

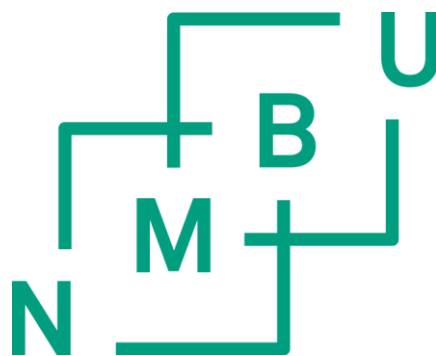
Microbial interactions – effects on virulence in Enterohaemorrhagic *Escherichia coli* (EHEC)

Philosophiae Doctor (PhD) Thesis

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Oslo, April 2015.

ABBREVIATIONS

| | |
|------------|--|
| AE | Attaching and effacing |
| CFU | Colony forming unit |
| DAEC | Diffusely adherent <i>E. coli</i> |
| <i>eae</i> | Gene encoding intimin |
| EAEC | Enteroggregative <i>E. coli</i> |
| EAHEC | Enteroggregative-haemorrhagic <i>E. coli</i> |
| EHEC | Enterohaemorrhagic <i>E. coli</i> |
| EIEC | Enteroinvasive <i>E. coli</i> |
| EPEC | Enteropathogenic <i>E. coli</i> |
| ETEC | Enterotoxigenic <i>E. coli</i> |
| Gb3 | Globotriaocylceramide |
| HC | Haemolytic colitis |
| HUS | Haemolytic uremic syndrome |
| IVOC | <i>In vitro</i> organ culture |
| LEE | Locus of enterocyte effacement |
| LPS | Lipopolysaccharide |
| MMC | Mitomycin C |
| OM | Outer membrane |
| PFU | Plaque forming unit |
| QS | Quorum sensing |
| STEC | Shiga toxin-producing <i>E. coli</i> |
| Stx | Shiga toxin |
| Tir | Translocated intimin receptor |
| TTSS | Type three secretion system |

LIST OF PAPERS

Paper I

The gut bacterium *Bacteroides thetaiotaomicron* influences the virulence potential of the Enterohemorrhagic *Escherichia coli* O103:H25

PLOS ONE (2015)

Hildegunn Iversen, Toril Lindbäck, Trine M. L'Abée-Lund, Norbert Roos, Marina Aspholm and Lotte S. Arnesen.

Paper II

Commensal *E. coli* Stx2 lysogens produce high levels of phages after spontaneous prophage induction

Frontiers in Cellular and Infection Microbiology (2015)

Hildegunn Iversen, Trine M. L'Abée-Lund, Marina Aspholm, Lotte P. Stenfors Arnesen and Toril Lindbäck

Paper III

Menadione reduces mitomycin C induction of Shiga toxin 2 production in EHEC

Manuscript

Hildegunn Iversen, Marina Aspholm, Erik M. Ræder, Trine L'Abée-Lund, Lotte P. Stenfors Arnesen and Toril Lindbäck

SUMMARY

Enterohaemorrhagic *Escherichia coli* (EHEC) is an emerging food borne pathogen associated with a number of outbreaks worldwide, and is a serious public health threat. The symptoms of the disease can range from mild diarrhoea to severe disease, such as haemorrhagic colitis (HC), and can result in life-threatening systemic complications such as haemolytic uremic syndrome (HUS). The O157:H7 serotype is traditionally associated with severe outbreaks, however, non-O157 serotypes are becoming a significant public health concern especially in European countries and in Australia. In Norway, an outbreak of EHEC disease of serotype O103:H25 (NIPH-11060424) showed particularly high rates of HUS.

The main goal of this study was to improve knowledge regarding interactions between EHEC strains and commensal bacteria in a setting where some of the environmental factors characteristic of the gastrointestinal tract were represented. To do so, the effect of co-culture/spent medium from various bacteria on gene expression in EHEC strains, the effect of the vitamin K-homologue menadione on Shiga toxin 2 (Stx2) production, and the interactions between Stx2 bacteriophages and various *E. coli* strains were investigated. The latter were studied in order to obtain more information about the host range of the Stx2 phage.

The results presented in this thesis demonstrate that contact and signalling between microbes influence virulence and global gene expression in pathogenic *E. coli*. When the EHEC strain NIPH-11060424 was co-cultured with a common human intestinal commensal (*Bacteroides thetaiotaomicron*), its virulence potential was affected. A number of genes involved in colonisation were up-regulated, and an increased adherence to eukaryotic cells was observed using an *in vitro* cell line. At the same time, the expression of Stx2, the main virulence factor in EHEC, was suppressed during co-culture with *B. thetaiotaomicron*. When the EHEC strain was cultured in spent medium from *B. thetaiotaomicron*, as well as other commensal strains, a decrease in Stx2 production was observed indicating that several bacteria produce a substance that inhibits Shiga toxin 2 production. The presence of inhibitory molecules from commensals has been demonstrated in other studies as well; however, the identity of the inhibitory substance has not yet been revealed. The vitamin K homologue menadione was also shown to exert an inhibitory effect on mitomycin C (MMC) induced Stx2 production via down-regulation of the bacterial SOS-response. Follow-up experiments identified a lower level of

intracellular MMC in menadione-treated cells compared to the control group, thus indicating that menadione induces a cross-protection for MMC. Considering that enteric bacteria synthesize vitamin K homologues and additional inducers of oxidative stress, it is likely that such compounds secreted by the intestinal microbiota may have an impact on the pathogenicity of EHEC in the gut.

When a group of commensal *E. coli* strains isolated from healthy children was co-incubated with recombinant Stx2-phages originating from NIPH-11060424, 40 % were infected lysogenically. Surprisingly, some of these lysogens produced more bacteriophages than the original EHEC strain, both spontaneously and when induced with MMC or hydrogen peroxide (H₂O₂), indicating that they would produce high levels of Shiga toxin if infected with the wild-type Stx2 phage. As high levels of Shiga toxin in the intestinal environment is associated with severe disease in humans, the susceptibility of commensal *E. coli*, as well as the ability to release phages/toxin in the intestine as a response to infection by toxin-encoding phages, may be important for disease development.

Bacterial life processes and pathogenicity are practised under conditions where several strains and species are present, offering synergies as well as competition for space, nutrition and receptors. Overall, the level of complexity among the different residents in the gut is high. This study illustrates that contact between commensals and EHEC as well as the presence of products produced by commensals, influence gene- and virulence expression in EHEC.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Enterohemoragisk *Escherichia coli* (EHEC) er en voksende gruppe matbårne patogener som er assosiert med mange utbrudd og er regnet som et alvorlig folkehelseproblem. Symptomer kan variere fra mild diare til alvorlig sykdom, som utvikling av blødende tykktarmbetennelse, som videre kan føre til livstruende systemiske komplikasjoner som hemolytisk uremisk syndrom (HUS). Tradisjonelt er det serotype O157:H7 som har vært assosiert med alvorlige utbrudd, men en rekke serotyper er nå regnet som et folkehelseproblem spesielt i Europa og i Australia. I Norge hadde vi et EHEC-utbrudd grunnet en serotype O103:H25 (NIPH-11060424), hvor HUS raten var usedvanlig høy.

Hovedmålet i denne studien var å øke kunnskapen om interaksjonene mellom EHEC-stammer og ulike kommensale bakterier, under betingelser der noen av miljøfaktorene karakteristisk for gastrointestinaltrakten var representert. Effekten av samkultur/supernatant fra ulike bakterier på virulensen i ulike EHEC-stammer, effekten av vitamin K-homologen menadione på Shigatoksin 2 (Stx2) produksjon, og interaksjonene mellom Stx2-bakteriofager og ulike *E. coli*-stammer ble undersøkt. Sistnevnte ble utført for å øke kunnskapen om smitteevnen til Stx2-bakteriofagen.

Resultatene presentert i denne avhandlingen viser at kontakt og signalisering mellom mikrober har innflytelse på det globale genuttrykket og særlig på uttrykk av virulensgener i patogene *E. coli*. Da EHEC-stammen NIPH-11060424 ble dyrket i samkultur med en human kommensal bakterie (*Bacteroides thetaiotaomicron*) ble virulenspotensialet endret. En rekke gener involvert i kolonisering ble oppregulert, og man observerte økt adheranse til eukaryote celler ved bruk av *in vitro* cellekultur. Samtidig ble genuttrykket av Shigatoksin 2, som er regnet som den viktigste virulensfaktoren for EHEC, nedregulert ved samkultur med *B. thetaiotaomicron*. Når EHEC-stammen ble dyrket i brukt medium/supernatant fra *B. thetaiotaomicron*, samt fra andre kommensale stammer, ble det registrert en nedgang i Stx2-produksjonen, noe som gir en indikasjon på at flere ulike bakterier produserer en substans som hemmer Stx2-produksjon. Tilstedeværelsen av hemmende molekyler fra kommensaler har vært demonstrert i andre studier, men identiteten til dette stoffet er fortsatt ukjent. Vitamin K-homologen menadione viste seg også å hemme mitomycin C (MMC)-indusert Stx2-produksjon via nedregulering av bakteriens SOS-respons. Videre eksperimenter viste at det var lavere intracellulær konsentrasjon av MMC i menadione-behandlede

bakterieceller sammenlignet med ubehandlede celler, noe som indikerer at menadione inducerer kryss-beskyttelse for MMC. Med tanke på at tarmbakterier produserer vitamin K homologer samt andre stoffer som kan indusere oksidativt stress, kan man forestille seg at disse, og andre, ulike substanser produsert av den intestinale mikrobiota kan påvirke patogenesen til EHEC i tarmen.

Da et utvalg av kommensale *E. coli* isolert fra barn ble dyrket i kontakt med en rekombinant Stx2-fag med opphav i den norske utbruddsstammen NIPH-11060424, ble 40% smittet lysogent. Noen av disse lysogene stammene produserte flere bakteriofager enn den originale EHEC stammen både spontant og etter induksjon med MMC eller hydrogenperoksid (H₂O₂), noe som indikerer at disse stammene har potensiale til produksjon av store mengder Shigatoksin hvis smittet med den originale Stx2-fagen. Da høy konsentrasjon av Shigatoksin i den tarmsystemet er forbundet med mer alvorlig sykdomsforløp i EHEC-infeksjoner, er mottakeligheten av kommensale *E. coli* for smitte med toksin-kodende fager, samt evnen til å frigjøre fag/toksin i tarmen, viktig for videre sykdomsforløp.

Livsprosesser og patogenisitet hos bakterier blir praktisert under forhold hvor en stor mengde bakteriearter og ulike stammer er tilstede, noe som gir synergiske interaksjoner i tillegg til konkurranse om plass, ernæring og reseptorer. Totalt sett så er kompleksiteten og interaksjonen mellom de ulike bakteriene i tarmen stor. Denne studien illustrerer at kontakt mellom kommensale bakterier og EHEC samt tilstedeværelse av produkter produsert av kommensaler påvirker genuttrykket og virulensen i EHEC.

INTRODUCTION

ESCHERICHIA COLI (E. COLI)

Escherichia coli is a member of the *Enterobacteriaceae* family and is a Gram-negative, facultative anaerobic rod. *Enterobacteriaceae* are grouped within the gamma subdivision of the phylum *Proteobacteria* ¹. The German researcher Theodor Escherich discovered the bacterium in 1885 and the genus is named after him ². The bacterium is a member of the normal intestinal flora of humans and animals, and most isolates are non-pathogenic. However, several pathogenic strains exist, causing a range of diseases in their hosts such as various forms of diarrhoeal disease, urinary tract infections, septicaemia and meningitis ^{3,4}. The gastrointestinal types of pathogenic *E. coli* were originally divided into seven categories/pathotypes according to their different virulence mechanisms: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusively adherent *E. coli* (DAEC) and adherent invasive *E. coli* (AIEC) ^{4, 5}. However, a new category – the enteroaggregative haemorrhagic *E. coli* (EAHEC) - was suggested as a new pathotype due to an outbreak in Germany of an *E. coli* strain of serotype O104:H4. The EAHEC strain evolved from an EAEC O104:H4 strain which acquired a Shiga toxin 2-encoding bacteriophage ⁶. Pathogenic *E. coli* that are not involved in diarrhoeal disease are termed extra-intestinal *E. coli* (ExPEC) and they will not be described any further in this thesis.

The general term for an *E. coli* strain producing Shiga toxin without any association with disease in humans, is Shiga toxin producing *E. coli* (STEC) or Verotoxin-producing *E. coli* (VTEC) ⁴. EHEC strains are characterised by the ability to produce Shiga toxins, and often possess the pathogenicity island termed the locus of enterocyte effacement (LEE), or other proteins involved in adhesion to intestinal epithelium, and they have been involved in human disease ⁵.

Pathogenic *E. coli* are major contributors to diarrhoeal disease worldwide. In developing countries, the pathotype ETEC is often involved in diarrhoeal disease in children ⁷. In Norway, the pathotype most often involved in diarrhoeal disease is EPEC ⁸ and this bacterial agent is mainly transmitted from person to person via the faecal-oral route, but food borne transmission has been reported as well ^{9, 10}. The number of food borne infections due to

EHEC has increased steadily in Norway over the last decades⁸. EPEC colonises the small intestine causing gastroenteritis in small children (< 2 years) while EHEC colonises the large intestine, and infection is associated with serious clinical outcomes⁴.

Enterohaemorrhagic *E. coli* (EHEC)

From the initial discovery of STEC until date, more than 400 serotypes have been identified but only a subset of these serotypes are associated with human disease^{5, 11, 12}. Historically, serotyping was the most important tool for identification of pathogenic *E. coli* strains; however, with more knowledge obtained regarding the pathogenicity and the presence of virulence genes characterizing the different *E. coli* strains, *E. coli* strains are now classified according to both serotype and to the presence of these specific virulence genes. According to the revised Kauffman scheme, *E. coli* are serotyped based upon their O (somatic), H (flagellar), and K (capsular) surface antigen profiles, and the arrangement of O and H antigens describes the serotype of a specific strain^{13, 14}.

The first described outbreak of EHEC disease occurred in the US in 1982, and was caused by the presence of EHEC bacteria of serotype O157:H7 in undercooked hamburgers¹⁵. The strain EDL933 was isolated in this outbreak and is since recognised as a reference strain for O157:H7. The sources of EHEC in the initial outbreaks were meat products. Today, any product that has not been heat-treated, such as vegetables, fruits, water, and unpasteurized juices or milk, or products contaminated with ruminant feces, can be potential sources of EHEC disease⁹. Moreover, transmission can occur directly from animal to human, and also between humans. The transmission route of the bacterial agent is faecal-oral transmission, and disease requires a notably low infectious dose⁵. The major reservoir for pathogenic EHEC strains is domestic ruminants; however, pathogenic strains have been found in other animals such as deer, wild boars, swine, rabbits, dogs, cats, rodents and even insects¹⁶. Some EHEC strains have only been isolated from humans (sorbitol-fermenting O157:H7 and O104:H4) suggesting that humans may be the reservoir for these particular strains^{17, 18}.

The O157:H7 serotype is highly associated with outbreaks in North-America, while non-O157 related outbreaks are on the rise in other parts of the world particularly in Australia and the European countries¹⁹. However, the number of non-O157 EHEC strains increases steadily in the US representing between 20-50% of the EHEC diseases annually in the US²⁰. The O-

groups most often associated with EHEC-disease in addition to O157 among humans are O26, O45, O103, O111, O121 and O145^{21, 22}. The clinical picture of non-O157 EHEC disease is similar to that of the O157 cases. However, the non-O157 strains are a heterogeneous group and therefore, the clinical manifestation varies in the different serotypes¹⁹. According to a study performed in Denmark, serotypes O157 and O103 were risk factors for haemorrhagic colitis (HC)²³.

The symptoms of EHEC infection can range from mild diarrhoea to more serious complications such as haemolytic colitis (HC) and haemolytic uremic syndrome (HUS)^{4, 9, 22, 24}. The pathogenesis of the disease is not completely understood, but the development of the various haematological and renal impairments is associated with Shiga toxins, most likely in combination with other toxins⁵.

EHEC outbreak in Norway

In 2006 Norway experienced a nationwide food borne outbreak caused by an EHEC strain of serotype O103:H25 (NIPH-11060424)²⁵. The majority of those affected were children (< 8 years) and the source was found to be fermented mutton sausage. In this outbreak, the HUS rate was particularly high (exceeding 60 %) indicating that this strain could be particularly virulent²⁵. However, only two out of eleven patient isolates, and none of the food isolates, were *stx2* positive. *stx*-negative isolates are described previously in other EHEC cases and are believed to be offspring of EHEC strains that have lost the toxin genes during the infection process²⁶. As all the food samples were *stx*-negative, the possible loss of toxin genes during infection is not likely. The *stx*-negative isolates in the Norwegian outbreak carried an Stx2 related phage in the same insertion site, and both variants, the one with the Stx2-phage and the *stx*-negative one, had most likely been present in the contaminated food source²⁷. *E. coli* O103:H25 serotype had previously not been associated with EHEC outbreaks but only sporadic cases of disease^{28, 29}. Interestingly, the genome of NIPH-11060424 seems to be related to the genome of the highly virulent EAEC O104:H4 strain which was responsible for the large outbreak in Germany in 2011. In addition, these two strains carry very similar Stx2-phages with a DNA sequence identity of 90%, which could point toward a mutual origin of these phages^{27, 30}.

Horizontal gene transfer and evolution of EHEC

More than 100 million years ago, the genus *Escherichia* diverged from the genus *Salmonella*³¹. Horizontal gene transfer is responsible for acquisition of most of the virulence factors in pathogenic *E. coli*. The prototype serotype of STEC/EHEC pathogenic *E. coli* strains is O157:H7, which, since its discovery in 1982, has become a well-known human pathogen. Sequencing studies suggest that O157:H7 evolved from the EPEC-strain O55:H7 by minor genetic changes approximately 50 years ago³². The LEE pathogenicity island, playing a central role in EPEC/EHEC virulence, has a G+C content of 38.3% which is lower than the rest of the *E. coli* chromosome (50.8%), indicating that this pathogenicity island most likely become established in *E. coli* by means of horizontal gene transfer. Studies have found that the LEE pathogenicity island has different insertion sites in various EPEC and EHEC strains, indicating that LEE has inserted on several occasions during evolution³³.

The fact that an EAEC strain could gain a Shiga toxin-converting phage from an STEC strain, generating a new pathotype – the enteroaggregative-enterohaemorrhagic *E. coli* (EAHEC), illustrates the evolutionary process of pathogenic *E. coli* strains³⁴, and emphasizes the unpredictable nature of pathogens in mobility of genes.

EHEC virulence factors and characteristics important in the gastrointestinal tract

The definition “the capability to enter a host, replicate and persist on locations that are unapproachable to other commensals” captures the basic concept of bacterial virulence³⁵. There is an on-going debate regarding which aspects of virulence that should be included in the definition. Wassenar *et al.* suggest a division of virulence factors into three groups: true virulence genes, virulence associated genes, and virulence life-style genes³⁶. True virulence genes are described as genes encoding factors not present in non-pathogenic organisms. These virulence factors interact directly with the host, and are in charge for any damage caused during the infection process. Virulence associated genes encode factors that are involved in the regulation of true virulence genes, and virulence life-style genes encode factors involved in colonisation of a host, or survival inside a particular host.

The effect of virulence factors in a complex environment such as the gastrointestinal tract, where numerous intercellular interactions occur, is difficult to predict/imitate in experimental conditions (*in vitro*). The ability for any given pathogen to adhere to the host mucosa is a critical step in pathogenesis³⁵. Several enteric pathogens have the ability to alter the host cell machinery to improve colonisation or gain access to the cytoplasm of the epithelial cells. Some of the effectors involved in these processes are quite complex systems, such as the type three secretion systems (TTSS) of many enteric pathogens. These systems manipulate the epithelial cell by interfering with signalling pathways, causing abnormalities in the actin skeleton of the affected cell³⁷. EHEC bacteria are not invasive, but change the host cell machinery through TTSS, injecting effector proteins that interfere with tight junctions and ion channels, leading to diarrhoea^{4, 38}. Diarrhoea results from the altered absorption of ions and solutes due to damage to the intestinal epithelium, with the subsequent movement of water to re-establish the previous state of osmolarity³⁹. The diarrhoea assists the pathogens dispersal by spreading infective organisms into the environment to reach new hosts. The TTSS used by EHEC strains will be described in more detail in the section on the Locus of enterocyte effacement (LEE).

Shiga toxin

The main virulence factor of EHEC, involved in the development of HC and HUS in humans, is the Shiga toxin (Stx)⁴. The Shiga toxin was first recognised in *Shigella dysenteriae* and was linked with the symptoms of bacillary dysentery⁴⁰. In 1983 it was reported that similar toxins were produced by *E. coli* strains⁴¹. Shiga toxin-encoding genes have been found in other bacterial species as well, including *Citrobacter*, *Acinetobacter*, *Campylobacter* and *Aeromonas*⁴²⁻⁴⁶. Stxs are classified into two main groups, Stx1 and Stx2, based upon identities/similarities at the amino acid level. Various sub-groups of each group exist. Stx1 is almost identical to Shiga toxin from *Shigella dysenteria* serotype 1, but differs serologically from Stx2. Stx2 is a more heterogeneous group including a number of sub-types, and some of these subtypes have been found to be associated with higher virulence^{47, 48}.

Stx1 and Stx2 toxins have the same mode of action (Figure 1)⁴⁹. Shiga toxins are AB₅ exotoxins made up of one enzymatically active A subunit and five B subunits that attach to specific globotriaocylceramid glycosphingolipid (Gb3) receptors on the eukaryotic cell⁵⁰⁻⁵². These receptors are present in most eukaryotic cells but are particularly abundant in human

renal tissue ⁵³. After binding of the holotoxin followed by internalisation in endosomes ⁵⁴, the toxin is transported into the trans-Golgi network where the A subunit is cleaved into an active A1 subunit and an StxB-associated A2 subunit ⁵⁵. In the endoplasmic reticulum, the A1-subunit detaches from the holotoxin due to protease activity ⁵⁶. The enzymatically activated A1- subunit moves into the cytoplasm and exerts its cytotoxic action by cleavage of the 28S rRNA, leading to inhibition of protein synthesis in the host, with resulting cell death ⁵⁷. AB₅ toxins are mainly found in Gram-negative bacteria ⁵⁸.

In *E. coli*, the Shiga toxins are encoded by lambdoid bacteriophages ^{59, 60}, while in *Shigella* strains the Shiga toxins are chromosomally encoded ⁶¹. Phage-induced cell lysis seems to be the key mechanism for release of Shiga toxin from the bacterial cell, as no secretion system has been found so far ⁶²⁻⁶⁴. However, Shimizu and colleagues suggest that there is an Stx2 secretion system in EHEC ^{65, 66}. Interestingly, Stx1 and Stx2 have different distributions in the bacterial cell. Stx1 is associated with the periplasmic fraction of the cell, while Stx2 seems to be located in the extracellular fraction, indicating differences in translocation systems for these toxins ^{49, 65, 66}. The promoters of *stx1* and *stx2* are different; a promoter that senses environmental iron concentrations regulates Stx1 production, while Stx2 production is dependent upon various phage-inducing agents ^{63, 64, 67}.

In human infection, Shiga toxins are synthesized in the large intestine and are most likely disseminated via the blood circulation to reach the target organs ^{9, 68}. As the epithelial cells in the colon appear to lack Gb3 receptors, it is not clear how the toxin gets access to the systemic circulation from its release in the intestinal lumen ⁶⁸⁻⁷⁰. Unfortunately, there are no existing suitable animal models to reveal the mechanisms used for the translocation of the toxins across the epithelium layer. It is suggested that Shiga toxin exits the lumen of the gastrointestinal tract due to damage of the mucosa caused by the bacterial colonisation and by the presence of toxin, thus getting access to the blood stream ⁵².

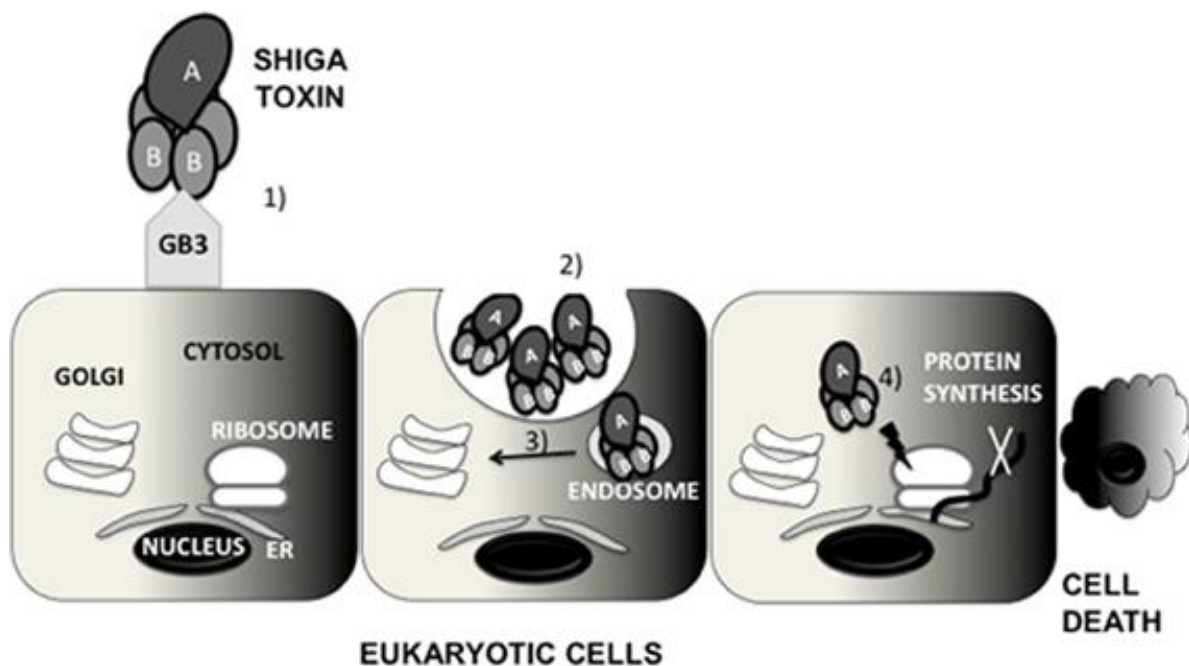


Figure 1. Mechanism of action of Shiga toxin. 1. The B-subunits bind to Gb3 receptors on the eukaryotic cell. 2. Stx is internalized by endocytosis. 3. Stx is transported into the trans-Golgi network where the A subunit is cleaved into an active A1 subunit and an StxB-associated A2 subunit. 4. The activated A1-subunit exerts its cytotoxic action by cleavage of the 28S rRNA, thus inhibiting protein synthesis resulting in cell death. Reprinted with permission under the Creative Commons Attribution License ⁷¹.

The locus of enterocyte effacement (LEE)

The capacity of EHEC strains to adhere to the intestinal mucosa is a crucial contributor to virulence ^{4, 72}. Various adherence genes believed to be important in the pathogenesis of EHEC disease are located on a 35.6 kb pathogenicity island named Locus of Enterocyte Effacement (LEE), which was first described in an EPEC-strain ⁷³. Attachment of EHEC to host intestinal cells is associated with the destruction of the intestinal mucosa and the assembly of a highly organized cytoskeletal structure termed an attaching and effacing (AE) lesion (reviewed in ⁷⁴). Pathogens harbouring LEE are termed AE pathogens, and include EHEC and EPEC, and also the murine pathogen *Citrobacter rodentium* ⁷³.

AE lesions are characterised by intimate adherence of bacteria to the host cell membrane resulting in damage to microvilli, reorganization of the host cell cytoskeleton and formation of a distinctive platform underneath the attached bacteria ^{73, 75} (Figure 2 and 3). These lesions are produced with the aid of components of TTSS, combined with a close interaction between the adhesion protein intimin and the receptor for intimin termed Tir (translocated intimin receptor) and a number of effector proteins ⁷⁶⁻⁷⁸. So far, more than 20 intimin types have been described. Intimin shares genetic homology with a protein named invasins produced by *Yersinia pseudotuberculosis*, which is involved in interaction with epithelial cells ⁷⁹. The Tir receptor, encoded in LEE, is translocated via the TTSS and inserted into the epithelial cell membrane, thereby becoming available for interaction with intimin. Intimin can also interact with nucleolin, a protein encoded by the eukaryotic host cell. Studies have shown that the binding affinity for nucleolin is similar to that of Tir ^{80, 81}. In fact, eukaryotic cells increase the expression of nucleolin in response to Shiga toxin, thus contributing to augmented colonisation of EHEC via an intimin-nucleolin association ⁸².

The LEE encoded proteins EspA, EspB and EspD are structural parts of a translocon that transports a selection of effector proteins into the host cell ^{83, 84}. EspA is involved in the initial adhesion of bacteria to the host cell and is also the major component of the translocation apparatus for Tir and other effector proteins. EspB and EspD form a pore in the host cell membrane and import effector proteins into the host cell ⁸³⁻⁸⁵ (Figure 4). A number of effector proteins are involved in the formation of the AE lesions, exerting their action by interfering with signalling pathways in the host cell through conversion of the actin cytoskeleton ⁸⁶.

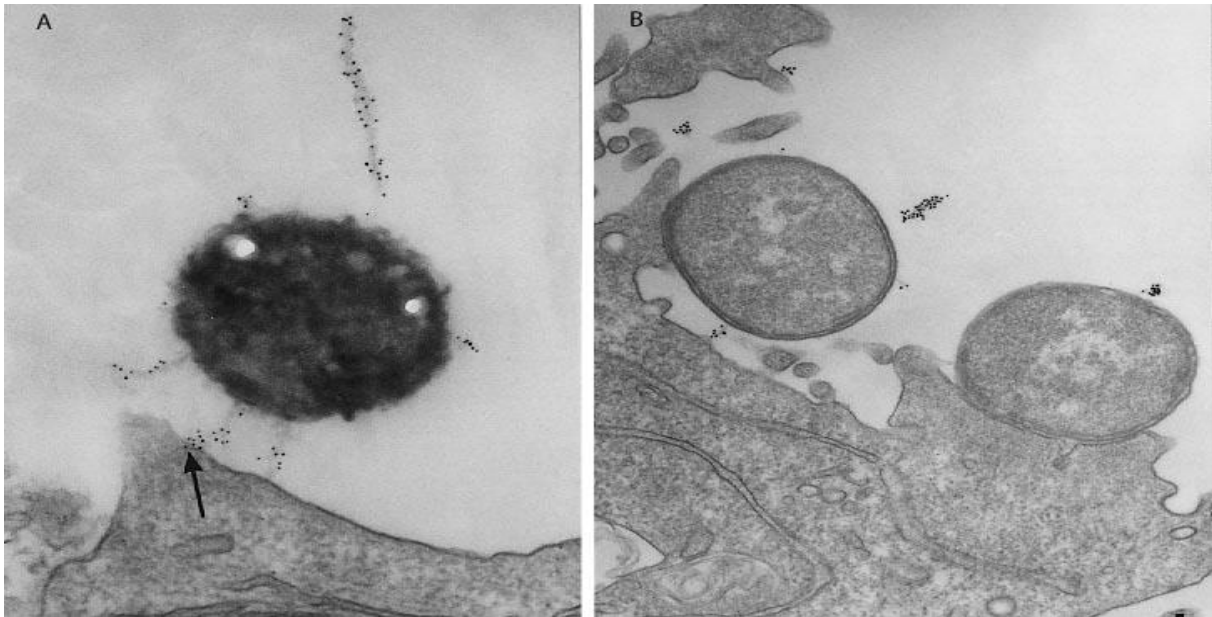


Figure 2. Transmission electron micrographs (TEM) showing EHEC cells immunogold-labelled with EspA antiserum after incubation with Hep-2 cells. A. EspA filaments connect bacteria with the epithelium (arrow). B. AE lesion in section. Reprinted with permission from John Wiley and Sons ⁸⁴.

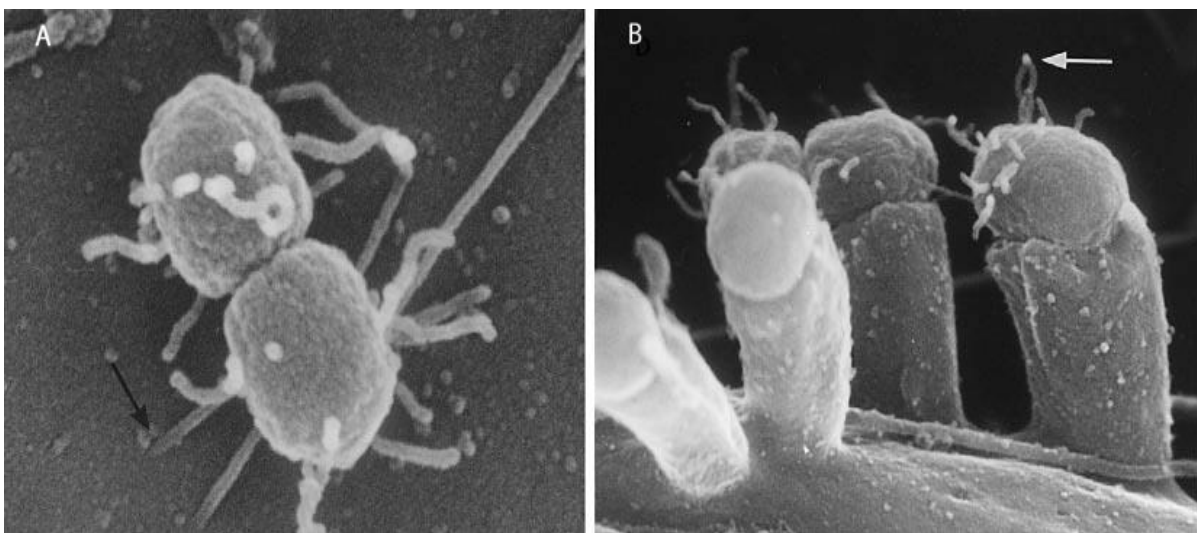


Figure 3. Scanning electron micrographs (SEM) showing EHEC incubated with HeLa cells. A. Picture demonstrates that EspA filaments connect bacteria and the epithelium (arrow). B. SEM-image of AE lesions. EspA filaments are still present on the bacterial surface (arrow). Reprinted with permission from John Wiley and Sons ⁸⁴.

The LEE-encoded TTSS also transports effector proteins that are encoded outside the LEE. In EHEC, 43 functional genes encoding non-LEE effectors have been found so far⁸⁷, and among these proteins EspFu/Tccp might be the most important one regarding the formation of AE lesions in EHEC strains⁸⁸. EspFu/Tccp is involved in the activation of Wiskott-Aldrich syndrome protein (N-WASP) via an indirect interaction with Tir, leading to actin polymerization in the host cell facilitated by the actin related protein 2/3 (Arp 2/3) complex⁸⁹. The TTSS genes (*esc* genes) in LEE are conserved between all AE pathogens. Genes represented by other parts of the LEE are more divergent⁹⁰. The majority of the non-LEE-encoded effectors differs in the various EHEC and EPEC serotypes, indicating varying infection strategies among the pathogens⁹¹. Not all STEC-strains possess LEE, indicating that other mechanisms are involved in the process of attachment to epithelial cells in these particular strains⁵.

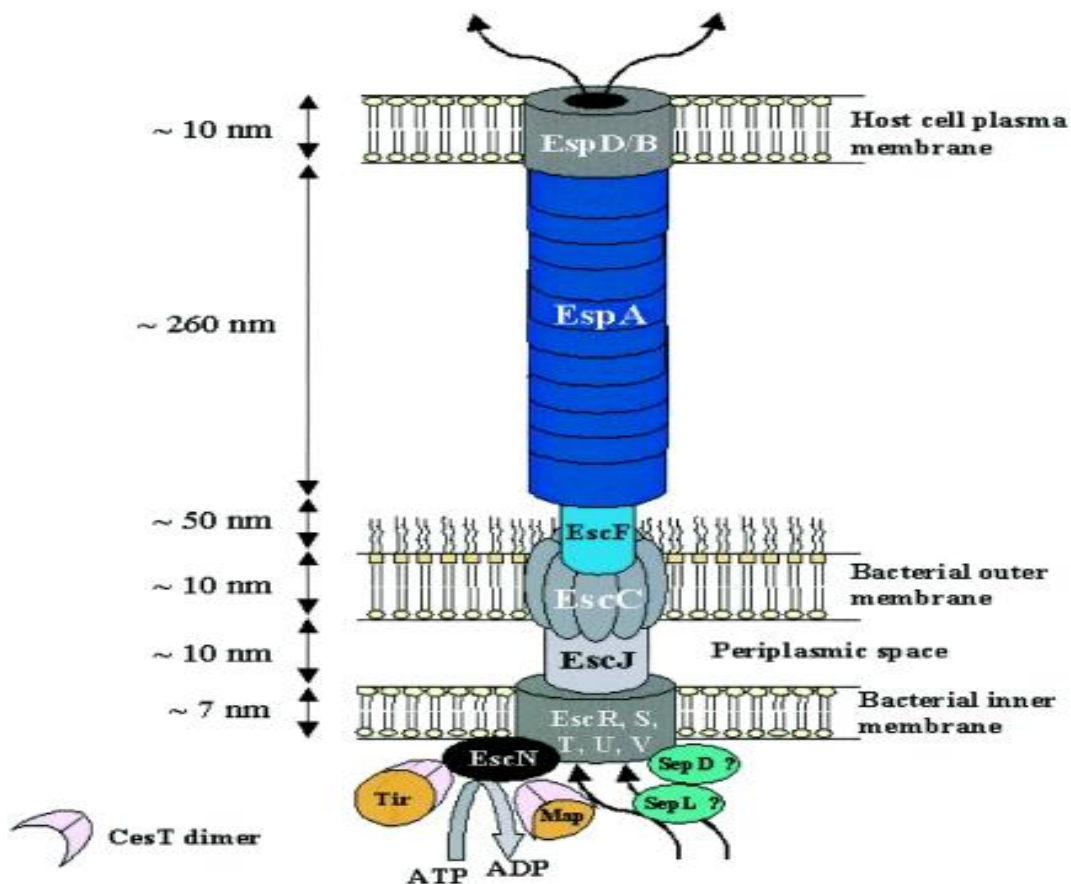


Figure 4. Schematic presentation of the EPEC/EHEC type three secretion system. Reprinted with permission from American Society for Microbiology (ASM)⁹².

