EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office via the User Portal:
https://wwws.esrf.fr/misapps/SMISWebClient/protected/welcome.do

Deadlines for submission of Experimental Reports

Experimental reports must be submitted within the period of 3 months after the end of the experiment.

Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report")*,
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

	Experiment title: Nanoscale follow up of antibiotics segregation in Gram negative bacteria	Experiment number: LS-3028
Beamline: ID16A	**Date of experiment**: from: 1/12/2021 to: 5/12/2021	**Date of report**: 20/02/2022
Shifts: 15	**Local contact(s)**: Dr Peter Cloetens	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

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Report:

- Objective & expected results: -

Increasing antibacterial resistance is both a scientific and a political top priority. The effectiveness of antibiotics depends on their ability to penetrate and accumulate into bacteria until they reach a threshold concentration sufficient to bind to their target and inhibit its activity[1]. However, the envelope of Gram-negative bacteria represents a barrier to this accumulation. On one hand, the outer membrane, with its outer layer exclusively made up of lipopolysaccharide, is impermeable to the diffusion of small polar molecules and antibiotics penetrate through the narrow channel of the porins (OmpF and OmpC in *Escherichia coli* and related enterobacteria). On the other hand, transmembrane efflux pumps are able to recognize and expel a wide range of antibiotics. Major efflux pumps belong to the Resistance, Nodulation, cell Division (RND) superfamily and share a common tripartite architecture with an active (proton-motive-force-dependent) drug transporter in the inner membrane, a channel forming protein in the outer membrane and a periplasmic linker protein. Not surprisingly, multidrug-resistant clinical strains often exhibit porin loss associated with overproduction of efflux pumps. For several years, MCT and the DISCO Beamline at SOLEIL have developed approaches based on the intrinsic fluorescence properties of certain antibiotics such as fluoroquinolones to quantify their accumulation in bacteria. Typically, spectrofluorimetry analysis of bacterial lysates is used to determine the intracellular antibiotic concentration in a bacterial population, while deep-UV (DUV) fluorescence microscopy is used to monitor the accumulation of an antibiotic into single intact bacteria in real-time[2]. However, the limited resolution of DUV microscopy (to approximately 150 nm) and the lack of fluorescence calibration impairs the possibility to obtain details on subcellular localization and intracellular quantification of the antibiotics inside the bacterial cells.

Antibiotic-metal complexes as novel fluoroquinolone derivatives could be an alternative to conventional drugs. Numerous studies regarding the interaction between quinolones and metal cations have been reported and reviewed in the literature. In particular, Requimte laboratory (University of Porto) has reported the study of quinolone-metal and quinolone-metal-1,10-phenanthroline complexes (phen, a nitrogen donor heterocyclic ligand, Fig. 1B)[3]. Interestingly, the copper ternary species are stable at physiological concentrations and retained

their antibacterial activity against *E. coli*. Ciprofloxacin (Cip) is a potent and widely prescribed fluoroquinolone. Fluorescence-based approaches showed that Cip is an efflux pump substrate, which accumulates twice as much in *E. coli* cells in the absence of the major efflux pump AcrB[4]. In this proposal, we followed metal-intracellular localization using nano-XRF. Bacterial cell cultures of AG100, wild-type and AG100A, *ΔacrB* were treated or untreated to 5 µM of CuCip ternary complex during 15 minutes, as usually performed with unlabelled Cip.

- Results and the conclusions of the study: -

Experiments were performed under vacuum (~1e^{-7} mbar) at room temperature on the Nano-Imaging beamline ID16A. The X-ray excitation energy was 17 keV, and all relevant elements were detected using their K-level emission lines. Nano-XRF measurements were performed with a step size of 20 nm and a dwell time of 50ms. The summed spectrum recorded with a 6-element silicon drif detector (Sensortech, UK) was fitted with the open source sofware PyMCA. The absolute calibration to the elemental areal density (ng/mm^2) had previously been determined by a thin film standard (AXO Dresden GmbH).

2D maps have been recently weighted (Figure 1A) to obtain localization of elements. We will next calculate the average areal density corresponds to the mean inside the cells of the amount per unit surface of a given element (mostly P and Cu). The total intracellular amount of this element corresponds to the integral of the areal density over the cell area (surface). The total amount is equal to the average areal density multiplied by the area covered by the cell. Ideally, each condition should have been was measured by nano-XRF at least in 10 individual bacterial cells. However, we obtained less then 10 measurements with the chosen step size of 20 nm for AG100 incubated in the presence of CuCip due to a piezo-motor issue at the very beginning of the experiment.

