Bacterial colonization of the infantile bowel and the ileal pouch

with focus on *Escherichia coli*

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2010

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Doctorial thesis. Department of Infectious medicine, Clinical Bacteriology Section,
Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Sweden.

ISBN 978-91-628-8210-5
E-published: http://hdl.handle.net/2077/22936

Printed by Intellecta Infolog AB
Göteborg, Sweden 2010

ABSTRACT

The colonic microbiota is a source of inflammatogenic and potentially pathogenic bacteria, but also a source of immune maturation signals to the infant. Here, we have investigated the normal colonic microbiota, with focus on *E. coli* in Swedish and Pakistani newborn infants, as well as the microbiota of the ileo-anal pouch in colectomized patients. The aim was to identify factors that contribute to long-term persistence of *E. coli* strains in the microbiota.

E. coli strains can be divided into four phylogenetic groups, of which most strains causing extraintestinal infections belong to the B2 group. These strains also carry an array of virulence-associated genes often located on chromosomal regions, termed pathogenicity-associated islands (PAIs). We have previously showed that B2 strains carrying certain adhesins and virulence markers have increased capacity to persist in the microbiota of Swedish infants.

Patients colectomized due to ulcerative colitis who received a continent pouch constructed from ileum were followed for 3 years with respect to adaptation of the microbiota. There was a gradual change in the microbiota, shown as a gradual rise in the ratio of anaerobic to facultative bacteria from 1:1 in ileostomal to 400:1 in the pouch after 3 years, which did not differ significantly from the ratio in normal colonic microbiota cultured in parallel (1000:1). The counts of facultative bacteria were considerably higher in the pouch content than in control faeces during the first year after connecting the pouch to faecal flow. *Klebsiella* and *E. coli* were very common in ileostomal samples but *Klebsiella* isolation rate isolation rate declined drastically, while *E. coli* stayed high in the pouch. Among anaerobic bacteria, bifidobacteria isolation rates increased rapidly over time reaching 88 % i.e. similar as in controls after 4 months, while *Bacteroides* did not reach the levels seen in controls until 10 months after closure. However, population levels of anaerobes in general, and bifidobacteria and *Bacteroides* in particular, remained considerably lower in pouch faeces than in control faeces.

E. coli capable of persisting in the gut microbiota of Swedish infants for >12 months carried a range of pathogenicity islands (e.g. PAI I, II_{CFT703}, $IV_{536,}$ $II_{J96,}$ and PAI_{usp}) while intermediate (1-11 m), or transient (< 3 w) colonizers had fewer of these traits. Although *E. coli* isolated from the ileal pouch most often belonged to phylogenetic group A (p = 0.006), group B2 strains were better at persisting and were more often found on biopsies, i.e. in the mucosa-adherent population. Long-term persisters also carried a range of virulence genes. Group B2 strains from pouches significantly more often carried the *sfaD/E* gene, than did B2 strains from the colon of healthy individuals.

In Pakistani infants, persistence in the bowel microbiota was associated with *papC* and *iutA*, but not B2 origin. Compared with B2 strains from Swedish infants, Pakistani B2 strains significantly less often carried several virulence genes (*fim H*, *papC*, *papG class III*, *sfaD/E*, *neuB*, *hlyA*) and the high pathogenicity island (PAI IV_{536}).

Our studies suggest that the bigger arsenal of virulence factor genes for extra-intestinal infections the longer *E. coli* can reside in the gut/pouch microbiota. However, different human populations differ in their *E. coli* composition and their traits favouring persistence in the gut microbiota.

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. Östblom AE, Bengtsson J, Barkman C, Öresland T, Börjesson L, Simrén M, Wold AE and Adlerberth I. **A longitudinal study of the ileal pouch microbiota using quantitatively culture.** *In manuscript*

II. Östblom AE, Adlerberth I, Wold AE and Nowrouzian FL. ***Escherichia coli* pathogenicity island-markers and capacity to persist in the infant's commensal microbiota.** *Submitted*

III. Östblom AE, Karami N, Nowrouzian FL, Adlerberth I, Lundstam U, and Wold AE. ***sfaD/E* and other virulence genes are enriched in *Eshcerichia coli* persisting in the ileal pouch microbiota.** *In manuscript*

IV. Nowrouzian FL, Östblom AE, Wold AE and Adlerberth I. **Phylogenetic group B2 *Escherichia coli* strains from the bowel microbiota of Pakistani infants carry few virulence genes and lack the capacity for long-term persistence.** *Clin Microbiol Infect* 2009; 15: 466–472

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ABBREVIATIONS

CD	Crohn's disease
CFU	Colony forming units
GEIs	Genomic islands
IBD	Inflammatory bowel disease
IPAA	Ileal pouch anal anastomosis
MLVA	Multiple-locus variable-number tandem repeats analysis
PAIs	Pathogenicity islands
RAPD	Random amplified polymorphic DNA
UC	Ulcerative colitis

INTRODUCTION

THE GASTROINTESTINAL TRACT

The gastrointestinal tract comprises of the mouth, oesophagus, stomach and the small and lager intestine. It offers a stable environment for some bacteria to thrive in. However, these sites vary widely in pH level, nutrient content, O_2 levels etc, which is reflected by vast differences in bacterial population levels and composition at the different locations.

THE SMALL AND LARGE INTESTINE

The small intestine has a rapid peristalsis. Within short time (3-5 h) the contents emptied from the stomach reaches the colon. Here, the contents normally remain 30-60h. The small intestine is the main site of digestion, and proteolytic enzymes, bile and pancreatic juice are excreted into the lumen. Together, about 9.0 litres of fluid enters the small intestine every day. Secretion of alkaline fluids and bicarbonate ions raises the pH which is low (5.7-6.4) in the proximal part, where the highly acidic stomach contents are ejected, to 7.3-7.7 in the ileum. Water and nutrients are absorbed all along the small intestine (7 m). To increase the absorptive surface of the small intestine, the mucosa is folded and finger-like projections, villi, are stretched out into the lumen. In addition, the surface of each enterocyte has numerous projections, microvilli. These extensions give the small intestine an area of at least 200 m^2.

The large intestine absorbs 1-2 l of water a day. Instead of villi extending out in the lumen, the large intestine has narrow invaginations, crypts. These are lined by enetrocytes and mucus secreting goblet cells. The lack of villi makes the total surface much smaller compared to the small intestine, and it is about 0.12 m^2. Except from host derived nutrients for microbes, such as mucins and cells shed from the epithelium, a considerable amount of undigested carbohydrates reaches the colon each day.

The mucus gel layer is a lubricant protecting the epithelial against damage and dehydration (91) and a barrier against bacterial access to epithelial cells (230) but probably also the site of bacterial colonization (Fig. 1a). Mucus consists mainly of water (>95 %) and mucins which are large glycoproteins secreted by goblet cells, they consists of a peptide backbone with O-linked oligosaccharides. The peptide backbones have regions of variable number tandem repats (VNTR), sequences of amino acids with a high proportion of serine and threonine. These VNTR is the attachment site of O-linked glycosylation and are highly glycosylated regions (219), giving the glycoproteins a “bottle brush” appearance (Fig. 1b, c). Secretory IgA, lysozymes and defensins are dispersed in the mucus layer. S-IgA provides attachment of bacteria, but does not kill the bacteria and does not cause inflammation.

Introduction

Mucins can be linear or branched and neutral or acidic. In the colon the mucins are often acidic, terminating with sialic acid and/or sulphate (137, 141, 142). It has been suggested that sulphate groups protect mucins against bacterial degradation, as mucins in areas with a high bacterial load also are highly sulphated (188). MUC2 a secreted mucin is predominant in the large intestine (77) and its oligosaccharides are more heavily sialyated in the small intestine and heavily sulphated in the large intestine (in rat) (111).

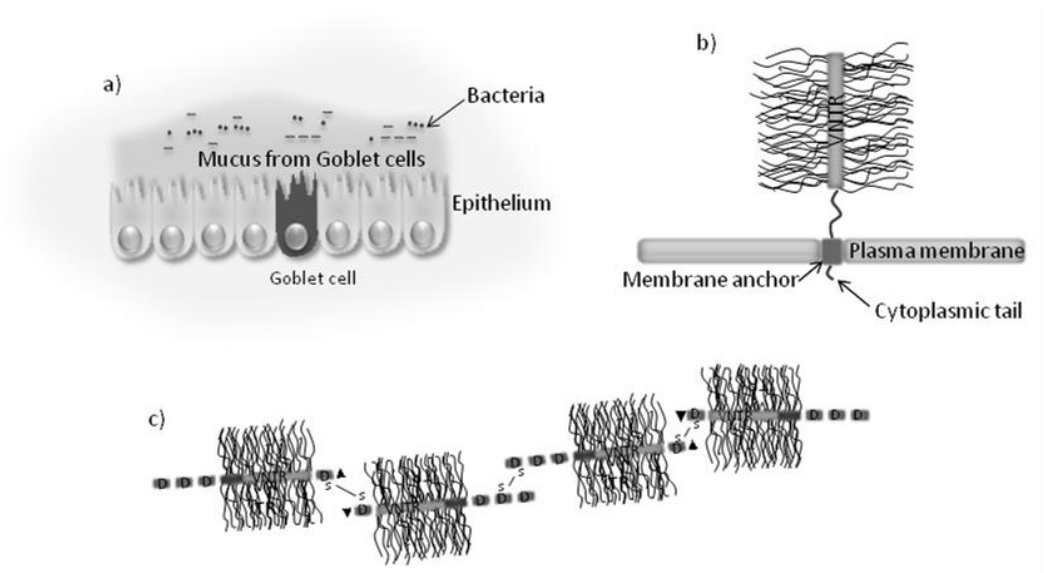

Figure 1. Mucus is a viscous gel of glycoproteins and water, acting as a lubricant and protecting the eptihelium against damage from intestinal contents and a) prevents bacteria to reach the epilium. b) The glycoprotein forms a "bottle brush" structure. MUC 1 is the only human membrane anchored mucin molecule known. c) All other mucins are secreted and disulfide bridges forming oligomeric mucins.

THE GASTROINTESTINAL MICROBIOTA

THE ADULT MICROBIOTA

Most microbes that are ingested are killed by stomach acid. In comparison, the newborn infant produce much less acid in the stomach, this facilitates its colonization. From the acidic stomach they are swept away through the small intestinal tract where they are showered by bile and enzymes. In the colon, contents move at a slower pace and a highly anaerobic milieu prevails. These results in very different bacterial communities in the small intestine compared to the large intestine.

The small intestinal microbiota

Cultivation studies have concluded that there is a gradient of aerobic bacteria in the small bowel, 10^4-10^5 in the jejunum to 10^7-10^8 in the distal ileum. Anaerobes are not that commonly found and in lower counts than aerobes, 10^2-10^5 in the upper small bowel and 10^5-10^7 in the ileum (223).

In the age of molecular microbiology it is confirmed that facultative anaerobes are the most abundant in jejunum. The bacteria mostly found in the gut content are lactobacilli, streptococci, *Enterococcus*, γ-proteobacteria, which include the *Enterobacteriaceae*. (86) The mucosa in the distal ileum as well as colon and rectum is dominated by *Bacteroides*. No major difference is seen between these locations (232), but slightly higher densities of bacteria and fewer bifidobacteria in the mucosa of terminal ileum than in colon (8).

The large intestinal microbiota

All three domains of life are found in the colon of adult humans, i.e. bacteria, archaea and eukarya. Bacteria reach the highest density and are the most studied group. They reach population numbers of 10^{14}, i.e. 10^{11}-10^{12} CFU/g (ml) faeces and thereby outcompete our own cells by a factor of 10. Despite the high density, only 8 of 55 known bacterial phyla are found in the human gastrointestinal tract, and 5 of these are rare (reviewed in (13)). Between 500 – 1000 bacterial species have been estimated to be able to inhabit the human gut. Table 1 shows the main bacterial phyla and groups found in the human colonic microbiota. Some report a discrepancy between the microbiota found in contact with the mucosa and the faecal microbiota (244). Others claim that bacteria do not come in contact with the epithelial cells but rather are trapped in the mucus, and that the same micobiota is found in biopsies as in faeces (230).

Short-chain fatty acids, primarily acetate, propionate and butyrate are produced within the intestinal lumen by bacterial fermentation of mainly undigested carbohydrates. Butyrate is an important energy source for colonic epithelial cells and may also have an anticarcinogenic and anti-inflammatory potential (reviewed (84)).

Table 1. The main bacterial groups and selected species found in human colonic microbiota.

Bacterial phyla and groups	Species that are common or otherwise important
Facultative bacteria	
Proteobacteria	
Enterobacteriaceae	*Escherichia coli*, *Klebsiella* spp, *Enterobacter* spp. *Citrobacter* spp, *Proteus* spp.
Firmicutes	
sreptococci	
enterococci	*Enterococcus faecalis, E. faecium*
Anaerobic bacteria	
Firmicutes	
clostridial cluster XIVa (*C. coccoides* group)	*Eubacterium rectal*[1], *Roseburia faecis*[1], *R. intestinalis*[1]
clostridial cluster IV (*C. leptum*)	*Faecalibacterium prausnitzii*[1],
clostridial cluster XVIII	
clostridial cluster IX	
clostridial cluster XI	*C. difficile*
clostridial cluster XVI	
clostridial cluster I	*Clostridium perfringens, C. butyricum, C. tetanii, C. botulinum*
lactobacilli	Lactobacillus acidophilus group, e.g. *L. gasseri, L. paracasei, L. rhamnosus*
Bacteroidetes	
Bacteroides	*B. fragilis, B. thetaiotaomicron, B. ovatus*
Actinobacteria	
Bifidobacterium	*Bifidobacterium adolescentis, B. catenulatum, B. longum, B. bifidum, B. rectale*
Atopbium cluster	
Fusobacteria	*Fusobacterium necrophorum*

Firmicutes

According to 16S based methods, there is no doubt that the adult human intestinal microbiota is dominated by the phylum *Firmucutes* (52, 89, 220).

Clostridia

Clostridia were originally defined as Gram-positive anaerobic rods forming spores. Spores are formed in drought and harsh environments and clostridia can therefore spread, e.g. via

air. *Clostridium* contains more than 100 species. It is genetically a very heterogeneous group, consisting of 19 clusters, which nowadays also contain non-spore-forming organisms (34).

Cluster XIVa (*C. coccoides* group) dominates in the colon of adults, with about 25-60% of total clones (52, 89, 220). *Roseburia* and *Eubacterium* are important butyrate producers, *Roseburia intestinalis and E. rectale* are two examples from cluster XIVa and their related sequences makes up about 7 % of the total bacterial diversity (10).

Cluster IV (*C. leptum* group) is the second largest group in the adult colon (52, 89, 220). *Faecaliumbacterium prausnitzii* is an important member of this cluster, which was transferred from the phylum *Fusobacteria* to cluster IV in 2002 by Duncan *et al*. (49).

Cluster I is the overall largest group including both pathogens such as *C. tetani* and *C. botulinum* and opportunistic pathogens such as *C. perfringens* and more harmless members, such as *C. butyricum* (34). *C. perfringens* is not uncommon in the normal intestinal microbiota (144, 191) but also the most frequently isolated clostridia from clinical specimens, such as food-borne gastroenteritis and enteritis necroticans (38). *C. difficile* is one other potential pathogen, belonging to cluster XI, and is found in the infantile microbiota (5, 191) but as the microbiota becomes more complex it often disappears (62). It is a common cause of antibiotic associated diarrhea and may cause the life-threatening disease pseudomembraneous colitis (70). The cause of both conditions is treatment with broad-spectrum antibiotics, which kills competing anaerobes and gives *C. difficil*e the chance to expand and produce toxins that cause inflammation and damage to the gut mucosa.

Lactobacilli

Lactobacilli belong to the class: *Bacilli*, order: *Lactobacillales*, family: *Lactobacillaceae* and are anaerobic rods or coccobacilli, with varying oxygen tolerance. They are members of Lactic Acid Bacteria (LAB), a functional group of Gram-positive, catalase negative, bacterial species that produce lactic acid as the main end-product of the fermentation of carbohydrates (61). This lowers the pH and makes the environment hostile for other bacteria; which is exploited in the use in fermentation of food. They are almost ubiquitous: found in all environments where carbohydrates are available, such as food (dairy products, fermented meat, sour dough, fruits and beverages), respiratory, gastrointestinal and genital tract of humans and animals, in sewage and plant material.

Lactobacilli can be isolated from approximately 80 % of adults faeces, but often in low counts (62) and is often used in probiotics and are considered to promote “god health”.

Enterococci

Enterococci are Gram-positive, facultative anaerobes found as single cocci or in chains. They belong to class: *Bacilli*, order: *Lactobacillales*, family: *Enterococcaceae*. Enterococci are also considered as members of the Lactic Acid Bacteria (LAB) group, they produce bacteriocins and are found in different sorts of food and are used as well as probiotics, starter and protective cultures and feed supplements (113).

Introduction

Enterococcus faecalis and *E. faecium* are the most common species in the intestinal microbiota of which the former is more prevalent (235). Despite that it is a normal member of the gastrointestinal tract, it is a rather common nosocomial pathogen, favoured by their inherent resistance to many commonly used antibiotics. Urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infections, bacteraemia and neonatal sepsis are examples of infections caused by enterococci, of which urinary tract infections is the most common infection (180).

Staphylococci

Staphylococci belong to class: *Bacilli*, order: *Lactobacillales*, family: *Staphylococcaceae.* They are Gram-positive, facultative anaerobes, cocci, which are normally found on the skin and mucous membranes.

S. aureus, is a common and feared pathogen causing skin and wound infections, abscesses, osteomyelitis, septic arthritis, and septicaemia. *S. aureus* is separated from a range of species, e.g. *S. epidermis*, *S. hemolyticus* etc. collectively referred to as coagulase negative staphylococci (CoNS) by its production of coagulase. Staphylococci are generally not considered as gut microbes. *S. aureus* was isolated in faeces of 24 % of Swedish healthy women (131). In Sweden CoNS are nowadays the first colonizers of the infantile intestine and *S. aureus* is as common as *E. coli* in the first 6 months (5).