Regulation of LEE

The LEE genes are organised into five operons (LEE1-LEE5) encoding 41 genes⁹³(Figure 5). The regulation of LEE is complex, involving several regulators encoded in the LEE, including Ler (LEE-encoded regulator), GrlA (global regulator of LEE activator), and GrlR (global regulator of LEE repressor). LEE expression is also under influence of global regulators encoded outside LEE. The regulation of LEE takes place at both the transcriptional and post-transcriptional level⁹⁴.

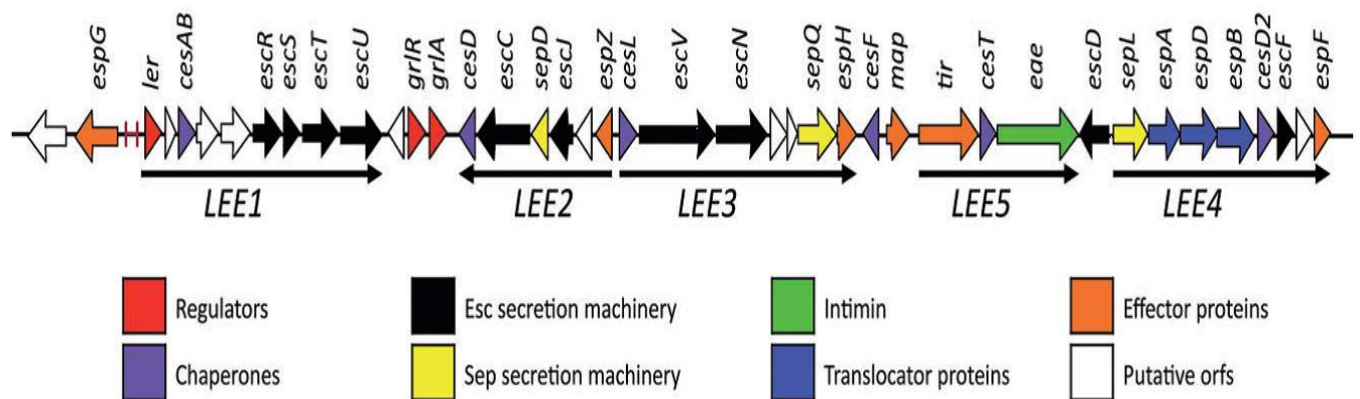


Figure 5. The genetic organization of the locus of enterocyte effacement (LEE). Reprinted with permission from John Wiley and sons⁹¹.

Diverse environmental conditions such as temperature, pH, and osmolarity affect the expression of LEE-encoded genes^{95, 96}. Studies have demonstrated up-regulation of LEE genes in response to contact with epithelial cells⁹⁷. Additionally, the population density has been shown to be important, revealing that LEE genes are influenced by communication between bacteria via quorum sensing (QS)⁹⁸. Hormones and other signalling molecules from the host also influence the expression of LEE genes⁹⁹. Furthermore, the expression of LEE genes is affected by the fluctuating nutrient levels in the gastrointestinal tract, and several studies have shown various mechanisms involved in this regulatory process¹⁰⁰⁻¹⁰².

Ler

Ler is the chief transcriptional LEE regulator and is itself controlled by other regulators (Figure 6) ^{103, 104}. Ler is located in LEE1 and its activity is involved in the stimulation of LEE2-LEE5- and the *grlRA*-operon, and at the same time, Ler suppresses expression of the LEE1-operon ¹⁰³. Ler works rather as a transcriptional de-repressor than as an activator, by removing the repressor activity applied by the global regulator H-NS. The regulatory repressor activity by H-NS is achieved by formation of a nucleorepressor complex that most likely captures RNA polymerase ^{105, 106}. The binding of Ler interrupts this complex, leading to reactivation of transcription. It is suggested that Ler regulates its own activity according to the concentration of Ler at a given time period ¹⁰³.

The GrlA-GrlR regulatory system

GrlA is a positive regulator of LEE1 and subsequent Ler activation, while GrlR acts as a negative regulator. These genes are co-transcribed from the *grlRA* operon ¹⁰⁷. The stimulatory effect of GrlA on Ler activity is inhibited by the action of GrlR ¹⁰⁸. GrlA most likely possesses two functional domains: one domain is involved in the interaction with GrlR, while the other acts as a transcriptional activator of the *ler* promoter ¹⁰⁹. The GrlA-GrlR complex is also involved in the regulation of flagellar genes ¹¹⁰.

Quorum sensing and its involvement in LEE regulation

QS is the mechanism by which bacteria communicate via chemical compounds released by the local bacterial population ¹¹¹. QS allows bacteria to assess the bacterial population density and to regulate the gene expression in response to it. In 1999, Sperandio *et al.* discovered that QS regulates the LEE pathogenicity island in EHEC and EPEC-strains through autoinducer 2 (AI2) ¹¹². It was later discovered that the actual QS molecule involved in the regulation of LEE was autoinducer 3 (AI3) ⁹⁸. The production of AI3 is not restricted to EHEC strains; the resident human microflora can also produce this aromatic compound ^{98, 113}. The sensor histidine kinase QseC, produced by EHEC, senses AI3 and triggers a cascade of events leading to activation of the transcription factor QseA. QseA activates *ler* with the successive activation of the LEE operons ¹¹³⁻¹¹⁶.

There is also a crosstalk between the eukaryotic hormones (adrenaline and noradrenaline) and the QS system conducting the communication to reach an inter-kingdom level from host to bacteria ^{117, 118}.

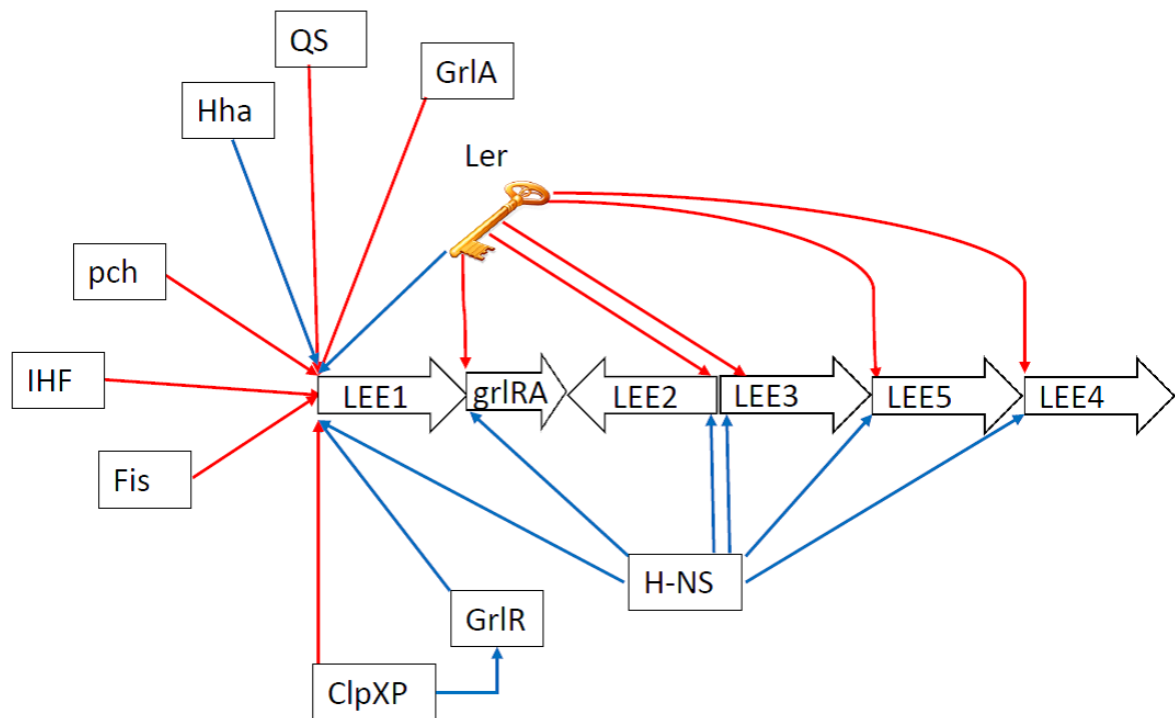


Figure 6. Simplified regulation model of the LEE pathogenicity island. Red arrows indicate activation while repression is represented by blue arrows.

The bacterial outer membrane permeability and antibiotic resistance

The outer membrane (OM) of Gram-negative bacteria performs an important role for the bacterium, protecting it from harmful substances and at the same time acting as a selective barrier. Outer membrane proteins (OMPs) have important functions for Gram-negative bacteria regarding responses to environmental changes such as nutrient limitations, oxygen tension, osmolarity, and antibiotics ¹¹⁹⁻¹²¹.

Various transcription factors are involved in the adaptation of bacteria to environmental threats such as pH changes, toxic substances, oxidative stress, and antimicrobial compounds.

MarA, SoxS and RobA are transcription factors involved in the regulation of responses by bacteria when exposed to environmental stressors¹²². The multiple antibiotic resistance (*mar*) locus consists of two operons, the *marC* and *marRAB*. A number of substances have been shown to induce the *marRAB* operon such as various antibiotics, plumbagin and menadione, among others^{123,124}. The *mar* locus is involved in resistance to a number of antibiotics^{125,126} and its activity is regulated by other transcriptional regulators such as RobA and SoxS¹²⁷. MarA up-regulate the multidrug efflux system AcrAB-TolC that encodes a bacterial efflux system¹²⁸, and indirectly causes decreased influx via the up-regulation of *micF* which encodes an antisense RNA¹²⁹. The natural function of the AcrAB-TolC efflux pump is believed to be efflux of bile salts as *E. coli* is exposed to high levels of bile salts in the gastrointestinal tract¹³⁰. The outer membrane porin OmpF is negatively regulated by *micF*. OmpF is one of the larger non-specific porins of *E. coli*, and has been found to play an important role in the penetration of several antibiotics^{125,131}. As porins offer a conduit through the OM for hydrophilic antibiotics, a decline in the capability to enter a cell for these antibiotics could lead to resistance. For the development of such antibiotic resistance in a bacterial cell, coordinated alterations in several porins and drug efflux pumps is required¹²⁰.

BACTERIOPHAGES

Bacteriophages are viruses that infect bacteria. They are genetic entities that are dependent on a living cell (obligate intracellular parasites) in order to carry out their replication cycle¹³². The credit for the discovery of bacteriophages is given to the French-Canadian scientist Felix D'Hèrelle, who was the first to announce the presence of a virus being parasitic on bacteria, and the first to characterise the agent as a bacteriophage¹³³.

The estimated number of bacteriophages in the biosphere is 10^{31} , thus bacteriophages are the most abundant organism present on Earth^{134,135}. The highest proportion of bacteriophages is located in the ocean, which is not surprising, considering the fact that the ocean covers more than 70% of the Earth's surface¹³⁶. There is limited knowledge of the diversity of bacteriophages in the human gastrointestinal canal. However, the first metagenomic study revealed the presence of abundant numbers of uncharacterised bacteriophages in the gastrointestinal tract of mammals¹³⁷. Bacteriophages seem to be present in most ecosystems, and their presence has been found to be important for the development of pathogens¹³⁸.

The International Committee on Taxonomy of Viruses (ICTV) determines the classification system for bacteriophages. Bacteriophages are classified according to morphology of the head and tail (short/long/contractile/non-contractile), and the nature of their nucleic acid (RNA/DNA, single-stranded/double-stranded) ¹³⁹.

Bacteriophage life cycles

The life cycle of a bacteriophage consists of several stages. Viral infections start with binding of the virus onto the host cell surface (adsorption), which is the key stage in the infection process ¹⁴⁰. Since bacteriophages are non-motile, the process of adsorption relies on random diffusion of the bacteriophages in order to find a host to infect. The number of collisions rises as the concentration of each component increases. When a bacteriophage has succeeded in adsorption onto the host surface, it most likely manages to inject its DNA or RNA into the host, as the correlation between adsorption and infection is quite high ¹⁴¹. At this point, there are two different outcomes of infection: lytic and lysogenic infection.

Bacteriophages are classified into two broad groups based upon their life cycle: virulent and temperate phages ¹⁴². Virulent phages can only enter the lytic cycle, while temperate phages replicate using both lytic and lysogenic cycles, thus having two lifestyles (Figure 7). The metabolic state of the cell will decide which of the two cycles are initiated ¹³⁵.

In the lytic cycle, the bacteriophage infects the bacterium and takes control over the replication apparatus. This leads to the production of progeny bacteriophages with subsequent lysis of the cell. Most temperate bacteriophages use this phage-induced bacterial lysis to release their progeny ¹³².

In the lysogenic cycle, the bacteriophage incorporates into the chromosome of the bacterial host where the bacteriophage then exists in a dormant state (a pro-phage) ¹⁴³. A successful lysogenic event can occur only if the phage genome integrates into the bacterial chromosome. The expression of a specific repressor that suppresses the late phage genes is crucial to achieve this condition. Additionally, phage integrase, which is regulated by gene products activated in the lysogenic pathway, and host recombinases (e.g. integration host factors), which are not regulated by any phage gene, must recognize homologous sequences present in the phage DNA and in the bacterial genome ¹⁴³. Pseudolysogeny has also been described,

where the bacteriophage exists as a plasmid instead of incorporating its genome into the host chromosome¹⁴⁴.

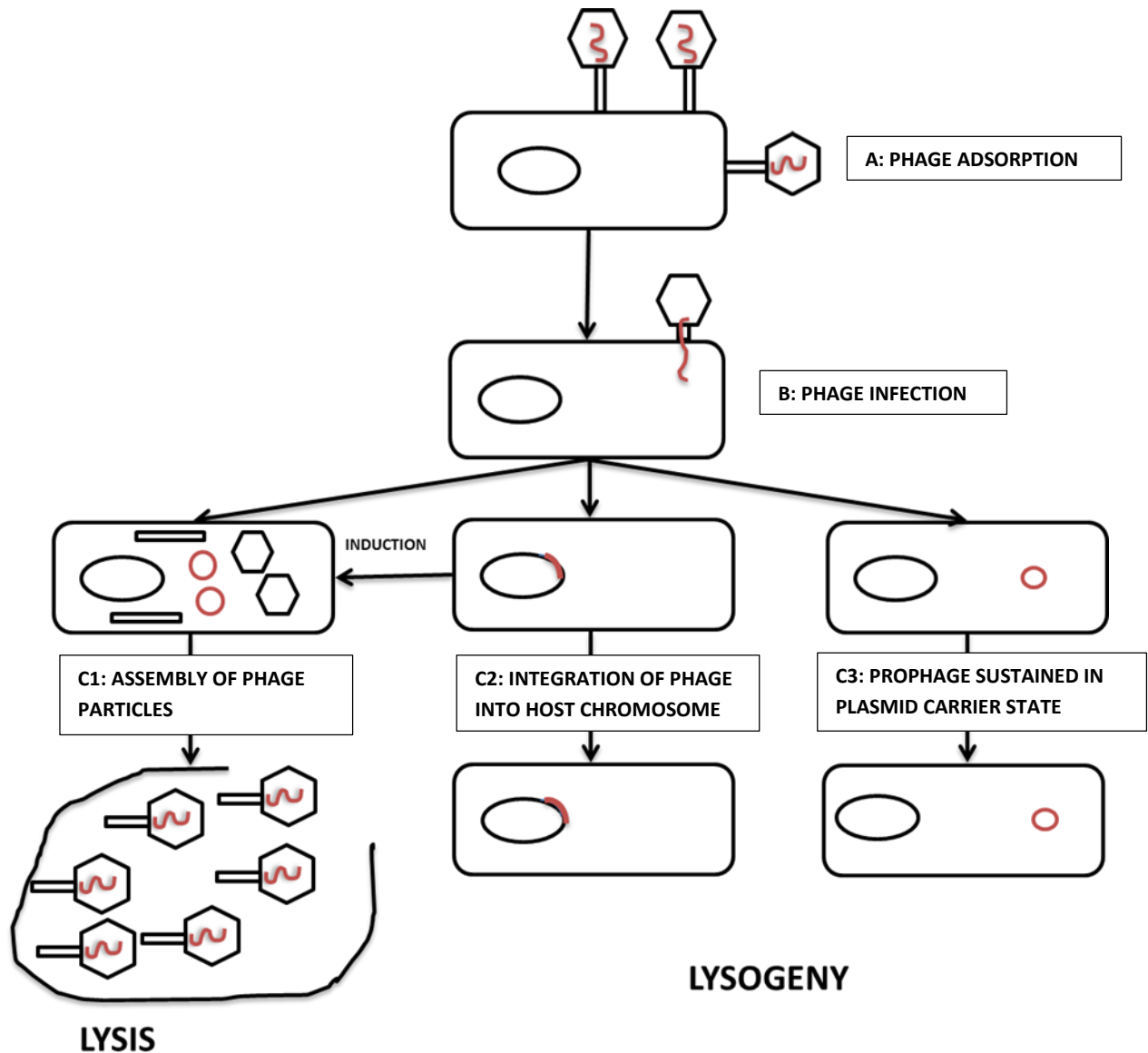


Figure 7. Figure showing the life cycles of bacteriophages. Phage adsorption (A). Phage infection (B). True virulent phages only follow the lytic cycle thus leading to a final lysis of the host cell and the release of bacteriophage particles (C1). Temperate phages can replicate using both life cycles. During the lysogenic cycle, most temperate phages integrate into the chromosome of the host (C2), however, some phages enter a pseudotemperate lifestyle, existing as pro-phages in a plasmid carrier state (C3). Figure is modified with permission under the Creative Commons Attribution License¹³⁵.

Bacteriophage infection

In Gram-negative bacteria, the receptors used by bacteriophages for binding to host cells may be cell surface proteins and porins and various lipopolysaccharide (LPS) sub-components¹⁴⁰. Stx2-phages are tailed phages, and the adsorption process with recognition of the host is dependent upon the tail-end of the specific phage. The adsorption machinery is evolving rapidly thus contributing to the host range of each phage¹⁴⁵. The details regarding molecular mechanisms involved in establishing infection of bacterial cells with Stx2-phages are not known. One study reports that Stx-converting phages only infect *E. coli* strains with rough LPS layer¹⁴⁶. This could point toward the possibility that deficiency in O side chain could increase phage infection and there are reports of differences in O side chain and susceptibility to phage infection^{147, 148}. It is demonstrated that Stx-phages with short tails use the conserved outer membrane protein YaeT (BamA) for adsorption onto host cells¹⁴⁹.

The lambda phage and the genetic switch

As mentioned, temperate bacteriophages can replicate using lytic and lysogenic cycles. The lambda phage is used as an example to explain the biology of temperate bacteriophages in more detail¹⁴³. The lambda phage has served as a tool for the study of various important principles in biology and is one of the best studied models used in molecular biology.

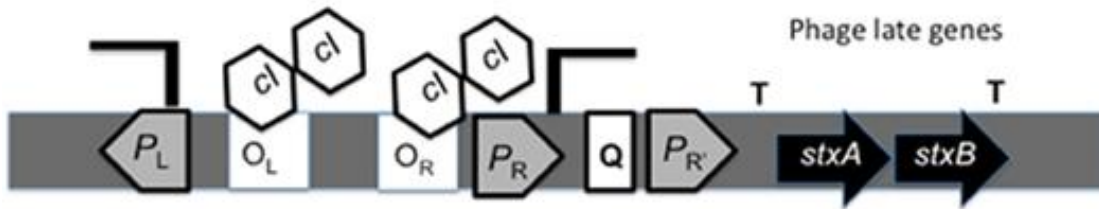
The initial stages of lambda phage growth are identical in lytic and lysogenic cycle. However, the decision, whether to enter lytic or lysogenic growth, is made early in the infection process. The choice is regulated by various repressor proteins, which are encoded on the phage genome. The early genes are expressed from two promoters: P_L and P_R. One of the transcripts expressed from P_L encodes a protein N with the ability to render RNA polymerase unable to identify transcription termination signals¹⁵⁰. The default state of temperate phages is the lytic mode of growth¹⁵¹.

In the lysogenic cycle, one of the regulatory proteins termed CII makes the polymerase transcribe the global repressor *cI* from an alternative promoter - the P_{RE} (promoter for repressor establishment) with the resultant production of repressor proteins. The levels of repressor proteins accumulate suppressing further activation of the lytic pathway and finally

lead to an inhibition of the activity of the Q protein with the subsequent entry into the lysogenic state ¹⁵¹. When the repressor CI is bound to O_R, it inhibits the activity of Cro (control of repressor and other genes) while at the same time stimulating activity of its own gene – *cI* (Figure 8). The concentration of the repressor in a lysogen is high, ensuring that repressors that detach from the operator sites are replaced by repressors nearby. The lysogenic state is very stable as long as inducing agents are absent ¹⁴³.

In the presence of inducing agents, the bacterial cell produces the SOS-response protein RecA. RecA cleaves the repressor proteins thus deactivating their activity (Figure 8) ¹⁵². As concentration of repressor drops, the rate of repressor synthesis declines. This is followed by the expression of *cro* and the gene encoding the positive regulator N involved in the early stages of the lytic phase. Cro is an important lytic regulator and controls further events leading to lytic development during both pro-phage induction and phage infection ^{138, 151}. Cro represses the activity of P_L and P_R promoters after the initial burst of synthesis, thus activating the lytic mode ¹⁵³. The protein Q is involved in inhibiting the termination of a specific transcript which indirectly leads to the expression of late phage genes including toxin genes, genes involved in lysis and assembly and packaging of viral particles ¹⁵⁴. At some point, there is enough Q present to activate the late phage genes involving the production of protein coats for the phages, and finally the lysis genes, thus releasing phages from the bacterial cell ¹⁵⁵.

Quiescent state - phage lysogenic cycle



Induced state - phage lytic cycle

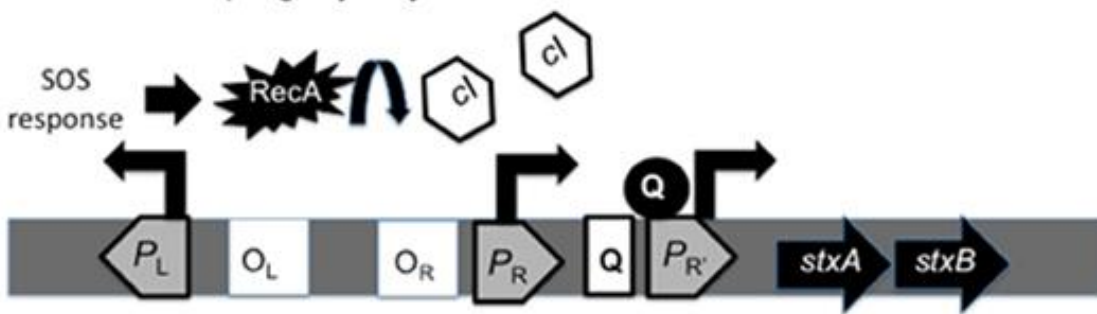


Figure 8.

Quiescent state: The bound repressor CI inhibits activity of the phage by silencing the two promoters P_R and P_L . When Q is not present, the transcript from $P_{R'}$ terminates at the first terminator located downstream of the promoter.

Induced state: RecA cleaves the phage repressor CI leading releasing P_R and P_L repression leading indirectly to the transcription of the antiterminator Q that modulate transcription from $P_{R'}$ making the transcripts resistant to termination, thus genes downstream of these terminators are transcribed, including *stx*-, lysis- and head/tail-genes. Figure is reprinted with permission under the creative common attribution license ⁷¹.

The evolutionary origin of bacteriophage-encoded virulence factors

Many of the virulence factors present in bacteria today most likely stem from the time when bacteria and other unicellular organisms lived together, before further coevolution between

higher eukaryotes and bacteria began ¹³⁸. However, the presence of pro-phages in most bacterial genomes indicates that bacteriophages were also a part of this initial evolutionary process ^{138, 156}. In fact, new sequencing technology has revealed that the presence of pro-phages in bacterial genomes represents the most important source of genetic diversity and variation in relation to virulence traits in pathogenic bacteria ^{157, 158}. A number of virulence factors could originally have developed as a means to infect unicellular eukaryotic predators, and studies have confirmed that some virulence factors are in fact active against unicellular predators ¹⁵⁹.

Stx-encoding bacteriophages

The Stx-encoding bacteriophages are part of the lambda phage family of temperate phages where the general genomic organisation and the regulatory circuits are similar ¹⁶⁰. The genomes of Stx2-encoding bacteriophages contain double-stranded linear DNA (47-70 kB) ¹⁶¹. The Stx2-encoding genome is organised into modules where each module represents genes encoding specific purposes for the life cycle of the phage ¹⁶². A high degree of recombination occurs between lambdoid phages resulting in the production of mosaic phage genomes ¹⁴⁵. Any phage encoding *stx* is characterised as an Stx-phage. Therefore, the virion morphology of Stx-phages is diverse (Figure 9). Lambdoid phages express the global repressor protein CI that represses all phage genes while the phage is integrated as a pro-phage. In the lambda phage model, the CI protein provides immunity for superinfection thus protecting the lysogen from incoming isogenic phages ¹⁴³. However, new discoveries have shown that EHEC lysogens often harbour more than one isogenic Stx2-phage, hence immunity for superinfection is not the case for Stx2-phages ¹⁶³⁻¹⁶⁵.

Stx-encoding bacteriophages are, as opposed to bacteria, more resistant to bactericidal processes used by the food industry such as various irradiation procedures including UV, but also thermal and high hydrostatic pressure treatment procedures ¹⁶⁶⁻¹⁶⁸. In that way, Stx-encoding bacteriophages ensure that *stx* genes within the bacteriophage are better protected than if existing as a pro-phage in the more fragile bacterial cell. Studies demonstrate the presence of free Stx-phages in the environment (both terrestrial and aquatic environments) and in various food products ^{169, 170}.

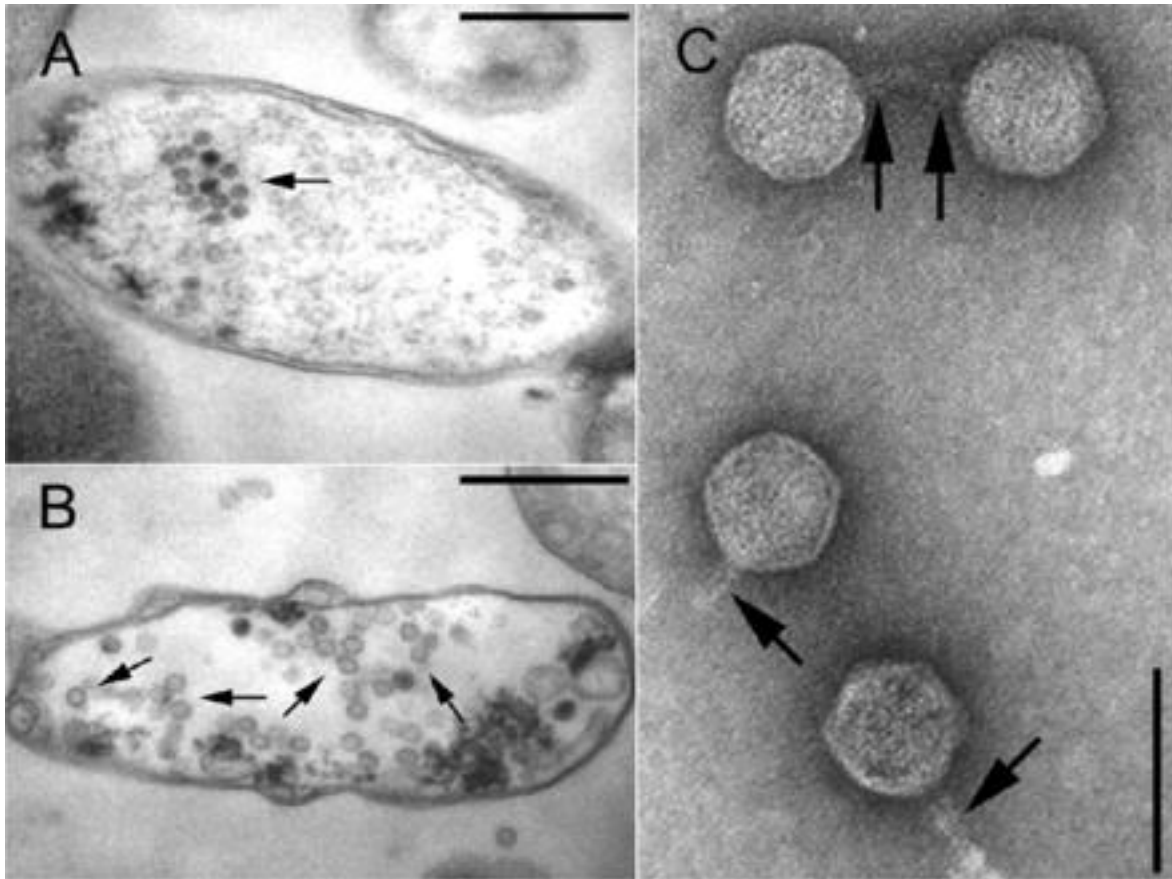


Figure 9. Transmission electron microscope (TEM) pictures of Stx2-phages. A and B. Ultrathin section of two EHEC cells with Stx2-phages (arrows) in the cytoplasm. C. TEM of Stx2-phages with short tails (arrows) and a hexagonal head. Reprinted with permission from American Society for Microbiology (ASM) ¹⁷¹.

Induction of Stx-encoding phages in the human gut

In the human gut, free Stx-encoding bacteriophages are in close contact with a remarkable number of bacterial cells, including commensal *E. coli*. Transduction of sensitive commensal flora both *in vitro* and *in vivo* has been demonstrated in a number of studies ¹⁷²⁻¹⁷⁷. A recent study indicates that free infectious Stx2-phages are present in a high percentage in human faeces (62 %) ¹⁷⁸.

As the genes encoding Shiga toxins are located within pro-phages present in STEC strains, the expression of these genes is dependent upon an inducing signal. Induction occurs when the bacterium harbouring the pro-phage experiences DNA damage with subsequent activation of the SOS-response ^{179, 180}. The SOS-response is a global response initiated as a result of

damage to the bacterial DNA ¹⁸¹. Under normal growth conditions, the SOS-genes are controlled by the transcriptional repressor LexA. However, in response to damaged DNA the RecA protein becomes activated by the presence of single-stranded DNA. The activated form of RecA cleaves LexA with the resulting derepression of a number of SOS-genes ¹⁸¹. The total number of SOS-inducible genes was found to be 43 according to a study performed by Courcelle *et al.* ¹⁸². The bacterial SOS-response causes a cascade of events finally leading to induction of pro-phage, followed by phage and Shiga toxin production, and lysis of the bacterial cell ^{183, 184}. Without an induction signal, only a small part of the population undergoes induction, a process referred to as spontaneous induction ¹⁸⁵.

Various compounds activate the SOS response in bacteria, triggering induction of pro-phages. Commonly used inducing agents include UV irradiation and Mitomycin ¹⁴³. It is not known whether inducing agents are present in the intestinal environment to trigger pro-phage induction and subsequent amplification of Shiga toxin. Hydrogen peroxide (H₂O₂) released from neutrophils has been found to cause oxidative stress, which activates the bacterial SOS-response. This in turn induces pro-phages, which consequently leads to increased Stx2 production *in vitro* ¹⁸⁶⁻¹⁸⁸. While the bacterial hosts die as a result of production of this toxin, there is seemingly no selective pressure against toxin-producing bacteria, indicating the existence of some beneficial characteristic linked to Shiga toxin production.

As neutrophils are present in the gut and participate in the immune response during infection with EHEC, the effect of H₂O₂ on pro-phage induction is interesting. It is proposed that this mechanism dates back to a time where bacteria and predators lived in close proximity and mammals were not the primary target for Shiga toxin ¹⁸⁹. This theory assumes that the induction of pro-phages takes place in only a small part of the *E. coli* population, and suggests that Shiga toxin has a protective effect against unicellular predators. Studies confirm the release of Shiga toxin by lysogens when in contact with eukaryotic predators releasing hydrogen peroxide ^{190, 191}. Hydrogen peroxide in this case acts as a signal for the lysogen, conveying the information that a predator is present, thus triggering the induction process in a small percentage of the lysogens with subsequent toxin production, and at the same time saving the life of the majority of the bacterial population. In light of these findings, the native role of Shiga toxin production could be to protect bacteria against predators but with an unintentional similar protective effect when exposed to the immune system in the intestine.

Stx-encoding bacteriophages have been found to be spontaneously induced more frequently than other phages from the lambdoid family^{66, 192}. There seems to be a lower threshold for induction as lower amounts of RecA are necessary to trigger the induction pathway. Studies performed with 933W (Stx2-phage originating from EDL933) lysogens reveal a reduced concentration of repressor molecules in these lysogens when compared to other lysogens not encoding Stx¹⁹³.

THE HUMAN GUT MICROBIOTA

A great number of bacterial species live in the human gastrointestinal tract, creating a complex ecosystem that has an impact on both the normal host physiology and the host's susceptibility to disease^{194, 195}. It is predicted that the number of bacteria in the human body outnumber human cells by a factor of 10^{16} ¹⁹⁶. Furthermore, new inhabitants of the gastrointestinal tract are steadily being reported, so the diversity of the intestinal microbiota is ever-changing¹⁹⁷. The greatest numbers of bacteria are located in the large intestine¹⁹⁸, where the density of microorganisms is estimated to be around 10^{12} microbes/gram¹⁹⁹ (Figure 10). The microbiota of the large intestine is quantitatively dominated by the two bacterial phyla Bacteroidetes (17-60%) and Firmicutes (35-80%)^{198, 200, 201}. A number of other phyla are represented, including Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria^{201, 202}. The large intestine also comprises archaeal species such as the methanogenic *Methanobrevibacter smithii*, an organism with the important role of removing redundant hydrogen during bacterial fermentation processes²⁰². The gut microbiota is involved in the fermentation of complex polysaccharides into short-chain fatty acids (SCFA) used as an energy source by the host. The metabolism of potentially harmful substances, such as bilirubin and bile acid, is also performed by the gut microbiota²⁰³. Additionally, the gut microbiota contributes to the production of vitamins and essential amino acids^{203, 204}. Notably, the microbiota plays an important role during development of the immune system²⁰⁵.

The gastrointestinal tract of humans is sterile at birth but within 48 hours, the initial intestinal flora has established, containing around 10^8 microbes per gram^{206, 207}. The initial population consists mainly of facultative anaerobes including *E. coli* and *Streptococcus* followed by the arrival of *Bacteroides*, *Clostridium* and *Bifidobacterium* within the first week of life

(reviewed in ²⁰⁸). Later in life, the diet will affect the composition of the microbiota ^{207, 209}. Around the age of three, the gut microbiota reaches a level of complexity comparable to the adult microbiota ²¹⁰⁻²¹². The gut microbiota seems to be relatively stable between individuals at phylum level but varies at the species level, and seems to be stable for long time intervals within different individuals ^{202, 213} ¹⁹⁵. Even though the diversity of gut microbiota is large between individuals, the functional gene profiles seem to be similar ¹⁹⁵. There has been a revolution in the molecular methods utilized to reveal the landscape of the human microbiota. Along with the development of high-throughput next generation sequencing methods, more knowledge of the human gastrointestinal microbiota has been acquired ^{197, 214}.

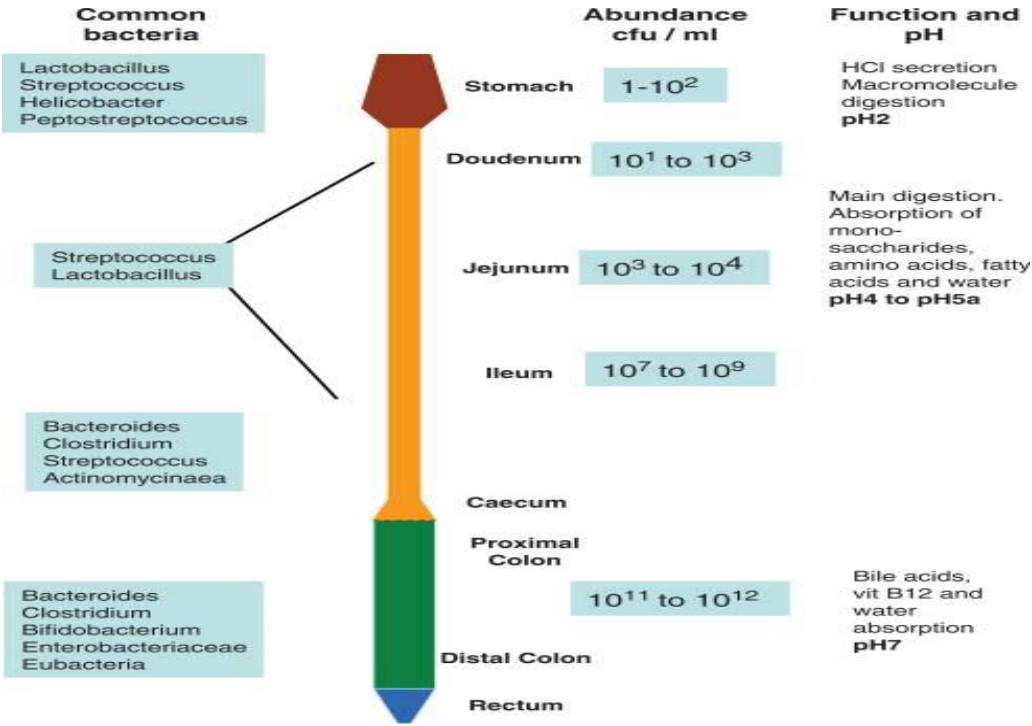


Figure 10. A schematic presentation showing the variation in microbial numbers and composition of the lower gastrointestinal tract. Reprinted with permission under the Creative Commons Attribution License ²¹⁵.

