Engineering of a Human Commensal Bacterium for the Controlled *in vivo* delivery of Immunomodulatory Proteins

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ABSTRACT

Inflammatory bowel disease (IBD) is a significant public health problem in western societies, affecting nearly 1 in 1000 individuals. The aetiology is not fully understood although immune system dysfunction and hypersensitivity to the intestinal microbiota plays a significant role in the pathogenesis of IBD. Several immunoregulatory agents are being used in the treatment of human IBD to control dysregulated immune responses. Growth factors such as keratinocyte growth factor (KGF-2) and transforming growth factor (TGF-β) are an important immunoregulatory and epithelial growth factor and potential therapeutic proteins for IBD. However, due to protein instability in the upper gastrointestinal tract it is difficult to achieve therapeutic levels of these proteins in the injured colon when given orally. Furthermore, short half-life necessitates repeated dosage with large amounts of the growth factor that may have dangerous side effects, such as vascularisation of non-target tissues or growth of tumours. Hence, the importance of temporal and spatial control of growth factor delivery. The work described in this thesis have overcome these issues by engineering the human commensal gut bacterium, *Bacteroides ovatus* to produce human KGF-2 or TGF-β_1 (BO-KGF or BO-TGF) in a regulated manner in response to the dietary plant polysaccharide, xylan. The successful application of BO-KGF or BO-TGF to the treatment and prevention of dextran sodium sulphate (DSS) induced murine colitis is presented here as well.

Continuous administration of xylan in drinking water to BO-KGF or BO-TGF treated mice resulted in a significant improvement of DSS-induced colitis; reducing weight loss, improving stool consistency, reducing rectal bleeding, accelerating healing of damaged colonic epithelium, reducing inflammatory cell and neutrophil infiltration, reducing expression of pro-inflammatory cytokines and promoting production of mucin-rich goblet cells in colonic crypts. These beneficial effects are comparable and in most cases superior to that achieved by conventional steroid therapy. This novel drug delivery system also had a significant prophylactic effect, limiting the development of intestinal inflammation both clinically and histopathologically.

The ability to regulate heterologous protein production by *B. ovatus* using xylan is both unique and an important safety feature of this drug delivery system. An

added advantage of using *B. ovatus* as a drug delivery vehicle is its anaerobic nature that provides a natural inbuilt biosafety feature that is lacking in other recombinant drug delivery bacteria. The use of genetically engineered *B. ovatus* for the controlled and localized delivery of epithelial growth promoting and immunomodulatory proteins has potential clinical applications for the treatment of various diseases targeting the colon including IBD.

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aa	amino acid
A260	absorbance at 260 nm
Ab	antibody
APC	antigen presenting cell
Ap^r	ampicillin resistant
ATP	adenosine triphosphate
BHI	Brain-Heart-Infusion
bp	base pair
CD	Crohn's disease
CFU	colony forming unit
COX	cyclooxygenase
CTAB	hexadecyltrimethylammonium bromide
DAB	3,3'-Diaminobenzidine
DAI	disease activity index
DC	dendritic cell
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNase I	deoxyribonuclease I
dNTP	deoxynucleoside triphosphate
DSS	dextran sodium sulphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoSorbent assay
Erm^r	Erythromycin resistant
Ep-CAM	epithelial cellular adhesion molecule
FBS	foetal bovine serum
FGF	fibroblast growth factor
5'RACE	rapid amplification of 5' complementary DNA ends
GMP	genetically modified probiotic
HRP	horse radish peroxidase
HTAB	hexadecytrimethylammonuim bromide
IBD	inflammatory bowel disease

IEC	intestinal epithelial cells
IEL	intestinal epithelial lymphocytes
INF	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
ITF	intestinal trefoil factor
Kan^r	kanamycin resistant
kbp	kilo base pair
KGF	keratinocyte growth factor
kDa	kilodalton
LB	Luria-Bertani
LP	lamina properia
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MPO	myeloperoxidase
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
OD$_{600}$	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PP	Pyere's patch
PRR	pattern recognition receptors
RBS	ribosome binding site
RNase A	ribonuclease A
RPMI	Roswell Park memorial institute tissue culture medium
RT-PCR	reverse transcription polymerase chain reaction
SAP	shrimp alkaline phosphatase
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
spp.	species

TBE	tris/borate/EDTA
TE	tris/EDTA
tet^r	tetracycline resistant
TIS	transcription initiation site
TGF	transforming growth factor
TNBS	2,4,6-trinitrobenzene sulfonic acid
Th	T helper cells
TLRs	toll-like receptors
TNF	tumour necrosis factor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER (1)

INTRODUCTION

1.1   Anatomy of the Gut

The gut (*alimentary canal*) is a musculo-membranous tube, about 9 metres long, extending from the mouth to the anus, and lined throughout its entire extent by mucous membrane. In addition to its function in digestion, nutrient transport, water and electrolyte exchange, the mucosal layer lining the gut forms a physical and immunological barrier between the host and the external environment. The gut is divided embryologically into three distinct regions, the foregut, from mouth to duodenum, the mid-gut, the jejunum and ileum up to the first half of transverse colon and the hindgut, the rest of the colon.

The gut has a uniform general histology composed of 4 concentric layers: *mucosa, submucosa, muscularis externa* (the external muscle layer) and *adventitia or serosa*. The mucosa is the innermost layer of the gut and is responsible for digestion and immunological function. The total mucosal gastrointestinal surface in an adult human can extend to 200-300 m^2 (Kato & Owen, 1999), the largest area in the body in contact with the external environment. The mucosa is further divided into three layers (Fig 1.1):

  I.   Epithelium: a single layer of Intestinal Epithelial Cells (IEC) attached to a basement membrane. This layer is covered by mucus.

 II.   Lamina propria (LP): thin connective tissue layer that supports the epithelium of the gut and contains the immune cells.

III.   Muscularis mucosa: is the thin layer of smooth muscle found in most parts of the gastrointestinal tract located outside the lamina propria.

1.2   Intestinal Epithelial Cells

Intestinal epithelial cells (IEC) originate from and are maintained by a small number of pluripotent stem cells in crypts and have a life span of 2-4 days (Creamer, 1967). It is composed of columnar absorptive cells, goblet cells, paneth cells and
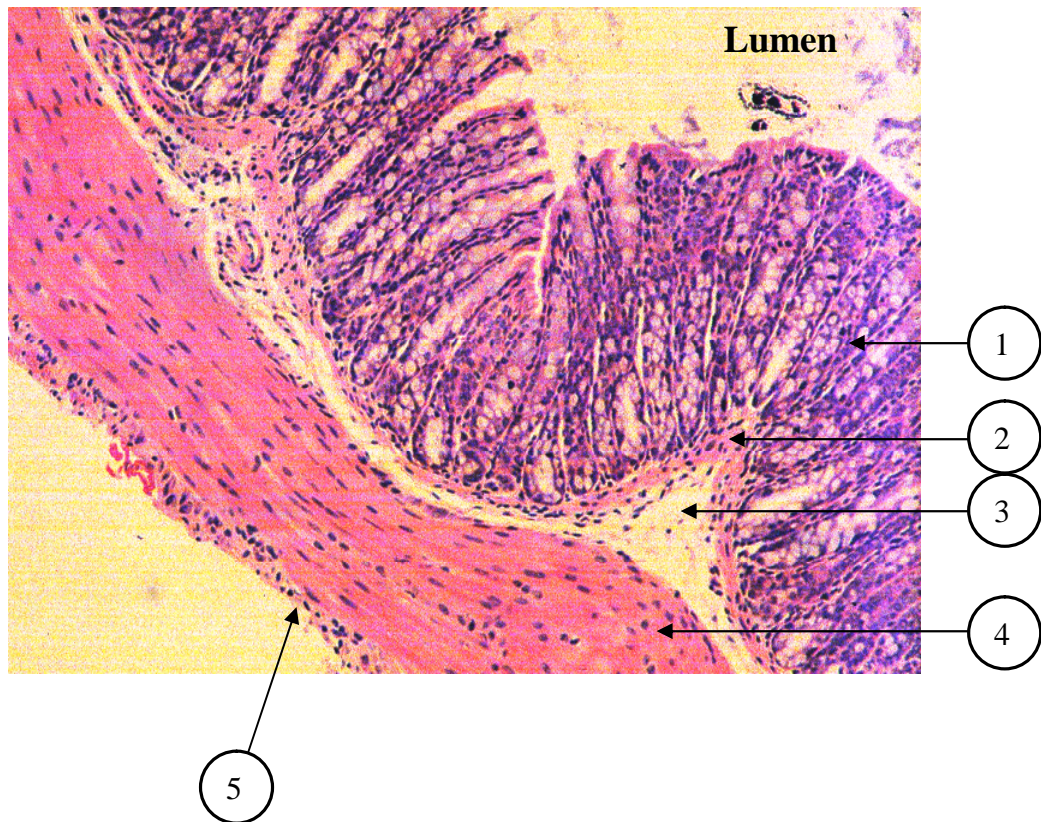
Figure 1.1   Anatomical layers of the colon

A section of murine colon stained with Haematoxylin and Eosin illustrating the layers of the gastrointestinal tract (Magnification 200X). 1- Single layer of *epithelial cells* runs the length of the gastrointestinal tract and is the initial point of contact with luminal content. 2- Directly under the epithelial cells and the space between the crypts in the *lamina propria* (LP). This area contains the vascular network and immune cells. Underneath the LP there is a thin layer of smooth muscle called the *muscularis mucosa*. Collectively the above layers are referred to as the *mucosa*. 3- *submucosa*. 4- *muscularis externa* (the external muscle layer) 5- *adventitia or serosa.*

enteroendocrine cells. Between these cells lie the intra-epithelial lymphocytes (IELs). In addition to forming a physical barrier, the epithelial lining also forms a functionally interactive barrier that is crucial for the development of the innate immune response. It is also central to the induction and expression of the mucosal adaptive immune response (Fig 1.2).

The columnar absorptive cell*s* (enterocytes) constitute the majority of the mucosal epithelium comprising > 80% of the IECs (Chang & Leblond, 1971). They undergo various stages of maturation as they migrate from crypts to the luminal surface. Enterocytes are connected with each other by junctional complexes (Boyer & Thiery, 1989). Their surfaces are covered with glycocalyx and a thick mucus layer, which is produced by the goblet cells. Paneth cells are commonly located in the crypts of the small intestine. They have various secretary granules that contain lysozyme, tumour necrosis factor (Keshav *et al.*, 1990) and cryptdin (Ouellette *et al.*, 1989). These granules are thought to prevent proliferation of the crypt microorganisms by their strong antimicrobial action. Enteroendocrine cells are distributed throughout the gut epithelium and comprise less than 1% of the IECs (Tsubouchi & Leblond, 1979). They are essentially endocrine cells that secrete specific hormones essential for the regulation of intestinal secretions and motility.

Intra epithelial lymphocytes (IELs) are located in the epithelial layer. The average number of IELs/100 absorptive cells is 20 in normal adult human jejunum and decreases distally in the gut (Dobbins, 1986). IELs are separated from IECs by 10-20 nm spaces and they do not form junctional structures with adjacent epithelial cells. However, Ep-CAM may provide physical interaction between IELs and IECs for intact barrier function (Nochi *et al.*, 2004). Almost all IELs are CD3^{+}. Among these cells, 5-15% are CD4^{+} (helper phenotype) and the rest are CD8^{+} (cytotoxic phenotype). Although the T cell receptors that mediate antigen recognition are composed predominantly of *αβ*-chains, the proportion of γδ-positive lymphocytes is much larger than in the peripheral blood (Jarry *et al.*, 1990). The exact immunological functions of IELs remain unknown, however, they have been shown to be involved in both the innate and acquired phases of mucosal immunity (Hayday *et al.*, 2001)

Follicle-associated epithelium (FAE): is a monolayer that covers the intestinal lymphoid apparatus. It is generated within the crypts from the stem cells and is characterised by the presence of specialised M cells (described later).

1.3   Functions of the IECs

Digestion and absorption of nutrients are the primary functions of IECs. In addition IECs have the following functions:

i. Physical Barrier

Like all mucosal surfaces, the intestine forms a barrier that separates the external environment, i.e., the gut lumen, from the protected internal milieu. It restricts the passive transepithelial movement of hydrophilic solutes. The main structural components for this barrier are **microvilli** of the brush borders and the **tight junctions** (Sansonetti, 2004). Each cell is encircled at the apicolateral boundary by the tight junction proteins, for example, occludin (Furuse *et al.*, 1993) and claudin (Furuse *et al.*, 1998) which incompletely seals the paracellular space.

The epithelial physical barrier is reinforced by the presence of a layer of glycocalyx which is just above the epithelial cells. It contains various enzyme and non-enzyme proteins, all of which are important for digestion and absorption of nutrients. Glycocalyx is formed from mucins that bind the apical membrane of IECs (Sansonetti, 2004).

An additional system of protection is provided by a thick layer of mucous, comprising diverse mucins. Mucous is secreted by Goblet cells and contains albumin, immunoglobulin, α1-antitrypsin, lysozyme, lactoferrin and EGF (Snyder & Walker, 1987). Several mucin genes have been identified, of which *muc*2 is predominately expressed in the colon and *muc*3 in the small intestine (Chang *et al.*, 1994). It has been shown that probiotic agents are able to inhibit enteropathogen adherence to intestinal epithelial cells through their ability to increase expression of *muc*2 and *muc*3 (Mack *et al.*, 1999). Important functions of the mucus layer are to form a semipermeable protective barrier and to help accelerate the repair of intestinal damage, particularly through intestinal trefoil factor (ITF) which further

maintains the physical barrier integrity. ITF which is secreted onto the lumenal surface by Goblet cells has an important role in protecting the intestinal mucosa from a variety of insults by promoting re-establishment of mucosal integrity (by restitution) after injury (Efstathiou *et al.*, 1998). The protective importance of ITF was demonstrated by ITF-null mice which developed more pronounced widespread colonic ulceration on exposure to DSS compared to wild-type mice (Mashimo *et al.*, 1996).

ii. Innate Immunity

Innate immunity is broadly defined as the immune response which comprises all mechanisms that resist infection but do not require specific recognition of pathogens. Intestinal epithelial cells, particularly Paneth cells, are the major producers of multiple peptides and proteins with antimicrobial activity in the intestine. The most abundant and diverse of these are the defensins (Ouellette, 1999). Other peptides such as cathelicidin, bactericidal/permeability-inducing protein, resistin-like molecule and lectins are all important microbicidal molecules that kill bacteria or interfere with their attachment to epithelial cells (Dann & Eckmann, 2007). Antimicrobial peptides have broad activity *in vitro* against Gram-positive and Gram-negative bacteria (Sansonetti, 2004), and their expression in the gastrointestinal tract is either constitutive or inducible. For example, *β*-defensin1 is synthesized constitutively in the surface epithelium of the colon (Zhao *et al.*, 1996) whereas the major inducible *β*-defensins2 and 3 are expressed only in the case of infection or inflammation (O'Neil *et al.*, 1999; Wehkamp *et al.*, 2003). Discovery of an expanding set of antimicrobial effectors supports the evolutionary importance of innate intestinal defences against microbial threats.

Attachment of enteric pathogens to epithelial surfaces results in generation of a signalling cascade which directs the trafficking of neutrophils in the basolateral-to-apical direction across the intestinal epithelium. Among the events stimulated by such pathogen-host interactions is the release of chemotaxins (such as IL-8) that guide neutrophils into the subepithelial compartment and subsequently across epithelia into the luminal compartment where they can exert their antimicrobial function (McCormick *et al.*, 1998). Furthermore, after infection, the IECs undergo

activation of the NF-κβ and activator protein-1 (AP-1) pathways (Tato & Hunter, 2002). This leads to expression of several pro-inflammatory cytokines and chemokines that coordinate the innate immune response.

iii. Adaptive Immunity

In addition to efficient physical and innate immune barrier functions, the intestinal epithelium takes an active part in the induction of adaptive immune responses at the mucosal surface. To achieve this function two components are needed: Antigen presentation and lymphoid cells. Antigen presentation is achieved by IECs, M-cells and dendritic cells (DC). T cells are abundant in all mucosal immune compartments, including gut-associated lymphoreticular tissue (GALT), the epithelial layer and the LP, and are distinct from peripheral T cells.

Pathways of antigen uptake in gastrointestinal tract

IECs: In order for IECs to act as antigen presenting cells (APCs) they must be able to internalise and process antigens. Pinocytosis is the major means by which IECs sample soluble antigens (Telega *et al.*, 2000; Warshaw *et al.*, 1971; Zimmer *et al.*, 2000). However, facilitated antigen uptake (transport of Ig-bound antigen) can take place, as well, by transcytosis, namely by CD23 (IgE FcRn) and IgG FcRn (Berin *et al.*, 1997; Dickinson *et al.*, 1999). IECs express molecules involved in the antigen processing system such as MHC class II, which is expressed constitutively predominantly in the small bowel (Lin *et al.*, 2005). Peptide antigens are processed and presented on MHC class II molecules to CD4^{+} T lymphocytes, however, in the absence of inflammation, less efficient antigen presentation takes place via class II molecules (Hershberg *et al.*, 1997). In fact, in the absence of inflammation, IECs do not express co-stimulatory molecules (CD80, CD 86), and therefore, antigen uptake will result in the induction of anergy or tolerance in a subset of CD4^{+} T cells in the intestinal mucosa (Hershberg & Mayer, 2000). Co-stimulatory molecule expression was found to be stimulated by pro-inflammatory cytokines (Nakazawa *et al.*, 1999).

Furthermore, non-classical MHC I or class Ib molecules (CD1d, MICA and MICB) were identified in IECs (Balk *et al.*, 1991; Groh *et al.*, 1996). They function in IECs antigen presentation to CD8^{+} T cells. Previous studies have suggested that

antigens presented by IECs result in the activation of CD8$^+$ regulatory T-cell subset in a non-classical MHC I molecule (CD1) restricted manner (Campbell *et al.*, 1999); later it was shown that neutralisation of HLA class I molecules by antibodies did not inhibit IEC induced proliferation of CD8$^+$ T cells (Mayer & Shlien, 1987). Recent data suggested that an additional molecule, gp180, can physically associate with CD1d and deliver signals to CD8$^+$ T cells (Yio & Mayer, 1997). In contrast to conventional APCs, IECs appear to selectively activate CD8$^+$ suppressor T cells (Mayer & Shlien, 1987).

M cells are specialised epithelial antigen presenting cells that constitute more than 10% of the cells covering the dome region of the FAE (Bockman & Cooper, 1973). M cells are characterised by the absence of surface microvilli, invaginated basolateral membrane and form "pockets", which harbour infiltrating lymphocytes (Regoli *et al.*, 1995). One of the major functions of M cells is the uptake and transport of antigens from the gut lumen to the underlying mucosal immune system; this is facilitated by reduced glycocalyx on the apical surface (Frey *et al.*, 1996). M cells are able to transport proteins, bacteria, viruses and non-infectious particles from the apical membrane to the basolateral surface. They provide consistent monitoring for potentially harmful agents (such as pathogens) while discriminating these from harmless food and non-pathogenic antigens. Therefore, M cells are known as the "sentinels" of the GI immune system (Miller *et al.*, 2007). Due to their low lysosome content, M cells can transport from the intestinal lumen with little degradation (Owen *et al.*, 1986).

There are contradictory reports about major histocompatibility complex (MHC) class II molecules expression in M cells. MHC II was found to be expressed in small bowel M cells, but not colonic M cells (Allan *et al.*, 1993; Brandtzaeg & Bjerke, 1990). However, colonic M cells express ICAM-1 (Ueki *et al.*, 1995) and cathepsin E, which is typically expressed on APCs (Finzi *et al.*, 1993), they also produce the pro-inflammatory cytokine interleukin 1 (IL-1) (Pappo & Mahlman, 1993).

Dendritic Cells (DC) is the third group of APCs in intestinal mucosa. Mucosal DCs extend dendrites (by opening the tight junctions between epithelial cells) into the intestinal lumen and continuously sample commensal and pathogenic microorganisms (Niess *et al.*, 2005). Furthermore, DCs located in the intraepithelial

pockets below the IECs detect incoming pathogens, phagocytose and process them, then present them to naïve T cells in secondary lymphatic organs such as PPs and mesenteric lymph nodes (MLNs) (Iwasaki & Kelsall, 1999). Intestinal mucosal DCs have a pivotal role in directing the migration of effector T cells to the intestine (Mora *et al.*, 2003) and the induction of oral tolerance (Viney *et al.*, 1998).

Lymphoid tissue

Effector sites for gut mucosal immune responses include lymphocytes scattered throughout the epithelium and lamina propria. These lymphoid cells are distributed throughout the digestive tract either as visible aggregates (Peyer's patches, in small intestine) or as single lymphoid follicles (in colon and rectum) (Sansonetti, 2004). These extranodal lymphoid tissues associated with intestinal mucosa are known as gut-associated lymphoreticular tissue (GALT).

There are two lineages of T cells; those that bear $\alpha\beta$ T-cell receptors (TCR) and those that bear $\gamma\delta$ TCR. In the intestinal epithelium the proportion of $\gamma\delta$ positive lymphocytes is much larger than in the peripheral blood (Jarry *et al.*, 1990). $\gamma\delta$ IELs in the gut epithelium appear to regulate epithelial homeostasis. Mice that lack $\gamma\delta$ IELs display severe disease in two different inflammatory bowel disease models owing to defects in KGF-1 mediated epithelial repair (Chen *et al.*, 2002; Inagaki-Ohara *et al.*, 2004). Further Studies of $\gamma\delta$ IEL-deficient mice have shown that $\gamma\delta$ T cells are protective against infection by many bacteria, viruses and parasites. This protective role may be explained by maintaining tight junction integrity and epithelial barrier function (Aude *et al.*, 2001; Dalton *et al.*, 2006; Roberts *et al.*, 1996).

It should be stressed that the adaptive immune response of the intestine is always oriented towards tolerance. The presence of regulatory T cells (TH3) is characteristic of the LP. These cells inhibit the activation, differentiation and proliferation of other T cells by IL-10 or TGF-β dependent mechanisms (Garside & Mowat, 2001).
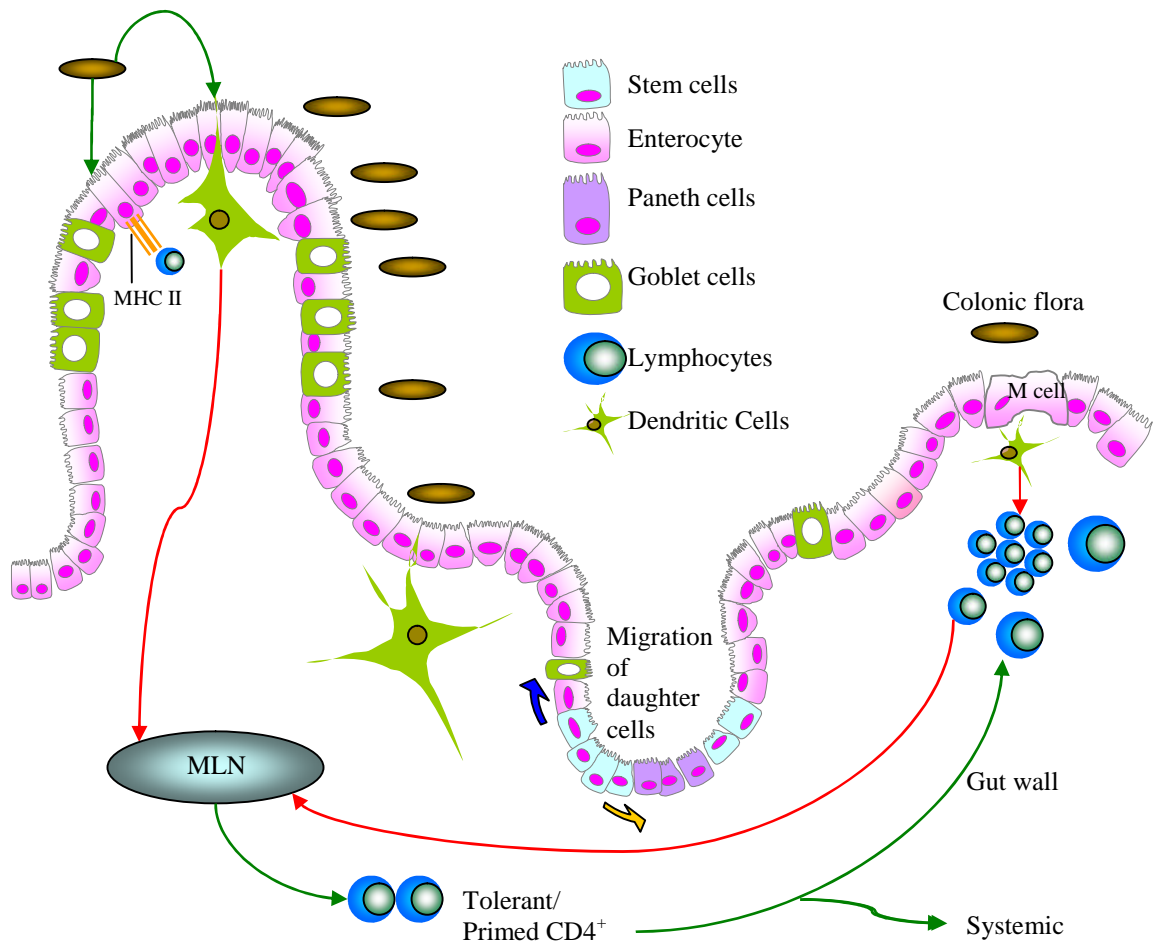
Figure 1.2   Schematic representation of the intestinal epithelial cells (IEC) and their functions. This picture demonstrates the various IECs and immune cells of the lamina propria. The predominant epithelial cells are *Enterocytes* and *Goblet cells* that form the flat mucosa with perpendicular pits (crypts). Enterocytes migrate from *Stem cells*, they undergo various stages of maturation as they migrate from crypts to the luminal surface (blue arrow) where they are sequestrated whereas *Paneth cells* migrate into the crypt base (yellow arrow) they are mainly found in the small intestine and sparsely in the colon. Mucosal *Dendritic Cells* extend dendrites into the intestinal lumen and continuously sample commensal and pathogenic microorganisms. *M cells* are specialised epithelial antigen presenting cells characterised by pocket formation which harbours infiltrating lymphocytes. *Intra epithelial lymphocytes* (IELs) are located in the epithelial layer.

1.4   Oral tolerance

The physiological response to food antigens and commensal flora is the induction of a state of specific immunological unresponsiveness (oral tolerance). Breakdown of this response would be deleterious and lead to an exaggerated inflammatory response. Two principal immunological mechanisms have been implicated in oral tolerance: clonal deletion/anergy and T cell mediated suppression. Clonal deletion of CD4$^+$ T cells via apoptosis *in vivo* has been demonstrated following high doses of OVA in Peyer's patches of TCR-transgenic mice (Chen *et al.*, 1995). Nevertheless 'non-physiological' large doses of antigen were required to demonstrate such effects. Another report showed no evidence of deletion in mice fed more conventional tolerogenic doses of OVA protein (Sun *et al.*, 1999). However, high doses of antigen given to normal mice are believed to induce clonal anergy (Doron Melamed, 1993). It has been shown by adoptive transfer of TCR-transgenic T cells that Ag-specific T cells persist *in vivo* after feeding tolerogenic doses of OVA, but are unresponsive to restimulation with antigen *in vitro* (Van Houten & Blake, 1996). Non-responsiveness due to feeding antigen results in the induction of anergy.

An alternative to clonal anergy of antigen specific T cells is the induction of regulatory T cells (T$_{reg}$) which maintain tolerance via suppression of naïve T cells. Initially, it was shown that suppressor T cells were CD8$^+$ (Miller *et al.*, 1993). Oral tolerance, however, can be induced in CD8-knockout mice (Hornquist *et al.*, 1996), but not in CD4-depleted mice (Barone *et al.*, 1995; Desvignes *et al.*, 1996). From the available data, both CD8$^+$ and CD4$^+$ T cells can serve as oral tolerance effector cells, albeit through different mechanisms. In addition, CD4$^+$ T cells may play both an inductive and effector role (Fujihashi & McGhee, 1999). It appears that oral antigens can produce antigen non-specific suppressor T cells, or "bystander suppression", i.e., suppression of responses to antigens that are totally unrelated to antigen inducing the suppressor cells (Miller *et al.*, 1991). These regulatory T cells can inhibit TH1 cell mediated immunopathologies such as encephalomyelitis and colitis (Groux *et al.*, 1997; McGeachy *et al.*, 2005). The mechanism by which T$_{reg}$ produce their effect is through production of inhibitory cytokines such as TGF-β and IL-10. However, all mechanisms may be acting at once, as repetitive oral administration of OVA induced tolerance, which appears to be composed of clonal

deletion/anergy and TGF-β_1 mediated active suppression (Omata *et al.*, 2005). It is possible that a "break" in these mechanisms leads to intestinal inflammation such as IBD.

Role of transforming growth factor-beta (TGF-β)

Transforming growth factor beta (TGF-β) was first identified as a protein secreted from sarcoma cells that promoted normal rat kidney cells to grow in soft agar (Moses *et al.*, 1981). TGF-β inhibits epithelial cell growth and regulates a diverse array of cellular functions unrelated to cellular transformation (Serra, 2002).

The TGF-βs are first synthesized as large precursor proteins with a signal sequence and a large pro-domain. They are secreted in an inactive, latent form consisting of the 25-kDa mature peptide in association with the N-terminal pro-domain of the precursor protein (Fig 1.3). Proteolytic processing of the precursor to yield mature TGF-β occurs at the cleavage site immediately preceding the mature 25 kDa dimer (Gentry *et al.*, 1988; Nunes *et al.*, 1996). TGF-β isoforms (TGF-β1, 2 and 3) have a high degree of homology in the mature peptide (60–70% amino acid identity) and they signal through the same serine/threonine kinase type I and type II cell surface receptors (Wrana *et al.*, 1994). Upon binding, the receptor complex is activated by phosphorylation and propagates the signal inside the cell through phosphorylation of receptor regulated mediators (R-Smads) which include Smad-1, 2, 3, 5 and 8. Activated R-Smads then form heterodimeric complexes with Smad-4. These heterodimeric complexes translocate to the nucleus, where they control gene expression. Inhibitory Smads (Smad6 and Smad7) have been identified that block the activation of these pathway-restricted Smads (Heldin *et al.*, 1997).

TGF-β is a pleiotropic cytokine with unique and potent immunoregulatory properties. It is produced by every leukocyte lineage, including lymphocytes, macrophages and dendritic cells, and acts both in an autocrine and paracrine manner (Fujihashi & McGhee, 1999). Mice deficient for TGF-β have a dramatic phenotype, and develop multiple inflammatory changes throughout the body leading to organ failure and death within 3 weeks of life (Shull *et al.*, 1992). This makes it difficult to
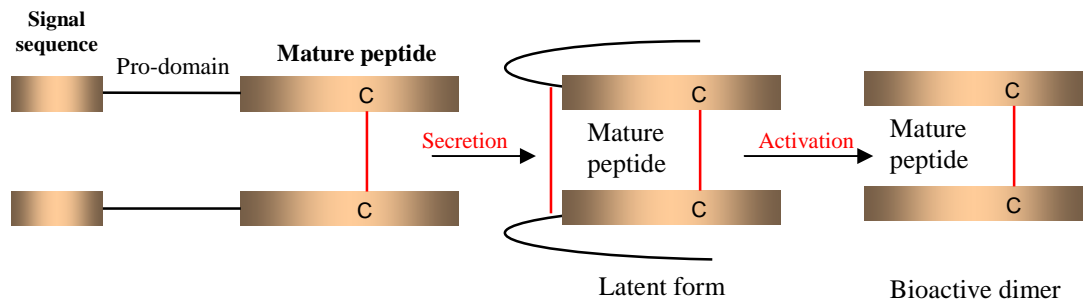
Figure 1.3   Structure of the TGF-*β* molecule

Transforming growth factor-*β* (TGF-*β*) is synthesized as a large precursor protein which has a signal sequence, pro-domain and mature peptide. Two TGF molecules are held together to form a homodimer by intermolecular disulfide bonds from the conserved cysteine residues. When TGF is secreted the signal sequence is removed by proteolytic cleavage. The pro-domain remains associated with the dimer, resulting in a latent protein complex (LAP complex). Bioactive TGF is released when the pro-domain is removed by a protease.

perform investigation over longer period of time. Recently, mice that have defects in TGF-β signalling pathways were created, these displayed a spontaneous autoimmune disease characterized by inflammatory infiltration in several organs (Gorelik & Flavell, 2000) or enhanced antigen-induced airway inflammation (Nakao *et al.*, 2000), suggesting that perturbation of the balance of this cytokine may contribute to immunopathology.

The most pronounced effect of TGF-β on immune system cells is antiproliferative. TGF-β inhibits the proliferation of T and B lymphocytes, thymocytes and NK cells. It was shown that TGF-β is a potent inhibitor of the proliferation of T cells typically in the G1 phase (Morris *et al.*, 1989a). This inhibition is thought to be mediated through induction of cell cycle inhibitors, p27KIP1 and p21CIP1 (Wolfraim *et al.*, 2004) or reduced expression of certain genes associated with cell proliferation (c-myc) (Coffey *et al.*, 1988). Furthermore, TGF-β is a strong reversible inhibitor of T helper (CD4) cell differentiation and prevents the differentiation of Th1 through suppression of transcription factor T-bet (Gorelik *et al.*, 2002; Sad & Mosmann, 1994). This inhibitory effect can be blocked by interferon-γ through upregulation of Smad-7 (the inhibitor of TGF-β signalling) (Ulloa *et al.*, 1999). Similar to Th1, TGF-β inhibits Th2 differentiation by suppression of transcription factor GATA-3 (Gorelik *et al.*, 2000) (Fig 1.4). Furthermore, TGF-β suppresses the development of $CD8^{+}$ T cell cytotoxic function (Ranges *et al.*, 1987). On the other hand, recent evidence indicates that naïve peripheral T cells may acquire a regulatory phenotype (T_{reg}) under the influence of TGF-β through induction of transcription factor FoxP3 (Chen *et al.*, 2003). T_{reg} were able to suppress inflammation in murine models of asthma, transplantation and colitis. This suppression has been shown to be TGF-β dependent (Becker *et al.*, 2006). An essential role for TGF-β signalling in maintenance of the peripheral T_{reg} cell subset or in limiting Th1 and /or Th2 could be a reason for the fatal autoimmune lesions observed in $tgfb1^{-/-}$ mice (Rubtsov & Rudensky, 2007).

Many lines of evidence implicate TGF-β in the pathogenesis of autoimmune disease. Studies of experimental allergic encephalomyelitis (EAE) and collagen induced arthritis in rodents demonstrated that systemic administration of TGF-β suppressed the symptoms of the disease whereas anti-TGFβ enhanced the disease process (Racke *et al.*, 1991; Thorbecke *et al.*, 1992). In the gut, deficiency of TGF-β

signalling contributes to the development of IBD and maintenance of TGF-β signalling may be important in regulating immune homeostasis in the intestine (Hahm *et al.*, 2001). For example, transfusing naive CD4^{+}CD45RB^{hi} T cells into SCID mice causes colitis, that can be prevented by the simultaneous infusion of memory CD4^{+}CD45RB^{lo} T cells. The protective effect of the memory RB^{lo} cells is inhibited by anti–TGF-β but not anti–IL-4 (Powrie *et al.*, 1996). Rectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) also induces IBD-like colitis, with infiltration of Th1-type T cells. However, feeding haptenated protein abrogates this Th1 responsiveness and increases production of TGF-β by lamina propria T cells (Neurath *et al.*, 1996) and disease is exacerbated in tolerant mice by treatment with anti–TGF-β antibody. Furthermore, TGF-β production is increased in inflamed mucosa of patients with Crohn's disease and Ulcerative colitis (Babyatsky *et al.*, 1996) and the frequency of CD4+CD25+ T_{reg} cells increases in inflamed IBD mucosa compared with non-inflamed mucosa (Maul *et al.*, 2005). Accordingly, an elevated CD4+ CD25+ may be a valuable index of remission in patients with IBD (Cuadrado *et al.*, 2008). Interestingly, a spectrum of growth factors and cytokines have been demonstrated to enhance epithelial cell restitution and improve intestinal wound healing through a TGF-β dependent pathway (Sturm & Dignass, 2008).

The well-characterised abilities of TGF-β to promote healing, as well as its potent immunosuppressive effects, have provided the basis for the use of TGF-β as a potential therapeutic agent in colitis. Intranasal administration of a TGF-β_1 plasmid prevented the development of Th1 mediated experimental colitis (Kitani *et al.*, 2000). The main downside, however, of TGF-β treatment is the fibrogenic effect attributed to this growth factor that ultimately results in fibrosis which may develop into colonic obstruction (Vallance *et al.*, 2005). To overcome this effect a non-fibrogenic isoform, TGF-β_3, was used in a hamster model of chemotherapy-induced oral mucositis. Application of TGF-β_3 reduced the severity and duration of the resulting mucositis (Sonis *et al.*, 1994). However, clinical trials using TGF-β_3 to treat oral mucositis was not efficient probably due to inactivation by proteases found in the fluid in the ulcer base (Foncuberta *et al.*, 2001).

While restoration of the proper flux of TGF-β by administration of ligand may alleviate the pathology, accomplishing this in only the affected tissue or cell type will be difficult. Therefore, normal homeostatic actions of TGF-β in

uncompromised cells may also be altered by treatment agents leading to unwanted and unexpected complications. For example, in a phase I trial for treatment of chronic progressive multiple sclerosis, intravenous administration of active TGF-β_2 (Calabresi *et al.*, 1998) resulted in anaemia and reversible nephrotoxicity in some patients. In addition, TGF-β has a role in carcinogenesis, where TGF-β can have tumour suppressor, as well as pro-oncogenic activities (Wakefield & Roberts, 2002). An ideal treatment therefore, would involve restoring TGF-β signalling locally in affected epithelial cells necessitating the development of specific delivery systems. Furthermore, active TGF-β has a short half-life (2-3 minutes) (Wakefield *et al.*, 1990), making it necessary for recombinant cytokine to be given frequently enough to replenish the level in the circulation every few minutes.
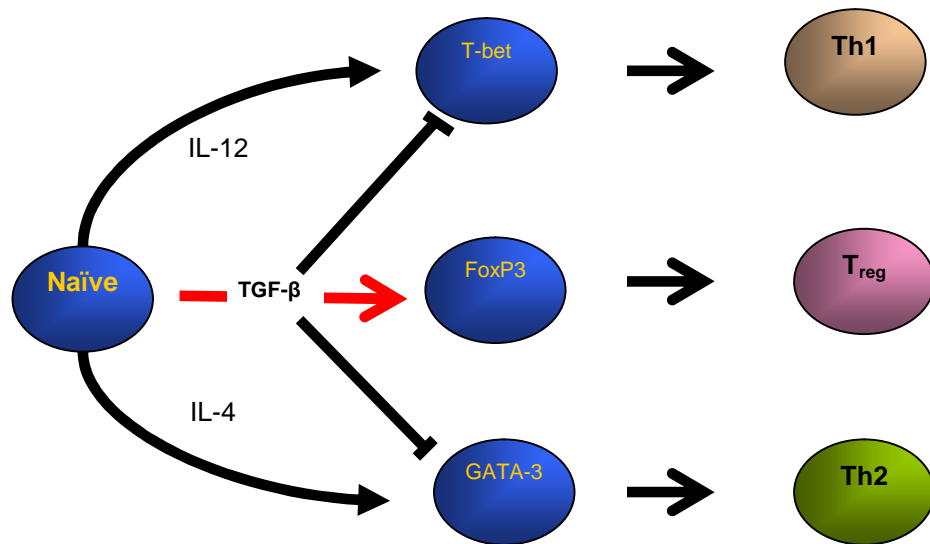
Figure 1.4   Mechanisms by which TGF-β regulates T cell differentiation.

Naïve T cells differentiate towards TH1 or TH2 effector cells upon optimal stimulation by either IL-12 or IL-4, respectively. TGF-β blocks this differentiation by suppressing the expression of the transcriptional master-regulators T-bet and GATA-3, respectively. Instead TGF-β promotes the expression of the Treg specific transcription factor FoxP3 leading to T cells with an induced regulatory phenotype.

1.5   Epithelial cells homeostasis

Epithelial cells are constantly renewed in order to maintain a protective barrier and to defend the host against environmental challenge. $\gamma\delta$IELs in the gut appears to regulate epithelial homeostasis. Mice that lack $\gamma\delta$IELs display severe disease in two different inflammatory bowel disease models owing to a defect in keratinocyte growth factor-1 (KGF-1) mediated epithelial repair (Chen *et al.*, 2002; Inagaki-Ohara *et al.*, 2004). Transfer of $\gamma\delta$IEL to C$\delta^{-/-}$ mice ameliorated TNBS-induced colitis, which correlated with a decrease of IFN-γ and TNF-α production and an increase of TGF-β production by IEL. Data indicate that $\gamma\delta$IEL play important roles in controlling IBD by regulating mucosal T cell activation cooperated with EC function, suggesting that enhancement of regulatory $\gamma\delta$T cell activity is a possible therapy for colitis (Inagaki-Ohara *et al.*, 2004).

Role of KGF-2 (FGF-10)

Keratinocyte growth factor-2 (KGF-2) is a member of the fibroblast growth factor (FGF) family. FGFs constitute a family of at least 19 structurally related polypeptides that play key roles during development and morphogenesis (Martin, 1998). Human FGF-7 was purified from a human embryonic lung fibroblast line as an epithelial mitogen (Aaronson *et al.*, 1991). FGF-7 was termed KGF-1 because of its predominant activity on this cell type. A few years later a structurally and functionally similar growth factor was isolated (FGF-10) (Emoto *et al.*, 1997; Igarashi *et al.*, 1998), this was termed KGF-2. The KGF-2 gene is located at the 5p12-p13 locus of chromosome 5. The cDNA of human *kgf-2* encodes a protein of 208 amino acids with high sequence homology (95.6%) to rat FGF-10, and 57% homology to the human KGF-1 protein. Human FGF-10 as well as rat FGF-10 has a hydrophobic amino terminus of approximately 40 amino acids, which may serve as a signal sequence. Recombinant human FGF-10 (amino acids 40 to 208, approximately 19 kDa) showed mitogenic activity for foetal rat keratinizing epidermal cells, but essentially no activity for fibroblasts (Emoto *et al.*, 1997). Heparin was found to be inhibitory for KGF-1 mitogenic activity, and stimulatory for KGF-2 (Igarashi *et al.*, 1998). The deduced protein contains 2 putative glycosylation sites, and several putative phosphorylation sites (Bagai *et al.*, 2002).

Both glycosylated and non-glycosylated KGF-1 were capable of stimulating DNA synthesis in quiescent Balb/MK mouse epidermal keratinocytes (Hsu *et al.*, 1998). The effect of glycosylation is not yet known in KGF-2, but apparently bacterially produced, non-glycosylated, KGF-2 is biologically active (Igarashi *et al.*, 1998).

KGF is expressed in fibroblasts of the urothelium lamina propria (Bagai *et al.*, 2002), lung , pancreas and gastrointestinal tract (Ye *et al.*, 2005). The cellular origin of the KGFs remain ambiguous but fibroblasts and T lymphocytes were identified by *in situ* hybridization (Finch *et al.*, 1996) as a possible source. FGFs stimulate cells by interacting with cell surface tyrosine kinase receptors (Jaye *et al.*, 1992). However, KGF is unique among FGFs in that it interacts only with a specific isoform of FGFR2, designated FGFR2b or KGFR, which is expressed exclusively by epithelial cells (Miki *et al.*, 1992). KGFR transcripts were found to be present throughout the entire gastrointestinal tract (Housley *et al.*, 1994). They display a basolateral polarized distribution on the cell surfaces in the Caco-2 monolayer cell line. These receptors are expressed mainly on the more differentiated cells located on the upper portion of the intestinal crypt, and are able to proliferate in response to the two ligands of KGFR, KGF-1 and KGF-2 (Visco *et al.*, 2004). KGF-2 binds the same receptors as KGF-1 but with higher affinity (Igarashi *et al.*, 1998). Taken together, these observations imply that KGF-1 and 2 are an endogenous paracrine effector for a variety of epithelial cells.

KGF-1 and 2 were found to be strongly upregulated in injured skin (Jameson *et al.*, 2002). This temporal modulation of FGF and KGFRs provides strong evidence for the functional involvement of FGF in human skin re-epithelialisation (Marchese *et al.*, 1995). This function was further proved by severe delay in wound healing and re-epithelialisation in transgenic mice expressing a dominant-negative FGFR2-IIIb in keratinocytes (Werner *et al.*, 1994). Unexpectedly, incisional wounds healed normally in mice lacking FGF-7 (Guo *et al.*, 1996), suggesting that KGF-1 deficiency was compensated by another KGFR2-IIIb ligand which is KGF-2 (Jameson *et al.*, 2002). These findings suggest that FGFs are crucial regulators of wound repair. A recent study showed that a single injection of KGF DNA accelerated wound closure in mice (Marti *et al.*, 2004). Topical KGF-2 is currently in clinical trials for the treatment of venous ulcers (Robson *et al.*, 2001).

In addition to the skin, KGFs appears to play a crucial role in the repair of other tissues and organs. A strong upregulation of this growth factor was observed after injury to the kidney, bladder, stomach, pancreas and intestine (Werner, 1998). KGF-1 over expression was reported in IBD patients in the lamina propria cells of non-immune origin (Finch *et al.*, 1996). Both KGF-1 and 2 were able to reduce the severity and extent of the murine gut mucosal injury induced by TNBS, DSS or Indomethacin (Han *et al.*, 2000; Miceli *et al.*, 1999; Zeeh *et al.*, 1996). Furthermore, KGF-1 was successfully used in pre-clinical models of mucositis induced by radiation and/or chemotherapy (Dorr *et al.*, 2005; Farrell *et al.*, 2002).

In clinical trials, however, high doses of recombinant systemic KGF-2 was needed for a longer period to achieve therapeutic effect in active ulcerative colitis (Sandborn *et al.*, 2003b). These high doses may not be tolerated and hence systemic administration may not be the optimal way of delivery. Therefore, an alternate route of delivery has been tried. Rectal administration was used to deliver EGF (another member of the FGF family) to treat ulcerative colitis (Sinha *et al.*, 2003). The convenience of using the rectal route for ill patients is questionable. Oral route is probably more appropriate. However, development of growth factors is hampered by its instability, as manifested by rapid aggregation and chemical modification on exposure to extreme environmental factors such as high temperature, low pH (Zhang *et al.*, 1995) and protease rich environments. Protein stability makes the oral route not ideal, as these proteins have to pass the extreme conditions of the stomach. Therefore, the development of more sophisticated delivery systems for this rapidly expanding class of therapeutic agents has not kept pace. The use of genetically-modified bacteria to deliver biologically active, therapeutic molecules directly to the gut mucosa has become an increasingly intensive area of investigation as part of the search for new therapeutic options for intestinal disorders such as IBD.

1.6 Bacteria in the Gut

The normal human large intestine contains an enormous indigenous microbiota consisting of bacteria of many genera and hundreds of species. They may number more than 10^{14} bacteria comprising over 1000 species. Gram-negative and Gram-positive, facultative, microaerophilic and strictly anaerobic species are present (Egert *et al.*, 2006; Whitman *et al.*, 1998). The total population of these organisms

increases distally in the gastrointestinal tract until the terminal ileum where bacterial diversity and number is almost similar to that of the colon where it contributes to 60% of the faecal mass (O'Hara & Shanahan, 2006). Despite DNA fingerprinting and cloning-sequencing technology which have provided a view of the diversity profile of microbial ecosystems based on comparisons of 16S rRNA genes, a significant number of organisms remain unclassified (Muyzer *et al.*, 1993). However, anaerobes comprise the majority of bacteria in the human colon; the most numerically predominant of these are members of the genus *Bacteroides* which comprise over 50% of the flora (Croucher *et al.*, 1983; Poxton *et al.*, 1997). Most of the inferred organisms from 3 subjects by 16S rRNA analysis were members of the *Firmicutes* and *Bacteroidetes* phyla (Eckburg *et al.*, 2005). In another report, three phylogenetic groups contained 95% of the clones: the *Bacteroides* group, the *Clostridium coccoides* group, and the *Clostridium leptum* subgroup (Suau *et al.*, 1999). The samples in the above studies were obtained either surgically or enoscopically and therefore subjects were exposed to pre-procedure mechanical and chemical preparations which may influence flora composition. Table 1.1 shows the bacterial genera yield from 141 person (Finegold *et al.*, 1983).

Table 1.1 Indigenous Bacterial Genera in human faeces (Finegold *et al.*, 1983)

Genus	Predominance (%)*	No**
Bacteroides	**99.3**	**11.3 (9.2-13.5)**
Fusobacterium	18.4	18.4 (5.1-11.0)
Butyrivibrio	0.7	7.5
Clostridium	100	9.8 (3.8-13.1)
Actinomyces	7.8	9.2 (5.7-11.1)
Propionibacterium	9.2	8.9(4.3-12.0)
Bifidobacterium	74.0	10.2 (4.9-13.4)
Eubacterium	94.0	10.7 (5.0-13.3)
Lactobacillus	78.0	9.6 (3.6-12.5)
Streptococcus	34.0	10.3(7.0-12.6)
Ruminicoccus	45.0	10.2(4.6-12.8)

*of 141 person tested yielding species of genus listed. ** Mean and range of $\log_{10}$ of the number of microorganisms/g faeces

Gut organisms are involved in numerous metabolic activities in the colon, including fermentation of carbohydrates, utilisation of nitrogenous substances, and biotransformation of bile acids and other steroids (Hentges, 1989). This complex metabolic activity recovers valuable energy and absorbable substances for the host, and provides energy and nutrients for bacterial growth. Furthermore, the intestinal microflora adhere to the surface of epithelial cells and mucin associated with the intestinal wall, with *Bacteroides* being the most common anaerobic colonizer (Croucher *et al.*, 1983). By coating the walls of the intestine, it is believed that the microflora prevents transient bacteria from binding the intestinal surface, and the transients are subsequently lost with the luminal contents during peristalsis. Therefore, *Bacteroides* and other anaerobes provide an additional benefit to their host in excluding pathogenic organisms from colonizing the intestine (van der Waaij *et al.*, 1971). In addition, gut microflora are believed to be engaged in cross talk with cytokine networks, and this communication may inhibit intestinal inflammation for example, *Bacteroides thetaiotaomicron* has been shown to modulate expression of genes involved in several important intestinal functions, including improvement of barrier function (Hooper *et al.*, 2001). Therefore, enteric bacteria form a natural defence barrier and exert numerous protective, structural and metabolic effects on the epithelium. These benefits have been shown in comparative studies of germ-free and colonised animals. Germ-free animals are more susceptible to infections, have reduced digestive enzyme activity and cytokine production (Shanahan, 2002). Furthermore, the gut microbiota has been shown to influence the development and function of the gut mucosal immune system which becomes tolerant to or fails to mount a response to the gut bacteria (Savage, 1999). A breakdown in immune tolerance to members of the microbiota has been shown to be a contributing factor in the pathogenesis of IBD (Baumgart & Carding, 2007).

Bacteroides is currently the only genus of Gram-negative anaerobic commensal which can be manipulated genetically. Complete genome sequences are available for some *Bacteroides* species such as *B. thetaiotaomicron* (Xu *et al.*, 2003). Some Bacteroides species can utilize xylan as a sole source of carbohydrate (Salyers *et al.*, 1981). The best studied xylan degrading *Bacteroides* species is *B. ovatus*. *B. ovatus* is a major commensal colonic Gram-negative bacterium in humans and rodents (Savage, 1999). Xylan utilization genes of *B. ovatus* have been partially

cloned (Whitehead & Hespell, 1990) and was shown that the xylanase (*xyl*I) gene is part of the same operon, of which only one other gene has been fully identified, xylosidase-arabinosidase (*xsa*). It has been further shown that this operon is under the control of a xylan-inducible promoter (Weaver *et al.*, 1992), although the promoter has not been identified. Disruption of the xylan utilization gene resulted in reduced growth rate of *B. ovatus* on xylan (Weaver *et al.*, 1992) and affected its ability to colonise the mice colon (Carding SR, personal communication). Our group have previously engineered *B. ovatus* to produce and secrete biologically active murine interleukin-2 (IL-2) in response to the dietary polysaccharide xylan (Farrar *et al.*, 2005) However, insertion of the IL-2 gene into the known part of the xylanase operon disrupted transcription of the xylanase genes. Lack of xylanase production by the recombinant organisms may result in a competitive disadvantage when introduced into the gut.

1.7   Xylan

Hemicellulose or xylan is a major component (20-40%) of plant material (Thompson, 1993). Xylans are chemically complex, and highly branched, they frequently have acetyl groups attached by ester linkages. Only very small amounts of xylans are degraded by the human digestive system (Hespell & Whitehead, 1990). Therefore, several bacterial systems have evolved to degrade xylans. In the human colon, *B. ovatus* is a major xylanolytic bacterium and the best studied xylan degrading *Bacteroides* species (Salyers *et al.*, 1981; Weaver *et al.*, 1992). For the bacterial system to degrade the xylan backbone, first it needs to break the substituents such as the acetyl group using the enzyme acetylesterase then endoxylanase releases the xylooligosaccharides this then followed by xylosidases which cleave the oligomeric fragments to xylose (Thompson, 1993). Therefore, xylanase genes are genetically related to acetylases and xylosidases such as the *B. ovatus* and *Prevotella bryantii* xylanase systems (Miyazaki *et al.*, 2003; Weaver *et al.*, 1992). However, it is not yet known how much xylan is present in the regular human diet.

Xylooligosaccharides (XO) which are the main soluble products from xylan hydrolysis, have a prebiotic effect when ingested as part of the diet (Moure *et al.*,

2006). *In vitro* evaluation of commercial prebiotic oligosaccharides concluded that XO increased the number of *Bifidobacteria* (Rycroft *et al.*, 2001) and *Lactobacillus* spp. (Ross Crittenden *et al.,* 2002). Furthermore, XO was reported to reduce concentrations of secondary bile acids which are considered tumour promoters (Zampa *et al.*, 2004). Besides, the colonic XO fermentation leads to the production of CO_2, H_2, short chain fatty acids (SCFA) and lactate. The latter may be further metabolised systemically or locally to provide energy for the host. A number of health effects have been reported for SCFA, including improvement in bowel function, calcium absorption, lipid metabolism and reduction of the risk of colon cancer (Hsu *et al.*, 2004; Scheppach *et al.*, 2001). Controlled administration of XO may help avoid intestinal disorders such as constipation, IBD and diarrhoea (Moure *et al.*, 2006). Therefore, XO can be mixed with other prebiotics to achieve synergistic effects or make part of synbiotic preparations together with probiotic microorganisms.

1.8   Inflammatory Bowel Disease (IBD)

1.8.1   Definition

IBD is an idiopathic chronic inflammation of the lining of the gut. Ulcerative colitis (UC) and Crohn's disease (CD) represent the two main types of inflammatory bowel disease. UC causes diffuse mucosal inflammation in the colon, and CD causes a patchy transmural inflammation that may affect any part of the gastrointestinal tract. Although, the aetiology remains unknown, the current leading hypotheses for the aetiology of IBD emphasizes genetic predispositions to dysregulation of the gastrointestinal immune system (Podolsky, 2002).

1.8.2   Epidemiology

In general, the highest incidence rates and prevalence for both CD and UC have been reported from northern Europe, the United Kingdom and North America, which are the geographic regions that have been historically associated with IBD (Loftus, 2004). Studies of migrant populations suggest that differences may be more

related to lifestyle and environmental influences than true genetic differences. Prevalence equals over time with migration to other geographic areas (Niv *et al.*, 2000). In the UK, the incidence for CD is currently approximately 2 cases per 100,000 person-years, and for UC is 6-8 cases per 100,000 person-years (Carter *et al.*, 2004) (www.patient.co.uk), and the prevalence for CD is 122 and for UC 214 cases per 100,000 persons (Loftus, 2004).

1.8.3   Predisposing factors

IBD results from the interplay between multiple genetic and environmental risk factors. Therefore, a specific genetic factor is neither necessary nor sufficient to develop the disease.

i. Genetic

There is ample evidence that CD and UC are, in part, the result of a genetic predisposition.

Familial aggregation: a positive family history is still the largest independent risk factor for the disease. The risk of IBD is highest in first-degree relatives of an IBD proband but more distant relatives are also at increased risk. People with CD have a first-degree relative with CD in 2.2–16.2% of cases and with IBD in 5.2–22.5% of cases. People with UC have a first-degree relative with UC in 5.7–15.5% of cases, and with IBD in 6.6% to 15.8% (Baumgart & Carding, 2007; Russell & Satsangi, 2004).

Disease aggregation in twins: The strongest evidence of genetic factors contributing to IBD comes from concordance studies in twins (Orholm *et al.*, 2000; Thompson *et al.*, 1996; Tysk *et al.*, 1988). From the above studies, the concordance rates for monozygotic twins are 36% for CD and 16% for UC, and for dizygotic twin pairs are 4% for both diseases. These twin studies demonstrate a greater genetic influence for CD compared with UC. It is clear, from these studies, that the risk of IBD is significantly higher in family members, with twins being the highest risk group, followed by first-degree relatives.

Susceptibility gene: Susceptibility genes in IBD were studied by two strategies; genome-wide screening and positional cloning techniques. Nine susceptibility genes have been identified so far, named IBD1-9, some loci seem specific to CD (e.g., IBD1 or NOD2 on 16q) or UC (e.g., IBD2 on 12q), others seem to confer susceptibility to inflammatory bowel disease overall (Gaya *et al.*, 2006). CARD15/ NOD2 is widely recognized as a susceptibility gene for CD, and accounts for 10-15% of patients with this disease (Hugot *et al.*, 2001). The risk of developing CD for mutated individuals has been estimated to be 17 fold (95% CI 10.7-27.2) higher than normal control (Economou *et al.*, 2004). How these mutations give rise to susceptibility of CD is not fully understood, however, unresponsiveness of this innate immune receptor results in overactivation of the mucosal immune system to components of commensal intestinal microbiota and the development of inflammatory lesions (Baumgart & Carding, 2007). Genes of the major histocompatibility complex are implicated as important inherited determinants of susceptibility to UC and may also influence the pattern of disease (Satsangl *et al.*, 1996). A significant positive association between HLA-DR2 and UC has been reported (odds ratio 2.00, 95% CI 1.5–2.63) (Yap *et al.*, 2004). In contrast expression of DR4 and Drw6 is protective against UC (Satsangl *et al.*, 1996).

ii. Environmental

Geographic: epidemiological studies suggest a gradient of IBD incidence exist from north to south (Loftus, 2004). Different incidence rates could also result from different genetic backgrounds of the residents of the different parts of the world.

Appendecectomy: several studies suggested that appendecectomy both reduces the likelihood of developing UC and gives rise to a milder disease phenotype in UC (Feeney *et al.*, 2002; Radford-Smith *et al.*, 2002). The opposite is true for CD, with appendecectomy being associated with an increasing risk of developing stricture (Cosnes *et al.*, 2006).

Microbial: *Mycobacterium paratuberculosis,* measles virus and *Listeria monocytogenes* have been implicated in the pathogenesis of IBD (Ardizzone *et al.*, 1999). Differences in microbial ecology in individuals with IBD and normal

individuals may somehow contribute to the disease. Patients with active IBD have got significantly less anaerobes and *Lactobacillus* (Fabia *et al.*, 1993). In experimental models of IBD, colitis results from an abnormal immune response to a normal flora antigenic stimulus (Sadlack *et al.*, 1993), and it does not develop in a germ-free environment (Taurog *et al.*, 1994). The finding that mucosal mononuclear cells from patients with IBD proliferate when exposed to autologous intestinal bacteria supports the concept that bowel flora may act as antigenic stimuli in IBD (Duchmann *et al.*, 1995). Further evidence comes from the ability of narrow and broad spectrum antibiotics to prevent experimental colitis (Hoentjen *et al.*, 2003).

Life style: **Smokers** are less likely to develop UC. In families with siblings affected by either UC or CD, cigarette smoking continues to demonstrate a protective role against UC (Bridger *et al.*, 2002). In contrast, smoking aggravates the course of CD, and smoking cessation appears to be an effective therapeutic intervention in CD (Cosnes, 2004).

The traditional low incidence of IBD in developing countries might be related to **socioeconomic changes** affecting hygiene (Gent *et al.*, 1994). Excessive sanitation might limit exposure to environmental antigens and impair the functional maturation of the mucosal immune system and induction of immune tolerance, which results in an inappropriate immune response when re-exposed to these antigens later in life.

Psychological stress has been reported to increase disease activity in IBD. Recent well designed studies have confirmed that adverse life events, chronic stress, and depression increase the likelihood of relapse in patients with quiescent IBD (Mawdsley & Rampton, 2005).

1.8.4 Immunopathology

Most students studying the pathogenesis of IBD have adopted the view that the disease is due to a dysfunctional interaction between the bacterial microflora of the gut and the mucosal immune system. Evidence suggests that several pathways might result in activation of the inflammatory cascade.

Human studies have shown a lowered epithelial resistance and increased permeability of the inflamed and non-inflamed mucosa in CD and UC (Gitter *et al.*,

2001; Soderholm *et al.*, 2002). The break of intact barrier function may provide entrance for excess commensal bacterial antigens to provide sufficient stimuli via the TLR system and induce inflammation. On the other hand the barrier defect may allow the entry of a large number of commensal organisms which can induce an immune response sufficiently robust to initiate inflammation (Hermiston & Gordon, 1995).

