Investigating enteroaggregative *Escherichia coli* virulence factors in human intestinal infection

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Abstract

Enteroaggregative *Escherichia coli* (EAEC) are a major cause of diarrhoeal illness in children, travellers, and the immunocompromised, and associated with foodborne outbreaks worldwide. However, EAEC is a heterogeneous pathotype with frequent asymptomatic carriage and a diversity of virulence factors. Previous studies have been unsuccessful in identifying genetic virulence markers. In this study, two complimentary approaches were applied using intestinal infection models to investigate bacterial factors contributing to EAEC pathogenesis in the human gut.

Firstly, the influence of intestinal environmental signals on the expression of putative EAEC virulence genes was evaluated using a vertical diffusion chamber (VDC). Aerobic oxygen levels increased expression of the adhesins aggregative adherence fimbriae II (AAF/II) and *E. coli* common pilus, the colonisation factor dispersin, and the global transcriptional activator AggR in prototype strain 042. Furthermore, adherence to polarised T84 intestinal epithelial cells significantly enhanced the expression of adherence factors (AAFs and dispersin), toxins (HlyE, EAST-1, Pet) and the Pic mucinase. This induction required host cell binding and was independent of AggR regulation. Based on these findings, it is proposed that EAEC adherence factors are induced by proximity to the oxygen diffusion gradient across the gut epithelium, while epithelial cell contact activates expression of further virulence factors.

As an alternative approach to identify EAEC pathogenicity markers, virulence-associated phenotypes and genotypic profiles were determined for EAEC sequence types associated with disease (ST40) or carriage (ST31). ST40 isolates exhibited significantly higher biofilm formation and adherence to T84 cells and human colonic biopsies. The genotype comparison identified differences in virulence genes associated with epithelial colonisation and induction of host inflammatory responses between both sequence types.

Overall, this project has revealed that EAEC virulence gene expression is modulated by intestinal environmental signals and identified phenotypic and genotypic traits specific for EAEC sequence types associated with disease or carriage.

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A/E	Attaching and effacing
AA	Aggregative adherence
AAF	Aggregative adherence fimbriae
Aap	Anti-aggregation protein / dispersin
AE	Aerobic
AIEC	Adherent-invasive *E. coli*
AN	Anaerobic
APEC	Avian pathogenic *E. coli*
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cdt	Cytolethal distending toxin
CFU	Colony forming units
CK8	Cytokeratin-8
C_T	Cycle threshold
DA	Diffuse adherence
DAEC	Diffusely-adherent *E. coli*
DAPI	4',6-diamidino-2-phenylindole (DNA stain)
ddH_2O	distilled deionised water
DEC	Diarrheagenic *E. coli*

DMEM	Dulbecco's modified Eagle's medium
EAEC	Enteroaggregative *E. coli*
EAST-1	EAEC heat-stable toxin 1
ECM	Extracellular matrix
ECP	*E. coli* common pilus
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
GBRU	Gastrointestinal bacteria reference unit
GIT	Gastrointestinal tract
HIV	Human immunodeficiency virus
HlyE	Haemolysin E
Hra1	Heat-resistant agglutinin
HUS	Haemolytic uraemic syndrome
IBS	Irritable bowel syndrome
IID	Infectious intestinal disease
IL	Interleukin
IVOC	*In vitro* organ culture
LA	Localised adherence
LB	Luria Bertani

LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
MA	Microaerobic
MAL-ED	Malnutrition and enteric disease
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
NMEC	Meningitis-associated *E. coli*
NTC	Non-template control
ONC	Overnight culture
pAA	Aggregative adherence plasmid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pet	Plasmid encoded toxin
PHE	Public Health England
Pic	Protein involved in colonisation
qPCR	Quantitative reverse transcription PCR
rpm	Revolutions per minute
RSB	Reducing sample buffer
RT	Room temperature
RTC	Reverse-transcription control
Sat	Secreted autotransporter toxin
SCEM	Simulated colonic environment medium

SE	Standard error (of the mean)
SEM	Scanning electron microscopy
ShET1	*Shigella* enterotoxin 1
SIEM	Simulated ileal environment medium
SNP	Single nucleotide polymorphism
SPATE	Serine protease autotransporters of Enterobacteriaceae
SRST2	Short read sequence typing 2 tool
ST	Sequence type
Stx	Shiga toxin
T3S(S)	Type III secretion (system)
TEER	Transepithelial electrical resistance
TIF	Type I fimbria
T_m	Melting temperature
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
VDC	Vertical diffusion chamber
VFDB	Virulence factors of bacterial pathogens database
WGS	Whole-genome sequencing

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CHAPTER ONE

INTRODUCTION

1.1: Enteroaggregative *Escherichia coli*

In the field of microbiology, *Escherichia coli* are a paradigm for the versatility of a bacterial species. *E. coli* are the most abundant facultative anaerobes in the human gastrointestinal tract (GIT) and the majority of strains are harmless commensals (Kaper *et al*., 2004). It is estimated that individuals are colonised by an average of five *E. coli* strains within their intestinal microbiota, where they participate in nutrient processing and scavenging oxygen in symbiosis with strictly anaerobic species (Maltby *et al*., 2013, Apperloo-Renkema *et al*., 1990). Commensal *E. coli* also contribute to resistance against enteric pathogens, through diverse mechanisms including adherence exclusion, restriction of nutrients such as carbon sources and iron, and secretion of antimicrobial compounds (Sassone-Corsi and Raffatellu, 2015). In addition to a beneficial role in the human microflora, *E. coli* strains have long held a unique importance as the predominant bacterial model and tool for molecular biology, to the extent that the laboratory strain *E. coli* K12 was amongst the first published full genomes (Blattner *et al*., 1997, Luo *et al*., 2011).

However, a minority of *E. coli* strains have acquired virulence genes during evolution which confer the ability to cause disease in a human host. Such pathogenic strains are diverse in genetics and colonisation niches and have been categorised on emergence into groups termed pathotypes (**Figure 1.1**) (Croxen *et al*., 2013, Kaper *et al*., 2004). The first identified pathogenic *E. coli* strains were isolated from cases of infant diarrhoea in the United Kingdom (UK) in the 1940s and represented what was later recognised as the pathotype enteropathogenic *E. coli* (EPEC) (Bray, 1945). Other diarrheagenic *E. coli* (DEC) pathotypes which colonise the human GIT are: enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC, which includes *Shigella*), enterotoxigenic *E. coli* (ETEC), and diffusely-adherent *E. coli* (DAEC). A further enteric pathotype is adherent-invasive *E. coli* (AIEC), although this group has been implicated in inflammatory bowel disease rather than diarrhoeal illness specifically. *E. coli* virulence is not restricted to the gut, with the two major extraintestinal pathotypes being uropathogenic *E. coli* (UPEC), associated with bladder and urinary tract infections,

and neonatal meningitis-associated *E. coli* (NMEC), which colonises the cardiovascular system and brain.

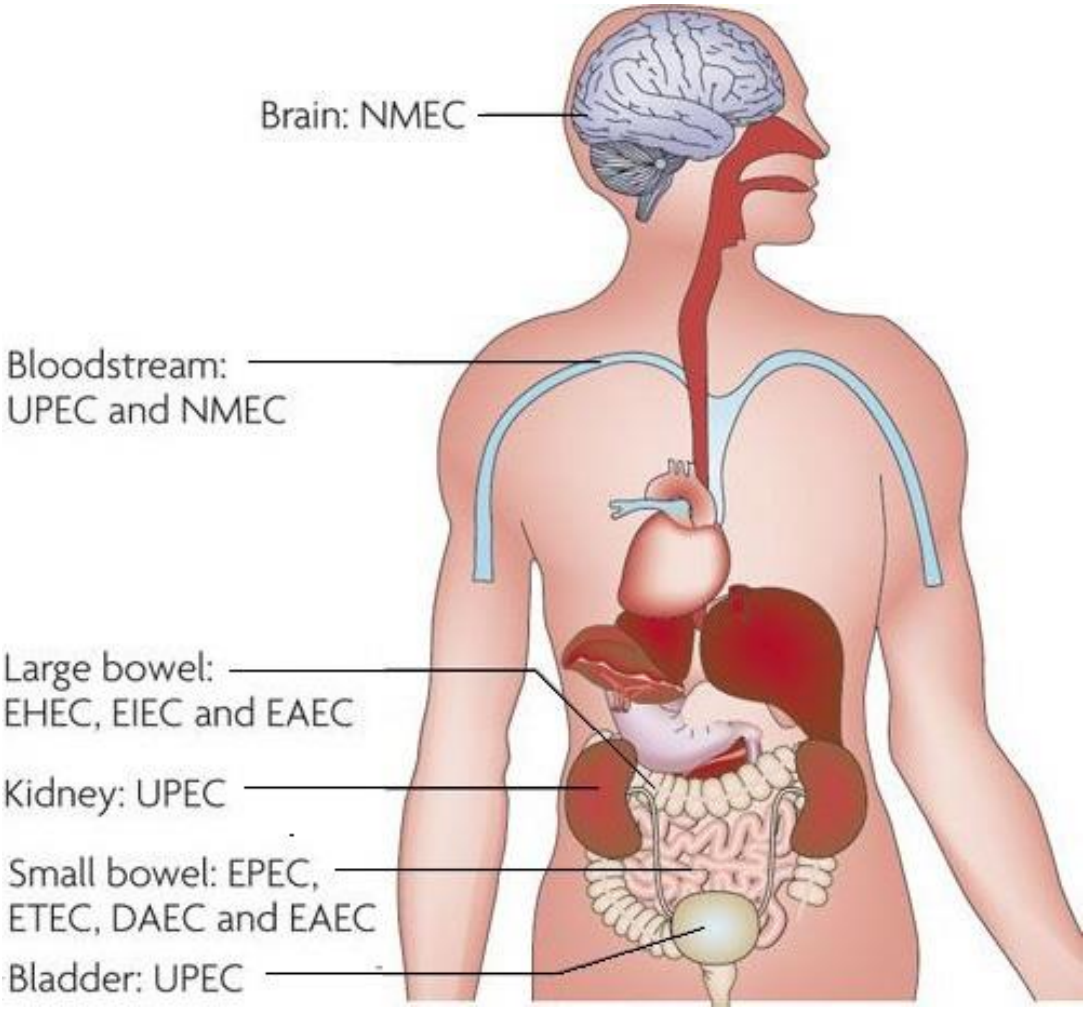

Figure 1.1 Colonisation sites of *E. coli* pathotypes. Modified from (Croxen and Finlay, 2010)

DEC are highly genetically diverse global pathogens. For example, one study of 1196 clinical DEC isolates using Multi-Locus Sequence Typing (a typing method discussed in detail in Chapter 1.3.4), identified 579 distinct sequence types grouped into 27 clonal complexes (Yu *et al*., 2018). The population structure is also diverse, with some sequence type groups restricted to specific pathotypes while others are highly heterogeneous for different pathotypes (**Figure 1.2**). Genetic diversity has also been demonstrated using pulsed-field gel electrophoresis, such as a collection of DEC from human, animal and environmental sources in India which contained 52 unique pulsotypes for 59 recovered DEC pathotype samples (Dhaka *et al*., 2016).

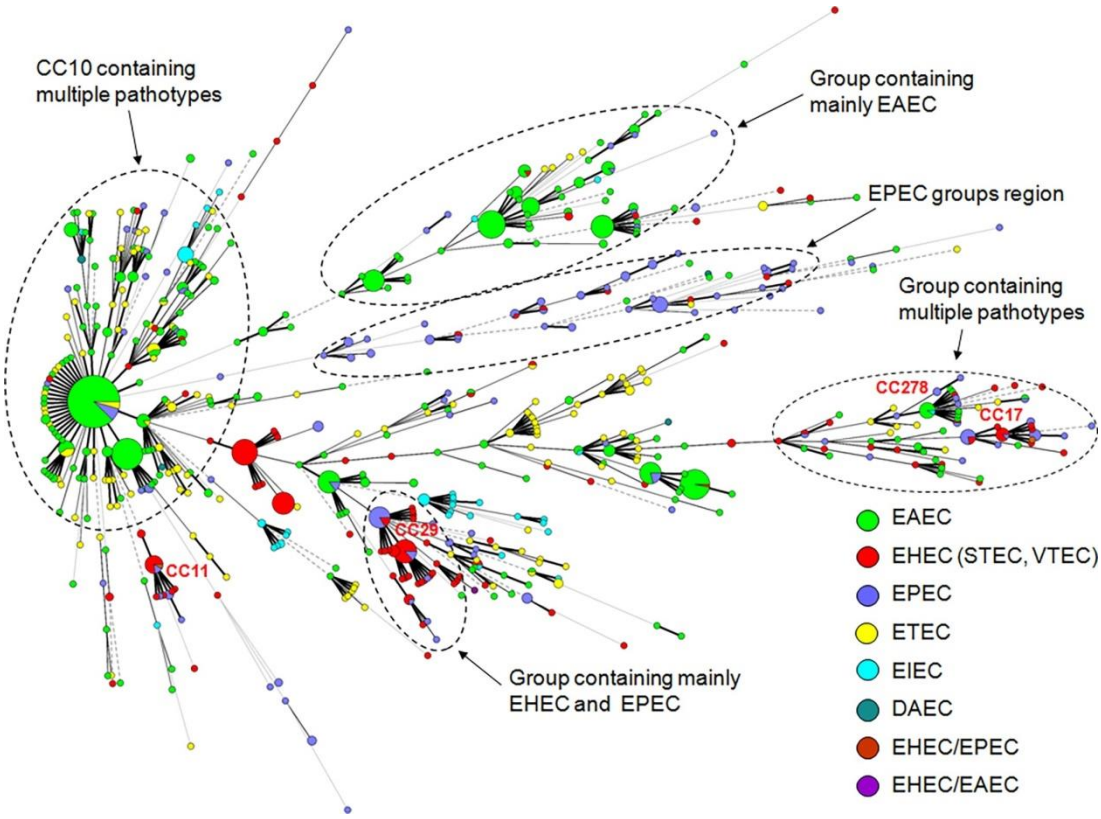

Figure 1.2 Diversity of diarrheagenic *E. coli*. Minimum spanning tree analysis of 1196 human isolates by multi-locus sequence typing. Connecting lines indicate six (thick lines), five (thin), four (dashed) or three to one (dotted) shared alleles, out of seven (Yu *et al*., 2018).

EAEC is a relatively recent pathotype, only reported as a distinct group since the late 1980s (Nataro *et al*., 1987). However, it has been increasingly recognised as an important emerging enteric pathogen, associated with diarrhoeal illness in populations worldwide and frequently the most common bacterial pathogen identified in diarrhoeal stool samples during epidemiological studies (Croxen *et al*., 2013, Huang *et al*., 2006b). Although improvements in sanitation, nutrition, and medical care have seen global deaths due to diarrhoea decrease by an estimated 20% between 2005 and 2015, there remains an annual 1.31 million deaths, including approximately 500,000 children under the age of five years, due to a largely preventable disease (Troeger *et al*., 2017). Additionally, repeated or chronic enteric infections are associated with long-term clinical disadvantages, including malnutrition, a weakened immune system, and impaired growth and development (Guerrant *et al*., 2008). The EAEC pathotype has been specifically associated with

persistent diarrhoeal illness in children and the immunocompromised, representing a major health burden (Lima *et al*., 2017b, Rogawski *et al*., 2017).

Many aspects of EAEC pathogenesis remain unclear, and the pathotype has not been as well characterised as other DEC such as EPEC and EHEC. This is largely due to the genetic heterogeneity of EAEC as a whole, which includes significant variability between strains in pathogenicity and putative virulence genes (Hebbelstrup Jensen *et al*., 2014). This chapter will provide an overview of the current understanding of EAEC infection biology, including clinical epidemiology, reported virulence mechanisms, and the strengths and limitations of available infection model systems for this pathotype.

1.2: Emergence

The classical definition of EAEC is based on the ability to form the characteristic "stacked-brick" aggregative adherence (AA) phenotype on cultured HEp-2 cells, with this pattern playing an important role in the original determination of the pathotype (Villaseca *et al*., 2005, Lacroix, 2008). The initial observations of distinct phenotypes of HEp-2 cell adherence by enteropathogenic serotypes of *E. coli* date back to research by Cravioto *et al*. in 1979, with some EPEC forming microcolonies described as localised adherence (LA) (Cravioto *et al*., 1979). However, other *E. coli* strains also adhered to HEp-2 cells with a phenotype described as diffuse adherence (DA), which was subsequently further subdivided into separate AA and true diffuse categories (**Figure 1.3**) (Nataro *et al*., 1987, Nataro and Kaper, 1998). It was a result of these findings that enteropathogenic strains displaying AA were suggested as a new category of DEC. The original name 'enteroadherent-aggregative *E. coli*' was eventually shortened to enteroaggregative *E. coli*, with the abbreviations EAggEC or EAEC alternatively used in the literature.

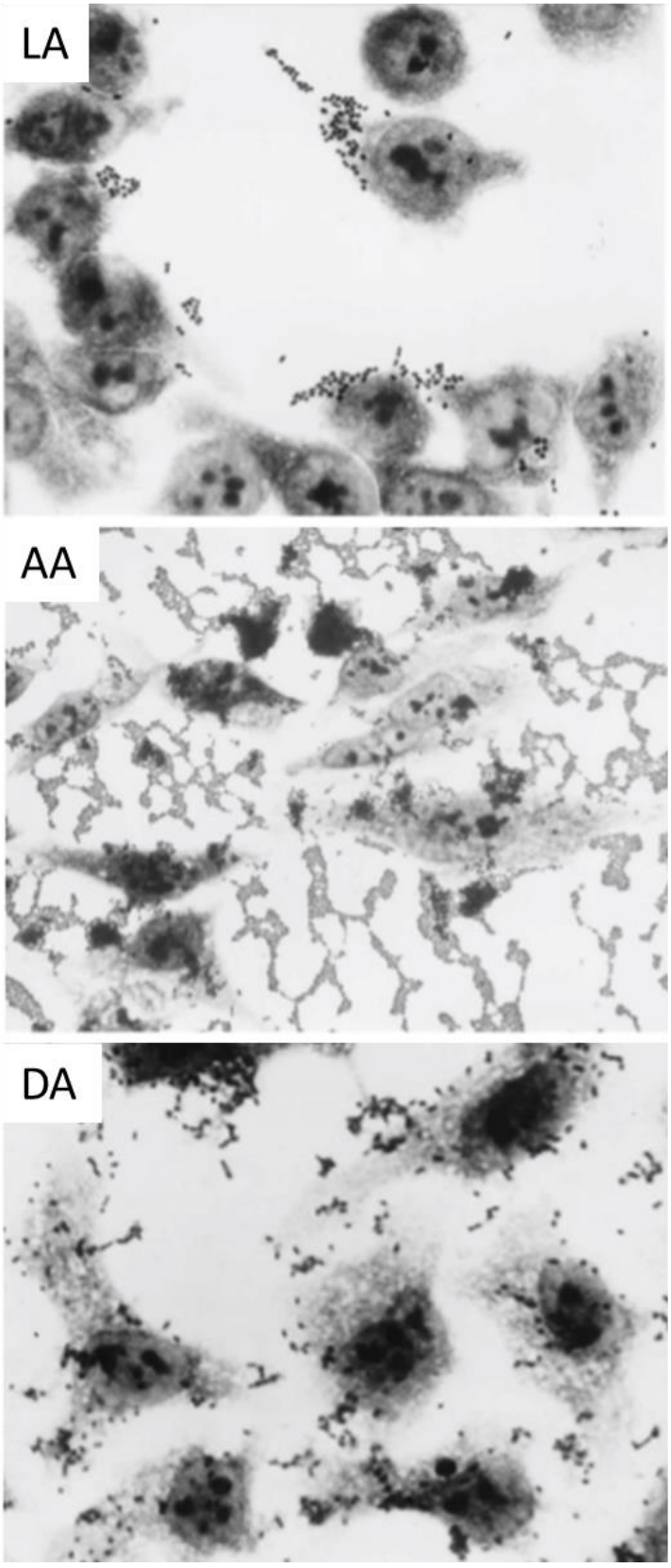

Figure 1.3 Adherence patterns of diarrhoeagenic *E. coli* on HEp-2 cells. The EAEC pathotype is characterised by the aggregative adherence (AA) phenotype on cultured HEp-2 cells, distinct from the localised adherence (LA) pattern of microcolonies associated with EPEC and the diffuse adherence (DA) phenotype of DAEC. Adapted from (Polotsky *et al*., 1997)

An evolutionary relationship has been determined for certain DEC pathotypes. An example is the strong evidence that EHEC emerged from an EPEC background. Both groups share virulence features such as the formation of attaching/effacing (A/E) lesions on intestinal epithelial cells and the locus of enterocyte effacement (LEE) pathogenicity island of virulence genes associated with the A/E phenotype (Mellies *et al*., 2007). EHEC strains have evolved from EPEC by the gain of additional virulence factors, most notably the acquisition of phage-encoded Shiga toxin genes (Reid *et al*., 2000). The evolutionary history of EAEC is less well-defined. Phylogenetic analysis of EAEC populations has determined that the characteristic AA is a convergent phenotype, arising independently in multiple lineages and selected by survival advantages in the human host environment. While certain lineages are prevalent in multiple global locations, indicative of clonal expansion, the heterogeneity of EAEC is linked to the parallel evolution of the AA phenotype in lineages with diverse genotypes and virulence (Chattaway *et al*., 2014b, Okeke *et al*., 2010).

1.3: Epidemiology

It is extremely difficult to accurately determine the incidence of any enteric pathogen due to the challenges of determining causative agents of gastrointestinal illness and the under-reporting of such disease in the community (Flint *et al*., 2005). However, since its description as a separate DEC pathotype it has been increasingly recognised that EAEC is a major contributor to the global burden of diarrhoeal disease (Huang *et al*., 2006a, Walker *et al*., 2013). Epidemiological studies in diverse geographical locations have consistently identified EAEC as one of the most common causes of diarrhoeal illness in children and travellers worldwide, in many cases being isolated more frequently than any other DEC (Rajendran *et al*., 2010, González *et al*., 1997, Sarantuya *et al*., 2004). In addition, the overall impact of EAEC disease may be greatly underestimated, as it is associated with persistent diarrhoea which is a risk factor for malnutrition and compromised immunity (Roche *et al*., 2010). The global incidence of EAEC is unclear, as it is not commonly screened for in global disease burden studies unlike established pathogens such as Rotavirus,

Shigella, *Campylobacter,* and *Salmonella* species. However, the epidemiological prevalence of EAEC strains associated with gastrointestinal illness suggests a major contribution to the annual estimate of 2.39 billion global episodes of diarrhoea (Troeger *et al*., 2017)

1.3.1: Association with disease in global populations

The importance of EAEC as an enteric pathogen was originally not well recognised. Following the identification of EAEC as a separate DEC pathotype, epidemiological studies resulted in initial uncertainty on the true pathogenicity of such strains. While some early case studies failed to show a significant association between EAEC infection and diarrhoea (Gomes *et al*., 1989), others concluded that EAEC was likely an enteric pathogen playing a causal role for diarrhoea in children (Paul *et al*., 1994, Bhatnagar *et al*., 1993). A stronger association was eventually established by Nataro *et al*. when a volunteer study was conducted where the prototype strain 042 elicited diarrhoea in adults. However, three other strains also isolated from infant diarrhoeal cases failed to induce any disease response, showing the heterogeneity of EAEC pathogenicity (Nataro *et al*., 1995).

Over the last 20 years, many case-control and cohort studies have increasingly shown both the prevalence of EAEC carriage and an association with diarrhoea. For example, a case-control study performed between 2003 and 2006 in children in South India found that EAEC was the most common pathotype of DEC detected in both clinical cases and control groups, although isolates expressing 3 specific virulence markers (*aap*, *aggR*, and *aat*) were significantly associated with diarrhoea (Rajendran *et al*., 2010). In 2006, Huang *et al*. published a meta-analysis examining the existing data. This confirmed that EAEC was significantly associated with acute diarrhoeal illness in many subpopulations including: children and adults in developing regions, children in industrialised regions, international travellers to developing regions, and adults with human immunodeficiency virus infection residing in developing regions (Huang *et al*., 2006b). As such, although the pathotype is highly heterogeneous, EAEC can be considered an important causal agent of diarrhoeal disease.

Many of the epidemiological surveys in the existing literature focus on the level of EAEC infection in developing countries. EAEC has repeatedly been demonstrated as a significant contributor to diarrhoeal illnesses in diverse geographical locations. For example, a study of 513 infant patients between 1993 and 1995 in Caracas, Venezuela found that EAEC was the second-most commonly isolated enteropathogen (26.9% of patients) after Rotavirus (González *et al*., 1997). A case-control study of 587 children with diarrhoea and 249 age-matched healthy controls in Vietnam identified EAEC as the predominant bacterial pathogen (although again less frequent than Rotavirus) and most associated with disease in infants below two years of age (Vu Nguyen *et al*., 2006). More recently, the malnutrition and enteric disease (MAL-ED) longitudinal study screened stools from over 2092 infants from eight global low-income regions during the first two years of life. EAEC was isolated at least once from 94.8% of the cohort, and was associated with intestinal inflammation and growth reduction (Rogawski *et al*., 2017). Often studies have shown a particularly significant association between EAEC and chronic or persistent diarrhoea, usually defined as lasting more than 14 days (Lima *et al*., 1992, Bhan *et al*., 1989). While therapies for acute diarrhoea continue to improve in developing countries, persistent illness remains a great concern, especially in infants (Fang *et al*., 1995, Troeger *et al*., 2017, Kotloff *et al*., 2013). In general, EAEC appears to be an important emerging threat in paediatric diarrhoea. This is evident in the results of the meta-analysis by Huang *et al*., which estimated EAEC to be the cause of acute diarrhoeal illness in children for 4% of cases in industrialised nations, and 15 % in developing countries (Huang *et al*., 2006b, Huang *et al*., 2006a).

EAEC is also being increasingly recognised as a prevalent enteropathogen in industrialised nations. An early case-control study established that this pathotype was both more prevalent and more strongly associated with diarrhoea than EPEC in Scandinavia (Bhatnagar *et al*., 1993). Since then, similar results have been obtained in many developed countries, including the UK, Austria and Japan (Knutton *et al*., 2001, Presterl *et al*., 1999, Itoh *et al*., 1997). As in lower income regions, EAEC is most commonly associated with acute and persistent diarrhoea in infants and children (Itoh *et al*., 1997, Knutton *et al*., 2001, Tobias *et al*., 2015, Chan *et al*., 1994). However, EAEC cases in adult patients are also significant as demonstrated

by a Swedish study which identified EAEC as the second-most frequently isolated DEC (after ETEC) in cases of adult diarrhoea (Svenungsson *et al*., 2000).

Another population associated with EAEC-induced diarrhoea are travellers from industrialised countries to developing regions (Jiang *et al*., 2002, Adachi *et al*., 2002a). This was established in a large study by Adachi *et al*., which found that EAEC was isolated from 26% of travellers' diarrhoea cases studied in areas of Mexico, Jamaica, and India. This made it second only to ETEC in prevalence, and it was proposed that EAEC may account for a large proportion of the previously recorded cases with an unknown pathogen that still responded well to antimicrobial therapy (Adachi *et al*., 2001). In a study of travellers returning to Finland from subtropical destinations, EAEC colonisation was significantly associated with ongoing travellers' diarrhoeal symptoms (Laaveri *et al*., 2016). EAEC has also been reported as prevalent in traveller's diarrhoea cases in children (Pouletty *et al*., 2018).

A further group which may be affected by EAEC infection are immunocompromised patients. Wanke *et al*. previously investigated EAEC prevalence in human immunodeficiency virus (HIV)–infected adults with diarrhoea, as compared to healthy controls. This study found EAEC to be significantly associated with diarrhoeal illness in this population, and interestingly EAEC infection was strongly linked to the stage of HIV disease progression and corresponding lower CD4 cell counts (Wanke *et al*., 1998). Although further studies are needed, EAEC has been previously associated with higher isolation frequency and greater symptomatic severity in acquired immunodeficiency syndrome patients, as compared to the healthy adult population (Rossit *et al*., 2009).

1.3.2: Transmission and reservoirs

EAEC is primarily transmitted via the faecal-oral route (Hebbelstrup Jensen *et al*., 2014, Kaur *et al*., 2010). Therefore, most cases likely arise from consumption of either contaminated food products or drinking water. A number of investigations have shown that EAEC can remain viable for long periods in various conditions

outside the human host, thereby increasing their danger as food or drink-related pathogens. For example, viable EAEC were recovered from inoculated bottles of mineral or spring water after more than 60 days (Vasudevan *et al*., 2003). Studies have also found EAEC to be prevalent in standing water, both in developing and industrialised regions (Sidhu *et al*., 2013, Akter *et al*., 2013). Additionally, a recent publication identified the EAEC virulence marker *aggR* in as many as 69% of samples taken from rivers in South Africa (Ndlovu *et al*., 2015).

As with many enteropathogens, food handling is believed to be a major source of EAEC infections. This is supported by findings in adult volunteer studies where a relatively high inoculum (approximately 10^{10} bacteria) was required for prototype strain 042 to cause diarrhoea, which makes food and water consumption more likely modes of transmission than environmental exposure (Nataro *et al*., 1995, Huang *et al*., 2006a, Huang and DuPont, 2004). While the infectious dose has been well-characterised for EPEC and EHEC (10^{8}-10^{10} and less than 100 bacteria, respectively), this has not been defined for EAEC strains in general due to the heterogeneity of virulence for clinical isolates (Mellies *et al*., 2007). A number of known outbreaks of diarrhoeal illness associated with virulent EAEC strains have been linked to contaminated food and water sources. These include a restaurant serving unpasteurised cheese in Italy, villagers drinking from an open well in South India, and a large outbreak affecting 2,697 children in Japan at 16 schools all using the same supplier of cooked lunches (Scavia *et al*., 2008, Pai *et al*., 1997, Itoh *et al*., 1997). In addition, the most publicised EAEC outbreak so far, the 2011 O104:H4 outbreak in Germany, likely originated from contaminated fenugreek sprouts often consumed raw in salads (Beutin and Martin, 2012).

Contaminated food and water sources are also suggested to cause many cases of EAEC-induced travellers' diarrhoea. For example, a cross-sectional study of table sauces in Guadalajara, Mexico, and Houston, Texas found a significantly higher prevalence of viable *E. coli* at the former location, of which EAEC was the most common pathotype of DEC identified (Adachi *et al*., 2002b). A recent study in South Africa investigated the prevalence of DEC in diverse sources including milk, irrigation water, vegetables, and street food. This determined that EAEC was the most abundant pathotype, and the authors suggested that it may represent the

leading cause of DEC-associated food and water-borne enteric infection in the country (Aijuka *et al*., 2018). Another consideration is the role of asymptomatic human carriers. A study of food handlers at Kenyan hotels concluded that they represented a risk for transmitting EAEC to traveller via shedding and food contamination (Oundo *et al*., 2008).

The main reservoir of pathogenic EAEC is generally accepted to be humans (Hebbelstrup Jensen *et al*., 2014, Okeke, 2009). Animal reservoirs have been established for some pathogenic *E. coli* subtypes, including the carriage of EHEC by cattle and other ruminant livestock, yet have not been conclusively demonstrated for EAEC (Ferens and Hovde, 2011). Large studies of farmed animals including ruminants have failed to show any association with EAEC carriage (Cassar *et al*., 2004, Orden *et al*., 2017). Low levels of EAEC have been isolated from an abattoir setting in South Africa, yet no EAEC were identified amongst 10,618 *E. coli* isolates screened from slaughterhouse effluents in France (Tanih *et al*., 2015, Bibbal *et al*., 2014). A recent study of DEC prevalence in humans, food, and livestock in Japan determined that the EAEC marker *aggR* was only associated with humans, supporting a predominantly human reservoir for the pathotype (Wang *et al*., 2017). Similarly, *aggR*-positive *E. coli* were associated with humans but not farmed chickens in South Vietnam (Trung *et al*., 2016). EAEC strains previously isolated from animal faeces often displayed major genetic differences to typical human pathogenic strains, although the possibility of animal-related strains becoming further adapted for human infection cannot be discounted (Uber *et al*., 2006). Plants can also act as reservoirs of human pathogenic enterobacteria, often with a direct link to harvested produce entering the food chain (Holden *et al*., 2009). Although it is unknown if this is a common transmission route for EAEC, studies have shown that several clinical strains are able to bind crops such as spinach and salad leaves using established adherence factors (Berger *et al*., 2009, Nagy *et al*., 2016). The prevalence of EAEC in standing water and irrigation sources also supports agricultural contamination as a contributory factor in faecal-oral transmission of pathogenic EAEC (Ndlovu *et al*., 2015, Sidhu *et al*., 2013, Aijuka *et al*., 2018).

1.3.3: Recent developments and hybrid strains

The heterogeneity of EAEC has been recognised from an early stage, associated with the theory that diverse factors confer the shared AA phenotype in different strains (Nataro and Kaper, 1998, Nataro *et al*., 1995). One distinction commonly used in the literature is the classification of EAEC strains as typical or atypical EAEC based on the presence or absence of the AggR regulator or pAA plasmid (Hebbelstrup Jensen *et al*., 2014). Some case studies have found a positive association between typical strains of EAEC and pathogenic effects, suggesting that they represent the most clinically relevant class of EAEC (Dudley *et al*., 2006a, Jiang *et al*., 2002). However, human disease is also caused by some atypical strains, and the prevalence of virulence factors is often different from typical EAEC (Andrade *et al*., 2017, Elias *et al*., 2002). The use of typical EAEC markers including AggR for general EAEC identification also introduces bias towards the association of typical strains with virulence (Jenkins *et al*., 2006). This distinction between typical and atypical EAEC is therefore too simplistic to account for the heterogeneity of the pathotype. More recently, the advances in sequencing and bioinformatic approaches for studying pathogen populations have been predicted to allow a clearer definition of pathogenic and non-pathogenic subpopulations within EAEC in the future (Robins-Browne *et al*., 2016).

Another aspect of EAEC research that has become more prominent in the last decade is the evolution of hybrid-pathotype strains. This interest has been largely driven by the *E. coli* O104:H4 outbreak in 2011 (**Figure 1.4**). During the late spring of that year, a large-scale outbreak of enteric disease occurred in central Europe, primarily in regions of Germany. This led to over 3800 reported cases of gastrointestinal illness, but the situation was complicated by an unusually high number of patients developing haemolytic uraemic syndrome (HUS), a disease most commonly associated with the Shiga toxin of EHEC. This syndrome is characterised by a combination of haemolytic anaemia, thrombocytopenia (platelet loss), and subsequent acute renal failure, and can cause up to 10% mortality (Corrigan and Boineau, 2001). While HUS is predominantly associated with infants, adults were mostly affected in this outbreak with approximately 850 recorded cases of HUS and 54 deaths (Hebbelstrup Jensen *et al*., 2014, Karch *et al*., 2012). This incident was

notable for both the overall scale and high mortality rate, especially for developed nations, and received major media coverage.

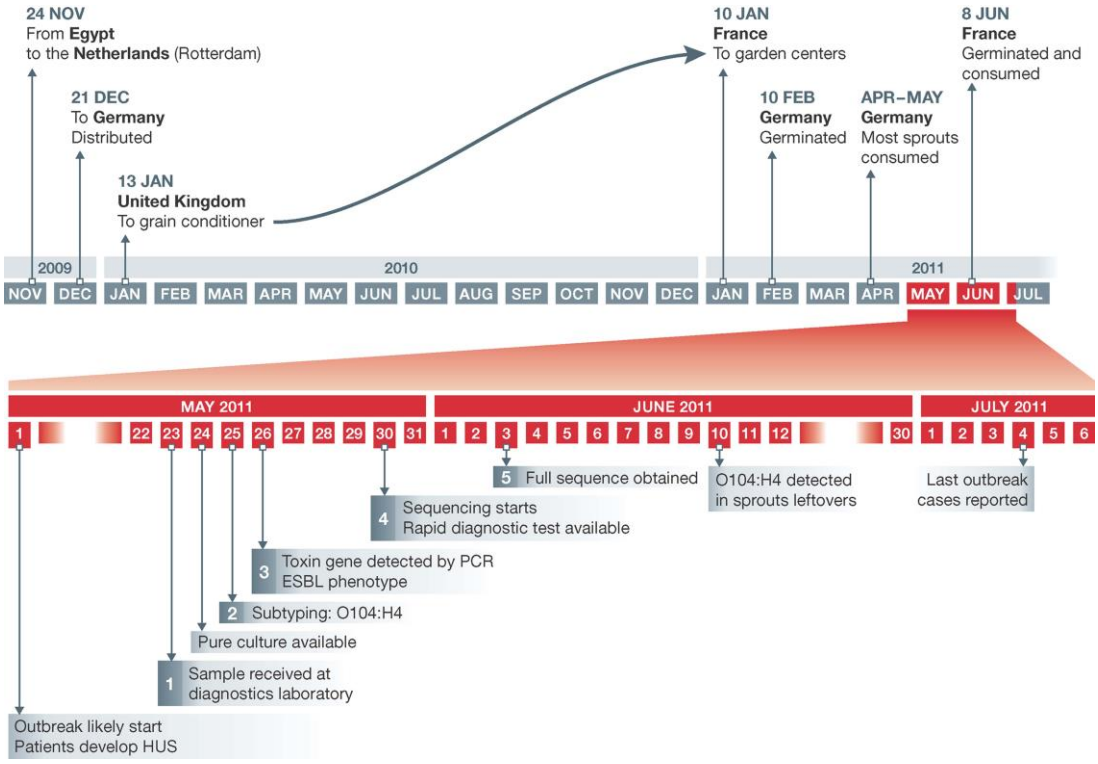

Figure 1.4 Timeline of 2011 *E. coli* O104:H4 outbreak. (Karch *et al*., 2012)

Within weeks of the first cases, microbiological laboratories began to complete work on sequencing and typing the causative pathogen. Serotyping classified the isolated strains as O104:H4 *E. coli* (Mellmann *et al*., 2011, Bielaszewska *et al*., 2011). This serotype had been identified in at least 8 previous infections in Europe and Korea, which had formerly been attributed to EHEC strains (Karch *et al*., 2012). However, investigators subsequently determined that the German outbreak strain was negative for intimin (encoded by *eae*), a key virulence factor in typical EHEC involved in intimate attachment and the formation of A/E lesions (Donnenberg *et al*., 1993). Records in Germany between 1996 and 2010 showed that less than 4% of previous HUS cases were caused by *eae*-negative isolates, demonstrating that

the 2011 outbreak strain represented an uncommon *E. coli* pathogen (Karch *et al*., 2012).

By early June, full sequencing results were available and indicated that the outbreak was the result of an EHEC-EAEC hybrid. Whilst lacking *eae*, the strain contained the *stx2* gene encoding Shiga toxin 2 (Stx2), as well as additional EHEC associated genes such as the adhesin *iha* (Bielaszewska *et al*., 2011). However, a range of molecular markers for typical EAEC strains were also present, including the aggregative adherence fimbriae AAF/I, the global regulator AggR, a mucinase Pic, dispersin, and the enterotoxin ShET1 (Muniesa *et al*., 2012, Bielaszewska *et al*., 2011). A picture therefore developed of a pathogen with an EAEC origin which had acquired *stx2* and other EHEC virulence genes via horizontal gene transfer.

Although rare, other incidents of Stx-producing EAEC hybrids have been described, albeit on a smaller scale than the 2011 outbreak. For example, an outbreak in France in 1992 resulted in 10 cases of HUS in children caused by an Stx2-positive EAEC strain of serotype O111:H2 (Morabito *et al*., 1998). More recently, an isolated case of HUS in Northern Ireland in 2012 was attributed to an Stx-producing EAEC O111:H21 strain (Dallman *et al*., 2012). Research into the evolutionary history of the 2011 O104:H4 strain identified a similar O104:H4 case in 2001, but lacking a plasmid conferring ceftazidime resistance (Karch *et al*., 2012). Interestingly, the outbreak strain had 99.8% nucleotide sequence similarity to a Shiga toxin–negative EAEC O104:H4 strain (55989) isolated in 1995 from an HIV-positive patient with diarrhoea in Central Africa; therefore giving a rough time window for the acquisition of the prophage encoding Stx2 (Beutin and Martin, 2012).

The 2011 O104:H4 strain caused the largest outbreak of Stx-producing *E. coli* ever recorded, and was one of the most serious microbiological incidents in the modern era of Western medicine (Muniesa *et al*., 2012). The virulence of this strain is a likely result of the unusual combination of the pAA plasmid mediating AA and the Stx2-encoding phage linked to kidney damage and HUS. It has been proposed that EAEC virulence mechanisms contributed to epithelial barrier disruption, and therefore greater penetration of the Stx2 toxin (Boisen *et al*., 2015).

This outbreak demonstrates that combinations of existing pathotypes, utilising the exceptional ability of virulence genes to spread between *E. coli* strains, may cause the rise of new pathogens. The scale of this incident has resulted in renewed interest in EAEC research (Beutin and Martin, 2012). Furthermore, the example of the 2011 outbreak shows how the acquisition of certain genetic elements can make EAEC a much greater threat for adults in developed countries, a population in which EAEC is less frequently considered.

1.3.4: EAEC typing

While increasingly considered outdated, *E. coli* research has traditionally used serotyping as a standard practise for characterisation of strains. This uses a numerical scheme assigned to specific surface antigens: the O antigen of the lipopolysaccharide (LPS) layer, H antigen of flagellin, and also the K antigen of capsular polysaccharide (Orskov and Orskov, 1992). However, as fewer reference laboratories had the capabilities for K antigen typing, the O:H serotype has historically been the gold standard in *E. coli* typing (Fratamico *et al*., 2016). While developed in the 1940s, serotyping has remained popular as a technique applicable across all *E. coli* species which allows sufficient discrimination for differentiating taxonomic groups and tracking pathogenic outbreaks (Jenkins, 2015, Kauffmann, 1947). It has been effective for determining virulent lineages in the study of DEC. A prominent example is the serotype O157:H7 which has been identified as the major EHEC group associated with diarrhoeal illness (Nguyen and Sperandio, 2012, Weintraub, 2007). Unfortunately, serotyping has proven less useful in the study of EAEC as no O:H group dominates or specifically associates with disease; therefore it has been limited to specific outbreak identification (Scavia *et al*., 2008, Hebbelstrup Jensen *et al*., 2014). This is partly the result of the genetic heterogeneity of the pathotype, with one major UK epidemiological study identifying 47 distinct EAEC serotypes (Jenkins *et al*., 2006). The issue is compounded by the highly aggregative phenotype of EAEC as auto-agglutination causes a high proportion of strains to be classed as non-typeable (Weintraub, 2007).

E. coli have also been previously analysed by phylogroup (A, B1, B2, C, D, E, F), where isolates can be assigned by triplex PCR (Clermont *et al*., 2013). Some subgroups of *E. coli* are highly associated with phylogroups, such as extraintestinal pathogenic *E. coli* with groups B2 and D, or commensal *E. coli* with group A (Croxen *et al*., 2013). However, EAEC have been found to be widely distributed across these phylogroups, a further indicator of the phylogenetic diversity of the pathotype and parallel evolution of the aggregative adherence phenotype (Escobar-Páramo *et al*., 2004, Hebbelstrup Jensen *et al*., 2014)

Another common typing scheme in bacterial epidemiology is Multi-Locus Sequence Typing (MLST), which was developed for *Neisseria meningitidis* in the 1990s but is now applied to various pathogens including *E. coli* (Maiden *et al*., 1998, Larsen *et al*., 2012). An allelic profile of core gene sequences, amplified by polymerase chain reaction (PCR), is used to type strains against defined sequence types (STs). The standard *E. coli* MLST scheme uses 7 loci: *adk, fumC, gyrB, icd, mdh, purA* and *recA* (Chattaway *et al*., 2014b). MLST can be particularly effective for investigating population genetics and discriminating between lineages in larger data sets (Okeke *et al*., 2010).

Both serotyping and sequence typing have benefited from the advances in genome sequencing technologies. For serotyping, determining antigen alleles from genetic sequences circumvents the practical limitations of maintaining at least 188 O and 53 H antisera and specialised facilities for traditional antigen testing (Jenkins, 2015, Joensen *et al*., 2015, Ingle *et al*., 2016). Similarly, *in silico* MLST has become increasingly efficient with the improving cost-effectiveness of whole-genome sequencing (WGS), as opposed to traditional PCR amplification (Larsen *et al*., 2012). WGS is also allowing the development of new typing approaches, such as core genome MLST. This technique performs genome-wide gene-by-gene allele comparisons for typically 1500 to 4000 genes conserved within a species, allowing enhanced resolution versus conventional MLST schemes (Kimura, 2018).

The standard typing methods for epidemiology have been effective for identifying EAEC outbreaks and lineages, but the heterogeneity of the pathotype is a challenge for determining pathogenic subgroups. However, ongoing advances using WGS in

combination with typing techniques may improve discriminatory power for future studies and allow pathogenic and non-pathogenic EAEC strains to be distinguished (Robins-Browne *et al*., 2016, Chattaway *et al*., 2013).

1.4: Pathology

1.4.1: Diagnosis of EAEC

The characteristic AA phenotype of bacteria incubated with cultured HEp-2 cells was the original defining feature for classification of EAEC as a separate pathotype and remains the nominal gold-standard for EAEC diagnosis. The main feature of this phenotype is the stacked-brick adherence pattern, with bacteria binding not only to the HEp-2 cells, but also to the surface of the culture dish (**Figure 1.5**) (Cennimo *et al*., 2007). However, the HEp-2 adherence assay has drawbacks due to the necessary time, practical expertise, and cell culture facility requirements (Weintraub, 2007). It has been demonstrated that formalin-fixed HEp-2 cells can be used for many weeks with a similar sensitivity and specificity to the standard assay, which offers extra practicality and reduces the risk of contamination, but this has not been widely adopted (Miqdady *et al*., 2002). In practice, epidemiological identification of EAEC is now typically performed by molecular biology techniques.

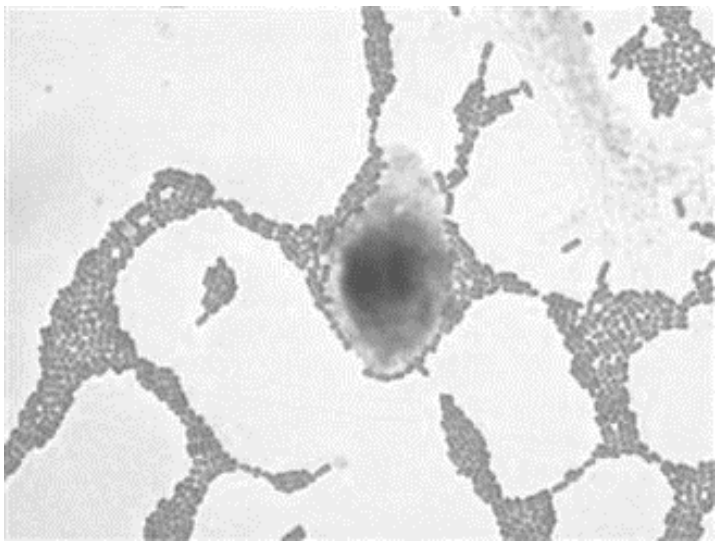

Figure 1.5 Aggregative adherence phenotype of EAEC on HEp-2 cells. The EAEC aggregate in a "stacked-brick" pattern on both the cultured epithelial cells and the underlying abiotic surface. (Boisen *et al*., 2013)

The use of PCR assays for diagnosis of infective disease agents is well established and has advantages for rapid detection of microbes (Yang and Rothman, 2004). However, the use of PCR with EAEC has been complicated by the genetic heterogeneity of the pathotype. Many studies have used the DNA probe CVD432, targeting the *aatA* gene sequence (encoding a membrane transporter of the dispersin protein). This which was originally developed by Baudry *et al*. for Southern blotting based on a specific fragment of the 042 pAA (Baudry *et al*., 1990). Although the CVD432 probe has been widely used for its high specificity, its sensitivity is highly variable (15% to 89%) (Cennimo *et al*., 2007). Other studies have used a range of potential target genes as markers for EAEC, including virulence genes such as *aap, astA*, and *aaiA*, and the transcriptional regulator gene *aggR* (Jenkins *et al*., 2006, Andrade *et al*., 2014). However, as many putative virulence genes are poorly conserved amongst all EAEC and also found in other pathotypes, it is difficult to design a specific assay using PCR alone (Weintraub, 2007). Despite this, PCR approaches are widely used due to their cost-effectiveness especially when screening large sample collections. Multiplex PCR against multiple EAEC-associated genes is often used to improve the sensitivity of EAEC identification, whilst real-time PCR can allow a more rapid detection of the bacteria (Bischoff *et al*., 2005, Bouzari *et al*., 2005, Cerna *et al*., 2003).

Other basic diagnostic options have been proposed based on EAEC-associated phenotypes. One simple method for detection is a "clump-formation" test, based on the visible auto-agglutination of EAEC in culture broths. While crude, it has a reported sensitivity and specificity of approximately 90% and was recommended as a potential EAEC detection tool following a study in Japan (Iwanaga *et al*., 2002). A quantitative biofilm assay has also been described, utilising optical density to identify rapid bacterial aggregation in a microtiter plate, and may be useful as a quick initial screening tool in regions lacking more specialised facilities (Wakimoto *et al*., 2004).

For now, the HEp-2 adherence assay remains the nominal gold standard for reliable confirmation of EAEC, although many reference laboratories mostly use PCR techniques for efficiency of identification. The development of improved molecular diagnostic tools could improve future research of this pathotype, particularly for specific epidemiological analysis of pathogenic EAEC strains, but will require further understanding of the key factors in EAEC pathogenesis (Hebbelstrup Jensen *et al*., 2014).

1.4.2: Clinical symptoms of EAEC infection

The heterogeneity of EAEC strains results in great variation in the clinical symptoms of infection, including frequent asymptomatic carriage. This diversity in disease has been proposed to also depend on factors such as host genetic susceptibility and immune response, as well as the number of bacteria initially ingested (Kaur *et al*., 2010). Two major infectious intestinal disease studies in the UK (IID1 and IID2) reported that a quarter of EAEC-positive individuals were asymptomatic (Chattaway *et al*., 2013). This was also observed in traveller's diarrhoea, where a study of 382 travellers to the tropics identified EAEC in 50% of diarrhoeal samples but also in 28% of asymptomatic controls (Laaveri *et al*., 2016).

Gastrointestinal illness caused by EAEC can present with a range of symptoms (**Table 1.1**), although watery diarrhoea (less frequently containing mucus and/or blood) is most prevalent. Accompanying symptoms can include dehydration,

abdominal pain, vomiting, nausea, and low-grade fever (Kaur *et al*., 2010, Kahali *et al*., 2004, Nataro *et al*., 2006). However, many studies focus on EAEC strains isolated from hospitalised patients. As EAEC is not typically screened for in community health services, especially in developing countries, infections with milder symptoms are likely to be underreported (Okeke, 2009). Onset of diarrhoea following ingestion of the pathogen may be rapid, with a human volunteer study by Nataro *et al*. reporting an average incubation period of 14 hours for affected subjects (Nataro *et al*., 1995). However, some typical EAEC-associated foodborne outbreaks have reported longer incubation times of 33-50 hours before symptoms, while the 2011 O104:H4 outbreak had an estimated median incubation time of 8 days (Scavia *et al*., 2008, Itoh *et al*., 1997, Frank *et al*., 2011).

Characteristic	No. (%) of hospitalized patients
Stool consistency	
Watery	91 (75.2)
Mucoid	5 (4.1)
Bloody	4 (3.3)
Loose	22 (18.2)
Clinical symptoms	
Vomiting	78 (64.5)
Fever (temp, >38.8°C)	24 (19.8)
Abdominal pain	55 (45.5)
Dehydration status	
Severe	52 (43.0)
Moderate	65 (53.7)
None	4 (3.3)
Duration of diarrhea before admission to the hospital	
Up to 24 h	103 (85.1)
>24 to 48 h	12 (9.9)
>48 h	6 (5.0)

Table 1.1 Clinical characteristics of EAEC infections in India (Kahali *et al*., 2004)

EAEC infections can cause both acute and chronic diarrhoeal illness. Persistent EAEC diarrhoea has mostly been associated with children, especially in developing countries (Fang *et al*., 1995, Cravioto *et al*., 1991, Henry *et al*., 1992). However, further epidemiological studies have disputed this, with only low rates of persistent diarrhoea (> 14 days) reported for EAEC infections (Lima *et al*., 2013b, Pabst *et al*., 2003). A specific role of chronic diarrhoea as an important disease trait for EAEC infection compared to other DEC may have been overestimated by earlier studies of the pathotype (Hebbelstrup Jensen *et al*., 2014). Chronic infection by enteric pathogens such as EAEC has been linked to malnutrition in children, both as a risk factor for initial infection due to mucosal damage, and also as a resulting effect of persistent diarrhoeal illness (Nataro and Kaper, 1998, Roche *et al*., 2010). Children under 60 months of age in Kenya with clinical wasting (defined as mid-upper arm circumference </= 125 mm) had a significantly greater rate of EAEC infection and more severe disease and dehydration (Tickell *et al*., 2017). EAEC was one of multiple pathogens associated with infant malnutrition in a case-control study in Bangladesh, after adjusting for sociodemographic factors, and EAEC infections were more prevalent in malnourished children in North-eastern Brazil (Platts-Mills *et al*., 2017, Lima *et al*., 2017a). While EAEC is suggested to mostly cause acute illness in otherwise healthy adults, immunocompromised individuals such as HIV-positive patients also have a significant association with chronic diarrhoea (Wanke *et al*., 1998, Samie *et al*., 2007).

Aside from the initial gastrointestinal symptoms of infection, EAEC has been linked to additional long-term effects. Studies have shown an association between the presence of EAEC in stools of children and a subsequent impairment of growth. This also occurs in children colonised without symptomatic illness, suggesting EAEC-induced malnutrition is at last partially independent of diarrhoea (Steiner *et al*., 1998, Guerrant *et al*., 2008). The MAL-ED study associated EAEC infections with reduced body length by 2 years of age, and proposed that maintained intestinal inflammation may stunt growth without diarrhoea (Rogawski *et al*., 2017). A similar observation of growth shortfalls and under-nutrition induced by EAEC has been demonstrated in a murine model (Roche *et al*., 2010). Sobieszczańska *et al*. have suggested an additional link between EAEC and irritable bowel syndrome (IBS)

(Sobieszczańska *et al*., 2007). They determined a highly significant increase in EAEC isolates in IBS patients, and theorised that a persistent infection of this pathotype could contribute to IBS development through induced mucosal inflammation. However, there is currently insufficient evidence to indicate any causative correlation.

Although EAEC is primarily a foodborne enteric pathogen, some strains have shown an ability to cause extraintestinal infection. The most common causative agent of urinary tract infections has long been established as uropathogenic *E. coli* (UPEC). However, while UPEC dominates in community-acquired cases, hospital-acquired infections can be caused by *E. coli* strains normally associated with intestinal colonisation, including EAEC (Toval *et al*., 2014). An increase in such observations, including an outbreak of EAEC-associated urinary tract infections in Copenhagen, has led some researchers to suggest that a UPEC/EAEC hybrid pathotype may be an evolving clonal group specialised for this extraintestinal niche (Chattaway *et al*., 2014a, Olesen *et al*., 2012).

While most cases of EAEC infection do not result in life-threatening illness, some strains have been able to cause more serious complications. Most notable is the association between certain EAEC strains and the development of haemolytic-uraemic syndrome (HUS). HUS has been historically linked to EHEC, with the pathogenesis dependent on the expression of Shiga toxins (Melton-Celsa and O'Brien, 2014). However, there have been multiple examples of HUS associated with EAEC strains that have acquired Shiga toxins, including cases in HIV-positive patients in the Central African Republic, and minor outbreaks in France and Japan in 1996 and 1999, respectively (Mossoro *et al*., 2002, Chattaway *et al*., 2013). However, the risk of Shiga toxin-producing EAEC received greater recognition in the wake of the major 2011 O104:H4 outbreak in Europe, as previously described in Chapter 1.3.3.

1.4.3: Clinical therapy

In the majority of cases of EAEC-associated diarrhoea, the infection is self-limiting (Huang *et al*., 2004). As a result, oral rehydration therapy is commonly applied, although the link between EAEC and chronic disease can make this less suitable alone in cases with persistent symptoms (Kaur *et al*., 2010). Antimicrobial therapy is frequently given empirically for severe diarrheal illness before pathogen identification and is likely for EAEC infection due to the aforementioned limitations in diagnosis. Certain classes of antibiotics have proven effective against most EAEC strains, with a susceptibility to fluoroquinolones often highlighted (Glandt *et al*., 1999, Cennimo *et al*., 2007). Evidence from two clinical trials, using ciprofloxacin and rifaximin, showed a significant reduction in post-treatment diarrhoea duration in comparison to placebo controls (Infante *et al*., 2004, Glandt *et al*., 1999). However, antibiotics are not recommended for treatment of infections caused by Stx-producing EAEC. Many of the antibiotics available for use against gastrointestinal infections, such as mitomycin C or quinolones, can induce bacterial SOS responses and trigger additional Stx expression and therefore an enhanced risk of HUS (Karch *et al*., 2012, Kimmitt *et al*., 1999). The O104:H4 outbreak strain demonstrates increased Stx2 production in response to ciprofloxacin (Bielaszewska *et al*., 2012). While supportive rehydration therapy is currently used for confirmed infections by Stx-producing *E. coli*, new therapeutics in development include monoclonal antibodies against the toxin itself (Melton-Celsa and O'Brien, 2014).

