Figure S1. Resistance acquisition does not induce in vitro growth defects in presence of glucose. P0 and P8 isolates upon selection with TAT-RasGAP$_{317-326}$ **(A)**, polymyxin B **(B)** or tetracycline **(C)** were grown overnight in LB and diluted to an OD_{600} of 0.01 in fresh LB supplemented with 0.4% glucose. Growth was then assessed by OD_{600} measurement each 30 minutes for a total of 16 hours. Values are the average of three experiments. Error bars represent standard deviation of the three replicates.

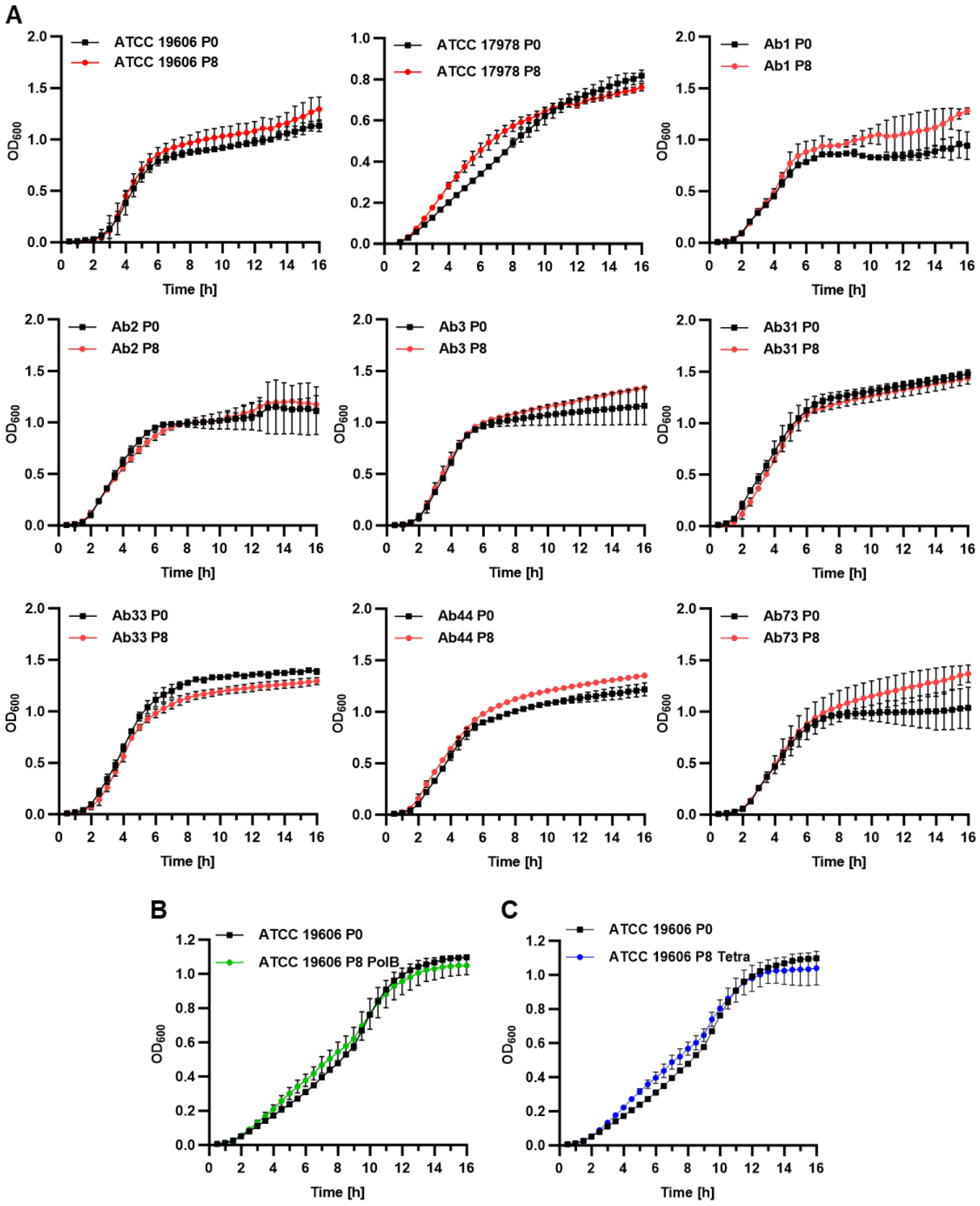

Figure S2. **Resistance acquisition does not induce in vitro growth defects in presence of increased salt concentration.** P0 and P8 isolates upon selection with TAT-RasGAP$_{317-326}$ **(A)**, polymyxin B **(B)** or tetracycline **(C)** were grown overnight in LB and diluted to an OD_{600} of 0.01 in fresh LB containing 2% of NaCl. Growth was then assessed by OD_{600} measurement each 30 minutes for a total of 16 hours. Values are the average of three experiments. Error bars represent standard deviation of the three replicates.

