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**Characterisation of adhesion of a probiotic  
bacterium *Lactobacillus rhamnosus* HN001  
to extracellular matrix proteins and the  
intestinal cell line Caco-2**

A thesis presented to Massey University in partial fulfilment of the requirement for the  
degree of Master of Science in Microbiology

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## ABSTRACT

This study focuses on *Lactobacillus rhamnosus* HN001, a potential candidate for use as a probiotic. Probiotics are microorganisms that can exert a beneficial effect on a host. It is believed that the ability of a probiotic to colonise gastrointestinal surfaces is important in its ability to exert a beneficial effect on the host. In order to do so, it is thought the microorganism must be able to adhere to molecules found on intestinal cells. HN001 has been shown to adhere to human intestinal cell lines (Gopal *et al.*, 2001). This study characterises the molecular species involved in the adherence of HN001 to intestinal molecules and cell lines, which may be important in the ability of HN001 to exert health benefits in a host.

Both liquid and solid-phase binding assays were used to characterise HN001 binding to extracellular matrix (ECM) components found in intestinal tissues. Of the ECM components investigated, HN001 bound fibronectin with the highest affinity. This interaction was specific, saturable and dependent on the growth phase of HN001. HN001 bound immobilised fibronectin in preference to soluble fibronectin through a protein-dependent interaction. HN001 was also found to bind to the N-terminal heparin binding domain of fibronectin and the C-terminal part of the first type III repeat in the fibronectin molecule (III<sub>1</sub>-C). HN001 adhered to the human intestinal cell line, Caco-2, in a dose-dependent manner that was enhanced by a pH-sensitive factor present in the spent culture supernatant.

Since fibronectin-binding was identified as a possible mechanism for adherence of HN001 to intestinal tissues, HN001 genome DNA sequence was examined for genes encoding putative fibronectin-binding proteins. Fbl (Fibronectin-binding like) was identified through its similarity to fibronectin-binding proteins from *Streptococcus pneumoniae* (Holmes *et al.*, 2001) and *S. pyogenes* (Courtney *et al.*, 1994). Fbl was expressed by a GST fusion system and used to compete with HN001 adhesion in liquid-phase binding assays to ascertain its function. Since difficulties were experienced when expressing and purifying soluble Fbl, an insertional disruption of the *fbl* gene was created and its phenotype investigated in liquid-phase, solid-phase and Caco-2 binding assays to determine Fbl function.



## ABBREVIATIONS

BLAST	Basic Local Alignment Search Tools
BSA	Bovine Serum Albumin
cFn	Cellular fibronectin
cfu	Colony forming units
CIII	C-terminal part of the first type III repeat in fibronectin
cpm	Counts per minute
Da	Dalton
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra Acetic Acid Disodium salt
ELISA	Enzyme-Linked Immunosorbent Assay
Em	Erythromycin
Fbl	Fibronectin-binding-like protein
Fn	Fibronectin
FRC	Fonterra Research Centre
FPLC	Fast Performance Liquid Chromatography
GST	Glutathione S-transferase
HRP	Horseradish Peroxidase
IAA	Iso Amyl Alcohol
IPTG	Isopropylthio- $\beta$ -D-galactoside
LAB	Lactic acid bacteria
LTA	Lipoteichoic acid
NEB	New England Biolabs
OD	Optical density
ORF	Open reading frame
ori	Origin of replication
PCR	Polymerase chain reaction
pFn	Plasma fibronectin
SCS	Spent culture supernatant
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sFn	Super fibronectin
Tm	Melting temperature
TMB	3,3',5,5' tetramethylbenzidine
WT	Wild type

# TABLE OF CONTENTS

ABSTRACT	i
ABBREVIATIONS	ii
TABLE OF CONTENTS	iii
LIST OF FIGURES	ix
LIST OF TABLES	xi
 <b>CHAPTER ONE: INTRODUCTION</b>	 <b>1</b>
 <b>1.1 Lactic Acid Bacteria</b>	 <b>1</b>
1.1.1 General characteristics of lactic acid bacteria	1
1.1.2 Lactobacilli	1
1.1.2.1 Surface characteristics of lactobacilli	3
 <b>1.2 Probiotic bacteria</b>	 <b>5</b>
1.2.1 Definition	5
1.2.2 Selection of probiotic strains	5
1.2.3 <i>L. rhamnosus</i> HN001 (DR20™)	7
 <b>1.3 Gastrointestinal surfaces available for bacterial adhesion</b>	 <b>9</b>
1.3.1 Why is adhesion important to bacteria?	9
1.3.2 The gastrointestinal surface	9
1.3.3 The extracellular matrix and basement membrane	13
1.3.3.1 Fibronectin	14
1.3.4 The Caco-2 cell line	16
1.3.4.1 Adhesion of lactobacilli to Caco-2 cells	16
 <b>1.4 Mechanisms of adhesion of bacteria</b>	 <b>17</b>
1.4.1 General model for adhesion of bacteria	17
1.4.2 Bacterial adhesins	18
1.4.2.1 Fibronectin-binding proteins	19
1.4.2.2 Characteristics of cell surface proteins	20
 <b>1.5 Characterised adhesins of <i>Lactobacillus</i> species</b>	 <b>21</b>
 <b>1.6 Aims of this study</b>	 <b>23</b>
 <b>CHAPTER 2: MATERIALS AND METHODS</b>	 <b>24</b>
 <b>2.1 Bacterial strains and plasmids</b>	 <b>24</b>

<b>2.2</b>	<b>Media and Buffers</b>	<b>24</b>
2.2.1	Media	24
2.2.2	Buffers and Solutions	28
2.2.3	Protein solutions	32
<b>2.3</b>	<b>Bacteriological methods</b>	<b>34</b>
2.3.1	Culture conditions	34
2.3.2	Storage of strains	35
2.3.3	Determination of bacteria numbers	35
2.3.4	Preparation of HN001 spent culture supernatant	35
<b>2.4</b>	<b>DNA manipulation</b>	<b>36</b>
2.4.1	Preparation of plasmid DNA from <i>E. coli</i> DH5 $\alpha$	36
2.4.2	Preparation of total genomic DNA from HN001	36
2.4.3	Purification of genomic DNA from HN001	37
2.4.4	Storage of DNA	38
2.4.5	Quantitation of DNA	38
2.4.6	Agarose gel electrophoresis of DNA	38
2.4.7	Restriction digestion of DNA	39
2.4.8	Sequencing genomic DNA	39
<b>2.5</b>	<b>PCR amplification of DNA</b>	<b>40</b>
2.5.1	Oligonucleotide primer design	40
2.5.2	PCR amplification of DNA targets	43
2.5.3	Purification of PCR products	44
2.5.4	Sequencing of PCR products	44
<b>2.6</b>	<b>Characterisation of <i>fbl</i> in silico</b>	<b>44</b>
2.6.1	Use of ORF prediction tools, BLASTP and sequence alignments	44
2.6.2	Use of Motif search engines	45
<b>2.7</b>	<b>Cloning of DNA into plasmid vectors</b>	<b>45</b>
2.7.1	Vectors	45
2.7.2	Primer design and PCR amplification for cloning into plasmid vectors	46
2.7.3	Ligation of inserts into plasmid vectors	46
2.7.4	Transformation of <i>E. coli</i> DH5 $\alpha$ or TOP10 cells	47
2.7.5	Screening of transformants	47
<b>2.8</b>	<b>Expression of gene products</b>	<b>48</b>
2.8.1	Expression conditions	48
2.8.2	Lysis of <i>E. coli</i> cells	48
2.8.2.1	Sonication	48

2.8.2.2	Freeze/thaw	49
2.8.2.3	French Press	49
2.8.3	Protein gel electrophoresis	49
<b>2.9</b>	<b>Purification of fusion proteins</b>	<b>50</b>
<b>2.10</b>	<b>Insertional disruption of gene targets</b>	<b>51</b>
2.10.1	Vectors for insertional disruption	51
2.10.2	Transformation of HN001 cells	51
2.10.3	HN001 colony PCR	52
<b>2.11</b>	<b>Southern Blot</b>	<b>52</b>
2.11.1	Southern transfer	52
2.11.2	Probe labelling and Hybridisation	53
2.11.3	Washing and Signal detection	54
<b>2.12</b>	<b>Preparation of crude HN001 cell envelope</b>	<b>54</b>
<b>2.13</b>	<b>Immunoblotting</b>	<b>55</b>
2.13.1	Inoculation of rabbits	55
2.13.2	Preparation of SDS-PAGE gels for Western Blotting	55
2.13.3	Western transfer	56
2.13.4	Immunodetection	56
<b>2.14</b>	<b>Purification of IgG from anti-serum</b>	<b>57</b>
2.14.1	Purification of IgG using FPLC	57
2.14.2	Desalting and Storage of purified IgG	58
<b>2.15</b>	<b>Liquid-phase binding assays for ECM proteins</b>	<b>58</b>
2.15.1	<sup>125</sup> I labelling of proteins	58
2.15.2	<sup>125</sup> I fibronectin binding assays	59
<b>2.16</b>	<b>Solid-phase binding assays</b>	<b>60</b>
2.16.1	Coating microtitre plates with protein or bacterial cells	60
2.16.2	Binding of bacteria to immobilised proteins	60
2.16.3	Detection of HN001 cells by ELISA	60
2.16.3.1	Crisscross serial dilution	61
2.16.4	Detection by radioisotope labelling	61
2.16.5	Detection by Crystal violet staining	62
2.16.6	Detection by Phase contrast microscopy	62
2.16.7	Detection by OD <sub>403</sub>	63
2.16.8	Glass slide binding assays	63

<b>2.17</b>	<b>Binding to Caco-2 cells</b>	<b>64</b>
2.17.1	Growth and Maintenance of the intestinal cell line Caco-2	64
2.17.2	Estimation of number of Caco-2 cells per well in 6-well plate	64
2.17.3	Adhesion assay	65
2.17.3.1	Metabolic labelling of HN001 grown on MRS agar plates	65
<b>2.18</b>	<b>Pre-treatment of HN001 cells</b>	<b>65</b>
2.18.1	Protease treatment	65
2.18.2	Periodate treatment	66
<b>CHAPTER THREE:</b>	<b>RESULTS</b>	<b>67</b>
<b>3.1</b>	<b>Liquid-phase binding assays for intestinal molecules</b>	<b>67</b>
3.1.1	HN001 binds soluble fibronectin	67
3.1.2	HN001 binding to <sup>125</sup> I-fibronectin is saturable	68
3.1.3	HN001 binding to <sup>125</sup> I-fibronectin is dependant on growth phase	70
3.1.4	HN001 binding to <sup>125</sup> I-fibronectin is specific	70
<b>3.2</b>	<b>Solid-phase binding assays for intestinal molecules</b>	<b>73</b>
3.2.1	Detection of rabbit anti-HN001 cell envelope antibody titre by Immunoblotting	73
3.2.2	Purification of rabbit anti-HN001 cell envelope IgG	75
3.2.3	Optimisation of detection of HN001 cells	76
3.2.4	HN001 binding to extracellular matrix components in solid-phase	77
3.2.5	Dose-dependent binding of HN001 to fibronectin	78
3.2.6	Binding of HN001 to different fragments of fibronectin	79
3.2.7	Specificity of interaction between HN001 and fibronectin	81
3.2.8	Evaluation of different methods to detect HN001 cells	84
3.2.8.1	Crystal violet detection	84
3.2.8.2	OD <sub>405</sub> detection	85
3.2.8.3	Phase-contrast microscopy detection	85
3.2.8.4	Slide-binding assays	85
3.2.8.5	Radioisotope labelling	85
3.2.9	Binding of protease and periodate treated cells to fibronectin	87
<b>3.3</b>	<b>Caco-2 adhesion assays</b>	<b>89</b>
3.3.1	There are approximately $7 \times 10^6$ Caco-2 cells per adhesion assay	89
3.3.2	Dose-dependent adhesion of HN001 to Caco-2 cells	89
3.3.3	A factor in HN001 SCS promotes HN001 adhesion to Caco-2 cells	91
3.3.4	Role of HN001 spent culture supernatant in fibronectin binding	93
3.3.5	Competition of HN001 adhesion to Caco-2 with IgG	94
3.3.6	Competition of HN001 adhesion to Caco-2 by fibronectin	96

3.3.7	Adhesion of surface-treated HN001 cells to Caco-2 cells	98
<b>3.4</b>	<b>Characterisation of <i>fbl</i></b>	<b>99</b>
3.4.1	Identification of Fbl as a putative fibronectin-binding protein	99
3.4.2	Sequencing of <i>fbl</i>	99
3.4.3	In silico characterisation of <i>fbl</i>	99
3.4.3.1	Use of BLASTP and sequence alignments	99
3.4.3.2	Use of motif search engines	104
3.4.4	Biochemical characterisation of Fbl function	105
3.4.4.1	Expression of Fbl	105
3.4.4.2	Purification of Fbl fusion proteins	108
3.4.4.2	Competition of HN001 binding to <sup>125</sup> I-fibronectin with Fbl4-GST	110
3.4.5	Genetic characterisation of Fbl function	111
3.4.5.1	Creation of integration construct	111
3.4.5.2	HN001 colony PCR	112
3.4.5.3	Southern Blot	112
3.4.5.4	Phenotype of HN001Δ <i>fbl</i>	116
<b>CHAPTER FOUR:</b>	<b>DISCUSSION</b>	<b>119</b>
4.1	HN001 binds fibronectin	119
4.2	HN001 binds Caco-2 cells	123
4.3	Characterisation of Fbl	129
4.3.1	Is Fbl likely to be a fibronectin-binding protein?	129
4.3.2	Characterisation of Fbl function	131
<b>CHAPTER FIVE:</b>	<b>SUMMARY AND FUTURE DIRECTIONS</b>	<b>133</b>
4.1	Summary	133
5.2	Future directions	134
<b>REFERENCES</b>		<b>136</b>
<b>APPENDIX 1: FORMULAE USED</b>		<b>147</b>
<b>APPENDIX 2: MAPS OF VECTORS USED</b>		<b>148</b>
<b>APPENDIX 3: <i>IN SILICO</i> CHARACTERISATION</b>		<b>150</b>

Sequence assembly of <i>fbl</i>	150
Prediction of a signal peptide	150
BLASTP results	151
Dot Plot results	152
<b>APPENDIX 4: ENUMERATION OF CACO-2 CELLS</b>	<b>153</b>



## LIST OF FIGURES

Figure 1 Representation of the typical cell wall architecture of lactobacilli	4
Figure 2 Surface of small intestine of humans	10
Figure 3 Surface of the large intestine of humans	11
Figure 4 Diagram of an epithelial cell	12
Figure 5 Structure of fibronectin	15
Figure 6 Alignment of the fibronectin-binding repeat region (R2)	20
Figure 7 HN001 binding to <sup>125</sup> I-fibronectin is saturable	71
Figure 8 Stationary-phase binds more <sup>125</sup> I-Fn than exponential-phase HN001	71
Figure 9 HN001 binding to <sup>125</sup> I-fibronectin is specific	72
Figure 10 Antiserum has high reactivity to HN001 cell envelope at 1/1000 dilution	74
Figure 11 Purification of rabbit anti-HN001 cell envelope IgG	75
Figure 12 Immunoblot	76
Figure 13 HN001 binds three forms of fibronectin in the solid-phase	77
Figure 14 Dose-dependent binding of HN001 to fibronectin	78
Figure 15 Structure of plasma fibronectin	79
Figure 16 HN001 binds CIII and p30 fragments of fibronectin	80
Figure 17 Super fibronectin and CIII disrupt HN001 binding to immobilised plasma fibronectin	82
Figure 18 CIII disrupts HN001 binding to super fibronectin (sFn)	83
Figure 19 Super fibronectin has small effect on HN001 binding to CIII	83
Figure 20 Crystal violet staining not suitable for detecting HN001 cells	84
Figure 21 Slide-binding assay	86
Figure 22 Protease treatment of HN001 cells decreases binding to fibronectin	87
Figure 23 Periodate oxidation of HN001 cell surfaces increases non-specific binding	88
Figure 24 Dose-dependent adhesion of HN001 to Caco-2 cells	90
Figure 25 A factor in HN001 SCS promotes adhesion of HN001 to Caco-2 cells	92
Figure 26 HN001 spent culture supernatant does not have a positive influence on fibronectin binding	93
Figure 27 IgG appears to increase HN001 adhesion to Caco-2 cells	95
Figure 28 No support for role of fibronectin in HN001 adhesion to Caco-2 cells	97
Figure 29 <i>fbl</i> genomic region	100
Figure 30 Predicted amino acid sequence of Fbl	101
Figure 31 Multiple sequence alignment of Fbl against PavA and Fbp54	104
Figure 32 Plasmid maps of pFbl2 and pFbl4	105
Figure 33 Expression of soluble Fbl2-GST	106
Figure 34 Expression of soluble Fbl4-GST	107
Figure 35 Purification of Fbl2 by cleavage	108
Figure 36 Purification of Fbl4-GST by elution	109
Figure 37 Role of Fbl4 in fibronectin binding unclear	110

Figure 38 Plasmid map of pBeryFbl1	111
Figure 39 HN001 colony PCR	113
Figure 40 Southern Blot	114
Figure 41 Disruption of <i>fbl</i> did not decrease HN001 binding to $^{125}$ I-Fn	116
Figure 42 HN001D/ <i>fbl</i> and WT HN001 have similar binding to intestinal molecules in the solid-phase	117
Figure 43 Disruption of <i>fbl</i> does not decrease adhesion of HN001 to Caco-2 cells	118
Figure 44 Proposed model of action of factor in HN001 SCS	126

## LIST OF TABLES

Table 1 Bacterial strains used in this study	25
Table 2 Plasmids used in this study	26
Table 3 Oligonucleotide primers used in this study	41
Table 4 $^{125}\text{I}$ -Fn binding of various strains	69
Table 5 Predicted and actual restriction fragments detected in Southern Blot	115
Table 6 Summary of the BLASTP results (top five)	151

## CHAPTER ONE: INTRODUCTION

### 1.1 Lactic Acid Bacteria

#### 1.1.1 General characteristics of lactic acid bacteria

The genera that belong to the lactic acid bacteria (LAB) group include *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. The genus *Bifidobacterium* shares some of the typical features of LAB and is often considered in the same context as LAB even though they are phylogenetically distinct from the group (Axelsson, 1998). LAB are Gram-positive microorganisms that produce lactic acid as a major product during fermentation of carbon sources. During fermentation, LAB generate energy by substrate-level phosphorylation. This is due to their inability to synthesise heme, which is an important component of the respiration mechanism. If hematin is added to the growth media, there is evidence that a cytochrome-like respiratory chain is formed (Gaudu *et al.*, 2002). LAB are utilised in the production of fermented foods because the lactic acid produced imparts a unique flavour on the food produced and can act as a preservative for the food. In addition, other metabolic products of LAB are important flavour constituents of many fermented foods (Urbach, 1995). LAB are also found in the gastrointestinal tract of animals and humans as part of the indigenous microflora where they are considered to be commensal or symbiotic (Klaenhammer, 1995).

#### 1.1.2 Lactobacilli

The genus *Lactobacillus* is heterogenic, with the G + C % content of the DNA (34 – 53%) considered to be twice the normal span accepted for a single genus (Bergey, 1977; Schleifer *et al.*, 1983). The cellular morphology of lactobacilli is mostly regular and rod-shaped, varying in length from 0.5-1.2 X 1.0-10.0µm. The rods are usually long and slender but sometimes short, bent rods occur. Lactobacilli are non-sporulating cells, chain forming, and are usually non-motile, although some lactobacilli are motile with peritrichious flagella. A few strictly anaerobic species of lactobacilli exist but the majority are facultative anaerobes and growth is generally enhanced by 5% CO<sub>2</sub>

(Bergey, 1977). Lactobacilli are chemoorganotrophs and require a rich complex media. Each species within the *Lactobacillus* genus has its own characteristic nutritional requirements. Species in the *Lactobacillus* genus can be divided into three metabolic groups: obligatory homofermentative – the main product is lactic acid (more than 85% lactic acid), obligatory heterofermentative – the products are ethanol, CO<sub>2</sub> and lactic acid (approximately 50% lactic acid), or facultatively heterofermentative – show both fermentation patterns. Generally, at least half of the carbon-based end product is lactic acid (Bergey, 1977). Lactobacilli can also produce acetic acid and hydrogen peroxide, which along with lactic acid may inhibit the growth of pathogens *in vivo* (Lidbeck *et al.*, 1993). The optimum growth temperature for lactobacilli is between 30-40°C. Lactobacilli cells are aciduric, with an optimal pH for growth at less than 5.5. Both of these characteristics clarify why lactobacilli can naturally occur in a wide range of habitats including various fermenting plant and animal products, and the mouth, vagina and intestinal tract of animals and humans. Lactobacilli are also found in many cultured dairy products (Bergey, 1977). Recently there has been an increase in the use of certain *Lactobacillus* species as probiotic organisms (described in Section 1.2). Lactobacilli are not regarded as significant pathogens, however some *Lactobacillus* species have been implicated in bacteraemia and endocarditis, especially in patients with an underlying medical condition, and in dental decay (Becker *et al.*, 2002; Carretto *et al.*, 2001; Farina *et al.*, 2001; Ishibashi *et al.*, 2001; Olano *et al.*, 2001; Presterl *et al.*, 2001; Richard *et al.*, 2001; Salminen *et al.*, 2002; Zhang *et al.*, 2001).

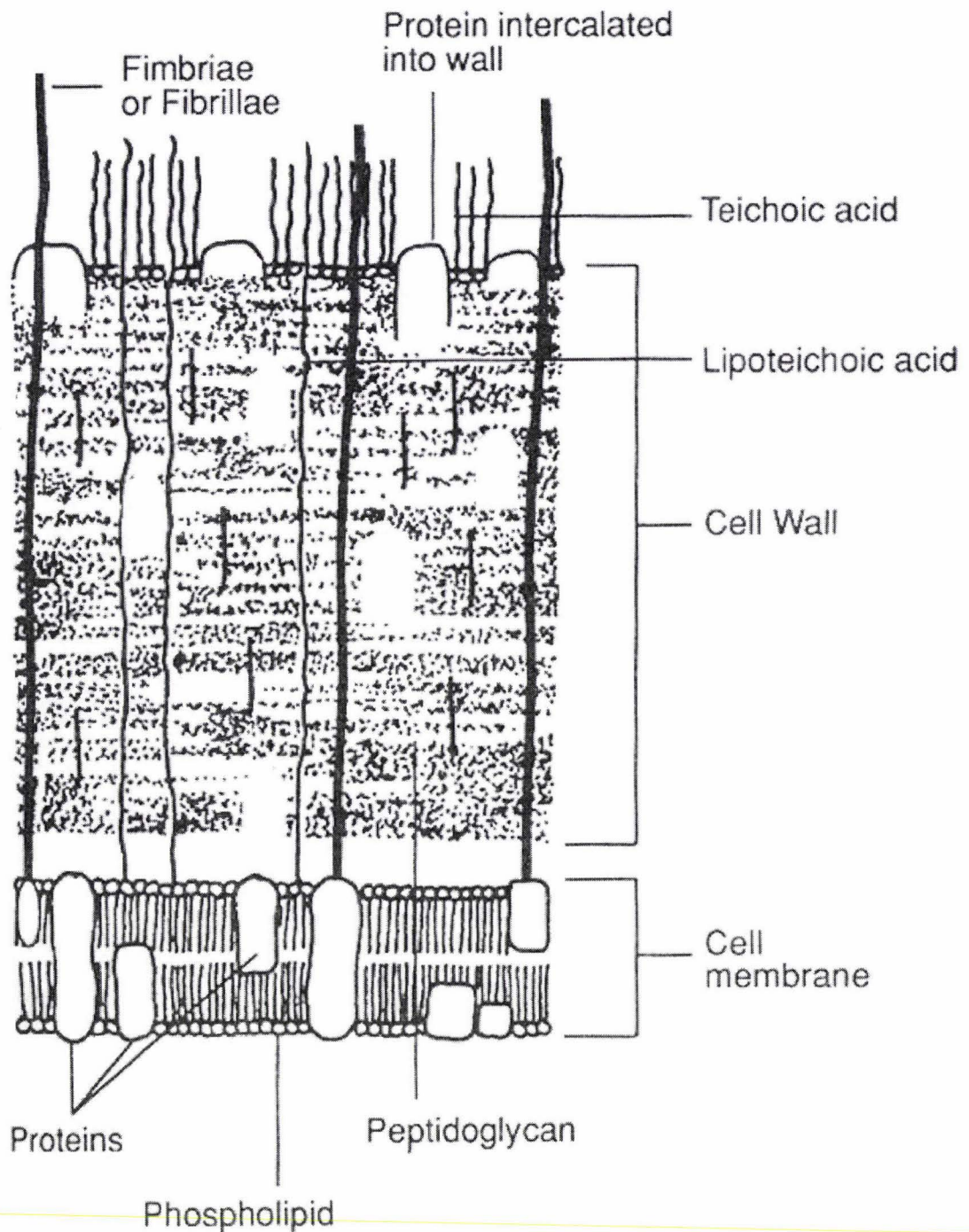
*Lactobacillus* genomes (generally 2 – 3 megabases) are smaller than the genome of *Escherichia coli* (4.7 megabases). Plasmids are found in most *Lactobacillus* isolates (Klaenhammer, 1995). Promoter mapping by primer-extension analysis showed that the lactobacilli -35 and -10 regions closely resembled those of *Escherichia coli* and *Bacillus subtilis*. The region upstream of the -35 motif, the UP element, is also conserved in lactobacilli (McCracken *et al.*, 2000). Phage-related DNA sequence and insertion sequences have been found in lactobacilli (Brandt *et al.*, 2001; Shimizu-Kadota *et al.*, 1985). Some *Lactobacillus* strains are amenable to *in vitro* genetic manipulation by the introduction of DNA via electroporation. Many *Lactobacillus* species have been shown to be transformed with plasmids at a reasonable frequency by electroporation (Kullen *et al.*, 2000). With a reasonable frequency of transformation,



integration of DNA via homologous recombination in *Lactobacillus* species has been performed successfully in several cases (Kullen *et al.*, 2000; Russell *et al.*, 2001).

