additional genomes of the same MLST type in GenBank.

RNA-Seq experiments. The 57 strains characterized in reference 17 were grown to mid-log phase at 37°C in LB broth. Cells were harvested and preserved with RNAprotect (Qiagen) until extraction with UltraClean RNA isolation kits (MoBio). cDNA libraries were constructed with ScriptSeq Complete Gold kits (Epicentre Biosciences) and were sequenced on an Illumina HiSeq instrument. Reads from each strain were mapped to the corresponding genome assembly and RPKM (number of mapped reads per kilobase of gene length per million total mapped reads) values were calculated in CLC (version 7.0.4). Genes with significantly different RPKM values were identified using the Significant Analysis for Microarray (SAM) (24) statistical analysis component of Multiexperiment Viewer (MeV version 4.9 [www.tm4.org]), where Col^r and Col^s strains represented the two unpaired classes.

Complementation assay. To determine whether the mutation in *crrB* was necessary to confer colistin resistance, a pUC-19-derived plasmid containing the wild-type *crrB* gene as well as the upstream and downstream flanking sequences was introduced into UHKPC26 and UHKPC28. To generate a plasmid vector with a zeocin marker that could be used for selection of transformants in the MDR background of these strains, the *bla* gene in the pUC19 vector was replaced with the open reading frame of the resistance gene for zeocin. Specifically, by using the primers Zeo_ORF_F and Zeo_ORF_R (primer sequences are shown in Table S1 in the supplemental material), a 412-bp PCR fragment representing the open reading frame (ORF) of the zeocin resistance gene was amplified from a pAF6-derived plasmid (25). A vector fragment ending with the *bla* promoter and terminator, but not extending into the *bla* ORF, was amplified as a 1,857-bp fragment from pUC19 using the primers Zeo-pUC19_F and Zeo-pUC19_R. These fragments were purified using a PCR clean-up kit (ZymoResearch) and combined using Gibson assembly (26). The assembly product was used for transformation of NEB5α *Escherichia coli* cells (New England BioLabs). Plasmids were prepared from the transformants using a miniprep kit (Qiagen) and confirmed using restriction analysis with SmaI (New England BioLabs).

To clone a fragment containing the *crrB* gene into the zeocin plasmid, a 2,125-bp fragment was amplified from the genomic DNA sample of the Col^s strain UHKPC27. The *crrB* locus is wild type in this strain. This amplification was first performed using Q5 polymerase and the primers MW_F_5Phos and MW_R. The amplified fragment was purified using a PCR purification kit (ZymoResearch) and further amplified using PrimeSTAR MAX polymerase (Clontech) and the primers MW_F and MW_R. The amplified fragment included the *crrAB* ORFs and the 5′ and 3′ flanking sequences. The zeocin vector was amplified using PrimeSTAR Max polymerase and the primers pUC19_MW_GA_F and pUC19_MW_GA_R. The amplified vector and insert fragments were purified using a PCR purification kit and used in Gibson assembly. The assembly mixture was used to transform NEB5α cells. Plasmids were purified from the transformants and confirmed using restriction analysis with HinfI and Sanger sequencing.

Electroporation of UHKPC26 and UHKPC28 was conducted as described previously (27) except that >1 μg of DNA was introduced into the transformation mixture and cells were recovered in 500 μl low-salt LB broth. The transformed cells were selected on an agar plate containing low-salt LB broth and 1,250 μg/ml zeocin. The presence of this plasmid in the colonies was confirmed using PCR with the primers Jct_Up_F and Jct_Up_R, where the amplification of the product in this PCR required the junction between the *crrA* upstream sequence and the vector to be present.

A broth microdilution assay was used to determine the MIC of UHKPC26 and UHKPC28 strains carrying the empty vector or the vector with the *crrAB* genes. Overnight cultures of UHKPC26 and UHKPC28 grown in cation-adjusted Mueller-Hinton broth (MHB) plus 1,250 μg/ml zeocin were washed, resuspended in MHB broth, and inoculated into

TABLE 1 *Klebsiella pneumoniae* strains and predicted Col^r-associated mutations

Strain	GenBank accession no.[a]	SRA accession no.[b]	MLST[c]	Location[d]	Colistin MIC (μg/ml)[e]	KPC type[c]	Col^s parent	Mutated gene[g]	Protein accession no.	Mutation
UHKPC45	ARVO00000000	SRR900179	258b	Cleveland OH, USA	4	3		*mgrB*		Deletion of 863 bp starting at *mgrB38*
UHKPC52	ARVN00000000	SRR921382	258a	Cleveland OH, USA	32	None[f]	UHKPC27	*mgrB*		IS1 family IS in *mgrB* with adjacent deletion, missing first 14 bp of *mgrB*
UHKPC81	APVQ00000000	SRR900190	234	Cleveland OH, USA	4	2		*mgrB*		IS4 family IS inserted at *mgrB23*
VAKPC280	APVZ00000000	SRR900205	258a	Cleveland OH, USA	16	2		*mgrB*		IS5 family IS inserted at *mgrB41*
280_1220	ARSQ00000000	SRR900126	258a	Northeast Ohio, USA	16	2		*mgrB*		IS5 family IS inserted with adjacent deletion; full *mgrB* deletion
UHKPC179	ARSM01000000	SRR900157	15	Cleveland OH, USA	16	None[f]	UHKPC57	*mgrB*	EPO88337	C28Y substitution
DMC1316	ARSB00000000	SRR900140	258b	Detroit, MI, USA	1	3		*phoQ*	EPO27247	D434N substitution
UHKPC26	APVT00000000	SRR900163	258a	Cleveland OH, USA	16	2		*crrB*	EOY97329	L94M substitution
UHKPC28	ARRU00000000	SRR900165	258a	Cleveland OH, USA	16	2		*crrB*	EPN94815	Q10L substitution
UHKPC27	APVR00000000	SRR900164	258a	Cleveland OH, USA	0.25	2		NA		Isogenic Col^s strain
UHKPC57	ARPR00000000	SRR900182	15	Cleveland OH, USA	0.5	2		NA		Isogenic Col^s strain

[a] Accession number for the genomic sequence.
[b] Accession number for the RNA-seq data set.
[c] Multilocus sequence type (MLST) and bla_{KPC} type determined as described in reference 15.
[d] Geographic location of source hospital.
[e] Determined by Etest.
[f] Plasmid present in the paired Col^s strain but not in the Col^r strain.
[g] NA, not applicable.

