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ANALYSIS OF GENE FUNCTION IN PSYCHROTROPHY ADAPTATION OF SERRATIA PLYMUTHICA RVH1

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ABSTRACT

Exposure to low temperature imposes stress that can be lethal to microbial life. However microorganisms have evolved mechanisms to survive and adapt to extremely low temperature through physiological and structural adjustment in their genome. Psychrotrophs or psych-tolerant bacteria are adapted to survive at low temperature (0^0C) but their optimal growth is achieved at temperature range between 20-30^0C. Psychrotrophs therefore pose a big challenge in food safety and stability especially when it is coupled with refrigeration as mean of food preservation. Understanding the underlying mechanisms of adaptation to psychrotrophy is thus important.

Previous study which resulted in four putative psychrotrophy mutants revealed that one mutant had transposon insertion in the open reading frame of *basys04238* gene with unknown function. This research therefore aimed to confirm the role of *basys04238* gene in psychrotrophy adaptation of *Serratia plymuthica* RVH1, and its relation to acid adaptation and osmotic tolerance by first confirming the defect in psychrotrophy adaptation of the mutant. Growth analysis of the mutant revealed retardation in growth at 4^0C and 10^0C in comparison to the wild type strain. This growth retardation was confirmed to be due to the mutation of *basys04238* gene as revealed by the complementation experiment. In addition, this study also showed that the *basys04238* play no role in acid adaptation and osmotic tolerance of *Serratia plymuthica* RVH1. However, combination of high salt concentration (more than 3%) and low temperature (4°C) had severe effect on the growth of the mutant.

THE IDENTIFICATION OF THE PROTEIN RESPONSIBLE OF FALSE POSITIVES IN *PLASMODIUM FALCIPARUM* CIRCUNSPOROZOITE ELISA

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ABSTRACT

Plasmodium falciparum malaria, a vector-borne disease caused by the bite of *Anopheles* mosquitoes is a public health problem worldwide. Vector control remains as an efficient method to block malaria transmission. Vector incrimination, being a crucial factor for efficient vector control, nowadays mainly relies on detection of the parasite's circumsporozoite protein in ELISA. However, false positivity in *P. falciparum* Circumsporozoite ELISA has been reported in Africa and Southeast Asia. The principal objective of this research is to determine the nature of the agent that provokes false positivity in Pf CSP ELISA through *in silico* analysis of Pf CSP, Next Generation Sequencing of bacterial 16s rDNA, detection of Microsporidia parasites in mosquitoes and isolation of the protein responsible for false positivity. **MATERIALS AND METHODS:** *In silico* analysis of Pf CSP was performed through BLAST search. Primers Bakt_341F/Bakt_805R attached to Illumina® adaptors were used to amplify bacterial 16s rDNA in 10 false positive samples from Vietnam and Cambodia, 1 laboratory reared *An. stephensi* and 2 true negative mosquitoes. Samples were identified into 16s rDNA database from GenBank with the Galaxy workflow modified for metagenomic studies. Cross-reaction of Microsporidia in Pf CSP ELISA was evaluated with *Aedes aegypti* infected with *Vavraia culicis* and *Edhazardia aedis.* Microsporidia were detected by specific amplification of 18s rDNA. Protein capture from 6 false positive samples was carried out using Dynabeads® M270 Epoxy antibody coupling kit and capture monoclonal antibodies 2A10. Isolated protein was separated through SDS-PAGE and identified by MALDI TOF/TOF analysis. **RESULTS:** *In silico* analysis of Pf CSP demonstrates some degree of identity with the domain Bacteria and a disordered NANPL region present in the Microsporidia *E. aedis*. Amplification and sequencing of 16s rDNA of false positive samples did not indicate a single species of bacteria present in the majority of false positive samples analyzed and absent in negative controls. Microsporidia did not show cross reactivity in Pf CSP ELISA and only one sample was found positive in head/thorax and abdomen for detection of Microsporidia by PCR. MALDI TOF/TOF analysis showed that actin cross-reacted with capture monoclonal antibodies in Pf CSP ELISA. **DISCUSSION**: *In silico* and laboratory analysis can not confirm Microsporidia, nor one single bacterial species as the cross-reactive protein. The remarkable diversity of species supports the hypothesis that more than one bacterial species could cross react with monoclonal antibodies in Pf CSP ELISA. The finding that the actin protein can be recognized by anti-Pf CSP monoclonal antibodies supports the animal origin theory of the causative agent of false positivity. Identification of this agent remains unclear, nevertheless important insights are provided towards a feasible explanation of false positivity in Pf CSP ELISA.

NANOBODIES AS THERAPEUTIC TOOLS AGAINST SHIGA TOXIN

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is a pathogenic strain of *E. coli* that causes gastrointestinal diseases both in animals and humans. STEC associated diseases manifest as bloody diarrhea with hemorrhagic colitis (HC). However, in some advanced stages, they can lead to life-threatening complications such as the hemolytic-uremic syndrome (HUS) and neural disorder. The main virulence factor underlying the HC and HUS is Shiga toxin (Stx), an AB_5 toxin with two variants of Stx: Stx1 and Stx2. The A subunit has the RNA *N*-glycosidase activity that cleaves a specific *N*-glycosidic bond in the 28S rRNA, and therefore stop translation of protein (Johannes and Römer, 2010) whereas the B subunit is the binding part of Stx and therefore for interacts with membrane receptors Gb3/Gb4 of the target sensitive cells. Despite increasing effort for preventing the EHEC infections and it complications, the treatment of infection is still limited to supportive care. However, several strategies have been elaborate to fight against STEC and completely block the cytopathic effects of the toxins via production of vaccine. Hence, the goal of this research was to generate nanobodies against the Stx via immunization of dromedary with the StxB subunits or Stx toxoids. For that purpose, we first amplified the stxB from the total DNA of clinical *E. coli* isolates, and then, we attempted to clone and express *stxB* in successively in pDEST14 and pGV5403 plasmids vectors. Unfortunally, we did not see expression of the StxB in *E. coli* DH5α. We planned another alternative strategy based on the Stx toxoids for immunizing lama in order to generate specific anti Stx-nanobody. Hence, total genomic DNA from the above mentioned *E. coli* strains was used as template to amplify the wild type *stx* operons. The Stx toxoids were produced by introducing site-specific mutations in the *stxA* gene of the *stx* operons (Y77S for Stx2A and E167Q/G170R for Stx1A) using two consecutives overlap extension PCRs. The mutant *stx* were cloned in pGV540 vector, under the control of P_{lac} promoter. The SDS-PAGE and Western blot analysis showed no expression in *E. coli* DH5α. The Stx toxoids and Stx wild type were cloned and expressed in pDEST14 plasmid, under the control of strong P_{T7} promoter, and Stx2 toxoid was purified from *E. Coli* C43 (DE3) host by anions exchange column chromatography. The Western blot analysis of the extracts showed a low level of Stx toxoid production. Since we observed low production of the Stx proteins from the *stx* operons controlled by the P_{lac} or the P_{T7} promoters, we decided to clone and express the Stx toxin and the Stx toxoids behind a less strong and tightly regulated promoter such as the arabinose-inducible promoter (P_{BAD}). Hence, after only 3 h induction with 0.2% arabinose, we

observed a better expression level than from the P_{lac} or the P_{T7} promoters. The *in vitro* cytotoxicity of Stx1/Stx2 toxoids and toxins (in pDEST14), Stx2e purified toxoids and Stx2e toxin was tested on HeLa cells. The pure Stx2e toxin showed cytotoxic effects on HeLa cells till 1/10^5 dilution. The purified Stx2e toxoid, the Stx toxin or the Stx toxoid (in pDEST14) extracts did not show any cytotoxic effects, which is consistent with previous result obtained when the StxB subunit or the Stx toxoids were cloned and express in pDEST14.
In conclusion, we have amplified and cloned the *stxB* genes in different plasmid vectors but
we could not express and purify it for generating nanobodies against wild type Stx1 or Stx2.We also constructed and expressed the Stx1 and Stx2 toxoids (s*txA* mutants) in plasmid vectors, albeit at a low to very low level of expression. A lot of obstacles that are still unclearoccurred during several attempts to express the cloned genes (*stxB*, *stx* wild type and *stx* mutant operons) in plasmid vectors harboring different promoters.