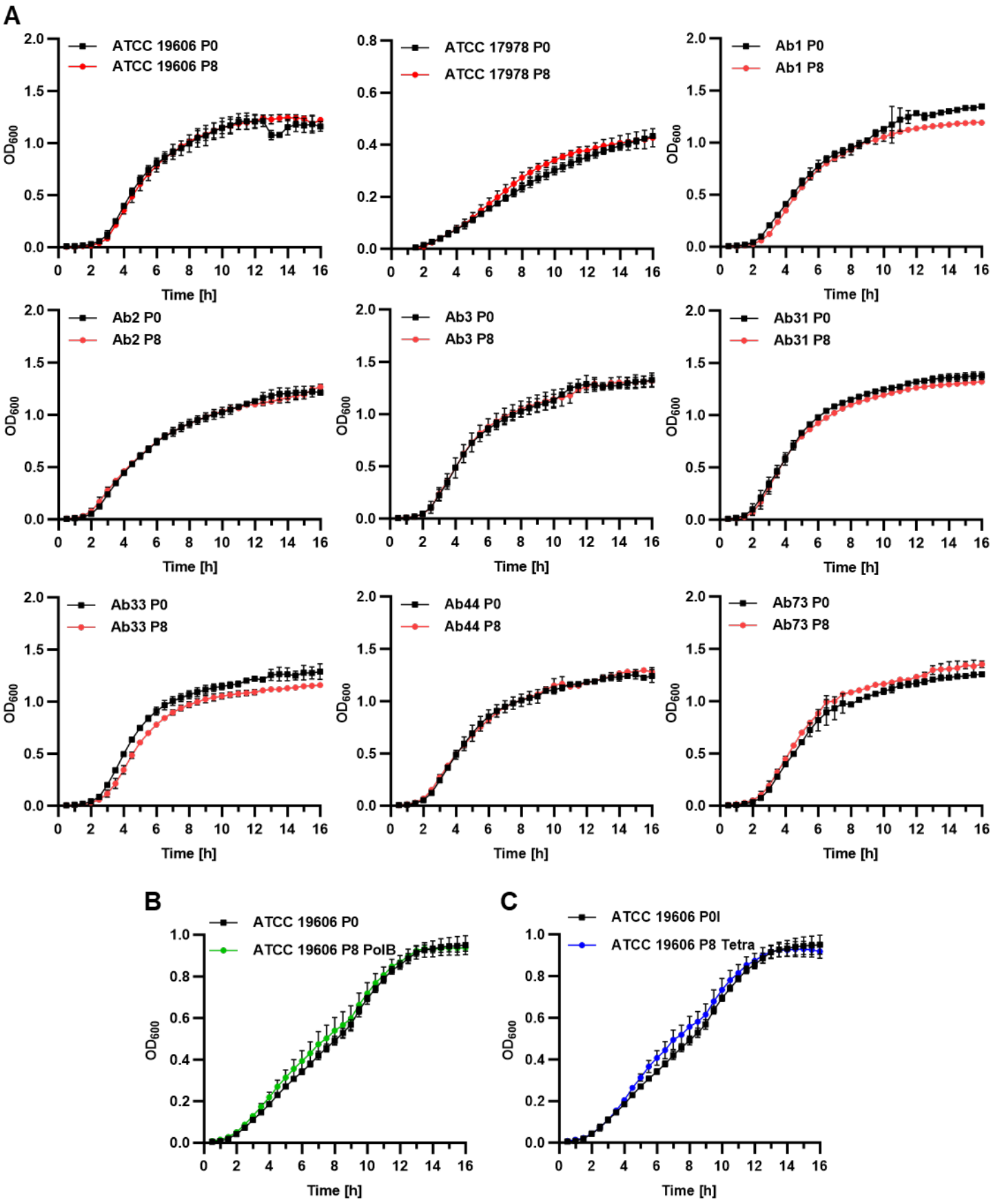

Figure S3. Resistance acquisition does not induce morphology in vitro. P0 and P8 isolates upon selection with TAT-RasGAP$_{317-326}$, polymyxin B or tetracycline were grown overnight in LB, diluted to an OD_{600} of 0.1 in fresh medium and grown for 2 hours. Morphology of the different isolates was then assessed by microscopy.

