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F4 fimbriae and its adhesin FaeG as a mucosal carrier for a heterologous antigen or peptide

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List of abbreviations

ABTS	2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate)
ADWG	Average daily weight gain
AEC	Anion exchange chromatography
APC(s)	Antigen-presenting cell(s)
ARF	ADP-ribosylating factor
ASC(s)	Antibody-secreting cell(s)
BCA	Bicinchoninic acid reaction
cAMP	Cyclic AMP
CD	Cluster of differentiation
ConA	Concanavalin A
Cpm	Counts per minute
CT	Cholera toxin
DC(s)	Dendritic cell(s)
dpi	Days post infection
dppi	Days post primary immunization
dpc	Days post challenge
E. coli	*Escherichia coli*
EDTA	Etylenediaminetetraacetaat
ETEC	Enterotoxigenic *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ELIspot	Enzyme-linked immuno spot
F4R	F4 receptor
FCS	Foetal calf serum
BSA	Bovine serum albumin
GuHCl	Guanidine hydrochloride
HSA	Human serum albumin
IFN-γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IPP	Ileal Peyer's patches
IPTG	Isopropyl-β-D-thiogalactoside
IS	Insertion sequence
JPP	Jejunal Peyer's patches
MC(s)	Monomorphonuclear cell(s)
MLN	Mesenteric lymph node
LT	Heat-labile enterotoxin
LP	Lamina propria
MAb	Monoclonal antibody
MHC II	Major histocompability complex class II
OD	Optical density
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood monomorphonuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PP	Peyer's patches

rFaeG	Recombinant FaeG
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SI	Stimulation index
SLT-IIv	Shiga-like toxin type II variant
SP	Spleen
ST	Heat-stable enterotoxin
TGF-β	Transforming growth factor β
Th	T-helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
VTEC	Verotoxigenic *Escherichia coli*

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Introduction

Since the development of the cowpox (*Vaccinia*) vaccine by Jenner in 1796, vaccination has been able to protect both humans and animals against several diseases and even to eliminate some pathogens. The majority of the vaccines in use today are against systemic pathogens or toxins, despite the fact that intestinal infections are still a major cause of disease in both humans and animals. Protection against intestinal pathogens is obtained by the induction of pathogen-specific secretory IgA (sIgA) in the mucosae. While the parenteral route of vaccination is effective in the development of systemic immunity, it generally does not extend to the mucosal surfaces. In addition, the secretion of antigen-specific sIgA in the mucosae is maximal near the induction site. Therefore, the development of systems for vaccination via the oral route is an essential prerequisite for the stimulation of protective immunity against the majority of enteric pathogens of both man and animals.

Despite the need for oral vaccine technology, progress in this area has been rather slow. Indeed, inactivated vaccines are in some cases not effective, attenuated live vaccines could potentially be dangerous and oral delivery of non-replicating antigens deals with physiological and immunological obstacles. Indeed, most antigens that are taken up orally are digested due to the low gastric pH and the presence of proteases. As a consequence, effective oral immunization usually requires the repeated administration of very large quantities of antigen. Oral administration of large doses of non-replicating antigens can sometimes induce a short-lived mucosal IgA immune response, but often develops a systemic state of immunological hyporesponsiveness, called oral tolerance. On the other hand, receptor-mediated uptake of orally administered antigens mostly induces an antigen-specific mucosal immune response. Therefore, these mucosa-binding antigens can serve as mucosal carriers, inducing a mucosal immune response against coupled heterologous antigens following oral administration of antigen-carrier complexes.

It has been demonstrated that oral administration of newly weaned piglets with purified F4 fimbriae induced a receptor-mediated F4-specific antibody response, protecting these piglets against a subsequent $F4^+$ ETEC challenge. However, F4 fimbriae have no mucosal adjuvant effect to co-administered antigens, in contrast to

Escherichia coli produced heat-labile enterotoxine (LT) and *Vibrio cholerae* produced cholera toxin (CT).

It is interesting to analyse the potential of both virulence factors of $F4^+$ ETEC, F4 fimbriae and LT enterotoxin (or the related CT), in the simultaneous induction of an immune response against coupled or co-administered heterologous antigens and against $F4^+$ ETEC infections in pigs. To determine the potential of F4 fimbriae or recombinant produced F4 fimbrial adhesin FaeG as mucosal carrier to coupled heterologous antigens, knowledge on the F4 fimbrial biogenesis and the use of fimbriae as carrier is needed. In addition, to study if CT can act synergistically with F4 or FaeG as mucosal carrier to induce an immune response against the coupled antigen following oral administration, knowledge on the uptake and immunomodulating effects of CT is necessary. Therefore, Part I of this thesis represents the present knowledge on the use of fimbriae as carrier (Chapter 1), the F4 fimbrial biosynthesis (Chapter 2), and the toxic and immunomodulatory effects of LT and CT (Chapter 3).

PART I

INTRODUCTION

Chapter 1

Fimbriae as immunogens and carrier molecules

1.1. Introduction

The role of *Escherichia coli* as a pathogen has been well known for many years and many strains of *E. coli* have an inherent capacity to cause disease (Table 1). The most frequent infection caused in humans by *E. coli* is that of the urinary tract (Wullt, 2003). Enteropathogenic and enterotoxigenic strains of *E. coli* cause gastroenteritis, especially in developing countries (Presterl et al., 2003; Ratchtrachenchai et al., 2004), and are responsible for many cases of traveler's diarrhoea (Clarke, 2001). In animals, enterotoxigenic and verotoxigenic *E. coli* are known to cause severe diarrhoea and oedema disease in young animals.

The ability of many pathogenic bacteria to adhere to specific host tissues is a factor of primary importance in causing disease. Specific adherence allows bacteria to resist and circumvent the flushing and cleaning mechanisms that protect many epithelial surfaces in humans and animals. In addition, adhesion determines the site of microbial infection. However, the pathogenic potential of an *E. coli* strain also depends on other determinants such as its ability to produce toxins.

Fimbriae and pili are long proteinaceous surface appendages that allow adhesion of pathogenic bacteria to their specific receptor. Pili (e.g. P, type 1, F6) are more rigid structures with a diameter of about 7-8 nm and have an axial hole, whereas fimbriae (e.g. F4, F5, F18) are thin and flexible structures with a diameter of about 2-4 nm without axial hole (Fig. 1) (De Graaf and Mooi, 1986). They are composed of repeating major subunits and some additional minor subunits. In most fimbriae (e.g. F17, F18) and pili (e.g. P, type 1), a minor subunit, mostly located at the tip, mediates adhesion (Lund et al., 1987; Krogfelt et al., 1990; Smeds et al., 2001; Buts et al., 2003a). However, in F4 and F5 fimbriae, the major subunit functions as the adhesin (De Graaf et al., 1980; Bakker et al., 1992a).

Table 1: Characteristics of fimbrial and non-fimbrial adhesins of *E. coli*

Fimbriae or pili (alternative name)	Morphology	Diameter (nm)	Pathogenicity	Host	Receptor
Type 1 (F1)	R	7	U	H,P,A	α-D-mannose
CFA/I (F2)	R	7	D	H	NeuAc-GM2, human erythrocyte sialylglycoprotein
CFA/II (F3)			D	H	Asialo-GM1
CS1	R	7			
CS2	R	7			
CS3	CL	2-3			
Dr	R	7	D and UTI	H	Decay accelerating factor (CD55)
F4 (K88)	F	2.1	D	P	Contains Galα(1-3)Gal
F5 (K99)	F	5	D	B,O,P	Neu5Gc-α(2-3)Galp-β(1-4)Glcp-β(1-1)ceramide
F6 (P987)	R	7	D	P	14-20 kDa glycoprotein
P (F7-16)	R	7	UTI	H,A	Galα(1-4)Gal containing glycolipids
F17	F	3.4	D and S	B	NAcGlc
F18	F	4.5	D and OD	P	α-L-fucose-(1-2)-β-D-galactose-(1-4)- β-NAcGlc
F41	F	3.2	D	B,O,P	NAc-Gal, Gal
F1C	R	7	UTI	H	NAc-Gal, Gal, glycophorin
CS31A	CL	2	D	B	Unknown
CFA/III	R	7-8	D	H	Unknown
CFA/IV			D	H	Unknown
CS4	R	6			
CS5	F or R	2 or 5-6			
CS6	NF				
CS7	R	5			
S	R	7	UTI	H	NeuAc-α(2-3)Galβ(1-3)GalNAc

F, fimbriae; R, pili; CL, capsule like; NF, non fimbriaeted; A, avian;B, bovine; H, human; O:ovine; P:porcine; UTI, urinary tract infection; D, diarrhoea; S, septicemia; OD, oedema disease; U, unknown. References : Imberechts et al., 1992; Smyth et al., 1994; Rippinger et al., 1995; Mol and Oudega, 1996; Van Loy et al., 2002; Snoeck et al., 2004d.

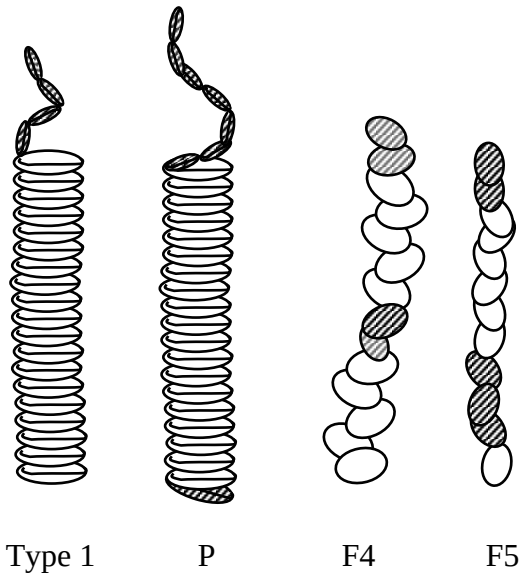


Figure 1 : Schematic representation of type 1 pili, P pili, F4 fimbriae and F5 fimbriae. Major subunit (white), minor subunit (shaded)

Infection with fimbriae-expressing ETEC induces fimbriae-specific antibodies (Evans et al., 1978; Verdonck et al., 2002). Moreover, these antibodies were shown to provide protection against a subsequent homologous ETEC infection by inhibiting attachment of the bacteria to the host cells (Nagy et al., 1978; Levine et al., 1984; Cravioto et al., 1990). Therefore, the ability of purified fimbriae to induce protective fimbriae-specific antibodies is of significant importance for the development of vaccines.

1.2. Parenteral immunization with fimbriae

Parenteral immunization of mice with purified P pili induces pili-specific antibodies in the urinary tract, which provide protection against a homologous challenge infection (O'Hanley, 1990; Roberts et al., 1994). However, the sequence variability in the major subunit of both P and type 1 pili leads to variation in antigenicity among clinical isolates (Abraham et al., 1988; Vandemaele et al., 2003a and 2003b). In addition, the predominant immune response is directed against the major subunit that composes more than 99% of the total protein mass of the pilus structure (Lund et al., 1988; Langermann et al., 1997). The low level of antibodies against the minor subunit and adhesin cannot protect against a subsequent

heterologous challenge infection (Lund et al., 1988; Langermann et al., 1997). In contrast, immunization with recombinant type 1 adhesin subunit FimH (Langermann et al., 1997, 2000 and 2001; Palaszynski et al., 1998), the recombinant FimH receptor-binding domain (Thankavel et al., 1997) or complexes of FimH with its chaperone FimC (Meiland et al., 2004) induce a protective antibody response in mice and monkeys. However, the role of type 1 fimbriae in *E. coli*-induced pathogenesis seems to differ between animal species. Recent studies with denatured recombinant FimH and the bioactive FimH156 lectin domain reported the induction of FimH-specific antibodies following vaccination of chickens, but they could not protect the chickens against a homologous *E. coli* infection (Kariyawasam et al., 2004; Vandemaele F., unpublished data). This is consistent with the observation that a *fimH* deletion mutant of an avian pathogenic *E. coli* colonized the chicken trachea better than did the wild type strain (Arné et al., 2000), suggesting that type 1 pili are not necessary for APEC-induced pathogenesis. On the other hand, recombinant PapG-induced antibodies provided protection to chickens against subsequent homologous and heterologous APEC challenge (Kariyawasam et al., 2004). Moreover, the conservation of the adhesin among P pili of different clinical isolates supports its application as subunit vaccine against P pilus expressing *E. coli* (Vandemaele et al., 2003b).

In agreement, parenteral immunization of pigs with F4 fimbriae induces a fimbriae-specific systemic antibody response, which is mainly directed against the adhesin since the major F4 fimbrial subunit FaeG also constitutes the adhesin (Bianchi et al., 1996; Van der Stede et al., 2002b). Moreover, the induced antibodies block the fimbriae-receptor interaction (Yokoyama et al., 1992; Van den Broeck et al., 1999c; Sun et al., 2000). As a consequence, vaccination of dams with F4 fimbriae produces fimbriae-specific antibodies in colostrum and milk which subsequently protects their suckling piglets from $F4^+$ ETEC-induced neonatal diarrhoea (Rutter and Jones 1973; Rutter et al., 1976; Logan and Meneely, 1981; Nagy et al., 1985; Moon and Bunn, 1993; Osek et al., 1995; Barman and Sarma, 1999). Identical results are obtained with F5 fimbriae, as their major subunit FanC also constitutes the fimbrial adhesin (Morgan et al., 1978; Nagy et al., 1978 and 1980; Acres et al., 1979; Jacobs et al., 1987). Alternatively, fimbriae-specific antibodies produced by intramuscular immunization of chickens, extracted from egg yolk and then used to passively immunize pigs were shown to result in protection against a subsequent challenge

infection with pathogenic *E. coli* expressing homologous fimbriae (Yokoyama et al., 1992; Imberechts et al., 1997b; Zuniga et al., 1997; Jin et al., 1998; Marquardt et al., 1999; Hennig-Pauka et al., 2003; Kariyawasam et al., 2004).

Despite the induction of systemic F4-specific antibody responses following parenteral immunization, no fimbriae-specific antibody secreting cells appear in the mucosa (Bianchi et al., 1996; Van der Stede et al., 2003). Bianchi et al. (1996) even observed that parenteral immunization of pigs with F4 fimbriae induced F4-specific suppression. However, parenteral immunization of pigs with purified F4 in the presence of adjuvants that modulate a systemically induced immune response towards a mucosal immune response, reduces the faecal $F4^+$ *E. coli* excretion following challenge (Van der Stede et al, 2002b and 2003). This suggests that parenteral immunization under the right circumstances may have applications for the induction of a mucosal immune response.

1.3. Mucosal immunization with fimbriae

Protection against a mucosal infection can be achieved by the induction of a mucosal antigen-specific IgA response. The importance of IgA in mucosal secretions consists of its ability to agglutinate bacteria and neutralize antigens (Kilian et al., 1988; Wold et al., 1990). The ability of IgA to block the adherence of bacteria to host mucosal epithelial cells has been demonstrated (Wold et al., 1990; McGhee et al., 1992). In addition, IgA antibodies are more effective in inhibiting bacterial adherence than IgG of the same affinity as a result of its charge, extensive glycosylation and resistance to proteolysis (Magnusson et al., 1982; McGhee et al., 1992).

Migration of B cells induced in nasal-associated lymphoid tissue and bronchus-associated lymphoid tissue to the gut lamina propria is negligible and results in a negligible level of intestinal IgA antibodies. This is in contrast with B cells induced in the gut-associated lymphoid tissue (Brandtzaeg et al., 1999; Ogra et al., 2001). Thus, to induce mucosal fimbriae-specific antibodies preventing ETEC-adhesion in the small intestine, oral immunizations are supposed to be most successful. However, it is known that oral immunization may alter the immunogenicity of antigens by effects of the acid environment of the stomach, presence of proteases and the flushing action of the intestines. Furthermore, the intestinal mucosal immune system only induces an immune response against

pathogenic antigens, whereas harmless antigens tend to induce oral tolerance (Stokes and Bailey, 2000; Garside and Mowat, 2001).

Oral immunizations with intact or encapsulated CFA fimbriae induce poor mucosal IgA antibody responses. This has been suggested to be in part due to the alteration of CFA fimbrial antigens upon exposure to low pH in the gastro-intestinal tract (Evans et al., 1984; Schmidt et al., 1985; Edelman et al., 1993; Guillobel et al., 2000; Byrd and Cassels, 2003). In contrast, F4 and F5 fimbriae are stable in the presence of proteolytic enzymes and although F5 dissociates at pH 2.5, the subunits are still able to bind their receptor (Gabor et al., 1997; Snoeck et al., 2004a). In agreement, oral immunization of $F4R^+$ piglets with purified F4 fimbriae induces mucosal F4-specific antibodies, able to protect against a subsequent $F4^+$ ETEC challenge (Van den Broeck et al., 1999a and 1999b). The presence of the F4 receptor is necessary since oral F4 immunization of $F4R^-$ piglets does not result in the induction of an F4-specific mucosal immune response (Van den Broeck et al, 1999b). Nevertheless, a secondary systemic F4-specific antibody response appears in $F4R^-$ piglets following oral and subsequent intramuscular immunization with purified F4 (Van den Broeck et al., 2002). Van den Broeck et al. (2002) suggest that purified F4 fimbriae act as normal food antigen in the absence of the F4R since identical immunization with a same low dose of OVA induces a similar secondary antigen-specific antibody profile. However, the F4-specific antibody-response appears earlier than the OVA-specific response, which is likely due to a better intrinsic immunogenicity of the polymeric F4 antigen compared to monomer OVA. Oral co-administration of OVA and F4 did not enhance an OVA-specific antibody response, suggesting that F4 has no adjuvant function (Van den Broeck, unpublished data).

In contrast to F4 fimbriae, oral immunization of piglets with purified F18 fimbriae does not induce a protective antibody response, even when using a 30-times higher dose of F18 fimbriae (Verdonck et al., unpublished data) or following incorporation of F18 fimbriae in microparticles (Felder et al., 2000). The fact that the minor subunit FedF functions as the adhesin of F18 fimbriae (Smeds et al., 2001 and 2003) may at least partly explain the observed difference with F4 immunizations.

1.4. Use of fimbriae as carrier molecules

Fimbriae are attractive structures to use as carriers in vaccine design due to their polymeric character, their ability to adhere to specific receptors on mucosal

surfaces, their presence on the surface of bacteria and the possibility to prepare them in large amounts.

A first approach to use fimbrial subunits as carrier molecules includes insertion of heterologous epitopes. This is mostly performed by insertion of epitopes in known hypervariable domains (Thiry et al., 1989; Bakker et al., 1990; van Die et al., 1990; Bousquet et al., 1994) or predicted surface-exposed domains of fimbrial proteins (Hedegaard and Klemm 1989; Pedersen et al., 1991; Steidler et al., 1993; Stentebjerg-Olesen et al., 1997; Yakhchali and Manning, 1997; Gao et al., 2001). However, random linker insertion mutagenesis can be used to identify new insertion sites in fimbrial subunits (Rani et al., 1999).

The peptides that have been inserted in fimbrial subunits of *E. coli* are mainly immunogenic virulence antigens (Table 1). However, none of these chimeric fimbriae has been purified and subsequently analysed on its ability to induce a protective antibody response against a subsequent challenge infection. In addition to *E. coli* fimbrial subunits, fimbrial subunits of *Bacteroides nodosus* (Jennings et al., 1989), *Pseudomonas aeruginosa* (Hertle et al, 2001) and *Salmonella* (McEwen et al., 1992; White et al., 1999; Luna et al., 2000) as well as the flagellin subunit of flagella (Westerlund-Wikström, 2000) have been used as carriers for heterologous epitopes.

Table 1 : Overview of heterologous peptides inserted in fimbrial subunits of *E. coli.*

Fimbrial subunit	Heterologous epitope	Length epitope	Expression fimbriae	Adhesion	Induction antibodies	Reference
FimA (Type 1)	HBsAg	9	+	+	-	Hedegaard and Klemm, 1989
	HBsAg	20	-	-	-	Hedegaard and Klemm, 1989
	VP1 (FMDV)	19	+	+	+	Hedegaard and Klemm, 1989
	VP1 (polio)	11	+	+	+	Hedegaard and Klemm, 1989
	CT-B	34	+	+	+	Stentebjerg-Olesen et al., 1997
FimH (Type 1)	HbsAg	56	+	+	+	Pallesen et al., 1995
	CT-B	34	+	+	+	Pallesen et al., 1995
ClpG (CS31A)	TGEV-S	9	+	ND	+	Bousquet et al., 1994
	TGEV-S	10	+	ND	+	Bousquet et al., 1994
	$TGEV\text{-}S_{363\text{-}371}$ $TGEV\text{-}S_{522\text{-}531}$ tandems	up to 51	+	ND	ND	Méchin et al., 1996
	$TGEV\text{-}S_{363\text{-}371}$ $TGEV\text{-}S_{522\text{-}531}$	25	+	ND	+	Der Vartanian et al., 1997
CstH(CS3)	ST	15	+	ND	ND	Yakhchali and Manning, 1997
	LT-B	20	+	ND	ND	Yakhchali and Manning, 1997
	VP1 (FMDV)	12	+	ND	+	Gao et al., 2001
	c-Myc	10	+	ND	+	Gao et al., 2001
PapA (P)	FMDV	9	+	ND	+	van Die et al., 1988
	SPA	58	+	ND		Steidler et al., 1993
	GnRH	10	+	ND	+	van der Zee et al., 1995
FaeG (F4)	IP 1	12	+	ND	±	Thiry et al., 1989
	IP 2	7	+	ND	ND	Thiry et al., 1989
	SH	14	+	ND	ND	Thiry et al., 1989
	GP	11	+	ND	ND	Bakker et al., 1990
	VP1 (FMDV)	11	+	ND	+	Bakker et al., 1990
	HIV-1	11	+	ND	+	Bakker et al., 1990
FasA (F6)	HSV-1 gD	9	+	+	+	Rani et al., 1999
	HSV-1 gD	8	+	+	ND	Rani et al., 1999
	TGEV-S	10	+	+	+	Rani et al., 1999

ND, not determined; VP1 capsid protein of type 1 polio-virus; FMDV, foot-and-mouth disease virus; HBsAg, hepatitis B surface antigen; IP, peptide derived from influenza; GP, peptide derived from a pilin subunit of *Neisseria gonorrhoeae*; HSV 1, human immunodeficiency virus type 1; SH, somatostatin hormone; TGEV, transmissible gastroenteritis virus; GnRH, gonadotropin releasing hormone; ST, heat-stable enterotoxins; LT-B, heat-labile enterotoxins subunit B; CT-B, cholera toxin subunit B; SPA, *Staphylococcus aureus* protein A

The length of the inserted heterologous epitopes is limited as the folding and stability of fimbrial subunits may not be disturbed. Therefore, a second approach to use fimbriae as carriers for heterologous epitopes is to conjugate them. For instance, F5 has been chemically coupled to 6-methylprednisolone (Bernkop-Schnurch et al., 1997) and both F5 and F6 have been chemically linked to dinitrophenyl and bovine serum albumin (BSA) (Russell-Jones, 2001). Feeding fimbriae-BSA complexes to mice generates an anti-BSA antibody response. However, the ratio of fimbrial carrier for heterologous antigen and the chemical coupling both influence the immune response against both carrier and heterologous antigen (Kirkley et al., 2001; Russell-Jones, 2001). Therefore, recent approaches consists of constructing genetic fusions between fimbrial adhesins and heterologous antigens (Batisson et al., 2000a and 2000b; Zavialov et al., 2001).

1.5. Conclusion

F4 fimbriae are highly immunogenic molecules when administered parenteral or mucosal. Moreover, oral F4 immunization of pigs results in a protective FaeG-specific immune response. Therefore, it would be worthwhile to determine the potential of F4 fimbriae or its adhesin FaeG to function as a mucosal carrier molecule, inducing antibodies against a coupled or fused heterologous antigen or peptide.

Chapter 2

Expression and assembly of F4 fimbriae by *Escherichia coli*, a molecular overview[1]

[1] *Based on : Verdonck F, Cox E and Goddeeris BM. 2004. F4 (K88) fimbriae expressed by porcine enterotoxigenic Escherichia coli, an example of an eccentric fimbrial system ? J. Mol. Microbiol. Biotechnol., accepted.*

2.1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains that express F4 fimbriae are an important cause of diarrhoea in newborn and weaned piglets (Gyles, 1994). The fimbriae mediate adhesion of bacteria to F4-receptors on porcine small intestinal epithelial cells, the initial step in colonization and disease (Jones and Rutter, 1972).

2.2. Genetic organisation of the F4 operon

Fimbriae are encoded by operons or gene clusters that are either located on a plasmid or on the bacterial chromosome. The F4 operon is located on a plasmid with a ranging molecular weight of 50 to 177 MDa (Shipley et al., 1978; de Graaf, 1990; Wasteson and Olsvik, 1991; Bertin, 1992; Mainil et al., 1998). The smaller plasmids were nonconjugative, whereas larger F4-containing plasmids were conjugative (Shipley et al., 1978). Therefore, F4 expression by *E. coli* can be spontaneously lost (∅rskov and ∅rskov, 1966). Mainil et al. (1998) found that F4 operon containing plasmids are mainly mono- or bireplicon plasmids. It is interesting to note that the genes encoding fimbriae and toxins of ETEC are often located on the same plasmids (Tschäpe et al., 1987; de Graaf, 1990; Mainil et al., 1992 and 1998; Gyles, 1994; Smyth et al., 1994). In addition, this plasmid also encodes the enzyme that enables bacteria to utilize raffinose (Raf) as energy source (∅rskov and ∅rskov, 1966; Shipley et al, 1978). The F4 and Raf genes are not closely linked, but separated by 30 kbp (Mooi et al., 1979). The association of F4 and Raf genes might endow porcine ETEC with a selective advantage, which could explain the tendency of $F4^+$ ETEC strains to dominate the *E. coli* flora in the porcine intestinal flora (Hinton et al., 1985).

The F4 operon (Fig. 1) contains genes encoding the regulatory proteins FaeA and FaeB, the usher FaeD, the chaperone FaeE, the minor subunits FaeC, FaeF, FaeH, FaeI and the major subunit FaeG that constitutes also the adhesin (Van den Broeck et al., 2000). It is not clear whether FaeJ is expressed at all (Bakker et al., 1992b).

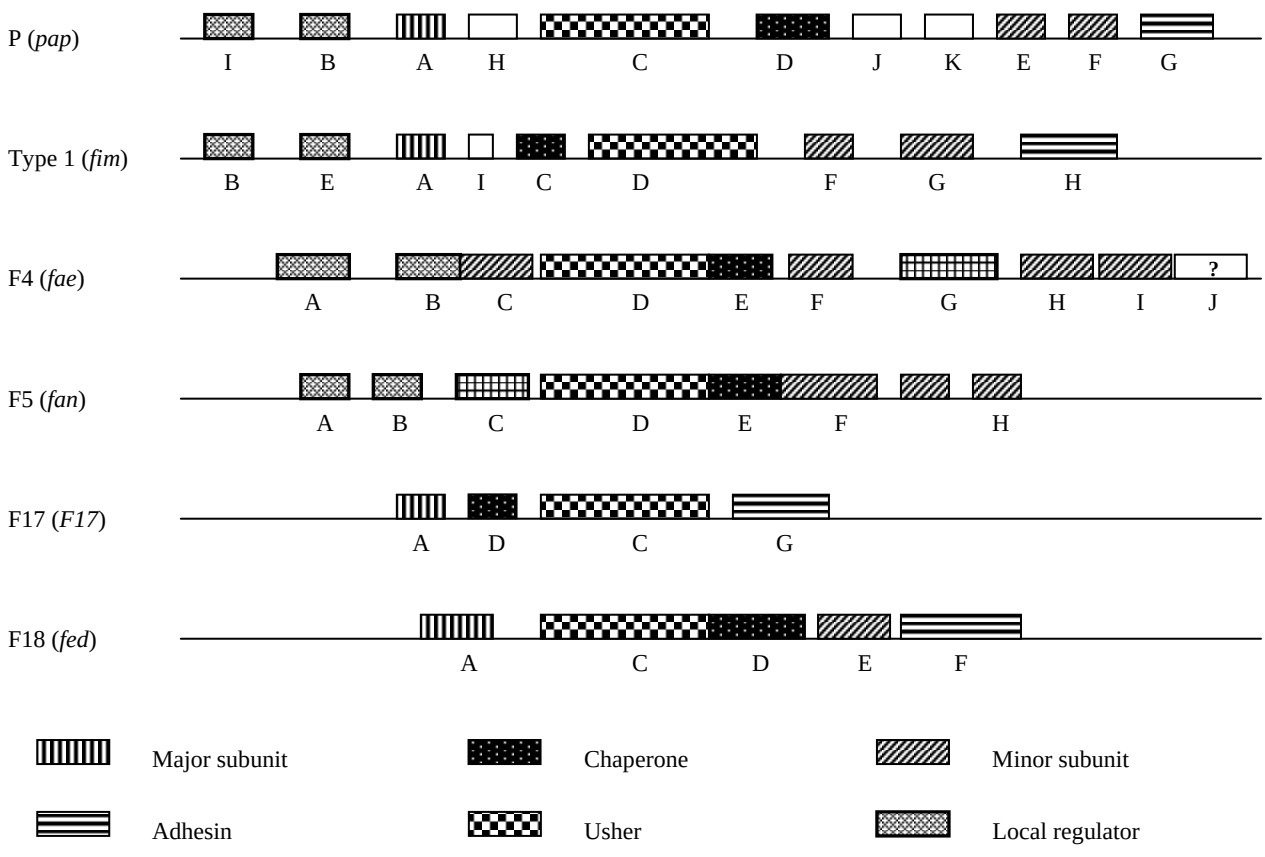


Figure 1 : Comparison of the gene clusters encoding the biogenesis of P pili, type 1 pili, F4, F5, F17 and F18 fimbriae. The boxes present genes. The function of the genes is indicated by the shading of the boxes. The letters below the boxes are symbols used for the genes.

Fimbrial operons share more or less the same genetic organisation (Fig. 1). However, the organisation of the F4 operon shows some differences in comparison with other fimbrial operons. First, the location of *faeC* in the F4 operon corresponds with the location of the major subunit gene in F5, F6, P, S and type 1 fimbrial systems (Mol and Oudega, 1996). In addition, FaeC is the only protein of the *fae* gene locus that shows the characteristics that are common to major subunits of other fimbrial systems. Indeed, FaeC is about 16 kDa, shows homology at the N- and C-terminal ends with other major fimbrial subunits (e.g. the major subunits FanC (Roosendaal et al., 1984), FimA (Klemm, 1984), and PapA (Baga et al., 1984)) and is the only protein encoded by the F4 operon that contains the conserved cysteine residues. It seems likely that FaeC used to be the major subunit of F4 fimbriae, but that during evolution this role has been taken over by the adhesin FaeG (Oudega et al., 1989).

Second, the *faeG* gene encoding the major subunit of F4 fimbriae is located upstream of the gene encoding the usher, in contrast to most gene clusters analysed to date (Pap, Type 1, S, F5, F17, F18, F6, CFA-I) (Imberechts et al., 1992; Mol and Oudega, 1996; Buts et al., 2003a). Only the operons encoding the F4-related F41 and CS31A fimbriae have an organization similar to that of F4 (Moseley et al., 1986; Casey et al., 1990; Girardeau et al., 1991; Martin, 1996).

Third, the major fimbrial subunit FaeG and the minor subunits FaeH and FaeI are much larger than the corresponding subunits of most other fimbrial subunits.

2.3. Regulation of F4 expression

The biosynthesis of fimbriae is influenced by growth rate (Jacobs et al., 1985; Van Verseveld et al., 1985; Van der Woude et al., 1990; Blomberg et al., 1991 and 1993a; Payne et al., 1993) and environmental conditions such as carbon source (Shipley et al., 1978; Blomberg et al., 1991; White-Ziegler et al., 2000), temperature (Mooi et al., 1979 and 1985; Göransson, 1984; Klemm, 1985; Göransson et al., 1990; Schmoll et al., 1990; White-Ziegler et al., 1990, 1992, 2000 and 2002; Gally et al., 1993; Huisman et al., 1994;), pH (Van Verseveld et al., 1985; White-Ziegler et al., 2000; Schwan et al., 2002), osmolarity (Spears et al., 1986; Göransson et al., 1990; White-Ziegler et al., 2000; Schwan et al., 2002), oxygen levels (White-Ziegler et al., 2000) and presence of alanine or leucine (Girardeau et al., 1982b; De Graaf and Mooi, 1986; Martin et al., 1991; Braaten et al., 1992; Gally et al., 1993). Indeed, pathogenic bacteria only fully express virulence factors like fimbriae when the conditions are appropriate for adherence and subsequent colonization (Pourbakhsh et al., 1997; Zhao et al., 1997; Struve and Krogfelt, 1999). To this end, the bacteria have regulatory systems at their disposal that enable them to recognize the environmental circumstances and to adapt fimbriae biosynthesis according to these conditions. In the case of F4 fimbriae, expression is optimal at a temperature of 37°C, a pH ranging from 6.5 to 8.0, reflecting the intestinal conditions, and at the end of the exponential growth (Jacobs et al., 1985; Mooi and de Graaf 1985; Van Verseveld et al., 1985; Blomberg, 1991; Payne et al., 1993). This regulation of fimbrial expression is mediated by several factors like global regulators, local regulators, crosstalk between fimbriae and posttranscriptional mechanisms.

2.3.1. Global regulators

Global regulators control the expression of a variety of operons. The global regulators that affect fimbriae biosynthesis are leucine-responsive regulatory protein (Lrp) (Braaten et al., 1991 and 1992; Huisman et al., 1994 and 1995; van der Woude et al., 1994 and 1995; Nou et al., 1995), the DNA methylating enzyme deoxyadenosine methylase (Dam) (Blyn et al., 1990; Göransson et al., 1990; Braaten et al., 1991 and 1994; van der Woude and Low, 1994; Huisman and de Graaf, 1995), the histone-like protein (Göransson et al., 1990; Jordi et al., 1992, Olsen et al., 1994; Atlung et al., 1997; Olsen et al., 1998; White-Ziegler et al., 1998 and 2000), integration host factor (Dorman et al., 1987; Eisenstein et al., 1987) and catabolite gene activator protein (Baga et al., 1985; Göransson et al., 1989; Forsman et al., 1992). However, little is known about the exact influence of these global regulators on F4 expression.

2.3.2. Local regulators

Local regulators are encoded by a fimbrial operon of which they subsequently regulate the expression. The local regulators of the F4 operon are FaeA and FaeB. Two IS*1* insertion sequences (IS*1*(A) and IS*1*(B)) are found between *faeA* and *faeB*, which are independently inserted in the regulatory region of the F4 operon (Fig. 2). In the *fas* operon encoding the genes for the biogenesis of 987P fimbriae, insertions of IS*1* were found to turn on adjacent genes, thereby permitting a cryptic promoter sequence to function (Klaasen et al., 1990). However, the IS*1*(A) insertion in the F4 operon was shown to only have a very limited effect on the level of *faeA* transcription (Huisman et al., 1994). Indeed, the putative original *faeA* promoter is suggested to be separated from the FaeA coding region by IS*1*(A), whereafter transcription of *faeA* became directed from the IS*1*(A) sequence upstream of the gene. Upstream of *faeB*, a putative promoter region is detected, with –35 and –10 regions resembling a typical σ^{70} promoter (Bakker et al., 1991b). The activity of the *faeB* promoter determines not only the FaeB expression, but also the level of F4 fimbriae expression since no significant promoter activity was observed in the non-coding regions between other *fae* genes downstream of *faeB* (Mooi et al., 1981 and 1986; Huisman et al., 1994, Verdonck, unpublished data).

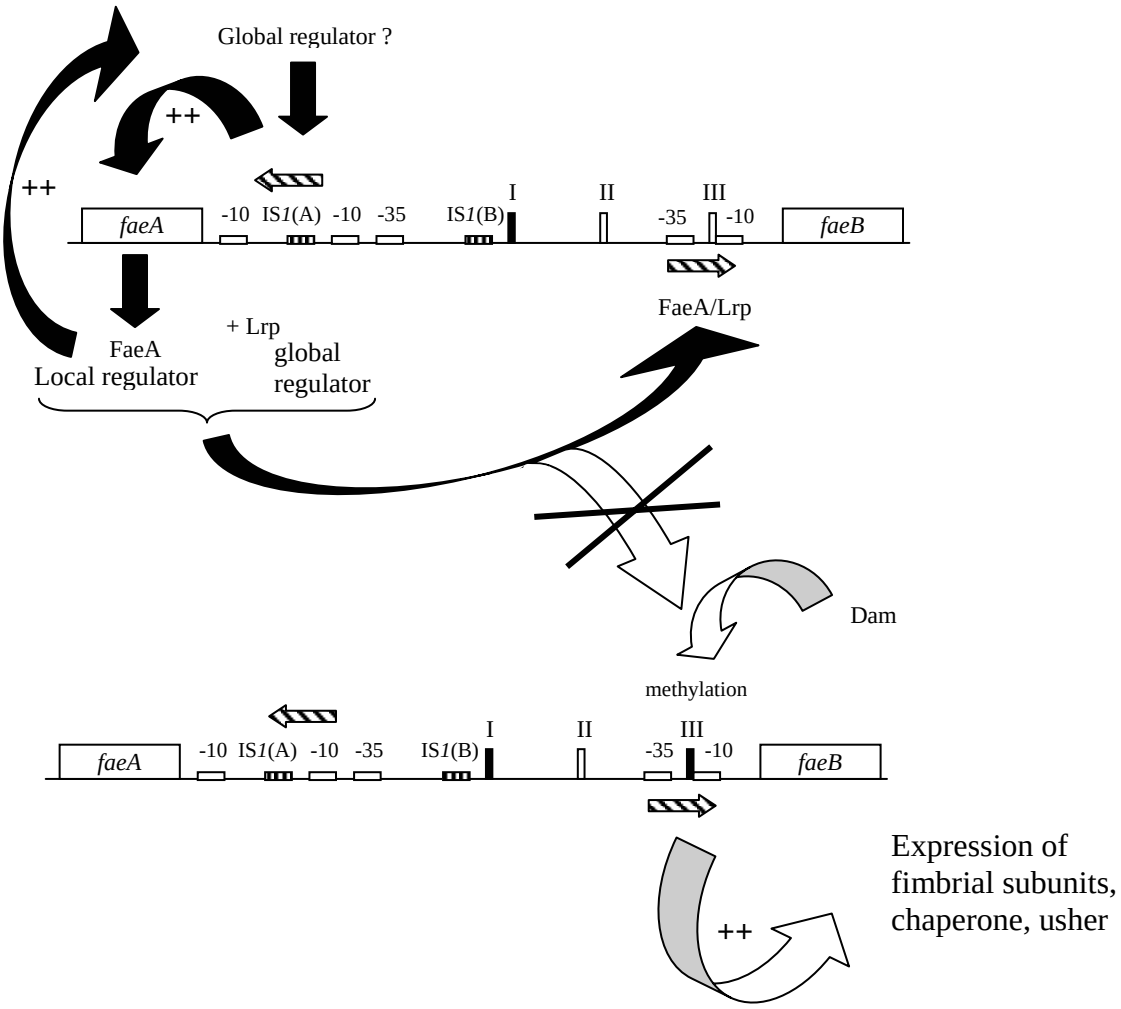


Figure 2 : The regulation of F4 expression is regulated by competition between Dam and Lrp, FaeA or Lrp/FaeA to bind to $GATC^{631}$ (symbol III). Binding of Dam results in methylation of $GATC^{631}$ and subsequent expression of the F4 fimbrial subunits, whereas binding of Lrp, FaeA or Lrp/FaeA inhibits transcription of the F4 open reading frame. A more detailed explanation can be found in the text.

The expression of *faeA* is positively controlled by an autoregulation mechanism of FaeA, whereas this protein represses F4 expression from the *faeB* promoter (Fig. 2) (Huisman et al., 1994). On the other hand, mutations in *faeB* had no effect on F4 fimbriae production (Huisman et al., 1994 and 1995). Since the FaeB homologue of P pili preferentially binds sequences upstream of the promoter of the FaeA homologue in P pili and stimulates transcription of the latter (Forsman et al., 1989; Göransson et al., 1989), Huisman et al. (1995) suggest that the effect of FaeB is probably absent because of the disruption of the original *faeA* promoter region by the two IS*1* insertion sequences.

The *fae* operon contains two conserved GATC-sites ($GATC^{497}$ and $GATC^{600}$) between the regulatory *faeA* and *faeB* genes (Huisman et al., 1994; Bakker et al.,

1991b). GATC-sites has been shown to play an essential role in the regulation of several fimbrial operons, for instance those encoding P, F1845, CS31A and S fimbriae (Blyn et al., 1990; Bilge et al., 1993; Morschhäuser et al., 1993; Martin, 1996; Hale et al., 1998). GATC-sites are important target sites of methylation by Dam, influencing DNA transcription. The region containing the $GATC^{497}$ (symbol I in Fig. 2) *in vivo* is always methylated, whereas $GATC^{600}$ (symbol II in Fig. 2) is non-methylated and this independent of the presence of FaeA (Huisman and de Graaf, 1995). In contrast to other fimbrial operons, an additional third GATC site ($GATC^{631}$; symbol III in Fig. 2) is present in the F4 operon (Huisman et al., 1994). $GATC^{631}$ methylation is variable *in vivo*. When this site is methylated, Lrp, FaeA or, most likely, Lrp/FaeA cannot bind to the region encompassing this site, resulting in F4 biosynthesis (Fig. 2). On the other hand, when it is not methylated, Lrp, FaeA or Lrp/FaeA can bind, resulting in a subsequent reduction of fimbriae production. Therefore, F4 expression in *E. coli* is carefully balanced mainly by the methylation status of $GATC^{631}$ (Huisman et al., 1994 and 1995). Perhaps, the methylation status of $GATC^{631}$ is regulated by competition between Dam and Lrp, FaeA or Lrp/FaeA to bind this site since competition between Dam and Lrp to bind a GATC site in the P pilus regulatory region is reported to influence P pilus expression (Hale et al., 1998), but further experiments are needed to confirm or deny this hypothesis.

Some additional differences are reported in the regulation of F4 fimbriae in comparison with other fimbrial systems. First, the expression of F4 fimbriae is not regulated by phase variation, a process by which individual cells switch between expression (phase ON) and non-expression (phase OFF) states (Blomfield, 2001). This is even in contrast to the F4-related CS31A fimbriae (Crost et al., 2003). Second, FaeA interacts with Lrp to suppress the F4 fimbriae production, whereas the FaeA homologue of P pili, S and F1845 fimbriae bind with Lrp to induce fimbriae expression (Baga et al., 1985; Van der Woude et al., 1992 and 1994; Braaten et al., 1994; Huisman and de Graaf, 1995). The opposite activity of these regulators is likely the result of the binding of these regulators at different locations in the respective operons (Huisman et al., 1994 and 1995; Kaltenbach et al., 1995; Nou et al., 1995; van der Woude et al., 1995).

2.3.3. Regulatory crosstalk between fimbriae

A third mechanism that controls fimbrial expression is crosstalk between fimbrial operons. For instance, when *faeA* is inserted in the F5 operon, FaeA functions as a repressor on F5 fimbrial biosynthesis (Bakker et al., 1991b). In contrast, FaeB has no influence on type 1 phase switching (Holden et al., 2001). Probably, type 1 fimbriae and F4 fimbriae are non-communicating fimbrial systems as they are expressed under different environmental conditions (Huisman et al., 1994; Roesch et al., 1998). There is no information on crosstalk between F4 fimbriae and other fimbriae.

2.3.4. Post-transcriptional regulation

Only a low percentage of the surface located fimbrial proteins are minor proteins. The relative concentration of each subunit type in the periplasm is shown to be an important factor in fimbriae assembly (Jacob-Dubuisson et al., 1993), which fits with the observation that the major subunits of fimbriae are produced in much larger amounts than the minor subunits (Schembri et al., 2002).

(Post)-transcriptional regulation is supposed to determine the level of expression of the different F4 fimbrial proteins, since it is thought that only one polycistronic mRNA from *faeB* to *faeJ* is produced (Rosenberg et al., 1979). In several other fimbrial systems, gene expression is controlled by processing and differential degradation of mRNA (Baga et al., 1988; Bilge et al., 1993; Jordi et al., 1993; Morschhäuser er al., 1993; Hacker and Morschhäuser, 1994; Bricker and Belasco, 1999; Schembri et al., 2002). Most fimbrial transcripts terminate directly downstream from the major subunit genes (Baga et al., 1987; Morschhäuser et al., 1993) but the localization of *faeG* in the F4 operon suggests that this is not likely in the F4 operon (Fig. 1).

2.4. Biogenesis of F4 fimbriae

The assembly of F4 fimbriae can be divided into three distinct stages : translocation of the fimbrial subunits across the inner membrane, interaction of the fimbrial subunits with the chaperone in the periplasm and translocation of the fimbrial subunits across the outer membrane by the outer membrane usher.

2.4.1. Translocation of fimbrial subunits across the inner membrane

The N-termini of fimbrial subunits that take part in the fimbrial biogenesis harbour characteristic features of signal sequences that mediate translocation across the cytoplasmic membrane (Nielsen et al., 1997; Pugsley et al., 1997). Highly conserved signal sequences are present in the subunits of F4 fimbriae and the related CS31A and F41 fimbriae (Girardeau et al., 1991). A serine(Ser)-flanked cryptic consensus sequence forming an alpha-helical conformation in the hydrophobic region of the signal sequence, mediates the accessibility of the cleavage site to signal peptidase I (Der Vartanian et al., 1994; Pugsley et al., 1997) (Fig. 3).

FaeC 6 LACVFFLTGGGVSHA 20

FaeD 23 VMSAVLGSASVIA 35

FaeE 22 TLALMMTCQSAMA 34

FaeF 10 LVLSALSIQSALA 22

FaeG 6 IALAIAASAASGMAHA 21

FaeH 12 SAIISVALFYSAA 24

FaeI 8 LFAASLLPSCVLA 20 ↑

Figure 3 : Part of the signal sequences of the F4 fimbrial proteins containing the serine(S)-flanked consensus sequence (underlined) and the processing site (arrow). The number represents the amino acid residue position.

Once Fae subunits are translocated by the general secretory pathway into the periplasmic space (Dodd and Eisenstein, 1984; Pugsley et al., 1997), they probably interact with the F4 fimbrial chaperone FaeE. Indeed, FaeE seems capable to interact with the signal sequences of F4-fimbrial subunits as well as with their native form. The signal sequences of the F4 fimbrial minor and major subunits, except FaeC, contain a Ser-flanked segment near the peptide cleavage site (Fig. 3). In contrast, there is no Ser-flanked region in the chaperone FaeE signal sequence. This suggests that FaeE can be involved in the Ser-flanked sequence recognition and subsequently ameliorate the accessibility of the cleavage site to signal peptidase I. This is in agreement with the P pilus chaperone PapD that aids in the release of the pilin subunits from the inner membrane (Hultgren et al., 1989). Indeed, 80% of P pili subunits remained anchored to the inner membrane in the absence of their chaperone (Jones et al., 1997).

The C-terminal sequences of the fimbrial subunits are extremely hydrophobic (Klemm, 1981; Kuehn et al., 1993; Hung et al., 1996) and may insert into the cytoplasmic membrane. Alternatively, the C-terminus may be part of an edge strand of a β-sheet that lies on top of the membrane, inserting the hydrophobic side chains of its amino acid residues into the membrane. As a result, the C-terminus cannot interact with the chaperone. It is suggested that the P-pilus chaperone PapD recognizes a binding site on the P pilus subunit PapG separate from the C-terminus (Xu et al., 1995), triggering a conformational change in the subunit that results in exposure of its C-terminus to the chaperone (Hung et al., 1999b). This mechanism of interaction, suggests that subunits change their membrane-associated state directly to a chaperone-associated state in the periplasm (Soto et al., 1998; Thanassi and Hultgren, 2000b).

2.4.2. Interaction of fimbrial subunits with their chaperone FaeE in the periplasm

2.4.2.1. Donor strand complementation

The interaction between fimbrial subunits and their specific chaperone has been most intensively studied in the P- and type 1 pilus systems. Crystallography of the P pilus chaperone PapD revealed two globular domains with an immunoglobulin(Ig)-like fold (Holmgren and Bränden, 1992). These domains are oriented towards one another in a way that gives the molecule the overall shape of a

boomerang (Kuehn et al., 1993). The crystal structure of the PapD-PapK chaperone-subunit complex indicated that the PapK subunit had an incomplete Ig-like fold, lacking the C-terminal seventh β-strand G (Sauer et al., 1999). In the PapD-PapK complex, the chaperone donates its G_1β-strand to complete the Ig-fold of the PapK subunit (Fig. 4). This mechanism is called 'donor strand complementation'. However, an atypical Ig fold is produced as the donated strand runs parallel to the C-terminal strand F of PapK rather than anti-parallel in a typical Ig fold. The interaction of the type 1 FimC-FimH chaperone-adhesin complex was found to be identical to that of the PapD-PapK complex (Choudhury et al., 1999).

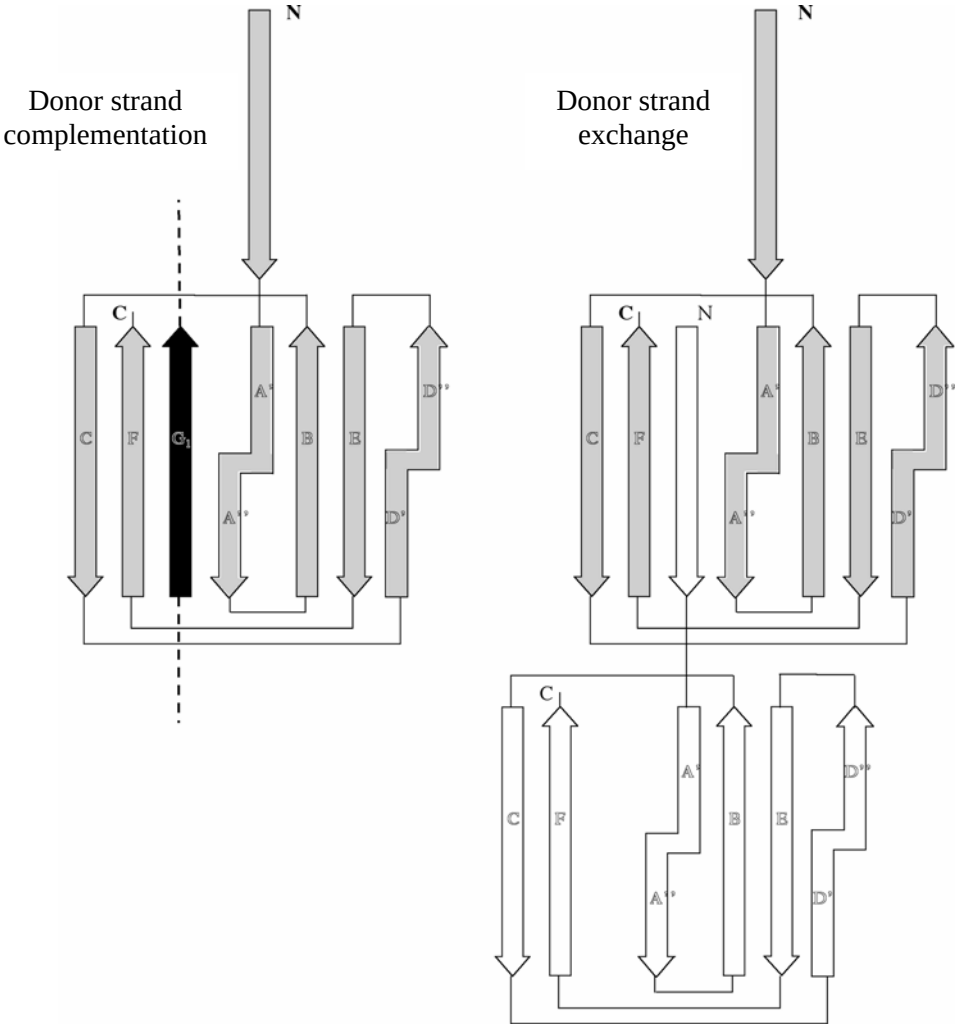


Figure 4 : Model for the molecular interactions between subunit and chaperone (donor strand complementation) or between two subunits (donor strand exchange). The C- and N-termini of the subunits are indicated, as well as the names of the β-strands. The names of the strands are according to the P-pilus subunits (according to Justice et al., 2003).

The crystal structure of the F4 fimbrial chaperone FaeE has not been elucidated to this moment. However, sequence alignment reveals that the functional important amino acid residues Arg8, Thr53, Arg68, Glu83, Lys112, Arg116, Gly167, Met172 and Asp196 of PapD are conserved in FaeE (Slonim et al., 1992; Kuehn et al., 1993; Hung et al., 1999a). Alternating hydrophobic amino acid residues were found in FaeE (Ile99, Val101 and Leu103) at the same position as alternating hydrophobic residues found in the $G_1\beta$-strand of PapD (Leu103, Ile105 and Leu107). The presence of these conserved amino acid residues in FaeE suggests that a donor strand complementation mechanism may also be involved in F4 biogenesis.

During donor strand complementation, the alternating hydrophobic amino acid residues of the PapD $G_1\beta$-strand bind to a region composed of alternating hydrophobic amino acid residues at the C-terminus of each P-pilus subunit (Hultgren et al., 1989, Kuehn et al., 1993; Sauer et al., 1998 and 2000). In line with this typical mechanism of donor strand complementation, most Fae subunits constitute such conserved C-terminus flanked by a glycine at position –14 from the C-terminus and a penultimate tyrosine, which may allow their interaction with FaeE (Soto et al., 1998). However, two Fae subunits, FaeF and FaeC, do not interact with FaeE. The carboxyl-terminal structure of FaeF does not match with the consensus sequence for chaperone binding (Fig. 5), which perhaps may explain the lack of interaction between FaeE and FaeF (Mol et al., 2001). The C-terminus of FaeC is in reasonable agreement with the consensus sequence, but some specific differences are apparent. The alanine at position –9 is specific for FaeC and differs from the residues in the other subunits. In

FaeC : **G**E**Y**S**G**A**L**T**F**V**V**T**Y**Q

FaeF : SYRGNLQI**A**LQVED

FaeG : TQ**W**S**A**P**L**N**V**A**I**T**Y**Y

FaeH : AR**W**Q**A**G**L**N**V**T**V**TVQ

FaeI : ER**W**R**V**S**L**P**V**S**I**E**Y**Q

FaeJ : KR**W**Q**G**N**L**T**P**V**V**V**Y**F

Figure 5 : The C-termini of the F4 fimbrial subunits. The conserved alternating hydrophobic amino acid residues are indicated (bold symbols).

addition, the negatively charged glutamic acid residue at position –13 and the positively charged lysine residue at –16 are quite unique at these positions. These changes may possibly explain the absence of interaction with FaeE (Mol et al., 2001).

A second site of interaction between PapD and PapK during donor strand complementation was found at the 3_{10}C-helix in the center of PapK (Sauer et al., 1999). A similar region was also found in the center of the F4 fimbrial adhesin FaeG and probably serves to interact with the chaperone FaeE (Gaastra et al., 1983; Verdonck et al., 2004c).

It is noteworthy that all fimbrial operons express their own chaperone, suggesting that the fimbrial chaperones bind their subunits in a sequence dependent manner (Kuehn et al., 1993; Bonci et al., 1997). In agreement with this, FaeE cannot replace the F5 fimbrial chaperone FanE in F5 fimbrial biosynthesis and vice versa (Bakker et al., 1991a).

2.4.2.2. FaeE functions as dimer

Gel filtration experiments combined with protein cross-linking analysis and a biophysical approach in which the rotation diffusion coefficient of purified FaeE was determined, indicate that FaeE functions as a homodimer (Mol et al., 1994 and 1996b). Indeed, heterotrimeric complexes, consisting of two subunits of FaeE on the one hand and one subunit of FaeG, FaeH or FaeI on the other hand, are present in the periplasm (Mooi et al., 1983; Mol et al., 1994 and 1995). This characteristic of FaeE is unique to all fimbrial chaperones. Although, PapD and the S fimbrial chaperone SfaE were also found as dimers, albeit only transiently when they are not engaged in binding to subunits (Hung et al., 1999b; Knight et al., 2002), Walse et al. (1997) could not detect PapD dimers or higher aggregates in solution, indicating that the dimer observed in the crystal was an artefact caused by crystal packing. Studies using hybrids of FaeE and the K99 chaperone FanE revealed that the N-terminal domain of FaeE determines the dimerization of the protein (Bakker et al., 1991a). Indeed, although FaeE has 9 amino acids more in comparison to PapD and 18 to 19 amino acid residues in comparison with several other fimbrial chaperones (Holmgren et al., 1992), truncation of the 19 C-terminal amino acid residues of FaeE did not influence FaeE dimerization or F4 biogenesis (Mol et al., 1996a).

2.4.2.3. Chaperone-subunit complexes are folded native-like and prevent subunit degradation and premature polymerisation

The fimbrial subunits exist in a native-like folded state in the chaperone-subunit complex. For instance, the periplasmic complex of FaeG and FaeE is able to agglutinate erythrocytes (Mol et al., 2001) and is recognised by monoclonal antibodies directed against conformational epitopes of F4 fimbriae (Bakker et al., 1991a). This observation is in agreement with other fimbrial systems of which chaperone-adhesin complexes bind to their receptor (Kuehn et al., 1991; Mahmood et al., 2000; Zhou et al., 2001). However, expression of most fimbrial subunits also needs the periplasmic disulfide isomerase DsbA that catalyses disulfide bound formation (Jacob-Dubuisson et al., 1994a; Hung and Hultgren, 1998), but FaeG does not have cysteine residues (Klemm, 1981).