Bacteroidetes (previously Cytophaga-Flavobacterium-Bacteroides)

Bacteroidetes is the largest group of gut bacteria after *Firmicutes* and make up 16 – 31 % of the phylotypes found in the gut microbiota by molecular methods (52, 89, 220). They are Gram-negative strict anaerobic rod-shaped bacteria and have a large ensemble of genes involved in acquiring and metabolizing carbohydrates (reviewed in (13)). *Bacteroides* can degrade a wide range of carbohydrates and the major end products from carbohydrate metabolism are succinate, propionate and acetate.

B. fragilis is common in the gastrointestinal tract, it is also a common cause of anaerobic bacteraemia (38).

Proteobacteria

Proteobacteria are common but usually not dominant in the intestinal microbiota (205). *Enterobacteriaceaea* is a family of Gram-negative, rod shaped, facultative anaerobes belonging to class Gammaproteobacteria and order *Enterobacteriales*. *Escherichia* (e.g. *E. coli*), *Klebsiella*, *Enterobacter, Citrobacter* and *Proteus* are normal members of the human microbiota. *E. coli* is common in most adults whereas *Klebsiella* and *Enterobacter* are more common in neonates (2) but not in adults (62).

E. coli is the most common cause of urinary tract infections, but also *Klebsiella*, *Enterobacter*, and *Proteus* can cause both uncomplicated cystitis and pyelonephritis (190).

Fusobacteria

Fusobacteria are anaerobic, Gram-negative, pleomorpic and/or filamentous rods, of which *Fusobacterium nucleatum* consistently demonstrate a fusiform morphology with tapering ends. They comprise of 14 species and are normal members of the oral cavity microbiota, were they can co-aggregate with other species and are important in plaque formation (235). Using culture independent methods, *Fusobacteria* in the colonic microbiota are found in few hosts (52, 89, 220), and in low abundance, < 1 % (52, 171).

F. nucleatum is the *Fusobacterium* spp. most often isolated from clinical specimens, for example upper respiratory, genital and gastrointestinal tract infections (38).

Actinobacteria

Class: *Actinobacteria*, subclass: *Actinobacteriadae*, order: *Bifidobacteriales*, family: *Bifidobacteraiceae*, genus: *Bifidobacterium*.

Bifidobacteria

Bifidobacteria are Gram-positive, anaerobic (some are aerotolerant) bacteria with bifid (Y-shaped) morphology when grown on some media. They can be found in six different ecological niches, the human intestine, oral cavity, insect intestine, sewages and food.

The genus comprise of 30 species and *B. catenulatum,* is the most common and found in almost all adults followed by *B. longum* and *B. adolescentis* (140). However, there is a discrepancy between culture dependent and culture independent methods. Actinobacteria/bifidobacteria are cultivated in number up to 10^{8-9} , and make up about 3% of total bacterial populations using FISH (64) but are not found in the mucosa (89, 233). They are reported only to make up a minor part of the faecal microbiota (52) using cloning and sequencing. This may relate to low copy number of the 16S rRNA gene.

The genus *Bifidobacterium* is traditionally listed as Lactic Acid Bacteria (LAB), but is poorly phylogenetically related to genuine LAB (61). They have extremely low pathogenic potential and are often used as probiotics.

Others

Verrucomicrobia is a phylum common in soil, and contribute up to ~10 % of total bacterial 16S rDNA in soil (195). *Akkermansia municipala* is the dominating mucin degrading bacteria, of this phylum, in the human intestine (42). Wang *et al.* found *Verrucomicrobia* to account for 6 % of the clones in colonic biopsies (232) and Eckburg *et al.* found all *Verrucomicrobia* sequences to be *Akkermansia munciphila* (52).

The *Lentisphaerae* phylum was proposed in 2004, found to make up a minor < 1% of the bacterial community in the Pacific and Atlantic Ocean (31). The phylum includes *Victivallis vandensis* found in human faeces (243).

Introduction

Yeasts, mainly *Candida* species, are found in intestinal microbiota of 35 - 40 % of healthy humans (62).

ESTABLISHMENT OF THE MICROBIOTA

The intestine of a newborn is sterile and the colonization process starts during birth when the infant is exposed to bacteria from the vagina and maternal intestine. Microbial establishment in the gastrointestinal tract and colonization is related to a number of environmental and host-related factors, including delivery mode, feeding pattern and bacterial load of the immediate environment. Still, a general pattern of colonization and succession can be described. Bacteria usually appear in the faeces of the infant within a few hours. The high oxygen content prevents obligate anaerobes to expand and the classical first colonizers are facultative anaerobes, such as enterococci, *Enterobacteriaceae* and streptococci (139, 191), such bacteria can perform both aerobic and anaerobic metabolism and can replicate in both oxygen-rich and completely anaerobic environments. A recent study has shown that the "classical" pattern has changed in Sweden and staphylococci are now more frequently found than *E. coli* in the infantile gut during the first two months of life (5). Although *E. coli* is the only member of the *Enterobacteriaceae* family found in sufficient numbers of adults, infants are commonly colonized by, *Klebsiella* and *Enterobacter* as well (2). Whereas *E. coli* is a strict gut colonizer, found only in faeces of man and (other) animals, other members of the *Enterobacteraiceae* family are common in nature, e.g. on plants and fresh vegetables.

As oxygen is consumed by the facultative bacteria, obligate anaerobes expand. Anaerobes colonize the intestine within the first week, especially bifidobacteria, followed by *Bacteroides* and clostridia (139, 191). *Clostridium perfringens* is the most commonly found clostridia in infants (144, 191), but *C. difficile* is also rather common (5, 191). Over time the number of anaerobic species increase and the microbiota becomes more complex. Within two years the micobiota have established and is quite similar to the adult microbiota (54, 217).

These studies are all based on cultivation of the faecal microbiota. Few studies have been done using DNA based methodology, and molecular studies have shown that at two months of age the discrepancy between cloning and sequencing is not very significant (231). The major difference may be that certain *Ruminococcus* spp. are detected by DNA based methodology (59, 231).

ESCHERICHIA COLI

E. COLI, A NORMAL INHABITANT IN OUR INTESTINAL MICROBIOTA

Escherichia coli, or *Bacterium coli commune* (the common colon bacillus), as it was called when first described by Escherich in 1885, is a Gram-negative rod-shaped bacterium, belonging to the family *Enterobacteriaceae. E. coli* is widely distributed in the intestine of humans and animals and is the predominant facultative anaerobe in the bowel, but still a minor part of the total microbiota.

Some *E. coli* strains cause disease in the intestine, e.g. EHEC, enterohemorrhagic *E. coli,* ETEC*,* enterotoxigenic *E. coli* etc. (108). These are not members of the normal microbiota. Other *E. coli* strains whose normal habitat are the intestine can cause opportunistic infections when introduced into extraintestinal sites, mainly urinary tract and infant septicaemia and meningitis but also wound infections, septic arthritis and osteomyelitis are seen.

E. coli is the most common cause in urinary tract infections (167). Pyelonephritis is the most severe form, and is caused by bacteria entering into the kidneys, where they cause intense inflammation characterized by high-grade fever. Cystitis, infection of the urinary bladder is the less severe form and the bacteria can also establish without symptoms (asymptomatic bacteriuria). *E. coli* can also cause neonatal septicaemia and meningitis (167). The isolates responsible for urinary tract infections in a given individual often match the rectal isolates from the same person (147, 239).

E. coli has a clonal genetic population structure (162) made up mainly by four phylogenetic groups: A, B1, B2 and D (88). Strains isolated from extraintestinal sites belong mainly to the B2 group and to a lesser extent to the D group (22). Both of these groups have a higher prevalence of extraintestinal virulence determinants than group A and B1 (178).

VIRULENCE FACTORS

A pathogen is an organism that bears ("gen") suffering ("pathos") upon another organism, this term is most commonly used to refer to infectious organisms. Virulence derives from the Latin word “virulentus” (virus = poison), meaning full of poison, and is the degree of pathogenicity of an organism. To colonize extraintestinal sites, such as the urinary tract, the bacteria have some obstacles to overcome. The bacteria have to evade the innate immune response, prevent being flushed away and also retrieve important nutrients, such as iron. Traits that aid the bacteria to overcome these problems and to cause infections are called virulence factors (Fig. 2).

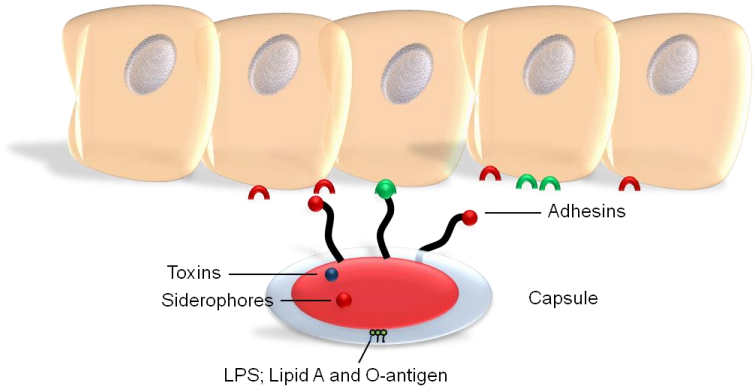

Figure 2. Examples of virulence factors involved in extra-intestinal infections by *E. coli*

Lipopolysaccharide (endotoxins)

Lipopolysaccharides are a constituent of the outer leaflet of the outer-membrane of Gram-negative bacteria (Fig. 3). It consists of an O-specific polysaccharide chain, a core oligosaccharide and a lipid component anchored in the membrane, termed Lipid A. Bacteria with a complete lipopolysaccharide side chain are termed smooth and those lacking a part of it; rough. The Lipid A part is responsible for the toxic and inflammatogenic action of LPS, the O-polysaccharide side chains of LPS can sterically hinder the access of complement components to the bacterial membrane (183). Traditionally pathogenic *E. coli* are classified by their O-antigen, the K-antigen (capsule antigen) and their major flagella protein component (flagellin) the H antigen. Approximately 175 *E. coli* O-antigens are described today (199) of which strains of O-serotype O1, O2, O4, O6, O7, O18 and O75 are most often associated with urinary tract infections (167).

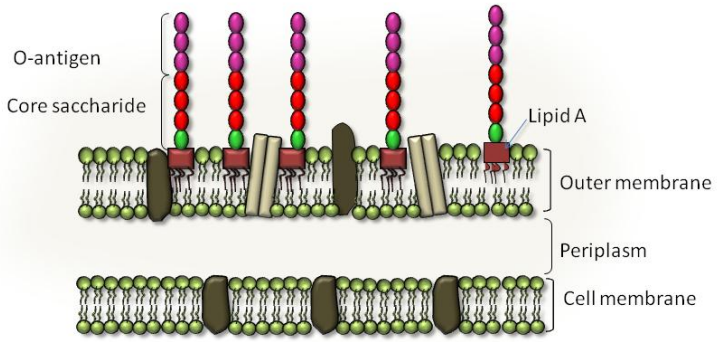

Figure 3. The outer layer of the outer membrane of Gram-negative bacteria consists to a large extent of lipopolysaccharides. Lipid A, the anchor to the membrane is the inflammatogenic part, whereas the O-polysaccharide LPS can sterically hinder the access of complement components to the bacterial membrane and induces specific antibody production.

Capsules

Capsules are composed of linear polymers of repeating carbohydrate subunits that sometimes include amino acids or lipid components. They are very hydrophilic and coat the cell, thereby protecting the cell from phagocytosis (90).

Over 80 capsule types (K antigen) are described (167) and a few of these are enriched among infectious strains. K1 is found in more than 80 % of *E. coli* in neonatal meningitis and is commonly found in neonatal septicaemia and childhood pyelonephritis (102, 187). It is an α-2,8-linked linear polymer of sialic acid (NeuNAc), sialic acid is found on the surface of mammalian cells and the K1 polysaccharide is only weakly immunogenic (212). K5 is a linear polymer of 4-linked α-N-acetyl glucosamine and 4-linked β-glucuronic acid, and resembles heparin, which is probably why it is only weakly immunogenic (234). It is associated with urinary tract infections and septicaemia (103).

Fimbriae and adhesins

Bacterial adherence to host cells is a first step in colonization. It prevents the bacteria to be swept along by normal flow of body fluids, such as intestinal contents, urine etc. Furthermore, adherence places the microbe close to the mucosal surface, where nutrients and oxygen are plentiful. For example, it has been shown that only *E. coli* cells that are in close contact with the mucosa are replicating; those in the lumen are dormant because a lack of nutrients in this site (228).

Fimbriae are filamentous organelles carrying adhesins that recognize carbohydrates and sometimes proteins exposed on host cell surfaces. Bacterial adhesins are very specific in their recognition. Figure 4 shows a schematic fimbriae and examples of *E. coli* fimbriae and cell types they adhere to, are shown in Table 2.

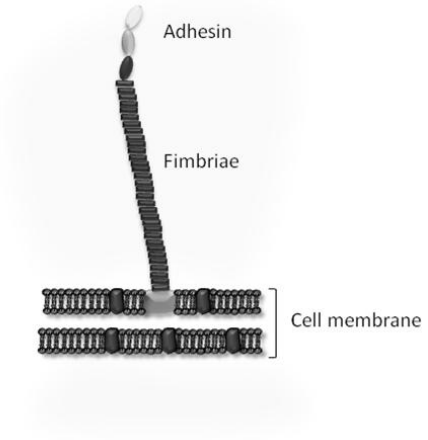

Figure 4. A schematic figure of a fimbriae and its adhesins.

Table 2. Examples of adhesins and the celltypes they adhere to: *in vivo* and *in vitro* studies.

Adhesin	Human celltypes it adheres to	Reference
Type 1 fimbriae	bladder epithelium, Tamm-Horsfall glycoprotein, buccal cells, vaginal cells, colonic and ileal enterocytes	(3, 58, 130, 163, 166, 238)
P fimbriae	kidney, urinary bladder, urethra, colonic and ileal enterocytes	(3, 119)
S fimbriae	Bladder and kidney epithelium, brain endothelium, colonic and ileal enterocytes	(3, 117, 121, 181)
F1C fimbriae	Collecting ducts and distal tubules of kidneys, renal tubulus cells	(12, 114)

Type 1 fimbriae

Type 1 fimbriae were first described by Duguid as early as 1955 (48). They bind to mannose-containing receptors on glycoproteins and mediate adherence to various cell types (Table 2). Type 1 fimbriae are encoded by the *fim* gene cluster and fimH is the actual adhesin responsible for the binding (123). Type 1 fimbriae bind to mannose-containing oligosaccharide chains on secretory IgA that is abundant on mucosal surfaces (237).

Its main biological role may be to provide adhesion to mucus in the large intestine (168). Type 1 fimbriae are commonly found on *E. coli* isolated from faeces of healthy individuals (Table 3).

P fimbriae

The name P fimbriae, “Pyelonephritis-associated pili” comes from the high prevalence among strains that cause pyelonephritis (104). They are strongly associated with urinary tract infections, in particular pyelonephritis (94). P fimbriated *E. coli* adhere to cells in the urinary tract as well as the intestine (Table 2).

P fimbriae are encoded by the *pap* operon, and *papC* codes for an outer membrane assembly protein /usher channel (225) and *papG* for the adhesin recognizing Galα (1-4) Galβ moieties in the globoseries of membrane glycolipids (135). The *papG* adhesin occurs in three different varieties, termed class I, II and III. They bind to the same Galα 1→4 Gal moiety, but this is recognized when present in globotriacocylceramide (GbO_3), globoside (GbO_4) and the Forssman antigen (GbO_5), respectively (218).

The class II allele is primarily associated with human pyelonephritis and bacteraemia while class III (*prs*) papG allele is common in human cystitis and genitourinary infections in dogs (93, 96, 98, 172). The class I allele is rare, little is known about its role in disease and commensal colonization. P fimbriae are not as common in faecal *E. coli* from healthy persons as type 1 fimbriae (Table 3).

S fimbriae

S fimbriae are named by its binding to terminal sialyl-galactoside residues (118). S fimbriae are the most important adhesins in neonatal meningitis (120) but are also common among strains causing urinary tract infections (169). S fimbriated strains adhere to a variety of cells (Table 2).

S fimbriae genes are cloned from two pathogenic *E. coli*, a uropathogenic isolate*, E. coli* 563 (sfaI) (82) and a meningitis isolate, *E. coli* IHE3034 (sfaII) (81). The sfa II variety adhere more strongly to human colonic and ileal cells than does the sfa type I (3). The major sequence differences between the two varieties are found in the sfaA subunit (81) which makes up the fimbrial shaft (202), wheras the *sfaS* which codes for the specific adhesin (202) have a quit similar sequence (81). *sfaD/E* presumably involved in transport and assembly of the fimbrial subunits (203).

Genes for S fimbriae are less common among *E. coli* in faeces from healthy humans than are both type 1 fimbriae and P fimbriae (Table 3).

F1C fimbriae

F1C fimbriae are closely related to S fimbriae and the gene clusters *sfa* and *foc* are similar in many aspects, but the adhesins differ in receptor specificity (170). F1C recognizes galactosylceramide containing glycolipids (12, 112) and adheres to cells in the kidney cultured renal tubulus cells (114) (Table 2). It is found in 14 - 30% of *E. coli* causing urinary tract infections and is rare, 0 – 7 %, among faecal *E. coli* isolates (Table 3) (175, 211).

Exo-toxin

Hemolysin

Many Gram-positive and Gram-negative bacteria produce hemolysins, i.e. toxins that lyses red blood cells. α-hemolysin is a secreted toxin and the most commonly produced in *E. coli* (29) encoded by the *hlyA* gene (74).

Hemolysin A forms pores in host cells in a Ca^{2+} dependent manner (24) and cell lysis occurs when levels of hemolysin are high (18). Hemolysin lyses erythrocytes from all mammals and fish (186) and is cytotoxic to other cells, as well, including leukocytes (67). The advantage for the bacterium is thought to be the release of nutrients from destructed host cells, including iron, which is necessary for bacterial growth.