#### **1.1.2.1 Surface characteristics of lactobacilli**

Cell surface characteristics of lactobacilli are strain specific, and they can be hydrophilic, with a slightly negative charge at alkaline pH, or hydrophobic, with a neutral charge (similar affinity to cationic and anionic exchange resins) (Gusils *et al.*, 2002; Pelletier *et al.*, 1997). Teichoic, lipoteichoic, and teichuronic acids as well as peptidoglycan all contribute to the negative charge of Gram-positive bacteria (Ofek *et al.*, 1994). A diagram of typical cell wall architecture of lactobacilli is included in Figure 1. The cell wall of a Gram-positive bacterium has a thick peptidoglycan layer, generally exposed to the environment, and teichoic acid or teichuronic acid (Ofek *et al.*, 1994). Lipoteichoic acids are ribitol or glycerolphosphate polymers covalently linked with glycolipids (Gusils *et al.*, 2002). Fimbriae have been observed on vaginal isolates of lactobacilli (McGroarty, 1994). Fimbriae are non-flagellar filaments on the surface of bacteria that may be responsible for adhesive properties of the bacteria. Fimbriae are thought to be anchored in the cytoplasmic membrane, and extend to the exterior environment through the peptidoglycan layer (Ofek *et al.*, 1994). Lactobacilli can also have an S-layer, which is a proteinaceous layer, arrayed in geometric forms, located outside the boundary of the cell wall (Ofek *et al.*, 1994). Some *Lactobacillus* species have capsules, which are acidic polysaccharides (or polypeptides) that are secreted by bacteria but remain cell-bound (Ofek *et al.*, 1994).



**Figure 1 Representation of the typical cell wall architecture of lactobacilli**

Diagram adapted from Ofek and Doyle (1994).



## 1.2 Probiotic bacteria

### 1.2.1 Definition

Probiotic organisms are defined as: “a preparation of, or a product containing, viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host” (Schrezenmeir *et al.*, 2001). However, several beneficial health effects can be duplicated using non-viable probiotic organisms (Ouwehand *et al.*, 1998) and cell-wall components of probiotic organisms (Schrezenmeir *et al.*, 2001).

### 1.2.2 Selection of probiotic strains

Dunne *et al.* (1999) state that in order to fulfil the criteria for the selection and assessment of probiotic bacteria, the bacterial strain should:

- Be non-pathogenic
- Be resistant to means of delivery to the host and to gastric acid and bile
- Adhere to gastrointestinal tissue
- Have a beneficial effect on the host

Non-pathogenic bacteria chosen as probiotic candidates are often of human origin or are those utilised in the food industry (such as lactic acid bacteria). The potential probiotic organism should not be contaminated with pathogenic organisms and it should have GRAS status (Generally Recognised As Safe) (Dunne *et al.*, 1999; Klaenhammer *et al.*, 1999). The ability of the bacterial strain to resist gastric acid and bile and to adhere to gastrointestinal tissue *in vitro* is thought to relate to the ability of the bacterial strain to survive and colonise the site of application *in vivo*, if only transiently. Other factors the bacterial strain must overcome *in vivo* are interactions between microbes (competition for nutrients etc.) and the action of the host immune system (Salminen *et al.*, 1998).

Most importantly, a probiotic bacterial strain should exert a beneficial effect on the host. A possible beneficial effect of probiotics is interference with pathogen attachment to intestinal surfaces. Mechanisms involved in interference of pathogen attachment could include competition for nutrients and host cell-binding sites, production of factors that inhibit toxin-receptor interactions, and the killing of pathogens by the

production of acid, bacteriocins or hydrogen peroxide (Reid *et al.*, 2001). Also, some probiotic bacteria have been found to modulate the host immune system, which may lead to eradication of pathogens. Probiotic bacteria have been shown to enhance humoral immune responses, stimulate non-specific host resistance to pathogens and to down regulate hypersensitivity reactions. A detailed description of modulation of immune responses by probiotic bacteria is beyond the scope of this introduction and is covered by two recent reviews (Cross, 2002; Isolauri *et al.*, 2001). Commensal bacteria also seem to have an effect on the host immune system. Studies in germfree animals that lack indigenous microflora show that in the absence of intestinal microflora, the intestinal immune system is underdeveloped and intestinal morphology is different from animals with intestinal microflora (Lu *et al.*, 2001). Also, probiotics may reduce the severity of food allergies (where the host immune system responds to food antigens with an inflammatory response). The action of some probiotics results in a down regulation of inflammatory machinery and stimulation of a Th-1 type response, which prevents inflammatory responses when food antigens are reintroduced (Paganelli *et al.*, 2002).

Several studies in the past decade have suggested that dairy products fermented with certain strains of probiotics are able to lower cholesterol levels. The mechanisms proposed for this effect include physiological actions of the end products of short-chain fatty acid fermentation, cholesterol assimilation by the bacteria, cholesterol binding to the cell wall, and enzymatic deconjugation of bile acids. A recent study showed that several lactic acid bacteria and bifidobacteria strains can remove cholesterol from culture medium (Pereira *et al.*, 2002).

The use of probiotics to treat or alleviate the symptoms of several gastrointestinal conditions, including lactose maldigestion, antibiotic-associated diarrhoea, gastroenteritis, traveler's diarrhoea, various intestinal infections, irritable bowel syndrome, inflammatory bowel disease, and colon cancer, has been demonstrated (Marteau *et al.*, 2001). Research on potentially probiotic strains is vast and has been described in several recent reviews (Hart *et al.*, 2002; Reid, 1999; Reid *et al.*, 2002; Sanders *et al.*, 2001).



*L. rhamnosus* GG is a commercially used probiotic strain that is amongst the most thoroughly researched. *L. rhamnosus* GG inhibits the adherence of a pathogenic *E. coli* O157:H7 to HT-29 epithelial cells (Mack *et al.*, 1999). GG regulates mucin gene expression in the Caco-2 intestinal cell line model, which may be the mechanism by which GG decreases pathogen invasion of Caco-2 cells (Mattar *et al.*, 2002). *L. rhamnosus* GG has a dose- and duration-dependant effect on the proliferative activity of B and T lymphocytes (Kirjavainen *et al.*, 1999). In addition, enhanced IL-10 production was observed when *L. rhamnosus* GG was administered in a clinical trial. *L. rhamnosus* GG can also remove the carcinogenic aflatoxin AFB1 from the intestinal lumen (El-Nezami *et al.*, 2000; Kankaanpaa *et al.*, 2000). There have been numerous other studies investigating possible beneficial roles of *L. rhamnosus* GG, for a review see Reid (1999).

### **1.2.3 *L. rhamnosus* HN001 (DR20™)**

HN001, originally isolated from cheese, was identified as a probiotic candidate through tests that first assessed the ability of the bacteria to withstand conditions similar to the human gastrointestinal tract, and then determined the safety of the bacteria, and the beneficial health impacts of the bacteria on a host.

HN001 was shown to have high viability at low pH and at high bile concentrations. HN001 was also compared with the commercial probiotic strains, *L. rhamnosus* GG and *L. acidophilus* LA-1, and was found to have similar acidity and bile tolerance (Prasad *et al.*, 1998).

The safety of HN001 was studied in mice, which were fed different doses of the bacteria for 7 days. No clinically adverse signs, like a change in feed or water uptake, weight gain, or bacteria in the spleen were detected in mice (Shu *et al.*, 1999). The effect of HN001 on various parameters of the intestinal tract histology, haematology, and intestinal mucosal morphology of the mice were studied by feeding mice HN001 cells for 4 weeks and then taking tissue and blood samples. No adverse effects were found on the haematology, blood biochemistry, intestinal tract histology, intestinal morphology (villus height, crypt depth, epithelial cell height and mucosal thickness), or the incidence of bacterial translocation (Zhou *et al.*, 2000a; Zhou *et al.*, 2000b; Zhou *et al.*, 2001). Consumption of an HN001-containing milk product was shown to

transiently alter the lactobacilli and enterococcal numbers in the faeces of the consumers without having an effect on the numbers of other bacteria present in the faeces. An exception was one subject, where HN001 was detected 2 months after application (Tannock *et al.*, 2000). This extensive characterisation of the safety of HN001 suggests it is safe to be consumed in high numbers, as was expected for a microbial species with a history of safe use in food manufacture.

Adhesion of HN001 to intestinal cell lines compared favourably to that found for the commercial probiotic strains *L. rhamnosus* GG and *L. acidophilus* LA1. Adhesion to the mucus-secreting cell line HT29-MTX, was found to be 2-3 times higher than for Caco-2 and HT-29, indicating that mucus may play an important role in the adhesion of HN001 (Gopal *et al.*, 2001). Concentrated spent culture supernatant (SCS) from HN001 was found to reduce culturable numbers of *E. coli* O125:H7, and also reduce the adhesion and invasiveness of this pathogen to Caco-2 cells. The action of HN001 SCS was possibly due to a synergistic action of lactic acid and proteinaceous substances (Gopal *et al.*, 2001).

Gill *et al.* (Gill *et al.*, 2000) showed that by feeding mice HN001 cells for 28 days the natural and acquired immune response was enhanced. Specifically, a significant increase in the level of phagocytic activity of peripheral blood leucocytes and the peritoneal macrophages was seen and higher amounts of interferon- $\gamma$  were produced, compared with the control mice. Consumption of HN001 was found to increase the proportion of polymorphonuclear cells showing phagocytic activity and the relative level of natural killer (NK) cell tumour killing activity (Sheih *et al.*, 2001). HN001 has also been shown to protect against *Salmonella typhimurium* infection. HN001-fed mice challenged with *S. typhimurium* had greater food and water uptake and weight gain; they produced higher titres of serum and intestinal tract anti-*Salmonella* antibodies; and showed greater survival of infection compared to mice not treated with HN001 (Gill *et al.*, 2001c). A recent study has shown that HN001 can also protect against *E. coli* O157:H7 infection in a murine challenge infection model. HN001-fed mice showed greater survival; had lower bacterial translocation rates; and showed higher intestinal anti-*E. coli* IgA responses and blood leucocyte phagocytic activity compared to mice not treated with HN001 (Shu *et al.*, 2002). HN001 was also shown to enhance the production of both Th1 and Th2 cytokines (involved in the immune response to



allergies) in mice that had been primed with ovalbumin (Cross *et al.*, 2002). Two studies have shown that the immuno-enhancing properties of HN001 are dependent on the dose of HN001, and HN001 has active immuno-enhancing properties when used as an additive or as a fermentative agent. Heat-killed HN001 produces different effects compared to live cells (Gill *et al.*, 2001a; Gill *et al.*, 2001b). These studies on HN001 indicate that the organism fulfils the criteria widely accepted for a probiotic organism.

### 1.3 Gastrointestinal surfaces available for bacterial adhesion

#### 1.3.1 Why is adhesion important to bacteria?

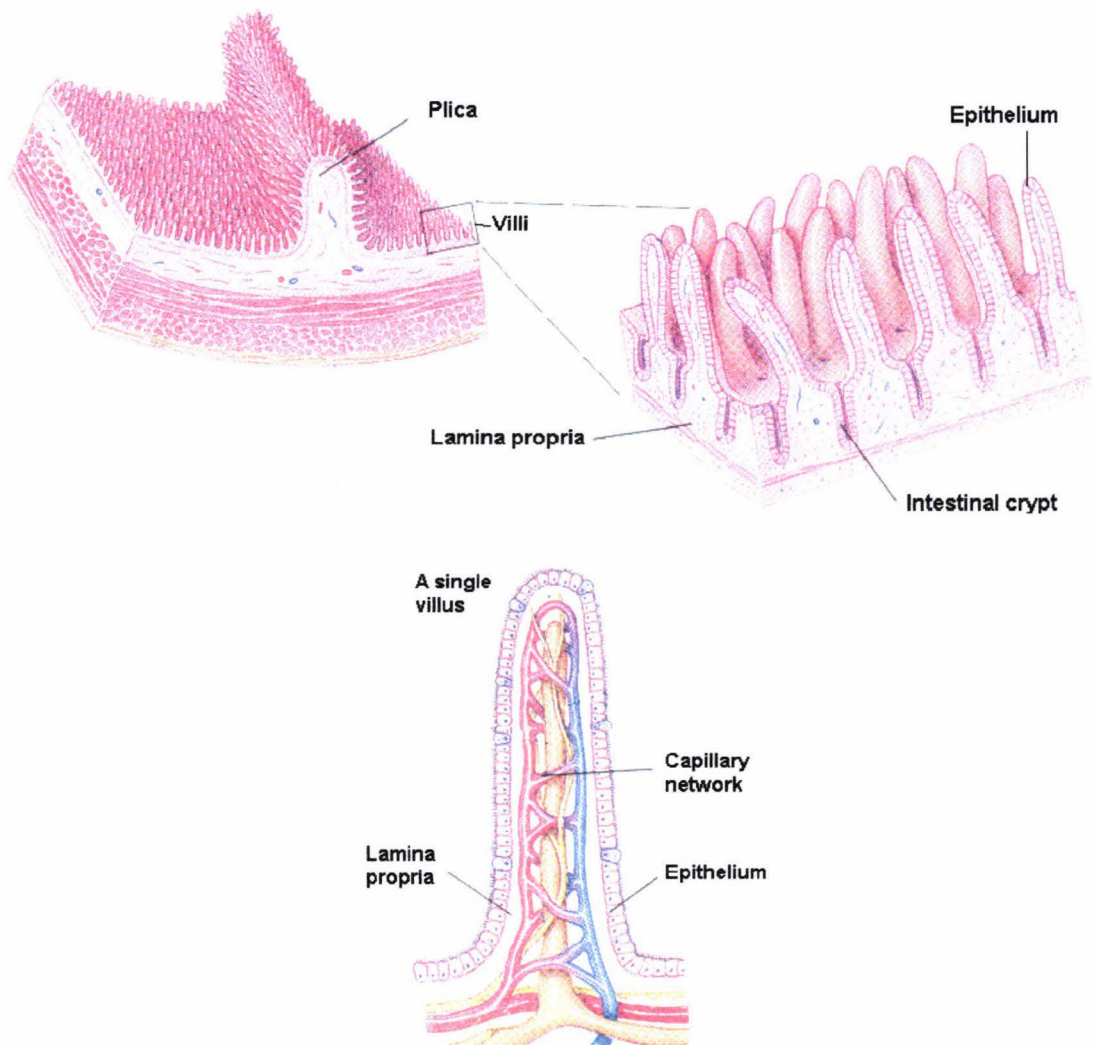
One of the main advantages gained by a bacterium adhering to tissue surfaces is the ability to withstand mechanical cleaning mechanisms (eg. secretions) of the host, thereby allowing growth of bacteria at the surface. Also, adherence has been found to increase bacterial resistance to deleterious agents (eg. antibodies) in comparison to bacteria in suspension, perhaps due to restricted diffusion near the bacterial cell-host cell interface (Ofek *et al.*, 1994).

#### 1.3.2 The gastrointestinal surface

The gastrointestinal tract of humans can be divided into 6 regions: the oral cavity, the oesophagus, the stomach, the small intestine, the large intestine (colon), and the rectum. This introduction will describe the surface of the small intestine and the large intestine of humans, and three cell types present in intestinal surfaces: epithelial cells, M cells and goblet cells, since these regions are colonised by microorganisms, and thus are relevant to this study.

The small intestine is the primary location where food is enzymatically digested and water, nutrients, vitamins and ions are absorbed. The surface of the small intestine has a series of folds, called plicae, which are convoluted into innumerable villi. This increases the absorptive surface of the intestine. The villi are lined by columnar epithelial cells and contain a tissue core, made up of the lamina propria (where cells of the immune system are located) and a capillary network. At the bases of villi, the entrances to intestinal crypts are located, where goblet cells eject mucus into intestinal surfaces and new generations of columnar epithelial cells and goblet cells are produced.

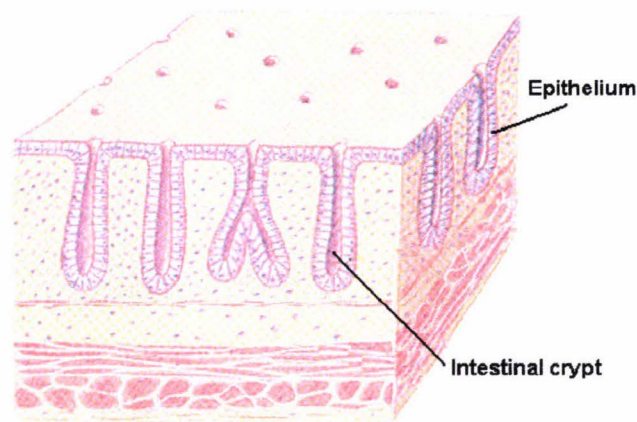
The new cells are continually displaced towards the intestinal surface (Martini, 1992). See Figure 2 for a diagram of the surface of the small intestine.



**Figure 2 Surface of small intestine of humans**

The top left diagram shows the surface of the small intestine with plicae. The top right diagram shows detail of the surface of the small intestine with villi. The bottom diagram is a diagram of a single villus, showing the epithelium and the tissue core. All diagrams are reproduced from Martini (1992).

The large intestine is where water is absorbed and indigestible materials are dehydrated and compacted in preparation for elimination. Also, vitamins that have been liberated through bacterial action are absorbed. The surface is much thinner than the surface of the small intestine and there are no villi present on the surface. The intestinal crypts are deeper than in the small intestine. The epithelium is similar to the epithelium lining the villi of the small intestine except the large intestine epithelium contains more goblet cells (Martini, 1992). For a diagram of the surface of the large intestine see Figure 3.



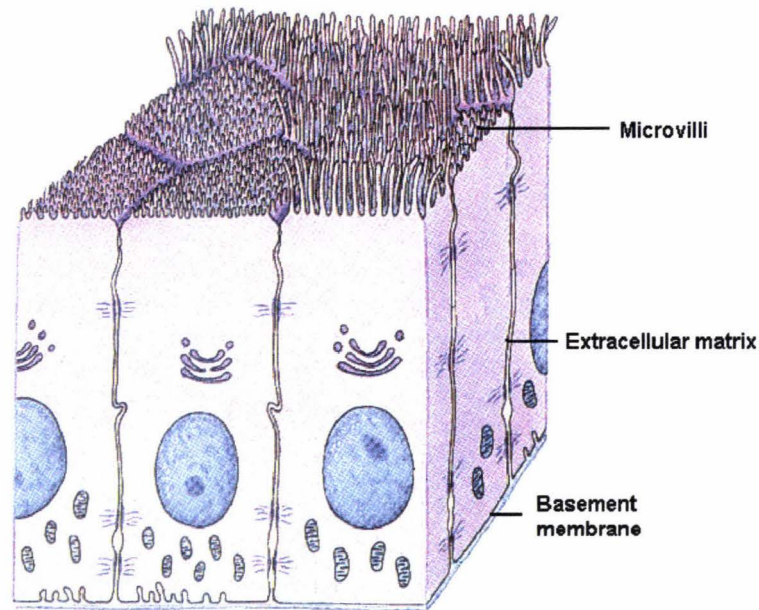
**Figure 3 Surface of the large intestine of humans**

This diagram of the large intestine shows the lack of villi and the deep intestinal crypts. The diagram is reproduced from Martini (1992).

The epithelium lining the gastrointestinal tract has two main functions: it allows absorption of gastrointestinal contents and it is an essential barrier to potential harmful substances and pathogens within the intestinal lumen (Dignass, 2001). Epithelial cells in intestinal surfaces have a brush border at the apical surface of the cell. The brush border consists of minute microvilli and is densely populated with glycoproteins and glycolipids, which form a glycocalyx. The glycocalyx is thought to serve as a barrier to microbes reaching the epithelial cell surface but can also function as a receptor for microbial adhesins. A protective layer of mucus covers the glycocalyx. The basement membrane, a sheet of specialised extracellular matrix, is located at the basolateral surface of the epithelial cell and is made up of two parts. The layer closest to the epithelium is called the basal lamina. The second part is the reticular lamina and gives the basement membrane its strength (Lussier *et al.*, 2000; Martin *et al.*, 1993).

Extracellular matrix is also located between adjacent epithelial cells and underneath epithelial cells, and is sealed off from the lumen by a tight junction (Martini, 1992). See Figure 4 for a diagram of an epithelial cell. Section 1.3.3 describes the extracellular matrix and basement membrane in more detail.





**Figure 4 Diagram of an epithelial cell**

Diagram of a typical epithelial shows microvilli (found on intestinal epithelial cells), the extracellular matrix (between adjacent epithelial cells) and the basement membrane (underlying epithelial cells). This diagram is adapted from Martini (1992).

Membranous epithelial cells (M cells) are located throughout the gastrointestinal surface as follicular-associated epithelium that overlay tissue of the immune system. M cells are like normal epithelial cells except they have no mucus and glycocalyx coating at their apical surface, no typical brush border and lymphocytes closely associated at their basolateral surface. They are involved in sampling and transporting antigens from the lumen to lymphoid tissue. It is thought that due to the lack of protective structures on their surface, M cells are often utilised by pathogenic bacteria as a pathway for invasion (Lu *et al.*, 2001; Neutra *et al.*, 1999).

Goblet cells are unicellular exocrine glands that are scattered among epithelial cells. They produce mucus, which is primarily made up of mucins (large glycoproteins). Mucins consist of a peptide backbone containing alternating glycosylated and non-glycosylated domains. Mucus acts to lubricate tissue surfaces, trap microorganisms and act as a barrier to microorganisms reaching the epithelial cell surface (Deplancke *et al.*, 2001; Martini, 1992).

### 1.3.3 The extracellular matrix and basement membrane

The ECM and the basement membrane are biologically active tissue, which serve for the attachment of the host's cells. However, it can also be utilised by microorganisms for attachment. In a healthy organism, the ECM and the basement membrane are only thought to be exposed to bacteria in the lumen during the sloughing off of epithelial cells, as the epithelial cells and tight junction between the cells protect these tissues from the intestinal lumen. However, tissue trauma can expose these ECM components to bacteria. This is often the mechanism by which opportunistic pathogens can cause complications to disease (Patti *et al.*, 1994).

The major constituents of the basement membrane are laminin, collagen type IV and heparan sulphate (Lussier *et al.*, 2000; Martin *et al.*, 1993). Prevalent molecules in the ECM include fibronectin, various collagen types, laminin, tenascin and heparan sulphate (Korhonen *et al.*, 2000; Lussier *et al.*, 2000; Patti *et al.*, 1994). Fibronectin, which is of particular interest in this study, is described in more detail in Section 1.3.3.1, and the other components commonly found in basement membranes and the ECM are described below.

Laminin, one of the major glycoproteins of basement membranes, is important for the structure of the basement membrane by formation of networks with collagen type IV, entactin and heparan sulphate (Dubreuil *et al.*, 2002; Martin *et al.*, 1993). Heparan sulphate is a proteoglycan, made up of a core protein with polysaccharide chains (Yurchenco *et al.*, 1993). As well as being a component of the basement membrane, heparan sulphate is also a component of cell surface-membrane-associated proteoglycans (Dubreuil *et al.*, 2002). Collagen type IV, the major component of basement membranes, is a glycoprotein that forms a complex matrix structure by self-association. Entactin is a sulphated glycoprotein found in basement membranes. It binds tightly to laminin to form a stable, non-covalent complex. Entactin also binds collagen type IV and therefore, may help to stabilise the complex basement membrane structure (Martin *et al.*, 1993; Yamada, 1991).

Tenascin is an unusually large glycoprotein made up of six similar subunits joined at their N-terminus to form a star shaped complex. Tenascin contains numerous

fibronectin type III repeats (described in Section 1.3.3.1) and is found to bind to certain proteoglycans and fibronectin. It is located in the ECM, especially around the edges of healing wounds (Yamada, 1991). A less prevalent glycoprotein of the ECM, vitronectin, is sometimes found in association with fibronectin in the ECM (Yamada, 1991). Vitronectin is also found in serum as a soluble protein. Thrombospondin is another less prevalent glycoprotein located in the ECM. Thrombospondin also binds several ECM molecules and can interact with cells (Yamada, 1991). There are other components of intestinal tissue not described here, which are beyond the scope of this study.

