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ABSTRACTS

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Phage therapy of avian pathogenic *Escherichia coli* (APEC) : identification of bacteriophage receptors

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ABSTRACT

Extra-intestinal *Escherichia coli* strains, the avian pathogenic *E. coli* (APEC), are responsible for causing colibacillosis, an invasive infection of poultry with the respiratory tract as a route of entry, due to their invasive ability. Colibacillosis is responsible for significant economic losses in poultry industries worldwide. The increasing antibiotic resistance acquired by these strains, as well as the restrictions to the antibiotic usage, have encouraged the search of new solutions to control these severe infections. Bacteriophages, viruses infecting exclusively bacteria, have been proposed as valuable alternatives to antibiotics, based on their capacity to infect and destroy the bacteria, also termed as bacteriophage therapy.

In this study, the goal was to characterize genes encoding the receptors for phages φ2, φ6 and φ7, which could be applied in a bacteriophage cocktail for prevention or treatment of colibacillosis. Mutagenesis was carried out, by use of mini-TnphoA2, to generate mutants of the APEC strain CH2 that are resistant to bacteriophages φ2, φ6 or φ7. We utilized the inverse PCR technique and direct PCR to localize and identify the insertion site of the transposon mini-TnphoA2. Sequence analysis of the amplified fragments showed that bacteriophage φ2 resistant mutants carried mutations occurred at different positions in the *ompF* gene, encoding the membrane porin 1a (OmpF). Transposon-tagged mutants resistant to phages φ6 and φ7 were difficult to obtain, due to the high frequency of spontaneous mutants and the lack of a system for linkage analysis. One bacteriophage φ7 resistant mutant carried a mini-TnphoA2 insertion in gene *wejN,* that encodes for a glycosyl transferase of the *E. coli* serogroup O78 O- antigen gene cluster. This is an interesting observation, because CH2 is an O78 strain and phages φ6 and φ7 infect only O78 strains. It therefore seems likely that the *wejN* gene contributes to the synthesis of the phage receptor

Analysis of fimbriae specific for O157 EHEC strains

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ABSTRACT

EHEC O157:H7 is a highly virulent enteric pathogen that is acquired by ingestion of contaminated food or water, and healthy cattle and sheep sporadically harbor *E. coli* O157:H7 in their gastrointestinal tract and shed the bacteria in their feces. *E. coli* O157:H7 is the major STEC serotype involved in sporadic cases and outbreaks of HC and HUS worldwide. Pathogenic *E. coli* strains possess morphologically distinct structures adherence factors, called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes and are thread or hair-like structures that radiate out from the bacterial surface and present adhesive moieties known as adhesins that recognize specific host cell receptors and allow them to colonize sites in the host that *E. coli* does not normally inhabit. There are 16 predicted fimbrial operons on the chromosome of *E. coli* O157:H7, and these clusters generally share a standard genetic organization and contain regulators, major and minor subunit genes as well as the chaperone and usher genes. Fimbrial operons classically have a minor gene encoding adhesins that mediate the binding specificity of the fimbriae. For most fimbriae no information is available on their targets in the host. The majority of the predicted fimbrial clusters are at least partially conserved in other *E. coli* strains. However, four of the fimbrial loci, loc1, loc6, loc12 (*lpf1*) and loc13 (*lpf2*) are mainly found in *E. coli* O157:H7 strains. Of the unique clusters, two (loc12 (*lpf1*) and loc13 (*lpf2*)) have a high similarity to the long polar fimbriae (*lpf*) first described in *S. Typhimurium*. In this study, these specific fimbrial gene clusters, together with the full length self-complementing adhesins and the lectin domain of the adhesins were cloned and expressed. We obtained expression for the C-terminally his-tagged full length self-complementing loc1 and loc12 clones encoding the loc1 and loc12 adhesins and the his- and HA-tagged lectin domain of loc1 adhesin. Large scale expression of the C-terminally his-tagged lectin domain of the loc1 adhesin was achieved for both its native and selenomethionine-derivatized lectin domains (residues 1-168). Candidate crystals were obtained from the different crystal screening kits and the structure was solved for selenomethionine-derivatized lectin domain at a resolution of 1.89 Å. The structure revealed that the fold exists of an α-helix capped against the β-sheet and in addition two disulphide bridges are located at the top and stabilize both the loop, which is protruding from the tip of the lectin domain, and also the N-terminal end of the lectin domain. Therefore, this study contributed to the identification and structure determination of a novel adhesin. We also succeeded in obtaining self-complementing constructs expressing the loc1 and loc12 adhesins. Additionally, identification of the eukaryotic receptors for these individual fimbrial adhesins and determining the structural basis of host cell recognition by the fimbrial adhesins is the following major step to tackle.

