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CHARACTERIZATION OF *ESCHERICHIA COLI* COLONIZING THE GASTROINTESTINAL TRACT AND URINARY TRACT CATHETERS

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To my family,

ABSTRACT

Commensal and pathogenic bacteria express biofilm behaviour which is thought to alter bacteria-host interactions and contribute to colonization and persistence. In the current thesis, the epidemiology of biofilm formation in gastrointestinal commensal *Escherichia coli* and *E. coli* isolated from urinary catheters is described. Further on, in selected commensal isolates the impact of biofilm formation on the interaction with a gastrointestinal epithelial cell line is investigated. The contribution of selected genes to biofilm formation of *E. coli* on catheter surfaces is investigated.

Gastrointestinal commensal *E. coli* were commonly able to express a biofilm behaviour (adherence, pellicle formation and clumps) which was characterized by the expression of the extracellular matrix components curli fimbriae and cellulose as it can be assessed by a distinct colony morphotype on Congo Red agar plates. Expression of the exopolysaccharide cellulose and curli fimbriae was positively regulated by the master regulator of biofilm formation, the transcriptional regulator CsgD, in the commensal strain TOB1. However, in the probiotic *E. coli* strain Nissle 1917, cellulose production was uncoupled from CsgD expression.

The biologic roles of cellulose and curli fimbriae were investigated by studying the interactions between commensal bacteria and the human gastrointestinal epithelial cell line HT-29. Curli fimbriae expressed by the commensal strain TOB1 enhanced adherence to, internalization by and IL-8 production of the cell line HT-29. Expression of cellulose, which is co-expressed with curli fimbriae in TOB1 at 37 ºC diminished adherence, internalization and IL-8 production. The role of cellulose is different in Nissle 1917. Although expression of cellulose by Nissle 1917 at 37 ºC also suppressed internalization by HT-29 cells, cellulose production had a positive effect on adherence of Nissle 1917 to intestinal epithelial cells and the production of IL-8 in response to the bacteria.

Most bacteria isolated from urinary tract catheters had the capability to adhere to polystyrene surfaces in laboratory medium. However, adherence was highly dependent on environmental conditions and changed with the culture media and the surface. Urine as growth medium generally leads to a decrease in adherence. Bacteria adhered less to the surface of silicone catheters than silicone coated latex catheters. A species dependent pattern of adherence was also observed. *Pseudomonas* spp. isolates adhered more to the catheter surface than isolates of the other species. Adherence by *E. coli* strain was correlated with presence of the *fluA* gene, expression of rough LPS and type 1 fimbriae, but not with the expression of cellulose and curli fimbriae.

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CONTENTS

1 Introduction 1
1.1 Commensal gastrointestinal flora 1
1.1.1 Characteristics of commensal flora 1
1.1.2 Commensal *Escherichia coli* 1
1.1.3 Nissle 1917 – a probiotic *E. coli* strain 2
1.2 The intestinal mucosa 2
1.2.1 Composition and function of intestinal mucosa lining 2
1.2.2 Bacteria-mucosa interaction 3
1.3 Urinary tract infection 4
1.3.1 Urinary tract infection 4
1.3.2 Catheter associated urinary tract infection 4
1.4 Bacterial typing methods 4
1.4.1 Serotyping 5
1.4.2 Phylogenetic typing by PCR analysis 5
1.4.3 Typing by pulsed-field gel electrophoresis 5
1.5 Biofilm 5
1.5.1 Biofilm formation by commensal *E. coli* 6
1.5.2 Biofilm formation by uropathogenic *E. coli* 6
1.5.3 Extracellular structures in biofilm development 7
1.5.4 Cyclic-di-GMP in biofilm formation 10
1.5.5 Rdar morphotype system 11
1.5.6 Virulence factors in commensal and uropathogenic *E. coli* 12
1.6 Bacterial-host interaction 13
1.6.1 Adherence and internalization 13
1.6.2 Host immune response 13
2 Aims of The Thesis 14
3 Material and Methods 15
3.1 Patient populations 15
3.1.1 Study I 15
3.1.2 Study IV 15
3.2 Laboratory methods 15
3.2.1 Bacterial strains and growth conditions 15
3.2.2 Molecular typing methods 16
3.2.3 Phylogenetic typing 16
3.2.4 Pulsed-field gel electrophoresis typing 17
3.2.5 Detection of virulence factors 17
3.2.6 Morphotype screen 17
3.2.7 Knock-out of genes and cloning 18
3.2.8 Assessment of adherence 18
3.2.9 Internalization assay 19
3.2.10 IL-8 activation by *E. coli* 19
3.2.11 Assessment of biofilm formation 19
3.2.12 Biofilm formation on catheters in urine medium 19
4 Results and Discussion 21
4.1 Study I 21

4.1.1 Commensal strains express curli fimbriae and cellulose 21
4.1.2 Expression of curli fimbriae tends to be at 28°C................21
4.1.3 Morphotype can be an indicator of biofilm formation........22
4.1.4 Role of curli fimbriae and cellulose in biofilm formation ..22
4.1.5 General characterization of the commensal strains............23
4.2 Study II..23
4.2.1 Role of cellulose and curli fimbriae in adherence..............23
4.2.2 Role of cellulose and curli fimbriae in internalization........24
4.2.3 Invasion capacity of commensal *E. coli* isolates................24
4.2.4 Role of cellulose and curli fimbriae in inducing IL-8.........24
4.2.5 Flagellin requires curli fimbriae for IL-8 response 25
4.2.6 IL-8 response triggered by commensal isolates of *E. coli* ..26
4.3 Study III 26
4.3.1 Nissle 1917 produces cellulose and curli fimbriae.............26
4.3.2 Curli fimbriae, but not cellulose is regulated by CsgD.......26
4.3.3 Regulation of cellulose biosynthesis by c-di-GMP............26
4.3.4 Interaction between Nissle 1917 and HT-29 cells..............27
4.4 Study IV and preliminary results: 27
4.4.1 Characterization of bacteria colonizing urinary catheters...27
4.4.2 Bacteria constituting biofilm on urinary catheters 28
4.4.3 Time of catheterization impacts recovered species............28
4.4.4 Visualization of biofilm formation on urinary catheters.....28
4.4.5 Biofilm and adherence formation 29
4.4.6 Influence of urine on adherence to polystyrene surface......29
4.4.7 Influence of urinary catheters on biofilm formation 29
4.4.8 Biofilm formation by *E. coli* isolates from catheters 31
5 Conclusions: 33
6 Acknowledgements 34
7 References...37

LIST OF ABBREVIATIONS

Ag43	Antigen 43
bas	brown and smooth
bdar	brown, dry and rough
CAUTI	Catheter-Associated Urinary Tract Infection
CR	Congo Red
c-di-GMP	bis-(3'-5')-cyclic dimeric guanosine monophosphate
IL-8	Interleukin 8
LB	Luria-Bertani
LPS	Lipopolysaccharide
PAMPs	Pathogen-Associated Molecular Patterns
pas	pink and smooth
PCR	Polymerase Chain Reaction
pdar	pink, dry and rough
PFGE	Pulsed-Field Gel Electrophoresis
PRRs	Pattern Recognition Receptors
ras	red and smooth
rdar	red, dry and rough
saw	smooth and white
SC	Silicone Catheter
SCLC	Silicone Coated Latex Catheter
TLR	Toll-Like Receptor
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infection

1 INTRODUCTION

1.1 COMMENSAL GASTROINTESTINAL FLORA

1.1.1 Characteristics of commensal flora

"Commensal", originates from the Latin word "commensalis," which means "at the table together". It generally refers to a relationship between two organisms which coexist without imposing detrimental effects on each other, but also without having obvious benefits. The gastrointestinal tract of a healthy human being is colonized by a highly diversified community of commensal microorganisms, mainly bacteria, but also archae and eukarya. In total number of cells, the microbial population exceeds the number of cells in the body by a factor of 10 and consists of more than 500 bacterial species [1-3].

As going distal, the number and diversity of bacteria that colonizes the gastrointestinal tract increases. Whereas 10^1 to 10^3 colony forming units per ml are found in the stomach and the proximal small intestine (duodenum), the bacterial number increases in the distal small intestine and reaches 10^{11} to 10^{12} colony forming units per ml in the large intestine (colon) [4]. The majority of the bacteria in the large intestine are anaerobes whereby anaerobic bacteria outnumber aerobic bacteria by a factor of 100 [5].

The gastrointestinal tract of the neonate is sterile, but is colonized within hours by bacteria acquired from the mother and environmental sources [6]. The commensal bacteria enter the gastrointestinal tract through the mouth either with food or drink, or are transferred by direct person-to-person contact.

Although called commensals, the community of microorganisms in the gastrointestinal tract elicits important functions that benefit its host. Thereby, the beneficial effects of the gastrointestinal flora go beyond the gastrointestinal system as the microorganisms contribute to organ development and the development of the immune system. In the gastrointestinal tract the gut flora contributes to the development of the mucosa by e.g. tightening the epithelia barrier. The gut flora has also metabolic functions as it synthesizes vitamins and processes dietary residues. Last, but not least, the microbial flora has a protective function as it provides a natural defence barrier against invading pathogenic organisms [7] [2] [4].

1.1.2 Commensal *Escherichia coli*

Escherichia coli is a Gram-negative, facultative anaerobic bacterium which is about 2 µm long and 0.5 µm wide. Phylogenetically, *E. coli* belongs to the γ- proteobacteria. Together with bifidobacteria, *E. coli* and other enterobacteria are the first colonizers of the gastrointestinal tract of newborns [6]. After establishment of a stable microbial population, *E. coli* is a minor component of the commensal flora, however, with up to 10^8 cells/ml it reaches significantly high concentrations [8, 9]. Those high absolute numbers make the gastrointestinal tract a major source for spread of potentially pathogenic *E. coli* to susceptible sites [10, 11]. Besides being a commensal bacterium, *E. coli* is also a major pathogen of humans [12]. When a group of *E. coli* isolates can cause a distinct disease, this group of E coli isolates is called a pathovar. A pathovar of

E. coli harbours a distinct set of virulence factors that makes the isolates capable to cause a distinct disease phenotype, e.g. the urinary tract infection (UTI) [13]. Commensal *E. coli* do generally not expressing virulence factors. On the other hand, if the commensal flora is out of balance or the host is immunocompromised, translocation of predominant *E. coli* over the gut epithelial cell lining can occur [14] and cause sepsis in susceptible individuals [15-17]. Predominance of *E. coli* is also observed in early and chronic ileal lesions of Crohn´s disease, an inflammatory disease of the bowel [18, 19].

1.1.3 Nissle 1917 – a probiotic *E. coli* strain

According to the definition, probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [20]. Probiotics are applied with the aim to protect the host against invading pathogens, to change the composition of the residual microflora to suppress the production of toxic compounds and to stimulate or regulate the immune system. Probiotics protect the host against pathogens by, e.g. interference with both the growth and the virulence mechanisms of pathogens [21-27].

Nissle 1917 is a probiotic *E. coli* strain, which was discovered in World War I since it protected an individual against infectious gastroenteritis [28], but isolates of Nissle 1917 have also been found recently in populations of pigs [29].

Nissle 1917 exerts adverse effects against a range of gastrointestinal pathogens such as *Salmonella*, *Candida albicans*, *Yersinia enterocolitica*, *Shigella flexneri*, *Listeria monocytogenes* and adherent-invasive *E. coli* [30-32] In practical terms, Nissle 1917 has been reported to protect the intestine of newborn infants against colonization by microbial pathogens [33]. The mechanisms how Nissle 1917 protects against pathogens are unknown, however contact with the epithelial cells is not required [30].

Besides protection against infections with intestinal pathogens, Nissle 1917 has been proposed to counteract chronic inflammatory conditions. Consequently, Nissle 1917 has been proposed for the therapy of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis [34-36]. It has been shown that Nissle 1917 elicits a distinct immune response in gastrointestinal epithelial cells through the production of flagellin as it e.g. induces the proinflammatory cytokine IL-8 and stimulates production of the human antimicrobial peptide β-defensin 2 (hBD-2) [37]. In addition, Nissle 1917 demonstrates immunomodulatory properties as it regulates central T cell function in different cell culture models resulting in an upregulation of the secretion of regulatory cytokines, in reduced secretion of proinflammatory cytokines and apoptosis of T cell subpopulations [37-44]. Since Nissle 1917 shows high adherence to host cells *in vitro* [31] this feature can contribute to the colonization of the gastrointestinal tract *in vivo*.