However, as with most bacterial pathogens the issue of developing antibiotic resistance must be considered. While susceptibility patterns naturally vary by geographic region, high levels of resistance in EAEC have been reported for a number of major antibiotics including ampicillin, tetracycline and chloramphenicol (Huang *et al*., 2006a). Multi-drug resistance is also on the rise in certain areas. For example, a study in rural South India found over 75% of isolated EAEC strains were resistant to more than 3 of a panel of common antimicrobial agents tested (Raju and Ballal, 2009). Recent screening of all DEC pathotypes (of which EAEC was most prevalent) isolated from children with diarrhoea in Nairobi, Kenya, found more than 50% resistance to the following antibiotics: ampicillin, trimethoprim/sulfamethoxazole, streptomycin, gentamicin, kanamycin,

ciprofloxacin, chloramphenicol, and tetracycline (Nyanga *et al*., 2017). Antimicrobial resistance is also observed in asymptomatic carriage, with 56% of EAEC isolates from a healthy elderly population in China being positive for extended spectrum beta-lactamases (Wang *et al*., 2015). It has been proposed that resistance has grown most rapidly in enteric pathogens in areas such as South and South-East Asia due to poorer control of antimicrobial use. The availability of antibiotics for self-treatment for symptoms such as diarrhoea without a prescription or medical consultation is likely a driving factor (Nguyen *et al*., 2005).

Some research has been performed into alternative therapies for EAEC infection. Some of the most interesting findings have been reported for the effect of the antimicrobial protein lactoferrin. In general, lactoferrin exhibits antibacterial activity by sequestering iron to inhibit the growth of microorganisms, as well as disrupting the outer membrane of Gram-negative bacteria by inducing the release of LPS (González-Chávez *et al*., 2009). *In vitro*, lactoferrin has been shown to inhibit EAEC adherence to HEp-2 cells and biofilm formation, via disruption of AAF binding (Ochoa *et al*., 2006). In addition, a clinical trial of Japanese children given lactoferrin supplements found a significant reduction in vomiting and diarrhoeal illness without any effect on Rotavirus incidence, suggesting a protective effect against bacterial enteric pathogens in general (Egashira *et al*., 2007). The inclusion of recombinant human lactoferrin and lysozyme in oral rehydration therapy during a study of Peruvian children caused a significant decrease in diarrhoea duration and relapse (Zavaleta *et al*., 2007). It is unclear if supplements such as lactoferrin could be viable alternatives in antimicrobial therapy against virulent EAEC, but growing evidence suggests a beneficial effect during enteric disease.

Bacteriophage therapy has been proposed as an alternative to antibiotic usage. Such treatments are currently applied in areas of Eastern Europe and Russia, yet their effectiveness remains unconfirmed in the wider medical community (Sulakvelidze *et al*., 2001). Bacteriophages specific for an EAEC strain reduced biofilm formation on abiotic surfaces and were able to infect bacterial aggregates on epithelial cells and persist in murine intestines (Maura *et al*., 2012). It has also been proposed that candidate bacteriophages could be selected rapidly from existing libraries as a response to outbreaks such as the 2011 O104:H4 event

(Merabishvili *et al*., 2012). Research is expanding for phage therapy to target *E. coli* pathogens, although *in vivo* animal studies and clinical trials for intestinal infections have demonstrated only modest effects (Bolocan *et al*., 2016). Bacteriophages able to lyse EHEC O157:H7 *in vitro* improved clearance of the bacteria in a murine model, but caused only a limited reduction of colonisation in cattle and no effect in sheep (Sheng *et al*., 2006). Furthermore, a randomised clinical trial of phage therapy (including a commercial Russian coliphage product) for microbiologically diagnosed *E. coli* diarrhoea, mostly ETEC and EAEC, observed no improvement in clinical outcomes including diarrhoea duration, stool frequency, and vomiting (Sarker *et al*., 2016). As such, the applicability of bacteriophage therapy for clinical EAEC cases remains undetermined.

The heterogeneity of DEC has hindered the development of effective vaccines. However, recent progress has been made using major colonisation factors as antigens which could confer protection against the majority of virulent ETEC strains (von Mentzer *et al*., 2014). Although EAEC has a particularly high degree of heterogeneity, it has been proposed that similar approaches could also be effective for this pathotype. One potential option is exploiting the adhesins specific to EAEC, as incorporating all of the AAF family adhesins as antigens could be successful against many (but not all) virulent strains (Boisen *et al*., 2008). The dispersin protein involved in epithelial dispersal of adhesins is also highly immunogenic and well conserved, and has therefore been suggested as a possible vaccine candidate (Nataro *et al*., 1995, Huang *et al*., 2006a, Karam *et al*., 2017). Other research has identified protective antigens from the core *E. coli* genome, such as the outer-membrane protein YncE, which could be used in combination with pathotype-specific antigens for future vaccine development against DEC (Moriel *et al*., 2016).

1.5: Mechanisms of EAEC virulence

The pathogenesis of EAEC is likely variable, reflecting the heterogeneity of the pathotype. However, the generally accepted course of infection can be summarised by the following stages: Adherence to the intestinal epithelium, colony expansion

and biofilm formation, toxin-induced epithelial damage and host inflammatory response (**Figure 1.6**).

Due to the exceptional aggregative properties of EAEC, it is proposed that initial epithelial adherence is enhanced by auto-aggregation of bacteria in the gut lumen which benefits initial colony establishment (Hebbelstrup Jensen *et al*., 2014). Aggregative adherence at the mucosal surface is multi-factorial and includes a number of adhesins and other putative virulence factors. Once EAEC colonisation is established, the secretion of bacterial toxins and other serine protease autotransporters of Enterobacteriaceae (SPATEs) induces tissue damage and immunological host responses (Kaur *et al*., 2010). Inflammation is also a key factor of symptomatic infection and is triggered by host-pathogen interactions including toxins and flagella (Kong *et al*., 2015, Harrington *et al*., 2005). In chronic infections associated with EAEC, it is suggested that dense biofilm formation contributes to the resistance to clearance by the immune system and subsequent persistent diarrhoea (Weintraub, 2007).

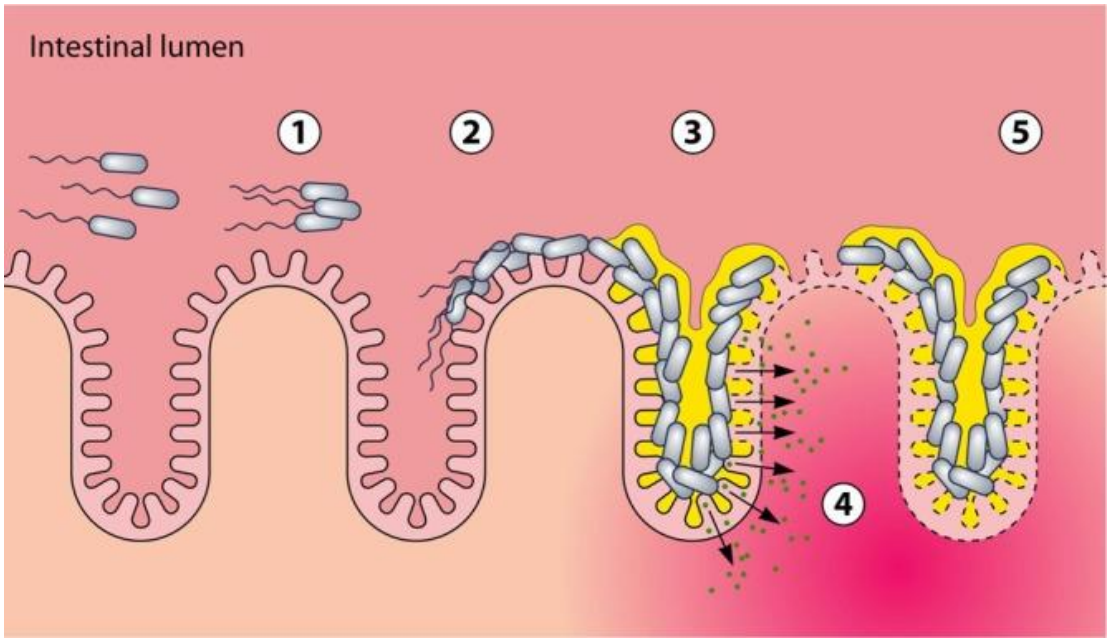

Figure 1.6 Stages of EAEC pathogenesis. 1. Agglutination of bacteria in the lumen. 2. Aggregative adherence to the mucosal surface. 3. Colony expansion and biofilm formation. 4. Release of toxins causing tissue damage and inflammation, resulting in diarrhoeal symptoms. 5. In persistent cases, establishment of additional biofilm and resistance to clearing by immune responses (Hebbelstrup Jensen *et al*., 2014).

Many putative virulence factors have been described for pathogenic EAEC. A key feature of "typical" EAEC strains is the aggregative adherence plasmid (pAA) which encodes many of the virulence factors associated with the AA phenotype including the *aggR* transcriptional activator gene (Johnson and Nolan, 2009). The archetype of this virulence plasmid is that of prototype strain 042 (pAA2), but the pAA varies greatly between different EAEC strains in size (72-120 Kb) and genetic composition (Chaudhuri *et al*., 2010, Jonsson *et al*., 2017b). The AggR regulon (**Figure 1.7**) includes a set of virulence genes linked with EAEC pathogenesis, such as genes encoding AAFs, dispersin, and the toxin Pet (Dudley *et al*., 2006a, Cerna *et al*., 2003). Most of the regulon is located on the pAA plasmid, although AggR also regulates genes located on chromosomal islands, such as *pic* (Morin *et al*., 2013). Some studies have found a positive association between AggR and acute diarrhoea and inflammation caused by EAEC (Jiang *et al*., 2002, Hebbelstrup Jensen *et al*., 2017).

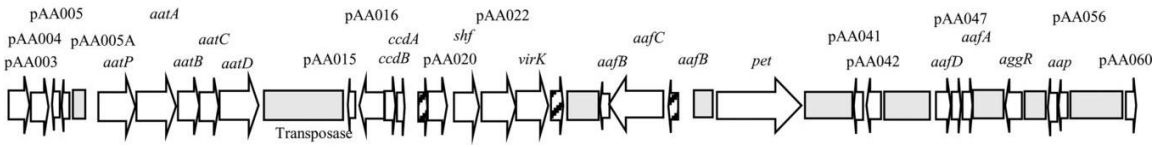

Figure 1.7 AggR regulon on the pAA2 of EAEC 042. (Morin *et al*., 2013)

While several virulence factors have been reported, many of these are only found in a minority of EAEC isolates. In this section, the proposed mechanisms for different aspects of EAEC pathogenesis will be reviewed, along with the associated virulence genes at each stage.

1.5.1: Epithelial adherence

One of the defining aspects of EAEC is the AA phenotype, as originally identified on cultured HEp-2 cells. This adherence is key for the initial colonisation of the intestinal mucosa and has been associated with a number of expressed adhesins. The major virulence factors responsible for AA are the aggregative adherence fimbriae (AAF) (**Figure 1.8**). These bundle-forming fimbriae were first identified in

the prototype strain 17-2; with this variant now termed AAF/I. Introduction of AAF/I into *E. coli* K12 strains successfully conferred an AA phenotype, demonstrating the importance of these fimbriae for EAEC adherence (Nataro *et al*., 1992). The frequently used prototype strain 042 was later found to express a distinct allele, AAF/II, which while functionally similar, includes a major pilin subunit with less than 25% amino acid identity with that of AAF/I (Nataro *et al*., 1995, Harrington *et al*., 2006). Subsequent research has increased the number of known AAF alleles to at least five, with a large degree of variation between the pilin subunits, but each EAEC strain typically only possesses a single allele (Dallman *et al*., 2014). Protein interaction studies have identified a number of potential host cell receptors for AAF attachment, which include fibronectin, laminin, collagen IV, and cytokeratin 8 (Izquierdo *et al*., 2014b).

The AAFs are related to the Dr family of fimbrial adhesins, which are important in UPEC and DAEC. Both groups of adhesins utilise usher and chaperone proteins with a high level of sequence conservation (Harrington *et al*., 2006). The AAF adhesins are encoded on the EAEC virulence plasmid pAA. However, while AAFs are important facilitators of the AA phenotype, many clinical EAEC isolates do not possess any known AAF alleles (Jonsson *et al*., 2015). In addition, a study using a rabbit infection model refuted that the pAA is absolutely required for *in vivo* colonisation by virulent EAEC, although this conclusion was based on tissue homogenates rather than direct evaluation of intestinal adherence (Munera *et al*., 2014). While it is possible that further uncharacterized AAFs may be involved, other adhesins are also thought to play a role in EAEC adherence.

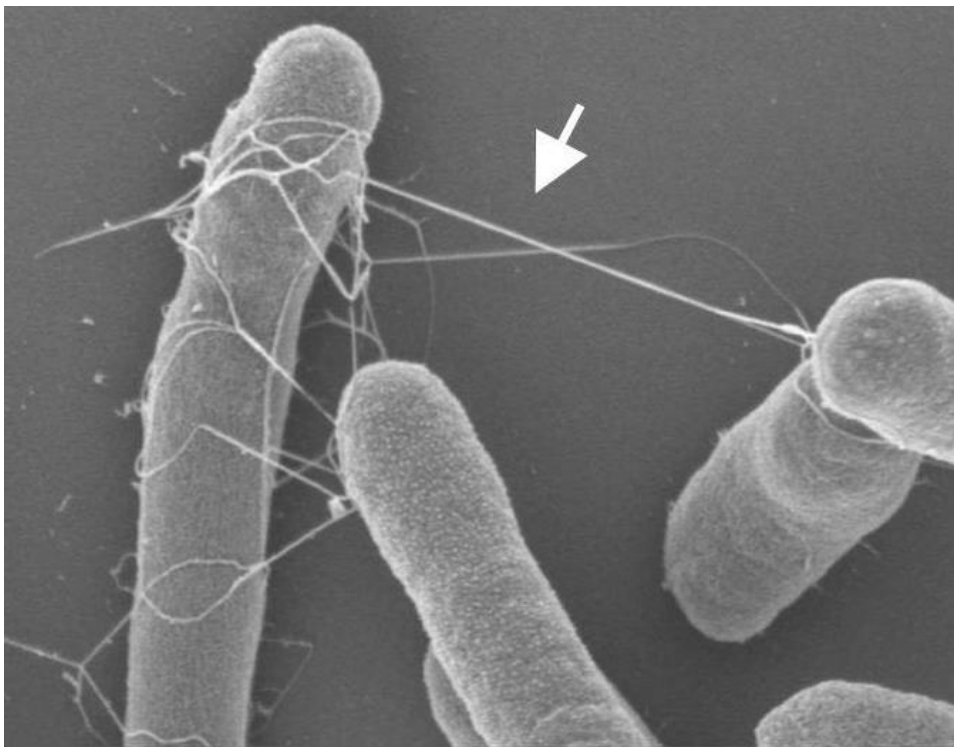

Figure 1.8 Electron micrograph of Aggregative Adherence Fimbriae connecting EAEC bacteria. The image shows EAEC strain 042, which expresses AAF/II. (Sheikh *et al*., 2002)

A common adhesin in various pathogenic and commensal *E. coli* is the *E. coli* common pilus (ECP), associated with enhanced bacterial adherence to cultured epithelial cells *in vitro* (Rendón *et al*., 2007). ECP is conserved in the majority of EAEC strains, with a study of 130 EAEC isolates determining the *ecpA* gene to be present in 96% of strains. The same study demonstrated that loss of *ecpA* significantly reduced adherence in AAF-negative but not AAF-positive EAEC strains, such as 042 (Avelino *et al*., 2010). It has therefore been suggested that ECP could be an important mediator of AA in some of the many EAEC strains lacking AAF adhesins.

Another common adhesin in Enterobacteriaceae is the type I fimbria (TIF), which plays an important role in the adherence of many commensal and pathogenic *E. coli* subtypes. It is encoded by the *fim* operon present in the majority of clinical EAEC isolates (Regua-Mangia *et al*., 2009). TIF knockouts and anti-TIF serum can inhibit the AA phenotype *in vitro*, suggesting TIF contribute to the adherence of EAEC strains (Moreira *et al*., 2003).

Alongside adhesins themselves, EAEC also express an additional virulence factor from the pAA plasmid which supports adherence. This is a 10.2-kDa protein termed anti-aggregation protein (Aap), now more commonly referred to as dispersin (Sheikh *et al*., 2002). Dispersin is non-covalently attached to the bacterial surface and associated with a greater dispersal of EAEC on epithelial surfaces. Structural studies suggest the protein binds to outer membrane LPS to mask its strong negative charge, allowing positively charged adhesins such as AAFs to effectively bind more distant sites and mediate the hyper-aggregative properties of EAEC, thus regulating the AA phenotype for optimal colonisation (Harrington *et al*., 2006, Velarde *et al*., 2007). Dispersin is exported to the bacterial surface by a specialized ABC transporter, encoded by the *aat* cluster also located on pAA (Nishi *et al*., 2003). Both dispersin and *aat* are well conserved in typical EAEC and have been used as markers for PCR-based identification of the pathotype (Jenkins *et al*., 2006).

Agglutinins have also been described for EAEC, with the heat-resistant agglutinin (encoded by *hra1*) characterised in 042. While Hra1 conferred enhanced auto-agglutination, biofilm formation and AA when expressed by laboratory *E. coli* strains, no effects were observed in 042 *hra1* deletion mutants. As such, Hra1 has been described as an auto-adhesion factor as well as a putative accessory adhesin (Bhargava *et al*., 2009). Recently, a study demonstrated that a hyper-aggregative phenotype exhibited by dispersin mutants was due to an unmasking of Hra1, not AAF. However, all three factors were important for colonisation of a *Caenorhabditis elegans* infection model, highlighting the interplay of multiple factors in EAEC adherence (Blanton *et al*., 2018).

While AAFs are specifically associated with EAEC and the AA phenotype, they are not expressed by all strains. It is apparent that AA is multi-factorial, which is connected to the genetic diversity of EAEC. In addition, many of the factors important for initial adherence also contribute to the development of biofilm.

1.5.2: Biofilm formation

Following the establishment of aggregative adherence to the intestinal epithelium, the next stage of EAEC pathogenesis is the formation of a biofilm. It is well established that biofilms confer a number of advantages to colonising bacteria, such as resistance to antimicrobial agents including antibiotics and host factors (Costerton *et al*., 1999, Xu *et al*., 2000). Furthermore, the ability of EAEC to form thick, aggregating biofilms likely contributes to persistent infection and prolonged diarrhoea often seen in clinical cases, especially in infants (Kaur *et al*., 2010, Nataro and Kaper, 1998).

Biofilm formation is a complex, multi-factorial process, but some factors have been identified as important for EAEC biofilms. In addition to their central role in aggregative adherence, AAF adhesins are also implicated in biofilm development (Berry *et al*., 2014). Biofilm formation by prototype strains 042 and 17-2 require expression of AAF (AAF/II and AAF/I, respectively), and non-pathogenic *E. coli* strains harbouring pAA constructs (containing AAF genes) are able to form biofilms (Sheikh *et al*., 2001).

AAF expression is regulated by the transcriptional activator AggR, yet there is some disagreement as to the overall importance of AggR in biofilm formation. While some studies have observed no significant correlation between AggR and biofilms (Sheikh *et al*., 2002), others have suggested that AggR is strongly associated with the phenotype (Mohamed *et al*., 2007b). However, AAF can be regulated by other factors including Fis, a nucleoid-associated protein involved in growth-phase dependent regulation of EAEC genes (Morin *et al*., 2010, Rossiter *et al*., 2011). Deletion of *fis* results in loss of biofilm formation and disruption of AggR and AAF expression, which are restored by *fis* complementation. It has therefore been suggested that Fis mediates biofilm formation via AggR regulation of AAF biogenesis (Sheikh *et al*., 2001). Although less well characterised than *fis*, a similar role has been detected for the *yafK* gene (Kaur *et al*., 2010). Mutations in *yafK* significantly reduced expression of AAF genes, adherence, and generation of biofilms (Sheikh *et al*., 2001).

However, as with the AA phenotype, many EAEC isolates form biofilms without possessing AAF alleles, suggesting that other factors play important roles. One candidate gene is *shf,* first described on the pAA2 plasmid of prototype strain 042. Mutations of *shf* resulted in significant disruption of biofilm formation, yet the bacteria retained an aggregative phenotype in liquid phase (Fujiyama *et al*., 2008). While the function of Shf is undetermined, the closest characterised homologue is the IcaB protein of pathogenic *Staphylococcus epidermidis*. IcaB mediates modifications of exopolysaccharides and promotes intercellular adhesion in bacterial biofilms (Vuong *et al*., 2004). The ECP adhesin, which has been proposed to contribute to AA in AAF-negative EAEC strains, is also a common factor associated with biofilm development by *E. coli* pathogens and commensals alike (Garnett *et al*., 2012).

Other putative regulators of biofilm formation are related to a type III secretion system (T3SS) designated ETT2, encoded at the *glyU* locus on the chromosome of EAEC strain 042, with potential effectors reported at the *selC* locus. These include EilA, a homologue of a transcriptional regulator HilA from *Salmonella enterica,* and Air, a homologue of invasin from *Yersinia*. Mutations in either *eilA* or *air* reduced epithelial adherence and biofilm abundance (Sheikh *et al*., 2006). It is suggested that EilA regulates the co-expression of ETT2 and *air*, while *air* is predicted to encode an outer membrane protein with roles as an agglutinin and possible accessory adhesin contributing to biofilm formation (Kaur *et al*., 2010, Sheikh *et al*., 2006).

A further example of AggR-independent biofilm-promoting factors are type IV pili, which contribute to bacterial adherence and twitch-motility in Gram-negative pathogens (Donnenberg *et al*., 1992). Dudley *et al*. have previously described a clinical EAEC isolate lacking the AggR regulon and containing a plasmid-encoded type IV pilus, which mediated both adherence and biofilm formation (Dudley *et al*., 2006a). Interestingly, up to 10 % of EAEC strains possessed similar pilus genes suggesting that type IV pili may be as prevalent as some AAF alleles.

1.5.3: Toxins and Serine Protease Autotransporters of *Enterobacteriaceae* (SPATEs)

The clinical manifestations of EAEC infection are varied but usually include watery diarrhoea, occasionally with blood and mucus components, resulting from expression of enterotoxins and cytotoxins (**Figure 1.9**) (Harrington *et al*., 2006). While the 2011 German outbreak isolate gained notoriety due to the association between the acquired Shiga toxin and risk of HUS, the pathogenesis of typical EAEC strains involves other virulence factors (Karch *et al*., 2012).

One enterotoxin that was discovered in EAEC strains is the plasmid-encoded toxin (Pet). It was originally isolated from the supernatant of outbreak strains in Mexico, and subsequently linked to cytotoxic effects on the mucosa of infected rats (Eslava *et al*., 1998). Moreover, infection of human colonic explants with prototype strain 042 resulted in mucosal abnormalities such as crypt dilation and cell rounding, which were Pet-dependent (Henderson *et al*., 1999b). Pet is a class I SPATE, a family of extracellular proteases consisting of a C-terminal domain required for translocation through the outer membrane and an N-terminal functional domain with enzymatic activity (Dautin, 2010). The SPATE family appears to be important in EAEC, with two studies respectively reporting 94.5% and 80% of typical strains contained genes for at least one SPATE (Boisen *et al*., 2009, Andrade *et al*., 2017). Studies suggest that following export by the autotransporter pathway, Pet is internalised by epithelial cells and degrades the structural protein fodrin (Boisen *et al*., 2009, Canizalez-Roman and Navarro-García, 2003). This causes cytoskeletal disruption, resulting in detachment, rounding and/or cell death (Betancourt-Sanchez and Navarro-Garcia, 2009). Expression of Pet is regulated by AggR, and there is also evidence that the transcriptional factors CRP and Fis act as co-activators for the toxin (Rossiter *et al*., 2011). While the activity of Pet is likely a contributing factor to EAEC pathogenesis, it is only found in a minority of strains, with studies reporting prevalence rates between 4.3% and 19.2% (Harrington *et al*., 2006, Andrade *et al*., 2017, Vila *et al*., 2000, Boisen *et al*., 2009).

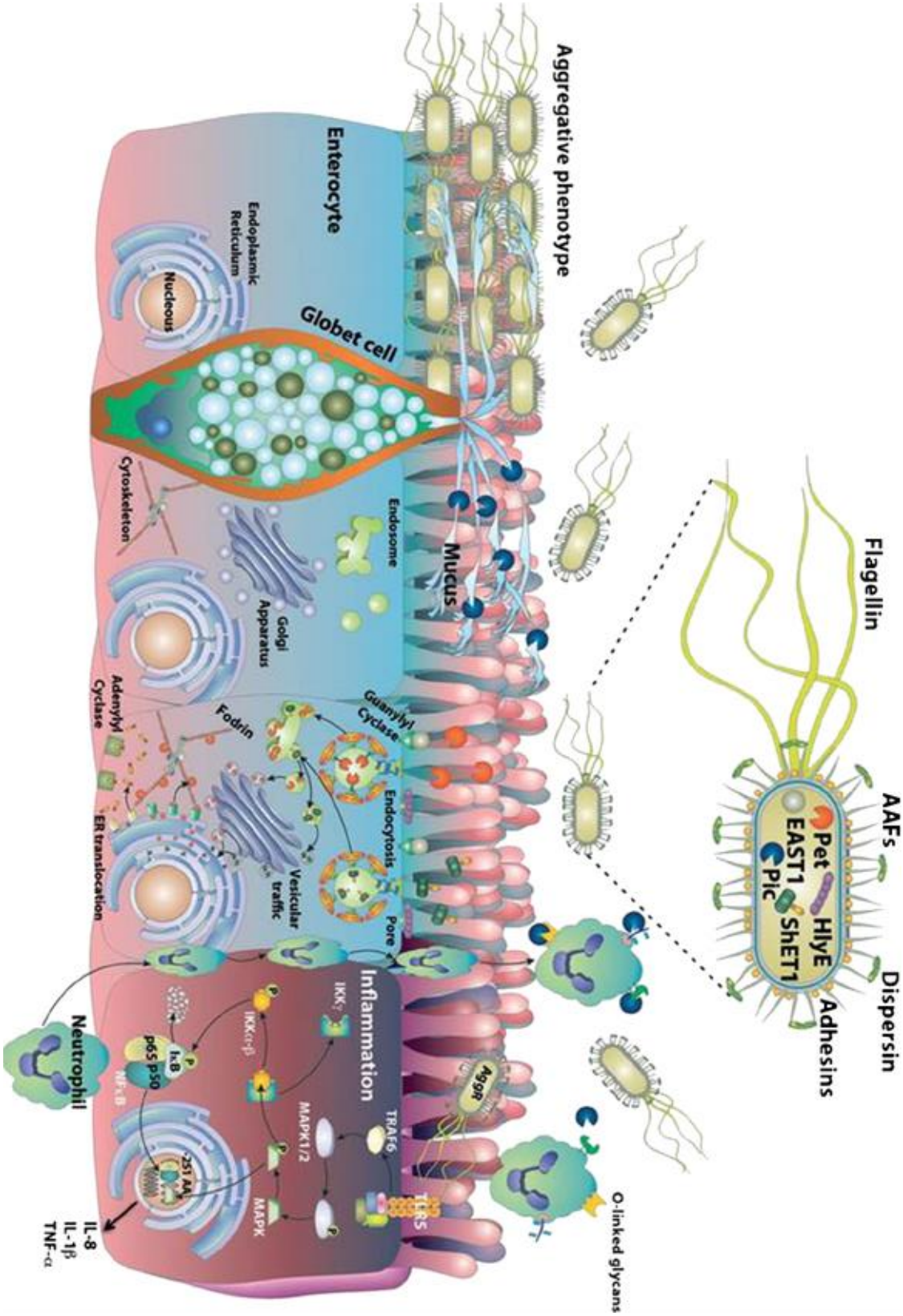

Figure 1.9 Targets of EAEC toxins and SPATEs contributing to tissue damage and inflammation (Estrada-Garcia *et al*., 2014)

Another SPATE cytotoxin linked to EAEC pathogenesis is the secreted autotransporter toxin (Sat), originally described in UPEC. Similar to Pet, Sat is only present in some EAEC strains, but has been detected in isolates from children with diarrhoea (Mendez-Arancibia *et al*., 2008). While it is absent in 042, Sat is one of the toxins expressed by the prototype strain 17-2 (Boisen *et al*., 2009). It has been proposed that Sat and Pet are functionally homologous, with similar proteolytic specificity for spectrin-like proteins and the epithelial cytoskeleton (Maroncle *et al*., 2006, Lievin-Le Moal *et al*., 2011). Other putative SPATE cytotoxins identified in a smaller proportion of EAEC strains, such as SigA and EspP, are typically associated with virulence of EHEC and *Shigella flexneri* (Boisen *et al*., 2009, In *et al*., 2013, Al-Hasani *et al*., 2009).

The EAEC heat-stable toxin 1 (EAST-1) was originally identified as a distinct EAEC virulence factor but has subsequently been detected frequently in other DEC pathotypes and *Salmonella* species (Savarino *et al*., 1996, Paiva de Sousa and Dubreuil, 2001). As with most EAEC-associated virulence factors, EAST-1 is not conserved in all strains, but is a common toxin and present in both 042 and 17-2 (Savarino *et al*., 1996, Nataro *et al*., 1995). Encoded by the *astA* gene, EAST-1 has been significantly associated with diarrhoea in case studies (Zamboni *et al*., 2004, Pereira *et al*., 2007). EAST-1 has similarities to the heat-stable enterotoxin STa of ETEC, and is proposed to function in a comparable way via interference of cyclic GMP signalling resulting in anion secretion and disrupted fluid homeostasis across the gut epithelium (Menard *et al*., 2004, Harrington *et al*., 2006). Although inconclusive in a mouse model, purified EAST-1 toxin causes sustained anion secretion from T84 cells and a secretory response in a rabbit ileal mucosa model (Savarino *et al*., 1991, Veilleux *et al*., 2008).

Some other putative virulence factors of EAEC are more commonly associated with other DEC. These include haemolysins, pore-forming cytotoxins associated with epithelial cell death in culture (Chaudhuri *et al*., 2010). One of these is α-haemolysin which is normally found in UPEC, but has been identified in some EAEC strains including 17-2 (Suzart *et al*., 2001). Prototype strain 042 expresses haemolysin E (encoded by *hlyE*), also known as cytolysin A (Chaudhuri *et al*., 2010). A role for HlyE in EAEC pathogenesis has not yet been determined, but the protein

forms a dodecameric transmembrane pore and is cytotoxic to cultured mammalian cells (Mueller *et al*., 2009). HlyE is prevalent in DEC but also found in non-pathogenic bacteria, so its importance for disease is uncertain (Navarro-Garcia and Elias, 2011). It is also unclear whether cytolethal distending toxin (Cdt) is a virulence factor of EAEC. While it is an important inducer of epithelial apoptosis for some DEC types such as EPEC, Cdt has only rarely been found in EAEC isolates (Albert *et al*., 1996, Mendez-Arancibia *et al*., 2008). Some studies have identified no Cdt-positive strains at all from clinical EAEC collections, suggesting that it is more important in EPEC than EAEC (Suzart *et al*., 2001, Patzi-Vargas *et al*., 2015).

Many EAEC strains possess a pair of virulence factors originating from *Shigella flexneri*. The first of these is a putative oligomeric toxin referred to as *Shigella* enterotoxin 1 (ShET1), which has been found in a range of EAEC isolates including 042 (Zamboni *et al*., 2004, Villaseca *et al*., 2005, Chaudhuri *et al*., 2010). ShET1 has been previously associated with intestinal fluid secretion using a rabbit model and is a possible contributor to the watery diarrhoea phenotype (Fasano *et al*., 1997, Schroeder and Hilbi, 2008). The mechanism for this effect is unclear as the enterotoxin does not affect predicted secretion mediators such as cAMP, cGMP and Ca^{2+}. However, a role in nitric oxide signalling has been suggested (Fasano *et al*., 1997, Harrington *et al*., 2006, Andrade *et al*., 2017).

The genes for ShET1 (*setA* and *setB*) are part of the same locus as another virulence factor, named 'protein involved in colonization' (Pic). Unusually, ShET1 is encoded completely within the *pic* gene on the opposite strand, so both factors are always present together. While not considered a cytotoxin, Pic has been characterised as a SPATE with potentially diverse biological functions (Henderson *et al*., 1999a). Pic exhibits mucinolytic activity which might promote penetration of the mucus layer and bacterial colonisation (Harrington *et al*., 2009). A putative role in resistance to complement proteins via Pic protease activity has also been proposed, but has not been well characterised (Henderson *et al*., 1999a). More recently, an additional secretagogue activity has been reported, as Pic was shown to induce intestinal mucus hypersecretion which may contribute to biofilm production and mucoid diarrhoea (Navarro-Garcia *et al*., 2010). Pic is relatively well conserved in EAEC, with a recent study determining it to be the most prevalent SPATE (present in 47% of

strains) in a clinical EAEC collection (Andrade *et al*., 2017). In a study of children in Peru, Pic was the only virulence factor out of 18 examined, which was statistically associated with diarrhoea (both acute and persistent), suggesting this SPATE is important for EAEC infection (Durand *et al*., 2016).

1.5.4: Inflammation

Intestinal inflammation is implicated as a contributory factor in EAEC diarrhoea, and case studies have frequently identified pro-inflammatory markers in patient stools, including lactoferrin and the cytokines interleukin (IL)-8 and IL-1β (Greenberg *et al*., 2002, Steiner *et al*., 1998). This effect can also be replicated *in vitro*, with EAEC strains inducing IL-8 release from Caco-2 cells. This was reported as the first example of an adherent, non-invasive pathogen causing cytokine release from intestinal epithelial cells (Steiner *et al*., 1998). The flagellin of EAEC strain 042 has been shown to stimulate IL-8 secretion via binding of Toll-like receptor 5 and activation of mitogen-activated protein kinases such as ERK-1/2, JNK and p38MAPK (Steiner *et al*., 2000, Khan *et al*., 2010).

In addition to flagellin, AAF/II induces IL-8 release from T84 intestinal epithelial cells infected with EAEC 042 (Harrington *et al*., 2005). This further highlights the central role of AAFs throughout EAEC pathogenesis, including adhesion, biofilm formation and pro-inflammatory response (Boll *et al*., 2012, Harrington *et al*., 2005). The inflammatory response characterised by IL-8 release leads to transepithelial migration of polymorphonuclear neutrophils as demonstrated by experiments *in vitro* and using a human intestinal xenograft mouse model (Boll *et al*., 2012). The AAF adhesins have also been implicated in inducing epithelial barrier dysfunction through disruption of tight junction components such as occludin and claudin-1 (Strauman *et al*., 2010). This may aid the translocation of neutrophils, with resulting epithelial damage from innate immune response activity or induced apoptosis suggested to contribute to diarrhoeal illness in some EAEC cases (Boll *et al*., 2012). In addition, studies using an EAEC mouse model observed an increased goblet cell concentration and mucus depletion following infection (Roche *et al*., 2010). This suggests an interaction between EAEC and goblet cells during the local response to

the pathogen, and may contribute to the manifestation of mucoid diarrhoea of some clinical cases (Hebbelstrup Jensen *et al*., 2014).

As well as inflammatory infectious diarrhoea during symptomatic infection, EAEC has been associated with prolonged subclinical inflammation. For example, in the MAL-ED study of over 2000 infants in low-resource settings, EAEC infection was associated with intestinal inflammation but not diarrhoeal symptoms (Rogawski *et al*., 2017). A recent study of enteric dysfunction in slum-dwelling children in Bangladesh determined that EAEC infection causes enhanced faecal levels of myeloperoxidase, a marker for intestinal inflammation and permeability (Fahim *et al*., 2018). It is possible that even in the absence of diarrhoea, EAEC colonisation can induce an inflammatory response with negative clinical outcomes such as malnutrition.

1.6: Influence of oxygen on bacterial virulence

Many enteric pathogens use different chemical signals in the intestinal environment to regulate virulence gene expression. Oxygen concentration has been shown to modulate the virulence of many bacterial pathogens in the GIT (Marteyn *et al*., 2011). The availability of oxygen varies greatly throughout the gut, with intestinal bacteria exposed to fluctuating conditions (**Figure 1.10**). Overall, there is a decreasing oxygen gradient along the length of the GIT as oxygen consumed with air during swallowing is diffused into the mucosal tissue or depleted by bacterial respiration. Non-invasive measurements in a live murine model determined a transition from approximately 7% oxygen in the stomach, to less than 0.5% in the distal colon (He *et al*., 1999).

However, there is also a radial oxygen gradient within the GIT. Due to oxygen diffusion from capillary blood supply across the intestinal epithelium, there is a steep increase in oxygen concentration relative to proximity to the mucosal surface (Albenberg *et al*., 2014). This is greatest in the small intestine, which has a superior mesenteric arterial blood supply than the colon. While the central lumen can be near to anoxic (< 0.1% oxygen), oxygen levels at the base of the villi are as high as

10-13% In the colon, the oxygen concentration at the mucosal surface is approximately 4-5% (Espey, 2013).

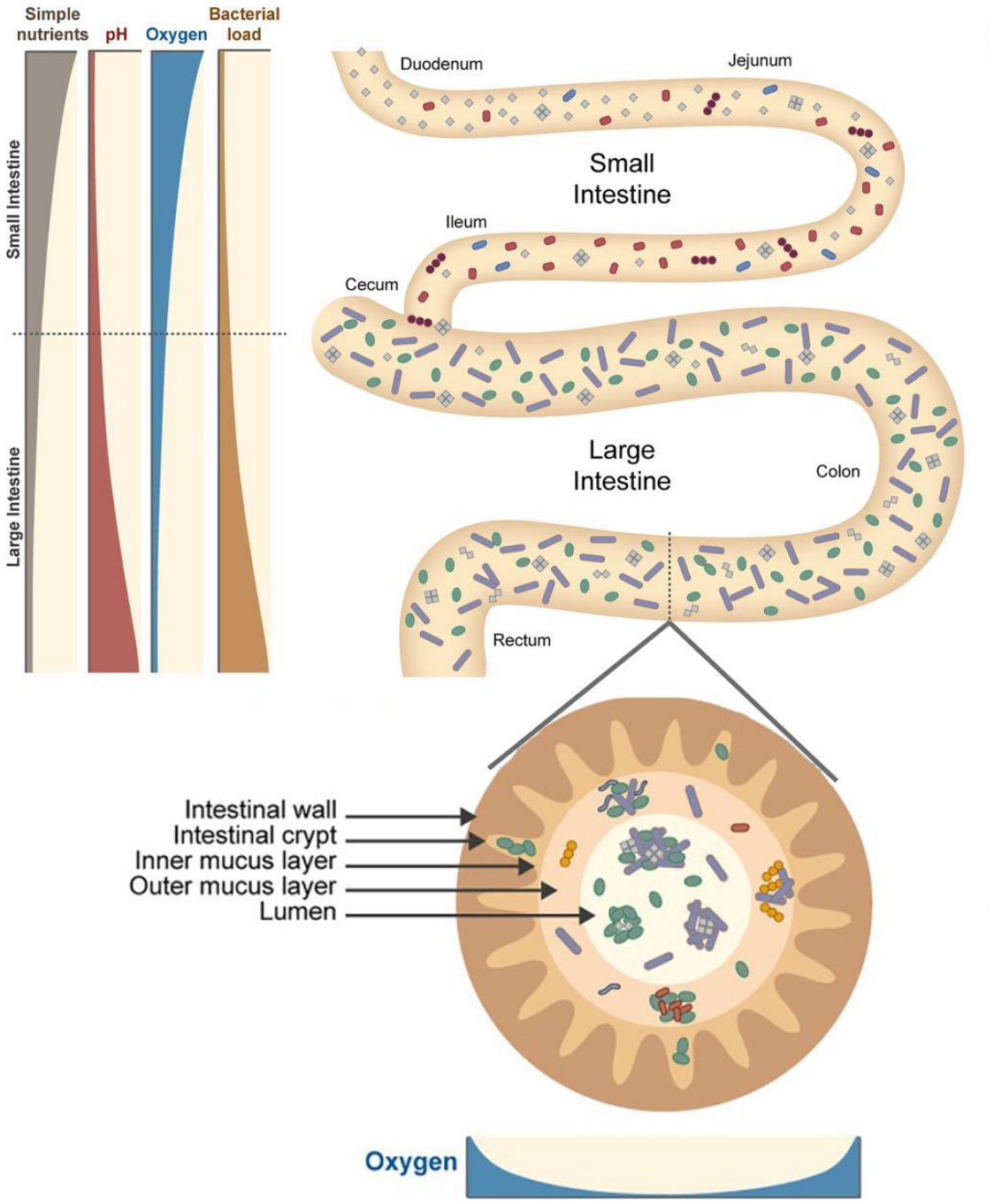

Figure 1.10 Environmental gradients in the human gastrointestinal tract. Adapted from (Pereira and Berry, 2017)

These differences in oxygen microenvironment have implications for the segregation of the intestinal microbiota. For example, the luminal gradient may act as a signal for aerotaxis of oxygen-tolerant bacteria into the mucus later, and an increase in oxygen diffusion during inflammation has been implicated in the bloom of potentially harmful facultative anaerobes associated with inflammatory bowel disease (Albenberg *et al*., 2014, Winter *et al*., 2013). The ability to adapt to fluctuations in oxygen availability is important for *E. coli* colonisation of the intestine. *E. coli* colonisation in mouse models requires both functional aerobic (AE) and anaerobic (AN) respiration, reflecting the variable oxygen levels encountered in the GIT of mammals (Jones *et al*., 2007). Oxygen gradients also modulate the direction of *E. coli* motility, and trigger fast nonmotile to motile transitions, with motility often associated with bacterial virulence (Douarche *et al*., 2009, Josenhans and Suerbaum, 2002)

Oxygen availability has been recognised as an important environmental signal for the modulation of virulence in several enteric pathogens. For example, *Salmonella* Typhimurium demonstrates increased host cell adherence and invasion at low oxygen concentrations (Marteyn *et al*., 2011). In addition, the FNR transcriptional regulator (involved in sensing of low oxygen environments) is required for full virulence of *Salmonella* in a murine model, and modifies expression of a T3SS required for pathogenesis (Fink *et al*., 2007). Microaerobic (MA) and AN growth of *Vibrio cholerae* enhances biofilm formation and expression of the Toxin co-regulated pilus virulence factor important for adherence (Marrero *et al*., 2009). A paper from 2010 elegantly described an oxygen-dependant virulence regulation system in *Shigella flexneri*: While an AN environment similar to that of the intestinal lumen enhances expression of a T3SS essential for cell invasion and virulence, it also causes an FNR-regulated suppression of effector protein secretion. Therefore, effector proteins accumulate within the bacterial cell until the suppression is removed by the oxygen gradient encountered in proximity to the mucosal surface, thereby restricting full T3SS activity to the precise site of action (Marteyn *et al*., 2010).

A similar role for oxygen sensing has been demonstrated in EHEC, where MA conditions enhanced maturation of the T3SS and adherence to polarized human

colon carcinoma cells (Ando *et al*., 2007, Schüller and Phillips, 2010, Carlson-Banning and Sperandio, 2016). The expression of Sfp adhesion fimbriae by sorbitol-fermenting EHEC O157:NM strains is also dependent on low oxygen tension (Müsken *et al*., 2008). However, there is currently a lack of research regarding the influence of oxygen on EAEC virulence.

1.7: Model systems for studying EAEC pathogenesis

1.7.1: EAEC prototype strains

Due to the great variety of EAEC, it is difficult to perform experimental studies which are representative of the whole pathotype. As a result, a few prototype strains have been frequently used in previous EAEC research as examples of typical EAEC. The most common prototype strain is 042, which was isolated from a case of infant diarrhoea in Lima, Peru in 1983 (Nataro *et al*., 1985). A major reason for the popularity of 042 was the adult volunteer study performed by Nataro *et al*. in 1995 where volunteers were fed 10^{10} colony forming units (CFU) of different EAEC isolates. While strains 17-2, JM221, and 34b failed to elicit any symptomatic response, strain 042 caused diarrhoea in 3 out of 5 adults, including one case of persistent diarrhoea over 7 days (Nataro *et al*., 1995). As a result of this, 042 subsequently became established as the major prototype strain for studying EAEC pathogenesis (Chaudhuri *et al*., 2010). However, the small size of the study groups (5 individuals per strain) limits the conclusions that can safely be made from this result. Additionally, the volunteers were given a gastric neutralisation treatment before inoculation to promote live bacteria reaching the gut, which in combination with the relatively large dose introduces further uncertainty regarding the true virulence of 042 (Nataro *et al*., 1995). It is possible that the focus on 042 may have caused a bias for subsequent attempts to identify different host factors important for EAEC-induced disease, as genes present in this strain have received greater research interest (Hebbelstrup Jensen *et al*., 2014).

Many EAEC virulence factors have been first identified in 042 including the archetypical pAA plasmid encoding AggR and dispersin (Sheikh *et al*., 2002, Morin *et*

al., 2013, Dudley *et al*., 2006b),the SPATEs Pic and Pet (Eslava *et al*., 1998, Henderson *et al*., 1999a), and the AAF/II fimbriae (Jonsson *et al*., 2017a, Czeczulin *et al*., 1997). In addition, the complete genome sequence of 042 was the first published for an EAEC strain (Chaudhuri *et al*., 2010).

Another EAEC prototype strain is 17-2 which was isolated during a study of infants with diarrhoea in Santiago, Chile in 1988 (Vial *et al*., 1988). Notably, it was in 17-2 that the first AAF variant (AAF/I) was identified in 1992. In that same study, 17-2 was given to 19 adult volunteers, of which only one subject experienced diarrhoea (Nataro *et al*., 1992). The same dose was used as in the 1995 volunteer study, where 17-2 caused no diarrhoea in a group of 5 subjects (Nataro *et al*., 1995). In this project, both 042 and 17-2 have been used as EAEC prototype strains. This allows the interpretation of the results in the context of previous findings with two common reference strains expressing AAF/I and AAF/II

1.7.2: *In vitro* models

Cell culture has been used extensively for the study of virulence mechanisms in gastrointestinal pathogens including DEC (Langerholc *et al*., 2011). An important cell type within EAEC research is the HEp-2 cell line, due to the importance of the AA phenotype on HEp-2 cells as the gold standard for identification of the pathotype (Cravioto *et al*., 1991). However, HEp-2 cells were originally established from an epidermoid carcinoma of the larynx, but have since been contaminated by the cervical carcinoma cell line HeLa (Lacroix, 2008). Therefore, they are not an ideal model for the intestinal epithelium and the specific study of host-pathogen interactions, aside from assays of general adherence and aggregation properties.

Two commonly used cell lines in this field are T84 and Caco-2. Both are derived from human colon carcinomas, yet display key differences in structure and function (Devriese *et al*., 2017). Caco-2 cells undergo spontaneous differentiation upon confluency to gain features reminiscent of small intestinal enterocytes. These include an apical brush border of microvilli comparable to that of small bowel epithelium (**Figure 1.11**), and increased expression of enterocyte-specific surface

enzymes (including sucrase, maltase, lactase, alkaline phosphatase, gamma-glutamyltransferase, aminopeptidase N, and dipeptidyl-dipeptidase IV) (Jumarie and Malo, 1991). In contrast, differentiated T84 cells resemble colonocytes, including a brush border of shorter irregular microvilli. T84 cells also express colonocyte-specific differentiation markers such as monocarboxylate transporter 1, and demonstrate a dose-dependent response to butyrate indicative of the colonocyte cell type (Devriese *et al*., 2017). In this project, T84 and Caco-2 cells have been utilised as *in vitro* models of the colonic and small intestinal epithelium, respectively.

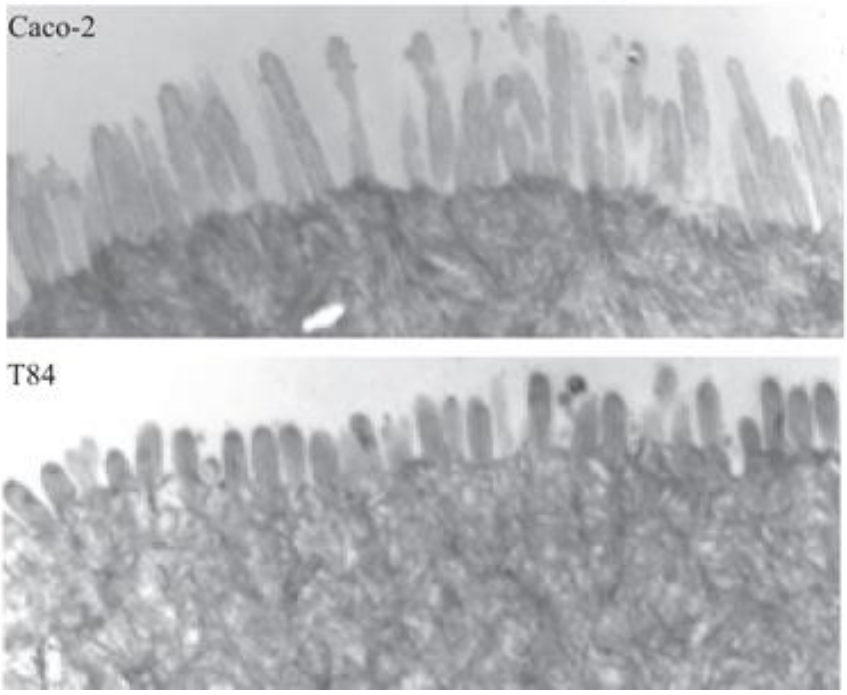

Figure 1.11 Brush border microvilli of T84 and Caco-2 cells. Transmission electron microscopy reveals that Caco-2 cells express longer microvilli on the apical cell surface. (Devriese *et al*., 2017)

Both Caco-2 and T84 have been used in previous EAEC research. For example, Nataro *et al.* demonstrated that T84 cells are a suitable model for EAEC pathogenesis due to a strong adherence of prototype strain 042 and observations of cytotoxicity induced by infection, which agreed with findings from *ex vivo* infections of human intestinal biopsies (Nataro *et al*., 1996). Caco-2 cells have been

used as model for intestinal colonisation by EAEC strains including 17-2, as well as demonstrating the induction of IL-8 release by epithelial cells in response to EAEC infection (Couto *et al*., 2007, Steiner *et al*., 1998, Steiner *et al*., 2000). Other human intestinal epithelial cell lines have been utilised in studies of EAEC pathogenesis. For example, HT29, a colon adenocarcinoma cell line, has been used to investigate EAEC adherence factors and the cytoskeletal effects of the Pet toxin (Navarro-García *et al*., 1999, Dudley *et al*., 2006a). Induction of IL-8 secretion by EAEC infection was demonstrated using cultured INT-407 cells, which were derived from an embryonic intestinal epithelium but, as with HEp-2, is now understood to be a HeLa-contaminated cell line (Khan *et al*., 2010).

In order to model the epithelial barrier, intestinal carcinoma cells can be cultured in conditions which promote cell polarisation. This allows the establishment of tight junctions which separate the apical and basolateral membrane, and results in a difference in membrane protein distribution between the two sides. Polarised cell culture can be performed using Transwell or Snapwell inserts, whereby intestinal epithelial cells are seeded onto a permeable membrane (often pre-coated with collagen for enhanced cell attachment) suspended in culture plate wells. When maintained at high cell density with frequent exchanges of culture media, the monolayers develop a columnar morphology and demonstrate high epithelial barrier function. Polarised T84 cells have been frequently used to study bacterial adherence, IL-8 release, and tight junction integrity during EAEC infection (Strauman *et al*., 2010, Harrington *et al*., 2005, Farfan *et al*., 2008). Polarised Caco-2 cells have been used along with T84 cells to compare adherence properties of prototype 042 to clinical EAEC isolates (Abe *et al*., 2001).

An *in vitro* model system called the vertical diffusion chamber (VDC) can be used in combination with polarised intestinal epithelial cells to simulate separate apical and basal conditions (**Figure 1.12**). The VDC is a dual compartment system similar to an Ussing chamber, with each side connected to independent gas flow inputs. By perfusing with either AE or AN gas mixture, cell culture media can be maintained in AE or MA conditions, respectively. When a polarised monolayer of intestinal epithelial cells is inserted between the compartments, an AE basal side can support the eukaryotic cells (mimicking oxygen supply from the blood) while the apical side

is inoculated with bacteria under the experimental conditions of interest. This allows for bacterial infection assays including control of conditions such as oxygen concentration which is not possible with standard *in vitro* cell culture models (Schüller and Phillips, 2010).

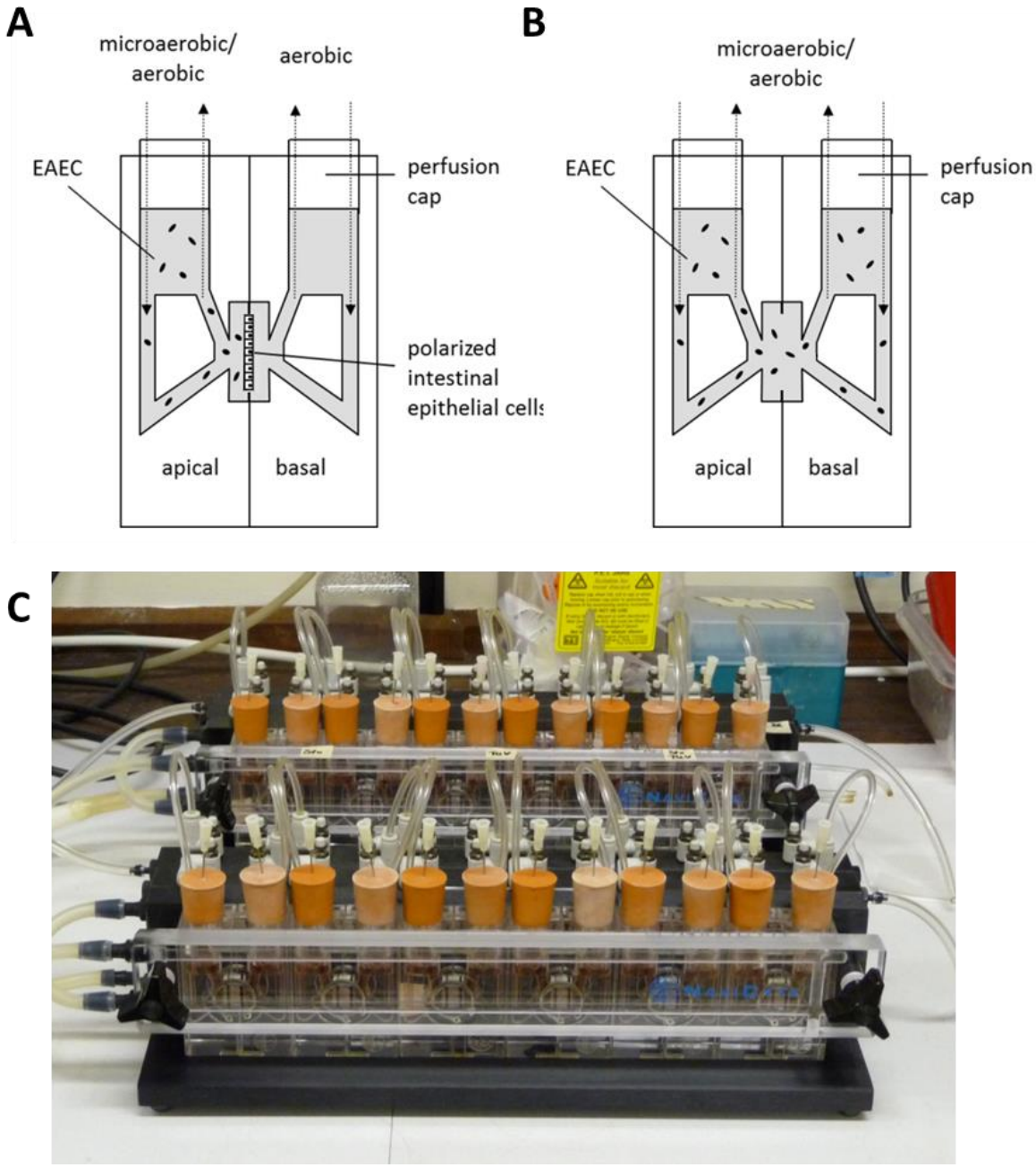

Figure 1.12 Vertical diffusion chamber apparatus. The two half chambers can be separated by a polarised intestinal epithelial cell monolayer for infections (A) or connected as one chamber for incubations of bacteria only (B). (C) Experimental setup of 12 independent chambers attached to a gas supply and mounted onto heating blocks. A and B adapted from (Tran *et al*., 2014)

The VDC system has been previously used to study interactions of EHEC with polarised T84 cells, and has demonstrated that MA conditions enhance EHEC T3S, adherence, and Shiga toxin translocation across the epithelium (Schüller and Phillips, 2010, Tran *et al*., 2014, Tran *et al*., 2018). The VDC system was first developed using polarised Caco-2 cells to study *Helicobacter pylori* infection under low oxygen conditions (Cottet *et al*., 2002). It has also been used to demonstrate enhanced *Campylobacter jejuni* invasion of polarised Caco-2 cells during MA incubation (Mills *et al*., 2012). *Clostridioides difficile* strains induce an enhanced cytokine response from polarised T84 cells under AN VDC conditions (Jafari *et al*., 2016, Anonye *et al*., 2018). However, to my knowledge the VDC model system has not previously been applied for the study of EAEC virulence mechanisms.

1.7.3: *Ex vivo* models

In vitro experiments with intestinal epithelial cells can be a practical and effective model for studying host-pathogen interactions. However, this approach has limitations such as that the monoculture of a single cell type does not replicate the complex structure of intestinal mucosal tissue and lacks important physiological features such as the mucus layer. Also, most cell lines are derived from cancer cells which exhibit a different metabolism and gene expression pattern compared to healthy tissue (Rajan *et al*., 2018). Therefore, primary cells and tissues are being increasingly used to study host-microbe interactions.