Keywords: 3D-structure, adhesin/lectin domain, EHEC O157:H7, fimbrial gene clusters.

Protein aggregation in plants as a tool to specifically knock-down proteins' functions

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Characterization of maize mutator lines and transgenic lines modulated for PARP1 gene expression for drought stress tolerance

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ABSTRACT

Poly (ADP-ribose) polymerases are a family of enzymes present in all eukaryotes except in yeast. In plants, PARPs have been linked to DNA repair, mitosis, innate immunity and stress responses. Upon induction of oxidative stresses such as drought PARPs are activated causing NAD+ breakdown and ATP consumption. The resynthesis of NAD+ requires high amount of energy resulting into depletion of cell energy which lead to cell death. The aim of this study was to improve drought stress tolerance in maize by down-regulation of the PARP1 gene through maintaining energy homeostasis. Two approaches were applied for down-regulation of endogenous PARP1 gene, hairpin construct and Mu insertional mutagenesis.

A total of 14 maize lines transformed with either hairpin (hp) construct 555-1-555 or 373-1-373 were analysed for stability of the T-DNA by using ammonium multiwell assay and PCR reaction. The results show that 10 lines had intact T-DNA and in 3 lines the T-DNA was not intact.

Mu insertional mutagenesis was used as a complementary approach for hairpin construct. The screening and characterisation of positive lines from UFMu00029 derived (Mutator insertion in ZmPARP1) segregant were done to check the Mutator insertion in the PARP1 gene. Out of 5 lines analysed, 2 lines were heterozygous, 2 homozygous and 1 negative. Since, Mu tend to excise from where it was inserted, the screening for Mutator excision events in PARP1 was done on 4 homozygous lines and 1 heterozygous lines using seeds showing high transposition activity. The seeds were selected using a visual marker at the bronze locus: purple spots and completely purple indicates somatic and germinal excision respectively. None of the lines analysed had excision events in the PARP1 gene which could mean that the visual marker on the kernel does not tell anything about excision events at the PARP1 gene. A QPCR analysis was done to check the expression level of the endogenous PARP1 gene in the Mu-positive and the control line. There was no significant difference in expression of endogenous PARP1 between the 2 Mu-positive lines and the control line (Mu-negative). Furthermore, we were able to determine the optimal concentration of methyl viologen required for detecting the difference in oxidative stress tolerance in the B104 and W22 genotypes which can be used in future experiments to test PARP1 knock-out or knock-down lines. Oxidative stress tolerance was analysed in Mutator insertion and transgenic lines (amiRNA construct) in the PARP1 gene by using a Methyl viologen assay.

DNA methylation in maize leaf development

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ABSTRACT

One of the most fascinating open questions in biology is how organ and organism size is controlled. The maize leaf offers an ideal experimental system to study leaf growth due to the linear organization of the growth processes. At the base of the leaf, cells are dividing (division zone, DZ) and they pass transiently through the transition zone (TZ) from DZ to the elongation zone (EZ) and undergo molecular reprogramming, changing from dividing to elongating cells. It was shown that the position of the TZ, which is regulated by the plant hormone gibberellic acid, is a main determinant of the size of the DZ and in turn the final organ size [1].

Here we examined the role of differential DNA methylation on gene expression along the leaf growth zone, by use of methylation sensitive-amplified fragment length polymorphism (MSAP) technique, covering about 6,25% of the CCGG sites present in the genome. Generally, more sequences were found to be decreased than increased in methylation when comparing TZ to DZ. Also, this method identified relatively few differentially methylated transposons, corresponding to their usually stable methylation to silence them. In genic regions, differential methylation was found to be highest at the 5' and 3' edges compared to the central gene body, opposite to what is known for stable methylation patterns [2]. The regions upstream and downstream of the genes seem to follow this pattern, with a highest degree of differential methylation close to the ATG and transcription stop site.

The effect of the differential methylation on the expression profiles, validated by Q-PCR, showed a correlation between methylation and expression levels. Taken together, methylation is an additional mechanism regulating the transition from cell division to cell expansion, at least in part by affecting transcription levels of novel identified leaf growth regulatory genes.