1.2 THE INTESTINAL MUCOSA

1.2.1 Composition and function of intestinal mucosa lining

The mucosa or mucous membrane is the innermost layer of the gastrointestinal tract, directly surrounding the lumen. It consists of an epithelium, a lamina propria, and a muscularis mucosa [45].

The epithelium consists of a single layer of epithelial cells. The epithelium provides not only the barrier between the 'outer world' and the body milieu, but is also the route for absorption of nutrients and selective exchange of information. In general, epithelial cells are responsible for absorption, protection, transcellular transport, sensation detection, and selective permeability and secretion. These functions are mediated by differentiated cell types such as the enterocytes, which perform absorption and the goblet cells, which secrete mucus.

The lamina propria is a layer of connective tissue that contains blood and lymph vessels, nerve cells and cells of the immune system. The muscularis mucosa is a layer of smooth muscles which keeps the mucosa in a constant state of agitation which facilitates the contact with the lumen

1.2.2 Bacteria-mucosa interaction

One of the important functions of the mucosa with respect to the commensal microorganisms in the lumen is to sense and respond to the presence of bacteria (commensal and pathogenic) and prevent bacterial overgrowth. Enterocytes, which have long been considered to be immunological inert, can sense the presence of bacteria and secrete chemokines and cytokines in response. M cells, specialized sampling cells in the epithelium, and dendritic cells collect bacteria for further processing of antigenic components [46]. Specialized cells such as Paneth cells exist in the small intestine and secrete antimicrobial agents such as the antimicrobial peptides as defensins and lysozyme [47]. Antimicrobial agents are also present in the mucus layer overlaying the epithelium, which prevents the direct contact between the commensal flora and the epithelial cell layer.

It is considered that *E. coli* stays in mucus layer [48-50].However, certain strains of *E. coli* manage to adhere to or invade mucosal cells [51]. Some pathogenic *E. coli* invaded colonic mucosal cells in a manner similar to *Shigella* spp. The ability of the invasion *in vivo* is paralleled by their ability to invade cultured cell lines *in vitro* [52, 53].

The intestinal mucosa must rapidly recognize detrimental pathogenic threats in the lumen and initiate adequate immune responses, but maintain hyporesponsiveness to the omnipresent commensals. Hereby, pattern recognition receptors (PRRs) allow immunosensory cells to discriminate between "self" and microbial "non-self". This discrimination is based on the recognition of broadly conserved molecular patterns on microbes such as cell membrane or cell wall components and specific patterns in the DNA [54]. A class of transmembrane PRRs called Toll-Like Receptors (TLRs) play a key role in microbial recognition, induction of antimicrobial and proinflammatory genes, and the control of adaptive immune responses. Up to now, 10 human TLRs have been identified which recognize a broad spectrum of microbial components [55]. For example, TLR2 is activated by peptidoglycan and lipoteichoic acid and TLR4 by the lipid A part of the lipopolysaccharide. TLR5 has been identified as the receptor for flagellin when epithelial cells were infected with *Salmonella* typhimurium [56, 57]. TLR5 is expressed in unpolarized intestinal epithelial cells and on the basolateral side of polarized intestinal epithelial cells *in vitro* [56, 58], while expressed on both poles of intestinal epithelial cells *in vivo* [59-61].

1.3 URINARY TRACT INFECTION

1.3.1 Urinary tract infection

UTI is a very common disease, which affects primarily women and can be community acquired or hospital (nosocomially) acquired. UTIs can be restricted to the bladder (lower UTIs) or can ascent to the upper urinary tract and the kidney (pyelonephritis), which is usually associated with more severe symptoms such as fever. *E. coli* is the most common pathogen causing UTIs. Other members causing UTIs belonging to the family *Enterobacteriaceae* include *Proteus mirabilis*, *Klebsiella, Enterobacter and Serratia*. *Pseudomonas aeruginosa* occurs more frequently in nosocomial UTIs. Among Gram-positive bacteria *Staphylococcus saprophyticus* is a causative agent of UTIs. The spectrum and frequency of species causing UTIs differs with the acquisition of the UTIs associated with catheterisation. In Sweden, it is estimated that 25-40% of nosocomial infections are related with the urinary tract. Those UTIs account for enormous costs for health system, by prolonging hospital stay (for example, 1-5 extra days in Sweden) and also are associated with increased mortality. The urinary tract is also the major origin for urosepticaemia and bacteraemia. The majority of bacteraemia were hospital acquired and about 40% of them originated from urinary tract as shown in a retrospective Danish study covering 25 years. Some kind of instrumentation i.e. catheterisation, was performed in the majority of patients prior to the bacteraemia [62].

1.3.2 Catheter associated urinary tract infection

Catheter-associated urinary tract infection (CAUTI) is UTI associated with the application of urinary catheter. CAUTIs are the most common nosocomial infections, accounting for up to 40% of all nosocomial infections and more than 1 million cases in US hospitals and nursing homes each year [63-65].

Catheterisation is one of the major risk factors that predispose the patients to UTIs, as the presence of an artificial device facilitates microbial colonisation of the device. Therefore, there is a 5-10% risk each day for patients undergoing urinary catheterisation to become colonized by microorganisms [66]. Consequently, the catheters of approximately 50% of patients are colonized within 10 days and long-term catheterisation leads to the colonisation of all patients [67]. Patients with the urinary catheter run an increased risk to develop UTIs and even urosepticaemia [68].

CAUTIs are major reservoir of antibiotic-resistant organisms in the hospital [69]. Tambyah and Maki reported that more than 90% of the patients with CAUTIs were asymptomatic with only 52% of the cases detected by the hospital physicians. There were no significant differences between patients with and without CAUTI in signs or symptoms referable to the UTIs [70, 71].

1.4 BACTERIAL TYPING METHODS

Typing refers to the classification of bacterial isolates from the same species according to phenotypic or molecular traits and is the basis of bacterial epidemiology. Epidemiologic typing systems are used to study the spread of bacteria and the pathogenesis of infections.

1.4.1 Serotyping

Serotyping aims at the determination of surface antigens by using a defined set of polyclonal or monoclonal antibodies. The current serotyping system for *E. coli* is based mainly on three types of antigen: the somatic (O) antigen which corresponds to terminal repetitive polysaccharide of the cell surface lipopolysaccharide (LPS), the capsular (K) antigen and the flagella (H) antigen. There are over 170 O-antigens, over 100 K-antigens and over 50 H-antigens. Certain serotypes of *E. coli* are associated with virulence properties. A small number of O-antigens were considered epidemiologically associated with UTIs [72-74]. Serotype of O4, O6, O14, O22, O75 and O83 account for 75% of *E. coli* strains causing UTIs [75].

1.4.2 Phylogenetic typing by PCR analysis

The population of *E. coli* strains can be subdivided into four phylogenetic groups (A, B1, B2, and D) [76, 77]. This classification of isolates into different phylogenetic groups has been first observed when reference collections of *E. coli* were typed by multi-locus enzyme electrophoresis [76] or ribotyping. Subsequently, biological functions could be correlated with the phylogenetic groups. For example, the virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D [78-80], whereas most commensal strains belong to group A. Clermont et al. developed a simple and rapid triplex PCR test [81] to determine the phylogenetic class of a specific isolate. The test assesses the presence or absence of marker genes *chuA* and *yjaA* and the DNA fragment TSPE4.C2.

1.4.3 Typing by pulsed-field gel electrophoresis

While phylogenetic typing classifies *E. coli* strains into four groups, typing by pulsed-field gel electrophoresis (PFGE) discriminates isolates on the strain level. PFGE is an electrophoresis method developed by Schwartz and Cantor in 1983 for the separation of DNA molecules up to 10 Mb [82]. When bacterial genomes are cut 10-40 times by rare cutting restriction enzymes, a clear pattern (characteristic fingerprint) of the genome is visualized by PFGE [83, 84]. Comparison of the fingerprint patterns allows conclusion about the relatedness of microbes.

1.5 BIOFILM

Biofilms are defined as “an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix” [85, 86]. Broadly interpreted, any aggregation of microorganisms can therefore be considered a biofilm. Many, if not most microbes, form biofilms at least at some stage of their life cycle [87]. The extracellular matrix of biofilms can consist of microbial produced material such as exopolysaccharide, proteins and DNA, but also include host components and mineral crystals. The nature of the biofilm and the degree of biofilm formation can vary greatly in an individual strain dependent on the environmental conditions such as the growth medium and surface [88-90]. Obviously for each species there exist conditions where

biofilm formation is favourable, while under other conditions biofilm formation is detrimental.

By being imbedded in a biofilm the microorganisms increase their resistance towards environmental stress, the immune response of the host and treatment with antibiotics. Specifically, non-specific defense mechanisms of the body such as the urine or intestinal content flow can be overcome by adhesion. On the other hand, immune components might also influence biofilm formation [91-93]. In addition, the structure of biofilm can facilitate transfer of resistance genes between different strains, resulting in the fact that biofilms express new, and sometimes more virulent phenotypes [94].

Biofilms can be beneficial or detrimental. The commensal flora on mucosal surfaces forms biofilms that protect against invading pathogens [95-97]. On the other hand, biofilms form easily on artificial surfaces and devices implanted into the host, which leads to infection of patients [98-100]

1.5.1 Biofilm formation by commensal *E. coli*

The genetic laboratory work horse *E. coli* K-12 is probably the most well-known commensal *E. coli* strain. Numerous genetic studies have been conducted in *E. coli* K-12 derivatives to investigate the nature and regulation of biofilm formation under laboratory conditions [101-104]. Useful information about e.g. extracellular matrix components required for biofilm formation, such as flagella, type 1 fimbriae, the antigen 43 (Ag43) and curli fimbriae [101, 102, 105, 106] and regulation of biofilm formation by e.g. the transcriptional regulator CsgD [106] has been created.

However, since *E. coli* K-12 has been handled in the laboratory since 1922 and biofilm formation is variable among *E. coli* K-12 isolates [105] the question remained whether a biofilm of *E. coli* K-12 is a representative model for biofilm formation of commensal *E. coli* isolates. Recent studies have shown, that there is a diversity of biofilm formation in commensal *E. coli* strains [107, 108]. And host components, such as secretory IgA and mucin aid biofilm formation of *E. coli* K-12 and environmental isolates *in vitro* [93, 109], which might aid biofilm formation in the gut.

1.5.2 Biofilm formation by uropathogenic *E. coli*

E. coli is the most common organism associated with infection of the urinary tract. The bacteria can colonize the urinary tract asymptomatically, cause UTIs or be a member of the catheter-associated bacterial community. Several studies indicate that biofilm formation is a factor required for successful colonization and persistence. Indeed, type 1 fimbriae, which have been shown to be required for biofilm formation *in vitro*[102], are crucially important in the establishment of UTIs [110]. On the other hand, biofilm-like pods in bladder epithelial cells were observed which help to explain how *E. coli* UTIs strains can persist under pressure from both host defenses and antibiotics [111]. The formation of pods in bladder epithelial cells also required type 1 fimbriae [112]. Also Ag43, which has been shown to contribute to biofilm formation in *E. coli* K-12 [102], promoted long-term persistence in the urinary bladder [113].

The amount and the nature of the biofilm formed by strains colonizing the urinary tract are not clearly defined. Early study report that, although the amount of biofilm formation varies significantly among *E. coli* isolates, there is no increase in biofilm

formation in pathogenic strains [114]. However, a standard laboratory medium was used in this study. Another study using urine as a growth medium showed that strains from asymptomatic bacteriuria are better biofilm-formers than UTIs strains [115]. However, using catheters as a surface UTIs strains outcompeted the strains from asymptomatic bacteriuria [116]. Some components contributing to biofilm formation of UTIs strains have been identified [117, 118].

1.5.3 Extracellular structures in biofilm development

In *E. coli*, biofilm formation has been intensively studied and various cell surface appendages are required to achieve a mature biofilm [119]. Flagella, type 1 fimbriae, curli fimbriae, polysaccharide-rich matrix (cellulose, colanic acid, and poly-β-1,6-*N*-acetylglucosamine) and short adhesins such as Ag43 and conjugative plasmid pili have been shown to be required for biofilm formation [101, 102, 108, 113, 120-127].