The highly folded chaperone-subunit complexes prevent degradation of the fimbrial subunit. Indeed, *faeE* deletion mutants do not contain FaeG subunits, whereas the FaeG precursor is not affected (Mooi et al., 1982). Also type 1 and P pilus subunits are misfolded and unstable in the absence of their chaperone or the missing strand and collapse into off-pathway aggregates that are proteolytically degraded (Tewari et al., 1993; Bullitt et al., 1996; Barnhart et al., 2000). It is reported that the presence of unfolded PapG subunits stimulates transcription of the periplasmic protease DegP by activating the Cpx, σ^E and BaeSR modulator pathways (Jones et al., 1997; Hung et al., 2001; Raffia and Ravioli, 2002). In agreement, DegP degrades FaeG subunits in the absence of its chaperone FaeE (Bakker et al., 1991a). In the case of the P-pilus subunit PapA, the conserved C-terminal strand is exposed to the solvent in the absence of the chaperone and activates the DegP protease, although it is not a cleavable substrate for DegP. The preferential cleavage sites occur between paired hydrophobic amino acids in three separate regions of PapA (Jones et al., 2002).

Another function attributed to the FaeE chaperone is to prevent polymerisation of the F4 fimbrial subunits in the periplasm (Mooi et al., 1983), which is in accordance with observations made in other fimbrial systems (Kuehn et al., 1993, Bullitt et al., 1996; Thanassi and Hultgren, 2000a). Indeed, Bakker et al. (1991a) reported the presence of FaeG multimers containing conformational epitopes in *degP/faeE* double deletion mutants. On the other hand, the P pilus chaperone PapD is necessary to assemble adhesive P-pili *in vivo* because less than 10% of the P pilus

PapG subunits recovered from periplasmic extracts of a *degP*/*PapD* double mutant strain bind to their receptor (Jones et al., 1997; Sauer et al., 2002).

2.4.3. Translocation of fimbrial subunits across the outer membrane

2.4.3.1. The F4 fimbrial usher FaeD

During assembly of F4 fimbriae, the chaperone-subunit complexes are targeted to the outer membrane usher FaeD (Van Doorn et al., 1982). This protein has a high content of glycine and serine residues, in agreement with the ushers of type 1 pili, P pili and several other fimbrial systems (Kuehn et al., 1994). In addition, FaeD contains four conserved cysteine residues, two at the N-terminus and two at the C-terminus of the proteins (Schifferli and Alrutz, 1994).

Two folding models propose an amino-terminal periplasmic domain of 126 (Harms et al., 1999) or 133 (Valent et al., 1995) amino acid residues for FaeD respectively. Deletion mutations in this domain did not alter the localization or stability of the usher, but inhibited fimbriae biosynthesis. The central domain contains 22 (Harms et al., 1999) to 24 (Valent et al., 1995) membrane spanning β-strands. The carboxyl-terminal periplasmic domain is predicted to contain 167 (Valent et al., 1995) or 255 (Harms et al., 1999) amino acid residues and is proposed to be important for the structural organisation of the protein as well as for its functioning in fimbriae biosynthesis (Harms et al., 1999).

2.4.3.2. Formation of the initiation complex

FaeD is involved in the translocation of the fimbrial subunits across the outer membrane and for the anchoring of the fimbrial structure to the outer membrane (Mooi et al., 1983; de Graaf and Mooi, 1986) (Fig. 6). It is not clear how the ordered assembly of F4 fimbrial subunits is accomplished, but difference in the relative affinities of the various subunits towards FaeD probably play an important role in this process. A direct interaction of FaeC with the 215 amino-terminal amino acid residues of FaeD has been shown to occur which is not the case for other F4 fimbrial subunits (Mol et al., 2001). It is therefore likely that FaeC is the first protein that interacts with FaeD in the process of fimbriae biosynthesis, especially since this subunit is present at the tip of the fimbriae (Oudega et al., 1989). In type 1 and P pili, the relative affinity of each chaperone-subunit complex for the usher correlates with the final position of

the respective subunit in the pilus (Dodson et al., 1993; Jacob-Dubuisson et al., 1993; Saulino et al., 1998). In addition, it is discovered that the information necessary for targeting the chaperone-subunit complex to the usher resides mainly in the subunit protein (Barnhart et al., 2003; Nishiyama et al., 2003).

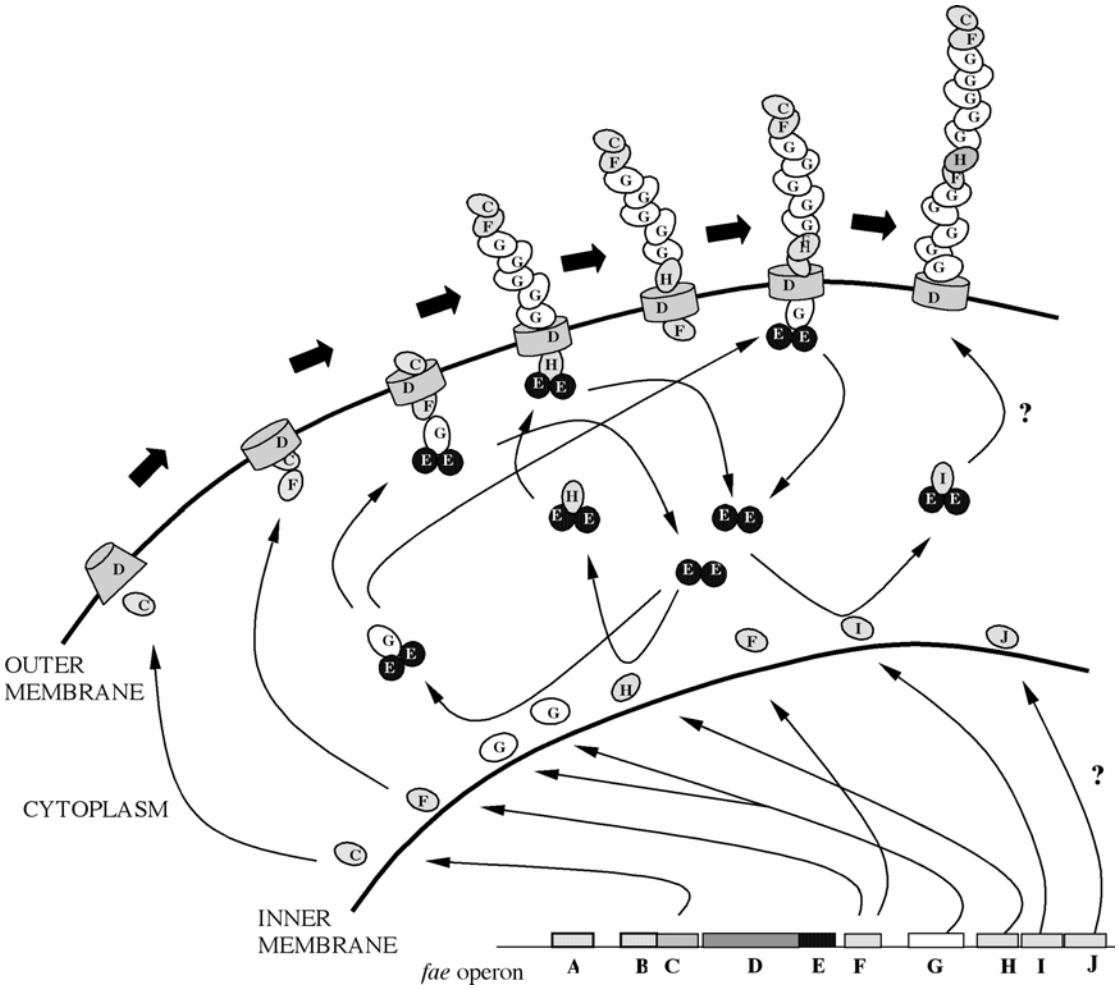


Figure 6 : Expression of the F4 fimbrial subunits and subsequent assembly of F4 fimbriae. The chaperone FaeE transports the subunits, except FaeC and FaeF, to the outer membrane usher FaeD where they are incorporated in the growing fimbriae. Thereafter, the chaperone FaeE recycles in the cytoplasm to transport another subunit. A more detailed explanation on the F4 fimbrial assembly can be found in the text. (Based on Van den Broeck et al., 2000)

In the P pilus, binding of the PapD-PapG chaperone-adhesin complex to the PapC usher alters the conformation of the latter and stabilizes it in an assembly-competent structure allowing initiation of pilus assembly (Saulino et al., 1998).

Probably, this also happens with the F4 fimbrial usher FaeD as the protease sensitivity of FaeD alters in the presence or absence of F4 fimbrial subunits (Valent et al., 1995). After binding of the F4 fimbrial tip subunit FaeC, FaeF binds the FaeD-FaeC complexes, resulting in the formation of the initiation FaeD-FaeC-FaeF complexes (Fig. 6) (Mol et al., 2001). FaeF functions as adaptor molecule, coupling the major subunit FaeG to the tip subunit FaeC. At regular intervals, the minor subunits FaeF, FaeH and probably also FaeI are incorporated in the F4 fimbrial structure (Bakker et al., 1992b).

2.4.3.3. Subunit-subunit interaction

In several fimbrial systems, the mechanism of interaction between subunits relies on the highly conserved N-terminus of the subunits. This N-terminal amino acid sequence contains an alternating hydrophobic amino acid residue motif that is analogous to the "G" β-strand of the chaperone and is exposed in chaperone-subunit complexes (Soto et al., 1998; Choudhury et al., 1999; Sauer et al., 1999; Zavialov et al., 2002). Such alternating hydrophobic amino acid residues are also present in the F4 fimbrial subunits. Moreover, the N-terminus of FaeG is very conserved between the three F4 antigenic variants (Gaastra et al., 1983; Verdonck et al., 2004c). Therefore, like for several fimbrial systems, the subunit-subunit interactions between F4 fimbriae are also supposed to rely on the mechanism of donor strand exchange (Barnhart et al., 2000; Knight et al., 2000). This mechanism is best characterized in P pili and type 1 fimbriae of *E. coli* and in F1 fimbriae of *Yersinia pestis* (Soto et al., 1998; Barnhart et al., 2003; Zavialov et al., 2003a and 2003b) (Fig. 4). During assembly of these fimbriae, the "G" β-strand of the chaperone is exchanged for an N-terminal extension from the subunit of an incoming chaperone-subunit complex. The incoming N-terminus during donor strand exchange runs anti-parallel to the subunit "F" β-strand, in contrast to the "G" β-strand of the chaperone. So, the subunit undergoes a topological transition from a non-canonical to a canonical Ig fold, resulting in more closed conformation of the groove and driving subunit assembly into a fiber (Sauer et al., 2002; Barnhart et al., 2003; Zavialov et al., 2003b). These energetically favourable conformational changes during pilus assembly are very important since the mode of action of fimbrial chaperones is independent of cellular energy (Jacob-Dubuisson et al., 1994b).

2.4.3.4. Translocation of subunits across the outer membrane

The mechanism of translocation of F4 fimbrial subunits across the outer membrane is not determined. Thanassi et al. (1998) showed that the P-pilus usher PapC forms an oligomeric channel. This channel, probably a hexamer, has a diameter of approximately 2 nm and is large enough to accommodate a pilus subunit or the linear form of the pilus (Saulino et al., 2000). Similarly, in type IV pilus biogenesis, large oligomeric structures are found that consist of 10-12 monomers with a large central diameter of approximately 5.3 nm (Bitter et al., 1998). However, Harms et al. (1999) did not detect oligomeric FaeD complexes. Possibly, the putative quaternary structure of FaeD in the outer membrane is less stable or FaeD is different from the other molecular ushers studied so far in that it does not function as an oligomer. Indeed, the estimated copy number of FaeD per cell equals the number of fimbriae per cell (Mooi et al., 1981) and does not support a model in which the functional FaeD unit in the outer membrane contains 6 or 10-12 FaeD subunits.

2.5. The major subunit FaeG mediates F4-receptor binding

Although the adhesin of most fimbriae is a minor subunit located at the tip, the adhesin of F4 fimbriae is the major subunit FaeG. The receptor-binding site of FaeG has not been exactly characterised at the moment. The three F4 antigenic variants F4ab, F4ac and F4ad show small differences in the amino acid sequence of their adhesin FaeG (Guinée and Jansen, 1979; Mooi et al., 1979; Bakker et al., 1992a). The epitopes a1 to a7 are common epitopes and are present on two or on all three antigenic different FaeG subunits, whereas each of the three antigenic different FaeG subunits has a variant-specific “b”, “c” or “d” epitope (van Zijderveld et al., 1990). Although conserved common regions in FaeG are suggested to be involved in F4 receptor binding, the variant-specific epitopes mediate, at least partly, the binding of the three F4 antigenic variants to different receptors (Wilson and Hohmann, 1974; Jacobs et al., 1987; Bakker et al., 1992a; Jin and Zhao, 2000; Sun et al., 2000). Indeed, six different porcine F4-receptor phenotypes can be distinguished based on the variability of the three F4 antigenic variants to adhere to porcine intestinal brush borders (Bijlsma et al., 1982; Baker et al., 1997).

In F4 fimbriae, the major subunit FaeG functions as adhesin and presents receptor-binding sites at different places along the fimbriae (Bakker et al., 1992a; Van den Broeck et al., 1999c). In contrast, the adhesins of P pili, type 1 pili and F17 are minor subunits that are only present at the tip. Moreover, these tip located adhesins have twice the molecular weight of the other fimbrial subunits as they are composed of an N-terminal lectin domain that mediates the receptor binding and a C-terminal pilin domain that couples the adhesin to the fimbrial shaft during assembly (Hultgren et al., 1989; Thankavel et al., 1997; Schembri et al., 2000; Dodson et al., 2001; Tanskanen et al., 2001; Hung et al., 2002; Buts et al., 2003a and 2003b; Merckel et al., 2003). The presence of a single adhesin subunit located at the tip of these fimbriae agrees with the lack of an N-terminal extension in the lectin domain and its resulting inability to mediate donor strand exchange with a preceding subunit (Hahn et al., 2002). The molecular weight of FaeG is not twice that of the other F4 fimbrial subunits and it is not clear to this time if FaeG is composed of two domains (Méchin et al., 1995).

2.6. Conclusion

In addition to characteristics that were identical to other *E. coli* fimbriae or pili, several unique mechanisms are discussed that are only present in F4 fimbrial induction, expression or assembly. Importantly, in contrast to the adhesins of most fimbrial systems, the F4 major fimbrial subunit FaeG also constitutes the adhesin. Therefore, the F4 fimbrial system could offer an opportunity to study the immunogenic potential of monomeric and multimeric adhesins.

Chapter 3

Heat-labile enterotoxin and cholera toxin : virulence factors and mucosal adjuvants

3.1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) colonize the small intestine, release their heat-labile (LT) and/or heat-stable (ST) enterotoxins and induce diarrhoea. Similarly, colonization of the small intestine by *Vibrio cholerae* and secretion of cholera toxin (CT) results in diarrhoea. Besides functioning as a toxin, both LT and CT function as mucosal adjuvants.

There are two major antigenic variants of LT, LT-I and LT-II, which do not cross-react immunologically (Nataro and Kaper, 1998). LT-I is highly similar to CT, whereas LT-II differs in amino acid sequence and carbohydrate binding specificities (Jobling and Holmes, 1991). The human (hLT-I) and porcine (pLT-I) variants of LT-I differ only by four amino acids (Domenighini et al., 1995) and in their binding to paragloboside (Karlsson et al., 1996). LT-I is further referred as LT. LT-II is not discussed in this review since its role in disease has not been established.

3.2. Toxin structure

The amino acid sequences of LT and CT are approximately 80% identical, resulting in similar three-dimensional structures (Fig. 1) (Dallas and Falkow, 1980; Sixma et al., 1991 and 1993; Zhang et al., 1995a and 1995b). The receptor-binding part is comprised of five identical B subunits that noncovalently associate into a highly stable pentameric ring (Cheesman et al., 2004). Each B-subunit (10.5 kDa) has a receptor-binding place but pentamerization improves receptor-binding (De Wolf et al., 1981a and 1981b; Iida et al., 1989; Streatfield et al., 1992; Jobling and Holmes, 2002). The A-subunit (27 kDa) is a single polypeptide comprised of two major domains (A1 and A2) that are linked by a surface exposed loop which contains a site for proteolytic cleavage and a single disulfide bond which bridges the cleavage site (Sixma et al., 1991; Spangler 1992; Zhang et al., 1995a and 1995b). The A1-peptide contains an ADP-ribosyltransferase active pocket that binds NAD and catalyses ADP ribosylation of Gs_{α} (Gill and King, 1975) and the A2-peptide non-covalently anchors the A subunit to the central cavity of the B pentamer (Sixma et al, 1991; Tinker et al., 2003). The C-terminal motif of A2 protrudes from the pentameric B-subunit on the side that binds the cell surface (Zhang et al., 1995a and 1995b).

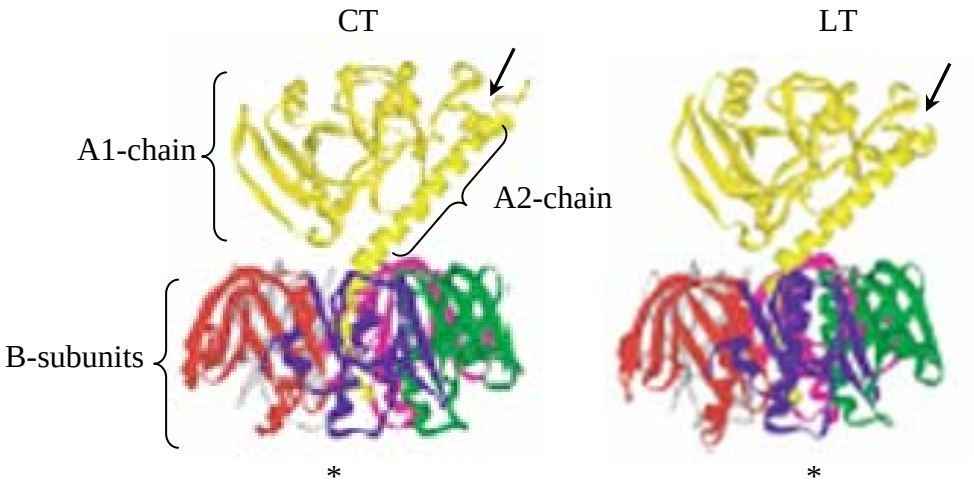


Figure 1 : Crystal structures of cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxins (LT). The A1- and A2-chains are linked by a flexible loop (not resolved in the crystal structure) that contains a proteolytic cleavage site subtended by a disulphide bond. Arrows mark the site of the loop and proteolytic cleavage. The K(R)DEL motif is indicated by an asterisk. (Figure obtained from W.I. Lencer and W. Hol).

3.3. Toxin expression and assembly

Chromosomal genes of *V. cholerae* encode CT (Vasil et al., 1975; Mekalanos et al., 1983; Miller and Mekalanos, 1984), whereas the genes encoding LT in *E. coli* are plasmid borne (So et al., 1978; Dallas et al., 1979). They both belong to the family of the AB_5 toxins, characterized by an enzymatically active A subunit and five receptor binding B subunits (Fig. 1) (Merrit and Hol, 1995). The genes for the A and the B subunits are transcribed in a single mRNA (Dallas et al., 1979; Spicer et al., 1981; Finkelstein And LoSpalluto, 1987) and expression of excess B over A subunits is the result of a more efficient ribosome-binding site of the B subunit (Mekalanos et al., 1983).

The A and B subunits are synthesized in the cytoplasm and co-translationally transported to the periplasm, where assembly of the AB_5 holotoxin takes place (Holmes and Twiddy, 1983; Hirst and Holmgren 1987a; Hardy et al., 1988; Streatfield et al., 1992). In contrast to CT (Finkelstein et al., 1970; Richardson andNoftle, 1970), LT is not secreted but remains in the periplasm or is located on the surface of LT-producing bacteria (Wensink et al., 1978; Kunkel and Robertson, 1979; Hirst et al., 1984; Horstman and Kuehn, 2000 and 2002; Tauschek et al., 2002).

Furthermore, the A subunit of CT (CT-A) is activated by a *V. cholerae*-encoded protease (Booth et al., 1984), whereas the A subunit of LT (LT-A) is nicked by proteases in the intestinal lumen (Clements and Finkelstein, 1979).

3.4. Toxin receptors

Receptor binding is critical for the biological effects of CT and LT. The main receptor for both LT-B and CT-B is GM1-ganglioside [Gal(β1-3)GalNAc(β1-4)(NeuAc(α2-3))Gal(β1-4)Glc(β1-1)ceramide], a glycosphingolipid found ubiquitously on the surface of mammalian cells (Lencer et al., 1992). Hydrogen bond interactions within each of five pockets formed by the B-subunit pentamer allow CT-B and LT-B to cross-link GM1 with extremely high affinity for GM1 (dissociation constants of 7.3×10^{-10} and 5.7×10^{-10} for LT-B and CT-B, respectively) (Kuziemko et al., 1996; Turnbull et al., 2004). In addition, both toxins interact with ganglioside-GD1b and LT-B also binds with lower affinity to polyglycosylceramides, asialo-GM1, GM2 and polylactosamine-containing glycoproteins (Holmgren et al., 1982; Fukuta et al., 1988). After toxin binding to intact cells, there is a lag time of 15 to 60 min before adenylate cyclase is activated (Gill and King, 1975). The lag time is necessary to allow the A1 peptide to translocate through the membrane and to come into contact with the Gs protein.

3.5. Mechanism of toxin action

LT and CT bind with their B subunits to GM1 at the cell surface, resulting in the association of toxin with specialized membrane microdomains rich in cholesterol and glycosphingolipids, known as lipid rafts (Orlandi, 1993 and 1998; Wolf et al., 1998 and 2002; Shogomori and Futerman, 2001a and 2001b). The toxin is endocytosed by invagination of the plasma membrane, forming pits that can be non-coated or coated with clathrin on their cytosolic surface (Shogomori and Futerman, 2001a and 2001b; Parton et al., 1994a and 1994b; Schnitzer et al., 1995; Nichols et al., 2001; Le and Nabi, 2003; Singh et al., 2003). The pits invaginate into the cell and pinch off to form (clathrin-coated) vesicles. Following internalisation, the vesicles shed their coat (if present) and are able to fuse with early endosomes. Then, the toxin moves via early and recycling endosomes into the trans-Golgi (Tran et al., 1987; Parton et al., 1994; Schnitzer et al., 1996; Henley et al., 1998) (Fig. 2). After

subsequent dissociation of the A and the B subunit (Fig. 2), the A subunit undergoes retrograde transport to the endoplasmic reticulum (ER), while the B subunit recycles from the Golgi compartment to late endosomes and lysosomes (Cosson and Letourneur, 1994; Bastiaens et al., 1996; Majoul et al., 1996 and 1998; Orci et al., 1997; Aoe et al., 1998; Richards et al., 2002; Chen et al., 2002a). The movement of the A-subunit from the Golgi to the ER is facilitated by a short, four-amin acid ER-sorting signal (KDEL in CT-A, RDEL in LT-A; Fig. 1), present at the C-terminus of the A-subunit (Cieplak et al., 1995b; Lencer et al., 1995b; Bastiaens et al., 1996; Majoul et al., 1996; Sandvig et al., 1996; Henley et al., 1998). In the Golgi network the specific membrane-bound receptor ERD2 binds to the ER-sorting signal, resulting in package of the A-subunit into special transport vesicles that are transferred to the ER (Pelham, 1991). In the ER, disulphide isomerase reduces the disulphide bond between the A1- and A2-fragments (Majoul et al., 1997; Orlandi, 1997). Subsequently, the A1-peptide behaves as a misfolded protein that exits the ER via reverse translocation through the sec61p complex and reaches the cytosol (Hazes and Read, 1997; Schmitz et al., 2000; Teter et al., 2002; Winkeler et al., 2003).

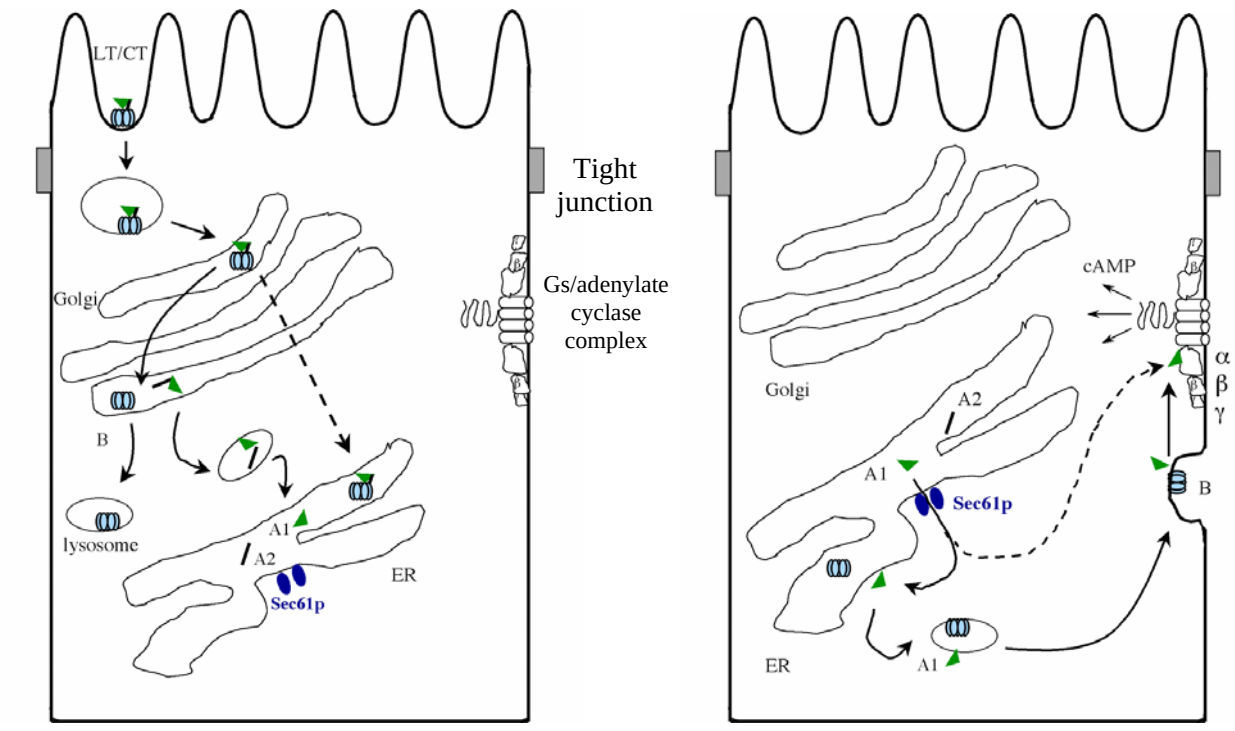


Figure 2 : Schematic representation of LT and CT internalisation (left) and induction of cAMP production (right). ER, endoplasmic reticulum.

Alternatively, Lencer et al. (2001) support the view that the B subunit would be the transport vesicle for the A subunit and that the A and B subunits of the holotoxin only dissociate when they have reached the ER (Fig. 2) (Tsai et al., 2001; Fujinaga et al., 2003). In this alternative model, the B subunit is not unfolded, remains membrane associated and moves to the basolateral membrane by traffic back out the secretory pathway by anterograde vesicles (Lencer et al., 1995b).

The events following A1-peptide translocation to the cytosol that lead to binding of the A1-peptide to the basolateral located $Gs_{\alpha\beta\gamma}$ complex, remain incompletely defined. A first possible mechanism (Fig. 2) is that the A1-peptide diffuses through the cytosol and escapes from degradation by the proteasome due to the absence or near absence of lysines in the toxic peptide, together with its ability to rapidly refold (Rodighiero et al., 2002). Lysine residues are targets for covalent addition of ubiquitin, a peptide typically required for proteasome-dependent degradation. It is however not clear how the A1-peptide is subsequently targeted to the Gs/adenylate cyclase complex. A second potential mechanism (Fig. 2) is that the A1-peptide remains associated to the cytosolic side of the ER membrane following sec61p-mediated translocation and reaches the cytoplasmic surface of the basolateral membrane by membrane traffic back out the secretory pathway. In support of this mechanism, the A1-peptide binds *in vitro* with the family of GTP-binding ADP-ribosylating factors (ARF) that are involved in vesicular trafficking (Tsai et al., 1988; Stevens et al., 1999; Jobling and Holmes, 2000; Zhu et al., 2001a and 2001b). Indeed, initiation of vesicle formation occurs when a membrane protein interacts with the ARF-GDP complex and catalyses the release of GDP and the binding of GTP. The ARF-GTP form mediates the assembly of cytosolic coat proteins (coatomers) to membranes, inducing budding of the transport vesicles (Donaldson and Klausner, 1994; Boman and Kahn, 1995; Moss and Vaughan, 1995). Fusion of basolaterally-targeted vesicles with the plasma membrane delivers the translocated A1-peptide to a site near the Gs/adenylate cyclase complex on the cytosolic surface of the basolateral membrane.

The A1-peptide catalyses the transfer of the ADP-ribose moiety of NAD to a specific arginine residue of the basolaterally located G protein Gs_{α}. The Gs_{α} protein also contains a GTP binding site and an intrinsic GTPase activity (Hepler and Gilman, 1992). Binding of GTP to the Gs_{α} leads to dissociation of Gs_{α} from the $Gs_{\beta\gamma}$ dimer

(Kahn and Gilman, 1984) and subsequent increased affinity of Gs_α for adenylate cyclase. The resulting activation of adenylate cyclase continues until the intrinsic GTPase activity hydrolyzes GTP to GDP, thereby inactivating Gs_α and adenylate cyclase. However, ADP-ribosylation of Gs_α by the A1-peptide inhibits the hydrolysis of GTP to GDP, thus leaving the adenylate cyclase constitutively activated and causing an increase in cAMP (Cassel and Selinger, 1977). Increased cAMP activates cAMP-dependent protein kinase A, which in turns phosphorylates the regulatory domain of cystic fibrosis transmembrane conductance regulator (CFTR) located in the apical epithelial cell membranes (Barrett and Keely, 2000). This CFTR is the major chloride channel activated by LT and CT (Sears and Kaper, 1996; Thiagarajah et al., 2004). The net result is stimulation of Cl^- secretion from secretory crypt cells and inhibition of NaCl absorption by villous tip cells (Field et al., 1972). The net movement of electrolytes into the lumen results in a transepithelial osmotic gradient that causes water secretion into the lumen. The massive volume of water overwhelms the absorptive capacity of the intestine, resulting in diarrhoea.

The activation of adenylate cyclase leading to increased cAMP and subsequent altered ion transport is the most reported mode of action of CT. However, data from several research groups suggest that the increased levels of cAMP and subsequent A kinase activation may not explain all of the secretory effects of CT. There is evidence that prostaglandins (Bennett, 1971; Beubler et al., 1989; Bearcroft et al., 1996; Rocha et al., 2003), secretion of mucin from goblet cells (Forstner et al., 1981; Jarry et al., 1994; Flach et al., 2004) and the enteric nervous system (Cassuto et al., 1981; Lundgren, 1988; Banks et al., 2004) are also involved in the response to CT.

3.6. LT and CT immunomodulating effects

CT as well as LT are potent immunogens and induce antigen-specific sIgA and serum IgG antibody responses (Elson and Ealding 1984a and 1984b; Spangler, 1992; Nakagawa et al., 1996; Takahashi et al., 1996). Moreover, both toxins can act as adjuvants for the enhancement of mucosal and serum antibody responses to mucosal co-administered protein antigen (Clements et al., 1988; Holmgren et al., 1993; Di Tommaso et al., 1996; Okahashi et al., 1996; Takahashi et al., 1996; Yamamoto et al., 1997; Yamamoto et al., 1998; Namikoshi et al., 2003; Imai et al., 2004). Indeed, LT and CT were found to induce long-term memory to itself and to co-

administered protein antigens (Lycke et al., 1986 and1991; Vajdy and Lycke, 1992; Kamiya et al., 2001). Although high levels of toxin-specific IgA at the inductive site reduce the adjuvant effect, they do not inhibit the mucosal adjuvanticity of LT or CT in a subsequent immunization (Tamura et al., 1989 and 1997; Wu and Russel 1994). However, it has been shown that CT must be administered by the same route and at the same time for induction of immunity against the target antigen (Elson and Ealding, 1984). The induced mucosal immune response is best at the mucosal site directly exposed to the antigen and the adjuvant (Pierce and Cray, 1982). Homing of lymphocytes to the mucosal site of activation is probably enhanced by CT-B since a recent study reports that CT-B increased expression of the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) on endothelial cells in cultured human gastric explants (Lindholm et al., 2004). Binding of MAdCAM-1 to its exclusive ligand, integrin $\alpha 4\beta 7$, on lymphocytes represents a tissue-specific homing mechanism for the intestine and gut-associated lymphoid tissue (Briskin et al., 1997).

An important strategy for utilizing the immune-stimulatory properties of LT and CT has been the use of the non-toxic B subunits. The results obtained with LT-B and CT-B alone as mucosal adjuvant are highly inconsistent. Studies have shown that neither LT-B nor CT-B enhances immune responses to mucosally co-administered protein antigens (Douce et al., 1995; Rappuoli et al., 1999; Yamamoto et al., 1999; Hirai et al., 2000), whereas some other reports have suggested that LT-B and CT-B display mucosal adjuvant activity when (large doses) given with proteins by the nasal route (Hazama et al., 1993; Douce et al., 1997; de Haan et al., 1998a and 1998b; Rappuoli et al., 1999; Rask et al., 2000; Larsson et al., 2004) or when LT-B or CT-B is directly conjugated to the antigen itself (Rappouli et al., 1999; Rask et al., 2000; Larsson et al., 2004). However, it is interesting to note that the holotoxin stimulates stronger responses on a dose for dose basis following intranasal delivery compared to the B subunit (Douce et al., 1997; Kang et al., 2003). On the other hand, there are studies that report the use of recombinant LT-B and CT-B subunits to induce tolerance following oral delivery, but only in the complete absence of holotoxin (Williams et al., 1999). Hereto, the B-subunits need to be directly coupled to the antigen so that it can function as a carrier. Following GM1-receptor-mediated uptake, the antigen reaches immature antigen presenting cells, resulting in the induction of TGF-β-secreting regulatory T cells (Sun et al., 2000b).

Despite the fact that LT and CT are of the most potent mucosal adjuvants, exactly how these toxins exert their immunomodulatory effect is poorly characterized. Following intestinal administration of LT and CT, both toxins bind intestinal epithelial cells, which subsequently secrete IL-1, IL-6, IL-10 and IL-1Rα (Hansson et al., 1984; Bromander et al., 1993; McGee et al., 1993; Vervelde et al., 1998; Soriani et al., 2002). Although this may be of significance for the observed adjuvant effect, further experiments are necessary to define the exact influence of intestinal epithelial cells in enterotoxin adjuvanticity. In addition with the interaction with intestinal epithelial cells, interaction of LT and CT with different leukocytes is thought to be of major importance for mediating the adjuvant effect. LT and CT have been shown to be present within monomorphonuclear cells in the lamina propria and both toxins are transported by M cells into Peyer's patches following oral application (Hansson et al., 1984; Kraehenbuhl and Neutra, 2000). The influence of both toxins on different leukocytes will be discussed below and schematically presented in Figure 3.

3.6.1. Influence on dendritic cells (DCs)

There is emerging evidence that DCs are one of the principal cell types that mediate the adjuvant effect of both LT and CT *in vivo* (Fig. 3). These toxins induce phenotypic and functional maturation of DCs, promoting upregulated expression of MHCII, B7.1 and B7.2 costimulatory molecules, downregulation of CD40 and ICAM-1 expression and increased secretion of IL-1β (Gagliardi et al., 2000; Bagley et al., 2002; Martin et al., 2002; Jang et al., 2003; Eriksson et al., 2003; Lavelle et al., 2003 and 2004; Petrovska et al., 2003). Recent observations indicate that luminal CT attracts DCs to the intestinal epithelial layer, where DCs seem to take up luminal antigens (Rescigno et al., 2001; Lycke, 2004). LT-treated DCs also have an improved ability to present protein antigen (Petrovska et al., 2003). In addition, CT induces a cAMP-dependent upregulation of the chemokine receptors CXCR4 and CCR7 (Gagliardi et al., 2000 and 2003), enabling the migration of DCs to lymph nodes to interact with naive T cells (Iwasaki and Kelsall, 2000; Salusto et al., 2000; Shreedhar et al., 2003). *In vitro*, CT-maturated DCs are able to prime native $CD4^+/CD45RA^+$ T cells, driving their polarization towards the Th2 phenotype (Gagliardi et al., 2000). The fact that CT-treated DCs inhibit the expression of the Th1-response promoting cytokine IL-12 has been implicated as the mechanism by which this toxin mediates

the polarization of Th2 cells (Braun et al., 1999; Gagliardi et al., 2000; Bagley et al., 2002; de Jong et al., 2002; Lavelle et al., 2003). The CT-mediated maturation of DCs for priming of a Th2 response requires catalytically active holotoxin, since recombinant CT-B or catalytically inactive holotoxin did not cause significant maturation of human DCs (Gagliardi et al., 2000; Shreedhar et al., 2003). This is in agreement with the mentioned capacity of CT-B to induce oral tolerance against a coupled antigen, in contrast to CT.

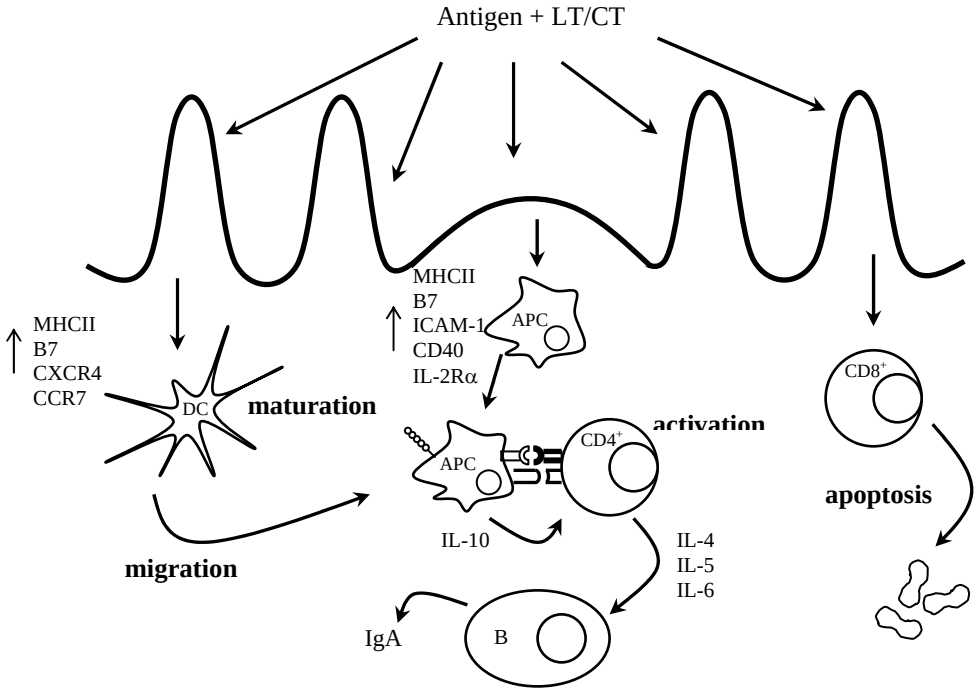


Figure 3 : Immunomodulating effects of LT and CT

3.6.2. Influence on B cells

Binding of the toxins to B cells leads to the upregulated expression of MHCII, B7.1 and B7.2, CD40, ICAM-1 and IL-2Rα (Fig. 3) (Anastassiou et al., 1990; Francis et al., 1992; Agren et al., 1997; Nashar et al., 1997 and 2001; Papadimitriou et al., 1997, Bone et al., 2002; Martin et al., 2002). This activation of B cells enhances their role as MHC II-restricted antigen presenting cells and favours the induction of Th2-dominated responses. *In vitro* studies indicate that CT facilitates B-cell switching to IgA through the action of TGF-β1 and increases the effects of IL-4 and IL-5 on IgG1 and IgA synthesis in lipopolysaccharide(LPS)-triggered spleen B cells (Lebman et al.,

1988; Lycke et al., 1989 and 1990; Kim et al., 1998). Intraduodenal application of CT is also shown to enhance isotype switching of Peyer's patch B cells to secrete IgA and IgG (Lebman et al., 1988). This IgA induction is independent of the A subunit (Wu and Russell, 1993; Stok et al., 1994; Kim et al., 1998). In contrast to the *in vivo* immunostimulatory effect of LT, *in vitro* incubation of murine B lymphoma cells inhibits their APC function in a cAMP-dependent way by increasing the intracellular pH and reducing antigen degradation (Tanaka et al., 1999).

3.6.3. Influence on macrophages

Treatment of both primary macrophages and macrophage cell lines with CT holotoxin suppresses production of TNF-α in response to LPS (Cong et al., 2001; Burkart et al., 2002; Chen et al., 2002b). Recent studies have revealed that CT-B may also suppress production of other proinflammatory cytokines. The expression of IL-6 is influenced by altering the MAPK signalling pathways (Chen et al., 2002b) and suppression of IL-12 production is due to inhibition of transcription of both the IL-12 p35 and p40 chains (Braun et al., 1999). In addition, NO production is also reduced by CT treatment since the synthesis of NO requires signalling by TNF-α (Cong et al., 2001). These studies demonstrate that CT inhibits innate immunity at the early steps of infection. The enzyme and binding activity of LT differentially affect the production of pro- and anti-inflammatory cytokines. The LT mutant LTK63 that is devoid of enzymatic activity stimulates IL-12 and TNF-α production by macrophages, whereas the LT holotoxin and the LT mutant LTR72, which retains partial enzymatic activity, suppressed LPS-induced IL-12 production (Ryan et al., 2000). These distinct modulatory effects of the receptor binding and enzyme activity of LT may be related to their different effects on signaling pathways. The B subunits of LT bind to the GM1, leading to the acidic sphingomylinase-mediated activation of NF-κB (Ballou et al., 1996; Truitt et al., 1998). NF-κB controls the transcription of a number of genes involved in inflammatory responses such as IL-12 (Baeuerle and Henkel, 1994). On the other hand, the suppression of IL-12 by enzymatically active toxins is likely due to the higher mentioned accumulation of intracellular cAMP, since it has been reported that cAMP has inhibitory effects on the production of inflammatory cytokines by macrophages (Parry and Mackman, 1997).

Similarly to B cells, CT and LT upregulate the expression of B7.2 (B7.1 by enzyme inactivated mutant LT) on macrophages that in turn leads to the co-stimulation of $CD4^+$ T cells activated via the T cell receptor-CD3 complex (Cong et al., 1997; Foss et al., 1999b; Yamamoto et al., 1999 and 2000; Ryan et al., 2000; Martin et al., 2002). The toxins also induce the secretion of IL-10 by macrophages, favouring the induction of Th2 responses (Feng et al., 2000; Ryan et al., 2000). In addition to the mentioned reduction of IL-12 secretion by macrophages, CT also reduces responsiveness of T cells to the Th1-response promoting IL-12 by inhibiting expression of surface receptor chains IL-12Rβ1 and IL-12Rβ2 (Braun et al., 1999). Furthermore, CT induces macrophages to produce membrane-associated and secreted IL-1, and enhances peptide presentation (Lycke and Strober, 1989; Bromander et al., 1991; Matousek et al., 1996; Foss et al., 1999b and 1999c). In contrast to these studies but in agreement with toxin effect on B cells and DCs, it has been shown that CT and LT inhibit antigen processing of soluble native antigen by macrophages (Matousek et al., 1998; Damiani and Colombo, 2001). Moreover, *in vitro* treatment of macrophages with CT resulted in a significant increased recycling of the phagosomal compartment to the cell membrane (Damiani and Colombo, 2001).

3.6.4. Influence on $CD4^+$ T cells

The mentioned negative influence of both toxins on antigen processing resulted in a hypothesis that both toxins could have a mitogenic effect on Th2 cells (Ryan et al., 2000), analogous to the effect of pertussis toxin on Th1 cells (Ryan et al., 1998). However, the addition of CT and LT to *in vitro* cultures of Peyer's patch $CD4^+$ T cells that were purified using a magnetic cell sorter system, reduces T cell proliferation (Yamamoto et al., 1999 and 2000; Lopes et al., 2000), suggesting that the *in vivo* toxin-induced T cell priming is due to indirect effects.

Studies with Th1 and Th2 cell lines as well as studies with positively selected T cell populations demonstrate that CT significantly enhances IL-10 production and selectively inhibits proliferative responses and IFN-γ synthesis of Th1 clones (Munoz et al., 1990; Yamamoto et al., 1999; Lavelle et al., 2004; Ozegbe et al., 2004). In addition, CT is shown to abrogate IL-12R expression by T cells (Braun et al., 1999). Moreover, the initial event induced by CT in $CD4^+$ T cells involves the upregulation of IL-4 (Vajdy et al., 1995; Okahashi et al., 1996; Yamamoto et al., 2000). This

results in the secretion of IL-5, IL-6 and IL-10, which provide helper signals for the induction of antigen-specific sIgA as well as serum IgG1, IgA and IgE antibody responses in mouse models (Hornquist and Lycke, 1993; Xu-Amano et al., 1993; Marinaro et al., 1995). In contrast, LT induces both Th1- and Th2-responses with subsequent mucosal sIgA as well as serum IgG1, IgG2a and IgA antibody responses (Takahashi et al., 1996). The LT-induced Th2-response is largely IL-4 independent (Yamamoto et al., 2000). This differences in CT and LT holotoxin adjuvanticity is suggested to rely on differences in the A subunit (Ryan et al., 1999 and 2000; Bowman and Clements, 2001) or the B subunit (Boyaka et al., 2003).

3.6.5. Influence on $CD8^+$ T cells

Hornquist et al. (1996) reported that $CD8^+$ T cells are not required for the mucosal adjuvant effect of CT. Indeed, LT and CT induce apoptosis of $CD8^+$ T cells (Fig. 3) (Elson et al., 1995; Nashar et al., 1996; Yankelevich et al., 1996; Truitt et al., 1998; Kim et al., 2001; Soriani et al., 2001; Salmond et al., 2002 and 2003). However, indirect activation of $CD8^+$ cytotoxic T cells has been reported and probably relies on the toxin-induced stimulation of B7-1 expression on DCs (Porgador et al., 1998; Simmons et al., 1999 and 2001; Jang et al., 2003; Eriksson et al., 2004).

3.6.6. Influence on intestinal permeability to macromolecules

Besides the influence of LT and CT on leukocytes, Lycke et al. (1991) found that CT increases the intestinal permeability to fluorescein-labeled dextran particles of 3 kDa. This suggests that CT acts on the epithelial layer to increase permeability to macromolecules, thereby delivering antigen into the underlying lamina propria and generating an immune response. Similar to CT, LT is reported to increase protein uptake (Verma et al., 1994 and 1995). Verma and co-workers (1994) suggested a change in tight junctional permeability following LT administration, which may be due to a change in the cytoskeletal microfilaments.

However, the influence of LT and CT on intestinal permeability to macromolecules is controversial since there are also reports arguing against it. For instance, no increased uptake of orally administered hen egg lysozyme into the peripheral circulation was found (Nedrud and Sigmund, 1991). Furthermore, no polyclonal increase in antibody production was observed in the lamina propria after

CT is introduced (Jackson et al., 1993), nor is there any increase in antibody responses to food antigens (Nedrud and Sigmund, 1991).

3.7. Conclusion

LT and CT are highly similar molecules which both function as virulence factor in *E. coli* and *V. cholerae* infections respectively and have the capacity to function as a mucosal adjuvant to co-administered antigens. Their immunomodulatory effects are suggested to be due to an increased antigen uptake of the co-administered antigens, a better induction of an antigen-specific immune response and an increased homing of antigen-specific effector cells to the original site of activation. However, further research will be necessary to elucidate the controversial hypotheses concerning the mechanisms of toxin endocytosis and adjuvanticity.

PART II

AIMS OF THE STUDY

Aims of the study

Oral immunizations with soluble non-replicating antigens most often result in oral tolerance. However, oral immunization of F4-receptor positive ($F4R^+$) pigs with F4 fimbriae induces an F4-specific immune response protecting pigs against a subsequent challenge with $F4^+$ enterotoxigenic *Escherichia coli* (Van den Broeck et al.,1999b). In $F4R^-$ pigs, F4 fimbriae seem to act as a normal food antigen (Van den Broeck et al., 2002), indicating that the F4-induced immune response is receptor-dependent.

The aim of the present work was to determine the potential of F4 fimbriae or its adhesin FaeG to function as a mucosal carrier for inducing antibodies against a coupled heterologous antigen or fused heterologous peptide.

For the F4 fimbriae, the following questions were addressed :

- Are F4 fimbriae conserved among $F4^+$ *E. coli* field isolates ? In addition, are F4-specific antibodies induced by fimbriae isolated from the $F4^+$ ETEC reference strain GIS26 able to inhibit binding of $F4^+$ *E. coli* field isolates to the F4 receptor ?
- Is the great immunogenicity of F4 fimbriae unique or does it also exist for F18 fimbriae ?
- Is it possible to screen the multimeric character and receptor-binding capacity of F4 fimbriae in a fast, specific and sensitive manner ?
- Are F4 fimbriae able to induce an antibody response against the coupled model antigen human serum albumin, following oral immunization of pigs ? Has cholera toxin (CT) the potential to improve the mucosal carrier effect of F4 fimbriae following oral co-administration ?

For the FaeG adhesin, the following issues were studied :

- Is there a correlation between F4 bioactivity and its binding with an FaeG-specific monoclonal antibody ?
- Can the F4 fimbrial adhesin FaeG be produced in an *E. coli* expression system, retaining its F4R-binding conformation ? Furthermore, is there a

difference in the F4-specific immune response induced following oral administration of rFaeG or purified F4 fimbriae ?

- Has recombinant FaeG the potential to function as a mucosal carrier to a N-terminally fused heterologous peptide in the presence or absence of CT ?

PART III
EXPERIMENTAL STUDIES

Chapter 4

Conserved regions in the sequence of the F4 fimbrial adhesin FaeG, suggest a donor strand mechanism in F4 assembly and the usefulness of FaeG in a vaccine against F4$^+$ enterotoxigenic *Escherichia coli*[1]

[1] *Based on : Verdonck F, Cox E, Schepers E, Imberechts H, Joensuu JJ, Goddeeris BM. 2004c. Conserved regions in the sequence of the F4 fimbrial adhesin FaeG suggest a donor strand mechanism in F4 assembly. Vet. Microbiol., accepted.*

Abstract

To use FaeG subunits in an oral vaccine against F4$^+$ enterotoxigenic *E. coli*, it is necessary to determine the conservation of the adhesin subunit. Hereto, the *faeG* sequence was determined of 21 F4ac$^+$ *E. coli* field isolates from piglets with diarrhoea and subsequently compared with these of the reference strain GIS26 and previously reported FaeG sequences. The FaeG amino acid sequence was 96-100% homologue within each F4 serotype, but only 92% and 88% when the F4ab and F4ad serotypes were compared with the F4ac serotype. The conserved regions of the adhesin suggest a donor strand mechanism in F4 fimbriae assembly as reported for type 1 fimbriae and P pili. On the other hand, antibodies induced by purified GIS26 F4 fimbriae immunization were able to inhibit binding of all 21 isolates. In conclusion, the results of the reported experiments support the study of FaeG in an oral subunit vaccine against F4$^+$ *E. coli* infections.

4.1. Introduction

F4 fimbriae are frequently detected on enterotoxigenic *Escherichia coli* (ETEC) strains causing neonatal or post-weaning diarrhoea in pigs (Wilson et al., 1986; Harel et al., 1991). They are long thin proteinaceous appendages radiating from the surface of the bacterium to a length of 0.1 – 1 μm (Stirm et al., 1967), peritrichously distributed in numbers of 100 to 1000 per bacterium (Ottow, 1975; Klemm, 1985). Their flexible structure allows them to adhere to the F4 receptor (F4R) on brush borders of small intestinal enterocytes, resulting in colonization of the small intestinal mucosa (Jones et al., 1972). Subsequently, the heat-labile enterotoxin (LT), heat-stable enterotoxin a and/or b (STa, STb) produced by these strains induce a secretory diarrhoea (Nataro and Kaper, 1998).

F4 fimbriae are composed of the major subunit FaeG and the minor subunits FaeC, FaeF, FaeH and FaeI (Oudega et al., 1989; Bakker et al., 1992b). The major subunit is also the adhesin (Bakker et al., 1992a; Van den Broeck et al., 1999c), whereas for other *E. coli* fimbriae a minor subunit is involved in the adhesion (Mol and Oudega, 1996). In the latter *E. coli* fimbrial systems, the adhesins are reported as conserved proteins, whereas the major subunits are described as variable (Smeds et al., 2003; Vandemaele et al., 2003a and 2003b).

Immunization of mice with recombinant subunits of the conserved type 1 adhesin FimH was reported to protect against a subsequent uropathogenic *E. coli* infection (Langermann et al., 1997). In agreement, oral immunization of newly weaned piglets with recombinant FaeG induced an F4-specific mucosal and systemic immune response, significantly reducing $F4^+$ *E. coli* excretion following challenge (Verdonck et al., 2004a). However, before using the FaeG subunit as a vaccine, the conservation of FaeG has to be determined. Therefore, the *faeG* sequences of 21 $F4^+$ *E. coli* isolates from piglets with diarrhoea were determined and compared with the *faeG* sequence of the $F4^+$ ETEC reference strain GIS26 and nine reported *faeG* sequences.

4.2. Material and methods

4.2.1. Bacterial strains

Twenty *E. coli* strains were isolated from animals that died from a neonatal (N) or post-weaning (PW) $F4^+$ ETEC infection. They were collected on nineteen different pig farms in Flanders (Table 1). The 5/95 isolate was isolated in Finland and obtained from J. Joensuu, whereas strain GIS26 was used as reference strain. All strains were cultured on brain heart infusion agar (Oxoid, Basingstoke, Hampshire, England) for 18 hours at 37°C, whereafter the expression of F4ac fimbriae was tested using the c-epitope specific monoclonal antibody (MAb) CVI F4ac-5 (ID-DLO, Lelystad, The Netherlands) (van Zijderveld et al., 1990).

To isolate F4 fimbriae or to determine the F4R binding, all strains were cultured during 18 h in Tryptone Soya Broth (TSB, Oxoid) at 37°C and 85 rpm. The bacteria were collected by centrifugation and washed with phosphate-buffered saline (PBS) (150mM, pH 7.4). The concentration of the bacteria was determined by measuring the optical density of 10-fold dilutions of the bacterial suspension at 660nm (OD_{660}). An OD_{660} of 1 equals 10^9 viable bacteria/ml, as determined by counting colony forming units.

4.2.2. Serotyping, haemolysis and antibiotic resistance

Serotyping was performed by agglutination with specific antisera directed against somatic antigens O138, O139, O141, O147 and O149 (Orskov et al, 1977).

The β-haemolytic phenotype of the isolated strains was determined by growing the bacteria 18 h at 37°C on blood (5% sheep red blood cells) agar plates (Difco Laboratories, Becton Dickinson, Le Pont de Claix, France).

Diffusion sensitivity testing was conducted for the following antibiotics : amoxycillin-clavulanic acid (AC), chloramphenicol (CM), tetracycline (TC), sulfonamide-trimethoprim (TSU), apramycin (AP), ceftiofur (CF), minocycline (M), spectinomycin (SP) and nalidixin acid (NAL).

4.2.3. PCR assays

Multiplex PCR was performed to detect the presence of LT, STa, STb enterotoxin and the verotoxin (VT) coding gene (Bosworth and Casey, 1997). *faeG* was amplified from the isolates by PCR (Fig. 1) using the primers FaeGS1GMF (5'-GGACTGAGGATTAATCTAGATAGTGATGCAAAACATCCG-3') and FaeGS1GMR (5'-CGTATCAATAATAAATTGGGAGCTCATCACGAC-3') to include the signal sequence. PCR was performed in a PTC-100^{TM} (MJ Research, Watertown, USA) using Supertaq (HT Biotechnology Ltd, Cambridge, England) and the resulting PCR product was purified using the Qiaquick PCR purification Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions.

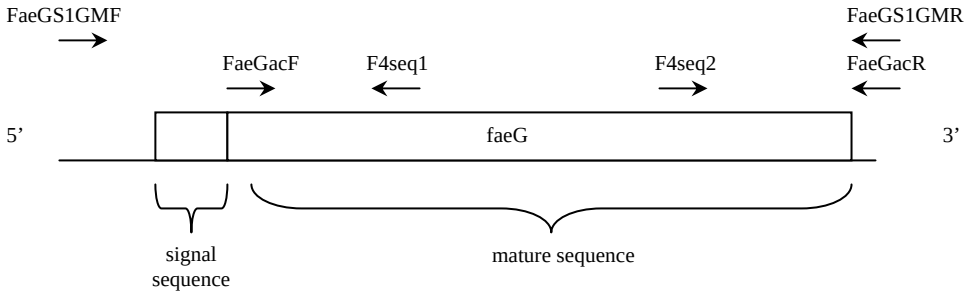


Figure 1 : Position of the primers used to sequence *faeG* of F4^{+} *E. coli* isolates.

4.2.4. Sequencing and DNA analysis

The nucleotide sequences of *faeG* genes were determined by the dideoxynucleotide chain termination method of Sanger using the ABI PRISM Bigdye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). To sequence the *faeG* signal sequence of isolates GIS26, IMM 02,

IMM 03 and IMM 14, purified PCR fragments were used in combination with the FaeGS1GMF primer. To identify the sequence coding for the mature FaeG (Fig 1), purified PCR fragments were used in combination with the primers FaeGacF (5'-GACGACGACAAGATTGCACATGCCTGGATGACTGG-3'), FaeGacR (5'-GAGGAGAAGCCCGGTAATAAATTGGCAGCTCATCACG-3'), F4seq1 (5'-GCTTCTTTGGTTCGGCCTAAC-3') and F4seq2 (5'-CTCTTGTTGACGTCGCAGGTT-3'). The sequencing reaction was performed in a PTC-100^{TM} following the manufacturers protocol. The resulting products were purified using the Qiagen Dye Ex kit (Qiagen) and sequenced on a Genetic Analyzer 3100 (Applied Biosystems) according to the manufacturers manual. The chromatograms of the sequences were visualized using the CHROMAS 2.0 software (Technelysium Ltd., Australia) and the DNA sequences were analysed and transformed to amino acid sequences using the DNAMAN version 5.0 (Lynnon Biosoft, Vaudreuil, Canada).