EXPLORING THE POTENTIAL OF THE SPLICE LEADER SEQUENCE (SL-RNA) FOR ACCURATE MOLECULAR DIAGNOSIS OF HUMAN AFRICAN TRYPANOSOMIASIS

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ABSTRACT

We have developed the baseline of a reverse transcriptase real-time PCR assay for detecting the SL RNA of *Trypanosoma brucei*. SL RNA specific cDNA was generated using a specific primer (cSL) targeting a highly conserved region in the SL RNA sequence. The real-time PCR was conducted with cSL primer and a forward primer (SL-F) targeting the *Trypanozoon* specific region in the SL RNA sequence. SYBR Green I was used as a fluorescent reporter during real-time PCR. Using synthetic SL RNA molecules as a target the assay show a lower detection limit of 4 fM or 2408 SL RNA molecules. When we applied the assay on human blood spiked with know numbers of *T. b. gambiense* LiTat1.3 parasites, the detection limit was 10 trypanosomes per 100 L blood. Analytical specificity experiments showed that the assay only detects the *Trypanozoon* parasites and remains negative with other *Trypanosoma* or *Leishmania* parasites. We also quantified the number of SL RNA molecules in one single *T. brucei* cell, which is about 9000 molecules. In this study we showed for the first time thediagnostic potential of the SL RNA for accurate detection of *T. brucei* parasites. We delivered the proof-of-principle and assessed the analytical sensitivity and specificity in experimentally prepared blood specimens.

Key words: human African trypanosomiasis, spliced leader RNA, reverse transcription,
real-time PCR, *Trypanozoon*

IDENTIFICATION OF GENES RESPONSIBLE FOR CONVEYING 2-DEOXY (D) GLUCOSE (2DOG) RESISTANCE TO A MUTANT STRAIN OF A BREWER'S YEAST

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ABSTRACT

In the past decades, Saccharomyces cerevesiae has been a useful eukaryotic model organism in fundamental research because it is easy to work with (ease by which experimental crosses can be performed) and has a genome that has been fully sequenced. In recent years Saccharomyces cerevisiae has become one of the most favored production organisms in industrial biotechnology. With the discovery of some strains that bear industrially relevant phenotypic traits, there has been great interest within the brewery and other industries and amongst yeast scientists to identify the underlying genes responsible for these phenotypic traits. Identification of such genes will lead to engineering of improved yeast strains.

This project involves the identification of genes responsible for conveying 2-deoxy (D) glucose (2DOG) resistance to a mutant strain of a brewer's yeast. We have reasons to believe that this property is linked to an increased fermentative capacity observed in this mutant strain. The mutant strain grows in the presence of 2-deoxy (D) glucose while the wild type strain does not grow in the presence of 2-deoxy (D) glucose. This is explained by the glucose repression mechanism which is a process whereby in the presence of glucose, the gene expression of a variety of enzymes involved in using other carbon sources is shut down (2-deoxy (D) glucose has this same glucose repression effect as glucose).

Most phenotypic traits of industrial interest are heritable, just like diseases in humans, are caused by multiple genetic loci known as quantitative trait loci (QTL). Mapping of these QTLs is an approach which is fairly straightforward in identifying their genetic basis. This method aims at the allocation of the genetic determinants to regions in the genome.

We have identified and isolated segregants with the 2DOG resistance property; we have then crossed these segregants with laboratory strains which do not have this property and their progeny selected for the presence of the 2DOG resistance. Up to now, we have selected 60 segregants having the 2DOGR property and 20 segregants which do not have the 2DOGR property. We shall apply pooled-segregant whole genome sequence analysis to map all the quantitative trait loci (QTL) determining this 2DOG resistance.

GENERATION OF NANOBODIES FOR THE DEVELOPMENT OF SCHISTOSOMA DIAGNOSTIC TOOL

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ABSTRACT

Schistomiasis is a worldwide disease affecting mostly rural populations. Even though the official known annual death rates are not as high as those of malaria or HIV, the disease causes severe debilitations seriously compromising the quality of life of millions of people who are affected by the disease. This has negative economic repercussions on the community. The schistosomes of medical importance include *Schistosoma japonicum, S. mansoni* and *S. haematobium*.

Limitations associated with the current diagnostic test for schitosomiasis such as low sensitivity, high overhead cost and impracticability in remote setting among others calls for development of alternative test. This study investigated the use of Nanobody for developing a tool for diagnosis for schistosomiasis suited for use in remote areas. It aimed at showing a proof of concept for possibility of developing a simple lateral flow kit for schistosomiasis that detects presence of soluble egg antigen (SEA) released in the host's blood during infection, using *S. japonicum* as a model species. Conventional antibodies have been used to develop dipsticks, but they usually test for anti-schistosome antibodies and they also tend to cross-react with other helminthes infections. So, nanobodies are being explored because of their specificity that is attributed to their long CDR 3 regions. This allows unique binding to deep-seated epitopes within an antigen, with very high affinity. SEA is being targeted because it is probably specific to schistosomes hence circumventing cross-reactivity with other human infective helminthes.

For this study, six nanobodies with affinity for SEA were generated starting from mRNA of SEA immunized alpaca. The six nanobodies bearing a C-terminal histidine tag were shown to bind SEA coated on maxisorp 96 well ELISA plate (Nunc™) ELISA. In this binding assay, Nb1 and Nb19 showed better binding to SEA than Nb2, 7, 8 and 17. The successfully produced, biotinylated versions of Nb1, 7 and 8 did not show significant binding to SEA coated on ELISA plate. With this, the capability of a Nb to be used as part of a diagnostic test for schistosomiasis based on SEA was demonstrated. However, since this test was performed on egg lysate coated on ELISA plate, the result obtained is only the beginning. Indeed, the desired test format for a diagnostic test is a sandwich system for detection of parasite antigen floating in human blood, preferentially performed on a lateral flow format, rather than an ELISA format. Therefore, Future selection of a good binding partner for either Nb1 or Nb19 will have to be performed as a continuation of the work presented here.

GENETIC PARAMETERS OF PSOROPTIC MANGE IN BELGIAN BLUE CATTLE

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ABSTRACT

Increasing susceptibility of Belgian Blue Breed to Psoroptic mange is a big problem confronting the meaty industry in Belgium. Psoroptic mange is the skin disease caused by Psoroptes ovis mites. The disease causes important economic losses to the meat industry and suffering of the sick animals. Environmental factors and the animal genotypes were important for the increasing susceptibility of Belgian Blue Breed to Psoroptic mange. The extents of Psoroptic mange problems in dual purpose Belgian Blue farms were examined by questionnaire survey and farm visits.