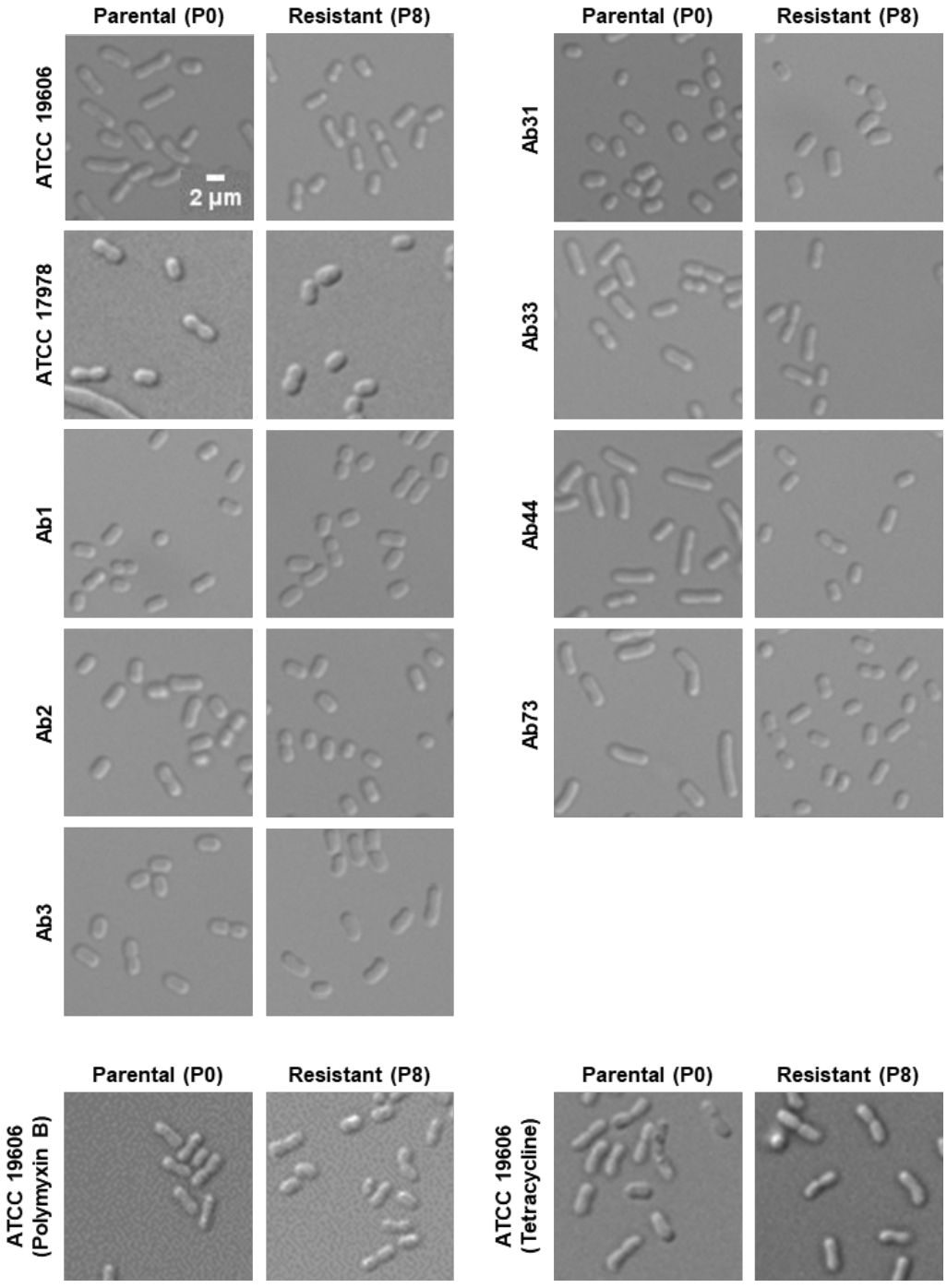

Figure S4. The effect of resistance acquisition on the structure of the biofilm was assessed by live-dead staining followed by Z-stacks acquisition with a confocal microscope. Reconstruction of Z projections are shown on the top and on the right for each strain. This figure is a complement to Figure 3.