4.2.5. In vitro adhesion to the F4R

The *in vitro* adhesion of the *E. coli* isolates to the F4R was determined in triplicate using small intestinal villi from three strong F4R$^+$ and one F4R$^-$ pig, as described by Van den Broeck et al. (1999c). The villi were washed and suspended in PBS supplemented with 1% (wt/vol) D-mannose to prevent adhesion of *E. coli* by type 1 pili. Subsequently, 4.10^8 *E. coli* were added to an average of 50 villi in a final volume of 0.5 ml and incubated for 1 hour while being gently shaken. Villi were examined by phase-contrast microscopy at a magnification of 600 and adhesion of bacteria was quantified by counting the number of bacteria adhering along a 50 µm villous border at 20 different places, after which the mean bacterial adhesion per 250 µm villous brush border length was calculated. Binding of GIS26 to F4R$^+$ villi (mean of 72.5, 73.5 and 84 bacteria per 250 µm for the three used F4R$^+$ villi) was used as reference and set at 100 %, whereafter the binding percentage of the isolates was calculated in comparison with GIS26 F4R-binding. No binding of the isolates to the F4R$^-$ villi was observed.

4.2.6. Isolation of F4 fimbriae

The F4 fimbriae were purified as described by Van den Broeck et al. (1999c). Briefly, fimbriae were isolated by homogenising the bacterial suspension of *E. coli* using an Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany), followed by a purification using two centrifugation steps and an additional precipitation of the supernatans with 40% (wt/vol) ammonium sulphate. Thereafter the pellet was dissolved and dialysed overnight against ultra pure H_2O.

4.2.7. Multimeric FaeG character of F4 fimbriae

The presence of FaeG multi- or monomers in purified F4 fimbriae of the different isolates, was determined by SDS-PAGE followed by Coomassie staining or Western blot using the FaeG-specific MAb IMM01 (Van der Stede et al., 2002b) as described by Van den Broeck et al. (1999c). The samples were diluted in 60 mM TrisHCl pH 6.8, 2% SDS, 10% glycerol and 0.02% bromophenol blue and loaded on SDS-PAGE without heating.

4.2.8. Transmission electron microscopic analysis (TEM)

E. coli grown in TSB as described above, were examined using a TEM2085 transmission electron microscope (FEI, Eindhoven, The Netherlands) after rotary shadowing or negative staining with 2% uranylacetate as described by Imberechts et al. (1996).

4.2.9. MALDI-TOF mass spectrometry (MS)

To identify the proteins in the 50 kDa and 75 kDa protein bands in heat denaturated and non-heat denaturated purified GIS26 F4 fimbriae respectively (Fig. 4), these protein bands were excised from a SDS-PAGE, incubated with trypsin and analysed by MALDI-TOF MS (Bruker Reflex IV). The obtained peptide mass fingerprint was then used for identity searching using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). The score of the identity match is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 56 are significant ($P<0.05$).

4.2.10. Motility assay

The presence of flagella on *E. coli* isolates was analysed using a motility assay (Schmitt et al., 2001). Motility was assessed by stabbing motility agar (1 % (wt/vol) tryptone (Oxoid), 0.5% (wt/vol) NaCl and 0.35% (wt/vol) agar (Oxoid), pH 7.4) with the bacterial strain and after incubation at 37°C for 18 h observing the agar for migration of bacteria away from the point of inoculation.

4.2.11. Brush-border fragment (BBF) ELISA

A BBF-ELISA was developed to analyse if the antibodies, induced by oral immunization of purified GIS26 F4 fimbriae, were able to inhibit *in vitro* adhesion of other F4ac$^+$ *E. coli* isolates to the F4R. To obtain BBF, jejunal enterocytes of pigs were isolated as described by Verdonck et al. (2004b). The enterocytes were homogenized by squeezing them 20 times through an 18G needle. The homogenate was centrifuged at 27,000 g for 30 min whereafter the pellet was collected. This procedure was repeated twice and finally BBF were stored at –20°C until use in ELISA.

A 96-well microtiter plate (NUNC, Maxisorp, Immuno Plates, Roskilde, Denmark) was coated with the FaeG-specific MAb IMM01 at a concentration of 1 μg/ml in PBS. After 2 h incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0,2% (v/v) Tween®80. Purified F4 fimbriae were diluted in ELISA dilution buffer (PBS + 0,05% (v/v) Tween®20) to a concentration of 50 μg/ml and incubated for 1 h at 37°C. Thereafter, the plates were incubated with serum from a pig orally immunized with GIS26 F4 fimbriae (F4-specific titer 1280) or serum from an F4-seronegative pig (F4-specific titer < 10), again for 1 h at 37°C. Subsequently, F4R$^+$ or F4R$^-$ BBF were washed three times in PBS (400g, 10 min) and diluted to 1 mg/ml in PBS containing 5% (w/v) non-fat dry milk, were brought onto the plate for 1 h at 37°C. BBF-specific rabbit serum optimally diluted in ELISA dilution buffer was used for 1 h at 37°C. Thereafter, pig anti-rabbit HRP-conjugated serum (Dako, Denmark), optimally diluted in ELISA dilution buffer, was added. Following 1 h incubation at 37°C, an ABTS solution containing H_2O_2 was added for 1 h incubation at 37°C whereafter the optical density was spectrophotometrically measured at 405 nm (OD_{405}). Between each incubation step, the plates were washed three times with washing buffer (PBS + 0,2% (v/v)

Tween®20), except after incubation with BBF when the plates were washed three times with an extra salt-containing buffer (PBS with 0,3 M NaCl + 0,2% (v/v) Tween®20).

4.3. Results

4.3.1. Phenotyping of isolates

All isolates were β-haemolytic and resistant to one or more antibiotics (Table 1). Nineteen of the 22 isolates belonged to the serotype O147, whereas 5/95 and GIS26 were O149 and isolate IMM 24 was not typable with the used serotype-specific antibodies. Furthermore, all isolates except IMM 24 bound the F4R *in vitro* and contained the genes coding for the enterotoxins LT and STb. Indeed, IMM 24 did not express F4 fimbriae as no agglutination with F4-specific MAb was observed and no F4 fimbriae could be obtained following purification. In addition, IMM 24 contained verotoxin instead of enterotoxin coding genes. However, this isolate did contain the *faeG* gene.

Table 1 : Analysed *E. coli* isolates

strain	reference	infection	toxines	F4R-binding	antibiotic resistance	motility	accession number
IMM 01	BA752	N	LT STb	72,5 ± 16,2	TSU, TC	-	AJ616237
IMM 02	BA2	PW	LT STb	62,8 ± 6,8	TSU, TC	-	AJ616238
IMM 03	761	PW	LT STb	76,3 ± 8,4	TSU, TC, AC, AP	-	AJ616239
IMM 04	2000/00256/1	PW	LT STa STb	34,9 ± 9,8	AC, CM, TC, TSU	-	AJ616240
IMM 05	2000/00295/1	PW	LT STb	57,3 ± 7,8	CM	-	AJ616241
IMM 06	2000/00727/1	PW	LT STa STb	64,1 ± 18,7	CM	-	AJ616242
IMM 07	2000/03083/1	PW	LT STb	105,3 ± 21,7	CM	-	AJ616243
IMM 08	2000/03229/1	PW	LT STb	97,0 ± 3,9	AP,TSU	++	AJ616251
IMM 09	2000/03876/1	PW	LT STa STb	18,1 ± 9,6	CM, TC, TSU	-	AJ616244
IMM 10	2000/05726/1	N	LT STa STb	80,6 ± 17,6	AC, AP, CF, CM, M, TC	-	AJ616245
IMM 12	2000/06526/1	PW	LT STa STb	34,7 ± 4,6	TC	-	AJ616246
IMM 13	2000/06528/1	PW	LT STa STb	26,7 ± 6,8	AP, CM,TC, TSU	-	AJ616247
IMM 14	2000/06529/1	PW	LT STa STb	73,0 ± 20,0	AP,CM, TC, TSU	-	AJ616256
IMM 16	2000/08690/1	N	LT STb	24,9 ± 4,2	CM, TSU	-	AJ616248
IMM 17	2000/08690/2	N	LT STb	41,8 ± 6,7	CM	-	AJ616249
IMM 18	2000/11176/1	PW	LT STa STb	63,0 ± 13,4	SP, TC, TSU	-	AJ616250
IMM 21	BH 1197	PW	LT STb	90,3 ± 5,1	TSU, TC, AC	+	AJ616252
IMM 24	BH 1053	PW	VT	0,0 ± 0,0	TSU, TC, AC	+	AJ616253
IMM 26	BH 1433	PW	LT STb	75,3 ± 9,7	TSU, TC	+	AJ616254
IMM 30	BH 1389	PW	LT STb	89,2 ± 4,9	TSU, TC, SP	++	AJ616255
5/95	-	N	LT STb	59,8 ± 3,4	TC	+	AY437806
GIS26	-*	-	LT STa STb	100	TSU, TC	+++	AJ616236

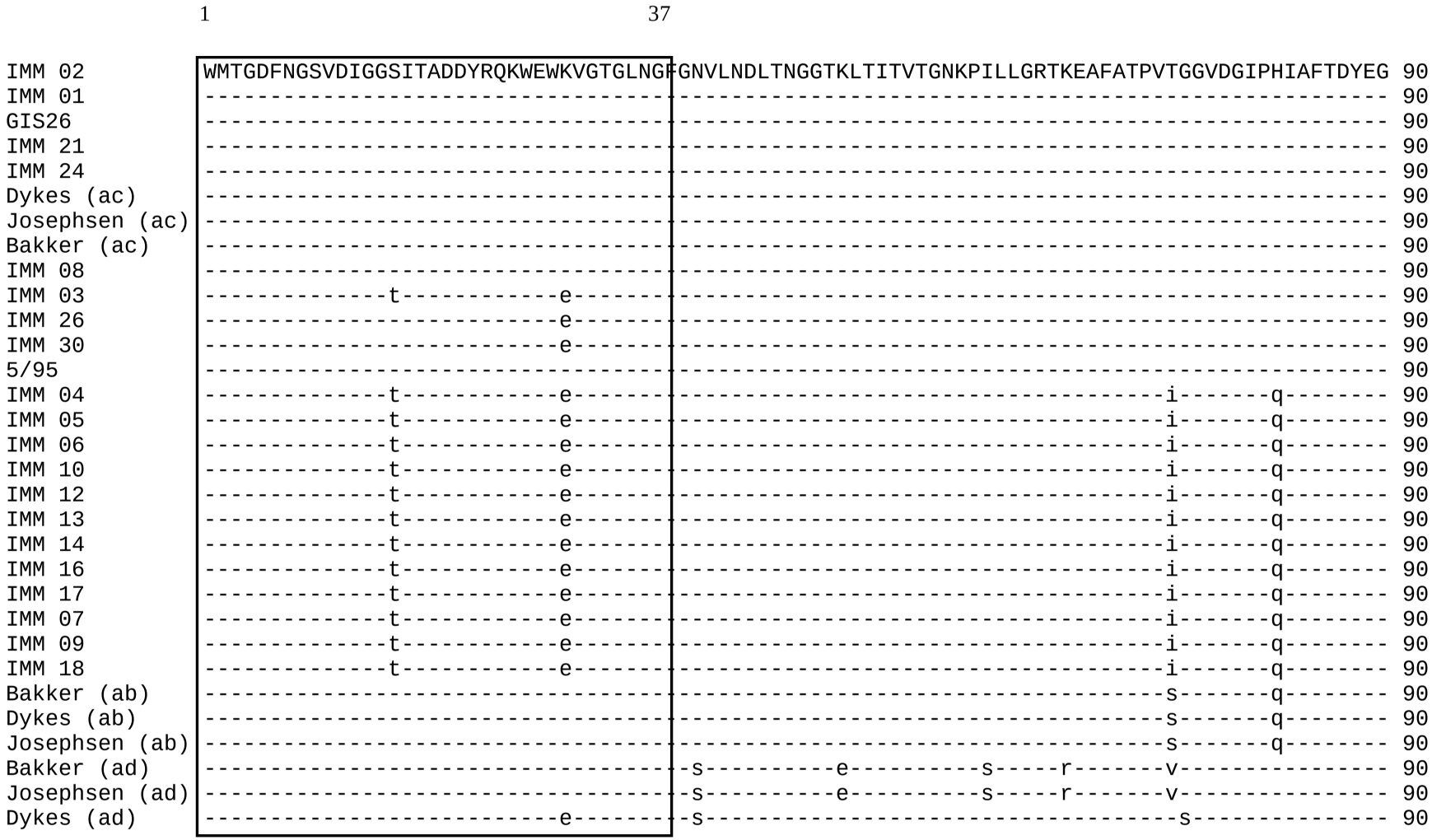
* Strain GIS26 was obtained from Smith Kline RIT, Genval, Belgium

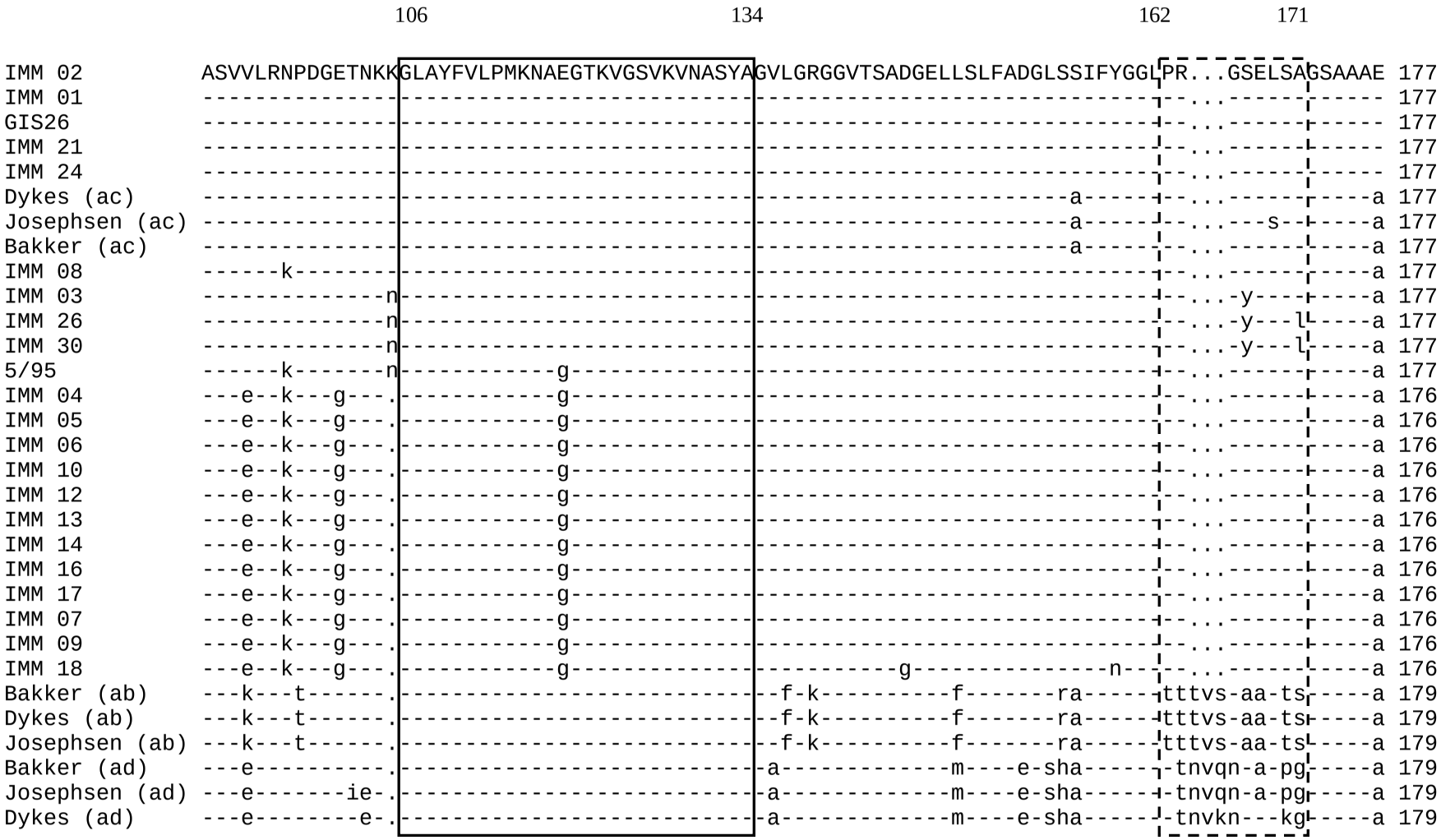
Neonatal (N) or post-weaning (PW) *E. coli* infection; Non-typable serotype (NT) (serotype not belonging to the serotypes tested); Heat-labile (LT), heat-stable a (STa) or b (STb) enterotoxin, verotoxin (VT). Percentage F4 receptor binding in comparison with GIS26 ± SD; amoxycillin-clavulanic acid (AC); chloramphenicol (CM); tetracycline (TC); sulfonamide-trimethoprim (TSU); apramycin (AP); ceftiofur (CF); minocycline (M); spectinomycin (SP); nalidixin acid (NAL)

4.3.2. Analysis of FaeG sequence

The *faeG* nucleotide sequence of all the F4ac$^+$ *E. coli* isolates was determined to elucidate the degree of conservation. The nucleotide sequence coding for the FaeG signal sequence was identical for all the tested isolates, whereas mutations occurring at 29 different positions were detected in the 786 nucleotides coding for the mature FaeGac protein. Following deduction of the protein sequences from the obtained nucleotide sequences, silent mutations were seen at 4 positions each occurring in only one isolate. Most frequent nucleotide substitutions caused replacements by amino acids, which changed the hydrophobic, charged or polar character of the side chain.

As shown in Figure 2, conservation was found in the central (106-134) as well as the N- and C-terminal region (1-37 and 236-262, respectively). Furthermore, the regions 162-171 and 206-216 were conserved in F4ac$^+$ ETEC strains, but variable between the three antigenic variants as observed following comparison with the reported FaeG amino acid sequences of F4ab and F4ad strains (Josephsen et al., 1984; Dykes et al., 1985; Bakker et al. 1992a). In agreement, analysis of the identity between mature FaeG amino acid sequences of the isolates reported in this study and the previously reported FaeG sequences, revealed that the FaeG amino acid sequence is 96-100% identical within each antigenic variant, but only 92 and 88% when the mature FaeG amino acid sequence of F4ab and F4ad antigenic variants are compared with that of the F4ac antigenic variant, respectively (Fig. 3). Interesting to note is the observation that 12 of the 21 F4ac isolates revealed a deletion of amino acid residue 105, identical to the FaeG sequence of F4ab and F4ad antigenic variants. Moreover, at 5 positions amino acid residues differed from earlier reported F4ac isolates, but were identical to the corresponding amino acid residue in F4ab and/or F4ad sequences.


1
37
IMM 02 WMTGDFNGSVDIGGSITADDYRQKWEWKVGTGLNGFGNVLNDLTNGGTKLTITVTGNKPILLGRTKEAFATPVTGGVDGIPHIAFTDYEG 90
IMM 01 ---------- 90
GIS26 ---------- 90
IMM 21 ---------- 90
IMM 24 ---------- 90
Dykes (ac) ---------- 90
Josephsen (ac) ---------- 90
Bakker (ac) ---------- 90
IMM 08 ---------- 90
IMM 03 ----------t----------e---------- 90
IMM 26 ----------e---------- 90
IMM 30 ----------e---------- 90
5/95 ---------- 90
IMM 04 ----------t----------e----------i-------q-------- 90
IMM 05 ----------t----------e----------i-------q-------- 90
IMM 06 ----------t----------e----------i-------q-------- 90
IMM 10 ----------t----------e----------i-------q-------- 90
IMM 12 ----------t----------e----------i-------q-------- 90
IMM 13 ----------t----------e----------i-------q-------- 90
IMM 14 ----------t----------e----------i-------q-------- 90
IMM 16 ----------t----------e----------i-------q-------- 90
IMM 17 ----------t----------e----------i-------q-------- 90
IMM 07 ----------t----------e----------i-------q-------- 90
IMM 09 ----------t----------e----------i-------q-------- 90
IMM 18 ----------t----------e----------i-------q-------- 90
Bakker (ab) ----------s-------q-------- 90
Dykes (ab) ----------s-------q-------- 90
Josephsen (ab) ----------s-------q-------- 90
Bakker (ad) ----------s----------e----------s-----r-------v---------- 90
Josephsen (ad) ----------s----------e----------s-----r-------v---------- 90
Dykes (ad) ----------e---------s----------s---------- 90


106
134
162
171
IMM 02 ASVVLRNPDGETNKKGLAYFVLPMKNAEGTKVGSVKVNASYAGVLGRGGVTSADGELLSLFADGLSSIFYGGLPR...GSELSAGSAAAE 177
IMM 01 ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
GIS26 ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 21 ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 24 ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
Dykes (ac) ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
Josephsen (ac) ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
Bakker (ac) ----------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 08 ------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 03 ----------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 26 ----------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 30 ----------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
5/95 ------k-------n-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 177
IMM 04 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 05 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 06 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 10 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 12 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 13 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 14 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 16 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 17 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 07 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 09 ---e--k---g---.-----------g-------------a-----f-k----------f-------ra-----tttvs-aa-ts-----a 176
IMM 18 ---e--k---g---.-----------g-------------a-----g----------n-------ra-----tttvs-aa-ts-----a 176
Bakker (ab) ---k---t------.----------f-k----------f-------ra-----tttvs-aa-ts-----a 179
Dykes (ab) ---k---t------.----------f-k----------f-------ra-----tttvs-aa-ts-----a 179
Josephsen (ab) ---k---t------.----------f-k----------f-------ra-----tttvs-aa-ts-----a 179
Bakker (ad) ---e----------.----------a-------------m----e-sha-----tnvqn-a-pg-----a 179
Josephsen (ad) ---e-------ie-.----------a-------------m----e-sha-----tnvqn-a-pg-----a 179
Dykes (ad) ---e--------e-.----------a-------------m----e-sha-----tnvkn---kg-----a 179

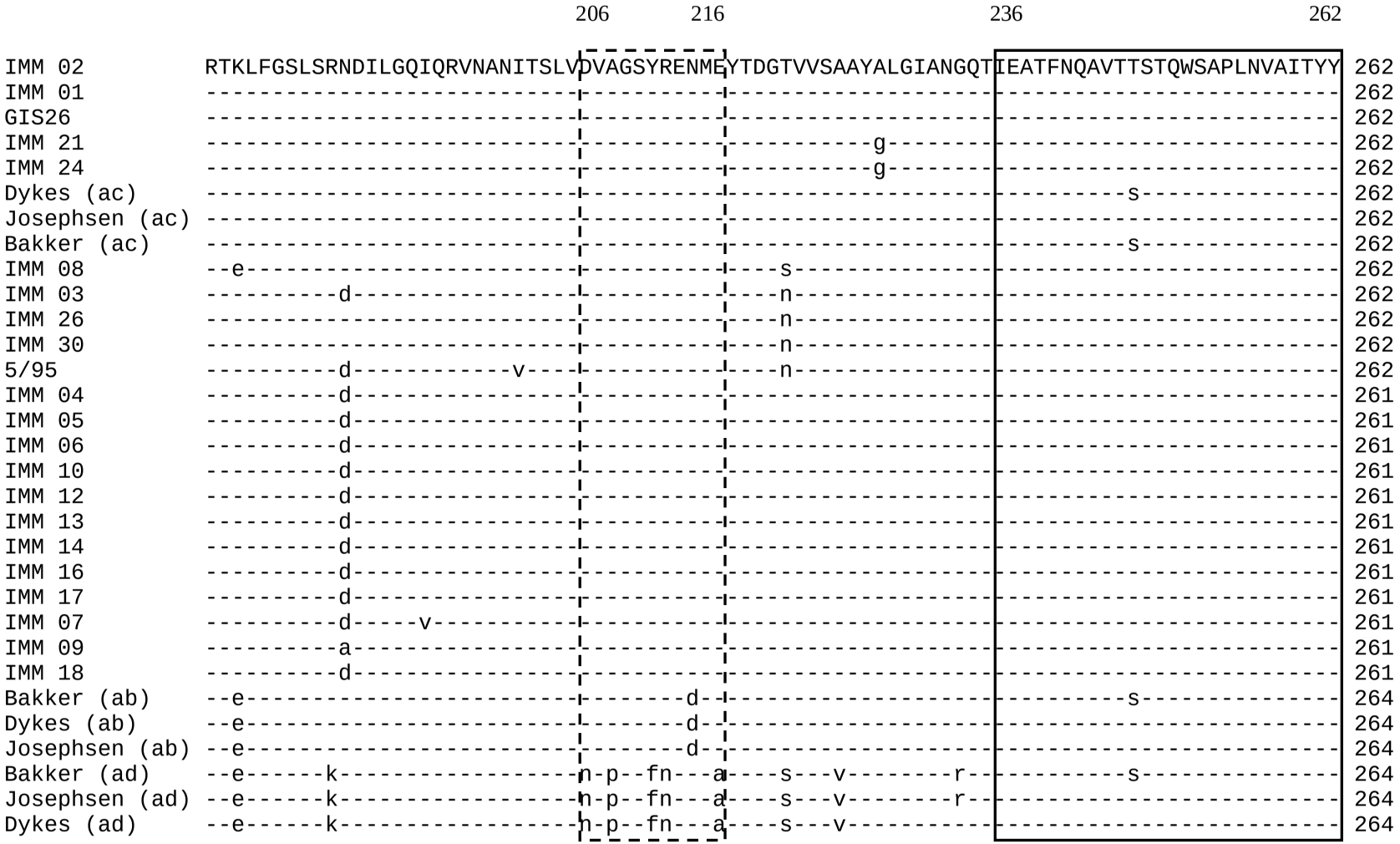


Figure 2 : Alignment of the amino acid sequence of mature FaeG from the isolates studied here and sequences reported by Dykes et al. (1985), Josephsen et al. (1984), Bakker et al. (1992a). Conserved regions (1-37, 106-134 and 236-262) and variable regions (162-171 and 206-216) are indicated by a box and a dotted box, respectively.

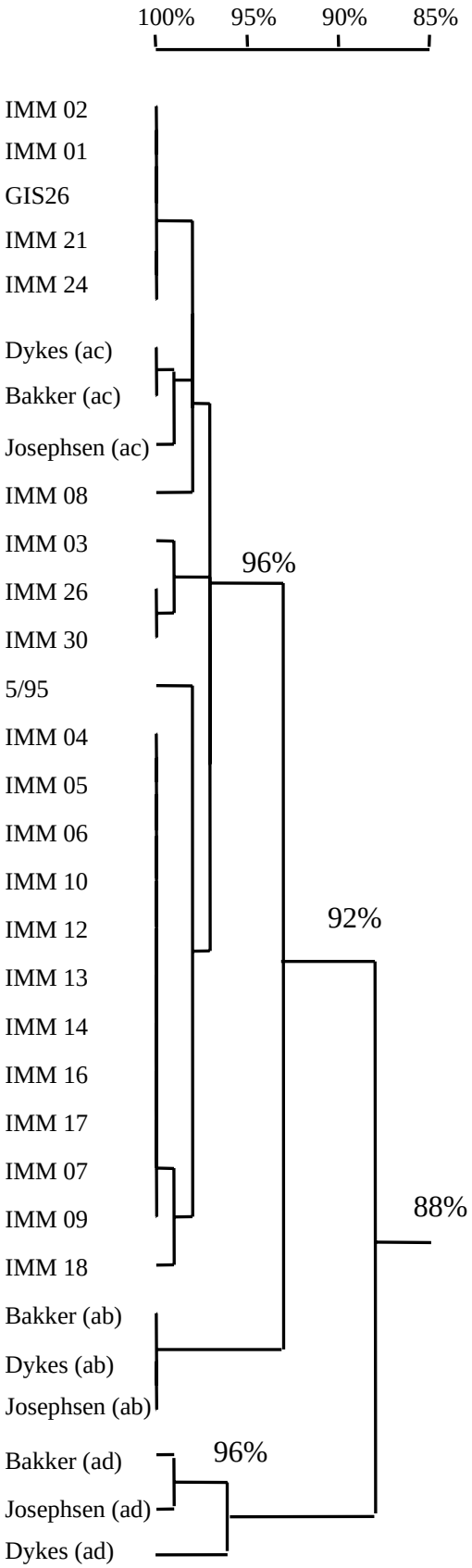


Figure 3 : Identity tree of the multiple FaeG alignment at protein level from the isolates studied here and sequences reported by Dykes et al. (1985), Josephsen et al. (1984), Bakker et al. (1992a). The percentages indicate the degree of identity.

4.3.3. Mono- and polymer

The F4 fimbriae of all isolates except of IMM 24, were purified for characterization. SDS-PAGE and Coomassie staining of heat-denaturated F4 fimbriae revealed a band of 27 kDa. This 27kDa band was identified as the FaeG monomer because it was recognized by the FaeG-specific MAb IMM01 in Western blot. On the other hand, a second band of 50 kDa was observed in several isolates, but this band was not recognized by the MAb IMM01 (data not shown). The 50 kDa band could reach up to 25-40% of the total protein amount in purified GIS26 F4 fimbriae and was identified as flagellin following MALDI-TOF MS analysis (score 301). Subsequently, the presence of flagella was analysed on all isolates using a motility assay. Seven of the 21 isolates appeared to express flagella (Table 1), but the GIS26 was most positive. Electron microscopy confirmed the presence of flagella on GIS26.

The mono- or polymeric nature of FaeG in purified F4 fimbriae was determined by SDS-PAGE of native purified fimbriae followed by Coomassie staining or Western blot. Only the FaeG monomer band of 27 kDa was detected in the F4 fimbrial sample of the 5/95 isolate, whereas a ladder pattern of FaeG multimers was observed for the other F4ac^{+} *E. coli* isolates (Fig. 4). This ladder pattern indeed consisted of FaeG polymers since MALDI-TOF MS analysis confirmed that the 75 kDa band of non-heat denaturated purified GIS26 F4 fimbriae contained FaeG (score 160). To determine whether 5/95 FaeG depolymerised due to SDS-PAGE or whether the FaeG monomers were already present following the fimbrial purification, 5/95 F4 fimbriae were analysed by TEM. A low number of fimbriae could be detected in between a huge amount of protein aggregates for 5/95 fimbriae (Fig. 4). In contrast, EM analysis of purified GIS26 F4 fimbriae revealed a lot of fimbriae with only a low amount of aggregates.

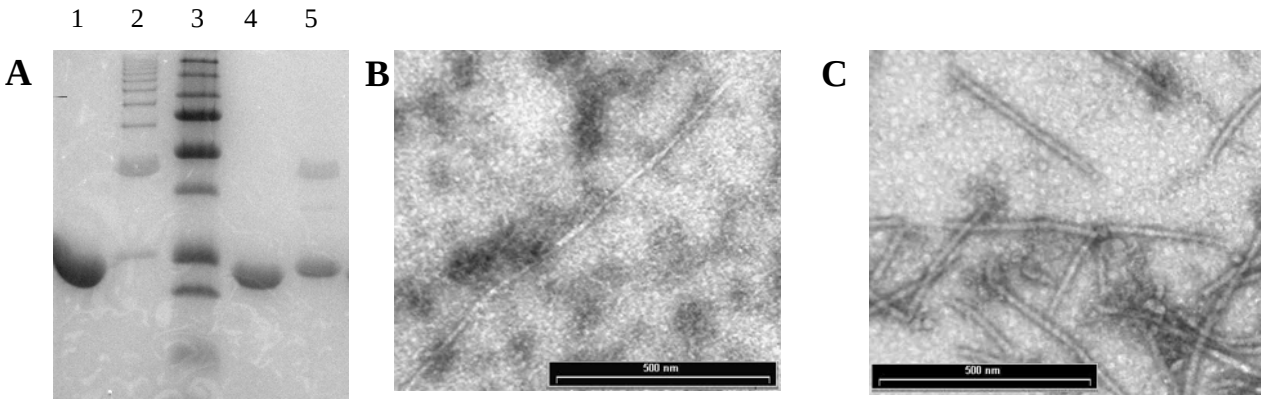


Figure 4 : (A) Coomassie stained SDS-PAGE of non heat-denaturated purified 5/95 (lane 1) and GIS26 (lane 2) F4 fimbriae, molecular weight marker : 250, 150, 100, 75, 50, 37, 25, 20, 15 kDa (lane 3), heat-denaturated purified 5/95 (lane 4) and GIS26 (lane 5) F4 fimbriae. (B) Electron micrograph of purified 5/95 F4 fimbriae (same sample as in A). (C) Electron micrograph of purified GIS26 F4 fimbriae (same sample as in A).

4.3.4. BBF ELISA

A BBF-ELISA was developed to analyse if antibodies induced by oral immunization of purified GIS26 F4 fimbriae were able to inhibit *in vitro* adhesion of other F4ac$^+$ *E. coli* isolates to the F4R. Binding of purified F4 from all isolates except IMM 24 was observed to BBF isolated of F4R$^+$ villi, whereas only low OD_{405}-signals (0.09 to 0.17) were obtained in the presence of F4R$^-$ BBF (data not shown). Addition of serum from a pig that was orally immunized with purified GIS26 F4 fimbriae, reduced the OD_{405}-signals to the level observed with F4R$^-$ BBF (Fig. 5). In contrast, serum without F4-specific antibodies was not inhibiting the interaction between the F4R and the F4 fimbriae. So, it was shown that antibodies induced by purified GIS26 F4 fimbriae were able to inhibit adhesion of 20 different F4ac$^+$ ETEC isolates to F4R$^+$ villi.

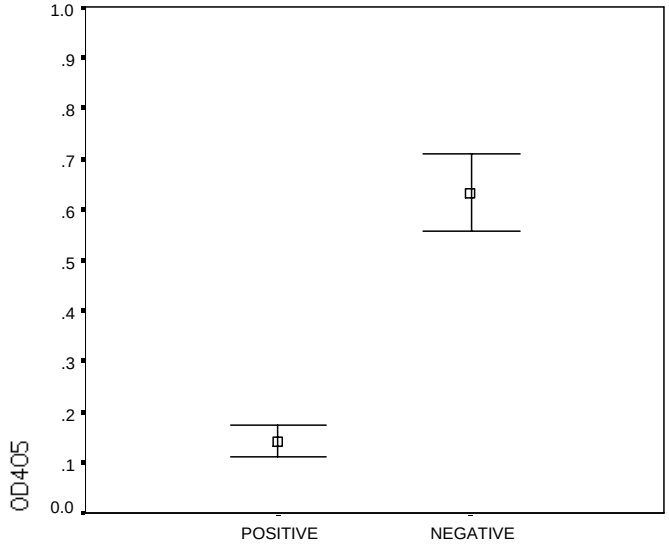


Figure 5 : The OD_{405}-values obtained following brush border fragment (BBF) ELISA, represent binding of purified F4 from all $F4^+$ *E. coli* isolates except IMM 24 to $F4R^+$ BBF, in the presence of serum with (positive) or without (negative) F4-specific antibodies.

4.4. Discussion

In the present study, the conservation of the F4 fimbrial adhesin FaeG sequence was evaluated. Twenty-one different $F4^+$ *E. coli* were isolated from piglets with neonatal or post-weaning diarrhoea. These field strains were haemolytic and encoded the enterotoxins LT, STa and STb, in accordance to other reports on pathogenic $F4^+$ ETEC isolates (Wilson et al., 1986; Osek, 2000; Frydendahl, 2002).

The FaeG amino acid sequence of the 21 $F4ac^+$ *E. coli* field isolates, the GIS26 reference strain and the strains reported in literature, were 96% identical. Similar high degrees of sequence identity have been reported for the adhesins of F18 fimbriae (97%; Smeds et al., 2003; Tiels, unpublished results) and type 1 fimbriae (98%; Sokurenko et al., 1994; Vandemaele et al., 2003a), whereas the degree of sequence identity appeared lower for the F17 fimbrial lectin domain (90%; Buts et al., 2003a) and the P pilus adhesin (87%; Vandemaele et al., 2003a)**.** However, in contrast to F4 fimbriae, the adhesins of all these fimbriae are minor subunits and the degree of sequence identity for their respective major subunits is often lower (Garcia et al., 1992; Vandemaele et al., 2003a). In the case of type 1 fimbriae, six variable regions are present in the major subunit, resulting in a 90% degree of sequence identity between different isolates (Vandemaele et al., 2003a). Garcia et al. (1992) reported

only 46.5% sequence identity between the major subunit PapA of different antigenic P pilus variants.

In comparison with the degree of FaeG sequence identity within F4ac strains, lower identity levels were found between FaeG of F4ac strains and of F4ab and F4ad strains (92 and 88%, respectively). The amino acid sequence differences between the three antigenic variants are mainly located between amino acid residues 162-174 and 206-216. However, these regions are conserved within the F4ac isolates, indicating a possible importance in F4R-binding. Indeed, this region has been reported to be a receptor-binding site (Bakker et al., 1992a) and to be the cause of differences in receptor binding among the three F4 antigenic variants (Bijlsma et al., 1982).

In accordance with Gaastra et al. (1983), it was observed that the signal sequence, the N- and C-terminal regions (amino acid residue 1-37 and 236-262, respectively), as well as a central region of FaeG (106-134) were very conserved for the three F4 antigenic variants. These regions are probably very important for the structure and the function of the subunit as they are also conserved in subunits of other fimbriae such as type 1 fimbriae and P pili (Soto and Hultgren, 1999). The FaeG signal sequence has probably a regulatory function in FaeG production. Hultgren et al. (1999) reported that release of the mature PapG adhesin from its signal sequence by signal peptidase I is enhanced by its interaction with the PapD chaperone. Furthermore, the FaeG signal sequence is conserved in F4-related fimbriae F41 and CS31A (Girardeau et al., 1991), but different compared to other fimbrial systems.

The N-terminal region of FaeG contains an alternating hydrophobic sequence. Crystal structures of subunits from type 1 and P pili and the lectin domain of the F17 fimbrial adhesin revealed a folding like an immunoglobulin(Ig)-domain, but lacking the C-terminal strand (Choudhury et al., 1999; Sauer et al., 1999; Buts et al., 2003a). The missing strand results in a hydrophobic groove along the surface of the subunit. However, the Ig-like domain structure of fimbrial subunits is stabilized by a β-strand of the chaperone in the periplasm (donor strand complementation) or by the N-terminal β-strand of a subunit in the fimbria (donor strand exchange). Therefore, changing the N-terminal sequence of fimbrial subunits inhibits the polymerisation of the subunits into a fimbria (Zavialov et al., 2003b). However, this cannot likely explain the observed weak interaction of FaeG subunits in 5/95 fimbriae, since its sequence only differs by the presence of a valine residue at position 201 but this amino acid has similar properties as the isoleucine found in all other isolates. The

weaker interaction between 5/95 FaeG subunits is still unknown, and may be due to a combination of certain amino acid residues at specific positions.

An alternating hydrophobic amino acid sequence was also observed in the FaeG C-terminus, containing a penultimate Tyr. In other fimbrial subunits like type 1 fimbriae and P pili, the C-terminal alternating hydrophobic sequence is reported to interact with the chaperone in the periplasm (Kuehn et al., 1993; Soto et al., 1998). The penultimate Tyr is critical for the conformational stability of the protein and for its interaction with the chaperone (Simons et al., 1990; Bullitt et al., 1996; Krasan et al., 2000; Ogunniyi et al., 2002). However, a serine at position 14 from the carboxyl terminal motif is present in FaeG, in contrast to a glycine in most fimbrial subunits (Soto and Hultgren, 1999). Nevertheless, we suggest that the donor strand complementation system may also work in the F4 fimbrial system. Indeed, the critical amino acid residues that are needed for proper functioning of the P-pilus chaperone PapD are also present in the F4-fimbrial chaperone FaeE (Bakker et al., 1991; Slonim et al., 1992; Holmgren et al., 1992; Kuehn et al., 1993; Hung et al., 1999a). Moreover, the C-terminal alternating hydrophobic sequences are present in the F4 fimbrial subunits FaeC, FaeG, FaeH and FaeI that interact with the chaperone FaeE (Mol et al., 2001).

A third conserved region was determined in the centre of FaeG. This region was found to be highly similar to the corresponding region of the P-pilus subunit PapK, which is coding for a part of the B β-strand and of the loop between the 3_{10}C α-helix. This 3_{10}C α-helix forms part of a site that interacts with the chaperone PapD (Sauer et al., 1999).

Isolate IMM 24 did not express F4 fimbriae and did not adhere to porcine enterocytes although the gene encoding FaeG was present. Probably, this could be due to a failure at another location in the F4 operon, like the gene encoding the chaperone or the usher. No study was performed on differences in adhesion efficiency among the other *E. coli* isolates or their purified F4 fimbriae to porcine enterocytes, because differences in numbers of fimbriae per cell and average lengths of fimbriae could occur and have an effect on adhesion. In addition, some isolates were found to express flagella and although flagella are not supposed to bind to the apical side of enterocytes (Gerwitz et al. 2001a and 2001b), these are known to influence the motility of an isolate and can subsequently enhance the likelihood to adhere (Jones et al., 1992; Schmitt et al., 2001).

The widely spread prevalence of F4$^+$ *E. coli* isolates (Nagy et al., 1990; Harel et al., 1991; Osek and Svennerholm, 1991; Wray et al., 1993; Garabal et al., 1997; Van den Broeck et al., 1999d; Frydendahl, 2002) and their increasing ability to resist antibiotics (Amezcua et al., 2002; Bischoff et al., 2002; Lanz et al., 2003; Noamani et al., 2003), stress the need to develop a vaccine against F4$^+$ ETEC. F4 fimbriae of the F4$^+$ ETEC reference strain GIS26 seem to be good vaccine candidates. Indeed, the results of the present study indicate that the F4 fimbrial adhesin FaeG is conserved and that F4 purified from GIS26 induce FaeG-specific antibodies which were able to inhibit F4R-binding of 20 different F4$^+$ *E. coli* isolates. In addition, the current results confirm the multimeric FaeG character of F4. Although the polymeric FaeG character of F4 fimbriae could be an advantage to improve the FaeG immunogenicity, FaeG monomers may perhaps bind more F4R than F4 fimbriae on a molar base. Therefore, further research on the usefulness of FaeG polymers or FaeG monomers in an oral vaccine against homologous and heterologous F4$^+$ *E. coli* infections would be worthwhile.

Chapter 5

Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain[1]

[1] *Based on : Verdonck F, Cox E, Van Gog K, Van der Stede Y, Duchateau L, Deprez P, Goddeeris BM. 2002. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic Escherichia coli strain or an F18 verotoxigenic Escherichia coli strain. Vaccine 20:2995-3004.*

Abstract

To develop a vaccine against *Escherichia coli* (*E. coli*) induced postweaning diarrhoea and oedema disease, insights in the induction of the protective immune response following infection with these pathogenic *E. coli*'s is needed. Therefore, the fimbriae-specific antibody response of newly weaned pigs following infection with the SLT-IIv producing $F18^+$ verotoxigenic *E. coli* (VTEC) (strain 107/86) was compared with the response following an infection with LT producing $F4^+$ enterotoxigenic *E. coli* (ETEC) (strain GIS26). $F4^+$ ETEC were able to colonize the gut very soon after infection since peak excretion of $F4^+$ *Escherichia coli* (*E. coli*) bacteria was seen 2 days post infection (dpi), but was already disappeared 7 dpi. On the other hand, $F18^+$ VTEC infection resulted in a slower colonization of the gut as the peak excretion of $F18^+$ *E. coli* was observed between 3 and 5 dpi, but this colonization remained longer as $F18^+$ *E. coli* were detected till 9 dpi in feces. Furthermore, this fast colonization pattern of $F4^+$ ETEC is accompanied with the presence of F4-specific antibodies in mucosal tissues and serum from 4 dpi onward, with maximal amounts of F4-specific IgA in the jejunal lamina propria and serum 7 dpi. In contrast, F18-specific IgA was only readily detected in the jejunal lamina propria 15 dpi and showed a maximum serum titer 21 dpi. Besides this faster induction and higher antibody response, the switch from IgM to IgA and IgG was also earlier following the $F4^+$ ETEC infection.

5.1. Introduction

Intestinal infections with *Escherichia coli* (*E. coli*) are an important cause of diarrhoea and mortality in humans in developing countries and in domestic animals. Especially in pigs, enterotoxigenic *E. coli* (ETEC) infections immediately after birth (neonatal diarrhoea) and ETEC or verotoxigenic *E. coli* (VTEC) infections after weaning (postweaning diarrhoea or oedema disease) are responsible for significant economical losses due to diarrhoea, growth retardation and mortality. The infections are mainly caused by $F4^+$ or $F18^+$ ETEC or by $F18^+$ VTEC (Nagy et al., 1990; Wittig et al., 1995), which have two important virulence factors : fimbriae and toxins. The fimbriae allow adhesion to specific receptors on small intestinal villi and consequently colonization can occur (Jones and Rutter, 1972; Bertschinger et al., 1990). The F4 receptor (F4R) and F18R are different, based on comparative *in vitro* adhesion studies

(Nagy et al., 1997) and on the localization on different porcine chromosomes (Gibbons et al., 1977; Vögeli et al., 1996). The presence of the F4R and F18R is genetically determined by an autosomal dominant gene and their absence makes pigs resistant to infection (Rutter et al., 1975; Bertschinger et al., 1993). Following colonization, ETEC strains produce heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa) and/or STb (Morris and Sojka, 1985) which induce a secretory diarrhoea (Nataro and Kaper, 1998). VTEC strains produce the Shiga-like toxin type II variant (SLT-IIv) (Marques et al., 1987; MacLeod et al., 1991), a vasotoxin that acts on vascular endothelial cells resulting in oedema and subsequent neurological signs including ataxia, recumbency and paddling movements, eventually leading to death (Clugston et al., 1974).

$F4^+$ ETEC infections mainly occur the first week after weaning, whereas $F18^+$ VTEC infection occurs between a week and two weeks post weaning. Colonization begins after adhesion of the bacteria with their fimbriae to the small intestine and stops when anti-fimbrial antibodies can be detected in the intestinal lumen of the infected pig (Yokoyama et al., 1992; Imberechts et al., 1997b; Zuniga et al., 1997; de Geus et al., 1998; Van den Broeck et al., 1999b). To protect weaned pigs, an oral vaccine must be developed since parenteral immunization does not induce protective immunity at mucosal surfaces (Bianchi et al., 1996). Van den Broeck et al. (1999b) reported that oral immunization of weaned pigs with purified F4 fimbriae protects them against subsequent $F4^+$ ETEC infection. For $F18^+$ *E. coli* however, only an infection has been reported to induce protective F18-specific antibodies at the small intestinal mucosa (Sarrazin and Bertschinger, 1997; Bertschinger et al., 2000). In the present study, the kinetics and localization of the immune response following an infection with $F4^+$ ETEC or $F18^+$ VTEC were compared. Insights in the mucosal immune response following infection with these pathogenic *E. coli*'s can be helpful in the development of an effective vaccine.

5.2. Material and methods

5.2.1. Pigs

Conventional bred pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks and subsequently housed in isolation units, fed at libitum and treated orally with colistine (Promycine pulvis, VMD, Berendonk, Belgium, 150,000 U/kg of body

weight/day) to prevent *E. coli* infections. One week post weaning, eighteen F18-seronegative and F18-receptor positive ($F18R^+$) pigs were infected with an $F18^+$ VTEC strain and twenty F4-seronegative and $F4R^+$ pigs were infected with an $F4^+$ ETEC strain.

5.2.2. Bacterial inoculum

The VTEC strain 107/86 (O139:K12:H1, $F18ab^+$, $SLT\text{-}IIv^+$) and the ETEC strain GIS26 (O149:K91, $F4ac^+$, LT^+ STa^+ STb^+) were cultured during 18 hours in Tryptone Soya Broth at 37°C and 85 rpm. The bacteria were collected by centrifugation (2,000 x g, 35 minutes, 4°C) and washed with phosphate-buffered saline (PBS, 150 mM, pH 7.4). The concentration of the bacteria was determined by measuring the optical 10-fold dilutions of the bacterial suspension at 660 nm (OD_{660}). An OD_{660} of 1 equals 10^9 bacteria per ml, as determined by counting colony forming units. Subsequently, the concentration of the bacteria was adjusted to 10^{10} bacteria per ml.

5.2.3. Purification of F18 fimbriae

A protocol to purify F18 fimbriae was developed. Bacteria of the *E. coli* strain 107/86 were cultured in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) at 37°C and 85 rpm for 18 hours. Subsequently, the bacteria were collected by centrifugation (3,000 x g, 35 minutes) and washed in PBS, after which the F18 fimbriae were isolated by heat shock (60°C for 20 minutes). Larger fragments were removed by centrifugation (10,000 x g, 20 minutes) and the supernatant was further purified by a subsequent centrifugation (20,000 x g, 40 minutes), both at 4°C. The solubilized F18 fimbriae were precipitated with 20% (wt/vol) ammonium sulphate and the pellet was dissolved and dialysed overnight against ultra pure H_2O.

5.2.4. Purification of F4 fimbriae

The F4 fimbriae were purified as described by Van den Broeck et al. (1999c). Briefly, fimbriae were isolated by homogenising the bacterial suspension of strain *E. coli* GIS26 using an Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany), followed by a purification using two centrifugation steps as for F18, a

precipitation step with 40% (wt/vol) ammonium sulphate. Thereafter the pellet was dissolved and dialysed overnight against ultra pure H_2O.

5.2.5. PCR of F18R linked FUT1

In order to select $F18R^+$ pigs, PCR amplification of the F18R linked FUT1 gene on isolated DNA of blood leukocytes was performed as described by Meijerink et al. (1997).

5.2.6. In vitro villous adhesion assay

The presence of F18R was confirmed and the presence of F4R was determined by the *in vitro* villous adhesion assays previously described (Van den Broeck et al., 1999c). Briefly, a 15-cm-long intestinal segment was excised of the mid jejunum at the moment of slaughter. The segment was washed twice with PBS and once with Krebs-Henseleit buffer (160 mM, pH 7.4) containing 1% vol/vol formaldehyde at 4°C. Subsequently, the villi were scraped from the mucosa and suspended in the same solution. Before use, the villi were washed 4 times in Krebs-Henseleit buffer without formaldehyde whereafter they were resuspended in PBS supplemented with 1% (wt/vol) D-mannose (Fluka, Sigma-Aldrich, Bornem, Belgium) to prevent adhesion by type 1 pili. Subsequently 4 x 10^8 $F18^+$ or $F4^+$ *E. coli* were added to an average of 50 villi in 0.5 ml buffer and incubated by room temperature for 1 hour while gently shaking. Then, villi were examined by phase-contrast microscopy at a magnification of 600 and the adhesion of bacteria was evaluated quantitatively by counting the number of bacteria adhering along a 50 µm villous brush border at 20 randomly selected places, after which the mean bacterial adhesion was calculated. Adhesion of more than 5 bacteria per 250 µm villous length was noted as positive (Cox and Houvenaghel, 1993).

5.2.7. Experimental procedure

5.2.7.1. VTEC experiment

On 2 consecutive days, the 5-week-old $F18R^+$ pigs (n = 18) were sedated using Stresnil (40 mg/ml; Janssen-Cilag, Berchem, Belgium), whereafter the acidic gastric pH was neutralized by intragastrical administration of 62 ml $NaHCO_3$ (1.4% (wt/vol) in distilled water) using a stomach tube. Fifteen to thirty minutes later, 10^{11} $F18^+$

VTEC (in 10 ml PBS) were intragastrically inoculated using a stomach tube. Faecal excretion of the $F18^+$ *E. coli* and presence of diarrhoea were determined daily from 2 till 10 days after the first inoculation (dpi). The kinetics and the localization of the antibody response was analysed by enumerating the F18-specific antibody secreting cells (ASCs) in peripheral blood, mesenteric lymph nodes, jejunal and ileal Peyer's patches, jejunal lamina propria, mesenteric lymph nodes and spleen 0, 4, 7, 11 and 15 dpi, from 3, 3, 3, 2 and 2 pigs respectively. Tissues were taken following euthanasia of animals by intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. At euthanasia, duodenal, jejunal and ileal contents were also sampled for measuring F18-specific IgA. Furthermore, F18-specific serum IgM, IgA and IgG were determined 0, 4, 7, 11, 15, 21 and 25 dpi, from 18, 15, 11, 7, 5, 3 and 3 pigs respectively. Finally, jejunal villi were isolated for the *in vitro* villous adhesion assay.

5.2.7.2. ETEC experiment

The experimental procedure was similar as for the VTEC experiment with minor differences. Firstly, 5-week-old $F4R^+$ pigs (n = 20) were intragastrically inoculated on two consecutive days with 10^{11} $F4^+$ ETEC. Secondly, the faecal excretion of the $F4^+$ *E. coli* was daily determined until day 7 after infection. The kinetics and the localization of the antibody response were analysed 0, 4, 7, 11 and 15 dpi, using 4, 3, 3, 3 and 4 pigs respectively. F4-specific serum IgM, IgA and IgG were determined 0, 4, 7, 11, 15, 21 and 25 dpi, from 20, 15, 12, 9, 5, 2 and 2 pigs respectively.

5.2.8. Samples

5.2.8.1. Serum

Blood was taken form the jugular vein. After 18 hours incubation at room temperature, serum was collected, inactivated at 56°C during 30 minutes and subsequently treated with kaolin (Sigma, Sigma-Aldrich) to decrease the background reading in ELISA (Van den Broeck et al., 1999a). Subsequently, the serum was diluted in ELISA dilution buffer (PBS + 0.05% (vol/vol) Tween®20 (Merck, Hohenbrunn, Germany) + 3% (wt/vol) bovine serum albumin (BSA; Sigma)) to obtain a final serum dilution of 1/10.

5.2.8.2. Faeces

Faecal samples were examined for the presence of $F18^+$ (VTEC experiment) or $F4^+$ *E. coli* (ETEC experiment). Therefore, 1% (wt/vol) suspensions of faecal samples in PBS were prepared at 4°C. Of these suspensions, four serial 10-fold dilutions were made in PBS and 50 µl drops of each dilution were spread onto plain blood agar plates. The plates were incubated at 37°C for 24 hours. $F18^+$ or $F4^+$ *E. coli* were identified using dot blotting as previously described (Van den Broeck et al., 1999b). F18 producing *E. coli* strains were detected using an F18-specific rabbit antiserum followed by incubation with a swine anti-rabbit immunoglobulin horseradish peroxidase (HRP)-conjugated antiserum (Dako, Denmark), the F4 producing *E. coli* strains were detected using an HRP-conjugated F4-specific Mab (IMM01; Van der Stede et al., 2002b). Binding of conjugate was visualized with a 3-amino-9-ethylcarbazole containing substrate solution. The developed brown-red dots were counted and the average within both groups was calculated. Results are presented as the mean number ± standard error of the mean (SEM) of excreted *E. coli* per gram feces.

5.2.8.3. Intestinal contents

Intestinal contents of duodenum, jejunum and ileum were twofold diluted in PBS + 20% foetal calf serum (FCS (vol/vol) + penicillin (100 IU/ml) + streptomycin (100 µg/ml) + 0.2% (vol/vol) Tween®20 and incubated at 56°C for 30 minutes. Afterwards, the samples were centrifugated at 4°C and 9,500 x g. The supernatant was centrifugated once again and stored at -70°C until analysis.

5.2.8.4. Peripheral blood monomorphonuclear cells (MC)

Peripheral blood MC were isolated as previously described (Van den Broeck et al., 1999a). Briefly, peripheral blood MC were isolated by density gradient centrifugation and erythrocytes were lysed in ammonium chloride (0.74% [wt/vol]). After washing at 4°C, the pelleted cells were resuspended at 10^7 cells/ml in leukocyte medium [RPMI-1640 (GIBCO BRL, Paisley, Scotland) containing FCS (10% [vol/vol]) (Seromed, International Medical, Berlin, Germany), 2-mercaptoethanol (5 x 10^{-5} M) (GIBCO BRL), non-essential amino acids (GIBCO BRL), Na-pyruvate (100 µg/ml) (GIBCO BRL), L-glutamine (292 µg/ml) (GIBCO BRL), penicillin (100

IU/ml) (GIBCO BRL), streptomycin (100 µg/ml) (GIBCO BRL) and kanamycin (100 µg/ml) (GIBCO BRL)].

5.2.8.5. Spleen and mesenteric lymph node MC

At the moment of slaughter, a part of the spleen and some mesenteric (jejunal and ileal) lymph nodes were aseptically dissected. After removing surrounding fat from the specimen, the MC were isolated by teasing the tissue apart, followed by lysis of erythrocytes in ammonium chloride. After washing, the pelleted cells were resuspended at 10^7 cells/ml in leukocyte medium.

5.2.8.6. Jejunal lamina propria MC

At the moment of slaughter, an intestinal segment without Peyer's patches was excised of the jejunum and washed with PBS at room temperature. The segment was cut in 2-cm-long pieces which were washed with RPMI-1640 (GIBCO BRL) + 10 mM HEPES (GIBCO BRL) + 5% (vol/vol) FCS. Then, the pieces were washed two times with PBS without calcium and magnesium (CMF buffer) at room temperature and once with warm (37°C) CMF buffer supplemented with EDTA (3.7% (wt/vol)) and 0.94 M dithiotreithol (GIBCO BRL). Subsequently, the 2-cm-long pieces were cut in very small pieces and incubated in RPMI-1640 + 10 mM HEPES + 2% (vol/vol) FCS + 0.015% (wt/vol) collagenase (SERVA, Polylab, Antwerp, Belgium) + 0.01% (wt/vol) DNase I (Boehringer Mannheim, Brussels, Belgium) for 30 minutes at 37°C and 200 rpm. The obtained cell suspension was filtered through cell collectors of 200, 150 and 80 mesh (Sigma). The isolated cells were washed and resuspended in leukocyte medium at 10^7 cells/ml.

