SUPPORTING INFORMATION

Subcellular quantification of uptake in Gram-negative bacteria

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1. Table S1: Assays for quantification of compound uptake in bacteria

Assay	Description	Compound modification	Reference
MIC	Determine minimum inhibitory concentration (MIC) by broth microdilution protocol	None; result depends on uptake and target affinity	1,2
Residual activity of antibiotic in medium	Use supernatant of antibiotic treated culture to determine the residual activity in the agar diffusion test	None; result depends on uptake and target affinity	3
Radiometry	Introduce radioactive label into compound of interest. Incubate bacteria and monitor incorporation of radioactivity by filtration	^{55}Fe ^{3}H ^{14}C	4-8
Growth recovery assay	Applied for siderophore-like compounds. Bacterial mutants without endogenous siderophores only grow under iron-limiting conditions if the exogenous siderophore is internalized and may serve as iron carrier	None; depends on the compounds ability to rescue growth under nutrient limiting conditions	2,9
Photometric monitoring of b-lactamase activity	Monitor permeability to β -lactams: Rate of hydrolysis by periplasmic β-lactamase from sonicated vs intact cells yields cryptic indices (CIs). Decrease of specific absorption recorded. Bacterium expressing plasmid-encode b-lactamase needed.	None; restricted to β-lactam with specific absorption	10,11
Fluorescence-based	Monitor accumulation of autofluorescent compound/ antibiotic (fleroxacin for example)	None; limited to naturally fluorescent compounds	7,12
Fluorophore-based (in cells)	Conjugate compound of interest to fluorescent dye and monitor accumulation by fluorescence reader/flow cytometry/microscope	Fluorescent dye	9,13-15
Fluorogenic dye -based	Conjugate compound of interest to fluorogenic dye. Generate bacteria expressing the fluorescence activator protein. Monitor accumulation by fluorescence readout, flow cytometry or microscopy	Fluorogenic dye	9
(Micro-) Spectrofluorimetry	Measuring intracellular accumulation of fluoroquinolones	None; limited to naturally fluorescent compounds	16
LC-MS (in cells)	Quantify compound concentration by LC-MS/ MS. Appropriate controls and washing procedure important.	None	7,17-20
LC-MS (in supernatant)	Quantify residual compound concentration by LC-MS/MS in bacterial supernatants	None	21

2. **Table S2: Quantification of ciprofloxacin, trimethoprim, tetracycline and erythromycin in subcellular compartments of *E. coli*.**

	total amounts [pg]				concentration ng/ml			
	wt		ΔTolC		wt		ΔTolC	
Ciprofloxacin	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**
PP	277	13	402	19	221	10	320	15
CP	592	50	1974	236	37	3	124	15
M	45	33	133	53	57	42	171	68
∑ Fractions	914	23	2510	202	-	-	-	-
WC	750	35	2588	348	42	2	144	19
Ciprofloxacin (4°C)								
PP	147	73	256	2	117	58	203	1
CP	91	21	703	37	6	1	44	2
M	16	5	98	18	21	7	126	23
∑ Fractions	254	49	1057	56	-	-	-	-
WC	181	41	1184	37	10	2	66	2
Trimethoprim								
PP	130	19	130	7	103	15	104	6
CP	356	16	460	73	22	1	29	5
M	0	0	0	0	0	0	0	0
∑ Fractions	485	32	591	74	-	-	-	-
WC	400	85	593	17	22	5	33	1
Tetracycline								
PP	629	35	772	62	500	28	613	49
CP	4451	435	4274	677	280	27	269	43
M	610	25	785	100	781	32	1005	128
∑ Fractions	5690	449	5831	606	-	-	-	-
WC	6791	609	8452	812	378	34	471	45
Erythromycin (10 min)								
PP	36	12	15	2	29	9	12	1
CP	230	25	530	40	14	2	33	2
M	7	1	6	0	9	2	8	1
∑ Fractions	273	28	550	39	-	-	-	-
WC	200	10	619	62	11	1	34	3
Erythromycin (1h)								
PP	5	1	21	1	4	1	17	0
CP	160	5	893	20	10	0	56	1
M	7	1	8	0	8	1	11	0
∑ Fractions	172	4	923	35	-	-	-	-
WC	233	22	1001	17	13	1	56	1

