ABSTRACTS ORAL PRESENTATIONS BaMa SYMPOSIUM 2018

BaMa-001

Unravelling the specific interaction of wall teichoic acid with antibodies using a new WTA-bead model

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INTRODUCTION

Antibiotic resistance is an increasing problem, with methicillin resistant *Staphylococcus aureus* as a prime example. This calls for novel strategies to target bacterial infections. A predominant component of the *S. aureus* cell wall is wall teichoic acid (WTA). WTA is critical for bacterial physiology, antibiotic resistance and host interaction through multiple human immune receptors, such as mannose binding lectin, langerin and antibodies. Anti-WTA antibodies are protective in mouse models and have been successfully used for targeted delivery of antibiotics to kill intracellular *S. aureus* persisters. *S. aureus* WTA consists of a polyribitol-phophate (RboP) backbone, which is covalently attached to peptidoglycan. WTA can be modified by *N*-acetylglucosamine (GlcNAc), which is linked in α-orientation by TarM or in β-orientation by TarS. Human anti-WTA antibodies specifically recognize either the α- or β-GlcNAc. However, the structural requirements for IgG binding, complement activation and phagocytosis are currently unknown. Here, we developed a bead model to study the interaction between human IgG and synthetic WTA in a purified manner. Information from these structure-function studies helps the rational optimization of existing monoclonal antibodies (mAbs) for therapeutic applications.

METHODS

Chemically synthesized biotinylated RboP hexamers or 12-mers were enzymatically modified *in vitro* by recombinant TarM or TarS. These structures were coated on streptavidin beads and stained with six different GlcNAc-specific IgG1 mAbs or pooled human serum and analysed by flow cytometry, in order to measure antibody and complement activation as measured by C3 and C4 deposition.

RESULTS

Recombinant TarM and TarS glycosylated synthetic WTA RboP molecules of both lengths and GlcNAc modifications were recognized by respective anti-α-GlcNAc and anti-β-GlcNAc mAbs. Antibody binding increased with increased RboP polymer length for α-GlcNAc but not for β-GlcNAc beads. Screening pooled human serum, we observed that anti-β-GlcNAc IgG antibodies are 10-fold more abundant than anti- α-GlcNAc IgG antibodies, but antibodies specific for unglycosylated WTA were undetectable. This corresponds with previous work done in an ELISA setting on purified WTA molecules. Both α-GlcNAc and β-GlcNAc WTA, but not unglycosylated WTA activated the complement system as measured by C4 deposition, indicating that antibody binding to synthetic WTA activates complement.

CONCLUSION

This new WTA bead model is a novel tool to study immune receptor interactions with WTA. It allows for good control over WTA length, modifications and density. We show that the chemically synthesized RboP molecules can be (1) enzymatically modified with GlcNAc, (2) recognized by WTA-specific antibodies in human serum and (3) trigger complement activation via the classical and/or lectin pathway. We will use this model to compare WTA-specific antibodies in plasma from patients and healthy controls. In the future, this model can be used as a tool for identification and optimization of therapeutic antibodies directed against *S. aureus* WTA, replacing antibiotics and thereby help in the battle against resistance.

BaMa-002

Enrichment of a novel sulfate-reducing bacteria from volcanic areas

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Volcanic and geothermal sites are known to emit sulfur compounds, mainly hydrogen sulfite. Limited research is done towards the microbial involvement in the sulfur cycles within these ecosystems. Within this research, novel sulfate-reducing bacteria are enriched. Sulfate-reducing bacteria are anaerobic microorganisms that use sulfate as terminal electron acceptor in their metabolism and reduce it to H_2S. They are omnipresent in anoxic habitats, where they have an important role in the sulphur as well as in the carbon cycle.

The geothermal site on Pantelleria Island, Italy, is characterized by geothermal gas emissions of CH_4, CO_2, H_2, NH_3, and H_2S. Analysis of the soil composition showed high concentrations of sulfate. These soil samples are used to enriched a culture of Sulfate-reducing bacteria, using H_2 as electron donor and CO_2 as carbon source. Two autotrophic cultures are enriched at 50 °C and 70 °C, both showing activity.