Patients with IBD have a different pattern of TLR expression from normal individuals. IEC of normal mucosa constitutively expressed TLR3 and TLR5, while TLR2 and TLR4 were only barely detectable. TLR3 was significantly downregulated in IEC in active CD and TLR4 was strongly upregulated in both UC and CD (Cario & Podolsky, 2000). Changes in TLR expression and signalling may contribute to changes in the host response to bacterial antigens seen in colitis (Singh *et al.*, 2005). An upregulation of NOD2 has also been reported in inflamed mucosa of IBD patients which appears to play a key role against bacteria by triggering a host defence response through the activation of the transcription factor NF-κβ and a consequent proinflammatory cytokine production. This might compromise the ability of the host to eliminate invasive and pathogenic microbes resulting in chronic inflammation (Berrebi *et al.*, 2003; Rosenstiel *et al.*, 2003; Stronati *et al.*, 2008).

Another recognised defect in the mucosa of IBD people is the disturbed antigen presentation function. Dendritic cells may incorrectly recognise commensal bacteria and induce a Th1 proinflammatory immune response, due to microbial recognition receptor up-regulation (Hart *et al.*, 2005). Atypical APC may become potent effector T-cell activators in people with IBD. IEC (non-professional APC) acquire an activated phenotype with increased histocompatibilty molecule expression in the presence of interferon-γ and TNFα (Cruickshank *et al.*, 2004).

The balance of regulatory and effector T cells is disturbed in IBD. When the disease is active, effector T cells (Th1 and Th2) predominate over regulatory T cells leading to the release of inflammatory cytokines which stimulate other immune cells such as macrophages and NK T cells and further augment inflammation (Baumgart & Carding, 2007; Martin *et al.*, 2004).

The other major hypothesis of pathogenesis of IBD is a problem with the microflora, which induces a pathologic response from normal immune system. The

reduction in mucosa-associated *Bifidobacteria* and increase in *E. coli* and clostridia in patients with IBD supports the hypothesis that bacterial imbalance may contribute to its pathogenesis (Mylonaki *et al.*, 2005). *E. coli* was detected more frequently and in greater numbers in samples from patients with IBD than in samples from control. The frequency and numbers, however, were not related to the severity of the disease (Fujita *et al.*, 2002). Overall, any reported differences in phylogenetic groups between patients and controls were not sufficiently reproducible to justify the conclusion that the microflora was associated with IBD (Prindiville *et al.*, 2004).

1.8.5   Current Therapy of IBD

The currently available therapeutic armamentarium for IBD includes agents that reduce inflammation during active disease and are illustrated in Table 1.2.

Table 1.2   Summary of current medical therapy for IBD

Medical Therapy	Clinical use	Mode of action
Aminosalicylate (Sulfasalazine, Mesalazine and 5-aminosalicylate (5-ASA))	Mild-moderate active CD, maintain remission in UC	They act on epithelial cells to moderate the release of lipid mediators, cytokines and reactive oxygen species (Carter *et al.*, 2004)
Corticosteroids	Moderate-severe active CD and UC	Suppress inflammation by several mechanisms, including decreased transcription of multiple inflammatory genes and increased transcription of anti-inflammatory genes (Barnes, 1998)
Immunomdulators Thiopurines (6-MP, AZA)	For both active disease and maintaining remission	Inducing T cell apoptosis by modulating cell signalling (RacI) (Tiede *et al.*, 2003)
Methotrexate	Induce remission in CD	Anti-inflammatory effect in colitis is unknown
Cyclosporine	Induce remission in refractory colitis	Inhibit calcineurin, preventing clonal expansion of T-cells (Hawthorne, 2003)
Infleximab	Active and fistulating CD	Chimeric anti-TNF monoclonal antibody with potent anti inflammatory effect (Rutgeerts *et al.*, 2004)
Antibiotics	fulminant colitis and toxic megacolon mild-moderate active CD	Change the composition of bacterial flora

1.8.6   Investigational therapies for IBD

i. Non-Biologic therapy

Immunomodulatory agents used for transplant and autoimmune disease have been investigated for potential activity in IBD, such as Thalidomide, Tacrolimus and Mycophenolate mufetil. These agents were promising after initial trials. More randomised double blinded controlled trials, however, are needed to evaluate the efficacy (Ehrenpreis *et al.*, 1999; Neurath *et al.*, 1999; Sandborn *et al.*, 2003a).

ii. Biologic therapy

Recent advances in our understanding of the pathophysiology of inflammation and in bioengineering have led to new therapeutic concepts targeting almost every aspect of the inflammatory process and help with the restitution of mucosal integrity. Table 1.3 illustrates most recent investigational therapeutics for IBD. Further details on future therapeutics was reviewed by Baumgart and Sandborn (Baumgart & Sandborn, 2007).

iii. Probiotic therapy

Evolution of Probiotic therapy for IBD

As early as 1907 Elie Metchinkoff attributed a beneficial effect on human health to the consumption of *Lactobacillus* as present in yogurt (Metchinkoff, 1907). Currently, live non-pathogenic microbial supplements that colonise the gut while providing benefits to the host known as probiotics. The manipulation of intestinal microbiota has received little attention, and the few studies addressing this option have been carried out with antibiotics. Due to the possible role of indigenous microbiota in the pathogenesis of IBD (Darfeuille-Michaud *et al.*, 2004), the external manipulation of its composition using probiotic organisms seems to be promising therapeutic form.

Table 1.3   Summary of Biologic investigational therapy for IBD

Compound	Target	Class	References
CDP571	TNF	Humanised mAb	(Feagan *et al.*, 2005)
MLN02 Natalizumab	α4β7-integrin	Humanised mAb	(Feagan *et al.*, 2005) (Sandborn *et al.*, 2005)
Alicaforsen	ICAM-1	Antisense oligonucleotide	(Yacyshyn *et al.*, 2002)
Repifermin (KGF2)	Epithelial cells	Growth factor	(Sandborn *et al.*, 2003b)
Epidermal growth factor	Epithelial cells	Growth factor	(Sinha *et al.*, 2003)
Sagramostim	GM-CSF	Growth factor	(Korzenik *et al.*, 2005)
Interleukin-10	IL-10	Recombinant human cytokine	(Fedorak *et al.*, 2000)
Daclizumab	IL-2	Humanised mAb	(Van Assche *et al.*, 2006)
ABT-874	IL-12	Humanised mAb	(Mannon *et al.*, 2004)
Cathelicidin	Innate immunity	Antimicrobial peptide	(Tai *et al.*, 2007)
Interferon-α	INF-α	Recombinant INF	(Tilg *et al.*, 2003)
Interleukin-11	IL-11	Recombinant human cytokine	(Herrlinger *et al.*, 2006)
Rosiglitazone	PPAR-γ	PPAR- γ agonist	(Lewis *et al.*, 2008)

Favier and colleagues (Favier *et al.*, 1997) cultured and counted the colonic microflora in faeces from patients with active Crohn's disease. *Bifidobacteria* numbers were significantly reduced in patients compared with healthy controls and bacterial enzyme activities, especially ß-D-galactosidase, were also decreased in faecal extracts from Crohn's disease patients, correlated with the decrease of bifidobacteria counts. Another investigation (Fabia *et al.*, 1993) had analysed colonic mucosa associated microflora in patients with active and inactive ulcerative colitis. A significant decrease in the number of anaerobic bacteria and anaerobic Gram negatives was shown in patients with active ulcerative colitis. Data from previous studies suggested that probiotic administration can help restore microbial homoeostasis in the gut, down-regulate intestinal inflammation, and ameliorate the disease (Bai & Ouyang, 2006). Several studies in animal IBD models have yielded encouraging results, the most promising results obtained in preventing colitis after

administration of *Lactobacillus reuteri*, *L.plantarum,* non-pathogenic *E.coli* (serotype O6) or *Bifidobacterium* (Thompson-Chagoyan *et al.*, 2005).

A number of trials in patients with IBD report successful use of probiotics. Probiotic strains used with there clinical effects are illustrated in Table 1.4.

Table 1.4   Summary of probiotic strains in clinical use

Probiotic strain	Clinical effect	Reference
E.coli Nissle 1917	Reduce the risk of relapse and reduce steroid requirement in CD patients	(Rembacken *et al.*, 1999)
Lactobacillus GG	Improve gut barrier function and clinical status in children with CD.	(Gupta *et al.*, 2000)
	No benefit in inducing or maintaining remission in CD.	(Schultz *et al.*, 2004)
Bifidobacterium infantis	protected the gut epithelial layer including the PP from being invaded by *Bacteroides vulgatus*	(Shiba *et al.*, 2003)
Combined preparation of:	Reduce relapse in patients with pouchitis.	(Gionchetti *et al.*, 2000)
Lactobacillus		
Bifidobacteria	Maintenance treatment of UC	(Venturi *et al.*, 1999)
Streptococcus salivarius		

The possible therapeutic effects of probiotics on inflammatory bowel diseases are summarised in Figure 1.5. They may imply alteration of the microbial content and displacement of noxious bacteria by modifying pH or producing antibacterial compounds, such as the low-molecular-weight antimicrobial compounds in the culture filtrate of *L. plantarum* and antibacterial compounds described in the culture supernatant of *Bifidobacterium* strains (Lievin *et al.*, 2000; Niku-Paavola *et al.*, 1999). Displacement of bacteria may be a consequence of physical competition for binding, such as the inhibitory effect of adherent human bifidobacterial strains against colonization by a number of diarrheagenic bacteria (*E. coli* 0157; *S. typhimurium*) and viruses (murine and rhesus *Rotavirus*) (Duffy, 2000). Some bacteria can induce cytokine production by epithelial and immune cells (Lammers *et al.*, 2002) or interfere with the pro-inflammatory IκB/NFκB signaling pathway in intestinal epithelial cells by blockade of IκB-α degradation and may indeed provide a way to modulate local immune response (Neish *et al.*, 2000). Other probiotics may lead to an increase in local IgA levels which may also contribute to enhancement of mucosal resistance against gastrointestinal infections for example

Lactobacillus GG promotes IgA-secreting plasma cell numbers in CD patients (Malin *et al.*, 1996). A notable feature of *L. plantarum* is its ability to generate nitric oxide as a side product of arginine catabolism which could be the reason behind probiotic effects of this organism (Jónsson *et al.*, 1983). Furthermore, probiotic bacteria, such as *bifidobacteria*, can sequester toxins and drain these from the intestine (Oatley *et al.*, 2000).

Genetically modified probiotic (GMP)

The exploitation of microbes is no longer limited to their role as live probiotics or as cell factories for production of human therapeutics. Commensal and food grade bacteria can be engineered for delivery of anti-inflammatory cytokines or other biologically active molecules and vaccines to the gut. *Lactococcus lactis* was engineered to coexpress tetanus toxin (TTFC) and either murine IL-2 or IL-6 (Steidler *et al.*, 1998). This system was the first example of the use of a cytokine secreting bacterial vaccine to enhance immune responses and pointed the way to experiments which tested the therapeutic effect of this mode of cytokine delivery. Schotte *et al.*, took this step further and designed the *L. lactis* system that secrete biologically active IL-10 and proposed this as a possible way of delivering IL-10 to the inflamed bowel in IBD (Schotte *et al.*, 2000). Efficacy of *L. lactis* secreting IL-10 have been demonstrated within the gut in a murine model of IBD (Steidler *et al.*, 2000). Human phase I clinical trial with IL-10 producing *L. lactis* for Crohn's disease has opened new avenues for the use of transgenic bacteria as delivery vehicles (Braat *et al.*, 2006). This trial demonstrated the feasibility of using genetically modified bacteria for mucosal delivery of therapeutic proteins in human being. Importantly, biological containment was successfully demonstrated in Braat's trial. However, it is too early to draw a conclusion about its clinical efficacy as there was not enough clinical data in this study. Other examples of GM microbes include the delivery of trefoil factor to promote healing of the inflamed gut and single-chain antibodies for pathogen specific passive immunity (Kruger *et al.*, 2002).

Although effective, the *L. lactis* system has significant drawbacks. Chief among these is the issue of safety. Despite being designed in a way so that the transgenic bacteria cannot live in the absence of thymidine synthetase, transgenic *L.*

lactis can still survive in the thymidine deficient media for more than 72 hours. Trace amounts of thymidine present in the culture media would increase the cell viability to more than 100 hours (Steidler *et al.*, 2003). A second safety issue is the constitutive cytokine secretion by the *L. lactis* system. Expression of IL-10 is driven by the constitutive lactococcal P1 promoter (Schotte *et al.*, 2000). Lack of regulation of the production of the therapeutic molecules can cause unwanted side effects. Thirdly, *L. lactis* is non-colonising bacterium, therefore, repeated dosing is required to achieve therapeutic levels of the secreted cytokines. The GM *L. lactis* used in the treatment of murine IBD was administered in daily gavages of the animals and in a human trial it was given as twice daily dosage.
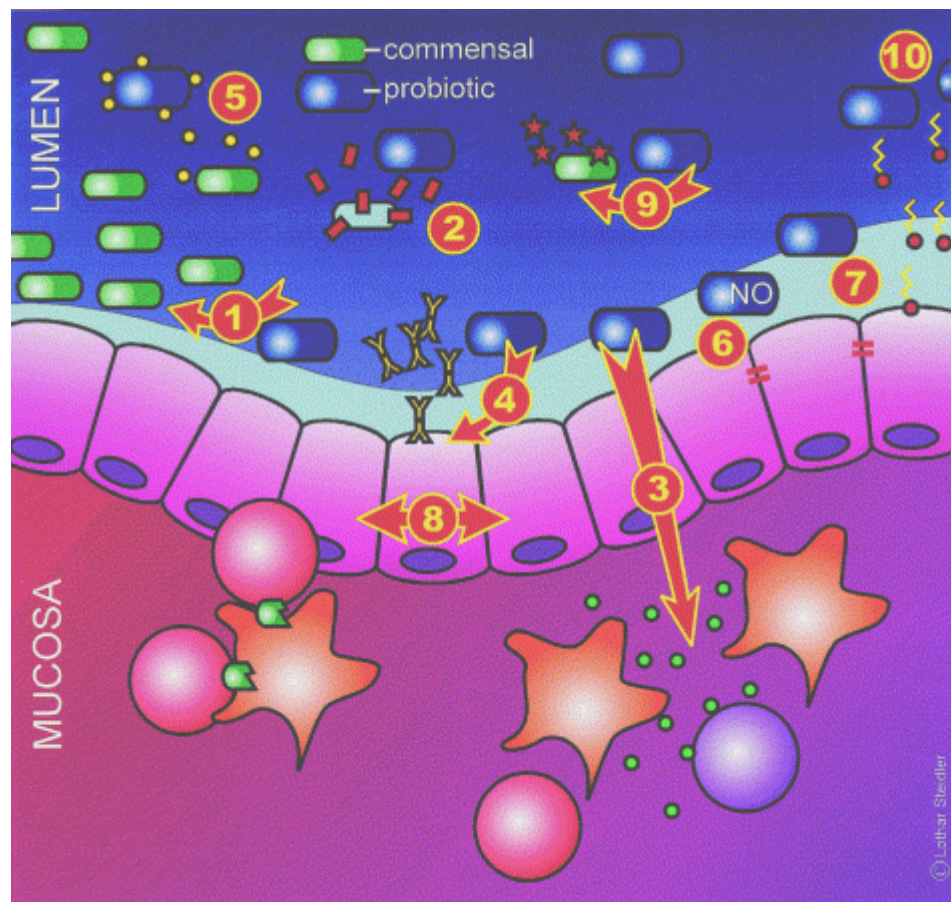
Figure 1.5 Possible mechanisms of probiotic activity. Probiotic strains can displace nocuous bacteria by competitive binding (1) or killing/growth inhibition by antibacterial compounds or lowering of the pH (2). Some probiotics have defined influence on the host immune cells such as the induction of cytokine production (3) or the increase in local IgA secretion (4). Probiotic bacteria can sequester toxins and drain these from the intestine (5). Probiotics can influence epithelial and tissue integrity by low-dose NO synthesis (6), stimulation of mucus production (7), enhancing gut epithelial cell proliferation (8), inhibition of endogenous carcinogen production (9) and providing nutrients by short chain fatty acid production (10). (Adapted from microbes and infection (Steidler, 2001)

1.9   Animal models of IBD.

Since the first description of a colitis model in 1961, 63 models have been described (Hoffmann *et al.*, 2002-2003). Although human studies have improved our knowledge about IBD in recent years, most advances in our understanding of IBD pathogenesis came from IBD animal models. Despite the varying nature of these models, the concept that they have in common support greatly the view that environmental factors affecting genetically susceptible hosts are responsible for the induction of IBD. However, the exact aetiology of IBD remains unknown.

An ideal animal model for investigating IBD pathogenesis and treatment should closely resemble the human disease and develop intestinal inflammation without gene targeting or immunological manipulation. In spite of the better understanding of mucosal pathology none of the currently available models resembles in all aspects the human IBD. In general, animal models of intestinal inflammation can be divided into 4 categories: (i) Spontaneous models (ii) Inducible models (iii) Genetic models (iv) Adaptive transfer models.

Murine models represent the most useful experimental animal systems because the mouse genome has been fully characterised and murine immunological reagents are readily available (Pizarro *et al.*, 2003).

Inducible DSS murine colitis is the most commonly used model (Elson *et al.*, 2005). Addition of DSS (2-10% w/v) to the drinking water can induce colitis in a variety of animals, including rats and mice (Kitajima *et al.*, 1999). Within 2-3 days of administration, initial findings are crypt loss followed by inflammatory process and loss of the surface mucosal epithelial cells. Several lines of evidence indicate that the principal instigator of DSS colitis is the consequence of damage to the epithelium, perhaps via direct toxicity (Kitajima *et al.*, 1999). This model has been useful in providing proof of concept for therapeutic interventions in a simple, reproducible and relatively inexpensive model (Pizarro *et al.*, 2003). Other commonly used models of murine colitis are illustrated in table 1.5.

Table 1.5   Commonly used animal models of intestinal inflammation and IBD

Model	Pathology	Mode of injury	Reference
Spontaneous models			
Cotton top tamarin	Acute/Chronic UC	Stress associated with captivity leads to decrease mucous production and increase gut permeability	(Wood *et al.*, 1998)
C3H/HejBir mice	Acute/Chronic CD	Impaired immune response to TLR ligands that translate to increased T-cell responses to bacterial antigen.	(Sundberg *et al.*, 1994)
SAMP1/Yit mice	Chronic CD	Alteration of epithelial cells results in activation of pathogenic $CD4^+$ T cells reactive to bacterial antigen.	(Kosiewicz *et al.*, 2001)
Inducible models			
Oxazolone	Acute UC	Th2 driven colitis; affect mucosal layer of the distal colon.	(Boirivant *et al.*, 1998)
TNBS	Acute/Chronic CD	Th1 mediated, TNBS act as hapten	(Morris *et al.*, 1989b)
Indomethacin	Acute/Chronic CD	Inhibits protective prostaglandins and prostacyclin.	(Elson *et al.*, 1995)
DSS	Acute/Chronic UC	Damage to the epithelium via direct toxicity lead to Th1 (acute) or Th1/Th2 (chronic) colitis.	(Okayasu *et al.*, 1990)
Genetic models			
$IL-2^{-/-}$	UC	Colitis mediated via $CD4^+$ T cells producing IFN-γ and IL-12 or failure to develop $CD4^+CD25^+$ T_{Reg} subset.	(Sadlack *et al.*, 1993)
$IL-10^{-/-}$	Acute/Chronic CD	Colitis mediated via $CD4^+$ T cells producing IFN-γ and IL-12	(Kühn *et al.*, 1993)
$TNF^{\Delta ARE}$	Chronic CD/ arthritis	Excessive TNF-α production	(Kontoyiannis *et al.*, 1999)
Adaptive transfer model			
$CD4^+CD45RB^{hi}$ into SCID mice	CD	Transfer of effector T cells, in the absence of T_{Reg} cells	(Powrie *et al.*, 1994)
Bone marrow into Tgε26 mice	Chronic UC	Abnormal intra thymic T cell development leads to IL-12 dependant inflammation	(Holländer *et al.*, 1995)

1.10   Rationale for thesis

Several immune-regulatory agents, such as corticosteroids, 5-ASA, and anti-TNF-α have been used for the treatment of human IBD to control the dysregulated immune response. However, there is no cure for this disease, and these immune-regulatory agents can cause destructive side effects. Therefore, various other agents have been tried for IBD treatment; of these are the cytokines and growth factors. Growth factors have various biological actions, such as enhancement of cell proliferation, modulation of cell differentiation, and acceleration of cell migration. They play an essential role in regulating differential epithelial cell functions to preserve normal homeostasis and integrity of the intestinal mucosa (Dignass, 2001). Therefore, they might serve as an alternative therapy for patients with IBD.

Keratinocyte growth factor (KGF) and Transforming Growth Factor-*β* (TGF-*β*) are two growth factors well studied for their role to promote healing of injured intestinal epithelium (Sturm & Dignass, 2008). Systemic administration of these cytokines may not be the optimal way of delivery, because of tissue specificity and risk of dangerous side effects. Therefore, an alternate route of delivery should be tried. Rectal administration was used to treat ulcerative colitis (Sinha *et al.*, 2003) but the convenience of using rectal route for these ill patients is questionable. Oral route is probably more appropriate. However, protein stability makes the oral route not ideal, as these proteins have to pass through the extreme conditions of the stomach. The short *in vivo* half-lives, the physical and chemical instability, and the low oral bioavailability of proteins currently necessitate their administration by frequent injections of protein solutions.

The scope of probiotic therapy has recently been redefined by the development of genetically modified probiotic (GMP) such as the *L. lactis* system which has been engineered to secrete IL-10 for the treatment of colitis (Steidler *et al.*, 1995; Steidler *et al.*, 1998; Steidler *et al.*, 2000). Although effective, GMP strains such as *L. lactis* have significant drawbacks including lack of regulation of the production of the therapeutic molecule and biosafety and environmental contamination concerns. To attempt to overcome the drawbacks of the *L. lactis* system, we have utilized *Bacteroides ovatus*, a major **commensal** anaerobic colonic Gram-negative bacterium in humans, as a potentially improved genetically-modified probiotic bacterium. Firstly, the delivery of protein by *B. ovatus* can be **controlled**

by the xylan induction system (Farrar *et al.*, 2005). Secondly, this system is naturally **contained** because of the anaerobic nature of *B. ovatus* (shown in chapter 3).

1.11 Hypothesis

Strains of the commensal gut bacterium *Bacteroides ovatus* genetically engineered to produce growth factor under the control of xylan can be used to induce healing of inflamed intestinal epithelium and restore barrier function (Figure 1.6).

1.12 Specific aims

1. Identification of *B. ovatus* putative xylanase operon promoter

2. Generation of recombinant strains of *B. ovatus* that produce TGF-β_1 or KGF-2 under the control of xylanase promoter and demonstrate that growth factors can be produced by the recombinant *B. ovatus in vivo* upon exposure to xylan.

3. To use the recombinant *B. ovatus* for the treatment and prevention of murine model of colitis.
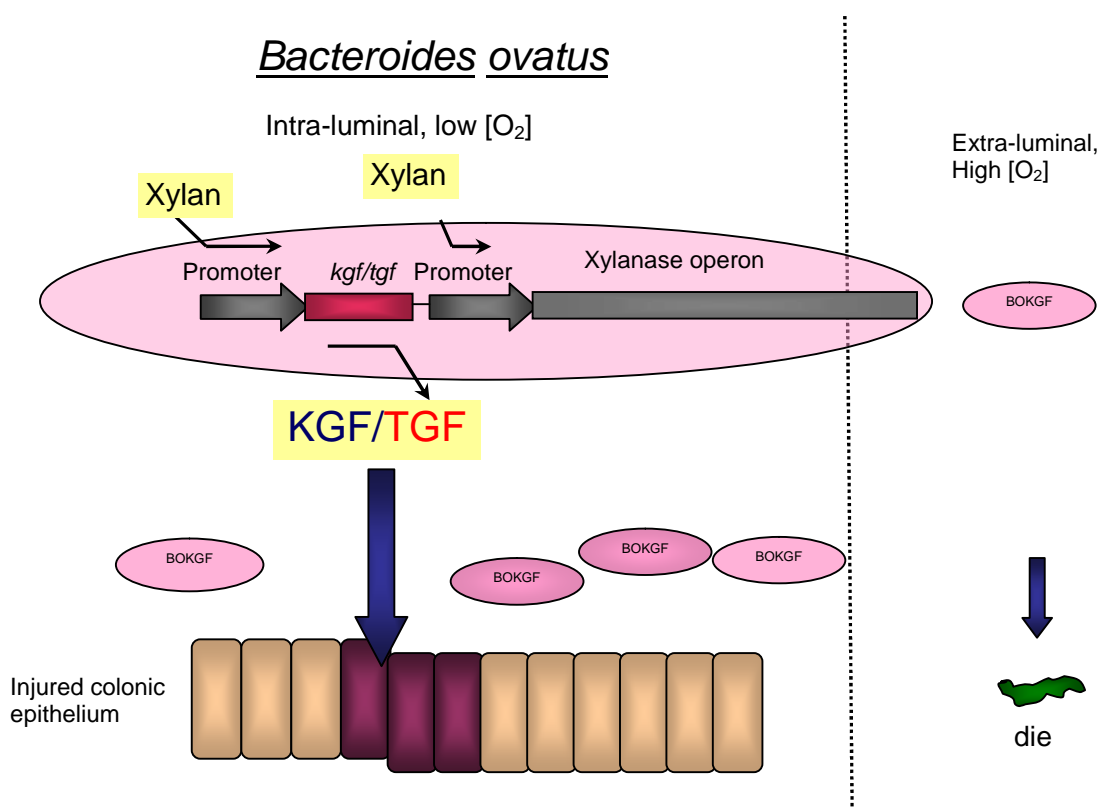
Figure 1.6 **Diagrammatic representation of the proposed *B. ovatus* xylan controlled protein delivery system.**

Bacteroides ovatus is a predominant *commensal* colonic microbiota with the ability to colonise the bowel, therefore, repeated administration is not needed. *B. ovatus* can ferment the xylan and the xylanase promoter is to be cloned upstream of the recombinant mature protein gene (*kgf* or *tgf* in this case) and drive (*control*) the transcription of the gene only in the presence of xylan. Once secreted the recombinant protein will induce healing of the injured bowel mucosa. After healing is completed xylan supplementation is removed from the diet and the protein production is halted. In addition the anaerobic nature of *B. ovatus* is a natural biosafety and containment system that will prevent spread to the environment.

CHAPTER (2)

MATERIALS AND METHODS

2.1   Microorganisms and Plasmids

A list of bacterial strains and plasmids used in this study can be found in Appendix 1.

2.2   Sterilisation and Centrifugation

Media, heat stable solutions and other equipment were sterilized by autoclaving at 15 lb in^{-2} at 121°C for 20 minutes. Heat labile solutions were filter sterilized through 0.2µm pore size Minisart filters (0.2 µm pore, Sartorius AG).

Fermentas benchtop centrifuger was used for small volumes and Sorvall GSA rotor was used for volumes more than 2 ml.

2.3   Antibiotics

Stock solutions were prepared in sterile distilled water unless otherwise stated and stored at -20°C. Antibiotics were added to liquid media immediately prior to use and to solid media immediately prior to dispensing into Petri dishes. Table 2.1 illustrates antibiotics used and their stock and working concentrations.

Table 2.1   Working and stock concentrations of antibiotic solutions used in this study.

Antibiotic	Stock Conc. (mg ml^{-1})	Working Conc. (µg ml^{-1})
Ampicillin	100 (in H_2O)	100
Gentamicin	40 (in H_2O)	200
Kanamycin	25 (in H_2O)	50
Trimethoprim	10 (in Methanol)	200
Tetracycline	10 (in Methanol)	1 or 10

2.4   Other media additives

Glucose

A stock solution (100 mg ml^{-1}) was prepared in distilled water then filter sterilised and stored at +4˚C. Glucose was used at various working concentrations as stated.

Hemin

A stock solution (10 mg ml^{-1}) was prepared in 0.15 M NaOH, filter sterilised and stored at +4°C. Hemin was used at a working concentration of 10 µg ml^{-1} (0.001% w/v).

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)

X-gal (80 mg) was dissolved in 2 ml dimethylformamide and stored at -20°C. Just before use, 40 µl of X-gal solution was spread aseptically on each solid media plate.

Xylan

Oat spelt xylan (Sigma) was used as a source of xylan throughout this study. A stock solution (100 mg ml^{-1}) was prepared in warm distilled water (55˚C), stirred for 1 h then centrifuged at 6000g for 5 min. The supernatant was autoclaved and stored at +4°C. Just before use, xylan was added to the media in various working concentrations as stated.

2.5   Culture Media

All media components were dissolved in distilled water, prepared and autoclaved (unless otherwise stated) in Schott Duran 500 ml screw cap bottles. Liquid culture media were prepared as described and any heat labile solutions were added prior to use. Solid culture media were prepared by adding agar

(Bacteriological agar number [1]; Oxoid) 1% (w/v) prior to autoclaving. Following cooling to 50°C any necessary heat labile solutions were added, and the media dispensed into sterile 9 cm diameter plastic Petri dishes (Philip Harris Ltd.).

2.5.1   Luria-Bertani (LB)

Tryptone (5g), yeast extract (2.5 g) and NaCl (5 g) were added to 500 ml distilled water and sterilised by autoclaving.

2.5.2   Brain Heart Infusion-hemin (BHI-hemin)

Brain Heart Infusion (18.5 g) was added to distilled water (500 ml) and sterilised by autoclaving. Just before use, hemin was added at a final concentration of 10 µg ml^{-1} (0.001% w/v).

2.5.3   SOB medium

Magnesium salts solution

$MgSO_4.7H_2O$ (24.65 g) and $MgCl_2.6H_2O$ (20.33 g) were dissolved in distilled deionised water made up to 100 ml and filter sterilised into suitable aliquots then stored at -20°C.

Media preparation

The following components were added to 494 ml distilled deionised water and sterilised by autoclaving

Tryptone	20 g
Yeast extract	5 g
NaCl (1M)	5 ml
KCl (1M)	1.25 ml

Just before use, glucose (5 ml; 2 M) and magnesium salt solution (5 ml) were added.

2.5.4 *Bacteroides* **Routine Growth Medium (RGM)**

The following components were added to 500 ml distilled water, pH adjusted to 6.7 then filter sterilised (Minisart, 0.2 µm pore, Sartorius AG) into 10 ml aliquots.

K_2HPO_4	0.146 g
KH_2PO_4	0.12 g
$(NH_4)_2SO_4$	0.24 g
NaCl	0.24 g
$MgSO_4.7H_2O$	0.05 g
$CaCl_2.2H_2O$	0.032 g
Na_2CO_3	2 g
Cysteine-HCl	0.3 g
Tryptone	0.5 g
Yeast Extract	0.25 g
Resazurin	0.5ml of 0.1% (w/v) solution
Acetic acid	810 µl
Propionic acid	302 µl
Butyric acid	155 µl
Isobutyric acid	46µl
Valeric acid	54µl
Isovaleric acid	54µl
2-Methylbutyric acid	55µl

Just before use, hemin 0.001% (w/v) and carbon sources (glucose and/or xylan at various concentrations) were added.

2.6   General Buffer Solutions

Tris-HCl (1M, pH 8.0)

Tris (12.11 g) was dissolved in distilled deionised water, the pH adjusted to 8.0 with HCl (11.46 M) and the volume made up to 100ml.

Tris/EDTA (TE buffer)

Tris-HCl (1 ml; 1 M, pH 8.0) and EDTA (0.2 ml, 0.5 M, pH 8.0) were added to distilled deionised water and the volume made up to 100 ml.

Phosphate buffered saline (PBS)

NaCl (0.8 g), K_2HPO_4 (0.12 g) and KH_2PO_4 (0.034 g) were dissolved in distilled water, made up to 100 ml and sterilised by autoclaving.

2.7 Storage of viable bacteria

For long-term storage, bacteria were grown aerobically or anaerobically depending on growth requirements. A single colony was picked up and resuspended in 1 ml PBS with 40% (v/v) glycerol. Resuspended cells were stored in a sterile, DNase and RNase free tubes and stored in liquid nitrogen (-196°C).

2.8 Standard culture conditions

Aerobic incubation

Cultures on solid media were incubated at 37°C. Liquid cultures were incubated in an orbital incubator (Sanyo Gallenkamp, 160 rpm). Incubation was for 18-20 h unless otherwise indicated.

Anaerobic incubation

Both solid and liquid cultures were incubated in a tightly sealed container that contained Genbox anaer sachets (Biomerieux, France) at 37°C. Incubation was for 18-20 h unless otherwise indicated.

2.9   Preparation of *E. coli* competent cells

Materials

RF1

RbCl (1.21 g), $MnCl_2.4H_2O$ (0.99 g), potassium acetate (0.29 g), $CaCl_2.2H_2O$ (0.15 g) and glycerol (15 g) were dissolved in distilled deionised water and the pH adjusted to 5.8 with acetic acid (0.2 M). The volume was made up to 100 ml and the solution filter sterilised.

RF2

3-[N-Morpholino]propanesulphonic acid (MOPS; 0.21 g), RbCl (0.12 g), $CaCl_2.2H_2O$ (1.1 g) and glycerol (15 g) were dissolved in distilled deionised water and the pH adjusted to 6.8 with NaOH (0.1 M). The volume was made up to 100 ml and the solution filter sterilised.

Procedure

E. coli DH5α or J53/R751 were used to prepare competent cells; single colonies were selected from overnight fresh culture of LB plates to inoculate 10 ml of SOB broth in a universal bottle. This was incubated aerobically for 16-18 h at 37°C with gentle agitation. This was then inoculated into 100 ml of SOB (in a 2L shake flask) to an OD_{600} of 0.05 and incubated at 37°C with moderate agitation until an OD_{600} of 0.3-0.5 was reached. The culture was chilled on ice for 15 min and cells were harvested by centrifugation (10000 rpm, 4°C, Sorvall GSA rotor) for 10 min. The pellet was resuspended in 33 ml of ice cold RF1 and placed on ice for 15 min then the cells were harvested by centrifugation and resuspended in 8 ml of ice cold RF2, this was placed on ice for another 15 min. Aliquots (100 µl) of cells were placed into sterile pre-chilled microcentrifuge tubes, snap frozen and stored in liquid nitrogen at -196°C until use.

2.10   Transformation of *E. coli* competent cells

Frozen competent cells were thawed on ice. The plasmid DNA was added to the competent cells at 5% (v/v) and held on ice for 30 min. Uptake of DNA from the surrounding buffer was facilitated by a heat shock at 42°C for 30-40 sec followed by 10 min held on ice. SOB (900µl) medium was then added and the tube was incubated at 37°C for 1.5-2 h with shaking. The cell suspension was spread on LB plates supplemented with appropriate antibiotics for vector selection; x-gal was added if the vector was appropriate for white/blue screening. The plates were then incubated for 16-18 h at 37°C.

2.11   Isolation of Bacterial Genomic DNA

For isolation of Genomic DNA from *B. ovatus*, the NaCl/CTAB (hexadecyltrimethylammonium bromide) method was used (Wilson, 1997).

Cells were harvested from 10 ml of overnight culture by centrifugation at maximum speed for 5 min. The cells were resuspended in 565 µl TE buffer. SDS (10% w/v) 30 µl and Proteinase K (20 mg ml^{-1}) 3 µl were added, mixed by inversion and incubated at 37°C for 1 h. Sodium chloride (5M) 100 µl was added and mixed before the addition of 80 µl of CTAB/NaCl solution (10% w/v CTAB in 0.7 M NaCl) then incubated for 10 min in a 65°C water bath. RNase A (10 mg ml^{-1}) was added at 0.5 µl for each 100 µl preparation and incubated for 30 min at 37°C. DNA was extracted by adding an equal volume of phenol/chloroform/iso-amyl alcohol, the supernatant was decanted into a fresh tube. DNA was precipitated by adding cold Isopropanol to 60% (v/v) then harvested by centrifugation at 14000g for 30 min. The DNA pellet was washed twice with 70% (v/v) ice cold ethanol and dried by lyophilisation for 5 min.

2.12   Preparation of plasmid DNA from *E. coli*

Plasmid DNA from *E. coli* was prepared using the Wizard® Plus SV Minipreps DNA purification system (Promega) according to manufacturer's instructions. The protocol is briefly described below.

Cells were harvested from 1-5 ml of overnight culture (supplemented with appropriate antibiotics) by centrifugation at 14000g for 1-2 min. The cells were resuspended in 250 µl suspension buffer by vortexing and lysed by adding 250 µl cell lysis solution and incubated for 5 min at room temperature. Alkaline protease (10 µl) was then added, mixed and incubated for 5 min at 22°C. Following lysis 350 µl of neutralisation solution was added and mixed by inversion. The lysate was cleared by centrifuging the preparation for 10 min at 14000g. The cleared lysate was purified by minicolumn and washed with washing solution. The bound plasmid DNA was eluted into a fresh microcentrifuge tube with 100 µl nuclease free water. Plasmid DNA was stored at -20˚C until use.

2.13   Purification of DNA

DNA was purified from small volumes by phenol:chloroform (1:1) extraction. The protocol is described briefly below.

An equal volume of phenol:choloroform was added to DNA solution in a microcentrifuge tube and mixed by inversion. The tube was centrifuged at 14.000 rpm for 5 min and the aqueous upper phase was transferred to a fresh tube. Sodium acetate (3 M, 10% v/v) or Ammonium acetate (7.5 M, 50% v/v) was added and mixed by inversion. Two volumes of ice cold 100% ethanol was added and mixed. The tube was kept at -20°C for 30 min and then centrifuged at 14000g for 30 min. The supernatant was removed very gently and the pellet washed twice with 70% ethanol. The pellet was allowed to dry and dissolved in either distilled deionised water or TE buffer as stated.

2.14   Quantitation of DNA

DNA solutions were quantified spectrophotometrically by measuring the absorbance at a wavelength of 260 nm (A_{260}). The concentration of DNA was calculated using the equation: A_{260} of 1 equates to 50 µg ml^{-1} of double stranded DNA. DNA solutions were also quantified by subjecting samples to agarose gel electrophoresis alongside λ *Hind*III DNA standards (Fermentas) which contains known quantities of DNA according to manufacturer's instructions.

2.15   Restriction endonuclease digestion of DNA

Small scale digestions were carried out using the following protocol:

DNA solution   1-2 µg

Reaction buffer (10x)   1.5 µl

Restriction endonuclease (8-12 U µl^{-1})   1   µl

Distilled deionised water was added to make the volume up to 15 µl. The reaction was carried out at 37°C for 1-4 hours. For double digests the reaction buffer was chosen according to enzyme activity in the specific buffer. The buffer that gave the highest activity for both enzymes was chosen.

2.16   Dephosphorylation of DNA

Shrimp alkaline phosphatase (SAP, Promega) was used to catalyse the removal of 5' phosphate groups from DNA ends. Dephosphorylation of vector DNA prior to ligation prevents self annealing of the vector thereby increasing the efficiency of cloning DNA fragments. Following restriction endonuclease digestion, vector DNA was gel purified before being dephosphorylated. The reaction mixed as follows:

DNA solution   26µl

SAP buffer (10 x)   3µl

SAP (1 U µl^{-1})   1µl

The reaction was carried out at 37°C for 30 min then SAP was inactivated by incubation at 65°C for 20 min.

2.17   Ligation of DNA

T4 DNA ligase catalyses the formation of phosphodiester bonds between the 3' hydroxyl and 5' phosphate termini of double stranded DNA. The vector/insert ratio varies depending on the sizes of the insert and vector. The following formula was used to calculate the optimal amount of insert DNA

$$\frac{\text{ng of vector } \mathbf{X} \text{ kb size of insert}}{\text{kb size of vector}} \; \mathbf{X} \text{ molar ratio of insert/vector} = \text{ng of insert}$$

The reaction mixed as follows:

Vector DNA	x ng
Insert DNA	y ng
T4 ligase buffer (5x)	4 µl
T4 DNA ligase (Invitrogen)	0.5-1 U
Sterile distilled water	to 20 µl

Ligation reactions were carried out at 16°C for 16 h.

2.18   PCR (general protocol)

PCR materials were stored at -20°C and thawed and mixed on ice before use. *Taq* DNA polymerase (Reddymix, ABgene) was used in amplification of small targets or when the sequence accuracy did not matter.

The reaction mixed as follows:

Template DNA	variable
Forward primer	1 µM
Reverse primer	1 µM
Reddymix	12.5 µl

Nuclease free water was added to make the volume up to 25 µl. the total mixture was immediately placed in the thermocycler (Light Cycler, Idaho Technology Inc.) and run using the cycling conditions given in Table 2.2.

Table 2.2   Cycling conditions of the PCR reaction used for general purposes.

	Temperature (°C)	Time	Cycle No.
Initial denaturation	94	2 min	x1
Denaturation	94	40 sec	
Annealing	55	30 sec	x35
Elongation	72	Variable	
Final elongation	72	5 min	x1

2.19   Agarose gel electrophoresis

Materials

Agarose gel electrophoresis was performed using Mini-Sub^{TM} or Wide Mini Sub^{TM} cells (Bio-Rad).

Tris-Borate-EDTA (TBE; 10x)

Tris (54 g), boric acid (27.5 g) and EDTA (20 ml; 0.5M, pH 8.0) were dissolved in distilled water and the volume made up to 500 ml.

Protocols

i. Preparation and electrophoresis of agarose gels

For a 1% (w/v) gel, agarose (0.3 g) was added to 30ml TBE (1x). The gel components were boiled to dissolve the agarose. After cooling to 50°C, the gel was poured into the prepared base and allowed to set. The gel was placed in electrophoresis cell filled with TBE (1x) to submerge the gel.

DNA sample was mixed with gel loading buffer (6:1 v/v) (Fermentas) and loaded into the submerged wells. The samples were electrophoresed at a constant voltage 70-100 V for 30-60 min (PowerPac 300, Bio-Rad).

ii. Visualisation of DNA

Gels were stained by soaking in a solution of ethidium bromide (EtBr, 2 µg ml^{-1}, Sigma) for 15 min. DNA was visualised using a shortwave UV transilluminator (White/UV TMW-20, UVP).

iii. Gel extraction of DNA

Following gel staining, the required fragment was cut with a sterile scalpel. The DNA was extracted from the gel using Wizard$^{®}$SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Briefly, membrane binding solution was added at 10 µl per 10 mg of agarose gel slice and incubated at

65˚C for 10 min with intermittent vortexing. The dissolved gel mixture or prepared PCR product was transferred to the SV Minicolumn and incubated for 1 min at 22˚C, then centrifuge at 14000g for 1 min. Bound DNA was washed twice with membrane wash solution and eluted with 50 µl nuclease free water.

2.20   Southern blot and visualisation of DNA

2.20.1 Southern blotting of DNA by capillary transfer

Materials

SSC (20x)

NaCl (87.66 g) and Sodium citrate (44.11 g) were dissolved in distilled deionised water. The pH was adjusted to 7.0 and the volume made up to 500 ml. SSC (10x, 6x and 2x) were obtained by diluting SSC (20x) with distilled deionised water.

Denaturation buffer

NaCl (43.83 g) and NaOH (10 g) were dissolved in distilled deionised water and the volume made up to 500 ml.

Neutralisation buffer

Tris (60.55 g) and NaCl (43.83 g) were dissolved in distilled deionised water. The pH was adjusted to 7.4 with HCl (11.46 M) and the volume made up to 500 ml.

Protocol

DNA to be blotted was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The gel was soaked in excess HCl (0.25 M) for 10 min to depurinate the DNA. Following a brief rinse in distilled deionised water, the gel was soaked in denaturation buffer with constant agitation for 45 min.

The gel was rinsed in distilled water then soaked in neutralisation buffer with constant agitation for 30 min. The gel was soaked in a fresh neutralisation buffer for a further 15 min.

A glass support was placed in a large pyrex dish and the dish was filled with SSC (10x). A piece of 3 mm blotting paper (Whatman) was placed over the support, so that the edges either side were in contact with the buffer, to act as a wick. When the paper was fully wetted, air bubbles were removed from between the wick and support. The agarose gel was placed inverted onto the centre of the wick and the remaining exposed wick was covered in cling film. A piece of positively-charged nylon membrane (Boehringer Mannheim) slightly larger than the gel was placed on top of the gel followed by three pieces of 3 mm blotting paper and finally a stack (approximately 10 cm high) of tissue paper towels. A 1 kg weight was applied to the stack to provide an even pressure and the DNA was allowed to transfer for 20 h (Figure 2.1).

Following transfer, the position of the gel wells was marked on the membrane and the membrane was rinsed in SSC (6x) for 5 min. The gel was re-stained with ethidium bromide to ensure DNA transfer was complete. The DNA was fixed to the membrane by exposure to U.V. using a shortwave transilluminator (White/UV TMW-20, UVP) for 3 min. The membrane was then ready for hybridisation.
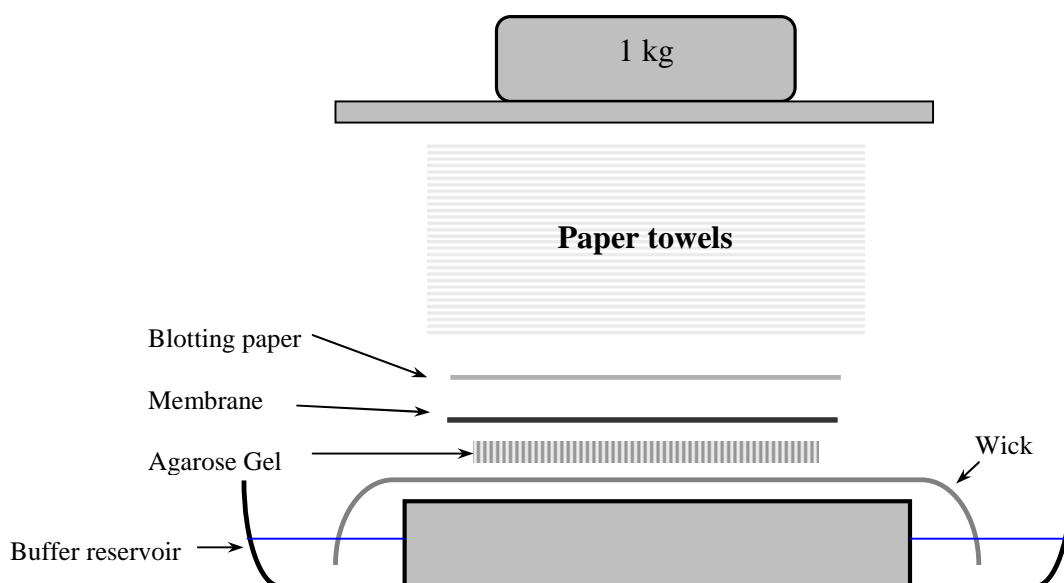
Figure 2.1   Arrangement of apparatus for Southern blotting

2.20.2 Non-radioactive labelling of DNA probe

Fragments of DNA to be used as a probe were labelled with digoxigenin (DIG)-11 UTP by random priming [DIG High Prime Labelling and Detection Starter Kit I (Boehringer Mannheim)] according to manufacturer's instructions. Briefly, template DNA (0.01-3 µg in a total volume of 16 µl in a 0.5 ml microcentrifuge tube) was denatured by heating to 100°C in a boiling water bath for 10 min followed by quick chilling in an ice bath. DIG-High Prime labelling mix (4µl) was added to the denatured DNA then briefly centrifuged to collect the content at the bottom of the tube. The reaction was incubated at 37°C for 20 h and was stopped by addition of 2 µl EDTA (0.2 M, pH 8.0) then stored at -20°C until use.

The labelling efficiency was determined by applying 1 µl of a series of dilutions of DIG-labelled DNA onto a DIG Quantification Teststrip (Boehringer Mannheim). The DNA was detected with anti-digoxigenin-Ab conjugate and compared to a DIG Control Teststrip containing known amount of labelled DNA.

2.20.3 DNA Hybridisation

Materials

Hybridisation buffer

Sterile distilled deionised water (64 ml) was carefully added in two portions to the DIG Easy Hyb Granules and dissolved by stirring immediately for 5 min at 37°C.

Hybridisation solution

DIG-labelled DNA probe was denatured by boiling for 5 min and rapidly cooled on ice/water. Denatured DIG-labelled DNA probe was diluted to ~25 ng ml^{-1} in pre-warmed (68°C) DIG Easy Hyb (3.5 ml/100 cm^2 membrane).

Wash buffer 1

SSC (50 ml; 20x) and SDS solution (5 ml; 10%, w/v) were added to distilled deionised water (445 ml)

Wash buffer 2

SSC (2.5 ml; 20x) and SDS solution (5 ml; 10%, w/v) were added to distilled deionised water (492.5 ml).

Protocol

The membrane carrying the DNA was laid on top of a piece of hybridisation mesh (Hybaid). The membrane/mesh was rolled up and placed in a hybridization bottle (HB-OV-BS, Hybaid). DIG Easy Hyb (Hybridisation buffer) was preheated to 41°C and used to prehybridise the membrane/mesh for 30 min with gentle agitation in a rotating hybridization oven (Mini 10, Hybaid). The membrane was then incubated in hybridization solution at 41°C for 16-18 h.

Following hybridization, stringency washes were carried out with constant agitation. The membrane was washed twice in wash buffer 1 at room temperature for 5 min, then twice in wash buffer 2 at 68°C for 15 min each. After post-hybridisation washes immunodetection of the probe was performed.

2.20.4 Immunological detection of DNA probes

Materials

Wash buffer

Maleic acid (5.81 g) and NaCl (4.38 g) were dissolved in distilled deionised water, the pH was adjusted to 7.5 with NaOH; then Tween 20 was added (0.3% v/v) and the volume made up to 500 ml.

Maleic acid buffer

Maleic acid (5.81 g) and NaCl (4.38 g) were dissolved in distilled deionised water, the pH was adjusted to 7.5 with solid NaOH and the volume made up to 500 ml.

Blocking solution

Blocking solution (10x, Boehringer Mannheim) was diluted 1:10 with maleic acid buffer.

Detection buffer

Tris (6.06 g) and NaCl (2.92 g) were dissolved in distilled deionised water, the pH was adjusted to 9.5 with HCl (1 M) and the volume made up to 500 ml.

Colour substrate solution

Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) 200 µl was added to 10 ml detection buffer.

Protocol

After hybridization and stringency washes, the membrane was rinsed briefly (1-5 min) in washing buffer. The membrane was then incubated in blocking solution at 22°C for 30 min. Anti-digoxigenin antibody conjugated to alkaline phosphatase was diluted 1:5000 in 20 ml blocking solution (150 mU ml^{-1}). The membrane was incubated in antibody solution at 22°C for 30 min. Unbound antibody was removed by incubating the membrane in washing buffer twice (15 min each). The membrane was equilibrated in 20 ml detection buffer for 5 min then was incubated in a freshly prepared colour substrate solution in the dark until the desired colour density was reached. The reaction was stopped by washing the membrane in distilled water for 5 min. The membrane was allowed to air-dry and was stored in the dark at 22°C.

2.21   Cloning of the putative *B. ovatus* xylanase operon

2.21.1 Inverse-PCR

i. Preparation of template for PCR

The GenBank sequence (BOU04957) of the xylanase precursor genes of *B. ovatus* V975 (Whitehead, 1995) were analysed using restriction enzyme finder software (NEBcutter V2.0). Two identified enzymes were selected that cut upstream of the probe, each recognize a 6 base-pair (bp) sequence of DNA; *Kpn*I (GGTACC) cuts at 609 bp and *Hind*III (AAGCTT) cuts at 2994. These enzymes were used to digest 0.5 µg of freshly prepared *B. ovatus* DNA to completion. Digested DNA was separated on 1% (w/v) agarose gel then transferred to nylon membrane by Southern blotting.

The 600 bp DNA fragment flanked by *Eco*RI and *Kpn*I was excised from plasmid pOX1 and used as a probe after labelling with DIG (above) for hybridisation in Southern blotting. This probe identified the restriction site flanking the known sequence.

B. ovatus DNA was digested to completion by *Hind*III. The reaction was stopped by incubation at 65°C for 20 min. Digested DNA was diluted x50 (final concentration 0.5 µg ml^{-1}) and self-ligated to produce circular DNA. At this low ligation concentration, more than 90% of the ligated DNA is present as monomeric circles (Collins & Weissman, 1984). The DNA from ligation reaction was phenol:chlorophorm purified and precipitated with ethanol (as previously described) then resuspended in nuclease free H$_2$O and used in subsequent PCR reactions.

ii. PCR

Enzyme mix containing *Taq* DNA polymerase and *Tgo* DNA polymerase, a thermostabe DNA polymerase with proofreading activity [Expand long template PCR system, Roche applied sciences] was used to amplify the circular DNA. Ingredients were added to a thin-walled 0.5 ml tube as follows:

Template DNA	200-300 ng
dNTPs	500 µM
Forward primer	600 nM
Reverse primer	600 nM
$MgCl_2$	1.75 mM
Sterile nuclease free H_2O	up to 50 µl

The cycling conditions used for the above reaction shown in Table 2.3

Table 2.3 Cycling conditions used for the PCR reaction to amplify long DNA template of the I-PCR

	Temperature (°C)	Time	Cycle No.
Initial denaturation	94	2 min	x1
Denaturation	94	10 s	
Annealing	50	30 s	x10
Elongation	68	6 min	
Denaturation	94	15 s	
Annealing	50	30 s	x20
Elongation	68	6 min	
Final elongation	68	10 min	x1
Cooling	4	Unlimited time	

2.21.2 Blunting of I-PCR fragment and 5' phosphorylation

The Expand long template PCR system (Roche) used to amplify long DNA fragment in I-PCR results in a mixture of blunt ends and 3' adenine overhangs on the PCR product. Cloning of these Expand PCR fragments with conventional vector systems is not very efficient and often not possible and therefore, it was necessary to blunt and phosphorylate the 5' ends (using T4 DNA polymerase and T4 polynucleotide kinase (Fermentas) in one working step, in the following reaction:

Purified PCR product	1-2 µg
T4 DNA polymerase buffer (10 x)	5 µl
dNTPs (2 mM each)	2.5 µl
T4 polynucleotide kinase buffer (10 x)	5µl
ATP 1mM	5 µl
T4 DNA polymerase	1 µl
T4 polynucleotide kinase	1 µl

Volume was made up to 50 µl with nuclease free water. The reaction was incubated at 37°C for 30 min. DNA was phenol:chlorophorm purified and precipitated with ethanol as illustrated in section 2.13.

2.21.3 Cloning of I-PCR product

The blunt ended I-PCR product was cloned into pCR®-blunt using the Zero Blunt PCR cloning kit (Invitrogen) as described in manufacturers instructions. Briefly the reaction was as follows:

Insert DNA (blunt PCR product)	350 ng
pCR®-Blunt (25 ng)	1µl
Buffer (10x)	1µl
T4 DNA ligase	1µl
Nuclease free water	up to 10 µl

The reaction was incubated at 16°C for 1 h and then used to transform competent *E. coli* as described previously. Transformed cells were selected on LB plates supplemented with 50 µg ml^{-1} kanamycin. Cloning of blunt PCR fragment into pCR®-blunt disrupts expression of the *lac*Zα-*ccd*B gene permitting growth of only positive recombinants upon transformation (Bernard *et al*., 1994).

Transformants were analysed by restriction endonuclease digestion of the recombinant plasmid to identify the correct size fragments on agarose gel electrophoresis. The cloned insert was then sequenced using the primer walking technique (DNA Sequencing Service, Dundee) and the sequence was analysed using

ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequence was searched against the sequence database using the BLAST server.
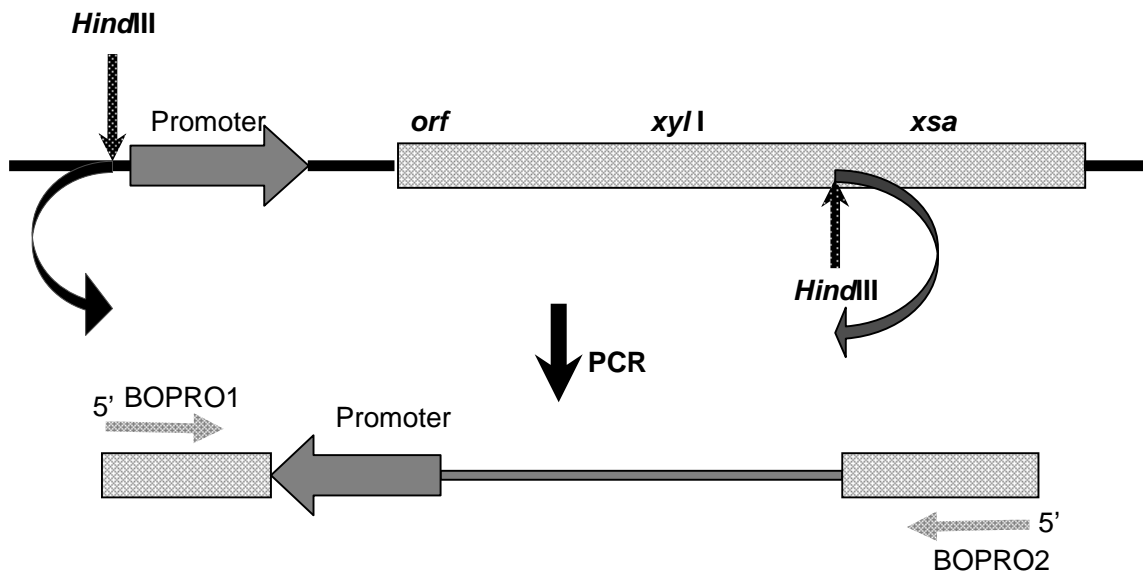
Figure 2.2   Diagrammatic representation of the Inverse-PCR technique used to clone xylanase promoter.

Inverse-PCR is used to clone unknown DNA regions when sequence information is available for upstream or downstream of the region of interest. It involves enzymatic digestion of the genomic DNA with restriction endonuclease that cut inside the known sequence and upstream of the putative promoter. Digested DNA ligated under conditions that promote monomeric circular DNA. The resulting circular DNA is then used as template for PCR reaction utilising the known sequence to design forward and reverse primers. *Hind*III cuts inside the xylanase (*xyl*I) gene and 6.5 kbp there downstream. The *Hind*III digested *B. ovatus* genomic DNA was circularised and then amplified using primers BOPRO1 and BOPRO2. *orf*, open reading frame. *xsa*, bifunctional xylosidase-arabinosidase gene.

2.22   Conjugation

The following conjugation protocol was used to transfer pBT2 plasmid into *Bacteroides* utilizing helper plasmid R751 which facilitates mobilisation between *E. coli* and *Bacteroides*. The promoter sequence and part of the upstream gene was utilized for the homologous recombination.

Cloned pBT2 plasmid was transformed into competent *E. coli* J53/R751 and transformants were selected on LB medium supplemented with trimethoprim and kanamycin. Transformant *E. coli* J53/R751/pBT2 (Donor) was subcultured into LB broth containing trimethoprim and kanamycin until OD_{600} was 0.2. At the same time *B. ovatus* V975 (Recipient) was cultured into BHI broth supplemented with Hemin (0.001%) until OD_{660} was 0.2. Donor and recipient strains were mixed at a ratio 0.2:1 and centrifuged for 1 min at 1000g. The pelleted cells were washed with 1 ml BHI-Hemin and re-centrifuged for 1 min at 1000g. The pellet was resuspended in 75 µl BHI-Hemin and cells were spotted onto 0.45 µm sterile nitrocellulose filters placed on the surface of a BHI-Hemin agar plate. The plate was incubated aerobically at 37˚C for 16-18 h. The filters were transferred into plastic universal containing 3 ml BHI-Hemin and vortexed to resuspend the cells. The cells were incubated at 37˚C for 1 h. The cells were plated onto selective medium plates (BHI-hemin-gentamicin-tetracycline) and incubated anaerobically at 37˚C for 2-3 days. Possible transconjugants were subcultured on the selective medium and tested by colony PCR for the presence of heterologous genes.

2.23   RNA Analysis

All steps were carried out using RNase/DNase free molecular grade solution and plastics

2.23.1 RNA Extraction

SV Total RNA Isolation system (Promega) was used for total RNA isolation from prokaryotic and eukaryotic cells as per manufacturer instructions. Briefly, for bacterial RNA isolation, 1×10^9 cells were centrifuged and the pellet was resuspended in 75 µl TE buffer. Lysozyme (2 mg ml^{-1}) was added to make the

volume up to 100 µl. The mixture was incubated for 5 min at 22˚C. Lysis Buffer (RLA) 75 µl was added and mixed by inversion followed by 350 µl of SV RNA Dilution Buffer and mixed by inversion. Ethanol (95% v/v) 200 µl was added and mixed by pipetting. The lysate was transferred into spin column assembly and washed once with SV RNA Wash Solution. DNase was added on column and incubated for 15 min at 22˚C. DNase was inactivated by 200 µl DNase Stop Solution and the column washed twice with SV RNA Wash Solution. RNA was eluted with RNase free water and stored at -70˚C until use.

For tissue RNA isolation, 30 mg of mid-colonic tissue was quickly cut into small pieces with a sterile blade. The tissue was transferred into a tube containing 175 µl RNA Lysis Buffer and homogenized using a syringe and a 23g needle. RNA Dilution Buffer 350 µl was added and mixed by inversion. The tube was placed on a heating block at 70°C for 3 min then centrifuged for 10 min at 14000g. The cleared lysate solution was transferred into a fresh microcentrifuge tube and ethanol (95% v/v) 200 µl was added and mixed by pipetting This mixture was transferred into the Spin Column Assembly. The rest of the procedure was the same as the bacterial RNA preparation mentioned above.

2.23.2 Quantitative analysis of RNA

Two microlitres of the eluted RNA was used to determine the RNA quantity and purity using a spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, USA) the absorbance was read at 260, 230 and 280 nm. The yield of total RNA obtained was determined at A_{260}. The purity of RNA was estimated by the ratio of A_{260}/A_{280} and A_{260}/A_{230}. A ratio in the range of 1.8-2.2 was acceptable.