Bacteroidetes

Bacteria belonging to the phylum Bacteroidetes are abundant members of the human intestinal microbiota ²⁰². The phylum establishes in the gastrointestinal tract early in life and performs metabolic modifications important for the host ²¹⁶. The genus *Bacteroides* (family *Bacteroidaceae*) comprises various gastrointestinal bacterial species and is the most stable constituent of the gastrointestinal inhabitants in healthy adults ^{202, 217}. *Bacteroides thetaiotaomicron* has a great ability to use nutrients present in the human gut. Genome sequencing revealed that *B. thetaiotaomicron* has an extensive carbohydrate utilization system and can metabolize simple carbohydrates as well as complex sugars ²¹⁸. The genus contains 172 glycosyl hydrolases which is more than any other gut commensal, emphasizing that this bacterium is highly adapted for a life in the human gut.

The gut microbiota and its defence mechanisms against pathogens

Colonisation resistance

Colonisation resistance has been defined as the inhibiting effect of the indigenous microbiota on bacterial pathogens in the gastrointestinal system ²¹⁹. Colonisation resistance is achieved by the competition for nutrient sources and attachment sites between the microbiota and the invading pathogen ²²⁰. When the commensal bacterium *B. thetaiotaomicron* is co-cultured with *Eubacterium rectale*, the epithelial cells increase the production of mucosal glycans because of signals received from *B. thetaiotaomicron*. Since only *B. thetaiotaomicron* is able to digest these glycans, the growth of *B. thetaiotaomicron* is favoured on the expense of the competitor ²²¹.

Antimicrobial peptides including colicins

The production of antibacterial substances by bacteria can improve their capacity to compete with other bacteria in the gastrointestinal tract. There are a multitude of antibacterial substances produced by bacteria such as antibiotics, metabolic by-products (ammonia and

hydrogen peroxide) and bacteriocins. Bacteriocins are peptides produced by most archaea and bacteria. They have a narrow target spectrum, and the producer is immune for its specific bacteriocin. The bacteriocins produced by *E. coli* are separated in two main groups: the colicins and the microcins (reviewed in ²²²). Colicins are plasmid-encoded proteins consisting of three specific domains: the colicin protein, an immunity protein and a protein involved in lysis. Microcins are encoded on the chromosome or on plasmids and these toxins are produced and actively secreted from the bacteria.

Immunomodulation

The gut microbiota plays an important role in the development of the intestinal mucosal immune system. Through direct interference with the intestinal epithelium, the microbiota affects both the innate and adaptive immune system ^{223, 224}.

The gastrointestinal epithelial defence system against invading pathogens

The mucosal immune system is challenged with two contradictory responsibilities. The system has to avoid the overgrowth and entry of pathogens into the systemic circulation, and at the same time, be tolerant to the existing microbiota and avoid unwarranted activation of the systemic immune system ²²⁵.

As a first order of defence, the intestinal epithelium protects the host from pathogens by providing a physical barrier. In addition, a layer of mucus, produced by Goblet cells, covers the intestinal epithelial cells. In the large intestine, the mucus layer is divided into two separate layers; a loosely arranged outer layer which is colonized by commensal bacteria, and a dense, unpopulated inner layer in close proximity to the epithelial cells ^{226, 227}. Mucus is composed of mucins, trefoil peptides, and surfactant phospholipids. Mucins consist of monomers of glycoproteins linked by disulfide bridges ²²⁸. The layer of mucus creates a defense barrier protecting the enterocytes from damage caused by microbes. Additionally, the specialized epithelial cells secrete various antimicrobial compounds into the mucus layer contributing to the protection of the enterocytes. The secretory action of the epithelial cells is often triggered by the presence of the microbiota ^{229, 230}.

M-cells are highly specialised cells that sample pathogens from the luminal side, transporting them across the epithelial layer, and present them for immune cells, with the subsequent triggering of adaptive immune responses ²³¹. Additionally, IgA produced by B-cells, is translocated and released on the apical surface of the epithelial cells where they act primarily by blocking access of the pathogens to receptors on the gut epithelial lining ²²³. Toll-like receptors (TLRs) are also distributed along the gastrointestinal epithelial cell layer. These receptors identify microbes via conserved microbe-associated molecular patterns (MAMPs) and pro-inflammatory genes are induced in response ²³². The various defence systems utilized by the intestinal epithelium are summarized in Figure 11.

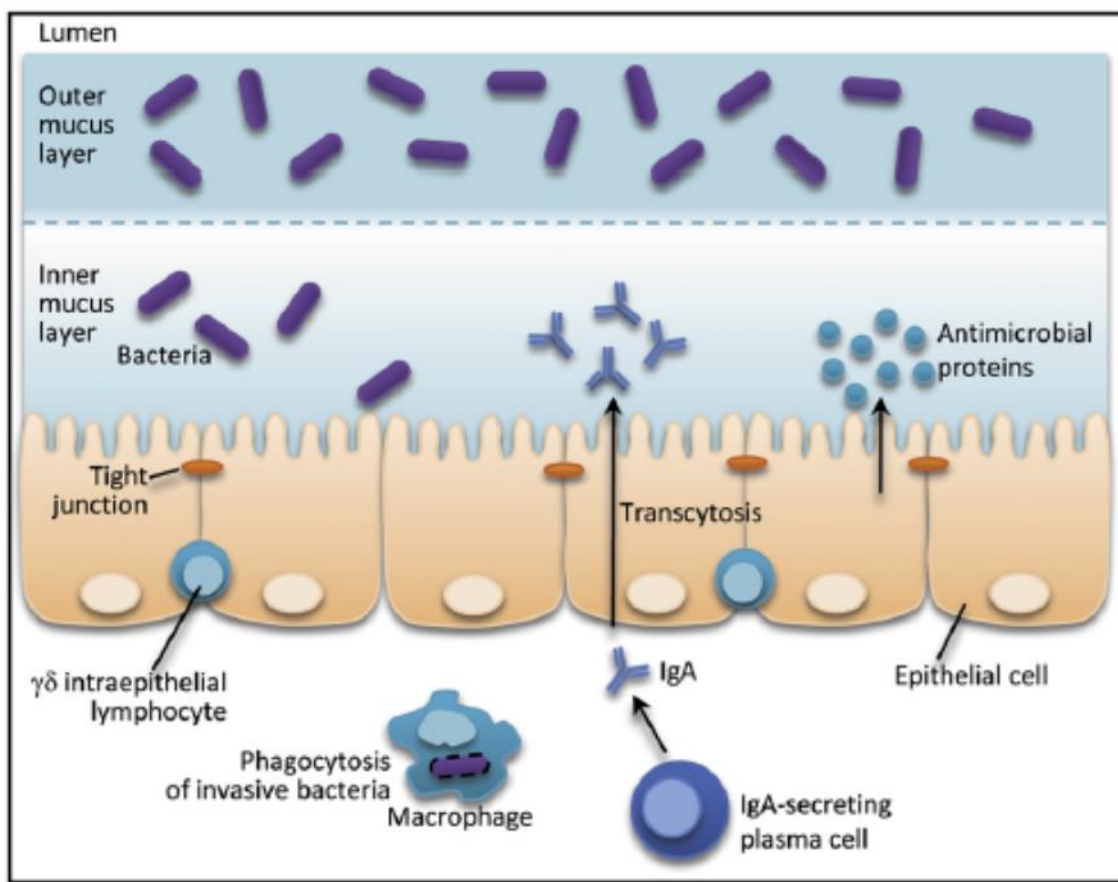


Figure 11. The defence system in the gastrointestinal tract. Reprinted with permission from Elsevier ²³³.

The strategies used by pathogens to overcome the defence mechanisms

Pathogenic bacteria have developed a number of strategies to avoid the defence mechanisms exerted by the microbiota and the host immune system, in order to get access to the host. Motile bacteria get access to the epithelial cells by swimming through the protective mucus layer²³⁴. Pathogens may possess genes that allow them to survive within macrophages thus gaining access to host tissues²³⁵. There are also pathogens that exploit existing signalling pathways in the host for invasive purposes²³⁶. For instance, EHEC and EPEC strains alter host cell signalling pathways via an intimate adherence to the host followed by injection of effector proteins via TTSS¹¹³.

MICROBIAL COMMUNITY INTERACTIONS

Traditionally, studies have focused on the individual bacterium and the way it interacts with its host, without including the natural environment of the pathogen. The natural habitat for most bacteria is often a community where several strains and species are present²³⁷. Well-documented intra-species communication models such as QS have proved that bacterial communication via chemical signals occurs²³⁸. According to genome sequencing studies, various bacteria appear to have an enormous capacity to produce small signal molecules²³⁹. Studies also indicate that a great number of bacteria produce small signal molecules with important roles in intra- and interspecies communication^{240, 241}.

Methodological advances, including various high-throughput sequencing technologies, have made it possible to view the pathogen in its biotic environment, as presented in the pathobiome concept introduced by Vayssier-tausat *et. al* (2014). They emphasize the importance of knowing the community surrounding the pathogen, including whether the community affects the expression and transmission of virulence²⁴². Pathogens often relate to more than one ecosystem, and it can be difficult to predict which ecosystem best simulates the given pathogen's natural environment. For example, a pathogen like EHEC can exist freely in the environment for a while, remain in the GI-system of ruminants for a period of time, and/or enter the human GI tract and cause disease. Pathogens have to adapt to the various ecosystems they encounter in a host or in a vector, but also when located outside the host or vector^{243, 244}.

The battle for nutrients is often the main issue involved in bacterial competition ²⁴⁵. The production of antibacterial substances in this setting may act as a means to increase growth on the expense of other bacteria. Cooperation should be the dominating trait for microbial groups of identical genotypes, while competition should dominate between group members of different genotypes using the same substances for energy, from an evolutionary point of view ²⁴⁶. If the microbes share the same resources, the degree of competition is defined by the level of intersection of this particular resource niche ²⁴⁷. The various interaction systems between bacteria are categorised into two separate groups: contact dependent- and contact-independent interactions.

Contact-dependent interactions

During the last decades, it has been revealed that bacteria actually interact broadly with each other. Bacteria use contact-dependent systems for intercellular signalling, and for the transport of effectors into neighbouring bacterial cells as well as into eukaryotic cells. The literature describes several approaches for the bacterium to create direct cell-to-cell contact. Until now, four main groups of cell surface structures involved in collaborative communication between bacterial cells are described (type III-VI secretion systems) ²⁴⁸. The type III secretion system was described in the section covering the LEE pathogenicity island. The Type IV secretion systems deliver DNA and/or proteins mainly between bacterial cells (conjugation) and this system is also utilized to deliver bacterial toxins into eukaryotic cells ²⁴⁹. The type VI secretion system delivers effectors mainly to bacterial cells but also to eukaryotic cells and demonstrates similarity to the phage injection systems ²⁵⁰. The type V secretion system mediates contact-dependent growth inhibition and is present in certain *E. coli* strains. The growth inhibition occurs by binding of a beta-helical surface structure onto a receptor on a susceptible target strain. This binding triggers a signal that in turn leads to growth inhibition of the target strain ²⁵¹.

Contact-independent interaction

Contact-independent interactions are defined as an association between bacterial cells that are dependent upon diffusible factors. The well-known process of QS is one example, regulating a number of processes including virulence gene expression, bioluminescence, sporulation,

biofilm formation, competence and cellular differentiation ²⁵². Studies indicate that sub-inhibitory concentrations of antibiotics produced by bacteria can also act as signalling molecules, and that their natural role as growth inhibitors has been slightly misinterpreted ²⁵³. An alteration in the expression of bacterial virulence genes in response to sub-inhibitory concentrations of antibiotics has been demonstrated ²⁵⁴.

KNOWLEDGE GAPS

Traditionally, bacterial characteristics have been studied in pure cultures. However, enteric pathogens residing in the gastrointestinal system live in an environment where interactions between various partners are expected. Recent studies have revealed that bacteria produce a number of secondary metabolites acting as signalling molecules. Common laboratory conditions might not support the production of these substances, as the production seems to depend on the presence of other microbes, or specific nutrient conditions. In this thesis, co-culturing of various bacteria is performed in order to observe altered behaviour/expression of genes including virulence genes in EHEC in a more realistic setting, simulating the natural environment of enteric pathogens – the gastrointestinal tract.

The nationwide food borne outbreak by EHEC O103:H25 (NIPH-11060424) in Norway had an interesting and unfortunate feature; the unusually high HUS rate in this outbreak (exceeding 60%) indicated that this strain could be particularly virulent, but whether this was due to the Stx-phage, the Shiga toxin, the regulation of these, or other factors, are not known.

The expansive distribution of Shiga toxin-encoding genes points toward the fact that Stx-phages are effective in sharing their genes with other bacterial species. However, the host-range of the Stx-phages is not known in detail and most likely, more than one receptor is involved in the dissemination of Stx-phages. The demonstration that an EAEC strain could gain an Stx-phage, thus creating a new pathotype - the EAHEC - which was responsible for the large outbreak in Germany in 2011, illustrates the expansive nature of Stx-phages. Interestingly, the genome of NIPH-11060424 bears a resemblance to this highly virulent EAHEC genome of serotype O104:H4.

AIMS OF STUDY

Main objective:

The main objective of the present study was to expand the knowledge on the effects of interactions between EHEC strains and commensals in a setting where some environmental factors characteristic of the gastrointestinal tract were represented. The effects of co-culture and spent medium from various bacteria on global and virulence gene expression in EHEC were investigated, and additionally, the effects of the vitamin K-homologue menadione on Stx2 production were explored.

A secondary objective was to explore the interactions occurring between Stx2 bacteriophages and various *E. coli* strains in an attempt to achieve a better understanding of the host range of the Stx2-phage.

Sub-objectives:

1. To describe the transcriptional response in combination with functional studies when a virulent EHEC strain was co-cultured with *B. thetaiotaomicron*, a predominant human commensal strain (Paper I)
2. To gain information concerning the factors that may contribute to virulence in the growing group of EHEC strains of varying serogroups, also outside the most known O157 serogroup (Paper I, Paper II).
3. To observe the effect on transcriptional responses of toxin genes and the effect on growth when EHEC strains were co-cultured with various bacteria, or cultured in compounds produced by various bacteria (Paper I, unpublished results).
4. To evaluate the susceptibility of a selection of commensal *E. coli* to infection by an Stx2-phage creating lysogens, and measuring the potential for Shiga toxin amplification indirectly via phage production by these lysogens (Paper II)
5. To explore the effect of menadione on Mitomycin C-induced Stx2 production in EHEC strains of two different serotypes (Paper III).

SUMMARY OF PAPERS

Paper I

The gut bacterium *Bacteroides thetaiotaomicron* influences the virulence potential of the Enterohemorrhagic *Escherichia coli* O103:H25

PLOS ONE (2015)

Hildegunn Iversen, Toril Lindbäck, Trine M. L'Abée-Lund, Norbert Roos, Marina Aspholm and Lotte S. Arnesen.

In order to explore the interspecies interactions between EHEC and a human intestinal commensal, the global gene expression profile of EHEC O103:H25 (EHEC NIPH-11060424) co-cultured with *B. thetaiotaomicron* (CCUG 10774) or grown in the presence of spent medium from *B. thetaiotaomicron* was determined. Microarray analysis revealed that approximately 1% of the EHEC NIPH-11060424 genes were significantly up-regulated both in co-culture (30 genes) and in the presence of spent medium (44 genes), and that the affected genes differed between the two conditions. In co-culture, genes encoding structural components of the type three secretion system were among the most affected genes with an almost 4-fold up-regulation, while the most affected genes in spent medium were involved in chemotaxis and were more than 3-fold up-regulated. The operons for type three secretion system (TTSS) are located on the Locus of enterocyte effacement (LEE) pathogenicity island, and qPCR showed that genes of all five operons (LEE1-LEE5) were up-regulated. Moreover, an increased adherence to HeLa cells was observed in EHEC NIPH-11060424 exposed to *B. thetaiotaomicron*. Expression of *stx2* genes, encoding the main virulence factor of EHEC, was down-regulated in both conditions (co-culture/spent medium). These results show that expression of EHEC genes involved in colonisation and virulence is modulated in response to direct interspecies contact between cells, or to diffusible factors released from *B. thetaiotaomicron*. Such interspecies interactions could allow the pathogen to recognize its predilection site and modulate its behaviour accordingly, thus increasing the efficiency of colonisation of the colon mucosa, facilitating its persistence and increasing its virulence potential.

Paper II

Commensal *E. coli* Stx2 lysogens produce high level of phages after spontaneous prophage induction

Frontiers in cellular and infection microbiology (2015)

Hildegunn Iversen, Trine M. L'Abée-Lund Marina Aspholm, Lotte S. Arnesen and Toril Lindbäck

Shiga toxin 2 (Stx2) is the major virulence factor of EHEC and is critical for development of HUS. The genes encoding Stx2 are carried by lambdoid bacteriophages and the toxin production is tightly linked to the production of phages during lytic cycle. It has previously been suggested that commensal intestinal *E. coli* could amplify the production of Stx2 phage and contribute to the severity of disease. In this study we examined the susceptibility of intestinal commensal *E. coli* strains to the Stx2-converting phage ϕ 734, isolated from a highly virulent EHEC O103:H25 outbreak strain (NIPH-11060424). Among 38 intestinal commensal *E. coli* strains from healthy children below five years, 15 were lysogenized by the ϕ 734 phage, whereas lytic infection was not observed. Three of the commensal *E. coli* ϕ 734 lysogens were tested for stability, and appeared stable and retained the phage for at least 10 cultural passages. When induced to enter lytic cycle by H₂O₂ treatment, 8 out of 13 commensal lysogens produced more ϕ 734 phages than NIPH-11060424. Strikingly, five of them even spontaneously (un-induced) produced higher levels of phage than the H₂O₂ induced NIPH-11060424. An especially high frequency of HUS (60%) was seen among children infected by NIPH-11060424. Based on our findings, a high Stx2 production by commensal *E. coli* lysogens cannot be ruled out as a contributor to the high frequency of HUS during the outbreak in 2006.

Paper III

Menadione reduces mitomycin C induction of Shiga toxin 2 production in EHEC

Manuscript

Hildegunn Iversen, Marina Aspholm, Erik Ræder, Lotte P. Stenfors Arnesen, Trine L'Abée-Lund and Toril Lindbäck

Abstract

The genes encoding Stx2 are carried by lambdoid bacteriophages, and their expression requires activation of the phage lytic cycle. Induction of the bacterial SOS response seems to be the most efficient way to activate the Stx pro-phage. Several studies have shown that various chemical components as well as factors secreted by the human microbiota and by some probiotic bacterial strains inhibit Stx synthesis. Here, we demonstrate that menadione (vitamin K3) reduces mitomycin C (MMC)-induced Stx2 production in EHEC. High-performance liquid chromatography–tandem mass spectrometric (HPLC-MS/MS) was used to demonstrate that the presence of menadione leads to reduced accumulation of MMC within the EHEC cells. It has previously been shown that treatment of *E. coli* with menadione leads to activation of the MarA SoxS Rob regulon, which promotes resistance to multiple antibiotics by altering expression of outer membrane porins (OMPs) and efflux pumps. We show that the presence of menadione leads to altered transcript levels of the OMP encoding genes *ompF* and *ompW*, and the *acrA* and *tolC* genes encoding structural components of the AcrAB-TolC efflux pump in EHEC. We suggest that the altered expression of outer membrane porins and efflux pumps results in a decreased intracellular MMC concentration and a subsequent weaker induction of the SOS-response and thereby a lower Stx2-production.

RESULTS AND DISCUSSION

General discussion

This thesis describes interactions between EHEC and commensal bacteria. Most of the experiments were performed with an EHEC strain of serotype O103:H25 (NIPH-11060424). The EHEC O157:H7 serotype has been linked to numerous outbreaks of food borne disease and extensively studied. An important aspect of this thesis was to increase the knowledge of factors that may contribute to the virulence in non-O157 EHEC strains.

In the human gut, EHEC interacts with numerous commensal bacteria. The effects of commensal bacteria or compounds produced by these bacteria on the growth and virulence potential of EHEC, and the mobility of virulence genes (via Stx-phages) from EHEC to commensal *E. coli* strains were explored in this thesis. The study the pathogen's response when in contact with other microbes may provide important knowledge about its *in vivo* behaviour. The results obtained during this study are presented in paper I-III and discussed in the sections below.

Growth kinetics in EHEC co-cultures

In paper I, the EHEC strain O103:H25 (NIPH-11060424) and *B. thetaiotaomicron* were co-cultured in different ratios (1:1 and 1:100) and additionally, the strains were cultured in spent medium from each other. Since *E. coli* in general only digest simple carbohydrates, EHEC in the intestines depends on anaerobes, such as members of the *Bacteroidaceae* family, for degradation of polysaccharides in the mucus layer²⁵⁵. As EHEC growth was not stunted by *B. thetaiotaomicron*, nor by its spent medium (paper I), EHEC did indeed receive the needed resources for growth even through exponential phase. Curtis *et al.* (2014) described the growth kinetics of EHEC (of serotype O157:H7) co-cultured with *B. thetaiotaomicron*, and found that *B. thetaiotaomicron* apparently had a growth advantage when co-cultured with EHEC compared to monoculture¹⁰⁰. This is in discrepancy with our results as we found that a co-culture between *B. thetaiotaomicron* and EHEC in the ratio 1:1 actually inhibited the growth of *B. thetaiotaomicron*. However, the culture conditions were different in the two studies; Curtis *et al.* used minimal media (low glucose DMEM), while rich BHI medium was used in our study. Additionally, the initial bacterial concentration used in our study was lower

than in Curtis' study, which may have caused the observed differences. The inhibition of *B. thetaiotaomicron* growth in co-culture with EHEC indicates that EHEC is more successful when nutrients are abundant, or that EHEC secretes inhibitory molecules in this particular environment.

The *cit* operon, involved in citrate fermentation, was the operon which was quantitatively most affected according to microarray results (Paper I). The expression of *cit* genes was increased both in co-culture and in the presence of spent medium, when compared to individual growth. This change in carbon source utilization could have been caused by carbon limitation due to nutritional competition between *Bacteroides* and *E. coli*. However, since the genes for citrate fermentation were also up-regulated when *E. coli* was cultured in spent medium, competition for nutrients is not the most likely reason as the level of nutrients in both cultures (pure culture/spent medium) was equalized. Since carbon availability is of vital importance for bacterial growth and virulence, several strategies have been developed by bacteria to adapt to alterations in carbon source composition ²⁵⁶. Interestingly, a similar change in *cit* expression was observed by Nouaille *et al.*, who observed an up-regulation of the *cit* operon in *Staphylococcus aureus* co-cultured with *Lactococcus* ²⁵⁷.

***B. thetaiotaomicron* influences expression of LEE genes in EHEC**

Paper I presents the transcriptomic response of an EHEC strain co-cultured with *B. thetaiotaomicron* examined by microarray, which revealed increased expression of TTSS genes and adhesion genes located within the LEE pathogenicity island. In contrast, when NIPH-11060424 was cultured in spent medium from *B. thetaiotaomicron*, no difference in LEE gene expressions was observed.

Gene regulation of virulence regulons is complex, and regulation of LEE is no exception. Some interactions between EHEC strains and *B. thetaiotaomicron* have previously been described in the literature. Pacheco *et al.* (2012) found that an EHEC strain of serotype O157:H7 utilizes fucose to regulate its virulence expression in the gastrointestinal tract ¹⁰². *B. thetaiotaomicron* cleaves fucose from mucin, resulting in an increased concentration of fucose in the mucus layer ^{258, 259}. EHEC senses the increased fucose levels via a two-component fucose sensing system termed FusKR, and down-regulates expression of LEE-encoded genes. QS is also involved in this gene regulatory cascade; when EHEC reaches the

epithelial surface, the QS systems become activated by noradrenaline released by host cells, thus triggering the expression of LEE genes. At the same time, the repressed expression of *fusKR*, when EHEC has moved away from the mucus layer, indirectly activates the LEE. The expression of LEE genes should be optimal when EHEC is in close contact with the epithelium, and not when the bacterium is located in the mucus layer. The FusKR mediated regulation secures that the virulence genes are only expressed when needed, preventing unnecessary loss of energy and cellular resources. We were not able to identify any homologues to the fucose-sensing genes in NIPH-11060424, and the FusKR system has previously been found only in EHEC strains of serotype O157:H7 and in EPEC strains. Considering that non-O157 EHEC are on the rise worldwide, the fucose-sensing mechanism is probably not indispensable in EHEC pathogenesis. However, it is possible that other genes are involved in similar fucose-sensing processes in non-O157 strains.

A number of studies indicate that the levels of certain glycans influence the virulence and colonisation abilities of certain EHEC strains. It has been shown that gluconeogenic conditions activate the expression of LEE genes^{101, 102}. Furthermore, Curtis *et al.* (2014) demonstrated that *B. thetaiotaomicron* in co-culture with an EHEC strain of serotype O157:H7 enhances the colonisation abilities by increasing LEE gene expression. They proposed that the modulation of virulence gene expression in EHEC co-cultured with *B. thetaiotaomicron* was caused by changes in the metabolic landscape, and that the resultant gluconeogenic environment produced by *Bacteroides* activated LEE gene expression. The transcription factor termed Catabolite repressor activator (Cra) was suggested to be a key regulator in this process¹⁰⁰. However, according to our microarray results described in paper I, the gene encoding the Cra transcription factor was not significantly up-regulated in NIPH-11060424 in co-culture, suggesting genetic and functional differences between EHEC strains/serotypes. The experimental procedure in Curtis' study differed from our study, as described above, which could explain the differences observed in gene regulation between the two different EHEC strains. The LEE operon was highly up-regulated when NIPH-11060424 was co-cultured with *B. thetaiotaomicron*, even in rich medium, indicating that a change in carbon nutrition utilization might not be the only way EHEC augment its colonisation abilities.

Transmission electron microscopy (TEM) pictures revealed a very close association between NIPH-11060424 and *B. thetaiotaomicron* (Figure 12) suggesting that cell-to-cell contact, or increased local concentration of signal compounds provided by *Bacteroides* in close

proximity, may trigger the enhanced LEE gene expression (unpublished results). Additionally, we observed that direct contact with *B. thetaiotaomicron* led to increased adhesion of EHEC to HeLa epithelial cells. We suggest that the close contact between the EHEC strain and *B. thetaiotaomicron* may act as a niche specific signal, priming EHEC for enhanced adherence to the colonic mucosa and consequent efficient colonisation of its mammalian host.

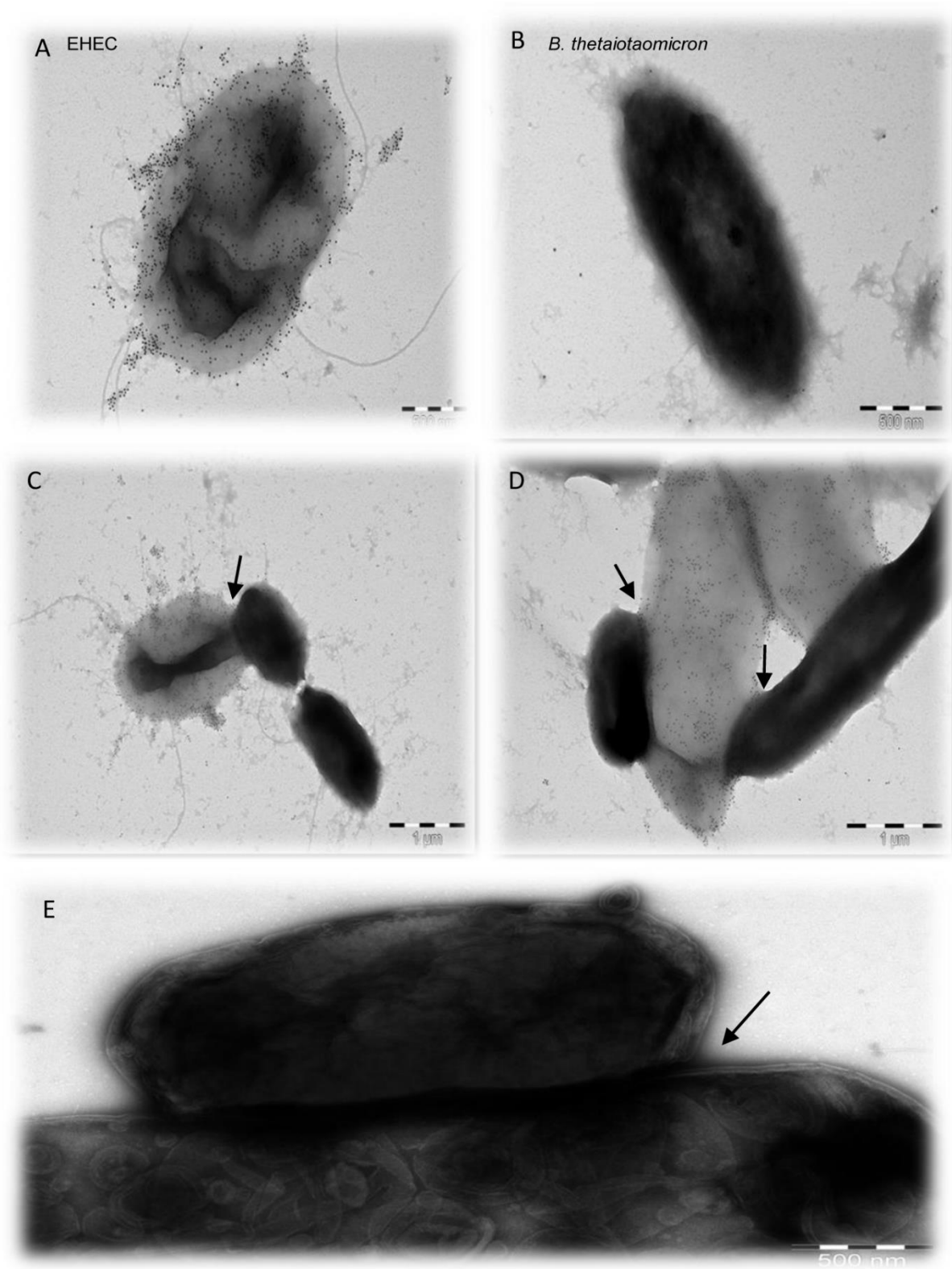


Figure 12. Transmission electron microscopy (TEM) images of NIPH-11060424 in co-culture with *B. thetaiotaomicron* and in pure culture. TEM images of uranylacetate negatively

stained cells showing intimate association between NIPH-11060424 and *B. thetaiotamicron*. To differentiate the bacterial species, NIPH-11060424 was immunogold labelled using anti O103 antibodies. A: NIPH-11060424 in pure culture, B: *B. thetaiotamicron* in pure culture, C and D: NIPH-11060424 co-cultured with *B. thetaiotaomicron*, E: NIPH-11060424 co-cultured with *B. thetaiotaomicron* (no labelling). The close interaction between EHEC NIPH-11060424 and *B. thetaiotaomicron* is indicated by arrows in picture C, D and E.

Photo: Norbert Roos, Marina Aspholm and Hildegunn Iversen (unpublished results).

Whether bacterial species other than *B. thetaiotaomiron* were able to influence the expression of the LEE genes when co-cultured with EHEC was investigated in paper I. We found that *C. perfringens* did not influence LEE gene expression, whereas *B. fragilis* increased LEE gene expression to the same level as *B. thetaiotaomicron*. Similarly, Curtis *et al.* (2014) have shown that *Enterococcus faecalis* increased LEE gene expression when co-cultured with EHEC ¹⁰⁰. They emphasized that *E. faecalis* produces large amounts of succinate ²⁶⁰ and that the resulting gluconeogenic environment most likely induced the increased expression of LEE genes. Interestingly, Curtis *et al.* demonstrated, using an *in vivo* infection model with a Stx-producing *C. rodentium* strain, that mice reconstituted with *B. thetaiotaomicron* presented with increased pathology of the distal colon (including oedema, vasculitis, destruction of crypts and apoptosis) when compared to mice depleted of their gut microbiota. This finding supports that the increased expression of LEE genes in EHEC co-cultured with *B. thetaiotaomicron* would presumably take place *in vivo* as well, leading to increased virulence potential of EHEC.

Spent medium from *B. thetaiotaomicron* influences the motility of EHEC

Motility appears to be important for initiation of infection for enteric pathogens. The flagellar system in motile bacteria somewhat resembles a TTSS, made up of a molecular motor in the inner membrane, and an export channel (the hook-basal body complex) anchored to the rotating flagellum ²⁶¹. A signal transduction cascade sensing the environment outside the bacterium is linked to the molecular motor, and regulates the motility of the bacteria ²⁶². For bacteria carrying both the TTSS and the flagellar system, it seems that these systems are counter-regulated ¹¹⁰. The flagellar system has been suggested to be active during the initial phases of infection for enteric pathogens, where movement towards host cells is important ²⁶³.

²⁶⁴. Later in the infection process, the flagellar activity is replaced by an active TTSS, which is involved in expression of adherence proteins. In paper I, we demonstrate that EHEC displayed decreased motility when cultured in spent medium from the commensal bacterium *B. thetaiotaomicron*, indicating that molecules secreted by *B. thetaiotaomicron* have an impact on the flagellar motor. The reason behind the apparent reduced motility in spent medium as compared to co-culture is difficult to elucidate without further experiments. Follow-up experiments are required to compare expression of virulence traits, such as colonisation abilities, in spent medium versus in co-culture.

The host range of Stx2-encoding bacteriophages

Stx-phages are a heterogeneous group, and the diversity is believed to be a result of recombination events occurring as phages travel between different hosts ¹⁴⁵. The expansive dissemination of Shiga toxin-encoding genes indicates that Stx-phages are successful in sharing their genes between different bacterial genera and species ^{44, 45, 162, 174, 177}.

In paper II, we wanted to explore the host range of an Stx2-phage from a disease outbreak. Thirty-eight commensal *E. coli* strains from children younger than 5 years old were tested for susceptibility to infection by an Stx2-phage isolated from EHEC NIPH-11060424 ²⁵. The phage infection experiments were performed using a modified version of the Stx2-phage in which *stx2* was replaced by a chloramphenicol resistance gene (*cat*) ²⁶⁵. About 40 % (15/38) of the commensal *E. coli* strains were susceptible to lysogenic infection and these lysogens were able to produce infective bacteriophages. As the *stx2* genes are controlled by the phage late gene promoter, and Stx2 is produced during the lytic phage cycle, there is a strong correlation between phage production and Stx2 production ^{62, 64}.

According to a study performed by Gamage *et al.* (2004), 35 % (14/40) of commensal *E. coli* strains were sensitive to lysogenic infection by a recombinant Stx2-phage from EDL933, and only 10% were lysogenized with a recombinant Stx2-phage isolated from another EHEC strain ¹⁷⁴. The results obtained by Gamage *et al.* (2004) indicate that different Stx2-encoding phages have different host ranges. Additionally, a selection of the commensal *E. coli* strains showed amplified Stx2 production caused by lytic infection of the 933W phage ¹⁷⁴. In these phage infection experiments, the commensal *E. coli* strains were incubated for 24 hours

before Stx2 production was measured. Thus, the amplified toxin levels could be the result of lysogens spontaneously entering the lytic cycle.

In contrast, none of the commensal *E. coli* strains in our study (paper II) were infected lytically with the Stx2-encoding phage. However, they did incorporate the Stx2-phage into their genome and produced infectious bacteriophages, as confirmed by a plaque assay, when induced with hydrogen peroxide (H₂O₂) and MMC, but also during non-inducing conditions. The laboratory *E. coli* strain C600 was lysogenized with the wild type Stx2-phage. The resulting lysogen produced 1000 times more bacteriophages, and experienced a 40-fold increase in Stx2 production, when compared to the original EHEC strain. We were not able to isolate a commensal *E. coli* strain that was lysogenized with the wild type Stx2-phage. However, we believe that the increased phage production observed in lysogens with the recombinant Stx2-phage strongly reflects the potential for amplified Stx2 production in the commensal strains. Since Shiga toxin production is linked to phage induction and subsequent cell lysis and death, the ability to transfer the burden of toxin production onto the commensal *E. coli* strains is a strategy that would benefit EHEC survival. Lysogeny is believed to be the favoured phage lifestyle in the gastrointestinal tract due to the low level of nutrients present and the lack of susceptible hosts^{266,267}. These results could imply that lysogenic infection of commensal *E. coli* strains is important for development of severe disease, such as HC and HUS, during EHEC infection. The differences in Stx2-phage susceptibility observed in our study could explain why some individuals may be more prone to illness when exposed to EHEC bacteria.

In paper II, we observed that when the recombinant phages were produced by a commensal lysogen, they had a broader host range compared to when they were produced in the original outbreak strain (NIPH-11060424). This may suggest that passage of a phage through a host can modify the phage, thus affecting its host range.

Does phage genotype influence bacteriophage and Stx2 production in lysogens?

Wagner *et al.* (1999) produced several isogenic lysogens by infecting a laboratory *E. coli* strain with different Stx2-encoding phages, and observed varied phage production and Stx2 production in these different lysogens²⁶⁸. This study indicates that the phage genotype influences toxin expression in lysogens. Phages possess different integration recombinases

thus the variation in toxin production observed in these lysogens could reflect the differences in phage integration sites. The authors also suggested that repressor activity in the different phages influenced the phage and toxin production in the various lysogens. Difference in toxin production was reduced by induction with MMC, and this supports that different repressor activity in the various phages are the reason for the observed variance under uninduced conditions. The clinical strains that originally harboured the phages produced less toxin than the isogenic lysogens. As the clinical strains most likely possessed additional phages, the reduced Stx2 production might be a result of interaction between the different phage repressor systems ²⁶⁹.

The lysogens described in paper II varied in their ability to produce bacteriophages during spontaneous induction and when induced with H₂O₂ or MMC. MMC is commonly used in experimental settings as an inducing agent for *in vitro* Stx2 production for EHEC strains; however, H₂O₂ may represent a more likely inducing agent in the gastrointestinal tract. Interestingly, five of the commensal lysogens produced more phages when induced with H₂O₂ than after induction with MMC. Some of the commensal lysogens produced 1000 times more phages than the original EHEC strain did when induced with H₂O₂ or MMC. Additionally, most of the lysogens produced 10-1000 times more phages than the original EHEC in non-induced conditions. These findings contradict the idea that phage genotype exclusively determines phage and toxin production in lysogens, since the lysogens with identical bacteriophages behaved differently with regard to phage production, and thus most likely also toxin production. According to a study performed by Fogg *et al* (2012), double lysogens seemed to be more sensitive to induction than single lysogens. They found that the lysogenic carriage of two isogenic Stx2-phages within the same genome led to an increase in Stx2 expression under both inducing- and non-inducing conditions ²⁷⁰. However, other studies report a decrease in toxin production in the presence of more than one lysogenic Stx2-phage ^{269, 271}. The high level of phage production by some of the lysogens described in paper II could be due to the presence of more than one Stx2-phage in the genome but we did not investigate whether the various lysogens were single or double lysogens. However, these processes are complex and most likely involve bacterial factors in addition to characteristics of the given bacteriophage.

