Characterization of Asymptomatic Bacteriuria (ABU) *Escherichia coli* Isolates: virulence traits and host-pathogen interactions

(Charakterisierung der Virulenzeigenschaften und Wirt-Pathogen-Interaktionen von asymptomatischer Bakteriurie (ABU) *Escherichia coli* Isolaten)

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Table of Contents

1. Summary

Urinary tract infection (UTI) is one of the most serious health problems worldwide. It accounts for a million hospital visits annually in the United States. Among the many uropathogenic bacteria, uropathogenic *Escherichia coli* (UPEC) is the most common causative agent of UTI. However, not all *E. coli* that inhabit the urinary tract can cause UTI. Some of them thrive for long periods of time in the urinary bladder without causing overt symptoms of infection. This carrier state is called asymptomatic bacteriuria (ABU).

E. coli ABU isolates can live in the host without inducing host response due to deletions, insertions and point mutations in the genome leading to the attenuation of virulence genes. They therefore behave in the same way as commensals. Since bacteria that inhabit the urinary tract are said to originate from the lower intestinal tract and ABU behave in a similar way as commensals, this study compared various phenotypic and genotypic characteristics of ABU and commensal *E. coli* fecal isolates. The two groups did not show a strict clustering with regards to phylogenetic lineage since there appears to be overlaps in their distribution in some clonal complexes. In addition, it was observed that the UPEC virulence genes were more frequently inactivated in ABU than in fecal isolates. Hence, ABU tend to have less functional virulence traits compared to the fecal isolates.

The ABU model organism *E. coli* 83972 which is known not only for its commensal behavior in the urinary bladder but its ability to outcompete other bacteria in the urinary tract is currently being used as prophylactic treatment in patients who have recurrent episodes of UTI at the University Hospital in Lund, Sweden. The pilot studies showed that upon deliberate long-term colonization of the patients with *E. coli* 83972, they become protected from symptomatic UTI.

In this study, the phenotypic and genotypic characteristics of eight re-isolates taken from initially asymptomatically colonized patients enrolled in the deliberate colonization study

who reported an episode of symptoms during the colonization period were investigated. Two out of the eight re-isolates were proven to be a result of super infection by another uropathogen. Six re-isolates, on the other hand, were *E. coli* 83972. The urine re-isolates confirmed to be *E. coli* 83972 were phenotypically heterogeneous in that they varied in colony size as well as in swarming motility. Four of these re-isolates were morphologically homogenous and similar to the parent isolate *E. coli* 83972 whereas one of them appeared phenotypically heterogenous as a mixture of smaller and normal-sized colonies. Still another re-isolate phenotypically resembled small colony variants. Meanwhile, three of the six re-isolates did not differ from the parent isolate with regards to motility. On the other hand, three exhibited a markedly increased motility compared to the parent isolate. Transcriptome analysis demonstrated the upregulation of a cascade of genes involved in flagellar expression and biosynthesis in one of the three motile re-isolates. However, upon further investigation, it was found out that the expression of flagella had no effect on bacterial adhesion to host cells *in vitro* as well as to the induction of host inflammatory markers. Thus, this implies that the increased motility in the re-isolates is used by the bacteria as a fitness factor for its benefit and not as a virulence factor. In addition, among the various deregulated genes, it was observed that gene regulation tends to be host-specific in that there is no common pattern as to which genes are deregulated in the re-isolates. Taken together, results of this study therefore suggest that the use of *E. coli* 83972 for prophylactic treatment of symptomatic UTI remains to be very promising.

1. Zusammenfassung

Harnwegsinfektionen (HWI) sind weltweit ein ernstes Gesundheitsproblem, auf welches allein in den USA jährlich ca. eine Million Krankenhausbesuche entfallen. Innerhalb der Gruppe uropathogener Bakterien stellen die uropathogenen *Escherichia coli* (UPEC) die wichtigsten Verursacher akuter Harnwegserkrankungen dar. Interessanterweise führen nicht alle *E. coli* Varianten, die den Harnweg besiedeln, zwangsläufig zu HWI. Einige von ihnen sind in der Lage, die Harnblase über einen langen Zeitraum zu kolonisieren ohne Symptome einer HWI auszulösen. Dieses Phänomen wird als asymptomatische Bakteriurie (ABU) bezeichnet.

Die Eigenschaft von *E. coli* ABU-Isolaten innerhalb des Wirtsorganismus leben zu können, ohne eine deutliche Wirtsabwehrreaktion hervorzurufen, ist unter anderem bedingt durch Deletionen, Insertionen und Punktmutationen im bakteriellen Genom und der daraus resultierenden Inaktivierung einiger Virulenzgene. Ihre Lebensweise ist daher mit der kommensaler Organismen vergleichbar. Da die den Harnweg besiedelnden Bakterien mit hoher Wahrscheinlichkeit ihren Ursprung im unteren Darmtrakt haben und sich die ABU-Isolate ähnlich der Kommensalen verhalten, wurden in dieser Arbeit zahlreiche phäno- und genotypische Charakteristika von ABU-Isolaten mit denen kommensaler *E. coli*-Fäkalisolate verglichen. Für diese beiden Gruppen konnte hinsichtlich ihrer phylogenetischen Abstammung keine strikte Clusterbildung festgestellt werden, da ihre Verteilung in einigen klonalen Komplexen Überlappungen aufwies. Es zeigte sich jedoch, dass die UPEC-Virulenzgene in den ABU-Isolaten häufiger inaktiviert vorlagen als in den Fäkalisolaten. Demzufolge scheinen die ABU-Isolate weniger funktionale Virulenzeigenschaften zu besitzen als die Fäkalisolate.

Der Modell-ABU Stamm *E. coli* 83972 ist sowohl für sein kommensales Verhalten in der menschlichen Blase bekannt, als auch für seine Fähigkeit, andere Bakterien aus dem Harntrakt zu verdrängen. Er wird gegenwärtig am Universitätsklinikum in Lund (Schweden) als prophylaktisches Therapeutikum bei der Behandlung von Patienten mit rezidivierenden HWI eingesetzt. In Pilotstudien konnte gezeigt werden, dass eine

vorsätzliche Langzeit-Kolonisierung von Patienten mit *E. coli* 83972 zum Schutz vor symptomatischen HWI führt.

In der vorliegenden Arbeit wurden die phäno- und genotypischen Charakteristika von acht Patienten-Reisolaten dieser Langzeitstudie untersucht. Die Reisolate wurden aus zunächst asymptomatisch kolonisierten Patienten isoliert, die allerdings im Verlauf der Langzeit-Kolonisierung über eine Reihe von Symptomen klagten. Zwei dieser acht Reisolate waren nachweislich das Resultat einer Superinfektion mit einem anderen uropathogenen Bakterium. Die restlichen sechs Reisolate konnten jedoch als *E. coli* 83972 identifiziert werden. Für diese sechs Reisolate war eine phänotypische Heterogenität zu beobachten, die sich zum einen in variierender Koloniegröße, zum anderen in unterschiedlichem Schwärmverhalten zeigte: Vier der Reisolate entsprachen morphologisch dem Ausgangsstamm *E. coli* 83972, wohingegen ein Reisolat phänotypisch abweichend als Mischung von kleineren und normal-großen Kolonien in Erscheinung trat. Ein weiteres Reisolat ähnelte phänotypisch sogenannten „Small Colony Variants“. Das Schwärmverhalten betreffend unterschieden sich indessen drei der sechs Reisolate vom Ausgangsstamm. Sie zeigten im Vergleich eine erhöhte Motilität. In einem dieser drei motilen Reisolate konnte mittels Transkriptomanalyse die hochregulierte Expression einer Reihe von Genen, welche für die Flagellenexpression und -biosynthese verantwortlich sind, aufgezeigt werden. Weiterführende *in vitro*-Untersuchungen ergaben jedoch, dass diese erhöhte Flagellenexpression weder einen verstärkenden Effekt auf die bakterielle Adhäsion an die Wirtszellen hat, noch in der Wirtszelle die Bildung von Entzündungsmarkern induziert. Dieses Ergebnis impliziert, dass die erhöhte Motilität der Reisolate als Fitnessfaktor und nicht als Virulenzfaktor zu betrachten ist.
Ferner führte die genauere Analyse der deregulierten Gene zu der Annahme, dass die Genregulation in den Reisolaten wirtsspezifisch ist, da sich kein übereinstimmendes Muster bezüglich der Deregulation abzeichnete.

Unter Berücksichtigung aller Ergebnisse dieser Studie lässt sich abschließend sagen, dass die Verwendung des *E. coli* Stammes 83972 als prophylaktisches Therapeutikum bei der

Behandlung von symptomatischen HWI weiterhin als sehr vielversprechend angesehen werden kann.

2. Introduction

2.1. Urinary Tract Infection

Urinary tract infection (UTI) is a serious health problem that affects millions of people worldwide (Stamm and Norrby, 2001). It is known to be the most common bacterial infection. In fact, UTI accounts for millions of clinical visits and hospitalizations annually.

UTI is more common in women, with almost half of all women incurring one episode of UTI during their lifetime (Foxman, 2002; Guyer *et al*., 2001). Among all UTI-causing organisms, *Escherichia coli* remains to be the most predominant (Croxen and Finlay, 2010; Ronald, 2002; Guyer *et al*., 2001). Moreover, uropathogenic *E. coli* (UPEC) is responsible for 70-90 % of the estimated 150 million UTIs diagnosed annually in the United States (Stamm and Norrby, 2001).

Three clinical types of UTI exist. Cystitis is confined to the urinary bladder. It is characterized by dysuria, urgency, frequency of urination, and sometimes suprapubic pain. Acute cystitis should not be accompanied by acute phase reactants or cytokines in the serum, but there is pyuria (*i.e.* leukocytes in the urine), and IL-6 and IL-8 levels in urine may be elevated (Svanborg and Godaly, 1997).

On the other hand, the more serious condition, acute pyelonephritis, ascends to the kidneys (Pace, 2000). It is accompanied by fever, flank pain, and general malaise. Investigation in the laboratory shows presence of pyuria, elevated acute phase reactants in serum (C reactive protein [CRP], erythrocyte sedimentation rate [ESR]), elevated levels of cytokines in serum and urine, and later increased levels of antibacterial IgA antibodies in urine and serum (Svanborg and Godaly, 1997). It is a severe systemic infection usually caused by UPEC clones with virulence genes clustered on pathogenicity islands (Mabbett *et al*., 2008).

A third type, known as asymptomatic bacteriuria (ABU), resembles commensalism due to its ability to thrive in the urinary bladder without causing symptoms in the host. (Mabbett *et al*., 2008).

2.2. Asymptomatic bacteriuria strain 83972

A certain proportion of apparently healthy individuals have bacteria in their urine in the absence of any clinical symptoms. This is called asymptomatic bacteriuria (ABU). ABU is characterized by the presence of 10^5 CFU/ml of one type of bacteria in a culture of clean voided midstream urine isolated from an individual without symptoms of a urinary tract infection or fever (Costerton *et al*., 1995). ABU is commonly detected only at screening because it is not accompanied by any of the symptoms seen for acute pyelonephritis and acute cystitis (Figure 1). The laboratory findings vary. In addition, the patients may have low level cytokine responses and leukocytes in urine, or they may have no host response to infection. (Svanborg and Godaly, 1997). ABU may be found in 2.7 % of women aged between 15 and 24 years and increases to 20 to 50 % in women above an age of 80 years. In men the prevalence of ABU is considerably lower, but increases to 6 to 20 % above the age of 80 years (Stein and Fünfstück, 2000).

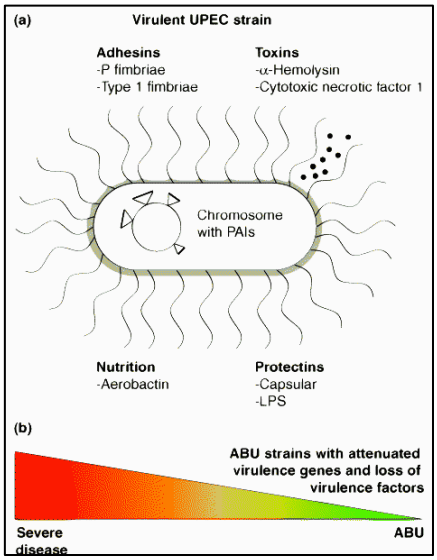

Figure 1. **Differences between UPEC and ABU**. (a) Fully virulent UPEC possess virulence factors while (b) ABU have become attenuated due to gene mutations and gene loss (adapted from Svanborg *et al*., 2006).

The most studied *Escherichia coli* asymptomatic bacteriuria isolate is ABU *E. coli* isolate 83972, which was first discovered to have colonized a young Swedish school girl for three years (Lindberg et al. 1978). It has several outstanding characteristics that sparked the interest of clinical researchers. It has the ability to persist in the human bladder for extended periods without provoking host response (Mabbett *et al.,* 2008). This is due to the fact that its virulence-associated adhesin genes have become attenuated giving rise to poor immune detection by the host. It also has the capability to outcompete other bacteria in the urinary tract (Klemm *et al.*, 2006). ABU thus represents a successful adaptation, because the bacteria can persist without competition in a niche with a rich nutrient source, often for several years (Svanborg *et al*. 2006). With these characteristics, *E. coli* 83972 is being used in deliberate colonization studies for prophylactic purposes in patients with recurrent urinary tract infection (Wullt *et al.*, 2001, Sunden et al., 2006, 2010).

2.3. Uropathogenic and Commensal *Escherichia coli*

UPECs differ from non-pathogenic *E coli* variants by the presence of virulence factors which contribute to their ability to cause disease (Blum-Oehler *et al*., 2002). They acquire traits that distinguish them from commensal *E. coli* strains. Characteristically, UPEC strains produce alpha/hemolysin and cytotoxic necrotizing factor 1, P fimbriae, aerobactin, exhibit serum resistance, and are encapsulated (Tóth *et al*., 2000). UPECs have clustered sets of virulence genes, termed pathogenicity islands (PAI). PAIs are heterogenous pieces of DNA characterized by an unusual genetic organization, comprising virulence genes, mobility and regulatory genes or pseudogenes as well as genes or open reading frames with unknown functions (Hacker *et al.*, 1997).

As a commensal, *E. coli* inhabits the intestines and feces of warm-blooded animals and reptiles. It is the predominant aerobic organism in the vertebrate gut. As in any commensal relationship, *E. coli* commensals derive nutrients from the host. In turn, they can prevent host colonization by pathogens by inducing colonization resistance in the

host. They are able to do this by production of bacteriocins and through other mechanisms (Tenaillon *et al*., 2010).

2.4. Virulence Factors of Uropathogenic *Escherichia coli*

2.4.1. Adhesins

The ability to adhere to a wide variety of biotic and abiotic surfaces is a feature which can promote bacterial survival and is one of the key virulence functions of many pathogens. Adhesion is mediated by distinct surface structures called adhesins (Gerlach and Hensel, 2007). For the invading bacterium, the ability to sense the host tissue depends on an adhesin protein at the tip of the pilus (Lund *et al*., 1987; Hultgren *et al*., 1989).

Adhesins are required for colonization of host mucosal tissues such as the urinary tract and establishment of the infectious process. In addition, adhesins and their cellular receptors also play an important function during subsequent stages of the infectious processes, such as during the formation of intracellular bacterial reservoirs for subsequent infection cycles, induction of cell signaling and induction of the innate immune response (Bouguénec, 2005). Most commensal and pathogenic bacteria interacting with eukaryotic hosts express adhesive molecules on their surfaces that promote interaction with host cell receptors or with soluble macromolecules. Although bacterial attachment to epithelial cells may be beneficial for bacterial colonization, adhesion may come at a cost because bacterial attachment can also stimulate immune cell infiltration, activation, and phagocytosis, which facilitate bacterial clearing (Kline *et al*., 2009). UPEC strains have been shown to express a number of different fimbrial adhesins including P, F1C, S, M, Dr and type 1 fimbriae (Bahrani-Mougeot *et al*., 2002).

In the urinary tract, adherence is considered a virulence trait. It has been proposed to play a critical role for the establishment of bacteriuria. Among the many adhesins, P fimbriae have the strongest association with acute disease severity, being present in at least 90 %

of acute pyelonephritis but only 20 % of ABU. In contrast, type 1 fimbriae can be expressed by more than 90 % of both commensal and uropathogenic *E coli*. The disease association of other adhesin genes, on the other hand, is less studied (Bergsten *et al*., 2005).

2.4.2. Flagella

Flagella are filamentous appendages required for bacterial chemotaxis and motility. They are rotary structures driven from a motor at the base, with the filament acting as a propeller. The flagellum consists of three major substructures: the filament, the hook and the basal body. The filament is typically about 20 nm in diameter and usually consists of thousands of copies of a single protein called flagellin. Less commonly the filament is composed of several different flagellins. (Shapiro, 1995; Bardy *et al*., 2003). The polymerized sub-units of flagellin in *E coli* are encoded by *fliC*. In UPEC, the mutation of *fliC* can lead to loss of flagellation and motility (Lane *et al*., 2007).

Flagellin signaling at the epithelium stimulates production of cytokines and chemokines responsible for the recruitment of immune cells to the site of pathogen entry. In addition, expression of TLR5 by immune cells such as polymorphonuclear neutrophils, natural killer (NK) cells, and monocytes and macrophages permits further amplification of the innate immune response (Honko and Mizel, 2005). A study by Ivison *et al*. (2010) showed that bacterial flagellin triggers inflammation in mammalian cells via TLR5 and that release of the chemokine IL-8 in response to flagellin involves NF-κB, p38 MAP kinase, and phosphatidylinositol 3-kinase (PI3K).

Apart from their functions in motility, flagella also contribute to efficient colonization of the urinary tract (Lane et al., 2007). In fact, the flagella of enteropathogenic *E. coli* mediate adhesion to epithelial cells *in vitro* (Girón *et al*., 2002). In addition, flagella, specifically the flagellin, could stimulate interleukin-8 (IL-8) production in T84 bladder cells (Zhou *et al*., 2003). In fact, it causes IL-8 release from several epithelial cell lines (Khan *et al*., 2004).

2.4.3. Extracellular Polysaccharides

Bacterial cell surface polysaccharides, such as lipopolysaccharides (LPS) and capsular polysaccharides (CPS), are important in processes critical for bacterial pathogenicity such as bacterium–host interactions, resistance to serum-mediated killing, and regulation of the host immune response (Robbins *et al*., 1980; Moxon and Kroll, 1990). Expression of CPS and LPS in part dictates the pathogenic potential of Gram-negative bacteria. Moreover, LPS and groups 2 and 3 CPS play major roles in the pathogenesis of infection. Capsules and LPS are considered as virulence factors that protect *E. coli* against various host bactericidal defenses (Russo, 2002).

LPS is a unique glycolipid found in the outer membrane of Gram-negative bacteria. It is made up of lipid A, a core oligosaccharide region and a serotype- specific O antigen (Russo, 2002). LPS enhances inflammatory response due to its effects on cytokine synthesis. In addition, it is involved in the resistance of the pathogen to the killing effect of the host serum (Emődy, 2003).

Capsular polysaccharides, on the other hand, are linear polymers of repeating carbohydrate subunits that sometimes also include a prominent amino acid or lipid component. They coat the cell, interfering with O antigen detection and protecting the cell from host defense mechanisms (Johnson, 1991). Capsules provide protection against phagocytic engulfment and complement bactericidal effect in the host. Moreover, certain capsular types such as K1 and K5 show a mimicry to tissue components which prevents humoral immune response by the infected host (Emődy, 2003). In *E. coli* Nissle 1917, the K5 capsule expressed on the cell surface was found to be able to be important in mediating interactions with intestinal epithelial cells as well as induction of chemokine gene expression (Hafez *et al*., 2009). In addition, the K5 capsule was also found to stimulate expression of Toll-Like Receptor (TLR) 5, CD14, MyD88 and TRIF together with the induction of Interleukin-8 expression in epithelia cells (Hafez *et al*., 2010).

2.4.4. Toxins

Toxins are exported virulence factors among UPEC of which the most important is α-hemolysin (Smith, 1963). Alpha-hemolysin is a pore-forming toxin targeting not only erythrocytes but also leukocytes as well as endothelial and renal epithelial cells (Keane *et al*., 1987). Another UPEC toxin is the cytotoxic necrotizing factor 1 (CNF1) which interferes with polymorphonuclear phagocytosis and evokes apoptotic death of bladder epithelial cells (Caprioli *et al*., 1987; Fiorentini *et al*., 1997). In addition, the cytolethal distending toxin (CDT) was also detected among UPEC. This toxin causes apoptosis of host cells (Lai *et al.*, 2000).

2.5. Bacteria and Host Immune Response

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and non-self. In mammals the immune system can be divided into two branches: "innate immunity" and "adaptive immunity" (Takeda *et al.*, 2003) (Figure 2). The first line of defense against infectious diseases is the innate immune response. This is mediated by white blood cells, cells that phagocytose and kill the pathogens, and those that synthesize inflammatory mediators and cytokines (Aderem and Ulevitch, 2000).

The innate host response reflects the reactivity of mucosal sites to microbial attack. Commensal organisms often fail to evoke this response; thus, the mucosal barrier remains inert to these microbes and to their secreted products, and a state of asymptomatic carriage is established. The pathogens, in contrast, are equipped to break the inertia of the mucosal barrier by triggering a host response (Bergsten *et al*., 2004).

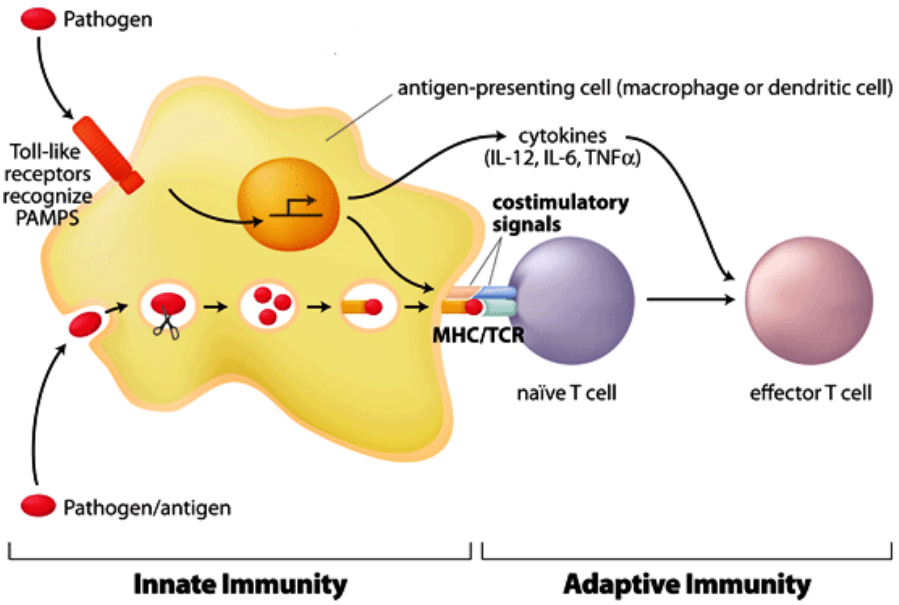

Figure 2. **Innate immune response** involves (1) activation of antigen-presenting cells (APCs) through recognition of pathogens or pathogen-associated molecular patterns (PAMPs) by receptors such as toll-like receptors (TLRs). (2) This leads to the production of inflammatory cytokines and expression of co-stimulatory molecules on the cell surface. **Adaptive immune response** involves (3) presentation of antigens on APCs to T lymphocytes by major histoocmpatibility complex (MHC). (4) Activated T lymphocytes become further differentiated to effector T lymphocytes by stimulation with cytokines. (Adapted from http://research4.dfci.harvard.edu/innate/innate.html).

2.5.1. Interleukin-6 (IL-6)

Mucosal surfaces are primary sites of encounter between bacteria and the human host. Mucosal response to microbial challenge includes the production of cytokines. (Agace *et al*., 1993). IL-6 is a part of the human mucosal response to Gram-negative UTIs (Hedges *et al*., 1991). It is a cytokine produced by a variety of cells and acts on a wide range of tissues, exerting growth-inducing, growth-inhibitory and differentiation-inducing effects, depending on the nature of the target cells (Hirano *et al*., 1990). Deliberate colonization of the human urinary tract with *E. coli* stimulated the secretion of IL-6 into the urine without concomitant elevation of the serum IL-6 levels. It is suggested that urinary IL-6 is of local origin and that mucosal cells were activated directly by bacteria to produce cytokines (Hedges *et al*., 1991; Hedges *et al*., 1992). Moreover, IL-6 is also demonstrated to be activated as part of lipopolysaccharide-induced mucosal and systemic responses to Gram-negative bacterial infections (de Man *et al*., 1989).

IL-6 functions in several different ways. It activates hepatocytes to secrete acute-phase reactants, stimulates mucosal B lymphocytes, and acts as an endogenous pyrogen (Hedges *et al*., 1992). Additional functions of IL-6 are listed in Table 1.

Table 1. Various functions of IL-6 (Adapted from Hirano *et al*., 1990)

Function	Cell/Process
Induction	B-cell differentiation Acute phase proteins in liver cells IL-2 and IL-2 receptor expression Myeloid leukemic cell lines differentiation to macrophages Maturation of megakaryocytes as a thrombopoietic factor Mesangial cell growth Neural differentiation of PC12 (pheochromocytoma) cells Keratinocyte growth
Growth Promotion	Myeloma/plasmacytoma/hybridoma cells
Proliferation and Differentiation	T cells|
Cell Growth Inhibition	Myeloid leukemic cell lines
Enhancement	IL-3-induced multipotential colony cell formation in hematopoietic stem cells

In 1992, Hedges *et al.* compared urine and serum concentrations of IL-6 in women with acute pyelonephritis or asymptomatic bacteriuria. They found out that IL-6 activity was elevated in urine samples from most bacteriuric women, regardless of the severity of infection. However, elevated IL-6 serum levels occurred only in patients with acute pyelonephritis. The spread of IL-6 to the circulation in patients with acute pyelonephritis may contribute to the elevation of fever and C-reactive protein characteristic of the disease.

2.5.2. Interleukin-8 (IL-8)

Infection or injury of the body results in inflammation. A hallmark of this response is the recruitment of neutrophils from the blood to the injured tissue. This process is directed by chemotactic polypeptides of 8–14 kDa, so-called chemokines. About 40 human chemokines are known today (Zlotnik and Yoshie, 2000). The founding member of the C-X-C subfamily of chemokines is interleukin-8 (IL-8) (Baggiolini and Lewis, 1992; Wolff *et al*., 1998; Utgaard *et al*., 1998; Hoffmann *et al*., 2002).

IL-8 localizes intracellularly in the Golgi apparatus (Wolff *et al*., 1998). It is produced in several tissues by phagocytes and mesenchymal cells upon infection, inflammation, ischemia, trauma, among others. It activates integrin-mediated adhesion of neutrophils at the site of infection (Baggiolini and Lewis, 1992; Utgaard *et al*., 1998). In addition, it acts as a chemotactic factor for T-cell subsets and basophils enabling neutrophils to release lysosomal enzymes, undergo a respiratory burst and degranulate (Tullus *et al*., 1994). One of the most remarkable properties of IL-8 is the variation of its expression levels. In healthy tissues, IL-8 is barely detectable, but it is rapidly induced by ten- to 100-fold in response to proinflammatory cytokines such as tumor necrosis factor or IL-1, bacterial or viral products, and cellular stress (Hoffmann *et al*., 2002).

2.5.3. TLR5

Toll-like receptors (TLRs) are the receptors of the innate immune system. These receptors recognize antigen patterns (like LPS in Gram-negative bacteria) (Utgaard *et al*., 1998). They are expressed by a variety of primary cells and cell lines that participate in innate and adaptive immune responses (Honko and Mizel, 2005). They are type I transmembrane receptors which recognize conserved molecular patterns of microbial origin. Extracellular surface TLRs including TLR2, TLR4, and TLR5 recognize bacterial lipoproteins, lipopolysaccharide (LPS), and flagellin, respectively. On the other hand, endosomal TLRs such as TLR3, TLR7 / 8, and TLR9 sense dsRNA, ssRNA, and CpG motif from bacterial and viral DNA (Akira *et al*., 2006). Activation of TLRs by specific ligands leads to activation of NF-κB, mitogen-activated protein kinases or interferon regulatory factors via myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent (Toll/Interleukin-1 receptor domain-containing adaptor protein inducing interferon b-dependent) pathways. This in turn produces proinflammatory cytokines or type I interferons (Akira *et al*., 2006; Lee and Kim, 2007).

TLR5 is responsible for the detection of flagellin, the major protein constituent of bacterial flagella. It specifically recognizes the constant flagellin domain D1, which is

relatively conserved among different species (Hayashi *et al*., 2001). TLR5 is expressed by epithelial cells, monocytes, and immature dendritic cells.

2.6. Bacterial Adaptation at the Genomic Level

Bacteria have to live and survive under continuously changing environmental conditions and are therefore compelled to adapt to them (Ziebuhr *et al*., 1999). In the process, they acquire new traits that lead to the development of new bacterial variants. These bacterial variants may become a new generation of pathogens that arose from former non-pathogens.

The rate of adaptation remains controversial to date and probably diverse. However, evolution among organisms could be classified as either "microevolution", when it is very rapid, *i.e.* taking place within days or weeks, yet already resulting to new variants, or "macroevolution", when the process proceeds over longer periods of time, leading to the development of new variants of organisms, new species and sometimes even to the emergence of new genera (Barrick *et al*., 2009; Ziebuhr *et al*., 1999).

Virulence of pathogenic organisms depends mostly on the various genetic changes that occur within the organism. These changes may be acquired as an adaptation to the host environment to enable the organism to become more fit to live and survive. These changes lead to genome evolution of the organism. Genome evolution pertains to both acquisition and loss of genetic information. Several mechanisms are involved in DNA uptake and reduction of genomic content. Both could result from mutations or horizontal gene transfer (Donnenberg, 2002).

Point mutations, genetic rearrangements and horizontal gene transfer are major driving forces of bacterial evolution (Schubert *et al*., 2009). Point mutations can be generated by slipped-strand mispairing, resulting in expression or non-expression of particular genes (Leathart and Gally, 1998). On the other hand, genetic rearrangements may result from deletions in the bacterial chromosome which could play a major role during bacterial

pathogenesis as well as insertion of so-called insertion sequence (IS) elements. IS elements are small mobile DNA units that encode features necessary for their own mobilization (Ziebuhr *et al*., 1999). Since point mutations and genetic rearrangements only lead to slow evolutionary development, they are most likely unable to create novel genetic determinants. In contrast, during horizontal gene transfer, substantial amounts of DNA are introduced into and deleted from the chromosome effectively changing the life style of the bacterial species. When transfer of large DNA elements including plasmids, phages and genomic islands (*i.e.* PAIs) occur, it could lead to a new bacterial pathotype since the incorporation of a PAI can transform a normally benign organism into a pathogen. (Schubert *et al*., 2009).

Aside from the modification of structural and regulatory genes, antimicrobial resistance can also result from insertions, deletions and point mutations in the genome (Musser, 1995). Specific resistance exhibits clear associations with virulence genes (Boerlin *et al*., 2005). Both virulence factors and resistance determinants are subject to intrastrain genetic and phenotypic variation (Ziebuhr *et al*., 1999).

Bacterial adaptation to novel environments is dependent on the rate of genetic variation and on how selection acts on the genetic variation formed (Nilsson, 2005). Therefore, as the environment changes, the organism tries to cope up with the changes in order to be fit for survival.

2.7. Aims of the Study

Phenotypic and genotypic characterization of bacterial isolates that have been re-isolated from *in vivo* growth conditions leads to a better understanding of the way they interact with their host and their immediate environment and how this may affect genome plasticity. Although the model ABU isolate 83972 is well characterized, other ABU isolates remain uncharacterized. Thus, this study aimed to describe the geno- and phenotypic traits of a collection of ABU isolates from Lund, Sweden in the context of asymptomatic colonization of the bladder and host-bacterium interaction. In addition, due

to the general commensal-like behavior of ABU with regards to interaction with the host, it was also of interest to compare them to commensals, in this case, fecal isolates from healthy carriers without any history of urinary tract infection.

One specific aim of this study was to determine on the genomic level the molecular mechanisms involved in the evolution of ABU isolates and thus characterize bacterial factors involved in host-bacterium interaction and bacterial adaptation to growth in the urinary bladder. Furthermore, even if *E. coli* 83972 is successfully being used as prophylactic treatment for recurrent UTI in Lund, Sweden, the fitness of the strain for this purpose should be further and carefully investigated. Rarely has there been any report of symptomatic episodes among patients who are stably colonized with ABU isolate 83972. However, there had been few among them who complained of symptom experience during *E. coli* 83972 colonization. Therefore, this study also aimed to compare *E. coli* 83972 re-isolates from patients who experienced symptoms and look at the possible reasons why they occurred.

3. Materials

All materials used in this study are listed below.

3.1. Strains

Table 2. Bacterial strains used in this study.

Strain	Description	Reference
CFT073	Clinical isolate from pyelonephritis, O6:K2:H7	(Mobley *et al.*, 1990)
E. coli MG1655	F-, γ-, *ilvG*, *rfb*-50 *rph*-1	(Blattner *et al.*, 1997)
E. coli Nissle 1917	Probiotic *Escherichia coli* strain, O6:K5:H7	(Nissle, 1918)
SW102 λ pir	Recombineering strain EL250 *gal* $^{+}$, λ pir lysogen	(Warming et al., 2005)
83972	Asymptomatic bacteriuria isolate	(Lindberg and Winberg, 1976)
74 ABU Isolates (1963-2037)	Asymptomatic bacteriuria isolates	(Svanborg C, Lund)
39 fecal isolates (2038-2076)	Asymptomatic bacteriuria isolates	(Svanborg C, Lund)
BU05 17629	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU05 42290	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU04 50907	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU04 40368	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU04 41631	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU04 63630	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU04 64941	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU04 68088	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU05 35364	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
FIM 636	83972 strain derivate – human colonization study, symptomatic	(Wullt B, Lund)
BU05 13813 (AS)	83972 strain derivate – human colonization study, asymptomatic	(Wullt B, Lund)
83972Δ*fliC*	83972 *fliC* deletion mutant	This study
83972Δ*papGX::*CFT073*papGX_cat*	83972 *papGX* deletion mutant complemented with functional *papGX*	This study

3.2. Oligonucleotides

Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Table 3. Oligonucleotides used in this study.

Primer Name	**Sequence (5´→3´)**	**Application**	**Length (bp)**
ES3_for	agt cat acc taa atg aat aac tgt aat tac gga agt gat ttc tga tga aag tgt agg ctg gag ctg ctt	deletion of *papGX* from 83972	69
ES4_rev	ctt ttg cct gaa gct atc cgg cat act cag gca ttt cac gct tta tga cat atg aat atc ctc ctt agt tcc ta	deletion of *papGX* from 83972	74
pKD3_papG_for	cct ttt tgc gtg gcc agt gcc aag ctt gca tgc aga ttg cag cat tac aca tga aaa aat ggt tcc cag ctt tg	pKD3 recombination of *papGX*	74
pKD3_papX_rev	gaa agt ata gga act tcg aag cag ctc cag cct aca caa tcg ctc aag aca ctt tat gag ctg aca tca tca ag	pKD3 recombination of *papGX*	74
F13	gtc ata ccc aaa tga ata act gta att acg gaa gtg att tct gat gaa aaa atg gtt ccc agc ttt gtt a	recombination of *papGX* from CFT073 into 83972	73
F14b	ttc ttt tgc ctg aag cta tcc ggc ata ctc agg cat ttc acg ctt cat ggt cca tat gaa tat cct cct tag ttc c	recombination of *papGX* from CFT073 into 83972	76
CFT_papG_for	ttc tga tga aaa aat ggt tcc c	screening and sequencing, *papGX-cat* fusion construct	22
papG_revOP	tca tga gca gcg ttg ctg aac c	screening and sequencing, *papGX-cat* construct	22
CFTpapXrev	gag ctg aca tca tca aca tcc gcc	screening and sequencing, *papGX*	24
CFTpapXmid	ggg tct ttg ttt ttt tgg tgt ct	screening and sequencing, *papGX*	25
papX rev	ggt tta tga gct gac atc atc aag	screening and sequencing, *papGX*	24
papX_for_seq	ctg atg cgc gct tgt aca ca	screening and sequencing, *papGX*	20
papEFf	gca aca gca acg ctg gtt gca tca t	screening and sequencing, *papGX*	25
c2	gat ctt ccg tca cag gta gg	screening and sequencing, *papGX*	20

Table 3.- Continued

Primer Name	**Sequence (5´→3´)**	**Application**	**Length (bp)**
c1	tta tac gca agg cga caa gg	screening and sequencing, *papGX*	20
cat3	cat atg aat atc ctc ctt agt tcc ta	screening and sequencing, *papGX*	26
cat5	gtg tag gct gga gct gct	screening and sequencing, *papGX*	18
F1	agc cat cga caa att ggt cgt a	screening and sequencing, *papGX*	22
F3	tca agc tgc tga gac cag gtg g	screening and sequencing, *papGX*	22
F2	gtc ttg caa tct ccc ctt ccg t	screening and sequencing, *papGX*	22
F5	gca gat taa cat cag ggg aaa t	screening and sequencing, *papGX*	22
F6	gtc gct gta ctg gct gat gtg c	screening and sequencing, *papGX*	22
F7	cag aaa tca ctt ccg taa tta c	screening and sequencing, *papGX*	22
Flic_DW_cat-ff	cgc agc gca tca ggc aat ttg gcg ttg ccg tca gtc tca gtt aat cag gtg tgt agg ctg gag ctg ctt	deletion of *fliC* from 83972	69
Flic_DW_cat-rev	gac ggg tgg aaa ccc aaa acg taa tca acg act tgc aat ata gga taa cgc ata tga ata tcc tcc tta gtt cct a	deletion of *fliC* from 83973	76
fliC_Ktr_ff	ccg ttt ctg cag ggt ttt ta	screening, *fliC* deletion from 83972	20
fliC_Ktr_rev	ata ctt gcc atg cga ttt cc	screening, *fliC* deletion from 83972	20
chuA_for	gaa agc tgc gca tcg cgt tg	qRT-PCR	20
chuA_rev	aat ggt gaa acc gcg ccg aa	qRT-PCR	20
papA_for	tgt tga tgc tcc atg cag ca	qRT-PCR	20
papA_rev	acc aga aac cct tgg acc ac	qRT-PCR	20
fliA_for	cga gcg tgg aac ttg acg a	qRT-PCR	19
fliA_rev	cgc tac ctc agt ttc cgt gg	qRT-PCR	20
rfaG_for	ccg ctt tct ctg gca gac tg	qRT-PCR	20
rfaG_rev	ctg ctg gaa gcg ata act gca	qRT-PCR	21

Table 3.- Continued

Primer Name	Sequence (5´→3´)	Application	Length (bp)
narI_for	cgtc atc ccc aac acc agg	qRT-PCR	18
narI_rev	gtg caa cga cta ccg gag c	qRT-PCR	19
frmA_for	tgc att tgc tcc cgg taa acc	qRT-PCR	21
frmA_rev	gca ctc tgc ggt gta aag c	qRT-PCR	19
rrnB_for	aac tga gac acg gtc cag act	qRT-PCR	21
rrnB_rev	tta acg ctt gca ccc tcc gt	qRT-PCR	20
IL6_for	gca ctg gca gaa aac aac ct	qRT-PCR	20
IL6_rev	tca aac tcc aaa aga cca gtg a	qRT-PCR	22
IL8_for	cca aca cag aaa tta ttg taa agc	qRT-PCR	24
IL8_rev	cca aca cag aaa tta ttg taa agc	qRT-PCR	21
GAPDH_for	tga acc acc aac tgc tta gc	qRT-PCR	20
GAPDH_rev	tga acc acc aac tgc tta gc	qRT-PCR	21

For a complete list of the primers used for the detection of virulence genes, please see Johnson and Stell (2000) and for those used for determination of the phylogenetic group see Clermont *et al.* (2000).