About 50 % of urinary tract infection isolates carry *hlyA* and the percentage increase with disease severity (138).

Siderophores

Iron is absolutely essential for many prokaryotic and eukaryotic cellular functions. *E. coli* uses iron for oxygen transport and storage, DNA synthesis, electron transport and metabolism of peroxides. Iron is limited in many environments, and in mammalians iron is usually in a complex with host proteins (haemoglobin, ferritin, transferin and lactoferrin) that bind with high affinity.

Siderophores are high-affinity extracellular ferric chelators which are first secreted by bacterial cells to scavenge Fe^{3+} from host iron-binding proteins. Figure 5 shows a schematic siderophore system. This siderophore- Fe^{3+} complex is then taken up by a specific outer membrane receptor protein on the bacterial surface and the iron is released intracellular for use in the bacteria. Examples of siderophores common in *E. coli* are enterobactin, salmochelin, yersiniabactin and aerobactin. These are especially prevalent in uropahogenic *E. coli* (94).

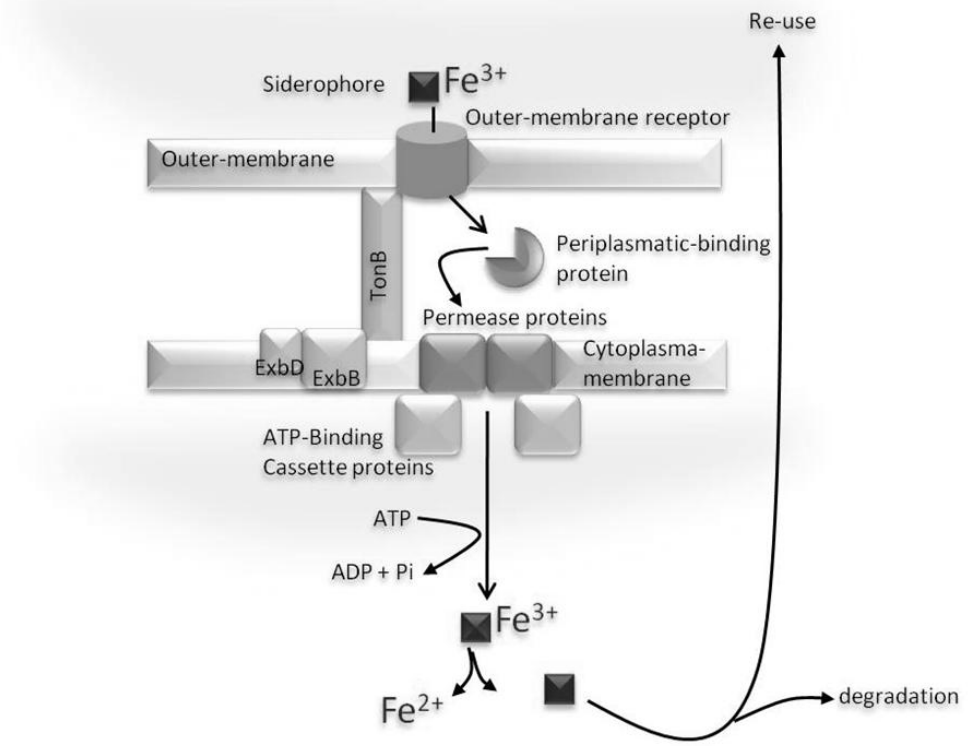

Figure 5. A schematic picture of a siderophore system.

Table 3. Virulence factor genes in this thesis and their prevalence in faeces from healthy humans.

Investigated genes	Virulence factor	Function	Prevalence in faecal strains (%)**
fimH	Type 1 fimbriae	Mannose specific adhesin (48)	71-92
papC	P fimbriae	Outer membrane assembly protein /usher channel (225)	20-37
papG	P fimbriae	Adhesin recognizing Galα (1-4) Galβ moiety (135)	
papGI	P fimbriae	Preferentially binds globotriaosylceramide (GbO_3) (218)	
papGII	P fimbriae	Preferentially binds globoside (GbO_4) (218)	16-25
papGIII	P fimbriae	Preferentially binds Forssman antigen (GbO_5) (218)	3-8
sfaD/E	S/F1C fimbriae	Possibly biogenesis, minor subunit and transport for SFA/FOC (203)	0-26
hlyA	α-hemolysin	The hemolysin protein (74)	8-23
iutA	Aerobactin	Outer membrane receptor (39)	20-55
neuB	K1 capsule	Sialic acid synthase (11)	17-27
kfiC	K5 capsule	Glycosyltransferase (177)	5-8
malX	PAI I_{CFT073}(78)*	Phosphotransferase system enzyme II (184)	28-61
usp	PAI_{USP} (124)*	Presumed bacteriocin (173)	24-51

* used as markers for theses PASs
**based on the following studies (15, 65, 97, 105, 148, 153, 154, 196).

THE FLEXIBLE GENE POOL

Despite the fact that *E. coli* populations have a clonal structure (206) a large amount of genetic material can be exchanged between clones. The so called core genome codes for essential metabolic functions whereas the “flexible gene pool” or the “pan-genome”, codes for proteins that might be beneficial under certain circumstances.

The genome size in naturally occurring *E. coli* isolates can differ by up to 1Mb, ranging from approximately 4.5 to 5.5 Mb (17). This is primarily due to the insertion or deletion of a few large chromosomal regions, with overall gene order maintained between different strains (189).

The overall G+C content between bacterial species can differ significantly; but within a species the base composition is quite conserved. Therefore, regions of atypical C+C content relative to the relative genome can be identified as horizontally transferred DNA (127, 161). According to Touchon *et al*. who annotated the genome of 20 commensal and pathogenic *E.*

coli, the average *E. coli* genome contains 4721 genes, of these the core genome contains 1976 genes, and the pan-genome contains 17838 genes (227).

The flexible gene pool includes mobile or formerly mobile genetic elements, such as insertion sequences, transposons, integrons, plasmids and prophages as well as large unstable regions “genomic islands” (69).

Horizontal transfer

Horizontal gene transfer contributes to the diversification and adaptation of microorganisms. Transfer of large DNA blocks can occur through three different mechanisms: transformation, conjugation and transduction.

Transformation is uptake of free DNA directly from the environment. Parts of the foreign DNA are degraded but some can be incorporated into the host genome. Naturally transformable bacteria acquire a physiological state which enables transformation, termed “competence” (30).

Conjugation is cell to cell transfer of DNA. DNA is injected through a specialized apparatus that consists of a translocation channel spanning the membrane. This tube-like structure is termed a pilus (pilus = hair) in Gram-negative bacteria (30). Most of the identified conjugative systems are carried on plasmids, but they may also be encoded by chromosome-borne mobile genetic elements (MGEs). The latter referred to as integrative and conjugative elements (ICEs) (240).

Transduction is the transfer of DNA from one bacterium to another via viruses infecting bacteria, so called bacteriophages. The DNA is carried as passengers in their genome.

Genomic islands

Genomic islands (GEIs) are DNA sequences of atypical G+C content, which are capable of integration into the chromosome of the host and excision and transfer into another host. Genomic islands are suggested to have different evolutionary origins, such as conjugative transposons or integrative and conjugative elements, conjugative plasmids and prophages (101). Some are not mobile any longer; many genomic islands may in fact be defective integrative and conjugative elements (240).

Certain features are often seen in genomic islands, coupled to their mobility. Some tRNA genes represent hot spots for integration of foreign DNA. The 3´end of tRNA genes is often identical to the attachment site for bacteriophages and thereby integration site of certain plasmids and phages (185). Many genomic islands are inserted in the 3’ end of tRNA genes (201).

Some genomic islands are flanked by Direct repeat (DR) sequences are usually between 16 to 20 bp of perfect or nearly perfect sequence repetition. They are frequently homologous to phage attachment site and are probably generated during the integration of mobile genetic elements into the host. Direct repeat sequences acts as recognitions sequences for enzymes involved in excision of mobile elements and probably contribute to genetic instability (80).

Genomic islands are often flanked by insertion sequences (IS elements). These are small mobile genetic elements, capable of transposing within and between prokaryotic genomes. They provide sites of inverted repeats at which homologous recombination can occur, and can mediate incorporation of mobile genetic elements but can also contribute to excision (80).

Genomic islands often include traits such as sucrose and aromatic compound metabolism (68) mercury resistance and siderophore synthesis (126). According to their gene content they are often described as pathogenicity, symbiosis, metabolic, fitness or resistance islands (46, 201).

Pathogenicity islands (PAIs)

The concept of pathogenicity islands was originally founded by Hacker *et al.* in the late 1980s (79). A pathogenicity island is a genomic island bearing genes coding for virulence factors, and thereby contributes to the virulence of the host. They are present in a wide range of both Gram-positive and Gram-negative bacteria. Table 4 shows common features of PAIs according to the definition by Hacker and Figure 6 shows the general structure of a PAI.

Table 4. Common features of pathogenicity islands

Large distinct chromosomal regions (10 kb to more than 100 kb)
G +C contents differs from core genome
Present in pathogens, absent in benign relatives
Contains virulence genes
Inserted adjacent to tRNA genes
Frequently associated with mobile genetic elements, i.e., presence of DR and/or IS elements
Cryptic or functional integrases and tansposases
Chromosomally integrated conjugative transposons, plasmids and phages
Genetic instability (if functional mobility genes are present)
Mosaic structure

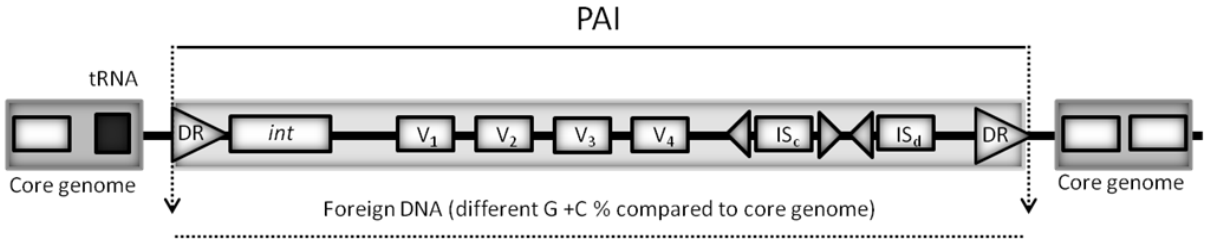

Figure 6. The general structure of a pathogenicity island (PAI).

PAIs found in uropathogenic E. coli

E. coli strains CFT073, J96 and 536 are archetypes of uropathogenic *E. coli*. The genome of these strains has been extensively investigated and a number of PAIs have been identified in these strains (Table 5).

Table 5. Examples of Pathogenicity islands in common uropathogenic *E. coli* strains and their virulence factor genes

Pathogenicity island	Virulence associated genes	Reference
I_{CFT073}	α-hemolysin, P fimbriae, aerobactin	(107)
II_{CFT073}	P fimbriae, iron-regulated genes	(182)
I_{536}	α-hemolysin,, F17-like fimbriae, CS12-like fimbriae	(45)
II_{536}	Hek adhesin, P-related fimbriae, alpha-hemolysin, hemaglutinin-like adhesin	(45)
III_{536}	S fimbriae, an iron siderophore system,	(45)
IV_{536}	Yersiniabacin siderophore system	(45)
II_{J96}	α-hemolysin, Prs-fimbriae cytoxoxin necrotizing factor	(21, 23)
PAI_{usp}	Uropathogenic specific protein	(124)

usp

Kurazono *et al.* discovered a DNA fragment associated to strains causing urinary tract infections which they designated “uropathogenic specific protein” (124). The sequence shows homology to S-pyocins and is possibly a bacteriocin (173). A possible virulence mechanism is not suggested, but *usp* is shown to contribute to infection in a mouse-model for urinary tract infections (241) and is associated to strains causing urinary tract infections (124).

VIRULENCE FACTORS AND PERSISTENCE OF *E. COLI* IN THE COMMENSAL MICROBIOTA

In 1943 Wallick and Stewart (229) showed that *E. coli* of different antigenic types can either be isolated from many consecutive samples of an individual or just appear briefly to soon disappear again. Based on the fact that *E. coli* have many different antigenic types Sears *et al*. assumed that when the same antigenic type was isolated from the same or consecutive samples it belonged to the same strain (204). He stated that **"one cannot escape the conclusion that *the E. coli* flora of the human bowel is made up of two kinds of strains, those which establish themselves firmly and continue to multiply over extended periods of time and those which are found only in a single or a few successive specimens"**. He designated the two kinds of strains as **resident** and **transient** (204). In 1992, Wold *et al.* (236) showed that resident strains from Swedish school girls with asymptomatic bacteriuria display uropathogenic characteristics, i.e. they express P fimbriae, adhere to colonic epithelial cells and are more likely to express an uropathogenic O serotype than transient strains. Similarly, resident *E. coli* from Pakistani infants showed higher mannose-resistant adherence, than transient strains (7). Our group has then shown in different human cohorts that genes for various virulence factors, such as P fimbriae, type 1 fimbriae, aerobactin, hemolysin and capsule K1 and K5, are associated to the ability to persist in the human colon (153, 154, 156). Further, resident strains are more likely than transient strains to belong to phylogenetic group B2 (155, 157).

THE ILEAL POUCH

ULCERATIVE COLITIS

Inflammatory bowel disease (IBD) comprises several related conditions characterized by relapsing intestinal inflammation due to (an) unknown cause(s). Ulcerative colitis (UC) is the most common IBD followed by Crohn's disease (CD). The incidence of IBD is higher in highly economically developed countries in North America, Northern and Western Europe compared to Asia, Africa and South America. The aetiology of IBD is unknown but is believed to involve inherent factors (genetic susceptibility), an immune response to the commensal microbiota and environmental triggers (Fig. 7). It has been proposed that IBD is linked to hygienic conditions (reviewed in (133)).

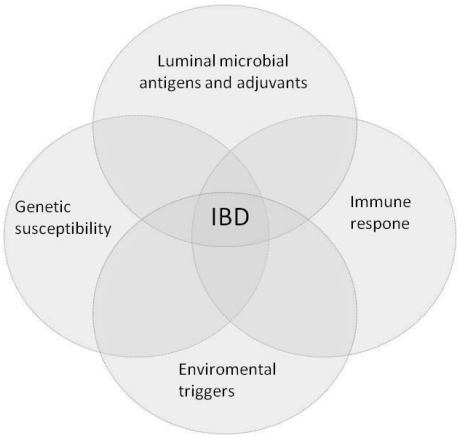

Figure 7. The aetiology of IBS is unknown but is believed to involve inherent factors (genetic susceptibility), a immune response to the commensal microbiota and environmental triggers.

Ulcerative colitis only affects the mucosa of the rectum and colon, the inflammation often starts in the rectum and spreads upwards. In some cases; the entire colon/large bowel is affected. Bloody stools, fever, malaise, weight loss and pain are common symptoms. Inflammatory bowel disease, especially UC, is a strong risk factor for colorectal cancer, and the risk increases with increased duration and extent of disease (51, 53, 71). In cases of malignant transformation, or when the disease does not respond to treatment, removal of the colon and rectum may be necessary.

Proctocolectomy and ileal pouch anal anastomosis

Ileal pouch-anal anastomosis (IPAA) has become the standard procedure for preservation of continence after removal of colon and rectum due to UC. In this procedure the ileum is constructed as a reservoir (pouch) and attached to the anal canal (Fig. 8).

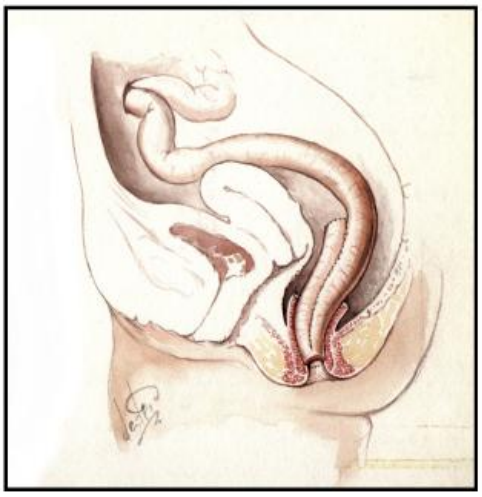

Figure 8. When the colon is removed due to UC, a pouch can be constructed of the lower part of the ileum. This is attached to the anal canal and continence is preserved.

Approximately 100 persons in Sweden receive an ileal-anal anastomosis each year. The most common cause is UC. Another cause is familial adenomatous polyposis (FAP), a condition carrying high risk of developing into colon cancer.

Most patients experience a good function and quality of life after ileal-anal anastomosis due to ulcerative colitis (60) and the functional outcome at 1 and 20 years after IPAA is shown in Table 6. The patients pass a median of 6 stools per day and medication with Loperamid is very common, in order to slow down peristalsis and reduce stool frequency (83). Inferior function can include urgency, defined as the inability to hold the stools for longer than 30 minutes, and varying degrees of incontinence (the need to use pad).

Table 6. Functional outcome of patients with ileal-pouch anal anastomosis because of ulcerative colitis.

	Follow-up (years)	
	1 (n=1511)	20 (n=251)
Mean stool frequency		
Per day	5.7	6.4
Per night	1.5	2.0
Stool consistency (% of patients)		
Liquid	7	12
Semisolid	62	72
Solid	31	16
Can distinguish gas from stool (%)	76	76
Pad use (%)	34	50
Medication use (%)	53	49

Data adapted from (83).

Transformation of the mucosa in the ileal pouch

When the lower part of the small intestine (the ileum) is made into a reservoir the mucosa undergoes some distinct changes, so called colonic metaplasia.

