DanCARD

ANNUAL SCIENTIFIC REPORT

2013

The Danish Centre for Antibiotic Research and Development

STATENS SERUM

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| CONTACTS

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| PREFACE

The DanCARD project is now in its fourth year. The long publication list reveals the large amount of data within this field which the project has supported. The list to date includes 30 articles in peer-reviewed journals and 39 abstracts along with several scientific reports and theses. Four of the DanCARD enrolled PhDs have now finished.

Numerous compounds have been synthesised and tested for *in vitro* and *in vivo* antimicrobial effect as well as cytotoxicity and several interesting candidate molecules have been identified, which are currently being tested further. Additionally, the project has gained substantial knowledge on important matters, including antimicrobial activity, treatment optimization, resistance mechanisms, new antibiotic structures, antimicrobials in nanoparticles and microbial lifestyle.

The project has inspired additional funding within this field, including the research projects UC-Care and ENABLE as well as the training program TRAIN-ASAP. This will empower the research to continue after 2015. We are looking forward to following these new initiatives.

Project Head
Professor Niels Frimodt-Møller

| SCIENTIFIC REPORTS FROM COLLABORATORS

Karen A. Krogfelt
Department of Microbiology and Infection Control, Statens Serum Institut (SSI)

The maggot defensin Lucifensin
Maggots are used for treatment of extreme chronic wounds. Serine protease produced by *Lucilia sericata* (blowfly) was characterized. The peptide was recombinantly produced and purified by SSI and Novozymes A/S in collaboration. The effect of the protease was tested on blood and it was shown to induce fibrinolysis. The work was done in collaboration with *LUMC, Leiden, The Netherlands and Lund University, Sweden.* This work resulted in a publication in PLOS-one.

Bacteria in wounds
Pseudomonas aeruginosa isolates from chronic wounds were biochemically characterised. Based on this, production of bacterial communication signals and associated virulence factors were described (Bachelor project, Neşe Celik).

Mette E. Skindersoe was trained at Finsenlaboratoriet in growing artificial human skin equivalents (HSE) for wound healing and infection studies. These HSEs were used to investigate the effect of sterile filtered *Pseudomonas aeruginosa* supernatants on skin and wound healing. Interestingly it was found that not only did *P. aeruginosa* sterile supernatants impair healing, but also that a strain expressing bacterial communication signals was much more virulent than a "silent" strain. Work is currently ongoing at Queensland University of Technology, Brisbane, Australia (in the Tissue Repair and Regeneration Research group) with another human skin model to investigate the effect of different bacteria and treatment types further. Moreover, it is expected that reconstructed human skin may be used to test novel topical agents (antimicrobials) developed within DanCARD for cytotoxicity.

H. pylori infection is a major cause of gastric cancer. The uPAR (urokinase Plasminogen Activator Receptor) system is thought to play an important role in tumour progression and metastasis, but also in healing of wounds. We have contributed to a study showing that *H. pylori,* at a very early stage of infection, induce expression of the uPAR protein *in vitro* as well as *in vivo*. This link between *H. pylori* infection and the expression of the tumour/metastasis linked uPAR protein may be important for development of new strategies to prevent gastric cancer. The work was performed in collaboration with the Plough group at Finsenlaboratoriet (Submitted to Infection and Immunity).

The study mentioned above about uPAR and infection inspired a new collaboration between Statens Serum Institut and Finsenlaboratoriet. Two student projects during the last year have shown that wound isolates of *P. aeruginosa* exhibit huge diversity with regard to cytotoxicity (Master project, June Lissa Hansen and Bachelor project, Neşe Celik).

Cytotoxicity and antimicrobial activity
The antibacterial activity of a lysophospholipid is under investigation. Continued work on the antibacterial activity of the lysophospholipid MPPA has shown:

1) that this compound can be linked to the surface of titanium implant mimicks
2) that coating of the slides with MPPA and derivatives thereof significantly reduce adhesion of *Staphylococcus aureus*
3) electron microscopy supports the findings above, and indicate that bacteria on coated slides produce less matrix material

The project was performed in collaboration with Jason Mansell, University of the West of England, who has performed linking of the compounds to the titanium slides. This project is still ongoing.