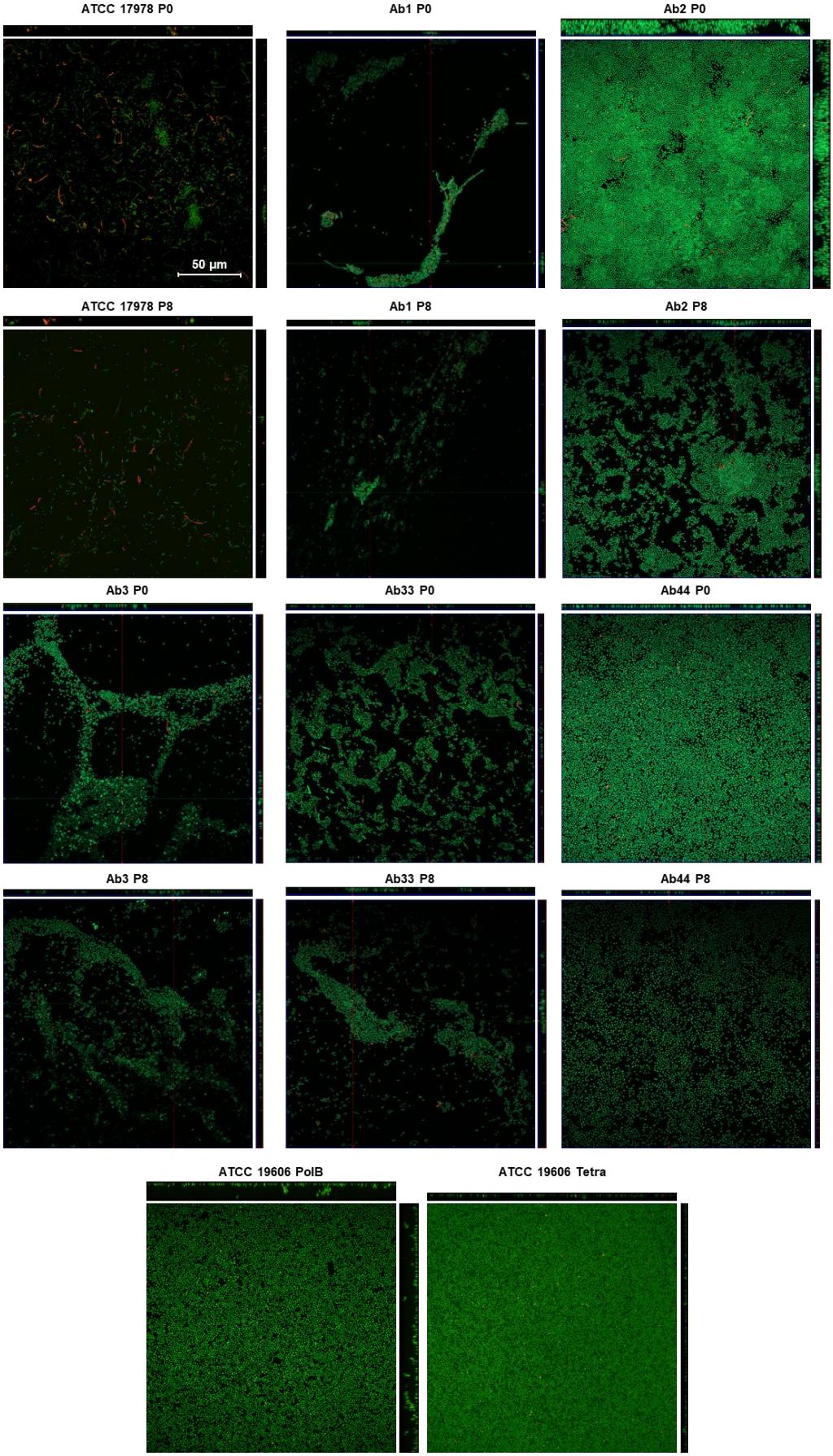

Figure S5. MIC of TAT-RasGAP$_{317-326}$ (Black) and polymyxin B (Red) of all resistant strains selected in this study.
MICs were measured on the indicated strains in duplicates. Black lines represent the mean of the replicates.

