IMI1 Final Project Report Public Summary

Project Acronym: Translocation

Project Title: Molecular basis of the bacterial cell wall permeability

Grant Agreement: 115525
Project Duration: 01/01/2013 – 30/06/2018

Executive summary

1. Project rationale and overall objectives of the project

The TRANSLOCATION project is part of the IMI's "New Drugs for Bad Bugs" (ND4BB) initiative (www.ND4BB.eu) which was designed to address scientific and financial challenges associated with antibacterial drug discovery and development. Within the ND4BB platform, TRANSLOCATION focuses on fundamental bottlenecks originating during the early stages of Gram-negative antibacterial drug discovery and on the challenge of sharing antibacterial R&D data broadly to enable the community to build future work on existing results.

From an antibacterial drug discovery point of view, TRANSLOCATION focuses on understanding and overcoming the low permeability of the Gram-negative bacterial cell envelope which can severely limit a drug's ability to reach its target. The objective is to increase the understanding of how small molecules (e.g. drugs) penetrate and are effluxed out of Gram-negative bacteria and to create and validate tools and assays that can be used to improve the design of new drugs to treat resistant Gram-negative infections. To facilitate data sharing, TRANSLOCATION will create and populate a repository of antibacterial data and the framework to allow the analysis of that data to establish best practices for future antibacterial drug discovery efforts.

TRANSLOCATION achieved these objectives by:

- Combining theoretical and experimental techniques, determining the structure, function, presence, and underlying molecular mechanisms of recognition and transport by bacterial outer membrane proteins associated with small molecule uptake or efflux (e.g. porins, siderophore receptors, other uptake systems, efflux systems).
- Surveying the data sharing needs of the antibacterial R&D community and the constraints of different institutions to share data in a straightforward manner and using this feedback to design a novel software solution linking multiple open-source solutions to create a single system that can address the broad needs of the community.

2. Overall deliverables of the project

The deliverables of the TRANSLOCATION project can be distilled into five key themes:

- Novel tools and assays to study drug penetration into bacteria;
- Understanding the molecular mechanisms for transit into and out of bacterial cells;
- Identification of novel bacterial uptake systems;
- Creation and population of a database to broadly share antibacterial R&D data;
- Recommendations and best practices based on shared data.

The key outputs of TRANSLOCATION are tools and knowledge about how drugs enter or exit bacteria rather than, for example, the discovery or progression of specific drugs to treat bacterial infections. As such, dissemination of results in the form of peer-reviewed publications, presentations at

international conferences, and workshops designed to engage TRANSLOCATION partners and others within the scientific community should be viewed as an overarching goal of the project.

3. Summary of progress versus plan since last period

WP1: Development of assays to study penetration and efflux in multi-resistant Gram-negative pathogens:

A major focus of TRANSLOCATION is to provide novel tools and assays to study drug penetration into bacteria. Prior to setting up such assays we need to identify the main components in the outer cell wall of bacteria that may be involved in such processes. In the fifth reporting period our proteomics group continued a close collaboration with clinical teams to quantify the membrane protein distribution of bacteria during active infection. In 2018 the first manuscript on the protein distribution in *Acinetobacter baumannii* (*A. baumannii*) was submitted. We improved sensitivity for analysis of proteins from *Pseudomonas aeruginosa* (*P. aeruginosa*) obtained from patients to levels of the current gold standard for clinical microbiology. Following this, the expression of heavy-metal uptake-related proteins in *P. aeruginosa* was determined in both rodent infection models and isolates from patients, demonstrating where such expression patterns showed good overall agreement These results provide a comparison of the proteins present (or absent) in the *P. aeruginosa* bacterial membrane in human infections and rodent models of infection. Coupled with information about which protein(s) might be important for uptake a specific drug, these comparisons can be used to assess the predictability of rodent infection models and the human clinical situation.