MHB with a series of colistin concentrations. The Col^s strain UHKPC27 was used as a control.

Nucleotide sequence accession numbers. Sequence reads are available from the Short Read Archive at NCBI under accession numbers SRR896011, SRR900124 to SRR900127, SRR900132 to SRR900135, SRR900138 to SRR900144, SRR900151 to SRR900158, SRR900160 to SRR900169, SRR900178 to SRR900184, SRR900187 to SRR900191, SRR900193, SRR900198, SRR900199, SRR900202 to SRR900208, and SRR921382.

RESULTS

Genomic characterization. Nine independent clinical strains representing three MLST sequence types (15, 234, and 258) were previously sequenced as part of a survey of *K. pneumoniae* strains in Midwestern U.S. hospitals (17) (Table 1). The most common mechanism of Col^r involved *mgrB* alteration, a previously identified Col^r mechanism in *K. pneumoniae* (6, 28) (Table 1). MgrB is a negative regulator of PhoQ, and inactivation leads to overexpression of *phoPQ* (9). Six strains had independent mutations of *mgrB*. Four strains experienced *mgrB* disruption mediated by different classes of insertion sequence (IS) families inserted at different positions in the *mgrB* gene. The MgrB amino acid substitution in UHKPC179 (C28Y) occurred at a cysteine residue previously identified as involved in a key disulfide bond in MgrB (29); thus, the substitution of a tyrosine here likely interferes with its ability to repress PhoQ. Genome analysis indicated that an 863-bp deletion resulted in loss of the entire *mgrB* gene and an adjacent ORF from UHKPC45. Two of these strains (UHKPC52 and UHKPC179) had a matched Col^s isolate (UHKPC27 and UHKPC57, respectively) from the same patient obtained before the initiation of colistin treatment. In each case, the *mgrB* gene was intact in the parental isolates and disrupted in the Col^r isolate.

A second resistance mechanism was identified as a mutation in the *phoQ* gene in DMC1316. The PhoQ substitution (D434N) in the cytoplasmic GHKL domain (ATP-lid loop) in DMC1316 is likely critical for phosphate transfer to PhoP (30).

Strains UHKPC26 and UHKPC28 did not have any mutations in known colistin resistance pathways, but each strain possessed an independent mutation in the histidine kinase component of a previously uncharacterized two-component regulatory system, which we designate CrrAB to signify colistin resistance regulation. In each case, the "wild-type" allele is observed in Col^s strains of ST258 (e.g., locus H239_3061 from strain UHKPC45) (Table 1). This histidine kinase gene (*crrB*) and an adjacent response regulator (*crrA*) are present in all *K. pneumoniae* ST258 strains with the exception of UHKPC06, where an insertion sequence (IS) event resulted in the deletion of the region, and in many other *K. pneumoniae* strain types (Fig. 2A). It appears that all *K. pneumoniae* strains have either the *crr* genes or an IS-mediated deletion or substitution of the region. The *crrAB* genes and the two flanking genes (Fig. 1C) do not have clear orthologs in *E. coli* or *Salmonella*, but orthologs of these genes are present several *Enterobacter* genomes but in a different genomic context than in *K. pneumoniae*.

Because no matched Col^s parental strains were available for UHKPC26 or UHKPC28, we considered whether other variants in these strains might be involved in the Col^r phenotype by identifying SNVs present in these strains relative to closely related Col^s strains. The only shared nonsynonymous mutation in the two strains was in MenB (A244T), a naphthoate synthase protein that is part of the menaquinone biosynthesis pathway. Strain UHKPC28 had an additional mutation in RstA (F166Y), the response regu-