Recent emerging models are the human intestinal organoid, derived from embryonic or pluripotent stem cells, and colonoids or enteroids, generated from adult stem cells by isolating intestinal crypts (from the colon and small intestine, respectively) (Hill and Spence, 2017). Using growth-factor enriched media, it is possible to grow three-dimensional “mini-intestines” with an epithelial layer containing all major intestinal cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) (Poole *et al*., 2018, Sato *et al*., 2011). These have been proposed as a promising new model for host-pathogen interaction studies, and have been used for research with pathogens including *Salmonella* species, *Clostridioides difficile*, and Rotavirus (Sun, 2017, Forbester *et al*., 2015, Zachos *et al*., 2016). Infection of

human colonoids with EHEC resulted in actin network remodelling and tight junction disruption which was mediated by the serine protease EspP (In *et al*., 2014).

In addition, EAEC adherence (prototypical strain 042 and clinical isolates from diarrheal cases) has been examined in human enteroids derived from different regions of the human intestine, showing colonisation of duodenal, ileal and colonic tissue, but very little adherence to jejunal enteroids. This suggests that EAEC strains may have adapted for greater adherence to specific niches in the GIT. Donor-dependent differences were also reported, suggesting that host susceptibility is an important factor in EAEC colonisation (Rajan *et al*., 2018). While the technology is still developing, intestinal organoids may be a useful model for future study of EAEC and other enteric pathogens.

An *ex vivo* model which has a longer history of use for studying EAEC pathogenesis is *in vitro* organ culture (IVOC) of endoscopic mucosal intestinal biopsy samples. In brief, intestinal epithelial biopsies are obtained from consenting donors undergoing endoscopy procedures. These samples can then be maintained in a specific IVOC medium for infection assays with bacteria, which contains both cell culture (DMEM) and tissue culture (NCTC-135) media and newborn calf serum to support tissue survival *in vitro* (**Figure 1.13**) (Fang *et al*., 2013).

IVOC studies with EAEC have previously revealed novel details regarding infection of the human GIT by the pathotype. One such finding is an apparent intestinal tissue tropism of greater colonisation of the colon than the small intestine by prototype EAEC strains. For example, 042 has previously demonstrated a greater than ten-fold higher colonisation of colonic than small bowel mucosa, as enumerated by scanning electron microscopy analysis (Nataro *et al*., 1996). Higher levels of adherence to colonic biopsies compared to the ileum and jejunum have also been reported for prototype strains 17-2 and JM221 and EAEC isolates from infant diarrhoea cases (Hicks *et al*., 1996). Another IVOC study of 44 clinical EAEC isolates observed consistent colonisation to colonic tissue for all strains, which was absent for experiments with duodenal biopsies (Knutton *et al*., 1992).

IVOC of duodenal biopsies inoculated with clinical EAEC isolates from paediatric diarrhoeal cases in India demonstrated a significant IL-8 response mediated by toll-like receptor-5 activation (Gupta *et al*., 2016). This is evidence to support previous results from cell culture assays (Steiner *et al*., 2000), representing a pro-inflammatory response to EAEC by *ex vivo* epithelial tissue as well as cells in monoculture. EAEC has also been associated with an induction of mucus secretion by the intestinal epithelium, including experiments with an *in vivo* Rat model where mucus hypersecretion was dependent on the Pic protein (Navarro-Garcia *et al*., 2010). This is supported by IVOC findings where mucus accumulation and emptying of goblet cells were observed following EAEC infection (Nataro *et al*., 1996, Hicks *et al*., 1996).

Previous studies have also investigated potential cytotoxic effects of EAEC infection using IVOC, although findings are inconsistent. Some studies report no mucosal abnormalities following infection, in comparison to non-infected control tissue (Knutton *et al*., 1992). However, others have observed indicators of tissue pathology including dilated crypt openings and increased cell rounding and extrusion (Nataro *et al*., 1996, Hicks *et al*., 1996) or possible microvillous effacement (Andrade *et al*., 2011). Such changes in tissue structure have been proposed as evidence of cytotoxicity linked to known EAEC toxins such as EAST1 and HlyE, yet this has not been demonstrated experimentally (Hicks *et al*., 1996). The uncertainty between different IVOC studies may be linked to the heterogeneity of the different EAEC clinical and prototype strains that were used, or aspects of host susceptibility in different donor populations. A further confounding factor may be inconsistency in the definition of “abnormal” tissue appearance between investigators performing qualitative microscopical analysis.

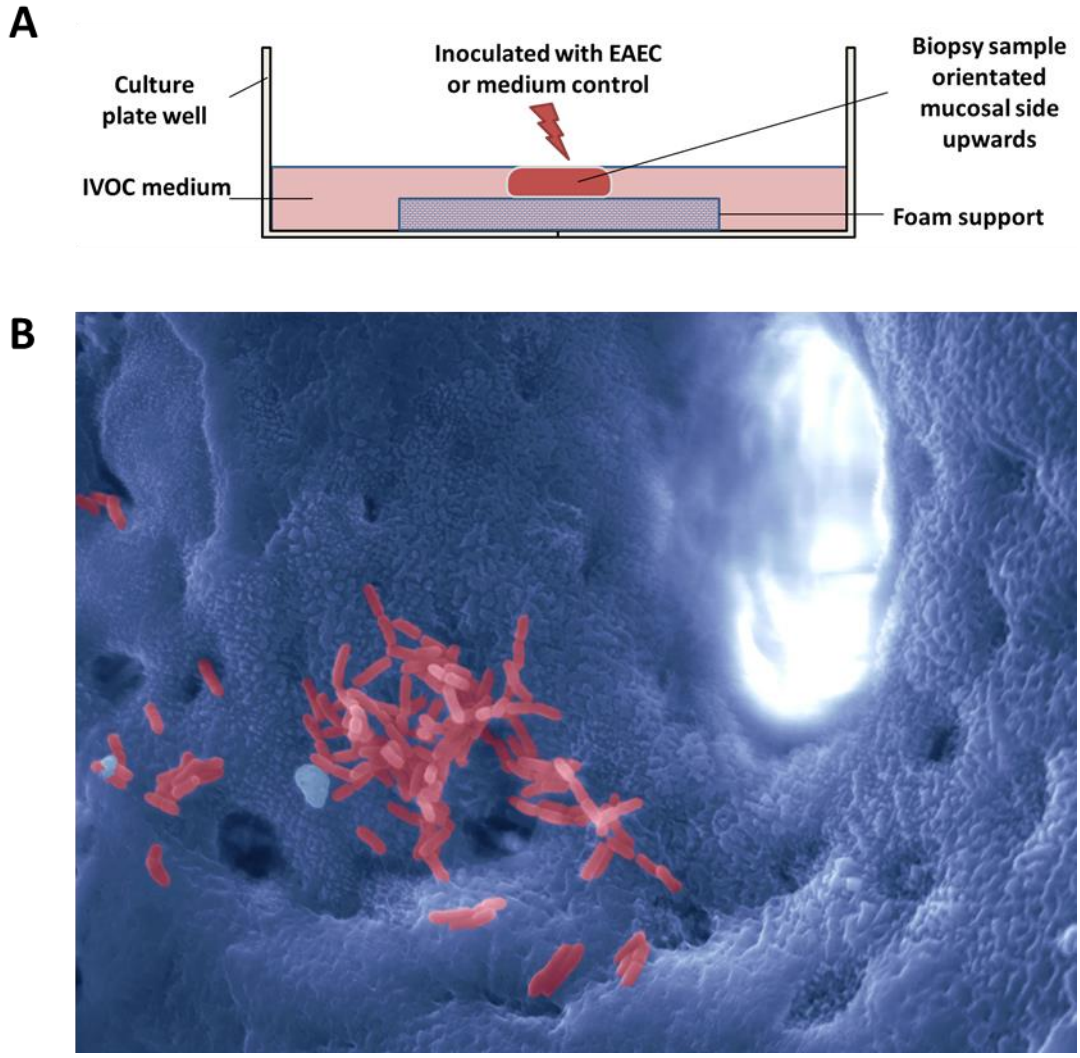

Figure 1.13 *In vitro* organ culture. (A) Experimental set-up for the incubation of a biopsy tissue sample with EAEC in a culture plate well. (B) Representative image of a colonic crypt (blue) with adherent EAEC (red) using the IVOC model. (False-coloured scanning electron micrograph; generated in this study)

As human tissue is difficult to obtain, IVOC has also been performed using animal tissue. For example, Braga *et al.* demonstrated that EAEC isolates from infant diarrhoeal cases in Brazil displayed greater adherence to rabbit colonic mucosa than ileal mucosa (Braga *et al*., 2017). Previous studies have also used rabbit ileal tissue in combination with an Ussing chamber to elucidate the enterotoxigenic effect of the EAST-1 toxin (Savarino *et al*., 1991, Savarino *et al*., 1993). Similarly, the cytotoxicity of the EAEC Pet toxin has been demonstrated using rat jejunal tissue mounted in a Ussing chamber (Henderson *et al*., 1999b).

IVOC is a powerful experimental model as it allows the study of host-pathogen interactions using native intestinal tissue with representative structure and complexity (Fang *et al*., 2013). However, there are practical limitations to the model, not least the availability of human tissue samples and variability of results due to different donor backgrounds. Furthermore, maintenance of tissue samples requires high oxygenation to delay ischemia and cell death, thereby preventing studies of EAEC infection under physiologically relevant low oxygen tension. Even under high oxygen pressure, the tissue can only be cultured for a limited time without deterioration (typically 4 to 24 hours), preventing investigation of long-term pathogenic effects (Benam *et al*., 2015, Law *et al*., 2013, Schüller *et al*., 2004).

1.7.4: Animal models

In vivo model systems are required to study enteric pathogenesis in a complex intestinal environment by taking into account the influence of an active immune system, interactions with the host microbiota, and the specific physical and chemical environment of the gut (Law *et al*., 2013). Animal models also allow to study long-term effects of bacterial infection, which is especially important for pathogens associated with chronic disease and outcomes such as malnutrition (Weintraub, 2007). However, *in vivo* studies are time and labour-intensive, alongside disadvantages regarding cost and the difficulties of biological containment of live animals for research with pathogenic strains. The complexity of animal models also limits the study of specific host-pathogen interactions (Benam *et al*., 2015). Another major obstacle is that pathogens adapted to the human host, such as EAEC and EPEC, often do not produce disease in animals (Law *et al*., 2013, Philipson *et al*., 2013).

Some of the earliest *in vivo* experiments of EAEC infection used a ligated intestinal loop model (**Figure 1.14**). This allows the incubation of bacteria within a confined intestinal section in a live animal, and the surgical removal of the affected tissue and fluids for analysis. Injection of rabbit and rat ileal loops with EAEC produced adherent colonies, as well as limb paralysis and fatal complications in some rabbits

suggesting toxin production by EAEC and contributing to the characterisation of the Pet cytotoxin (Vial *et al*., 1988, Navarro-García *et al*., 1999).

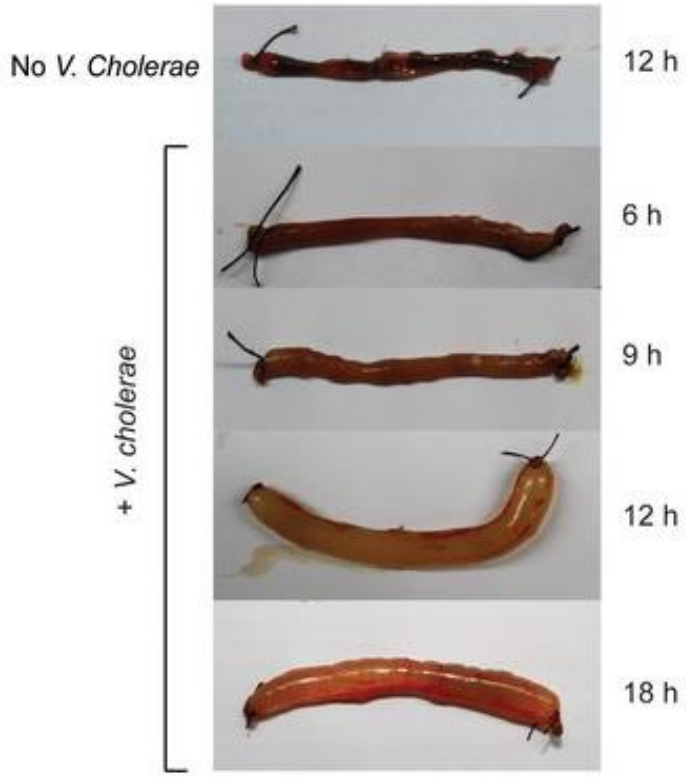

Figure 1.14 Ligated ileal loop model. Ligated ileal segments are injected with live bacteria or purified toxin for different time periods, before excision and analysis of tissue pathology. These are representative images of the model, in this case showing how the activity of the cholera toxin secreted by *Vibrio cholerae* (not EAEC) causes increasing fluid accumulation within murine ileal segments over time. Adapted from (Sawasvirojwong *et al*., 2013).

As EAEC is strongly associated with disease in infant and paediatric populations, neonatal and weanling mouse models have been developed. These also have a limited microbiota providing less colonisation resistance and immature immune systems, so are more susceptible to infection by pathogens (Roche *et al*., 2010). Neonatal mice challenged with EAEC displayed growth shortfalls dependent on bacterial dose, as well as stool shedding and histological tissue changes such as inflammatory damage and increased goblet cell density. However, infected adult mice did not demonstrate sustained stool shedding or weight loss, suggesting that the murine model replicates the increased vulnerability of infants to EAEC infection

reported in human epidemiological studies (Huang *et al*., 2006b). Interestingly, when a protein-poor diet was fed to weaned mice, EAEC-infected animals displayed impaired growth and increased stool shedding, which correlates with previous associations of clinical EAEC disease with malnourishment (Rogawski *et al*., 2017). However, a limitation of this model is a lack of symptomatic disease including diarrhoea.

Other murine models use microbiota depletion or genetic immunodeficiency to increase susceptibility of adult mice to bacterial infection. Adult mice treated with streptomycin for 48 hours prior to challenge were reliably colonised with EAEC at higher levels than untreated controls. Gastric neutralisation was also performed before oral inoculation to increase viable bacteria reaching the intestines. This model was used for *in vivo* competition assays which demonstrated the importance of dispersin and Pic for EAEC colonisation (Harrington *et al*., 2009). A similar murine model was utilised to investigate the role of the transcriptional regulators AggR and Fis in EAEC infection (Morin *et al*., 2010). However, while this model allows EAEC colonisation in adult mice, the infection does not induce clinical symptoms or histopathological abnormalities, and therefore poorly reflects human inflammation and disease (Philipson *et al*., 2013).

In addition, human intestinal xenografts in mice have been used to study EAEC infection. Human foetal intestinal tissue was implanted in severe-combined immunodeficient mice, and EAEC were inoculated intraluminally. Evaluation of tissue damage and transepithelial migration of polymorphonuclear neutrophils following infection with wild-type or mutant strains revealed that both depended on functional AAF expression (Boll *et al*., 2012). However, this approach is limited by the availability of foetal tissue samples as well as an inherently abnormal immune response.

Currently one of the best animal models reflecting EAEC infection in humans are gnotobiotic piglets. Germ-free 24-hour-old piglets challenged with EAEC demonstrated AA to the intestinal epithelium, severe diarrhoea and even mortality in some cases (Tzipori *et al*., 1992). However, the piglet model is limited by the cost and poor scalability compared to rodent models, as well as a lack of large animal

biosafety level 2 research facilities and genetic manipulation tools in pigs. As such, the gnotobiotic piglet model has not been widely used in EAEC research despite the promising initial studies. More recently, germ-free piglets were used to compare the virulence of the O104:H4 German outbreak strain against EHEC O157:H7, with the former strain unexpectedly causing only mild symptoms and no systemic disease (Wöchtl *et al*., 2017). This contrasts with the severe disease outcomes in humans during the 2011 outbreak, demonstrating the difference in EAEC virulence even in an animal model with a well-established similarity in gastrointestinal physiology and the immune system (Philipson *et al*., 2013).

Animal models are important for understanding pathogenesis in a complete intestinal system, yet are currently lacking for EAEC research due to limitations in replicating human symptomatic disease and immunological responses for a host-specific pathotype. The development of new *in vivo* models, or the refinement of existing approaches, may be necessary for the study of EAEC virulence in the future.

1.8: Summary

EAEC is an important global enteric pathogen, particularly associated with children, travellers, and the immunocompromised. It is also a causative agent of foodborne outbreaks worldwide and highly prevalent in diarrhoeal cases. Epithelial colonisation is mediated by AA, which remains the gold standard phenotype for EAEC diagnosis, although in practice PCR is now almost exclusively used for identification. However, AA is a multi-factorial phenotype, and EAEC strains are highly heterogenous in genetic background and virulence. Many putative virulence factors characterised to date are only present in a minority of strains and their importance in EAEC pathogenesis remains uncertain. Additional research using physiologically relevant infection models is needed to identify the factors and mechanisms which define EAEC pathogenesis and lead to the identification of reliable markers for the pathogenic subpopulations of this heterogenous *E. coli* pathotype.

1.9: Aim, hypotheses and objectives of this study

The overarching aim of this PhD project was to determine the underlying factors of EAEC pathogenicity in humans. The hypotheses underlying this work were that:

- Intestinal environmental signals such as oxygen concentration and host epithelial interactions modulate expression of EAEC virulence factors.
- Different subgroups of EAEC demonstrate phenotypic and genotypic differences which relate to differences in pathogenicity.

These have been investigated by addressing the following objectives:

A. Establish relevant human intestinal model systems to investigate EAEC virulence.
B. Determine the influence of intestinal environmental factors (low oxygen levels and host cell adherence) on the expression of putative EAEC virulence genes.
C. Compare phenotypic virulence traits (adherence and biofilm formation) and virulence gene profiles of EAEC isolates from sequence types associated with disease (ST40) and asymptomatic carriage (ST31).

Objectives A and B are addressed in Chapter 3, and objective C in Chapter 4.

CHAPTER TWO

MATERIALS AND METHODS

2.1: Bacterial strains and growth conditions

A full list of the wild-type *E. coli* strains used for this study is provided in **Table 2.1**. In addition, two 042 mutant strains were obtained from Douglas Browning (University of Birmingham): a knockout mutant of *aggR* and a re-complemented strain with *aggR* expressed on a pBAD30 plasmid (Sheikh *et al*., 2002, Sheikh *et al*., 2001). All experimental handling of viable EAEC strains was performed at containment level 2 throughout the project.

Frozen bacterial stocks were prepared by dilution of 700 µL of overnight cultures (ONC; in Luria Bertani Lennox (LB) broth at 37 °C) with 300 µL sterile 50% glycerol (Thermo Fisher Scientific) in distilled deionised water (ddH_2O) and stored in cryotubes (Sarstedt) at -80 °C. Bacteria from glycerol stocks were subsequently streaked out with a sterile pipette tip on LB-agar plates, incubated overnight at 37 °C, and stored for up to 1 month at 4 °C. Bacteria were passaged no more than 3 times before reversion to glycerol stocks to prevent accumulation of mutations. For infections, bacteria were inoculated from agar plates and grown as standing ONC in 2 mL LB broth at 37 °C. Antibiotic selection was used for culture of mutant strains. ONC of 042 Δ*aggR* contained 50 µg/mL kanamycin, while 042 Δ*aggR* pBAD30::*aggR* was selected with a combination of 50 µg/mL kanamycin and 100 µg/mL ampicillin.

Strain	**Disease case or asymptomatic control**	**MLST Sequence Type**	**Clinical Source**	**Year**	**Country**
Prototypes (a)					
042	Case	31	N/A	1985	Peru
17-2	Case	10	N/A	1994	Chile
Clinical isolates (b)					
E099975	Control	31	IID1	1994	UK
E103617	Case	31	IID1	1994	UK
E104931	Control	31	IID1	1994	UK

E104940	Control	31	IID1	1994	UK
E104946	Case	31	IID1	1994	UK
E104967	Case	31	IID1	1994	UK
E104969	Control	31	IID1	1994	UK
E107526	Case	31	IID1	1994	UK
E107759	Control	40	IID1	1995	UK
E109907	Control	40	IID1	1995	UK
1091	Case	40	IID2	2008	UK
1337	Case	40	IID2	2008	UK
146052	Case	40	Sporadic	2015	UK
153145	Case	40	Sporadic	2015	UK
221654	Case	40	Sporadic	2016	UK
232736	Case	40	Sporadic	2016	UK

Table 2.1 EAEC Strains used in this study. The prototype strains were originally isolated in previous studies (Nataro *et al*., 1985, Vial *et al*., 1988). The clinical isolate collection contains strains from two previous UK infectious intestinal disease studies (IID1 and IID2) and sporadic cases collected through Public Health England surveillance (Chattaway *et al*., 2014b).

2.2: Cell culture

2.2.1: Resurrection of cell lines

Cell line stocks prepared in cell culture medium containing 5% dimethyl sulfoxide (Sigma) were kept in liquid nitrogen storage (vapour phase, -190 °C) for long term preservation. To initiate cultures from frozen stocks, cells were rapidly thawed by partial submersion of the cryotube in warm water. The cell suspension was transferred into 5 mL of pre-warmed cell culture medium, and centrifuged at 100 x *g* for 7 min (Eppendorf 5810-R) to remove residual dimethyl sulphoxide. The supernatant was discarded, and cell pellet resuspended in 7 mL culture medium and transferred to a 25 cm^2 culture flask.

2.2.2: Culture conditions and passaging of cell lines

The human colon carcinoma cell line Caco-2 (ATCC HTB-37) and cervix carcinoma/HeLa contaminated cell line HEp-2 (ATCC CCL-23) were grown in Dulbecco's Modified Eagle's Medium with high glucose (DMEM; Sigma D5671) supplemented with 1x non-essential amino acids, 10% foetal bovine serum and 4 mM L- glutamine (Sigma). The human colon carcinoma cell line T84 (ATCC CCL-248) was cultured in DMEM/F-12 (Sigma D4621), supplemented with 10% foetal bovine serum and 2.5 mM L-glutamine.

Cells were grown in 25 cm^2 culture flasks (Sarstedt) and maintained at 37 °C in a 5% CO_2 atmosphere. All media used for routine cell culture was warmed to 37 °C prior to use. Cells were used for experiments within a window of approximately 15-20 passages before culture was restarted from liquid nitrogen stocks. Cells were routinely grown to confluence before passage and/or seeding for experiments.

When confluent, the cell culture medium was removed from the flask and the cell layer washed once with sterile phosphate buffered saline (PBS), and once with approximately 0.5 mL of 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution (Sigma). A further 0.5 mL of the trypsin solution was applied, and the cells incubated at 37 °C until cell detachment from the flask was observed. An incubation time of 5-10 min was required for detachment of Caco-2 and Hep-2 cells, and 15-30 min was required for T84 cell detachment. The cells were vigorously resuspended in 4.5 mL of culture medium to deactivate the trypsin, and then transferred to new culture flasks at a suitable dilution ratio. To maintain a passage approximately every 7 days, standard dilution ratios used were: 1:10 for Caco-2, 1:5 for T84, 1:10 for Hep-2 (relative to concentration before passage).

2.2.3: Determination of cell counts and seeding for infection assays

Cells were seeded for experimental use during the passaging process. Following trypsinisation and thorough resuspension of detached cells, 50 µL of cell suspension was diluted 1:1 with trypan blue solution (Sigma) and loaded into a Neubauer haemocytometer (Hawksley; depth 0.1 mm) for cell counting. Viable cells

(distinguishable by exclusion of blue stain) were counted under an inverted light microscope (Zeiss Invertoskop ID03), with the cell concentration (cell per mL) determined as the average count of two haemocytometer fields multiplied by 10^4. The volume of cell suspension required for seeding was calculated as:

Seeding volume (mL) = total number of cells required / cell concentration (cells per mL)

This volume was then diluted to the required volume of culture medium for seeding.

For standard infection experiments in 24-well plates (Sarstedt), cells were seeded onto sterile circular coverslips (13 mm diameter, Academy Science Products) for staining and microscopy, or directly onto well surfaces for all other infection assays. To achieve confluence in 7 days, cells were seeded at a density of 1.5 x 10^5 cells/mL for T84, 1.2 x 10^5 cells/mL for Caco-2, and 1.0 x 10^5 cells/mL for HEp-2 cells. Well plates were cultured at 37 °C in a 5% CO_2 atmosphere until confluency, and the culture medium was exchanged the day before infection.

2.2.4: Simulated Intestinal Media

Simulated ileal environment medium (SIEM) and simulated colonic environment medium (SCEM) were prepared according to previously published compositions (**Table 2.2**) (Polzin *et al*., 2013, Müsken *et al*., 2008). The media were autoclaved to ensure sterility, except for the D-glucose which was added from a 20x stock solution using filter-sterilisation. Media were adjusted to pH 7.0 using a Corning-240 pH meter. For SIEM, pancreatin from porcine pancreas (Sigma) instead of the individual specified digestive enzymes (α-amylase, lipase, trypsin, and chymotrypsin) was added to SIEM aliquots immediately before use to prevent loss of enzymatic activity during storage of stock solutions.

Simulated ileal environment medium		Simulated colonic environment medium	
Bacto tryptone	5.7 g/L	Bacto tryptone	6.25 g/L
D-glucose	2.4 g/L	D-glucose	2.6g/L
NaCl	6.14 g/L	NaCl	0.88 g/L
KH_2PO_4	0.68 g/L	KH_2PO_4	0.43 g/L
$NaHCO_3$	1.01 g/L	$NaHCO_3$	1.7 g/L
NaH_2PO_4	0.3 g/L	$KHCO_3$	2.7 g/L
Bile salts #3 (cholic and deoxycholic acid)	5.6 g/L	Bile salts #3 (cholic and deoxycholic acid)	4.0 g/L
Pancreatin (8 x USP)	1% (w/v)		

Table 2.2 Composition of simulated intestinal media Adapted from (Polzin *et al*., 2013, Müsken *et al*., 2008).

2.2.5: Determination of cell viability

The viability of T84 or Caco-2 cells following exposure to different culture conditions was evaluated using Trypan Blue staining (Sigma). Cells were washed with PBS, and a 50% solution of Trypan Blue stain in PBS was added. The cells were examined under an inverted light microscope (Zeiss Invertoskop ID03). Cell viability was assessed by evaluating dye exclusion, with the amount of non-stained (viable) cells compared to stained (dead) cells.

2.2.6: Culture of polarised cells in Transwells and Snapwells

For infection assays requiring a polarised cell monolayer, cells were seeded onto either Transwell or Snapwell permeable filter inserts (12 mm diameter, 0.4 μm pore polyester membrane; Corning Costar) at a density of 5×10^5 cells/insert for T84 and 2×10^5 cells/insert for Caco-2 cells. Prior to seeding, the inserts were collagen

coated to aid cell polarisation. Lyophilized rat tail collagen type I (Sigma) was dissolved in 0.1 M acetic acid and used to prepare a working solution of 50 µg/mL collagen in 60% ethanol in ddH_2O. 200 µL were added to each insert and left under sterile condition until all liquid had evaporated.

From day 4 after seeding, the culture medium was exchanged every 2 days to prevent acidification. At each medium exchange, the transepithelial electrical resistance (TEER) of the cell monolayers was measured to monitor polarisation. TEER was determined using an EVOM2 resistance meter with an STX2 electrode applied for Transwells, and an Endohm-24SNAP electrode for Snapwells (WPI). A stable TEER of greater than 1000 $\Omega \times cm^2$ for T84 and 500 $\Omega \times cm^2$ for Caco-2 cells indicated full differentiation and was typically reached after 10-14 days of growth.

To reduce the risk of contamination during repeated medium changes and TEER measurements, the culture medium for Transwells and Snapwells was supplemented with 100U/mL penicillin and 0.1 mg/mL streptomycin (Sigma). Antibiotics were removed the day before infection.

2.3: Vertical diffusion chamber

Snapwells inserts with polarised monolayers of T84 cells were inserted between the apical and basal compartments of a vertical diffusion chamber (VDC) (**Figure 2.1**; Harvard Apparatus). Each compartment was filled with 4 mL of pre-warmed non-supplemented DMEM/F-12, and chambers were mounted in a heating block pre-warmed to 37 °C by a circulating water bath (Grant Instruments). Each half-chamber was connected to a gas manifold supplying AE (5% CO_2 in air) or AN (5% CO_2, 5% H_2, 90% N_2) gas mixture; perfusion providing an AE or MA culture medium condition. After a 30 min equilibration period, the apical compartment was inoculated with $1x10^7$ EAEC, and incubated for 2-6 hours. At the end of the assay, apical media and T84 monolayers were harvested for further analysis. The disassembled chambers were sterilised between experiments by submersion in Presept Effervescent Disinfectant (Advanced Sterilization Products) for at least 1 hour.

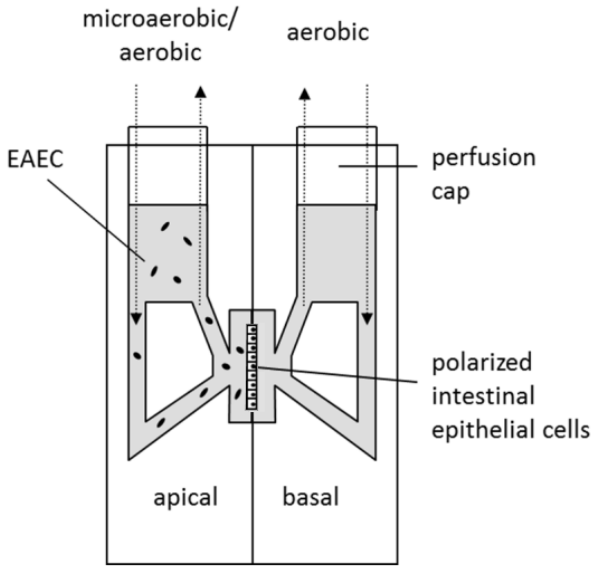

Figure 2.1 Diagram of vertical diffusion chamber incorporating a polarised T84 cell monolayer. Image modified from (Tran *et al*., 2014).

For assays without T84 cells, an empty Snapwell insert (filter removed) was used to connect both compartments of the chamber, and apical and basal compartments were gassed with the same AE or AN gas mixture (**Figure 2.2**). The total volume of 8 ml of culture medium was inoculated with $2x10^7$ EAEC.

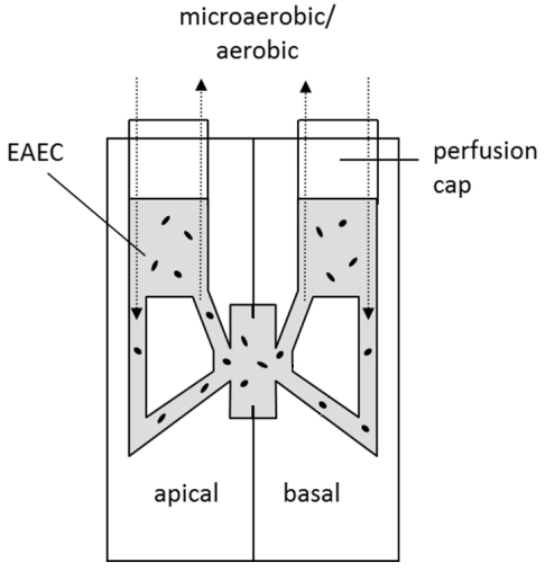

Figure 2.2 Diagram of vertical diffusion chamber with connected compartments without T84 cells. Image modified from (Tran *et al*., 2014).

The concentration of oxygen over time within the VDC was evaluated using an ISO2 isolated dissolved oxygen meter and oxygen electrode (WPI) inserted into the media. Bacterial growth within chambers was measured by determining optical density at 600 nm of 500 µL aliquots of media using a spectrophotometer (Uvikon XS, NorthStar Scientific Ltd.).

2.4: Quantification of bacterial adherence

Confluent monolayers of T84 or Caco-2 cells were grown in a 24-well plate, and wells were inoculated with 10 µL (≈1x10^7 CFU) ONC of EAEC isolates. The plate was incubated at 37 °C in a 5% CO_2 atmosphere for 2-5 hours. At the end of infection, the cell layer was washed with sterile PBS three times to remove non-adherent bacteria. Cells were lysed in 1% Triton X-100 in PBS for 10 min, and serial dilutions of lysates in sterile PBS were plated on LB agar plates. In addition, serial dilutions of the inoculum were plated. Agar plates were incubated at 37 °C overnight, and CFU were counted the following day. Counts of bacterial adherence were normalised against the inoculum concentration.

Adherence assays were also performed using the VDC system. Infection of polarised T84 cells was set up as previously described (section 2.3). A two-stage incubation was used, whereby chambers were incubated for either 2 hours, or 4 hours with media exchanged after 2 hours to reduce bacterial density. After removal from the chambers, the Snapwells were washed three times with PBS and bacterial adherence was determined as described above.

2.5: Transwell assay for cell contact dependence

An experiment was designed to determine if the influence of T84 cells on EAEC virulence gene expression required physical contact or proximity to secreted factors. T84 cells were grown to confluence in a 12-well culture plate. Infection assays were performed with 10 µL (≈1x10^7 CFU) EAEC inoculated from ONC in 3 conditions: directly into wells with a confluent T84 monolayer, into Transwell

inserts in wells containing only DMEM/F-12 media, or into Transwell inserts separated from a T84 monolayer by the permeable membrane (**Figure 2.3**). The plate was incubated for 3 hours at 37 °C in a 5% CO_2 atmosphere, and bacteria were then harvested for gene expression analysis.

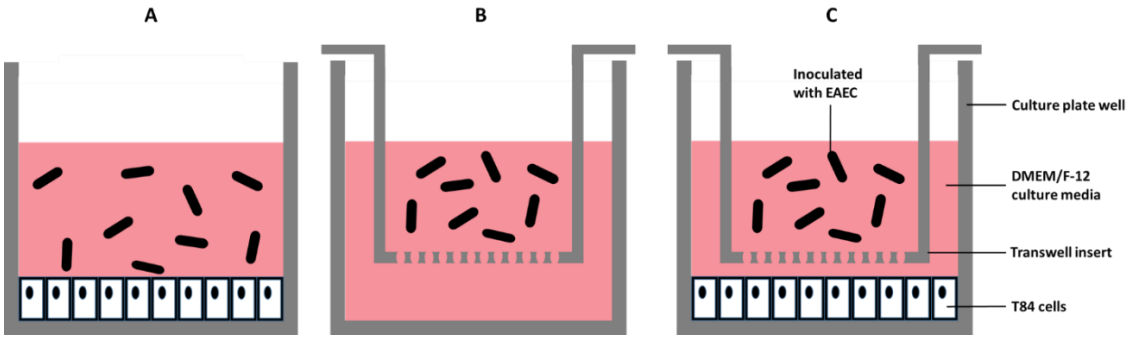

Figure 2.3 Diagram of conditions used for cell contact dependence assay. (A) EAEC were incubated for 3 hours in culture wells with confluent T84 cells, and adherent and non-adherent bacteria were harvested separately. (B) EAEC were harvested after incubation within a Transwell insert without T84 cells. (C) EAEC were harvested after incubation within a Transwell insert in wells containing confluent T84 cells.

2.6: *In vitro* organ culture of human intestinal biopsy tissue

2.6.1: Ethical approval and sample collection

This study was performed under ethical approval from the University of East Anglia Faculty of Medicine and Health ethics committee (ref 2010/11-030), with biopsies collected through the Norwich Biorepository (National Ethics Service approval; ref 08/h0304/85+5). All samples were obtained from patients recruited via the Gastroenterology Department of the Norfolk and Norwich University Hospital. Patients were undergoing routine colonoscopy for investigation of GI related symptoms or as part of the NHS bowel cancer screening programme. Informed written consent was obtained prior to donation of samples, using The Norwich Biorepository Consent Form v.15 (**Appendix 2**). Patients were excluded from the study if they met any of the following criteria: diagnosis of inflammatory bowel disease, infection with Human Immunodeficiency Virus or Hepatitis B virus, medication with blood thinning drugs.

Up to 8 biopsies were collected from an individual patient, with the tissue removed using an endoscope grasp capture forceps operated by a medically trained endoscopist. Biopsies were only collected from regions without macroscopic pathologies. Samples were collected from the second part of the duodenum (upper endoscopy) or from the terminal ileum, transverse colon, or sigmoid colon (colonoscopy). Upon removal, samples were immediately transferred into IVOC medium, transported to the laboratory, and processed within an hour.

2.6.2: Culture and infection of biopsies

Biopsies were cultured in IVOC medium, prepared from a solution of NCTC-135 (0.94 g), sodium bicarbonate (0.22 g), and D-(+)-mannose (1 g) in 90 mL ddH_2O (all Sigma). This solution was filter sterilised with a 0.45 µm syringe filter (Sartorius Stedim), and added to 90 mL DMEM and 20 mL newborn calf serum (Sigma) in a sterile glass bottle. IVOC medium was stored at 4 °C, and small aliquots in plastic bijoux (Ramboldi) taken to the hospital for transport of collected samples.

IVOC of intestinal biopsies was performed as previously by Knutton *et al.* (Knutton *et al*., 1987), with some modifications. Biopsies were initially examined for size and quality under a dissecting microscope (Zeiss Stem SV8), and where necessary dissected into pieces of approximately 5 mm^2 surface area. Samples were orientated with the mucosal side facing upwards onto foam supports (Simport) in a 12-well culture plate (**Figure 2.4**). Approximately 700 µL of IVOC medium was added to each well, and adjusted to saturate the foam support and allow only a thin film of medium to cover the biopsy without full submersion. Subsequently, 25 µL of EAEC ONC ($2.5x10^7$ CFU) or LB medium were applied directly to the mucosal surface of the biopsy. Samples were incubated on a rocking platform at 37 °C in a 5% CO_2 atmosphere for up to 8 hours. Media were exchanged at 4 and 6 hours post-inoculation to prevent bacterial overgrowth and maintain the pH. Following infection, biopsies were transferred into PBS, washed twice by vigorous shaking to remove mucus and non-adherent bacteria, and processed for microscopic analysis.

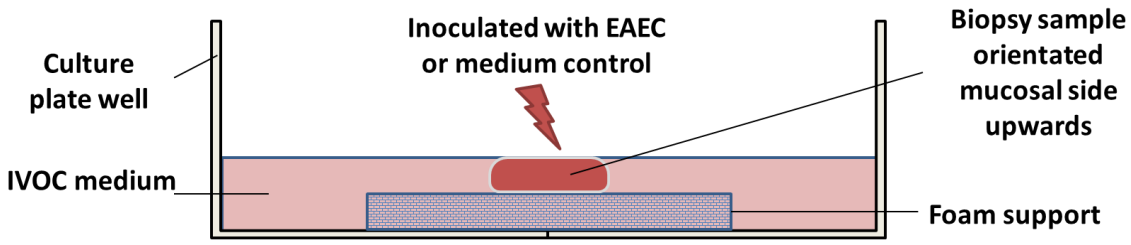

Figure 2.4 *In vitro* organ culture (IVOC)

2.7: Scanning Electron Microscopy

2.7.1: Sample preparation

Biopsy samples were fixed in 2.5% glutaraldehyde (Agar Scientific) in PBS overnight at 4 °C, washed twice in PBS, and dehydrated by sequential incubations in 30%, 50%, 70%, 90%, and 100% (twice) acetone in ddH_2O for 15 min on a rocker. The specimens were dried by a 10 min incubation in tetramethylsilane (Sigma) on a rocker and air-dried until evaporation of all residual liquid.

Processed samples were mounted onto aluminium stubs (TAAB Laboratory Equipment Ltd.) using conducive silver paint (Agar Scientific). Biopsies were orientated under a dissecting microscope with the mucosal surface facing upwards. The samples were then transported to the Environmental Analysis Laboratory of the UEA School of Environmental Sciences, where they were sputter-coated with gold (Polaron SC7640, Quorum Technologies) and imaged with a JSM 4900 LV scanning electron microscope (JEOL).

2.7.2: Semi-quantitative analysis of biopsy colonisation

Bacterial colonisation of biopsies during IVOC was scored against a relative scale based on frequency and size of adhering EAEC colonies observed by SEM. Colony size and frequency were both scored on a scale of 0-4, and the additive value used as an overall colonisation score for each biopsy. A score of 0 indicated no observed adherent bacteria.

For colony size:

1 = isolated bacteria or very small aggregates, < 10 bacteria

2 = small defined colonies, approx. 10-100 bacteria

3 = medium to large defined colonies, approx. 100-1000

4 = very large colonies, approx. >1000 bacteria

For colony frequency:

1 = approx. <10 % colonised crypts

2 = approx. 25 % colonised crypts

3 = approx. 50 % colonised crypts

4 = approx. >50 % colonised crypts

2.8: Immunofluorescence staining

Samples were fixed for immunostaining with 3.7% formaldehyde in PBS for 10-20 min at RT (Acros Organics) or by incubation in ice-cold ethanol for 15 min. For occludin staining, samples were pre-extracted in ice-cold 0.05% Triton X-100 in PBS for 2 min (Sigma) before fixation.

Fixed samples were blocked and permeabilised with 0.1% Triton X-100 and 0.5% bovine serum albumin (BSA) in PBS for 20 min (Sigma). Samples were incubated with primary antibodies (**Table 2.3**) diluted in 0.5% BSA in PBS for 1 hour at RT. The samples were washed in PBS for 10 min on a rocking platform, and subsequently incubated in Alexa Fluor 488 or 568-conjugated IgG (Life Technologies) for 30 min at RT in the dark. When a counterstain was required, DNA and filamentous actin were labelled with 4',6-diamidino-2-phenylindole (DAPI; 1:5000, Roche) and fluorescein isothiocyanate-conjugated phalloidin (1:200, Sigma), respectively, for 20 min at RT. Samples were washed in PBS for a minimum of 30 min following the final antibody incubation, and mounted on glass microscopy slides (R & L Slaughter) in Vectashield

mountant (Vector Laboratories) to reduce photo-bleaching. Slides were stored at 4 °C in the dark, and imaged with an Axio Imager 2 microscope (Zeiss).

When performing immunofluorescence staining for bacteria from liquid culture, 10 µL of sample was pipetted onto lysin-coated microscopy slides (Agar Scientific) inside an area encircled by liquid repellent slide-marker PAP pen (Sigma), and allowed to air dry. Samples were fixed and stained as described above.

Primary antibody	**Animal source**	**Dilution factor**	**Incubation temperature**	**Supplier**
Anti-*E. coli*	Goat	1:200	RT	Abcam
Anti-occludin	Rabbit	1:10	On ice	Life Technologies

Table 2.3 Primary antibodies used for immunofluorescence staining

2.9 Giemsa Staining

Coverslips were washed with PBS and fixed using a 15 min incubation in 70% methanol at room temperature (RT). The samples were stained using 10% Giemsa Modified Solution (Sigma) in PBS for 20-30 min at RT. The Giemsa solution was removed, and samples washed with dH_2O to remove excess stain. Coverslips were air-dried and mounted onto slides using Depex mounting medium (VWR) or clear nail varnish. Samples were observed under bright field or phase-contrast illumination as appropriate (Axio Imager 2 microscope, Zeiss).

2.10: Analysis of gene expression using quantitative reverse transcription PCR (qPCR)

All following protocols for analysis of RNA and cDNA were performed using RNase/DNase-free tubes, pipette tips and reagents.

2.10.1: RNA stabilisation and differential lysis

A 10% phenol-EtOH solution was prepared on ice (Sigma). Non-adherent bacteria in apical media were harvested and immediately mixed with phenol-EtOH solution to a 2% phenol final concentration, to deactivate biochemical reactions and preserve RNA. The samples were incubated for 30 min on ice, and centrifuged at 13000 rpm for 10 min at 4 °C (Eppendorf 5810-R). The supernatant was discarded, and cell pellets were snap-frozen on dry ice and stored at -80 °C.

For RNA stabilisation of bacteria adhered to T84 cells in culture-plate wells or on Snapwell filters, cells were washed with DMEM/F-12 medium and incubated on ice with 2% phenol / 20% ethanol / 3% Triton X-100 in DNase/RNase free water for 30 min to stabilise RNA and lyse the eukaryotic cells. Cells were detached by scraping with a pipette tip, and lysates were transferred to Eppendorf tubes. Bacterial pellets were collected by centrifugation (13000 rpm for 10 min at 4 °C), and snap-frozen on dry ice and stored at -80 °C.

2.10.2: RNA extraction

Total RNA was extracted from bacterial samples using the RNeasy Mini Kit (Qiagen). Samples were thawed on ice and bacterial pellets resuspended in 50 mg/mL lysozyme in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and vortexed for 30 sec initially, followed by 10 sec of vortexing every min for 5 min. Samples were treated with 350 µL of RLT buffer supplemented with 3.5 µL β-mercaptoethanol (Sigma) and briefly vortexed, followed by the addition of 250 µL of ethanol. After transfer to a Qiagen spin column, RNA was isolated according to the manufacturer's instructions for bacterial samples. Genomic DNA was removed by on-column digestion with RNase-free DNase I, following the manufacturer's protocol (Qiagen). RNA was eluted in 30 µL of RNase-free water and stored at -20 °C prior to analysis and further processing.

2.10.3: Analysis of RNA quality and quantity

RNA samples were evaluated using a NanoDrop spectrophotometer (ND-1000 & 2000, Thermo Scientific). RNA concentration was determined from absorbance at 260 nm. The absorbance ratios at 260/280 nm and 260/230 nm were used to assess protein and salt contamination, respectively.

RNA integrity was evaluated using agarose gel electrophoresis (**Figure 2.5**). RNA samples were run on a 1.5% agarose gel in TBE buffer (10.8 g/L Tris, 5.5 g/L boric acid, 2 mM EDTA, pH 8.0, in dH_2O; Sigma). Electrophoresis was performed at 100 V constant for 20 min, and the gel was stained with ethidium bromide solution (0.01% in dH_2O) for 15 min on a rocking platform. RNA was visualised under UV light with a U:Genius gel imager (Syngene), and distinct bands for bacterial 16S and 23S ribosomal RNA were taken as an indicator for RNA integrity.

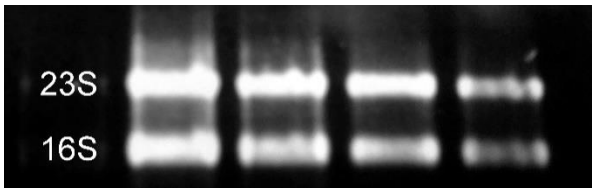

Figure 2.5 Agarose gel electrophoresis of isolated bacterial RNA. Each column represents an individual sample.

2.10.4: cDNA synthesis

Sample RNA was converted to complementary DNA (cDNA) using qScript cDNA supermix (Quanta Biosciences). The reaction mix consisted of 4 µL of the supermix and a concentration-dependent volume containing 1 µg of RNA, made up to a total volume of 20 µL with RNase-free water. Synthesis of cDNA was performed according to the manufacturer's instructions using a thermal cycler (Biometra Professional Trio). The cycling program was set as: 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C. The obtained cDNA sample was diluted with 20 µL sterile nanopure water and kept at 4 °C for short-term use or at -20 °C for long-term storage.

2.10.5: Primer design

All primers used in this study were designed using publicly available EAEC gene sequences published on the NCBI GenBank database. The primer oligonucleotides were supplied by Sigma Genosys. The primers were designed using both the Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) software, with accepted sequences requiring both software outputs to meet the following parameters:

- Primer sequence length: 15-25 base pairs
- Melting temperature (T_m): 58-63 °C
- GC content: 20-80%
- Self-complementarity score: < 8
- Amplicon length: 60-150 base pairs
- Amplicon GC content: < 60%

Additionally, the primers were also evaluated for predicted formation of primer dimers or secondary structures. For each gene target, the T_m of forward and reverse primers did not differ more than 1 °C. The primers used in this study are detailed in **Table 2.4**. The lyophilised oligonucleotides were resuspended in RNase-free water at a stock concentration of 100 µM, aliquoted and stored at -20 °C. Working solutions for use in qPCR were prepared from 5 µL of both the forward and reverse primer 100 µM stock solutions in 90 µL sterile nanopure water.

Gene	**Primer sequence (5'-3')**	**T_m (°C)**	**GC %**
gyrA (housekeeper)	CCGAAGTTACCCTGACCGTC	60.11	60
	GGTGACTCGGCGGTTTATGA	60.11	55
mdoG (housekeeper)	AATGCGTTGGTTGAGTGCTG	59.69	50
	CCCGGCTAAGGATTGAGCTT	59.82	55
gapA (housekeeper)	GGCTCCGCTGGCTAAAGTTA	60.11	55
	CCGCGCCAGTCTTTATGAGA	60.18	55

rpoB (housekeeper)	GTGGTGAAACCGCATCTTTT	60	45
	CGATGTACTCAACCGGGACT	60	55
aggR	AATTCGGACAACTACAAGCATCT	58.42	39.13
	CAACAGCAAATCCATTTATCGCA	58.58	39.13
pic	AATGCCCTGTCACTTCCCAG	59.96	55
	TCGCTGAAAGACGCTGACTT	59.97	50
hlyE	GGCTATCTAACGCCAGCAGT	59.89	55
	GCATCCGCCCAGAAAGACAT	60.75	55
aap	CGGGTCCACATTATCTGCGT	60.18	55
	TGGCATCTTGGGTATCAGCC	59.82	55
aafA	ACACCGGCTACAAATCGTGA	59.68	50
	TTGACCGTGATTGCCTTCCC	60.61	55
aggA	GACAATCCGCCTCACCGTTA	60.11	55
	AGACCCTTGCACCGCTTTTA	59.89	50
pet	TGAACTCGATGGCCTTGACC	60.04	55
	CCGGACTCAAACATGGCAGA	60.32	55
astA	GACGGCTTTGTAGTCCTTCCA	60.00	52.38
	GAAGGCCCGCATCCAGTTAT	60.18	55
cyoA	CCAGACCACAGCTTCCACTT	59.82	50
	TTCCCGCAATCTTGATGGCT	59.75	55
cydB	ACACTGGTCTGTTTCGCACT	59.82	50
	GTGGGTTAGAGGCTGCGTAA	59.75	55

Table 2.4 Primers designed and used in this study

2.10.6: qPCR

The reaction for qPCR was prepared by mixing 1 µL of cDNA with 3 µL RNase-free water, 0.1 µL internal reference dye, 5 µL SYBR Green Jumpstart Taq ReadyMix (Sigma), and 1 µL of primer pair working solution (final primer concentration of 1 µM). Two control samples were also prepared: A non-template control (NTC) with the cDNA replaced by water, and a reverse-transcription control (RTC) replacing cDNA with the equivalent amount of RNA had reverse-transcription not been performed (equivalent to 1/40 * volume containing 1 µg RNA). Samples were loaded in duplicate in a 96-well PCR plate (semi-skirt, Sarstedt) on ice, separated by a layer of cling film to prevent contact of the plate exterior with water or other contaminants. Plates were sealed with transparent sealing film (Sarstedt) and briefly centrifuged to ensure all reagents were collected at the well base (4000 rpm, 1 min, Eppendorf 5810-R).

The qPCR reaction was performed using the ABI 7500 real-time PCR system (Applied Biosystems), with the following cycling parameters:

1. 1 cycle:
    a. 95 °C, 2 min – initial denaturation
2. 40 cycles:
    a. 95 °C, 30 sec – denaturation
    b. 60 °C, 30 sec – annealing
    c. 72 °C, 35 sec – elongation (and SYBR signal data collection)
3. 1 cycle:
    a. 72 °C, 5 min – extended elongation (if higher quantity of amplicons required for further analysis such as gel electrophoresis)
4. 1 cycle: - dissociation curve analysis
    a. 95 °C, 15 sec
    b. 65 °C, 60 sec
    c. 95 °C, 15 sec
    d. 60 °C, 15 sec

At the end of the qPCR run, Taqman 7500 SDS software was used to set the absorbance value for the cycle threshold (C_T) and to generate dissociation curves.

2.10.7: Primer validation

The amplification efficiency of the designed primer pairs was determined by amplifying a two-fold serial dilution of cDNA from samples known to be positive for the gene target. The C_T values were log transformed and plotted against the dilution factor as a scatter graph. The gradient for each primer pair was used to calculate the primer efficiency according to the formula:

$$\text{Amplification efficiency (\%)} = (10^{(-1/\text{gradient})}) * 100$$

The efficiency of each primer pair was evaluated, with minimal variation between primer pairs and proximity to 100% considered optimal.

Primer specificity was confirmed by dissociation curve analysis during the qPCR run, with a single peak indicating a single amplification product. In addition, agarose gel electrophoresis confirmed the expected amplicon sizes.

2.10.8: Relative quantification of gene expression (ΔΔCT method)

Relative gene expression changes were calculated from qPCR data using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). C_T values for each sample were normalised for total cDNA concentration by subtraction of the geometric mean C_T value of two *E. coli* reference genes, glucans biosynthesis protein G (*mdoG*) and DNA gyrase (*gyrA*), to determine a normalised value referred to as ΔC_T. The fold-expression change between two samples of interest can then be calculated from the difference in ΔC_T between the treated sample and non-treated control sample, a value termed $\Delta\Delta C_T$, by the following formula:

$$\text{Fold-expression} = 2^{-\Delta\Delta CT}$$

For quality control, the C_T values for the RTC and NTC controls were assessed, and a C_T difference of greater than 5 compared to cDNA sample values (equivalent to a 32-fold difference in transcript abundance) was considered acceptable.

2.11: Western Blot

For quantification of protein expression, non-adherent bacteria were pelleted by centrifugation of culture media (4000 rpm, 10 min, Eppendorf 5810-R), whilst bacteria adherent to T84 cells were harvested by incubation of the cell monolayer for 20 min in ice-cold lysis buffer (50 mM HEPES pH 7.4, 50 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail (1:200, Sigma). Non-adherent bacterial pellets were resuspended in 1x reducing sample buffer (RSB), while 5x RSB was added to lysates for a 1x final concentration (**Table 2.5**). Samples were heat denatured at 100 °C for 5 min and electrophoresed in 15% SDS-polyacrylamide gels in 1x running buffer using a Mini-PROTEAN Tetra Cell device (Bio-Rad) for 60 min at 200 V, 50 mA, 10 W (**Tables 2.5 & 2.6**). Proteins were transferred to Polyvinylidene difluoride membranes (VWR) by wet-blotting at 100V constant for 60 min in blotting buffer (**Table 2.5**).

All following incubations were performed on a rocking platform unless otherwise stated. The membranes were blocked in 5% skimmed milk powder in TBS/0.05% Tween-20 (TBST), at RT for 60 min. After a 10 min wash in TBST, membranes were incubated with primary antibody diluted in TBST overnight at 4 °C. Primary antibodies were rabbit anti-dispersin (1:5000; provided by Christopher Icke, University of Birmingham) and rabbit anti-AAF/I (1:2000, provided by Ulrich Dobrindt, University of Münster). After a TBST wash, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200,000, Sigma) for 45 min. A final 30 min wash with TBST was used to remove non-bound antibody. The membranes were developed using enhanced chemiluminescence reagents (Immobilon Western, Millipore) and imaged with a FluorChem E Imager (ProteinSimple). ImageJ Fiji software was used for densitometric analysis of imaged blots.

Reducing Sample Buffer (RSB)

Glycerol	2.5 mL
Sodium dodecyl sulphate (SDS)	0.5 g
2M Tris pH 6.8	625 µL
Dithiothreitol	0.39 g
Bromophenol blue	5 mg
dH2O	1.9 mL

4x Stacking Gel Buffer (SGB) pH 6.8

0.5M Tris	60.5 g/L
SDS	0.4%

4x Running Gel Buffer (RGB) pH 8.8

1.5M Tris	181.6 g/L
SDS	0.4%

10x Running Buffer pH 8.3

0.25M Tris	30.3 g/L
2M Glycine	144.0 g/L
SDS	1%

Blotting Buffer pH 8.8

200mM Glycine	14.4 g/L
25mM Tris	3.0 g/L

TBS Tween 0.05%

5M NaCl	30 mL/L
1M Tris pH 8.0	10 mL/L
Tween-20	0.5 mL/L

Table 2.5 Reagents for SDS-PAGE and Western Blotting

Reagent	**Running gel (15%)**	**Stacking gel (3%)**
4x RGB/SGB	2.5 mL	1.25 mL
40% acrylamide/bis-aa	5.0 mL	500 µL
dH_2O	2.5 mL	3.2 mL
10% Ammonium persulfate	50 µL	50 µL
TEMED	10 µL	5 µL

Table 2.6 Components for 15% SDS-polyacrylamide gel

2.12: Biofilm formation assay

EAEC ONCs in LB were diluted 1:100 in DMEM, mixed by vortexing, and 100 µL of culture was added to the wells of a 96-well microplate (Sigma). The plate was incubated at 37 °C in a 5% CO_2 atmosphere for 48 hours. After removal of the medium, wells were washed twice with sterile water and stained with 0.1% crystal violet in water for 10 min (Sigma). Excess stain was removed with two washes in sterile water. Plates were left to air-dry, before 30% acetic acid in water was added to each well to solubilise the stain. The absorbance at 595 nm was measured using a Benchmark Plus microplate spectrophotometer, normalised against a blank well control (Bio-Rad).

For imaging biofilm density, 10 µL of EAEC ONC was added to wells of a 24-well culture plate (Sigma) containing 1 mL DMEM and a sterile circular coverslip (13 mm

diameter, Academy Science Products). The plate was incubated at 37 °C in a 5% CO_2 atmosphere for 48 hours. The coverslips were washed twice with sterile water and stained with 0.1% crystal violet in water for 10 min as above. Excess stain was removed by washing with sterile water and coverslips air-dried before mounting to glass microscopy slides (R & L Slaughter) with Depex mounting medium (VWR) for bright field microscopy (Axio Imager 2 microscope, Zeiss).

2.13: Sequencing and Bioinformatics

All clinical isolates from ST40 and ST31 were sequenced using Illumina platforms. Sequencing was performed by Gemma Kay (John Wain group; University of East Anglia, Faculty of Medicine and Health) and the Genomic Servicing Unit and Gastrointestinal Bacteria Reference Unit (GBRU) at PHE. The genome of prototype EAEC strain 042 has previously been published (Chaudhuri *et al*., 2010).

