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.

ESRF	**Experiment title:** Metal imaging in Gram-negative bacteria: insights into mode of action of antibiotics	**Experiment number**: LS-3131
Beamline: ID16A	**Date of experiment**: from: 17/11/2022 to: 21/11/2022	**Date of report**: 28/02/2023
Shifts: 12	**Local contact(s)**: Dr. Marina Eckermann	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Stephan Göttig, Institute of Medical Microbiology and Infection Control, Hospital of Goethe University, Frankfurt, Germany (Main Proposer)

Celia Romao, Universidade Nova de Lisboa UNL, Instituto de Tecnologia Quimica e Biologica, Oeiras, Portugal

Manuela Tietgen, Institute of Medical Microbiology and Infection Control, Hospital of Goethe University, Frankfurt, Germany

Report:

Justification of the project

The dissemination of multidrug-resistant bacteria is a global threat of healthcare systems and novel therapeutic options are urgently needed as demanded by the WHO. We hence studied the repurposed drug nitroxoline (Nx) as a novel therapy option for infections with multidrug-resistant bacteria. We observed a high activity of Nx against *Escherichia coli* and other relevant pathogens. However, the mode of action of Nx is unclear.

Objectives

Based on our previous results, we hypothesized that Nx could act as an ionophore by transporting divalent metal ions (especially Fe, Zn, Mn) into *E. coli*. High amounts of Nx could perturb metal homeostasis leading to subsequent cell death. We indeed verified the accumulation of metal ions within the cells by employing inductively coupled plasma mass spectrometry (ICP-MS). However, metal localization inside the cell is unknown. For bacteria like *Deinococcus radiodurans*, X-ray fluorescence (XRF) revealed that intracellular metal ions are not evenly distributed but stored in highly organized intracellular compartments, and this was essential for resistance against metal stress [1]. Since metal homeostasis is obviously crucial for the mode of action of Nx, we reasoned that XRF nanoimaging would be the most suited technique to study the localization of intracellular metal ions after Nx treatment of *E. coli.* We hence applied for beam-time at beamline ID-16A.

We aimed to achieve the following objectives:

(1) Impact of Nx on bacterial cell morphology
(2) Confirmation of intracellular metal data obtained by ICP-MS
(3) Novel insights into role of other biological relevant metals
(4) Quantification of metals in Nx-resistant *E. coli*
(5) Subcellular distribution of metals upon exposure of Nx

Execution of experiments

At our institute, a Nx-resistant *E. coli* (Nx-R) was generated by evolution experiments for comparison purposes with an isogenic Nx-susceptible *E. coli* strain (Nx-S). *E. coli* strains were treated with Nx or DMSO as a control for different time points (0, 15 min, 1 h, 6 h) and Nx concentrations (0; 1 µg/mL; 10 µg/ml) and stored in liquid nitrogen. Antibiotic effect and cell numbers were quantified by live cell plating.

At ESRF, cells were thawed and cell density adjusted with PBS after control by inverted light microscopy. Silica membranes were spotted with 10 µL of bacterial suspension and subsequently frozen in liquid nitrogen (plunge freezing). Additionally, bacterial samples were freshly prepared (cultivation and Nx treatment) at the microbiological facility building (EMBL) due to moderate quality of various freeze plunged samples (e.g. ice crystals, low cell density, perturbed surface).

XRF experiments were performed under cryo-conditions to obtain spectra and quantify elements by their K-level emission lines. Low-resolution and fast-position mapping by combined X-ray phase contrast and XRF coarse scans in low-dose mode (6.1×10^{10} ph/s) were performed using a scan step size of 300×300 nm^2 and a dwell time of 100 ms to identify bacteria. Thereafter, XRF fine scans in high-dose mode (2.49×10^{11} ph/s) were done with a step size of either 30×30 or 15×15 nm^2 and a dwell time of 50 ms to obtain quantitative elemental density maps. After fitting and normalization of data, mean intracellular elemental area density were recorded for elements (e.g. Zn, Fe, Ca) and weighted 2D maps were created.

Justification of beam time: The requested time of 12 shifts was continuously and comprehensively utilized. We were able to measure seven samples even though duration of measurement took longer than planned and despite several time-consuming technical obstacles (e.g. beam dumps, optimization of freeze plunging).

Results

Cell morphology: Comparison of Nx treated and untreated Nx-S *E. coli* revealed a significant reduction of the cell length after treatment with 10 µg/ml Nx from 4.3 ± 0.8 µm to 3.1 ± 0.4 µm.

Quantification of biologically relevant metals: Data indicate an increase of manganese, zinc and (to a lesser extent) iron after Nx-treatment (Figure 1). Thereby, the ICP-MS data could be validated by XRF and further confirm our working hypothesis. Notably, we observed an increase of copper, which had not been investigated by ICP-MS. Since copper is almost found exclusively in the periplasm of Gram-negative bacteria [2], we computed an in-house script with the help of our local contact Dr. Marina Eckermann to distinguish between copper content in the periplasm versus the cytoplasm. Copper content in the periplasm was indeed 8-fold higher in Nx treated cells compared to untreated *E. coli*, whereas increase of copper in the cytoplasm was only 2.5-fold (Figure 1B). This points to a novel, yet unknown role of copper in the mode of action of Nx. Importantly, this profound periplasm-specific enrichment was not observed for other metals.

Due to several time-consuming technical obstacles (e.g. quality of freeze plunged membranes, inhomogeneous cell morphology after 6 h and high Nx treatment, several beam dumps), we could just briefly analyse two samples of with Nx-R with only 1-5 scanned cells each. Comparison of treated and untreated Nx-R show a Nx-induced decrease of intracellular metal ions, indicating that Nx resistance is caused by a high activity of efflux pumps (Figure 1C). Further measurements with different Nx concentrations and incubation times could provide new insights into the resistance mechanism and deletion mutants could be used to identify the efflux pumps involved.