C. elegans
C. elegans was used as a model to screen compound libraries for identification of specific compounds targeting virulence and innate immunity. (Henrik Jakobsen PhD-thesis, Jacobsen AS BSc thesis)

Antiadhesive compounds
We have finalized the development of a novel assay for adhesion quantification of *Helicobacter pylori* (the causative agent of stomach ulcers). An article about the method, including a ready-to-go protocol, is under revision for publication in the journal Helicobacter. Using this assay we have also screened the adhesive capacity of extracts from tea, mushrooms and fish and an article about this has been submitted to Int J Med Microb. The work was conducted in collaboration with Rigshospitalet.

Cranberry juice is known as natural medicine in preventing urinary tract infections. A model for studying biofilm formation on catheters under flow was established (Steffen L. Jørgensen, Master thesis) and effect of cranberry juice on biofilm was tested (Natasja Nystrup, research project)

In collaboration with the University of Utrecht, polyvalent sugars were used to inhibit a *Streptococcus* infection in an animal model (Paper published in Biology 2013).

Luca Guardabassi,
Department of Veterinary Disease Biology, HEALTH, University of Copenhagen (UCPH-HEALTH)

At SUND-DVDB, the two general aims of DanCARD are to i) rationalize use of existing antibiotics in veterinary medicine and ii) develop non-antibiotic alternatives for prevention and treatment of bacterial infections in animals. The project started at this institution later compared to other partners (fall 2012) and has progressed without major deviations from the original plan. Additional funding by an EU project and a national research center funded by the University of Copenhagen and coordinated by Prof. Luca Guardabassi (see section 8) has allowed extension of the original plan and inclusion of new research activities that were not described in the application. The results achieved at this stage of the project can be summarized as follow.

The postdoc Marco Minoia has implemented his study on the effects of antimicrobial drugs on conjugation of epidemic plasmids encoding extended-spectrum beta-lactamase (ESBL). The ultimate goal of this study is to generate knowledge to develop ESBL plasmid conjugation inhibitors that can be administered in concomitance with cephalosporins, thereby preventing or limiting horizontal plasmid transfer in the gut microbiota of treated patients. The study started in October 2012. The first step was to elucidate the effects of therapeutic concentrations of cephalosporins on conjugation of a widespread IncI1 plasmid encoding CMY-2, a largely diffused ESBL type in poultry and poultry meat products in Denmark and in many other EU countries. To follow in real-time how the IncI1 plasmid transfer changes in response to cefotaxime, promoters of the genes responsible for plasmid transfer (*tra* operons) were fused with a gfp-marker. Results show that transfer genes activity and plasmid conjugation frequency rose significantly in presence of therapeutic concentrations of cefotaxime, whereas they were affected by neither subtherapeutic concentrations of cefotaxime nor by other cephalosporins used in hospitals (cefuroxime) and veterinary practice (cephalexin and ceftiofur) regardless of their concentrations. Preliminary results show that other plasmids transfer might also be influenced by therapeutic concentrations of cefotaxime depending on the MIC of ESBL-producing strains. This observation is very interesting as it suggests that this third generation cephalosporin may favour spread of ESBLs not only by antibiotic selective pressure, but also by enhancing plasmid transfer during therapy. Understanding of the genes/proteins involved in the regulation of this mechanism will reveal potential targets for development of ESBL plasmid conjugation inhibitors, which could be included in the formulation of cefotaxime to reduce its impact on the gut microbiota of patients during therapy.

After the first year of her PhD, Angelika Schoster has got a position as Senior Clinical Lecturer at the University of Zurich but has continued the PhD programme at KU-SUND. Her project focuses on the possible use of probiotics as tools for control and prevention of intestinal diseases. Based on in vitro activity against *Clostridium difficile* and *Clostridium perfringens*, five commercial probiotic strains belonging to *Lactobacillus* (n=4) and *Bifidobacterium* (n=1) were selected for further in vitro and in vivo testing. Four of them were shown to reduce *C. perfringens* beta2 toxin expression by 9-10-fold and one additionally reduced expression of alpha toxin. A probiotic prototype for equine use containing a mixture of these strains was manufactured in collaboration with the industrial partner Clerici-Sacco Group. The probiotic prototype was administered to 5 neonatal foals without adverse effects, and the effect of the probiotic compared to a placebo on the composition of the gastrointestinal microbiome is currently being analyzed using metagenomic sequencing. Further, the prototype is being tested in a randomized placebo controlled clinical trial in neonatal foals, to determine whether probiotic administration may reduce the incidence of diarrhea and antibiotic use in horse farms (ongoing in 2014).