Preliminary 16S rRNA gene analysis of the 50 °C sample shows that it might be a novel genus of sulfate-reducing bacteria, belonging to the phylum of Firmicutes. Trough further genomic analyzation, targeting the 16S ribosomal RNA and functional marker genes such as *dsrAB* and *aprBA*, and physiology studies, we hope to identify and characterize these novel strains.

The culture is taken from an extreme environment, characterized by high temperature and low pH. Its special adaptations to acidic conditions and its capability to reduce sulfate could be applied in an biotechnological application such as sustainable clean-up of acidic mining water.

BaMa-003

The effect of mucin on the production of novel ganglioside mimicking structures by Guillain-Barré syndrome-related Campylobacter jejuni strains with the serotype HS:19

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Introduction: *Campylobacter jejuni* strains that produce sialylated lipooligosaccharides (LOS) can cause the neurological disease Guillain-Barré syndrome (GBS). In GBS, antibodies to sialylated LOS, raised during infection, cross-react with similar ganglioside epitopes on human peripheral nerves resulting in immune-mediated nerve damage. The risk of GBS after an infection with *C. jejuni* serotype HS:19 is estimated to be at least 6x higher than the normal risk. In search for genes or gene clusters that could explain this increased risk, we recently sequenced the genomes of fifteen *C. jejuni* strains with the HS:19 serotype. Subsequent comparative genomics resulted in the identification of a novel gene cluster containing an unique sialyltransferase, combined with genes involved in sulfatase activation, sulfate uptake and sulfate incorporation. Sulfatase activation is shown to be dependent on sulfate sources such as mucin or heparin which are abundantly present in the intestine, the natural habitat of *C. jejuni*. We hypothesized that strains with the HS:19 serotype can incorporate sulfate in the outer-core LOS leading to the production of novel sulfated ganglioside mimicking structures. We assessed whether growth of *C. jejuni* in the presence of mucin enhanced reactivity against serum of a patient and increased recognition by the lectins Siglec-1 and Siglec-7.
Methods: *C. jejuni* strains with the HS:19 serotype and controls were grown on Mueller Hinton plates with or without 0.01% mucin derived from porcine stomach. Motility was assessed on low agar (0.4%) culture plates. To determine reactivity to patient serum, Western blotting was performed on bacterial lysates that were either untreated or treated with proteinase K. To test for sulfatase activity, strains were cultured to log-phase under microaerophilic and anaerobic conditions in Mueller Hinton broth with or without addition of 0.5% mucin or 0.5% heparin. Sulfatase activity of bacterial lysates was addressed by measuring the release of sulfate from the substrate *p*-nitrophenyl sulfate, in a spectrophotometer at OD450. The binding capacity of *C. jejuni* strains to Siglec-expressing cells was assessed by flow cytometry using FITC-labeled *C. jejuni.*
Results: Growth on mucin significantly enhanced the motility of *C. jejuni* strains GB11 (HS:2, $p = 0.01$) and GB60 (HS:19, $p = 0.04$) compared to non-mucin cultured strains. The addition of mucin clearly altered the behavior of *C. jejuni* but in a serotype independent manner. Current tested conditions did not show an effect on serum reactivity. Also, no sulfatase activity could be detected in any of the conditions tested. Growth on 0.01% mucin did have a minor effect on the binding of HS:19 strain GB28 to Siglec-1-expressing cells.
Conclusion: Growth in the presence of mucin enhanced bacterial motility and slightly increased the binding of a *C. jejuni* strain with the HS:19 serotype to Siglec-1. No difference in serum reactivity, or sulfatase activity was observed under the current test conditions. Antibodies against sulfated glycan structures are statistically associated with GBS. This suggests that sulfated antigens are produced by micro-organisms that cause GBS. Future research will focus on optimization of the experiments by increasing mucin concentrations during bacterial growth and using other sulfate donors.

