Supplementary Information: Integrated transcriptomic and metabolomic mapping reveals the mechanism of action of ceftazidime/avibactam against Pan-Drug Resistant *Klebsiella pneumoniae*

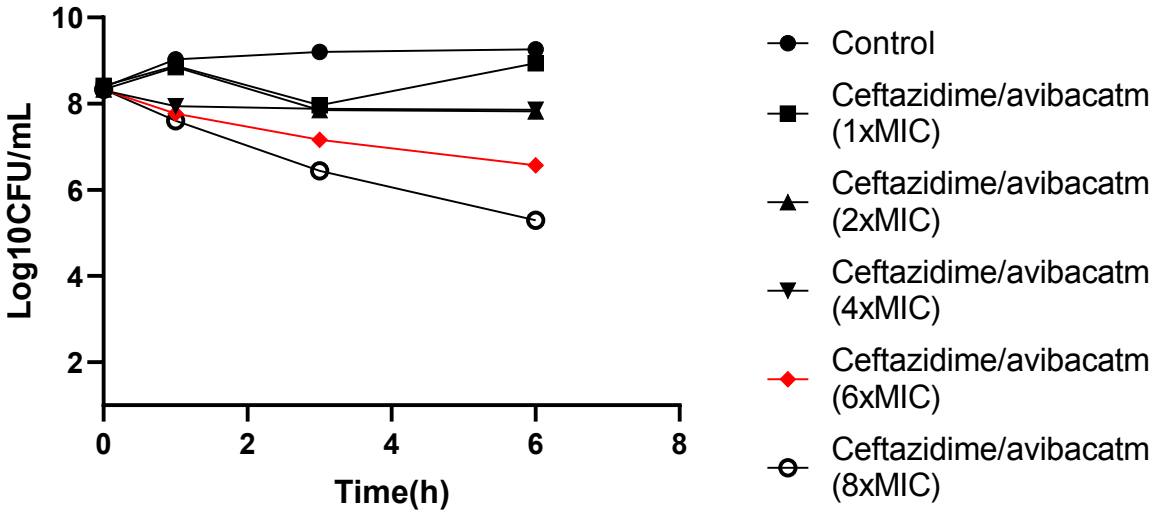
Maytham Hussein,[1] Rafah Allobawi,[1] Jinxin Zhao,[1] Heidi Yu,[1] Stephanie L. Neville,[2] Jonathan Wilksch,[2] Labell J.M. Wong,[1] Mark Baker,[3] Christopher A. McDevitt,[2] Gauri G. Rao,[4] Jian Li,[1*] Tony Velkov[1]*

Affiliations: [1]Monash Biomedicine Discovery Institute, Department of Microbiology, Monash University, Clayton, Victoria, 3800, Australia; [2]Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, Victoria,3010, Australia;[3]Discipline of Biological Sciences, Priority Research Centre in Reproductive Biology, Faculty of Science and IT, University of Newcastle, University Drive, Callaghan, NSW, 2308, Australia;[4]Division of Pharmacotherapy and Experimental Therapeutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7355, USA.

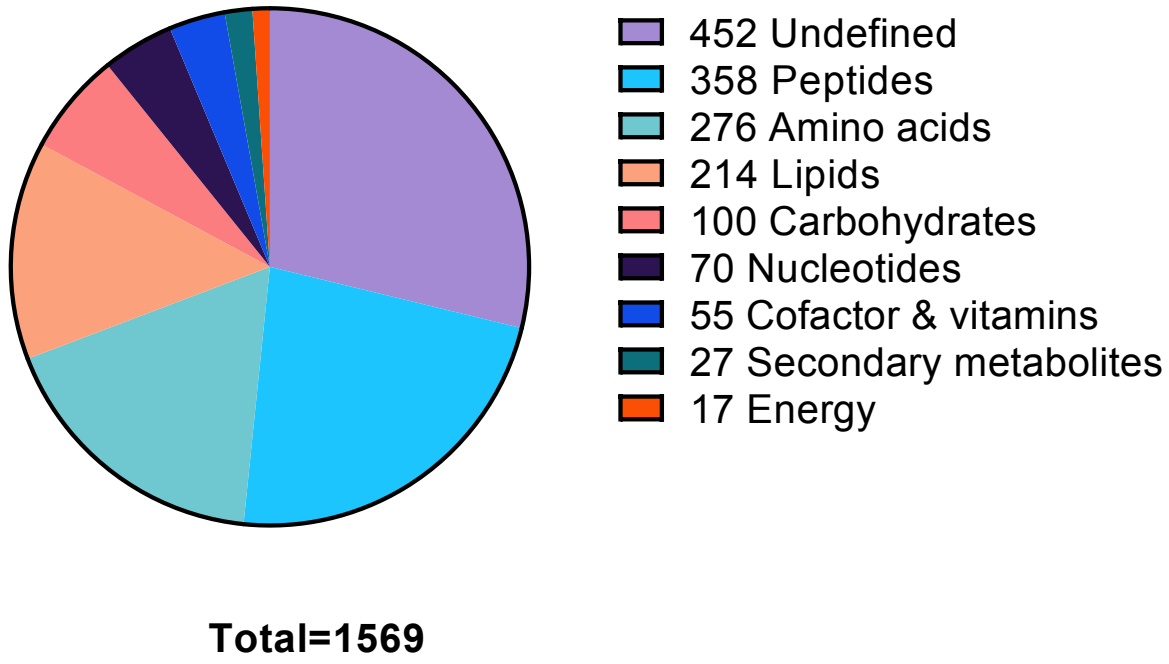
*Corresponding authors: Tony.velkov@monash.edu; tony.velkov@unimelb.edu.au OR Jian.li@monash.edu