Many of the bioinformatic analyses were performed using tools accessed via the MRC Cloud Infrastructure for Microbial Bioinformatics (MRC CLIMB) platform (Connor *et al*., 2016). Determination of Multi-Locus Sequence Type (MLST) was achieved using the Short Read Sequence Typing 2 tool (SRST2; (Inouye *et al*., 2014)) and the Galaxy computational biology platform. SRST2 was also used for *E. coli* serotype prediction and virulence factor genotyping. *Escherichia* virulence gene sequences were obtained from the Virulence Factors of Bacterial Pathogens database (VFDB; http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Escherichia). Local Basic Local Alignment Search Tool (BLAST) analysis and *in-silico* PCR were performed using BioEdit and FastPCR, respectively (Kalendar *et al*., 2017, Hall, 1999). Core genome alignment and single-nucleotide polymorphism (SNP) detection was performed using Parsnp (Treangen *et al*., 2014), and phylogenetic trees presented using Phandango (Hadfield *et al*., 2018).

2.14: Statistics

GraphPad Prism Version 6 software was used for statistical analysis throughout this project. For the comparison of two groups, parametric student's *t*-test was used for cell line experiments and the non-parametric Mann-Whitney test was used for experiments with biopsies. Parametric analysis of variance (ANOVA) was performed for comparison of three or more groups, with Dunnett's multiple comparison test used for gene expression experiments. All gene expression data were $\log_{10}$ transformed prior to statistical analysis. A P value of < 0.05 was considered significant, and degrees of statistical significance are presented as follows: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$.

CHAPTER THREE

Oxygen and host cell interaction modulate EAEC virulence gene expression

Collaborative work:

Biopsies provided by Alison Prior, Vivienne Cook, Anups De Silva, and Bernard Brett

SEM support provided by Bertrand Lézé

042 *aggR* mutant strains provided by Douglas Browning

A manuscript containing work from this chapter has been accepted for publication in *Cellular Microbiology*

3.1: Introduction

Essential virulence factors have not been established for EAEC pathogenesis, unlike other *E. coli* pathotypes; for example, the type III secretion system (T3SS) required for typical EHEC virulence (Schüller and Phillips, 2010, Kaper *et al*., 2004). However, many putative EAEC virulence factors have been identified, linked to different aspects of human intestinal infection. EAEC is associated with high levels of aggregation, including self-agglutination and on biological surfaces. The aggregative adherence (AA) pattern of EAEC on cultured HEp-2 cells is a defining phenotype of this pathotype and has been associated with a family of adhesins termed aggregative adherence fimbriae (AAF). Many, but not all, typical EAEC possess a single AAF allele, with five variants (AAF/I to AAF/V) identified so far (Jonsson *et al*., 2015, Berry *et al*., 2014, Kaur *et al*., 2010). Other adhesins have been implicated in EAEC adherence, including a non-specific *E. coli* fimbria named the *E. coli* common pilus (ECP) (Avelino *et al*., 2010, Rendón *et al*., 2007). AA has also been connected to dispersin (encoded by *aap*), a small surface protein which is suggested to mask charge interactions between fimbriae and affect spatial organisation with neighbouring bacterial cells (Blanton *et al*., 2018, Sheikh *et al*., 2002).

The symptomatic diarrhoea caused by EAEC infection is likely a result of tissue damage and inflammation induced by the expression of bacterial toxins. Two such virulence factors produced by some EAEC isolates are the pore-forming toxin haemolysin E (HlyE), and a heat-stable enterotoxin EAST-1 (encoded by *astA*) linked to disruption of cGMP signalling and anion secretion from epithelial cells (Navarro-Garcia and Elias, 2011, Savarino *et al*., 1993). A serine protease autotransporter of Enterobacteriaceae (SPATE) called plasmid encoded toxin (Pet) has also been associated with EAEC virulence, causing spectrin cleavage and cytoskeletal disruption (Betancourt-Sanchez and Navarro-Garcia, 2009, Eslava *et al*., 1998). A further SPATE found in many EAEC isolates is the mucinase Pic, which has been linked to both mucin digestion and induction of mucus hypersecretion by intestinal cells (Harrington *et al*., 2009, Navarro-Garcia *et al*., 2010). Many of these described putative virulence genes are located on the EAEC virulence plasmid pAA, including *astA*, *aap*, *pet*, and the AAF-encoding genes. The pAA plasmid also encodes a

transcriptional activator AggR, which regulates multiple virulence genes located on the pAA plasmid and the bacterial chromosome (Morin *et al*., 2013).

One obstacle to further understanding the mechanisms underlying EAEC pathogenesis is a lack of suitable *in vivo* models. Previous animal models used to investigate EAEC infection include gnotobiotic piglets, and infant, antibiotic-treated, or immunocompromised (genetically or through nutritional restriction) mice. The piglet model is limited by scalability, cost and availability of large animal biosafety level 2 facilities, while the murine models do not replicate aspects of clinical human disease including overt diarrhoea (Philipson *et al*., 2013). As such, most EAEC studies rely on *in vitro* infection models. Human intestinal epithelial cell lines such as T84 and Caco-2 have been utilised for investigating bacterial interactions with the host epithelium. However, there remains some uncertainty regarding the ability of prototype EAEC strains to colonise these cell lines. Strain 042 has shown effective colonisation of T84 cells but poor adherence to Caco-2 (Nataro *et al*., 1996, Strauman *et al*., 2010), while 17-2 has been reported to colonise Caco-2 cells but has not been studied using the T84 cell line (Steiner *et al*., 1998, Couto *et al*., 2007). In addition, previous studies have used *in vitro* organ culture of human intestinal biopsies which demonstrated EAEC adherence to small intestinal and colonic epithelium. However, the extent of colonisation of specific strains and biopsy sites has differed between studies (Knutton *et al*., 1992, Nataro *et al*., 1996, Andrade *et al*., 2011).

While the GIT is often considered AN, oxygen availability varies greatly between sites. There is a decreasing longitudinal oxygen gradient along the length of the human gut, but a radial oxygen gradient is also present due to oxygen diffusion across the epithelial barrier (Espey, 2013, Albenberg *et al*., 2014). Oxygen has previously been recognised as an environmental signal for the regulation of virulence in several enteric pathogens, including microaerobiosis enhancing host cell adherence and invasion by *Salmonella* Typhimurium and regulating the expression of virulence factors such as the Toxin co-regulated pilus and cholera toxin in *Vibrio cholerae* (Marteyn *et al*., 2011, Lee and Falkow, 1990, Marrero *et al*., 2009). In addition, low oxygen levels enhanced EHEC T3S and adherence to

polarised colonic epithelial cells (Schüller and Phillips, 2010). However, the effect of oxygen availability on EAEC virulence has not been previously characterised.

In addition to oxygen, contact with the host epithelium can also stimulate expression of virulence factors by enteric pathogens (Stones and Krachler, 2016). For example, the expression of key virulence factors encoded on the locus of enterocyte effacement (LEE) pathogenicity island in EHEC is activated by mechanosensation via GrlA regulation, in response to host cell attachment and fluid shear force (Alsharif *et al*., 2015). While many studies have investigated putative EAEC virulence factors and whether they contribute to adherence, any resulting effect of host cell contact on regulation of virulence-associated genes has not been characterised.

The objectives of this study were to:

- Characterise the adherence and colonisation of the prototypical EAEC strains 042 and 17-2 on human intestinal epithelial cell lines and intestinal biopsy samples
- Establish a microaerobic VDC system as a model for EAEC infection of polarised intestinal epithelial cells
- Determine the effect of oxygen levels and adherence to host cells on expression of EAEC virulence genes
- Investigate the importance of the transcriptional regulator AggR for the response of EAEC to these environmental signals

3.2: Results

3.2.1: Prototype EAEC strains adhere to T84 and Caco-2 intestinal cell lines

The human colon carcinoma cell lines T84 and Caco-2 were used for the characterisation of EAEC colonisation to intestinal epithelia *in vitro*. Both cell lines can form polarised monolayers in culture, with columnar cell shape, intact tight junctions, and high trans-epithelial electrical resistance (TEER) (Hidalgo *et al*., 1989,

Tran *et al*., 2014). T84 cells were used as a model for the colonic epithelium and have previously been employed for investigating EAEC infection (Nataro *et al*., 1996). The Caco-2 cell line, whilst colonic in origin, was used as a model for small-intestinal enterocytes. Features including expression of brush border enzymes and similar proteomic profiles to cells of the small intestine have led to the wider use of Caco-2 cells as an *in vitro* small bowel model for both pharmacological and microbial infection studies, including EAEC (Meunier *et al*., 1995, Lenaerts *et al*., 2007, Steiner *et al*., 2000).

To investigate the colonisation of EAEC on T84 and Caco-2, cells grown on coverslips were infected with EAEC prototype strains 042 and 17-2 for 5 hours, and bacterial colonisation was visualised by Giemsa or immunofluorescence staining (**Figure 3.1**). Giemsa staining showed that both 042 and 17-2 adhered to T84 and Caco-2 monolayers by forming large bacterial aggregates on the cell surface, consistent with the AA phenotype. However, while adherent 042 were largely confined to these aggregates, some diffuse colonisation of 17-2 could also be observed separate from aggregative colonies. This morphological difference was consistent for both intestinal cell lines.

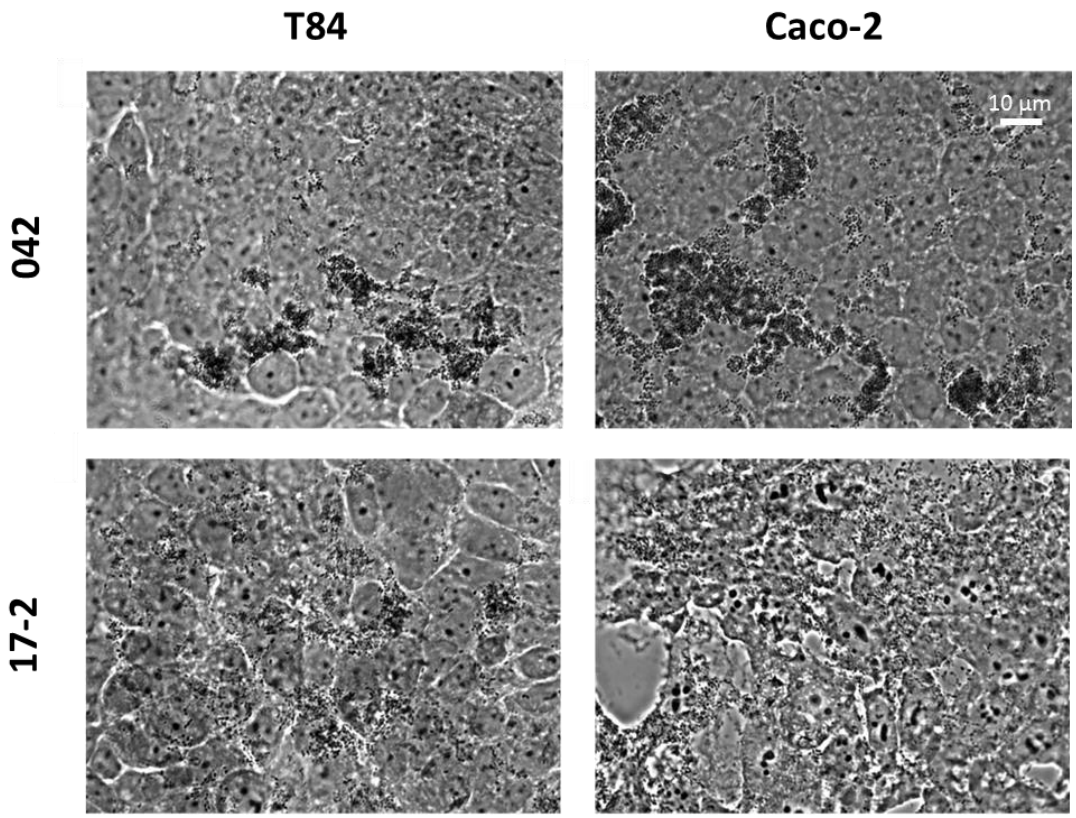

Figure 3.1 EAEC colonisation of intestinal cell lines. The EAEC strains 042 and 17-2 colonised confluent monolayers of T84 and Caco-2 cells (Multiplicity of infection (MOI) = 10 bacteria/cell, incubated for 5 hours). However, 042 demonstrated a denser colony morphology. Representative images of five experiments in duplicate, imaged by phase-contrast microscopy.

Colonisation patterns of EAEC 042 and 17-2 on T84 and Caco-2 cell monolayers were also confirmed by immunofluorescence staining (**Figure 3.2**). While the *E. coli* antibody detected both EAEC strains, some of the larger colonies observed after 5 hours of infection were only partially stained. The limited penetration of the antibodies was evident when DAPI was used as a DNA counterstain.

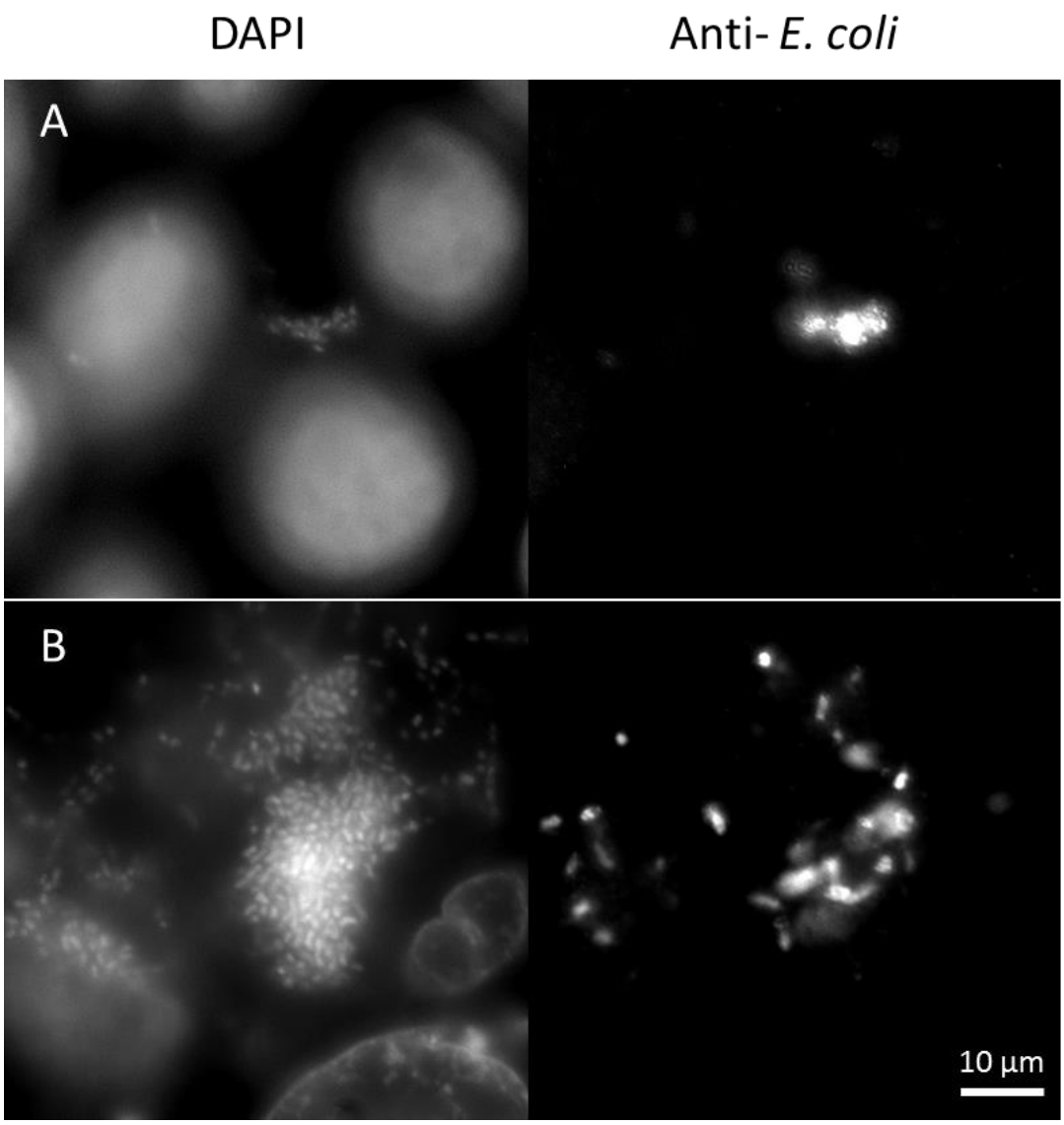

Figure 3.2 Immunofluorescence staining of EAEC colonies on intestinal epithelial cell lines. EAEC were incubated with confluent T84 or Caco-2 cells for 5 hours and stained for DNA (DAPI) and bacteria (anti-*E. coli*). Larger colonies demonstrated only partial staining, as evident by the DAPI counterstain. Representative images are of 17-2 colonisation of T84 (A) and Caco-2 (B) cells. (MOI = 10 bacteria/cell).

In addition to qualitative observations by microscopy, colonisation of the cell lines by 042 and 17-2 was quantified. Confluent T84 and Caco-2 monolayers were infected with EAEC for 2 or 5 hours. Cell layers were washed and lysed, and serial dilutions of the lysates plated on LB agar for colony counting. Colony counts were normalised by also plating out the inoculum for each experiment.

Quantification of colony forming units confirmed the previous observation that both EAEC strains could effectively adhere to both cell lines (**Figure 3.3**). There was

no significant difference in colonisation between T84 and Caco-2 monolayers at either time-point. Adherence of 17-2 was consistently higher than that of 042, but this difference was only significant for the T84 cell line at 2 hours of infection ($P < 0.05$). Overall, the quantitative and qualitative data suggests that EAEC prototype strains 042 and 17-2 can both adhere to monolayers of T84 or Caco-2 cells, but with minor differences in microcolony morphology and abundance.

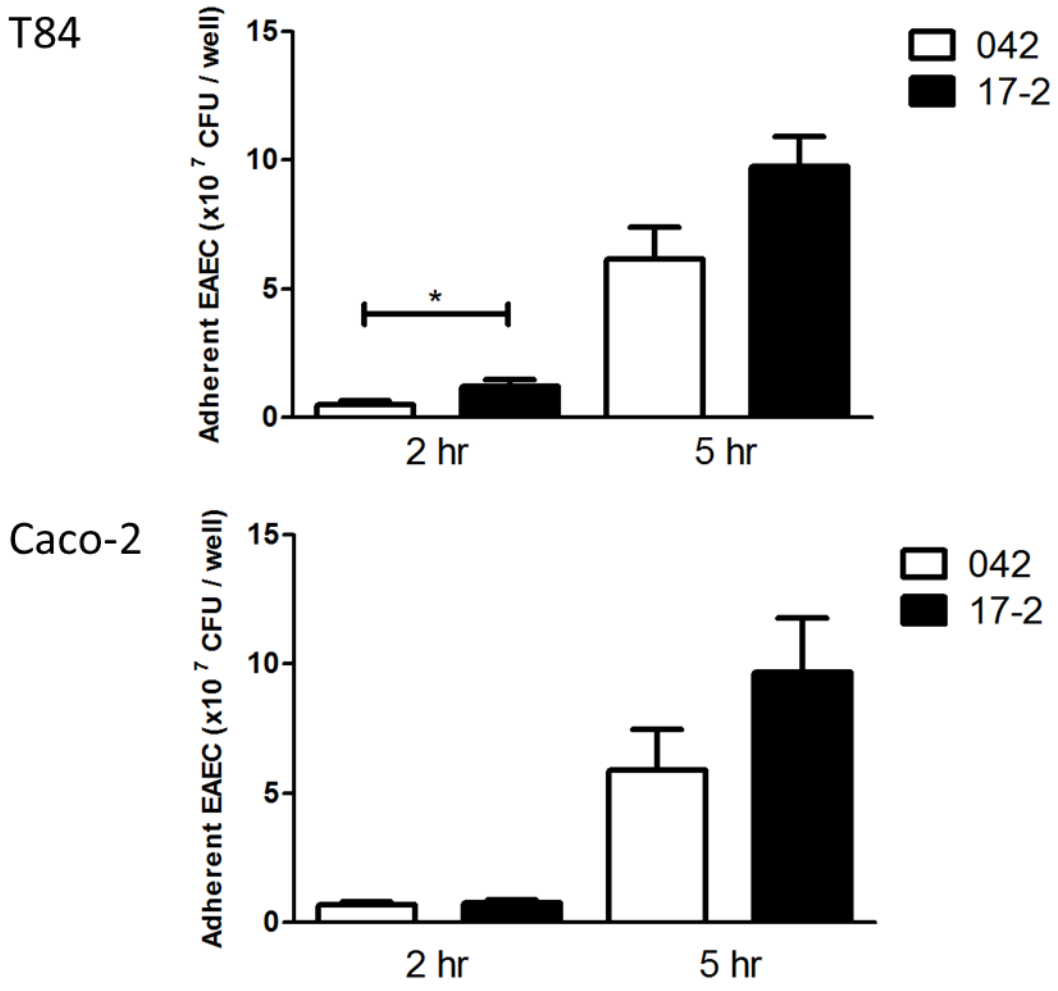

Figure 3.3 Colonisation of EAEC 042 and 17-2 to intestinal cells. Colonisation of confluent monolayers of (A) T84 and (B) Caco-2 was quantified by plate colony counting after 2 and 5 hours of infection (MOI = 10 bacteria/cell) and was normalised against the initial inoculum. Data are shown as means ± SE of three independent experiments performed in duplicate. *P < 0.05

3.2.2: Intestinal cell lines do not tolerate simulated intestinal bacterial media

This study aims to investigate the influence of the gut environment on EAEC, and one approach is to simulate physiological conditions in the experimental media. Simulated ileal environment medium (SIEM) and simulated colonic environment medium (SCEM) have previously been used to study virulence gene and proteome expression in EHEC (Polzin *et al*., 2013, Müsken *et al*., 2008). However, these studies were performed without the presence of intestinal epithelial cells. To investigate the tolerance of Caco-2 and T84 cells to incubation in simulated intestinal media, confluent monolayers were incubated in either full simulated media (Caco-2 in SIEM, T84 in SCEM) or with specific media components for 5 hours. Cell viability was assessed by Trypan Blue staining and microscopy. In addition, immunofluorescence staining for actin and occludin was performed to visualise the integrity of the cytoskeleton and tight junction network, respectively.

Incubation of Caco-2 and T84 cells in complete SIEM and SCEM, respectively, resulted in total cell detachment and loss of viability. Therefore, incubations were performed comparing normal non-supplemented cell culture medium (DMEM for Caco-2, DMEM/F-12 for T84) to the Bacto Tryptone used as the base media for SIEM and SCEM, and separate addition of bile salts or pancreatin at different concentrations, lysozyme, and the remaining soluble salts (NaCl, KH_2PO_4, $NaHCO_3$, NaH_2PO_4, $KHCO_3$). As expected, DMEM and DMEM/F-12 incubation maintained an intact monolayer of fully viable cells on the coverslips. In contrast, Bacto Tryptone alone caused partial loss of viability for both cell lines, with partial cell detachment for Caco-2 and disrupted cell morphology for T84. The addition of bile salts was also found to be toxic to the intestinal cells at >600 µM for Caco-2 and >200 µM for T84. These are far lower bile salt concentrations than the 6.6 mM in SIEM and 4.7 mM in SCEM as published for bacterial culture.

Due to the poor tolerance of the cells to Bacto Tryptone alone, incubations were performed using DMEM or DMEM/F-12 as the base media. This did not improve the tolerance of either cell line to bile salts. For Caco-2 cells, 1% (w/v) pancreatin was used to introduce digestive enzymes to the medium. However, this caused major

detachment of the monolayer from the coverslip even at a reduced 0.1% concentration, although any residual cells were seen to be viable when stained with Trypan Blue. However, the lysozyme concentration specified for SIEM had no negative effect on Caco-2 cells, and the cell lines tolerated the other soluble salts in the SIEM and SCEM recipes.

Immunofluorescence staining of cell monolayers on coverslips supported the results of the viability assays (**Figure 3.4**). When T84 and Caco-2 cells were incubated in DMEM/F-12 or DMEM respectively, a well organised actin network and intact tight junctions were observed. Bacto tryptone induced a partial loss of tight junction integrity as seen by occludin staining, as well as visible disorganisation of the actin cytoskeleton. The addition of SIEM/SCEM soluble salts caused no observable change to actin and occludin networks compared to DMEM and DMEM/F-12 controls, matching the tolerance seen with viability staining. Due to complete cell detachment in the presence of bile salts or pancreatin, immunofluorescence staining and microscopy could not be performed for these samples.

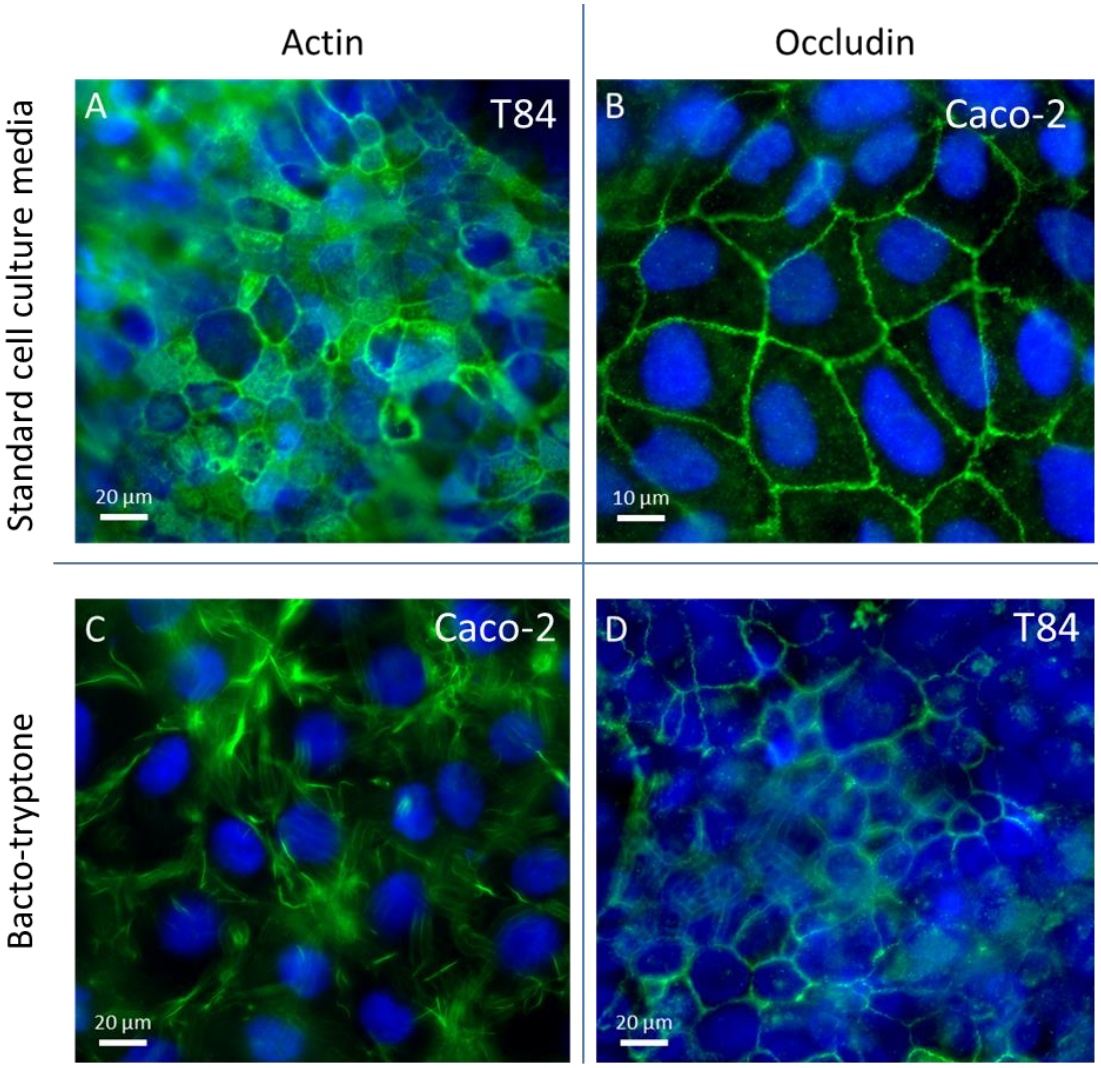

Figure 3.4 Immunofluorescence staining of T84 and Caco-2 cells to determine monolayer integrity. Confluent monolayers of T84 or Caco-2 cells were incubated for 5 hours with cell culture media (DMEM/F-12 and DMEM respectively) or Bacto-tryptone, and immunofluorescence staining performed for actin or occludin (green) and cell nuclei (blue). In cell culture media, cells formed an actin brush border (A; T84) and intact tight junctions containing occludin (B; Caco-2). Bacto-tryptone alone caused disruption of actin (C; Caco-2) and partial loss of tight junction integrity (D; T84). Representative images for experiments performed in triplicate.

In summary, while SIEM and SCEM have been used for incubations of bacteria in previous studies, most of the components including bile salts and digestive enzymes were poorly tolerated by T84 and Caco-2 cells. As soluble salt concentrations in SIEM/SCEM were generally similar to those in DMEM and DMEM/F-12, subsequent infection assays in this project were performed in DMEM (Caco-2) or DMEM/F-12 media (T84).

3.2.3: EAEC strains 17-2 and 042 adhere to human colonic but not small intestinal tissue

The use of *in vitro* organ culture (IVOC) allows the study of interactions between bacteria and human intestinal epithelial tissue *ex vivo*. This has greater physiological relevance than standard cell culture, conserving the complex tissue structure and diversity of differentiated cells, and avoiding the carcinoma background of cell lines (Fang *et al*., 2013). Previous studies using IVOC have suggested that EAEC can colonise tissue from both the small and large intestine, but certain strains demonstrated significantly greater adherence to colonic tissue (Andrade *et al*., 2011, Hicks *et al*., 1996). To investigate the ability of 042 and 17-2 to colonise the human intestinal mucosa and detect any tissue tropism, IVOC experiments were performed using biopsies taken from adult patients at four sites of the GIT: duodenum (second/descending part), terminal ileum, transverse colon, and sigmoid colon. The biopsies were infected with EAEC 042 or 17-2 for 6-7 hours and examined by scanning electron microscopy (SEM) for bacterial colonisation.

Both prototype strains colonised colonic biopsies, with extensive bacterial adherence observed to the epithelial surface, particularly around the edge of the crypts (**Figure 3.5**). Similar colonisation levels were observed on sigmoidal and transverse colon. As previously described for the adherence to T84 and Caco-2 cell lines, 042 formed distinct dense aggregates, while 17-2 established both large colonies and areas with a more diffuse pattern of adherence. Minimal colonisation by 042 and 17-2 was observed for IVOC of small intestinal tissue. Neither strain consistently adhered to biopsies from the terminal ileum, with only infrequent individual bacteria or small aggregates visible by SEM on some samples. No adherent EAEC were detected on duodenal biopsies (**Table 3.1**). These findings demonstrate that both 042 and 17-2 preferentially colonise human colonic mucosa.

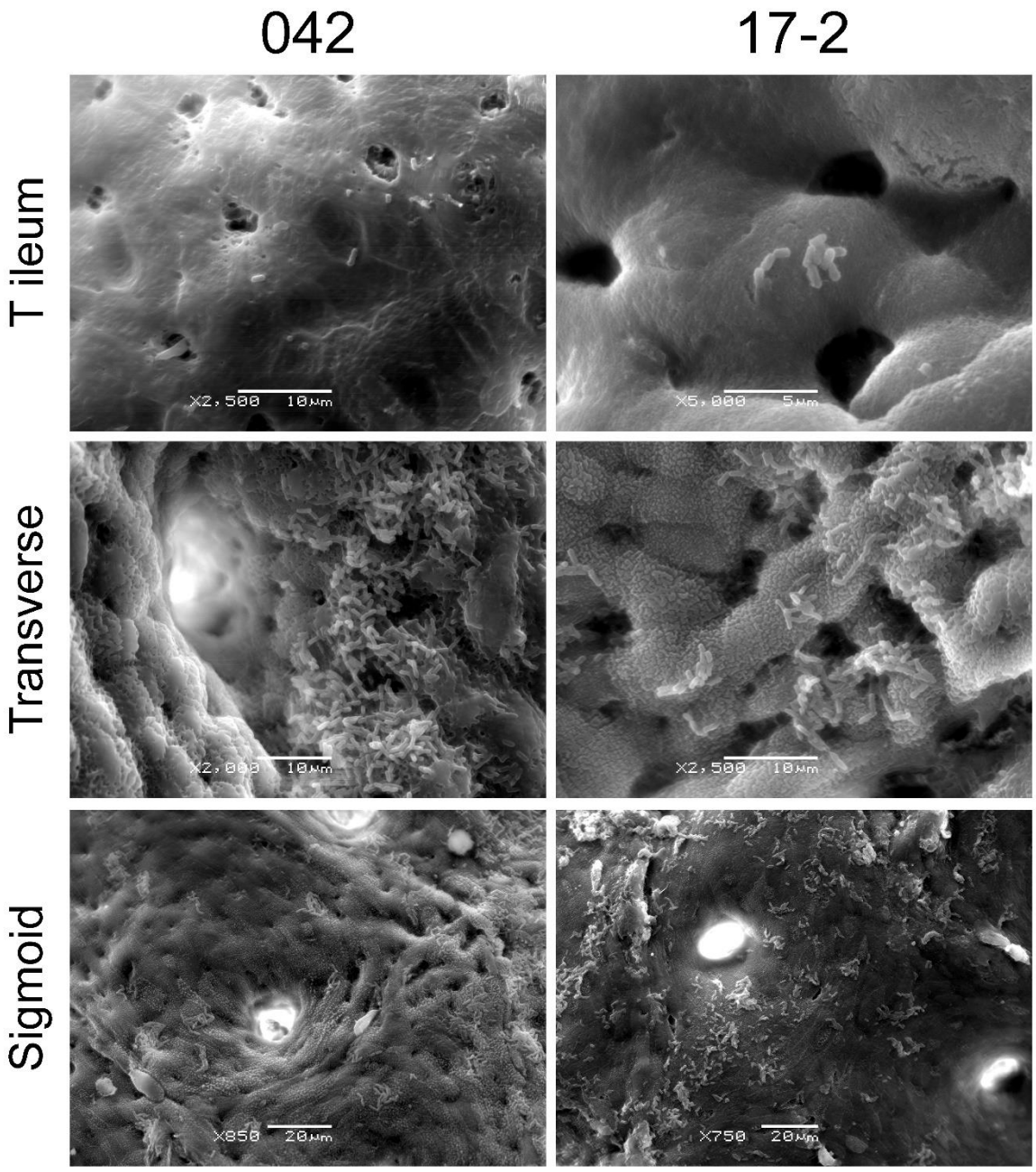

Figure 3.5 EAEC colonisation of human intestinal tissue. Scanning electron microscopy of human biopsies from the terminal ileum, transverse and sigmoid colon infected with EAEC 042 or 17-2 for 7 hours. Bars = 5 µm (top right panel), 20 µm (bottom panels) or 10 µm (all other panels). Shown are representative images of three experiments performed in duplicate.

Region	EAEC strain	
	042	17-2
Duodenum	0/8 (0)	0/8 (0)
Terminal ileum	4/7 (57)*	3/7 (43)*
Transverse colon	6/7 (86)	4/7 (57)
Sigmoid colon	6/7 (86)	2/6 (33)

Table 3.1 EAEC adherence to human intestinal biopsies. Data are presented as number of biopsies with adherent aggregates/total number of biopsies (percentage). *Single bacteria or very small aggregates only.

3.2.4: Establishment of a microaerobic VDC system

The vertical diffusion chamber (VDC) system has been previously used by the Schüller research group to investigate the influence of oxygen concentration on pathogenesis of EHEC (Tran *et al*., 2014). Before use of this model for the study of EAEC infection, the tolerance of the human intestinal cells to incubation in this model was determined, as well as characterisation of the oxygen condition in the VDC.

To investigate the stability of polarised epithelial monolayers in the VDC, T84 and Caco-2 cells were seeded onto collagen-coated Snapwells and differentiated for 10-14 days. The culture medium was exchanged every 2 days, and the trans-epithelial electrical resistance (TEER) was recorded with a stable TEER above a threshold of 1000 $\Omega \times cm^2$ for T84 or 500 $\Omega \times cm^2$ for Caco-2 cells indicating full polarisation. The Snapwell inserts containing the polarised cell monolayer were incubated in the VDC for 4 hours, with or without EAEC and in either an AE or MA conditions. Epithelial barrier integrity was investigated by comparing the TEER for each Snapwell before and after incubation (**Figure 3.6**). For T84 cells, incubation in the VDC in non-supplemented DMEM/F-12 caused a decrease in TEER in AE and MA conditions and

with or without bacteria, but the TEER remained above the integrity threshold of 1000 $\Omega \times cm^2$. Additionally, neither apical MA conditions or presence of EAEC caused a significant decrease in barrier integrity. However, Caco-2 cells poorly tolerated the VDC incubation, with high levels of cell detachment from the Snapwell filter. As previous IVOC experiments demonstrated preferential adherence of EAEC 042 and 17-2 to colonic tissue, all subsequent experiments were carried out using the T84 cell line.

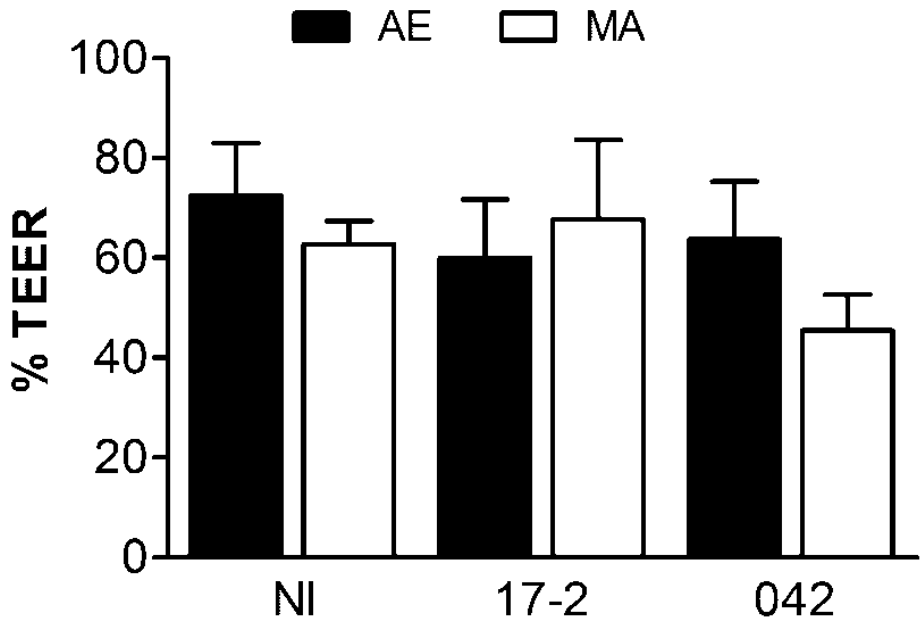

Figure 3.6 Epithelial barrier function during VDC incubation. Epithelial barrier function of polarised T84 cells was evaluated by TEER and is expressed as resistance after infection (4 hours, MOI = 20 bacteria/cell) with 042 or 17-2 or a non-infected (NI) control, relative to resistance before infection. Incubations were performed under aerobic (AE) or microaerobic (MA) conditions. Caco-2 cells detached from Snapwell filters, therefore preventing TEER measurement post-infection. Data shown as mean ± SE from three independent experiments.

To visualise bacterial colonisation and host cell morphology, polarised T84 cells infected with 042 or 17-2 for 4 hours under AE or MA conditions were stained for *E. coli*, actin, and cell nuclei and evaluated by immunofluorescence microscopy. To improve penetration of the anti-*E. coli* antibody, cells were fixed with ethanol rather than formaldehyde solution, and complete staining of EAEC colonies was

achieved using this method with no limitation. As shown in **Figure 3.7**, both EAEC strains demonstrated extensive colonisation of the epithelial cell surface. In addition, actin staining showed that the polarised T84 monolayers remained visibly confluent with an organised actin network and intact brush border. Some EAEC colonies were associated with areas of actin disruption, although it could not be determined if this was pre-existing and promoted binding or was a result of EAEC adherence.

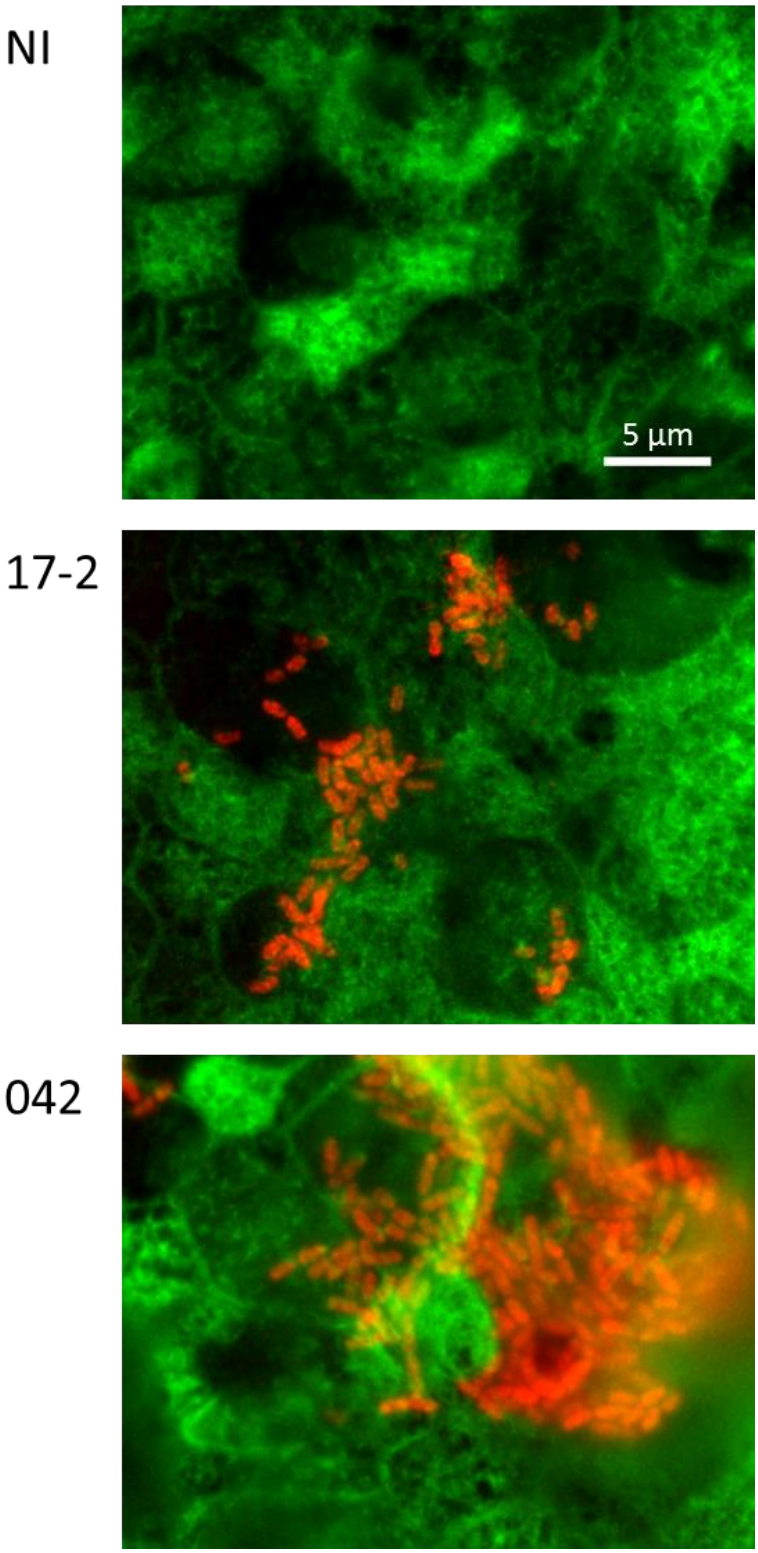

Figure 3.7 Colonisation of polarised T84 monolayers in the VDC. Polarised T84 cells were incubated with 042 or 17-2 or non-infected control (NI) for 4 hours in the VDC (MOI = 20 bacteria/cell). Aggregative adherence and epithelial integrity was visualised by immunofluorescence staining for EAEC (red) and actin (green). Data shown as representative images of four independent experiments.

To adapt the VDC system for EAEC experiments, oxygen concentrations during bacterial growth were characterised (**Figure 3.8**). This was achieved by measuring dissolved oxygen concentrations using an oxygen electrode probe over the course of a 4-hour incubation with EAEC, without T84 cells (with the probe calibrated relative to non-inoculated AE and MA chambers). In MA chambers, initial dissolved oxygen concentration was 3-4 %, but was fully depleted by 4 hours. For AE chambers, bacterial growth in the media caused oxygen levels to decrease from approximately 21% to 15% by 3 hours, and by 4 hours to 10 % and 5 % for 042 and 17-2, respectively.

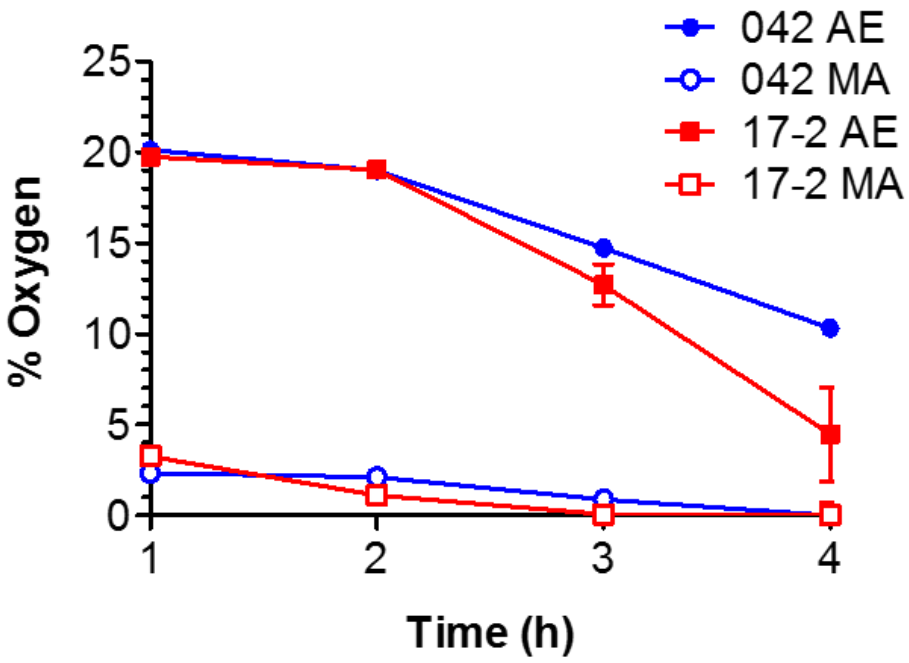

Figure 3.8 Oxygen concentration during VDC incubation. Dissolved **o**xygen concentrations during bacterial incubation over 4 hours were determined as percentage of atmospheric pressure, using an oxygen electrode probe. Data is shown as mean ± SE for three independent experiments.

To determine if these conditions induce changes in EAEC respiration pathways, bacteria were first isolated after 3 and 4 hours of incubation. After RNA extraction, the expression of the respiratory enzyme genes *cyoA* and *cydB* was determined by qPCR (**Figure 3.9**). While *cyoA* encodes a subunit of the lower oxygen affinity cytochrome O oxidase complex which is preferentially expressed in AE

environments, the product of *cydB* forms part of the high oxygen affinity cytochrome D oxidase, which is upregulated in conditions below approximately 5% oxygen (Cotter *et al*., 1990). For 042 and 17-2, AE conditions caused an induction of *cyoA* ($P < 0.05$ for 17-2; $P < 0.0001$ for 042) and downregulation of *cydB* ($P < 0.01$ for 042) compared to MA conditions. This demonstrated a distinct difference in expression of genes involved in EAEC respiration in response to the relative oxygen availability between AE and MA chambers

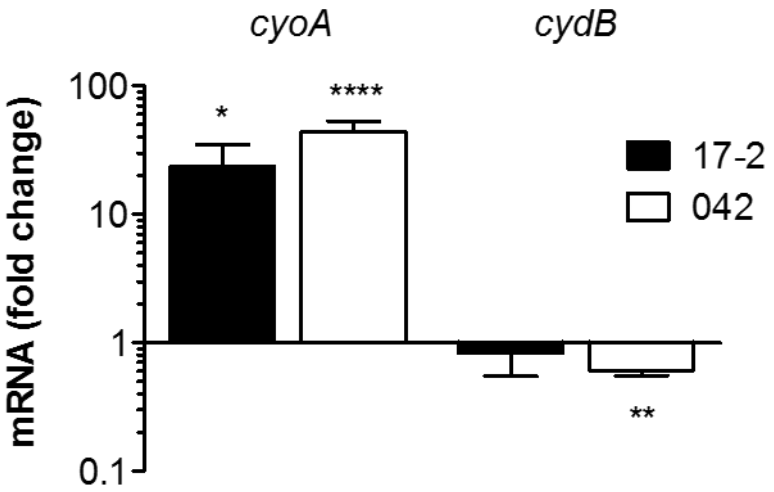

Figure 3.9 Relative expression of cytochrome oxidase genes. Bacterial respiration status after 3 hours of incubation was evaluated by qPCR gene expression analysis of low affinity cytochrome *bo3* oxidase (*cyoA*) and high affinity cytochrome *bd* oxidase (*cydB*). Gene expression is indicated as fold change under aerobic (AE) versus microaerobic (MA) conditions. Data was normalised against expression of housekeeper genes *gyrA* and *mdoG*. Data are shown as mean ± SE from three independent experiments in duplicate (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

3.2.5: Strain-specific modulation of growth and adherence by oxygen

The oxygen levels in AE and MA chambers during VDC incubation were shown to induce the use of different terminal oxygen respiration enzymes in 042 and 17-2, validating that these are distinct experimental conditions which affect EAEC gene expression. To determine the effect of oxygen availability on EAEC growth rate, strains 042 or 17-2 were incubated for up to 6 hours under AE or MA conditions in the absence of T84 cells and bacterial growth was evaluated by optical density at 600 nm. As shown in **Figure 3.10**, faster bacterial replication was observed for 17-2

than 042 in both oxygen conditions. Both strains exhibited a significantly slower growth rate during MA incubation ($P < 0.01$ for 042; $P < 0.0001$ for 17-2), with an approximate two-fold reduction in optical density compared to AE condition by 6 hours.

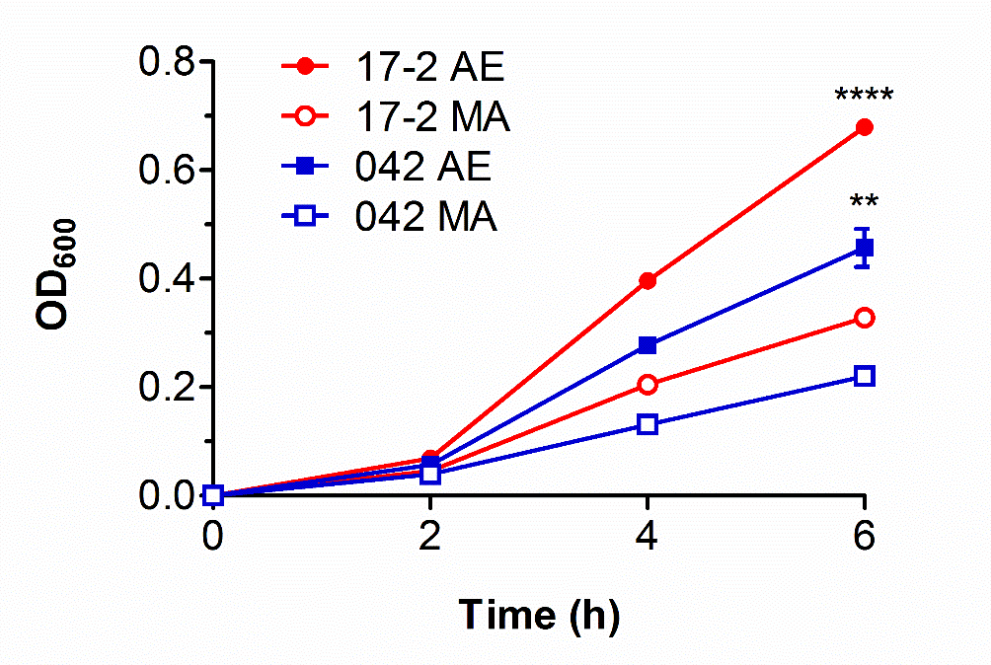

Figure 3.10 Bacterial growth during VDC incubation. EAEC were incubated in the VDC system without host cells under aerobic (AE) or microaerobic (MA) conditions for 6 hours. Bacterial growth was quantified by optical density (600 nm). **P < 0.01, ****P < 0.0001 versus MA conditions.

The influence of oxygen concentrations on EAEC adherence to T84 cells was also investigated (**Figure 3.11**). VDC experiments were performed with polarised T84 cells incubated with 042 or 17-2, followed by lysis of the T84 monolayer and plating of a serial dilution of lysate on LB agar for colony counting analysis. Initial assays evaluated adherence after 2 hours to determine initial colonisation. However, neither strain demonstrated a significant change in adherence rate when comparing AE to MA infection.

To determine if oxygen influenced colonisation over a longer time period, an additional series of VDC experiments were performed whereby after 2 hours incubation of polarised T84 cells with EAEC, apical media were exchanged with sterile DMEM/F-12. After incubation for a further 2 hours, cell-associated bacteria

were quantified as above. At this longer time-point, the adherence of 17-2 was significantly higher under AE than MA conditions ($P < 0.01$), whereas no difference was observed for strain 042 (**Figure 3.11**).

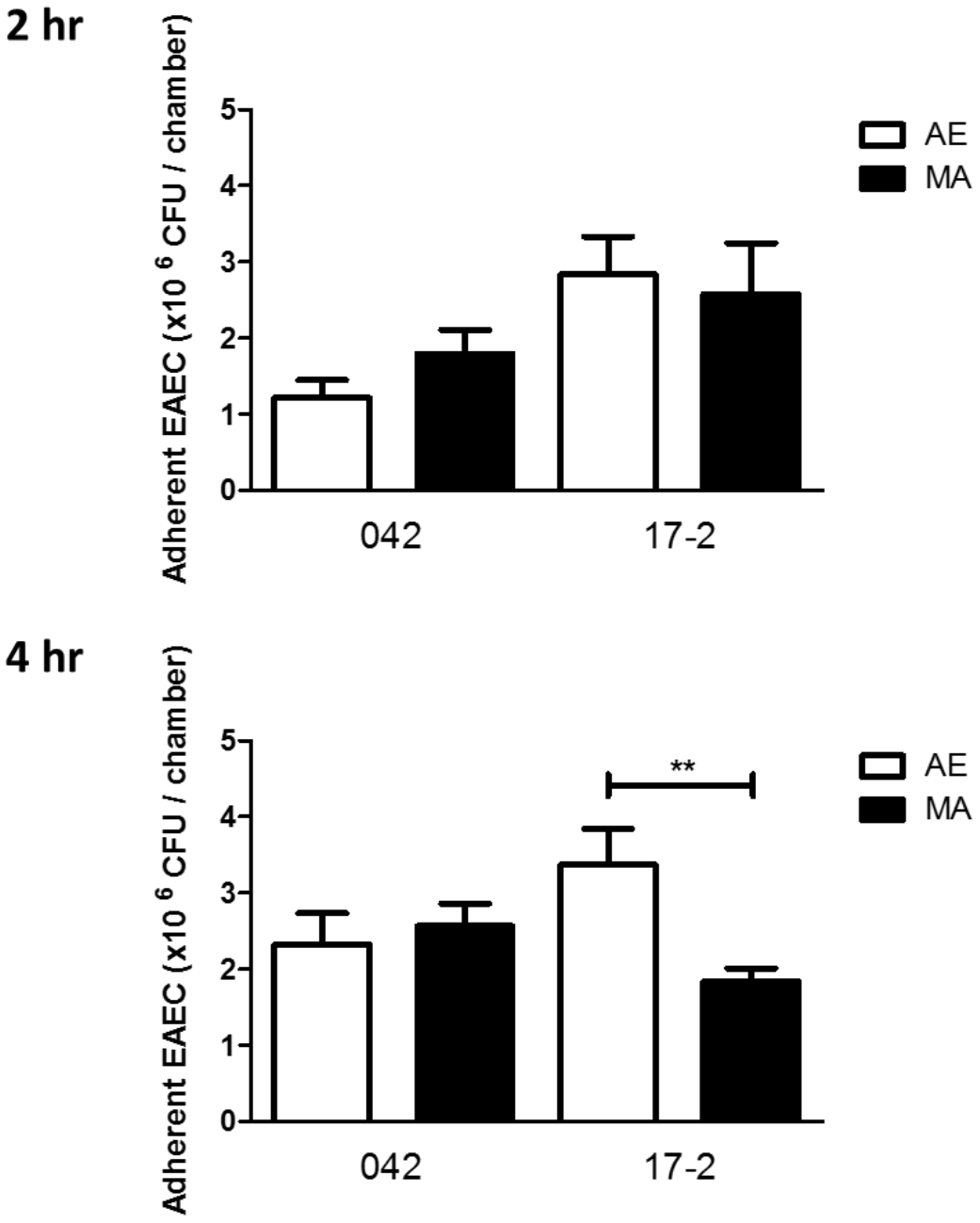

Figure 3.11 Influence of oxygen levels on EAEC colonisation of polarised T84 cells. EAEC were incubated with polarised T84 cells (MOI = 20 bacteria/cell) under aerobic (AE) or microaerobic (MA) apical conditions for 2 or 4 hours including a medium exchange after 2 hours. Colonisation was quantified by plate colony counting and was normalised against the initial inoculum. Data are shown as means ± SE of four independent experiments performed in triplicate (**$P < 0.01$).