Marit Maaland has entered the third year of her PhD study on optimization of veterinary therapy with older antibiotics. In vitro and in vivo PK/PD data of tetracyclines, nitrofurantoin and phenicols were generated to optimize treatment regimens in dogs. As these drugs are not critically important in human

medicine and the current use is limited in small animal medicine, this research is expected to impact both animal health (by providing new therapeutic options) and public health (by reducing veterinary use of critically important antibiotics in human medicine). The results show that some of these antibiotics are promising candidates for treatment of canine infections caused by multidrug-resistant (MDR) bacteria. The project has so far led to new canine breakpoints for doxycycline, and dosage recommendations of minocycline use against MRSP infections in dogs. As a spin-off of the study, new veterinary breakpoints for doxycycline were approved by the Clinical Laboratory Standards Institute (CLSI). Additional PK/PD studies on nitrofurantoin and phenicols are currently carried out to enhance use of these older antibiotics in human and veterinary medicine, respectively. As a result of this work, two manuscripts have been published and two additional manuscripts are in preparation. Marit is expected to defend her PhD thesis in the fall of 2014, exactly the 3 years after the start of the PhD study.

The study investigating the use of peptoids as topic antimicrobial drugs continues in collaboration with the EU project TRAIN-ASAP (www.train-asap.eu). A fellow supported by this project is continuing the work that was initiated by the postdoc Peter Damborg in the first part of DanCARD. A new formulation is presently tested by acute toxicity and skin susceptibility tests using a murine and a rabbit model, respectively. Moreover, another in vivo study of clinical efficacy will be performed to evaluate the clinical and microbiological outcome of new formulation in a murine skin infection model (the first experiment failed due to presumptive problems in the formulation used). In case of positive results, a patent application will be submitted in 2014 by Prof. Paul Robert Hansen and Prof. Luca Guardabassi. A large pharmaceutical company in the animal health sector (Zoetis) has shown interest in this project.

Finally, the bioinformatic analysis of the genome sequences of selected Syphoviridae bacteriophages able to lyse multidrug-resistant strains of the veterinary pathogen *Staphylococcus pseudintermedius* is being completed in the first part of 2014 and a publication describing this work is in preparation. The advantage of this approach is that the phages under study have a predominant activity against methicillin-resistant *S. pseudintermedius* strains and limited effect on methicillin-susceptible strains. As such phage therapy appears to be a promising alternative to conventional antibiotic therapy for management of dog skin infections caused by resistant strains without harming the commensal flora.

DanCARD has facilitated additional funding in the area of antimicrobial drug R&D at SUND-DVDB and other DanCARD partner institutions, therefore contributing to strengthen Danish research in this area. At least three projects have been achieved directly or indirectly based on the results of DanCARD and more broadly as a consequence of the enforcement of this research area at SUND-DVDB:

1) Starting from January 2012, L. Guardabassi is the coordinator of a EU Initial Training Network (ITN) dedicated to "Training and Research AImed at Novel Antibacterial Solutions in Animals and People" (TRAIN-ASAP) (www.train-asap.eu). This training project supports 12 PhD students and involves 7 partners including two SMEs and a large pharmaceutical industry (Zoetis, ex Pfizer Animal Health). TRAIN-ASAP provides a number of synergies and opportunities for collaboration with DanCARD, including research collaborations between fellows and organization of joint training events and workshops. The introductory TRAIN-ASAP course in October 2012 was attended by several DanCARD fellows. A joint international symposium has been scheduled in Copenhagen for 2015.