BaMa-004

Identification of parasitic nematodes using 18S rDNA, 28S rDNA and 5s rDNA molecular markers

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Animals can carry a number of different parasites. Some of these parasites can cause a risk to human health when eaten. When a human is infected with a parasite derived from an animal, it is called a zoonotic infection. *Trichinella* is a zoonotic nematode that can be present in the larval stage in the straight muscle tissue of animals. In the Netherlands, all pork, horse and wild boar meat intended for human consumption is screened for *Trichinella*. Because of the zoonotic relevance, it is important to monitor the presence of parasites like *Trichinella* in wildlife animals in The Netherlands.
In wildlife, *Trichinella* or parasites morphologically resembling *Trichinella* can be present, but microscopic identification can be problematic. Therefore, identification by means of molecular techniques would be an efficient way to identify unknown larval nematodes. This research aims to find molecular markers to distinguish the different nematodes.
A selection of wildlife nematodes was tested with primer pairs on different molecular markers (18S rDNA, CO1 rDNA, 5S rDNA, 28S rDNA, ITS2 rDNA and ITS1-5.8S-ITS2 rDNA). Consequently, PCR products were sequenced. All primers were already developed at the National Institute for Public Health and Environment (RIVM).
Most of the selected nematode gave a positive PCR result on the 18S rDNA marker. In addition, all of the selected Ascarididae yielded PCR products with a 28S rDNA primer pair. The ITS2 rDNA and ITS1-5.8S-ITS2 rDNA primer pairs could not be for any of the selected nematodes.
None of the molecular markers could be used for simultaneous identification of all selected nematode. Nevertheless, a selection of primer pairs directed at 18S rDNA, 28S rDNA and 5S rDNA allows identification of most intramuscular nematodes found in wildlife to genus level and some even to species level.
Concluding, a combination of molecular markers is necessary for the identification of unknown wildlife nematode.

BaMa-005

The role of *Streptococcus pyogenes* LytR in resistance to human Group-IIA Secreted Phospholipase A2.

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Introduction: Group A *streptococcus* (*Streptococcus pyogenes*; GAS) is a Gram-positive commensal bacterium. Nonetheless, GAS can cause mild infections such as pharyngitis or impetigo as well as invasive life-threatening infections such as necrotizing fasciitis or toxic shock syndrome. GAS is able to cause these severe infections due to its ability to evade the human immune system. For example, GAS is intrinsically highly resistant to the antimicrobial enzyme Group-IIA Secreted Phospholipase A_2 (sPLA$_2$-IIA).
sPLA$_2$-IIA is a potent acute phase protein that kills Gram-positive bacteria by hydrolyzing the phospholipids within the bacterial membrane. Recently, our group performed a GAS transposon mutant library screen to identify genes involved in sPLA$_2$-IIA resistance. This screen revealed that transposon mutants of *lytR* are more susceptible to sPLA$_2$-IIA mediated killing. LytR is a member of the LytR-CpsA-Psr (LCP) protein family, a conserved family of cell wall assembly proteins in Gram-positive bacteria. The main characterized function of LCP proteins is their ability to anchor cell wall glycopolymers such as wall teichoic acid (WTA), lipoteichoic acid (LTA) and capsular polysaccharides to the cell envelope. Therefore, these proteins are critical for cell envelope assembly and virulence. Little is known about the function of LytR in GAS. In this study, we set out to confirm the role of LytR in sPLA$_2$-IIA resistance and unravel the underlying molecular mechanism.
Methods: The bactericidal effect of sPLA$_2$-IIA was assessed using classical plating assays using recombinant sPLA$_2$-IIA and a clinically relevant GAS M1T1 serotype as well as an isogenic *lytR* deletion mutant. Binding of sPLA$_2$-IIA to bacterial strains was performed using a specific anti-sPLA$_2$ antibody. Differences in sPLA2-IIA cell wall penetration were obtained by time-course studies using the DNA dye SYTOX.
Results: We confirmed that a GAS *lytR* deletion mutant is approximately x-fold more susceptible to sPLA$_2$-IIA mediated killing compared to the GAS parent strain, which corresponded to an increased influx of SYTOX. In contrast, binding experiments show a decreased amount of sPLA$_2$-IIA on the surface of GAS Δ*lytR* compared to wild-type bacteria.
Conclusion: GAS LytR contributes to high resistance phenotype of GAS against sPLA$_2$-IIA, which has not been described previously. Decreased detection of sPLA$_2$-IIA to the surface of the *lytR* deletion mutant suggests an increased ability to penetrate through the cell wall as corroborated by results from SYTOX experiments. The function of GAS LytR in cell wall remodeling is currently unknown; further experiments aim to reveal the function of LytR within GAS and the underlying molecular mechanism in sPLA$_2$-IIA resistance.