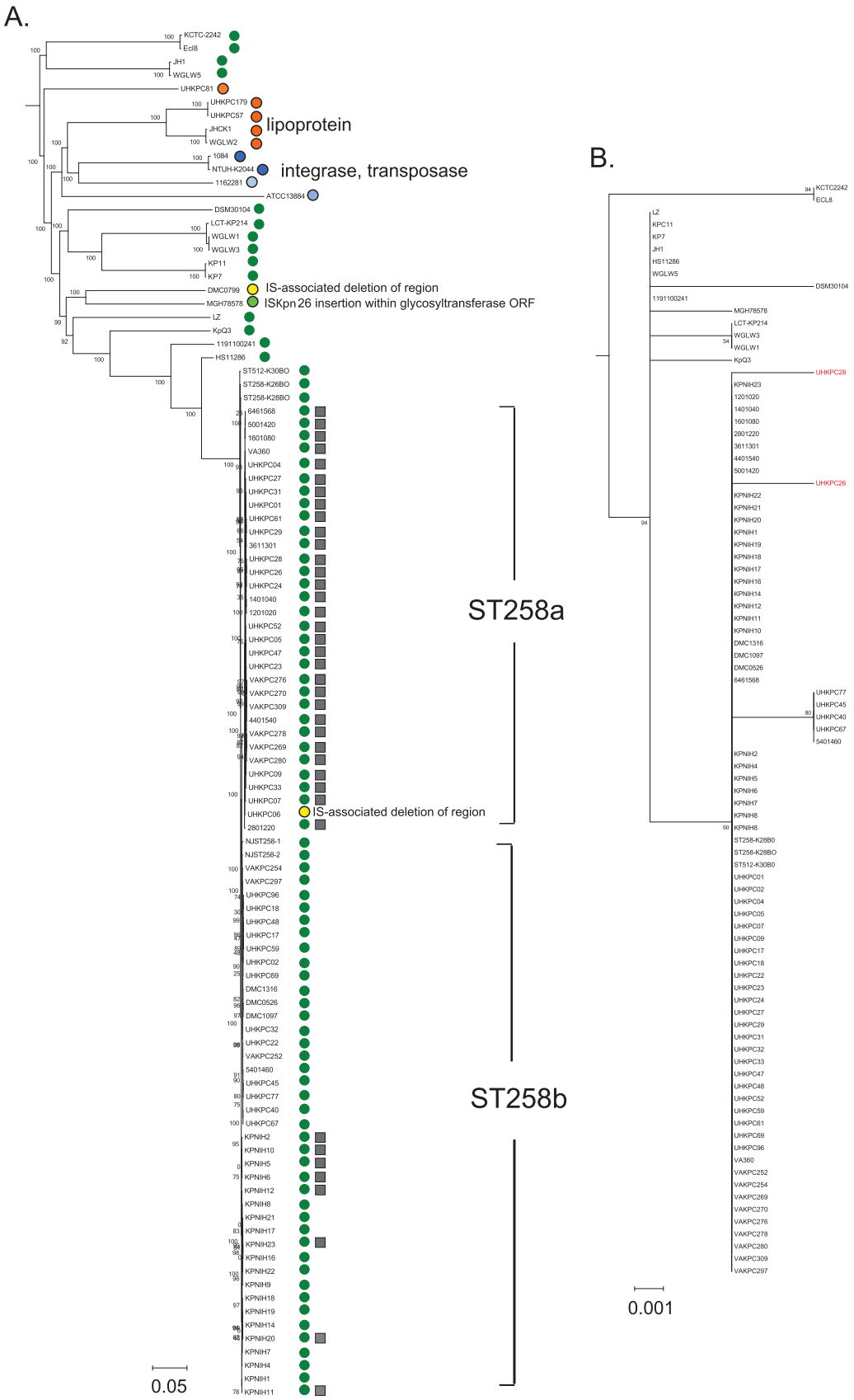

FIG 2 Comparative analyses of the *crrAB* region. (A) Presence of *crrAB* plus upstream (conserved hypothetical membrane protein ORF) and downstream (glycosyltransferase-like ORF) region among *K. pneumoniae* strains shown with a genome-wide SNV-based phylogeny constructed using kSNP output. Shared circle color indicates shared genome content and organization; gray squares indicate the presence of a shared IS*Kpn26* insertion upstream of the conserved hypothetical membrane protein H239_3059. (B) Maximum-likelihood tree of protein sequence alignment of CrrB (353 amino acids) in publicly available *K. pneumoniae* genomes.

TABLE 2 RPKM values for differentially expressed genes or those that have been previously associated with PhoPQ or PmrAB regulons in other organisms