QTL Mapping for Endoreduplication in *Arabidopsis thaliana*

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ABSTRACT

Endoreduplication is a nuclear polyploidization process that results in multiple, uniform copies of chromosomes; it is common in plants and animals. This process, amplifying genome without chromatin condensation, segregation or cytokinesis, results in what appears to be multiple, uniform copies of the nuclear DNA. Endoreduplication is an area of interest for this study because it has implications for crop production, increasing metabolic activity and the adaptation of plants to changing environments, this could come up with remedies for the global challenges of food and energy. The objective of this study is to search Quantitative trait genes (QTGs) which are responsible for the natural variation of endoreduplication. To achieve the objective of the study the trait value was quantified using flowcytometry; then the endoreduplication index (EI) value was plotted in a graph against their frequency to see pattern of distribution and variation of EI with in RIL mapping population. Once such variation was identified in the graph, the next step is to make heritability analysis to see whether the cause of variability is genetic or not. After that, polymorphic genomic regions (QTL) associated with the variation in EI was detected using phenotypic data, molecular markers and linkage map used as input data in Genstat v14. For the sack of efficient genetic dissection of complex traits, parental accession differs in trait of interest, and their segregating RIL progeny were used. Finally, three QTL for Bay x Sha mapping population and two QTL for Sorbo x Gy mapping population was detected. Then further expression analysis for the candidate gene was performed to identify differential expression. Based on the expression analysis CYCD3;3 and CCDKA;1 shows variation among the two parental lines Bay and Sha. The expression of CYCD3;3 decreases significantly ($P<0.001$) across the growth stage 1.02, 1.04, 1.06 and 1.08 in the same way variation. Studies on trichome cell indicates that, overexpression of CYCD3 favours for mitotic cell division, to the contrary mutant cycd3 increases endoreduplication (Schnittger, A. et al. 2002; Dewitte, W. et al. 2007). From this we can understand that compare to Bay allel of CYCD3;3, the Shahdara allele of CYCD3;3 could have relatively a negative impact on the onset of endoreduplication . In contrast with CYCD3; 3, the expression of CDKA;1 does not vary significantly during the development of the first leaf pair.

The selection of a neuraminidase inhibitor escape mutant of a low pathogenic avian influenza virus and phenotypical characterization

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ABSTRACT

Avian influenza is one of the most contagious and devastating poultry diseases. Many different influenza subtypes exist, categorized according to the nature of combination of two glycoproteins – hemagglutinin (H) and neuraminidase (N).

Avian influenza is a difficult disease to prevent and contain because it is able to disguise itself as a new agent, never seen before because it can evolve quickly and regularly produces new strains through antigenic drift, antigenic shift, insertion, deletion and recombination which are also known as evolutionary strategies of avian influenza.

Avian influenza viruses, like other RNA virus populations, are recognized as complex distributions of mutant genomes in a population structure known as quasispecies.

Since 1997, avian influenza virus has been detected in humans. Up to date HPAI H5N1 virus remains an avian virus and has not become a pandemic virus, but it is undergoing continual evolving new clades of viruses. Oseltamivir, most known neuraminidase inhibitor, was granted FDA Approval for the treatment and prophylaxis of influenza and stored in many countries as an important part of preparation against pandemic influenza infection.

This study was aimed to see what happens with a clonal virus when being put under strong selection pressure in vitro as opposed to what would happen in an animal or human treated and to study the evolution of the antiviral escape mutants selected.

The influenza virus A/Italy/mallard/401/2005 (H5N1) cloned by plaque assay was used in this study. Variants of the cloned A/Italy/mallard/401/2005 (H5N1) virus with reduced susceptibility to the neuramidase inhibitor Tamiflu were selected in vitro by passaging the virus in CEF cells in the presence of inhibitor. Three passages were performed and variant containing one amino acid substitution was early detected after the first passage. This substitution was not recorded before for the neuraminidase lacking activity established in the second and third passage without the appearance of new mutation. The variants after three passages showed significant decrease in neuraminidase activity however their susceptibility to oseltamivir by neuraminidase inhibition assay were not much different. This result indicates that there must be a compensating mechanism diminishing the virus dependence on neuraminidase activity. This mechanism highlights the important role of balanced HA – NA activities which were showed to determine the susceptibility of avian influenza A virus to neuraminidase inhibitors.