Questionnaires were sent to 62 farms to assess the status of Psoroptic mange and the general husbandry practice (environmental factors) in the farms. 2 farms were visited where blood sampling, skin scrapings and filling in the individual animal forms were done during the visits. Presence of Psoroptes ovis was examined from skin scrapings and animal genotyping was done from blood samples. Animal genotypes were analysed using Microsoft Excel software. Fisher's exact tests were used to analyse the importance of environmental factors in relation to Psoroptic mange in dual purpose Belgian Blue farms.

Out of the 62 sent questionnaires, 23 questionnaires were completely filled (37%). 11 farms (18%) were contacted by phone and answered a short questionnaire. Information of a total of 34 out of 62 farms was obtained (55%). The herd prevalence of Psoroptic mange in the dual purpose Belgian Blue farms was 41%.

Analysis of data showed that homozygous muscle hypertrophy (mh/mh) genotype was found in majority of cattle (71%) followed by heterozygous muscle hypertrophy (mh/wt) genotype (20%) and lastly homozygous wild type (wt/wt) genotype (9%). Animals with mh/mh genotype were found to be more susceptible to Psoroptic mange compared to animals with mh/wt and wt/wt genotypes. The environmental factors were found to have no influence to increased Psoroptic mange susceptibility in dual purpose Belgian Blue farms (P-values > 0.05) at significance level of 5%.

EXPRESSION ANALYSIS OF *mrr* IN *Escherichia coli* K-12

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ABSTRACT

The *mrr* gene of *Escherichia coli K-12* is a laterally acquired gene which encodes a cryptic type IV restriction endonuclease called Mrr, with specificity for methylated DNA, whose *in vivo* endonuclease activity could be triggered by heterologous expression of certain known methyltrasferases or high pressure stress, leading to double stranded break in the host chromosome and activation of the SOS response. However, several aspect of the Mrr protein, such as its biological role in a cell, mechanism of action or *in vitro* enzymatic activity, has not been elucidated well. Moreover, the transcriptional and translational regulations of *mrr* remain to be studied. In this study, an attempt was made to analyze the expression of *mrr* and to explore any trans acting gene that specifies a regulatory protein for its transcription, using reporter gene assay and transposon mediated random knockout approaches. A single copy of transcriptional promoter fusion, *mrr* promoter to *lacZ* was constructed at its native location in the genome of *E.coli* K12, using the lambda Red recombinase system. Both *in vivo* and *in vitro* β-galactosidase activity assays indicated synthesis of functional protein from *mrr* promoter in the absence of foreign methyltrasferase or high pressure stress. Quantitative *lacZ* activity has been measured to evaluate the strength of *mrr* promoter in kinetic way and the result revealed an expression in a growth rate-dependent manner, the peak being in late exponential phase. At present, genetic screening of candidate random knockout strain from large number of colonies in the library and subsequent analysis of the insertion did not result in a genuine mutation associated with regulation of *mrr* gene. This study contributed to the general design of the experiment and provided information on how some false positive results may be managed in construction of strain and the screening of mutants after random transposon knockout. Further screening and other methods such as methods of measuring mRNA level should be used to correlate the mRNA transcript in combination of the method discussed here to validate the present study.

Key words: *gene expression, lacZ, random knockout, regulatory proteins, transcriptional*
fusion

REPLICATION CHARACTERISTICS OF ASIAN AND EUROPEAN HIGHLY VIRULENT PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (HIGH FEVER PRRSV) IN PORCINE MONOCYTIC CELLS AND SEARCH FOR A NEW RECEPTOR

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(Not submitted)

GENOTYPING OF *Mycobacterium tuberculosis* COMPLEX ISOLATES FROM NEW AND RETREATMENT CASES OF SELECTED WEST AFRICAN COUNTRIES

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ABSTRACT

A cross-sectional study was conducted at the Institute of Tropical Medicine from October, 2012 to July, 2013 on six hundred five *Mycobacterium tuberculosis* complex isolates that originated from West African countries to assess the genotypic distribution and population structure of the strains and also to analyze the association of drug susceptibility profiles between *M. tuberculosis* and *M. africanum* lineages. *Mycobacterium tuberculosis* complex isolates were genotyped using spoligotype analysis and were compared with the international spoligotype databases. A total of 170 different spoligo-patterns were identified among the study samples. Sixty of these spoligopatterns had not been previously identified in these databases (designated ‘orphan’), while the remaining 110 different spoligo-patterns had been previously reported (shared types). In this study, 501 (82.8%) of the *M. tuberculosis* complex isolates were genotypically clustered (66 clusters containing 2 to 137 isolates per cluster). We identified the six major *M. tuberculosis* complex lineages of human relevance in the region, with predominance of lineage 4 (Euro-American), accounting for 508 (84%) of the total study isolates and 128 of the total spoligotypepatterns. Within lineage 4, the LAM10_CAM clade was predominant (26.3% of the study isolates). Most other isolates belonged to the Haarlem-; *M. africanum* West Africa 1 and 2-; Beijing-; Indo-Oceanic-; and ill-defined T-clades. Analysis of drug susceptibility test profiles between *M. tuberculosis* (lineages 1-4) and *M. africanum* West African1&2 (lineages 5 and 6) clades showed no statistically significant difference (61.8% vs. 76.5%; OR =0.50; 95% CI, 0.22- 1.12; p=0.086) for first-line anti-tuberculosis drugs. Given the phylogenetic relatedness of lineages 1, 5 and 6, also classified as ‘ancestral’, in contrast to ‘modern’ lineages 2, 3 and 4, we added a secondary analysis on differences in drug resistance between ancestral and modern *M.*

tuberculosis. We identified significant lower levels of drug resistance, both in new- (29.7% of 'modern' isolates showed any resistance vs. 10.9% in 'ancestral' isolates; OR= 0.29; 95% CI, 0.11-0.75; p=0.007) and re-treatment cases (66.2% vs. 41.2%; OR= 0.36; 95% CI, 0.13-1.00; p= 0.042).The genotypic diversity of the *M. tuberculosis* complex population structure identified in this study was high and all lineages were identified in the selected West African countries, whereas *M. africanum* West Africa 1 and 2 clades, were not identified among isolates from the Central African Republic. Moreover, the prevalence of *M. africanum* (overall 5.6%) was lower than expected from genotype results from other countries. Future research should be encouraged on investigations of genotype diversity to arrive at a high resolution mapping of the *M. tuberculosis* population structure in the region, as well as further exploration of association between lineages and anti-tuberculosis drugs susceptibility in West African countries and the underlying biology of such differences.
Key Words: Clades; First-line anti-tuberculosis drugs; Genotypes; *Mycobacterium tuberculosis* complex; Phylogeography; Population structure; Spoligotyping, West African countries.

EVOLUTION OF BETA-LACTAMASE IN CERTAIN SALMONELLA SEROTYPES

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ABSTRACT

Salmonella Typhimurium is one of the most important causes of gastrointestinal disease humans. Nowadays, there are an increasing number of reports on resistance to cephalosporins which is jeopardising the effectiveness of the antimicrobial treatments against this pathogen. The aim of this study is to decipher the current spread of ESBLs enzymes in *S*. Typhimurium from different sources (poultry, pigs) in Belgium.

A total of 59 cephalosporin resistant *S*. Typhimurium isolates were collected from 2011 to 2012 in Belgium. They were characterized by susceptibility testing and macro-restriction analysis followed by pulse field gel electrophoresis (PFGE). β-lactam resistance genes were characterised by isoelectric focusing (IEF) and PCR analysis followed by sequencing.