KEYWORDS. Ceftazidime-avibactam, *K. pneumoniae*, Antimicrobial resistance, Metabolomics, Transcriptomics.

Short Title: Ceftazidime/avibactam multi-omics study

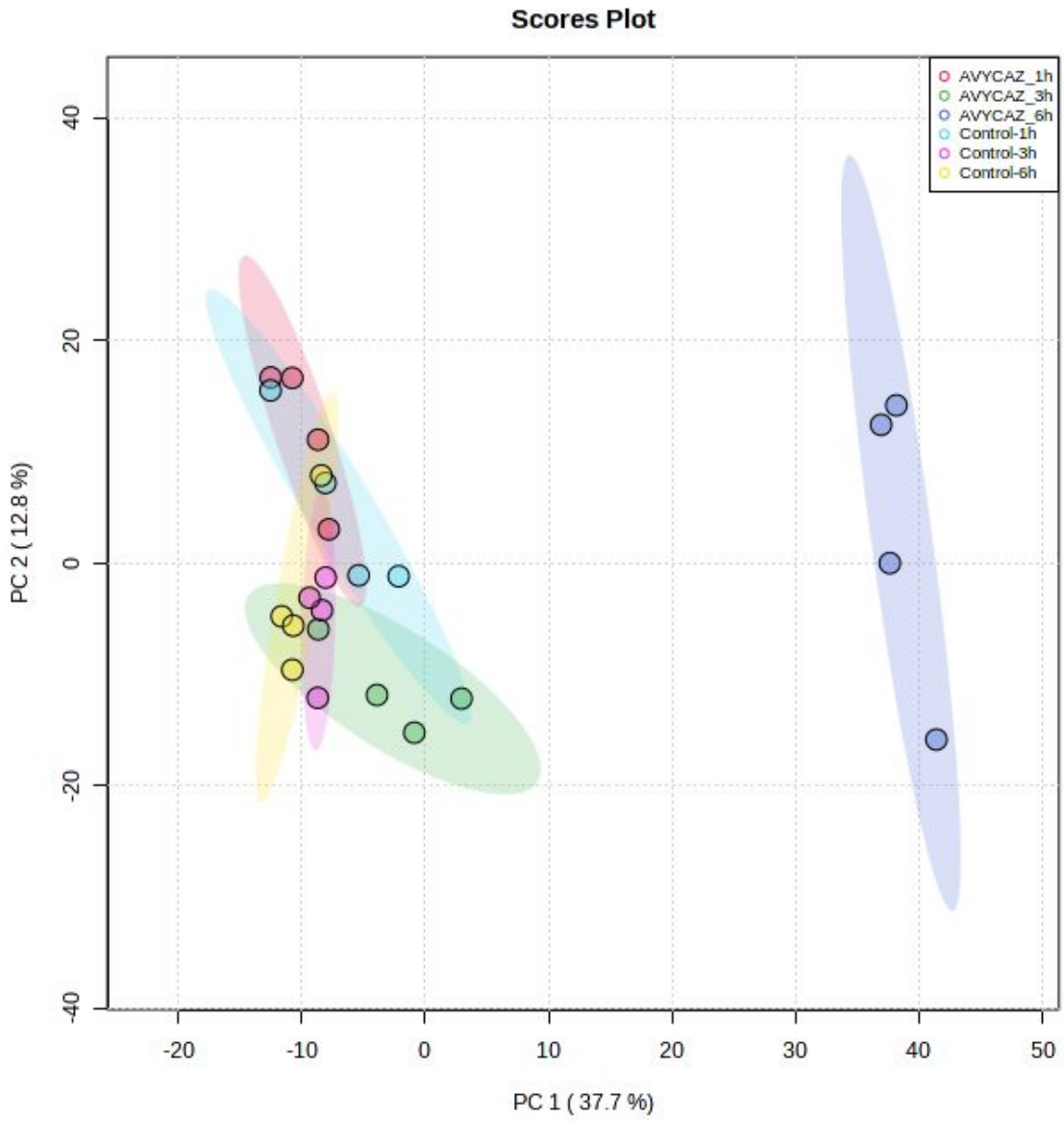


Supplementary Figure S1. Time-kill curve for different concentrations of ceftazidime/avibactam [1xMIC (mg/L): 8/4; 2xMIC (mg/L): 16/4; 4xMIC (mg/L): 32/4; 6xMIC (mg/L): 48/4; 8xMIC (mg/L): 64/4] against high inoculum size (~10^8 CFU/mL) *K. pneumoniae* FADDI-KP070 