5.2.8.7. Peyer's patches MC

At the moment of slaughter, jejunal and ileal Peyer's patches were excised from the intestine. The isolated tissue was washed at room temperature with PBS and CMF buffer and subsequently incubated with CMF buffer supplemented with EDTA for 15 minutes at 37°C and 200 rpm as described before. Then, Peyer's patches MC were collected by scraping the Peyer's patches at room temperature, washing the cells and resuspending the cells at 4°C in leukocyte medium at 10^7 cells/ml.

5.2.9. ELISA's for F18- or F4-specific IgM, IgA and IgG

An indirect ELISA was developed to detect F18-specific serum IgM, IgA and IgG titers in serum and IgA titers in intestinal content. Purified F18 fimbriae were coated at a concentration of 1.56 µg/ml in coating buffer (carbonate-bicarbonate buffer, 50 mM, pH 9.4) on a 96-well microtiter plate (NUNC, Maxisorp Immuno Plates, Roskilde, Denmark). After 2 h incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2% (vol/vol) Tween®80. The serum or intestinal contents were diluted in ELISA dilution buffer (PBS + 0.05% (vol/vol) Tween®20 + 3% (wt/vol) BSA) and series of twofold dilutions, starting from 1/10 and 1/2 respectively, were made. The plates were incubated for 1 hour at 37°C. Thereafter, optimal dilutions of anti-swine IgM-, IgA- or IgG-specific MAb (Van Zaane and Hulst, 1987) were added to the wells for 1 h at 37°C. Subsequently, rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) optimally diluted in the ELISA dilution buffer and supplemented with 2% (vol/vol) pig serum was brought on the plate for 1 h at 37°C. An ABTS solution containing H_2O_2 was added and after 1 hr incubation at 37°C the optical density was spectrophotometrically measured at 405 nm (OD_{405}). The plates were washed three times with ELISA washing buffer (PBS + 0.2% (vol/vol) Tween®20) between each incubation step. The IgM, IgA and IgG cut-off values were calculated as the mean OD_{405}-value of all sera (dilution 1/10) at day 0, increased with 3 times the standard deviation. The antibody titer was the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value.

For detection of F4-specific antibodies, the indirect ELISA described by (Van den Broeck et al., 1999a) was used. Briefly, the wells of a 96-well microtiter plate were coated with the F4-specific monoclonal antibody (IMM01) at a concentration of 1µg/ml coating buffer. Subsequently, purified F4, the treated sera or intestinal contents, optimal dilutions of biotinylated-swine-specific IgM, IgA and IgG MAb and HRP-conjugated streptavidin (GIBCO BRL) were added. Incubation times and conditions were similar as for F18.

5.2.10. Elispot assays for antigen-specific IgM, IgA and IgG antibody-secreting cells

F18 or F4 coated plates were prepared as described above. Thereafter, MC suspensions at a concentration of 10^6 cells/ml leukocyte medium were added (100 µl/well), and plates were incubated for 14 hours at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, the cells were removed by three washes with ELISA washing buffer and wells were sequentially incubated with anti-swine IgM, IgA and IgG Mab, rabbit anti-mouse antibodies (Dako) coupled to biotin and HRP-conjugated streptavidin (Dako) each time for 1 hour at 37°C. Between each step, the plates were washed with ELISA washing buffer. Subsequently, a substrate solution, consisting of 4 volumes of 3-amino-9-ethylcarbazole (AEC) (Sigma) working solution (0.67 ml AEC stock solution (0.4% (wt/vol) in dimethylformamide) in 10 ml Na acetate (0.1 M, pH 5.2) + 10 µl 30% H_2O_2) and 1 volume of 3% (wt/vol) low-melting-point agarose (BIOzym, Landgraaf, The Netherlands) was added. After overnight incubation in the dark at room temperature, spots were counted with an inverted microscope. For each MC suspension, spots in 3 wells (10^6 MC/well) were counted to obtain the number of isotype-specific ASCs per 3 x 10^5 MC. Results are presented as the mean number of ASCs per 10^6 MC ± SEM.

5.2.11. Statistical analysis

Statistical analysis (SAS, version 8) was done using mixed models with pig as random effect and the ETEC/VTEC strains, time and the interaction between ETEC/VTEC strains and time (categorized) as fixed effects. Firstly these fixed effects were tested for significance and secondly, the ETEC/VTEC infection was compared at each of the time points separately, adjusting for multiple comparison by Bonferoni, with each individual comparison being tested at $\alpha = 0.05$ divided by the number of time points.

5.3. Results

5.3.1. Clinical signs and excretion of F18$^+$ VTEC or F4$^+$ ETEC

In the VTEC experiment, eighteen 5-week-old F18-seronegative but F18R$^+$ pigs were intragastrically infected with F18$^+$ VTEC, whereas in the ETEC experiment, twenty 5-week-old, F4-seronegative but F4R$^+$ pigs were infected with F4$^+$ ETEC. The presence of the F18R was demonstrated by PCR and confirmed by the *in vitro* villous adhesion assay. Indeed, all PCR-positive pigs were shown to be receptor positive (data not shown). Similarly, the presence of the F4R on enterocytes of all the pigs was demonstrated in the *in vitro* villous adhesion assay (data not shown).

Following the F18$^+$ VTEC infection only three pigs had diarrhoea 1 dpi, but following the F4$^+$ ETEC infection ten pigs had severe watery diarrhoea 2 dpi, six pigs 3 dpi and three pigs 4 dpi.

Furthermore, two pigs died following the F18$^+$ VTEC infection, one 6 dpi and another 10 dpi, without obvious clinical signs of disease. F18$^+$ *E. coli* was isolated from their intestinal tract and hemorrhagic intestines and swollen mesenteric lymph nodes were found. In the F4 infection experiment, one of the pigs with severe diarrhoea died 3 dpi. F4$^+$ *E. coli* was isolated from the intestinal content of this pig, but postmortem examination revealed no pathologic signs.

F4$^+$ and F18$^+$ *E. coli* excretion were statistically significant different ($p = 0.0008$) and the F4$^+$ *E. coli* excretion evolved differently over time than F18$^+$ *E. coli* excretion (significant time-strain interaction, $p < 0.0001$). Before infection, the examination of the faecal samples for F18$^+$ and F4$^+$ *E. coli* was negative. Following infection, F18$^+$ *E. coli* were detected in pigs of the VTEC experiment from 2 till 9 dpi with a peak excretion 3 dpi (9.9×10^7 F18$^+$ *E. coli* / g feces), whereafter the amount of F18$^+$ *E. coli* gradually decreased (Fig. 1). The pigs of the ETEC experiment excreted 2 dpi a very high number of F4$^+$ *E. coli* (5.97×10^8 F4$^+$ *E. coli* / g feces). Subsequently, this number rapidly decreased and F4$^+$ *E. coli* could not be detected anymore from 7 dpi (Fig. 1). Moreover, the excretion of F4$^+$ *E. coli* was significant lower ($p < 0.0001$) compared with the excretion of F18$^+$ *E. coli* at 6 and 7 dpi.

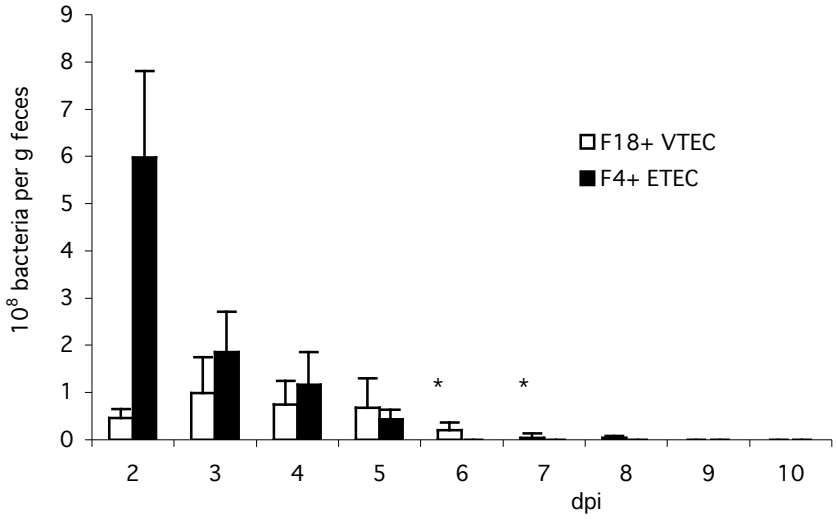


Figure 1 : Mean faecal F18$^+$ VTEC and F4$^+$ ETEC excretion per gram feces (± SEM) (* P<0.0001)

5.3.2. Localization of the fimbriae-specific antibody response following infection

In order to localize the induced antibody response following the F18$^+$ VTEC and the F4$^+$ ETEC infection, the number of fimbriae-specific IgM, IgA and IgG ASCs was determined in bone marrow, peripheral blood, spleen, mesenteric lymph nodes, jejunal and ileal Peyer's patches and jejunal lamina propria. There were no F18-specific ASCs present at the moment of infection (0 dpi), but F4-specific background levels of IgM ASCs (Fig. 2) were detected at the moment of infection in the spleen (9 IgM ASCs per 10^6 MC) and the mesenteric lymph nodes (25 IgM ASCs per 10^6 MC) as previously also observed by Van den Broeck et al. (1999a).

F18-specific IgM ASCs could already be detected 4 dpi. At that moment the mean numbers of IgM ASCs (Fig. 2) peaked in the spleen (15 IgM ASCs per 10^6 MC) whereas the peak occurred 3 days later in the mesenteric lymph nodes (7.5 IgM ASCs per 10^6 MC) and the ileal Peyer's patches (3.3 IgM ASCs per 10^6 MC) and 7 days later in the jejunal Peyer's patches (23 IgM ASCs per 10^6 MC), lamina propria (7 IgM ASCs per 10^6 MC), peripheral blood (6.6 IgM ASCs per 10^6 MC) and bone marrow (0.83 IgM ASCs per 10^6 MC). In the F4$^+$ ETEC experiment, the spleen contained the highest number of F4-specific IgM ASCs (100 per 10^6 MC) 4 dpi,

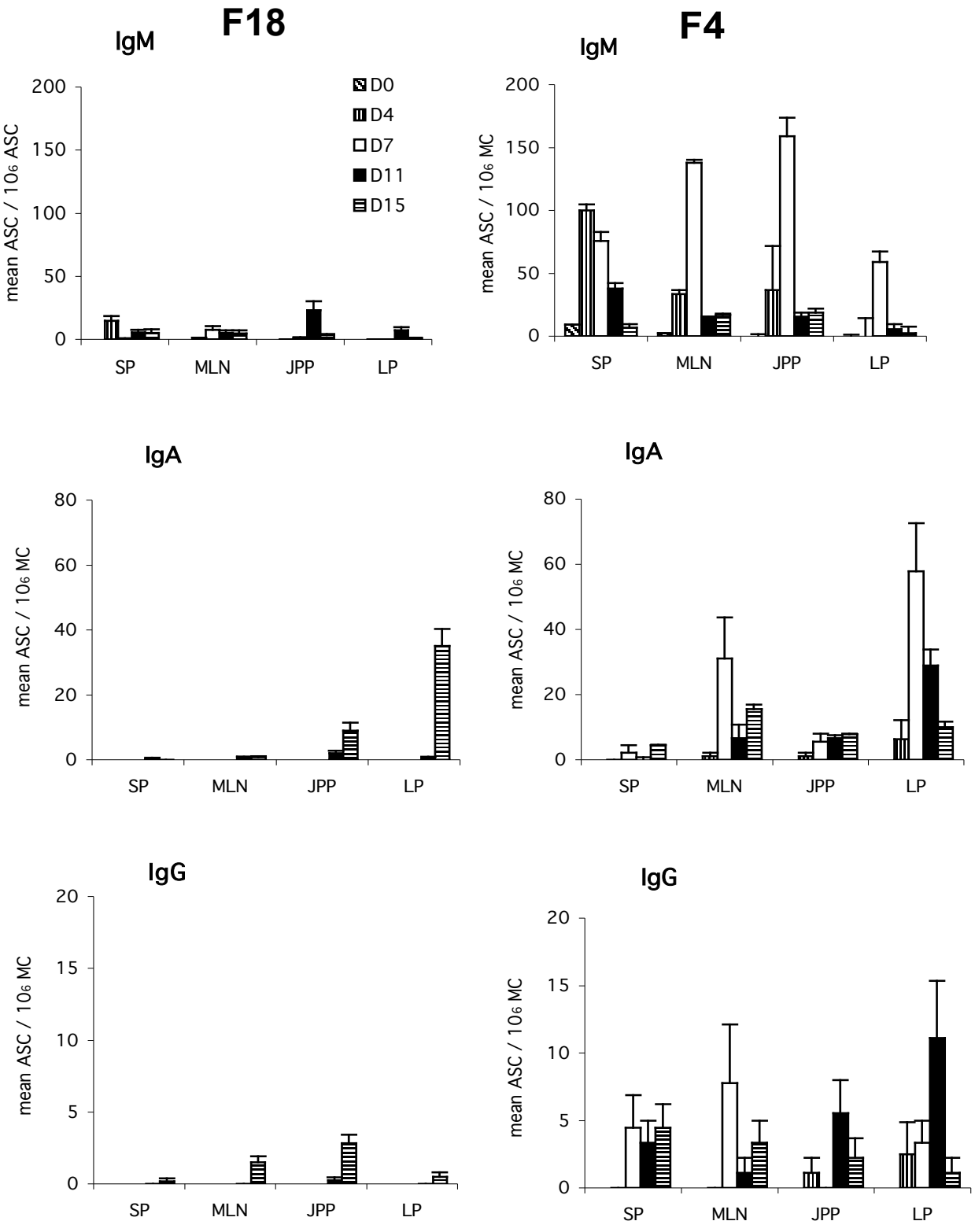


Figure 2 : Mean F18-specific IgM, IgA and IgG and F4-specific IgM, IgA and IgG ASC per 10^6 MC (± SEM) in spleen (SP), mesenteric lymph node (MLN), jejunal Peyer's patches (JPP), and lamina propria (LP) at 0, 4, 7, 11 and 15 days post infection (dpi).

whereas the other lymphoid tissues revealed maxima 7 dpi with the highest number in the jejunal Peyer's patches (159 per 10^6 MC) (Fig. 2). However, the numbers F4-specific IgM ASCs were several times higher than for F18-specific IgM ASCs.

F18-specific IgA ASCs appeared later in the immune response. The first IgA ASCs were found in most tissues 11 dpi, but only 15 dpi substantial numbers of IgA ASCs were present in the jejunal Peyer's patches (9 IgA ASCs per 10^6 MC) and in the lamina propria (35 IgA ASCs per 10^6 MC) (Fig. 2). In contrast to the $F18^+$ VTEC infection, F4-specific IgA ASCs (Fig. 2) could already be detected 4 dpi in peripheral blood, the mesenteric lymph nodes, jejunal and ileal Peyer's patches and jejunal lamina propria. Furthermore, the F4-specific IgA ASCs number already peaked in these tissues 7 dpi, except for the jejunal Peyer's patches where a gradual increase was observed until 15 dpi (7 IgA ASCs per 10^6 MC). In the spleen and bone marrow F4-specific IgA ASCs were first seen 7 dpi, but remained low in number. Furthermore, the numbers of fimbriae-specific IgA ASCs were generally higher following the $F4^+$ ETEC infection than following the $F18^+$ VTEC infection.

In accordance to the late appearance of F18-specific IgA, F18-specific IgG ASCs appeared in most tissues 15 dpi, but their mean numbers were almost 10 times lower than for IgA (0 to 28 IgG ASCs per 10^5 MC) (Fig. 2). However, F4-specific IgG ASCs (Fig. 2) were already observed in most tissues 4 dpi, but in general these numbers remained low. The highest numbers of F4-specific IgG ASCs per 10^6 MC were observed 7 dpi in the mesenteric lymph nodes (8) and 11 dpi in the jejunal lamina propria (11) and the jejunal Peyer's patches (6).

5.3.3. Fimbriae-specific serum antibody responses following infection

The F18-specific serum antibody response following the $F18^+$ VTEC infection was low (Fig. 3). F18-specific serum IgM only slightly increased till 7 dpi and decreased again from 15 dpi to reach the background level 21 dpi. Following the $F4^+$ ETEC infection, F4-specific serum IgM peaked 7 dpi whereafter it gradually decreased again to baseline values. F4-specific IgM was however significantly higher ($p < 0.0001$) 4, 7 and 11 dpi in comparison with F18-specific IgM.

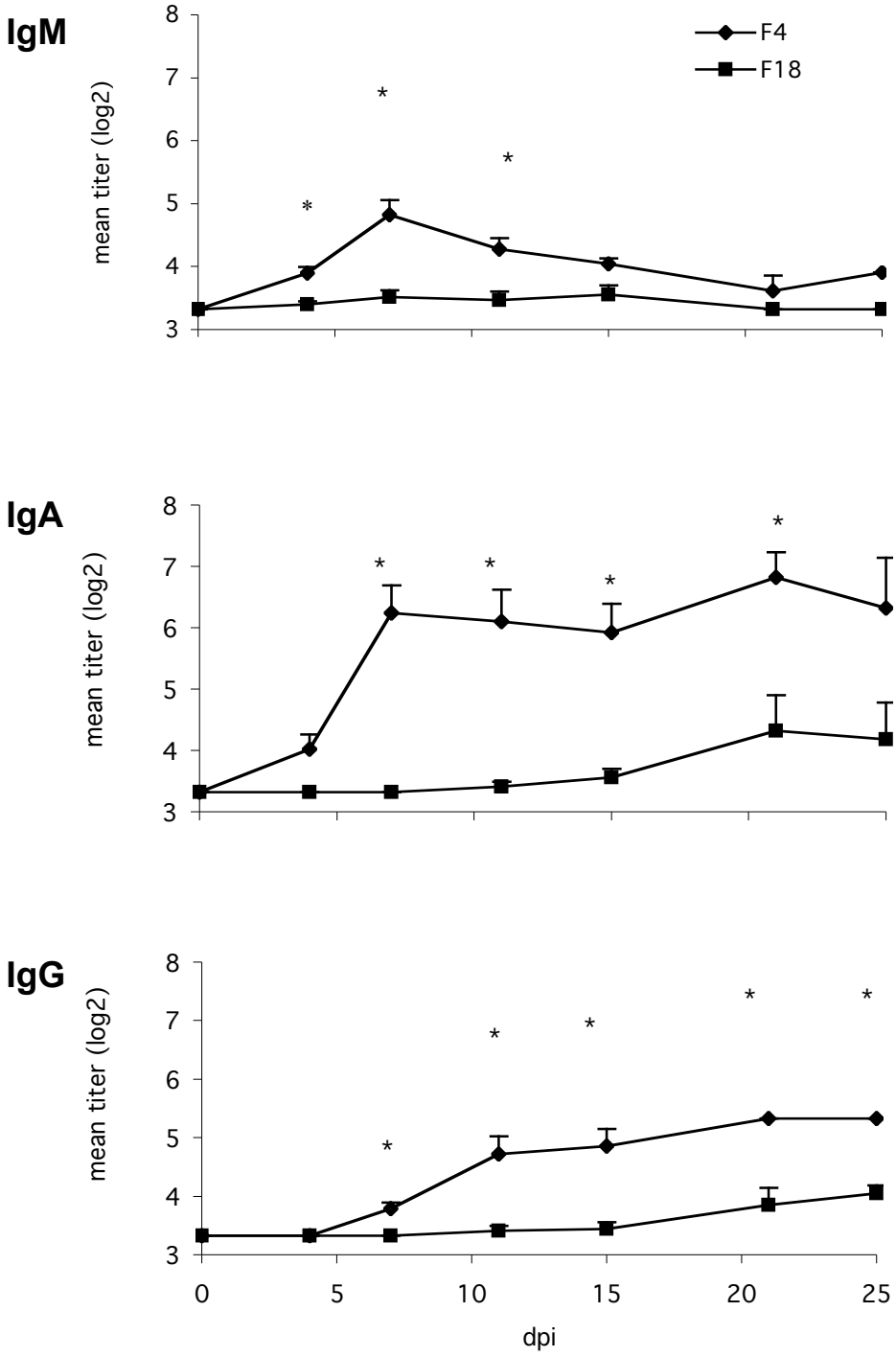


Figure 3 : Mean F4- and F18-specific IgM, IgA and IgG serum antibody titers (± SEM) at 0, 4, 7, 11, 15, 21 and 25 days post infection (dpi) (* P<0.0009).

F18-specific serum IgA and IgG were first detected 11 dpi (Fig. 3). Thereafter, IgA reached a maximum on 21 dpi whereas IgG was still increasing 25 dpi. On the other hand, F4-specific IgA and IgG were already detected 4 and 7 dpi to reach a plateau on 7 and 11 dpi, respectively. Besides the faster appearance, F4-specific IgA and IgG responses were also significantly higher ($p < 0.0009$) than F18-specific IgA and IgG responses from 7 to 25 dpi.

5.3.4. Fimbriae-specific IgA in intestinal contents following infection

At euthanasia, the intestinal contents of duodenum, jejunum and ileum were collected for determining fimbriae-specific IgA. F18-specific IgA appeared relative late (11 to 15 dpi) in the jejunum and ileum. It could not be detected in duodenal content (Table 1). Only in 1 pig IgA was already detected 7 dpi in the jejunal content. The F4-specific IgA appeared earlier than the F18-specific IgA : two out of three jejunal samples were already positive 4 dpi (Table 1). Furthermore, IgA was not restricted to jejunum and ileum as most duodenal samples were also positive from 7 dpi onwards.

Table 1 : F18- and F4-specific IgA titers in intestinal contents of the duodenum, jejunum and ileum at 0, 4, 7, 11 and 15 days post infection (dpi).

F18	duodenum	jejunum	ileum
0 dpi	0	0	0
(n=3)	0	0	0
	0	0	0
4 dpi	0	0	0
(n=3)	0	0	0
	0	0	0
7 dpi	0	0	0
(n=3)	0	0	0
	0	2	0
11 dpi	0	0	4
(n=2)	0	0	2
15 dpi	0	4	2
(n=2)	0	32	128

F4	duodenum	jejunum	ileum
0 dpi	0	0	0
(n=4)	0	0	0
	0	0	0
	0	0	0
4 dpi	0	4	0
(n=3)	0	0	0
	0	4	0
7 dpi	16	4	8
(n=3)	8	0	32
	8	32	8
11 dpi	0	0	0
(n=3)	32	128	4
	64	64	8
15 dpi	32	32	ND
(n=4)	32	ND	4
	4	32	ND
	64	128	16

5.4. Discussion

In the present study, infection of newly weaned pigs with $F18^+$ VTEC and $F4^+$ ETEC resulted in a significant different excretion of $F18^+$ and $F4^+$ *E. coli*. In accordance with previous studies (Bertschinger and Pohlenz, 1983; Bertschinger et al., 1990; Nagy et al., 1992), the peak excretion following the $F4^+$ ETEC infection was observed 2 dpi, whereas 1 to 3 days later for the $F18^+$ VTEC infection the maximal excretion occurred. Furthermore, the excretion of $F4^+$ *E. coli* disappeared 7 dpi whereas the $F18^+$ *E. coli* could be detected till 9 dpi. A reason for the slower colonization of the $F18^+$ VTEC than $F4^+$ ETEC is not known. A factor that could play a role is the adhesion to the receptor. For F4 fimbriae, the adherent subunit is the major subunit whereas for F18 fimbriae this is a minor subunit of which the localization in the fimbrial structure is not known (Bakker et al., 1992a; Smeds et al., 2001). This difference could account for a weaker adhesion of $F18^+$ *E. coli* to the enterocytes brush border receptor. In the *in vitro* villous adhesion assay, the $F18^+$ *E. coli* strain 107/86 always showed a weak adhesion (maximum 53 bacteria/250 μm brush border), in comparison with the $F4^+$ *E. coli* strain GIS26 (> 80 bacteria/250 μm brush border). Other factors that could influence this adhesion are the amount of fimbriae expressed by the bacteria, the number of receptors on the surface of enterocytes, the strength of the binding and environmental factors influencing the interaction.

The rapid colonization of the intestine with $F4^+$ ETEC resulted in a fast F4-specific mucosal immune response. Indeed, more than 30 F4-specific IgM ASCs per 10^6 MC were seen 4 dpi in the mesenteric lymph nodes, the jejunal Peyer's patches and the spleen followed only four days later in the mucosal tissues by maximal amounts of F4-specific IgA ASCs. On the other hand, the slower $F18^+$ VTEC colonization induced a slower F18-specific mucosal immune response. First detection of F18-specific IgM ASCs occurred 4 to 7 dpi and maximal amounts of F18-specific IgA ASCs were observed only on 15 dpi. It is reported in several studies that fimbriae-specific mucosal antibodies prevent colonization (Yokoyama et al., 1992; Imberechts et al., 1997b; Zuniga et al., 1997; de Geus et al., 1998; Van den Broeck et al., 1999b). As a consequence, the rapid appearance of F4-specific ASCs in the lamina propria and F4-specific IgA in the small intestinal content resulted in an early decrease in faecal excretion of $F4^+$ *E. coli* (6 dpi), whereas the slower response

against F18 fimbriae was accompanied with a longer excretion of the $F18^+$ strain (9 dpi). This slower immune response following the $F18^+$ VTEC infection and the corresponding longer excretion increases the risk on spreading of an $F18^+$ VTEC infection. This might explain why in a recent seroprevalence study 93% of the farms were F18-positive (Verdonck et al., 2003), whereas only 65% of the farms were F4-positive (Van den Broeck et al., 1999d).

In order to develop an oral vaccine against *E. coli* induced postweaning diarrhoea and oedema disease, it is important to elucidate why the $F4^+$ ETEC infection is inducing such a rapid and high immune response.

It has been demonstrated that oral immunization of pigs with purified F4 fimbriae induces a protection against $F4^+$ ETEC infections (Van den Broeck et al., 1999b), whereas oral immunization of pigs with purified F18 fimbriae does not (unpublished data). In agreement, Felder et al. (2001) reported that oral immunization of pigs with poly(lactide-co-glycolide) microspheres containing F18 fimbriae could not induce significant F18-specific serum antibodies, nor reduced $F18^+$ *E. coli* colonization following a challenge infection. These findings indicate that F18 is less immunogenic than F4 when given via the oral route. Here, the same mechanisms responsible for the differences in colonization might give an explanation.

Furthermore, the $F4^+$ ETEC strain GIS26 produces heat-labile (LT) and heat-stable (STa, STb) enterotoxins, whereas the $F18^+$ VTEC strain 107/86 expresses the verotoxin SLT-IIv. In contrast with SLT-IIv, the LT enterotoxin is known to possess adjuvant properties (Rappuoli et al., 1999). Several studies reported increased IgA and IgG but not IgM responses following an immunization with antigen in the presence of LT (Guidry et al., 1997; Hartman et al., 1999; de Haan et al., 2001). So, the adjuvanticity of LT is a second important factor that could be involved with the rapid F4-specific immune response and the early switch from IgM to IgA and IgG. Indeed, in different lymphoid tissues the F4-specific IgM ASCs following $F4^+$ ETEC infection reached 4 days earlier their maximum number, which was more than twice as high as following oral F4 immunization with purified F4 fimbriae (without LT) (Van den Broeck et al., 1999a). Similar, the maximal amounts of F4-specific IgA and IgG ASCs were seen 4 days earlier and were ten times higher following $F4^+$ ETEC infection as opposed to oral immunization with purified F4 fimbriae. One should however also consider that LPS has also adjuvant properties, which might influence fimbriae-specific antibody responses in *E. coli* infections.

The reported results have useful implications for the development of an oral vaccine against *E. coli* induced postweaning diarrhoea and oedema disease. Oral immunization of newly weaned pigs with purified F4 fimbriae is already reported to induce a protective mucosal immune response against $F4^+$ ETEC challenge **(**Van den Broeck et al., 1999b). However, in the case of post-weaning diarrhoea a very rapid immune response is needed since oral vaccination of the suckling piglets is probably impossible and infection with ETEC strains occurs during the first week after weaning. The results of the present study show that immune response against F4 can occur within 4 days following infection. So, factors such as dose of the antigen and the use of LT enterotoxin as oral adjuvant have to be considered for ameliorating the response against purified fimbriae. In case of F18 fimbriae, the antibody response is mainly directed against the major FedA subunit and not against the minor FedF subunit, which is responsible for adhesion. Therefore, vaccination with FedF in the absence of FedA could improve protection. Based on reported results of the recombinant adhesin FimH of type 1 fimbriae of uropathogenic *E. coli*, use of recombinant FedF adhesins could be envisaged. Indeed, vaccination of mice and monkeys with recombinant minor subunit FimH protects them against challenge with uropathogenic *E. coli*, whereas the antibodies evoked with purified type 1 fimbriae were directed primarily at the nonconserved major FimA subunit (Levine et al., 1982; Pecha et al., 1989; Langermann et al., 1997 and 2000; Thankavel et al., 1997). On the other hand, protection against VTEC infections has also been reported by immunization of pigs with recombinant SLT-IIv (Bosworth et al., 1996; Johansen et al., 1997; Makino et al., 2001). However, although these vasotoxin-specific antibodies can block the toxic effects of the SLT-IIv (Johansen et al., 2000), they will not influence the colonization of the intestine and subsequently the spread of the VTEC bacteria. Therefore, the induction of a protective F18-specific mucosal immune response against $F18^+$ *E. coli* infections remains preferable.

Chapter 6

The interaction of F4 fimbriae with porcine enterocytes as analysed by surface plasmon resonance[1]

[1] *Based on : Verdonck F, Cox E, Vancaeneghem S, Goddeeris BM. 2004b. The interaction of F4 fimbriae with porcine enterocytes as analysed by surface plasmon resonance. FEMS Immunol. Med. Microbiol., in press.*

Abstract

Fimbriae often play a prominent role in anchoring bacterial cells to host tissue and mediate the first step in pathogenesis. As a consequence, there is a continuous development of new strategies to block the binding of fimbriae to their specific receptor on host cells. The present study demonstrates the specific interaction of F4 (K88) fimbriae and porcine enterocytes using a real-time biomolecular interaction analysis system (BIAcore® 3000), based on the principles of surface plasmon resonance (SPR). This method offers new opportunities to screen therapeutics for prevention of adhesion and subsequent disease without receptor purification.

6.1. Introduction

$F4^+$ ETEC are an important cause of neonatal and post-weaning diarrhoea in piglets. The F4 fimbriae allow attachment to F4R on enterocytes, enabling the bacteria to colonize the small intestine and to produce heat-labile (LT) and/or heat-stable enterotoxins (STa, STb), resulting in diarrhoea (Gyles, 1994).

F4 fimbriae are long proteinaceous appendages radiating from the surface of the bacteria and occur as three antigenic variants: F4ab, F4ac and F4ad (∅rskov et al., 1964 ; Guinée and Jansen, 1979). The differences between these variants were found to be only located in the major F4 fimbrial subunit FaeG, which is also the adhesin. Conserved regions of FaeG form the “a” epitopes, whereas a variable region forms the “b”, “c” or “d” epitope (Bakker et al., 1992a). With these 3 variants, 6 porcine phenotypes (A through F) can be distinguished with regard to brush border adhesiveness (Bijlsma et al., 1982; Baker et al., 1997). The phenotypic difference between pigs has been shown to be genetic in origin, being inherited in a Mendelian way, with adhesion dominant over non-adhesion (Sellwood and Kearns, 1979).

In vitro F4-mediated adhesion to isolated porcine small intestinal brush border membranes (Sellwood et al., 1975; Valpotic et al., 1989a), small intestinal enterocytes (Isaacson *et al.,* 1978) or isolated villi (Girardeau, 1980) correlates with *in vivo* villous adhesion and colonization by $F4^+$ ETEC. Furthermore, an indirect ELISA (Valpotic et al., 1989b) and a Western blot (Willemsen and de Graaf 1992; Erickson et al., 1992) are described to distinguish F4 adhesive and non-adhesive pigs. However, these assays analyse

the interaction between fimbriae and their receptor in a static way, in contrast to the *in vivo* situation. Therefore, the aim of the present study was to determine the interaction of host cells and fimbriae in a non-static situation and without previous receptor purification. Hereto, the interaction of F4 receptor positive ($F4R^+$) and $F4R^-$ enterocytes with F4 fimbriae was determined in a continuous flow using the BIAcore® 3000 (Uppsala, Sweden), a real-time biomolecular interaction analysis system based on the principles of surface plasmon resonance (SPR).

6.2. Material and methods

6.2.1. Isolation of F4 fimbriae

F4ac fimbriae of the *E. coli* strain GIS26 (O149:K91:F4ac, $LT^+STa^+STb^+$) were isolated by homogenizing a GIS26 bacterial suspension. Subsequently, fimbriae were purified by anion exchange chromatography using a Bio-Scale Q5 column (BIO-RAD Laboratories) as described by Van den Broeck et al. (1999c). The protein concentration was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as standard (ICN Biomedicals, Belgium) and the purity of the purified F4 fimbriae was assessed using a Coomassie stained 15% SDS-PAGE and the ImageMaster 1D prime software (Amersham Pharmacia Biotech, Belgium).

6.2.2. In vitro villous adhesion assay

The *in vitro* villous adhesion assay has been described by Van den Broeck et al. (1999c). Adhesion of more than 30 bacteria per 250 µm brush border length was noted as strong, less than 30 bacteria per 250 µm brush border length meant weak adhesion, and less than 5 was regarded as negative (Cox et al., 1991).

6.2.3. Enterocyte isolation

Enterocytes of nine pigs were isolated from the jejunum since the F4R is present in high amounts in the mid-small intestine (Cox and Houvenaghel, 1993; Chandler et al., 1994).

Hereto, a 1-m-long intestinal segment was excised of the mid jejunum at the moment of slaughter. The segment was washed in Krebs-Henseleit buffer (160 mM, pH 7.4) containing 1% (vol/vol) formaldehyde, whereafter the segment was filled with EDTA-buffer (phosphate buffered saline (PBS; pH 7.4, 150 mM) + 0.01M

EDTA, pH 6.8). The ends were ligated and the segment was incubated 30 minutes on ice in a sucrose-buffer (PBS + 0.3 M sucrose, pH 6.8). The content of the segment was collected and the segment was refilled again with sucrose-buffer and incubated for 5-10 minutes at room temperature. The content of the segment was collected and centrifugated (10 min, 200 g, 4°C). Subsequently, the pellet was resuspended in 10 volumes EDTA-buffer, pushed gently through an 18G needle and centrifugated (10 min, 200 g, 4°C). Finally, the pellet was resuspended in Krebs-Henseleit buffer.

6.2.4. Immobilization of F4 fimbriae on sensor chip

The interaction between F4 fimbriae and the F4R on pig enterocytes was analyzed with the Biacore®3000 biosensor (Uppsala, Sweden) using the F1 chip. This has a short carboxymethyldextran surface to allow immobilization of F4 fimbriae via free NH_2 using an amine coupling kit (Biacore, Uppsala, Sweden) containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*'-[(3-dimethylamino)-propyl]-carbodiimide hydrochloride (EDC) and ethanolamine-HCl. Hereto, 50 µl of a mixture of NHS (290 µg) and EDC (190 µg) was injected at a flow rate of 5 µl/ml at 25°C to activate the dextran matrix on the sensor chip, followed by purified F4 at a concentration of 100 µg/ml in 10 mM sodium acetate buffer pH 3.5 until the amount (mol) of immobilized F4 equalized 2000 resonance units (RU). Thousand RU corresponds to a change in the surface concentration of 1 ng/mm^2 (Fägerstam *et al.*, 1992 ; Stenberg *et al.*, 1991). Subsequently, the remaining active sites of the matrix were blocked with ethanolamine-HCl (1 M) and washed with HBS-EP (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005 % (v/v) polysorbate 20). One flow cell was activated and subsequently blocked without immobilization of F4 fimbriae and served as blanco. All samples used in further experiments were diluted in HBS-EP buffer, which also served as running buffer during the experiments.

6.2.5. Analysis of the F4R phenotype of enterocytes

Enterocyte suspensions were injected at a concentration of 5.10^5 cells/ml and a flow rate of 5 µl/min during 240 seconds at 25°C. The binding of the enterocytes was monitored and registered as a sensogram (plot of RU versus time). To regenerate flow cells after binding of enterocytes to immobilized F4 fimbriae, 25 mM NaOH + 100 mM CHAPS (3-[(3-cholamiindopropyl)dimethylammonio]-1-propane-sulphonate,

Sigma) was used. The experiments were always performed in duplicate and revealed identical results.

6.2.6. Determining the specificity of F4R$^+$ enterocyte binding

The first approach was to block the c-epitope of immobilized F4 fimbriae. This epitope is involved in binding the F4R. Hereto, the F4ac-specific monoclonal antibody (MAb) CVI F4ac-5 (van Zijderveld et al., 1990; ID-DLO, Lelystad, The Netherlands) was diluted to concentrations of 1 µg/ml and 10 µg/ml and injected with a flow rate of 5 µl/min during 240 seconds. Subsequently, enterocytes of the F4R$^+$ pigs were injected as described above.

The second approach was to pre-incubate the F4R$^+$ enterocytes with 0, 50, 100, 200, 500 or 1000 µg/ml F4 fimbriae, while gently shaking. After 1 h pre-incubation at room temperature, a gentle short spin centrifugation was performed. Subsequently, the supernatant was discharged, enterocytes were adjusted to the start volume (5.10^5 cells/ml) and injected as described above.

The third approach was to destroy the carbohydrates of F4R$^+$ enterocytes, which are reported to be part of the F4R (Erickson et al., 1992; Grange et al., 2002). Hereto, periodate oxidation of carbohydrates was performed by treating enterocytes for 1 h at room temperature with 0.2 M sodium acetate (pH 4.5) containing 10 mM sodium metaperiodate (Erickson et al., 1992). In addition, the enterocytes were similarly treated with 0.2 M sodium acetate (pH 4.5) as a control. After treatment, the enterocytes were washed three times with running buffer and injected as described above. All these experiments were performed in duplicate and revealed identical results.

6.3. Results

To determine the interaction of F4 fimbriae with the F4R on intestinal villi using SPR, purified F4 fimbriae were immobilized on a sensor chip F1. A dose dependent binding of the c-epitope specific MAb to F4 fimbriae was observed. This MAb has been shown to inhibit the binding of F4$^+$ ETEC to F4R$^+$ villi in an *in vitro* villous adhesion assay since the c-epitope is involved in the interaction with the F4R (Bakker et al., 1992a; Van den Broeck et al., 1999c). An irrelevant porcine IgG-specific MAb of the same isotype (23.3.1b, Van Zaane and Hulst, 1987) was not able

to bind immobilized fimbriae and this up to a concentration of 100 μg/ml (data not shown).

Nine piglets were tested for F4R presence in an *in vitro* villous adhesion assay (Table 1). Subsequently, the enterocytes of three $F4R^-$ pigs (pigs 4, 5 and 6), one weakly $F4R^+$ pig (pig 2) and five strong $F4R^+$ pigs (pigs 1, 3, 7, 8 and 9) were examined in SPR for their F4-specific binding to immobilized F4 fimbriae. As shown in figure 1, strong $F4R^+$ enterocytes were able to bind to the F4 fimbriae (1500 to 2400 RU). This interaction was stable since no dissociation was observed when flushing with running buffer. On the other hand, no interaction was observed using weakly $F4R^+$ or $F4R^-$ enterocytes.

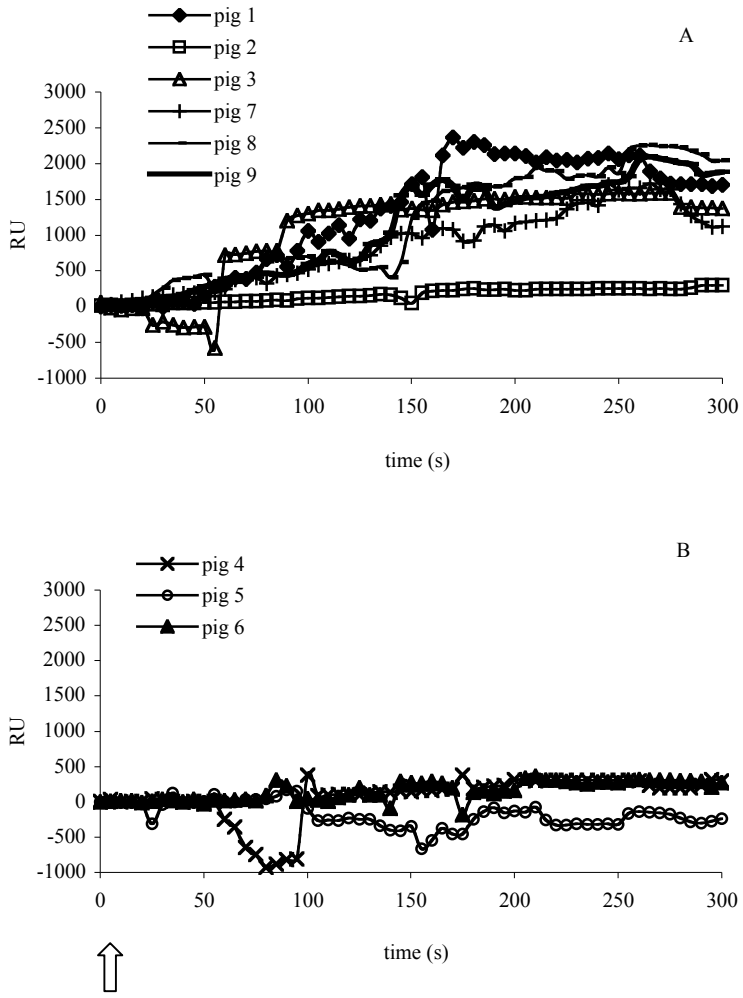


Figure 1 : Sensogram showing the real-time interaction of enterocytes with immobilized F4ac fimbriae. Enterocytes of pigs that were adherent in the *in vitro* villous adhesion assay are shown in A, whereas the non-adherent are shown in B. White arrow : injection of enterocytes.

Table 1 : Results of the *in vitro* villous adhesion assay.

Pig number	Mean number of adhering F4ac$^+$ ETEC bacteria per 250 µm length of villous brush border
1	43.2
2	8.7
3	41.5
4	0.2
5	0.5
6	1.3
7	40.7
8	45.5
9	35.5

To prove that the observed binding between F4R$^+$ enterocytes and immobilized F4 fimbriae was F4-specific, the c-epitope specific MAb was injected over the immobilized F4 fimbriae to block the F4R, whereafter the enterocytes were injected. Figure 2 shows that a concentration of 10 μg/ml MAb was able to inhibit the adhesion of F4R$^+$ enterocytes to the immobilized F4 fimbriae. This inhibition was dose dependent as a 10-fold lower amount of the MAb could not inhibit the adhesion completely.

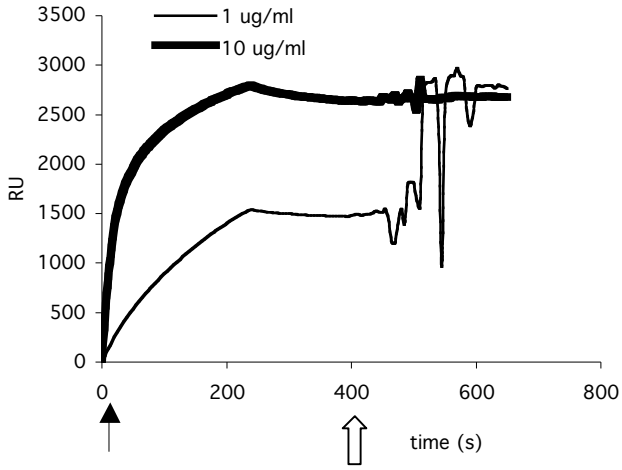


Figure 2 : Sensogram showing the interaction of the c-epitope specific MAb CVI F4ac-5 (1 and 10 µg/ml) with immobilized F4 fimbriae and the effect of MAb binding on subsequent binding of enterocytes. Injection of the MAb (black arrow), injection of F4R$^+$ enterocytes (white arrow).

A second way to test the specificity of binding between immobilized F4 fimbriae and F4R$^+$ enterocytes, was to pre-incubate the enterocytes with different concentrations of purified F4 fimbriae. F4 fimbriae at a concentration of 50, 100 and 200 μg per ml inhibited the adhesion of enterocytes to immobilized F4 in a dose-dependent way, resulting in 51.5 ± 6.5, 40.8 ± 8.2 and 8.3 ± 1.6 % binding of F4R$^+$ enterocytes to immobilized F4, respectively (Fig. 3).

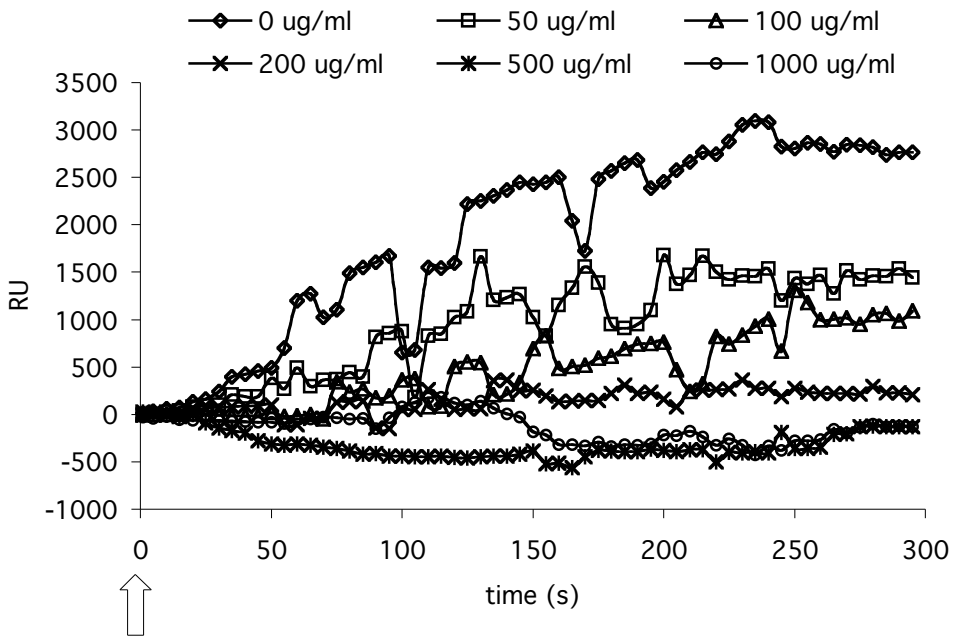


Figure 3 : Sensogram showing the interaction of F4R$^+$ enterocytes, pre-incubated with different concentrations of purified F4 (0, 50, 100, 200, 500 or 1000 µg/ml), with immobilized F4 fimbriae. White arrow: injection of enterocytes.

It has previously been demonstrated that a carbohydrate moiety on the enterocytes is involved in the interaction between F4 fimbriae and the F4R (Erickson et al., 1992; Grange et al., 2002). Therefore, a third way to test the specificity of the obtained signal was to disrupt the carbohydrate molecules of the enterocytes with sodium metaperiodate. Figure 4 shows that incubation of F4R$^+$ enterocytes in sodium acetate buffer reduced the binding ability to F4 fimbriae, but that binding was completely lost following sodium metaperiodate treatment.

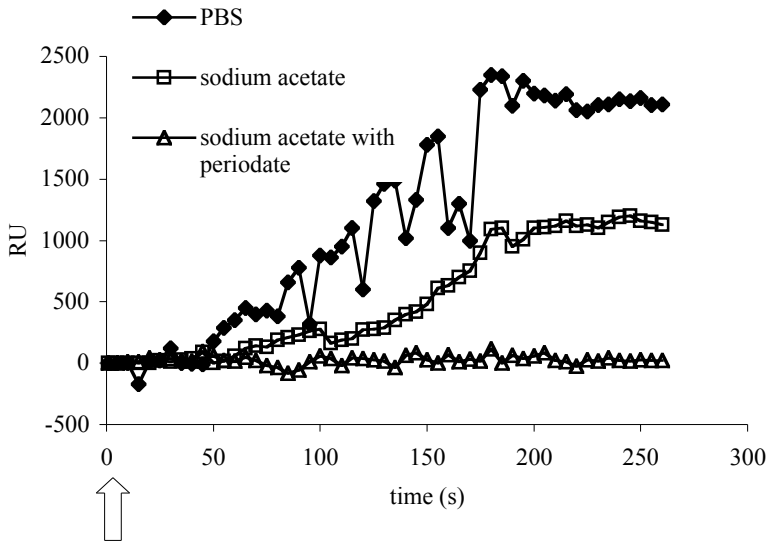


Figure 4 : Sensogram showing the interaction of F4R$^+$ enterocytes, previously incubated in PBS, sodium acetate buffer or sodium acetate buffer supplemented with sodium metaperiodate, with immobilized F4 fimbriae. White arrow: injection of enterocytes.

6.4. Discussion

Results demonstrate that the SPR technique is useful to determine the F4-specific interaction between fimbriae and small intestinal enterocytes isolated from the mid jejunum of pigs. Indeed, immobilized F4 fimbriae retain enough free receptor-binding c-epitopes to allow binding of strong F4R$^+$ enterocytes. The specificity of the binding is proven by blocking the interaction in three different ways. First, a MAb specific for the c epitope is able to inhibit the binding in a dose-dependent manner. Indeed, Bakker et al. (1992a) already demonstrated that there is at least a partial overlap between the receptor-binding site of the F4 fimbriae and the c epitope. Second, incubation of F4R$^+$ enterocytes with purified F4 fimbriae is able to inhibit a subsequent adhesion to immobilized F4 fimbriae in a dose dependent way. These results are comparable to those seen in the *in vitro* villous adhesion assay described by Van den Broeck et al. (1999c). Third, adhesion of F4R$^+$ enterocytes to immobilised F4 is inhibited following oxidation of the enterocyte carbohydrates using sodium metaperiodate. Indeed, it is already reported that the binding activity of F4 to the F4R is dependent on the presence of a carbohydrate structure (Erickson et al., 1992; Grange et al., 2002).

On the other hand, no interaction was observed with $F4R^-$ or weakly $F4R^+$ enterocytes. In agreement, we observed in previous infection experiments that pigs with less than 15 bacteria per 250 µm brush border length in the *in vitro* villous adhesion assay, excreted only very few bacteria for 1 to 2 days following challenge with the pathogenic $F4^+$ ETEC strain GIS26 (Verdonck et al., 2002; Vancaeneghem S, unpublished data). In the Biacore system, the binding strength between the few receptors on the weakly $F4R^+$ enterocytes and the immobilized F4 fimbriae is probably so low that the enterocytes are flushed away by the flow of the running buffer. The used flow rate in the flow cell corresponds to 8.33 cm/min, but flow rates in the small intestine of pigs, which are fed ad libitum, can reach 20.6 ± 2.4 cm/min (Devinder et al., 1986). It is therefore likely that the biosensor can mimic the *in vivo* situation more than an *in vitro* villous adhesion assay.

The biosensor has some well known advantages: the method is less subjective, is adaptable to automatization for processing a large number of samples and is also less time-consuming than the immunoblotting technique or the *in vitro* villous adhesion assay. Furthermore, the use of SPR to determine the interaction between adhesins and receptors on eukaryotic cells allows easy screening for blocking agents or receptor analogues to prevent infection. Indeed, one of the strategies to prevent $F4^+$ ETEC-induced diarrhoea in neonatal or weaned piglets, is to prevent attachment of the bacteria to the intestinal F4R. Proteases (Chandler et al., 1994; Mynott et al., 1996), antibodies (Yokoyama et al., 1992; Jin et al. 1998) or lactic acid bacteria (Blomberg et al., 1993b; Ouwehand and Conway, 1996) have been used to inhibit the interaction between F4 fimbriae and the F4R and subsequent disease.

In conclusion, this study shows that SPR can be used to distinguish strong $F4R^+$ pigs from weak $F4R^+$ or $F4R^-$ pigs and offers new opportunities to study the interaction between F4 fimbriae and the F4R. Furthermore, SPR opens new perspectives to screen therapeutics for prevention of ETEC adhesion.

Chapter 7

F4 fimbriae as carrier and cholera toxin as adjuvant synergistically improve the induction of a HSA-specific immune response following oral immunization of pigs[1]

[1] *Based on : Verdonck F, De Hauwere V, Bouckaert J, Goddeeris BM, Cox E. F4 fimbriae as carrier and cholera toxin as adjuvant synergistically improve the induction of a HSA-specific immune response following oral immunization of pigs. Submitted.*

Abstract

Receptor-mediated uptake of orally administered antigen can lead to an antigen-specific immune response, whereas oral administration of most other non-replicating soluble antigens results in the induction of oral tolerance. In the present study, it is shown that fimbriae purified from an $F4^+$ enterotoxigenic *Escherichia coli* strain can function as a mucosal carrier molecule for the model antigen human serum albumin (HSA). Oral immunization of pigs with glutaraldehyde coupled F4/HSA conjugates induced a HSA-specific immune response. This mucosal carrier function of F4 fimbriae was improved following oral co-administration of the F4/HSA conjugates with the mucosal adjuvant cholera toxin (CT) to $F4R^+$ pigs, since both humoral and cellular HSA-specific responses were significantly increased. In comparison with $F4R^+$ pigs, the HSA-specific response was reduced following oral F4/HSA+CT immunization of $F4R^-$ pigs. This indicates that F4 fimbriae as carrier and CT as adjuvant synergistically improve the induction of a HSA-specific immune response following oral immunization of pigs.

7.1. Introduction

Most bacterial and viral infections of man and animals begin by the interaction of the pathogen with mucous membranes. It is generally accepted that protection of these mucosal surfaces is largely mediated by local production of IgA (Porter et al., 1974; Mestecky and McGhee, 1987). To induce the secretion of antigen-specific IgA in the intestine, oral immunization is needed since parenteral immunization is not very effective for the induction of IgA (Bianchi et al., 1996; Van der Stede et al., 2002b). However, oral immunization with most soluble non-replicating antigens results in oral tolerance (Weiner, 2001). On the other hand, receptor-dependent uptake of soluble antigen by epithelial cells can result in an antigen-specific mucosal and systemic immune response. Indeed, oral immunization of F4-receptor positive ($F4R^+$) pigs with purified F4 (K88) fimbriae induces an intestinal F4-specific antibody response protecting pigs against a subsequent challenge with $F4^+$ enterotoxigenic *Escherichia coli* (ETEC) (Van den Broeck et al., 1999b). In $F4R^-$ pigs, F4 fimbriae behave as a normal food antigen (Van den Broeck et al., 2002).

Fimbriae are attractive structures to be used as carriers in vaccine design due to their polymeric character, their high immunogenicity, their ability to bind to specific receptors, the presence on the surface of bacteria and the possibility to prepare them in large amounts. F4 fimbriae have the same properties since they are surface exposed (Ørskov and Ørskov, 1961), immunogenic (Rutter and Jones, 1973), easy to purify in large amounts (Van den Broeck et al., 1999c; Wong et al., 2003), able to bind to the F4R on porcine small intestinal enterocytes (Sellwood et al., 1975) and are multimeric structures of the major fimbrial subunit FaeG that constitutes the adhesin and some minor subunits (Oudega et al., 1989). Moreover, the potential carrier function of F4 fimbriae has already been demonstrated by the induction of antibodies against a heterologous epitope that was inserted in the variable region of FaeG (Thiry et al., 1989; Bakker et al., 1990). However, the length of the inserted heterologous epitopes is limited as the folding and the stability of the fimbrial subunits may not be disturbed. On the other hand, fimbriae are able to function as carriers for larger heterologous antigens when chemically coupled. Indeed, F5 and F6 fimbriae were already reported to have the potential to function as a mucosal carrier molecule, inducing an antibody response against chemically conjugated bovine serum albumin following oral immunization of mice (Russell-Jones, 2001).

The aim of the present study was to determine for the first time the potential of F4 fimbriae to act as a mucosal carrier molecule, inducing a mucosal and systemic immune response against a chemically conjugated antigen following oral immunization in pigs. In the present study, the model antigen human serum albumin (HSA) was chosen since HSA is not known to interact with a specific receptor. In addition, it was determined whether the mucosal adjuvant cholera toxin (CT) could improve the mucosal immune response against the F4-conjugated HSA. The mechanisms of CT adjuvanticity are not completely known but include an enhanced antigen presentation by a variety of cell types, promotion of the isotype switch to IgA and an influence on cytokine production and T cell activation (Holmgren et al., 2003).

7.2. Material and methods

7.2.1. Purification of F4 fimbriae

F4ac fimbriae of the *E. coli* strain GIS26 (O149:K91:F4ac, $LT^+STa^+STb^+$) were isolated by homogenizing a GIS26 bacterial suspension. Subsequently, fimbriae were purified by anion exchange chromatography using a Bio-Scale Q5 column (BIO-RAD Laboratories) as described by Van den Broeck et al. (1999c). The protein concentration was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as a standard (ICN Biomedicals, Belgium). The purity of the purified F4 fimbriae was assessed using a Coomassie stained 15% SDS-PAGE and the ImageMaster 1D prime software (Amersham Pharmacia Biotech, Belgium).