Most *S*. Typhimurium isolates were multi-resistant. None of these isolates were positive for OXA, CMY, SHV, ACC-1, PER-1, DHA genes. Sequencing analysis showed the following ESBLs: TEM-52 (n=14), CTX-M-1 (n=7), CTX-M unidentified (n=9), and NSBLs namely TEM-1 (n=6) and TEM-206 (n=15). Some strains having an MIC below the *S*. Typhimurium breakpoint were positive for an ESBL encoding gene and in some strains with high cephalosporin MICs, no gene could be detected.

There were three main PFGE clusters compromising a total of 25 patterns. In conclusion, there are still some problems with the breakpoint for cephalosporins and *Salmonella* Typhimurium: some strains classified as susceptible contained a resistance gene. In some strains, no gene associated with cephalosporin resistance could be found. Especially TEM-52 and CTX-M-1 are widely distributed in different *Salmonella* Typhimurium clones.

STUDYING THE GENETIC DIVERSITY OF ACETIC ACID TOLERANCE IN *Saccharomyces cerevisiae* USING WHOLE-GENOME SEQUENCING

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ABSTRACT

The polygenic analysis of acetic acid tolerance remains an important challenge in industrial bio-ethanol production. Acetic acid is one of the major inhibitors for lignocellulosic fermentation and it is more feasible to use acetic acid tolerant yeast strains for fermentation instead of lowering acetic acid concentrations in hydrolysates, since removing acetic acid is a costly and cumbersome process. Currently, the industrial yeast strain Ethanol Red is commercially used for bioethanol production. However, this strain is not suitable for second generation bioethanol production because of its high sensitivity to acetic acid. The main aim of this study was to screen for yeast strains that carry unidentified genes that confer acetic acid tolerance, in order to develop an acetic acid tolerant Ethanol Red strain. To this end, a quick and efficient method was developed to quickly screen for the presence of novel acetic acid tolerance alleles. In this method, three selected genes, *XXX1, XXX2* and *XXX3*, were marked with the antibiotic resistance genes NatMX, HphMX and KanMX in the inferior parent ER18, a segregant from Ethanol Red. The marked ER18 was subsequently crossed with newly identified acetic acid tolerant yeast strains. After phenotypic selection, tolerant segregants were tested for the presence or absence of these marker genes by checking their growth on YPD plates with antibiotics geneticin (KanMX), nourseothricin (NatMX) and hygromycin (HphMX). From this screening, it could be concluded that the newly identified acetic acid tolerant strains rely on other genes for acetic acid tolerance than the genes identified previously. The novel QTLs can in the future be mapped by pooled-segregant whole-genome sequencing analysis, followed by reciprocal hemizygosity analysis to further confirm the causative genes. These causative genes from different acetic acid tolerant yeast strains can subsequently be introduced and combined in Ethanol Red to improve the performance for second generation bioethanol production.

LEAVES GROWTH: COMBINING PHENOTYPICAL AND TRANSCRIPTOMIC DATA

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ABSTRACT

Worldwide, the population is dramatically increasing and as a result, the demand for food will also increase proportionally. Therefore, the production of crops should increase to feed the huge population. Maize, the world's third most important cereal grain for mankind after wheat and rice, could greatly contribute in feeding the population.

In this study, the maize leaf was used as a model to study organ size control taking the advantage of its big size and the linear organization of cell division and expansion in its growth zone. In order to understand how growth is coordinated at the different cell layers in the leaf, we examined the transcriptomics and growth characteristics of the upper epidermis, mesophyll, and lower epidermis cells.

The kinematic analysis revealed that drought resulted in a high percentage reduction (26%) in GA20ox plants indicating their poor response to drought. We confirmed the growth enhancing characteristics of GA20ox. We found non-significant difference between the WT and GA20ox plants under both well watered and drought conditions for LER and cell length. Therefore, based on our results, it is difficult to phenotype between the GA20ox and WT plants.

Transmission electron microscopy was used to study the developmental progress of the cellular and organelle components along the growth zone of the maize leaf. The vacuolization occurred earlier in the epidermis, denoting that epidermal cells differentiated earlier than mesophyll. In the epidermis, plastid number was high at the leaf base but gradually decreased throughout the division zone, while in the mesophyll it showed an increase until 0.5cm and then gradually decreased till stabilization. Both differentiation and plastid development show that two independent developmental programs drive mesophyll and epidermis growth.

To study the underlying molecular processes, laser capture microdissection (LCM) was applied to specifically harvest the three different cell types for transcriptome analysis. RNA sequencing showed that 13347 (51%) genes were expressed in all the three cell layers and identified 375, 5577 and 522 genes as specific to the upper epidermis, mesophyll and lower epidermis cells, respectively. The mesophyll specific genes contained an enrichement of photosynthetic genes, providing proof of concept of the cell type specific sampling by LCM. By mRNA in situ hybridization, marker

genes specific for the mesophyll and the epidermis were confirmed. GO analysis revealed that protein kinase activity is involved in lower epidermis and genes encode the regulation of cellular transcription are expressed in mesophyll. The GRAS family of transcription factors showed a remarkable expression profile in the three cell types revealing that they are responsible for some differences between the developments of the cell types.

To conclude, the combination of LCM with transcript profiling using RNA-seq is a powerful tool in the identification of cell type specific transcripts. The transcriptome data found in this study is useful resource for future gene discovery that controls leaf size in maize.

INDUCIBILITY OF BANANA PROMOTERS BY STRESS SIGNALLING COMPOUNDS AND *M. fijiensis*

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ABSTRACT

Although Black Leaf Streak Disease (BLSD) caused by *Mycosphaerella fijiensis* can be controlled effectively by fungicide application, the continuous employment of fungicides poses serious drawbacks. The cultivation of BLSD resistant varieties developed by genetic engineering is a better alternative. As part of such strategy to obtain banana plants with durable resistance against *M. fijiensis Musa*-derived promoters that are BLSD-responsive need to be investigated.

In this study, the baseline activity and inducibility of four BLSD-responsive and *Musa*-derived candidate promoters (P_{BC2094}, P_{BC349}, P_{BC784} and P_{BC307}) were assessed by different stress signaling compounds (ABA, Juglone and MeJA) under *in vitro* conditions and after *M. fijiensis* infection. Candidate promoters were evaluated qualitatively by histochemical staining and quantitatively by fluorometric GUS assay. Moreover, the presence of the candidate promoters in the transformed lines was confirmed by PCR amplification.

Results revealed that the selected transformed lines contain their respective candidate promoters. The candidate promoters except P_{BC307} responded to ABA, MeJA and Juglone, however upregulation of their activity was only observed after MeJA and Juglone treatment (only for P_{BC784}) as indicated by their average GUS activity. Changes were not statistically different and one of the possible causes might be the small number of lines evaluated. In addition, qualitative and quantitative analyses of their baseline activity revealed that candidate promoters P_{BC2094} and P_{BC349} exhibited

weak to high GUS staining intensity and moderate GUS activity (100-1000 pmol MU/h/μg protein). On the other hand, candidate promoters P_{BC784} and P_{BC307} exhibited weak to moderately low GUS staining intensity and weak GUS activity (<50 pmol MU/h/μg protein). Also, the activity of candidate promoters P_{BC2094}, P_{BC784} and P_{BC307} were expressed and localized in the vascular bundles of the leaf while activity of P_{BC349} were expressed both in the leaf and root tissues, but more localized in the root tip. Lastly, candidate promoter P_{BC2094} did not exhibit induced activity by *M. fijiensis* after 13 days of infection.
This study recommends further investigation of the functionality of the candidate promoters under stressed and non-stressed conditions with larger number of normal plants and not with off-types as was the case (in vitro and in vivo).