at 1, 3 and 6 h. Data are mean values of three independent cultures, and vertical bars represent the standard deviations. Error bars are too small to appear in the graphs. The red curve represents the selected concentration (ceftazidime/avibactam MIC= 8/4 mg/L) for the omics experiments.

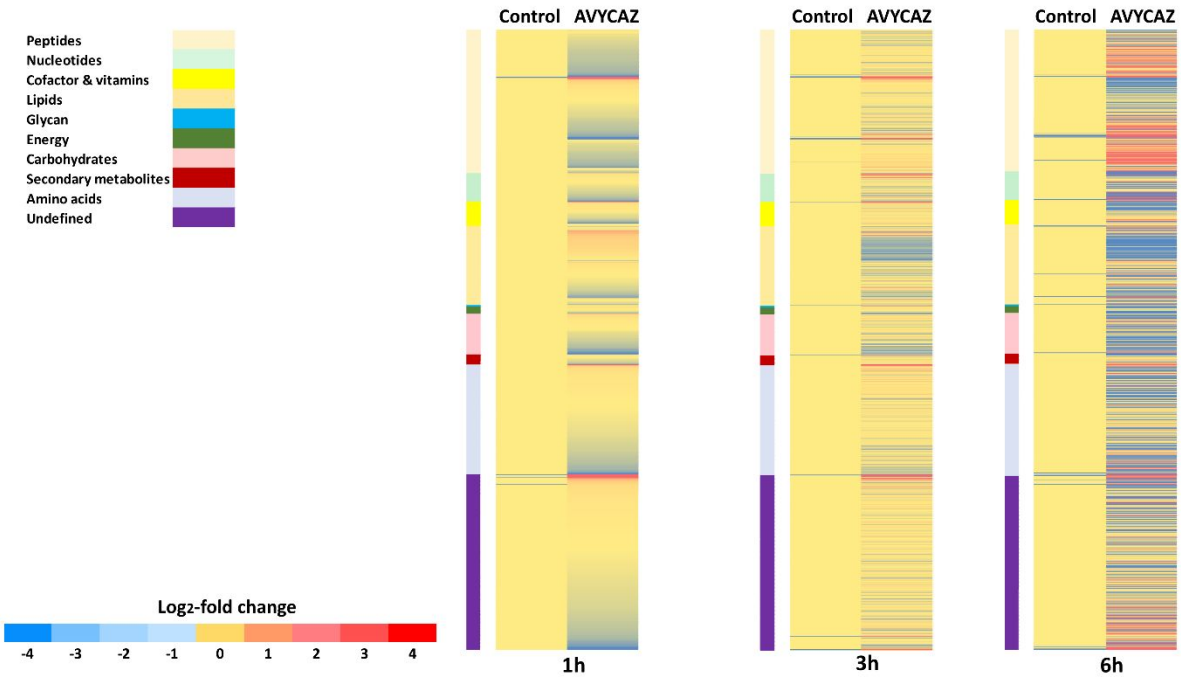


Supplementary Figure S2. Total acquired metabolites detected through comprehensive profiling and the proportion of each metabolite class.