7.2.2. Conjugation of F4 fimbriae and HSA

HSA (Sigma, Bornem, Belgium) was conjugated to purified F4 fimbriae (F4/HSA) in a molar ratio (HSA to FaeG subunits) of 0.5:1, 1:1, 2:1, 4:1 as described by Vervelde et al. (1998) with minor modifications. Briefly, HSA and purified F4 were dissolved in 0.1 M phosphate buffer pH 8.0. Glutaraldehyde was slowly added to the mixture until a concentration of 0.5, 1, 2 or 4 mM was reached. Subsequently, the mixture was stirred for 2.5 h at room temperature. Thereafter, the reaction was stopped by adding glycine in a final concentration of 60 mM and by stirring the solution for another 45 min. Finally, the solution was dialysed for 18 h against PBS (27.5 mM NaCl, 0.54 mM KCl, 2 mM Na_2HPO_4, 0.4 mM KH_2PO_4, pH 7.4) at 4°C. Similar procedures were used to couple HSA to HSA (HSA/HSA) and F4 to F4 (F4/F4).

7.2.3. Characterization of conjugates

7.2.3.1. Analysing coupling of F4 to HSA

To analyse the effectiveness of the different conjugation conditions on cross-linking F4 to HSA, the conjugated samples were analysed in ELISA. The wells of 96-well microtiter plates (NUNC, Maxisorp Immuno Plates, Roskilde, Denmark) were coated with the F4-specific monoclonal antibody (MAb) IMM01 (Van der Stede et al., 2002b) or a HSA-specific polyclonal antibody (Serotec, Kidlington, England). Blocking and washing were performed according to the procedure described by Van

den Broeck et al. (1999a). Thereafter, serial dilutions of the conjugates starting from 1/10 in PBS supplemented with 0.05% Tween®20 were added to both the F4- and the HSA-specific antibody coated plates and plates were incubated for 1 h at 37°C. Purified F4 fimbriae, HSA (both starting at 1 mg/ml) and plain conjugation buffers were used as controls. After several washes, the F4- and HSA-specific antibody-coated plates were incubated with optimal dilutions of peroxidase-conjugated HSA- and F4-specific antibodies respectively for 1 h at 37°C. After several washes, an H_2O_2-containing ABTS solution was added and the optical density was spectrophotometrically measured at 405 nm (OD_{405}) following 30 and 60 minutes of incubation at 37°C.

7.2.3.2. Real-time interaction of conjugates with enterocytes

The interaction between the conjugates and the F4R on small intestinal pig enterocytes was analysed with the BIAcore®3000 biosensor (Uppsala, Sweden) as described in chapter 6. Enterocytes of two $F4R^-$ and two $F4R^+$ pigs were used, with a mean binding of 0.2, 1.3, 43.2 and 45.5 $F4^+$ ETEC bacteria per 250 μm length of villous brush border respectively in the *in vitro* villous adhesion assay. The HSA-specific polyclonal antibodies were immobilized in one flow cell of a F1 chip using a 10 mM sodium acetate buffer pH 4.8 until the amount (mol) of immobilized antibodies equalized 13,000 resonance units (RU). Thousand RU corresponds to a change in the surface concentration of 1 ng/mm^2 (Fägerstam et al., 1992). Another flow cell on the same chip was activated and subsequently blocked without immobilization of antibodies and served as control.

F4/HSA conjugates were injected at a flow rate of 5 μl/min during 120 seconds at 25°C. Thereafter, enterocyte suspensions prepared as described in chapter 6 were injected at a concentration of 5.10^5 cells/ml and a flow rate of 5 μl/min during 240 seconds at 25°C. Binding of conjugates and enterocytes was monitored and registered as a sensogram (plot of RU versus time). To regenerate flow cells at the end of each experiment, 25 mM NaOH was used. The experiments were performed in duplicate. The duplicates gave similar results.

7.2.3.3. Determination of the hydrodynamic radius

The hydrodynamic radii of F4, HSA or conjugates were determined using dynamic light scattering (DLS). Following centrifugation of the samples at 18,000 g for 60 minutes at 4°C, 30 µl was injected into a flow cell of the Laser-spectroscatter (RiNA Netzwerk RNA-Technologien, Berlin, Germany) at room temperature and illuminated by a 30-mW, 660-nm-wavelength, solid-state laser. Data were collected of 10 measurements, 20 seconds each. Two independent experiments were performed, resulting in identical results.

7.2.4. Experimental procedure

7.2.4.1. Pigs

Twenty F4- and HSA-seronegative, conventionally bred pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks, transported to the experimental facilities at the faculty and subsequently housed in isolation units with water and feed ad libitum. These pigs were treated orally with colistine (Promycine pulvis, VMD, Berendonk, Belgium, 150,000 U/kg of body weight/day) from 2 days before till 3 days after weaning to prevent *E. coli* infections due to transport and handling.

7.2.4.2. Immunization

One week post weaning (0 days post primary immunization, dppi), pigs were orally immunized with the F4/HSA conjugate in the absence (F4/HSA group, n=6) or presence of 50 µg CT (F4/HSA+CT group, n=6) or with the HSA/HSA conjugate in the absence (HSA/HSA group, n=3) or presence of 50 µg CT (HSA/HSA+CT group, n=5). In fact, CT was added or not to 2 ml of the conjugation solutions (containing 2 mg purified F4 and/or 4.8 mg HSA) which was subsequently adjusted with PBS to a final volume of 10 ml. The pigs were orally immunized at 0, 1 and 2 dppi, which is referred as the first oral immunization. The pigs were orally immunized for a second and a third time at 16 and 35 dppi, respectively. From three hours before till 2 h after oral immunization, all animals were deprived of food and water. At 48 dppi, all animals were intramuscularly (IM) immunized with 1 mg HSA to observe priming of the systemic immune system against HSA by the oral immunizations. Hereto, HSA

was dissolved in 0.5 ml PBS and suspended in an equal volume of incomplete Freund's adjuvant (DIFCO Laboratories, Detroit, USA).

7.2.4.3. Kinetics of F4-, HSA- and CT-specific immune response

F4- and HSA-specific serum IgA, IgG and IgM antibodies were determined 0, 7, 16, 21, 24, 35, 38, 42, 48, 52, 55, 62, 69 and 77 dppi**,** whereas F4- and HSA-specific IgA mucosal antibodies were analysed in saliva 0, 16, 24, 35 and 41 dppi. Serum samples of 0, 16, 35 and 48 dppi were also tested for the presence of CT-specific antibodies. The F4-specific lymphocyte proliferation was examined 42 and 55 dppi and the HSA-specific lymphocyte proliferation was determined 42, 55 and 66 dppi.

7.2.4.4. Weight and average daily weight gain (ADWG)

All pigs were weighed at 0, 3, 7, 15 and 24 dppi. The daily weight gain of each pig was calculated at 3, 7, 15 and 24 dppi by calculating weight gain between two subsequent measurements divided by the number of days between both subsequent measurements. Subsequently, the average daily weight gain (ADWG) per group was calculated ± SEM.

7.2.5. Samples

To determine F4-specific serum and mucosal antibodies, serum and saliva were sampled as described by Van der Stede et al. (2002b).

In order to analyse the F4- and HSA-specific lymphocyte proliferation, blood was collected from the jugular vein and peripheral blood monomorphonuclear cells (PBMC) were isolated as described by Van den Broeck et al. (1999a).

At the end of the experiment, the pigs were euthanised and jejunal villi were isolated for determining the presence or absence of the F4R as described by Van den Broeck et al. (1999c).

7.2.6. ELISA for F4-, HSA- and CT-specific antibodies

For quantifying the F4-specific IgM, IgA and IgG antibody response, the indirect ELISA described by Van den Broeck et al. (1999a) was used and serial

dilution of serum and saliva samples were tested, starting from 1/10 and 1/2.5, respectively.

The HSA-specific IgM, IgA and IgG antibody titers were determined with the indirect ELISA as described by Van der Stede et al. (2001), with some modifications. Briefly, the wells of a 96-well microtiter plate (NUNC®, Polysorb Immuno Plates, Roskilde, Denmark) were coated with HSA at a concentration of 30 μg/ml in PBS. After 2 h incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2 % Tween®80. Subsequently, the plates were incubated for 1 h at 37°C with twofold serial dilutions of the samples in ELISA dilution buffer (PBS + 0.05% Tween®20), followed by the swine-specific IgM, IgA and IgG MAb (Van Zaane and Hulst, 1987) and peroxidase-conjugated rabbit-anti-mouse polyclonal antibodies (Dako, Denmark) supplemented with 2% (vol/vol) pig serum. Finally, ABTS and H_2O_2 were used as chromogen and substrate and the optical density was spectrophotometrically measured at 405 nm (OD_{405}).

An identical ELISA with minor modifications was used to determine the CT-specific antibodies. The wells of microtiter plates (NUNC®, Polysorb Immuno Plates) were coated with 5 μg/ml CT (Sigma) in PBS and an optimal dilution of horseradish peroxidase conjugated rabbit-anti-swine polyclonal antibodies (Dako) was used as conjugate.

The cut-off values were calculated as the mean OD_{405}-value of all sera (dilution 1/10) or saliva (dilution 1/2.5) at day 0, increased with 3 times the standard deviation. The antibody titer was the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value. The cut-off values for F4-specific serum IgM, IgA, IgG and saliva IgA and IgM were 0.48, 0.26, 0.43, 0.31 and 0.35 respectively. The cut-off values for HSA-specific serum IgM, IgA, IgG and saliva IgA and IgM were 0.20, 0.12, 0.15, 0.25 and 0.28 respectively, whereas the cut-off value for the CT-specific serum antibodies was 0.24.

7.2.7. Presence of the F4R

The presence or absence of the F4R on the brush border of small intestinal enterocytes was determined on isolated intestinal villi as described by Van den Broeck et al. (1999c). Adhesion of more than five $F4^+$ *E. coli* per 250 μm villous length was noted as positive (Cox and Houvenaghel, 1993).

7.2.8. F4- and HSA-specific lymphocyte proliferation

The PBMC were diluted to a concentration of 5.10^6 cells/ml in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), kanamycin (100 μg/ml), L-glutamin (200 mM), sodiumpyruvate (100 mM), non-essential amino acids (100 mM), β-mercaptoethanol (5.10^{-5} M) and 5% (vol/vol) F4-seronegative serum. Subsequently, the isolated PBMC (5.10^5 cells/well) were incubated in medium without (negative control) or with purified F4 (final concentration of 10 μg/ml), HSA (final concentration of 100 μg/ml) or concanavalin A (final concentration of 10 μg/ml, positive control) to determine their F4- and HSA-specific proliferation as described by Van der Stede et al. (2002a and 2003). The results are presented as F4- and HSA-specific stimulation index (SI), obtained by dividing the mean counts per minute (cpm) of the F4- and HSA-stimulated cultures, respectively, by the mean cpm of the non-stimulated cultures. Mean background levels of about 350, 620 and 510 cpm were obtained in medium samples at 42, 55 and 66 dppi, whereas positive controls of concanavalin A reached 130,000, 150,000 and 150,000 cpm respectively.

7.2.9. Statistical analysis

Statistical analysis (SPSS 10.0 for Windows) of antibody titers was done using General Linear Model (Repeated Measures Analysis of Variance), adjusting for multiple comparison by Bonferoni. Differences between groups in F4- or HSA-specific cell proliferation were analysed for statistical significance using the Kurskal-Wallis test. Differences in the ADWG between the groups were tested for statistical significance using a two-sample T-test. $P<0.05$ was considered as statistically significant.

7.3. Results

7.3.1. Characterization of F4/HSA conjugates

Optimal covalent binding between F4 and HSA was obtained at a molar ratio of 1:1 using 4 mM glutaraldehyde. Indeed, the F4/HSA molecules obtained at this ratio were able to interact with both a F4- or HSA-specific antibody coating and were subsequently detected by HSA- or F4-specific peroxidase labelled antibodies, respectively, resulting in OD_{405}-values of 1.47 and 1.7 at a 1/10 dilution of the F4/HSA conjugates. In addition**,** these F4/HSA conjugates were able to bind $F4R^+$ but not $F4R^-$ enterocytes as determined by surface plasmon resonance (Fig. 1). Other conjugation conditions resulted in lower OD_{405}-values or in a reduced F4R-binding (data not shown). In further experiments, only the optimally conjugated F4/HSA was used.

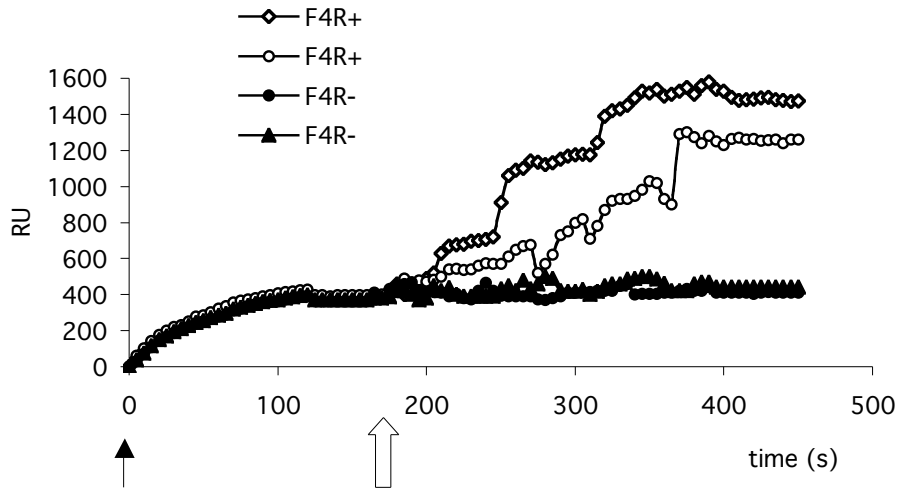


Figure 1 : Sensogram showing the interaction of the F4/HSA conjugate with immobilized HSA-specific antibodies and subsequent with F4-receptor positive (white symbols) and F4-receptor negative (black symbols) porcine enterocytes. Black arrow, F4/HSA injection; White arrow, injection of enterocytes.

Binding of HSA to purified F4 fimbriae was also confirmed by determining the hydrodynamic radii of the F4/HSA conjugates using the DLS technique (Fig. 2). DLS measurements of non-conjugated HSA molecules revealed a single group of particles with a radius of 4 to 7 nm (Fig. 2B). Measurements of purified F4 fimbriae showed different particles with a radius ranging between 10 and 100 nm (Fig. 2A).

When HSA and F4 were mixed (F4+HSA), particles were detected with a radius of 4 to 7 nm as well as particles with a ratio of 10 to 100 nm (Fig. 2C). Conjugation of HSA to F4 fimbriae resulted in a clear reduction of the 4 to 7 nm particle group (Fig. 2D), suggesting that HSA molecules were indeed coupled to F4. Since the linear F4 fimbriae are measured as ellipsoid spheres by the DLS technique, conjugation of HSA molecules along the linear F4 fimbriae will result in a similar ellipsoid sphere and not in particles with a larger radius. SDS-PAGE and Coomassie staining of F4/HSA samples also showed a decrease in free HSA molecules as compared to samples of mixed F4 and HSA (data not shown). In contrast to the F4/HSA conjugation, coupling F4 to F4 and HSA to HSA seemed less effective since the hydrodynamic radii of the conjugates are similar to the uncoupled molecules (Fig. 2E and 2F). However, the glutaraldehyde treatment of F4 fimbriae seemed to result in the covalent linkage of its subunits since incubation of F4/F4 at 95°C for 5 minutes did not reduce the polymeric character of F4, whereas heating of non-coupled F4 resulted in the desintegration of F4 fimbriae as only its adhesin FaeG could be observed following SDS-PAGE and Coomassie staining (data not shown).

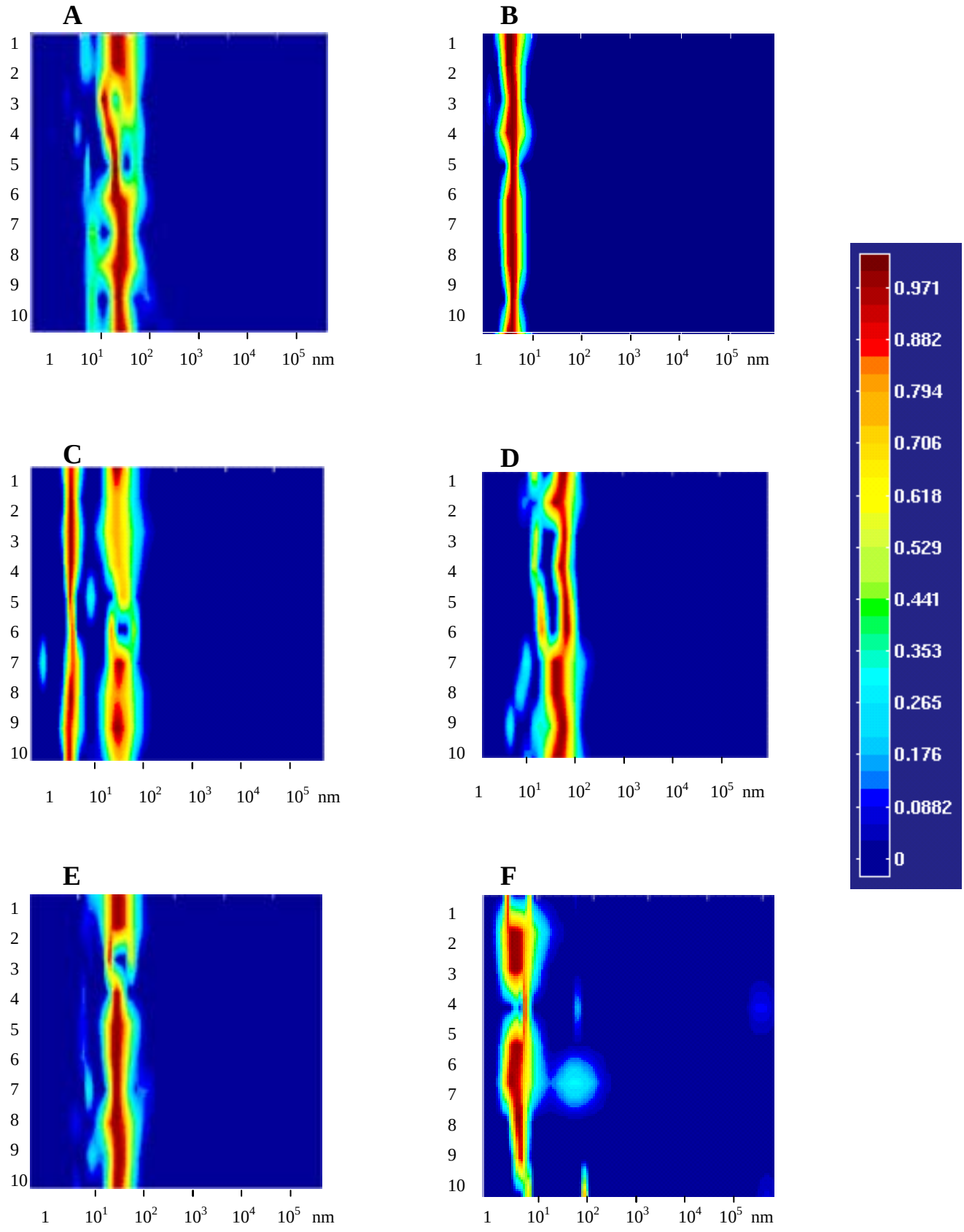


Figure 2 : Distribution of the hydrodynamic radii (nm) of the molecules present in F4 (A), HSA (B), F4+HSA (C), F4/HSA (D), F4/F4 (E) or HSA/HSA (F) samples as determined by the dynamic light scatter method and presented using a color gradient. Each sample was measured ten times (Y-axis).

7.3.2. Induction of F4-specific serum antibodies

The *in vitro* villous adhesion assay revealed that seventeen pigs were $F4R^+$ whereas three pigs were $F4R^-$. As a consequence, the six pigs that were orally immunized with F4/HSA conjugates and CT were divided in two subgroups of three pigs each : three $F4R^+$ pigs (F4/HSA+CT group) and three $F4R^-$ pigs (F4/HSA+CT $F4R^-$ group).

As expected, pigs orally immunized with HSA/HSA in the presence or absence of CT (HSA/HSA+CT and HSA/HSA groups, respectively) did not have a F4-specific antibody responses. The $F4R^+$ pigs immunized with plain F4/HSA only showed F4-specific IgA and IgG responses after the third oral immunization (Fig. 3), which became significantly higher ($P \leq 0.031$) than the background in the HSA groups 42 and 48 dppi. On the other hand, oral co-administration of CT to F4/HSA in $F4R^+$ pigs (F4/HSA+CT group) significantly increased the F4-specific antibody response. Indeed, a primary antibody response already occurred 7 days following the first oral immunization (7 dppi) with first IgM (log2 titer 4.85) and subsequently IgA and IgG antibodies, which reached maximal log2 titers of 8.32 and were significantly higher than these of other groups during a period of about 2 months ($P \leq 0.046$).

In $F4R^-$ pigs, CT adjuvanticity also induced F4-specific antibodies following oral co-administration with F4/HSA (F4/HSA+CT $F4R^-$ group) from one week following the second oral immunization onwards (Fig. 4). Moreover, this immunization resulted in a faster appearance of F4-specific IgA and IgG titers than in $F4R^+$ pigs immunized with plain F4/HSA (F4/HSA group). However, the F4-specific IgG and IgA serum antibody titers in the F4/HSA+CT $F4R^-$ group reached similar levels as in the F4/HSA group from the third oral immunization onwards and was significantly lower as compared to $F4R^+$ pigs immunized with F4/HSA + CT (F4/HSA+CT group). These results demonstrated that CT functions as mucosal adjuvant in pigs and that the highest F4-specific response is obtained following mucosal targeting of F4 in the presence of CT.

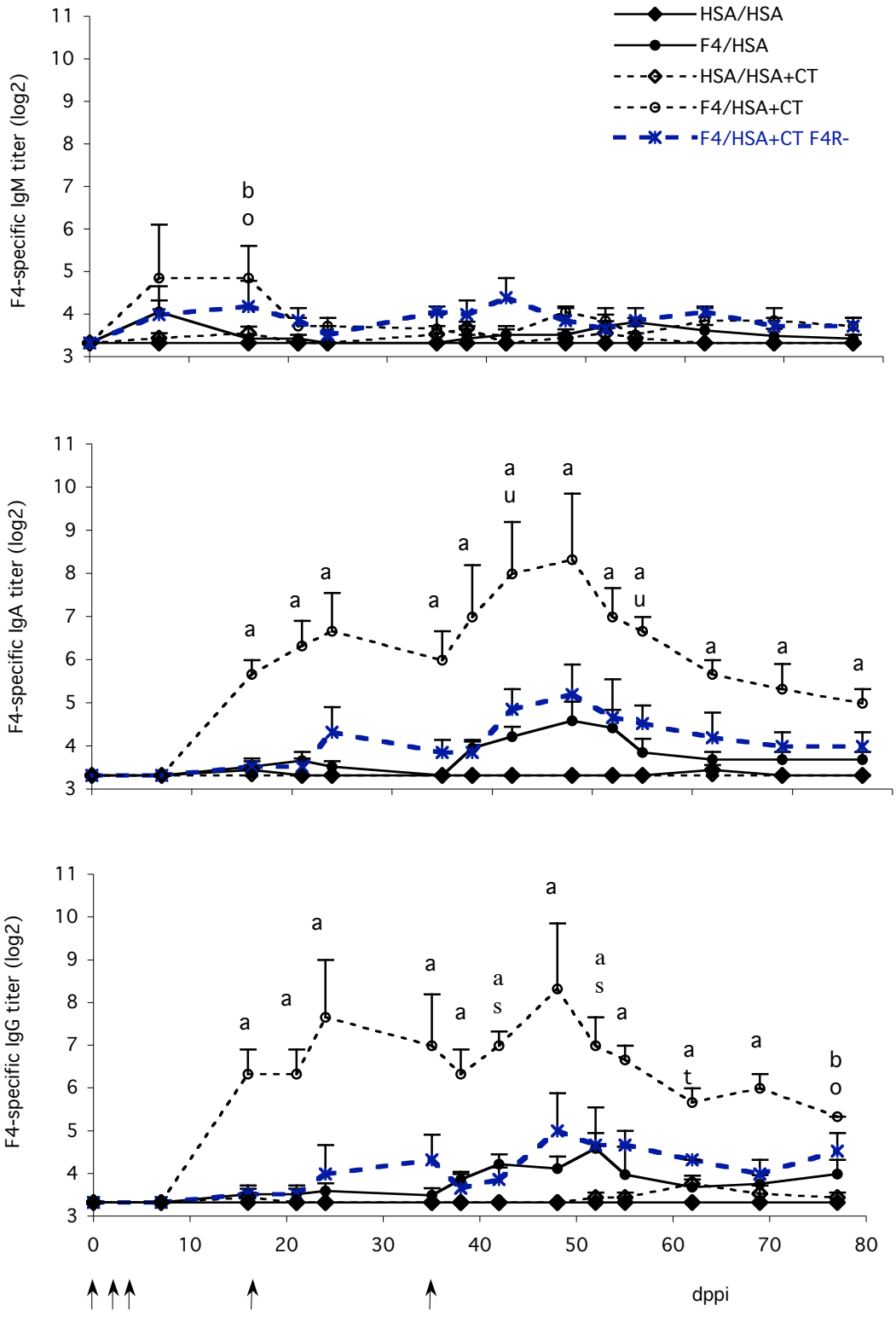


Figure 3 : Mean F4-specific IgM, IgA and IgG serum antibody titers (± SEM) of immunized pigs. The animals of the F4/HSA+CT F4R$^-$ group are F4R$^-$, whereas all other animals are F4R$^+$. Significant difference (P<0.05) between F4/HSA+CT and F4/HSA, HSA/HSA, HSA/HSA+CT, F4/HSA+CT F4R$^-$ (a), between F4/HSA+CT and HSA/HSA+CT (b), between F4/HSA+CT and HSA/HSA, F4/HSA (o), between F4/HSA and HSA/HSA, HSA/HSA+CT (s), between F4/HSA+CT F4R$^-$ and F4/HSA, HSA/HSA (t), between F4/HSA+CT F4R$^-$ and HSA/HSA, HSA/HSA+CT (u). Black arrow, oral immunization.

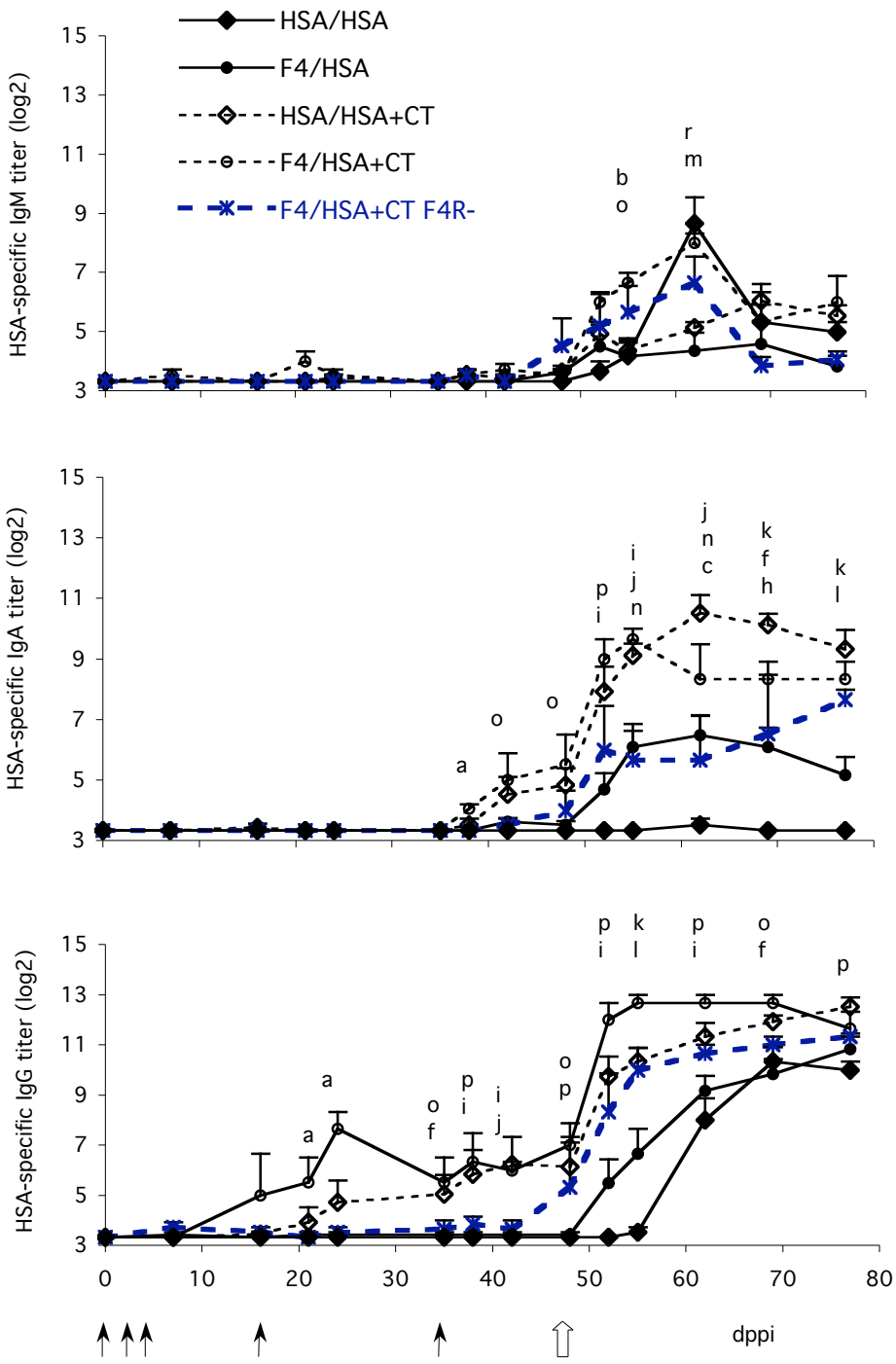


Figure 4 : Mean HSA-specific IgM, IgA and IgG serum antibody titers (± SEM) of immunized pigs. Significant difference (P<0.05) between F4/HSA+CT and HSA/HSA, HSA/HSA+CT, F4/HSA, F4/HSA+CT F4R$^-$ (a), between F4/HSA+CT and HSA/HSA+CT (b), between F4/HSA+CT and HSA/HSA (c), between HSA/HSA+CT and F4/HSA (f), between HSA/HSA+CT and F4/HSA+CT F4R$^-$ (h), between F4/HSA+CT and F4/HSA, F4/HSA+CT F4R$^-$, HSA/HSA (i), between HSA/HSA+CT and F4/HSA, F4/HSA+CT F4R$^-$, HSA/HSA (j), between HSA/HSA and F4/HSA+CT, F4/HSA+CT F4R$^-$, HSA/HSA+CT, F4/HSA (k), between F4/HSA and F4/HSA+CT, F4/HSA+CT F4R$^-$, HSA/HSA+CT, HSA/HSA (l), between HSA/HSA and F4/HSA, HSA/HSA+CT (m), between F4/HSA and HSA/HSA (n), between F4/HSA+CT and HSA/HSA, F4/HSA (o), between HSA/HSA+CT and HSA/HSA, F4/HSA (p), between F4/HSA+CT and HSA/HSA+CT, F4/HSA, F4/HSA+CT F4R$^-$ (r). Black arrow, oral immunization; white arrow, intramuscular HSA immunization

7.3.3. Induction of HSA-specific serum antibodies

7.3.3.1. Oral immunization

To analyse the mucosal carrier function of F4 for the conjugated HSA, the HSA-specific antibody response was determined. As expected, no HSA-specific antibodies were observed following oral immunization of pigs with HSA/HSA (HSA/HSA group) (Fig. 4), nor were HSA-specific serum antibodies detected following oral administration of F4/HSA conjugates to $F4R^+$ pigs (F4/HSA group).

On the other hand, oral co-administration of HSA/HSA or F4/HSA with CT (HSA/HSA+CT and F4/HSA+CT group, respectively) induced a HSA-specific IgG and IgA serum antibody response in $F4R^+$ pigs around the moment of the second oral immunization and following the third oral immunization, respectively (Fig. 4). However, conjugation of HSA to F4 enhanced the induction of a HSA-specific response since significantly higher HSA-specific IgG and IgA titers were observed in the F4/HSA+CT group as compared to the HSA/HSA+CT group following the second and the third oral immunization, respectively. Later on, the HSA-specific IgG titer in the HSA/HSA+CT group reached the level of the F4/HSA+CT group at the moment of the third oral immunization (35 dppi) and remained at this level till the IM immunization at 48 dppi. The titers in the CT-supplemented groups were significantly higher than for the groups without CT (F4/HSA and HSA/HSA groups) following the third oral immunization (between 38 and 48 dppi).

In $F4R^-$ pigs orally immunized with F4/HSA+CT (F4/HSA+CT $F4R^-$ group), a HSA-specific IgG serum response was only observed two weeks following the third oral immunization (Fig. 4). These data indicate that the fast induction of a HSA-specific serum response following oral co-administration of CT and F4/HSA in $F4R^+$ pigs (F4/HSA+CT group) resulted from a complementary effect of CT as adjuvant and F4 as mucosal carrier. A remarkable observation was the faster appearance of HSA-specific serum IgG in the HSA/HSA+CT group than in the F4/HSA+CT $F4R^-$ group.

7.3.3.2. Intramuscular HSA immunization

At 48 dppi, the pigs were IM immunized with HSA to determine whether oral immunization had primed the animals systemically against HSA (Fig. 4). A significant increase of the HSA-specific serum IgM titer between 7 and 14 days post

IM immunization (55 and 62 dppi) was only observed in the HSA/HSA group, confirming a primary antibody response in this group. The kinetics of HSA-specific serum IgG confirms a primary response (Fig. 4), whereas no HSA-specific serum IgA response could be detected in the HSA/HSA group.

In contrast to a primary response in the HSA/HSA group, secondary responses were detected in the four other groups with significant increases in HSA-specific serum IgG titers directly following IM immunization (between 48 and 52 dppi) ($P \leq 0.025$). This resulted in a significantly higher IgG antibody titer in these four groups than in the HSA/HSA group one week following IM immunization (55 dppi). However, gradual differences in priming were observed between these four groups as evidenced by the different kinetic of the HSA-specific serum IgG response. Indeed, a HSA-specific serum IgG titer of 1280 (log2 titer > 10) was reached at 29, 14, 7 and 4 days following IM HSA immunization in the F4/HSA, F4/HSA+CT F4R$^-$, HSA/HSA+CT and F4/HSA+CT groups, respectively. A similar kinetic pattern was obtained for the HSA-specific IgA serum response. These observations are in agreement with those following oral immunization, confirming that conjugation of HSA to F4 improved the targeting of HSA to the gut associated lymphoid tissue following oral administration and that CT functioned as a mucosal adjuvant to orally co-administered antigens. In addition, it is interesting to notice that HSA-specific serum IgA also boosted in the F4/HSA group upon IM HSA immunization, This observation revealed the priming of the gut-associated immune system by HSA that was conjugated to F4, in contrast to non-mucosae-targeted HSA/HSA.

7.3.4. F4- and HSA-specific IgA response in saliva

The presence of F4-specific serum antibodies in F4/HSA, F4/HSA+CT and F4/HSA+CT F4R$^-$ groups following oral immunization corresponded with the presence of F4-specific IgA in saliva (Fig. 5). The HSA-specific IgA in saliva was only detected in the F4/HSA+CT group one week after the second oral immunization (24 dppi)**.** At that moment, no HSA-specific IgA could be detected in serum. The induced HSA-specific IgA in saliva was also significantly higher in the F4/HSA+CT group than in the HSA/HSA, F4/HSA and F4/HSA+CT F4R$^-$ groups one week following the third oral immunization (42 dppi) ($P \leq 0.016$).

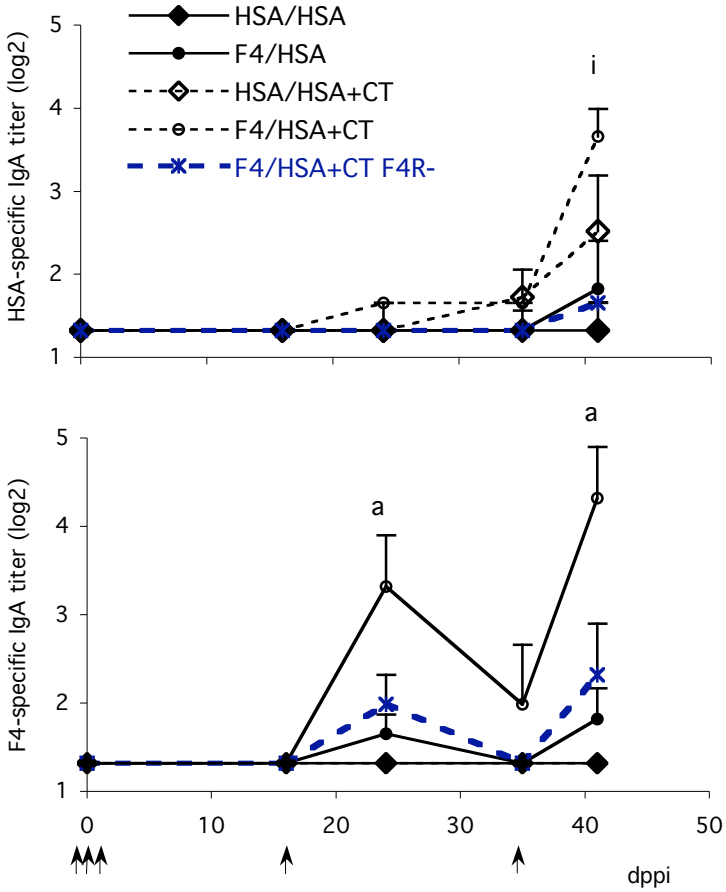


Figure 5 : Mean F4- and HSA-specific IgA antibody titer (± SEM) in saliva of immunized pigs. The animals of the F4/HSA+CT F4R$^-$ group are F4R$^-$, whereas all other animals are F4R$^+$. Significant difference ($P<0.05$) between F4/HSA+CT and HSA/HSA, HSA/HSA+CT, F4/HSA, F4/HSA+CT F4R$^-$ (a), between F4/HSA+CT and F4/HSA, F4/HSA+CT F4R$^-$, HSA/HSA (i). Black arrow, oral immunization.

7.3.5. Induction of a F4- and HSA-specific lymphocyte proliferation

The F4-specific cellular immune response is in accordance with the humoral antibody response one week following the third oral immunization (42 dppi) in that the F4-specific cellular immune response was significantly higher in the F4/HSA and F4/HSA+CT groups than the background levels of the HSA/HSA and HSA/HSA+CT groups ($P\leq0.020$) (Fig. 6). A similar situation was observed 55 dppi, but then the SI were lower suggesting a decreased F4-specific cellular immune response.

The HSA-specific lymphocyte proliferation was low after the third oral immunization as well as one week after the IM HSA immunization (maximal mean SI

of 2.3 and 2.8, respectively) (Fig. 6). However, the HSA-specific lymphocyte proliferation was significantly increased (P≤0.022) in all groups except the HSA/HSA group three weeks after IM immunization. At that moment, the HSA-specific SI was significant lower in the HSA/HSA group as compared to the HSA/HSA+CT, F4/HSA+CT and F4/HSA+CT F4R$^-$ groups (P≤0.030), resembling the HSA-specific antibody response.

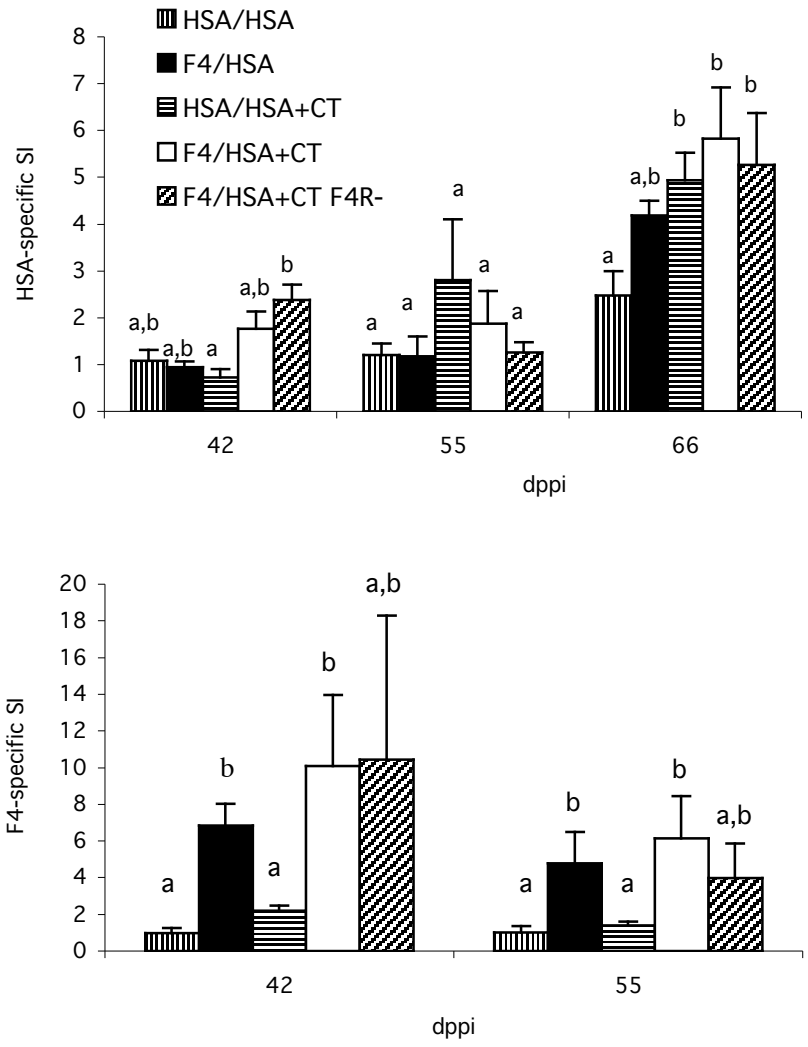


Figure 6 : Mean F4- and HSA-specific stimulation index (SI) (± SEM). of immunized pigs. The animals of the F4/HSA+CT F4R$^-$ group are F4R$^-$, whereas all other animals are F4R$^+$. Bars with a different letter are significantly different (P<0.05).

7.3.6. Induction of CT-specific serum antibodies

The use of CT as mucosal adjuvant resulted in the improvement of the antigen-specific immune responses as shown above. CT also induced toxin-specific antibodies (Fig. 7). The use of CT did not result in diarrhoea or a reduced weight gain (data not shown).

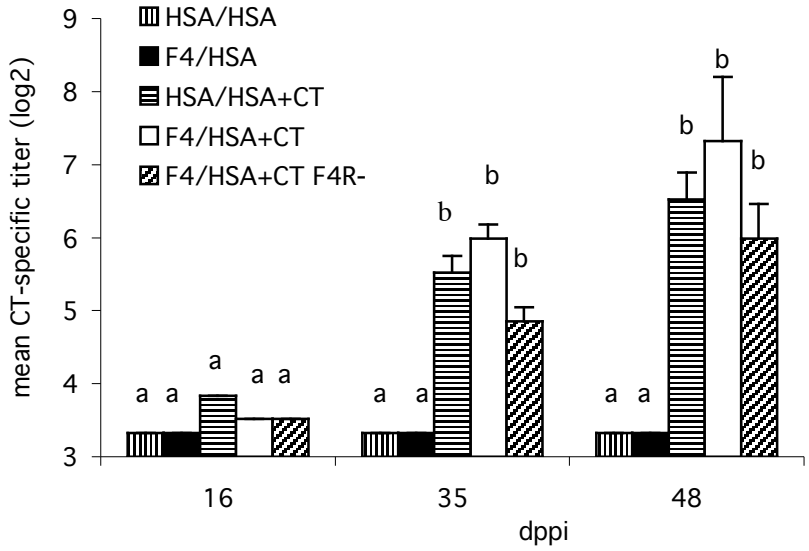


Figure 7 : Mean CT-specific serum antibody titer (± SEM) of immunized pigs. Bars with a different letter are significantly different (P<0.05).

7.4. Discussion

It is generally accepted that oral immunization with non-replicating antigens that do not bind to the intestinal mucosae, does not result in the induction of antigen-specific antibodies in serum or at mucosae (Foss and Murtaugh, 1999a; Van den Broeck et al., 2002; Lauterslager et al., 2003). Oral immunization of animals with proteins can lead to either oral tolerance or priming of the systemic immune system, depending on the dose given (Lamont et al., 1989; Mowat, 1995; Van den Broeck et al., 2002). In the present study, oral immunization of pigs with HSA-HSA at a dose of 4.8 mg per immunization did not result in oral tolerance, nor in a clear priming of the systemic immune response since a primary HSA-specific serum antibody response was observed following IM HSA immunization.

Mice orally immunized with antigen in the presence of CT showed an enhanced induction of the antigen-specific humoral and cellular immune response

(Clarke et al, 1991; Smith et al., 2002; Lauterslager et al., 2003). This has also been demonstrated in guinea pigs, chickens and rabbits (McKenzie and Halsey, 1984; Debard et al., 1996; Girard et al., 1999). The present study is the first to show that CT is also a mucosal adjuvant for an orally administered non-mucosa-targeted antigen in pigs. Indeed, the observed high HSA-specific serum IgG antibody titer following oral co-administration of antigen and CT indicate an antigen-specific priming of the systemic immune response. This observation is in line with previous studies using CT in mice, rabbits, guinea pigs and chickens (McKenzie and Halsey, 1984; Debard et al., 1996; Benedetti et al., 1998; Girard et al., 1999; Lauterslager et al., 2002). Foss et al. (1999a), however, did not observe an antigen-specific immune response following oral co-administration of CT with non-mucosa-targeted albumin or keyhole limpet hemocyanin. This argues that the dose of antigen and the immunization schedule are probably very important since these were the major differences with the present study.

The results of the present study indicate that F4 fimbriae can act as a mucosal carrier molecule for HSA, inducing a HSA-specific priming of the mucosal and systemic immune system. Indeed, the IM HSA immunization resulted in a secondary immune response in the orally F4/HSA immunized pigs as evidenced by a low HSA-specific IgM response and a rapidly increasing HSA-specific IgG titer which reached a high titer. A primary HSA-specific antibody response appeared in the HSA/HSA immunized animals with a high HSA-specific IgM response and a slower IgG response reaching lower titers than in the F4/HSA immunized animals. This priming of a HSA-specific immune response following F4/HSA immunization is probably not due to an adjuvant effect of F4 since co-administration of F4 with ovalbumin did not induce an ovalbumin-specific immune response (Van den Broeck, unpublished data). The conjugation of HSA to purified F4 fimbriae in a 1:1 molar ratio resulted in F4/HSA complexes that are still able to bind to the F4R, as shown by the interaction of the complexes with $F4R^+$ enterocytes using a biosensor. This suggests that the F4-mediated binding of F4/HSA allows a more efficient uptake of the conjugated HSA across the intestinal epithelial barrier. Indeed, a recent study in our lab showed that F4 fimbriae can be taken up in $F4R^+$ pigs by both enterocytes and M cells thus reaching antigen presenting cells such as porcine SWC-3 positive myeloid cells in the lamina propria (Snoeck et al., 2004b). The F4-dependent uptake of F4/HSA in $F4R^+$ pigs reaches the gut-associated lymphoid tissue as evidenced by the appearance of HSA-specific serum IgA in the F4/HSA group following the IM booster immunization, in

contrast to the HSA/HSA immunized pigs. It has been reported that mucosal priming followed by a systemic booster immunization enhances not only the antigen-specific mucosal response but also the systemic immune response (Vajdy et al., 2003).

A negative effect of the conjugation of HSA to F4 fimbriae is the lower F4-specific antibody response following the oral immunization with F4/HSA in the present study in comparison with oral immunization with purified F4 in a previous study (Van den Broeck et al., 1999b), despite the similar experimental design. This could be due to a reduced capacity of F4/HSA to bind the F4R since further increasing the molar amount of HSA conjugated to F4 clearly reduced the F4R binding in the biosensor assay. The conjugation could influence binding as shown for the dietary lectin wheat germ agglutinin (WGA). Increasing the molar ratio of WGA in a WGA-albumin conjugation results in increased binding of the WGA-albumin complexes to Caco-2 cells (Gabor et al., 2002). A second factor influencing the immunogenicity could be the covalent linkage of adjacent FaeG subunits in the F4 fimbriae due to the glutaraldehyde-mediated conjugation, making the F4 fimbriae more rigid and perhaps influencing their immunogenicity. It has been reported that chemical conjugation of antigens can inhibit their degradation and subsequently reduce antigen presentation and induction of an antigen-specific immune response (Kobayashi et al., 2003).

Oral co-administration of F4/HSA and CT significantly increased the F4-specific serum IgA titer, whereas HSA/HSA with CT feeding did not significantly induce HSA-specific serum IgA antibodies. Oral co-administration of CT with other fimbriae like CFAI and CS6 also resulted in the induction of higher fimbriae-specific IgG and IgA serum responses and in higher amounts of faecal IgA (Byrd and Cassels, 2003). In addition, the results of the present study show that the addition of CT significantly enhanced the immune response against the antigen that was targeted to the mucosa by F4 following the oral immunization. Indeed, significantly higher HSA-specific serum IgG titers are present in $F4R^+$ pigs immunized with F4/HSA+CT as compared to HSA/HSA+CT immunization. In addition, oral F4/HSA+CT immunization results in significantly higher HSA-specific antibody titers in $F4R^+$ than in $F4R^-$ pigs. Thus, conjugation to F4 and addition of CT enhance synergistically the HSA-specific antibody response. This is in agreement with studies that report higher antigen-specific antibody responses following oral co-administration of CT with

mucosa-targeted antigen as compared to non-mucosa-targeted antigen (McKenzie and Halsey, 1984; Czerkinsky et al., 1989; Van der Heijden et al., 1991).

In $F4R^-$ pigs, oral co-administration of F4/HSA with CT results in a significantly lower HSA-specific serum IgG antibody titer as compared to oral co-administration of HSA/HSA with CT in $F4R^+$ pigs. This difference in HSA-specific immune response is not due to a different efficiency of CT in both groups as the CT-specific antibody response is identical in both groups. Frey et al. (1996) observed that smaller molecules could better pass the intestinal mucus layer than larger molecules. Probably, the smaller size of the HSA/HSA molecules that was observed in the DLS experiments could enable the uptake of these molecules through the intestinal mucus layer as compared to the larger F4/HSA molecules. Indeed, CT is suggested to attract dendritic cells to the epithelial cells lining the gut mucosa, subsequently enhancing uptake of luminal antigens (Lycke, 2004). In addition, Verma et al. (1994) reported that CT increases the intestinal permeability. Therefore, a more efficient passage of HSA/HSA through the intestinal mucus than of F4/HSA probably could result in a faster induction of an HSA-specific immune response.

Nevertheless, the use of F4 fimbriae as mucosal carrier to heterologous antigens could open new perspectives in the development of a vaccine that simultaneously protects pigs against the widely spread $F4^+$ ETEC and other enteropathogens such as rotavirus or verotoxigenic *E. coli*. In contrast to the insertion of heterologous epitopes in the variable region of FaeG (Thiry et al., 1989; Bakker et al., 1990), conjugation of antigens to fimbriae is not restricted to short peptides. In addition, conjugates can be obtained that retain their F4R-binding capacity and subsequently can be used as mucosal carrier molecule, whereas exchanging the variable region of FaeG seems to inhibit its F4R-binding (Bakker et al., 1992a). Perhaps, further improvement of the mucosal carrier capacity of F4 fimbriae could be obtained by altering the molar ratio of F4 to HSA in the conjugate (Russell-Jones, 2001). On the other hand, fusion of a heterologous antigen to recombinant fimbrial adhesin subunits could be used as mucosal carrier system (Batisson et al., 2000a and 2000b). Indeed, fusion of a heterologous peptide to the F4 fimbrial adhesin FaeG induces a peptide-specific antibody response following oral immunization of pigs with this fusion protein (Verdonck et al., 2004a). Perhaps, the fimbrial adhesin FaeG could be a better mucosal carrier than F4 fimbriae as no HSA-specific antibodies were found in this study following oral F4/HSA immunization of $F4R^+$ pigs. However, it is

not known at the moment if FaeG has the potential to function as mucosal carrier for large antigens.

In conclusion, the results of the present study show the potential of F4 fimbriae to induce an immune response against a coupled heterologous antigen following oral administration in pigs. In addition, this potential of F4 fimbriae to function as a mucosal carrier can be improved by the adjuvant effect of CT. The combined use of CT as mucosal adjuvant and F4 fimbriae as mucosal carrier could open new perspectives in the development of vaccines against other enteropathogens in pigs.

Chapter 8

Altered bioactivity of purified F4 fimbriae by anionic detergents, temperature and pH: correlation with a monoclonal antibody based ELISA[1]

[1] *Based on : Verdonck F, Snoeck V, Goddeeris BM, Cox E. Binding of a monoclonal antibody positively correlates with bioactivity of the F4 fimbrial adhesin FaeG. Submitted*

Abstract

To develop a vaccine, a bioactive F4-receptor (F4R) binding FaeG molecule is required that binds to the F4R following oral immunization and induces a FaeG-specific immune response. The present study reports the altered binding of the FaeG-specific monoclonal antibody IMM01 with bioactive versus non-bioactive F4 fimbrial adhesin FaeG. The correlation of altered IMM01 binding with altered FaeG bioactivity, enables the use of an IMM01-based ELISA as a fast, specific and sensitive *in vitro* selection for potent F4 or (recombinant) FaeG antigen formulations, useful in an $F4^+$ ETEC vaccine.

8.1. Introduction

Today, no commercial vaccine exists against post-weaning diarrhoea. However, orally administered F4 fimbriae bind to the F4R in the small intestine and induce local secretion of F4-specific IgA (Van den Broeck et al., 1999a) that inhibit subsequent colonization of $F4^+$ ETEC (Van den Broeck et al., 1999b). Incorporation of F4 fimbriae in pellets or microspheres (Snoeck et al., 2003) or coupling of F4-fimbriae to an adjuvant could favour the induction of a protective mucosal F4-specific immune response and/or decrease the amount of antigen needed for immunization. On the other hand, the use of recombinant FaeG (rFaeG) subunits could perhaps be an alternative to the purified F4 fimbriae to induce a mucosal F4-specific antibody response. Recently, expression of rFaeG is reported in tobacco (Huang et al., 2003; Joensuu et al., 2004) and in *Escherichia coli* (Verdonck et al., 2004a).

To analyse the usefulness of formulations containing purified F4 fimbriae or rFaeG to induce a mucosal F4-specific immune response, or to study their capacity as mucosal carrier for heterologous antigens, it is important to analyse their bioactive properties in a fast, specific and sensitive way. First, multimerization of FaeG will be important, as the multimeric nature of an antigen is reported to induce better protection following immunization than its monomeric nature (Miller et al., 1998). Second, F4 fimbriae or FaeG subunits need to bind to the F4R as purified F4 fimbriae only induce an F4-specific mucosal immune response when they bind to the F4R on small intestinal villi

(Van den Broeck et al., 1999b). At the moment, the inhibition adhesion assay described by Van den Broeck et al. (1999c) is the most accurate system to test the binding of F4 fimbriae or FaeG subunits to the F4R. However, this assay is not very sensitive and is labour intensive. Haemagglutination assays are also reported to analyse adhesive properties of FaeG adhesins, but it is presently not known if this interaction reflects the interaction between FaeG and the F4R on intestinal villi (Gibbons et al., 1977; Bakker et al., 1992a). Furthermore, haemagglutination assays are not useful in the presence of contaminating molecules like detergents in the sample. On the other hand, the conformation of FaeG can be analysed using conformational epitope-specific monoclonal antibodies (MAb) (Bakker et al., 1992a).

The aim of the present study was to determine the bioactivity of F4 fimbriae following incubation in a number of conditions, often used during protein purification and refolding, in encapsulation of antigens or during coupling procedures. Subsequently, an ELISA was developed that correlated the optical density of analysed F4 fimbrial samples with their bioactive properties in a fast, sensitive and specific manner.

8.2. Materials and methods

8.2.1. Isolation of F4 fimbriae

The F4 fimbriae were purified as described by Van den Broeck (1999c). Briefly, fimbriae were isolated by homogenizing the bacterial suspension of strain *E. coli* GIS26 using an Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany), followed by a purification using two centrifugation steps and a precipitation step with 40 % (w/v) ammonium sulphate. Thereafter, the pellet was dissolved and dialyzed overnight against ultra-pure H_2O.

8.2.2. Incubation conditions

The protein concentration of purified F4 fimbriae was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as a standard (ICN Biomedicals, Belgium). The purity of the purified F4 fimbriae was assessed in a

Coomassie stained 15 % SDS-PAGE with the ImageMaster 1D prime software (Amersham Pharmacia Biotech, Belgium).

Purified F4 fimbriae were incubated in different conditions to analyse conformational changes of F4 fimbriae or to study the influence of frequently used chemicals or physical conditions on its receptor-binding capacity, multimerization and conformation. Therefore, 500 µg of purified F4 fimbriae were incubated for 30 minutes at room temperature (25°C) in a total of 500 µl reaction volume, whereafter the treated F4 sample was dialyzed overnight against PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4°C. In this way, F4 fimbriae were incubated in PBS supplemented with final concentrations of 5; 1.5; 0.5 or 0.15 % (v/v) SDS, Triton-X-100, saponin or Tween®20. Furthermore, 500 µg of purified F4 fimbriae were incubated for 30 minutes at room temperature in 500 µl 50 mM TrisHCl-buffer with/without urea or guanidine hydrochloride (GuHCl) at a final concentration of 1, 2, 3 or 4 M. Additionally, F4 fimbriae were also incubated in PBS with adjusted pH values (using 1M HCl) of 2, 4, 6, 8 or 10 in the presence/absence of 0.05 % SDS. Finally, F4 fimbriae were incubated 5 minutes at room temperature (25°C), 50, 70, 85 or 100°C. All samples were also made without the addition of purified F4 fimbriae (further indicated as 'treatment solution') and analysed to determine the direct influence of the treatment solutions on the used test systems. Each condition of treating purified F4 samples were individually performed three times and were always dialyzed overnight against PBS following treatment.