SALMONELLA AND BIOFILMS

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ABSTRACT
According to National Institute of Health, more than 80% of bacterial infections in human are due to biofilms (Schachter 2003). Biofilm is a mode of bacterial growth which is described as sessile and organised communities of micro-organism formed on living and non-living solid surfaces (J. W. Costerton 1999). A major aspect of biofilms is their adherent nature; moreover they are resistant to antibiotics (Nickel et al. 1985; Sutherland 2001). *Salmonella* biofilms are not exception in this case. They have been discovered to colonize and adhere to various surfaces (Steenackers et al. 2012), and they are known to cause major havoc in the food industry (Vestby et al. 2009). Apart from antibiotics, *Salmonella* biofilms are also resistant to disinfectants (biofilm inhibitors) which are meant to combat them (Brooks and Flint 2008).
In order to study the mode of action of these inhibitors, an evolutionary experiment was carried out for *Salmonella* Typhimurium ATCC14028 (WT) in the presence of AI-243 biofilm inhibitor as a top to bottom approach. After 54 cycles, an evolved strain (BWSM) which was able to form biofilms in the presence of the inhibitor emerged. Via whole genome sequencing of BWSM, causal mutations were identified in six genes. The aim of this present study was functional characterization of genes (STM14_0676, *rlpB*, *dinI*, *ycfU* and *rpoA*) in imparting resistance and in biofilm formation in the evolved strain. Over-expression mutants of the five genes were constructed in *Salmonella* Typhimurium ATCC14028 (WT) and were subsequently subjected to various assays such as; bioscreen, biofilm and phenotypic assay.
Results obtained from bioscreen assay indicate that OV of *rpoA* and STM14_0676 leads to high planktonic growth as compared to WT and other mutants in a poor nutrient media, which is known to promote biofilm growth. Moreover, results obtained from biofilm assay shows that OV of *rpoA* leads to the lowest biofilm growth under normal conditions while ΔSTM14_0676 results to the highest biofilm growth as compared to WT and other mutants in the presence of 75μM of AI-243.

Interestingly, OV of *rpoA* led to the lowest biofilm growth in the controls, thus same results were expected in the treated set-up. On the contrary, OV of *rpoA* led to moderate biofilm growth equivalent to WT and higher compared to other mutants (except ΔSTM14_0676) in the treated set-up.
STM14_0676 and *rpoA* therefore, play a role in imparting resistance against the biofilm inhibitor and in biofilm formation in BWSM. Their role in biofilm formation is through curli biosynthesis while resistance is achieved through co-ordination of porins and efflux pumps, indicating that these genes employ the same pathways to accomplish these roles in BWSM.

NANOBODIES AS A DIAGNOSTIC TOOL IN ANIMAL TRYPANOSOMIASIS CAUSED BY *Trypanosoma vivax*

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ABSTRACT

The animal trypanosomiasis is a tropical disease that affects mainly livestock and wild animals in Africa and South America. The two major causal agents involved in animal trypanosomiasis are *Trypanosoma vivax* and *Trypanosoma congolense*. The lack of an efficient diagnostic test applicable in field hinders the effective control of the disease. The search for potential parasite antigens that can be targeted, together with the promising nanobody (VHH) technology might help. The aim of this work was to evaluate 1) weather or not the trans-sialidases are a good target for diagnostic of the disease, 2) if the VHHs are a good tool for diagnostic of animal trypanosomiasis and 3) the best immunization/ panning approach to find antigen-specific binders. As a **first approach**, 2 alpacas were immunized with pure recombinant *T. vivax* and *T.congolense* trans-sialidases (rTSs). 2 VHHs libraries were constructed and screened using the rTSs through phage display technology. The specific binders against the *T. vivax* and *T.congolense* rTSs were selected and tested in ELISA. Although all the binders were able to recognize the rTSs in ELISA, it wasn't possible to detect the enzyme in plasma of infected mice, maybe due its low abundance. However using a GFP fused □-rTcoTS nanobody, it seems that the protein is being expressed in the parasite even though this is not conclusive, further optimization and evaluation of the tests used are required to conclude whether or not TS is a suitable target for the diagnosis of the disease. In a **second approach** a new library was constructed from an alpaca immunized with *T. vivax* lysate to generate a diverse pool of VHHs against multiple trypanosome proteins. Although binders were selected after panning against the lysate, in ELISA, most of them were as well recognizing mice proteins, indicating that lysate was contaminated. This approach stresses the impairments to select parasite-specific binders from the lysate.

Nevertheless, this library remains as a rich source of binders against multiple parasite proteins. To take advantage of this rich library, in the **last approach** the library was screened through phage display using the *T. vivax* rTS, as a "proof of principle". From this library we were able to select anti- rTS specific binders. This strategy could be used to retrieve several binders against other antigens from one single lysate library, without requirement of new immunizations. This would have considerable implications both in ethical and financial aspects of the immunization procedures.

BINDING AND FUNCTIONAL PROPERTIES OF GLUCAGON-LIKE PEPTIDE-1- RECEPTOR LIGANDS AND POSITIVE ALLOSTERIC MODULATORS IN CHINESE HAMSTER OVARY CELLS

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(Not submitted)

Hsp65 SEQUENCING FOR IDENTIFICATION OF NON-TUBERCULOUS MYOBACTERIA: OPTIMIZATION AND EVALUATION

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ABSTRACT

This study consisted of analysing the physical, structural, and chemical makeup of two nanobodies (NbAahIF12 and NbAahII10C/S) that have very similar pharmacokinetic/dynamic (PK/PD) properties; each is able to neutralize a very toxic protein from the scorpion venom of *Androctonus australis hector* (AahI and AahII respectively). Interestingly their tissue biodistribution and clearance from the blood via the kidneys are different when administered in mice or rats (Hmila *et al*., 2012; 2008).
An ideal situation for the application of nanobody-based probes for in vivo diagnosis would be to engineer Nanobodies that do not stick onto the kidney tissues but rapidly go into the bladder. These can then later be eliminated easily as urine. In the first part of this project we confirmed that the two Nanobodies were cleared out of the kidneys differently. The second part was to find which factors might be responsible for this major difference. After investigative their respective 3D conformation, we realized that the two Nanobodies are structurally very similar. Nevertheless, their biochemical properties such as overall charge, pH and hydrophobicity, were different. Finally we decided to interchange corresponding CDRs of both Nanobodies by grafting. Our aim was to have 14 different Nanobody chimeras. Ultimately, 9 chimeric Nanobodies were generated, sequenced,

produced and purified via IMAC and size exclusion chromatography. We encountered some difficulties during the construction of the chimeric DNA fragments, their production and purification, and labelling with 99m-Technetium. Successfully labelled Nanobodies were administered systemically in anaesthetized male rats. Dynamic scintigraphy was performed and images were used to evaluate the pattern of renal clearance. A quantitative analysis was carryout by measuring the percentage injected activities in the kidneys and the bladder.
We successfully confirmed that the two original Nanobodies targeted to the toxins of *Androctonus australis hector* (Aah) have different clearance patterns from the kidneys into the bladder. Our preliminary results also show that grafting of all three CDRs from one Nanobody onto the other alters the overall clearance from the kidneys, suggesting that amino acids within the CDRs instead of nanobody framework determine kidney clearance. Due to issues with nanobody labelling, further investigations will be required to conclude which CDR(s) is/are mostly affecting the clearance of Nbs via the kidneys.