UHKPC45 locus[a]	Description	gene	Mean[b]	SD[c]	crrB mut UHKPC 26	crrB mut UHKPC 28	phoQ mut DMC_ 1316	mgrB del UHKPC 45	mgrB IS UHKPC 81	mgrB IS 280_ 1220	mgrB IS VAKPC 280	mgrB IS UHKPC 52	mgrB mut UHKPC 179
H239_1608	PhoP/PhoQ regulator protein MgrB	mgrB	13.8	6.1	14.4	11.5	17.1	NA	NA	NA	NA	NA	15.5
H239_1721	polymyxin resistance protein PmrD	pmrD	19.3	11.8	30.8	13.5	261.7	74.8	88.5	104.6	53.2	27.8	44.6
H239_3747	lipid A phosphoethanolamine transferase PmrC	pmrC	15.2	11.5	57.6	97.9	5.6	4.5	6.2	9.5	5.0	5.3	4.3
H239_3748	response regulator receiver domain protein PmrA	pmrA	25.1	15.0	103.4	151.2	14.9	12.0	20.6	18.1	7.1	13.2	8.2
H239_3749	histidine kinase domain protein PmrB	pmrB	23.7	13.4	117.9	206.0	9.3	30.4	37.8	9.7	4.8	16.9	19.8
H239_3592	response regulator receiver domain protein PhoP	phoP	65.3	36.1	105.2	95.9	399.8	212.3	314.6	260.1	210.8	132.0	272.4
H239_3591	virulence sensor histidine kinase PhoQ	phoQ	103.0	38.9	158.4	185.7	474.4	372.5	458.7	449.3	241.0	237.6	440.3
H239_1901	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	pmrH	51.5	38.5	147.7	177.8	722.1	193.4	202.2	449.9	340.6	150.0	250.1
H239_1900	glycosyltransferase, group 2 family protein	pmrF	237.1	161.8	840.6	832.5	3081.3	872.1	918.8	2785.3	1285.0	625.2	972.6
H239_1899	bifunctional UDP-glucuronic acid decarboxylase	pmrI	273.9	135.8	1877.6	1911.6	2770.3	1606.1	1156.4	1998.1	1381.1	1444.2	1392.8
H239_1898	polysaccharide deacetylase family protein	pmrJ	144.0	78.6	732.7	814.3	1597.3	756.1	930.3	1212.2	753.1	687.1	707.7
H239_1897	undecaprenyl phosphate-alpha-4-amino-4-deoxy-L-arabinose arabinosyl transferase	pmrK	89.4	57.6	579.0	1160.3	611.6	1000.9	749.4	482.3	319.2	869.9	744.5
H239_1896	4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit ArnE	pmrL	7.8	5.8	29.7	38.6	77.5	41.0	34.8	42.1	33.1	30.3	31.8
H239_1895	4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit ArnF	pmrM	7.2	5.0	15.7	38.7	90.3	23.2	42.3	69.4	35.1	18.3	12.8
H239_3145	outer membrane lipoprotein, membrane integrity	slyB	123.5	43.5	223.0	231.3	544.8	308.8	356.4	201.4	431.4	270.7	272.7
H239_4881	magnesium-importing ATPase, mgtA	mgtA	15.0	8.3	41.6	81.8	73.4	91.7	130.1	84.9	38.3	135.9	137.9
H239_3675	undecaprenyl-pyrophosphatase	ybjG	19.5	11.2	21.8	22.8	107.1	58.8	104.5	95.8	54.0	57.7	75.6
H239_3448	transfers a palmitate chain from a phospholipid to lipid A	pagP	52.8	29.2	37.3	55.5	238.8	130.5	286.1	254.1	178.7	109.2	73.6
H239_3395	lipid A hydroxylase	lpxO	40.5	18.0	103.6	27.7	134.6	107.7	356.3	73.2	87.3	100.2	120.4
H239_3059	glycosyltransferase, TupA-like ATPgrasp protein		1.6	3.5	1887.4	493.6	0.6	1.0	NA	1.6	0.9	3.0	NA
H239_3060	histidine kinase domain protein	crrB	26.0	13.5	247.2	81.3	33.8	13.6	NA	52.4	26.1	42.0	NA
H239_3061	response regulator receiver domain protein	crrA	47.4	28.5	207.1	62.9	52.0	22.1	NA	76.6	37.5	70.2	NA
H239_3062	conserved hypothetical membrane protein		1.3	1.2	91.9	13.4	0.9	0.5	NA	1.4	0.7	2.7	NA
H239_1912	divalent cation transporter		29.7	24.0	106.4	255.7	149.4	616.1	303.5	151.6	62.4	184.9	642.8
H239_4284	cation diffusion facilitator family transporter		1.2	1.0	7.3	6.4	7.2	4.9	19.5	5.4	2.6	8.4	3.1
H239_4045	high-affinity nickel-transport protein, NicO-like		4.8	1.9	7.7	13.1	26.8	25.8	29.1	11.3	6.9	10.5	18.8
H239_4046	ABC type transporter		13.4	6.3	34.2	28.5	76.5	67.6	112.4	31.0	25.2	42.7	78.2
H239_2134	alkyl guanine transferase I, transcriptional regulator	ada	9.7	3.0	18.7	16.5	32.0	34.2	37.5	8.5	15.4	28.7	37.2
H239_2135	thiamine biosynthesis lipoprotein, FAD binding	apbE	36.5	13.0	37.1	53.5	144.6	131.7	228.8	106.4	72.1	78.5	139.5
H239_1012	efflux transporter, RND family, MFP subunit		2.0	1.4	4.5	7.9	19.9	8.3	26.0	3.8	4.8	6.0	16.4
H239_1011	conserved hypothetical protein		5.2	5.3	2.2	1.8	35.6	2.8	164.0	8.3	11.7	1.7	21.0
H239_1010	P-type ATPase, Mg2+ transport		7.3	4.0	16.5	27.2	82.0	27.3	110.4	17.2	19.0	16.7	65.1
H239_2234	chloride transporter, ClC family		7.0	3.7	15.1	19.5	48.1	46.5	27.9	14.7	15.8	25.2	22.1
H239_4639	MacB-like periplasmic core domain protein	macB	18.0	7.6	16.7	42.8	79.9	58.5	84.9	53.8	24.2	56.4	63.1
H239_4640	macrolide-specific efflux protein MacA	macA	135.0	56.2	211.4	293.5	548.2	400.2	852.5	406.5	171.9	480.8	609.6
H239_1992	nucleotide sugar dehydrogenase	ugd	494.9	428.4	150.1	108.0	720.6	1280.9	210.6	126.9	288.6	145.3	327.2
H239_2650	transcriptional regulatory protein RstA	rstA	20.9	11.3	21.7	18.2	18.0	23.7	30.8	14.4	18.2	16.9	30.8
H239_2647	histidine kinase protein RstB	rstB	3.4	2.1	4.5	3.7	3.4	5.6	3.7	2.1	0.7	2.4	2.7
H239_4615	UDP-GlcNAc:Und-P GlcNAc-1-P transferase	wecA	31.6	12.7	7.3	14.7	35.9	22.9	20.6	25.1	31.5	16.5	13.4

[a]A representative locus ID for each gene is given from the K. pneumoniae UHKPC45 genome sequence (ARVO01000000). [b]Mean and [c]standard deviation (SD) are given for all ColS strains (n=45). Locus IDs colored orange are predicted to be part of the PhoPQ regulon. RPKM values colored green are significantly different (p<0.05) and >2-fold higher in ColR strains than the mean value in ColS strains. Yellow indicates significantly different (p<0.05) but <2-fold different RPKM value. NA: gene not present in genome.

[a] A representative locus ID for each gene from the *K. pneumoniae* UHKPC45 genome sequence (ARVO01000000) is given. Locus IDs in orange are predicted to be part of the PhoPQ regulon.

[b] Means and standard deviations are given for all Col^s strains ($n = 45$). RPKM values in green are significantly different ($P < 0.05$) and >2-fold higher in Col^r strains than the mean value in Col^s strains. Yellow indicates significantly different ($P < 0.05$) but <2-fold-different RPKM values. NA, gene not present in the genome.

[c] SD, standard deviation.

lator component of RstAB, and a mutation in a transmembrane domain of WecA (G134A), which is involved in cell envelope synthesis. The complete list of sequence differences between UHKPC24, UHKPC26, and UHKPC28 genomes and the reference genome HS11286 are given in Table S2 in the supplemental material. None of these candidates were deemed to be stronger candidates as the Col^r mutation than the *crrB* mutations.

Transcriptome characterization. Two primary patterns of changes in gene expression were observed in Col^r strains relative to Col^s strains (Table 2). Common to both patterns was increased expression of the *pmrHFIJKLM* operon relative to Col^s strains. This operon encodes genes responsible for the biosynthesis of Ara4N and modification of the lipid A component of LPS with this moiety. The six strains with *mgrB* modifications and the one *phoQ* mutant strain shared a pattern characterized by elevated *phoPQ* expression relative to Col^s strains with no difference in *pmrCAB* transcript levels. The mutational spectrum in *mgrB* suggests loss-of-function mutations in this negative regulator of *phoPQ*, and each strain showed higher *phoPQ* expression levels than Col^s strains from the collection. Of these strains, all but UHKPC52 also exhibited significantly elevated transcript levels for *pmrD*.