Erythromycin (3h)								
PP	9	0	42	13	7	0	33	10
CP	243	16	2037	755	15	1	128	27
M	8	1	31	3	10	1	40	4
∑ Fractions	260	15	2110	428	-	-	-	-
WC	308	21	1602	49	17	1	89	3
Erythromycin (6h)								
PP	15	2	47	3	12	1	37	2
CP	672	51	1815	152	42	3	114	10
M	28	2	66	3	36	2	84	4
∑ Fractions	715	54	1928	154	-	-	-	-
WC	1039	45	2830	198	58	3	158	11
Erythromycin (12h)								
PP	128	10	164	21	102	8	130	17
CP	2191	292	2896	77	138	18	182	5
M	73	15	85	16	93	19	109	21
∑ Fractions	2392	549	3145	95	-	-	-	-
WC	3831	221	4330	431	214	12	241	24
Erythromycin (24h)								
PP	56	1	104	23	45	0	82	19
CP	3715	213	4219	454	234	13	265	29
M	69	4	76	6	88	5	97	8
∑ Fractions	3840	213	4398	475	-	-	-	-
WC	3326	28	4223	701	185	2	235	39

3. Table S3: Triple Quadrupole MS-MS detection parameters for all compounds analyzed in this study.

	Q1 (mass) [g/mol]	Q3 (mass) [g/mol]	Time [msec]	Declustering potential [V]	Entering potential [V]	Collision energy [V]	Collision cell exit potential [V]
Ciprofloxacin							
quantifier	332.04	314.2	50	111	10	27	16
qualifier	332.04	231.2	50	111	10	49	12
Erythromycin							
quantifier	734.411	158.1	50	66	10	31	32
qualifier	734.411	576.4	50	66	10	25	32
Trimethoprim							
quantifier	291.112	230.2	50	1	10	31	12
qualifier	291.112	261.2	50	1	10	33	14
Tetracycline							
quantifier	445.148	410.1	50	66	10	25	22
qualifier	445.148	427.1	50	66	10	15	30
Caffeine							
quantifier	195.116	138.1	50	81	10	27	10
qualifier	195.116	110.1	50	81	10	31	6

4. Figure S1: CFU-counts of untreated and CIP-treated (c=100 ng/ml) E. coli WT.

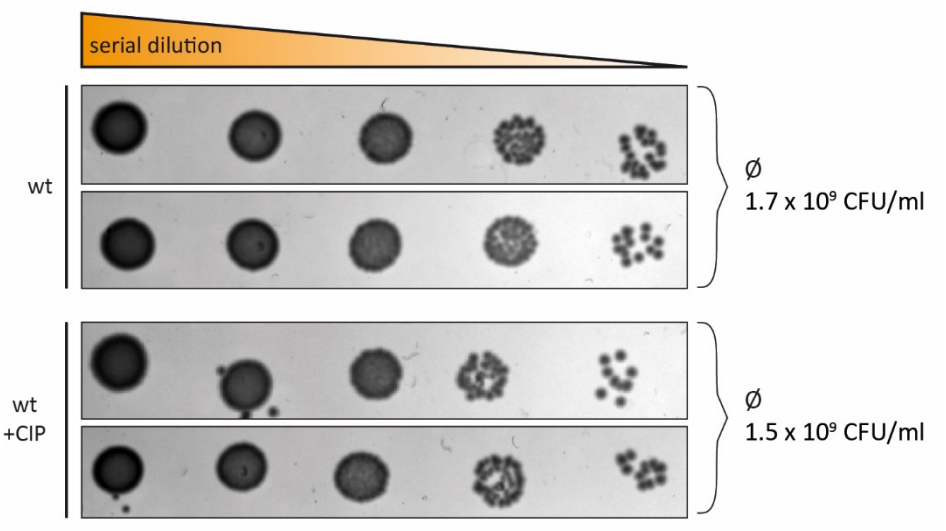

Figure S1: CFU-counts of untreated and CIP-treated (c=100 ng/ml) *E. coli* WT.