BaMa-006

Variability of *Salmonella* during enrichment according to ISO 6579:1-2017

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Introduction
Salmonella is a gram-negative micro-organism which causes salmonellosis, the second most reported foodborne disease in the European Union. *Salmonella* can be detected with ISO method 6579:1-2017, which consists of non-selective pre-enrichment in Buffered Peptone water (BPW) followed by selective enrichment in Rappaport-Vassiliadis Soya Peptone Broth (RVS) and a second medium. BPW could consist different peptone sources, like casein, beef and yeast extracts. Non-selective enrichment in BPW aims to let sub-lethally cells recover and increase to detectable level. It is important that low concentrations of *Salmonella* can be detected in a product, even when cells are sub-lethally injured by for example draught-stress. Furthermore, there are indications that cells numbers decrease during selective enrichment in RVS, so it is crucial for *Salmonella* to grow in this step, even when there is a competitive micro-organism in the same enrichment. This research aims to compare peptone sources during enrichment of stressed cells, and to verify detection of a variety of *Salmonella* strains in the presence of competitive flora.
Methods
20 strains of *Salmonella* were enriched based on ISO method 6579:1-2017. Before enrichment, the *Salmonella* strains were sub-lethally injured by applying draught stress. This was done by submerging filter paper in a watery suspension of *Salmonella*, drying this paper 48h in a 37 °C incubator and blending it to obtain powder. This powder was rehydrated in BPW with different peptone sources. Growth and recovery were measured at 600 nm with the Bioscreen and by plate counting. To determine growth in selective enrichment, 20 healthy *Salmonella* strains were incubated in BPW for 24 hours in 37 °C After that, the strains were transferred from the BPW to RVS and incubated for 24 hours at 41.5 °C. The concentration of cells in RVS were determined by plating serial dilutions on XLD agar, while competitor *E. faecalis* was plated on Slanetz and Bartley agar.
Results
All 20 strains of *Salmonella* could be resuscitated in all types of BPW after drying. The Bioscreen results show that BPW containing casein as peptone source led to slower growth than beef and yeast–based peptones. However, the plating experiment had not a significant outcome concerning the peptone source. Counts of *Salmonella* strains were not reduced in RVS during 24 hours incubation. However, two strains showed an initial reduction in the first 8 hours of incubation, similar to reduction of competitor *E. faecalis* in this selective enrichment medium. One *Salmonella* strain was barely able to grow in RVS and counts after 24 hours were similar to the initial concentration.
Conclusions
Drying cells on filter led to stressed but viable *Salmonella*

Casein-based peptone results in slower growth than yeast- and beef-based peptones for pre-enrichment of 20 draught stressed *Salmonella* strain, as determined by optical density.
The concentration of the 20 *Salmonella* strains was stable or increased during 24 hours incubation in selective enrichment medium RVS.
Two strains deceased after 8 hours with *faecalis* as competitor in the RVS, one of them barely showed growth in 24 hours.

BaMa-007

Microbiological causes of untreated dysuria in men and women in South Africa: diverse aetiology and antimicrobial resistance

