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# Influence of the host response on *stx2* expression by Enterohemorrhagic *E. coli* (EHEC)

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## Summary

Enterohemorrhagic *Escherichia coli* (EHEC) is an intestinal pathogen that can cause hemorrhagic colitis and hemolytic-uremic syndrome (HUS) in humans. These severe diseases are linked to the production and release of Shiga toxins (Stx) which are encoded on prophages integrated in the bacterial genome (*stx1/stx2*). In this work I focused on s*tx2* expression which is tightly regulated and closely linked to the bacterial SOS response and induction of the phage lytic cycle. Not all EHEC-infected patients develop hemorrhagic colitis and only some suffer from a progression to HUS. The risk factors for developing HUS remain unclear as well as the signals triggering Stx2 production in the human gut. This work aimed to identify risk factors for progression to HUS in infected patients. We hypothesized that inflammatory processes in the gut could promote Stx2 production in EHEC and therefore worsen the clinical outcome of patients. *In vitro* experiments have shown that polymorphonuclear neutrophils (PMN) and reactive oxygen species (ROS) have an influence on Stx2 production (Wagner et al., 2001a). The aim of my thesis was to validate these findings by using a novel reporter strain and to investigate the effect of other host-derived factors on *stx2* expression *in vitro*.

To study *stx2* expression in response to various host-derived stimuli, I used a set of well-characterized *stx2* transcriptional reporter strains. These reporters carry the gene for Gaussia luciferase (*gluc*) inserted into the *stx2* locus, thereby downgrading the EHEC strain from biosafety level (BSL) 3 to BSL 2. This system allows the quantification of Gaussia luciferase (Gluc) activity instead of Stx2 in the culture supernatant and is therefore a safe and fast assay to study the influence of various host and environmental factors on *stx2* expression. This assay was used to elucidate the effect of PMNs on *stx2* expression. I observed an activation of *gluc* expression by PMNs. This inducing effect was not mediated by $H_2O_2$ as $H_2O_2$ levels in activated PMNs were in the µM range and too low to cause this effect. The reporter strain, when exposed to higher $H_2O_2$ concentrations, showed higher Gluc activity. However, this was caused by bacterial killing, lysis and increased release of Gluc in the culture supernatant. Hence, the mechanisms by which PMNs stimulate Gluc/Stx2 production or release remain unknown.

Furthermore, the effects of serum and complement on *gluc*/*stx2* expression were investigated. It was observed that serum increased Gluc activity while bacterial survival was negatively affected. Effects of serum in the Gluc assay were primarily caused by bacterial lysis as inhibition of protein synthesis did

not lead to an alteration of released Gluc. One of the key factors in serum to mediate bacterial lysis is the complement system. Serum from mice deficient in complement factor C3 showed that effects of serum on bacterial survival were mainly caused by the complement system.

In conclusion, several effector mechanisms of the innate immune response were found to be triggers of *stx2* expression and/or toxin release. It was confirmed that PMNs have an inducing effect. Moreover, complement-dependent bacterial lysis was found to increase toxin release from bacteria. Together, the data support the idea that the inflammatory response increases Stx2 levels in the gut.

## Zusammenfassung

Enterohämorrhagische *Escherichia coli* (EHEC) sind pathogene Bakterien, die der Auslöser einer hämorrhagischen Kolitis und des hämolytisch-urämischen Syndroms (HUS) sein können. Diese schweren Erkrankungen sind eng mit der Produktion und Freisetzung des Shiga-Toxins (Stx) verbunden. Dieses Toxin wird auf Prophagen kodiert (*stx1/stx2*), die in das bakterielle Genom integrieren. In meiner Arbeit fokussierte ich mich auf *stx2*, dessen Expression streng reguliert und eng mit der bakteriellen SOS-Antwort und der Induktion des lytischen Zyklus des Phagen verknüpft ist. Nicht alle Patienten, die mit EHEC infiziert sind, entwickeln eine hämorrhagische Kolitis und nur bei wenigen Patienten zeigt sich ein Fortschreiten zum HUS. Die Risikofaktoren für diese Entwicklung sowie die Faktoren, die die Stx2 Produktion im Gastrointestinaltrakt begünstigen, sind bislang weitestgehend unklar. Das Ziel meiner Doktorarbeit war, Risikofaktoren für die Entwicklung eines HUS bei mit EHEC infizierten Patienten zu identifizieren. Die zugrundeliegende Hypothese war, dass entzündliche Prozesse im Darm die bakterielle Produktion von Stx2 verstärken und damit den klinischen Verlauf der Patienten verschlechtern können. *In vitro* Experimente haben gezeigt, dass polymorphkernige neutrophile Granulozyten (PMNs) und reaktive Sauerstoffspezies (ROS) einen Einfluss auf die *stx2* Expression haben (Wagner et al., 2001a). Das Ziel meiner Dissertation war, diese Ergebnisse mit einem neuen Reportersystem zu validieren und den Effekt anderer Wirtsfaktoren auf die *stx2* Expression *in vitro* zu untersuchen.

Um den Einfluss von Wirtsfaktoren auf die Expression von *stx2* zu untersuchen, habe ich transkriptionelle Reporterstämme verwendet. Die Reporter tragen das Gen für die Gaussia Luciferase (*gluc*) im Lokus von *stx2*, wodurch die biologische Sicherheitsstufe von 3 auf 2 hinabgesetzt werden konnte. Die Messung der Aktivität der Gaussia Luciferase (Gluc) erlaubte es mir, ein sicheres und schnelles Nachweissystem für die Bestimmung des Einflusses verschiedener Wirtsfaktoren auf die Expression von *stx2* zu etablieren. Dieses Nachweissystem wurde zunächst dafür genutzt, den Einfluss von PMNs auf die Bildung und Freisetzung von Stx2 zu untersuchen. Inkubation mit PMNs führte zur Steigerung der Gluc-Aktivität des Reporterstamms. Dies wurde nicht durch $H_2O_2$-vermittelte Induktion der *stx2* Expression ausgelöst, denn die $H_2O_2$-Konzentration der aktivierten PMNs lag nur im µM-Bereich und war damit zu niedrig, um einen Effekt auf die Gluc-Aktivität auszuüben. Höhere $H_2O_2$ Konzentrationen führten zu einer gesteigerten Gluc-Aktivität. Allerdings war dies durch das Abtöten der Bakterien und die Freisetzung des Reporterenzyms durch Lyse bedingt. Zusammenfassend konnte gezeigt werden, dass PMNs eine Verstärkung der Expression von *stx2* bewirkten und dadurch zur

Pathogenese des HUS beitragen können. Die ursächlichen Mechanismen bleiben aber Gegenstand weiterer Forschung.

Des Weiteren habe ich den Effekt von Serum auf die Expression von *stx2* untersucht. Dabei wurde beobachtet, dass Serum die Aktivität der Gluc gesteigert hat und zum Abtöten der Reporterbakterien führte. Die Steigerung der Gluc-Aktivität wurde vorrangig durch Lyse und nachfolgende Gluc-Freisetzung in den Kulturüberstand bedingt. Serum hatte keinen Einfluss auf die Genexpression, da eine Hemmung der Proteinsynthese keine Änderung der Serum-vermittelten Steigerung der Aktivität zeigte. Einer der im Serum enthaltenen Schlüsselfaktoren für die bakterielle Lyse ist das Komplementsystem. Die Inaktivierung bestimmter Komplementfaktoren zeigte, dass die Effekte von Serum auf das bakterielle Überleben durch das Komplementsystem bedingt waren.

Zusammenfassend konnte ich in meiner Arbeit zeigen, dass verschiedene Komponenten der angeborenen Immunantwort für die Expression von *stx2* beziehungsweise für die Freisetzung von Stx2 relevant sind. Es konnte bestätigt werden, dass PMNs die Expression von *stx2* induzieren. Außerdem habe ich festgestellt, dass die Freisetzung von Stx2 durch das Komplementsystem gefördert wird. Insgesamt legen meine Daten nahe, dass entzündliche Prozesse im Darm die Menge an Stx2 erhöhen können.

## List of abbreviations

| | |
|---|---|
| °C | Degree Celsius |
| AE | Attaching and effacing (lesions) |
| ANOVA | One-way analysis of variance |
| Bp | Base pair |
| BSL | Biosafety level |
| $CD_{50}$ | 50% cell death |
| CFU | Colony forming units |
| Cm | Chloramphenicol |
| CNS | Central nervous system |
| CTZ | Coelenterazine |
| D | Day |
| Da | Dalton |
| DCFDA | Dichlorofluorescin diacetate |
| $ddH_2O$ | Distilled water |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethylsulfoxid |
| *E. coli* | *Escherichia coli* |
| EHEC | Enterohemorrhagic *E. coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| EtOH | Ethanol |
| FCS | Fetal calf serum |
| Fluc | Firefly luciferase |
| Gb3 | Globotriaosylceramide |
| Gluc | Gaussia luciferase |
| H | Hours |
| $H_2O_2$ | Hydrogen peroxide |
| HRP | Horseradish peroxidase |
| HS | Human serum |
| HUS | Hemolytic-uremic syndrome |
| LB | Luria-Bertani |
| LEE | Locus of enterocyte effacement |
| LPS | Lipopolysaccharide |
| MAC | Membrane attack complex |
| Min | Minutes |
| MitC | Mitomycin C |
| MOI | Multiplicity of infection |
| MS | Mouse serum |

List of abbreviations

| | |
|---|---|
| MW | Molecular weight |
| NO | Nitric oxide |
| o.d. | Over day |
| o.n. | Over night |
| $OD_{600}$ | Optical density at 600nm |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| Pen-Strep | Penicillin-Streptomycin |
| PMA | Phorbol-12-myristate-13-acetate |
| PMN | Polymorphonuclear neutrophil |
| RCF | Relative centrifugal force |
| ROS | Reactive oxygen species |
| RPM | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| SD | Standard deviation |
| Sec | Seconds |
| SMAC | Sorbitol MacConkey agar |
| STEC | Shiga toxigenic *E. coli* |
| Stx | Shiga toxin |
| T3SS | Type 3 secretion system |
| Tir | Translocated intimin receptor |
| TMA | Thrombotic microangiopathy |
| u | Units |
| wt | Wild type |

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# 1. Introduction

## 1.1 Epidemiology

*Escherichia coli* (*E. coli*) is a versatile and well-studied microorganism that has a broad host range (Blount, 2015). On the one hand, this facultative anaerobic bacterium is a member of the common microbiota (Lloyd-Price et al., 2016). Commensal *E. coli* resides in the mucus layer of the mammalian gut and is beneficial for its host by producing vitamin K and vitamin B12 (Bentley and Meganathan, 1982; Lawrence and Roth, 1996). On the other hand, a few strains harbor virulence factors and cause numerous diseases, mainly diarrheal and urogenic problems (Kaper et al., 2004). Regarding the intestinal diseases, there are various relevant pathotypes with different features – for example enterohemorrhagic *E.coli* (EHEC), enterotoxigenic *E.coli* (ETEC) and enteroaggregative *E.coli* (EAEC) (Kaper et al., 2004). The focus in this work lies on Shiga toxigenic *E. coli* (STEC) that release at least one type of Shiga toxin (Stx) (Donnenberg and Whittam, 2001). These toxins were initially identified by Kiyoshi Shiga in *Shigella dysenteriae* serotype I (Shiga, 1898 as cited in Fraser et al., 2004). In 1977, it was discovered that some *E. coli* strains are able to produce cytotoxins that kill Vero cells (Konowalchuk et al., 1977). Six years later, in 1983, it was described that those toxins produced by *E. coli* are similar to Shiga toxins produced by *Shigella dysenteriae* serotype I (O'Brien, 1983). A subtype of STEC is called Enterohemorrhagic *E. coli* (EHEC). EHEC is associated with various serotypes and can cause severe diseases like hemolytic-uremic syndrome (HUS) (Orth and Würzner, 2006).

Epidemiology of EHEC-associated diseases in different countries is highly heterogeneous. In Germany, 2226 cases of EHEC were reported in 2018 (Robert-Koch-Institut, 2018). Epidemiological studies showed that children and elderly people are especially threatened by EHEC infections (Gould et al., 2009). The highest incidence of EHEC infection and HUS is found among children under 5 years (Gould et al., 2009). HUS is one of the leading causes of acute kidney failure in children (Williams et al., 2002). In Germany, HUS has an annual incidence of 3 per 100.000 children below 5 years and 53% of acute renal failures among children are associated with HUS (Büscher and Hoyer, 2010; Schaefer, 2014). Elderly people, the other threatened age group, have the highest risk of dying due to an EHEC infection, even in the absence of HUS (Gould et al., 2009).

The major reservoir of EHEC is the intestine of cattle, sheep, goats and other ruminants (Goldwater and Bettelheim, 2012). Therefore, close contact to ruminants and uncooked meat are major routes of transmission (Robert-Koch-Institut, 2011b; Schaefer, 2014). Other sources of EHEC infections include

municipal water, swimming water, milk, salami, lettuce and radish sprouts (Tarr et al., 2005). Most cases of EHEC are sporadic or appear in small clusters but every now and then bigger outbreaks are registered (Tarr et al., 2005). A crucial feature of EHEC is its extremely low infectious dose. Required colony forming units (CFU) range from 1-100 (Paton and Paton, 1998 as cited in Welinder-Olsson and Kaijser, 2005). The most common among the various EHEC serotypes is O157:H7 (Böhnlein et al., 2016). However, non-O157:H7 strains can also be triggers of HUS and get more and more important (Böhnlein et al., 2016; Helwigh, 2015). In 2011, northern Germany suffered from an outbreak caused by a non-O157:H7 strain (Muniesa et al., 2012). During this outbreak 3.842 people were infected with EHEC, 855 developed HUS and 53 died which makes it the EHEC outbreak with the highest number of HUS cases so far (Pacheco and Sperandio, 2012; Robert-Koch-Institut, 2011a).

## 1.2 Clinical course of EHEC infections

After EHEC infection, clinical symptoms appear after 2-12 days with an average incubation time of 3 days (Bell et al., 1994 as cited in Tarr et al., 2005). Initially, most patients suffer from watery diarrhea and simultaneous cramping pain (Obrig, 2010a). Other common symptoms are nausea and vomiting while fever is present in only 30% of cases (Scheiring et al., 2008). After another 1-3 days the colitis progresses and results in bloody diarrhea (90%) (Ostroff et al. 1989 as cited in Tarr et al., 2005). In 85-90% of the cases the symptoms subside after 5-8 days (reviewed in Grisaru, 2014). However, 5-10% among adults and about 15% of children under 10 years develop a systemic complication 5-13 days after the onset of the bloody diarrhea: hemolytic-uremic syndrome (HUS) (Jandhyala et al., 2013; Tarr et al., 2005). In most cases, HUS is a self-limitating disease and results in full recovery (Garg et al., 2003). However, up to 5% of the patients with HUS die (Gould et al., 2009; Pacheco and Sperandio, 2012). Different EHEC strains show a variable clinical course. Typical EHEC infection with O157:H7 usually provides a clinical course as described above. In contrast, the strain of the German outbreak in 2011 (O104:H4) deviated from this course (Frank et al., 2011) (**Figure 1-1**). The median incubation time after ingestion was longer (8 days) and many patients developed bloody diarrhea without preceding watery diarrhea (Frank et al., 2011). Furthermore, O104:H4 caused illness predominantly in adults and showed an enhanced rate of patients developing HUS (22% vs. 10%) (Frank et al., 2011; Menne et al., 2012).

HUS causes a thrombotic disorder and belongs to the group of thrombotic microangiopathies (TMA) (Tarr et al., 2005). HUS was first described in 1953 and is defined by the triad of **1.**) non-immune (=Coombs-negative) hemolytic anemia, **2.**) thrombocytopenia with platelet count of less than $150x10^9$/l and **3.**) acute renal failure with serum creatinine above the 95$^{th}$ percentile for sex and age (Gasser et al., 1955). The production and release of Shiga toxin (Stx) is a prerequisite for the development of HUS (Karpman, 2012). After Stx gets into systemic circulation, it binds to its receptor globotriaosylceramide (Gb3) (Lingwood, 2003). Biologically active Stx receptors can be found in high amount in the kidney's glomerular endothelial cells, microvascular endothelial cells and proximal tubular epithelial cells (Tarr et al., 2005). The combination of high amount of Gb3 and high blood flow explains why the kidneys are the most affected organs during HUS what results in glomerular and tubular kidney damage (Obrig, 2010a; Obrig and Karpman, 2012).

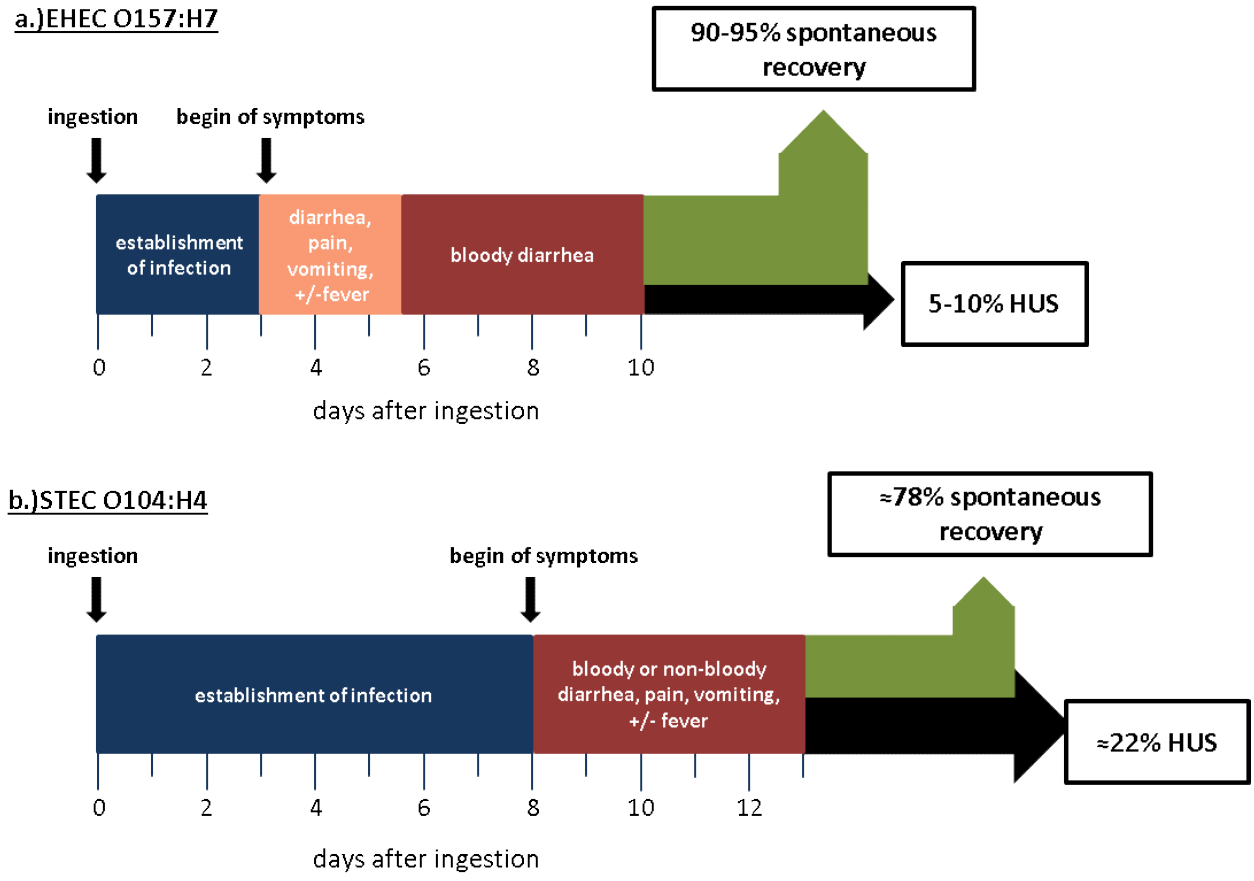


**Figure 1-1 Comparison of the clinical course of EHEC O157:H7 and STEC O104:H4 among adult patients.** EHEC O157:H7 shows initial watery diarrhea and vomiting after an incubation time of approximately three days. After some days of bloody diarrhea most patients recover spontaneously and only 5-10% progress to HUS. In contrast, STEC O104:H4 shows a longer incubation time, instant bloody diarrhea and more often a progression to HUS. Adapted from (Jandhyala et al., 2013).

Thrombotic microangiopathy and cell injury are main components of HUS pathogenesis (Tarr et al., 2005). Stx-mediated cell damage leads to the release of endothelial factors and the formation of thrombi consisting of platelets and fibrin (Corrigan and Boineau, 2001; Louise and Obrig, 1995). The thrombosed small vessels in the kidney cause decreased renal perfusion and kidney damage (Louise and Obrig, 1995). The renal impairment results in clinical signs like oligoanuria and edema as well as in laboratory changes including electrolyte imbalances (Garg et al., 2003; Scheiring et al., 2008). Thrombocytopenia is caused by the above described consumption of thrombocytes for formation of thrombi and leads to petechiae (Corrigan and Boineau, 2001). Erythrocytes get fragmented during their passage through the thrombosed vessels (Petruzziello-Pellegrini and Marsden, 2012). The fragmented erythrocytes, so termed schistocytes, are detected and eradicated by the reticuloendothelial system what leads to anemia (Corrigan and Boineau, 2001).

Microvascular cells in different organs and neural tissue of the central nervous system carry a various amount of Gb3 (Obata et al., 2008; Obrig, 2010b). This explains the affection of extrarenal sites like mesentery, heart, pancreas and the central nervous system (CNS) (Boyer and Niaudet, 2011; Jandhyala et al., 2013). Cerebral involvement is the major complication of HUS and an important factor regarding mortality (Siegler, 1994). The affection of the CNS can cause a broad range of symptoms including lethargy, irritability, strokes, seizures and coma (Nathanson et al., 2010; Tarr et al., 2005). Frequency of symptoms varies among different EHEC strains. For example, the outbreak strain of 2011 (O104:H4) did not only show a high rate of HUS but also a high incidence of neurological symptoms (Jansen and Kielstein, 2011).

## 1.3 Diagnosis of EHEC infection

A step-by-step approach is recommended for diagnosis of EHEC infection and HUS. Most significant clinical indicators of an EHEC infection are bloody diarrhea (70%), abdominal cramps and vomiting (Mead and Griffin, 1998; Scheiring et al., 2008). Acute bloody diarrhea and acute gastroenteritis among children should always be a reason to think of an EHEC infection (Mead and Griffin, 1998; Robert-Koch-Institut, 2011b). The typical clinical signs of HUS like oliguria, anuria, pallor and petechiae should be checked in the clinical examination (Corrigan and Boineau, 2001; Scheiring et al., 2008). Blood analysis can detect the typical combination of anemia, thrombocytopenia and increased retention values (Gasser et al., 1955). The anemia appears normochromic-normocytic with elevated reticulocytes and decreased hemoglobin. Fragmented cells (schistocytes) can be found in the blood

smear (Corrigan and Boineau, 2001). Thrombocytopenia is moderate in most cases but can get as low as 5000/µl (Corrigan and Boineau, 2001).

Microbial diagnosis of EHEC infections focuses on the identification of bacteria and the direct detection of Stx. Indications for microbial diagnostics include diarrhea among children below the age of six years, visible bloody stool, detection of hemorrhagic colitis via endoscopy, established HUS, contact persons of patients with HUS and pediatric patients with acute renal failure (Robert-Koch-Institut, 2011b). EHEC strain O157:H7 can be detected in stool by plating on sorbitol MacConkey agar (SMAC) (March and Ratnam, 1986). SMAC offers sorbitol as carbon source. While commensal *E. coli* can ferment sorbitol, *E. coli* O157:H7 does not have this ability and can be identified as colorless colonies (Tarr et al., 2005). However, some non-O157:H7 strains ferment sorbitol and can not be detected by plating on SMAC (Werber et al., 2008). Besides plating, immunological methods like enzyme-linked immunosorbent assays (ELISA), latex agglutination and immunofluorescence are used for characterizing EHEC serotypes by detecting certain antigens (Bloch et al., 2012). In recent years, molecular methods like polymerase chain reaction (PCR) and real-time PCR became more and more popular. Today, the detection of Stx genes via PCR is established as the most important step of diagnosis (Bloch et al., 2012; Robert-Koch-Institut, 2011b). Direct detection of Stx is the second approach of microbial diagnosis of an EHEC infection. For this purpose, ELISA of *E. coli* cultures is recommended (Robert-Koch-Institut, 2011b). In Germany, diagnosis and even suspicion of HUS as well as direct or indirect detection of EHEC are notifiable. Patients are infectious as long as EHEC can be detected in stool samples – especially children can show a prolonged contagiousness (Robert-Koch-Institut, 2011b).

## 1.4 Management of EHEC-infected patients

Treatment of EHEC-caused disease and life-threatening HUS is challenging. Administration of antibiotics is controversial and no specific therapy has yet been described as successful (Bruyand et al., 2018). It is supposed that antibiotics promote the production and release of Stx and therefore increase the risk of developing HUS (Wong et al., 2000; Zhang et al., 2000). A recent meta-analysis showed an association between the use of antibiotics and progression to HUS in EHEC-infected patients (Freedman et al., 2016). However, certain antibiotics may not increase the risk of developing HUS or even decrease it (Bielaszewska et al., 2012; Mühlen et al., 2020). For example, azithromycin was found to decrease Stx production under certain terms (McGannon et al., 2010). Furthermore, it has to be considered that non-O157:H7 EHEC strains may react differently to treatment with certain

antibiotics (Bielaszewska et al., 2012; Corogeanu et al., 2012). Treatment with ciprofloxacin during the German outbreak decreased the risk of developing HUS (Geerdes-Fenge et al., 2013). However, this could be biased as most of the patients during this outbreak did not receive antibiotics (Rahal et al., 2015). In summary, findings about antibiotics as causal treatment option are inconsistent and no clear recommendation for antimicrobial treatment can be given (Freedman et al., 2016). Therefore, supportive measures are the main strategy of treatment and should be initiated as soon as possible. Gastrointestinal symptoms like abdominal pain and cramps that are predominate in the early phase of EHEC infection are recommended to be treated with opioids like morphine (Bitzan, 2009). Non-steroidal anti-inflammatory medication should be avoided to prevent a decrease of renal perfusion (Murray and Brater, 1993). Antimotility agents like narcotics or anticholinergics enhance the risk of a progression to HUS or neurological symptoms and should therefore not be administered (Tarr et al., 2005).

The main focus of the symptomatic treatment lies on the kidneys. Regulation of fluid levels and electrolytes is essential in therapy of EHEC-infected patients and prevention of HUS (Ake et al., 2005; Hickey et al., 2011). Dehydration is associated with a higher mortality (Grisaru et al., 2017). Acute renal replacement methods are often applied during HUS (Goldwater and Bettelheim, 2012). 50-70% of children with HUS require renal replacement therapy and especially peritoneal dialysis is often used (Garg et al., 2003). Due to the wide availability of dialysis, the prognosis of the renal impairment has improved significantly (Scheiring et al., 2008). Plasma exchange has been used for adults suffering from HUS to reduce bacterial toxins, prothrombiotic factors and inflammatory mediators (Karpman, 2012). However, no significantly better outcome of patients treated with this method during the German outbreak of 2011 has been detected (Menne et al., 2012). Tight monitoring of hemoglobin, hematocrit and platelet count is necessary for evaluating hemolysis and thrombocytopenia (Goldwater and Bettelheim, 2012). About 80% of patients with HUS require erythrocyte transfusions that should be administered cautiously to prevent hypervolemia and hypertension (Tarr et al., 2005). Platelet transfusions are regarded critically due to the risk of worsening thrombosis and should only be administered if the patients show clinically significant bleeding (Tarr et al., 2005). Inhibition of the complement system with the monoclonal antibody eculizumab can be considered in severe cases of HUS (Menne et al., 2012). However, during the German outbreak no clear benefit of the use of eculizumab was found (Menne et al., 2012).

The prognosis of EHEC infections improved remarkably due to better supportive therapy and intensive care (Garg et al., 2003). The renal impairment is nowadays only rarely the reason for death

due to of availability and effectiveness of dialysis (Scheiring et al., 2008). Fatality is rather caused by insufficient prevention and therapy of extra-renal damages, especially neurological complications (Oakes et al., 2006). Approximately 30% of surviving patients retain health problems with variable severity in the follow-up (Garg et al., 2003; Rosales et al., 2012). Possible long-time sequelae include arterial hypertension, chronic renal failure, neurological impairment, diabetes mellitus and pancreatitis (Grisaru, 2014; Scheiring et al., 2008).

## 1.5 EHEC pathogenicity

Colonization of EHEC takes place in the distal ileum and the colon (Croxen and Finlay, 2010). A key mechanism of EHEC colonization is its ability to form attaching and effacing (AE) lesions of the intestinal epithelial layer (Nataro and Kaper, 1998). The mechanisms of the formation of AE lesions were initially found in another *E. coli* pathotype, enteropathogenic *E. coli* (EPEC) (reviewed in Pacheco and Sperandio, 2012). Similar to EPEC, EHEC triggers a massive remodeling of the cytoskeleton which leads to the effacement of microvilli and formation of "pedestal-like" structures (Kaper et al., 2004). AE lesions result in a close attachment to epithelial cells and enhanced pathogen colonization (Nataro and Kaper, 1998). The ability to form AE lesions requires the presence of a pathogenicity island called locus of enterocyte effacement (LEE) (McDaniel et al., 1995). This genetic locus encodes its own regulator (*ler*) and numerous proteins that are responsible for forming AE lesions (Mellies et al., 1999; Pacheco and Sperandio, 2012). LEE encodes the adhesion molecule Intimin and its host cell receptor Tir (translocated Intimin receptor) (Jerse et al., 1990; Kenny et al., 1997). Furthermore, a type 3 secretion system (T3SS) and effector proteins for translocation and signal transduction (e.g. EspA and EspB) are encoded on this genomic island (reviewed in Pacheco and Sperandio, 2012). The T3SS translocates the bacterially-encoded effectors into the host cell which leads to modulations in the intracellular compartment (Eaton et al., 2017). In summary, EHEC uses LEE-encoded effector proteins to attach to epithelial host cells and to affect cellular functions (reviewed in Navarro-Garcia, 2014). Therefore, these proteins are an important component of EHEC pathogenesis as they promote EHEC colonization and disease development (reviewed in Barnett Foster, 2013).