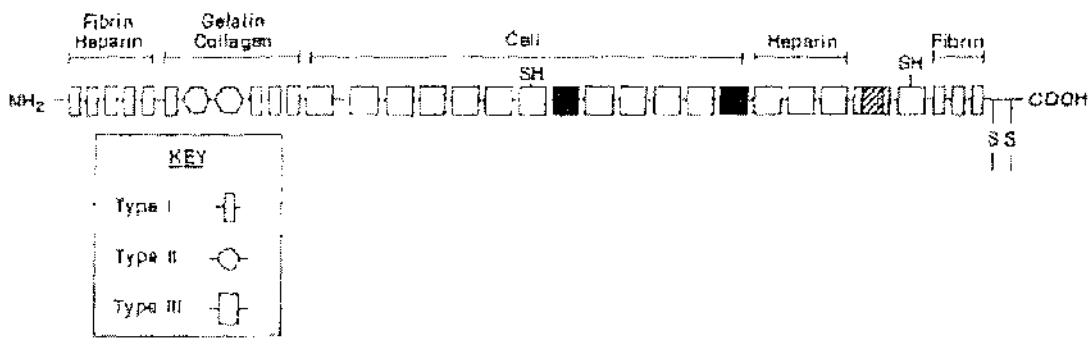
### **1.3.3.1 Fibronectin**

Fibronectin is a large glycoprotein found on cell surfaces, in the ECM, and in extracellular fluid (eg. plasma). In plasma, it is a heterodimer consisting of two 220kDa monomers joined by a disulphide bond at the C-terminus. "Cellular" fibronectins are produced by a wide variety of cell types that secrete them and often organise them into extensive extracellular matrices (Ruoslahti, 1988; Sharma *et al.*, 1999). Type III repeats found in fibronectin (described in more detail below) have been implicated in the formation of a fibronectin matrix assembly (Hocking *et al.*, 1994; Hocking *et al.*, 1996). An *in vitro* form of matrix fibril fibronectin (super fibronectin) is formed when a fragment from the first type III repeat of fibronectin (described below) is added to plasma fibronectin. This fragment induces spontaneous disulphide crosslinking of the molecule into multimers of high relative molecular mass, which resemble matrix fibrils. This matrix form of fibronectin appears to be functionally distinct from plasma fibronectin (Morla *et al.*, 1994). Fibronectin has many roles, including helping mediate cell adhesion, embryonic cell migration, and wound healing (Ruoslahti, 1988). Some of these roles are mediated through the interaction between fibronectin and integrins, which are exposed on the surface of many animal cell types (Ruoslahti, 1988). The  $\alpha 5 \beta 1$  integrin is known as a specific fibronectin receptor (Lussier *et al.*, 2000). Fibronectin has also been shown to interact with other integrins, including  $\alpha M \beta 2$  (Lishko *et al.*, 2003).

Fibronectin contains distinct, functionally active polypeptide domains specialised for binding to specific cell surface receptors, as well as to other extracellular matrix molecules. Each fibronectin monomer consists of homologous modules of 40-90 amino

acids, classed as type I, II or III repeats (Ruoslahti, 1988). The N-terminal 29kDa fragment of fibronectin contains five type I “finger” modules and binds heparin and fibrin. It has also been shown to be involved in fibronectin matrix assembly (Huff *et al.*, 1994). The 45kDa collagen (gelatin)-binding domain contains the only type II repeats in the fibronectin molecule (Pickford *et al.*, 1997; Sharma *et al.*, 1999). The 75kDa DNA/cell binding domain contains several type III repeats. The C-terminal heparin/fibrin-binding domain contains both type III and type I repeats (Sharma *et al.*, 1999; van der Flier *et al.*, 1995). See Figure 5 for a diagram of the structure of fibronectin.

The fibronectin type III repeat was found to be of particular interest in this study. It is an approximately 100 amino acid domain, is the most prevalent type of repeat in the fibronectin molecule, and is also found in many other proteins (Bork *et al.*, 1992). Structural analysis has shown that the type III repeats form a  $\beta$ -sandwich with four  $\beta$ -strands on one side and three on the other. Type III repeats are involved in fibronectin matrix assembly and also in integrin binding via the sequence RGD in the tenth type III repeat, and via the synergy site (PHSRN) in the ninth type III repeat (Altroff *et al.*, 2001; Hocking *et al.*, 1996; Sharma *et al.*, 1999).



**Figure 5 Structure of fibronectin**

This diagram shows one monomer of the fibronectin heterodimer. The labels on the fibronectin molecule refer to the ligand bound by the region indicated. The type of repeat present in the fibronectin molecule is indicated by a symbol (shown in the key). This diagram is adapted from Ruoslahti (1988).

#### **1.3.4 The Caco-2 cell line**

The Caco-2 intestinal cell line has been used extensively to model the interactions of pathogenic, commensal and probiotic bacteria with intestinal surfaces. The Caco-2 cell line was established from human colonic adenocarcinoma (Fogh *et al.*, 1977). A large number of studies have characterised the properties of the Caco-2 cell line. These studies have shown that Caco-2 cells differentiate into a monolayer formation *in vitro*, with each cell having a well-differentiated brush border on the apical surface. The cells are polarised and have a columnar structure (Gilbert *et al.*, 2000; Rousset, 1986). Thus, the Caco-2 monolayer closely resembles the structure and function of the normal gastrointestinal epithelium. Electron microscopy was used to show that bacteria can adhere to Caco-2 cells via the brush border on the apical surface of Caco-2 cells (Sarem *et al.*, 1996). A description of studies on lactobacilli adhesion to Caco-2 cells is included in Section 1.3.4.1.

##### **1.3.4.1 Adhesion of lactobacilli to Caco-2 cells**

Little is known about mechanisms involved in lactobacilli adherence to intestinal surfaces. However, several studies of lactobacilli adhesion to Caco-2 cells have given insight to the possible mechanism of adherence to intestinal surfaces. Key studies are described below.

Adherence of *L. acidophilus* BG2FO4 and NCFM/N2 to Caco-2 monolayers was decreased by protease treatment of the bacterial cells, as opposed to protease treatment of *L. gasseri* ADH, which either had no effect or increased adhesion. Treatment with periodate, which oxidises cell surface carbohydrates, significantly reduced the adhesion of *L. gasseri* ADH, had a moderate effect on the adhesion of *L. acidophilus* BG2FO4 and had no effect on the adhesion of *L. acidophilus* NCFM/N2. These results indicate that *L. acidophilus* NCFM/N2 adherence to Caco-2 cells is mainly mediated through bacterial cell surface proteins; *L. gasseri* ADH adherence is mediated through carbohydrate groups and *L. acidophilus* BG2FO4 adherence is a result of both protein and carbohydrate mediated interactions (Greene *et al.*, 1994).

Cell-surface associated lipoteichoic acid (LTA) was found to act as an adhesion factor in the interaction between *L. johnsonii* La1 and Caco-2 cells. The difference between

the adherent strain *L. johnsonii* La1 and a non-adherent strain was investigated by using murine monoclonal antibodies reactive specifically against La1. They were found to be against a non-proteinaceous compound, which was characterised to be LTA. A competition assay in which purified LTA inhibited the binding of La1, confirmed the involvement of LTA in the adhesion of *L. johnsonii* La1 to Caco-2 cells (Granato *et al.*, 1999).

In one study, the *in vitro* interaction between *L. rhamnosus* GG and Caco-2 cells was investigated in the presence of normal faecal microflora, in an attempt to more closely model what may happen *in vivo*. The normal microflora added was not found to influence adhesion of *L. rhamnosus* GG (Ouwehand *et al.*, 1999). The addition of *L. casei* NY1301 was found to increase adhesion of *L. gasseri* NY0509 to Caco-2 cells, indicating that non-pathogenic bacteria may act synergistically to promote adhesion to intestinal surfaces (Azuma *et al.*, 2001).

Finally, several studies have shown that *Lactobacillus* species can compete and reduce adhesion of pathogenic bacteria to Caco-2 cells. *L. acidophilus* LB was found to decrease both adhesion to, and invasion of, Caco-2 cells by *Salmonella typhimurium*, *Escherichia coli* (enteropathogenic), *Yersinia pseudotuberculosis* and *Listeria monocytogenes* (Coconnier *et al.*, 1993). A recent study has shown that *L. acidophilus* LB has a protective effect against brush border lesions caused by a diarrhoeagenic *E. coli* strain (Lievin-Le Moal *et al.*, 2002). Competition of adhesion of enteropathogens was shown for *L. acidophilus* LA1 (Bernet *et al.*, 1994), *L. rhamnosus* GG and *L. casei* Shirota (Lee *et al.*, 2000) and *L. crispatus* JCM 8779 and *L. reuteri* JCM 1081 (Todoriki *et al.*, 2001).

## 1.4 Mechanisms of adhesion of bacteria

### 1.4.1 General model for adhesion of bacteria

Adhesion can involve ionic interactions, hydrogen-bonding, hydrophobic interactions, and coordination complexes involving multivalent ions (Ofek *et al.*, 1994). To facilitate adhesion, bacterial cells position adhesins on their surfaces. Bacterial adhesins are described in Section 1.4.2. Adhesion mechanisms are thought to operate independently and sequentially, possibly setting the stage for subsequent adhesion events to other cell

surface ligands (Patti *et al.*, 1994). Adherence to the surface of a healthy gastrointestinal tract usually occurs in two phases: (1) adhesion to the mucus layer covering epithelial cells, and then (2) adhesion to the epithelial surface. Invasive pathogens can also then invade the epithelial cells in a third adhesion phase. If the extracellular matrix is exposed in the gastrointestinal surface, it can also be a target for adhesion (described in Section 1.3.3) (Ofek *et al.*, 1994).

#### **1.4.2 Bacterial adhesins**

Interest in the mechanisms by which pathogenic bacteria colonise the human gastrointestinal tract and cause disease has led to the identification of genes controlling adherence and invasion in a variety of pathogenic bacterial species. In contrast, there have been few studies investigating the mechanisms by which normal intestinal microflora establish and maintain colonisation. General characteristics of bacterial adhesins are described below with a few examples. Description of all the bacterial adhesins characterised is beyond the scope of this introduction. Some examples of fibronectin-binding proteins are described in Section 1.4.2.1 and the general characteristics of cell-surface proteins from Gram-positive organisms are described in Section 1.4.2.2. All characterised adhesins of lactobacilli are described in Section 1.5.

Adhesins may be composed of proteins, lipids, polysaccharides, or phosphodiester-containing polymers (lipoteichoic acids) (Ofek *et al.*, 1994). An example of lipoteichoic acid acting as an adhesin is described in Section 1.3.4.1. Adhesins may be located in fimbriae, cell walls (peptidoglycan), or in loosely attached components (capsule) (Ofek *et al.*, 1994). See Figure 1 for a diagram of the Gram-positive cell surface. Peptidoglycan may act as an adhesin, but its main function in adhesion appears to be in supporting or forming a matrix for adhesins. Apart from surface-associated components, bacteria may also secrete soluble components that associate with the bacteria to promote adhesion (Ofek *et al.*, 1994).

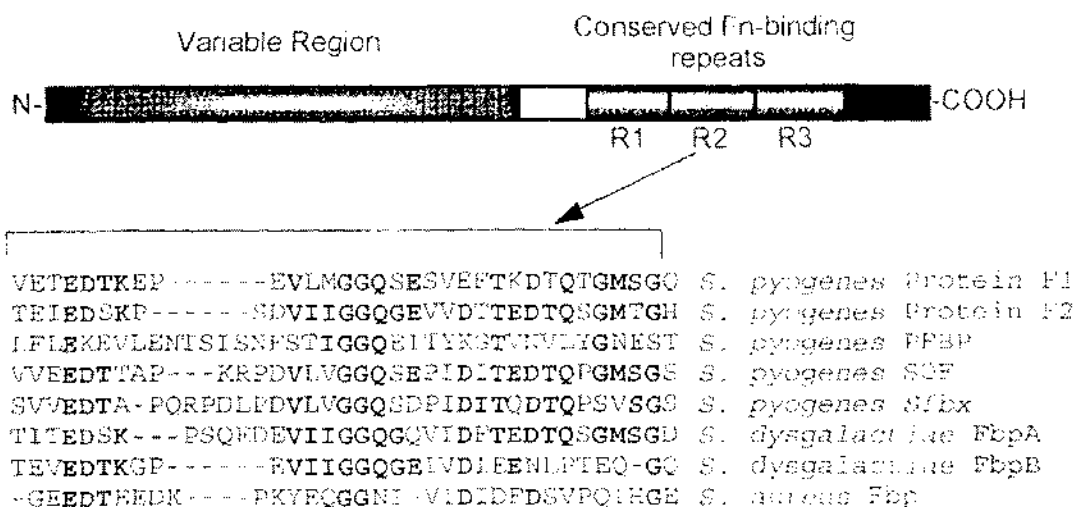
MSCRAMMs (microbial surface components recognising adhesive matrix molecules) are a class of cell surface adhesins that specifically interact with ECM ligands. To be classified as a MSCRAMM the interaction with the ECM ligand must have high affinity and must be specific. This means lectin-type adhesins are not classified as



MSCRAMMs, as many types of carbohydrates can interfere with the interaction. MSCRAMMs are thought to be involved in the virulence of some pathogens (Patti *et al.*, 1994). Many examples of MSCRAMMs exist and a number of these are described in several reviews (Joh *et al.*, 1999; Patti *et al.*, 1994; Westerlund *et al.*, 1993). Fibronectin-binding proteins are a class of MSCRAMM and are described in Section 1.4.2.1. There are many other characterised adhesins that bind to components on intestinal surfaces other than those in the extracellular matrix. Examples of well-studied adhesins that bind ligands other than extracellular matrix components are the M proteins from *Streptococcus pyogenes*, an important human pathogen. M proteins are one of the major virulence factors of *S. pyogenes* and can bind a range of ligands, including fibrinogen, CD46, galactose, fibronectin, laminin, fucose/fucosylated glycoproteins and sialic acid containing ligands, dependant on the type of M protein. M proteins have also been shown to mediate adhesion to HEp-2 tissue culture cells and keratinocytes (Courtney *et al.*, 2002).

#### **1.4.2.1 Fibronectin-binding proteins**

A large number of fibronectin-binding proteins have been identified in the Gram-positive *Streptococcus* and *Staphylococcus* species. Fibronectin-binding proteins identified in *Streptococcus pyogenes* include Protein F1 (Hanski *et al.*, 1992), Protein F2 (Jaffe *et al.*, 1996), Pfbp (Rocha *et al.*, 1999), Serum opacity factor (Sof) (Rakonjac *et al.*, 1995), Fba (Terao *et al.*, 2001), Sfb (Talay *et al.*, 1994), and Fbp54 (Courtney *et al.*, 1994). Five of these proteins, Protein F1, Protein F2, Pfbp, Sof and Sfb, have a common fibronectin-binding peptide motif that is repeated in tandem in the protein (Figure 6). This repeat is weakly conserved in the fibronectin-binding region of Fbp54 (Courtney *et al.*, 2002). PavA is a fibronectin-binding protein from *S. pneumoniae* that has 67% identity to Fbp54 (Holmes *et al.*, 2001). Fibronectin-binding proteins identified in *Staphylococcus aureus* include FnbpA and FnbpB (Flock *et al.*, 1987; Froman *et al.*, 1987), ECM protein-binding protein (Emp) (Hussain *et al.*, 2001), and ECM-binding protein homologue (Ebh) (Clarke *et al.*, 2002). FnbpA and FnbpB both have the fibronectin-binding peptide commonly found in fibronectin-binding proteins from *S. pyogenes* (Joh *et al.*, 1999). Fibronectin-binding proteins from a variety of bacteria are described in a review by Joh *et al.* (Joh *et al.*, 1999).



**Figure 6 Alignment of the fibronectin-binding repeat region (R2) found in some Gram-positive fibronectin-binding proteins**

This diagram is adapted from Courtney (2002).

#### 1.4.2.2 Characteristics of cell surface proteins

The anchoring of proteins on the cell surface requires a mechanism to allow the proteins to traverse the plasma membrane and become anchored to the cell surface. Proteins to be secreted or surface exposed are often secreted via the general secretion pathway. A signal peptide at the N-terminus of the protein acts as a signal to targeting factors, which direct the protein to the translocation site. The signal peptide is then cleaved off during secretion across the plasma membrane (Fekkes *et al.*, 1999; Izard *et al.*, 1994).

There are several mechanisms that have been identified in Gram-positive bacteria to anchor proteins to cell surfaces. The LPXTG motif has been identified as part of the major mechanism by which surface proteins of Gram-positive organisms are anchored to the cell wall. The LPXTG motif is recognised by the enzyme sortase, which cleaves the motif and covalently links the protein to the cell wall. The LPXTG motif is preceded by a hydrophobic membrane-spanning region and a positively charged tail, which is thought to act as a stop-secretion signal so the protein remains in the cell membrane. Before the LPXTG motif there is normally a region rich in proline and glycine residues, which are thought to promote a more extended conformation allowing

the protein to span the cell wall (Navarre *et al.*, 1999). Transmembrane anchors are characterised by a hydrophobic stretch of amino acids. Lipoprotein anchors are characterised a cysteine residue immediately to the C-terminal side of the signal sequence cleavage site possessing the motif LXXC (Sutcliffe *et al.*, 1995; Turner *et al.*, 1997). Also, there are surface-exposed proteins associated with lipotechoic acid and the choline residues attached to teichoic acid. Attachment of proteins to choline residues appears to be specific to *Streptococcus pneumoniae* (Cossart *et al.*, 2000). The AcmA anchor is characterised by repeated amino acid sequences separated by stretches of amino acids rich in serine, threonine and asparagine residues and associates with peptidoglycan in the bacterial cell wall. Finally, surface-layer-protein anchors are characterised by a conserved C-terminal third found in surface-layer proteins, which is thought to interact with the cell wall (Leenhouts *et al.*, 1999).

### 1.5 Characterised adhesins of *Lactobacillus* species

This study begins to characterise the molecular species involved in the adherence of HN001 to intestinal molecules and cell lines. Several studies in the past decade have identified adhesive components of various *Lactobacillus* species.

S-layer proteins are present in several, but not all *Lactobacillus* species. An S-layer protein extracted from *L. crispatus* JCM 5810 was found to bind collagen type IV (Toba *et al.*, 1995). Further study on S-layers from *L. crispatus* JCM 5810 revealed a gene called *chsA* that encodes a protein that appears to mediate adhesiveness to collagens. CbsA has typical features of a bacterial S-layer protein, with a conserved C-terminal region, and was shown to bind both collagen type I and IV (Sillanpaa *et al.*, 2000). A recent study has mapped the domains in CbsA involved in binding collagens and laminin, in anchoring the protein to the cell wall, and in self-assembly (to form the S-layer) by deletion analysis of His-tagged peptides and by heterologous expression on *L. casei* (Antikainen *et al.*, 2002). Further functional analysis shows that CbsA on *L. crispatus* JCM 5810 cell surfaces seems to inhibit pathogenic *E. coli* from adhering to laminin and Matrigel (reconstituted basement membrane preparation) (Horie *et al.*, 2002). The S-layer, SlpA, from *L. brevis* ATCC 8287 has both intestinal cell line-binding and fibronectin-binding functions (Hynonen *et al.*, 2002). An S-layer from *L. acidophilus* was found to increase adhesion to avian intestinal epithelial cells (Schneitz

*et al.*, 1993). However, several other S-layer proteins identified from *Lactobacillus* species are as yet uncharacterised (Boot *et al.*, 1993; Boot *et al.*, 1995; Callegari *et al.*, 1998; Lortal, 1992; Ventura *et al.*, 2002).

Affinity purification was used to purify collagen type I-binding proteins of *L. reuteri* NCIB 11951. Two proteins were purified, which were found to bind to labelled collagen type I (Aleljung *et al.*, 1994). One of these collagen type I-binding proteins, Cnb, had similarity to the solute binding component of bacterial ABC transporters. Cnb is thought to be part of an ABC transporter, because an open reading frame found upstream of the *cnb* gene encodes a protein with 48% identity to the ATP binding component present in ABC transporters (Roos *et al.*, 1996). A protein from *L. fermentum* RC-14 with anti-adhesive properties against *Enterococcus faecalis* was found to have 100% identity to the N-terminal sequence of Cnb, and thus is probably a homologue of Cnb (Heinemann *et al.*, 2000). A recent study on adhesins of *L. acidophilus* CRL 639 has shown that fibronectin binds to a 15kDa protein, and collagen type I binds to proteins of 45 and 58kDa (Lorca *et al.*, 2002).

Mucin-binding proteins have been purified from two different *Lactobacillus* species recently. Mub, from *L. reuteri* 1063, encodes a cell-surface exposed protein that adheres to pig mucus components. Mub was also detected in the growth medium (Roos *et al.*, 2002). A protein from *L. fermentum* 104R was found to bind both small intestine mucus and gastric mucin. This protein was also detected in growth medium after 24hr of growth. The amino acid sequence of this protein has little sequence similarity with previously published sequences (Rojas *et al.*, 2002).

The identification of lactobacilli with adhesins specific to molecules found in gastrointestinal tissue indicates that the bacteria may have mechanisms that allow adhesion to intestinal surfaces *in vivo*. Adhesion to intestinal surfaces may then promote colonisation of the gastrointestinal tract and potentially allow probiotic *Lactobacillus* strains to exert a beneficial effect on the host. However, nothing is known about the relevance of these characterised adhesins *in vivo*, so no conclusions can be reached about whether adhesins on lactobacilli cell surfaces contribute to their ability to act as a probiotic.

## 1.6 Aims of this study

The hypothesis formulated for this study is that HN001 interacts with human intestinal molecules via specific interactions between its surface components (adhesins) and ligands in intestinal tissues. The aims of this study are:

- To characterise adhesion of HN001 to intestinal molecules and cell lines.
- To characterise the molecular species involved in the adherence of HN001 to intestinal molecules and cell lines.

There is a large gap in the understanding of the molecular mechanisms by which normal intestinal microflora and probiotic bacteria can adhere to the intestinal surface. As mentioned in Section 1.2, adhesion to intestinal surfaces is thought to be an important characteristic of probiotic organisms as it prevents the elimination of the microbe by host mechanisms, therefore allowing the probiotic to exert beneficial effects in the host. Also, adhesion by probiotic bacteria is thought to inhibit the adhesion of pathogenic bacteria by blocking or masking specific receptors that pathogens use to adhere to intestinal surfaces.

The characterisation of HN001 adhesion to fibronectin is of interest in this study as fibronectin is one of the predominant ligands in the extracellular matrix. Fibronectin-binding may be relevant to HN001 adhesion *in vivo* where fibronectin is probably exposed to bacteria at the sites of potential pathogen invasion, both through tissue trauma and sloughing off of epithelial cells. Also, only two proteins from *Lactobacillus* species have been shown to have fibronectin-binding function (Section 1.5), leaving a large gap in the understanding of the molecular mechanism by which lactobacilli adhere to fibronectin.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 Bacterial strains and plasmids**

Bacterial strains used in this study are listed in Table 1 and were cultured as outlined in Section 2.3.1. Plasmids used in this study are listed in Table 2.

### **2.2 Media and Buffers**

#### **2.2.1 Media**

Unless stated, all media was sterilised by autoclaving at 121°C for 15 min. Media solutions were made up with MilliQ water. Solid media was cooled to 50°C before addition of antibiotic and pouring of plates. Liquid media was cooled to ambient temperature before use. Sterilised, uninoculated liquid media was stored at ambient temperature. Uninoculated plates were stored at 4°C.

#### **Brain Heart Infusion (BHI) broth and BHI agar**

BHI broth contained 37g of dehydrated BHI broth (Difco) in 1 litre MilliQ water. BHI agar contained 15g/l agar (Becton Dickinson) in BHI broth. When required, erythromycin was supplemented at a concentration of 150µg/ml in molten solid or liquid media (cooled to at least 50°C).

#### **Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM)**

13.5g dehydrated DMEM (Gibco™, Invitrogen) was added to 900ml sterilised, ambient temperature MilliQ water. NaHCO<sub>3</sub> (50ml 7.5 % w/v solution) (Gibco™, Invitrogen) was added and the medium was adjusted to pH 7.4. The total volume was made up to 1 litre and 200ml aliquots were sterilised by membrane filtration (Stericup™, Millipore). DMEM with fetal bovine serum (FBS) and non-essential amino acids (NEAA) was prepared by adding FBS (Gibco™, Invitrogen) to 20% and 100× NEAA (Gibco™, Invitrogen) to 1× (final concentration). DMEM with 1× Antimycotic Antibiotic was prepared as for DMEM with FBS and NEAA except 100× Antimycotic Antibiotic (Gibco™, Invitrogen) was added to 1× (final concentration).