3.3. Plasmids

All plasmids used during this study are listed below.

Table 4. Plasmids used in this study.

Plasmid	Description	Reference
pKD46	*repA*101 (ts), *araBp-gam-bet-exo* (α red recombinase under the contol of *araB* promotor), Ap^R *(bla)*	(Datsenko and Wanner, 2000)
pKD3	*oriRγ,* Ap^R, *cat*-gene flanked by FRT sites	(Datsenko and Wanner, 2000)
pKD4	*oriRγ,* Ap^R, *npt*-gene flanked by FRT sites	(Datsenko and Wanner, 2000)
pCP20	Yeast Flp recombinase gene (FLP, *aka exo*) ts-*rep*, Ap^R, Cm^R	(Datsenko and Wanner, 2000)

3.4. Chemicals and solutions

Protein Work:

4 x Protein Sample Buffer

40 mM	Tris/HCl, pH 8.0
4 mM	EDTA
8 %	SDS
40 %	Glycerol
0.004 %	Bromophenol blue
	Aqua bidest

10 % SDS solution

100 g	SDS
900 ml	H_2O

- pH adjusted to 7.2 and volume to 1 L by filling up with H_2O
- sterilized by autoclaving

10 x Running Buffer (1 L)

30 g	Tris
188 g	Glycine
10 g	SDS

1M Tris (1 L)

121.1 g	Tris base
800 ml	H_2O

- pH adjusted to 7.4 and volume to 1 L by filling up with H_2O

Coomassie Brilliant Blue Stain

25 %	Isopropanol
10 %	Acetic acid
0.04 %	Coomassie brilliant blue stain
	H_2O

Destaining Solution

5 %	Acetic acid
7.5 %	Methanol
	H_2O

Semi-Dry Blot Buffer

48 mM	Tris
39 mM	Glycine
0.0375% (w/v)	SDS

- dissolved in 800 ml H_2O
- filled with H_2O up to 1 L mark
- 20 % methanol added prior to use

Block Buffer

2 %	Milk powder in TBS or
3 %	BSA in TBS

Incubation Buffer

0.2 %	Milk powder in TBS or
0.3 %	BSA in TBS

20 x TBS

1 M	Tris/HCl, pH 7.5
4 M	NaCl

TBS-T

1 x	TBS
0.1 %	Tween-20

TE Buffer

10 mM	Tris/HCl, pH 8.0
1 mM	EDTA

Membrane Staining Solution (Western Blot)

50 ml	1x TBS
3 ml	0.3 % 4-chloro-l-naphtol in methanol
80 µl	H_2O_2

LPS Silver Nitrate Staining

2 x Fixation Solution

250 ml	Isopropanol
70 ml	Acetic acid

Periodate Solution

0.87 g	Na-m-periodate ($NaIO_4$)
100 ml	1 x Fixation solution

Silver Nitrate Solution

1.4 ml	1 M NaOH
1 ml	NH_3 (33%)
70 ml	dH_2O
1.25 ml	20 % (w/v) $AgNO_3$

Developing Solution

100 ml	2.5 % (w/v) Na_2CO_3
27 µl	Formaldehyde

DNA Work:

6 x DNA Loading Buffer

0.25 %	Bromophenol blue
50 % (w/v)	Glycerol
1 mM	EDTA

TAE buffer, 50 x

242 g Tris base

57.1 ml Glacial acetic acid

22.6 g EDTA

- H_2O added to a final volume of 1 L

Ethidiumbromide stock solution

10 mg/ml in TE (10 mM Tris/HCl, pH 7.5; 1 mM EDTA)

DNA Polymerases:

- *Taq* DNA Polymerase (Invitrogen, Qiagen)
- Go*Taq*® DNA Polymerase (Promega)
- Red *Taq* (Sigma)
- DAP Goldstar Polymerase (Eurogentec)
- Phusion Polymerase (Finnzymes)

Biofilm Assay and Cell Culture:

10 x PBS (1L)

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

- dissolved in 800 ml H_2O
- pH set to 7.4
- filled with H_2O up to 1 L mark

1 x PBS

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

800 ml Distilled H_2O

- sterilized by autoclaving for 20 minutes

Pulsed Field Gel Electrophoresis:

NDE Buffer

20 mm Hepes

50 mm B-glycerophosphate

15 mm $MgCl_2$

20 mm EGTA

5 mm NaF

2 mm DTT

5 mm Leupeptin

5 mm Pepstatin

2 mg/ml Aprotinin

0.5 mm Sodium orthovanadate

10 mm Ammonium molybdate

10 mg/ml Sodium 4-nitrophenylphosphate,

- with PMSF added to 0.5 mm final concentration immediately before use, pH 7.4

SE buffer

10 mM Tris-HCl, pH 7.5

25 mM EDTA, pH 7.5

TE buffer

75 mM NaCl

1 mM EDTA, pH 7.5

NDS buffer

1 % N-laurylsarcosine

500 mM EDTA, pH 9.5

2 mg/ml Proteinase K

LGT buffer

10 mM	Tris-HCl, pH 7.5
10 mM	$MgCl_2$
0.1 mM	EDTA, pH 7.5

3.5. Media and antibiotics

All media used were autoclaved for 20 min at 120 °C unless stated otherwise. Supplements for media and plates were sterile filtered through a 0.22-µm pore filter and added after cooling down the media to <50 °C.

Liquid Media

Lysogeny Broth (LB)

10 g	Casein hydrolysate
5 g	Bacto-yeast extract
5 g	NaCl

- H_2O_{bidest} added to a final volume of 1 L
- autoclaved and stored at 4 ºC until use

Pooled Human Urine

Human urine was collected from an equal number of healthy male and female volunteers, pooled and sterilized by filtration. Sterile urine was stored at 4 °C not longer than one week until use.

Solid Media

LB Agar (1L)

5 g	Yeast
10 g	Trypton
5 g	NaCl
15 g	Agar

- H_2O_{bidest} added to a final volume of 1 L

- autoclaved and cooled to 55 ºC
- poured on plates and stored at 4 ºC until use

Motility Agar Plates

LB Plates (1 L)

5 g	Yeast
10 g	Trypton
5 g	NaCl
3 g	Agar

- H_2O_{bidest} added to a final volume of 1 L

Urine Plates (1 L)

3 g	Agar
0.2 L	0.9 % NaCl
0.8 L	Pooled human urine

- agar and NaCl were first prepared and autoclaved before addition of pooled human urine

Agar Plates for Phenotypic Assays

Aerobactin and Colicin:

Solution I. Tris Succinate Medium (1 L)

5.8 g	NaCl
3.7 g	KCl
0.15 g	$CaCl_2$ x 2 H_2O
0.1 g	$MgCl_2$ x 6 H_2O
0.1 g	NH_4Cl
0.142 g	Na_2SO_4
0.272 g	KH_2PO_4
12.1 g	Tris (hydroxymethyl) aminomethane
4.5 g	Succinic acid, pH 7.4

- 20 min at 121 °C

Solution II

200 ml	Solution I
2.4 g	Agar
1 g	Casamino acids
0.2 g	Peptone

- 20 min at 121 °C

- cooled to 50 °C and added the following components:

Aerobactin Assay:

2 ml	0.2 % Thiamine
1200 µl	25 mm alpha-alpha-dipyridyl
1 ml	OD_{600}=0.6 *E. coli* strain LG1522

Colicin Inhibition Assay:

2 ml	0.2% Thiamine
1 ml	OD_{600}=0.6 *E. coli* strain DH5α

Congo Red: No NaCl (800 ml)

32 mg	Congo Red
16 mg	Coomassie Red + Brilliant Blue (R250, Roth)
8 g	Peptone
4 g	Yeast Extract
15 g	Agar

Blood Agar:

LB agar plate containing 5 % (v/v) Sheep Blood, Oxoid GmbH

MacConkey Agar:

BBL™ MacConkey Agar, Beckton Dickinson

Chromogenic Agar:

Brilliance™ UTI Clarity™ Agar, Oxoid

Calcofluor Plate:

CFW fluorescent brightener (F-3543, Sigma)

Antibiotics:

When appropriate, media and plates were supplemented with the antibiotics listed below using the indicated concentrations. Stock solutions were sterile-filtered and stored at -20 °C until use.

Table 5. Antibiotics used in this study.

Antibiotic	**Stock concentration**	**Solvent**	**Working concentration**
Chloramphenicol (Cm)	50 mg ml^{-1}	EtOH	20 µg ml^{-1}
Ampicillin (Amp)	100 mg ml^{-1}	dH_2O	100 µg ml^{-1}
Kanamycin (Kan)	50 mg ml^{-1}	dH_2O	50 µg ml^{-1}

3.6. Electrophoresis markers

To determine the size of DNA and protein fragments in agarose gels and membranes, the GeneRuler™ 100 bp DNA Ladder (MBI Fermentas) and GeneRuler™ 1 kb DNA Ladder (MBI Fermentas), Lambda Ladder PFG Marker (New England BioLabs) and PageRuler™ Plus Prestained Protein Ladder (MBI Fermentas) were used (Figure 3).

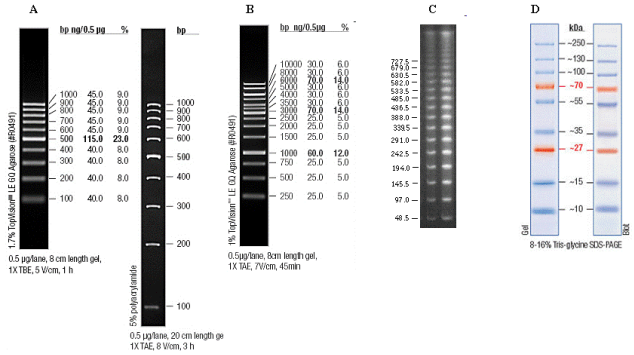

Figure 3. **DNA and Protein Markers used in this study**. A. GeneRuler™ 100 bp DNA Ladder B. GeneRuler™ 1 kb DNA Ladder C. Lambda Ladder PFG Marker D. PageRuler™ Plus Prestained Protein Ladder.

3.7. Kits and enzymes

Enzymes used in this study were purchased from the following companies in Germany: New England Biolabs (Frankfurt am Main), Invitrogen (Karlsruhe), MBI Fermentas (St. Leon-Rot), Roche Diagnostics (Mannheim), Gibco BRL (Eggenstein), Merck (Darmstadt), Oxoid (Wesel), GE Healthcare/Amersham Biosciences (Freiburg), R & D Systems (Wiesbaden), Roth (Karlsruhe).

The following commercial kits were used:

- Plasmid Mini and Midi kit, QIAGEN (Hilden, Germany)
- PCR purification kit, QIAGEN (Hilden, Germany)
- Gel extraction kit, QIAGEN (Hilden, Germany)
- RNeasy kit, QIAGEN (Hilden, Germany)
- ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems (Foster City, USA)
- ECL™ Direct Acid Labeling and Detection System, and ECL™ advance system GE Healthcare/Amersham Biosciences (Freiburg, Germany)
- OpArray Hybridization Buffer Kit, Operon (Cologne, Germany)
- Superscript III Reverse Transcriptase, Invitrogen ((Foster City, USA)
- RevertAid ™ First Strand cDNA Synthesis Kit, Fermentas (St. Leon-Rot, Germany)
- Human CXCL8/IL-8 Immunoassay, R & D Systems (Wiesbaden, Germany)
- Quantikine HS Human IL-6 ELISA, R & D Systems (Wiesbaden, Germany)

3.8. Antibodies

The following antibodies were used for Western Blot, immunoblotting and agglutination:

- rabbit polyclonal antibody against purified P-related P-fimbriae (Dr. Salam Kahn)
- rabbit polyclonal antibody against purified S-fimbriae (Dr. Salam Kahn)
- rabbit polyclonal antibody against *flaA*, flagellin sub-unit in *Legionella pneumophila*

- rabbit polyclonal antibody against poly-N-acetyl glucosamine (PIA)

3.9. Technical equipment

Autoclaves	Integra Bioscience, H+P Varioclav
Balances	IL-180, Chyo Balance Corp
	Kern 470
	Ohaus Navigator
Bioanalyzer	Agilent Technologies, 2100expert
Centrifuges	Beckmann J2-HC® JA10 and JA20 rotors
	Beckmann L-70 Ultracentrifuge
	Heraeus Sepatech Megafuge1.OR
	Heraeus Sepatech Biofuge 13R
	Hettich Mikro20
Clean bench	NUAIRE, Class II, type A/B3
Documentation	BioRad GelDoc2000 + MultiAnalyst Software V1.1
Electrophoresis systems	BioRad
Electroporator	Gene Pulser, BioRad
Hybridization oven	HybAid Mini 10
Incubators	Memmert Tv40b (30 °C)
	Heraeus B5050E (37 °C)
Magnetic stirrer	Heidolf MR3001K
Microarray scanner	GenePix 4000B, Molecular Devices
Microarray hybridization chamber	Scienion
Micropipettes	Eppendorf Research 0.5-10 µl, 2-20 µl
	20-200 µl, 100-1000 µl
Microwave AEG	Micromat
Power supplies	BioRad Power Pac 300
PCR Thermocycler	Biometra T3
pH meter	WTW pH 525
Photometer	Pharmacia Biotech Ultrospec 3000

	Thermo Scientific NanoDropTM 1000
Phosphoimager	Amersham Biosciences, Typhoon 4600
Real Time PCR Thermocycler	Bio Rad, CFX96
Scanner	HP ScanJet IIcx
Shakers	Bühler TH30 SM-30 (37°C, 150 rpm)
	Innova 4300, New Brunswick Scientific (37°C, 220 rpm)
	Innova 4230, New Brunswick Scientific (30°C, 220 rpm)
Speedvac	Savant SC110
Thermoblocks	Liebisch
Vaccum pump	Univac Uniequipe
Videoprinter	Mitsubishi Hitachi, Cybertech Cb1
Vortexer	Vortex-Genie 2^{TM} Scientific Industries
UV-Crosslinker	BioRad
Waterbath	GFL 1083, Memmert

4. Methods

The methods used in this study followed standard protocols described in the Molecular Cloning Manual (Sambrook *et al*., 1989) unless stated otherwise. Specific details of each method are found in Section 8.2, Appendices.

4.1. Pheno- and genotypic characterization of ABU isolates and fecal isolates from healthy individuals

74 *E. coli* ABU isolates and 39 fecal *E. coli* isolates from healthy individuals without any history of urinary tract infection from Sweden collected by C. Svanborg were sent from Lund, Sweden for characterization. The phylogenetic groups (*E. coli* group of reference strains, ECOR) of the isolates were typified (Clermont *et al*., 2000). In addition, multi-locus sequence typing was performed to identify the sequence type of the isolates. The presence of various virulence-associated genes of extraintestinal pathogenic *Escherichia coli* was also determined through multiplex and single polymerase chain reactions (PCRs) (Johnson and Stell, 2000). Additionally, phenotypic expression of important virulence-associated traits (α-hemolysin, curli and cellulose, K5 capsule, aerobactin, colicin as well as type 1-, P- and S/F1C-fimbria) was compared between the ABU and fecal isolates.

4.1.1. Phylogenetic Classification (E. coli group of reference strains, ECOR)

A multiplex PCR (Taq DNA Polymerase, Invitrogen) which amplifies a combination of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TspE4.C2) of *E. coli* was used for the determination of the phylogenetic group (A, B1, B2, and D) of the isolates (Figure 4) (Clermont *et al*., 2000). The PCR products were separated on a 1 % gel by electrophoresis.

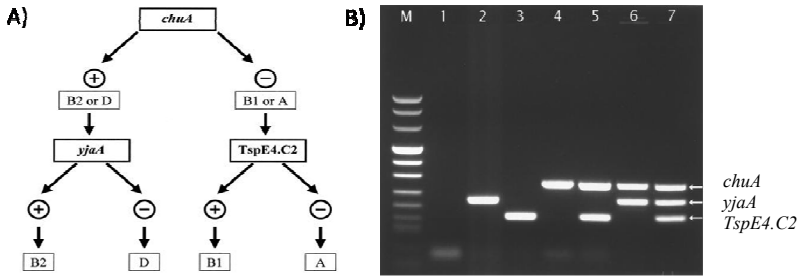

Figure 4. Triplex PCR profiles specific for the four *E. coli* phylogenetic groups. Each combination of *chuA* and *yjaA* gene and DNA fragment TspE4.C2 amplification allowed the determination of the phylogenetic group of a given strain; B) Lanes 1 and 2, ECOR group A; lane 3, ECOR group B1; lanes 4 and 5, ECOR group D; lanes 6 and 7, group ECOR B2. Lane M, DNA size marker (Clermont, 2000).

4.1.2. Multi-Locus Sequence Typing

Multi-locus sequence typing (MLST) was done for the isolates studied using seven *E. coli* housekeeping genes that are evenly spread over the chromosome. The seven housekeeping genes included according to the Achtman MLST (http://www.shigatox.net/mlst) were *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). Internal fragments of these house-keeping genes were amplified using PCR and concomitantly sequenced. The DNA sequences obtained were then used for allocation to different clonal groups. The MLST analysis was done in cooperation with the Institute for Hygiene, Westfälische Wilhelms-Universität Münster, Germany).

4.1.3. Determination of Virulence-Associated Genes

Multiplex and single PCRs (GoTaq® DNA Polymerase, Promega) were performed for the detection of extraintestinal pathogenic *Escherichia coli*-related virulence genes of the isolates. The multiplex PCR consists of 29 genes (Johnson and Stell, 2000) and the single

PCRs which were conducted for verification include genes encoding for siderophore aerobactin and hemolysin.

4.1.4. Polymerase Chain Reaction

Polymerase chain reaction is a method used to amplify a selected DNA fragment. It involves three steps. First of all, the template DNA is heat-denatured to separate its complementary strands. Then, primers anneal to the DNA on both sides of the sequence (5' and 3'). Finally, DNA polymerase extends the primers and new copies of the fragment are generated, the amount depending on the number of cycles (Prescott, 2002).

The following PCR mixes are used for PCR:

4.1.4.1. Standard PCR

Taq DNA Polymerase (Invitrogen, Qiagen)

2 µl	10 x reaction buffer
2 µl	20 mM dNTP mix
0.6 µl	25 mM $MgCl_2$
1 µl	10 pM primer solution 1
1 µl	10 pM primer solution 2
1 µl	100 ng template DNA or bacterial cells
0.05 µl	*Taq* DNA polymerase

Add double distilled H_2O for a total of 50 µl

Go*Taq*® DNA Polymerase (Promega)

1 µl	dNTPs
0.25 µl	*Taq* polymerase
2 µl	Primer mix
4 µl	$MgCl_2$
1 µl	100 ng template DNA or bacterial cells

10 µl PCR buffer

Add double distilled H_2O for a total of 50 µl

Red *Taq* (Sigma)

12.5 µl *Taq* polymerase

9.5 µl Water

2 µl Primer mix

1 µl DNA template

PCR reactions were carried out with the following conditions in a thermal cycler:

***Taq* DNA Polymerase (Invitrogen, Qiagen) and Red *Taq* DNA Polymerase (Sigma)**

1. 94 °C 3 min
2. 94 °C 30 sec
3. 55-63 °C 30 sec
4. 72 °C 1 min/kb* go back to step 2 and repeat 24 or 34 times
5. 72 °C 10 min
6. 16 °C ∞

Go*Taq*® DNA Polymerase (Promega)

1. 95 °C 2 min
2. 95 °C 30 sec
3. 55-63 °C 30 sec
4. 72 °C 1 min/kb* go back to step 2 and repeat 24 or 34 times
5. 72 °C 5 min
6. 16 °C ∞

4.1.4.2. PCR with proof-reading polymerases

For cloning and sequencing, polymerases with 3' → 5' proof-reading activity was used in order to prevent misincorporations during extension. The composition of the PCR mixes is given below.

DAP Goldstar Polymerase (Eurogentec)

5 µl	10 x Opti buffer
5 µl	20 mM dNTP mix
3.5 µl	50 mM $MgCl_2$
1 µl	10 pM primer solution 1
1 µl	10 pM primer solution 2
1 µl	100 ng μl^{-1} template DNA
0.5 µl	DAP Goldstar polymerase

Add double distilled H_2O for a total of 50 µl

Phusion Polymerase (Finnzymes)

10 µl	5 x HF or GC buffer
1 µl	20 mM dNTP mix
1.5 µl	50 mM $MgCl_2$ (optional)
1 µl	10 pM primer solution 1
1 µl	10 pM primer solution 2
1 µl	100 ng μl^{-1} template DNA
0.5 µl	Phusion polymerase

Add double distilled H_2O for a total of 50 µl

PCR reactions were carried out with the following thermal cycling profiles:

DAP Goldstar Polymerase (Eurogentec)

1. 95 °C	2 min
2. 95 °C	45 sec
3. 54 – 60 °C	45 sec
4. 72 °C	1 min /1kb * go back to step 2 and repeat 24 or 34 times
5. 72 °C	10 min
6. 16 °C	∞

Phusion Polymerase (Finnzymes)

1. 98 °C	30 sec
2. 98 °C	10 sec
3. 45 – 72 °C	30 sec
4. 72 °C	30 sec /1kb * go back to step 2 and repeat 24 or 34 times
5. 72 °C	10 min
6. 16 °C	∞

After running a PCR reaction, the PCR products were run on a 1 or 2 % agarose gel in an electrophoresis machine (Appendix 8.2.1). A list of the primers used can be found in Section 3.2, Materials.

4.1.5. Phenotypic Analysis of Important Virulence-Associated Traits

Bacterial samples used for the assays were prepared either by making a 50 µl saline bacterial suspension or an overnight culture. Each sample was spot-inoculated into the plate using a sterile pipette tip.

Hemolysin Production Assay

Upon preparation of the bacterial samples, they were spot inoculated on blood agar plates. Afterwards, they were incubated for 16 h at 37 °C. Hemolysin production was determined by the presence of a clear haemolytic halo around the spot.

Aerobactin Production Assay

Upon preparation of the bacterial samples, they were spot inoculated on the plates and incubated for 48 h at 37 °C. Production of aerobactin was visualized by the presence of growth zones of indicator strain LG1522 around the colonies.

Colicin Inhibition Assay

Upon preparation of the bacterial samples, they were spot inoculated on the plates. They were then incubated for 24 h at 37 °C. Assessment was done afterwards by the presence of clear zones of growths of inhibition of indicator strain DH5α around colonies of tested strains.

Congo Red Staining Assay

Upon preparation of the bacterial samples, they were spot inoculated on Congo Red plates. Incubation was done for 48 h at RT, 30 °C and 37 °C. Bacterial staining by the red dye was assessed. Red, dry and rough colonies indicate expression of curli and cellulose, white ones of cellulose and brown of curli.

K5 Capsule Expression

Bacterial samples were streaked out horizontally on LB agar plates. Next, a K5 phage lysate (10^8 $PFUml^{-1}$) preparation was vertically streaked out into individual bacterial streaks made on the plates. Plates were incubated overnight at 37 °C. Lysis of bacterial cells grown on the plates indicated expression of K5 capsule.

Fimbrial Expression

Agglutination assays were done to determine expression of type 1, P- and S- fimbriae among the isolates.

To determine the expression of type 1 fimbria, bacteria were statically grown overnight. The following day, 20 µl of overnight culture was pipetted onto a glass slide with 20 µl of 10 mg/ml yeast in sterile 0.9 % NaCl (with or without 2 % mannose). After thorough mixing, agglutination of yeast cells was observed. *E. coli* 536 was used as positive control while 83972 wt was used as negative control.

Next, for the detection of P-fimbrial expression, bacteria grown on LB agar plates were mixed with 30 µl α-Prf antibody on a glass slide. Agglutination was observed after mixing using *E. coli* 536 and J96 as positive controls and MG1655 as negative control.

Finally, for the determination of S-fimbrial expression, bacteria grown on LB agar plates were mixed with 30 µl α-F1C antibody on a glass slide. Agglutination was observed after mixing using *E coli* 536 positive control and MG1655 as negative control.

4.2. Characterization of ABU re-isolates from symptomatic episodes

Eight 83972 re-isolates taken from patients stably colonized with ABU 83972 wt at the Lund University Hospital, Lund, Sweden were analyzed. The said patients reported an episode of symptoms at the time of collection. Primarily, the phylogenetic groups (*E. coli* group of reference strains, ECOR) of the isolates were typified (Clermont *et al*., 2000). Then, the presence of various virulence-associated genes of uropathogenic *E. coli* was determined through multiplex and single polymerase chain reactions (PCRs) using primers previously used by Johnson and Stell (2000). In order to verify the presence of some genes, other primers were also used. On the other hand, phenotypic expression of the genes was verified through spot-inoculation of bacterial suspension or overnight culture of the samples in various agar plate preparations (*i.e.* blood agar for α-hemolysin, Congo red agar for curli and cellulose, agar with growth inhibiting strains for aerobactin and colicin) and agglutination tests using specific antibodies (*i.e.* for fimbrial expression). Moreover, the presence of the 1.2 kb cryptic plasmid and fimbrial gene deletion characteristic of *E. coli* 83972 were also determined using PCR. Finally, to analyze the genomic structure of the 83972 re-isolates, their genomic DNA was subjected to pulsed-field gel electrophoresis (PFGE) after *AvrII* restriction. Further analysis of the re-isolates were done using motility assays, SDS-PAGE and Western Blot analysis, immuno dot blot, autoaggregation assay, biofilm assay, adhesion experiments, lipopolysaccharide (LPS) analysis, quantification of inflammatory markers secretion and expression, microarray experiments and qRT-PCR.

4.2.1. Genotypic and Phenotypic Analysis of the Re-isolates

Determination of phylogenetic group, virulence-associated genes and phenotypic traits of the re-isolates was done in the same way as described for the ABU isolates and fecal isolates collected from healthy individuals.

4.2.2. Verification of Re-isolates as 83972

E. coli 83972 has two determining characteristics, the presence of the 1.2 kb cryptic plasmid and deletion within the type 1 fimbria gene cluster. For this reason, the re-isolates were verified as 83972 by performing PCR amplifying these using primers pABU_for (5'-acatagatccctcatgcggtg -3') and pABU_rev (5'-ctgcggtgttacagcgatgg-3') and fim_for (5'-tctaatccgcatgctgagagc-3') and fim_rev (5'-tgacctgtgcagtaccacgag-3') for the cryptic plasmid and fimbrial gene deletion, respectively. In addition, the re-isolates were streaked out on Brilliance™ UTI Clarity™ agar (Oxoid) and MacConkey agar plates. Sequencing of the 16S rRNA was also done for verification in one re-isolate.

4.2.3. Pulsed Field Gel Electrophoresis (PFGE)

To determine the restriction pattern of the re-isolates, PFGE (Appendix 8.2.2) was performed. Bacterial samples were grown overnight in LB medium and mixed with 2 % low melting agarose gel. The gels were washed with TE buffer for 2 weeks until transparent. When ready, the gels were digested with *AvrII* (Finnzymes) for 1 h and and run on gel for 20 h.

Digestion of agarose plugs containing DNA was done as follows:
LGT agarose blocks containing the high molecular weight DNA were transferred into a new Eppendorf tube. Blocks were pre-incubated 1 h at 50 °C in 1 ml 1 × NEB buffer. Restriction was carried out for 3 h at 37 °C in a 150 µl overall reaction mixture containing 1 x restriction buffer and 30 U restriction enzyme. When recommended by manufacturer, BSA was added.

10 × NEB buffer
50 mM NaCl
10 mM Tris-HCl
10 mM MgCl
1 mM DTT

4.2.4. Analysis of Motility and Flagellar Expression

Motility Test

Overnight cultures were stabbed into the middle of motility agar (0.3 %) plates through an inoculating needle. Plates were incubated for 16 h at 37 °C. Motility was then assessed by measuring the migration of the bacteria through the agar by zone of growth. Results are expressed (in mm) as the mean ± standard deviation of triplicate colonies from three independent experiments.

Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot

Re-isolates that expressed motility on swarm agar plates (LB and urine) were further analyzed with regards to motility trait and flagellar expression. First, bacterial cultures of the samples were prepared by growing overnight and standardized to OD_{600} = 0.1 the following day as starter culture. Bacteria were grown statically in pooled human urine in a 1:100 (bacterial inoculum: urine) ratio at 37 °C until they reached an OD_{600} = 0.3-0.4. The samples were centrifuged for 5 minutes at 4000 rpm and used for SDS-PAGE (Appendix 8.2.3). Following this, Western blot analyses (Appendix 8.2.4) of the samples were conducted using a polyclonal anti-serum raised against the flagellin of *Legionella pneumophila*.

4.2.5. Transcriptome analysis of the Re-isolates

To analyze the role of increased motility and other traits of re-isolates BU04 63630, BU05 35364 and FIM 636 for the possible induction of host innate immune response, a transcriptome comparison upon *in vitro* growth of the re-isolates with that of the parent strain 83972 was conducted. Expression profiling is a technique used to study the relative amounts of all transcripts at a given time of sample collection. This allows monitoring of the expression level of every single gene detectable by the array.

Custom-tailored oligonucleotide glass microarrays (Operon Biotechnologies, Inc.) were used for expression profiling. A single Operon *E. coli* Custom 55156017 array contains 10816 longmer oligonucleotide probes covering the complete genomes of six *Escherichia coli* strains (6 genomes and four plasmids). The number of open reading frames (ORFs) or genes represented is as follows: 4269 ORFs of non-pathogenic *E. coli* K-12 strain MG1655, 5306 ORFs of enterohemorrhagic *E. coli* O157:H7 strain EDL933, 5251 ORFs of enterohemorrhagic *E. coli* O157:H7 strain Sakai, 5366 ORFs of uropathogenic *E. coli* strain CFT073, 322 ORFs of uropathogenic *E. coli* strain 536, 448 ORFs of uropathogenic *E. coli* strain UTI89, 3 genes of EHEC plasmid OSAK1, 10 genes of EHEC plasmid pO157_Sakai, 97 genes of EHEC plasmid pO157_EDL933 and genes of UPEC plasmid pUTI89. In addition, the array also includes a number of positive and negative controls. Each probe contains an amino linker at the 5' end. Probes were spotted as single spots in 32 blocks (4 columns, 8 rows), each block with 18 columns x 19 rows.

First, the samples were grown statically in pooled human urine at 37 °C until they reach mid-logarithmic phase. Samples were then treated with RNA Protect (Qiagen) followed by extraction of total RNA (Appendix 8.2.5). Next, traces of DNA were removed by RNAse-free DNAse treatment (Appendix 8.2.6). For probe synthesis, 10 µg of total RNA was reversed transcribed with direct incorporation of fluorescently-labelled nucleotides Cy3- and Cy-5-dCTP (Appendix 8.2.7). Following this, 160 pmol each of Cy3- and Cy5-labelled probes were used for hybridization of oligonucleotide microarrays (Appendix 8.2.8). The microarray hybridizations per strain pair were performed in triplicates.

Hybridized and washed slides were scanned using a GenePix Model 4000B Microarray Scanner (Axon Instruments Inc., USA) with a resolution of 5 µm pixel size. The excitation frequencies of the two lasers were 532 nm and 635 nm. The gain settings for the photomultiplier tubes were adjusted to use the entire dynamic range of the instrument and to get comparable fluorescence yields in both channels. Images of Cy3 and Cy5 signals were recorded as 2 layer 16 bit TIFF files and analyzed using the GenePix Pro 6.0 software.

For each experiment, at least three independent hybridizations were performed, one with a dye switch. After removal of bad quality spots (if less than 70 % of foreground pixels were below background intensity plus 2 standard deviations in both channels or if the signal to noise ratio was below 3 in both channels or if the difference between ratio of medians and regression ratio exceeded 20 % in one of the channels) the remaining intensities were saved as gpr output data file. For statistical validation and further analysis the Acuity 4.0 (Molecular Devices, USA) software was used. For all data, the local background was subtracted from the intensity values of each spot on the array and normalized by both linear ratio-based methods and non-linear Lowess including print-tip groups. For statistical significance, one sample t-test was applied and the resulting data set was exported to Microsoft Excel. Hierarchical clustering of genes for visualization of expression patterns was performed with the CLUSTER software (Eisen *et al.*, 1998). The output was displayed with the software TREEVIEW (Eisen *et al.*, 1998).

For data analysis, a cut-off value of 1.7 was used although the commonly used threshold value is two-fold (DeRisi *et al.*, 1997; Wildsmith and Elcock, 2001). Several studies have shown that a lower cut-off ranging from 1.4 to 1.74 can be used reliably if the results are reproducible in more replicates (Perez-Amador *et al.*, 2001).

Microarray results were then confirmed by real-time PCR analysis with selected candidate genes.

4.2.6. Quantitative Real-Time PCR

To evaluate expression of single genes, a quantitative Real-Time PCR (qRT-PCR) approach was used. This method employs polymerase chain reaction to amplify gene transcripts in presence of the SYBR Green I dye (BioRad). This fluorescent dye intercalates into double stranded DNA and emits signals collected by the optical camera within the MyiQ cycler (BioRad). The number of cycles needed to reach a certain fluorescent signal threshold (CT) was used to calculate transcript levels. Primers for selected genes were designed using the FastPCR software (Ruslan Kalendar, Institute for Biotechnology, University of Helsinki, Finland) with the following parameters: product length range from 190 to 300 nt and annealing temperature 57 - 59 °C. Before their use for expression profiling, the different primer pairs were checked for amplification efficiency with pooled cDNA from different experiments. Only primer pairs with amplification efficiency of at least 90 % were used. For copy number estimation, cDNA samples derived from the reverse transcription reaction were 100-fold diluted in dH_2O and the reaction mix was prepared (for one reaction) as follows:

12.0 µl cDNA
12.5 µl SYBR Green Mix, 2 ×
0.25 µl Primer 1 (10 µmol)
0.25 µl Primer 2 (10 µmol)

The following thermal cycling profile was used:

95 °C	3 min	
95 °C	30 sec	
60 °C	30 sec	(40 cycles)
72 °C	20 sec	
95 °C – 57 °C	30 sec	(melting curve)

All PCR reactions were done in triplicates in three independent experiments.

4.2.7. Cell Adhesion Assay

Cell adhesion assays were conducted to compare the re-isolates with their parent strain 83972 with regards to their interaction with human bladder epithelial cell line T24. First, the eukaryotic cells were seeded in 24-well tissue culture plates (2.2×10^5 cells/well) and incubated overnight at 37 °C and 5 % CO_2. Meanwhile, 10^7 bacteria of an overnight culture were co-cultivated with the eukaryotic cells for 3 h. *Citrobacter freundii* 3009 was used as positive control. After 3 washing steps with PBS (pH 7.4), the T24 cells were lysed in 0.1 % TritonX-100 for 15 min. Appropriate dilutions of the inoculum and cell lysates were plated on LB agar and incubated overnight at 37 °C. Colony forming units were counted the following day.

4.2.8. IL-8 and IL-6 Concentrations

IL-8 and IL-6 concentrations were measured using the Quantikine® Kit (R & D Systems). The kit works in the same way as sandwich Enzyme-linked Immunosorbent Assay (ELISA). Human kidney cells A498 were seeded in 24-well tissue culture plates (2.2×10^5 cells/well) and incubated overnight at 37 °C and 5 % CO_2. Next, 10^7 bacteria of an overnight culture were co-cultivated with the eukaryotic cells for 3h. Cell culture supernates were collected, particles removed by centrifugation and diluted according to manufacturer's protocol. The assay followed immediately or cell culture supernates were stored in aliquots at -20 °C until use in the assay. Absorbance was read in a microplate reader at 450 nm with a wavelength correction set to 550 nm.

4.2.9. Autoaggregation Assay

Five milliliters of each sample was grown in Lysogeny Broth (LB) medium during the day for 6h at 37 °C with shaking. Following this, 100 µl of the culture was grown in 5 ml LB overnight under the same conditions. The next day, the optical density of the samples was adjusted to OD_{600}= 3 in 3 ml LB. Cultures were left standing overnight at room

temperature. The following day, optical density was measured at 600 nm to quantitate aggregation of cells at the bottom of the tube.

4.2.10. Biofilm Assay in Urine

Bacteria were grown overnight in LB medium at 37 °C with shaking. The following day, filter-sterilized pooled human urine was inoculated (1:100) with the overnight bacterial culture. Next, 160 µl of the inoculum was pipetted into 96-well U-bottom flexible plates (BD Falcon), 8x per sample. Plates were incubated statically at 37 °C for 48 h. Afterwards, the medium was removed and plates were washed twice with 1 % PBS followed by drying at 65 °C for 10 min. The plates were then stained with 0.1 % crystal violet for another 10 min. Next, plates were washed twice with 1 % PBS and dried at 65 °C for 10 min. Absorbed crystal violet was eluted using 180 µl acetone-ethanol (1:5), pooled and diluted 1:10. Finally, optical density was measured at 580 nm.

4.2.11. Polysaccharide Intercellular Adhesin (PIA) Immuno Dot Blot

Polysaccharide intercellular adhesin (PIA) is a biofilm exopolysaccharide made of *N*-acetylglucosamine that is found in many bacterial biofilm-forming pathogens. In order to detect its expression among the re-isolates, an immuno dot blot assay was performed. Overnight cultures of bacteria were grown and blotted onto a nitrocellulose membrane. The membrane was washed and PIA was detected using antibody raised against PIA in *Staphylococcus aureus* (Appendix 8.2.9).

4.2.12. Growth Curve Experiment

Growth of the bacterial isolates was compared to 83972 wt by growing them in LB and pooled human urine overnight and inoculating a 30 ml fresh medium the following day with the overnight culture. Optical density was measured at 600 nm every hour for 8 h and overnight. Experiment was repeated three times using different batches of urine.

4.2.13. Analysis of Lipolysaccharide Phenotype

Bacterial cells were first grown overnight on agar plates. Next, the cells were scraped off from the agar plates, transferred into Eppendorf tubes and weighed. The cells were resuspsended in distilled water to a final concentration of 1 mg cells/30 µl. This is then mixed with 10 µl 4 x SDS sample buffer and incubated at 100 °C for 10 minutes. Next, the samples were briefly cooled prior to addition of 20 µl proteinase K (100 µg in 1X SDS sample buffer). The samples were then incubated at 60 °C for an hour. 30 µl of this was run on denaturing SDS gel overnight at 8 mA. Afterwards, the gel was stained with silver nitrate for visualization of bands (Appendix 8.2.10).

4.3. Reconstitution of a functional papGX gene from CFT073 into the 83972 chromosome

4.3.1. Complementation of papGX gene

A *papGX*-83972 deletion mutant (83972Δ*papGX*) was generated using lamda red homologous recombination technique (Datsenko and Wanner, 2000). Briefly, the *cat* casette of pKD3 was amplified using primers ES3 and ES4 (Section 3.2, Materials), with overhangs homologous to the 5´and 3´regions of the 83972 *papGX* gene. Next, the PCR product was purified using QIAquick PCR Product Purification Kit (Qiagen™) (Appendix 8.2.11) and transformed into electrocompetent 83972 cells carrying the pKD4 plasmid. Transformants were subsequently screened using PCR and Southern Blot analysis.

Meanwhile, 74-mer primers pKD3_papG_for and pKD3_papX_rev (Section 3.2, Materials) containing 50 bp overhangs complementary to a region in the pKD3 upstream of the chloramphenicol cassette were used to amplify the *papGX* fragment from CFT073 (Phusion Polymerase, New England Biolabs™). The resulting PCR product was purified using QIAquick PCR Product Purification Kit (Qiagen™). Following this, plasmid pKD3 was linearized by restriction with *BmgBI* (New England Biolabs™) for 1.5 h, followed by

a 30 min dephosphorylation with Antartic phosphatase (New England Biolabs™) at 37 °C. The plasmid was then gel purified and precipitated using ethanol. This was co-transformed with the *papGX* fragment into *E. coli* SW102 λ pir electrocompetent cells by electroporation. Selection of transformants was done on LB agar plate supplemented with chloramphenicol (20 μg/ml) at 30 °C. Screening for the correct clone was made by PCR and verified by sequencing.