Subcellular distribution of metals upon exposure of Nx: Two-dimensional mapping of intracellular metal localization revealed an accumulation of iron in specific areas in particular in the control without Nx treatment throughout the cell; in comparison, calcium or zinc, which seems to be evenly distributed in the cells (Figure 1 A). Preliminary data showed that localization of iron cluster disappeared after Nx treatment. This might indicate that Nx disrupts organization of metals in bacteria. To analyse this in detail we aimed to generate 3D-XRF tomograms of Nx treated and untreated cells. However, due to computational issues during XRF fine scans in high-dose mode the measurement could not be finished.

Conclusions

Using XRF nanoimaging, we demonstrated that the bacterial cell size decreased significantly in response to Nx treatment. We confirmed the Nx dependent accumulation of several metal ions in *E. coli*, which clearly supports the ionophore hypothesis on the mode of action of Nx. We furthermore, observed an increase of copper in the periplasm, which we will validate in functional assays. Our imaging data furthermore suggest that iron is present in certain compartments of the cell. However, due to the lack of 3D mapping, no further conclusions can be drawn and should hence be further investigated. Due to time constraints, the data on Nx-R are derived from few cells, but indicate that Nx leads to reduced intracellular metal content. Further analysis would gain new insights regarding the resistance mechanism. Moreover, we gained important insights on sample preparation and Nx treatment conditions of bacteria for XRF.

We think that these data are encouraging and provide important new insights in the mode of action of Nx. The data are however not complete: (a) most of the results are derived from few cells, (b) the Nx-R strain could not be tested entirely, (c) and a 3D tomogram is missing. We would hence like to apply for a continuation proposal.

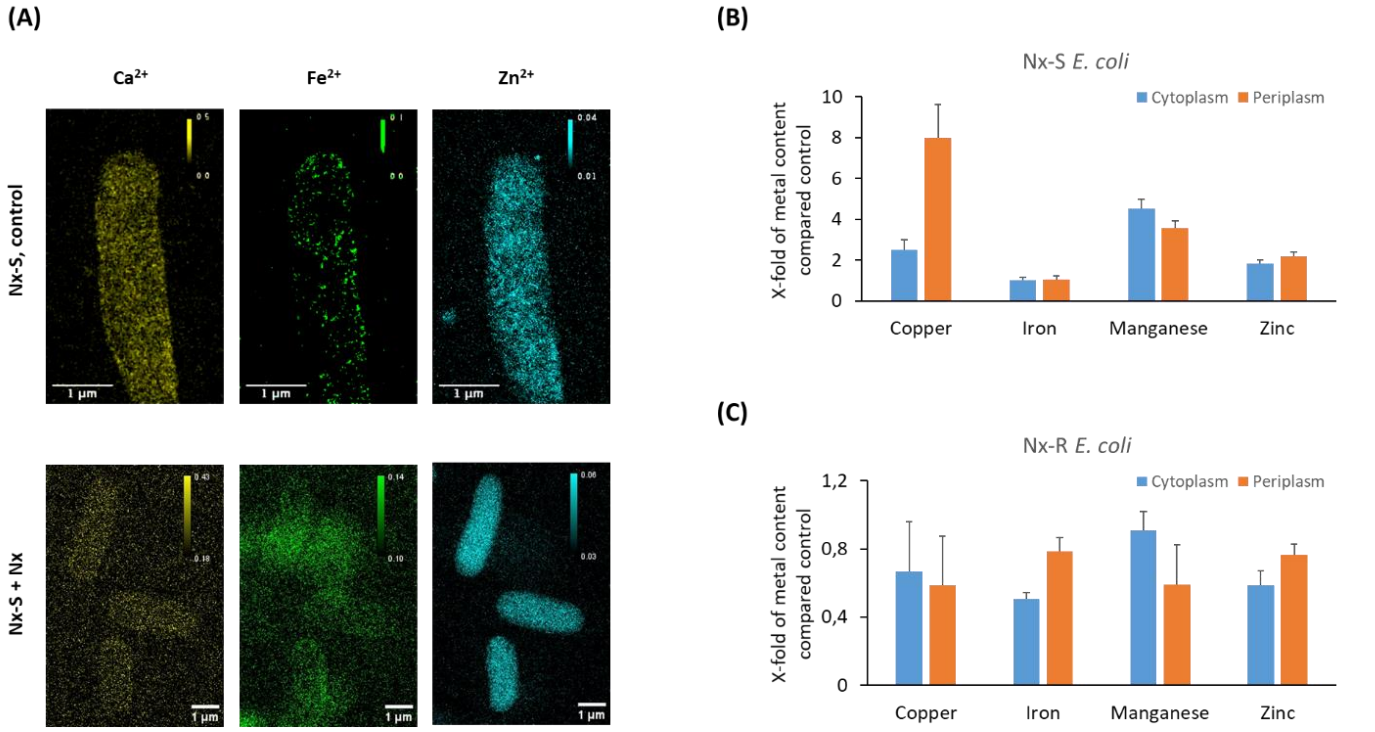

Figure 1. Impact of Nx on metal content in *E. coli*. (A) 2D mapping for calcium, iron and zinc of Nitroxolin-susceptible E. coli (Nx-S) treated with DMSO as control or 1µg/ml Nx for 15 minutes. (B) Analysis of the metal content of treated Nx-S. (C) Analysis of the metal content of treated Nx-R.

References

[1] Santos SP et al (2019) *Sci Reports* 9, 17217
[2] Imlay JA (2014) *J Biol Chem* 10; 28121-8