Some degree of chronic inflammation, mainly manifestation as increased density of plasma cells and lymphocytes is seen in all functional pouches, according to some studies (149, 160, 210) whereas others report such changes in about half of the patients (40, 152). The mucosa undergoes villous atrophy and crypt hyperplasia (40, 149, 152, 160, 210) in other words, the villi disappear and the crypts become deeper, giving the ileal mucosa a colon-like appearance, i.e. colonic metaplasia. Some studies indicate a correlation between inflammation and colonic metaplasia (66) whereas others do not (40). There is also a shift in the mucin composition from predominance of sialomucins to sulphomucins. This is seen in 16 – 50 % of the pouches (19, 149, 210). These changes are probably due to faecal stasis, since they do not occur until the pouch is exposed to the faecal stream (41).

Pouchitis

Pouchitis is an idiopathic non-specific inflammation of the pouch, and the most common late complication of IPAA. It affects about 20 – 59 % of patients within 5 years after surgery (60, 134, 208). The diagnosis should not be based solely on symptoms, but also include endoscopy and histology (208). Symptoms and clinical findings are shown in Table 7. Villous atrophy and crypt hyperplasia is greater in pouchitis than in a healthy pouch (40, 66).

Table 7. The features of pouchitis, inflammation in the ileal reservoir

	Pouchitis
Definition	Inflammation of the ileal reservoir
Symptoms	Frequency, urgency, liquid consistency of stools, anorexia, rectal bleeding, low-grade fever, extraintestinal manifestations
Endoscopic findings	Mucosal oedema, contact bleeding, mucosal haemorrhage, ulcerations
Histological findings	Marked acute and chronic inflammatory infiltration, ulceration, increased crypt depth, marked villous atrophy

It is unclear whether pouchitits represents a reactivation of the immunological response involved in UC or if it is an entirely new form of inflammatory bowel disease. It is common in UC patients and rare in patients with a pouch due to familial adenomatous polyposis (37, 134, 210). Furthermore, the morphological features of pouchitis resembles that seen in the colon in UC (210), which speaks for a reactivation of the original disease. On the other hand, smoking does not decrease the risk of pouchitis (92) contrary to UC (16, 226), which points to a discrepancy between these two inflammatory conditions. Pouchitis usually responds well, as opposed to UC, to the two antibiotics, metronidazole and ciprofloxacin, which are active against anaerobes and the *Enterobacteriaceae* family, respectively (76, 145, 209), and suggests a bacterial involvement.

THE MICROBIOTA IN IBD/ULCERATIVE COLITIS

It is now generally accepted that the commensal microbiota is involved in the immunological reaction in IBD, and there are four theories for pathogenesis. 1. Pathogenic bacteria. 2. An abnormal composition of the micobiota. 3. A defective mucosal barrier functions and microbial killing. 4. A defective immunoregulation.

Patogenic bacteria

Pathogenic bacteria are mostly suggested as a cause of Crohn's disease. Examples of suggested bacteria are *Mycobacterium avium* subspecies *paratuberculosis*,

adherent/invasive *E. coli*, toxin-producing *Clostridium difficile* and enterotoxigenic *Bacteroides fragilis* (reviewed in (198)).

Dysbiosis

A deranged microbiota – dysbiosis – might cause IBD, be a consequence, or both. Many studies have investigated the microbiota in IBD. Some have investigated the mucosa, others faecal samples. Some studies compare active vs disease in remission, others UC vs Crohn's disease, still others IBD vs healthy controls.

In IBD as a group, a decrease in mucosa associated *Firmicutes* (clostridial cluster XIVa and IV or *Eubacterium*) and in faecal *Firmicutes* in active IBD has been shown (63, 171, 214). The *Clostridium coccoides* group was reduced in active UC vs healthy controls (215).

Some reported a reduction in *Bacteroides* (63, 171, 224), whereas other reported a *Bacteroides fragilis* biofilm close to the mucosa as the main feature of IBD (222). Members of the phylum *Bacteroidetes* were more prevalent in CD than in UC patients (20).

A skewed ratio of anaerobes to aerobes, (63) and a higher number of *Enterobacteriaceae* (122) have been found in IBD. Others find no difference in composition but higher counts of mucosa associated bacteria in IBD than in healthy controls (221).

It is reported that the species richness increase from controls to noninflamed mucosa, in fully inflamed it decline to lower than haelthy controls (207), whereas others show no difference in inflamed vs non-inflamed tissues (20, 75).

THE MICROBIOTA IN THE ILEAL POUCH

In comparsion to an ileostomy, the faecal content of the ileal pouch have more bacteria per gram of contents, more anaerobes, such as *Bacteroides* and bifidobacteria, and a greater ratio of anaerobes to aerobes (136, 151, 197). In addition, sulfate-reducing bacteria can be detected in 80 % of pouches in patients with UC, but not in pouches constructed in patients colectomized due to familial adenomatous polyposis or stomal effluents from UC patients (47).

When Almeida *et al.* cultivated mucus from patients with an ileal pouch in patients operated because of UC, they found that *Veillonella* was the genus most often isolated bacteria 2 month after closure (90 %) followed by *Enterobacter*, *Klebsiella* and *Staphylococcus* in 70 %. Eight months after surgery, *Veillonella* and *E. coli* were found in 50 % of the pouches, followed by *Enterobacter*, *Klebsiella*, *Staphylococcus* and *Peptococcus* in 40 % each (9).

One study compared pouch patients to healthy controls by terminal-restriction fragment length polymorphism (T-RFLP). Samples were taken from the ileostomy, and faecal samples were taken at two occasions, once before 2 years and once at least 2 years after closure. They found that the T-RFLP pattern in the ileal pouch had a time-dependent decrease in

"ileal" and increase in some of the "colonic" fragments. For example *C. coccoides* group was increased over time (116).

Using DNA-based methods on mucosal samples, two patients were followed over time, they were sampled before construction and closure, and 1, 3 and 12 months after closure. Patient A had a microbiota dominated by Gram-positive bacteria at all occasions. Before surgery *Turibacter sanguinis* dominated and clostridia cluster XI (the *C. difficile* cluster) was found. The prevalence of clostridal cluster XIVa (*C. coccoides* group) was 33 % at 1 mo and increased to 76.5 % of the clones at 12 mo after surgery. Patient B was dominated by *Bacteroides* (40% of clones) before surgery. At 1 mo cluster XIVa was the most common (69 %), while γ-proteobacteria dominated at 3 mo (58 %), at 12 mo *Bacteroides* were almost back to the level before surgery (57).

THE MICTOBIOTA IN POUCHITIS

It is likely that the microbiota plays a role in pouchitis. Pouchitis responds to antibiotics such as metronidazole and ciprofloxacin (76, 145, 209) and to treatment with probiotics such as VSL#3® (72) or for maintenance of remission (73, 146) after initial antibiotic therapy/treatment.

When the faecal microbiota has been compared in patients with pouchitis and those with a well functioning pouch some contradictive results have been obtained. Some report no difference in composition (115, 160), although one of these studies reported a non significant higher aerobic and lower anaerobic counts in stools from pouchitis (115).

Others have reported higher counts of aerobes (152) in combination with lower counts of anaerobes (192), indicating that inflammation is associated with an overall decrease in anaerobe/facultative ratio. Less bifidobacteria and lactobacilli and more *Clostridium perfringens* (192) and higher counts of sulphate-reducing bacteria (164) have been reported in pouchitis compared to healthy pouches. Gosselink *et al.* found lower anaerobes, higher aerobes, more *C. perfringens* and haemolytic *E. coli* during pouchitis compared to pouchitis free periods in the same patient (76).

E. COLI AND INFLAMMATORY BOWEL DISEASE

The virulence of *E. coli* strains colonizing the bowel of IBD has been studied. Early on, Cooke found that haemolytic and necrotizing *E. coli* was more common in UC than in healthy controls (35) associated to an active disease rather than disease in remission (36). It seemed though as these strains followed rather than preceded relapse of colitis (36), which speaks against an actual cause.

Dickinson *et al.* found an increased incidence of *E. coli* adhesive and invasive properties in faecal samples from UC patients, both during active disease and during remission compared

to controls (43). *E. coli* from stools of UC with active disease adhered to buccal epithelial cells in a mannose resistant manner to a greater extent than those from controls (27), Crohn's disease or UC in remission (26). In contrast, Hartley *et al*. (85) found no difference in adhesion properties between *E. coli* isolates from mucosa active or inactive UC, or controls. In this study, *Enterobacteriaceae* in general and *E. coli* in particular, were isolated less frequently and in lower number from patients with active colitis than controls.

More recently, a higher proportion of B2 and D groups from the bowel mucosa of IBD patients compared to controls was shown (122). Petersen *et al*. showed that *E. coli* of group B2 were isolated more often from the mucosa of IBD patients than healthy controls, and that B2 with virulence genes were more often found in active than inactive disease (176). Contrary to this, one study found that the colonic microbiota of IBD patient were dominated by phylogenetic group A followed by D, as was the case in controls. However, *E. coli* with >1 adhesive/virulence determinant were significantly enriched in UC than Crohn's disease and controls (200).

AIMS

The aims of the present study were:

- To study the composition and establishment of the microbiota in the ileal pouch after proctocloectomy due to ulcerative colitis:
    - if possible, relate any differences in the microbiota to pouch function.
- To study some chosen virulence associated traits in *Escherichia coli* with different capacity to persist in three human cohorts:
    - the relation between pathogenicity island markers and the capacity of *E. coli* to persist in the gut of Swedish infants.
    - the virulence factor gene pattern of *E. coli* in the ileal pouch microbiota in relation to healthy individuals and the ability to persist.
    - to investigate the phylogenetic distribution in resident and transient *E. coli* in Pakistani infants.

MATERIAL & METHODS

STUDY COHORTS

THE ILEAL POUCH MICROBIOTA MICROBIOTA

Eighteen Swedish patients with an ileal pouch because of ulcerative colitis, and a control group of 16 healthy Swedish adults

THE *E. COLI* MICROBIOTA

1. One hundred and thirty Swedish infants
2. Twenty two Pakistani infants
3. Eighteen Swedish patients with an ileal pouch

ILEAL POUCH PATIENTS

Twenty one consecutive patients (10 female) with ulcerative colitis who underwent proctocolectomy with subsequent ileal pouch anal- anastomosis were included in this study. The median duration of disease before colecomy/proctocolectomy was 4 years and the patients median age at pouch surgery was 38 (range 21 – 59) years. Ninteen of the ileal pouches were constructed at Sahlgrenska University Hospital, Gothenburg, and the remaining two were constructed at NÄL Hospital, Uddevalla. Three of the patients were excluded at an early stage (due to a total lack of follow-up data). Another patient dropped out after 8 months, but was included in the analyses until this time-point. Informed consent was obtained and the Ethics Committee of Gothenburg University approved the study.

All patients received single doses of antibiotics prior to surgery. This was either cefuroxim + metronidazole or sulfamethoxazole + trimethoprim + metronidazole. One patient recived doxycycline at 4 months (not related to the pouch), metronidazole at 11 and ciprofloxacin at 12 months after closure, due to problems with the pouch. Other five patients recived antibiotics prescribed at outpatient clinics not connected with the study centers: two recived flucloxacillin and ciprofloxacin, respectively, 1 mo after opening the pouch for faecal flow, one recived norfloxacin 3 months after surgery, one received norfloxacin at 7 months and one received doxycycline at 8 months.

Fifteen healthy individuals (12 female) were included as controls. Their median age was 36 years (range 24 – 55). None of the controls had taken antibiotics during at least one month preceding inclusion in the study.

SAMPLING METHODOLOGY

The patients were followed with regular sampling of the pouch microbiota during three years after IPAA surgery. The first sample was collected from the stoma one to seven days before construction of the pouch. A second sample was taken one to seven days before closure. The two patients who had a one step procedure and the two patients who were converted from an ileorectal anastomosis did not contribute stomal samples. Thereafter, a faecal sample was collected by the patient once each month during the first year. During the second year samples were collected every third month and finally a sample was taken after three years (Fig 9).

Biopsies were taken from the pouch as part of the routine clinical controls, before and at 1, 6 and 12 months, 2 and 3 years after closure. They were immediately placed in 1 ml of pre-reduced peptone water and transported under anaerobic conditions to the laboratory.

The controls were sampled at a single occasion only.

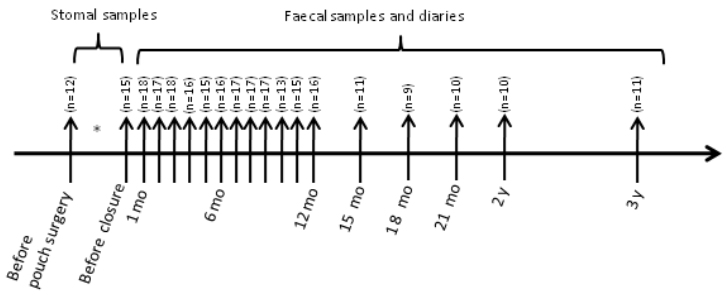

Figure 9. Eighteen patients who underwent surgery and received an ileal pouch due to ulcerative colitis were followed over three years. Stomal samples were collected before construction and before closure. Thereafter, faecal samples were taken at regular intervals and a diary was filled in by the patient the week before handing in the faecal sample. *The median time between pouch construction and closure was 3 months (range 0 – 11.5 months). The numbers of samples obtained at each time-point are shown in brackets. Clinical and endoscopic examinations are indicated by red time arrows.

CULTIVATION METHODOLOGY

Stomal samples were collected by hospital staff or patients and freshly voided faeces were collected by the patient. Samples were placed in a sterile tube or Petri dish in a plastic bag in which an anaerobic atmosphere was created (AnaeroGen Compact, Oxoid Ltd, Basingstoke, UK). The samples were kept refrigerated until transported to the laboratory, where they were serially diluted and cultured on non-selective and selective media under aerobic and anaerobic conditions within 24 hours after sampling, the procedure is shown in Figure 10. Selective media, time of incubation and typing methods are shown in Table 8.

The biopsies were sonicated for 2 min and thereafter incubated on a shaker, in room temperature for 5 min. The liquid was serially diluted and cultivated in the same manner as the faecal samples (Fig. 10 and Table 8).

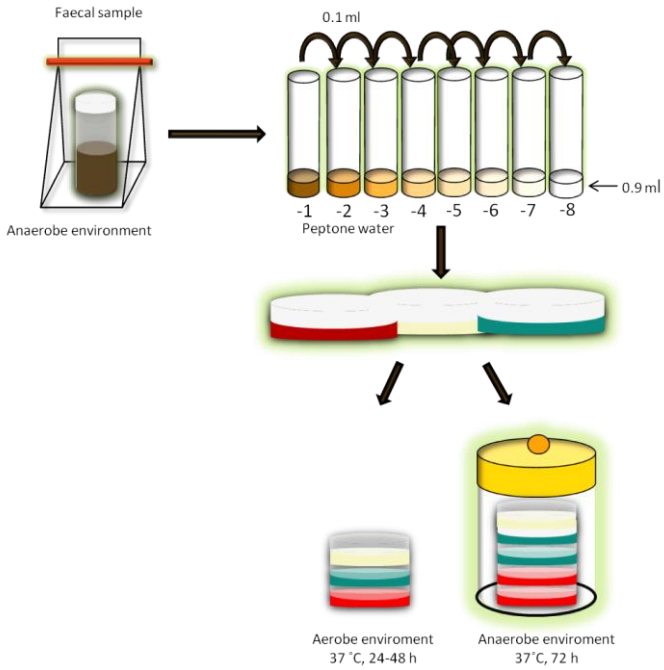

Figure 10. The cultivation procedure of stomal content and faecal samples from patients with an ileal pouch because of ulcerative colitis.

Colonies of different morphology were enumerated, Gram-stained and subcultured for further identification by biochemical or genetic tests (5, 174, 216) (Table 8). The limit of detection was 330 ($10^{2.52}$) colony-forming units (CFU)/g faeces. Anaerobic bacteria were tested for aerobic growth and sparse aerobic growth was accepted for Gram-positive rods resembling *Bifidobacterum* or *Lactobacillus* spp. The total anaerobic and facultative anaerobic population counts were calculated from nonselective media incubated under anaerobic or aerobic conditions, respectively (5).

Table 8. Culture media and conditions of incubation as well as bacterial groups detected

Medium	Time (d)	Culture conditions	Bacteria	Bacterial typing
Colombia blood	1	Aerobic	Total facultative bacteria	
Drigalski bok	1	Aerobic	*Enterobacteriaceae*	API20E
Staphylococcus agar	2	Aerobic	*Staphylococcus* aureus CoNS	gram-staining catalase coagulase
Entercoccsel agar	1	Aerobic	*Enterococcus* spp.	gram-stained appearance esculine hydrolysis
Saboraud	2	Aerobic	yeast	gram-stained appearance
Brucella blood	3	Anaerobic†	Total anaerobes***	
	3	Anaerobic	Spore formers (*Clostridium*)****	RAPID32A
BBE* agar	3	Anaerobic	*Bacteroides fragilis* group	RAPID32A
Beerens agar	3	Anaerobic	*Bifidobacterium* spp.	Genus specific real-time PCR (174)
Rogosa		Anaerobic	lactobacilli	PCR (216)
CCFA**	3	Anaerobic	*C. difficile*	RAPID32A

The table shows media and culture conditions for the bacterial groups, further described in (5).
* Bacteroides bile esculin agar.
** Cycloserine cefoxitin fructose egg yolk agar.
*** Isolates growing aerobically were not included in the counts.
**** Cultured in jars using BBL GasPak anaerobic system (Becton Dickinson Microbiology Systems, Sparks, NV).
‡Fore spore forming bacteria (clostridia), faeces diluted 1:10 was mixed with 99% ethanol and incubated on a shaker at room temperature for 30 min to kill vegetative cells, where after the sample was diluted and plated.

Clinical investigations and diaries

Clinical and endoscopic examinations were performed at 1, 6, 12, 24 and 36 months after closure. At these occasions pouch function was assessed with a detailed questionnaire as previously described (165). Number of bowel movements, urgency (meaning the inability to hold the stools for longer than 30 minutes), evacuation difficulties, leakage, perianal soreness, medication, diet restrictions and impact on social functioning were registered. A summarized score ranging from 0 to 15 (15 being the worst) was calculated (165).