ISOLATION AND MOLECULAR CHARACTERIZATION OF HIV FROM SEMEN AND BLOOD SAMPLES OF SEROPOSITIVE MEN VISITING THE HOSPITAL FACILITY OF ITG

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ABSTRACT

Sexual transmission of HIV involves semen and accounts for 70-80% of all the HIV transmission globally. Seminal HIV occurs and is thought to disseminate as cell-free or cell-associated forms. It however stands unresolved whether it is the cell-free or the cell-associated seminal HIV that is responsible for the transmission. To provide insight in solving this, we endeavored to isolate the infectious seminal HIV and characterize both in vitro and molecular aspects of the isolated virus.
We set out to optimize the seminal HIV isolation procedure, isolate both the cell-free and the cell-associated HIV, identify a cell line that best works with the isolated virus, compare the viral burden and the efficiency of isolation of seminal HIV and finally to evaluate the in vitro evolution that happens with the isolated seminal HIV.
To achieve the above objectives, we obtained semen samples from therapy naïve HIV seropositive men visiting the hospital facility of Institute of Tropical Medicine in Antwerp. The samples were processed by isolating the cell free and cell associated HIV using 19% Nycodenz gradient centrifugation and using the CD44 micro-beads. The isolated virus was propagated in four cell lines; two adherent cell lines (TZM-bl and GHOST cells) and two suspension cell lines (Jurkat and CEM-SS cells). Single gene sequencing was performed on one of the samples to elucidate the in-vitro evolution of the isolated seminal HIV.

Our results showed that seminal HIV could be successfully be isolated and perfectly propagated in cell culture cells with an 18% isolation success rate. Among the four cell lines used, GHOST cells gave the best results. Transferring infected cells during propagation gave better results compared to transferring the supernatants. In addition, transfer of infected cells to fresh culture cells as opposed to fresh medium only produced better results. The successful isolations had the highest seminal viral loads and surprisingly even higher than blood viral loads. However, there was a weak correlation between the seminal viral loads and blood viral loads ($r = 0.27$). The seminal HIV did not show much in vitro evolution at least after four passages in culture cell lines.

Our data shows the best isolation and in vitro manipulations of the isolated seminal HIV. These findings will go a long way in helping to work with seminal HIV, which will help in understanding the sexual transmission of HIV-1, a consequence that will allow coming up with effective strategies and targetable interventions to curb the spread of AIDS pandemic.

IN VITRO DNA BINDING STUDIES WITH THE TRANSCRIPTIONAL REGULATOR ArgP OF *Escherichia coli*

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ABSTRACT

ArgP has been shown to be a member of LysR-type family, which is crucial, both *in vivo* and *in vitro* for the transcription of gene encoding L-arginine exporter *argO* (YggA).

argO encodes arginine exporter (it might also be used to export canavanine, a plant-derived metabolite and toxic analogue of arginine). Also *lysP* (encodes LysP a specific amino acid transporter for L-lysine) is another gene identified to be controlled by ArgP. *In vitro* studies indicated that ArgP binds to *lysP* promoter/operator region to mediate it transcription activation, absence of ArgP, or addition of lysine result in very low *lysP* expression.

EMSA experiments were performed to measure the binding affinity of ArgP with mutated downstream and upstream (substitution mutation of AT to AT, CG or GC) subsites of *lysP* regulatory region. The mutated *lysP* control region was compared with wild type *lysP* control region in EMSAs. With the analysis of EMSAs we demonstrated that ArgP has reduced binding affinity to *lysP* mutants (substitution mutation of TA to either AT, CG or GC) when compared with the wild type *lysP*. Comparing the binding affinity of ArgP to *lysP* mutated at (TA substituted by either AT, CG or GC) the downstream and upstream binding boxes it was observed that there is reduction in the binding affinity of ArgP in relation to the downstream binding box, while in the upstream binding box, changes related to binding affinity were smaller. This may be because the downstream binding box is more important for binding of ArgP to the *lysP* than the upstream. For the *argO* there is little increase in binding affinity of ArgP to *argO* in the presence of lysine while no differences

observed in the presence of arginine when compare with the condition in which none of the cofactor was present .

DNase I footprinting and Cu-phenanthroline in gel footprinting experiments were performed to analyze the binding of ArgP on *argO* and *lysP* operator regions, in the absence of cofactor, and in the presence of 5mM L-arginine or 5mM L-lysine. Generally in both the two footprinting experiments a similar zone of protection about 56 bps long, extending from -72 to -23 with regard to transcription start (+1) were identified. In the presence of arginine a less protected zone overlapping -35 promoter element was demonstrated. In the presence of lysine another less protected zone situated at the center of the control region just close to the -35 promoter element was observed. We therefore reported that ArgP bind less tightly at downstream part of the *argO* promoter and more tightly at upstream part of the promoter in the presence of arginine. The reason for this, less binding of the downstream part of *argO* operator by ArgP in the presence of arginine, may be to allow RNA polymerase to recognize, bind, and form stable ternary complexes. Indeed, arginine is required for ArgP mediated activation of *argO* transcription *in vivo.* Presence of less protected area at the central part of the operator in the presence lysine indicates the formation of slightly different ArgP-*argO* complexes. It may prevent the RNA polymerase from leaving promoter (promoter clearance). Hyperreactive sites were observed in Cu-phenanthroline footprinting at positions -50 and -51 on the top strand and positions -52, -53 and -54 on bottom strands in the presence of arginine only. This suggested that when arginine bind ArgP a DNA-Protein complexes is form that results in the bending of DNA possibly to allow interaction of the protein dimers to form an active tetramer that can activate transcription. When ArgP protected region against DNase I on both *lysP* and *argO* control regions where compared it showed that, in *argO* the protected zone overlaps the -35 promoter element, this is not for *lysP*.

To further study the binding of ArgP to *argO* control region, premodification binding interference technique was used (in the absence of cofactor and in the presence of lysine and or arginine) to identify those bases that are critical for an efficient binding of ArgP to *argO* promoter/operator region and to reveal potential effects of DNA conformability on complex formation. We have demonstrated that all the negative interference effects with or without cofactor observed in these experiments, are found within -20 and -71 with respect to transcription start (+1). This is consistent with DNase I and Cu-phenanthroline footprinting in this work. It was observed that negative interference effects are concentrated more at the upstream part of the promoter mostly within the T-N_{11}-A conserved motif than the downstream parts. Region approximately between -38 to -51 with respect to transcription start (+1) appeared not important for bind of ArgP to the *argO* operator. Because there is no significant negative interference effects present there, may be because the ArgP protein bind *argO* operator as a tetramer such that one dimer subunit bind the downstream and the other dimer subunit upstream of the promoter leaving this region (-38 to -51) in between the two subunit. These correspond to region where hyperreactive sites (-50,-51 on the top strand and -52, -53, -54 bottom strand) were observed in Cu-phenanthroline in gel footprinting discussed above.