Curli fimbriae

Curli fimbriae (also called thin aggregative fibers in *Salmonella typhimurium*) are proteinaceous components of *E. coli* biofilms [123, 128, 129]., Curli fimbriae are present and expressed by many *E. coli* strains [130] and also in other *Enterobacteriaceae*, such as *Salmonella* spp. , *Shigella*, *Citrobacter*, and *Enterobacter* spp. [131].

Curli fibers mediate binding to a range of substances among them host factors such as fibronectin [132], the MHC-I complex [133] and also the dye Congo red (CR) [134] and the exopolysaccharide cellulose [123].

In *E. coli* K-12, the biosynthesis of curli fimbriae is achieved by the two divergently transcribed operons *csgDEFG*-*csgABC* [127, 128], separated by a 513-bp intergenic region. The *csgABC* operon encodes curli subunit genes whereby CsgA is the major subunit of curli fimbriae. The gene *csgD* encodes a transcriptional response regulator of the LuxR superfamily, which is required for the activation of curli biosynthesis [135]. Curli expression in *E. coli* MC4100 and YMel, *E. coli* K-12 derivatives, is highly regulated by environmental conditions such as temperature [128, 136].

Cellulose

As the most abundant organic renewable polymer carbon resource in nature, cellulose can be produced by plants, some animals, algae, fungi, flagellates and bacteria [137]. In bacteria, it is generally produced as an extracellular component for mechanical and chemical protection. When cellulose is expressed by the plant pathogen *Agrobacterium tumefaciens* and the plant symbiont *Rhizobium* spp, cellulose facilitated adhesion of the bacteria to the host tissue. [138].

Cellulose is another main component contributing to the extracellular matrix of *E. coli* biofilms [123, 129]. In *E. coli* and other *Enterobacteriaceae* cellulose biosynthesis genes are encoded by the two *bcs* (bacterial cellulose synthesis) operons: *bcsABZC* and *bcsEFG*. The catalytic subunit of the cellulose synthase is encoded by *bcsA*. Cellulose biosynthesis requires allosteric activation of the cellulose synthase by the small molecule c-di-GMP (see **1.5.4**). BcsA binds c-di-GMP on its C-terminal end by the

PilZ domain [139, 140]. Apart from this, very little is known about the biosynthesis of cellulose in *E. coli*.

Ag43

Ag43 is a self-recognizing autotransporter adhesin and expressed in large amounts with approximately 50,000 copies per cell. It is associated with cell aggregation and biofilm formation in commensal and uropathogenic *E. coli* [113]. The expression of Ag43 by UPEC has also been demonstrated in human urine [141]. Ag43 is suggested to contribute to survival and persistence of UPEC during prolonged UTIs [111].

Ag43 is encoded by the *flu* gene. The UPEC strain CFT073 has two copies of the Ag43-encoding *flu* gene, *fluA* and *fluB*. The two *flu* genes share 91% conservation at the nucleotide sequence level. Recently Ag43a (but not Ag43b) was reported to contribute to pathogenesis of UTIs by enhancing long-term persistence in the urinary bladder [113].

Ag43 is composed of two domains: the passenger domain (α-domain) and the transporter module (β-domain) [142]. Once expressed on the bacterial surface, the α-domain can be cleaved off, but remains in contact with the β-domain via noncovalent interactions. Therefore, the α-domain can be released from the surface of the cells under denaturing conditions such as brief heating to 60°C.

Type 1 fimbriae

Type 1 fimbriae are rod-shaped adhesive surface organelles, which are the most common adhesins found in both commensal and pathogenic *E. coli* isolates as well as in other *Enterobacteriaceae* [143]. Type 1 fimbriae are multi-functional macromolecules and can mediate mannose-sensitive binding of bacteria to erythrocytes, epithelial cells, and leukocytes [119]. Analysis of biofilm formation showed that type 1 fimbriae are critical for initial attachment of bacterial to abiotic surfaces [102, 144]. In pathogenic strains of *E. coli*, Type 1 fimbriae are involved in adherence to mucosal cells as the first step in the pathogenic process [145-147].

The *fim* gene cluster encodes genes required for the biogenesis of type 1 fimbriae. The fimbrial shaft is primarily composed of the FimA subunit [148]. Although the FimH protein is a minor component found at intervals along the fimbrial shaft [149] and also at the tip [150], it is the actual receptor-binding molecule which recognizes D-mannose-containing structures on cells [149]. Sequence differences in the *fimH* gene confer different receptor binding specificities for Type 1 fimbriae [151, 152]. The ability of the FimH binding to monomannosyl residues strongly correlates with their ability to facilitated adhesion of *E. coli* to uroepithelial cells [153].

LPS

LPS is the main outer leaflet of the outer membrane of Gram-negative bacteria. It contributes to the structural integrity of the bacteria, and protects bacteria from the action of bile salts, hydrophobic antibiotics, and complement activation [154]. LPS is an endotoxin and elicits a strong immune responses in human and animals, with a diversity of causative or modulating effects in a number of disease processes [155, 156].

LPS is a large molecule consisting of three parts: the highly hydrophobic lipid A, the core oligosaccharide and the repeating polysaccharide (O) side chains. Lipid A is a disaccharide with multiple fatty acid tails reaching into the membrane. The core oligosaccharide is attached to lipid A and composed of conserved sugars. The

polysaccharide side chain, also called O-side chain, is referred to as the O-antigen and ligated to the core polysaccharide. Lipid A-core oligosaccharide and O-polysaccharide chain are formed by independent assembly pathways [157-159]. The WaaL protein, encoded by *waaL* gene, is the only known ligase involved in the ligation of pre-assembled O-polysaccharide to Lipid A-core oligosaccharide in *E. coli*. This occurs at the periplasmic face of the plasma membrane, prior to translocation of completed smooth LPS to the outer membrane [159]. When presenting full length O-chains, the LPSs are referred to as smooth (as also the colony morphology appears on the agar plate). When the O-polysaccharide chains are absent, the LPSs are referred to as rough [155-157] The deletion of *waaL* results in a rough colony morphology consistent with the loss of O antigen ligase activity [160].

Flagellin

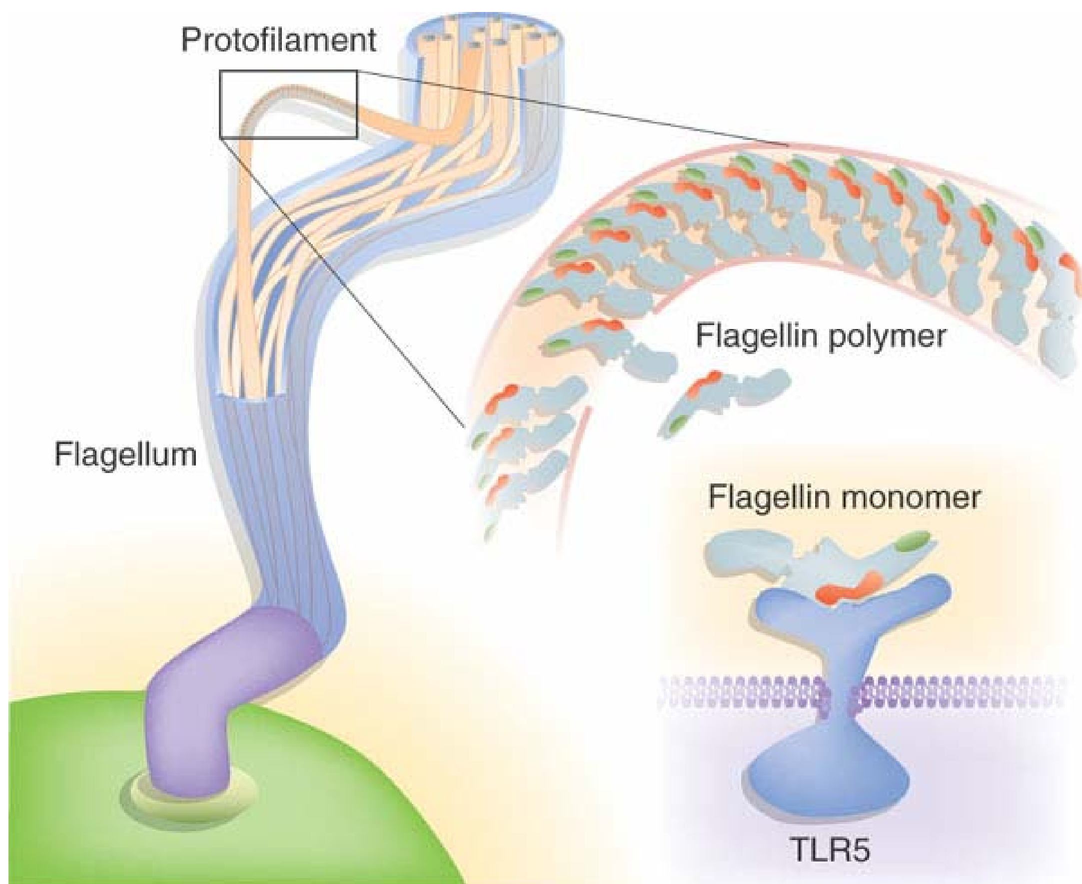

Figure 1. Flagellin binding to TLR5 for IL-8 induction [161]**.**

The flagellum is composed of 11 protofilaments. Each protofilament is nearly exclusively a polymer of flagellin. The monomers are packed through relatively small but deeply buried axial interactions between the concave (green) and convex (red) surfaces of the D1 domain. TLR5 recognizes the flagellin monomer at the very same surface of the convex, normally hidden in the filament, and activates the immune system.

Bacterial flagella are protein structures present on the cell surface. They are approximately 10-15 nm in diameter and 2-15 µm in length. In *E. coli* flagella are peritrich, this means there are multiple (up to 20) flagella distributed all over the bacterial cell. Flagella are key organs for bacterial motility such as swimming and swarming [162] and exert important function in biofilm formation [119].

Flagellin, encoded by gene *fliC,* is the monomer subunit protein polymerizing up the flagella filament [162, 163]. Flagellin is secreted by a type III secretion system and passed through the central channel of the growing filament to be assembled in a helical structure at the distal end. A capping structure promotes polymerization at the tip and prevents the extended release of subunits into the environment [163, 164].

Bacterial flagellin is one important protein target of both the innate and the adaptive immune system. It is one of the microbe specific molecular structures (also called pathogen-associated molecular patterns or PAMPs). In the extracellular milieu, flagellin but not flagella is detected by TLR5 (Figure 1) [161, 165, 166] and in the cytoplasm by Ipaf to activate the innate immune system [163]. Expressed in pathogenic and commensal *E. coli*, it has been reported as a major determinant triggering IL-8 production in gastrointestinal epithelial cells [60, 167-169]. However, there is amino acid sequence variability in the flagellin within the *E. coli* species [170]. Different flagellin proteins therefore can induce a variable immune response [171].

1.5.4 Cyclic-di-GMP in biofilm formation

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is emerging as a novel global second messenger in bacteria, which regulates bacterial biofilm formation and virulence as well as interactions between the microorganisms and their eukaryotic hosts and other phenotypes [172, 173]. Almost ubiquitous in bacteria, the GGDEF and EAL domain proteins are involved in synthesis and hydrolysis of c-di-GMP, respectively.