In UHKPC26 and UHKPC28, the two strains with mutations in *crrB*, a distinct transcriptional pattern was observed, characterized by elevated transcription of *pmrCAB*, with no difference in *phoPQ* RPKM values compared to Col^s strains. The sequences of the *pmrCAB* locus and flanking regions in these strains were identical to those in other ST258 strains. Expression levels were higher in UHKPC26 and UHKPC28 for *crrB*, a gene encoding an adjacent conserved hypothetical membrane protein, and a gene encoding an adjacent putative glycosyltransferase than in other Col^r and Col^s strains (Table 2 and Fig. 1C). In UHKPC26, the mutation is in the HAMP domain of the histidine kinase. In contrast, the histidine kinase mutation in UHKPC28 affects the predicted signal peptide domain, and transcript levels of the response regulator *crrA* were unaffected. Neither *rstA* nor *wecA*, two other genes with sequence variants in UHKPC28, had altered expression in UHKPC28 relative to Col^s strains.

Other components of the PhoPQ regulon, such as *slyB* (encoding an outer membrane lipoprotein) and *mgtA* (encoding a magnesium-importing ATPase), were upregulated in all Col^r strains (31–33), but *pagP* (encoding an outer membrane protein that adds a palmitate chain to lipid A), *lpxO* (encoding a dioxygenase that modifies lipid A) (34, 35), and *ybjG* (encoding a putative undecaprenyl pyrophosphate phosphatase) (36) were upregu-

TABLE 3 Complementation of *crrB* mutations

Strain	MIC (μg/ml)[a]
UHKPC27	<0.5
UHKPC26	>16
UHKPC26 + vector	>16
UHKPC26 *crrAB*	<0.5
UHKPC28	>16
UHKPC28 + vector	>16
UHKPC28 *crrAB*	<0.5

[a] Determined by broth microdilution in cation-adjusted Mueller-Hinton broth.

lated only in the *mgrB* and *phoQ* mutant strains. Other genes with altered transcription include those associated with cation transport, membrane integrity, and the *macAB* efflux transporters (Table 2). Expression of *pmrD* was elevated in some strains yet was not correlated with expression of *pmrCAB* under these experimental conditions for strains with *mgrB* or *phoQ* alterations. RPKM values for *mgrB* for strains with full-length sequences did not vary between Col^r and Col^s strains, indicating that the *phoQ* mutation in DMC1316 and related increased expression did not activate the feedback inhibition mediated by MgrB. No correlation was observed between the colistin MIC and the genetic mechanism of resistance: the MIC of strains with *mgrB* mutations ranged from 4 to 32 μg/ml. This could indicate that variation in additional genes, including genes in the *phoPQ* regulon, may modulate the resistance phenotype.

Confirmation of the role of *crrB* in colistin resistance. To confirm that the *crrB* mutations in UHKPC26 and UHKP28 were necessary to confer colistin resistance, we cloned the wild-type *crrAB* genes from UHKPC27 into a pUC19-derived vector carrying a zeocin resistance marker and introduced this plasmid into the Col^r strains. The UHKPC27 sequence is identical to that in UHKCP26 and UHKPC28 except for the hypothesized Col^r-associated mutations. Colistin susceptibility was restored to UHKPC26 and UHKPC28 cells carrying the *crrAB* plasmid but not to cells with the vector alone (Table 3).

DISCUSSION

Two distinct transcription profiles were observed; common to both was increased expression of the Ara4N pathway. Previous studies have shown linkage between Col^r and *pmrCAB* expression (6, 12) and cross talk between *pmrAB* and *phoPQ* (37). The strains reported here demonstrate upregulation of either the *pmrAB* or *phoPQ* genes, but not both in the same Col^r strain. The identification of the *crr* genes as additional regulators of colistin resistance expands the number of known genes that modulate this phenotype and highlights multifaceted ways that cells respond to antimicrobial peptide challenge.

The CrrAB proteins are not orthologs of other TCRS linked to PhoPQ or cell envelope stress response, such as RcsAB (38), RstAB (32), EvgAS (36), CpxAR (39), and BaeSR (40). Comparative analyses indicate that the *crrAB* region is variably present in *K. pneumoniae*, although it is present in nearly all ST258 strains (green circles in Fig. 2) and is found in other *Klebsiella* and *Enterobacter* species, albeit in different genomic contexts. In *Enterobacter* sp. strain SST3, a plant endophyte, the region is adjacent to the *sap* (sensitivity to antimicrobial peptides) operon (41). In *K. pneumoniae* genomes without these genes, either a lipoprotein of unknown function is present at this genomic location, or a phage-related integrase and transposase are present. The GC content of this region is approximately 40%, much lower than the average GC content of >50% for the *K. pneumoniae* chromosome, suggesting that it was laterally acquired. Genomic analysis also suggests that IS events are reshaping the region. ST258a strains described in reference 17 all have the same IS*Kpn26* insertion upstream of the conserved hypothetical protein (H239_3062), which also resulted in the deletion of 468 bp and truncation of an adjacent ABC transporter (H239_3063). Interestingly, eight ST258b strains described in reference 42 carried the same IS, IS*Kpn26*, inserted at the same chromosomal position, suggesting a recombination event at this location. A phylogeny of CrrB is largely congruent with the SNV-based whole-genome phylogeny (Fig. 2B).

Both UHKPC26 and UHKPC28 had significantly elevated expression of an adjacent conserved hypothetical protein (H239_3059), a putative glycosyltransferase with a TupA-like ATP grasp domain (PF14305), which is predicted to be involved in surface polysaccharide biosynthesis, and an adjacent uncharacterized conserved membrane protein (H239_3062). These strains also had elevated expression of both the *pmrH* and *pmrCAB* operons, which encode the Ara4N and pETN pathways of LPS modification. Our working hypothesis is that CrrAB induces expression of the glycosyltransferase-like protein which transfers an as-yet-unidentified sugar to lipid A phosphate in a manner analogous to PmrC (Fig. 1B). The basis for the upregulation of *pmrCAB* has not been established. It is possible that CrrB, the novel TCRS histidine kinase, directly phosphorylates PmrA or that a yet-to-be-identified connector protein, perhaps the conserved hypothetical membrane protein (H239_3062), facilitates feedback between the two systems.