WP2: Understanding the impact of Porin structure and intrinsic permeability

We solved six new porin structures from Enterobacteriaceae, OmpF/OmpC orthologs, defining a common fingerprint for this family of porins in the closely related species *Escherichia coli (E. coli), Enterobacter cloacae, Enterobacter aerogenes* and *Klebsiella pneumoniae (K. pneumoniae)*. Analysing the structures allowed us to develop and test a virtual tool in the form of a scoring function to predict the permeability of small molecules through porins from Enterobacteriaceae. Importantly this model not only predicted porin-based permeability, it also offered a physical mechanism and explanation for the observed permeability trends. Thus, starting from simple properties we can predict its permeability through the eight porins, opening the way to the screening of virtual libraries for identifying molecules with optimal permeation.

We solved the first crystal structure of the outer membrane protein MlaA, revealing a novel structure wherein three MlaA molecules are bound to a trimer of OmpF. This led to a working model to describe how MlaA, which plays a critical role in the maintenance of the asymmetry of the bacterial outer membrane, functions. This functional model further clarifies how MlaA could play a role in Gram-negative drug discovery as disruption of this system would be expected to reduce or remove the one of the barrier to drug penetration. In collaboration with WP3 we solved the structure of several siderophore receptors from *P. aeruginosa* (PiuA, PiuD, PirA, PfeA) and *A. baumannii* (PiuA, PirA, BauA). We obtained a co-crystal for PfeA in complex with enterobactin; for BauA in complex with

preacinetobactin, showing an asymmetric 2:1 antibiotic-metal ion complex. This was confirmed using Nuclear Magnetic Resonance spectroscopy (NMR), validating this approach for obtaining structures of metal ion-siderophore complexes. The NMR approach, in combination with molecular modelling, was used to determine the probable structure of the complex of the siderophore containing drug BAL30072 with iron. The structural information obtained from complexes such as these is of fundamental importance to use top design new drugs that can take advantage of these receptor systems uptake systems for antibiotics.

We developed a protocol to quantify the permeation of charged compounds through porins in a quantitative manner, as opposed to previous technologies based on the assumption that intermittent blockage of the ion flux through porins by drugs was directly related to drug translocation. As this approach requires only one parameter to get a qualitative result, good throughput can be achieved. A more sophisticated approach requires bi-ionic condition (high solubility of the compound, large volume) and provides quantitative turnover numbers. Finally, we were able to enhance the resolution by engineering a second translocation barrier into OmpF (OmpC and CymA are ready to be measured). This allows the quantification of transport of uncharged molecules in in micromolar concentrations. Together, these technology advances allow, under specific circumstances, the measurement of porin-mediated drug transport through the bacterial membrane, potentially, along with the complementary techniques developed in WP1, can be used to prioritize work on more promising drug scaffolds.

WP3: Pro-drugging permeability (hijacking bacterial transport mechanisms)

We synthesized tricatechol-linezolid conjugates able to enter very efficiently into the *P. aeruginosa* periplasm and demonstrated conjugate-mediated transport of 300 to 2000 iron ions (and therefore antibiotic)/bacterium/min. Unfortunately, these compounds appear to stay localized to the periplasm and further optimization is needed to transport the antibiotics across the inner membrane where the site of action of linezolid sits. Our data also showed that these tricatechol-linezolid conjugates can induce the expression of the enterobactin-dependent iron uptake pathway in *P. aeruginosa* (i.e. promoting the pathway they use to enter the bacteria) and repress the production of the endogenous siderophores pyoverdine and pyochelin, even in growth systems like epithelial cell infection assays. These studies provide evidence that the tricatechol-siderophore mimics are capable of transporting payloads through one of the bacterial membranes, although additional work would be needed in order to fully utilize this approach.

We previously demonstrated that PiuA and PirA are the main transporters for currently available siderophore-drug conjugates in *P. aeruginosa* and *A. baumannii*. During this reporting period, we analysed the regulation of the Piu and Pir systems in *P. aeruginosa*. We identified the two-component system PirRS as a major regulator of both systems. While PirR directly or indirectly regulates the basal-level transcription of *piuA*, both PirR and PirS are required for the inducible expression of *pirA*. We identified the polyphenol quercetin as a natural substrate and inducer of *pirA*. Addition of µM concentrations, quercetin increased the susceptibility to siderophore drug conjugates by 4-8 fold through increased expression of the *pirA* transporter gene. Further, we identified four additional TonB-dependent receptors, which increased the susceptibility to siderophore-drug conjugates, by

individually overexpressing 28 TonB-dependent receptors. The identification of quercetin's interaction with *pirA* and the further understanding of the bacterial iron-uptake system in general could potentially be used to design more effective siderophore mimics in future drug discovery efforts.