Holotomography microscopy has been performed on one coarse field of AG100A exposed to CuCip, which is expeted to deliver nanoscale, label-free and can be combine with fluorescence for state-of-the-art spatiotemporal resolution as well as high molecular specificity. Fluotomography has also been performed on two coarse field corresponding to AG100 and AG100A exposed to CuCip for 3D localization of the elements. The latter data require 3D reconstruction and have not been fully analyzed yet.

The amount of CuCip quantified by nano-XRF will be compared to that of otained by spectrofluorimetry in the same conditions at SOLEIL.

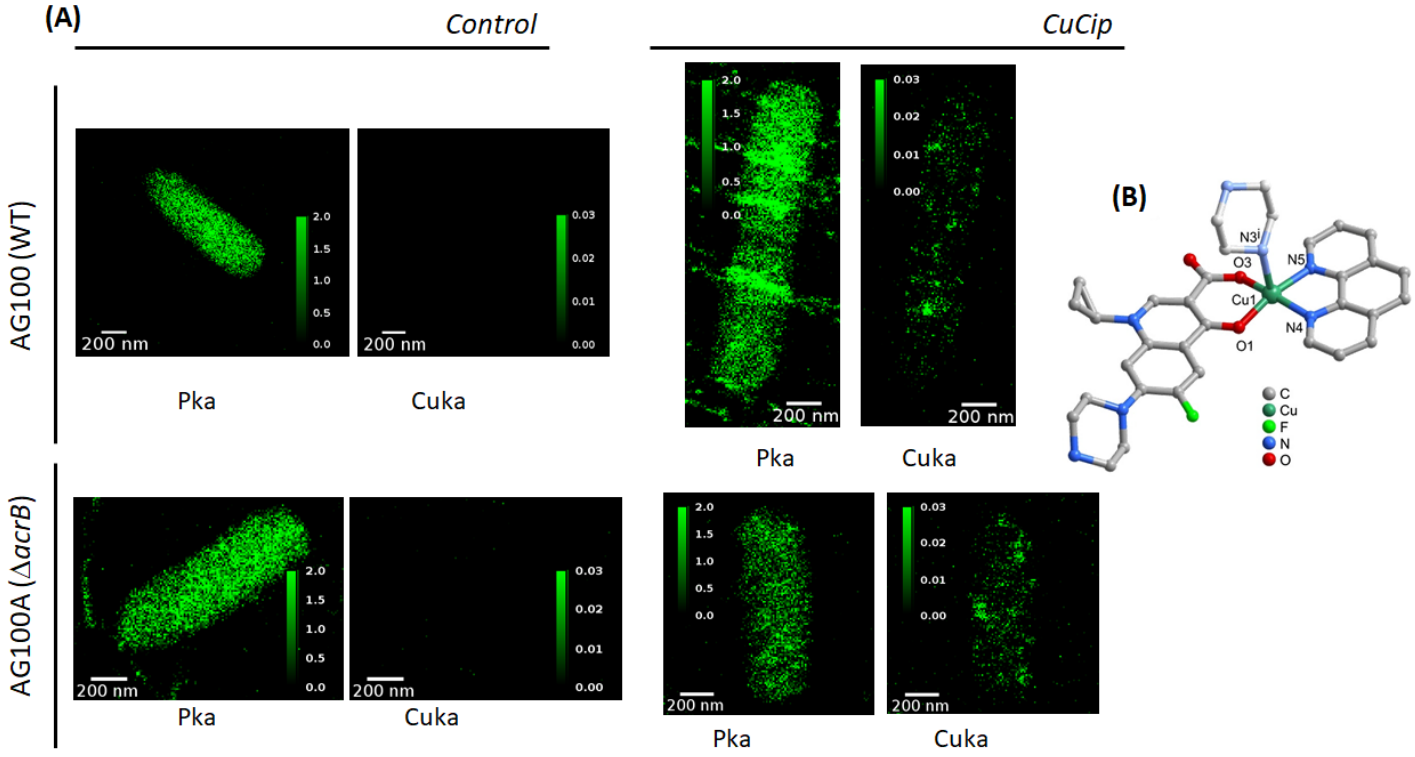

Figure 1: (A) Localisation of elements in *E. coli* K12 cells by X-ray fluorescence nano-imaging. Elements mapping was performed on cultures of AG100 and AG100A were treated or untreated with 5 µM of CuCip during 15 min. Phosphorus (Pka) and copper-bound (Cuka) X-ray fluorescence indicate the bacterial nucleoid and the antibiotic-metal complex, respectively. Elemental areal density quantification bar (ng/mm2) for each element is presented. **(B)** Representation of the cationic ternary complex [CuCip(phen)]+**.**