2) Starting from October 2013, L. Guardabassi is the Principal Investigator of the University of Copenhagen Research Center for Control of Antibiotic REsistance (UC-Care) (www.uc-care.ku.dk), which includes participation by other 3 DanCARD partners affiliated to the University (Anders Løbner-Olesen, Henrik Franzyk and Paul Robert Hansen). N. Frimodt-Møller is an external collaborator and a member of the Scientific Advisory Board of UC-Care. This research center is an extension of the DanCARD framework with inclusion of clinical and humanistic studies. The center supports 5 postdocs and over 10 PhD students with focus on translational research bridging the gaps between basic science, clinical research, patient care and food safety. UC-Care is intended to last beyond the 4 years funded by the University of Copenhagen and become a self-sustainable research center financed through academia-industry partnerships. UC-Care will also participate in the organization of the joint DanCARD international symposium in 2015, contributing to increase the capacity and impact of this event.

3) Following the isolation of phages displaying lytic activity against MRSP, two postdoctoral grants on phage induction and phage lysins were funded by the Danish Council for Independent Research (Technology and Production Sciences) and the Sapere Aude Award for young elite researchers. Arshnee Moodley is the grant holder of these projects. The project has started in 2013 and will end in 2015.

Paul Robert Hansen
Department of Natural Sciences and Environment, HEALTH, University of Copenhagen (UCPH-HEALTH)

The work in our lab has been aimed at identifying new anoplin analogs which selectively target Gram negative species such as *E. coli*. To this end, we have synthesized and purified more than 65 anoplin analogs and subsequently characterized them with regard to their activity against five microorganisms in collaboration with SSI. Furthermore, their hemolytic activities have been determined. Several peptides with unprecedented activity and specificity towards *E. coli* relative to other microorganisms as well as human red blood cells have been identified.

In collaboration with Dr. Wimmer´s group at University of Aalborg (AAU), we have shown that the process of optimization of anoplin analog properties should abide certain rules; the balance between charged and hydrophobic residues is important, and while the size of the hydrophobic residues governs membrane affinity, the polar amino acid composition determines antimicrobial specificity.

Applying statistical design and analysis strategies to the biological data obtained from the anoplin analogs has revealed that mathematical relations exist between amino acid sequence and biological activity. In collaboration with Dr. Christian Ritz, University of Copenhagen, we have shown that biological activity measurements from a designed subset of amino acid combinations can be used to reliably predict the biological activities of other combinations. This makes synthesis of all amino acid combinations unnecessary, thus shortcutting to the peptide with desired properties, saving time and money.

We have found that very discrete modifications of a central amino acid can dramatically affect biological activity. This has been investigated in collaboration with Dr. L. Thøgersen, University of Aarhus using molecular dynamics simulations in a system of water, ions and lipid bilayers resembling either the bacterial membrane or the red blood cell membrane. A clear effect of the subtle modifications is evident, observable as varying mechanisms of peptide attack on the membranes. A relation between the rotational behavior of the varied central amino acid side chain and the hemolytic activity has been identified, thus possibly providing atomic-level explanation of the hemolytic properties of peptides in general.

In collaboration with Dr. Peter Damborg and Professor Luca Guardabassi, University of Copenhagen, 10 peptide-peptoid hybrids were synthesized and tested against *E. coli*, *S. aureus* and *S. pseudintermedius* as potential veterinary antibiotics. Two of these compounds were tested against 50 *S. pseudintermedius* and *Pseudomonas aeruginosa* isolates. Time kill curves showed that the peptide-peptoid hybrid killed the bacteria at 4 x MIC within 30 and 120 min respectively. These compounds are now being developed into veterinary antibiotics in the project FP7-PEOPLE-2011-ITN TRAIN-ASAP ("Training Research AImed at Novel Antibacterial Strategies in Animals and People").

Jens K. Munk successfully defended his Ph.D.-thesis on October 25, 2013.

Two master students are doing a related project at the Department of Drug Design and Pharmacology.

Hanne Mørck Nielsen & Henrik Franzyk
Department of Pharmacy & Department of Drug Design and Pharmacology, HEALTH, University of Copenhagen (UCPH-HEALTH)

The research performed at Department of Pharmacy at UCPH-HEALTH comprises development of new antibacterial drugs as described in WP2a and b (*Incorporation of antimicrobial compounds in nanoparticles* and *Improvement of drug delivery at the target site*, respectively).