(A)

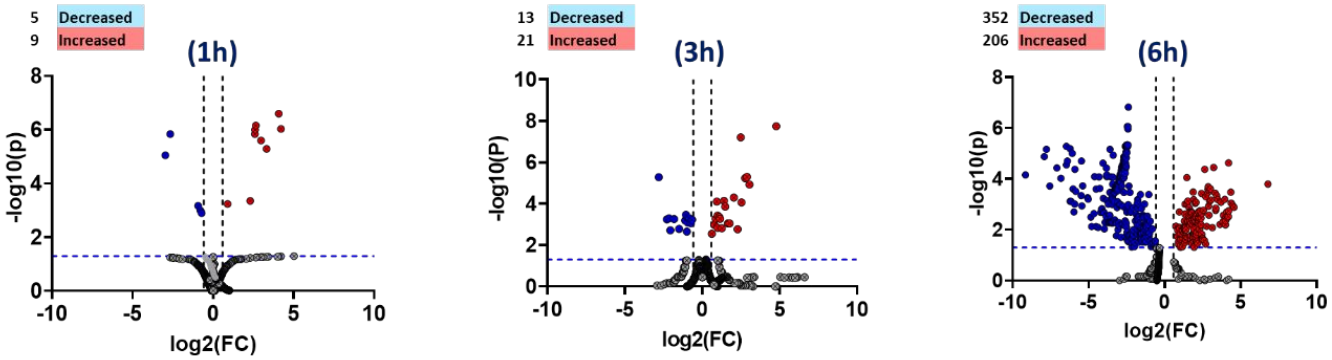

Scores Plot
AVYCAZ_1h
AVYCAZ_3h
AVYCAZ_6h
Control-1h
Control-3h
Control-6h
PC 2 (12.8 %)
PC 1 (37.7 %)
40
20
0
-20
-40
-20
-10
0
10
20
30
40
50

(B)

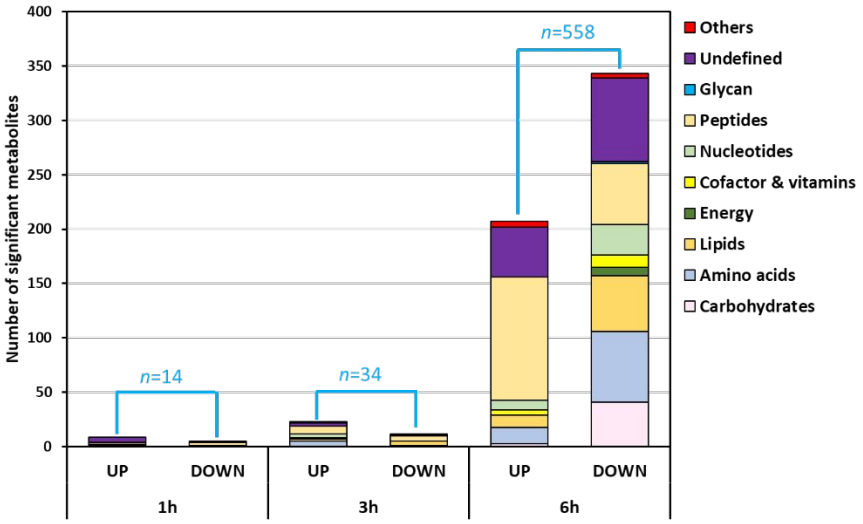


Supplementary Figure S3. (A) PCA plots for metabolite levels from *K. pneumoniae* FADDI-KP070 samples treated with ceftazidime/avibactam at 1,3 and 6 h. Each data set represents a total of 8 samples of 4 biological replicates of each condition. Orange = control; blue = ceftazidime/avibactam. **(B)** Heatmap profiles of *K. pneumoniae* FADDI-KP070 with hierarchical clustering of all identified metabolites after treatment with ceftazidime/avibactam at 1,3, and 6 h. AVYCAZ= ceftazidime/avibactam; control= untreated samples