Next, primers F13 and F14b (Section 3.2, Materials) were used for the amplification of the p*apGX-cat* construct in the pKD3 plasmid with homologous overhangs to the *tnpA* and *papF* regions of the 83972 chromosome (Phusion Polymerase, New England Biolabs™). The PCR fragment was gel-purified (Gel Extraction Kit, Qiagen™) (Appendix 8.2.11) and used for transformation into 83972Δ*papGX* electrocompetent cells carrying the pKD46 plasmid via homologous recombination (Datsenko and Wanner, 2000).

Clones were screened using PCR and verified by sequencing. To test for fitness of the mutant strain, growth curve experiments (see 4.2.12) were performed. In addition, phenotypic assay for the presence of P-fimbriae (see 4.1.5) was also carried out.

4.3.2. Mutant Curing from Antibiotic Resistance Cassette

Electro-competent cells of the mutant strain were transformed with plasmid pCP20 (Amp/Cm) and allowed to recover for 2 h by shaking. They were then plated on agar supplemented with antibiotics. Plates were incubated at 30 °C overnight. Four to eight colonies were picked from each plate and individually re-plated on Amp-agar followed by overnight incubation at 30 °C. This was repeated three consecutive times. Next, single colonies were plated on LB-agar and incubated overnight at 42 °C. Single colonies were picked from the 42 °C culture and plated on LB-agar followed by overnight incubation at 37 °C. Finally, single colonies were plated on Amp, Cm, Kan and LB-agar and incubated overnight at 37 °C.

4.3.3. Southern Blot Analysis

For verification of the mutants, Southern Blot hybridization and analysis was performed. First, chromosomal DNA was isolated. Following this, the DNA was hybridized to a membrane, probed with a specific PCR product and detected by chemiluminescence on an X-ray film (Appendix 8.2.12).

4.3.4. Plasmid and Chromosomal DNA Isolation

Bacterial samples were grown overnight. Following this, plasmid was isolated using the QIAGEN Plasmid Midi Kit (Qiagen™) (Appendix 8.2.13). On the other hand, chromosomal DNA was isolated by using phenol with prior treatment with lysozyme and proteinase K (Appendix 8.2.14).

4.3.5. DNA Sequencing

Verification of transformants on plates following electroporation was carried out by DNA sequencing. The following components and program are used for the sequencing reaction:

Sequencing Reaction Mix

0.5 µl DNA
2 µl 5 x Buffer
2 µl Premix
1 µl Primer
4.5 µl dH_2O

Sequencing PCR Program

94 °C 3 min
94 °C 30 sec
58 °C 15 sec
60 °C 4 min * go back to step 2 and repeat 34 times

The following components were mixed in a 1.5 ml Eppendorf tube following sequencing reaction:

10 µl Sequencing reaction
80 µl ROTIPURAN® Low organic water (Roth ™)
10 µl 3 M Sodium acetate, pH 4.8
250 µl 100% ethanol (Roth ™)

The mixture is then centrifuged for 15 min at 13000 rpm, room temperature (RT). The supernatant was removed and the pellet was dried. This was followed by washing with 70 % ethanol then vortexing. Next, it was centrifuged for 5 min at 13000 rpm, RT. Again, the supernatant was removed and the pellet was dried. Afterwards, this was centrifuged for 5 min at 13000 rpm, RT to dry completely. Finally, 25 µl HiDi Formamide was added. This was then loaded into the sequencing machine. Data were analyzed using the Vector NTI® software (Invitrogen).

4.3.6. Preparation of Electro-competent Cells

For recombination experiments, electro-competent cells were prepared by inoculating 50 ml of LB medium with 500 µl fresh overnight culture of bacteria. The cells were grown (and induced with arabinose when necessary) with vigorous shaking at the appropriate temperature until they reach an optical density (OD_{600}) = 0.7. Next, the cells were harvested by centrifugation in a cold rotor at 4000 x g_{max} for 15 min after chilling on ice for 15 min. The cells were washed three times with ice cold water after which they were resuspended in 1 ml cold 10 % glycerol. Finally after centrifugation, the cells were resuspended in 150 µl cold 10 % glycerol and aliquots of 40 µl were pipetted into 1.5 ml Eppendorf tubes.

4.3.7. Preparation of Glycerol Stocks of Bacterial Strains

To make conserve stocks of bacterial strains, overnight cultures of the bacteria were grown. Then, to 1 ml of the culture, 300 µl of 87% glycerol was added and mixed. Finally, the stocks were stored at -20°C until use.

4.3.8. Digestion of DNA with Restriction Endonucleases

DNA was dissolved in dH_2O and mixed with 0.2 vol 10 x reaction buffer and 1 U of restriction enzyme per 1 µg of DNA. The final volume of the sample was 15 µl for plasmid DNA and 50 µl for genomic DNA. The reaction mixture was incubated at 37 °C depending of the specific requirements of the enzyme (stated by supplier). Plasmid DNA was digested for one to two hours and genomic DNA at least for three hours, or eventually overnight. When appropriate, the reaction mix was stopped by heat inactivation (20 min at 65 °C).

4.4. In silico analysis

For standard sequence comparison and similarity searches, the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) homepage was used. For alignments of nucleotide and amino acid sequences, the BioEdit sequence alignment editor V7.0.1 and Vector NTI V7.0 were used. Genome comparison was performed using the Artemis Comparison Tool (ACT) Release 4 of the Sanger Institute.

4.5. Statistical analysis

Comparisons of $\chi 2$ P value between the ABU and fecal group of isolates were done using SPSS statistical software. Differences in IL-6 and IL-8 secretions among the re-isolates were compared using ANOVA.

5. Results

5.1. Pheno- and genotypic characterization of ABU isolates and fecal isolates from healthy individuals

In characterizing various isolates, it is important to have a look at virulence factors since they play a major role in the induction of host response. Thus, the presence of various virulence genes and their phenotypic expression were tested among 74 ABU and 39 fecal isolates in a strain collection from Lund, Sweden. Although this study deals more on ABU isolates, fecal isolates were included to ascertain the relatedness of ABU to commensal isolates due to the former's commensal-like behavior.

5.1.1. Phylogenetic Classification

To find out whether ABU isolates are more related to pathogenic or commensal isolates, a triplex PCR was performed. *E. coli* strains fall into four main phylogenetic groups (A, B1, B2, and D) (Clermont *et al*., 2000). Results show that among the 74 ABU isolates studied, 51 % and 5 % belong to Groups B2 and D which are groups that typically include virulent extra-intestinal strains, respectively, whereas 38 % and 5 % belong to typical commensal ECOR Groups A and B1, respectively (Figure 5). This shows that more than half of all the ABU isolates included in the study are related to a typical pathogenic group.

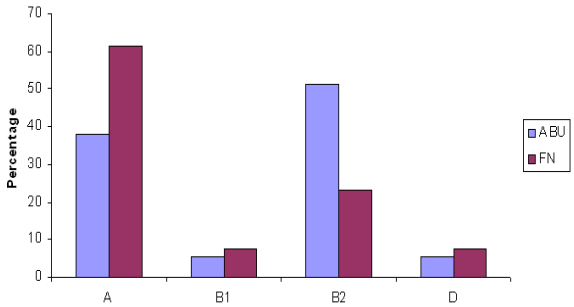

Figure 5. ECOR Grouping of the various isolates.
FN- fecal isolates.

In addition to ECOR phylogenetic classification, multi-locus sequence typing (MLST) was also carried out in collaboration with the Institute for Hygiene (University of Münster) according to the MLST system established by Mark Achtman using seven housekeeping genes namely *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif).

Results show that ABU isolates belong to multiple clonal groups. With a few exceptions, ABU and fecal isolates belong to different clonal lineages. ABU isolates are frequently clustered within clonal complex (CC)73 and CC10. Some others belonged to CC14 and sequence type (ST)53. ABU isolates were also found to share the same cluster as the fecal isolates in ST59 and ST95 (Figure 6).

A study by Wirth *et al*. (2006) showed that UTI strains as well as those with O25:H1/O6:H1 serotype clustered in CC73. On the other hand, those which mostly belong to phylogenetic group A as well as a few entoinvasive/entoaggregative *E. coli* (ETEC/EAEC) pathogens were grouped under CC10. Meanwhile, CC14 was majorly comprised of UPEC and UTI strains whereas CC95 was found to be composed of a combination of APEC, sepsis/UTI/neonatal bacterial meningitis (NBM), O1/O2/O18:K1 and OMP 6/9 strains.

As shown in Figure 6, no strict separation in clustering within clonal complexes can be observed between ABU and fecal isolates as they were both sometimes found within the same complex.

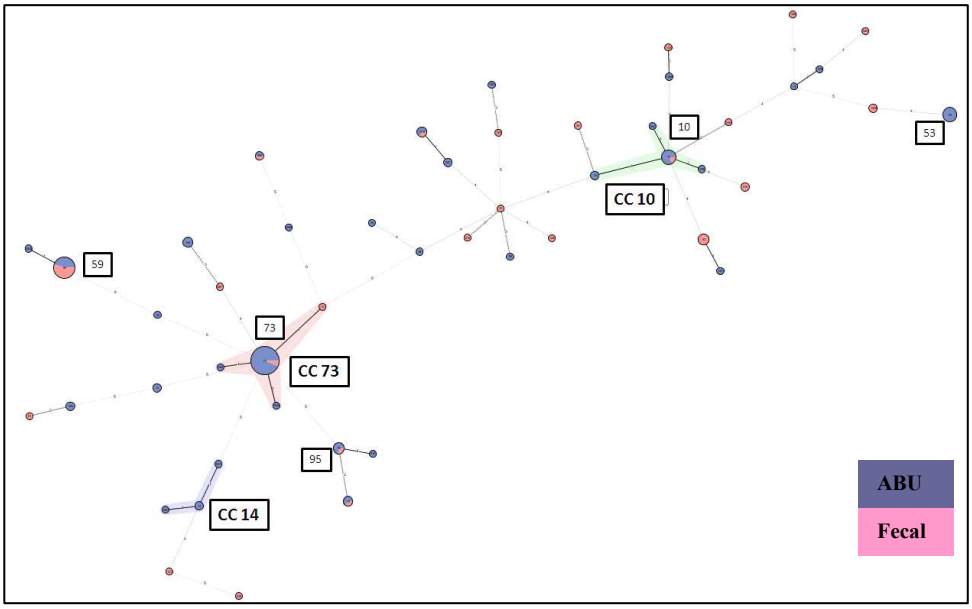

Figure 6. Minimum spanning tree (SeqSphere) based on MLST analysis (Achtman).
Major sequence types and clonal complexes are indicated by boxes.

5.1.2. Distribution of Virulence Factors

A series of multiplex and single PCRs were perfomed using primers previously used by Johnson and Stell (2000). The prevalence of 30 defined virulence genes commonly associated with extra-intestinal pathogenic *E. coli* (ExPEC) was determined (Table 5). In addition, phenotypic assays that correspond to the expression of these genes were also conducted (Table 6).

5.1.2.1. Genotypic Traits

Toxin Genes

Of the three exported virulence factors studied, namely *hlyA* (α-hemolysin), *cnf*1 (cytotoxic necrotizing factor) and *cdtB* (cytolethal distending toxin), ABU isolates harbored only *hlyA* and *cnf*1. The toxin encoded by the *cnf*1 gene interferes with polymorphonuclear phagocytosis and evokes apoptotic death of bladder epithelial cells

(Fiorentini *et al*., 1997). Fecal isolates, in comparison, were found to have only the *hlyA* gene. None of the isolates, however, were found to have the *cdt* gene which encodes for a toxin that causes apoptosis of host cells (Emődy *et al*., 2003) (Figure 7A). The *hlyA* gene, encoding a pore-forming toxin (Emődy *et al*., 2003), was more prevalent among ABU than fecal isolates with 20.27 % of them harboring the gene versus 2.56 % among fecal isolates.

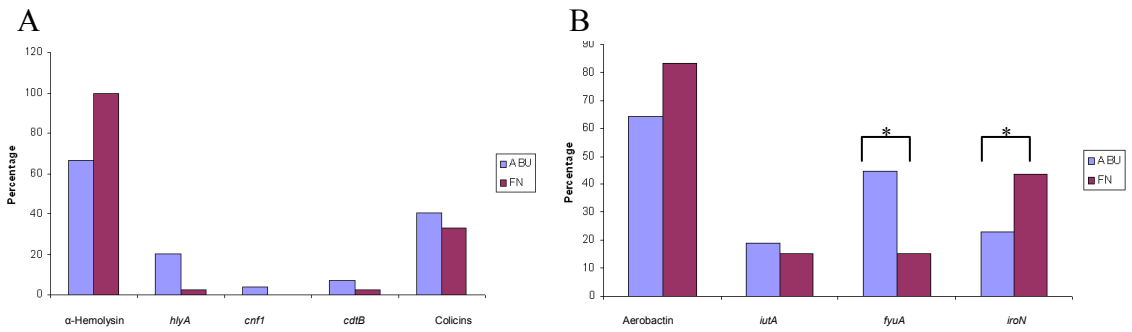

Figure 7. (A) Toxin genes (B) Genes involved in iron-uptake. FN- fecal isolates. Significant difference between ABU and FN is indicated by an asterisk (p <0.05).

Siderophore Systems

During growth under iron-limiting conditions, bacterial mechanisms are required to compete successfully for iron in the host. Low molecular weight siderophores like aerobactin, enterobactin and yersiniabactin are involved in this process (Guerinot, 1994; Braun *et al.*, 1998; Schubert *et al*., 2002). Siderophores are responsible for iron uptake and transport. Free iron is very limited in the human urinary tract. Due to this, there is a tight competition between the host and infectious bacteria for this essential element.

The presence of three siderophore genes namely *fyuA* (yersiniabactin), *iutA* (aerobactin receptor; inorganic iron transport and metabolism) and *iroN* (salmochelin receptor and adhesin) was tested for all the isolates. Results showed that the yersiniabactin gene cluster was more prevalent in ABU isolates (44.6 %) compared to their fecal counterparts (p = 0.002). The *fyuA* gene, encoding the yersiniabactin receptor, is one of the most upregulated genes in biofilm. It is highly important for biofilm formation in iron-poor

environments such as human urine (Hancock *et al.*, 2008). Only 23 % of the ABU isolates, however, had the *iroN* gene compared to 43.6 % of the fecal isolates ($p = 0.024$) (Figure 7B).

Capsules

Polysaccharide capsules make up the outermost layer of the cell. Hence, it may mediate direct interactions between the bacterium and its immediate environment and has been implicated as an important factor in the virulence of many animal and plant pathogens. They are known to function in prevention of cell desiccation, adherence and resistance to both specific and non-specific host immunity (Roberts, 1996). There are several groups of K capsules. In this study, the presence of two K capsule groups, group II and group III, was determined through the presence of the *kpsMT* genes which make up the third of the three regions of the K capsule determinants. K1 and K5 are capsular types under group II.

In the investigation of the presence of capsules, it has been found that 47.30 % of the ABU isolates and 30.77 % of the fecal isolates have group II capsules. 35.14 % of the ABU isolates harbored the gene for K5 capsule which is frequently associated with symptomatic UTI (Herias *et al*., 1997).

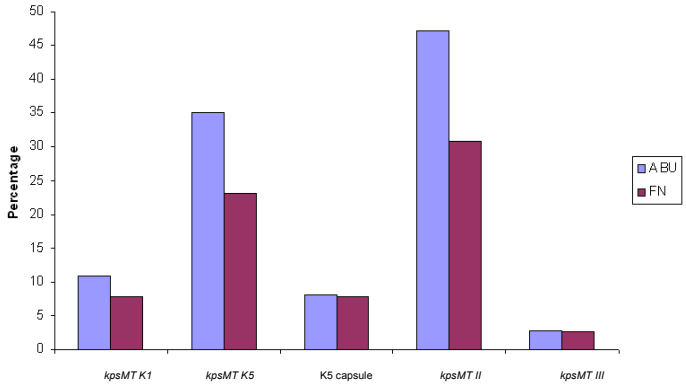

Figure 8. K capsule genes present among the isolates. FN- fecal isolates.
Significant difference between ABU and FN is indicated by an asterisk ($p < 0.05$).

Fimbriae

Fimbriae, located at the surface of the cell, are very important inducers of host response. Presence of the *fimH* gene which encodes the mannose-specific adhesion sub-unit of type 1 fimbria, the adhesin genes *focG/sfaS* of the *sfa/foc* operon encoding for F1C/S fimbria and genes encoding for some regions of the *pap* operon encoding for P fimbria in the various isolates had been determined. Data show that most of the isolates harbored the *fimH* gene (87.84 % ABU and 92.31 % fecal) and was thus the most prevalent virulence gene in both groups. Meanwhile, more ABU isolates had the *sfaS* gene (13.52 % versus 2.56 % for fecal isolates). In addition, only 2.56 % of the fecal isolates harbored the *sfa/focDE* and *focG* genes. With regards to P-fimbria, 28.38 %, 29.73 % and 28.38 % of the ABU isolates harbored the *papAH*, *papEF* and *papC* genes versus 15.38 %, 15.38 % and 12.82 % in the fecal isolates, respectively.

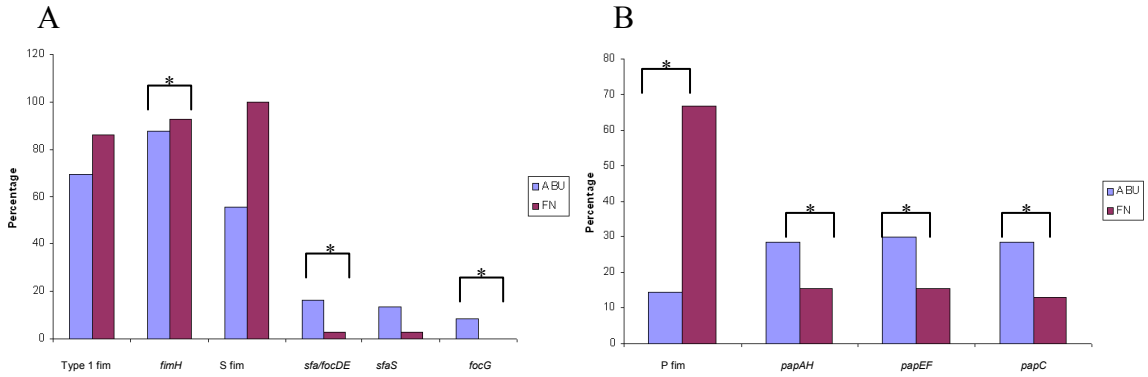

Figure 9. Fimbrial genes present in the various isolates. (A) Fimbrial genes (B) P-fimbrial genes. FN- fecal isolates. Significant difference between ABU and FN is indicated by an asterisk (p <0.05).

Miscellaneous Virulence Genes

Other genes included in the multiplex PCR reaction included a pathogenicity island (PAI) marker *malX, cvaC* (colicin V), *ibeA* (brain microvascular endothelial cell invasion), *bmaE* (M-agglutinin), *rfc* (participates in O4 LPS biosynthesis), *traT* (contributes to serum survival), *gafD* (N-acetyl-D-glucosamine-specific fimbrial lectin) and *afa/draBC* (afimbrial adhesin). Most of them were more prevalent among ABU isolates except *bmaE* and *rfc,* which were both more prevalent in fecal isolates ($p = 0.001$).

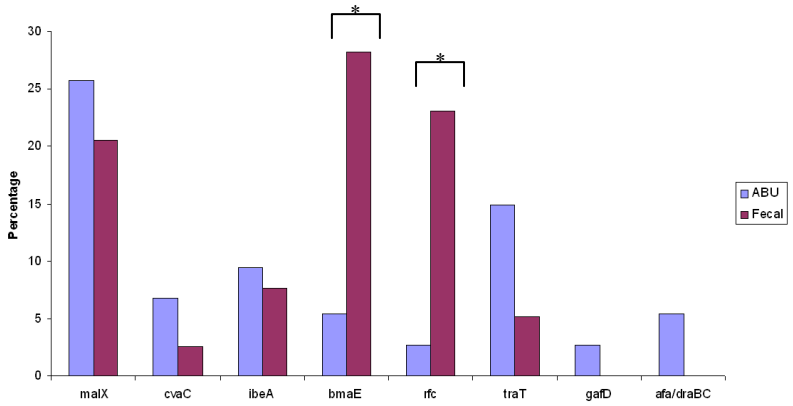

Figure 10. Miscellaneous virulence genes. FN- fecal isolates. Significant difference between ABU and FN is indicated by an asterisk ($p < 0.05$).

All genes included in the PCR were found among the ABU isolates whereas *cnf1* (cytotoxic necrotizing factor), *papG alleleI*, *papG alleleIII*, *papGI* (papG fimbrial adhesin), *sfa/focDE*, *focG*, *gafD* and *afa/draBC* were not found among the fecal isolates.

Table 6. Distribution of Virulence Genes among ABU and Fecal Isolates

Gene	ABU (n = 74) n (%)	Fecal (n = 39) n (%)	p-value*
hlyA	15 (20.27)	1 (2.564)	0.324
cnf1	3 (4.05)	0 (0)	0.080
cdtB	5 (6.8)	1 (2.56)	0.345
iutA	14 (18.92)	6 (15.38)	0.054
fyuA	33 (44.6)	6 (15.4)	0.002
fimH	65 (87.84)	36 (92.31)	0.0
papAH	21 (28.38)	6 (15.38)	0.001
papEF	22 (29.73)	6 (15.38)	0.001
papC	21 (28.38)	5 (12.82)	0.001
papG allele I	2 (2.70)	0 (0)	0.300
papG allele II	15 (20.27)	4 (10.26)	0.007
papG allele III	5 (6.8)	0 (0)	0.023
papG I	5 (6.8)	0 (0)	0.097
papG II,III	10 (13.51)	1 (2.56)	0.005
sfa/focDE	12 (16.22)	1 (2.56)	0.001
sfaS	10 (13.52)	1 (2.56)	0.435
focG	6 (8.11)	0 (0)	0.047
kpsMT K1	8 (10.81)	3 (7.69)	0.463
kpsMT K5	26 (35.14)	9 (23.08)	0.081
kpsMT II	35 (47.30)	12 (30.77)	0.192
kpsMT III	2 (2.703)	1 (2.56)	0.965
malX	19 (25.68)	8 (20.51)	0.541
cvaC	5 (6.76)	1 (2.56)	0.345
ibeA	7 (9.46)	3 (7.69)	0.190
bmaE	4 (5.41)	11 (28.2)	0.001
rfc	2 (2.703)	9 (23.08)	0.001

Table 6. - Continued

Gene	ABU (n = 74) n (%)	Fecal (n = 39) n (%)	p-value*
traT	11 (14.9)	2 (5.13)	0.123
gafD	2 (2.703)	0 (0)	0.302
afa/draBC	4 (5.41)	0 (0)	0.141
iroN	17 (23)	17 (43.6)	0.024

**Pearson's Uncorrected Chi-Square Test*

5.1.2.2. Phenotypic Traits

To complement investigation of virulence genes present among the isolates, phenotypic tests were also conducted. Among these were tests for hemolysis, aerobactin and colicin production, K5 phage lysis, ability to bind Congo red dye, and agglutination tests.

Hemolytic Activity

To check the ability of the isolates to express α-hemolysin, they were plated on sheep agar plates. Growth on the plates indicated that not all isolates that harbored the *hlyA* gene were able to lyse erythrocytes. 66.67 % of the ABU isolates that harbored the *hlyA* gene and 100% of the fecal isolates express α-hemolysin (Figure 7A; Table 7).

Iron Uptake

The isolates were grown on plates containing bacterial strain LG1522 to look at their ability to produce aerobactin. The presence of growth zones of LG1522 around the colonies indicated a positive result. In the same way that not all those that harbored the gene for α-hemolysin expressed it, not all the isolates that harbored the *iutA* gene, both for ABU and fecal groups, were shown to produce aerobactin, *i.e.* only 64.29 % and 83.33 % of them, respectively (Figure 7B; Table 7).

Colicin Production

Colicins are bacterial toxins that kill *E. coli* and related cells (Gökçe and Lakey, 2003). To look at colicin production among the isolates, they were grown on plates containing indicator strain DH5α. The presence of zones of inhibition around colonies of tested strains indicated colicin production. Results show that 40.5 % of ABU isolates were able to produce colicins that kill indicator strain DH5α whereas 33.3 % of fecal isolates did.

K5 lysis

An assay whereby a K5 bacteriophage lysate (kindly provided by Ian Roberts, University of Manchester) could be used to lyse bacterial cells to show expression of K5 capsule was conducted. If the cells were lysed, it meant that the bacteria contained binding sites recognized by the K5 bacteriophage and were thus positive for K5 capsule to which the K5-specific bacteriophages bound. Of the 26 (35.14 %) ABU isolates that harbored the *kpsMTK5* gene, only 6 (8.10 %) were able to express K5 capsule as shown by the lysis assay (Figure 8; Table 7).

Fimbrial and Cellulose Expression

Fimbriae facilitate the colonization of the urinary tract and promote *E. coli* colonization and persistence in the colon or vagina which acts as reservoir for ascending infection in the urinary tract. Of all the three major types of fimbriae (type 1, S- and P-fimbria), type 1 fimbria was the most common in pathogenic and even less virulent strains. It enhances bacterial survival, stimulates mucosal inflammation and promotes bacterial invasion. P fimbria, on the other hand, is recognized as a key determinant in promoting the virulence of *E. coli* in UTI.

In this study, results showed that 69.23 % of ABU isolates expressed type 1 fimbria, making it one of the most highly expressed virulence traits. This is in affirmation to PCR results showing that *fimH*, encoding for this fimbria, was the most common of all the

virulence genes tested in ABU. S-fimbriae were also commonly expressed among ABU as well as the the fecal isolates that harbored any of the genes encoding for S-fimbriae (55.56% and 100%, respectively). Finally, only 14.29 % of the ABU isolates and 66.67 % of the fecal isolates that harbored any of the genes encoding for P-fimbriae functionally expressed P-fimbria as shown by agglutination tests using yeast cells and antibodies raised against the fimbriae (Figure 9; Table 7).

Meanwhile, the bacterial isolates were also grown on Congo red agar to determine curli fimbriae and cellulose production. The assays revealed that 62.16 % of ABU produced curli and 43.24 % expressed cellulose while 71.79 % of the fecal isolates produced curli and 56.41 % manifested cellulose production at room temperature. On the other hand, 35.13 % of ABU produced curli and 75.67 % expressed cellulose while 35.89 % and 53.84 % fecal isolates produced curli and cellulose respectively at 30 °C. Finally, 95.94 % of ABU expressed curli at 37 °C as compared to 30.77 % among the fecal isolates and 71.79 % of ABU and 12.16% fecal isolates manifested cellulose production (Figure 11; Table 7).

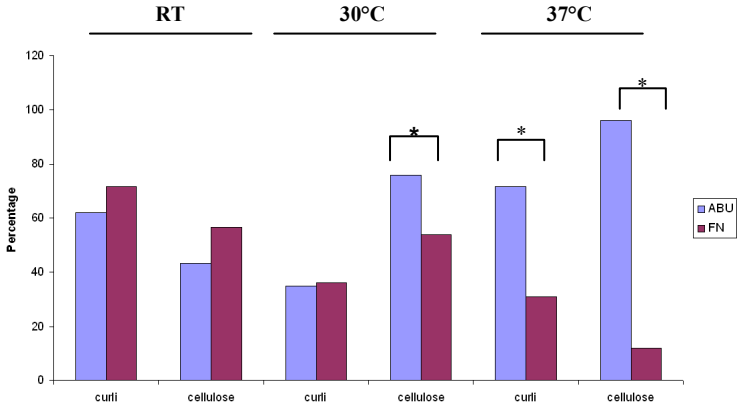

Figure 11. Curli fimbriae and cellulose expression detected by growth on Congo red agar at room temperature (RT), 30 °C and 37 °C. FN- fecal isolates. Significant difference between ABU and FN is indicated by an asterisk (p <0.05).

Table 7. Phenotypic Characteristics of ABU and Fecal Isolates

Phenotype		n**	ABU n (%)	n**	Fecal n (%)	p-value*
α-Hemolysin		15	10 (66.67)	1	1 (100)	0.486
Aerobactin		14	9 (64.29)	6	5 (83.33)	0.394
Colicins			30 (40.5)		13 (33.3)	0.453
Type 1 fimbria		65	45 (69.23)	36	31 (86.11)	0.060
P- fimbria		21***	3 (14.29)	6***	4 (66.67)	0.010
S-fimbria		9***	5 (55.56)	1***	1 (100)	0.389
K5 Capsule		26	6 (8.108)	9	3 (7.692)	0.544
Curli Fimbriae	RT		46 (62.16)		28 (71.79)	0.306
	30°C		26 (35.13)		14 (35.89)	0.936
	37°C		53 (95.94)		12 (30.77)	0.000
Cellulose	RT		32 (43.24)		22 (56.41)	0.183
	30°C		56 (75.67)		21 (53.84)	0.018
	37°C		71 (71.79)		5 (12.16)	0.000

**Pearson's Uncorrected Chi-Square Test*

*** n = the number of isolates that harbor the gene for the phenotype. When not indicated, computation of percentage was based on the total number of isolates (i.e. ABU, n=74; FN, n=39).*

**** n = the mean number of the genes harbored by the isolates that correspond to the phenotype when there is more than one gene tested for the phenotype (e.g. for S fimbria - sfaS/focDE, sfaS and focG).*

5.2. Characterization of ABU re-isolates from symptomatic episodes

In addition to the ABU isolates sampled from healthy individuals, eight other ABU isolates taken from patients stably colonized with *E. coli* 83972 as prophylactic treatment for recurring urinary tract infection were also characterized. Glycerol stocks of these eight re-isolates namely BU05 17629, BU05 42290, BU04 50907, BU04 41631, BU04 63630, BU04 68088, BU05 35364 and FIM 636 were sent from the Lund University

Hospital, University of Lund, Sweden to the Institute for Molecular Infection Biology, University of Würzburg, Germany, as materials for characterization. These isolates were re-isolated from asymptomatically colonized patients that reported very rare cases of symptoms while undergoing prophylactic treatment.

5.2.1. Phylogenetic Classification

The eight re-isolates were primarily phylogenetically classified. According to results, 25 % of the re-isolates namely BU05 17629 and BU05 42290 belonged to group A while the remaining 75 % belonged to group B2, the same group to which parent strain *E. coli* 83972 belongs.

5.2.2. Verification of Re-isolates as *E. coli* 83972

To verify whether the eight re-isolates were ABU isolate 83972 or not, 20 colonies were randomly picked from overnight-grown samples on agar plate at 37 °C. Following this, all 20 colonies from each re-isolate were subjected to PCR to detect the presence of the cryptic plasmid and the internal deletion of the type 1 fimbrial gene cluster characteristic of the parent strain. Results showed that 75 % of them were positive for the PCR while 25 %, namely BU05 17629 and BU05 42290, were not.

In addition to PCR, the re-isolates were grown in chromogenic agar (Brilliance™ UTI Clarity™ agar (Oxoid)). *Brilliance* UTI Clarity Agar is a non-selective, differential agar which provides presumptive identification of the main pathogens which cause infection of the urinary tract. This medium contains two chromogenic substrates which are cleaved by enzymes produced by *E. coli*, *Enterococcus* spp. and coliforms. Of the two chromogens included in the medium, one is metabolized by β-galactosidase, an enzyme produced by *E. coli*, which yields pink colonies. The other is cleaved by β-glucosidase enzyme activity, allowing the specific detection of enterococci which form blue or turquoise colonies (http://www.oxoid.com/UK).

All re-isolates produced a pink color on plate except BU05 17629 which grew as green colonies. This implied that BU05 17629 was a pseudomonad. On the other hand, BU05

42290 even though not ABU isolate 83972, was *E. coli*. Moreover, interestingly, five of the 20 colonies of BU04 50907 grew as very small, transparent colonies in chromogenic agar (Figure 12) wherein *E. coli* should grow as pink colonies. In addition, they grew as very small colonies in sheep blood agar. To verify that they were *E. coli*, sequencing of the 16S rRNA region was done. The sequencing data obtained verified that these isolates were indeed *E. coli*.

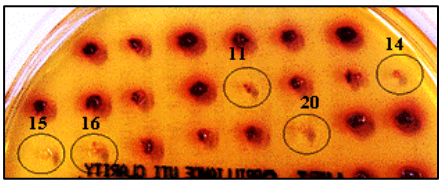

Figure 12. Five BU04 50907 colonies growing almost transluscent and with morphology similar to small colony variants in chromogenic agar.

The re-isolates were also grown on MacConkey agar. MacConkey is known as an "indicator medium". It distinguishes the Gram-negative bacteria that can ferment the sugar lactose (Lac+) from those that cannot (Lac-). Lactose is utilized by the Lac+ ones producing acid which results in the appearance of red/pink colonies. On the other hand, Lac- ones cannot utilize lactose and this leads to the formation of white/colorless colonies. Except for BU05 17629, all re-isolates grew pink on MacConkey agar (Figure 13).

All those re-isolates confirmed to be *E. coli* 83972 were further investigated.

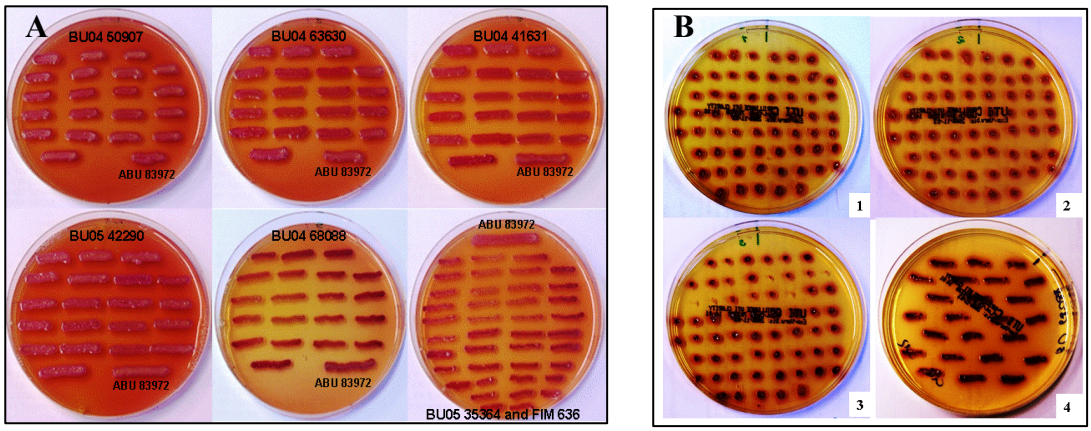

Figure 13. Confirmation of the re-isolates as ABU isolate 83972. (A) 20 colonies of each re-isolate grown on MacConkey agar at 37 °C. (B) 20 colonies of each re-isolate grown on chromogenic agar at 37 °C. (1) BU04 41631 and AS; (2) BU05 42290 and BU05 35364; (3) BU04 50907, BU04 63630 and FIM 636; (4) BU04 68088.

5.2.3. Morphology of the Re-isolates

Growth of the re-isolates on different agar media showed that all of them, except BU04 50907 and BU04 68088, exhibited homogeneous colony morphology with regards to size and appearance. The two aforementioned re-isolates exhibited heterogeneous colony morphology on agar plates. BU04 50907 grew relatively small, resembling small colony variants (SCV) or normal, resembling ABU isolate 83972 wt. BU04 68088, on the other hand, grew as small colonies, the size of which was bigger than BU04 50907 resembling SCV, or normal like ABU isolate 83972 wt.

5.2.4. Genotypic and Phenotypic Analysis of the Re-isolates

The re-isolates were characterized using standard genotypic and phenotypic assays. With the exception of aerobactin and colicin phenotypic assays, similar tests were performed for all re-isolates as done to those that belong to the isolates collection taken from healthy individuals. In addition, motility test was also conducted by observing swarming on soft agar. A summary of the various phenotypic and genotypic characteristics of the re-isolates can be found in Tables 8 and 9.

5.2.5. Virulence Genes

E. coli 83972 harbors virulence genes characteristic of uropathogenic *Escherichia coli* (UPEC). However, it does not express most of these genes. PCR results showed that all re-isolates were similar to isolate 83972 wt in harboring the various virulence genes included in the PCR with the exception of BU05 17629 which did not harbor the *fimH* or any of the *pap* genes and was not at all *E. coli*.

5.2.6. Phenotypic Traits

Curli Fimbria and Cellulose

Based on the assay conducted, re-isolates BU04 63630, BU04 41631 and BU05 35364 produced cellulose at 37 °C while BU04 68088 and BU04 50907 expressed both curli and cellulose. On the other hand, BU04 63630, BU04 68088 and FIM 636 manifested cellulose production at 30 °C. To verify the results of the Congo red assay, the re-isolates were also grown on calcofluor (fluorescent brightener 28) plates. FIM 636 was positive for cellulose production at 30 °C (Table 9; Figure 14).

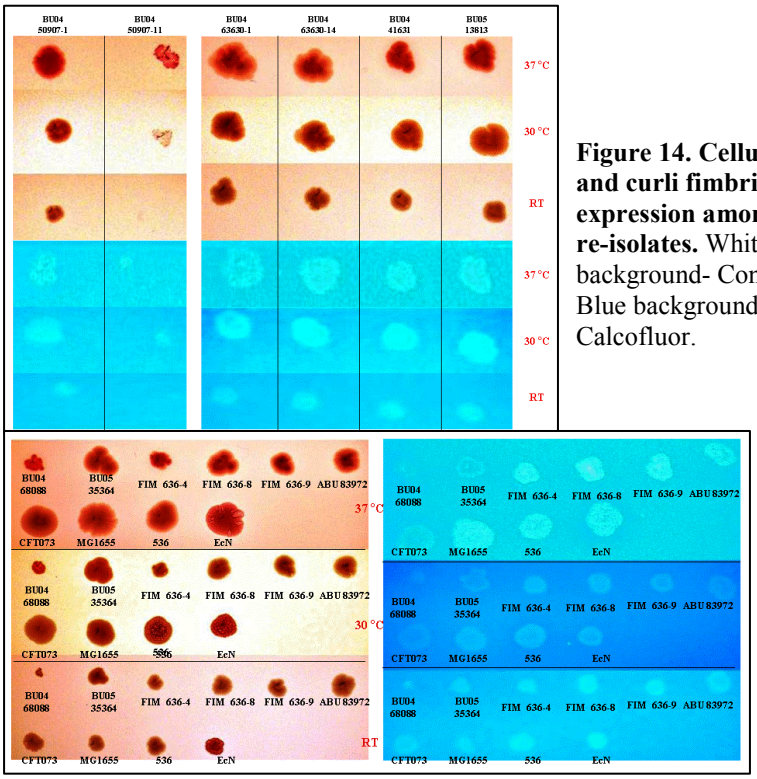

Figure 14. Cellulose and curli fimbriae expression among the re-isolates. White background- Congo Red; Blue background- Calcofluor.

Autoaggregation Assay

Autoaggregation is the cell-cell interaction of bacteria belonging to the same strain (Khemaleelakul *et al*., 2006). The ability of bacterial cells to autoaggregate is important in establishing biofilms and is thus a known virulence trait. To find out whether the re-

isolates have the ability to autoaggregate, they were grown on LB medium and left overnight at room temperature. The following day, the appearance of the cells as a result of clumping together was observed. The assay conducted showed that there was no difference among the re-isolates and ABU isolate 83972 wt with regards to their ability to autoaggregate in LB medium (Figure 15).