The research performed at Department of Drug Design and Pharmacology at UCPH-HEALTH has concerned development of new antibacterial peptidomimetics within WP1 (Development of novel antibiotics).

Part of the research deals with evaluating the bacterial killing effect of antimicrobial peptides as well as cell penetrating peptides with potential antimicrobial effect. The microbiological evaluation of the synthesized compounds was partly performed in collaboration with SSI and Hvidovre Hospital (Anne Sandberg-Schaal and Niels Frimodt-Moller).

For the ongoing activities, collaborations with researchers in the UK and in US have been established. Several manuscripts will be submitted to peer-reviewed journals within the coming year.

At Department of Pharmacy, two PhD students have been employed, and the projects are still ongoing. From these projects, currently one full-

length manuscript has been published in *BBA Biomembranes* (Bahnsen *et al.*, 2013) and three peer-reviewed abstracts have been prepared and accepted for poster presentations at the Annual Meeting of Controlled Release 2013 (Water *et al.*, 2013; and Bahnsen *et al.*, 2013) the Annual Meeting of Controlled Release (Water *et al.* 2014). Further, a popular scientific manuscript has been published in *Lægemiddelforskning* (Bahnsen *et al.*, 2012). Two master students (2012, 2013) and an Erasmus student (2013) have been associated to the project (all completed).

At Department of Drug Design and Pharmacology, one PhD student has been employed, and this PhD project was finalized in the autumn of 2013. In this PhD project we have conducted the first comparative study of several different peptidomimetic backbones displaying the same sequence of side chains (Jahnsen *et al.*; J. Med. Chem. 2012).

In collaboration with Department of Microbiology and Immunology at UBC, one of these peptidomimetics (HDM-4) was examined regarding: (i) immunomodulatory activity, (ii) anti-biofilm activity, and (iii) direct antibacterial activity (Jahnsen *et al.*; Chem & Biol. 2013). In addition to the already reported high potency against multidrug-resistant *E. coli* strains, HDM-4 was found to display antimicrobial activity against other Gram-negative pathogens comprising *Pseudomonas aeruginosa*, *Salmonella enterica*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*. The mechanism of action of HDM-4 was shown to involve permeabilization of the outer membrane and partial depolarization of the inner membrane at its minimal inhibitory concentration (MIC). Moreover, it was demonstrated that HDM-4 was distributed widely in the bacterial cells when these were challenged with concentrations above the MIC. This multi-modal action of HDM-4 was reflected in the finding that it was less prone to resistance development as compared to several known single-target antibiotics. Also, HDM-4 exhibited anti-biofilm activity at sub-MIC levels against *E. coli*, *P. aeruginosa*, *S. enterica*, and *A. baumannii*. Furthermore, HDM-4 modulated the immune response by inducing the release of chemoattractants (e.g. IL-8) from human peripheral blood mononuclear cells. Finally, the compound suppressed LPS-mediated inflammation by reducing the release of the pro-inflammatory cytokines IL-6 and TNF-α.

Another library of twelve compounds, displaying chain lengths of 10-14 residues, have been evaluated for their antibacterial activity (against six bacteria including multidrug-resistant strains) and cytotoxicity towards both cancer-related (immortalized) and benign cells (six cell lines in total) in order to determine the optimal chain length. Both chiral and achiral peptoid residues were incorporated into these peptidomimetics.

Omitting *α*-chirality in the side chains of the peptoid residues proved to reduce the propensity of the compounds to adopt secondary structure, and this feature was correlated to decreased cytotoxicity. Furthermore, optimization of the length of these peptidomimetics with an alternating cationic-hydrophobic design proved to be a powerful tool for enhancing the selectivity against Gram-negative pathogens over benign mammalian cells. Thus, 12-mer lead compounds with a high selectivity towards killing of clinically important multidrug-resistant *E. coli* were identified (Jahnsen *et al.*; submitted to J. Med. Chem.).