Production and release of Shiga toxins (Stx) are essential steps for progression to HUS (Karpman, 2012). Two types of Stx have been described: Shiga toxin 1 (Stx1) and 2 (Stx2) (Strockbine et al., 1986). Both are $AB_5$ toxins that consist of one A-subunit and five B-subunits (reviewed in Obrig, 2010a) (**Figure 1-2**). In this thesis I focused on Stx2 which is more often connected with a severe course of infection (reviewed in Melton-Celsa, 2014). EHEC releases Stx into the gut lumen in the

surroundings of enterocytes (Schüller, 2011). Several mechanisms how Stx gets from the gut into systemic circulation are being discussed but so far no definite answer can be given (Schüller, 2011). Stx might pass the gut barrier by internalization via endocytosis or transcellular processes (Malyukova et al., 2009). Gastrointestinal M-cells might play a role in the translocation of Stx into systemic circulation (Schüller, 2011). Bacteria may be transferred by M-cells to macrophages where Stx production might take place (Etienne-Mesmin et al., 2011). Furthermore, it is suggested that the inflammatory response and the influx of polymorphonuclear neutrophils (PMNs) during EHEC infection might promote the loosening of the gut barrier and increase the translocation of Stx through enterocytes (Hurley et al., 2001). Attachment of Stx to the surface of PMNs has been detected which might be essential for the transport of Stx to its effector organs (Brigotti et al., 2011).

The Stx receptor is the ganglioside globotriaosylceramide (Gb3) that is expressed on renal glomerular endothelial cells, renal cortical tubular, vascular endothelial cells and neural tissue (reviewed in Jandhyala et al., 2013). The amount of reactive Gb3 on a cell correlates with its sensitivity to Stx (Obrig, 2010a). The expression and distribution of the receptors underlie differences among groups of patients (Lingwood, 1996). For example, children possess a higher Gb3 amount in glomeruli that are considerably involved in HUS (Lingwood, 1994). Recent studies have detected that receptors for certain Stx subunits and small amounts of Gb3 are present in intestinal tissue (Zumbrun et al., 2010). Furthermore, it has been detected in mouse models that Stx promotes intestinal cell damages (Békássy et al., 2011). Therefore, Stx production by EHEC is not only responsible for damages in the kidney, the brain and other organs but also for the promotion of hemorrhagic colitis through local damage in the gut (Eaton et al., 2017).

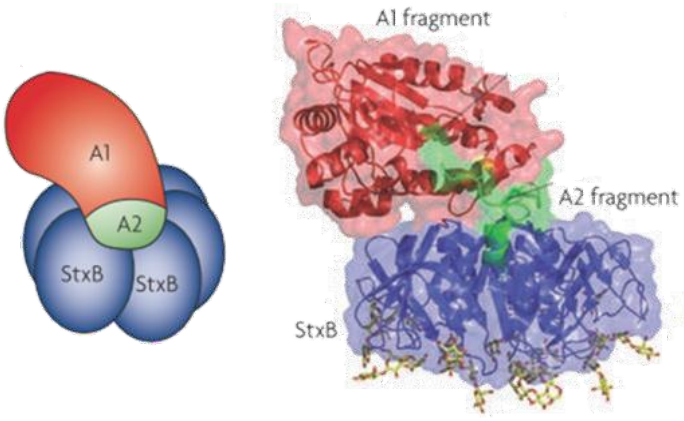


**Figure 1-2 Shiga toxin structure** Shiga toxin is an $AB_5$ toxin, consisting of one A-subunit (red and green) and five B-subunits (blue). Figure adapted from (Bergan et al., 2012)

Endocytosis of Stx is induced after the binding of the B-subunits of Stx to Gb3 (Ling et al., 1998). The internalization of the complex leads to the formation of an endosome within the cytoplasm (reviewed in Chan and Ng, 2016). This structure is afterwards transferred through the Golgi apparatus to the endoplasmic reticulum (ER) of the cell (Sandvig et al., 1992). Then, the A-subunit is detached from the B-subunits and gets released into the cytoplasm of the cell where it gets in contact with the ribosomes (reviewed in Tam and Lingwood, 2007). The A-subunit is an N-glycosidase that removes a single adenine base of the 28S ribosomal ribonucleic acid (rRNA) within the large ribosomal subunit (Endo et al., 1988). The ribosome gets inactivated and translation is stopped what consequently leads to the death of the host cell and induction of proinflammatory and proapoptotic pathways (reviewed in Obrig, 2010b).

## 1.6 Bacteriophages

The question arises how *E. coli* has acquired genes encoding for Stx. The most common theory is that *E. coli* acquired the genes via a bacteriophage from *Shigella dysenteria* serotype I (Allison, 2007). Bacteriophages are viruses that infect bacteria and were discovered in 1915 (reviewed in Campbell, 2003). It is suggested that there are more than $10^{30}$ phages in the biosphere (Brussow and Hendrix, 2002). Phages are heterogeneous organisms and classified into 10 families considering their morphology and genetic organization (reviewed in Brussow and Hendrix, 2002). Shiga toxins are usually encoded on prophages related to the λ family that are therefore called lambdoid phages (reviewed in Tyler et al., 2005). Among the group of Stx phages a variability regarding host range and pathogenicity can be found (Wagner et al., 1999). In the following sections I will focus on Stx2 phages as Stx2 is more often associated with a severe course of infection (reviewed in Melton-Celsa, 2014 and Wagner et al., 1999). Furthermore, the German outbreak of 2011 was caused by a strain (O104:H4) that had a lambdoid prophage encoding *stx2* inserted in its genome (reviewed in Muniesa et al., 2012).

Lambdoid phages belong to the group of temperate phages that have the potential of a lysogenic and a lytic development (**Figure 1-3**) (reviewed in Gamage et al., 2004). The majority of cells infected by a temperate phage immediately enter the lytic mode and only a small fraction of cells establishes a lysogenic state (De Paepe et al., 2014). In the lysogenic state the phage is integrated into the bacterial genome and is called prophage (De Paepe et al., 2014). It replicates with its host cell but the genes encoded by the phage remain silent and, in case of EHEC, *stx*2 is not expressed (reviewed in Friedman and Court, 2001). The activation of the prophage is called induction and it starts the lytic mode with

expression of virulence genes in the phage's genome (Wagner et al., 2001b). During the lytic cycle replication of the phage's DNA takes place by using bacterial cell components. New phage particles are produced, assembled and released from their host cell via lysis which leads to bacterial cell death (reviewed in De Paepe et al., 2014).

The *stx2* genes are encoded in the late-gene region of the phage between the genes for the anti-terminator Q and the genes for proteins that are important for cell lysis (reviewed in Neely and Friedman, 1998). Normally, the cI repressor binds to both sides of the operator. Thereby, the activity of the early phage promotors (Pr and Pl) is suppressed (Neely and Friedman, 1998). Consequently, most of the genes necessary for production and release of Stx2 are not being transcribed and the prophage remains silent as long as sufficient amounts of cI repressors are present **(Figure 1-4 Phage**

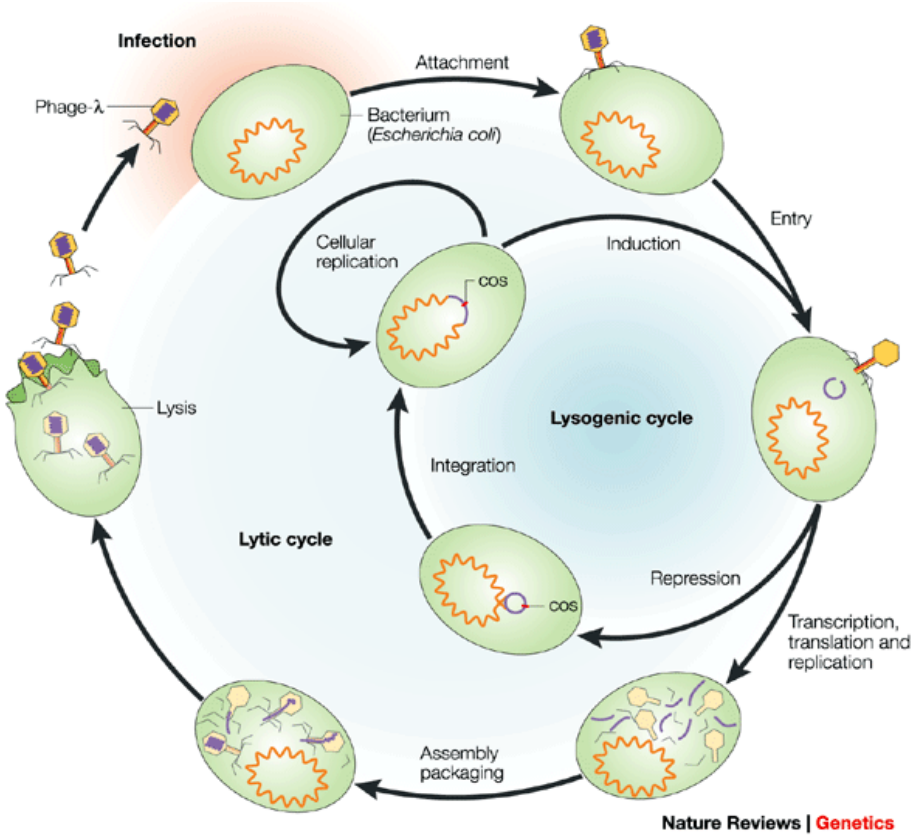


**Figure 1-3 Lytic and lysogenic phage development** After attachment to the surface of the cell two different modes of infection are possible. In lysogenic infection the phage's genetic information gets integrated into the genome of the host and remains silent until induction. Lytic infection leads to DNA replication and production of new phage particles. Finally, lysis of the host cell releases the phage particles. Figure taken from (Campbell, 2003).

**lysogenic mode)** (Ptashne, 1992). DNA damage starts the bacterial SOS response which leads to the activation of RecA protease (Muhldorfer et al., 1996). The bacterial SOS response is an intriguing bacterial survival mechanism that occurs when single-stranded DNA fragments are generated (reviewed in Žgur-Bertok, 2013). Any trigger of the bacterial SOS response is a potential inducer of prophages (Los et al., 2010). Well-described triggers of the bacterial SOS response include oxidative stress, changes of temperature and certain antibiotics (reviewed in De Paepe et al., 2014). RecA's major effect is the cleavage and detachment of the cI repressor (Little, 2005). The absence of this repressor promotes the expression of genes encoding the anti-terminator proteins called N and Q (Friedman Di, 2006). Q binds to the phage promotor Pr' which allows phage induction and replication of *stx2* to take place (Roberts et al., 1998). Finally, the lysis genes *S* and *R* downstream of *stx2* are expressed (Neely and Friedman, 1998) **(Figure 1-4 Phage lytic induction).** Lysis releases Stx2 and phage particles that can infect further bacteria after the initial host is lysed and destroyed (McGannon et al., 2010). If lysis genes were deleted, Stx2 would remain within the bacterial cell (Shimizu et al., 2009). Apart from prophage induction following bacterial SOS response, a small fraction of cells undergoes a spontaneous prophage induction, at least under laboratory growth conditions (Livny and Friedman, 2004). This might further enhance Stx2 levels.

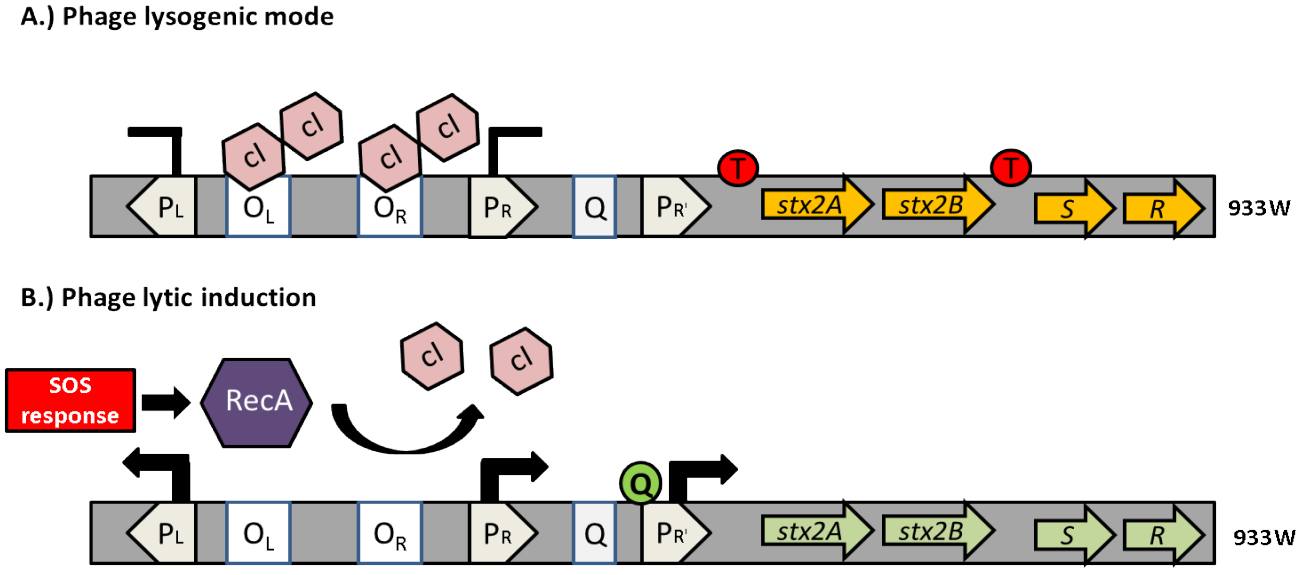


**Figure 1-4 Regulation of *stx2* expression in the course of bacterial lysis.** Phage promotor region in the lysogenic (A) and the lytic (B) phage. The cI repressor bound to operator sites $O_L$ and $O_R$ inhibits transcription of the promoters $P_L$ and $P_R$. The presence of a terminator (T) downstream of $P_R$' inhibits transcription of *stx2A* and *stx2B* and the phage remains quiescent. When the SOS response is triggered, RecA cleaves CI, stops $P_R$ repression and allows expression of genes for antiterminator Q. Antiterminator Q binds $P_R$' and leads to transcription of *stx2* genes and lysis genes *S* and *R*. Adapted from (Pacheco and Sperandio, 2012).

## 1.7 Regulation of *stx2* expression

Stx2 production and release are regulated by several environmental factors like temperature, bacterial growth, quorum sensing, reactions of the immune system and external factors like antibiotics (reviewed in Pacheco and Sperandio, 2012). In the following section, some of these factors shall be described in further detail.

**Induction of *stx2* expression under laboratory conditions** Two common methods for prophage induction are treatment with UV irradiation and certain antibiotics (Muhldorfer et al., 1996). The strong inducing effect of mitomycin C (MitC) has been described extensively (reviewed in Muhldorfer et al., 1996; Zhang et al., 2000). This substance is an antibiotic and antitumor drug that intercalates between DNA strands and triggers the bacterial SOS response (reviewed in Verweij and Pinedo, 1990). Although UV irradiation and MitC are optimal for stimulation of Stx2 production *in vitro*, they are no plausible explanation for Stx2 production during EHEC infections as they are commonly not present in the human gut (Los et al., 2010).

**The influence of the microbiota and colicins on *stx2* expression** The entirety of all microorganisms colonizing the human gut is called intestinal microbiota (Kamada et al., 2013). An estimated number of $10^{13}$-$10^{14}$ microbes can be found in the human gut (reviewed in Gill et al., 2006). These commensals have engaged in complex interactions among each other and with the host and have a profound impact on health and disease (reviewed in Clemente et al., 2012). Treatment with antibiotics causes a major disturbance of its composition and may increase the spread of antibiotic resistance genes (Dethlefsen et al., 2008; Sommer et al., 2009). Other factors that modulate the gut microbiota include changes in diet, various diseases, intake of probiotics and drugs (reviewed in Stecher, 2015). The healthy microbiota is an important factor for defying pathogens by inhibiting infection and colonization (Stecher, 2015). It was shown in mouse models that the absence of commensals is associated with increased Stx2 levels (Gamage et al., 2006). This might be caused by a higher survival of EHEC in the absence of commensal *E.coli* strains (Gamage et al., 2006). Commensal *E.coli* can affect the growth of EHEC by consuming organic acids, amino acids and other nutrients that EHEC requires (reviewed in Kamada et al., 2013). Furthermore, commensal bacteria can produce toxins (bacteriocins) that are able to inhibit the colonization of pathogens (reviewed in Hammami et al., 2013). Bacteriocins produced by commensal *E. coli* strains are called colicins (Pugsley and Oudega, 1987). Colicins have a narrow spectrum of activity and affect only related *E. coli* strains with the purpose to outcompete them (Schamberger and Diez-Gonzalez, 2002). In conclusion, the healthy

microbiota offers many functions that prevent or reduce the dimension of an EHEC infection (Kamada et al., 2013).

**Polymorphonuclear neutrophils (PMNs) are a key factor in inflammatory responses to EHEC infections** (reviewed in Karpman and Stahl, 2014). PMNs are among the first cells attacking pathogens during infections of the gut (Licznerska et al., 2016). However, it has been described that PMNs can be a trigger of Stx2 production by EHEC and might therefore worsen the outcome of EHEC infections (Wagner et al., 2001a). Activation of PMNs leads to degranulation and release of proteases like elastase that promote tissue damage (reviewed in Karpman and Stahl, 2014). Furthermore, PMNs are among the most important sources of antimicrobial products like reactive oxygen species (ROS) and free radicals that are produced during oxidative burst (reviewed in Freitas et al., 2009). ROS are an important defense mechanism but can also promote tissue damage (Mohanty et al., 1997). ROS have been described as inducer of the bacterial SOS response (Imlay and Linn, 1987). One of the major components of ROS produced by PMNs is hydrogen peroxide ($H_2O_2$) (reviewed in Rhee, 1999). Los and colleagues showed that $H_2O_2$ served as an *in vitro* inducer of prophages (Los et al., 2010).

## 1.8 New therapeutic developments in disease treatment

As previously described, treatment of HUS today is far from optimal. Therefore, new strategies in treatment are being investigated. Some of the most promising results are mentioned in this section. However, none of the following ideas could make its way into clinical praxis so far. Some researchers tried to create ligands that mimic the Stx receptor Gb3. The idea was to capture and neutralize Stx in the gut lumen before it gets into systemic circulation (reviewed in Goldwater and Bettelheim, 2012). A placebo-controlled study among children that were treated with a Stx1 and Stx2 binding ligand did not result in an improved clinical outcome and could therefore not prove effectiveness of this idea (Trachtman et al., 2003). Furthermore, vaccines against EHEC have been investigated (reviewed in Mühlen and Dersch, 2020). Proposed strategies included vaccination of children and cattle with recombinant virulence proteins (reviewed in Goldwater and Bettelheim, 2012). Cattle are the major reservoir for EHEC O157:H7 and successful vaccination of cattle would be a promising method to prevent EHEC infections in humans (Serna IV and Boedeker, 2008). Several vaccines for cattle have been tested and were proven to be an effective method (Varela et al., 2013). The focus of human vaccine development lies on different antigens that include Stx, Intimin and the O-specific polysaccharide of EHEC O157:H7 (Ahmed et al., 2006; O'Ryan et al., 2015). In mouse models, vaccination with subunits of Stx1 and Stx2 led to decreased colonization of EHEC and decreased

bacterial shedding (Zhang et al., 2011). A combined vaccination against Stx2, Intimin and the bacterial protein EspA appeared to be more efficient than vaccinations against just one of these factors (Gu et al., 2009). However, animal models can only partially replicate EHEC infection and disease in humans (reviewed in O'Ryan et al., 2015). The number of EHEC-infected patients is rather low which makes phase III trials difficult to conduct (Mühlen and Dersch, 2020). So far, active immunization against EHEC is not available and remains the subject of further investigation (Goldwater and Bettelheim, 2012).

Passive immunotherapy against Stx has also been considered. A great number of possible antibodies for prevention of HUS has been investigated in animal models (reviewed in Pacheco and Sperandio, 2012). In a mouse model it was shown that administration of a solution with a humanized monoclonal antibody against Stx2 could neutralize 90% of the activity of Stx2 (Mukherjee et al., 2002). Furthermore, neutralizing antibodies can disturb bacterial colonization and decrease bacterial shedding (Mohawk et al., 2010). Another study showed that monoclonal antibodies against Stx2 can reduce the accumulation of Stx2 in the kidney (Sheoran et al., 2012). In conclusion, usefulness of new therapy approaches could be reported under laboratory conditions and in animal experiments but clinical translation of these findings is still far away (reviewed in Mühlen and Dersch, 2020).

## 1.9 Towards a better understanding of environmental factors influencing EHEC pathogenicity

Many details of EHEC infection and pathogenicity are still not completely understood. As no causal therapy for EHEC infections is available, it is important to get a better understanding of the underlying mechanisms. In the following part, luciferase reporter strains and mouse models shall be described as new and promising methods. As mentioned earlier, it is difficult to study EHEC infections in animal models as many animals do not develop disease when infected with EHEC (reviewed in Ritchie, 2014). The characteristic combination of AE lesions and Stx-associated damage can not be modelled in conventional murine models (reviewed in Mallick et al., 2012). In light of this shortcoming, Mallick and colleagues constructed a pathogen with improved characteristics by using the murine pathogen *Citrobacter rodentium (C. rodentium)* (Mallick et al., 2012). This strain normally lacks genes for Stx2 production but is able to induce AE lesions in mice. The Stx-encoding phage Φ1720a-02 *Rz::cat* was transduced into *Citrobacter rodentium* DBS100 to create the new strain *C. rodentium* DBS770. Mice infected with *C. rodentium* DBS770 actually developed AE lesions in their intestine as well as Stx-

associated kidney damage (Mallick et al., 2012). Work with *C. rodentium* has become more and more important in EHEC research over the last years (reviewed in Crepin et al., 2016).

Fluorescent (e.g. GFP, RFP) and luminescent (e.g. firefly luciferase, Gaussia luciferase) reporters have increasingly been used in the last decades to observe biological processes (reviewed in Weissleder and Ntziachristos, 2003). In this work, I focused on the broad group of luciferase enzymes. The Stx2 encoding locus can be replaced with a luciferase reporter gene. Instead of Stx2, luciferase is produced and released via bacterial lysis. Thereby, these luciferase strains are a safe alternative for quantification of Stx2 production. The function of luciferase enzymes is to catalyze chemical reactions involving oxygen that lead to emission of photons (reviewed in Tannous et al., 2005). Several luciferases are known, including luciferases from bacteria, fungi, insects and marine organisms (reviewed in Tannous et al., 2005). Three well-characterized types of luciferases are firefly luciferase (Fluc) from the North American firefly (*Photinus pyralis*) (Deluca and McElroy, 1978), luciferase from *Renilla reniformis* (Lorenz et al., 1991) and Gaussia luciferase (Gluc) from *Gaussia princeps* (Szent-Gyorgyi et al., 1999). *Gaussia princeps* is a marine copepod that can be found in warm water all over the world (Suárez-Morales, 2007). These organisms might use their luciferase as defense against predators (reviewed in Verhaegent and Christopoulos, 2002).

The substrate of Gluc is coelenterazine (CTZ) and Gluc does not require ATP for the light-producing reaction (Tannous et al., 2005) (**Figure 1-5**). Gluc catalyzes the oxidative decarboxylation of CTZ what leads to the emission of light with a wave length of 460nm (Verhaegent and Christopoulos, 2002). It offers more than 1000-fold higher luminescence levels than firefly luciferase (Tannous et al., 2005).

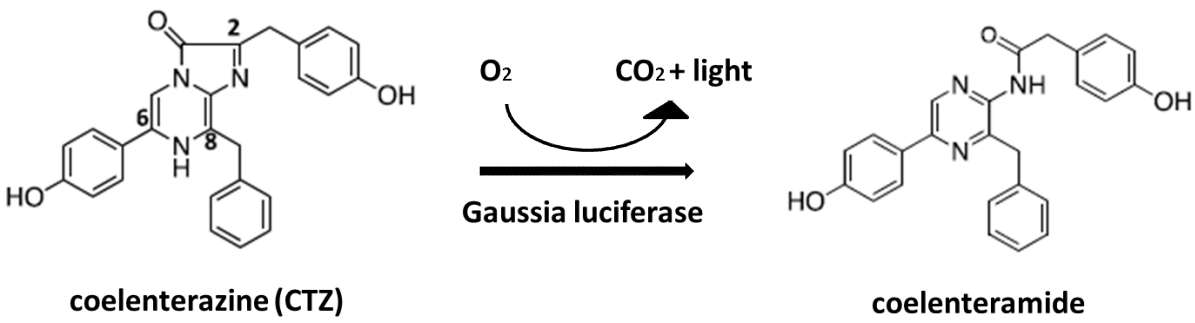


**Figure 1-5 Chemical structure of coelenterazine (CTZ) and luminescence reaction** Gaussia luciferase (Gluc) is an enzyme that catalyzes the oxidative decarboxylation of coelenterazine (CTZ) to coelenteramide. This reaction leads to the emission of light (Verhaegent and Christopoulos, 2002). Figure adapted from (Inouye et al., 2013).

Gluc is highly stable under different temperatures and different pH conditions which makes it superior to other luciferase enzymes (Wiles et al., 2005).

Gluc has primarily been used as a reporter gene in microorganisms like *Candida albicans* (Enjalbert et al., 2009), *Mycobacterium tuberculosis* (Andreu et al., 2010) and *Chlamydomonas reinhardtii* (Shao and Bock, 2008). Rathnayaka and colleagues showed the potential usefulness of Gaussia luciferase as luminescence reporter in Enterobacteriaceae (Rathnayaka et al., 2010). **Figure 1-6** shows details of Gluc reporter strains used in this thesis. Stx2-encoding loci are replaced by *gluc*. This renders the strain from biosafety level 3 (BSL3) to BSL2. Two different reporter strains were generated. In the strain *E. coli stx::gluc* only *stx2A* has been replaced by *gluc* while *stx2B* and lysis genes *S* and *R* are still intact. In contrast, *stx2A* and *Stx2B* as well as the lysis genes have been replaced by *gluc* in *E. coli stx2 sr::gluc*. This strain is therefore unable to release produced Gluc by expression of lysis genes.

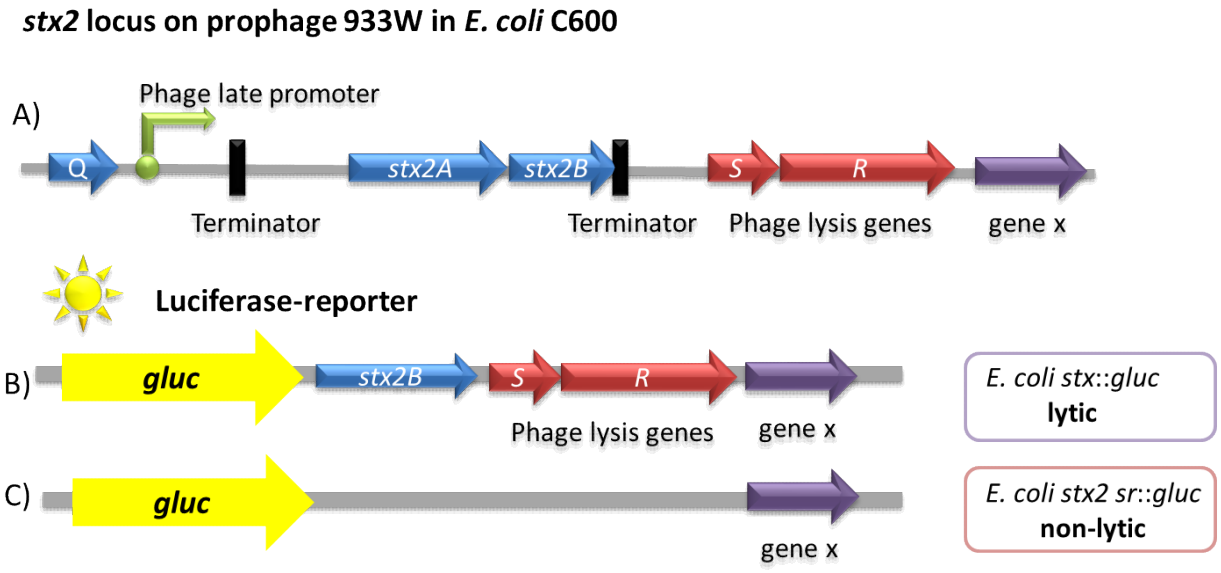


**Figure 1-6 Construction of Gaussia luciferase reporter strains** In *E. coli stx::gluc* the gene *stx2A* was replaced by a gene encoding for Gaussia luciferase (*gluc*) whereas *stx2B* and lysis genes *S* and *R* remained intact. Therefore, this strain was lysis-proficient. *E. coli stx2 sr::gluc* is non-lytic. For construction of this strain *stx2A*, *stx2B* and lysis genes *S* and *R* were replaced by *gluc*.

# 2. Objectives

Shiga toxin (Stx) producing *Escherichia coli* strains are clinically and economically important intestinal pathogens. The latest outbreak in Germany in 2011 has shown that more research into EHEC pathogenesis is urgent as the available treatment options are insufficient. Stx is the trigger of bloody diarrhea and hemolytic-uremic syndrome (HUS). Not all EHEC-infected patients develop hemorrhagic colitis and only some suffer from a progression to HUS. The risk factors for developing HUS remain vague as well as the mechanisms that trigger Stx production in the human gut. The primary aim of this thesis is to characterize host factors that might influence *stx2* expression. Inflammation in the gut may be an important trigger that has not yet been investigated in detail.

1.) The first aim is to establish a *stx2* reporter assay to compare the influence of different host factors on *stx2* expression. Therefore, different culture media and conditions shall be tested to establish a highly sensitive and reproducible reporter assay.

2.) Previously published work showed that polymorphonuclear neutrophils (PMNs) have an inducing effect on the production of Stx2 (Wagner et al., 2001a). Therefore, the second major objective is to investigate the influence of PMNs on Stx2 production and to elucidate the underlying mechanisms.