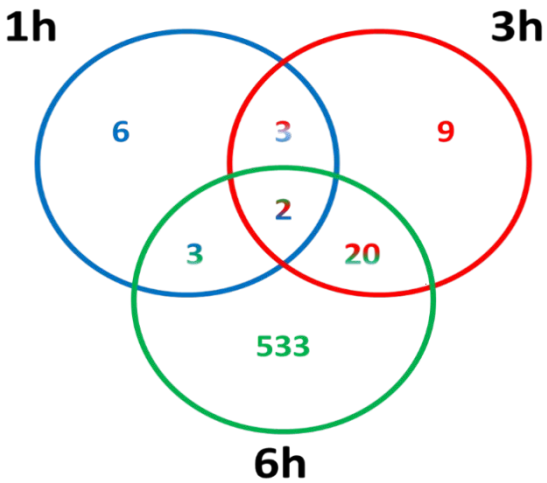
(A)


5 Decreased
9 Increased
(1h)
-log10(p)
log2(FC)
13 Decreased
21 Increased
(3h)
-log10(P)
log2(FC)
352 Decreased
206 Increased
(6h)
-log10(p)
log2(FC)

(B)

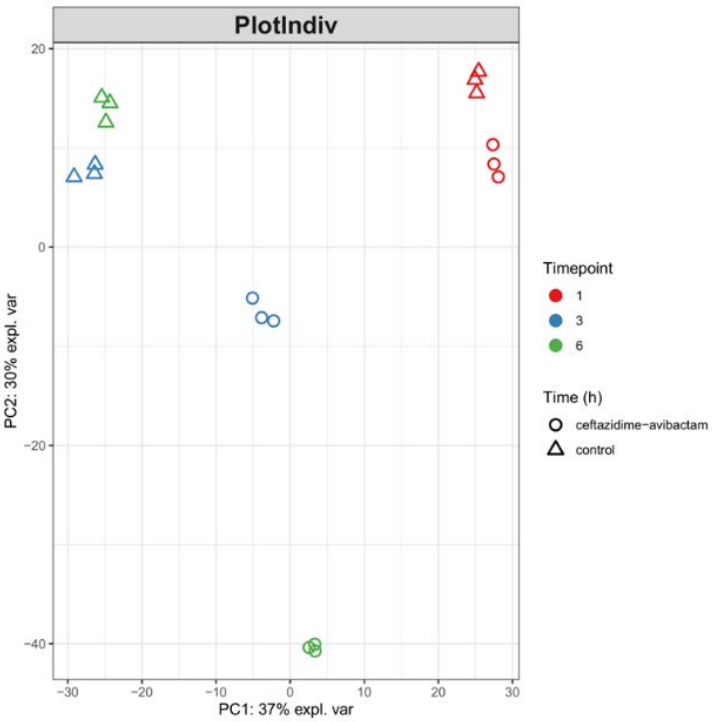

Number of significant metabolites
n=14
n=34
n=558
UP
DOWN
1h
3h
6h
Others
Undefined
Glycan
Peptides
Nucleotides
Cofactor & vitamins
Energy
Lipids
Amino acids
Carbohydrates

(C)

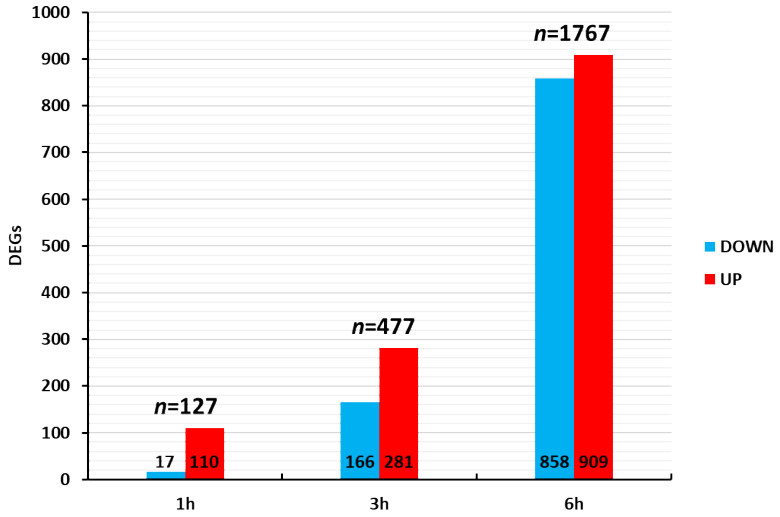

1h
3h
6h
6
3
9
2
3
20
533

Supplementary Figure S4. (A) Volcano plots depicting total number of significant metabolites after ceftazidime/avibactam treatment of *K. pneumoniae* FADDI-KP070 at 1, 3, and 6 h. **(B)** Summary of significantly changed metabolites of *K. pneumoniae* FADDI-KP070 from different categories following ceftazidime/avibactam treatment at 1, 3, and 6 h. Changes ($\log_2FC \geq 0.59$, $p < 0.05$). **(C)** Venn diagrams showing the number of metabolites significantly affected by each treatment for *K. pneumoniae* FADDI-KP070 at 1, 3, and 6 h. Significant metabolites were selected with ($\log_2FC \geq 0.59$, $p < 0.05$).