WP4a: Structure-Intracellular Accumulation Relationships via fluorescence and other methods

We developed concepts and methods to investigate (i) the impact of drug influx and efflux, and (ii) the impact of the antibiotic physicochemical properties on their intracellular accumulation/activity. Combined spectrofluorimetry (micro- and kinetic-) and mass spectrometry approaches were used to quantify and/or visualize the intracellular accumulation of fluoroquinolones in bacterial cells expressing various efflux level. The data generated using these technologies have helped to define a SICAR index (Structure Intracellular Concentration Activity Relationship) and RTC2T (Resident Time Concentration Close to Target) for different antibiotics, both being ways to quantitatively describe a drug's ability to accumulate in Gram-negative bacteria. These can be further used to correlate the rate of antibiotic accumulation and real-time activity of antibiotics.

WP4b: Structure-function rules regulating the interaction of antimicrobial compounds with bacterial efflux pumps

Although the specific role of efflux systems in clinical multidrug resistance is under debate, these molecular machines are key in contributing to bacterial survival in the preliminary phase of antibiotic treatment, potentially permitting the development of more specific resistance mechanisms. Thus, the investigation of the dynamic structure-function relationship of bacterial efflux pumps is important to limit the impact of these molecular machines. We focussed our attention primarily on the RND (Resistance-Nodulation-cell Division) transporters of *E. coli* and *P. aeruginosa*, characterizing physico-chemical properties of the putative binding sites of AcrB, AcrD, MexB, and MexY. Note that our crystallographic team was able to identify a new affinity site for fusidic acid and other lipophilic carboxylated drugs in AcrB. Additionally, improvement of crystallization techniques (e.g. purification and crystallization of a truncated AcrB transporter) has been reported, which will increase the throughput of structural studies. Finally, we extended our research to MdfA, a member of the MFS (Major Facilitator Superfamily) family, to gain a more accurate picture of the interplay between different efflux systems.

The main goal of this joint experimental-computational effort was to dissect the different steps of compound-transporter interaction (uptake, binding, transport), including the role of solvent, and to gain insights into structural and dynamic features affecting these steps. Our results have provided a basis for further work to define a more general Structure Activity Relationship (SAR) of drug removal from bacteria via efflux based on both experimental and computational methods. Additionally, we are developing a microscopic map of the protein residues which directly contact drugs during their transit

through efflux pumps which could lead to a better understanding of how to design drugs which are better retained inside the bacterium.

WP5: Understanding penetration and efflux via modelling and simulation

After having improved the Kinetic Monte Carlo (KMC) scheme by including features that could account for information available in consortium and literature (for example, different entropic contributions and multiple occupation of the affinity sites), we performed KMC simulations on the free energy profile associated with the transport of doxorubicin by AcrB. These simulations took into account the impact of the functional rotation on the transport rate by AcrB. The results support a previous hypothesis that transport of drugs by this pump is associated with relatively high energy barriers. The inclusion of the free energy profile related to the translocation through TolC will be a valuable step for a more quantitative picture of the process and will allow computationally determined rates to be compared with experimental measurements, helping to identify an overall SAR for transport of drugs by RND efflux systems.