Suppression of Stx2 production

A number of studies have shown that chemical components, as well as factors produced by the human microbiota and some probiotic bacterial strains, inhibit Stx synthesis²⁷²⁻²⁷⁵. In paper I, we demonstrated that Stx2 production of EHEC NIPH-11060424 was significantly inhibited in *B. thetaiotamoicron* co-culture or when the strain was cultured in spent medium from *B. thetaiotamoicron*. An inhibitory effect of *B. thetaiotaomicron*, as well as other commensals, on Stx2 production at the transcriptional level has been reported previously in another EHEC serotype²⁷³. The inhibitory substance has been assumed to be a peptide of molecular mass below 3 kDa, but the chemical identity of this substance is as yet unknown. We investigated whether inhibition of Stx2 production could follow a dose-dependent pattern by varying the ratios between our EHEC strain and *B. thetaiotaomicron* in co-culture. We observed that the initial concentration of *B. thetaiotaomicron* was important in order to achieve a repression of Stx2 production. When the ratio between *B. thetaiotamoicron* and EHEC was 1:1 (10^7 CFU/ml), a high level of Stx2 suppression was observed, however, less suppression was observed when the initial concentrations of both strains were 10^6 CFU/mls (Figure 5, Paper I). This may indicate that the production of molecules that inhibit toxin-production is dependent on the density of the *B. thetaiotamoicron* culture.

Curtis *et al.* observed an increased *stx2* expression when an EHEC strain of serotype O157:H7 was co-cultured with *B. thetaiotaomicron* compared to monoculture in late-exponential phase¹⁰⁰. In paper I, the level of *stx2* transcripts was not significantly different in co-culture compared to pure culture in mid-exponential phase. However, the culture conditions (uninduced versus induced cultures), sampling procedure (6 hours versus 3 hours) and the culture medium (DMEM vs BHI) differed between the two studies, which could explain the dissimilar results obtained.

The ability of other commensal bacteria to repress Stx2 production in EHEC NIPH-11060424 was also investigated in Paper I and in our unpublished, preliminary results (Figure 13). Stx2 production was repressed when the EHEC strain was grown in spent medium from various bacteria including *B. fragilis*, *C. perfringens*, *Bacillus cereus*, *S. aureus* and commensal *E. coli*. These results indicate that the inhibitory factor might be a molecule of universal nature, produced by many bacteria. Notably, when the EHEC strain was cultured in spent medium from the skin bacterium *S. aureus*, less inhibition was observed.

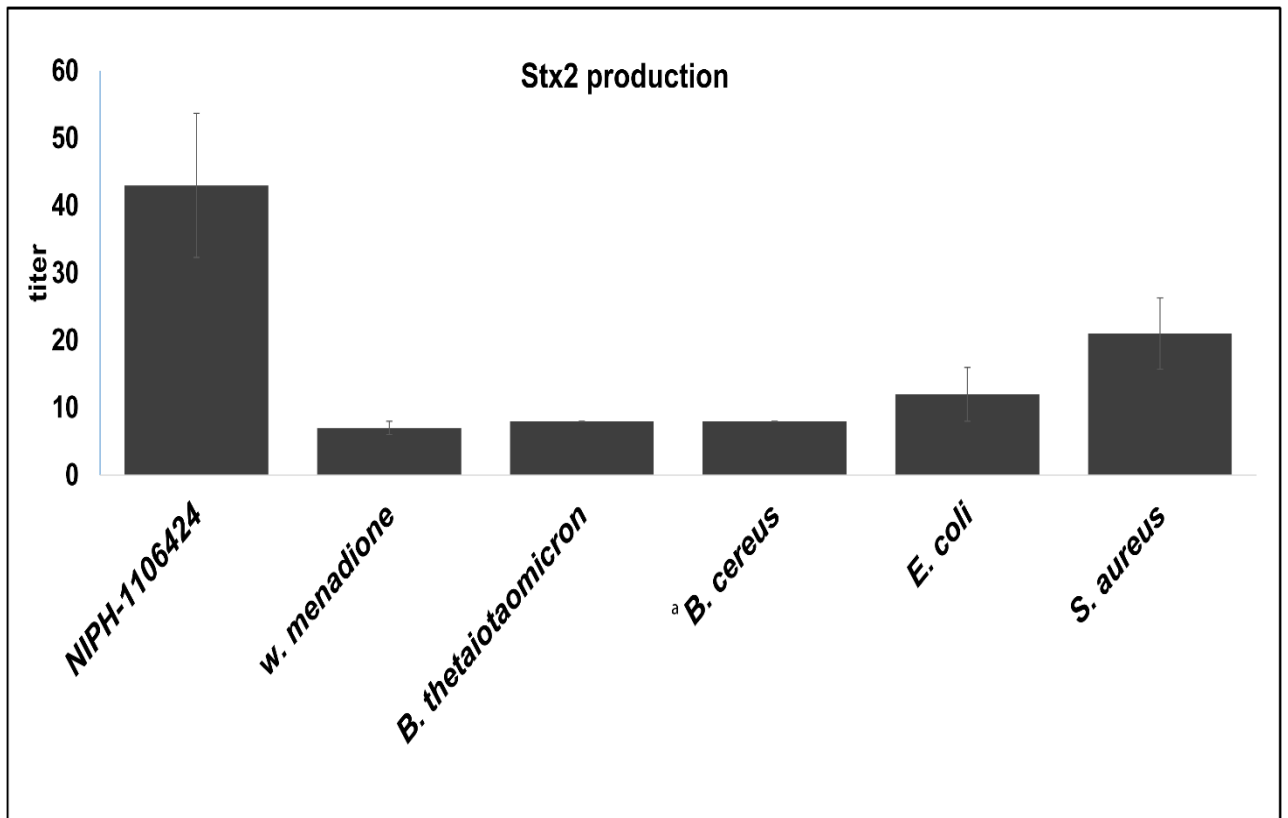


Figure 13. The effect of spent medium from *B. thaitaomicron*, *B. cereus*, *E. coli* (commensal) and *S. aureus* and the vit. K homologue menadione (20 µg/ml) on Stx2 production. The cultures were induced with MMC at OD = 0.5 and the samples were harvested 24 hours after induction during anaerobic conditions. Data represent the mean +/- SEM of three independent experiments, a – only one replicate.

As Stx production is phage-regulated, substances that inhibit induction of phages will also repress Stx production. Since Stx2 overexpression is associated with severe human disease, the prevention of phage induction is an innovative method to combat the pathogenicity of EHEC ^{276, 277}. Considering that EHEC harbours Stx-phages that are activated by certain antibiotics, the use of antibiotics for treatment of EHEC infections is controversial due to concerns about triggering the SOS response and the subsequent increase in Stx2 production ²⁷⁸⁻²⁸⁰. Therefore, anti-induction drugs are promising candidates for preventing serious complications in relation to EHEC disease. The direct inhibition of specific virulence factors is a novel method in the fight against microbes ²⁸¹⁻²⁸³.

In paper III, we demonstrate that menadione, a synthetic vitamin K-derivative with redox-cycling properties, suppresses Stx2-production in MMC-induced EHEC cultures.

Interestingly, the level of inhibition exerted by adding menadione (20 ug/ml) to NIPH-11060424 is similar to the inhibition observed when the EHEC strain is cultured in spent medium from various bacteria (Figure 13). Since many enteric bacteria secrete various vitamin K homologs and other redox-cycling substances, the observed suppression of Stx2 production may be due to such compounds. It is assumed that there are inducing agents present in the intestinal environment that trigger pro-phage induction and subsequent Stx production. *In vitro* experiments have demonstrated that oxidative stress caused by H₂O₂ released from neutrophils, and also produced by bacteria, activates the bacterial SOS-response and consequently induces pro-phages leading to increased Stx2 production¹⁸⁶⁻¹⁸⁸. Additionally, an *in vivo* mouse model has demonstrated that Stx2-phages are induced in the intestinal environment; however, the induction agent has not been identified¹⁷⁹. The inhibitory activity of menadione seems to involve a reduced bacterial SOS-response as the level of RecA is reduced in menadione-treated samples compared to control samples.

Outer membrane permeability and multidrug efflux systems

The effect of menadione on bacterial cells has been associated with oxidative stress²⁸⁴⁻²⁸⁶. The oxidative stress caused by menadione activates various regulons (including MarRAB, SoxRS and Rob) that regulate a number of efflux pumps and outer membrane porins^{286, 287}. Gram-negative bacteria have a selective permeability barrier that protects against toxic substances and at the same time provides the influx of nutrients into the cell²⁸⁸.

The presence of menadione decreases Stx2 production in MMC-induced EHEC as demonstrated in paper III. Genes encoding efflux pumps (AcrA-TolC) and outer membrane porins (OmpF and OmpW) showed altered expression in the presence of menadione. Furthermore, the intracellular MMC concentration was lower in cells grown in the presence of menadione. We propose that the decreased level of OmpF in menadione-treated cells in combination with the increase in the multidrug efflux pump AcrAB-TolC, could result in decreased accumulation of MMC in the bacterial cells. In that way, menadione works as an anti-induction compound leading to suppressed Stx2 production in MMC-induced EHEC cells. However, the suggestion that the presence of the vitamin K homologue menadione might increase antibiotic resistance of pathogenic *E. coli* strains by increasing the adaptive

response to antibiotics, does raise concerns regarding drug resistance mechanisms in the gastrointestinal environment.

We suggest that the suppressed Stx2 production was achieved by an altered membrane permeability induced by menadione, affecting the intracellular MMC concentration and thereby influencing the bacterial SOS response. Why the intracellular concentration of MMC in EDL933 was higher than in NIPH-1106424, both in the control samples and the samples treated with menadione, is uncertain. It was also noticed that EDL933 produced higher levels of Stx2 as compared to NIPH-11060424 (measured both on transcriptional level and at the protein level), when induced with MMC (Paper III). This could indicate that the EDL933 strain is more susceptible to induction with MMC, and therefore a more efficient Stx2 producer (at the time point the samples were analysed). The reason for this discrepancy could be regulatory differences related to the Stx2-phage genome in the two EHEC strains.

Virulence regulation in O157 versus non-O157 EHEC

The number of non-O157 EHEC infections are increasing worldwide ^{19, 20}. The clinical manifestations of non-O157 are wide ranging, as many serotypes are represented, however the severity of disease induced by non-O157 strains is now recognized as comparable to O157-strains ²⁸⁹.

To summarize, several differences were observed when comparing EDL933 (representing O157:H7) and NIPH-11060424 (representing O103:H25). EDL933 was a more efficient Stx2 producer when induced with MMC compared to NIPH-11060424. The Stx2 production in both strains was equally inhibited by vitamin K3 (menadione) (Paper III). Additionally, both strains demonstrated a decreased Stx2 production when co-cultured with or cultured in spent medium from, various bacteria ²⁷³ (Paper I, unpublished results). There seems to be some differences regarding regulation of LEE genes/operons. NIPH-11060424 (and other non-O157 strains) does not possess the two-component fucose-sensing system (FusKR) involved in the regulation of LEE ¹⁰². Additionally, expression of the transcription regulator Cra differed in NIPH-11060424 compared to EDL933, however, as described previously the culture conditions were different in the two studies thus it is difficult to do a comparison ¹⁰⁰. Since the number of strains examined in this study is limited, it does not allow any conclusions as to whether the observed differences in virulence regulation observed are due to strain variation or serotype differences.

Methodological considerations

In vitro* versus *in vivo

In order to gain a better understanding of the human gastrointestinal environment, various *in vitro* models have been developed to simulate the microbial conditions encountered in, for instance, the large intestine. The existing *in vitro* models range from simple batch culture models to more advanced multistage continuous culture systems. The continuous culture systems provide a way to simulate the large intestinal system of humans. This culture system is controlled, and can therefore avoid fluctuating pH-levels and redox potentials over time, while still allowing for control of nutrient levels ²⁹⁰. All growth kinetic experiments in this thesis were performed using batch culture conditions. However, since the cultures were grown for less than 24 hours, the negative effects of nutrient exhaustion and fluctuating pH-levels were reduced. The use of a continuous culture system might be a better option in order to simulate the *in vivo* conditions in the large intestinal tract but, unfortunately, this model system was not available at our laboratory.

The intestinal environment is highly complex and cannot be fully simulated using *in vitro* systems. Main limitations of *in vitro* models include that host immune responses are not taken into account and nor are the effects of the neuroendocrine system ²⁹⁰. Therefore, co-colonisation studies using animals might have added valuable information to this study. On the other hand, a number of studies have used animal models (mouse/rat/pig) but extrapolation of the results from these studies to humans is questionable due to differences in the composition of the microbiota in humans as compared to animals ¹⁹⁶. Attempts to solve this problem have been made by using germfree animal models possessing human microbiota ²²⁵.

Model systems for AE lesions

Abundant literature covers the formation of EHEC AE lesions using tissue culture cells as well as various *in vivo* animal models, yet the demonstration of formation of these lesions in human epithelium *in vivo* is lacking ²⁹¹. We used a HeLa cell line to investigate the

attachment of EHEC to epithelial cells since AE lesions are known to form in various cell lines including Hep-2 and HeLa cells ^{84, 292}. HeLa cells are not polarised, and are quite different from gastrointestinal cells, but are however commonly used as a model for AE lesions ²⁹¹. Polarized cell lines of gastrointestinal origin that forms tight junction and brush borders such as T84, Caco-2 and HT29 might better reflect the gastrointestinal epithelium, although, for the demonstration of AE lesions, non-polarised cell lines should be sufficient ^{291, 293}. *In vitro* organ culture (IVOC) of human intestinal epithelium has demonstrated AE lesions on the terminal ileum and the colon, and there are differences regarding which cellular pathways are activated when using cell lines versus IVOC ²⁹⁴⁻²⁹⁷. Some of these differences could be caused by the use of different oxygen levels in the various systems, as it has been shown that high oxygen levels actually repress AE lesion formation on both *in vitro* cell lines and IVOC of human intestinal epithelium ²⁹⁷. The level of oxygen tension near intestinal cells has been shown to affect the adhesion abilities (via TTSS and fimbriae) of some EHEC strains and several studies have demonstrated that microaerobic conditions increase the colonisation ability of EHEC ²⁹⁷⁻²⁹⁹.

Stx2 regulation *in vivo* versus *in vitro*

There is a lack of knowledge on how Shiga toxin expression is regulated *in vivo* since no animal models effectively reproduces the typical human EHEC infection ³⁰⁰. Therefore, most studies are performed *in vitro*. The effect of the oxygen levels upon virulence gene expression is an important aspect to consider when studying enteric pathogens, as levels of oxygen vary between niches in the human gut; anaerobic conditions in the lumen and microaerobic conditions at the mucosal surface ^{301, 302}. During infection EHEC will most likely be located at the mucosal surface where the atmosphere is microaerobic (~1.5% oxygen), thus, the strict anaerobic conditions used in our co-culture experiments may not reflect the *in vivo* conditions accurately. The level of Stx production was significantly lower in anaerobic conditions compared to aerobic conditions as presented in paper I and paper II. It would be interesting to observe how the different EHEC strains behave during microaerobic conditions in regards to the ability to produce Stx. Previous studies have shown that the atmosphere in the gastrointestinal tract influences the virulence potential of EHEC strains ^{302, 303}. It has been demonstrated that microaerobic conditions suppressed Stx production in EHEC strains, but increased the translocation of the Stx across the epithelial layer ³⁰³.

In all studies in this thesis, a VTEC-RPLA-kit was used to measure Shiga toxin production. This kit semi-quantifies toxin levels, and thus does not quantify the exact concentration of toxin. However, the main goal of our study was to compare relative toxin production under different conditions, so for our experiments, the VTEC-RPLA kit was sufficient. It would be interesting to examine precise toxin concentrations under the different conditions.

Microarray versus RNA sequencing

In paper I, the global transcriptomic response of the pathogenic EHEC strain in co-culture with *B. thetaiotaomicron*, or in contact with spent medium from *B. thetaiotaomicron*, was analyzed using a microarray chip. When designing the study, the possibility of using a probe-independent approach using RNA sequencing was considered. The advantages of using RNA sequencing would be the possibility to measure gene expression in both organisms at high resolution, covering transcripts from non-coding areas as well. Digital quantification is another feature that increases the dynamic range, leading to increased sensitivity when using RNA sequencing compared to microarray. This increase in sensitivity, and the fact that the RNA sequencing method is probe independent, could have lead to the discovery of novel transcripts/genes. RNA sequencing was not used in this work because of the higher cost of the experiment at the time the experiment in paper I was planned. RNA sequencing would also have produced a great amount of data, requiring adequate bioinformatics competence/in depth data analysis.

Future perspectives

- ✓ Co-culture studies using colonic biopsy (*in vitro* organ culture (IVOC) systems) samples from humans
- ✓ Follow-up experiments regarding the contact-dependent up-regulation of LEE genes
- ✓ Comparison of susceptibility to Stx2-phage infection of commensal *E. coli* isolated from children to commensal *E. coli* isolated from adults
- ✓ Investigation of the effect of other vitamin K variants on Stx2 production in EHEC strains such as MK-10 and MK-11 which are produced by *B. thetaiotaomicron*.

MAIN CONCLUSIONS

The main conclusions from this study were:

- ✓ The expression of EHEC genes involved in metabolism, colonisation, and toxin production was modulated in response to direct contact with *B. thetaiotaomicron* and soluble factors released from *B. thetaiotaomicron*.
- ✓ Virulence gene expression and regulation differ between O157 strains and non-O157 strains.
- ✓ The EHEC O103:H25 (NIPH-11060424) demonstrated increased production of the TTSS protein EspA and increased adhesion to HeLa cells when grown in co-culture with *B. thetaiotaomicron*.
- ✓ An intimate interaction between EHEC O103:H25 (NIPH-11060424) and *B. thetaiotaomicron* when co-cultured was indicated, using transmission electron microscopy (TME).
- ✓ A number of chemotaxis and flagellar genes were up-regulated when EHEC O103:H25 was cultured in spent medium from *B. thetaiotaomicron*, and a decrease in motility was observed.
- ✓ The expression of *stx2* genes was down-regulated when various EHEC strains were co-cultured or grown in spent medium from various commensal bacteria (including *B. thetaiotaomicron*) and accordingly the levels of Stx2 production were decreased.
- ✓ The redox-cycling drug menadione suppresses Stx2 production in MMC-induced EHEC strains due to the altered expression of porins and efflux pumps.
- ✓ Commensal *E. coli* show different susceptibilities for lysogenic infection by a recombinant Stx2-encoding bacteriophage.
- ✓ The lysogenic commensal *E. coli* strains produce significantly more bacteriophages after induction than the wild type strain EHEC O103:H25 (NIPH-11060424). This result indicates that phage-susceptible commensal bacteria could amplify Stx2 production profoundly in the gastrointestinal tract.
- ✓ None of the commensal *E. coli* strains were susceptible for lytic infection by the *stx2*-encoding bacteriophage, indicating that lysogenic infection is the predominant infection cycle in the gastrointestinal system.

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SCIENTIFIC PAPERS I-III

PAPER I

The gut bacterium *Bacteroides thetaiotaomicron* influences the virulence potential of the Enterohemorrhagic *Escherichia coli* O103:H25

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Hildegunn Iversen, Toril Lindback, Trine M. L'Abée-Lund, Norbert Roos, Marina Aspholm and Lotte S. Arnesen.

RESEARCH ARTICLE

The Gut Bacterium *Bacteroides thetaiotaomicron* Influences the Virulence Potential of the Enterohemorrhagic *Escherichia coli* O103:H25

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Abstract

Enterohemorrhagic *E. coli* (EHEC) is associated with severe gastrointestinal disease. Upon entering the gastrointestinal tract, EHEC is exposed to a fluctuating environment and a myriad of other bacterial species. To establish an infection, EHEC strains have to modulate their gene expression according to the GI tract environment. In order to explore the interspecies interactions between EHEC and an human intestinal commensal, the global gene expression profile was determined of EHEC O103:H25 (EHEC NIPH-11060424) co-cultured with *B. thetaiotaomicron* (CCUG 10774) or grown in the presence of spent medium from *B. thetaiotaomicron*. Microarray analysis revealed that approximately 1% of the EHEC NIPH-11060424 genes were significantly up-regulated both in co-culture (30 genes) and in the presence of spent medium (44 genes), and that the affected genes differed between the two conditions. In co-culture, genes encoding structural components of the type three secretion system were among the most affected genes with an almost 4-fold up-regulation, while the most affected genes in spent medium were involved in chemotaxis and were more than 3-fold up-regulated. The operons for type three secretion system (TTSS) are located on the Locus of enterocyte effacement (LEE) pathogenicity island, and qPCR showed that genes of all five operons (LEE1-LEE5) were up-regulated. Moreover, an increased adherence to HeLa cells was observed in EHEC NIPH-11060424 exposed to *B. thetaiotaomicron*. Expression of *stx2* genes, encoding the main virulence factor of EHEC, was down-regulated in both conditions (co-culture/spent medium). These results show that expression of EHEC genes involved in colonization and virulence is modulated in response to direct interspecies contact between cells, or to diffusible factors released from *B. thetaiotaomicron*. Such interspecies interactions could allow the pathogen to recognize its predilection site and modulate its behaviour accordingly, thus increasing the efficiency of colonization of the colon mucosa, facilitating its persistence and increasing its virulence potential.

Introduction

The human intestinal tract is colonized by a huge number of commensal microbes and the composition and importance of this microbiota in human health and disease have been studied increasingly the last years. It is now known that the intestinal microbiota is central to the health of the host by influencing the metabolism, physiology, nutrition and immune function [1,2]. The intestinal microbiota also plays an important role as a protective barrier against pathogenic microorganisms by competing for nutrition and attachment sites on the epithelium, boosting the host's gut- and systemic immune response, and producing various antibacterial substances [3]. To overcome this hurdle, enteric pathogens must have mechanisms to interact and cope with the resident microbial community and with numerous host- and environmentally derived stressors that affect their functionality and pathogenic processes. In fact, their pathogenicity is the net result of numerous interactions with either their microbial environment or their host (reviewed in [4,5]).

The dominating bacteria in the distal human gastrointestinal tract are Bacteroidetes (17–60%) and Firmicutes (35–80%) under normal conditions, suggesting that these phyla have important functions in the host [6–8]. Other common phyla in the gastrointestinal tract are the Proteobacteria, Actinobacteria and Euryarchaeota [9]. Bacteria of the *Bacteroidaceae* family are significant contributors to polysaccharide degradation and uptake in the human gut, and members of the *Bacteroides* family produce a high number of glycosyl hydrolases compared to other commensals [10]. *B. thetaiotaomicron* cleaves fucose from host glycans, which results in free fucose available in the gut lumen [11]. Some pathogenic *E. coli*, e.g. enterohemorrhagic *E. coli* (EHEC) O157 carry a fucose-sensing two-component signal transduction system which senses free fucose and this mechanism is involved in the timing of virulence and metabolic gene expression [12]. It has also been shown that *B. thetaiotaomicron* (and other commensals) produces an unidentified extracellular molecule, of molecular mass below 3 kDa, which inhibits the production of Shiga toxin 2 (Stx2) in EHEC O157:H7 [13]. However, the overall gene expression profile of an EHEC in co-culture with the predominant human commensal *B. thetaiotaomicron* has, to our knowledge, not been explored.

EHEC is associated with increasing numbers of disease outbreaks and sporadic cases of human disease worldwide [14,15]. The disease is characterized by initial diarrhoea, sometimes followed by bloody diarrhoea, and can occasionally progress to the serious, life-threatening condition hemolytic uremic syndrome (HUS) [16]. EHEC disease is characterized by a low infectious dose, which may be associated with elevated acid tolerance [17] and most likely, competitive abilities in order for EHEC to survive in the intestinal environment and colonize the epithelial cells lining the terminal ileum and colon [17–19].

Pathogenesis of EHEC is mediated by multiple mechanisms, but the main virulence factor is the Shiga toxin [20]. The genes encoding Shiga toxin are carried in the genomes of lambdoid bacteriophages, and toxin expression is controlled by a promoter present in the phage genome [21,22]. In contrast to commensal *E. coli* strains, which live in the mucus layer, EHEC is found in close contact with the epithelium [23]. Attachment of EHEC to intestinal cells is associated with the destruction of microvilli and the formation of a highly organized cytoskeletal structure termed an attaching and effacing (AE) lesion [24]. Central in these key events is the LEE. LEE is a pathogenicity island encoding proteins which play an important role in initial attachment to enterocytes, and in the translocation of effector proteins (EspD, EspB and Tir) into the host. LEE harbours genes encoding a type three secretion system (TTSS), the adhesin intimin and the intimin receptor Tir (translocated intimin receptor) (reviewed in [24]). The formation of the AE-lesion is an important step in the EHEC infection process, and the structure is made after translocation of Tir into the host cell via the TTSS system [25,26]. The interaction

between Tir and intimin modulates multiple host signalling cascades that lead to actin polymerization creating the characteristic AE-lesions [27,28].

Studies regarding adaptive regulation of genes in EHEC have mainly focused on changes in expression of virulence genes under varying growth conditions [29–31], in the presence of eukaryotic cells [32], and in co-cultures with probiotic bacterial species [33,34]. It has also been shown that the intestinal microbiota affects virulence gene expression in EHEC O157:H7 [13]. Although a large number of studies have focused on understanding how single pathogens interact with their host, huge information gaps remain regarding the ecology of the intestinal microbiota and its interactions with pathogenic bacteria. Elucidating these interactions is of importance as it provides knowledge concerning the mechanisms of the pathogens' ability to persist in their host and cause disease.

In the present study, we investigated the global gene expression profile, using microarray technology and qPCR, of the highly virulent outbreak strain EHEC O103:H25 (EHEC NIPH-11060424) in co-culture or grown in the presence of spent medium from *B. thetaiotaomicron* (the type strain—VPI-5482).

Results

Growth of EHEC NIPH-11060424 is not affected in co-culture with *B. thetaiotaomicron*

To explore interspecies interactions between *B. thetaiotaomicron* and the EHEC strain, the growth kinetics of EHEC NIPH-11060424 grown in pure culture and in co-culture with *B. thetaiotaomicron* were examined. As shown in Fig. 1A, the growth kinetics of EHEC NIPH-11060424 was not affected in co-culture with *B. thetaiotaomicron* when the ratio of the initial concentrations between the species was 1:100 (EHEC NIPH-11060424: *B. thetaiotaomicron*). However, *B. thetaiotaomicron* was inhibited when co-cultured with EHEC NIPH-11060424 under the same condition. After 5 hours in co-culture, a growth deceleration-phase in *B. thetaiotaomicron* was observed compared to pure *B. thetaiotaomicron* culture. After 24 hours in co-culture, the growth of *B. thetaiotaomicron* was inhibited 12-fold compared to the growth pattern in pure culture ($P < 0.05$). When the two strains were co-cultured using equal initial concentrations (10^6 CFU ml⁻¹), the growth inhibitory effect on *B. thetaiotaomicron* was more evident and statistically significant growth inhibition was observed in all time intervals except at time point zero ($P < 0.05$) (Fig. 1B). When *B. thetaiotaomicron* was cultured in spent medium from EHEC NIPH-11060424, *B. thetaiotaomicron* had a prolonged lag-phase however *B. thetaiotaomicron* managed to regain its growth pattern so the biological significance of this extended lag-phase is not known (Fig. 1C). The growth of EHEC NIPH-11060424 was inhibited at time point 4 hours when cultured in spent medium from *B. thetaiotaomicron* and *B. fragilis*, again the biological significance of this inhibition is difficult to evaluate (Fig. 1D).

Global gene expression profiles of EHEC NIPH-11060424 in co-culture with *B. thetaiotaomicron* and grown in spent medium from *B. thetaiotaomicron*

Thirty EHEC NIPH-11060424 genes (0.8% of genes represented on the array) were significantly up-regulated and 25 genes (0.65%) were down-regulated when EHEC NIPH-11060424 was co-cultured with *B. thetaiotaomicron*, compared to when cultured alone (at OD = 0.5). In spent medium from *B. thetaiotaomicron*, 44 EHEC NIPH-11060424 genes (1.14%) were significantly up-regulated and 37 genes (0.95%) were down-regulated (at OD = 0.5). The location of affected genes was equally distributed throughout the genome.

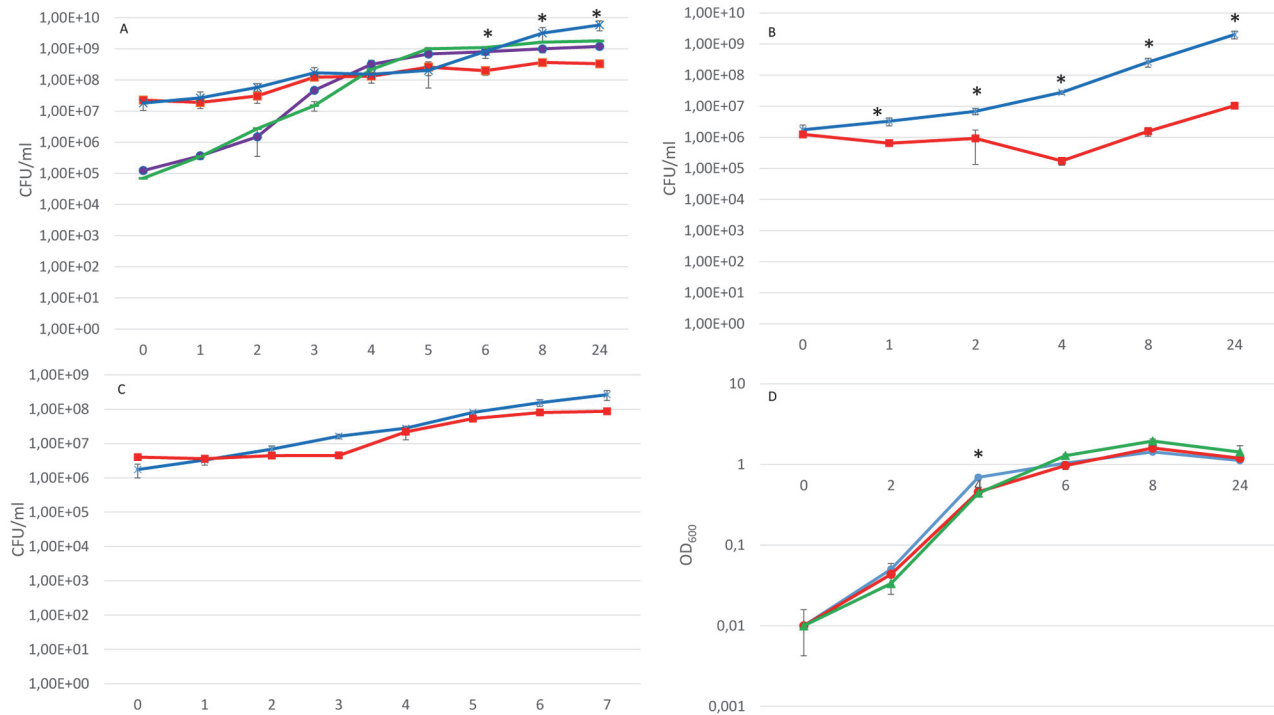


Fig 1. Growth kinetics of EHEC NIPH-11060424 and *B. thetaiotaomicron* in co-culture and when grown in spent medium. (A) Growth kinetics of *B. thetaiotaomicron* and EHEC NIPH-11060424 grown in pure and co-culture (1:100) under the same conditions as used for microarray analyses. Symbols: purple ● EHEC NIPH-11060424 in co-culture with *B. thetaiotaomicron* (1:100), green — EHEC NIPH-11060424 in pure culture, red ■ *B. thetaiotaomicron* in co-culture with EHEC NIPH-11060424 (100:1), blue x *B. thetaiotaomicron* in pure culture (B) Kinetics of growth *B. thetaiotaomicron* in pure culture and in co-culture with EHEC NIPH-11060424 using equal initial concentrations of the two strains. Symbols: blue x *B. thetaiotaomicron* in pure culture, red ■ *B. thetaiotaomicron* in co-culture with EHEC NIPH-11060424 (C) Kinetics of growth of *B. thetaiotaomicron* in spent medium from EHEC NIPH-11060424. Symbols: red ■ *B. thetaiotaomicron* in spent medium from EHEC NIPH-11060424, blue x *B. thetaiotaomicron* in pure culture (D) Kinetics of growth of EHEC NIPH-11060424 in spent medium from *B. thetaiotaomicron* and *B. fragilis*. Symbols: red ● EHEC NIPH-11060424 in spent medium from *B. thetaiotaomicron*, green ▲ EHEC NIPH-11060424 in spent medium from *B. fragilis*, blue ● EHEC NIPH-11060424 in pure culture (control). Data represent mean +/- standard error of the mean (SEM) of three independent experiments.

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A selection (>2-fold) of affected genes was assigned to functional groups, based upon gene annotations, using KEGG databases [35,36]. Altered gene expression in three main functional groups was observed: virulence/adhesion, chemotaxis and metabolism (S1, S2 and S3 Files). In co-culture, 13 genes located in the LEE pathogenicity island were up-regulated almost 4-fold compared to pure culture (S1 File). In spent medium, genes involved in chemotaxis were up-regulated more than 3-fold compared to pure culture (S2 File). Thirteen genes involved in metabolism were affected in both groups (co-culture and spent medium at OD = 0.5) and the extent of regulation was similar in the two groups. After induction with Mitomycin C, the *stx2* genes along with associated phage-genes were equally down-regulated in both conditions (S3 File). A brief description of differential expression of genes involved in other functions can be found in S6 File.

Co-culture with *B. thetaiotaomicron* increases expression of LEE genes and increases adherence of EHEC NIPH-11060424 to HeLa cells

Expression of thirteen genes located within the LEE was significantly up-regulated in the microarray analysis when EHEC was co-cultured with *B. thetaiotaomicron* (S1 File). In contrast, when EHEC NIPH-11060424 was grown in spent medium from *B. thetaiotaomicron*, no up-regulation of the TTSS genes was observed (S2 File). Among the significantly (≥ 2 -fold) up-

Table 1. Comparison of gene expression values obtained with microarray and qPCR.

| Gene | Culture condition | Fold change Microarray | Fold change qPCR | Gene description |
|-------------------------|-------------------|------------------------|------------------|---------------------------------------|
| LEE-genes | | | | |
| <i>ler</i> | Co-culture | NA | 13.3 | Transcription regulator |
| <i>escU</i> | Co-culture | 2.6 | 3.1 | TTSS structure protein |
| <i>grlA</i> | Co-culture | 3.2 | 4.3 | positive regulator |
| <i>escJ</i> | Co-culture | 2.3 | 11.3 | TTSS structure protein |
| <i>escV</i> | Co-culture | 1.7 | 7.3 | translocator EscV |
| <i>espA</i> | Co-culture | NA | 4.8 | Translocon EspA |
| <i>eae</i> | Co-culture | NA | 10.6 | Intimin |
| <i>tir</i> | Co-culture | NA | 10.9 | Translocated intimin receptor |
| <i>escF</i> | Co-culture | NA | 4.4 | TTSS structure protein |
| Chemotaxis genes | | | | |
| <i>cheY</i> | Spent medium | 3.32 | 9.1 | Chemotaxis regulator |
| <i>motA</i> | Spent medium | 2.5 | 5.5 | Flagellar motor protein |
| <i>motB</i> | Spent medium | 2.6 | 6.3 | Flagellar motor protein |
| <i>cheZ</i> | Spent medium | 3.3 | 4.6 | Chemotaxis regulator |
| <i>cheB</i> | Spent medium | 3.06 | 4.5 | Fused chemotaxis regulator |
| Metabolic genes | | | | |
| <i>citD</i> | Co-culture | 4.5 | 37 | citrate lyase acyl carrier subunit |
| <i>potE</i> | Co-culture | -3.2 | -1.2 | putrescine/proton symporter |
| <i>glpD</i> | Co-culture | -2.95 | -1.4 | sn-glycerol-3-phosphate dehydrogenase |

NA- not available

A Pearson correlation coefficient of 0.67 was obtained when comparing the fold changes obtained by microarray and qPCR.

Boldface values represent significant changes (P-value ≤ 0.05)

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regulated LEE genes in co-culture were the *Escherichia* secretion components (*escR*, *escS*, *escT* and *escU*) encoding structural parts of the basal body of the TTSS. The Ler (LEE encoded regulator) protein is the major transcriptional regulator of LEE and is encoded by the first gene in the LEE1 operon [37]. The global regulator of *ler* activation (GrlA), which binds to the LEE1 promoter and activates *ler* expression, was also significantly up-regulated.