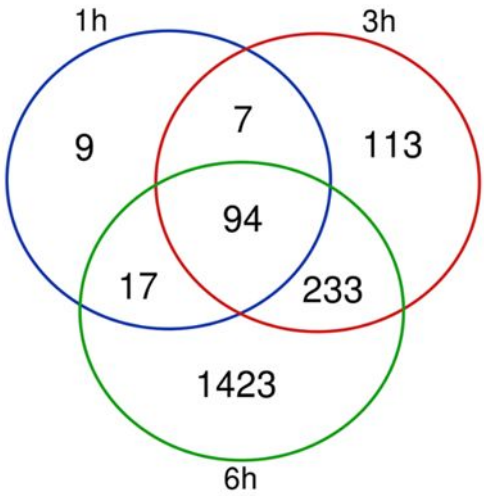
(A)


PlotIndiv
20
0
−20
−40
PC2: 30% expl. var
−30
−20
−10
0
10
20
30
PC1: 37% expl. var
Timepoint
1
3
6
Time (h)
ceftazidime−avibactam
control

(B)



(C)



Supplementary Figure S5. (A) PCA plots for *K. pneumoniae* FADDI-KP070 transcriptome after ceftazidime/avibactam at 1,3, and 6 h. Each data set represents a total of 68 samples of 3 biological replicates of each condition. Triangle = control; circle = ceftazidime/avibactam. **(B)** The total numbers of differentially expressed genes (DEGs) of *K. pneumoniae* FADDI-KP070 after ceftazidime/avibactam treatment at 1, 3 and 6 h. **(C)** Venn diagrams showing the number of differentially expressed genes (DEGs) induced by ceftazidime/avibactam treatment of *K.*

pneumoniae FADDI-KP070 at 1, 3, and 6 h. Significant DEGs were selected with ($log_2FC \geq 0.59$, FDR < 0.05).

Supplementary Table S1. Antibiogram of the clinical isolate *K. pneumoniae* FADDI-KP070. The susceptibility breakpoints are based on the latest versions of EUCAST and CLSI guidelines [1, 2].

Antibiotic	Susceptibility	MIC Value (mg/L)
Ceftazidime/avibactam	S	8/4
Colistin	R	64
Polymyxin B	R	32
Amikacin	R	>64
Amoxicillin/ Clavulanic Acid	R	>32
Ampicillin	R	>32
Cefazolin	R	>64
Cefepime	R	16
Cefoxitin	R	>64
Ceftazidime	R	>64
Ceftriaxone	R	>64
Ciprofloxacin	R	>4
Gentamicin	R	>16
Meropenem	R	32
Minocycline	I	8
Norfloxacin	R	>16
Nitrofurantoin	R	256
Piperacillin/Tazobactam	R	>128
Rifampicin	NA	32

Ticarcillin/Clavulanic Acid	R	>128
Tobramycin	R	>128
Trimethoprim	R	>16
Trimethoprim/Sulfamethoxazole	R	>320

R=Resistant; I= Intermediate; S= Susceptible; NA= Not available

Supplementary Table S2. Differentially expressed genes (DEGs) of *K. pneumoniae* FADD-KP070 after ceftazidime/avibactam treatment. The table presents annotation information for DEGs across all time points, with focus on pathways including cell envelope biosynthesis downstream pathways (peptidoglycan, lipopolysaccharide and *O*-antigen biosynthesis), as well as central carbon metabolism [glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and electron transport chain (ETC)], lysine biosynthesis and bacterial membrane lipids. *Note:* The table aims to provide pathway insights rather than distinguishing between up-regulated and down-regulated genes.