Yet another array of peptidomimetics was prepared with the purpose of examining the effect of end-group modification of the N-terminus. Testing of their antibacterial activity revealed that selectivity between Gram-negative and Gram-positive bacteria may be interchanged depending on the type of moiety introduced. Remarkably, introduction of lipophilic moieties at the *N*-terminus resulted in analogues with high activity against multidrug-resistant *Staphylococcus aureus* and *Enterococcus faecium*. Despite increased toxicity against murine fibroblasts and human umbilical vein endothelial cells, the optimized peptidomimetics exhibited significantly improved selectivity. Initial animal studies using a mouse skin infection model indicated that a 100-fold reduction in the bacterial count in the infected wound was possible by application of a gel containing one of the two most cell-selective compounds.

Reinhard Wimmer
Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University (AAU)

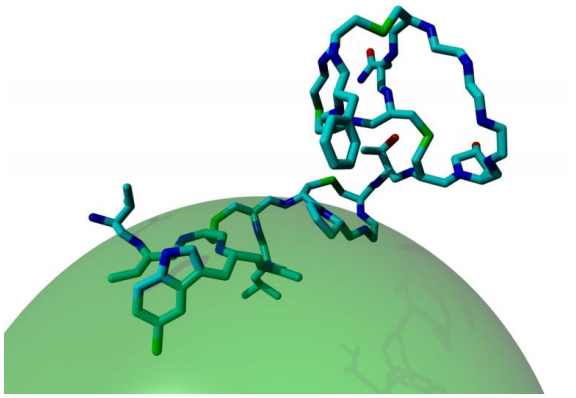
Structures of new antibiotics

Based on an extensive dataset with activity data and structure information of mutants of anoplin a set of guidelines was proposed for the development and improvement of α-helical antimicrobial peptides. The data showed why some approaches previously used fail, and shows new ways of designing modifi-

cations in these peptides. The work is currently under review at a scientific journal.

Structures of peptoids
Peptoid derivatives of maculatin were synthesized in collaboration with Paul R. Hansen and NMR studies on the peptoids showed that the variants exhibit *cis-trans* equilibria around the peptide bond leading to the N-substituted glycine. The structures were determined for a purely *trans* and a mixed *cis:trans* peptoid. Based on the results, guidelines for the successful insertion of peptoid residues can be given. We are currently preparing the manuscript.

Structure and membrane interaction of the lantibiotic NAI-107
NAI-107, also known as microbisporicin or 107891, is a new lantibiotic produced by the actinomycete Microbispora. NAI-107 is active against multi-drug resistant Gram-positive pathogens. In collaboration with a German and an Italian research group, the mode of action of NAI-107 and its structure in micelles (Fig.) was determined. NAI-107 binds to micelles with its N-terminal nisin-homologous part. It interacts with the cell wall precursor lipid II in a 2:1 stoichiometry. The work has been accepted for publication at J.Biol.Chem.



Anders Løbner Olesen
Department of Biology, Functional genomics, BIO, University of Copenhagen (UCPH-BIO)

In the past 6 months we have primarily been focused on purification and optimization of the peptides selected as inhibitors of the DnaN-DnaN interaction.

We are optimizing the selected peptides by substituting the amino acids with alanine one at a time in each peptide. We have at the present moment substituted every amino acid in two of the peptides targeting DnaN. We are currently measuring the effect of these substitutions on the DnaN-DnaN interaction using the BTH system.

We have constructed a plasmid with increased expression of cyclic peptides *in vivo*. We will use this plasmid for purification of cyclic peptides from *E. coli*. When sterilized supernatant from cultures expressing active peptides is added to growing cultures of *S. epidermidis* we observe inhibition of growth. Thus at least part of the cyclic peptide is secreted into the supernatant of the *E. coli* culture. We will try to purify the peptides from culture supernatants. By addition of culture supernatants to growing cultures of *S. epidermidis* we have a quick assay to test newly selected and optimized peptides for activity against this strain. We will use this assay together with the BTH system to optimize the selected peptides. In addition to purification of the cyclic peptides from culture supernatants we are currently optimizing the chemical synthesis of the peptides in collaboration with Paul Robert Hansen, KU and Håvard Jenssen, RUC.

We have used the culture supernatant from *E. coli* expressing active peptides to select a number of resistant mutants by addition of culture supernatant to growing cultures of *S. epidermidis*. One of the resistant mutants has a mutation in the target gene, DnaN, while the mutation in the rest of the strains has not yet been identified. These contain a wt dnaN gene.