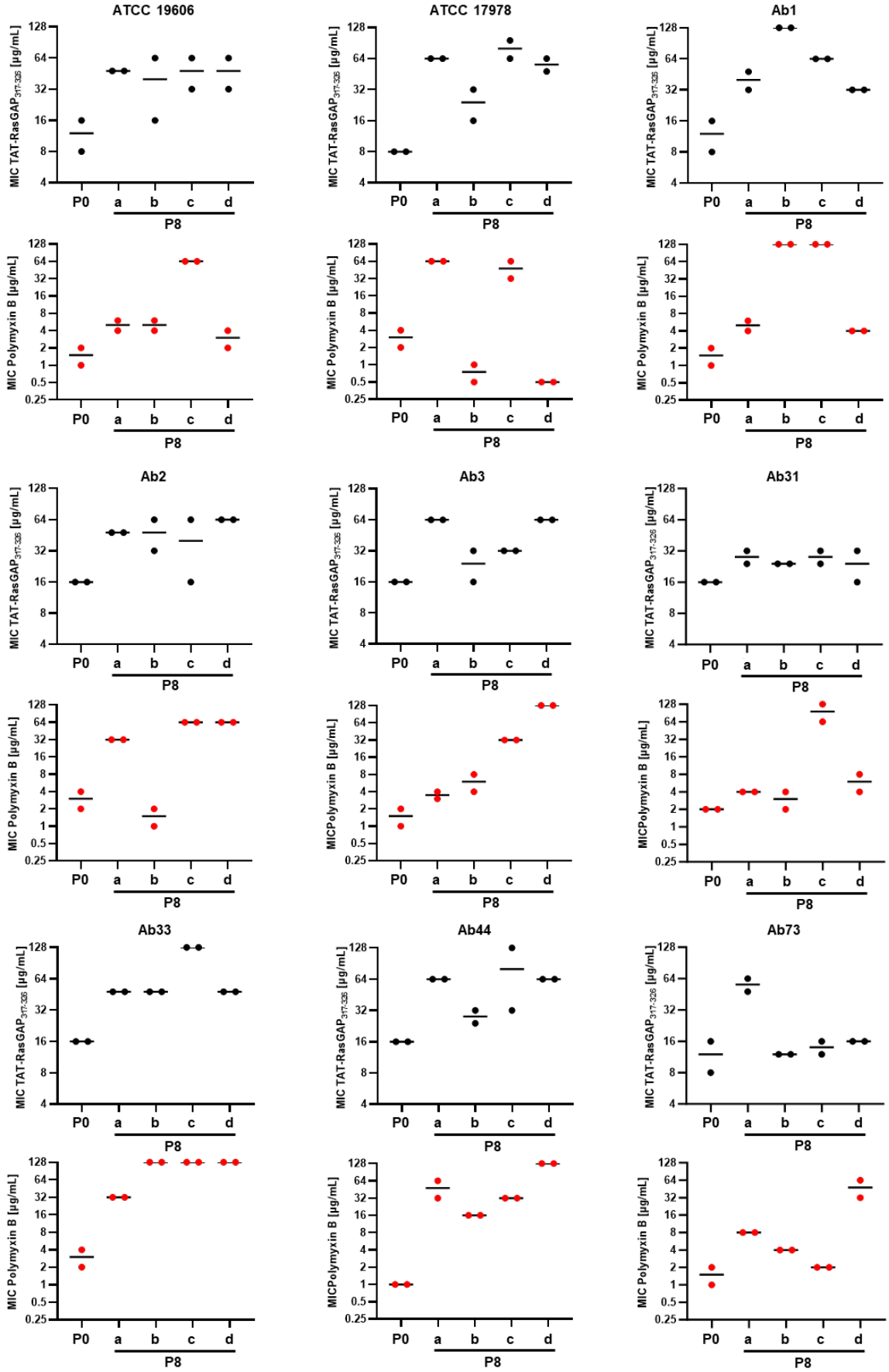

Figure S6. Cross-resistance to polymyxin B is stable upon 8 passages in absence of drug selection. ATCC19606 parental strain and selected resistant isolates b, c and d were passaged in absence of any antimicrobial agents for a total of 8 passages. MICs of TAT-RasGAP$_{317-326}$ and polymyxin B before (Resistant) and after (Back-selection) the 8 passages were measured.