Pathway	Protein name	Gene
Peptidoglycan	UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase	*murG*
	UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase	*murE*
	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase	*murA*
	N-acetylmuramic acid 6-phosphate etherase	*murQ*
	UDP-*N*-acetylmuramate-L-alanine ligase	*murC*
	UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase	*murF*
	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase	*murD*
	Penicillin-binding protein 1A	*mrcA*
	Penicillin-binding protein 1B	*mrcB*
	Penicillin-binding protein 2	*mrdA*
	Penicillin-binding protein	*dacA*
β-lactam resistance	β-lactamase class A SHV-2a	*bla (shv2)*
	β-lactamase SHV-4	*bla (shv4)*
	β-lactamase-like protein	*ytnP*
	Putative β-lactamase	*nylB*
LPS	Lipid A biosynthesis lauroyltransferase	*lpxL*
	Tetraacyldisaccharide 4'-kinase	*lpxK*
	Fe(2+)/alpha-ketoglutarate-dependent dioxygenase LpxO	*lpxO*
	UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase	*lpxD*
	Acyl-[acyl-carrier-protein]-UDP-N-acetylglucosamine O-acyltransferase	*lpxA*
	ATP-dependent zinc metalloprotease FtsH	*ftsH*
	LPS-assembly lipoprotein LptE	*lptE*

	Permease	*lptG*
	Lipopolysaccharide export system permease protein LptF	*lptF*
	LPS-assembly protein LptD	*lptD*
***O*-antigen assembly**	UDP-glucose 4-epimerase	*galE*
	UTP--glucose-1-phosphate uridylyltransferase	*galU*
	UDP-glucose 6-dehydrogenase	*ugd*
	UDP-galactopyranose mutase	*glf*
	O-antigen export system permease protein RfbA	*rfbA*
	Rhamnosyl transferase	*rfbN*
	UDP-galactopyranose mutase	*rfbD*
	dTDP-4-dehydrorhamnose 3,5-epimerase	*rfbC*
	O-antigen export system ATP-binding protein RfbB	*rfbB*
	Bifunctional protein GlmU	*glmU*
Glycolysis	Glucose-6-phosphate isomerase	*pgi*
	Phosphofructokinase	*pfkB*
	Fructose-1,6-bisphosphatase	*glpX*
	Glyceraldehyde-3-phosphate dehydrogenase	*gapA*
	Enolase	*eno*
PPP	Phosphogluconate dehydratase	*edd*
	6-phosphogluconolactonase	*ybhE*
TCA Cycle	Pyruvate dehydrogenase E1 component	*aceE*
	Acetyltransferase component of pyruvate dehydrogenase complex	*aceF*
	Citrate synthase	*gltA*
	Malate dehydrogenase	*mdh*
	Probable malate:quinone oxidoreductase	*mqo*
	Fumarate hydratase class I	*fumB*
	Fumarate reductase flavoprotein subunit	*frdA*
	Succinate--CoA ligase [ADP-forming] subunit alpha	*sucD*
	Oxoglutarate dehydrogenase (succinyl-transferring)	*sucA*
	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	*sucB*
	Aconitate hydratase	*acnA*
	Aconitate hydratase B	*acnB*
ETC	NADH-quinone oxidoreductase subunit N	*nuoN*
	Succinate dehydrogenase cytochrome b556 subunit	*sdhC*
	ATP synthase epsilon chain	*atpC*
Lysine biosynthesis	Diaminopimelate decarboxylase	*lysA*
	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	*murE*
	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	*murF*
	4-hydroxy-tetrahydrodipicolinate reductase	*dapB*
	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	*dapD*
	Succinyl-diaminopimelate desuccinylase	*dapE*
	Diaminopimelate epimerase	*dapF*
	Acetylornithine/succinyl diaminopimelate aminotransferase	*argD*

	Aspartate-semialdehyde dehydrogenase	*asd*
Bacterial membrane lipids	Glycerol dehydrogenase	*gldA*
	Glycerol kinase	*glpK*
	Glycerol-3-phosphate dehydrogenase	*glpA*
	1-acyl-*sn*-glycerol-3-phosphate acyltransferase	*plsC*
	Phosphate acyltransferase	*plsX*
	Outer membrane lipoprotein Blc	*blc*

References

1. EUCAST, Clinical breakpoints-breakpoints and guidance. **2023**.
2. Weinstein MP, Lewis JS, II. 2020. The Clinical and Laboratory Standards Institute Subcommittee on Antimicrobial Susceptibility Testing: background, organization, functions, and processes. J Clin Microbiol 58:e01864-19.