The patients also filled in a diary during the week preceding every sampling occasion after closure. The data registered were the following; number of stools, stool viscosity, number of bloody stools, urgency, leakage, daily dose of Loperamide.

E. COLI COLLECTIONS

PAKISTANI INFANTS

This group included 22 infants born in 1984 in the urban slum of Lahore. They were delivered at home by traditional birth attendants and followed by regular sampling during their first six months of life (4).

Samples of the rectal microbiota were obtained using a cotton-tipped swab every second day during the first week, weekly during the first month, and monthly until six months of age. The swab was streaked over a small part of a Drigalski agar plate, the inoculate was spread to maintain free-lying colonies and the plates were incubated at 37°C over night. The three last free-lying colonies were picked, to ensure the inclusion of the dominant *E. coli* strain and identified by biotyping. The *E. coli* isolates was typed to the strain level by multilocus enzyme electrophoresis (MLEE) (4).

SWEDISH INFANTS

The 130 Swedish infants were born in 1998-2001 at the Sahlgrenska University Hospital, Göteborg, Sweden. They were part of a prospective birth-cohort study examining the relation between the intestinal colonization pattern and allergy development, the ALLERGYFLORA study (5, 6). *E. coli* strains (n = 149) from 70 of these have previously been described regarding *E. coli* colonization pattern, association between certain virulence factor genes, phylogenetic group distribution, and persistence (154, 157). Here in a larger cohort, carriage of eight markers for PAI I-IV$_{536}$, PAI II$_{J96}$, PAI I, II$_{CFT073}$ and PAI$_{usp}$ in the intestinal *E. coli* strains were identified. Phylogenetic group distribution was further determined.

E. coli strains were isolated and quantified in stools as previously described (5). In brief, a rectal swab was obtained at 3 days of age and cultured semi-quantitatively under aerobic condition for facultative bacteria. Faecal samples were collected at 1, 2, 4, and 8 weeks, and at 6 and 12 months of age. They were diluted serially and cultured on Drigalski´s agar for isolation of *Enterobacteriaceae*. One to six colonies with different morphologies were isolated from each positive culture and enumerated, subcultured, speciated using bio-typing (API20E, API Systems SA, La Balme les Grottes, Montalieu-Vercieu, France), and stored at -70°C. Colony forming units of the same morphology within each sample was regarded as the same strain. After species identification *E. coli* isolates were analyzed by Random Amplified Polymorphic DNA (RAPD) profiling. Isolates from a particular infant that exhibited RAPD patterns differing by no more than three weak bands were considered to be the same strain (154). Popultaion counts (CFU/g faeces) of individual *E. coli* strains in each sample were calculated after strain typing of the isolates.

E. COLI IN THE ILEAL POUCH

E. coli were isolated according to the description above (ileal pouch microbiota paper I).

E. coli strain typing

RAPD

All *E. coli* isolates were typed to the strain level by Random Amplified Polymorphic DNA as described for the Swedish infants (154).

MLVA (BR)

In parallel with the RAPD analysis, isolates from two patients were analyzed by multiple-locus variable-number tandem repeats analysis (MLVA). There was a 95 % concordance between the methods, when analysing 21 isolates. Only one RAPD type was expanded into two by MLVA. MLVA was also used in three patients where certain strains yielded ambiguous RAPD pattern. MLVA was performed as previously described (132) with a few modifications. Briefly, amplification of seven variable-number of tandem repeats (VNTR) were performed using three sets of multiplex PCRs. PCR 1 detected the regions CVN003 and CVN014, PCR 2 amplified the regions CVN001, CVN004, CVN007 and CVN015 and PCR 3 amplified CVN002. Primers and their concentrations are shown in Table 9.

The amplicon sizes were determined using an ABI PRISM 310 genetic analyzer (Applied Biosystems) with pop 4 gel, 5s injection time and running conditions of 40 min at 15 kV. The fragment lengths were analyzed by GeneMapper, version 4 (Applied Biosystems). The number of tandem repeats for each locus was determined as previously described (132).

CLASSIFICATION OF STRAINS DUE TO THEIR TIME OF PERSISTENCE

In the Pakistani infants the strains were defined as resident of they were isolated on at least two sampling occasions at least three weeks apart, and as transient if only isolated once or over a shorter period than 3 weeks. If a scheduled sampling was missed an a strains only found once before of if a strain was found only in the last sample, it could not be defined as resident or transient (4).

Strains from the Swedish infants and the ileal pouch were grouped according to their time of persistence in the microbiota: 1) those found only once, termed “transient”, 2) those isolated in 2 – 11 monthly samples were termed “intermediate colonizers”, and 3) those isolated for a period of ≥ 12 month were termed “long time colonizers”. The time of colonization for the biopsy strains were defined from their occurrence in another biopsy sample or in a faecal sample at another occasion. Some strains could not be grouped using these definitions, i.e. those appearing in the first or last sample and did not persist for ≥ 12 mo were unclassified.

PCR FOR PHYLOGENETIC GROUP, VIRULENCE FACTOR GENES AND PATHOGENICITY ISLAND MARKERS

DNA extraction

To extract genomic DNA, a small amount of bacteria was suspended in Tris-EDTA buffer (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and heated at 95°C for 10 min. The mixture was centrifuged for 5 min at room temperature and supernatants were used for PCRs reactions. DNA extraction method was identical in all PCR assays.

PCR

Table 9 shows all primers and their concentrations used in the studies.

Phylogenetic group distribution was determined using triplex PCR as described by Clermont *et al*. amplifying the genes c*huA* and y*jaA*, and the DNA fragment *tspE4.C2* (32).

The carriage of the following virulence genes was detected by three sets of multiplex PCRs: The first PCR detected *papC* (P fimbriae), *sfaD/E* (S fimbriae) and *draA* (Dr hemagglutinin), the second detected the three known varieties of the P-fimbrial adhesion genes *papG* (class I-III) and the third detected *neuB* (capsule K1), *kfiC* (capsule K5), *iutA* (aerobactin), and *hlyA* (hemolysin) (153). The gene *fimH* (type 1 fimbriae) was detected in a single PCR (100).

PAI IV_{536} was identified by amplifying the genes *fyuA* and *irp2* (33). The presence of PAI I_{CFT073} was confirmed using primers detecting *malX* as previously described (100). The PCR conditions used have been described elsewhere (159).

PAI I and II in *E. coli* 536, PAI II_{CFT073} and PAI III_{536} and PAI II_{J96} were identified by a two duplex and a single PCR respectively, primers have been published by Sabaté *et al.* (193).

HotStarTaq master mix (Qiagen, Spånga, Sweden) was used in all PCR protocols except for PAI I_{536} and II_{536} and PAI II_{J96} where *Taq* DNA polymerase (Roche diagnostics, Bromma, Sweden) was being used.

E. coli strains, CFT073, 536, J96 served as positive controls for the PAIs. All products were separated by electrophoresis in agarose gels and visualized by staining with ethidium bromide.

A real-time PCR was developed to detect *usp*. Primers and a probe were designed using Primer Express® Software 3.0 (Applied Biosystems, Stockholm, Sweden) based the sequence for *usp* in *E. coli* strain Z42 as retrieved from DDBJ, accession number AB056434 (150). The real-time PCR was verified in 100 strains assessed using the conventional primers described by Nakano *et al*. (150). There was 99 % accordance. Strain Z42, Z13 and C10 (150) served as positive controls for PAI markers.

Material & Methods

Table 9. The virulence genes locate on the different pathogenicity islands and primers used for detection of the PAI markers and the virulence associated genes *malX* and *usp*.

	Primer name	Sequence (5' -3')	Reference	Primer concentration
RAPD				
		GTGATCGCAG	(155)	
MLVA				
	CVN003-F	5′-**6FAM**-AAAAATCCGGATGAG***W***TGGTC-3′		0.8 µM
	CVN003-R	5′-TTGCGTTGTCAGTAATTTGTTCAG-3′	(132)	0.8 µM
	CVN014-F	5′-**6FAM**-TCCCCGCAATCAGCAA***M***ACAAAGA-3′		0.2 µM
	CVN014-R	5′-GCAGC***R***GGGACAACGGAAGC-3′	(132)	0.2 µM
	CVN001-F	5′-**HEX**-AACCGGCTGGGGCGAATCC-3′		0.2 µM
	CVN001-R	5′-GGCGGCGGTGTCAGCAAATC-3′	(132)	0.2 µM
	CVN004-F	5′-**HEX**-***M***GCTGCGGC***R***CTGAAGAAGA-3′		1.2 µM
	CVN004-R	5′-CCCGGCAGGCGAAGCATTGT-3′	(132)	1.2 µM
	CVN007-F	5′-**6FAM**-ACCGTGGCTCCAG***Y***TGATTTC-3′		0.2 µM
	CVN007-R	5′-ACCAGTGTTGCGCCCAGTGTC-3′	(132)	0.2 µM
	CVN015-F	5′-**TET**-TAGGCATAGCGCACAGACAGATAA-3′		0.2 µM
	CVN015-R	5′-GTACCGCCGAACTTCAACACTC-3′	(132)	0.2 µM
	CVN002-F	5′-**TET**-AACCGTTATGAA***R***G***R***AAGTCCT-3′		0.2 µM
	CVN002-R	5′-TCGCCCAGTAAGTATGAAATC-3′	(132)	0.2 µM
Phylogenetic groups				
	ChuA.1	GACGAACCAACGGTCAGGAT		0.8 µM
	ChuA.2	TGCCGCCAGTACCAAAGACA	(32)	0.8 µM
	YjaA.1	TGAAGTGTCAGGAGACGCTG		0.8 µM
	YjaA.2	ATGGAGAATGCGTTCCTCAAC	(32)	0.8 µM
	Tsp4C2.1	GAGTAATGTCGGGGCATTCA		0.8 µM
	TspE4C2.2	CGCGCCAACAAAGTATTACG	(32)	0.8 µM
Gene				
papC	pap 1	GACGGCTGTACTGCAGGGTGTGGCG		0.45 µM
	pap 2	ATATCCTTTCTGCAGGGATGCAATA	(128)	0.45 µM
sfaD/E	sfa 1	CTCCGGAGAACTGGGTGCATCTTAC		0.45 µM
	sfa 2	CGGAGGAGTAATTACAAACCTGGCA	(128)	0.45 µM
Dr	Dr-321f	GCCAACTGACGGACGCAGCAC		0.45 µM
	Dr-550r	CCCCAGCTCCCGACATCGTTTTT	(153)	0.45 µM
papGI	j96-193f	TCGTGCTCAGGTCCGGAATTT		0.45 µM
	j96-653r	TGGCATCCCCCAACATTATCG	(95)	0.45 µM
papGII	ia2-383f	GGGATGAGCGGGCCTTTGAT		0.45 µM
	Ia2-572r	CGGGCCCCCAAGTAACTCG	(95)	0.45 µM
papGIII	prs-198f	GGCCTGCAATGGATTTACCTGG		0.45 µM
	prs-455r	CCACCAAATGACCATGCCAGAC	(95)	0.45 µM
neuB	K1-1011f	CTACCCCTTTTGACGAAGAC		0.45 µM
	K1-1504r	ACACACCTGACCCCAATAC	(153)	0.45 µM
kfiC	K5-1756f	GCCACCAACTGTCGCAAAA		0.45 µM
	K5-2564r	TGTCGCCCAAACAAAAAGATT	(153)	0.45 µM
iutA	aer-851f	GGCTGGACATCATGGGAACTGG		0.45 µM
	aer-1152r	CGTCGGGAACGGGTAGAATCG	(99)	0.45 µM
hlyA	hly1	AACAAGGATAAGCACTGTTCTGGCT		0.45 µM
	hly2	ACCATATAAGCGGTCATTCCCGTCA	(242)	0.45 µM
fimH	fimH f	TGCAGAACGGATAAGCCGTGG		0.5 µM
	fimH r	GCAGTCACCTGCCCTCCGGTA	(100)	0.5 µM

PAI markers				
PAI IV_{536}	fuyA.1	TGATTAACCCCGCGACGGGAA		0.45 µM
	fuyA.2	CGCAGTAGGCACGATGTTGTA	(33)	0.45 µM
	irp2.1	AAGGATTCGCTGTTACCGGAC		0.45 µM
	irp2.2	TCGTCGGGCAGCGTTTCTTCT	(33)	0.45 µM
PAI I_{CFT073} (malX)	RPAi f	GGACATCCTGTTACAGCGCGCA		0.45 µM
	RPAi r	TCGCCACCAATCACAGCCGAAC	(100)	0.45 µM
PAI II_{CFT073}	cft073.2Ent1	ATGGATGTTGTATCGCGC		0.1 µM
	cft073.2Ent2	ACGAGCATGTGGATCTGC	(193)	0.1 µM
PAI III_{536}	sfaAI.1	CGGGCATGCATCAATTATCTTTG		0.5 µM
	sfaAI.2	TGTGTAGATGCAGTCACTCCG	(44)	0.5 µM
PAI I_{536}	I.9	TAATGCCGGAGATTCATTGTC		0.5 µM
	I.10	AGGATTTGTCTCAGGGCTTT	(193)[a]	0.5 µM
PAI II_{536}	orf1up	CATGTCCAAAGCTCGAGCC		0.5 µM
	orf1down	CTACGTCAGGCTGGCTTTG	(193)[a]	0.5 µM
PAI II_{J96}	hlyd	GGATCCATGAAAACATGGTTAATGGG		0.5 µM
	cnf	GATATTTTTGTTGCCATTGGTTACC	(125)	0.5 µM
usp	USPf	CGGCGCATATTGCTGATAAAT	*	0.2 µM
	USPr	CGCCTCGCGAAACTCATC		0.2 µM
	probe[b]	ACGGGAGTTTAAAACC		0.2 µM

MLVA:Text in bold describes the dye used to color the primer; text in bold and italic describes the degenerate bases with IUPAC nomenclature.

[a] the author had selected primers from http://www.uni-wuerzburg.de/infektionsbiologie.

[b] a MGB-probe with FAM™ as the reporter dye was used

[c] primers were designed using Primer express software, the sequence for this gene in *E. coli* strain Z42 was retrieved from DDBJ, accession number AB056434 (150)

VIRULENCE AND PAI-MARKER SCORES

For *E. coli* from the Swedish infants and pouch patients, a virulence score was generated from carriage of the virulence factor genes: *fimH*, *papC*, *sfaD/E*, *iutA*, *hlyA*, *kfiC*, *neuB*, *malX, usp* and *fuyA/irp2*. The score ranged from 0 – 9, since the capsule type cannot be both K1 (*neuB)* and K5 (*kfiC)*.

STATISTICAL METHODS

Frequencies were compared using Fisher's exact test and exact test was used for trend analyses (linear by linear association). Virulence scores and population counts from patients and controls were compared using Mann-Whitney U-test. Comparisons between faecal samples from the IPAA patients were performed using Wilcoxon matched-pairs signed rank test (SPSS software Package 15.0).

Logistic regression using stepwise selection was performed to evaluate the relative contribution of different bacterial traits to colonic persistence (SPSS software Package 15.0).

Multivariate analysis was performed using the SIMCA-P (Umetrics AB, Umeå, Sweden). Principal component analysis (PCA) was used to reveal clustering of patients and healthy

controls based on their *E. coli* microbiota and its composition with respect to phylogenetic group distribution and the bacterial determinants screened for.

Projections to latent structures by means of partial least square (PLS) is a linear multivariate data analysis technique which can handle collinarity between the variables. PLS analysis was performed to investigate the relation between persistence in the ileal pouch and the characters screened for using Simca-P+ 12.0 (Umetrics, Umeå, Sweden). Persistence, i.e the time of persistence of each strains in the microbiota was used as the Y variable and the virulence factor genes, PAI markers, the phylogenetic groups and the virulence score as X variables. Confidence intervals were calculated using default settings in SIMCA-P 12.0.

RESULTS & COMMENTS

THE MICROBIOTA IN THE ILEAL POUCH (PAPER I)

We followed 18 patients in whom an ileal pouch was constructed, most often in a two step procedure. The entire colon had been removed on average 10 months before the first surgery, in all cases due to ulcerative colitis.

PREVALENCE OF MAJOR BACTERIAL GROUPS IN THE POUCH MICROBIOTA

The contents of the pouch were cultured quantitatively for the presence of major groups of facultative and anaerobic bacteria. The proportions of individuals colonized by some major bacterial groups, at selected time points are shown in Table 10. The p_{trend} and the arrow indicate if there was a significant trend in the colonization rate of the bacterial group.

Among the facultative anaerobes, *Klebsiella* was the one that showed a major change. *Klebsiella* was significantly more often isolated from the two stomal samples compared to the controls. Isolation rate of *Klebsiella* declined significantly over time ($p_{trend} < 0.0001$) after closure of the pouch.

Several anaerobic groups became more prevalent in the pouch content with time. *Bacteroides* was quite uncommon in the stomal samples and declined even more the first months after IPAA surgery. We attribute this disappearance to the antibiotic treatment that was done in conjunction with each surgical procedure. Metronidazole, which kills Gram-negative anaerobes (e.g. *Bacteroides*) and also many Gram-positive anaerobes, was part of the treatment. From five months after surgery isolation rates of *Bacteroides* steadily ($p_{trend} < 0.001$) increased and 10 months after surgery it no longer deviated from the controls. Bifidobacteria were more common than *Bacteroides* in the stomal samples and seemed not to be affected by the surgical procedures. After connecting the pouch it increased rapidly ($p_{trend} < 0.001$) and 4 months after surgery it was no longer different from control faecal samples. Lactobacilli never differed from the control group, still there was a non-significant tendency towards higher colonization rate over time ($p_{trend} = 0.056$). Clostridia were isolated by alcohol treatment, and spore-forming counts were calculated. Clostridia were common in the ileal samples and isolation rates did not change after taking the pouch into use. *C. perfringens* was a commonly isolated clostridial species. There was a significant trend towards lower isolation rate of *C. perfringens* ($p_{trend} = 0.021$). *C. difficile* was isolated on selective media. It was not present in ileal samples and unusual in pouch faecal samples.