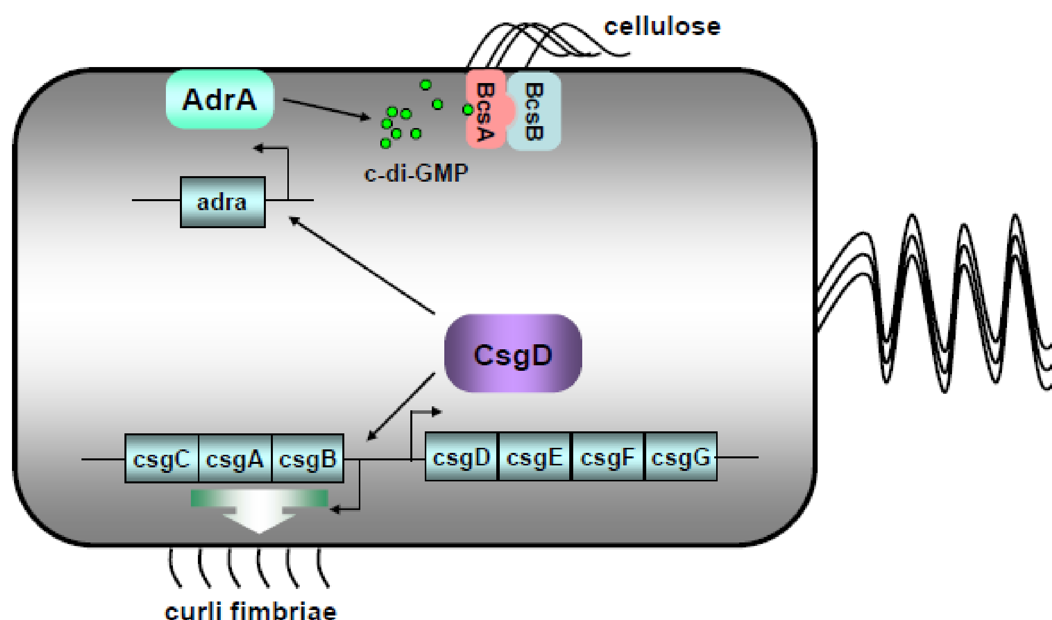

Figure 2. CsgD up-regulates expression of the extracellular matrix components curli fimbriae and cellulose

In most *E. coli* strains and other *Enterobacteriaceae* CsgD activates the production of curli fimbriae and cellulose. Cellulose production required CsgD-dependent transcriptional activation of the di-guanylate cyclase AdrA which produces the small molecule c-di-GMP. The c-di-GMP is an allosteric activator of the cellulose synthase.

Biofilm formation in *S. typhimurium* and *E. coli* is regulated by c-di-GMP. Thereby, c-di-GMP regulates the expression of the major regulator of biofilm formation, the transcriptional regulator CsgD [172, 174]. Further on, biosynthesis of the extracellular matrix components cellulose and curli fimbriae was reported to be regulated by CsgD [123, 135]. Curli fimbriae are directly regulated by CsgD. On the other hand, CsgD activated AdrA, a GGDEF domain protein with diguanylate cyclase activity under many culture conditions (Figure 2) [123, 135, 175]. Subsequently, AdrA produced c-di-GMP activates cellulose biosynthesis [135, 176]. However, expression of CsgD and activation of cellulose biosynthesis is uncoupled under certain environmental conditions and in certain strains. Cellulose biosynthesis can also be activated by the GGDEF domain protein YedQ whose expression is independent of CsgD [108].

1.5.5 Rdar morphotype system

Rdar is the abbreviation for "red, dry and rough" and was used to describe a characteristic colony morphotype on agar plate supplemented with the dye CR for *S.* Typhimurium, *E. coli* and some other enterobacteria (Figure 3) [123, 124, 129]. This morphotype is elaborated when cultured bacteria strongly express the two extracellular matrix components cellulose and curli fimbriae, which have a strong non-covalent affinity to bind to CR. When the expression of cellulose and curli fimbriae is less pronounced, the colony displays a "red, dry and smooth" morphotype, abbreviated as "ras". The absorbance spectrum is different when CR binds to cellulose or curli fimbriae. Expression of only cellulose by the bacteria appears as a pdar (pink, dry and rough) morphotype, while expression of only curli produces a bdar (brown, dry and rough) morphotype (Figure 3). Similarly to rdar and ras, less pronounced phenotypes are reported as bas (brown and smooth) and pas (pink and smooth). When neither cellulose nor curli fimbriae are expressed, there is a white and smooth colony phenotype named as saw (smooth and white) (Figure 3) [123, 124, 129]. The morphotype system is a simple tool to screen for the expression of the extracellular matrix components cellulose and curli fimbriae.

Figure 3. Rdar morphotype system in the commensal strain *E. coli* TOB1 on CR plate [107]**.** Expression of both cellulose and curli fimbriae led to the rdar morphotype; the white and smooth colony (saw morphotype) indicated no expression of cellulose or curli fimbriae; expression of only cellulose appeared as pdar, expression of only curli produced a bdar morphotype.

1.5.6 Virulence factors in commensal and uropathogenic *E. coli*

Pathogenic *E. coli* strains are the main cause of bacterial diarrhea and extra-intestinal infections mostly including UTIs, meningitis and septicemia. The commensal *E. coli* are distinct from pathogens with a lower frequency of virulence traits such as adherence factors and toxins [48]. The different physiopathological behavioral patterns of *E. coli* can be associated with distinct virulence factors. These virulence factors frequently cluster on distinct pathogenicity islands [177, 178].

The adhesins are considered to be crucial virulence factors which allow particular clones of *E. coli* to colonize the urinary tract. Although epidemiological studies suggested that the presence of adhesins such as P fimbriae enhances the colonization capacity in the intestine [179, 180], no fimbrial adhesion has been shown to be exclusively required for colonization. P fimbriae, encoded by the *pap* (pyelonephritis-associated pili) operon, are expressed in the majority of *E. coli* strains causing upper UTIs [181]. They promote ascending UTIs by recognizing the α-D-galactopyranosyl -(1-4)-β-D-galactopyranoside disaccharide presenting in the globoseries of glycolipids located in the human kidney as well as on erythrocytes [182-184]. The sequence variation of the PapG-adhesin at the tip classifies P fimbriae as class I–III with different binding characteristics, whereby *E. coli* with class II PapG adhesin preferentially bind globoside and dominate in UTIs [185-187].

Type 1 fimbriae (see **1.5.3**) are mainly associated with cystitis and confer binding to uroplakins, α-D-mannosylated proteins, which are abundant in the bladder [188, 189]. They play no role in gut colonization [190, 191].

S-fimbrial family of adhesins, encoded by *sfa* operon, are frequently expressed in extraintestinal pathogenic *E. coli* strains [192, 193]. A subtype of the S-fimbrial family, F1C-fimbriae, preferentially expressed by UTIs isolates [193], bind to epithelial and endothelial cells from the kidney and lower urinary tract [194].

Dr haemagglutinin (*draA*), also called Dr adhesin, is a mannose-resistant adhesin. *E. coli* strains harboring Afa/Dr adhesins have been found to be associated with UTIs (pyelonephritis, cystitis, and asymptomatic bacteriuria) and with various enteric infections [195, 196]. In addition, S fimbriae and Dr haemagglutinin can also bind to human intestinal epithelial cells, and therefore could contribute to intestinal persistence [197].

Besides adhesins, UPEC strains have additional virulence factors that collectively increase their ability to cause disease. α-haemolysin is encoded by *hly* operon. Although up to 12% of commensal *E. coli* strains could be haemolytic, the haemolysis phenotype is mainly associated with pathogenic *E. coli* strains causing UTIs and bacteriaemia. The haemolysis phenotype occurs in 20% of asymptomatic bacteriuria, 40% of cystitis, 49% of pyelonephritis, and 39% of bacteriaemia [198].

The capsule help *E. coli* strains to compete for the establishment of colonization in the intestine *in vivo* [199]. The capsule provides protection against phagocytic engulfment and complement mediated bactericidal effect in the host. Certain capsular types (K1 and K5) show a molecular mimicry to tissue components, preventing a proper humoral immune response by the infected host [200-202]. The combination of the P fimbriae, K1 (encoded by *neuB* gene) or K5 (encoded by *kfiC* gene) capsule and aerobactin (*iutA*) were reported to be associated with persistence of *E. coli* strains in the human intestine [203].

1.6 BACTERIAL-HOST INTERACTION

1.6.1 Adherence and internalization

Above mentioned adhesins and some virulence factors help bacteria colonize on host mucosa during the first stage of adherence or attachment to a eucaryotic cell or tissue surface. By adherence and/or further internalization to host cells, the bacteria try to overcome the pressure from the host defenses. Bacterial adherence to mucosal surfaces requires the participation of two factors: a receptor and a ligand. The receptors are usually specific carbohydrate or peptide residues on the eucaryotic cell surface. The adhesins are the bacterial ligands, typically a macromolecular component of the bacterial cell surface, which interact with the host cell receptors. Besides the adhesin, bacterial LPS and capsule, determining physicochemical surface properties, are critical virulence factor for adherence [204, 205]. Interestingly, bacterial motility and production of flagella are reported as virulence factors that favour adherence and internalization into eukaryotic cells [206-208].

By adhesion to and invasion into host cells, pathogens access to niches to replicate and to disseminate within the infected host. When invading into host cells, they further manage to avoid immune clearance. This is a two way interaction involving the two players "cross talk", whereby pathogenesis is in part due to altered the host cell function to facilitate the invasion [209]. Many pathogenic *E. coli* express surface adhesins or secretory toxins to persuade host cells to take up the bacteria into the cell by modulating the cytoskeleton actin and microtubule system. The cytoskeletal actin filaments functions in coordination with the microtubules system to rearrange cell shape [210].That leads to an uptake of the bacteria into normally non-phagocytic cells such as epithelial cells [209, 211, 212].

1.6.2 Host immune response

The presence of bacteria triggers innate and adaptive immune response in the human intestine. Besides being a mechanical barrier to the invaders, epithelial cells are directly part of the immune system. They are able to produce substances toxic to bacteria such as nitric oxide [213-216] and defensin [217-219], and to express a characteristic profile of chemokines and cytokines to recruit and activate effector cells to clear the infecting bacteria [220-222]. Interleukin 8 (IL-8) is a considerable proinflammatory cytokines secreted from the epithelial cells for its chemoattraction and activation of neutrophils and T lymphocytes [223-225].

2 AIMS OF THE THESIS

The general aim of this thesis is to evaluate the epidemiology of biofilm formation in commensal and uropathogenic *E. coli* and determine the role of biofilm formation in bacterial-host interactions.

The specific aims of the study are:

1. To characterize biofilm formation in gastrointestinal commensal *E. coli in vitro*.
2. To investigate the role of major biofilm components on interaction between commensal *E. coli* and the gastrointestinal epithelial cell line HT-29.
3. To characterize expression of the biofilm matrix components cellulose and curli fimbriae in the probiotic strain *E. coli* Nissle 1917 and evaluate the role of cellulose on the interaction between Nissle 1917 and the gastrointestinal epithelial cell line HT-29.
4. To characterize biofilm formation in bacteria colonizing urinary tract catheters *in vitro.*

3 MATERIAL AND METHODS

The major principles and central methods used in the papers and manuscripts presented in this thesis will be described in this section. Detailed information is provided in the method parts of the attached publications or manuscripts and will not be repeated here.

3.1 PATIENT POPULATIONS

3.1.1 Study I

As a commensal bacterium of the human gastrointestinal tract, *Escherichia coli* is symbiosis with its host. Commensal *E. coli* strains are generally considered to be different from pathogenic *E. coli* strains. However, biofilm formation has not been investigated in commensal *E. coli* strains. To investigate this physiological trait, we collected commensal *E. coli* strains from a group of healthy people. The isolates were collected from eleven members of three family households, to investigate whether there was any isolate transfer between members of a family. For statistical purposes, faecal swabs were collected from 10 additional anonymous individuals. To compare with pathogenic *E. coli*, 20 *E. coli* strains were isolated from urine samples of UTI patients.

3.1.2 Study IV

In order to understand the factors required for bacterial colonisation of urinary tract catheters with respect to biofilm formation, bacterial isolates were collected from urine and catheter samples from outpatients undergoing catheterization. The isolates were collected on the occasion when the urinary catheter was removed. A closed drainage system has been used for all patients. Urine and urinary catheter samples were obtained from 43 patients, 41 male and 2 female, from a total of 45 episodes. Median patient age was 80 years (range 51 to 97 years). 20 patients had all silicone catheters (SCs), 19 silicone coated latex catheters (SCLCs) and 6 patients had a different type of urinary catheter. The median time of the carriage of the current urinary catheter was 1½ months. The total catheterization time was 3 months (range 1 day to 6 years) and 42 % were short time catheterizations (< 30 days). The remaining 26 patients were on long time catheterization (30 days - 6 months). The main indications for catheterisation were prostate hyperplasia (42 %) and prostate cancer (38 %) followed by urine retention due to other causes (9 %). No selection was made with respect to underlying disease. In order to see the innate spectrum of colonisation species, a prerequisite was no antibiotic treatment during the last week before sampling.