8.2.3. Multimeric FaeG character of F4 fimbriae

The multimeric nature of an antigen is reported to induce better protection following immunization than its monomeric nature (Miller et al., 1998). Therefore, the presence of FaeG multi- or monomers following incubation of F4 fimbriae was determined by SDS-PAGE followed by Western blot using the F4-specific MAb IMM01 (Van der Stede et al., 2002b) as described by Van den Broeck (1999c). The samples were diluted in the same volume of 60 mM TrisHCl pH 6.8, 2 % SDS, 10 % glycerol and 0.02 % bromophenol blue and loaded without heating the samples. The SDS concentration in the gel and the running buffer is 0.1%.

8.2.4. Turbidity analysis

The turbidity of a protein solution gives an indication of the amount of protein aggregates of a sufficient size to scatter visible light. Turbidity measurements were performed with a spectrophotometer (Ultrospec 4000, Pharmacia Biotech) at 550 nm (OD_{550}) (Andersen, 2002), immediately after each of the incubations (done to induce conformational changes of F4) as well as after overnight dialysis to see if aggregates (dis)appeared.

8.2.5. Bioactivity

The bioactivity of the treated F4 samples is correlated with its ability to bind to the F4R. Therefore, the binding of treated F4 fimbriae to the F4R was determined by the *in vitro* competitive villous adhesion assay, a variant of the method described by Van den Broeck et al. (1999c). Briefly, villi of F4R positive ($F4R^+$) or F4R negative ($F4R^-$) pigs were washed four times in Krebs-Henseleit buffer (160 mM, pH 7.4) whereafter they were resuspended in PBS supplemented with 1 % (w/v) D-mannose (Fluka, Sigma-Aldrich, Bornem, Belgium) to prevent adhesion by type 1 pili. Subsequently, 4 x 10^8 $F4^+$ *E. coli* and the test sample (200 µg treated F4 fimbriae or the treatment solution without F4 as negative control) were added to an average of 50 villi. The suspension was adjusted with PBS and D-mannose to a final volume of 0.5 ml containing 1 % (w/v) D-mannose. This suspension was incubated at room temperature for 1 h while gently shaking. Then, the adhesion of bacteria was evaluated quantitatively by counting the number of bacteria adhering along a 50 µm villous brush border at 20 randomly selected places using phase-contrast microscopy at a magnification of 600x, after which the mean bacterial adhesion per 250 µm villous brush border was calculated. The percentage inhibition was calculated in comparison with $F4R^+$ villi incubated only with $F4^+$ *E. coli*. Villi of three different $F4R^+$ pigs and one $F4R^-$ pig as negative control were used and the analyses were performed in triplicate.

8.2.6. ELISA

An indirect ELISA was developed to analyse the conformation of FaeG. Therefore, a 96-well microtiter plate (NUNC, Maxisorp Immuno Plates, Roskilde,

Denmark) was coated with an optimal concentration of F4-specific swine polyclonal antibodies in PBS. This polyclonal antibody will bind native as well as denaturated F4. After 2 h incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2 % (v/v) Tween®80. Following blocking, series of two-fold dilutions (10 to 0.005 µg⁄ml) of native and treated F4 fimbriae in ELISA dilution buffer (PBS + 0.05 % (v/v) Tween®20 + 3 % (w/v) BSA) were incubated for 1 h at 37°C. In addition, native F4 was similarly diluted in the treatment solutions to determine their direct influence on the assay. Thereafter, an optimal dilution of the FaeG-specific monoclonal antibody (MAb) IMM01 was added to the wells for 1 h at 37°C. Subsequently, rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) optimally diluted in ELISA dilution buffer and supplemented with 2 % (v/v) pig serum was brought onto the plates for 1 h at 37°C. An ABTS solution containing H_2O_2 was added and after 1 h incubation at 37°C the optical density was spectrophotometrically measured at 405 nm (OD_{405}). The plates were washed three times with ELISA washing buffer (PBS + 0.2 % (v/v) Tween®20) between each incubation step. The treated samples were independently analysed three times and data of a representative analysis are shown.

8.3. Results

Purified F4 fimbriae were incubated at different temperatures to induce conformational changes in the FaeG adhesins. Indeed, the bioactivity of the F4 fimbrial adhesin FaeG decreased following incubation of F4 fimbriae at high temperatures: F4 fimbriae incubated at 70°C still inhibited the adhesion of $F4^+$ *E. coli* to $F4R^+$ villi by 89.4 ± 4.5 %, whereas F4 fimbriae incubated at 85°C and 100°C only inhibited by 34.4 ± 14.8 % and 7.7 ± 6.8 %, respectively (Table 1). Western blotting of purified F4 fimbriae incubated at 85°C or higher temperatures revealed also a decreased polymerisation of the FaeG adhesins (Fig. 1), whereas turbidity measurements suggested the presence of non-ordered protein aggregations as evidenced by their increase in OD_{550} (Table 1). However, these aggregates did not precipitate, neither influence the concentration of F4 in solution. Indeed, the protein concentration in the top fraction of an F4 fimbrial sample incubated 5

minutes at 95 °C is identical directly following heat treatment and mixing as compared to 3 hours later.

Table 1 : Effect of different incubation conditions for F4 fimbriae on formation of aggregates (OD_{550} ± standard deviation, SD) (directly following incubation or following dialysis of the test samples) and on inhibition of adhesion of $F4^+$ *E. coli* adhesion to $F4R^+$ villi (% ± SD) (after dialysis).

Incubation conditions*	OD_{550} after		Percent inhibition of $F4^+$
	incubation	dialysis	ETEC to $F4R^+$ villi
25°C	0.056 ± 0.005	0.008 ± 0.002	92.4 ± 3.4
50°C	0.053 ± 0.004	0.008 ± 0.001	91.2 ± 3.6
70°C	0.055 ± 0.006	0.007 ± 0.001	89.4 ± 4.5
85°C	0.262 ± 0.022	0.347 ± 0.025	34.4 ± 14.8
100°C	0.375 ± 0.037	0.466 ± 0.046	7.7 ± 6.8
0.15% SDS	0.049 ± 0.004	0.062 ± 0.006	86.8 ± 4.1
0.5% SDS	0.025 ± 0.010	0.013 ± 0.007	78.6 ± 7.8
1.5% SDS	0.007 ± 0.005	0.005 ± 0.003	54.7 ± 9.0
5% SDS	0.005 ± 0.003	0.004 ± 0.003	13.1 ± 6.3
pH2	1.342 ± 0.069	0.081 ± 0.034	87.0 ± 7.1
pH4	0.583 ± 0.021	0.050 ± 0.008	85.3 ± 4.6
pH6	0.090 ± 0.009	0.061 ± 0.012	91.2 ± 5.3
pH8	0.078 ± 0.007	0.051 ± 0.009	90.7 ± 4.7
pH10	0.069 ± 0.012	0.032 ± 0.013	89.6 ± 6.2
pH2 0.05% SDS	0.510 ± 0.037	0.004 ± 0.004	15.7 ± 3.3
pH4 0.05% SDS	0.032 ± 0.005	0.006 ± 0.005	24.0 ± 8.1
pH6 0.05% SDS	0.015 ± 0.003	0.006 ± 0.002	88.9 ± 4.4
pH8 0.05% SDS	0.013 ± 0.004	0.007 ± 0.003	89.8 ± 5.2
pH10 0.05% SDS	0.013 ± 0.005	0.004 ± 0.002	87.9 ± 5.6

* 500 µg purified F4 fimbriae in 500 µl during 30 minutes

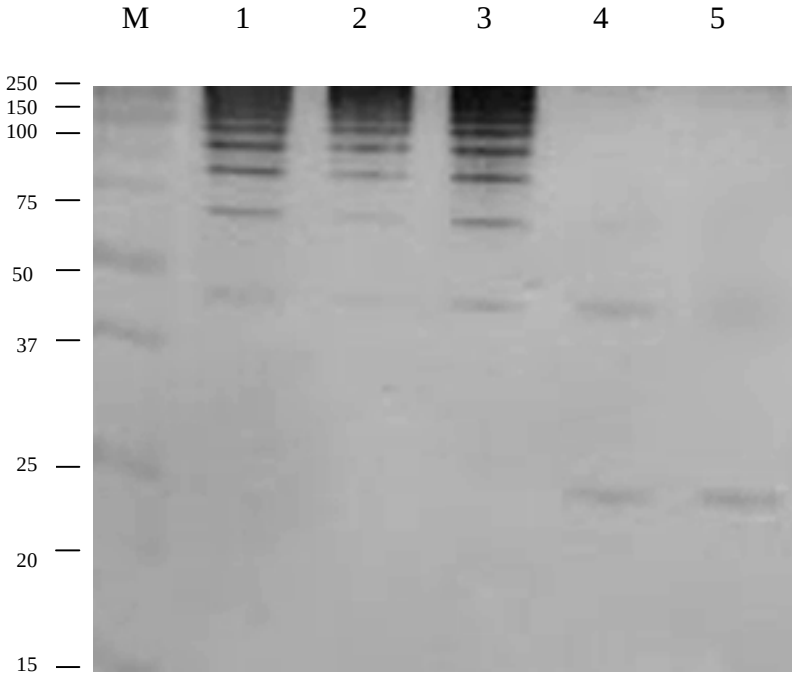


Figure 1 : Multimeric FaeG character of F4 fimbriae incubated for 5 minutes at 25°C (1), 50°C (2), 70°C (3), 85°C (4) and 100°C (5), determined in Western blot using the IMM01 MAb. M = molecular weight marker.

Subsequently, interaction of the F4-specific MAb IMM01 with serial dilutions of these temperature treated F4 fimbriae were tested in ELISA. Purified F4 fimbriae incubated at 25 to 70°C resulted in near parallel sigmoid OD_{405}-curves, characterized by an identical steep linear phase and a plateau phase decreasing with increasing incubation temperature (Fig. 2). However, F4 fimbriae incubated at a temperature of 85°C or higher, resulted in sigmoid OD_{405}-curves with a very low plateau phase and a linear phase with a flat slope. These flat sigmoid OD_{405}-curves indicate a lower affinity of the MAb for the high-temperature treated F4 fimbriae, most likely due to a conformational change in the epitope recognized by the MAb.

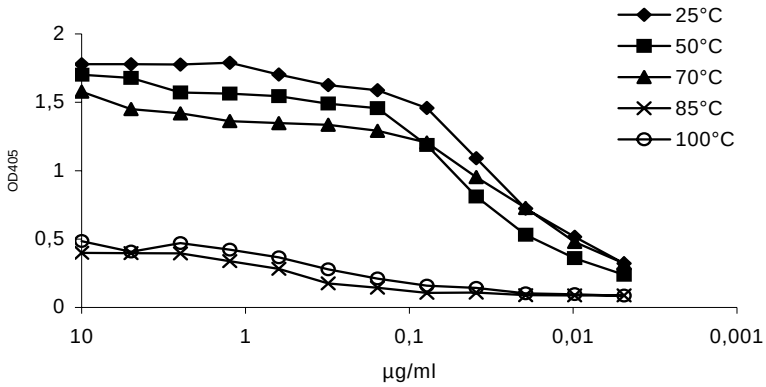


Figure 2 : Binding of the FaeG-specific MAb IMM01 with F4 fimbriae incubated for 5 minutes at 25°C, 50°C, 70°C, 85°C and 100°C.

F4 fimbriae were analysed following treatment with urea, GuHCl, detergents or at different pH as these conditions are often used during purification and refolding of proteins, in encapsulation of antigens or during coupling procedures (Singh and O'Hagan, 1998; De Bernandez, 2001; Tsumoto et al., 2003). Incubation with non-ionic detergents, urea or GuHCl did not influence the ELISA reactivity as well as the multimerization and the bioactivity of the treated F4 samples following dialysis (data not shown). However, incubation of F4 fimbriae with increasing SDS concentrations, resulted in a gradually altered binding of the IMM01 MAb to the treated F4 (Fig. 3) as no influence of the treatment solution containing SDS was observed. Indeed, conditions with 0.15 % and 0.5 % SDS revealed both sigmoid OD_{405}-curves with a decreased plateau phase but a quite parallel and steep linear phase as compared with native purified F4 fimbriae incubated without SDS. Higher concentrations of SDS resulted in linear-like**,** low value OD_{405}-curves. Furthermore, the incubation of F4 fimbriae with SDS reduced also its multimeric character (Fig. 4) and its bioactivity (% inhibition) in a SDS concentration-dependent manner (Table 1). However, the influence of SDS concentrations below 1 % on the multimeric character of F4 could not be determined by SDS-PAGE since the preparation of the samples resulted in a minimal final SDS-concentration of 1%. In agreement with the ELISA, no influence of the treatment solution on the *in vitro* competitive villous adhesion assay was noticed. Aggregate formation, as evidenced by turbidity, was however not increased (Table 1).

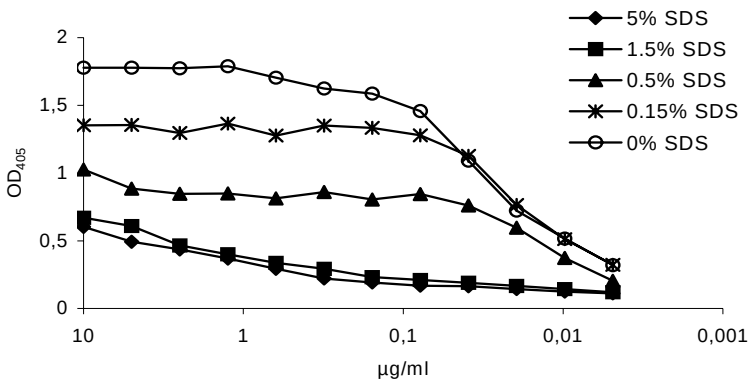


Figure 3 : Binding of the FaeG-specific MAb IMM01 with F4 fimbriae incubated for 5 minutes at 25°C or treated 30 minutes with 0.15 %, 0.5 %, 1.5 % and 5 % SDS, followed by overnight dialysis against PBS.

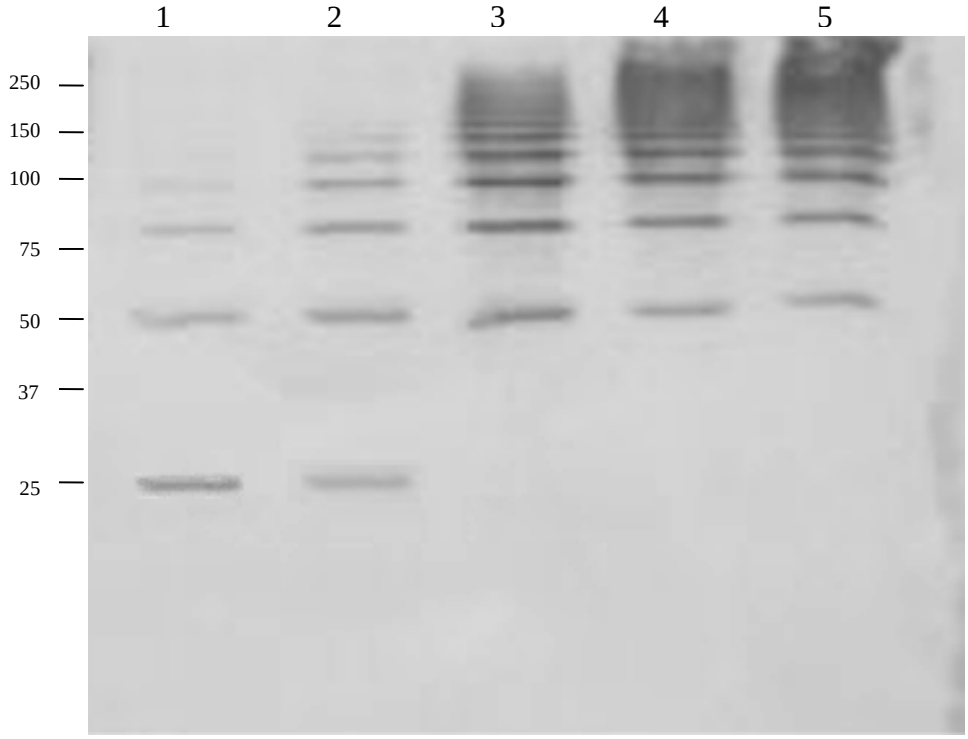


Figure 4 : Multimeric FaeG character of F4 fimbriae treated 30 minutes with 5 % SDS (1), 1.5 % SDS (2), 0.5 % (3), 0.15 % (4) or incubated for 5 minutes at 25°C (5) and both followed by overnight dialysis against PBS, determined in Western blot using the IMM01 MAb. M = molecular weight marker

Following incubation of F4 fimbriae at pH2 and 4, the turbidity increased, suggesting aggregation of proteins (Table 1). After dialysing these samples, aggregation disappeared and interaction of the treated F4 with the IMM01 MAb was identical as compared to native F4 fimbriae. Indeed, sigmoid OD_{405}-curves were obtained in ELISA identical to those obtained with F4 fimbriae incubated at pH 7.4 (Fig. 5). Furthermore, FaeG multimerization was present with little more FaeG mono- and dimers and the bioactivity was only slightly decreased as compared to non-incubated purified F4 fimbriae. These results suggest a reversible conformational change of F4 fimbriae in the presence of a low pH environment. In a subsequent experiment, F4 fimbriae were incubated at different pH in the presence of SDS at a concentration (0.05 %) that is not influencing characteristics of purified F4 fimbriae on its own. Following dialysis, samples of the pH2 SDS and the pH4 SDS conditions revealed linear instead of sigmoid OD_{405}-curves (Fig. 5), could only inhibit the binding of $F4^+$ *E. coli* to the F4R by 15.7 ± 3.3 % and 24 ± 8.1 % respectively and showed reduced FaeG polymerisation (mono- to trimers and mono- to hexamers, respectively) as compared to non-incubated purified F4 fimbriae and the other pH conditions in the presence of 0.05 % SDS.

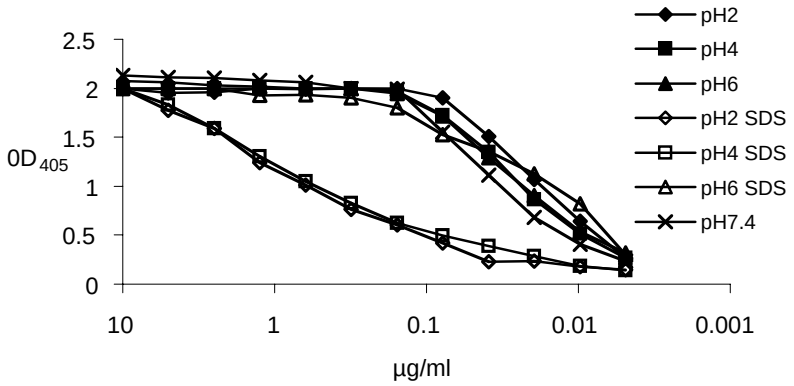


Figure 5 : Binding of the FaeG-specific MAb IMM01 with F4 fimbriae incubated for 5 minutes at 25°C (pH7.4) or 30 minutes at pH2, pH4, pH6 in the presence/absence of 0.05 % SDS, both followed by overnight dialysis against PBS.

8.4. Discussion

F4 fimbriae purified from the $F4^+$ ETEC strain GIS26 seem to be very stable proteins since they retain their multimeric FaeG character and their F4R binding following incubation in the presence of 5 % (v/v) non-ionic detergents, 4 M urea or GuHCl and at temperatures up to 70°C. In agreement, treatment of F4ab fimbriae with 2 M urea at 55°C is reported to remove all minor fimbrial subunits, but without dissociating and affecting the adhesive properties of the fimbriae (Oudega et al., 1989; Bakker et al., 1992a). Also fimbriae of other bacteria are reported to be stable protein structures. For example, dissociation of type 1 pili requires 8.6 M GuHCl (Eshdat et al., 1981). In the present study, irreversibly reduced F4 bioactivity is observed following incubation with SDS at concentrations of 1.5 % or higher and at temperatures of 85 °C or higher. The high denaturation temperature of FaeG is associated with a high stabilizing Gibbs energy (Knörle and Hubner, 1995). However, the *Yersinia pestis* F1 capsular antigen is reported to dissociate after heating in the absence and presence of 0.1 % SDS, but to reassociate upon cooling and removal of SDS by dialysis against PBS, respectively (Miller et al., 1998). This difference between F4 fimbriae and F1 antigen is probably due to structural differences and not due to a different multimerization mechanism. Indeed, the multimerization of FaeG subunits in F4 fimbriae as well as that of the non-fimbrial F1 antigen, is mediated by the conserved chaperone-subunit pathway (Sauer et al., 2000; Zavialov et al., 2003b; Chapter 4).

Exposure of type 1 pili to low pH does not affect the pili (McMichael and Ou, 1979), but incubation of purified F4 fimbriae at low pH results in a reversible conformational change of fimbriae. In agreement, a number of research groups use this phenomenon as the basis for a method for purification of F4 fimbriae (Erickson et al., 1994; Jin et al., 1998). However, this reversible conformational change became irreversible and stabilised in the presence of 0.05 % SDS, a concentration which did not affect the bioactivity at neutral pH. The addition of SDS itself is not harmful because samples were always dialyzed against PBS following treatment and no influence was observed in the analysis of control solutions. Furthermore, the results of Miller et al.

(1998) indicated that the bioactivity of F1 antigen can be altered following incubation with SDS, but can be restored following dialysis against PBS.

The suggested conformational change of F4 fimbriae at low pH is probably not very detrimental for the fimbrial characteristics *in vivo*, since only very limited digestion of F4 fimbriae was observed following 2 h incubation in the presence of pepsin at pH 2 (Snoeck et al, 2004a). In agreement, oral administration of purified F4 fimbriae can still immunize $F4R^+$ pigs, indicating that the stomach is not destroying the binding capacity of F4 (Van den Broeck et al., 1999a en 1999b).

To develop an F4 fimbrial vaccine against $F4^+$ ETEC-induced diarrhoea in post-weaning piglet or to use the adhesin as a carrier molecule (Schembri et al., 1999), it is important to determine the bioactive-related characteristics of the F4 fimbrial adhesin FaeG in a specific and fast manner. Results of the present study reveal a correlation of FaeG bioactivity and binding of the FaeG-specific MAb IMM01, as no direct influences of the used treatment solutions on the ELISA or the *in vitro* competitive adhesion assay were observed. Therefore, reduced binding of the adhesin with its receptor and a reduced multimerization of FaeG adhesins seems to be correlated with altered interaction of the IMM01 MAb with FaeG. However, this altered binding by IMM01 MAb upon antigen denaturation cannot be interpreted as an indication of complete antigen denaturation. It can only be considered as an indication that the conformational modifications that have occurred are important enough to disrupt the specific spatial arrangement necessary for antibody binding and bioactivity. The MAb IMM01 is able to block the binding of $F4^+$ *E. coli* of the F4ab, F4ac and F4ad antigenic variants to the F4R (Van der Stede et al., unpublished data). Furthermore, the IMM01 MAb binds to fimbriae of more than 25 haemolytic $F4ac^+$ *E. coli* strains isolated from pigs with post-weaning diarrhoea (Verdonck et al, unpublished data). Thus, the epitope recognized by the IMM01 MAb is a conserved ‘a’-epitope present in all three different F4 antigenic variants.

The interaction of FaeG with the F4R is not fully characterized, but both conserved and variable amino acids seem to contribute to the receptor-binding site (Bakker et al., 1992a). It is not clear if the receptor-binding site and the multimerization site are identical or located near to each other, as the protein structure of FaeG is not elucidated at the moment. Therefore, the present study cannot discriminate if the

observed reduction in binding of treated F4 fimbriae to the F4R is due to conformational changes in the receptor binding epitope or due to the reduced polymerisation of FaeG adhesins.

In conclusion, purified F4 fimbriae are very stable proteins but their bioactivity was observed to be reduced following incubation with SDS at concentrations of 1.5 % or higher, at temperatures of 85°C or higher and at pH 4 or lower. This reduced F4R-binding and reduced FaeG multimerization of treated F4 fimbriae correlated with optical density determined in a newly developed ELISA using the FaeG-specific MAb IMM01. This assay will enable a fast, specific and sensitive *in vitro* selection for potent F4 or (r)FaeG antigen formulations, useful in an $F4^+$ ETEC vaccine.

Chapter 9

Bioactive recombinant F4 fimbrial adhesin FaeG monomers induce a fimbriae-specific immune response following oral immunization of piglets and function as mucosal carrier to an N-terminal fused peptide[1]

[1] *Based on : Verdonck F, Cox E, Van der Stede Y, Goddeeris BM. 2004a. Oral immunization of piglets with recombinant F4 fimbrial adhesin FaeG monomers induces a mucosal and systemic F4-specific immune response. Vaccine, in press.*

Abstract

The importance of adhesins in the pathogenicity of several bacteria resulted in studies on their usefulness in vaccines. In this study, the gene of the F4(K88)-fimbrial adhesin FaeG of the pathogenic enterotoxigenic *Escherichia coli* (ETEC) strain GIS26 was cloned in the pET30Ek-LIC vector and expressed with an N-terminal His- and S-tag in the cytoplasm of BL21(DE3). Recombinant FaeG (rFaeG) subunits were isolated from insoluble cytoplasmic aggregates and refolded into a native-like F4R-binding conformation. Indeed, the presence of conformational epitopes was shown by ELISA and the ability to bind the F4R was observed by inhibiting the adhesion of $F4^+$ ETEC to $F4R^+$ villi with increasing concentrations of native-like refolded rFaeG subunits. The rFaeG subunits appear as monomers, whereas the purified F4 fimbriae are multimers. Oral immunization of newly weaned piglets with native-like rFaeG induced a mucosal and systemic F4-specific immune response, significantly reducing $F4^+$ *E. coli* excretion from 2 till 5 days following challenge infection. However, improvement of stability and immunogenicity of rFaeG is necessary since a higher F4-specific response was obtained following immunization with purified F4 fimbriae. Furthermore, the presence of antibodies against the N-terminal fused His- and S-tag containing peptide support the use of (r)FaeG as a mucosal carrier.

9.1. Introduction

The importance of fimbriae as colonizing factors in the pathogenesis of ETEC induced diseases, led to the development of fimbriae-based vaccines. Rutter and Jones (1973) reported that parenteral vaccination of sows with fimbriae protected suckling piglets via passive lactogenic immunity. However, in the postweaning period, an active intestinal mucosal immune response is required. Since parenteral vaccination tends to stimulate the systemic rather than the mucosal immune system (Moon and Bunn, 1993; Bianchi et al., 1996; Van der Stede et al., 2003), it seems worthwhile to develop oral vaccines containing fimbriae that stimulate the intestinal mucosal immune system.

Van den Broeck et al. (1999a and 1999b) reported that oral immunization of weaned piglets with purified F4 fimbriae induced a protective FaeG-specific immune response in $F4R^+$ piglets. On the other hand, purified F4 seemed to behave as a normal food antigen in $F4R^-$ pigs (Van den Broeck et al., 2002). In contrast to F4 fimbriae, oral immunization with most soluble antigens induces an immunohyporesponsive state, named oral tolerance (Strobel and Mowat, 1998; Stokes and Bailey, 2000). However, the conjugation of an antigen to a mucosal carrier molecule can result in the induction of an antigen-specific immune response (Holmgren et al., 1993; Foss and Murtaugh, 1999b; Ogra et al., 2001).

The purpose of the present study was to determine whether recombinant FaeG (rFaeG) could be obtained in native-like conformation in the absence of the chaperone FaeE, allowing oral immunization of pigs and induction of an FaeG-specific immune response. In addition, it was analysed if rFaeG could be used as a mucosal carrier that is able to induce an immune response against an N-terminal fused heterologous peptide following oral immunization.

9.2. Material and methods

9.2.1. Bacterial inoculum

The ETEC strain GIS26 (serotype O149:K91, $F4ac^+$, $LT^+STa^+STb^+$) was cultured during 18 h in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) at 37°C and 85 rpm. The bacteria were collected by centrifugation and washed with phosphate-buffered saline (PBS) (150mM, pH 7.4). The concentration of the bacteria was determined by measuring the optical density of 10-fold dilutions of the bacterial suspension at 660nm (OD_{660}). An OD_{660} of 1 equals 10^9 viable bacteria/ml, as determined by counting colony-forming units.

9.2.2. Purification of F4 fimbriae

F4 fimbriae were purified from an *E. coli* GIS26 bacterial suspension as described by Van den Broeck et al. (1999c). The purity of the purified F4 fimbriae was assessed using a Coomassie stained 15% SDS-PAGE and the ImageMaster 1D prime software

(Amersham Pharmacia Biotech, Belgium). The protein concentration of purified F4 fimbriae was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as a standard (ICN Biomedicals, Belgium), taking into account the purity of the purified F4 fimbriae. Furthermore, rFaeG or purified F4 fimbriae were identified by Western blot using the FaeG-specific MAb IMM01 (Van der Stede et al., 2002b) as described by Van den Broeck et al. (1999c).

9.2.3. pETFaeG/7 construction

faeG without its signal sequence was amplified from purified chromosomal DNA of strain GIS26 by the polymerase chain reaction (PCR), using the primers FaeGac Forw (5'-GACGACGACAAGATTGCACATGCCTGGATGACTGG-3') and FaeGac Rev (5'-GAGGAGAAGCCCGGTAATAAATTGGCAGCTCATCACG-3'). The resulting product was purified (Quantum Prep PCR Kleen Spin Columns, BioRAD and made sticky by treating with T4 DNA polymerase (Promega, Madison, USA) in the presence of dATP. Subsequently, the sticky fragment was ligated in the pET-30 Ek/LIC vector (Novagen, Madison, USA) and transformed by heat shock in Nova Blue Singles competent cells (Novagen) according to the manufacturers instructions. The nucleotide sequence of purified plasmid (Midiprep, Qiagen) of the selected pETFaeG/7 clone was determined by the Sanger dideoxy chain termination method using the ABI PRISM Big Dye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (ABI) and the FaeGac and T7 primer sets (T7 forward : TAATACGACTCACTATAGGG, T7 reverse: GCTAGTTATTGCGCGG), following the manufacturers protocol. After the cycle sequencing reaction, the products were sequenced on a 377 ABIPRISM automated DNA sequencer according to the manufacturers manual. The chromatograms of the sequences were visualised using the CHROMAS 2.0 software (Technelysium Ltd., Australia) and the DNA sequences were analysed using the DNAMAN version 5.0 (Lynnon Biosoft). Finally, BL21(DE3) host cells (Novagen) were transformed by heat shock with the selected construct pETFaeG/7.

9.2.4. rFaeG expression

The rFaeG expressing BL21(DE3)[pETFaeG/7] *E. coli* were grown overnight in Luria broth (LB; Life Technologies, Paisley, Scotland) with 30 μg/ml kanamycin at 28°C. Afterwards, the bacteria were diluted 100 times in fresh LB supplemented with kanamycin and incubated at 28°C, 200 rpm to an OD_{660} of 0,2 - 0,3. So, the bacteria could grow and the expression of rFaeG in the absence of induction was reduced. rFaeG expression was subsequently induced by adding 1mM isopropyl-β-D-thiogalactoside (IPTG, Sigma) to the cultures, which were further incubated during 4 hours at 37°C and 200 rpm.

9.2.5. Purification of different fractions of E. coli

Spheroplasts of BL21(DE3)[pETFaeG/7] *E. coli* were prepared as described by Lindberg et al. (1989). In addition, the method described by Ausubel et al. (1989) was used to purify proteins from different fractions of *E. coli*. Briefly, 10^{10} induced bacteria were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The resulting supernatant is the '*medium fraction*'. The pellet was resuspended in 30 ml Tris-HCl buffer (30 mM, pH 8) supplemented with 20% sucrose. Thereafter, EDTA was supplemented to a final concentration of 1 mM for 10 min at room temperature. The bacteria were collected by centrifugation at 10,000 x g and 4°C for 10 min. The pellet was resuspended in 30 ml ice-cold 5 mM $MgSO_4$, shaken slowly for 10 min on ice and centrifugated again. The supernatant was the '*periplasmic fraction*', the pellet contained the shocked cells and was resuspended for 15 min at 30°C in 4 ml of cold 20 mM Tris-HCl pH 7.5, supplemented with 100 μg/ml lysozyme. Subsequently, the bacterial suspension was sonicated 20 seconds with power level 5, duty 50% (Vibra Cell, Sonics & Materials Inc., Danbury, Conneticut, USA) and centrifugated for 10 minutes by 14,000 x g at 4°C. The resulting supernatant was the '*soluble cytoplasmatic fraction*', whereas the '*insoluble cytoplasmatic fraction*' (pellet) was washed twice with 20 mM Tris-HCl pH 7.5 and finally resuspended in 1.5 ml Tris-HCl buffer containing 6M urea or PBS + 0.5 % SDS**.** Following overnight dialysis against PBS at 4°C, rFaeG of the urea- or SDS-refolded insoluble cytoplasmic fraction (urea- and SDS-refolded rFaeG, respectively) was

used in tests to analyse its conformation or was used to immunize animals. The medium and periplasmic fraction were concentrated using saturated ammonium sulphate prior to SDS-PAGE.

9.2.6. Determination of epitopes on rFaeG by ELISA

An indirect ELISA was developed to determine the presence of different conformational epitopes on rFaeG. A 96-well microtiter plate (NUNC, Maxisorp Immuno Plates, Roskilde, Denmark) was coated with polyclonal F4-specific swine antibodies, optimally diluted in PBS. After 2 h incubation at 37°C, the remaining binding sites were blocked overnight at 4 °C with PBS supplemented with 0.2% (w/v) Tween®80. Then, the plates were incubated for 1 h at 37°C with serial two-fold dilutions of rFaeG, F4 fimbriae or heat-denaturated F4 fimbriae (5 min, 95°C) in ELISA dilution buffer (PBS + 0.05% (v/v) Tween®20 + 3% (w/v) BSA). Thereafter, optimal dilutions (determined in an assay with purified F4 fimbriae) of FaeG-specific MAb (IMM01, Van der Stede et al., 2002b; CVI-F4ac-5, CVI-F4ac-6 and CVI-F4ad-3, van Zijderveld et al. 1990) were added to the wells for 1 h at 37°C. Subsequently, rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) optimally diluted (1/1000) in ELISA dilution buffer and supplemented with 2% (v/v) pig serum was brought on the plate for 1 h at 37°C. An ABTS solution containing H_2O_2 was added and the optical density was spectrophotometrically measured at 405 nm (OD_{405}). Between each incubation step, the plates were washed three times with ELISA washing buffer (PBS + 0.2% (v/v) Tween®20).

9.2.7. In vitro villous adhesion assays

The presence or absence of the F4R on the brush border of small intestinal enterocytes was determined on isolated intestinal villi as described by Van den Broeck et al. (1999c).

The F4R-binding capacity of F4 fimbriae and rFaeG was analyzed by an *in vitro* competitive villous adhesion assay. Briefly, an average of 50 villi of $F4R^+$ or $F4R^-$ pigs were incubated while gently shaking at room temperature with 800, 400, 200, 100, 50, 25, 12.5 or 1.25 µg/ml F4 fimbriae or rFaeG together with 4 x 10^8 $F4^+$ *E. coli* in a final volume of 0.5 ml. The suspension contained 1% (w/v) D-mannose (Fluka, Sigma-

Aldrich, Bornem, Belgium) to prevent adhesion by type 1 pili. The adhesion of bacteria to villi was evaluated quantitatively by counting the number of bacteria adhering along a 50 µm villous brush border at 20 randomly selected places by phase-contrast microscopy at a magnification of 600x. Then, the mean bacterial adhesion per 250 µm villous brush border was calculated. The percentage inhibition of adhesion was calculated for each sample by comparing with $F4R^+$ villi incubated with only the $F4^+$ *E. coli*.

To demonstrate that rFaeG was responsible for the inhibition of adhesion of $F4^+$ *E. coli* to $F4R^+$ villi, 200 µg/ml SDS-refolded rFaeG was pre-incubated with 1 µg/ml of the c-epitope specific MAb CVI-F4ac-5 to block their adhesion to the F4R. After incubation with the $F4R^+$ villi and washing, $F4^+$ *E. coli* were added and the inhibition percentage was determined. Similarly, the inhibiting capacity of an irrelevant swine IgG-specific MAb of the same isotype (clone 23.3.1b) (Van Zaane and Hulst, 1987) was tested at a concentration of 10 µg/ml as a control.

Villi of three different $F4R^+$ pigs were used and all analyses were performed in triplicate. $F4R^-$ villi of one pig served as negative control. These villi never bound $F4^+$ ETEC.

9.2.8. Oral immunization experiment

Twenty-nine, $F4R^+$ and F4-seronegative conventionally bred pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks, transported to the experimental facilities at the faculty and subsequently housed in isolation units where they obtained water and food at libitum. These piglets were treated orally with colistine (Promycine pulvis, VMD, Berendonk, Belgium, 150,000 U/kg of body weight/day) from 2 days before till 3 days after weaning to prevent *E. coli* infections due to transport and handling.

One week post weaning, thirty-two pigs were orally given 20 mg rabeprazolum (Pariet, Janssen-Cilag, Berchem, Belgium) on 3 consecutive days and again 15 days post primary immunization (dppi) to neutralize the acidic gastric pH and thus reducing the possible alteration of fimbrial antigens during exposure to the low pH (Snoeck et al., 2004a). Twenty-four hours following each rabeprazolum ingestion (0, 1, 2 and 16 dppi), the pigs were orally immunized with 2 mg purified F4 fimbriae (F4 group, n=10) or 8 mg SDS-refolded rFaeG (rFaeG (SDS) group, n=9) in 10 ml PBS. Three animals were orally

immunized with 8 mg urea-refolded rFaeG (rFaeG (urea) group, n=3) in 10 ml PBS with 2M urea to reduce the formation of rFaeG aggregates. Ten animals received PBS (PBS group) and served as negative control. Three hours before till 2 h after gastric pH neutralization or immunization, each animal was deprived of food and water.

One week following the booster immunization (23 dppi), the local mucosal F4-specific immune response was analysed in two pigs of the F4, the rFaeG (SDS) and the PBS groups and in all three pigs of the rFaeG (urea) group by enumerating the F4-specific IgA, IgG and IgM antibody-secreting cells (ASCs) in mesenteric lymph nodes (MLN), jejunal and ileal Peyer's patches (JPP and IPP) and jejunal lamina propria (LP). In addition, F4-specific IgA and IgM antibodies were determined in duodenal, jejunal and ileal contents. Tissues and intestinal content were sampled following euthanasia of animals by intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. The remaining animals were orally challenged with the virulent $F4^+$ ETEC strain GIS26 as previously described (Cox et al., 1991) with minor modifications. Briefly, pigs were orally pre-treated at 21 and 22 dppi with 300 mg florfenicol (Nuflor, Schering-Plough, Brussels, Belgium) to decrease colonization resistance. At 24 dppi, pigs were sedated with Stressnil (40 mg/ml; Janssen-Cilag, Berchem, Belgium), after which the gastric pH was neutralized by intragastrical administration of 62 ml $NaHCO_3$ (1.4% (w/v) in distilled water). Fifteen to thirty min later, 10^{10} $F4^+$ ETEC in 10 ml PBS was given intragastrically. Faecal samples were taken daily for determining the excretion of $F4^+$ ETEC from challenge till 7 days post challenge (31 dppi). Furthermore, FaeG-specific serum IgA, IgG and IgM were determined 0, 7, 16, 24, 27, 31, 37 and 45 dppi. Three weeks following challenge (45 dppi), the remaining pigs were euthanatised. Jejunal villi were isolated of all euthanatised pigs to confirm the presence of F4R as described by Van den Broeck et al. (1999c).

9.2.9. Samples

Serum and contents of duodenum, jejunum and ileum were sampled as described by Van den Broeck et al. (1999a) and Verdonck et al. (Chapter 5), respectively.

$F4^+$ *E. coli* were enumerated in faecal samples by dot blotting using the FaeG-specific MAb IMM01 as previously described (Van den Broeck et al., 1999b). The

resulting brown-red dots were counted and the average within each group was calculated. Results are presented as the mean number ± standard error of the mean (SEM) of excreted *E. coli* per gram faeces.

At the moment of slaughter, mesenteric (jejunal and ileal) lymph nodes, a mid jejunal intestinal segment without Peyer's patches, and jejunal and ileal intestinal segments with Peyer's patches were sampled. Subsequently, monomorphonuclear cells (MC) were isolated as described by Verdonck et al. (chapter 5) and finally resuspended at 10^6 cells/ml.

9.2.10. ELISA for FaeG-specific IgM, IgA and IgG

For detection of FaeG-specific antibodies, the indirect ELISA described by Van den Broeck et al. (1999a) was used. The IgM, IgA and IgG cut-off values were calculated as the mean OD_{405}-value of all sera (dilution 1/10) at day 0, increased with 3 times the standard deviation. In case of intestinal contents, the IgA and IgM cut-off values were calculated as the OD_{405}-value of the dilution buffer, increased with 3 times the standard deviation. The antibody titer was the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value.

9.2.11. Elispot assays for FaeG-specific IgM, IgA and IgG ASCs

FaeG-specific IgM, IgA and IgG ASCs were detected as described by Van den Broeck et al. (1999a). For each MC suspension, spots in 5 wells (10^5 MC/well) were counted to obtain the number of isotype-specific ASCs per 5 x 10^5 MC. Results are presented as the mean number of ASCs per 10^6 MC ± SEM.

9.2.12. Analysis of His- and S-tag-specific antibodies

To analyse the usefulness of rFaeG as mucosal carrier molecule, the presence of His- and S-tag-specific antibodies was determined in serum at 24 dppi using the His-S-SctW fusion protein (Geens et al., unpublished data) that was kindly provided by Tom Geens et al. (Ghent University). The His-S-SctW protein is encoded by pET-HTSctW that is obtained from ligation of the *Chlamydia psittaci* type III secretion protein SctW in the pET-30 Ek/LIC vector. The His-S-tag fragment was cleaved from SctW using

enterokinase (Sigma) according to the manufacturers instructions. Thereafter, the His-S-tag-fragment and SctW were separated using a 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane as described by Van den Broeck et al. (1999c). Following overnight blocking, the membranes were firstly incubated with undiluted serum samples, secondly with peroxidase-conjugated rabbit-anti-swine polyclonal antibodies (Dako, Denmark) and thirdly with a 3-amino-9-ethylcarbazole containing substrate solution to visualize binding of the conjugate in accordance to the procedure described by Van den Broeck et al. (1999c).

9.2.13. Statistical analysis

Statistical analysis (SPSS 10.0 for Windows) of serum antibody titers and $F4^+$ *E. coli* excretion (log-values) was done using General Linear Model (Repeated Measures Analysis of Variance), adjusting for multiple comparison by Bonferoni. $P < 0.05$ was considered as statistically significant.

9.3. Results

9.3.1. Expression of rFaeG

Since high yield expression of several recombinant proteins in the cytoplasm of *E. coli* are reported (Makrides, 1996), *faeG* from the pathogenic $F4^+$ ETEC strain GIS26 was cloned in the pET-30 Ek/LIC vector without its signal sequence to obtain expression in the cytoplasm of BL21(DE3). The cytoplasm allows extensive protein accumulation and the more reducing environment as compared to the periplasm was not expected to be harmful because FaeG does not contain disulfide bounds (Cornelis, 2000). rFaeG has a molecular weight of 32 kDa due to the presence of N-terminal His- and S-tag, whereas native FaeG is 27 kDa. As shown in Figure 1, rFaeG was found in the pellet fraction of induced BL21(DE3)[pET-FaeG/7] spheroplasts, and not in the supernatant containing the periplasmic proteins. Indeed, immunoblotting of proteins from the soluble and insoluble cytoplasmic fractions of induced BL21(DE3)[pET-FaeG/7] using the FaeG-specific MAb IMM01, indicated that rFaeG was expressed in the cytoplasm, but only in an insoluble form. Approximately 95 mg rFaeG was obtained following purification of the insoluble

cytoplasmic fraction of 10^{12} induced bacteria, with a purity of 90-94%. On the other hand, mechanical sharing of the same amount of GIS26 resulted in a protein mixture containing 1.7 mg F4 fimbriae with a purity of 75% (Van den Broeck et al., 1999c).

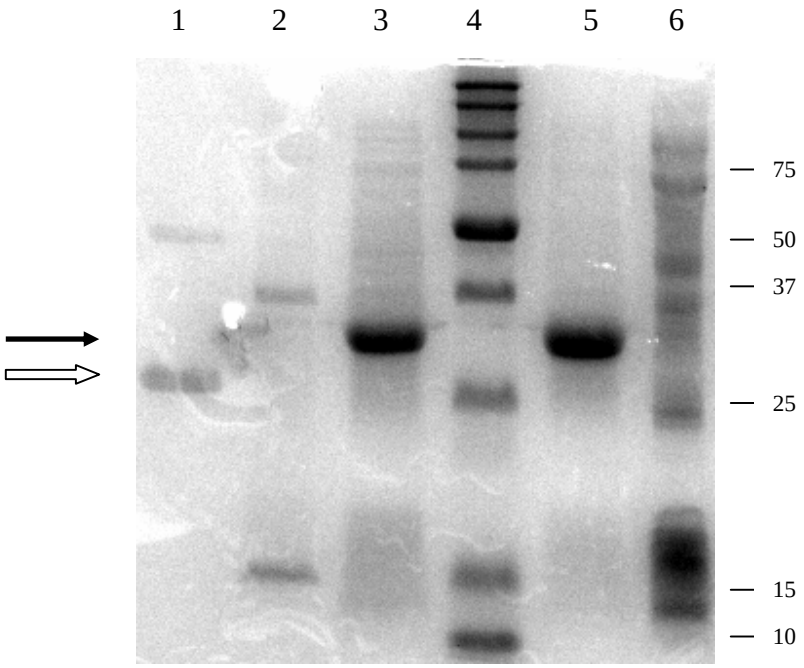


Figure 1 : Coomassie stained SDS-PAGE of isolated fractions of induced BL21(DE3)[pETFaeG/7] : heat denaturated purified F4 fimbriae of GIS26 (lane 1), supernatant of spheroplast preparation (lane 2), pellet of spheroplast preparation (lane 3), molecular weight marker (lane 4), insoluble cytoplasmic fraction (lane 5), soluble cytoplasmic fraction (lane 6). Native FaeG (white arrow), rFaeG (black arrow).

9.3.2. Optimalisation of rFaeG folding

Purification and folding of rFaeG were optimised using an ELISA based on the FaeG-specific MAb IMM01 (Chapter 8). The epitope recognized by IMM01 is not defined but adhesion of $F4^+$ ETEC to $F4R^+$ villi is inhibited by IMM01 (Y. Van der Stede, unpublished data). Figure 2 shows that serial dilutions of heat-denaturated and native fimbriae, resulted in non-parallel OD_{405}-lines. This must be due to a difference in affinity of F4 fimbriae and heat-denaturated F4 fimbriae for IMM01. Refolding of rFaeG using SDS resulted in a conformational structure that reacted similarly in this ELISA with IMM01 as native purified F4 fimbriae (parallel slopes**,** Fig. 2). However, to obtain

identical OD_{405} values, the protein concentration of rFaeG had to be 75 times higher than the F4 fimbriae concentration. This great difference between rFaeG and F4 fimbriae is probably an overestimation due to a higher signal for multimers than for monomers in the used ELISA. In contrast, refolding of rFaeG using urea did not result in OD_{405}-lines parallel to this of native F4 fimbriae and removal of the denaturating agent caused aggregation of the rFaeG. Also the use of guanidine hydrochloride (GuHCl), non-ionic detergents (Tween, Triton-X-100), sonication or altered pH (pH 2 till 10) was not successful in refolding rFaeG and is therefore not discussed further. Furthermore, the SDS-refolded rFaeG as well as purified F4 fimbriae bound the c-, a6- and a7-specific MAb's **(**CVI-F4ac-5, CVI-F4ac-6 and CVI-F4ad-3), whereas urea-refolded rFaeG did not. The fimbrial c epitope is reported to constitute at least partially the F4ac receptor-binding site (Bakker et al., 1992a).

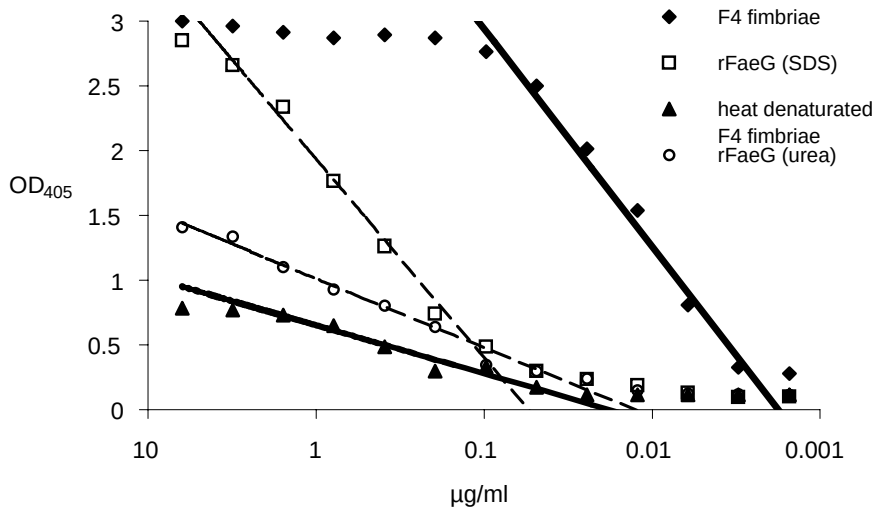


Figure 2 : OD_{405}-lines represent the interaction of the FaeG-specific monoclonal antibody IMM01 with purified F4, heat-denaturated purified F4 fimbriae, SDS- or urea-refolded rFaeG.

The ability of rFaeG monomers to bind the F4R was evaluated in a competitive villous adhesion assay. Intestinal villi of $F4R^+$ pigs were incubated with different concentrations of F4 fimbriae or rFaeG and with 4.10^8 $F4^+$ ETEC. A concentration dependent inhibition of $F4^+$ ETEC adhesion to the F4R for both SDS-refolded rFaeG and

F4 fimbriae was observed (Fig. 3). However, 94.7 ± 4.93 % inhibition was obtained at a concentration of 800 μg/ml rFaeG, whereas 200 μg/ml F4 fimbriae already resulted in 97.7 ± 3.21 % inhibition. In contrast, heat-denaturated F4 fimbriae and rFaeG refolded using urea, GuHCl, non-ionic detergents, sonication or altered pH were not inhibiting $F4^+$ ETEC binding to $F4R^+$ villi.

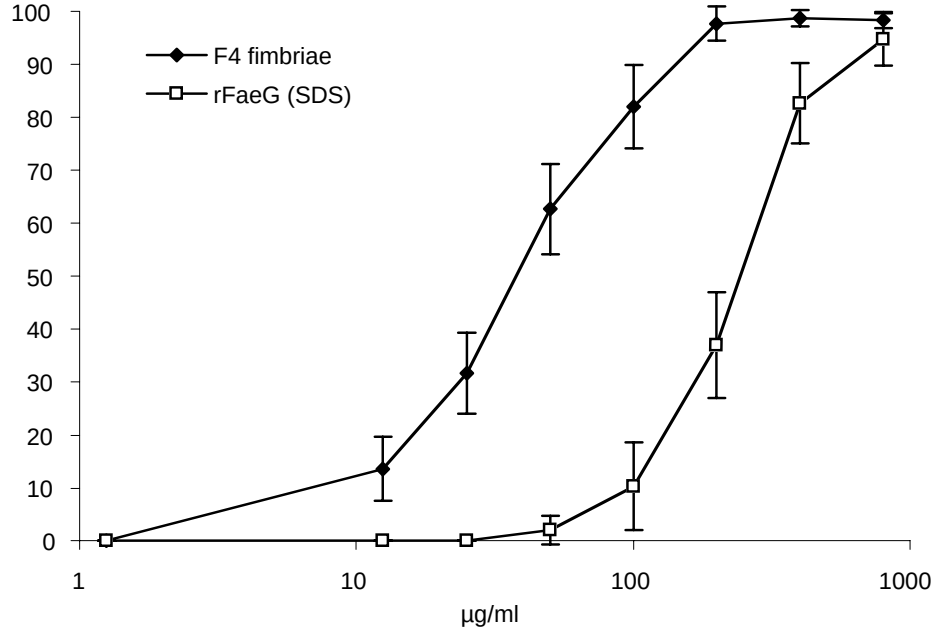


Figure 3 : Percentage inhibition of $F4^+$ ETEC adhesion to $F4R^+$ intestinal villi by competition with purified F4 fimbriae and SDS-refolded rFaeG.

Preincubation of SDS-refolded rFaeG with MAb CVI F4ac-5, decreased the inhibition of $F4^+$ ETEC adhesion from 37 % to 5.2 ± 4.1 %, indicating that the inhibition of adhesion is due to binding of rFaeG to the F4R. Indeed, pre-incubation with an irrelevant MAb had no effect (32 % adhesion).

9.3.3. rFaeG monomers

As expected, rFaeG was only found as monomers in Western blot using the IMM01 MAb. Conversely, a ladder pattern consisting of FaeG multimers was observed when F4 fimbriae were not heat-denaturated (Fig. 4). The polymeric FaeG structure of F4 fimbriae disintegrated into its FaeG monomers following heat-denaturation in the presence of SDS.

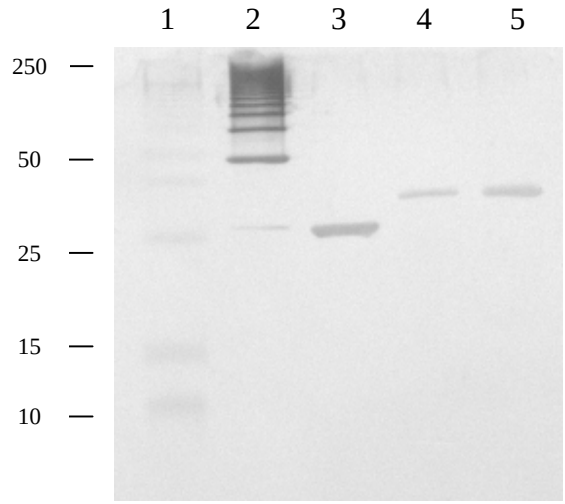


Figure 4 : Western blot of purified F4 fimbriae and SDS-refolded rFaeG using the FaeG-specific monoclonal antibody IMM01 : molecular weight marker (lane 1), purified F4 fimbriae (lane 2), heat-denaturated purified F4 fimbriae (lane 3), rFaeG (lane 4), heat-denaturated rFaeG (lane 5).

9.3.4. Oral immunization with rFaeG and challenge infection

The capacity of rFaeG to induce an FaeG-specific mucosal immune response upon oral administration to newly-weaned piglets was determined. Based on the *in vitro* inhibition assays wherein four times more SDS-refolded rFaeG than F4 fimbriae was needed to inhibit the adhesion of $F4^+$ ETEC to the F4R, a four times higher dose of rFaeG (8 mg) was used than F4 fimbriae (2 mg) to immunize the animals.

From 7 and 16 dppi onwards, FaeG-specific IgM, IgG and IgA antibodies were detected in the F4 group and the rFaeG (SDS) group respectively, whereas no antibodies were observed in the PBS group (Fig. 5). The F4 immunization induced the highest response with all titers significantly higher ($p \leq 0.017$) 16 and 24 dppi as compared to the

response with all titers significantly higher ($p \leq 0.017$) 16 and 24 dppi as compared to the PBS group and the IgM titer at 16 dpi ($p=0.002$) as well as the IgA titer at 16 ($p=0.012$) and 24 dppi ($p=0.002$) significantly higher than for the rFaeG (SDS) group. The SDS-refolded rFaeG immunization induced a weak antibody response and was only significantly higher ($p=0.017$) for IgM at 24 dppi in comparison with the PBS group, whereas urea-rFaeG immunization induced no FaeG-specific antibodies.

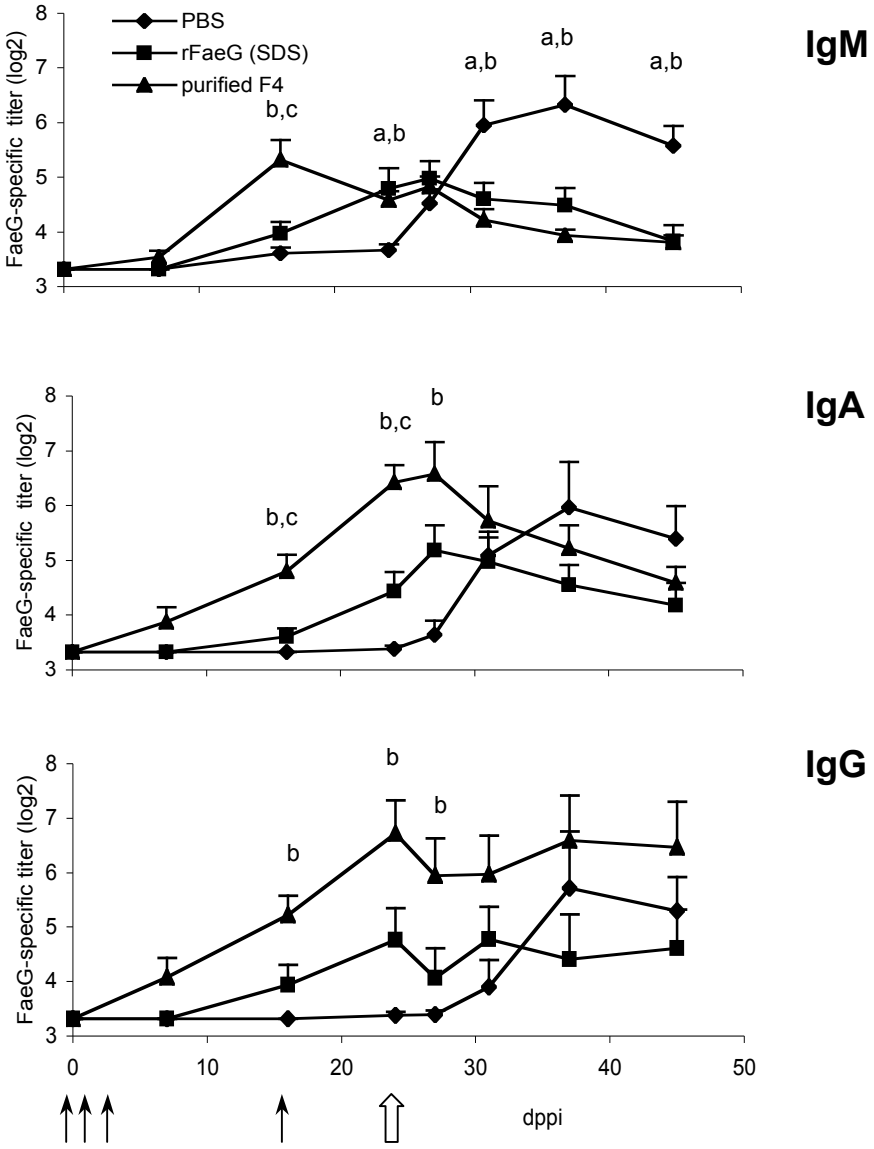


Figure 5 : Mean FaeG-specific IgM, IgA and IgG serum antibody titers (± SEM) of weaned pigs immunized with PBS, SDS-refolded rFaeG or purified F4 fimbriae at 0, 7, 16, 24, 27, 31, 37 and 45 days post primary immunization (dppi). Significant difference ($p<0.05$) between PBS and rFaeG (a), between PBS and F4 group (b) and between rFaeG and F4 (c). Black arrow, immunization; white arrow, $F4^+$ ETEC challenge.