Results & Comments

Table 10. The prevalence of major bacterial groups in the ileal pouch of patients with ulcerative colitis monitored over time. The table shows the frequency in the stomal samples before constructing and connecting the pouch and the highest and lowest value between 1 month and 3 years.

	Colonization rate (%)							
	Controls	Stomal samples		Pouch samples (month after closure)				
Bacterial groups		Constr.	Closure	1	12	24	36	p_{trend}[a]
Facultatives								
E. coli	94	75	87	94	88	80	82	
Klebsiella spp	6	75**	87**	50*	31	30	18	< 0.0001 ↓[b]
Other *Entero-bacteriaceae*	13	17	40	11	31	40	20	
enterococci	100	94	100	100	100	100	100	
S.aureus	27	31	19	11	25	30	0	
CoNS	47	69	63	67	50	80	45	
Yeast	75	63	44	56	56	40	27	
Anaerobes								
Bacteroides	94	43**	13***	6***	63	70	60	< 0.001 ↑[b]
bifidobacteria	100	50**	53**	56**	91	80	90	< 0.001 ↑[b]
lactobacilli	87	58	64	83	73	70	90	0.056 ↑[b]
clostridia	93	83	63	94	93	63	100	
C. perfringens	38	38	40	50	19	0	44	0.021 ↓[b]
C. difficile	6	0	0	11	6	11	0	

[a]The p_{trend} value in the table indicates that there is a significant trend in any direction and [b] the arrow indicates the direction in the specific group. Comparisons were made between controls and patients at different time points using Fisher's exact test. Stars indicate the level of significance; * $p < 0.05$, ** $p < 0.01$ ***, $p < 0.001$.

BACTERIAL COUNTS AND THE RATIO OF ANAEROBIC TO AEROBIC BACTERIA IN THE POUCH MICROBIOTA

We found that both total and anaerobic bacterial counts increased approximately 10 fold within a month after closure, but did not increase thereafter. On all sampling occasion were the counts higher in faecal pouch samples than in stomal samples. However, the population counts never reached the levels found in faecal samples from controls, during the whole study ($p = 0.01$, at all times).

The counts of facultative anaerobes were somewhat higher in pouch samples obtained during the first 10 months than in faeces from controls on several occasions. Thereafter, facultative population levels seemed to decrease. In the three year sample, counts of facultative anaerobes were significantly lower than in the stomal sample collected before construction of the pouch ($p=0.039$).

Thus, the microbiota of the pouch became more and more dominated by anaerobes. The normal colonic microbiota is strongly anaerobic and in faecal samples from healthy controls the ratio of anaerobic to facultative anaerobes bacterial counts was approximately 1000:1. In the stomal samples collected from the patients before surgery, facultative anaerobes were instead more common than anaerobes (anaerobes/facultative anaerobes ratio of approximately 1:2). The ratio in the pouch samples increased over time, and at three years after surgery it was no longer significantly different from control faeces (Fig. 1h, paper I).

POPULATION COUNTS OF MAJOR BACTERIAL GROUPS IN THE POUCH MICROBIOTA

The counts of *Enterobacteriaceae* in colonized patient samples were initially quite high, i.e. significantly higher than in control samples at many occasions during the first year. In the 2 year sample the counts started to decrease and were significantly lower than in the first month after closure ($p = 0.01$) (Fig 2b, paper I). *E. coli,* which is a member of the *Enterobacteriaceae* family, followed the same pattern but started to decline already at 4 months after closure. In the 2 and 3 year samples, counts were significantly lower than at one month after closure. Counts of *Klebsiella* decreased after some months. Only one control individual harboured *Klebsiella* in the faeces in low counts. The counts of enterococci were significantly higher in the pouch samples than in the control samples at all occasions until 1 year after surgery ($p = 0.029$). The counts declined over time and were significantly lower after 3 years than in the first month after connecting the pouch (Fig. 2e, paper I). Population counts of *S. aureus* and CoNS did not differ significantly from counts in the control group and did not change much over time. The same was true for yeast.

Results & Comments

Bacteroides were rarely found in the stomal samples and, when found, showed rather low counts (~10^7, Fig. 3e, paper I). The population did not increase very much after closure of the pouch. During the entire study period, the population levels in pouch faeces varied between 4.24 and 8.52 log CFU/g, and never reached the counts observed in the control samples, 9.26 log CFU/g faeces on average. No tendency towards increased counts over time was observed (Fig. 3f, paper I). Bifidobacterial counts were low in the two stomal samples, before construction and closure, 7.23 and 6.03 log CFU/g respectively. After closure, the bifidobacterial counts rose quickly. However, the counts remained lower in the pouch patients than in the healthy controls (Fig. 3f, paper I) and no tendency towards increased counts over time was observed (Fig. 3f, paper I). Lactobacilli were frequently found in the ileostomal and the pouch samples. Counts were at least as high as in normal faeces. Upon closure of the pouch, population levels were stagnant or increased marginally (Fig. 3g paper I) and were actually significantly higher in pouch samples at 1 and 12 months than in control faecal samples faecal ($p < 0.05$). The median counts of clostridial spores never deviated significantly from the counts in control faeces, but seemed to decline in the pouch effluents after the first 12 months (Fig. 3h, paper I). *C. perfringens* reached counts ranging from 3.12 to 6.40 log CFU/g (median stoma = 3.86, pouch = 4.52) in patient samples, similar to the counts in controls (4.52 log CFU/g) on average. Median counts of *C. difficile* in colonized patients varied between 2.52 and 6.34 log CFU/g (median pouch = 3.47), while the counts in the faeces of single colonized control individual was 3.37 log CFU/g.

THE POUCH MICROBIOTA IN RELATION TO POUCH FUNCTION

Patients were investigated clinically and a score reflecting pouch function was constructed from a questionnaire (165). At 12 months, 12 patients contributed both functional score and microbiological data. A score of ≥ 7 signifies poor pouch function. Three patients had a score of ≥ 7 and those also fulfilled clinical and endoscopic criteria of pouchitis. Two patients had a score of 6, indicating a rather poor function, but without fulfilling the clinical and endoscopic criteria for pouchitis. The remaining 7 patients had a score of ≤ 4, indicating a good function.

Patients with poor pouch function (n = 5) (score ≥ 6) were compared with patients with good pouch function (n = 7)(score ≤ 4). Regarding composition of the microbiota, patients with poor function had lower ratio of anaerobic to facultative bacterial counts (14:1) than those with good function (40:1), ($p = 0.41$). They also tended to have lower bacterial counts, (8.95 vs 9.52 log CFU/g, respectively, $p = 0.15$), due to lower counts of strict anaerobes (8.56 vs 9.26 log CFU/g, respectively, $p = 0.15$). Their facultative bacterial counts were at least as high (8.45 vs 8.22 log CFU/g, $p = 0.46$), compared to patients with good function. The proportions of patients in the two groups colonized with different bacteria are shown in Table 11. Bifidobacteria were found in all good functioning pouches but only in two of the patients with reduced function ($p = 0.045$). The same trend was seen regarding lactobacilli, 88 % vs 40 % in good vs poor function, respectively ($p = 0.22$) and yeast, 71 % vs 20 %, ($p = 0.24$).

Table 11. Bacterial colonization rate in faecal samples obtained at 12 months after connecting the pouch in patients with a well functioning pouch (score ≤ 4) and those with reduced pouch function (score ≥ 6).

	% bacterial carriage rate		
	Pouch function*		
Bacterial group	Poor (n=5)	Good (n=7)	P value
Enterobacteriaceae	100	100	
E. coli	60	100	
Klebsiella	40	29	
Other	20	29	
enterococci	100	100	
S. aureus	40	14	
CoNS	60	43	
yeast	20	71	
Bacteroides	60	71	
bifidobacteria	40	100	0.045
lactobacilli	40	88	
clostridia	80	100	
C. perfringens	0	29	
C. difficile	20	0	

*defined as a score based on, number of bowel movements, urgency, evacuation difficulties, leakage, perianal soreness, medication, diet restrictions and impact on social functioning (165).

THE *E. COLI* MICROBIOTA (PAPER II, III & IV)

This thesis focused on *E. coli* as a member of the commensal gut microbiota. This was studied in different human populations and the numbers of *E. coli* strain isolated from each cohort are shown in Table 12.

Table 12. *E. coli* strain collections studied in this thesis.

Cohort	No of strains	Paper	Reference if previously published
Swedish infants (n = 130)	273	II	70 infants (154)
Ileal pouch patients faecal strains (n = 18)	92	III	
Ileal pouch, biopsy strains	33	III	
Healthy controls (n = 16)	68	III	(158)
Pakistani infants (n = 22)	158	IV	(4)

THE FAECAL *E. COLI* MICROBIOTA

Phylogenetic distribution

The distribution of phylogenetic groups differs between different human populations (50) and our group has shown that B2 is the dominant group in healthy Swedish infants (155). Here, we confirm in a larger group of infants that the microbiota of the Swedish infants is dominated by B2 and A is somewhat more common than D and B1. The microbiota of health Swedish adults was fairly similar to that of Swedish infants.

This contrasts sharply with the *E. coli* microbiota of the Pakistani infants which was dominated by phylogenetic group A, that made up approximately half of all the strains. B2 was the least common group, whereas B1 and D had fairly similar proportions (Fig 11).

Patients with an ileal pouch had an *E. coli* microbiota dominated by group A strains. Thus, they had significantly higher proportion of group A strains compared to healthy control (42 % vs 21 %, p = 0.006), while the controls instead had significantly more B2 strains (47 % vs 27 %, p = 0.012). (Fig 11).

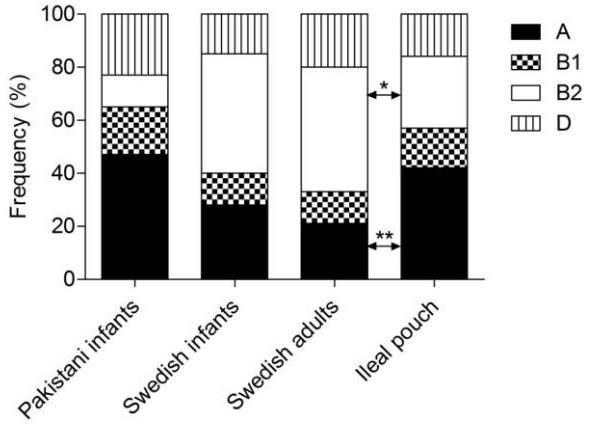

Figure 11. The figure combines data from different studies, some of which are previously published. The phylogenetic distribution among faecal *E. coli* from in four different cohorts is shown, Pakistani infants, 0-6 months old (159), Swedish infants, 0-12 months old (155), the ileal pouch of patients with ulcerative colitis and a healthy control group of Swedish adults.

Changes in phylogenetic distribution in the ileal pouch over time

The phylogenetic distribution in the ileal pouch differed significantly from that found in the faeces from the healthy controls, as shown above. But when investigating the phylogenetic distribution of the *E. coli* population at different time points the picture changed (Fig. 12). In the stomal samples 25 strains were isolated. B2 made up 48 % similar to the proportions in faeces from both Swedish infants (45 %) and healthy adults (47 %). After closure the A group expanded, while the B2 group decreased. Twelve months after surgery the distribution was almost back as in the stomal sample, with a dominance of B2 (50 %) followed by A (25 %), B1 (19 %) and D (6 %).

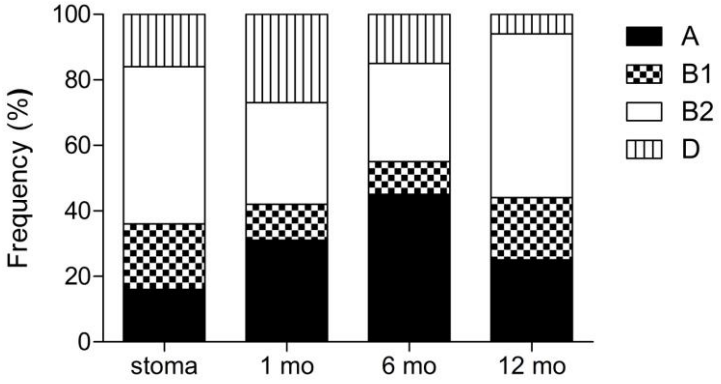

Figure 12. The phylogenetic distribution of *E. coli* strains in the ileal pouch of patients with ulcerative colitis at different time points. Stomal samples (n = 25), 1 (n = 26), 6 (n = 20) and 12 months after surgery (n = 16).

Virulence factor genes and PAI markers in phylogenetic group B2

B2 strains are known to carry an array of different virulence genes and PAIs (22, 178). However, at least 9 subgroups are found, and a high polymorphism is observed in the B2 flexible gene pool (129). Here, we found that B2 strains from different cohorts differed in virulence gene pattern. Thus, B2 strains from Pakistani infants significantly less often carried many of the investigated virulence factor genes than B2 from Swedish infants, e.g. *fimH*, *papC*, *papG* class III, *sfaD/E*, *neuB*, *hlyA* and markers for the PAI HPI/IV_{536} (Fig 13a).

We also found that B2 strains obtained from stools of patients with an ileal pouch differed from those isolated from faeces of healthy controls (Fig. 13b). Hence, B2 from the pouch more often carried *sfaD/E*, PAI II_{CFT073}, PAI I_{536} and PAI II_{J96} than B2 from normal faeces. ($p<0.0001$, $p=0.005$, $p=0.02$ and 0.04, respectively). Instead, B2 from the healthy controls more often carried *iutA* and *papGII* than pouch B2 strains ($p=0.001$ and $p=0.02$, respectively).

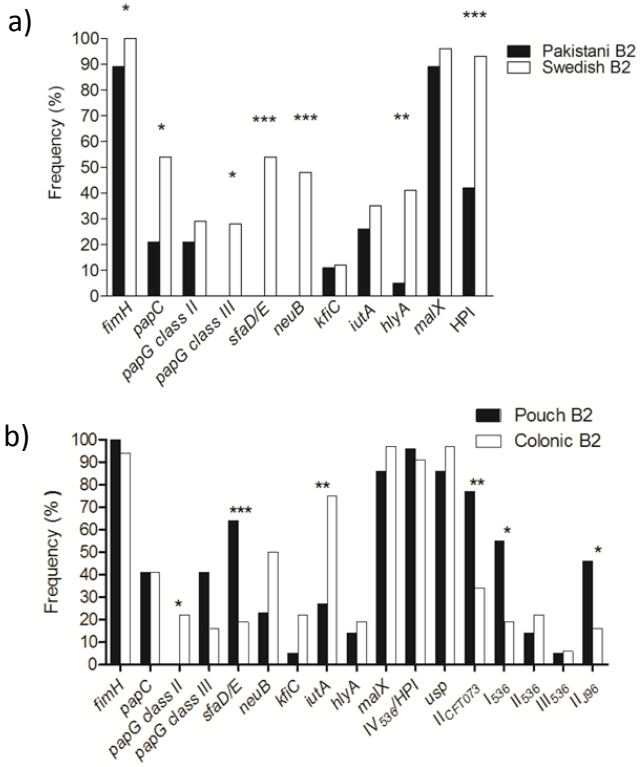

Figure 13. Prevalence of certain virulence factor genes and pathogenicity island markers in *E. coli* group B2 strains were compared a) Pakistani B2 strains (n = 19) were compared to B2 (n = 69) from Swedish infants previously published (155). b) B2 strains from the ileal pouch were compared to B2 strains from Swedish controls. Comparisons were made using Fisher's exact test. Prevalence significantly reduced in Pakistani as compared to Swedish B2 is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Thus, the B2 phylogenetic group is not homogenous and particular subgroups within this group may be especially fit for colonization of the large intestine or ileal pouch, respectively.

PERSISTENCE OF *E. COLI* STRAINS IN THE COLONIC AND POUCH MICROBIOTA

The *E. coli* strains were categorized depending on their ability to persist in the colonic microbiota. Table 13 shows the number of strains with different colonization capacity isolated from Pakistani and Swedish infants and the ileal pouch. As seen from the table, *E. coli* in Pakistani infants have a short colonization time and most strains are present for < 3 weeks in their microbiota. In contrast, most *E. coli* strains obtained from Swedish infants are resident, for either an intermediate or long period.

Table 13. *E. coli* strains of different colonization capacity in three different human populations.

	Number of strains		
Cohort	Transient strains (< 3 wk)	Resident strains (> 3 wk)	
Pakistani infants	118	23	
	Transient strains (< 3 wk/< 4 wk*)	Intermediate strains (1 – 11 mo)	Long-term colonizers (≥ 12 mo)
Swedish infants	21	47	32
Ileal pouch	28	27	8

*Three weeks were used for the definition of transient strains in the Swedish infants and 4 weeks for the definition of transient strains in the ileal pouch.

E. coli phylogenetic groups and capacity to persist in the microbiota

The phylogenetic distribution is strongly linked to the capacity to persist in the gut. Thus, our group has previously shown that B2 is more common in resident than transient strains in Swedish school girls (157) and in the Swedish infants presented here (155) when defining the strains as transient or resident using 3 weeks as the break-point. Here we show that the phylogenetic group B2 is more prevalent the longer the strains persist in both the Swedish infants ($p_{trend} < 0.0001$) and the ileal pouch ($p_{trend} < 0.0001$). B1 decreased in relation to time of persistence in the Swedish infants (Fig. 14a) but not in the ileal pouch (Fig. 3a paper III). There was also a non-significant trend for A to decline in comparison to how long the strains reside in the colon of the Swedish infants ($p_{trend} = 0.06$)(Fig. 14a), and A declined significantly in the ileal pouch ($p_{trend} < 0.0001$)(Fig. 3, paper I). In sharp contrast, we demonstrate that B2 strains are not at all better colonizers than strains of other phylogenetic groups in Pakistani infants (Fig. 14b).