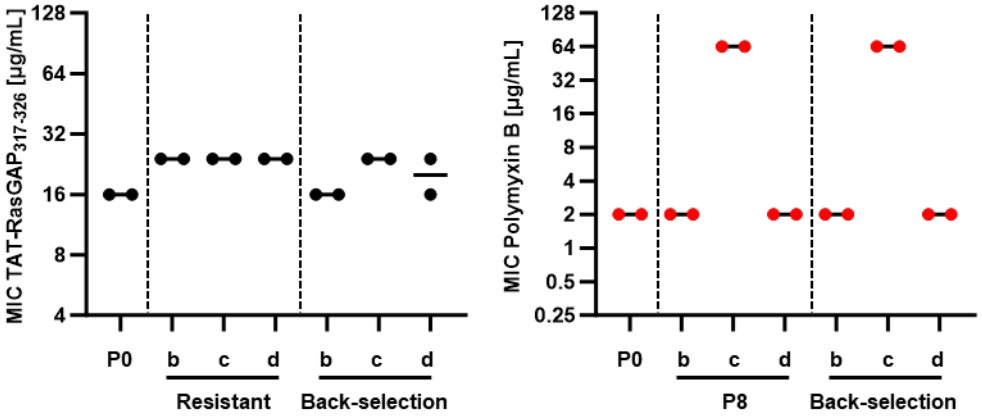

Table S1: Mutations detected in resistant strains, selected with TAT-RasGAP$_{317-326}$ (TAT), polymyxin B (PolB) or tetracycline (Tetra) from Tables 2 and 3. Type of mutations, as well as the targeted gene and the amino acid changes induced are shown. Ref nt: reference nucleotide, Mut nt: mutated nucleotide, Aa: amino acid.