5. Figure S2: LC-MS/MS-generated standard curves for CIP, TMP, TET and ERY.

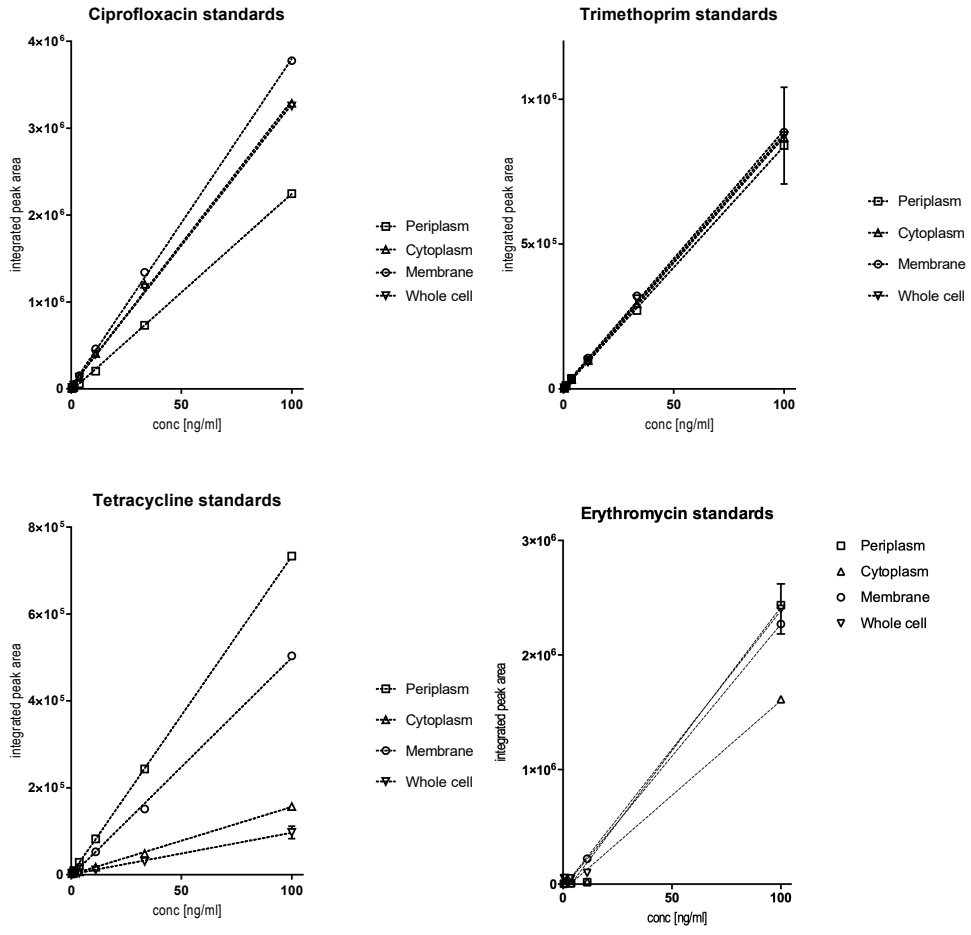

Figure S2: LC-MS/MS-generated standard curves for CIP, TMP, TET and ERY.

6. CFU count assay

CFU counts were determined by spot platting 2 µl of 1:10 serial dilutions on a 12cm x 12cm square LB agar plate. Briefly, samples were generated as described in the 'treatment with antibiotics' section, and 10 µl were serially diluted in 90 µl of PBS in a 96-well microtiter plate. From each well, 2 µl were carefully dropped onto the LB agar plate with a Bravo Automated Liquid-Handling Platform (Agilent Technologies, Santa Clara, CA, USA). Images were taken with a ChemiDoc XRS (BioRad, Hercules, CA, USA) and processed with the Image Lab software (BioRad, Hercules, CA, USA).

7. Kinetic measurements in filter plate for whole cells

E. coli cells (wildtype or ΔTolC) were grown in LB medium from a starting OD_{600} of 0.1 at 37°C and 140 rpm until they reached OD_{600}=1.0. Harvesting of cells was done by centrifugation for 9 min at room temperature (RT) and 4.500 x g, and the supernatant was discarded. The pellet was washed once and resuspended in NaPi-Buffer (50 mM NaH_2PO_4 + Na_2HPO_4; 5 mM $MgCl_2$; pH 7.0) to obtain an OD_{600} = 5.0. The concentrated bacterial solution was kept shaking at 37°C for 5 min and 100 µL (3.9 $x10^8$ cells / 100µl) were transferred to every well of a 96-well filter-plate (MultiScreen®HTS-HV, 0.45µm hydrophilic; Merck Millipore, Tullagreen, IRL) preincubated at 37°C. Bacterial cells were incubated for different periods of time with 25 µl of antibiotic stock solution (0.5 µg/ml), resulting in a compound concentration of 100 ng/ml, and kept shaking at 37°C and 350 rpm in a ThermoMixer® C (Eppendorf GmbH, Hamburg, Germany). Incubation was stopped by the fast removal of the supernatant with a vacuum manifold (15 seconds) and by washing the cells twice with 200µl of ice-cold NaPi-Buffer with a Bravo Automated Liquid-Handling Platform (Agilent Technologies, Santa Clara, CA, USA).Immediately after, cells were lysed using 200µl of ice-cold 80% MeOH in water (v/v), followed by incubation for 30 min at RT at 450 rpm. Lysates were filtered via centrifugation at 2250 x g and 4°C for 15 min onto a deep well receiver plate. Cell debris were further lysed by adding 200 µl of ice-cold ACN for 30 min, followed by centrifugation onto same receiver plate. Then, 320 µl of supernatant were transferred to a 96-well V-shaped plate and dried by vacuum centrifugation in a Refigerated CentriVap with -50°C Cold Trap (Labconco, Kansas, MO, USA). Dried pellets were resolved in a volume of 40 µl containing 10 ng/ml caffeine as an internal standard. Standards of compounds were prepared directly in respective bacterial matrix, obtained from cells lysed the same way as for the assay.