3.) Besides PMNs, the aim of this thesis is to investigate the role of other immune defense mechanisms in the induction of *stx2* by using the novel reporter assay.

# 3. Materials and Methods

## 3.1 Materials

### 3.1.1 Bacteria strains

**Table 1. *E. coli* strains**

| ***E. coli* strains** | **Lab-internal strain number** | **Genotype** | **Reference** |
|---|---|---|---|
| *E. coli* C600 | | *E. coli* C600 *F⁻ tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ⁻* | (Appleyard, 1954) |
| *E. coli* C600W34 | | *E. coli* C600 K12 lysogen of 933W | (O'Brien et al., 1984) |
| *E. coli stx::gluc* | JLG 5 | *E. coli* C600W34 *stx2A::gluc*$^{M43LM100L}$*aphT*, Kan$^{R}$ | Unpublished |
| *E. coli stx2 sr::gluc* | JLG 6 | *E. coli* C600W34 *stx2 S R::gluc*$^{M43LM100L}$*aphT*, Kan$^{R}$ | Unpublished |

**Table 2. *C. rodentium* strains**

| ***C. rodentium* strains** | **Lab-internal strain number** | **Genotype** | **Reference** |
|---|---|---|---|
| *C. rodentium DBS100* | | *C. rodentium* Prototype TMCH isolate, ATCC 51459, original biotype 4280 | (Schauer and Falkow, 1993) |
| *C. rodentium DBS770* | | *C. rodentium* DBS100 lysogen of ϕ1720a Δ*Rz::cat*, Cm$^{R}$ | (Mallick et al., 2012) |
| *C. rodentium stx2 sr::gluc* | MBK 22 | *C. rodentium* DBS770 *stx2 S R::gluc*$^{M43LM100L}$*aphT*, Kan$^{R}$ | Unpublished |
| *C. rodentium stx::gluc* | MBK 23 | *C. rodentium* DBS770 *stx2A::gluc*$^{M43LM100L}$*aphT*, Kan$^{R}$ | Unpublished |

### 3.1.2 Chemicals and antibiotics

**Table 3. Chemicals**

| Item | Supplier |
|---|---|
| Ampliflu | Sigma-Aldrich (Munich) |
| Ampuwa | Fresenius KABI (Bad Homburg) |
| Bacto™ Agar | Oxoid (St.Leon-Rot) |
| Coelenterazine (CTZ) native | Synchem (Felsberg) |
| Crystal violet | Sigma-Aldrich (Munich) |
| DCFDA | Sigma-Aldrich (Munich) |
| $ddH_20$ | Fresenius KABI (Bad Homburg) |
| DMEM (Dulbecco's modified eagle medium) | Thermo Fisher Scientific (St.Leon-Rot) |
| DMSO | Roth (Karlsruhe) |
| Ethanol (EtOH) 96% | Roth (Karlsruhe) |
| Fetal calf serum (FCS) | Thermo Fisher Scientific (St.Leon-Rot) |
| Formalin | Sigma-Aldrich (Munich) |
| Heparin | Thermo Fisher Scientific (St.Leon-Rot) |
| HRP (horseradish peroxidase) | Sigma-Aldrich (Munich) |
| Hydrogen peroxide ($H_2O_2$) solution | Sigma-Aldrich (Munich) |
| L-Glutamine (L-Glut) | Thermo Fisher Scientific (St.Leon-Rot) |
| Methanol | Roth (Karlsruhe) |
| NaCl | Roth (Karlsruhe) |
| PBS<br>(Dulbecco's phosphate buffered saline) | Thermo Fisher Scientific (St.Leon-Rot) |
| Percoll plus | GE Healthcare (Munich) |
| Phorbol-12-myristate-13-acetate (PMA) | Sigma-Aldrich (Munich) |
| Protease Inhibitor Cocktail (P8465) | Sigma-Aldrich (Munich) |
| Tris-EDTA | MP Biomedicals (Eschwege) |
| Tris-HCl | Roth (Karlsruhe) |
| Trypan blue solution (0.4%) | Sigma-Aldrich (Munich) |
| Trypsin (1x) | Thermo Fisher Scientific (St.Leon-Rot) |
| Tryptone | Oxoid (St.Leon-Rot) |

| | |
|---|---|
| Yeast extract | Roth (Karlsruhe) |

**Table 4. Antibiotics**

| Antibiotic | Supplier | Final concentration |
|---|---|---|
| Chloramphenicol (Cm) | Roth (Karlsruhe) | 30µg/ml |
| Kanamycinsulfate | Roth (Karlsruhe) | 30µg/ml |
| Mitomycin C (MitC) | Roth (Karlsruhe) | 0.5µg/ml |
| Penicillin/Streptomycin (Pen-Strep) | Thermo Fisher Scientific (St.Leon-Rot) | 100U/ml |

### 3.1.3 Media and reagents

**Table 5. Luria-Bertani (LB) medium**

| Component | Per liter medium |
|---|---|
| NaCl | 5g |
| Yeast extract | 5g |
| Tryptone | 10g |

All components were dissolved in $ddH_2O$ and autoclaved.

**Table 6. LB agar**

| Component | Per liter medium |
|---|---|
| NaCl | 5g |
| Yeast extract | 5g |
| Tryptone | 10g |
| Bacto™ Agar | 15g |

All components were dissolved in $ddH_2O$ and autoclaved.

**Table 7. Gluc assay buffer**

| Component | For 50 ml aliquots | Final concentration |
|---|---|---|
| Tris-HCl (1M, pH8) | 0.5ml | 10mM |
| NaCl (58.44g/mol) | 1.7532g | 0.6M |
| Tris-EDTA (0.5M pH8) | 0.1ml | 1mM |
| Ampuwa | fill up to 50ml | |

Adjusted to pH 7.8 with Tris-HCl. Gluc assay buffer aliquots of 50ml were stored at -20°C.

### Coelenterazine (CTZ)

CTZ (stock: 423.46g/mol) was dissolved in methanol to yield a stock concentration of 10mM. Aliquots of 50µl were stored at -80°C for long-term storage and at -20°C for a maximum of 4 weeks. For the production of Gluc assay substrate solution (see below), aliquots of CTZ were defrosted and directly used.

### Gluc assay substrate solution

Liquid cold CTZ was diluted in Gluc assay buffer (4°C) to yield a concentration of 12.5µM. Final concentration of CTZ in the Gluc assay was 10µM (10µl sample+40µl Gluc assay substrate solution with 12.5µm CTZ).

### Protease Inhibitor Cocktail (PIC)

5ml PIC (Sigma P8465) was dissolved in 1ml DMSO and 50µl aliquots were stored at -20°C. For experiments PIC was defrosted and mixed with Gluc assay buffer at a ratio of 1:25.

### 3.1.4 Equipment

**Table 8. Devices**

| | |
|---|---|
| BioPhotometer | Eppendorf (Wesseling-Berzdorf) |
| Centrifuge 5430R | Eppendorf (Wesseling-Berzdorf) |
| Certomat$^{R}$ | B.Braun Biotech International (Melsungen) |
| CLARIOstar plate reader | BMG Labtech (Ortenberg) |
| $CO_2$ incubator CB210 | Binder (Tüttlingen) |
| Magnetic stirrer MMS-3000 | A. Hartenstein (Würzburg) |
| Microscope Axiovert 25 | Zeiss (Oberkochen) |

| | |
|---|---|
| Mini-spin plus | Eppendorf (Wesseling-Berzdorf) |
| Multifuge X3R centrifuge | Thermo Fisher (St.Leon-Rot) |
| Thermomixer comfort | Eppendorf (Wesseling-Berzdorf) |
| Tissue lyzer | Quiagen (Hilden) |
| Vortex Genie 2 | Scientific Industries (USA) |

**Table 9. Further equipment**

| | |
|---|---|
| 15ml plastic tubes, V-bottom | Greiner Bio-One (Frickenhausen) |
| 50ml plastic tubes, V-bottom | Greiner Bio-One (Frickenhausen) |
| 96 F nontreated, white microwell SH plate | Thermo Fisher scientific (St. Leon-Rot) |
| Butterfly needle | Sarstedt (Nürnbrecht) |
| Cell Carrier 96, black clear bottom, TC treated, 96-well | PerkinElmer (Rodgau) |
| Desderman | Schülke (Telgte) |
| Disposable cuvettes | Brand (Wertheim) |
| Eppendorf tubes for OD-measurement | Eppendorf (Wesseling-Berzdorf) |
| Glass beads | Sigma-Aldrich (Munich) |
| Multichannel pipet | Gilson (Limburg) |
| Neubauer improved counting chamber, 0.0025m², 0.1mm depth | Brand (Wertheim) |
| Spin X-UF Spinning tubes | Corning (Wiesbaden) |
| Syringe (20ml) | Braun (Melsungen) |
| T75 flakes | Thermo Fisher Scientific (St. Leon-Rot) |
| TC Platte 96 Well, Standard, F | Sarstedt (Nürnbrecht) |
| Tissue Culture OrPlates, 96 well plate, flat bottom, transparent | Orange Scientific (Braine-l'Alleud, Belgium) |

## 3.2 Methods

### 3.2.1 Gaussia luciferase (Gluc) assay

**Growth of bacterial strains for *in vitro* assays**

Bacteria streaked from -80°C glycerol cryostocks were grown overnight (o.n.) on LB agar with appropriate antibiotics at 37°C. The next day, 2-3 bacterial colonies were inoculated in 5ml LB medium supplemented with appropriate antibiotics in Erlenmeyer flakes. These o.n. cultures of Gaussia luciferase reporter strains and controls were grown for at least 12h in LB medium at 37°C on a wheel rotor. In the morning, overday (o.d.) cultures were generated by diluting the o.n. cultures to an $OD_{600}$ of approximately 0.1 (1:20 of overnight cultures) and afterwards grown until an $OD_{600}$ of 0.5 (incubating for about 2 hours). Then, cultures were transferred into plastic tubes and centrifuged for 5min at 4°C at 5,000g. A culture with an $OD_{600}$ of 0.5 contains approximately $2x10^8$ *E. coli*/ml. For most experiments it was aimed to use ~$5x10^7$ bacteria/well in a volume of 50µl ($1x10^9$ bacteria/ml). Bacterial pellets were therefore resuspended in PBS or LB medium to yield a concentration of $1x10^9$ bacteria/ml by adjusting $OD_{600}$ to 2.5 (approximately 1/5 of the original volume). Bacteria were then inoculated to different conditions in a 96-well flat-bottom plate (TC Platte 96 Well, Standard, F by Sarstedt) and incubated at 37°C shaking for 4h.

**Generation of positive controls for the Gaussia luciferase assay**

Positive controls were generated via stimulation of *E. coli stx::gluc* with mitomycin C (MitC). Bacteria were grown as described before but MitC at a final concentration of 0.5µg/ml was added. Bacteria were grown for 4h on a wheel rotor at 37°C. Afterwards, cultures were centrifuged and the supernatant was stored at -20°C for further use. These samples were used in every luminescence measurement as positive control.

**Quantification of luciferase in the culture supernatant**

At indicated time points, 50µl reporter strain culture from each well of the 96-well plate was transferred into another 96-well plate using a multichannel pipet. This plate was centrifuged at 4°C for 5min with 3,828g (=4,700rpm). 40µl supernatant was carefully transferred to a 96-well flat-bottom plate (TC Platte 96 Well, Standard, F by Sarstedt) by tilting the plate and slow pipetting with a multichannel pipet. The remainder of the supernatant (approximately 10µl) was left to make sure that the pellets were not aspired. 10µl supernatant was directly transferred into a white-opaque 96-well plate (96 F nontreated, white microwell SH plate by Thermo-Fischer) to determine the

luminescence levels of released Gaussia luciferase (Gluc) with the protocol below. The remainder of the supernatant and the pellets were stored at -20°C for further use.

Evaluation of luminescence levels was performed with the CLARIOstar plate reader and a standardized protocol with two injectors. 40µl Gluc assay buffer (with CTZ concentration 12.5µM) was injected into each well of the white-opaque 96-well plates (Thermo-Fischer) containing 10µl of the reporter culture supernatant. Afterwards, luminescence was detected at 560nm emission wavelength. It was observed that high luminescence in a well could lead to falsely high luminescence levels in neighbor wells. Therefore, quenching of luminescence signals was achieved by injection of 100µl EtOH 97% per well and 3sec of 500rpm orbital shaking directly after measurement of the luminescence signal.

**Table 10. Luciferase assay CLARIOstar setting**

| | |
|---|---|
| Top optic<br>No. of multichromatics: 1<br>Gain = 3600<br>Focal height = 11mm<br>Measurement start time = 2sec<br>No. of intervals = 1<br>Measurement interval time = 1sec | **Injection timing:**<br><br>Measurement start time = 2sec<br>Injection Gluc assay buffer start time = 0.2sec<br>Injection 96% EtOH start time = 3.4sec<br>Shake mode: Double orbital<br>Frequency of shaking: 500rpm<br>Shake time: 3s |

**Quantification of Gluc in bacterial pellets**

Bacterial pellets in 96-well flat-bottom plates were thawed on ice and resuspended in cold 80µl Gluc assay buffer. Afterwards, the suspension was transferred into 1.5ml Eppendorf tubes. 20µl of 0.1mm glass beads (covered with Gluc assay buffer + PIC 1:25, pipetted up and down thoroughly) was added to the samples. Bacteria were lysed in a pre-cooled tissuelyser for 5min at 50Hz at 4°C. Afterwards, lysed samples were centrifuged for 5min at 14,000g at 4°C. 10µl supernatant was cautiously taken and transferred into a white-opaque 96-well plate (96 F nontreated, white microwell SH plate by Thermo-Fischer) for measurement of luminescence levels. Measurement with the CLARIOstar plate reader was performed using the same protocol as the measurement of luminescence levels in the supernatant (described above).

### 3.2.2 Monitoring of growth of the reporter strain

Optical density at 600nm ($OD_{600}$) was measured in two different ways. 500µl bacterial culture was taken and transferred into disposable cuvettes. Afterwards, $OD_{600}$ was measured with the Bio Photometer (Eppendorf) using PBS or LB medium as a blank.

For bacterial growth experiments in 96-well plates, $OD_{600}$ was automatically measured in the CLARIOstar plate reader with a standardized protocol.

**Table 11. $OD_{600}$ CLARIOstar setting**

Discrete wavelength, No. of wavelengths: 1
Wavelength: 600nm
Path length correction on – Volume: 200µl, Length: 5.34mm
Settling time: 0.1sec
No. of flashes per well: 22

### 3.2.3 Determining bacterial colony forming units (cfu)

**Standard plating**

Serial 1:10-dilutions with LB medium were generated. Afterwards 100µl of each dilution was plated on LB agar plates containing kanamycin. Plates were incubated in the dark at 37°C overnight. The next day, colonies were counted and colony forming units (CFU) per well or per ml were calculated using the weighted arithmetic mean (Jarvis, 2016).

Calculation:

$$\overline{x} = \frac{1}{\mathrm{d}_1} \times \frac{(\sum c_1 + \sum c_2 + \sum c_3 + \cdots + \sum c_z}{n_1 + \frac{n_2}{a} + \frac{n_3}{a^2} + \cdots + \frac{n_z}{a^{z-1}}}$$

Where: $\sum c_1$ = the total colony count on all ($n_1$) plates at the lowest countable dilution ($d_1$); $\sum c_2$ = total colony count on all (n2) plates at the next countable dilution (d2) with dilution factor = a; etc. (Jarvis, 2016).

**Spotting method**

This method was performed using a multichannel pipet. 20µl of each well of a column in a 96-well flat-bottom plate (Sarstedt) was taken and transferred into the first column of another 96-well flat-bottom plate (Sarstedt) containing 180µl PBS in each well. Then, a 1:10 dilution series (with PBS) in the 96-well plate was performed. 5µl of these dilutions was taken with a multichannel pipet and

transferred to an LB agar plate. On each plate 4x4 spots were placed. Sufficient space (approximately 1.5cm) between the spots was necessary to avoid mixture that could falsify the results. Plates were incubated at 37°C overnight. The next day, the spots were evaluated and it was observed at which dilution single colonies could be counted. Colonies were counted for this and higher dilution steps and CFU per well or per ml were calculated using the weighted arithmetic mean mentioned above.

### 3.2.4 Isolation of polymorphonuclear neutrophils (PMNs)

Protocol adapted from Hjorth et al., 1981. All steps were performed at room temperature. 40µl heparin was added to a 20ml syringe. Afterwards, 20ml of blood was drawn from a healthy donor using a butterfly needle. The blood was uniformly distributed in three 15ml tubes (approximately 6-7ml/tube). Only one of these tubes was centrifuged for 5min at 1,000g. This centrifugation step separated the blood into formed elements and plasma. Afterwards, only the plasma was taken and equally divided into the two remaining tubes. This step was performed carefully to prevent mixing. Those two tubes were then abandoned for 1h at room temperature.

In the meantime isotonic percoll was prepared. Therefore, 15ml percoll was delivered into a 50ml tube. 0.135g NaCl was added and completely dissolved (until no more crystals could be seen). Afterwards, two different concentrations were prepared in 15ml tubes: **a.)** 74% percoll (7.4ml percoll isotonic + 2.6ml PBS) and **b.)** 55% percoll (5.5ml percoll isotonic + 4.5ml PBS).

These tubes were stored at 4°C before generating the gradient. The gradients were prepared in 15ml tubes. 4ml 74% percoll was transferred into a 15ml tube and carefully overlaid with 3ml 55% percoll. Finally, 2ml plasma was layered on top of the generated percoll gradient. Careful and slow pipetting was necessary to avoid mixing. This tube (74% percoll, 55% percoll, plasma) was centrifuged for 20min at 600g without brake. After centrifugation, an interphase containing the PMNs could be seen. The supernatant above this interphase was slowly extracted using a Pasteur pipette. Afterwards, the interphase was taken and transferred into a 50ml tube containing 40ml PBS.

The tube was then centrifuged for 5min at 300g without brake. Afterwards, PMNs could be found at the bottom of the tube. For further use, the supernatant was disposed and the cells were suspended in 1ml PBS. Finally, cell number and viability were determined with a Neubauer counting chamber – for this step 10µl PBS containing the cells was mixed with 90µl Trypan blue solution (Sigma-Aldrich) and cells were counted via microscopy. The number of cells in all 4 quadrants was counted.

Afterwards, this number was divided by 4 and multiplied by $10^4$ to calculate the cells/ml in the suspension. Finally, PMNs were diluted in PBS to obtain the desired cell number for the experiments.

### 3.2.5 Gluc assay with polymorphonuclear neutrophils (PMNs)

200µl PBS containing PMNs in the required cell number was incubated with 50µl media (LB medium or PBS) containing bacteria at the required concentration in a 96-well flat-bottom plate (Sarstedt). Conditions were pre-defined and are depicted in the results section. For the course of the experiment, the 96-well plate was stored at 37°C + $CO_2$ (5%). At defined time points, samples were taken for further analysis in the luciferase assay. Therefore, 50µl of the culture in the 96-well plates was transferred into a new 96-well flat-bottom plate (Sarstedt). These plates were centrifuged for 5min at 4°C with 3,828g (=4700rpm). Afterwards, the supernatant was taken and transferred into another 96-well plate. 10µl supernatant was directly transferred into a white-opaque 96-well plate (Thermo-Fischer) to determine the luminescence levels of released Gluc (see 3.2.1). The plate with the remaining pellet and the plate with the remainder of the supernatant were stored at -20°C for later analysis.

### 3.2.6 Preparation of human serum (HS)

All steps were performed at room temperature. 20ml blood was taken from a healthy donor using a butterfly needle without use of an anticoagulant. Afterwards, the blood was centrifuged for 5min at 1,000g at 21°C. After the centrifugation, the serum fraction was taken und transferred to another plastic tube. The serum was stored at -20°C until being used.

For the assays, serum was diluted with PBS to the desired concentration. 200µl serum was mixed with 50µl PBS containing Gluc reporter strains in 96-well plates (Sarstedt). At certain time points, samples were harvested to determine luminescence levels (as described above) and CFU/well by plating. Furthermore, $OD_{600}$ of the culture was measured at the same time points.

### 3.2.7 Preparation of mouse serum (MS)

Serum of C57 Bl/6 mice (purchased from Janvier, Le-Genest-Saint-Isle) and $C3^{-/-}$ mice (Wessels et al., 1995) was stored at -80°C. This serum was defrosted and used for assays with Gluc reporter strains. The mouse serum was diluted with PBS to acquire different concentrations. 100µl serum in the desired concentration was transferred into each well of a 96-well flat-bottom plate (Sarstedt) and

incubated with Gluc reporter strains at 37°C for 4h. At certain time points, samples were taken for measuring luminescence levels as well as for plating. Additionally, $OD_{600}$ was measured.

### 3.2.8 Heat inactivation of serum

For heat inactivation, serum was filled in Eppendorf tubes and incubated in the Thermomixer comfort for 1h at 65°C. Afterwards, the tubes were centrifuged and the supernatants were used for experiments.

### 3.2.9 Gluc assay with hydrogen peroxide ($H_2O_2$)

$H_2O_2$ (Stock: 30%, 8.818M, Sigma-Aldrich) was diluted in plastic tubes in PBS at different concentrations. Afterwards, 200µl of the serial dilution was transferred into a 96-well flat-bottom plate (Sarstedt) and incubated with Gluc reporter strains (in 50µl PBS, as previously described) for at least 4h. Therefore, the total volume of each well was 250µl. Samples were taken at several time points and analyzed as described above.

### 3.2.10 Quantification of ROS (Reactive oxygen species)

**Amplex Red Assay (Adapted from Zhou et al., 1997)**

5g Ampliflu (257.24g/mol; Sigma-Aldrich) was dissolved in 1.94ml DMSO (Roth) to generate a stock solution of 10mM. This stock was divided into 50µl aliquots and stored at -80°C. Ampliflu is oxygen-sensitive and long exposure to $O_2$ should therefore be avoided.

1mg HRP (horseradish peroxidase, 52u/mg Sigma-Aldrich) was diluted in 1ml PBS to a concentration of 52U/ml. For the assay a concentration of 0.1U/ml was required. Therefore a 1:520 dilution with PBS was performed.

**Table 12. Reaction mixture for Amplex Red assay**

| Component | Amount | Final concentration |
|---|---|---|
| PBS | 5ml | |
| Ampliflu (10mM) | 25µl | 50µM |
| HRP (52U/ml) | 10µl | 0.1U/ml |

Optional: addition of 5µM phorbol-12-myristate-13-acetate (PMA) for stimulation of the oxidative burst of PMNs.

100µl of this mixture was pipetted into each well of a transparent 96-well flat-bottom plate (Cell carrier 96, PerkinElmer). Afterwards, the plate was pre-incubated for at least 10min at 37°C.

20µl PBS containing the pre-defined number of isolated PMNs (see protocol above) was added. Negative controls (PBS only) were always included. Afterwards, the plate was incubated in the CLARIOstar plate reader at 37°C and fluorescence (excitation: 555nm, emission 590nm) was measured every 10min during the course of 2h with a standardized protocol.

**Table 13. Amplex Red assay CLARIOstar setting**

Focal height: 6.9mm, No. of multichromatics: 1, top optic
Gain: 1000
Excitation: 555-10, Dichroic: 572.5, Emission: 590-10
Well Scanning: Orbital, Diameter: 4mm
Settling time: 0sec, No. of flashes per well: 12

**DCFDA (Dichlorofluorescin diacetate) assay (Adapted from Bass et al., 1983)**

5mg DCFDA (487.29 g/mol; Sigma-Aldrich) was dissolved in 1.03ml DMSO to acquire a 10mM stock. Afterwards, the stock was divided into 50µl aliquots and stored at -80 °C. For the assay, a final concentration of 50µM DCFDA was required. Therefore, a 1:200 dilution with PBS was created. 100µl DCFDA was transferred to transparent 96-well flat-bottom plates (Cell carrier 96, PerkinElmer). Afterwards, 20µl PBS containing PMNs at different concentrations was added. The plates were incubated in the CLARIOstar plate reader and fluorescence (excitation: 490nm, emission: 525nm) was measured every 10min in the course of 2h.

**Table 14. DCFDA assay CLARIOstar setting**

Focal height: 4.8mm, No. of multichromatics: 1, bottom optic
Presets: Alexa Fluor 488, Gain: 1250
Excitation: 490-10, Dichroic: 507.5, Emission: 525-10
Well Scanning: Orbital, Diameter: 4mm
Settling time: 0sec, No. of flashes per well: 12

### 3.2.11 Size exclusion centrifugation

Using "Spin-X-UF"-tubes (Corning), serum was divided into two fractions, regarding molecular weight (cut-off: 5kDa or 100kDa). 5ml serum was transferred into the size exclusion columns. These tubes were centrifuged for 30min at 5,000g. Afterwards, both the filtrate and the supernatant were taken and used in the reporter assays as described above.

### 3.2.12 Vero cell assay

**Vero cell assay – procedure (adapted from Konowalchuk et al., 1977)**

Vero cells (Vircell, reference number: FTVE, lot number: 11VE151) were grown in T75 flakes containing a cell medium of 5% DMEM (Thermo-Fischer) + Penicillin/Streptomycin (100U/ml) + L-Glutamine (200U/ml). Cells were grown until they were 100% confluent. Cells were washed with 20ml PBS and afterwards PBS was aspired again. Next, 2ml of 0.25% trypsin (Thermo-Fischer) was added to detach the cells. Trypsin was left for at maximum 2min and afterwards aspired again. Now, cells were harvested in 10ml of medium (DMEM, 5% FCS/L-Glut/Pen-Strep). Then, cells were counted using a Neubauer counting chamber. Therefore, 10µl medium containing Vero cells was mixed with 90µl Trypan blue solution (Sigma-Aldrich) and cells were counted via microscopy. Cells in several fields of the chamber were counted and the median number of cells per field was calculated. Afterwards cell number/ml was calculated. In each 96-well tissue culture plate (Orange Scientific), 2 million cells were seeded. Therefore, cells were diluted to the appropriate concentration. Different supplements were prepared for the Vero cell assay. Therefore, *E. coli* strain C600W34 was incubated in different media (serum, PBS or LB medium with or without MitC) in various concentrations to stimulate Stx2 production. At certain time points, samples were centrifuged with filter tubes for 5min with 10,000g. A dilution series of these supernatants was created in 96-well tissue culture plates (Orange Scientific). Finally, 100µl media containing $2x10^4$ Vero cells was pipetted into each well of these plates with the dilution series of the supernatants. The plates were incubated for 3d in the $CO_2$ incubator at 37°C with 5% $CO_2$.

**Analysis of Vero cell stimulation**

The Vero cell assay was evaluated in two ways:

a.) First, a visual microscopic inspection was performed at certain time points to evaluate under which condition and until which dilution Vero cells were still viable. Dead cells were identified based on detachment.

b.) Second, optical density was measured after staining with crystal violet (Adapted from (Gentry and Dalrymple, 1980; Tran et al., 2014)). After 3 days in the incubator, the supernatants in the plates were carefully discarded. 50µl formalin (2%) was added to each well using the multichannel pipet to fix the cells. After 2min the fluid was gently flicked. Now, 50µl of a mixture of crystal violet (0.13%), ethanol (5%) and formalin (2%) was added to each well for 2min. Afterwards, the fluid was once again gently

flicked out of the plate. Then, the wells were washed with 100µl Ampuwa for 3min and afterwards the fluid was removed again. 150µl 50% ethanol was added and plates were gently shaken. Finally, absorbance (595nm) for each well of these plates was determined using the CLARIOstar plate reader to quantify cells stained by crystal violet. In the result section, the highest dilution at which at least 50% of Vero cells were killed ($CD_{50}$) is depicted as reciprocal value ($1/CD_{50}$).

### 3.2.13 Statistical analysis

Statistical analysis of data obtained from the *in vitro* experiments was performed by using one-way analysis of variance (ANOVA) or unpaired student's t-test with Graphpad Prism Version 5.01. P-values less than 0.05 (2-tailed) were considered as statistically significant: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). P-values>0.05 were considered not significant (ns).

# 4. Results

## 4.1 Establishing a luciferase reporter assay to quantify Stx2 release from *E. coli*

Laboratory handling of most Shiga toxin 2 (Stx2)-producing bacteria puts the researcher at risk of severe infection. EHEC possesses a combination of various virulence factors including Stx, attaching and effacing (AE) lesions and a type 3 secretion system. Therefore, experiments have to be performed under biosafety level 3 (BSL3) conditions. This complicates the conduction of medium- or high-throughput assays to characterize the effect of different supplements on *stx2* expression. Therefore, it was intended to establish an alternative method to quantify Stx2 production by the pathogens. Previously, BSL2 EHEC reporter strains for *stx2* expression were constructed in our laboratory. Stx2 genes were replaced with the gene of Gaussia luciferase (*gluc*) as described in **part 1.9 of the introduction**. The advantages of these reporter strains are **1.)** downgrading to BSL2 and decreased infection risk for investigators and **2.)** use of Gaussia luciferase (Gluc) as straightforward detection method compared to ELISA-based detection of Stx2.

Two different *E. coli* reporter strains with *E. coli* C600 background were used: **1.)** *E. coli stx::gluc*, in which the *gluc* gene is inserted in the *stx2A* locus. This strain harbors phage lysis genes and lyses upon induction of the phage lytic mode. Therefore, Gluc gets released via lysis and its activity can be measured in the supernatant of the bacterial culture. **2.)** *E. coli stx2 sr::gluc* in which *stx2* and phage lysis genes *S* and *R* are replaced by *gluc*. In previous work of the Stecher lab, it was shown that phage-induced lysis is abrogated in this reporter strain (unpublished). As EHEC lacks a transport mechanism for Stx2 and is absolutely dependent on lysis (Pacheco and Sperandio, 2012), it was expected to find increased luminescence levels in the pellet of the bacterial culture (indicating production of Gluc) but not in the culture supernatant (release).