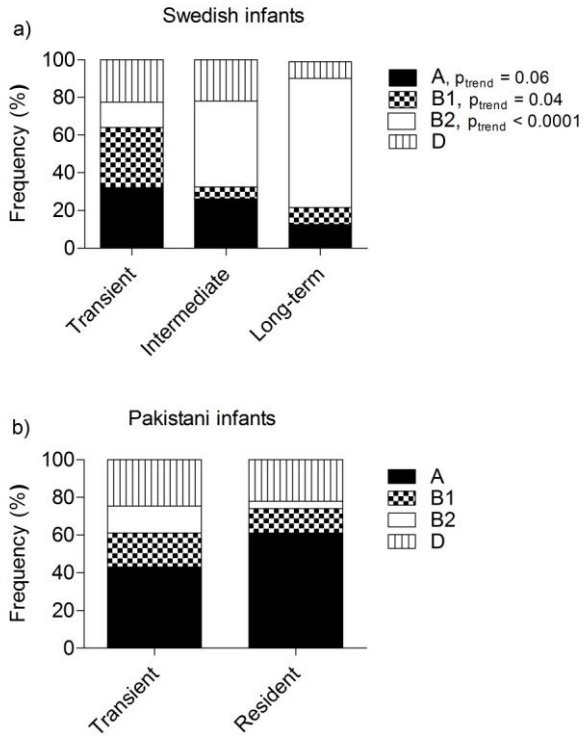

Figure 14. The phylogenetic distribution of *E. coli* differing in capacity to persist in the gut microbiota of three human populations, a) Swedish infants, b) Pakistani infants. Comparisons are made by Exact test, trends are detected by linear by linear association.

Virulence/PAI marker score in *E. coli* and persistence

A virulence score was generated from the number of virulence associated genes carried by each strain. The score was based on carriage of the genes: *fimH*, *papC*, *sfaD/E*, *iutA*, *hlyA*, *kfiC*, *neuB*, *malX, usp* and *fuyA/irp2*. The score ranged from 0 – 9, since the capsule type cannot be both K1 (*neuB)* and K5 (*kfiC)*.

The score was compared between strains of various capacity to persist in the microbiota. The long-term colonizers of the ileal pouch carried much more of the investigated virulence factor genes than strains with intermediate colonization capacity, (average 6.5 vs 1, p = 0.0002). No difference was seen between transient strains and those with intermediate colonization capacity in the ileal pouch microbiota (average 1 vs 1) (Fig. 15a). In the Swedish infants there was a slight difference between transient and intermediate strains (1 vs 3 virulence factor genes, p = 0.048). Long-term colonizers had 6 virulence factors on average of 6 compared to the 3 in intermediate strains (p = 0.002) (Fig. 15b).

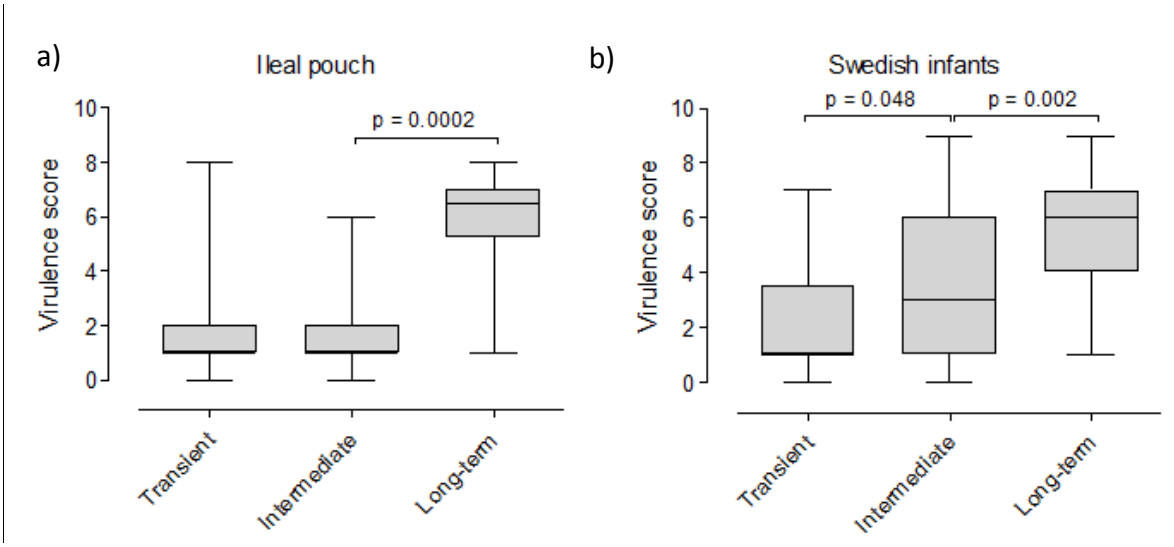

Figure 15. *E. coli* virulence factor score and the capacity to persist in the ileal pouch microbiota of patient with ulcerative colitis or in the microbiota of Swedish infants 0-12 mo. The following virulence markers were included: *fimH*, *papC*, *sfaD/E*, *iutA*, *hlyA*, *kfiC*, *neuB*, *malX, usp* and *fuyA/irp2* .

Association between individual virulence factor genes, PAI markers and persistence

Univariate analysis

Of the 8 PAI markers investigated in the microbiota of Swedish infants, five had a significant increase in relation to the time each strain colonized the bowel (Fig. 16) These were: IV_{536}, *usp*, I_{CFT073}, II_{CFT073} and II_{J96} ($p_{trend} < 0.000$, $p_{trend} < 0.000$, $p_{trend} < 0.000$, $p_{trend} = 0.003$ and $p_{trend} < 0.001$, respectively). The frequencies are shown in figure 1, paper II.

As seen in Table 14, many of the virulence factor genes and PAI markers were associated with the capacity to persist in the ileal pouch microbiota. We used Fisher's excact test for non-random distribution and linear by linear association for trend analysis to identify these traits.

In view of the many analysis performed, we only take p values of ≤ 0.01 into account. Using this rather rigorous approach, we could identify *sfaD/E*, *usp*, *malX* and the marker for PAI I_{536} as associated with the capacity to persist in the pouch microbiota (Table 14).

Results & Comments

Table 14. Virulence factor genes and PAI markers associated with the capacity of *E. coli* strains to persist in the ileal pouch microbiota in patients proctocolectomized due to ulcerative colitis.

	% of strains with gene				
Virulence factor gene/PAI marker	Transient (n = 27)	Intermediate (n = 25)	Long-term (n = 8)	P Fishers	P_{trend}
fimH	74	84	100		
papC	7	4	33		
papGII	0	4	0		
papGIII	7	0	38	0.007	
sfaD/E	7	8	63	0.002	0.004
hlyA	0	4	25		
kfiC	0	0	25		
neuB	4	8	25		
iutA	11	8	50		
usp	11	12	88	0.000	0.000
IV_{536}	37	28	88	0.01	
I_{CFT073} (*malX*)	7	16	75	0.000	0.000
II_{CFT073}	19	8	75	0.001	
II_{J96}	7	0	38	0.007	
I_{536}	7	0	63	0.000	0.004
II_{536}	0	0	13		
III_{536}	0	0	0		

Comparisons were mad using Fishers exact test for non-random distribution and exact test, linear by linear association for trend analysis. Stars indicate the level of significance, ** $p < 0.01$ and *** $p < 0.001$.

Multivariate logistic regression

We used a multiple logistic regression with stepwise selection and in this model only transient strains and long term colonizers were included.

When the ileal pouch strains were modelled, all virulence factor genes and PAI markers were included, e.g. *fimH*, *papC*, *sfaD/E*, *iutA*, *hlyA*, *kfiC*, *neuB* and *usp,* and markers for PAI I-II_{CFT073}, PAI II_{J96} and PAI I-IV_{536}, as well as phylogenetic group B2. In this model, *usp* was the only trait that attained significance (O.R 58.3, CI 95 % 5.22 – 652, p = 0.001). In a logistic regression with stepwise selection including only the adhesins (*fimH*, *papC* and *sfaD/E*), *sfaD*/E was the only variable that attained significance (OR 21.8, CI 2.85 – 165, p = 0.001).

In a similar model for the PAI-markers (*usp,* PAI I-II_{CFT073}, PAI II_{J96} and PAI I-IV_{536}) and phylogentic group B2 was included as explanatory variables for persistence in the Swedish infants, PAI I_{CFT073} was the only variable that rendered significant independent contribution (O.R: 16.2, 95 % CI: 3.8-68, p<0.000).

Projections to latent structures (PLS)

Classical multivariate methods, such as linear or logistic regression, require that all investigated explanatory variables are independent from one other. Virulence genes are strongly clustered and associated to phylogentic groups.

PLS regression analysis was used to investigate the relative strength of the associations of virulence factor genes, PAI markers and phylogentic group B2 with persistence in the colonic microbiota of Swedish infants and the ileal pouch. As seen in figure 3a, paper II and 4a paper III, both PLS analysis yielded a one-component model. For both cohorts long-time colonizers were mainly positioned on the positive axis of the component, while transient strains were most often found on the negative axis of the component. In the Swedish infants intermediate-colonizers were scattered across both axes, whereas in the ileal pouch they clustered together with the transient strains. In other worlds, the intermediate strains in the Swedish infants were dissimilar, whereas in the ileal pouch they were similar to the transient strains.

The bacterial determinants that generated the separation are shown in the scatter plots (Fig 3b, paper II and 4b, paper III). According to the model, variables located on the right side of diagram (with positive values on the Y axis) were associated with long-term colonizers, while traits enriched in transient strains appear on the left (with negative values on the Y axis). The taller the bar, the greater the trait's estimated contribution to persistence and the smaller the error bar, the more reliable the estimate. For both cohorts, the PAI score/virulence score was the variable strongest associated to long-term colonizers.

In the Swedish infants, group B2 origin, PAI I, $II_{CFT073,}$ PAI $IV_{536,}$ PAI II_{J96} and PAI_{USP} were obviously associated with persistence, while markers for PAI I-III_{536} contributed less to persistence (Fig. 3b, paper II). Phylogenetic group origins A, B1 and D were negatively associated to persistence.

In the ileal pouch all examined virulence factor genes and PAI markers as well as phylogenetic group B2 were associated with persistence. The variables being closest associated with persistence were PAI I_{CFT073} (*malX*), *usp*, PAI I_{536}, *sfaD/E* and B2. Phylogenetic groups A and B1 were negatively associated with persistence (Fig 4b, paper III).

BIOPSY STRAINS FROM THE ILEAL POUCH (PAPER III)

It has been shown that a position close to the mucosa is favorable for *E. coli* (228), which is a bacterium capable of aerobic metabolism.

We compared *E. coli* isolated from the pouch biopsies obtained in conjuntion with clinical examination of the pouch and the faecal samples of the same patients. Regarding phylogenetic distribution, we could not demonstrate any difference. The biopsy strains slightly more often than faecal strains belonged to the "pathogenic" B2 (37 % vs 27 %) and D groups (27 % vs 16 %), but less often to A (30 % vs 42 %) and B1 (6 % vs 15 %).

Strains from biopsies significantly more often carried the gene *usp* than faecal strains (47 % vs 25 %, p=0.04) and had a significantly higher median virulence score compared to faecal strains (2, range 0-9 vs 1 range 0-8, p=0.03).

Furthermore, strains obtained from biopsies had a higher proportion of long-term colonizers compared to the faecal strains (Fig 16).

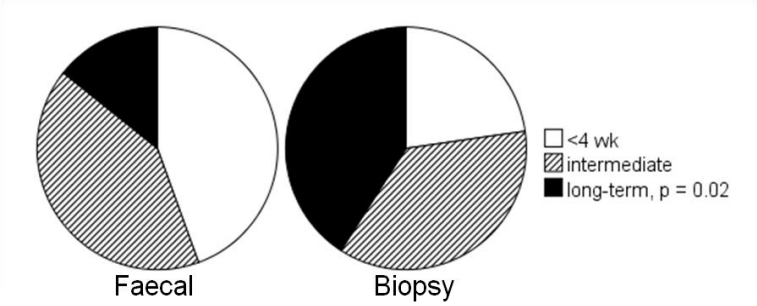

Figure 16. The proportions of transient, intermediate and long-term colonizing *E. coli* derived from faecal and biopsies from the ileal pouch.

DISCUSSION

Proctocolectomy with subsequent ileal pouch anal-anastomosis is an established surgical treatment when the colon has to be removed, mostly due to aggressive or long-standing ulcerative colitis. After closure of the protective stoma and opening the pouch for faecal flow, adaptive changes defined as “colonic transformation” occur in the ileal pouch mucosa. This is likely due to an increased bacterial load and faecal stasis (41). The pouch microbiota has been described as more colon-like than the ileostomal microbiota (151, 197, 213) and colonization by e.g. *Bacteroides* has been linked to morphological physiological changes in the gut mucosa (151). In the present study, we have investigated how and when these changes in microbial population occur in the ileal pouch. The aim was to define the base-line conditions of the normal microbiota transitions. Although, many studies exist that have compared the microbiota between the pouch and the ileostoma, or between the functioning and non-functioning ileal pouch, we are only aware of three previous longitudinal studies of the pouch microbiota, from UC patients. Two use molecular methods (e.g. T-RFLP and cloning and sequencing), the first comprised 2 patients, sampled at five occasions up to one year after closure (57). The second comprises of 49 patient and 31 healthy controls. The patients were sampled three times and the last sample taken > 2 years after closure (116). A third study has cultivated mucus at surgery and at 2 and 8 months after closure (9). We followed 18 patients with ulcerative colitis with regular sampling and quantitative cultivate of important facultative anaerobic and anaerobic bacteria for three years after closure.

The patients defecate approximately 5 times a day (83) and the contents do not remain as long as in the normal colon. This does not permit the highly anaerobic conditions characterizing the normal colon to develop, and limits the expansion of strictly anaerobic bacterial populations. Accordingly, it was the anaerobic bacteria that did not reach the levels found in the normal colon.

First, an immediate increase in total bacterial counts was seen after closure, due to an increase in counts of anaerobic bacteria in pouch as compared to stomal samples. The stomal contents pass through the small intestine at the normal rapid pace and transit through this organ and empty through the stoma. The pouch provides a more static milieu is favourable for the expansion of anaerobic bacteria. Thus, an increase in the anaerobic to aerobic ratio was evident already one month after closure. However, the ratio of anaerobes to aerobes also increased over time in the pouch microbiota, and three years after surgery it was (400:1) no longer significantly different from that in faecal samples from healthy controls (1000:1). This increase over time was due to successively reduced counts of facultative anaerobes. This scenario shows similarities with the establishment of the colonic microbiota in infants, where facultative anaerobes initially dominate the flora, but are later suppressed in response to the establishment of an increasingly diverse anaerobic microbiota (1). An increase in the ratio of anaerobic to aerobic bacteria in the pouch microbiota has been observed also in other studies (151, 197, 213). The total bacterial counts in the ileal pouch samples were approximately five times higher than in ileostomal samples. This increase in bacterial numbers occurred soon after closure, and thereafter the counts

remained relatively stable around 10^9 CFU/g faeces. This is still considerably lower than normal faeces, 10^{10} CFU/g faeces.

Among anaerobes, lactobacilli and clostridia were already present in the stomal samples and immediately reached the same population levels as seen in normal faecal samples from healthy controls. Clostridia are a diverse group of bacterial species, with the ability to form spores which can survive in various environments. Thus, clostridia are easily acquired from environmental sources which is seen e.g. in babies delivered by caesarean section. They are not exposed to their mothers' microbiota and show delayed acquisition of several bacterial groups, but are instead quickly colonized by clostridia (5). We also explored specifically colonization by two potentially pathogenic clostridial species, *Clostridium perfringens* and *C. difficile*, because they could be suspected to cause inflammation and, thereby, contribute to poor reservoir function and pouchitis. However, neither *C. perfringens* nor *C. difficile* were more common or numerous in pouch patients than in normal control faeces. Lactobacilli are common inhabitants also in the normal small intestinal microbiota, and seemed to thrive especially well in the pouch milieu, as they at times reached higher population levels than in control faeces. Thus, both lactobacilli and clostridia are evidently already in place to expand in the pouch as soon a relatively static and anaerobic milieu is created.

Regarding *Bacteroides*, the situation was quite different. They were found in less than half of the ileostomal samples and were even less prevalent in pouch faeces the first months after closure. One possibility is that this depends on the antibiotic treatment in conjunction with the two surgical procedures, when the pouch is constructed, and thereafter when the stoma is closed and the pouch opened for faecal flow. Metronidazole, which is active against *Bacteroides*, was administered to all patients. It took quite long for *Bacteroides* to establish, by 12 months approximately 70 % of the individuals with pouches had *Bacteroides* in their faeces. Bifidobacteria represented an intermediate colonization pattern. It was present in approximately half of the stomal sample. However, colonization rate increased rather rapidly and after 6 months almost all individuals had acquired these bacteria. Notably, neither *Bacteroides* nor bifidobacteria reached the population counts observed in faecal samples from healthy controls. This shows that these typical faecal bacteria are adapted to the colonic milieu and that the pouch environment does not provide a completely optimal milieu. The slow acquisition of especially *Bacteroides*, but also bifidobacteria, points to a scarcity of these types of bacteria in the everyday environment. In parallel, we have demonstrated a very slow acquisition of *Bacteroides* in Swedish infants born today (5), compared with studies performed, e.g. in the 1980s (191, 217) and we have suggested that circulation of these typical faecal bacteria has declined in parallel to improved hygienic standards.

Facultative anaerobes are bacteria equipped for aerobic metabolism, but which also can live and replicate under anaerobic conditions. In the latter case they perform glycolysis and accumulate lactate. Typical faecal facultative anaerobes include *E. coli*, which belongs to the *Enterobacteriaceae* family and the Gram-positive enterococci. Less prevalent are staphylococci, which are mainly adapted to colonize the skin.