The first part of the LEE pathogenicity island (LEE1) encoding the basal body of TTSS is highly conserved between various EHEC strains. However, genes located downstream (LEE2-LEE5) are less conserved and hence prone to weak hybridization in the microarray assay as the array consisted of EHEC O103:H2 12009 probes. Indeed, several of the probes encoding genes located in the LEE2-LEE5 were removed from the dataset due to a mean log2 signal below baseline. Due to these missing microarray data, a selection of genes (*ler*, *escJ*, *escV*, *espA*, *eae*, *tir* and *escF*) representing each of the LEE1-LEE5 operons was analysed by qPCR. The qPCR results revealed that all representative genes from the various LEE-operons were up-regulated significantly in co-culture compared to pure culture (Table 1 and Fig. 2). While differential expression of *ler* was insignificant in the microarray analysis qPCR revealed a significant difference in *ler* expression in co-culture compared to pure culture. The expression of the adhesin protein intimin and its concomitant receptor Tir were up-regulated more than 10-fold, while the genes encoding the major needle subunit of the TTSS EspA and the TTSS needle protein EscF were up-regulated almost 5-fold. In accordance with these results Western blot analysis showed that the level of EspA was increased in co-culture culture relative to pure culture (Fig. 3A). Antiserum against the other TTSS components was unavailable, and hence changes

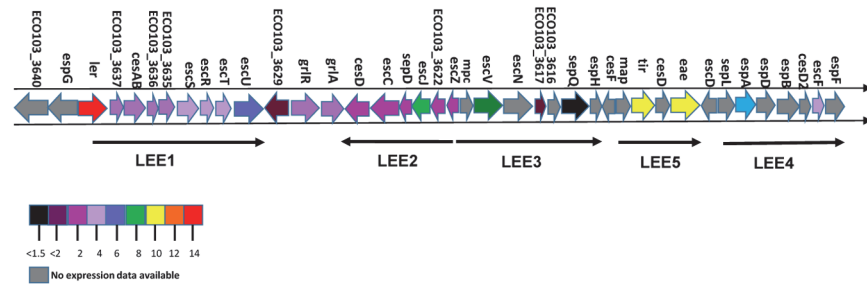


Fig 2. ORF map of the LEE locus to demonstrate differences in gene expression in co-culture compared to pure culture. The ORF map was generated based upon the relative expression ratio of co-culture: pure culture obtained in microarray and qPCR analysis and the graphic presentation of the genetic organization of the LEE pathogenicity island was adapted from Garmendia *et al.* [25].

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in protein levels by Western blot analysis were not measured. Furthermore, a quantitative adhesion assay using HeLa cells demonstrated significantly increased adhesion properties of co-cultured EHEC NIPH-11060424 relative to EHEC NIPH-11060424 grown in pure culture (3B).

To investigate whether the increased expression of TTSS genes was restricted to contact with the *B. thetaiotaomicron*, EHEC NIPH-11060424 was co-cultured with two other gastrointestinal species, *Bacteroides fragilis* and *Clostridium perfringens*, and expression of *escU* was determined using qPCR. Interestingly, *escU* was significantly up-regulated in co-culture with *B. fragilis* but not with *C. perfringens* (Fig. 4).

B. thetaiotaomicron repressed *stx2* expression in EHEC NIPH-11060424

Microarray revealed a more than 3-fold down-regulation of *stx2* gene expression and various phage-associated genes in mitomycin C induced co-cultures of EHEC NIPH-11060424 and *B. thetaiotaomicron*, relative to EHEC NIPH-11060424 in pure culture. A similar down-regulation of *stx2* genes (including other phage-associated genes) was observed when EHEC NIPH-11060424 was cultured in spent medium from *B. thetaiotaomicron* (S3 File). The decrease in *stx2* expression levels under these conditions was verified by using the VTEC-RPLA kit (Fig. 5A). Reduced Stx2 production was also observed when EHEC NIPH-11060424 was cultured in spent medium from *B. fragilis* and *C. perfringens* (S5 File).

To investigate whether the reduced toxin level was due to inhibition of phage production, a plaque assay was performed demonstrating a significant decrease in bacteriophage titre when EHEC NIPH-11060424 was co-cultured with *B. thetaiotaomicron*, relative to pure culture (Fig. 5B).

Altered expression of chemotaxis genes and motility in spent medium

When EHEC NIPH-11060424 was grown in spent medium from *B. thetaiotaomicron*, a number of chemotaxis and flagellar genes, including *cheY*, *cheA*, *cheZ*, *cheR*, *motB* and *fliS*, were up-regulated (S2 File). In addition, genes encoding chemoreceptors for dipeptides and aspartate (*tap* and *tar*) showed higher expression when EHEC NIPH-11060424 was cultured in spent medium. These chemoreceptors are called methyl-accepting chemotaxis proteins (MCPs) and the binding of an attractant or repellent stimulates cytoplasmic proteins influencing the rotation of the flagella [38]. In concordance, *lrhA*, encoding the transcriptional regulator LrhA, a repressor of flagellar, motility and chemotaxis genes, showed lower expression (S2 File). In the

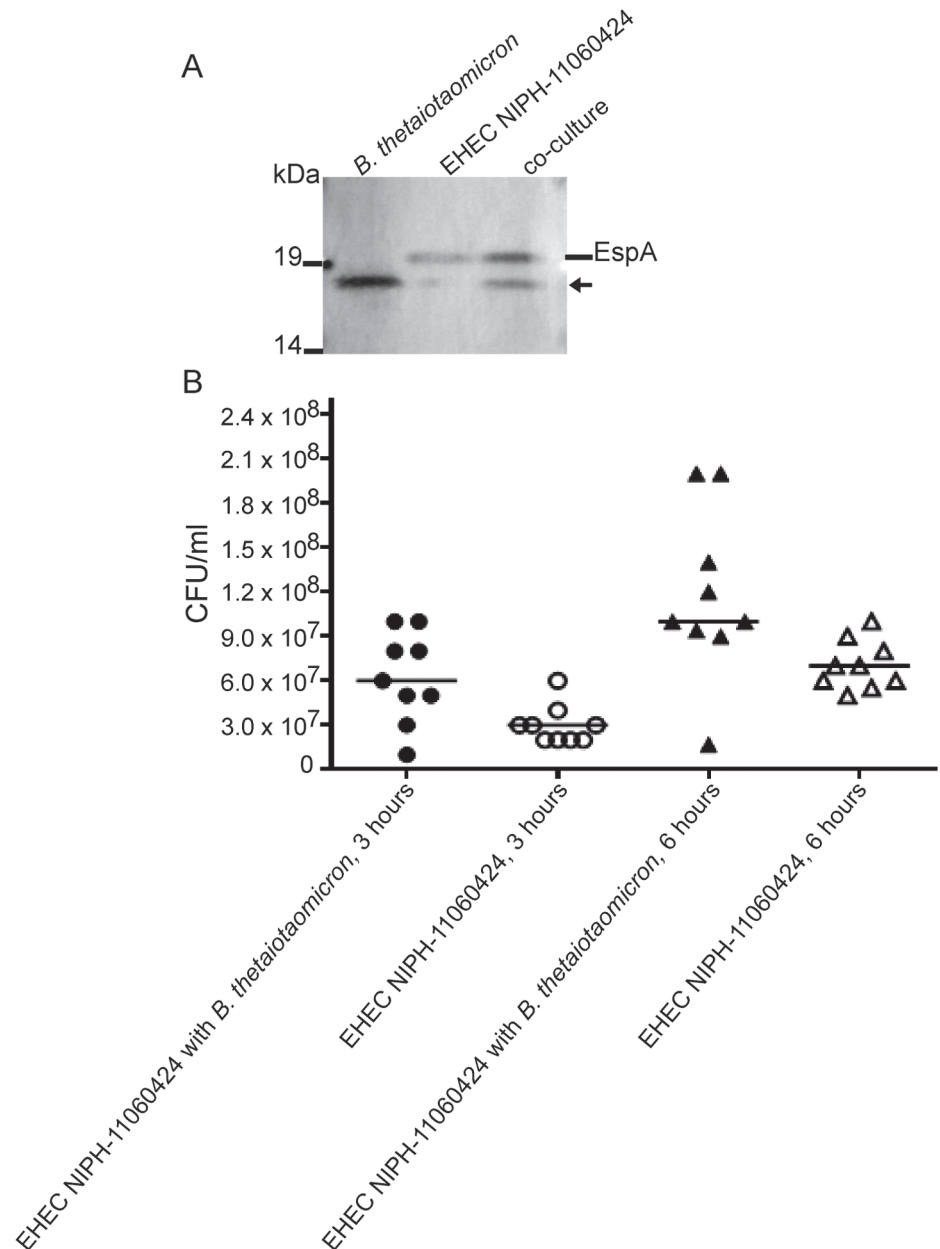


Fig 3. Adherence to HeLa cells, and expression of EspA in co-culture relative to pure culture. (A) Western blot of samples of total cell extracts from pure culture of *B. thetaiotaomicron* (lane 1), pure culture of EHEC NIPH-11060424 (lane 2), and *B. thetaiotaomicron* EHEC NIPH-11060424 co-culture (lane 3) using anti-EspA monoclonal antibodies. The arrow represents an unknown protein present in *B. thetaiotaomicron*. The results shown are representative of three independent biological and technical replicates. (B) The scatter plot shows the adherence of EHEC NIPH-11060424 in co-culture with *B. thetaiotaomicron* compared to pure culture of EHEC NIPH-11060424 to HeLa cells after 3 and 6 hours of incubation. The data are representative of three independent experiments with 3 technical replicates (n = 9). The vertical line illustrates the median of each group. The Mann-Whitney non-parametric test was used for comparison of groups. P < 0.05 was considered statistically significant.

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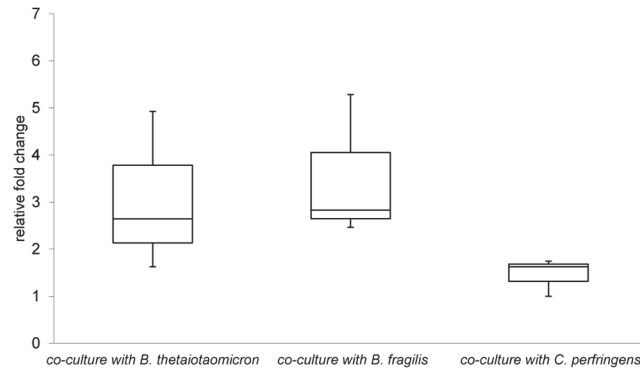


Fig 4. The expression of *escU* determined by qPCR in various conditions. The figure shows relative expression of *escU* when EHEC NIPH-11060424 is grown in co-culture with *B. thetaiotaomicron*, *B. fragilis* and *C. perfringens* compared to growth in pure culture. Boxes show the upper (75%) and the lower (25%) percentiles of the data. Whiskers indicate the highest and the lowest numbers.

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agar-motility assay the presence of spent medium from *B. thetaiotaomicron* decreased EHEC motility 4-fold (Fig. 6A (1) and 6B) while the presence of spent medium from *B. fragilis* did not affect EHEC motility significantly (Fig. 6A (2) and 6B). Before performing motility assays, EHEC NIPH-11060424 was cultured in spent medium from *B. thetaiotaomicron* and *B. fragilis*

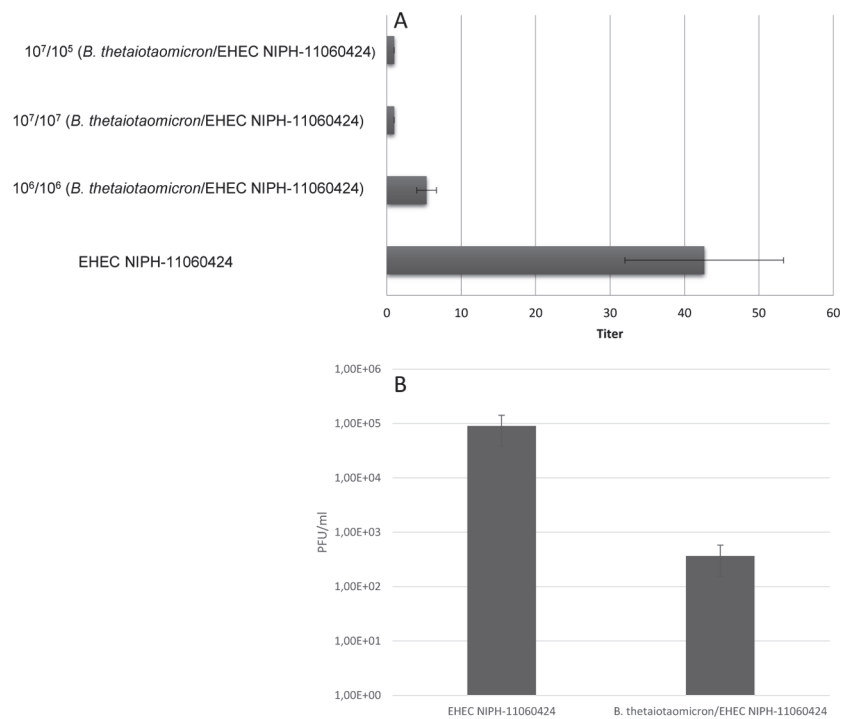


Fig 5. The effect of co-culture on Stx2 production and release of bacteriophages. (A) Stx2 production by EHEC NIPH-11060424 was measured by reverse passive latex agglutination (RPLA) test. EHEC NIPH-11060424 was co-cultured with *B. thetaiotaomicron*, using different initial bacterial concentrations. The reciprocal of the highest twofold serial dilution causing latex agglutination was recorded as the titre. Data represent means \pm standard errors of the mean (SEM) from three independent experiments. (B) Plaque assay demonstrating production of fewer bacteriophages when EHEC NIPH-11060424 was co-cultured with *B. thetaiotaomicron* compared to pure culture.

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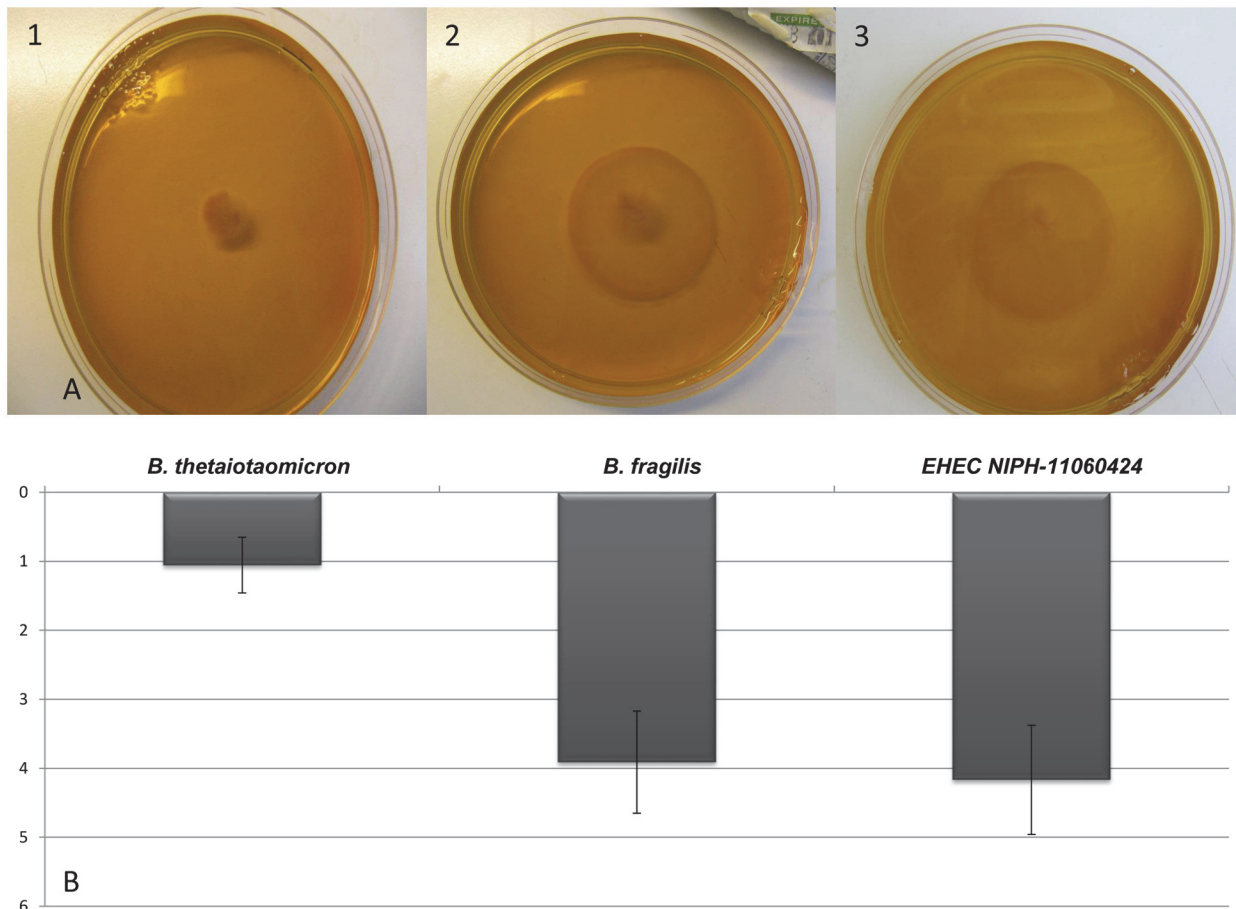


Fig 6. The motility of EHEC NIPH-11060424 in spent medium from various commensals. (A) Motility of EHEC NIPH-11060424 grown in the presence of spent medium from *B. thetaiotaomicron* (1), *B. fragilis* (2) and in pure culture (filter-sterilized water) (3). (B) The size (cm) of the growth halo in the motility assay upon exposure to spent medium from *B. thetaiotaomicron* and *B. fragilis*. Results are given as means of three experiments, with bars showing standard error of the mean (SEM).

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to exclude any growth inhibition exerted by these strains and similar to what was observed earlier, the growth of EHEC NIPH-11060424 was generally not influenced by the presence of spent medium from these two bacterial strains (Fig. 1D).

To investigate whether chemotaxis genes of EHEC NIPH-11060424 were up-regulated in response to intestinal commensals other than *B. thetaiotaomicron*, EHEC NIPH-11060424 was cultured in spent medium from *B. fragilis* and *C. perfringens*, and gene expression levels of *cheY* were investigated by qPCR. The results revealed that *cheY* was significantly up-regulated when EHEC NIPH-11060424 was cultured in spent medium from *B. thetaiotaomicron*. However, no significant change in expression of *cheY* was observed when EHEC NIPH-11060424 was grown in spent medium from *B. fragilis* and *C. perfringens*, indicating that there is some degree of species specificity in this response (Fig. 7).

Differently expressed metabolic genes in co-culture and in spent medium

Among the 60 genes that were differently expressed (>2-fold) in co-culture, *citD* was one of the most affected genes, with a 4.5 fold up-regulation. In fact, the whole operon involved in

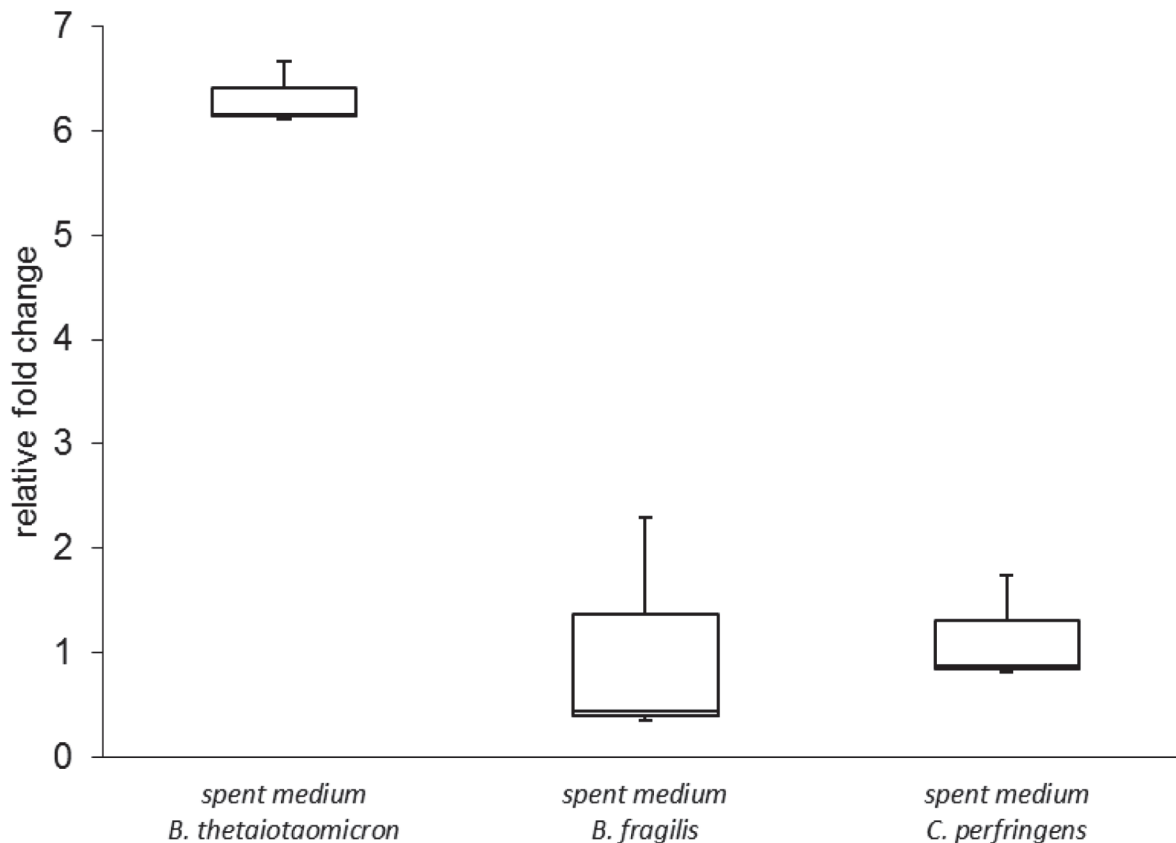


Fig 7. The expression of *cheY* determined by qPCR in various conditions. The figure displays the relative expression of *cheY* when EHEC NIPH-11060424 is cultured in spent medium from *B. thetaiotaomicron*, *B. fragilis* and *C. perfringens* compared to growth in BHI. Boxes show the upper (75%) and the lower (25%) percentiles of the data. Whiskers indicate the highest and the lowest numbers.

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citrate fermentation (*cit* operon) was up-regulated both when EHEC NIPH-11060424 was grown in co-culture (S1 File) and when grown in spent medium from *B. thetaiotaomicron* (S2 File). However, the majority of affected metabolic genes (13/19), excluding the *cit* operon, were down-regulated in co-culture compared to pure culture. In the presence of spent medium from *B. thetaiotaomicron*, 12 metabolic genes and the *cit* operon were up-regulated, while 15 metabolic genes were down-regulated. The differences in expression profile of metabolic genes expressed in co-culture and in spent medium indicate that EHEC NIPH-11060424 responds differently to direct contact with *B. thetaiotaomicron* cells compared to exposure to components present in spent medium from *B. thetaiotaomicron*. Six metabolic genes (*speF*, *tnaA*, *dsdA*, *fixC*, *srlA* and *srlE*) were affected in both co-culture and in spent medium, all down-regulated 2-fold ($P < 0.05$), except *speF*, which was down-regulated 4-fold in spent medium ($P < 0.05$).

Confirmation of microarray data with qPCR

To confirm the altered expression patterns observed in the microarray experiments, qPCR was carried out for selected genes representing each affected group. Each assay was performed on three biological replicates and included triplicate PCR of the samples, negative no-template controls, and the reference gene whose expression was similar during all experimental

conditions (co-culture/pure culture/spent medium). 12 representative genes were selected as presented in [Table 1](#). The changes in expression levels from the microarray experiments were confirmed, showing a correlation coefficient of ~ 0.7 (Pearson correlation coefficient) between array results and qPCR ([Table 1](#)). The *citD* gene is highly up-regulated both on the microarray and in the qPCR analysis, however the fold change obtained in the microarray for this particular gene, seems for some reason to be underestimated. As the dynamic range of microarray technology is lower compared to that of qPCR, especially after normalization, most genes are expected to have a higher fold-change in the qPCR analysis compared to the microarray analysis [[39–41](#)]. Primers for qPCR did not react with cDNA generated from pure cultures of *B. thetaiotaomicron* and therefore cross-hybridization was not an issue for these genes.

Discussion

Among key findings in this study of co-culture of a gut commensal and a major enteric pathogen were that the expression of three main groups of virulence genes and virulence related phenotypes were significantly affected. Among these, the expression of TTSS and adhesion associated genes located within the LEE pathogenicity island were affected by direct contact between *B. thetaiotaomicron* and EHEC NIPH-11060424, while the altered expression of several chemotaxis genes was independent of cell-to-cell contact.

The LEE pathogenicity island is arranged into 5 operons termed LEE1-LEE5 consisting of more than 40 genes essential for EPEC and EHEC virulence [[26](#)]. The TTSS, formed as a syringe-needle nanomachine able to secrete proteins directly into the host cells, is one of the most complex secretion systems in bacteria [[42](#)]. The assembly of the TTSS machinery is a multistep process coordinated by a sequential up-regulation of the TTSS genes [[25](#)]. The basal anchoring structure (syringe) is built first and serves as a secretion machine for the needle components [[25](#)]. EspA has a dual role in this process, forming filamentous structures involved in initial adhesion of bacteria to the host cell and at the same time building the translocation apparatus for Tir and other effector proteins into the host cell [[43](#)].

According to the microarray results, genes within LEE1 and LEE2 encoding the basal body of the TTSS and the regulators GrlA and GrlR, were up-regulated in co-culture compared to pure culture. However, qPCR analysis revealed that all LEE operons were affected in co-culture compared to pure culture, suggesting that the whole LEE pathogenicity island was activated in response to contact with *B. thetaiotaomicron* ([Fig. 2](#)). Several of the regulators of LEE genes were up-regulated in co-culture including GrlA, GrlR and the major regulator Ler. Ler positively regulates the expression of LEE2-LEE5 and negatively regulates LEE1 [[26,44](#)]. The *grlA* and *grlR* constitute a transcriptional unit encoding GrlA (positive regulator) and GrlR (negative regulator) for LEE1 [[45](#)]. The increase of the EspA protein in co-culture compared to pure culture and the concomitantly increased adhesion on HeLa cells indicate that contact with *B. thetaiotaomicron* in fact increases the virulence potential of the EHEC-strain.

The regulation of LEE gene expression is complex, as it is receptive to several environmental signals, including population status (via quorum sensing), temperature, nutrients and physiological state of cell (growth phase) [[46–48](#)]. Previous studies have shown that some EPEC- and EHEC-strains use fucose to regulate the virulence via a two-component fucose-sensing system (FusKR) [[12,49](#)]. *B. thetaiotaomicron* cleaves fucose from mucin in the gastrointestinal tract resulting in increased fucose availability in the mucus layer [[11](#)]. Increased fucose levels lead to repression of LEE-encoded genes in some strains. This fucose-sensing system is exclusively found in the O157:H7-serotype and its progenitor EPEC O55:H7 serotype [[12](#)]. The specialised fucose-sensing genes were not found in the genome of EHEC NIPH-11060424, which supports the fact that differences exist between O157 and non-O157-strains. Some EHEC strains thus

suppress expression of LEE-genes when present in the mucus layer with *B. thetaiotaomicron* [12,49], while the results of the present study show that EHEC increases LEE-gene expression when co-cultured with *B. thetaiotaomicron*. This illustrates the fine-tuned balance between expression and repression of genes. The suppression of LEE-genes when present in the mucus-layer perhaps prevents these specific EHEC-strains spending energy expressing virulence in a location where virulence is not necessary. It has not yet been described if, or how, non-O157 EHEC strains may use strategies to conserve energy in the human gut. Non-O157 EHEC strains have a different evolutionary history compared to O157 and represent a heterogeneous group consisting of strains with varying virulence potential [14,50,51].

LEE gene expression in an EHEC-strain of serotype O157:H7 has been demonstrated to increase in a gluconeogenic environment. The transcription factors, KdpE involved in general bacterial homeostasis and the catabolite repressor/activator protein Cra, were demonstrated to be involved in this glucose-regulated process of LEE-gene expression [52]. According to our microarray, no significant differences in the expression of *kdpE* or *cra* genes were observed in our O103:H25 strain when comparing the expression of these genes in co-culture to pure culture. Njoroge *et al.* also demonstrate increased expression of the type three secretion protein EspA when EHEC O157:H7 is co-cultured with *B. thetaiotaomicron* [52]. The authors hypothesize that the increased expression could be a result of quorum sensing (QS) as previous studies have demonstrated the production of autoinducers by the intestinal microbiota [53].

Bacteria have several communication systems, allowing them to sense the presence of potential competitors or partners [54,55]. QS is used by bacteria to modulate gene expression patterns based upon population density. The process is mediated by diffusion of signal molecules (auto-inducers) that bind to appropriate receptors on target bacteria [55,56]. We observed no alteration in expression of any of the known QS genes (*qseC* and *qseB*) involved in LEE regulation in EHEC-strains, in co-culture compared to pure culture. Since the EHEC cell density was similar under both conditions, this was not unexpected. It has been demonstrated that quorum sensing signal molecules produced by EHEC and commensal *E. coli* influence expression of LEE-genes and it has been suggested that other intestinal bacteria influence expression of LEE-genes as well [53,57,58]. However, since the LEE-genes were not affected when EHEC NIPH-11060424 was cultured in the presence of spent medium from *B. thetaiotaomicron*, the up-regulation of LEE-genes in this experiment is most likely not a result of QS.

Recently, contact-dependent signaling systems utilized by bacteria have been found. These systems are used in both intra- and inter-species signaling involving symbiotic and antagonistic interactions [59,60]. An intra-species contact-dependent growth inhibition (CDI) mechanism (type V secretion system) has been described in *E. coli*, where the growth of one *E. coli* strain is suppressed when it comes in contact with another *E. coli* strain [61]. Notably, increased expression of LEE genes was observed only in co-culture, and not when EHEC NIPH-11060424 was grown in spent medium from the *Bacteroides* strain, suggesting that cell to cell contact, or increased local concentration of signal compound(s) provided by *Bacteroides* in close proximity, might trigger the enhanced LEE-gene expression. Interestingly, expression of *escU* was also significantly up-regulated when EHEC NIPH-11060424 was co-cultured with *B. fragilis* but not in co-culture with *C. perfringens*, indicating that the observed effect on TTSS gene transcription may only be triggered by specific bacterial species. The increase in the expression of TTSS genes in a confined bacterial co-culture was surprising, as the TTSS is currently known to secrete bacterial effectors into eukaryotic cells. Based upon these findings we propose that the interaction with *B. thetaiotaomicron* may act as a niche specific signal, priming EHEC for increased adherence to enterocytes and subsequent efficient colonization of its host.

The chemotactic behaviour of bacteria relies on chemosensory adjustment of the activity of the flagellar motor (tumbling/smooth swimming) [62]. The overall direction of movement is

determined by whether repellents or attractants are present [63]. The default state of *E. coli* is smooth swimming achieved by counter-clockwise rotation of flagella [64]. The cytoplasmic response regulator CheY monitors the direction of flagellar rotation and depending on its phosphorylation state, can either be an activator or inhibitor of smooth swimming [38]. Overall, we observed that major parts of the chemotactic apparatus, from chemo-sensing to motility, were transcriptionally up-regulated by spent medium from *B. thetaiotaomicron* compared to pure cultures. Phosphorylated CheY interacts with flagellar rotation causing an increase in tumbling activity (increased clockwise bias) thus inhibiting the motility of EHEC NIPH-11060424 when grown in spent medium from *B. thetaiotaomicron*. These findings indicate that *B. thetaiotaomicron* might secrete motility inhibiting factor(s). Spent medium from *B. fragilis*, another intestinal member of the *Bacteroidaceae* family did not decrease the motility of the EHEC-strain, suggesting that the effect is species specific. Interestingly, the increased expression of motility and chemotaxis genes was not seen in co-cultures of EHEC NIPH-11060424 and *B. thetaiotaomicron* but occurred only in the presence of spent medium. Flagella are believed to be important in the early stages of infection contributing to the localization of EHEC close to the epithelial surface [65,66]. However, the regulation of both LEE-genes and flagellar genes should be strict as the simultaneous expression of both groups of genes could impede adhesion [67]. Therefore, the different expression profiles of LEE and chemotaxis genes in co-culture compared to spent medium seen in our study, might relate to a temporal control of virulence factors during EHEC's positioning to closer contact with the epithelium and a changed closeness with *B. thetaiotaomicron*.

The genes encoding the Stx2 toxin are carried by a bacteriophage and thus, expression of the toxin genes is controlled by the bacteriophage itself [22]. Prophage induction, Stx2 production and subsequent cell lysis for release of toxin are believed to occur in the intestine as a result of innate immune effectors working as inducers (e.g. neutrophils producing H₂O₂) [68]. In this study mitomycin C (MMC) was used as inducing agent in both co-culture and spent medium experiments. Even after MMC induction, transcription of *stx2* and a number of regulatory bacteriophage genes were strongly repressed in both co-culture with *B. thetaiotaomicron* and in spent medium compared to pure EHEC NIPH-11060424 culture. The reduced Stx2 level and phage number measured in co-culture and in spent medium supported the microarray results. The observed decrease in *stx2* transcription also occurred when EHEC NIPH-11060424 was grown in spent medium from the intestinal commensals *B. fragilis* and *C. perfringens* (S5 File). These data are in compliance with results presented by De Sablet and co-workers, showing that *B. thetaiotaomicron* and other commensals in the gastrointestinal tract produced an unidentified substance with inhibitory effect on Stx2 production in EHEC O157:H7 [13]. However, our data show that the repression of Shiga toxin production also takes place in EHEC of other serotypes than O157:H7. Together, the present and previous findings indicate that the suppression of prophage induction, leading to reduced toxin levels, might be a result of a more universal mechanism, not specific to *B. thetaiotaomicron*. Considering that intestinal commensals are exposed to numerous bacteriophages in the gastrointestinal tract [69], a system which suppresses prophage induction would most likely be beneficial for maintenance of the microbial community.

The expression of a large number of metabolic genes in EHEC was affected both by co-culture and growth in the presence of spent medium from *B. thetaiotaomicron*. The competition for nutritional resources, both quantitatively and qualitatively, is a central point in bacterial relations [70], and therefore, the observed effect on metabolic gene expression in co-culture is not surprising. The predominant intestinal bacterium *B. thetaiotaomicron* is a primary fermenter thus its activity in the gut will affect the rest of the microbial community [11]. Most metabolic genes were down-regulated in co-culture apart from the *cit* genes and malate

dehydrogenase (*mdh*, up-regulated nearly 2-fold). The *cit*-operon is involved in catabolism of citrate under anaerobic conditions, and is important for anaerobic growth [71]. A similar change in expression of citrate metabolic genes have previously been observed by Nouaille *et al.* [72], who reported an up-regulation of the *cit* operon in *Staphylococcus aureus* co-cultured with *Lactococcus*. The *cit* operon was also up-regulated when EHEC NIPH-11060424 was grown in the presence of spent medium from *B. thetaiotaomicron*. As EHEC growth is not attenuated by the presence of *B. thetaiotaomicron*, or by its secreted components, the resources needed for growth are obviously met. And yet, in co-culture as well as in spent medium from *B. thetaiotaomicron*, EHEC NIPH-11060424 changes expression of several metabolic genes, some similarly and some differentially between the two conditions, probably indicating a metabolic modulation in response to the changed conditions.

Conclusions

In summary, the expression of EHEC NIPH-11060424 genes involved in metabolism, colonization and virulence is modulated in response to direct contact with *B. thetaiotaomicron* and to soluble factors released from *B. thetaiotaomicron*. In the presence of spent medium from *B. thetaiotaomicron*, a number of chemotaxis and flagellar genes were up-regulated and a decrease in motility was observed. The expression of Stx phage genes, including the Shiga toxin (Stx) genes, was down-regulated in mitomycin C induced co-culture/spent medium and accordingly the levels of Stx production and phage release were decreased. Genes encoding the TTSS and other factors involved in adherence to host cells were up-regulated in direct contact with *B. thetaiotaomicron*. We also show that direct contact with *B. thetaiotaomicron* leads to increased expression of the TTSS protein EspA and increased adhesion to epithelial cells. Based on our findings, we propose that direct contact with *B. thetaiotaomicron* could function as a niche specific signal that primes EHEC for a more efficient interaction with the host cells thus increasing its virulence potential.

Methods

Bacterial strains

The bacterial strains used in this study are listed in [Table 2](#).

Growth conditions

In co-culture experiments EHEC NIPH-11060424 and *B. thetaiotaomicron* (CFU ratio 1:100) were grown in modified BHI (BHI (OXOID, UK) with added yeast extract 5 g l⁻¹, menadione

Table 2. Bacterial strains used in this study.

| Bacterial strain | Serotype/ID | Source | Reference |
|---------------------------------|-------------|-------------------------|------------|
| EHEC NIPH-11060424 ^a | O103:H25 | EHEC | [90] |
| EHEC 12009 | O103:H2 | EHEC | [50] |
| <i>B. thetaiotaomicron</i> | | CCUG 10774 ^b | [91] |
| <i>B. fragilis</i> | | Isolated from horse | This study |
| <i>C. perfringens</i> | | DSM756 | [92] |
| DH5α <i>E. coli</i> | | Laboratory strain | [89] |

^aSynonym with NVH-734

^bAlso designated VPI-5482

Table 3. Overview workflow for microarray experiment.

| Culture condition | CFU Ratio | Sample time point experiment | Induction | Biological replicates |
|--|-----------|----------------------------------|-----------|-----------------------|
| Pure culture EHEC NIPH-11060424 | NA | mid-logarithmic phase (OD = 0.5) | No | >3 |
| Co-culture EHEC NIPH-11060424/ <i>B. thetaiotaomicron</i> | 1/100 | mid-logarithmic phase (OD = 0.5) | No | 3 |
| Pure culture EHEC NIPH-11060424 in spent medium from <i>B. thetaiotaomicron</i> | NA | mid-logarithmic phase (OD = 0.5) | No | 3 |
| Induced pure culture EHEC NIPH-11060424 | NA | 3 hours after induction | MMC | 3 |
| Induced co-culture EHEC NIPH-11060424/ <i>B. thetaiotaomicron</i> | 1/100 | 3 hours after induction | MMC | 3 |
| Induced culture EHEC NIPH-11060424 in spent medium from <i>B. thetaiotaomicron</i> | NA | 3 hours after induction | MMC | 3 |

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1 mg l⁻¹ and haemin, 5 mg l⁻¹ anaerobically at 37°C [73]. Anaerobic conditions were achieved using an anaerobic work station (Whitley A35 Anaerobic Workstation, Don Whitley Scientific, West Yorkshire, UK). The pure EHEC NIPH-11060424 and *B. thetaiotaomicron* cultures were set up under identical conditions. *B. thetaiotaomicron* was cultured in modified BHI in an anaerobic atmosphere at 37°C for 24 hours followed by centrifugation at 4500 RPM for 15 minutes and the spent medium was filter sterilized through 0.2 µm filters (Minisart, Sartorius Stedim Biotech, Goettingen, Germany). For the experiments in spent medium, EHEC NIPH-11060424 was grown in modified BHI supplemented with spent medium from the *B. thetaiotaomicron* cultures harvested at 24 hours (1 volume of supernatant:1 volume of 2x modified BHI). For reference, EHEC NIPH-11060424 was grown as pure culture in modified BHI under identical conditions. The pH in spent medium, used for culturing of EHEC, was checked and adjusted to 7 when necessary. For both co-culturing and growth in the presence of spent medium, sample collection was performed at two time points: mid-logarithmic phase (OD = 0.5) and 3 hours after induction with mitomycin C (MMC). The induction with MMC was performed to simulate phage induction in the gastrointestinal tract. Hydrogen peroxide released by neutrophils is believed to activate the bacterial SOS-response subsequently triggering Shiga toxin production [68]. Withdrawn samples were mixed with methanol (500 µl culture/ 500 µl methanol) and kept at -80°C before isolation of RNA. For each condition, three independent biological replicates were established (overview of experiment workflow, see Table 3).