UHKPC26 and UHKPC28 do carry other single-nucleotide variants (SNVs) compared with Col^s strains (see Table S1 in the supplemental material). RstA has been shown to be induced by PhoPQ in other Gram-negative pathogens (32, 43); however, the variant seen in UHKPC28 is unlikely to have contributed to the Col^r phenotype as neither *rstAB* nor *phoPQ* expression was altered in UHKPC28. Furthermore, the UHKPC26 sequence was identical to Col^s strains across this region, and the expression profiles are similar between the two strains. An additional variant was observed in a transmembrane region of WecA, which is involved in O-antigen biosynthesis, in strain UHKPC28 but is not predicted to be a key residue (44–46). However, the UHKPC26 *wecA* RPKM value was significantly lower than that in Col^s strains even though there was no mutation in this gene. An examination of a potential role for WecA in LPS modification and a better characterization of what regulates its expression is needed to better understand any potential role in Col^r.

Other transcription changes common to Col^r strains included increased expression of cation transporters and other efflux pumps, which could be a response to altered membrane charge and difficulty in transporting cations through a LPS layer with a more neutral charge. Other genomic changes include the loss of bla_{KPC} plasmids in UHKPC52 and UHKPC179 which were present in a closely related strain in the case of UHKPC52, or isogenic paired strain UHKPC57 in the case of UHKPC179. The loss of these plasmids suggests that there may be a fitness cost associated with acquiring colistin resistance that is partially mitigated by the plasmid loss, and this should be more rigorously explored with formalized fitness comparisons.

This study identified transcriptional changes of Col^r strains during growth without colistin. Further experiments to examine how colistin exposure alters gene expression may provide additional insight into the resistance mechanism(s). Analysis of LPS and lipid A structures may identify novel modifications in UHKPC26 and UHKPC28. In addition, formal analysis of the fitness cost of resistance along with follow-up experiments to examine how the observed LPS modifications alter *K. pneumoniae* host interaction is important to understand the impact of colistin resistance on virulence.

Conclusions. The combination of genomic and transcriptomic analysis revealed three genetic mechanisms conferring colistin resistance with distinct global gene expression profiles depending on the genetic mechanism. Interestingly, we found that each strain possessed unique mutations predicted to confer colistin resistance. Although the sample collection was not designed to be comprehensive or represent a formal epidemiological study, the absence of repeat observations of specific mutations suggests that independently derived Col^r strains of *K. pneumoniae* may be more common than patient-to-patient spread of resistant strains. Moreover, the constellation of changes that can lead to a "final common phenotype" will challenge the future development of cationic antimicrobial peptides and molecular diagnostics, as targets may vary across strains. The fact that mutations were found in genes without *E. coli* orthologs highlights the importance of studying colistin resistance across the range of clinically significant pathogens.

ACKNOWLEDGMENTS

This project was funded in part by the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Department of Health and Human Services, under contract no. HHSN272200900007C. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Numbers UM1AI104681 (R.A.B. and D.V.D.), R01AI072219 and R01AI063517 to R.A.B. and R01GM094403 to M.D.A. This study was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, the Veterans Affairs Merit Review Program Award 1I01BX001974 and the Geriatric Research Education and Clinical Center VISN 10 to R.A.B. In addition, K.K. is supported by the National Institute of Allergy and Infectious Diseases (NIAID); DMID Protocol Number: 10-0065; D.V.D. was awarded support from the Clinical and Translational Science Collaborative of Cleveland and award UL1TR000439 from the National Center for Advancing Translational Sciences (NCATS) component of the NIH and NIH roadmap for Medical Research.

We thank the JCVI sequencing group for producing Illumina sequence data.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Veterans Administration.