The first step was the establishment of an assay to confirm the functionality of the reporter strains by detecting reporter activity under *stx2* inducing conditions. Until then, luciferase assays were only performed in tubes. As this was unsuitable for a medium-throughput assay, we decided to scale the assay down to a 96-well format. More conditions could be tested at the same time and luminescence levels in the 96-well plates could easily be measured using the CLARIOstar 96-well plate reader. For the establishment of the assay, bacteria were grown in LB medium as this is the standard growth medium for *E. coli*. As the gene of Gaussia luciferase (*gluc*) has been inserted in the *stx2* locus, the

increase of Gaussia activity represents an increased activity of the *stx2* promotor. It was decided to use mitomycin C (MitC) for stimulating the reporter strains due to the fact that this antibiotic is a well-characterized stimulatory agent for *stx2* expression in EHEC (Zhang et al., 2000)(**Introduction part 1.7**). Previous data obtained in our laboratory showed that upon MitC treatment reporter activity increases over time and peaks at around 7h after addition of MitC (data not shown). A pre-culture to synchronize bacterial growth in the logarithmic growth phase was used as compromise between signal strength and feasible timing. Therefore, overday cultures of reporter strains were grown for 2h until an $OD_{600}$ of 0.5. Then, bacterial $OD_{600}$ was adjusted to obtain a bacterial cell number of $5x10^7$/well. Bacteria were then (time point 0) incubated with MitC at a sublethal concentration of 0.5µg/ml in 96-well plates. Every hour, samples were taken and centrifuged. Then, luminescence levels were measured in the supernatants. Already at earlier time points (after 3-5h) a strong signal was detected. Furthermore, the lysis process that is activated during prophage induction becomes apparent at around 3h of incubation (data not shown). Therefore, we decided to test samples after 4h of incubation and also analyze an early sample, at 0.5h, to assess the baseline activity (**Figure 4-1 A**).

### 4.1.1 The Gluc assay in LB shows high luminescence levels and extensive bacterial growth

The assay showed excellent sensitivity and a high dynamic range (with more than 5 orders of magnitude). Stimulation with MitC led to a significant increase in luminescence signal for both strains, thus *stx2* expression was activated as expected (**Figure 4-1 B and C**). Luminescence levels are depicted for both strains with or without stimulation with MitC. LB without bacteria is depicted as a control for background luminescence. The lytic strain *E. coli stx::gluc* provided 20-fold higher luminescence levels in the supernatant compared to the non-lytic strain *E. coli stx2 sr::gluc* (**Figure 4-1 B**). Like Stx2, Gaussia luciferase (Gluc) is not actively exported out of the bacterial cell and is only released if the cell lyses (reviewed in Shimizu et al., 2009). As the lysis gene knockout strain (*E. coli stx2 sr::gluc)* does not have the ability to lyse via phage lysis, less Gluc activity was measured in the supernatant. However, still a significant increase in luminescence could be detected for this strain. We assumed that the toxic effect of MitC is sufficient to kill a fraction of the bacteria. Therefore, Gluc is set free due to the disturbance of the cell wall integrity. An increase of luminescence levels could also be seen without stimulation with MitC, especially for *E. coli stx::gluc*. This could be explained by spontaneous prophage activity in a fraction of the bacteria, an effect that has previously been described (Livny and Friedman, 2004).

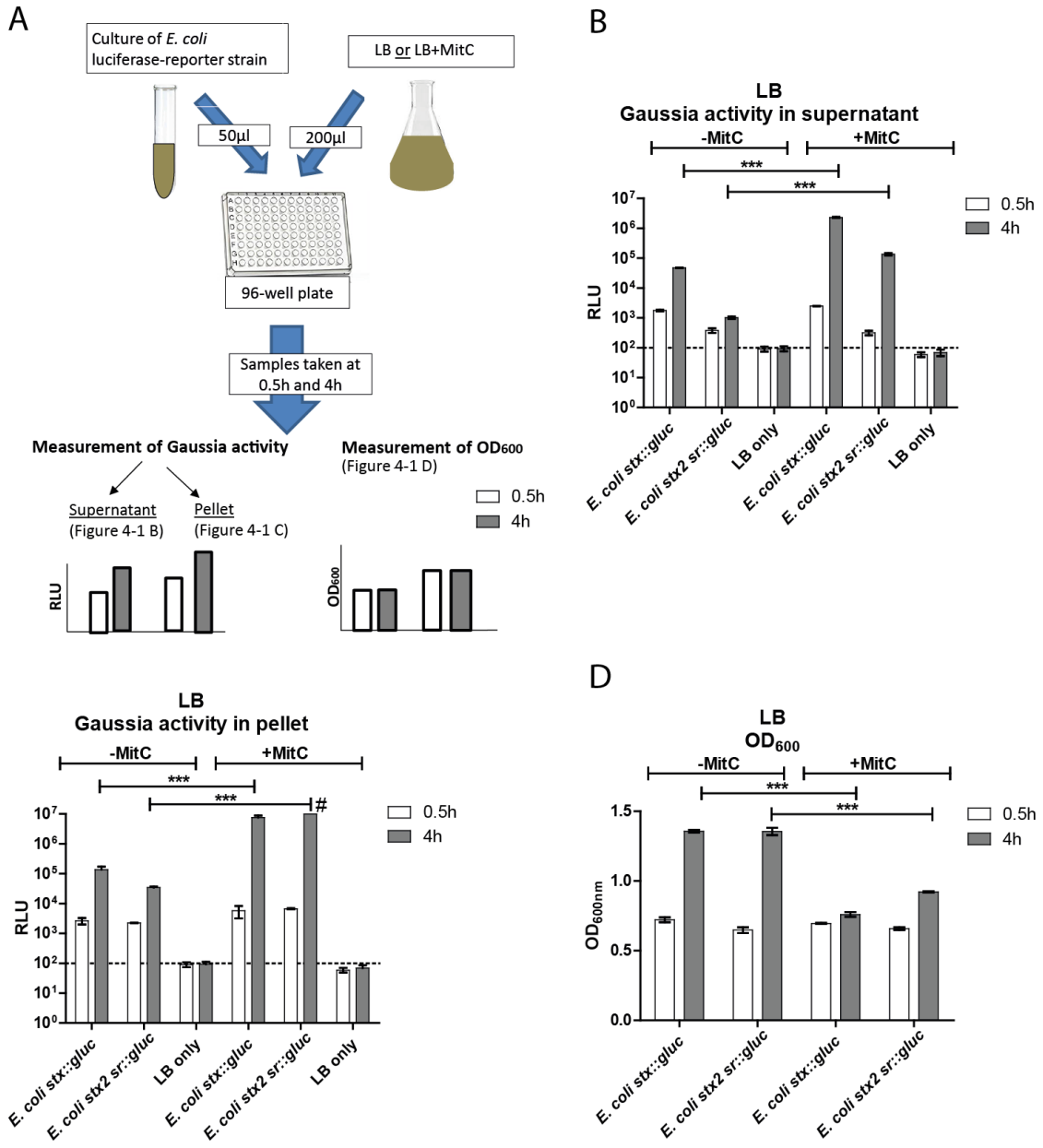


**Figure 4-1 Gaussia luciferase (Gluc) reporter assay of reporter strains grown in LB** Setup of the experiment (**A**): Precultures of *E.coli* luciferase-reporter strains *stx::gluc* (lysis-proficient) and *stx2 sr::gluc (*lysis-deficient) were grown in LB medium until mid-log phase ($OD_{600}$ of approximately 0.5). After centrifugation, bacteria were suspended in LB to a final concentration of $1x10^9$ bacteria/ml. 50µl containing $5x10^7$ bacteria were transferred into a 96-well plate. 200µl of either LB alone or LB containing mitomycin C (MitC) at a final concentration of 0.5µg/ml was added. Samples were taken after 0.5h and 4h after incubation at 37°C while shaking. Gaussia activity was measured as described in the method section in the culture supernatants (**B**) and in the bacterial pellets (**C**) and is depicted as RLU (relative luminescence units) per well. Additionally, optical density at 600nm ($OD_{600}$) was determined after 0.5h and 4h (**D**). Dotted lines: Background luminescence levels of the assay (LB only). #=saturated. Data is shown as means ± standard deviation – results are representative for three independent experiments measured in triplicates. Statistical analysis was performed to compare MitC-treated and non-treated strains at the 4h time point by using the t-test. Levels of significance: ns p>0.05, * p<0.05, **p<0.01, ***p<0.001.

For the same conditions and strains, luminescence levels were measured in the pellets to detect Gluc that was produced within the bacterial cells but not (yet) released (**Figure 4-1 C)**. Stimulation with MitC led to a significant increase in luminescence for both strains what confirmed the functionality of both strains to produce Gluc. An increase of reporter activity was found for both strains even without MitC stimulation – this supported the notion that spontaneous prophage induction and growth effects had an impact. Bacterial growth was measured for both strains via determination of the optical density at 600nm ($OD_{600}$) (**Figure 4-1 D**). The initial $OD_{600}$ of all experiments was approximately 0.5. At the 0.5h time point, growth of both conditions (with or without MitC) was comparable. After 4h, growth of MitC-treated strains was restricted whereas unstimulated strains showed logarithmic growth and increase of $OD_{600}$. Stimulation with MitC induced bacterial lysis and therefore led to bacterial death. Phage-mediated lysis requires a certain amount of time (3-5h). This explains why effects on bacterial growth are seen at the 4h time point in this experiment.

The reporter assay in LB revealed some drawbacks. Luminescence levels increased considerably even without MitC stimulation (**Figure 4-1 B and C**). This increase is presumably explained by bacterial growth and spontaneous induction of the phage lytic development. Hence, it is difficult to determine whether the increased reporter activity is due to bacterial growth effects or to prophage induction and lysis caused by MitC stimulation. This is a clear limitation of the Gluc reporter assay in LB.

### 4.1.2 Performing the Gluc assay in PBS limits growth-dependent interference

After discovering that the Gluc assay in LB involves problems based on bacterial growth, it was decided to establish the assay in PBS in order to restrict bacterial growth due to nutrient limitation. The setup of the experiment was the same as explained in **Figure 4-1 A** with the exception that PBS was used instead of LB medium. Generally, it could be seen that luminescence levels in PBS were lower than in LB (**Figure 4-2**). Nevertheless, a significant prophage induction by MitC was detected in the supernatant (**Figure 4-2 A**) and the pellet **(Figure 4-2 B)** of both reporter strains (*E. coli stx::gluc* and *E. coli stx2 sr::gluc*). In the supernatant of the lytic strain, a 17-fold increase of luminescence levels was triggered by MitC. After 4h, the lytic strain provided a higher luminescence level than the not-lytic strain in the supernatant – proving that bacterial lysis is a key step in releasing luciferase and Stx2. In the pellets, a significant increase in luminescence upon MitC stimulation was detected for both strains after 4h. This indicated that the stimulation with MitC was effective.

The obvious advantage of conducting the assay in PBS was the significant reduction of growth of the reporter strains and therefore growth-related interferences with Gluc production (**Figure 4-2 C).** The initial $OD_{600}$ of 0.5 of the unstimulated strains remained constant over the course of time. MitC stimulation led to a decrease of $OD_{600}$ of the lysis-proficient strain *E. coli stx::gluc (***Figure 4-2 C***)*. This was in accordance with the expression of lysis genes for release of Gluc. Increase of Gaussia activity in this experiment could not be explained by bacterial growth effects but only by MitC-stimulated

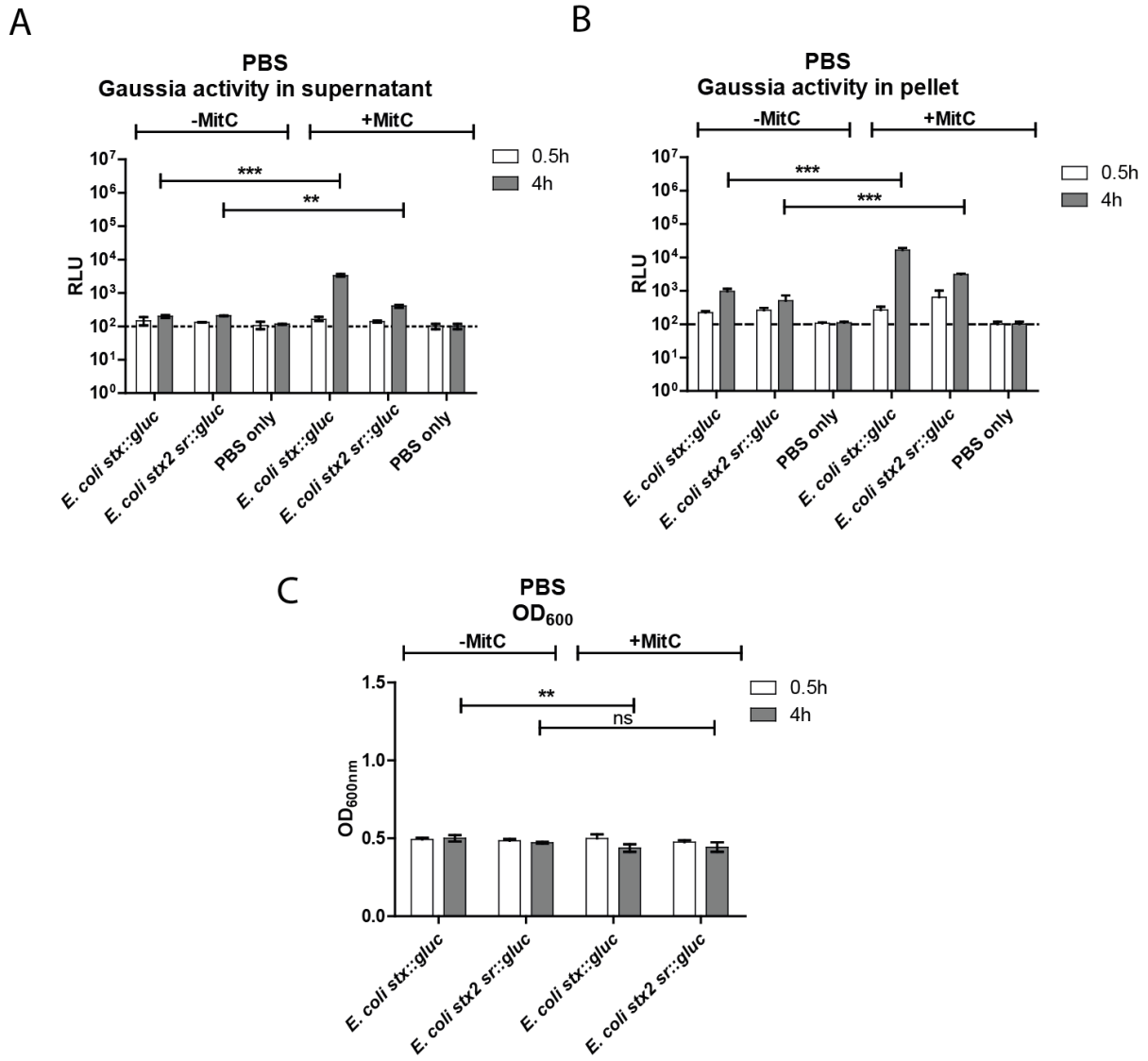


**Figure 4-2 Gaussia luciferase (Gluc) reporter assay in PBS** The experiment was performed as described above in Figure 4-1 A. Only this time bacteria were suspended in PBS. Gaussia activity was measured as described in methods in the supernatant (**A**) and in the pellets (**B**) and is depicted at RLU per well. Additionally, optical density at 600nm ($OD_{600}$) was determined (**C**). Data is shown as means ± standard deviation – results are representative for three independent experiments measured in triplicates. Statistical analysis was performed to compare MitC-treated and non-treated strains at the 4h time point by using the t-test.

prophage induction. Therefore, it was decided to conduct further experiments using the PBS-based assay as *gluc/stx2* induction should be detected as precisely as possible.

Concluding, an assay with Gluc reporter strains was established to study *gluc/stx2* expression. Conducting the assay in LB led to a high increase in luminescence levels due to concomitant bacterial growth. This way, stimulation of *gluc*/*stx2* expression could not be analyzed precisely as bacterial growth also led to an increase of Gluc/Stx2 activity over time. Bacterial growth in PBS was restricted and therefore allowed uncoupling growth from increase in reporter activity. Therefore, we decided to use PBS as medium for further experiments.

## 4.2 Exposure to polymorphonuclear neutrophils (PMNs) induces *stx2* expression

After establishing the assay, the next aim was to reproduce and confirm a result that has previously been shown by Wagner and colleagues. This research group determined by ELISA that exposure to isolated human blood PMNs causes an increase of Stx2 production (Wagner et al., 2001a). For my experiment, human PMNs were isolated and suspended in PBS. Afterwards, PMNs were incubated with Gluc reporter strains for 4h. This time, the lytic *E. coli stx::gluc* (**Figure 4-3 A**) and a lytic *Citrobacter rodentium* strain carrying the reporter gene (**Figure 4-3 B**), were used. *C. rodentium* is a pathogen that is used for studying EHEC infections in mouse models (Mallick et al., 2012)(**Introduction part 1.9)**. *C. rodentium stx::gluc* has comparable characteristics as *E. coli stx::gluc*. *stx2A* is replaced by the *gluc* gene and release of Gluc is dependent on lysis. The two strains were chosen to detect whether effects were reproducible irrespective of the reporter strain background.

Gaussia activity of reporter strains exposed to different numbers of PMNs was evaluated (**Figure 4-3)**. Furthermore, negative controls (PMNs and PBS without reporter bacteria) were included to detect

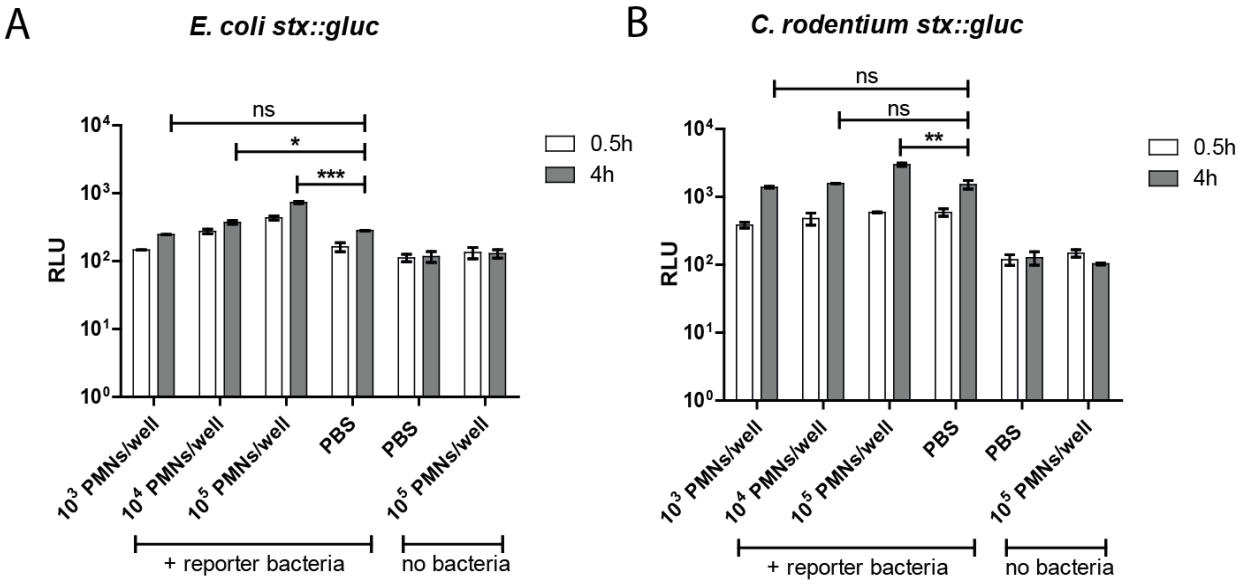


**Figure 4-3 PMNs cause a significant but slight increase in luminescence** PMNs were isolated and suspended in PBS. Different numbers of PMNs ($10^3$-$10^5$ PMNs/well) were incubated with or without *E. coli* luciferase-reporter strain *stx::gluc* (**A**) or *C. rodentium* luciferase-reporter strain *stx::gluc* (**B**) for 4h in a 96-well plate ($5x10^7$ bacteria/well). Samples were taken at different time points. Gaussia activity was measured in the culture supernatants after centrifugation and is depicted as RLU per well. Data is shown as means ± standard deviation – results are representative for three independent experiments measured in duplicates. ANOVA and Tukey's test were performed to compare selected treatment conditions. Only selected comparisons are depicted. [MOI/well: Bacteria/PMN=50.000, 5000 and 500]

interfering effects between PBS, PMNs and the Gluc substrate coelenterazine (CTZ). A significant increase of Gluc production and release could be seen for both reporter strains when incubated with a PMN concentration of $10^5$ per well after 4h (**Figure 4-3 A and B**). In comparison to the signal levels after stimulation with MitC (17-fold higher than bacteria in PBS only), the effects of PMNs were rather subtle (2.6-fold). Lower numbers of PMNs ($10^3$/well) and the negative controls did not cause a significant increase of Gluc activity. In conclusion, PMNs induced *gluc* expression in the Gluc reporter assay and we could confirm the results of Wagner et al.

## 4.3 Hydrogen peroxide impairs bacterial viability and promotes bacterial lysis and Gluc release

After observing that PMNs induce *gluc/stx2* expression, I set out to determine the underlying mechanisms of the effects of PMNs in the Gluc reporter assay. PMNs are a source of reactive oxygen species (ROS) (Freitas et al., 2009). Therefore, I next investigated if *gluc*/*stx2* expression is induced by PMN effector molecules. Hydrogen peroxide ($H_2O_2$) is one of the major components of ROS produced and released by PMNs (reviewed in Rhee, 1999). $H_2O_2$ is produced by human cells via generation of the superoxide anion radical that is afterwards transformed to $H_2O_2$ (McCord and Fridovich, 1969). It is an important part of the defense against microbial intruders but can also cause tissue damage (reviewed in Halliwell et al., 2000). The impact of this chemical agent was investigated in the Gluc reporter assay. It has previously been shown that $H_2O_2$ is a prophage inductor. Los and colleagues described that the optimal $H_2O_2$ concentration for prophage induction is in the low mM-range (Los et al., 2010). It was intended to reproduce these results in the Gluc assay.

### 4.3.1 $H_2O_2$ interferes with Gluc enzyme activity at concentrations above 20mM

Before studying the effects of $H_2O_2$ on reporter strains, we intended to determine the effect of $H_2O_2$ on Gaussia luciferase or its substrate coelenterazine (CTZ). Detection of luminescence is dependent on the oxidative decarboxylation of CTZ by Gluc (Verhaegent and Christopoulos, 2002). If $H_2O_2$ had a destructive impact on the enzyme or its substrate, results could be biased. For this aim, the supernatants of MitC-stimulated positive cultures were taken. These samples offered reporter activity at the highest possible level and were created to confirm the efficacy of the assays as positive controls. Different $H_2O_2$ concentrations were added to these supernatants in 96-well plates. After 4h,

luminescence was measured in the supernatants and compared to the condition not exposed to $H_2O_2$ (**Figure 4-4 A**).

As expected, the supernatants of the positive controls that were not exposed to $H_2O_2$ offered high signal levels. $H_2O_2$ concentrations ≤ 20mM did not lead to a significant alteration of the high luminescence levels of the positive controls (**Figure 4-4 B**). Concentrations of $H_2O_2$ > 20mM, however, inhibited Gluc activity as a significant decrease in luminescence could be observed. Thus, with the Gluc assay we could not evaluate if $H_2O_2$ concentrations > 20mM had an effect on *gluc*/*stx2* expression due to the destroying impact of these concentrations on Gluc. Concluding, only $H_2O_2$ concentrations ≤ 20mM did not interact with the Gluc enzyme or its substrate CTZ and could therefore be evaluated in the Gluc assay.

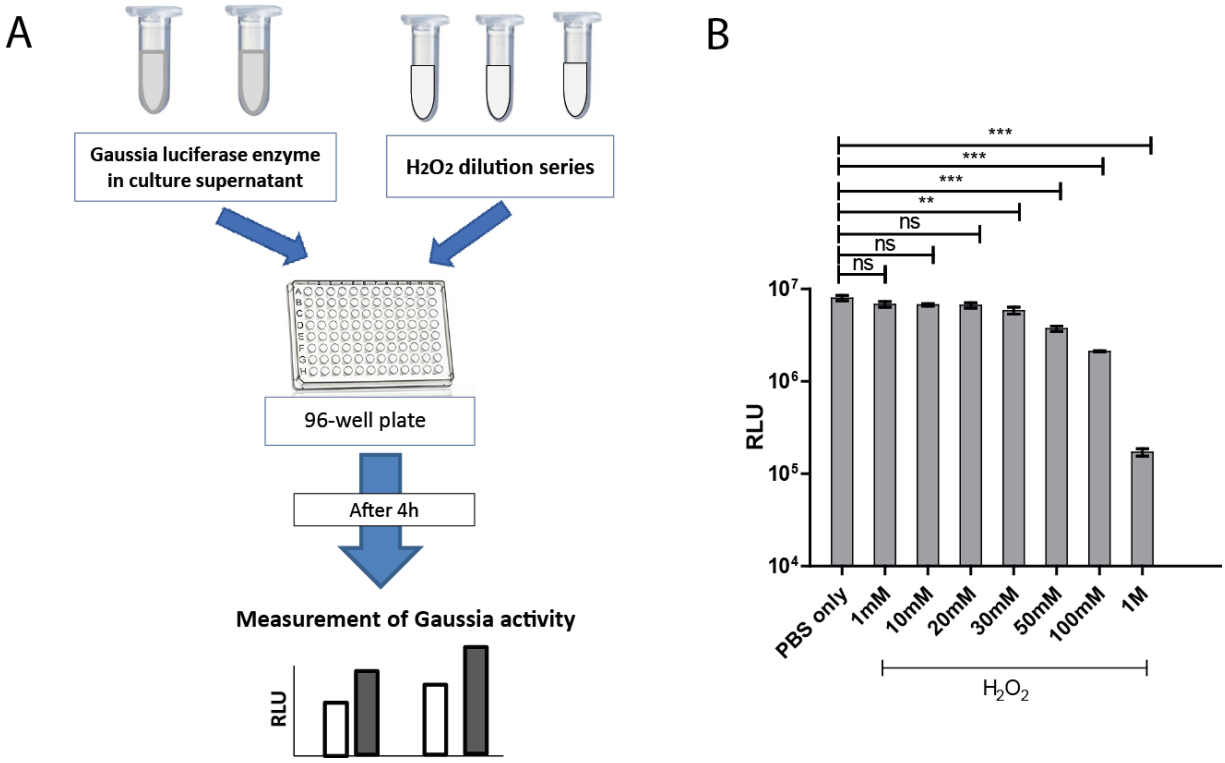


**Figure 4-4 Effects of hydrogen peroxide on Gaussia luciferase** Setup of the experiment (**A**): Gaussia luciferase positive controls (supernatants of *E. coli stx::gluc* stimulated with MitC) were exposed to different concentrations of hydrogen peroxide ($H_2O_2$) for 4h in a 96-well plate. After 4h, the samples were centrifuged and Gaussia activity was measured in the supernatants and is depicted as RLU per well (**B**). ANOVA and Tukey's test were performed to compare the effect of different concentrations. Data is shown as means ± standard deviation – results are representative for two independent experiments measured in duplicates.

### 4.3.2 $H_2O_2$ leads to bacterial lysis and release of the Gluc reporter

Next, the influence of $H_2O_2$ on bacterial survival and luminescence levels was determined. The setup of the Gluc assay with $H_2O_2$ was similar to **Figure 4-1 and 4-2** with the exception that bacteria were exposed to different $H_2O_2$ concentrations instead of LB or PBS with MitC. Two methods for determining the effects of $H_2O_2$ on bacterial survival were used (**Figure 4-5)**. First, $OD_{600}$ was measured. For this purpose, bacteria in PBS that were not exposed to $H_2O_2$ were compared to those exposed to different $H_2O_2$ concentrations. A concentration-dependent impact on bacterial survival was found (**Figure 4-5 A)**. The more $H_2O_2$ was added, the lower was the $OD_{600}$. These effects could already be seen after 0.5h but were enhanced at the 4h time point. Furthermore, culture samples were taken and colony forming units (CFU) *E. coli* per well were determined (**Figure 4-5 B)**. After 4h, significantly fewer live bacteria could be detected for all $H_2O_2$ concentrations in comparison to bacteria not exposed to $H_2O_2$. The 20mM $H_2O_2$ concentration caused a decrease of CFU/well below the limit of detection (LOD). This is in accordance to findings that bacteria are killed by $H_2O_2$ in the mM-range (Winterbourn et al., 2016).

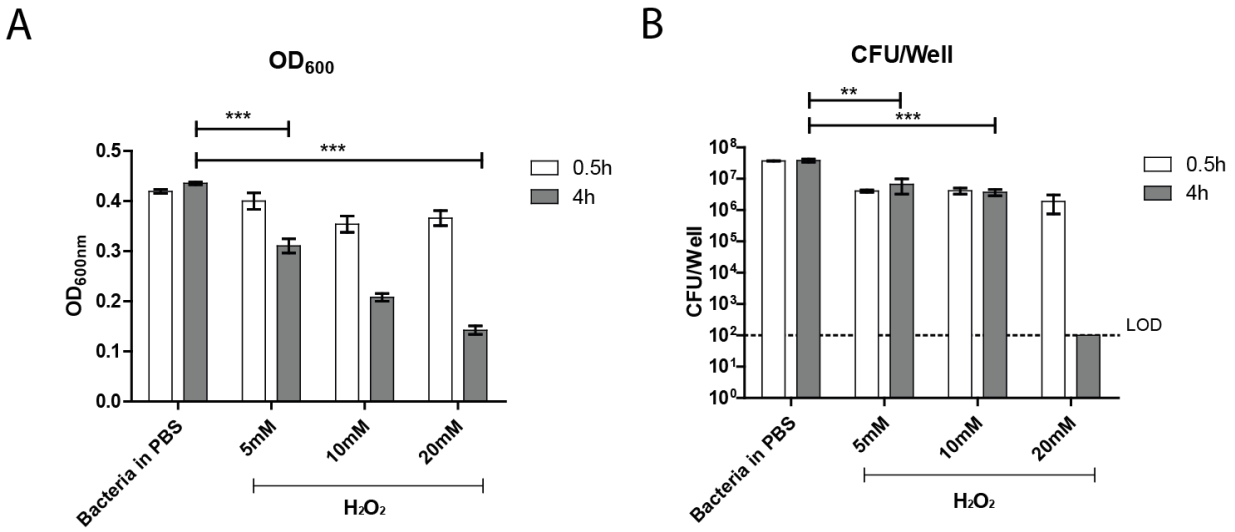


**Figure 4-5 Survival of bacteria is affected by different concentrations of hydrogen peroxide** *E. coli* luciferase-reporter strain *stx::gluc* was grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards bacteria were centrifuged and suspended in PBS to a concentration of $5x10^7$ bacteria/well (=$1x10^9$ bacteria/ml). Bacteria were exposed to different concentrations of hydrogen peroxide ($H_2O_2$) for 4 hours in a 96-well plate. The $OD_{600}$ was measured at different time points (**A**). At the same times samples were taken and CFU *E. coli*/well were determined by plating (**B**). Results are representative for three experiments of triplicate cultures. ANOVA and Tukey's test were performed to compare treatment conditions – only selected comparisons are depicted.