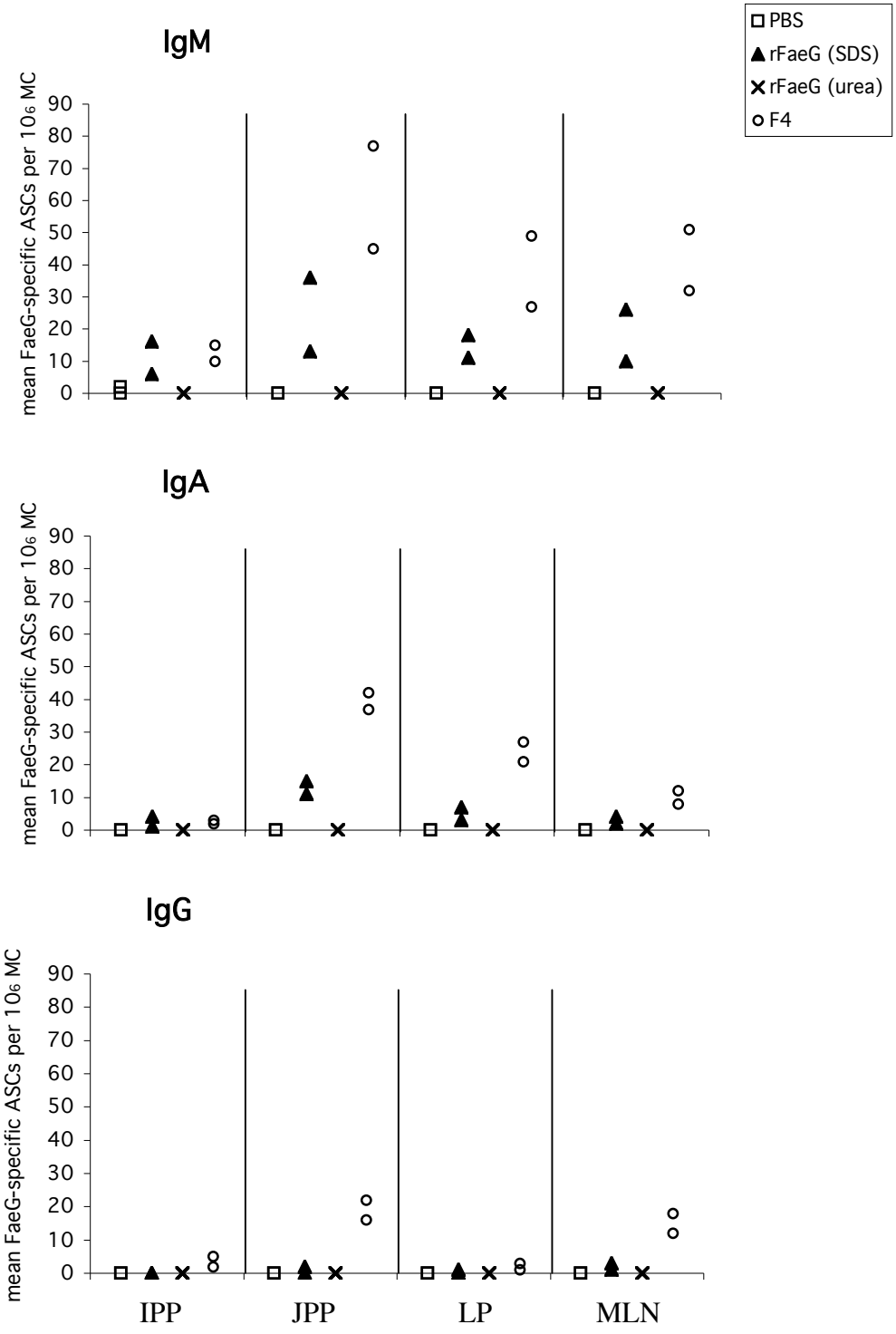


Figure 6 : Mean FaeG-specific IgM, IgA and IgG ASCs per 10^6 MC in ileal Peyer's patches (IPP), jejunal Peyer's patches (JPP), lamina propria (LP) and mesenteric lymph nodes (MLN) of weaned pigs immunized with PBS (n=2), SDS-refolded rFaeG (n=2), urea refolded rFaeG (n=3) or purified F4 fimbriae (n=2) at 23 dppi.

One day before the challenge infection, the mucosal FaeG-specific immune response was analysed by determining the number of FaeG-specific IgA, IgG and IgM ASCs in MLN, JPP, IPP and LP and the presence of FaeG-specific IgA and IgM in intestinal contents of three piglets of the rFaeG (urea) group and two piglets of the F4, PBS and rFaeG (SDS) groups. As shown in Figure 6, IgA and IgM FaeG-specific ASCs were detected in all four tissues of piglets from the F4 and the rFaeG (SDS) groups, whereas a background signal of two FaeG-specific IgM ASCs per 10^6 MC was observed in the IPP of one piglet from the PBS group. However, the numbers of IgA and IgM FaeG-specific ASCs found in JPP, LP and MLN of the F4 group were higher (twice or more) than in the rFaeG group whereas IPP showed similar low numbers for both groups. On the other hand, FaeG-specific IgG ASCs were only clearly detected in JPP (22 and 16 per 10^6 MC) and MLN (18 and 12 per 10^6 MC) of the F4 immunized piglets. No FaeG-specific ASCs were found in the rFaeG (urea) group.

In agreement with these results, FaeG-specific IgA and IgM were observed in small intestinal contents of rFaeG (SDS) as well as of F4 immunized piglets, with the highest titers in the F4 group (Table 1). These results indicate that oral immunization of pigs with rFaeG in an F4R-binding conformation induces an FaeG-specific mucosal antibody response, which is weaker than the immunization with F4 fimbriae.

Table 1: FaeG-specific IgM and IgA titers in contents of the duodenum, jejunum, and ileum of weaned pigs immunized with PBS (n=2), SDS-refolded rFaeG (n=2), urea-refolded rFaeG (n=3) or purified F4 fimbriae (n=2) at 23 dppi.

	IgM			IgA		
	duodenum	jejunum	ileum	duodenum	jejunum	ileum
PBS	<2	<2	ND	<2	<2	ND
	ND	<2	<2	ND	2	<2
rFaeG (SDS)	2	32	<2	<2	32	<2
	ND	16	32	ND	16	16
rFaeG (urea)	<2	<2	<2	<2	<2	<2
	<2	<2	<2	<2	<2	<2
	ND	<2	<2	ND	<2	<2
F4	128	256	<2	64	128	64
	ND	128	<2	ND	512	128

ND = not determined

To see if this response provided protection against an F4$^+$ ETEC infection, piglets of the PBS, F4 and rFaeG (SDS) groups were challenged 24 dppi with the F4$^+$ ETEC strain GIS26 and excretion of F4$^+$ *E. coli* was daily analysed from 1 till 7 days post challenge (dpc) (Fig. 7). In the rFaeG (SDS) group, F4$^+$ *E. coli* excretion was detected till 3 dpc, whereas excretion in the PBS control group lasted till 6 dpc and was significantly higher ($p \leq 0.003$) as compared to the rFaeG (SDS) group from 2 till 5 dpc. However, the F4$^+$ ETEC excretion in the rFaeG (SDS) group was significantly higher ($p<0.001$) than in the F4 group 1 and 2 dpc. Indeed, immunization of weaned piglets with purified F4 resulted in protection against infection as only a low amount of F4$^+$ *E. coli* (1.34 log10 F4$^+$ *E. coli* per g faeces) was excreted 1 dpc.

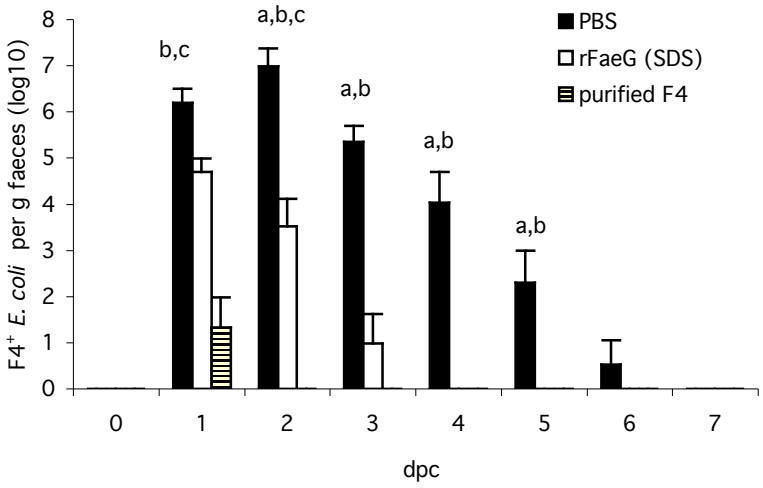


Figure 7 : Mean faecal F4$^+$ *E. coli* (log 10) per gram faeces (± SEM) following F4$^+$ ETEC challenge of weaned pigs immunized with PBS (n=8), SDS-refolded rFaeG (n=7) or purified F4 fimbriae (n=8) at 0 to 7 days post challenge (dpc). Significant difference ($p<0.05$) between PBS and rFaeG (a), between PBS and F4 group (b) and between rFaeG and F4 (c).

The protection in the F4 group and the absence of protection in the PBS group were reflected in their serum antibody responses. Upon challenge, the PBS group (Fig. 5) showed a primary systemic FaeG-specific response with relatively high IgM titers peaking 14 dpc, at the same time as the IgG and IgA titers (5.72 and 5.97, respectively). In contrast, the F4 group did not show an antibody boost response after challenge : IgM and IgA decreased and IgG did not change much. In the rFaeG (SDS) group, the boost response upon challenge was also missing, as observed in the F4 group. Thus, the

infection in the F4 and the rFaeG (SDS) group was not able to induce a secondary response, confirming the lack of colonization and multiplication by $F4^+$ *E. coli*.

9.3.5. The use of rFaeG as mucosal carrier

To explore the usefulness of SDS-refolded rFaeG as mucosal carrier molecule, the presence of His-S-tag-specific antibodies was analysed in serum of two animals per group at 24 dppi. As shown in Figure 8, serum of SDS-refolded rFaeG immunized piglets bound to the His-S-SctW fusion protein but not to the SctW. Serum samples of F4 immunized piglets and PBS control piglets did not react with His-S-SctW or SctW, whereas His-S-SctW-specific control serum bound to both proteins. Therefore, we suggest that SDS-refolded rFaeG can be used as a mucosal carrier molecule to induce an immune response against a fused heterologous antigen following oral immunization with the fusion protein.

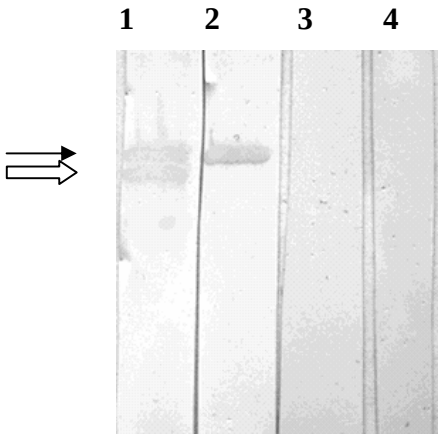


Figure 8 : Western blot of enterokinase digest from His-S-SctW using His-S-SctW-specific control serum (lane 1), serum of pig immunized with SDS-refolded rFaeG (lane 2) or purified F4 fimbriae (lane 3) or serum of a PBS control pig (lane 4). Black arrow : His-S-SctW protein (47.4 kDa); White arrow : SctW protein (43 kDa).

9.4. Discussion

The importance of adhesins in the pathogenicity of several bacteria resulted in studies on their usefulness in vaccines (Langermann et al., 1997 and 2000; Thankavel et al., 1997; Barnhart et al., 2000). Recently, oral immunization with recombinant pneumococcal surface adhesin A (rPSA) of *Streptococcus pneumoniae* induced systemic and mucosal rSPA-specific antibody responses, suggesting the utility of recombinant adhesins for mucosal immunizations (Seo et al., 2002). The present study reports the induction of a fimbriae-specific as well as a heterologous-antigen specific immune response following oral immunization of piglets with the SDS-refolded rFaeG fimbrial adhesin containing an N-terminal His- and S-tag peptide.

The adhesin FaeG of the pathogenic $F4^+$ ETEC strain GIS26 was cloned and expressed in the cytoplasm of BL21(DE3). Purification of cytoplasmic rFaeG inclusion bodies in denaturating conditions resulted mostly in aggregation of rFaeG during refolding or removal of the denaturating agents. Similarly, high level expression of the type 1 fimbrial adhesin FimH and the P-pili adhesin PapG, resulted in inclusion bodies in the cytoplasm and in aggregation when the denaturant urea was removed by dialysis (Kariyawasam et al., 2002). X-ray diffractions of type 1- and P-pili subunits revealed an immunoglobulin-like domain, but lacking the C-terminal β-strand. However, the groove along the surface of these subunits is filled by a β-strand of the chaperone in the periplasm or by a β-strand of a subunit in the fimbriae (Choudhury et al., 1999; Sauer et al., 1999). The conformation of the F4 fimbrial adhesin FaeG and the chaperone FaeE are not known at the moment, but sequence alignment with other fimbrial subunits and chaperones suggest a similar interaction. Therefore, a hydrophobic core of rFaeG could have caused the aggregation during refolding.

The capacity of rFaeG to successfully assemble in a native-like receptor binding conformation in the presence of SDS was somewhat surprising since SDS is a known denaturating agent. However, FaeG and FimH subunits eluted from a SDS-polyacrylamide gel were reported to bind the F4R and mannose respectively (Van den Broeck et al., 1999c; Tewari et al., 1993). In addition, several recombinant proteins with a high hydrophobic character or a high tendency to aggregate were also successfully

refolded in the presence of SDS (Juri et al., 2001; Niebla et al., 2001; Ohnishi and Kameyama, 2001; Tsetlin et al., 2002). Perhaps, the masking of hydrophobic protein interfaces by detergent molecules may explain the reduced aggregation (Schrooyen et al., 2001).

The SDS-refolded rFaeG appeared a good candidate vaccine against $F4^+$ ETEC infections since it was able to bind to the F4R and to induce in rabbit antibodies, which could inhibit $F4^+$ *E. coli* adhesion (data not shown). The conformation of the refolded rFaeG is important to bind the F4R and to induce a FaeG-specific immune response since FaeG-specific ASCs were detected in intestinal lymphoid tissues of piglets immunized with SDS-refolded rFaeG, but not with urea-refolded rFaeG. This result confirms the need for receptor-binding to induce an FaeG-specific immune response following oral immunization (Van den Broeck et al., 1999c).

Piglets immunized with SDS-refolded rFaeG showed a FaeG-specific systemic and mucosal antibody response that significantly reduced the $F4^+$ *E. coli* excretion from 2 till 5 days following challenge infection. However, rFaeG immunization did not prevent infection as the F4 immunization did. Indeed, F4 immunized pigs excreted only few $F4^+$ *E. coli* on 1 dpc, probably some remainder of the orally inoculated $F4^+$ *E. coli* and showed higher serum and mucosal antibody responses than the rFaeG immunized pigs. It is important to note that it did not matter whether rFaeG or purified F4 fimbriae was used in the ELISA, as similar titers were obtained.

The observed difference in the FaeG-specific immune response following oral immunization with F4 fimbriae versus SDS-refolded rFaeG can be multifactorial. Firstly, N-terminal fusion of the His- and S-tag to rFaeG is probably not favourable to form rFaeG polymers since the N-terminal strand of Fim and Pap subunits is reported to contribute in subunit-subunit interactions (Sauer et al., 1999, Choudhury et al., 1999). Indeed, rFaeG was shown to appear as monomers whereas purified F4 fimbriae have polymeric structures mainly composed of FaeG subunits (Bakker et al., 1992a). At the moment, differences in the interaction of monomeric versus polymeric *E. coli* adhesins with their receptor were not analysed and a number of questions remain. However, in the case of *Porphyromonas gingivalis*, the inhibitory effect of recombinant major subunit monomers on binding of the bacteria to their receptor is higher than that of the purified

native fimbriae on a molar basis (Sharma et al., 1993). On the other hand, it is well known that multimeric structures are more immunogenic as compared to monomers (Jackson et al., 1997).

Secondly, the stability of SDS-refolded rFaeG is probably low since storage is best at 4°C and activity was found to decrease over time (>50% reduction after 72 h). Similarly, the lectin and pilin domain of FimH can both fold chaperone independently, but the thermodynamic stability of the pilin domain is very low and leads to unfolding of the pilin domain even in the absence of denaturant (Vetsch et al., 2002). However, the piglets were treated with rabeprazolum to neutralize the gastric pH at the moment of immunization, in order to prevent effects resulting from differences in resistance to low pH and proteolytic degradation between rFaeG and purified F4.

Fimbriae have been used to display heterologous peptides because of their strong immunogenicity, strong adhesive properties and ease of purification (Klemm and Schembri, 2000). Unfortunately, the inserted peptides had to be short and the constructed F4 fimbriae lost their receptor-binding epitope (Thiry et al., 1989; Bakker et al., 1990). In contrast, the results of the present study indicate that a N-terminal fusion with a His- and S-tag containing 4.8 kDa peptide sequence to FaeG was not detrimental for its binding to the F4R. Moreover, the presence of antibodies against this His-S-tag peptide suggest that rFaeG can be used as mucosal carrier to induce an immune response against N-terminal fused peptides. In agreement, the major subunit ClpG of the F4-related CS31A fimbriae was coupled to peptides containing all or part of the *E. coli* human heat-stable enterotoxin (STh), by fusing the STh-encoding DNA sequence to the 5’ or 3’ extremity of the *clpG* gene. These ClpG-STh subunits were exported efficiently but none formed hybrid CS31A-STh fimbriae at the cell surface of *E. coli* (Batisson et al., 2000a and 2000b). However, the potential of rFaeG or other fimbrial adhesins to function as a mucosal carrier to antigens remains to be confirmed.

In conclusion, rFaeG can be produced in a correct bioactive form which is, by oral immunization, able to partly protect piglets against $F4^+$ ETEC infection and functions as mucosal carrier to an N-terminal fused peptide. This opens new perspectives to simultaneously immunize pigs against $F4^+$ ETEC infections and other intestinal pathogens. However, improvement of the stability and immunogenicity of rFaeG must be

considered since immunization with purified F4 fimbriae resulted in a more robust FaeG-specific response.

Chapter 10

Mucosal carrier potential of recombinant F4 fimbrial adhesin FaeG is significantly improved following oral co-administration with cholera toxin in pigs[1]

[1] *Based on :Verdonck F, Snoeck V, Goddeeris BM, Cox E. Mucosal carrier potential of recombinant F4 fimbrial adhesin FaeG is significantly improved following oral co-administration with cholera toxin in pigs. Submitted.*

Abstract

Oral immunization of both humans and animals with non-replicating soluble antigens often results in the induction of oral tolerance. However, receptor-dependent uptake of orally administered soluble antigens can lead to the induction of an antigen-specific immune response. Indeed, oral immunization of pigs with recombinant FaeG (rFaeG), the adhesin of the F4(K88) fimbriae of enterotoxigenic E. coli, induces an F-specific humoral and cellular immune response. This response is accompagnied with a reduction in the excretion of $F4^+$ enterotoxigenic *Escherichia coli* (ETEC) following challenge. In addition, rFaeG has the potential to function as a mucosal carrier since oral immunization of pigs with rFaeG induces antibodies against an N-terminally fused His- and S-tag. To improve the immune response against FaeG and the carried peptide, rFaeG was orally co-administered with the mucosal adjuvant cholera toxin (CT). Oral immunization of pigs with rFaeG and CT significantly enhanced the immune response against the peptide since significantly higher His-S-tag-specific antibodies were detected. In addition, the co-administration significantly improved the F4-specific humoral and cellular immune response and significantly reduced the fecal $F4^+$ *E. coli* excretion following challenge infection as compared to rFaeG-immunized pigs. In conclusion, the results of the present study suggest that fimbrial adhesins can be used as a mucosal carrier for inducing an immune response against antigens, which normally are not immunogenic.

10.1. Introduction

The induction of an antigen-specific mucosal antibody response is needed to protect both humans and animals against an intestinal infection (Porter et al., 1974; Bloom and Boedeker, 1996). However, oral administration of most non-replicating antigens (e.g. food antigens) results in oral tolerance (Strobel and Mowat, 1998). One of the exceptions is the oral immunization of F4-receptor positive ($F4R^+$) pigs with F4 fimbriae, which induces a protective FaeG-specific intestinal antibody response (Van den Broeck et al., 1999b). In $F4R^-$ pigs, F4 fimbriae act as a normal food antigen (Van den Broeck et al., 2002). We recently demonstrated that this unique F4R-based uptake of antigen can be used to induce an antibody response against a heterologous peptide that is fused to the N-terminus of the F4 fimbrial adhesin FaeG (Chapter 9). On the

other hand, the FaeG-specific response was weaker following oral immunization with rFaeG than with purified F4, resulting in a decreased protection against an $F4^+$ ETEC challenge.

The interest of the present study was to improve the FaeG- and heterologous-specific immune response following co-administration of rFaeG with the mucosal adjuvant cholera toxin (CT) (Ogra et al., 2001), since this rFaeG carrier system could open new perspectives to simultaneously induce a mucosal immune response against FaeG and against antigens of other enteropathogens. In mice, CT enhances co-stimulation (Cong et al., 1997) and promotes Th2 cytokine responses with induction of antigen-specific serum IgG and mucosal IgA (Marinaro et al., 1995). In pigs, the oral administration of CT is non-toxic at an oral dose of 100 μg and is reported to enhance the induction of an antigen-specific immune response to co-administered antigens that are targeted to the gut-associated lymphoid tissue by coupling to CT-B (Foss and Murtaugh, 1999a). However, there is no information whether oral co-administration of an antigen and CT, without conjugation of the antigen to CT or its B-subunit, improves the antigen-specific immune response in pigs.

In the present study, it was determined if the use of CT could improve the induction of an immune response against the FaeG carrier as well as against the N-terminal fused His-S-tag peptide following oral co-administration of newly weaned pigs with rFaeG.

10.2. Material and methods

10.2.1. Bacterial inoculum

The ETEC strain GIS26 (serotype O149:K91, $F4ac^+$, $LT^+STa^+STb^+$) was cultured during 18 h in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) at 37°C and 85 rpm. The bacteria were collected by centrifugation and washed with phosphate-buffered saline (PBS) (150mM, pH 7.4). The concentration of the bacteria was determined by measuring the optical density of 10-fold dilutions of the bacterial suspension at 660nm (OD_{660}). An OD_{660} of 1 equals 10^9 viable bacteria/ml, as determined by counting colony-forming units.

10.2.2. Purification of F4 fimbriae

F4 fimbriae were purified as described by Van den Broeck et al. (1999c). These fimbriae were used in the FaeG-specific antibody ELISA. Isolated F4 fimbriae were also further purified by anion exchange chromatography (AEC) using a Bio-Scale Q5 column (BIORAD, Eke, Belgium) (indicated as AEC purified F4), subsequently sterilised by filtration through an 0.2 μm filter and used to induce F4-specific proliferation. The percent purity of the isolated F4 and the AEC purified F4 fimbriae was assessed using a Coomassie stained 15% SDS-PAGE and the ImageMaster 1D prime software (Amersham Pharmacia Biotech, Belgium). The F4 fimbrial protein concentration was calculated from the total protein concentration as determined using the bicinchoninic acid (BCA) reaction with bovine serum albumin (BSA) as standard (ICN Biomedicals, Belgium) and taking into account the percentage of F4 fimbriae on the total protein concentration.

10.2.3. rFaeG expression and refolding

Recombinant FaeG (rFaeG) containing an N-terminal fused His- and S-tag was expressed and refolded as described in chapter 9. Briefly, BL21(DE3)[pETFaeG/7] *E. coli* were grown overnight in Luria broth (LB; Life Technologies, Paisley, Scotland) with 30 μg/ml kanamycin at 28°C. Afterwards, the bacteria were diluted 100 times in fresh LB supplemented with kanamycin and incubated at 28°C, 200 rpm to an OD_{660} of 0.2 – 0.3. rFaeG expression was subsequently induced by adding 1mM isopropyl-β-D-thiogalactoside (IPTG, Sigma) to the cultures, which were further incubated during 4 hours at 37°C and 200 rpm. Subsequently, the insoluble cytoplasmatic fraction was isolated and resuspended in PBS + 0.5 % SDS**.** Following overnight dialysis against PBS at 4°C, the concentration of rFaeG was determined using the BCA-reaction and used to immunize animals.

10.2.4. Experimental procedure

Twenty-one, $F4R^+$ and FaeG-seronegative, conventionally bred pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks, transported to the experimental facilities at the faculty and subsequently housed in isolation units were they obtained water and food ad libitum. These pigs were treated orally with colistine (Promycine pulvis, VMD, Berendonk, Belgium, 150,000 U/kg of body weight/day)

from 2 days before till 3 days after weaning to prevent *E. coli* infections due to transport and handling.

One week post weaning, all pigs were orally given 20 mg rabeprazolum (Pariet, Janssen-Cilag, Berchem, Belgium) on 3 subsequent days to block the gastric HCl production. They also received rabeprazolum 15 days post primary immunization (dppi). Twenty-four hours following each rabeprazolum ingestion (0, 1, 2 and 16 dppi), the pigs were orally immunized with 50 µg CT (CT group, n=7), 4 mg rFaeG (rFaeG group, n=7) or 4 mg rFaeG + 50 µg CT (rFaeG+CT group, n=7) in 10 ml PBS. Each animal was deprived of food and water from three hours before till 2 h after gastric pH neutralization or immunization. One week following the booster immunization (23 dppi), the F4-specific proliferation of peripheral blood monomorphonuclear cells (PBMC) was determined.

At 24 dppi, the animals were orally challenged with the virulent $F4^+$ ETEC strain GIS26 as previously described (Cox et al., 1991) with minor modifications. Briefly, pigs were orally pre-treated at 21 and 22 dppi with 300 mg florfenicol (Nuflor, Schering-Plough, Brussels, Belgium) to decrease colonization resistance. Pigs were sedated with Stressnil (40 mg/ml ; Janssen-Cilag, Berchem, Belgium), after which the gastric pH was neutralized by intragastrical administration of 62 ml $NaHCO_3$ (1.4% (w/v) in destilled water). Fifteen to thirty minutes later, 10^{10} $F4^+$ ETEC (GIS26) in 10 ml PBS was given intragastrically. Faecal samples were taken daily to determine the excretion of $F4^+$ *E. coli* from challenge till 8 days post challenge (dpc, 31 dppi). Furthermore, FaeG-specific IgA, IgG and IgM and CT-specific (total) antibodies were determined in serum at 0, 7, 16, 24, 28, 31, 38 and 49 dppi, whereas FaeG-specific IgA was analysed in saliva at 0, 16 and 24 dppi. His-S-tag specific (total) serum antibodies were determined in serum at 0 and 24 dppi. Three weeks following challenge (49 dppi), the remaining pigs were euthanatised and jejunal villi were isolated to confirm the presence of the F4R.

All pigs were weighed at 0, 3, 7, 15, 24, 31 and 49 dppi. The daily weight gain of each pig was calculated at 3, 7, 15, 24, 31 and 49 dppi by substracting the weight of two subsequent measurements, and dividing the difference by the number of days between both measurements. Subsequently, the average daily weight gain (ADWG) per group was calculated ± standard error of the mean (SEM).

10.2.5. Samples

To determine antigen-specific serum and mucosal antibodies, serum and saliva were sampled as described by Van den Broeck et al. (1999a) and Van der Stede et al. (2002a), respectively.

$F4^+$ *E. coli* were enumerated in faecal samples by dot blotting using the FaeG-specific MAb IMM01 as previously described (Van den Broeck et al., 1999b). The resulting brown-red dots were counted and the average within each group was calculated. Results are presented as the mean number ± SEM of excreted $F4^+$ *E. coli* per gram faeces.

At the end of the experiment, jejunal villi were isolated of all euthanatised pigs to confirm the presence of the F4R as described by Van den Broeck et al. (1999c). Adhesion of more than five $F4^+$ *E. coli* per 250 μm villous length was noted as positive (Cox and Houvenaghel, 1993).

10.2.6. ELISA for FaeG-, CT- and His-S-tag-specific antibodies

For detection of FaeG-specific antibodies, the indirect ELISA described by Van den Broeck et al. (1999a) was used. An identical ELISA which only differed in the coating step and conjugate was used to determine CT-specific antibodies : the wells of microtiter plates (NUNC, Polysorp Immuno Plates, Roskilde, Denmark**)** were coated with 5 μg/ml CT (Sigma) in PBS and an optimal dilution of horseradish peroxidase conjugated rabbit polyclonal antibodies (Dako, Denmark) was used as conjugate. The FaeG- and CT-specific cut-off values were calculated as the mean OD_{405}-value of all sera (dilution 1/10) or saliva (dilution 1/2) samples at day 0, increased with 3 times the standard deviation (cut-off values of the FaeG-specific serum IgM, IgA, IgG and mucosal IgA and CT-specific ELISA were 0.32, 0.17, 0.31, 0.28 and 0.21 respectively). The antibody titer was the inverse of the highest dilution that still had an OD_{405} higher than the calculated cut-off value.

To analyse the capacity of rFaeG to function as mucosal carrier molecule, the presence of His-S-tag-specific antibodies was determined in serum at 24 dppi using the His-S-SctW fusion protein (Geens et al., unpublished data) that was kindly provided by Tom Geens et al. The His-S-SctW protein is encoded by pET-HTSctW that is obtained from ligation of the *Chlamydia psittaci* type III secretion protein SctW in the pET-30 Ek/LIC vector. His-S-SctW was purified with Ni-NTA agarose beads

according to the manufacturers instructions (Qiagen, Madison, US) and shown to be 91 % pure following SDS-PAGE and Coomassie staining. The purified His-S-SctW protein was dialysed overnight at 4°C against PBS and used to coat the wells of microtiter plates (NUNC, Polysorp Immuno Plates) at a concentration of 20 μg/ml in PBS. Incubation times, blocking condition and washing procedures of this ELISA were identical as described by Van den Broeck et al. (1999a). The serum samples were added in series of twofold dilutions in ELISA dilution buffer (PBS + 0.2% Tween®20 + 3% BSA), starting from the dilution 1/4. Then, an optimal dilution of horseradish peroxidase conjugated rabbit polyclonal antibodies (Dako) was used as conjugate. Finally, ABTS and H_2O_2 were used as chromogen and substrate and the OD_{405} was spectrophotometrically determined. The His-S-tag-specific cut-off value (0.42) was calculated as the mean OD_{405}-value of all sera (dilution 1/4) at day 0, increased with 3 times the standard deviation.

10.2.7. F4-specific proliferation

Blood was collected from the jugular vein and PBMC were isolated as described by Van den Broeck et al. (1999a). The PBMC were diluted to a concentration of 5.10^6 cells/ml in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), kanamycin (100 μg/ml), L-glutamin (200 mM), sodiumpyruvate (100 mM), non-essential amino acids (100 mM), β-mercaptoethanol (5.10^{-5} M) and 5% (vol/vol) FaeG-seronegative serum. Subsequently, the isolated PBMC were incubated in medium (negative control) or medium with purified F4 (final concentration of 10 μg/ml), or concanavalin A (final concentration of 10 μg/ml, positive control) to determine their F4-specific proliferation as described by Van der Stede et al. (2003). The results are presented using the F4-specific stimulation index (SI), obtained by dividing the mean counts per minute (cpm) of the F4-stimulated cultures by the mean cpm of the non-stimulated cultures. Mean background levels of about 700 cpm were obtained in medium samples, whereas positive controls of concanavalin A reached 170,000 cpm.

10.2.8. Statistical analysis

Statistical analysis (SPSS 10.0 for Windows) of antibody titers and $F4^+$ *E. coli* excretion (log-values) was done using General Linear Model (Repeated Measures

Analysis of Variance). Differences between groups in F4-specific cell proliferation and ADWG were analysed for statistical significance using the One-way Anova. $P < 0.05$ was considered as statistically significant.

10.3. Results

10.3.1. FaeG-specific serum antibody response following oral immunization

Oral immunizations of newly weaned pigs with rFaeG (rFaeG group) induced FaeG-specific serum IgM in all animals, resulting in a significantly higher titer 7 dppi as compared to the background value in animals immunized with CT (CT group; P=0.002) (Fig. 1). However, oral immunization of pigs with rFaeG in the presence of CT (rFaeG+CT group) resulted in significantly higher FaeG-specific IgM serum titers than in the rFaeG (16 dppi; P=0.004) groups. The oral booster immunization 16 dppi induced low FaeG-specific serum IgA and IgG titers in the rFaeG group, whereas the FaeG-specific IgM titer was significantly higher (P=0.011) 24 dppi as compared to the background value in the animals immunized with CT alone. The highest FaeG-specific serum antibody titers following booster immunization were obtained in the rFaeG+CT group, with significantly higher titers than in the rFaeG group (IgG; P=0.038) and the CT group (IgM, IgA, IgG; $P \leq 0.024$) 24 dppi.

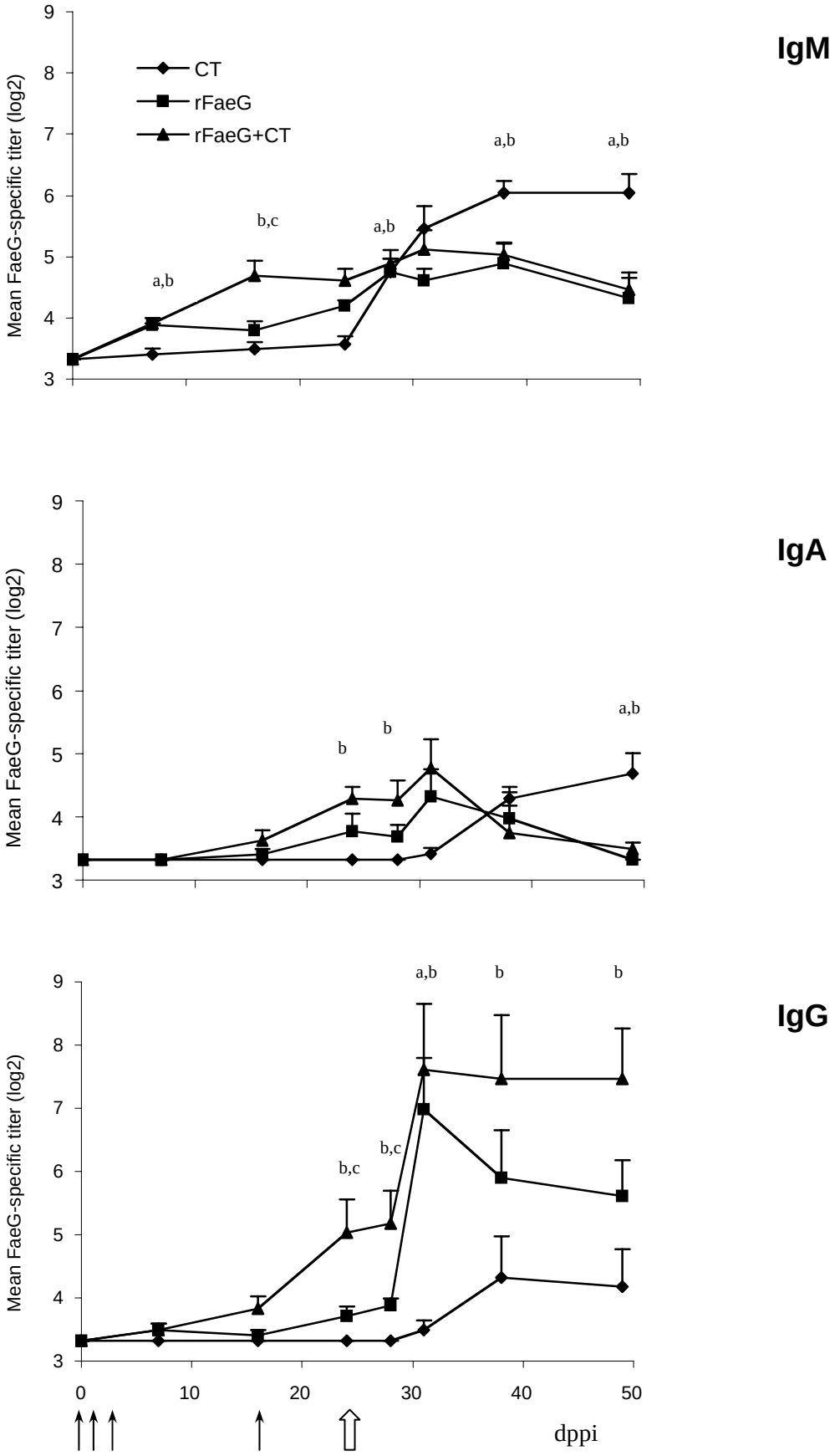


Figure 1: Mean FaeG-specific IgM, IgA and IgG serum antibody titers (± SEM) at 0, 7, 16, 24, 28, 31, 38 and 49 days post primary immunization (dppi) of pigs orally immunized with cholera toxin (CT, n=7), rFaeG (n=7) or rFaeG and cholera toxin (rFaeG+CT, n=7). Significant difference ($P < 0.05$) between CT and rFaeG (a), between CT and rFaeG+CT (b), between rFaeG and rFaeG+CT (c). Black arrow : immunization; white arrow : challenge.

10.3.2. His-S-tag-specific serum antibody response following oral immunization

Besides inducing FaeG-specific serum antibodies following oral rFaeG immunization, rFaeG functioned as a mucosal carrier for the N-terminal fused His-S-tag. Indeed, one week following oral booster immunization (24 dppi), His-S-tag-specific antibodies were detected in the rFaeG group but not in the CT group (Fig. 2). However, oral co-administration of rFaeG and CT resulted in significantly higher His-S-tag-specific serum titers than in the CT and the rFaeG groups (P=0.003 and P=0.047 respectively).

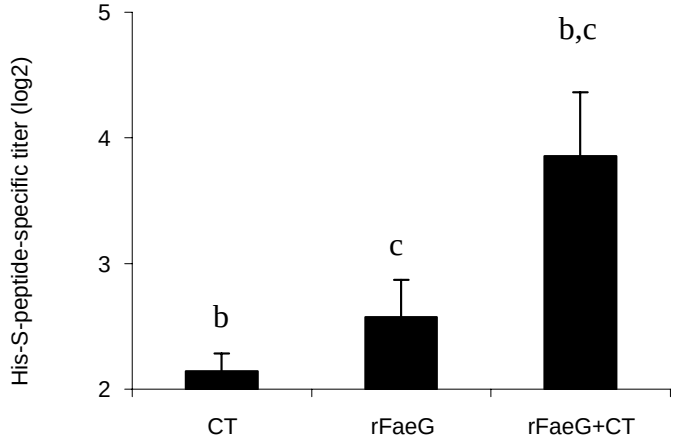


Figure 2 : Mean His-S-specific serum antibody titers (± SEM) at 24 days post primary immunization (dppi) of pigs orally immunized with cholera toxin (CT, n=7), rFaeG (n=7) or rFaeG and cholera toxin (rFaeG+CT, n=7). Significant difference ($P < 0.05$) between CT and rFaeG (a), between CT and rFaeG+CT (b), between rFaeG and rFaeG+CT (c).

10.3.3. CT-specific serum antibody response following oral immunization

The use of CT in the oral immunizations induced a CT-specific systemic immune response, whereas no CT-specific serum antibodies were detected in the non-CT immunized rFaeG group (Fig. 3). One week following booster immunization (24 dppi), the CT-specific antibody titer of both CT immunized groups was significantly higher as compared to the rFaeG group ($P \leq 0.016$). On the other hand, the dose of CT used, did not result in diarrhoea (data not shown) or growth retardation (Fig. 4) of the pigs.

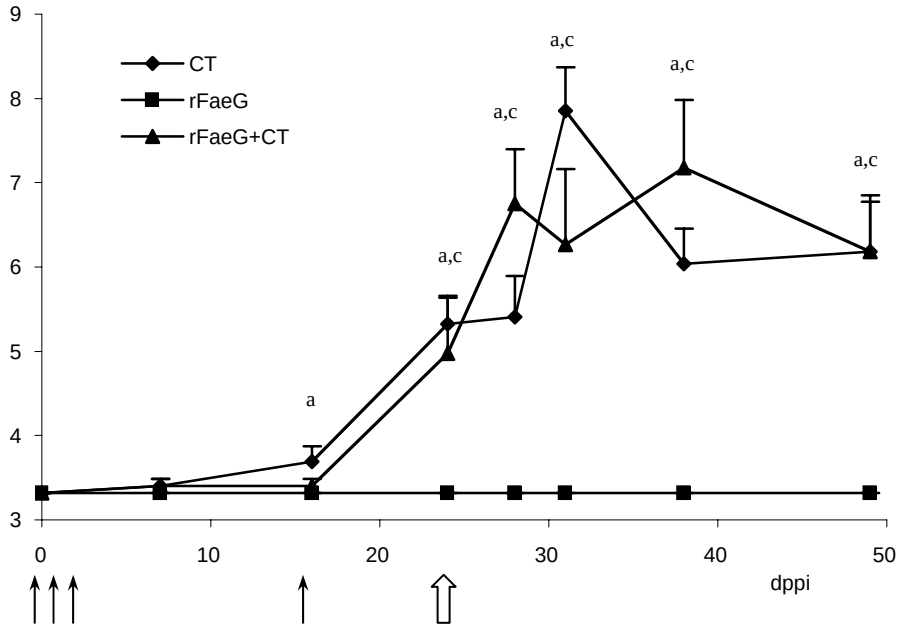


Figure 3 : Mean CT-specific serum antibody titers (± SEM) at 0, 7, 16, 24, 28, 31, 38 and 49 days post primary immunization (dppi) of pigs orally immunized with cholera toxin (CT, n=7), rFaeG (n=7) or rFaeG and cholera toxin (rFaeG+CT, n=7). Significant difference ($P < 0.05$) between CT and rFaeG (a), between CT and rFaeG+CT (b), between rFaeG and rFaeG+CT (c). Black arrow : immunization; white arrow : challenge.

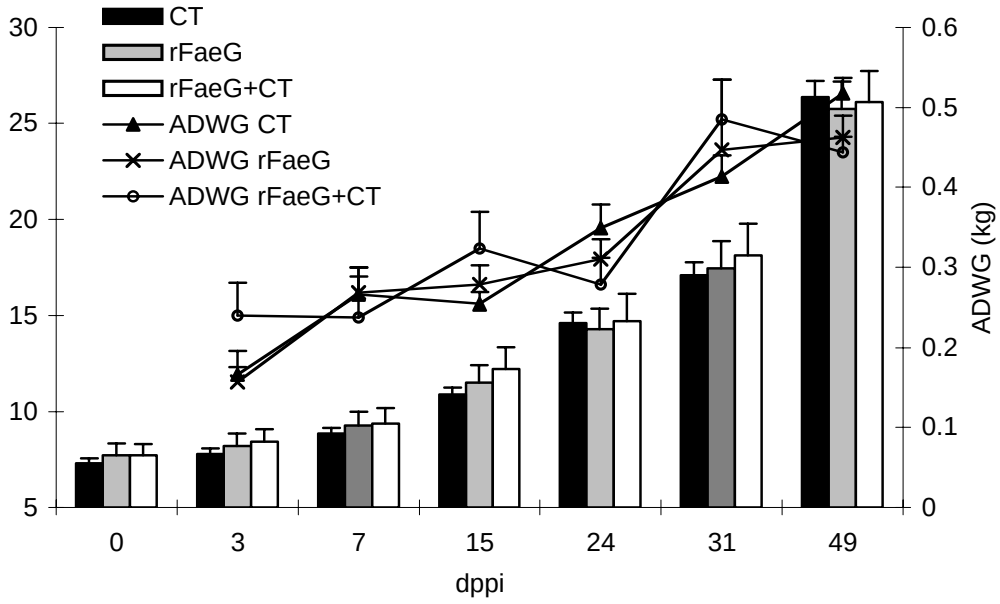


Figure 4 : Mean weight at 0, 3, 7, 15, 24, 31 and 49 days post primary immunization (dppi) and average daily weight gain (ADWG) at 3, 7, 15, 24, 31 and 49 dppi of pigs orally immunized with cholera toxin (CT, n=7), rFaeG (n=7) or rFaeG and cholera toxin (rFaeG+CT, n=7).

10.3.4. F4-specific cell proliferation following oral immunization

The observed adjuvanticity of CT on the F4-specific immune response to co-administered rFaeG is not restricted to the antibody response, as also a significantly higher F4-specific cell proliferation was observed in the rFaeG+CT group as compared to both other groups 23 dppi (Fig. 5; P≤0.036). Oral immunization of pigs with rFaeG alone induced an F4-specific cell proliferation that was significantly higher as compared to the CT immunized pigs (P=0.021).

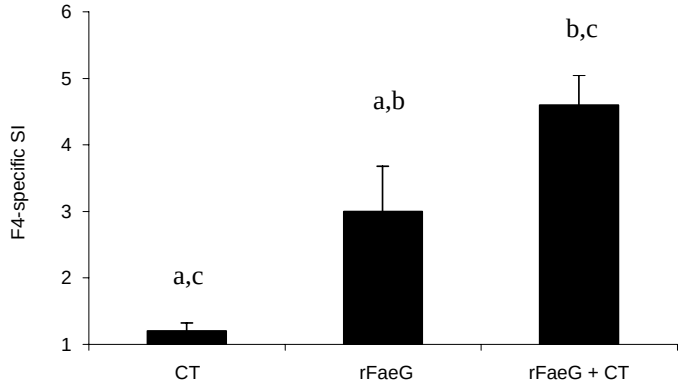


Figure 5 : Mean F4-specific stimulation index (SI) (± SEM) at 23 days post primary immunization of pigs orally immunized with cholera toxin (CT, n=7), rFaeG (n=7) or rFaeG and cholera toxin (rFaeG+CT, n=7). Significant difference (P < 0.05) between CT and rFaeG (a), between CT and rFaeG+CT (b), between rFaeG and rFaeG+CT (c).

10.3.5. FaeG-specific mucosal antibody response following oral immunization

Oral rFaeG immunization of newly weaned pigs resulted in the secretion of very low amounts of FaeG-specific antibodies in saliva. In the rFaeG group, an insignificant increase of FaeG-specific IgA was seen from 16 to 24 dppi (log2 titer 1.08 and 1.25, respectively). In the rFaeG+CT group, significantly higher FaeG-specific IgA was found as compared to both other groups both 16 and 24 dppi (log2 titer 1.33 and 1.74 respectively, P≤0.047).

10.3.6. F4+ E. coli excretion following challenge

To determine if the induced FaeG-specific immune response was able to protect against an F4+ ETEC infection, the pigs were challenge infected 24 dppi. Daily enumeration of the faecal F4+ *E. coli* excretion (Fig. 6) revealed >10^7 bacteria per g faeces in the CT group till 4 dpc, whereafter the excretion gradually decreased till 8 dpc. The excretion of the rFaeG-immunized animals was similar to that of the CT group until 2 dpc, after which it decreased faster to become significantly lower from 4 till 7 dpc (P≤0.016). However, animals immunized with rFaeG in the presence of CT already showed a significantly reduced F4+ *E. coli* excretion in comparison with the CT group but also the rFaeG group from day 1 post challenge onwards until 7 and 6 dpc, respectively (P≤0.004). Despite these differences in F4+ *E. coli* excretion, no significant differences were observed in faecal consistency (data not shown) and ADWG (Fig. 4) between the groups.

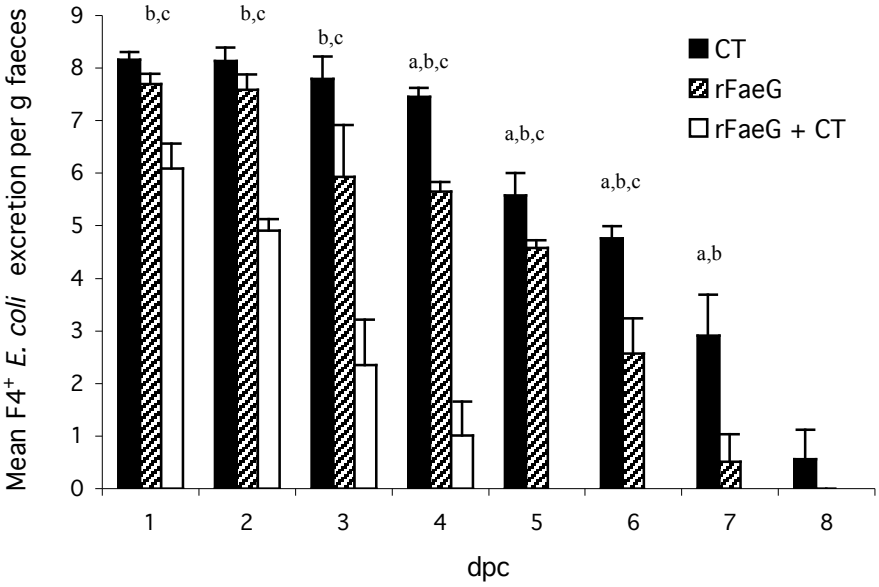


Figure 6 : Mean faecal F4+ *E. coli* excretion (log 10) per gram faeces (± SEM) of pigs orally immunized with cholera toxin (CT, n=7), rFaeG (n=7) or rFaeG and cholera toxin (rFaeG+CT, n=7). Significant difference (P < 0.05) between CT and rFaeG (a), between CT and rFaeG+CT (b), between rFaeG and rFaeG+CT (c).

10.3.7. FaeG-specific serum antibody response following challenge

In agreement with the high $F4^+$ *E. coli* excretion in the CT group, the challenge infection induced a primary FaeG-specific immune response in the CT immunized group (Fig. 1). Indeed, an FaeG-specific IgM serum antibody response appeared 28 dppi (4 dpc) and was significantly higher than for both rFaeG immunized groups on 38 and 49 dppi ($P \leq 0.019$), whereas the FaeG-specific IgA and IgG serum antibodies were only detected from 38 dppi onwards. On the other hand, FaeG-specific IgG of both rFaeG immunized groups showed a secondary response upon challenge with titers significantly higher as compared to the CT group 31 dppi ($P \leq 0.043$). Thereafter, IgG titers decreased again in the rFaeG group, but not in the rFaeG+CT group where they remained significantly higher than in the CT group (38 and 49 dppi; $P \leq 0.037$).

10.3.8. CT-specific serum antibody response following challenge

Following challenge infection of CT-immunized animals with the LT-producing $F4^+$ ETEC strain GIS26, CT-specific antibodies remained increasing until 4 or 7 dpc (rFaeG+CT and CT groups, respectively; Fig. 3) and then reached a plateau. The increase of the CT-specific antibody titer following ETEC infection could suggest a booster of the CT-response. On the other hand, challenge infection could not induce CT-specific serum antibodies in animals immunized with rFaeG alone, so that the observed increase in both other groups could still have been due to the CT immunization 16 dppi.

10.4. Discussion

The results of the present study show that oral immunization of newly weaned pigs with rFaeG results in an FaeG-specific mucosal and systemic immune response, in agreement with previous experiments (Chapter 9). However, the results of the present study indicate that oral co-administration of rFaeG with CT improves the induction of an FaeG-specific immune response in pigs. Indeed, the addition of the mucosal adjuvant CT induces faster and higher FaeG-specific antibody titers in serum

as well as F4-specific cell proliferation in the rFaeG+CT group, as compared to the animals immunized with rFaeG alone. The mechanisms underlying the observed mucosal adjuvanticity of CT are not clear, but there is growing evidence that the establishment of an environment leading to an enhanced antigen presentation is important (Porgador et al., 1998; Gagliardi and De Magistris, 2003). In pigs, CT increases CD80-CD86 expression and induces IL-1 expression by macrophages (Foss et al., 1999c). In addition, several studies in mice also report a CT-mediated optimised antigen presentation, influencing the induction or regulation of an antigen-specific immune response (Cong et al., 1997; Yamamoto et al., 1999; Jang et al., 2003).

The high FaeG-specific serum IgG titers and the detection of FaeG-specific IgA in saliva samples in the rFaeG+CT group, are in agreement with the induction of antigen-specific IgG (mainly IgG1) and IgA in the serum and mucosal secretions respectively, following oral co-administration of different heterologous antigens with CT in mice (Xu-Amano et al., 1994; Marinaro et al., 1995; Cong et al., 1997; Kim et al., 1998). The low levels of FaeG-specific IgA in saliva samples suggest diffusion of FaeG-specific IgA from serum to saliva, instead of a local production of FaeG-specific IgA (Vaerman et al., 1997). On the other hand, oral immunization of pigs with rFaeG has been shown to induce FaeG-specific antibody secreting cells in the gut associated lymphoid tissue (Chapter 9). Secretion of higher FaeG-specific IgA levels in the small intestinal lumen following rFaeG+CT immunization could occur since CT induces a predominant Th2-response (Xu-Amano et al., 1994; Marinaro et al., 1995) and stimulates IgA isotype switching (Kim et al., 1998) in mice. This could explain the better inhibition of $F4^+$ ETEC colonization following challenge of the rFaeG+CT immunized animals as compared to rFaeG immunized animals. Indeed, a correlation between the presence of FaeG-specific IgA in the small intestinal lumen and a reduction of the $F4^+$ ETEC colonization has been reported (Porter et al., 1974). Other influences of CT on leukocytes or intestinal epithelial cells that could mediate the induction of high FaeG-specific cellular and humoral immune response in the rFaeG+CT group (Holmgren et al., 2003) cannot be confirmed nor excluded with the results of the present study.

Despite the improved FaeG-specific immune response, pigs orally immunized with rFaeG and CT are not fully protected against a subsequent $F4^+$ ETEC challenge. Therefore, further improvement of the rFaeG immunization protocol is necessary. Perhaps, a higher rFaeG dose is necessary and/or the refolding of rFaeG must be

further optimised. Refolded rFaeG is reported to bind the F4R and to have the conformational epitopes a6, a7 and c in common with purified F4 fimbriae (Chapter 9). However, if the rFaeG folding is not totally identical to that of purified F4, rFaeG could induce less neutralizing antibodies or antibodies with a lower affinity to the native structure. Indeed, conformational changes in antigens are reported to influence its immunogenicity in terms of both affinity and titer (Subramanian et al., 2001; Titball and Williamson, 2001; Joyce et al., 2002). On the other hand, the effect of the challenge infection in the present study was more severe as compared to a previous experiment (Chapter 9). Therefore, pigs need a higher FaeG-specific immune response to inhibit the $F4^+$ ETEC colonization in this study.

Results of the present study show that oral administration of CT to pigs results in the significant induction of CT-specific serum antibodies. In addition, Foss and Murtaugh (1999a) observe CT-B specific IgA and IgG in jejunal mucus and saliva of pigs that are orally immunized with CT. As could be expected, the CT-specific immune response did not reduce $F4^+$ ETEC colonization since $F4^+$ *E. coli* excretion resembled that of identically infected non-immunized pigs (Chapter 9). However, cross-reactivity is reported between antibodies against the related enterotoxins CT and LT (Svennerholm et al., 1983; Clements et al., 1988). In humans, the presence of high anti-LT antibody titers is also shown not to be protective against ETEC infections (Levine et al., 1979; Cravioto et al., 1990). Therefore, the induced CT-specific antibodies are likely to reduce or even inhibit the toxic effect of $F4^+$ ETEC produced LT during challenge infection. Indeed, oral vaccines successful in protecting humans against ETEC-induced diarrhoea contain the cholera toxin B-subunit to induce anti-toxin antibodies (Peltola et al., 1991; Savarino et al., 1999; Hall et al., 2001).

Foss and Murtaugh (1999a) suggest that the mucosal adjuvanticity of CT in pigs needs mucosal targeting of the added heterologue antigen, as an antigen-specific immune response is only observed when the co-administered heterologue antigen is coupled to CT-B. The significantly higher His-S-tag-specific antibody titer in the rFaeG+CT pigs as compared to the rFaeG immunized pigs, shows that CT can act in pigs as a mucosal adjuvant of a heterologous antigen that is targeted to the mucosae by other systems than binding to the CT receptor GM1. There are advantages that may promote the use of the F4 fimbrial adhesin FaeG as mucosal carrier of heterologous antigens in combination with the mucosal adjuvant CT in pigs. Indeed, CT improves not only the antibody response against the adhesin but also against the coupled

heterologous antigen. Furthermore, the absence of competition between adjuvant and carrier for receptor binding could be an advantage to enhance the immune response, since they bind to different receptors. Further experiments are needed to confirm the potential of this system to obtain a simultaneously protective mucosal immune response against the widely spread pathogenic ETEC strains and other enteropathogens in pigs such as rotavirus or verotoxigenic *E. coli*. However, F4 fimbrial adhesins will not be able to function as universal mucosal carriers in pigs since F4 behaves as a normal food antigen in $F4R^-$ pigs (Van den Broeck et al., 2002).