REFERENCES

1. **van Duin D, Kaye KS, Neuner EA, Bonomo RA.** 2013. Carbapenem-resistant Enterobacteriaceae: a review of treatment and outcomes. Diagnostic Microbiol Infect Dis **75:**115–120. http://dx.doi.org/10.1016/j.diagmicrobio.2012.11.009.
2. **Bogdanovich T, Adams-Haduch JM, Tian GB, Nguyen MH, Kwak EJ, Muto CA, Doi Y.** 2011. Colistin-resistant, Klebsiella pneumoniae carbapenemase (KPC)-producing Klebsiella pneumoniae belonging to the international epidemic clone ST258. Clin Infect Dis **53:**373–376. http://dx.doi.org/10.1093/cid/cir401.
3. **Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, Venditti M, Bordi E, Capozzi D, Balice MP, Tarasi A, Parisi G, Lappa A, Carattoli A, Petrosillo N, SEERBIO-GRAB Network.** 2013. High rate of colistin resistance among patients with carbapenem-resistant Klebsiella pneumoniae infection accounts for an excess of mortality. Clin Microbiol Infect **19:**E23–E30. http://dx.doi.org/10.1111/1469-0691.12070.
4. **Marchaim D, Chopra T, Pogue JM, Perez F, Hujer AM, Rudin S, Endimiani A, Navon-Venezia S, Hothi J, Slim J, Blunden C, Shango M, Lephart PR, Salimnia H, Reid D, Moshos J, Hafeez W, Bheemreddy S, Chen TY, Dhar S, Bonomo RA, Kaye KS.** 2011. Outbreak of colistin-resistant, carbapenem-resistant Klebsiella pneumoniae in metropolitan Detroit, Michigan. Antimicrob Agents Chemother **55:**593–599. http://dx.doi.org/10.1128/AAC.01020-10.
5. **Falagas ME, Kasiakou SK.** 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis **40:**1333–1341. http://dx.doi.org/10.1086/429323.
6. **Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM.** 2013. In vivo emergence of colistin resistance in Klebsiella pneumoniae producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. Antimicrob Agents Chemother **57:**5521–5526. http://dx.doi.org/10.1128/AAC.01480-13.
7. **Lopez-Camacho E, Gomez-Gil R, Tobes R, Manrique M, Lorenzo M, Galvan B, Salvarelli E, Moatassim Y, Salanueva IJ, Pareja E, Codoner FM, Alvarez-Tejado M, Garcillan-Barcia MP, De la Cruz F, Mingorance J.** 2014. Genomic analysis of the emergence and evolution of multidrug resistance during a Klebsiella pneumoniae outbreak including carbapenem and colistin resistance. J Antimicrob Chemother **69:**632–636. http://dx.doi.org/10.1093/jac/dkt419.
8. **Luo SC, Lou YC, Rajasekaran M, Chang YW, Hsiao CD, Chen C.** 2013. Structural basis of a physical blockage mechanism for the interaction of response regulator PmrA with connector protein PmrD from Klebsiella pneumoniae. J Biol Chem **288:**25551–25561. http://dx.doi.org/10.1074/jbc.M113.481978.
9. **Lippa AM, Goulian M.** 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet **5:**e1000788. http://dx.doi.org/10.1371/journal.pgen.1000788.
10. **Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA.** 2009. Resistance to colistin in Acinetobacter baumannii associated with mutations in the PmrAB two-component system. Antimicrob Agents Chemother **53:**3628–3634. http://dx.doi.org/10.1128/AAC.00284-09.
11. **Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N.** 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the pmrAB two-component regulatory system. Antimicrob Agents Chemother **55:**3370–3379. http://dx.doi.org/10.1128/AAC.00079-11.
12. **Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P.** 2014. Resistance to colistin associated to a single amino acid change in protein PmrB among Klebsiella pneumoniae of worldwide origin. Antimicrob Agents Chemother **58:**4762–4766. http://dx.doi.org/10.1128/AAC.00084-14.
13. **Kim SY, Choi HJ, Ko KS.** 2014. Differential expression of two-component systems, pmrAB and phoPQ, with different growth phases of Klebsiella pneumoniae in the presence or absence of colistin. Curr Microbiol **69:**37–41. http://dx.doi.org/10.1007/s00284-014-0549-0.
14. **Lesho E, Yoon EJ, McGann P, Snesrud E, Kwak Y, Milillo M, Onmus-Leone F, Preston L, St Clair K, Nikolich M, Viscount H, Wortmann G, Zapor M, Grillot-Courvalin C, Courvalin P, Clifford R, Waterman PE.** 2013. Emergence of colistin-resistance in extremely drug-resistant Acinetobacter baumannii containing a novel pmrCAB operon during colistin therapy of wound infections. J Infect Dis **208:**1142–1151. http://dx.doi.org/10.1093/infdis/jit293.
15. **Cannatelli A, Pilato VD, Giani T, Arena F, Ambretti S, Gaibani P, D'Andrea MM, Rossolini GM.** 2014. In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing Klebsiella pneumoniae associated with low-dosage colistin treatment. Antimicrob Agents Chemother **58:**4399–4403. http://dx.doi.org/10.1128/AAC.02555-14.
16. **van Duin D, Perez F, Rudin SD, Cober E, Hanrahan J, Ziegler J, Webber R, Fox J, Mason P, Richter SS, Cline M, Hall GS, Kaye KS, Jacobs MR, Kalayjian RC, Salata RA, Segre JA, Conlan S, Evans S, Fowler VG, Jr, Bonomo RA.** 2014. Surveillance of carbapenem-resistant *Klebsiella pneumoniae*: tracking molecular epidemiology and outcomes