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Introduction
There is a large burden of genitourinary tract infections (UTI) in rural South Africa, a setting with limited access to microbiological diagnostics. Dysuria is a common symptom but, for various clinical and psychosocial reasons, many individuals do not access healthcare and remain untreated or uncured. The objective of this study is to determine the microbiological causes of untreated UTI to inform policy design and empirical treatment guidelines.
Methods
This analysis is part of a larger cross-sectional study that is conducted in rural Mopani District, South Africa. We mobilised men and women (≥ 18 years) with untreated dysuria to visit their primary healthcare (PHC) facility on a designated day; mobilisation was done through posters, ward-based outreach teams and traditional leaders. Following informed consent, history was taken, physical examination conducted and urine specimens obtained for dipstick, microscopy, bacterial culture with antimicrobial susceptibility determination and molecular testing for sexually transmitted infections (STI) using routine microbiological methods.
Results
We present an interim analysis of data from 45 patients (30 female and 15 male) recruited at three PHC facilities in the district. The median age of participants was 49 years (range 19-82) and approximately half of them was treated for UTI before. The reported duration of symptoms varies from one week up to several years. Based on the facility's catchment population, at least 1:600 people in these three communities has untreated symptoms of dysuria.
UTI was confirmed by dipstick and microscopy in all except one participant (98%). Bacterial aetiology of dysuria was established in 21/45 patients (47%), 26 (58%) had an STI diagnosed (including 10 with concurrent positive bacterial culture), 2 (4.4%) had yeast infection and aetiology was unclear in 6 (13%). *Enterobacteriaceae* infection was the most common bacterial aetiology (16/21; 76%) followed by *Enterococcus faecalis* (n=4). Most of the *Enterobacteriaceae* isolates (76%) were resistant to amoxicillin, the first-line antibiotic for UTI in our area. Seven isolates (33%) were resistant to cotrimoxazole, two to Augmentin and only one to ciprofloxacin. Only one strain, of *Morganella morganii,* produced extended spectrum beta-lacatamase. Among 26 patients with STI, *Trichomonas vaginalis* (62%) was the most common followed by *Chlamydia trachomatis* (24%), *Mycoplasma genitalium* (19%) and *Neisseria gonorrhoeae* (14%). All except for two of the 16 STI cases with negative concurrent bacterial culture were attributed to *T. vaginalis* (n=11) and *M. genitalium* (n=3) infection. Six of these were in men with longstanding persistent dysuria despite treatment.
Conclusion
There is a large unmet need for UTI care in rural South Africa as demonstrated by our mobilisation strategy. The aetiological mix of bacterial infection and STI makes it difficult to define an all-inclusive empirical treatment regimen. Considering the high frequency of amoxicillin resistance, a switch to ciprofloxacin would be appropriate in our area. *Trichomonas vaginalis* and *Mycoplasma genitalium* infection warrant attention as aetiological agents of dysuria in our region. This applies especially to men with longstanding dysuria not responding to initial treatment as, in the absence of diagnostics, these men are unlikely to receive empirical treatment that covers these infections.

BaMa-008

Tn-seq as a method to discover new vaccine targets for nontypeable *Haemophilus influenzae*

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Introduction Nontypeable *Haemophilus influenzae* (NTHi) is a gram-negative opportunistic pathogen that usually resides asymptomatically in the upper respiratory tract, but can also cause disease when it infects other body parts like the middle ear or the lower respiratory tract. Middle ear infection or otitis media affects mainly children, while infection of the lower respiratory tract occurs often in adult patients with chronic obstructive pulmonary disease (COPD). In both patient groups infection with NTHi causes malaise, high healthcare costs and increased use of antibiotics. Currently, no effective vaccine is available against NTHi since the *H. influenzae* type B conjugate vaccine protects only against encapsulated *H. Influenzae*. In this study, we aimed to identify new potential vaccine targets by using transposon sequencing (Tn-seq), a high throughput sequence-based method for evaluating fitness of a transposon mutant library for survival during infection.