3.2.6: Oxygen induces virulence gene expression in EAEC 042

To investigate the expression of putative EAEC virulence genes in response to oxygen availability, relative qPCR analysis and the $\Delta\Delta C_T$ method were applied (Livak and Schmittgen, 2001). Prior to this, the primers designed for this study were analysed for specificity and amplification efficiency. Primer specificity was determined for each primer pair by performing qPCR with cDNA generated from sample RNA of either 042 or 17-2 (depending on which strain contained the target gene), with the qPCR cycling parameters containing a dissociation curve analysis step. The resulting heat dissociation curves contained only single peaks for each primer pair, suggesting the generation of a single amplicon (**Figure 3.12 A**). The size of the amplification products was determined by agarose gel electrophoresis alongside a molecular-weight size marker (**Figure 3.12 B**). For each primer pair, a single band was observed to further confirm the presence of a specific amplification product, and the band positions were found to match the expected size of the amplicon. These results validated the designed primer pairs as specific for the target gene sequences.

Relative qPCR analysis using the $\Delta\Delta C_T$ method requires validation of sufficiently high amplification efficiencies for all primer pairs. To determine this, qPCR was performed with a two-fold serial dilution of cDNA. The resulting C_T values were plotted against dilution factor, allowing the amplification efficiency to be calculated from the gradient for each primer pair (**Figure 3.13**). The range of amplification efficiencies all exceeded 95%, above the 90% threshold recommended for use in experimental qPCR analysis (**Table 3.2**) (Svec *et al*., 2015). Values greater than the theoretical maximum of 100% may be due to inhibitors carried over from sample or reagents, as this is more pronounced in more concentrated cDNA samples and can skew the linear fit (Svec *et al*., 2015)

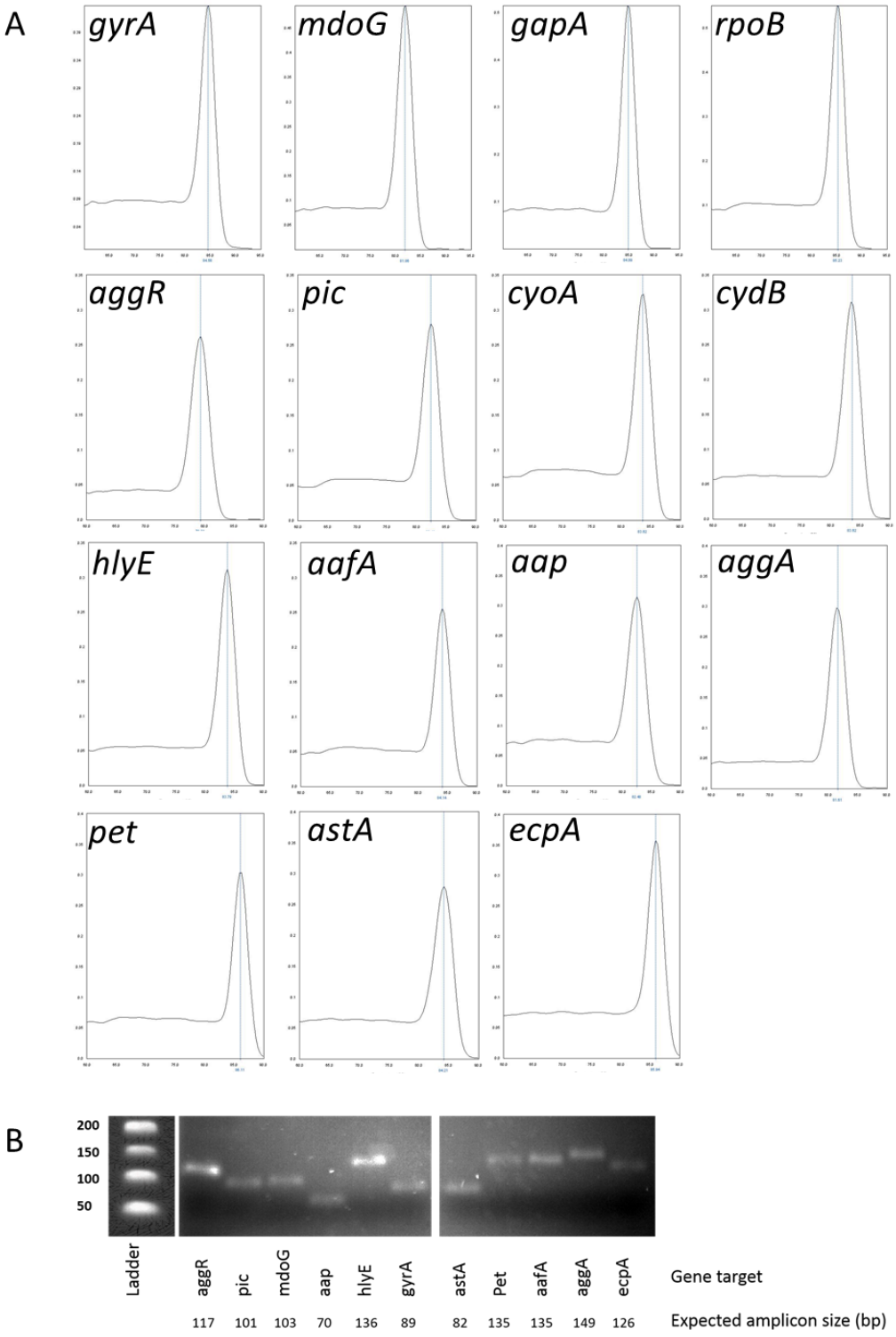

Figure 3.12 Validation of primer specificity. (A) Dissociation curves were generated for each primer pair during qPCR amplification. Single peaks indicate that primers amplified a single amplicon. Curves are representative of all qPCR experiments with these primers. (B) Agarose gel electrophoresis of qPCR products. Image is representative of two independent experiments in duplicate.

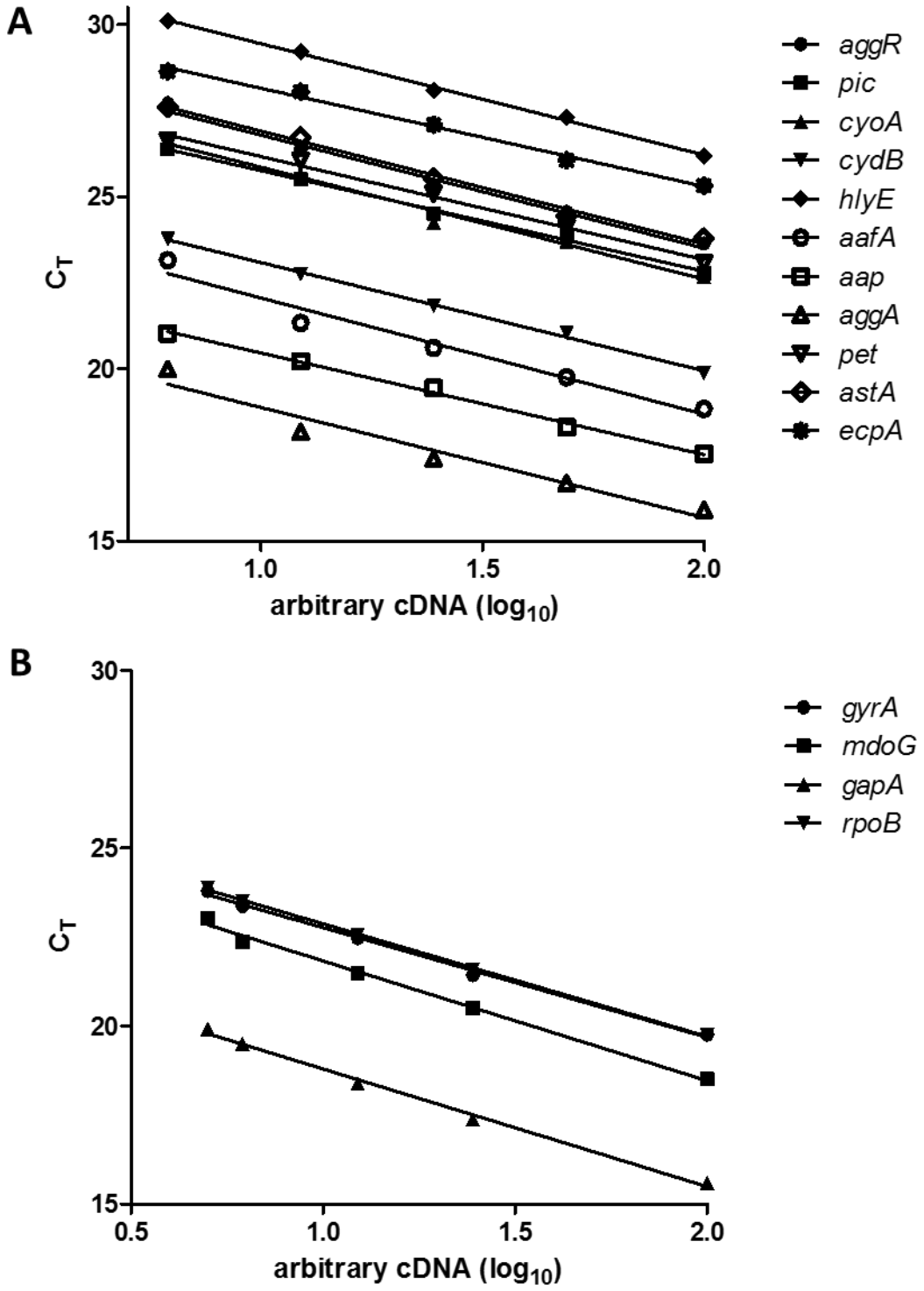

Figure 3.13 Validation of primer efficiency. qPCR was performed using a two-fold serial dilution of cDNA for each primer pair for (A) virulence genes and (B) reference genes. The resulting cycle threshold (C_T) values were plotted against cDNA concentrations, to determine the gradient for calculation of primer efficiency.

Primer target	Negative gradient	Primer Efficiency %
gyrA	3.09	110.7
mdoG	3.37	98.0
gapA	3.3	100.9
rpoB	3.17	106.8
aggR	3.29	101.3
Pic	2.93	119.4
cyoA	3.26	102.7
cydB	3.15	107.7
hlyE	3.24	103.5
aafA	3.38	97.6
aap	2.96	117.7
aggA	3.2	105.4
pet	2.99	116.0
astA	3.28	101.8
ecpA	2.85	124.3

Table 3.2 Primer efficiencies. Cycle threshold (C_T) values were plotted against cDNA concentrations for a two-fold serial dilution, with the gradient of the linear fit for each primer pair (**Figure 3.13**) used to calculate amplification efficiency.

The normalisation of relative qPCR data required the use of reference *E. coli* genes, and it was therefore important that the expression of these genes remained consistent between experimental conditions. Four potential reference genes were selected: *gapA* (glyceraldehyde phosphate dehydrogenase), *rpoB* (β subunit of bacterial RNA polymerase), *gyrA* (DNA gyrase), and *mdoG* (glucans biosynthesis protein G, glycan metabolism). qPCR was performed using cDNA from strains 042 and 17-2 after a 4-hour VDC incubation under AE and MA conditions (**Figure 3.14**). The C_T values indicated that *gapA* expression was possibly affected by oxygen level, whilst *rpoB* showed different expression levels in the two EAEC strains. In contrast, *gyrA* and *mdoG* showed consistent expression levels under all conditions and were therefore selected as reference genes for subsequent qPCR analysis.

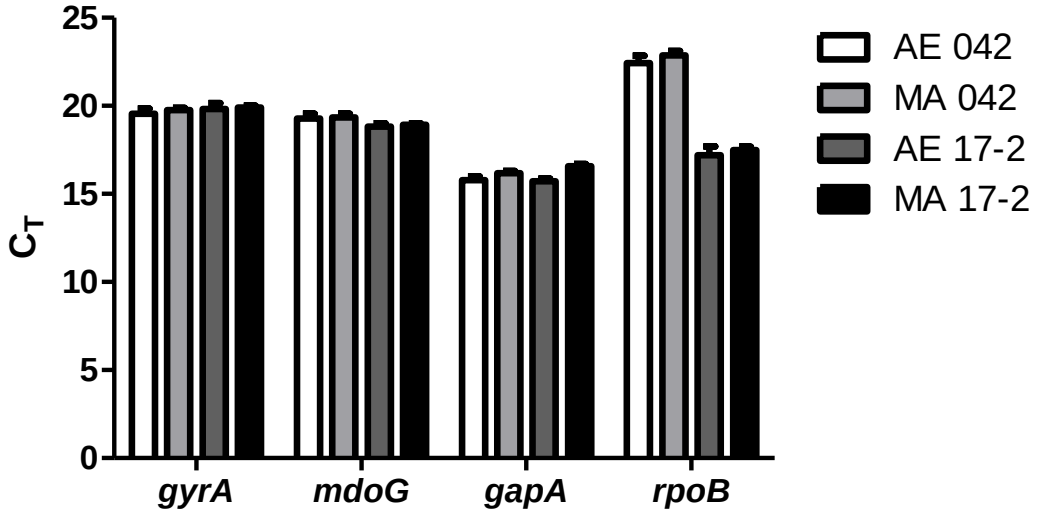

Figure 3.14 Effect of oxygen levels on expression of selected *E. coli* reference genes. qPCR was used to analyse expression of four *E. coli* genes in 042 and 17-2 after a 4-hour VDC incubation in aerobic (AE) or microaerobic (MA) conditions. Data are shown as mean ± SE for three experiments in duplicate.

After optimisation of the qPCR protocol, the effect of oxygen availability on EAEC virulence gene expression was investigated by incubating strains 042 and 17-2 for 3 hours in the VDC system under AE or MA conditions, without T84 cells. This time point was chosen to avoid oxygen depletion in the AE chambers, as previously

evaluated in Chapter 3.2.4. The bacteria were immediately treated with phenol-ethanol to stabilise RNA, cDNA was synthesised, and gene expression was analysed by qPCR. Relative virulence gene expression under AE compared with MA conditions was evaluated using the $\Delta\Delta C_T$ method (**Figure 3.15 A**). For 042, oxygen induced a significant two-fold upregulation in expression of *aggR*, *aap*, *aafA*, and *ecpA* ($P < 0.05$ to $P < 0.01$). Both *aap* and *aafA* are regulated by the transcription regulator AggR. There was no significant difference in gene expression for the remaining virulence factors in 042 when comparing AE and MA conditions. For strain 17-2, none of the examined genes were significantly upregulated by oxygen. However, *aggR*, *aap*, and the corresponding AAF gene in 17-2, *aggA*, demonstrated a fold change in expression of greater than 1, but this did not reach statistical significance.

To determine if the presence of host cells influenced the response of EAEC to oxygen, this analysis of relative virulence gene expression was repeated for non-adherent EAEC in apical VDC compartments containing polarised T84 cells (**Figure 3.15 B**). The results were similar to those observed in the absence of T84 cells, with no significant changes in gene expression for 17-2, and upregulation of *aggR*, *aap*, *aafA*, and *ecpA* by oxygen for 042. The only observable difference was that expression of *astA* was significantly increased under AE versus MA incubation in the presence of T84 cells ($P < 0.01$). Overall, this shows that the VDC system can be used as a model system to investigate the effect of oxygen levels on EAEC virulence gene expression. The data demonstrates that oxygen causes a strain-specific upregulation of certain virulence genes for 042, independent of the presence of T84 cells.

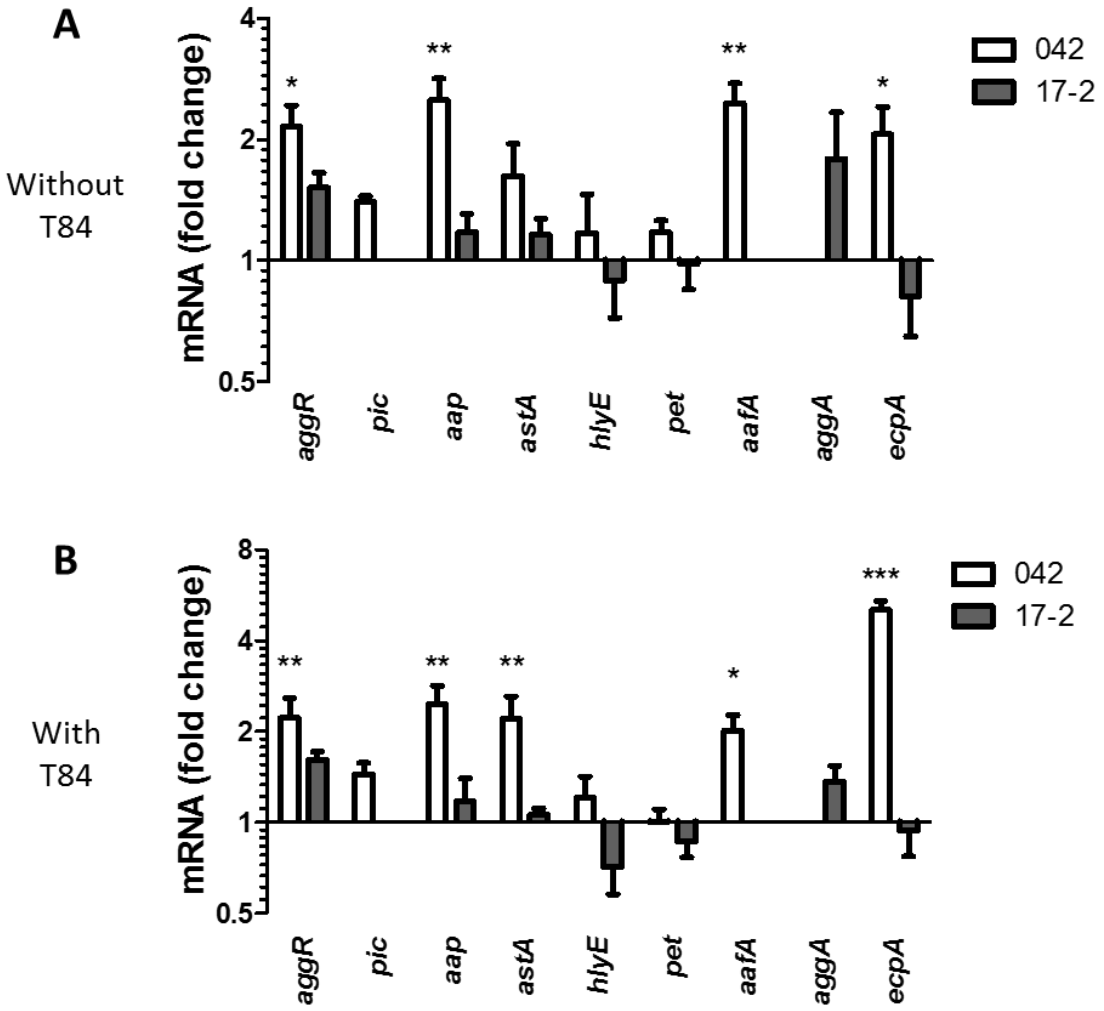

Figure 3.15 Oxygen enhances virulence gene expression in EAEC 042. (A) EAEC strains 17-2 or 042 were incubated in the VDC for 3 hours under aerobic (AE) or microaerobic (MA) conditions. Relative expression of selected virulence genes was determined by qPCR and is indicated as fold change under AE versus MA conditions. Data are shown as mean ± SE for five independent experiments. (B) Polarised T84 cells were infected with strains 17-2 or 042 for 3 hours (MOI = 20 bacteria/cell) under AE or MA conditions in the VDC. Expression of selected virulence genes by non-adherent bacteria was determined by qPCR. Data was normalised against expression of housekeeper genes *gyrA* and *mdoG.* Data are shown as mean ± SE for three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001

3.2.7: Adherence to host cells enhances EAEC virulence gene expression

To examine the effect of adherence to human colonic epithelial cells on virulence gene expression, polarised T84 monolayers were incubated for 3 hours in the VDC system following apical inoculation with 042 or 17-2. At the end of infection, the

apical medium was immediately treated with phenol-ethanol to stabilise bacterial RNA, while the T84 Snapwells were removed and differential lysis performed to deplete eukaryotic cells and preserve bacterial RNA. Relative qPCR analysis with the $\Delta\Delta C_T$ method was used to directly compare virulence gene expression between adherent and non-adherent bacteria from the same chamber.

The qPCR analysis revealed that adherence to the polarised T84 monolayer increased the expression of most of the investigated virulence genes in both 042 and 17-2 ($P < 0.05$ to $P < 0.0001$) (**Figure 3.16**). For both strains, the largest change in relative gene expression was recorded for EAST-1 toxin gene *astA*, which saw a 4-fold upregulation for 17-2 and 8-fold increase for 042. Whilst the aggR-regulated genes *aap* and *aafA*/*aggA* were also increased in adherent bacteria, *aggR* itself was not upregulated in 042 and showed a relatively small increase in expression for 17-2 in MA chambers. The relative expression of each gene in response to T84 contact was determined for both AE and MA apical conditions, with the results mostly unaffected by oxygen availability. The only exceptions were a significant upregulation of *hlyE* in 042 only under AE incubation, and *aggR* and *ecpA* in 17-2 for MA conditions only.

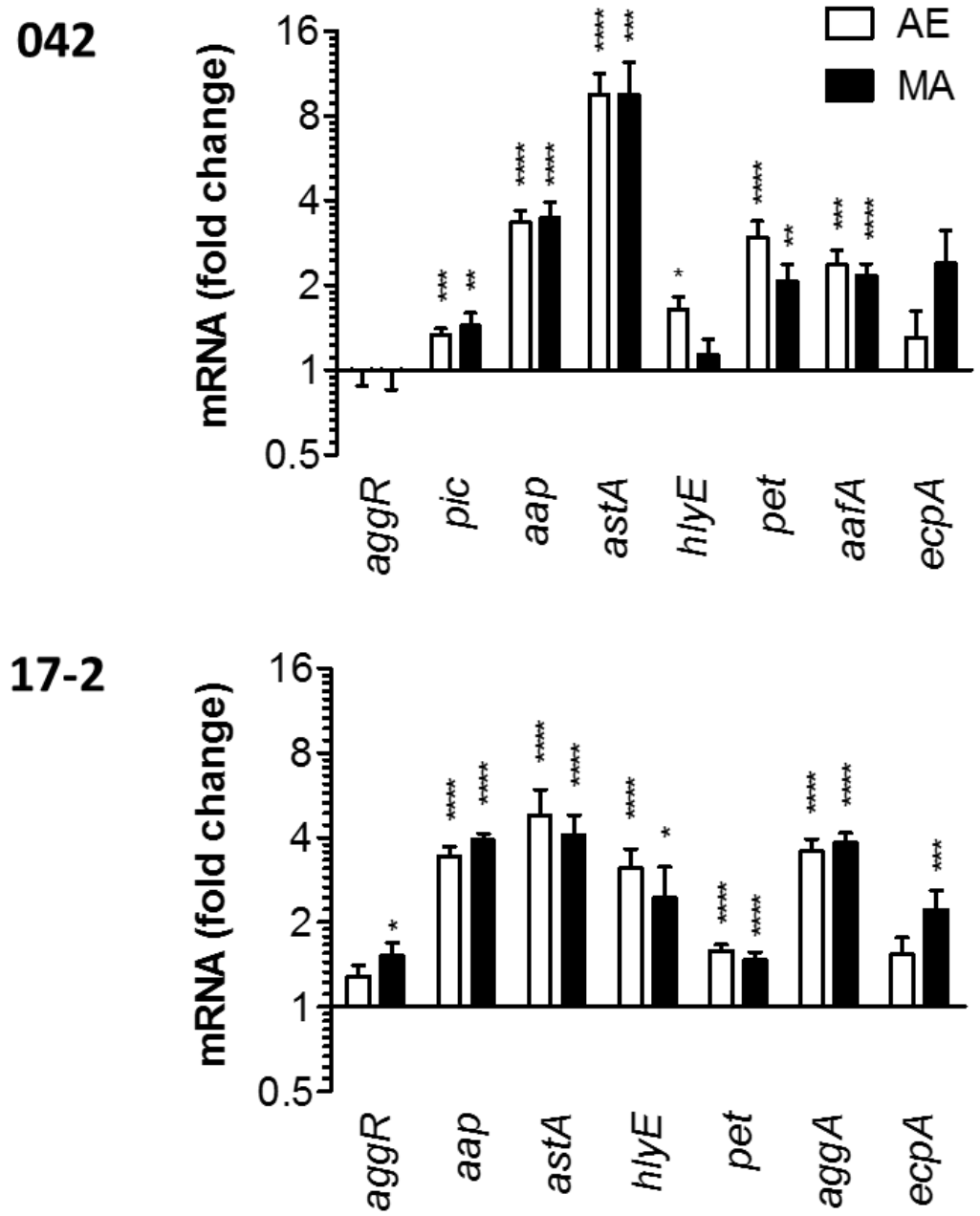

Figure 3.16 EAEC virulence gene expression is enhanced by host cell contact. Polarised T84 cells were infected with EAEC 042 or 17-2 (MOI = 20 bacteria/cell) and maintained under aerobic (AE) or microaerobic (MA) conditions for 3 hours. Relative expression of selected virulence genes in adherent and non-adherent EAEC was determined by qPCR and is indicated as fold change in adherent versus non-adherent bacteria. Data was normalised against expression of housekeeper genes *gyrA* and *mdoG.* Data are shown as mean ± SE for five independent experiments in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.2.8: Virulence gene induction by T84 cells is contact-dependant

The induction of bacterial gene expression by host cells may be mediated by proximity to secreted host factors, or by physical contact mechanisms such as receptor binding (Stones and Krachler, 2016). To determine which of these options was responsible for the virulence gene upregulation observed for adherent EAEC, confluent T84 cells were grown in a 12-well culture plate and inoculated as follows: 1) T84 monolayers were directly inoculated with EAEC and incubated for 3 hours, after which adherent and non-adherent bacteria were processed for qPCR analysis of relative virulence gene expression. 2) A sterile Transwell insert was added to wells with T84 cells, and bacteria incubated for 3 hours within the insert. Virulence gene expression was analysed by qPCR against a negative control of bacteria in a Transwell insert in a well without T84 cells. In this approach, EAEC were exposed to T84 proximity and any secreted factors, but the 0.4 µM Transwell filter prevented direct contact with the host cells (see **Figure 2.3**).

Relative gene expression was determined for *astA*, *aap*, *pet*, and *aafA* (042) or *aggA* (17-2) (**Figure 3.17**). When comparing gene expression for adherent and non-adherent bacteria from the direct inoculation method, this simpler model partially reproduced the previous VDC results. Adherence caused increased expression of *aap* and *aafA*/*aggA* for both strains, and *astA* for 17-2, although the fold-change was less than observed in the VDC model ($P < 0.05$ to $P < 0.0001$). The change in relative *astA* expression was not significant for 042, while neither strain demonstrated an upregulation of *pet* by T84 adherence.

For the Transwell insert model, relative gene expression was compared between bacteria incubated with and without a T84 monolayer within the culture well. None of the virulence genes analysed were upregulated in the presence of T84 cells. These results indicate that proximity to T84 cells alone does not cause increased virulence gene expression, and that this effect is therefore dependent on physical host cell contact.

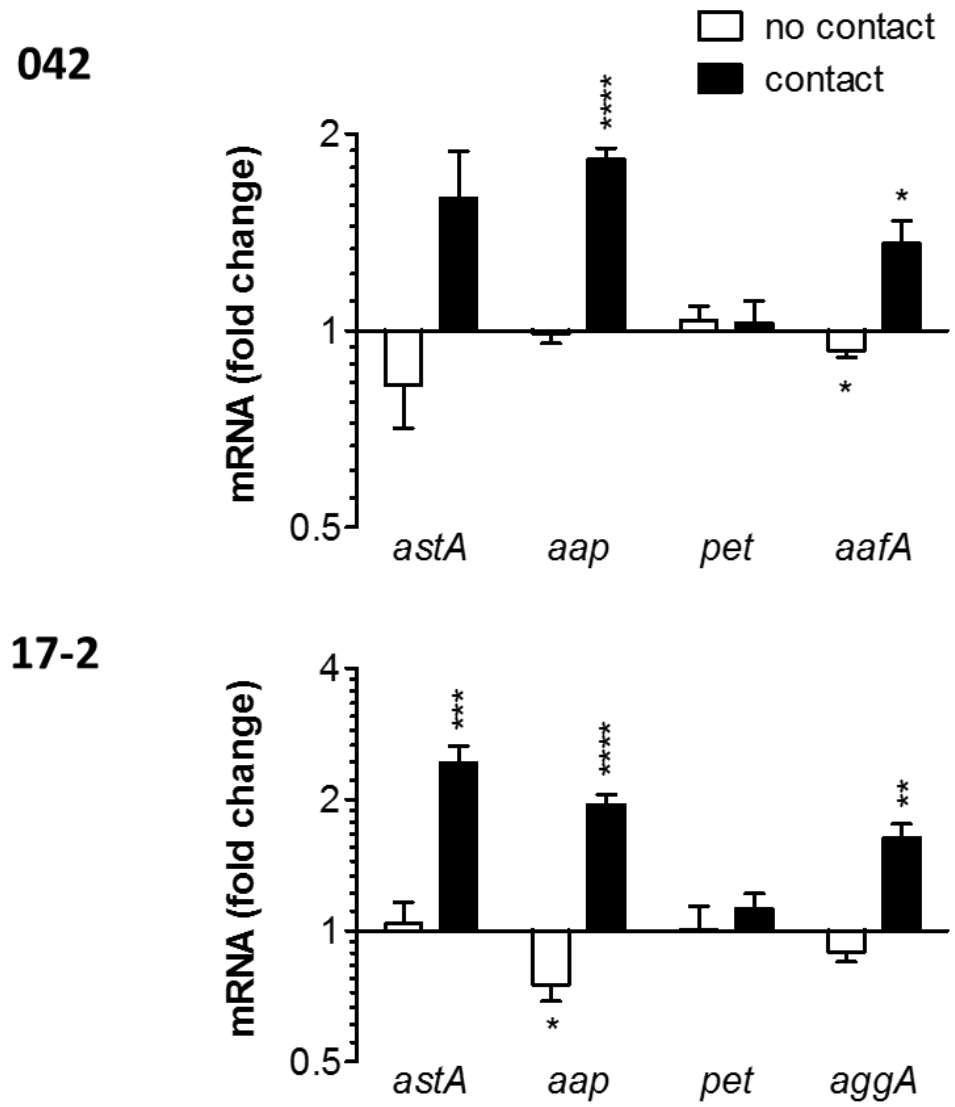

Figure 3.17 EAEC virulence gene induction by T84 cells is contact dependent. Confluent T84 cells were grown in 12 well plates, and 042 or 17-2 were added directly to the cells (MOI = 5 bacteria/cell) or prevented from direct cell contact by insertion of a porous Transwell insert. After 3 hours, expression of selected virulence genes was by quantified by qPCR and is expressed as fold change in EAEC in Transwell inserts with and without T84 cells (no contact) or in adherent versus non-adherent EAEC in plates without Transwells (contact). Data was normalised against expression of housekeeper genes *gyrA* and *mdoG*. Data are shown as mean ± SE for three independent experiments in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.2.9: Adherence to host cells upregulates dispersin protein expression

As adherence to T84 cells induced an increase in virulence gene expression in EAEC, experiments were designed to investigate if this upregulation was observable at the

protein level. Polarised T84 monolayers were incubated in the VDC with 042 or 17-2 under AE conditions. An 042 *aggR* deletion mutant was also included as a negative control for 042. Non-adherent bacteria were collected by centrifugation of apical media, while the T84 monolayer was lysed to harvest adherent bacteria. Western blots were performed to compare protein levels between adherent and non-adherent bacterial samples from the same chambers, using antibodies against dispersin (*aap*) and AAF/I (*aggA*).

Visualisation of protein bands by chemiluminescence revealed that for 042, levels of dispersin appeared to be greater in adherent than non-adherent bacteria at all time points (**Figure 3.18 A**). This was quantified by densitometric analysis of band intensity, with normalisation against GroEL expression as a measure of total bacterial protein abundance (**Figure 3.18 B**). This was chosen as a control as the GroE chaperone system is stably expressed in *E. coli* unless induced by heat shock(Rudolph *et al*., 2010). Dispersin expression was higher in adherent 042, although the increase was only significant at 5 hours incubation ($P < 0.01$). The results were more variable with 17-2, and no significant change in dispersin was observed at any time point (**Figure 3.19**). While Western blot analysis was also performed for AAF/I in strain 17-2, the resulting antibody signal was too weak for reliable analysis. Overall, these experiments suggest that at least in the case of 042 dispersin, the observed effect of T84 adherence on gene expression translates into a significant increase in protein expression.

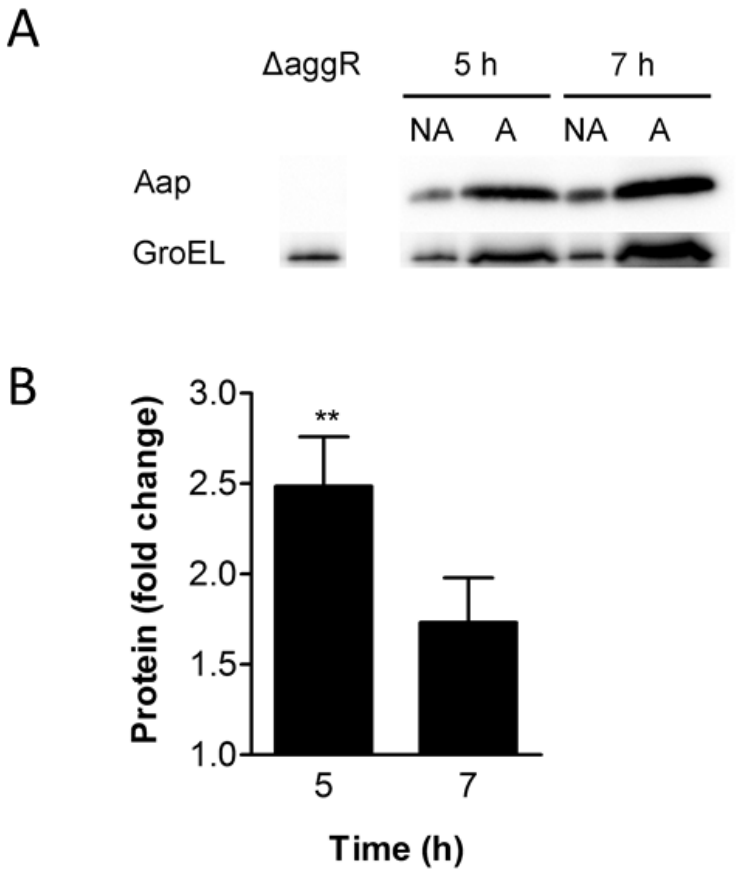

Figure 3.18 Expression of dispersin by 042 is increased by T84 adherence. (A) Polarised T84 cells were infected with strain 042 (MOI = 20 bacteria/cell) for up to 7 hours under AE conditions. Expression of dispersin (Aap) in adherent (A) and non-adherent bacteria (NA) was determined by Western blot analysis. Bacterial lysates of an isogenic *aggR* mutant (ΔaggR) were included as negative control, and GroEL expression was used to normalise total protein amounts. Image is representative of four independent experiments. (B) Band intensities were quantified with ImageJ and protein expression is indicated as fold change in adherent versus non-adherent bacteria. Data are shown as mean ± SE for four independent experiments. ** $P < 0.01$.

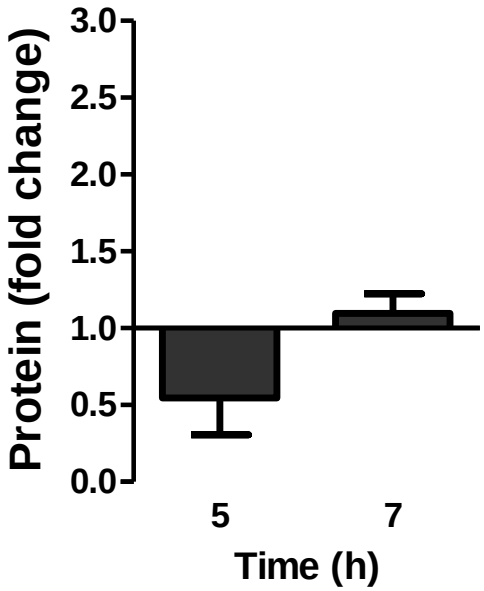

Figure 3.19 Expression of dispersin is not induced by T84 adherence for 17-2. Polarised T84 cells were infected with strain 17-2 (MOI = 20 bacteria/cell) for up to 7 hours under AE conditions. Expression of dispersin was determined by Western blot analysis and band intensities were quantified with ImageJ. Protein expression is indicated as fold change in adherent versus non-adherent bacteria. Data are shown as mean ± SE for four independent experiments.

3.2.10: Dependence of virulence gene induction on AggR regulation

The transcriptional regulator AggR modulates the expression of multiple EAEC virulence genes and is often described as promoting pathogenesis (Morin *et al*., 2013, Huttener *et al*., 2018, Harrington *et al*., 2006). The results of the VDC experiments in this study demonstrated an upregulation of *aggR* by oxygen in strain 042, but no change induced by T84 adherence. To investigate the importance of AggR for the virulence gene expression in response to oxygen and host cell contact, experiments were performed using an isogenic 042 *aggR* deletion mutant and the corresponding complemented strain (kindly provided by Douglas Browning, University of Birmingham; first published in (Sheikh *et al*., 2001, Sheikh *et al*., 2002)).

The mutant strains were initially validated by the observation of aggregation and colonisation phenotypes (**Figure 3.20**). Wild-type, *aggR* deletion, and complemented 042 strains were incubated in DMEM/F-12 medium for 4 hours,

with or without confluent T84 cells. Wild-type 042 formed large, dense aggregates in liquid culture, while the deletion mutant displayed no auto-agglutination with only individual bacteria observed. Following arabinose-induced expression of *aggR*, the complemented strain displayed some aggregation, although this was not fully restored to wild-type levels. On T84 cells, the wild-type strain demonstrated AA. When *aggR* was deleted, no aggregation occurred and only low levels of individual adherent bacteria were observed. Complementation of *aggR* partially reversed this effect, with AA observed but at a lower density than wild-type 042 colonies.

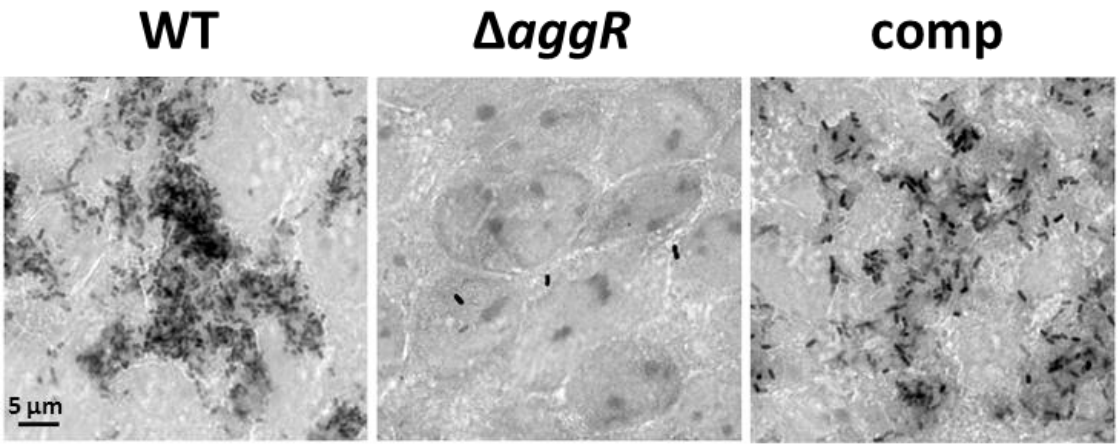

Figure 3.20 Aggregative adherence phenotype requires AggR. Confluent T84 cells in 24 well plates were infected (MOI = 10 bacteria/cell) with EAEC 042 wild-type (A), Δ*aggR* mutant (B) and complemented strain (C) for 4 hours and treated with Giemsa stain. Shown are representative images of two experiments performed in duplicate.

To evaluate the effect of AggR on total virulence gene expression irrespective of signals such as oxygen and adherence, the relative expression of *aggR*, *astA*, *aap*, and *aafA* was determined for bacteria when comparing the deletion mutant and complemented strain against wild-type 042 (**Figure 3.21**). Gene expression was analysed by qPCR following a 3-hour VDC incubation in AE conditions, without host cells. Plasmid complementation of *aggR* caused a small increase in expression of all four genes, significant for *aap* and *aafA* ($P < 0.05$ and $P < 0.01$, respectively). Deletion of *aggR* caused a strong significant decrease in *aap* and *aafA* expression ($P < 0.001$ and $P < 0.0001$, respectively), which are known to be AggR regulated (Morin *et al*., 2013). The deletion mutant did not have decreased expression of *astA* compared to wild-type 042.

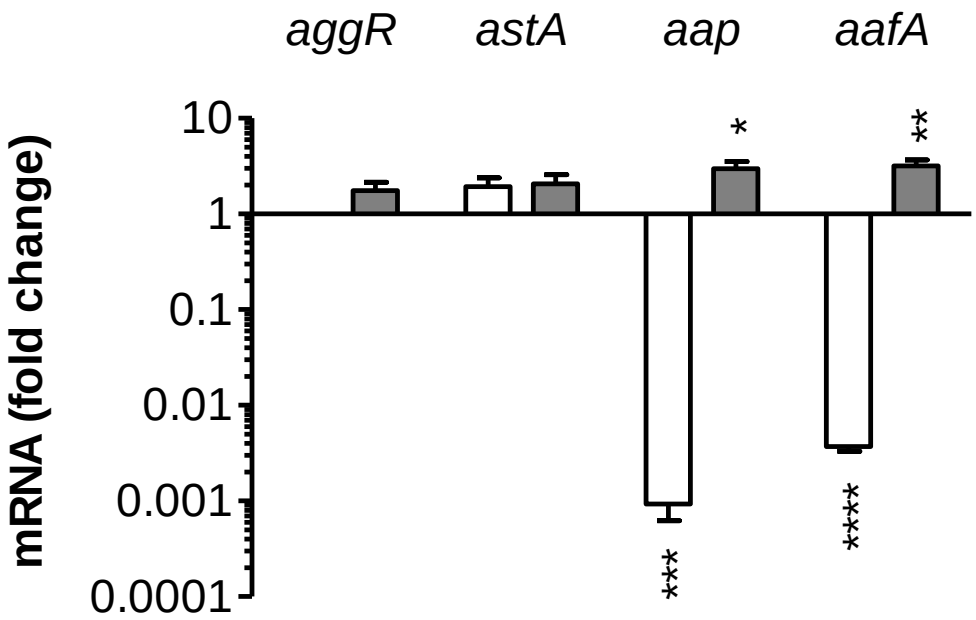

Figure 3.21 Relative gene expression in mutant strains compared to wild-type 042. Virulence gene expression following a 3-hour incubation in the VDC of non-adherent bacteria was quantified by qPCR and is expressed as fold change in Δ*aggR* mutant (white) and complemented strain (grey) versus wild-type. Data was normalised against expression of housekeeper genes *gyrA* and *mdoG.* Data are shown as mean ± SE for four independent experiments in triplicate, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

The effect of oxygen on expression of *aggR*, *astA*, *aap*, and *aafA* was determined by incubating 042 wild-type, ΔaggR and the complemented strain under AE or MA conditions in the VDC for 3 hours, without host cells. Arabinose was added to induce plasmid-derived *aggR* expression for the complemented strain. Relative qPCR analysis showed increased expression by oxygen of all 4 genes, although this only reached statistical significance for *aggR* in this set of experiments (P < 0.05) (**Figure 3.22**). When *aggR* was deleted, oxygen did not induce upregulation of the aggR-regulated genes *aap* and *aafA*, as well as *ast*A. The increased gene expression in AE versus MA conditions was restored for *astA* and *aap* in the complement. However, data variability caused the difference between wild-type and mutant strains to not reach statistical significance for these genes.

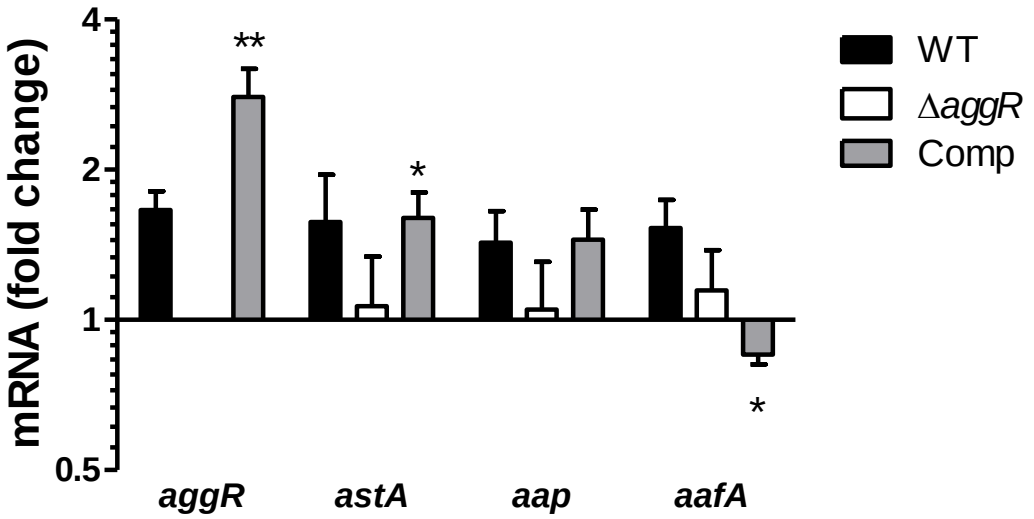

Figure 3.22 Influence of oxygen on virulence gene expression in 042 *aggR* mutants. Wild-type 042 (WT), Δ*aggR* mutant and complemented strain (Comp) were incubated in the VDC for 3 hours under AE or MA conditions. Relative expression of selected virulence genes was determined by qPCR and is indicated as fold change under AE versus MA conditions. Data was normalised against expression of housekeeper genes *gyrA* and *mdoG*. Data are shown as mean ± SE for four independent experiments. *P < 0.05, **P < 0.01

To determine if AggR regulation is involved in the response of 042 virulence gene expression to host cell adherence, confluent T84 monolayers grown in 24-well plates were incubated with 042 wild-type, *aggR* deletion, or *aggR* complement strains for 3 hours and qPCR analysis was performed for *aggR*, *astA*, *aap*, and *aafA* (**Figure 3.23**). The loss of *aggR* did not abolish the effect of T84 adherence, as no significant difference was determined for relative gene expression of *astA*, *aap,* or *aafA* between 042 wild-type and Δ*aggR*. However, the results for the *aggR* complemented strain were unreliable and not consistent with either the wild-type or *aggR* deletion strain.

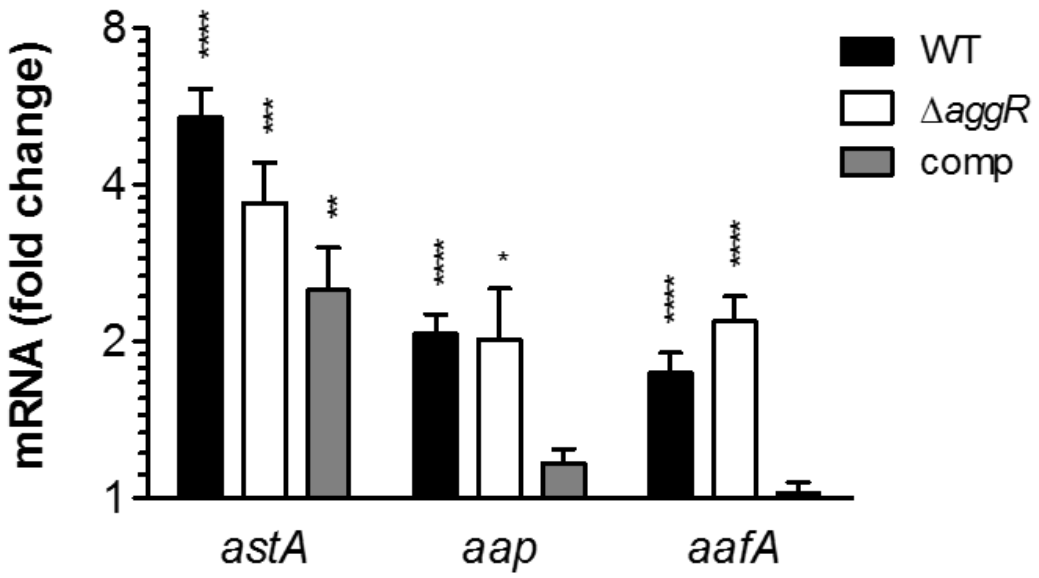

Figure 3.23 Host cell-induced virulence gene expression is not dependent on AggR. Confluent T84 cells in 24 well plates were infected (MOI = 10 bacteria/cell) with EAEC 042 wild-type (WT), Δ*aggR* mutant and complemented strain (Comp) for 4 hours. Transcription of selected virulence genes in adherent and non-adherent EAEC was determined by qPCR and is indicated as fold change in adherent versus non-adherent bacteria. Data was normalised against expression of housekeeper genes *gyrA* and *mdoG*. Data are shown as mean ± SE for four independent experiments in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Overall, the results using these mutant strains suggest that the upregulation of 042 virulence gene expression by adherence to T84 cells is not dependent on AggR regulation. While a trend was observed for the requirement of *aggR* for the increase in relative virulence gene expression caused by oxygen, this was not statistically significant and remains undetermined.

3.3: Discussion

As EAEC is a heterogenous pathotype, most fundamental research of virulence has been performed using prototype strains. Two of the most commonly used strains are 042 and 17-2, both isolated from South American paediatric diarrhoeal cases in the 1980s. 042 was isolated during a case study in Lima, Peru in 1983, while 17-2 was obtained from an infant with diarrhoea in Santiago, Chile (Nataro *et al*., 1985,

Vial *et al*., 1988). In an adult human volunteer study, 042 induced diarrhoeal illness while other EAEC strains including 17-2 had no effect (Nataro *et al*., 1995). Animal models of infection have also been used, with 042 colonising the murine intestine but only inducing diarrhoea in combination with interventions such as zinc deficiency (Bolick *et al*., 2014, Roche *et al*., 2010). Rabbits orally infected with 17-2 were colonised in the terminal ileum and colon, but did not develop diarrhoeal symptoms (Kang *et al*., 2001). The genomes of these two prototype strains have also been investigated. Both 042 and 17-2 are typical EAEC possessing a pAA plasmid and the transcriptional regulator AggR, as well as putative EAEC virulence genes such as dispersin, the mucinase Pic, and the toxins EAST-1 and HlyE (Morin *et al*., 2013, Chaudhuri *et al*., 2010, Weintraub, 2007, Cerna *et al*., 2003). However, there are also notable differences such as the Pet toxin encoded only by 042, and different AAF variants (AAF/I in 17-2 and AAF/II in 042) (Nataro *et al*., 1992, Chaudhuri *et al*., 2010, Suzart *et al*., 2001).

As there is a lack of suitable *in vivo* models for EAEC infection, this study applied complimentary *in vitro* and *ex vivo* models to investigate 042 and 17-2 intestinal infection. (Philipson *et al*., 2013). In particular, the ability of these strains to colonise different sites in the GIT was evaluated. Additionally, the adaptation of EAEC for enteric pathogenesis was investigated using molecular biology techniques to determine the effect of intestinal environmental signals on virulence gene expression.

3.3.1: EAEC colonisation of intestinal epithelial cells and tissue

A key step in EAEC pathogenesis is adherence to the intestinal epithelium and the formation of bacterial aggregates. The *ex vivo* colonisation of human intestinal biopsies by 042 and 17-2 was investigated using the IVOC model. This has strong biological relevance compared to traditional cell culture, avoiding the abnormalities in metabolism and structure exhibited by cancer-derived cell lines (Rajan *et al*., 2018). Tissue samples were taken from the second part of the duodenum, terminal ileum, transverse colon, and sigmoid colon to compare EAEC adherence. SEM revealed that both strains colonised colonic biopsies, with some regions displaying

extensive colony coverage across multiple crypts. Strain 042 colonised a higher proportion of biopsies from the transverse and sigmoid colon (both 86%) than 17-2 (57% and 33%, respectively). This suggests 042 has a greater ability to infect adult colonic mucosa, which would correlate with 042 but not 17-2 causing diarrhoea in adult volunteers, albeit in a very limited small cohort study (Nataro *et al*., 1995). It was also observed that 042 often demonstrated denser and more distinct colonies than 17-2. The contrast between strong colonisation and complete absence of adherent bacteria observed for biopsies from different donors supports how host susceptibility may contribute to the heterogeneity of EAEC virulence. This could include factors such as genetic backgrounds, immune response, microbiota composition, and nutritional status (Kaur *et al*., 2010).

Neither 042 or 17-2 formed adherent aggregates on small intestinal biopsies. While some terminal ileal biopsies were associated with individual bacteria or very small aggregates, no larger established colonies were observed on any occasion. No adherent EAEC were found on duodenal biopsy samples. This apparent tissue tropism is supported by previous IVOC studies for EAEC, where consistent colonisation of colonic tissue fragments was reported, compared to either no bacteria or much lower adherence on small bowel regions infected with 17-2 (Knutton *et al*., 1992, Hicks *et al*., 1996) or 042 (Andrade *et al*., 2011, Nataro *et al*., 1996). While the experiments herein used tissue samples from adults, some of the previous IVOC studies demonstrated similar results with biopsies from children. This suggests that despite the greater clinical association of EAEC with paediatric diarrhoeal illness, the tissue tropism for colonic colonisation is consistent during aging. In agreement with human *ex vivo* data, an *in vivo* rabbit infection model also reported 17-2 was recoverable throughout the colon but not in the duodenum, although 17-2 was present in the terminal ileum in this case (Kang *et al*., 2001).

There are conflicting conclusions on cytotoxicity, with some studies observing no mucosal abnormalities (Knutton *et al*., 1992), while others reported enlarged crypt openings and cell extrusion (Hicks *et al*., 1996) or possible microvilli effacement (Andrade *et al*., 2011). In this study, any similar observations were rare for infected colonic biopsies, and not deemed sufficiently distinct from uninfected control tissue to support a cytotoxic bacterial effect. As these reported cytotoxic indications are

based on microscopical observations, it is probable that differences in individual interpretation contribute to the lack of consensus.

In parallel with IVOC experiments, two well-established human intestinal epithelial cell lines were used; T84 and Caco-2. Although both are colon carcinoma-derived cell lines, the differences in their fine structure and function have led to their use as models for different GI tissue types. T84 cells exhibit structural similarity to colonocytes and form highly polarised columnar epithelia upon confluency (Madara *et al*., 1987). Conversely, confluent Caco-2 cells spontaneously differentiate into a structure reminiscent of small intestinal enterocytes. This includes the development of a small intestinal brush border and the expression of specific surface enzyme markers such as sucrase, maltase, lactase, alkaline phosphatase, gamma-glutamyltransferase, aminopeptidase N, and dipeptidyl-dipeptidase IV (Jumarie and Malo, 1991). T84 cells do not undergo such differentiation, maintain shorter microvilli and express colonic markers such as monocarboxylate transporter 1, responsible for improved barrier function in response to butyrate exposure (Devriese *et al*., 2017). Due to these distinctions, T84 and Caco-2 cells were used in this study as culture models for the colonic and small intestinal epithelium, respectively.

It was demonstrated that 042 and 17-2 colonised both T84 and Caco-2 cell monolayers, with similar efficiency at most time points. When quantified by CFU counting of cell lysates plated on LB agar, the level of adherence was only significantly different at 2 hours incubation on T84 cells. While not statistically significant, adherence was consistently higher for 17-2 than 042 with both cell lines, although this may be due to the faster growth rate of 17-2 in cell culture media rather than superior cell adhesion. These prototype strains have been previously studied in interaction with T84 and Caco-2 cells, with some discrepancies. T84 cells have been proposed as a suitable colonic model for 042 infection due to similar adherence and possible cytotoxicity compared to observations in IVOC, yet the same study reported poor adherence of 042 to Caco-2 cells (Nataro *et al*., 1996). This disagreement with the findings presented here may be due to a difference in analysis techniques. While this study determined colonisation qualitatively by immunofluorescence, phase-contrast, and bright-field microscopy, and

quantitatively by plating, Nataro *et al.* used SEM which involves more rigorous processing protocols. In addition, several subclones of the Caco-2 cell line are available which differ in properties. In contrast to 042, strain 17-2 has been shown to effectively colonise Caco-2 cells in multiple publications (Steiner *et al*., 1998, Couto *et al*., 2007). However, to my knowledge the adherence of 17-2 to T84 cells has not previously been characterised.

While 042 and 17-2 demonstrated similar levels of colonisation, a morphological difference was observed on both cell lines, as was also previously described with IVOC. While both strains formed adherent aggregates on the surface of the cell monolayers, a denser aggregation was frequently observed for 042 compared to 17-2. In addition, 042 bacteria were mostly confined to aggregates only, whereas 17-2 demonstrated a more diffuse adherence pattern alongside the aggregative colonies for some samples. While this did not affect the quantity of adherent bacteria in these experiments, these observations do suggest possible differences in adherence mechanisms.