2.24   Rapid amplification of 5' complementary DNA ends (5' RACE)

Although primer extension analysis is commonly employed for 5' end mapping of RNA, it is less sensitive and requires radioactive labelled primers. Therefore, 5'RACE was used to identify the transcription initiation site (TIS). Figure 2.3 illustrates the 5'RACE procedure. Briefly, it was performed as follows:

Reverse transcription: Bacterial RNA was treated with DNaseI (DNA-free, Ambion). DNA-free RNA was used for first strand DNA synthesis in the following reaction:

RNA	1-2µg
SP1 primer (12.5 µM)	1 µl
dNTP (2.5 mM each)	2 µl

The above was mixed in a 0.2 ml tube, spun at 12000g then incubated at 70˚C for 5 min then quenched on ice for 5 min. Then the following was added:

RTase buffer (10x)	2 µl
RNase inhibitor (Promega)	1 µl
RTase (NEB)	1 µl

The reaction volume was made up to 20 µl with nuclease free water and incubated at 42˚C for 1 h. The reaction was terminated by further incubation at 90˚C for 10 min.

To remove excess salts, PCR Purification kit (Qiagen) was used as per manufacturer protocol. Briefly, PB buffer was added to the tube (x5 the volume) then applied to the column. The column was centrifuged for 30 sec, washed with 750 µl PE buffer. The flow-through was discarded and the column was further centrifuged for 1 min. cDNA was eluted in 30 µl elution buffer.

Addition of Poly(A) tail to first strand cDNA: Homopolymeric A-tail was added to the 3' end of first strand cDNA using recombinant Terminal Transferase (TdT) and dATP.

The following was added into a sterile microcentrifuge tube on ice:

Purified cDNA sample	15 µl
Reaction buffer (10x)	5 µl
dATP (100 µM)	1.5 µl
$CoCl_2$ (1.5 mM)	2.5 µl

The components were mixed and briefly spun at 12000g. The reaction was incubated at 94˚C for 3 min then chilled on ice and briefly spun at 12000g. Terminal

Transferase (TdT) 1 µl was added and the reaction was incubated at 37˚C for 20 min then 70˚C for 10 min to heat inactivate the TdT.

PCR amplification of poly(A)-tailed cDNA: The tailed cDNA was directly amplified by PCR using *Taq* DNA polymerase in the following reaction:

dA-tailed cDNA	12 µl
Anchor d(T) primer (12.5 µM)	1 µl
SP1 primer (12.5 µM)	1 µl
Reddymix (ABgene) (2x)	25 µl
Nuclease free water	up to 50 µl

The cycling conditions used are shown in Table 2.4

Table 2.4   Cycling conditions used in PCR reaction of the 5'RACE procedure

	Temperature (°C)	Time	Cycle No.
Initial denaturation	94	2 min	x1
Denaturation	94	40 sec	
Annealing	55	30 sec	x35
Elongation	72	1 min	
Final elongation	72	5 min	x1

A second and third nested PCR was performed on the first PCR product with gene specific primers (SP2 and SP3) located further inside the gene. The same cycling conditions were used but the annealing temp was increased to 58 °C.

The product of the third nested PCR was gel purified and used for cloning into pCR®4-TOPO (TOPO-TA Cloning kit, Invitrogen) in the following reaction:

Gel purified PCR product	3 µl
pCR4-TOPO	1 µl
Salt solution	1 µl
Nuclease free water	1 µl

The reaction was incubated for 5 min at 22˚C, transformed into *E. coli* competent cells and selected on LB plates supplemented with kanamycin. The plasmid was isolated from transformants, checked by agarose gel electrophoresis after digestion with suitable restriction endonuclease and sequenced by primer walking (DNA Sequencing Service, Dundee).
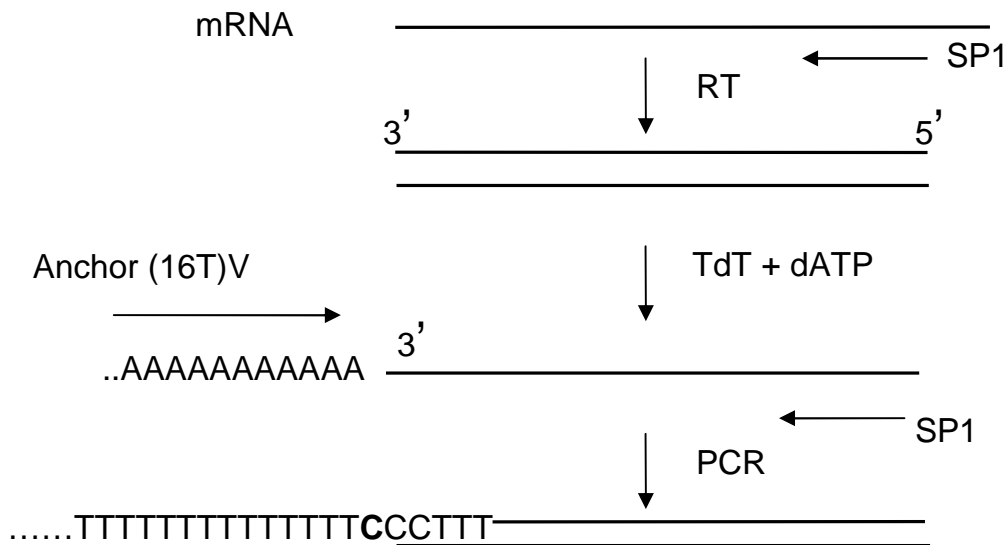
Figure 2.3   Diagrammatic representation of the 5'RACE technique

Total RNA used as template for cDNA formation using single gene specific primer (SP1) in the reverse direction. A poly-A tail was then added to the 3' end of the cDNA by terminal transferase (TdT). Polyadenylated cDNA was used as template in a PCR with primers, anchor d(T) and SP1, and the resulting product subjected to a second and third rounds of PCR with an anchor forward primer and nested reverse primers SP2 and SP3 respectively. The PCR product was cloned and sequenced to identify the transcription initiation site (TIS) in bold. V=G, A or C.

2.25   DNA first strand synthesis (Reverse transcription)

Reverse transcription of RNA was performed in a sterile microfuge tube as follows:

RNA solution	0.5-2 µg
random hexanucleotide primers (Sigma)	4 µM
dNTP (2.5 mM each)	4 µl
Nuclease free water	up to 17 µl

The reaction was heated for 3-5 min at 70˚C, then spun at 12000g and placed on ice for further 2 min. The following were then added:

Buffer (10x)	2 µl
M-MuLV Reverse Transcriptase (NEB)	1 µl

This was incubated at 42˚C for 1 h then 90˚C for 10 min to inactivate the enzyme. Samples were used immediately or stored at -80˚C.

2.26   Semiquantitative RT-PCR

For each reaction, the following mixture was used

cDNA	1-2 µl
Reddymix (ABgene)	12.5 µl
Forward primer	500 nM
Reverse primer	500 nM
Nuclease free water	up to 25 µl

The cycling conditions of these reactions were similar to those used in general PCR reactions. PCR products were visualised by agarose gel electrophoresis. For normalisation eight 4-fold dilutions of cDNA were assessed for *gyr*A expression of *B. ovatus*. The same dilutions were assessed for the test gene expression.

2.27   Real time RT-PCR

Real Time PCR was carried out using SYBR Green detection system (MyiQ Single-Color Real-Time PCR Detection System, Bio-Rad). Primers were designed using Primer3 freeware V 0.4.0 (http://fokker.wi.mit.edu/primer3/input.htm). A list of primers used in real-time PCR are illustrated in Appendix 2. Initially the designed primer sets were tested to optimise the concentrations. Optimal primer concentration mix should obtain the lowest CT value and no primer dimer on melting curve analysis. The standard curve method was used to quantitate the results obtained by real-time RT-PCR. In this method, a standard curve is first constructed from cDNA samples expressing the target gene. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations.

After synthesis of cDNAs, 2 µl was removed from each reaction and pooled to be used as standards. This was then diluted 20-fold by adding ddH$_2$O. This was the x1 standard. From this a series of dilutions (x3, x9, and x81) were made. These dilution series provided the template cDNA for the standard curve wells. The remaining cDNA sample was diluted 30-fold to be used as template cDNA for the PCR reaction. Once all cDNA dilutions had been made, the Q-PCR plate was set up.

The reaction mixture for the real time PCR was constructed as follows:

SYBR Green master mix (Sigma)	12.5 µl
Forward primer	200-500 nM
Reverse primer	200-500 nM
Template cDNA	5 µl
Nuclease free water	up to 25 µl

The cycling conditions used for the real time PCR reactions are illustreated in Table 2.5.

Table 2.5   Cycling conditions used in Real-time PCR reactions

Cycle	Repeat	Step	Time (min)	Tm (˚C)
1	1	1	3:00	95
2	40	1	0:30	95
		2	0:30	60*
		3	0:30	72
3	1	1	0:30	95
4	1	1	0:30	55
5	82	1	0:10	55**

*Annealing temperature depends on the primer

** Temperature increased by 0.5 ˚C on each repeat of the cycle

The last 3 cycles are melting curve steps

The threshold cycle (Ct) was determined with optical system software (V 1.0, Bio-Rad) and the starting quantity (SQ) was calculated by the software using the standard curve. The mean starting quantity of a test gene from duplicates (SQ Mean) was divided by the mean starting quantity of the house keeping gene from duplicates of the same sample (SQ Mean) to obtain the relative gene expression of that sample.

2.28   KGF-2 and TGF-β_1 ELISA

Microwells (Nunc Maxisorp) were coated with 100 µl per well of capture antibody diluted in coating buffer (Na_2CO_3 (0.35 g) and $NaHCO_3$ (0.84 g) were dissolved in 100 ml ddH$_2$O, pH= 9.5). The plate was sealed and incubated for 16-18 h at 4˚C. Wells were aspirated and washed 3 times with 300µl/well Washing buffer (Tween-20 0.05% (v/v) in PBS). After the last wash, the plate was inverted and blotted on absorbent paper to remove any residual buffer. Wells were blocked with 300 µl/well blocking buffer (FBS 10% (v/v) in PBS) and incubated for 1 h at 22˚C followed by 3 washes with washing buffer. Standards, samples and control were pipetted into appropriate wells in triplicate (100 µl well[-1]) and the plate was incubated for 2 h at 22˚C. Wells were aspirated and washed 5 times with Washing buffer. Detection antibody diluted in assay diluent was added (100 µl well[-1]) and the

plate was incubated for 1 h at 22˚C followed by 7 washes with Washing buffer. Substrate solution (equal volumes of tetramethylbenzidine (TMB) and hydrogen peroxide) was added 100 µl well^{-1} and incubated for 30 min at 22˚C in the dark. The reaction was stopped by adding 50µl well^{-1} stop solution (2N H_2SO_4) and absorbance at 450 nm (OD_{450}) was measured within 30 min using a microplate reader (SpectraMax 340PC, USA).

The mean absorbance for each triplicate was calculated. The mean zero standards was subtracted from standards, samples and controls. The standard curve was plotted for the log-log of the standard concentrations and absorbance and linear regression equation was obtained using Microsoft Excel software (v.2003). The log concentrations were calculated for the samples and controls by applying their log absorbance to the equation. The results then returned into its exponents and corrected for dilution factor if used.

2.29 SDS-PAGE and Western blotting.

2.29.1 Protein separation by SDS-PAGE

Materials

Non-reducing sample buffer

Sodium dodecyl sulfate (SDS) 1.2 g, Glycerol 3 g, Coomassie blue 5 mg and 750 µl of 2M Tris/HCl solution (pH 7.0) (final conc. 150 mM) were added to 10 ml ddH_2O. Three volumes of ddH2O were added when used for precipitated protein.

Gel buffer (3x)

Tris-base 18.15 g, HCl (36M) 1.37 ml and SDS 0.15 g were added to 50 ml ddH_2O and stored at 22˚C.

Running buffer and gel preparation

Running buffer ingredients are shown in Table 2.6 and resolving and stacking gels in Table 2.7

Table 2.6   Ingredients and concentrations of the SDS-PAGE running buffer

	Cathode buffer	Anode buffer
Tris (M)	1	1
Tricine (M)	1	---
HCl (M)	---	0.225
SDS (%)	1	---
pH	8.25	8.9

Table 2.7   Ingredients and concentrations of the SDS-PAGE resolving and stacking gels

	Resolving Gel (15%)	Stacking Gel (4%)
Gel Buffer (3x)	10 ml	3 ml
Acrylamide 30% (w/v)	16 ml	1.6 ml
Glycerol	3 ml	----
TEMED	10 µl	9 µl
APS 10% (w/v)	100 µl	90 µl
ddH2O	890 µl	7.4 ml

Protocol

Casting the gel

Bio-Rad Protean II mini equipment was used to prepare both resolving and stacking gel. Briefly, 15% (w/v) resolving gel was freshly prepared and inserted into the cast. Water-saturated butane was laid on the top of the resolving gel to level the surface. The cast was incubated for 30 min at 22˚C. Polymerised resolving gel was then overlaid directly with freshly prepared 4% (w/v) stacking gel with the comb inserted. The gel was incubated at room temperature for 30 min.

Sample preparation and electrophoresis

Overnight bacterial culture was sheered through a 23G needle then centrifuged for 10 min at maximum speed. The supernatant was filtered through 0.45 µm filter. The protein was precipitated by adding 4 volumes of ice cold acetone then held for 60 min at -20°C. The protein was retrieved by centrifugation for 10 min and the pellet was allowed to dry for 30 min. The pellet was resuspended in non-reducing sample buffer. Protein (30-40 µg) was loaded into each well. In addition 15 µl prestained protein ladder (NEB) was loaded to facilitate orientation and identification of protein bands. The gel was electrophoresed in running buffer at constant 150V until the bromophenol blue mark had run off the end of the gel (approximately 2 h).

2.29.2 Silver staining

The SDS-PAGE gel was stained with silver nitrate as follows: The gel was incubated for 30 min in fixing solution (Methanol 50% v/v, Acetic acid 10% v/v and 100 mM ammonium acetate) then washed twice (30 min each) with dH_2O. The gel was sensitised by incubation with 0.005% (w/v) sodium thiosulfate ($Na_2S_2O_3$) for 30 min followed by incubation with 0.1% (w/v) silver nitrate for 30 min, then washed for seconds with dH_2O. Finally, the gel was incubated with developer (Formaldehyde 0.036% v/v and sodium carbonate 2% w/v) for 2-3 min. Development was stopped by incubating the gel in 50 mM EDTA for 30 min.

2.29.3 Electroblotting

The Immobilon membrane (PVDF membrane, Millipore, UK) was washed in methanol for 3 seconds then immersed in water for 2 min to displace the methanol. The membrane was then incubated in transfer buffer (Tris-base 3 g, Glycine 14.4 g and 200 ml methanol were added to 800 ml ddH_2O, transfer buffer was freshly prepared and kept at 4°C until use) for 2-3 min. Separated proteins were transferred from the gel to the membrane submerged in transfer buffer for 1 h at a constant 150 V at 4°C.

2.29.4 Immunoblot

After electroblotting the membrane was washed twice briefly in washing buffer (Tris-base 2.42 g, NaCl 8.8 g and 500 µl Tween-20 were added to 1 L dH$_2$O) then held overnight at 4°C in blocking buffer (5% (w/v) skimmed milk powder in washing buffer). The blocked membrane was then washed 5 times (10 min each) and held with biotinylated primary monoclonal anti-TGFβ_1 antibody (1:250) with HRP (1:250) in blocking buffer overnight at 4°C with shaking. The blotted membrane then washed 5 times with washing buffer and blotted on tissue paper then incubated at 22°C for 1-2 min with equal quantities of ECL solution 1 and 2 (Amersham-Pharmacia) and exposed to X-Ray film (Kodak) for 10-20 seconds in a developing cassette. X-Ray film was developed using compact X4 automatic X-Ray film processor (Xograph, Kodak).

2.30   TGF-β_1 Bioassay

TGF-β1 bioassay was performed by P. Dilger (Division of Immunobiology, National Institute for Biological Standards and Control, Potters Bar, UK) and was based on the ability of TGF-β1 to inhibit IL-5 induced proliferation of the erythroleukaemia cell line, TF-1.

TF-1 preparation: the human TF-1 cell line was grown in RPMI 1640 medium supplemented with 5% (w/v) heat inactivated foetal calf serum (FCS), 100 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin, and 2 ng ml^{-1} recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) in a humidified atmosphere containing 5% (v/v) CO$_2$ at 37°C. Cultures were passaged when they reached a density of 2 x 10^5 cells ml^{-1} and diluted to a density of 5 x 10^4 cells ml^{-1}.

TGF Bioassay: A dilution series of samples was prepared in 100 µl volumes in 96-well microtiter plates. TF-1 cells were used when they had reached a cell density of 2 $\times$ 10^5 cells ml^{-1}. The cells were washed twice in RPMI 1640 medium and resuspended in RPMI 1640 containing 5% (v/v) FCS, 50 µg ml^{-1} streptomycin and 100 U ml^{-1} penicillin and 4 ng ml^{-1} rhIL-5 at a density of 1 $\times$ 10^5 cell ml^{-1}. Cells were added to the microtitre plate at 100 µl well^{-1}.

The microtitre plate was incubated at 37°C, in a 5% (v/v) CO$_2$ humidified atmosphere for 48 h. To assess cell proliferation, 0.5 µCi^{-1} of [^{3}H] thymidine ([^{3}H]

Tdr) was added per well and the microtitre plate returned to the incubator for a further 4 h. Cells were harvested onto filter mats and the radioactivity incorporated into DNA was measured using a scintillation counter. A dose-response curve of cpm versus dilution of standard or unknown was plotted, and the amounts of TGF-β in the samples were determined from standard curve linear regression analysis of cpm versus log standard concentration.

In neutralization experiments, purified chicken anti-TGFβ antibody (1:200 dilutions) was incubated with the appropriate samples for 1 hour at 37°C before the addition of cells.

2.31   KGF-2 (FGF-10) Bioasay

All manipulations involving tissue culture were performed in a class II laminar flow safety cabinet.

Normal human keratinocytes (NHK, Invitrogen) were used in an epithelial cell proliferation assay as previously described (Shoucheng *et al*., 1998). NHK were grown on collagen-coated flasks (Greiner-Bio-one) in keratinocyte serum-free media (K-SFM) supplemented with 5 ng ml^{-1} epidermal growth factor (EGF, Gibco), 100 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin (Sigma) at 37˚C in a humidified atmosphere of 5% (v/v) CO_2. At passage 4 when they reached 75-80% confluence, NHK were used for the proliferation assay. NHK were washed 3 times, 5 min each, with PBS and harvested with 1 ml trypsin/EDTA (Sigma) for 7-8 min. Trypsin inhibitor (Sigma) was added to final concentration 500 µg ml^{-1} the cells were retrieved by centrifugation at 430 g for 10 min. Cell pellets were resuspended in fresh K-SFM supplemented 100 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin, but not EGF. The NHK were cultured in 96 well plates (Costar) at 5×10^4 cell ml^{-1}, recombinant KGF-2 (Sigma) was added for the standard curve wells (concentration ranging from 0 to 40 ng ml^{-1}) or dilutions of *Bacteroides* culture supernatant (from BO-KGF and BO V975) concentrated using 5000 MWCO Amicon Ultra columns (Millipore) with and without affinity purified anti-KGF-2 neutralizing polyclonal antibody 2 µg ml^{-1} (MBL International). After incubation for 4 days at 37˚C, 10 µl of 5 mg ml^{-1} Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma) was added to each well. Cells were incubated for further 4 h and supernatant was removed. The crystal

formazan product was dissolved with 200 µl DMSO (Sigma). The Optical Density (OD) at 570 nm was measured using a microplate reader (SpectraMax 340PC, USA). The quantity of biologically active KGF-2 in each dilution was estimated from linear regression of the standard curve.

2.32   Animal Experiments

2.32.1 Animals

This study was carried out under project licence (PPL 40/2784) issued by the Home Office in conjunction with animals scientific procedures Act, 1986. All mice were housed under specific pathogen free conditions at University of Leeds and were between 6 and 8 weeks of age. All mice were C57Bl/6 strain. They were supplied with water and food *ad libitum*. Xylan reduced diet was obtained from Special Diets Services (829053–AIN 93M (P), SDS, Denmark) and was tested by the manufacturer for the xylan content by gas-liquid chromatography (GLC). The estimated quantity was 0.7% (w/w). The estimated quantity of xylan in the regular rodents' diet was 10% (B&K Universals). When needed, xylan (Sigma) was supplemented to the drinking water at 30 mg ml^{-1}. Xylan was prepared as follows: xylan was added to distilled water (30 mg ml^{-1}) and was left on stirrer for 2 h with heating to 60°C. The non-dissolved precipitate was removed by centrifugation at 4000g.

Bacteroides from an overnight culture was centrifuged for 5 min at 4000 g Cells were washed with 1 ml PBS and retrieved by centrifugation. Bacterial cells were resuspended in PBS at 2×10^8 CFU per 200 µl. Samples of the gavage solution were spread on BHI-Hemin plates supplemented with tetracycline for estimation of CFU. Viable bacterial cells were given to mice by oro-gastric gavage using a 22G x 1" stainless steel needle. A volume of 200 µl was introduced into the stomach.

2.32.2 Induction of colitis

Acute colitis was induced by Dextran Sodium Sulfate (DSS). DSS (2.5% w/v) was added to the drinking water *ad libitum* for 5 days. Usually by day 3-4 all

animals had weight reduction, by day 5 they developed severe rectal bleeding and watery diarrhoea. Figure 2.4 summarises the treatment and prevention protocols described below.

i. Treatment protocol

There were 6 treatment groups (n=8 in each group) for each recombinant *Bacteroides* strain tested. All animals were fed a xylan reduced diet 24-48 h before the start of the experiment. Xylan-reduced diet continued until the end of the experiment. DSS (2.5% w/v) started on day 0 and continued for 5 days. On day 5, DSS was replaced with normal drinking water or drinking water supplemented with xylan for groups 2 and 4 (see below). The treatment groups were categorised as following:

Group 1: DSS control (DSS Ctrl.). Mice received DSS for 5 days then switched to normal water until the end of the experiment. Oro-gastric gavage of 200 μl PBS was given on days 5, 7 and 9.

Group 2: *B. ovatus* V975 and xylan control (BO V975+X). Mice received DSS for 5 days, then xylan supplemented water until the end of the experiment. Oro-gastric gavage of $2x10^8$ CFU of BO V975 was given on days 5, 7 and 9.

Group 3: Recombinant strain without xylan treatment (BOKGF-X or BOKGF-X). Mice received DSS for 5 days, then normal water until the end of the experiment. Oro-gastric gavage of $2x10^8$ CFU of BO-KGF or BO-TGF was given on days 5, 7 and 9.

Group 4: Recombinant strain with xylan treatment (BOKGF+X or BOKGF+X). Mice received DSS for 5 days, then xylan supplemented water until the end of the experiment. Oro-gastric gavage of $2x10^8$ CFU of BO-KGF or BO-TGF was given on days 5, 7 and 9.

Group5: Dexamethasone control (Steroid). Mice received DSS for 5 days then switched to normal water until the end of the experiment. Dexamethasone (Colvasone, Norbrook) was given daily 3 μg g^{-1} s.c.

Group 6: Normal control (Normal Ctrl). Mice received normal drinking water throughout the experiment.

ii. Prevention protocol

There were 5 treatment groups (n=8 in each group) for each recombinant strain tested. Animals were fed a xylan-reduced diet 24-48 h before the start of the experiment. Xylan-reduced diet continued until the end of the experiment. DSS (2.5% w/v) started on day 0 and continued for 5 days. Xylan was added to the drinking water of groups 2 and 4. On day 5, animals were sacrificed. The prevention groups were categorised as following:

Group 1: DSS control (DSS Ctrl.). Mice received DSS for 5 days. Oro-gastric gavage of 200 μl PBS was given on days 0, 2 and 4.

Group 2: *B. ovatus* V975 and xylan control (BO V975+X). Mice received DSS water supplemented with xylan for 5 days until the end of the experiment. Oro-gastric gavage of $2x10^8$ CFU of BO V975 was given on days 0, 2 and 4.

Group 3: Recombinant strain without xylan treatment (BOKGF-X or BOKGF-X). Mice received DSS for 5 days until the end of the experiment. Oro-gastric gavage of $2x10^8$ CFU of BO-KGF or BO-TGF was given on days 0, 2 and 4.

Group 4: Recombinant strain with xylan treatment (BOKGF+X or BOKGF+X). Mice received DSS for 5 days, then xylan supplemented water until the end of the experiment. Oro-gastric gavage of $2x10^8$ CFU of BO-KGF or BO-TGF was given on days 0, 2 and 4.

Group 5: Normal control (Normal Ctrl). Mice received normal drinking water throughout the experiment.
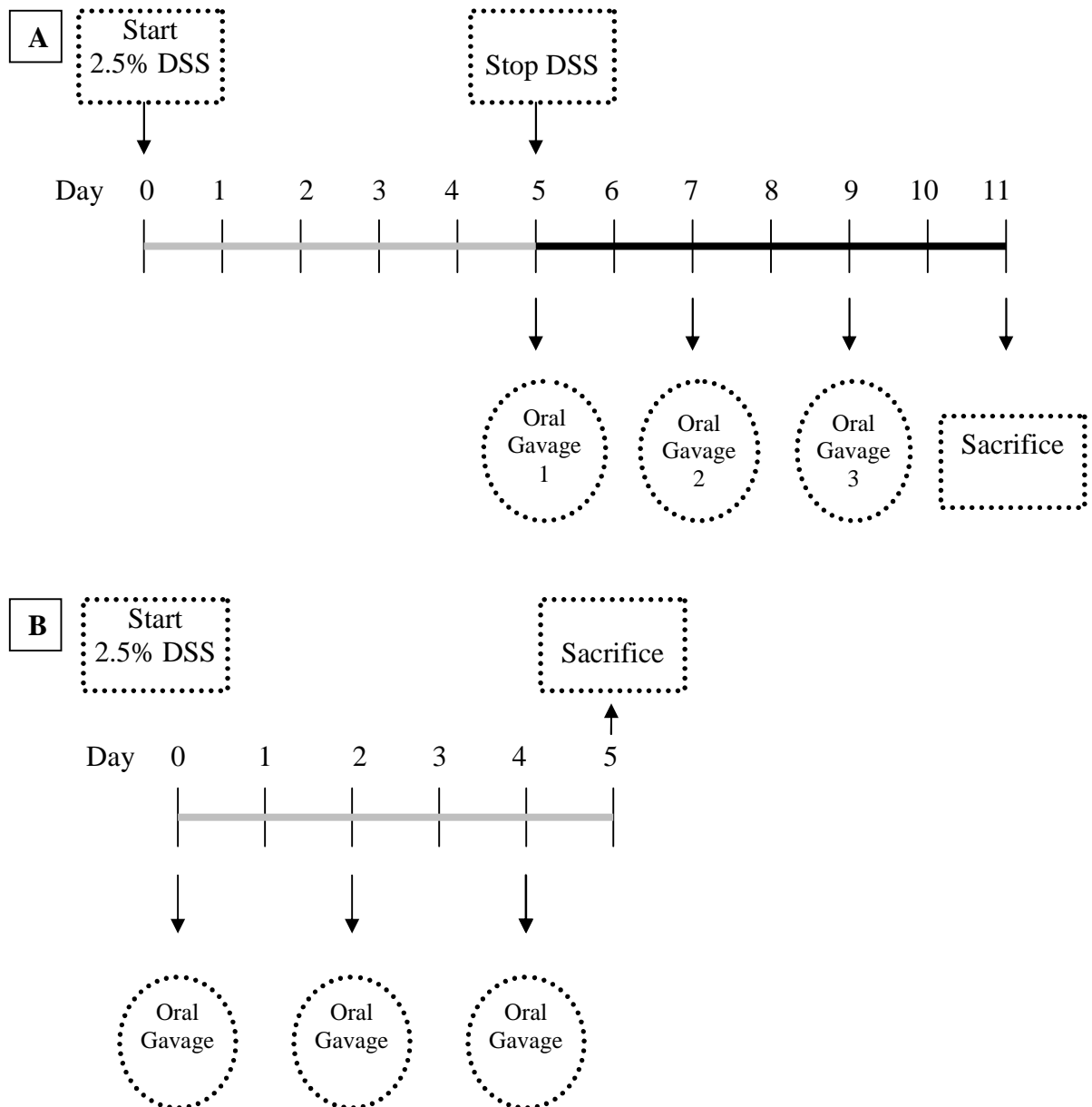
Figure 2.4   Diagram illustrating the treatment and prevention protocols for DSS-induced colitis by xylan controlled *B. ovatus* protein delivery (A) Treatment protocol: DSS (2.5%) was given for 5 days to induce colitis in C57Bl/6 mice. On day 5 DSS was replaced by normal water or water containing xylan, Mice were given BO V975, BO-KGF or BO-TGF by oro-gastric gavage. Treatment effect was assessed clinically from day 5 to day 11 and pathologically at necropsy. **(B) Prevention protocol**: oro-gastric gavage of BO V975, BO-KGF or BO-TGF was started simultaneously with DSS (2.5%) at day 0. Prophylactic effect was assessed clinically until day 5 and pathologically at necropsy.

2.32.3 Disease activity index score (DAI)

Mice were weighed and observed daily for rectal bleeding and stool consistency. If the stool was free of frank bleeding, it was tested for the presence of occult blood by test strips (Peroheme 40-C, VWR International). The disease activity index score was the sum of the three scores (Weight reduction, stool consistency and faecal blood content). Table 2.8 shows the scoring system used to grade the three clinical entities.

Table 2.8   Parameters of disease activity index (DAI) used to assess the clinical signs of colitis.

Weight reduction		Stool consistency		Faecal blood	
Score	Feature	Score	Feature	Score	Feature
0	None	0	Normal	0	No Blood
1	1-5%	1	Mucus covered stool	1	Occult bleeding
2	5-10%	2	Loose stool	2	Slight bleeding
3	10-15%	4	Watery diarrhoea	4	Gross bleeding
4	>15%				

2.32.4 Pathological grading of inflammation

At autopsy colonic length was measured and macroscopic inflammation graded as to the percent colonic involvement as follows: 0, none; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; 4, >75%.

To assess the microscopic grade of inflammation, 3 sections from each mouse colon were taken 1, 2 and 3 cm away from the anal verge and immediately fixed in 10% (v/v) buffered formalin saline. Tissue sections were embedded in paraffin and stained with H&E (Processing of specimens was carried out by the Department of Pathology at Leeds Institute of Molecular Medicine). Sections were blindly graded by specialist pathologist (Dr N Scott, St James's University Hospital). The grading was repeated blindly by the author. The score from each mouse represents the average of both readings. Each reading represents an average of three sections scores. The scoring system used in this study is shown in Table 2.9.

Table 2.9   Parameters of histological scores used to assess colonic sections stained with H&E.

Criteria	Score	Description
Crypt Injury	0	None
	1	Crypt epithelial injury/flattening ± necrotic debris in crypt lumen
	2	Erosion in < 50% mucosal thickness with basal crypt preserved
	3	Ulceration in > 50% of mucosal thickness or Crypt epithelium completely destroyed, but superficial epithelium and muscularis mucosa intact
	4	3 and superficial epithelium destroyed
	5	Ulceration involving submucosa (muscularis mucosa) or deeper (transmural)
Extent of injury	0	None
	1	Focal
	2	Multifocal ($\geq$ 2 areas)
	3	Diffuse ($\geq$ 50% circumference)
Chronic Inflammatory cell Infiltrate	0	None
	1	Mild
	2	Moderate
	3	Severe
Acute Inflammatory cell infiltrate	0	None
	1	Mild
	2	Moderate
	3	Severe
Goblet cell depletion	0	None
	1	Mild/Moderate
	2	Severe

2.32.5 Goblet cells counting

Alcian blue stains acid mucosubstances and mucins. It was used to stain colonic goblet cells, which contain mucins. Strongly acidic mucosubstances will be stained blue, nuclei will be stained pink to red, and cytoplasm will be stained pale pink. The average number of goblet cells per crypt was obtained by counting 3 fields from each section, averaging the values obtained from 3 sections of each mouse.

Protocol

Deparaffinized and hydrated 5mm sections were stained in Alcian blue solution (Alcian blue 1 g in 100 ml of 3% v/v acetic acid solution, pH=2.5 with acetic acid) for 30 min followed by washing in running tap water for 2 min then sections were rinsed in distilled water. The sections were counterstained in nuclear fast red solution (Aluminium sulphate 5 g dissolved in 100 ml water then nuclear fast red 0.1 g added slowly, boiled and allowed to cool) for 5 min and washed in running tap water for 1 min then dehydrated through 95% (v/v) alcohol, cleared in xylene and mounted using DePex (BDH).

2.32.6 Immunostaining

Formalin fixed, paraffin embedded tissues were cut into 5 µm thick sections and placed on Superfrost Plus slides (BDH) then incubated for 16-18 h at 37°C. The sections were de-waxed and rehydrated sequentially in xylene, absolute alcohol and running tap water. Endogenous peroxidases were blocked with 3% (w/v) H_2O_2 in Methanol for 15 min then tissues were washed in tap water. Antigens were retrieved in a pressure cooker using Antigen retrieval solution (Vector labs) for 2 min then washed in PBS for 5 min. Endogenous biotins were quenched in Avidin/Biotin blocking reagents (Vector), washed in PBS for 5 min then blocked in Casein block solution (Vector) diluted 1:10 in PBS. Sections were incubated for 2 h at 22°C with primary antibody (Monoclonal Rat anti-mouse Ki-67, clone TEC-3, Dako) diluted 1:80 in antibody diluent (Zymed) or Isotype control (Rat IgG2a, Caltag) diluted 1:10 in antibody diluent. Sections were then washed for three 5 min periods in PBS followed by secondary antibody incubation (Biotinylated Rabbit anti-Rat IgG Vector, diluted 1:200 in antibody diluent) for 30 min, then washed for three 5 min

periods in PBS. Sections were incubated with streptavidin (Dako) for 30 min then washed for three 5 min periods in PBS. The colour was developed by incubation with DAB and chromogen (Dako) for 10 min then washed in tap water. Sections were incubated with copper sulphate solution (2% w/v) for 3 min then counterstained in Mayers Haematoxylin followed by Scott's tap water substitute and rinsed in tap water. Finally, sections were dehydrated in ethanol, cleared in xylene and mounted using DePex (BDH).

2.32.7 Myeloperoxidase (MPO) Assay

Materials

Working buffer (50 mM potassium phosphate, pH=6.0)

K_2HPO_4 (1M) 13.2 ml and KH_2PO_4 (1M) 86.8 ml were mixed before use and diluted 20 times in ddH_2O.

Protocol

Mid-colonic tissue (50 mg) was placed into a 14 x 100 mm plastic test tube and sample buffer (0.5 ml) (0.5% w/v hexadecytrimethylammonuim bromibe (HTAB) in working buffer) was added. Tissue was homogenized on ice then additional sample buffer was added to give a final concentration of 1 ml of buffer per 50 mg of tissue. The samples were then sonicated (Soniprep 150) on ice for 30 sec and freeze-thawed three times. The samples were centrifuged at maximum speed for 2 min. and supernatant was transferred into labelled eppendorf tubes. Supernatant (20 µl) from the homogenized tissue sample was placed into duplicate wells of a 96-well plate. Development Reagent (200 µl) (working Buffer 40 ml, H_2O_2 (1% v/v) 1µl and *O*-dianisidine dihydrochloride 6.7 mg) was added to each well. Wells with development reagent only served as a blank. The assay develops at different rates depending on the amount of MPO in the sample. Absorbance was measured at 450 nm immediately after adding the Development Reagent, and then measurement continued at 1 minute intervals.

Catalysis of 1 mole H_2O_2 gives a change in absorbance (A) = 1.13 x 10^4 min^{-1}, therefore, cleavage of 1 µmole H_2O_2 gives A = 1.13 x 10^{-2} min^{-1}, which corresponds to 1 U of MPO as previously defined (Bradley *et al.*, 1982).

Units of MPO in a well = (Y/X) x 1.13 x 10^{-2}

Y= OD_2-OD_1, X= time between OD_1 and OD_2

To convert to Units of MPO per mg tissue, Units MPO per well were divided by the number of mg of tissue added to each well. If the tissue:buffer ratio is 50 mg ml^{-1}, then using the recommended 20 µl of sample there would be MPO extract from 1 mg of tissue in each well.

2.32.8 Colonic Cytokine expression analysis

Cytokine (IL1β, IL-6, TNF-α, ITF, Cox-2, TGF-β1 and KGF-2) expression was analysed by real-time PCR. Colonic RNA was isolated using SV total RNA isolation kit (Promega) as detailed under RNA analysis (section 2.23.1). Real-Time Primers used in these reactions are illustrated in Appendix 2.

2.33   Statistics

Statistical software SPSS v.14.0 (USA) was used for statistical analysis. All graphs were presented as means, with error bars denoting standard error of the means (SEM). Data obtained was assessed using Kolmogorov Smirnov calculation in order to demonstrate if the data was parametric or not. If the results were parametrically distributed, the Student's *t*-test was used for comparison within groups or single factor ANOVA for comparison between groups. Non-parametric data was assessed using Mann-Whitney U test for comparison within groups or Kruskal-Wallis H test for comparison between groups. An associated *p*-value of <0.05 was considered significant.

CHAPTER (3)

IDENTIFICATION OF *B. ovatus* PUTATIVE XYLANASE OPERON PROMOTER

3.1 Introduction

Hemicelluloses or xylans are a major component of plant cell wall polysaccharides. These polysaccharides can not be degraded by digestive enzymes produced by human and other animals. However, colonic microflora has a major role in their degradation by the expression of xylanolytic enzymes. Fermentation products of these polysaccharides formed by the colonic microflora may be utilized by humans. The best studied human colonic xylanolytic bacterium is the obligatory anaerobic *Bacteroides ovatus*, which can utilize xylan as a sole source of carbohydrate (Salyers *et al.*, 1981; Whitehead & Hespell, 1990). Two genes at the 3' of the *B. ovatus* xylan utilization operon were previously cloned (Whitehead & Hespell, 1990), one gene encoded xylanase (*xyl*I) and the other encoded the bifunctional xylosidase-arabinosidase (*xsa*). It was then shown that both genes are part of the same operon which could comprise more genes. This operon is under the control of a xylan-inducible promoter and the disruption of the xylan utilization operon resulted in reduced growth of *B. ovatus* on xylan (Weaver *et al.*, 1992). However, the number of genes in this operon and the promoter driving the production of xylanase remain unidentified.

Our group has previously utilized this operon to enable murine IL-2 to be produced by *B. ovatus* in response to xylan *in vitro* (Farrar *et al.*, 2005). However, insertion of the IL-2 gene into the known part of the xylanase operon disrupted the xylanase genes. Lack of xylanase production by the recombinant organisms may result in a competitive disadvantage when introduced into the gut. To generate strains of *B. ovatus* capable of producing therapeutic proteins in a xylan-inducible manner without disruption of the xylanase operon, the promoter of this operon must be used. This chapter describes the cloning of *B. ovatus* genomic DNA fragment carrying the 5' of the xylan utilization genes and the upstream promoter region, the sequencing of these genes and the analysis of the DNA sequences. Partial molecular and functional characterisation of the putative promoter is described here as well.

3.2   Aims and Experimental design

3.2.1   Identification of the xylanase operon and its promoter.

Experimental design:

```
┌─────────────────────────────────────────┐
│     Isolate B. ovatus genomic DNA        │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────────────────────────┐
│  Digest the genomic DNA to completion using variouse          │
│  restriction endonuclease. Identify the fragments that carry   │
│  the operon and the putative promoter by Southern blot         │
│  hybridization. Choose the restriction enzyme for subsequent   │
│  steps.                                                        │
└─────────────────────────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────────────────────────┐
│  Perform self-ligation on the digested genomic DNA using      │
│  conditions that promote monomeric circularisation            │
└─────────────────────────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────────────────────┐
│  Amplify the circular DNA product using primers from       │
│  known sequences (Inverse-PCR).                            │
└─────────────────────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────────────────────┐
│  Clone the PCR product into cloning vector and sequence it. │
└─────────────────────────────────────────────────────────┘
```

3.2.2   Characterisation of the xylanase promoter

Experimental design:

Identify the putative promoter and operon regions by ORF finder software

Produce mutant *B.ovatus* strains by insertion mutation in the first gene of the xylanase operon (BOΩG1) and study its growth in xylan containing medium.
Prove by RT-PCR that the genes in the putative xylanase operon are transcribed from the same promoter

Determine the TIS site by 5'RACE
Clone the promoter sequence upstream of the reporter gene *xyl*E

Characterise the promoter by real-time PCR to determine regulation of the putative promoter

3.3 Results

3.3.1 Cloning of the putative *B. ovatus* xylanase operon

B. ovatus genomic DNA was isolated by NaCl/CTAB method (Fig 3.1A). Various restriction endonucleases were used to digest 3 µg of the genomic DNA to completion. Digested DNA was blotted onto nitrocellulose membrane using Southern method, and hybridized with *Eco*RI-*Kpn*I 600bp DNA fragment from pOX1 (Appendix 1 and Fig 3.1B) after labelling with digoxigenin. Southern hybridisation identified a 6.5 kb *Hind*III fragment containing the *xyl*I gene and an upstream non-characterised region (Fig 3.2). To amplify the fragment upstream of the known sequence I-PCR was used. Briefly, it involves circularisation of the digested DNA, by self-ligation, at proportionately low ligation concentration (1 µg ml^{-1}) to promote formation of monomeric circular DNA. DNA was recovered by ethanol precipitation and 200 ng was used to amplify the fragment of interest using a mixture of proofreading *Tgo* and *Taq* DNA polymerase (Roche), forward BOPRO1 and reverse BOPRO2 primers at an annealing temperature of 52 ˚C (Fig 3.3). The PCR product was gel purified, treated with T4 DNA polymerase and T4 polynucleotide kinase in a one-step blunting and 5' phosphorylation procedure to generate blunt end PCR products then cloned into pCR®-Blunt for sequencing. Sequencing was carried out by the Dundee sequencing service by primer walking (Fig 3.4, 3.5, 3.6, 3.7) and assigned GenBank accession no EU334491.

The obtained sequence was analysed by ORF finder software of the National Centre for Biotechnology Information (NCBI) website http://www.ncbi.nlm.nih.gov/gorf/gorf.html to predict the putative ORFs and the possible promoter upstream of the fully sequenced operon. The *xyl*I and *xsa* genes previously described (Whitehead, 1995) were identified as was the ORF2 upstream of *xyl*I that had previously been partially sequenced. This ORF2 comprised 1413 bp encoding a protein of 471 aa with a predicted molecular mass of 52.4 kDa (Fig 3.7). This protein was identified as a sodium/sugar symporter with 64% identity to a putative sodium symporter gene of *Prevotella bryantii* and 38% identity to the xyloside transporter of *Lactococcus lactis*. A second ORF (ORF1) of 1656 bp was identified further upstream encoding a protein of 552 aa with a predicted molecular

mass of 62.1 kDa (Fig 3.6). This protein was identified as a putative sialic acid-specific 9-O-acetylesterase. As no further ORFs were identified the xylanase operon of *B. ovatus* comprises 4 genes (Fig 3.4).
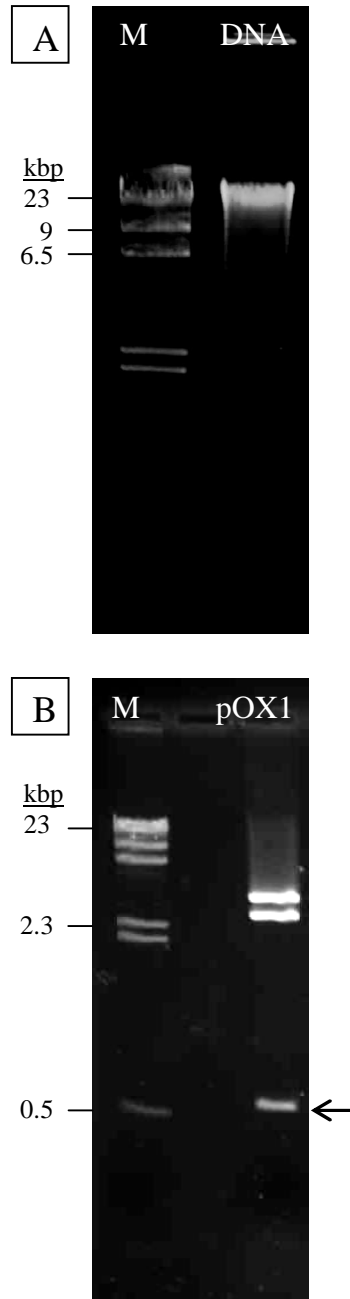
Figure 3.1   Agarose gel elecrophoresis of *B. ovatus* genomic DNA and pOX1. **(A) *B. ovatus* genomic DNA**. *B. ovatus* was cultured on BHI-Hemin and DNA was isolated by NaCl/CTAB method **(B) pOX1** (Appendix 1) digested with *EcoR*I and *Kpn*I. 600 bp *EcoR*I-*Kpn*I fragment (arrow) was gel extracted and labelled with digoxinin then used as a probe in the Southern blot hybridisation. M; λ *Hind*III molecular weight standards.
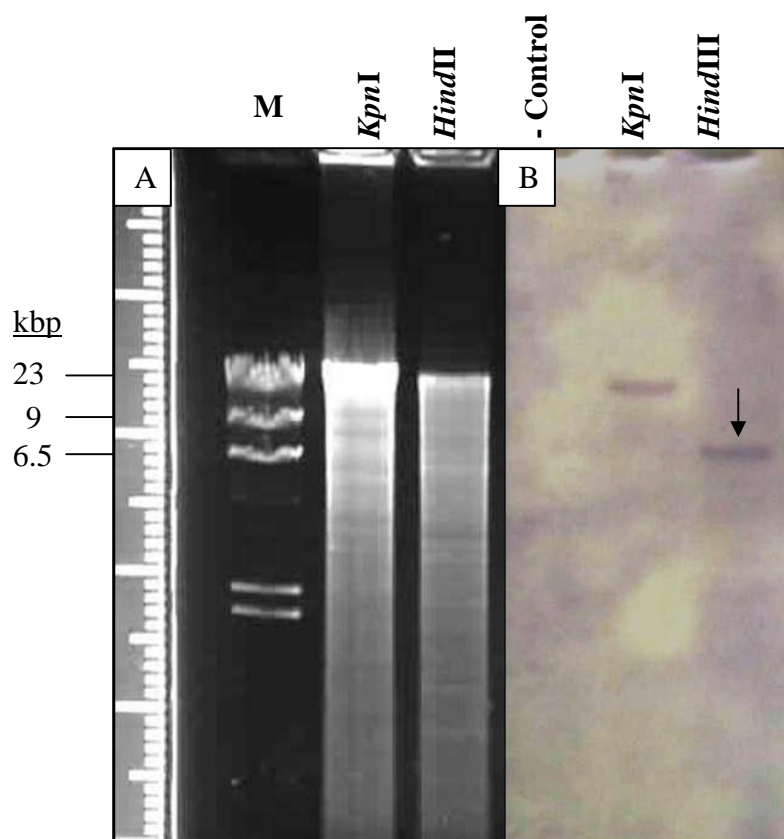
Figure 3.2   Southern blot hybridisation analysis of *B. ovatus* V975 genomic DNA with DIG labelled *Eco*RI-*Kpn*I 600bp DNA fragment from pOX1 (A) Electrophoresed *B. ovatus* genomic DNA subjected to enzymatic digestion using restriction endonuclease as shown. (Lane M, λ *Hind*III molecular weight standards) **(B)** Detection of the blotted DNA with DIG High Prime Labelling and Detection Starter Kit I. Following DNA transfer to nitrocellulose which corresponds to restriction enzymes used in **(A)** the 6.5 kb *Hind*III fragment to which the *xyl*I probe hybridised is highlighted by the arrow on the Southern blot. This fragment was expected to consist of the genes in the xylanase operon and the promoter. (–Control, negative control)
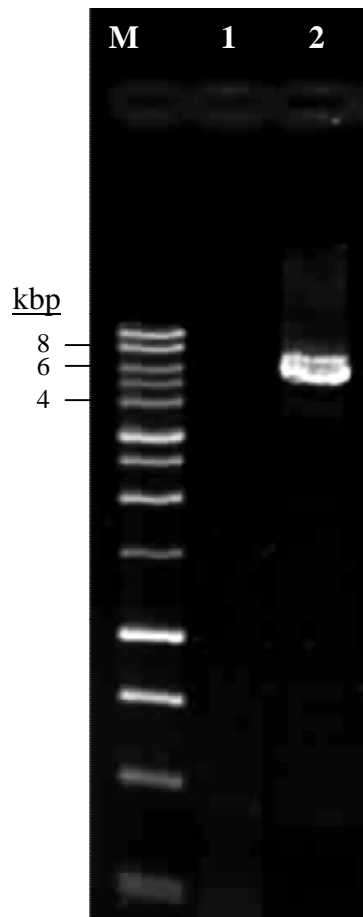
Figure 3.3   Agarose gel of inverse-PCR product. 1% (w/v) Agarose gel stained with ethedium bromide. (M) 1 kbp DNA marker. 1, negative control PCR (water control). 2, 6.5 kbp PCR product was gel purified and exposed to one-step blunting and 5' phosphorylation procedure, subcloned into pCR®-blunt and sequenced by primer walking method.
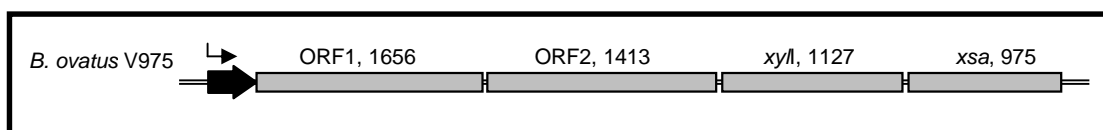
Figure 3.4 **Schematic representation of the xylan utilization genes in *B. ovatus* V975.** Genes estimated sizes indicated in bp. ORF, open reading frame; *xyl*I, xylanase I; *xsa*, xylosidase/arabinosidase. The promoter is indicated by black arrow.

```
  1   GGAATAAATG ATTTTATAAC GATTAAAGAA GGGGAGAGTG AGGGTTACAG

 51   TAGTTGTTAT TATCTTGGAG TGGATCAAAT GAATAACAAT AACTACTGTA

101   ATCCGCTCGT TCTAAAACTT TCCCTTTCCT CTTGTTTATC GGTGGTTTCA

151   GCTTGAATGT AACATTGCAA CAAGTTTTTG TAACATACGT AAACGATGAT

201   GTAACAAGCG AGTAATGATT TGAATCAGAA TTTCTGGGTA AGTGGGAAGA

251   AACTTCCTAA GTTTGCAAAG TGGAATCCCG ATGGATTTCT TCTCTTGCTA

301   AATTATCATG AAATAAAGAC ATATAAAAGA AAGACAGAAT GAATAAGTAT
```

Figure 3.5 **Nucleotide sequence of *B. ovatus* V975 putative xylanase promoter.** Putative Ribosome Binding Site (RBS) is shown in bold. The initiation and stop codons are underlined in bold, Transcription initiation site (TIS) is highlighted.

```
                    M   N   K   Y   W   F   Y   K   V   G   L   V   V   V
   1 ATATAAAAGAAAGACAGAATGAATAAGTATTGGTTTTATAAGGTAGGGTTAGTAGTTGTG
  21 F   L   C   F   A   L   L   G   G   A   K   V   K   L   P   T   L   V   S   D
  61 TTCCTTTGCTTCGCCTTGTTGGGCGGAGCAAAGGTTAAACTTCCGACTCTTGTTTCCGAC
  41 G   M   V   L   Q   R   G   E   P   V   N   I   W   G   T   A   D   P   D   E
 121 GGAATGGTACTTCAGCGTGGGGAACCTGTCAATATCTGGGGAACGGCTGATCCTGATGAA
  61 T   V   D   I   T   F   L   K   K   K   Y   K   T   V   G   D   V   Q   G   N
 181 ACCGTTGATATAACTTTCCTGAAAAAGAAATATAAGACTGTTGGGGATGTACAAGGTAAC
  81 W   K   V   T   L   P   I   L   K   A   G   G   P   Y   T   M   A   I   N   D
 241 TGGAAAGTGACTTTGCCTATATTGAAAGCTGGCGGACCTTATACGATGGCCATTAATGAT
 101 I   E   L   K   D   I   L   I   G   D   V   W   V   C   S   G   Q   S   N   M
 301 ATCGAATTAAAGGATATTCTTATTGGCGATGTTTGGGTATGTTCGGGACAGTCGAATATG
 121 E   L   P   V   S   R   V   T   D   R   F   R   D   E   I   S   T   D   S   N
 361 GAATTGCCTGTTTCACGGGTTACAGATCGTTTTCGCGATGAAATATCTACGGACAGTAAC
 141 Y   P   M   V   R   Y   I   K   T   P   L   L   Y   N   F   H   A   P   Q   A
 421 TATCCGATGGTACGCTATATAAAAACACCTCTGCTCTATAATTTTCATGCTCCGCAGGCA
 161 D   I   P   G   I   S   W   Q   A   M   T   P   E   N   V   M   P   F   S   A
 481 GATATTCCGGGAATTTCCTGGCAAGCGATGACTCCTGAAAATGTGATGCCTTTCTCTGCT
 181 L   A   Y   F   A   K   D   V   Y   Q   K   T   K   V   P   V   G   I   I
 541 TTGGCCTATTTCTTCGCTAAAGATGTCTATCAAAAGACAAAGGTTCCGGTAGGAATCATA
 201 N   S   S   V   G   G   S   P   V   E   A   W   I   S   E   G   G   L   K   P
 601 AATTCCAGTGTCGGAGGTTCACCGGTAGAAGCGTGGATCAGTGAGGGAGGGTTGAAGCCT
 221 F   P   F   Y   L   N   E   K   R   I   Y   E   S   D   D   L   M   E   S   M
 661 TTTCCATTTTATTTGAATGAAAAGCGTATCTATGAGTCAGACGATTTGATGGAGTCGATG
 241 K   K   E   R   K   K   S   H   A   W   N   V   A   L   F   Q   G   D   K
 721 AAAAAAGAGGAGAGGAAGAAAAGTCATGCCTGGAATGTGGCGTTGTTTCAGGGAGATAAA
 261 G   M   H   E   A   T   P   W   Y   A   A   D   Y   D   D   S   N   W   T   E
 781 GGGATGCATGAGGCTACCCCTTGGTATGCTGCCGATTATGATGATAGCAATTGGACAGAA
 281 T   D   L   F   T   S   G   W   A   T   N   G   L   N   T   V   N   G   S   H
 841 ACAGATTTGTTTACTTCCGGCTGGGCAACAAACGGACTGAATACCGTCAATGGCTCCCAC
 301 W   F   R   K   D   F   Q   V   S   A   Q   Q   A   G   E   K   A   T   L   R
 901 TGGTTCCGTAAAGACTTTCAGGTGTCTGCACAACAGGCGGGAGAGAAAGCGACTCTTCGT
 321 L   G   C   I   V   D   A   D   S   V   Y   V   N   G   T   F   V   G   T   V
 961 TTGGGATGCATCGTAGATGCAGATTCGGTCTATGTAAATGGCACATTTGTGGGGACTGTC
 341 S   Y   Q   Y   P   P   R   I   Y   T   I   P   A   G   L   L   K   A   G   K
1021 TCTTATCAGTATCCTCCCCGTATCTACACCATTCCTGCCGGATTGTTGAAAGCCGGAAAA
 361 N   T   I   T   I   R   L   F   S   Y   G   G   R   P   Q   F   V   K   E   K
1081 AATACAATAACCATACGCCTTTTCAGTTATGGCGGTCGTCCTCAATTTGTAAAGGAAAAG
 381 P   Y   K   I   L   F   G   K   G   Q   P   E   K   G   E   S   E   I   N   L
1141 CCTTATAAAATCCTTTTCGGAAAAGGTCAGCCGGAAAAAGGAGAATCGGAGATCAATTTG
 401 E   G   S   W   K   Y   H   L   G   A   P   M   P   A   A   P   G   Q   T   A
1201 GAGGGGAGTTGGAAATATCATCTCGGTGCTCCTATGCCCGCTGCTCCGGGACAAACGGCT
 421 F   H   Y   K   P   T   G   L   Y   N   A   M   I   A   P   L   L   N   Y   T
1261 TTTCATTATAAACCCACAGGACTGTATAATGCAATGATTGCTCCTTTGCTGAACTATACG
 441 V   S   G   V   I   W   Y   Q   G   E   S   N   V   S   R   R   N   E   Y   K
1321 GTATCTGGTGTTATCTGGTATCAGGGAGAATCGAATGTCTCACGCAGAAATGAGTATAAA
 461 D   L   L   T   A   M   I   S   D   W   R   Q   R   W   N   K   S   D   M   P
1381 GACTTGTTGACGGCTATGATTAGCGATTGGAGACAACGATGGAATAAGTCGGATATGCCT
 481 F   Y   I   I   E   L   A   D   F   L   S   P   T   D   K   G   G   R   T   A
1441 TTCTATATCATTGAGCTGGCGGATTTCCTTTCACCCACAGATAAAGGAGGACGCACTGCC
 501 W   A   E   F   R   K   A   Q   A   E   V   A   D   T   N   K   N   V   T   L
1501 TGGGCGGAATTCCGGAAAGCGCAGGCGGAAGTAGCCGATACAAATAAAAATGTTACTCTG
 521 I   K   N   S   D   L   G   E   W   N   D   I   H   P   L   D   K   K   T   L
1561 ATTAAAAATAGTGATTTAGGAGAATGGAATGATATTCATCCATTGGATAAAAAGACGCTA
 541 G   Q   R   V   A   A   A   I   L   I   E   M   N   T   K   N   R   K   *
1621 GGGCAACGAGTGGCAGCAGCTATCTTGATAGAAATGAATACGAAAAACAGAAAATGACCA
```

Figure 3.6   Nucleotide sequence of 1ˢᵗ ORF of Xylanase Operon (1656 nt), encoding a protein of 552 aa with a predicted molecular mass of 62.1 kDa. This protein was identified as a putative sialic acid-specific 9-O-acetylesterase. The start and stop codons are underlined in bold. Amino acids are shown using the single letter code, the stop signal is represented by an asterisk (*).

```
   1 GGGCAACGAGTGGCAGCAGCTATCTTGATAGAAATGAATACGAAAAACAGAAAATGACCA
                M   E   N   I   K   T   N   E   A   K   G   F   Y   K   L
  61 ATTTATCTATTTTATGGAGAATACAATAAAGACCAATGAGCGAAAGGTTTCTATAAACT
  41 S   W   L   Q   R   I   G   F   G   S   G   D   L   A   Q   N   L   I   Y   Q
 121 CTCTTGGCTTCAACGTATAGGATTCGGTTCCGGTGATTTGGCGCAAAACCTTATTTACCA
  61 T   V   C   M   Y   L   L   I   F   Y   T   N   V   Y   G   L   K   P   E   V
 181 GACCGTATGTATGTATCTGCTGATTTTTTATACCAATGTATATGGACTTAAACCGGAAGT
  81 A   A   V   M   F   L   I   V   R   I   A   D   V   L   W   D   P   L   V   G
 241 GGCAGCCGTGATGTTTCTTATTGTCAGGATAGCGGATGTCCTTTGGGATCCTCTGGTGGG
 101 A   F   V   D   K   H   N   P   K   L   G   K   Y   R   S   Y   L   I   W   G
 301 TGCTTTCGTCGATAAACACAATCCTAAACTAGGTAAATACCGTTCATATCTTATTTGGGG
 121 G   I   P   L   T   G   F   A   I   L   C   F   W   N   G   F   S   G   S   L
 361 AGGAATTCCGCTGACTGGTTTTGCTATTCTTTGTTTTTGGAACGGCTTTTCGGGTTCACT
 141 F   Y   A   Y   F   T   Y   V   G   L   S   M   C   Y   T   L   I   N   V   P
 421 GTTCTATGCCTATTTCACTTACGTTGGATTATCCATGTGTTATACATTGATTAATGTGCC
 161 Y   G   A   L   N   A   S   L   T   R   D   T   N   E   I   T   V   L   T   S
 481 TTATGGAGCACTGAATGCGTCACTTACCCGCGATACGAATGAAATCACGGTGTTGACGTC
 181 V   R   M   F   L   A   N   L   G   G   L   A   V   A   Y   G   I   P   I   L
 541 AGTGCGTATGTTTCTTGCCAATTTGGGTGGTTTGGCTGTGGCATACGGTATTCCGATACT
 201 V   K   V   L   S   P   D   G   K   I   N   T   A   S   A   N   A   W   F
 601 GGTGAAGGTGTTGTCTCCCGATGGTAAAATCAATACTACTGCATCTGCTAACGCATGGTT
 221 I   T   M   T   I   Y   A   V   I   G   L   A   L   L   M   F   C   F   N   Q
 661 TATTACGATGACTATTTATGCTGTTATCGGATTGGCGTTATTGATGTTCTGCTTTAACCA
 241 T   K   E   R   V   V   M   D   Q   E   E   T   S   K   V   K   V   S   D   L
 721 GACGAAGGAGCGTGTGGTTATGGATCAGGAGGAGACATCTAAAGTAAAAGTGTCCGACTT
 261 W   V   E   F   C   R   N   K   P   L   R   I   L   A   F   F   I   T   A
 781 GTGGGTAGAATTTTGTAGAAATAAACCTTTGCGTATTTTGGCGTTCTTTTTCATTACTGC
 281 F   A   M   M   A   I   G   N   S   A   G   S   Y   Y   M   I   Y   N   V   R
 841 TTTTGCAATGATGGCGATTGGTAATTCTGCCGGTTCATATTATATGATTTATAATGTACG
 301 A   P   E   M   L   P   Y   F   M   A   L   G   S   I   P   A   F   I   F   M
 901 TGCACCGGAGATGTTACCTTATTTCATGGCCTTGGGCTCGATACCCGCATTCATTTTCAT
 321 P   M   V   P   A   I   K   R   A   I   G   K   K   Q   M   F   Y   V   F   L
 961 GCCGATGGTACCTGCCATTAAACGTGCCATTGGAAAAAAGCAAATGTTTTATGTATTCCT
 341 S   V   A   I   L   G   M   A   L   L   Y   I   I   S   V   V   P   V   L   K
1021 TTCAGTCGCTATATTGGGTATGGCATTGCTGTATATTATTTCTGTGGTTCCGGTACTCAA
 361 T   Q   I   W   L   V   F   V   A   Q   F   I   K   S   T   G   V   I   I   A
1081 AACGCAGATATGGTTGGTCTTTGTGGCGCAGTTCATAAAATCGACGGGAGTCATTATTGC
 381 T   G   Y   M   W   A   L   V   P   E   V   I   S   Y   G   E   Y   T   H   G
1141 GACAGGGTATATGTGGGCTTTGGTTCCCGAAGTAATTTCGTATGGCGAATATACTCATGG
 401 K   R   I   S   G   I   V   N   A   L   T   G   I   F   Y   K   A   G   M   A
1201 TAAACGTATTTCGGGTATAGTCAATGCTTTGACTGGTATTTTCTATAAAGCGGGAATGGC
 421 L   G   G   V   V   P   G   L   V   M   A   F   V   G   F   D   Q   T   N   E
1261 TCTTGGAGGAGTTGTACCGGGACTTGTTATGGCTTTTGTCGGATTCGACCAGACAAATGA
 441 V   S   Q   S   P   F   A   E   Q   G   I   L   W   L   V   A   V   I   P   A
1321 AGTGTCACAATCGCCTTTTGCCGAACAGGGAATACTGTGGCTCGTAGCCGTTATTCCGGC
 461 L   L   L   L   V   A   M   F   I   I   S   K   Y   E   L   E   D   N   V   I
1381 GTTGTTGCTTTTGGTCGCTATGTTCATTATTTCTAAATATGAATTGGAAGATAATGTGAT
 481 D   N   I   N   E   E   I   E   S   R   C   K   K   G   E   *
1441 TGACAATATAAATGAGGAGATAGAATCGCGCTGTAAAAAAGGCGAATAGATAAGATAGTA
```

Figure 3.7 **Nucleotide sequence of 2nd ORF of Xylanase Operon** (1413 nt), encoding a protein of 471 aa with a predicted molecular mass of 52.4 kDa. This protein was identified as a sodium/sugar symporter. The start and stop codons are indicated in bold. Amino acids are shown using the single letter code, the stop signal is represented by an asterisk (*).