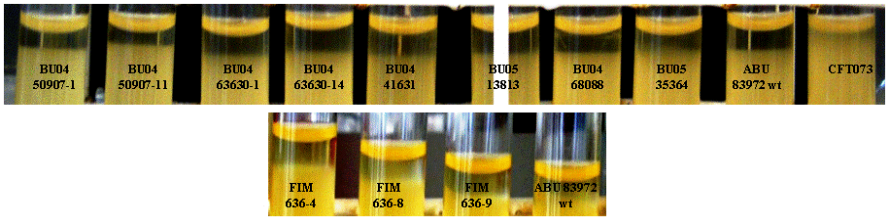

Figure 15. Autoaggregation of re-isolates in LB medium. BU05 13813 is an asymptomatic re-isolate used for control.

Biofilm Assay in Urine

Based on the assays conducted BU04 41631, BU04 63630 (clones 1 and 14) and FIM 636 (clones 8 and 9) could form biofilm equal to ABU isolate 83972 wt. On the other hand, all the other re-isolates could form less biofilm (Figure 16).

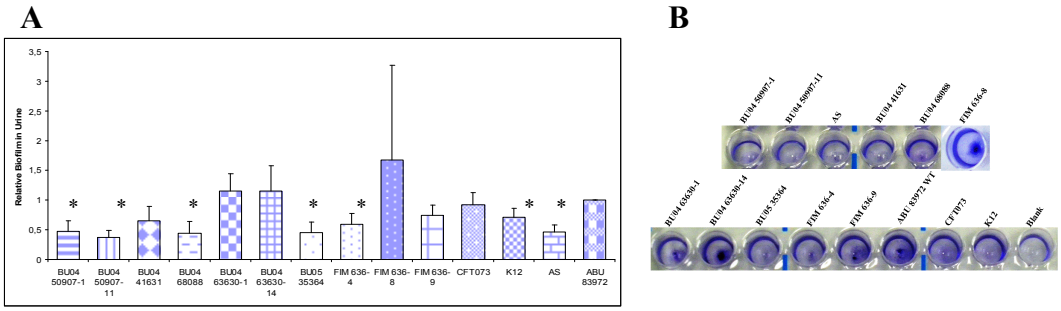

Figure 16. Biofilm formation among the different re-isolates in pooled human urine at 37 °C. (A). Biofilm formation of the tested isolates standardized to that of ABU isolate 83972 wt. (B) Biofilm formation in pooled human urine. Results significantly different from ABU isolate 83972 wt are indicated by an asterisk. (p <0.05). Error bars shown are standard deviations of three independent experiments. AS is an asymptomatic re-isolate.

Polysaccharide Intercellular Adhesin (PIA) Immuno Dot Blot

The polysaccharide intercellular adhesin (PIA) is a biofilm-related exopolysaccharide made of *N*-acetylglucosamine that is found in staphylococci and many other bacterial biofilm-forming pathogens (Vuong and Otto, 2008). In *E. coli,* this β-1,6-GlcNAc polysaccharide promotes surface binding, intercellular adhesion, and biofilm formation (Wang *et al*., 2004). For quantification of PIA among the eight re-isolates immuno dot blot assay was conducted. Results show that only the motile re-isolate BU05 35364 produced more PIA than ABU isolate 83972 wt (Figure 17).

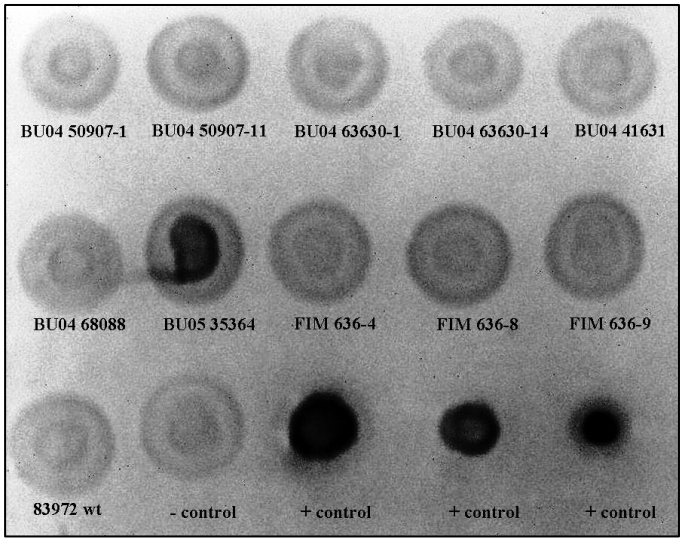

Figure 17. Detection of the poly-GlcNAc extracellular polysaccharide by immuno-blot of the different re-isolates using antiserum against PIA in *Staphylococcus aureus* raised in rabbit. *Staphylococcus* strains RP62A and TM 300 were used as positive and negative controls, respectively.

Lipopolysaccharide (LPS)

Isolation of lipopolysaccharide (LPS) from cells grown on agar plates revealed that all re-isolates exhibited a rough LPS phenotype similar to ABU isolate 83972 wt. The intensity of the bands produced by the re-isolates was also similar to the parent isolate. Rough LPS

has shorter or no O-side chain. On the other hand, smooth LPS has full-length O-side chains, such as in case of UPEC strain 536. In this study, all re-isolates expressed no longer O-side chain and thus exhibited a similar LPS phenotype as isolate 83972 wt (Figure 18).

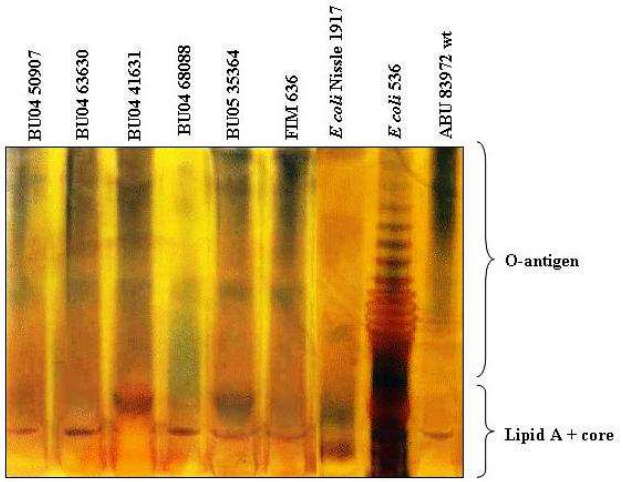

Figure 18. Phenotypic analysis of lipopolysaccharide among the various re-isolates using silver nitrate staining.

K5 Phage Lysis

Capsules inhibit the opsonizing activities of complement, decreasing the elimination by phagocytes, or escape the immune response by mimicking host molecules thereby increasing bacterial virulence (Bayer and Bayer, 1994; Boulnois and Roberts, 1990; Cross *et al*., 1990). The *E. coli* K1 and K5 capsules are frequently found among isolates causing extraintestinal infections. K1 is more commonly associated with neonatal septicemia and K5 with sepsis and urinary tract infection (Cross *et al*., 1990). ABU isolate 83972 wt and all the re-isolates except BU04 63630 and BU05 35364 were found to express K5 capsule by lysing of cells with a K5 bacteriophage lysate (Figure 19).

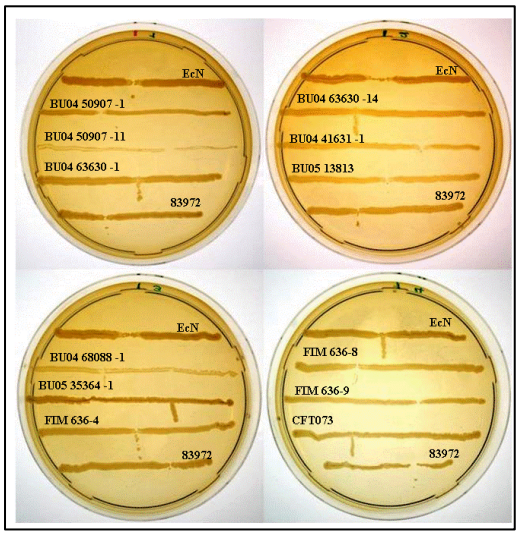

Figure 19. K5 lysis assay using K5 bacteriophage lysate. *E. coli* Nissle (EcN) 1917 was used as positive control.

Analysis of Motility and Flagella Expression

Expression of flagella is one of the virulence traits of UPEC. Therefore, to analyze whether or not this UPEC trait is expressed in the 83972 re-isolates, motility tests on soft LB or urine agar followed by confirmation by SDS-PAGE and Western Blot analysis were done.

Motility Test

Among the six re-isolates tested on soft agar, three re-isolates stood out with regards to motility expression. Marked motility was observed in BU04 63630, BU05 35364 and FIM 636. Eighteen out of 20 colonies of BU04 63630 were found to be more or as equally motile than/as that of *E. coli* 83972 wt. On the other hand, all 20 colonies of BU05 35364 were found to be either equally or more motile as/than ABU isolate 83972 wt. Finally, FIM 636 displayed a mixture of less, equally and more motile colonies compared to the parent strain (Figure 20 and Tables 7A-C). An average of 32 % of the

colonies tested for FIM 636 were found to be more motile in urine and LB agar compared to ABU isolate 83972 wt.

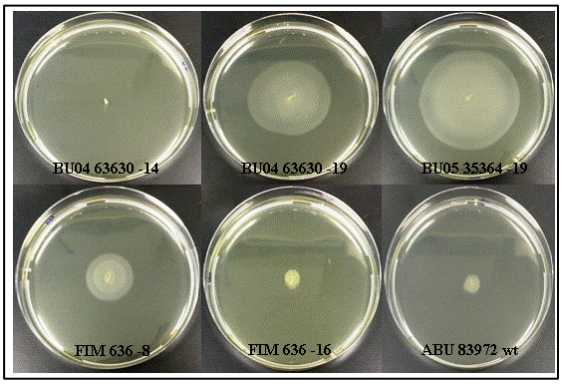

Figure 20. Motility on urine plates. The swarming ability of the re-isolates varied from being non-motile, less motile, equally motile and more motile compared to ABU isolate 83972 wt.

Table 8. Average Swarming Diameter of Motile Re-isolate BU04 63630

Clone	Motility (mm) Urine agar	Comparison with ABU isolate 83972 wt*	Motility (mm) LB agar	Comparison with ABU isolate 83972 wt*
1	34	↑	18	↓
2	10	↓	5	↓
3	43	↑	23	↓
4	16	=	28	↑
5	43	↑	48	↑
6	25	↑	15	↓
7	43	↑	45	↑
8	44	↑	48	↑
9	56	↑	38	↑
10	26	↑	30	↑
11	37	↑	25	=
12	39	↑	25	=
13	55	↑	48	↑
14	4	↓	10	↓
15	46	↑	43	↑
16	35	↑	45	↑
17	58	↑	38	↑
18	39	↑	43	↑
19	58	↑	35	↑
20	31	↑	28	↑

*Values for ABU isolate 83972: Urine = 16 mm; LB= 25 mm. ↑ refers to an increased motility, ↓ refers to a decreased motility, = refers to equal motility with respect to ABU isolate 83972 wt.

Table 9. Average Swarming Diameter of Motile Re-isolate BU05 35364

Clone	Motility (mm) Urine agar	Comparison with ABU isolate 83972 wt*	Motility (mm) LB agar	Comparison with ABU isolate 83972 wt*
1	37	↑	55	↑
2	57	↑	65	↑
3	49	↑	40	↑
4	53	↑	60	↑
5	48	↑	65	↑
6	54	↑	68	↑
7	50	↑	65	↑
8	52	↑	58	↑
9	41	↑	65	↑
10	42	↑	53	↑
11	24	↑	65	↑
12	39	↑	65	↑
13	45	↑	63	↑
14	41	↑	42	↑
15	35	↑	50	↑
16	49	↑	49	↑
17	33	↑	47	↑
18	35	↑	60	↑
19	43	↑	48	↑
20	16	=	48	↑

*Values for ABU isolate 83972: Urine = 16 mm; LB= 25 mm. ↑ refers to an increased motility, ↓ refers to a decreased motility, = refers to equal motility with respect to ABU isolate 83972 wt.

Table 10. Average Swarming Diameter of Motile Re-isolate FIM 636

Clone	Motility (mm) Urine agar	Comparison with ABU isolate 83972 wt*	Motility (mm) LB agar	Comparison with ABU isolate 83972 wt*
1	4	↓	31	↑
2	4	↓	0	↓
3	17	↑	0	↓
4	6	↓	44	↑
5	15	↓	29	↑
6	12	↓	21	↓
7	10	↓	26	↑
8	23	↑	41	↑
9	7	↓	0	↓
10	26	↑	0	↓
11	5	↓	18	↓
12	11	↓	15	↓
13	11	↓	28	↑
14	9	↓	18	↓
15	12	↓	30	↑
16	7	↓	21	↓
17	17	↑	20	↓
18	9	↓	33	↑
19	16	=	0	↓
20	19	↑	18	↓

*Values for ABU isolate 83972: Urine = 16 mm; LB= 25 mm. ↑ refers to an increased motility, ↓ refers to a decreased motility, = refers to equal motility with respect to ABU isolate 83972 wt.

SDS-PAGE and Western Blot

Western Blot results showed that there was an increased amount of flagellin detectable in the more motile re-isolates (*i.e.* BU05 35364 and BU04 63630) compared to FIM 636 which contained a mixture of colonies that are non-motile or only as equally motile as ABU isolate 83972 wt. As control, a *fliC* deletion mutant was constructed from ABU isolate 83972 wt using the lamda red homologous recombination technique (Datsenko and Wanner, 2000). The *fliC* gene encodes the flagellin protein which is the major component of the flagella. The deletion mutant generated a very faint band in the Western Blot assay (Figure 21).

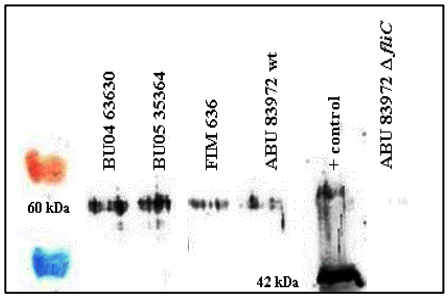

Figure 21. Detection of flagellin in the motile re-isolates through Western Blot. Flagellin expressed in the different 83972 re-isolates were detected using a polyclonal anti-*flaA* serum raised in rabbits against the *Legionella* flagellin FlaA.

Adhesion Assay

Results of the adhesion assays showed that none of the re-isolates had the ability to adhere more to T24 bladder cells compared to ABU isolate 83972 wt (Figure 22).

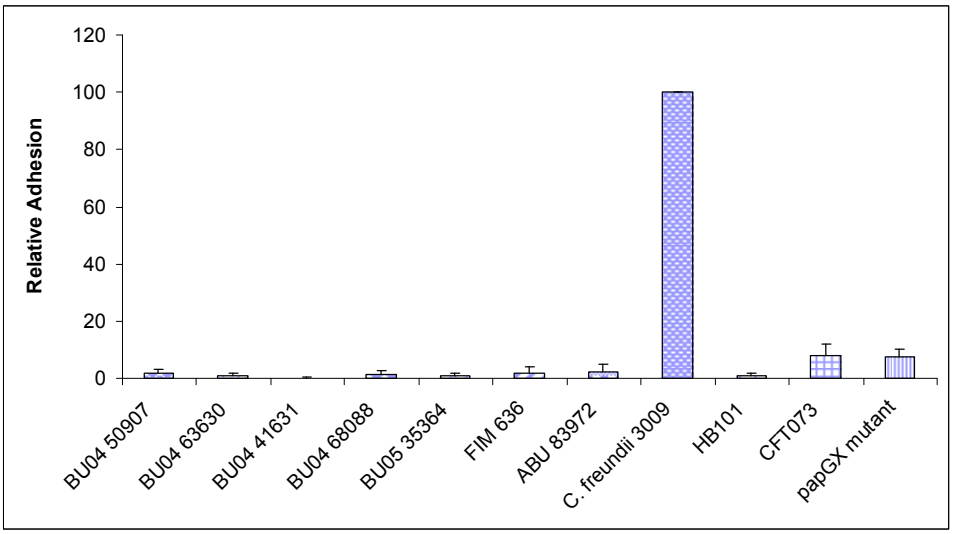

Figure 22. Adhesion of re-isolates to T24 bladder cells. Standard (100%) was set for positive control *Citrobacter freundii* 3009. The *Citrobacter* strain possesses a high internalization capacity. In contrast, *E. coli* HB 101 is internalized only with a low number of bacterial cells and was used as negative control strain. The *papGX* mutant is *E. coli* 83972 complemented with a functional *papG* gene from *E. coli* CFT073. Adhesion levels of the re-isolates were not significantly different from ABU isolate 83972 wt. Error bars shown are standard deviations of three independent experiments.

5.2.7. Restriction Pattern Analysis

The genome structure of the re-isolates was further characterized through pulsed field gel electrophoresis (PFGE). The genomic *AvrII* restriction fragment pattern of all six re-isolates proven by other tests to be ABU isolate 83972 were identical to that of ABU isolate 83972 wt (Figure 23). On the other hand, those that had been confirmed to be not *E. coli* 83972 show completely different genomic restriction profiles (Figure 24).

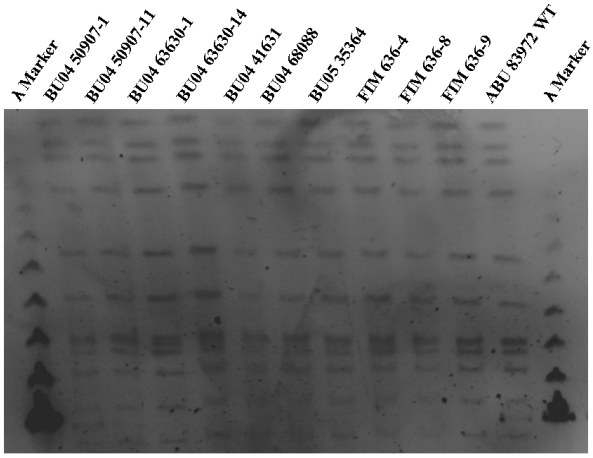

Figure 23. Comparison of the genomic fingerprints of the various re-isolates confirmed as ABU isolate 83972 using PFGE following digestion with *AvrII*.

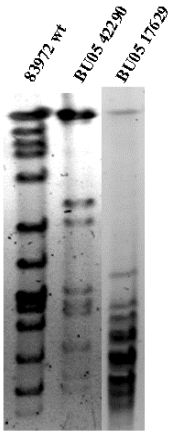

Figure 24. Genomic fingerprints of re-isolates confirmed not to be *E. coli* 83972 by PFGE following digestion with *AvrII*. BU05 42290 was found to be *E. coli* but not ABU isolate 83972 and BU05 17629 was found to be *Pseudomonas spp.*.

5.2.8. Growth characteristics

Analysis of growth in different media showed that all the re-isolates had relatively similar growth rates in urine as well as in LB compared to ABU isolate 83972 wt. Exceptions were BU04 50907-1 and BU04 50907-11 resembling small colony variants and BU04

68088, a mixture of small and big colonies, which grew relatively slow (Figure 25). It is also interesting to note that re-isolate BU04 68088 did not grow at all in M63 medium.

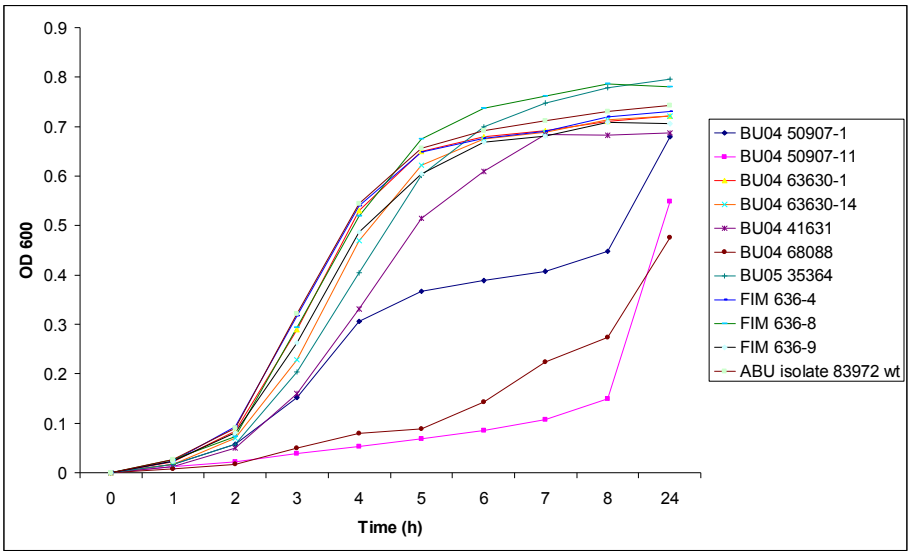

Figure 25. Growth curve of the different re-isolates in urine at 37 °C. Different numbers following strain designations pertain to different colonies of the same re-isolate (*i.e.* BU04 50907-1 is a normal-sized colony while BU04 50907-11 resembles SCV. BU04 63630-1 is a motile colony while BU04 63630-14 is non-motile. FIM 636-8 is more motile than *E. coli* 83972 wt while FIM 636-9 is non-motile. FIM 636-4 is non-motile in urine agar but motile in LB agar). Data shown are average results of three independent experiments.

Table 11. Primary characteristics of re-isolates from symptomatic episodes compared to parent strain

Sample ID	Patient ID	Chrom Agar	pABU	fim del	IL-6 (pg/ml)	IL-8 (pg/ml)	PMN ($x10^4$)	Remarks
ABU isolate 83972 wt		*E. coli*	+	+	-	-	-	83972
BU05 17629	HK1	*Pseudomonas*	-	-	3	378	28	superinfection, *Pseudomonas*
BU05 42290	SN1	*E. coli*	-	-	36	2781	69	*E. coli* but not 83972
BU04 50907 (Clone 1)	BJK2	*E. coli*	+	+	66	3268	960	83972
BU04 50907 (Clone 11)	BJK2	*E. coli*	+	+	66	3268	960	83972
BU04 41631	IJ2	*E. coli*	+	+	18	4706	240	83972
BU04 63630 (Clone 1)	BJK3	*E. coli*	+	+	107	551	800	83972
BU04 63630 (Clone 14)	BJK3	*E. coli*	+	+	107	551	800	83972
BU04 68088	RN2	*E. coli*	+	+	8	431	-	83972
BU05 35364	BO2	*E. coli*	+	+	300	7500	20000	83972
FIM 636 (Clone 4)	KA	*E. coli*	+	+	41	982	1.39E+06	83972
FIM 636 (Clone 8)	KA	*E. coli*	+	+	41	982	1.39E+06	83972
FIM 636 (Clone 9)	KA	*E. coli*	+	+	41	982	1.39E+06	83972

**Data for IL-6, IL-8 and PMN levels were provided by B. Wullt from Lund, Sweden. Levels were detected using routine ELISA test on urine samples from patients. Different numbers following strain designations pertain to different colonies of the same re-isolate. Please see Figure 25.*

Table12. Summary of genotypic and phenotypic characteristics of the re-isolates

Sample ID	ExPEC PCR	Autoaggregation	Motility*	Biofilm*	K5 capsule	PIA	Cellulose	Curli
ABU isolate 83972 wt	+	+	+	+	+	-	30°C	30°C
BU05 17629	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
BU05 42290	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
BU04 50907 (Clone 1)	+	+	-	-	+	-	37°C	37°C
BU04 50907 (Clone 11)	+	+	-	-	+	-	37°C	37°C
BU04 41631	+	+	-	<	+	-	-	-
BU04 63630 (Clone 1)	+	+	>	>	-	-	30°, 37°C	-
BU04 63630 (Clone 14)	+	+	-	>	-	-	30°, 37°C	-
BU04 68088	+	+	-	-	+	-	30°, 37°C	37°C
BU05 35364	+	+	>	-	-	+	-	-
FIM 636 (Clone 4)	+	+	-	-	+	-	30°C	-
FIM 636 (Clone 8)	+	+	>	>	+	-	30°C	-
FIM 636 (Clone 9)	+	+	-	=	+	-	30°C	-

**Re-isolates data relative to ABU isolate 83972 (equal, more or less compared to ABU isolate 83972 wt; n/a- no further tests were conducted).*

Different numbers following strain designations pertain to different colonies of the same re-isolate. Please see Figure 25.

5.2.9. Transcriptome analysis of the Motile Re-isolates

The various phenotypic analyses revealed differences in expression of virulence traits among the different re-isolates. In order to evaluate the changes among selected re-isolates in the transcriptional level, microarray experiments were performed. Transcriptome analysis was conducted for the three motile re-isolates namely BU04 63630, BU05 35364 and FIM 636. Results showed that deregulation of genes was unique for each of the re-isolates since not a lot of genes were commonly de-regulated in all three or any two of them. A total of 220 genes were de-regulated in the three re-isolates. Of these, 193 were upregulated and 27 were downregulated (Figures 27 and 28).

Most genes that were upregulated in BU04 63630 encode for phages and cell structures. On the other hand, most of the upregulated genes in BU05 35364 are involved in transport processes. Finally, for FIM 636, most of the upregulated genes function for cell processes which include adaptation and protection. Meanwhile, most of the downregulated genes in all three re-isolates are involved in cellular processes (Figure 26).

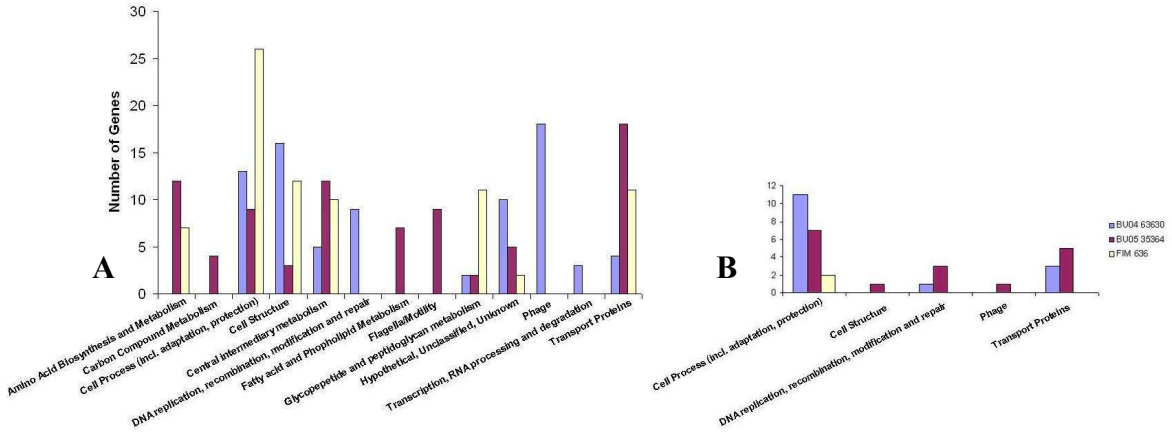

Figure 26. Distribution of the deregulated genes among the motile re-isolates into functional groups.
(A). Upregulated genes. (B). Downregulated genes.

5.2.9.1. Deregulated Genes among the Re-isolates

Motility/Chemotaxis

Flagella enhance the fitness of UPEC throughout the urinary tract. They are thought to contribute to UPEC virulence during urinary tract infection since they play putative roles in adherence, ascension, biofilm formation and dispersal (Wright *et al*., 2005). ABU isolate 83972 does not swarm when grown on soft LB/urine-agar. However, three of the re-isolates showed marked motility compared to parent strain ABU isolate 83972 and these were included in the transcriptome analysis.

The microarray data revealed that BU05 35364 which was 100 % motile (*i.e.* 20/20 colonies grown in soft agar exhibited swarming) had significantly upregulated genes involved in flagellar assembly and biosynthesis (*flgA* and *flgDEFG, flhA* and *fliA, fliG* and *fliO*). In comparison, BU04 63630 which was 90 % motile (*i.e.* 18/20 colonies grown in soft agar exhibited swarming) had only the flagellar motor protein *motA* upregulated. Meanwhile, the mixed motile and non-motile FIM 636 was shown to have the fumarate reductase involved in anaerobic respiration and flagellar motility upregulated.

Resistance/Adaptation

Changes in the environment call for adaptation which could bring about stress in an organism. The microarray data showed that there were several genes involved in resistance to various conditions and coping up mechanisms which had been upregulated among the re-isolates.

For instance, in FIM 636, genes involved in formic acid metabolism (*frmRAB*), formate dehydrogenase (*fdnGHI*) and nitrate reductase (*narHIJ*, which forms a respiratory chain with formate dehydrogenase, and *napAGBC*) as well as those involved in reduction of nitrite to ammonia (*nirBDC*) and those that act as scavengers of toxic by-products of nitrogen metabolism (*hcp/hcr*) were significantly upregulated.

Meanwhile, BU04 63630 have genes involved in acid resistance (*gadA* and *gadB*, *hdeAB, cadB, slp*), RNA polymerase sigma factor (*rpoA, rpoE* and *rpoS*), osmotic stress (*osmB*) and SOS response to DNA damage (*recA* and *recN, lexA, yebG, psb1, sulA, dinB* and *dinI, ruvB, umuD,* and *rus*) upregulated.

In BU05 35364, on the other hand, few genes were upregulated such as those involved in heat shock (*groEL* and *groES*). The gene *degP* involved in survival at high temperature was upregulated in all three re-isolates.

Adhesins

ABU isolate 83972 harbors a non-functional *pap* gene cluster encoding P fimbriae, and a truncated *fim* gene cluster encoding type 1 fimbriae (Klemm *et al*., 2005).

According to the microarray data, FIM 636 was shown to have several upregulated adhesin genes such as *papACDEFHK* (P fimbria), *focA* and *focC*, and *sfaB* and *sfaD* (F1C). The P-fimbrial genes *papAF* was also upregulated in BU05 35364 while only *papA* was upregulated in BU04 63630.

Polysaccharides

The cell walls of gram-negative bacteria are made up of polysaccharides specifically lipopolysaccharide (LPS). LPS makes up the major part of the outer leaflet of the outer membrane of *E. coli*. Its two main components are the lipid A core that imparts most of the biological effects of the LPS and the O-antigen which is important in the colonization of the host and resistance to complement-mediated killing as well as cationic antimicrobial peptides which are key elements of the innate immune system. (Ullrich, 2009). Meanwhile, mucoid (slimy) strains contain a polysaccharide capsule that surrounds the whole cell, which may have a role in virulence. The capsular polysaccharides and LPS represent the first line of defense against complement and bacteriophages (Varki *et al.*, 2009).

Several LPS genes such as *rfaGPIJY*, *waaV* and *waaW* were found to be significantly upregulated in BU05 35364. In addition, N-acetyl glucosamine-related genes such as *glmUS* and *lpxAB* were also upregulated in this re-isolate.

Toxins

Bacterial toxins are a major group of virulence factors. Two types of toxins exist, the first being the endotoxin lipid A. The second type, called exotoxins are those secreted by the cell.

Based on the microarray results, toxin genes which were upregulated were *cnf1* (cytotoxic necrotizing factor) in BU05 35364 and BU04 63630 as well as colicin-related genes *cvpA* and *cirA* in BU05 35364, and *ECP_0113* in all three re-isolates.

Iron

BU05 35364 was shown to have iron-uptake related genes which were upregulated such as the genes involved in heme utilization (*chuATUWXY*) and those involved in the enterochelin biosynthetic pathway (*entCA*).

Other Genes

Other genes which were upregulated in any one or two or all of the three isolates were those involved in transport and secretion, amino acid biosynthesis, several enzymes, ribosomal proteins, phage related proteins and a number of hypothetical proteins.

5.2.9.2. Changes in gene expression in individual re-isolates

Alterations in the transcriptome of re-isolate BU04 63630 relative to ABU isolate 83972 wt

Most of the genes with known functions which were highly upregulated in re-isolate BU04 63630 are involved in SOS response to DNA damage. The *recA* gene, which is a part of the SOS regulon induced by inhibition of DNA replication as a result of DNA damage, was the most upregulated gene in re-isolate BU04 63630 (Appendix Table 11). Interestingly, another gene in the SOS regulon, *recN*, which is responsible for recombinatorial repair, was also upregulated. In addition to these two, other genes involved in SOS response such as *lexA*, *ruvB*, *dinI, dinB, sulA, yebG* and *umuD* were also upregulated. Other genes which were upregulated in BU04 63630 are those involved in acid resistance and the remaining ones either have unknown functions or are phage-related.

In contrast, a group of significantly downregulated genes found in re-isolate BU04 63630 code for phospho-transferase transport systems (PTS) (Appendix Table 18). The phosphotransferase systems or PTS are distinct transport systems used by bacteria to take up numerous carbohydrates and convert them into their respective phosphoesters during transport where the source of energy is from phosphoenolpyruvate (Deutscher *et al.*, 2006). PTS transports many sugars into bacteria, including glucose, mannose, fructose and cellobiose. It is also involved in metabolic and transcriptional regulation (Saier, 2001).

Alterations in the transcriptome of re-isolate BU05 35364 relative to ABU isolate 83972 wt

BU05 35364, the re-isolate which was found to be 100 % motile in soft agar swarming assay, was shown to have a group of genes involved in flagellar expression and biosynthesis significantly upregulated (Appendix Table 12). In addition, it was also found to have other groups of significantly upregulated genes which include those that

are involved in LPS synthesis, N-acetyl glucosamine biosynthesis, heme utilization, colicin production, enterochelin biosynthetic pathway, maltose transport and amino acid biosynthesis. There were, however, not so many significantly downregulated genes in this re-isolate which could be grouped together except those involved in galactose ABC transporter (*mglAB*) and formate-dependent nitrite reductase (*nrfABC*) (Appendix Table 19).

Alterations in the transcriptome of re-isolate FIM 636 relative to ABU isolate 83972 wt

The most highly upregulated genes in re-isolate FIM 636 were those that make up the entire formic acid metabolism (*frmRAB*) gene cluster (Appendix Table 13). The *frmA* gene encodes for a glutathione-dependent alcohol dehydrogenase which, together with *frmB*, is involved in the metabolism of endogenously formed formaldehyde as well as detoxification of exogenous formaldehyde (Gutheil *et al*., 1997). Interestingly, this alcohol dehydrogenase is also involved in protection against nitrosative stress (Liu *et al*., 2001). Other upregulated genes in re-isolate FIM 636 include those involved in formate dehydrogenase, nitrate reductase, fumarate reductase, P- and F-fimbriae, PTS (mannose) and amino acid biosynthesis. On the contrary, only one gene, a predicted hydrolase was shown to be downregulated in FIM 636 (Appendix Table 20).

5.2.9.3. Common changes in gene expression among the re-isolates

Common alterations in the transcriptome of both re-isolate BU04 63630 and BU05 35364 relative to ABU isolate 83972 wt

Only two genes having similar function, *i.e.* encoding for 50S ribosomal protein were upregulated for both BU04 63630 and BU05 35364 (Appendix Table 14). The other commonly upregulated genes have unrelated functions.

Common alterations in the transcriptome of both re-isolate BU04 63630 and FIM 636 relative to ABU isolate 83972 wt

The microarray expression data showed several genes to be upregulated in both BU04 63630 and FIM 636. Two of these genes are involved in biofilm formation (Appendix Table 15). Noteworthy, however, is that in the biofilm assay, none of the re-isolates were observed to form significantly stronger biofilm compared to ABU isolate 83972 wt. Other genes upregulated in both re-isolates are those that play a role in glucarate transport, as well as 30S and 50S ribosomal protein synthesis.

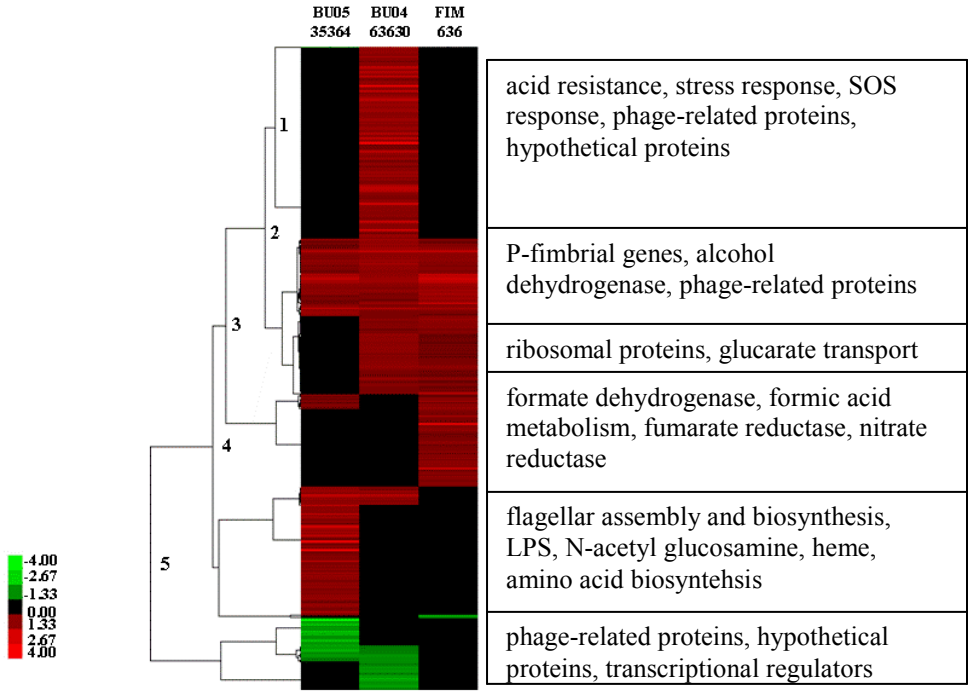

Figure 27. Hierarchical cluster analysis of all de-regulated genes in the motile re-isolates BU05 35364, BU04 63630 and FIM 636 relative to their parent strain ABU isolate 83972 wt. The strains were grown *in vitro* at 37 °C in pooled human urine. Each bar represents one gene and its expression level corresponds to the color bar on the bottom left hand side. Numbers from 1 to 5 indicate five sub-clusters of commonly regulated genes in one re-isolate. The datasets for each strain are mean values of the expression ratio from at least three independent microarray experiments. Genes without statistically significant changes in their expression pattern (p>0.05) are shown in black.

Common alterations in the transcriptome of all three re-isolates relative to ABU isolate 83972 wt

Most of the genes that were commonly upregulated in all three re-isolates have no known function. Only the P-fimbrial gene *papA* was shown to be significantly upregulated in all three re-isolates.

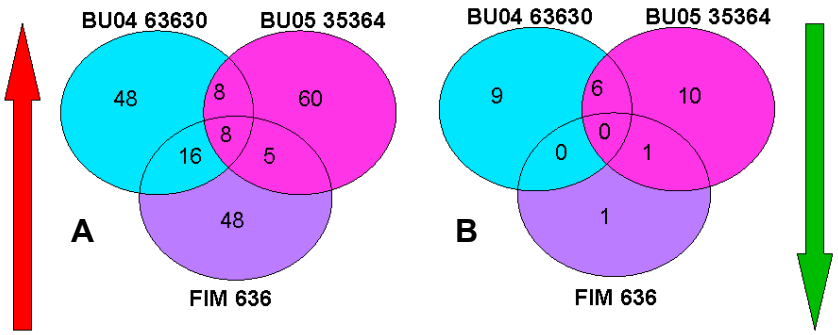

Figure 28. Venn Diagram of De-regulated Genes. (A). Upregulated. (B). Downregulated.

5.2.10. Verification of microarray results by quantitative RT-PCR

To validate the data generated from the microarray experiments, quantitative real time PCR was performed. Six genes (*fliA*, *chuA*, *rfaG*, *frmA*, *narI* and *papA*) shown to be upregulated in any one of the re-isolates were randomly selected and used. Meanwhile, the *rrnB* gene encoding for 16S rRNA was used as positive control.

Based on the results gathered, the only gene with a remarkable increase in expression level was *fliA*, which yielded a 160-fold higher expression in BU05 35364 compared to ABU isolate 83972. In addition, *chuA* and *rfaG* also showed significantly higher expression levels (6- and 5-fold, respectively) for re-isolate BU05 35364. As for the other genes included in the experiments, *frmA* and *narI* showed a 4- and 6-fold increase in expression levels for re-isolate BU04 63630, respectively. It can be noted that *frmA* was significantly upregulated in FIM 636 in the microarray experiments but this was not

confirmed in quantitative RT-PCR. Meanwhile, *papA* was upregulated in both BU05 35364 and FIM 636 (28- and 9-fold respectively) (Figure 29).