WP6: ND4BB Information Centre: governance structure and software development

As described, previously, the Information Centre team experienced significant challenges with respect to the amount of data deposition compared to what was originally anticipated. This situation is reflective of the wider situation when it comes to securing access to proprietary scientific data from commercial organisations and academic partners and then being able to properly combine multiple separate data sources to facilitate wider scale analyses. In the field of antibacterial drug discovery, a similar approach to the InfoCentre was initiated by the Pew Charitable Trust (SPARK, see: http://www.pewtrusts.org/en/research-and-analysis/articles/2017/08/information-sharing-platform-to-fill-knowledge-gaps-impeding-antibiotic-innovation). Together with the Collaborative Drug Discovery, Inc., the Pew created an infrastructure ready to host in-vitro data of compounds with antibacterial activity. Likewise, a parallel initiative was launched by the Drugs for Neglected Diseases Initiative (DNDi) / Global Antibiotic Research & Development Partnership (GARDP) Antimicrobial Memory Recovery & Exploratory Programme (see: https://www.gardp.org/programmes/amrp/), seeking to identify historical know-how and data sets which could be used by a cohort of experts to inform decision making around new antibacterial discovery projects. In this reporting period, the InfoCentre team has established strong connections with both of these initiatives. From these interactions, it is clear that a bottleneck for all three has been achieving the release of data sets, which are not already in the public domain, from both industrial and academic sources. One contributory factor inhibiting release was identified as the considerable effort involved for each organisation to prepare data so that it was in suitable for release and mobilisation. For example, organisations tended to have variety of difficult to work with data formats (e.g. paper records, non machine-readable scanned pdf's, data archived on tape, etc) as well as diversely applied assay ontology standards (e.g. different terminologies used to describe what should be similar assay endpoints), or extent of metadata available (incomplete or non-available assay protocol information) The InfoCentre team has

addressed the issue of facilitating data mobilisation from a strategic perspective by co-initiating with the European Bioinformatics Institute the IMI consortium FAIR+, which will create a toolbox for making data from IMI projects and EFPIA companies FAIR (**F**indable, **A**ccessible, **I**nteroperable and **R**eusable). Application of the FAIR methods to existing data will systematically facilitate data mobilisation and access. Promoting interoperability through the application of common ontologies will allow multiple data sets to be analysed in parallel, allowing the ambition of integration from several Pharma based data providers to be achieved. Although the InfoCentre solution was obtained, in the form of a linked database that could handle all expected data types (e.g. drug structures, in vitro and in vivo screening data and summarized clinical trial data), full implementation was not achieved due to the limited amount of data received for deposition. Importunately the framework of the InfoCentre can be reused for other efforts as well.

The team successfully accomplished the formal shutdown of the Electronic Lab Notebook (ELN) system that had been used in TRANSLOCATION. Importantly, the results stored in the ELN will be sustained for future use and access as one partner (Jacobs University Bremen) is planning to maintain their license to the ELN for the foreseeable future. In addition, backup copies of all ELN files are on storage at the Fraunhofer, ensuring that the results recorded in the ELN will be available to researchers in the future.

To maintain the ND4BB Information Centre beyond the lifetime of TRANSLOCATION we identified a number of additional possible partners/options to sustain the framework created in this project. A preliminary sustainability plan for the ND4BB Information Centre was outlined, extending to external, non-antimicrobial research areas. Discussions to implement a version of the InfoCentre as a Use Case for the Medical Data Space (a Fraunhofer Initiative) was made and now this solution forms the basis for current management of the data until wider European-scale programs begin at the end of 2018. To be effective on a wider European level, developing long term best practice requires that (i) data are accessible to the scientists with the research questions and (ii) data sets can be accessed and analysed in parallel to complementary public data sets. In cooperation with ELIXIR, the European Infrastructure for life science informatics, the InfoCentre team identified a sustainability solution process for long cloudification of the InfoCentre databases under the EU open Science Cloud project “EU-OS Life”. This project, with a total volume of 25M Euro, was successfully granted in Q3 2018 and will go live in Q1 2019. These steps will ensure that the InfoCentre framework and software created as part of TRANSLOCATION will be utilized in other EU projects.

WP7: Combining R&D experience to develop best practice and avoid duplication

Since only a small amount of InfoCentre data was available for analysis, the work of WP7 was focussed on the data analysis team looking at lung infection models. In-vivo data from two companies was available for analysis, for a range of treatments involving standard antibiotic drugs. However, no new insights could be drawn from this limited data set due to the differences between the experimental protocols involved and the limited capacity to initiate further prospective studies to confirm the performance of the models and widen the scope of data sets. This subject overall (validation and translation of in vivo infection models to clinical data) would be a potentially interesting subject for a future, more focused PPP project. The organisational model for working with the InfoCentre data sets

in WP7 was also sub-optimal as it relied only upon scientists associated with the project to work with the data. It became clear that a broader group of expert data users was necessary to be engaged in order to align the research goals of scientists with what the data could offer in terms of source of new insights. As a result of these findings, strong links with other groups with similar objectives (e.g. the Pew and GARDP initiatives) were established so that once the suitable data were FAIRified within FAIR+ and then made available on the OpenScience cloud that there would be explicit linkages to experts willing and able to work on the data. Again, this learning has been included in future planned initiatives for AMR (antimicrobial resistance) data sharing and community-wide exploitation of historical and new data sets.