Functional characterization of candidate Black Sigatoka responsive banana promoters

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In-vitro propagation of spermatogonial stem cells

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ABSTRACT

The aim of this study was to evaluate the current method used to propagate human spermatogonial stem cell (SSC) in-vitro by characterizing the types of cells in culture after the first and third passages which corresponded to approximately six and sixteen weeks respectively. The types of cells in culture were analyzed by RT-PCR and immunofluorescence for the expression of germ cell and somatic cell markers. All the markers used to identify SSCs (GFRα1, SSEA1, OCT4, PLZF and C-KIT) were under-expressed compared to whole testis. No expression was detected for MAGEA4 by RT-PCR and immunufluorescence. Only C-KIT, a pre-meiotic germ cell marker was increased from passage one to three. The meiotic marker BOLL was undetectable while CREM and Acrosin-expressing germ cells decreased steadily. The Sertoli cell specific marker SOX9 was not detected by RT-PCR even though double immunufluorescence for inhibin α and StAR showed that Sertoli cells were present in culture. In addition, only vimentin was over-expressed in both passages. Immunofluorescence further revealed that αSMA positive cells constituted a great part of the feeder layer. The Sertoli and Leydig cells were both present in the culture but peritubular myoid cells were predominant. The in-vitro SSC culture described in this experiment did not allow SSC proliferation. Because C-KIT expression is increased after P1 and P3, the SSCs might be differentiating or de-differentiating. Further experiments are necessary to confirm this.

Optimization of flow cytometry and qPCR techniques to measure a direct effect of antiretroviral therapy on immunosenescence in HIV patients

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ABSTRACT

Recent studies have shown that effective treatment has greatly increased the life expectancy of HIV patients but those on long-term treatments present an aged immune phenotype when compared to those of the same age without the disease. This accelerated decline in immune competence is termed premature immunosenescence or accelerated immune aging. In these individuals, there is a quantitative restoration of their immune cells, but some immune dysfunctions persist decreasing the quality of immune recovery. Persistent chronic immune activation and continuous viral replication are two factors that have been shown to contribute substantially leading to immune exhaustion, T cell dysfunction and increase in comorbidities.

Studies on zidovudine (a nucleoside reverse transcriptase inhibitor), formally known as azidothymidine (AZT) shows that it reduces viral replication but does not stop it. These studies also show a mitochondrial-induced toxicity with the drug as it interferers with mitochondrial DNA polymerase action, impairing mitochondrial function and enhancing the risk of oxidative stress leading to accelerated telomere shortening. There is therefore, a need in drug combination therapies that will not only control the virus effectively, but improve the quality of the immune system.

T-cell Receptor excision circles and telomeres are known markers of immune aging. Several studies involving real time PCR and flow cytometry have been used to quantify both biomarkers. Some have stated the potentials of these biomarkers to measure cell aging as they progressively decline in peripheral blood with age.

The main aim of this study was to have tools that can be used to measure the direct effect of antiretroviral drugs on immune cell aging. The specific objectives were: to evaluate the different biomarkers, test the precision and accuracy of the different techniques, and see if they correlate with physiological age. The final specific objective was to compare both techniques and see if both biomarkers correlate with each other.

Peripheral blood mononuclear cells (PBMC) from “healthy” buffy coats of known ages and sex were used to test both techniques. We employed real-time PCR based on SYBR Green I and TaqMan for the relative quantification of TRECs and Dako telomere PNA kit/FITC for flow cytometry for telomere measurement. Both biomarkers were measured in CD4+ T cell population isolated by positive selection of magnetically labeled cells.

Our results showed no significant correlation between telomere length and age. We observed a correlation between TRECs and age. Though TRECs showed a significant correlation with age, it was not strong enough to distinguish between individuals of similar ages. A longitudinal study is recommended for further optimization of both techniques in order to state clearly the potentials of both markers to measure immune cell age.

Key words

T-cell receptor excision circles, human immunodeficiency virus, immunosenescence, flow-FISH, qPCR.

Identification of *Plasmodium falciparum* drug resistance markers to artemether lumefantrine and dihydroartemisinin piperaquine

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ABSTRACT

Background: The artemisinin combination therapies such as Artemether Lumefantrine (ALu) and dihydroartemisinin-piperaquine (DHA-PQ) are recommended for the treatment of uncomplicated *P.falciparum* malaria after the development of high failure rates to most existing monotherapies. Despite present high efficacy of ALu and DHA-PQ, their widespread deployment may select for drug resistant/tolerant parasites strains. Therefore, molecular surveillance of potential resistance markers associated with ACTs resistance is crucial for monitoring antimalarial drug efficacy and protect ACTs against the spread of resistant strains. This study aims to identify potential markers for ACTs resistance following treatment with ALu and DHA-PQ in the following three genes i) chloroquine resistance transporter gene (*Pfcrt*), ii) the multidrug resistance gene 1 (*Pfmdr1*), iii) *PfATPase*6 (putative marker for artemisinin resistance) gene.

Methods: Samples collected from a 42-day drug efficacy trial carried out in Burkina-Faso (2005- 2006) were retrospectively analyzed. DNA was extracted from bloodspots collected on filter paper. *Pfcrt* codon 76 and *Pfmdr1* codon 86 were genotyped using PCR–RFLP in 272 isolates at day 0 and day of recurrence (n=61) .*Pf*ATPase6 polymorphisms were analyzed in recrudescent isolates (n=20) and corresponding day 0 samples following either ALu or DHA-PQ treatment. PCR products were sequenced and then aligned with 3D7 control strain.