8. Figure S3: Concentration dependence of CIP uptake

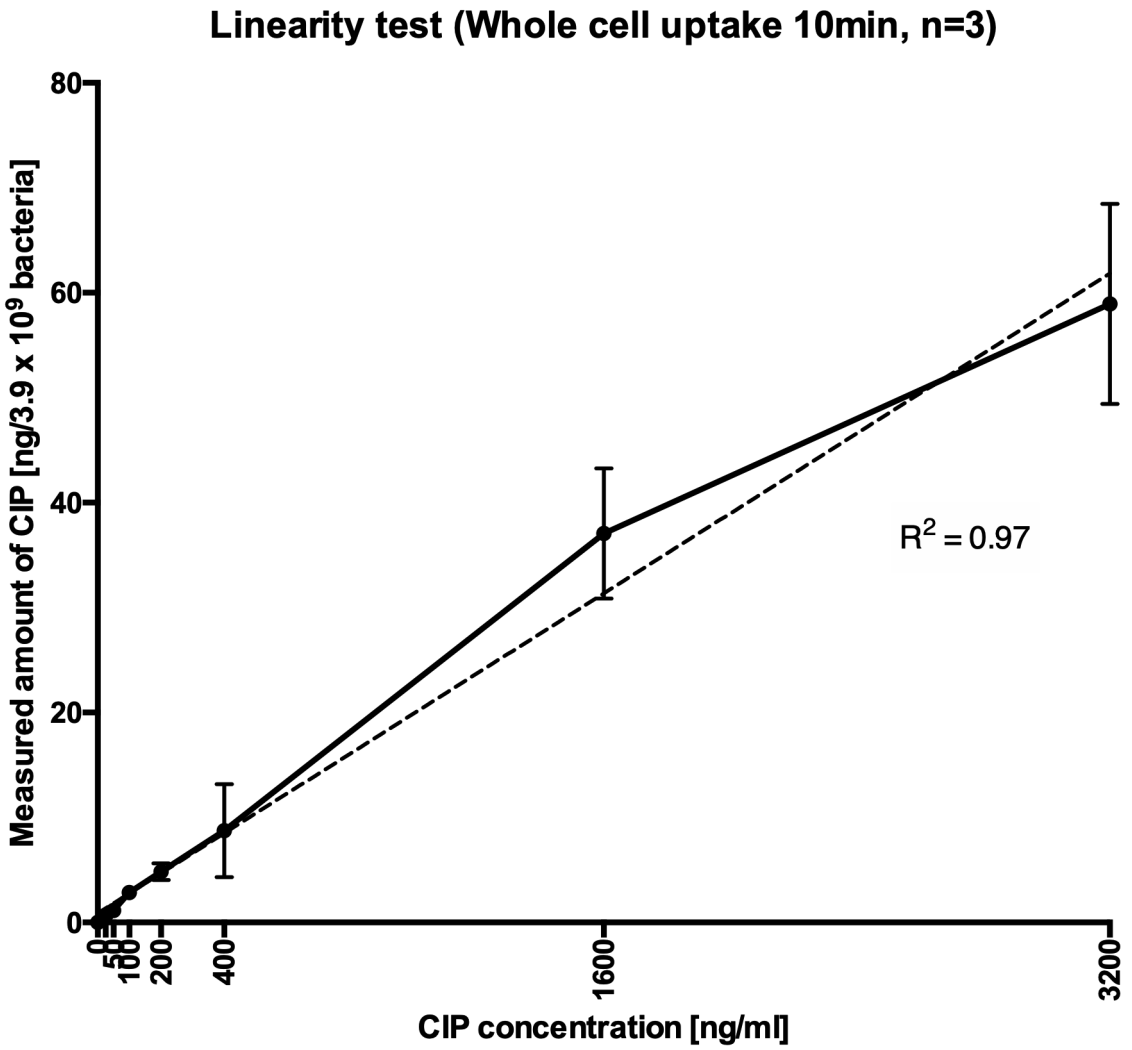

Figure S3: Concentration dependence of CIP uptake. *E. coli* ΔTolC cells were incubated with 25-3200 ng/ml of CIP for 10 min (n = 3), and the the samples were processed as outlined under 'Generation of whole cell lysates' in the Materials and Methods section. CIP uptake was found to be linear over that concentration range with a coefficient of determination of $R^2 = 0.97$.

9. Figure S4: Time-dependent uptake of CIP, TMP, and TET in unfractionated whole cells.