To confirm that all 4 genes were part of the same operon, the first gene of the operon (putative acetyl esterase) was inactivated through insertion disruption. A 509 bp region from ORF1 in the xylanase operon was PCR amplified using primers GENE1F1 and GENE1R1 and the PCR product was cloned into pCR2.1 (Appendix 1, Invitrogen) according to the manufacturer's instructions. The resultant plasmid was digested with *Bam*HI and *Sst*I, and the genomic insert recovered and ligated into the *Bacteroides* suicide vector pCQW1 (Feldhaus *et al.*, 1991) digested with the same enzymes. The suicide vector was transferred into *B. ovatus* by conjugation and transconjugates (BOΩG1) were selected on gentamicin (200 µg ml^{-1}) and tetracycline (10 µg ml^{-1}) plate (BOΩG1 was generated by T. Whitehead). BOΩG1 demonstrated reduced growth on xylan similar to that seen with disruption of the *xyl*I gene (Weaver *et al.*, 1992). Growth in medium containing glucose was unaffected (Fig 3.8A). RT-PCR analysis of the mutant showed that the *xsa* gene of the xylanase operon was not expressed when grown on xylan (Fig 3.8B). Furthermore, *B. ovatus* V975 RT-PCR of the junctional regions between ORF1 and ORF2, ORF2 and *xyl*I, *xyl*I and xsa genes produced PCR products of expected sizes (Primers used JUNC1-F/JUNC1-R, JUNC2-F/JUNC2-R, JUNC3-F/JUNC3-R respectively) (Fig 3.9). Together, these results confirmed that there are 4 genes which constitute the xylanase operon under the control of a single promoter. *xsa* was considered the last gene in this operon as previously reported (Weaver *et al.*, 1992).

3.3.2 Identification of the putative xylanase operon promoter

A region of non-coding DNA upstream of the first gene (ORF1) in the xylanase operon was predicted to contain the xylanase operon promoter. A possible RBS (ATATAA) was located 12 bp upstream of the ATG initiation codon of the operon (Fig. 3.5) which was homologue to the first 5 bp of the putative consensus sequence of *P. bryantii* (Miyazaki *et al.*, 2003). The *xyl*E gene was used as the reporter gene to characterise the xylanase promoter. The promoterless *xyl*E gene was isolated from pLEC23 (Coyne *et al.*, 2003) by digestion with *Bam*HI and *Pst*I and cloned into pUC18 digested with same enzymes to produce pUC-*xyl*E. The putative *B. ovatus* xylanase operon promoter was PCR-amplified using primers XYLP-f and XYLP-rb, with *Bam*HI sites added at both ends and cloned into pCR-blunt (Invitrogen). The promoter was retrieved with *Bam*HI and cloned into the *Bam*HI

site in pUC-*xyl*E upstream of *xyl*E in both forward (ON) and reverse (OFF) orientations. Orientations were confirmed by sequencing. The promoter-*xyl*E fragment was isolated by digestion with *Hind*III and *Kpn*I and cloned into pBT2 then transferred by conjugation to *B. ovatus* utilizing *E.coli* J53/R751 and integrated into the chromosomal DNA by homologous recombination in the promoter region. Cloning of the putative promoter region upstream of the promoterless *xyl*E gene in the forward (ON) orientation in *E. coli* resulted in yellow colonies when sprayed with 0.5 M catechol (i.e. catechol 2,3-dioxygenase activity). In contrast, no enzyme activity was detected when the promoter was in the reverse (OFF) orientation. It was not possible, however, to detect XylE enzyme activity among any of the *Bacteroides* recombinants using optimal culture conditions. Therefore, *xyl*E reporter gene expression was analysed by RT-PCR using primers XYLE-F and XYLE-R. As seen in (Fig 3.10), *xyl*E transcripts were only detected in *B. ovatus* containing the promoter-*xly*E reporter in the forward (ON) orientation confirming that this region of DNA was able to drive transcription in *B. ovatus*.

3.3.3   Identification of transcriptional initiation site (TIS)

TIS was identified by 5'RACE. This involves total RNA isolation then formation of cDNA using a single primer in the reverse direction 200-300 bp from the ATG start codon. Poly-A tail was then added to the 3' end of the cDNA by terminal transferase (TdT). Polyadenylated cDNA was used as template in a PCR reaction with the primers, anchor d (T) and SP1, and the resulting product subjected to a second and third round of PCR with an anchor forward primer and nested reverse primers SP2 and SP3 respectively. The PCR product was cloned into pCR4-TOPO vector (Appendix 1) and sequenced to identify the TIS.

The RACE reaction products were analysed by agarose gel electrophoresis (Fig 3.11A). A single RACE reaction product was visualized on ethidium bromide-stained agarose gel after two rounds of nested PCR reactions. The sequence of the RACE product was determined and the TIS was identified at a cytosine residue 216 bp upstream of the ATG start codon (Fig. 3.5). Additional RT-PCR assays were performed to confirm the identity of the putative TIS and to determine if additional TIS existed upstream. No PCR products were obtained using a primer XYLP (Fig

3.11B) upstream of the TIS, which contrast with the large amount of product detected using the same reverse primer in combination with a forward primer complimentary to the putative TIS (Fig. 3.11B and 3.11C). The DNA sequence adjacent to the TIS revealed motifs of the general AT rich character, including TTCTAA centred on -9 and TGTAAT centred on -23. No inverted repeat sequences indicative of binding sites for one or more regulatory proteins were found in the promoter sequence. Close inspection of the putative xylanase promoter sequence in Fig 3.4 revealed the presence of a *Bacteroides* consensus promoter (Bayley *et al.*, 2000) at -74 relative to the ATG translational start codon. It is possible that this represents a second TIS, however, as only one TIS was identified by 5'RACE this promoter may not be active under the conditions used in our experiments. Therefore, the TIS identified in this study is likely to be the primary TIS.
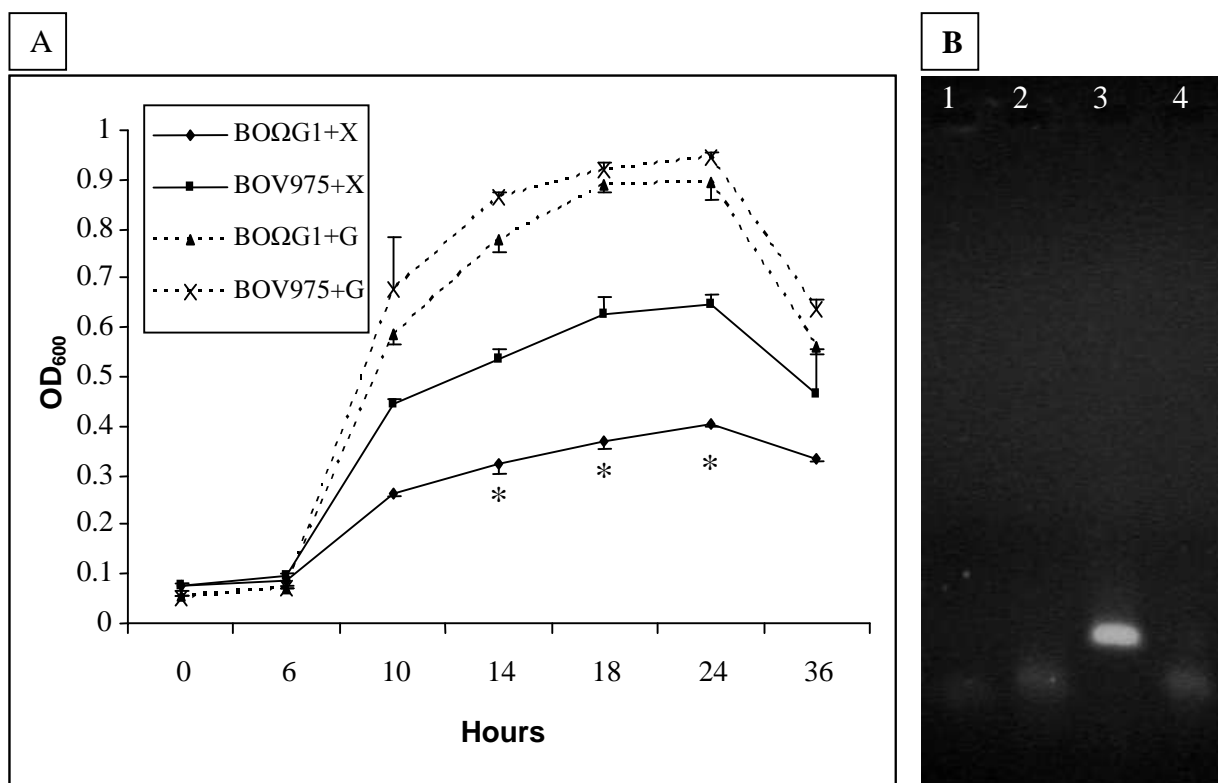
Figure 3.8   Growth curve analysis and RT-PCR of BOΩG1 grown with and without xylan (**A**) Wild type *B.ovatus* V975 and xylanase operon mutant strain (BOΩG1) were grown in RGM supplemented with Glucose 0.1% w/v (+G) or Xylan 0.05% w/v (+X) and the OD_{600} was monitored over 36 hours to assess the influence of xylanase operon mutation on growth in xylan media. Growth of BOΩG1 was reduced on xylan containing medium, whereas growth on glucose containing medium was unaffected. This observation indicates that the ORF1 is part of the same operon of the *xyl*I. Data shown are means (±SEM) from triplicates of two separate experiments. (* $p<0.05$) (**B**) Agarose gel. RT-PCR of *xsa* mRNA (using primers Xsa-F and Xsa-R) from BOΩG1 grown on RGM supplemented with xylan 0.05% w/v. 1, Water control. 2, PCR on RNA template. 3, positive control (*gyr*A). 4, *xsa* RT-PCR. *xsa* was not expressed when this mutant grown on xylan containing medium. These results indicate that the genes identified upstream the xylanase gene *xyl*I in *B.ovatus* V975 are part of the same operon.
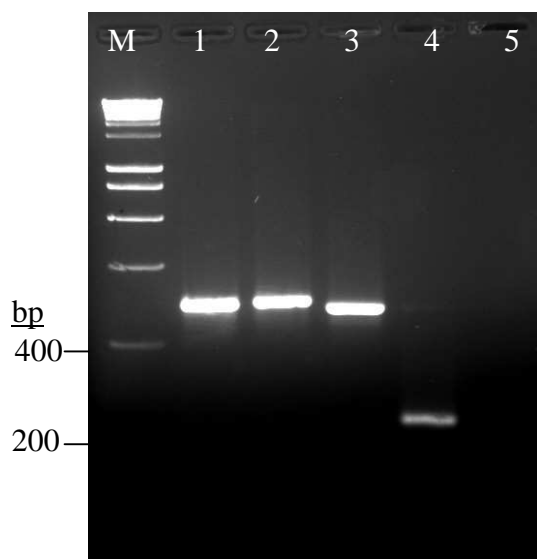
Figure 3.9   Junctional RT-PCR of *B.ovatus* V975 mRNA grown on RGM supplemented with glucose 0.1% (w/v) and xylan 0.05% (w/v). *B. ovatus* RNA was isolated and reverse transcribed into cDNA. PCR was performed to detect products at the junctional regions between the genes. M, DNA marker. 1, transcript of junction between ORF1 and ORF2 (primer set; JUNC1-F and JUNC1-R). 2, transcript of junction between ORF2 and *xyl*I (primer set; JUNC2-F and JUNC2-R). 3, transcript of junction between *xyl*I and xsa (primer set; JUNC3-F and JUNC3-R). 4, *gyr*A RT-PCR product (+control). 5, No cDNA control (-control). This result confirms that the 4 genes comprising the xylan utilization operon are transcribed from a single promoter.
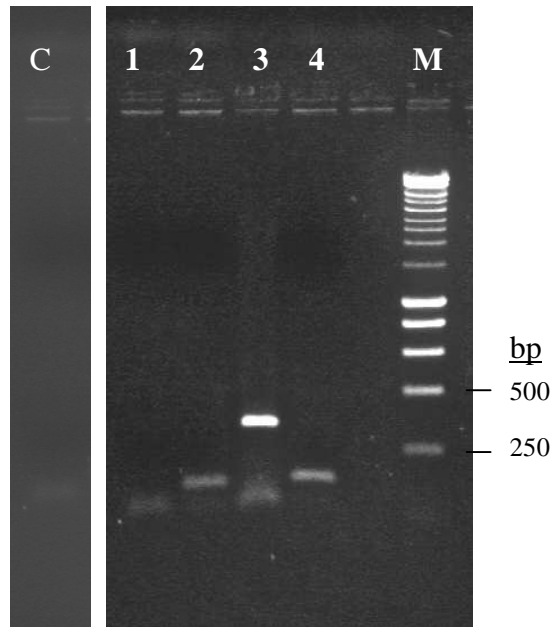
Figure 3.10 *xyl*E **RT-PCR transcribed from xylanase promoter in** *B. ovatus*. The putative *B. ovatus* xylanase operon promoter was integrated into the *B. ovatus* chromosome upstream of the *xyl*E in either the forward (ON) or reverse (OFF) orientation and RT-PCR was used to detect transcriptional activation after stimulation with xylan (0.05% w/v) for 2 h (primers used XYL-F and XYLE-R) 1, *BOXylE* (OFF); 3, *BOXylE* (ON); 2 and 4 *gyrA* expression; M, 1 kbp DNA ladder. C, no cDNA control**.** This result shows that the xylanase promoter drives *xyl*E expression in *B. ovatus*.
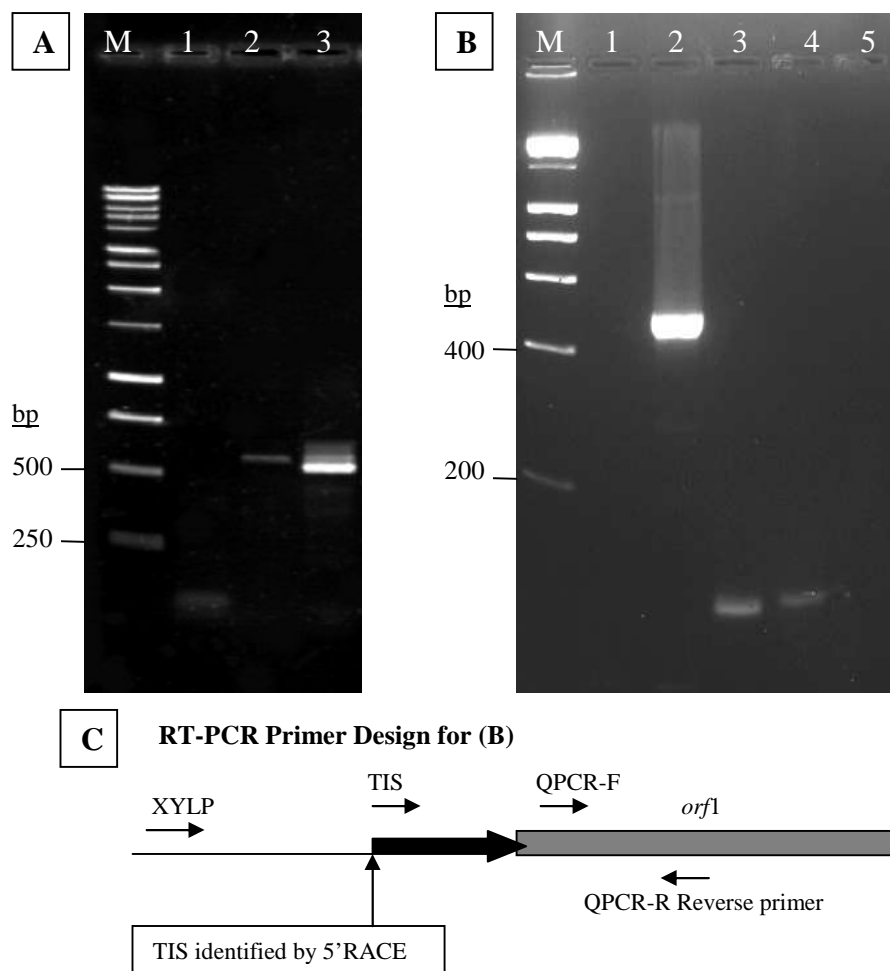
Figure 3.11   Identification of the transcription initiation site (TIS) of the *B. ovatus* V975 xylanase operon promoter by 5'RACE. (**A**) The TIS was identified by 5'RACE using total bacterial RNA. RACE products were amplified in three nested RT-PCR products and visualised by agarose gel electrophoresis. M; 1 kbp DNA ladder. 1; 5' end DNA amplification product using reverse primer SP1 and non-specific primer anchor-d(T). 2; PCR product of DNA in lane 1 using nested internal reverse primer SP2 and non-specific primer anchor. 3; Repeat PCR on DNA in lane 2 using nested internal primer SP3 and anchor primer. The amplicon in Lane 3 was excised, cloned and sequenced to identify the related TIS. (**B**) RT-PCR of *B.ovatus* xylanase operon mRNA using various forward primers to confirm the identity of the xylanase promoter. 1; XYLP forward primer, 2; TIS forward primer, 3; QPCR2-F forward primer, QPCR2-R reverse primer used in 1, 2 and 3. 4; *gyr*A positive control, 5; no cDNA control. (**C**) Locations of primers used in (B).

3.3.4   Functional analysis of the xylan utilization promoter

Real-time RT-PCR of ORF1 was used to quantify expression of the xylanase operon in the wild-type *B. ovatus* V975 and to determine the growth conditions under which the xylanase promoter is active. *B. ovatus* was grown in RGM supplemented with 0.001% (w/v) hemin and varying concentrations of glucose and/or xylan. Real-time PCR was carried out using SYBR green (Sigma) and primers QPCR2-F and QPCR2-R for ORF1, and GYRA-F and GYRA-R for the housekeeping gene *gyrA*. PCR results were analyzed using the standard curve method to determine relative gene expression compared to *gyr*A gene. The level of promoter activity increased by >2-fold with increasing concentrations of xylan up to 0.05% (w/v) (Fig 3.12A). At higher xylan concentrations promoter activity was reduced. Maximum levels of xylanase operon expression occurred within 2 h of exposure to xylan and persisted for up to 24 h after the initial exposure to xylan (Fig 3.12B). Glucose suppressed xylan-induced promoter activity at concentrations as low as 0.025% (w/v) (Fig 3.12C).
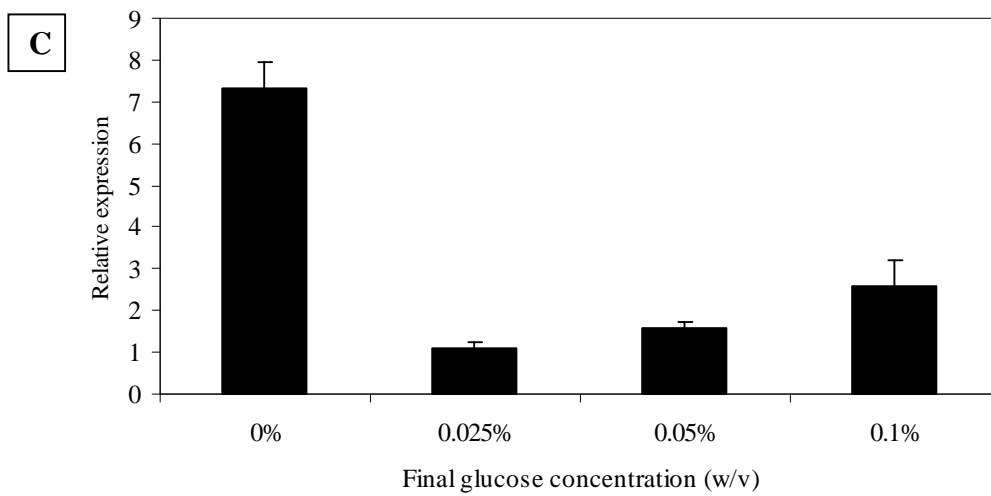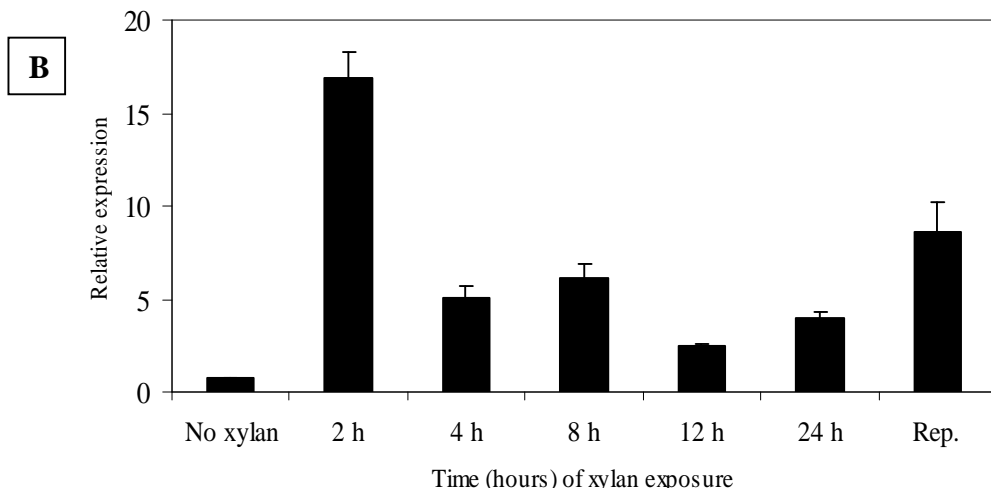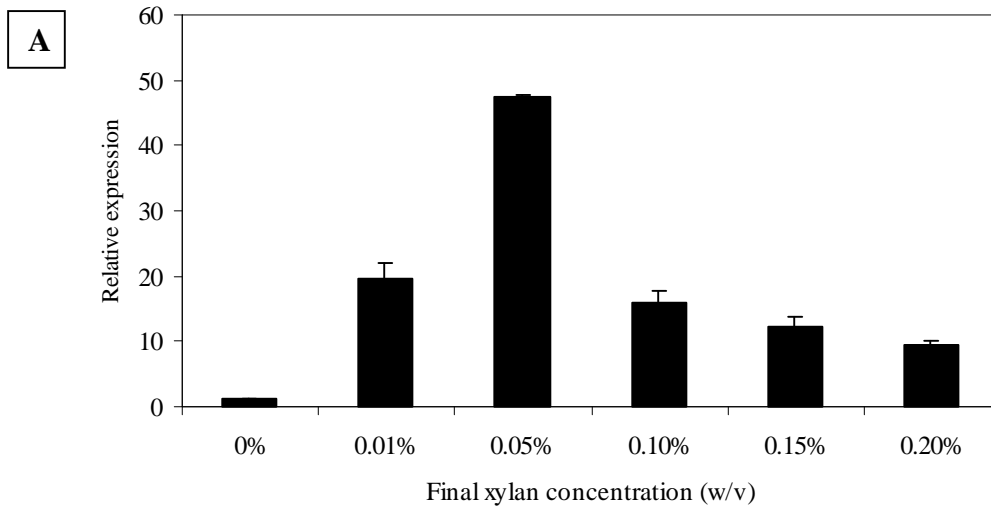
Figure 3.12   Functional characterisation of the xylanase promoter. The effect of xylan concentration (**A**), xylan exposure time (**B**) and presence of glucose (**C**) on activation of the xylanase operon in wild type *B. ovatus* V975 was determined by quantitative RT-PCR using primers specific for ORF1 region of the xylanase operon and *gyrA* as a reference gene. In (**A**) bacteria were exposed to increasing concentrations of xylan for 24 h prior to analysis. In (**B**) bacteria were exposed to 0.05% (w/v) xylan for the indicated times. The effect of repeated exposure to xylan on expression of the xylan operon was assessed by adding xylan (0.05% w/v) to the growth media at 24, 8 and 4 h (Rep.). In (**C**) bacteria were incubated with 0.05% (w/v) xylan in the presence of increasing concentrations of glucose for 24 h prior to analysis. For all experiments standard curve method was used to determine the level of ORF1 gene expression relative to *gyr*A as a reference gene. The results represent the mean (±SEM) of triplicate determination from one of three experiments.

3.4   Discussion

This chapter describes the identification of the putative xylanase operon promoter from *B. ovatus* V975 and is the first promoter to be identified in this organism. We have demonstrated that this promoter is functional in *E. coli* and is regulated by xylan in a dose dependent manner. Glucose was found to be a strong repressor for this promoter.

The inverse PCR permits the rapid amplification of regions of unknown sequence flanking a specified segment of DNA (Ochman *et al.*, 1988). This procedure was used to "walk" outside a region of known sequence without resorting to conventional cloning techniques. Two new genes were sequenced that completed the xylanase operon. These encode a putative sodium/sugar symporter and sialic acid-specific 9-O-acetylesterase, both of which would be expected to complement the activity of the *xyl*I and *xsa* gene products in the degradation of xylan. Grass xylans frequently have acetyl groups attached by ester linkages (Hespell & Whitehead, 1990) that would be removed by the esterase and the symporter would mediate the transport of liberated xylose and arabinose residues into the cell.

Attempts to identify the TIS using fluorescent labelled reverse primers were unsuccessful. Therefore, RACE was used, a more sensitive approach to identify the putative TIS (Braun & Stein, 2004). The TIS of the xylanase promoter was located well upstream of the translation initiation codon at -216. This large distance is not unusual and has been described for bacterial genes in other species, for example, *Porphyromonas gingivalis* (Jackson *et al.*, 2000) and *Prevotella loescheii* (Manch-Citron *et al.*, 1999), which is closely related to *Bacteroides* spp. This is important when this promoter is to be used for the controlled expression of heterologous or homologous genes. The sequences at positions -10 or -35 upstream of the start site did not resemble any known RNA polymerase recognition sequences in *E. coli*, but, given the large difference in consensus sequences that have been identified in *E. coli* and other species, *B. ovatus* may have unique RNA polymerase recognition sequences. This possibility is supported by the unconventional promoter motifs of *Bacteroides fragilis* that have been previously identified (Bayley *et al.*, 2000). However, no similarity with *B. fragilis* consensus sequences could be identified. This may not however be entirely unexpected since the absence of these consensus sequences in the *rec*A and *sod* genes of *B. fragilis* (Bayley *et al.*, 2000) suggest that

these consensus sequences may not strictly apply to all *Bacteroides* promoters. To define the regulatory region(s) in the promoter a detailed deletion and point mutation analysis is required.

The reporter gene *xyl*E was used successfully in *Bacteroides fragilis* in plasmid pLEC23 (Coyne *et al.*, 2003). Therefore, *xyl*E gene was used to characterise the xylanase promoter. However, XylE enzyme activity was undetectable after integration into chromosomal DNA. It is unlikely that XylE is not functional in *B. ovatus* as it was shown to be functional in *B. fragilis*, however, it was integrated into expression plasmid vector (Coyne *et al.*, 2003). The lack of XylE expression in *B. ovatus* recombinants may be a consequence of expression from a single gene copy integrated into the chromosomal DNA compared to multiple plasmid-encoded copies in *E. coli* transformants and *B. fragilis* producing high levels of catechol dioxygenase activity. Therefore, real-time PCR technique using RNAs derived from bacteria subjected to altered growth environments was used to identify the optimal conditions for xylanase expression. This technique has been used in the past for bacterial operon characterisation in different growth conditions (Machado *et al.*, 2006). The xylanase operon promoter was up-regulated by xylan and expression increased with increasing xylan concentration up to 0.05% (w/v). Expression was however, reduced at higher xylan concentrations and by the addition of glucose. Glucose repression has been previously described for the *Streptomyces lividans* xylanase promoter (Chen & Westpheling, 1998). However, the direct repeat or inverted repeat sequences present in the *S. lividans* promoter are not present in the *B. ovatus* xylanase promoter. Furthermore, the *B. ovatus* xylanase promoter reaches maximal expression 2-4 hours after induction. These features may be utilized by *B. ovatus* to improve the efficiency of polysaccharide digestion and maximise the nutritional effect.

B. ovatus xylan utilization can be used to direct the controlled production of mammalian protein (Farrar *et al.*, 2005). As a result of the work presented here, the xylanase promoter can be utilized to control protein production with the intact xylanase operon, increasing the prospect of using engineered *B. ovatus* strains in probiotic regimens.

CHAPTER (4)

ENGINEERING OF *B. ovatus* TO SECRETE BIOLOGICALLY ACTIVE GROWTH FACTORS UNDER THE CONTROL OF XYLAN.

4.1 Introduction

Soluble growth factors that can improve the intestinal barrier function, such as keratinocyte growth factor (KGF) or Transforming Growth Factor (TGF) are of interest as potential therapeutic agents for IBD. If administered orally or by injection, only a fraction of the active components reaches the intended target site. This is not only an inefficient way to deliver drugs, but more importantly, it means that patients are often subject to a spectrum of unpleasant side effects resulting from the high levels of the drugs in otherwise healthy tissues and organs of the body. Furthermore, some of these cytokines such as TGF-β_1 are short lived, rendering them inactive when they reach the site of action such as the gut. The use of enemas for the localised delivery of therapeutics has gained interest. This method however, is unpleasant for the seriously ill patients, especially when the colon is actively inflamed, and is not suitable for day to day routine.

For all these reasons and others related to long term medication that is often the fate of patients suffering from IBD, alternative approaches for localised delivery of therapeutics were designed. Gene transfer was one approach, which was conducted by administration of plasmids carrying a cloned gene. TGF-β has been cloned into a plasmid under the control of CMV promoter which was used to abrogate the establishment of TNBS colitis when given intranasally in a single dose (Kitani *et al.*, 2000). Another example is the recombinant adenovirus vector carrying exogenous cytokine genes (IL-4 and IL-10) (Barbara *et al.*, 2000). A drawback of these methods may be the high amount of plasmid DNA required, and when the adenoviral vectors are administered through systemic route this leads often to high expression of the recombinant gene in the liver and spleen which shows lack of organ specificity.

The feasibility of delivering cytokines in a localised manner by recombinant *Lactococcus lactis* was investigated (Steidler *et al.*, 1998). *L. lactis* was engineered

to deliver biologically active IL-10, a key factor in the down regulation of immunity to the intestinal mucosa. Daily treatment with IL-10 secreting *L. lactis* was successfully used to treat and prevent murine colitis (Steidler *et al.*, 2000). In considering the applications of this approach to treat human IBD several issues need to be addressed. First, *L. lactis* is not a human commensal bacterium and does not colonise the human colon, hence repeated administrations of recombinant strains would be needed to maintain therapeutic levels of the cytokine in the intestine. Second, the production of the protein by *L. lactis* is not controlled in terms of dosage or stopping production if needed. Unregulated, constitutive expression of immunologically active molecules, such as that obtained with *L. lactis* may have adverse effects. Finally, the use of genetically modified probiotics (GMP) in medicine raises legitimate concerns about environmental biosafety. A biological containment system was designed for the *L. lactis* recombinants, however, these genetically engineered bacteria can still survive up to 200 hours in the environment (Steidler *et al.*, 2003). This gives them enough time to spread through the environment.

To address these issues and to further develop gut bacterial delivery systems, a novel second generation of GMP has been developed in this study. The human commensal colonic Gram negative, anaerobic, *Bacteroides ovatus* has been genetically engineered to produce and secrete mammalian cytokines under the control of the xylanase promoter, which is only activated in the presence of xylan. The advantages of this system are, firstly, *B. ovatus* is part of human microbiota with ability to colonise the human bowel. Secondly, the unique feature of xylan utilization by *B. ovatus* is used to control the recombinant cytokine production in the bowel. Thirdly, the anaerobic nature of *B. ovatus* is an indigenous environmental safety and containment character, as it looses its viability quickly upon exposure to the environment.

This chapter describes the production of recombinant strains of *B. ovatus* that secrete TGF-β_1 or KGF-2 in the presence of the polysaccharide, xylan. Human cytokines produced by recombinant *B. ovatus* was detected *in vitro* and *in vivo* after xylan supplementation and they are biologically active in *in vitro* bioassays.

4.2   Aims and Experimental design

4.2.1   Production and characterisation of recombinant *B. ovatus* strains secreting KGF-2 or TGF-*β*₁ under the control of xylan

i. Construction of KGF and TGF secreting *B. ovatus*

The coding region of human keratinocyte growth factor-2 (KGF-2) and transforming growth factor-β_1 (TGF-β_1) was PCR-amplified from a cDNA clone (MRC Geneservices, UK. GenBank Acc: BC069561, IMAGE: 7262177 for KGF-2 and GenBank Acc: BE 255274, IMAGE: 3356605 for TGF-β_1) using *Pfu* DNA polymerase (Promega). *Bacteroides fragilis* enterotoxin secretion signal sequence was cloned upstream the cytokine coding sequence in this reaction. Primers used were KGF-f, KGF-r and TGF-f, TGF-r for KGF and TGF respectively. Primers were flanked by *Nde*I/*Bam*HI for KGF and *Nde*I/*Hin*dIII for TGF cloning. The *B. ovatus* xylanase operon promoter was PCR-amplified using primers XYLP-f and XYLP-rn. They are flanked by *Nde*I and *Bam*HI. The promoter, KGF-2 and TGF-*β* PCR products were cloned into pCR®-Blunt to create plasmids pCR-Pro, pCR-KGF and pCR-TGF respectively. *kgf-2* and *tgf* were removed from pCR-KGF and pCR-TGF by digestion with *Nde*I and *Hin*dIII then ligated into *Nde*I-*Hin*dIII digested pCR-Pro to generate pCR-PKGF and pCR-PTGF respectively. The promoter-*kgf*-2 fragment was then retrieved by digestion with *Bam*HI and cloned into the *Bacteroides* suicide vector pBT2 digested with *Bam*HI to produce pBT-KGF, and the promoter-*tgf* fragment was isolated by digestion with *Xba*I/*Hin*dIII and cloned into the pBT2 digested with same enzymes to produce pBT-TGF (Fig 4.1, see pCR-blunt map appendix 1). pBT-KGF and pBT-TGF were transformed into *E. coli* J53/R751 and selected on LB-Trimethoprim plates. *E.coli* containing cloned pBT was then mated with *B. ovatus* V975 by triparental conjugation (Materials and Methods). Transconugates were selected on BHI-Hemin plates containing Gentamicin and Tetracycline. Confirmation of genome integration was done by colony PCR.
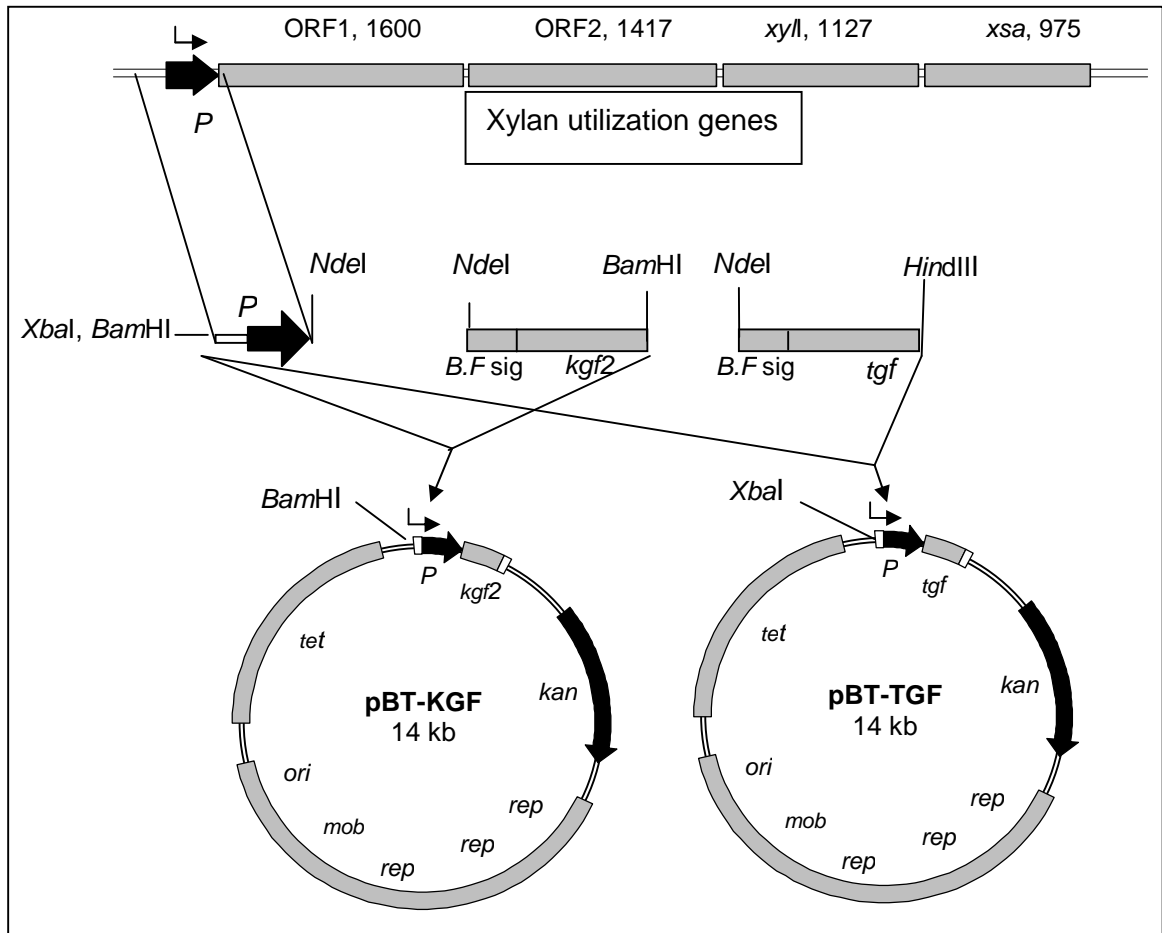
Figure 4.1 **Diagram of the strategy used to create recombinant *B.ovatus* strains that secret KGF or TGF under the control of xylan.** Schematic representation of the xylan utilization genes with estimated sizes indicated in bp. ORF, open reading frame; *xyl*I, xylanase I gene; *xsa*, xylosidase-arabinosidase gene. The xylanase promoter with 3' sequence of the upstream gene and the mature human KGF-2 or TGF-β_1 gene proceeded by the *B. fragilis* enterotoxin secretion signal (*B.F* sig) sequence were PCR amplified, cloned into pCR-blunt and subcloned into pBT2 to create pBT-KGF and p BT-TGF respectively. Only the restriction sites used for cloning are shown. Tetracycline (*tet*) resistance was used for selection in *B. ovatus* and kanamycin (*kan*) resistance for selection in *E. coli*. *oriV*, origin of replication; *repA, repB, repC* encode replication functions with *mob* required for mobilization from *E. coli* to *B. ovatus*.

ii. Detection of *Bacteroides* heterologous gene transcript

RT-PCR was performed to detect recombinant and native gene transcripts. *B. ovatus* was grown anaerobically on RGM-Hemin supplemented with 0.1% (w/v) glucose and 0.05% (w/v) xylan. Xylan was added to the medium 2-3 hours before RNA preparation. RNA was prepared using Total RNA isolation kit (Promega) and treated twice with DNaseI, once on column and then off column (Ambion). The RNA was checked for DNA contamination by performing PCR on RNA template. RNA was transcribed into cDNA using M-MuLV Reverse Transcriptase (NEB). PCR was performed using 2 µl cDNA and the following primer sets (KGF-f/KGF-r, TGF-f/TGF-r, GYRA-f/GYRA-r, QPCR2-f/QPCR2-r).

iii. Growth of recombinant *B.ovtus* on xylan and glucose

To assess if the cloning had affected the ability of the recombinant *Bacteroides* to grow on xylan, BO-KGF and BO-TGF were grown on RGM-Hemin supplemented with 0.1% (w/v) glucose or 0.05% (w/v) xylan and incubated anaerobically at 37˚C for 36 hours. Wild type *B. ovatus* V975 was used as control strain. Growth was monitored by measuring the OD_{600}.

iv. *In vitro* viability of recombinant *B.ovatus*

The anaerobic character of *B.ovatus* is an inherent environmental safety feature. *Bacteroides* is thought to be aerotolerant. To assess the recombinant *Bacteroides* tolerance to environmental O_2 tension, BO-KGF was grown on RGM-Hemin supplemented with 0.1% (w/v) glucose and 0.05% (w/v) xylan for 24 h at 37˚C anaerobically. The culture was then exposed to room atmosphere and left for 7 days. The culture was sampled daily, diluted in BHI media and spread on BHI-Hemin agar plates supplemented with tetracycline. The colonies were counted at 48 hours and the number corrected for dilutions to obtain the CFU ml^{-1}.

4.2.2 Detection of recombinant growth factors produced by *B. ovatus* under xylan control *in vitro* and *in vivo*.

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to quantify levels of KGF-2 or TGF-β_1 produced by recombinant strains of *B. ovatus*. Briefly, 96 well ELISA plates (Nunc Maxisorp) were coated overnight at 4°C with 100 µl of capture monoclonal anti-KGF2 antibody (R&D systems) (3.3µg ml^{-1}) or anti-TGF-β_1 antibody (BD Bioscience) diluted in sodium carbonate coating buffer (pH 9.5). Plates were washed 3 times with PBS/Tween then blocked with 300 µl 10% (v/v) foetal bovine serum in PBS. After 3 washes with PBS/Tween, 100 µl of culture supernatant diluted in blocking solution was added to each well and the plates were incubated at 22°C for 2 h then washed 5 times with PBS/Tween. Detection was with 100 µl of biotinylated anti-KGF-2 antibody (0.3 µg ml^{-1}) (ProSci incorporated, Canada) or biotinylated anti-TGF-β_1 antibody (BD Biosciences) and 1:250 streptavidin-HRP (BD Biosciences). The plates were washed 7 times then substrate (1:1 v/v of 2,2'-azino-di 3-ethylbenzthiazoline-6-sulfonate) (BD Biosciences) was added and incubated for 30 min at 22°C in the dark. The reaction was stopped by adding 50µl of 2N H$_2$SO$_4$. A standard curve was obtained from serial dilutions of recombinant KGF-2 (Sigma) or TGF-β_1 (BD Biosciences). Samples were assayed in duplicate.

For *in vivo* cytokine production assay, C57Bl/6 mice (6-8 weeks) were kept on xylan-reduced diet (SDS, UK). *B. ovatus* V975, BO-KGF or BO-TGF were given by oro-gastric gavage at 2x10^8 CFU. Xylan (30 mg ml^{-1}) was added to drinking water to induce *in vivo* KGF-2 or TGF-β_1 production. 24 h after gavage, mice were sacrificed and colonic content were retrieved, weighed and mixed with 500µl working solution (PBS containing 1µg ml^{-1} protease inhibitors cocktail and 1mM PMSF (Sigma). The mixture then centrifuged for 2 min and supernatant used in ELISA.

4.2.3 Growth factors produced by *B.ovatus* are biologically active.

i. KGF-2 Bioassay: KGF-2 bioactivity was assessed using human primary keratinocytes (NHK) (Invitrogen) in an epithelial cell proliferation assay as previously described (Shoucheng *et al.*, 1998). Briefly, NHK was grown until 80%

confluent in K-SFM supplemented with 5ng ml^{-1} EGF (Epidermal growth factor) (Gibco), 100 U ml^{-1} penicillin and 0.5mg ml^{-1} streptomycin (Sigma) at 37˚C in a humidified atmosphere of 5% CO_2. NHK were washed 3 times with PBS and cultured in 96 well plates (Costar) in K-SFM containing dilutions of recombinant KGF-2 (Sigma) to obtain a standard curve (concentration ranging from 0 to 40 ng ml^{-1}) or BO-KGF culture supernatant concentrated using 5000 MWCO Amicon Ultra columns (Millipore) with and without affinity purified anti-KGF-2 neutralising polyclonal antibody (2μg ml^{-1}; MBL International). After 4 days incubation at 37˚C, 10 μl of 5 mg ml^{-1} MTT (Thiazolyl Blue Tetrazolium Bromide) (Sigma) was added to each well. Cells were incubated for further 4 h and supernatant was removed. The crystal formazan product was dissolved with 200 μl DMSO (Sigma) and shaking for 10 min. The OD_{570} was measured using microplate reader (SpectraMax 340PC, USA). The quantity of biologically active KGF-2 in each dilution was estimated from linear regression equation of OD against standards. Samples were tested in triplicates.

ii. TGF-β_1 bioassay: Only dimeric TGF has biological activity (Brunner *et al.*, 1992). Therefore, SDS-PAGE and western bloting was performed on BO-TGF culture supernatant to determine the structure and molecular weight of BO-TGF produced TGF-β_1. Briefly, BO-TGF was cultured in RGM-Hemin supplemented with 0.1% (w/v) glucose and 3 hours before supernatant collection xylan was added to final concentration of 0.05% (w/v). Proteins were precipitated with acetone, resuspended in 500 μl non-reducing loading buffer then electrophoresed on 16% (w/v) tricine SDS-PAGE gel and transferred onto PVDF membranes (Millipore). TGF-β_1 was detected using biotinylated anti-TGF-β_1 antibody (BD Biosciences) and streptavidin-HRP, then incubated with chemiluminescent substrate and visualised on X-ray film.

Biological activity of TGF-β_1 in BO-TGF conditioned media was assayed by P G Dilger (National Institute for Biological Standards and Control, Potters Bar, UK) based on the ability of TGF-β_1 to inhibit IL5-induced proliferation of the erythroleukaemia cell line, TF-1. This assay is sensitive to less than 500 fg ml^{-1} of TGF-β_1. The assay can be made specific for TGF-β_1 by including neutralising antibodies for TGF-β_1. Briefly, the BO-TGF conditioned media or TGF-β_1 standards

were serial diluted and added to TF-1 cells in 96 well plates containing RPMI 1640, 5% (v/v) FBS, 100 mg ml^{-1} streptomycin and 100 U ml^{-1} penicillin and 4 ng ml^{-1} rhIL-5 at a density of 1×10^{5} cell ml^{-1}. Cells were incubated at 37°C for 48 h. To assess cell proliferation, [^{3}H] thymidine was added to each well and the microtitre plate returned to the incubator for a further 4 h. The radioactivity incorporated into DNA was measured using a scintillation counter. A dose-response curve of cpm versus dilution of standard or unknown was plotted, and the amounts of TGF-β_1 in the samples were determined from standard curve linear regression analysis of cpm versus standard concentration. In neutralisation experiments, purified chicken anti-TGF-β_1 (1:200 dilutions) was incubated with the appropriate samples for 1 h at 37°C before the addition of cells.

4.3   Results

4.3.1   Utilization of the xylanase promoter for the controlled production of human KGF-2 or TGF-β_1 by *B. ovatus*

A strain of *B. ovatus* capable of producing and secreting human KGF-2 or TGF-β_1 in a xylan-inducible manner was constructed. The regions of the *kgf-2* and *tgf-β_1* genes encoding the 170 aa and 112 aa mature proteins were cloned downstream of the xylanase promoter in pCR$^{\circledR}$-Blunt to generate the plasmid pCR-PKGF and pCR-PTGF respectively (Fig 4.1 and 4.2). The *B. fragilis* enterotoxin secretion signal sequence was introduced at the 5' of the growth factor coding sequence to mediate secretion of the recombinant protein. The use of an *Nde*I site for cloning resulted in 3 bp change (AGA to CAT) in the non-coding region which was outside the predicted RBS of the promoter and was not expected to affect translation. The promoter-KGF and promoter-TGF fragments were gel extracted after digestion with *BamH*I and *Hin*dIII/*Xba*I and cloned into the suicide vector pBT2 to create pBT-KGF and pBT-TGF, respectively (Fig 4.1). Cloned pBT2 was transferred to *B. ovatus* V975 by homologous recombination to create BO-KGF and BO-TGF. The presence of recombinant genes in the transconjugates was confirmed by colony PCR using primers specific for the cloned genes (Fig 4.3A).

The integrity of the xylanase operon region in these recombinant strains was confirmed by the presence of ORF1 transcript when cultured on xylan-containing media (Fig 4.4). Furthermore, these recombinant strains produced growth that was identical to the wild type *B. ovatus* V975 when grown on xylan or glucose containing media (Fig 4.5A and B).

4.3.2   Detection of heterologous gene transcript

To confirm that the recombinant strains of *B.ovatus* (BO-KGF and BO-TGF) transcribed the cloned gene from the xylanase promoter, end-point RT-PCR was performed. In the absence of xylan, hardly any visible PCR products could be detected. However, after 2 h of xylan exposure, strong PCR products of the corresponding gene size were detected (Fig 4.3B). After normalisation of the

amount of cDNA, it was estimated that there was a 1000-fold increase in the transcript level after xylan exposure.

4.3.3   Containment of recombinant *B.ovatus*

Spread of the genetically modified organisms to the environment is an ethical and biosafty concern. The anaerobic nature of *B.ovatus* was utilized in this recombinant strain, making its spread less likely. Viability of BO-KGF was reduced by more than 50% after 48 h of exposure to air with virtually almost no viable bacteria detected after 96 h (Fig 4.6).
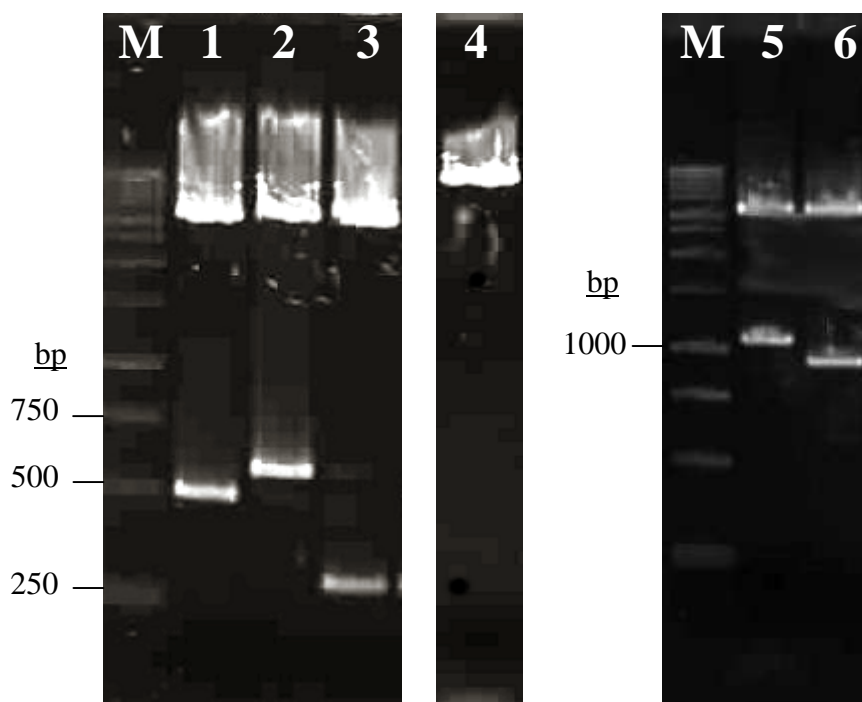
Figure 4.2   Agarose gel of pCR-PKGF and pCR-PTGF formation steps.
Sequence of xylanase promoter and mature region of *kgf-2*, *tgf-β*₁ were PCR
amplified and cloned into pCR-blunt to produce pCR-XYLP, pCR-KGF and pCR-
TGF. Plasmids were digested with *Bam*HI/*Nde*I for pCR-XYLP, *Hin*dIII/*Nde*I for
pCR-KGF and pCR-TGF (1, 2 and 3 respectively). pCR-XYLP was digested with
*Hin*dIII/*Nde*I (4), and same enzymes were used to cut the *kgf* and *tgf-b* inserts and
cloned them downstream of the xylanase promoter in the pCR-XYLP to produce
pCR-PKGF (5, digested with *Bam*HI) and pCR-PTGF respectively (6, digested with
*Hin*dIII/*Xba*I). M; DNA ladder.
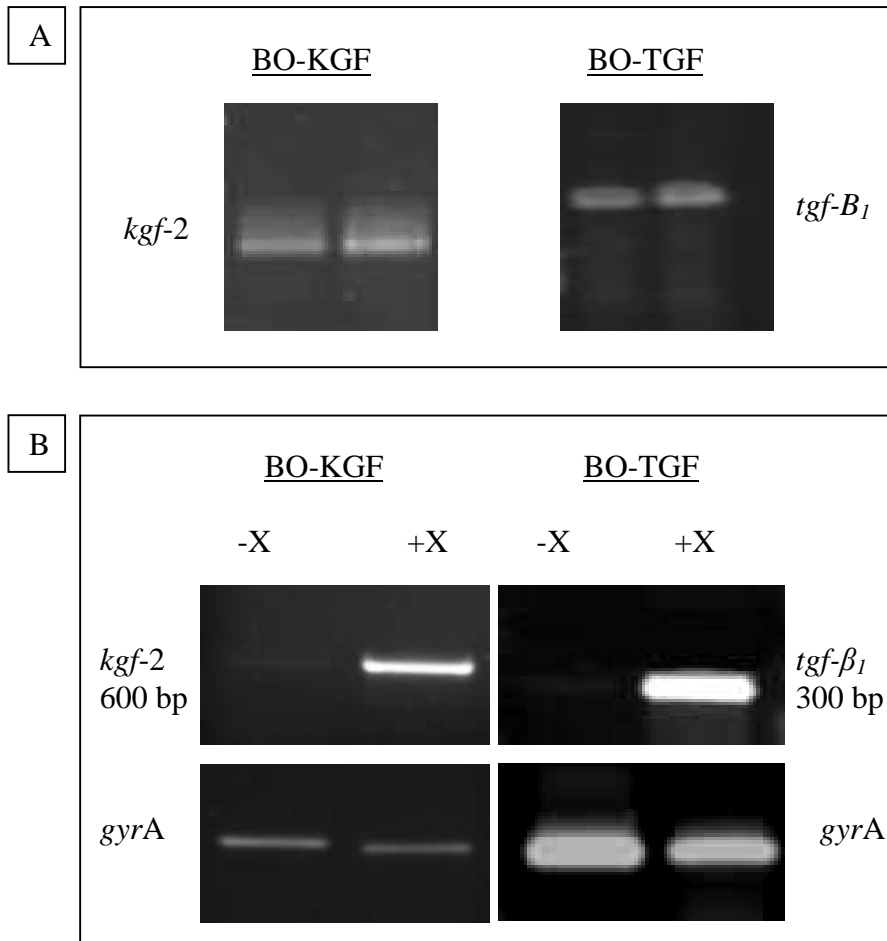
Figure 4.3 **Agarose gel of BO-KGF and BO-TGF colony PCR and RT-PCR (A) Colony PCR of BO-KGF/BO-TGF transconjugates:** Two colonies were picked from selection plates 3 days after conjugation. PCR was performed using primers specific for the *kgf-2* and *tgf-β* cloned genes. **(B) Increased expression of KGF-2 and TGF-β_1 mRNA in response to xylan**. Recombinant strains BO-KGF and BO-TGF were grown for 24 h in RGM-Hemin without xylan (-X). Xylan was added 2 h before collection of cells (+X). Cells were harvested, total RNA extracted and RT-PCR performed to detect KGF-2 and TGF-β_1 transcript. *gyr*A was used as a positive control.
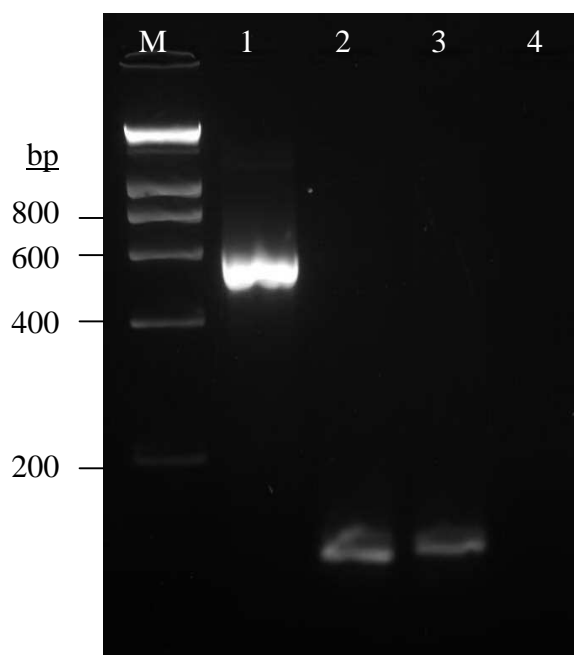
Figure 4.4   Agarose gel of xylanase operon analyses in BO-KGF by RT-PCR.
BO-KGF cultured in RGM-Hemin supplemented with glucose 0.1% (w/v), xylan
0.05% (w/v) was added 2 h before collection of cells. 1; cloned gene (*kgf-2*)
expression, 2; ORF1 gene of the xylanase operon expression, 3; gyrA expression
(+control), 4; negative control. M; DNA marker. Results show that that the xylanase
operon is intact after cloning of the *kgf* downstream the xylanase promoter.
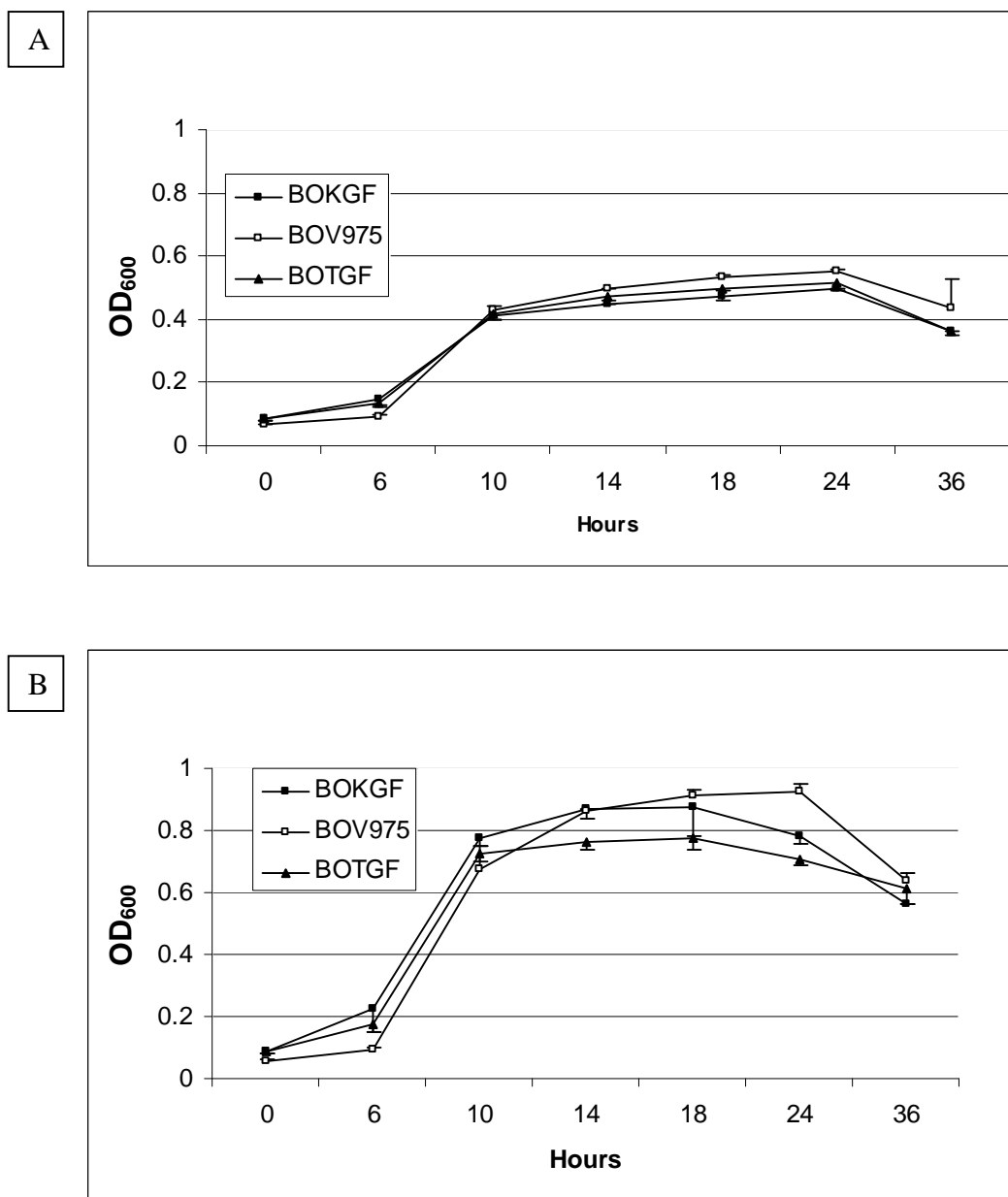
Figure 4.5   Growth of BO-KGF and BO-TGF on xylan or glucose containing media. Wild type *B.ovatus* (BO V975) and recombinant strains BO-KGF and BO-TGF were grown on RGM-Hemin supplemented with either 0.05% (w/v) xylan (**A**) or 0.1% (w/v) glucose (**B**) as a carbon source. Growth (OD_{600}) of BO-KGF and BO-TGF was identical to BOV975 on xylan and glucose. Data shown are means (±SEM) from duplicates of two independent experiments.
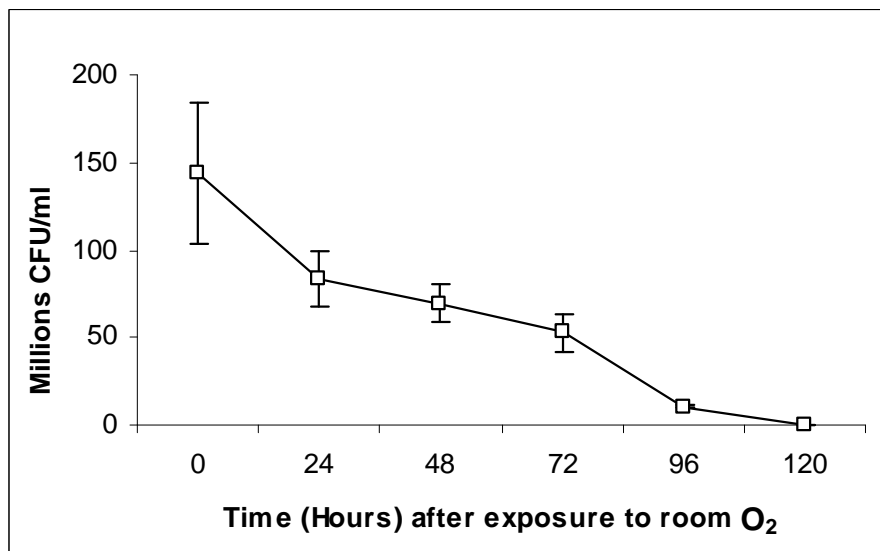
Figure 4.6   Viability of recombinant *B. ovatus* in O$_2$ containing environment.
BO-KGF was cultured into RGM-Hemin supplemented with 0.1% (w/v) glucose
and 0.05% (w/v) xylan. The culture was incubated in the laboratory and sampled
daily for viable cells. CFU was determined by serial dilution and plating on BHI-
Hemin plates supplemented with tetracycline, colonies were counted 48 h after
plating and corrected for dilution factor. BO-KGF lost 50% of its viability 48 h after
incubation in room air oxygen and more than 90% after 96 h. Data shown are means
(±SEM) from triplicates of two experiments.