One relevant difference between 17-2 and 042 is the expression of different AAF variants, AAF/I and AAF/II, respectively. The AAF operons have well-conserved usher and chaperone genes, but the structural pilin subunit has high sequence diversity between variants (Boisen *et al*., 2008). While the relative adherence characteristics between each type have not been clearly defined, they are known to affect phenotypes. Different AAFs show variation in agglutination of erythrocytes from different species, suggesting differences in receptor recognition (Berry *et al*., 2014). On a structural level, AAF/V has been shown to possess an inserted helix which is predicted to prevent the fibronectin binding common to the other four subunits (Jonsson *et al*., 2017a). The phenotypic difference between variants is further evidenced by a study which demonstrated that AAF/V caused significantly greater HEp-2 cell adherence and biofilm formation than AAF/III when cloned into the same laboratory *E. coli* strain (Jonsson *et al*., 2015). There is also evidence that the AA phenotype of EAEC is likely to be a multi-factorial process. As well as AAFs, the dispersin protein, type I fimbriae, heat-resistant agglutinin 1, and the *E. coli* common pilus are amongst the virulence factors associated with EAEC AA (Blanton *et al*., 2018, Moreira *et al*., 2003, Avelino *et al*., 2010). While the factors responsible

for the morphological differences observed between 042 and 17-2 in this study remain undetermined, this further demonstrates the heterogeneity of EAEC strains.

The results of the IVOC infection experiments with 042 and 17-2 suggest that EAEC primarily colonise the colon. This emphasises the value of using *ex vivo* tissue models in addition to *in vitro* cell line experiments, as the EAEC strains did not demonstrate greater adherence to the colonocyte-like T84 cells than the small intestinal enterocyte model, Caco-2. As the key binding targets for EAEC adherence are poorly understood, it may be that despite the enterocyte-like differentiation of confluent Caco-2 cells, they retain elements of their colonic background which allows EAEC colonisation (Izquierdo *et al*., 2014a, Devriese *et al*., 2017). This would explain why the cell culture results do not reflect the tissue tropism observed in the IVOC infection model.

3.3.2: Intolerance of intestinal epithelial cell lines to simulated intestinal media components

Various simulated intestinal fluids have previously been designed to mimic the conditions of the human intestinal environment for studies such as the effect on drug structure and uptake (Ingels *et al*., 2004, Doak *et al*., 2010). Versions have also been used for microbiology, including the influence of a simulated gut environment on pathogenic *E. coli*. Two previously described compositions were selected; simulated colonic environment medium (SCEM) and simulated ileal environment medium (SIEM). Each contains biologically relevant concentrations of salts, bile acids (cholic and deoxycholic acid) and glucose, as well as additional digestive enzymes in SIEM. One study used these media to investigate the intracellular proteome of an EHEC O157:H7 strain under different simulated conditions, revealing that the environment affected expression of nucleotide biosynthesis pathway proteins, virulence factors and stress response genes (Polzin *et al*., 2013). SIEM and SCEM have also been used to study the Sfp fimbriae of EHEC O157:NM, which are not expressed under standard laboratory growth conditions (Müsken *et al*., 2008). This study planned to evaluate the effect of these simulated conditions

on EAEC colonisation of the intestinal epithelium. However, SIEM and SCEM had previously been used for bacterial culture alone, so experiments were first performed to determine if these media could support co-culture with intestinal epithelial cells. Simulated intestinal fluids have been used with Caco-2 cells previously, but these did not contain bioactive components such as bile salts or digestive enzymes (Ingels *et al*., 2004). Therefore the tolerance was tested of enterocyte-like Caco-2 cells and colonocyte-like T84 cells to SIEM and SCEM, respectively.

As complete simulated media caused total cell detachment and loss of viability, individual media components were tested for cytotoxicity. Using increasing concentrations of bile salts (cholic acid and deoxycholic acid), toxic effects were observed with concentrations above 400-600 µM for Caco-2 cells and 200 µM for T84 cells. This agrees with previous studies where a selection of bile acids, including cholic and deoxycholic acids, caused cytotoxicity in Caco-2 cells at concentrations higher than 400 µM. In addition, changes in tight junction structure and barrier function, and disorganisation of occludin were reported similar to the observations presented here (Raimondi *et al*., 2008). Moreover, deoxycholic acid exhibited cytotoxic effects on T84 cells above a concentration of 200 µM, which agrees with the data in this study (Niamh *et al*., 2009).

The bile acid concentrations specified for SIEM and SCEM were 6.63 mM and 4.73 mM, respectively. This is within some estimates of the physiological range for bile acids in the human small intestine (Hamilton *et al*., 2007). However, bile acids undergo active reabsorption in the distal small intestine, with as little as 2-5% entering the colon (Niamh *et al*., 2009, Li and Chiang, 2014). The relatively high concentration of bile acids in SCEM may therefore be inaccurate when compared to physiological colonic conditions. While Caco-2 cells undergo differentiation and present features of small intestinal enterocytes, both T84 and Caco-2 are colon carcinoma-derived cell lines. As such, the bile acid cytotoxicity demonstrated in these experiments may be explained by the inability of colonic epithelial cells to tolerate prolonged exposure to the higher bile acid concentrations of the small intestinal environment. In addition, these cell lines do not secrete a mucus layer,

which likely protects intestinal cells from bile acid toxicity *in vivo* (Niv *et al*., 1992, Navabi *et al*., 2013, Shekels *et al*., 1996)

The published composition of SIEM includes the addition of the digestive enzymes α-amylase, trypsin, chymotrypsin, and lipase (Polzin *et al*., 2013). However, in this study these components were replaced due to concerns of loss of enzymatic activity during media storage between experiments. The alternative of adding the enzymes to aliquots of unsupplemented SIEM before each assay was not practical due to the small volumes required. Instead, 1% (w/v) pancreatin, which is extracted from porcine pancreas and contains a biologically relevant mixture of mammalian digestive enzymes, was added to SIEM aliquots immediately before use (Markell *et al*., 2017). However, the inclusion of pancreatin in SIEM caused major detachment of Caco-2 cells from coverslips even at ten-fold reduced concentrations. Interestingly, any residual cells were viable by Trypan Blue staining, suggesting disruption of focal cell adhesions by enzymatic action of pancreatin, without loss of cell viability. Pancreatin is typically used for *in vitro* food digestion models rather than cell culture (Hur *et al*., 2011). It has been successfully utilised in infections of stem cell-derived human intestinal organoids by Rotaviruses, but this is a non-adherent model so cell detachment would not be observed (Finkbeiner *et al*., 2012). One study previously used pancreatin to simulate gastric digestion of potentially harmful proteins, which were subsequently tested on Caco-2 monolayers. However, the pancreatin enzymes were deliberately heat-inactivated before samples were incubated with the Caco-2 cells to avoid potential alterations of monolayer integrity and viability (Markell *et al*., 2017). Therefore, the inclusion of digestive enzymes in SIEM is suitable for investigating effects on bacteria alone, but causes problems in combination with adherent cell culture due to the enzymatic disruption of cell attachments.

Not all SIEM and SCEM components were poorly tolerated, with lysozyme and the various specified salt concentrations causing no observed cytotoxicity or cell detachment. However, the exclusion of multiple important components would have reduced the relevance of the simulated conditions, as well as complicating any comparison of results with previous publications. Additionally, even using a simulated medium modified for intestinal epithelial tolerance could induce

underlying changes in the cells, potentially influencing interactions with EAEC and making comparisons to data produced using standard cell culture more challenging. It was also noted that the standard culture media for T84 and Caco-2 cells (DMEM/F-12 and DMEM, respectively) contain comparable salt concentrations to the simulated media specifications (**Appendix 3**). Overall, it was determined that the simulated intestinal media were not suitable for further use in this study.

3.3.3: Oxygen as a regulatory signal for EAEC virulence

One of the major benefits of the VDC as an *in vitro* culture model is the ability to control oxygen levels in the media through perfusion of aerobic (AE) or anaerobic (AN) gas mixtures. As the VDC system has not previously been used for the study of EAEC, oxygen levels during bacterial incubation were determined with an oxygen sensitive electrode probe before establishment of the infection protocol. As expected, bacterial growth caused depletion of oxygen over time, especially for the faster-replicating strain 17-2. The initial dissolved oxygen concentration in microaerobic (MA) chambers was in the range of 1.5-2.5%, which is comparable to the estimates of 1-2% in previous studies using the VDC model (Schüller and Phillips, 2010). It also corresponds to estimates for the human gut epithelial surface of 2-4% oxygen, with a steep gradient approaching AN conditions for the central lumen (Zeitouni *et al*., 2016).

In addition to oxygen measurements, *E. coli* respiration was analysed by qPCR analysis of terminal cytochrome oxidase expression. The *cyo*A gene is part of the cytochrome o oxidase complex used by *E. coli* under AE growth, while *cydB* is a subunit of the high-affinity cytochrome d oxidase complex which is expressed under MA conditions and shown to be important for *E. coli* colonisation in the mammalian intestine (Tseng *et al*., 1996, Jones *et al*., 2007). Oxygen limitation causes the transition from cytochrome o oxidase to cytochrome d oxidase as the major terminal oxidase for *E. coli* respiration, controlled by the ArcA global regulator (Alexeeva *et al*., 2003). When the relative expression of these genes was compared between bacteria incubated in AE or MA VDC compartments for 3 hours, *cyoA* was downregulated by microaerobiosis as expected. The expression of *cydB* was

increased for EAEC in MA chambers, but not as strongly as the decrease in *cyoA*. This may be partially explained by the dissolved oxygen measurements, which showed that the chambers initially maintained under MA conditions began to approach full AN status by 3 hours of bacterial growth. As the cytochrome d oxidase complex is expressed strictly in MA conditions, a transition to AN respiration may have occurred. This switch, regulated by the FNR system, would account for the relatively small upregulation of *cydB* observed (Sawers, 1999, Tseng *et al*., 1996). Combined with the measurements of dissolved oxygen concentrations, this data confirms that the AE and MA conditions as set up in the VDC system maintain a distinct difference in oxygen availability throughout the incubation, and that this induces related gene expression changes. As oxygen was also further depleted during AE incubation of 17-2 by 4 hours, a 3-hour incubation period was selected for investigating changes in EAEC gene expression in response to oxygen availability.

After establishment of oxygen levels during EAEC incubation under AE and MA conditions, growth and relative virulence gene expression for EAEC were evaluated. As expected for a facultative anaerobe, EAEC replication was greater in AE versus MA conditions as AE respiration allows more energy efficient metabolism and growth in *E. coli* (Jones *et al*., 2007). Analysis by qPCR revealed that for strain 042, *aggR*, *aap*, *aafA*, and *ecp*A were upregulated under AE conditions. The results were consistent for non-adherent EAEC incubated in chambers with polarised T84 cells, although for 042 the EAST-1 toxin gene *astA* was also significantly upregulated in AE chambers.

Dispersin and AAF/II are both part of the AggR regulon, and these three genes were induced to a similar degree, suggesting an AggR-mediated response to oxygen. When an 042 *aggR* deletion mutant and complemented strain were investigated, absence of *aggR* caused a non-significant decrease in the induction of *aap* and *aafA* by oxygen compared to the wild-type strain, which was restored by complementation for *aap*. However, *astA* expression, which is not reported as AggR-regulated, was also affected in the knockout strain. These experiments suggest that AggR signalling may be involved in the transcriptional response of 042

to oxygen availability, but uncertainties in the data support an influence from other unidentified factors.

How oxygen-sensing may influence AggR expression remains unknown. AggR itself has been linked to positive regulation by a DNA-binding protein Fis, and is negatively regulated by the *E. coli* global regulator HN-S, both of which are nucleoid proteins associated with gene transcription in rapidly growing cells (Morin *et al*., 2010, McLeod and Johnson, 2001). While there is some evidence of cross-talk between Fis and the ArcA/FNR redox-sensing transcription factors, the link between oxygen availability and AggR remains to be investigated (Cameron *et al*., 2013).

The *E. coli* common pilus (ECP) is not reported to be under AggR regulation but was also induced by oxygen. Some precedence for oxygen regulation of *ecpA* has been reported in EHEC, where protein expression was higher in low oxygen levels than full aeration, but lowest during AN incubation (Rendón *et al*., 2007). This corresponds with reduced *ecpA* gene expression by 042 in the MA chambers in this study, which were close to AN after 3 hours. This suggests ECP expression is enhanced in oxygen conditions similar to the mucosal interface, but the regulatory mechanism is unknown. The *astA* gene is also not under AggR regulation and has not been previously linked to oxygen sensing. However, *astA* expression was only significantly upregulated by oxygen in the presence of T84 cells. This may be linked, as *astA* was the most strongly induced virulence factor by T84 adherence, and shedding of bacteria from adherent 042 aggregates might confound results for non-adherent bacteria.

Unlike 042, the prototype strain 17-2 did not demonstrate significant changes in expression of any of the investigated virulence genes in response to oxygen. The AggR associated genes (*aggR*, *aap*, *aggA*) showed an increase in expression under AE conditions, but this did not reach statistical significance. This could be a lack of response for 17-2 or could indicate different kinetics for this strain. It is unclear why the response to oxygen availability differs between 042 and 17-2, but this is further evidence of the heterogeneity intrinsic to EAEC as a whole.

While the effects of oxygen on EAEC virulence have not been previously characterised, oxygen has been reported as an important signal for other enteric

pathogens (Marteyn *et al*., 2011). In many cases, MA conditions similar to the low oxygen availability of the GIT have been reported to enhance aspects of virulence. For example, *Salmonella* Typhimurium demonstrated increased adherence and invasion of canine kidney epithelial cells at low oxygen tension (Lee and Falkow, 1990). In addition, enterocyte binding, mucus penetration, and intracellular macrophage survival in mice were enhanced for *S*. Typhimurium after oxygen-restricted growth (Singh *et al*., 2000). For *Salmonella*, the oxygen-sensing FNR regulator is implicated in modulating virulence gene expression including the T3SS on *Salmonella* pathogenicity island 1, and FNR mutants are severely attenuated in murine infection (Fink *et al*., 2007). Low oxygen levels also enhanced maturation of the T3SS and secretion in EHEC, along with T3SS-associated adherence to epithelial cells (Schüller and Phillips, 2010, Ando *et al*., 2007, Carlson-Banning and Sperandio, 2016). The expression of Sfp adhesion fimbriae by sorbitol-fermenting EHEC O157:NM strains is also dependent on low oxygen tension (Müsken *et al*., 2008).

There is also evidence of pathogens using the oxygen diffusion gradient across the intestinal epithelium as a specific signal for activation of virulence systems. A previous study on *Shigella flexneri* showed that oxygen-starvation enhanced expression of a T3SS essential for cell invasion and virulence, y*et al*so caused an FNR-regulated suppression of effector protein secretion. This resulted in accumulation of virulence proteins inside the bacterial cell which was relieved by the oxygen gradient in proximity to the mucosal surface, thereby restricting full T3SS activity to the precise site of action (Marteyn *et al*., 2010). It has been speculated that oxygen gradient sensing may also contribute to chemotaxis and motility in *Salmonella* within the mucus layer (Bärbel *et al*., 2008). In *E. coli*, the enhancing effect of oxygen gradients on bacterial motility has been described (Douarche *et al*., 2009). It is possible that mucosal oxygen diffusion could modulate EAEC migration towards the epithelial surface in addition to influencing virulence gene expression.

3.3.4: Host cell contact as a regulatory signal for EAEC virulence

Previous IVOC studies presented here and by others suggested preferential EAEC binding and colonisation of the colon (Andrade *et al*., 2011, Nataro *et al*., 1996, Hicks *et al*., 1996). Therefore, polarised T84 monolayers were chosen as a model for the colonic epithelium in this part of the study. Epithelial barrier function of polarised T84 monolayers was monitored during incubation by measuring the transepithelial electrical resistance (TEER). While an initial drop in TEER was observed after mounting of the Snapwell inserts (possibly due to the switch to non-supplemented cell culture media and physical stresses within the chamber), infection with 042 or 17-2 did not cause a significant loss of barrier function compared to non-infected monolayers over a 3h time period, which agrees with previous work using polarised T84 cells infected with 042 (Strauman *et al*., 2010). Therefore, while 042 adherence may disrupt the T84 monolayer integrity over time, barrier function was sufficiently stable during the 3 hours of the infection assay.

The relative expression of nine EAEC virulence genes was determined for adherent versus non-adherent bacteria from the same inoculated VDC compartments. Phenol-ethanol treatment was used to stabilise mRNA at the experimental end-point, which has been demonstrated to efficiently preserve gene expression profiles in *E. coli* (Bhagwat *et al*., 2003). In contrast to the regulatory effect of oxygen in 042, adherence enhanced expression of not only colonization-associated virulence genes but also those encoding toxins and SPATEs (*astA*, *hlyE*, *pet*, and *pic*) for both strains. The effect of adherence on gene expression was largely consistent in both AE and MA conditions, suggesting that this is linked to separate signalling pathways than the response to oxygen. This also rules out the effect of hypoxia-induced changes to epithelial cells, such as membrane alterations, cytoskeletal rearrangements, or endoplasmatic reticulum stress (Zeitouni *et al*., 2016). Such changes have been previously associated with modulating virulence of enteropathogenic bacteria, with hypoxic stress decreasing internalisation of *Shigella flexneri* but enhancing invasion of HT-29 enterocyte cells by *Listeria monocytogenes* and some *E. coli* (Lima *et al*., 2013a, Wells *et al*., 1996).

While AggR-regulated factors such as dispersin and the AAFs were induced by adherence, no change in expression of *aggR* itself was observed for adherent 042. This result suggests that unlike the response of 042 to oxygen, enhanced EAEC virulence gene expression following epithelial adherence may be independent of AggR regulation. An *aggR*-deletion mutant in 042 demonstrated loss of the AA phenotype on T84 cells and showed a more than 100-fold decreased expression of the AggR-regulated genes *aap* and *aafA*, but not *astA* (not part of the AggR regulon), compared to wild-type 042. A plasmid complement restored expression of *aggR*, *aap*, and *aafA* to above wild-type levels. However, deletion of *aggR* did not abolish the relative upregulation of *astA, aap,* and *aafA* by T84 adherence, supporting the hypothesis that this effect is independent of AggR signalling. Unexpectedly, T84 adherence did not upregulate *aafA* or *aap* expression in the complemented strain. The pBAD30 plasmid used has sensitive promoter induction and a reported medium-high copy number (10-12 copies per cell), resulting in increased expression of plasmid-encoded genes (Sheikh *et al*., 2006, Guzman *et al*., 1995). AggR is a transcriptional regulator with many genetic interactions, so increased expression may disrupt other important pathways (Morin *et al*., 2013). The potential disadvantages of overexpression for loss of physiological relevance and detrimental effects on the bacteria were briefly discussed when the pBAD plasmids were originally constructed (Guzman *et al*., 1995). In addition, glucose acts as a repressor for the araBAD promoter on this plasmid, with the relatively abundant glucose concentration in DMEM/F12 medium therefore potentially complicating the stability of expression levels and outcomes for the complemented strain (Guzman *et al*., 1995, Siegele and Hu, 1997)

Many enteric pathogens use chemical cues from the intestinal environment to regulate virulence gene expression. EHEC is a well characterised example, shown to modulate virulence in response to various environmental factors such as acid stress, microbiota metabolites, and even host hormones (Foster, 2013). Often these signals are secreted by the host epithelium. For example, EHEC O157:H7 recognises epinephrine and norepinephrine using two adrenergic kinases, QseC and QseE, leading to enhanced LEE gene expression, bacterial motility, Shiga toxin production, and A/E lesion formation on epithelial cells (Njoroge and Sperandio, 2012).

Similarly, A/E lesions and expression of virulence regulators (including Ler, QseC, and QseE) and Shiga toxin are all increased in EHEC in response to ethanolamine, which is generated by turnover of both host epithelial cells and the microbial flora (Kendall *et al*., 2012). However, EHEC virulence is also mediated by physical interactions with the host epithelium. Mechanosensation is necessary for full LEE expression via GrlA regulation, dependent on both strong surface attachment and fluid shear forces (Alsharif *et al*., 2015, Islam and Krachler, 2016).

This study demonstrated that direct bacterial contact with the host epithelium was required for enhanced EAEC virulence gene expression as separation of bacteria from the host cells by a Transwell membrane did not cause any changes in transcription. Notably, induction of *astA, aap,* and *aafA*/*aggA* expression in adherent EAEC was less pronounced in experiments conducted in 12-well plates compared with the VDC system. This may be caused by differences in interactions with the less polarised T84 cells in wells compared to the highly-polarised monolayers on Snapwells in the VDC model, such as the expression and apical localisation of potential binding receptors (Navabi *et al*., 2013). Additionally, the well plate model is a static incubation with gravity promoting bacterial settling on the epithelial surface, while the gas flow through VDC compartments causes more dynamic conditions. Fluid shear forces are important in the mechanosensation described for adherent EHEC, so this may also contribute to the greater gene expression change for adherent EAEC in the VDC model (Alsharif *et al*., 2015).

This data suggests that the observed effect of T84 adherence on EAEC virulence gene expression is dependent on mechanical contact rather than sensing of chemical components. Previous studies have shown that adherence of *Vibrio cholerae* to intestinal epithelial INT 407 cells induces two major virulence factors, cholera toxin and the toxin-coregulated pilus, as well as the virulence regulatory gene *toxT*. Interestingly, *toxT* induction was independent of the conventional *toxT* activators ToxR and TcpP, which is similar to the apparently AggR-independent gene expression in this work (Dey *et al*., 2013).

While many EAEC adherence factors have been described, the binding receptors have yet to be fully characterised. However, some possible interactions have been

identified. The AAF genes are similar in organisation to the Afa/Dr family of *E. coli* adhesins which bind to extracellular matrix (ECM) components. Previous studies have shown that 042 adheres to surfaces coated with ECM proteins, that AAF/II demonstrates binding to fibronectin specifically, and that fibronectin interactions also involve integrin α5β1 (Farfan *et al*., 2008, Izquierdo *et al*., 2014a). However, the ECM is localised at the basement membrane and is not typically accessible to enteric bacteria, although there is evidence that some ECM proteins including fibronectin may be exposed to the apical environment during inflammation (Walia *et al*., 2004). Interestingly, AAFs induce inflammation in intestinal epithelial cells during EAEC infection (Harrington *et al*., 2005, Boll *et al*., 2012). Cytokeratin-8 (CK8) has also been identified as a potential EAEC receptor and binding site for AAF/II (Izquierdo *et al*., 2014b). The accessibility of bacteria to CK8 *in vivo* is unclear, as it is usually associated with intermediate filaments underlying the apical brush border of polarised intestinal epithelial cells (Coch and Leube, 2016). In contrast, many cancer-derived cell lines used in bacterial adherence studies express CK8 on the plasma membrane surface, which may limit the physiological relevance (Gires *et al*., 2005). A target for EAEC binding that is expressed on the intestinal epithelial surface is the mucin glycoprotein MUC1 (McGuckin *et al*., 2011). Interactions between AAFs and MUC1 enhance EAEC adhesion and cause a pro-inflammatory response (Boll *et al*., 2017). Increased MUC1 levels are associated with *Citrobacter rodentium* infection in the murine colon (a model for EHEC and EPEC pathogenesis), and previous studies have shown that the EAEC Pic mucinase increases mucus secretion and promotes intestinal colonization and growth in the presence of mucin (Lindén *et al*., 2008, Harrington *et al*., 2009, Navarro-Garcia *et al*., 2010). To my knowledge, none of these binding interactions have been associated with modulating EAEC virulence gene expression.

3.4: Summary

The results of this study can be interpreted as evidence of two separate environmental signals that allow EAEC to adapt for intestinal colonisation (**Figure 3.24**). The first is a response to oxygen, which occurs as the bacteria approach the mucosal surface. From the more AN lumen, enteropathogenic bacteria encounter an oxygen gradient established by diffusion of oxygen across the epithelial barrier from the bloodstream (Marteyn *et al*., 2011). This relative increase in oxygen concentration acts as a signal to prime the bacteria for adherence. Correspondingly, the data for EAEC 042 demonstrates an oxygen-induced and AggR-regulated upregulation of key virulence factors for initial epithelial adhesion: the adhesins AAF/II and the *E. coli* common pilus, and dispersin.

Once physical contact is established with colonic epithelial cells, the expression of further EAEC virulence factors is induced. As well as adherence-related genes, this includes upregulation of genes linked to colony spatial organisation (*aap*), modulation of the mucus layer (*pic*), and secreted toxins (*astA*, *hlyE*, *pet*) (Navarro-Garcia *et al*., 2010, Velarde *et al*., 2007). The data suggests the induction of virulence gene transcription at this stage is independent of AggR regulation. Subsequently, colony expansion and interactions between host cells and EAEC factors such as flagella and toxins can induce inflammation and symptomatic diarrhoeal illness (Steiner *et al*., 2000).

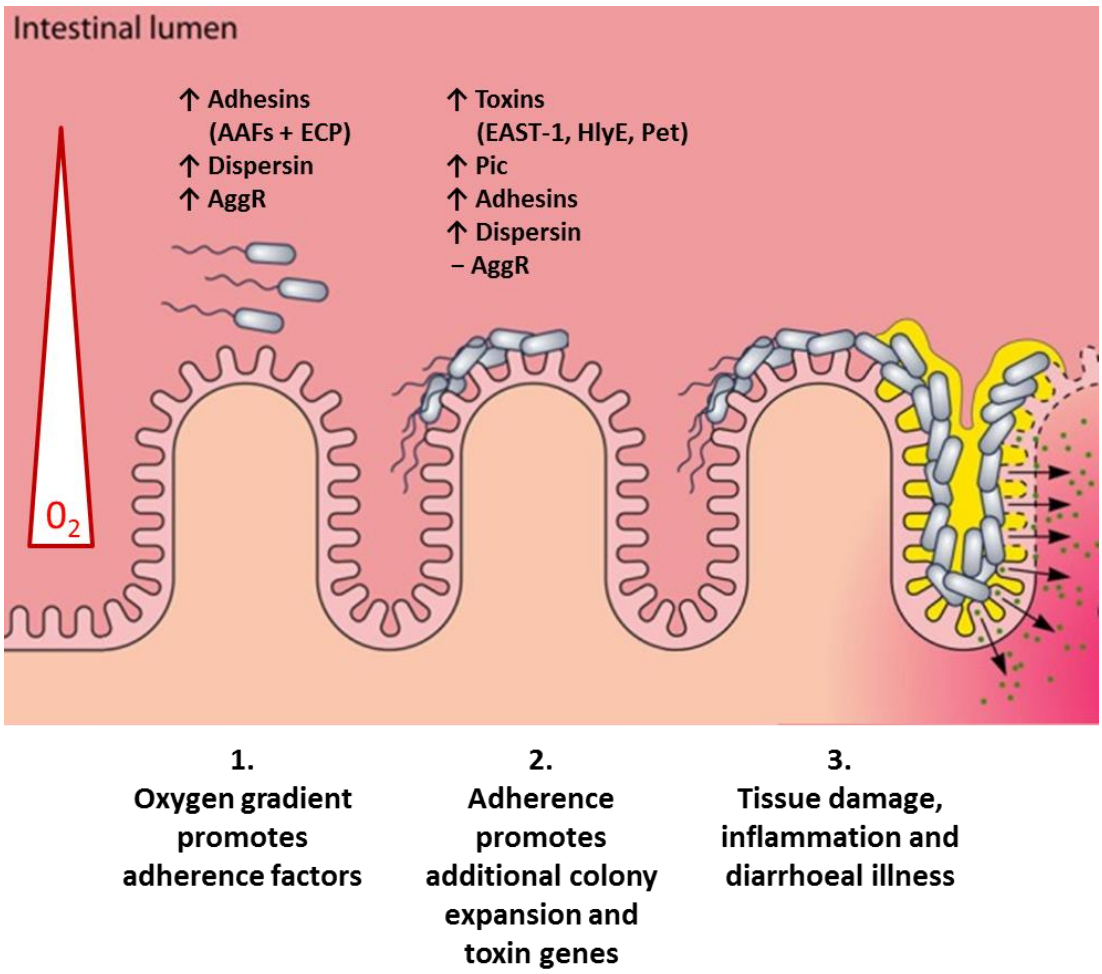

Figure 3.24 Proposed model for EAEC response to oxygen and host epithelial contact during infection. Yellow region represents biofilm development and green particles represent toxin secretion. Adapted from (Hebbelstrup Jensen *et al*., 2014)**.**

CHAPTER FOUR

Phenotypic and genotypic analysis of EAEC sequence types associated with disease or carriage

Collaborative work:

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SEM support provided by Bertrand Lézé

Whole-genome sequencing performed by Gemma Kay and John Wain, and Genomic Servicing Unit and Gastrointestinal Bacteria Reference Unit at Public Health England

Bioinformatic support provided by Rob Kingsley, Matt Bawn, and Oliver Charity

4.1: Introduction

A major limitation for monitoring EAEC pathogens is a lack of virulence markers. While a number of virulence genes have been associated with EAEC pathogenesis, many are only present in a minority of clinical strains. In addition, the importance of specific virulence factors for an EAEC strain to cause enteric disease rather than asymptomatic carriage remains unclear (Okhuysen and DuPont, 2010, Bafandeh *et al*., 2015). Correlations of putative virulence genes such as *astA*, *aap*, *pic,* and *aggA* with diarrhoea have been reported, but these associations are often inconsistent between studies (Pereira *et al*., 2007, Zamboni *et al*., 2004, Bafandeh *et al*., 2015, Hebbelstrup Jensen *et al*., 2017). For example, one case-control study with 128 EAEC strains found no statistical difference in the prevalence of any of 11 major EAEC virulence factor genes between cases and controls (Elias *et al*., 2002). Also, while a virulence gene may be identified in an isolate's genome, it may not be expressed under physiological conditions or contribute to *in vivo* pathogenesis.

To investigate these relationships, this study aimed to compare EAEC strains from case-control studies and outbreaks for differences in virulence-associated phenotypes *in vitro*, as well as analysing genotypic differences in putative virulence genes. The phenotypes evaluated included biofilm formation and the HEp-2 adherence assays as models of bacterial aggregation, with the latter established as the gold-standard diagnostic tool for EAEC. The *in vitro* adherence of the clinical isolates to a human colonic epithelial cell line, and *ex vivo* colonisation of human colonic biopsies, was also investigated. These are experimental models for the colonisation of the epithelium of the human colon, a key part of typical EAEC pathogenesis (Hebbelstrup Jensen *et al*., 2014).

Traditionally, serotyping has been widely applied for investigating pathogenic *E. coli*, remaining in use due to the applicability across pathotypes and the ability to use serotypes for functions such as outbreak tracking. While EHEC O157:H7 is an example of a serotype strongly associated with virulence, serotyping has not proven effective for distinguishing pathogenic strains for EAEC. Additionally, performing phenotypic serotyping is labour-intensive and typically limited to specialised reference laboratories (Jenkins, 2015, Fratamico *et al*., 2016). A more recent

alternative typing scheme is Multi-Locus Sequence Typing (MLST), first developed for *Neisseria meningitidis*, but now applied to many pathogens including *E. coli* (Maiden *et al*., 1998, Larsen *et al*., 2012). This technique uses the allelic profile of seven core gene fragments, traditionally amplified by PCR, to type matching strains into corresponding sequence types (STs). Although lacking the discriminatory power to differentiate individual strains, MLST can be a powerful tool for investigating population genetics. One such study investigated the STs present in a collection of 564 clinical EAEC isolates, sourced from multiple case-control cohort studies as well as sporadic and outbreak strains (Chattaway *et al*., 2014b). A key finding was that certain STs had statistically significant higher or lower association with disease than the overall collection (**Figure 4.1**). In particular, ST40 had an increased ratio of disease case strains versus control strains, while ST31 had a higher proportion of asymptomatic carriage. These groups were selected for further characterisation in this study.

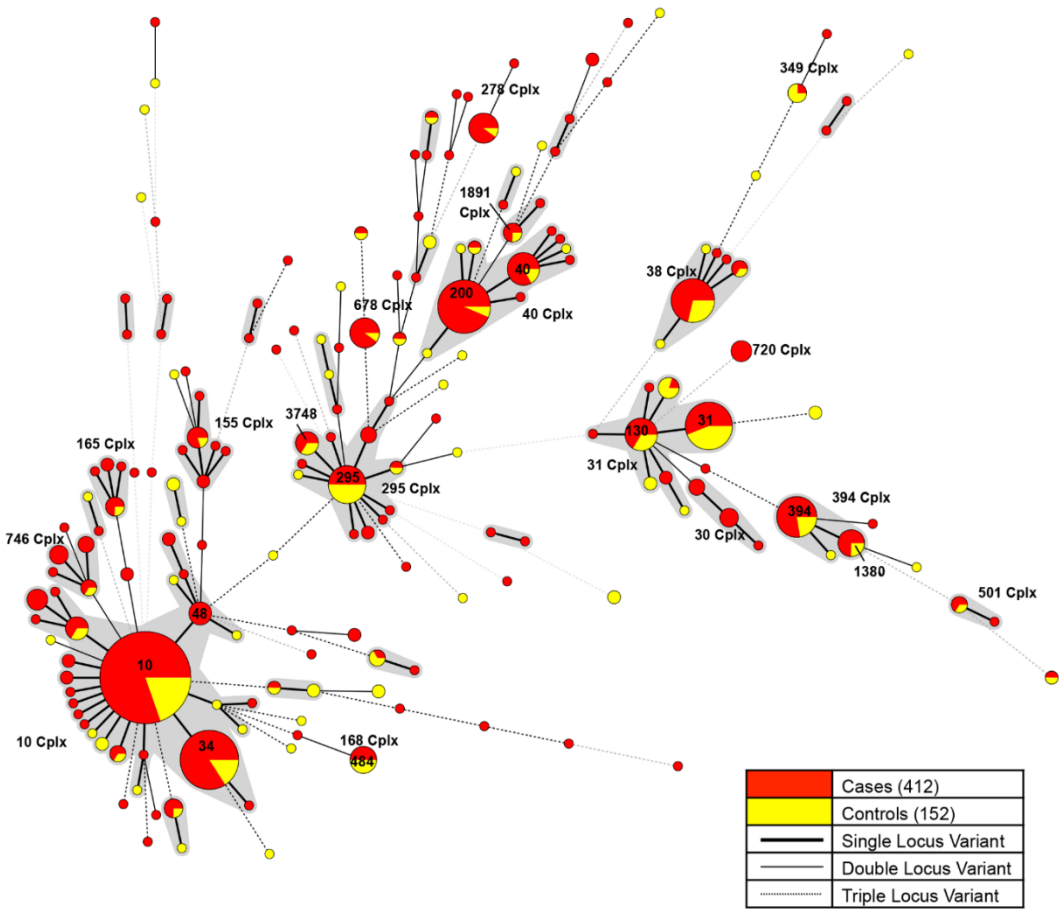

Figure 4.1 Minimal spanning tree of 564 clinical EAEC isolates. (Chattaway *et al*., 2014b)

Following the previous MLST study using these EAEC strains, the collection has since been genome sequenced in collaborations with Public Health England (PHE) and Norwich Medical School. This allows the use of bioinformatic tools to compare the virulence gene profile of ST40 and ST31 strains. In addition, sequencing data can be exploited for *in silico* typing approaches, including serotyping, MLST, and phylogenetic analysis, which can reveal additional detail compared with traditional phenotype-based techniques alone (Fratamico *et al*., 2016).

The objectives of this study were to:

- Investigate if strains from ST40 and ST31, which differ in their epidemiological association with disease, demonstrate differences in virulence-associated phenotypes *in vitro*
- Use whole genome sequencing data to evaluate serotype, ST, and phylogenetic variation of these strains
- Identify genotypic differences in virulence genes between ST40 and ST31 strains

4.2: Results

4.2.1: Virulence-associated *in vitro* phenotypes

The first part of this project aimed to use *in vitro* and *ex vivo* models to investigate any phenotypic difference between EAEC strains from ST40, epidemiologically associated with disease, and ST31, associated with carriage. Eight ST31 strains (4 isolated from cases of diarrhoea and 4 controls) and eight ST40 strains (6 cases and 2 controls) were investigated in this study (see **Table 2.1** for full sources).

4.2.1.1: Adherence to HEp-2 cells

Many of the ST40 and ST31 strains were isolated from case-control studies and classified as EAEC by PCR probes only. Therefore, all strains were first confirmed as EAEC using the gold-standard diagnostic tool, the HEp-2 adherence assay. Prototype EAEC strains 042 and 17-2 were also included as positive controls. HEp-2

cells were incubated with bacteria for 3 hours, and the adherence phenotype for each strain was evaluated by microscopy.

All EAEC isolates from this study adhered to HEp-2 cells and the underlying coverslip, but differences in morphology were observed between STs (**Figure 4.2 and 4.3**). The prototype EAEC strains 042 and 17-2 demonstrated typical aggregative adherence (AA), with dense bacterial aggregates and a "stacked-brick" colonisation pattern. ST40 strains demonstrated AA comparable to 042 and 17-2, including large aggregative colonies. However, ST31 isolates exhibited more variation in the adherence pattern. Although some areas of AA were seen for all strains, aggregates were typically smaller and less dense than observed for ST40 and there were also regions of more diffuse colonisation. In addition, ST40 strains had an observably higher level of total adherence than ST31. Overall, all strains displayed the AA phenotype on HEp-2 cells to confirm the EAEC classification, although differences in adherence were observed between the STs.

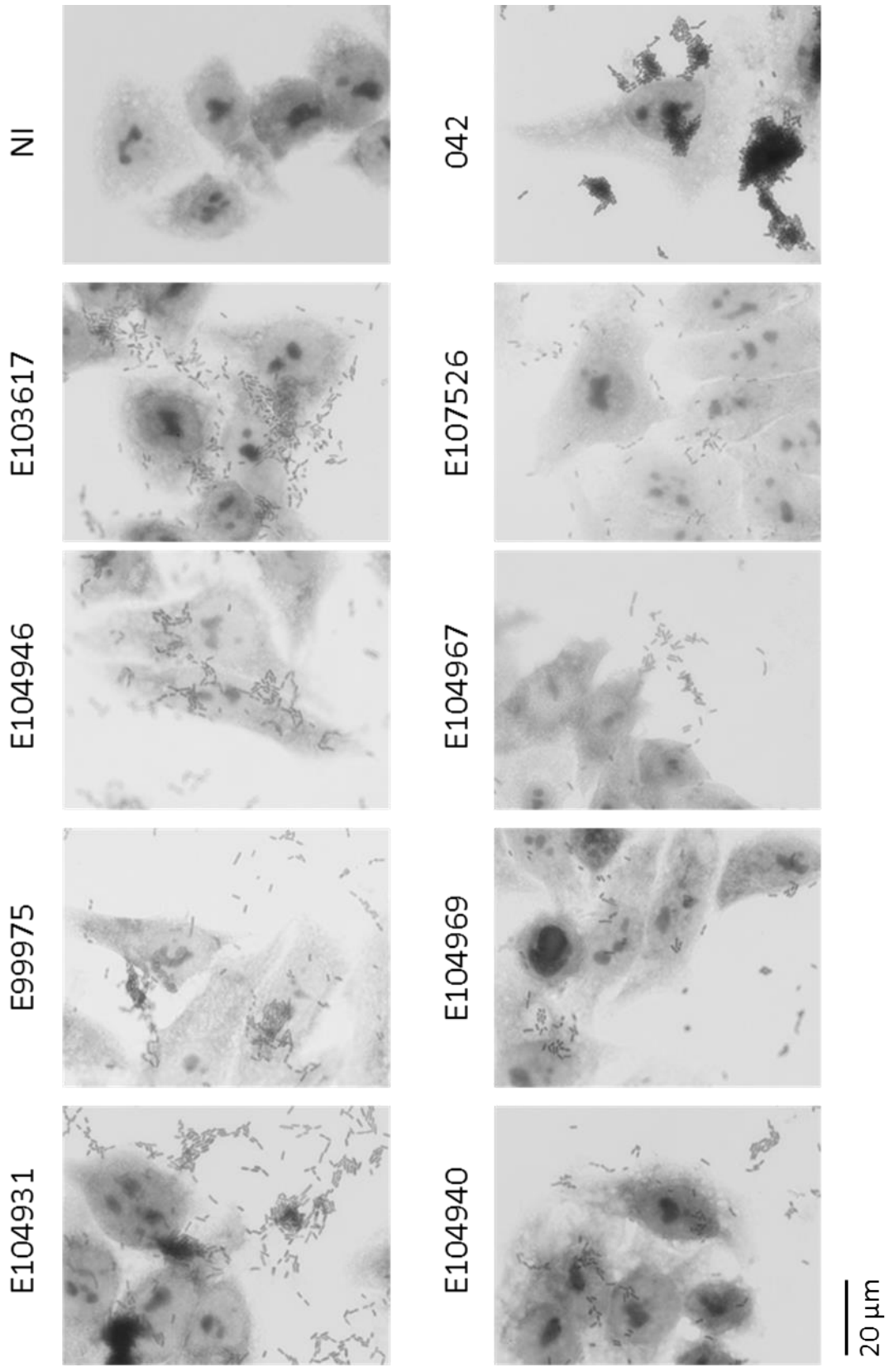

Figure 4.2 Aggregative adherence phenotype of ST31 strains. HEp-2 cells were incubated with ST31 EAEC for 3 hours (MOI = 20 bacteria/cell), and the colonisation phenotype evaluated by microscopy. Prototype EAEC strain 042 was included as a positive control. Images are representative of experiments performed in duplicate.

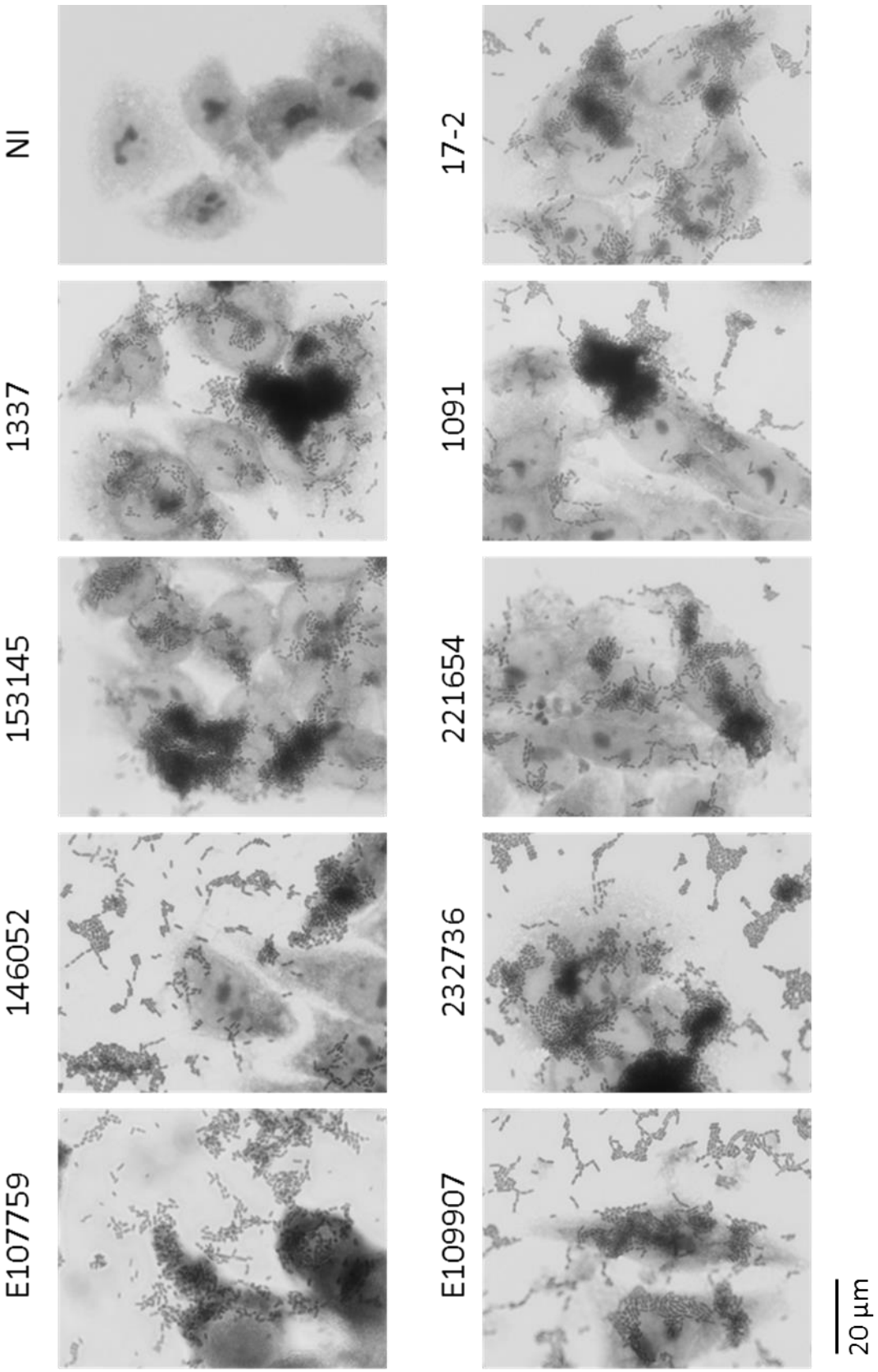

Figure 4.3 Aggregative adherence phenotype of ST40 strains HEp-2 cells were incubated with ST40 EAEC for 3 hours (MOI = 20 bacteria/cell), and the colonisation phenotype evaluated by microscopy. Prototype EAEC strain 17-2 was included as a positive control. Images are representative of experiments performed in duplicate.

4.2.1.2: Biofilm formation

Biofilm formation by the EAEC isolates was assessed using a crystal violet assay, which is a widely used method for determining bacterial aggregation (O'Toole, 2011). EAEC were incubated for 48 hours in a 96-well microtiter culture plate and crystal violet stain used to quantify bacterial density by absorbance. The incubation conditions used (37 °C in DMEM) had previously been reported to induce maximal biofilm formation for the EAEC prototype strain 042 (Sheikh *et al.*, 2001). Strain 042 was used as a positive control and produced denser biofilms than all the clinical isolates. Overall, ST40 strains demonstrated significantly higher biofilm formation than those from ST31 ($P < 0.05$) (**Figure 4.4**). Only one ST40 isolate (221654) produced an average absorbance below 0.2, while only one strain from ST31 (E104931) exceeded this value. In addition, biofilm formation by case and control isolates did not differ significantly for either ST. EAEC were also incubated with glass coverslips for 48 hours as above and stained with crystal violet for microscopy (**Figure 4.5**). The observed biofilm density was consistent with the quantitative data, with ST40 strains exhibiting greater bacterial aggregation than ST31 isolates.

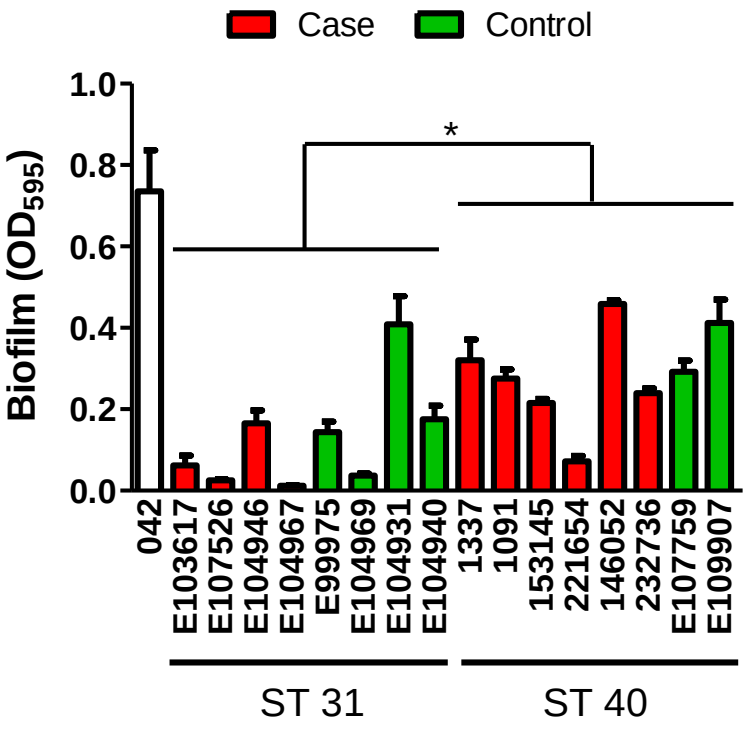

Figure 4.4 Biofilm formation by EAEC isolates. Bacteria were incubated in 96-well plates at 37 °C for 48 hours and treated with crystal violet stain. Biofilm density was quantified by absorbance at 595 nm (OD_{595}). Data are shown as mean ± SE for 3 experiments, each with 5 technical replicates. * $P < 0.05$.

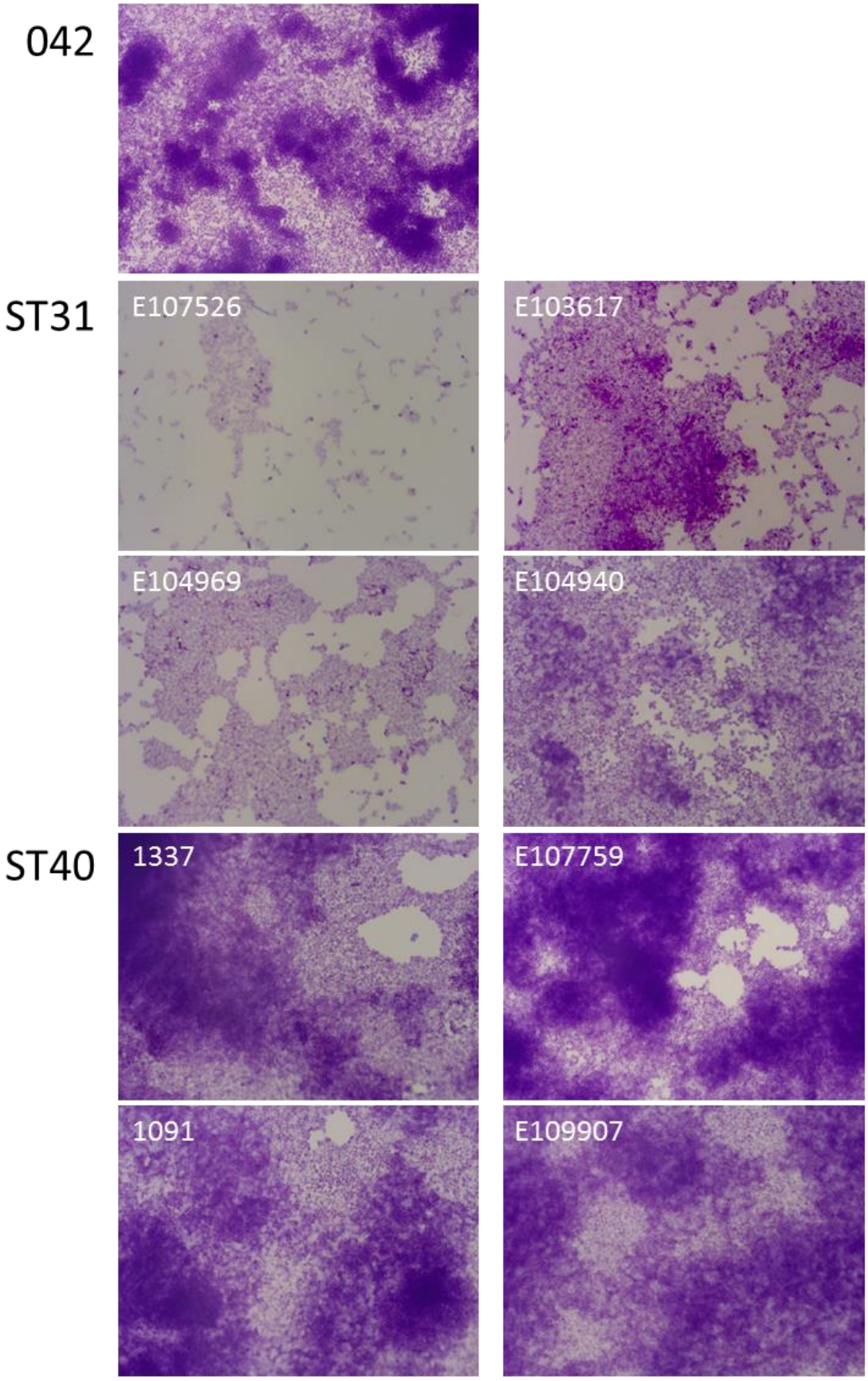

Figure 4.5 EAEC biofilm on abiotic surface. EAEC were incubated with a glass coverslip for 48 hours and stained with crystal violet for bright-field light microscopy (representative images of ST40 and ST31 strains, and 042 prototype strain as positive control).

4.2.1.3: Adherence to T84 intestinal epithelial cells

To ascertain whether EAEC strains from ST40 and ST31 differ in their ability to colonise intestinal epithelial cells, a cell culture model of infection was used. Confluent cells of the T84 colon carcinoma-derived cell line were incubated for 2 hours with EAEC. Bacterial adherence was quantified by plating of lysates on LB agar and CFU counting, normalised against inoculum concentration.

All isolates adhered to T84 cells. However, ST40 isolates demonstrated significantly higher adherence than those from ST31 ($P < 0.001$) (**Figure 4.6**). EAEC strain 042 was also evaluated as a positive control. All ST40 strains adhered to the T84 monolayer to a comparable or greater extent than 042 in these assays. Conversely, all ST31 isolates exhibited lower adherence than either the positive control or any individual ST40 example. When isolates from a case or control source were compared within STs, no significant differences were detected.

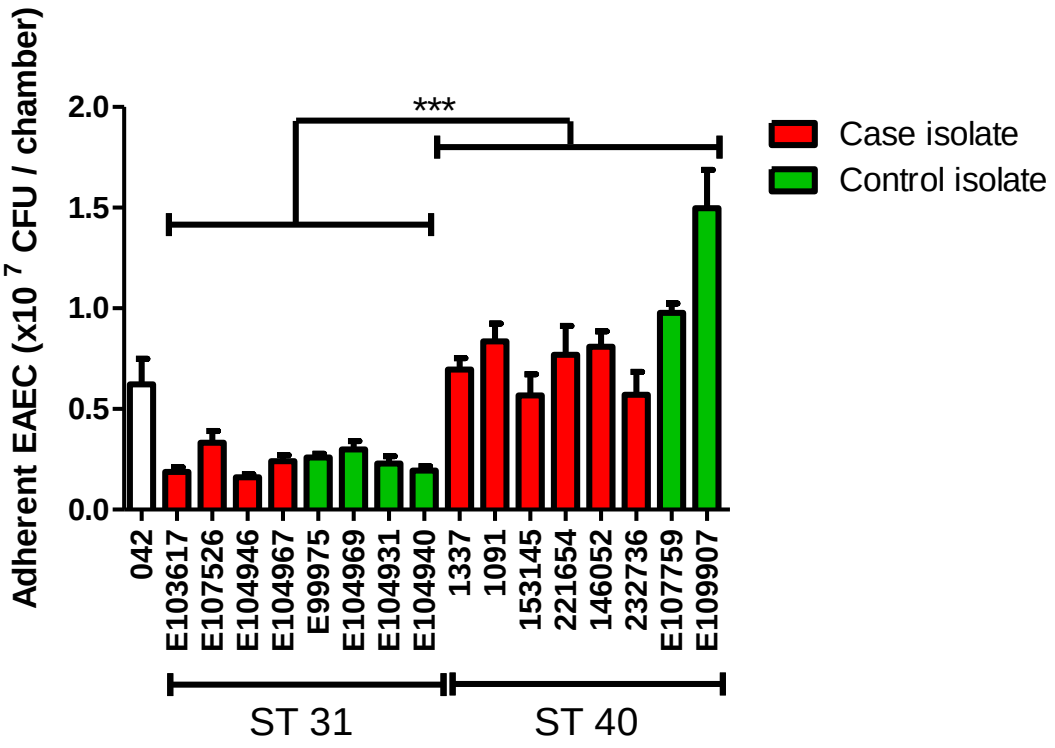

Figure 4.6 Adherence of EAEC to T84 cells. Confluent T84 cells were incubated with EAEC (MOI = 10 bacteria/cell) for 2 hours. Adherent bacteria were quantified by plating of cell lysates on LB agar and counting colony forming units (CFU). Adherence was normalised against the concentration of the inoculum. Data are shown as mean ± SE for three independent experiments, in duplicate. *** $P < 0.001$

4.2.1.4: Colonisation of human colonic biopsies

Having demonstrated a significant difference in the ability of ST31 and ST40 EAEC strains to adhere to T84 cells, the colonisation of human intestinal epithelial tissue was next investigated. This aimed to determine if the difference between the STs was maintained for adherence to *ex vivo* mucosa with greater physiological relevance than cell culture. Therefore, IVOC experiments were performed using sigmoid colonic biopsies obtained from consenting patients undergoing endoscopy at the Norfolk and Norwich University Hospital. The sigmoid colon was selected as prototype EAEC strains 042 and 17-2 demonstrated greater adherence to colonic than small bowel biopsies (see Chapter 3.2.3), and sigmoid colonic samples were more consistently available from both sigmoidoscopy and colonoscopy procedures. The mucosal surface of the biopsy samples was inoculated with EAEC and incubated for 7 hours on a rocking platform. Each strain was assessed using biopsies from at least 3 different donors.

Initially, evaluation of the abundance of adherent bacteria was performed using immunofluorescence staining with an anti-*E. coli* antibody and co-staining of epithelial cells for actin and cell nuclei (**Figure 4.7**). However, while the anti-*E. coli* antibody did successfully stain some adherent EAEC colonies, it was difficult to resolve the bacteria for accurate quantification. In addition, non-specific staining was observed in many samples, which could not be satisfactorily distinguished from stained bacteria.

Therefore, scanning electron microscopy (SEM) was employed for evaluation of IVOC samples. After incubation with EAEC, biopsies were washed in PBS to remove mucus and processed for SEM (**Figure 4.8**). Absolute quantification of adherent bacteria was not deemed feasible as each biopsy sample varied in size, and extensive EAEC colonisation was often observed beyond the limits for reliable counting. Therefore, a semi-quantitative analysis was performed whereby each sample was scored against a defined numerical scale for both colony size (see **Figure A1.1** for representative images) and frequency of colonisation. The combined score of the two features was used as a colonisation score for each biopsy.