E. coli and enterococci both thrived very well in the pouch milieu, where they reached higher counts than both in the ileum and the normal colonic microbiota. These bacteria have the

capacity to coexist with a diverse anaerobic microbiota, but their populations levels gradually decrease when anaerobic microbiota complexity increases, as seen during acquisition of the faecal microbiota in newborn infants. Thus, *E. coli* population levels in infants are higher in the first months, when the anaerobes are not so numerous, and decrease by approximately 1 log unit over the first year of life (5). The same is true for enterococci (5). A similar phenomenon was seen in the pouch, which shows that the competition from anaerobic bacteria increases with time. This process took time, as indicated by the finding that e.g. counts of enterococci was still significantly higher in pouch samples obtained one year after IPAA than in faeces from healthy controls.

In summary, this study clearly demonstrates the sequential acquisition of a colon-like microbiota in the ileal pouch during the first years after IPAA surgery. During this time span, the microbiota changed from a dominance of aerobic or facultative anaerobic bacteria to a clear dominance of anaerobic bacteria. A dramatic change occurs during the first year, especially with the rise in prevalence of *Bacteroides* and to some extent bifidobacteria. During the two following years the changes were chiefly characterized by a slow but steady decrease in aerobic and facultative anaerobic population counts. The limitation of this study was that we used culture, which only permits detection of anaerobic bacteria that survive handling for some time outside the strict anaerobic bowel milieu. Although all samples were kept and transported under anaerobic conditions, we did not employ anaerobic culture-box technique. Further, we only had limited number of selective media for isolation of anaerobes (BBE for Bacteroides, Beerens for bifidobacteria and Rogosa for lactobacilli). Thus, for example *Eubacterium, Roseburia* and *Faecalibacterium,* which belong to the *Firmicutes* and are known member of the normal colonic microbiota were not quantified. Stored faecal samples will later be analyzed by the non-culture dependent technique T-RFLP to further investigate the diversity and permit detection of such strict anaerobic bacteria. These analyses will complement our culture-based study. The advantage of the culture based approach is that it gives the population size of the bacterial groups and specific strains can be analyzed regarding different traits, and that minor populations (> $10^{2.52}$ CFU/g faeces) can be identified, provided selective media are used.

The *E. coli* microbiota was the focus of more detailed studies in which individual strains were tracked during the development of the pouch/colonic microbiota, and characterized regarding phylogenetic group, virulence factor genes and pathogenicity islands (PAIs). PAIs are genomic parts in the bacterial chromosome which includes genes associated to virulence and are thought to be acquired trough horizontal transfer between bacteria. The *E. coli* microbiota of four different human populations; Swedish and Pakistani infants, the patients with an ileal pouch in whom the overall microbiota had been characterized, and healthy adult Swedish controls, was investigated in this way.

The *E. coli* microbiota of 22 Pakistani infants were born in the urban slum of Lahore in 1984 and delivered at home by traditional birth attendants. All but one of the infants, were breastfed, still they also received feeds such as honey and water, fresh milk etc. before breastfeeding was initiated. Samples of the rectal flora were obtained using cotton tipped swabs (4). One hundred and thirty Swedish infant were born between 1998 and 2000, from

urban households, living in high-standard houses or apartments. Seventy percent of the infants were exclusively breastfed until 4 months of age (5). *E. coli* were isolated from quantitative cultures of faecal samples, in a manner identical as for the 18 Swedish adults who were proctocolectomized because of ulcerative colitis and received an ileal pouch at some time point between 2003 and 2005. Their *E. coli* microbiota was compared to that of a healthy control group of Swedish adults, sampled in the late 90s as a control group to a previous published study (158). Their microbiota was sampled using monthly rectal swabs during a period of six months.

The phylogenetic distribution of commensal *E. coli* differed vastly between the different populations studied. Phylogenetic group A dominated in the Pakistani infants but also in the ileal pouch of patients colectomized due to ulcerative colitis. Phylogenetic group B2, which is known to be the most common *E. coli* group to cause extraintestinal infections (22, 178) dominated in the healthy Swedish infants and adults. A climate and geographic dependent difference in phylogenetic origin of human commensal *E. coli* strains has been suggested, where group A strains are more common in individuals living in tropical zones and B2 are more common in temperate zones (55). However, these differences most likely reflect differences in socioeconomic level and hygienic standards, rather than to actual climate differences. For example, the Pakistani infants examined, lived in an urban slum environment where, animals such as goats and poultry are common in households, and with exceptionally poor sanitation (oral communication with I Adlerberth).

Group A and B2 seem to dominate among humans (56), while *E. coli* strains from animals mostly belong to phylogenetic group A and B1 (14, 28, 56). Animal contact and poor food hygiene therefore is likely to involve exposure to strains of the A and B1 groups. In the Pakistani infants, only 38 % became colonized by an *E. coli* found in the mother's microbiota at delivery, still 90 % were colonized by week two in life (4).

The Swedish society has been by a successively reduced exposure to common faecal bacteria, as evident by a successively delayed *E. coli* acquisition and reduced strain turn-over rate (5). In parallel, we have observed a shift in phylogenetic distribution among *E. coli* from Swedish individuals in the last 40 years: in a collection of rectal *E. coli* strains from Swedish school girls, sampled in the 70s, group A dominated (157), while group B2 dominated in the faecal *E. coli* flora of infants sampled in the last 10 years (159) and in healthy Swedish adults examined in the present thesis. We therefore believe that group B2 strains are superiorly adapted to survival and long-term persistence in the human large bowel (155, 157) and represent the truly human-adapted *E. coli*. However, as B2 strains did not appear to be better colonizers than other phylogenetic groups in Pakistani infants. This is likely due to the fact that the Pakistani infants carry less virulence factor genes, and probably belong to a less virulent subgroup of B2 (129).

The microbiota in the ileal pouch was dominated by group A strains and its composition significantly differed from that of normal faeces of healthy Swedish adults. We can think of two possible explanations for this, antibiotic treatment or limited competition due to a poorly developed anaerobic microbiota.

The examined patients had taken different types of antibiotics in conjunction to surgery or for other diseases. These included antibiotics active against *E. coli*, such as norfoxacin and ciprofloxacin. Since strains belonging to phylogenetic group A and D tend to be more resistant to antibiotics than group B2 (25, 109, 194) we cannot exclude that antibiotic treatment in the pouch patients favored group A and D strains. Accordingly, there was a non-significant tendency of a higher proportion of B2 strains in patients who had not received antibiotics (33 % vs 19 %, p = 0.16) (unpublished data). Still, most of the long-term resident strains appeared already in the stomal samples and were obviously not eradicated by the antibiotic treatments given in relation to the surgical procedures. Furthermore, three of the eight patients carrying long-term colonizing strains received antibiotics as outpatients (ciprofloxacin, norfloxacin and doxycycline); this did not result in loss of the strain, two of which belonged to group B2 and one to the phylogenetic group B1 (unpublished data).

Another alternative would relate to the lower complexity of the pouch microbiota in progress compared to the colonic microbiota. During the three years that we examined the pouch patients, there was a progressive increase in the ratio of anaerobes to aerobes, but the thousand fold difference seen in the normal colon was not quite reached. It is therefore possible that the pouch microbiota during the first period after surgery permitted strains of intermediate colonization capacity, such as A and D strains, to expand, but these strains were not able to compete with B2 strains and when the microbiota are more complex.

Interestingly, we found evidence of this hypothesis. Thus, the phylogenetic distribution of the *E. coli* microbiota changed over time in the ileal pouch. In the ileostomal samples, group B2 dominated. One month after closure A and B2 were found in equal proportions, at six months A dominated, while B2 dominated again at twelve months. Accordingly, *E. coli* acquired the highest population number between 1 and 6 months after closure, were after they decreased in numbers. We believe that *E. coli* found in the ileostoma were actually resident in the terminal ileum, because their population numbers were on average 50 times higher in the ileal effluents than were those of the closely related genus *Klebsiella*. Survival in the quickly moving contents of the small intestine probably requires good adherence capacity and other traits that are linked to the virulence profile. After closure, a relatively stationary milieu is created, which may permit less competent strains to persist for some time. When the microbiota becomes more complex, strong colonization skills would again become a necessity.

Despite the fact that A strains were allowed to expand in the pouch microbiota, long-term colonizing capacity was strongly linked to phylogenetic group B2 and carriage of an array of virulence factor genes and pathogenicity islands, as was the case in the Swedish infants. Thus, the word of Sears coined in the 1950s seem equally true today: “one cannot escape the conclusion that *the E. coli* flora of the human bowel is made up of two kinds of strains, those which establish themselves firmly and continue to multiply over extended periods of time and those which are found only in a single or a few successive specimens”. Long-term colonizers differed in practically every aspect from short and intermediate-term colonizers. A notable exception was the *E. coli* from the Pakistani infants, where B2 seemed not to be associated with persistence. We chiefly believe this is due to a difference in the nature of “Pakistani” and “Western” B2:s. Thus, Pakistani B2:s overall carried fewer of the virulence

genes investigated. B2 is known to be a heterogeneous phylogenetic group and at least 9 subgroups are found, and a high polymorphism observed in the B2 flexible gene pool (129). In this light, it is likely that different subgroups are selected for in different environment. For some reason, less armored B2 are selected for in the Pakistani surrounding, where the environment is crowded with various strains. In Sweden and probably other countries with high socioeconomic standard, where the colonization of *E. coli* in the infant is nowadays delayed (5), highly armored B2 are apparently in favor.

Another fact that should also be taken into account is that the "resident" strains from Pakistani infants colonized during quite a short time-span (4). There was a constant rapid turn-over of strains in their microbiota, testifying the enormous load of environmental bacteria. Actually, only a single strain in that *E. coli* collection was present over the entire observation period (3 d-6 mo) (4).

A large number of traits could be associated to persistence, as seen in uni-variate analysis. However, as demonstrated by multivariate analysis (PCA and PLS) that take clustering of variables into account revealed the lack of relevance with such an approach. Practically all virulence factor traits are so tightly clustered that their contribution could not be disentangled. Furthermore, it can be questioned whether this would be meaningful as it was clear from our data that the mere number of virulence traits was the factor most strongly linked to long-term success. Instead, one may speculate that long-term colonizers of the human gut are in a position where they can assemble more virulence/colonization genes from other members of the gut flora. Our group have recently demonstrated transfer of a plasmid conferring antibiotic resistance between two strains colonizing simultaneously in the gut microbiota of an infant (110). PAIs show evidence of being transferred between strains (GC content, transposon genes, IS elements etc.) and since *E. coli* is a strict gut colonizer not found elsewhere in nature, this must hence happened during colonization in the gut microbiota, of man and/or animal.

As we have previously hypothesized, the array of virulence genes characterizing *E. coli* of group B2 has probably mainly evolved to enable this subgroup to persist in its natural niche, the colon/ileum of man.

Never less, some virulence traits may deserve special mention. Among adhesins, we have seen in all examined strain collections that P fimbriae (*papC*) are associated to persistence (153, 154, 157). It is also demonstrated that P fimbriae contribute to colonization of the gut in gnotobiotic rats (87) and P fimbriated *E. coli* adhere well to ileal and colonic epithelial cells (3, 236).

A novel finding was that *sfaD/E* was associated to persistence i the ileal pouch, contrary to our other studies (153, 154, 157). The gene *sfaD/E* recognizes both S fimbriae and F1C fimbriae (128). We need first to examine whether the *sfaD/E* gene is connected with sialic acid binding adhesins, or represent F1C fimbriae. If the *sfaD/E* genes are part of the *sfa* locus encoding S fimbriae, one may speculate that the advantage of this adhesin lies in the capacity to adhere to sialyated mucin. The oligosaccharides are more heavily sialyated in the small intestine and more sulphated in the large intestine (rat) (111) and small intestinal mucins can histochemically be classified as sialomucins and the colonic mucins as a mixture

of sialomucins and sulphomucins (179). Approximately 50 % of the the ileal poch are dominated by sialomucins (40, 210). Since S fimbriae binds to sialyl containing receptors (118) it is tempting to speculate that this association is due to the mucin composition in this particular cohort. To claim this hypothesis, we need to look for the gene coding for the adhesin of S fimbriae.

The uropathogenic-specific protein is a relatively newly found virulence associated gene (124) and a presumed bacteriocin (173) which is highly associated B2 strains (106). Despite the fact that it contributes to infectivity but not lethality in a mouse model for urinary tract infections (241), it is not farfetched to believe that the true biological significance of such a trait is to provide an advantage in the bacterium's normal habitat, the colon.

The more virulence genes the *E. coli* strains carry, the longer they colonize the ileal pouch and the colon of infants. A slight difference is seen though. In the ileal pouch the intermediate strains do not differ from the transient strains at all, whereas in the infantile microbiota there is a slight difference already between intermediate strains and transient strains. This may indicate that it is easier for *E. coli* to colonize the ileal pouch for a semi-long time, compared to the infantile gut. It is possible that this is an indication that the complexity of the microbiota in the ileal pouch is strongly reduced during the first year after surgery. This especially concerns the anaerobic microbiota, e.g. *Bacteroides* and perhaps even other more strictly anaerobic bacteria not detected by us. However, the long-term colonizers in both cohorts differ significantly from the intermediate strains, indicating that it takes an *E. coli* with an arsenal of colonizing factors to inhabit the intestinal tract for at least one year.

In conclusion, we have shown that the larger amount of virulence factor genes for extraintestinal infections/colonization factors *E. coli* are equipped with, the longer they can reside in the ileal pouch and the colon. There is a profound difference between the long-term colonizing strains, which are mainly found the Swedish cohorts, compared to those colonizing for a shorter time.

ACKNOWLEDGEMENT

Ett stort tack till min huvudhandledare ***Agnes Wold***! Först och främst för att gav tillfälle att doktorera, och för dina stora visioner. Du är en högljudd person med varmt hjärta, det har varit roligt att få möjlighet att genomföra detta och att lära känna dig.

Tack till mina bihandledare, ***Forough***, introducerad mig till *E. coli* och virulens faktorernas värld och ***IA*** för ditt stöd och stora kunnande om bakterier och även för hjälp med läsande av manuskript.

Ett speciellt tack till ***Eva Å*** för våra oförglömliga resor och alla samtal om livet. Det har varit ovärderligt. Istanbul och Cluj-Napoca var fantastiska äventyr!!! Tack även för allt som du har lärt mig på labbet, ni "damer" har lärt mig allt jag kan!

Stort tack till dig, ***Ingela K***, till att börja med för att du har lärt mig att odla och läsa plattor och för labbjobbet du har utför åt mig. Men också för att du är så trevlig och omtänksam.

Jolanta, vår glamorösa filmstjärna på labbet, tack för alla recept och roliga samtal.

Tack ***Fei*** för våra resor till Norge och Amsterdam. Vem skulle man hellre fira midsommar med än dig? Tack även för vårt samarbete, jag lär mig massor när jag jobbar med dig.

Anna S, det har varit ett nöje att lära känna dig och dela skrivrum i nöden. Du är min föregångare och ledstjärna i doktorerande och jag vet inte hur jag skulle ha tagit mig igenom all byråkrati utan din hjälp. Tack för alla skratt när man inte orkar skriva mer!

Ett varmt tack till ***Nahid Karami***, för hjälp och samtal om stammar och kloner. Men framför allt för ditt varma skratt och dina kramar när de behövs som mest. Ett stort tack till ***Erika***, inte minst för läsandet av min ram under sena nätter. ***Cillan***, tack för din värme och alla roliga samtal. ***Susanne S*** för våra träningsrundor i Rudalen. ***Anna M*** för genomförd vasaloppsresa och roliga tillställningar utanför jobbet. ***Sofia*** för dina uppmuntrande ord och glada skratt. ***Marianne***, för din schyssta humor.

Tack till ***Jonas Bengtsson*** som har hjälp mig att försöka förstå "pouchen" och snällt svarat på mina frågor, tack även till alla andra på Östra som är involverade i BR-studien och sliter med prover år ut och år in.

Tack till våra nya vänner, ***Emma***, ***Anna H***, ***Erica & Magdalena***. Det är skoj att ni kommit hit.

Lena Svensson & Anna-Lena Stenfeldt, jag säger bara "fjällräven classic", WE ROCK!!!

Ett stort tack till alla andra som är eller som har varit på 6:e våningen, ni har gjort min tid här till ett sant nöje.

Jag vill även tacka mina nära och kära som förgyller min vardag och lyssnar när jag krisar!

Först vill jag tacka min kära ***mamma*** för att du alltid stöttat mig och trott på mig. Vad jag än har hittat på, så har du alltid trott att jag ska klara av det. Tack för ditt stöd mamma!

Mormor & Sven, tack för stöd och support genom livet.

Astrid, tack för all hjälp genom åren.

Pappa, för att du har gett mig intresset för friluftsliv, trots att jag aldrig lärt mig tycka om fiske.

Alexander & Vendela, mina kära små änglar som förgyller mitt liv minst en vecka per år. Jag tackar även Liseberg för att ni vill komma hit;) Tack även till min kära kusin Karin för att du finns.

Sofia Carlshamre, Jag säger bara R och tack för att du finns! Vem skulle jag annars dricka rödvin och diskutera livet med?

Gänget, Karin & Kalle, Olof, (Sofia igen), Jenny och David, hur skulle jag annars fira nyår och semester?

Tina, för att du gör världen lite roligare och lite mer intressant. ***Åsa***, för långa joursamtal på telefonen. ***Petra*** för dina uppmuntrande tillrop.

Maria R, för klätterkurser och fjällturer med trevligt sällskap. ***Marlene & George*** för roligt friluftsliv och goda middagar. ***Helena & Micke*** för skratt, fester och mat. ***Linnéa*** för timslånga telefonsamtal och analyser.

Mina kära hundvakter som fått livet att gå ihop, med speciellt omnämnande av ***Marcus***, ***Nina*** och ***Therese***.

Sist men inte minst så vill jag tacka ***Mikael***. Tack för att du har tagit dig in i mitt liv och stått ut under den sista tiden av mitt doktorerande.

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