To investigate whether differences in gene expression observed in the microarray experiments were also induced by other commensals in the human colon, EHEC NIPH-11060424 was co-cultured with or grown in spent medium from *B. fragilis* and *C. perfringens*. *B. fragilis* was chosen as it is a close relative of *B. thetaiotaomicron*. *C. perfringens* was selected as it is not related to *B. thetaiotaomicron*, it is a Gram-positive bacteria and a representative for the Firmicutes, which is the other main phyla present in the human intestine. The gene expression of LEE genes was represented by measuring the *escU* expression when *B. fragilis* and *C. perfringens* were co-cultured with EHEC NIPH-11060424. The chemotaxis gene *cheY* was chosen to investigate whether spent medium from other bacteria exerted the same effect as spent medium from *B. thetaiotaomicron* on chemotaxis/motility. The same protocol as described for *B. thetaiotaomicron* was used for qPCR.

Growth conditions in growth kinetic experiments

For the growth kinetic experiments with EHEC NIPH-11060424 and *B. thetaiotaomicron*, a CFU ratio of 1:100 and 1:1 between the two species was used. In order to quantify viable

bacterial cells from pure cultures and co-cultures, bacteria were enumerated on the basis of CFU ml⁻¹ on LB-agar and Bacteroides Bile Esculin Agar (BBE-agar, Becton, Dickinson and company, Maryland, USA) at serial time points. The LB-agar was incubated aerobically to ensure growth of EHEC and BBE-agar was incubated anaerobically to ensure growth of *B. thetaiotaomicron*. Furthermore, growth kinetics was determined for EHEC grown in spent medium from *B. thetaiotaomicron* and *B. fragilis* as described above. For these latter experiments growth kinetics were determined by measuring optical density (OD) at 600 nm. All experiments were performed independently three times.

RNA isolation and cDNA synthesis

Total RNA was extracted using a Purelink RNA mini kit (Life technologies, Carlsbad, California). DNA was removed using the Turbo DNA-free kit (Invitrogen) according to the manufacturer's instructions. RNA quantity (A₂₆₀) and purity (A_{260/280}) were measured in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was determined using Agilent 2100 bioanalyzer.

cDNA for microarray experiments was synthesized according to a recommended protocol from NimbleGen (NimbleGenUser's Guide) and was performed at the Microarray resource center in Tromsø [74]. For labeling of the cDNA samples, the NimbleGen protocol was followed using direct Cy3-cDNA labelling. For qPCR, cDNA was synthesized from 500 ng RNA using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems) according to the manufacturer's instructions in 20 µl reactions.

Design of microarray

Changes in gene expression of EHEC NIPH-11060424 in response to *B. thetaiotaomicron* (co-culture) or secreted products from *B. thetaiotaomicron* (spent medium) were investigated using microarray. Since the complete genome sequence of EHEC NIPH-11060424 was not available at the onset of the study, EHEC O103:H2 strain 12009 [50] was chosen as the template for the array design. An *in silico* comparison was performed between the EHEC NIPH-11060424 strain and EHEC 12009, demonstrating a very high degree of similarity [75]. A custom-made Nimblegen GeneChip containing 91% (5054/5541) of the open reading frames (ORFs) of *E. coli* 12009 was made for this experiment (www.nimblegen.com). Additionally, an *in silico* genome comparison of *B. thetaiotaomicron* (VPI-5482) and EHEC 12009 was performed to determine the genetic relatedness between the species, revealing a low genetic similarity. RNA from *B. thetaiotaomicron* in pure culture was isolated and converted into cDNA along with the other samples to investigate the possibility of cross-hybridization. No hybridization was observed for samples obtained from pure cultures of *B. thetaiotaomicron* confirming that cross-hybridization of genes belonging to *B. thetaiotaomicron* is most likely not an issue.

The array design was a 12-plex custom design with 135K probes. Nine probes were selected per transcript and a total of 43838 probes was produced, including 3 replicates per probe. The empty space was filled with random (negative) probes.

Hybridization and data analysis of microarray

The hybridizations of the mRNA samples were performed at the Microarray resource center in Tromsø, Norway (MRCT). The arrays were read with a GenePix 4000B scanner. The array data were processed using the R statistical language Bioconductor oligo package [76,77]. The oligo package Robust Multichip Average (RMA) normalization algorithm was used. RMA method was recommended by the manufacturer. RMA normalization included background subtraction, quantile normalization and summarization. To further reduce technical variations

the ComBat function of the Bioconductor Surrogate Variable Analysis (SVA) was applied [78,79]. Probes with a mean log₂ signal under 8 were removed from the dataset (23% of the probes). Comparison was carried out using the Bioconductor linear models for microarray data (Limma) package [80]. Normalization and comparison R scripts are available upon request. A fold-change ≥ 2 was set as the threshold for differential expression. The gene expression data are accessible in the NCBI Gene Expression Omnibus [81] through GEO Series accession number GSE44790.

Gene expression changes measured by q-PCR

mRNA levels for selected genes relative to *gapA* (glyceraldehyde-3-phosphate dehydrogenase) were determined by real-time PCR (qPCR). Five microliters of a 1:100 dilution of the cDNA reaction were used as template for qPCR amplification in 25 μ l final volumes containing 12.5 μ l of Power SYBRgreen PCR master mix with premixed ROX (Invitrogen) and 200 nM of each primer. Primer pairs were designed using Primer3plus [82]. The primers used for qPCR are listed in supplementary materials (Additional S4 file). qPCR amplification was performed using a StepOne system (Applied Biosystems). The thermal cycling conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence was monitored during each extension phase, and a melting curve analysis was performed after each run to confirm the amplification of specific transcripts. Again, to reveal any cross-hybridization, a selection of significantly up-regulated or down-regulated genes (from array) was tested for cross-hybridization with selected qPCR primers on pure cultures of *B. thetaiotaomicron*.

Changes in gene expression are presented as the differences between treated EHEC NIPH-11060424 in co-culture or in spent medium from various commensals compared to untreated controls. The slope of the standard curve and PCR efficiency for each primer pair were determined by amplifying serial dilutions of the target sequence (S4 File). The results were analyzed using Pfaffl method in combination with the Relative Expression Software Tool (REST) 2009 [83,84].

SDS-PAGE and immunoblotting

Whole cell lysates for immunoblotting were made from equal numbers of EHEC NIPH-11060424 cells from pure culture and co-culture with *B. thetaiotaomicron*. Whole cell lysate was also prepared for *B. thetaiotaomicron* in pure culture. Samples were taken at OD₆₀₀ = 0.5, and Procedures for SDS-PAGE and immunoblotting are described in [85]. The antiserum used to detect EspA is a monoclonal antibody directed against the EspA protein (tcgBIOMICS, Aachen, Germany). The primary antibody was used at 1:1000 dilution. Anti-mouse IgG, biotinylated whole antibody (from goat, GE Healthcare, UK) was used as secondary antibody. The result is representative of three independent biological and technical replicates.

In vitro adherence assays

Analysis of EHEC NIPH-11060424 binding to HeLa cells was performed as described previously [86]. Briefly, HeLa cells were cultured and propagated in MEM with 10% fetal bovine serum according to standard protocols. Prior to the assay, 24-well tissue culture plates were prepared, with HeLa cells cultured at 37°C in 5% CO₂ for 48 hours and then washed 3 times in 1 ml PBS. The bacterial test cultures were grown anaerobically at 37°C until mid-logarithmic phase (OD = 0.5) and 100 μ l of 10⁷ CFU were added to each well of HeLa cells, and incubated for 3 hours and 6 hours at 37°C in a 5% CO₂ environment. Unbound bacterial cells were removed by washing the wells three times with PBS. The HeLa cells were then lysed, using 0.1% TritonX, followed by preparation of serial dilutions of cell suspension onto LB plates. The plates were

incubated aerobically for 24 hours at 37°C and the CFU representing bacteria bound to HeLa cells was determined.

Semi-quantification of Stx2 levels by VTEC-RPLA kit

VTEC RPLA-toxin detection kit (Oxoid Limited, Basingstoke, UK) was used to determine Stx2 expression levels. The assay was performed according to the manufacturer's instruction. The amount of sample in each test well was reduced 2-fold at each dilution. The reciprocal of the highest dilution causing latex agglutination was verified as the titre.

Plaque assay

To compare phage production in co-culture (EHEC NIPH-11060424 and *B. thetaiotaomicron*) and pure culture (EHEC NIPH-11060424), the amount of bacteriophages was examined 24 hours after induction with MMC as described by previously [87]. The induced cultures were centrifuged for 10 minutes at 3900 x g and the supernatant was sterilized using 0.2 µm filters (Minisart, Sartorius Stedim Biotech). In order to remove possible colicins, trypsin was added to the filter-sterilized culture supernatant to a final concentration of 0.1 mg mL⁻¹, and incubated for 1 hour at 37°C [88]. The presence of bacteriophages was confirmed by a plaque assay [89]. Briefly, 100 µl of trypsinated phage filtrate was mixed with 900 µl of DH5α culture (OD ~ 0.3) and 3 ml LB soft agar (0.7%) containing (10 mM) CaCl₂ and poured onto a LB-agar plate. Plaques were counted after overnight incubation at 37°C.

Motility assay

Motility assays were performed as described previously [86] with minor modifications. Motility agar plates were prepared by mixing one volume spent medium from *B. thetaiotaomicron* and *B. fragilis* with one volume 2XBHI and agar to a final concentration of 0.3%. Overnight cultures of EHEC NIPH-11060424 were inoculated into modified BHI-medium and grown into mid-exponential phase (OD = 0.5) at 37°C in an anaerobic work station. Five microliters of the culture were placed in the middle of each motility plate. The diameter of the motility halos was measured after 16 hours incubation at 37°C under anaerobic atmosphere. Three motility plates were used for each condition and the experiment was repeated with three independent cultures.

Statistics

For the growth kinetic data, a t-test was done using SigmaPlot (Systat Software, San Jose, CA). A P-value <0.05 was considered statistically significant.

For the adhesion assays, the Mann-Whitney non-parametric test was used for comparison of groups (Graphpad prism). A P-value <0.05 was considered statistically significant.

Data analysis for qPCR was carried out with the Relative Expression Software Tool (REST) 2009 using the pairwise fixed randomization test. A P-value <0.05 was considered statistically significant.

Supporting Information

S1 File. Relative change in expression of genes of EHEC NIPH-11060424 (co-culture versus pure culture). Summary of changes in expression of selected categories of genes in microarray analysis of EHEC NIPH-11060424 in co-culture with *B. thetaiotaomicron* relative to EHEC NIPH-11060424 in pure culture. (DOCX)

S2 File. Relative change in expression of genes of EHEC NIPH-11060424 (spent medium versus pure culture). Summary of changes in expression of genes of selected categories in EHEC NIPH-11060424 cultured in spent medium from *B. thetaiotaomicron* relative to pure culture.

(DOCX)

S3 File. Relative change in expression of genes of EHEC NIPH-11060424 (co-culture and spent medium after induction). Summary of changes in expression genes in microarray analysis of EHEC NIPH-11060424 in co-culture with *B. thetaiotaomicron* and spent medium relative to when EHEC NIPH-11060424 is cultured alone (3 hours after induction with MMC).

(DOCX)

S4 File. Overview of primers used for qPCR,

(DOCX)

S5 File. Inhibition of Stx2 production when EHEC NIPH-11060424 is cultured in spent medium from *C. perfringens* and *B. fragilis*.

(DOCX)

S6 File. Other affected genes.

(DOCX)

Author Contributions

Conceived and designed the experiments: HI TL TLL NR MA LSA. Performed the experiments: HI TL MA. Analyzed the data: HI TL TLL MA LSA. Contributed reagents/materials/analysis tools: TL NR MA LSA. Wrote the paper: HI TL TLL MA LSA.

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S1 File. Relative change in expression of genes of EHEC NIPH-11060424 (co-culture versus pure culture).

| | <i>Gene ID</i> | <i>Gene description</i> | <i>OD=0.5 log₂ratio</i> |
|-------------------------------|--------------------|---|--|
| <i>LEE-genes</i> | | | |
| | <i>escS</i> | TTSS structure protein | 3.75 |
| | <i>escR</i> | type III secretion system protein | 3.60 |
| | <i>escT</i> | TTSS structure protein | 3.51 |
| | <i>ECO103_3635</i> | TTSS component | 3.43 |
| | <i>ECO103_3635</i> | TTSS component | 3.40 |
| | <i>ECO103_3636</i> | TTSS component | 3.24 |
| | <i>ECO103_3637</i> | TTSS component | 3.19 |
| | <i>grlA</i> | positive regulator | 3.16 |
| | <i>grlR</i> | negative regulator | 3.04 |
| | <i>escU</i> | TTSS structure protein | 2.62 |
| | <i>ECO103_3622</i> | putative TTSS component | 2.37 |
| | <i>escJ</i> | TTSS structure protein | 2.31 |
| | <i>espZ</i> | TTSS secreted effector | 2.12 |
| | <i>ECO103_2063</i> | TTSS secreted effector NleG-like protein | 1.87 |
| | <i>escC</i> | TTSS structure protein | 1.77 |
| | <i>ECO103_2223</i> | TTSS secreted effector NleF-like protein | 1.75 |
| | <i>ECO103_2292</i> | TTSS secreted effector EspO-homolog | 1.74 |
| <i>cit-operon</i> | | | |
| | <i>citD</i> | citrate lyase subunit gamma | 4.5 |
| | <i>citC</i> | citrate lyasesynthetase | 4.18 |
| | <i>citG</i> | triphosphoribosyl-dephospho-CoA synthase | 3.85 |
| | <i>citT</i> | citrate/succinate antiporterCitT | 2.97 |
| | <i>citE</i> | citrate lyase. citryl-ACP lyase subunit | 2.72 |
| <i>Metabolic genes</i> | | | |
| | <i>glpD</i> | glycerol-3-phosphate dehydrogenase | -2.95 |
| | <i>trxC</i> | thioredoxin 2 | -2.80 |
| | <i>fixC</i> | oxidoreductase | -2.74 |
| | <i>fixX</i> | 4Fe-4S ferredoxin-type protein | -2.59 |
| | <i>speF</i> | ornithine decarboxylase isozymeSpeF. inducible | -2.53 |
| | <i>hemF</i> | coproporphyrinogen III oxidase | -2.34 |
| | <i>hmp</i> | nitric oxide dioxygenase | 2.30 |
| | <i>hcp</i> | hydroxylamine reductase | 2.28 |
| | <i>srlE</i> | glucitol/sorbitol-specific enzyme IIB component | -2.27 |
| | <i>srlA</i> | glucitol/sorbitol-specific enzyme IIC component of PTS | -2.25 |
| | <i>fixB</i> | putative electron transfer flavoproteinFixB | -2.18 |
| | <i>hyuA</i> | D-stereospecific phenylhydantoinase | -2.10 |
| | | putative serine/threonine kinase | 2.08 |
| | <i>bcp</i> | thioredoxin-dependent thiol peroxidase | 2.06 |
| | <i>aes</i> | acetyl esterase | -2.05 |
| | <i>hcr</i> | HCP oxidoreductase. NADH-dependent | 2.04 |

S1 File. Relative change in expression of genes of EHEC NIPH-11060424 (co-culture versus pure culture).

| | | | |
|---------------------------------------|---------|--|--------------|
| <i>dsdA</i> | 8473987 | D-serine ammonia-lyase | -2.03 |
| <i>tnaA</i> | 8476946 | tryptophanase | -2.02 |
| <i>mdh</i> | 8475058 | malate dehydrogenase | 1.97 |
| <i>Stress response</i> | | | |
| <i>yjiX</i> | 8478262 | hypothetical protein | 5.00 |
| <i>yjiY</i> | 8478276 | putative inner membrane protein | 3.74 |
| <i>cspA</i> | 8475637 | major cold shock protein | 2.96 |
| <i>yjiA</i> | 8474488 | putative GTP-binding protein | 2.81 |
| <i>cspG</i> | 8474963 | Cold shock protein | 1.95 |
| <i>dinQ</i> | 8475537 | damage inducible protein | 1.89 |
| <i>Cell membrane/cell wall</i> | | | |
| <i>ytfE</i> | 8474984 | regulator of cell morphogenesis and cell wall metabolism | 2.77 |
| <i>ECO103_3792</i> | 8474267 | putative membrane-associated, metal-dependent hydrolase | 2.29 |
| <i>Transporters</i> | | | |
| <i>potE</i> | 8476302 | putrescine transporter | -3.21 |
| <i>yaaU</i> | 8475286 | transporter | -2.12 |
| <i>mntH</i> | 8473988 | manganese/divalent cation transporter | -2.08 |
| <i>tnaB</i> | 8475956 | low affinity tryptophan transporter | -2.07 |
| <i>tonB</i> | 8477474 | membrane spanning protein | -2.00 |
| <i>tehA</i> | 8477515 | potassium-telluriteethidium and proflavin transporter | 1.92 |

**boldface values represent significant changes ($P \leq 0.05$)*

S2 File. Relative change in expression of genes of EHEC NIPH-11060424 (spent medium versus pure culture).

| Category/ Gene symbol | Gene ID | Gene description | OD=0.5 log₂ratio |
|----------------------------------|----------------|--|--|
| Motility/chemotaxis | | | |
| <i>cheY</i> | 8474890 | chemotaxis regulatory protein | 3.32 |
| <i>cheZ</i> | 8475307 | chemotaxis regulator | 3.26 |
| <i>cheR</i> | 8474886 | chemotaxismethyltransferase | 3.14 |
| <i>cheB</i> | 8474889 | chemotaxis-specific methylesterase | 3.06 |
| <i>tar</i> | 8476885 | methyl-accepting chemotaxis protein II | 2.65 |
| <i>motB</i> | 8474885 | flagellar motor protein | 2.61 |
| <i>tap</i> | 8474887 | methyl-accepting protein IV | 2.58 |
| <i>cheW</i> | 8474834 | purine-binding chemotaxis protein. cheW | 2.58 |
| <i>yhjH</i> | 8478074 | yhjH EAL domain containing protein involved in flagellar function | 2.44 |
| <i>fliS</i> | 8475313 | flagellar protein | 2.43 |
| <i>fliT</i> | 8475328 | flagellar biosynthesis protein | 2.36 |
| <i>cheA</i> | 8474888 | chemotaxis protein | 2.31 |
| <i>motA</i> | 8476045 | flagellar motor protein | 2.15 |
| <i>flgK</i> | 8475301 | flagellar hook-associated protein | 1.96 |
| <i>lrhA</i> | | DNA-binding transcriptional repressor LrhA of flagellar. motility and chemotaxis genes | -1.7 |
| cit-operon | | | |
| <i>citC</i> | 8474899 | citrate lyasesynthetase | 4.54 |
| <i>citD</i> | 8474900 | citrate lyase subunit gamma | 4.11 |
| <i>citG</i> | 8474903 | triphosphoribosyl-dephospho-CoA synthase | 4.03 |
| <i>citE</i> | 8474901 | citrate lyase. citryl-ACP lyase subunit | 2.72 |
| <i>citX</i> | 8474902 | 2'-(5''-triphosphoribosyl)-3'-dephospho-CoA:apo- citrate lyase | 2.65 |
| <i>citT</i> | 8476579 | citrate/succinate antiporter | 2.28 |
| Other metabolic genes | | | |
| <i>speF</i> | 8476360 | ornithine decarboxylase | -4.21 |
| <i>lamB</i> | 8475913 | lamB maltose outer membrane porin | -4.04 |
| <i>malM</i> | 8475811 | maltose regulonperiplasmic protein | -3.49 |
| <i>adiA</i> | 8474613 | biodegradative arginine decarboxylase AdiA | 3.44 |
| <i>otsB</i> | 8476239 | trehalose-6-phosphate phosphatase | 3.42 |
| <i>sdaB</i> | 8476752 | L-serine deaminase II | -2.91 |
| <i>otsA</i> | 8477658 | trehalose-6-phosphate synthase | 2.89 |
| <i>sufC</i> | 8476870 | cysteine desulfurase ATPase component | 2.83 |
| <i>cadA</i> | 8478227 | lysine decarboxylase 1 | -2.79 |
| <i>tdcB</i> | 8476900 | threonine dehydratase | -2.58 |

S2 File. Relative change in expression of genes of EHEC NIPH-11060424 (spent medium versus pure culture).

| | | | |
|---------------------------------------|---------|--|--------------|
| <i>tnaA</i> | 8476946 | tryptophanase | -2.52 |
| <i>sufA</i> | 8476868 | iron-sulfur cluster assembly scaffold protein | 2.51 |
| <i>sufD</i> | 8476873 | cysteine desulfurase activator complex subunit | 2.38 |
| <i>dsdA</i> | 8473987 | D-serine dehydratase | -2.37 |
| <i>fixC</i> | 8475284 | putative oxidoreductase | -2.22 |
| <i>sufS</i> | 8476871 | bifunctional cysteine desulfurase/selenocysteinylase | 2.2 |
| <i>srlE</i> | 8476837 | glucitol/sorbitol-specific enzyme IIB component | -2.16 |
| <i>gadA</i> | 8475413 | glutamate decarboxylase A. PLP-dependent | 2.15 |
| <i>sufB</i> | 8476869 | cysteine desulfurase activator complex subunit | 2.14 |
| <i>astB</i> | 8474770 | succinylargininedihydrolase | -2.08 |
| <i>malQ</i> | 8475533 | 4-alpha-glucanotransferase | -2.08 |
| <i>astD</i> | 8474767 | succinylglutamicsemialdehyde dehydrogenase | -2.07 |
| <i>srlA</i> | 8476018 | glucitol/sorbitol-specific enzyme IIC component of PTS | -2.05 |
| <i>talA</i> | 8475907 | transaldolase A | 2.05 |
| <i>ECO103_1575</i> | 8477526 | putative acetyltransferase | 2.03 |
| <i>ECO103_5185</i> | 8474519 | putative exclusion protein | -2 |
| <i>poxB</i> | 8475889 | pyruvate dehydrogenase | 2 |
| <i>Stress response</i> | | | |
| <i>yjiX</i> | 8478262 | hypothetical protein | 2.63 |
| <i>yjiY</i> | 8478276 | putative inner membrane protein | 1.8 |
| <i>Cell membrane/cell wall</i> | | | |
| <i>ompF</i> | 8474760 | outer membrane protein F | -2 |
| <i>Transporters</i> | | | |
| <i>potE</i> | 8476302 | putrescine transporter | -4.50 |
| <i>proV</i> | 8476180 | glycine betaine transporter ATP-binding subunit | 4.32 |
| <i>proW</i> | 8476407 | glycine betaine transporter membrane protein | 4.18 |
| <i>proX</i> | 8476408 | glycine betaine transporter periplasmic subunit | 4.11 |
| <i>malK</i> | 8475909 | maltose/maltodextrin transporter ATP-binding protein | -3.6 |
| <i>cadB</i> | 8474836 | lysine/cadaverine antiporter | -3.23 |
| <i>malE</i> | 8475910 | maltose ABC transporter periplasmic protein | -2.92 |
| <i>sdaC</i> | 8477886 | putative serine transporter | -2.48 |
| <i>xylF</i> | 8477131 | D-xylose transporter subunit | -2.4 |
| <i>tnaB</i> | 8475956 | low affinitytryptophan transporter | -2.39 |

S2 File. Relative change in expression of genes of EHEC NIPH-11060424 (spent medium versus pure culture).

| | | | |
|------|---------|---|-------|
| tdcC | 8476901 | threonine/serine transporter | -2.2 |
| ydhC | 8477564 | inner membrane transport protein YdhC | 2.12 |
| yqcE | 8477883 | putative transporter | -2.1 |
| yhdW | 8478015 | putative periplasmic binding transport protein | -1.98 |
| yhdX | 8478016 | putative transport system permease protein | -1.98 |
| glcA | 8474210 | glycolate transporter | -1.96 |
| garP | 8475428 | putative (D)-galactarate transporter | -1.95 |

**boldface values represent significant changes ($P \leq 0.05$)*

S3 File. Relative change in expression of genes of EHEC NIPH-11060424 (co-culture and spent medium after induction).

| Category/ Gene symbol | Gene ID | Gene description | 3 hrs after ind./ log ₂ ratio |
|--------------------------|---------|----------------------------------|--|
| in co-culture | | | |
| <i>ECO103_2069</i> | 8473571 | tail assembly protein | -4.23 |
| <i>stx2a</i> | 8473941 | Shiga toxin 2 subunit A | -4.04 |
| <i>ECO103_5159</i> | 8478281 | integrase | -3.81 |
| <i>ECO103_5203</i> | 8474537 | endolysin | -3.75 |
| <i>ECO103_2839</i> | 8473935 | antirepressor | -3.73 |
| <i>ECO103_0518</i> | 8473152 | phage regulatory protein CII | -3.5 |
| <i>ECO103_1197</i> | 8473307 | endolysin | -3.3 |
| <i>stx2b</i> | 8473940 | Shiga toxin 2 subunit B | -3.23 |
| <i>ECO103_0533</i> | 8473167 | lipoprotein Bor | -3.11 |
| <i>ECO103_2326</i> | 8473737 | endolysin | -3.01 |
| <i>ECO103_0794</i> | 8473244 | minor tail protein | -2.96 |
| <i>ECO103_5181</i> | 8474515 | antirepressor protein Cro | -2.94 |
| <i>ECO103_5212</i> | 8474546 | head protein/prohead protease | -2.94 |
| <i>ECO103_2846</i> | 8475737 | late gene regulator Q | -2.83 |
| <i>ECO103_2325</i> | 8473736 | antirepressor | -2.71 |
| <i>ECO103_2850</i> | 8473946 | helicase | -2.29 |
| <i>recX</i> | 8474632 | regulatory protein RecX for RecA | -2.09 |
| <i>ECO103_2864</i> | 8473960 | exonuclease | -2.04 |
| in spent medium | | | |
| <i>stx2a</i> | 8473941 | Shiga toxin 2 subunit A | -4.16 |
| <i>stx2b</i> | 8473940 | Shiga toxin 2 subunit B | -3.61 |
| <i>ECO103_2878</i> | 8473974 | antirepressor | -3.42 |
| <i>ECO103_0533</i> | 8473167 | lipoprotein Bor | -3.33 |
| <i>ECO103_2069</i> | 8473571 | tail assembly protein | -3.04 |
| <i>ECO103_1197</i> | 8473307 | endolysin | -2.79 |
| <i>ECO103_2326</i> | 8473737 | endolysin | -2.7 |
| <i>ECO103_2325</i> | 8473736 | antirepressor | -2.67 |
| <i>ECO103_0794</i> | 8473244 | minor tail protein | -2.64 |
| <i>ECO103_2864</i> | 8473960 | exonuclease | -2.53 |
| <i>ECO103_2846</i> | 8475737 | late gene regulator Q | -2.49 |
| <i>ECO103_2850</i> | 8473946 | helicase | -2.33 |
| <i>ECO103_5212</i> | 8474546 | head protein/prohead protease | -2.3 |

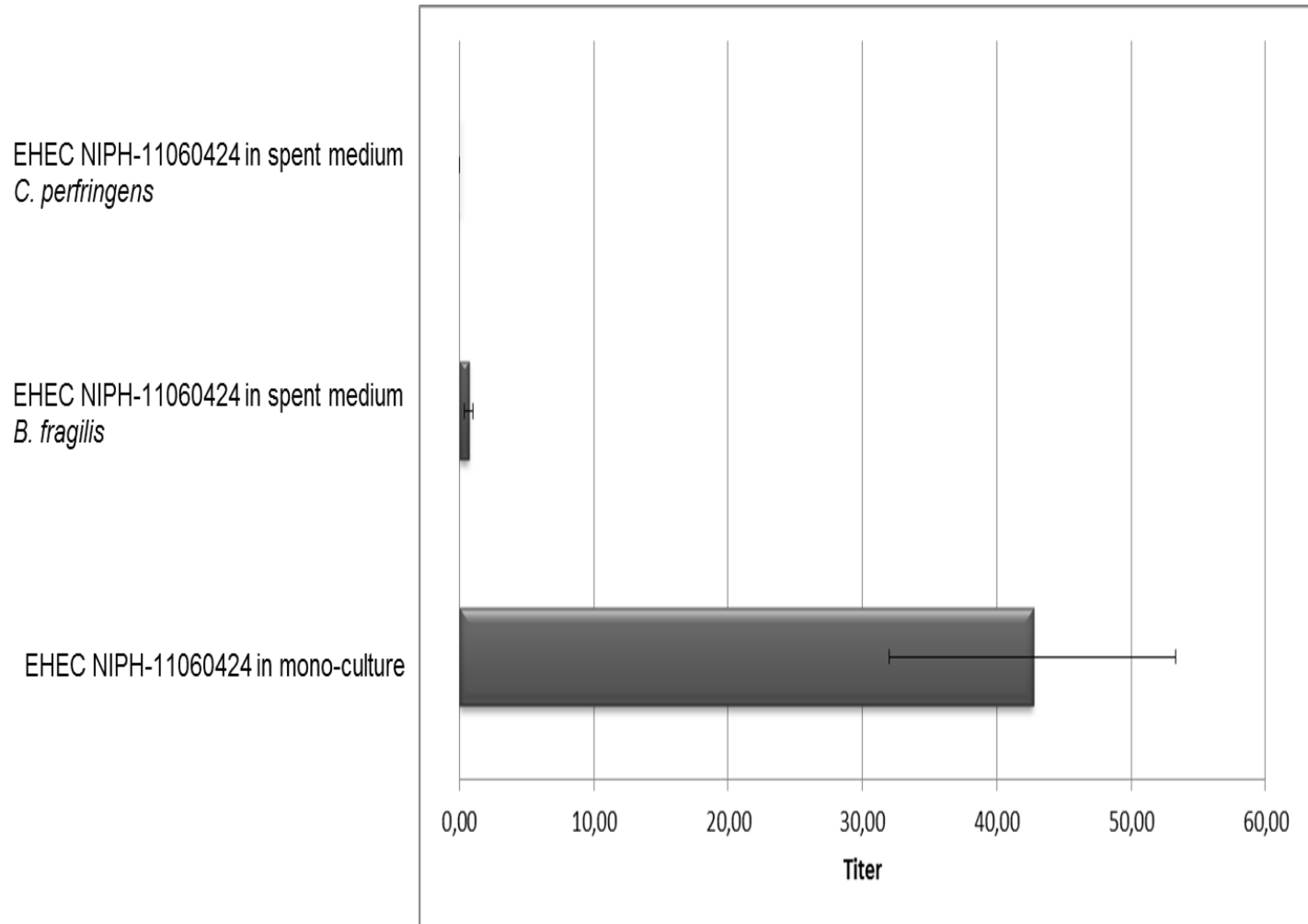
S4 File. Overview of primers used for qPCR,

| Gene | PRIMER SEQUENCES | | Slope ^a | % Eff ^b |
|-------------|-----------------------|---------------------------|--------------------|--------------------|
| | Forward (5' to 3') | Reverse (5' to 3') | | |
| <i>gapA</i> | AGGTCTGATGACCACCGTTC | AACGGTCAGGTCAACTACGG | -3.3 | 99.7 |
| <i>grlA</i> | GCCGAAGCATTTCGGTATAAA | TTTTCTTTTTGGTCCGGTTG | -3.3 | 99.5 |
| <i>escU</i> | CCGTCGCCTTCATTCTGAGA | GATCACGGTTGATTTTTTGATGTTA | -3.2 | 92.1 |
| <i>Ler</i> | ATGTGCCTGATGATGGACTC | AGGTCTGCCCTTCTTCATTG | -3.1 | 88 |
| <i>escJ</i> | TAGCACCATCGGTCATTTCAG | ACATATTACCCGTCCTGTCCTG | -3.2 | 84 |
| <i>escV</i> | CGCACTAATGCTCGTAATGG | TTCCAAGCTATCCCCAACAG | -3.2 | 88 |
| <i>escF</i> | AACGCTGAGTGATTCTGTGC | AATATTGCTGAACCGCGAAC | -3.4 | 97.8 |
| <i>espA</i> | CGCTTGAGCTGAAAATAGCTG | AACGCTGAGTGATTCTGTGC | -3.4 | 95 |
| <i>eae</i> | ACATTATGGAACGGCAGAGG | AAGCGGGAGTCAATGTAACG | -3.1 | 88 |
| <i>tir</i> | ATCAACAGCTTCCAGCGTTC | TTGTAGGATCATCCGGTTCC | -3.2 | 96 |
| <i>cheY</i> | CAGGCGGTTATGGATTTGTT | AATGGCTTCACCACATAGCC | -3.3 | 99.6 |
| <i>cheZ</i> | AAATCGTCCACCTGATCCTG | AGTTGCTGATGGTGCTGTTG | -3.7 | 86.7 |
| <i>cheB</i> | TGGTAATTTGCGCCACTACG | GGTTAAAGAAGCCGAAGACG | -3.3 | 99.3 |
| <i>motA</i> | GCTGGAATAGAGCGTTTTGC | ATGCAGTGCGTCAAAGTCAC | -3.3 | 97.4 |
| <i>motB</i> | CAGGGGGAAGTGAATAAGCA | TTCTAAACATCGGGCGATTG | -3.1 | 91 |
| <i>citD</i> | TGAGTCTGGGGATGTGATGA | CTTCCAGTCGTGCACGTAAA | -3.6 | 90 |
| <i>glpD</i> | CATCAATGGTGCTGGTATCG | GTTTTGAACTGGCGGAAGAG | -3.1 | 90.4 |
| <i>trxC</i> | CGTTTGTACCCATTGTCAGG | TCTCCGTCAAACAAGTCGTG | -3.3 | 83 |
| <i>potE</i> | AACCAAGGAAGGTCACGATG | TCAGAAGGTCGCTAATGTGC | -3.6 | 91 |

^aSlope was calculated from the regression line in the standard curve

^bEfficiency was calculated using the slope of the regression line in the standard curve

S5 File. Inhibition of Stx2 production when EHEC NIPH-11060424 is cultured in spent medium from *C. perfringens* and *B. fragilis*



[S6 File](#). Other affected genes.

Other affected genes

The presence of *B. thetaiotaomicron* did not result in a major stress response for the EHEC strain. However, some genes involved in stress responses including cold shock genes, as well as genes involved in cell wall- and cell membrane synthesis were up-regulated in EHEC NIPH-11060424 grown in co-culture or in the presence of spent medium from *B. thetaiotaomicron* relative to pure culture.

PAPER II

Commensal *E. coli* Stx2 lysogens produce high level of phages after spontaneous prophage induction

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**Hildegunn Iversen, Trine M. L'Abée-Lund Marina
Aspholm, Lotte S. Arnesen and Toril Lindback**



Commensal *E. coli* Stx2 lysogens produce high levels of phages after spontaneous prophage induction

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Enterohemorrhagic *E. coli* (EHEC) is a food-borne pathogen that causes disease ranging from uncomplicated diarrhea to life-threatening hemolytic uremic syndrome (HUS) and nervous system complications. Shiga toxin 2 (Stx2) is the major virulence factor of EHEC and is critical for development of HUS. The genes encoding Stx2 are carried by lambdoid bacteriophages and the toxin production is tightly linked to the production of phages during lytic cycle. It has previously been suggested that commensal *E. coli* could amplify the production of Stx2-phages and contribute to the severity of disease. In this study we examined the susceptibility of commensal *E. coli* strains to the Stx2-converting phage ϕ 734, isolated from a highly virulent EHEC O103:H25 (NIPH-11060424). Among 38 commensal *E. coli* strains from healthy children below 5 years, 15 were lysogenized by the ϕ 734 phage, whereas lytic infection was not observed. Three of the commensal *E. coli* ϕ 734 lysogens were tested for stability, and appeared stable and retained the phage for at least 10 cultural passages. When induced to enter lytic cycle by H₂O₂ treatment, 8 out of 13 commensal lysogens produced more ϕ 734 phages than NIPH-11060424. Strikingly, five of them even spontaneously (non-induced) produced higher levels of phage than the H₂O₂ induced NIPH-11060424. An especially high frequency of HUS (60%) was seen among children infected by NIPH-11060424 during the outbreak in 2006. Based on our findings, a high Stx2 production by commensal *E. coli* lysogens cannot be ruled out as a contributor to the high frequency of HUS during this outbreak.

Keywords: EHEC, Stx2, bacteriophage lambda, lysogen, commensal *E. coli*

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) causes disease with manifestations ranging from mild diarrhea to severe illness comprising hemorrhagic colitis (HC) (Riley et al., 1983) and hemolytic uremic syndrome (HUS) (Karmali et al., 1983, 1985). The *E. coli* serotype O157:H7, which caused the first described EHEC outbreak in 1982 (Riley et al., 1983) is so far the best described EHEC serotype. However, non-O157:H7 serotype strains have been implicated in a number of outbreaks and sporadic cases of HC and HUS (Luna-Gierke et al., 2014). In Europe, *E. coli* serotypes such as O103:H25 and O104:H4 have caused severe outbreaks (Schimmer et al., 2008; Beutin and Martin, 2012).