Strain	Contig n#	Position	Ref nt	Mut nt	Mutation type	Gene name	Aa change	Gene ID	Predicted gene product	Protein_ID	Aa length
19606_TAT	33	19751	C	A	Missense	*ompA*	A63S	bakta_11390	Outer membrane protein	Q6RYW5.1	356
19606_PolB	7	37849	G	T	missense	*mlaF*	Q96H	bakta_03235	Toluene tolerance protein	QXV68106.1	272
19606_PolB	34	41531	A	T	missense	*pmrB*	M145K	bakta_11690	Sensor histidine kinase	QXV68398.1	444
19606_Tetra	7	60285	G	C	missense	*rpsJ*	V57L	bakta_03345	30S ribosomal protein S10	QXV68128.1	103
19606_Tetra	33	37873	C	A	stop_gained		E139*	bakta_11470	Methyltransferase	QXV68296.1	305
17978_TAT	1	244138	C	T	missense	*pmrA*	A14V	CHUV_01160	DNA-binding response regulator	QFQ06527.1	224
17978_TAT	1	244155	T	G	missense	*pmrA*	L20V	CHUV_01160	DNA-binding response regulator	QFQ06527.1	224
Ab1_TAT	3	18949	T	A	missense	*tauE*	E71V	CHUV_00935	Sulfite exporter	QXV68055.1	258
Ab1_TAT	5	27308	A	C	missense		T260P	CHUV_01620	Hypothetical protein	QXV70740.1	333
Ab1_TAT	7	1245	TTCAC	T	frameshift	*uhpC*	S67fs	CHUV_02195	nitrate transmembrane transporter	QXV69291.1	449
Ab1_TAT	10	21166	T	C	missense	*topA*	V791A	CHUV_03150	DNA topoisomerase	QXV70887.1	878
Ab1_TAT	20	36632	G	T	missense	*yaaA*	T142N	CHUV_05580	peroxide stress protein	QXV68890.1	257
Ab1_TAT	45	14463	C	T	missense	*bauA*	A391T	CHUV_09155	TonB-dependent siderophore receptor	QXV68708.1	757
Ab1_TAT	51	3253	C	T	missense		G64E	CHUV_09845	DUF2237 domain-containing protein	QXV69640.1	127
Ab1_TAT	63	5267	G	A	missense	*mdh*	T184I	CHUV_11155	malate dehydrogenase	QXV68186.1	328
Ab1_TAT	110	5006	T	G	missense		D185E	CHUV_14570	phage capsid	No homologue	368
Ab1_TAT	124	1494	T	G	missense		V458G	CHUV_15175	Trimeric autotransporter adhesin	QXV70340.1	2258
Ab1_TAT	141	2607	CGTGATG	C	inframe_deletion	*czcD*	H167_H168del	CHUV_15935	Co/Zn/Cd efflux system component	QXV71353.1	329
Ab1_TAT	166	4646	C	CA	frameshift		D369fs	CHUV_16785	PucR family transcriptional regulator	No homologue	1443
Ab1_TAT	191	2689	C	A	missense		M132I	CHUV_17385	Hypothetical protein	QXV70197.1	358
Ab1_TAT	191	2696	T	C	missense		E130G	CHUV_17385	Hypothetical protein	QXV70197.1	358
Ab1_TAT	191	2718	C	T	missense		V123I	CHUV_17385	Hypothetical protein	QXV70197.1	358
Ab1_TAT	242	434	CCGGTCGGCTTGAG	C	frameshift		A205fs	CHUV_18135	GNAT family N-acetyltransferase	No homologue	220
Ab1_TAT	261	261	C	CA	frameshift	*proC*	L78fs	CHUV_18360	pyrroline-5-carboxylate reductase	QXV70080.1	271
Ab1_TAT	279	198	C	T	missense		A301V	CHUV_18495	lipid A phosphoethanolamine transferase	QXV68396.1	533
Ab1_TAT	291	280	T	C	missense		V86A	CHUV_18570	tape measure protein (phage)	QXV68840.1	1637
Ab1_TAT	291	396	C	T	missense		L125F	CHUV_18570	tape measure protein (phage)	QXV68840.1	1637
Ab2_TAT	3	22478	A	C	missense	*pmrB*	T187P	CHUV_00950	Sensor histidine kinase	QXV68398.1	444
Ab2_TAT	399	26	C	T	missense		M112I	CHUV_19810	DUF4265 domain-containing protein	QXV70250.1	148
Ab3_TAT	58	22402	C	A	missense		V14L	CHUV_13180	AAA family ATPase	No homologue	552
Ab3_TAT	58	22414	C	A	missense		V10L	CHUV_13180	AAA family ATPase	No homologue	552
Ab3_TAT	156	64	G	T	missense		S286Y	CHUV_18030	Integrase family protein	QXV69689.1	410
Ab33_TAT	61	18919	G	A	missense	*pmrB*	P233S	CHUV_13230	Sensor histidine kinase	QXV68398.1	444
Ab44_TAT	53	7269	G	GA	frameshift	*acyC*	M452fs	CHUV_10515	Adenylate/Guanylate cyclase	QXV69577.1	489
Ab44_TAT	133	6748	T	A	missense	*pmrB*	I164F	CHUV_16740	Sensor histidine kinase	QXV68398.1	444
Ab73_TAT	23	26937	AT	A	frameshift	*pldA*	I80fs	bakta_04850	Phospholipase	QXV69240.1	383
Ab73_TAT	32	10879	A	C	missense	*bamA*	S693R	bakta_06200	Outer membrane assembly protein	QXV69181.1	835
Ab73_TAT	35	24475	A	T	missense	*lptC*	V146E	bakta_06675	LPS export ABC transporter periplasmic protein	QXV70013.1	182
Ab73_TAT	101	4454	T	A	stop_gained	*plcD*	L266*	bakta_13280	Phospholipase	QXV68216.1	541

Table S2: Mutations detected in cross-resistant strains that do not have mutations in *pmrAB* (Ab2c, Ab3c) from Table 4. Type of mutations, as well as the targeted gene and the amino acid changes induced are shown. Ref nt: reference nucleotide, Mut nt: mutated nucleotide, Aa: amino acid.