Søren Molin
Center for Systems Microbiology, Technical University of Denmark
(DTU)

We have investigated the expression of small regulatory RNAs (sRNAs) in *Pseudomonas aeruginosa* in a variety of different conditions, which include exposure to the most commonly used antipseudomonal antibiotics. During the last year we have specifically investigated the role of the sRNA OsiS, which was recently identified in a genome-wide search of sRNAs. OsiS is highly transcribed during oxidative stress conditions, including treatment with ciprofloxacin and hydrogen peroxide. We have shown that 30 min of induction of the expression of OsiS results in a significant downregulation in the levels of PhrS. PhrS is another sRNA that activates the translation of the quorum sensing (QS) regulator PqsR under low oxygen concentrations, which in turn activates the synthesis of the PQS operon. In fact, many of the downregulated genes after 45 min of induction of OsiS are PqsR-dependent. These results indicate that OsiS provides a link between oxygen levels and QS, by downregulating the levels of the sRNA PhrS during oxidative stress conditions. We hypothesize that the interaction between the two sRNAs is by direct base-pairing, with a predicted recognition site of OsiS at the highly conserved-region of PhrS. However, more experiments are required to know the exact nature of the interaction between these two sRNAs. OsiS is a sRNA that responds to oxygen levels in bacteria, such as PhrS in *P. aeruginosa*, RgsA in *P. fluorescens*, OxyS, FnrS and MicF in *E. coli*, and RliB in *L. monocytogenes*. During infection, *P. aeruginosa* is subject to constant shifts in oxygen tension due to the human airway structure, biofilm formation, antibiotic treatment and the host immune response. We infer that *P. aeruginosa* most likely needs a tight and fine-tuned regulation of the mechanisms necessary to survive during adaptation to an environment with such fluctuating levels of oxygen tension as the one inside the host, and that the sRNA OsiS is involved in this kind of regulation by responding to high oxygen tension and regulating the levels of the sRNA PhrS.

Niels Frimodt-Møller & Klaus Skovbo Jensen
Department of Clinical Microbiology, Copenhagen University Hospital, Hvidovre
Department of Biomedical Sciences, HEALTH, The University of Copenhagen (UCPH-HEALTH)

IS-PRO Analysis of faeces
The purpose of this study, performed in collaboration with Dr. Paul Savelkoul, Netherlands,
is to elucidate the impact of common antibiotics on the indigenous flora of mice.
A pilot study has been performed in which Dicloxacillin and Clindamycin were given to mice, subcutaneously once a day for three consecutive days. Faeces were taken directly from mice on day 1, 3 and 5 (day 1 being the first day of treatment) and IS-Pro analysis performed. IS-Pro is a High-throughput molecular fingerprinting of the intestinal microbiota. The analysis is based on PCR in which the variation in length of the 16S-23S rDNA regions is used to classify the microflora into specific phyla. The results from the pilot study indicate that IS-Pro can in fact be used to analyse antibiotics general impact on the microflora. Thus, a study including five antibiotics will be performed in 2014.

Case control study of urinary tract infection (UTI), from general practises
To investigate risk- as well as bacteriological factors related to UTI in general practises (GP), caused by ESBL-producing *E. coli* (ESBL).

Prospective collection of *E. coli* isolates in urine samples, submitted for investigation from patients with UTI. We randomly included 98 cases: Patients with ESBL producing *E. coli* and compared them to 3 control groups: 174 patients with non-ESBL producing *E. coli,* but resistant to at least one groups of antimicrobials (R), 177 patients with fully susceptible *E. coli* (S) and 200 patients with negative urine cultures (N).

Admissions to hospitals have been obtained from The Danish Health and Medicines Authority and antimicrobial prescription one year prior to the UTI, for all included patients, obtained from the Statistics Denmark. Preliminary results described below.

The proportion of patients > 65 y old, were for ESBL: 50%, R: 51 %, S: 37% and N: 19% ($P < 0.05$). Hospitalizations, as $\geq$ one admission, within one year before UTI: 47% for ESBL, 31% for R, 27% for S and 19% for N, respectively ($P < 0.05$) and hospitalization after UTI: 36% for ESBL, 29% for R, 26% for S and 39% for N, respectively (P = NS). This investigation is on-going.