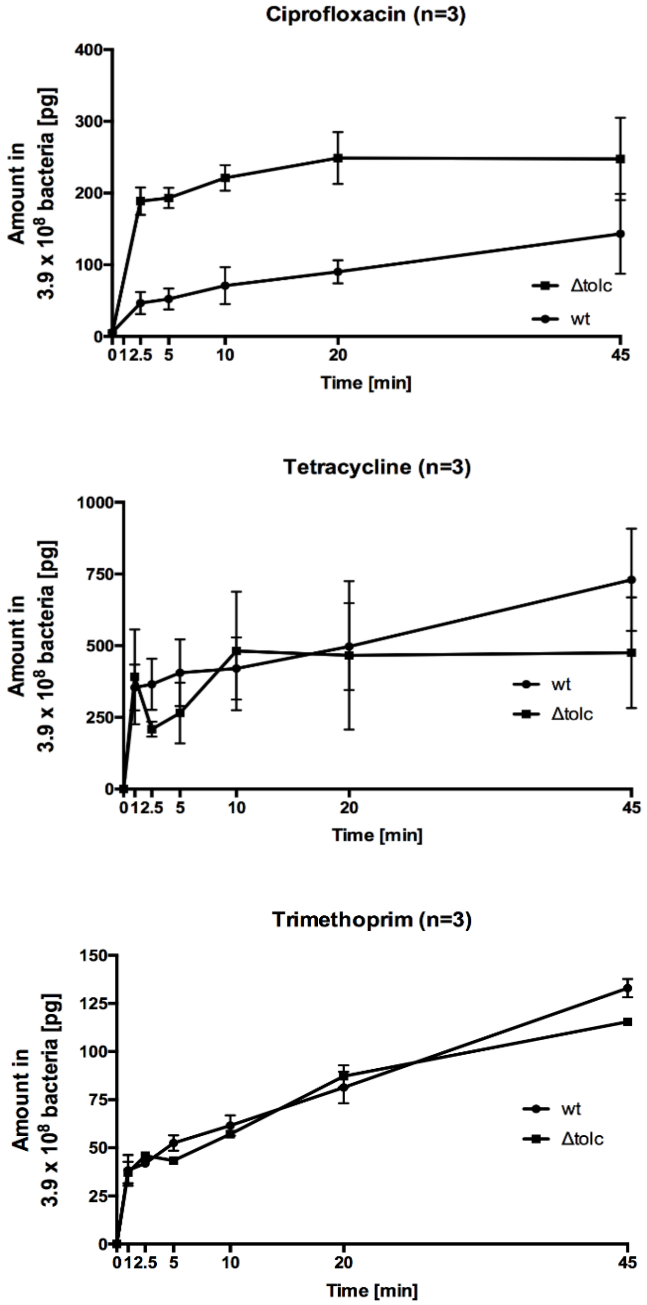

Figure S4: Time-dependent uptake of CIP, TET, and TMP in whole cells. *E. coli* cells (wildtype or ΔTolC) were incubated with 100 ng/ml of antibiotics for defined periods of time. The incubation was stopped by fast filtration using a vacuum manifold, and the amount of antibiotics in 3.9 x 10^8 bacteria was quantified by LC/MS/MS. The detailed assay procedure is given in paragraph 7 of the supplementary information.

10. References

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