**Table 1 Bacterial strains used in this study**

Strain	Relevant characteristics	Source/Reference
<i>Escherichia coli</i>		
DH5 $\alpha$	Negative control for fibronectin binding Host for propagation of plasmids	Invitrogen
TOP10	Host for propagation of plasmids	Invitrogen
AA13	DH5 $\alpha$ / pGex-6p-3 Control for pGex expression	This study
AA14	TOP10 / pBAD/HisB Control for pBAD expression	This study
AA1	DH5 $\alpha$ / pFbl1	This study
AA2	DH5 $\alpha$ / pFbl2	This study
AA3	DH5 $\alpha$ / pFbl3	This study
AA4	DH5 $\alpha$ / pFbl4	This study
AA5	DH5 $\alpha$ / pFbl5	This study
AA6	DH5 $\alpha$ / pFbl6	This study
AA7	TOP10 / pFbl7	This study
AA8	TOP10/ pFbl8	This study
AA9	TOP10/ pFbl9	This study
AA10	TOP10/ pFbl10	This study
AA11	TOP10/ pFbl11	This study
AA12	DH5 $\alpha$ /pBeryFbl1	This study
<i>Helicobacter pylori</i> CCUG 17874	Positive control for laminin binding	IMBS <sup>a</sup> , MU <sup>b</sup> (Trust et al., 1991)
<i>Lactobacillus rhamnosus</i>		
HN001	Probiotic candidate	FRC CC <sup>c</sup> /B3122
HN001Dfbl	HN001 with a disrupted fbl gene	This study FRC CC/B3165
<i>Lactococcus lactis</i> MG1363	Heterologous antigen used in ELISA	FRC CC/B0037
<i>Staphylococcus aureus</i>		
8325-4	Positive control for fibronectin binding	IMBS, MU
ATCC 25923	Positive control for fibronectin binding	FRC micro testing facility

<sup>a</sup>IMBS = Institute of molecular bioscience

<sup>b</sup>MU = Massey University

<sup>c</sup>FRC CC = Fonterra Research Centre culture collection

**Table 2 Plasmids used in this study**

<b>Plasmid</b>	<b>Relevant characteristics</b>	<b>Source/Reference</b>
pGex-6p-3	Expression vector that produces a GST fusion protein	Invitrogen
pFbl1	pGex-6p-3 + nucleotides crsp.* to Fbl aa 1-557	This study
pFbl2	pGex-6p-3 + nucleotides crsp. to Fbl aa 66-557	This study
pFbl3	pGex-6p-3 + nucleotides crsp. to Fbl aa 1-361	This study
pFbl4	pGex-6p-3 + nucleotides crsp. to Fbl aa 1-156	This study
pFbl5	pGex-6p-3 + nucleotides crsp. to Fbl aa 66-361	This study
pFbl6	pGex-6p-3 + nucleotides crsp. to Fbl aa 66-156	This study
pBAD/HisB	Expression vector that produces a His-tagged protein	Invitrogen
pFbl7	pBAD/HisB + nucleotides crsp. to Fbl aa 1-557	This study
pFbl8	pBAD/HisB + nucleotides crsp. to Fbl aa 1-361	This study
pFbl9	pBAD/HisB + nucleotides crsp. to Fbl aa 1-156	This study
pFbl10	pBAD/HisB + nucleotides crsp. to Fbl aa 66-361	This study
pFbl11	pBAD/HisB + nucleotides crsp. to Fbl aa 66-156	This study
pBery-1	Suicide vector that creates a targeted gene disruption	FRC
pBeryFbl1	pBery-1 + <i>fbl</i> nucleotides 502-1095	This study

\*nucleotides crsp. (corresponding) to Fbl is the DNA sequence encoding the indicated predicted amino acid sequence.



**Lactobacilli recovery broth**

MRS broth/20mM MgCl<sub>2</sub>/2mM CaCl<sub>2</sub> was prepared to the required concentration from 1M MgCl<sub>2</sub> and 1M CaCl<sub>2</sub> stock solutions and MRS broth.

**Luria-Bertani (LB) broth and LB agar**

LB broth contained 10g tryptone, 5g yeast extract and 5g NaCl per litre MilliQ water. LB agar contained 15g/l agar (Becton Dickinson) in LB broth. When required, ampicillin was supplemented at a concentration of 150µg/ml in molten solid or liquid media (cooled to at least 50°C).

**MRS broth and MRS agar**

MRS broth contained 55g of dehydrated MRS broth (Difco) in 1 litre MilliQ water. MRS agar contained 15g/l agar (Becton Dickinson) in MRS broth. When required, erythromycin was supplemented at a concentration of 2.5µg/ml in molten solid or liquid media (cooled to at least 50°C).

**M17 broth and M17 agar**

M17 broth contained 37.25g of dehydrated M17 broth (Difco) in 1 litre MilliQ water. After autoclaving and cooling to ambient temperature, 20% w/v glucose was added to 1%. M17 agar contained 15g/l agar (Becton Dickinson) in M17 broth. After autoclaving and cooling to 50°C, M17 agar was supplemented with 1% w/v glucose.

**SOC medium**

SOB medium/0.36% (v/v) glucose/10mM MgCl<sub>2</sub>/10mM MgSO<sub>4</sub> was prepared by adding 20g tryptone, 5g yeast extract, 0.6g NaCl and 0.2g KCl per litre MilliQ water. After autoclaving and cooling to ambient temperature, SOC medium was prepared to the required concentration from 1M MgCl<sub>2</sub>, 1M MgSO<sub>4</sub> and 20% (w/v) glucose stock solutions and SOB medium.

**Tryptic Soy Broth (TSB) and TSB agar**

TSB contained 30g of dehydrated TSB (Difco) in 1 litre MilliQ water. TSB agar contained 15g/l agar (Becton Dickinson) in TSB.

### **2.2.2 Buffers and Solutions**

Buffers and solutions requiring sterilisation were autoclaved at 121°C for 20min or passed through a 0.22µm filter. MilliQ water and Molecular biology grade reagents were used when possible. All buffers and solutions were stored at ambient temperature unless stated otherwise.

#### **Agarose gels (0.7-1%)**

0.7-1% agarose was prepared by adding 0.7-1g agarose (Sigma) to 100ml 1× TAE buffer and heating until dissolved. Low-melt agarose was prepared in the same manner except, low-melt agarose (SeaPlaque® agarose, FMC BioProducts) was used instead of normal agarose.

#### **Binding Buffer**

0.2M sodium phosphate buffer, pH 7.0

30.5ml 0.2M Na<sub>2</sub>HPO<sub>4</sub> and 19.5ml 0.2M NaH<sub>2</sub>PO<sub>4</sub> were mixed and the pH of the solution was checked (should be pH 7.0). 10× buffer was diluted to 1× (20mM) by the addition of MilliQ water.

#### **Buffer A**

50mM NaCl/30mM Tris-HCl, pH 8.0/5mM EDTA, pH 8.0

#### **Buffer B**

25% (w/v) sucrose/50mM Tris-HCl, pH 8.0/1mM EDTA, pH 8.0/

20µg/ml lysozyme (Sigma)/20µg/ml mutanolysin (Sigma)

Buffer was stored without lysozyme and mutanolysin at ambient temperature.

Immediately before use, lysozyme and mutanolysin was added to the required concentration from 200mg/ml lysozyme and 100µg/ml mutanolysin stock solutions.

#### **Buffer T**

0.05M Tris-HCl-0.1M NaCl, pH 8.0

0.05M Tris solution was made up in MilliQ water and adjusted to pH 8.0 with HCl or NaOH. NaCl was then added to 0.1M.

**Chloroform: Iso-amyl alcohol (24:1)**

Solution was prepared by adding 24 parts chloroform to 1 part iso-amyl alcohol.

**0.1M Citrate-phosphate-0.1M NaCl, pH 4.5**

54.5ml 0.1M Citric acid was added to 45.5ml 0.2M Na<sub>2</sub>HPO<sub>4</sub> and the pH of the solution was checked. If the solution was lower than pH 4.5, more 0.2M Na<sub>2</sub>HPO<sub>4</sub> was added. If the solution was higher than pH 4.5, more 0.1M Citric acid was added. Solid NaCl was then added to a final concentration of 0.1M.

**Coomassie protein stain**

0.1% (w/v) Coomassie brilliant blue R-250/40% (v/v) Methanol/10% (v/v) Acetic acid  
Stain was prepared by dissolving Coomassie blue in methanol and stirring for 30 min. MilliQ water and acetic acid were then added.

**Denaturation solution**

1.5M NaCl/0.5M NaOH

**Depurination solution**

0.25M HCl

**Destain buffer**

40% (v/v) Methanol/10% (v/v) Acetic acid

**6× DNA loading buffer**

50% (v/v) Glycerol/0.25% (w/v) Bromophenol blue/0.25% (w/v) Xylene cyanol

**DNase-free RNase A**

RNase A (Sigma) was made up to 10mg/ml in 10mM Tris-HCl, pH 7.5/15mM NaCl, heated at 100°C for 15min, cooled to ambient temperature and stored at -20°C in aliquots.

### **DNaseI**

DNase I (Sigma) was made up to 1mg/ml in 10mM Tris-HCl, pH 7.5/150mM NaCl/1mM MgCl<sub>2</sub>. When DNase I was dissolved, an equal volume of glycerol was added, mixed gently and stored in aliquots at -20°C.

### **Glutathione elution buffer**

10mM reduced glutathione in 50mM Tris-HCl, pH 8.0

The elution buffer was stored at -20°C in aliquots.

### **Hybridisation buffer**

To the required amount of hybridisation buffer, solid NaCl was added to a final concentration of 0.5M and blocking reagent (supplied with ECL™ direct nucleic acid labelling and detection systems kit, Amersham) was added to 5% (w/v). The buffer was mixed for 1 hr and then heated to 42°C before use.

### **Neutralisation solution**

1.5M NaCl/0.5M Tris-HCl was adjusted to pH 7.5 with HCl or NaOH.

### **PBS-based buffers**

**10× PBS:** 0.01M KH<sub>2</sub>PO<sub>4</sub>/0.1M Na<sub>2</sub>HPO<sub>4</sub>/1.37M NaCl/0.027M KCl

**1× PBS, pH 7.4:** Dilute 10× PBS to 1× with the addition of MilliQ water (pH does not need adjustment)

**1× PBST:** Add Tween 20 to 0.1% (v/v) in 1× PBS, pH 7.4

**1× PBS + protease inhibitors:** 1 complete, mini, EDTA-free protease inhibitor cocktail tablet (Roche) was added to 10ml 1× PBS, pH 7.4. EDTA, pH 8.0 was added to 2mM (final concentration) and used immediately.

### **Phenol saturated with 50mM Tris, pH 8.0**

A 500g bottle of phenol crystals was heated at 50°C until melted, hydroxyquinoline was added to 0.1% (w/v), 250ml 50mM Tris-HCl, pH 8.0 was added and stirred until mixed. Once the two phases had separated, the clear top phase was discarded, 250ml 50mM Tris-HCl, pH 8.0 was added, mixed and the top phase was discarded. One hundred ml 50mM Tris-HCl, pH 8.0 was added. The saturated phenol was stored at 4°C.

**PreScission cleavage buffer**

50mM Tris-HCl, pH 7.0/150mM NaCl/1mM EDTA

Immediately before use, dithiothreitol (DTT) was added to a final concentration of 1mM.

**Primary wash buffer**

0.4% (w/v) SDS/0.5× SSC

**2× SDS-PAGE sample buffer**

The following solutions were mixed together and stored at -20°C: 1.25ml 0.5M Tris-HCl, pH 6.8, 2ml 10% (w/v) SDS, 5ml MilliQ water, 1ml Glycerol, 0.5ml β-mercaptoethanol and 0.25ml 0.1% (w/v) Bromophenol blue.

**5× SDS-running buffer**

0.125M Tris/0.96M Glycine/0.5% (w/v) SDS

The solution was confirmed to be pH 8.3 (not adjusted). The buffer was stored at 4°C and diluted to 1× with MilliQ water when needed.

**20× SSC**

0.3M Na<sub>3</sub>citrate/3M NaCl

The solution was adjusted to pH 7.0 with HCl or NaOH. The stock SSC was diluted to different concentrations (10×, 6× and 5×) with the addition of MilliQ water.

**50× TAE**

50mM Tris-HCl/0.11% (v/v) glacial acetic acid/1mM EDTA

50× TAE was made up in MilliQ water and adjusted to pH 8.0 with HCl or NaOH. 1× TAE was prepared by diluting 50× TAE to 1× in MilliQ water.

**10× TBS**

0.25M Tris/0.144M NaCl

The solution was confirmed as pH 8.0 (not adjusted). 10× TBS was diluted to 1× with MilliQ water.



**TE buffer, pH 8.0**

10mM Tris-HCl, pH 8.0/1mM EDTA, pH 8.0

**Transfer buffer**

48mM Tris/39mM Glycine/20% (v/v) Methanol

The buffer was confirmed to be between pH 9.0 and 9.4 (pH not adjusted).

**V buffer**

0.5M Sucrose/7mM potassium phosphate buffer, pH 7.4/1mM MgCl<sub>2</sub>

**Water-saturated isobutanol (1:1)**

Add equal volumes of MilliQ water and isobutanol. Mix by shaking vigorously and stand to allow the layers to separate. Isobutanol is the top layer.

**Western blocking solution**

1% (w/v) non-fat milk powder (Anchor) in 1× TBS

**Western developing solution**

Chloronaphthol (30mg) was dissolved in 10ml ice-cold methanol. 1× TBS (50ml) and 33μl hydrogen peroxide solution (approx. 30%, BDH) was added and the solution was used immediately.

**2.2.3 Protein solutions**

Unless stated, all protein solutions were stored at 4°C. Protein solutions were diluted to 100μg/ml in 1× PBS, pH 7.4 (Section 2.2) except for collagen I and collagen IV, which were diluted in 0.1M acetic acid.

**100mg BSA/ml PBS**

Bovine serum albumin (Molecular Biology Grade, Sigma) was prepared to 100mg/ml in 1× PBS, pH 7.4 (Section 2.2).

**20mg BSA/ml PBST**

Bovine serum albumin (Molecular Biology Grade, Sigma) was prepared to 20mg/ml in 1× PBST (Section 2.2).

### **Cellular fibronectin**

Cellular fibronectin (from human foreskin fibroblasts, Sigma F-6277) was prepared to 1mg/ml by adding 0.5ml MilliQ water to a 0.5mg bottle and incubating at ambient temperature for 30 min.

### **Collagen I**

A piece of collagen I (Sigma C-9791) was weighed and dissolved in an appropriate volume of 0.1M acetic acid (to give a solution of 2mg collagen I/ml) at ambient temperature for 2 hr. The solution was then left to further dissolve at 4°C overnight before being centrifuged at 6000×g for 5 min (fixed angle rotor) to pellet undissolved protein. The  $A_{280}$  of the supernatant was measured to determine protein quantity (1mg collagen I /ml =  $A_{280}$  0.066).

### **Collagen IV**

Collagen IV (from human placenta, Sigma C-5533) was prepared to 1mg/ml by adding 5ml 0.05M acetic acid to a 5mg bottle and incubating at 4°C overnight. Collagen IV was quantitated with the Bio-Rad protein assay (outlined in Section 9.1).

### **Fibronectin (plasma)**

Fibronectin (from human plasma, Sigma F-2006) was prepared to 1mg/ml by adding 1ml MilliQ water to a 1mg bottle fibronectin and incubating at 37°C for 30 min. The concentration was checked by measuring  $A_{280}$  in a quartz cuvette against a water blank (1mg/ml plasma fibronectin =  $A_{280}$  1.387). 10× PBS (Section 2.2) was then added to 1× final concentration.

### **Heparan Sulphate**

Heparan sulphate (Sigma H-4777) came as a 0.59mg/ml solution, which was stored at -80°C until needed.

### **Laminin**

Laminin (Sigma L-2020) came as a 1mg/ml solution, which was stored at -80°C until needed.

## **Mucin**

Mucin (Sigma M-1778) was dissolved in 1× PBS, pH 7.4 (Section 2.2) to give a 4mg mucin/ml solution (at ambient temperature for 1hr). Mucin was quantitated with the Bio-Rad protein assay (outlined in Section 9.1).

## **Super fibronectin**

Super fibronectin (Sigma S-5171) came as a 2mg/ml solution.

## **Vitronectin**

Vitronectin (Sigma V-8379) was prepared to 0.25mg/ml in sterile water and dissolved at ambient temperature for 1 hr.

## **2.3 Bacteriological methods**

### **2.3.1 Culture conditions**

*Lactobacillus rhamnosus* HN001 and other lactobacilli were routinely cultured in MRS broth (Section 2.2.1) in a stationary 37°C waterbath or on MRS agar plates (Section 2.2.1). The plates were placed inside an anaerobic chamber (GasPak® System, Becton Dickinson) with anaerobic generator (Anaerocult® A, Merck) or in a sealed plastic bag with anaerobic generator (Pouch-Anaero, BioMerieux). The anaerobic chambers or bags were placed in a 37°C incubator. Anaerobic conditions were confirmed by indicator strips (BBL™ Dry Anaerobic indicator strips, Becton Dickinson). A 1% inoculum of stationary-phase HN001 was used to culture subsequent HN001 cultures. For use in binding assays, HN001 was cultured from a freshly grown stationary-phase culture. HN001 strains with erythromycin resistance were cultured in the presence of 2.5µg/ml erythromycin in either MRS broth or MRS agar plates. For use in binding assays, HN001 strains with erythromycin resistance were cultured without erythromycin. *Escherichia coli* was routinely cultured in LB broth (Section 2.2.1) or BHI broth (Section 2.2.1) at 37°C with shaking at 200 rpm. Solid culture was grown on LB agar plates (Section 2.2.1) and BHI agar plates (Section 2.2.1) at 37°C in aerobic conditions. Antibiotics were included in the medium where appropriate as follows: strains with ampicillin resistance were selected for with 150µg/ml ampicillin in LB

broth or LB agar plates and strains with erythromycin resistance were selected for with 150µg/ml erythromycin in BHI broth or BHI agar plates. *Lactococcus lactis* MG1363 was routinely cultured in M17 broth supplemented with 1% glucose (Section 2.2.1), at 30°C in a stationary waterbath or on M17 agar plates (Section 2.2.1) at 30°C (ambient temperature). *Staphylococcus aureus* ATCC 25923 and *S. aureus* 8325-4 were routinely cultured in TSB (Section 2.2.1) or in BHI broth at 37°C with shaking at 200 rpm or on TSB agar plates (Section 2.2.1) in aerobic conditions at 37°C.

### **2.3.2 Storage of strains**

Long term storage of bacterial strains was achieved by adding sterile glycerol to 20% (v/v) in 1ml fresh overnight liquid culture. The aliquots were stored in cryogenic vials at -80°C until required. Aliquots were revived by adding a 1% inoculum of frozen stock to fresh medium and then incubating as described in Section 2.3.1. Short-term storage of bacterial strains was achieved by storing solid plates and bottles or tubes of culture at 4°C.

### **2.3.3 Determination of bacteria numbers**

The approximate number of HN001 cells in a given culture was determined from OD<sub>650</sub> of the culture (Ultrospec® II, LKB Biochrom). The relationship between HN001 cfu/ml and OD<sub>650</sub> had been previously determined (OD<sub>650</sub> 1.0 = 1×10<sup>8</sup> cfu/ml). The approximate number of *E. coli* DH5α and *S. aureus* 8325-4 cells in a given culture were determined by OD<sub>650</sub> (Ultrospec® II, LKB Biochrom). The number of bacterial cells corresponding to a particular OD<sub>650</sub> was determined by plating serial dilutions of cells and counting the resulting colonies (grown as outlined in Section 2.3.1).

### **2.3.4 Preparation of HN001 spent culture supernatant**

Spent culture supernatant was prepared by harvesting an 18 hr HN001 culture (grown as outlined in Section 2.3.1) at 6000×g for 10min (GSA, Sorvall®, Du Pont) and retaining the supernatant.

## 2.4 DNA manipulation

### 2.4.1 Preparation of plasmid DNA from *E. coli* DH5 $\alpha$

Plasmid DNA from *E. coli* DH5 $\alpha$  was prepared by using the QIAprep® Spin Miniprep kit (QIAGEN), which uses a modified alkaline lysis method (Birnboim *et al.*, 1979). If larger quantities of plasmid were required (eg. for transformation of HN001) the QIAGEN plasmid Maxi kit (QIAGEN) was used. Cultures were grown with antibiotic selection for maintenance of the plasmid as described in Section 2.3.1. Three ml of stationary phase culture was harvested for DNA extraction when using the miniprep kit. Forty ml of stationary phase culture was harvested for DNA extraction when using the maxiprep kit. DNA extraction was performed as outlined in the manufacturer's instructions (QIAGEN). Briefly, cells were disrupted by alkaline lysis. The bacterial lysate was cleared by centrifugation and then applied to a silica-gel membrane (in a column). After washing the column to remove excess salt, plasmid DNA was eluted from the column. In the maxiprep kit, DNA in the eluate was precipitated by isopropanol, pelleted by centrifugation and resuspended in 10mM Tris, pH 8.0.

### 2.4.2 Preparation of total genomic DNA from HN001

Total genomic DNA was prepared by a method based on a streptococci DNA extraction method (Anderson *et al.*, 1983). Twenty ml of a fresh 18 hr culture was harvested by centrifugation at 10000 $\times$ g for 10 min (GSA rotor, Sorvall®, Du Pont), resuspended in 20ml fresh MRS broth (Section 2.2.1) and incubated in a 37°C waterbath for 2 hr. The 20ml culture was then harvested by centrifugation (as above) and washed twice in Buffer A (Section 2.2.2). The pellet was resuspended in 0.5ml Buffer B (Section 2.2.2). The suspension of cells was then incubated at 37°C for 45 min to lyse the cells. 0.5ml of 0.25M EDTA, pH 8.0 was then added and incubated for 5 min at ambient temperature. 200 $\mu$ l of 20% (w/v) SDS was then added and incubated at 65°C until the solution cleared (or 90 min if no clearing occurred). Addition of 10 $\mu$ l 20mg proteinase K/ml PBS and incubation at 65°C for 15 min degraded the proteins in the solution. Proteins were extracted from the solution by addition of an equal volume of phenol saturated with 50mM Tris, pH 8.0 (Section 2.2.2) plus 200 $\mu$ l chloroform. The solution was mixed and incubated at ambient temperature for 5 min to allow proteins to be collected into the organic/aqueous phase boundary. This mixture was then centrifuged

at 2700×g for 10 min (Megafuge 1.0, Heraeus Sepatech GmbH) to separate the two phases. The aqueous (upper) phase was transferred to a clean tube and the phenol extraction was repeated. The aqueous phase from the second phenol extraction was transferred to a clean tube and an equal volume of chloroform:iso-amyl alcohol (IAA) (24:1) (Section 2.2.2) was added. The mixture was centrifuged 2700×g for 10 min (Megafuge 1.0) and the aqueous phase was transferred to a clean tube. The chloroform:IAA extractions were repeated until no protein appeared at the organic/aqueous interface. Crude DNA in the final aqueous phase was precipitated with an equal volume of ice-cold isopropanol at -20°C for at least 4 hr or overnight. DNA was harvested by centrifugation at 15000×g for 10 min (MicroMax®, IEC) and the supernatant was aspirated. The open tubes were placed at 37°C for approximately 10 min to evaporate the residual isopropanol. Crude DNA was then purified as outlined in Section 2.4.3.

#### **2.4.3 Purification of genomic DNA from HN001**

The crude DNA pellet was dissolved in 200µl TE, pH 8.0 (Section 2.2.2) by gentle flicking of the tube. Residual RNA contamination was removed by adding DNase-free RNaseA to a final concentration of 100µg/ml (Section 2.2.2) and incubating at 37°C for 30 min. The crude DNA was then further deproteinated with 20µl 20mg proteinase K/ml PBS and 0.2% (w/v) SDS (final concentration) at 37°C for 30 min. After this incubation, the SDS concentration was adjusted to 0.5% (w/v) and the proteins in the mixture were extracted by addition of an equal volume of phenol saturated with 50mM Tris, pH 8.0 (Section 2.2.2) plus 20µl chloroform. The solution was mixed until an emulsion formed and then incubated at ambient temperature for 30 min. The two phases were separated by centrifugation at 3300×g for 5 min (MicroMax®, IEC) and the aqueous phase was transferred to a clean tube. An equal volume of chloroform:IAA (24:1) (Section 2.2.2) was added to the aqueous phase, mixed and centrifuged at 3300×g for 5 min (MicroMax®). The aqueous phase was transferred to a clean tube and further extracted with an equal volume of chloroform:IAA if required. Purified DNA in the aqueous phase was precipitated by addition of two volumes of -20°C 95% (v/v) ethanol at -20°C for at least 1 hr. The precipitated DNA was harvested by centrifugation at 15000×g for 10 min (MicroMax®). The supernatant was aspirated and



the DNA pellet dried by placing the open tube at 37°C for approximately 10 min. The pellet was then dissolved in 100µl 10mM Tris, pH 8.0 by gentle flicking of the tube.

#### **2.4.4 Storage of DNA**

Once DNA was fully dissolved, it was stored at -20°C.

#### **2.4.5 Quantitation of DNA**

Genomic DNA was quantitated by two methods. 5µl of dissolved DNA was added to 995µl sterile water and the absorbance at 260nm ( $A_{260}$ ) was measured in a quartz cuvette against a water blank (Ultrospec® II, LKB Biochrom). The formula used to determine DNA concentration from the  $A_{260}$  value is included in Appendix 1. Genomic DNA was also quantitated by applying 1µl of undiluted DNA (or a dilution of DNA) and several known amounts of  $\lambda$  DNA (Invitrogen) on a 0.7 % agarose gel (Section 2.2.2) and comparing intensity of fluorescence. Plasmid DNA was quantified by applying 1µl of undiluted DNA (or a dilution of DNA) and a mass ladder (Invitrogen) on a 0.8% agarose gel and comparing intensity of fluorescence. Electrophoresis, staining and visualisation of agarose gels were carried out as outlined in Section 2.4.6.