3.2 LABORATORY METHODS

3.2.1 Bacterial strains and growth conditions

Routinely, *Enterobacteriaceae* and *Pseudomonas* spp. were maintained in Luria-Bertani (LB) broth; *Staphylococci, Enteroco*cci and *Streptococci* were maintained in Brain Heart Infusion medium. Biofilm formation is a bacterial behaviour that varies significantly with the species and culture conditions. Based on earlier studies [135, 226], specific media considered to be optimal for the expression of biofilm formation were

chosen for the different species : LB without salt broth was used for *E. coli* and other *Enterobacteriaceae;* LB broth for *Pseudomonas*; Brain Heart Infusion medium supplemented with 2% glucose and 2% sucrose or Tryptic Soy Broth medium supplemented with 1% of glucose for *Staphylococci, Enterococci* and *Streptococci*.

In order to study biofilm development under conditions close to natural conditions, bacteria were also grown in artificial and human urine. Artificial urine was prepared according to the recipe by [227]. The human urine was pooled from midstream urine of 4 healthy male individuals. The individuals had not taken any medication for at least two weeks.

3.2.2 Molecular typing methods

Molecular typing of bacterial strains was applied in order to determine the genetic relationships among the isolates on the phylogenetic and the strain level [76].

3.2.3 Phylogenetic typing

Triplex PCR is carried out in order to test for the presence or absence of genes or genomic fragments *chuA*, *yjaA* and TspE4-C2 [81]. With this triplex PCR classification of *E. coli* into the four phylogenetic groups A, B1, B2 and D can be performed. The decision tree for phylogenetic classification is shown in the Figure 4. This method was applied for all *E. coli* strains isolated and investigated in this thesis.

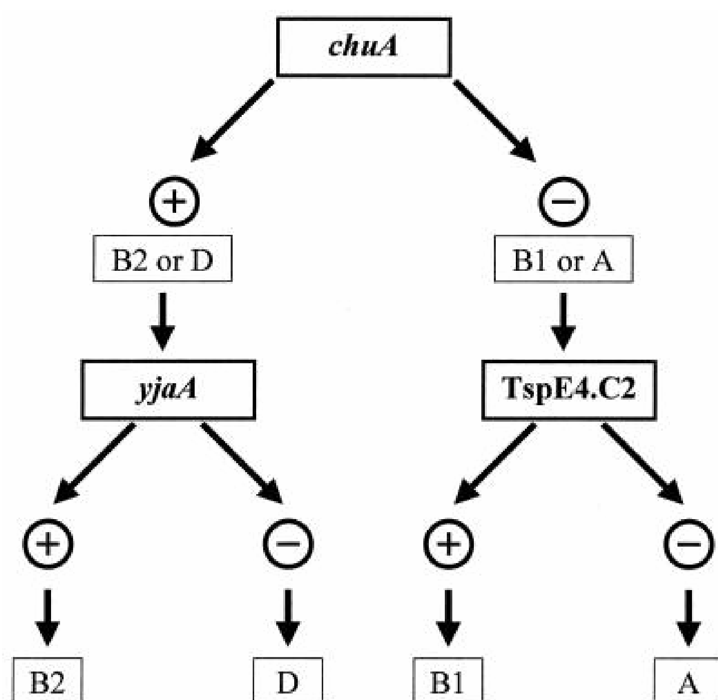

Figure 4. Dichotomous decision tree to determine the phylogenetic group [81]
Strains were tested by Triplex PCR. The phylogenetic groups of *E. coli* strains were deduced by the results of PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2.

3.2.4 Pulsed-field gel electrophoresis typing

PFGE typing is used to resolves the genetic distance between bacteria in a species on strain level. PFGE typing is an electrophoresis based method which separates DNA molecules of large size. For analysis, the bacterial DNA needs to be cut by rare-cutting restriction enzymes, which, resolved by PFGE, will give a characteristic banding pattern for each strain. This typing method is very sensitive and is therefore called macrorestriction fingerprint analysis.

For *E. coli*, a suitable restriction enzyme, which cuts the *E. coli* genome into approximately 35 fragments is *Xba*I [228]. In this thesis, PFGE was used to demonstrate the similarity of strains between members of the same families showing potential strain transfer.

3.2.5 Detection of virulence factors

Virulence factors refer to “the properties that enable a microorganism to establish itself on or within a host of a particular species and enhance it potential to cause disease” [229]. The presence of a combination of virulence factors provides important insights into the potential of bacteria to cause diseases. Adherence factors, so called adhesins, are an important group of virulence factors, since adherence of the organisms is an essential step in the bacterial pathogenesis leading to infection. They are also important in biofilm formation. In order to draw a picture of the pathogenic potential of commensal *E. coli* and *E. coli* colonizing urinary catheters, the strains were screened for the presence of genes encoding for a variety of adhesins.

In addition, the presence of virulence factors associated with the pathogenesis of UTIs was investigated. The presence of genes encoding for virulence factors was determined by the presence of a characteristic PCR product using specific primers. Virulence factors investigated were P fimbriae (presence of *papC* and *papG*), S fimbriae (*sfaD*, *sfaE*), Dr haemagglutinin (*draA*), the capsule K1 (*neuB*), capsule K5 (*kfiC)*, aerobactin (*iutA*) and haemolysin (*hlyA*) (for **study I)** [203]; P fimbriae (*papC*) and PapG adhesin (classes I, II and III), type 1 fimbriae adhesins (*fimH*), haemolysin (*hlyA*) and cell surface protein Ag43 (*flu*) (for **study IV)** [113, 203].

3.2.6 Morphotype screen

CR has a strong non-covalent binding affinity to cellulose and curli. When the dye is added to the medium in agar plates, the colour of the colony growing on the plate is changed depending on the expression of cellulose and/or curli. Therefore, expression of cellulose or curli by *Salmonella* typhimurium are indicated by the following morphotypes on agar plates [135]: rdar (red, dry and rough colony; cellulose$^+$ and curli$^+$), pdar (pink, dry and rough; cellulose$^+$ and curli$^-$), bdar (brown, dry and rough; cellulose$^-$ and curli$^+$) and saw (smooth and white colony; cellulose$^-$ and curli$^-$). Less pronounced expression of the matrix shows light phenotypes like ras (red and smooth), bas (brown and smooth) and pas (pink and smooth). This referred to basic morphotypes detected in *S*. Typhimurium [135].

Calcofluor (fluorescent brightener 28) is a non-specific fluorochrome with the ability to bind to β-(1,3) and β-(1,4)-glucans among them cellulose [230]. Calcofluor

added to the medium in agar plates serves as an indicator of cellulose production. Calcofluor binding can be detected as colonies fluorescence under a 366 nm UV light source. Fluorescence is judged visually using control stains with defined Calcofluor binding capacity as comparison. The control strains are knockout mutants of S. Typhimurium expressing individual matrix components [135].

The morphotype screening system can also be applied to *E. coli* in the same way as it has been developed for *S.* typhimurium. However, this phenotypic method has to be complemented by molecular methods to give definite information about the presence or absence of extracellular matrix components.

3.2.7 Knock-out of genes and cloning

In order to investigate the function of a gene, mutational analysis of the gene in its chromosomal location required. Datsenko & Wanner developed a fast and efficient methodology to directly disrupt targeted gene by homologous recombination using 40-60 bp of homologous regions [231].

Bacteria are transformed with PCR fragments encoding a selectable marker and 40-60 nt of flanking DNA on the 5' and 3' end. The flanking DNA is homologous to the 5' and 3' sequences of the gene on the chromosome. In this thesis, all targeted genes were disrupted by applying this technique.

Transformation and recombination of PCR products occurs efficiently due to the presence of the λ Red system, which encodes three genes among them the phage λ Red recombinase. The λ Red system has to be provided on low copy number helper plasmids. Plasmid pKD46 (with ampicillin resistance as the selection marker) was originally developed for this procedure [231, 232]. However, as clinical isolates are often resistant to ampicillin, other plasmids had been developed in the literature (pTP223 (TetR) [232]). Since some selected *E. coli* strains isolated from catheters were resistant to both ampicillin and tetracycline, plasmid pZHL7 (KmR) was applied. pZHL7 was constructed from pKD46 by inserting a *kan* cassette (kanamycin resistance gene) into the *bla* (ampicillin resistance) gene.

3.2.8 Assessment of adherence

Adherence is the first step in bacterial colonization and infection. At the intestinal mucosa, adherence to the intestinal epithelium is believed to aid the persistence of commensal bacteria [233]. Adherence to intestinal epithelia cells is also a pathogenic trait as it is found under disease conditions and triggers host response and precedes cellular invasion [234]. The human colon adenocarcinoma cell line HT-29 offers a convenient and widely used experimental system to study bacteria-epithelial cell interaction.

HT-29 cells were grown to confluency on glass coverslips deposited into a 24-well plate. Afterwards, FITC labeled bacterial cells were added with a multiplicity of infection of 17. After 1.5 h of incubation, the glass cover slips were rinsed with phosphate-buffered saline and fixed with 4% formaldehyde. The number of bacteria that adhered to HT-29 cells was determined by counting the bacteria under a fluorescence microscope.

3.2.9 Internalization assay

Related to this study, uptake of bacteria into intestinal epithelial cells with the help of curli fimbriae was demonstrated for both commensal and pathogenic *E. coli* strains [235, 236]. In order to disclose the role of cellulose and curli fimbriae in the interaction with intestinal epithelial cells, selected commensal *E. coli* strains were investigated for internalization by intestinal epithelial cells. HT-29 cells were seeded into 24-well tissue culture plates and incubated until full confluency, and then infected with bacteria at a multiplicity of infection of 17. After 3 hours incubation, the cells were washed. Extracellular bacteria were killed by 1 hour gentamicin treatment and cells were lysed with Triton X-100. Appropriate dilutions were plated and the internalization rate was defined as the percentage of the original inoculum that resisted treatment with gentamicin.

3.2.10 IL-8 activation by *E. coli*

Commensal bacteria, among them *E. coli*, can trigger proinflammatory response in gastrointestinal epithelial cells [7] [60]. IL-8 is a proinflammatory cytokine secreted by epithelial cells upon sensing of bacteria. The role of the two extracellular matrix components cellulose and curli fimbriae on the induction of IL-8 was investigated. Therefore, IL-8 was assayed in culture supernatants after HT-29 cells had been challenged by bacteria for 3 hours. IL-8 induction in cultured intestinal epithelial cells had been reported to be triggered by monomeric flagellin secreted by pathogenic or commensal *E. coli* [60, 167-169].

3.2.11 Assessment of biofilm formation

The biofilm forming ability of bacterial strains was screened in 96-well polystyrene microtiter plates (Nunc). An overnight culture of the bacterial strain was diluted 1:10 in 200 µl medium in the microtiter plates well. Biofilm formation was allowed to occur for 24 h at 37°C or 48 h at 28°C. In liquid culture bacterial cells can usually form three types of biofilms through adherence, clumps and pellicle formation. Most isolates formed an adherent biofilm, whereby the bacterial cells adhered to the wall or bottom of the wells. Adherent biofilm were visualized by staining with 0.4% crystal violet. Biofilm that precipitates or floats in the culture media, but does not attach forms visible clumps. A bacterial film at the air-liquid interface is called a pellicle. Clumps and pellicle formation were inspected visually, and scores were assigned according to the amount of the biofilm. The sum of the scores from pellicle (2x), clumps (1x) and adherence (1x) represented the overall biofilm formation capacity of a bacterial strains [107]. Alternatively, adherence can be quantified by dissolving the crystal violet bound by adherent bacteria in 250 µl 100% (v/v) DMSO and measuring the absorbance.

3.2.12 Biofilm formation on catheters in urine medium

Urine is the growth medium for strains isolated from catheters and urine. It is poorly understood how urine influences biofilm formation. To investigate biofilm formation in human urine, artificial urine and pooled human urine was used instead of laboratory

medium in the above biofilm screen model with modifications. According to the literature, the material in the urine can form a conditioning film before bacteria attach [237]. Therefore, the 96-well microtiter plates were preincubated with 200 µl human urine for 16 h to allow the conditioning film (if there was any) to be formed. After inoculation with target bacteria and further growth for 24 hours, biofilm was formation was scored as described.

To investigate biofilm formation on catheters, bacterial adherence was allowed to occur on catheters: the catheter was sterilely cut into small pieces and immersed in pooled human urine in the wells of a microtiter plate. After incubation for 24 h, bacterial biofilm was allowed to develop for 24 or 48 hours at 37°C. In the staining control without bacteria crystal violet bound significantly to the catheter surface. This background staining was so high that there was usually insufficient discrimination between a catheter inoculated with bacteria and the control. Therefore, the adherence to the catheter surface was investigated by scanning electron microscopy.