4.3.4 *In vitro* detection of recombinant KGF-2 and TGF-β_1

BO-KGF, BO-TGF and wild type *B. ovatus* V975 were grown in medium with or without xylan. Xylan (0.05% w/v) was added at 4 h or at 24 h before sample collection to medium. Cultures supernatant were assayed for human KGF-2 and TGF-β_1 by ELISA. KGF-2 was detected in the culture supernatant of BO-KGF grown with xylan (680 pg ml^{-1}) at concentrations 7 fold higher than that produced by BO-KGF cultured in the absence of xylan (99 pg ml^{-1}) ($p<0.01$) and 2 fold higher than when xylan was added for 4 h (336 pg ml^{-1}) ($p<0.05$) (Fig. 4.7A). This shows that KGF-2 acumulated in the culture supernatant when the xylan was left for longer period. Likewise, TGF-β1 was detected in culture supernatant of BO-TGF grown with xylan (260 pg ml^{-1}) and this was double the amount preduced when xylan was added for 4 h (113 pg ml^{-1}) ($p=0.14$) and 100 times the amount when BO-TGF was cultured without xylan (3 pg ml^{-1}) ($p<0.01$) (Fig 4.7B).

4.3.5 *In vivo* KGF-2 and TGF-β1 production

BO-KGF and BO-TGF were given to C57Bl/6 mice by oro-gastric gavage to assess if they produced KGF-2 and TGF-β1 respectively *in vivo* after induction by xylan. Mice were kept on xylan-reduced diet. When needed, xylan was administered via the drinking water. Relevant recombinant cytokine was detected in the colon of C57BL/6 mice maintained on a xylan-containing diet after oral administration of BO-KGF (16 ng gm^{-1} KGF-2 in colonic content) or BO-TGF (11 ng gm^{-1} TGF-β_1 in colonic content) at levels significantly higher than in mice given recombinant strains but maintained on a xylan-reduced diet (7 ng gm^{-1} of KGF-2, 4 ng gm^{-1} of TGF-β_1, $p<0.01$), as well as in non-treated mice and in mice administered wild type BO V975 maintained on a xylan-containing diet (Fig. 4.8). Collectively, these findings are consistent with the ability of BO-KGF and BO-TGF to produce KGF-2 and TGF-β_1 *in vivo* in a xylan controlled manner.
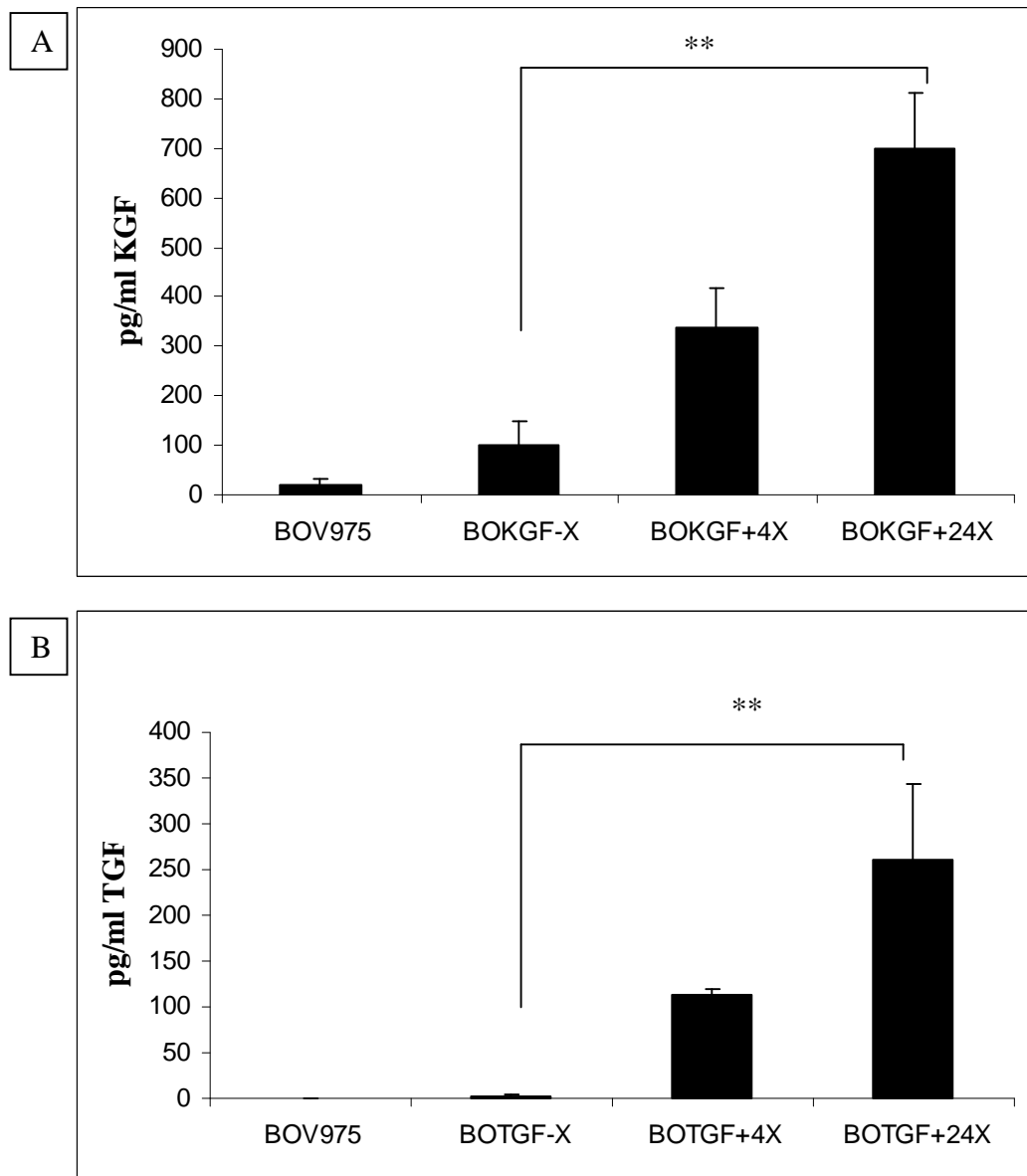
Figure 4.7   Quantitation of KGF-2 and TGF-β_1 in culture supernatant of BO-KGF and BO-TGF in the presence and absence of xylan. Levels of recombinant cytokine produced in culture supernatant of BOV975, BO-KGF **(A)** and BO-TGF **(B)** as determined by ELISA. BO-KGF and BO-TGF was cultured in the absence (-X) or in the presence of 0.05% (w/v) xylan for 4 h (+4X) and 24 h (+24X) and BOV975 was cultured in the presence of xylan. KGF-2 and TGF-β_1 were produced *in vitro* when xylan was present in the media of relevant strains, and the cytokine accumulate in the media on longer exposure to xylan. Data presented are means (± SEM) from three experiments.**p<0.01.
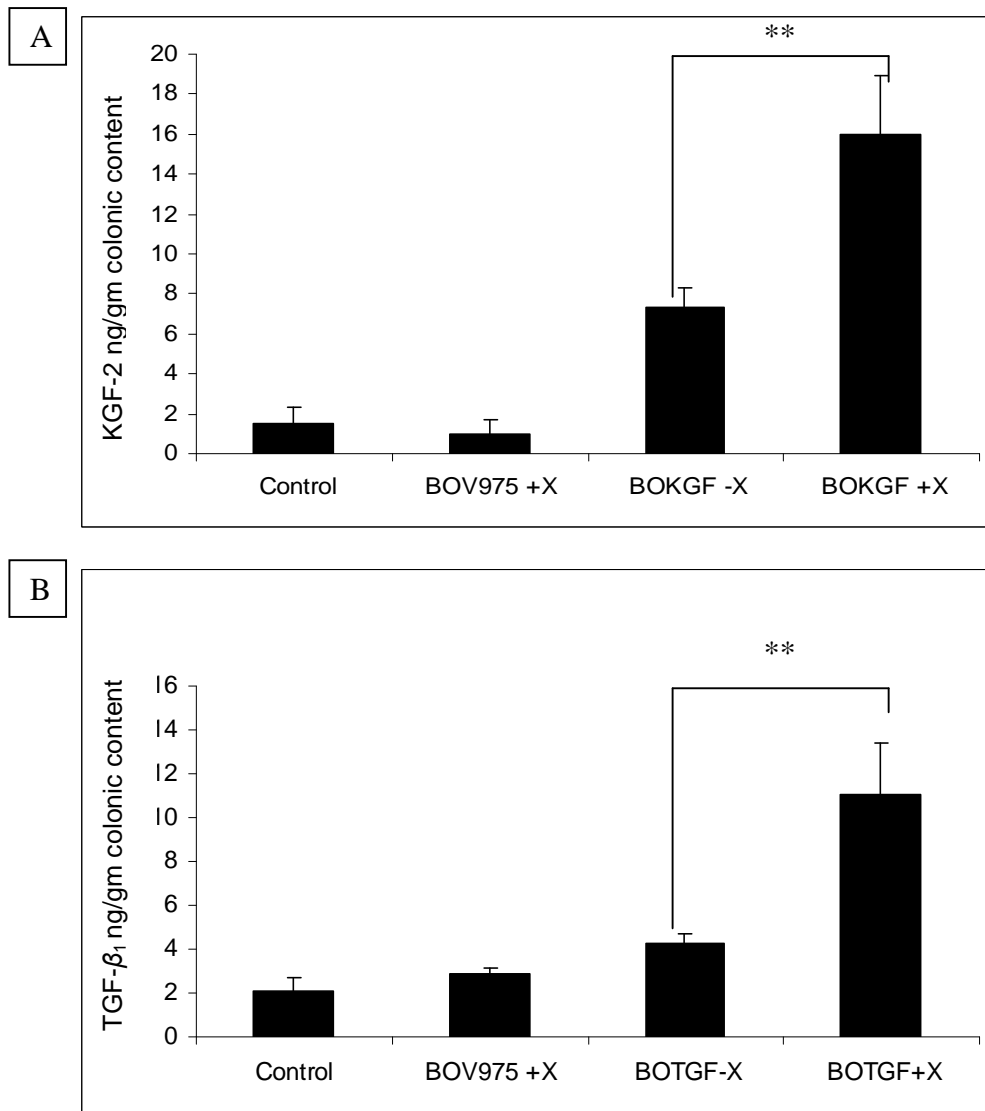
Figure 4.8   Levels of recombinant cytokine produced in C57Bl/6 mice colon 24 h after single gavage of *B. ovatus* V975, BO-KGF and BO-TGF as determined by ELISA. BOV975, BO-KGF and BO-TGF were cultured in the absence of xylan, 10^8 CFU were given to C57Bl/6 mice kept on xylan reduced diet (-X), xylan was added to the drinking water at 30 mg ml^{-1} (+X). 24 h later the colonic contents were taken and processed for KGF-2 (**A**) and TGF-β1 (**B**) assay. Control mice received no treatment. KGF-2 and TGf-β1 were detected in the colon of mice fed the relevant recombinant strains when xylan was present in the water. Data presented are means (± SEM) from 3 experiments for the control groups and 6 experiments for the treatment groups, **p<0.01.

4.3.6 TGF-β_1 produced by BO-TGF is dimeric

Only dimeric TGF has biological activity (Brunner *et al.*, 1992). Therefore, SDS-PAGE and western blot was performed on BO-TGF culture supernatant to determine the structure of BO-TGF expressed TGF-β_1. Western blot analysis of the BO-TGF culture supernatatnt under non-reducing condition identified a 25 kDa protein (Fig 4.9), corresponding to the dimeric TGF-β_1 produced by mamilan cells (Frolik *et al.*, 1983). The control recombinant TGF-β_1 was 12.5 kDa.

4.3.7 *In vitro* biological activity

BO-KGF: Culture supernatants from BO-KGF grown in the presence of xylan induced proliferation of NHK cells. The inclusion of anti-KGF-2 antibodies in the supernatant blocked NHK proliferation demonstrating that the growth promoting activity in culture supernatant of BO-KGF was due to KGF-2 (Fig 4.10). These results demonstrate that BO-KGF produces biologically active KGF-2 in a xylan inducible manner. Supernatants from BO V975 control strain demonstrated no biological activity. Levels of biologically active KGF-2 ranged between 50-150 pg ml^{-1} which is less than estimated amount of KGF-2 in culture supernatant of BO-KGF detected by ELISA.

BO-TGF: TGF-β_1 produced by BO-TGF was able to suppress IL-5 induced proliferation of TF-1 cells and therefore was biologically active (Fig 4.11). Biological activity was not detected in supernatants from BO V975. Neutralising the inhibition of proliferation by the addition of an anti-TGF antibody, demonstrated that the growth inhibition activity in culture supernatants of BO-TGF was specifically due to TGF-β_1 (Fig 4.11). The level of biologically active TGF-β1 detected in BO-TGF supernatant ranged between 50-110 pg/ml which was almost less than half of the TGF-β_1 levels detected by ELISA.
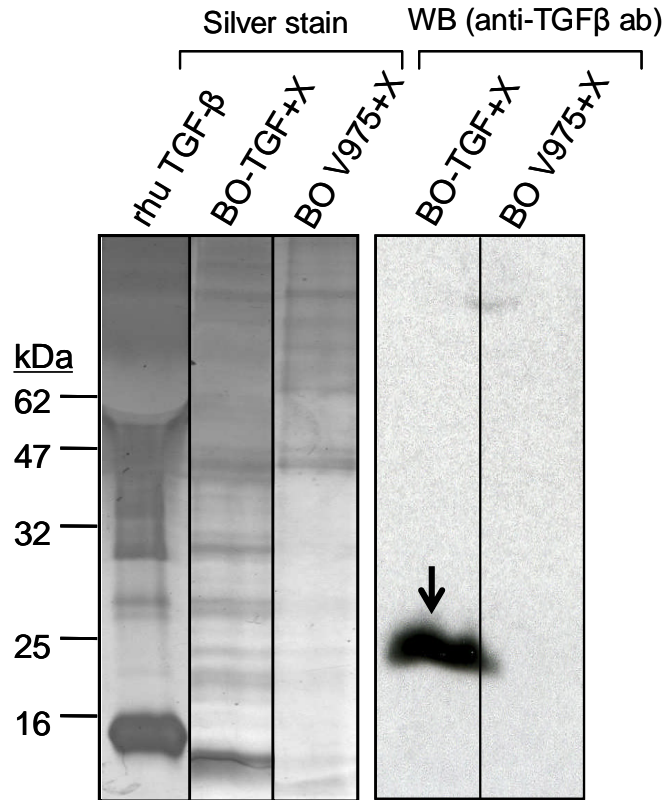
Figure 4.9   SDS-PAGE and western blot of BO V975 and BO-TGF culture supernatant. BO V975 and BO-TGF were grown on RGM-Hemin supplemented with 0.1% (w/v) glucose and Xylan 0.05% (w/v). Proteins in culture supernatant were precipitated then resuspended in non-reducing loading buffer and separated on 16% (w/v) SDS gel. Gels were either silver stained or Western blot using biotinylated anti-TGF-β_1 antibody to detect TGF-β_1. Sizes shown in kDa as predicted by a prestained protein ladder (NEB). Solid arrow indicates the 25 kDa TGF-β_1 protein present in BO-TGF supernatant cultured in the presence of xylan.
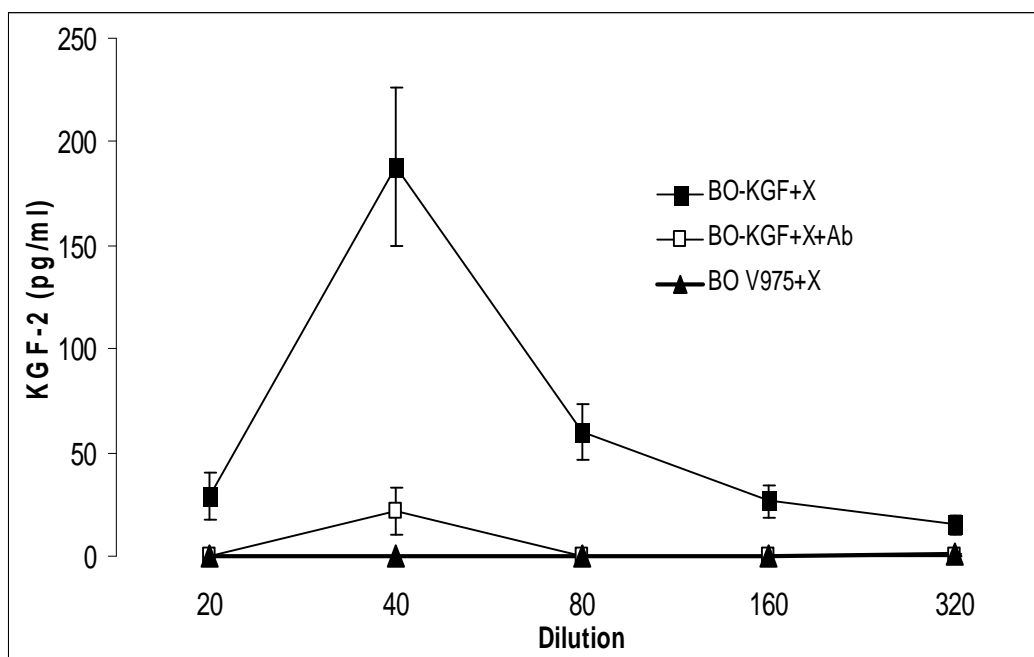
Figure 4.10   Bioassay of KGF-2 in culture supernatants of BO-KGF grown with xylan. Proliferation of human keratinocyte (NHK) cells induced by KGF-2 was measured by the uptake of MTT following incubation with doubling dilutions of culture supernatant of BO-KGF cultured with xylan (BOKGF+X), anti-KGF antibody with culture supernatant of BO-KGF cultured with xylan (BOKGF+X+ Ab). Wild type BO V975 cultured with xylan (BOV975+X). Data are means (± SEM) representative from triplicates of two experiments
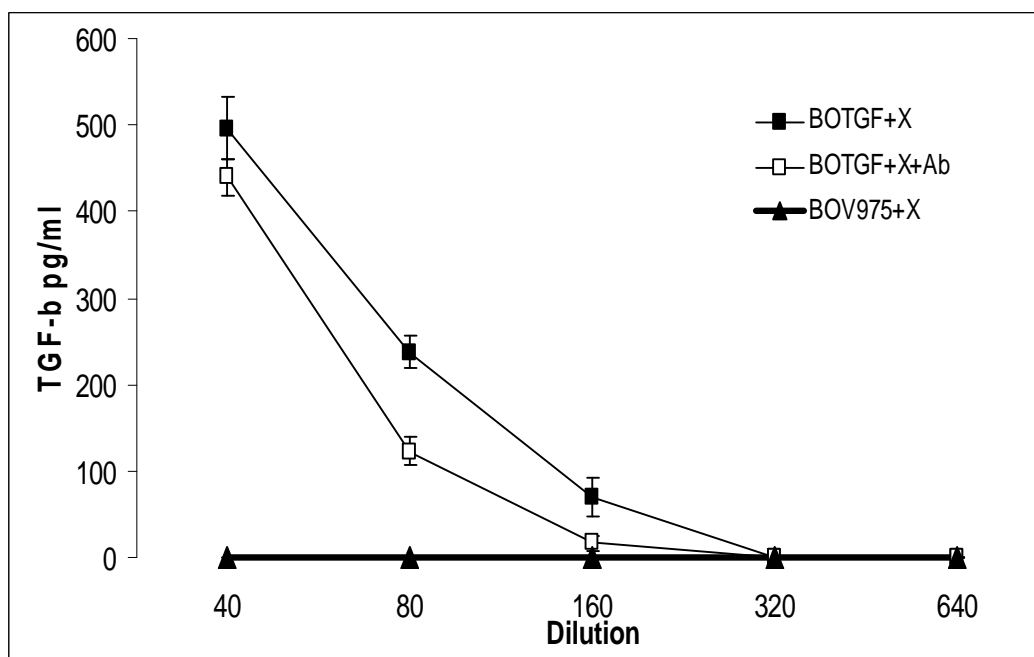
Figure 4.11   Bioassay of TGF-β_1 in culture supernatants of BO-TGF grown with and without xylan. Inhibition of IL-5 induced proliferation of TF-1 cells by TGF-β was measured by the uptake of [3H] thymidine following incubation with doubling dilutions of supernatant of BO-TGF cultured with xylan (BOTGF+X); anti-TGF antibody with supernatant of BO-TGF cultured with xylan (BOTGF+X+Ab); Wild type BO V975 culture with xylan (BOV975+X). Data are means (± SEM) from triplicates of two independant experiments.

4.4   Discussion

B. ovatus xylan utilization was used to direct the controlled production of the murine cytokine IL-2 *in vitro* (Farrar *et al.*, 2005). Disruption of the xylan utilization gene resulted in reduced growth rate of *B. ovatus* and the xylanase mutant *Streptomyces lividans* when grown with xylan (Arhin *et al.*, 1994; Weaver *et al.*, 1992) and affected its ability to colonise the mice colon (Carding, unpublished observations). As a result of the work presented here, the integrity of the xylanse operon is maintained, increasing the prospect of using engineered *B. ovatus* strains in probiotic regimens. The cloning of *kgf* and *tgf* into the *Bacteroides* genome was done utilizing the promoter sequence to avoid any disruption to the xylanase operon. To optimise conjugation, the homologous sequence was extended to include part of the upstream gene which obviously has to be mutated by this procedure. To confirm that the xylanase gene remains intact after cloning, the first gene of the xylanase operon was shown to be expressed in the recombinant strains. Fortuitously, mutation of the upstream gene did not affect the xylanase operon expression. In addition, the growth rate of the recombinant strains on xylan or glucose was similar to wild type *B.ovatus*.

The *Bacteroides* utilized in this system was originally isolated from the human colon. Therefore, it is expected that the recombinant strains would be able to colonise the human colon. The engineered *Bacteroides* delivery system is expected to address the issues raised by other recombinant bacteria that are to be used for mucosal delivery of therapeutic proteins. It was shown that recombinant *B.ovatus* lost 50% of its viability 48 h after exposure to environmetal oxygen. Further containment strategy used in this system was the integration of the cloned gene into genomic DNA. Recombinant genes can either be transcribed from a copy incorporated into plasmid DNA or genomic DNA. Using plasmids for the production of recombinant genes can give higher amounts of protein, as multiple copies of plasmids inside a single bacterium will transcribe the cloned gene more than a single copy. On the other hand, plasmids are unstable and can be lost from the bacterium if grown in an environment that lacks selection antibiotics and furthermore, the plasmids can transfer into other organisms resulting in spread of the recombinant genes in uncontrolled manner. Whereas cloning of genes into genomic DNA overcomes these problems.

The xylan controlled protein delivery by *Bacteroides* makes it easier to stop the protein production by asking the patient to stop taking the xylan containing diet. Xylan may be present in the normal diet. However, it may only be present in very small quantity which may not be enough to induce the cloned gene expression. Indeed, this area needs to be addressed before human utilization of this system.

The feasibility of using the xylanase promoter to control the expression of human genes has been demonstrated here using human KGF-2 and TGF-β_1 as test proteins. To control human cytokine expression the mature part of the protein was positioned downstream the xylanase promoter and translationally fused to the *B. fragilis* enterotoxin secretion signal sequence. This signal sequence was shown previously to mediate secretion of recombinant protein into the extracellular environment (Farrar *et al.*, 2005). It has been shown that KGF and TGF were present in culture supernatant and in the mouse colonic environment when xylan was added to the culture medium and to drinking water respectively. Due to the poor tolerance of xylan-free diet by the mice it was not possible to determine the levels of KGF-2 and TGF-β_1 produced by BO-KGF and BO-TGF in the complete absence of xylan. It is possible therefore that the increase in KGF-2 and TGF-β_1 above baseline (control) levels in treated mice maintained on xylan-reduced diet reflect low levels of induction by residual xylan in the diet.

The correct folding of the human cytokines was a major concern. KGF has Asn-linked glycosylation site in its amino acid sequence (Finch *et al.*, 1989) and when expressed in mammalian cells is glycosylated (Rubin *et al.*, 1989). However, biologically active recombinant KGF-1 was produced in an *E.coli* expression system (Ron *et al.*, 1993), and commercially available KGF-2 is also produced in bacterial systems. Bacterially expressed recombinant KGF was substantially more active than the native molecule (Ron *et al.*, 1993). Therefore, it is likely that *Bacteroides* expressed KGF will be biologically active. In this chapter it was proven that the culture supernatant of BO-KGF induced proliferation of human keratinocytes. This proliferation was selectively blocked by incorporation of affinity purified anti-KGF-2 antibody.

On the other hand, TGF-β_1 is a homodimeric molecule, two TGF molecules held together to form a dimer by intermolecular disulfide bonds from the conserved cysteine residues. It was proven that only dimeric TGF has biological activity

(Brunner *et al.*, 1992). The production of disulfide-containing eukaryotic proteins in bacterial systems is often complicated by low expression levels and the formation of inclusion bodies. The highly reducing intracellular environment of *E.coli* makes disulfide bond formation difficult to achieve (Hwang *et al.*, 1992). The *E.coli* expression system was successful to produce recombinant mature TGF-β_1 in monomeric form (Gao *et al.*, 2001). However, this TGF-β_1 monomer has no biological activity, and it needs oxidative refolding to gain biological activity (Tuan *et al.*, 1996). Little is known, however, about *Bacteroides* expression of complex eukaryotic proteins. Therefore, it was necessary to characterise TGF-β_1 produced by BO-TGF to identify whether it is monomeric or dimeric. Surprisingly, the culture supernatant of BO-TGF contained 25 kDa protein and it was not possible to identify the monomeric TGF-β at 12.5 kDa possibly due to short-half life leading to degradation during preparation or that monomeric TGF-β was produced in very little amount below the detection limit of the western blot. This finding may imply a novel ability of the *B. ovatus* for oxidative protein folding, and formation of dimer molecules. The culture supernatant of BO-TGF did contain biologically active protein as assayed by a previously optimised bioassay (Randall *et al.*, 1993). Thus, the recombinant expression of TGF-β_1 in bacteria established in this study may be a promising tool for production of large quantities of biologically active TGF-β_1.

TGF-β may be partly responsible for fostering aberrant wound healing and the tissue damage caused by scaring and the pathological consequences has been termed the "dark side" of tissue repair (Border & Ruoslahti, 1992). Therefore, it is important to control its delivery and bioavailability to advance its use as a potential therapeutic agent. The biologically active TGF-β1 produced *in situ* by *B. ovatus* may be useful clinically in the management of gut ulceration with reduced scaring effect since its production is temporary and will cease with removal of xylan from the gut.

In recombinant *B. ovatus* strains, an ATG codon was added to the 5' end of the mature protein sequence in order to facilitate translation. Consequently, a methionine residue was present on the N-terminus of the mature protein. The results of the bioassay demonstrated that this did not ablate the biological activity of the protein. Likewise, secretion of KGF and TGF directed by the *B. fragilis* enterotoxin secretion signal sequence did not eliminate the biological activity of the protein. The higher the concentration of culture supernatant proved inhibitory to the indicator cell

lines, hence it was measured in the 1/40 dilution of BO-KGF and BO-TGF supernatant. However, both KGF-2 and TGF-β levels detected by bioassay were almost half of the levels detected by ELISA. This discrepancy could be due to the short half-life of bioactive protein or it may be not all protein is correctly folded to have biological activity.

In summary, these studies advance the genetically modified bacterial delivery systems by developing a novel system for the controlled *in vivo* delivery of biologically active human proteins. Next aim is to test this system *in vivo* in an experimental animal model of intestinal inflammation and epithelial injury to determine the efficacy of this type of therapeutic delivery.

CHAPTER (5)

TREATMENT AND PREVENTION OF ACUTE COLITIS BY
RECOMBINANT *B. ovatus* PRODUCING HUMAN GROWTH FACTOR
(KGF-2 OR TGF-β_1) IN RESPONSE TO XYLAN

5.1   Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and Ulcerative colitis (UC), are a significant public health problem in Western societies, affecting nearly 1 in 1000 individuals (Loftus, 2004). Human IBDs are characterised by inflammation in the large and/or small intestine associated with uncontrolled innate and adaptive immunity against normal constitutes, including commensal bacteria and various microbial products (Baumgart & Carding, 2007), yet the aetiology is not fully understood. Several immune-regulatory agents have been used for the treatment of human IBD to control the dysregulated immune response. However, there is no cure, and these immune-regulatory agents can cause destructive side effects. Therefore, various other agents have been tried for IBD treatment; of these are the cytokines and growth factors (Han *et al.*, 2000; Sandborn *et al.*, 2003b).

A number of growth factors are expressed in the gastrointestinal tract, including members of epidermal growth factor (EGF), transforming growth factor (TGF) and the fibroblast growth factor (FGF) family. They play an essential role in regulating differential epithelial cell functions to preserve normal homeostasis and integrity of the intestinal mucosa (Dignass, 2001). Therefore, they might serve as an alternative therapy for patients with IBD.

Keratinocyte growth factor (KGF), for example, is a member of the FGF family. Its expression increases in the mucosa and submucosa of the affected areas of patients with IBD, whereby the degree of KGF over expression correlated with the degree of inflammation in the bowel mucosa (Finch *et al.*, 1996). This led to the belief that KGF helps preserve the integrity of damaged epithelial surfaces (Chen *et al.*, 2002). Other studies showed that KGF stimulates the proliferation of epithelial cells, ameliorates murine colitis and promotes healing of intestinal ulceration when

given systematically (Han *et al.*, 2000; Zeeh *et al.*, 1996). Recombinant KGF ameliorates radiation-induced oral mucositis (Farrell *et al.*, 2002). In clinical trials, high doses of recombinant systemic KGF-2 was needed for longer period to achieve therapeutic effect in active ulcerative colitis (Sandborn *et al.*, 2003b), these high doses may not be tolerated.

Another example is the transforming growth factor-β (TGF-β). TGF-β signalling helps maintain normal intestinal homeostasis to commensal luminal enteric bacteria (Haller *et al.*, 2003). Inadequate TGF-β signalling in T cells has been implicated in the pathogenesis of various diseases including IBD (Monteleone *et al.*, 2004). TGF-β has been found, *in vitro*, to exert potent effects on the intestinal epithelium and play an important role in reconstitution of epithelial integrity after injury (Ciacci *et al.*, 1993). Transgenic mice over expressing dominant-negative TGF-βRII were more susceptible to DSS-induced colitis and exhibited impaired recovery from colitis (Beck *et al.*, 2003). Studies of experimental murine colitis have provided evidence that TGF-β play a counter regulatory role in the Th1 pathway, leading to inflammation. It has been shown that TNBS colitis can be prevented by feeding haptenated protein before the time of TNBS administration, such prevention is accompanied by the appearance of TGF-β producing T cells and is abrogated by administration of anti–TGF-β (Neurath *et al.*, 1996). Similarly, in the SCID transfer model of colitis, co-administration of anti–TGF-β prevents the ability of CD45RB^{lo} T cells to prevent colitis induced by CD45RB^{hi} T cells (Powrie *et al.*, 1994; Powrie *et al.*, 1996). Furthermore, probiotic strains VSL#3 ameliorate Th-1 murine colitis by TGF-β dependant pathway (Di Giacinto *et al.*, 2005). These studies highlighted the potential use of TGF-β for the treatment of IBD. It was shown that intranasal delivery of plasmid DNA encoding active TGF-β_1 in a murine TNBS-induced colitis, was highly effective both in preventing and in ameliorating established colitis (Kitani *et al.*, 2000). However, in the same study recombinant TGF-β administered intraperitonealy did not have this effect. This ineffective systematic TGF-β delivery may be due to its short half-life and/or inadequate dosage. In another study, blocking of the inhibitory Smad7 by antisense oligonucleotides enabled endogenous TGF-β to down-regulate the proinflammatory response in IBD (Monteleone *et al.*, 2001).

Systemic administration of these cytokines may not be the optimal way of delivery. Therefore, an alternate route of delivery is needed. Rectal administration was used to deliver EGF to treat ulcerative colitis (Sinha *et al.*, 2003) the convenience of using rectal route for these ill patients is questionable. Oral route is probably more appropriate. However, development of KGF is hampered by its instability, as manifested by rapid aggregation and chemical modification on exposure to extreme environmental factors such as high temperature, low pH (Zhang *et al.*, 1995) and a protease rich environment. Protein stability makes oral route not ideal, as these proteins have to bypass the extreme conditions of the stomach. The short *in vivo* half-lives, the physical and chemical instability, and the low oral bioavailability of proteins currently necessitate their administration by frequent injections of protein solutions (Putney & Burke, 1998).

The use of GM bacteria to deliver biologically active, immunomodulatory molecules directly to the gut has become an increasingly intensive area of investigation as part of the search for new therapeutic options for chronic intestinal disorders such as IBD. Proof of principle studies using *Lactococcus lactis* have shown that this organism can produce and secrete biologically active human cytokines and can be used to successfully treat intestinal inflammation in animal models of IBD (Steidler *et al.*, 1995; Steidler *et al.*, 1998; Steidler *et al.*, 2000). Although effective, GMP strains such as *L. lactis* have significant drawbacks including lack of regulation of the production of the therapeutic molecule and biosafety and environmental contamination concerns. To overcome the drawbacks of the *L. lactis* system, we have utilized *Bacteroides ovatus*, a major **commensal** anaerobic colonic Gram-negative bacterium in humans, as a potentially improved GMP bacterium. Firstly, the delivery of protein by *B ovatus* can be **controlled** by the xylan induction system (Farrar *et al.*, 2005; Hamady *et al.*, 2008). Secondly, this system is naturally **contained** because of the anaerobic nature of *B. ovatus* (shown in Chapter 3). The obvious advantage of this method of delivery is that the bacterium is administered less frequently, and protein production can be controlled by the patient or treating physician.

The purpose of the studies presented in this chapter is to test the ability of KGF or TGF producing *B. ovatus* to repair intestinal epithelial injury in a xylan dependent manner of experimental mouse model of colitis.

5.2 Aims and experimental design

5.2.1 Treatment of DSS-induced colitis by BO-KGF and BO-TGF

Six-eight week-old male C57BL/6 mice were fed standard laboratory chow and tap water *ad libitum*. Twenty four hours prior to treatment mice were fed a xylan reduced diet (SDS, UK). In the treatment protocol acute colitis was induced in groups of mice (n=8) by addition of 2.5% (w/v) DSS (molecular weight, 35–45 kDa; MP Biomedicals) in the drinking water for 5 days and then changed to either water alone or water supplemented with xylan (30 mg ml^{-1}). Bacteria (2 x10^8 CFU in 0.2 ml PBS) were administered by oro-gastric gavage. Groups of mice (n=8 each) received BO-KGF, BO-TGF or wild type BO V975 on alternate days from day 5 to day 11. Control groups received PBS (DSS Ctrl), dexamethasone (3 mg/kg s.c. once daily, Steroid) or received no treatement (Normal Ctrl). At day 11 all animals were euthanised.

5.2.2 Prevention of DSS-induced colitis by BO-KGF and BO-TGF

In the prevention protocol bacterial gavage and xylan were started at the same time as DSS administration (2.5% w/v), which was continued for 5 days. Bacteria (2 x10^8 CFU in 0.2 ml PBS) were administered by oro-gastric gavage. Groups of mice (n= 8 each) received BO-KGF, BO-TGF or wild type BO V975 at day 0 and on alternate days for 5 days. Control groups received PBS (DSS Ctrl) or received no treatement (Normal Ctrl). Xylan was added to the drinking water of one group of animals receiving BO-KGF, BO-TGF and to the group receiving BO V975. At day 5 all animals were euthanized.

5.3   Methods

5.3.1   Disease activity index (DAI)

For both treatment and prevention experiments disease activity was monitored by DAI. It was based upon the sum of the scores assigned to weight loss, stool consistency (diarrhoea) and faecal blood. Diarrhoea was scored as normal, 0; mucus covered stool, 1; loose stools, 2; or watery diarrhoea, 4. Blood in stool was scored as normal, 0; occult bleeding, 1; slight bleeding, 2; or gross bleeding, 4. Weight loss was scored as none, 0; 1–5%, 1; 5–10%, 2; 10–15%, 3; or >15%, 4.

5.3.2   Pathological analysis

At autopsy colonic length was measured and macroscopic inflammation graded as to the percent colonic involvement as follows: 0, none; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; 4, >75%.

The colon was sampled at 1, 2 and 3 cm from the anal verge. Tissue samples were fixed in formalin and paraffin-embedded tissue sections were stained with H&E. Specimens were scored microscopically (Chapter 2) with the total colitis score being the sum of the five microscopic scores. The final score for individual colon is the average of scores of 3 samples. To exclude bias, the histological score was determined in a blind manner twice, by a specialist colorectal pathologist (Dr. N Scott) and by the author. Final score represent the average of both readings. Macroscopic score was added to the microscopic score to obtain pathology score.

For goblet cell quantitation sections of colon were stained with alcian blue and the nuclei were counterstained with nuclear fast red. An average of goblet cells per crypt was obtained by counting 3 fields from each section, 3 sections in each mouse and averaged.

5.3.3   Immunohistochemistry

Paraffin-embedded colon sections were incubated with monoclonal rat anti-ki67 antibody (DAKO). Bound antibody was visualized using a biotin-conjugated polyclonal rabbit anti-rat IgG (Vector) and a streptavidin horseradish peroxidase

conjugate and subsequent development with 3, 3'-diaminobenzidine (DAB) chromogen (DAKO). The sections were counterstained with hematoxylin. The average number of ki-67 positive cells per crypt was obtained by counting 3 fields from each section, averaging the values obtained from 3 sections of each mouse.

5.3.4   Real-Time PCR

Total RNA was isolated from colonic tissue using the SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. First-strand complementary DNA was synthesized using oligo d(T) primer with M-MuLV reverse transcriptase (New England Biolabs) according to the manufacturer's instructions. Real-time quantitative PCR was performed using an iCycler (Bio-Rad) with SYBER Green Jump Start Taq ready mix (Sigma) using the following conditions: 95°C for 3 min followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. A melting curve was obtained to verify the presence of a single amplicon. Primers used were: TNF-α-f/TNF-α-r, IL-1β-f/IL-1β-r, IL-6-f/IL-6-r, TGF-β_1-f/TGF-β_1-r, KGF-2-f/KGF-2-r, ITF-f/ITFr, COX-f/COX-r and β-actin-f/β-actin-r (Appendix 2). β-actin was used as reference for normalization. Quantification of mRNA was performed using the standard curve method with the starting quantity of the gene then normalized to the amount of β-actin product for each condition to determine gene expression relative to β-actin gene.

5.3.5   Myeloperoxidase assay

Myeloperoxidase (MPO) activity in the middle colon tissue was measured as described in Materials and Methods (2.32.7). Data are expressed as Units of MPO per gram of colon tissue.

5.4   Results

5.4.1   BO-KGF   and   BO-TGF   accelerate   healing   of   acute   intestinal inflammation in a xylan controlled manner

Acute colitis was induced in adult mice by 2.5% (w/v) DSS in the drinking water for 5 days after which mice were given *ad libitum* drinking water or water supplemented with xylan.   Bacteria were administered by oro-gastric gavage on alternate days starting at day 5 until the endpoint of the experiment at day 11. Prior to treatment all animals had rectal bleeding accompanied by diarrhoea after 5 days of DSS exposure. Treatment of DSS-colitis by BO-KGF and BO-TGF had a marked beneficial effect on disease progression and recovery as determined by both clinical and pathological criteria.

i. Xylan controlled growth factor delivery reduced DAI.

Weight loss was significantly less in BO-KGF treated animals given xylan (11-13%) than in animals given BO-KGF but no xylan (21-23%) or BO V975 and xylan (23-24%) and, in DSS-treated animals that received no bacteria (22%) ($p<0.05$) (Fig 5.1A). DAI scores were also significantly lower in mice treated with BO-KGF and xylan ($p< 0.01$) compared with other treatment groups (Figure 5.2A). The reduction in the disease activity was comparable if not better than that in steroid treated animals (Fig 5.2A).

Likewise, weight loss was less in BO-TGF-treated animals given xylan (14-16%) than in animals given BO-TGF but no xylan (21%) or BO V975 and xylan and in non-treated DSS control. This reduction was not statistically significant ($p=0.25$) (Fig 5.1B). However, DAI scores were significantly lower in mice treated with BO-TGF and xylan ($p< 0.05$) compared with other treatment groups (Fig 5.2B). The reduction in the disease activity was better than that in steroid treated animals (Fig 5.2B).
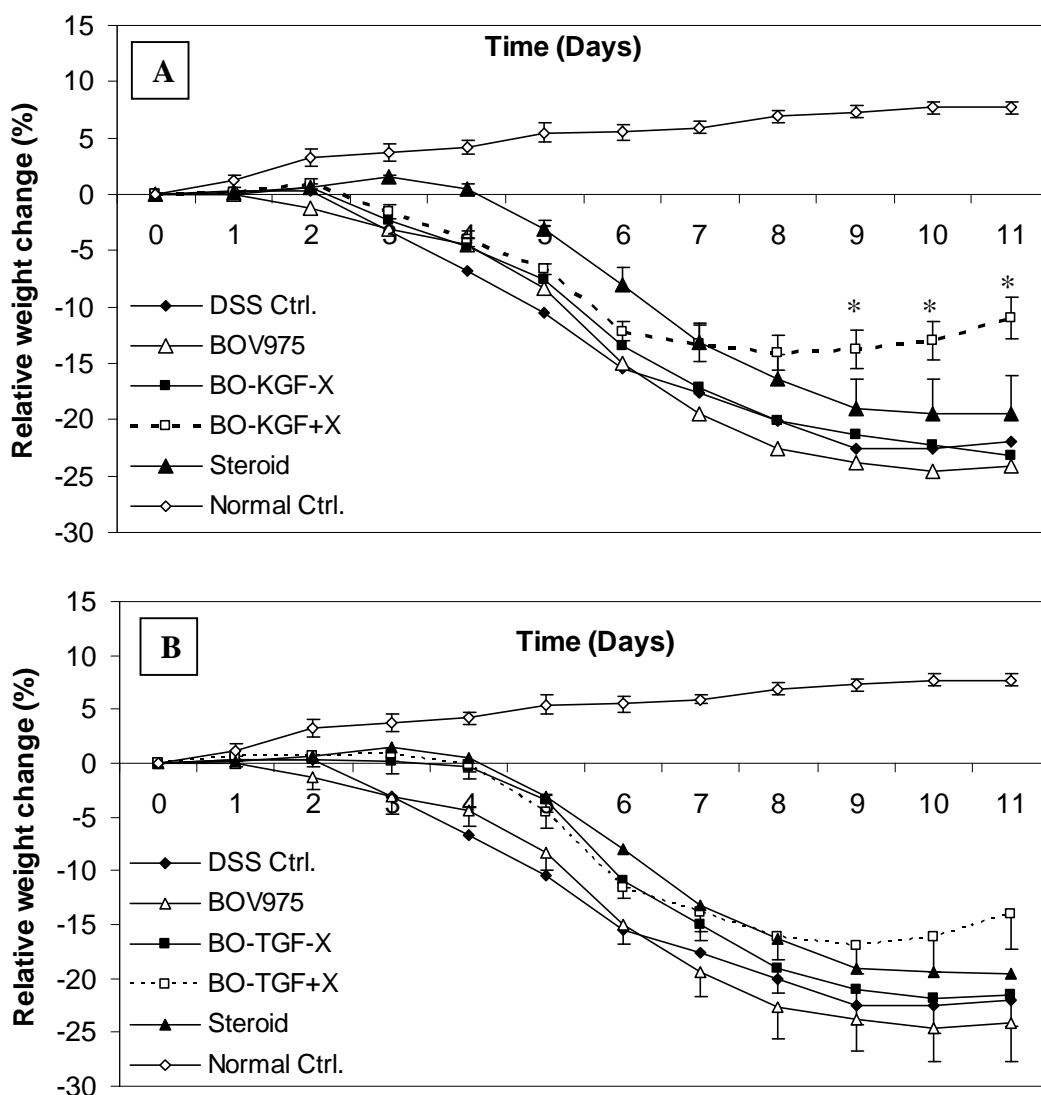
Figure 5.1 **Effect of BO-KGF or BO-TGF and xylan treatment on weight loss of DSS-induced colitis.** DSS was administered to adult mice via drinking water for 5 days after which it was withdrawn and replaced with either normal drinking (-X) or water containing 30 mg ml^{-1} xylan (+X). At day 5, 7 and 9 post DSS administration mice were gavaged with ~10^8 CFU of either wild type *B. ovatus* (BO V975), KGF-producing *B. ovatus* (BO-KGF) (**A**) or TGF-producing *B. ovatus* (BO-TGF) (**B**) . Control groups comprised animals that received no bacteria (DSS Ctrl), steroid treated (Steroid) and normal healthy animals (Normal Ctrl); n= 8 in each group. Body weight was determined at day 0 and daily after DSS administration up to 11 days. *p<0.05 comparing BO-KGF+X group with BO-KGF-X, BOV975+X and DSS Ctrl groups.
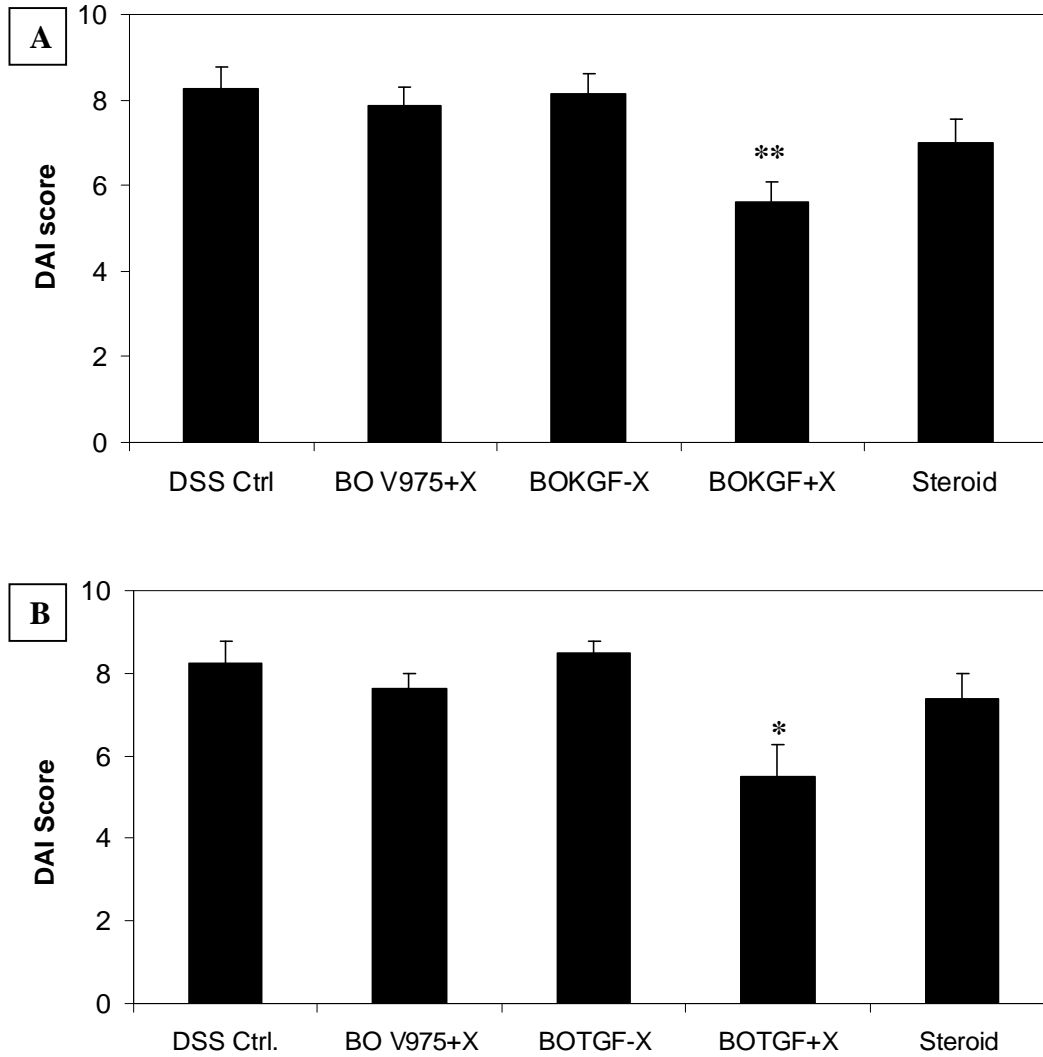
Figure 5.2   Effect of BO-KGF or BO-TGF and xylan treatment on disease activity index scores (DAI) of DSS-induced colitis. DAI scores were based on cumulative scores for extent of weight loss, stool consistency and faecal blood presented at day 11 as described in Materials and Methods section. **p<0.01 comparing BO-KGF+X group with DSS Ctrl, BOV975+X and BO-KGF-X groups. *p<0.05 comparing BO-TGF+X group with BO-TGF-X, BOV975+X and DSS Ctrl groups. (n=8 in each group)

ii. Xylan controlled growth factor delivery reduced DSS associated colonic shortening:

Reduction in colon length was used as an independent parameter of inflammation (Okayasu *et al.*, 1990), which in BO-KGF and xylan treated animals associated with the improved clinical results. The colon of animals treated with BO-KGF and xylan (7.5 cm ± 0.14) were significantly longer than those in animals treated with BO-KGF alone (6.8 cm ± 0.22; 10% shorter, $p<0.05$), BO V975 and xylan (6.7 cm ± 0.2; 12% shorter, $p<0.01$), DSS control (6.5 cm ± 0.16; 14% shorter, $p<0.01$) or, in animals treated with steroids (6.8 cm ± 0.2; 10% shorter, $p<0.05$) (Fig 5.3A and B).

Similarly, the colon of animals treated with BO-TGF and xylan (7.6 cm ± 0.19) were significantly longer than those in animals treated with BO-TGF alone (6.3 cm ± 0.16; 17% shorter, $p<0.001$), BO V975 and xylan (12% shorter, $p<0.01$), DSS control (14% shorter, $p<0.001$) or in animals treated with steroids (10% shorter, $p<0.05$) (Fig 5.3A and B).

iii. Xylan controlled growth factor delivery reduced MPO activity.

MPO activity, a marker of neutrophil infiltration and activity, was reduced by >65% in BO-KGF and xylan treated animals compared to non-treated DSS colitic animals ($p<0.001$). It was also reduced by >50% compared to animals treated with BO-KGF in the absence of xylan ($p<0.001$) and in animals treated with BO V975 and xylan ($p<0.001$) (Fig 5.4). MPO activity was comparable between xylan treated BO-KGF colonised group and steroid treated animals ($p=0.8$)

Likewise, MPO activity was reduced by BO-TGF and xylan treatment by more than 72% compared to non-treated colitic animals ($p<0.001$). It was also reduced by >60% compared to animals treated with BO V975 in the presence of xylan ($p<0.01$) However, MPO activity was not significantly different in BO-TGF and xylan treated animals as compared with non xylan treated peers ($p=0.5$) and steroid treated group ($p=0.8$) (Fig 5.4)
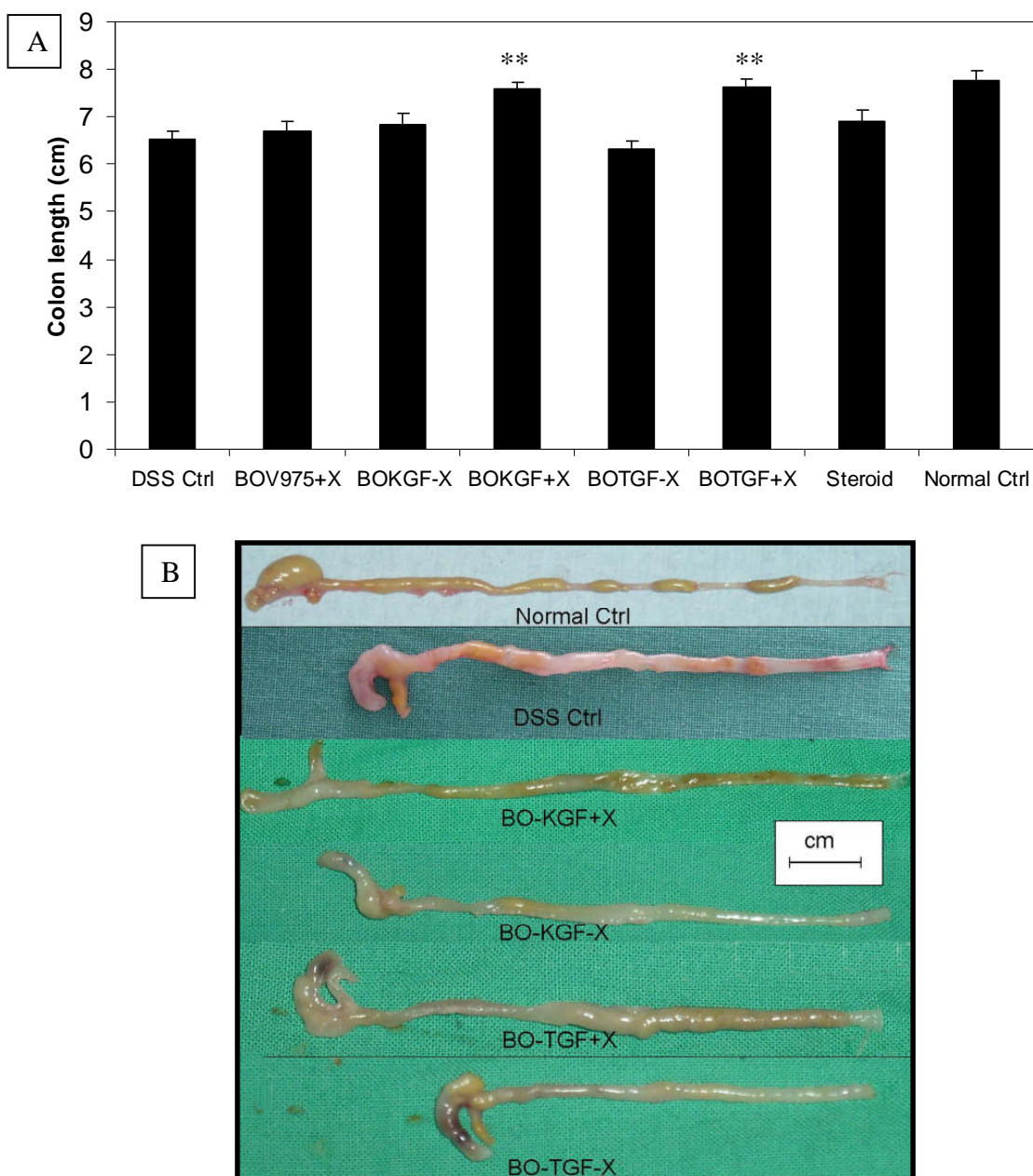
Figure 5.3   Effect of BO-KGF or BO-TGF and xylan treatment on colonic length after DSS-induced colitis. Colonic length was measured from ileocecal junction to the anal verge at necropsy (on day 11). Data are expressed as means ±SEM (n=8 in each group). (A) Colonic length was significantly longer in the groups treated with BO-KGF or BO-TGF and xylan **p<0.01 compared with other treatment groups. (B) Representative pictures of the gross colonic appearance at day 11 after treatment of DSS colitis by BO-KGF or BO-TGF with (+X) or with out (-X) xylan. BO-KGF+X and BO-TGF+X colons appears longer and grossly less inflamed than colons of BO-KGF-X and BO-TGF-X or DSS Ctrl colons.
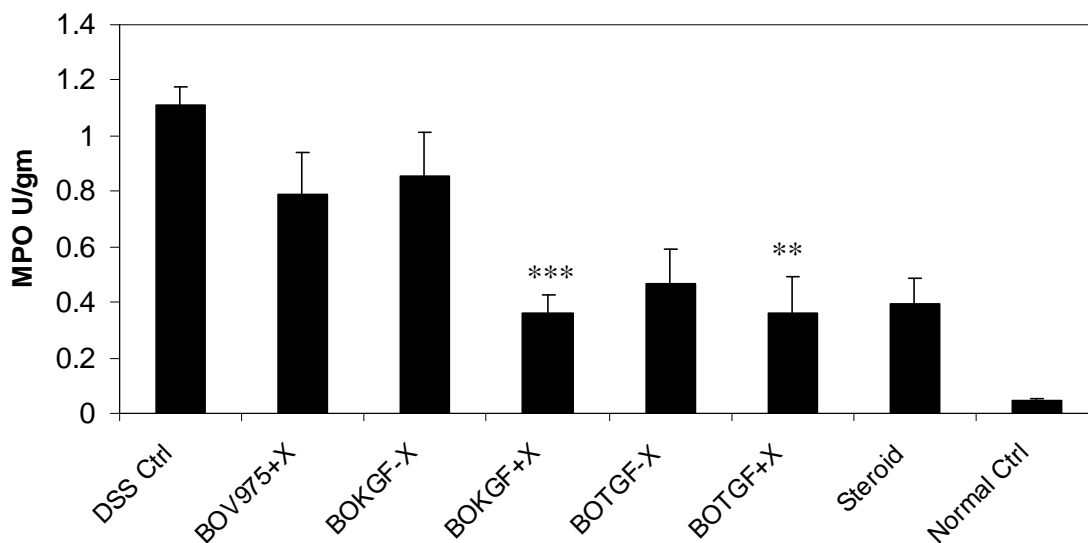
Figure 5.4   Therapeutic effect of BO-KGF or BO-TGF and xylan treatment on MPO activity after DSS-induced colitis. DSS was given for 5 days. At day 5 revert to normal drinking water (-X) or water supplemented with 30 mg ml^{-1} xylan (+X). Oro-gastric gavage of BO V975, BO-KGF or BO-TGF was given at day 5, 7 and 9. MPO levels from mice colons measured on day 11. Data are expressed as means ± SEM (n= 8 in each group). ***p<0.001 compared with non-treated mice with DSS colitis (DSS Ctrl), BO V975 plus xylan treated mice with DSS-colitis and BO-KGF without xylan treated mice with DSS colitis. **p<0.01 compared with non-treated mice with DSS colitis, BO V975 plus xylan treated mice with DSS-colitis.

iv. Xylan controlled growth factor delivery accelerates colonic epithelial healing and reduces inflammatory infiltrate.