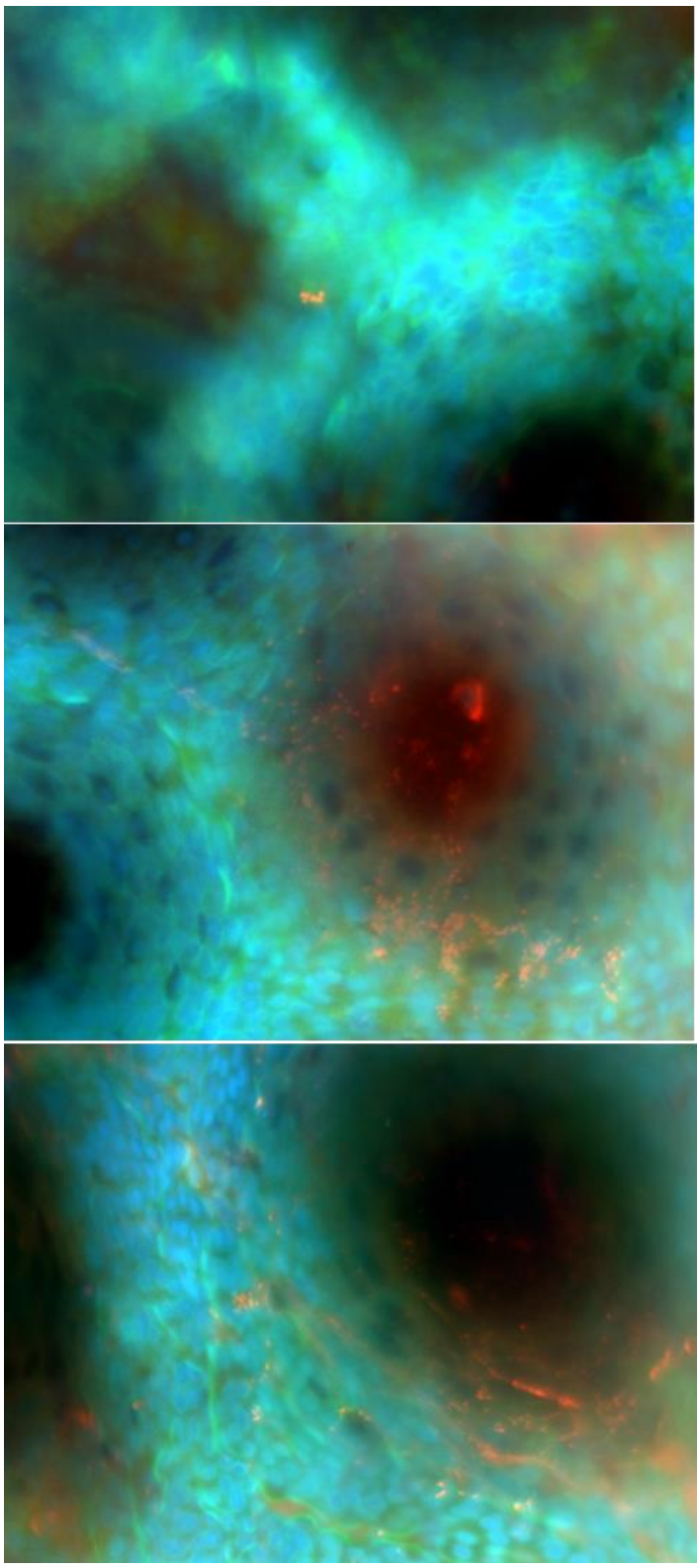

Figure 4.7 Immunofluorescence staining of colonic biopsies infected with EAEC. Biopsies were incubated with EAEC for 7 hours. Biopsy samples were stained for cell nuclei (blue), actin (green) and *E. coli* (red), and imaged by immunofluorescence microscopy. Representative images of three independent experiments, in duplicate.

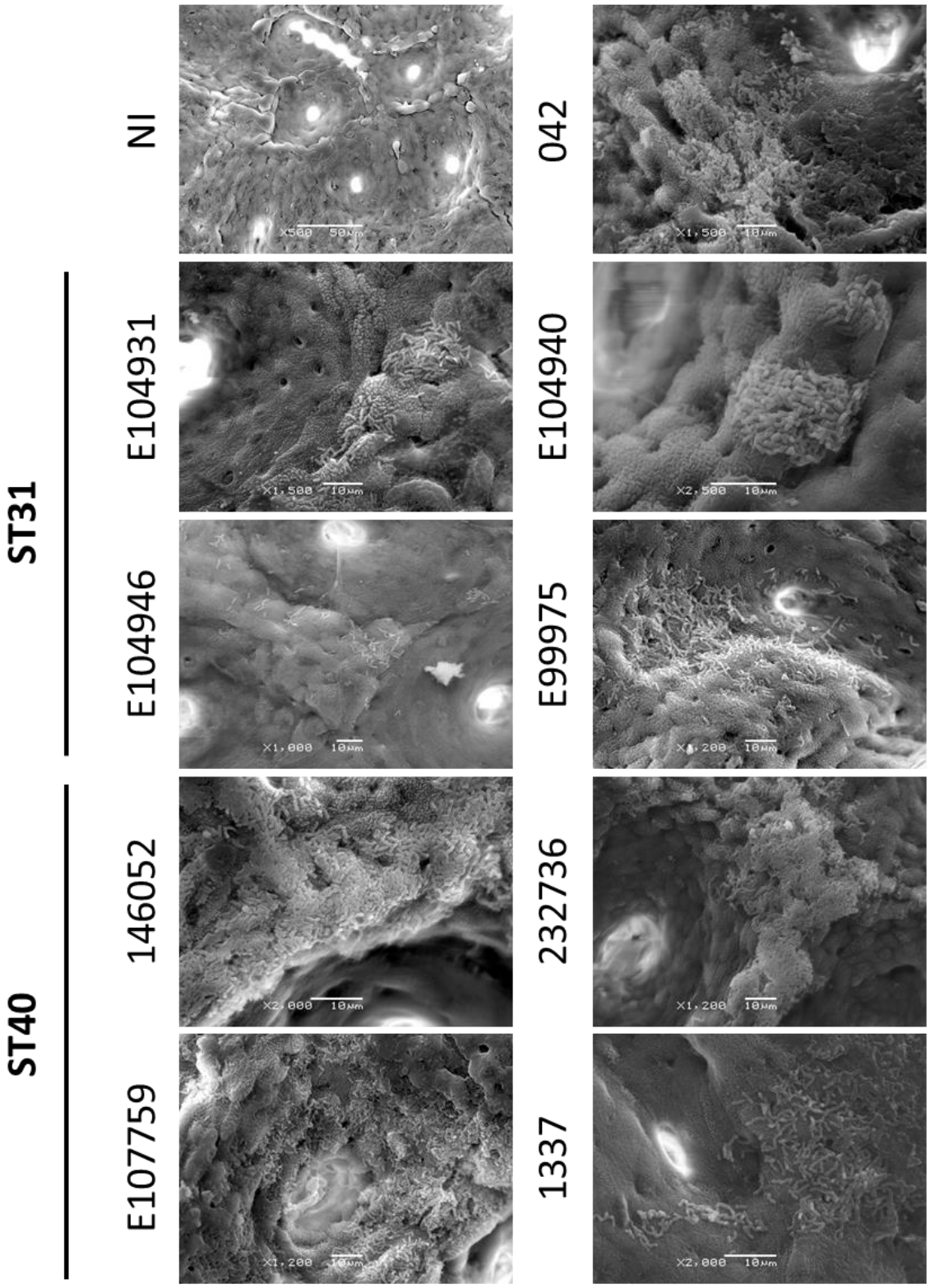

Figure 4.8 Scanning electron micrographs of EAEC colonisation of colonic biopsies. The biopsies were incubated with EAEC for 7 hours and then washed for removal of the mucus layer. Samples were fixed and dehydrated for evaluation by SEM. The images are representative of three independent experiments in duplicate.

As observed for T84 cells, ST40 isolates colonised colonic tissue to a comparable or greater extent than the positive control, 042. While ST40 adherence was relatively consistent for all strains, greater variation was observed in the ability of ST31 strains to colonise biopsy samples (**Figure 4.9**). Some ST31 isolates demonstrated a median colonisation score comparable to the 042 control, whereas others showed low levels of mucosal binding. Also apparent was that many individual biopsies had no observable bacteria for ST31, while all biopsies infected with ST40 isolates had a score of at least 2. No adherent bacteria were ever observed for ST31 strain E104967.

When comparing the STs overall, ST40 strains exhibited significantly higher colonisation levels of human colonic biopsies than ST31 strains ($P < 0.01$). Within the ST groups, no significant difference was evident between case and control isolates for ST40, while control isolates displayed higher adherence than case isolates for ST31 ($P < 0.01$).

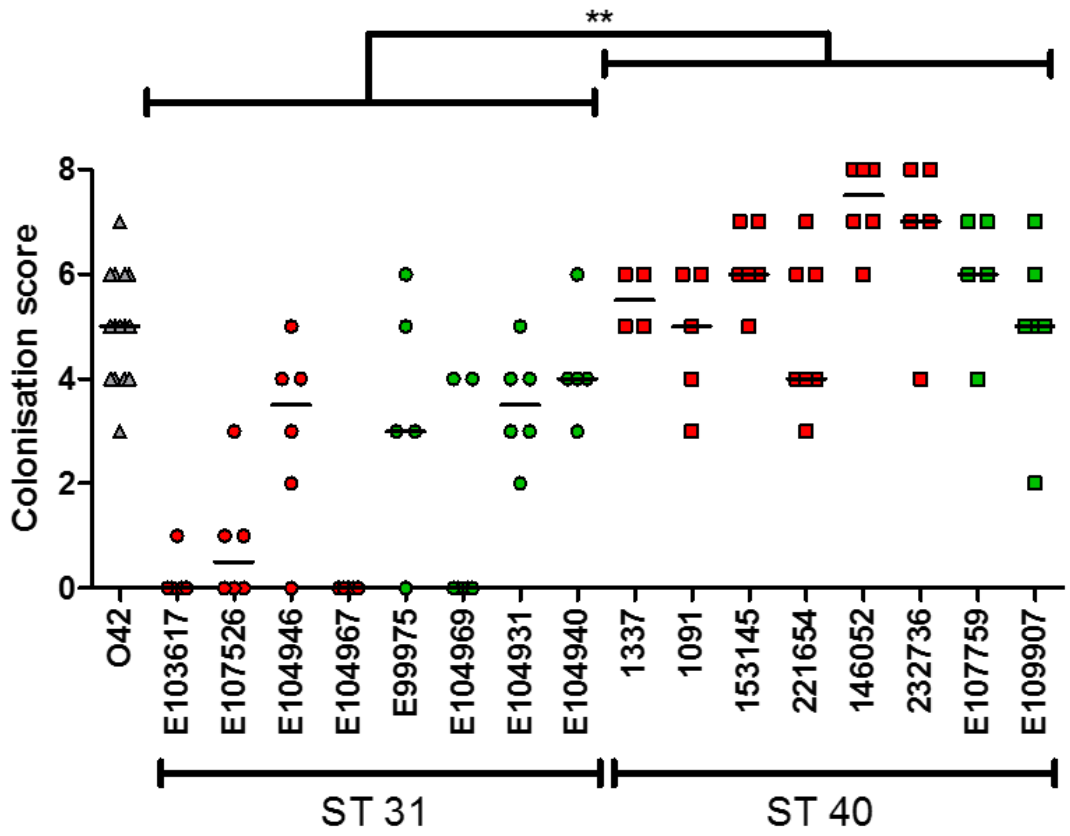

Figure 4.9 Colonisation of colonic biopsies by EAEC. Biopsies from the sigmoid colon were incubated with EAEC for 7 hours, and bacterial colonisation was evaluated by SEM. Biopsies were additively scored for size and frequency of observed colonies on a 0-4 scale. Red and green represent case and control isolates, respectively. Triangles are individual biopsies for the 042 positive control, circles are ST31, and squares are ST40. Horizontal bar represents median score. Data are shown for three independent experiments, in duplicate. *** $P < 0.01$.

4.2.2: Genotypic characterisation of ST40 and ST31 strains

Whole-genome sequencing (WGS) has recently been performed for the EAEC strains investigated in the project. This was undertaken as part of a larger collaborative work to sequence all of the EAEC collection at PHE, with the sequencing performed by Gemma Kay (John Wain group; University of East Anglia, Faculty of Medicine and Health) and the Genomic Servicing Unit and GBRU at PHE, using the Illumina Mi-Seq and Hi-Seq platforms respectively. The available genetic data was utilised in this study to compare the EAEC putative virulence gene distribution between ST40 and ST31 strains, as well as use bioinformatic approaches to analyse strain typing and phylogeny.

4.2.2.1: Confirmation of multi-locus sequence type

The STs of the EAEC isolates used in this project were previously determined by multi-locus sequence typing (MLST) by PCR for seven *E. coli* gene targets (*adk*, *fum*C, *gyr*B, *icd, mdh*, *pur*A and *rec*A) (Chattaway *et al*., 2014b). To confirm the original ST assignments, *in silico* MLST was performed using the WGS data for all 16 strains.

Two different bioinformatic tools were used. In the first method, the sequence data was analysed with the MLST tool incorporated in the Galaxy computational biology platform. In addition, the Short Read Sequence Typing for Bacterial Pathogens (SRST2) tool was utilised, and the sequencing reads for each strain were analysed against a custom fasta sequence database containing the allele sequences for each of the MLST target genes. The combination of aligned alleles for each strain was then converted to ST according to previously defined MLST profiles. Both tools gave corroborating results confirming that all strains belonged to ST40 or ST31 as initially assigned (**Table 4.1**). The Galaxy MLST tool identified strain E103617 as ST31 but indicated a higher level of uncertainty than for the rest of the group. However, the SRST2 output confirmed the ST for this strain.

Previously assigned ST	Strain	SRST2 MLST	Galaxy MLST
ST31	E103617	31	31?
	E107526	31	31
	E104946	31	31
	E104967	31	31
	E99975	31	31
	E104969	31	31
	E104931	31	31
	E104940	31	31
ST40	1337	40	40
	1091	40	40
	153145	40	40
	221654	40	40
	146052	40	40
	232736	40	40
	E107759	40	40
	E109907	40	40

Table 4.1 *In silico* sequence typing of EAEC isolates

4.2.2.2: *In silico s*erotyping and core genome phylogeny

These EAEC strains were originally isolated from different epidemiological studies and disease cases and not all had been previously fully characterised by classical antigen serotyping. Therefore, in addition to confirming the MLST results for all of the ST40 and ST31 strains, the sequencing data was also used to analyse the serotypes present in these two groups. WGS of the strains allowed *in silico* serotype prediction by alignment of defined O and H antigen genes (**Table 4.2**). This was performed using the SRST2 tool against a fasta sequence database containing the sequences for each characterised antigen allele.

For the ST40 group, all strains were identified as positive for the H21 flagellin. This matched previous antigen typing for most strains other than 1091 and 1337, which had been serotyped as H11. The LPS antigen was identified as O111 for all ST40 strains other than E107759 (O127) and 146052 (unidentified). The ST31 strains were identified as belonging to two serotype groups. Four strains (E104931, E104969, E103617, E104940) were serotyped as O130 and H26 or H27. Strain E107526 was also positive for H27 but could not be matched to any O antigen allele. The remaining three ST31 isolates (E104946, E104967, E99975) were identified as O15:H18.

ST	Strain	Previous antigenic serotyping	*In silico* serotyping
31	E107526	O?:H?	O?:H27
31	E104931	O?:H?	O130:H26
31	E104969	O130:H25	O130:H26
31	E103617	O130:H27	O130:H26
31	E104940	O130:H27	O130:H27
31	E104946	O?:H?	O15:H18
31	E99975	O15:H18	O15:H18
31	E104967	O?:H?	O15:H18
40	146052	0154:H21	O?:H21
40	232736	O111:H21	O111:H21
40	1091	O111ac:H11	O111:H21
40	221654	O111:H21	O111:H21
40	153145	O111:H21	O111:H21
40	1337	O111ac:H11	O111:H21
40	E109907	O111ab:H-	O111:H21
40	E107759	O?:H?	O127:H21

Table 4.2 *In silico* serotyping of EAEC strains. The *in silico* serotype was analysed using the SRST2 tool with whole-genome sequences for each strain. The previous serotype data generated by antigen testing was provided by the GBRU at PHE (ab and ac represent polysaccharide minor subtypes of O111).

To further explore the diversity of these 16 EAEC strains, a phylogenetic analysis was performed (**Figure 4.10**). A core genome alignment was undertaken using the Parsnp tool, with the clinical isolate sequences analysed against 042 as a reference genome. This software generates a multi-alignment of all genomes and identifies single-nucleotide polymorphisms (SNPs) within the core genome of all strains. This data set is then used for reconstruction of the whole-genome phylogeny. The resulting phylogenetic tree was processed and visualised using the Phandango software. Overall, this data demonstrates the ST31 and ST40 strains are from highly distinct lineages, and that the ST31 strains contain multiple serotype groups.

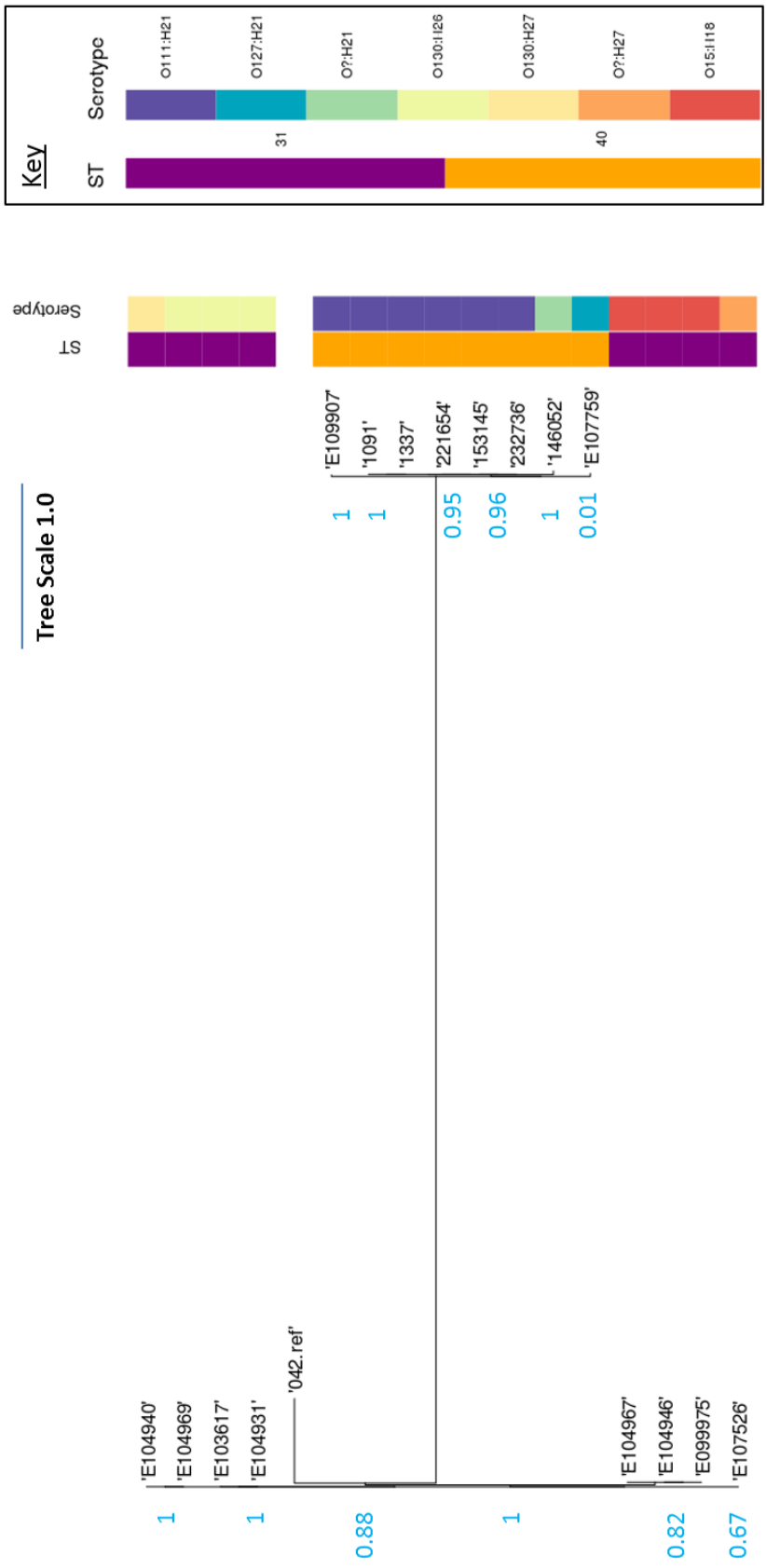

Figure 4.10 Core genome phylogenetic tree of EAEC strains. Bootstrap confidence values are shown in blue for each branch. Tree scale is sequence substitutions per site.

4.2.2.3: Genotype profiles for putative EAEC virulence factors

As the previous experiments had demonstrated a difference in virulence-associated phenotypes between ST31 and ST40 strains, the genotypes of these groups were next investigated for putative virulence factors. In particular, the distinct presence or absence of virulence genes was determined between the two STs. Initially, a specific subset of virulence genes was selected for investigation which corresponded to the EAEC-associated virulence factors studied in Chapter 3 of this project, with the addition of all AAF structural subunit alleles: *aggR, pic, aap, astA, hlyE, pet, ecpA, aggA, aafA, agg3A, hdaA,* and *aaf5A*.

To minimise the risk of experimental bias, multiple tools were used in parallel to analyse the presence of virulence genes in the sequence data for each strain: (1) Basic Local Alignment Search Tool (BLAST) was applied to search for virulence gene query sequences in genome assemblies, using both a custom local BLAST search (BioEdit software) and the BLAST function provided by the Galaxy bioinformatics platform. (2) *In silico* PCR was performed with genome assemblies using the FastPCR software tool to predict any amplification for previously used PCR primer sequences for each gene. (3) Finally, the SRST2 tool was applied to perform alignments of the virulence gene sequences, using sequencing read files instead of assemblies.

While there was some variation in specificity, the results for most virulence genes were largely consistent across all tools utilised. As SRST2 has been reported to outperform assembly-based methods for accurate gene detection, the results of method 3 was used for further analysis (Inouye *et al*., 2014) (**Table 4.3**). The data from methods 1 and 2 are presented in **Figure A1.2** in **Appendix 1**.

Table 4.3 Virulence gene profile for ST40 and ST31 EAEC strains. Alignments performed from sequence reads using SRST2 software. * = alignment below 90% identity threshold.

The genes *pic*, *aap*, and *hlyE* were conserved in all 16 strains. The *astA* gene (EAST-1 toxin) was present only in ST40 strains. In addition, all ST40 genomes contained *ecpA*, while only 2 of the 8 ST31 strains were positive for this gene. The other major difference observed was in the AAF types. While ST40 strains were associated with *aaf5A* (AAF/V), the ST31 genomes aligned with *agg3A* (AAF/III) or *aggA*, (AAF/I). For the SRST2 tool, the coverage threshold had to be reduced from the 90% default value for the *aggA* alignment to be reported in these strains. The ST31 AAF variants corresponded with the previously identified serotypes, AAF/III with O15:H18 and AAF/I with O130:H26/27.

This subset contained some of the major putative virulence genes for EAEC, but many other genes have been associated with the pathotype. In addition, other *E. coli* virulence factors not specific to EAEC may be of importance in pathogenesis. Therefore, the investigation of genotypical differences between ST40 and ST31 strains was expanded from the initial specific set of virulence factors to a much larger genetic database. The Virulence Factors of Bacterial Pathogens database is an online resource containing sequence data for potential virulence genes. This resource was used to generate an *Escherichia* database of 2686 genes from 134 different strains, which were aligned against the sequencing data for each of the 16 EAEC strains using the SRST2 tool. This identified 302 genes present in at least one strain. This data set was then manually curated for target genes identified only in one ST and not the other (**Table 4.4**). The results contain many additional genes of interest, as well as the previously identified EAST-1 and AAF genes.

Sequence type (serotype)	Gene / operon	Predicted function
ST31	EC042_4532	Putative type VI secretion protein
	air/eaeX	Adhesion
	aslA	Periplasmic sulfatase
	chuA, S-Y	Heme utilisation
	ehaB	Adhesion
	kpsD	Capsule
	pkgA	Putative type III secretion effector
	sitABCD	Iron/Manganese uptake
ST31 (O130)	*aggBCD*	Accessory genes for AAF/I
ST31 (O130) & ST40	*gspC-M*	Cryptic type II secretion pathway
ST31 (O15:H18)	*agg3A*	AAF/III structural subunit
	hlyABCD	Alpha-haemolysin
	papA-K, X	P pilin, adhesion
ST31 (O15:H18) & ST40	*agg3BCD*	Accessory genes for AAF/III
ST40	*astA*	EAST-1 toxin
	cfaABCD	CS1 fimbriae, adhesion
	ehaA	Adhesion
	hcp	Missing designation in VFDB
	stgCD	Stg fimbriae, adhesion
	ycbF, R-V	Predicted chaperone-usher fimbriae, adhesion

Table 4.4 Distinct putative EAEC virulence genes for ST40 and ST31 strains. This table lists the putative virulence genes identified as specific to ST31 or ST40 genomes only, and/or in specific ST31 serotypes.

Overall, the use of bioinformatic tools with the sequenced EAEC genomes identified differences in virulence gene profiles which may be associated with the difference in virulence-associated phenotypes between ST40 and ST31 strains.

4.3: Discussion

This research was designed to investigate the difference between EAEC STs with different epidemiological associations with disease or carriage, as established in a previous MLST study (Chattaway *et al*., 2014b). As previous studies have not established specific EAEC virulence genes as reliable markers of virulence, this work analysed phenotypes as indicators of functions involved in pathogenesis. Therefore,

eight strains were screened for each of the ST40 and ST31 complexes for an *in vitro* study of virulence-associated phenotypes alongside bioinformatic analysis of WGS data. The prototype strain 042, which is the best characterised and most frequently used EAEC strain in laboratory studies of infection, was utilised as a positive control throughout. 042 has also been previously identified as an ST31 strain (Okeke *et al*., 2010, Chattaway *et al*., 2014b). While both ST10 and ST40 were significantly associated with disease in the previous MLST publication, ST40 was chosen for this study due to the high strain diversity of the ancestral lineage ST10 which includes many different *E. coli* pathotypes (Yu *et al*., 2018, Chattaway *et al*., 2014b).

Of the 16 clinical strains investigated (**Table 2.1**), most were originally isolated from two large cohort studies of diarrhoeal illness in the UK (Chattaway *et al*., 2014b). All eight ST31 strains and two ST40 isolates were collected during the English case-control Infectious Intestinal Disease Study (IID1). Between 1993 and 1995, bacteria were isolated from a total of 4,664 stool specimens collected from cases of clinically significant intestinal infectious disease, or from age and sex matched healthy controls within the study cohort (2,443 cases and 2,221 controls) (Amar *et al*., 2007, Sethi *et al*., 1999). A further two ST40 strains were isolated during the Second Study of Infectious Intestinal Disease in the Community (IID2). This was a case-only study undertaken between 2008 and 2009, which produced a dataset from 3,966 stools from individuals with diarrhoea (O'Brien *et al*., 2010). Both IID1 and IID2 used a PCR probe (CVD432) specific for the anti-aggregation transporter gene *aatD* for identification of EAEC (Chattaway *et al*., 2013, Baudry *et al*., 1990). The remaining ST40 strains were derived from sporadic diarrhoea cases catalogued by the Gastrointestinal Bacteria Reference Unit of Public Health England. While some subsets of strains from this EAEC collection have been studied for the presence of virulence and antimicrobial resistance genes, none have previously been analysed for *in vitro* phenotypes (Chattaway *et al*., 2017).

4.3.1: ST40 strains demonstrate greater aggregation and adherence than ST31 strains

4.3.1.1: Biofilm formation

A phenotype often associated with EAEC pathogenesis is the strong formation of biofilms (Mohamed *et al*., 2007b). The association of biofilm formation with bacterial pathogenesis has been well described. In particular, biofilm communities have enhanced resistance to clearance by immune system responses, environmental stresses, and antimicrobial therapeutics (Costerton *et al*., 1999, Xu *et al*., 2000). Biofilms are important during human infection with *Salmonella* and many *E. coli* pathotypes, enhancing resistance and persistence on biological surfaces such as epithelial cells, and abiotic surfaces such as implanted medical devices (Steenackers *et al*., 2012, Sharma *et al*., 2016). EAEC has been associated with exceptional biofilm formation, with studies of clinical EAEC isolates demonstrating significantly greater quantified biofilm density compared to other *E. coli* isolates (Mendez-Arancibia *et al*., 2008, Mohamed *et al*., 2007b, Wakimoto *et al*., 2004). This has led to the theory that mucosal biofilm development contributes to EAEC persistence and symptomatic disease, especially in chronic infections in infants and immunocompromised patients (Nataro and Kaper, 1998, Kaur *et al*., 2010). The role of biofilm expression as a virulence-associated factor for EAEC has a longstanding consensus in the field (Sheikh *et al*., 2001, Weintraub, 2007, Huang *et al*., 2004). This would support the results showing increased biofilm formation in ST40 strains as indicative of increased virulence potential compared to ST31.

In addition to a likely role in pathogenesis, biofilm formation can be important for EAEC persistence in the food chain. For example, biofilm formation and associated virulence factors such as AAF/I promote the colonisation of fresh produce, including the type of fenugreek sprouts contaminated as the source of the O104:H4 outbreak in 2011 (Borgersen *et al*., 2018, Nagy *et al*., 2016). The enhanced resistance characteristics of biofilms are also associated with the difficulty of safely disinfecting fresh produce for many pathogens, including pathogenic *E. coli* (Yaron and Römling, 2014). Therefore, the greater biofilm potential displayed by ST40

strains could correlate with increased exposure to consumers and contribute to higher rates of disease, independent of pathogenesis.

However, biofilm formation is a multi-factorial process, and several virulence factors including the transcriptional activator AggR and adhesins AAFs, ECP, and Air are reported to be involved in EAEC biofilm formation (Garnett *et al*., 2012, Sheikh *et al*., 2001, Sheikh *et al*., 2006, Mohamed *et al*., 2007b). There is potential for functional redundancy, so presence or absence of single factors may not be reliable for prediction of biofilm phenotype. It is also possible that the factors which contribute to biofilm development *in vitro* do not function the same way *in vivo* on biological surfaces (Hebbelstrup Jensen *et al*., 2014). Furthermore, some epidemiological studies have found similar levels of biofilm production in EAEC isolates from cases and controls (Mohamed *et al*., 2007b). Notably, a virulent ST131 EAEC clonal group in Japan caused disease despite displaying low biofilm potential *in vitro* (Imuta *et al*., 2016).

Biofilm formation was analysed using the well-established microtiter plate assay, with crystal violet staining used to quantify bacterial density. Although this is a static model which does not allow the development of mature biofilm properties as effectively as flow cell systems, the microtiter plate assay is suitable for the study of early stages of biofilm formation (O'Toole, 2011, Merritt *et al*., 2005). Additionally, as EAEC develop denser biofilms in this model than other *E. coli* pathotypes, it has been proposed as a simple initial screening tool for EAEC (Wakimoto *et al*., 2004). Prototype 042, included as a positive control, exhibited higher biofilm formation than all 16 clinical isolates. This is unsurprising, as 042 is known to produce dense biofilms and has been used as a model for studies investigating the effect of temperature on EAEC biofilm growth (Hinthong *et al*., 2015). Strain 042 was also recently used to demonstrate that deletion of *aggR* or *aafA* inhibits the establishment of biofilm (Huttener *et al*., 2018). Importantly, the growth conditions used in this assay (37 °C in DMEM) match those identified as optimal for 042 biofilm development in a previous study (Sheikh *et al*., 2001).

4.3.1.2: Colonisation of intestinal epithelial cells

While the relevance of biofilm formation for EAEC virulence is uncertain, adherence to mucosal surfaces is likely involved in development of diarrhoea. Previous studies have used IVOC and enteroid models to determine that the colon is the major colonisation site for enteric infection, as EAEC strains demonstrate lower levels of adherence to small bowel tissue (Knutton *et al*., 1992, Rajan *et al*., 2018, Braga *et al*., 2017). Additionally, in this project greater colonisation of colonic biopsies than small intestinal biopsies was observed for the prototype EAEC strains 042 and 17-2 (Chapter 3.2.3). The colonocyte-like cell line T84 has previously been established as a suitable *in vitro* model of EAEC infection (Nataro *et al*., 1996). Therefore, both human colonic biopsies and T84 cells were used to investigate the adherence of ST40 and ST41 isolates to human intestinal mucosa.

The colonisation of colonic biopsies by the clinical EAEC isolates was analysed semi-quantitatively using SEM. ST40 isolates demonstrated significantly greater IVOC colonisation than those of ST31. All ST40 strains displayed similar or greater median colonisation scores than the positive control (O42), while the ST31 group displayed less consistent adherence including one strain, E104967, which demonstrated no observable adherent bacteria. A corresponding difference in adherence was observed for T84 monolayers, using CFU counting from plated lysates for absolute quantification. There was less variation between strains within STs for this model, and the increased adherence of the ST40 group was more clearly pronounced compared to the IVOC data. This may be attributed to the relative simplicity of a single cell-type culture model opposed to whole ex-vivo tissue, especially considering the likely variability of interactions with biopsies from different donors. Biopsy colonisation may involve more complex interactions, such as additional receptors for adhesin binding and the presence of a mucus layer, with differences in related virulence factors contributing to the greater variety between strains (Fang *et al*., 2013). The semi-quantitative nature of the IVOC analysis compared to the absolute quantification of T84 adherence may also be a factor in this difference. However, a greater ability for colonic epithelial adherence was determined in ST40 versus ST31 strains for both models. This confirms a phenotypic difference between

the STs that is therefore not attributable to an individual model artefact, such as the carcinoma background of T84 cells.

Intestinal epithelial adherence is proposed as a key step in EAEC pathogenesis (Izquierdo *et al*., 2014b, Harrington *et al*., 2006). In addition to a role in subsequent biofilm formation, auto-agglutination of EAEC bacteria has also been suggested as occurring in the lumen and enhancing the initial adhesion to the mucosal surface (Hebbelstrup Jensen *et al*., 2014). Therefore, the aggregation phenotype may also be directly relevant to epithelial colonisation during infection, connecting the biofilm and adherence assay results here presented. There are many potential genetic factors behind these phenotypical differences, which are discussed in further detail in chapter 4.3.2 and 4.3.3. In brief, the AAF adhesins are strongly associated with both adherence and biofilm formation in EAEC pathogenesis (Farfan *et al*., 2008, Jonsson *et al*., 2017a, Shamir *et al*., 2010, Hinthong *et al*., 2015). The virulence factor dispersin is also important, due to its function in masking electrostatic interactions to allow proper dispersal of adhesins such as AAFs during AA (Blanton *et al*., 2018, Velarde *et al*., 2007). The AAFs and dispersin are regulated by the transcriptional activator AggR, which is essential for aggregative adherence in typical EAEC strains (Harrington *et al*., 2006, Hebbelstrup Jensen *et al*., 2014). Other factors associated with the adherence of EAEC include the SPATE Pic, which has mucinolytic and mucus secretion regulatory functions that are suggested to aid mucosal colonisation and subsequent biofilm formation (Harrington *et al*., 2009, Navarro-Garcia *et al*., 2010). Other adhesins, such as the *E. coli* common pilus, are linked to EAEC adherence, especially for the subpopulations which lack a known AAF (Avelino *et al*., 2010). All of the above virulence factors were included in the analysis of ST40 and ST31 genomes for specific EAEC virulence-associated genes, as discussed in Chapter 4.3.2. The broader genotype comparison performed using the VFDB resource (see 4.3.3) also included further genes of interest for biofilm and adherence phenotypes.

A further phenotype associated with the pathogenesis of EAEC is the induction of an immune response. An undergraduate placement student in this research group, Leah Bundy, recently studied the same ST40 and ST31 strains for the effect of incubation with T84 cells on secretion of IL-8. An enzyme-linked immunosorbent

assay approach was used to demonstrate that the ST40 strains induced a significantly greater IL-8 response than ST31 isolates ($P < 0.001$) (**Appendix 1, Figure A1.3**) (Bundy, 2018). Inflammatory cytokines including IL-8 and IL-1 have previously been reported for clinical cases of EAEC infection (Greenberg *et al*., 2002, Steiner *et al*., 1998). As an inflammatory response is proposed as a key factor in EAEC-related diarrhoeal illness, this phenotypic difference between the STs supports the epidemiological association of ST40 with disease and ST31 with carriage (Hebbelstrup Jensen *et al*., 2014). Two bacterial factors linked to immunogenicity of EAEC are AAFs and flagella, both of which differed between ST31 (AAF/I and AAF/III; H18/26/27) and ST40 (AAF/V; H21) strains (Harrington *et al*., 2005, Savar *et al*., 2013, Yanez *et al*., 2016).

Overall, the results of these *in vitro* virulence-associated phenotype assays support an intrinsic phenotypic difference between the ST40 and ST31 groups. ST40 demonstrated a significantly greater adherence to intestinal epithelial cells, increased biofilm-like aggregation, and induction of IL-8 secretion from host cells. Therefore, this phenotype data supports the previous epidemiological findings of greater pathogenicity for ST40 versus ST31 strains, and suggests some of the underlying mechanistic differences (Chattaway *et al*., 2014b). It is often proposed that the current definition of EAEC as a pathotype includes both pathogenic and non-pathogenic subpopulations, contributing to the heterogeneity of virulence (Chattaway *et al*., 2013, Okeke and Nataro, 2001). This comparison of ST40 and ST31 supports the possibility of distinct variation in virulence for different EAEC lineages, although this may be further complicated by the effect of host susceptibility.

4.3.2: Differences in specific putative virulence genes

All sixteen of the ST40 and ST31 strains evaluated in this study have been genome sequenced using Illumina platforms. This sequencing data was available for the investigation of genotypic differences, including the virulence gene profiles of each strain. Initially, this was focused on a specific set of virulence genes associated with aspects of EAEC pathogenesis (*aggR, pic, aap, astA, hlyE, pet, ecpA, aggA, aafA*).

These target genes were described in detail in Chapter 3, where their transcriptional response to intestinal environmental signals was characterised for prototype strains 042 and 17-2. The query sequences for each gene (**Appendix 1.1**) were obtained from the published genome of prototype EAEC strain 042, other than the AAF/I gene *aggA* from strain 17-2 (Chaudhuri *et al*., 2010). In addition to *aafA* (AAF/II) and *aggA* (AAF/I), the major subunit genes for the remaining AAF variants III-V (*agg3A*, *hdaA*, and *aaf5A*) were included (Bernier *et al*., 2002, Jonsson *et al*., 2015, Boisen *et al*., 2008).

Multiple bioinformatics tools were used to determine the virulence gene profiles of each strain from WGS data, which gave largely consistent outputs (**Appendix 1, Figure A1.2**). However, the results obtained using the SRST2 tool were chosen for further analysis. This software is specifically designed to perform alignments from short-read Illumina sequencing data against databases of target genes, such as virulence or antimicrobial resistance genes (Inouye *et al*., 2014). It uses raw sequencing reads, which gives an advantage over the other methods which require genome assemblies. This is due to the quality of an assembly limiting the sensitivity and efficiency of alignments, and therefore SRST2 has previously been demonstrated to outperform assembly-based approaches (Inouye *et al*., 2014, Clausen *et al*., 2016). In addition, the *in silico* PCR method is reliant on the quality of primer design as well as assembly quality.

When the presence and absence of these specific virulence genes was determined for the ST31 and ST40 strains in this study, the most obvious difference was that all ST40 isolates possessed the *astA* gene, while this was completely absent from the ST31 group. This gene encodes the EAST-1 toxin, a heat-stable enterotoxin first identified in prototype EAEC strain 17-2 (Savarino *et al*., 1993). Due to homology of the 38-amino acid EAST-1 peptide with the enterotoxic domain of heat-stable enterotoxin a (STa) of ETEC, it has been proposed to share a similar activity (Menard *et al*., 2004). Stimulation of guanylate cyclase is predicted to disrupt regulation of cGMP and cAMP signalling in host epithelial cells, leading to abnormal fluid secretion in the intestines (Ménard and Dubreuil, 2002). While EAST-1 positive strains have been associated with paediatric disease, intestinal fluid accumulation was not induced by EAST-1 in *in vivo* mouse and gnotobiotic piglet models (Menard

et al., 2004, Vila *et al*., 1998, Ruan *et al*., 2012). While *astA* may theoretically contribute to increased symptomatic infections caused by ST40 strains compared to ST31, it has not been associated with adherence and would not account for the observed phenotypic differences between the STs. The value of *astA* as a marker for EAEC has been previously proposed, due to a prevalence in atypical EAEC lacking other markers such as *aggR* or *aap* (Jenkins *et al*., 2006). However, it should also be considered that *astA* is not restricted to EAEC, having been identified in multiple diarrhoeagenic *E. coli* pathotypes and some *Salmonella* strains, so would need to be used alongside specific EAEC targets (Paiva de Sousa and Dubreuil, 2001, Yatsuyanagi *et al*., 2003).

Another distinction between ST40 and ST31 genotypes was the AAF profile of the strains. ST40 isolates exclusively possessed the most recently identified AAF/V variant. Three of the ST31 strains encoded AAF/III, while it was predicted that the remaining ST31 isolates contained AAF/I. However, the alignment with the query *aggA* sequence was below the default 90% coverage threshold for the SRST2 tool (although greater than 80%), but the remaining *aggB-D* genes of the AAF/I operon were later identified in these ST31 strains, which supports the identification. While the accessory genes are highly conserved, there is precedence for sequence variation in the major subunit for other AAFs, such as an 83-100% identity range in AAF/V from different strains (Jonsson *et al*., 2015). This supports that this alignment in ST31 does represent AAF/I, and not a novel AAF variant. Similar results were obtained in a previous study of clinical EAEC isolates in Japan, where the majority of ST40 strains expressed AAF/V (Imuta *et al*., 2016). Only one ST31 strain was included which was positive for AAF/I, thereby matching the major ST31 subgroup in these results. The AAF variants are widely distributed in EAEC, with no one variant dominating overall in previous studies, although many strains also lack all currently identified AAF genes (Suzart *et al*., 2001, Boisen *et al*., 2008). It remains to be determined if particular AAF variants confer a greater potential to cause disease, and therefore could be used as markers of EAEC virulence. Interestingly, one study has reported that AAF/V causes significantly higher HEp-2 adherence and biofilm formation than AAF/III when expressed by the same laboratory *E. coli* strain, which agrees with the results presented here (Jonsson *et al*., 2015). This could be linked to

mechanistic differences, as AAF/V possesses a specific and conserved mutation that abolishes binding to fibronectin. As AAF/V is still prevalent in clinical isolates, it was suggested that this mutation either enhances binding to alternative receptors or aids evasion of the host immune response (Jonsson *et al*., 2017a). Further study is required to investigate if the AAF type is relevant for the intrinsic virulence potential of EAEC strains. Interestingly, when the sequencing data was used for serotype prediction, the AAF variants aligned with the serotype profiles. In ST31, O130 and O15 were associated with AAF/I and AAF/III, respectively, while AAF/V-positive ST40 strains belonged mostly to serotype O111:H21.

The comparison of virulence gene profiles also revealed that *ecpA* was present in all ST40, but only in 25% of ST31 isolates. ECP is a common adherence factor present in commensal and pathogenic *E. coli* types (Rendón *et al*., 2007). It is widespread in EAEC, although experiments using *ecpA* deletion mutants suggest it is most important for adherence in AAF-negative strains (Avelino *et al*., 2010). The presence of ECP alone did not result in greater adherence within the ST31 group, as the ECP-positive isolate E104931 exhibited relatively poor mucosal colonisation and biofilm formation. While ECP may be a contributory factor in the stronger general adherence displayed by ST40 versus ST31 strains, this data does not suggest the presence of ECP alone increases virulence-associated phenotypes for individual strains. In addition, the high prevalence of ECP across many pathogenic and non-pathogenic *E. coli* strains suggests that it is of limited relevance for identifying specific virulent EAEC risks (Stacy *et al*., 2014, Saldaña *et al*., 2009, López-Banda *et al*., 2014).

4.3.3: Diversity of *E. coli* virulence factors and core genome

To identify additional virulence-associated genes differing between ST31 and ST40 genomes, the Virulence Factors of Bacterial Pathogens database (VFDB) was utilised. This online resource has been collecting functional and genetic data on virulence factors associated with many families of bacterial pathogens since 2004 (Chen *et al*., 2005). In recent years, it has been specifically enhanced for data quality and usability as a tool for bioinformatic mining (Chen *et al*., 2016). At the

time of use, the *Escherichia* database contained 2686 putative virulence genes, of which 302 genes aligned with the sequences of at least one of the 16 EAEC isolates in this study. This data set was manually analysed to identify genes which differed between the STs.

This analysis confirmed the presence of *astA* in ST40 strains only, as well as the three AAF/III-positive ST31 isolates. The accessory genes of the AAF/III operon (*agg3B-D*) were also present in all ST40 isolates, which initially appears contradictory to their predicted AAF/V association. However, when AAF/V was discovered it was determined that while the *agg5A*-encoded subunit had a novel sequence, aside from a conserved signal peptide, the operon uses the same accessory factors as AAF/III (Jonsson *et al*., 2015).

Multiple virulence-associated *Escherichia* genes were found in either ST40 or ST31 exclusively and were subsequently researched for any previously described functions which may contribute to the phenotypic differences between these STs.

4.3.3.1: ST40 specific virulence genes

Amongst the VFDB genes exclusively found in the ST40 strains were several additional adherence systems. These included genes of the *cfa* operon, which encodes the CFA/I fimbriae. This is the prototypical rigid rod-shaped fimbria widely expressed by ETEC, and present in approximately a third of strains in one large dataset (Wolf, 1997). CFA fimbriae are important for ETEC adherence to human intestinal mucosa and erythrocytes (Nataro and Kaper, 1998, Levine, 1981, Sakellaris *et al*., 1999). However, this family is usually strongly associated with the ETEC pathotype only, and to my knowledge have not been previously reported in EAEC (Wurpel *et al*., 2013). The full *stg* operon was also present in the ST40 strains, while ST31 isolates lacked the important usher and chaperone genes *stgC/D* (Forest *et al*., 2007). Stg fimbria are typically associated with the *Salmonella enterica* serovar Typhi, where they are involved in epithelial attachment and survival in macrophages (Forest *et al*., 2007, Berrocal *et al*., 2015). However, an orthologous Stg operon has also been reported in an avian pathogenic *E. coli* (APEC) strain and

UPEC isolates, which when cloned into an *E. coli* K12 strain caused enhanced adherence to human epithelial cells (Lymberopoulos *et al*., 2006). Genes of the *ycb* operon were also present in ST40 strains. This operon is poorly understood as it is not expressed under normal laboratory conditions, but is predicted to encode chaperone-usher fimbriae with adherence properties to abiotic surfaces (Korea *et al*., 2010). A version of the operon has been described in EHEC O157:H7, where it contributed to intestinal epithelial cell adherence. However, that study identified the *ycbQ* gene as the key structural fimbrial subunit, which is absent in the ST40 strains, therefore the operon may not be functional in ST40 EAEC strains (Samadder *et al*., 2009).

A further virulence gene which may contribute to ST40 adherence is *ehaA*. This encodes a type V secretion system autotransporter associated with outer membrane expression in Gram-negative bacteria (Henderson *et al*., 2004). EhaA was identified as prevalent in EHEC O157:H7 and other serotypes using conserved autotransporter motifs. Overexpression of EhaA in *E. coli* K12 resulted in increased bacterial aggregation, enhanced biofilm formation, and adhesion to bovine rectal epithelial cells (Wells *et al*., 2008, Tseng *et al*., 2014). However, deletion of *ehaA* from wild-type EHEC did not decrease biofilm formation, so the biological importance of this putative adhesin remains to be determined (Wells *et al*., 2008).

Additional ST40 genes included a *gsp* operon. The *gsp* genes are part of a type II secretion pathway first identified as a cryptic operon in *E. coli* K12 (Francetic and Pugsley, 1996). These genes were also found in ST31 strains of the O130 serotype. Type II secretion has been linked to many functions in Gram-negative bacteria, including virulence protein export, but the activity of the *gsp* genes is unclear in this context (Cianciotto, 2005). A *hcp* gene was also identified in ST40 genomes, however the designation of this gene was missing in the VFDB database. This could refer to the gene encoding the hybrid cluster protein, a hydroxylamine reductase involved in oxidative and nitrosative stress responses, or the hemolysin coregulated protein, a putative type VI secretion system effector first associated with *Vibrio cholerae* which has a homologue identified in EAEC 042 (Wolfe *et al*., 2002, Williams *et al*., 1996, Dudley *et al*., 2006b). Alignments of the ST40 WGS data against query gene sequences for these targets were inconclusive.

The VFDB resource allowed the identification of many genes found only in the ST40 strains used in this study. Many of these have functions relevant to the investigated virulence-associated phenotypes, including adherence fimbriae which could contribute to the greater colonisation potential of the ST40 isolates. However, it is important to note that while these genes were found in the genome sequences, their expression may be restricted to specific environments, and some of the targets may be part of incomplete operons and thus be inactive systems in these isolates.

4.3.3.2: ST31 specific virulence genes

The virulence-associated gene profiles from the VFDB alignment were also analysed for genes found only in ST31 strains. One difference was the presence of *ehaB* in all the ST31 isolates, whilst the ST40 genomes contained *ehaA* as previously discussed. Both EhaA and EhaB are autotransporter proteins associated with adhesion and biofilm formation, with similarly high prevalence in EHEC (Wells *et al*., 2008, Farfan and Torres, 2012). Conserved paralogues of *ehaB* have been identified across many *E. coli* pathotypes, including EAEC. However, unlike *ehaA*, the introduction of *ehaB* to a laboratory *E. coli* strain did not promote adherence to Caco-2 and bovine lung and rectal epithelial cells (Wells *et al*., 2009). The same study showed that EhaB binds the ECM proteins collagen I and laminin. While both EhaA and EhaB are common in pathogenic *E. coli*, differences in their activity or specificity could potentially contribute to phenotypic differences between ST31 and ST40 alongside other adherence factors such as AAF variant.

An EAEC-specific factor associated with ST31 strains was Air. The *air* gene, encoding the enteroaggregative immunoglobulin-repeat protein, was previously termed *eaeX* as an unknown outer membrane protein gene (Ren *et al*., 2004). Air is a large surface adhesin with repeated bacterial immunoglobulin domains and secreted via a T3SS. It shares homology with invasin from *Yersinia* and intimin from EPEC. When *air* was deleted from 042, the mutant strain demonstrated reduced biofilm formation and impaired HEp-2 cell adherence (Sheikh *et al*., 2006). As such, the presence of Air in ST31 but not ST40 strains is surprising, as the ST31 was associated

with lower biofilm formation and cell adhesion in this study. However, the role of Air *in vivo* remains to be established as, unlike other established EAEC genes *aap* and *pic*, deletion of *air* did not affect EAEC colonisation of mice (Harrington *et al*., 2009).

As well as adherence systems, genes connected to nutrient acquisition were identified in the ST31 genome sequences. The *sitABCD* operon is an iron/manganese transport system and a member of the periplasmic binding protein-dependent ATP-binding-cassette (ABC) family of metal transporters (Sabri *et al*., 2008). First identified in *Salmonella enterica* Typhimurium, Sit homologues are found in many enterobacteria including pathogenic *E. coli* (Zhou *et al*., 1999). As well as metal ion transport, the *sit* operon has also been associated with resistance to oxidative stress in APEC (Sabri *et al*., 2006). The loss of the *sit* operon has been linked to attenuated virulence in *in vivo* infection models of *Salmonella* Typhimurium and APEC but has not been studied in EAEC (Sabri *et al*., 2008, Janakiraman and Slauch, 2000). The ST31 strains also encoded the *E. coli chu* heme utilisation operon. This includes a heme outer membrane receptor (*chuA*), a periplasmic chaperone and an ABC-transporter system (*chuTUV*) and a heme oxygenase (*chuS*) releasing free iron for bacterial use (Suits *et al*., 2005). This operon has been reported in EHEC O157:H7 and UPEC strains (Porcheron *et al*., 2013). The Chu system is a homologue of the Shu heme uptake transporter system described in *Shigella dysenteriae* (Wyckoff *et al*., 2005). However, as EAEC is typically a non-invasive pathogen it is unclear if heme exploitation significantly contributes to virulence. The Sit and Chu systems may contribute to ST31 growth in the intestinal environment, but their importance for virulence in EAEC is less clear compared to other enteric pathogens (Porcheron *et al*., 2013).

Other ST31 specific genes have poorly characterised functions in relation to potential EAEC virulence determination. The *aslA* gene encodes a periplasmic sulfatase which has been associated with invasion of brain microvascular endothelial cells. This has been studied in relation to the penetration of the blood-brain barrier by neonatal meningitis *E. coli* (Hoffman *et al*., 2000). Previously described in DEC including EHEC O157 and EAEC 042, *pkgA* encodes a phosphorylase-kinase-like glucoamylase which is associated with the *E. coli* T3SS-2,

and proposed as a potential effector protein (Zhou *et al*., 2014). Due to homology with eukaryotic phosphorylase kinases, PkgA has been suggested to act by disrupting glycogen metabolism in host cells (Pallen, 2003). Transposon insertion mutagenesis identified a PkgA homologue in EHEC O26:H$^-$ which promoted intestinal colonisation in calves (van Diemen *et al*., 2005). Further study of PkgA function is needed to understand if it may act as an EAEC virulence factor. There is also uncertainty to the relevance of *kps* operon genes in ST31. This operon is responsible for the production of the polysialic acid capsule common in NMEC and some UPEC strains (King *et al*., 2007, Johnson *et al*., 2008). All ST31 isolates possessed *kpsD*, encoding a periplasmic protein involved in polysialic acid membrane transport (Wunder *et al*., 1994). However, only three of the eight strains contained *kpsM*, and none possessed *kpsT*, with the rest of the operon not currently included in the VFDB. This suggests that the Kps system is incomplete and likely inactive in ST31, especially as this capsule type is typically only associated with extraintestinal pathogenic *E. coli* and not EAEC (Park *et al*., 2009, Johnson and Russo, 2002). Finally, the EC042_4532 gene is part of a large uncharacterised locus identified in 042 with predicted homology to type VI secretion systems, but the function is otherwise unknown (Chaudhuri *et al*., 2010).

A more specific difference was the presence of the complete *hlyABCD* operon in O15:H18 ST31 strains only. This operon encodes the alpha-haemolysin toxin and associated activation and export machinery (Herlax *et al*., 2010). Although typically associated with extraintestinal infections, alpha-haemolysin is expressed by some EAEC strains including 17-2 and induces macrophage cell death *in vitro* (Fernandez-Prada *et al*., 1998). These genes are seemingly unrelated to the virulence phenotypes tested in this study, with the O15:H18 group correspondingly demonstrating no phenotypical differences to the other ST31 strains, but it remains possible that alpha-haemolysin could contribute to EAEC virulence *in vivo*. The O15:H18 serotype also contained the *pap* operon encoding the P fimbriae mostly associated with UPEC. All the genes required for fimbrial expression, *papA-K*, were present (Kuehn *et al*., 1992). P fimbriae have been associated with urinary tract infection in a human volunteer study, but also enhance intestinal colonisation and persistence for commensal *E. coli* (Wullt *et al*., 2000, Herías *et al*., 1995, Adlerberth

et al., 1998). The O15:H18 ST31 strains also contain *papX*, which is associated with repressing flagellar motility during expression of fimbriae (Simms and Mobley, 2008). If the *pap* operon is expressed it could contribute to intestinal epithelial adherence, however the O15:H18 ST31 strains did not significantly differ from the other ST31 isolates during *in vitro* adherence and colonisation experiments in this project.

4.3.3.3: Core-genome phylogeny

A core-genome phylogenetic analysis was also performed, using alignment against 042 as a reference genome (Chaudhuri *et al*., 2010). This revealed that the ST40 and ST31 strains are distinct lineages with a high degree of separation in core-genome single nucleotide polymorphisms. The ST40 strains clustered together on one branch of the phylogenetic tree, supporting the serotype data which suggested that the 8 strains of this ST represented a single related group. The ST31 strains were more closely aligned to the 042 reference genome, which has been previously identified as ST31 (Okeke *et al*., 2010, Chattaway *et al*., 2014b). The ST31 strains were separated into branches matching the two identified serotype groups (O130 and O15), with E107526 (O?:H27) demonstrating further separation corresponding with the unknown O-antigen.

The use of core-gene SNPs has been reported as an effective approach for inferring phylogeny due to the highly clonal nature of *E. coli*. It has been shown to separate lineages from a mixed *E. coli* population, accurately fitting with MLST assignment (Kaas *et al*., 2012). It also corresponds well with the results of traditional phylotyping as performed by multiplex PCR (Gordon *et al*., 2008). Interestingly, analysis of variation in core *E.* coli genes has suggested that many of the genes used in MLST schemes have relatively low diversity, which may limit the resolution for distinguishing strains (Kaas *et al*., 2012). However, it is unlikely that MLST schemes will be modified, due to the development of WGS-based typing techniques with greater resolution power and cost-effectiveness (Larsen *et al*., 2012)

4.3.4: Whole-genome sequencing and *in silico* typing

The clinical EAEC isolates in this study had previously been sequence typed using PCR-based MLST (Chattaway *et al*., 2014b). With the genome sequences available, it was possible to reconfirm the assignment of STs for each strain by using *in silico* analysis, by searching the sequence data for the MLST alleles as opposed to PCR amplification. This was performed twice using SRST2 and Galaxy platform tools, with both giving matching results to the pre-existing ST assignments. This demonstrates the effectiveness of *in silico* MLST in combination with WGS of clinical isolates, which with the ever-decreasing expense of sequencing is reported as more efficient and cost-effective than traditional PCR MLST (Larsen *et al*., 2012). This has evolved further in the last few years, as core genome MLST has been developed. This exploits the scale of available data from WGS by comparing genome-wide gene-by-gene alleles from typically 1500 to 4000 genes conserved within a species, thereby allowing far greater resolution than the 5 to 7 alleles used for conventional MLST. However, the standard MLST schemes are predicted to remain useful for the foreseeable future, due to their established widespread application in modern molecular epidemiology systems (Kimura, 2018).