Classification of ESBL and non-ESBL E. coli isolates from UTI, from General practice

The purpose of the study was to use isolates collected as part of "Case control study of urinary tract infection (UTI), from general practises" to investigate the clonal composition of mono-resistant (non-ESBL), ESBL-producing and fully susceptible *E. coli* populations, using two characterization methods.

From the case-control collection of clinical *E. coli* isolates we included three groups of isolates: 94 isolates of non-ESBL-producing *E. coli,* but resistant to at least one class of antimicrobials (R-*E. coli*) and 94 isolates of fully susceptible *E. coli* (S-*E. coli*). These 188 *E. coli* isolates were compared to the 98 ESBL-producing *E. coli* isolates (ESBL-*E. coli)*. An in-house developed abbreviated multilocus variable number of tandem repeat analysis (MLVA) was performed by PCR. All isolates were analysed and assigned a MLVA-code. At least one isolate from each identified MLVA-cluster was chosen for multilocus sequence typing (MLST), meaning that all MLVA-codes were correspondingly characterized by MLST.

We found a total of 44 and 64 different MLVA codes in R-*E. coli* and S-*E. coli*, respectively. In contrast, we only found 22 different MLVA codes in the ESBL-group. Likewise, we identified a total of 27 and 38 ST´s in R-*E. coli* and S-*E. coli*, respectively, while only 19 different ST´s were seen in the ESBL-group. Nine ST´s were found in more than one group. The prevalence of ST131 in the three populations varied with 17% in R-*E. coli*, merely 2% in S-*E. coli* and of the ESBL isolates, 51% proved to be ST131.

PKPD studies of older antibiotics in an experimental animal model

The following older antibiotics with excellent effect against multidrug resistant (e.g. ESBL) Enterobacteriaceae have not been studied for their optimal PKPD, i.e. to estimate the optimal dosing regimens in treatment of urinary tract infections: nitrofurantoin, fosfomycin, and ciprofloxacin. The in vitro work includes time-kill curves. PK studies were performed in mice by administering single boluses either orally (nitrofurantoin) or subcutaneously (fosfomycin and ciprofloxacin). Drug concentrations in urine and blood were measured by HPLC (nitrofurantoin) or bioassays (fosfomycin and ciprofloxacin). From the PK data obtained PK/PD studies were designed to include dosage regimens varying in their PK parameters (C_{peak}, $T>MIC_{24h}$, AUC/MIC_{24h}). The mouse urinary tract infection model was used for the PK/PD studies investigating the effect of 5-10 different dosages for each antimicrobial. Analysis of the experimental data includes the sigmoidal model (Hill's equation) and nonlinear regression. For each antibiotic a scientific paper will be submitted.

| COMMUNICATION

Peer-review articles:

1. **Bahnsen, J.S., Franzyk, H., Sandberg-Schaal, Nielsen, H.M.**
   Antimicrobial and cell-penetrating properties of penetratin analogs: Effect of sequence and secondary structure.
   BBA Biomembranes (2013) 1828: 223-232.
2. **Bojer, M.S., Jakobsen, H., Struve, C., Krogfelt, K.A., and Løbner-Olesen, A.**
   Lack of hfq attenuates virulence of several Escherichia coli pathotypes towards Caenorhabditis elegans.
   Microbes and Infection, 14, (2012) 1034-1039
3. **Fazzini, R.B., Skindersoe, M., Bielecki, P., Puchaka, J., Givskov, M., and Martins dos Santos, V.**
   Protoanemonin: a new natural quorum sensing inhibitor that selectively activates iron starvation response.
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   Anja Sloth Nielsen
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2. **Delima Sebamalairashan**
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3. **Frederikke Fliedner** (Bachelor Thesis)
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4. **Jesper Søborg Bahnsen**
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5. **Mads Erlin Nygaard Kristensen**
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6. **Maria H. Michaelsen**
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7. **Mikkel Calum**
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8. **Monica Hermann**
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9. **Natasja M Nystrup.**
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10. **Paula Melo Paulon Hansen**
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11. **Ping Fang**
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