EHEC strains possess a range of colonization and virulence factors that facilitate infection and contribute to development of disease. Shiga toxin (Stx) is recognized as one of the main virulence factors in enterohemorrhagic disease caused by *E. coli* and all EHEC strains produce one or both of the Shiga toxins Stx1 and Stx2 (Scotland et al., 1985). Stx2 has been shown to be far more potent (as quantified by LD₅₀ in mice) than Stx1 and patients infected with the latter are much less likely to develop serious illness than those infected by the former (Tesh et al., 1993; Friedrich et al., 2002). After being produced in the large intestine the Stx toxin passes through the epithelial cells and is disseminated via the blood stream to the target organs. Stx

binds specifically and with high-affinity to the glycosphingolipid receptor globotriaosylceramide (Gb3) which is highly expressed in kidney cells (Jacewicz et al., 1986; Hughes et al., 2000; Okuda et al., 2006; Shimizu et al., 2007; Shin et al., 2009). After binding to the receptor, Stx is translocated into the cytosol where it causes cell damage by inhibiting protein synthesis (Sandvig and van Deurs, 2000). The Stx induced cell damage appears to be central in the pathogenic events leading to HUS and occasionally chronic kidney disease (Obrig, 2010; Obrig and Karpman, 2012).

About 15 years ago, several *E. coli* and *Shigella* strains were lysogenized with labeled Stx2 phages *in vitro* (Schmidt et al., 1999; James et al., 2001), and successful *in vivo* transduction experiments with Stx derivative phages have also been reported (Acheson et al., 1998; Toth et al., 2003). When a bacterial cell is infected by an Stx-encoding phage, two different pathways are possible (Allison, 2007). During lytic infection, the phage DNA exists as a separate molecule within the cell and utilizes the host machinery to express its genes and to produce large amounts of new phage particles until the host cell bursts. The other outcome is lysogenic infection, where the phage genome is integrated into the chromosome as a prophage, and is replicated along with the host genome. The phage can remain in the lysogenic state as long as the phage genes are repressed. It has been shown in several studies that the *stx2* genes are controlled by the phage late gene promoter, and that phage production is

tightly linked to production of Stx toxin (Neely and Friedman, 1998; Unkmeir and Schmidt, 2000; Zhang et al., 2000; Wagner et al., 2002). Upon induction, the prophage can switch from the lysogenic state to the lytic cycle, accompanied by production of Stx and new phage particles (Herold et al., 2004; Waldor and Friedman, 2005). Several physical and chemical agents may act as prophage-inducing agents and all share the ability to activate the bacterial SOS response, mainly due to DNA damage (Kimmitt et al., 2000; Erill et al., 2007). Mitomycin C has often been used as prophage-inducing agent in studies of EHEC, however, H₂O₂ has been shown to be an effective prophage-inducer (Loś et al., 2009, 2010) and its presence in the gut may also increase Stx production (Wagner et al., 2001).

It has been reported that phages present in the gastrointestinal tract tend to enter the lysogenic pathway more often than the lytic pathway (Reyes et al., 2012). Factors like the number of infecting phages per bacterial cell and cell size prior to infection have been shown to influence whether the host will lyse or become lysogenic (St-Pierre and Endy, 2008). However, the mechanisms that determine the cell fate following phage-infection are complex and not fully understood.

Previous studies have shown that Stx-phages display a diverse host range, and also infect commensal *E. coli* (Wagner et al., 1999; Muniesa et al., 2003; Gamage et al., 2004). Gamage et al. (2004) demonstrated that commensal *E. coli* infected with Stx2 phages from *E. coli* O157:H7 were able to produce Stx2 and possibly increase the pathogenic potential of EHEC during infection. The contribution of commensal *E. coli* flora to Stx production was also demonstrated in a mouse model infected with *E. coli* O157:H7, where Stx was more commonly detected in mice colonized with *E. coli* sensitive to the Stx-phage than mice colonized with *E. coli* resistant to the Stx phage (Gamage et al., 2006). Children are particularly susceptible to EHEC infections and development of HUS (Tarr et al., 2005; Gyles, 2007). In 2006, Norway experienced a foodborne EHEC outbreak comprising 17 cases where all patients, except one (an adult aged 18), were children. The outbreak had an HUS frequency of 60%, which is extremely high, and all HUS patients were less than 9 years old (Schimmer et al., 2008). Due to the high HUS frequency, the causative strain, *E. coli* O103:H25 (NIPH-11060424), was considered to be particularly virulent (Schimmer et al., 2008). The strain was later shown to be closely related to the *E. coli* O104:H4 strain causing a large outbreak in Germany in 2011 (L' Abée-Lund et al., 2012). However, the genetic and phenotypic features underlying the extraordinary high virulence of the Norwegian outbreak strain are not yet known.

In this study, we examine the susceptibility of commensal *E. coli* isolates from young children to the Stx2-converting phage (ϕ 734) from the 2006 Norwegian outbreak strain. We address the commensal *E. coli* strains sensitivity for lytic and lysogen infection and their ability to contribute to ϕ 734 phage production and thereby Stx2 production.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PHAGES

The bacterial strains used in this study are listed in Table 1 and Supplementary Table 1. The commensal *E. coli* strains

were isolated from fecal samples from healthy Norwegian children below 5 years of age in the years 2009–2014. All strains tested negative against Test Serum Anti-Coli O 103:K- and Anti-Coli O157:K- in agglutination tests (SIFIN, Germany). EHEC O103:H25 NIPH-11060424 is a highly virulent strain which caused a severe outbreak in Norway in 2006 (Schimmer et al., 2008; L' Abée-Lund et al., 2012). The phage infection experiments in this study were performed using the Stx2-converting phage ϕ 734 from NIPH-11060424 (L' Abée-Lund et al., 2012) or the recombinant version of this phage (Table 1). The recombinant phage ϕ 734Cm in which *stx2A* is replaced by the chloramphenicol resistance gene (*cat*) was constructed by Dr. Muniesa, University of Barcelona, Spain, as described by Serra-Moreno et al. (2006). *E. coli* DH5 α was used as a propagating strain for determination of phage concentration. A stable lysogen of the laboratory strain *E. coli* C600 carrying ϕ 734 (C600: ϕ 734) was created by infecting *E. coli* C600 with ϕ 734. The lysogen was identified by PCR using the *stx2* primers listed in Table 2.

PREPARATION OF PHAGE FILTRATES FOR PHAGE INFECTION EXPERIMENTS

E. coli strains carrying either ϕ 734 or ϕ 734Cm were grown in Lysogeny broth (LB) to mid-exponential growth phase

Table 1 | *E. coli* strains and bacteriophages used in the study.

| Bacterial strains and phage | Characteristics | References |
|---|--|--|
| <i>E. coli</i> LABORATORY STRAINS | | |
| C600 | K-12 derivate | Appleyard, 1954 |
| DH5 α | K-12 derivate | Hanahan, 1985 |
| EHEC STRAINS | | |
| NIPH-11060424 | Human isolate, Norwegian outbreak strain 2006, O103:H25. Possesses the Stx2-phage ϕ 734 and the phi-like phage TL-2011b | Schimmer et al., 2008; L' Abée-Lund et al., 2012 |
| COMMENSAL <i>E. coli</i> ISOLATES | | |
| NVH-1034-NVH-1042, NVH-1064-NVH-1094 | Child isolates ($n = 38$) non-O103/O157 | This study |
| L1034-L1042, L1064-L1094 | ϕ 734 Cm lysogens of the commensal <i>E. coli</i> isolates with corresponding numbering | |
| RECOMBINANT <i>E. coli</i> STRAINS | | |
| NIPH-11060424: ϕ 734 Cm | ϕ 734 Cm lysogen in NIPH-11060424 ^a | This study |
| C600: ϕ 734 Cm | ϕ 734 Cm lysogen in C600 ^a | This study |
| C600: ϕ 734 | ϕ 734 lysogen in C600 ^a | This study |
| BACTERIOPHAGES | | |
| ϕ 734 | Stx2-converting phage from NIPH-11060424 (GenBank acc no JQ011318.1) Synonym name is TL-2011c | L' Abée-Lund et al., 2012 |
| ϕ 734 Cm | ϕ 734 Δ <i>stx2A::cat</i> | This study |

^aThe strains were stable.

Table 2 | PCR primers used in the study.

| Primers | Sequence (5'-3') | References |
|-------------------|----------------------|-------------------------|
| stx2 forward | GCGTTTTGACCATCTTCGT | Muniesa and Jofre, 1998 |
| stx2 reverse | ACAGGAGCAGTTTCAGACAG | Muniesa and Jofre, 1998 |
| cat forward | GGGCGAAGAAGTTGTCCATA | This study |
| cat reverse | TACACCGTTTTCCATGAGCA | This study |
| phi-phage forward | GCGGTCATGAAAACAAACCT | This study |
| phi-phage reverse | AGGCGGCAGGATTATCAAG | This study |

(OD₆₀₀ = 0.3–0.5) and then left non-induced or induced by addition of either Mitomycin C (MMC) (0.5 µg/ml) or H₂O₂ (1.5 mM). The cultures were then further incubated overnight at 37°C followed by centrifugation for 10 min at 4500 rpm and sterile-filtrated using 0.22 µm filters (Millex-GP, Millipore, Bedford, MA). The phage concentration in the bacteria-free filtrate was determined by plaque assay using *E. coli* DH5α as a propagating strain. In order to remove any colicins, Trypsin (Sigma) was added to the phage-filtrate to a final concentration of 0.1 mg/ml followed by 1 h incubation at 37°C (Gordon and O'Brien, 2006).

PLAQUE ASSAY

A plaque assay was used to determine the concentration of infective phage particles in the phage filtrates. A volume of 100 µl of phage filtrate was mixed with 900 µl of *E. coli* DH5α culture (OD₆₀₀ = 0.3) containing 10 mM CaCl₂, and then further incubated without agitation for 30 min. After incubation, the samples were mixed with 2.5 ml 0.7% LB agar and poured onto LB agar plates containing 10 mM CaCl₂. The plates were incubated overnight at 37°C and plaques were counted. The phage concentration is given as plaque forming units/ml (PFU/ml).

Hybridization of plaques in *E. coli* DH5α lawn was performed to confirm that *E. coli* DH5α was susceptible to φ734 and φ734 Cm (see the Materials and Methods below). The results showed that 100% of the plaques were positive for the corresponding probe, either *stx2A* or *cat*. Thus, *E. coli* DH5α was used to quantify the number of phages in the phage filtrates.

PLAQUE HYBRIDIZATION

Plaque hybridization was performed according to a standard procedure (Datz et al., 1996; Sambrook and Russell, 2001) using Hybond-N+ membranes (Amersham Pharmacia Biotech). The membranes were hybridized against a DIG labeled PCR amplified probe (primers shown in Table 2). Labeling of probe and hybridization were performed using the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. The hybridization temperature used for all experiments was 56°C.

LYTIC PHAGE INFECTION

To test for susceptibility to lytic infection, 5 µl of φ734 phage-filtrate was spotted on LB soft agar plates with 10 mM CaCl₂, containing commensal *E. coli* strains, *E. coli* C600 or *E. coli* DH5α.

The concentrations of the φ734 phage-filtrates used in the spot assay were 10⁶ PFU/ml when propagated on NIPH-11060424 and 10⁹ PFU/ml when propagated on *E. coli* C600. The LB plates were incubated overnight at 37°C. The susceptibility to lytic infection among the commensal *E. coli* strains were additionally tested using the plaque assay where the recipient culture had a cell density of 1 × 10⁸ CFU/ml (OD₆₀₀ = 0.3) and the φ734 phage concentration was either 1 × 10⁸ PFU/ml or 1 × 10⁵ PFU/ml, giving a multiplicity of infection (MOI) of 1 and 0.001, respectively.

LYSOGEN INFECTION

The recombinant φ734 Cm phage was used to test commensal *E. coli* strains for susceptibility to lysogenic infection as described previously (Schmidt et al., 1999). The commensal *E. coli* strains were tested for Cm sensitivity prior to the experiment, and all strains were found sensitive. Colonies growing on LB plates containing 25 µg/ml of chloramphenicol were considered to be lysogens. Lysogens from each commensal strain were named by adding the prefix L to their wildtype number. Phage filtrate from 13 lysogens (Table 3) was prepared to examine their phage production by plaque assay using DH5α as a recipient strain. The stability of the φ734 Cm phage containing lysogens was tested by culturing lysogens in LB without antibiotic selection for 10 passages. After each passage, dilutions of the cultures were spread onto LB plates with chloramphenicol to examining the level of bacteria carrying φ734 Cm.

SEMI-QUANTIFICATION OF Stx2 LEVELS BY VTEC-RPLA KIT

A VTEC RPLA-toxin detection kit (Oxoid Limited, Basingstoke, UK) was used to determine the Stx2 production by NIPH-11060424 and C600:φ734. The assay was performed according to the manufacturer's instruction. The amount of sample in each test well was reduced 2-fold at each dilution. The Stx2 titer was defined as the reciprocal of the highest dilution causing latex agglutination.

WESTERN BLOT

Proteins were separated by electrophoresis using the NuPAGE Novex Bis-Tris gel systems (Invitrogen) and SeeBlue Plus2 Pre-Stained Standard (Invitrogen) as molecular weight marker. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore) according to standard protocols (Harlow and Lane, 1988). Stx2 in culture supernatants was detected using monoclonal antibodies against Stx2 (STX2-11E10, TOXIN TECHNOLOGY, INC., Sarasota, FL) diluted 1:1000. Biotin-conjugated anti-mouse antibodies from goat (Amersham Biosciences) were used as secondary antibodies (1:3000). A complex of streptavidin (Bio-Rad) and biotinylated alkaline phosphatase (Bio-Rad) was used at a dilution of 1:3000 prior to development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

STATISTICAL ANALYSIS

Student's *t*-test was used to determine significant differences between groups. A *P* ≤ 0.05 was considered significant.

Table 3 | Susceptibility of 38 commensal *E. coli* strains to lysogenic infection by the ϕ 734 Cm phage.

| <i>E. coli</i> isolates | ϕ 734 Cm from NIPH-11060424: ϕ 734 Cm MOI 0.005 | ϕ 734 Cm from C600: ϕ 734 Cm MOI 0.005 | ϕ 734 Cm from C600: ϕ 734 Cm MOI 0.5 ^a | ϕ 734 Cm from NVH-1090: ϕ 734 Cm MOI 0.5 |
|-------------------------|---|--|---|--|
| NVH-1034 | -/- | -/- | -/- | -/- |
| NVH-1036 | -/- | -/- | -/- | -/- |
| NVH-1037 | -/- | -/- | 40/20 | 30/50 |
| NVH-1038 | -/- | -/- | -/- | -/- |
| NVH-1039 | -/- | -/- | -/- | -/- |
| NVH-1040 | -/- | -/- | -/- | -/- |
| NVH-1041 | -/- | -/- | -/- | -/- |
| NVH-1042 | -/- | -/- | -/- | -/- |
| NVH-1064 | -/- | 200/10 | 10/90 | 300/200 |
| NVH-1065 | -/- | -/10 | 200/600 | 20/1000 |
| NVH-1066 | -/- | 20/- | 200/30 | 30/20 |
| NVG-1067 | -/- | -/- | 200/10 | 40/100 |
| NVH-1068 | -/- | -/- | -/- | -/- |
| NVH-1069 | -/- | -/- | -/- | -/- |
| NVH-1070 | -/- | -/- | -/- | -/- |
| NVH-1071 | -/- | -/- | -/- | -/- |
| NVH-1072 | -/- | -/- | -/- | -/- |
| NVH-1073 | -/- | -/- | -/- | -/- |
| NVH-1074 | -/- | -/- | -/- | -/- |
| NVH-1075 | -/- | -/- | -/- | 500/20 |
| NVH-1076 | -/- | -/- | -/- | -/- |
| NVH-1077 | 30/100 | -/- | 1200/400 | 30/700 |
| NVH-1078 | 2000/100 | 500/2000 | 10000/8000 | 3000/8000 |
| NVH-1079 | -/- | -/- | -/- | -/- |
| NVH-1080 | -/- | -/- | -/- | -/- |
| NVH-1081 | -/- | -/- | 10/50 | 30/20 |
| NVH-1083 | -/- | -/- | -/- | -/- |
| NVH-1084 | -/- | -/- | 30/30 | -/- |
| NVH-1085 | -/- | -/- | -/- | 30/200 |
| NVH-1086 | -/- | 70/- | 50/300 | 100/20 |
| NVH-1087 | -/- | -/- | -/- | -/- |
| NVH-1088 | -/- | 10000/50000 | 10000/10000 | 8000/9000 |
| NVH-1089 | -/- | -/- | -/- | -/- |
| NVH-1090 | 40/200 | 100/400 | 4000/3000 | 3000/2000 |
| NVH-1091 | -/- | -/- | -/- | -/- |
| NVH-1092 | -/- | -/- | -/- | -/- |
| NVH-1093 | -/- | -/- | 60/40 | 30/80 |
| NVH-1094 | -/- | -/- | -/- | - |

The phage was propagated on three different strains (the original outbreak strain NIPH-11060424, the laboratory *E. coli* strain C600 and the commensal *E. coli* strain NVH1090), and two different concentrations of phages (MOI 0.005 and MOI 0.5) were used. The results are presented as the number of lysogens/ml. Two replicates were performed for all conditions.

-, no lysogens detected.

^alysogens made under this condition were selected for further examination of phage production (Figure 2).

RESULTS

SUSCEPTIBILITY OF COMMENSAL *E. COLI* STRAINS TO LYTIC INFECTION BY THE Stx2-CONVERTING PHAGE ϕ 734

Thirty-eight commensal *E. coli* strains were tested for susceptibility to lytic infection by ϕ 734 or ϕ 734 Cm propagated on

either in EHEC NIPH-11060424 or on the laboratory strain *E. coli* C600. None of the commensal *E. coli* strains were susceptible to lytic infection by any of the two phages propagated on NIPH-11060424 or *E. coli* C600 at any of the tested concentrations. Previous studies have shown that NIPH-11060424 carries a phi-like phage (TL-2011b) in addition to the Stx2 phage (L' Abée-Lund et al., 2012). This phi-like phage is 53% identical to bacteriophage Φ V10, a temperate phage that specifically infects *E. coli* of serogroup O157:H7 (Perry et al., 2009). TL-2011b was shown by spot assay and following hybridization using a phi-phage specific probe to infect *E. coli* of serogroup O103:H25, while none of the commensal strains tested were susceptible for lytic infection by this phage (Supplementary Table 2). This indicates that phage TL-2011b is serotype specific.

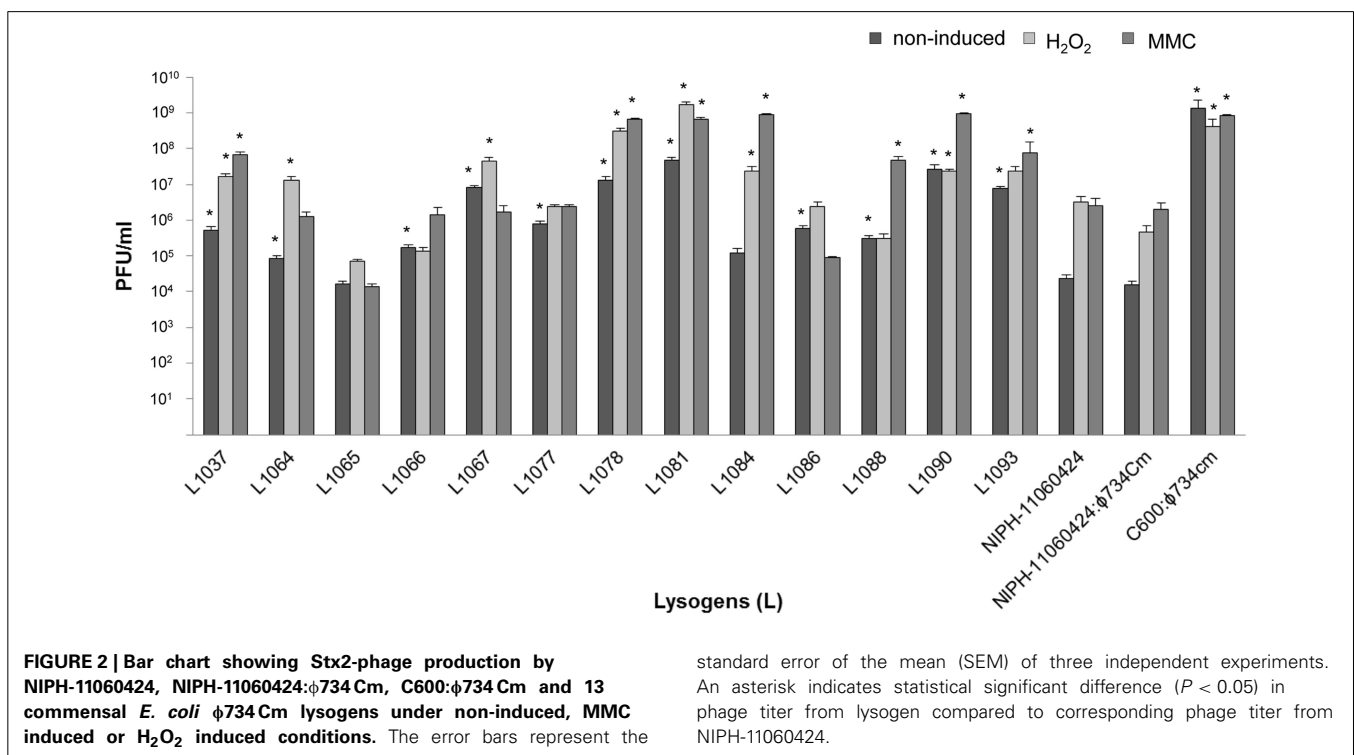
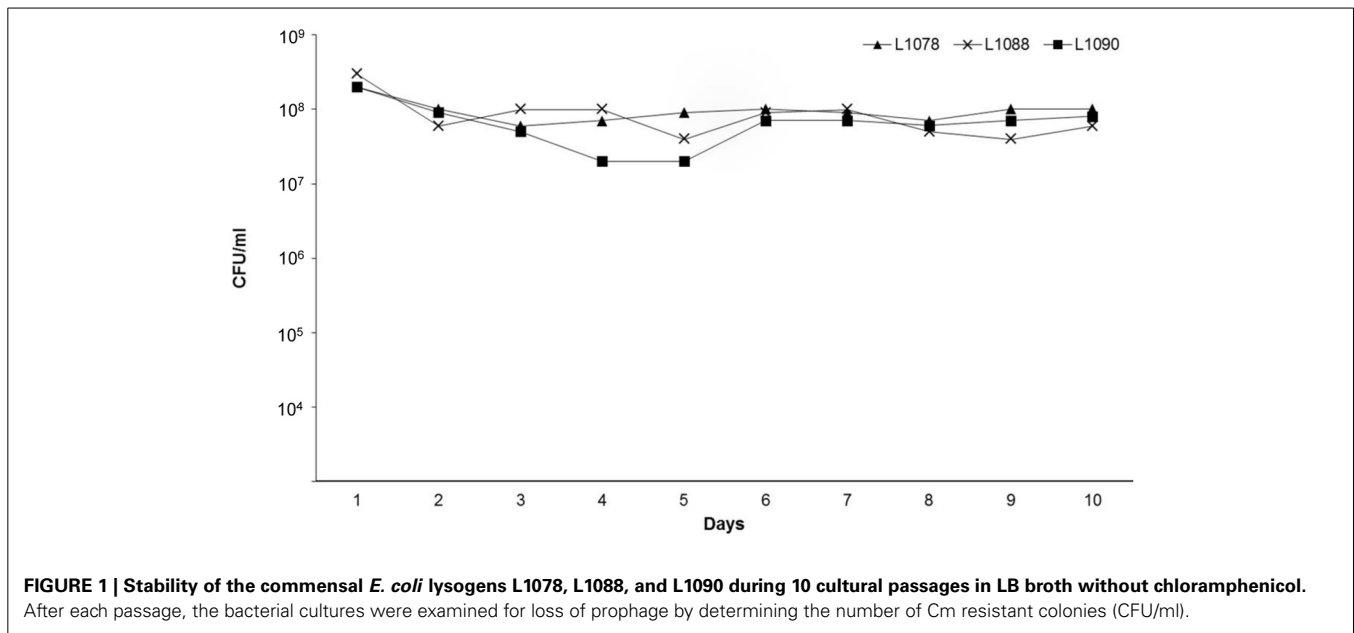
SUSCEPTIBILITY OF COMMENSAL *E. COLI* STRAINS TO LYSOGENIC INFECTION BY THE Stx2-CONVERTING PHAGE ϕ 734 Cm

A total of 15 out of 38 (39%) commensal *E. coli* isolates were susceptible to lysogenic infection by ϕ 734 Cm (Table 3). The number of lysogenic cells recovered varied considerably, from 10 CFU/ml to 10^4 CFU/ml, between the different isolates. Two of the tested isolates (*E. coli* NVH-1078 and *E. coli* NVH-1088) seemed particularly susceptible to the ϕ 734 Cm phage. The bacterial host in which the phage was produced also influenced the lysogenicity, as 8% (3/38) of the commensal isolates were susceptible to lysogenic infection by ϕ 734 Cm propagated on NIPH-11060424 while 18% (7/38) was susceptible to ϕ 734 Cm propagated on C600 when the multiplicity of infection were the same (MOI of 0.005) (Table 3). Within isolates, the number of lysogens increased with increasing phage concentration. The number of strains susceptible to ϕ 734 Cm propagated on C600 increased from 18 to 34% (13/38) when the MOI was increased from 0.005 to 0.5 (Table 3). When the commensal isolates were infected with ϕ 734 Cm, propagated on the commensal lysogenic *E. coli* strain 1090 at an MOI of 0.5, the number of strains susceptible to lysogenic infection increased to 39% (15/38) (Table 3).

The level of Cm resistant colonies remained constant during all cultural passages of the three lysogens (L1078, L1088, and L1090) that were tested for stability (Figure 1). This shows that ϕ 734 Cm was stably maintained in the commensal hosts.

PHAGE PRODUCTION BY ϕ 734 Cm LYSOGENS UNDER NON-INDUCED CONDITIONS AND FOLLOWING TREATMENT WITH MMC OR H₂O₂

The 13 commensal *E. coli* isolates that were susceptible to lysogenic infection by ϕ 734 Cm propagated on *E. coli* C600 were selected for further studies (Table 3). These isolates were tested for phage production during spontaneous (non-induced) prophage induction and after induction with mitomycin C (MMC) or H₂O₂ (Figure 2). There was no difference in phage production between NIPH-11060424 carrying the original ϕ 734 phage and NIPH-11060424 carrying ϕ 734 Cm. Twelve out of 13 commensal lysogens (L1037, L1064, L1066, L1067, L1077, L1078, L1081, L1084, L1086, L1088, L1090, and L1093) produced significantly more phages than NIPH-11060424 under one or more of the tested conditions. The remaining commensal lysogen (L1065) produced less Stx2-phages compared to NIPH-11060424. The differences between non-induced and induced phage production



(either by MMC or H₂O₂) were less than 2 log for all lysogens except L1084, which showed one of the highest MMC induced phage productions (10⁹ phages/ml). The non-induced culture of NIPH-11060424:φ734Cm produced about 2 log less phages than the MMC or H₂O₂ induced cultures, which produced approximately equal numbers of phages. All the commensal *E. coli* lysogens produced more than 10⁴ phages/ml without induction, and L1081 and L1090 produced nearly as much as 10⁸ phages/ml

in the non-induced cultures (Figure 2). Three lysogens (L1065, L1067, and L1086) produced either equal amounts or more phages in the non-induced cultures than in the MMC induced cultures. These lysogens also showed 1–2 log greater phage production after induction with H₂O₂ than with MMC. Prior to the experiments, all the commensal *E. coli* strains were tested for the ability to produce phages after MMC induction by testing the culture filtrates in plaque assay (data not shown). Three of

the commensal stains (NVH-1064, NVH-1077, and NVH-1086) carried MMC inducible phages naturally, of which none were of Stx type. The level of phage production in these strains was negligible ($<10^3$ PFU/ml) compared to the phage production after ϕ 734 Cm infection ($>10^5$). Furthermore, the naturally carried phages formed larger plaques compared to the characteristic pinpoint plaques formed by the Stx2-phages (results not shown) which made them easy to exclude when counting plaques formed by ϕ 734.

PHAGE PRODUCTION AND Stx2 EXPRESSION BY *E. COLI* C600: ϕ 734

While the production of phages was approximately 3 log higher in C600: ϕ 734 than in NIPH-11060424 after MMC induction (Figure 3A), the Stx2 titer indicated that Stx2 production was 40 times higher in *E. coli* C600: ϕ 734 than in NIPH-11060424 (Figure 3B). Western blot analysis of the phage filtrates confirmed the high Stx2 production by C600: ϕ 734 (Figure 3C).

DISCUSSION

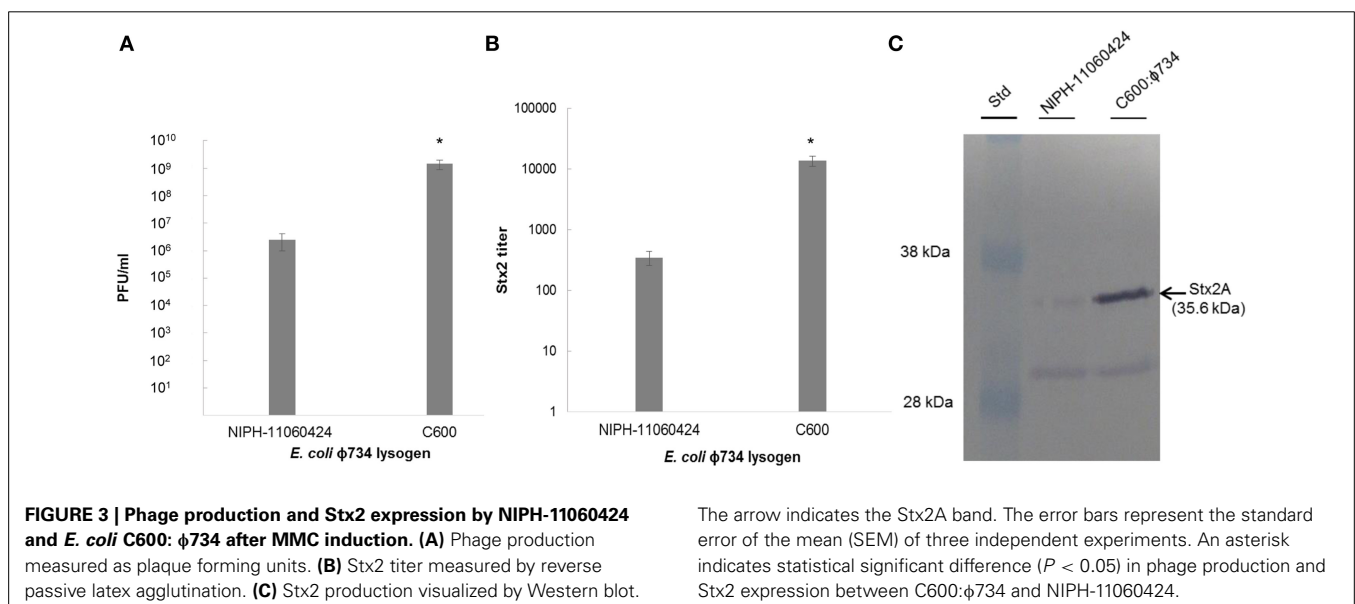
Children are usually more susceptible to EHEC infections and development of HUS than other groups. While some individuals exposed to the bacteria become ill others carry the bacteria asymptomatically, and the reason for this is still unknown. There is increasing evidence that commensal *E. coli* strains infected with Stx2-converting phages can contribute to Stx production in the intestine, and thereby increase the pathogenicity during EHEC infection (Gamage et al., 2003, 2004, 2006; Toth et al., 2003; Cornick et al., 2006). In this report, we provide results which suggest that some commensal *E. coli* have the potential to be significant producers of Stx and could have contributed to the extraordinary pathogenicity of strain NIPH-11060424 during the Norwegian 2006 EHEC outbreak.

We showed that 39% of commensal *E. coli* isolates from children were susceptible to lysogenic infection by a chloramphenicol resistant derivative of ϕ 734. No lytic infection of the commensal *E. coli* isolates was observed which is consistent with the low rate

of lytic infection by Stx2-encoding phages observed in other studies (Schmidt et al., 1999; James et al., 2001; Gamage et al., 2004; Reyes et al., 2012). The lysogenic infection rate observed here is comparable to the rates reported in other studies (Gamage et al., 2004). Gamage et al. (2004) found that 35% of *E. coli* isolates were susceptible to lysogenic infection by the Stx2-converting phage W933. The *E. coli* isolates tested in that study were of both clinical and non-clinical origin from animals and humans, and were therefore distinct from our study population. Recently, Tozzoli et al. (2014) showed that *E. coli* isolates representing the main *E. coli* pathogroups [enterotoxigenic *E. coli* (EPEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAaggEC) and extraintestinal pathogenic *E. coli* (ExPEC)] were susceptible to infection by Stx2-phages. However, in contrast to the commensal *E. coli* isolates studied here, the pathogenic *E. coli* strains were only able to carry the Stx2-phages transiently (Tozzoli et al., 2014).

In accordance with other studies, we observed that an increased MOI resulted in an increased formation of lysogens (Zeng and Golding, 2011). However, we also observed that the strain used for ϕ 734 Cm phage production influenced the susceptibility of the recipient strain to lysogenic infection. When ϕ 734 Cm was produced in either *E. coli* strain C600 or L1090 it seemed to tolerate a broader host range compared to when it was produced in NIPH-11060424 (Table 3).

Phage production by strains NIPH-11060424 and NIPH-11060424: ϕ 734 Cm was very similar under all tested conditions (Figure 2), indicating that replacing *stx2A* with the chloramphenicol resistance gene (*cat*) did not influence the behavior of the phage. The selective marker was convenient in the phage experiments, as it made retrieval of lysogens more feasible, but, the recombinant phage was of course unsuitable in experiments for Stx production. Unfortunately, due to the relatively low infection rate, we were not able to isolate a commensal *E. coli* strain lysogenized by the wild-type ϕ 734 phage. However, we were able to retrieve the ϕ 734 phage in *E. coli* C600 (C600: ϕ 734). This

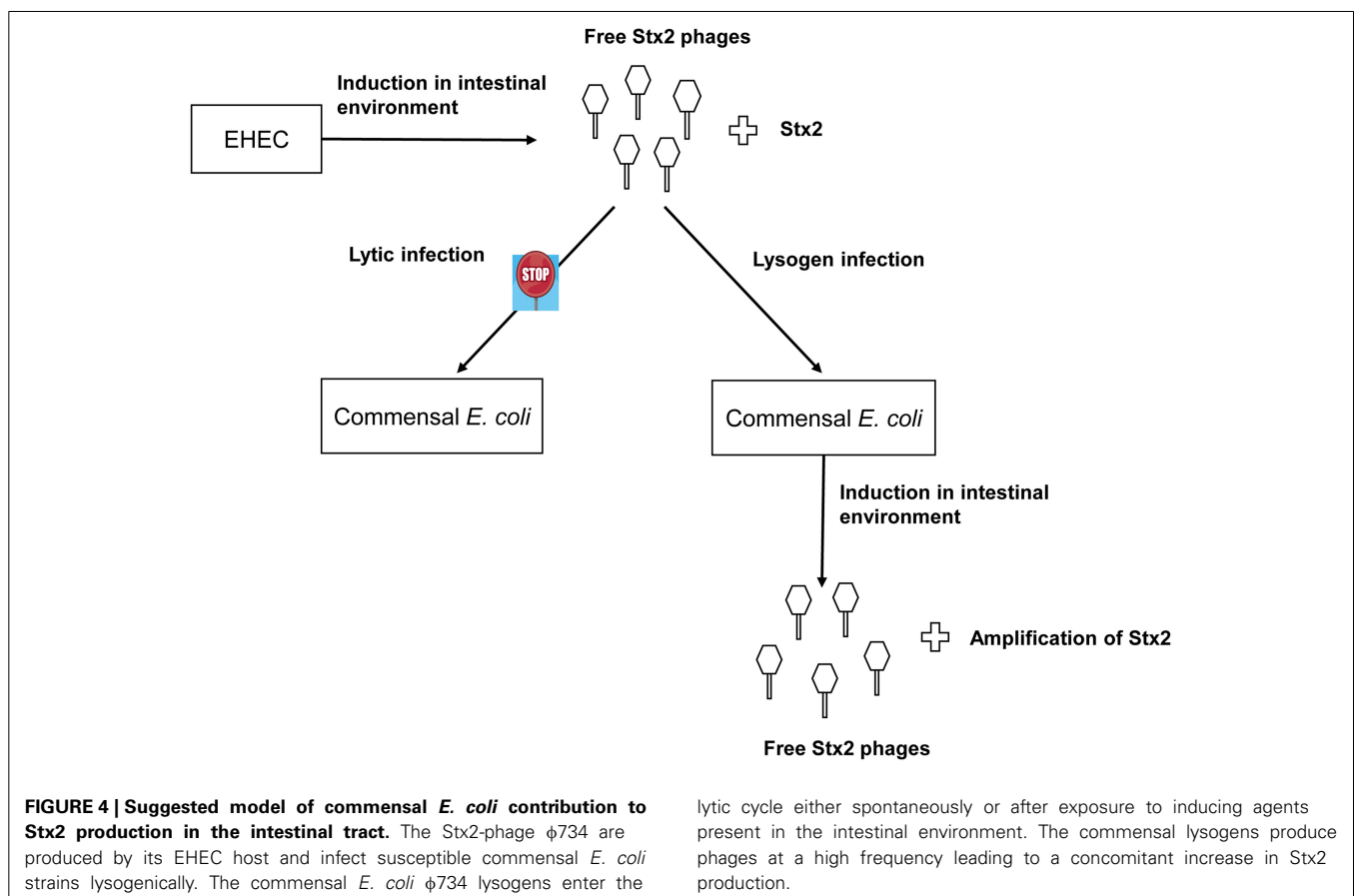


lysogen enabled determination of Stx2 production in another genetic background than the original EHEC outbreak strain. Under the same conditions, *E. coli* C600:φ734 produced about 1000 times more Stx2-converting phage than the original EHEC outbreak strain, and about 40 times more Stx2 (Figure 3). Stx2 measurements could not be done in the commensal *E. coli* lysogens, however, based on the close linkage between phage production and toxin synthesis (Neely and Friedman, 1998; Unkmeir and Schmidt, 2000; Zhang et al., 2000; Wagner et al., 2002) we assume that the number of phage produced in these lysogens will mirror the amount of Stx2 that would have been produced by the native Stx2-converting phage. A similar discrepancy between increased phage-production compared to increased Stx2 production has been shown earlier by Zhang et al. (2000), where ciprofloxacin induction of an O157:H7 strain resulted a 1000 fold increase in phage production while the Stx2 production only increased 58 fold.