Serotyping is a further surveillance technique which can benefit from WGS. By using *in silico* tools to identify specific O-antigen and flagellin genes from sequence data, serotypes can be determined without performing labour-intensive conventional antisera testing. Serotyping from genetic data circumvents traditional limitations such as bacterial autoagglutination or lack of antigen expression *in vitro* (Robins-Browne *et al*., 2016). Traditional serotyping can also be fallible due to sera quality variation and the risk of cross-reactivity during testing (Ballmer *et al*., 2007). Here, SRST2 was used to perform serotype prediction from sequence reads for the 16 clinical EAEC isolates, an approach which has been previously successful for studying serotype distribution between pathotypes including EPEC, ETEC, and UPEC (Ingle *et al*., 2016). As previously discussed, the results identified two separate serotype groups for ST31 and one related group for ST40. Interestingly, the ST31 serotypes correlated with differences in AAF type, although they did not exhibit differences in *in vitro* adherence phenotypes. Overall, the *in silico* serotyping provided greater detail than previous traditional serotyping performed for these

isolates, and identified H-antigens for all strains and O-antigens for all but two. Unexpectedly, the predicted H-antigens did not always match the previous results obtained using antisera, although it remains to be determined if this discrepancy is due to limitations of the *in silico* analysis or the previous serotyping. This data is a small-scale example of the usefulness of *in silico* serotyping in combination with WGS as a pathogen surveillance tool, which is predicted to increasingly replace traditional serotyping (Robins-Browne *et al*., 2016).

Serotyping can be useful for predicting pathotypes due to some specific associations, such as the predominance of O157:H7 for disease cases of EHEC, as well as established applications for public health surveillance and outbreak detection (Fratamico *et al*., 2016). However, EAEC strains are highly heterogenous in serotype. For example, the IID1 study found at least 40 O-serogroups for identified EAEC isolates (Okeke and Nataro, 2001). The serotypes of the clinical isolates in this study have been previously reported in infection. For example, O111 (identified for ST40 strains in this study) are a serogroup associated with paediatric diarrhoea, including EHEC, EPEC, and EAEC. Within this group, H21 is one of the most common accompanying flagellin antigens in disease cases (Alikhani *et al*., 2011). The serotypes here identified for ST40 strains have also been previously associated with AAF/V, with the *aaf5A* gene sequence used for bioinformatics alignment in this study acquired from an O127:H21 strain (GenBank: AB571097.1), and a reported O111:H21 *aaf5A* gene (GenBank: AB513347.1) has a high sequence identity. The virulence risk of this serotype is further highlighted by a UK household outbreak of O111:H21 EAEC in 2012. This strain was identified as ST40 and positive for AAF/V, but had also acquired a gene for Shiga toxin (Stx2c) (Dallman *et al*., 2012). This raised obvious parallels to the 2011 O104:H4 *E. coli* outbreak, and the danger of emerging EAEC/STEC hybrid strains (Navarro-Garcia, 2014). The serotypes predicted for the ST31 strains in this project were also reported in previous EAEC infection studies. For example, O15:H18 was the most prevalent EAEC serotype in children of less than 5 years of age, which were hospitalized for acute gastroenteritis in Israel (Tobias *et al*., 2015). In addition, EAEC O15:H18 strains have been previously identified containing alpha-haemolysin genes, a combination also identified in this study (Beutin *et al*., 2005).

Altogether, this work used *in silico* typing tools with WGS data to confirm MLST and serotyping for these clinical EAEC isolates. This demonstrated both the efficiency and advantages of this approach versus traditional laboratory typing. On-going advances in typing and surveillance are likely to significantly contribute to improving the detection of virulent EAEC subgroups within this heterogenous pathotype (Robins-Browne *et al*., 2016).

4.3.5: Host Susceptibility

In addition to comparing virulence-associated phenotypes and genotypes between STs, the data was also analysed to determine if strains isolated from disease cases had a more virulent phenotype than those from asymptomatic carriage controls, within the same ST. It was found that case isolates did not demonstrate stronger aggregation or colonisation than control isolates for any of the investigated phenotypes. In fact, the control strains exhibited higher biopsy colonisation than case isolates for ST31 ($P < 0.01$). However, the limitations of a small sample size should also be considered, especially as only two control strains were available for ST40.

These results suggest the importance of host susceptibility for symptomatic EAEC infection. The heterogeneity of EAEC for causing disease, as well as variability of symptoms, has been well described (Okeke, 2009, Estrada-Garcia and Navarro-Garcia, 2012). While this may be associated with the genetic diversity of the pathotype, the high occurrence of asymptomatic carriage is also likely to be related to individual susceptibility to pathogenesis (Estrada-Garcia *et al*., 2014). This further complicates the study of EAEC overall, and any attempts to distinguish between the proposed pathogenic and non-pathogenic subtypes (Chattaway *et al*., 2013).

Some possible mechanisms for host susceptibility to enteric pathogens including EAEC have been identified (Flores and Okhuysen, 2009). Often these consist of SNPs in host immune system components (Estrada-Garcia and Navarro-Garcia, 2012). For example, CD14 is a component of the Toll-like receptor 4 complex with recognition for bacterial LPS and involved in the innate immune response in the intestinal

mucosa. Specific polymorphisms in the CD14 gene were found to significantly influence the susceptibility of adults to traveller's diarrhoea, with EAEC and ETEC identified in patient stool (Mohamed *et al*., 2011). A similar effect has been reported for lactoferrin, an iron-binding protein which can inhibit growth of enteric pathogens and disrupt surface-bound virulence factors including AAFs to reduce EAEC adherence and biofilm formation *in vitro* (Ochoa *et al*., 2006, Ochoa and Cleary, 2009). A novel SNP in the lactoferrin gene was associated with significantly increased risk of traveller's diarrhoea (Mohamed *et al*., 2007a). An additional study of traveller's diarrhoea identified a SNP in osteoprotegerin, an immunoregulatory protein within the tumour necrosis factor receptor superfamily. Osteoprotegerin was produced by T84 cells in response to diarrhoeagenic *E. coli* infection, and the SNP increased the risk of diarrhoea in an adult traveller cohort (Mohamed *et al*., 2009). EAEC infection induces production of the cytokine interleukin-8 (IL-8) in *in vitro* models and faecal samples (Steiner *et al*., 1998, Greenberg *et al*., 2002). A polymorphism in the IL-8 promoter has been shown to increase both the chance of EAEC-associated diarrhoea and subsequent faecal IL-8 concentration (Jiang *et al*., 2003). However, these studies have focused on otherwise healthy adult travellers, and there remains uncertainty whether single gene SNPs contribute significantly to disease susceptibility overall (Estrada-Garcia and Navarro-Garcia, 2012).

Beyond host genetics, other factors may influence the development of symptomatic disease in individual EAEC infections. The role of specific populations of the intestinal microbiota in the prevention of infectious disease is the topic of extensive research (McKenney and Pamer, 2015). Specific interactions have been reported in other *E. coli* pathotypes, including *Lactobacillus reuteri* inhibition of EPEC adherence, and mixed microbial communities associated with preventing EHEC attaching/effacing lesions (Walsham *et al*., 2016, Liu *et al*., 2015). However, while similar protective interactions are likely during EAEC infection, they have yet to be studied in detail.

A more established factor in the development of EAEC-associated disease is a compromised immune system. This has been well-studied in the context of HIV-positive populations, which alongside malnourished children was one of the initial patient groups to be significantly associated with EAEC as an enteric pathogen

(Huang *et al*., 2006b). Many studies have found HIV-positive individuals to be at increased risk of EAEC-associated diarrhoea, with symptomatic severity also linked to progression of HIV disease (Mossoro *et al*., 2002, Samie *et al*., 2007, Wanke *et al*., 1998). Diarrhoeagenic *E. coli* are also a risk for immunosuppressed patients such as those undergoing cancer therapies, with EAEC previously associated with acute and chronic infections in this population (Chao *et al*., 2017)

Another predisposing factor may be co-infection with other enteropathogens. The large UK IID1 and IID2 studies found an association of EAEC infection with the presence of other enteric bacterial pathogens such as *Campylobacter* and norovirus (Chattaway *et al*., 2013). A paediatric gastroenteritis study in Israel also reported especially severe diarrhoea for co-infections of EAEC and norovirus (Tobias *et al*., 2015). Data from a multisite birth cohort study (Malnutrition and Enteric Diseases (MAL-ED) study) across sites in Asia, Africa, and Latin America found no negative effect of subclinical EAEC infection alone, but co-infection with other enteropathogens caused significant infant growth deficiencies (Lima *et al*., 2017b). It has been proposed that while true pathogenic EAEC variants can cause illness in isolation, other less virulent EAEC subtypes may affect disease severity only in combination with other pathogens (Chattaway *et al*., 2013). This would be a confounding factor contributing to the heterogeneity of data on EAEC virulence.

As case isolates did not outperform carriage isolates for virulence-associated phenotypes in this study, the data supports the theory that host susceptibility is a major factor for the development of symptomatic disease following EAEC colonisation. However, this does not diminish the significance of the difference in phenotypes observed between ST40 and ST31. Conversely, the heterogeneity of individual susceptibility to EAEC infection enhances the value of characterising subtypes with an intrinsic increased disease risk overall, as well as the study of genotypes for potential molecular markers for improving identification of virulent EAEC.

4.3.6: Implications for identifying EAEC virulence risk

While the primary aims of this study focused on the comparison of two specific STs of EAEC clinical isolates, the techniques applied in this investigation provide some insight into both the challenges and opportunities for ongoing EAEC research in general. The heterogeneity of the pathotype is well established, which is a challenge for the identification of pathogenic strains against a background of frequent asymptomatic EAEC carriage (Hebbelstrup Jensen *et al*., 2014).

The original classification of EAEC is based on its aggregative adherence phenotype on HEp-2 cells, which still represents the gold-standard diagnostic tool (Kaur *et al*., 2010). This diagnostic technique requires time-consuming cell culture and specialised facilities and expertise, practically restricting the use of the assay to a limited number of sufficiently equipped reference laboratories (Weintraub, 2007). The basis on phenotypical observations is also subjective and may introduce inconsistency between different practitioners. Despite suggestions for improving the ease of the HEp-2 assay, such as the use of storable pre-prepared formalin-fixed HEp-2 monolayers, many studies instead rely on the use of molecular techniques (Miqdady *et al*., 2002). One of the most common is the CVD432 probe, which targets the dispersin transporter operon *aat* (Baudry *et al*., 1990). Alternatively, multiplex PCR approaches are used to identify the plasmid-borne genes *aggR* and *aap*, or the chromosomal *aaiC* gene (Croxen *et al*., 2013, Cerna *et al*., 2003).

The majority of ST40 and ST31 strains used in this study were identified as EAEC using the CVD432 probe as part of the IID1 and IID2 studies (Chattaway *et al*., 2013). Therefore, the HEp-2 adherence assay results are novel for these isolates. The HEp-2 adherence assay is considered insufficient for distinguishing between pathogenic and non-pathogenic EAEC (Estrada-Garcia and Navarro-Garcia, 2012). However, differences were observed in the adherence phenotypes of the ST40 and ST31 groups. HEp-2 cells are not an intestinal epithelial cell type, so colonocyte-like T84 cells and sigmoid colonic biopsies were used as physiologically relevant models (Nataro *et al*., 1996). The data presented here demonstrated that adherence to T84 cells consistently distinguished between ST40 and ST31 strains. This suggests that intestinal epithelial cell culture assays may have value for identifying EAEC

subgroups with greater adherence as a risk indicator of potential virulence. However, this has the same practical limitations as the HEp-2 assay, requiring sufficient cell culture facilities and expertise.

AA has since been shown to be a multi-factorial phenotype, contributing to the genetic variety of the pathotype (Moreira *et al*., 2003). As such, the use of probes against single genes which are frequent in typical EAEC overall, will inevitably identify strains with heterogenous virulence phenotypes. Attempts to determine genes which are a marker for EAEC virulence by direct comparison of genomes for case and control isolates have so far proven unsuccessful. Therefore, this project has used the alternative approach of investigating phenotypic differences between groups of strains with epidemiological associations with disease or carriage, in combination with analysing virulence genotypes.

4.4: Summary

In this part of the study, it is demonstrated that ST40 strains, previously associated with a higher epidemiological rate of disease, display stronger virulence-associated phenotypes than ST31 strains, associated with asymptomatic carriage. These phenotypes encompassed elements of intestinal epithelial adherence and biofilm formation, which are considered key steps in EAEC pathogenesis (Kong *et al*., 2015). While the evidence supports an intrinsic difference in virulence between the two STs, clinical isolates from disease cases did not outperform carriage control isolates. This potentially indicates the importance of host susceptibility for disease outcome, although a larger sample size is needed to confirm this conclusion.

Bioinformatic analysis allowed confirmation and further clarification of the sequence typing, serotyping, and core-genome phylogeny of these 16 EAEC isolates. This demonstrates the power of *in silico* tools in combination with WGS as an alternative to traditional lab-based typing approaches (Jenkins, 2015). Genotype comparisons were also performed which have identified a number of virulence genes which differ between ST40 and ST31. These include adhesins (such as AAFs and ECP) which may contribute to the different colonisation phenotypes observed.

Collaborators (Lisa Crossman and John Wain, UEA) are currently expanding this genotypic comparison to all 51 ST40 and ST31 isolates available through PHE to determine if the differences in genes identified here are maintained in a larger collection of strains. Future study is also needed to characterise the importance of these targets, such as applying molecular biology to investigate the effect of introducing or deleting the genes into strains on *in vitro* virulence phenotypes.

It is increasingly recognised that EAEC pathogenicity cannot be predicted by any single factor, and that virulence is dependent on a range of contributory genes (Estrada-Garcia *et al*., 2014). There also remains the risk of pathotype crossover events, such as the gain of EHEC factors including Shiga toxin in the deadly O104:H4 outbreak, despite the EAEC background strain demonstrating relatively average virulence phenotypes (Haarmann *et al*., 2018). The advances in the use of WGS for epidemiology are hoped to allow improved definition of pathogenic subpopulations within the heterogenous EAEC pathotype (Robins-Browne *et al*., 2016).

The AA exhibited by EAEC is multi-factorial and associated with many putative virulence genes. These findings have demonstrated that a phenotypical analysis identifies functional differences between EAEC isolates. This allows for future targeted screening of candidate genes which may be responsible for the phenotypic results. The factors identified in this study may therefore be relevant for identifying EAEC virulence markers in the future.

CHAPTER FIVE

CONCLUSIONS

The aim of this PhD project was to investigate the enteric pathogenesis of EAEC. The first part of the study involved the characterisation of interactions between prototype EAEC strains and intestinal epithelial models, and determination of the effect of host cell adherence and oxygen concentration on virulence gene expression. The second part of this project focused on determining functional phenotypes and corresponding genotypes in EAEC sequence types associated with disease or carriage in order to identify markers of EAEC pathogenicity. This chapter summarises the major findings of this study and their impact, as well as proposes future research opportunities arising from this work.

5.1: Prototype EAEC colonisation and virulence gene regulation

The relevant site of intestinal EAEC infection is an important consideration for studies of host-pathogen interactions. In the first part of this project, *in vitro* and *ex vivo* intestinal epithelial models were evaluated for suitability to investigate EAEC virulence. This has demonstrated that two prototype EAEC strains, 042 and 17-2, adhere with similar efficiency to the human intestinal epithelial cell lines T84 and Caco-2, used as models for colonocytes and small intestinal enterocytes, respectively. This contrasts with a previous study which concluded only T84 cells were suitable for colonisation by strain 042 (Nataro *et al*., 1996), and is also the first reported example of strain 17-2 adhering to T84 cells to my knowledge.

The use of the IVOC model with biopsies from different parts of the human intestine revealed a tissue tropism for EAEC colonisation of colonic tissue agreeing with previous IVOC studies. Reports of EAEC colonisation of small intestinal biopsies are less consistent in the literature, and data herein supports the suggestion that EAEC can colonise the colonic epithelium more effectively (Andrade *et al*., 2011, Nataro *et al*., 1996, Knutton *et al*., 1992, Hicks *et al*., 1996). The predominant association of EAEC colonisation with colonic tissue in the physiologically relevant IVOC model allowed an informed selection of colonocyte-like T84 cells for experiments in the VDC system.

This study represents the first use of the VDC model for EAEC research, and has presented evidence of separate regulatory effects of epithelial cell contact and oxygen concentration on the expression of virulence genes. It is proposed that the increasing oxygen diffusion gradient in proximity to the mucosal surface induces the expression of virulence factors enhancing EAEC adherence to epithelial cells, such as the adhesins AAF/II and the *E. coli* common pilus, and dispersin. Physical contact with the colonic epithelium then stimulates increased expression of additional virulence genes. As well as adherence-related genes, this includes genes involved in colony spatial organisation (*aap*), manipulation of the mucus layer (*pic*), and secreted toxins (*astA*, *hlyE*, *pet*). Importantly, the data suggests that the latter response is independent of the transcriptional activator AggR, often considered a key virulence regulator in EAEC.

While transcriptional changes were significant for many virulence genes, increased protein expression was only demonstrated for dispersin by 042 in response to T84 cell contact. Therefore, future work could determine the effect of these signals on translation of other virulence factors and investigate if further regulation affects any changes at the protein level. Oxygen concentration only influenced gene expression in strain 042 and not 17-2, suggesting strain specificity or differences in transcription kinetics. It would be interesting to determine the effect of oxygen availability on additional EAEC strains, such as representative isolates from the ST40 and ST31 groups studied in the other part of this project.

Follow-up studies could aim to identify the mechanisms involved in the transcriptional changes reported in this work. As it was shown that the increased gene expression in adherent bacteria is dependent on physical contact, the T84 cell receptors responsible for adherence need to be determined. Epithelial surface factors associated with EAEC binding include cytokeratin-8 and MUC1 (Izquierdo *et al*., 2014b, Boll *et al*., 2017). Candidate epithelial cell receptors for EAEC should be investigated in future work for a role in regulation of virulence factor expression, for example by gene silencing by RNA interference or blocking of receptors with specific antibodies. Furthermore, physical signals such as fluid shear forces have been associated with mechanosensation by EHEC and require future study in EAEC (Alsharif *et al*., 2015). In addition, further research is needed to characterise the

molecular mechanisms involved in the response of EAEC to oxygen and epithelial adherence. According to results presented in this study, the AggR transcriptional activator is implicated in oxygen-dependent virulence gene expression, and has been previously associated with the ArcA/FNR redox-sensing transcription factors (Cameron *et al*., 2013). Future work could experimentally investigate the potential role of these factors by determining the effect of molecular biology interventions on the induction of EAEC virulence genes in response to oxygen or T84 adherence.

The VDC system allowed for the investigation of EAEC responses to oxygen concentrations, which would not be possible with traditional cell culture models. However, this model does not address other physiologically relevant conditions in the human gut such as the presence of bile salts and digestive enzymes, which were not tolerated by the intestinal epithelial cell lines used. One limitation may be the lack of a protective mucus layer, which could be addressed by developing a mixed culture of T84 cells with a higher mucin-secreting type such as the goblet cell-like LS174T cell line. Future studies of EAEC virulence genes associated with pathogenesis would also benefit from greater use of *in vivo* experiments, particularly for elucidating functions related to long-term effects such as malnutrition or sustained inflammatory responses. However, this is currently limited by a lack of suitable animal models which exhibit symptomatic enteric disease in response to EAEC infection.

5.2: Association of EAEC phenotype and genotype with epidemiological disease or carriage

EAEC is a genetically heterogenous pathotype, likely related to the ability of multiple factors to confer the defining aggregative adherence phenotype. It is also recognised that EAEC contains diverse strains including pathogenic and non-pathogenic groups (Chattaway *et al*., 2013). Previous studies demonstrated only limited success in identifying specific virulence genes significantly associated with EAEC pathogenicity, and their findings were often inconsistent. It is likely that EAEC pathogenesis relies on combinations of biological functions, which can each be

affected by different factors, thereby limiting the use of single genes as absolute virulence markers (Estrada-Garcia *et al*., 2014). Therefore, the rationale for the second part of the study was to evaluate EAEC strains for *in vitro* phenotypes relevant to pathogenesis followed by analysis of WGS data for related genotypic differences.

This work built on a previous MLST study of clinical EAEC isolates, which identified an epidemiological association of ST40 strains with higher rates of disease and ST31 strains with asymptomatic carriage (Chattaway *et al*., 2014b). In this project, it has been shown that ST40 strains exhibit significantly greater adherence to T84 intestinal epithelial cells, colonisation of colonic biopsies, and formation of biofilm, in comparison to ST31 strains. Additionally, a final year undergraduate student in this group demonstrated that ST40 isolates induced higher levels of IL-8 secretion from T84 cells. Therefore, the epidemiological association of the ST40 group with disease correlates with the experimental results for phenotypes related to EAEC pathogenesis, with T84 adherence exhibiting the most distinctive difference between STs.

While screening for virulence-associated phenotypes could be effective for predicting the pathogenic risk of EAEC strains, such assays are impractical for rapid and cost-effective public health applications. However, the characterisation of virulence gene profiles associated with the phenotypic differences may identify markers that can be used for screening by PCR or genome sequencing approaches. Analysis of WGS data for the ST40 and ST31 strains used in this study determined genotypic differences with relevance for EAEC biological functions. These include adhesins associated with colonisation phenotypes, and bacterial surface factors implicated in induction of host inflammatory responses.

A limitation of this study was the relatively small sample size (8 strains from each ST). However, a current collaboration with Dr Lisa Crossman, UEA, is expanding the genotypic comparison to include all available EAEC ST40 and ST31 genomes sequenced at UEA and PHE (currently 51 total isolates)

While putative virulence genes differing between ST40 and ST31 were identified, there remains uncertainty regarding their expression and importance for *in vitro*

phenotypes. Therefore, future work should also focus on determining the functional influence of these virulence factors. Molecular biology studies could be performed to introduce, overexpress, or delete target genes in EAEC strains and determine if virulence-associated phenotypes are altered. It would also be interesting to investigate if specific interventions can abolish the significant differences between ST40 and ST31 isolates, such as changing the AAF variants.

Finally, this work applied bioinformatics tools for the *in silico* typing of EAEC strains using WGS data. The results of *in silico* MLST analysis were consistent with the previous ST assignments performed using PCR. The use of genome sequences to predict serotypes revealed greater detail than was available from laboratory antigen testing. However, there were some inconsistencies in assigned antigens, so further research is needed into the relative accuracy of *in silico* serotyping. It is likely that further advances in sequencing and bioinformatic technologies will continue to improve the options available for characterising populations of pathogenic bacteria. This is predicted to also affect the future definition of *E. coli* pathotypes, which is of particular relevance for a group as heterogenous as EAEC (Robins-Browne *et al*., 2016).

5.3: Summary

In summary, work from this PhD project has discovered novel regulation of EAEC virulence factors implicated in pathogenesis by signals associated with the human gastrointestinal environment. Oxygen concentration and physical contact with host epithelial cells both modulate the expression of virulence genes and might serve to restrict transcription to intestinal niches advantageous for colonisation. This study has also demonstrated the potential of screening clinical EAEC isolates for virulence-associated phenotypes. ST40 strains, epidemiologically associated with a higher rate of disease, were compared to ST31 strains, associated with asymptomatic carriage. The ST40 isolates exhibited greater adherence to *in vitro* and *ex vivo* colonic epithelium models, increased formation of biofilm, and higher induction of pro-inflammatory IL-8 secretion from host cells. Also presented are

genotypic differences between the STs related to pathogenic functions, including adhesins, toxins, and flagella. The findings of this project contribute to the understanding of EAEC pathogenesis in the human host and provide new targets for the future development of virulence markers for high-risk EAEC strains.

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APPENDIX ONE

SUPPLEMENTARY DATA

Collaborative work:

Interleukin-8 ELISA experiments performed by Leah Bundy and Stephanie Schüller

Appendix 1.1: Virulence gene query sequences

>*aggR* Sequence ID: FN554767.1
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>*ecpA* FN554766.1

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Appendix 1.2: Supplementary figures

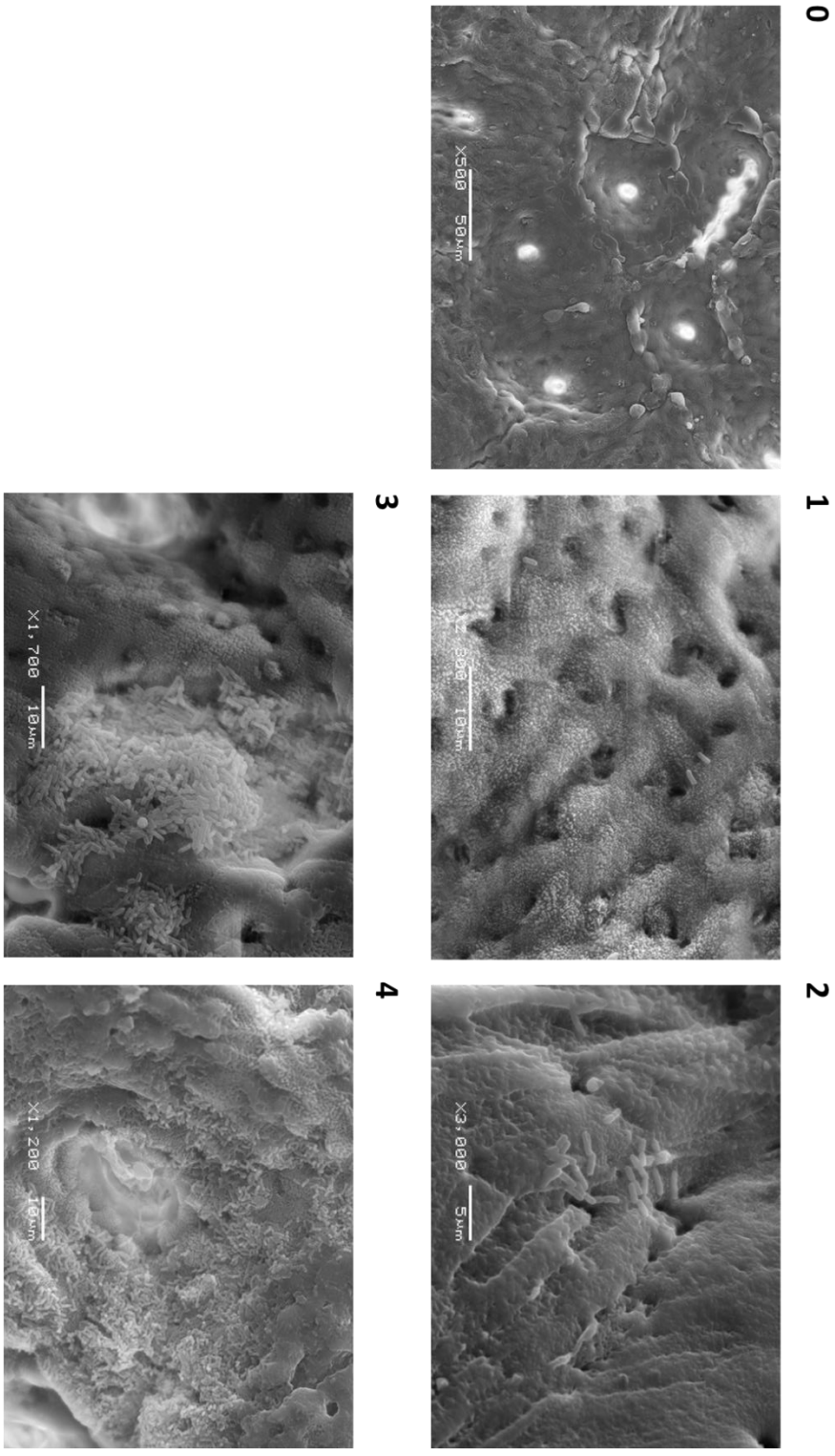
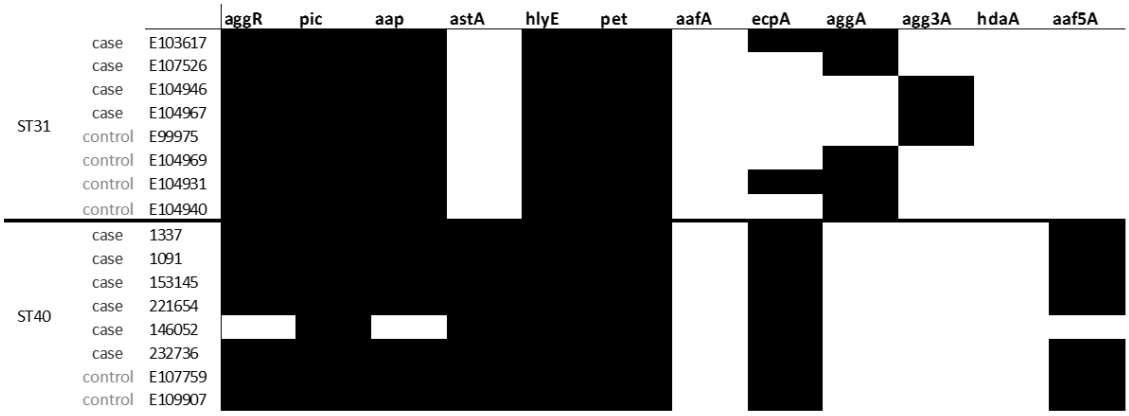
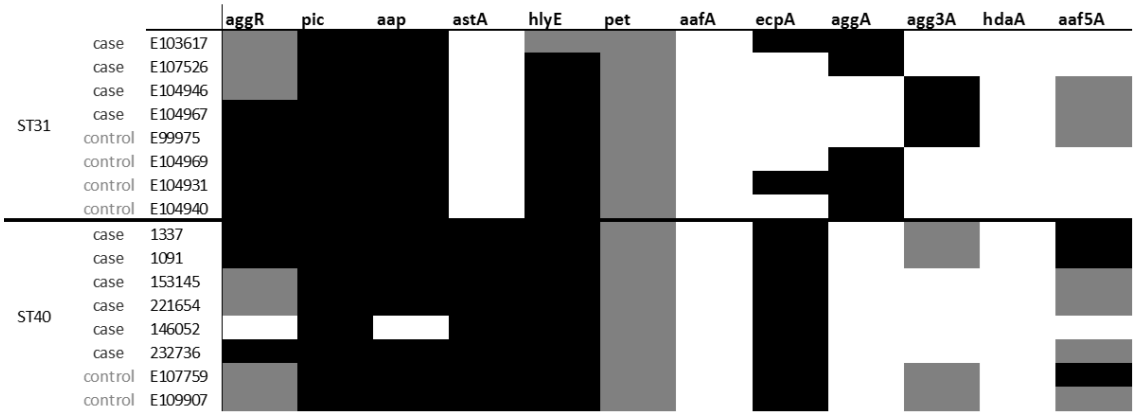


Figure A1.1 The scoring scale for EAEC colony size on human colonic biopsies.
Representative images showing colonisation scale for each score. 0 = no bacteria; 1 = isolated bacteria or very small aggregates, < 10 bacteria; 2 = small defined colonies, approximately 10-100 bacteria; 3 = medium to large defined colonies, approximately 100-1000 bacteria; 4 = very large colonies, approximately >1000 bacteria. Images obtained by scanning electron microscopy of sigmoidal colonic biopsies incubated with EAEC for 7 hours.

BLAST (local)



BLAST (Galaxy)



In silico PCR

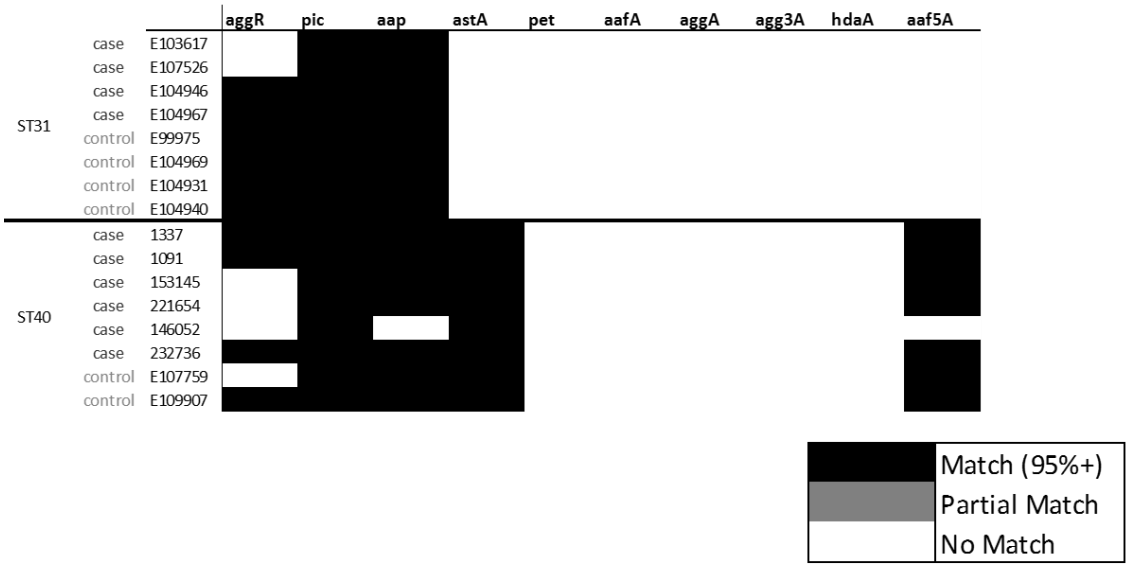


Figure A1.2 Virulence gene profiles identified by alternative tools. BLAST alignment was performed for all ST40 and ST31 strains using the query virulence gene sequences in Appendix 1.1. *In silico* PCR was performed using primer sequences obtained from Marie Chattaway (GBRU at PHE)

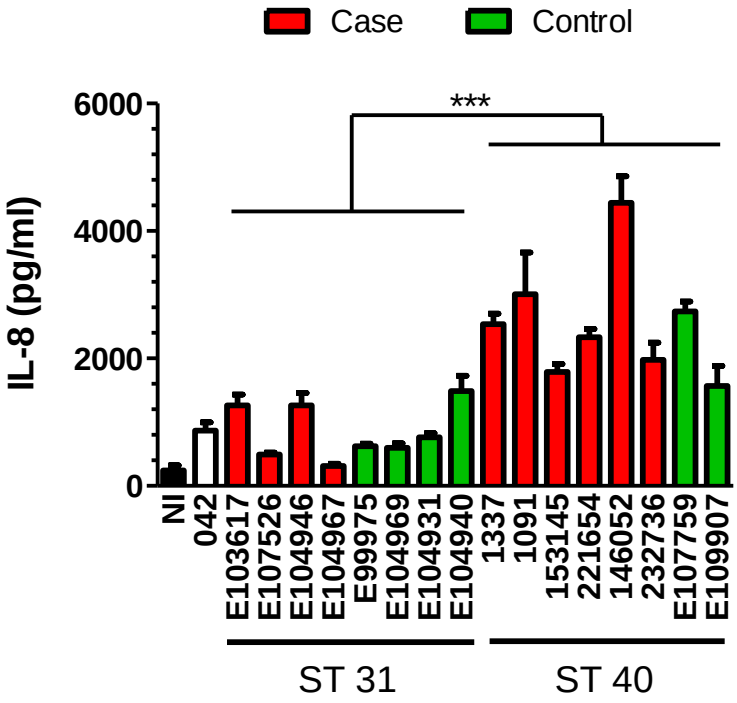


Figure A1.3 Induction of IL-8 secretion from T84 cells by EAEC. Confluent T84 cells were incubated with EAEC for 3 hours (MOI = 7.5 bacteria/cell). Bacteria were killed with gentamicin (50 µg/mL), and IL-8 concentrations in supernatants were determined by ELISA after 24 hours. Data is shown for three independent experiments, in duplicate. *** P < 0.001. Th work was performed by Leah Bundy and Stephanie Schüller (Bundy, 2018).

APPENDIX TWO

NORWICH BIOREPOSITORY

PATIENT CONSENT FORM

The Norwich Biorepository

The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from an adult for research

Information sheet for patients - Version 15 (21 February 2014)

Thank you for considering giving a sample for biomedical research. This information sheet provides a brief summary to help you to understand what this means and involves.

There is a consent form after the information sheet. It is important that you complete and sign it, if you decide to give us a sample. Please complete all parts of the consent form.

Doctors and other health professionals often take samples (which may be blood, small biopsies, or something else) from patients to help tell us what is wrong and how best to treat it. Larger pieces of tissue or whole organs may be removed by surgeons from some patients as part of their treatment.

Quite often, some of the test sample or surgical tissue is left over at the end. The left-over sample can be used by other doctors or researchers in special experiments to learn more about illness, how it happens and how to treat it, and sometimes to help make new medicines.

We would like to do some experiments on any left-over sample or surgical tissue, once your tests are finished. To do that, we need your permission and signed consent.

Samples donated (given) to the Norwich Biorepository are not:

- Normally used in animal research. It will be made clear to you if animal research is an integral part of the project for which we are seeking a donation.
- Used in cloning experiments. However, the Biorepository would consider the use of donations in non-reproductive cloning experiments based on their scientific value and in the context of prevailing law and ethical standards. It will be made clear to you if cloning experiments are part of the research project for which we are seeking a donation.

If you want to help us, please sign the form at the end of this document.

Continued.....

If you give permission for a sample to be taken –

- The Hospital will own the sample.
- The sample may be stored, usually in a deep freezer, until it is used. The freezer is referred to as a tissue bank in the consent form.
- Nobody involved in the research will know where the sample has come from.
- The sample will be used only in experiments that are ethical and to help other people. Please see the section entitled 'Scientific and ethical approval' below to understand what we mean by *ethical*.
- Your donated sample(s) and any genetic material derived from it (them) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing. *Whole genome sequencing* means reading your total DNA code (your genetic blueprint) in a single assessment.
- We might give some of or all the sample to other doctors or researchers for their experiments, if they are ethical and to help other people. Some of these people might work in companies in this country or abroad.
- Data derived from your sample(s) may be placed anonymously in an international database to be used in future research. While we will take all possible steps to maintain your anonymity and protect your privacy, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.
- We will keep some facts about you on our Biorepository database.
- Although these facts might be given to the research doctors or scientists to help their experiments, we will NOT tell them your name or other details that would let them know who you are.
- Doctors in the Hospital might also read your hospital records to help them understand what the doctors or scientists find out in the experiments. This is possible because your hospital records can be linked to the anonymous research sample without loss of confidentiality as far as the researchers are concerned. If the research results are important for you, it might be possible using this linkage to feed back the information to your doctor, so that any appropriate action can be considered.

Your treatment will not be affected in any way, if you do not feel able to say yes to our request for a research sample.

The next sections give you some more detailed information. If you have any questions, please do not hesitate to ask the person who is seeking your permission.

WHAT WILL HAPPEN

Tissue, blood or other samples taken from you for diagnosis and/or treatment of your condition will be sent to the Pathology Laboratories, where they will be tested to decide exactly what they are and whether any further treatment will be necessary. This is a standard part of treatment. **Only as much tissue or fluid as is needed will be removed.**

Continued.....

MEDICAL RESEARCH AND WHY THIS PROGRAMME IS IMPORTANT

When all the routine tests have been done, if any samples are left over, with your consent, they could be donated (given) for use in medical research. As part of a research programme which now includes the Norfolk and Norwich University Hospitals NHS Foundation Trust, the James Paget University Hospitals NHS Foundation Trust, the University of East Anglia (UEA), and the Institute of Food Research (IFR), some of the sample or material extracted from it will be stored in the Norwich Biorepository (usually in a special deep freezer) for use by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease, and to develop new methods of prevention or new treatments for the benefit of future patient care. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care, in which case there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your hospital records, including case notes, as part of this research in order to relate the research findings to the clinical outcome. It is important to be able see how you progress after the tissue or other samples you have donated (given) have been used in the research project(s). They will not give your name to those doing the research.

The research may also involve training doctors and researchers in scientific medicine, and may lead to higher qualifications for them (e.g., PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS

If you agree, we may send stored material or products derived from it to other approved tissue banks or companies in this country or abroad to support their research programmes or the research programmes of those companies' clients. Such outside organisations will provide financial support for the Norwich Biorepository (our tissue bank), to help it recover its operating costs. We are not, however, allowed to sell tissue or other samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL

The Norwich Biorepository acts as a custodian of the samples it holds. It releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been approved by a properly constituted Research Governance Committee before it starts. It must also be approved by a Research Ethics Committee or on behalf of the Research Ethics Committee that oversees the work of the Norwich Biorepository under the terms of the Biorepository's own Research Ethics Committee approval. That committee is the Cambridge East Research Ethics Committee. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participants and issues of consent and confidentiality. We will release samples to commercial companies only if they work to appropriate ethical and scientific standards.

Continued.....

DONATING EXTRA SAMPLES FOR RESEARCH

In certain circumstances you may be asked by the doctor treating you (or by a doctor, research nurse or nurse practitioner working with him/her) to consider donating (giving) tissue or other samples in addition to those to be removed as part of your diagnostic investigation or treatment. **Such extra samples will be taken only if you give your consent and if their removal does not cause you any harmful effects now or in the future.**

YOUR RIGHTS

If your samples are stored, information about your case will be kept on a computer in the Norwich Biorepository. This will help us understand what your illness was like and relate what we find in experiments to what happens to patients. Under the Data Protection Act, you are entitled to ask to see what is recorded about you by applying to the Chairman of the Norwich Biorepository Committee, Norfolk & Norwich University Hospital, c/o Dept. of Cellular Pathology, The Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records and any information needed for research purposes will be made anonymous before it is given to the researcher.

The researchers will not be able to find out your name or any personal details about you from the information that they receive.

You will have the opportunity to discuss with a doctor issues relating to the possible use of your samples for research purposes. He or she will answer any questions you may have.

MAKING A DONATION (GIFT) OF TISSUE AND/OR OTHER MATERIAL FOR RESEARCH

If you decide that you want your tissue, etc., to be stored in the Norwich Biorepository and used for research purposes, you will be asked to sign a special Consent Form confirming your decision and stating that you have read and understood this sheet. When you sign the form you will give the ownership of the tissue or other samples to the Norfolk & Norwich University Hospitals NHS Foundation Trust. The tissue or other samples will then belong to the Trust, which will store it for an indefinite period of time and will able to decide how it should be used for research. It will also have the right to dispose of unused stored material in an appropriate legal and ethical manner following normal procedures.

If you do not want your tissue to be stored in the Norwich Biorepository, please tell us and do NOT sign the special Consent Form.

If you do not sign this form, the tissue or other samples will still be sent to the laboratory to undergo those tests that are necessary for your care but they will not be used for research purposes. All unused tissue from your procedure will be disposed of using normal hospital methods. We will respect your decision and it will not affect in any way the treatment you receive.

Continued.....

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I agree (Please initial small box) that the following tissue or other material may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

List samples for research:	

I also agree that (Please initial small boxes, as appropriate):

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("the Trust")	
The Trust may store these samples in a tissue bank / biorepository	
The Trust may use these samples at its discretion in properly approved research programmes	
The Trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes	Yes / No
My genetic material and donated sample(s) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing	
Information about my case may be kept on the Norwich Biorepository database	
Anonymous data derived from my sample(s) may be placed in an international database for future research	
Such information may be passed in an anonymous form to persons outside the Trust In connection with research and may be published with any research findings	
I agree that appropriately qualified staff employed by the Trust may review my hospital Medical records, including case notes, as appropriate, for the purposes of research using the donated samples	
These samples may be used in ethically approved animal research	Yes / No
These samples may be used in ethically approved cloning research	Yes / No

Continued.....

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I confirm that:

1) **I have read and understand the Information Sheet for Patients, Version 15, dated 21 February 2014**
2) **The issues have been explained to me, and that I have had the opportunity to ask questions.**

Signed _____ (Patient) Date _____

I have explained the request for tissue for research purposes and have answered such questions as the patient has asked.

Signed_____ Print name _____
Medical / Nursing Practitioner

Date _____

CONSENT FOR TAKING EXTRA SAMPLES FOR RESEARCH

Please initial the appropriate box for each item:	YES	NO
In addition to the removal of tissue, blood or other fluid samples as a necessary part of my procedure, I also consent to the removal of additional tissue, blood or other fluid samples from the operation site during my procedure PROVIDED THAT SUCH REMOVAL CAUSES ME NO HARM now or in the future, is limited to what I and the doctor treating me (or a research nurse or nurse practitioner delegated by him/her) have discussed and agreed, and which is specified below. * *Please initial appropriate box*		
***Please list additional samples for research:**		

Signed _____ (Patient) Date _____

I have explained the request for the donation (gift) of extra tissue and/or other samples for research purposes and have answered such questions as the patient has asked.

Signed_____ Print name_____
Medical / Nursing Practitioner

Date _____

APPENDIX THREE

CELL CULTURE MEDIA PRODUCT INFORMATION

This study used DMEM D5671 and DMEM/F12 D6421 throughout

3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Dulbecco's Modified Eagle's Medium (DME)

Many modifications of Eagle's Medium have been developed since the original formulation appeared in the literature. Among the most widely used of these modifications is Dulbecco's Modified Eagle's Medium (DME).

DME is a modification of Basal Medium Eagle (BME) that contains a 4-fold higher concentration of amino acids and vitamins, as well as additional supplementary components. The original DME formula, first reported for culturing embryonic mouse cells, contained 1,000 mg/L of glucose. An alteration with 4,500 mg/L glucose is optimal in cultivating certain cell types.

	D5546	D5648	D5671	D5796	D5921	D4947	D6046	D6171
	[1×]	[powder]	[1×]	[1×]	[1×]	[1×]	[1×]	[1×]
COMPONENT	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L
Inorganic Salts								
$CaCl_2$	0.2	0.2	0.2	0.2	0.265	0.265	0.2	0.265
$Fe(NO_3)_3 \cdot 9H_2O$	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
$MgSO_4$	0.09767	0.09767	0.09767	0.09767	0.09767	0.09767	0.09767	0.09767
KCl	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
$NaHCO_3$	3.7	—	3.7	3.7	3.7	3.7	3.7	3.7
NaCl	6.4	6.4	6.4	6.4	6.4	6.4	6.4	4.4
NaH_2PO_4	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109
Amino Acids								
L-Alanyl-L-Glutamine	—	—	—	—	—	—	—	—
L-Arginine • HCl	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.084
L-Cystine • 2HCl	0.0626	0.0626	0.0626	0.0626	0.0626	0.0626	0.0626	0.0626
L-Glutamine	—	0.584	—	0.584	—	0.584	0.584	—
Glycine	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
L-Histidine • HCl • H_2O	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042
L-Isoleucine	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.105
L-Leucine	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.105
L-Lysine • HCl	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.146
L-Methionine	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
L-Phenylalanine	0.066	0.066	0.066	0.066	0.066	0.066	0.066	0.066
L-Serine	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042
L-Threonine	0.095	0.095	0.095	0.095	0.095	0.095	0.095	0.095
L-Tryptophan	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
L-Tyrosine • 2Na • $2H_2O$	0.10379	0.10379	0.10379	0.10379	0.12037	0.12037	0.10379	0.10379
L-Valine	0.094	0.094	0.094	0.094	0.094	0.094	0.094	0.094
Vitamins								
Choline Chloride	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Folic Acid	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
myo-Inositol	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072
Niacinamide	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
D-Pantothenic Acid • ½Ca	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Pyridoxal • HCl	—	0.004	—	—	—	—	—	—
Pyridoxine • HCl	0.00404	—	0.00404	0.00404	0.00404	0.00404	0.00404	0.00404
Riboflavin	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004
Thiamine • HCl	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Other								
D-Glucose	1.0	4.5	4.5	4.5	1.0	1.0	1.0	4.5
HEPES	—	—	—	—	—	—	—	5.958
Phenol Red • Na	0.0159	0.0159	0.0159	0.0159	—	—	0.0159	0.0159
Pyruvic Acid • Na	0.11	—	—	—	—	—	0.11	—
ADD								
Glucose	—	—	—	—	—	—	—	—
L-Glutamine	0.584	—	0.584	—	0.584	—	—	0.584
$NaHCO_3$	—	3.7	—	—	—	—	—	—

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Product Information

Dulbecco's Modified Eagle's Medium (DME)/ Ham's Nutrient Mixture F-12

The use of serum-free media has expanded as researchers investigate the conditions for successful culture of a variety of cell lines. Instead of serum, the media are supplemented with a defined combination of nutrients, growth factors, and hormones. Variation of the supplements and their concentrations are specific for the type of cell under study.

The media often used for these defined studies contain a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12 Nutrient Mixture. HEPES buffer may be included in the formulations at a final concentration of 15 mM to compensate for the loss of buffering capacity incurred by eliminating serum.

	D0547	D0697	D2906	D6421	D6434	D8900	D8062	D8437	D9785
	[powder]	[1×]	[powder]	[1×]	[1×]	[powder]	[1×]	[1×]	[powder]
COMPONENT	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L
Inorganic Salts									
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.00000618	—	—	—	—	—	—	—	—
NH_4VO_3	0.00000058	—	—	—	—	—	—	—	—
$CaCl_2$	0.1166	—	0.1166	—	—	0.1166	—	—	—
$CaCl_2 \cdot 2H_2O$	—	0.1545	—	0.1545	0.1545	—	0.1545	0.1545	—
$CuSO_4 \cdot 5H_2O$	0.0000013	0.0000013	0.0000013	0.0000013	0.0000013	0.0000013	0.0000013	0.0000013	0.0000013
$Fe(NO_3)_3 \cdot 9H_2O$	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005
$FeSO_4 \cdot 7H_2O$	0.000417	0.000417	0.000417	0.000417	0.000417	0.000417	0.000417	0.000417	0.000417
$MgCl_2 \cdot 6H_2O$	0.0612	0.0612	0.0612	0.0612	0.0612	0.0612	0.0612	0.0612	—
$MgSO_4$	0.04884	0.04884	0.04884	0.04884	0.04884	0.04884	0.04884	0.04884	—
$MnSO_4$	0.000000151	—	—	—	—	—	—	—	—
$NiCl_2$	0.00000012	—	—	—	—	—	—	—	—
KCl	0.3118	0.3118	0.3118	0.3118	0.3118	0.3118	0.3118	0.3118	0.3118
$NaHCO_3$	—	1.2	—	1.2	1.2	—	1.2	1.2	—
NaCl	6.996	6.996	6.996	6.996	6.996	6.996	6.996	6.996	6.996
$NaSiO_3 \cdot 9H_2O$	0.0000142	—	—	—	—	—	—	—	—
Na_2SeO_3	0.00000519	—	—	—	—	—	—	—	—
Na_2HPO_4	0.07102	0.07102	0.07102	0.07102	0.07102	0.07102	0.07102	0.07102	0.07102
NaH_2PO_4	0.0543	0.0543	0.0543	0.0543	0.0543	0.0543	0.0543	0.0543	0.0543
$SnCl_2 \cdot 7H_2O$	0.00000011	—	—	—	—	—	—	—	—
$ZnSO_4 \cdot 7H_2O$	0.000432	0.000432	0.000432	0.000432	0.000432	0.000432	0.000432	0.000432	0.000432
Amino Acids									
L-Alanine	0.00445	0.0045	0.00445	0.0045	0.00445	0.00445	0.00445	0.0045	0.00445
L-Alanyl-L-Glutamine	—	0.54253	—	—	—	—	—	—	—
L-Arginine • HCl	0.1475	0.1475	0.1475	0.1475	0.1475	0.1475	0.1475	0.1475	0.1475
L-Asparagine • H_2O	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075
L-Aspartic Acid	0.00665	0.00665	0.00665	0.00665	0.00665	0.00665	0.00665	0.00665	0.00665
L-Cystine • 2HCl	0.01756	0.03129	0.01756	0.03129	0.03129	0.01756	0.03129	0.03129	0.01756
L-Cysteine • HCl • H_2O	0.03129	0.01756	0.03129	0.01756	0.01756	0.03129	0.01756	0.01756	0.03129
L-Glutamic Acid	0.00735	0.00735	0.00735	0.00735	0.00735	0.00735	0.00735	0.00735	0.00735
L-Glutamine	0.365	—	0.365	—	—	0.365	0.365	0.365	—
Glycine	0.01875	0.01875	0.01875	0.01875	0.01875	0.01875	0.01875	0.01875	0.01875
L-Histidine • HCl • H_2O	0.03148	0.03148	0.03148	0.03148	0.03148	0.03148	0.03148	0.03148	0.03148
L-Isoleucine	0.05447	0.05447	0.05447	0.05447	0.05447	0.05447	0.05447	0.05447	0.05447
L-Leucine	0.05905	0.05905	0.05905	0.05905	0.05905	0.05905	0.05905	0.05905	—
L-Lysine • HCl	0.09125	0.09125	0.09125	0.09125	0.09125	0.09125	0.09125	0.09125	—
L-Methionine	0.01724	0.01724	0.01724	0.01724	0.01724	0.01724	0.01724	0.01724	—
L-Phenylalanine	0.03548	0.03548	0.03548	0.03548	0.03548	0.03548	0.03548	0.03548	0.03548
L-Proline	0.01725	0.01725	0.01725	0.01725	0.01725	0.01725	0.01725	0.01725	0.01725
L-Serine	0.02625	0.02625	0.02625	0.02625	0.02625	0.02625	0.02625	0.02625	0.02625
L-Threonine	0.05345	0.05345	0.05345	0.05345	0.05345	0.05345	0.05345	0.05345	0.05345
L-Tryptophan	0.00902	0.00902	0.00902	0.00902	0.00902	0.00902	0.00902	0.00902	0.00902
L-Tyrosine • 2Na • $2H_2O$	0.05579	0.05579	0.05579	0.05579	0.05579	0.05579	0.05579	0.05579	0.05579
L-Valine	0.05285	0.05285	0.05285	0.05285	0.05285	0.05285	0.05285	0.05285	0.05285

	D0547	D0697	D2906	D6421	D6434	D8900	D8062	D8437	D9785
	[powder]	[1×]	[powder]	[1×]	[1×]	[powder]	[1×]	[1×]	[powder]
COMPONENT	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L
Vitamins									
D-Biotin	0.0000035	0.0000035	0.0000035	0.0000035	0.0000035	0.0000035	0.0000035	0.0000035	0.0000035
Choline Chloride	0.00898	0.00898	0.00898	0.00898	0.00898	0.00898	0.00898	0.00898	0.00898
Folic Acid	0.00266	0.00266	0.00266	0.00265	0.00266	0.00266	0.00266	0.00266	0.00266
myo-Inositol	0.0126	0.0126	0.0126	0.0126	0.0126	0.0126	0.0126	0.0126	0.0126
Niacinamide	0.00202	0.00202	0.00202	0.00202	0.00202	0.00202	0.00202	0.00202	0.00202
D-Pantothenic Acid • ½Ca	0.00224	0.00224	0.00224	0.00224	0.00224	0.00224	0.00224	0.00224	0.00224
Pyridoxal • HCl	0.002	—	0.002	—	0.002	0.002	0.002	—	0.002
Pyridoxine • HCl	0.000031	0.002031	0.000031	0.002031	0.002031	0.000031	0.002031	0.002031	0.000031
Riboflavin	0.000219	0.000219	0.000219	0.000219	0.000219	0.000219	0.000219	0.000219	0.000219
Thiamine • HCl	0.00217	0.00217	0.00217	0.00217	0.00217	0.00217	0.00217	0.00217	0.00217
Vitamin B_{12}	0.00068	0.00068	0.00068	0.00068	0.00068	0.00068	0.00068	0.00068	0.00068
Other									
D-Glucose	3.15	3.15	3.15	3.15	3.15	3.15	3.15	3.15	3.15
HEPES	—	3.5745	3.5745	3.5745	3.5745	3.5745	—	3.5745	3.5745
Hypoxanthine	0.0021	0.00244	0.0021	0.00244	0.00244	0.0021	0.00244	0.00244	0.0021
Linoleic Acid	0.000042	0.000042	0.000042	0.000042	0.000042	0.000042	0.000042	0.000042	0.000042
Phenol Red • Na	0.00863	0.00863	—	0.00863	—	0.00863	0.00863	0.00863	—
Putrescine • 2HCl	0.000081	0.000081	0.000081	0.000081	0.000081	0.000081	0.000081	0.000081	0.000081
Pyruvic Acid • Na	0.11	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.055
DL-Thioctic Acid	0.000105	0.000105	0.000105	0.000105	0.000105	0.000105	0.000105	0.000105	0.000105
Thymidine	0.000365	0.000365	0.000365	0.000365	0.000365	0.000365	0.000365	0.000365	0.000365
ADD									
$NaHCO_3$	1.2	—	1.2	—	—	1.2	—	—	1.2
$CaCl_2 \cdot 2H_2O$	—	—	—	—	—	—	—	—	0.1545
L-Glutamine	—	—	—	0.365	0.365	—	—	—	0.365
L-Leucine	—	—	—	—	—	—	—	—	0.05905
L-Lysine • HCl	—	—	—	—	—	—	—	—	0.09125
$MgCl_2 \cdot 6H_2O$	—	—	—	—	—	—	—	—	0.0612
$MgSO_4$	—	—	—	—	—	—	—	—	0.04884
L-Methionine	—	—	—	—	—	—	—	—	0.0172