WP8: Translocation – project management, collaboration, ethics, and dissemination

We spent significant efforts in the last year of the project both exploring options for sustainability and ensuring appropriate dissemination of results. For sustainability, we canvased the broader EFPIA community (and related industry or other partners that might have been interested in sponsoring a follow IMI-project). Unfortunately, although scientific interest in the area was high, the challenge of committing enough in-kind resources to result in a project of appropriate focus and scope was a challenge and efforts in this direction were stopped. Other plans to keep core members of the TRANSLOCATION team together have been explored and several smaller proposals (e.g. US National Institutes of Health (NIH), Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), or various national calls) are being explored or have been submitted (see also sustainability points under WP6/7 above).

In terms of dissemination, the fifth annual meeting was held in June 2017 at the Helmholtz Centre for Infection Research in Braunschweig. During this annual meeting / workshop both oral presentations and posters were included from project partners and other academic experts in the area. Also, several members of TRANSLOCATION participated in a workshop at Oxford in May 2017.

Finally, to maximise the delivery potential of TRANSLOCATION we executed our final amendment of the Description of Work (DoW) in the fall of 2017. This amendment further focused the research efforts of the WPs and extended the project by 6 months to allow completion of various aspects of the science and efficient use of the funding provided.

4. Significant achievements since last report

Given the complexity of the project we focussed our resources in this period on the most promising directions of research. An extension of six months allowed us to combine various tasks to give a more integrated picture of the results. For example, we combined quantitative proteomics with high resolution structure and functional investigation to obtain quantitative numbers of the uptake of small molecules through individual channels. In this period, we initiated a study in collaboration with ND4BB project ENABLE (see: http://nd4bb-enable.eu/), investigating in more detail the reason behind a failed approach, and another study to validate a novel uptake system, both involving multiple public partners and significant input from an EFPIA partner. These close collaborations on well-defined questions

allowed the rapid build-up of expertise from the large reservoir available within the project which otherwise would not have been possible.

Several areas of the project were able to finalize and report the results of multiyear / multi-partner efforts in the final reporting period, for example:

- Finalization of studies on the outer membrane protein distribution determined by proteomics in *A. baumannii*;
- Continuation of studies to determine the outer membrane protein distribution in *P. aeruginosa* from patient isolates;
- A working model was developed to describe how MlaA, which plays a critical role in the maintenance of the asymmetry of the bacterial outer membrane, functions;
- Cross validation of HPLC-mass spectrometry (LCMS) and fluorescence measurements of small molecule uptake into whole cells;
- Structural characterization of the stoichiometry of siderophore-metal binding and siderophore-siderophore receptor interactions;
- Scoring function to describe penetration through porins of Enterobacteriaceae;
- Quantification of the flux (not simply binding events) of charged molecules through porins;
- Utilized LCMS and fluorescence techniques to study the uptake of fluoroquinolones;
- Characterization of the physico-chemical properties of AcrB, AcrD, MexB, and MexY using molecular dynamics techniques;
- A framework for the sustainability of the InfoCentre was established;
- Results from the project were actively disseminated (>200 posters and presentations at scientific meetings (>80 in the final reporting period), >140 manuscripts published in peer-reviewed journals (>30 in the final reporting period)).

As in previous reporting periods, challenges in population of the InfoCentre with data remained and were noted by others attempting such efforts, such as the Pew Charitable Trust and their SPARK initiative. Two new sources of support for InfoCentre sustainability were secured in the past period. Firstly, enhancing the contents of the ND4BB InfoCentre data though fairification (IMI FAIR+ project) and secondly, making the data themselves interoperable with parallel public data and accessible via a sustainable cloud platform (EU-OS Life). This opens a new direction for providing long-term access to data until at least 2023. Further funding proposals in cooperation with GARDP, the Pew Charitable Trust, or JPIAMR are also in advanced development. Those initiatives will facilitate access to the data from and by their groups of experts.