To measure the influence of $H_2O_2$ on Gluc activity, samples of the reporter strain culture in the 96-well plates were taken at two time points (0.5h and 4h) and the supernatants were used for measurement of Gluc activity. A concentration-dependent effect of $H_2O_2$ in the Gluc assay could be seen over the course of time (**Figure 4-6 A)**. The 4h time point is shown in **Figure 4-6 B**. The highest increase in luminescence was observed in the range between 10-20mM. For those concentrations a significant increase of luminescence could be observed between 0.5h and 2h. Afterwards, only a slight further increase was detected. Lower (<10mM) $H_2O_2$ concentrations did not result in a significant reporter activity in the assay.

Furthermore, we wanted to rule out interactions between $H_2O_2$ and the Gluc substrate coelenterazine (CTZ). As mentioned before, Gluc triggers the oxidative decarboxylation of CTZ and this process leads to light emission (**part 1.9 of the introduction**). Luminescence levels of $H_2O_2$ dilutions that were not exposed to *E. coli stx::gluc* were measured to detect if $H_2O_2$ could trigger the oxidative decarboxylation of CTZ and therefore increase luminescence levels (**Figure 4-6 B**). It was found that $H_2O_2$ dilutions without reporter bacteria did not interfere with CTZ as luminescence levels remained low. Concluding, we observed that certain concentrations of $H_2O_2$ (10-20mM) trigger a significant increase of luminescence activity in the Gluc reporter assay. At the same time, bacterial growth and survival were affected when bacteria were exposed to those $H_2O_2$ concentrations.

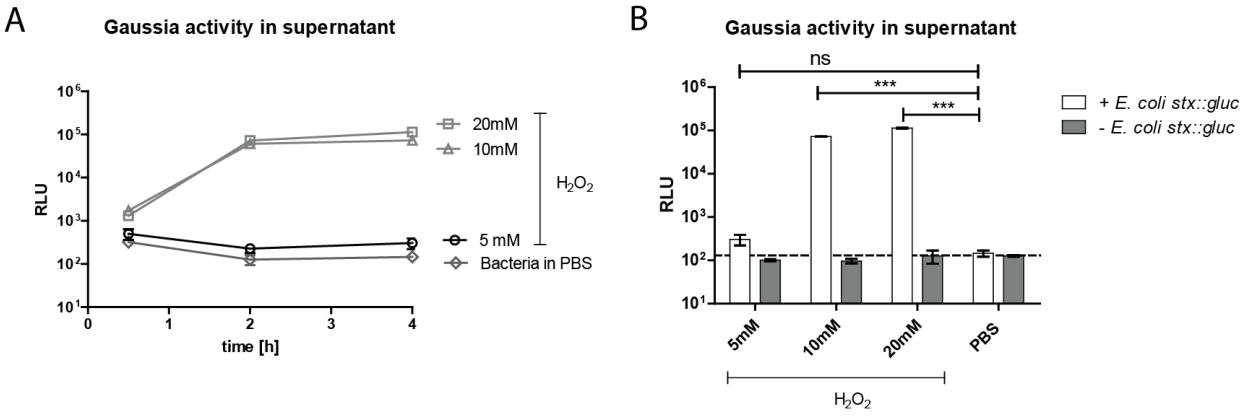


**Figure 4-6 Addition of hydrogen peroxide results in increase of Gaussia activity in culture supernatants** *E. coli* luciferase-reporter strain *stx::gluc* (lysis-competent) was grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards, bacteria were centrifuged and resuspended in PBS. Bacteria were exposed to different concentrations of $H_2O_2$ for 4h in a 96-well plate. Gaussia activity was measured in the supernatant after centrifugation of the samples and is represented as time course (**A**) and in comparison to $H_2O_2$ activity without bacteria and the background (dotted line: bacteria in PBS without addition of $H_2O_2$) after 4h (**B**). Gaussia activity is depicted as RLU per well. Results are representative for three experiments of triplicate cultures. ANOVA and Tukey's test were performed to compare treatment conditions – only selected comparisons are depicted

### 4.3.3 $H_2O_2$ does not have an inducing effect on *stx2* expression

I assumed that the observed increase of luminescence activity triggered by $H_2O_2$ could happen in two ways: Either $H_2O_2$ leads to bacterial lysis and concomitant Gluc release or, alternatively, to the induction of *stx2* gene expression and subsequent phage lysis. To distinguish between these two mechanisms, I determined if the observed increase of reporter activity after exposure to $H_2O_2$ was dependent on protein synthesis. Chloramphenicol (Cm) was used to inhibit bacterial protein synthesis. The minimal inhibitory concentration (MIC) for *E. coli stx::gluc* was determined to be 2µg/ml. First, bacteria were incubated with the MIC. Afterwards, bacteria were exposed to different $H_2O_2$ concentrations for 4h as in the experiments before. Inhibition of protein synthesis did not have an impact on Gaussia activity after 4h (**Figure 4-7 A)**. No significant alteration in luminescence was registered at any $H_2O_2$ concentration when bacteria were pre-treated with Cm. Especially the 10mM $H_2O_2$ concentration was of interest as it showed the highest reporter activity in the previous experiments. However, inhibition of protein synthesis with Cm did not alter luminescence levels. Again, $OD_{600}$ strongly decreased when bacteria were exposed to higher $H_2O_2$ concentrations but Cm did not have any altering impact (**Figure 4-7 B**). These results indicated that stimulation of Gaussia activity by $H_2O_2$ was not caused by prophage induction but rather through bacterial lysis. We assumed that Gluc was pre-formed within bacterial cells. Through lysis effects by $H_2O_2$ these pre-formed proteins were able to leave the bacterial cells.

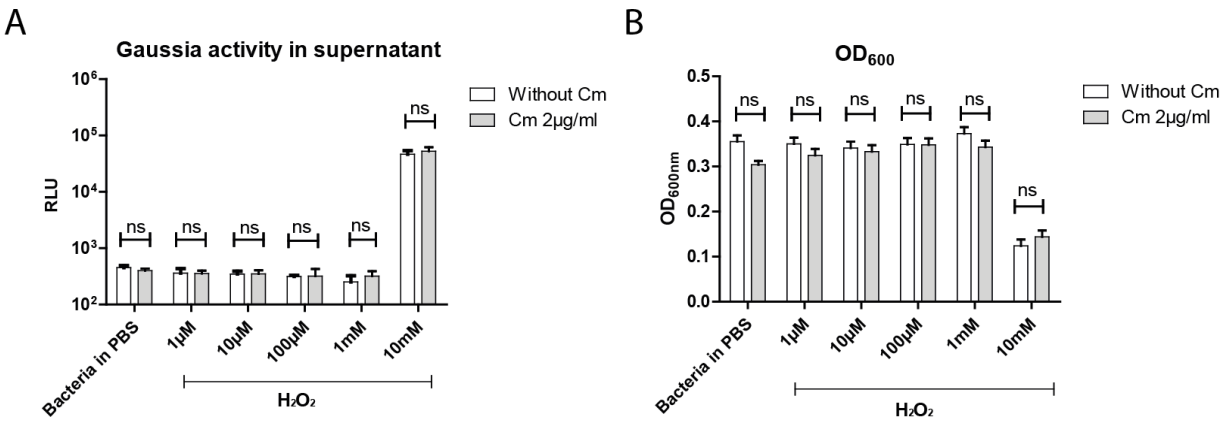


**Figure 4-7 Effects of hydrogen peroxide are not caused by gene induction** *E. coli* luciferase-reporter strain *stx::gluc* was grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards bacteria were centrifuged and suspended in PBS with or without chloramphenicol (Cm). After 30 minutes, bacteria were exposed to different concentrations of hydrogen peroxide ($H_2O_2$) for 4h in a 96-well plate. Gaussia activity was measured after 4h in the supernatant after centrifugation of the samples (**A**) and is depicted as RLU per well. At the same time $OD_{600}$ was determined (**B**). t-test was performed to compare bacteria without Cm treatment with those pre-treated with 2µg/ml Cm (determined as MIC). Data is shown as means ± standard deviation – results are representative for two independent experiments measured in duplicates.

According to these findings, $H_2O_2$ increased Gluc activity primarily via bacterial lysis and not by prophage induction. However, we assumed that it could be possible that the overall profound effects of cell lysis covered slight effects of $H_2O_2$ on actual prophage induction and gene expression. Therefore, it was investigated whether $H_2O_2$ concentrations lower than 10mM (1µM to 1mM) could possibly cause *gluc* induction while not being potent enough to lyse the cells. For that reason, a $\log_{10}$ dilution series of $H_2O_2$ was created. Afterwards, reporter strains were exposed to these concentrations for 4h. This time, activity was not only measured in supernatants but also in the pellets to detect produced Gluc within the cells (**Figure 4-8**). $H_2O_2$ concentrations below 10mM had no effect on Gaussia activity that would have indicated prophage induction. Concentrations < 10mM were therefore neither able to cause prophage induction nor to cause bacterial lysis.

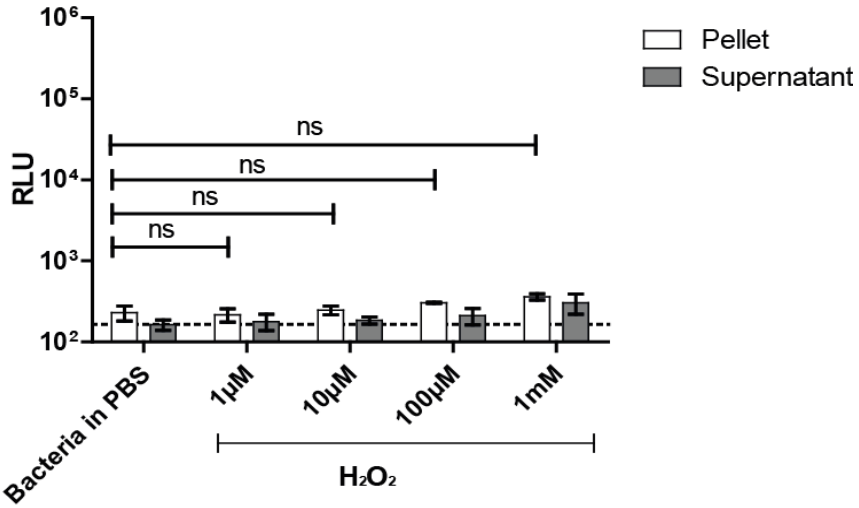


**Figure 4-8 Hydrogen peroxide concentrations below 10mM do not induce *stx2* expression** *E. coli* luciferase-reporter strain *stx::gluc* (lysis-competent) was grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards bacteria were centrifuged and suspended in PBS ($5x10^7$ bacteria/well). Bacteria were exposed to different concentrations of hydrogen peroxide ($H_2O_2$) for 4 hours in a 96-well plate. Gaussia activity was measured after 4h in the supernatant after centrifugation of the samples as well as in the pellets and is depicted as RLU per well. t-test was performed to compare luminescence levels in the pellets of bacteria exposed to various $H_2O_2$ concentrations with bacteria in PBS. Data is shown as means ± standard deviation – results are representative for three independent experiments measured in duplicates.

### 4.3.4 $H_2O_2$ leads to Gluc release in lysis-deficient reporter bacteria

$H_2O_2$ concentrations of 10-20mM led to an increase of Gluc activity that was not altered by inhibition of protein synthesis. Until now, only the lysis-competent *E. coli* strain *stx::gluc* was used for experiments investigating the effects of $H_2O_2$ in the Gluc assay. Therefore, it was intended to reproduce the effects of $H_2O_2$ with various reporter strains. For this aim, the non-lytic *E. coli* strain *stx2 sr::gluc* and the non-lytic *C. rodentium* strain *stx2 sr::gluc* were used and exposed to a $log_{10}$ dilution series of $H_2O_2$. These strains do not have the ability to lyse via phage lysis. However, if $H_2O_2$ mediates an increase of Gluc via bacterial lysis, these strains should also show an increased luminescence when exposed to $H_2O_2$. After 4h, the effects of $H_2O_2$ were comparable among all strains (**Figure 4-9 A).** All three reporter strains showed the highest luminescence levels when exposed to 10mM $H_2O_2$. Gaussia activity reached comparable levels in lytic and non-lytic strains. This was a further indication that prophage-independent lysis was the underlying mechanism. $H_2O_2$ concentrations below 10mM led to significantly lower reporter activity. Measurement of $OD_{600}$ revealed comparable results for the different strains (**Figure 4-9 B**). All strains started with an $OD_{600}$ of 0.5. After 4h, a significant decrease of $OD_{600}$ was observed after exposure to the $H_2O_2$ concentration of 10mM whereas $H_2O_2$ concentrations below 10mM did not cause a significant alteration of growth.

Taken together, 10mM $H_2O_2$ caused the highest increase of reporter activity in all experiments while causing a significant reduction of $OD_{600}$ at the same time. Therefore, the increase in luminescence was correlated with the decrease of $OD_{600}$ for the 10mM $H_2O_2$ condition. Inhibition of protein synthesis and use of prophage lysis-deficient reporter strains showed that there was no effect of $H_2O_2$ on *stx2* expression and prophage induction. Therefore, $H_2O_2$ mediates increase in Gluc activity via induction of prophage-independent lysis.

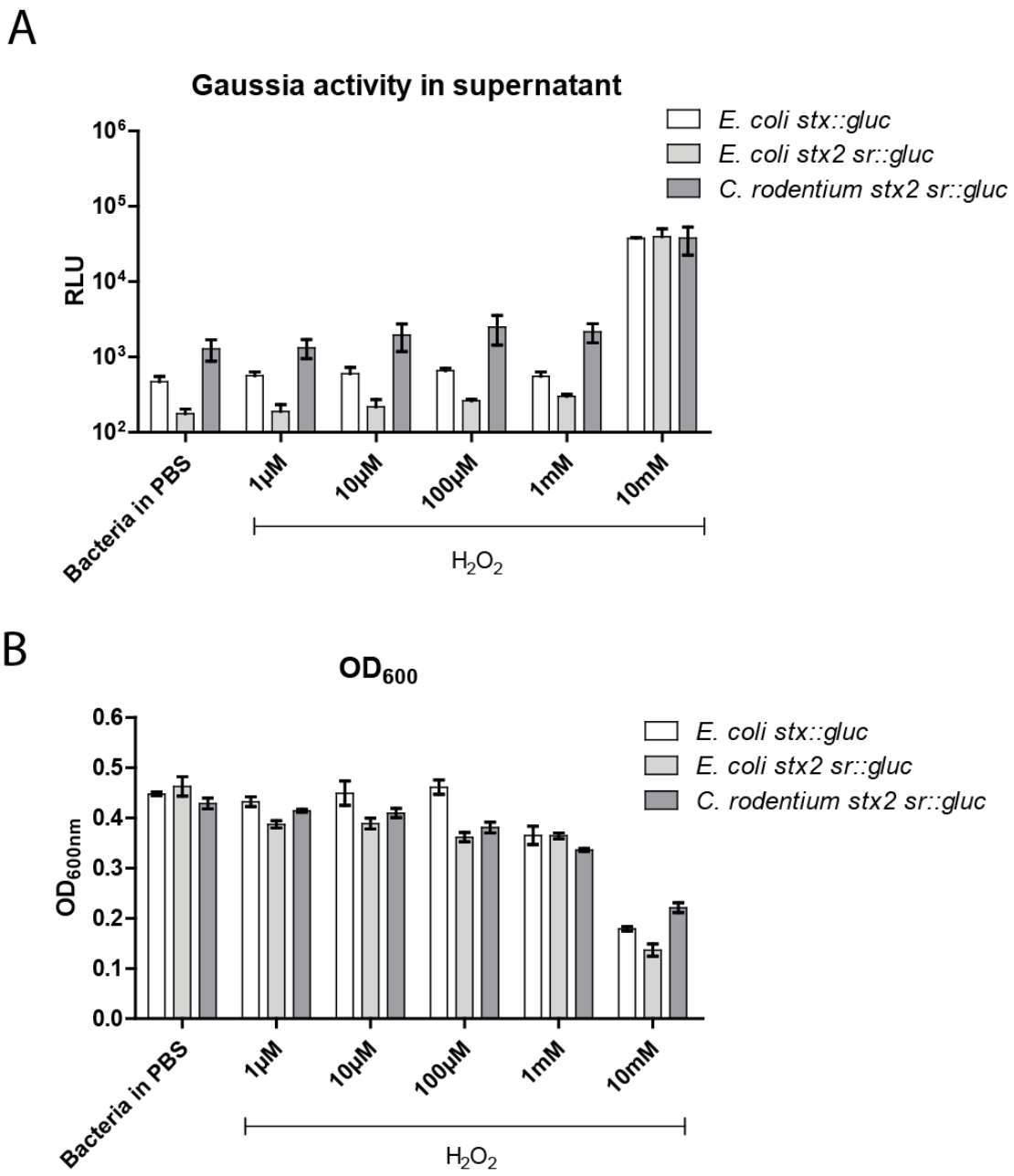


**Figure 4-9 Effects of hydrogen peroxide on lysis-competent and -deficient reporter strains** *E. coli* luciferase-reporter strains *stx::gluc* (lysis-competent) and *stx2 sr::gluc* (lysis-deficient) as well as *C. rodentium* luciferase-reporter strain *stx2 sr::gluc* (lysis-deficient) were grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards, bacteria were centrifuged and suspended in PBS. Bacteria were exposed to different concentrations of hydrogen peroxide ($H_2O_2$) for 4h in a 96-well plate. Gaussia activity was measured after 4h in the supernatant after centrifugation of the samples (**A**) and is depicted as RLU per well. At the same time, $OD_{600}$ of the cultures was determined (**B**). Data is shown as means ± standard deviation – results are representative for three independent experiments measured in duplicates.

## 4.4 Quantification of $H_2O_2$ produced by isolated human PMNs

The experiments with $H_2O_2$ showed that a concentration of at least 10mM is necessary for a significant release of Gluc and increase of luminescence levels. With this knowledge the next question was posed: What concentrations of $H_2O_2$ are produced by PMNs during oxidative burst *in vitro*? We continued by quantifying the production and release of $H_2O_2$ by human PMNs. For this aim, PMNs from human blood were isolated and used in two different assays.

### 4.4.1 The intracellular amount of $H_2O_2$ in human PMNs lies in the low µM range

Measurement of intracellular ROS is difficult because the intracellular oxidant products are instable and reactive (Jakubowski and Bartosz, 2000). We decided to use dichlorofluorescin diacetate (DCFDA) to measure the concentration of $H_2O_2$ produced by PMNs within the cells. This substance is widely used for measurement of cellular ROS production (Jakubowski and Bartosz, 2000). To become fluorescent, two steps are required after DCFDA enters the cell via diffusion: **a.)** The acetate groups of the substrate must be removed by intracellular esterases and **b.)** Oxidation takes place within the cell (Eruslanov and Kusmartsev, 2010). The presence of ROS leads to the transformation of DCFDA to the fluorescent dichlorofluorescein (DCF). Increase in fluorescence can afterwards be measured with a microplate reader.

PMNs were isolated from human blood and incubated in different numbers with DCFDA in 96-well plates. Negative controls of DCFDA without PMNs were included. In the course of 2h, fluorescence was measured at an excitation of 485nm and an emission of 520nm. After 2h, a significant increase in fluorescence could be detected for higher numbers of PMNs in comparison to wells without PMNs **(Figure 4-10 A)**. The more PMNs were added, the higher was the fluorescence detected. This development could also be observed over the course of time (**Figure 4-10 B)**. Highest numbers of PMNs ($5x10^6$/ml) showed a significant signal increase in the course of 2h. Furthermore, the oxidative burst of PMNs was stimulated by addition of 5µM phorbol-12-myristate-13-acetate (PMA). PMA is a well-described inductor of the PMN's oxidative burst (Chacko et al., 2013). It could be detected that unstimulated PMNs did not at all exhaust their ROS production ability as PMA stimulation led to a significant increase in fluorescence. After 1h, an 8-fold higher fluorescence signal was achieved via stimulation with PMA. After 2h, the assay was already saturated for the highest number of PMNs.

Finally, stimulation of PMNs in different conditions was compared **(Figure 4-10 C)**. Therefore, not only PMA but also bacteria (*E. coli* C600, $5x10^7$/well) were taken to stimulate PMNs. We hypothesized that the confrontation with bacteria could trigger an elevation of ROS production. Exposure to PMA and bacteria led to an increase in fluorescence in comparison to unstimulated PMNs (**Figure 4-10 C)**. Comparison with fluorescence levels triggered by different concentrations of $H_2O_2$ showed that the concentration of $H_2O_2$ produced by $5x10^6$ PMNs/ml was in the µM range (not depicted).

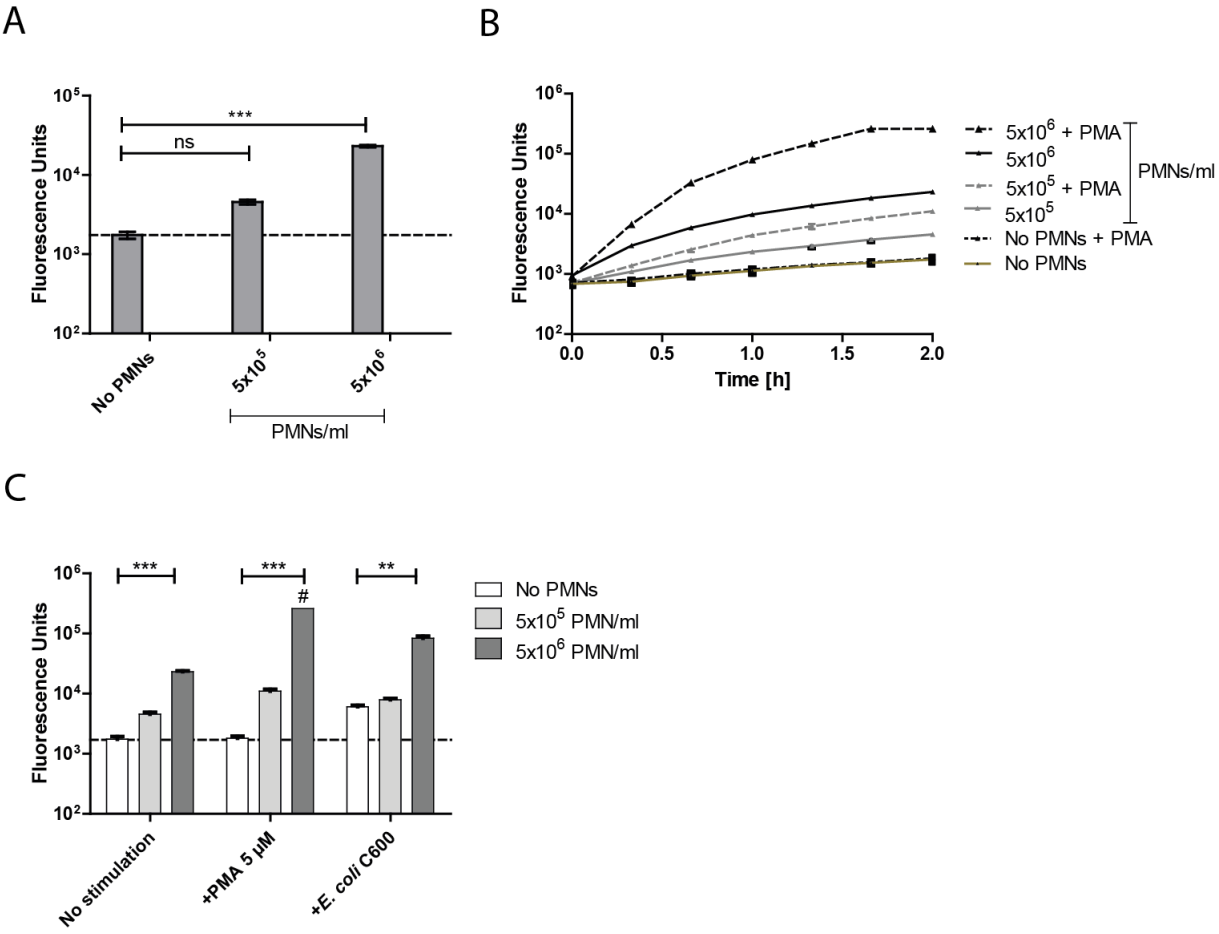


**Figure 4-10 Intracellular ROS production in PMNs** PMNs (polymorphonuclear neutrophils) were isolated and suspended in prewarmed PBS buffer containing 50µM DCFDA (dichlorofluorescin diacetate) solution for 30min. Afterwards, fluorescence units were measured at an excitation of 490nm and an emission of 525nm over the course of 2h. Fluorescence per well is depicted for different PMN numbers: after 2h (**A**), with or without additional stimulation of 5µM PMA over the course of time (**B**) and for three different conditions after 2h (**C**). (**A**) ANOVA and Tukey's test were performed. (**C**) t-test was performed to compare treatment conditions. Data is shown as means ± standard deviation. [Bacteria: $5x10^7$/well → MOI: 500 and 5000] # = saturated.

### 4.4.2 The amount of $H_2O_2$ released by isolated human PMNs is in the µM range

After the quantification of the intracellular concentration of $H_2O_2$ we wanted to detect the $H_2O_2$ concentration that is actually released by human PMNs. For this purpose, another substrate mix that included Ampliflu and horseradish peroxidase (HRP) was used as described in the methods (Zhou et al., 1997). Ampliflu is a normally colorless substrate that is transformed to red-fluorescent resorufin by $H_2O_2$ (reviewed in Mishin et al., 2010). Different numbers of PMNs were incubated with the detection solution for 2h. Fluorescence was measured over the course of the time. Additionally, PMNs were stimulated in two different ways: **a.)** by addition of 5µM PMA **b.)** by addition of bacteria (*E. coli* C600, $5x10^7$/well) (**Figure 4-11 A)**. After stimulation with PMA, a significant increase in fluorescence could be detected. Unstimulated PMNs and PMNs exposed to bacteria caused only a small increase of fluorescence levels. The condition with PMA stimulation was followed over the course of time and showed a dynamic increase in fluorescence only for the highest number of PMNs (**Figure 4-11 B).** Comparison with fluorescence levels triggered by different $H_2O_2$ concentrations showed that the extracellular $H_2O_2$ concentration is in the low µM range (not depicted). This was in accordance with previous findings that the amount of extracellular ROS is in the low µM range (Test and Weiss, 1984; Winterbourn, 2014). In summary, we showed that under the conditions tested, PMNs produce and release $H_2O_2$ concentrations in the µM range. However, $H_2O_2$ concentrations below 10mM did not stimulate Gluc production or release in the Gluc assay (**Figure 4-7** and **Figure 4-8**). This suggested that $H_2O_2$ concentrations produced by bacteria-exposed PMNs may not be sufficient for stimulating Stx2 production or release by EHEC.

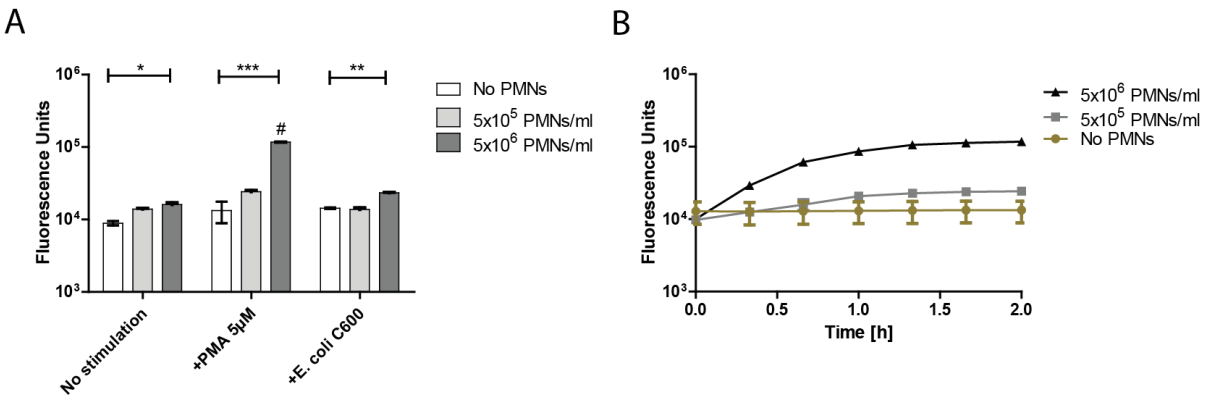


**Figure 4-11 Extracellular ROS production in PMNs** PMNs were isolated and suspended in a pre-warmed mixture of Amplex Red, HRP (horseradish peroxidase) and PBS. Afterwards, fluorescence units were measured at an excitation of 555nm and an emission of 590nm over the course of 2h. Fluorescence per well is depicted for different PMN numbers: with or without additional stimulation of PMA or bacteria after 2h (**A**) and with additional stimulation of 5µM PMA over the course of time (**B**). t-test was performed to compare treatment conditions. Data is shown as means ± standard deviation. [Bacteria: $5x10^7$/well → MOI: 500 and 5000] # = saturated.