Levels of accumulation of CuCip and unlabelled Cip are similar in *E. coli* using spectrofluorimetry (around 1.25 fold more in AG100A in comparison to AG100). Interestingly, although data are still waiting to be fully analysed, nano-XRF shows that CuCip (copper-bound fluorescence, Cuka, Fig. 1A) is not homogeneously distributed in *E. coli* but rather localized at the periphery of the bacterial nucleoid (phosphorus-bound fluorescence, Pka, Fig. 1A). This was even more clear in AG100A compared to AG100.
Quinolones target the bacterial gyrase, which introduces negative and relieves positive supercoils necessary for DNA replication reactions to initiate and progress. The inhibition of DNA synthesis by quinolones is due not to the inhibition of gyrase activity *per se* but to the stabilization of the gyrase-DNA cleavage complex. In *E. coli*, the majority of gyrase molecules are not active, with GyrA and GyrB subunits randomly distributed in the cytoplasm, as shown by immunogold labelling[5]. More recently, high-speed single-molecule fluorescence imaging in live *E. coli* demonstrated that most of GyrA foci colocalized with the replisome[6]. Since Cip only captures gyrases during catalysis, our preliminary results suggest that copper-bound fluorescence after CuCip exposure corresponds to the localization functional gyrases. To confirm this hypothesis, we will submit a continuation proposal to use a derivative mutant of AG100 that carry mutations in a gyrase region that confer resistance to quinolones (AG100*gyrA**). This region of GyrA has been termed the quinolone-resistance-determining region (QRDR). Here, we expect random distribution of copper-bound fluorescence into *E. coli* as CuCip will be able to bind GyrA. All together, these results will provide formal proof of evidence that partitioning of CuCip is consistent with the binding of the antibiotic to its physiological target "in motion", which is increased in the absence of active efflux and are expected to be published in a high-rank journal.

- Justification and comments about the use of beam time: -
Allocated beamtime of this proposal was 27 shifts. This was necessary to perform XRF-tomography experiments, which require a lot of time (around 6 hours each), on two independent samples; XRF-microscopy experiments on 4 different samples (two biological replicates of AG100 and AG100A ± CuCip) in order to obtain a minimum of 10 individual bacterial cells for each as well as holotomography microscopy on coarse fields selected from nano-XRF.

- Publication(s): -

1. Masi M, Réfregiers M, Pos KM, Pagès JM. Mechanisms of envelope permeability and antibiotic influx and efflux in Gram-negative bacteria. Nat Microbiol. 2017, 2:17001. doi: 10.1038/nmicrobiol.2017.1.
2. Vergalli J, Dumont E, Pajović J, Cinquin B, Maigre L, Masi M, Réfrégiers M, Pagés JM. Spectrofluorimetric quantification of antibiotic drug concentration in bacterial cells for the characterization of translocation across bacterial membranes. Nat Protoc. 2018,13:1348-1361. doi: 10.1038/nprot.2018.036.
3. Feio MJ, Sousa I, Ferreira M, Cunha-Silva L, Saraiva RG, Queirós C, Alexandre JG, Claro V, Mendes A, Ortiz R, Lopes S, Amaral AL, Lino J, Fernandes P, Silva AJ, Moutinho L, de Castro B, Pereira E, Perelló L, Gameiro P. Fluoroquinolone-metal complexes: a route to counteract bacterial resistance? J Inorg Biochem. 2014, 138:129-143. doi: 10.1016/j.jinorgbio.2014.05.007.
4. Vergalli J, Atzori A, Pajovic J, Dumont E, Malloci G, Masi M, Vargiu AV, Winterhalter M, Réfrégiers M, Ruggerone P, Pagès JM. The challenge of intracellular antibiotic accumulation, a function of fluoroquinolone influx versus bacterial efflux. Commun Biol. 2020, 3:198. doi: 10.1038/s42003-020-0929-x.
5. Thornton M, Armitage M, Maxwell A, Dosanjh B, Howells AJ, Norris V, Sigee DC. Immunogold localization of GyrA and GyrB proteins in *Escherichia coli*. Microbiology. 1994, 140:2371-82. doi: 10.1099/13500872-140-9-2371.
6. Stracy M, Wollman AJM, Kaja E, Gapinski J, Lee JE, Leek VA, McKie SJ, Mitchenall LA, Maxwell A, Sherratt DJ, Leake MC, Zawadzki P. Single-molecule imaging of DNA gyrase activity in living *Escherichia coli*. Nucleic Acids Res. 2019, 47:210-220. doi: 10.1093/nar/gky1143.