5. Scientific and technical results/foregrounds of the project

The key outputs of TRANSLOCATION are the tools and knowledge which have been actively shared with the community via publication. Some specific examples relative to each key deliverable are below.

Deliverable	Output	Summary

Novel tools and assays to study drug penetration into bacteria	• A protocol to detect fluorescent drug uptake at the single bacterium and population levels in *E. coli* was developed and validated. • Electroosmotic flow or reversal potential recording was established as a new technique to quantify penetration in combination classical electrophysiology • MS-based protocol for cpd uptake was designed and benchmarked against fluorescence-based method, showing good agreement	Several new techniques or assays were developed that can provide details information on the penetration of drugs either into bacteria or penetration through a bacterial membrane. These assays can be used to guide future efforts to discover Gram-negative antibiotics by providing quantitative data on penetration into bacteria. This could drive decisions making (e.g. choosing a template with intrinsically better penetration) and be used for validation of potentially higher throughput computational approaches to drug uptake.
Understanding the molecular mechanisms for transit into and out of bacterial cells	• The outer membrane proteome of *P. aeruginosa* and *A. baumannii* in different in vitro and in vivo conditions was characterized. • Approximately 40 new high-resolution X-ray structures of porins, siderophore receptors and other related proteins were solved • A variety of novel vectors to exploit the iron uptake systems were synthesized and studied • A scoring function to both predict and explain small molecule penetration through porins of Enterobacteriaceae was developed • Orthologues of the PiuA and PirA, Fe-receptors important for siderophore-conjugate antibiotic transport into Pseudomonas have been identified in *A. baumannii* • New protocol to increase the throughput of X-ray studies of efflux systems • Structural and dynamic features affecting uptake, binding, and transport of compounds by RND transporters • New insights in the ferri-enterobactin uptake pathway in *P. aeruginosa* • Proteome of *P. aeruginosa* grown in the presence or not of siderophore-linezolid conjugates in planktonic growth conditions of eukaryotic cell infection assays	The molecular structure of many bacterial membrane proteins potentially utilized in passive (e.g. porins) or active (e.g. iron-receptors) transport of drugs into bacteria in addition to efflux out of drugs from bacteria were determined. This information served as a starting point and was combined with additional experimental data to support the development of high level computational models to predict and understand the molecular basis of drug penetration into Gram-negative bacteria. These results can assist in the future design of drugs with potentially better penetration properties into bacteria. In addition, these structures serve as a broad repository of information for the broader scientific community.
Identification of novel bacterial uptake systems	• Improved understanding of the potential of both maltodextrin uptake and fatty acid uptake as routes for drug discovery • Synthesis of tris-catechol vectors able to transport the linezolid into *P. aeruginosa* periplasm and induce the expression of the enterobactin pathway	Nutrient uptake systems can provide a 'Trojan Horse) approach to delivering drugs into Gram-negative bacteria. For example, a nutrient mimic can be linked to an antibiotic, thus transporting the antibiotic into bacteria using the bacteria's own nutrient uptake pathways. We have extensively studied two less know systems (maltodextrin and fatty acid uptake) and synthesized new mimics for the well-studied iron uptake system. This information could serve as a starting point for new drug discovery efforts using these systems as an approach to improve penetration into Gram-negative bacteria.
Creation and population of a database to broadly share antibacterial R&D data	• A functional Information Centre was constructed by linking two existing platforms providing a database that could cover the full scope and needs for the InfoCentre. • Data governance and access procedures were established to ensure the secure deposition, utilization and management of both research and Intellectual Property (IP)-sensitive data in the InfoCentre	A database that can link chemical structure data, preclinical in vitro and in vivo data and potentially summarized clinical data was constructed as a data warehouse for ND4BB projects. Although challenges remain in the releasing of data, primarily due to IP constraints, the framework now exists for such data to compiled. The overall Information Centre

		platform could also be used in non-AMR projects to facilitate data sharing.
Recommendations and best practices based on shared data	• Considerable progress in this area was hindered by data-sharing challenges noted elsewhere. • Links were established to parallel initiatives working on similar efforts in AMR which of potential for long term usage of the data by expert teams	Although no clear recommendations were created, primarily due to lack of data, links to newer initiatives with similar goals were made.