## 4.5 Elucidating the effect of serum on *gluc* expression and Gluc release

While working on the effect of $H_2O_2$ and PMNs on Gluc/Stx2 release, we observed an increase of luminescence activity when reporter bacteria were exposed to fetal calf serum (FCS). EHEC causes a disruption of the intestinal barrier and many EHEC-infected patients develop bloody diarrhea (Tarr et al., 2005). So far, only few data on the interaction between serum components and EHEC is available. Therefore, we decided to investigate serum as another host factor in the Gluc assay. We assumed that the increase of luminescence levels in the Gluc reporter assay triggered by serum might be caused by: **a.)** an effect of serum on the Gluc enzyme **b.)** light emission independent of Gluc and CTZ **c.)** oxidative decarboxylation of CTZ by serum **d.)** enhancement of bacterial lysis and release of Gluc or **e.)** prophage induction and *gluc* expression. To dissect these effects we used the Gluc reporter assay and fetal calf serum (FCS), human serum (HS) and mouse serum (MS).

### 4.5.1 Non-diluted serum destroys Gluc activity

First of all, effects of human serum (HS) and fetal calf serum (FCS) on the Gluc enzyme were investigated. The purpose of this experiment was to detect if HS and FCS had a similar destructive effect on Gluc or its substrate coelenterazine (CTZ) as $H_2O_2$ concentrations above 20mM (**Figure 4-4**). Reporter strains that had been stimulated with MitC were used as positive controls. These positive culture supernatants generated highest luminescence levels and were incubated with different concentrations of HS (**Figure 4-12 B**) and FCS (**Figure 4-12 C**) for 4h in 96-well plates. PBS was used for dilution of HS and FCS as this buffer did not interfere with our assay. Furthermore, conditions with heat-inactivated sera were tested to detect if the heat-sensitive fraction of serum was responsible for the observed effects.

As expected, the supernatant of positive controls that were not exposed to sera exhibited high signal levels. In comparison to those controls, it was observed that undiluted HS and FCS caused a significant decrease in luminescence. This effect was less strong for the 10% diluted sera. The findings were comparable for both HS and FCS. Heat inactivation did not alter the impact of sera on Gluc. Concluding, we found that non-diluted serum inhibited Gluc activity while this effect was reduced for diluted serum. As we had previously observed an inducing effect of serum at lower concentrations, we decided to continue our investigations with serum at a concentration of 10%.

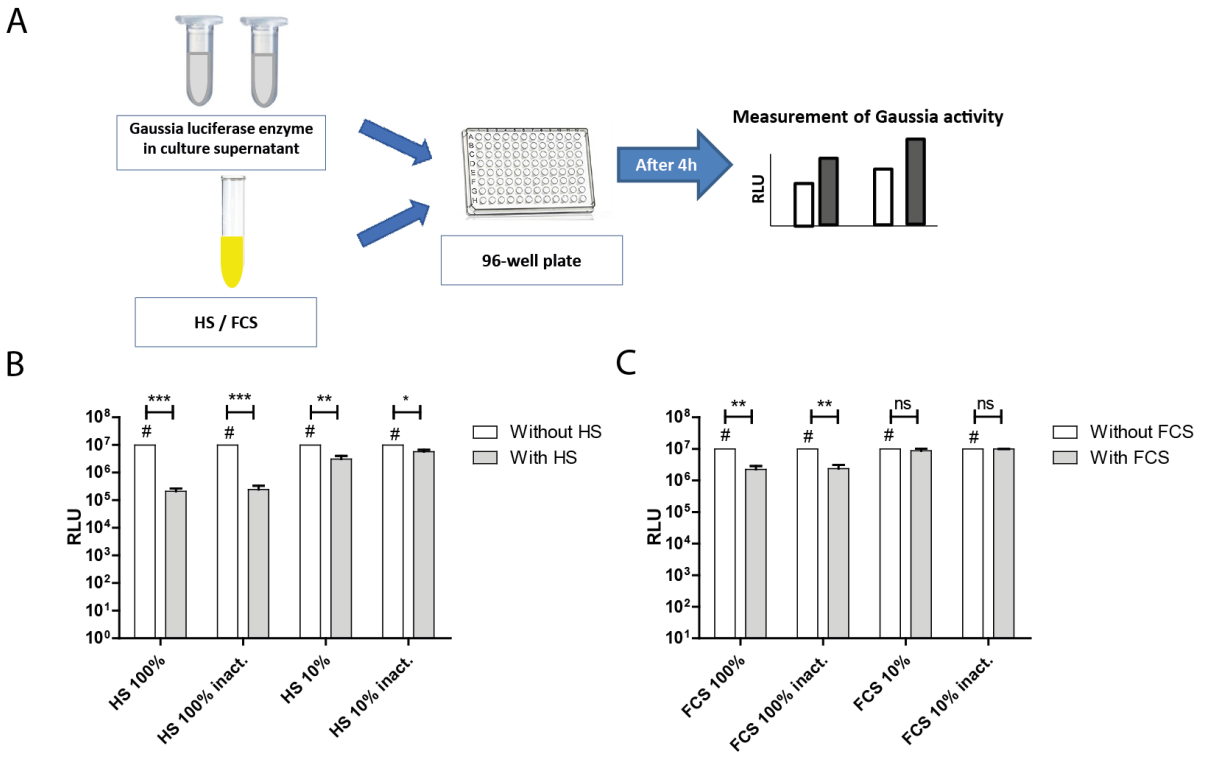


**Figure 4-12 Effects of HS/FCS on Gaussia luciferase** Setup of the experiment (**A**): Gaussia luciferase positive controls (LB+MitC, 4h, supernatant) were incubated with different concentrations of HS (**B**) and FCS (**C**) – afterwards, Gaussia activity was measured and is shown in comparison to the positive control. Gaussia activity is depicted as RLU per well. # = saturated. t-tests were performed to compare different treatment conditions – only selected results are depicted. Data is shown as means ± standard deviation.

### 4.5.2 Serum enhances conversion of CTZ to light

The next step was to examine if **a.)** serum could trigger light emission independent of Gluc and its substrate coelenterazine (CTZ) and **b.)** serum could trigger the decarboxylation of CTZ and therefore lead to light emission. For that purpose, luminescence levels of 10% diluted HS (**Figure 4-13 A)** and FCS (**Figure 4-13 B**) were measured before and after injection of CTZ and luminescence was compared to PBS. No significant increase of luminescence levels was observed without injection of CTZ. This proved that generation of luminescence in our assay was dependent on injection of CTZ. However, after injection of CTZ all conditions of HS and FCS showed increased luminescence levels compared to PBS. We assumed that some components of HS and FCS might interact with CTZ. A recent study showed that plasma proteins bind luciferase substrates what can alter the signal levels in luciferase

assays (Keyaerts et al., 2011). These interactions have to be taken in consideration when discussing the effects of serum in the Gluc assay.

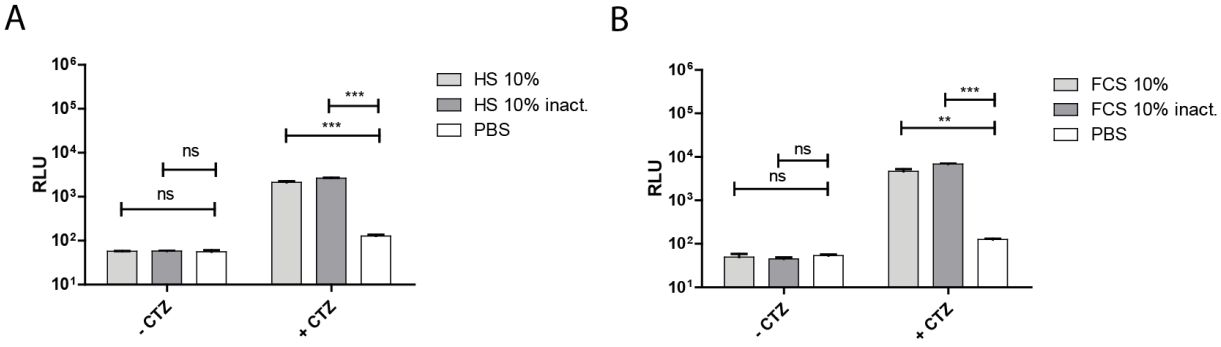


**Figure 4-13 Human serum and FCS interact with CTZ** 10% concentrations of human serum (HS) and fetal calf serum (FCS) were transferred into a 96-well plate. Additionally, heat-inactivated HS and FCS were used (65°C for 1h). After 4h, samples were taken, centrifuged and the supernatant was used for quantification of luciferase activity. Luminescence levels were measured before (-CTZ) and after (+CTZ) injection of CTZ. Gaussia activity is depicted as RLU per well. (**A**) Luminescence of HS conditions in comparison to PBS. (**B**) Luminescence of FCS conditions in comparison to PBS. ANOVA and Tukey's test were performed to compare different treatment conditions – only selected results are depicted. Data is shown as means ± standard deviation.

### 4.5.3 Dissecting the effects of FCS in the Gluc reporter assay

After showing that serum stimulated CTZ conversion, we aimed to determine the effects of FCS on Gluc activity, bacterial growth and survival. 10% FCS was used to analyze the impact of FCS on Gaussia-mediated luminescence levels. Again, heat inactivation of FCS at 65°C for 1h was performed for the assay. *E. coli stx::gluc* was incubated with FCS for 4h in 96-well plates. At two time points reporter activity was measured in the supernatants. 10% diluted FCS led to high luminescence levels in comparison to Gluc reporter strains incubated in PBS (**Figure 4-14 A)**. Heat inactivation was found to reduce the effect as less Gaussia activity was detected than for native FCS. Nevertheless, even heat-inactivated FCS led to significantly elevated levels compared to bacteria in PBS. Furthermore, it was again observed that blank FCS (without incubation with the Gluc reporter strain) caused elevated background luminescence levels by interaction with CTZ in comparison to blank PBS. However, this background luminescence triggered by FCS was significantly lower than luminescence levels of FCS incubated with Gluc reporter bacteria. We also analyzed changes of reporter activity over time (**Figure 4-14 B)**. Right at the beginning (after 0.5h) already a high level of Gaussia activity was detected. The activity increased slowly over the course of time until it reached its maximum after 4h.

10% diluted FCS caused 355-fold higher luminescence levels than reporter strain bacteria in PBS without addition of FCS after 4h.

It was not clear whether this strong increase was only caused by the effect of FCS on CTZ or also by its effect at a different stage in the assay. Therefore, the effect of FCS on survival and growth of Gluc reporter strains was investigated (**Figure 4-14 C and D**). To this end, $OD_{600}$ was measured at 600nm and CFU/well of selected samples were determined. The initial $OD_{600}$ of the bacteria was approximately 0.5. After 0.5h, no difference between bacteria incubated with FCS and bacteria in PBS could be observed. After 4h, however, it was detected that FCS led to a significant decrease in $OD_{600}$ (**Figure 4-14 C**). In contrast, heat-inactivated serum did not lyse the bacteria. This result suggested that a heat-sensitive fraction of FCS caused bacterial lysis and, at least partially, the increase in luminescence. The increase in luminescence levels was associated with lysis and bacterial death. This finding was in accordance with the mechanism of prophage induction after bacterial SOS response. Prophage induction does not only lead to Gluc production but eventually also to the expression of lysis genes. Plating of samples at two time points showed that FCS had a strong negative effect on bacterial viability (**Figure 4-14 D**). Even after 0.5h, the survival and growth of reporter strain bacteria in presence of FCS was clearly reduced. Until 4h, this did not further increase. Again, heat-inactivated FCS did not have a negative influence on survival. This was a further indication that a component of the inactivated fraction was responsible for the observed effect.

In conclusion, FCS caused a significant increase of luminescence activity. At the same time, FCS led to bacterial lysis, an effect that was alleviated by heat inactivation. FCS without reporter bacteria and heat-inactivated serum also led to significantly increased luminescence levels in comparison to PBS. However, those luminescence levels were significantly lower then those for reporter bacteria incubated with untreated FCS.

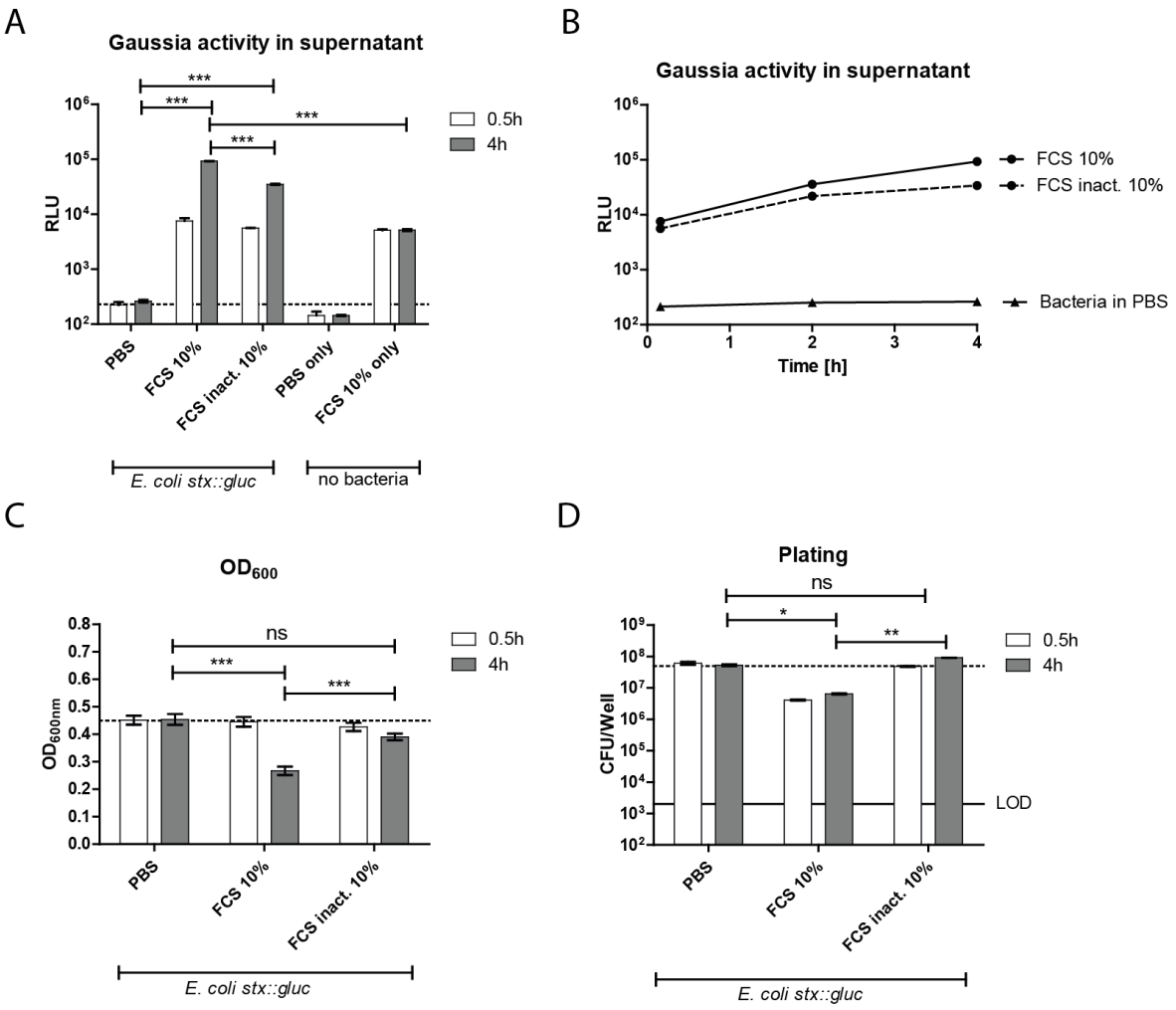


**Figure 4-14 Effects of fetal calf serum (FCS) on Gluc activity and bacterial growth and survival** *E. coli* luciferase-reporter strain *stx::gluc* was grown in 5ml LB medium until an $OD_{600}$ of 0.5. After centrifugation, bacteria were resuspended in PBS. 50µl PBS containing $5x10^7$ bacteria was transferred into a 96-well plate and incubated with different concentrations of fetal calf serum (FCS) for 4h. Additionally, heat-inactivated FCS (=FCS inact.) was used (65°C for 1h). At different time points, samples were taken, centrifuged and the supernatant was used for quantification of Gaussia activity and is depicted as RLU per well. (**A**) Luminescence at two time points in comparison to the background (Bacteria in PBS, dotted line). (**B**) Dynamics of the luciferase activity for different FCS concentrations. Additionally, $OD_{600}$ was measured at different time points (**C**). Furthermore, samples were taken and CFU *E. coli*/well were determined by plating (**D**). The results are shown in comparison to the background (bacteria in PBS, dotted lines). ANOVA and Tukey's test were performed to compare treatment conditions – only selected comparisons are depicted. Data is shown as means ± standard deviation – results are representative for three independent experiments measured in duplicates.

### 4.5.4 Human serum increases Gluc activity in the reporter assay

The next step was to investigate if human serum (HS) had a comparable effect on Gluc reporter strains as FCS. Therefore, blood was taken from a healthy donor and its serum fraction was used for experiments. All other experimental conditions were completely equal to the trials with FCS (**Figure 4–14**). Serum was diluted in PBS and afterwards incubated with *E. coli stx::gluc* in 96-well plates. Furthermore, heat inactivation was performed to identify the underlying mechanisms of the effects of HS on Gaussia reporter bacteria. HS led to a significant increase of Gluc activity in comparison to bacteria that were only exposed to PBS (**Figure 4-15 A**). Heat-inactivated HS triggered a significantly lower increase of luminescence levels. However, comparable to heat-inactivated FCS, signal levels remained quite high. Untreated HS interacted with CTZ and provided increased background luminescence levels, another similarity to the effects of FCS. In comparison to reporter bacteria incubated in PBS, the Gaussia activity of reporter bacteria exposed to the 10% serum dilution was 238-fold increased. High luminescence levels were already reached after 0.5h (**Figure 4-15 B)**. The bigger part of the impact of HS on Gaussia reporter bacteria took place until 2h. Afterwards, luminescence levels increased only slightly and reached their maximum at 4h.

Survival and bacterial growth were evaluated and results were comparable to FCS. Regarding $OD_{600}$, 10% serum had a remarkable effect on bacterial survival (**Figure 4-15 C**). Already after 0.5h, the initial $OD_{600}$ of 0.5 was decreased massively. HS caused a fast and complete lysis. In contrast, incubation of reporter bacteria with heat-inactivated serum led only to a small decrease of $OD_{600}$ after 0.5h. Afterwards, it seemed that bacteria exposed to heat-inactivated HS could even regrow as an increase of $OD_{600}$ could be detected. Plating showed similar results (**Figure 4-15 D**). Even after 0.5h, only few bacteria incubated with HS were alive and able to grow. After 4h, CFU *E. coli*/well for this condition were only slightly above our limit of detection. Heat-inactivated serum, in contrast, did not affect the bacterial ability to grow.

In summary, HS triggered a significant increase of luminescence while viability of bacteria was reduced. Heat-inactivated HS incubated with Gluc reporter bacteria and HS without bacteria also triggered an increase in luminescence levels. However, those signal levels were significantly lower compared to reporter strains incubated with untreated HS. The results were comparable to my previous finding on FCS. The next question was whether these results could be replicated with different reporter strains.

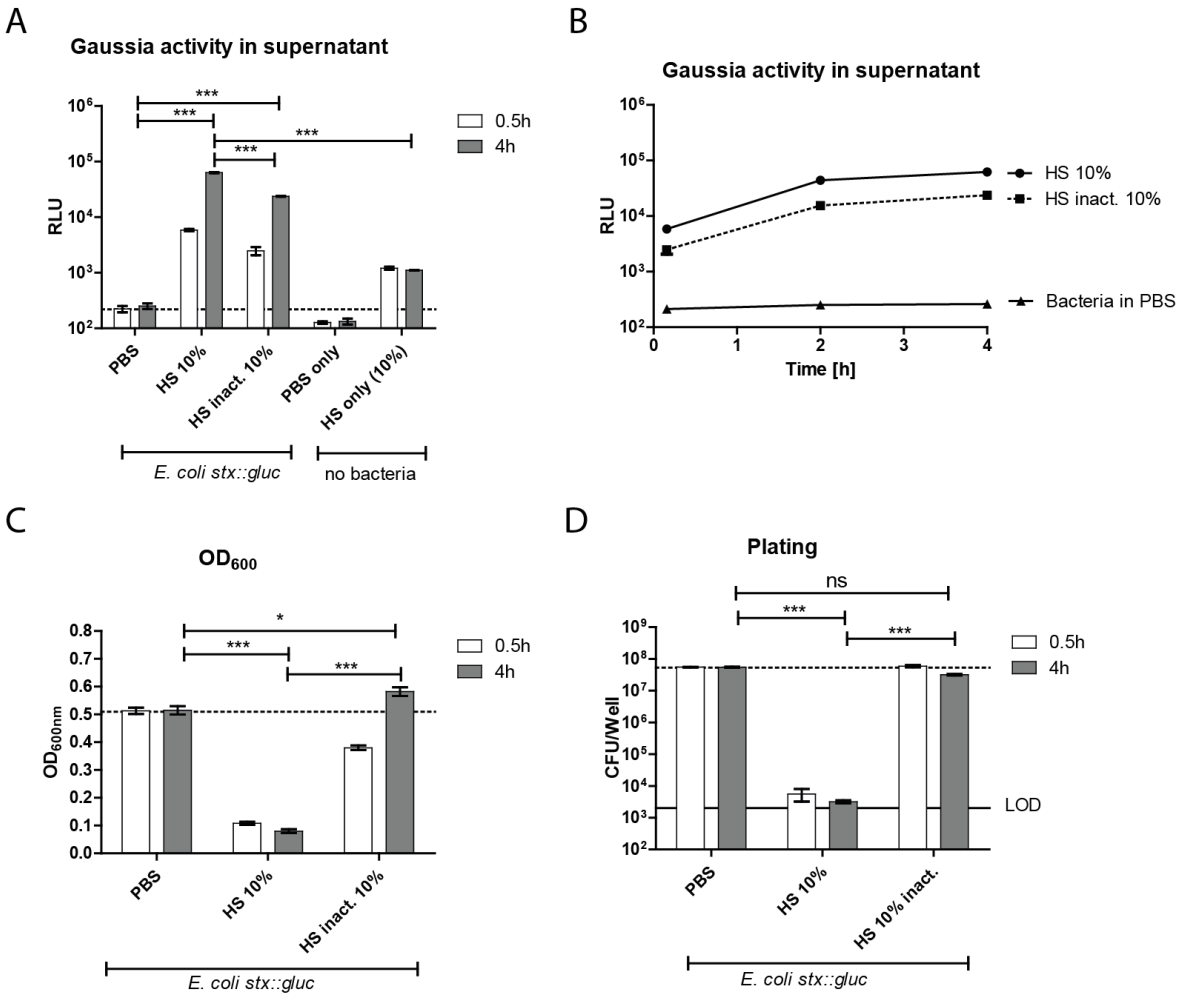


**Figure 4-15 Effects of human serum (HS) in the Gluc assay** *E. coli* luciferase-reporter strain *stx::gluc* was grown in 5ml LB medium until an $OD_{600}$ of 0.5. After centrifugation, bacteria were resuspended in PBS. 50µl containing $5x10^7$ bacteria were transferred to a 96-well plate and incubated with 10% diluted human serum (HS) for 4h. Additionally, heat-inactivated HS was used (65°C for 1h). At different time points samples were taken, centrifuged and the supernatant was used for quantification of the luciferase activity. Gaussia activity is depicted as RLU per well. (**A**) Luminescence at two time points in comparison to the background (bacteria in PBS, dotted line). (**B**) Dynamics of the Gluc activity for different serum concentrations. Additionally, $OD_{600}$ was measured at different time points (**C**). Furthermore, samples were taken and CFU *E.coli* per well were determined by plating (**D**). The results are shown in comparison to the background (bacteria in PBS, dotted lines). ANOVA and Tukey's test were performed to compare treatment conditions. Data is shown as means ± standard deviation – results are representative for three independent experiments measured in duplicates

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### 4.5.5 FCS and HS also increase Gluc activity in non-lytic reporter strains

We noticed that HS and FCS led to high luminescence levels in the Gluc assay while reducing bacterial growth and survival. So far, we could not distinguish whether the effects of serum in the Gluc assay were due to enhancement of bacterial lysis and release of Gluc or due to prophage induction and *stx2* expression. The next step was to extend our experiments with HS and FCS to another reporter strain. This time *E. coli stx::gluc* (lytic) and *E. coli stx2 sr::gluc* (non-lytic) were exposed to HS and FCS. Furthermore, bacterial growth was monitored by measurement of $OD_{600}$.

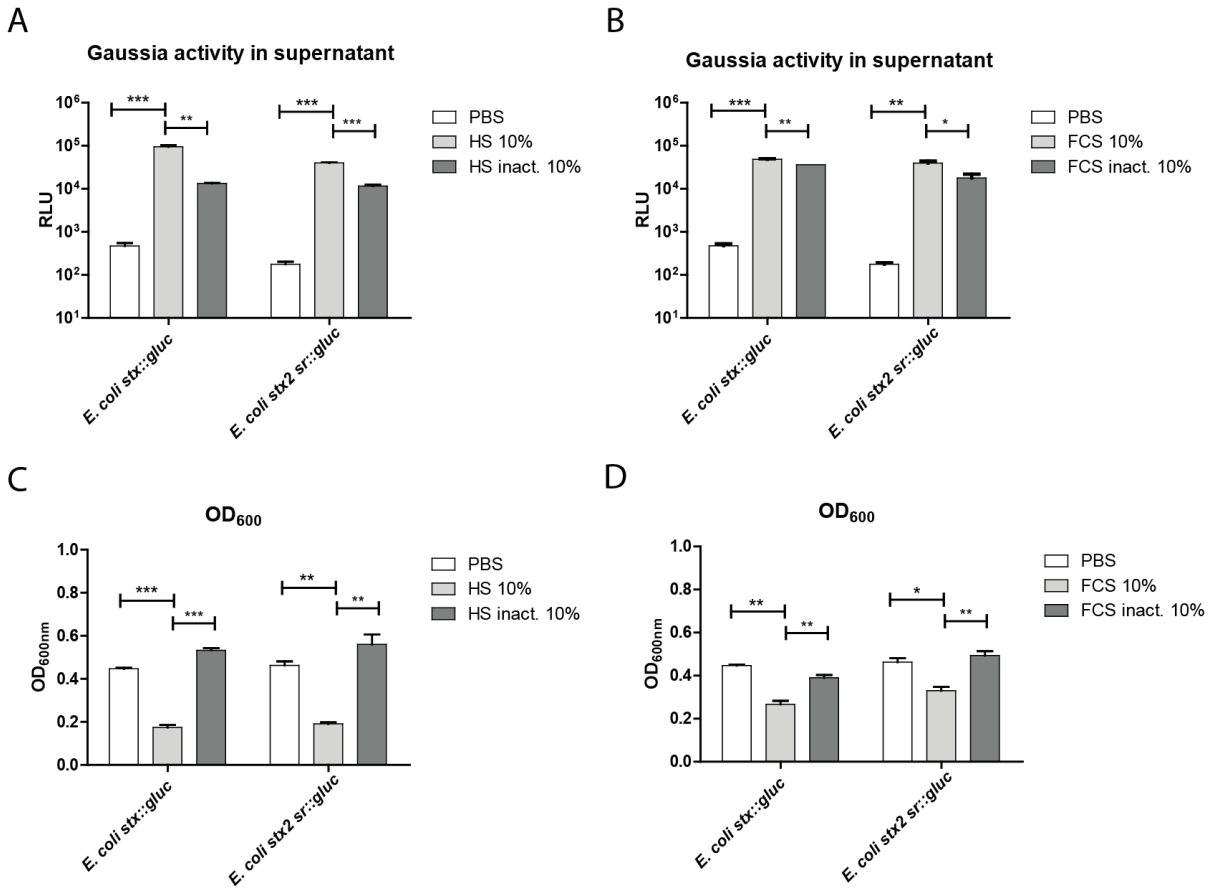


**Figure 4-16 Effects of HS and FCS on lysis-competent and -deficient reporter strains** *E. coli* luciferase-reporter strains *stx::gluc* (lysis-competent) and *stx2 sr::gluc* (lysis-deficient) were grown in 5ml LB medium until an $OD_{600}$ of 0.5. Bacteria were resuspended in PBS and transferred to a 96-well plate ($5x10^7$ bacteria/well). Bacteria were incubated with different concentrations of human serum (HS) and fetal calf serum (FCS) for 4h. Additionally, heat-inactivated (inact.) HS and FCS were used (65°C for 1h). After 4h, samples were taken, centrifuged and the supernatant was used for quantification of the luciferase activity. Gaussia activity is depicted as RLU per well. (**A+B**) Luminescence in comparison to bacteria in PBS. (**C+D**) $OD_{600}$ in comparison to bacteria in PBS. ANOVA and Tukey's test were performed to compare treatment conditions. Data is shown as means ± standard deviation – results are representative for three independent experiments measured in duplicates.

HS had a comparable impact on both *E. coli* strains (**Figure 4-16 A)**. The non-lytic strain showed significantly increased luminescence levels. However, the release of Gluc after prophage induction is absolutely dependent on expression of lysis genes. Therefore, not prophage induction but bacterial lysis by serum was considered the responsible mechanism. Even in the heat-inactivated state, a significant increase in luminescence was detected for both strains. Furthermore, $OD_{600}$ was measured for bacteria exposed to HS (**Figure 4-16 C**) and FCS (**Figure 4-16 D**). As shown in the previous experiments, HS and FCS caused a significant decrease of $OD_{600}$ after 4h compared to bacteria in PBS. Heat-inactivated serum, however, did not cause cell death and bacteria were still intact. In contrast, growth effects could be observed when bacteria were exposed to heat-inactivated serum (**Figure 4-16 C and D)**. The decrease in $OD_{600}$ caused by serum conditions was most probably caused by antibacterial effector mechanisms of serum, such as activity of the complement system.

In summary, it was found that serum of different species (HS, FCS) triggered an increase of Gaussia activity in the Gluc assay. At the same time, bacterial growth and survival were reduced. We found that a component of the heat-sensitive fraction was responsible for those effects as heat inactivation led to significantly better bacterial growth and survival and less Gaussia activity. As the non-lytic *E. coli* reporter strain was likewise affected, we concluded that FCS and HS caused an increase of luminescence levels by bacterial lysis and not by induction of phage lysis.