Results: The overall pre-treatment prevalence of the mutant *Pfcrt* 76T allele was 48.5% (n=130/268) as compared to 29.5% (n=79/268) of the wild type K76 allele and an additional 22.0% (59/286) isolates with mixed (K76+76T) genotype. Conversely, the pre-treatment prevalence of the wild type *Pfmdr1* N86 allele was higher (62.5% (n=170/272)) as the mutant *Pfmdr1* 86Y allele (18.1% (49/272)) and mixed alleles were found in 19.5% (53/272) of the day 0 samples. Comparison between pre- and post-treatment samples showed that the prevalence of the *Pfcrt* K76 allele increased from 30.3% to 79.2%, and the same trend was observed in new infections and recrudescent parasitaemia following treatment with either ALu or DHA-PQ. Similarly, the post-treatment prevalence of *Pfmdr1* N86 allele increased following ALu therapy (59.3% to 95.8%) in recurrent infections with similar trends in new infections and recrudescences. However, in the DHA-PQ arm the prevalence of *Pfmdr1* N86 remained stable after treatment (above 60%) in both new infections and recrudescences. We did not find a significant association between the *Pfcrt* gene polymorphism and treatment outcome following ALu (OR=0.90 [95% CI: 0.2-5.2], p= 0.91) or DHA-PQ treatment (OR=2.4 [95% CI: 0.5-11.3] p= 0.27), neither between the *Pfmdr1* mutations and treatment outcome (OR=0) and (OR=2.96 [95% CI: 0.92-9.57], p=0.07) respectively following ALu and DHA)). Further analysis of the pre- and post-treatment samples showed significant directional selection for *Pfcrt* K76 allele following ALu (p=0.001) and DHA-PQ (p=0.022), while there was no evidence for selection of *Pfmdr1* alleles in both arms. No *Pf*ATPase6 polymorphisms were detected.

Conclusion: We have shown high pre-treatment prevalence of *Pfcrt* 76T and *Pfmdr1* N86 alleles and a strong increase in post treatment prevalence of *Pfcrt* K76 following ALu and DHA-PQ treatment. *Pfmdr1* N86 allele increased following ALu treatment but remained stable after DHA-PQ treatment. The polymorphisms were not associated with treatment outcome but signify directional selection for *Pfcrt* K76 and *Pfmdr1* N86 post-treatment. *Pf*ATPase6 polymorphisms were not detected suggesting no *P. falciparum* resistance against artemisinins at the time of the study. Further studies are necessary to assess the role of PfATPase6 polymorphisms in artemisinin resistance.

The usage of bacteriophages in pharmaceutical delivery forms to develop new arms in the battle against multidrug-resistant bacteria

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ABSTRACT

Infections caused by antibiotic-resistant bacteria are of great concern to public health administrations worldwide. These infections are common among critically ill patients, who are already immunocompromised. However, the incidence of community-associated infections due to multidrug- resistant bacteria is on the rise. It seems the battle is turning in favour of these pathogens as efforts to combat them with new antibiotics seem futile. This highlights the fact that there is a real need for alternative therapeutic agents. In this line of thinking, bacteriophage therapy has received renewed attention in the West. Phages, bacterial specific viruses, are valid candidates for the treatment of antibiotic-resistant bacterial infections.

Though phage therapy has been used since 1919, phages have mostly been applied as suspensions. However, the development of other pharmaceutical dosage forms for phages, such as dry powder formulations, is crucial for the advancement of the application of phages as therapeutics. In cases such as infections of the respiratory system, bacteria can be secluded from the blood circulation by biofilms or mucous layers. Treatment using phage suspensions in such situations will be cumbersome, but the use of dry powder sprays can offer a better approach. Moreover, conversion to tablets, to tackle gastro-intestinal infections, is easier from the powder form.

This study focuses on bacteriophage large scale purification and spray drying of phage suspensions. The main purification tool used was fast protein liquid chromatography. The phages were bound on a monolith column and washed off with a Tris-HCl buffer solution. The process was optimized for the purification of lysates of *Staphylococcus aureus* phages Remus and Romulus via linear gradient and subsequent stepwise elutions. Phage powders were produced using *S. aureus* and *Pseudomonas aeruginosa* phages, Romulus and LUZ19 respectively. The phages were spray-dried with a sugar excipient (trehalose, lactose or dextran35). After determining the best excipient through titrations, the influence of the spray-drying parameters on the survival of the phages was researched. The infectivity of a LUZ19 powder was compared with the phage stock from which it was made using the killing curve method. Furthermore, the one day-, one week- and two month-4^{o}C storage stabilities of some LUZ19 powders made using different spray drying conditions were tested. In addition, the structural proteome of a newly isolated *Acinetobacter baumannii* phage, Aci4, was investigated by mass spectrometry.