6. Potential impact and main dissemination activities and exploitation of results

Antimicrobial resistance is a growing problem in the EU and globally and many government and non-government organizations have called for increased action. IMI's launch of the ND4BB program of projects, starting with TRANSLOCATION and COMBACTE (now COMBACTE-NET, see: https://www.combacte.com/) was a concrete step in to address some of the bottlenecks in antibacterial drug discovery and development. With respect to TRANSLOCATION specifically, the challenges of bacterial cell entry and the challenges of sharing data in the AMR space are recognized as bottlenecks for optimal antibacterial drug discovery. The results and learnings from TRANSLOCATION have and will continue to have significant impact on both of these areas, in addition to broader impact in the basic science landscape in the EU. For example:

- New assays or measurement techniques were developed which allow for better characterization of drug activity and/or uptake in bacteria, both in whole cells and in model systems, including detection of single molecule events;
- Determination of quantitation and structure of Gram-negative membrane proteins related to drug uptake which build on previous structural knowledge and can provide insights to molecular mechanisms of drug transport;
- Hypotheses and models for transport across the Gram-negative cell envelope which could be used to prioritize or optimise future series in antibacterial drug discovery;
- Improved understanding of the structural basis and regulation of iron-siderophore uptake and other uptake systems which can be used to assist in the design of new drugs using the Trojan Horse approach;
- New ligand binding sites and simulations to understand drug efflux with RND pumps which could provide insights to avoid or prevent efflux of new drugs;
- The ND4BB Information Centre was created, the framework of which will be transferred and sustained for use in other EU projects;
- Learnings from the challenges related collecting and collating antibacterial R&D data discovered and shared with groups formed after TRANSLOCATION potentially streamlining the approach taken by other efforts;

More generally the project as a whole has also assisted in the development of the EU as a leader in antibacterial basic research:

- The inclusion of many younger researchers in the project—fostering education and ensuring there will be a next generation of EU-based scientists to conduct antibacterial research;
- The SME GRIT42 (formerly GritSystems, see: http://grit42.com/) was established as direct result of the work undertaken in the technical development of the ND4BB InfoCentre. This enterprise was realised as spin-out from the EFPIA company, Lundbeck and the software made available for the InfoCentre leverages > 20 years of in-house development at this speciality Pharma company.

  In order to fully engage with the consortia, GRIT42 became a project partner of TRANSLOCATION soon after it was founded, which allowed close collaborative working with the wider team to a far greater degree than could be achieved through a "sub-contracting" arrangement. Through this exposure to a wide group of EFPIA and public partners, GRIT42 extended its overall software tools offering and has now secured new contracts with the Danish OPENSCREEN and Leo Pharma, demonstrating the utility of the software for both public and commercial organisations. GRIT42 is also contributing to the creation of new standards in life science data management through membership of the Pistoia Alliance. This is a key success story for TRANSLOCATION and shows its impact on the development of new and innovative SME's in Europe;

TRANSLOCATION has actively disseminated research results to allow for others in the field to benefit from learnings and challenges. Overall the project has presented >200 oral presentation and posters at national or international conferences and has >140 manuscripts published or submitted, including in high impact journals such as *Nature, Nature Microbiology, Nature Protocols*, *Nature Communications*, *Proceedings of the National Academy of Sciences*, *ACS Nano, ACS Infection Diseases, Journal of the American Chemical Society, Antimicrobial Agents and Chemotherapy*. Also, we have hosted five international workshops (>120 participants each), in conjunction with annual meetings, in which members of the project and the wider scientific community attended and shared information. An additional 40 manuscripts are in preparation and will be submitted after the formal end of the project, including a perspective that will summarize overall results and impact of TRANSLOCATION and critically evaluate the project's progress with the state of the art in the field. It is anticipated that this perspective will appear as one or more reviews in the journal *Drug Resistance Updates*.

7. Lessons learned and further opportunities for research

TRANSLOCATION had several ambitious objectives and although considerable progress was made in all areas, the idea that any single project or approach can 'solve the bacterial penetration challenge' is unrealistic. Importantly this area overall has seen a resurgence, judged by the number of papers that have appeared recently on the topics of measuring small molecule uptake in bacteria, models of drug penetration through bacterial membranes or pores, computer simulations, and information sharing activities in the antibacterial space. This points both to the importance of the problem and the need for additional work to reach real solutions.