#### **2.4.6 Agarose gel electrophoresis of DNA**

Various DNA fragments (PCR products, plasmids and total genomic DNA) were visualised by electrophoresis through agarose (0.7-1%) (Section 2.2.2), using 1× TAE buffer (Section 2.2.2). Samples were mixed with 1/6 volume of 6× DNA loading buffer (Section 2.2.2) before being loaded into the well of an agarose gel. Several molecular weight and mass ladders were used to determine quantity and molecular weight of DNA fragments run through agarose. Ladders used included: 1Kb+, supercoiled and  $\lambda$ /Hind III ladders (Invitrogen) for size determination; low mass and high mass ladders (Invitrogen) for quantitation of DNA. DNA fragments were separated by application of an electrical current of 100V for approximately 1 hr, in an electrophoresis apparatus (Sub-Cell® GT Agarose gel electrophoresis system, Bio-Rad). Agarose gels were stained with 0.6µg ethidium bromide/ml water for 15 min. Gels were briefly destained in distilled water before being visualised by eye through use of an UV Transilluminator (Ultraviolet products inc.). Gels were then photographed with a Polaroid MP-4 land camera (Polaroid) or by a Fluor-S® MultiImager (Bio-Rad).

#### **2.4.7 Restriction digestion of DNA**

All restriction digestion of DNA was performed following the same formula of ingredients:

1/10 vol	10× RE Buffer (supplied by company that supplied enzyme)
1-2 unit/s	Restriction Endonuclease (RE) (Invitrogen or NEB)
Required volume	DNA
Required volume	Sterile water to make up to required reaction volume

Each reaction was incubated in a 1.5ml tube in a floating rack in a waterbath at the recommended temperature for 1.5 to 2 hr. In the case of digestion of plasmid and genomic DNA, visualising DNA fragments by agarose gel electrophoresis (as outlined in Section 2.4.6) assessed the completeness of the reaction.

#### **2.4.8 Sequencing genomic DNA**

HN001 genomic DNA was sheared by forcing purified genomic DNA (prepared as outlined in Sections 2.4.2 – 2.4.3) through a 20-gauge needle 10 times. Oligonucleotide primers were designed to anneal to the region encoding the N-terminus of the gene so that sequencing reactions would cover the region of interest. Sequencing reactions were performed using ABIPrism® Big Dye™ Terminator V3.0 (Applied Biosystems) as outlined in the manufacturer's instructions. Briefly, distilled water (to a total reaction volume of 40µl) and 1µg sheared genomic DNA were mixed and incubated at 50°C for 1 hr in the GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems) to remove any secondary structure in the DNA. 16µl of Terminator Ready Reaction Mix and 10pmol primer were then added and mixed. The sequencing mixture was then subjected to 99 cycles of thermocycling in the GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems). The thermocycling protocol started with an initial denaturation of 95°C for 5 min. Then the sequencing reaction was subjected to 99 cycles of a denaturation step (95°C for 30 sec), an annealing step (55°C for 30 sec) and an extension step (60°C for 4 min). Sequencing products were purified by the addition of 3µl 3M sodium acetate pH 4.6, 62.5µl 95% ethanol and 14.5µl deionised water per 20µl of sequence reaction. This solution was mixed and incubated for 15min, then centrifuged for 20 min at 15000×g (Fixed angle rotor). The supernatant was aspirated and pellet washed with 250µl 70% (v/v) ethanol. After centrifugation for 5 min at

15000×g (Fixed angle rotor), the supernatant was aspirated and pellet air-dried. This purified reaction was either analysed by the ABI Prism 377-64 DNA sequencer at Massey University Sequencing Facility, Massey University, Palmerston North or by the MegaBACE DNA analysis system at Waikato DNA sequencing facility, Waikato University, Hamilton.

## 2.5 PCR amplification of DNA

### 2.5.1 Oligonucleotide primer design

Draft HN001 genome sequence was obtained prior to this study. Oligonucleotide primers were designed from HN001 genome contiguous (contig) sequences with the help of the computer program Omega (Oxford Molecular Ltd.). Primers were purchased from Sigma® Genosys (Sigma-Aldrich). Oligonucleotide primers used in this study are listed in Table 3.

**Table 3 Oligonucleotide primers used in this study**

Primer Name	Oligonucleotide Sequence 5' to 3'	Function of primer	Cloning system
AB2F01	AAAGCGAGCGGATCAATATG	Sequencing	N/A
AB2F02	GGCATGACGAGTATTGAAC	Sequencing	N/A
AB2F03	ACAGCAACAAGGCTATCTACG	Sequencing	N/A
AB2F04	ACCGACATTTGGCTGCAC	Sequencing	N/A
AB2F05	CCGCCGCCGGTCGACTCTTTTGACG GAATTTTTTCACC	Expression	pGex-6P-3
AB2F06	CCGCCGCCGGTCGACACGTTGCGG AAGTATTTTAACG	Expression	pGex-6P-3
AB2F07	CCGTGTTTGGTGAACGAAC	Sequencing	N/A
AB2F08	GCTTAACCTTAAGTCCCAT	Sequencing	N/A
AB2F09	TCTTTTATAGTCAGCAAGCG	Sequencing	N/A
AB2F10	CCGCCGCCGGTCGACGCCAGGAG CTTAATACCACG	Expression	pGex-6P-3
AB2F11	CCGCCGCCGCTCGAGAGCCCAGGA GCTTAATACCACG	Expression	pBAD/HisB
AB2F12	CCGCCGCCGCTCGAGAACGTTGCG GAAGTATTTTAACG	Expression	pBAD/HisB
AB2F13	CCGCCGCCGCTAGAGATTCGGAG CGGATTTATCAC	Insertional mutant	pBery-1
AB2R01	TCAAGGTAGTCGAGTTCTTC	Sequencing	N/A
AB2R02	ATCTCGAGCGTTATGGGCGTAAAG GAGA	Sequencing	N/A
AB2R03	CGGTAATGCACCGAATCG	Sequencing	N/A
AB2R04	CAATGATATGTTCTGAAGTGG	Sequencing	N/A
AB2R05	CCGCCGCCGGCGGCCCAATGAT ATGTTCTGAAGTGG	Expression	pGex-6P-3
AB2R06	CATTATCAGCATAGAAATTAGGC	Sequencing	N/A

AB2R07	GGATTAGCTGAGAGCAACAA	Sequencing	N/A
AB2R08	CCGCCGCCGGCGGCCGCTTGCTTA GGCGGCTCGACATA	Expression	pGex-6P-3
AB2R09	CCGCCGCCGGCGGCCGCTTGGCA AAATATTTCTGGGC	Expression	pGex-6P-3
AB2R10	CCGCCGCCGAAGCTTTTATTGCTTA GGCGGCTCGACATA	Expression	pBAD/HisB
AB2R11	CCGCCGCCGAAGCTTTTACTTGGCA AAATATTTCTGGGC	Expression	pBAD/HisB
AB2R12	CCGCCGCCGAAGCTTGCAATGATA TGTTCTGAAGTGGG	Expression	pBAD/HisB
AB2R13	CCGCCGCCGGAGCTCCTTGGCAA ATATTTCTGGGC	Insertional mutant	pBery-1
AB2R14	AACGTGGCGAATGAGATCA	Sequencing	N/A
AB2R15	CTTGCGTCCAGCTCGAATC	Sequencing	N/A
AB2R16	CTAAGGCCAAAGGCTGGTCAAC	Sequencing	N/A
ErmF01	TGTGGTATGGCGGGTAAG	Insertional mutant	pBery-1
ErmR01	ATAATGGGAGATAAGACGGTTC	Insertional mutant	pBery-1
pGex6p3F	CTGGCAAGCCACGTTTGG	Expression	pGex-6P-3
pGex6p3R	GGAGCTGCATGTGTCAGAGG	Expression	pGex-6P-3
pBADHisBF01	CGCAACTCTCTACTGTTTCTCC	Expression	pBAD/HisB
M13F	CCCAGTCACGACGTTGTAAAACG	Insertional mutant	pBery-1
M13R	AGCGGATAACAATTTACACAGG	Insertional mutant	pBery-1

### 2.5.2 PCR amplification of DNA targets

PCR amplification was performed using a GeneAmp® PCR System 9700 Thermal Cycler (PE Applied Biosystems). Amplification using *Taq* polymerase was performed using 2× PCR Master Mix (Roche), which includes *Taq* polymerase, buffer and deoxynucleotides. Amplification using *Pwo* polymerase was performed using *Pwo* polymerase and *Pwo* polymerase buffer (Roche). Most PCR reactions were first tested in a reaction volume of 25µl before being scaled-up. Small scale PCR reactions (25µl) usually contained 50ng of HN001 genomic DNA, 10pmol of each primer, 12.5µl PCR master mix and sterile water to make the volume up to 25µl. Large-scale PCR reactions were formulated at the same ratio as the small scale reaction. PCR amplification was achieved with 25-35 cycles of thermocycling. The thermocycling protocol started with an initial denaturation of 94°C for 5 min. Then the PCR reaction is subjected to 25-35 cycles of a denaturation step (94°C for 30 sec), an annealing step (2°C lower than the calculated  $T_m$  values of the primers used for 30 sec) and an extension step (72°C for 1 min per Kb of expected product size). The formula used to calculate  $T_m$  is included in Appendix 1. After the cycles are complete, the reaction is subjected to a further extension step of 72°C for 4 min to ensure that all products are fully synthesised to double-stranded DNA. When amplifying DNA template with *Pwo* polymerase, Hot-start PCR amplification was used. A lower reaction mixture was made that contained primers, deoxynucleotides (Invitrogen) and sterile water. The lower reaction mixture was added to a HotStart Reaction tube (Molecular BioProducts). The tubes were then heated in a GeneAmp® PCR System 9700 Thermal Cycler (PE Applied Biosystems) at 90°C for 30 sec to melt the wax inside the tube. The tubes were allowed to cool at ambient temperature to allow the wax to harden over the lower reaction mixture. An upper reaction mixture containing the DNA template, 10× PCR buffer and *Pwo* polymerase was assembled and added to the tubes after the wax had cooled. PCR amplification was achieved as outlined above. A negative control containing all reagents except template was performed for every reaction. Also, the first time a primer was used, a reaction with all reagents minus one primer was performed to ensure the PCR product obtained was specific to both primers.



### **2.5.3 Purification of PCR products**

PCR reactions for use in cloning were purified before restriction endonuclease digest. In reactions with a single PCR product, unincorporated nucleotides and oligonucleotide primers were removed by using the High Pure PCR Purification Kit (Roche) according to the manufacturer's instructions. Briefly, a binding buffer containing guanidine thiocyanate was added to the PCR product. DNA then bound specifically to glass fibres in a column. After two washes, DNA was eluted using a low salt solution. In reactions with several PCR products, the entire reaction was run on a low melt agarose gel (Section 2.2.2) at 80V. The gel was stained and visualised as outlined in Section 2.4.5. The required PCR product (determined from its size against the 1Kb+ ladder) was excised from the gel with a sterile scalpel and placed in a sterile plastic 1.5ml tube. The QIAEX® II Gel extraction kit (QIAGEN) was used to purify the DNA in the agarose slice according to the manufacturer's instructions. Briefly, the agarose slice was solubilised with a buffer containing chaotropic salt and DNA was adsorbed onto silica gel particles. DNA was eluted from the particles with a low salt solution. Purified PCR products were quantitated by electrophoresis against DNA mass ladders (Invitrogen) as outlined in Section 2.4.6.

### **2.5.4 Sequencing of PCR products**

The DNA sequences of PCR products were deduced using the dideoxy chain termination method. PCR products were sequenced by dye terminator chemistry (Sanger *et al.*, 1977) and analysed by the ABI Prism 377-64 DNA sequencer at the Massey University Sequencing Facility, Massey University, Palmerston North or by the MegaBACE DNA analysis system at Waikato DNA sequencing facility, Waikato University, Hamilton. A verified sequence of the HN001 contig with *fbl* was assembled from overlapping sequences from PCR products with the aid of the sequence assembly program Sequencher™ (Version 4.0.5, Gene Codes Corporation).

## **2.6 Characterisation of *fbl* in silico**

### **2.6.1 Use of ORF prediction tools, BLASTP and sequence alignments**

Draft HN001 genome sequence was obtained prior to this study. The prediction of ORFs in DNA sequence and the predicted amino acid sequence was determined using the computer program Omega 2.0 (Oxford Molecular Ltd.). The possible start codons

used were TTG, CTG, ATT, ATG and GTG. BLASTP was done through the Bionavigator website ([www.bionavigator.com](http://www.bionavigator.com)) using default settings (database: NCBI nr, matrix: BLOSUM62, expect cut off: 10 and word size: 3). Multiple sequence alignments were done using a Clustal W1.6 algorithm available through Omega 2.0. Dot Plots of protein sequences were performed using the BLOSUM62 scoring matrix (using default settings) in Omega 2.0. Dot Plots of DNA sequences were performed using the NA identity matrix (using default settings) in Omega 2.0.

### **2.6.2 Use of Motif search engines**

Motifs in the putative Fbl protein sequence were searched for using Prosite 15.0 available through the computer program Omega 2.0 (Oxford Molecular Ltd.) and Pfam 7.6 (<http://pfam.wustl.edu/>). The presence of a putative signal peptide was searched for using Signal P 2.0 available through the Bionavigator website ([www.bionavigator.com](http://www.bionavigator.com)).

## **2.7 Cloning of DNA into plasmid vectors**

### **2.7.1 Vectors**

The expression system pGex-6p-3 (Pharmacia Biotech) was used to express the Fbl gene product in *E. coli* DH5 $\alpha$ . The pGex expression system relies on induction of the *tac* promoter by addition of isopropylthio- $\beta$ -D-galactoside (IPTG) to induce expression of the cloned gene product. The inclusion of an internal *lacI*<sup>q</sup> gene (*lac* repressor) on the vector reduces leaky expression of the gene product until the inducer is added. The inducer binds to the repressor and allows expression of the gene product. An alternative expression system, pBAD/HisB (Invitrogen) was used to express the Fbl gene product in *E. coli* TOP10. pBAD/HisB is an expression system that relies on induction of the *araBAD* promoter by addition of L-arabinose to induce expression of the cloned gene product. The regulatory gene, *araC*, also located on the vector is a tight regulator of the *araBAD* promoter. The production of C protein from *araC* shuts off any leaky expression from the arabinose operon until the inducer (arabinose) binds to C protein (bound to the operon) and acts as an activator of transcription. Maps of these two expression vectors are included in Appendix 2. The vector used for insertional disruption of gene targets is described in Section 2.10.1 and included in Appendix 2.

### 2.7.2 Primer design and PCR amplification for cloning into plasmid vectors

Primers used for cloning into plasmid vectors are included in Table 3. All primers for cloning into plasmid vectors were designed with a 5' CCG clamp (repeated three times) followed by a restriction endonuclease site. Two different restriction endonuclease sites were used for a primer pair to ensure directional cloning. For an upstream expression primer, the restriction endonuclease site was followed by the 2<sup>nd</sup> to 9<sup>th</sup> codons of the coding sequence. For a downstream expression primer, a natural stop codon and the 7 codons preceding the stop codon followed the restriction endonuclease site. The inclusion of a stop codon in a downstream expression primer was additional to the stop codon that follows the multiple cloning site in pGex-6P-3. In addition, rrnBT<sub>1</sub> and T<sub>2</sub> transcriptional terminators follow the multiple cloning site in pBAD/HisB. Inserts for cloning into expression vectors were amplified by HotStart PCR with *Pwo* polymerase (as outlined in Section 2.5.2). For cloning into vectors other than expression vectors, PCR products for were amplified containing flanking restriction endonuclease sites with *Taq* polymerase (as outlined in Section 2.5.2).

### 2.7.3 Ligation of inserts into plasmid vectors

After both the insert and vector were digested with the appropriate restriction endonucleases (as outlined in Section 2.4.7), the insert was ligated into the vector using T4 DNA Ligase (New England Biolabs inc.) according to the manufacturer's instructions. To ensure optimal ligation of an insert into a vector, the molar ratios of insert and vector were calculated from the quantity and size of each fragment (see Appendix I for the formula used). Insert and vector were added to the ligation mix at a molar ratio of 3:1 (insert: vector). The approximate quantity of insert: vector used was 1µl: 5-15µl. The ligation mix was formulated as follows:

1/5 volume	5× Ligase buffer (NEB)
1 unit	T4 DNA Ligase (NEB)
Required volume	Digested insert DNA
Required volume	Digested vector DNA
Required volume	Sterile water to make up to required reaction volume

The ligation mix was incubated at 16°C overnight in a GeneAmp® PCR System 9700 Thermal Cycler (PE Applied Biosystems). Digested vector DNA alone was included as a negative control to assess the amount of recircularisation of vector DNA.

#### **2.7.4 Transformation of *E.coli* DH5α or TOP10 cells**

Chemically competent *E. coli* DH5α cells (MAX Efficiency® DH5α™ Competent cells, Invitrogen) were transformed with pGex ligation mixtures according to the manufacturer's instructions. *E. coli* TOP10 cells (One Shot® TOP10 Chemically competent *E. coli*, Invitrogen) were transformed with pBAD ligations according to the manufacturer's instructions. Generally 5µl of ligation mix was used to transform the two *E. coli* strains used. DNA and 50µl cells were incubated on ice for 30 min before being heat shocked at 42°C for 45 sec (30 sec at 42°C for TOP10 cells). The transformation mixture was then placed back on ice for 2 min before addition of 450µl SOC (250µl SOC for TOP10 cells) (Section 2.2.1). Cells were then incubated at 37°C in a shaking incubator for 1 hr. Several amounts of each transformation were plated on agar plates with antibiotic selection as outlined in Section 2.3.1. Transformation efficiencies were calculated for each set of plates (see Appendix 1 for formula used).

#### **2.7.5 Screening of transformants**

Transformants were screened for the presence of the correct insert in the plasmid vector by PCR using a primer annealing in the insert region and one within the vector region. This amplified a diagnostic fragment that indicated that the insert in the vector was *fbl* and that the insert was in the correct orientation. PCR was performed as outlined in Section 2.5.2, except no DNA was added. Colonies were picked off plates with a sterile tip, patched onto a fresh agar plate with antibiotic selection, and then resuspended in a tube containing PCR Master Mix, primers and sterile water. Clones that showed a positive result were streaked for single colonies on agar plates with antibiotic selection as outlined in Section 2.3.1. A single colony from the streak was used to inoculate 10ml broth as outlined in Section 2.3.1. A small-scale preparation of plasmid DNA was performed as outlined in Section 2.4.1. 5µl of the plasmid preparation was digested with one or two restriction endonucleases (Section 2.4.7) to provide several diagnostic fragments that were visualised by agarose gel electrophoresis (Section 2.4.6). Clones that showed expected patterns of DNA fragments were chosen for use in expression studies. These clones were stored as outlined in Section 2.3.2.

## 2.8 Expression of gene products

### 2.8.1 Expression conditions

Fusion proteins were expressed using the pGex expression system (Pharmacia Biotech) or the pBAD expression system (Invitrogen) according to the manufacturer's instructions. Briefly, a 2% inoculum of fresh stationary culture of an *E. coli* strain containing an expression vector (grown from glycerol stock or single colony) was added to 40ml LB broth with 150µg/ml ampicillin (Section 2.2.1) and cultured as outlined in Section 2.3.1 until it reached an OD<sub>600</sub> of 0.5. The inducer (IPTG or arabinose) was then added, the growth temperature was reduced, and the culture was then grown further for a set time period (Section 3.4.4.1). To monitor fusion protein expression, at the end of the induction 1ml of cells was removed and centrifuged at 15000×g for 1 min (MicroMax®, IEC). The supernatant was discarded and the cells were stored at -20°C until analysed by protein gel electrophoresis as outlined in Section 2.8.3. Several expression conditions including the amount of inducer added, the temperature of induction and the length of induction were varied to optimise the amount and solubility of protein produced. The solubility of the fusion protein was determined by lysing the cells at the end of induction (Section 2.8.2). The soluble and insoluble proteins were then visualised by SDS-PAGE (Section 2.8.3). Levels of protein expression were increased by increasing the amount of inducer added or by increasing the length of induction. If the protein expressed was found to be insoluble, the temperature of the induction, amount of inducer or time of induction or combination of these conditions were altered.

### 2.8.2 Lysis of *E. coli* cells

#### 2.8.2.1 Sonication

Sonication was used to mechanically disrupt the cells. Forty ml of *E. coli* was harvested by centrifugation at 6000×g for 5 min (GSA, Sorvall®, Du Pont). Cells were resuspended in 5ml 1× PBS buffer + protease inhibitors (Section 2.2.2) and sonicated with a microtip probe (3mm tapered probe, Vibra cell™, Sonics and Materials Inc.) at setting 3 for 5× 10 sec pulsed (50% duty cycle) and then setting 4 for 5× 10 sec pulsed (50% duty cycle). The sonicate was centrifuged at 12000×g for 10 min (SS-34,

Sorvall®, Du Pont) to separate soluble protein from insoluble cell contents. The supernatant (soluble fraction) was transferred to a clean tube and the pellet (insoluble fraction) was resuspended in 1× PBS buffer + protease inhibitors.

#### **2.8.2.2 Freeze/thaw**

One ml of *E.coli* was harvested by centrifugation at 3300×g for 5 min (MicroMax®, IEC) and resuspended in 1ml 1× PBS buffer + protease inhibitors (Section 2.2.2). The cells (in 1.5ml tubes) were suspended in liquid nitrogen for 10 sec and then incubated in a 37°C waterbath for 1 min. This cycle was repeated 8 times. The lysed cells were centrifuged at 13400×g for 10 min (MicroMax®). The supernatant (soluble fraction) was transferred to a clean tube and the pellet (insoluble fraction) was resuspended in 1ml 1× PBS buffer + protease inhibitors. If the lysate was viscous due to the presence of DNA, DNase I (Section 2.2.2) was added to 10µg/ml for 30 min at 37°C.

#### **2.8.2.3 French Press**

*E.coli* (500ml) was harvested at 6000×g for 10 min (GSA, Sorvall®, Du Pont) and resuspended in 30ml 1× PBS buffer + protease inhibitors (Section 2.2.2). The cells were passed twice through the French Press (FRENCH® Pressure cells and press, Spectronic Unicam) at 35,000 psi. The lysed cells were then harvested at 12000×g for 10 min (SS-34, Sorvall®, Du Pont) and the supernatant (soluble fraction) was transferred to a clean tube. The pellet (insoluble fraction) was resuspended 1× PBS buffer + protease inhibitors. If the cells did not lyse efficiently by any of the methods, cells were pre-treated with 100µg lysozyme/ml PBS (final concentration) for 5 min at ambient temperature before being lysed.

### **2.8.3 Protein gel electrophoresis**

Proteins were visualised by one-dimensional protein gel electrophoresis, based on an established method (Laemmli, 1970). An equal amount of 2× SDS-PAGE sample buffer (Section 2.2.2) and sample were mixed and incubated at 100°C for 5 min to denature proteins. The heat-treated samples and Rainbow™ coloured protein molecular weight markers (Amersham) were loaded in 12% acrylamide Ready Gels (Bio-Rad) and run in 1× SDS-running buffer (Section 2.2.2) according to the manufacturer's instructions. The gel apparatus was then dismantled and the gel stained for 1 hr with



Coomassie protein stain (Section 2.2.2). The gel was then destained with Destain buffer (Section 2.2.2) for approximately 1 hr. Protein bands were then visualised by eye and photographed with the Fluor-S® MultiImager (Bio-Rad).

## 2.9 Purification of fusion proteins

GST fusion proteins were purified according to the manufacturer's instructions (Pharmacia Biotech). A bacterial culture was grown and induced at optimal conditions previously determined (Section 2.8.1). Cells were lysed by sonication or French Press (Section 2.8.2) and a 1ml aliquot was set aside for SDS-PAGE analysis. Glutathione Sepharose slurry (100µl 50% v/v) was added to 5ml soluble fraction and incubated with gentle shaking for 30 min at ambient temperature. The matrix with bound protein was sedimented by centrifugation at 500×g for 5 min (Megafuge 1.0, Heraeus Sepatech GmbH). The supernatant containing unbound proteins was discarded except for a 1ml aliquot for SDS-PAGE analysis. The matrix was washed with 500µl 1×PBS, pH 7.4 (Section 2.2.2) and transferred to a 1.5ml tube. The matrix was sedimented at 500×g for 5 min (MicroMax®, IEC). The matrix was washed twice more (a total of 3 washes). A 10µl sample of the matrix was removed and kept for SDS-PAGE analysis.

For elution of the fusion protein with an intact GST tag, 50µl of Glutathione Elution buffer (Section 2.2.2) was added to the washed matrix. The matrix was resuspended gently and incubated at ambient temperature for 10 min. The matrix was then centrifuged at 500×g for 5 min (MicroMax®) and the supernatant kept in a clean 1.5ml tube. The elution and centrifugation steps were repeated twice and the 3 eluates were pooled. Aliquots of the eluates and a sample of the matrix after the elution steps were analysed by SDS-PAGE.