## 4.6 Investigating the mechanisms of the effect of serum on bacterial lysis and Gluc release

Our previous experiments demonstrated that serum had a significant impact on Gaussia activity. We could observe this effect with fetal calf serum (FCS) and human serum (HS). Furthermore, it was detected that both sera affected the survival and growth of the Gluc reporter strains. As described in the previous section, the effects of serum could be caused by different mechanisms. Non-diluted serum acted inhibitory on Gluc and could therefore not be monitored in the Gluc assay. Serum triggered oxidative decarboxylation of CTZ and led to an increased background activity in comparison to PBS. However, this could not entirely explain the marked increase in luminescence when reporter bacteria were exposed to serum. As incubation of non-lytic *E. coli* strains with serum also led to increased luminescence levels, it was clear that those inducing effects of serum on Gluc could not or at least not entirely be explained by prophage induction. Therefore, we aimed to determine the underlying mechanisms of the influence of serum on Gluc activity. Additionally, we wanted to identify which component of serum could be responsible for the observed effects.

### 4.6.1 Inhibition of protein synthesis does not have an impact of Gluc activity triggered by serum

For production and release of Stx2 via prophage induction and the phage lytic mode, expression of *stx2* and lysis genes is a prerequisite. The next step was to inhibit bacterial protein synthesis by treatment with chloramphenicol (Cm) to detect whether this would alter serum-mediated stimulation of luminescence. If *de novo* protein synthesis was dispensable, serum would more likely trigger the release of pre-formed Stx2 by lysis than by inducing *stx2* expression. For this experiment lysis-competent *E. coli* and *C. rodentium* Gluc reporter strains were used. The minimal inhibitory concentration (MIC) of Cm was determined to be 2µg/ml. Therefore, bacteria were resuspended in PBS with 2µg/ml Cm. Afterwards, reporter bacteria were exposed to 10% diluted human serum (HS) in 96-well plates for 4h. Other conditions such as PBS, LB and MitC were included as controls.

Inhibition of protein synthesis by Cm led to a significant decrease of Gaussia activity in the supernatants of Gluc reporter strains that were cultured in LB with MitC (**Figure 4-17 A und B)**. This finding was detected for both reporter strains and confirmed that production and release of Gluc after stimulation with MitC were dependent on protein synthesis. Furthermore, it proved that the applied Cm concentration was sufficient for inhibiting protein synthesis. In contrast, inhibition of protein synthesis did not alter the luminescence levels for reporter strains incubated with HS. For

both strains, luminescence levels did not significantly change despite pre-treatment with Cm. This indicated that release of Gluc triggered by HS was not due to prophage induction and reporter gene expression. Inhibition of protein synthesis did not alter luminescence levels of reporter strains in PBS but decreased Gaussia activity of *E. coli stx::gluc* incubated in LB. This was most likely due to restriction of growth.

Furthermore, $OD_{600}$ of both reporter strains was recorded for the same conditions (**Figure 4-17 C and D**). As observed before (**Figure 4-15**), 10% HS caused a strong decrease of $OD_{600}$ of the *E. coli* reporter strain. In contrast, the *C. rodentium* strain was more robust. Treatment with Cm did not alter the $OD_{600}$ of bacteria exposed to serum conditions. However, it could be shown for bacteria in LB that

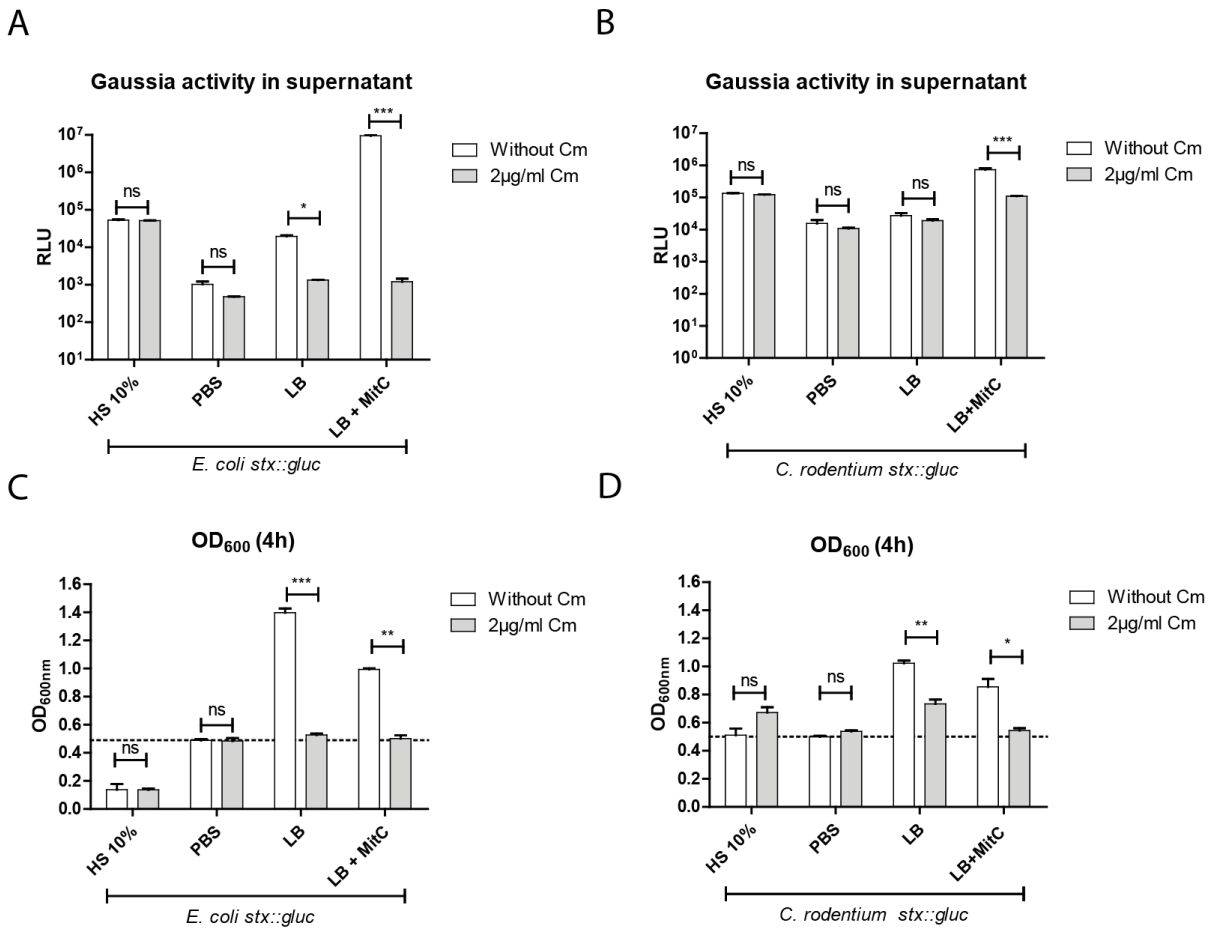


**Figure 4-17 Cm does not inhibit serum-mediated Gluc activity in the supernatant** *E. coli* luciferase-reporter strain *stx::gluc* (**A,C**) and *C. rodentium* luciferase-reporter strain *stx::gluc* (**B,D**) were grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards, bacteria were centrifuged and suspended in PBS with chloramphenicol (Cm). After 30 minutes, bacteria were incubated with different conditions (HS, PBS, LB, LB+MitC) in a 96-well plate for 4 hours. After this time samples were taken and centrifuged - the supernatants were used for detection of Gaussia activity that is depicted as RLU per well (**A, B**). Additionally, $OD_{600}$ was detected after 4 hours (**C, D**). t-test was performed to compare bacteria without treatment with Cm to bacteria treated with 2µg/ml Cm. Data is shown as means ± standard deviation.

inhibition of protein synthesis inhibited bacterial growth. $OD_{600}$ of those conditions was significantly decreased in both bacterial strains when pre-treated with Cm (**Figure 4-17 C and D**). In conclusion, Gluc activity triggered by serum was not lowered when bacteria were pre-treated with Cm to inhibit protein synthesis. This finding was a further indication that the serum-induced increase in luminescence levels was not caused by prophage induction and gene expression but by bacterial lysis.

### 4.6.2 Serum proteins with a molecular weight >100kDa are responsible for the increase of Gluc activity and decrease of bacterial viability

It was observed that serum-induced reporter activity is not, or at least not primarily, caused by gene induction but rather by bacterial lysis. Therefore, we wanted to identify the component of serum that was responsible for its impact in the Gluc assay. For this aim, we decided to size-fractionate serum using size exclusion columns with a molecular weight (MW) cut-off of 5kDa or 100kDa. Both the filtrate and the supernatant were taken and tested in the Gluc assay. Subsequently, the effect on luminescence and survival was probed.

First, blood was taken from a healthy donor and the serum fraction was filtrated with a size exclusion column with a MW cut-off of 5kDa. Afterwards, supernatant (>5kDa) and filtrate (<5kDa) were used in the Gluc assay in a 10% diluted (with PBS) manner. The dilution step was necessary as non-diluted serum acts inhibiting on Gluc (**Figure 4-12**). The lytic *E. coli* reporter strain *stx::gluc* was incubated with these conditions for 4h in a 96-well plate. Molecules <5kDa neither caused a significant increase in luminescence nor lysis, compared to bacteria in PBS after 4h (**Figure 4-18 A)**. In contrast, the fraction >5kDa caused elevated luminescence levels compared to bacteria in PBS. Background luminescence levels of serum without bacteria were measured to monitor interferences with the assay. Again, it could be observed that serum enhances light emission by interaction with CTZ. Furthermore, $OD_{600}$ was measured to monitor the influence of the serum fractions on bacterial growth (**Figure 4-18 B**). It was observed that the fraction >5kDa could explain the effects observed with non-filtrated serum. This fraction showed a negative effect on survival that is comparable to previously observed effects of serum (**Figure 4-15**).

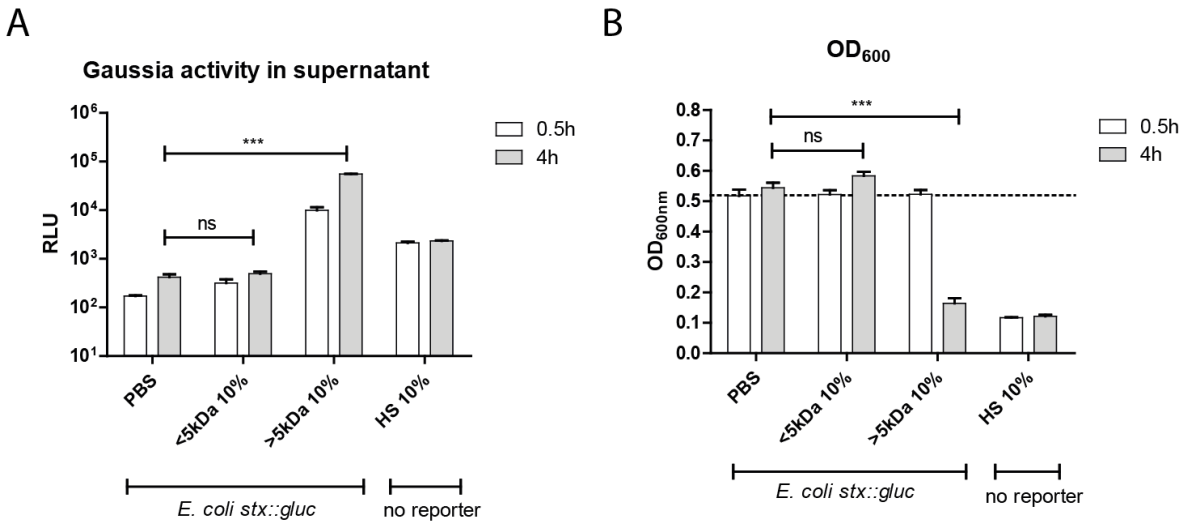


**Figure 4-18 Effects of serum on Gluc reporter strains are caused by serum proteins with a molecular weight (MW) above 5kDa** *E. coli* luciferase-reporter strain *stx::gluc* was grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards, bacteria were centrifuged and suspended in PBS medium. Spin-X size exclusion columns were used to fractionate serum with a cut-off of 5kDa. Bacteria were then incubated with a 10% dilution of the supernatant (>5kDa) and the filtrate (<5kDa) for 4h in a 96-well plate. At different time points samples were taken and centrifuged. Gaussia activity was measured in the supernatants (**A**) and is depicted as RLU per well. At the same time points, $OD_{600}$ was detected (**B**). ANOVA and Tukey's test were performed to compare treatment conditions. Data is shown as means ± standard deviation – results are representative for two independent experiments measured in duplicates.

Finally, we repeated this assay with size exclusion columns with the MW cut-off of 100kDa. Again both the supernatant (>100kDa) and the filtrate (<100kDa) were taken and used for evaluation in the Gluc assay. Increase of Gaussia activity in the culture supernatants was primarily caused by serum components >100kDa (**Figure 4-19 A)**. Again, the supernatants of reporter bacteria exposed to the 10% dilution showed maximal luminescence levels. In contrast, the fraction <100kDa did not cause a significant increase of Gluc activity compared to reporter bacteria in PBS.

The results for the fraction >100kDa were comparable to previous findings and significantly higher than the background luminescence levels of serum. Measurement of $OD_{600}$ was similar to previous results **(Figure 4-19 B**). For bacteria exposed to serum of the fraction >100kDa, a decrease in $OD_{600}$ was detected after 4h. In contrast, the fraction <100kDa did not cause a significant alteration of $OD_{600}$ in comparison to reporter bacteria exposed to PBS. In conclusion, we found that serum components >100kDa were responsible for serum-induced reporter effects: leading to an increase of luminescence levels and impairment of bacterial viability.

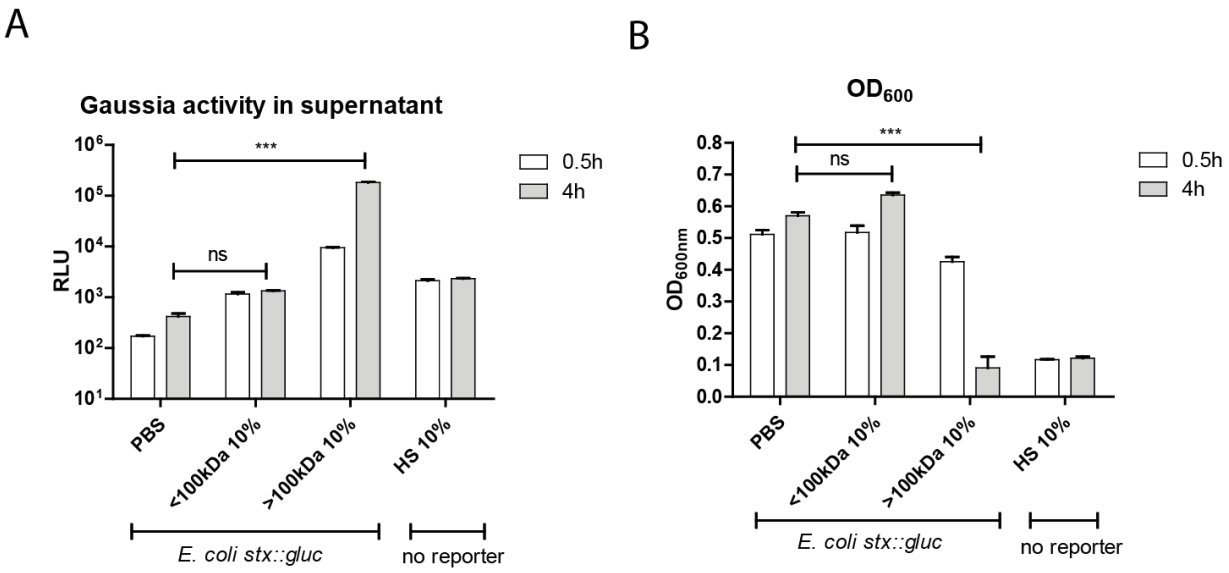


**Figure 4-19 Effects on luciferase-reporter strains are caused by serum proteins with a molecular weight (MW) above 100kDa** The experiment was performed as described in Figure 4-18. This time, Spin-X size exclusion columns with the MW cut-off of 100kDa were used. Gaussia activity in the supernatants (**A**) was measured and is depicted as RLU per well. At the same time points, $OD_{600}$ was detected (**B**). ANOVA and Tukey's test were performed to compare treatment conditions. Data is shown as means ± standard deviation – results are representative for two independent experiments measured in duplicates.

### 4.6.3 The complement system is responsible for the increase of Gluc activity and bacterial killing by serum

We continued to identify the serum component > 100kDa which caused the observed effects on Gluc activity and bacterial lysis. The prime candidate was the complement system. This enzyme cascade is composed of over 30 proteins and is involved in several immunological functions including chemotaxis, opsonization, phagocytosis and cell lysis. Upon activation, the complement system leads to the formation of the membrane attack complex (MAC) and lyses cells by generation of pores (reviewed in Ricklin et al., 2010). To determine the influence of the complement system on the Gluc reporter strains we used serum of C57 Bl/6 wild type (wt) mice (Bl/6) and compared it to serum of mice deficient in complement factor C3 ($C3^{-/-}$) (Wessels et al., 1995). C3 is a protein with a molecular weight of 185kDa and plays an essential role in both the classical and the alternative complement pathway (reviewed in Ricklin et al., 2016). A lack of C3 results in complex immunological diseases (reviewed in Erdei et al., 1991).

The experimental setup was generally the same as in previous experiments. Sera of wt mice and of $C3^{-/-}$ mice were used in two dilutions (10% and 1%). The sera were incubated with the reporter strain *E. coli stx::gluc* in 96-well plates. Gaussia activity in the supernatants and $OD_{600}$ were measured after 4h. Both dilutions of $C3^{-/-}$ serum led to a significantly lower reporter activity than serum of wt mice (**Figure 4-20 A)**. However, supernatants of bacteria that were exposed to $C3^{-/-}$ serum still generated elevated luminescence levels compared to bacteria in PBS and serum only with enzyme substrate CTZ. All serum conditions showed much higher Gaussia activity than conditions of serum without Gluc

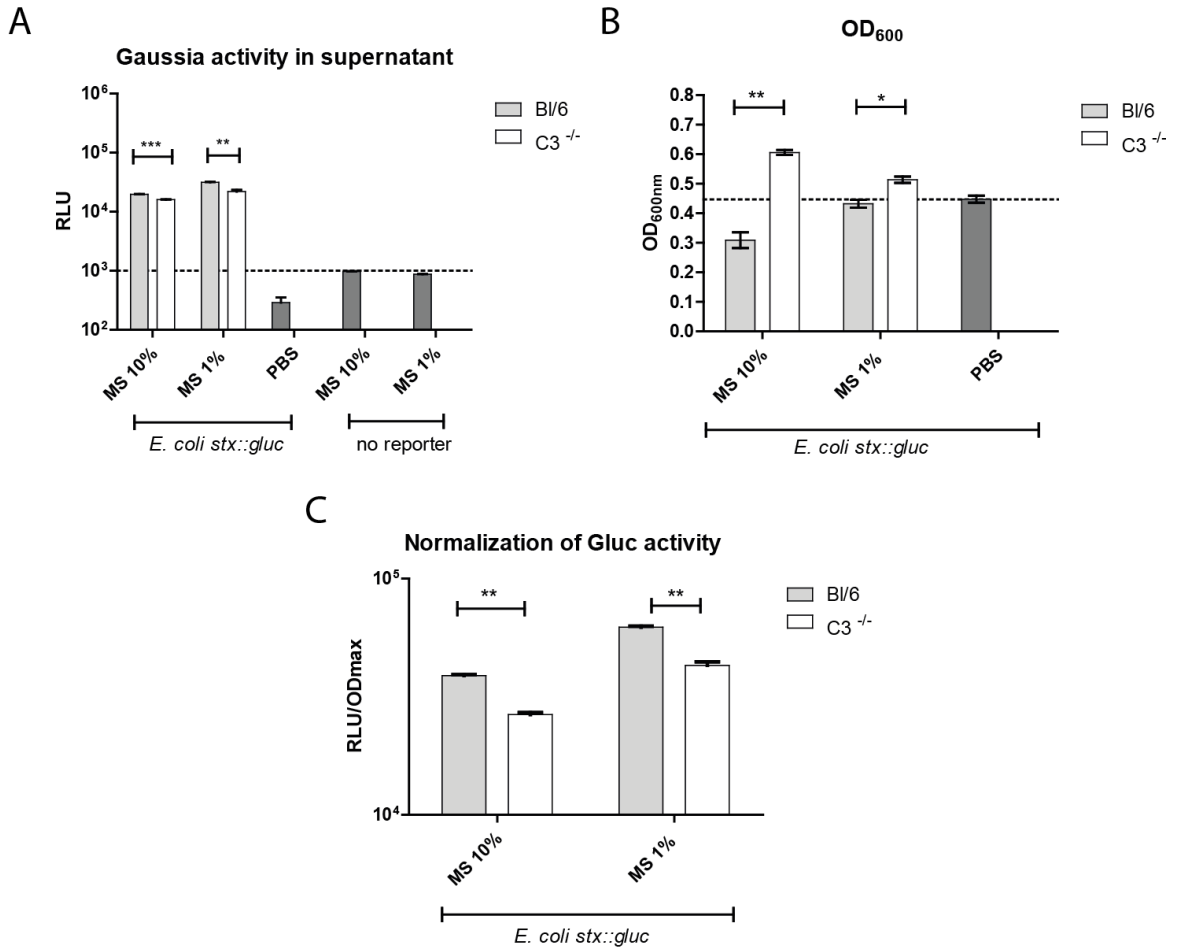


**Figure 4-20 Serum from $C3^{-/-}$ mice does not trigger bacterial lysis** *E. coli* luciferase-reporter strain *stx::gluc* was grown in 5ml LB medium until an $OD_{600}$ of 0.5. Afterwards bacteria were centrifuged and suspended in PBS. Bacteria ($5x10^7$/well) were incubated with different serum concentrations of either C57 Bl/6 (Bl/6) or $C3^{-/-}$ ($C3^{-/-}$) mice for 4h in a 96-well plate. After 4h samples were taken, centrifuged and luminescence levels were measured in the supernatants (**A**). Gaussia activity is depicted as RLU per well. Absorbance was detected after 4h (**B**). Furthermore, RLU was normalized to the maximum $OD_{600}$ (ODmax) that the reporter reached in the assay. This was the initial OD in case of Bl/6 MS (lysis was observed) and the final OD in $C3^{-/-}$ MS (no lysis was observed). (**C**). t-test was performed to compare the effects of treatment with different sera. Data is shown as means ± standard deviation.

reporter bacteria. Moreover, we found that the absence of a functional complement system significantly promoted growth of the reporter strain (**Figure 4-20 B)**. An increase of the initial $OD_{600}$ of approximately 0.5 could be observed after 4h. This was especially true for the 10% dilution. Serum of wt mice caused a significant decrease of survival whereas $C3^{-/-}$ serum promoted bacterial growth in the observed time. Similar results could be seen for the 1% dilution. We assumed that, due to the absence of complement-dependent killing mechanisms, nutrients in $C3^{-/-}$ MS promoted the growth of the reporter strain. Increase in $OD_{600}$ of the reporter strain contributed to elevated luminescence levels when bacteria were incubated with $C3^{-/-}$ MS. Therefore, we normalized the RLU values to the maximal $OD_{600}$ of the reporter strain. This was the initial $OD_{600}$ in case of the Bl/6 MS and the final $OD_{600}$ in case of the $C3^{-/-}$ condition. By normalizing the data this way a significantly higher Gluc activity was observed when reporter bacteria where exposed to Bl/6 MS compared to bacteria exposed to $C3^{-/-}$ MS. (**Figure 4-20 C**).

Concluding, serum of different species mediated an increase of luminescence in the Gluc assay and a decrease of bacterial growth and survival. We found that the increase of luminescence was triggered independent of prophage lysis as inhibition of protein synthesis did not alter the results and non-lytic strains were affected likewise. We found that the serum protein fraction >100 kDa was responsible for serum's influence on Gluc activity and bacterial survival. Experiments with serum of $C3^{-/-}$ mice showed that the complement system contributed to the increase of Gluc activity triggered by serum. Most importantly, it was a major factor mediating bacterial lysis.

## 4.7 Detection of Shiga toxin using the Vero cell killing assay

We observed that $H_2O_2$ and serum could lyse bacteria and increase phage-independent release of Gluc in the Gluc reporter assay. The question arose whether Stx2 could also be released via bacterial lysis. We therefore chose to use the Vero cell assay as method to quantify Stx2 toxicity. Vero cells are kidney cells from an African green monkey that were first isolated in 1962 (Yasumura, 1963). These cells are highly susceptible to Stx-mediated killing and are therefore used for Stx detection (Konowalchuk et al., 1977).

### 4.7.1 Vero cells as method to detect Shiga toxins

The assay was conducted in 96-well plates to test a dilution series of multiple substances at once. This time, I worked with a wild type *E. coli* strain harboring the Stx2 prophage (*E. coli* C600W34). *E. coli* C600W34 was grown with or without stimulation by MitC. Samples were taken at several time points and centrifuged. Afterwards, a 1:2 dilution series of these supernatants was created in 96-well plates. Vero cells were incubated with these supernatant dilutions for 3 days (d). The intention of this assay was to observe if and until which toxin dilution death of Vero cells could be detected. The more toxins in the supernatants, the higher was the dilution until which Vero cells were killed (**Figure 4-21 A**).

After 3 days, the plate was inspected whether bacterial toxins affected the survival and growth of Vero cells. Plates were evaluated via microscopy (**Figure 4-21 B**) and via measurement of absorbance after cellular staining with Crystal violet (**Figure 4-21 C**). It was determined at which dilution of the sample at least 50% of Vero cells in the wells were dead ($CD_{50}$). The $CD_{50}$ dilution of the sample is depicted as reciprocal value. The higher the $1/CD_{50}$, the more Stx2 was present. Stimulation of *E. coli* C600W34 with MitC triggered the bacterial SOS response and prophage induction and increased production of Stx2. The supernatants of these cultures contained high Stx2 levels and killed Vero cells until higher dilutions. The supernatants of bacteria without MitC also caused death of Vero cells but to a much lower extent. At an early time point after induction, bacteria treated with or without MitC released similar amounts of Stx2. This was in accordance with the finding that Stx2 production and release after prophage induction takes approximately 3h. From this time point on, a significant difference of $CD_{50}$ between supernatants of unstimulated bacteria and supernatants of bacteria treated with MitC could be observed. Toxin production and release increased until 7h after MitC stimulation. Afterwards, no further increase of Stx2 levels and therefore no higher $1/CD_{50}$ was found.

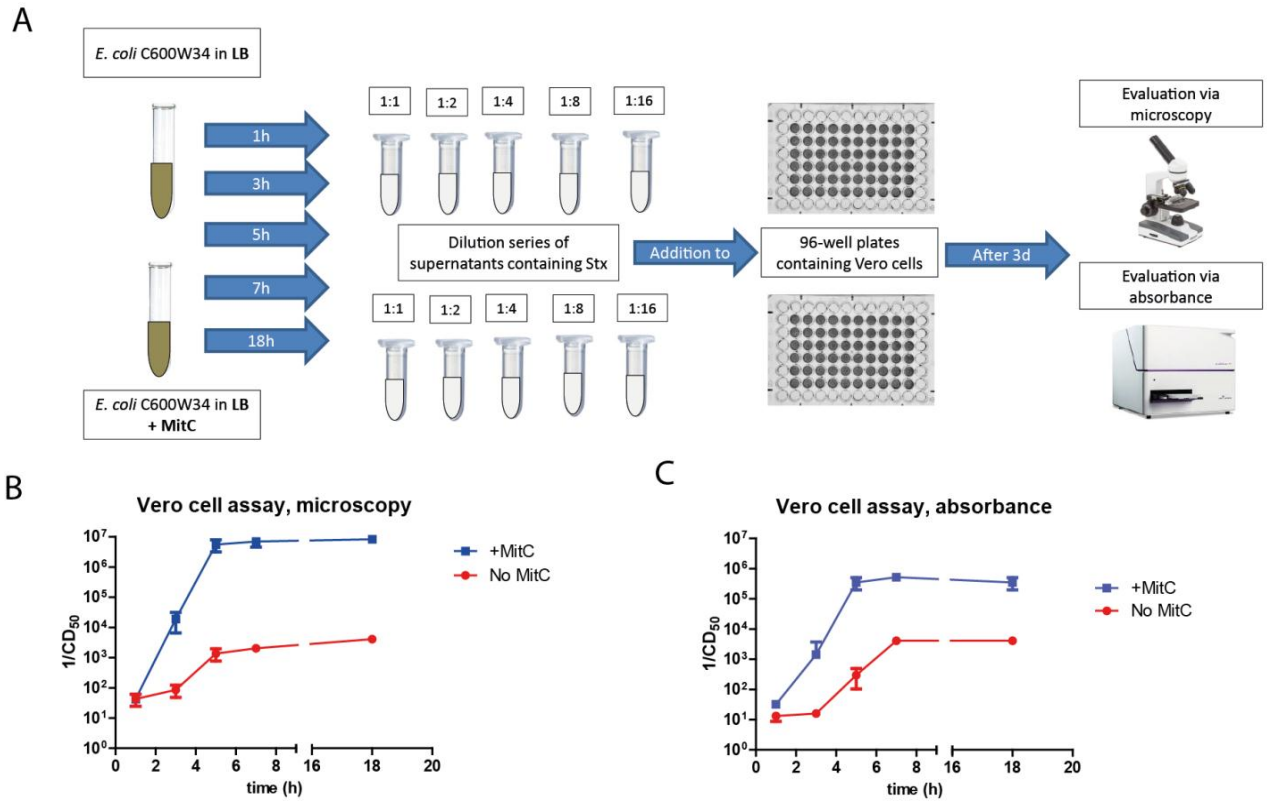


**Figure 4-21 Using the Vero cell assay for detection of Shiga toxin 2 (Stx2) production** Setup of the experiment (**A**): An overnight culture of Stx2-carrying strain *E. coli* C600W34 was grown in 5ml of LB medium until an $OD_{600}$ of 0.5. Afterwards, bacteria were diluted either with LB medium alone or with LB medium containing MitC (0.5µg/ml) to an $OD_{600}$ of 0.1. Bacteria were then incubated shaking at 37° C for 18h. At various time points samples were taken und centrifuged with filter tubes. These supernatants were incubated in 1:2 dilutions (with PBS) in 96-well plates with Vero cells ($2x10^4$ cells/well) for 3d. Afterwards, analysis was done in two different ways: (**B**) Detection of 50% dead cells ($CD_{50}$) via microscopy by two persons. (**C**) Fixation of the cells with formaldehyde (PFA) and staining with crystal violet. Then, cells were diluted with 50% EtOH and absorbance was measured. The highest dilution at which at least 50% of Vero cells were killed ($CD_{50}$) is depicted as reciprocal value. Data is shown as means ± standard deviation.