4 RESULTS AND DISCUSSION

In this thesis, biofilm formation of *E. coli* strains isolated from the gastrointestinal tract and the urinary tract was analyzed. Further on, the influence of biofilm formation, in particular the expression of curli fimbriae and cellulose, on the behavior of commensal *E. coli* strains in the interaction with the gastrointestinal epithelial cell line HT-29 was investigated. The preliminary results report about the components required for biofilm formation on urinary catheters. The first 3 studies focused on the commensal strains, while study IV and the preliminary results investigated strains from urinary catheters or urine.

4.1 STUDY I

Commensal *E. coli* are important components of the intestinal microbial flora present in almost any individual. Although biofilm formation was analyzed in bacteria colonizing many biological niches [100, 238-240], little was known about the ability of commensal *E. coli* strains to form biofilms and the components required for biofilm formation. Therefore, we characterized in this study a collection of 52 commensal *E. coli* isolates from 21 individuals with respected to their ability to form biofilms and investigated the expression of the extracellular matrix components curli fimbriae and cellulose.

4.1.1 Commensal strains express curli fimbriae and cellulose

In the collection of commensals, 74% of the strains belonged to four dominant morphotypes: rdar/rdar (morphotypes mentioned for 28°C/37°C), bdar/saw, saw/saw and rdar/bdar, while the most common morphotypes in the UTIs strain collection were rdar/saw and saw/saw. The "rdar/saw" was previously observed as the major morphotype in *S*. Typhimurium and *Salmonella enterica* serovar Enteritidis strains [129, 241-243]. In contrast, the rdar/saw morphotype was expressed at very low frequency (0.33 %) by commensal isolates of *E. coli*. Those observations could indicate that low level expression of both extracellular matrix components at ambient temperatures is a characteristic of a subset of enterobacterial pathovars, while a variable expression of curli fimbriae and cellulose is a characteristic of commensal *E. coli*.

In selected strains, Western blot confirmed the expression of curli fimbriae which was commonly correlated with the expression of the rdar (ras) and bdar (bas) morphotypes. Calcofluor binding was detected for isolates with rdar (ras) or pdar (pas) colony morphology, indicating that cellulose was expressed by those isolates. A chemical assay applied in randomly selected isolates further proved the expression of cellulose.

4.1.2 Expression of curli fimbriae tends to be at 28°C

Both commensal and UTIs isolates tended to express curli fimbriae at 28°C rather than 37°C ($p<0.01$). It is consistent with earlier and later investigation on curli fimbriae [136]. This finding also supported that many environmental *E. coli* strains do not

express curli under conditions of high temperature, while septicemic strains do express curli under such conditions [236, 244].

Type 1 fimbriae and curli fimbriae are the only two fimbriae commonly present in commensal and pathogenic *E. coli* strains, while regulations of the expression of the two fimbriae by environmental conditions are different [245, 246]. For type 1 fimbriae, allelic variation of the tip adhesin FimH leads to tissue tropism of commensals and pathogens [247]. Whether curli fimbriae expressed by commensals and pathogens also show such discriminatory binding remains to be shown.

4.1.3 Morphotype can be an indicator of biofilm formation

Nineteen isolates with distinct morphotypes on CR agar plates were chosen to investigate the capacity of faecal strains to form biofilms in nutrient rich and poor media, LB without salt and M9 minimal medium. UTIs isolates and control strains with known biofilm behaviour were also used. Most of the faecal and UTIs strains showed only a low capacity to form biofilms in nutrient-defined medium. However, biofilm formation in LB medium without salt correlated with the colony morphotype on CR agar plates. Most strains expressing the rdar (ras) morphotype showed medium or high biofilm formation capacity, while strains expressing the saw morphotype only displayed low to medium biofilm-forming capacity. Bdar (bas) and pdar (pas) morphotype strains showed a lower capacity to form biofilms than rdar (ras) strains.

Thus, morphotype expression on CR agar plates is an indicator of biofilm formation. Biofilm formation was shown to be associated with the expression of cellulose and curli fimbriae by *E. coli*, *Salmonella* and other *Enterobacteriaceae* [123, 248, 249]. The two matrix components also contributed to biofilm formation of commensal *E. coli* in the current study.

4.1.4 Role of curli fimbriae and cellulose in biofilm formation

Strain TOB1 showed the rdar/rdar morphotype on CR plate, the most frequent morphotype in the current collection of commensal *E. coli* strains. TOB1 was chosen as a representative strain to investigate the contribution of the matrix components cellulose and curli fimbriae to biofilm formation. Therefore, isogenic mutants deficient in expression of the individual or both matrix components were created. Co-expression of cellulose and curli fimbriae in wild type TOB1 resulted in high biofilm formation at both 28°C and 37°C, while deletion of matrix components reduced biofilm formation. At 28 °C, the deletion of curli abolished adherence to the walls and pellicle formation. Clumps were still observed when only cellulose was expressed. Surprisingly, a pellicle was formed independently of cellulose and curli fimbriae in the *csgD* mutant at 37°C suggesting that additional components are required for pellicle formation. This is in contrast with the observation made in *S.* Typhimurium ATCC 14028 [135]. Other polysaccharides such as the homopolymer poly-β-1,6-*N*-acetyl-d- glucosamine or fimbriae such as type 1 fimbriae [102, 122] could be responsible for this phenomenon. In order to find the components required for pellicle formation, it would be best to perform random mutagenesis.

4.1.5 General characterization of the commensal strains

To investigate whether our collection of commensal strains is a representative collection with respect to commensal strains, the strains were characterized epidemiologically and the presence of genes encoding virulence factors was investigated. Firstly morphologically different isolates from the 11 family members were phylogenetically typed.

The genetic relationship was determined by grouping the strains in the phylogenetic groups A, B1, B2 and D. The majority of isolates were placed into group A (55 %), with 21% of isolates in group B2. This is consistent with earlier observation from a middle-European population in which commensal strains were distributed as: A (48%), B1 (22%), D (15%), B2 (15%), and with later observations in the Swedish population: A (57%) and B2 (5%) [233, 250]. A different phylogenetic distribution was found in pathogenic *E. coli* strains as follows: A (9%), B1 (2.5%), D (16%) and B2 (72%) [78, 80].

Duriez et al. also found that a subset of commensal strains gathered in group B2 and those were referred to as potentially virulent strains since they accumulated virulence factors like pathogenic strains [250]. The presence of multiple virulence factors in B2 strains was also observed in our study, while strains from other phylogenetic groups rarely harboured virulence factors.

The genetic relationship between strains was further classified on the individual level by PFGE. Although results revealed a high diversity of PFGE patterns, strains within family members frequently shared the same pulsed-field type indicating strain transfer.

4.2 STUDY II

Commensal *E. coli* strains express cellulose and curli fimbriae *in vitro* as demonstrated in Study I. In order to address the role of curli fimbriae and cellulose on the interaction of commensal bacteria with gastrointestinal epithelial cells, Study II was designed. The representative isolate TOB1 and its isogenic mutants were used to investigate the interaction between commensal *E. coli* and the gastrointestinal colonic carcinoma cell line HT-29. The extracellular matrix components curli fimbriae and cellulose affect adherence and internalization of the bacteria and IL-8 production by HT-29 cells. In addition, flagellin interacts with curli fimbriae to mediate IL-8 production.

4.2.1 Role of cellulose and curli fimbriae in adherence

Adhesion is essential for members of the commensal microflora in the intestine to be maintained. A large number of different fimbrial adhesins is described in bacterial-host interactions in pathogenic *E. coli* strains [251]. Here we demonstrated that curli fimbriae mediate adherence of commensal *E. coli* to HT-29 cells. The representative wild type strain TOB1 showed intermediate adherence to the cells, while the mutant expressing only cellulose and the mutant expressing neither cellulose nor curli fimbriae did not significantly adhere. The mutant expressing only curli fimbriae adhered significantly to the HT-29 cell line. Those results indicated that curli fimbriae, but not cellulose mediated adherence of the fecal commensal isolate to HT-29 cells. Co-

expression of cellulose in the presence of curli fimbriae decreased the adherence to HT-29 cells.

Although S-fimbrial adhesins (Sfa I or Sfa II), P fimbriae or type 1 fimbriae had been associated with the persistence of commensal *E. coli* isolates in the gastrointestinal tract [252, 253], commensal isolates are commonly characterized by the absence of the S-fimbrial adhesin and P fimbriae [107, 179]. On the other hand, curli fimbriae and type 1 fimbriae are present in almost all commensal isolates [107]. The expression of curli fimbriae might be one factor contributing to gastrointestinal persistence.

4.2.2 Role of cellulose and curli fimbriae in internalization

Curli-mediated invasion was previously observed in pathogenic *E. coli* and *S. enterica* strains [235, 236, 254, 255].. In this study it was demonstrated that curli fimbriae mediate internalization of commensal *E. coli* strains. The mutant expressing only curli fimbriae showed a 100-fold higher internalization rate than the wild type and mutants lacking expression of curli fimbriae. Since the wild type expresses curli fimbriae and cellulose, these observations indicated that cellulose expression prevented internalization mediated by curli fimbriae. In addition, the internalization rate of the representative isolates correlated with the amount of adherence.

4.2.3 Invasion capacity of commensal *E. coli* isolates

In order to investigate whether expression of curli fimbriae commonly correlated with enhanced invasion capacity, 11 fecal isolates with variable expression of the two extracellular matrix components curli fimbriae and cellulose were chosen [107] and investigated in the internalization assay. Two strains with bdar morphotype (indicating strong expression of curli fimbriae) were internalized at 10 and 1000-fold higher rate (0.003% and 0.082%) than strains (0.00001% to 0.0003%) with other morphotypes. This finding generalized the previous result that curli fimbriae increase the internalization of the commensal strains into the gastrointestinal epithelial cell line HT-29.

The gastrointestinal flora, in particular members of the family of *Enterobacteriaceae*, play an etiological role in triggering disease in the immunocompromised host [14, 256]. Whether curli-mediated adherence and invasion of these potential pathogenic *E. coli* involved in the pathogenesis remains to be investigated.

4.2.4 Role of cellulose and curli fimbriae in inducing IL-8

Besides acting as a physical barrier, the gastrointestinal epithelium is an immunosensory organ which responds to the microbiota [220]. Thereby, gastrointestinal epithelial cells respond to the gastrointestinal commensal microflora like *E. coli* isolates [7, 14, 60, 212]. We wanted to know whether curli fimbriae and cellulose play a role in triggering an immune response in gastrointestinal epithelial cells. Therefore induction of the proinflammatory cytokine IL-8 upon stimulation with TOB1

and its cellulose and curli deficient mutants was investigated. A high IL-8 production was found to be triggered by the curli expressing mutant of TOB1. Co-expression of cellulose and lack of extracellular matrix components diminished the induction of IL-8 in HT-29 cells. These finding suggested that curli fimbriae expressed by the commensal isolate TOB1 were able to trigger a proinflammatory response. Alternatively, since the stimulation of IL-8 production correlated with the invasion and adhesion properties of TOB1 derivatives, curli fimbriae may facilitate inflammatory response through one of these mechanisms.

4.2.5 Flagellin requires curli fimbriae for IL-8 response

Flagellin, the structural subunit of flagella, has been recognized as a major determinant of IL-8 induction from gastrointestinal epithelial cells triggered by pathogenic and commensal *E. coli* [60, 167-169, 212, 257], although components of adhesive fimbriae of *E. coli* were also shown to elicit a significant immune response in epithelial cells [258].

In order to elucidate the role of flagellin in the IL-8 induction observed with TOB1 and derivatives, the gene *fliC* encoding flagellin, was knocked out in the wild type strain and the mutant which only expressed curli fimbriae, but not cellulose (TOB1Δ*bcsA*). Deletion of flagellin in TOB1 lead to a similar amount of IL-8 production as in the wild type strain (which is relatively low to begin with), suggesting that flagellin did not contribute to the immune response triggered by TOB1.