Although the increase in expression levels of the genes tested were not very high, in general, the trend of upregulation of gene expression as determined by microarray results could be confirmed by quantitative RT-PCR.

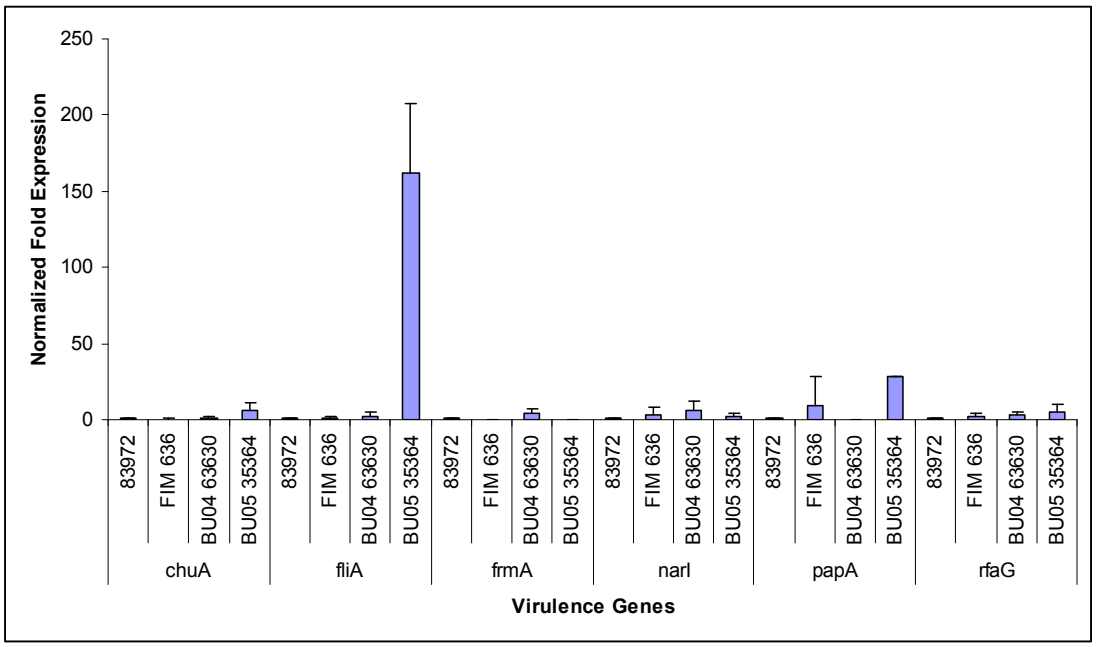

Figure 29. Real Time-PCR-based quantification of transcript levels of selected genes in ABU re-isolates. Relative expression of genes in the re-isolates was normalized to that of parent strain ABU isolate 83972. All experiments were performed in triplicate. Gene expression was standardized using the *rrnB* gene as positive control.

5.2.11. Induction of Inflammatory Markers by the Motile Re-isolates

Levels of IL-6 and IL-8 concentration as well as PMN influx in the bladder were routinely checked at every urine sampling time point to monitor patients enrolled in the colonization study. Patients that reported cases of symptoms showed considerable increase in the concentration of inflammatory markers at the specific time point where episodes of symptoms occurred as compared to time points when they reported no symptoms. Data show that there is a rising trend of inflammatory markers among the re-isolates from the beginning of inoculation to the time point wherein the patients reported to have experienced symptoms. In re-isolate FIM 636, however, there has been an increase in the inflammatory markers 40 days post inoculation which went down again and went up once more 192 days post inoculation (Appendix Figure 47). It was only after 192 days, however, that the patient reported having symptoms like fever of 38 °C and pain in the "lower region" of the urinary tract.

During the sampling point where the patients each reported symptoms, the motile re-isolate BU05 35364 induced very high levels of IL-6 and IL-8 compared to the other re-isolates. FIM 636, on the other hand, had a very high influx of PMN into the bladder. (Figure 30).

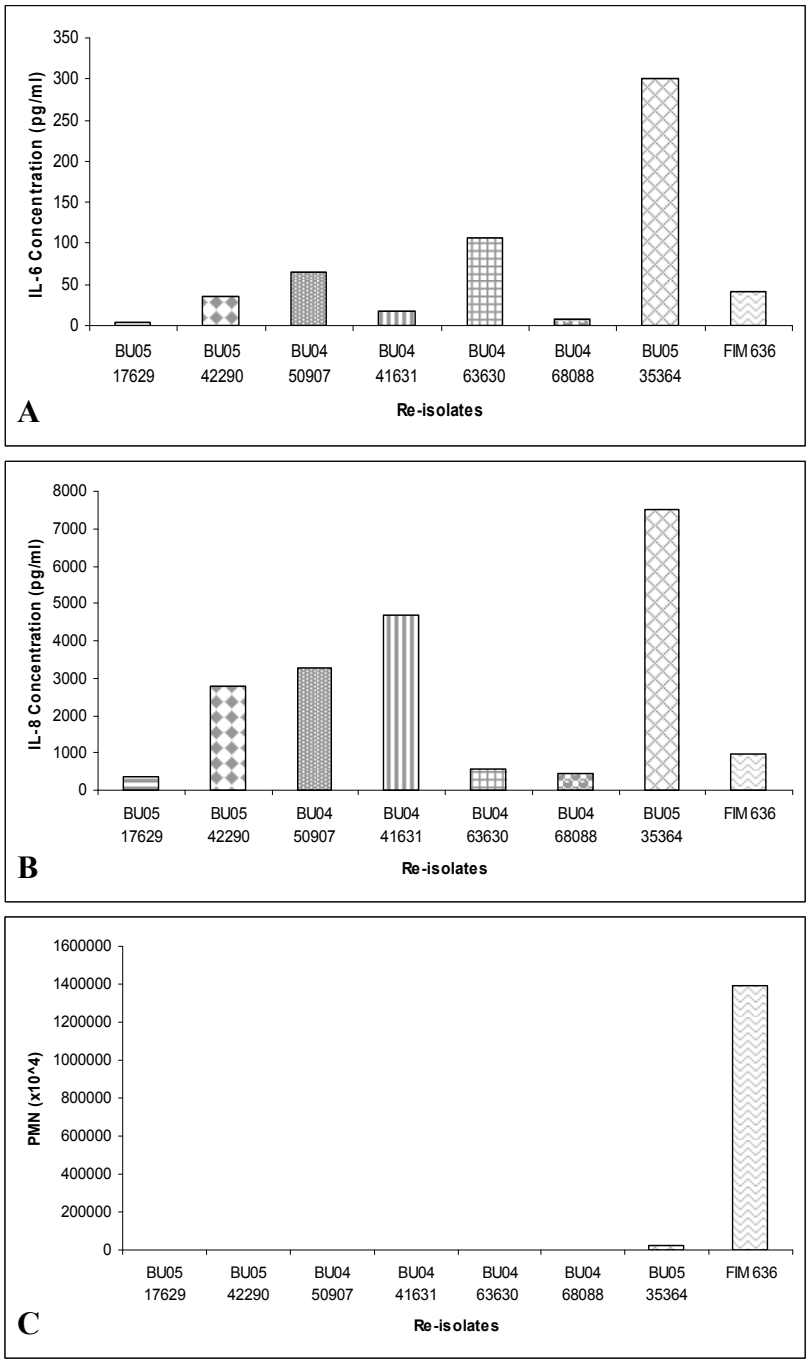

Figure 30. Host response parameters in urine samples collected from patients during episode of symptoms. A.) IL-6 concentration B.) IL-8 concentration C.) PMN influx into the bladder. Data kindly provided by Dr. B. Wullt, Lund, Sweden.

Meanwhile, to determine if there is a link between the marked motility of the re-isolates and the occurrence of symptoms, levels of secreted IL-6 and IL-8 in supernatant of A498 kidney cells co-incubated with the motile re-isolates for 3h were determined by ELISA. A498 kidney cells secrete Toll-like receptor (TLR) 5 which recognizes flagellin, the major protein sub-unit of the flagellum.

Results of the assays showed that there was no considerable difference in the ability of the motile re-isolates to induce host response (*i.e.* IL-6 and IL-8) compared to ABU isolate 83972 wt. Moreover, compared to the positive control *E. coli* Nissle 1917 super swimmer (*i.e.* a hyperflagellated strain), the re-isolates did not show significantly high secretion levels of both IL-6 and IL-8 (Figure 31). In addition, the IL-6 and IL-8 secretion levels generated from the flagellin (*fli*C) deletion mutant of ABU isolate 83972 did not at all vary from the wild type.

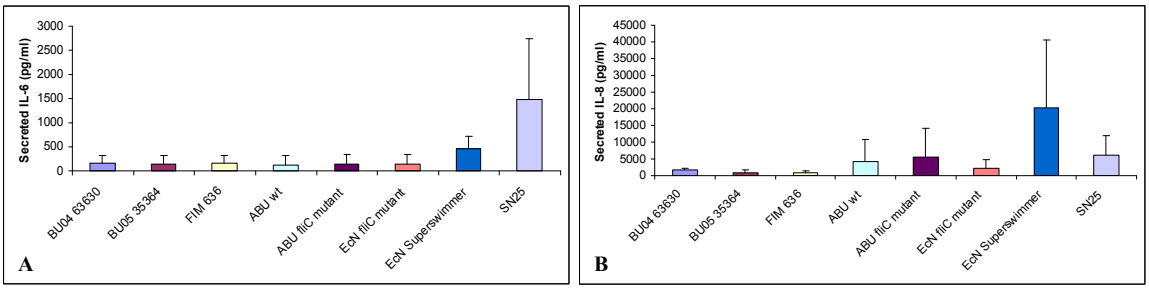

Figure 31. Levels of secreted (A) IL-6 and (B) IL-8 from A498 kidney cell supernatant after 3h co-incubation with re-isolates. The very motile *E. coli* Nissle strain (super swimmer) was used as positive control. SN25 is a motile *E. coli* 83972 re-isolate from a non-symptomatic episode used in another study. Error bars shown are standard deviations of five independent experiments.

In addition to checking the secreted IL-6 and IL-8 levels in cell culture supernatants after co-incubation with the motile re-isolates to see if flagella play a role in induction of immune host response, the transcriptional expression of these inflammatory markers was also checked. qRT-PCR was performed for lysates of A498 cells co-incubated with the motile re-isolate BU05 35364 at 0, 1 and 3h incubation periods. Results show that expression of IL-6 and IL-8 was not significantly increased in re-isolate BU05 35364. Increased expression levels, however, was observed with increased incubation time (Figure 32).

The experiment was performed three times under the same conditions. However, the results generated from the three independent experiments were variable and cannot be reproduced.

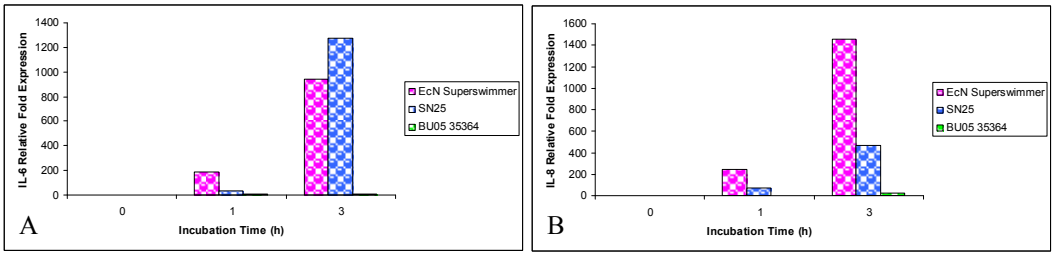

Figure 32. Transcriptional expression of (A) IL-6 and (B) IL-8 in A498 cells after 0, 1, and 3h co-incubation with motile re-isolate BU05 35364. The very motile *E. coli* Nissle strain (super swimmer) was used as positive control. SN25 is a motile *E. coli* 83972 re-isolate from a non-symptomatic episode used in another study. Gene expression was normalized to *E. coli* 83972 and standardized using the *GAPDH* gene as an internal control.

5.3. Reconstitution of a functional papG gene into the *E. coli* 83972 chromosome

P fimbriae are known to improve the establishment of bacteriuria and activate the innate immune response in animal models as well as the human urinary tract (Bergsten *et al.*, 2004; Wullt *et al.*, 2003, 2002, 2001). At the tips of the fimbriae is the PapG adhesin, which recognizes the α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside receptor epitope in the globoseries of glycolipids thus mediating binding (Bock *et al.*, 1985; Leffler and Svanborg-Edén, 1980; Lund *et al.*, 1987). *E. coli* 83972 contains *pap* gene sequences (Hull *et al.*, 1999) but has never been shown to express P fimbriae. However, Klemm *et al.* (2006) observed under transmission electron microscopy that the majority produced fimbriae but did not hemagglutinate human red blood cells (RBCs). In addition, the *pap* gene cluster in *E. coli* 83972 was attenuated by the acquisition of multiple point mutations, and as a consequence, the PapG adhesin was inactivated (Zdziarski *et al.*, 2008). Thus, ABU isolate 83972 is shown to produce P fimbriae but these are unable to bind to the receptor.

An ABU isolate 83972 mutant complemented with the *papGX* gene from the UPEC strain CFT073 was constructed. The *papGX* gene of the wild type was first deleted and

replaced with a *cat* cassette from the pKD3 plasmid. The antibiotic resistance cassette was cured through the plasmid pCP20. PCR and Southern Blot analysis verified the deletion (Figure 33). Following this, the deleted gene was replaced with the *papGX* gene from UPEC strain CFT073. First, the gene was amplified from CFT073 and afterwards introduced into a region of the pKD3 plasmid upstream of the antibiotic resistance *cat* cassette. Successful incorporation of the gene was verified by PCR and sequencing (Figure 34). Next, the *papGX* gene together with the *cat* cassette was amplified from the plasmid giving a 4-kb product. This was then successfully introduced into the chromosome as proven by PCR and DNA sequencing (Figure 35; Table 10). Finally, the functional expression of P-fimbria was tested by agglutination with sheep blood where the ABU isolate 83972 wt was shown to be incapable of agglutinating the blood cells while the constructed mutant (83972Δ*papGX*::CFT073*papGX*_cat) was able to (Figure 36). In addition, the *papG* complemented ABU isolate 83972 strain also adhered better to T24 cells compared to wild type. In fact, it adhered to the cells as much as UPEC strain CFT073 (See Figure 22, Section 5.2.6).

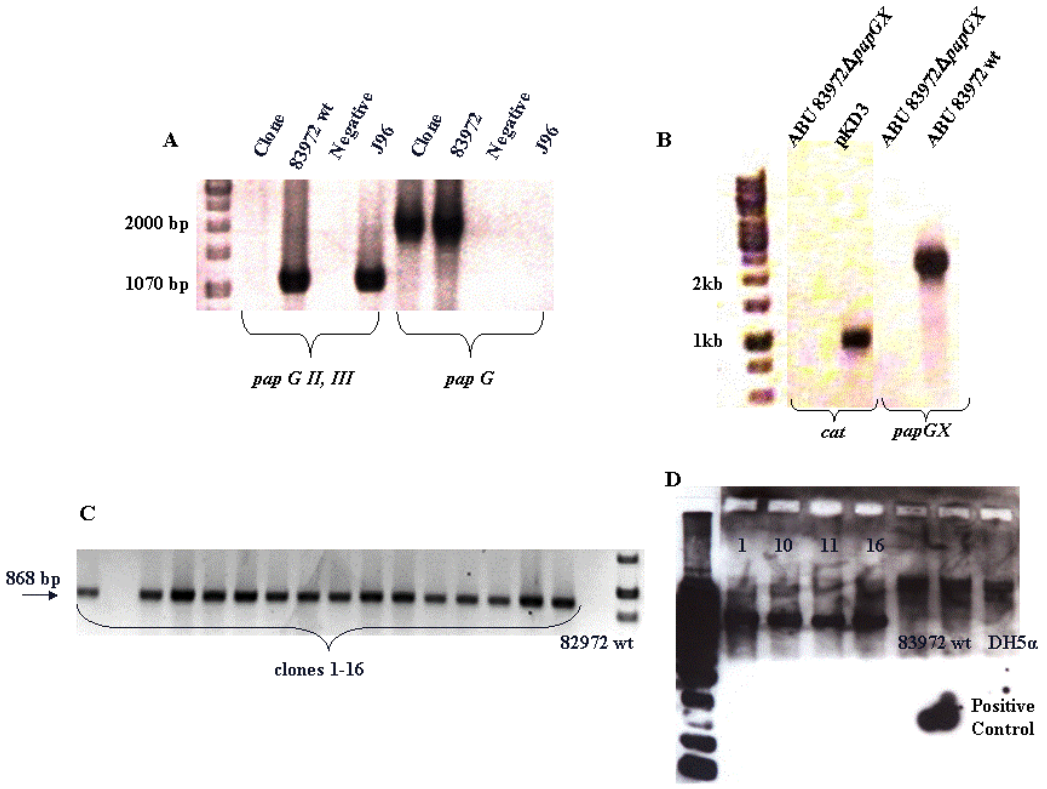

Figure 33. Reconstitution of a functional *papG* gene into the *E. coli* 83972 chromosome. (A)PCR screening for deletion of *papGX* inABU isolate 83972 using primers for the *papGX* gene, (B) curing of *cat* cassette using pCP20 plasmid, verified by PCR, (C) PCR screening for *cat* cassette integration in ABU isolate 83972, (D) Southern Blot analysis to verify the integration of *cat* cassette in ABU isolate 83972.

Figure 34. Introduction of *papGX* gene from CFT073 into the pKD3 plasmid. (A) PCR showing that the *papGX* gene is inserted into the plasmid, (B) PCR showing that *papGX* is inserted upstream of the *cat* cassette in pKD3.

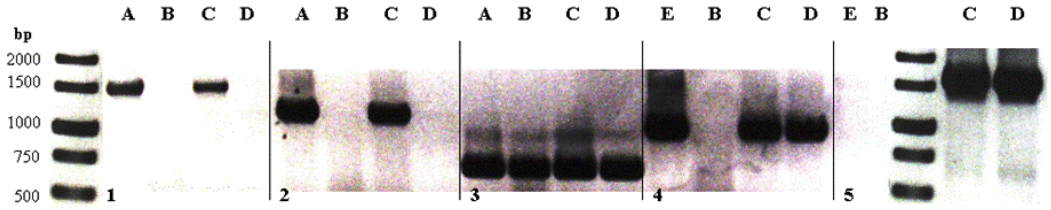

Figure 35. Screening for *E. coli* 83972 wt complemented with *papG* gene from CFT073. (A) CFT073, (B) ABU isolate 83972Δ*papGX*, (C) ABU isolate 83972Δ*papGX*::CFT073*papGX*_cat, (D) *papGX* in pKD3 plasmid, (E) pKD3 plasmid. (1) *papFG,* (2) *papG,* (3) *papX,* (4) *cat,* (5) *cat* and *papX.*

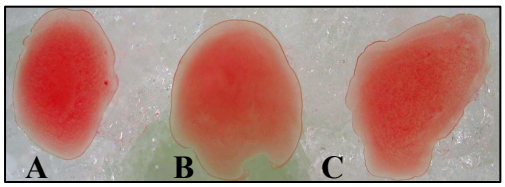

Figure 36. Agglutination assay using sheep blood cells. (A) ABU isolate 83972Δ*papGX*::CFT073*papGX*_cat, (B) *E. coli* 83972 wt, (C) *E. coli* CFT073.

Table 13. Summary of screening for correct reconstitution of the *papGX* gene from UPEC strain CFT073 into the *E. coli* 83972 chromosome

Bacterial Strain	***papFG***		***papG***		***papX***		***cat***		***cat_papX***	
	E	R	E	R	E	R	E	R	E	R
CFT073	+	+	+	+	+	+	_	n/a	_	n/a
ABU isolate 83972Δ*papGX*	_	_	_	_	+	+	_	_	_	_
ABU isolate 83972Δ*papGX*:: CFT073*papGX*_cat	+	+	+	+	+	+	+	+	+	+
papGX in pKD3 plasmid	_	_	_	_	+	+	+	+	+	+
pKD3 plasmid	_	n/a	_	n/a	_	n/a	+	+	_	_

**E- Expected; R- PCR result*

6. Discussion

The main focus of this study was to characterize the various genotypic and phenotypic traits and factors associated with *E. coli* asymptomatic bacteriuria isolates as well as re-isolates from patients stably colonized with *E. coli* 83972 for prophylactic treatment.

6.1. Pheno- and genotypic characterization of ABU isolates and fecal isolates from healthy individuals

6.1.1. Phylogenetic lineage of ABU isolates differs from commensal fecal isolates

Phylogenetic analyses showed that there are four main phylogenetic groups (A, B1, B2 and D) in which *E. coli* strains are classified (Herzer *et al.*, 1990; Selander *et al*., 1986). Virulent extra-intestinal strains belong mainly to group B2 and to a lesser extent, group D. On the contrary, many commensal strains belong to group A (Johnson and Stell, 2000).

ABU patients often carry the strain for long periods of time without producing symptoms, resembling commensalism. One would then expect them to be phylogenetically related to commensals. However, majority of the ABU isolates included in this study belonged to group B2 (Figure 5). This shows that more than half of all the ABU isolates were related to pathogenic *E. coli* since group B2 is from where pathogenic *E. coli* strains are chiefly derived (Johnson and Stell, 2000). A study by Mabbett *et al*. (2008) demonstrated that many ABU isolates are phylogenetically related to UPEC in that most of them belong to group B2. Furthermore, a comparative study between ABU isolates from catheterized versus non-catheterized patients by Watts *et al.* (2010) also showed that the majority of all isolates belong to group B2.

The genetic diversity of the isolates was further explored in this study. Multi-locus sequence typing (MLST) was carried out to examine if the isolates in the collection were

clonally related. MLST showed that ABU isolates belong to multiple clonal groups. In addition, ABU and fecal isolates belong to different clonal lineages but that there really is no strict delineation as to which clonal group they each belong to since there appears to be overlaps in the distribution of ABU and fecal isolates in some clonal complexes (Figure 6). This also indicates that ABU isolates represent a phylogenetically heterogenous group. Two thirds have most likely evolved from classical uropathogenic strains probably by reductive genome evolution while one third resembles commensals according to phylogeny (Zdziarski *et al.*, 2008).

In order to substantiate this genetic diversity, one should look at traits which are common or different among the isolates. Pathogens differ from commensals in that the former have evolved mechanisms that enable them to establish themselves at the site of infection and thereafter due to a cascade of virulence traits are recognized by the host. On the other hand, commensals may share the virulence properties of their pathogenic variants required for initial establishment at the site of infection but lack pathogenicity islands and genes that would trigger host response (Godaly *et al*., 2000; Müller *et al*., 2006).

6.1.2. Genotypic and phenotypic variations

Virulence is the ability of an organism to cause disease in the host. In *E. coli*, virulence results from the cumulative impact of one or several special properties, or virulence factors which serve to distinguish potential pathogens from harmless intestinal strains (Johnson, 1991). The expression of these special properties or virulence factors lies in the virulence genes carried by the organism.

In this study it was shown that all 30 virulence genes included in the PCR were found among the ABU isolates. On the other hand, eight of these genes were not carried by any of the fecal isolates. However, phenotypic assays conducted in this study revealed that there are cases when even if the gene is present in the isolate, it is not expressed. For instance, among ABU isolates, the most predominant gene *fimH*, which encodes the mannose-specific adhesin sub-unit of type 1 fimbriae, is present in most of the isolates,

but not functionally expressed by all of them that harbor the gene. The *fimH* gene is a part of the transcriptional unit *fimAICDFGH* (Korea et al., 2011). It specifically encodes for the adhesin sub-unit FimH, which functions for receptor recognition (Krogfelt, 1990).

Uropathogens are believed to originate predominately from the intestinal tract and later on ascend into the bladder to cause either symptomatic or asymptomatic bacteriuria. This is initially accomplished through bacterial adherence to the host environment (Reid and Sobel, 1987). Adherence is a property used by pathogenic organisms to enable them to attach to host structures and avoid being swept along by the normal flow of fluids and eliminated (Johnson, 1991).

In the case of type 1 fimbriae, successful colonization specifically of the bladder is aided by its ability to adhere to host cells. During urinary tract infection, the hydrodynamic forces brought about by the flow of urine post a challenge to the bacteria in successfully colonizing the bladder and from there establish a niche. It is known that adhesion to mucosal surfaces pave the way for the coming together of bacterial colonies to establish biofilms. Type 1 fimbriae appear to play a role in inter-bacterial binding and biofilm formation in the central parts of the epithelial cell lumen (Melican *et al*., 2011). The FimH adhesin has been shown to be instrumental in biofilm formation by *E. coli* K-12 under both static and hydrodynamic growth conditions *in vitro* (Pratt and Kolter, 1998; Schembri and Klemm, 2001). Furthermore, in a *fimH* deletion mutant, it was also demonstrated that biofilm-forming capacity was significantly reduced.

Type 1 fimbriae are expressed by many Gram-negative bacteria. They facilitate adherence to mucosal surfaces and inflammatory cells *in vitro*. Moreover, they were shown to increase the virulence of *E. coli* in the urinary tract by promoting bacterial persistence and enhancing the inflammatory response to infection. Thus, they are said to contribute to the pathogenesis of *E. coli* in the urinary tract (Connell *et al*., 1996). In addition, subunits of adhesive structures might act not only as adhesins but also as invasins. This is, for example, the case of the FimH adhesin (Bouguénec, 2005). FimH

appears to mediate the bacterial invasion of human bladder cells (Martinez *et al*., 2000; Mulvey *et al*., 2001; Mulvey, 2002).

Type 1 fimbriae are also found to be involved in phase-variation. It has been demonstrated that the virulence characteristic imparted by type 1 fimbriae is dependent on its switching "on" or "off". As demonstrated by Gunther *et al*. (2002), expression of type 1 fimbriae is regulated at the transcriptional level by a promoter situated on an invertible element, which can exist in one of two different orientations. The orientation of the invertible element that allows the expression of type 1 fimbriae is defined as "on," and the opposite orientation, in which no transcription occurs, is defined as "off." They found that UTI strains isolated from cystitis patients switched the invertible element to the "on" position where it remained. Meanwhile, strains from pyelonephritis patients turned the switch "on" early in infection and then tended to turn the switch "off" later. These results suggest that the switching of the invertible element itself contributes significantly to virulence in the urinary tract.

The various abilities conferred by expression of type 1 fimbriae among bacteria then lead to the question as to whether type 1 fimbriae are really a virulence factor or not. To shed light to this question, one has to look at how these fimbriae actually act upon the host cell. Virulence factors are defined as those components of an organism that determine its capacity to cause disease but are not required for its viability per se (Davis *et al*., 1990). They are molecules expressed and secreted by pathogens that enable them to colonize a niche in the host (which includes adhesion to cells), evade the host's immune response, suppress or inhibit the host's immune response, enter into and exit out of cells and obtain nutrition from the host. In addition, virulence factors are very often responsible for causing disease in the host as they inhibit certain host functions. (Peterson J, 1996; Chen *et al*., 2005).

In the case of the model ABU organism *E. coli* 83972, the type 1 fimbriae are non-functional due to a deletion in the *fim* region (Klemm *et al*., 2006). In the same way, more than half of the ABU isolates included in this study also fail to phenotypical express type 1 fimbriae. Since type 1 fimbriae enable the bacteria to colonize the host through

adhesion, it can be counted as a virulence factor. However, in general, since most ABU isolates still fail to express it and use it for this purpose, it probably does not count as a means by which ABU increases its virulence.

In addition to type 1 fimbriae, other genes encoding for virulence factors had been found present among the ABU isolates. Nevertheless, most of these genes are not phenotypically expressed. An earlier study showed that *E. coli* 83972 carry genes of the *pap*, *fim* and *foc* gene clusters, encoding P, type 1 and F1C fimbriae, respectively (Hull *et al.*, 1999). However, it was also demonstrated that it is unable to express functional P and type 1 fimbriae (Klemm *et al.*, 2006). Moreover, molecular epidemiology has shown that >60 % of ABU strains carry virulence genes but fail to express the phenotype (Plos *et al*., 1991, 1995). This implies that the virulence genes found in UPEC are also found among ABU although most of these genes are not functionally expressed and may thus have contributed to the ability of ABU to establish infection but not cause any symptom. The fecal isolates, on the other hand, do not possess all these genes with the toxin gene *cnf1* and fimbrial genes (some *pap* genes, *gafD* and *sfa/focDE)*, as well as *afa/draBC*) not found in any of the tested isolates. This is in affirmation to the finding that urinary isolates including ABU *E. coli* are found to typically have higher inferred virulence compared to fecal isolates from healthy individuals (Blanco *et al*., 1996; Johanson *et al*., 1993). In addition, virulence determinants were rarely detected for fecal isolates and those with virulence factors most frequently belonged to phylogenetic group B2, a rare group among commensal isolates (Duriez *et al*., 2001). According to Russo and Johnson (2000), commensal isolates of *E. coli* lack the specialized virulence traits present among intestinal and extra-intestinal pathogenic *E. coli*. Isolates are defined as extra-intestinal pathogenic *E. coli* through the presence of two or more of a specific subset of virulence genes which includes *papA*, *papC*, *sfa/foc*, *afa/draBC*, *iutA* and *kpSMTII* (Karisik *et al.*, 2007). Thus, it can be said that the difference between commensal isolates and extra-intestinal pathogenic *E. coli* lies in the virulence genes present. From the results, it can be deduced that ABU isolates are more closely related to extra-intestinal pathogenic *E. coli* which includes UPEC with regards to the virulence genes they possess but behave like

commensals in the urinary tract being “asymptomatic”, or in other words, carried by the host symptom-free.

The inability of ABU isolates to express functional virulence-associated determinants may result in successful urinary tract colonization without activation of local and systemic inflammatory response pathways. It was suggested that some ABU strains may arise by the attenuation of UPEC strains as an adaptation to growth in the urinary tract. This implies that the ABU isolates stemmed out from UPEC but due to survival mechanisms have adapted commensal lives (Mabbett *et al.*, 2009; Zdziarski *et al*., 2008).

Bacteria in the urinary tract are said to have originated from the gastrointestinal tract (Reid and Sobel, 1987. In the phylogenetic analyses conducted, it was shown that UPEC and ABU are derived from the same lineage. One could then deduce from here that there appears to be a transition, first of all, from a commensal bacterium in the gastrointestinal tract to a uropathogen in the urinary tract or an asymptomatic bacterium. Nonetheless, it is also possible that the uropathogen that originated from the gastrointestinal tract transformed itself back to a “commensal-like” bacterium in the course of time.

Genome plasticity plays a significant role in persistent infections. Among ABU and other persistent infections, prolonged growth in the host is accompanied by genomic alterations that result in bacterial attenuation which then contributes to bacterial adapatation to their host niche and a reduced activation of immune host responses (Dobrindt *et al*., 2010). For instance, reductive evolution and gene loss are well-known events in persistent UTI caused by UPEC. However, this is not only confined to UPEC but described to other persistent bacterial pathogens such as *Pseudomonas aeruginosa* or *Helicobacter pylori* as well. In the case of *P. aeruginosa*, long-term persistence in the cystic fibrosis (CF) lung is characterized by the selection of a variety of genotypes and phenotypes that typically descend from one infecting *P. aeruginosa* clone, a process known as adaptive radiation (Hogardt and Heesemann, 2010). This infection process is associated with extensive genetic adaptation and microevolution of the infecting bacteria (Jelsbak, 2007; Smith,

2006). A similar event was also observed for *H. pylori* wherein genes may be lost or gained during progression of infection (Oh, 2006).

6.2. Characterization of ABU re-isolates from symptomatic episodes

The eight re-isolates included in this study were taken from patients stably colonized with *E. coli* 83972 wt as prophylactic treatment for recurring UTI who reported episodes of symptoms during the inoculation period. This study aimed to compare re-isolates taken from patients stably colonized with ABU isolate 83972 who reported symptoms. The characterization of phenotypic alterations in *E. coli* 83972 re-isolates from symptomatic episodes may help in the improvement of the use of this strain for prophylaxis of symptomatic UTI.

Results demonstrated that out of the eight re-isolates, only two were not ABU isolate 83972 namely BU05 17629 and BU05 42290 which were found to be *Pseudomonas* and a different *E. coli*, respectively. This shows that both patients from which BU05 17629 and BU05 42290 were isolated most probably had a superinfection. *Pseudomonas* abounds in hospitals and the likelihood of an infection is not improbable. A 3-year study period documented that *Pseudomonas aeruginosa* is a common nosocomial pathogen. The most common sites for *P. aeruginosa* infection were the lower respiratory tract, urinary tract, blood stream, and surgical wounds. Nosocomial *P. aeruginosa* lower respiratory tract and blood stream infections were significantly associated with exposure to certain intensive care units, whereas *P. aeruginosa* urinary tract infections more commonly occurred on the neurology and neurosurgery services (Sherertz and Sarubbi, 1983). Since the patients enrolled in the deliberate colonization studies have frequent hospital visits due to illnesses associated with UTI, it is not unlikely for them to acquire nosocomial infections.

Whether the infection with another microorganism besides *E. coli* 83972 is nosocomial or not, however, is beyond the scope of this study.

6.2.1 Re-isolates are genetopically identical to parent strain E. coli 83972 but some vary in morphology

Even though the re-isolates were samples taken from patients that reported episodes of symptoms, their genomic content does not vary from the parent strain ABU isolate 83972. As revealed by the results of the genotypic tests, all investigated virulence-associated genes of uropathogenic *Escherichia coli* (UPEC) found in parent strain 83972 were also found among the re-isolates confirmed to be ABU isolate 83972 (Section 5.2.5). Furthermore, the DNA banding patterns of all the re-isolates were identical to ABU isolate 83972 (Figure 23) which confirms that there is no alteration at all which took place among the re-isolates relative to their parent strain (*i.e.* that they are still, in essence ABU isolate 83972).

6.2.1.1. Small colony variants (SCV)

Although the re-isolates showed to be completely identical to ABU isolate 83972 genotype-wise, deviation of one re-isolate from the typical morphology of ABU isolate 83972 wt was observed. Five out of 20 colonies from BU04 50907 showed small, atypical growth morphology in all media used. They grew transparent on chromogenic agar instead of the typical pink color indicating the ability to synthesize β-galactosidase, an enzyme produced by *E. coli*. However, 16S rRNA sequencing identified all five colonies to be *E. coli.* Furthermore, they carry the cryptic plasmid, have the *fim* deletion and showed a 100 % similar restriction pattern as ABU isolate 83972 in the PFGE. They also share 100 % of the UPEC-associated genes of ABU isolate 83972 wt. Interestingly, the unusual morphological characteristic of these colonies could be likened to small colony variants (SCV). SCVs are certain variants of bacteria that grow slowly on routine media and yield unexpectedly small colonies in comparison to the normally growing parent strains (Roggenkamp *et al*., 1998). Minute transparent colonies, then referred to as "dwarf-colony mutants" were isolated from the urine of a patient with an asymptomatic urinary infection (Borderon *et al.*, 1978). Furthermore, 16 metabolically deficient dwarf mutants of *E. coli* were isolated from urine culture and it appeared that the mutants were the true cause of urinary tract infection since there was leukocyturia and important

bacteriuria were obtained in pure culture (Borderon and Horodniceanu, 1978). It can then be deduced that SVCs could be isolated from urine samples of typical UTI patients and even those with ABU. However, whether the morphological alteration carries alongside it genotypic changes leading to the occurrence of symptoms among patients carrying ABU remains unclear.

6.2.2. Expression of virulence traits and host-response

Virulence traits in bacteria are known to induce several immune responses in the host that aid in bacterial clearance. Although the phenotypic assays showed that there were several virulence characteristics expressed by the re-isolates, most of them cannot be taken in the general context since they were not commonly expressed among all re-isolates. In fact, the only marked virulence factor common in at least three of the re-isolates is motility. The question then is whether bacterial factors (*i.e*. changes in the expression of virulence traits) induced the host-response among the patients who reported episodes of symptoms or that changes within the hosts themselves prompted the bacteria to undergo certain alterations in behavior and expression of virulence traits in order to cope up with the changes in their living environment.

6.2.2.1. Bacterial factors involved in symptomatic episodes

The establishment of symptomatic UTI by UPEC is enhanced by virulence factors such as adhesins (e.g. type1 and P fimbriae) and toxins (e.g. hemolysin) (Brzuszkiewicz *et al.*, 2006; Klemm and Schembri, 2000; Oelschlaeger *et al*., 2002). The ability of *E. coli* 83972, the best characterized ABU *E. coli* to date, to colonize the host without triggering immune response is owed to the fact that its virulence-associated adhesin genes have become attenuated giving rise to poor immune detection by the host (Klemm *et al.*, 2006). In addition, *E. coli* strains isolated from pyelonephritis show virulence factors more frequently than those from cystitis and asymptomatic bacteriuria (Blanco *et al*., 1996). Moreover, Zdziarski *et al*. (2008) suggested that some ABU isolates arise from virulent strains by attenuation of virulence genes while others are non-virulent and resemble commensal strains.

Meanwhile, results of this study showed that the various re-isolates possess various virulence characteristics. Based on the PCR conducted, all six re-isolates confirmed to be *E. coli* 83972 share 100 % similarity to ABU isolate 83972 wt with regards to presence of UPEC-associated virulence genes. In addition, the genomic restriction pattern did not show any difference between the re-isolates and the parent strain. However, the marked differences lie in the phenotypic expression of the virulence traits among them.

Primarily, as mentioned previously, assessment of swarming motility revealed three re-isolates to be motile (Figure 20; Table 12). However, among these three re-isolates, only BU05 35364 was completely motile. The other two were a mixture of motile and non-motile clones. This finding is in agreement with the result of the microarray analysis. Among the three re-isolates, upregulation of a cascade of genes involved in flagellar assembly and biosynthesis was observed only in re-isolate BU05 35364 (Figure 27). This was further confirmed by the results of qRT-PCR wherein only this re-isolate among all motile re-isolates showed a significantly high expression of the *fliA* gene involved in flagellar biosynthesis (Figure 29).

It has been suggested that flagella may be required for biofilm formation. Motility facilitated by flagella might be necessary to bring cells in close proximity with each other and the surface to which they will attach (Dunne, 2002; Pratt and Kolter, 1998). In addition, flagella, together with outer membrane proteins and fimbriae are required for initial bacterial adhesion during biofilm formation (Landini and Zehnder, 2002). However, c-di-GMP also plays a role in the expression of flagella and biofilm formation. Intracellular c-di-GMP positively regulates phenotypes such as sessility, biofilm formation, and expression of adhesive extracellular matrix (ECM) components. On the other hand, it negatively influences phenotypes such as motility and virulence in bacteria (Lee *et al*., 2010). This shows that motility and biofilm formation are diametrically affected by c-di-GMP and are thus mutually exclusive. This was observed among the motile re-isolates as none of them could form significantly stronger biofilm compared to their parent strain.

In fact, together with non-motile re-isolates BU04 50907-1 and 11, BU04 68088, motile re-isolates BU05 35364 and FIM 636-4 formed even significantly less biofilm compared to their parent strain. Biofilms may be important virulence factors (Mendez-Arancibia, 2008). Hancock *et al*. (2007) reported that ABU isolate 83972 has the ability to form stronger biofilms compared to UPEC and proposed that this is a favorable strategy for a successful ABU lifestyle. Biofilms provide a protected environment for survival under hostile conditions. They are composed of cells embedded within a complex glycocalyx-like matrix. Biofilms protect pathogens from attack by the immune system. This complicates chronic infections that are difficult to eliminate with antibiotic therapy. Bacterial biofilm infections are up to 1000-fold more resistant to antibiotics, biocides and hydrodynamic shear forces because sessile bacteria can withstand host immune responses more than their planktonic counterparts (Costerton *et al.*, 1995, 1999; Donlan and Costerton, 2002).