Colonic sections stained with H&E showed that treatment with BO-KGF and xylan reduced crypt damage inflammatory infiltrate scores by 42% compared to DSS control mice (p<0.001), by 38% to animals treated with BO-KGF alone (p<0.001) and by 34% to wild type strain BO V975 and xylan (p<0.01) (Fig 5.5A and 5.6). This therapeutic effect of BO-KGF and xylan was better than steroid treatment, but not significant (25%, p=0.054) (Fig 5.5A and 5.6). When the gross area of the colon involved in inflammation was scored and added to the microscopic histopathology score, the BO-KGF and xylan treated group remained significantly better than other treatment groups (p<0.001) (Fig 5.5B).

BO-TGF and xylan lowered the microscpic histopathology score as well, but improvement achieved with this treatment modality was not statisticaly significant (p=0.17) (Fig 5.5A and 5.6). However, when gross colonic involvement taken into account, the difference became significant (p<0.05). The pathology score of the BO-TGF and xylan treated animals was better than DSS control by 28% (p<0.05) and animals treated with BO-TGF alon by 29% (p<0.05) (Fig 5.5B).

v. Xylan controlled growth factor delivery suppress pro-inflammatory cytokines.

BO-KGF and xylan treatment reduced expression of pro-inflammatory cytokines in the inflamed colon. Compared to non-treated colitic mice expression of genes encoding TNF-α, IL-1β and IL-6 were all significantly lower after BO-KGF and xylan treatment (p<0.001) (Fig 5.7A). TNF-α, IL-1β and IL-6 mRNA levels were lower in BO-KGF and xylan treated animals compared to animals treated with BO-KGF alone (p<0.05), in addition, TNF-α and IL-1β mRNA levels were lower compared to BO V975 and xylan treated group (p<0.01) (Fig 5.7A).

Similarly, TNF-α, IL-1β and IL-6 mRNA levels were reduced by BO-TGF and xylan treatment compared to non-treated DSS control mice and mice treated with BO-TGF alone (p<0.05). Furthermore, TNF-α and IL-1β mRNA levels were lower in BO-TGF and xylan treated animals compared to animals treated with BO V975 and xylan (p<0.01) (Fig 5.7B).
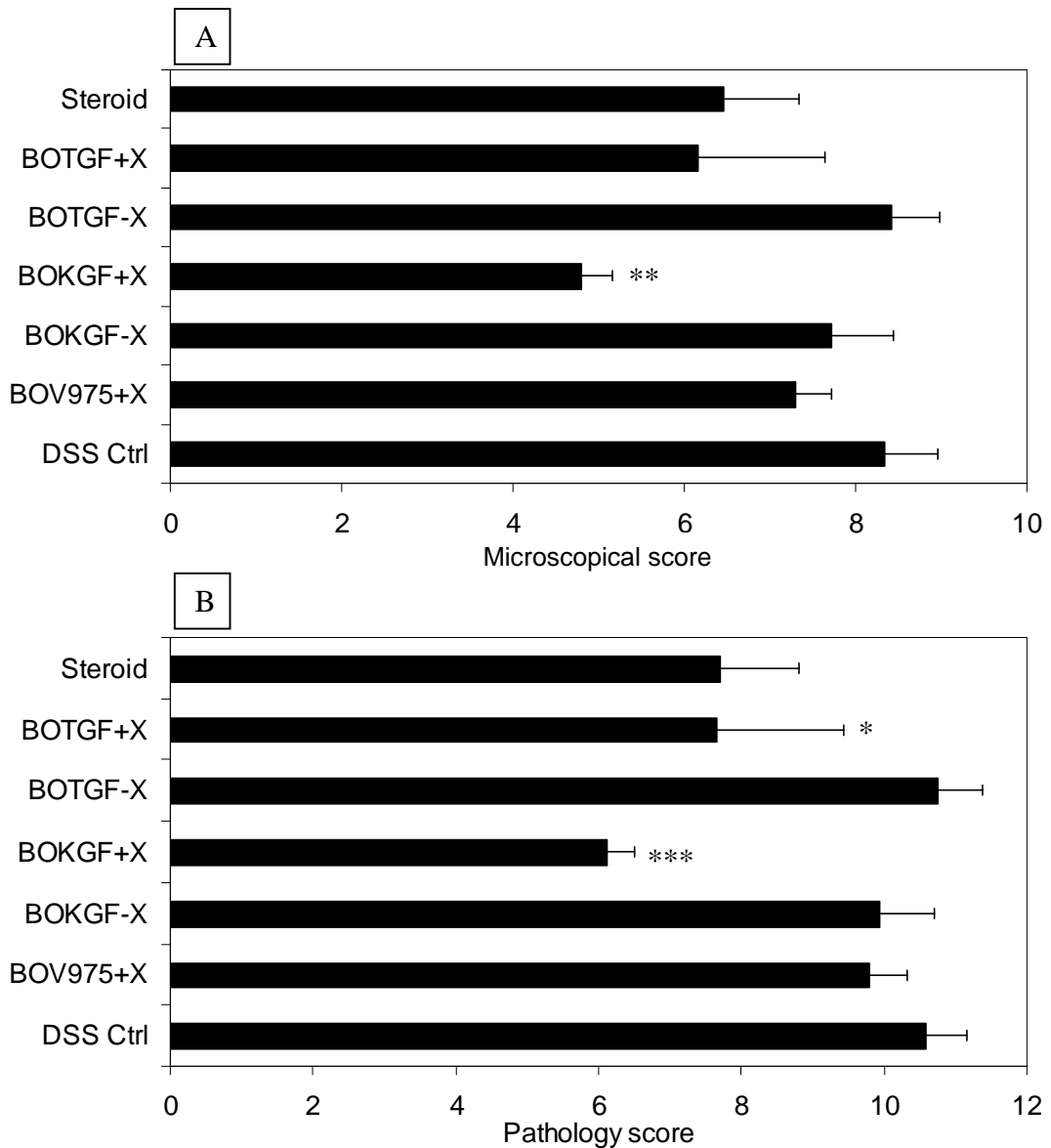
Figure 5.5   Effect of BO-KGF or BO-TGF and xylan treatment on pathology score. (**A**) Microscopical score was based on cumulative scores for severity and extent of crypt injury, acute and chronic inflammatory infiltrate and goblet cell depletion (Table 2.9). **p<0.01 compared to DSS Ctrl, BOV975+X and BO-KGF-X groups (n=8 each). (**B**) Pathology score was obtained by adding score for proportion of colon affected by inflammation to the microscpical score in (A) on the same groups of animals. *p<0.05 compared to DSS Ctrl and BO-TGF-X groups.***p<0.001 compared to DSS Ctrl, BOV975+X and BO-KGF-X group (n=8 in each group).

Normal Ctrl

DSS Ctrl

BOV975+X

Steroid

BO-KGF-X

BO-KGF+X

BO-KGF-X

BO-TGF+X
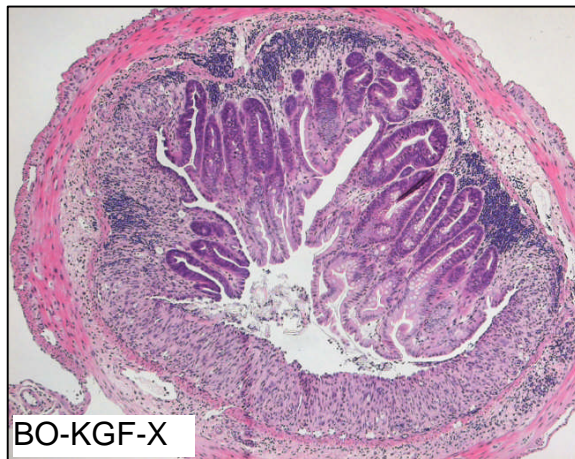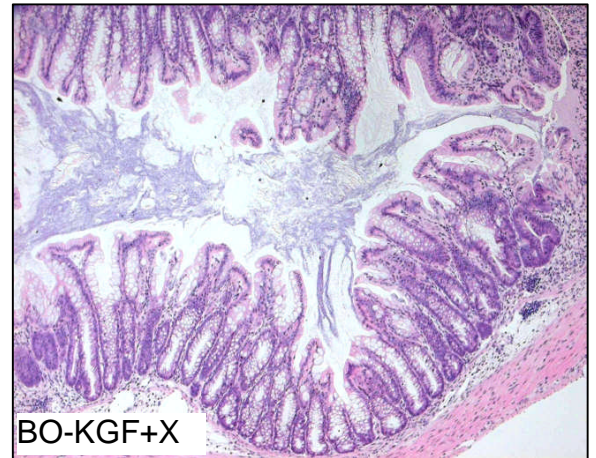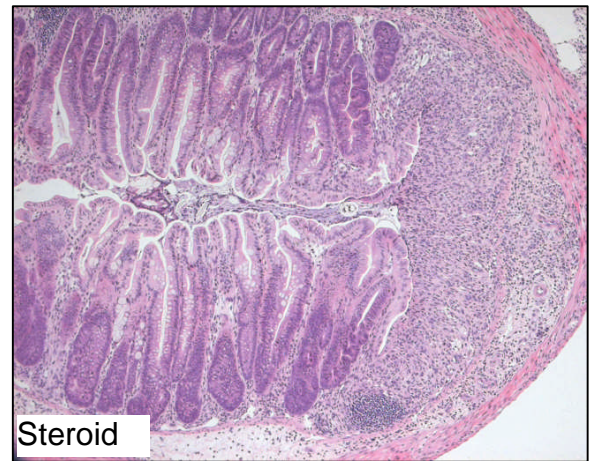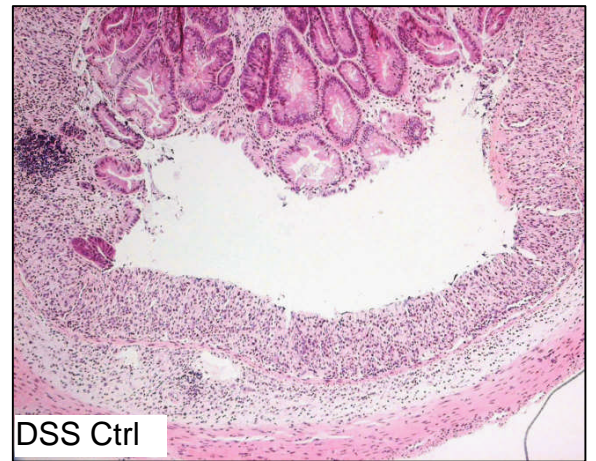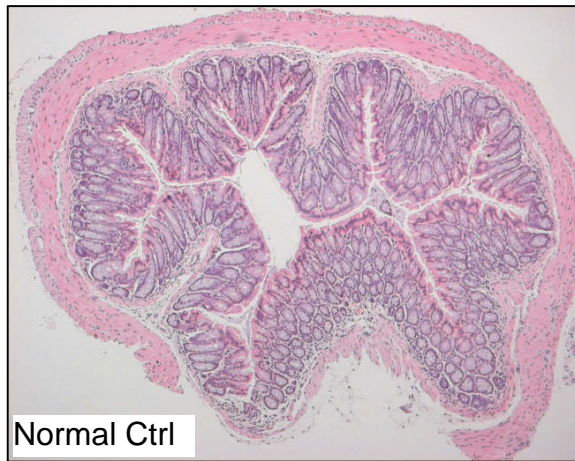
Figure 5.6   Histopathology of colitis in animals treated with BO-KGF and xylan. Representative photomicrographs of H&E stained sections of colon from normal healthy mice (Normal Ctrl) and from animals administered DSS alone (DSS Ctrl) or, DSS and treatment with wild type *B. ovatus* with xylan (BOV975+X), steroids (Steroids), KGF-producing *B. ovatus* in the presence (BO-KGF+X) or absence (BO-KGF-X) of xylan or TGF-producing *B. ovatus* in the presence (BO-TGF+X) or absence (BO-TGF-X) of xylan. Magnification, 100X. Colonic sections stained with H&E revealed severe crypt epithelium destruction, inflammatory cell infiltrates, submucosal oedema and severe goblet cells depletion in non-treated (DSS Ctrl), BO V975+X, BO-KGF-X and BO-TGF-X groups of mice. However, in colonic sections of mice treated with steroid, BO-KGF+X or BO-TGF+X, crypt regeneration and restoration of colonic mucosa were frequently observed and the inflammatory cellular infiltrate was limited and had a normal appearance.
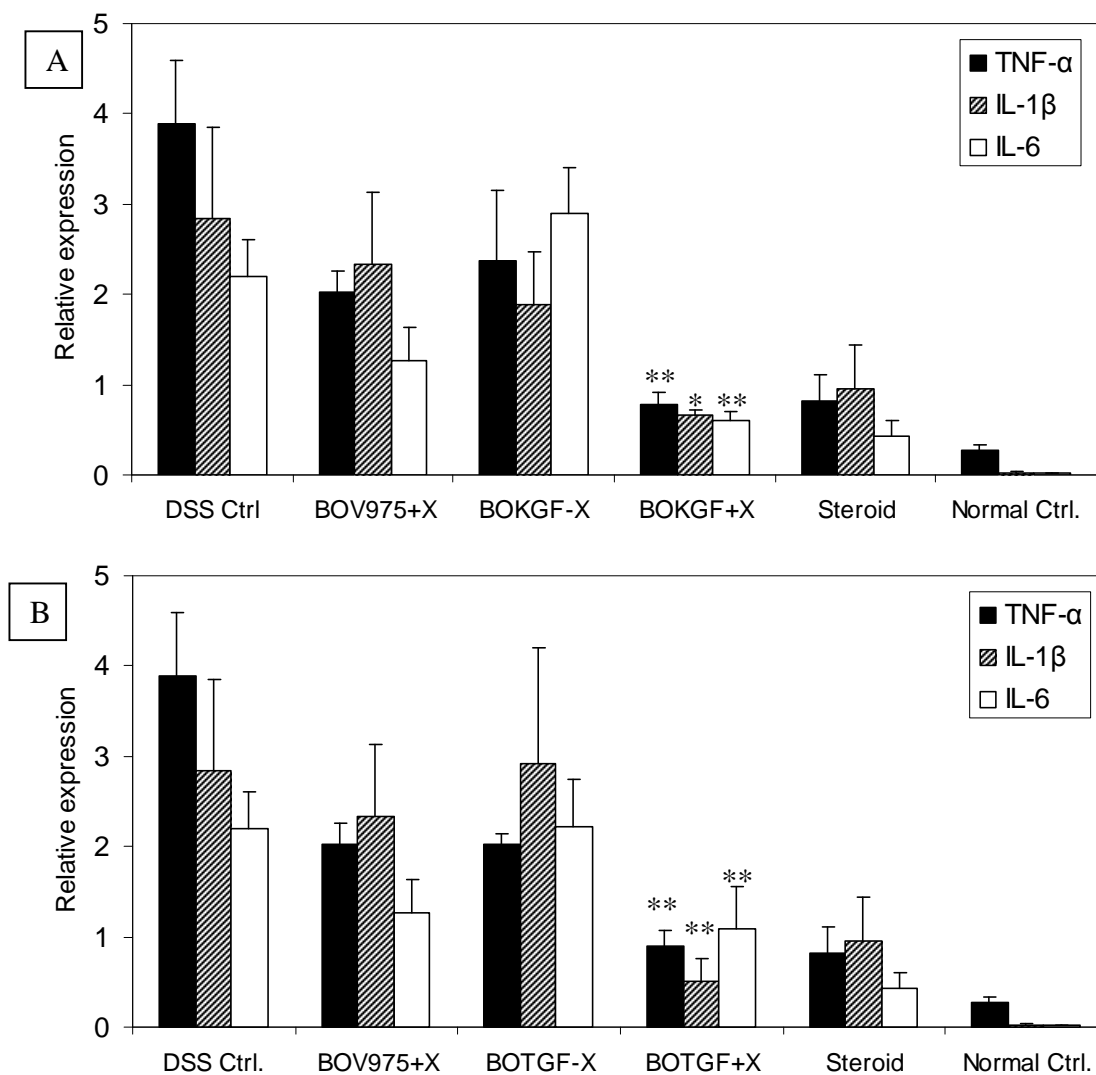
Figure 5.7   Effect of BO-KGF or BO-TGF and xylan treatment on colonic pro-inflammatory cytokine expression in DSS-induced colitis. The gene expression of the target molecule was determined by real-time PCR and was standardized against β-actin. Data are expressed as mean (±SEM) of RNA samples obtained from 6 animals in each group. **p<0.01 compared to DSS control, BO-KGF-X/BO-TGF-X and BOV975+X groups. *p<0.05 compared to DSS control and BO-KGF-X and BOV975+X groups.

The amount of mRNA encoding the pro-fibrogenic cytokine TGF-β_1 in the colonic mucosa of mice with DSS-induced colitis and treated with BO-KGF and xylan was significantly lower than DSS control mice and in mice treated with BO V975 and xylan ($p<0.01$) (Fig 5.8A). On the other hand treatment with xylan to BO-KGF colonized DSS-colitis mice has significantly upregulated intestinal trefoil factor (ITF) and reduced COX-2 mRNA levels as compared to DSS-control mice and mice treated with BO-KGF alone ($p<0.05$ and <0.01 respectively) (Fig 5.8B and C).

Levels of ITF mRNA was significantly increased and COX-2 was reduced by BO-TGF and xylan treatment ($p<0.05$) compared to non-treated DSS control mice, mice treated with BO-TGF alone and mice treated with BO V975 and xylan (Fig 5.9A and B)
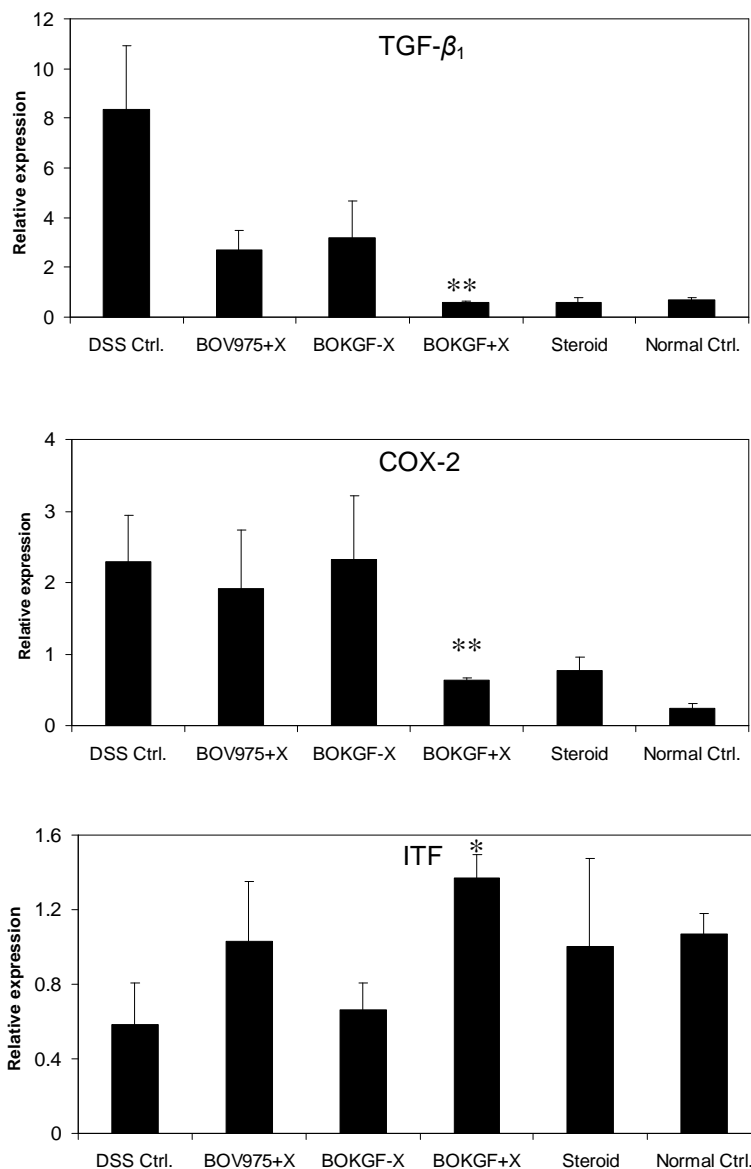
Figure 5.8   Effects of BO-KGF and xylan treatment on TGF-β_1, COX-2 and ITF transcript level in the colonic mucosa in DSS-induced colitis. The gene expression of the target molecule was determined by real-time PCR and was standardized against β-actin. Data are expressed as mean ($\pm$SEM) of RNA samples obtained from 6 animals in each group. **$p<0.01$ compared to DSS control, BO-KGF-X and BOV975+X groups. *$p<0.05$ to DSS control and BOKGF-X groups.
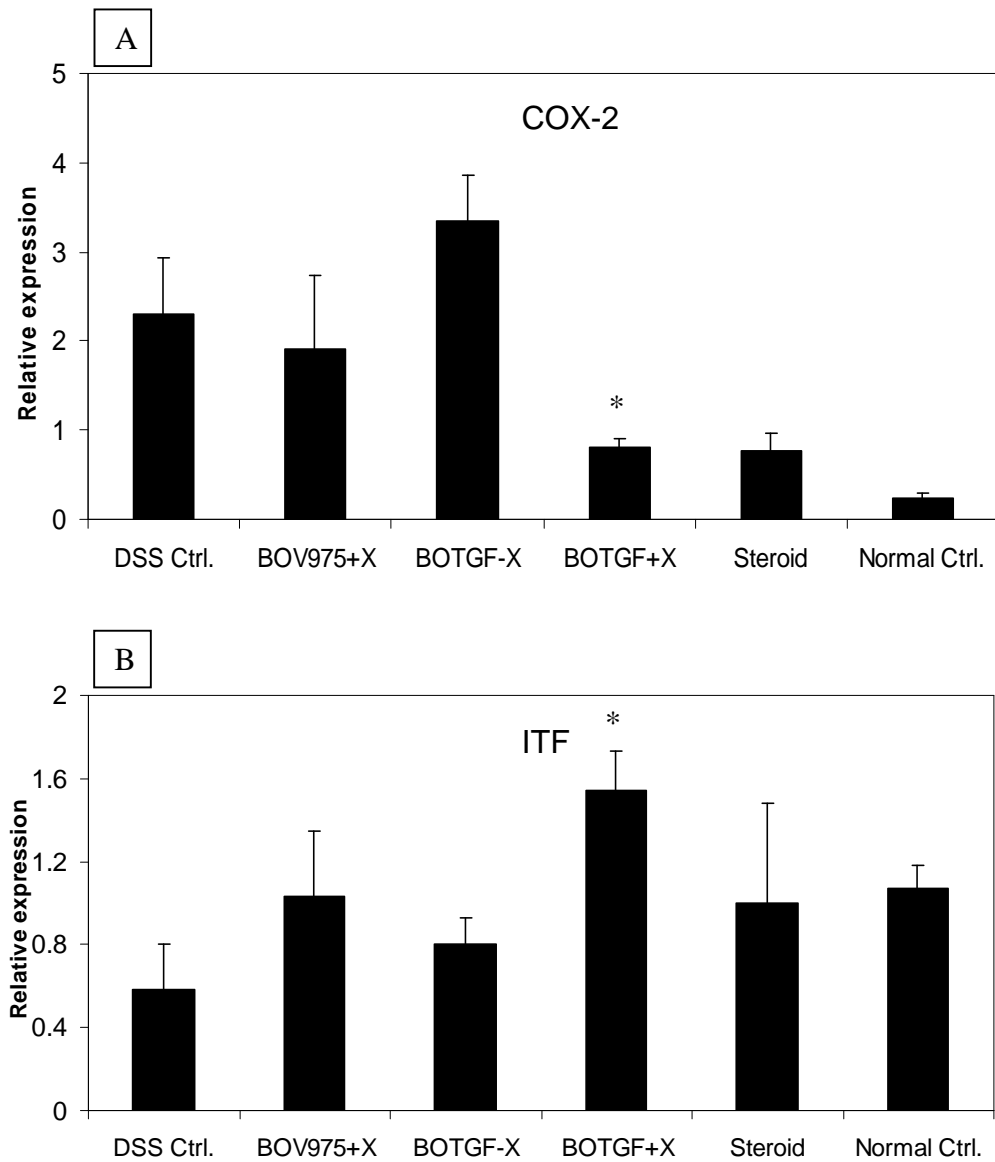
Figure 5.9   Effect of BO-TGF and xylan treatment on COX-2 and ITF transcript level in the colonic mucosa in DSS-induced colitis. The gene expression of the target molecule was determined by real-time PCR and was standardized against β-actin. Data are expressed as mean ($\pm$SEM) of RNA samples obtained from 6 animals in each group. *$p<0.05$ compared to DSS control, BO-TGF-X and BOV975+X groups.

5.4.2 Xylan controlled KGF-2 secretion by BOKGF promotes epithelial mitogenesis and mucin production

To determine if xylan induced KGF-2 secretion by BO-KGF influenced colonic epithelial cell proliferation, sections of colon were stained for Ki-67, a nuclear proliferation antigen. Compared to non-treated animals or animals receiving BO-KGF alone there was 30-40% increase in the number of Ki-67^{+} cells per crypt in animals treated with BO-KGF and xylan (p<0.05), consistent with increased levels of epithelial cell proliferation (Fig 5.10A and B). This increase in epithelial mitogenic activity was attributed to exogenous KGF-2 produced by BO-KGF rather than increased production of endogenous KGF-2 as KGF-2 mRNA expression in the colonic mucosa of BO-KGF treated was unchanged and comparable to that of non-treated animals (Fig 5.10C).

Mucin production was also increased as a result of BO-KGF and xylan treatment. There was a marked and significant increase in alcian blue-stained mucin-containing goblet cells in the colons of mice treated with BO-KGF and xylan compared to BO-KGF with no xylan treated mice, non-treated DSS control mice and healthy mice (p<0.001) (Fig 5.11A and B). Increased goblet cells is consistent with a beneficial effect on epithelial turnover and the upregulation of ITF mRNA levels in the colonic mucosa of BO-KGF and xylan treated animals (Fig 5.8C).
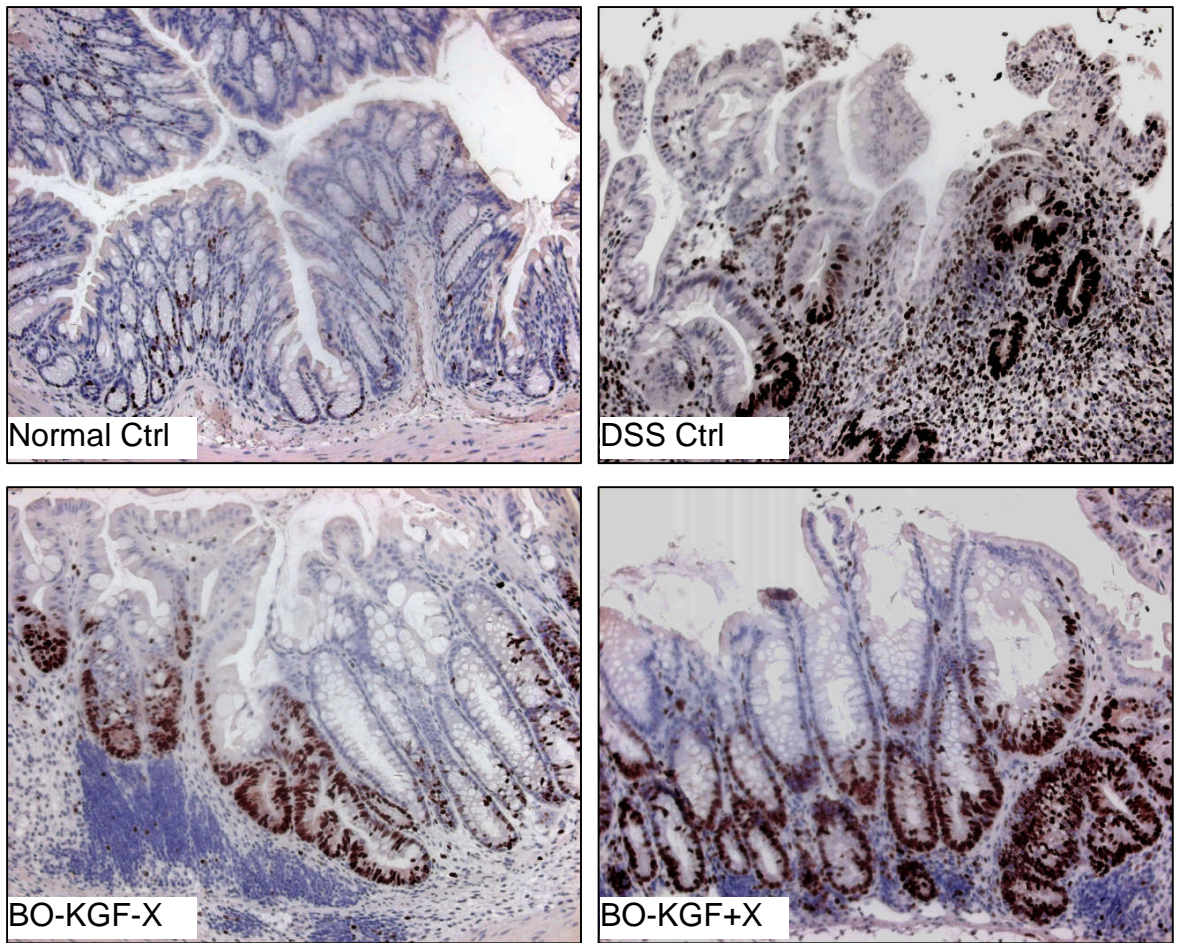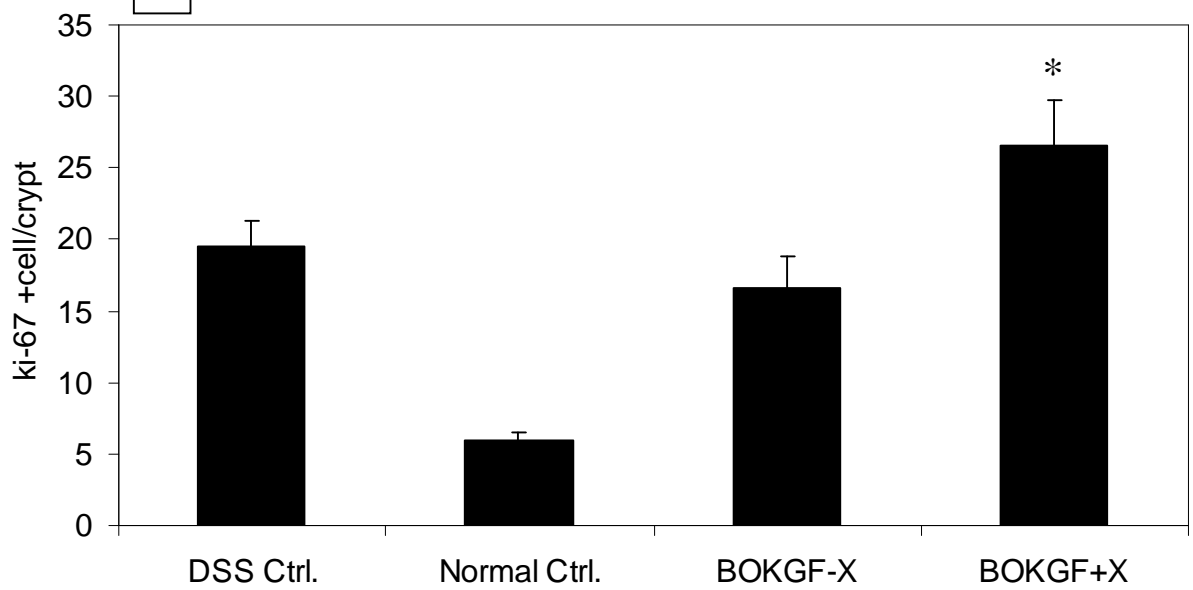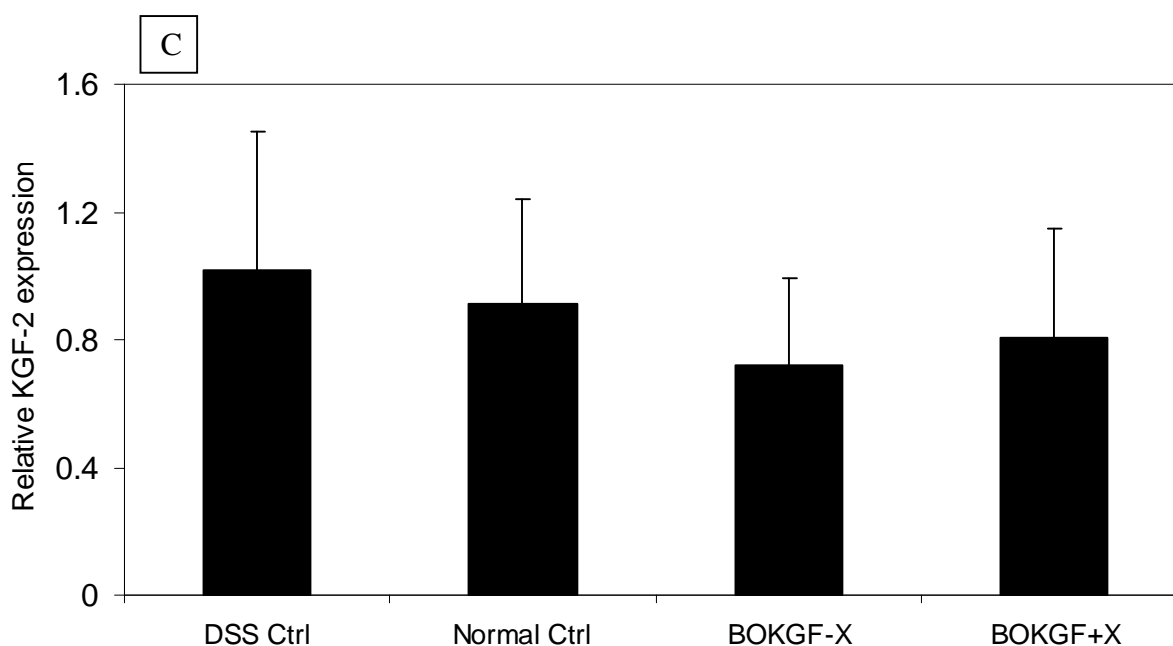
Figure 5.10   Effect of BO-KGF and xylan treatment on colonic epithelial cell proliferation and KGF-2 transcript level in DSS-induced colitis. DSS was administered to adult mice via drinking water for 5 days after which it was withdrawn and replaced with either normal drinking (-X) or water containing 30 mg ml^{-1} xylan (+X). At day 5, 7 and 9 post DSS-administration mice were gavaged with ~10^8 CFU BO-KGF. Control groups comprised animals that received no bacteria (DSS Ctrl) and normal healthy animals (Normal Ctrl); n= 8 n each group. (**A**) Colonic epithelial cell proliferation was assessed by staining sections of colon for ki-67 proliferation marker. The intensity of staining was more pronounce in the BO-KGF and xylan treated mice. Magnification 200X. (**B**) Ki-67$^+$ cells were enumerated by counting antibody-reactive cells in the crypt of at least 3 fields from each section and averaging the values obtained from 3 sections for each tissue sample. Data are expressed as means ± SEM. *p<0.05 comparing BO-KFG+X to DSS Ctrl and BO-KGF-X groups. (**C**) Endogenous KGF-2 mRNA level in the colonic tissues after DSS-induced colitis. The gene expression of the target molecule was determined by real-time PCR and was standardized against *β*-actin. Data are expressed as means ± SEM (n= 5 in each group). KGF-2 expression was not statistically different between the groups.
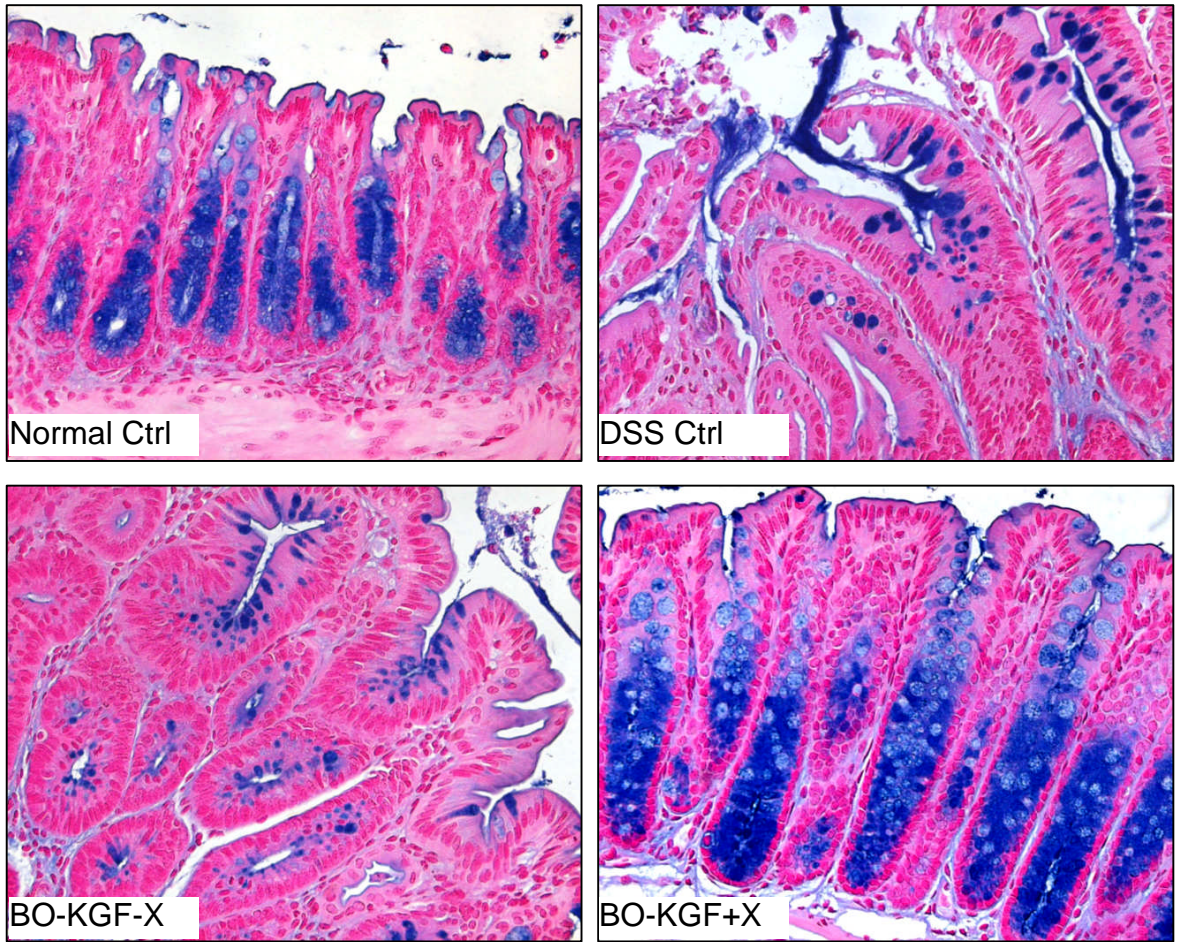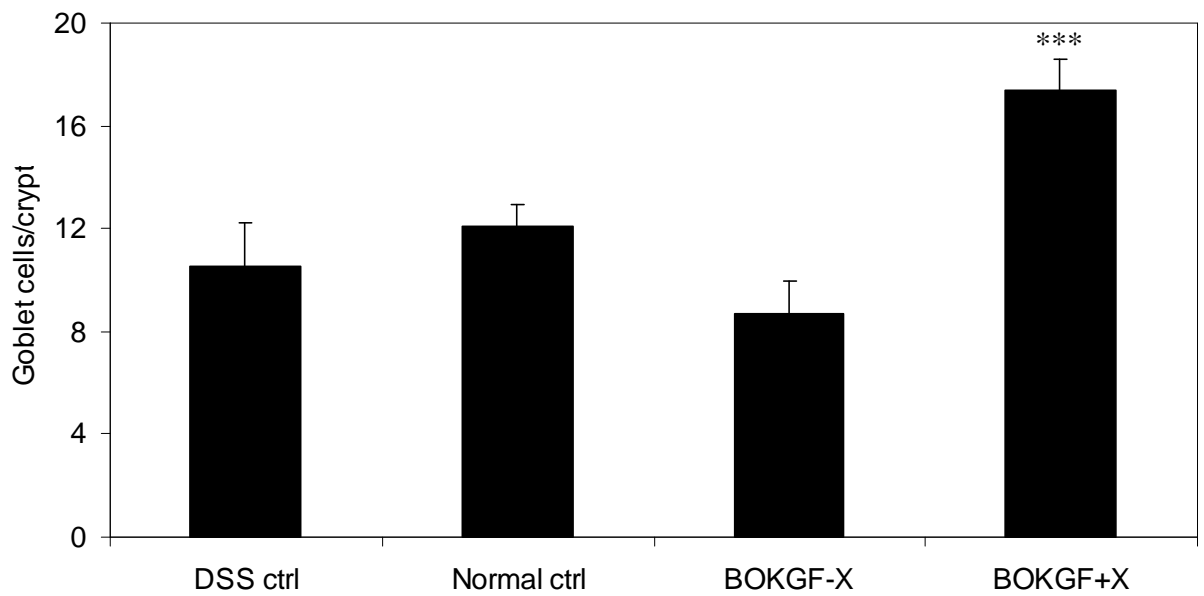
Figure 5.11 Effect of BO-KGF and xylan treatment on goblet cell regeneration in DSS-induced colitis. DSS was administered to adult mice via drinking water for 5 days after which it was withdrawn and replaced with either normal drinking (-X) or water containing 30 mg ml^{-1} xylan (+X). At day 5, 7 and 9 post DSS-administration mice were gavaged with ~10^8 CFU BO-KGF. Control groups comprised animals that received no bacteria (DSS Ctrl) and normal healthy animals (Normal Ctrl); n= 8in each group. (**A**) Mucin-producing goblet cells were identified in sections of colon by alcian blue staining and counterstaining with nuclear fast Red. The intensity of staining for acidic mucins (blue) was more pronounced in the BO-KGF and xylan treated mice and goblet cells extended along the entire length of the crypt. (**B**) Goblet cells were enumerated by counting the number of aclian blue stained cells per crypt in 3 fields of view from each section and averaging the values obtained from 3 sections for each tissue sample. Data are expressed as mean ± SEM. ***$p<0.001$ comparing BO-KGF+X to DSS Ctrl and BO-KGF-X groups.

5.4.3 BO-KGFand BO-TGF protect from DSS-colitis in a xylan controlled manner

BO-KGF: The ability of xylan-regulated KFG production by BO-KGF to affect the development of colitis was assessed by administering BO-KGF and xylan at the inception of DSS exposure. This dosing regimen produced a significant reduction in weight loss on days 2, 3 and 4 (2.7%) compared to non-treated animals (6.6%) and animals receiving BO-KGF alone (5.5%) or BO V975 and xylan (6.6%) ($p<0.05$) (Fig 5.12A).

BO-KGF and xylan treated animals had limited colon shortening as compared to BO-KGF alone, BO V975 and xylan and DSS control groups. The colons in the BO-KGF and xylan treated group (7.2 cm $\pm$ 0.08) were significantly longer than mice treated with BO-KGF alone (6.2 cm $\pm$ 0.2) (14%, $p<0.001$), mice treated with BO V975 and xylan (6.6 cm $\pm$ 0.2) (8%, $p<0.01$), non-treated DSS control group (6.2 cm $\pm$ 0.1) (14%, $p<0.001$) (Fig 5.13).

The level of neutrophil infiltration and activity, as determined by MPO activity, in the colon of BO-KGF and xylan treated animals was 70% less than in non-treated DSS controls and in animals treated with BO-KGF alone, and was 60% less than that seen in animals treated with BO V975 and xylan ($p<0.01$) (Fig 5.14A).

Histological analysis showed that treatment with BOKGF and xylan reduced depth and extent of crypt epithelium damage, acute and chronic inflammatory infiltrate and goblet cells depletion scores by more than 45% as compared with non-treated DSS control and mice treated with BO-KGF alone ($p<0.01$), histological score was 38% better than BO V975 and xyaln treated ($p<0.05$) (Fig 5.14B and 5.15).

Finally, TNF-α, IL-1β and IL-6 transcript levels were significantly lower in the colonic mucosa of BO-KGF and xylan treated animals compared to non-treated DSS controls animals, animals treated with BO V95 and xylan and BO-KGF alone ($p<0.05$) (Fig 5.16A). Collectively, these findings demonstrate that xylan-induced production of KGF-2 by BO-KGF has a prophylactic effect on the development of DSS-induced colitis.

BO-TGF: Likewise, BO-TGF and xylan treatment produced a significant effect on weight loss at days 2, 3 and 4 (2.4%) compared to non-treated animals and

animals receiving BO-TGF alone (7.2%) or BO V975 and xylan (p<0.05) (Fig 5.12B). Colons from BO-TGF and xylan treated animals were significantly longer than non-treated DSS control colons (6.8 cm ± 0.16 vs. 6.2 cm ± 0.1, p<0.01) and BO-TGF alone group (6.4 cm ± 0.07, p<0.01), it was longer than BO V975 and xylan treated animals (6.2 cm ± 0.18, p=0.5) but not significant (Fig 5.13A and B).

MPO activity, however, was not significantly reduced by BO-TGF and xylan treatment (p>0.05) (Fig 5.14A). Histological analysis showed that treatment with BO-TGF and xylan reduced epithelial damage, inflammatory infiltrate and goblet cells depletion scores by 35% as compared with BO-KGF alone group (p<0.05). The histology score was better than DSS control (>20%) and BO V975 with xylan treated mice (~10%) but was not statistically significant (p=0.09 and 0.644 respectively) (Fig 5.14B and 5.15).

Pro-inflammatory cytokines TNF-α and IL-1β transcript levels were significantly reduced by BO-TGF and xylan treatment as compared to other groups (p<0.05) and IL-6 transcript level was reduced as compared to non-treated DSS control (p<0.01) but not to BO V975 and xylan or BO-TGF alone treatment (Fig 5.16B).
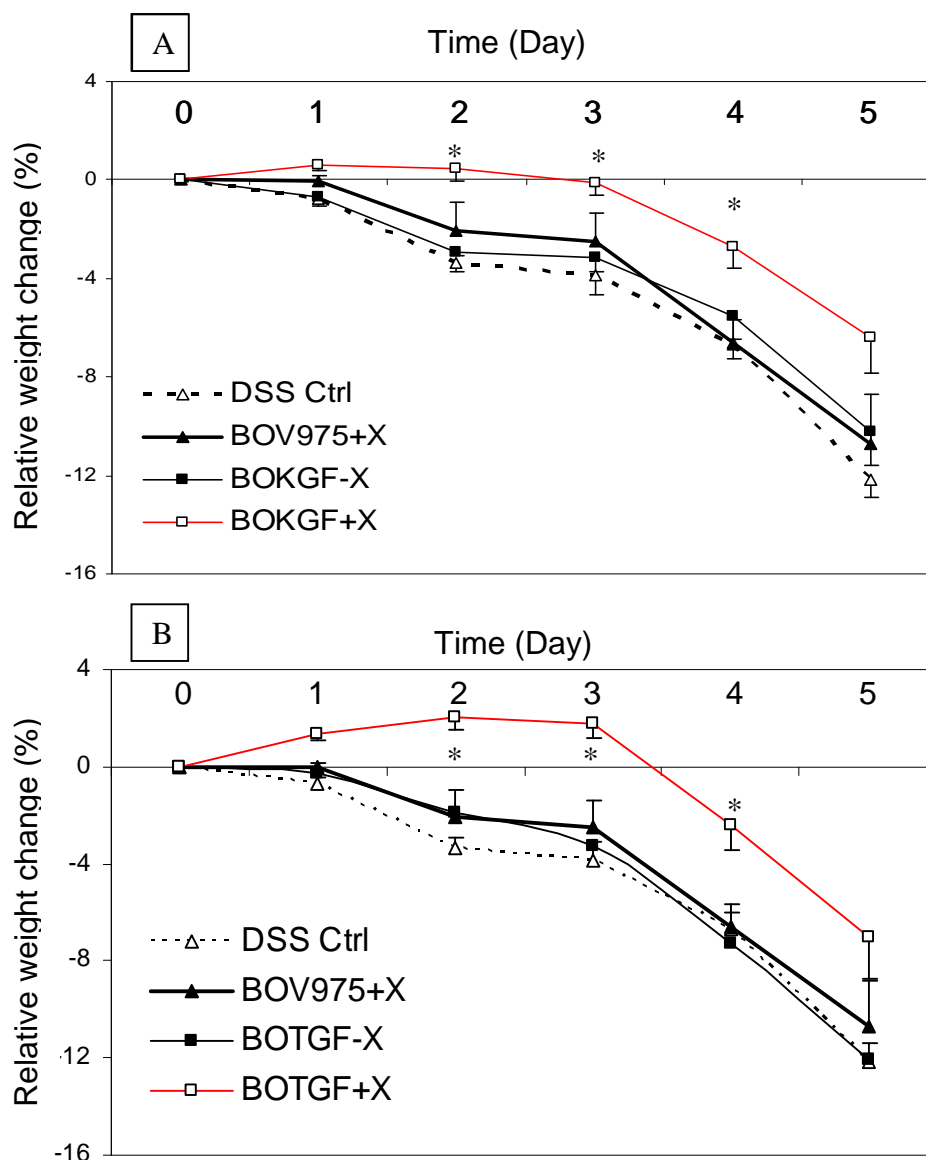
Figure 5.12   Prophylactic effect of BO-KGF (A) or BO-TGF (B) and xylan treatment on weight loss from DSS-colitis. At day 0 DSS was added to normal drinking water (-X) or water supplemented with 30 mg ml^{-1} xylan (+X) and continued for 5 days. Oro-gastric gavage of ~10^8 CFU of BO-KGF, BO-TGF or BO V975 was given at days 0, 2 and 4. Control groups comprised animals that received no bacteria (DSS Ctrl). Animals were sacrificed on day 5 (n= 8 in each group). Body weight was determined daily between day 0 and day 5. Data are expressed as mean ± SEM. *p<0.05 comparing BO-KGF+X or BO-TGF+X group with other groups.
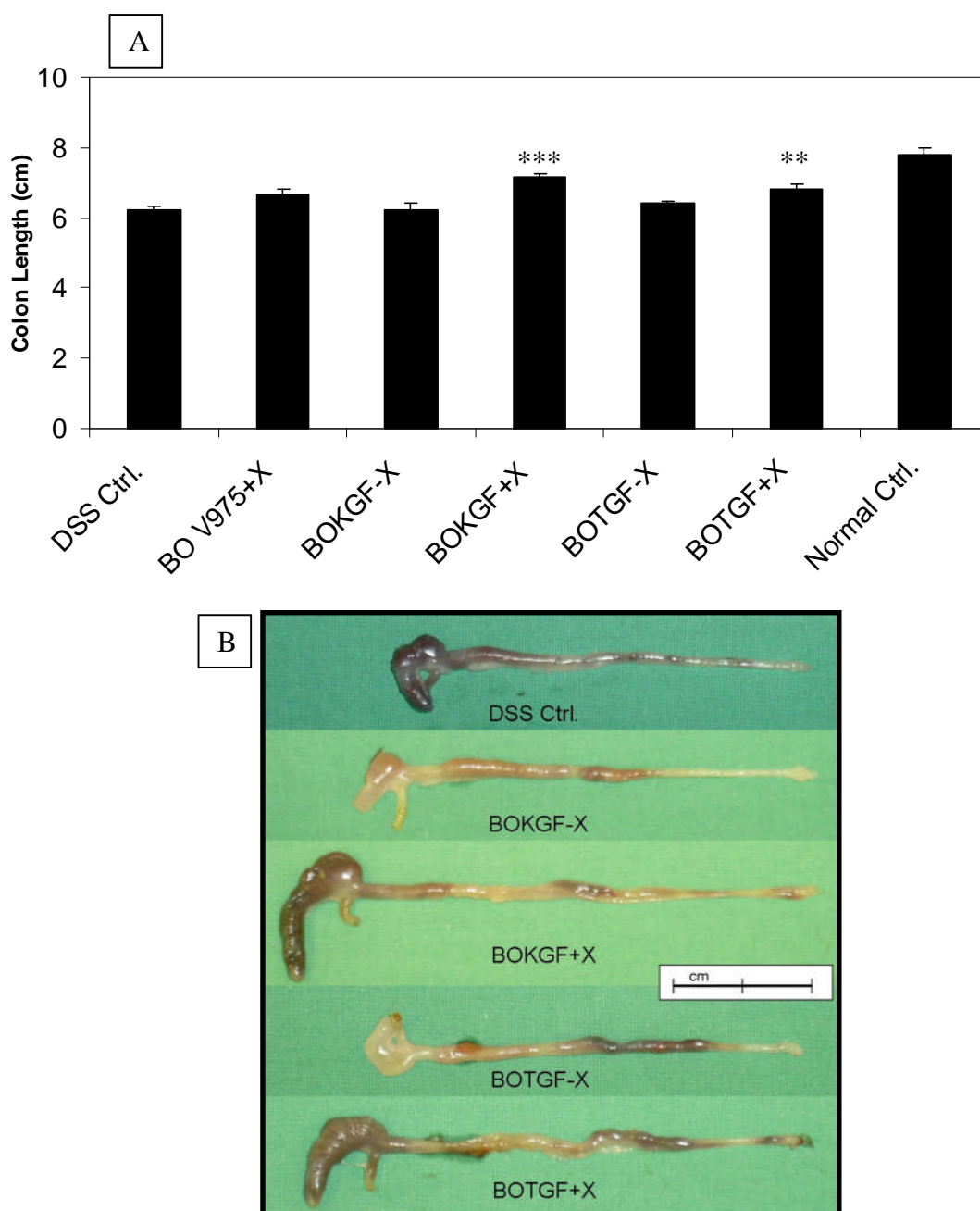
Figure 5.13 **Prophylactic effect of treatment with BO-KGF or BO-TGF and xylan on colon shortening associated with DSS-induced colitis. (A)** Colonic length was measured from ileocecal junction to the anal verge at necropsy (on day 5). Data are expressed as means ±SEM (n=8 in each group). ***p<0.001 compared to DSS Ctrl, BO V975+X and BO-KGF-X, **p<0.01 compared to DSS Ctrl and BO-TGF-X. **(B)** Representative macroscopic appearance of colons from prevention experiment. Colons treated with BO-KGF or BO-TGF but without xylan have shorter colons and grossly appeared more inflamed than colons from animals treated with recombinant *B. ovatus* and xyaln.
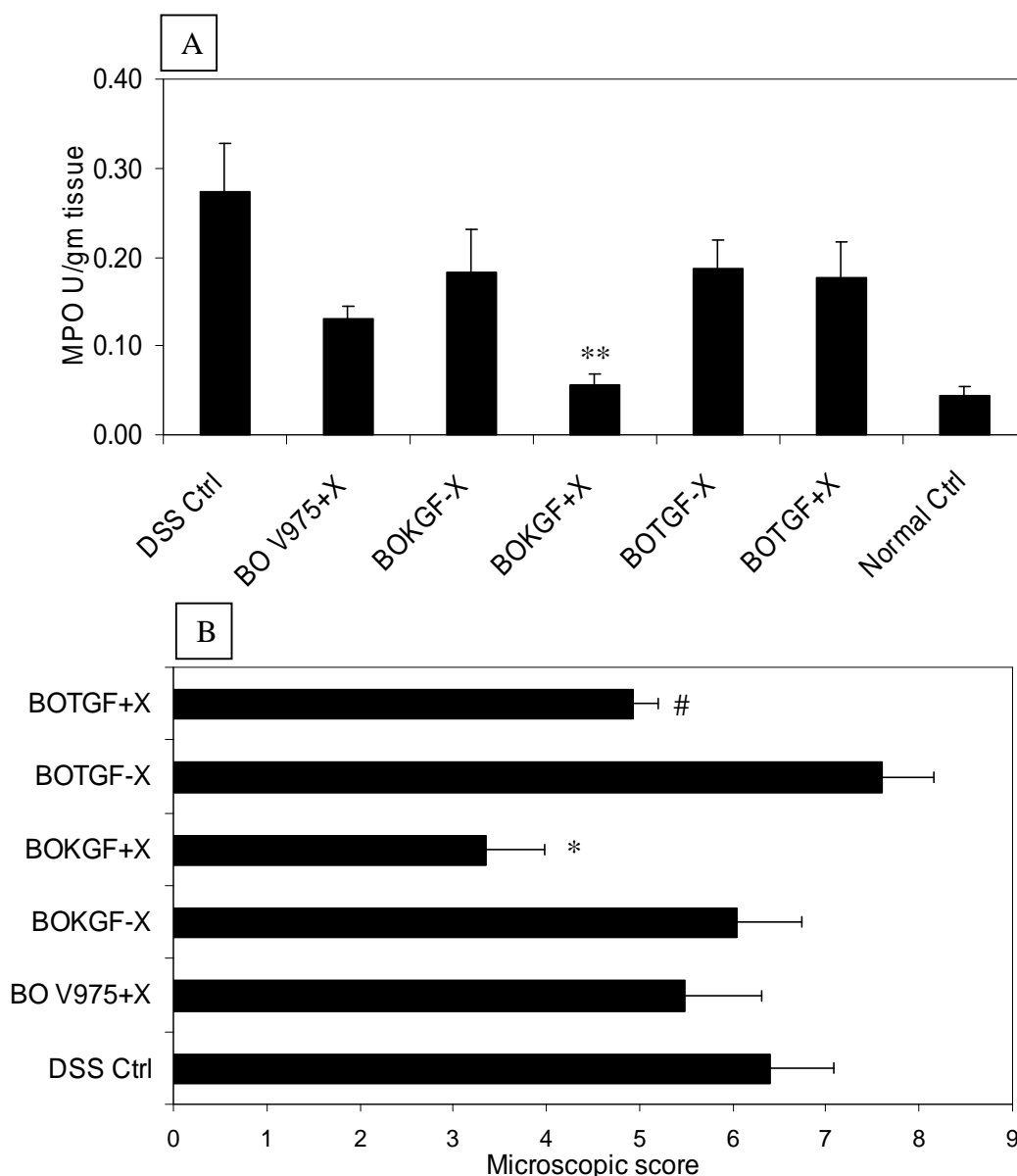
Figure 5.14   Prophylactic effect of treatment with BO-KGF or BO-TGF and xylan on colonic inflammation associated with DSS-induced colitis. (A) MPO activity assay. MPO activity in whole colonic homogenates are expressed as Units of activity per gram of tissue and represent the averaged values (±SEM) from 8 animals. **p<0.01 comparing BO-KGF+X group with DSS Ctrl, BOV975 and BO-KGF-X groups. **(B) Histopathological scores**. The score was based on cumulative scores for depth and extent of crypt injury, acute and chronic inflammatory infiltrate and goblet cell depletion seen in sections of colon at autopsy. Data are expressed as means ± SEM from 8 animals. *p<0.05 compared to DSS Ctrl, BO V975+X and BO-KGF-X. #p<0.05 compared to BO-TGF-X group.
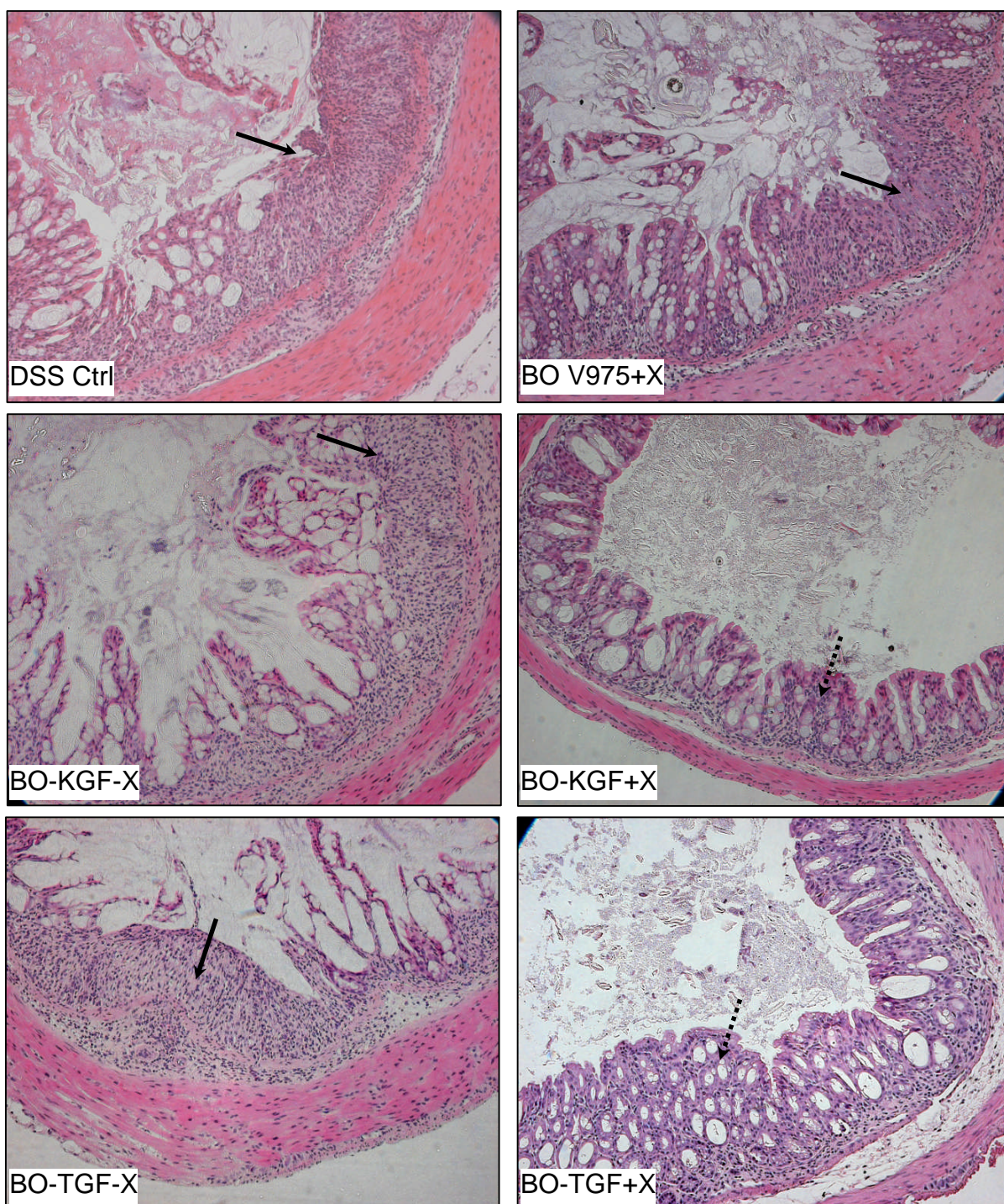
Figure 5.15   Histopathology of prophylactic effect BO-KGF or BO-TGF and xylan treatment on the development of DSS-colitis. Photomicrographs of H&E stained sections of colon from animals treated with BO-KGF or BO-TGF and xylan (+X) or with out xylan (-X) at the inception of DSS exposure. Control groups have DSS alone (DSS Ctrl) or, DSS and treated with BO V975 and xylan (BOV975+X), Magnification, 100X. Areas of crypt damage and inflammatory infiltrate pointed by **solid arrows**. Less inflammatory cell infltarte and crypt damage seen in the BO-KGF or BO-TGF and xylan treated mice, **interrupted arrows**.
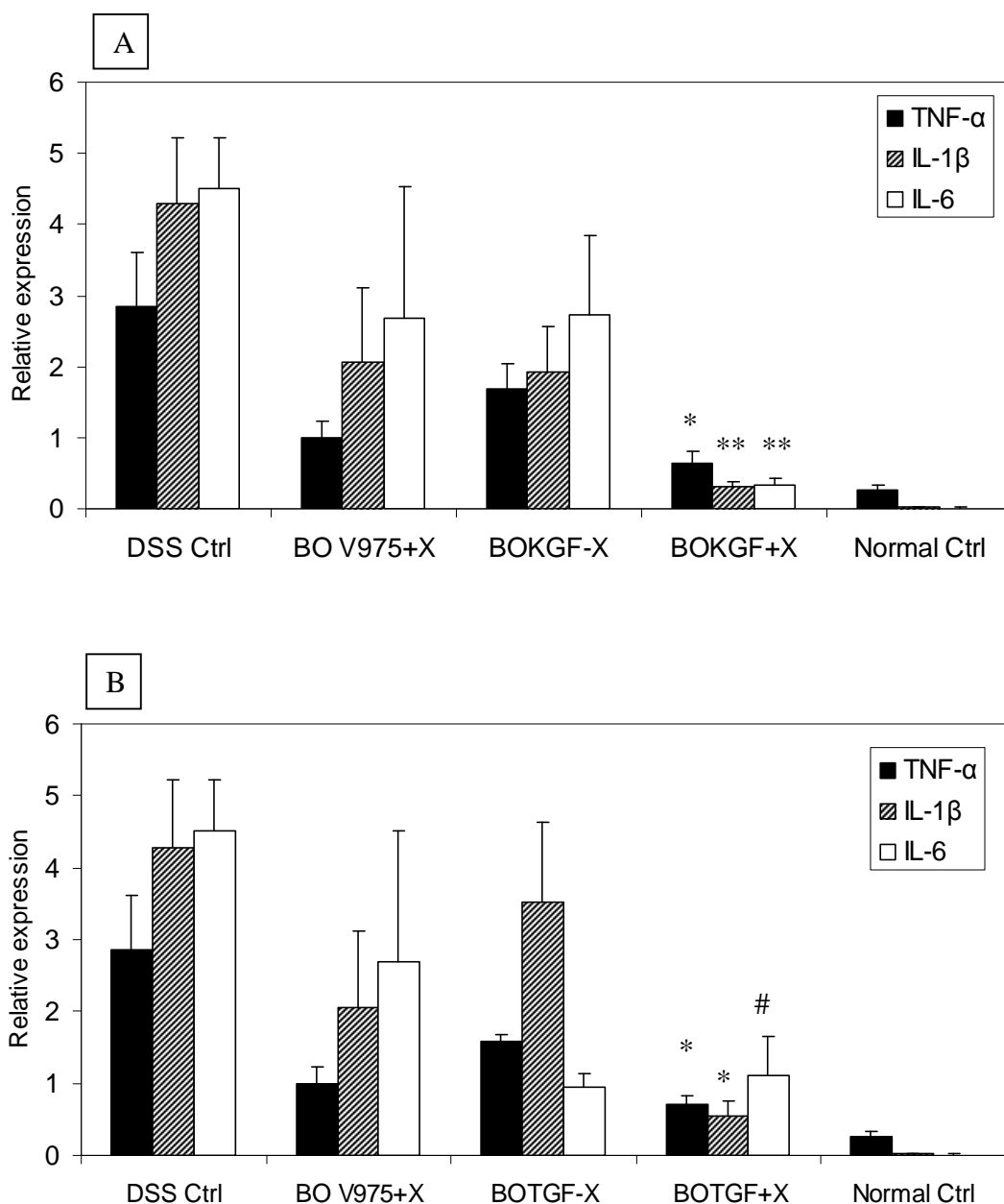
Figure 5.16 Prophylactic effect of BO-KGF (A) or BO-TGF (B) and xylan treatment on pro-inflammatory cytokines transcript levels. The level of TNF-α, IL-1β and IL-6 mRNA in the colonic mucosa at day 5 was determined by real-time PCR and by standardising values against β-actin. Data are expressed as means (±SEM) of RNA samples obtained from 5 animals in each group. *p<0.05 **p<0.01, comparing BO-KGF+X group with DSS Ctrl, BOV975+X and BO-KGF-X groups. #p<0.01 compared to DSS Ctrl group.