From our perspective most of our results could not be obtained without a larger 'community' of interdisciplinary researchers and industrial, academic, and small company perspectives that are available with a Public-Private Partnership (PPP) approach such as IMI. The advantage of a combination of skill sets and expertise, especially the integration of biophysical and theoretical methods side-by-side with more traditional microbiological approaches, was a foundational key to the success that TRANSLOCATION had. For example, the following would have been challenging if not impossible without the framework of a multi-year PPP:

- Close collaboration and sharing of ideas between 'competing' pharma companies;
- Iterative investigation requiring input from many disciplines over an extended period of time (i.e. scoring function porin work)—not generally possible with smaller, shorter or more focused grants;
- The setup of a critical mass of researchers that can be involved in similar projects with little or no 'ramp up' time;
- Identification of a collaboration protocol between academic and industrial partners;

Similarly, the combination of such a wide group of researchers also highlights challenges that appeared while undertaking the project and over the course of the project a number of learnings were realized that could be useful for future PPPs:

- Some approaches that appeared promising on paper were simply not able to deliver (as is often the case for novel approaches or technologies)—it was key that the project was able to constantly update plans based on the direction data was moving. We recommend that as much flexibility as practical should be built into project plans from the start, including formal review of progress / re-structuring at an appropriate point in the project;
- Sharing data and results, even within the project, was a challenge, despite putting in place tools (e.g. an Electronic Laboratory Notebook) to make this straightforward;
- Best practices for communication (meetings, sharing information, etc.) should be discussed, established at the outset, and monitored or refined at regular intervals to ensure appropriate communication across the project;
- Discovery does not happen according to a specific month—avoid project plans that 'back-load' deliverables to 'end of year' as it is neither realistic nor practical to have everything come in at once;
- Bringing together a group of researchers with distinct experience and backgrounds creates communication challenges (e.g. everyone hears what is being said, but interprets it differently)—these should be acknowledged at the start and efforts made to ensure everyone understands messages as intended;
- Broadly speaking, the more engaged partners were, the more they were both able to do for the project and the more they got out of the project. Therefore, we would recommend having a realistic view of how much effort is needed to make an impact and base projections on that (and if plans are for minimal effort, expect minimal output);
- Likewise, a uniform view of how the project should be tracked should be agreed up front—dealing with different sections of a project having different approaches to writing Deliverables,

becomes more complex as the project evolves. Similarly, it should be noted that real progress, rather than simply 'completing many deliverables' is the goal;

- WP Leaders (or similar roles, depending on project structure) are key conduits for information and for scientific management of the project. Without strong and engaged leaders in these positions work will flounder, and partners will drift. All efforts should be made to identify appropriate WP Leaders, empower these leaders to make real decisions and provide them with all tools and support (including e.g. funding for teleconference options and/or team travel as appropriate) to enable success;
- Transfer of researchers from one group to another (especially from, e.g. academics groups to EFPIA partners), even for a relatively brief time, is a powerful way to foster collaboration and improve engagement;

Potential new or continued areas of research to build upon TRANSLOCATION results include:

- Increase the throughput of structural and biophysical methods;
- Improve the quantitative readouts of drug flux measurements by combining different techniques;
- Utilize newly developed tools to understand structure-activity-relationship of small molecule penetration and efflux
- Using newly available tools, screen small/medium-sized collections of derivatives from several chemical series in order to push/test putative "rules of Gram(-) penetration"
- Create a data base of molecule with high permeability through the major outer membrane channel
- Suitable in-vitro activity data contained in the InfoCentre and FAIRified data from across the ND4BB projects will be included in the FAIRified data sets to be generated by FAIR+.
- Since analysing proprietary data on compound activities in isolation gives limited insight, continued research will amalgamate suitable in-vitro data from the InfoCentre with bioactivity data from the ChEMBL (see: https://www.ebi.ac.uk/chembl/) database in the EU Open Science Cloud (EU-OS LIFE Demonstrator 1)