However, since there is no increased biofilm formation among any of the re-isolates, it seems to be the opposite case for the re-isolates. Instead of being protected from incurring host response from the host, it is probable that by having a diminished ability to form biofilm, these weak biofilm-forming re-isolates may have enabled recognition by the host and reduced their fitness.

Secondly, motility is another major role of flagella (Pratt and Kolter, 1998). Although flagellar expression was shown in the Western Blot analyses conducted, microarray results revealed that only the homogeneously motile re-isolate BU05 35364 have upregulated flagella-related genes (Figure 21; Figure 27). In addition to its role in motility and biofilm formation, flagella are also considered to be contributors to virulence of UPEC (Wright *et al*., 2005). In 2005, Wright *et al*. hypothesized that they may contribute to UPEC virulence during urinary tract infection since they have putative roles in adherence, ascension, biofilm formation and dispersal. Moreover, they concluded that flagella enhance the fitness of UPEC throughout the urinary tract. Flagellum-driven motility allows bacteria to disseminate to sites more advantageous for colonization (Simms and Mobley, 2008). In a study by Lane *et al*. (2007), it was demonstrated that

flagella contribute to efficient colonization of the urinary tract. Hence, it can be deduced that increased motility and upregulated flagellar gene expression may have brought about an increased fitness quality to re-isolate BU05 35364.

Since flagella are considered virulence factors, they are able to induce host response. It has been reported by Zhou *et al*. (2003) that FliC, the flagellin of enteropathogenic *E. coli* (EPEC) stimulates interleukin-8 (IL8) production and release in T84 cells. Data of pro-inflammatory cytokine secretion among the re-isolates showed that there was no significant increase in expression levels of IL-6 and IL-8 among the motile re-isolates. In addition, the motile re-isolates were also not able to adhere better to bladder epithelial cells compared to the other re-isolates as well as the parent strain. (Figure 22; Figure 31). These results show that the induction of symptoms of UTI in the formerly asymptomatically colonized patients cannot be attributed to the presence of flagella in that it does not play a role in adhesion, biofilm formation nor increased host response. Flagella, however, are not only considered as virulence factors but also fitness factors. Since the motile re-isolates were confirmed to be flagellated, the expression of flagella is possibly a kind of adaptation to enable them to become more fit in the host environment. It was already reported by Zdziarski *et al*. (2010) that different host appear to personalize their microflora in that re-isolates taken from different patients showed a distinct pattern of genetic alterations in addition to random changes. In order for the bacteria to survive the selective host environment, they use various mechanisms to for adaptation.

It is interesting to note that the re-isolates taken from urine samples of patients were heterogeneous specifically when it comes to motility. Among the six re-isolates confirmed to be ABU 83972, three showed increased motility compared to the parent strain (Figure 20; Figure 21; Tables 8-10). Still, however, there is heterogeneity also among clones from the same re-isolate. For instance, re-isolate FIM 636 was observed to be a mixture of motile and non-motile clones and the degree of motility observed among clones which were motile also varied. This shows that it is not only the patients which tend to contribute to the variability of the re-isolates but that the clones within each re-isolate are in themselves also variable.

Third, BU04 63630 (clones 1 and 14) was also observed to be incapable of lysing the K5 bacteriophage lysate, indicative of inability to express K5 capsule. Capsules inhibit the opsonizing activities of complement, decreasing the elimination by phagocytes, or escape the immune response by mimicking host molecules thereby increasing bacterial virulence (Bayer *et al*., 1994; Boulnois *et al*., 1990; Cross *et al*., 1990). The *E. coli* K1 and K5 capsules are frequently found among isolates causing extraintestinal infections. K1 is more commonly associated with neonatal septicemia and K5 with sepsis and urinary tract infection (Cross *et al*., 1990). *E. coli* 83972 harbors the *kpsMT*K5 genes and is able to express K5 capsule which is a group 2 type capsule. In the same way, all the re-isolates also do except for BU04 63630 (clones 1 and 14) and BU05 35364. This particular change in capsular expression may be contributory to altered and increased host response. According to Clarke *et al*. (2000), expression of a polysaccharide capsule confers resistance to host immune defences and other adverse environmental conditions. Therefore, the absence of a functional K5 capsule in BU04 63630 (clones 1 and 14) and BU05 35364 could have triggered the host immune system to react and produce symptoms. The results showing that these two re-isolates do not express K5 capsule is in line with the findings of Valle *et al*. (2006) that group 2 capsules modulate bacterial adhesion and prevent biofilm formation in both gram-negative and gram-positive bacteria. Strains that express group 2 capsules exhibit antibiofilm activity. The capsular polysaccharide K-antigen, which is a surface-exposed polysaccharide polymer, plays an indirect role in biofilms by shielding of bacterial surface adhesin. The physicochemical properties displayed by the group 2 capsules might sharply alter bacterial ability to interact with surfaces and therefore drastically reduce adhesion. The notion that biofilm formation is evident among those that do not form group 2 capsules is true in re-isolate BU04 63630 (clones 1 and 14). This re-isolate was found to not express K5 capsule but formed biofilm.

Furthermore, a cascade of genes involved in LPS biosynthesis was also upregulated in BU05 35364. LPS is a known virulence factor. The connection between flagella and LPS was shown in a study by Parker *et al*. (1992). They found out that flagella formation is impaired in LPS-deficient strains which means that in order for flagella formation to

occur, LPS should be expressed. In this sense, it is true that there is upregulation of several flagella-related genes as well as those involved in LPS biosynthesis in the motile re-isolate BU05 35364. However, no increase in the expression of LPS was observed in this re-isolate compared to the rest, as well as the parent strain. In addition, genes involved in the production of another extracellular polysaccharide, N-acetyl glucosamine, were also upregulated in BU05 35364 which was also able to express it based on immunoblot assay. Poly-GlcNAc expression has been hypothesized to correlate with urovirulence (Cerca *et al.*, 2007).

It is known that exopolysaccharides such as the polysaccharide poly-*N*-acetylglucosamine also known as intercellular adhesin (PIA) play a crucial role in the formation of biofilms and biofilm resistance to antimicrobials and innate host defense (Vuong and Otto, 2008). The motile re-isolate BU05 35364 was found to be the only re-isolate capable of expressing PIA. However, even if flagella and PIA are associated with biofilm formation, BU05 35364 is not a stronger biofilm former compared to ABU isolate 83972 wt. According to Hall-Stoodley *et al.* (2004), strains with PIA might still fail to form biofilms if they are defective in initial adherence.

Finally, an upregulation of six P-fimbrial genes in FIM 636 was observed. ABU isolate 83972, although expressing P-fimbriae, fails to hemagglutinate human red blood cells (RBCs), bind to human uroepithelial cells as well as Galα1-4-Galβ-containing glycolipids (Klemm *et al.*, 2006). This means that even if P-fimbriae are expressed, they are non-functional. Although an increased transcriptional expression of *papA,* one of the genes in the *pap* operon in re-isolates BU05 35364 and FIM 636 was confirmed by qRT-PCR, the fact remains that their P-fimbriae are still non-functional as shown by hemagglutination assays.

Bacteria have the ability to adapt to changes in their environment. Dalhoff *et al.* (1985) stated that some characteristics of bacteria growing in an infected host may differ markedly from those growing in vitro. It has been shown that when grown under different growth conditions (*i.e. in vivo* and *in vitro*), *Staphylococcus auerus* grown *in vivo*

possessed enhanced virulence compared with their counterparts grown in vitro when injected intradermally into sheep, intraperitoneally into mice or infused into lactating mammary glands of ewes (Watson, 1982). This implies that bacteria grown *in vivo* differ in their pathogenicity from their counterparts grown *in vitro*. Dalhoff *et al*. (1985) further stated that bacteria frequently exhibit such a pronounced adaptive plasticity to a changing growth environment that the new characteristics acquired during growth *in vivo* are rapidly lost upon subculture *in vitro*. For instance, *E. coli* sequentially isolated from untreated patients suffering from asymptomatic bacteriuria have shown consecutive changes with respect to their serum sensitivity and spontaneous agglutinability (Lindberg, *et al.,* 1975). In addition, in another study, Ljungh & Wadstrom (1983) described that approximately 20% of the bacteria originally isolated from midstream urine samples were not fimbriated whereas after serial transfers under static conditions all isolates expressed fimbriae.

It would be good to take note that in this study, the bacterial isolates tested were re-isolated from urine samples of patients deliberately colonized with the parent isolates *E. coli* 83972 wt. Once they were re-isolated, they were then grown either in pooled human urine or LB broth *in vitro* for all experimental procedures conducted. It is then possible that there may be differences in the actual behavior and characteristics of the bacteria residing in the bladder of the patients compared to when they were re-isolated and grown *in vitro* for the experiments.

6.2.3.1. Host factors involved in symptomatic episodes

When there is a change in the environment and bacteria undergo stress, they respond by changing the pattern of gene expression. They either activate a set of genes necessary for survival or turn off genes which are not needed at a certain time. Often, this is accompanied by induction of virulence factors (Chowdhury *et al.*, 1996).

An example of stress encountered by bacteria is DNA lesion or damage. If there are lesions in the DNA, DNA replication is inhibited. This in turn induces the SOS response.

During SOS response, there is an increased expression of about 20 genes aimed at restoring the capacity of the chromosome to replicate. The synthesized proteins are induced at various time points. For instance, after the expression of *lexA*, an SOS repressor gene, *ruvB* and *dinI,* along with some other genes, the *recA* and *recB* genes which are involved in recombinatorial repair are known to be expressed next. Following this, translesion DNA synthesis takes place and *umuD* encoding an error-prone DNA polymerase as well as *sfiA* (also known as *sulA*) encoding a cell division inhibitor are two of the genes which are then expressed (Kuzminov, 1999). All aforementioned genes were shown to be upregulated in re-isolate BU04 63630 which implies that the re-isolate has encountered DNA lesion or damage which brought about the induction of the SOS response regulon.

E. coli, a neutrophilic bacterium, can survive extreme acid (pH <2.5) stress for several hours (Castanie-Cornet *et al*., 2010). There are several acid resistance systems in *E. coli*. One acid response system designated as the oxidative or glucose-repressed system was reported to require the alternative sigma factor RpOS (Castanie-Cornet *et al*., 1999). The *rpoS* gene encodes the alternative sigma factor σ^S, a subunit of RNA polymerase that acts as the master regulator of the general stress response in *E. coli*. In the microarray analysis, the *rpoS* gene was observed to be upregulated together with *rpoA* and *rpoE* in BU04 63630. It was reported that many gene products regulated by *rpoS* protect *E. coli* from adverse environmental conditions such as starvation, hyperosmolarity, oxidative damage, and UV radiation (Small *et al*., 1994). In addition to RNA polymerase sigma factor genes, the *gadA* and *gadB* genes were also shown to be upregulated in BU04 63630. These genes are involved in another acid resistance system based on glutamate decarboxylation by the GadA and GadB decarboxylases and the import of glutamate via the GadC membrane protein (Castanie-Cornet *et al*., 2010; Foster 2004). It is interesting to note that aside from these, other genes involved in resistance to low pH such as *hdeA* and *hdeB* were also upregulated in the same re-isolate. HdeA supports acid resistance in pathogenic enteric bacteria (Gajiwala and Burley, 2000). This implies that re-isolate BU04 63630 has undergone a drastic change in environmental pH.

The normal physiological range of urine pH values ranges from 4.8 to 7.4. In 2003, Cahill *et al.* showed that urine becomes alkalinized in its passage along the lower urinary tract. Urine acidification relative to that of arterial blood occurs initially as urine passes along the kidney nephron, such that renal pelvic urine is more acidic than that of the proximal tubule. Although urine has mainly an acidic pH and *E. coli* is a neutrophilic bacterium, the upregulation of genes for resistance to low pH suggests a change in the environmental milieu of the bacterial re-isolate to a more acidic one which it has to cope up with.

In addition to coping up with stress in the environment, bacteria also need to defy changes in the normally-occurring levels of toxins within the host. The *frmRAB* operon, shown to be upregulated in FIM 636, is induced by formaldehyde. In 2004, Herring and Blattner, demonstrated that the level of *frmR* transcript was induced by formaldehyde 215-fold over the level of non-induced cells. They suggested that it is most likely that the *frmRAB* operon encodes a complete pathway for degradation of formaldehyde produced endogenously as a by-product of demethylation reactions. In addition to these genes, a formate dehydrogenase transcript was also upregulated. Formate dehydrogenase catalyzes the oxidation of formate, a salt or ester of formic acid, to bicarbonate. Formic acid is the oxidizing product of formaldehyde while exposed to air. Formaldehyde can occur in very small amounts in the body. However, upregulation of genes related to formaldehyde in FIM 636 relative to ABU isolate 83972 suggests that there was probable increase in the levels of formaldehyde in the host to which the bacteria had been exposed.

Interestingly, aside from the upregulation of genes related to formic acid metabolism and formate dehydrogenase, genes related to nitrate reductase were also upregulated in FIM 636. Bacterial nitrate reductase reduces nitrates to nitrite (Lundberg *et al*., 2004). According to Stewart (1988), in the absence of oxygen, there is induction of respiratory nitrate reductase. *E. coli* is a member of the family *Enterobacteriaceae*, of which all members are facultative anaerobes (Brenner, 1984. In: Stewart, 1988). Facultative anaerobic bacteria use nitrate as an alternative electron acceptor to oxygen under hypoxic conditions (Lundberg *et al*., 2004). That means, they grow well in the absence of oxygen

when fermentable substrates are available. Moreover, they also grow with oxidizable substrates when environmental conditions allow for respiration. All enteric bacteria respire oxygen, nitrate, and fumarate, and most species can use other terminal electron acceptors as well (Brenner, 1984. In: Stewart, 1988).

As early as 1962, Itagaki *et al*. (In: Stewart, 1988) already showed that formate dehydrogenase is induced during anaerobic growth on nitrate, reinforcing the idea that formate is an important electron donor for nitrate reduction.

Taken together, since there is a unique pattern of phenotypic traits expressed by the re-isolates as well as the transcriptional profile of the three motile re-isolates, it can be speculated that the expression of virulence factors and other factors such as stress-resistance among the re-isolates could be attributed to the involvement of response mechanisms of the hosts and not only on the characteristics of the bacteria. It might very well be that the different hosts (*i.e.* patients included in the colonization studies) have different ways of responding to the introduction of ABU isolate 83972 into their system. Although ABU are said to be asymptomatic, they are still able to induce host response. There appears to be an increased chemokine production as well as neutrophil influx at the site of infection in ABU infections (Haraoka *et al*., 1999; Samuelsson *et al*., 2004). In this study, IL-6 and IL-8 levels were not significantly increased *in vitro* in A498 kidney cells upon inoculation with the motile re-isolates. However, the general trend of increase in the levels of these inflammatory markers was observed as incubation time increases (Figure 32). It can therefore be deduced that the longer time the bacteria remains in the host could lead to increased detection and response.

Moreover, even if the colonization studies have been going on for years and the patients have been stably colonized with ABU isolate 83972 without triggering any sign of infection, there may have been changes in the general-well being of the patients during the course of time (*i.e.* older age and possibly, physical deterioration and decrease in potency) which increased their susceptibility to even the slightest environmental stressor which aided the immune system to recognize ABU isolate 83972. This in turn, might

have paved the way for the bacteria to counter-act the changes in the host by undergoing changes in the expression of their own self-protecting factors.

6.3. Functional papG gene in the E. coli 83972 Chromosome

Genes of the P-fimbrial operon of ABU isolate 83972 are highly identical to UPEC strain CFT073 with the exception of *papA*, *papE* and *papG*, showing the greatest divergence (Klemm *et al.*, 2006). The PapG adhesin, located at the tips of the fimbriae, recognizes the α-d-galactopyranosyl-(1-4)-β-d-galactopyranoside receptor epitope in the globoseries of glycolipids and mediates binding (Leffler *et al.*, 1980; Lindberg *et al*., 1987). However, in ABU isolate 83972, although P-fimbria is expressed, it fails to hemagglutinate human red blood cells (RBCs), bind to human uroepithelial cells as well as Galα1-4Galβ-containing glycolipids (Klemm *et al.*, 2006).

In 2004, Bergsten *et al*. selected a strain that normally causes ABU and transformed it with a fully functional *pap* operon or a mutated, *papG*-deficient sequence. Their results demonstrated that PapG mediated adherence activates a mucosal host response.

In this study, the non-functional *papG* gene of ABU isolate 83972 wt strain was complemented with a functional *papG* gene from UPEC strain CFT073. Results showed that upon complementation, the non-functional P-fimbria of ABU isolate 83972 has restored its function. The strain which does not have the ability to hemagglutinate sheep blood cells was able to do so upon complementation (Figure 36). In addition, the complemented strain could adhere better to T24 bladder cells compared to the wild type strain (Figure 22). This just shows the significant role played by the *papG* gene in the functionality of P-fimbria in *E. coli* 83972.

6.4. Implications and Outlook

This study tried to address questions about the virulence traits and mechanisms present in ABU isolates. It tried to look at whether ABU isolates which mostly belong to the same phylogenetic group as UPEC strain CFT073 but behave like commensals have more similarities with commensal *E. coli* fecal isolates or if they have the tendency to harbor and express virulence traits in a similar fashion known among ExPEC strains. Using isolates from *in vivo* growth conditions, results of this study showed that ABU isolates generally possess virulence genes typically found among ExPEC strains but that they are mostly not expressed in the isolates. This verifies other researches already conducted on the virulence properties of ABU isolates (Watts *et al*., 2010; Mabbett *et al*., 2008; Hull *et al*., 1998). In these researches, ABU isolates were shown to possess genes, which are known to contribute to bacterial virulence. However, in this study, apart from looking at the virulence characteristics of ABU isolates alone, we also probed into the fact that since ABU isolates do not elicit symptoms on the part of the host, they therefore resemble commensals. It was already shown that some ABU strains arise from virulent strains by attenuation of virulence genes while others are non-virulent and resemble commensal strains. Through this finding it was proposed that virulence attenuation might constitute a general mechanism for mucosal pathogens to evolve toward commensalism (Zdziarski *et al*., 2008). We tried to look at the relatedness of ABU isolates to commensal fecal strains with regards to virulence properties. As a result, we were able to see that there is indeed not much significant difference between ABU and fecal isolates with regards to the virulence genes that they harbor and express.

Next, this study was able to show that some of the re-isolates taken from patients stably colonized with *E. coli* 83972 that experienced episodes of symptoms were able to express certain virulence-related traits not expressed by their parent strain. The fact that these virulence-related strains were expressed variably from one re-isolate to another suggests that the changes in the expression of these traits in the bacteria is not dependent on the changes that the bacteria could acquire over time from its environment but rather, traits acquired through host-driven and host-dependent factors. Changes in the host

environment might have required the bacteria to acquire certain traits that will enable them to be more fit in thriving within the host environment. Zdziarski *et al*. (2010) already reported that some of the adaptation mechanisms involved in the changes within the bacteria appear to be unique for each host, suggesting that the genomic identity of a bacterial isolate is flexible and relevant in a given host niche.

Most prominent among the virulence traits in the re-isolates is motility brought about by the flagella. It could be deduced that since motility is the only common virulence trait expressed in at least three of the re-isolates, the expression of flagella could have contributed to the induction of host response. However since the motile re-isolates, specifically BU05 35364, failed to adhere to host cells or even increase the levels of inflammatory markers secreted and expressed by the host cells show that motility among these re-isolates is most probably not a virulence trait at all. In contrast, it a means by which the bacteria are able to become more fit for survival in the host environment.

Finally, this study also showed that the reconstitution of the non-functional *papG* gene of *E. coli* 83972 with the *papG* gene of UPEC strain CFT073 was able to restore the functionality of the P-fimbria in that it was able to agglutinate sheep blood cells and adhere better to host cells. This shows that expression of the fimbrial adhesin alone could possibly lead to a change from an asymptomatic strain to one that could induce host response.

In summary, this work on the characterization of asymptomatic bacteriuria *E. coli* isolates could show the main similarities and differences between ABU and commensal fecal isolates, the significance of virulence traits expression and how they impact host-bacterium interactions, and whether or not these virulence traits are used by the bacterium for their benefit or that these may pose a threat to their detection by the host's immune system.

In conclusion, in order to better understand the role played by the host in the changes of virulence traits expression and other factors among the re-isolates, it would be useful to

do functional gene analysis in the future. In addition, it would be interesting to analyze the gene expression pattern initiated by the bacteria in the host (*i.e.* either *in vivo* via a mouse model or *in vitro* through cell culture experiments).

7. References

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8. Appendices

8.1. Legends to figures and tables

Figures:

Tables:

8.2. Detailed methodological approaches

Appendix 8.2.1. Agarose Gel Electrophoresis

In running PCR products from multiplex reactions, a gel concentration of 2 % was used. For other PCRs performed, 1% gel was used.

Procedure:

1. An appropriate amount of agarose was dissolved in 1x TAE buffer by boiling in an Erlenmeyer flask in a microwave oven and cooled.
2. The gel was casted when the Erlenmeyer flask was cool enough to be handled by pouring the gel to a gel mold.
3. When the gel has hardened, it was transferred to an electrophoresis machine. The samples were loaded on the slots.
4. The gel was run at 140 volts for 45 minutes.
5. After the run, the gel was stained in ethidium bromide after which a photograph was taken to visualize the bands.

Appendix 8.2 2. Pulsed Field Gel Electrophoresis

Procedure:

1. 4 ml (2x 2 ml) of an overnight culture was centrifuged at 13000 rpm, 2 min, RT
2. They were then washed 2x with 1 ml SE buffer (Note: Vortexed after the first wash and re-suspended with a pipette upon the second wash.) OD_{600}= 0.6-0.8 (proper dilutions were made when necessary).
3. The metal molds for the gel were prepared. (They were cooled at 4 °C prior to pouring of the gel).
4. 900 µl bacterial suspension was mixed with 0.9 ml 2 % low melting agarose gel dissolved in LGT Buffer.

5. The mixture was pipetted into the metal mould.
6. Once the cast hardened, half of the whole gel was placed into a 15 ml Greiner tube with 2.5 ml NDS solution. The gel was sliced into smaller pieces.
7. These were incubated overnight in a 50 °C water bath.
8. The following day, they were washed with TE Buffer every two hours and once a day everyday thereafter for 1-2 weeks.
9. The plugs were stored at 4 °C until use.
10. ***Restriction.*** Two pieces of agar plugs (cut further into about 1-3 mm size) were incubated in 1 ml NDE buffer (*Vorpuffer*) for 1 h at 50 °C. The buffer was warmed at the same temperature prior to this because it was stored at -20 °C.
11. Afterwards, the following components were used for restriction digestion at 37 °C for 1 h:

    1 x restriction buffer (*Spaltpuffer*), 15 µl in a 150 µl reaction mix
    1 µl enzyme (*XbaI* or *AvrII*)
    134 µl water

12. The agar plugs were loaded on gel and electrophoresis was done for 20h using 0.5 x TBE + urea (1 M, 100 µl/L) and 1% agarose gel.

    Program: Block 1, Initial time (5), Final time (50), Run time (20)
    Blocks 2 and 3 must be set at 0

Appendix 8.2.3. Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins on a gel based on their molecular size. SDS is a detergent with a C-12 chain containing an ionogen sulfo-group. When β-mercapthoethanol is added to the protein samples and heated, the S=S bonds between sub-units in the proteins are broken and the proteins loose their tertiary structures.

SDS-PAGE needs the following materials:

1x Running buffer

12 % Running gel

Stacking gel

Molecular marker

1. Preparation of the Gel

Procedure:

1. Two well-fitted glass plates were cleaned with ethanol to make one gel. The glass plates were clamped in place.
2. The running and stacking gels were prepared using small flasks. For two gels, the following amounts and chemicals were used:

Components	**Running Gel (12%)**	**Stacking Gel**
30 % Acrylamide Mix	4 ml	330 µl
Water	3.3 ml	1400 µl
SDS (10 %)	100 µl	20 µl
Tris (3 M, pH 8.8 for running gel & 1 M, pH 6.8 for stacking gel)	2.5 ml	250 µl
APS (10 %)	100 µl	20 µl
TEMED	4 µl	2 µl

3. The APS and the TEMED were added to the rest only when the gel was ready to be poured.
4. The gel was poured in between the two glass plates using a Pasteur pipette. Immediately, isopropanol was added to make the gel very straight.
5. The gel was then left at room temperature to polymerize for 15-30 minutes.
6. When the gel has set, the top was rinsed with distilled water. Excess water was removed by using a Whatmann filter paper.

7. The comb was inserted just a few centimeters above the level of the smaller glass plate to give room for the stacking gel.
8. The stacking gel was then poured and left for 15 minutes or more until it was set.

2. Running the Gel

Procedure:

1. The glass plates with the gel were transferred to the SDS-PAGE apparatus.
2. Running buffer was then poured into the tank. The combs were removed and the samples (boiled at 94 ^{o}C for 10 minutes and centrifuged to spin down) were loaded into the slots.
3. The lid was placed and the voltage set to maximum and amperage to 25 A per gel for 1 hour.
4. The electrophoresis machine was turned off when the gel has run until the end of the glass plates.

3. Protein Gel Staining

Procedure:

1. Gel was stained for 20 min in Coomasie Brilliant Blue (0.25 % CBB R-250, 0.25 % CBB G-250, 40 % methanol and 7 % acetate).
2. Then, it was destained 2 x 20 min in Destaining solution I (50 % ethanol, 10 % acetate).
3. Gel was further destained 2 x 20 min, also overnight or longer (5 % ethanol, 7.5 % acetic acid).

Appendix 8.2.4. Western Blot Analysis

Western blot is an immunodetection technique that uses antibodies to recognize specific proteins. This technique is normally preceded by separation of proteins on a polyacrylamide gel. The proteins are blotted on a nitrocellulose membrane followed by immunodetection.

1. Semi-dry Blotting

After performing SDS-PAGE, the proteins on the gel were transferred on a nitrocellulose membrane by semi-dry blotting. For this, the following materials were needed:

Semi-dry Blotting Buffer
Methanol
8 pieces of Whatmann filter paper
Nitrocellulose membrane (Immobilon-P, Millipore)
Electrophoresed gel

Procedure:

1. The Whatmann filter papers and nitrocellulose membrane were soaked in semi-dry blotting buffer for 5 minutes.
2. The necessary materials were arranged in the electrode plate of the blotting apparatus from top to bottom as follows: 3 pieces of Whatmann filter paper, electrophoresed gel, nitrocellulose membrane and 9 pieces of Whatmann filter paper.
3. The amperage was set to 32 mA per gel and blotting was done for 1 hour. After which, immunodetection followed.

2. Immunodetection of Western Blot

To detect proteins on the blot, specific antibodies are used. The primary antibody is used to bind to the antigen and the secondary antibody binds to the primary antibody for detection using a series of incubation and washing steps. The secondary antibody is conjugated to an enzyme that would react with a specific substrate to determine the presence of the antigen/s.

Procedure:

1. After semi dry blotting, the nitrocellulose membrane was blocked for one hour at room temperature or overnight at 4 ºC in 5 % skimmed milk in TBS-T.
2. The blot was then washed for 5 minutes, 3 times in TBS-T.
3. The blot was incubated with the primary antibody (anti-flaA raised in rabbit against *Legionella pneumophila*) in 2.5 % skimmed in a 1:1000 dilution for one hour.
4. Then it was washed again 2 times 10 minutes and 2 times 5 minutes in TBS-T.
5. The blot was incubated with the secondary antibody (anti-rabbit from goat or swine) in incubation buffer similarly used in step 3 in a 1:5000 dilution.
6. The blot was then washed 2 times 15 minutes and 4 times 5 minutes in TBS-T.
7. After washing, detection followed by staining the membrane with the Membrane Staining Solution for a few minutes. Once bands were visible, reaction was stopped in water.

Appendix 8.2.5. **Extraction of Total RNA**

When working with RNA, special care had to be taken in order to prevent contamination of the samples with exogenous RNases. Gloves were worn throughout the whole experiment and RNase free pipette tips and reaction tubes were used. For all buffers and solutions, water was pre-treated overnight with 0.1 % (v/v) diethylpyrocarbonate (DEPC) at 37 °C and autoclaved twice to remove remaining DEPC.

RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the supplied protocol. All steps of the RNeasy protocol were performed at room temperature (RT).

Procedure:

1. Bacteria were grown at 37 °C in 125 ml of pooled human urine until the optical density OD_{600} reached 0.15.
2. 50 ml of the urine culture was taken and centrifuged at 6000 rpm for 5 min.
3. The supernatant was removed and pellets were resuspended in 20 ml PBS and RNAprotect (Qiagen) 1:1 (v/v).
4. Samples were incubated at room temperature for 5 min and centrifuged at 6000 rpm for 10 min.
5. Bacterial pellets were either stored at -80 °C or bacterial RNA was immediately isolated. To continue, bacterial pellets were resuspended thoroughly in 100 µl of lysozyme-containing TE buffer (50 mg/ml) and incubated at 37 °C for 5 s with vortexing every 2 min.

***Appendix 8.2.6*. Removal of contaminating DNA by DNase treatment and RNA cleanup**

Contaminating DNA was removed from total RNA preparations by DNase I digestion (New England Biolabs).

Procedure:

1. 15 µg RNA in a final volume of 85 µl were mixed with 10 µl 10 x DNase I buffer and 10 µl RNase- free DNase I.
2. Samples were incubated for 1 h at 37 °C, followed by RNA cleanup using the RNeasy Mini kit (QIAGEN).
3. 350 µl RLT buffer supplemented without 10 µl ß-mercaptoethanol and 250 µl 100 % (v/v) ethanol were added to the DNase-treated RNA samples
4. They were then loaded into the purification columns.

5. After brief centrifugation, the columns were transferred to fresh collection tubes and washed twice with 500 µl RPE buffer.
6. Finally, RNA was eluted from the column in 30 µl nuclease-free water and run on Bioanalyzer for analysis of RNA quality and quantity.

Analysis of RNA Stability and Concentration (2100 Bioanalyzer, Agilent Technologies)

Procedure:

1. Samples are denatured at 94 °C for 2 min.
2. Next, 65 µl gel matrix and 1 µl dye are mixed, vortexed and centrifuged at 1000 rpm for 10 min.
3. Meanwhile, the Bioanalyzer pins were washed with 350 µl RNAseZap for 1 min followed by 350 µl water for 30 seconds.
4. 9 µl gel matrix is applied to the corresponding slots on the chip.
5. 5 µl of buffer is then pipetted into every slot on the chip except for the 3 slots for the gel matrix.
6. 1 µl of each sample is then loaded onto the slots.
7. The chip is vortexed and loaded into the Bioanalayzer.

Appendix 8.2.7. Reverse Transcription and cDNA labelling

Reverse transcription was performed using SuperScript III™ reverse transcriptase (Invitrogen) and the fluorescently labelled nucleotides Cy3- and Cy5-dCTP (Amersham Pharmacia, Freiburg, Germany).

Procedure:

1. For primer annealing, 10 µg total RNA was mixed with 1 µg of hexamer oligos in a total volume of 15 µl.

2. The annealing mix was heated for 10 minutes at 70 °C, then cooled down to room temperature for 5 min followed by brief centrifugation. In the meantime, the reaction mix composed of the following was prepared:

5x first strand buffer	8 µl
0.1x DTT	4 µl
Nucleotide mastermix	4 µl
RNaseOut	1 µl
SuperScript III^{TM} (200 U/µl)	1 µl
RNase free H_2O	4 µl

3. Then, 22 µl of the reaction mix was pipetted into a small PCR reaction tube together with 15 µl annealing mix
4. 4 µl Cy3- or Cy5-dCTP (1 mM) was added.
5. The total mix was incubated for 1h at 46 °C in a thermoblock.
6. After 25 min, another 1 µl of SuperScript III^{TM} reverse transcriptase (200 U/µl) was added.
7. The reaction was stopped by addition of 5 µl EDTA (500 mM).
8. To hydrolyze RNA, 10 µl NaOH (1 M) was added, followed by incubation at 65 °C for 15 min.
9. The reaction mixture was cooled down to room temperature.
10. 25 µl Tris-HCl (1M, pH 7.5) was added.
11. Labelled targets were purified using the Qiaquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

For generation of cDNA for qRT-PCR, after annealing of primers at 70 °C for 15 minutes, the tubes were cooled down for 5 min at room temperature. Afterwards, 22

µl transcription mix was added to each tube. The reaction mixture was incubated at 52 °C for 1 h in a thermoblock, followed by 15 min at 70 °C.

Appendix 8.2.8. Hybridization of Nucleotide Microarrays

1. Pre-hybridization (OpArray)

1. An appropriate volume of OpArray Pre-hybridization Solution was pre-warmed at 42°C for 30 min.
2. Slides were incubated in the pre-warmed solution using an array box (Scienion AG) with enough volume of buffer to soak them at 42°C for 1h.
3. Slides were washed for 5 min in Wash Solution 1 at room temperature. They were then transferred to double distilled water and rinsed for 30 seconds. This was repeated twice.
4. The slides were then centrifuged at 1000 rpm for 5 min to dry.

2. Hybridization (OpArray)

1. The slide was placed in the hybridization cassette.
2. Meanwhile, the labelling target solution was prepared by mixing 2 µl of 40 pmol Cy5-labelled targets with 2 µl of 40 pmol Cy3-labelled targets and 36 µl OpArray Hybridization Buffer.
3. The labelling target solution was denatured by incubating in a thermocycler at 65 °C for 5 minutes.
4. The solution was applied directly to the slides.
5. The cassette was then placed inside the hybridization of oven and left to hybridize for 14-16 hours.

3. Post-hybridization Washing (OpArray)

1. The post-hybridization washing solutions were prepared as follows:

Wash Solution 2:

50 ml OpArray Wash A

25 ml OpArray Wash B

to a final volume of 500 ml with sterile water

Wash Solution 3:

50 ml OpArray Wash A

to a final volume of 500 ml with sterile water

Wash Solution 4:

5 ml OpArray Wash A

to a final volume of 500 ml with sterile water

2. Wash Solution 2 was pre-warmed at 42 °C.
3. After hybridization, the slides were washed in the pre-warmed Wash Solution 2 at 42 °C for 10 min.
4. The slides were transferred to Wash Solution 3 and incubated at RT for 10 min.
5. The slides were transferred to Wash Solution 4 and incubated at RT for 5 min.
6. The slides were then centrifuged at 1000 rpm for 5 min to dry.

Appendix 8.2.9. Polysaccharide Intercellular Adhesin (PIA) Immuno Dot Blot

Procedure:

1. 20 ml overnight cultures of bacteria were grown statically at 37 °C. RP62A was used as positive control and TM 300 as negative control.
2. Optical density was measured the following day. OD_{600} was standardized to 6. Samples pelleted in 50 µl EDTA (0.5 M, pH8) by centrifugation.
3. Samples were heated at 100 °C for 5 minutes.
4. Samples were centrifuged at 13000 rpm for 10 minutes, room temperature.
5. 40 µl of the supernatant were taken and 5 µl Proteinase K (20 mg/ml) was added.

6. They were then incubated at 57 °C for 1 hour.
7. Samples were inactivated by heating for 5 minutes at 100 °C.
8. 5 µl each of the samples was gently and carefully pipetted on a nitrocellulose membrane.
9. The membrane was dried very well under the sterile hood.
10. The membrane was blocked in 5 % skimmed milk in 1 X TBS for 1 hour while shaking. As alternative, blocking overnight at 4 °C may also be done.
11. The membrane was incubated in the primary antibody (PIA), diluted 1:200 in 2.5 % skimmed milk in 1 x TBS-T for 1hour at room temperature while shaking. Alternatively, incubation may be done overnight at 4 °C.
12. The primary antibody was decanted and stored at -20 °C for further use (may be re-used up to 3 x).
13. The membrane was briefly washed 1x followed by 3x washing for 5 minutes each, in 1x TBS-T.
14. The membrane was then incubated in the secondary antibody diluted 1:5000 for 1 hour at room temperature.
15. The secondary antibody was decanted and store at -20 °C for further use (may be re-used up to 4x).
16. The membrane was briefly washed 1x followed by 3x washing for 5 minutes each, in 1x TBS-T.
17. For visualization of immunological interaction, the membrane was incubated in the developing solution for 2 minutes and immediately viewed under a chemiluminescence camera.
18. In addition, the blot may also be transferred to an X-ray film by incubation of the blot unto the film in the dark room, followed by development in the machine.

Developing Solution:

1 ml Solution A + 0.3 µl H_2O_2 (30 %) + 100 µl Solution B

100 ml Solution A + 3 µl H_2O_2 + 100 µl Solution B

Solutions:

Solution A: 200 ml 0.1 M Tris-HCl pH 8.6 + 50 mg Luminol (Sigma A4685) (stored at 4°C)

Solution B: 11 mg para-Hydroxycomarine acid (Sigma C9008) in 10 ml DMSO (stored in the dark at room temperature)

Appendix 8.2.10. LPS Isolation

Procedure:

1. Strains were grown on plate. Next, they were scraped off from the agar and transferred to an Eppendorf tube.
2. The cells were weighed and resuspended in water to a concentration of 1 mg cells/ 30 µl suspension.
3. 30 µl of sample (1 mg cells) was mixed with 10 µl 4x SDS sample buffer and incubated at 100 °C for 10 min.
4. This was then cooled briefly.
5. Afterwards, 100 µg proteinase K was dissolved in 1x SDS sample buffer. 20 µl of this was added into the samples and incubated at 60 °C for 1 h.
6. 30 µl of the LPS prep was used for electrophoresis in a 20 x 20 cm 15 % gel. The gel was run overnight (O/N) at 8 mA, RT.

Staining with $AgNO_3$

Procedure:

1. The gel was fixed O/N in 100 ml 1X fixation solution.
2. Next day, the fixation solution was replaced with 100 ml 1x periodate solution where the gel was incubated for 5 min.
3. The gel was washed 3x, 30 min each, with dH_2O.
4. Next, the gel was incubated for 10 min in $AgNO_3$.

5. The gel was washed 3x, 10 min each, with dH_2O.
6. Meanwhile, the developing solution was pre-heated to 60 °C. The gel was developed in this solution.
7. When bands appeared intense enough, the reaction was stopped by incubating in 50 mM EDTA solution for 10 min.
8. Finally, the gel was scanned.

Appendix 8.2.11. **PCR Product Purification**

PCR products were directly purified either using the QIAquick PCR Product Purification Kit (Qiagen™) or gel-purified by the Gel Extraction Kit (Qiagen™).

QIAquick PCR Product Purification

Procedure:

1. 5 volumes of Buffer PB were added to 1 volume of PCR sample and mixed.
2. DNA was bound into a QIAquick spin column by pipetting the sample into the column and centrifuging at 13000 rpm for 1 min.
3. The flow-through was discarded and the sample was washed with 0.75 ml Buffer PE and centrifuged at 13000 rpm for 1 min.
4. The flow-through was discarded and the column was dried by centrifugation at 13000 rpm for 1 min.
5. The column was placed in a clean 1.5 ml eppendorf tube. The DNA was eluted with 30 µl of water by centrifugation at 13000 rpm for 1 min.