In contrast, when flagellin was deleted in the curli-producing derivative of TOB1 a significantly decreased IL-8 level was observed compared to the parent strain TOB1Δ*bcsA* (where IL-8 production is relatively high). This finding suggested that the presence of flagellin contributed substantially to the immune response triggered by the curli producing TOB1 derivative. The IL-8 induction was at a similar low level in strains TOB1Δ*csgD* and TOB1Δ*bcsA*Δ*fliC*, indicating that neither flagellin nor curli fimbriae alone triggered a substantial IL-8 response in HT-29 cells.

In addition, purified curli fimbriae did not cause a significant enhancement of the immune response, while binding of purified flagellin to the purified curli fimbriae induced augmented IL-8 response in HT-29 cells (unpublished results). Therefore, binding of flagellin to curli fimbriae is responsible for the elevated IL-8 response of TOB3. A synergistic effect of flagellin bound to curli fimbriae on IL-8 induction has also been recently demonstrated for *Salmonella enterica* serovar Typhimurium [259].

Complementation of the *fliC* knock-out in TOB1Δ*fliC* and TOB1Δ*bcsA*Δ*fliC* with *fliC* of *Salmonella enterica* serovar Typhimurium induced a high level of IL-8 production in HT-29 cells. Therefore, overexpression of flagellin derived from *S.* Typhimurium could trigger an immune response independent of curli fimbriae in both *E. coli* strains.

Recently, it has been shown that curli fimbriae is a PAMP recognized by TLR2 [260]. In the system used in this study, no evidence for a PAMP function of curli fimbriae could be detected. It is not known which toll-like receptor, if any, recognizes the flagellin/curli fimbriae complex. Although TLR5 is the primary receptor for flagellin recognition and signaling, TLR2, TLR4 and gangliosides cooperate with TLR5 as receptors for flagellin binding and signaling [164].

4.2.6 IL-8 response triggered by commensal isolates of *E. coli*

In order to understand the variability in the IL-8 induction triggered by *E. coli* and its dependence on the presence of extracellular matrix components, 16 fecal *E. coli* isolates with different H-serotypes in combination with various expression patterns of cellulose and curli fimbriae were selected [107]. After 3 hours of co-incubation with HT-29 cells, most of the commensal *E. coli* isolates showed a low level of IL-8 production (< 0.20 ng/ml) with the exception of 3 flagella positive isolates (>0.45 ng/ml). Two of the strains (with an IL-8 induction of 0.98 ng/ml, and 0.47 ng/ml) showed strong expression of curli fimbriae. The other strain, Fec10, with high IL-8 induction (0.91 ng/ml) did not express any matrix components. In order to understand the cause of high IL-8 induction, the amino acid sequence of the flagellin of Fec10 and other commensal isolates was investigated. The amino acid sequence of the flagellin of Fec10 is more closely related to *Salmonella* than *E. coli*, especially in the motifs required for TLR5 mediated IL-8 induction Consequently, the co-expression of curli fimbriae and flagellin by commensal *E. coli* usually enhance the induction of immune response, although a high immune response can be caused by only expression of a unique flagellin.

4.3 STUDY III

The probiotic *E. coli* strain Nissle 1917 was found to exclusively express cellulose, but not curli fimbriae at 37ºC. In study III, the regulation of the extracellular matrix components in Nissle 1917 and the role of cellulose in interaction with the host were investigated using HT-29 cells.

4.3.1 Nissle 1917 produces cellulose and curli fimbriae

Nissle 1917 was characterized for the expression of cellulose and curli fimbriae on CR agar plates and Calcofluor plates. The results indicated that Nissle 1917 expressed cellulose at 28ºC and 37 ºC, and curli fimbriae at 28ºC.

4.3.2 Curli fimbriae, but not cellulose is regulated by CsgD

To investigate the regulation of curli fimbriae and cellulose in Nissle 1917, mutants in genes encoding for the transcriptional regulator CsgD, the di-guanylate cyclase AdrA and the cellulose synthase BcsA were created. The combined results confirmed that Nissle 1917 produces cellulose at 28ºC and 37 ºC. In addition, CsgD activated the production of curli fimbriae at 28ºC, but biosynthesis of cellulose at 28ºC and 37 ºC did not require CsgD and the di-guanylate cyclase AdrA.

4.3.3 Regulation of cellulose biosynthesis by c-di-GMP

To investigate whether c-di-GMP is required for cellulose biosynthesis in Nissle 1917, YhjH and STM1827, phosphodiesterases which degrade c-di-GMP were expressed in Nissle 1917. The expression of YhjH and STM1827 lead to diminished expression of the pdar morphotype, suggesting that cellulose biosynthesis in Nissle 1917 is dependent

on c-di-GMP production. This was further verified by the application of an *in vitro* assay for cellulose synthesis [261]. When c-di-GMP was added to membrane fractions of Nissle 1917, cellulose biosynthesis was stimulated approximately 10-fold higher than background level. The stimulation was not observed when c-di-GMP was added to cell extracts of a *bcsA* mutant of Nissle 1917. Consequently, c-di-GMP does indeed regulate cellulose biosynthesis in Nissle 1917.

4.3.4 Interaction between Nissle 1917 and HT-29 cells

Cellulose was required for adhesion to HT-29 cells

By using the HT-29 cell culture model, Nissle 1917 and its *csgD* knock-out demonstrated significant adherence to the gastrointestinal epithelial cells. Since expression of curli fimbriae has not been observed in Nissle 1917 at 37 °C, the role of cellulose was investigated in the interaction between Nissle 1917 and epithelial cells. A cellulose deficient mutant showed more than 7-fold diminished adherence to HT-29 cells, which indicated that cellulose increased adhesion of Nissle 1917 to HT-29 cells. This result was confirmed in studies *in vivo* using the ileal loop model in mice.

The role of cellulose in adhesion to HT-29 cells in Nissle 1917 is in contrast to the role of cellulose in the commensal strain TOB1 [171]. In TOB1, production of cellulose decreased the adherence to HT-29 cells.

Cellulose suppressed internalization of Nissle 1917 by HT-29 cells and triggered IL-8 production of HT-29 cells

The potential of Nissle 1917 to invade intestinal epithelial cells was described to be very low [31] and could be confirmed in this study. Deletion of cellulose production in Nissle 1917 enhanced invasion more than 8-fold when compared to the wild type. In the internalization assay, the role of cellulose is similar in Nissle 1917 and the commensal strain *E. coli* TOB1.

Nissle 1917 can induce production of the proinflammatory cytokine IL-8 in epithelial cells [262]. IL-8 production was reduced by 28% in the cellulose-deficient derivative. The diminished IL-8 production may be a consequence of the reduced adherence of the cellulose-deficient mutant.

In summary, the role of cellulose in bacterial-host interaction is dependent on the strain background in *E. coli.*

4.4 STUDY IV AND PRELIMINARY RESULTS:

4.4.1 Characterization of bacteria colonizing urinary catheters

Biofilm formation is considered the key factor in pathogenesis of catheter-associated UTIs/bacteriuria. The attachment to the catheter surface supports the persistence of the bacteria in the urinary tract [263]. Although there is no effective way to prevent biofilm development on urinary catheters during long-term catheterization [65, 263], attachment to the urinary catheter however has been poorly characterized. Knowledge about biofilm formation on the catheter surface will be helpful to find rational

approaches for prevention or inhibition of infection. In this study, the biofilm behaviour was characterised for a collection of bacterial isolates from catheter and urine samples.

4.4.2 Bacteria constituting biofilm on urinary catheters

Bacterial isolates were recovered on all but one urinary catheter. In 91 % (40/44) of the catheters, bacteria belonging to the same species were commonly detected in the urine. In total, 179 bacterial isolates were recovered from the urine (83 isolates) and catheter (96 isolates) samples. Gram-positive and Gram-negative bacteria were equally often found (49 % respectively). Multispecies colonization, ranging between 2 and 5 species, was observed in 69% (31/45) of the patients. From both catheter and urine, the 5 most common recovered species were *E. coli*, *Enterococcus faecalis*, coagulase negative staphylococci (CNS), *Klebsiella pneumoniae* and *Staphylococcus aureus*. The population found on the catheter is represented when urine isolates are collected.

Bacterial species were isolated with a different frequency from patients with the catheter associated UTIs/bacteriuria or uncomplicated UTIs/bacteriuria [264, 265]. Thereby, the spectrum of infecting organisms widely varied with the underlying diseases, populations, antimicrobial treatments and catheterization periods [265-270]. In this study, the majority of species recovered were associated with gut or skin flora. This finding indicated that the colonization of the catheter could be caused by the normal flora of the patients or the staff handling catheterization.

4.4.3 Time of catheterization impacts recovered species

CNS was recovered significantly more often in patients having the present catheter < 1 month (n=21), ($p<0.05$). On the other hand, *S. aureus* ($p=0.005$), *E. faecalis* ($p=0.02$) and *Enterococcus cloacae* ($p<0.01$) were more prevalent from patients having had the present catheter for longer time, ≥ 1 month (n=23). In patients with a total time on catheterization ≥ 6 months, regardless of number of catheter changes, *E. cloacae* were more commonly found as compared to shorter catheterization times ($p\leq0.01$). *E. coli* and *K. pneumoniae* were recovered independent of the time of catheterization.

In the present study we aimed to detect all culturable bacteria present on the catheter or in the urine, even bacteria that are normally not brought to the attention of the clinician, since they may aid biofilm formation of more virulent species. In short time catheterization, CNS was commonly found, which is in line with other investigations [268].

4.4.4 Visualization of biofilm formation on urinary catheters

Biofilm formation on urinary catheters from patients was investigated by electron microscopy. On 4 out of 5 catheters surfaces, biofilm consisted of a community of both rod and coccus-shaped bacteria embedded in an extracellular matrix. The extent of extracellular matrix varied between different biofilms. Host cell components like single leukocytes in association with near-by bacteria were found in the biofilm.

4.4.5 Biofilm and adherence formation

The central character of CAUTI/bacteriuria is biofilm formation on catheter surface, which continually seeds urine with detached bacteria [263, 271]. Many urinary pathogens have been found to form biofilms [107, 272-274]

In this study, 95% of the isolates showed a capability to form biofilm *in vitro* although the extent of biofilm varied within a species and between different species. *P. aeruginosa* isolates showed the highest ability to form biofilm and adherence among all tested species. Beside adherence, all *P. aeruginosa* strains (n=8) formed pellicles, bacterial communities at the air-liquid interface of a standing culture. Pellet formation was observed significantly more often by *P. aeruginosa* than all remaining tested species ($p<0.001$).

4.4.6 Influence of urine on adherence to polystyrene surface

Among species with more than five isolates recovered, two isolates from each species were selected for further studies. The two selected isolates represented strains with the highest and lowest capacity to adhere to the polystyrene surface when grown in laboratory media. The following species were included *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *E. faecalis*, *S. aureus* and CNS. When grown in human urine, the adherence of the selected strains with a high capacity to form biofilm decreased significantly ($p\leq0.001$) compared to laboratory media. Moreover, no difference in adherence was observed between the two representative isolates of *E. coli*, *K. pneumoniae*, *E. cloacae*, and CNS which previously adhered in high and low amounts in laboratory medium. The isolates of *P. aeruginosa, K. oxytoca, E. faecalis* and *S. aureus* retained their different ability to adhere to polystyrene in urine as compared to laboratory medium. This observation indicated that urine suppresses biofilm formation, although it served as culture medium for bacteria as previously reported [275].

4.4.7 Influence of urinary catheters on biofilm formation

Adherence to the surface of two commonly used catheter types was studied. In this model, bacteria were allowed to attach to a catheter segment submerged in human urine. The representative bacteria showed different properties of biofilm formation on the catheter surface as compared to the polystyrene surface when cultivated in the urine.

In general, more bacteria adhered to the surface of SCLCs than the SCs, although all selected isolates adhered to the surface of both SCs and SCLCs. A species-dependent adherence pattern on the catheter surface was found. Both *P. aeruginosa* strains showed the highest adherence capacity while most other species did not significantly attach after 24 h of incubation. The selected isolates from *P. aeruginosa, E. coli* and CNS were further tested when cultured for a longer time, 96 h, on SCLCs. *P. aeruginosa* still adhered more than *E. coli* and CNS ($p<0.05$ respectively). And the biofilm formation by *P. aeruginosa* was significantly higher on SCLCs compared to SCs already after 24 h ($p<0.001$). After 96 h, biofilm increased further on the SCLCs ($p<0.05$ *vs* 24 h), but not on SCs.