For cleavage of the fusion protein off the GST tag, 500µl PreScission cleavage buffer (Section 2.2.2) was added to the washed matrix. The matrix was centrifuged at 500×g for 5 min (MicroMax®) and the buffer was removed. PreScission cleavage buffer (48µl) and 2µl PreScission protease was added to the matrix and incubated at 4°C overnight. The matrix was sedimented by centrifugation at 500×g for 5 min (MicroMax®) and the supernatant was transferred to a clean 1.5ml tube. The matrix

was then washed 3 times with 50µl 1× PBS, pH 7.4, saving the supernatant from each wash. Aliquots of the total lysate, non-binding proteins, matrix with bound protein, eluate, matrix after elution, cleavage reaction, washes and a sample of the matrix after the cleavage and washes were analysed by SDS-PAGE as outlined in Section 2.8.3. Purified protein was quantified by the Bio-Rad Protein Assay, which is based on the method of Bradford (Bradford, 1976), according to the manufacturer's instructions (Bio-Rad). Briefly, a set of five BSA concentration standards was prepared. BSA standards (800µl of each), and three 800µl aliquots of purified protein were added to 200µl concentrated dye reagent each. The reactions were incubated at ambient temperature for 5 min and then the absorbance at 595nm was read in the U-2000 Double-beam spectrophotometer (Hitachi Ltd.).

## 2.10 Insertional disruption of gene targets

### 2.10.1 Vectors for insertional disruption

Insertional disruption of target genes was achieved using pBery-1 (FRC), which is designed to integrate specifically into the target gene in the HN001 genome. Integration is essential for survival of the pBery DNA as it lacks an origin of replication (ori) for *Lactobacillus* and it contains a strong erythromycin resistance gene for the selection of integrants in the HN001 genome. The plasmid can be maintained in *E. coli* as an autonomously replicating plasmid because it contains an *E. coli* origin of replication (ori). *E. coli* strains containing a pBery vector with a cloned *fbl* fragment were obtained as outlined in Sections 2.7.2 – 2.7.5. A large-scale preparation of the required plasmid was prepared as outlined in Section 2.4.2.

### 2.10.2 Transformation of HN001 cells

Transformation of HN001 cells was achieved by using a previously optimised method (Varmanen *et al.*, 1998). Electro-competent HN001 cells were cultured by adding 1.3ml fresh stationary HN001 culture to 100ml MRS broth (Section 2.2.1) supplemented with 11ml 25% w/v glycine. The culture was grown to an OD<sub>600</sub> of 0.3-0.5 before being harvested at 1500×g for 10 min (GSA, Sorvall®, Du Pont). Cells were washed three times in 20ml V buffer (Section 2.2.2) and then finally resuspended in 1ml V buffer. Aliquots of cells (100µl) were chilled on ice. Cells were then either

transformed with DNA immediately or frozen in a dry ice/ethanol bath and stored at -80°C.

Electro-competent HN001 cells were transformed with between 1µg and 20µg plasmid DNA per 100µl cells. Cells and the required amount of plasmid DNA were incubated on ice for 5 min. During this time, the cells were transferred to a disposable electroporation curvette (Gene Pulser® Curvette, Bio-Rad) on ice. The cells were then subjected to an electric pulse (200Ω, 1.5kV, 25µF) in the Gene Pulser™ (Bio-Rad). Immediately after the pulse, 900µl of lactobacilli recovery broth (Section 2.2.1) was added and the cells were incubated at 37°C for 3 hr in a stationary waterbath. After the incubation, aliquots of 150µl cells were plated on MRS agar plates with 2.5µg/ml erythromycin (Section 2.2.1). Transformants were grown as outlined in Section 2.3.1.

### **2.10.3 HN001 colony PCR**

Transformants were screened as outlined in Section 2.7.5. Colonies that appeared to be disruptants by PCR were streaked for single colonies on MRS agar plates with 2.5µg/ml erythromycin (Section 2.2.1). A single colony was used to inoculate 10ml MRS broth with 2.5µg/ml erythromycin (Section 2.2.1). The disruptant was grown as outlined in Section 2.3.1 and glycerol stocks were made as outlined in Section 2.3.2. Southern blots confirmed the insertion of pBery vectors in the correct gene locus. The Southern blot protocol used is outlined in Section 2.11.

## **2.11 Southern Blot**

### **2.11.1 Southern transfer**

Wild type HN001 and integrant DNA (prepared as outlined in Sections 2.4.2 – 2.4.3) were digested with three different restriction endonucleases as outlined in Section 2.4.7. All of the three digests, a small amount of λ/HindIII ladder and 1Kb+ ladder was loaded onto a 0.7% gel (Section 2.2.2) and briefly run at 100V until loading dye had migrated into the gel. The gel was then run at 10V overnight and visualised as outlined in Section 2.4.6. Southern transfer was set up according to the ECL™ direct nucleic acid labelling and detection system instruction manual (Amersham). The gel was processed for blotting by covering the gel with Depurination solution (Section 2.2.2)

and incubating with gentle shaking for 15 min at ambient temperature (until the blue DNA loading gel turned yellow). The gel was then rinsed with distilled water and incubated with Denaturation solution (Section 2.2.2) for 30 min with gentle shaking at ambient temperature (25 min after the DNA loading gel had turned blue again). Following denaturation, the gel was rinsed with distilled water and incubated with Neutralisation solution (Section 2.2.2) for 30 min with gentle shaking at ambient temperature. Finally, the gel was rinsed with distilled water.

DNA from the gel was transferred to Hybond-N+ nylon membrane (Amersham) by capillary blotting. Firstly, 4 sheets of 3MM paper (Whatman) were pre-wetted in 20× SSC (Section 2.2.2) and placed on top of each other in a glass dish. The gel was placed on top of the wet 3MM paper and covered with a piece of cling film (Gladwrap) with a gel-sized hole cut out of it. A piece of Hybond-N+ nylon membrane, cut to the size of the gel, was pre-wetted with distilled water and placed on top of gel carefully, as so not to introduce any air bubbles. Three sheets of 3MM paper, cut to the size of the gel and pre-wetted with 10× SSC (Section 2.2.2), were placed on top of the membrane. Finally, a stack of absorbent paper, cut to the size of the gel, was placed on top of the filter paper. A glass plate and a small weight were then added to the top of the stack and left overnight.

Once the transfer was complete, the capillary blot was dismantled and the membrane was rinsed in 6× SSC (Section 2.2.2) for 5 min. The blot was then dried on 3MM paper. DNA was cross-linked to the membrane through exposure to medium wave UV (300nm) for 1 min by placing the blot DNA side down, on a UV source (Mighty Bright, Hoefer Scientific Instruments). Membranes were either probed immediately or placed in a filter paper envelope and stored at ambient temperature in the dark until required.

### **2.11.2 Probe labelling and Hybridisation**

Hybridisation was performed according to the “Hybridisation and stringency washing in tubes” procedure described in the ECL™ direct nucleic acid labelling and detection systems instruction manual (Amersham). Briefly, the membrane was placed in a Pyrex glass tube and small amount of 5× SSC (Section 2.2.2) was added to wet the membrane

and discarded. Hybridisation buffer (Section 2.2.2), preheated to 42°C, was added to the tube and the membrane was prehybridised for 15 min at 42°C with constant rotation in a Midi Dual 14 oven (Hybaid). During the prehybridisation of the membrane the DNA probes were labelled as outlined in the ECL instruction manual. Briefly, DNA was denatured by heating at 100°C for 5 min. An equal volume of DNA labelling reagent was added to cooled DNA and the same volume of glutaraldehyde solution was added and incubated for 10 min at 37°C. After 15 min of prehybridisation, part of the prehybridisation solution was removed from the tube and the denatured probe was added to the solution, which was then placed back in the tube. Hybridisation was performed at 42°C overnight with constant rotation.

### **2.11.3 Washing and Signal detection**

High stringency washing was performed according to the “Hybridisation and stringency washing in tubes” procedure described in the ECL™ direct nucleic acid labelling and detection systems instruction manual (Amersham). Following hybridisation, the membrane was washed once in 75 ml 5× SSC (Section 2.2.2) for 5 min at 42°C with rotation. The 5× SSC was discarded and the blot was washed with 3× 1/3 tube full Primary wash buffer (Section 2.2.2) at 42°C (20 min for first wash, 10 min each for second and third wash), with rotation at 42°C to remove unbound probe. The blot was then removed from the tube and placed in a plastic container. The blot was washed twice by covering with 20× SSC (Section 2.2.2) and incubating at ambient temperature with gentle shaking for 5 min. Hybridised DNA was detected as described in the instruction manual (Amersham). Briefly, the secondary wash buffer was drained off the membrane and the blot was placed on a piece of cling film. An equal volume of Reagent 1 and Reagent 2 were mixed and added directly to the surface of the blot. After incubation with the mixture for 1 min at ambient temperature, excess reagents were poured off, the blot was wrapped in cling film and placed in a film cassette with a piece of X-ray film (Agfa). Several pieces of film were exposed for different time lengths.

### **2.12 Preparation of crude HN001 cell envelope**

Crude HN001 cell envelope preparations were prepared by French Press according to a previously optimised method (Christensson *et al.*, 2002). Two 250 ml cultures of

HN001 were grown for 18 hr to stationary phase (as outlined in Section 2.3.1). Cells from each culture were harvested at 6000×g for 10 min (GSA, Sorvall®, Du Pont) and resuspended in 20ml 1× PBS, pH 7.4 (Section 2.2.2). Cells were harvested again and cells from one culture were set aside as the 1× wash sample. The remaining cells were washed a further 7× to give a total of 8 washes. Each cell pellet was resuspended in 20ml 1× PBS, pH 7.4 before being disrupted with the French press using 3 passages at 35000 psi (FRENCH® Pressure cells and press, Spectronic Unicam). The cytoplasmic and envelope fractions were harvested by centrifugation at 27000×g for 15 min (SS-34, Sorvall®, Du Pont). The supernatant (cytoplasmic fraction) was stored at -80°C. The pellet (envelope fraction) was resuspended in 5ml 1× PBS, pH 7.4 and stored in 500µl aliquots at -80°C. The envelope proteins were visualised as outlined in Section 2.8.3.

## 2.13 Immunoblotting

### 2.13.1 Inoculation of rabbits

All the animal work in this study was done at the Institute of Veterinary Science, Massey University, Palmerston North. One rabbit was inoculated with 1× washed HN001 cell envelope and another rabbit was inoculated with 8× washed HN001 cell envelope. The rabbits were inoculated in the back, intramuscularly. The rabbits were inoculated with a series of three inoculations, a month to 6 weeks apart. The adjunct, Freund's incomplete was used to stimulate an immune response. During the course of the inoculations, serum was collected at regular intervals and Western Blotting was used to monitor the rabbit anti-HN001 cell envelope IgG titre in the serum.

### 2.13.2 Preparation of SDS-PAGE gels for Western Blotting

SDS-PAGE gels were prepared based on the method of Laemmli (1970). A gel sandwich was assembled with clean glass plates as per manufacturer's instructions (Mini-protean® II Dual slab cell, Bio-Rad). The resolving gel components were mixed and poured into the gel sandwich. For 2 mini-size 12% acrylamide gels: 6.7ml distilled water, 8ml Acrylamide/Bis-acrylamide 30%/2.67% (Bio-Rad), 5ml 1.5M Tris-HCl, pH 8.8, 200µl 10% (w/v) SDS, 100µl 10% (w/v) ammonium persulphate and 10µl TEMED (Gibco BRL) were mixed together. The resolving gel was layered with water-saturated isobutanol (1:1) (Section 2.2.2). The gel was left to polymerise for 30 min before the



isobutanol was poured off and the gel was rinsed with distilled water. Excess water was removed with a tissue. The stacking gel components were mixed and poured on top of the resolving gel. For 2 mini-size gels 6.05ml distilled water, 1.35ml Acrylamide/Bis-acrylamide 30%/2.67%, 2.5ml 0.5M Tris-HCl, pH 6.8, 100µl 10% (w/v) SDS, 50µl 10% (w/v) ammonium persulphate and 10µl TEMED were mixed together. A preparative comb with one small and one large well was then pushed into the stacking gel. The gel was left to polymerise for 30 min. Gels were stored wrapped in water-saturated tissue paper and plastic cling wrap at 4°C.

### **2.13.3 Western transfer**

Western transfer was performed according to the Trans-Blot® Semi-Dry Electrophoretic Transfer cell instruction manual (Bio-Rad). Gels were prepared for blotting by equilibrating in Transfer buffer (Section 2.2.2) for 15 min. A piece of nitrocellulose membrane (Bio-Rad) was cut to the size of the gel and equilibrated in transfer buffer for 15 min. Two pieces of gel-sized filter paper (Bio-Rad) per gel were soaked in transfer buffer. Electrophoretic transfer was performed as outlined in the instruction manual for the Semi-Dry Electrophoretic Transfer cell. Once the blot was assembled, proteins were transferred to the nitrocellulose membrane at 15V for 30 min. When the transfer was complete the blot was disassembled and the membrane was either probed immediately or stored at 4°C.

### **2.13.4 Immunodetection**

The blocking, washing and incubation with primary and secondary reactants in the immunodetection method was based on the method outlined in the ECL™ Western blotting instruction manual (Amersham). Each membrane was cut in half so part of it could be stored at 4°C (in case immunodetection needed to be repeated). The other half of each membrane was cut into 4 equal sized strips, labelled 1 to 4 with pencil and blocked overnight in Western blocking solution (Section 2.2.2) at 4°C. The membrane strips were removed from the blocking solution and placed into four different dilutions of 1° antibody (1:100, 1:500, 1:1000 and 1:5000 of anti-HN001 cell envelope anti-serum) in blocking solution. The blot containing 1× washed envelope sample was incubated with anti-serum from rabbit inoculated with 1× washed envelope; the blot containing 8× washed envelope sample was incubated with anti-serum from rabbit

inoculated with 8× washed envelope. Tubes were secured to a rocking platform (Bioblock Scientific) and the blots were incubated for 1 hr at ambient temperature with gentle rocking. Blots were then washed with 10ml 1× TBS (Section 2.2.2) for 3× 10 min with gentle rocking. Blots were then incubated with a secondary reactant (HRP-labelled Protein A, Amersham) at 1:1000 dilution for 1 hr at ambient temperature with gentle rocking. Blots were washed with 10ml 1× TBS for 3× 10 min with gentle rocking. Binding of protein A was detected using a colorimetric method as outlined below. Approximately 60ml Western developing solution (Section 2.2.2) was poured over the membrane strips in a plastic box and incubated for 5 min to 1 hr in the dark at ambient temperature until the blue-grey colour had developed. When sufficient colour had developed, the strips were washed in distilled water, visualised by eye and photographed with the Fluor-S® MultiImager (Bio-Rad). Developed membranes were stored in a paper envelope in the dark.

## 2.14 Purification of IgG from anti-serum

### 2.14.1 Purification of IgG using FPLC

IgG from rabbits (Section 2.13.1) were purified using FPLC (Amersham) with a HiTrap Protein G HP column (Amersham) according to the manufacturer's instructions. Since the binding capacity of the 5ml column is approximately 125mg and the approximate concentration of IgG in rabbit serum is 10mg/ml, 10ml of each type of antibody (rabbit 1× washed and 8× washed anti-HN001 cell envelope anti-serum) was run through the column. Ten ml of each type of antibody was thawed at ambient temperature and 1ml 10× Binding buffer (Section 2.2.2) was added to give a final concentration the same as the 1× Binding buffer (Section 2.2.2) for the purification. The antibody samples were then passed through a 0.45µm filter. The purification was performed as outlined in the instruction manual for the column (Amersham). Briefly, fraction collection tubes were prepared by adding 60µl 1M Tris-HCl, pH 9.0 to adjust the pH of the collected fractions to approximately neutral. The column was washed with 1× Binding buffer before samples were applied to the column. After the samples were applied, the column was washed again with 1× Binding buffer and 1ml fractions were collected. Protein content in the fractions was monitored by A<sub>280</sub> and A<sub>214</sub>. After non-binding material had passed through the column 0.1M glycine-HCl, pH 2.7

(Elution buffer) was applied to the column. Fractions were collected until the protein peak corresponding to IgG disappeared. The column was stripped with 0.1M glycine-HCl, pH 2.0 before being washed with 1× Binding buffer to restore neutral pH in the column. Once all serum samples had been processed the column was washed with 20% (v/v) ethanol, removed from the FPLC, capped tightly and stored at 4°C.

#### **2.14.2 Desalting and Storage of purified IgG**

Fractions from Section 2.14.1 that corresponded to the expected IgG peak were pooled and applied to a PD-10 desalting column (Amersham) according to the manufacturer's instructions. Briefly, the column was equilibrated with 25ml 1× PBS, pH 7.4 (Section 2.2.2). The fractions were then applied to the column in 2.5ml. If the peak was less than 2.5ml the volume was made up with 1× PBS, pH 7.4. If the peak was larger than 2.5ml more than one column was run. IgG was eluted from the column in 5× 1ml 1× PBS, pH 7.4. Fractions were collected in 1.5ml tubes and the  $A_{280}$  was measured to identify fractions containing IgG. The IgG fractions were pooled and  $A_{280}$  was measured to quantify the IgG (Section 3.2.2). The IgG was stored in 500µl aliquots at -80°C.

### **2.15 Liquid-phase binding assays for ECM proteins**

#### **2.15.1 $^{125}\text{I}$ labelling of proteins**

Proteins were labelled using the Iodobead method (Pierce). Iodobeads (Pierce) to be used in the iodination reaction were washed with 500µl 1× PBS, pH 7.4 per bead (Section 2.2.2). Beads were then blotted dry on clean tissue paper.

The iodination reaction was set up as follows:

1 iodobead

1× PBS, pH 7.4 to a final volume of 200µl (after the protein is added)

0.1mCi  $^{125}\text{I}$  (NaI in NaOH solution, Amersham) per 10µg protein

The components were mixed and incubated at ambient temperature for 5min. The protein to be labelled was then added (Section 2.2.3) and the mixture was incubated at ambient temperature for 15 min. The reaction was stopped by removing the solution from the iodobead. The iodobead was then washed with 300µl 1× PBS, pH 7.4 to

remove any attached protein. The PBS was removed from the bead and added to the tube with the labelled protein. The protein was then applied to a PD-10 column to remove unincorporated  $^{125}\text{I}$ . PD-10 columns (Amersham) were prepared by applying 25ml 1× PBS, pH 7.4 to the column. Once the PBS had run through, 10ml 10mg BSA/ml (Section 2.2.3) was applied to the column. The column was then washed with 10ml 1× PBS, pH 7.4. The 500µl reaction was then applied to the column with 2ml 1× PBS, pH 7.4. The eluate was discarded. The protein was eluted with 10 volumes of 500µl 1× PBS, pH 7.4. Fractions were collected in 2ml tubes and the protein peak and the unincorporated  $^{125}\text{I}$  peak were determined by counting the fractions in a Quickcount QC.4000 XER gamma counter (Bioscan). The first peak off the column (the protein peak) was pooled and 100mg BSA/ml (Section 2.2.3) was added to a final concentration of 10mg BSA/ml. The specific activity of the labelled protein was determined and if greater than  $1 \times 10^6 \text{ dpm}/\mu\text{g}$  protein it was used in binding assays. The labelled protein was stored at 4°C for a maximum of 2 weeks before being disposed of.

### **2.15.2 $^{125}\text{I}$ fibronectin binding assays**

The adhesion of bacteria to labelled proteins was investigated according to the liquid-phase binding assay of Trust et al. (1991). Bacterial strains were grown as outlined in Section 2.3.1. HN001 was used in stationary phase (18 hr); *S. aureus* 8325-4 and *E. coli* DH5α were used in exponential phase for the assay ( $\text{OD}_{650}$  0.5). Cells were harvested by centrifugation at 3000×g for 5 min (Megafuge 1.0, Heraeus Sepatech GmbH) and washed once with 1× PBS, pH 7.4 (Section 2.2.2) before being resuspended in 1× PBS, pH 7.4 at  $5 \times 10^9$  cfu/ml by pipetting at  $5 \times 10^9$  cfu/ml (Section 2.3.3). 100µl of each bacterial strain or 100µl 1× PBS, pH 7.4 (negative control) in 2ml plastic tubes was incubated with 20µl  $^{125}\text{I}$ -fibronectin (Section 2.15.1) (diluted to 1.5µg/ml in 10mg BSA/ml (Section 2.2.3)) for 1 hr at ambient temperature. The binding reaction was stopped with the addition of 1.5ml ice-cold 1× PBST (Section 2.2.2) and the cells and bound fibronectin was pelleted by centrifugation at 1350×g for 25 min (MicroMax®, IEC). The supernatant containing non-bound  $^{125}\text{I}$ -fibronectin was carefully aspirated and the pellets were counted in a Quickcount QC.4000 XER gamma counter (Bioscan). Total radioactivity added to each tube was measured by counting three aliquots of 20µl 1.5µg/ml  $^{125}\text{I}$  fibronectin. Percent radioactivity bound was then calculated for each bacterial strain. Each binding assay was done in triplicate.

## 2.16 Solid-phase binding assays

### 2.16.1 Coating microtitre plates with protein or bacterial cells

The coating of microtitre wells was based on the method of van der Flier *et al.* (1995). Wells of Immuno Maxi Sorp ELISA plates (NUNC) were coated with 100µl of 100µg/ml protein solution (Section 2.2.3) or 100µl of an appropriate amount of bacterial cells (Sections 2.3.1 and 2.3.3) by passive adsorption for 30 min at 37°C with gentle shaking (20 rpm). Bacterial cells for use in binding assays were harvested by centrifugation at 3000×g for 5 min (Megafuge 1.0, Heraeus Sepatech GmbH) and washed once with 1× PBS, pH 7.4 (Section 2.2.2) before being resuspended in 1× PBS, pH 7.4 by pipetting. The ELISA plate was then washed with 1× PBS, pH 7.4 according to a wash program in the Columbus 4.01 plate washer (Tecan). The wash program consisted of an aspiration step (to remove excess protein or bacterial cells) and then a series of five dispense/aspiration steps to leave the plate with no buffer in it. The wells were then blocked with 100µl 20mg BSA/ml PBS (Section 2.2.3) at 37°C for 1 hr (20 rpm) and then washed with 1× PBST (Section 2.2.2) with the five cycle wash program as above.

### 2.16.2 Binding of bacteria to immobilised proteins

Cells were grown as outlined in Section 2.3.1, prepared for binding assays as outlined in Section 2.16.1 and resuspended to  $5 \times 10^8$  cfu/ml in 20mg BSA/ml PBS (Section 2.3.3). The bacterial suspension (100µl) was added to a plate coated with protein (as outlined in Section 2.16.1) and incubated at 37°C for 90 min (20 rpm). Wells were washed with 1× PBST (Section 2.2.2) according to the five cycle wash program (as outlined in Section 2.16.1). Bound cells were then detected by one of the methods detailed in Sections 2.16.3 – 2.16.7.

### 2.16.3 Detection of HN001 cells by ELISA

ELISA detection of cells was based on a previously published method (Sylvester *et al.*, 1996). 100µl of an appropriate dilution of anti-HN001 cell envelope IgG (purified as in Section 2.14) in 20mg BSA/ml PBST (Section 2.2.3) was added to the wells and incubated for 1 hr at 37°C (20 rpm). Wells were washed with 1× PBST (Section 2.2.2) according to the five cycle wash program (as outlined in Section 2.16.1). 100µl of an

appropriate dilution of anti-rabbit IgG conjugated to HRP (Amersham) in 20mg BSA/ml PBST was added to the wells and incubated for 1 hr at 37°C (20 rpm). The dilutions of antibodies used were determined in the crisscross serial dilution outlined in Section 2.16.3.1. Wells were washed with 1× PBST according to the five cycle wash program. The TMB kit (Pierce) was used to detect the bound secondary antibody (as outlined in the manufacturer's instructions). Briefly, equal volumes of the peroxide solution and the peroxidase substrate (TMB) were mixed and 100µl of the mixture was added to the wells and incubated for at least 15 min. When sufficient colour had developed, the reaction was stopped with 100µl 2M H<sub>2</sub>SO<sub>4</sub>. The absorbance of each well was read in an Optimax tunable microtitre plate reader (Molecular devices) at 450nm. Data was exported as an Excel spreadsheet and analysed in Microsoft Excel (Microsoft).

#### **2.16.3.1 Crisscross serial dilution**

Wells of one Immuno Maxi Sorp ELISA plate (NUNC) were coated with HN001 cells (Section 2.16.1) at a specified concentration. Control wells were coated with *Lactococcus lactis* MG1363 cells at the same concentration (as outlined in Section 2.16.1). ELISA detection was then performed (as outlined in Section 2.16.3) with serial dilutions of primary (1°, rabbit anti-HN001 cell envelope IgG) antibody applied to one column each and serial dilutions of secondary (2°, anti-rabbit IgG conjugated to HRP) antibody applied to one row each at the appropriate stage of the ELISA protocol. A combination of 1° and 2° antibody dilutions that gave a linear signal in response to HN001 cells with minimal interaction with MG1363 was chosen for use in ELISA detection of HN001 cells.