Gel Extraction

Procedure:

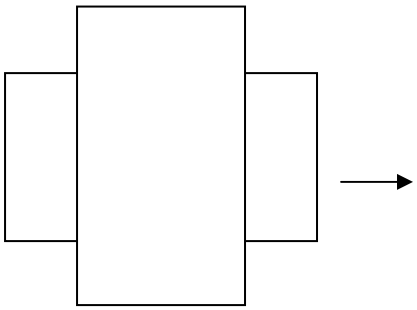
1. The gel fragment was excised using a clean sharp scalpel and the gel slice was weighed.
2. 3 volumes of Buffer QG were added to 1 volume of gel.
3. This was then incubated at 50°C for 10 min or until it was completely dissolved.
4. 1 gel volume of isopropanol was then added to the sample and mixed.
5. DNA was bound into a QIAquick column by applying 800 µl of sample followed by centrifugation.
6. The flow-through was discarded and 0.5 ml of Buffer QG was given to the column then centrifuged at 13000 rpm for 1 min.
7. The sample was washed with 0.75 ml Buffer PE and centrifuged at 13000 rpm for 1 min.
8. The column was again centrifuged at 13000 rpm for 1 min to dry.
9. The sample was eluted with 40 µl of water by centrifugation at 13000 rpm for 1 min.

Appendix 8.2.12. **Southern Blot Analysis**

Procedure:

1. 10 µg chromosomal DNA was subjected to restriction digestion with the proper enzyme then
2. Run on agarose gel.
3. The blotting apparatus consisting of a white tray (height should be sufficient just to hold enough solution but must not be too deep), plus a glass, a stack of gray tissue paper (about 10 cm and of equal size as gel) was prepared.
4. The gel was placed in denaturation solution (0.5 NaOH; 1.5 M NaCl) for 20 min, 2x with shaking.

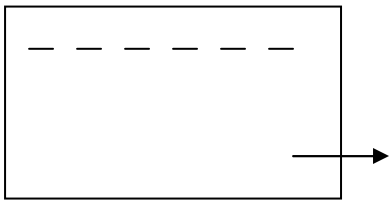
5. Afterwards, it was transferred into neutralizing solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 20 min, 2x with shaking.
6. Meanwhile, the following: 5x Whatmann paper (exactly the same size as gel), 1x Nytran Super Charge membrane were prepared.
7. The "Blotting Machinery" was constructed as follows:



8. Over the Whatman paper over the glass, 2x pre-wetted Whatman (remove air bubbles by rolling a Pasteur/glass pipette over it), the agarose gel (upside down), membrane, 3x Whatman and a stack of tissue paper (10 cm) were stacked on top of each other. A weight was put on top to facilitate transfer.
9. This was left to stand overnight.
10. The following day, the blot was marked with pencil by tracing through the holes of the gel (to serve as guide).
11. The blot was then washed 1x in 6x SSC for 5 min, with shaking.
12. Next, it is dried in Whatman paper until ready to use.

Note! Probe should be made ready. At least 3x of 50 µl of PCR volume for both strains of interest and a control should be available.

13. For cross-linking of the DNA into the membrane, 1 µl of probe was pipetted into the membrane. For example,



Then, in the UV chamber, C3 (for cross-linking through exposure to UV) was used for cross-linking.

Meanwhile, the hybridization buffer was prepared by thawing at 42 °C.

14. The membrane was transferred into two short shaking glass tubes (one part only with the DNA ruler and another with the samples to be probed). 25 ml of the thawed hybridization buffer was put into each.
15. This was then put in shaking incubator (set at 42 °C) and incubated while shaking for 1 hour. This is called pre-hybridization.
16. Next step was to label the probe. 100 ng DNA per ml of hybridization buffer in a final volume of 10 µl in dH_2O was denatured for 5 min at 94 °C and cooled immediately on ice for another 5 min. Then, 10 µl of labelling reagent and 10 µl gluteraldehyde were added. The mixture was incubated for 15 min at 37 °C and added to the hybridization reaction. This was incubated overnight at 42 °C.
17. The membrane was washed 2x for 20 min at 42 °C with wash solution I (0.5 x SSC; 0.4 % (w/v) SDS).
18. Then again, it was washed 2x for 10 min, RT, with wash solution II (2x SSC).
19. A bit of the remaining wash solution was removed by tapping the membrane on Whatman paper (Note! It should not be very dry though).
20. The membrane was incubated for 2 min in 2-3 ml each (depends on the size of the membrane) of detection solution I and detection solution II (ECL Kit). The two must be mixed well (1:1).

21. The membrane was dried a bit on Whatman paper and covered in a Saran wrap (avoid air bubbles).

Chemoluminescence was detected by exposure of the membrane to an X-ray film, developed in the dark room.

Appendix 8.2.13. **Plasmid Isolation**

Procedure:

1. Bacterial cultures were grown overnight at 37 °C.
2. 4 ml (2 x 2 ml) of the cultures were centrifuged for 2 min at 13000 rpm, room temperature (RT).
3. Pellets were resuspended with 150 µl Buffer 1 by vortexing. 30 µl lysozyme was added and incubated for 10 min, RT.
4. 300 µl Buffer 2 was added. The mixture was inverted 2x and incubated for 5 min, RT.
5. 225 µl Buffer 3 was added. The mixture was inverted 2x and incubated for 10 min on ice.
6. 200 µl Phenol-chloroform was added into the mixture followed by vortexing.
7. Mixture was centrifuged for 10 min, RT.
8. Supernatant was transfered into a new 1.5 ml Eppendorf tube. 100 % ethanol was added up to the brim of the tube.
9. It was then centrifuged for 30 min at 13000 rpm, RT. Supernatant was decanted.
10. 70 % ethanol was added up to the brim of the tube. This was followed by centrifugation for 10 min at 13000 rpm, RT. Supernatant was decanted.
11. The tube was spun for 1 min at 13000 rpm, RT to facilitate complete drying.

Appendix 8.2.14. **Chromosomal DNA Isolation (using Phenol)**

Procedure:

1. Overnight bacterial culture was grown in LB.
2. The sample was centrifuged for 15 minutes, 13000 rpm.
3. Pellet was resuspended in 500 µl Buffer 1 (50 mM Tris-HCl; 50 mM EDTA; pH 8.0) and
4. Incubated for 1 h or longer (-20 °C).
5. 50 µl lysozyme solution (freshly prepared: 10 mg/ml lysozyme in 0.25 M Tris-HCl, pH 7.5) was added into the cells and inverted.
6. Samples were incubated for 45 minutes on ice.
7. 100 µl step-solution was added while inverting.

   Step-solution: 0.5 % SDS
50 mM Tris-HCl, pH 7.5
0.4 M EDTA } + 1 mg/ml Proteinase K

8. The samples were incubated for 1-2 h in 50 °C water bath. 15 µl Proteinase K was added and then another 10 µl if it was still not clear.
9. Water was added up to the 1.5 ml mark (in a 2 ml microfuge tube) and inverted.
10. Phenol (equilibrated in Tris-HCl) was added up to the 2 ml mark and vortexed.
11. Samples were centrifuged for 15 minutes, 13000 rpm, RT.
12. The upper phase was removed using a blue pipette tip cut at the edge to widen the hole. (Only the thread-like part was taken and transferred to an eppendorf tube).
13. 0.1 volume 3 M Na acetate, pH 5.2 was added.
14. Next, 2.5 volume ethanol was added and this was mixed until clear then transfered into a white plastic tube.
15. Samples were washed 1 x in 100% ethanol, 1 x in 70 %. (Just inverted and removed).
16. They were then dissolved in 200 ml water.

8.3. Appendix Figures

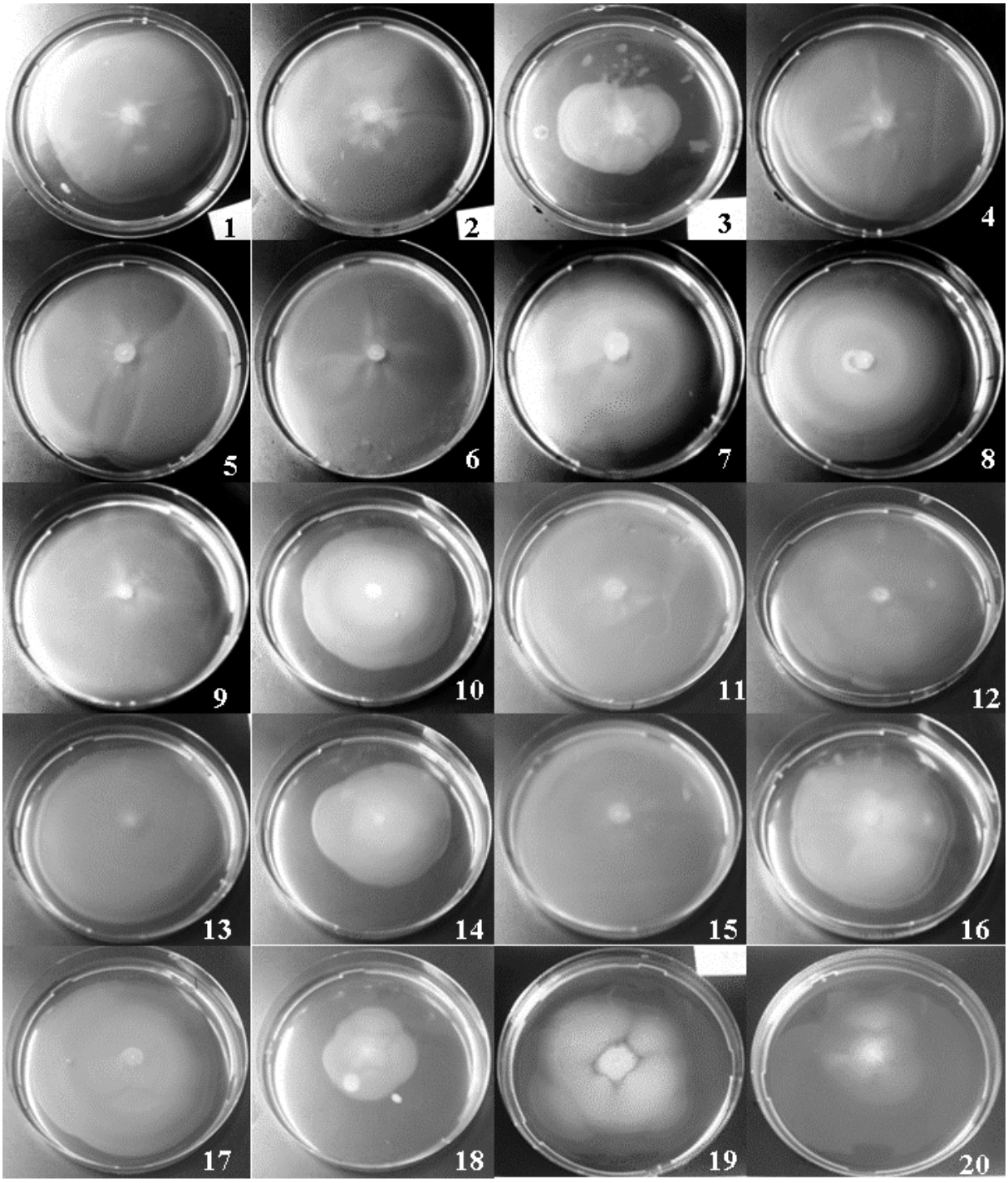


Figure 37. Motility of 20 colonies of BU05 35364 on LB swarm agar plates.

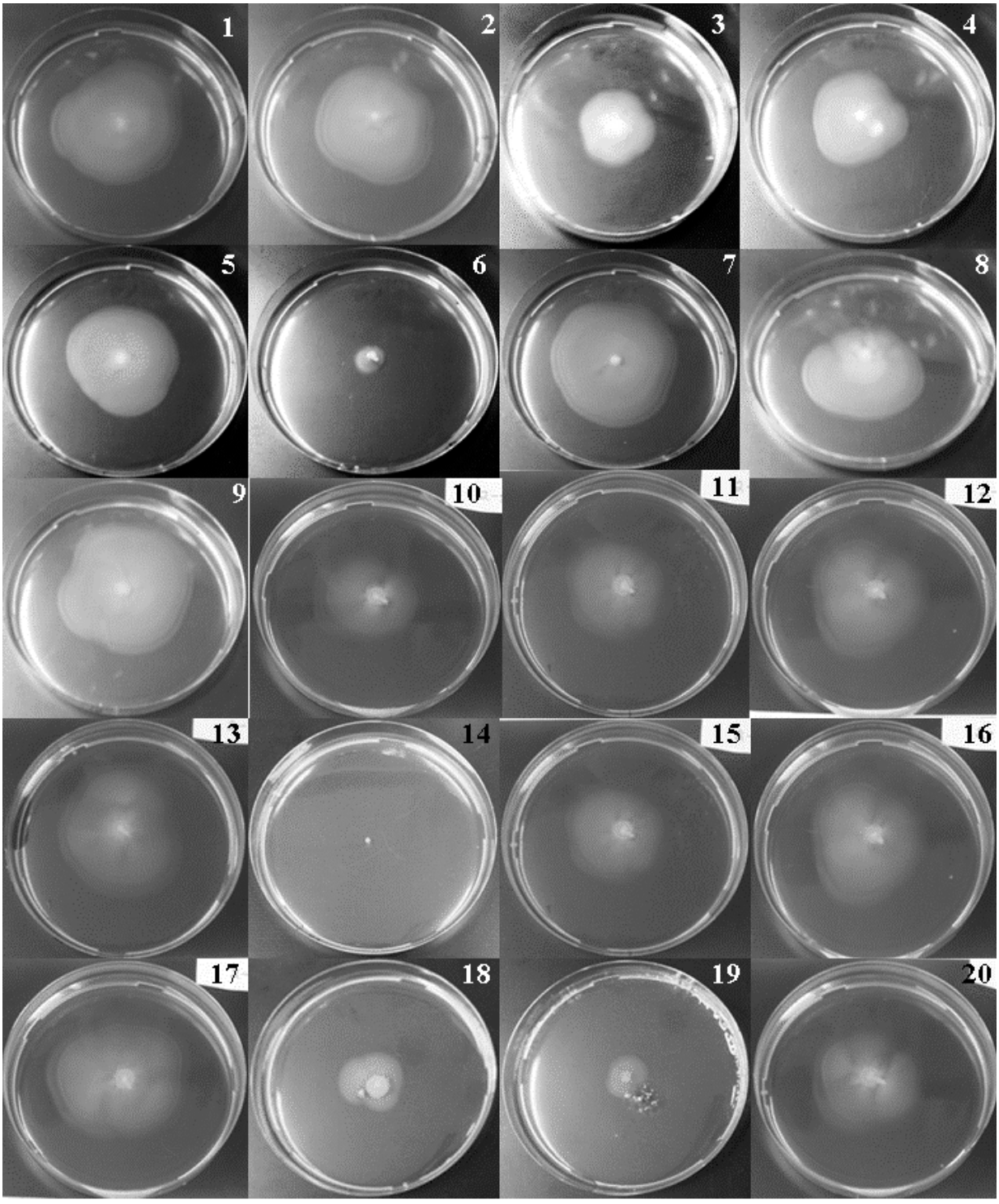


Figure 38. Motility of 20 colonies of BU04 63630 on LB swarm agar plates.

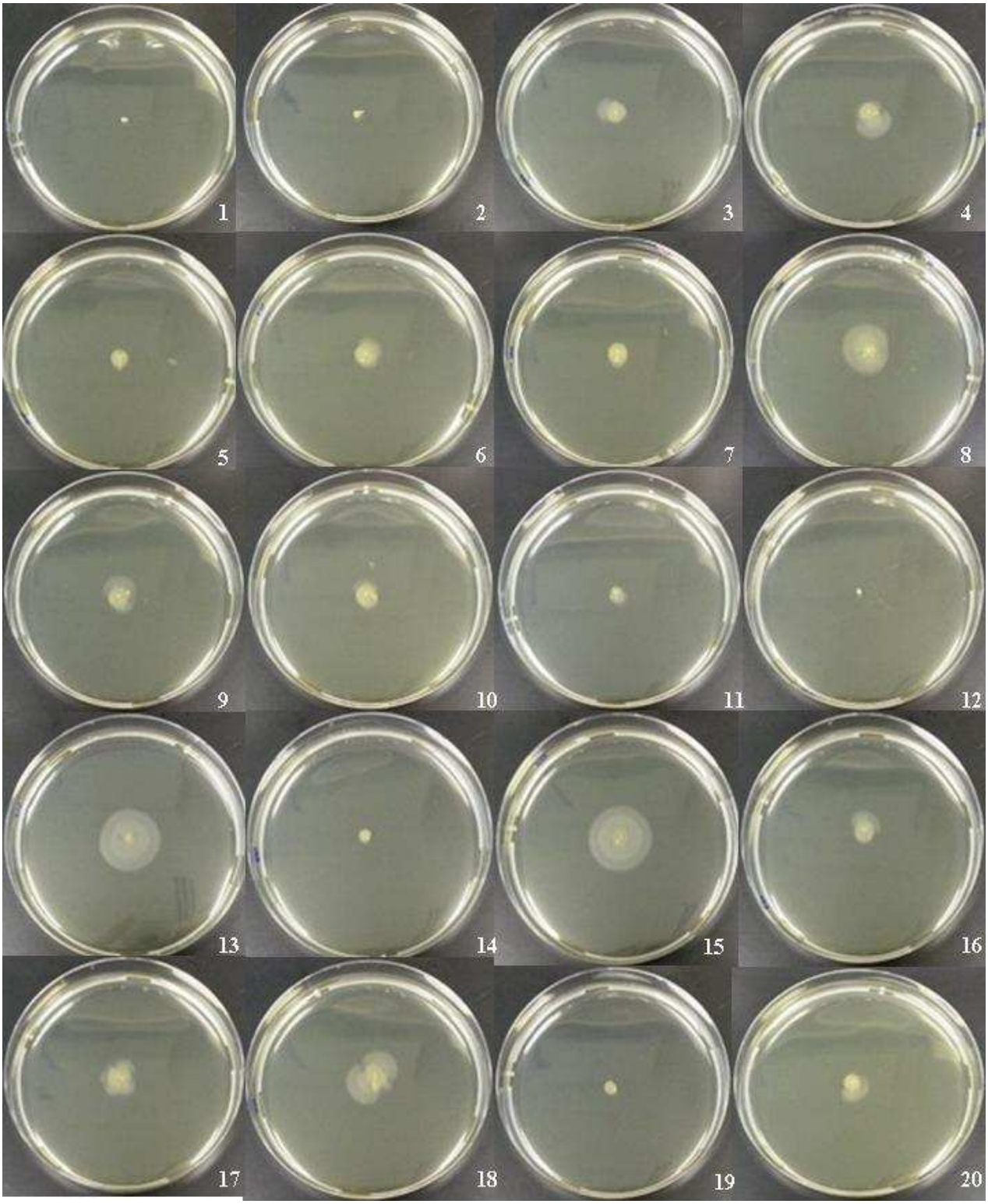


Figure 39. Motility of 20 colonies of FIM 636 on LB swarm agar plates.

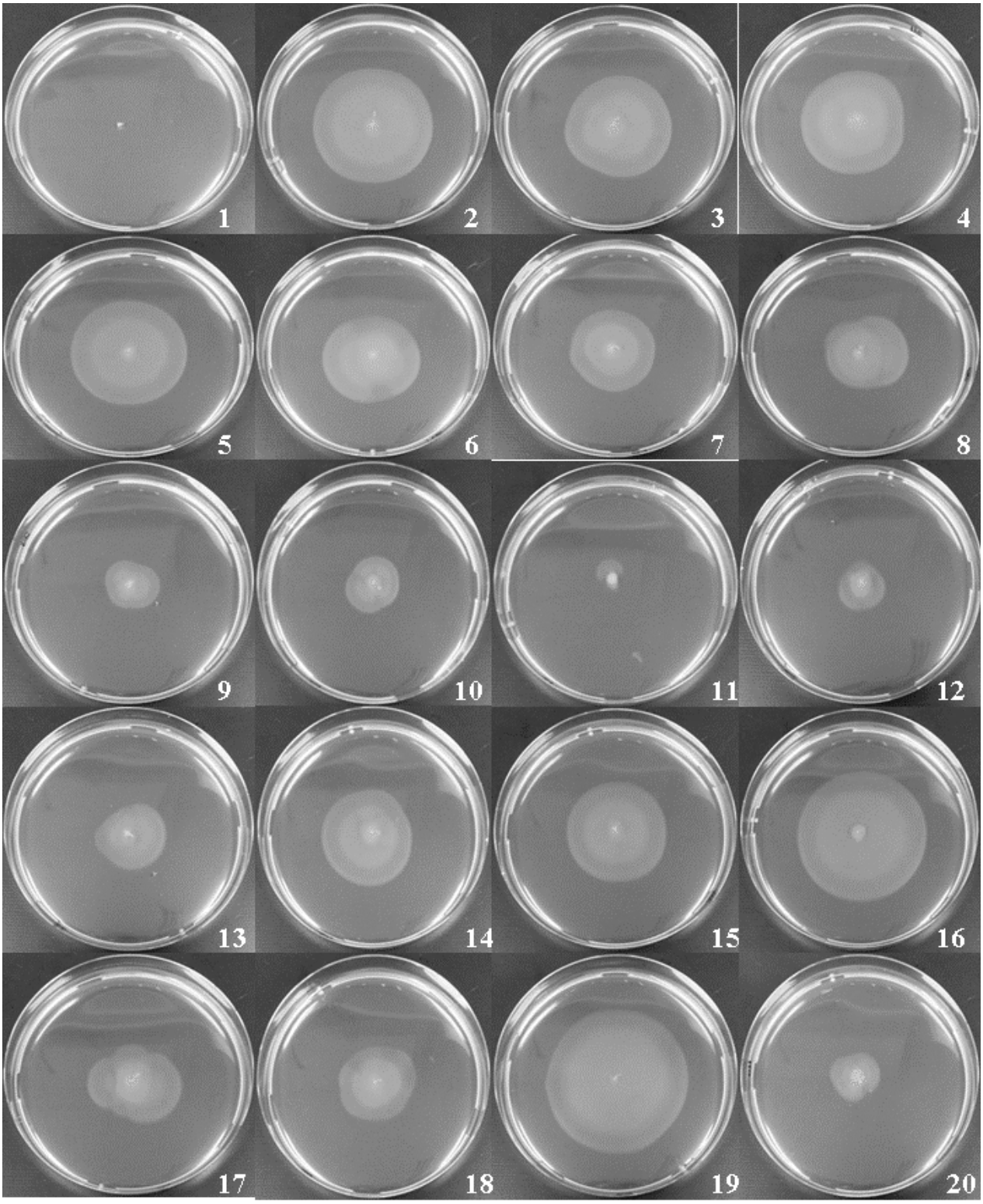


Figure 40. Motility of 20 colonies of BU05 35364 on urine swarm agar plates.

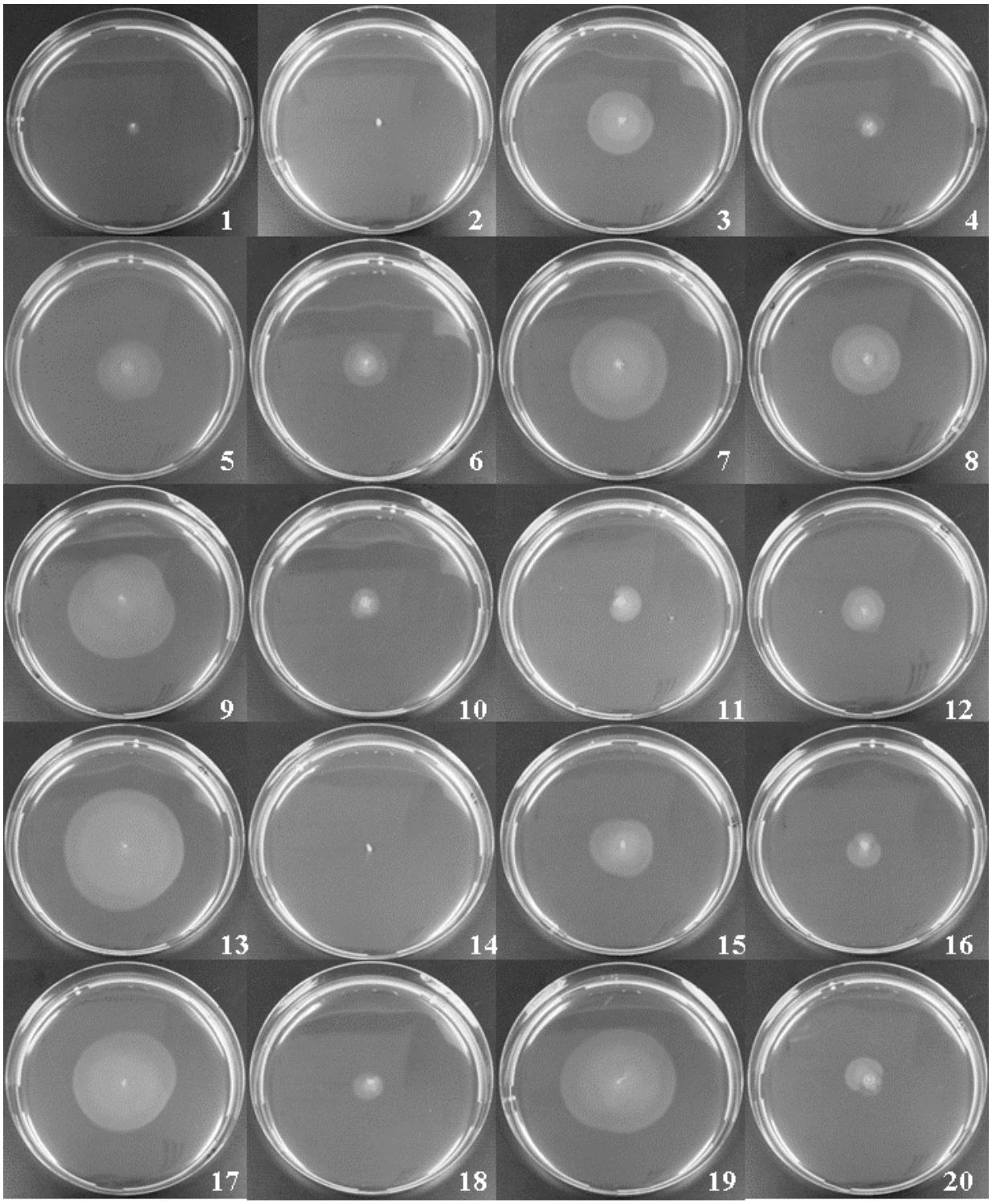


Figure 41. Motility of 20 colonies of BU04 63630 on urine swarm agar plates.

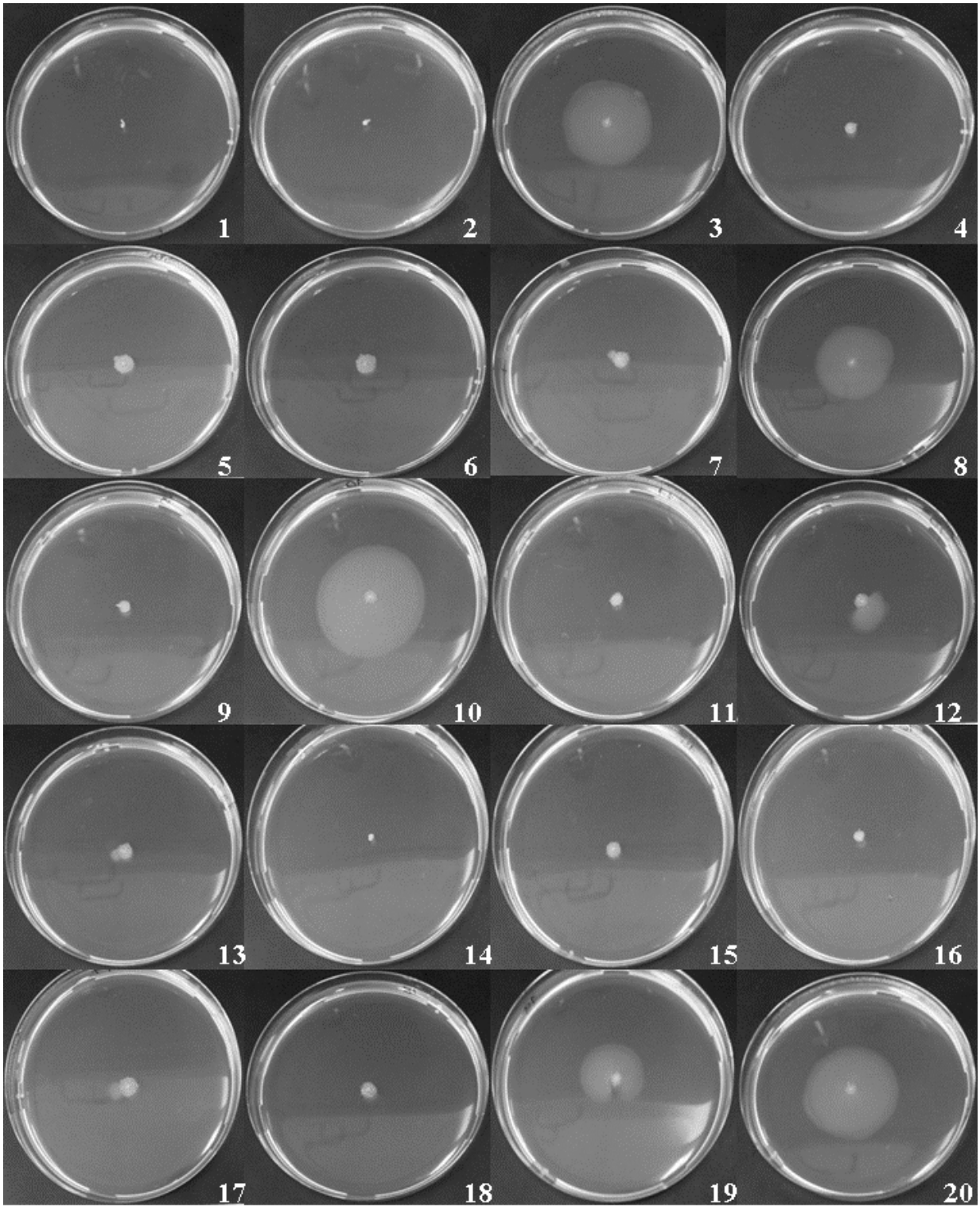


Figure 42. Motility of 20 colonies of FIM 636 on urine swarm agar plates.

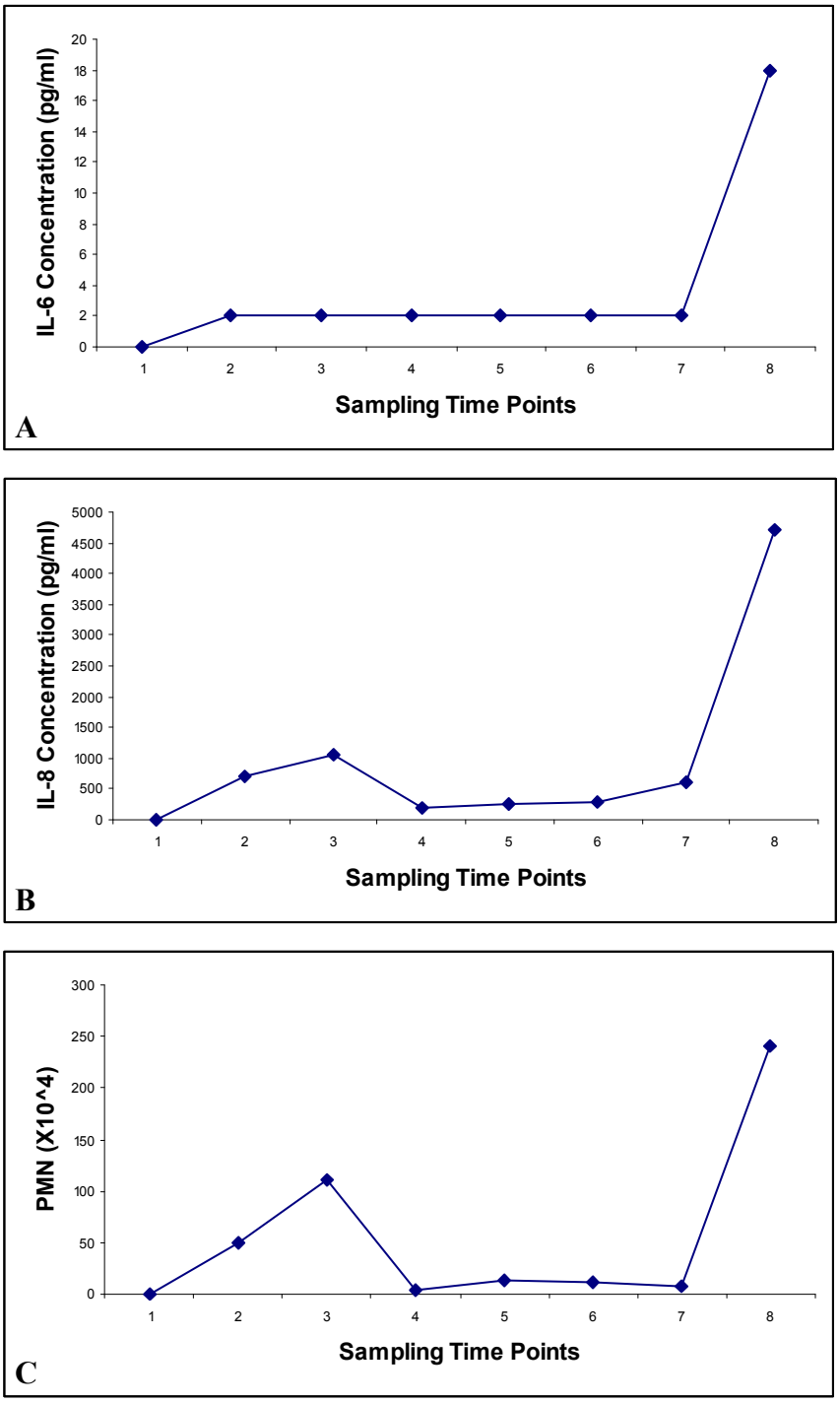


Figure 43. Host parameters of Patient 1 from which re-isolate BU 04 41631 was taken. Sampling Time Points (Number of days post-inoculation): 1- 0, 2- 11, 3- 40, 4- 76, 5- 111, 6- 140, 7- 185, 8- 192. BU 04 41631 was taken at time point 8. Data kindly provided by Dr. B. Wullt, Lund, Sweden.

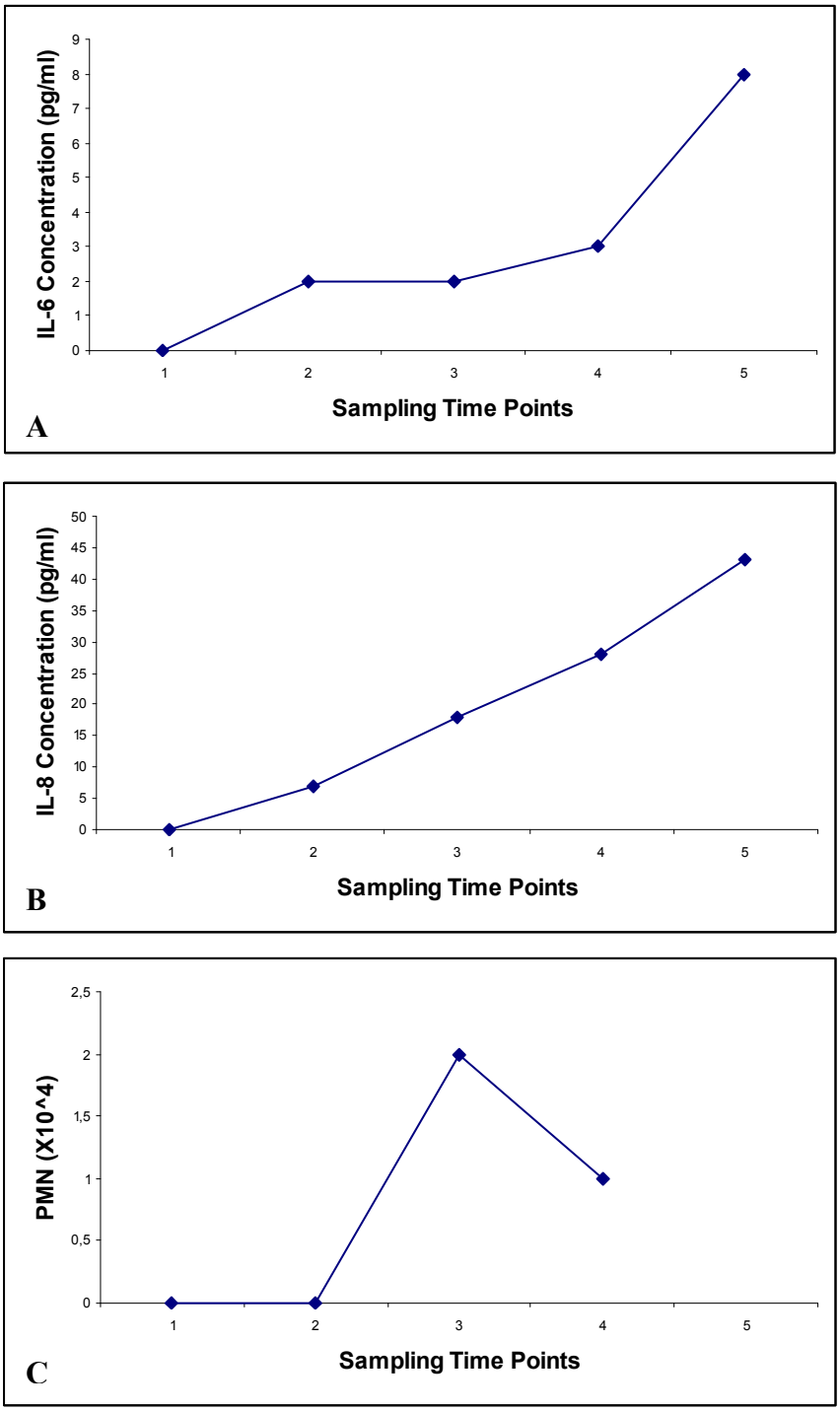


Figure 44. Host parameters of Patient 2 from which re-isolate BU 04 68088 was taken. Sampling Time Points (Number of days post-inoculation): 1- 0, 2- 25, 3- 53, 4- 88, 5- 104. BU 04 68088 was taken at time point 5. No data available for PMN influx for time point 5. Data kindly provided by Dr. B. Wullt, Lund, Sweden.