through a regional network. Antimicrob Agents Chemother **58:**4035–4041. http://dx.doi.org/10.1128/AAC.02636-14.
17. **Wright MS, Perez F, Brinkac L, Jacobs MR, Kaye K, Cober E, van Duin D, Marshall SH, Hujer AM, Rudin SD, Hujer KM, Bonomo RA, Adams MD.** 2014. Population structure of KPC-producing Klebsiella pneumoniae from Midwestern U.S. hospitals. Antimicrob Agents Chemother **58:**4961–4965. http://dx.doi.org/10.1128/AAC.00125-14.
18. **Davidsen T, Beck E, Ganapathy A, Montgomery R, Zafar N, Yang Q, Madupu R, Goetz P, Galinsky K, White O, Sutton G.** 2010. The comprehensive microbial resource. Nucleic Acids Res **38:**D340–D345. http://dx.doi.org/10.1093/nar/gkp912.
19. **Darling AC, Mau B, Blattner FR, Perna NT.** 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res **14:**1394–1403. http://dx.doi.org/10.1101/gr.2289704.
20. **Fouts DE, Brinkac L, Beck E, Inman J, Sutton G.** 2012. PanOCT: automated clustering of orthologs using conserved gene neighborhood for pan-genomic analysis of bacterial strains and closely related species. Nucleic Acids Res **40:**e172. http://dx.doi.org/10.1093/nar/gks757.
21. **Li H, Durbin R.** 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics **25:**1754–1760. http://dx.doi.org/10.1093/bioinformatics/btp324.
22. **Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup.** 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics **25:**2078–2079. http://dx.doi.org/10.1093/bioinformatics/btp352.
23. **Gardner SN, Hall BG.** 2013. When whole-genome alignments just won't work: kSNP v2 software for alignment-free SNP discovery and phylogenetics of hundreds of microbial genomes. PLoS One **8:**e81760. http://dx.doi.org/10.1371/journal.pone.0081760.
24. **Tusher VG, Tibshirani R, Chu G.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A **98:**5116–5121. http://dx.doi.org/10.1073/pnas.091062498.
25. **Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C.** 1999. Transformation of nonselectable reporter genes in marine diatoms. Mar Biotechnol **1:**239–251. http://dx.doi.org/10.1007/PL00011773.
26. **Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, III, Smith HO.** 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods **6:**343–345. http://dx.doi.org/10.1038/nmeth.1318.
27. **Fournet-Fayard S, Joly B, Forestier C.** 1995. Transformation of wild type *Klebsiella pneumoniae* with plasmid DNA by electroporation. J Microbiol Methods **24:**49–54. http://dx.doi.org/10.1016/0167-7012(95)00053-4.
28. **Gaibani P, Lombardo D, Lewis RE, Mercuri M, Bonora S, Landini MP, Ambretti S.** 2014. In vitro activity and post-antibiotic effects of colistin in combination with other antimicrobials against colistin-resistant KPC-producing Klebsiella pneumoniae bloodstream isolates. J Antimicrob Chemother **69:**1856–1865. http://dx.doi.org/10.1093/jac/dku065.
29. **Lippa AM, Goulian M.** 2012. Perturbation of the oxidizing environment of the periplasm stimulates the PhoQ/PhoP system in Escherichia coli. J Bacteriol **194:**1457–1463. http://dx.doi.org/10.1128/JB.06055-11.
30. **Marina A, Mott C, Auyzenberg A, Hendrickson WA, Waldburger CD.** 2001. Structural and mutational analysis of the PhoQ histidine kinase catalytic domain. Insight into the reaction mechanism. J Biol Chem **276:**41182–41190.
31. **Groisman EA.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol **183:**1835–1842. http://dx.doi.org/10.1128/JB.183.6.1835-1842.2001.
32. **Minagawa S, Ogasawara H, Kato A, Yamamoto K, Eguchi Y, Oshima T, Mori H, Ishihama A, Utsumi R.** 2003. Identification and molecular characterization of the Mg2+ stimulon of Escherichia coli. J Bacteriol **185:**3696–3702. http://dx.doi.org/10.1128/JB.185.13.3696-3702.2003.
33. **Lejona S, Aguirre A, Cabeza ML, Garcia Vescovi E, Soncini FC.** 2003. Molecular characterization of the Mg2+-responsive PhoP-PhoQ regulon in Salmonella enterica. J Bacteriol **185:**6287–6294. http://dx.doi.org/10.1128/JB.185.21.6287-6294.2003.
34. **Murata T, Tseng W, Guina T, Miller SI, Nikaido H.** 2007. PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of Salmonella enterica serovar Typhimurium. J Bacteriol **189:**7213–7222. http://dx.doi.org/10.1128/JB.00973-07.
35. **Gibbons HS, Reynolds CM, Guan Z, Raetz CR.** 2008. An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of Salmonella lipid A Biochemistry 47:2814–2825. http://dx.doi.org/10.1021/bi702457c.
36. **Eguchi Y, Okada T, Minagawa S, Oshima T, Mori H, Yamamoto K, Ishihama A, Utsumi R.** 2004. Signal transduction cascade between EvgA/EvgS and PhoP/PhoQ two-component systems of Escherichia coli. J Bacteriol **186:**3006–3014. http://dx.doi.org/10.1128/JB.186.10.3006-3014.2004.
37. **Cheng HY, Chen YF, Peng HL.** 2010. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in Klebsiella pneumoniae CG43. J Biomed Sci **17:**60. http://dx.doi.org/10.1186/1423-0127-17-60.
38. **Mouslim C, Groisman EA.** 2003. Control of the Salmonella ugd gene by three two-component regulatory systems. Mol Microbiol **47:**335–344. http://dx.doi.org/10.1046/j.1365-2958.2003.03318.x.
39. **Raivio TL.** 2014. Everything old is new again: an update on current research on the Cpx envelope stress response. Biochim Biophys Acta **1844:**1529–1541. http://dx.doi.org/10.1016/j.bbamcr.2013.10.018.
40. **Leblanc SK, Oates CW, Raivio TL.** 2011. Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of Escherichia coli. J Bacteriol **193:**3367–3375. http://dx.doi.org/10.1128/JB.01534-10.
41. **Gan HM, McGroty SE, Chew TH, Chan KG, Buckley LJ, Savka MA, Hudson AO.** 2012. Whole-genome sequence of Enterobacter sp. strain SST3, an endophyte isolated from Jamaican sugarcane (Saccharum sp.) stalk tissue. J Bacteriol **194:**5981–5982. http://dx.doi.org/10.1128/JB.01469-12.
42. **Snitkin ES, Zelazny AM, Thomas PJ, Stock F, NISC Comparative Sequencing Program, Henderson DK, Palmore TN, Segre JA.** 2012. Tracking a hospital outbreak of carbapenem-resistant Klebsiella pneumoniae with whole-genome sequencing. Sci Transl Med 4:148ra116. http://dx.doi.org/10.1126/scitranslmed.3004129.
43. **Nam D, Choi E, Kweon DH, Shin D.** 2010. The RstB sensor acts on the PhoQ sensor to control expression of PhoP-regulated genes. Mol Cells **30:**363–368. http://dx.doi.org/10.1007/s10059-010-0126-8.
44. **Al-Dabbagh B, Mengin-Lecreulx D, Bouhss A.** 2008. Purification and characterization of the bacterial UDP-GlcNAc:undecaprenyl-phosphate GlcNAc-1-phosphate transferase WecA. J Bacteriol **190:**7141–7146. http://dx.doi.org/10.1128/JB.00676-08.
45. **Amer AO, Valvano MA.** 2002. Conserved aspartic acids are essential for the enzymic activity of the WecA protein initiating the biosynthesis of O-specific lipopolysaccharide and enterobacterial common antigen in Escherichia coli. Microbiology **148:**571–582.
46. **Lehrer J, Vigeant KA, Tatar LD, Valvano MA.** 2007. Functional characterization and membrane topology of Escherichia coli WecA, a sugar-phosphate transferase initiating the biosynthesis of enterobacterial common antigen and O-antigen lipopolysaccharide. J Bacteriol **189:**2618–2628. http://dx.doi.org/10.1128/JB.01905-06.