Optimization of FPLC led to the conclusion that, 10% of 2M Tris-HCl buffer is enough to elute Remus and Romulus. In the spray drying experiments, trehalose was observed to be the best carrier, since its powders yielded the least phage loss. Moreover, among the four parameter-combinations used, the temperature of 85°C and nozzle airflow of 6l/min, gave the highest phage survival for both phages. The killing curve showed a diminished bactericidal activity of the LUZ19 powder. Additionally, the results of the storage stability tests indicated that most of the LUZ19 powders were stable after 2 months of storage at 4^{o}C. Furthermore, many of the Aci4 putative structural proteins were confirmed after the obtained ESI-MS/MS spectra was screened against a database containing all gene products of Aci4.

Identification of novel surface-expressed factors mediating virulence and biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Staphylococcus aureus is an important human opportunistic pathogen and is the leading causative agent of numerous hospital- and community-associated infections worldwide like; abscesses, septicemia, toxic shock syndrome, and endocarditis. However, *S. aureus* can also cause infections in other organisms, like bovine mastitis. In humans, its primary habitat is the moist squamous epithelium of the anterior nares. About 20% of the population are always colonized with *S. aureus*, 30% are intermittent carriers, and 50% never carry the organism. There is considerable evidence that carriage is an important risk factor for invasive infection (Foster, 2004; Peacock *et al*., 2001; Von Eiff *et al*., 2001).

The organism is capable of expressing a large number of virulence factors such as; cell wall proteins, membrane proteins and secreted proteins, which are all involved in the infection process. These virulence factors are modulated by many regulatory genes that belong to different pathways and networks but are interconnected. *S. aureus* like many other Gram-positive bacteria are known to secrete virulence factors into the extracellular milieu via the Sec translocon across the membrane by a mechanism which requires N-terminal signal peptides for recognition (Burts *et al*., 2008).

The emergence of multidrug resistant *S. aureus* strains like methicillin-resistant *S. aureus* (MRSA) which developed resistance to β-lactam antibiotics has challenged treatment of these infections. Therefore, genetic analysis of *S. aureus* has provided avenues for the discovery of new approaches to treat and manage *S. aureus* infections by understanding of virulence and pathogenesis of *S. aureus* at a molecular level, like identifying the bacterial virulence genes and then finally develop novel therapeutic strategies.

The aim of the study to which this thesis will contribute is to identify novel surface-expressed factors mediating virulence and biofilm formation in MRSA. Identifying factors involved in biofilm formation and virulence will aid our understanding of virulence mechanisms of recalcitrant MRSA and may further help in the development of more targeted therapeutic approaches for biofilm-associated infection due to MRSA.

In this study, *bursa aurealis*, a mariner-based transposon, was used for random mutagenesis and for the isolation of *S. aureus* mutants with defined insertion sites. Random mutations were generated in the *S. aureus* genome and then the transposon insertion position was determined by plasmid rescue methodology to identify the genes affected and their corresponding encoded proteins.

Application of nanobodies as probes in proximity ligation assay

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ABSTRACT

Proximity ligation asssays (PLA) are novel probe based techniques with high sensitivity at femtomolar level. Dual recognition of the target antigen by PLA probes (targeting different epitopes on the antigen) is a prerequisite for the formation of an amplifiable DNA template (readout). PLA was originally developed with classical antibodies. The large size of these antibodies limit their application in targeting very close epitopes in PLA and certain conserved epitopes are out of their reach. Difficulty to engineer this class of antibodies to specific functional probes cannot be left out.

Nanobodies are 10x smaller than the classical antibodies and their great affinity and specificity, ease of production and engineering makes them better candidates for PLA than classical antibodies. In this study, we aimed at exploiting these properties of Nanobodies to develop PLA on HER2.

To this end, we first selected two anti-HER2 Nanobodies (2Rb17c and 2Rs15d) from literature. Sequences of Nanobodies were reconfirmed by sequencing their plasmid constructs. We then expressed and purified the Nanobodies and then characterized them in terms of affinity and confirmed the absence of overlap between their epitopes. Tailored Nanobodies with a terminal cysteine residue were labeled with SMCC-oligonucleotides followed by two purification steps, IMAC and SEC. The labeled Nanobodies were then used to develop PLA on immobilized recombinant HER2-Fc protein. We demonstrated for the first time the labeling of Nanobodies with oligonucleotide. We also demonstrated for the first time that PLA can be done with Nanobodies. Our results open doors for development of novel HER2 based assay for improve selection of $HER2^{+}$ breast cancer patients.