Strain	Contig n#	Position	Ref nt	Mut nt	Mutation type	Gene name	Aa change	Gene ID	Predicted gene product	Protein_ID	Aa length
Ab2c_TAT	150	6550	C	T	start_lost	*crp*	M1?	CHUV_14980	Cyclic AMP receptor protein	QXV70134.1	235
Ab2c_TAT	223	6	T	G	missense	*basI*	I230L	CHUV_17560	acinetobactin biosynthesis phosphopantetheinyl transferase	QXV68718.1	251
Ab2c_TAT	223	21	C	A	missense	*basI*	V225L	CHUV_17560	acinetobactin biosynthesis phosphopantetheinyl transferase	QXV68718.1	251
Ab2c_TAT	223	146	C	A	missense	*basI*	C183F	CHUV_17560	acinetobactin biosynthesis phosphopantetheinyl transferase	QXV68718.1	251
Ab2c_TAT	243	3877	A	T	missense		F96I	CHUV_18000	hypothetical protein	QXV70077.1	114
Ab2c_TAT	284	28	T	A	missense		T289S	CHUV_18690	Hydrolase-4 domain-containing protein	No homologue	293
Ab2c_TAT	291	145	C	A	missense		D101Y	CHUV_18790	hypothetical protein	QXV70461.1	144
Ab2c_TAT	292	85	T	A	missense	*uvrA*	S2T	CHUV_18810	thiamine ABC transporter permease	QXV69420.1	753
Ab2c_TAT	303	1732	G	T	missense		A503E	CHUV_18960	hypothetical protein	No homologue	557
Ab2c_TAT	399	26	C	T	missense		M112I	CHUV_19810	DUF4265 domain-containing protein	QXV70250.1	148
Ab3c_TAT	58	22402	C	A	missense		V14L	CHUV_13180	AAA family ATPase	No homologue	552
Ab3c_TAT	58	22414	C	A	missense		V10L	CHUV_13180	AAA family ATPase	No homologue	552
Ab3c_TAT	156	64	G	T	missense		S286Y	CHUV_18030	Integrase family protein	QXV69689.1	410

Table S3: List of bacterial strains and oligonucleotides used in this study.

Bacterial species	Bacterial strain	Source	Internal reference
A. baumannii	ATCC 19606	ATCC	NJ629
A. baumannii	ATCC 17978	ATCC	NJ836
A. baumannii	Ab1	Heulot et al., 2017	NJ721
A. baumannii	Ab2	Heulot et al., 2017	NJ722
A. baumannii	Ab3	Heulot et al., 2017	NJ723
A. baumannii	Ab31	Leshkasheli et al., 2019	NJ749
A. baumannii	Ab33	Leshkasheli et al., 2019	NJ750
A. baumannii	Ab44	Leshkasheli et al., 2019	NJ751
A. baumannii	Ab73	Leshkasheli et al., 2019	NJ752
E. coli	DH5α pCasAb-apr	Wang et al., 2019	NJ823
E. coli	DH5α pSGAb-km	Wang et al., 2019	NJ824
E. coli	DH5α pSGAb-spe	Wang et al., 2019	NJ825

Oligonucleotides	Sequence	Purpose	Origin
PmrB_450_F	AAT TAT TTT ACC TTT TGC A	*pmrB* amplification and sequencing	This study
PmrB_950_R	ACA AAA CCT AAA TCG ATT T	*pmrB* amplification and sequencing	This study
PmrB_50_F	GTG TCA TCT TAG GTT GTA TTT	*pmrB* amplification and sequencing	This study
PmrB_1260_R	CTT ATC GAG AGT TAA AGT CC	*pmrB* amplification and sequencing	This study
PmrB_spacerF_17978	TAG TTT CAA TTG GTG TGA GTT CTT	targeted mutagenesis of *pmrB*	This study
PmrB_spacerR_17978	AAA CAA GAA CTC ACA CCA ATT GAA	targeted mutagenesis of *pmrB*	This study
PmrB_Donor	AGG TAA AAG CTC TTG AGG ATA ATC ATG TAC TTC AATTGG GGG GAG CTC TTC GGA ATC GCG TTC TTT TAA CTCATT TTT AA	targeted mutagenesis of *pmrB*	This study
pCasAb_5053F	AGT CTA ATA GAA TGA GGT CG	targeted mutagenesis of *pmrB*	Wang et al., 2019
pCasAB_5647R	TGA TAG AAC ATG TAA ATC GA	targeted mutagenesis of *pmrB*	Wang et al., 2019
M13R	CAG GAA ACA GCT ATG ACC	targeted mutagenesis of *pmrB*	Wang et al., 2019