In conclusion, the results of the present study show that the potential of rFaeG as mucosal carrier antigen to induce an antibody response against a coupled heterologous antigen, is improved following co-administration of rFaeG with CT in pigs. These results open new perspectives in the development of mucosal vaccines against enteric infectious diseases in pigs.

PART IV
GENERAL DISCUSSION

Chapter 11

General discussion, conclusions and perspectives

11.1. Characterization of F4 and its adhesin FaeG

Previous studies of our group have shown that oral immunization of pigs with purified F4 fimbriae results in a protective F4-specific immune response in $F4R^+$ pigs (Van den Broeck et al., 1999b). In $F4R^-$ pigs, F4 fimbriae seem to act as a normal food antigen (Van den Broeck et al., 2002). Therefore, the mucosal immunogenicity of F4 relies on its binding to the F4R on porcine small intestinal enterocytes. In agreement, soluble antigens like cholera toxin, heat-labile enterotoxin and some plant lectins that bind to enterocytes can induce an antigen-specific immune response following oral immunization, but most soluble non-replicating antigens lead to the induction of oral tolerance (Strobel and Mowat, 1998). The aim of the present work was to determine if F4 fimbriae of the $F4^+$ ETEC reference strain GIS26 or its adhesin FaeG could be used as a mucosal carrier molecule following oral immunization, inducing an immune response against a coupled/fused heterologous antigen in addition to a FaeG-specific immune response.

F4 fimbriae are composed of the minor subunits FaeC, FaeF, FaeH and FaeI and the major subunit FaeG that also constitutes the adhesin (Bakker et al., 1992a and 1992b). Fimbriae on the surface of bacteria can contain up to 1000 subunits, with a 10 times higher molar amount of the major subunit than of the minor subunits (Klemm, 1985). Probably, the fimbriae will break down in fragments due to the purification process, resulting in FaeG-containing multimers of different length (Chapters 4, 7 and 8). The hydrodynamic radii of most fimbriae in a sample of purified GIS26 F4 fimbriae is between 60 to 70 nm (Chapter 7), suggesting that the majority of purified F4 fimbriae contain at least 15 subunits since the hydrodynamic radii of monomeric rFaeG (without tags) is 3.8 nm (Bouckaert J., unpublished data). However, we were unable to determine the exact number of subunits in the purified fimbriae based upon these data since the multimeric F4 fimbrial structure can appear in linear or more winded forms depending on the medium conditions (Simons et al., 1994). This

multimeric character of purified F4 fimbriae is not unique to the GIS26 strain as it is also observed in 20 out of the 21-studied F4ac$^+$ *E. coli* field isolates (Chapter 4). Purification of other fimbriae like type 1 pili, P pili and F18 fimbriae also show polymeric structures (Abraham et al., 1988; Bullitt et al., 1996), but their adhesin is a minor subunit (Lund et al., 1985; Jones et al., 1995; Imberechts et al., 1996).

The multimeric character of the adhesin and major subunit FaeG distinguishes F4 fimbriae from other fimbriae like F18 where the adhesin is a minor subunit. This difference could at least partly explain the faster and more severe colonization and subsequent induction of a protective fimbriae-specific immune response following infection of pigs with the F4$^+$ ETEC strain GIS26 as compared to infection with the F18$^+$ VTEC strain F107/86 (Chapter 5). Furthermore, in contrast to F4 fimbriae (Van den Broeck et al., 1999b), oral immunizations with F18 fimbriae (Felder et al., 2000; Verdonck et al., unpublished data) do not lead to induction of a protective fimbriae-specific immune response. Parenteral immunization studies using type 1 fimbriae indicate that the predominant immune response against fimbriae with the adhesin as minor subunit, is directed against the non-adhesive major subunit that composes more than 99% of the total protein mass of the pilus structure (Lund et al., 1988; Langermann et al., 1997). These major-subunit-specific antibodies often cannot provide protection against infection such as is the case for the major subunits of type 1 pili since they are not conserved (Vandemaele et al., 2003a). On the other hand, the type 1 adhesin FimH is conserved (Sokurenko et al., 1994; Vandemaele et al., 2003a), but the low level of antibodies against this adhesive minor subunit cannot protect against a subsequent challenge infection (Lund et al., 1988; Langermann et al., 1997; Kariyawasam et al., 2002).

Since the adhesin FaeG is also the major subunit of F4 fimbriae, it was necessary to examine if FaeG is conserved or not. There are three different *faeG* allels encoding the adhesin of F4ab, F4ac or F4ad fimbriae (Guinee and Jansen, 1979), but F4ac$^+$ ETEC strains are most prevalent at different places all over the world (Westerman et al., 1988; Choi and Chae, 1999). Therefore, the FaeG amino acid sequence of the GIS26 reference strain and of 21 F4ac$^+$ *E. coli* field isolates was determined and subsequently compared with the FaeG amino acid sequence of F4 strains (F4ab$^+$, F4ac$^+$ or F4ad$^+$) reported in the literature. The percentage identity between FaeG of F4ac$^+$ strains was 96%, whereas the percentage identity between FaeG of F4ac$^+$ and this of F4ab$^+$ or F4ad$^+$ strains was 92 and 88% respectively

(Chapter 4). These degrees of sequence identity are in the range of percentages reported for other fimbrial adhesins such as F18 (Smeds et al., 2003; Tiels, unpublished results) and F17 (Buts et al., 2003a) and indicate that FaeG is as conserved as other fimbrial adhesins. The high percentage of identity among the adhesin of F4ac$^+$ strains gave a reason to believe that the F4ac fimbriae of one F4ac$^+$ *E. coli* strain could induce F4-specific antibodies that could inhibit adhesion of other F4ac$^+$ *E. coli* strains to the F4R. In chapter 4, this was confirmed for the GIS26 strain. Indeed, F4ac fimbriae purified from GIS26 induced F4-specific antibodies that blocked adhesion of the 20 tested F4ac$^+$ *E. coli* field isolates. Similarly, the degree of sequence identity between the adhesins of F4ac$^+$ strains and this of F4ab$^+$ or of F4ad$^+$ strains could also suggest blocking of F4ab$^+$ and F4ad$^+$ strains by F4ac-specific antibodies. Bijlsma et al. (1987) reported that oral administration of a particular F4$^+$ *E. coli* variant to pigs resulted in FaeG-specific antibody titers that were similar for the homologous as for the heterologous F4 variants. On the other hand, the three variants of FaeG have a different receptor specificity (reviewed in Van den Broeck et al., 2000) mediated at least in part by the variant-specific epitopes b, c and d (Bakker et al., 1992a; Sun et al., 2000b). Furthermore, of the 7 epitopes identified as the ‘a’ part of FaeG, only three ‘a’ epitopes are present in all three F4 variants, whereas the four other ‘a’ epitopes are only present in one or two F4 variants. This suggests that blocking of F4ab and F4ad strains by F4ac-specific antibodies will be incomplete if present at all. Nevertheless, Parry and Porter (1978) reported that antisera raised to F4ab and F4ac were cross-reactive with the heterologous fimbrial type and blocked binding of the heterologous and homologous fimbrial variants to porcine enterocytes. However Wilson and Hohmann (1974) could not demonstrate this cross-blocking. Further experiments are needed to elucidate these seemingly contradictory observations.

The high immunogenicity of F4 fimbriae is mediated by its multimeric character, but probably also by its high stability, certainly in the case of an oral immunization. Passage of immunogenic proteins through the gastro-intestinal tract mostly renders them non-immunogenic through the extreme low pH of the environment and the degradation by proteolytic enzymes in stomach (pepsin, gastricsin and chymosin) and small bowel (trypsin, chymotrypsin and some other pancreatic proteases) (Mayer, 2003). F4 fimbriae were shown to be very stable and to retain their immunogenicity in conditions reflecting those of the gastro-intestinal tract.

Indeed, incubation of F4 fimbriae at pH 4 or pH2 for 30 minutes results in a conformational change that can be restored by neutralizing the pH (Chapter 8). A significant reduction in the multimeric FaeG composition and slow digestion is only observed following incubation of F4 fimbriae for 2 h or longer in a simulated gastric fluid at pH 1.5 and 2 (Snoeck et al., 2004a). Oral immunization of pigs with purified F4 will not result in total destruction of the fimbriae since there is a fast transport of ingested material in the gastro-intestinal tract. Snoeck et al. (2003) orally administered pellets to piglets and found already 40% of the pellets in the small intestine 1.5 h following oral administration and a solution can leave the stomach even faster (Gregory et al., 1990). Furthermore, F4 fimbriae are very stable proteins since they retain their receptor-binding capacity and multimeric FaeG composition following heating up to 70°C, incubation in the presence of 0.5% SDS, 4M urea, 4M GuHCl or 5% non-ionic detergents (Chapter 8; Knörle and Hubner, 1995).

Purified F4 fimbriae of strain GIS26 are contaminated with flagellin (Chapter 4). Flagellin could induce a proinflammatory response during an infection when it would be able to interact with Toll-like receptor 5 (TLR-5), resulting in IL-8 expression (Gewirtz et al., 2001a and 2001b; Yu et al., 2003). In the human enterocyte cell lines T84, HT-29cI19A and Caco-2BBE and the Madin-Darby canine kidney cells, expression of TLR-5 is restricted to the basolateral membranes (Gewirtz et al., 2001a; Reed et al., 2002; Yu et al., 2003) so that if present on porcine enterocytes a similar location can be expected. Oral immunization with F4 most likely does not open the tight junction between enterocytes of the epithelial barrier since $F4^+$ ETEC infection of newly weaned pigs even did not increase the permeability of the porcine jejunal epithelium (Egberts et al., 1993). Therefore the presence of flagellin is not supposed to be of any importance for the immunogenicity of F4. Furthermore, the great immunogenicity of F4 is not due to an adjuvant effect of LT as this molecule was not detected in 5 different samples of GIS26 purified F4 fimbriae using an agglutination assay with a detection limit of 2 ng/ml (Verdonck, unpublished data). In addition, lipopolysaccharides (LPS) in the purified fimbrial preparation will not influence the FaeG-specific response following oral F4 immunization. Indeed, even though LPS are known immunostimulating agents when used systemically (Johnson, 1994), there are no studies reporting a significant immunostimulating effect of LPS when used orally with an antigen (Childers et al., 2000). This is most likely because LPS is already widely present in the gut. All these data confirm that the F4 fimbriae of

strain GIS26 are stable, immunogenic and are composed of multimers of a conserved FaeG adhesin, indicating that these fimbriae can be used in a mucosal vaccine against $F4^+$ ETEC infections.

11.2. Oral immunization with recombinant FaeG

Immunization with the recombinant type 1 adhesin FimH reduced *in vivo* colonization of the bladder mucosa by more than 99% in a murine cystitis model (Langermann et al., 1997). To determine the potential of the F4 fimbrial adhesin FaeG to induce a FaeG-specific immune response following oral immunization, recombinant FaeG was produced in the cytoplasm of *E. coli*. A cytoplasmatic expression was chosen since high yields can be obtained in the cytoplasm (Makrides, 1996) and periplasmic expression would require co-expression of the F4 fimbrial chaperone FaeE in the presence of the periplasmic protease DegP (Bakker et al., 1992a). The produced rFaeG was only present in cytoplasmic inclusion bodies (Chapter 9). Fimbrial adhesins of P pili, type 1 pili and F18 fimbriae expressed in an *E. coli* expression system, were also found in inclusion bodies (Vetsch et al., 2002; Karyawasam et al., 2002; Tiels, unpublished data). Fimbrial subunits have an immunoglobulin-like fold, missing the seventh β-strand (Sauer et al., 1999; Choudhury et al., 1999). This missing strand is complemented by the fimbrial chaperone during fimbrial biogenesis or by the N-terminal strand of a subsequent subunit in the fimbrial structure (Thanassi and Hultgren, 2000a). In the absence of this complementing strand, the subunit contains a hydrophobic groove on its surface (Barnhart et al., 2000), what could lead to the observed aggregation of monomeric FaeG subunits (Chapters 4, 8 and 9). The structure of FaeG is not elucidated at the moment, but we believe that a similar mechanism of subunit interaction is present as that found in other fimbriae. This is discussed in the chapters 2 and 4.

The refolding of rFaeG was screened with an ELISA using the FaeG-specific MAb IMM01 (Chapter 9), since the interaction of this MAb with F4 fimbriae correlated with the bio-activity of the fimbriae (Chapter 8). Refolding of rFaeG with SDS resulted in a conformation that contains conformational epitopes and binds the F4R in a specific and concentration-dependent manner, in contrast to refolding with for instance urea (Chapter 9). The potential of SDS to refold rFaeG was somewhat

surprising but is probably due to SDS-mediated shielding of the hydrophobic core of the FaeG subunits, reducing their non-specific aggregation. Heat treatment of F4 fimbriae resulted in aggregation and loss of the F4R-binding capacity (Chapter 8), but FaeG that was heat denaturated and subsequently eluted out of an SDS-PAGE could bind the F4R (Van den Broeck et al., 1999c). Tewari et al. (1993) also observed receptor binding capacity of the type 1 fimbrial adhesin FimH, following heat denaturation and subsequent elution out of an SDS-PAGE. The influence of SDS on protein conformation is concentration and protein dependent (Otzen and Oliveberg, 2002). Indeed, the protocol that was developed to refold rFaeG with SDS was not successful to refold the recombinant F18 fimbrial adhesin FedF (unpublished data, Tiels et al.).

The conformation of refolded rFaeG influences its binding to the receptor and subsequently also the induction of an FaeG-specific immune response since FaeG-specific ASCs were found in intestinal lymphoid tissues of pigs orally immunized with SDS-refolded rFaeG, but not with urea-refolded rFaeG (Chapter 9). This result confirms the need for receptor-binding to induce a FaeG-specific immune response following oral immunization (Van den Broeck et al., 1999b). Interestingly, Joensuu et al. (2004) reported the production of rFaeG subunits in tobacco, which bind the F4R without a refolding procedure. These plant-produced rFaeG subunits could be an alternative to the SDS-refolded rFaeG when further experiments are able to show that they can induce an FaeG-specific immune response following oral immunization.

Snoeck et al. (2004c) showed that uptake of F4 at the follicle associated epithelium via enterocytes and M cells could result in the induction of a FaeG-specific immune response in the (jejunal) Peyer's patches. In addition, F4 that binds $F4R^+$ enterocytes, enters the lamina propria (LP) and is at least partly taken up by local antigen-presenting cells (Snoeck et al., 2004b). Probably, these antigen-presenting cells migrate to the MLN where they could induce an FaeG-specific immune response since high numbers of FaeG-specific IgM ASCs were detected in MLN four to seven days following an $F4^+$ ETEC infection (Chapter 5). Then, FaeG-specific ASCs likely home to the intestinal effector sites meanwhile switching to the IgA isotype (McGhee et al., 1992), thereby explaining the high numbers of FaeG-specific IgA ASCs in the LP 7 to 11 days post $F4^+$ ETEC infection (Chapter 5). Oral rFaeG and F4 immunizations induce an FaeG-specific immune response that resembles the response induced following $F4^+$ ETEC infection (Chapters 9 and 10). Indeed, following $F4^+$

ETEC infection as well as following oral immunization with rFaeG or F4, higher amounts of IgM than IgA ASCs were found (Chapters 5 and 9). IgM is a more important immunoglobulin in the mucosal secretions of pigs than of other animals (Porter et al., 1974; Bianchi et al., 1999). It was only following oral rFaeG or F4 booster immunization that a clear antibody class switch from IgM to IgA was seen, resulting in a change in the IgM-IgA ASCs ratio (Chapters 9 and 10). As expected, mucosal FaeG-specific IgG responses are low but not absent (Bianchi et al., 1999; Yuan et al., 2001). This could suggest a role for the FcRn receptor, recently discovered in pigs (Claypool et al., 2004). This receptor can transport IgG bi-directionally through epithelial cells.

Although oral immunization of pigs with rFaeG or F4 are both able to induce an FaeG-specific antibody response, F4 immunization is more effective than rFaeG immunization since a higher FaeG-specific immune response and a better protection against a subsequent challenge is observed (Chapters 9 and 10). This difference in FaeG-specific response is probably due to a combination of factors. First, rFaeG are monomers, whereas purified F4 fimbriae are polymeric FaeG structures (Chapters 4 and 9). This difference may have consequences for the immunogenicity of the protein as discussed before. In addition, the different multimerization status could influence avidity for binding to the enterocytes. It is for instance well known that the binding strength of one binding site of an IgG towards a multimeric antigen is much lower than the avidity of a whole IgG antibody interacting with its two antigen-binding sites to this antigen (Lee et al., 2004). On the other hand, binding of SDS-refolded rFaeG to the F4R may be reduced due to repulsion of some negatively charged SDS molecules still present on the rFaeG by the negatively charged sialic acid and sulphate groups that are present in mucus oligosaccharides (Cone, 1999). Furthermore, it is possible that refolding of rFaeG results in a native-like structure that is not totally identical to native FaeG. Finally, the presence of the N-terminal His-S-tag does not inhibit F4R binding but may influence its binding. The reduced immunogenicity of rFaeG as compared to F4 is likely not a result of a difference in proteolytic degradation since the gastric HCl production was blocked at immunization. Furthermore, Joensuu et al. (2004) reported that their plant-produced rFaeG was as stable in simulated gastric fluid as compared to F4 fimbriae.

10.3. F4 and rFaeG as mucosal antigen carriers

Binding of F4 or rFaeG to the F4R on small intestinal enterocytes and their ability to induce an FaeG-specific immune response led to the question if F4 and/or rFaeG could be used as a mucosal carrier, inducing an antibody response against a coupled or fused heterologous antigen following oral administration. Lectins that bind enterocytes in mice were already reported as functional mucosal carriers, inducing an antibody response against a carried heterologous antigen when orally administered (Lavelle et al., 2000). However, the advantage of using FaeG as a mucosal carrier in pigs would be the simultaneous induction of FaeG- and heterologous-specific antibodies. This could open perspectives for simultaneously vaccinating pigs against the widely spread $F4^+$ ETEC and against another enteropathogen.

Insertion of epitopes in variable regions of FaeG has been reported to induce epitope-specific antibodies following parenteral immunization (Thiry et al., 1989; Bakker et al., 1990). However, these constructs are not able to bind the F4R since the variable regions are involved in the F4R binding (Bakker et al., 1992a). In addition, even though small epitopes can be inserted without disturbing the folding of the subunit, vaccination with one epitope of an antigen does not always result in protection against a pathogen (Fayolle et al., 2001; Jeon et al., 2002; Rodriguez et al., 2003).

The potential of F4 fimbriae to function as a mucosal carrier molecule was investigated by chemically coupling the model antigen HSA to the fimbriae. HSA has no receptor on enterocytes. The chemical conjugation of antigen and carrier was performed with glutaraldehyde since several studies reported the effective production of immunogenic antigen-carrier complexes, using this technique (McKenzie and Halsey, 1984; Hamajima et al., 1995; Fujiwara et al., 1999). Indeed, conjugation of F4 to HSA (F4/HSA) in a 1:1 molar ratio resulted in F4 fimbriae that were covalently bound to HSA and also retained their ability to bind to the F4R (Chapter 7). Oral immunization of pigs followed by two oral booster immunizations with F4/HSA conjugates primed the immune system against HSA as a secondary HSA-specific antibody response was observed when these animals were boosted intramuscularly with HSA. In contrast, a primary HSA-specific immune response was observed after the intramuscular immunization in pigs which orally received HSA/HSA following the same protocol as for F4/HSA (Chapter 7). These results indicate that F4 fimbriae

allow transport of conjugated HSA through the intestinal epithelial barrier. The presence of HSA-specific IgA antibodies in the F4/HSA group, but not in the HSA/HSA group following the intramuscular HSA boost immunization, suggest that translocated HSA reached the gut associated lymphoid tissue. Indeed, the induction of an antigen-specific immune response in mucosae-associated lymphoid tissue leads to the production of antigen-specific IgA (Murray et al., 1987). Russell-Jones (2001) reported the potential of F5 and F6 to function in mice as mucosal carrier for chemically conjugated DNP and BSA, but specific binding of the complexes to murine enterocytes was not proven and binding of $F5^+$ or $F6^+$ *E. coli* to murine enterocytes has not yet been demonstrated.

Although F4 fimbriae were shown to have the capacity to function as a mucosal carrier, this system was not very effective since the oral immunization induced only low FaeG-specific antibody titers and even no HSA-specific antibodies (Chapter 7). The F4-specific serum antibody response was significantly lower than following oral F4 immunization (Chapters 7 and 9). F4/HSA is a large molecule and its subunits cannot fall apart due to covalent linking by glutaraldehyde (Chapter 7). This could reduce the diffusion through the intestinal mucus layer as compared to purified F4 fimbriae. *In vitro* studies have suggested that the rate of diffusion of molecules through mucus progressively decreases with increasing molecular size (Desai et al., 1992). However, intestinal mucus is not totally impermeable for large proteins provided that their luminal concentration is sufficiently high (Flemström et al., 1999). An additional reason for the low immunogenicity of F4/HSA could be a reduction in the F4R-binding capacity by the conjugated HSA molecules as well as a mimicking of F4 epitopes. Furthermore, the chemical linker molecule used to bind HSA to F4 can have an effect on the immunogenicity of carrier and HSA antigen as demonstrated by Kirkley et al. (2001). Also the molar ratio of carrier and antigen in the conjugates can influence the immunogenicity (Russell-Jones, 2001). In addition, the presence of free HSA molecules in the F4/HSA conjugate solution could negatively influence the induction of a HSA-specific immune response since it rather induces tolerance (Stok et al., 1994).

A genetic fusion between FaeG and a heterologous peptide/antigen allows a strictly controlled coupling of both molecules and results in smaller molecules than antigen-F4 complexes. In addition, no free His-S-tag fragments are present in purified rFaeG. This could explain the higher immune response against the His-S-tag than

against HSA following oral rFaeG or F4/HSA immunization (Chapters 7, 9 and 10). However, this has to be confirmed for larger antigens and comparison can only be made correctly if the same antigen is conjugated to F4 as is conjugated to rFaeG.

10.4. Complementary effect of F4 and rFaeG as mucosal carrier with the mucosal adjuvant CT

To improve the immune response against the carried antigen, the F4/HSA or rFaeG complexes were orally co-administered with CT, one of the most potent mucosal adjuvants (Lycke, 2004). Supplementing CT induced a faster heterologous peptide/antigen-specific antibody response and significantly higher antibody titers than immunization without CT (Chapters 7 and 10). The improved heterologous-specific antibody response is due to a complementary effect of the carrier system and the adjuvant (Chapter 7). Based on a study on the uptake of F4 in intestinal loops (Snoeck et al., 2004b), we believe that the F4 or FaeG carrier allows an F4R-dependent uptake of a heterologous antigen or peptide by epithelial cells. Subsequently, this antigen could reach antigen-presenting cells (Snoeck et al., 2004b), resulting in an antibody response.

Oral co-administration of CT and antigen leads mainly to an increase of antigen-specific IgG titers in serum and IgA titers in saliva (Chapters 7 and 10). It is likely that oral co-administration of CT with an F4- or FaeG-based carrier-antigen complex improves the induction of a local antigen-specific immune response since oral immunization with both rFaeG (Chapter 9) and F4 fimbriae (Van den Broeck et al., 1999a) results in the induction of FaeG-specific ASCs in intestinal lymphoid tissues and CT is a known mucosal adjuvant (Lycke, 2004). Furthermore, HSA-specific IgA antibodies were detected 24 dppi in saliva of $F4R^+$ pigs immunized with F4/HSA and CT, whereas no HSA-specific IgA antibodies could be detected in the serum at that moment. In addition, several other studies showed the ability of CT to enhance mucosal as well as systemic immune responses against a mucosal co-administered antigen (Biet et al., 2003; Karlsen et al., 2003; Yasuda et al., 2003).

In contrast to the high toxicity of CT in humans (Sack et al., 2004), the use of 50 μg CT in pigs is not toxic and does not lead to growth retardation (Chapters 7 and 10; Foss and Murtaugh, 1999a). Moreover, induction of CT-specific antibodies could

cross-react with LT and inhibit its toxic effect following ETEC infection (Holmgren, 1973; Jacob et al., 1986). Indeed, no diarrhoea was observed in animals immunized with CT alone (Chapter 10), whereas non-immunized animals that were identically challenged developed diarrhoea (Van der Stede et al., 2003). So, the presence of CT- and FaeG-specific antibodies most likely results in better protection against $F4^+$ ETEC challenge than when only FaeG-specific antibodies are present, as has been reported by Francis and Willgohs (1991) for LT- and FaeG-specific antibodies. Therefore, it is worthwhile to determine if co-administration of the antigen-carrier system with LT has a similar or even better effect than with CT.

11.5. Main conclusions and future perspectives

The multimeric character, the stability and the conservation of the F4 fimbrial adhesin FaeG render the F4 structure very immunogenic and a good mucosal vaccine candidate against $F4^+$ ETEC infections. The results of the present thesis suggest that polymeric FaeG structures are more immunogenic following oral immunization than monomeric FaeG. However, there is no information available whether this is due to a higher avidity of binding to the F4R or due to the polymeric character of the antigen. It could be worthwhile to study the binding kinetics of (r)FaeG mono-, di- and higher FaeG-multimers to the F4R in a biosensor, their uptake by M cells and enterocytes in intestinal loops and their mucosal immunogenicity by orally immunizing $F4R^+$ pigs.

The main conclusion of this work is that both purified F4 fimbriae and rFaeG have the potential to function as a mucosal carrier, inducing an antibody response against a coupled/fused heterologous antigen/peptide. A genetic fusion of an antigen and a carrier molecule is favourable since there is no free antigen or carrier present and the conjugation is more controlled and thus more reproducible than in the case of chemical coupling. However, the potential of rFaeG to function as a mucosal carrier to an antigen needs to be confirmed. Hereto, further research is necessary to elucidate the structure of FaeG. This information could probably enable the production of a stable FaeG lectin domain or alternatively fusion of the missing seventh β-strand to complete its immunoglobulin fold, thus making a stable immunogen. As a consequence, these new FaeG constructs would circumvent SDS-refolding that could

influence immunogenicity of a fused heterologous antigen. Perhaps, the plant-produced rFaeG can be an alternative.

Several methods of antigen targeting to M cells are described that induce a mucosal antigen-specific immune response against non-replicating soluble antigens, but the quantity of material taken up from the intestine is often low (Clark et al., 2000; Nicoletti, 2000). The low number of M cells and their location in restricted sites of the intestinal wall probably cause this problem. Therefore, targeting antigens to enterocytes and subsequently to the intestinal lymphoid tissues is probably an alternative, which can be studied using the F4- or rFaeG-based carrier systems. However, the F4 system will not be able to function as universal mucosal carrier in pigs since some animals lack the F4 receptor. The development of a universal mucosal carrier will require the identification of molecules that bind to and can translocate through enterocytes of all pigs.

Summary

Intestinal infections are still an important cause of disease in both humans and animals. Fimbriae often play a prominent role in anchoring bacterial cells to host tissue and mediate the first step in pathogenesis. For instance, F4 fimbriae of $F4^+$ enterotoxigenic *Escherichia coli* (ETEC) bind to F4-receptors on small intestinal enterocytes of pigs, resulting in colonization of the small intestine. Subsequently, $F4^+$ ETEC secrete heat-labile (LT) and heat-stable (ST) enterotoxins that cause diarrhoea. As a consequence, there is a continuous development of new strategies to block the binding of fimbriae to their specific receptor on host cells.

Chapter 1 gives an overview of the most prevalent fimbriae of pathogenic *E. coli* strains in humans and animals. The focus of the chapter concerns the differences in fimbrial structures, the use of fimbriae in systemic and mucosal immunizations against fimbriated pathogens and the use of fimbriae as carrier systems. F4 fimbriae differ from most fimbriae in that the F4 fimbrial adhesin FaeG also constitutes the major subunit of the fimbriae. In addition, oral immunization of pigs with F4 fimbriae has been reported to result in the induction of a FaeG-specific immune response that protects piglets against a $F4^+$ ETEC infection. This is a remarkable finding since oral immunization with soluble non-replicating antigens often induces oral tolerance, which hampers the development of mucosal vaccines. Therefore, this finding opens the question if F4 fimbriae or its adhesin FaeG has the potential to function as a carrier molecule to induce immune response against a fused/coupled heterologous antigen/peptide.

Chapter 2 reviews the present knowledge on the genetic configuration of the F4 fimbrial operon *fae*, the regulation of subunit expression and the biogenesis of the fimbrial structure. In addition, the similarities and differences with well studied other fimbrial systems are discussed.

The enterotoxins are the second virulence factors of pathogenic ($F4^+$) ETEC strains. In the first part of chapter 3, the structure, receptor binding and the mechanism of action of both LT and the closely related cholera toxin (CT) are described. In the second part of chapter 3, the immunomodulatory effect of both toxins is reviewed with the focus on their influence on the different leukocyte populations.

Chapters 4 to 10 present the experimental work of this thesis. The objective was to answer the following questions :

- Are F4 fimbriae a good vaccine candidate to be used in a mucosal vaccine against an $F4^+$ ETEC infection ?

- Are F4 fimbriae or the F4 fimbrial adhesin FaeG able to induce an antibody response against a coupled/fused heterologous antigen/peptide following oral immunization of pigs ?

- Can the oral co-administration of the carrier-antigen/peptide complex with CT improve the heterologous antigen/peptide-specific immune response ?

In order to use F4 fimbriae or FaeG subunits in an oral vaccine against $F4^+$ ETEC, it is necessary to determine the conservation of the adhesin subunit. Therefore, in chapter 4 the *faeG* sequence was determined of 21 $F4ac^+$ *E. coli* field isolates from piglets with diarrhoea and subsequently compared with these of the reference strain GIS26 and previously reported *faeG* sequences from $F4ab^+$, $F4ac^+$ and $F4ad^+$ strains. The FaeG amino acid sequence was 96-100% homologous within each F4 serotype, but only 92% and 88% when the F4ab and F4ad serotypes were compared with the F4ac serotype. In addition, antibodies induced by purified GIS26 F4ac fimbriae immunization were able to inhibit binding of all 21 $F4ac^+$ *E. coli* field isolates. Further characterization of purified F4 fimbriae of GIS26 revealed the presence of flagellin and the multimeric character of the FaeG adhesin. This multimeric character was also found in 20 of the 21 $F4ac^+$ *E. coli* field isolates. In conclusion, the results of the experiments reported in this chapter support the usefulness of GIS26 F4 fimbriae in an oral vaccine against $F4^+$ *E. coli* infections.

In chapter 5, the fimbriae-specific systemic and mucosal immune response following infection with $F18^+$ VTEC (strain 107/86) was compared with the response following an infection with the $F4^+$ ETEC strain GIS26. These strains differ in both their virulence factors : the $F4^+$ ETEC strain has a major subunit as adhesin and secretes heat-labile enterotoxin (LT), whereas the $F18^+$ VTEC strain has a minor subunit as adhesin and secretes the shiga-like toxin II variant (SLT-IIv). Weaned

F18-seronegative F18-receptor positive piglets were infected with the F18ab positive strain and weaned F4-seronegative F4-receptor positive ($F4R^+$) piglets with the $F4^+$ strain. The peak excretion of bacteria was 2 days post primary infection (dpi) following the inoculation with the $F4^+$ ETEC strain and between 3 and 5 dpi after the $F18^+$ VTEC inoculation. ELISPOT assays enumerating the fimbriae specific IgM, IgA and IgG antibody secreting cells (ASCs) revealed high numbers of fimbriae-specific IgM ASCs in the spleen 4 dpi with both strains. F18-specific IgM ASCs were present 4 dpi in the mesenteric lymph nodes and from 7 dpi onwards in the Peyer's patches, whereas F4-specific IgM ASCs were detected in the mesenteric lymph nodes and the Peyer's patches 4 dpi. Besides the faster induction of an immune response following $F4^+$ ETEC infection compared with $F18^+$ VTEC infection, the switch from IgM to IgA and IgG was also earlier following the $F4^+$ ETEC infection. F4-specific IgA and IgG ASCs were detected from day 4 onwards, together with F4-specific IgA antibodies in serum and intestinal contents. F18-specific IgA and IgG ASCs and F18-specific IgA in serum and intestinal contents were found 11 dpi. The results of this study suggest that the multimeric adhesin character of F4 fimbriae and/or the immunomodulatory effect of LT enterotoxin may accelerate the mucosal immune response in the $F4^+$ ETEC infected piglets.

Chapter 6 describes the usefulness of a real-time biomolecular interaction analysis system (BIAcore® 3000), based on the principles of surface plasmon resonance (SPR) for the study of the interaction of F4 fimbriae and porcine enterocytes. Using this system, F4 fimbriae were found to interact with $F4R^+$ porcine enterocytes, whereas no interaction was observed with enterocytes that were typed $F4R^-$ or weakly $F4R^+$ based on an *in vitro* adhesion assay. Since the flow rate in the biosensor resembles the *in vivo* intestinal flow better as compared to the *in vitro* adhesion assay, it is likely that the biosensor can better mimic the *in vivo* situation. Furthermore, this method offers new opportunities to analyse the receptor-binding capacity of an F4-antigen conjugate or to screen therapeutics for prevention of ETEC adhesion.

The potential of F4 fimbriae to act as a mucosal carrier molecule for the model antigen human serum albumin (HSA), was examined in chapter 7. Oral immunization of pigs with glutaraldehyde coupled HSA molecules (HSA/HSA complexes; HSA/HSA group) did not result in the induction of a HSA-specific immune response,

in contrast to oral co-administration of HSA/HSA with CT (HSA/HSA/CT group). These results showed that CT acts as a mucosal adjuvant in pigs, which is in accordance with the reported CT adjuvant effect in other animals. Glutaraldehyde-conjugation of HSA to purified F4 fimbriae resulted in F4/HSA complexes that were able to bind the F4R. Oral immunization of F4-HSA in $F4R^+$ pigs (F4/HSA group) induced a HSA-specific immune response, however this immunization did not result in the detection of HSA-specific antibodies. Indeed, intramuscular HSA immunization induced a secondary response in the F4/HSA immunized pigs (F4/HSA group), as compared to a primary response in HSA/HSA immunized animals (HSA/HSA group). These data indicated that F4 fimbriae could function as a mucosal carrier to a chemically coupled heterologous antigen. In addition, oral co-administration of F4-HSA with CT (F4/HSA+CT group) to $F4R^+$ pigs induced HSA-specific antibodies in serum and saliva that were significantly higher than these in the F4/HSA and the HSA/HSA+CT groups. The high HSA-specific response seems to be due to a complementary effect of F4-mediated binding of HSA to $F4R^+$ enterocytes and the mucosal adjuvanticity by CT since a significantly lower HSA-specific antibody response is observed following oral immunization of $F4R^-$ pigs with F4/HSA and CT. This combined use of CT as mucosal adjuvant and F4 fimbriae as mucosal carrier system could open new perspectives in the development of vaccines against $F4^+$ ETEC and other enteropathogens in pigs.

In chapter 8, it was determined if altered interaction of F4 fimbriae with the FaeG-specific monoclonal antibody (MAb) IMM01 correlated with an altered bioactivity of F4 fimbriae. Hereto, purified F4 fimbriae were first treated in conditions that are known to alter protein folding. Indeed, the F4R-binding and FaeG multimerization of purified F4 fimbriae was observed to be reduced following incubation with SDS at concentrations of 1.5 % or higher and at temperatures of 85°C or higher. Incubation of purified F4 fimbriae at pH 4 or lower resulted in a reversible conformational change that became irreversible and stabilised in the presence of 0.05 % SDS, a concentration that did not affect the F4 fimbrial bioactivity at neutral pH. The altered bioactivity of treated F4 fimbriae correlated with optical density determined in ELISA using the FaeG-specific MAb IMM01. This fast and sensitive assay opens new perspectives to analyse the FaeG conformation following refolding of recombinant protein, the release of bioactive FaeG from microcapsules and the production of appropriate oral F4 subunit vaccines.

In chapter 9, the gene of the F4-fimbrial adhesin FaeG of the pathogenic $F4^+$ ETEC strain GIS26 was cloned in the pET30Ek-LIC vector and expressed with an N-terminal His- and S-tag in the cytoplasm of BL21(DE3). Recombinant FaeG (rFaeG) subunits were isolated from insoluble cytoplasmic aggregates and refolded into a native-like F4R-binding conformation using SDS. The presence of conformational epitopes was confirmed by ELISA and the ability to bind the F4R was confirmed by inhibiting the adhesion of $F4^+$ ETEC to $F4R^+$ villi with increasing concentrations of native-like SDS-refolded rFaeG subunits, in contrast to urea-refolded rFaeG. The rFaeG subunits appear as monomers, whereas the purified F4 fimbriae are multimers. Oral immunization of newly weaned piglets with native-like rFaeG induced a mucosal and systemic FaeG-specific immune response, whereas oral immunization with ureum refolded rFaeG did not induce FaeG-specific ASCs. In agreement, SDS-refolded rFaeG immunized pigs showed a significantly reduced $F4^+$ *E. coli* excretion from 2 till 5 days following challenge infection. However, improvement of stability and immunogenicity of (SDS-refolded) rFaeG will be necessary since rFaeG immunization resulted in a lower F4-specific response compared to immunization with purified F4 fimbriae. Furthermore, the N-terminal fusion of a His- and S-tag was not detrimental for binding the F4R, supporting the use of FaeG as mucosal carrier. Indeed, oral immunization of pigs with SDS-refolded rFaeG resulted in the production of His-S-tag specific antibodies as determined in Western blot. In conclusion, oral immunization with a native-like refolded recombinant FaeG fimbrial adhesin subunit of *Escherichia coli* induces a mucosal and systemic FaeG-specific immune response and rFaeG functions as a mucosal carrier inducing antibodies against a fused His-S-tag peptide.

Chapter 10 describes the results of oral co-administration of rFaeG and CT, to improve the immune response against FaeG and the N-terminally fused His-S-tag. Oral immunization of pigs with rFaeG and CT significantly enhanced the immune response against the heterologous peptide since significantly higher His-S-tag-specific antibodies were detected. In addition, the co-administration improved the FaeG-specific humoral and cellular immune response and significantly reduced the fecal $F4^+$ *E. coli* excretion following challenge infection as compared to rFaeG-immunized pigs. In conclusion, the results of this study show that fimbrial adhesins can be used as mucosal carrier for inducing an immune response against peptides, which normally are not immunogenic following oral administration.

The experiments in the current thesis demonstrated the multimeric character, the stability and the conservation of the F4 fimbrial adhesin FaeG, which make it very immunogenic and a good mucosal vaccine candidate against $F4^+$ ETEC infections. The main statement of the present work is that both purified F4 fimbriae and rFaeG have the potential to function as a mucosal carrier, inducing an antibody response against a coupled or fused heterologous antigen or peptide following oral immunization. However, the potential of rFaeG to function as a mucosal carrier to an antigen needs to be confirmed in further experiments. These F4- and rFaeG-based mucosal carrier systems, although restricted to $F4R^+$ pigs, enable to study the potential of enterocyte-targeting of antigens to induce a mucosal antigen-specific immune response, which perhaps could be an alternative to M-cell-targeting of antigens.

Samenvatting

Darminfecties vormen nog steeds een belangrijke oorzaak van ziekte bij mens en dier. Fimbriae zorgen voor de binding van bacteriën aan gastheerweefsel en zorgen zo de eerste stap in de pathogenese. Zo binden bijvoorbeeld F4 fimbriae van $F4^+$ enterotoxigene *Escherichia coli* (ETEC) aan F4-receptoren op dunne darm enterocyten van varkens, wat resulteert in kolonisatie van de dunne darm. Vervolgens zullen $F4^+$ ETEC hitte-labiele (LT) en hitte-stabiele (ST) enterotoxines secreteren die diarree veroorzaken. Het belang van fimbriae in de pathogenese leidt tot de voortdurende ontwikkeling van nieuwe methoden om binding van fimbriae aan hun specifieke receptor op gastheercellen te blokkeren.

Hoofdstuk 1 geeft een overzicht van de meest voorkomende fimbriae van pathogene *E. coli* stammen bij mens en dier. Dit overzicht richt zich voornamelijk tot de verschillen in fimbriële structuren, het gebruik van fimbriae in systemische en mucosale immunisaties tegen pathogenen met fimbriae als virulentiekenmerk en het gebruik van fimbriae als dragermoleculen. F4 fimbriae verschillen van de meeste andere fimbriae doordat het F4 fimbrieel adhesine FaeG tevens de meest voorkomende subeenheid is van de fimbriae. Daarenboven resulteert orale immunisatie van varkens met F4 fimbriae in inductie van een FaeG-specifieke immuunrespons die varkens beschermt tegen een $F4^+$ ETEC infectie. Dit is een opmerkelijke bevinding aangezien orale immunisatie met oplosbare niet-vermenigvuldigende antigenen meestal leidt tot de inductie van orale tolerantie, wat de ontwikkeling van mucosale vaccins belemmert. Deze unieke eigenschappen van F4 fimbriae brengen dan ook de vraag naar voor of F4 fimbriae of het F4 fimbrieel adhesine FaeG in staat is te werken als een dragermolecule die een immuunrespons kan induceren tegen een chemisch of genetisch gekoppeld heteroloog antigeen of peptide.

Hoofdstuk 2 geeft een overzicht van de huidige kennis in verband met de genetische configuratie van het F4 fimbrieel operon *fae*, de regulatie van de expressie van de verschillende fimbriële subeenheden en de biogenese van de fimbriële

structuur. Bijkomend worden gelijkenissen en verschillen tussen F4 en andere goed bestudeerde fimbriële systemen bediscussieerd.

Naast fimbriae fungeren enterotoxines als tweede virulentiefactor van pathogene ($F4^+$) ETEC stammen. In het eerste gedeelte van hoofdstuk 3 worden de structuur, de receptorbinding en het werkingsmechanisme van zowel LT als het sterk gelijkende cholera toxine (CT) beschreven. In het tweede gedeelte van hoofdstuk 3 wordt een overzicht gegeven van de immunomodulerende effecten van beide bacteriële toxines, waarbij vooral aandacht besteed wordt aan hun invloed op verschillende populaties witte bloedcellen.

De hoofdstukken 4 tot 10 stellen het experimenteel gedeelte van deze thesis voor. Deze thesis had tot doelstelling volgende vragen te beantwoorden :

- Zijn F4 fimbriae goede antigenen om te gebruiken in een mucosaal vaccin tegen een $F4^+$ ETEC infectie ?
- Zijn F4 fimbriae of het F4 fimbriële adhesine FaeG in staat te werken als mucosaal dragermolecule die na orale immunisatie van varkens een antistoffenrespons kan induceren tegen een chemisch of genetisch gekoppeld antigeen of peptide ?
- Kan gelijktijdige toediening van het drager-antigeen/peptide complex met CT de heterologe antigeen/peptide-specifieke immuunrespons verbeteren ?

Vooraleer F4 fimbriae of FaeG subeenheden in een oraal vaccin tegen $F4^+$ ETEC te gebruiken, is het noodzakelijk te onderzoeken of het adhesine geconserveerd is. Hiervoor werd in hoofdstuk 4 de *faeG* sequentie van 21 $F4ac^+$ *E. coli* veldisolaten van biggen met diarree bepaald en nadien vergeleken met deze van de referentiestam GIS26 en eerder gerapporteerde *faeG* sequenties van $F4ab^+$, $F4ac^+$ en $F4ad^+$ *E. coli* stammen. De FaeG aminozuursequentie was 96-100% identiek binnen elke F4 variant, maar enkel 92% en 88% wanneer de F4ab en de F4ad varianten werden vergeleken met F4ac. Tevens kunnen antistoffen die geïnduceerd werden met gezuiverde GIS26 F4ac fimbriae de binding van alle 21 $F4ac^+$ *E. coli* veldisolaten verhinderen. Verdere karakterisatie van gezuiverde F4 fimbriae van GIS26 toonde de aanwezigheid van flagelline aan evenals het multimeer karakter van het FaeG

adhesine. Dit multimeer karakter werd ook gevonden in 20 van de 21 F4ac$^+$ *E. coli* veldisolaten. De resultaten van de experimenten die in dit hoofdstuk worden beschreven suggereren het gebruik van GIS26 F4 fimbriae in een oraal vaccin tegen F4$^+$ *E. coli* infecties.

In hoofdstuk 5 werd de fimbriae-specifieke systemische en mucosale immuunrespons na een F18$^+$ verotoxigene *E. coli* (VTEC) (stam F107/86) infectie vergeleken met de respons na een infectie met de F4$^+$ ETEC stam GIS26. De F4$^+$ ETEC en de F18$^+$ VTEC stam verschillen in hun beide virulentiefactoren : de F4$^+$ ETEC stam heeft een major subeenheid als adhesine en expresseert het hitte-labiel enterotoxine (LT), terwijl de F18$^+$ VTEC stam een minor subeenheid als adhesine heeft en de shiga-achtige toxine II variant (SLT-IIv) secreteert. Gespeende F18-seronegatieve F18-receptor positieve biggen werden geïnfecteerd met de F18ab positieve stam en gespeende F4-seronegatieve F4-receptor positieve (F4R$^+$) biggen met de F4$^+$ stam. Een maximale excretie van bacteriën werd waargenomen 2 dagen na infectie (dpi) bij de F4$^+$ ETEC stam en tussen 3 en 5 dagen na F18$^+$ VTEC inoculatie. ELISPOT testen om het aantal fimbriae-specifieke IgM, IgA en IgG antistoffen secreterende cellen (ASCs) na te gaan, tonen hoge aantallen fimbriae-specifieke IgM ASCs in de milt 4 dpi bij beide stammen. F18-specifieke IgM ASCs waren aanwezig 4 dpi in de mesenteriale lymfeknopen (MLN) en vanaf 7 dpi in de Peyerse platen (PP), terwijl F4-specifieke IgM ASCs gedetecteerd werden in de MLN en de PP op 4 dpi. Naast de snellere inductie van een immuunrespons na F4$^+$ ETEC infectie in vergelijking met F18$^+$ VTEC infectie was er ook een snellere omschakeling van IgM naar IgA en IgG na F4$^+$ ETEC infectie. F4-specifieke IgA en IgG ASCs werden gedetecteerd vanaf 4 dpi, samen met F4-specifieke IgA antilichamen in sera en darminhouden. F18-specifiek IgA en IgG ASCs en F18-specifieke IgA werden 11 dpi gevonden in sera en darminhouden. De resultaten van deze studie suggereren dat het multimeer voorkomen van het adhesine in F4 fimbriae en/of het immunomodulatorisch effect van LT enterotoxine de mucosale immuunrespons mogelijks versnelt in de F4$^+$ ETEC geïnfecteerde biggen.

Hoofdstuk 6 beschrijft de mogelijkheid van een ‘real-time’ biomoleculair interactie analysesysteem (BIAcore® 3000), gebaseerd op de principes van

oppervlakte plasmaresonantie, voor de studie naar de interactie van F4 fimbriae en varkens enterocyten. Een binding van $F4R^+$ enterocyten aan F4 fimbriae kon worden waargenomen met dit systeem, maar er werd geen interactie gevonden tussen F4 fimbriae en enterocyten die als $F4R^-$ of zwak $F4R^+$ werden bestempeld in een *in vitro* adhesie test. Aangezien de vloeistofstroom in de biosensor deze van de *in vivo* situatie meer benadert dan de *in vitro* test is het mogelijk dat de biosensor de *in vivo* situatie beter nabootst. Bovendien biedt deze methode nieuwe mogelijkheden om de receptor-bindingscapaciteit van een F4 conjugaat te analyseren of om geneesmiddelen te zoeken voor de preventie van ETEC adhesie.

De mogelijkheid van F4 fimbriae om te werken als mucosale dragermolecule voor het modelantigeen humaan serum albumine (HSA) werd onderzocht in hoofdstuk 7. Orale immunisatie van varkens met glutaraldehyde gekoppelde HSA-moleculen (HSA/HSA complexen, HSA/HSA groep) resulteerde niet in de inductie van een HSA-specifieke immuunrespons, in tegenstelling tot een gelijktijdige orale toediening van HSA/HSA met CT (HSA/HSA+CT groep). Deze resultaten toonden dat CT werkt als mucosaal adjuvant in varkens, wat in overeenstemming is met het beschreven adjuvant effect in andere dieren. Glutaaraldehydeconjugatie van HSA aan gezuiverde F4 fimbriae resulteerde in F4/HSA complexen die in staat waren te binden aan de F4R. Orale immunisatie van $F4R^+$ varkens met F4/HSA (F4/HSA groep) induceerde een HSA-specifieke immuunrespons, hoewel deze immunisatie niet resulteerde in de inductie van HSA-specifieke antistoffen. Inderdaad, intramusculaire HSA immunisatie induceerde een secundaire immuunrespons in de F4/HSA geïmmuniseerde varkens (F4/HSA groep), terwijl een primaire respons werd geobserveerd in de oraal HSA/HSA geïmmuniseerde dieren (HSA/HSA groep). Deze gegevens tonen aan dat F4 fimbriae zouden kunnen werken als een mucosaal dragermolecule voor chemisch gekoppelde heterologe antigenen. Bovendien leidde de gelijktijdige orale immunisatie van $F4R^+$ varkens met F4/HSA en CT (F4/HSA+CT groep) tot de inductie van HSA-specifieke antistoffenresponsen in serum en speeksel die significant hoger waren dan deze in de F4/HSA en de HSA/HSA+CT groepen. De hoge HSA-specifieke respons lijkt een gevolg te zijn van een complementair effect van F4-afhankelijke binding van HSA aan $F4R^+$ enterocyten en de mucosale adjuvant eigenschappen van CT aangezien een significant lagere HSA-specifieke antistoffen respons werd waargenomen na orale immunisatie van $F4R^-$ varkens met F4/HSA en

CT. Het gelijktijdige gebruik van CT als mucosaal adjuvant en F4 fimbriae als mucosale dragermolecule zou nieuwe mogelijkheden kunnen openen in de ontwikkeling van vaccins tegen $F4^+$ ETEC en andere enteropathogenen in varkens.

In hoofdstuk 8 werd onderzocht of een veranderde interactie van F4 fimbriae met het FaeG-specifieke monoklonaal antilichaam (MAb) IMM01 in verband staat met een veranderde bioactiviteit van F4 fimbriae. Hiervoor werden gezuiverde F4 fimbriae eerst behandeld in condities die gekend zijn de opvouwing van eiwitten te veranderen. De F4R-binding en FaeG multimerizatie van gezuiverde F4 fimbriae was gereduceerd na incubatie met SDS aan een concentratie van 1.5% of hoger en een temperatuur van 85°C of hoger. Incubatie van gezuiverde F4 fimbriae bij pH 4 of lager resulteerde in een reversibele conformationele verandering die irreversibel werd in de aanwezigheid van 0.05% SDS, een concentratie die geen effect heeft op de F4 fimbriële bioactiviteit bij neutrale pH. De veranderde bioactiviteit van behandelde F4 fimbriae staat in verband met de optische densiteit bepaald in een ELISA die gebruik maakt van het FaeG-specifieke MAb IMM01. Deze snelle en gevoelige test opent dan ook mogelijkheden voor de analyse van FaeG-opvouwing na heropvouwing van recombinant eiwit, de vrijstelling van bioactief FaeG uit microcapsules en de productie van orale F4 subeenheid vaccins.

In hoofdstuk 9 werd het gen van het F4 fimbrieel adhesine FaeG van de pathogene $F4^+$ ETEC stam GIS26 gekloneerd in de pET30Ek-LIC vector en geëxpresseerd met een N-terminale His- en S-staart in het cytoplasma van BL21(DE3). Recombinant FaeG (rFaeG) subeenheden werden geïsoleerd uit onoplosbare cytoplasmatische aggregaten en heropgevouwen gebruik makend van SDS in een natief-achtige F4R-bindende conformatie. De aanwezigheid van conformationele epitopen in deze natief SDS-heropgevouwen rFaeG subeenheden werd bevestigd in ELISA en de mogelijkheid te binden aan de F4R werd bevestigd door inhibitie van adhesie van $F4^+$ ETEC aan $F4R^+$ villi met toenemende concentraties natief SDS-heropgevouwen rFaeG subeenheden. Anderzijds bleek ureum-heropgevouwen rFaeG niet aan de F4R te kunnen binden. De rFaeG subeenheden komen voor als monomeren, waar de gezuiverde F4 fimbriae multimeren zijn. Orale immunisatie van gespeende biggen met natief-achtig (SDS-heropgevouwen) rFaeG induceerde een mucosale en systemische FaeG-specifieke

immuunrespons, terwijl orale immunisatie met ureum-heropgevouwen rFaeG geen FaeG-specifieke ASC induceerde. Tevens kon worden vastgesteld dat varkens die geïmmunizeerd werden met SDS-heropgevouwen rFaeG een significant verlaagde $F4^+$ *E. coli* uitscheiding vertoonden van 2 tot 5 dagen na de challenge infectie. Verbetering van de stabiliteit en immunogeniciteit van (SDS-heropgevouwen) rFaeG is echter noodzakelijk aangezien deze na immunisatie aanleiding geven tot een lagere F4-specifieke antistoffenrespons in vergelijking met gezuiverde F4 fimbriae. Bovendien bleek de N-terminale fusie van een His- en S-staart niet schadelijk voor binding aan de F4R, wat het gebruik van rFaeG als mucosale dragermolecule suggereert. Inderdaad, orale immunisatie van varkens met SDS-heropgevouwen rFaeG resulteerde in de productie van His-S-staart-specifieke antistoffen zoals werd aangetoond in Western blot. Samenvattend stellen de resultaten van deze studie dat orale immunisatie van $F4R^+$ biggen met natief-achtig heropgevouwen rFaeG een mucosale en systemische FaeG-specifieke immuunrespons induceert en dat rFaeG kan werken als mucosale dragermolecule die antistoffen induceert tegen een genetisch gekoppeld His-S-peptide.

Hoofdstuk 10 beschrijft de resultaten van een gelijktijdige orale toediening van rFaeG en CT aan $F4R^+$ biggen om de immuunrespons tegen FaeG en het N-terminaal gekoppelde His-S-peptide te verbeteren. Orale immunisatie van varkens met rFaeG en CT verbeteren de immuunrespons tegen het heterologe peptide aangezien significant hogere His-S-staart-specifieke antistoffen werden gedetecteerd. Bijkomend werd er opgemerkt dat de gelijktijdige toediening van rFaeG en CT een verbeterde FaeG-specifieke humorale en cellulaire immuunrespons tot gevolg had, alsook een significant verlaagde uitscheiding van $F4^+$ *E. coli* na een challenge infectie in vergelijking met rFaeG geïmmunizeerde varkens. De resultaten van deze studie bevestigen dat fimbriële adhesines kunnen gebruikt worden als mucosale dragermolecule om een immuunrespons op te wekken tegen peptiden die normaal niet immunogeen zijn na orale toediening.

Het laatste hoofdstuk van dit proefschrift (Hoofdstuk 11) omvat de algemene discussie van de resultaten. De experimenten tonen het multimeer karakter, de stabiliteit en de conservatie van het F4 fimbrieel adhesine FaeG, waardoor het zeer immunogeen is en mogelijkheden biedt voor vaccins tegen $F4^+$ ETEC infecties. Het

belangrijkste besluit van het voorgestelde werk is dat zowel gezuiverde F4 fimbriae als rFaeG de capaciteit hebben te werken als een mucosale dragermolecule die in staat is na orale immunisatie een antistoffenrespons te induceren tegen een chemisch of genetisch gekoppeld heteroloog antigeen of peptide. Verder experimenteel onderzoek is echter nodig om de capaciteit van rFaeG als een mucosale dragermolecule voor antigenen aan te tonen. Deze mucosale dragersystemen gebaseerd op F4 en rFaeG, die weliswaar beperkt zijn tot varkens die tot het $F4R^+$ fenotype behoren, geven de mogelijkheid om de toepasbaarheid te onderzoeken van het richten van antigeen naar enterocyten en vervolgens een mucosale antigeen-specifieke immuunrespons op te wekken. Dit zou mogelijks een alternatief kunnen zijn voor het richten van antigenen naar M-cellen.

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Curriculum vitae

Frank Verdonck werd geboren op 7 september 1976 te Sint-Niklaas. In 1994 beëindigde hij zijn secundaire opleiding, richting Wetenschappelijke B, aan het Instituut Onbevlekt Ontvangen te Sint-Niklaas. In datzelfde jaar begon hij de studies Biologie aan de Universiteit Gent, waar hij in 1996 het diploma van kandidaat in de biologie behaalde met onderscheiding. Twee jaar later studeerde hij af als licentiaat in de biotechnologie met onderscheiding. Onmiddellijk daarna trad hij als assistent in dienst aan het Laboratorium voor Immunologie van de Huisdieren. Naast het begeleiden van de practica immunologie en het opvolgen van diagnosen, bestudeerde hij de mogelijkheid om F4 fimbriae of hun adhesine subeenheden te gebruiken als mucosale dragermoleculen bij varkens. Dit onderzoek werd uitgevoerd onder leiding van Prof. Dr. E. Cox en Prof. Dr. B. Goddeeris en leidde tot dit proefschrift. Tevens behaalde hij in 2004 het getuigschrift voor de doctoraatsopleiding in de diergeneeskundige wetenschappen. Frank is auteur of mede-auteur van meerdere wetenschappelijke publicaties.

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