The laboratory strain *E. coli* C600 lysogenized with φ734 Cm produced as much as 10^9 PFU/ml under non-induced conditions, which was the highest level of phage production observed during this study (Figure 2). Phage-production in the commensal *E. coli* φ734 Cm lysogens ranged from 10^4 to nearly 10^8 PFU/ml under both induced and non-induced conditions. This means that some commensal *E. coli* produced a considerably higher amount of Stx phage than NIPH-11060424, and also higher levels than EHEC O157:H7 EDL933, which produced about 10^6 PFU/ml

under identical non-induced conditions (Imamovic and Muniesa, 2012). The reason why different *E. coli* strains lysogenized by an identical phage, produce different amounts of phage is not known. However, the amount of phages produced is most probably dependent on the genetic background of the host strain e.g., the regulation of the SOS response and the phage repressor system in each strain will have an impact on phage production.

Since the Stx-prophage induction is closely linked to activation of the bacterial SOS-response and expression of host-encoded RecA protein (Fuchs et al., 1999; Kimmitt et al., 2000), the SOS-response inducing agent MMC is frequently used to activate the phage- and Stx production in EHEC (Fuchs et al., 1999; Schmidt et al., 1999; Muniesa et al., 2004). However, H₂O₂ may represent a more natural inducing agent, as it is produced in the gut as part of the innate immune response (Wagner et al., 2001). Five of the commensal *E. coli* φ734 Cm lysogens demonstrated higher phage production after H₂O₂ induction than after MMC induction. The levels of phage production in the non-commensal isolates NIPH-11060424, NIPH-11060424:φ734 Cm and C600:φ734 Cm were similar after H₂O₂ and MMC induction. The strong inducing capability by H₂O₂ seen in the commensal *E. coli* lysogens may have implications for disease, as H₂O₂ release occurs during *in vivo* EHEC infection (Wagner et al., 2001). Surprisingly, we also observed high production of phage in some of the lysogens under non-induced conditions. Five of the commensal *E. coli* φ734 Cm lysogens produced a higher amount of phage non-induced,



than NIPH-11060424 did under either H₂O₂ or MMC induced conditions.

The observed lack of lytic infection by the ϕ 734 phage in the commensal *E. coli* isolates contrasts the high level of non-induced phage production in the corresponding lysogens. However, together these results indicate that commensal *E. coli* strains might contribute to Stx2 production through first becoming lysogenized and then subsequently enter the lytic cycle at a high frequency (Figure 4). It has previously been reported that spontaneous induction occurs more readily in Stx-phages than in other lambdoid phages (Livny and Friedman, 2004; Aertsen et al., 2005; Shimizu et al., 2009). However, spontaneous induction to the extent observed in this study has, to our knowledge, not previously been reported.

Since efficient Stx production only occurs after prophage induction followed by lysis and death of the host cell, one may expect that EHEC carrying these phages will eventually die out. Recently, Loś et al. suggested that prophages are induced at a low frequency in the gut which does not compromise the persistence of the EHEC population (Loś et al., 2012). There are various repressor systems that interfere with phage production in strains carrying several prophages (Burz et al., 1994; Serra-Moreno et al., 2008). The lower production of phages by NIPH-11060424 compared to strain C600: ϕ 734 and several of the commensal *E. coli* lysogens may result from the presence of repressor systems originating from other prophages in the genome of NIPH-11060424. These repressor systems may act to keep a balance between the lysogenic and lytic infection and thereby benefit the survival of the EHEC population.

In conclusion, we observed that a high proportion of commensal *E. coli* is susceptible to infection by ϕ 734 Cm and that some isolates were infected at a higher frequency than others. The ϕ 734 Cm phage infected the commensal *E. coli* isolates only via the lysogenic pathway. Some of the commensal *E. coli* lysogens produced considerably higher amounts of phage particles than EHEC NIPH-11060424. These lysogens would also likely have produced high levels of Stx2 if they were lysogenized with the original Stx2-converting phage ϕ 734 as modeled in Figure 4. This study supports the hypothesis that Stx2-converting phages are able to infect commensal *E. coli* strains, and thereby enhance Stx2 production during EHEC infection. Together our data strongly endorse that Stx2-converting phages released from EHEC in the gut can lysogenize commensal *E. coli* and turn them into effective Stx producers and thus enhance the pathogenicity of the EHEC infection. Therefore, it would be interesting to examine commensal *E. coli* isolates from asymptomatic EHEC carriers and from EHEC triggered HUS patients for Stx phage susceptibility and for the presence of lysogenic Stx-phages.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the study, and to interpretation and analyses of the data. Hildegunn Iversen did the experiments and drafted the manuscript. Toril Lindbäck assisted in the experiments and in drafting the manuscript. Trine M. L' Abée-Lund, Lotte P. S. Arnesen and Marina Aspholm assisted in drafting the manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fcimb.2015.00005/abstract>

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Supplementary material

Table 1. *E. coli* strains used in the study

| <i>E. coli</i> strains | Serotype | Relevant characteristics | Reference |
|-------------------------------|---------------------|---------------------------------|---|
| NIPH-11060424 | O103:H25 | Norwegian EHEC outbreak, 2006 | (L'Abée-Lund et al., 2012; Schimmer et al., 2008) |
| C600 | <i>E. coli</i> K-12 | laboratory strain | (Appleyard, 1954) |
| DH5 | <i>E. coli</i> K-12 | laboratory strain | (Hanahan, 1985) |
| NVH-1034 | non-O103 | human commensal | This study |
| NVH-1036 | non-O103 | human commensal | This study |
| NVH-1037 | non-O103 | human commensal | This study |
| NVH-1038 | non-O103 | human commensal | This study |
| NVH-1039 | non-O103 | human commensal | This study |
| NVH-1040 | non-O103 | human commensal | This study |
| NVH-1041 | non-O103 | human commensal | This study |
| NVH-1042 | non-O103 | human commensal | This study |
| NVH-1064 | non-O103 | human commensal | This study |
| NVH-1065 | non-O103 | human commensal | This study |
| NVH-1066 | non-O103 | human commensal | This study |
| NVH-1067 | non-O103 | human commensal | This study |
| NVH-1068 | non-O103 | human commensal | This study |
| NVH-1069 | non-O103 | human commensal | This study |
| NVH-1070 | non-O103 | human commensal | This study |
| NVH-1071 | non-O103 | human commensal | This study |
| NVH-1072 | non-O103 | human commensal | This study |
| NVH-1073 | non-O103 | human commensal | This study |
| NVH-1074 | non-O103 | human commensal | This study |
| NVH-1075 | non-O103 | human commensal | This study |
| NVH-1076 | non-O103 | human commensal | This study |
| NVH-1077 | non-O103 | human commensal | This study |
| NVH-1078 | non-O103 | human commensal | This study |
| NVH-1079 | non-O103 | human commensal | This study |
| NVH-1080 | non-O103 | human commensal | This study |
| NVH-1081 | non-O103 | human commensal | This study |
| NVH-1083 | non-O103 | human commensal | This study |
| NVH-1084 | non-O103 | human commensal | This study |
| NVH-1085 | non-O103 | human commensal | This study |
| NVH-1086 | non-O103 | human commensal | This study |
| NVH-1087 | non-O103 | human commensal | This study |
| NVH-1088 | non-O103 | human commensal | This study |
| NVH-1089 | non-O103 | human commensal | This study |
| NVH-1090 | non-O103 | human commensal | This study |
| NVH-1091 | non-O103 | human commensal | This study |
| NVH-1092 | non-O103 | human commensal | This study |
| NVH-1093 | non-O103 | human commensal | This study |
| NVH-1094 | non-O103 | human commensal | This study |

| | | | |
|----------|----------|------------------------|-----------------|
| NVH-37 | O103 | human clinical isolate | This study |
| NVH-40 | O103:H7 | faeces, sheep | This study |
| NVH-317 | O103 | human clinical isolate | This study |
| NVH-660 | O103:H2 | human clinical isolate | This study |
| NVH-661 | O103:H25 | human clinical isolate | This study |
| NVH-662 | O103:H2 | human clinical isolate | This study |
| NVH-676 | O103:H2 | human clinical isolate | This study |
| NVH-677 | O103:H2 | human clinical isolate | This study |
| NVH-679 | O103:H2 | | This study |
| NVH-680 | O103:H8 | isolate from cattle | This study |
| NVH-681 | O103:H2 | isolate from cattle | This study |
| NVH-682 | O103 | | This study |
| NVH-729 | O103:H2 | | This study |
| NVH-735 | O103:H25 | food isolate | This study |
| NVH-806 | O103 | | This study |
| NVH-807 | O103:H2 | CB5500 | BfR, Germany |
| NVH-808 | O103:H2 | CB8086 | BfR, Germany |
| NVH-809 | O103:H2 | CB8956 | BfR, Germany |
| NVH-810 | O103:H2 | CB9286 | BfR, Germany |
| NVH-811 | O103:H2 | CB9469 | BfR, Germany |
| NVH-827 | O103:H25 | meat isolate | This study |
| NVH-829 | O103:H25 | meat isolate | This study |
| NVH-832 | O103:H25 | meat isolate | This study |
| NVH-834 | O103:H25 | meat isolate | This study |
| NVH-835 | O103:H25 | | This study |
| NVH-909 | O103:H21 | isolate from sheep | This study |
| NVH-927 | O103 | isolate from sheep | This study |
| NVH-1018 | O103:H25 | CDC 08-201 | CDC, Atlanta GA |
| NVH-1019 | O103:H25 | CDC 08-202 | CDC, Atlanta GA |

Supplementary material

Table 2. *E. coli* strains susceptible to lytic infection by three different phage filtrates in the spot assay. The number of tested strains is shown parentheses.

| <i>E. coli</i> strain | Phage filtrate | | |
|------------------------|--|---|--|
| | EHEC NIPH-11060424 original outbreak strain | EHEC NIPH-11060424 carrying ϕ 734Cm | <i>E. coli</i> C600 carrying ϕ 734Cm |
| Strain serotype | | | |
| O103 | 22 (30) | 22 (30) | 0 (30) |
| Commensals | 0 (38) | 0 (38) | 0 (38) |
| Non-O103/O157 | | | |

PAPER III

Menadione reduces mitomycin C induction of Shiga toxin 2 production in EHEC

Manuscript

Hildegunn Iversen, Marina E. Aspholm, Erik Ræder, Lotte P. Stenfors Arnesen, Trine L'Abée-Lund and Toril Lindbäck

III

Menadione reduces mitomycin C induction of Shiga toxin 2 production in EHEC

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Manuscript

Abstract

Shiga toxin (Stx), one of the main virulence factors produced by entero-haemorrhagic *Escherichia coli* (EHEC), is responsible for the severe conditions haemorrhagic colitis and haemolytic uremic syndrome. The genes encoding Stx2 are carried by lambdoid bacteriophages, and their expression requires activation of the phage lytic cycle. Induction of the bacterial SOS response seems to be the most efficient way to activate the Stx pro-phage. Several studies have shown that various chemical components as well as factors secreted by the human microbiota and by some probiotic bacterial strains, inhibit Stx synthesis. Here, we demonstrate that menadione (vitamin K3) reduces mitomycin C (MMC) induced Stx2 production in EHEC. High-performance liquid chromatography–tandem mass spectrometric (HPLC-MS/MS) was used to demonstrate that the presence of menadione leads to reduced accumulation of MMC within the EHEC cells. It has previously been shown that treatment of *E. coli* with menadione leads to activation of the MarA SoxS Rob regulon, which promotes

resistance to multiple antibiotics by altering expression of various outer membrane porins (OMPs) and efflux pumps. We show that the presence of menadione leads to altered transcript levels of the OMP encoding genes *ompF* and *ompW*, and the *acrA* and *tolC* genes encoding structural components of the AcrAB-TolC efflux pump in EHEC. We suggest that the altered expression of outer membrane porins and efflux pumps results in a decreased intracellular MMC concentration and a subsequent weaker induction of the SOS-response and thereby a lower Stx2-production.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) causes disease with manifestations ranging from mild diarrhea to severe illness comprising haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Kaper *et al.* 2004). EHEC possesses a range of virulence factors that facilitate infection, and Shiga toxin (Stx) is recognized as the main factor in the development of HUS (Tarr *et al.* 2005).

The Stx encoding genes are located on lambdoid bacteriophages carried in the EHEC genome as pro-phages. Upon induction, the pro-phage switches from the lysogenic state to lytic cycle, accompanied by production of Stx and new phage particles (Waldor and Friedman 2005). DNA-damaging agents such as mitomycin C (MMC) and hydrogen peroxide (H₂O₂), activate the bacterial SOS response and have been shown to be efficient inducers of phage- and Stx-production (Erill *et al.* 2007;Kimmitt *et al.* 2000;Los *et al.* 2010;Los *et al.* 2009). The level of activated RecA protein is increased when the SOS response is initiated in the cell. RecA cleaves the phage repressor CI which leads to a transcription cascade resulting in expression of *stx* and other phage-encoded genes (Fuchs *et al.* 1999;Neely and Friedman 1998).

Menadione is a synthetic naphthoquinone often referred to as vitamin K3. Many of its biological effects may be explained by its redox-cycling properties (Gu and Imlay

2011;Kumagai *et al.* 2012). Redox-cycling compounds are small molecules that can be repeatedly reduced and re-oxidized under physiological conditions. Reduction of menadione, by electrons from NADPH or NADH and the subsequent transfer of these electrons to O₂, generates superoxide anions (O₂^{•-}). It has previously been shown that exposure of *E. coli* to menadione activates the response to oxidative stress (Greenberg and Demple 1989).

MarA, SoxS and Rob are three closely related transcription factors that function in the bacterial adaptation to various environmental threats such as oxidative stress, toxic compounds, acidic pH and antimicrobial peptides (reviewed by (Duval and Lister 2013)). Exposure to a variety of chemical stressors and antimicrobial compounds can lead to activation of MarA, SoxS and Rob (Collao *et al.* 2013;Kwon *et al.* 2000;Li and Demple 1994;Pomposiello *et al.* 2001;Semchyshyn *et al.* 2005;Vasil'eva *et al.* 2001). Several studies have shown that these three transcriptional regulators bind to a degenerate consensus sequence and have considerable overlap in the genes they control. They have been shown to play a key role in multi-drug resistance by controlling various drug efflux pumps and outer membrane porins (Collao *et al.* 2013;Fernandez and Hancock 2012;Greenberg *et al.* 1990;Ma *et al.* 1993). For example, MarA and SoxS regulatory systems have been shown to control expression of *acrC* and *tolC* which encodes structural components of the AcrAB–TolC multidrug efflux system. This system is responsible for resistance against many antibiotics (Randall and Woodward 2002). MarA and Rob have been implicated in the regulation of *ompF* and *ompC*, encoding major outer membrane porins (OMPs) in *E. coli*, which reduced expression has been linked to resistance to multiple antibiotics (reviewed in Duval et al 2013).

Both MarA and SoxS have been shown to be induced by redox-cycling compounds such as menadione and plumbagin (Collao *et al.* 2013;Seoane and Levy 1995). It has been shown that exposure of *E. coli* to menadione activates the SoxRS mediated response to oxidative stress (Greenberg and Demple 1989;Tsaneva and Weiss 1990). In *Salmonella enterica* subsp.

enterica serovar Typhimurium (*S. Typhimurium*), menadione has been shown to activate MarA and SoxS and subsequently induce expression of the minor outer membrane porin OmpW (Collao *et al.* 2013). OmpW has been reported to function in osmoregulation, protection against oxidative stress and in resistance against host innate immune defence (Gil *et al.* 2007; Wu *et al.* 2013; Xu *et al.* 2005). Greenberg *et al.* (1990) reported that less OmpF protein was expressed in the presence of the redox cycling drugs paraquat and menadione.

MMC is a DNA damaging compound and a potent inducer of the bacterial SOS response and has therefore been widely used as a pro-phage inducing agent in scientific experiments (Raya and Hebert 2009). Previous studies have demonstrated that activation of *soxS* and *rob* is associated with increased resistance to MMC (Wei *et al.* 2001).

The present study is based on the hypothesis that pre-exposure of EHEC to menadione inhibits *stx2* expression by reducing the accumulation of MMC within the bacterial cells probably by reduced cell membrane permeability and by active efflux.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 1. The EHEC strains were grown in BHI (Oxoid Limited, Basingstoke, UK) at 37 °C with agitation (225 rpm). Menadione sodium bisulfite (Sigma Aldrich, St. Louis, MO) was used at a concentration of 20 µg/ml. Mitomycin C (MMC) (Sigma Aldrich, 0.5 µg/ml) was used to induce the SOS response in the cultures at OD₆₀₀ = 0.5 and the induced cultures were incubated in darkness.

Semi-quantification of Stx2 levels by VTEC RPLA kit

The VTEC RPLA toxin detection kit (Oxoid Limited, Basingstoke, UK) was used to determine Stx2 production in culture supernatants. The assay was performed according to the manufacturer's instruction. The cultures were induced by MMC at $OD_{600} = 0.5$ and the samples were harvested 4 hours after induction. The amount of toxin in each test-well was reduced 2-fold at each dilution. The reciprocal of the highest dilution causing latex agglutination was considered the titer. Statistics were performed using Student's *t*-test. A *p*-value < 0.05 was considered significant.

qPCR

qPCR was used to quantify the mRNA levels of *recA* and *stx2A* in MMC induced cultures two hours after induction after growth in BHI in the presence and absence of menadione. The mRNA levels of *acrB*, *tolC*, *ompF* and *ompW* in EHEC were determined from bacteria harvested at $OD_{600}=0.5$ after growth in BHI with and without menadione. Culture samples were mixed with methanol and kept at $-80\text{ }^{\circ}\text{C}$ before isolation of RNA. Total RNA was extracted using the Purelink RNA mini kit (Life technologies, Carlsbad, USA) and the DNA was removed using the Turbo DNA-free kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity (A_{260}) and purity ($A_{260/280}$) of purified RNA were measured in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and an Agilent 2100 bioanalyzer was used to assess the RNA quality. cDNA was synthesized from 500 ng RNA using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions.

mRNA levels for each gene were determined relative to the reference gene *gapA* (glyceraldehyde-3-phosphate dehydrogenase) by qPCR. Five microliters of a 1:100 dilution of the cDNA reactions were used as templates for qPCR amplification in 25 μl final volumes containing 12.5 μl of EXPRESS SYBR GreenER qPCR Supermix, with premixed ROX

(Invitrogen) and a primer concentration of 200 nM. The primers used for qPCR are listed in Table 2. qPCR amplification was done using a StepOne system (Applied Biosystems). The thermal cycling conditions were 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C, and 1 minutes at 60°C. Fluorescence was recorded during each extension phase, and a melting curve analysis was carried out after each run to verify the amplification of specific transcripts. Each assay was performed on three biological replicates and included triplicate PCR of the samples, negative no-template controls, and the reference gene. The slope of the standard curve and PCR efficiency for each primer pair were obtained by amplifying serial dilutions of the target sequence. The results were analyzed using Pfaffl method (Pfaffl 2001).

LC-MS/MS quantification of intracellular MMC

Cytoplasmic extracts were prepared from EHEC grown in BHI with and without menadione (20 µg/ml) at 37°C until $OD_{600}=0.5$. The cultures were then induced with MMC (0.5 µg/ml) for 20 min. 1.5 ml samples were harvested (18000 rcf for 1 min.) and washed twice in BHI. Prior to the last wash samples were diluted and plated on BHI agar for enumeration. The washed bacteria were lysed in 150 µl acetonitrile (100 %), and cell debris was removed by centrifugation for 15 min. at 18000 rcf. The supernatants were filtered through Amicon Ultra Centrifugal filters 30k (Millipore), diluted four times in acetonitrile - water (10/90 v/v) and aliquots of 5 µL were analyzed for MMC with LC-MS/MS as described previously (B'Hymer *et al.* 2015). The instrumentation used was an Agilent 1200 SL HPLC system equipped with an Agilent G6460 triple quadrupole mass spectrometer with an electrospray ion source, and the separation was done on an Agilent Zorbax Rx C18 column, 150 x 3.0 (ID) mm with 3.5 µm particles. Calibration standards were prepared with filtered cell extract matrix and diluted four times in acetonitrile - water (10/90 v/v) to final MMC concentrations of 0, 2, 5, 10, 50,

100 ng/ml. The calibration curve was forced through zero and was linear with correlation coefficient above 0.99.

RESULTS

Effects of Menadione on EHEC growth

To examine whether menadione affects growth, EHEC strains NIPH-11060424 and EDL933 were grown in presence or absence of menadione (Figure 1A). A concentration of 20 µg/ml menadione was used in further experiments as this concentration did not exert any inhibition of the growth of bacteria. None of the two strains were significantly affected by the tested concentration of menadione (Figure 1A). However, a dramatic drop in OD₆₀₀ was observed when cultures were induced with MMC at OD = 0.5 (Figure 1B). The drop in OD₆₀₀ is a result of cell lysis caused by the induction of the SOS-response activating the Stx2-phage followed by the production of toxin and phage particles and the expression of lysis genes. Notably, the cultures containing menadione were less prone to lysis after MMC induction (Figure 1B).

Menadione reduces MMC induced *stxA* and *recA* transcription and Stx2 production

In the presence of menadione, the expression of *stx2A* mRNA was reduced about 2-fold in NIPH-11060424 and almost 10-fold in EDL933 (Figure 2A). Quantification of Stx2 production revealed that both EHEC-strains produced about 4-fold less Stx2 in the presence of menadione (Figure 2B).

The level of *recA* mRNA in NIPH-11060424 and EDL933 was reduced about 2- and 6-fold, respectively, in presence of menadione (Figure 2A), suggesting that menadione affects induction of the SOS response since activation of SOS-response is accompanied by an increase in *recA* levels (Erill *et al.* 2007).

Menadione influences the intracellular MMC level

To investigate the possibility that menadione decreases the intracellular accumulation of MMC, and thereby leads to a weaker SOS response, the intracellular concentration of MMC was determined by LC-MS/MS. EHEC cultured in presence of menadione demonstrated a 57 % reduction in cytoplasmic MMC concentration compared to cultures without menadione (Table 3). For EHEC NIPH-11060424 the concentration was decreased 29 % in the presence of menadione compared to cultures without menadione. These data suggest that the presence of menadione results in MMC resistance in EHEC strains.

Menadione alters expression of genes encoding outer membrane proteins and efflux pumps in EHEC

To elucidate whether menadione prevents accumulation of MMC within the bacterial cells by altering the expression of efflux pumps and outer membrane porins, the effect of menadione on the mRNA levels of *acrB*, *tolC*, *ompF* and *ompW* was determined (Table 4). *ompF* was most affected by menadione, and demonstrated a more than 5-fold reduction in transcription level in EDL933 compared to cultures without menadione. Menadione treatment also resulted in about 3- and 2-fold increase in transcription levels of *acrA* and *tolC*, respectively. A 3-fold increase in the transcription level of *ompW* was also observed. In NIPH-1106424, similar changes in mRNA levels were observed, however the down-regulation of *ompF* was about 3-fold and *ompW* was up-regulated almost 6-fold.

Discussion

In this study, we demonstrate that menadione reduces Stx2 production in MMC-induced EHEC by reducing accumulation of MMC within the bacterial cells. We show that genes encoding efflux pumps and outer membrane porins including *acrA*, *tolC*, *ompF* and *ompW*, were regulated by the presence of menadione, and propose that the reduced intracellular concentration of MMC was due to altered membrane permeability and active efflux of MMC out of the EHEC cells.

Several studies throughout the years explored the effect of menadione on bacterial cells since the activity of menadione induces the bacterial response to oxidative stress (Greenberg *et al.* 1991; Greenberg and Demple 1989; Tamarit *et al.* 1998). The effect of menadione on transcription of the MarA SoxS Rob regulon results in increased bacterial resistance to many different cell damaging compounds. In this study, we observed a reduction in the intracellular MMC concentration when EHEC strains EDL933 and NIPH-11060424 were grown in presence of menadione. The observed reduction in the intracellular level of MMC suggests less influx and/or more efflux of MMC in the menadione treated EHEC cells and this is supported by the altered expression of efflux pumps and outer membrane porins measured in the menadione treated cells. The observed down-regulation of *ompF* in EHEC in response to menadione treatment is consistent with the results by Greenberg *et al.* 1990. OmpF is one of the larger non-specific porins of *E. coli*, and has been found to be important regarding the influx of antibiotics (Cohen *et al.* 1989; Jaffe *et al.* 1982; Kishii and Takei 2009; Tavio *et al.* 1999).

The up-regulation of *ompW* may also have contributed to the lower MMC concentration in the cells. In several studies, OmpW has shown to contribute to bacterial resistance to toxic compounds. It has been implicated in the efflux of paraquat in *S. Typhimurium* and in efflux of toxic quaternary cationic compounds in *E. coli* (Beketskaia *et al.* 2014; Gil *et al.* 2007). A ceftriaxone-resistant *S. Typhimurium* strain demonstrated decreased expression of *ompW*,

suggesting that OmpW could be involved in the uptake of this antibiotic (Hu *et al.* 2005). Thus, both increased and decreased levels of OmpW seem to contribute to resistance to toxic agents present in the surroundings. However, the detailed function of OmpW in promoting resistance to toxic compounds and the regulatory network affecting *ompW* expression is still partly unknown (Hu *et al.* 2005;Nandi *et al.* 2005;Xu *et al.* 2006).

The up-regulation of *ompW* observed in our study was considerably smaller than the menadione-induced up-regulation of *ompW* previously observed in *S. Typhimurium* (20 fold) (Collao *et al.* 2013). The different menadione concentrations used in these studies (20 µg/ml for EHEC versus 50 µg/ml for *S. Typhimurium*) may have contributed to the observed differences in expression levels. In addition, the patterns of OMP gene expression may differ between species.

The resistance-nodulation-cell division (RND) superfamily of efflux pumps is one of four important efflux pumps in Gram-negative bacteria used to actively transport chemical compounds out of the bacterial cell and the pumps also play an important role in the bacterial defense against toxic compounds (reviewed by (Fernandez and Hancock 2012)). The substrates of RND pumps comprises a great variety of substances such as antibiotics, biocides, bile salt, aromatic hydrocarbons, detergents and dyes. The best characterized RND pump in *E. coli* is the AcrAB-TolC efflux pump of multiple antibiotics, dyes, bile salts and detergents. In this study we observed an up-regulation of *acrA* and *tolC* expression in response to menadione. Tavio *et al.* (2010) studied two *in vitro* selected multidrug resistant *E. coli* mutants and reported that *tolC* and *acrB* were 3-6 times up-regulated in the mutants. They also observed a difference in MMC susceptibility between the mutants and their parental strains. This reinforce that AcrAB-TolC is involved in efflux of MMC, although several other genes belonging to the *marA/soxS/rob* regulon could be involved as well.

Unidentified factors secreted by the human gut microbiota have been shown to inhibit *recA* expression and Stx2 production in EHEC (Carey et al. 2008; de Sablet et al. 2009; Iversen *et al.* 2015). In this study we observed a reduced expression of *recA* in presence of menadione, however, we believe that the lower *recA* transcription is a consequence of the reduced intracellular concentration of MMC followed by a weaker induction of the SOS response. Considering that most bacteria produce vitamin K homologs and other inducers of oxidative stress, it is possible that factors secreted by the intestinal microbiota may influence the pathogenicity of EHEC in the gut by altering permeability of the membrane and active efflux activity and thereby the sensitivity towards pro-phage inducing agents.

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Table 1. Bacterial strains used in this study

| EHEC | Serotype | Characteristics | Reference |
|---------------|----------|-------------------|-------------------------------|
| NIPH-11060424 | O103:H25 | <i>stx2</i> | (Schimmer <i>et al.</i> 2008) |
| EDL933 | O157:H7 | <i>stx2, stx1</i> | (Perna <i>et al.</i> 2001) |

Table 2. Primers used in this study

| PRIMER SEQUENCES | | | | | |
|------------------|-----------------------|------------------------|--------------------|--------------------|--|
| Gene | Forward (5' to 3') | Reverse (5' to 3') | Slope ^a | % Eff ^b | |
| <i>stx2</i> | GAACGTTCCGGAATGCAAA | CCATTAACGCCAGATATGATGA | -3.4 | 98 | |
| <i>recA</i> | TTGACCTGGGCGTAAAAGAG | CGGTTTCCGGGTTATCTTTC | -3.1 | 90 | |
| <i>gapA</i> | AGGTCTGATGACCACCGTTC | AACGGTCAGGTCAACTACGG | -3.3 | 99.7 | |
| <i>ompF</i> | AAGGCGCTGACGCTCAAA | CGTATTTAAGACCCGCGAATG | -3.9 | 80 | |
| <i>ompW</i> | AGTGCCTTTGCGCATGAAG | TGGACGTACGGTTGCAGAAC | -3.3 | 98.4 | |
| <i>tolC</i> | TGCCGCCGATCGTGAT | TGGACTGCGCGCTTCAT | -3.4 | 95.4 | |
| <i>acrA</i> | CGACAGTGCGAAAGGTGATCT | CCGTCAATTGCGCGATATT | -3.4 | 96.2 | |

^aThe slope was calculated from the regression line in the standard curve

^bThe efficiency was calculated using the slope of the regression line in the standard curve

Table 3. Reduction of MMC levels in menadione-treated EHEC-strains.

| Bacterial strain | Intracellular levels of MMC (ng/ml) ^a | Reduction of MMC levels |
|---------------------------|--|-------------------------|
| EDL933 | 28 | NA |
| EDL933 w/menadione | 12 | 57 % |
| NIPH-11060424 | 7 | NA |
| NIPH-11060424 w/menadione | 5 | 29 % |

NA – not applicable

^aThe numbers are average of three independent biological replicates

Table 4. Effect of menadione on expression of diffusion porins and efflux pump encoding genes. Differences in transcription between cultures with and without menadione are expressed as fold change in mRNA level.

| Gene | NIPH-11060424 | EDL933 | Regulation |
|-------------|---------------|--------|----------------|
| <i>ompF</i> | -3.3 | -5.3 | Down-regulated |
| <i>ompW</i> | 5.9 | 3.1 | Up-regulated |
| <i>acrA</i> | 3.0 | 2.7 | Up-regulated |
| <i>tolC</i> | 3.0 | 2.0 | Up-regulated |

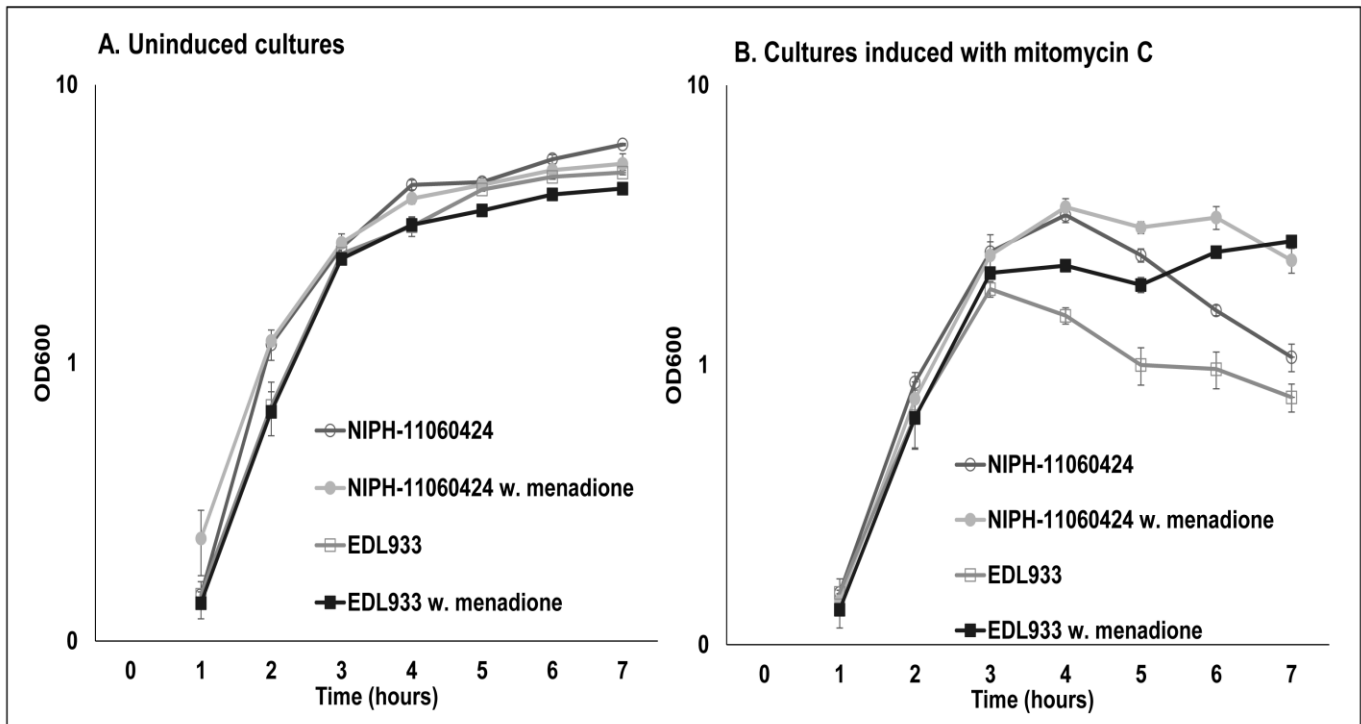


Figure 1. Kinetics of EHEC growth. A. Growth curves of EHEC NIPH-11060424 and EDL933 cultured in BHI medium with and without menadione (20 $\mu\text{g/ml}$). B. Growth curves of EHEC NIPH-11060424 and EDL933 cultured in BHI medium with and without menadione and induced with MMC at $\text{OD}_{600}=0.5$. Data represent the mean \pm SEM of three independent experiments.

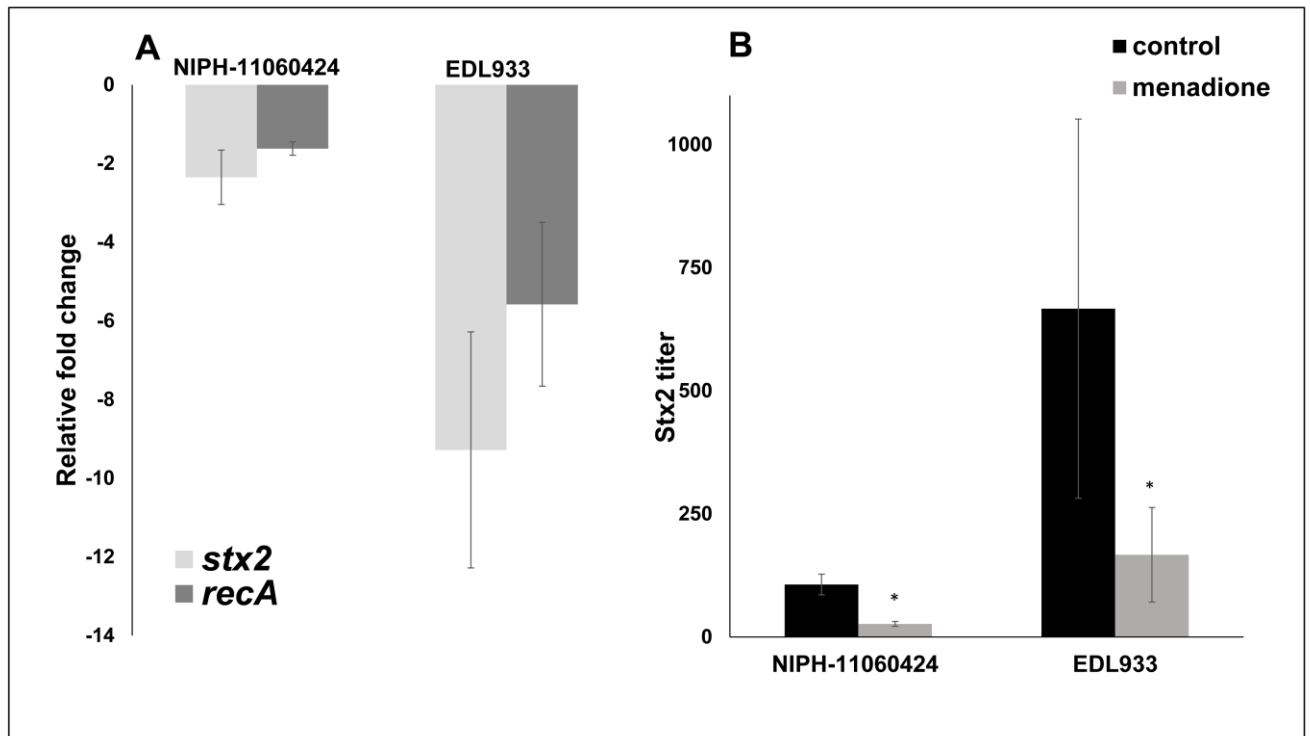


Figure 2. Effect of menadione on *stx2* and *recA* expression and Stx2 production.

A. Relative fold change in transcript levels of *stx2* and *recA* in menadione treated EHEC cultures compared to untreated cultures. Data represent the mean \pm SEM of three individual experiments. B. Stx2 production in menadione treated EHEC samples compared to control samples. Data represent the mean \pm SEM of three independent experiments. * $P < 0.05$ cultures with menadione vs. without menadione.