Methods To cover the genetic heterogeneity of NTHi, transposon insertion libraries were constructed in multiple strains. These libraries were grown in three *in vitro* models that mimic the lung environment: growth on normal human bronchial epithelial cells (NHBE cells), growth in apical wash from NHBE cells and growth in sputum isolated from COPD patients. For each model, the mutant libraries were grown in two conditions: 1) the challenge condition, in which the mutant library only had access to nutrients that were naturally present in the *in vitro* model and 2) the control condition, in which rich growth medium was added to the *in vitro* model, in order to ensure that the mutants were not dependent on the *in vitro* model for nutrient acquisition. After growth of the mutant libraries, the mutants were recovered for genomic DNA extraction and the frequency of each individual transposon mutant was determined by Tn-seq. Mutants that were able to grow in the control condition but failed to grow in the challenge condition possibly possessed a deletion in a gene that was essential to survive in the *in vitro* model. Identified conditionally-essential genes were validated through construction of gene deletion mutants in different NTHi strains.
Results We identified multiple genes encoding potential vaccine antigens. Genes *tdeA* and *emrA* were involved in drug and toxin export and were identified in multiple Tn-seq screens, as were genes *znuA*, *znuB* and *znuC* that code for a high affinity zinc uptake system. Genes of the Vacj/Yrb transport system were also identified in our screens. This system is involved in maintaining membrane stability and was already proven to be important in virulence in previous studies. Now we also show decreased growth in sputum of *vacJ/yrb* deletion mutants in two NTHi strains. All mentioned genes are interesting to characterize in more detail to identify their role in infection and to test their suitability as a vaccine antigen in follow-up experiments.
Conclusions 1) Conditionally essential genes were found in all Tn-seq screens. 2) Multiple genes were validated in growth experiments. 3) Genes of the Vacj/Yrb transport system were already proven to be important in virulence and appear to be essential for growth in sputum.

BaMa-009
Finding new strategies to improve *Trichoderma harzianum* lysing enzymes activity
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*Trichoderma harzianum*is most commonly known for its use as biocontrol agent in agriculture. It secretes lysing enzymes that degrade the cell wall of other fungi, thus releasing nutrients. This enzyme mixture(LEM) is commercially produced for the production of fungal protoplasts used for DNA transformations. However not all fungi can be protoplasted efficiently using these mixtures. Therefore it is important to develop a mixture with high enzymatic activity, which can be used on a wide variety of fungi.
Previous research in *Aspergillus niger* has shown that regions in the mycelium which are able to sporulate do not secrete proteins. Moreover, it was found that a mutant strain unable to sporulate produced a wider variety of enzymes. Therefore, it was hypothesized that elimination of sporulation in *T.harzianum* would lead to a higher secretion and a higher variety of lysing enzymes. As an alternative, we studied the possibility to complement the enzyme mixture with chitinases from another source. These enzymes are low in abundance in this mixture.
Methods
A wildtype *T.harzianum* strain was used that was provided by Wageningen University. To obtain non-sporulating mutants of *T.harzianum* UV-mutagenesis was used. Phenotypical characterization was done with light microscopy and SDS-page was performed to compare protein profiles of spent medium of wild-type and mutant strains. To confirm that mutants were actually *T.harzianum*, sequencing of the ITS region was done.
Chitinase activity in LEM was monitored on cell walls of *Agaricus bisporus, Lentinula edodus* and *Agrocybe* using a colorimetric assay. DNS reagent was used to quantify the concentration of reducing sugars and Reissig reagent to determine N-acetylglucosamine (monomer of chitin) concentration.
Results
Three non-sporulating mutants were obtained, all confirmed to be *T.harzianum* after sequencing their ITS region. Interestingly, all strains showed a different protein profile compared to the wildtype when grown on glucose or *A.bisporus* cell walls. One of the mutants obtained shows a higher and more complex secretion of proteins on glucose and cell walls after 3 days of growth. The other two mutants have a more similar complexity but show higher secretion of proteins of different molecular weights. Enzyme assays showed that a commercially available LEM(Sigma) has little to no chitinase activity, while a mixture produced at the eukaryotic microbiology group of Utrecht University did have this activity. Adding chitinase from *Streptomyces griseus* increased degradation of cell walls of *A.bisporus* by 40%. In contrast, no effect of adding chitinase was found when *L.edodus* cell walls were used as the substrate.

Conclusions 1. Sporulation seems to have an effect on enzyme secretion of *T. harzianum*
It was shown that fungi seem to have a different chitin content in their cell wall since the addition
of chitinase increases the activity of the LEM on *A. bisporus* but not on *L. edodus*
Commercially available LEM lack chitinase activity which could lead to poor protoplastation efficiency in fungi that have a high chitin content in their cell wall. Therefore adding chitinase to the LEM of *T. harzianum* can make it applicable to a broader range of fungi for protoplastation purposes.