### 4.7.2 Serum leads to Stx2 release from *E. coli* C600W34

Finally, we investigated the influence of serum on Stx2 release by *E. coli* in the Vero cell assay. For this, *E. coli* C600W34 was grown in LB with or without stimulation of MitC for 1h. Afterwards, bacteria were centrifuged and diluted either in serum (10%) or in PBS. Bacteria were then incubated for 30 minutes to enable release of Stx2. Afterwards, samples of the cultures were centrifuged and the culture supernatants were incubated with Vero cells in a 1:2 dilution series in a 96-well plate. After 3d, we evaluated the $CD_{50}$ (**Figure 4-22 A**).

In previous experiments, the impact of serum on our reporter strains was detected at early time points. Lysis of bacteria and increase in luminescence was observed after only 0.5h (**Figure 4-15**). The Vero cell assay confirmed these observations. Compared to PBS conditions, serum triggered an increased release of Stx2 in the MitC pre-stimulated cultures after only 0.5h. The supernatants contained more Stx2 and Vero cells were affected until higher dilutions (**Figure 4-22)**. This indicated that pre-stimulation of the bacteria and formation of Stx2 within the cells was necessary to obtain detectable amounts of released Stx2. In summary, the Vero cell assay showed that serum exposure leads to lysis of *E. coli* and concomitant release of active Stx2 in the culture supernatant.

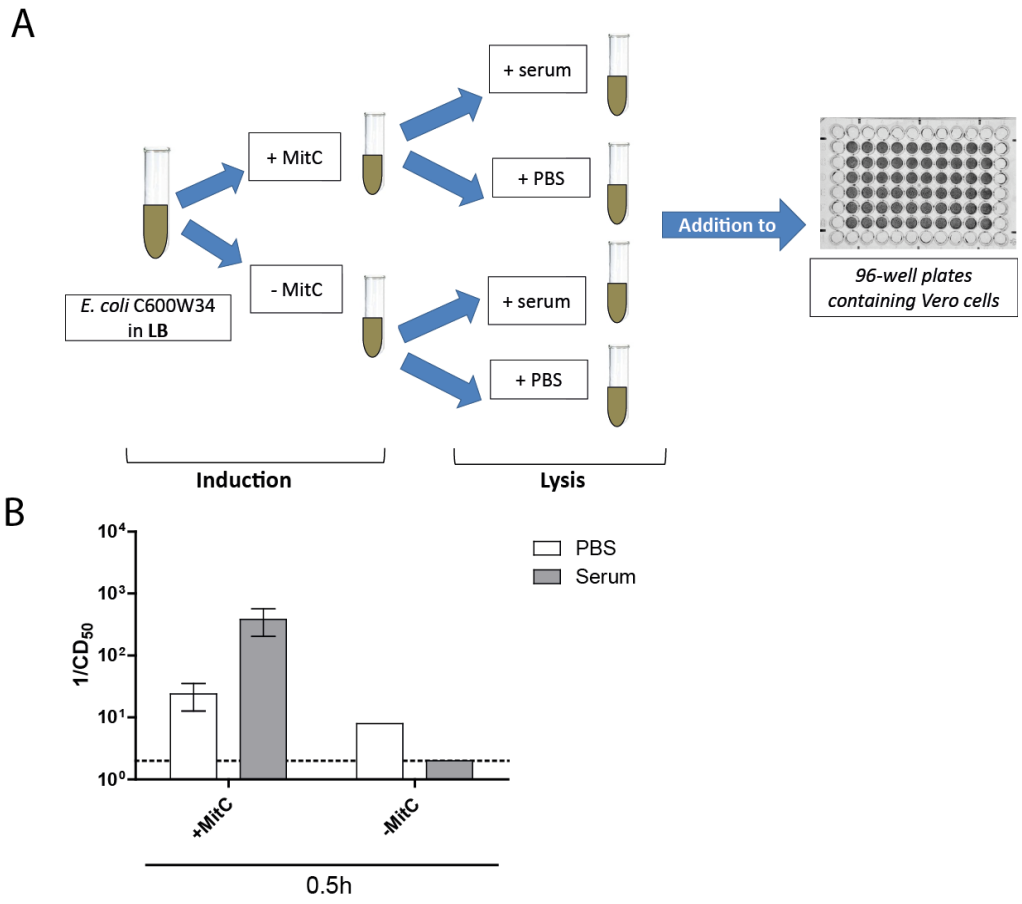


**Figure 4-22 Serum triggers the release of Shiga toxin2 (Stx2)** Setup of the experiment (**A**): Stx2-carrying strain *E. coli* C600W34 was grown in 5ml LB with or without MitC for 1h. Afterwards, bacteria were centrifuged and diluted in PBS or in human serum (10%). These cultures were grown for another 0.5h. Afterwards, cultures were centrifuged with filter tubes and bacterial supernatants were incubated with Vero cells ($2x10^4$ cells/well) for 3d in 96-well plates. Afterwards, analysis was done as described in Figure 4-21. The highest dilution at which at least 50% of Vero cells were killed ($CD_{50}$) is depicted as reciprocal value (**B**). Data is shown as means ± standard deviation.

# 5. Discussion

## 5.1 The luciferase-reporter assay for monitoring the influence of environmental and host factors on *stx2* expression and Stx2 release

EHEC infections can cause life-threating diseases in humans and new EHEC outbreaks are likely, due to the presence of the pathogen in zoonotic reservoirs. Currently, no causal treatment for EHEC infections is available as use of antibiotics remains controversial. Therefore, more research into EHEC pathogenesis is necessary on the basis of which improved patient management and optimized therapeutic strategies can be developed. So far, little is known about the interaction between inflammation in the human gut and s*tx2* expression. In this thesis, I investigated the influence of host defenses on *stx2* expression by EHEC and focused on the impact of PMNs, $H_2O_2$ and serum. For this purpose, a Gaussia luciferase (Gluc) reporter assay was established as a safe and fast method to elucidate the effect of host factors on *stx2* expression. With the Gluc reporter assay, I confirmed results of Wagner et al. that human PMNs activate *stx2* expression (Wagner et al., 2001a). Furthermore, I showed that $H_2O_2$ and serum release Shiga toxin by bacterial lysis. Based on this finding, we propose that Shiga toxin can be released in two ways: **a.)** by expression of *stx2* and subsequent phage lysis and **b.)** bacterial lysis mediated by host-dependent antimicrobial mechanisms. This finding may have important implications on the assessment of risk factors indicating HUS in infected patients. Furthermore, it suggests that besides *stx2* expression in the pathogen triggered by environmental factors also bacterial lysis can lead to increased levels of free active toxin in the gut. It remains to be shown to which extent these two mechanisms contribute to the pathogenesis of HUS.

The three main criteria for establishing a reporter assay for Stx2 production are: **1.)** safety for the investigator, **2.)** efficiency in testing and **3.)** faithful reflection of toxin production. Regarding safety for the investigators, the Gluc assay has obvious advantages. Genes for *stx2* are replaced with *gluc* and Gluc instead of Stx2 is produced upon activation of the prophage. The use of reporter strains leads to reclassification of the pathogen to risk group 2 (RG2) and investigators are not in danger of getting exposed to Stx2. Considering safety, the Gluc assay is therefore superior to methods that directly detect Stx2, for example ELISA and the Vero cell assay. ELISA is a common immunological method for testing stool samples from patients on Stx and has been established for diagnosis of Stx for a long time (reviewed in Karch et al., 1999 and Paton and Paton, 1998). However, conduction of ELISA bears disadvantages for *in vitro* studies as researchers are in danger of getting exposed to live

pathogens. However, Gluc is not the only safe reporter system that can be used for monitoring *stx2* expression. Another widely used reporter is the green fluorescent protein (GFP) (Aertsen et al., 2005; Wang et al., 2008). In contrast to Gluc, it is not a luminescence but a fluorescence-based reporter (reviewed in Close et al., 2011) and has the advantage of not requiring cofactors or substrates like coelenterazine (CTZ) (Kain et al., 1995). After application of excitation light, fluorescence reporters emit light of a certain wavelength (Troy et al., 2004). However, working with GFP is less efficient than working with Gluc because evaluation of fluorescence is performed via FACS. This is more time-consuming than evaluation of Gluc activity in 96-well plates via the Clariostar machine. Moreover, in contrast to a luminescence signal a fluorescence signal is not linear. This makes it a less valuable reporter system. A further disadvantage of GFP is auto-fluorescence of samples that can reduce the detection sensitivity and make interpretation of the results more difficult (reviewed in Billinton and Knight, 2001). One further reporter for monitoring the expression of *stx2* is *luxCDABE,* a bacterial luciferase operon from *Photorabdus luminescens* (Shimizu et al., 2011). Like GFP, *luxCDABE* has the advantage of not requiring a substrate for light emission. A disadvantage is a comparably lower dynamic range of the reporter compared to Gluc and an increased genetic instability of *luxCDABE* reporter bacteria as the metabolic burden to express the reporter system is quite high.

Its efficiency in testing is one of the major advantages of conducting the Gluc assay in 96-well plates. Several conditions can be tested at the same time and afterwards be evaluated in a fast and easy manner. This makes the Gluc assay more efficient and less time-consuming than cytotoxicity assays for example. In this thesis I also used the Vero cell assay (Konowalchuk et al., 1977)(**Figure 4-21**). This assay is a sensitive method for detecting Stx but has the disadvantage of being slow and time-consuming as it requires several days of incubation (reviewed in Karch et al., 1999).

The most important criterion is the faithful reflection of the toxin production. The Gluc assay reports Gluc production and release and is superior to molecular methods like RT-PCR that only detect *stx2* mRNA and not toxin production (Grys et al., 2009). Compared to other luciferases like firefly luciferase (Fluc), Gluc offers higher luminescence levels and is far more sensitive (Tannous et al., 2005). This is an essential feature when investigating small changes in toxin production, such as observed for the effect of PMNs on Stx2 production and release (**Figure 4-3**). Furthermore, Gluc exhibits increased stability compared to other luciferases when exposed to different temperatures or pH and it is not dependent on ATP (Tannous et al., 2005; Wiles et al., 2005). However, Gluc also has several disadvantages: I found that Gluc activity was decreased by high serum and high $H_2O_2$ levels (**Figure 4-4**, **Figure 4-12**). This effect may be caused by inactivation of Gluc or of its substrate

coelenterazine (CTZ). It has been described that CTZ is rather sensitive to environmental changes (Shimomura et al., 1993 as cited in Tannous et al., 2005). Furthermore, interactions between CTZ and serum proteins were described which can lead to changes in luminescence levels (Keyaerts et al., 2011). In experiments with serum I also observed that serum led to significantly elevated luminescence levels even in absence of Gluc reporter bacteria (**Figure 4-13**). Since environmental conditions can introduce bias to the results, results should be scrutinized to rule out artifacts before assay interpretation.

In conclusion, I showed that the Gluc reporter assay is a safe and sensitive medium-throughput assay for testing the influence of several host factors on *gluc* expression what represents the activity of the *stx2* promotor. This assay enables us to test host factors and chemicals and to compare their influence on reporter activity. However, it should be taken into consideration that the tested substances might interact with Gluc and its substrate CTZ. In the future, further host factors and therapeutic agents shall be tested in the Gluc assay. Possible candidates include hormones, antimicrobial peptides and further components of the human immune system. Furthermore, the Gluc assay can be an effective way to screen drug libraries on their influence on *gluc/stx2* expression. So far, the risk of antibiotics as therapy against EHEC infection is still unclear and no recommendation for antimicrobial treatment can be given (Freedman et al., 2016). For some antibiotic classes like fluorochinolones prophage induction is well described (Matsushiro et al., 1999). A recent study showed that quinolone antibiotics induce Stx2 production and death in a mouse EHEC model, while other antibiotics reduce mortality (Mühlen et al., 2020). Using the Gluc assay, the impact of several classes of antibiotics on reporter activity can be tested and compared. In a similar approach, Mühlen et al. used a *Citrobacter rodentium* mouse model to compare the impact of several classes of antibiotics on reporter activity. Therefore, antibiotics that do not promote *stx2* induction could be identified (Mühlen et al., 2020). Furthermore, other drugs (e.g. anti-inflammatory drugs, antihypertensive drugs) that are frequently used during EHEC infection might be tested on their influence on Gaussia reporter activity. In that way, we could evaluate which drugs can be applied safely during an EHEC infection without increasing the patient's risk for a severe course of infection and HUS.

## 5.2 Gluc is released in two different ways in the Gluc reporter assay

My experiments showed that there are two different ways how Gaussia luciferase or Stx2, respectively, gets released (**Figure 5-1**). **A.)** The "classical" pathway: expression of *gluc/stx2* after SOS response and prophage induction. Gluc is produced within bacterial cells and released after the expression of lysis genes (Neely and Friedman, 1998). This leads to phage-mediated bacterial lysis and the toxins reach the extracellular space. Any agent that triggers the bacterial SOS response can initiate the phage lytic mode (Los et al., 2010). Among well-described prophage inductors are antibiotics, UV light and hydrostatic pressure (De Paepe et al., 2014). Host factors like PMNs also trigger prophage induction (Wagner et al., 2001a). We confirmed this observation in the Gluc assay even though the underlying mechanism was not understood (**Figure 4-3**).

**B.)** Additionally, I suggest a second pathway: Release of Gaussia luciferase caused by antimicrobials acting on the bacteria from outside. Gluc/Stx2 that was already produced after prophage induction but not yet released can be set free in this manner. Furthermore, Stx storage within the periplasmic fraction of bacterial cells has been described – though only for Stx1 (Rahal et al., 2015). Bacterial lysis could release already produced or stored Gluc/Stx2 and lead to the increase of reporter activity in the Gluc assay. *In vivo*, bacteria might be stimulated by drugs, environmental or host factors to produce Stx2. Afterwards, other host factors like serum complement or antimicrobial peptides could mediate bacterial lysis additionally to phage-mediated lysis. Thus, even if phage lysis is prevented, antimicrobial-dependent bacterial lysis can enhance free Stx2 during infection. During colonization, EHEC is exposed to various host factors like the microbiota, the immune system und hormones. Several interactions between host factors and EHEC have been detected (reviewed in Karpman and Stahl, 2014). For example, bacteriocins are produced by commensal *E. coli* to outcompete related *E. coli* like EHEC (Schamberger and Diez-Gonzalez, 2002). Previously, it has been hypothesized that Stx2 production might be a defense strategy of EHEC to ensure its survival (Licznerska et al., 2016). Therefore, every host factor that threatens bacterial survival might enhance Shiga toxin levels. In my work, two of the tested agents led to enhanced Stx2 release by acting on the bacteria: $H_2O_2$ and complement could cause bacterial lysis *in vivo*. Exposure to $H_2O_2$ in concentrations between 10-20mM led to a strong increase of luminescence levels while causing cell death. Serum increased luminescence in a similar dimension like $H_2O_2$. Survival of lytic and non-lytic reporter strains was heavily affected and the complement system was found to be responsible for this effect. The Vero cell assay confirmed that serum led to an increased release of Stx2 in pre-stimulated bacteria in a short

amount of time in comparison to stimulated bacteria that were only exposed to PBS (**Figure 4-22**). This mechanism might well play a role in EHEC pathogenesis *in vivo* (**section 5.4**).

My work emphasizes that a complex interaction between host factors and bacteria contributes to EHEC pathogenesis and its severe consequences. Every patient reacts individually to an EHEC infection and not everybody develops dangerous complications like HUS (Tarr et al., 2005). Yet, we are far from understanding which factors are crucial for developing HUS or not. The Gluc reporter assay showed that both induction of phage lysis and release of Stx2 via bacterial lysis are involved in Stx2 release by EHEC. We suggest a bacterial-centered view of EHEC infections and HUS instead of just focusing on Shiga toxin pathogenesis. Several host factors and external factors like pharmaceutics could influence the amount of Stx2 in the gut. By identifying these factors, new treatment strategies could be developed.

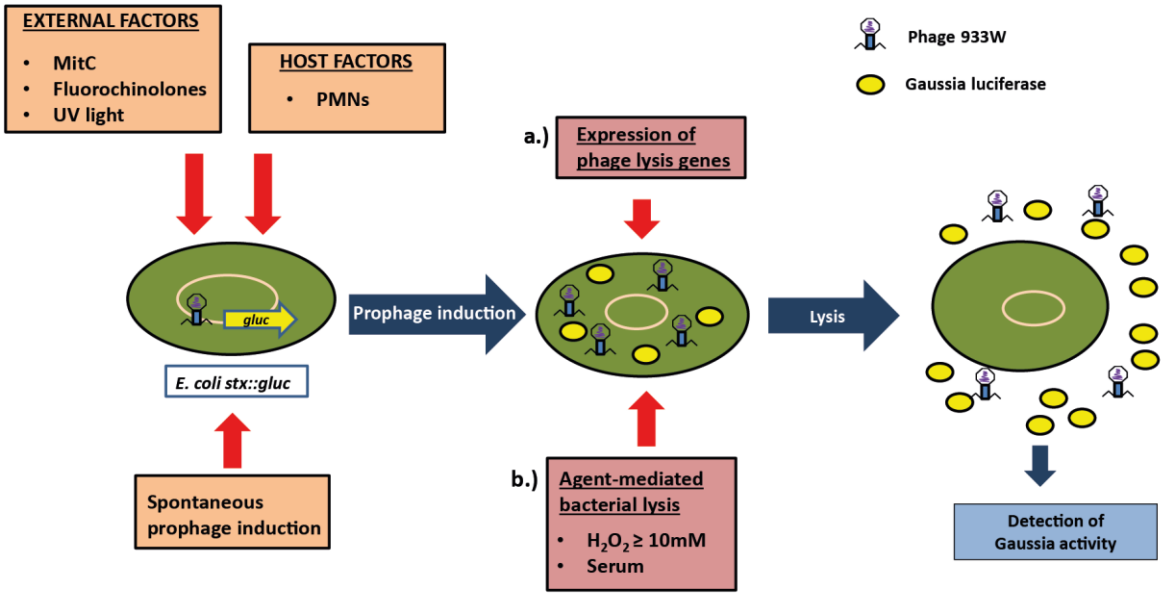


**Figure 5-1 Two mechanisms of Gluc/Stx2 release determined in this thesis** Prophage induction and expression of *gluc/stx2* can be triggered by environmental and host factors. Furthermore, spontaneous prophage induction has been described. After production of Gluc/Stx2, two different mechanisms of release are proposed: **a.)** phage lysis genes are expressed and Gluc/Stx2 gets released after bacterial lysis and **b.)** bacterial lysis mediated by factors like the complement system and higher concentrations of $H_2O_2$. Both mechanisms elevate Gluc levels (=Stx2 levels) in the supernatants which can be detected by increase in luminescence.

## 5.3 The influence of PMNs on *gluc/stx2* expression confirms their importance in EHEC pathogenesis

PMNs are among the first cells to reach the infected or inflamed gut and can be found at sites of inflammation within minutes (Sadik et al., 2011). The PMN count is often elevated during EHEC infections and is a predictor for the outcome of HUS patients (Robson et al., 1988; Walters et al., 1989). PMNs play a role in Stx pathogenesis even though they do not express the Stx receptor Gb3 (Macher and Klock, 1980). We observed that higher numbers of PMNs induced *gluc/stx2* expression (**Figure 4-3**). A 2-3-fold increase in luminescence was observed in comparison to Gluc reporter strains without exposure to PMNs. Wagner et. al. reported a 3-fold increase in Stx2 production when bacteria were exposed to PMNs as determined by ELISA. Thus, we could confirm these results (Wagner et al., 2001a). Depletion of PMNs decreased renal damage and lethality in a murine model (Fernandez et al., 2006). These findings indicate that PMNs play a crucial role in EHEC pathogenesis but the underlying mechanisms are not well understood. I assume that a 2-3-fold increase in Stx2 levels triggered by PMNs in the gut may well lead to a significant enhancement of Stx2-dependent clinical symptoms and of the risk of developing HUS. We compared the increase of luminescence triggered by PMNs with the strong inducing effect of Mitomycin C. MitC is one of the strongest known inducers *in vitro* and led to a 17-fold higher Gluc activity in my assay *(***Figure 4-2***)*(Zhang et al., 2000). When comparing the influence of PMNs and MitC in the Gluc assay, it must be taken into account that we analyzed PMNs only under laboratory conditions. *In vivo,* PMNs are likely in a more active state than the PMNs we used in the Gluc assay. Studies showed that Shiga toxin itself enhances neutrophilia (Fernandez et al., 2006). Moreover, it has been detected that Stx can increase the oxidative burst by PMNs (King et al., 1999). In my experiments, PMNs were only exposed to the Gluc reporter and might have been less activated as when exposed to EHEC that produce Stx2. The influence of PMNs on *stx2* expression is likely to be enhanced *in vivo*.

For determining the underlying mechanism of *stx2* induction by PMNs, I tested $H_2O_2$ in the Gluc assay. $H_2O_2$ is one of the major reactive oxygen species (ROS) that is released during oxidative burst (Rhee, 1999). Studies with *gfp* reporter bacteria showed that $H_2O_2$ in the low mM range triggers prophage induction (Los et al., 2009). We saw a significant increase of luminescence levels and decrease of bacterial viability when reporter bacteria were exposed to 10-20mM $H_2O_2$ (**Figure 4-6**). The decrease of bacterial survival was in accordance with the cytotoxic properties of $H_2O_2$ (Halliwell et al., 2000). However, inhibition of protein synthesis did not alter the luminescence levels and high Gluc activity

was also found in non-lytic reporter strains (**Figure 4-7**, **Figure 4-9**). Therefore, I assumed that the observed effects of $H_2O_2$ were caused by bacterial lysis and not by prophage induction. With the Gluc assay we could not confirm studies that showed prophage induction by $H_2O_2$. It has been described that $H_2O_2$ triggers a prophage induction in only 1.6% bacterial cells, compared to 10-30% by MitC (Los et al., 2012). I assume that the strong increase of luminescence levels triggered by $H_2O_2$ via bacterial lysis might have masked a potentially slight increase of luminescence by prophage induction.

Assays to quantify $H_2O_2$ concentrations by PMNs indicated that $H_2O_2$ is produced in the µM-range and therefore too low for increasing luminescence levels via bacterial lysis (**Figure 4-10** and **Figure 4-11**). This indicated that $H_2O_2$ was not or at least not predominantly responsible for *gluc/stx2* expression by PMNs. It must be considered that $H_2O_2$ was only tested *in vitro* and my assays did not test the influence of other ROS. *In vivo*, $H_2O_2$ is a substrate of other factors like myeloperoxidase to generate further antimicrobial agents like hydroxyl radicals and hypochlorous acid (HOCL) (Clifford and Repine, 1982). These antibacterial molecules and other agents like nitric oxide (NO) that are also released by PMNs during oxidative burst were not monitored in my assays. These agents may also reach high local concentrations at the site of infection and contribute to *stx2* expression *in vivo* and to the effects of PMNs in the Gluc reporter assay. NO has actually been described as an inductor of the bacterial SOS response (Lobysheva et al., 1999). NO was also found to increase killing of bacteria by $H_2O_2$ (Pacelli et al., 1995). Furthermore, exposure to bacteria leads to degranulation of PMNs and release of proteases like elastase (reviewed in Karpman and Stahl, 2014). Proteases act in concert with $H_2O_2$ to kill bacteria but also cause further tissue damage (Reeves et al., 2002). Proteases might contribute to the prophage inducing effects of PMNs *in vitro*.

Finally, my experiments could not reflect in *vivo* interactions of PMNs with signal molecules like chemokines that could enhance the effects of PMNs on *stx2* expression. Shiga toxin triggers an increased release of chemokines and interleukins by damaging cells (reviewed in Flagler et al., 2007). The local concentration of PMNs and released ROS might be much higher *in vivo* than in the Gluc assay (reviewed in Fang, 2011). This could enhance antimicrobial effects of ROS on bacteria. In summary, the underlying mechanisms of *stx2* expression triggered by PMNs are not resolved and will be the subject of follow-up studies. The interaction of various ROS and other antimicrobial mechanisms could contribute to *stx2* expression caused by PMNs. In my thesis, I only monitored the influence of one specific ROS, $H_2O_2$.

## 5.4 The influence of serum on reporter strains in the Gluc assay

Serum was found to have a broadly inducing effect in the Gaussia reporter assay. We found that this effect was partially attributable to oxidative decarboxylation of CTZ triggered by serum (**Figure 4-13**). Interferences between serum proteins and CTZ have been described in recent studies (Keyaerts et al., 2011). However, it was also shown that serum mediates complement-dependent lysis of the bacteria and release of Gluc in the supernatant (**Figure 4-15**). This indicates that EHEC exposed to complement during infection may lyse which concomitantly leads to enhanced release of Stx2 at the site of infection. So far, only few data on the influence of serum in the course of EHEC infections are available. It has previously been described that EHEC increases the permeability of intestinal epithelia by forming attaching and effacing lesions (Philpott et al., 1998). Furthermore, Shiga toxin triggers an intestinal inflammatory response and increases epithelial tissue damage by inhibition of protein synthesis (Békássy et al., 2011). The disruption of the epithelial barrier leads to bloody diarrhea that can be found in many patients with EHEC infections (reviewed in Tarr et al., 2005). Due to the increased gut permeability, it seems likely that serum proteins get in contact with EHEC in the gut. This could cause bacterial lysis and thereby enhance release of Shiga toxins. We found that the complement system was responsible for bacterial death and at least partially for the increase of luminescence in the Gluc assay (**Figure 4-20**). Likewise, Lee et al. stimulated their reporter strains with MitC and saw that additional exposure to serum complement led to a fast and significant increase of bioluminescence in their bacterial reporter (Lee et al., 2013). They used a reporter harboring a *recA::luxCDABE* transcriptional fusion and suggested that serum creates cell pores that allow large molecules to leave the cells. We assumed a similar mechanism in our Gluc reporter strains. Gluc is able to leave the cells after the complement system affects the bacterial cell integrity. Lee et al further noticed that heat inactivation of serum diminishes the bioluminescence activity. This is in accordance to our results that showed that heat inactivation of serum led to lower luminescence levels and better survival (**Figure 4-15**).

Exposure to serum caused a remarkable decrease of the survival of our reporter strains which we contributed to the effects of the complement system. Several studies showed that Shiga toxin activates the complement system (Morigi et al., 2011; Orth et al., 2009). This increased activity of the complement system in EHEC-infected patients could not be displayed in my experiments as I only tested blood samples of healthy donors. In a next step it would be interesting to use serum of patients with a generalized bacterial infection and a highly activated immune system in our Gluc assay and compare it to serum of healthy donors. However, it has to be taken into consideration that EHEC

strains might be more complement-resistant than the reporter strains in the Gluc assay. Several *E. coli* strains evolved resistance strategies against the complement system (reviewed in Abreu and Barbosa, 2017). For example, EHEC produces proteases that inactivate certain complement factors (Rasko et al., 2011). These resistance mechanisms against the complement system could prevent EHEC from being killed by serum in the human gut. Further studies with outbreak strains are required to detect if serum enhances Shiga toxin release. Controlled experiments with $C3^{-/-}$ mice could detect whether depletion of complement lowers Stx levels and improves the clinical outcome.

In conclusion, serum is likely to reach the gut of EHEC-infected patients at relevant concentrations and EHEC strains might be exposed to serum proteins. Therefore, these processes may well play a role the pathogenesis of EHEC infections. My experiments investigated interactions between EHEC and host defense mechanisms *in vitro.* In the future, it would be interesting to test the stool of patients with EHEC-infected patients on certain serum proteins and complement factors and correlate their quantity with HUS incidence. HUS develops approximately 5-13 days after the onset of the bloody diarrhea (Jandhyala et al., 2013; Tarr et al., 2005). Early identification of high amounts of serum proteins and complement factors in stool samples of patients with bloody diarrhea might help to identfy patients with an increased risk of developing HUS. This could be of therapeutical interest as the C5 complement inhibitor eculizumab is available as treatment option. Unfortunately, in the outbreak of 2011 no therapeutical benefit of this biological therapy was seen in EHEC patients (Menne et al., 2012). However, only patients that already developed a severe course of HUS were treated with ezulizumab and the effect of eculizumab on lowering the incidence of HUS was not investigated (Menne et al., 2012). Administration of ezulizumab before HUS induction could lower Stx levels and improve the patient's outcome. As no effective options to prevent HUS have been described so far, research of EHEC continues to be highly relevant.

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# List of publications

**Koeppel, M.B., Glaser, J., Baumgartner, T., Spriewald, S., Gerlach, R.G., von Armansperg, B., Leong, J.M., and Stecher, B. (2021).** Scalable Reporter Assays to Analyze the Regulation of *stx2* Expression in Shiga Toxin-Producing Enteropathogens. Toxins *13*, 534.

**Baumgartner, T., Glaser, J., Gerlach, R.G., Stecher, B., Koeppel, M.B. (2015).** Influence of host factors on *shiga toxin 2* (*stx2*) expression by Enterohemorrhagic Escherichia coli (EHEC). (Abstract for DZIF Autumn School 2015 and DGHM 2015)

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# Affidavit


**Eidesstattliche Versicherung**

Baumgartner, Tobias

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

**"Influence of the host response on *stx2* expression by Enterohemorrhagic *E. coli* (EHEC)"**

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 13.02.2022 Tobias Baumgartner