Electron microscopy confirmed that *P. aeruginosa* significantly adhered to SCLCs whereby large microcolonies with extensive production of extracellular matrix were formed (Figure 5), while the other species attached to a lesser extend with only some bacteria attaching to the inner surface of the SCLCs. It was reported that the fungus *Candida albicans* showed increased biofilm formation on the latex catheter surface than on all silicone surface [276]. Therefore, the culture media, the property of the catheter surface and the species determine adherence formation of clinical isolates.

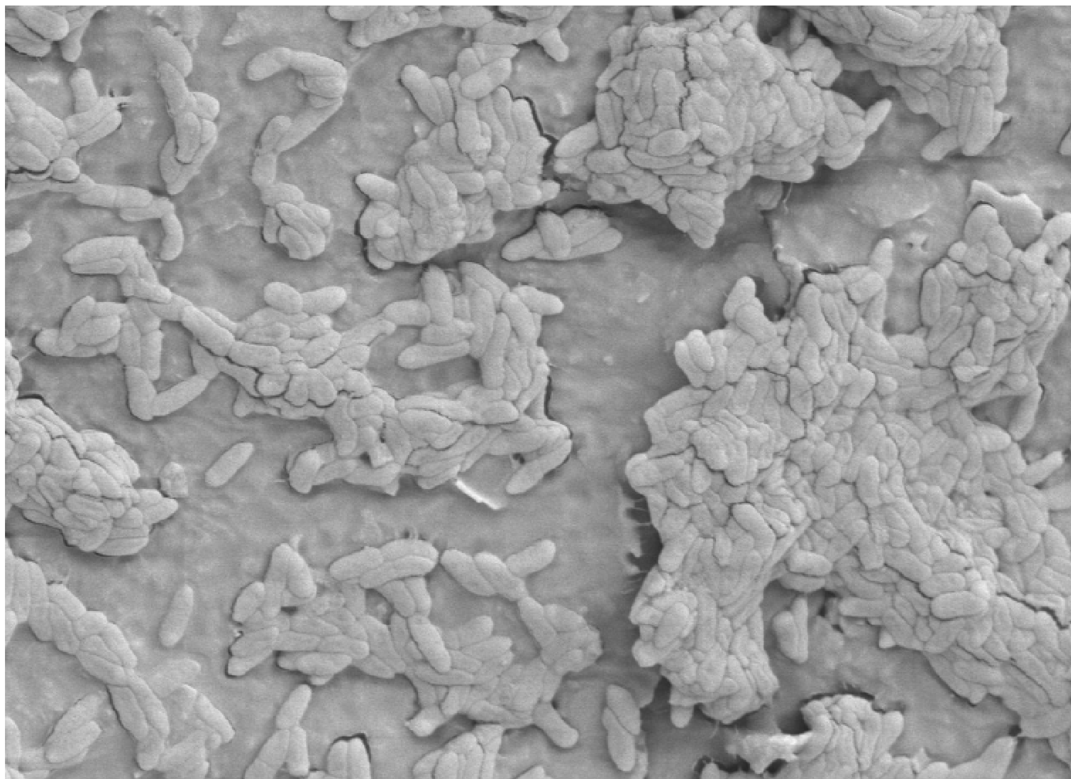

Figure 5. Biofilm formation by *P. aeruginosa* on SCLC
Scanning electron microscopy analysis of biofilms formed on the inner surface of the SCLC. Adherence of a representative *P. aeruginosa* strain incubated in human urine for 24 h without shaking at 37°C. (Courtesy of Heinrich Lünsdorf)

P. aeruginosa formed the highest amount of biofilm on polystyrene surface in culture medium and developed dramatically more adherence on both SCs and SCLCs in human urine when compared to the other species. This observation can explain why *P. aeruginosa* is more frequently isolated from CAUTI than from uncomplicated UTIs [265, 277, 278]. Many genes important for adherence to surfaces also contributed to virulence of *P. aeruginosa* in animal infection models [279, 280].

E. coli strains could adhere to some extend to both SC and SCLC surfaces, but adhered relatively poorly when compared to other tested species. However, Castonguay et al. observed that a non biofilm forming *E. coli* strain can develop a synergistic biofilm when co-cultured with adherence-proficient *Pseudomonas putida* [281]. In the current study, *E. coli* were often isolated together with other species from catheter surfaces. Those observations could help to explain why *E. coli* was more frequently isolated from uncomplicated UTIs (40.5%) than CAUTI (25.1%) [265]. However *E. coli* is still one of the most commonly recovered species from catheter samples. Therefore, biofilm formation by *E. coli* isolated from catheter surfaces requires further investigation.

4.4.8 Biofilm formation by *E. coli* isolates from catheters

Characterization of *E. coli* isolates

In total, 25 strains of *E. coli* were isolated from 12 patients. From 3 patients, the *E. coli* strains were only recovered from urine samples; from another 2 patients, 2 strains were isolated from urine and only 1 strain from catheter sample of each patient. Therefore, for 4 urine strains there is no counterpart from a catheter.

Strains isolated from catheters and urine shared the same morphotype, serotype, phylogenetic group, haemolysis activity, virulence pattern, swimming ability and similar biofilm formation ability, although the O-serotype could vary (Table 1).

Table 1. Characterization of *E. coli* strains recovered from urine and catheter samples of catheterized patients.

Patients	age	Strains	Serotype	Phylogenetic	prevelance of virulence genes						heamo lysin	Morphotype		Biofilm/ Adherenc	
					flu	*fluA*	*fluB*	*fimH*	*hly*	*papC*		28°C	37°C	28°C	37°C
1	97	UEB29	O86:H18	D	+	+	-	+	-	-	-	rdar	rdar	0/ 0	0.5/ 0.5
2	90	CEB25	Oraugh:H19	A	+	+	-	+	-	-	-	rdar	rdar	7/ 3	7/ 3
		UEB28	Oraugh:H19	A	+	+	-	+	-	-	-	rdar	rdar	7/ 3	7.5/ 3
3	84	CEB28	Ont:H-	A	-	-	-	+	-	+	-	saw	saw	0/ 0	0.5/ 0.5
		UEB32	Ont:H-	A	-	-	-	+	-	+	-	saw	saw	0/ 0	0/ 0
		UEB33	Oraugh:H-	A	-	-	-	+	-	+	-	saw	saw	1.5/ 0.5	2.5/ 0.5
4	81	UEB25	O150:H-	B1	-	-	-	+	-	-	-	saw	saw	0.2/ 0.2	0.5/ 0.5
5	80	CEB23	O40:H1	A	-	-	-	+	-	-	-	rdar	saw	2.5/ 0.5	0.5/ 0.5
		UEB26	O40:H1	A	-	-	-	-	-	-	-	rdar	saw	2.5/ 0.5	0.5/ 0.5
6	80	CEB22	Oraugh:H-	B2	+	-	-	+	+	+	+	rdar	saw	0.5/ 0.5	2.5/ 1.5
		UEB24	Oraugh:H-	B2	+	-	-	+	+	+	+	rdar	saw	0.5/ 0.5	1.5/ 0.5
7	69	UEB04	O5:H1	D	-	-	-	+	-	-	-	rdar	rdar	1/ 1	0/ 0
8	68	CEB27	O25:H4	B2	+	+	-	+	-	-	-	rdar	saw	3/ 2	0.5/ 0.5
		UEB31	O25:H4	B2	+	+	-	+	-	-	-	rdar	saw	1/ 1	0.2/ 0.2
9	65	CEB03	Ont:Hnt	A	+	-	+	-	-	-	-	saw	saw	0/ 0	0.2/ 0.2
		UEB02	Ont:Hnt	A	+	-	+	-	-	-	-	saw	saw	0/ 0	0/ 0
10	62	CEB01	O6:H1	B2	+	+	+	+	+	+	+	bas	bas	1/ 1	1/ 1
		CEB02	O6:H1	B2	+	+	+	+	+	+	+	bas	bas	2/ 2	1/ 1
		UEB01r	O6:H1	B2	+	+	+	+	+	+	+	ras	rdar	5/ 2	3/ 1
		UEB01s	O6:H1	B2	+	+	+	+	+	+	+	ras	ras	2/ 2	1/ 1
11	62	CEB29	O8:H25	B1	+	+	-	+	-	-	-	saw	saw	0.5/ 0.5	4/ 1
		UEB34	O8:H25	B1	+	+	-	+	-	-	-	saw	saw	0.5/ 0.5	1.5/ 0.5
		UEB35	Ont:Hnt	B1	+	+	-	+	-	-	-	mucoid	mucoid	1/ 1	4/ 1
12	58	CEB24	Oraugh:H18	D	+	+	-	+	-	-	-	bas	saw	2/ 2	2/ 2
		UEB27	O86:H18	D	+	+	-	+	-	-	-	rdar	ras	5/ 3	2/ 2

Factors associated with biofilm formation of catheter-associated *E. coli* isolates

A range of factors that contribute to biofilm formation of *E. coli* strains have been previously identified [119]. By correlating biofilm formation with the presence/expression factors required for biofilm formation in *E. coli*, factors contributing to biofilm formation in the present *E. coli* collection were identified.

Expression of type 1 fimbriae was reported to contribute to biofilm formation [102]. The universal expression of type 1 fimbriae in the current *E. coli* collection is indicated by mannose-sensitive agglutination of yeast cells, although extend of the agglutination was variable. Since the binding capability is determined by the amino acid sequence of the tip adhesion protein FimH [282], the sequence of the FimH was determined. A total of 11 amino acid exchanges in the FimH proteins were found. An alanine-to-valine substitution (A48V) existed in 3 strains which formed significantly more clumps ($p=0.001$) and total biofilm ($p<0.001$) at 37ºC than the other 13 isolates. This finding indicated that the A48V substitution could be important for the biofilm formation mediated by the FimH protein.

Expression of Ag43 encoded by the *fluA*, but not the *fluB* locus contributes to biofilm formation and intercellular adherence [113]. In accordance, isolates harbouring the *fluA* gene showed significantly more biofilm formation than isolates without the *fluA* gene.

Expression of LPS has been shown to affect biofilm formation in *E. coli* [283]. Isolates not expressing the O-antigen (rough strains) showed significantly higher total biofilm formation than isolates expressing the O-antigen. In detail, loss of O-antigen was associated with enhanced pellicle formation and adherence.

Role of biofilm genes in the adherence of *E. coli* to catheter surfaces

In order to confirm the role of biofilm genes on adhesion to catheter surfaces, isogenic mutants were created in selected strains to obtain *fluA*-deficient, O-antigen deficient, *fimH* deficient and *csgD*-deficient mutants, respectively. Since it was previously found that the medium (urine versus laboratory medium) and the surface (catheter surface versus polystyrene) affected adherence, adherence to catheters surfaces was investigated for bacteria grown in urine. The adherence was determined by evaluating pictures from scanning electron microscopy. Generally, all isolates and mutants show higher level of adherence on SCLCs than on SCs. The O-antigen deficient mutant adhered less to the surface of SCs, and the *fluA* mutant adhered less to both SCs and SCLCs. Interestingly, the *csgD* mutant showed enhanced adherence to both types of catheters. As a next step, the mutants need to be complemented with the genes on a plasmid to confirm that the observed phenotype is a consequence of the mutation.

5 CONCLUSIONS:

1. Gastrointestinal commensal *E. coli* were able to form biofilms which was associated with the expression of extracellular matrix components curli fimbriae and cellulose *in vitro*.
2. As extracellular matrix components of *E. coli* biofilms, the expression of curli fimbriae was correlated with enhanced adherence to, internalization in and IL-8 induction of human gastrointestinal epithelial HT-29 cells, while the production of cellulose diminished these effects.
3. Cyclic di-GMP, but not CsgD or AdrA activated cellulose biosynthesis in Nissle 1917. Production of cellulose mediated adhesion to and IL-8 production of gastrointestinal epithelial cell line HT-29.
4. Biofilm were formed *in vitro* by most bacteria colonizing urinary tract catheters, whereby the extent of adherence was influenced by the culture media and the surface. *Pseudomonas aeruginosa* formed dramatically more biofilm than other species under all analyzed circumstances. For *E. coli* isolates, the capability to form biofilms was associated with the presence of the *fluA* gene, expression of rough LPS and type 1 fimbriae. Bacteria adhered less to silicone catheters than to silicone coated latex catheters.

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