Isolation of characterization of serum albumin specific nanobody : Generic tool for drugs serum half-life extension

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ABSTRACT

Nanobody (Nb) is a smallest antigen binding domain derived from the heavy chain only antibodies (HCAbs) which occurs naturally in Camelidae (alpaca, dromedary and llama). HCAbs were discovered by serendipity in these animals and subsequently derivation of Nbs about 20 years ago. Afterwards, Nbs have been widely applied in many biotechnological procedures to design/develop diagnostics and therapeutics. However, the applications of Nb especially in development of therapeutics have been hampered by its short serum half-life due to fast clearance from blood circulation via the renal system. Nevertheless, some strategies have been designed and developed such as Nanobody conjugation to counteract this obstacle.

The present study aims to design and develop another strategy to counteract the short serum half-life of Nb's application in therapeutics. The main aim of our study was to develop a generic tool for drugs serum half-life extension. This goal was achieved by isolation and characterization of SA-specific Nbs. Once these Nbs are injected into blood circulation binds the SA, thus, increase the mass of a complex and remained longer in blood circulation system.

Alpaca was immunized with both human and mouse serum albumin (HSA and MSA) antigens. A successful immunization of alpaca was confirmed by immune response test using both IgGs and total plasma protein harvested from the collected blood sample. A blood sample was collected and Nb library constructed from the peripheral blood B-lymphocytes. The library size of 6.8×10^8 independent transformants was constructed, with the quality of about 60% correct insert size. Bio-panning technique was employed by phage display to select and enrich the positive binders, and then followed by ELISA to select the positive clones which was finally confirmed by sequencing. A total of 18 clones belonging into 9 different groups was obtained.

The expression and production of Nbs was done in *E. coli* WK6 system, and then purified by IMAC and SEC using HPLC (AKTAxpress) machine ready for subsequent experiments. Out of these 18 clones, 6 clones were cross-reactive between HSA and MSA antigens but they do not recognize other mammals SA such as BSA, DSA and ASA. The T200 Biacore instrument was used for affinity measurement of Nbs and the best Nbs based on their affinity, solubility and stability were selected for blood clearance analysis. These Nbs have a good affinity to their cognate antigens and also showed high %IA/TBV compared to the control Nb (BcII10).

We have successfully generated SA-specific Nbs and these novel molecules (Nbs) therefore, can be applied for drugs serum half-life extension and delivery of therapeutic agents to specific targets such as tumors, inflamed and/or diseased tissues.

Differentiation of mouse embryonic stem cells (MESC) towards endocrine pancreatic cells

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ABSTRACT

The pancreas is a heterogeneous gland with both exocrine and endocrine compartments. The exocrine pancreas is composed of clusters of secretory cells called acini that produce the digestive enzymes and release them into the duodenum through the pancreatic ducts. The endocrine pancreas is made up of individual islets of Langerhans that sense blood glucose levels and release hormones into the bloodstream to maintain proper glucose homeostasis. Each islet is composed of different cell types: α (glucagon-secreting), β (insulin-secreting), δ (somatostatin secreting), PP (pancreatic polypeptide-producing), and ε (ghrelin-secreting) cells. The hormones from alpha and beta cells act in an opposing fashion to maintain blood glucose homeostasis. Glucagon mobilizes glucose from peripheral tissues and therefore elevates blood glucose levels to prevent severe hypoglycemia during fasting, whereas insulin stimulates glucose storage and lowers blood glucose levels in the postprandial state. Diabetes results when insulin production by the pancreatic β-cells is unable to meet the metabolic demand of peripheral tissues such as liver, fat, and muscle. Diabetes mellitus is a devastating disease that, according to the WHO (World Health Organization), is expected to affect the lives of 380 million people by the year 2025. T1DM (Type 1 diabetes) is a disease characterized by autoimmune destruction of pancreatic *β*-cells, whereas T2DM (Type 2 diabetes) is due to systemic insulin resistance and inadequate insulin production by *β*-cells. Islet transplantation has the potential to treat type I diabetes mellitus. However islet transplantation therapy is limited due to the shortage of organ donors. Owing to their pluripotent property, embryonic stem cells hold a great promise as a surrogate source of beta cells to treat diabetes once their differentiation is properly controlled. We studied the in vitro differentiation of mouse embryonic stem cells towards pancreatic lineages by modulating several signalling pathways that operate in pancreas development. We performed differential gene expression analysis at definitive endoderm and pancreatic progenitor stages using qRT-PCR and we were able to detect increased expression of markers such as *Foxa2*, *Sox17*,*Gsc*, *Hnf1β* and *Cxcr4* at the definitive endoderm stage, and *Pdx1,NKx6.1,Ptf1a-p48*and *Ngn3*at the pancreatic progenitor stage. We also performed immunofluorescence analysis and were able to confirm the expression of several markers mentioned above at their respective differentiation stage. We finally performed western blot analysis that confirmed the protein expression profile of Foxa2 at the definitive endoderm and of Pdx1 and Nkx6.1 at the pancreatic progenitor stages. Based on these results, we speculated that pancreas progenitors can be generated from mouse embryonic stem cells (mESCs) using our protocol. We finally performed transplantation of pancreatic progenitors into the mouse to determine the endocrine potentials of these grafts at different stages.