#### **2.16.4 Detection by radioisotope labelling**

The binding of radiolabelled bacteria was investigated according to a previously published method (Cohen *et al.*, 1995). Bacterial cells were metabolically labelled by growing in MRS broth (Section 2.2.1) for 18 hr as outlined in Section 2.3.1 except 10µCi/ml each of 5, 6-<sup>3</sup>H-uracil and 8-<sup>3</sup>H-adenine (Amersham) were added to the medium. A 100µl aliquot of 5×10<sup>8</sup> cfu/ml labelled bacteria (Section 2.3.3) in 20mg BSA/ml PBS (Section 2.2.3) was used in the binding assay as outlined in Section 2.16.2. After the wash cycle, the adherent bacteria were released from the microtitre

plate by adding 100µl 1% (w/v) SDS in 0.1M NaOH (pre-heated to 65°C) to each well and incubating at 65°C for 1 hr. The contents of each well were pipetted up and down vigorously and transferred to a scintillation vial containing 10ml scintillation cocktail (BCS, Amersham). The radioactivity of each vial was measured using a liquid scintillation counter (1900TR, Packard). The specific activity of cultures (cpm/cfu) was determined by counting three aliquots of 100µl of cells (resuspended in 1% w/v SDS/0.1 M NaOH). The number of bacterial cells adhering in each well was calculated from the cpm bound and the specific activity of the bacterial culture.

#### **2.16.5 Detection by Crystal violet staining**

Crystal violet detection of cells was based on a previously published method (Mongodin *et al.*, 2002). To determine whether crystal violet staining would detect HN001, rows of wells of Immuno Maxi Sorp ELISA plates (NUNC) were coated with 100µl of dilutions of HN001 cells as outlined in Section 2.16.1 except wells were not blocked with BSA. After washing, the wells were dried and the cells were fixed to the plate with 100µl 2% v/v Formalin for 1 min. The formalin was removed and the wells were allowed to air dry for 10 min in a fume hood. The bound bacteria were stained with Crystal Violet stain (Difco) for 10 min. Wells were rinsed with 200µl distilled water to remove excess stain. Each row of the plate (containing a dilution series of cells) was washed with increasing washes of 1× PBST (Section 2.2.2) (from 1 wash to 4 washes). The stain was then released from the cells by incubating with 200µl SDS lysis solution (1% v/v SDS/5% v/v ethanol) at 37°C for 30 min. The absorbance was read at 595nm in an Optimax tunable microtitre plate reader (Molecular devices).

#### **2.16.6 Detection by Phase contrast microscopy**

To determine whether examination by phase contrast microscopy would detect HN001, rows of wells of Immuno Maxi Sorp ELISA plates (NUNC) were coated with 100µl of dilutions of HN001 cells as outlined in Section 2.16.1 except wells were not blocked and a cell wash manifold was used for the wash cycle (Tecan). A small layer of buffer was left over the bottom of the wells so the cells would not dry out. Randomly chosen fields from each well were visualised by eye by phase contrast microscopy (400× magnification) with an inverted microscope (Olympus CK2) and photographed using a



Nikon U-III camera. The average numbers of bacteria in each field were calculated from the photographs.

#### **2.16.7 Detection by OD<sub>403</sub>**

The detection of cells by OD<sub>403</sub> was based on a previously published method (Roos *et al.*, 2002). To determine whether detection by OD<sub>403</sub> would detect HN001, rows of wells of Immuno Maxi Sorp ELISA plates (NUNC) were coated with 100µl of dilutions of HN001 cells as outlined in Section 2.16.1 except wells were not blocked. Each row of wells were washed with an increasing number of washes of 1× PBST (Section 2.2.2) (ranging from 1 wash to 4 washes). After the final aspiration step the plates were allowed to air dry at 37°C for 30 min. The wells were then measured at 403nm in an Optimax tunable microtitre plate reader (Molecular devices).

#### **2.16.8 Glass slide binding assays**

Bacterial adhesion to coated glass slides was investigated based on a previously published method (Kukkonen *et al.*, 1993). Glass slides were coated with protein by incubating with 500µl 100µg/ml BSA or fibronectin (Section 2.2.3) at 37°C for 30 min in a moist chamber (to decrease evaporation). Slides were then placed in clean plastic box containing 50ml 1× PBST (Section 2.2.2) and were incubated at 37°C for 5 min (20 rpm). Most of the 1× PBST was removed from the slides and 50ml 1× PBST was added to the slides. The slides were incubated a further 5 min. The washing step was repeated once to give a total of three washes before the slides were blocked with 50ml 20mg BSA/ml PBST (Section 2.2.3) at 37°C for 30 min (20 rpm). The slides were washed as outlined above. The slides were then placed in a clean plastic box with 30ml  $1 \times 10^8$  cfu/ml bacterial culture in 1× PBS, pH 7.4 (Section 2.2.2) and incubated at 37°C for 90 min (20 rpm). The slides were washed as outlined above except the third wash was done in 1× PBS, pH 7.4 to decrease drying of the slide. The slides were then placed on a piece of tissue paper and a coverslip was placed over the region with the protein coating. Once the area surrounding the coverslip was dry, the coverslip was fixed in place using clear nail polish. The slides were visualised by phase contrast microscopy at 400× magnification (Optiphot, Nikon) and eight random fields of each slide were then photographed using a Nikon U-III camera.

## 2.17 Binding to Caco-2 cells

### 2.17.1 Growth and Maintenance of the intestinal cell line Caco-2

All cell culture work was done in a Class 2 Safety cabinet (HERAsafe®, Kendro Laboratory Products GmbH). Cell cultures were initiated by rapidly thawing a vial of frozen cells (at -80°C) and pipetting its contents into a 25CC flask (Nunc). Ten ml of DMEM with FBS and NEAA (Section 2.2.1) was added dropwise to the cells. The cells were incubated for 12-14 hr at 37°C in a 10% CO<sub>2</sub>-90% air atmosphere before the medium was replaced with fresh medium. Cells were routinely grown in DMEM as outlined above. When the cells were determined to be contaminant free (2 weeks without antimycotic antibiotic), DMEM with 1× Antimycotic Antibiotic (Section 2.2.1) was used to maintain the cell lines. The culture medium was replaced every 48 hr and cells were incubated at 37°C in a 10% CO<sub>2</sub>-90% air atmosphere. Monolayers of Caco-2 cells were prepared on 6-well plates (Falcon®, Becton Dickinson). Cells were seeded at a concentration of  $1.4 \times 10^4$  cells per cm<sup>2</sup> and were grown with 3ml DMEM with 1% antimycotic antibiotic per well. Cells were used for adherence assays after 15 days of growth.

### 2.17.2 Estimation of number of Caco-2 cells per well in 6-well plate

Caco-2 cell monolayers were grown as outlined in Section 2.17.1 except coverslips (Thermanox® 25mm, Nunc Inc.) were placed in bottom of well before inoculating with Caco-2 cells. After 15 days the medium was removed from wells and the monolayers were washed with 3ml 1× PBS, pH 7.4 (Section 2.2.2). Caco-2 cells were incubated for 2 min with enough safranin stain (Difco) to cover the bottom of the well, before excess stain was removed and the well was washed with 3ml 1×PBS, pH 7.4 twice. The coverslip with the stained monolayer was placed on a glass slide and a coverslip placed over it. Cells were visualised at 400× magnification (Optiphot, Nikon) and four random fields from two wells were photographed using a digital camera (Coolpix 995, Nikon). The number of Caco-2 cells in a field of view and the area of the field of view were used to calculate the approximate number of Caco-2 cells per well in a 6-well plate. See Appendix 4 for a sample calculation and photo.

### **2.17.3 Adhesion assay**

Adhesion of bacteria to Caco-2 cells was investigated according to a previously published method (Gopal *et al.*, 2001). HN001 was metabolically labelled by growing in MRS broth (Section 2.2.1) for 18 hr as outlined in Section 2.16.4. Cells were harvested by centrifugation at 4300×g for 5 min (SS-34, Sorvall®, Du Pont), washed once in 1× PBS, pH 7.4 (Section 2.2.2) and adjusted to  $1 \times 10^8$  cfu/ml (Section 2.3.3). Several 3ml aliquots of cells were then harvested and resuspended in either 3ml 1× PBS, pH 7.4 or other solutions as required. The Caco-2 cell monolayers were washed twice with 3ml 1× PBS (Cell culture grade, Gibco™ Invitrogen). Bacterial cells (3ml) were added to 3ml DMEM with FBS and NEAA (Section 2.2.1) and 2ml of this mixture was aliquoted into each of 3 wells of washed Caco-2 cells. The bacteria and Caco-2 cells were incubated at 37°C for 1 hr. The Caco-2 cell monolayer was then washed four times with 1× PBS, pH 7.4 before the Caco-2 cells were lysed with 500µl 0.1% (w/v) SDS in 0.1 M NaOH. The lysed cells were transferred to a scintillation vial with 10ml scintillation cocktail (BCS, Amersham). The vials were counted in a liquid scintillation counter (1900TR, Packard). The specific activity of cultures (cpm/cfu) was determined by counting three aliquots of 1ml of cells (resuspended in 0.1% w/v SDS/0.1 M NaOH). The number of bacterial cells adhering per 100 Caco-2 cells was calculated from the number of Caco-2 cells per well in a 6-well plate (as described in Section 2.17.2).

#### **2.17.3.1 Metabolic labelling of HN001 grown on MRS agar plates**

HN001 was grown on MRS agar plates (Section 2.2.1) as outlined in Section 2.3.1 with 10µCi/ml each of 5, 6-<sup>3</sup>H-uracil and 8-<sup>3</sup>H-adenine (Amersham) added to the MRS agar. Cells were harvested by scraping growth off the plate with a sterile pipette tip and resuspended in 10ml 1× PBS, pH 7.4 (Section 2.2.2).

## **2.18 Pre-treatment of HN001 cells**

### **2.18.1 Protease treatment**

A known amount of cells in 1× PBS, pH 7.4 (Section 2.2.2) was harvested by centrifugation at 4300×g for 5 min (SS-34, Sorvall®, Du Pont) and resuspended in a solution of 5mg pronase/ml PBS, 5mg proteinase K/ml PBS or 5mg trypsin/ml Buffer

T (Section 2.2.2). Cells resuspended in buffer alone were used as a no-treatment control. Cells were incubated at 37°C for 1 hr in a stationary waterbath. Cells were then harvested as before and resuspended in 1× PBS, pH 7.4. This wash step was repeated twice.

#### **2.18.2 Periodate treatment**

Bacterial cells were treated with periodate according to a previously published method (Greene *et al.*, 1994). A known amount of cells in 1× PBS, pH 7.4 (Section 2.2.2) was harvested by centrifugation at 4300×g for 5 min (SS-34, Sorvall®, Du Pont) and resuspended in a solution of 10mg sodium periodate/ml 0.1M citrate-phosphate-0.1M NaCl, pH 4.5 (Section 2.2.2). Cells resuspended in 10mg sodium iodate/ml 0.1M citrate-phosphate-0.1M NaCl, pH 4.5 and cells in the citrate buffer alone were used as non-oxidising controls. Cells were incubated at 37°C for 1 hr in a stationary waterbath. Cells were then harvested as before and resuspended in 1× PBS, pH 7.4. This wash step was repeated twice.

## CHAPTER THREE: RESULTS

### 3.1 Liquid-phase binding assays for intestinal molecules

The aim of this study was to characterise the molecular species involved in the adherence of *Lactobacillus rhamnosus* HN001 to the intestinal surface and extracellular matrix molecules and cell lines. Thus, the first objective was to investigate which intestinal molecules HN001 binds to at a significant level. Any significant interactions were then investigated to determine if they were specific and involved a finite number of receptors rather than being due to non-specific binding. Liquid-phase binding assays were used for this initial investigation because the technique is fast, convenient, and commonly used for this kind of investigation. Unless stated, all binding assays were performed at least twice on triplicate samples (six assays for each data point).

#### 3.1.1 HN001 binds soluble fibronectin

The binding of HN001 to  $^{125}\text{I}$ -fibronectin (Fn) was investigated according to the method outlined in Section 2.15.2. HN001 was found to bind  $^{125}\text{I}$ -Fn above background levels (no cells added) but did not bind a consistent percentage of the  $^{125}\text{I}$ -Fn added (Table 4). 10/13 assays showed binding above the 5% threshold generally considered to be significant (Trust *et al.*, 1991) with an average of approximately 7%  $^{125}\text{I}$ -Fn bound. This indicates that HN001 binds low but significant levels of soluble fibronectin. The binding values falling below the 5% threshold could not be correlated to a specific batch of  $^{125}\text{I}$ -Fn used in the binding assay (A, B or C in Table 1) and were attributed to variation in the assay. *Staphylococcus aureus* 8325-4 has characterised fibronectin-binding properties (Flock *et al.*, 1987) and was considered to be an appropriate positive control. *S. aureus* 8325-4 also did not bind a consistent amount of  $^{125}\text{I}$ -Fn (3% to 29%). However, was considered a satisfactory positive control for fibronectin binding (Table 4). The negative control, *E. coli* DH5 $\alpha$ , consistently did not bind  $^{125}\text{I}$ -Fn above background levels (Table 4) and was deemed a suitable non-binding control.

The binding of HN001 to  $^{125}\text{I}$ -laminin was also investigated in this study. No significant binding was found (data not shown) compared to the positive control *H. pylori* CCUG 17874. The binding of HN001 to several intestinal surface and

extracellular matrix molecules, including vitronectin, collagen I, collagen IV, and mucin in the liquid-phase has also been investigated by Dr. Michael Collett. No significant binding was found (personal communication) so these molecules were not further investigated.

### 3.1.2 HN001 binding to $^{125}\text{I}$ -fibronectin is saturable

The interaction between HN001 and  $^{125}\text{I}$ -Fn was further investigated to determine if the interaction is saturable. These data were used to determine if the amount of  $^{125}\text{I}$ -Fn added in subsequent assays was saturating HN001 cells and also to calculate the number of receptors by the maximum amount of Fn bound to HN001. The binding assay was performed as outlined in Section 2.15.2 except the amount of  $^{125}\text{I}$ -Fn added ranged from 0.03  $\mu\text{g}$  to 20  $\mu\text{g}$ . The amount of labelled protein binding to tubes without cells was determined for each concentration of added  $^{125}\text{I}$ -Fn and subtracted from the value obtained with tubes with added bacteria. The binding data obtained yielded a curve that indicated saturation at approximately 0.1  $\mu\text{g}$   $^{125}\text{I}$ -Fn bound to  $5 \times 10^8$  cfu HN001 (Figure 7).

A theory of bacterial interaction with receptors (Lee *et al.*, 2000) was used to calculate the approximate number of fibronectin receptors per cell and the affinity of the interaction. The method of Lee et al. (2000) could not be used because a plot of 1/fibronectin-bound versus 1/fibronectin added (based on the Lineweaver-Burk plot) did not agree with the data obtained from the saturation curve (data not shown), possibly due to variation between samples. Therefore, a different approach was taken to calculate the approximate number of receptors. The approximate number of Fn receptors was calculated as follows: the number of moles of Fn bound was calculated from  $n = m/\text{Mr}$  (approximate Mr of fibronectin is 450000g/mol) and the number of molecules bound was calculated using Avogadro's number (molecules =  $n \times 6.02214199 \times 10^{23}$ ). At a very rough approximation, HN001 cells were found to have 250 fibronectin receptors per bacterial cell.

$$\text{Moles (n)} = 1 \times 10^{-7} / 450000 = 2.22 \times 10^{-13} \text{ mol}$$

$$\text{Molecules} = 2.22 \times 10^{-13} \times 6.02214199 \times 10^{23} = 1.3369 \times 10^{11} \text{ molecules}$$

$$\text{Molecules per cfu} = 1.3369 \times 10^{11} / 5 \times 10^8 \text{ cfu} = 267 \text{ molecules per cfu}$$

**Table 4 <sup>125</sup>I-Fn binding of various strains**

Batch of <sup>125</sup> I-Fn	HN001*	<i>S. aureus</i> 8325-4*	<i>E. coli</i> DH5α*	No cells**
A	7.2 (1.1)	5.1 (1.3)	ND	1.8 (0.3)
A	3.6 (0.4)	18.8 (1.7)	2.3 (0.06)	ND <sup>b</sup>
A	2.7 (0.5)	10.4 (2.2)	2.3 (0.4)	ND
A	9.9 (0.6)	6.7 (0.2)	2.3 (0.3)	1.4 (0.07)
A	9.4 (1.2)	~	~	~
A	9.9 (0.4)	~	~	~
A	7.0 (1.1)	6.4 (0.07)	2.2 (0.5)	ND
A	8.4 (0.9)	3.7	2.2	ND
B	10.1 (1.4)	ND	ND	ND
B	5.7	2.9	1.6	1.0
B	9.6 (0.7)	21.2 (2.9)	7.7 (0.3)	1.9
C	8.4 (0.5)	28.7 (1.3)	ND	1.5 (0.2)
C	2.5 (0.2)	ND	ND	1.5 (0.1)
Average binding	7.3 (3.2)	#	3.0 (2.5)	1.5 (0.3)

\*Specifies the % of <sup>125</sup>I-Fn bound by the named strain (or the no cells control). The value indicates the mean of triplicate or duplicate samples. The value in brackets indicates +/- 2 standard errors of the mean (error was not calculated for experiments with only duplicate samples).

<sup>a</sup> No cells is the background binding level of labelled protein to the tube.

<sup>b</sup> ND, Not done

# Binding of *S. aureus* 8325-4 varied to a large degree so an average was not calculated.

~ Specifies the values for the controls are above (one set of controls for three triplicate HN001 samples).

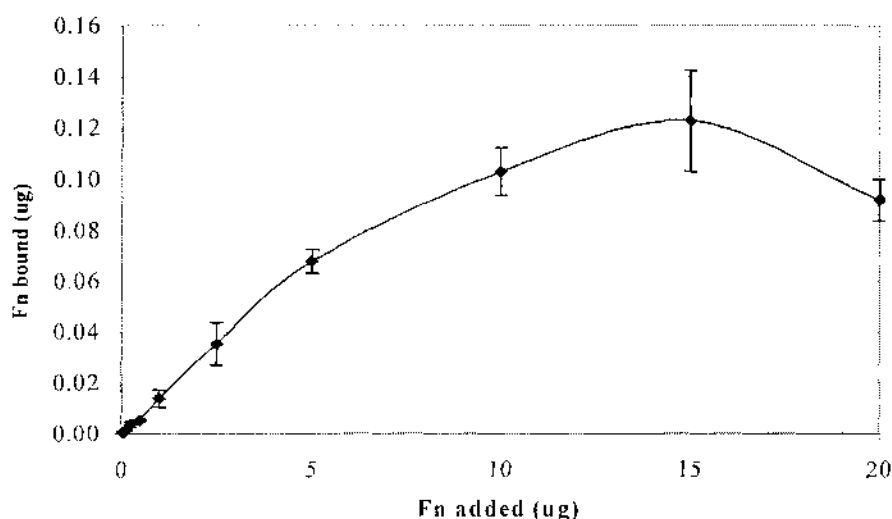
### **3.1.3 HN001 binding to $^{125}\text{I}$ -fibronectin is dependant on growth phase**

To determine the impact of growth phase on binding to fibronectin, the binding reaction was performed as outlined in Section 2.15.2 except that HN001 was grown for 6 hr (exponential phase sample) or for 18 hr (stationary phase sample). Cells were then adjusted to the same concentration and equal amounts were added in the binding assay. Stationary-phase HN001 was found to bind more  $^{125}\text{I}$ -Fn than exponential-phase HN001 (Figure 8).

### **3.1.4 HN001 binding to $^{125}\text{I}$ -fibronectin is specific**

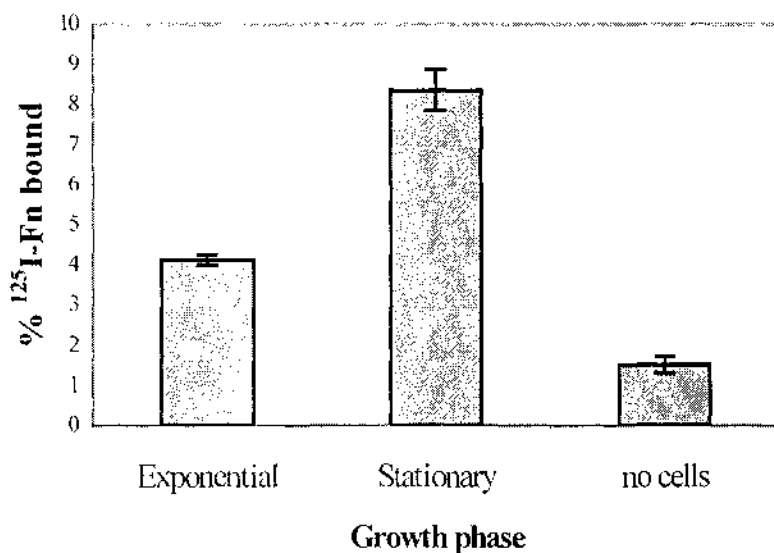
The specificity of the interaction between fibronectin and HN001 was investigated by competition with unlabelled fibronectin. A 1000-fold excess of unlabelled fibronectin was added along with the  $^{125}\text{I}$ -Fn in the binding reaction and the percent of bound  $^{125}\text{I}$ -Fn was determined as outlined in Section 2.15.2. A 1000-fold excess of BSA was used as a negative (no-competition) control. The unlabelled fibronectin reduced HN001 binding to  $^{125}\text{I}$ -Fn to 25% of the BSA control sample (75% inhibition) indicating that the interaction between HN001 and fibronectin is specific (Figure 9).





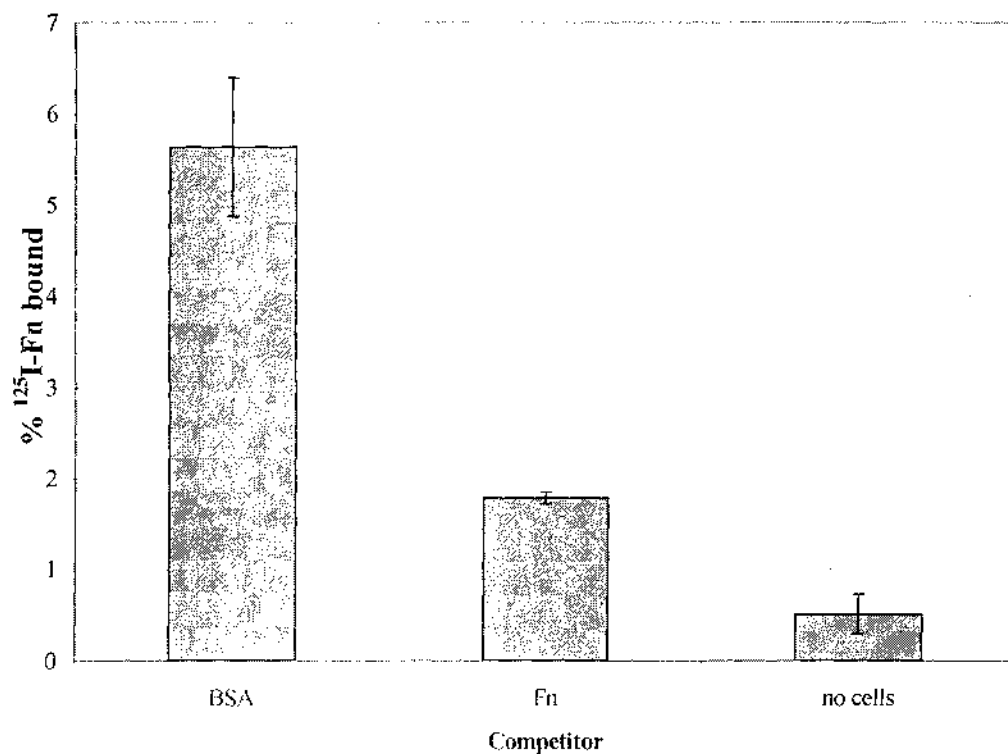
**Figure 7 HN001 binding to  $^{125}\text{I}$ -fibronectin is saturable**

$5 \times 10^8$  cfu HN001 were incubated with  $0.03 \mu\text{g}$  to  $20 \mu\text{g}$   $^{125}\text{I}$ -Fn (ug on axis label =  $\mu\text{g}$ ). HN001 was harvested by centrifugation and radioactivity associated with the cell pellet was measured. The data points are the mean of 3 samples (each assayed in two independent experiments). Error bars indicate  $\pm 2$  standard error of the mean.



**Figure 8 Stationary-phase binds more  $^{125}\text{I}$ -Fn than exponential-phase HN001**

$5 \times 10^8$  cfu HN001 from stationary-phase or exponential-phase were incubated with  $30 \text{ ng}$   $^{125}\text{I}$ -Fn. HN001 was harvested by centrifugation and radioactivity associated with the cell pellet was measured. The data points are the mean of 3 samples (each assayed in two separate experiments). Error bars indicate  $\pm 2$  standard error of the mean. The no cells sample was included as the background level of labelled protein binding to the tube.



**Figure 9 HN001 binding to <sup>125</sup>I-fibronectin is specific**

A 1000-fold excess of unlabelled fibronectin (Fn) or BSA was added with 30ng <sup>125</sup>I-Fn to 5×10<sup>8</sup> cfu HN001. HN001 was harvested by centrifugation and radioactivity associated with the cell pellet was measured. The data points are the mean of 3 samples (each assayed in two separate experiments). Error bars indicate +/- 2 standard error of the mean. The no cells sample was included as the background level of labelled protein binding to the tube.