5.5   Discussion

In this chapter, recombinant strains of *B. ovatus* producing biologically active growth factors under the control of the plant polysaccharide xylan were evaluated for their therapeutic and prophylactic effects on acute DSS-induced colitis in mice. Continuous administration of xylan to BO-KGF or BO-TGF treated mice resulted in significant improvement of colitis; reducing weight loss, improving stool consistency, reducing rectal bleeding, accelerating healing of damaged colonic epithelium, reducing inflammatory cell and neutrophil infiltration, reducing expression of pro-inflammatory cytokines and, promoting production of mucin-rich goblet cells in colonic crypts. These beneficial effects are comparable and in some cases better than that achieved by conventional steroid therapy. BO-KFG/BO-TGF and xylan treatment also had a significant prophylactic effect, limiting the development of intestinal inflammation both clinically and histopathologically. The use of genetically engineered *B. ovatus* for the controlled and localised delivery of epithelial growth promoting and immunomodulatory proteins has potential clinical applications for the treatment of various diseases targeting the colon including IBD. Regulation of heterologous protein production by recombinant *B. ovatus* using xylan is a novel and important safety feature of this drug delivery system. The recent identification of the *B. ovatus* xylan operon promoter (Chapter 3) (Hamady *et al.*, 2008) enabled human cytokine-producing strains to be generated.

The *Bacteroides* are dominant among commensal anaerobes and have been identified within the mucin layer coating the colonic mucosa (Croucher *et al.*, 1983) making them ideal for therapeutic protein delivery directly to the injured epithelium. The ability of the *Bacteroides* to utilize xylan as its sole energy source (Salyers *et al.*, 1981; Whitehead & Hespell, 1990) and the inability of the human gut to digest xylan (Cummings *et al.*, 2001) probably contributes to their predominance in the colonic microflora. It is likely that BO-KGF and BO-TGF delivers growth factors directly to the epithelium via diffusion or by receptors expressed on the basolateral surface of epithelial cells (Murphy *et al.*, 2004; Visco *et al.*, 2004) made accessible as a result of compromised barrier function and damaged epithelium in the inflamed colon. Part of the growth factor produced by BO-KGF and BO-TGF in the inflamed colon may also result from its release within the mucosa and lamina propria as a

result of bacterial translocation across damaged or denuded regions of epithelium (Saitoh *et al.*, 2002).

The prebiotic effect of xylan (Moure *et al.*, 2006) may also contribute to the therapeutic effect of BO-KGF and BO-TGF and xylan treatment (synbiotic) and help explain its beneficial affects in combination with wild type *B. ovatus* (BO V975) on DSS-colitis, in particular on reducing inflammatory cytokine expression (Figure 5.7 and 5.16). An added advantage of using *B. ovatus* as a drug delivery vehicle is its anaerobic nature that provides an important inbuilt biosafety feature that is an attribute lacking in other recombinant bacteria currently in use including *L. lactis* (Steidler *et al.*, 2003). Also, the concern of gene transfer and containment that is important in recombinant bacterial expression systems relying on extrachromosomal plasmids for expression of heterologous genes is much less of an issue with recombinant *Bacteroides* in which the growth factor gene is integrated into the bacterial chromosome.

KGF-2 and TGF-β are potent epithelial growth factors able to ameliorate colitis and promote healing of intestinal ulcerations when given systemically (Han *et al.*, 2000; Neurath *et al.*, 1996). However, due to protein instability in the acid- and protease-rich environment of the upper gastrointestinal tract, it is difficult to achieve therapeutic levels of these growth factors in the injured colon when given orally. In addition, bolus administration does not keep the protein localised necessitating repeated dosing with large amounts of the growth factor that may have dangerous side effects, such as vascularisation of non-target tissues or growth of tumours (Epstein *et al.*, 2001). Furthermore, active TGF-β has a short half-life (2-3 minutes) (Wakefield *et al.*, 1990), make it necessary for recombinant cytokine to be given frequent enough to replenish the level in the circulation every few minutes. The short biological half-life, lack of tissue-selectivity and the risk of carcinogenesis demand therefore, temporal and spatial control of growth factor delivery. As shown this is achievable through the delivery by *B. ovatus* controlled by the addition of xylan to the drinking water leading to enhanced epithelial cell proliferation and repair and, goblet cell production. The benefit of delivering KGF via *B. ovatus* versus a needle is evident in comparing the amount of cytokine required to lower mucosal MPO activity, which is an important inflammatory marker. Repeated systemic administration of 1 mg kg^{-1} over a 5 to 8 day period (20-100 µg animal^{-1}

day^{-1}) of recombinant KGF-2 reduces colonic mucosal MPO activity by up to 40% (Miceli *et al.*, 1999) which is still less than that achieved by BO-KGF (65%) producing ~5ng over a 24 h period in the presence of xylan (Fig 4.8). Therefore, with this system the protein is delivered in smaller doses which are unlikely to cause side effects.

Xylan induced KGF-2 production by *B. ovatus* has enhanced colonic epithelial cells proliferation. This was shown in this study by the increase in the number of Ki-67 positive cells per crypt after treatment of DSS-induced colitis. In the past, it has been difficult to verify intestinal cell proliferation *in vivo* in the DSS-colitis because the background level of colonic proliferation is high. However, an increase in the number of proliferating cells above this background level was shown in this study.

KGF may also act indirectly by increasing the production of other epithelial growth factors (Taupin & Podolsky, 2003). *B. ovatus*-delivered KGF appears to have other indirect effects on mucosal cells other than epithelial cells in the inflamed colon. The reduction in expression of pro-inflammatory cytokines that are mechanistically linked to DSS-colitis (Islam *et al.*, 2008; Jurjus *et al.*, 2004) in BO-KGF and xylan treated animals, in the absence of any evidence that KGF has direct effects on haematopoietic and immune cells, is an indirect effect and may be linked to improved epithelial barrier function and reduced exposure of underlying cells to enteric antigens. On the other hand, BO-TGF and xylan induced reduction of pro-inflammtory cytokines could be a result of direct anti-inflammatory effect of TGF-β and/or resumption of barrier function as an effect of restitution induced by TGF-β.

Cyclooxgenase (COX-2) catalyzes the first step in the synthesis of prostaglandins, which are "cytoprotective" in the gastrointestinal tract and one of the key molecules involved in the mucosal repair process (Simmons *et al.*, 2004). COX-2 mediated prostaglandin production contributes to the protection of colonic injury, and the inhibition of pro-inflammatory cytokine production by activated macrophages (Cohn *et al.*, 1997; Morteau *et al.*, 2000). However, data presented in this chapter showed that gene expression of COX-2 in mice treated with recombinant *B. ovatus* and xylan was lower than non-treated mice. These data are consistent with other anti-inflammatory therapies such as gamma-oryzanol, hrbFGF and curcumin which have significantly reduced COX-2 expression as well in DSS-

induced colitis (Camacho-Barquero *et al.*, 2007; Islam *et al.*, 2008; Matsuura *et al.*, 2005). These results were probably due to reduced inflammation in the colonic tissues of mice with DSS-colitis by xylan induced KGF-2 and TGF-β treatment leading to reduced COX-2 expression. Furthermore, expression of goblet cell-derived intestinal trefoil factor (ITF), which is a potent inducer of epithelial cell restitution (Taupin & Podolsky, 2003), was up-regulated by BO-KGF or BO-TGF and xylan treatment, which may be an added indirect mechanism for improved barrier function and reduced inflammation. The reduced expression of TGF-β_1 in the colonic mucosa of BO-KGF and xylan treated animals is of note considering that levels of this cytokine are used as a biomarker for IBD activity (Wiercińska-Drapało *et al.*, 2001). The reduced expression of TGF-β in BO-KGF and xylan treated animals is therefore, another indicator of successful treatment of the disease.

In summary, we have utilized a prominent member of the human colonic micorbiota for the controlled delivery of an epithelial growth factor for the effective therapeutic and prophylactic treatment of acute colitis. The results are promising and this approach may in the future be used to treat acute IBD as an additive or an alternative to current regimen. This drug delivery system may also have applications in the treatment of other bowel disorders.

CHAPTER (6)

DISCUSSION

Inflammatory Bowel Disease (IBD) is a group of disabling diseases affecting 0.2% of the population in developed countries. Current therapy is restricted to anti-inflammatory and immuno-suppressive drugs, which are not curative, and can cause destructive side effects. Soluble growth factors such as KGF and TGF-β are of interest as potential therapeutic agents for IBD as they can improve the intestinal barrier function and help maintain normal intestinal homeostasis to commensal luminal enteric bacteria, (Haller *et al.*, 2003; Zeeh *et al.*, 1996). When, administered orally, however, as recombinant proteins they are unstable and systemic administration requires high doses which increases the risk from unwanted side effects. Furthermore, these proteins have a short half-life (Wakefield *et al.*, 1990). In consideration of these problems alternative means of delivery have been considered of which delivery via live microorganisms has shown real promise. A strain of food-grade *L. lactis* engineered to secrete the anti-inflammatory cytokine IL-10 has been used to successfully treat murine DSS-colitis and spontaneously developed colitis in IL10-deficient mice (Steidler *et al.*, 2000).

In considering the application of this approach to treat patients several issues need to be addressed. First, *L. lactis* is not a human commensal bacterium and requires repeated administrations to maintain therapeutic levels of cytokine in the intestine. Second, cytokine production by *L. lactis* is constitutive and cannot be regulated increasing the risk of adverse effects. Third, the use of genetically modified probiotics (GMP) in medicine raises legitimate concerns concerning biosafety and environmental contamination. To address these issues and to further develop gut bacteria delivery systems, the work reported in this thesis has described the development of a novel second generation of GMP. The human commensal colonic Gram negative, anaerobic, *Bacteroides ovatus* has been genetically engineered to produce and secrete mammalian cytokines under the control of the xylanase promoter, which is activated in the presence of xylan.

The utility of this live bacteria-based drug delivery system was demonstrated by creating strains of recombinant *B. ovatus* that secrete KGF-2 or TGF-β_1 (BO-KGF or BO-TGF, respectively). The generation of these strains was made possible

by identification of the xylanase operon promoter. The xylanase promoter was cloned upstream of the *kgf* or *tgf* genes in a translation fusion manner utilizing *B. fragilis* enterotoxin secretion signals to facilitate secretion of the translated protein. BO-KGF and BO-TGF were shown to produce biologically active protein in the presence of xylan. Finally, these strains were utilized to treat and prevent acute murine DSS-colitis. Continuous administration of xylan to BO-KGF or BO-TGF treated mice resulted in significant improvement of colitis that was comparable and in some cases better than that achieved by conventional steroid therapy.

There are still, however, unanswered questions. Firstly, can the cytokines produced by *B. ovatus* cross the epithelial barrier into the systemic circulation? It is expected that some of the protein may be present in the circulation due to damaged/denuded epithelium in colitis. In addition, these proteins are relatively small and may cross via pinocytotic uptake or by receptor-mediated uptake. However, the amount of *Bacteroides* secreted recombinant protein in the colon is small compared to the amounts administered systematically to treat colitis (Chapter 5). It has been shown that recombinant IL-10 produced *in vivo* by *L. lactis* is taken up by the paracellular route in inflamed mucosal tissue (Waeytens *et al.*, 2008). This can improve the local action of recombinant protein in inflamed tissue and the efficiency of the treatment. This small amount of bacteria-delivered cytokine is not expected to induce unwanted side effects. On the other hand, it would be interesting to know if any recombinant cytokine present in the systemic circulation can have a therapeutic effect on systemic disorders and inflammation in other organs such as the liver.

Secondly, what is the optimal way of administering the recombinant bacteria to patients? Probiotic strains VSL#3 (mixture of 8 aerobic and anaerobic strains) is given as lyophilised powder (Chapman *et al.*, 2007). It is not clear if some of the VSL#3 preparation looses its viability during the preparation and/or administration. The GMP *L. lactis* had a significant reduction in viability after freeze-drying (Huyghebaert *et al.*, 2005) necessitating further genetic manipulation to increase their resistance to freeze-drying, bile toxicity and gastric acid (Termont *et al.*, 2006). Will lyophilisation be efficient in the case of *Bacteroides* given its anaerobic nature? Indications from animal experiments are that *B. ovatus* can survive transit through stomach and small intestine in sufficient numbers to have a therapeutic

effect in the colon. Furthermore, the dose required to achieve colonisation of the human colon or the frequency of dosage and whether there is a need for booster dose to keep the level of recombinant *Bacteroides* at therapeutic range needs to be established.

Thirdly, there are obvious ethical and public concerns about the use of GM products and bacteria specifically. Although GM *L. lactis* was used in a phase I trial in human (Braat *et al.*, 2006), it may be still a challenge to convince the ethical committees for approval to use GM *Bacteroides* in humans. In relation to biosafety, it has been shown that incorporation of the cloned gene into chromosomal DNA of *Bacteroides* make the transgene extremely stable with no demonstrated loss over 60 generations preventing lateral gene transfer. In contrast, plasmid-born transgenes are almost completely lost by 60 generations in the absence of antibiotic selection (Whitehead *et al.*, 1991). However, it may still be necessary to confirm the stability of *B. ovatus* transgene and the possibility of transfer to other bacteria. In addition, it will be important to identify the fate of the recombinant organisms in human faeces after being discharged to sewage as this condition will be different to the laboratory tested conditions in this work. In the future, optimisation of biosafety mechanisms may be needed to make recombinant *B. ovatus* more vulnerable when exposed to environment. For example, by targeting the superoxide dismutase (*sod*) gene to make the strains more susceptible to oxygen. *sod*-deficient mutants of aerotolerant *Bacteroides* exhibit a rapid viability loss immediately after exposure to air (Nakayama, 1994). Catalase is another target gene to induce rapid oxidative DNA damage in anaerobes (Takeuchi *et al.*, 1999). In addition, deletion or inactivation of the antibiotic selection markers in the recombinant strains could be another biosafety issue. However, one may argue that the antibiotic resistance genes used in this study are already prominent in more than 80% of the human colonic *Bacteroides* habitants (Shoemaker *et al.*, 2001).

The recombinant *Bacteroides* strains generated in this study were applied in a single model of acute colitis. A weaker therapeutic effect of BO-TGF strain was seen compared to BO-KGF (chapter 5). This may be due to the fact that TGF-β is more likely to affect chronic disease because of its action as a potent growth inhibitor of haematopoietic cells and lymphocytes as well as epithelial cells (Becker *et al.*, 2006). Initiation of epithelial regeneration is an important initial step towards

healing of colitis. In addition, variability in different colitis models has been previously noted with anti-TNF antibody therapy which showed little effect in acute model and more pronounced effects in chronic model of DSS-colitis (Kojouharoff *et al.*, 1997). Probiotic strains VSL#3 also had variable effects in different models of colitis (Shibolet *et al.*, 2002). Therefore, it may be useful to use different model of colitis to verify the therapeutic effect of KGF-2 and TGF-β delivered by *B. ovatus*. However, none of the models described in the literature are exact replicates of human IBD.

The well-characterised ability of TGF-β to promote wound healing, as well as its potent immunosuppressive effects (Miyazono, 2000; Sporn *et al.*, 1983) have provided the rationale for its therapeutic use in colitis. Intranasal administration of a plasmid encoding TGF-β_1 prevents the development of Th1-mediated experimental colitis (Kitani *et al.*, 2000). The downside of TGF-β however, is its fibrogenic effect. TGF-β1 has been implicated in certain fibrotic disorders such as such as pulmonary fibrosis, liver cirrhosis (Sanderson *et al.*, 1995) and indeed scar formation after wound healing. This scarring was reduced after TGF-β_1 neutralisation or after the exogenous addition of TGF-β_3 to cutaneous rat wounds (Shah *et al.*, 1995). Increased TGF-β_3 expression by inflamed ulcerative colitis myofibroblasts may have protective effect in this disease against fibrosis and stricture formation compared to reduced TGF-β_3 in Crohn's disease (McKaig *et al.*, 2002). Hence, TGF-β_3 may act as the anti-inflammatory non-fibrogenic member of TGF-β family. Therefore, TGF-β_3 could substitute for TGF-β_1 in treating colitis. TGF-β_1 however, is 100-fold more potent growth inhibitor of haematopoietic stem cells than TGF-β_2 and TGF-β_3 (Graycar *et al.*, 1989).

By introducing various other genes into the cassette that was created in this study it is possible to produce different strains of recombinant *B. ovatus* producing factors or cytokines to attack different illnesses. For example, our group is now working on strains that produce endostatin (anti-angiogenic factor) to treat colorectal cancer. A strain that can produce pancreatic enzymes will be novel treatment to patients with chronic pancreatitis, as well a strain that produces absorbable insulin to treat diabetes in human.

In conclusion, this study has demonstrated a novel mucosal drug delivery system. *B. ovatus* has been utilized to secrete biologically active growth factor under

the control of plant polysaccharide xyaln. Xylan administration to mice treated with BO-KGF or BO-TGF protected them from DSS-induced colitis and accelerated the healing of established colitis. These beneficial effects are comparable and in most cases superior to that achieved by conventional steroid therapy. This study supports the use of growth factors delivered by *B. ovatus* as a possible candidate in the prevention and treatment of human IBD. It provides new insights into the efficacy of KGF-2 or TGF-β in suppressing acute mucosal inflammation and efforts should now be directed to evaluate possible beneficial effect in inflamed human colons.

APPENDIX 1

BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

1. Bacterial strains used in this study

Strain	Genotype	Reference/Source
E. coli DH5α	*supE*44 Δ*lac*U169 (ø80*lac*ZΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(Hanahan, 1983)
E. coli J53/R751	F⁻ lac⁺ pro met nal Tpʳ; Contains self-transmissible IncPβ plasmid, R751	(Meyer & Shapiro, 1980)
E. coli HB101	F⁻ hsd S20 (r_b^-,m_b^-)leu B6 rec A13 ara-14 pro A2 lac Y1 thi-1 gal K2 rpsL 20 xyl-5 mtl-1 sup E44	(Boyer & Roulland-dussoix, 1969)
B. ovatus V975	Natural rifampicin resistant mutant of BO 0083	(Whitehead *et al*, 1990)

2.   Plasmids used in this study

Plasmid		Relevant characteristics	Source
pOX1	Ap^r	3.8 kb *Eco*R1 fragment of xylanase operon cloned in pUC18	(Whitehead *et al*, 1990)
pCR-blunt	Kan^r	Blunt PCR cloning vector	Invitrogen
pCQW-1	Erm^r	*E. coli-Bacteroides* suicide shuttle vector	(Feldhaus *et al*, 1991)
pCR2.1	Ap^r Kan^r	TA cloning vector	Invitrogen
pLEC23	Erm^r	*E. coli-Bacteroides* shuttle vector utilising *xyl*E as a reporter gene	(Coyne *et al*, 2003)
pUC18	Ap^r	Cloning vector	Fermentas
pBT2	Kan^r Tet^r	*E. coli-Bacteroides* suicide shuttle vector	(Tancula *et al.*, 1992)
pCR4-TOPO	Ap^r Kan^r	TA cloning vector	Invitrogen
pCR-pro	Kan^r	Xylanase operon promoter cloned in pCR-blunt	This study
pBT-xylE(ON)	Kan^r Tet^r	Xylanase promoter in the forward orientation upstream of the *xyl*E cloned in pBT2	This study
pBT-xylE(OFF)	Kan^r Tet^r	Xylanase promoter in the reverse orientation ustream of the *xyl*E cloned in pBT2	This study
pCR-KGF	Kan^r	Human *kgf-2* sequence downstream of *B. fragilis* secretion signals cloned in pCR-blunt	This study
pCR-TGF	Kan^r	Human *tgf-β* sequence downstream of *B. fragilis* secretion signals cloned in pCR-blunt	This study
pBT-PKGF	kan^r Tet^r	Xylanase promoter upstream of the *B. fragilis* secretion signals and human *kgf-2* cloned in pBT2	This study
pBT-PTGF	kan^r Tet^r	Xylanase promoter upstream of the *B. fragilis* secretion signals and human *tgf-β* cloned in pBT2	This study

3. Plasmids maps
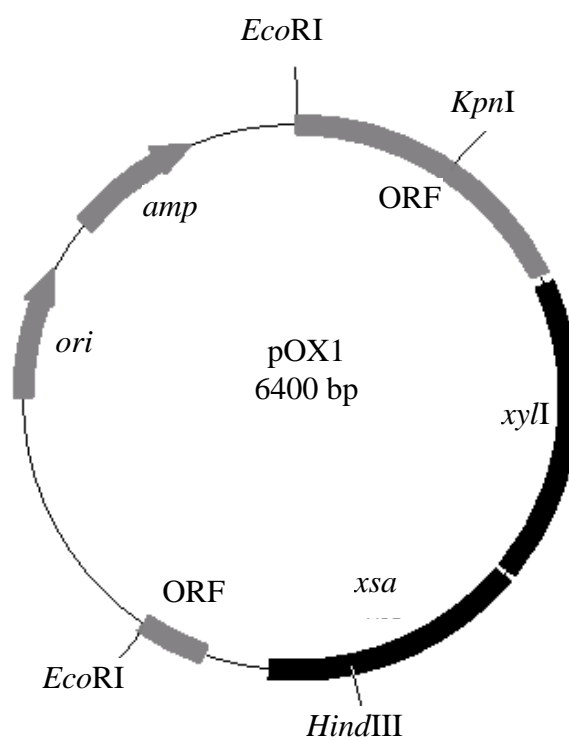
pOX1 was generated by T. Whitehead (Whitehead & Hespell, 1990). It is based on pUC18 with genomic DNA insert from *B.ovatus* carrying *xsa, xyl*I genes of the xylanase operon and an upstream partially identified ORF. This insert is 3.8 kbp and flanked by *Eco*RI sites (GeneBank accession no. U04957).
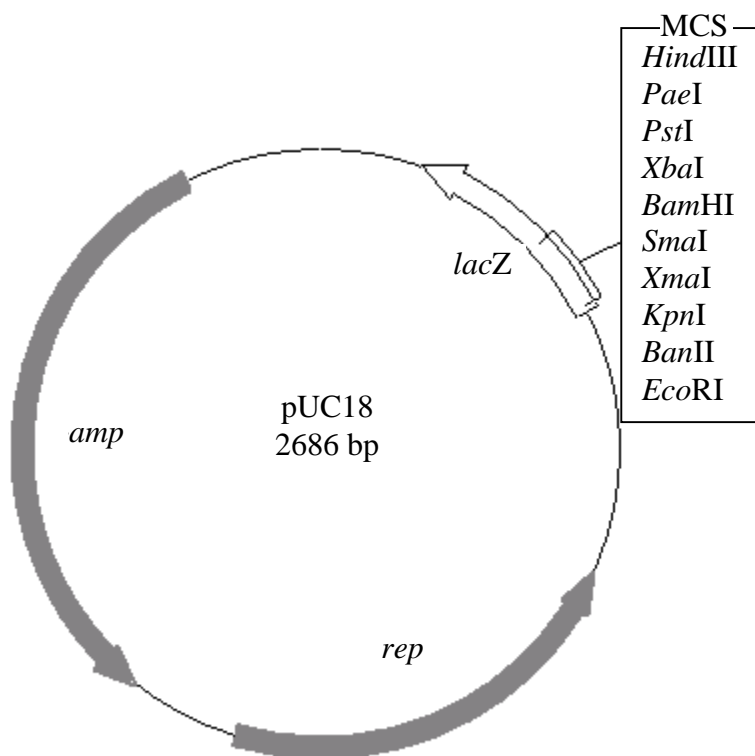
Multiple cloning site sequence is shown below with some of the restriction endonuclease.

*Eco*RI	*Kpn*I	*Bam*HI	*Xba*I	*Pst*I

G AATTCGAGCT CGGTACCCGG GGATCCTCTA GAGTCGACCT
GCAGGCATGC

*Hind*III

AAGCTT
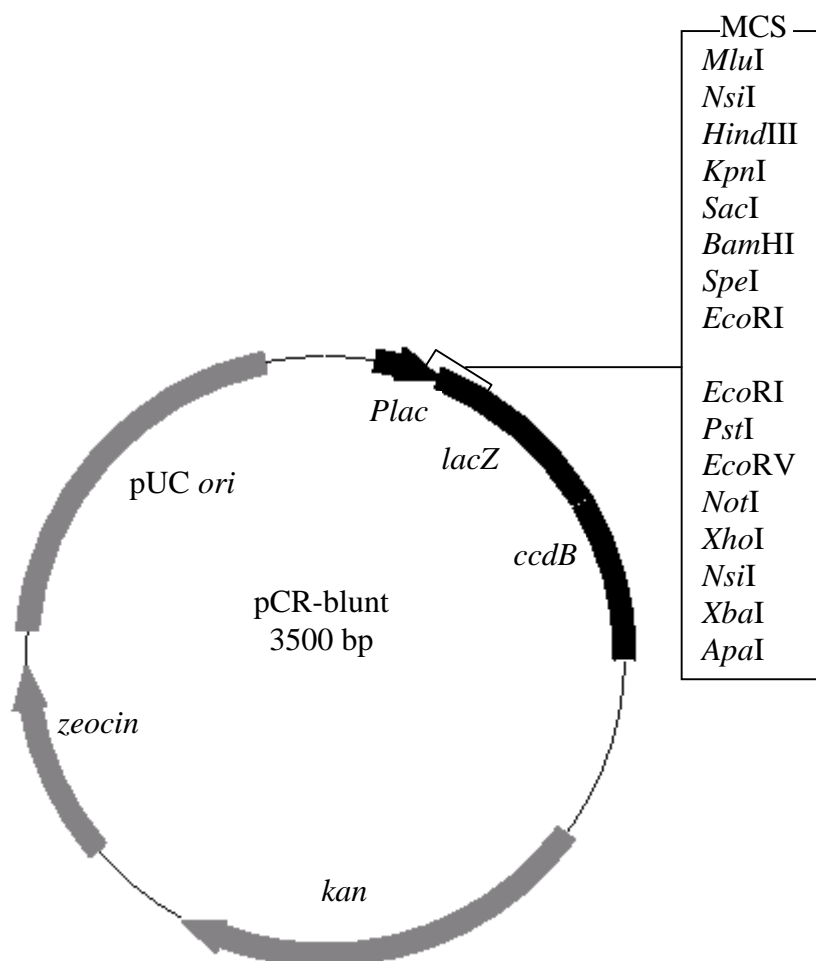
Multiple cloning site:

```
                                                                      Mlu I
CACACAGGAA  ACAGCTATGA  CCATGATTAC  GCCAAGCTAT  TTAGGTGACG
                         NsiI    HindIII        KpnI     SacI  BamHI
CGTTAGAATA  CTCAAGCTAT  GCATCAAGCT  TGGTACCGAG  CTCGGATCCA
SpeI                                EcoRI
CTAGTAACGG  CCGCCAGTGT  GCTGGAATTC  AGG-BLUNT  PCR  PRODUCT-
       EcoRI       PstI   EcoRV          NotI           XhoI       NsiI  XbaI
CCTGAATTCT  GCAGATCCAT  CACACTGGCG  GCCGCTCGAG  CATGCATCTA
         ApaI
GAGGGCCCAA  TTCGCCCTAT  AGTGAGTCGT  ATTACAATTC  ACTGGCCGTC


GTTTTACAAC  GTCGTGACTG  GGAAAACCCT  GGCGT
```

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MCS
*Hind*III
*Kpn*I
*Sac*I
*Bam*HI
*Spe*I
*Bst*XI
*Eco*RI

*Eco*RI
*Eco*RV
*Bst*XI
*Not*I
*Sst*I
*Xho*I
*Nsi*I
*Xba*I
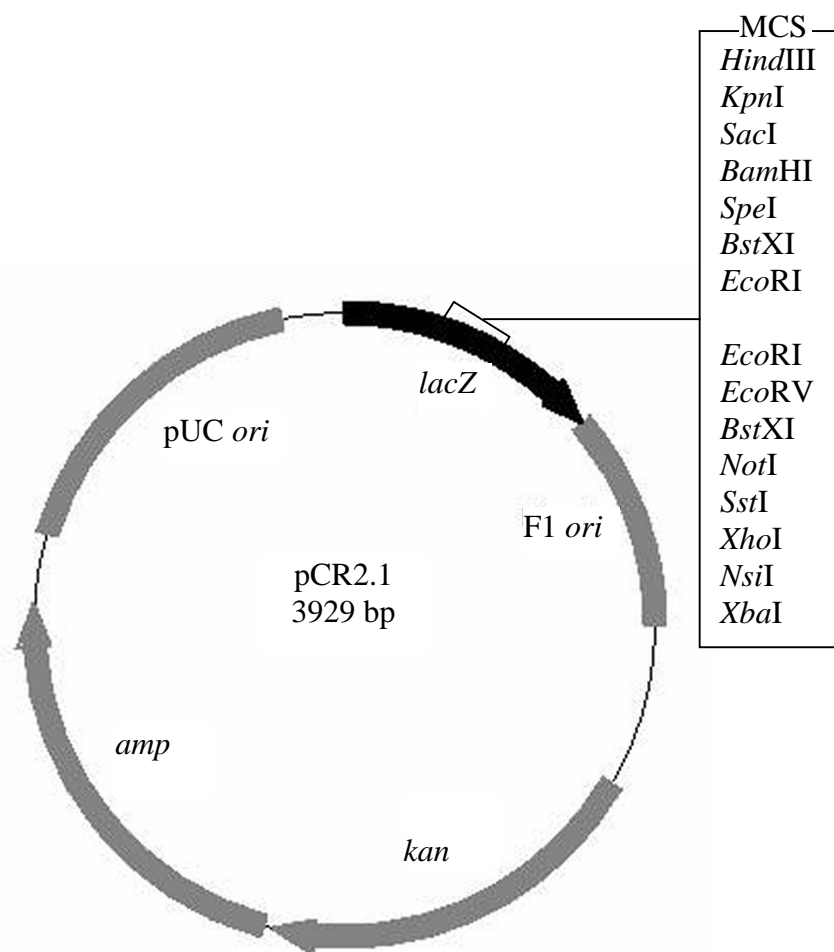
Multiple cloning site:

```
                              HindIII              KpnI      SacI BamHI
CAGGAAACAG CTATGACCAT GATTACGCCA AGCTTGGTAC CGAGCTCGGA
   SpeI                              EcoRI
TCCACTAGTA ACGGCCGCCA GTGTGCTGGA ATTCGGCT-PCR product-
        EcoRI       EcoRV              NotI       SstI
TAGCCGAATT CTGCAGATAA CCATCACACT GGCGGCCGCT CGAGCATGCA
XbaI          ApaI
TCTAGAGGGC CCAATTCGCC CTATAGTGAG TCGTATTACA ATTCACTGGC


CGTCGTTTTA CAACGTCGTG ACTGGGAAAA C
```
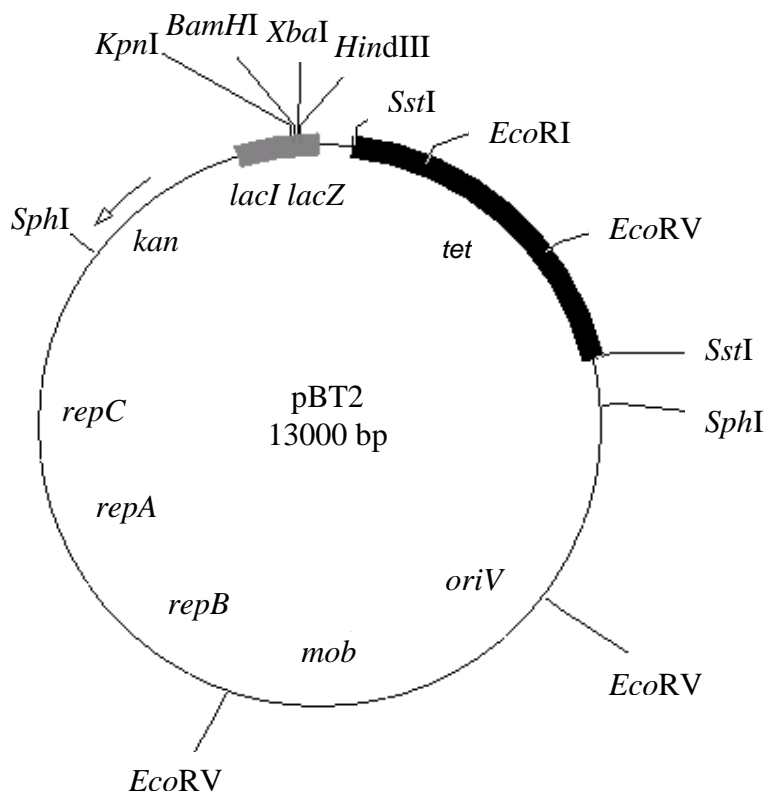
pBT2 is a suicide shuttle vector that transfer from *E. coli* to *Bacteroides* species by the IncPβ plasmid, R751. Tetracycline is used for selection of *Bacteroides* transconjugants and kanamycin resistance is used for selection of *E .coli.* MCS is the same as pUC18
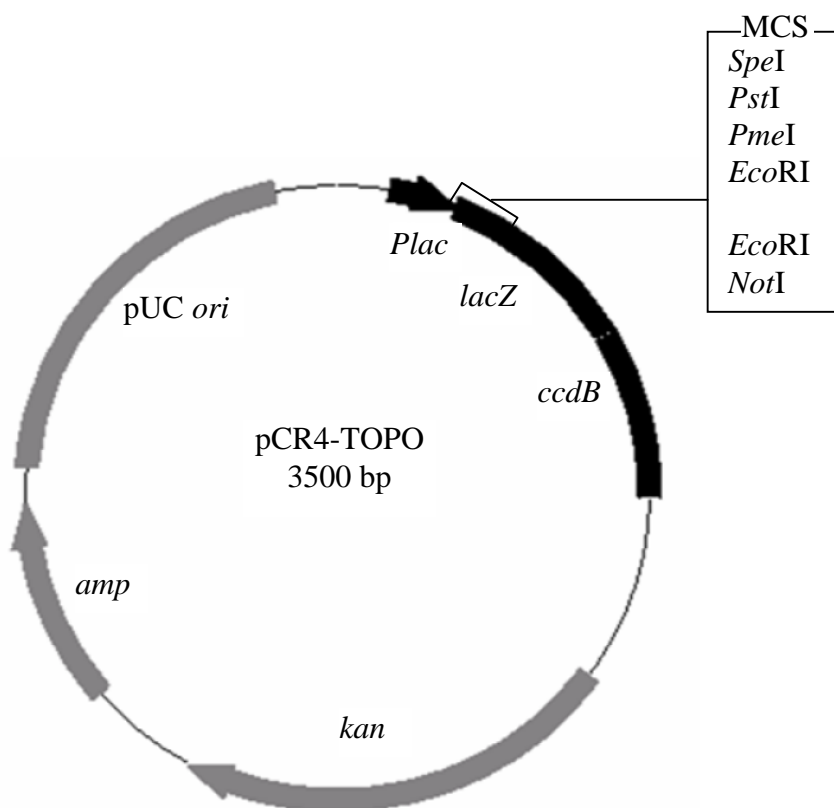
Multiple cloning site

```
ACAGCTATGA CCATGATTAC GCCAAGCTCA GAATTAACCCTCACTAAAGG
 SpeI                  PstI       PmeI      EcoRI
GACTAGTCCT GCAGGTTTAA ACGAATTCGC CCTT-PCR products-A
        EcoRI    NotI
GGGC GAATTCGCGG CCGCTAAATT CAATTCGCCC TATAGTGAGT
```

APPENDIX 2

OLIGNUCLEOTIDES PRIMERS USED IN THIS STUDY

Oligonucleotide pimers were synthesised by Sigma-Aldrich Genosys. Lyophilised primers were dissolved in nuclease free water to final concentration of 100 μM and stored at -20ºC. Bold oligonucleotides refer to cloned restriction enzyme and underline refer to *B. fragilis* secretion signals.

Name	5' to 3' Sequence	T_m(ºC)
BOPRO1	GAATAGCAAAACCAGTCAGCGG	55
BOPRO2	AGTTCCGGTAAGGATGTCGCA	55
GENE1F1	TTACTTCCGGCTGGGCAACAAA	55
GENE1R1	GACATTCGATTCTCCCTGATACCA	56
JUNC1-F	CGAGTGGCAGCAGCTATCTT	60
JUNC1-R	ACCAGAGGATCCCAAAGGAC	60
JUNC2-F	CTTTTGCCGAACAGGGAATA	60
JUNC2-R	CAAAGCCTTTTTCAGCGAAC	60
JUNC3-F	ATCGCAATTATCAGCCGAAG	60
JUNC3-R	ACCGTTCATCACATCATCCA	60
XYLE-F	ATTCACCATCCGGAAAAAGG	60
XYLE-R	GAGAATGCGGTCGTGGTAAA	60
XYLP	GATTAAAGAAGGGGAGAGTG	60
TIS	CCCTTTCCTCTTGTTTATCGGTG	60
SP1	CAATTCCATATTCGACTGTCCC	55
SP2	GAACATACCCAAACATCGCCAATAAG	60
SP3	CTTTAATTCGATATCATTAATGGCCATC	60
Anchor d(T)	CCACGCGTGAATTCGTCGACT$_{16}$V*	63
Anchor	CCACGCGTGAATTCGTCGAC	60
XYLP-f	GC**GGATCC**TGGGGAGTATCGGACAATG	63
XYLP-rb	GA**GGATCC**TCTGTCTTTCTTTTATATGTCTTTATTTC	60
XYLP-rn	GT**CATATG**GTCTTTCTTTTATATGTCTTTATTTCATG	60

Name	5' to 3' Sequence	T_m(°C)
TGF-f	GA**CATATG**AAGAATGTAAAGTTACTTTTAATGCTAGGA ACCGCGGCATTATTAGCTGCAGCCCTGGACACCAACTA	72
TGF-r	GTGGGATCCTCAGCTGCACTTGCAGGAGCG	68
KGF-f	GA**CATATG**AAGAATGTAAAGTTACTTTTAATGCTAGGA ACCGCGGCATTATTAGCTGCACAAGCCCTTGGTCAGGACA	74
KGF-r	GCGGGATCCCTATGAGTGTACCACCATTGGAAG	67
Xsa-F	GGAGGTACCATGAAAACAGAAAAAAGATA	60
Xsa-R	GTGGTACCTCATCTTTTCCCTCG	60
QPCR2-F	AATTCCAGTGTCGGAGGTTC	59
QPCR2-R	CAGCGACTCCATCAAATCGT	59
GYRA-F	TTCCGGATGTTAGAGATGGA	59
GYRA-R	CCAAGTACCTCACCCACGAT	59
TNF-α-f	ACGGCATGGATCTCAAAGAC	60
TNF-α-r	GTGGGTGAGGAGCACGTAGT	60
IL-1β-f	CAGGCAGGCAGTATCACTCA	60
IL-1β-r	TGTCCTCATCCTGGAAGGTC	60
IL-6-f	CCGGAGAGGAGACTTCACAG	60
IL-6-r	TCCACGATTTCCCAGAGAAC	60
TGF-*β*-f	GACTCTCCACCTGCAAGACC	60
TGF-*β*-r	GACTGGCGAGCCTTAGTTTG	60
KGF2-f	ACATTGTGCCTCAGCCTTTC	60
KGF2-r	ACCATGTCCTGACCAAGAGC	60
ITF-f	CTGTCACATCGGAGCAGTGT	60
ITF-r	CTCCTGCAGAGGTTTGAAGC	60
COX-f	TGCAGAATTGAAAGCCCTCT	60
COX-r	CCCCAAAGATAGCATCTGGA	60
β actin-f	CTTCTTCTTGGTATGGAATCCTG	60
β actin-r	GTAATCTCCTTCTGGATCCTGTC	60

*V=G, A or C

APPENDIX 3

NAMES AND ADDRESSES OF COMMERCIAL SUPPLIERS

Ambion

    Huntingdon, PE29 6XY

Amicon

    Amicon Ltd., Upper hill, Stonehouse, Gloucestershire GL10 2BJ

Applied Biosystems

    850 Lincoln Centre Drive, Foster City, CA 94404, USA

Autogen Bioclear

    Holly Ditch Farm, Calne, Wiltshire, SN11 0PY

BD

    Becton Dickinson Pharmingen, Between Towns Road, Oxford, OX4 3LY

BDH

    BDH Laboratory supplies, Poole, Dorset, BH15 1TD

Bioline

    Bioline Ltd, 16 The Edge Business Centre, London, NW2 6EW

Bio-rad

    Bio-rad Microscience Ltd, Bio-rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD

Biosera

    Biosera, 1 Acorn House, The Broyle, Ringmer, East Sussex, BN8 5NN

Caltag

    Caltag-Medsystems Ltd, PO Box 6139, Silverstone, Towcester, NN12 8GN

Dako

    DakoCytomation, Denmark House, Angel Drove, Ely, Cambridgeshire, CB7 4ET

Dundee University sequencing service

> The Sequencing Service, MSI Building, College of Life Sciences, University of Dundee, Dundee, Scotland, DD1 5EH

Fermentas

> Fermentas GmbH, Opelstrasse 9, 68789 St. Leon-Rot, Germany

GE Healthcare (formely known as Amersham Biosciences)

> GE Healthcare UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA

Greiner Bio-One Ltd

> Burnel way, Stroudwater business park, GL10 3SX, Stonehouse.

Invitrogen

> Invitrogen Ltd, Inchinnan Business Park, 3 Fountain Drive, Paisley, PA4 9RF

Leica

> Leica Microsystems, GmbH, Ernst-Leitz-Strasse 17-37, 35578 Wetzlar

MBL International

> 4 H Constitution Way, Woburn, MA 01801, USA

Molecular Devices

> 1311 Orleans Drive, Sunnyvale, CA 94089-1136, USA

MP Biomedicals

> 29525 Fountain Pkwy., Solon, OH 44139, USA

MRC geneservice

> 2 Cambridge Science Park, Milton Road, Cambridge, CB4 0FE

NEB

> New England Biolabs (UK) Ltd, 73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY

Norbrook Laboratories Ltd

   Station Works, Newry, County Down, BT35 6JP, Northern Ireland

Oxoid Ltd

   Wade Road, Basingstoke, Hampshire, RG24 8PW

Perkin Elmer

   PerkinElmer Ltd, 940 Winter Street, Waltham, Massachusetts, 02451, USA

Pierce Chemical Company, supplied through Perbio Ltd

   Perbio Science UK Ltd, Unit 9, Atley Way, North Nelson Industrial Estate,
   Cramlington, Northumberland, NE23 1WA

Promega

   Promega Ltd, Episilon House, Enterprise Road, Chilworth Research centre,
   Southhampton, S01 7NS

Qiagen

   Qiagen, Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ

R&D Systems Europe Ltd

   19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB

Roche Diagnostics Ltd.

   Charles Avenue, Burgess Hill, RH15 9RY, United Kingdom

Sanyo Gallenkamp

   Park home, Meridian east, Meridian business park, Leicester LE3 2UZ

Sarstedt

   Sarstedt Ltd, 68 Boston Road, Leicester, LE4 1AW

Serotec, also supplies Oxford Biotech Ltd

   Serotec Ltd, Endeavour House, Langford Business Park, Langford Lane,
   Kidlington, Oxford, OX5 1GF

Sigma-Aldrich

   Fancy Road, Poole, Dorset, BH17 7NH

Stratgene Europe

Gebouw California, Hogehilwegis, 1101 CB, Amersterdam, Netherlands

Whatmann supplied by SLS

Scientific Laboratory Supplies Ltd, Wilford Industrial Estate, Ruddington Lane Nottingham, NG11 7EP

Vector Laboratories

Vector Laboratories Ltd, 3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS

Zeiss

Carl Zeiss Ltd, PO Box 78, Woodfield Road, Welwyn Garden City, Hertfordshire, AL7 1LU

REFERENCES

Aaronson, S. A., Bottaro, D. P., Miki, T., Ron, D., Finch, P. W., Fleming, T. P., Ahn, J., Taylor, W. G. & Rubin, J. S. (1991). Keratinocyte growth factor. A fibroblast growth factor family member with unusual target cell specificity. *Ann NY Acad Sci* **638**, 62-77.

Allan, C. H., Mendrick, D. L. & Trier, J. S. (1993). Rat intestinal M cells contain acidic endosomal-lysosomal compartments and express class II major histocompatibility complex determinants. *Gastroenterology* **104**, 698-708.

Ardizzone, S., Bollani, S., Manzionna, G. & Bianchi Porro, G. (1999). Inflammatory bowel disease approaching the 3rd millennium: pathogenesis and therapeutic implications? *Eur J Gastroenterol Hepatol* **11**, 27-32.

Arhin, F. F., Shareck, F., Kluepfel, D. & Morosoli, R. (1994). Effects of disruption of xylanase-encoding genes on the xylanolytic system of Streptomyces lividans. *J Bacteriol* **176**, 4924-4930.

Aude, M. F., Yves, K., Elizabeth, M. K., Ghassan, G., David, H. M., Mark, M. D. & Yueh-hsiu, C. (2001). Attributes of γδ intraepithelial lymphocytes as suggested by their transcriptional profile. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 10261-10266.

Babyatsky, M. W., Rossiter, G. & Podolsky, D. K. (1996). Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. *Gastroenterology* **110**, 975-984.

Bagai, S., Rubio, E., Cheng, J. F., Sweet, R., Thomas, R., Fuchs, E., Grady, R., Mitchell, M. & Bassuk, J. A. (2002). Fibroblast growth factor-10 is a mitogen for urothelial cells. *J Biol Chem* **277**, 23828-23837.

Bai, A. P. & Ouyang, Q. (2006). Probiotics and inflammatory bowel diseases. *Postgraduate Medical J* **82**, 376-382.

Balk, S. P., Ebert, E. C., Blumenthal, R. L., McDermott, F. V., Wucherpfennig, K. W., Landau, S. B. & Blumberg, R. S. (1991). Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. *Science* **253**, 1411-1415.

Barbara, G., Xing, Z., Hogaboam, C. M., Gauldie, J. & Collins, S. M. (2000). Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut* **46**, 344-349.

Barnes, P. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci* **94**, 557-572.

Barone, K. S., Jain, S. L. & Michael, J. G. (1995). Effect of in Vivo Depletion of CD4+ and CD8+ Cells on the Induction and Maintenance of Oral Tolerance. *Cellular Immunology* **163**, 19-29.

Baumgart, D. C. & Carding, S. R. (2007). Inflammatory bowel disease: cause and immunobiology. *The Lancet* **369**, 1627-1640.

Baumgart, D. C. & Sandborn, W. J. (2007). Inflammatory bowel disease: clinical aspects and established and evolving therapies. *The Lancet* **369**, 1641-1657.

Bayley, D. P., Rocha, E. R. & Smith, C. J. (2000). Analysis of cepA and other Bacteroides fragilis genes reveals a unique promoter structure. *FEMS Microbiol Lett* **193**, 149-154.

Beck, P. L., Rosenberg, I. M., Xavier, R. J., Koh, T., Wong, J. F. & Podolsky, D. K. (2003). Transforming Growth Factor-{beta} Mediates Intestinal Healing and Susceptibility to Injury in Vitro and in Vivo Through Epithelial Cells. *Am J Pathol* **162**, 597-608.

Becker, C., Fantini, M. C. & Neurath, M. F. (2006). TGF-beta as a T cell regulator in colitis and colon cancer. *Cytokine & Growth Factor Reviews* **17**, 97-106.

Berin, M. C., Kiliaan, A. J., Yang, P. C., Groot, J. A., Taminiau, J. A. & Perdue, M. H. (1997). Rapid transepithelial antigen transport in rat jejunum: impact of sensitization and the hypersensitivity reaction. *Gastroenterology* **113**, 856-864.

Bernard, P., Gabant, P., Bahassi, E. M. & Couturier, M. (1994). Positive-selection vectors using the F plasmid ccdB killer gene. *Gene* **148**, 71-74.

Berrebi, D., Maudinas, R., Hugot, J. P. & other authors (2003). Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut* **52**, 840-846.

Bockman, D. E. & Cooper, M. D. (1973). Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. *Am J Anat* **136**, 455-477.

Boirivant, M., Fuss, I. J., Chu, A. & Strober, W. (1998). Oxazolone colitis: A murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *J Exp Med* **188**, 1929-1939.

Border, W. A. & Ruoslahti, E. (1992). Transforming growth factor-beta in disease: the dark side of tissue repair. *J Clin Invest* **90**, 1-7.

Boyer, B. & Thiery, J. P. (1989). Epithelial cell adhesion mechanisms. *J Membr Biol* **112**, 97-108.

Boyer, H. W. & Roulland-dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in Escherichia coli. *J Mol Biol* **41**, 459-472.

Braat, H., Rottiers, P., Hommes, D. W. & other authors (2006). A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol Hepatol* **4**, 754-759.

Bradley, P. P., Priebat, D. A., Christensen, R. D. & Rothstein, G. (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78**, 206-209.

Brandtzaeg, P. & Bjerke, K. (1990). Immunomorphological characteristics of human Peyer's patches. *Digestion* **46**, 262-273.

Braun, D. C. & Stein, D. C. (2004). The lgtABCDE Gene Cluster, Involved in Lipooligosaccharide Biosynthesis in Neisseria gonorrhoeae, Contains Multiple Promoter Sequences. *J Bacteriol* **186**, 1038-1049.

Bridger, S., Lee, J. C. W., Bjarnason, I., Jones, J. E. L. & Macpherson, A. J. (2002). In siblings with similar genetic susceptibility for inflammatory bowel disease, smokers tend to develop Crohn's disease and non-smokers develop ulcerative colitis. *Gut* **51**, 21-25.

Brunner, A. M., Lioubin, M. N., Marquardt, H. & other authors (1992). Site-directed mutagenesis of glycosylation sites in the transforming growth factor-beta 1 (TGF beta 1) and TGF beta 2 (414) precursors and of cysteine residues within

mature TGF beta 1: effects on secretion and bioactivity. *Mol Endocrinol* **6**, 1691-1700.

Calabresi, P. A., Fields, N. S., Maloni, H. W. & other authors (1998). Phase 1 trial of transforming growth factor beta 2 in chronic progressive MS. *Neurology* **51**, 289-292.

Camacho-Barquero, L., Villegas, I., Sánchez-Calvo, J. M., Talero, E., Sánchez-Fidalgo, S., Motilva, V. & Alarcón de la Lastra, C. (2007). Curcumin, a Curcuma longa constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis. *International Immunopharmacology* **7**, 333-342.

Campbell, N. A., Kim, H. S., Blumberg, R. S. & Mayer, L. (1999). The Nonclassical Class I Molecule CD1d Associates with the Novel CD8 Ligand gp180 on Intestinal Epithelial Cells. *J Biol Chem* **274**, 26259-26265.

Cario, E. & Podolsky, D. K. (2000). Differential Alteration in Intestinal Epithelial Cell Expression of Toll-Like Receptor 3 (TLR3) and TLR4 in Inflammatory Bowel Disease. *Infect Immun* **68**, 7010-7017.

Carter, M. J., Lobo, A. J. & Travis, S. P. L. (2004). Guidelines for the management of inflammatory bowel disease in adults. *Gut* **53**, v1-16.

Chang, S. K., Dohrman, A. F., Basbaum, C. B., Ho, S. B., Tsuda, T., Toribara, N. W., Gum, J. R. & Kim, Y. S. (1994). Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. . *Gastroenterology* **107**, 28-36.

Chang, W. W. & Leblond, C. P. (1971). Renewal of the epithelium in the descending colon of the mouse. I. Presence of three cell populations: vacuolated-columnar, mucous and argentaffin. *Am J Anat* **131**, 73-99.

Chapman, T. M., Plosker, G. L. & Figgitt, D. P. (2007). Spotlight on VSL#3 probiotic mixture in chronic inflammatory bowel diseases. *BioDrugs* **21**, 61-63.

Chen, C.-C. & Westpheling, J. (1998). Partial Characterization of the Streptomyces lividans xlnB Promoter and Its Use for Expression of a Thermostable Xylanase from Thermotoga maritima. *Appl Environ Microbiol* **64**, 4217-4225.

Chen, W., Jin, W., Hardegen, N., Lei, K.-j., Li, L., Marinos, N., McGrady, G. & Wahl, S. M. (2003). Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-{beta} Induction of Transcription Factor Foxp3. *J Exp Med* **198**, 1875-1886.

Chen, Y., Inobe, J.-i., Marks, R., Gonnella, P., Kuchroo, V. K. & Weiner, H. L. (1995). Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* **376**, 177-180.

Chen, Y., Chou, K., Fuchs, E., Havran, W. L. & Boismenu, R. (2002). Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* **99**, 14338-14343.

Ciacci, C., Lind, S. E. & Podolsky, D. K. (1993). Transforming growth factor beta regulation of migration in wounded rat intestinal epithelial monolayers. *Gastroenterology* **105**, 93-101.

Coffey, R. J., Jr., Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B. E. & Moses, H. L. (1988). Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Mol Cell Biol* **8**, 3088-3093.

Cohn, S. M., Schloemann, S., Tessner, T., Seibert, K. & Stenson, W. F. (1997). Crypt stem cell survival in the mouse intestinal epithelium is regulated by prostaglandins synthesized through cyclooxygenase-1. *J Clin Invest* **99**, 1376-1379.

Collins, F. S. & Weissman, S. M. (1984). Directional cloning of DNA fragments at a large distance from an initial probe: A circularization method. *Proc Natl Acad Sci U S A* **81**, 6812-6816.

Cosnes, J. (2004). Tobacco and IBD: relevance in the understanding of disease mechanisms and clinical practice. *Best Practice & Research Clinical Gastroenterology* **18**, 481-496.

Cosnes, J., Seksik, P., Nion-Larmurier, I., Beaugerie, L. & Gendre, J. P. (2006). Prior appendectomy and the phenotype and course of Crohn's disease. *World J Gastroenterol* **12**, 1235-1242.

Coyne, M. J., Weinacht, K. G., Krinos, C. M. & Comstock, L. E. (2003). Mpi recombinase globally modulates the surface architecture of a human commensal bacterium. *Proc Natl Acad Sci USA* **100**, 10446-10451.

Creamer, B. (1967). The turnover of the epithelium of the small intestine. *Br Med Bull* **23**, 226-230.

Croucher, S. C., Houston, A. P., Bayliss, C. E. & Turner, R. J. (1983). Bacterial Populations Associated with Different Regions of the Human Colon Wall. *Appl Environ Microbiol* **45**, 1025-1033.

Cruickshank, S. M., McVay, L. D., Baumgart, D. C., Felsburg, P. J. & Carding, S. R. (2004). Colonic epithelial cell mediated suppression of CD4 T cell activation. *Gut* **53**, 678-684.

Cuadrado, E., Alonso, M., de Juan, M. D., Echaniz, P. & Arenas, J. I. (2008). Regulatory T cells in patients with inflammatory bowel diseases treated with adacolumn granulocytapheresis. *World J Gastroenterol* **14**, 1521-1527.

Cummings, J. H., Macfarlane, G. T. & Englyst, H. N. (2001). Prebiotic digestion and fermentation. *Am J Clin Nutr* **73**, 415S-420.