Key words; Diabetes, *β*-cells, differentiation, transplantation, qRT-PCR, immunofluoresence, western blot

Development of an easy-to-use assay for detection of a multitude of pathogens in cerebrospinal fluid and faecal samples

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ABSTRACT

In the last decennia, clinical diagnostics have benefitted greatly from the advances in biotechnology and molecular biology, such as antibody detection and the polymerase chain reaction (PCR). Quantitative PCR (qPCR) has been established as a technique which can detect a DNA sequence specific for a certain pathogen with high sensitivity and selectivity. In this study, we report on the development of several singleplex and multiplex qPCR assays to detect genomic material from 14 viruses and 1 eukaryotic parasite. Ten of the viral agents (Herpes simplex virus 1, Herpes simplex virus 2, Varicella zoster virus, Epstein-Barr virus, Cytomegalovirus, Human enterovirus, Measles virus, Mumps virus, Rubella virus and Rabies virus) and the parasite (*Toxoplasma gondii*) cause serious infections of the central nervous system. The 4 remaining viruses (Adenovirus, Astrovirus, Norovirus and Rotavirus) all cause gastroenteritis.

The assays for all herpesviruses, Human enterovirus, Measles virus, Mumps virus and *Toxoplasma gondii* were successfully developed and several successful singleplex assays were combined into multiplex assays. The lower detection limits were ±2 - 3 copies per sample (singleplex) to 2,4 – 31 copies per sample (multiplex) for the herpesviruses, ±6,8*104 copies per sample for Human enterovirus (singleplex), ±2,3*104 copies per sample (singleplex) for Measles virus and ±1,7*106 copies per sample (singleplex) for Mumps virus. The detection limit for the *T. gondii* assay could not be determined, and assays for Rubella virus and Rabies virus could not be developed properly due to lack of positive samples. Furthermore, the multiplex assay containing the primers and probes for detection of Herpes simplex virus 1, Herpes simplex virus 2, Varicella zoster virus and Epstein-Barr virus was successfully performed on an automated device developed by Philips and Biocartis NV with little loss of sensitivity. The success of this assay means that in a near future, a rapid and accurate test could be commercially available for personalised diagnosis of these viruses.

Structural and functional characterization of long polar fimbriae

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ABSTRACT

Adhesin/fimbriae is one of the important virulence factors in *Escherichia coli*. One of the most prevalent adherence factors are chaperone/usher fimbriae or pili. Type 1 and P pili represent well-studied examples, responsible for the attachment to high mannose glycoproteins and globoside-series glycosphyngolipid receptors in bladder and kidney epithelium, respectively. Our research interested to study the long polar fimbriae adhesin from adherent-invasive *E.coli* (AIEC) strain LF82. The recent research highlighted that this adhesin specifically binds on the payer's patch. This fimbriae/adhesin (LpfD) is no sequence homology with structurally known adhesin. The aim of this project is to unravel the molecular structure of A1 long polar fimbriae (LpfA1) adhesin (LpfD) and find out the host receptor. The adhesin (LpfD) expressed in the periplasmic space of C43 (DE3) strain and purified by column chromatography. The native proteins are crystallized and collected data from beamline 124 at the Diamond synchrotron with the resolution of 2.8 A°. Now preparing selenomethionine labelled protein for structure determination by a single anomalous dispersion experiment. The additional information from glycan array, which is useful to, co-crystallized with this adhesin. This information is important to design drug against this adhesin.

Development of novel muscle-specific adeno-associated viral expression constructs for gene therapy of Duchenne muscular dystrophy

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For more information, please contact the promoter.