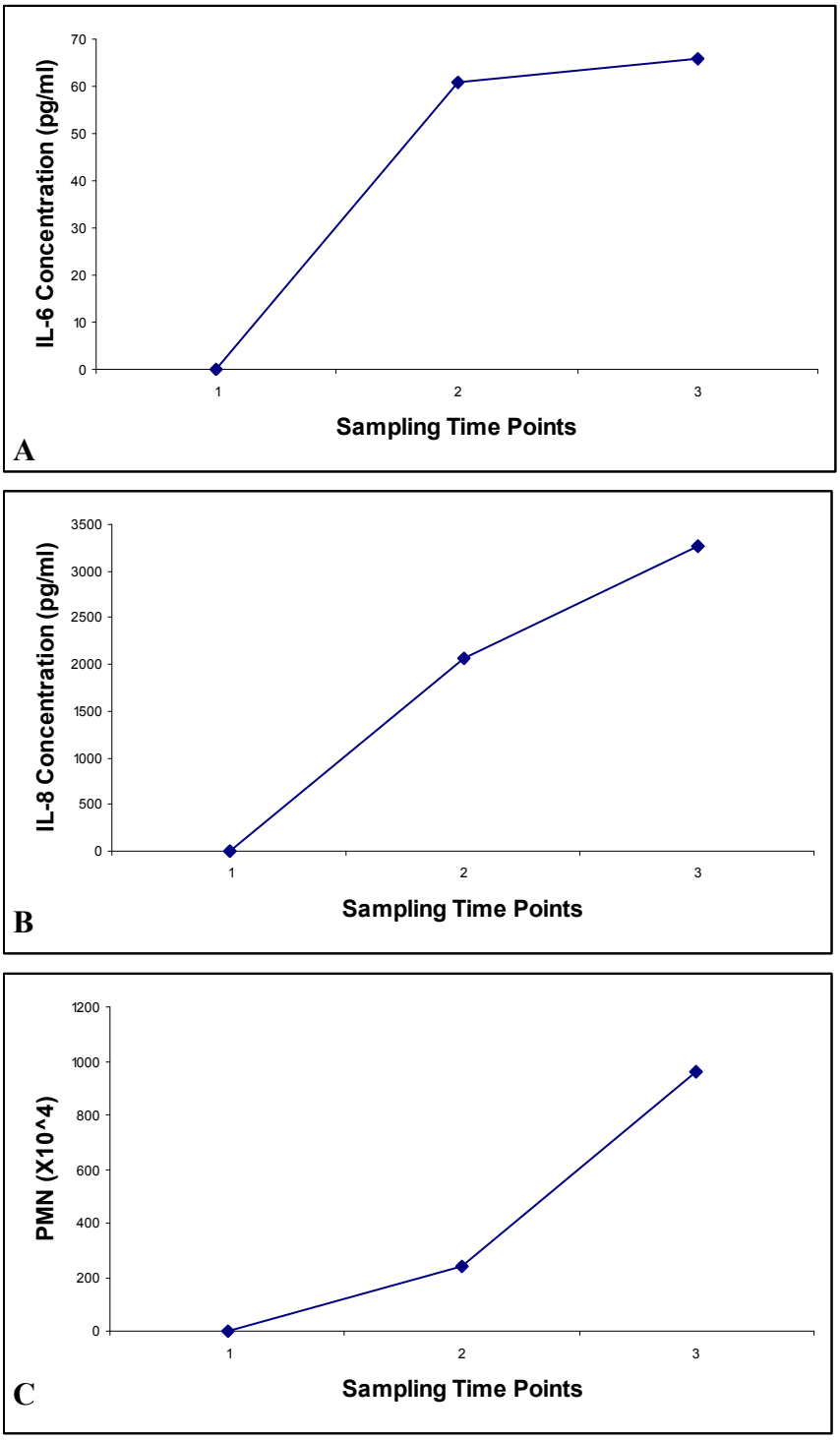
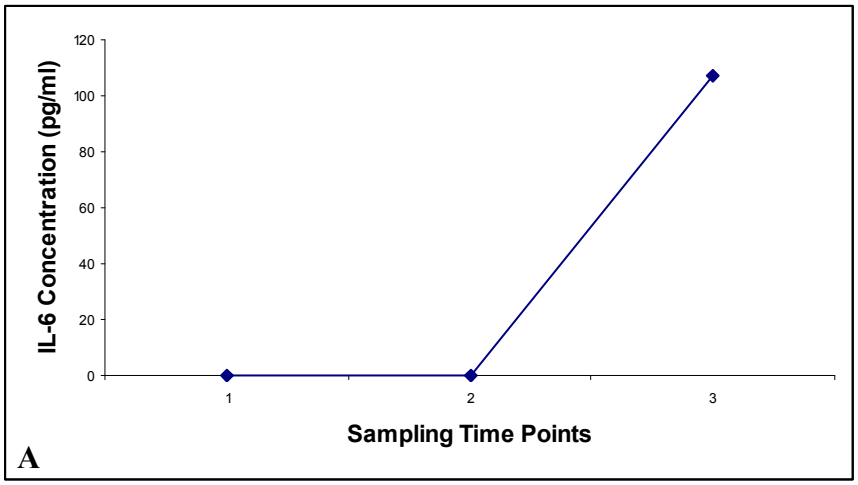
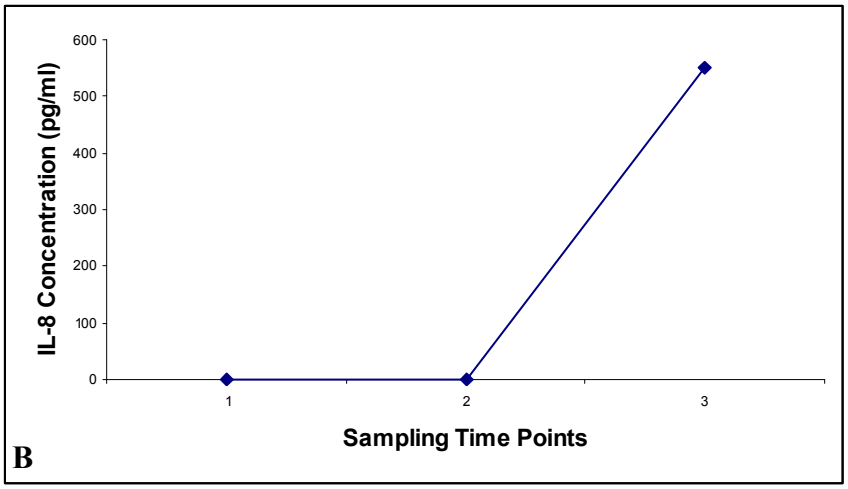


Figure 45. Host parameters of Patient 3 from which re-isolate BU 04 50907 was taken. Sampling Time Points (Number of days post-inoculation): 1- 0, 2- 3, 3- 20. BU 04 50907 was taken at time point 3. Data kindly provided by Dr. B. Wullt, Lund, Sweden.





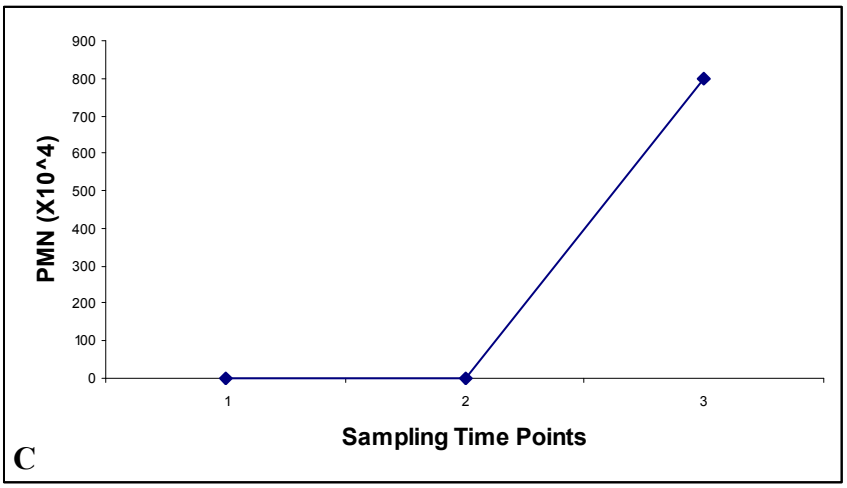


Figure 46. Host parameters of Patient 4 from which re-isolate BU 04 63630 was taken. Sampling Time Points (Number of days post-inoculation): 1- 0, 2- 1, 3- 19. BU 04 63630 was taken at time point 3. Data kindly provided by Dr. B. Wullt, Lund, Sweden.

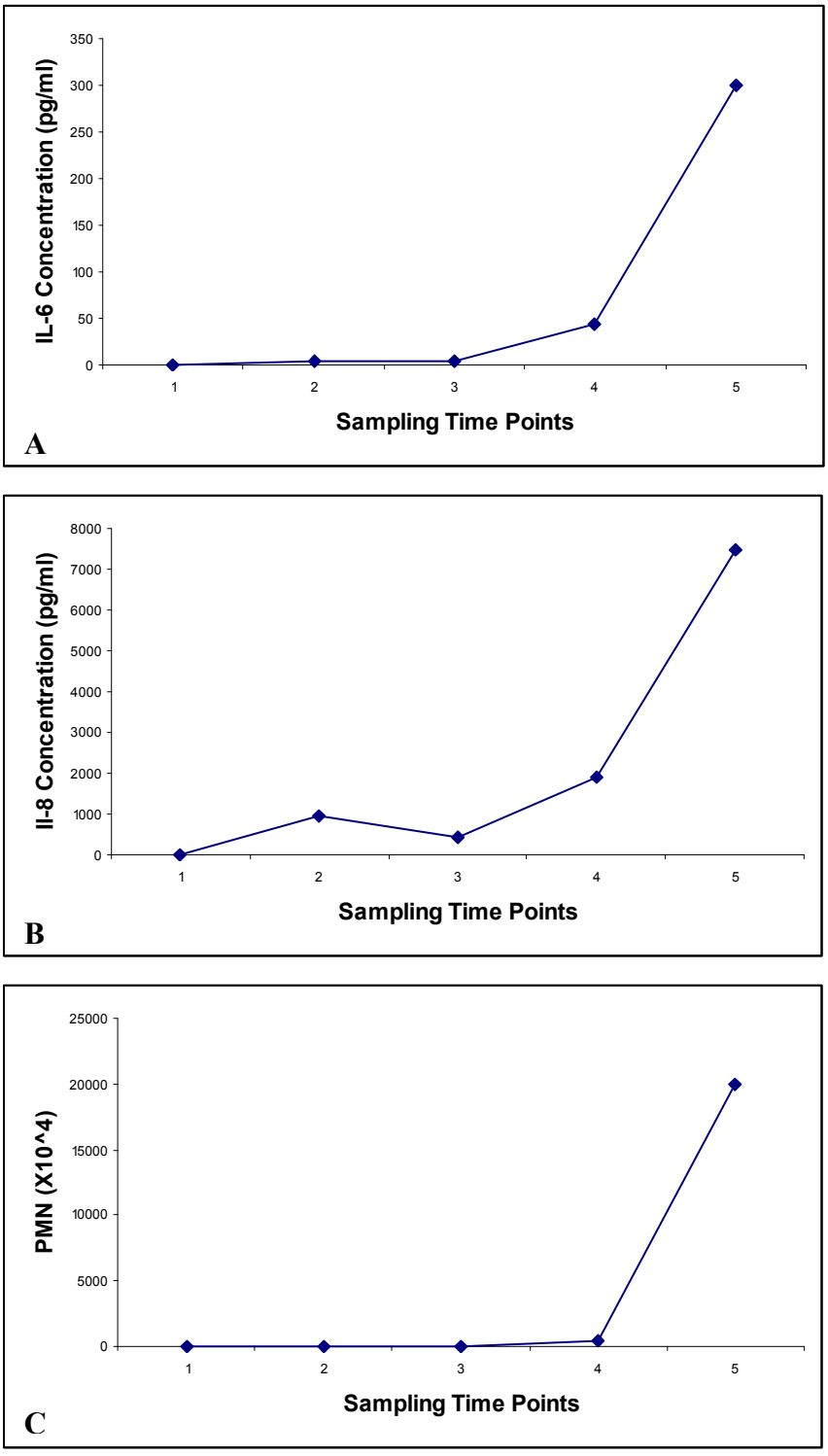


Figure 47. Host parameters of Patient 5 from which re-isolate BU 05 35364 was taken. Sampling Time Points (Number of days post-inoculation): 1- 0, 2- 12, 3- 40, 4- 63, 5- 67. BU 05 35364 was taken at time point 5. Data kindly provided by Dr. B. Wullt, Lund, Sweden.

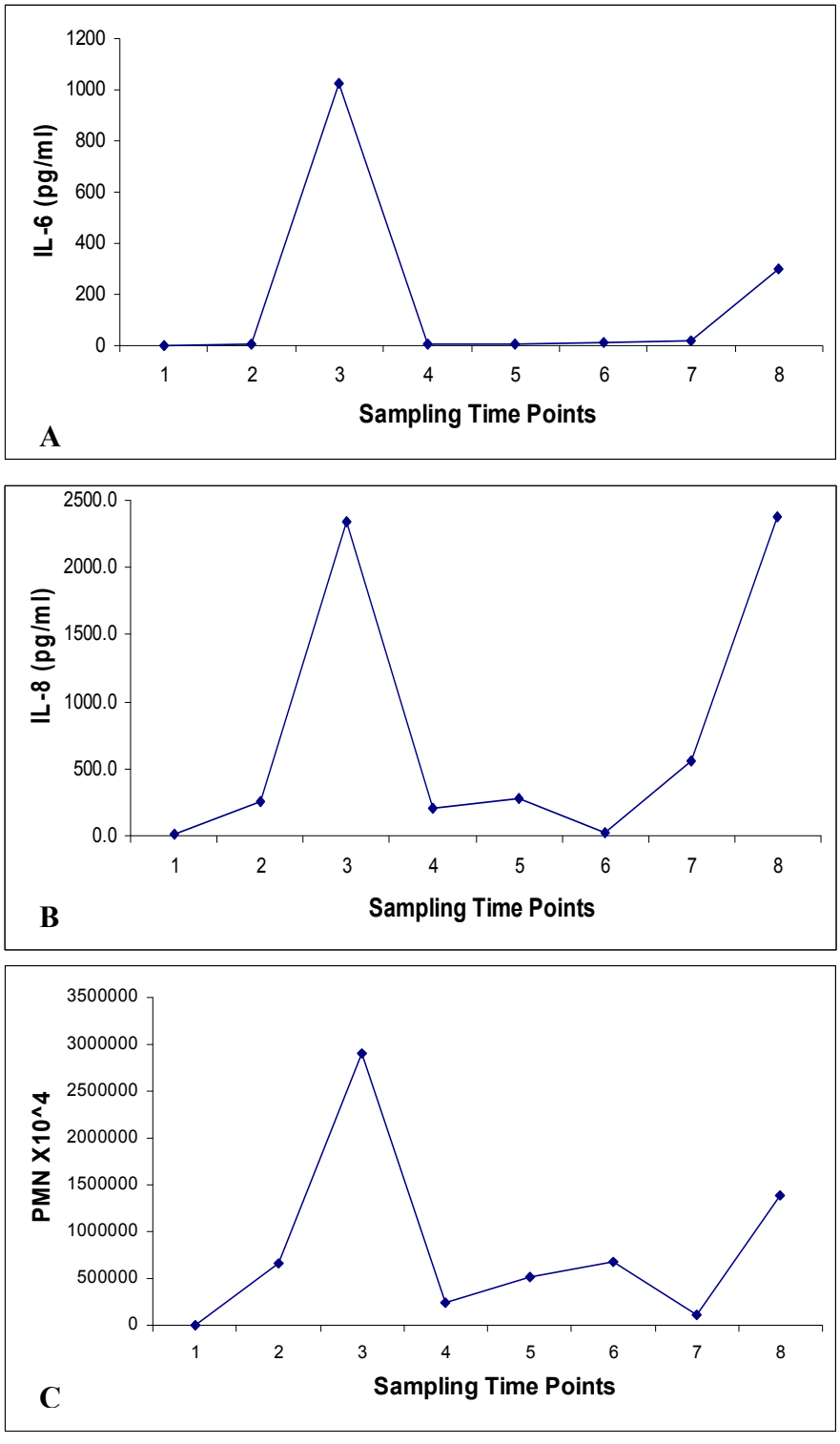


Figure 48. Host parameters of Patient 6 from which re-isolate FIM 636 was taken. Sampling Time Points (Number of days post-inoculation): 1- 0, 2- 1, 3- 2, 4- 7, 5- 14, 6- 28, 7- 49, 8- 59.5. FIM 636 was taken at time point 8. Data kindly provided by Dr. B. Wullt, Lund, Sweden.

8.4. Expression Profiling Data

Table 14. Upregulated genes in re-isolate BU04 63630

Gene	Function	Ratio	p-value
gadA	Acid resistance	1.012	0.016821
gadB	Acid resistance	1	0.018224
hdeA	Acid resistance	1.248	0.002506
hdeB	Acid resistance	1.062	0.006815
slp	Acid stress	1.182	0.008119
rpoA	RNA Polymerase, sigma factor	1.055	0.008119
rpoE	RNA Polymerase, sigma factor	1.046	0.005747
rpoS	RNA Polymerase, sigma factor	0.915	0.000314
recA	SOS response to DNA damage	3.196	0.001533
recN	SOS response to DNA damage	1.932	0.00274
lexA	SOS response to DNA damage	1.536	0.00932
yebG	SOS response to DNA damage	1.837	0.080393
sulA	SOS response to DNA damage	1.839	0.006357
dinB	SOS response to DNA damage	1.524	0.003321
dinI	SOS response to DNA damage	2.125	0.005379
ruvB	SOS response to DNA damage	1.197	0.001149
umuD	Response to DNA damage	1.67	0.007761
yphE	ABC Transporter	0.979	0.003161
yphF	ABC Transporter	1.12	0.013532
kdpA	Potassium Transport	0.932	0.002286
kdpB	Potassium Transport	1.136	0.001215
rpmC	50S ribosomal protein	1.115	0.000042
rpmD	50S ribosomal protein	1.029	0.017251
rpmI	50S ribosomal protein	0.911	0.00241
c1524	Unknown function	1.528	0.006826
c1525	Unknown function	1.472	0.001486
c1527	Unknown function	1.641	0.003696
c1528	Unknown function	1.607	0.003131
c1530 | c5144 | c3607	Unknown function	2.034	0.002908
c1531	Unknown function	1.362	0.004536
Z2360 | Z2135	Phage-related	2.027	0.004327
Z2361 | Z2134	Phage-related	1.502	0.001511
Z2362 | Z2133	Phage-related	1.407	0.007946
c1536	Phage-related	2.159	0.001151
c1538	Phage-related	1.95	0.007709
c1539	Phage-related	1.571	0.03906
c1541	Phage-related	1.214	0.001956
c1547	Phage-related	2.26	0.009778
c1550	Phage-related	1.581	0.007511
c1555	Phage-related	1.821	0.004958
c1570 | c1446	Phage-related	1.324	0.008774
c1571 | c1447	Phage-related	1.26	0.00663
c1574	Phage-related	2.027	0.00198
c1575 | c1451	Phage-related	1.697	0.004709

c1578	Phage-related	1.466	0.011361
c1584 | c3161	Phage-related	1.534	0.08459
c3162 | c1583	Phage-related	2.081	0.042295
c3163	Phage-related	1.48	0.000738

Table 15. Upregulated genes in re-isolate BU05 35364

Gene	**Function**	**Ratio**	**p-value**
groES	Chaperonin. heat-shock proteins	0.959	0.006171
flgA	Flagellar assembly and biosynthesis	1.481	0.031897
flgD	Flagellar assembly and biosynthesis	1.847	0.042864
flgE	Flagellar assembly and biosynthesis	1.413	0.018316
flgF	Flagellar assembly and biosynthesis	1.379	0.090894
flgG	Flagellar assembly and biosynthesis	1.716	0.046086
flhA	Flagellar assembly and biosynthesis	1.301	0.057089
fliA	Flagellar assembly and biosynthesis	3.55	0.067707
fliG	Flagellar assembly and biosynthesis	1.334	0.078135
fliO	Flagellar assembly and biosynthesis	1.068	0.000531
rfaG	Lipopolysaccharide	1.101	0.003292
rfaI	Lipopolysaccharide	1.311	0.003868
rfaJ	Lipopolysaccharide	1.249	0.015306
rfaP	Lipopolysaccharide	1.16	0.015306
rfaY	Lipopolysaccharide	1.368	0.00051
waaV	Lipopolysaccharide	1.191	0.010939
waaW	Lipopolysaccharide	1.31	0.002182
glmS	N-acetyl glucosamine	1.628	0.000975
glmU	N-acetyl glucosamine	1	0.002555
lpxA	N-acetyl glucosamine	1.407	0.009802
lpxB	N-acetyl glucosamine	0.952	0.003486
chuA	Heme	1.485	0.004442
chuT	Heme	1.26	0.016839
chuU	Heme	1.249	0.007451
chuW	Heme	1.277	0.011657
chuX	Heme	1.168	0.0219
chuY	Heme	1.162	0.017643
entC	Enterochelin biosynthetic pathway	1.021	0.000406
entA	Enterochelin biosynthetic pathway	1.049	0.018279
cvpA	Colicin production	3.492	0.000656
cirA	Colicin receptor, iron-siderophore complex uptake receptor	1.72	0.009339
malE	Maltose transporter	2.349	0.011769
malF	Maltose transporter	1.183	0.080574
malK	Maltose transporter	0.927	0.00219
malM	Maltose transporter	2.188	0.026
malP	Maltose transporter	2.141	0.013584
malQ	Maltose transporter	1.867	0.01209
malZ	Maltose transporter	0.976	0.058784
ompF	Outer membrane protein	1.663	0.001972

fabA	Dehydaratase	1.175	0.004356
fabZ	Dehydaratase	1.021	0.001472
fabB	Dehydaratase	1.107	0.000234
speA	Decarboxylase	1.288	0.00237
speB	Decarboxylase	0.9	0.000083
yqjG	Transferase	1.285	0.016022
yqjF	Oxidase	1.769	0.005481
proA	Amino acid biosynthesis	1.029	0.017441
proP	Amino acid biosynthesis	1.286	0.018709
proS	Amino acid biosynthesis	1.089	0.000408
proY	Amino acid biosynthesis	1.242	0.001179
glnA	Amino acid biosynthesis	0.992	0.00056
glnK	Amino acid biosynthesis	1.382	0.001173
glnL	Amino acid biosynthesis	1.014	0.007842
glnP	Amino acid biosynthesis	0.92	0.008164
glnQ	Amino acid biosynthesis	1.045	0.002131
thrA	Amino acid biosynthesis	1.176	0.025932
thrB	Amino acid biosynthesis	0.979	0.016287
thrL	Amino acid biosynthesis	0.889	0.076079
yhhW	Pirin homolog	1.201	0.013399
c4310	Unknown function	1.937	0.006885

Table 16. Upregulated genes in re-isolate FIM 636

Gene	**Function**	**Ratio**	**p-value**
frmA	Formic acid metabolism	2.653	0.027601
frmB	Formic acid metabolism	2.206	0.045425
frmR	Formic acid metabolism	1.942	0.026269
fdnG	Formate dehydrogenase	0.942	0.00034
fdnH	Formate dehydrogenase	1.283	0.008418
fdnI	Formate dehydrogenase	1.405	0.036096
narH	Nitrate reductase. reduces nitrate to nitrite	0.957	0.007741
narI	Forms a respiratory chain with formate dehydrogenase	1.318	0.028437
narJ	Forms a respiratory chain with formate dehydrogenase	1.159	0.000942
napA	Nitrate reductase	1.066	0.002103
napB	Nitrate reductase	1.071	0.002724
napC	Nitrate reductase	0.921	0.013884
napG	Nitrate reductase	1.121	0.002918
nirB	Reduces nitrite to ammonia	1.236	0.001582
nirC	Reduces nitrite to ammonia	0.898	0.003501
nirD	Reduces nitrite to ammonia	1.04	0.018341
hcp	Scavenger of toxic by-products of nitrogen metabolism	1.11	0.000099
hcr	Scavenger of toxic by-products of nitrogen metabolism	1.131	0.00182
yagU	Acid resistance	1.272	0.001399
uspA	Induced in response to a variety of stresses	1.254	0.002062
yfiA	Cold shock protein related to 30S	1.37	0.007329
frdB	Fumarate reductase, flagellar motility	1.118	0.003225
frdC	Fumarate reductase, flagellar motility	1.012	0.001462

papC_2 | papC	P fimbria	1.125	0.003409
papD_2	P fimbria	0.99	0.070153
PapE	P fimbria	1.434	0.082258
papK_2 | papK	P fimbria	1.233	0.019907
focA	F1C fimbria	1.455	0.00342
focC	F1C fimbria	1.016	0.007778
sfaB	F1C fimbria	1.09	0.004029
sfaD	F1C fimbria	1.034	0.006438
oppA	Oligopeptide transporter	1.197	0.000418
oppB	Oligopeptide transporter	0.952	0.00577
oppD	Oligopeptide transporter	0.957	0.000882
oppF	Oligopeptide transporter	1.003	0.000224
ompC	Outer membrane protein	1.296	0.000324
deoB	Phosphopentomutase	0.997	0.00036
deoD	Phosphopentomutase	0.957	0.007274
manX	Phosphotransferase system (PTS), mannose	0.912	0.030577
manY	Phosphotransferase system (PTS), mannose	0.924	0.006227
manZ	Phosphotransferase system (PTS), mannose	1.007	0.018686
leuB	Amino acid biosynthesis	1.361	0.000511
leuC	Amino acid biosynthesis	1.314	0.002602
leuD	Amino acid biosynthesis	1.261	0.000873
ilvC	Amino acid biosynthesis	1.008	0.000492
ilvH	Amino acid biosynthesis	0.999	0.001171
ilvN	Amino acid biosynthesis	0.965	0.006679
ilvY	Amino acid biosynthesis	0.815	0.001236

Table 17. Commonly upregulated genes in re-isolates BU04 63630 and BU05 35364

Gene	**Function**	**Ratio**	**Ratio**	**p-value**	**p-value**
		BU05 35364	**BU04 63630**	**BU05 35364**	**BU04 63630**
osmB	Osmotic stress, lipoprotein	1.996	1.92	0.021315	0.011682
groEL	Chaperonin, heat-shock proteins	1.205	1.212	0.029904	0.007058
cnf1	Cytotoxic necrotizing factor	1.364	0.944	0.049088	0.008995
ydhA	Lysozyme inhibitor, predicted lipoprotein	1.106	1.017	0.004189	0.011748
rpmB	50S ribosomal protein	1.43	1.299	0.014022	0.001217
rpmG	50S ribosomal protein	1.482	1.344	0.012841	0.001111
yohN	Unknown function	1.337	1.154	0.01608	0.004048
ypeC	Unknown function	1.332	1.609	0.027133	0.007874

Table 18. Commonly upregulated genes in re-isolates BU04 63630 and FIM 636

Gene	**Function**	**Ratio**	**Ratio**	**p-value**	**p-value**
		BU04 63630	**FIM 636**	**BU04 63630**	**FIM 636**
yjbE	Biofilm Formation	1.277	1.064	0.001571	0.000489
yqjD	Biofilm Formation	0.994	0.864	0.000166	0.010177
papH	P fimbria	1.021	1.936	0.002403	0.002768

gudD	Lyase and transferase	1.073	0.801	0.000271	0.004584
gudP	Lyase and transferase	1.293	0.906	0.000394	0.001035
rplF	50S ribosomal protein	0.964	0.881	0.001304	0.000326
rplO	50S ribosomal protein	1.208	0.974	0.001304	0.000326
rplQ	50S ribosomal protein	1.029	0.884	0.006106	0.002628
rplR	50S ribosomal protein	1.032	0.927	0.003437	0.000848
rplV	50S ribosomal protein	0.979	0.908	0.000892	0.001226
rplX	50S ribosomal protein	0.871	0.976	0.001673	0.007648
rpsC	30S ribosomal protein	0.981	0.948	0.000118	0.005782
rpsE	30S ribosomal protein	0.993	0.919	0.011663	0.001632
rpsH	30S ribosomal protein	0.965	0.887	0.008411	0.001092
rpsI	30S ribosomal protein	0.917	0.811	0.010743	0.000347
rpsN	30S ribosomal protein	0.976	0.96	0.00271	0.004157

Table 19. Commonly upregulated genes in re-isolates BU05 35364 and FIM 636

Gene	**Function**	**Ratio BU05 35364**	**Ratio FIM 636**	**p-value BU05 35364**	**p-value FIM 636**
dppA	Dipeptide transporter	0.801	1.103	0.012405	0.001699
dppB	Dipeptide transporter	0.901	0.805	0.05888	0.002997
yrdA	Transferase. hypothetical	1.076	1.208	0.031021	0.000603
eno	Phosphopyruvate hydratase	1.198	1.356	0.018329	0.003002
papF	P fimbria	1.243	1.752	0.018911	0.000383

Table 20. Commonly upregulated genes in re-isolates BU04 63630. BU05 35364 and FIM 636

Gene	**Function**	**Ratio BU05 35364**	**Ratio BU04 63630**	**Ratio FIM 636**	**p-value BU05 35364**	**p-value BU04 63630**	**p-value FIM 636**
yjgb	Alcohol dehydrogenase, RpoS regulon	1.094	1.036	1.426	0.0043	0.00085	0.000005
degP	Survival at high temperatures	0.876	1.382	0.835	0.0091	0.00445	0.015322
bipA	GTP-binding. colanic acid synthesis	1.501	1	1.019	0.0112	0.00891	0.000646
ECP_0113	Colicin production	1.558	1.015	1.059	0.0381	0.00424	0.02762
papA	P fimbria	0.918	1.556	2.056	0.0069	0.00119	0.002195
c0391	Integrase	1.315	1.226	1.207	0.0788	0.00007	0.002303
UTI89_C3360	Unknown function	1.239	1.019	1.071	0.0171	0.03126	0.000793
ytjA	Unknown function	1.041	1.413	1.147	0.0087	0.00026	0.003503

Table 21. Downregulated genes in re-isolates BU04 63630

Gene	**Function**	**Ratio**	**p-value**
dctA	dicarboxylate transport	-1.446	0.0282
mglA	galactose ABC transporter	-2.171	0.08096
mglB	galactose ABC transporter	-1.762	0.08532
malT	MalT-Maltotriose-ATP transcriptional activator	-1.765	0.01577

ygeV	putative transcriptional regulator	-1.586	0.05885
nrfA	formate-dependent nitrite reductase	-1.903	0.00841
nrfB	formate-dependent reduction of nitrite to ammonia	-1.326	0.0641
nrfC	enables formate dehydrogenase H to transfer electrons into an electrogenic electron-transfer chain	-1.182	0.08167
nmpC	Phage-related	-4.011	0.04418
ychH	Inner membrane protein	-1.881	0.06059

Table 22. Downregulated genes in re-isolates BU05 35364

Gene	**Function**	**Ratio**	**p-value**
agaB	Phosphotransferase System	-1.414	0.05281
agaV	Phosphotransferase System	-1.337	0.01381
agaW	Phosphotransferase System	-1.356	0.04572
srlE_1	Phosphotransferase System	-1.623	0.00907
srlR	Phosphotransferase System	-1.037	0.01128
gutM	transport and utilization of glucitol	-1.268	0.04317
yadD	predicted transposase	-1.575	0.00041
dgoD	galactonate dehydratase	-1.17	0.00075
dgoT	functions as a galactonate/proton symporter	-1.537	0.00075

Table 23. Downregulated genes in re-isolates FIM 636

Gene	**Function**	**Ratio**	**p-value**
ybiP	predicted hydrolase, inner membrane	-1.57	0.02598

Table 24. Commonly downregulated genes in re-isolates BU05 35364 and BU04 63630

		BU05 35364	**BU04 63630**	**BU05 35364**	**BU04 63630**
Gene	**Function**	**Ratio**	**Ratio**	**p-value**	**p-value**
lysU	lysine tRNA synthetase, inducible	-0.945	-1.342	0.036	0.0129
kbaZ	tagatose 6-phosphate aldolase 1, kbaZ subunit	-2.066	-1.026	0.09981	0.0106
ykgC	pyridine nucleotide-disulfide oxidoreductase	-1.042	-1.324	0.01502	0.0177
rbsA	ribose ABC transporter	-1.784	-1.024	0.00599	0.0003
rbsD	ribose ABC transporter	-1.45	-1.206	0.00359	0.0157
ybhD	predicted DNA-binding transcriptional regulator	-1.538	-1.474	0.0028	0.0418

Table 25. Commonly downregulated genes in re-isolates BU05 35364 and FIM 636

		BU05 35364	**FIM 636**	**BU05 35364**	**FIM 636**
Gene	**Function**	**Ratio**	**Ratio**	**p-value**	**p-value**
cspG	cold shock. response to stress	0.81	-1.677	0.0263	0.0006

8.5. Maps of Plasmids and Constructs

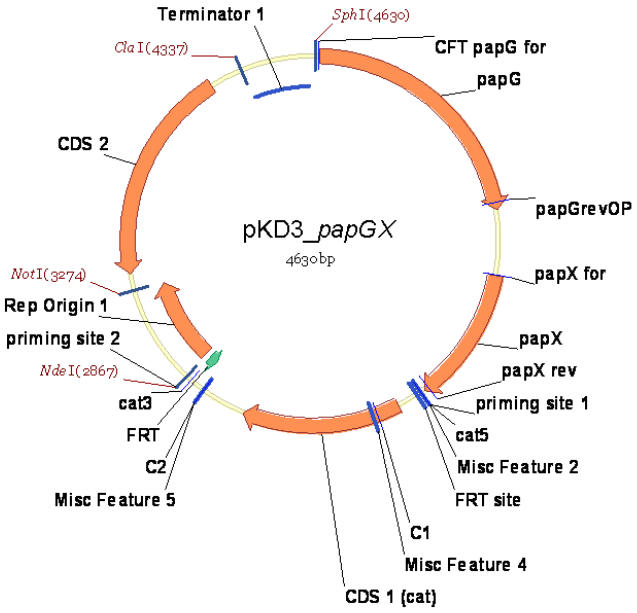


Figure 49. Plasmid construct pKD3 with *papGX* from CFT073 inserted upstream of *cat.* Primer binding sites are shown.

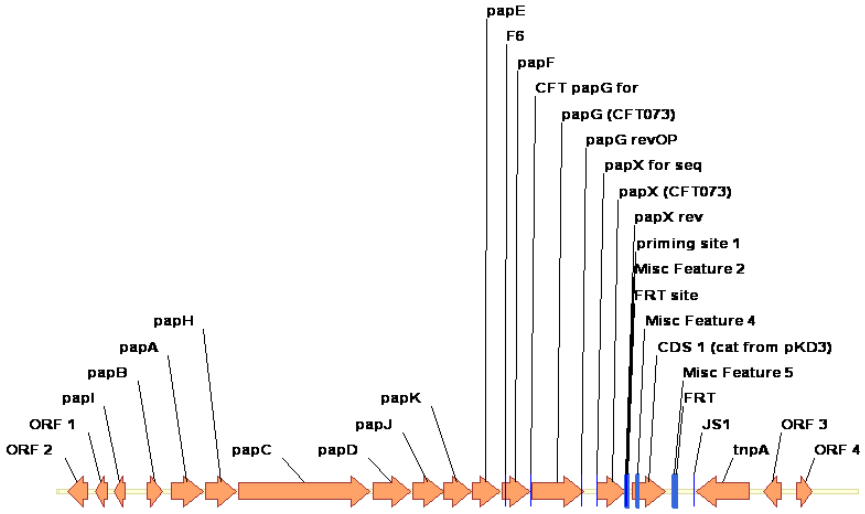


Figure 50. ABU 83972 with reconstituted *papGX* from CFT073. Primer binding sites are shown.

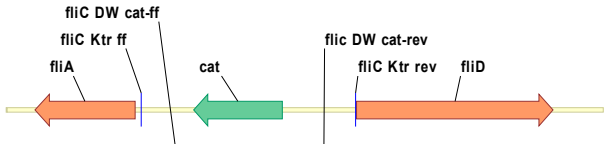


Figure 51. Flagellin gene (*fliC*) deleted from ABU 83972. Primer binding sites are shown.

8.6. Curriculum Vitae

Personal Data

Name	Ellaine Riciel del Pilar Salvador
Birthdate	August 26, 1978
Birthplace	San Jose City, Nueva Ecija, Philippines

Education

1991-1995	Saint Joseph School, San Jose City, Philippines High School Diploma Graduated Class Valedictorian and Gerry Roxas Leadership Awardee
1995-1996	Saint Louis University, Baguio City, Philippines
1996-1999	Central Luzon State University, Science City of Muñoz, Philippines Bachelor of Science in Biology, major in Zoology Awardee, Most Outstanding Biology Student
2001-2003	Central Luzon State University, Science City of Muñoz, Philippines Master of Science in Biology (18 credit units)
2003-2005	Wageningen University and Research Center Wageningen, the Netherlands Master of Science in Biotechnology, specialization in Cellular and Molecular Biotechnology Fellow, the Netherlands Organization for International Cooperation in Higher Education (NUFFIC)
June 2007	Start of PhD Dissertation at the Institute for Molecular Infection Biology, Julius-Maximillian University, Würburg, Germany Fellow, Graduate School of Life Sciences (German Excellence Initiative)

Practical Period

2002	Animal Biotechnology Laboratory Philippine Carabao Center Science City of Muñoz, Philippines
2004-2005	Laboratory of Virology Wageningen University and Research Center Wageningen, the Netherlands

8.7. Publications and Presentations

Publications

Salvador E, Wagenlehner F, Köhler C-D, Mellmann A, Hacker J, Svanborg C, Dobrindt U. 2011. Asymptomatic bacteriuria *E. coli* isolates from healthy individuals versus hospital patients: long-term bladder colonization selects for attenuated virulence phenotypes. Infection and Immunity. *Accepted.*

Dobrindt U, Zdziarski J, **Salvador E**, Hacker J. 2010. Bacterial genome plasticity and its impact on adaptation during persistent infection. International Journal of Medical Microbiology. 300 (6): 363-6.

Fischer H, Lutay N, Ragnarsdóttir B, Yadav M, Jönsson K, Urbano A, Al Hadad A, Rämisch S, Storm P, Dobrindt U, **Salvador E**, Karpman D, Jodal U, Svanborg C. 2010. Pathogen specific, IRF3-dependent signaling and innate resistance to human kidney infection. PLoS Pathogens 6(9): e1001109, doi:10.1371/journal.ppat.1001109.

Presentations

Salvador E, Hacker J, Svanborg C, Wullt B, Dobrindt U. 2008. Characterization of asymptomatic bacteriuria (ABU) *Escherichia coli* isolates: virulence traits and host-pathogen interactions. Summer School, Pathogen-Host Interactions at Cellular Barriers. Muenster, Germany. Poster Presentation.

Salvador E, Svanborg C, Wullt B, Hacker J, Dobrindt U. 2009. Characterizing *Escherichia coli* asymptomatic bacteriuria (ABU) what drives their successful silent colonization? 61st Annual Meeting of the DGHM (German Society for Hygiene and Microbiology). Göttingen, Germany. Poster Presentation.

Salvador E, Hacker J, Svanborg C, Wullt B, Dobrindt U. 2009. *Escherichia coli* Asymptomatic Bacteriuria (ABU) Isolates- what could have gone wrong?" 4th Students´ Meeting NoE EPG Graduate Academy, Palma De Mallorca, Spain. Poster and Poster Introduction Talk.

Salvador E, Hacker J, Svanborg C, Wullt B, Dobrindt U. 2010. Analyzing *E. coli* factors involved in host-bacterium interaction during asymptomatic colonization of the urinary bladder. 3rd Joint Conference of the DGHM (German Society for Hygiene and Microbiology) and VAAM (Association for General and Applied Microbiology). Hannover, Germany. Poster Presentation.

Salvador E. 2010. *E. coli* asymptomatic bacteriuria (ABU) factors involved in host-bacterium interaction during urinary bladder colonization. Graduate School of Life Sciences Fellows Symposium. Oral Presentation**.**

8.8. Abbreviations

µg	microgram
µl	microliter
ABU	asymptomatic bacteruria
Amp	ampicillin
APS	ammonium persulfate
bp	base pairs
BSA	bovine serum albumin
CFU	colony forming unit
Cm	chloramphenicol
del	deletion
DEPC	diethyl pyrocarbinate
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	deoxyribonucleotide phosphate
DTT	dithiothreitol
ECOR	*E. coli* group of reference strains
EDTA	ethylendiamine tetra-acetate
et al.	et altera (and others)
EtOH	ethanol
ExPEC	extra-intestinal pathogenic *E. coli*
Fim	type 1 fmbriae
g	gram
h	hour
ID	identification
i.e.	id est (this means)
IL 6	Interleukin 6
IL 8	Interleukin 8
Kan	kanamycin
kb	kilo bases
kDa	kilo Dalton
l	liter
LB	lysogeny broth
LPS	lipopolysaccharide acid
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
MLST	Multi Locus Sequence Typing
mm	millimeter
mM	millimolar
ng	nanogram
nt	nucleotides
OD	optical density
ORF	open reading frame
PAI	pathogenicity island
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PIA	polysaccharide intercellular adhesin
PMN	polymorphonuclear cell
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
qRT-PCR	real time PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
s	second
TBS	Tris buffered saline
TAE	Tris-acetate-EDTA
TLR	toll like receptor
U	unit
UPEC	uropathogenic *E.coli*
UTI	urinary tract infection
wt	wild type