AN *IN VIVO* STUDY OF BIOFILM FORMATION BY IMPORTANT HUMAN PATHOGENS

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ABSTRACT

The development of biofilms on medical devices such as endotracheal tubes (ETTs) is a considerable problem in intensive care units (ICU) and is associated with a high morbidity and mortality. The presence of ETT in patients with critical illness is one of the factors increasing the onset of ventilated associated pneumonia. In fact, the ETT impairs mucocilliary clearance of airways and disrupts the cough reflex. Together, they promote the accumulation of tracheobronchial secretions. With the biofilm formation on the ETTs, the development of VAP is more likely to affect those mechanically ventilated patients. A better understanding of the structure of the biofilm on the ETTs and the organization of the micro-organisms within are needed to improve our understanding on how these biofilms contribute to the onset of VAP and can eventually lead to a better treatment. The overall objective of this thesis was to study biofilm formation by *P. aeruginosa* isolates obtained from mechanically ventilated patients from the University Hospital of Antwerp (UZA) ICU. The ETTs obtained from patients in the ICU were examined for the presence of biofilms. The micro-organisms present on the ETTs were identified by plating and Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). From these, six *P. aeruginosa isolates* were identified. The genetic profile of these six isolates, was determined by multi locus sequence typing and were shown to belong to 4 different sequences types, one of which never before reported.

The isolates were further characterized for their biofilm formation properties in both static and dynamic assays, the presence of quorum sensing molecules and antimicrobial resistance profile, but these could not be correlated to their sequence type. To be able to study the structure of the biofilm in relation to the presence of certain organisms, Fluorescence in situ Hybridization (FISH) staining protocols needed establishment. Hybridization temperature, formamide concentration, the use of DNA versus PNA FISH probes with different labels were all investigated as well as a technique that enables the application of FISH staining on such complex biological samples. Initial successes were shown, but further optimization is warranted to allow the simultaneous application of a number of probes.

STRUCTURAL PROPERTIES OF PROTEIN SEGMENTS ENCODED BY SYNONYMOUS CONSTRAINED ELEMENTS AND ACCELERATED REGIONS OF THE HUMAN GENOME

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ABSTRACT

The catalogues of primate- and human-specific accelerated regions (PARs and HARs), and multi-functional genomic sites detected as synonymous constraint elements (SCEs) of the human genome have been considerably expanded recently by the publication of the results of the 29 Mammals Project. Our aim is to study the structural preferences of the protein segments encoded by these functionally or evolutionarily distinguished DNA regions. We believe that in case of multiple evolutionary constraints, the protein structure has to cope with the consequences stemming from the second function fulfilled by the coding DNA segment. These restrictions probably do not allow for the free exploration of the amino acid space in the affected segment of the protein, which could cause problems in terms of forming proper secondary structure elements and adopting a well-defined fold. Regarding evolutionarily accelerated regions that overlap protein-coding genes, the question is how protein structure and function can survive the increased rate of mutations in the given genomic region. A wide range of suitable bioinformatics methods were applied to analyze 577 PARs and 563 HARs, and 11882, 10757 and 8933 SCEs (9, 15 and 30 codons window sizes applied in their identification, respectively) from the protein structural aspect. Unfortunately, the majority of accelerated regions (ARs) were found to reside in non-protein-coding regions and consequently, we could only identify a small number of corresponding protein segments (3.81% and 4.62% of the total entries for PARs and HARs, respectively). Due to this, the AR derived protein segment datasets showed low statistical power that, together with contradictory results between HARs and PARs, prevented us from drawing any reliable conclusion on their structural properties. In the case of the SCEs, the large number of entries in each SCE dataset confers them a higher statistical power and, when comparing them with their equivalent reference sets, they showed a highly significant enrichment in predicted structural disorder and a bias towards low sequence complexity, while at the same time, a highly significant depletion in regular secondary structure elements and a tendency to reside outside annotated domain regions. These tendencies got stronger with decreasing detection window size (increasing resolution), demonstrating the selective and localized effect of multi-functionality on the implicated protein segments. In all, our results show that multi-functional DNA sequences tend to overlap with intrinsically disordered protein regions rather than with globular domains; suggesting that there might exist a strategic distribution of the encoded complexity within the coding regions of genomes.

ANALYSIS OF INSILICO DESIGNED ARTIFICIAL HUMAN CALPAIN INHIBITORS

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ABSTRACT

Calpains have drawn much attention because of their crucial role in many biological processes as well as in many diseases caused by abnormal calpain activity. Calpains are proteases that are regulated by calcium and calpastatin, an endogenous calpain inhibitor. Among calpain family members, m-calpain is ubiquitously expressed in mammalian cells and is the focus of many studies. Calpastatin is an intrinsically disordered protein (IDP), which is a new important class of proteins not only because of their flexibility in interacting with various partners but also because of their critical role in biological processes. The number of newly discovered IDPs is still increasing and they are very new in the field of protein design.

The aim of this thesis is to analyze the inhibitory effect of the *in silico* designed artificial IDPs against calpain activity. For this purpose, human m-calpain and human calpastatin domain 1 (hCSD1) were recombinantly produced and characterized. 2 artificial IDPs, which are expected to inhibit calpain activity as a designed property were purified. In addition, 27 artificial peptides, which were computationally designed to have the capacity of binding to the surface of m-calpain were purified. The interaction of hCSD1 and the 2 artificial IDPs with m-calpain was analyzed. In addition, the inhibition of calpain activity by hCSD1 and the artificial IDPs was tested. Experimental data showed that hCSD1 as a positive control interacts with m-calpain and inhibits calpain activity. Unfortunately, the artificial IDPs with the GST-tag did not show any interaction with calpain. Therefore, no inhibition of calpain activity by the artificial designed IDPs could be observed. From the data that was generated during this thesis, it was concluded that the artificial designed constructs need to be reevaluated for the aim of understanding why there was no interaction between the artificial IDPs with calpain nor inhibition of the calpain activity by the artificial IDPs.

ROLE OF D14 IN PLANT UNDER STRESS CONDITIONS

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ABSTRACT

Early responsive dehydration 10 and 14 proteins (ERD10 and ERD14) belongs to group 2 – Late embryogenesis abundant proteins (LEA proteins). They were also called the dehydrins proteins and were proved to be intrinsically disordered. Some

studies indicated that the dehydrins of several plants species played a role in protecting plants subjected to stress conditions such as: drought, salt and cold stresses. In the framework of this thesis: ERD10 and ERD14 of *Arabidopsis thaliana* were used to unreal the role of these dehydrins in stress tolerance of Arabidopsis. Arabidopsis plants with different expression levels of ERD10 and ERD14 were treated with several concentrations of mannitol (drought stress) and NaCl (salt stress). Under stress conditions, the overexpression plants expressed ERD10 and ERD14 much higher than wild type and knockout lines under the same conditions. The overexpression lines also showed better growth than the others subjected to stresses. The results presented a significant effects of ERD10 and ERD14 on plant development including plant growth and flowering time of plants under stress conditions. Especially, under two equiosmolar conditions mannitol and NaCl, ERD10 and ERD14 had significantly difference in their response to long period of stress treatments. The localization of ERD10 on the membrane of plant cells subjected to salt and cold stresses was also determined.

STRUCTURAL AND DISORDERED DOMAINS OF TWO LARGE TRANSCRIPTIONAL CO-ACTIVATORS, CBP AND p300; EXPRESSION, PURIFICATION AND ADDRESSING PUTATIVE INTERACTIONS AMONG THEM

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ABSTRACT

This study focuses on a partially disordered eukaryotic macromolecule, p300. This multidomain transcriptional co-activator plays pivotal roles in transcription regulation by its epigenetic modifying and reading activity, as well as signal co-integrator roles for its more than 400 identified partner proteins. This work is an integrant part of a project aiming to provide missing information about the structural background of the already discovered highly complex functional behavior of p300 protein.