Dalton, J. E., Cruickshank, S. M., Egan, C. E. & other authors (2006). Intraepithelial γδ Lymphocytes Maintain the Integrity of Intestinal Epithelial Tight Junctions in Response to Infection. *Gastroenterology* **131**, 818-829.

Dann, S. M. & Eckmann, L. (2007). Innate immune defenses in the intestinal tract. . *Curr Opin Gastroenterol* **23**, 115-120.

Darfeuille-Michaud, A., Boudeau, J., Bulois, P. & other authors (2004). High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* **127**, 412-421.

Desvignes, C., Bour, H., Nicolas, J. F. & Kaiserlian, D. (1996). Lack of oral tolerance but oral priming for contact sensitivity to dinitrofluorobenzene in major histocompatibility complex class II-deficient mice and in CD4+ T cell-depleted mice.Desvignes C, Bour H, Nicolas JF, Kaiserlian D. . *Eur J Immunol* **26**, 1756-1761.

Di Giacinto, C., Marinaro, M., Sanchez, M., Strober, W. & Boirivant, M. (2005). Probiotics Ameliorate Recurrent Th1-Mediated Murine Colitis by Inducing IL-10 and IL-10-Dependent TGF-{beta}-Bearing Regulatory Cells. *J Immunol* **174**, 3237-3246.

Dickinson, B. L., Badizadegan, K., Wu, Z., Ahouse, J. C., Zhu, X., Simister, N. E., Blumberg, R. S. & Lencer, W. I. (1999). Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. *J Clin Invest* **104**, 903-911.

Dignass, A. (2001). Mechanisms and modulation of intestinal epithelial repair. *Inflamm Bowel Dis* **7**, 68-77.

Dobbins, W. O. (1986). Human intestinal intraepithelial lymphocytes. *Gut* **27**, 972-985.

Doron Melamed, A. F. (1993). Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. *Eur J Immunol* **23**, 935-942.

Dorr, W., Bassler, S., Reichel, S. & Spekl, K. (2005). Reduction of radiochemotherapy-induced early oral mucositis by recombinant human keratinocyte growth factor (palifermin): Experimental studies in mice. *Int J Radiation Oncology Biol Phys* **62**, 881-887.

Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K. & Meyer zum Büschenfelde, K. H. (1995). Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *J Exp Med* **102**, 448-455.

Duffy, L. C. (2000). Interactions Mediating Bacterial Translocation in the Immature Intestine. *J Nutr* **130**, 432-.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005). Diversity of the Human Intestinal Microbial Flora. *Science* **308**, 1635-1638.

Economou, M., Trikalinos, T. A., Loizou, K. T., Tsianos, E. V. & Ioannidis, J. P. A. (2004). Differential Effects of NOD2 Variants on Crohn's Disease Risk and Phenotype in Diverse Populations: A Metaanalysis. *Am J Gastroenterol* **99**, 2393-2404.

Efstathiou, J. A., Noda, M., Rowan, A. & other authors (1998). Intestinal trefoil factor controls the expression of the adenomatous polyposis coli-catenin and the E-cadherin-catenin complexes in human colon carcinoma cells. *Proc Natl Acad Sci U S A* **95**, 3122-3127.

Egert, M., de Graaf, A. A., Smidt, H., de Vos, W. M. & Venema, K. (2006). Beyond diversity: functional microbiomics of the human colon. *Trends Microbiol* **14**, 86-91.

Ehrenpreis, E. D., Kane, S. V., Cohen, L. B., Cohen, R. D. & Hanauer, S. B. (1999). Thalidomide therapy for patients with refractory Crohn's disease: an open-label trial. *Gastroenterology* **117**, 1271-1277.

Elson, C. O., Sartor, R. B., Tennyson, G. S. & Riddell, R. H. (1995). Experimental models of inflammatory bowel disease. *Gastroenterology* **109**, 1344-1367.

Elson, C. O., Cong, Y., McCracken, V. J., Dimmitt, R. A., Lorenz, R. G. & Weaver, C. T. (2005). Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunological Reviews* **206**, 260-276.

Emoto, H., Tagashira, S., Mattei, M.-G. & other authors (1997). Structure and Expression of Human Fibroblast Growth Factor-10. *J Biol Chem* **272**, 23191-23194.

Epstein, S. E., Fuchs, S., Zhou, Y. F., Baffour, R. & Kornowski, R. (2001). Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. *Cardiovasc Res* **49**, 532-542.

Fabia, R., Ar'Rajab, A., Johansson, M. L., Andersson, R., Willén, R., Jeppsson, B., Molin, G. & Bengmark, S. (1993). Impairment of bacterial flora in human ulcerative colitis and experimental colitis in the rat. *Digestion* **54**, 248-255.

Farrar, M. D., Whitehead, T. R., Lan, J., Dilger, P., Thorpe, R., Holland, K. T. & Carding, S. R. (2005). Engineering of the gut commensal bacterium Bacteroides ovatus to produce and secrete biologically active murine interleukin-2 in response to xylan. *J Appl Microbiol* **98**, 1191-1197.

Farrell, C. L., Rex, K. L., Chen, J. N., Bready, J. V., DiPalma, C. R., Kaufman, S. A., Rattan, A., Scully, S. & Lacey, D. L. (2002). The effects of keratinocyte growth factor in preclinical models of mucositis. *Cell Prolif* **35**, 78-85.

Favier, C., Neut, C., Mizon, C., Cortot, A., Colombel, J. F. & Mizon, J. (1997). Fecal β-D-Galactosidase Production and Bifidobacteria Are Decreased in Crohn's Disease. *Digestive Diseases and Sciences* **42**, 817-822.

Feagan, B. G., Greenberg, G. R., Wild, G. & other authors (2005). Treatment of Ulcerative Colitis with a Humanized Antibody to the {alpha}4{beta}7 Integrin. *N Engl J Med* **352**, 2499-2507.

Fedorak, R. N., Gangl, A., Elson, C. O. & other authors (2000). Recombinant Human Interleukin 10 in the Treatment of Patients With Mild to Moderately Active Crohn's Disease. *Gastroenterology* **119**, 1473-1482.

Feeney, M. A., Murphy, F., Clegg, A. J., Trebble, T. M., Sharer, N. M. & Snook, J. A. (2002). A case-control study of childhood environmental risk factors for the development of inflammatory bowel disease. *Eur J Gastroenterol Hepatol* **14**, 529-534.

Feldhaus, M. J., Hwa, V., Cheng, Q. & Salyers, A. A. (1991). Use of an Escherichia coli beta-glucuronidase gene as a reporter gene for investigation of Bacteroides promoters. *J Bacteriol* **173**, 4540-4543.

Finch, P. W., Rubin, J. S., Miki, T., Ron, D. & Aaronson, S. A. (1989). Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* **245**, 752-755.

Finch, P. W., Pricolo, V., Wu, A. & Finkelstein, S. D. (1996). Increased expression of keratinocyte growth factor messenger RNA associated with inflammatory bowel disease. *Gastroenterology* **110**, 441-451.

Finegold, S., M., Sutter, V., L & Mathisen, G., E (1983). Anaerobic bacterial populations on normal and diseased human biopsy tissue obtained at colonoscopy. In *Human intestinal microflora in health and disease*, pp. 3-32. Edited by D. Hentges, J. New York: Academic press.

Finzi, G., Cornaggia, M., Capella, C., Fiocca, R., Bosi, F., Solcia, E. & Samloff, I. M. (1993). Cathepsin E in follicle associated epithelium of intestine and tonsils: localization to M cells and possible role in antigen processing. *Histochemistry* **99**, 201-211.

Foncuberta, M. C., Cagnoni, P. J., Brandts, C. H. & other authors (2001). Topical transforming growth factor-ß3 in the prevention or alleviation of chemotherapy-induced oral mucositis in patients with lymphomas or solid tumors. *J Immunother* **24**, 384-388.

Frey, A., Giannasca, K. T., Weltzin, R., Giannasca, P. J., Reggio, H., Lencer, W. I. & Neutra, M. R. (1996). Role of the glycocalyx in regulating access of

microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. *J Exp Med* **184**, 1045-1059.

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M. & Sporn, M. B. (1983). Purification and initial characterization of a type beta transforming growth factor from human placenta. *Proc Natl Acad Sci U S A* **80**, 3676-3680.

Fujihashi, K. & McGhee, J. R. (1999). TH1/TH2/TH3 cells for regulation of mucosal immunity, tolerance and inflammation. In *Mucosal Immunology*, pp. 539-558. Edited by J. Mestecky, M. Lamm, W. Strober, J. Bienenstock, J. R. McGhee & L. Mayer. USA: Academic press.

Fujita, H., Eishi, Y., Ishige, I., Saitoh, K., Takizawa, T., Arima, T. & Koike, M. (2002). Quantitative analysis of bacterial DNA from Mycobacteria spp., Bacteroides vulgatus , and Escherichia coli in tissue samples from patients with inflammatory bowel diseases. *Journal of Gastroenterology* **37**, 509-516.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S. & Tsukita, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* **123**, 1777-1788.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. & Tsukita, S. (1998). Claudin-1 and -2: Novel Integral Membrane Proteins Localizing at Tight Junctions with No Sequence Similarity to Occludin. *J Cell Biol* **141**, 1539-1550.

Gao, C. F., Kong, X. T., Gressner, A. M. & Weiskirchen, R. (2001). The expression and antigenicity identification of recombinant rat TGF-[beta]1 in bacteria. *Cell Res* **11**, 95-100.

Garside, P. & Mowat, A. M. (2001). Oral tolerance. *Seminars in Immunology* **13**, 177-185.

Gaya, D. R., Russell, R. K., Nimmo, E. R. & Satsangi, J. (2006). New genes in inflammatory bowel disease: lessons for complex diseases? *The Lancet* **367**, 1271-1284.

Gent, A. E., Hellier, M. D., Grace, R. H., Swarbrick, E. T. & Coggon, D. (1994). Inflammatory bowel disease and domestic hygiene in infancy. *The Lancet* **343**, 766-767.

Gentry, L. E., Lioubin, M. N., Purchio, A. F. & Marquardt, H. (1988). Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol Cell Biol* **8**, 4162-4168.

Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., PoggioliS, G., Miglioli, M. & Campieri, M. (2000). Oral Bacteriotherapy as Maintenance Treatment in Patients With Chronic Pouchitis: A Double-Blind, Placebo-Controlled Trial. *Gastroenterology* **119**, 305-309.

Gitter, A. H., Wullstein, F., Fromm, M. & Dieter Schulzke, J. (2001). Epithelial barrier defects in ulcerative colitis: Characterization and quantification by electrophysiological imaging. *Gastroenterology* **121**, 1320-1328.

Gorelik, L., Fields, P. E. & Flavell, R. A. (2000). Cutting Edge: TGF-{beta} Inhibits Th Type 2 Development Through Inhibition of GATA-3 Expression. *J Immunol* **165**, 4773-4777.

Gorelik, L. & Flavell, R. A. (2000). Abrogation of TGF[beta] Signaling in T Cells Leads to Spontaneous T Cell Differentiation and Autoimmune Disease. *Immunity* **12**, 171-181.

Gorelik, L., Constant, S. & Flavell, R. A. (2002). Mechanism of Transforming Growth Factor {beta}-induced Inhibition of T Helper Type 1 Differentiation. *J Exp Med* **195**, 1499-1505.

Graycar, J. L., Miller, D. A., Arrick, B. A., Lyons, R. M., Moses, H. L. & Derynck, R. (1989). Human transforming growth factor-beta 3: recombinant expression, purification, and biological activities in comparison with transforming growth factors-beta 1 and -beta 2. *Mol Endocrinol* **3**, 1977-1986.

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M. & Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A* **93**, 12445-12450.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. & Roncarolo, M. G. (1997). A CD4+T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737-742.

Guo, L., Degenstein, L. & Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev* **10**, 165-175.

Gupta, P., Andrew, H., Kirschner, B. S. & Guandalini, S. (2000). Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J Pediatr Gastroenterol Nutr* **31**, 453-457.

Hahm, K. B., Im, Y. H., Parks, T. W., Park, S. H., Markowitz, S., Jung, H. Y., Green, J. & Kim, S. J. (2001). Loss of transforming growth factor β signalling in the intestine contributes to tissue injury in inflammatory bowel disease. *Gut* **49**, 190-198.

Haller, D., Holt, L., Kim, S. C., Schwabe, R. F., Sartor, R. B. & Jobin, C. (2003). Transforming Growth Factor-{beta}1 Inhibits Non-pathogenic Gramnegative Bacteria-induced NF-{kappa}B Recruitment to the Interleukin-6 Gene Promoter in Intestinal Epithelial Cells through Modulation of Histone Acetylation. *J Biol Chem* **278**, 23851-23860.

Hamady, Z. Z. R., Farrar, M., Whitehead, R. H., Holland, K. T., Lodge, J. P. & Carding, S. R. (2008). Identification and use of the putative Bacteroides ovatus xylanase promoter for the inducible production of recombinant human proteins. *Microbiology*, (In press).

Han, D. S., Li, F., Holt, L. & other authors (2000). Keratinocyte growth factor-2 (FGF-10) promotes healing of experimental small intestinal ulceration in rats. *Am J Physiol Gastrointest Liver Physiol* **279**, G1011-1022.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. . *J Mol Biol* **166**, 557-580.

Hart, A. L., Al-Hassi, H. O., Rigby, R. J., Bell, S. J., Emmanuel, A. V., Knight, S. C., Kamm, M. A. & Stagg, A. J. (2005). Characteristics of Intestinal Dendritic Cells in Inflammatory Bowel Diseases. *Gastroenterology* **129**, 50-65.

Hawthorne, A. B. (2003). Ciclosporin and refractory colitis. *Eur J Gastroenterol Hepatol* **15**, 239-244.

Hayday, A., Theodoridis, E., Ramsburg, E. & Shires, J. (2001). Intraepithelial lymphocytes: exploring the Third Way in immunology. *Nat Immunol* **2**, 997-1003.

Heldin, C.-H., Miyazono, K. & ten Dijke, P. (1997). TGF-[beta] signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465-471.

Hentges, D., J (1989). Anaerobes as normal flora. In *Anaerobic infections in humans*, pp. 37-53. Edited by S. Finegold, M. & W. George, L. San Diego.: Academic Press.

Hermiston, M. L. & Gordon, J. I. (1995). Inflammatory Bowel Disease and Adenomas in Mice Expressing a Dominant Negative N-Cadherin. *Science* **270**, 1203-1207.

Herrlinger, K. R., Witthoeft, T., Raedler, A. & other authors (2006). Randomized, Double Blind Controlled Trial of Subcutaneous Recombinant Human Interleukin-11 *Versus* Prednisolone in Active Crohn's Disease. *Am J Gastroenterol* **101**, 793-797.

Hershberg, R. M., Framson, P. E., Cho, D. H., Lee, L. Y., Kovats, S., Beitz, J., Blum, J. S. & Nepom, G. T. (1997). Intestinal Epithelial Cells Use Two Distinct Pathways for HLA Class II Antigen Processing. *J Clin Invest* **100**, 204-215.

Hershberg, R. M. & Mayer, L. F. (2000). Antigen processing and presentation by intestinal epithelial cells - polarity and complexity. *Immunology Today* **21**, 123-128.

Hespell, R. B. & Whitehead, T. R. (1990). Physiology and Genetics of Xylan Degradation by Gastrointestinal Tract Bacteria. *J Dairy Sci* **73**, 3013-3022.

Hoentjen, F., Harmsen, H. J. M., Braat, H., Torrice, C. D., Mann, B. A., Sartor, R. B. & Dieleman, L. A. (2003). Antibiotics with a selective aerobic or anaerobic spectrum have different therapeutic activities in various regions of the colon in interleukin 10 gene deficient mice. *Gut* **52**, 1721-1727.

Hoffmann, J. C., Pawlowski, N. N., Kühl, A. A., Höhne, W. & Zeitz, M. (2002-2003). Animal Models of Inflammatory Bowel Disease: An Overview. *Pathobiology* **70**, 121-130.

Holländer, G. A., Simpson, S. J., Mizoguchi, E. & other authors (1995). Severe colitis in mice with aberrant thymic selection. *Immunity* **3**, 27-38.

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G. & Gordon, J. I. (2001). Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. *Science* **291**, 881-884.

Hornquist, E., Grdic, D., Mak, T. & Lycke, N. (1996). CD8-deficient mice exhibit augmented mucosal immune responses and intact adjuvant effects to cholera toxin. *Immunology* **87**, 220-229.

Housley, R. M., Morris, C. F., Boyle, W. & other authors (1994). Keratinocyte growth factor induces proliferation of hepatocytes and epithelial cells throughout the rat gastrointestinal tract. *J Clin Invest* **94**, 1764-1777.

Hsu, C.-K., Liao, J.-W., Chung, Y.-C., Hsieh, C.-P. & Chan, Y.-C. (2004). Xylooligosaccharides and Fructooligosaccharides Affect the Intestinal Microbiota and Precancerous Colonic Lesion Development in Rats. *J Nutr* **134**, 1523-1528.

Hsu, Y.-R., Hsu, E. W. J., Katta, V., Brankow, D., Tseng, J., Hu, S., Morris, C. F., Kenney, W. C. & Lu, H. S. (1998). Human Keratinocyte Growth Factor Recombinantly Expressed in Chinese Hamster Ovary Cells: Isolation of Isoforms and Characterization of Post-Translational Modifications. *Protein Expression and Purification* **12**, 189-200.

Hugot, J.-P., Chamaillard, M., Zouali, H. & other authors (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599-603.

Huyghebaert, N., Vermeire, A., Neirynck, S., Steidler, L., Remaut, E. & Remon, J. P. (2005). Development of an enteric-coated formulation containing freeze-dried, viable recombinant Lactococcus lactis for the ileal mucosal delivery of human interleukin-10. *Eur J Pharmaceutics Biopharmaceutics* **60**, 349-359.

Hwang, C., Sinskey, A. J. & Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257**, 1496-1502.

Igarashi, M., Finch, P. W. & Aaronson, S. A. (1998). Characterization of Recombinant Human Fibroblast Growth Factor (FGF)-10 Reveals Functional Similarities with Keratinocyte Growth Factor (FGF-7). *J Biol Chem* **273**, 13230-13235.

Inagaki-Ohara, K., Chinen, T., Matsuzaki, G., Sasaki, A., Sakamoto, Y., Hiromatsu, K., Nakamura-Uchiyama, F., Nawa, Y. & Yoshimura, A. (2004). Mucosal T Cells Bearing TCR{gamma}{delta} Play a Protective Role in Intestinal Inflammation. *J Immunol* **173**, 1390-1398.

Islam, M. S., Murata, T., Fujisawa, M., Nagasaka, R., Ushio, H., Bari, A. M., Hori, M. & Ozaki, H. (2008). Anti-inflammatory effects of phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice. *Br J Pharmacol* **154**, 812-824.

Iwasaki, A. & Kelsall, B. L. (1999). Freshly Isolated Peyer's Patch, But Not Spleen, Dendritic Cells Produce Interleukin 10 and Induce the Differentiation of T Helper Type 2 Cells. *J Exp Med* **190**, 229-240.

Jackson, C. A., Hoffmann, B., Slakeski, N., Cleal, S., Hendtlass, A. J. & Reynolds, E. C. (2000). A consensus Porphyromonas gingivalis promoter sequence. *FEMS Microbiol Lett* **186**, 133-138.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R. & Havran, W. L. (2002). A Role for Skin gamma delta T Cells in Wound Repair. *Science* **296**, 747-749.

Jarry, A., Cerf-Bensussan, N., Brousse, N., Selz, F. & Guy-Grand, D. (1990). Subsets of CD3+ (T cell receptor alpha/beta or gamma/delta) and CD3- lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur J Immunol* **20**, 1097-1103.

Jaye, M., Schlessinger, J. & Dionne, C. A. (1992). Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1135**, 185-199.

Jónsson, S., Clausen, E. & Raa, J. (1983). Amino acid degradation by a Lactobacillus plantarum strain from fish. *System Appl Microbiol* **4**.

Jurjus, A. R., Khoury, N. N. & Reimund, J.-M. (2004). Animal models of inflammatory bowel disease. *Journal of Pharmacological and Toxicological Methods* **50**, 81-92.

Kato, T. & Owen, R. L. (1999). Structure and function of intestinal mucosal epithelium. In *Mucosal Immunology*, pp. 115-132. Edited by P. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock & J. R. McGhee. San Diego: Academic Press,.

Keshav, S., Lawson, L., Chung, L. P., Stein, M., Perry, V. H. & Gordon, S. (1990). Tumor necrosis factor mRNA localized to Paneth cells of normal murine intestinal epithelium by in situ hybridization. *J Exp Med* **171**, 327-332.

Kitajima, S., Takuma, S. & Morimoto, M. (1999). Changes in Colonic Mucosal Permeability in Mouse Colitis Induced with Dextran Sulfate Sodium. *Exp Animals* **48**, 137-143.

Kitani, A., Fuss, I. J., Nakamura, K., Schwartz, O. M., Usui, T. & Strober, W. (2000). Treatment of Experimental (Trinitrobenzene Sulfonic Acid) Colitis by Intranasal Administration of Transforming Growth Factor (TGF)-{beta}1 Plasmid: TGF-{beta}1-mediated Suppression of T Helper Cell Type 1 Response Occurs by Interleukin (IL)-10 Induction and IL-12 Receptor {beta}2 Chain Downregulation. *J Exp Med* **192**, 41-52.

Kojouharoff, G., Hans, W., Obermeier, F., Männel, D. N., Andus, T., Schölmerich, J., Gross, V. & Falk, W. (1997). Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin Exp Immunol* **107**, 353-358.

Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F. & Kollias, G. (1999). Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. *Immunity* **10**, 387-398.

Korzenik, J. R., Dieckgraefe, B. K., Valentine, J. F., Hausman, D. F., Gilbert, M. J. & the Sargramostim in Crohn's Disease Study, G. (2005). Sargramostim for Active Crohn's Disease. *N Engl J Med* **352**, 2193-2201.

Kosiewicz, M. M., Nast, C. C., Krishnan, A., Rivera-Nieves, J., Moskaluk, C. A., Matsumoto, S., Kozaiwa, K. & Cominelli, F. (2001). Th1-type responses mediate spontaneous ileitis in a novel murine model of Crohn's disease. *J Clin Invest* **107**, 695-702.

Kruger, C., Hu, Y., Pan, Q. & other authors (2002). In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat Biotech* **20**, 702-706.

Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-274.

Lammers, K. M., Helwig, U., Swennen, E. & other authors (2002). Effect of probiotic strains on interleukin 8 production by HT29/19A cells. *Am J Gastroenterol* **97**, 1182-1186.

Lewis, J. D., Lichtenstein, G. R., Deren, J. J. & other authors (2008). Rosiglitazone for Active Ulcerative Colitis: A Randomized Placebo-Controlled Trial. *Gastroenterology* **134**, 688-695.

Lievin, V., Peiffer, I., Hudault, S., Rochat, F., Brassart, D., Neeser, J. R. & Servin, A. L. (2000). Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* **47**, 646-652.

Lin, X. P., Almqvist, N. & Telemo, E. (2005). Human small intestinal epithelial cells constitutively express the key elements for antigen processing and the production of exosomes. *Blood Cells, Molecules, and Diseases* **35**, 122-128.

Loftus, E. V. (2004). Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* **126**, 1504-1517.

Machado, P., Félix, R., Rodrigues, R., Oliveira, S. & Rodrigues-Pousada, C. (2006). Characterization and Expression Analysis of the Cytochrome bd Oxidase Operon from Desulfovibrio gigas. *Curr Microbiol* **52**, 274-281.

Mack, D. R., Michail, S., Wei, S., McDougall, L. & Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. *Am J Physiol* **276**, G941-950.

Malin, M., Suomalainen, H., Saxelin, M. & Isolauri, E. (1996). Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with Lactobacillus GG. *Ann Nutr Metab* **40**, 137-145.

Manch-Citron, J. N., Dey, A., Schneider, R. & Nguyen, N. Y. (1999). The Translational Hop Junction and the 5' Transcriptional Start Site for the Prevotella loescheii Adhesin Encoded by plaA. *Current Microbiology* **38**, 22-26.

Mannon, P. J., Fuss, I. J., Mayer, L. & other authors (2004). Anti-Interleukin-12 Antibody for Active Crohn's Disease. *N Engl J Med* **351**, 2069-2079.

Marchese, C., Chedid, M., Dirsch, O. R., Csaky, K. G., Santanelli, F., Latini, C., LaRochelle, W. J., Torrisi, M. R. & Aaronson, S. A. (1995). Modulation of keratinocyte growth factor and its receptor in reepithelializing human skin. *J Exp Med* **182**, 1369-1376.

Marti, G., Ferguson, M., Wang, J., Byrnes, C., Dieb, R., Qaiser, R., Bonde, P., Duncan, M. D. & Harmon, J. W. (2004). Electroporative transfection with KGF-1 DNA improves wound healing in a diabetic mouse model. *Gen Ther* **11**, 1780-1785.

Martin, B., Banz, A., Bienvenu, B., Cordier, C., Dautigny, N., Becourt, C. & Lucas, B. (2004). Suppression of CD4+ T Lymphocyte Effector Functions by CD4+CD25+ Cells In Vivo. *J Immunol* **172**, 3391-3398.

Martin, G. R. (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev* **12**, 1571-1586.

Mashimo, H., Wu, D. C., Podolsky, D. K. & Fishman, M. C. (1996). Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science* **274**, 262-265.

Matsuura, M., Okazaki, K., Nishio, A. & other authors (2005). Therapeutic effects of rectal administration of basic fibroblast growth factor on experimental murine colitis. *Gastroenterology* **128**, 975-986.

Maul, J., Loddenkemper, C., Mundt, P., Berg, E., Giese, T., Stallmach, A., Zeitz, M. & Duchmann, R. (2005). Peripheral and Intestinal Regulatory CD4+CD25high T Cells in Inflammatory Bowel Disease. *Gastroenterology* **128**, 1868-1878.

Mawdsley, J. E. & Rampton, D. S. (2005). Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* **54**, 1481-1491.

Mayer, L. & Shlien, R. (1987). Evidence for function of Ia molecules on gut epithelial cells in man. *J Exp Med* **166**, 1471-1483.

McCormick, B. A., Parkos, C. A., Colgan, S. P., Carnes, D. K. & Madara, J. L. (1998). Apical Secretion of a Pathogen-Elicited Epithelial Chemoattractant Activity in Response to Surface Colonization of Intestinal Epithelia by Salmonella typhimurium. *J Immunol* **160**, 455-466.

McGeachy, M. J., Stephens, L. A. & Anderton, S. M. (2005). Natural Recovery and Protection from Autoimmune Encephalomyelitis: Contribution of CD4+CD25+ Regulatory Cells within the Central Nervous System. *J Immunol* **175**, 3025-3032.

McKaig, B. C., Hughes, K., Tighe, P. J. & Mahida, Y. R. (2002). Differential expression of TGF-beta isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. *Am J Physiol Cell Physiol* **282**, C172-182.

Metchinkoff, E. (1907). *The prolongation of life: optimistic studies.* London.

Meyer, R. J. & Shapiro, J. A. (1980). Genetic organization of the broad-host-range IncP-1 plasmid R751. *J Bacteriol* **143**, 1362-1373.

Miceli, R., Hubert, M., Santiago, G., Yao, D.-L., Coleman, T. A., Huddleston, K. A. & Connolly, K. (1999). Efficacy of Keratinocyte Growth Factor-2 in Dextran Sulfate Sodium-Induced Murine Colitis. *J Pharmacol Exp Ther* **290**, 464-471.

Miki, T., Bottaro, D. P., Fleming, T. P., Smith, C. L., Burgess, W. H., Chan, A. M. & Aaronson, S. A. (1992). Determination of Ligand-Binding Specificity by Alternative Splicing: Two Distinct Growth Factor Receptors Encoded by a Single Gene. *Proc Natl Acad Sci U S A* **89**, 246-250.

Miller, A., Lider, O. & Weiner, H. L. (1991). Antigen-driven bystander suppression after oral administration of antigens. *J Exp Med* **174**, 791-798.

Miller, A., Jenny Zhang, Z., Sobel, R. A., Al-Sabbagh, A. & Weiner, H. L. (1993). Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein VI. Suppression of adoptively transferred disease and differential effects of oral vs. intravenous tolerization. *Journal of Neuroimmunology* **46**, 73-82.

Miller, H., Zhang, J., Kuolee, R., Patel, G. B. & Chen, W. (2007). Intestinal M cells: The fallible sentinels? . *World J Gastroenterol* **13**, 1477-1486.

Miyazaki, K., Miyamoto, H., Mercer, D. K., Hirase, T., Martin, J. C., Kojima, Y. & Flint, H. J. (2003). Involvement of the Multidomain Regulatory Protein XynR in Positive Control of Xylanase Gene Expression in the Ruminal Anaerobe Prevotella bryantii B14. *J Bacteriol* **185**, 2219-2226.

Miyazono, K. (2000). Positive and negative regulation of TGF-beta signaling. *J Cell Sci* **113**, 1101-1109.

Monteleone, G., Kumberova, A., Croft, N. M., McKenzie, C., Steer, H. W. & MacDonald, T. T. (2001). Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J Clin Invest* **108**, 601-609.

Monteleone, G., Pallone, F. & MacDonald, T. T. (2004). Smad7 in TGF-[beta]-mediated negative regulation of gut inflammation. *Trends in Immunology* **25**, 513-517.

Mora, J. R., Bono, M. R., Manjunath, N., Weninger, W., Cavanagh, L. L., Rosemblatt, M. & von Andrian, U. H. (2003). Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424**, 88-93.

Morris, D. R., Kuepfer, C. A., Ellingsworth, L. R., Ogawa, Y. & Rabinovitch, P. S. (1989a). Transforming growth factor-beta blocks proliferation but not early mitogenic signaling events in T-lymphocytes. *Exp Cell Res* **185**, 529-534.

Morris, G. P., Beck, P. L., Herridge, M. S., Depew, W. T., Szewczuk, M. R. & Wallace, J. L. (1989b). Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* **96**, 795-803.

Morteau, O., Morham, S. G., Sellon, R., Dieleman, L. A., Langenbach, R., O., S. & Sartor, R. B. (2000). Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. *J Clin Invest* **105**, 469-478.

Moses, H. L., Branum, E. L., Proper, J. A. & Robinson, R. A. (1981). Transforming Growth Factor Production by Chemically Transformed Cells. *Cancer Res* **41**, 2842-2848.

Moure, A., Gullón, P., Domínguez, H. & Parajó, J. C. (2006). Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochemistry* **41**, 1913-1923.

Murphy, S. J., Dore, J. J. E., Edens, M., Coffey, R. J., Barnard, J. A., Mitchell, H., Wilkes, M. & Leof, E. B. (2004). Differential Trafficking of Transforming Growth Factor-{beta} Receptors and Ligand in Polarized Epithelial Cells. *Mol Biol Cell* **15**, 2853-2862.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695-700.

Mylonaki, M., Rayment, N. B., Rampton, D. S., Hudspith, B. N. & Brostoff, J. (2005). Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis* **11**, 481-487.

Nakao, A., Miike, S., Hatano, M., Okumura, K., Tokuhisa, T., Ra, C. & Iwamoto, I. (2000). Blockade of Transforming Growth Factor {beta}/Smad Signaling in T Cells by Overexpression of Smad7 Enhances Antigen-induced Airway Inflammation and Airway Reactivity. *J Exp Med* **192**, 151-158.

Nakayama, K. (1994). Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of Porphyromonas gingivalis. *J Bacteriol* **176**, 1939-1943.

Nakazawa, A., Watanabe, M., Kanai, T., Yajima, T., Yamazaki, M., Ogata, H., Ishii, H., Azuma, M. & Hibi, T. (1999). Functional expression of costimulatory molecule CD86 on epithelial cells in the inflamed colonic mucosa. *Gastroenterology* **117**, 536-545.

Neish, A. S., Gewirtz, A. T., Zeng, H., Young, A. N., Hobert, M. E., Karmali, V., Rao, A. S. & Madara, J. L. (2000). Prokaryotic Regulation of Epithelial Responses by Inhibition of Ikappa B-alpha Ubiquitination. *Science* **289**, 1560-1563.

Neurath, M. F., Fuss, I., Kelsall, B. L., Presky, D. H., Waegell, W. & Strober, W. (1996). Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. *J Exp Med* **183**, 2605-2616.

Neurath, M. F., Wanitschke, R., Peters, M., Krummenauer, F., zum Buschenfelde, K. H. M. & Schlaak, J. F. (1999). Randomised trial of mycophenolate mofetil versus azathioprine for treatment of chronic active Crohn's disease. *Gut* **44**, 625-628.

Niess, J. H., Brand, S., Gu, X. & other authors (2005). CX3CR1-Mediated Dendritic Cell Access to the Intestinal Lumen and Bacterial Clearance. *Science* **307**, 254-258.

Niku-Paavola, M. L., Laitila, A., Mattila-Sandholm, T. & Haikara, A. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J Appl Microbiol* **86**, 29-35.

Niv, Y., Abuksis, G. & Fraser, G. M. (2000). Epidemiology of ulcerative colitis in Israel: a survey of Israeli Kibbutz settlements. *Am J Gastroenterol* **95**, 693-698.

Nochi, T., Yuki, Y., Terahara, K., Hino, A., Kunisawa, J., Kweon, M.-N., Yamaguchi, T. & Kiyono, H. (2004). Biological role of Ep-CAM in the physical interaction between epithelial cells and lymphocytes in intestinal epithelium. *Clin Immunol* **113**, 326-339.

Nunes, I., Munger, J. S., Harpel, J. G., Nagano, Y., Shapiro, R. L., Gleizes, P. E. & Rifkin, D. B. (1996). Structure and activation of the large latent transforming growth factor-beta complex. *Int J Obes Relat Metab Disord* **20**, S4-S8.

O'Hara, A. M. & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Rep* **7**, 688-693.

O'Neil, D. A., Porter, E. M., Elewaut, D., Anderson, G. M., Eckmann, L., Ganz, T. & Kagnoff, M. F. (1999). Expression and Regulation of the Human {beta}-Defensins hBD-1 and hBD-2 in Intestinal Epithelium. *J Immunol* **163**, 6718-6724.

Oatley, J. T., Rarick, M. D., Ji, G. E. & Linz, J. E. (2000). Binding of aflatoxin B1 to bifidobacteria in vitro. *J Food Prot* **63**, 1133-1136.

Ochman, H., Gerber, A. S. & Hartl, D. L. (1988). Genetic Applications of an Inverse Polymerase Chain Reaction. *Genetics* **120**, 621-623.

Okayasu, I., Hatakeyama, S., Yamada, M., Ohkusa, T., Inagaki, Y. & Nakaya, R. (1990). A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* **98**, 694-702.

Omata, N., Ohshima, Y., Yasutomi, M., Yamada, A., Karasuyama, H. & Mayumi, M. (2005). Ovalbumin-specific IgE modulates ovalbumin-specific T-cell response after repetitive oral antigen administration. *Journal of Allergy and Clinical Immunology* **115**, 822-827.

Orholm, M., Binder, V., oslash, rensen, T. I. A., Rasmussen, L. P. & Kyvik, K. O. (2000). Concordance of Inflammatory Bowel Disease among Danish Twins: Results of a Nationwide Study. *Scand J Gastroenterol* **35**, 1075 - 1081.

Ouellette, A. J., Pravtcheva, D., Ruddle, F. H. & James, M. (1989). Localization of the cryptdin locus on mouse chromosome 8. *Genomics* **5**, 233-239.

Ouellette, A. J. (1999). IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. *Am J Physiol* **277**, G257-261.

Owen, R. L., Apple, R. T. & Bhalla, D. K. (1986). Morphometric and cytochemical analysis of lysosomes in rat Peyer's patch follicle epithelium: their reduction in volume fraction and acid phosphatase content in M cells compared to adjacent enterocytes. *Anat Rec* **216**, 521-527.

Pappo, J. & Mahlman, R. T. (1993). Follicle epithelial M cells are a source of interleukin-1 in Peyer's patches. *Immunology* **78**, 505-507.

Pizarro, T. T., Arseneau, K. O., Bamias, G. & Cominelli, F. (2003). Mouse models for the study of Crohn's disease. *Trends in Molecular Medicine* **9**, 218-222.

Podolsky, D. K. (2002). Inflammatory Bowel Disease. *N Engl J Med* **347**, 417-429.

Powrie, F., Leach, M. W., Mauze, S., Menon, S., Caddle, L. B. & Coffman, R. L. (1994). Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553-562.

Powrie, F., Carlino, J., Leach, M. W., Mauze, S. & Coffman, R. L. (1996). A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* **183**, 2669-2674.

Poxton, I. R., Brown, R., Sawyerr, A. & Ferguson, A. (1997). Mucosa-associated bacterial flora of the human colon. *J Med Microbiol* **46**, 85-91.

Prindiville, T., Cantrell, M. & Wilson, K. H. (2004). Ribosomal DNA sequence analysis of mucosa-associated bacteria in Crohn's disease. *Inflamm Bowel Dis* **10**, 824-833.

Putney, S. D. & Burke, P. A. (1998). Improving protein therapeutics with sustained-release formulations. *Nat Biotech* **16**, 153-157.

Racke, M. K., Dhib-Jalbut, S., Cannella, B., Albert, P. S., Raine, C. S. & McFarlin, D. E. (1991). Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-beta 1. *J Immunol* **146**, 3012-3017.

Radford-Smith, G. L., Edwards, J. E., Purdie, D. M., Pandeya, N., Watson, M., Martin, N. G., Green, A., Newman, B. & Florin, T. H. J. (2002). Protective role of appendicectomy on onset and severity of ulcerative colitis and Crohn's disease. *Gut* **51**, 808-813.

Randall, L. A., Wadhwa, M., Thorpe, R. & Mire-Sluis, A. R. (1993). A novel, sensitive bioassay for transforming growth factor [beta]. *Journal of Immunological Methods* **164**, 61-67.

Ranges, G. E., Figari, I. S., Espevik, T. & Palladino, M. A. (1987). Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* **166**.

Regoli, M., Bertelli, E., Borghesi, C. & Nicoletti, C. (1995). Three-dimensional (3D-) reconstruction of M cells in rabbit Peyer's patches: definition of the intraepithelial compartment of the follicle-associated epithelium. *Anat Rec* **243**, 19-26.

Rembacken, B. J., Snelling, A. M., Hawkey, P. M., Chalmers, D. M. & Axon, A. T. R. (1999). Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *The Lancet* **354**, 635-639.

Roberts, S. J., Smith, A. L., West, A. B., Wen, L., Findly, R. C., Owen, M. J. & Hayday, A. C. (1996). T-cell alpha beta + and gamma delta + deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. *Proc Natl Acad Sci U S A* **93**, 11774-11779.

Robson, M. C., Phillips, T. J., Falanga, V., Odenheimer, D. J., Parish, L. C., Jensen, J. L. & Steed, D. L. (2001). Randomized trial of topically applied repifermin (recombinant human keratinocyte growth factor-2) to accelerate wound healing in venous ulcers. *Wound Repair Regen* **9**, 347-352.

Ron, D., Bottaro, D. P., Finch, P. W., Morris, D., Rubin, J. S. & Aaronson, S. A. (1993). Expression of biologically active recombinant keratinocyte growth factor. Structure/function analysis of amino-terminal truncation mutants. *J Biol Chem* **268**, 2984-2988.

Rosenstiel, P., Fantini, M., Bräutigam, K., Kühbacher, T., Waetzig, G. H., Seegert, D. & Schreiber, S. (2003). TNF-[alpha] and IFN-[gamma] regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* **124**, 1001-1009.

Ross Crittenden, Ojanen, S. K. S., Tenkanen, M., Fagerström, R., Mättö, J., Saarela, M., Mattila-Sandholm, T. & Poutanen, K. (2002). In vitro fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. *Journal of the Science of Food and Agriculture* **82**, 781-789.

Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. & Aaronson, S. A. (1989). Purification and Characterization of a Newly Identified Growth Factor Specific for Epithelial Cells. *Proc Natl Acad Sci U S A* **86**, 802-806.

Rubtsov, Y. P. & Rudensky, A. Y. (2007). TGF[beta] signalling in control of T-cell-mediated self-reactivity. *Nat Rev Immunol* **7**, 443-453.

Russell, R. K. & Satsangi, J. (2004). IBD: a family affair. *Best Practice & Research Clinical Gastroenterology* **18**, 525-539.

Rutgeerts, P., van Assche, G. & Vermeire, S. (2004). Optimizing anti-TNF treatment in inflammatory bowel disease. *Gastroenterology* **126**, 1593-1610.

Rycroft, C. E., Jones, M. R., Gibson, G. R. & Rastall, R. A. (2001). A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J Appl Microbiol* **91**, 878-887.

Sad, S. & Mosmann, T. R. (1994). Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J Immunol* **153**, 3514-3522.

Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C. & Horak, I. (1993). Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* **75**, 253-261.

Saitoh, S., Noda, S., Aiba, Y., Takagi, A., Sakamoto, M., Benno, Y. & Koga, Y. (2002). Bacteroides ovatus as the Predominant Commensal Intestinal Microbe Causing a Systemic Antibody Response in Inflammatory Bowel Disease. *Clin Diagn Lab Immunol* **9**, 54-59.

Salyers, A. A., Gherardini, F. & O'Brien, M. (1981). Utilization of Xylan by Two Species of Human Colonic Bacteroides. *Appl Environ Microbiol* **41**, 1065-1068.

Sandborn, W. J., Present, D. H., Isaacs, K. L. & other authors (2003a). Tacrolimus for the treatment of fistulas in patients with crohn's disease: a randomized, placebo-controlled trial. *Gastroenterology* **125**, 380-388.

Sandborn, W. J., Sands, B. E., Wolf, D. C. & other authors (2003b). Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: a

randomized, double-blind, placebo-controlled, dose-escalation trial. *Aliment Pharmacol Ther* **17**, 1355-1364.

Sandborn, W. J., Colombel, J. F., Enns, R. & other authors (2005). Natalizumab Induction and Maintenance Therapy for Crohn's Disease. *N Engl J Med* **353**, 1912-1925.

Sanderson, N., Factor, V., Nagy, P., Kopp, J., Kondaiah, P., Wakeield, L., Roberts, A. B., Sporn, M. B. & Thorgeirsson, S. S. (1995). Hepatic Expression of Mature Transforming Growth Factor {beta}1 in Transgenic Mice Results in Multiple Tissue Lesions. *Proc Natl Acad Sci U S A* **92**, 2572-2576.

Sansonetti, P. J. (2004). War and paece at mucosal surfaces. *Nat Rev Immunol* **4**, 953-964.

Satsangl, J., Farrant, J. M., Jewell, D. P., Bell, J. I., Welsh, K. I., Bunce, M. & Julier, C. (1996). Contribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory bowel disease. *The Lancet* **347**, 1212-1217.

Savage, D. C. (1999). Mucosal Microbiota. In *Mucosal Immunology* pp. 19-30. Edited by P. Ogra, J. Mestecky, M. Lamm, W. Strober, J. Bienenstock & J. McGhee. San Diego: Academic Press.

Scheppach, W., Luehrs, H. & Menzel, T. (2001). Beneficial health effects of low-digestible carbohydrate consumption. *Br J Nutr* **85**, S23-S30.

Schotte, L., Steidler, L., Vandekerckhove, J. & Remaut, E. (2000). Secretion of biologically active murine interleukin-10 by Lactococcus lactis. *Enzyme Microb Technol* **27**, 761-765.

Schultz, M., Timmer, A., Herfarth, H., Sartor, R. B., Vanderhoof, J. & Rath, H. (2004). Lactobacillus GG in inducing and maintaining remission of Crohn's disease. *BMC Gastroenterol* **4**, 5.

Serra, R. (2002). Transforming Growth Factor beta (TGF-b). *Encyclopedia of Life Sciences*.

Shah, M., Foreman, D. M. & Ferguson, M. W. (1995). Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* **108**, 985-1002.

Shanahan, F. (2002). The host-microbe interface within the gut. *Best Pract Res Clin Gastroenterol* **16**, 915-931.

Shiba, T., Aiba, Y., Ishikawa, H., Ushiyama, A., Takagi, A., Mine, T. & Koga, Y. (2003). The Suppressive Effect of Bifidobacteria on Bacteroides vulgatus, a Putative Pathogenic Microbe in Inflammatory Bowel Disease. *Microbiol Immunol* **47**, 371-378.

Shibolet, O., Karmeli, F., Eliakim, R., Swennen, E., Brigidi, P., Gionchetti, P., Campieri, M., Morgenstern, S. & Rachmilewitz, D. (2002). Variable response to probiotics in two models of experimental colitis in rats. *Inflamm Bowel Dis* **8**, 399-406.

Shoemaker, N. B., Vlamakis, H., Hayes, K. & Salyers, A. A. (2001). Evidence for Extensive Resistance Gene Transfer among Bacteroides spp. and among Bacteroides and Other Genera in the Human Colon. *Appl Environ Microbiol* **67**, 561-568.

Shoucheng, N., Chaoxiang, S., Khan, W. B., Benson, W., Lacey, D. L. & Knox, S. J. (1998). Effects of keratinocyte growth factor on the proliferation and radiation survival of human squamous cell carcinoma cell lines in vitro and in vivo. *Int J Radiat Oncol Biol Phys* **40**, 177-187.

Shull, M. M., Ormsby, I., Kier, A. B. & other authors (1992). Targeted disruption of the mouse transforming growth factor-[beta]1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-699.

Simmons, D. L., Botting, R. M. & Hla, T. (2004). Cyclooxygenase Isozymes: The Biology of Prostaglandin Synthesis and Inhibition. *Pharmacol Rev* **56**, 387-437.

Singh, J. C. I., Cruickshank, S. M., Newton, D. J., Wakenshaw, L., Graham, A., Lan, J., Lodge, J. P. A., Felsburg, P. J. & Carding, S. R. (2005). Toll-like receptor-mediated responses of primary intestinal epithelial cells during the development of colitis. *Am J Physiol Gastrointest Liver Physiol* **288**, G514-524.

Sinha, A., Nightingale, J. M. D., West, K. P., Berlanga-Acosta, J. & Playford, R. J. (2003). Epidermal Growth Factor Enemas with Oral Mesalamine for Mild-to-Moderate Left-Sided Ulcerative Colitis or Proctitis. *N Engl J Med* **349**, 350-357.

Snyder, J. D. & Walker, W. A. (1987). Structure and function of intestinal mucin: developmental aspects. *Int Arch Allergy Appl Immunol* **82**, 351-356.

Soderholm, J. D., Olaison, G., Peterson, K. H., Franzen, L. E., Lindmark, T., Wiren, M., Tagesson, C. & Sjodahl, R. (2002). Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease. *Gut* **50**, 307-313.

Sonis, S. T., Lindquist, L., Van Vugt, A., Stewart, A. A., Stam, K., Qu, G. Y., Iwata, K. K. & Haley, J. D. (1994). Prevention of Chemotherapy-induced Ulcerative Mucositis by Transforming Growth Factor {beta}3. *Cancer Res* **54**, 1135-1138.

Sporn, M. B., Roberts, A. B., Shull, J. H., Smith, J. M., Ward, J. M. & Sodek, J. (1983). Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo. *Science* **219**, 1329-1331.

Steidler, L., Wells, J. M., Raeymaekers, A., Vandekerckhove, J., Fiers, W. & Remaut, E. (1995). Secretion of biologically active murine interleukin-2 by Lactococcus lactis subsp. lactis. *Appl Environ Microbiol* **61**, 1627-1629.

Steidler, L., Robinson, K., Chamberlain, L., Schofield, K. M., Remaut, E., Le Page, R. W. F. & Wells, J. M. (1998). Mucosal Delivery of Murine Interleukin-2 (IL-2) and IL-6 by Recombinant Strains of Lactococcus lactis Coexpressing Antigen and Cytokine. *Infect Immun* **66**, 3183-3189.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. & Remaut, E. (2000). Treatment of Murine Colitis by Lactococcus lactis Secreting Interleukin-10. *Science* **289**, 1352-1355.

Steidler, L. (2001). Microbiological and immunological strategies for treatment of inflammatory bowel disease. *Microbes and Infection* **3**, 1157-1166.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P. & Remaut, E. (2003). Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. *Nat Biotech* **21**, 785-789.

Stronati, L., Negroni, A., Merola, P., Pannone, V., Borrelli, O., Cirulli, M., Annese, V. & Cucchiara, S. (2008). Mucosal NOD2 expression and NF-kappaB activation in pediatric Crohn's disease. *Inflamm Bowel Dis* **14**, 295-302.

Sturm, A. & Dignass, A. U. (2008). Epithelial restitution and wound healing in inflammatory bowel disease. *World J Gastroenterol* **14**, 348-353.

Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G. R., Collins, M. D. & Dore, J. (1999). Direct Analysis of Genes Encoding 16S rRNA from Complex Communities Reveals Many Novel Molecular Species within the Human Gut. *Appl Environ Microbiol* **65**, 4799-4807.

Sun, J., Dirden-Kramer, B., Ito, K., Ernst, P. B. & Van Houten, N. (1999). Antigen-Specific T Cell Activation and Proliferation During Oral Tolerance Induction. *J Immunol* **162**, 5868-5875.

Sundberg, J. P., Elson, C. O., Bedigian, H. & Birkenmeier, E. H. (1994). Spontaneous, heritable colitis in a new substrain of C3H/HeJ mice. *Gastroenterology* **107**, 1726-1735.

Tai, E. K. K., Wu, W. K. K., Wong, H. P. S., Lam, E. K. Y., Yu, L. & Cho, C. H. (2007). A New Role for Cathelicidin in Ulcerative Colitis in Mice. *Exp Biol Med (Maywood)* **232**, 799-808.

Takeuchi, T., Nakaya, Y., Kato, N., Watanabe, K. & Morimoto, K. (1999). Induction of oxidative DNA damage in anaerobes. *FEBS Letters* **450**, 178-180.

Tancula, E., Feldhaus, M. J., Bedzyk, L. A. & Salyers, A. A. (1992). Location and characterization of genes involved in binding of starch to the surface of Bacteroides thetaiotaomicron. *J Bacteriol* **174**, 5609-5616.

Tato, C. M. & Hunter, C. A. (2002). Host-Pathogen Interactions: Subversion and Utilization of the NF-{kappa}B Pathway during Infection. *Infect Immun* **70**, 3311-3317.

Taupin, D. & Podolsky, D. K. (2003). Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* **4**, 721-732.

Taurog, J. D., Richardson, J. A., Croft, J. T., Simmons, W. A., Zhou, M., Fernández-Sueiro, J. L., Balish, E. & Hammer, R. E. (1994). The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med* **180**, 2359-2364.

Telega, G. W., Baumgart, D. C. & Carding, S. R. (2000). Uptake and Presentation of Antigen to T Cells by Primary Colonic Epithelial Cells in Normal and Diseased States. *Gastroenterology* **119**, 1548-1559.

Termont, S., Vandenbroucke, K., Iserentant, D., Neirynck, S., Steidler, L., Remaut, E. & Rottiers, P. (2006). Intracellular Accumulation of Trehalose Protects Lactococcus lactis from Freeze-Drying Damage and Bile Toxicity and Increases Gastric Acid Resistance. *Appl Environ Microbiol* **72**, 7694-7700.

Thompson-Chagoyan, O. C., Maldonado, J. & Gil, A. (2005). Aetiology of inflammatory bowel disease (IBD): Role of intestinal microbiota and gut-associated lymphoid tissue immune response. *Clin Nutr* **24**, 339-352.

Thompson, J. A. (1993). Molecular biology of xylan degradation. *FEMS Microbiol Rev* **10**, 65-82.

Thompson, N. P., Driscoll, R., Pounder, R. E., Wakefield, A. J. & Bowel, I. (1996). Genetics versus environment in inflammatory bowel disease: results of a British twin study *BMJ* **312**, 95-96

Thorbecke, G. J., R, S., C, H. L., A, P. K., A, M. H. & M, A. P. (1992). Involvement of endogenous tumor necrosis factor alpha and transforming growth factor beta during induction of collagen type II arthritis in mice. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 7375-7379.

Tiede, I., Fritz, G., Strand, S. & other authors (2003). CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* **111**, 1133-1145.

Tilg, H., Vogelsang, H., Ludwiczek, O. & other authors (2003). A randomised placebo controlled trial of pegylated interferon {alpha} in active ulcerative colitis. *Gut* **52**, 1728-1733.

Tsubouchi, S. & Leblond, C. P. (1979). Migration and turnover of entero-endocrine and caveolated cells in the epithelium of the descending colon, as shown by radioautography after continuous infusion of 3H-thymidine into mice. *Am J Anat 1979 Dec;156(4):431-51* **156**, 431-451.

Tuan, T. L., Cheung, D. T., Wu, L. T., Yee, A., Gabriel, S., Han, B., Morton, L., Nimni, M. E. & Hall, F. L. (1996). Engineering, expression and renaturation of targeted TGF-beta fusion proteins. *Connect Tissue Res* **34**, 1-9.

Tysk, C., Lindberg, E., Jarnerot, G. & Floderus-Myrhed, B. (1988). Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* **29**, 990-996.

Ueki, T., Mizuno, M., Uesu, T., Kiso, T. & Tsuji, T. (1995). Expression of ICAM-I on M cells covering isolated lymphoid follicles of the human colon. *Acta Med Okayama* **49**, 145-151.

Ulloa, L., Doody, J. & Massague, J. (1999). Inhibition of transforming growth factor-[beta]/SMAD signalling by the interferon-[gamma]/STAT pathway. *Nature* **397**, 710-713.

Vallance, B. A., Gunawan, M. I., Hewlett, B., Bercik, P., Van Kampen, C., Galeazzi, F., Sime, P. J., Gauldie, J. & Collins, S. M. (2005). TGF-{beta}1 gene transfer to the mouse colon leads to intestinal fibrosis. *Am J Physiol Gastrointest Liver Physiol* **289**, G116-128.

Van Assche, G., Sandborn, W. J., Feagan, B. G. & other authors (2006). Daclizumab, a humanised monoclonal antibody to the interleukin 2 receptor (CD25), for the treatment of moderately to severely active ulcerative colitis: a randomised, double blind, placebo controlled, dose ranging trial. *Gut* **55**, 1568-1574.

van der Waaij, D., Berghuis-de Vries, J., M & Lekkerkerk Lekkerkerk-v (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J Hyg* **69**, 405-411.

Van Houten, N. & Blake, S. F. (1996). Direct measurement of anergy of antigen-specific T cells following oral tolerance induction. *J Immunol* **157**, 1337-1341.

Venturi, Gionchetti, Rizzello, Johansson, Zucconi, Brigidi, Matteuzzi & Campieri (1999). Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment Pharmacol Ther* **13**, 1103-1108.

Viney, J. L., Mowat, A. M., O'Malley, J. M., Williamson, E. & Fanger, N. A. (1998). Expanding Dendritic Cells In Vivo Enhances the Induction of Oral Tolerance. *J Immunol* **160**, 5815-5825.

Visco, V., Belleudi, F., Marchese, C., Leone, L., Aimati, L., Cardinali, G., Kovacs, D., Frati, L. & Torrisi, M. R. (2004). Differential response to keratinocyte

growth factor receptor and epidermal growth factor receptor ligands of proliferating and differentiating intestinal epithelial cells. *J Cell Physiol* **200**, 31-44.

Waeytens, A., Ferdinande, L., Neirynck, S., Rottiers, P., De Vos, M., Steidler, L. & Cuvelier, C. A. (2008). Paracellular entry of interleukin-10 producing <I>Lactococcus lactis</I> in inflamed intestinal mucosa in mice. *Inflamm Bowel Dis* **14**, 471-479.

Wakefield, L. M., Winokur, T. S., Hollands, R. S., Christopherson, K., Levinson, A. D. & Sporn, M. B. (1990). Recombinant latent transforming growth factor beta 1 has a longer plasma half-life in rats than active transforming growth factor beta 1, and a different tissue distribution.Wakefield LM, Winokur TS, Hollands RS, Christopherson K, Levinson AD, Sporn MB. *J Clin Invest* **86**, 1978-1984.

Wakefield, L. M. & Roberts, A. B. (2002). TGF-[beta] signaling: positive and negative effects on tumorigenesis. *Curr Opin Gene Develop* **12**, 22-29.

Warshaw, A. L., Walker, W. A., Cornell, R. & Isselbacher, K. J. (1971). Small intestinal permeability to macromolecules. Transmission of horseradish peroxidase into mesenteric lymph and portal blood. *Lab Invest* **25**, 675-684.

Weaver, J., Whitehead, T. R., Cotta, M. A., Valentine, P. C. & Salyers, A. A. (1992). Genetic analysis of a locus on the Bacteroides ovatus chromosome which contains xylan utilization genes. *Appl Environ Microbiol* **58**, 2764-2770.

Wehkamp, J., Harder, J., Weichenthal, M., Mueller, O., Herrlinger, K. R., Fellermann, K., Schroeder, J. M. & Stange, E. F. (2003). Inducible and Constitutive [beta]-Defensins Are Differentially Expressed in Crohn's Disease and Ulcerative Colitis. *Inflamm Bowel Dis* **9**, 215-223.

Werner, S., Smola, H., Liao, X., Longaker, M. T., Krieg, T., Hofschneider, P. H. & Williams, L. T. (1994). The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science* **266**, 819-822.

Werner, S. (1998). Keratinocyte Growth Factor: A Unique Player in Epithelial Repair Processes. *Cytokine & Growth Factor Reviews* **9**, 153-165.

Whitehead, T. R. & Hespell, R. B. (1990). The genes for three xylan-degrading activities from Bacteroides ovatus are clustered in a 3.8-kilobase region. *J Bacteriol* **172**, 2408-2412.

Whitehead, T. R., Cotta, M. A. & Hespell, R. B. (1991). Introduction of the Bacteroides ruminicola xylanase gene into the Bacteroides thetaiotaomicron chromosome for production of xylanase activity. *Appl Environ Microbiol* **57**, 277-282.

Whitehead, T. R. (1995). Nucleotide sequences of xylan-inducible xylanase and xylosidase/arabinosidase genes from Bacteroides ovatus V975. *Biochim Biophys Acta* **1244**, 239-241.

Whitman, W. B., Coleman, D. C. & Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc Natl Acad Sci U S A* **95**, 6578-6583.

Wiercińska-Drapało, A., Flisiak, R. & Prokopowicz, D. (2001). Effect of ulcerative colitis activity on plasma concentration of transforming growth factor beta1. *Cytokine* **14**, 343-346.

Wilson, K. (1997). Preparation of Genomic DNA from Bacteria. In *Curr Protocols Molecular Biol*. Edited by M. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith & K. Struhl. New York: John Wiley & Sons, Inc.

Wolfraim, L. A., Walz, T. M., James, Z., Fernandez, T. & Letterio, J. J. (2004). p21Cip1 and p27Kip1 Act in Synergy to Alter the Sensitivity of Naive T Cells to TGF-{beta}-Mediated G1 Arrest through Modulation of IL-2 Responsiveness. *J Immunol* **173**, 3093-3102.

Wood, J. D., Peck, O. C., Tefend, K. S., Rodriguez-M, M. A., Rodriguez-M, J. V., Hernandez-C, J. I., Stonerook, M. J. & Sharma, H. M. (1998). Colitis and Colon Cancer in Cotton-Top Tamarins (Saguinus oedipus oedipus) Living Wild in Their Natural Habitat. *Digestive Diseases and Sciences* **43**, 1443-1453.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. & Massague, J. (1994). Mechanism of activation of the TGF-[beta] receptor. *Nature* **370**, 341-347.

Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., Hooper, L. V. & Gordon, J. I. (2003). A Genomic View of the Human-Bacteroides thetaiotaomicron Symbiosis. *Science* **299**, 2074-2076.

Yacyshyn, B. R., Chey, W. Y., Goff, J. & other authors (2002). Double blind, placebo controlled trial of the remission inducing and steroid sparing properties of an ICAM-1 antisense oligodeoxynucleotide, alicaforsen (ISIS 2302), in active steroid dependent Crohn's disease. *Gut* **51**, 30-36.

Yap, L. M., Ahmad, T. & Jewell, D. P. (2004). The contribution of HLA genes to IBD susceptibility and phenotype. *Best Practice & Research Clinical Gastroenterology* **18**, 577-596.

Ye, F., Duvillié, B. & Scharfmann, R. (2005). Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. *Diabetologia* **48**, 277-281.

Yio, X. Y. & Mayer, L. (1997). Characterization of a 180-kDa Intestinal Epithelial Cell Membrane Glycoprotein, gp180. A candidate molecule mediating T cell-epithelial cell interactions. *J Biol Chem* **272**, 12786-12792.

Zampa, A., Silvi, S., Fabiani, R., Morozzi, G., Orpianesi, C. & Cresci, A. (2004). Effects of different digestible carbohydrates on bile acid metabolism and SCFA production by human gut micro-flora grown in an in vitro semi-continuous culture. *Anaerobe* **10**, 19-26.

Zeeh, J. M., Procaccino, F., Hoffmann, P. & other authors (1996). Keratinocyte growth factor ameliorates mucosal injury in an experimental model of colitis in rats. *Gastroenterology* **110**, 1077-1083.

Zhang, M. Z., Wen, J., Arakawa, T. & Prestrelski, S. J. (1995). A New Strategy for Enhancing the Stability of Lyophilized Protein: The Effect of the Reconstitution Medium on Keratinocyte Growth Factor. *Pharmaceutical Research* **12**, 1447-1452.

Zhao, C., Wang, I. & Lehrer, R. I. (1996). Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. . *FEBS Letters* **396**, 319-322.

Zimmer, K.-P., Buning, J., Weber, P., Kaiserlian, D. & Strobel, S. (2000). Modulation of antigen trafficking to MHC class II-positive late endosomes of enterocytes. *Gastroenterology* **118**, 128-137.