Proteins or regions of proteins that do not fold into stable three dimensional structures in native state are disordered. Due to the presence of such flexible disorder regions in between the folded domains of p300; chances of having inter-domain or linker-domain interactions were hypothesized. This study aims at discovering the existence of probable inter-domain or domain-linker interactions due to the flexibility imparted by the presence of disorder regions within the protein.

p300 has seven domains and in this study three zinc binding domains specifically ZZ, TAZ1, PHD and one fusion fragment covering Bromo-PHD of p300 were considered. These small fragments were expressed in different bacterial expression systems. The expression conditions, extraction and purification protocol were optimized for each small fragment. Purification of the proteins was performed primarily through Ni-affinity chromatography followed by size exclusion or ion-exchange chromatography, depending on the purity achieved in the first step. A crystallization screen was

performed for BromoPHD fusion protein. Interaction of purified small fragments with immobilized p300 was assessed in biolayer interferometry method by a Fortebio Octet machine.
The small fragments, ZZ, PHD, BromoPHD and TAZ1 were successfully expressed and purified. Notably the purification of PHD domain of p300 is the first time being reported in this study. Highest yield of protein was obtained for BromoPHD construct followed by ZZ and PHD; TAZ1 was found most aggregation prone protein. N-terminal His tag of ZZ and TAZ1 were successfully cleaved by enterokinase. Low affinity interactions of small fragments with immobilized p300 were observed; ZZ showed the highest affinity (KD=0.50 ±0.19 µM) followed by BromoPHD (KD=0.58 ±0.25 µM) and PHD (KD=10.61±2.13 µM). This study concludes with recommendations to map the weak binding sites of small fragments within the full length p300 that will contribute in outlining the structural complexity of the protein. The PHD domain could be further studied in solution NMR approach and the crystallization of BromoPHD can also provide information to resolve structure of still unknown PHD domain of p300.

MYCOPLASMA

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ABSTRACT

A real-time PCR, previously described in the literature, designed to characterize macrolide resistant *Mycoplasma pneumoniae* was validated and then retrospectively applied to a *M. pneumoniae* strain collection and samples from a biobanks known to be *M. pneumoniae* positive. The assay entails a duplex PCR amplifying of fragments from the 23S rDNA that might contain mutations which give rise to macrolide resistance, FRET-probes hybridize to the respective amplicons this is performed using Light Cycler 2.0(Roche Germany) and Tm analysis is subsequently performed and analysis using version 4.05 software.

The assay was validated for its specificity, sensitivity, accuracy, and reproducibility using reference strains and six well-characterized *M. pneumoniae* macrolide resistant strains or DNA-extracts from France and Leuven. The retrospective study was conducted on 110 strains isolated before resistance to macrolides was 1st reported and on 99 *M. pneumoniae* positive PCR clinical samples from the biobank.

4% of the clinical samples from the biobank showed mutations associated with macrolide resistance. To our knowledge, this is the earliest report on *M. pneumoniae* macrolide-resistance in Belgium with the first case dating from 2001.

DEVELOPMENT OF AN ASSAY FOR QUANTIFICATION OF LACTATE DEHYDROGENASE-ELEVATING VIRUS (LDV)

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ABSTRACT

LDV belongs to the *Arterivirus* family and is a mouse-restricted virus that causes a lifelong persistent infection in the blood. Mouse infections result in alterations in a number of physiological functions such as immunological responses and enzyme levels.
Detection of the virus by serology testing is unreliable and culture of the virus is limited. Since infected mice show elevated levels of lactate dehydrogenase, detection has relied in the past on indirect determination by enzyme level. However, hemolysis, tissue damage and freezing of plasma samples can also result in non-specific elevation of this enzyme, so false positive results are common by this method. Quantitative assay for LDV by using "LDH assay" is labor-intensive and many mice are required. Latex agglutination is another way to quantify LDV which has also its own limitation to work with including access of big machine which is not available everywhere. As LDV persistently circulates in the blood during infection, viral RNA can be detected by reverse transcription coupled PCR (RT/PCR) and then quantified by using qPCR.
Mice were infected by intraperitoneal injection (IP) of 2 x 10 5.5 ID 50/ml. Viral RNA was extracted from plasma and reverse-transcribed. Primers were determined by using Primer-blast software to amplify Open Reading Frame 7 (ORF7), encoding nucleocapsid protein VP1. Amplification products of the expected sizes were obtained by PCR amplification of ORF7 procedure (86 bp).
Quantification of virus was performed on plasma rather than whole blood. Using Rapid quantification of LDV virus cDNA by real-time PCR demonstrates that ORF7 expression was found to be at its peak on day 1 and then gradually decreased on day 2 and not detectable on day 4 and 7. This study also showed no increase in the level of LDV in SJLBGP1 mice.

INVESTIGATION OF THE USE VHHS, ALSO CALLED NANOBODIES, DERIVED FROM SINGLE-CHAIN ANTIBODIES OF CAMELIDAE FOR THE PROTECTION OF CHICKENS AGAINST COLONIZATION BY *C. jejuni*

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ABSTRACT

Campylobacter, a thermophilic and microaerophilic Gram-negative bacterium, is a major recognized cause of gastroenteritis in humans. It has been shown that, in both developed and developing countries, *Campylobacter* food-borne infections are the most predominant bacterial intestinal infection in humans.

The use of antibiotics in animal feeds to treat bacterial infection and colonization is not longer an acceptable option, as a result of antibiotic resistance which gave rise to resistant strains. Thus, there is urgency for innovative control measures that are acceptable to consumers and that can be used in the field. The use of pre- and probiotics, i.e. complex polysaccharides and strains of Lactic acid bacteria, has raised some hope.

Lactococcus lactis has great advantages over other bacterial species for *in vivo* protein delivery. It has an excellent safety status, through its long use in the dairy industry for the production of fermented food products. As a Gram-positive bacterium it does not possess endotoxic lipopolysaccharides (LPS), associated with Gram-negative bacteria such as *E. coli*.

The aim of this study was to investigate the use VHHs, also called Nanobodies, derived from single-chain antibodies of Camelidae for the protection of chickens against colonization by *C. jejuni*. In particular, anti-*Campylobacter jejuni* Nanobodies will be cloned and expressed, both as soluble proteins and covalently bound to the cell surface of *L. lactis* subsp. *cremoris* MG1363, by the anchor domain of protein A of *Staphylococcus aureus*.

After successful production and purification of the soluble VHHs protein, the binding of these Nanobodies to *C. jejuni* was assessed by immunofluorescence microscopy. The results of the immunofluorescence assay showed binding of Nanobodies Nb5, Nb15, Nb22, Nb23 and Nb34 to *C. jejuni* strain KC40, the strain that was used for the immunization of the llama from which the Nanobodies were cloned. In addition, Nanobodies Nb5 and Nb23 also bound other *C. jejuni* strains (KC64.1, KC 101, KC 96.1 and KC 84.2) and therefore probably recognize conserved epitopes.

We generated vectors for the expression of Nanobodies Nb5, Nb15 and Nb23 on the surface of L. lactis by the anchor domain of protein A of S. aureus. In the first experiment, L. lactis cells carrying these constructs did not agglutinate C. jejuni. Before tests can be performed in chickens, further studies on the presence and activity of the Nanobodies on L. lactis are therefore required.