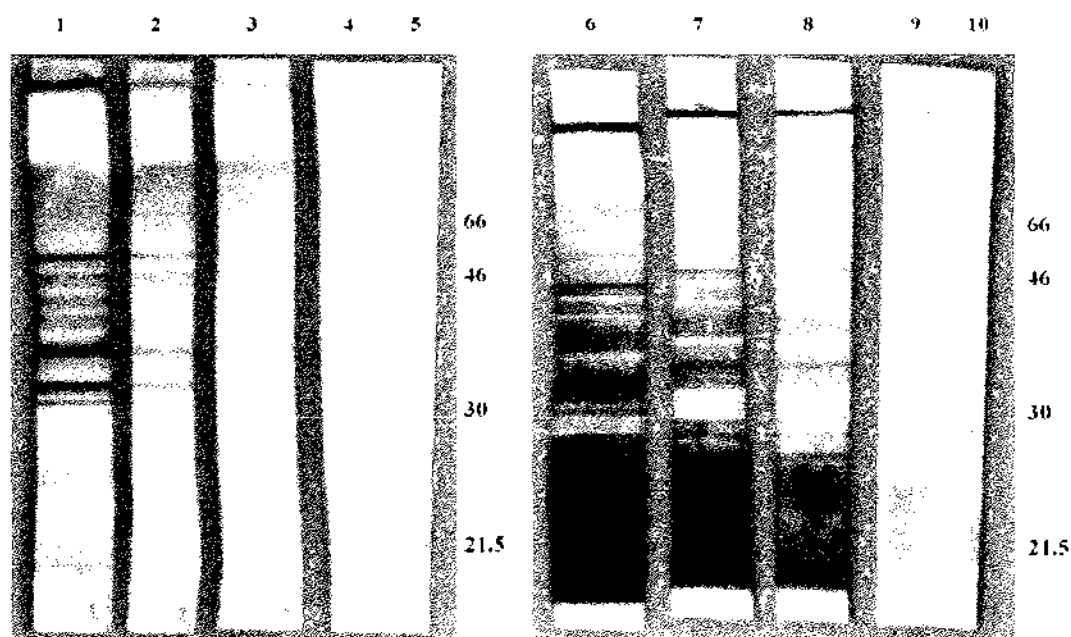
## 3.2 Solid-phase binding assays for intestinal molecules

Solid-phase binding assays were used to further characterise the molecular species involved in the adherence of *Lactobacillus rhamnosus* HN001 to intestinal surface and extracellular matrix molecules. Once the solid-phase binding assay method was developed, it was used in preference to liquid-phase binding assays because it probably more closely models the interaction between bacteria and immobilised molecules on intestinal cells. An ELISA technique was developed to detect HN001 cells in solid-phase binding assays. The results from the experiments used to develop the ELISA technique are outlined in Sections 3.2.1 – 3.2.3. Binding assays using ELISA detection of HN001 cells are outlined in Sections 3.2.4 – 3.2.7. Detection of HN001 by the ELISA technique is dependent on the binding of polyclonal antibodies to cell surface epitopes. It was felt that treatments that may alter the presence of these cell surface epitopes might alter detection of HN001 cells so other approaches to detect HN001 cells were investigated. The result of this investigation is described in Section 3.2.8 and the results of the HN001 cell surface treatment is described in Section 3.2.9. Unless stated all binding assays were performed at least twice on triplicate samples (six assays for each data point).

### 3.2.1 Detection of rabbit anti-HN001 cell envelope antibody titre by Immunoblotting

In order to develop a method to detect HN001 cells, antibodies were raised to the HN001 cell envelope. Rabbits were inoculated with either 1× washed or 8× washed HN001 crude cell envelope preparation (prepared by Dr. Paul O'Toole) as outlined in Section 2.13.1. The purpose of having one envelope sample washed more than the other was to hopefully expose more surface proteins by washing off surface carbohydrate. In this study HN001 cell envelopes were prepared to react with anti-HN001 cell envelope antibodies as outlined in Section 2.12. 1×-washed and 8×-washed HN001 envelope samples were each analysed on one preparative 12% SDS-PAGE gel. Envelope sample (20µl) was applied to the large well and 5µl of high molecular weight Rainbow™ coloured marker (Amersham) was applied to the small well on each gel (prepared as outlined in Section 2.13.2). Protein electrophoresis was performed as outlined in Section 2.8.3 except gels were not stained with Coomassie stain. Cell

envelope proteins were blotted onto a PVDF membrane and reacted with the rabbit antiserum as outlined in Sections 2.13.3 – 2.13.4. When blotted and exposed to the rabbit antiserum, many high molecular weight bands bound the antiserum indicating that they were antigenic (Figure 10). A large number of low molecular weight bands appeared in the 8× washed envelope sample that were not present in the 1× washed envelope sample. The presence of more low molecular weight proteins in the 8× washed sample could be due to proteolytic degradation during the multiple wash steps or the extra washing revealing low molecular weight proteins that were masked in the 1× washed sample. As a result of several inoculations of HN001 cell envelope over the course of the year, the rabbit antiserum had high affinity to HN001 cell envelope at 1/1000 dilution (Figure 10), which was considered high enough to purify IgG from the rabbit antiserum as outlined in Section 3.2.2.

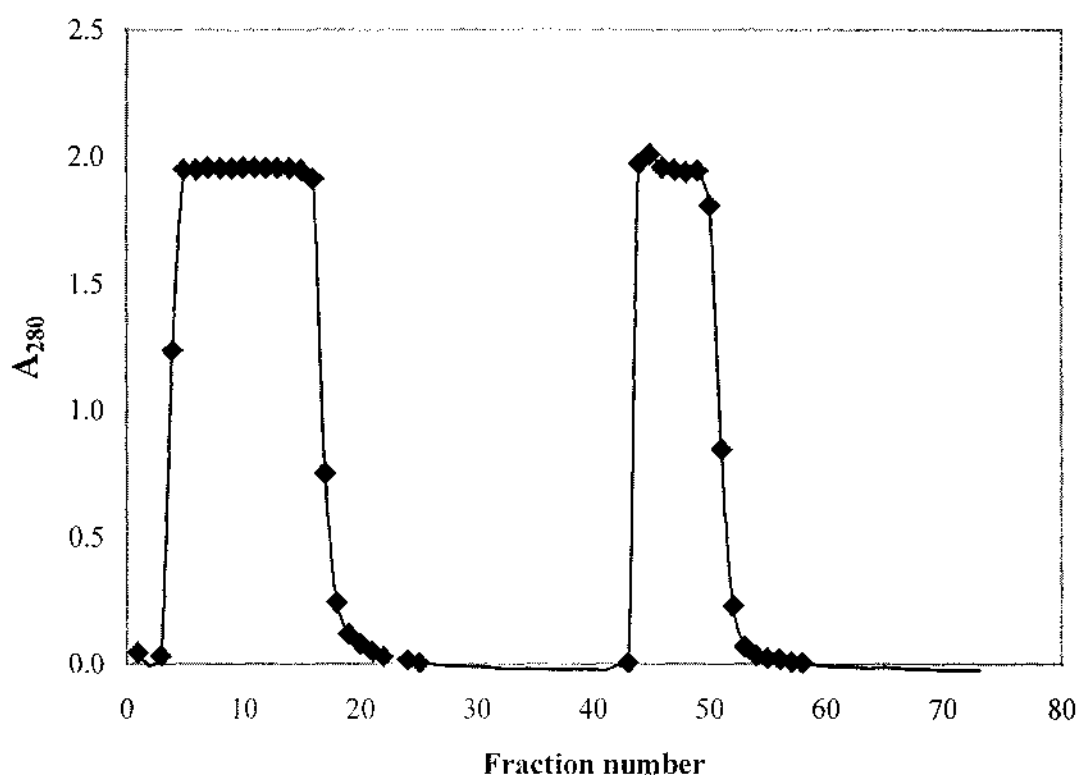


**Figure 10 Antiserum has high reactivity to HN001 cell envelope at 1/1000 dilution**

Two HN001 cell envelope preparations were blotted and probed with antiserum from a rabbit inoculated with HN001 cell envelope. 1× washed cell envelope was probed with antiserum from rabbit inoculated with 1× washed cell envelope. 8× washed cell envelope was probed with antiserum from rabbit inoculated with 8× washed cell envelope. Antibodies bound to blotted proteins were reacted with Protein A conjugated to HRP and bound HRP was detected by a colorimetric method. Lanes are: 1× wash 1:1/100 dilution, 2:1/500, 3:1/1000, 4:1/5000; 8× wash 6:1/100, 7:1/500, 8:1/1000, 9:1/5000, 5 and 10: Rainbow high molecular weight marker.

### 3.2.2 Purification of rabbit anti-HN001 cell envelope IgG

IgG was purified from rabbit antiserum to detect HN001 cells in ELISA-type assays. IgG was purified from rabbit antiserum using a Protein G column according to the method outlined in Section 2.14. During the purification process a peak corresponding to the expected peak for IgG was observed when the absorbance of fractions were monitored at 280nm (Figure 11). The IgG peak fractions were pooled, desalted, and the absorbance at 280nm measured to determine the approximate concentration of IgG in the solution. The formula:  $A_{280} 1.0 = 0.75\text{mg/ml IgG}$  was used to quantify the IgG (Harlow *et al.*, 1988). All types of IgG purified in this study had a final  $A_{280}$  of 1.9, which corresponds to approximately 1.4mg/ml IgG.

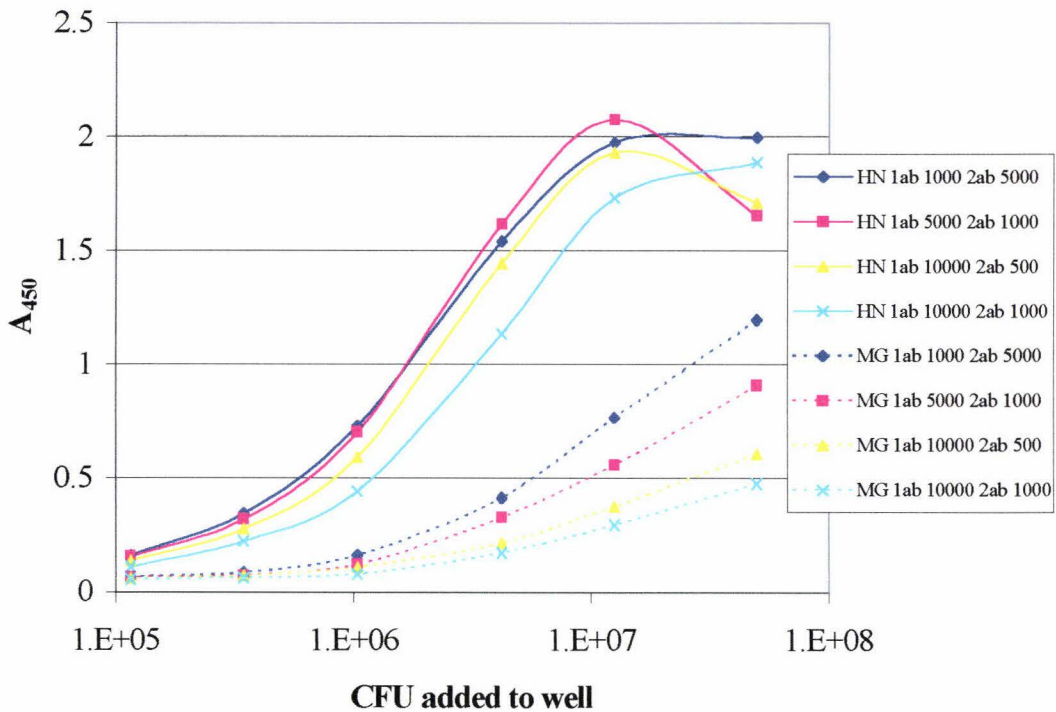


**Figure 11 Purification of rabbit anti-HN001 cell envelope IgG**

Purification of IgG from rabbit antiserum using a Protein G column run by FPLC. The first peak corresponds to non-binding material passing through the column. The second peak, corresponding to IgG, occurs soon after the elution buffer is run through the column.

3.2.3 Optimisation of detection of HN001 cells

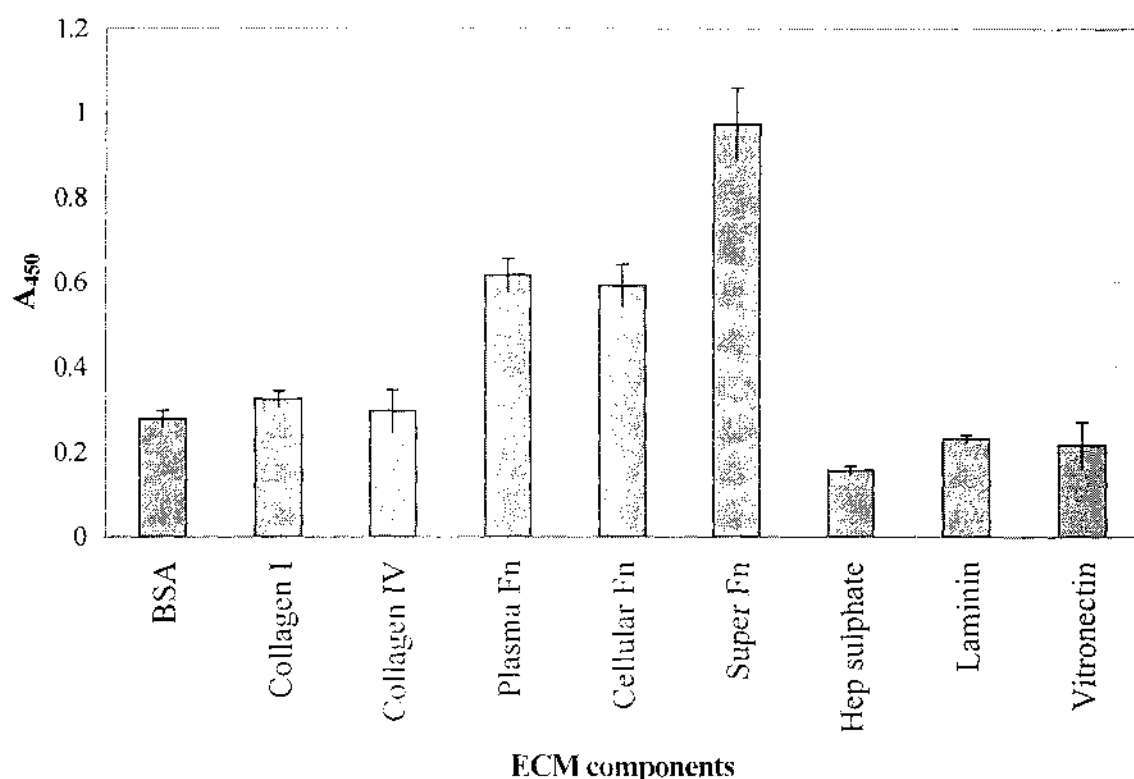
Firstly, criss-cross serial dilution ELISA (Section 2.16.3.1) was performed to determine several concentrations of 1° and 2° antibodies that quantitatively detect HN001 cells. Concentrations that quantitatively detected HN001 cells were further investigated against a larger cell range (Sections 2.16.1 – 2.16.3). A 1/5000 dilution of 1° Ab and a 1/1000 dilution of 2° Ab gave a linear response over a large cell range with minimal interaction with the heterologous antigen (Figure 12). This combination of antibody dilutions was selected to detect HN001 cells in binding assays with ELISA detection.



**Figure 12 1/5000 dilution of rabbit anti-HN001 cell envelope IgG and 1/1000 dilution of anti-rabbit IgG conjugated to HRP quantitatively detects HN001 cells**  
1.2×10<sup>5</sup>, 3.5×10<sup>5</sup>, 1.0×10<sup>6</sup>, 4.2×10<sup>6</sup>, 1.3×10<sup>7</sup>, 5×10<sup>7</sup> cfu HN001 were added per well and detected using ELISA with several different 1° and 2° antibody dilutions. Each data point is the mean of 2 samples. HN corresponds to wells coated with HN001 cells; MG corresponds to wells coated with MG1363 cells. 1ab corresponds to the dilution of primary antibody used; 2ab corresponds to the dilution of secondary antibody used.

### 3.2.4 HN001 binding to extracellular matrix components in solid-phase

To determine which extracellular matrix components (ECM) HN001 binds to, and to confirm the liquid-phase data, solid-phase binding to collagen I, collagen IV, plasma fibronectin, cellular fibronectin, super fibronectin, heparan sulphate, laminin and vitronectin was investigated. The binding assay was performed as outlined in Sections 2.16.1 – 2.16.3. BSA was included as a negative control protein that HN001 should not bind to. Binding to BSA was taken as the background, non-specific binding level in the assay. HN001 was found to bind significantly above background to three forms of fibronectin (Figure 13) confirming the liquid-phase data.

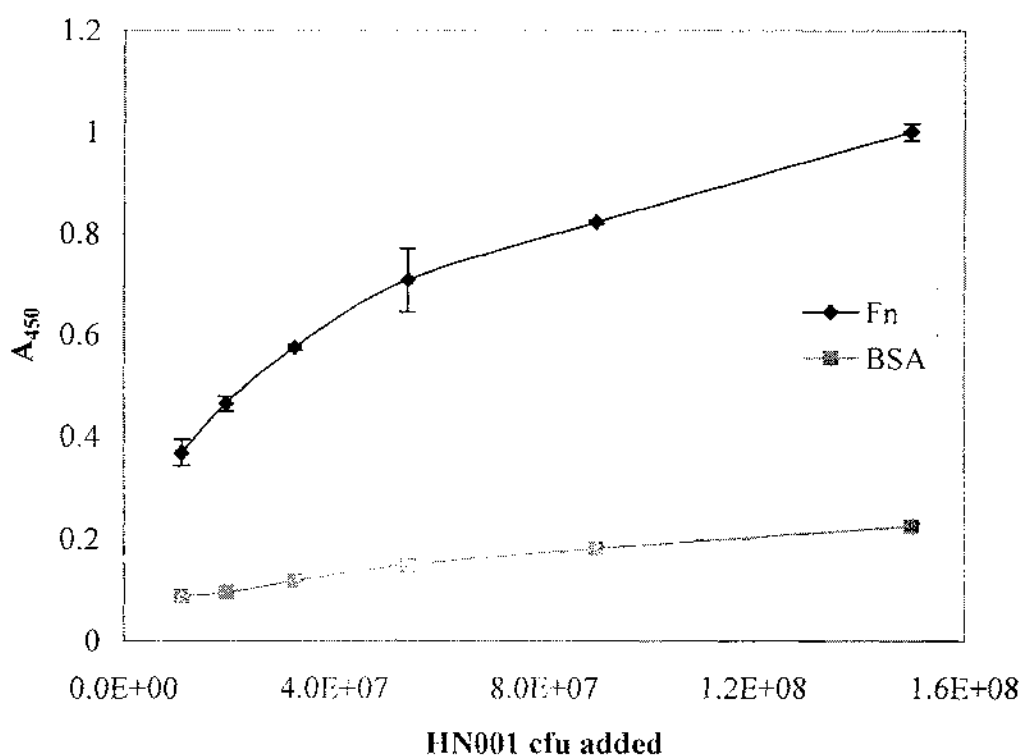


**Figure 13 HN001 binds three forms of fibronectin in the solid-phase**

HN001 binding to components of the ECM was investigated by incubating  $5 \times 10^7$  cfu HN001 in wells coated with various components. Bound HN001 cells were detected using ELISA. Each data point is the mean of 3 samples (assay performed once). Error bars indicate  $\pm 2$  standard error of the mean.

### 3.2.5 Dose-dependent binding of HN001 to fibronectin

The interaction of HN001 with fibronectin in the solid-phase was further investigated by determining if the interaction was dose-dependent with increasing HN001 cell concentration. The binding assay was performed as outlined in Sections 2.16.1 – 2.16.3 except that the amount of HN001 added ranged from  $1 \times 10^6$  to  $5 \times 10^9$  cfu. BSA coated wells were included as a negative control. The interaction between HN001 and fibronectin was found to be dose-dependent (Figure 14).  $5 \times 10^7$  cfu HN001 was used for subsequent assays.



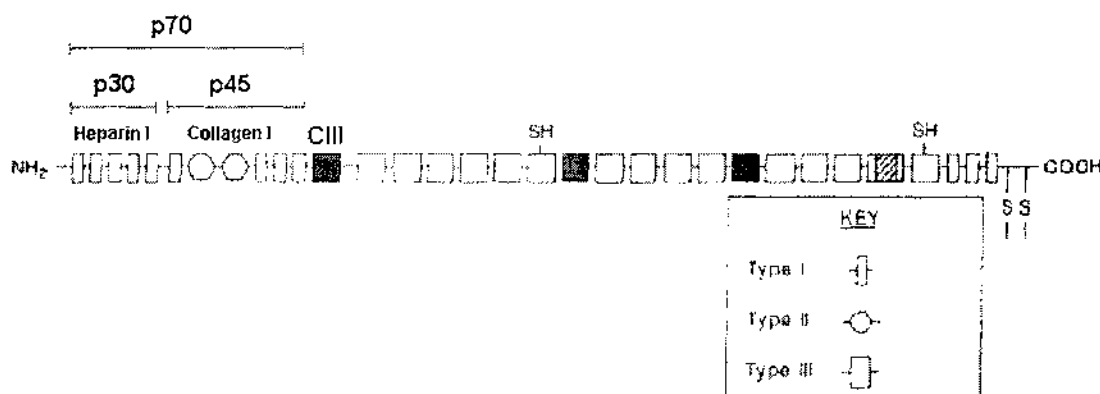
**Figure 14 Dose-dependent binding of HN001 to fibronectin**

Dose-dependent binding of HN001 to fibronectin in the solid-phase was investigated by incubating from  $1 \times 10^6$  to  $2 \times 10^8$  cfu HN001 in fibronectin and BSA-coated wells. Bound HN001 cells were detected by ELISA. Data points correspond to:  $1.1 \times 10^7$ ,  $1.9 \times 10^7$ ,  $3.2 \times 10^7$ ,  $5.4 \times 10^7$ ,  $9.0 \times 10^7$ , and  $1.5 \times 10^8$  cfu added. Each data point is the mean of 3 samples (each assayed in two separate experiments). Error bars indicate +/- 2 standard error of the mean.



### 3.2.6 Binding of HN001 to different fragments of fibronectin

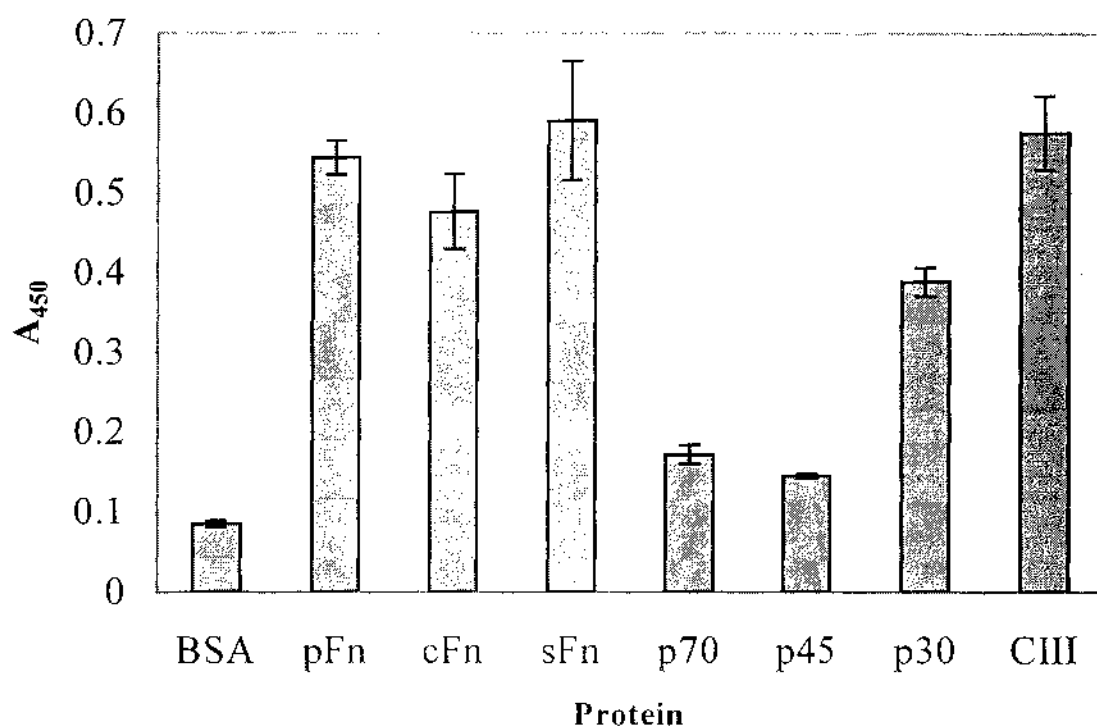
Fibronectin is a large dimer of approximately 440kDa (total mass); hence there are several potential binding sites within the molecule. The fibronectin domain structure specifying the fragments used in this study is shown in Figure 15.



**Figure 15 Structure of plasma fibronectin**

The diagram (adapted from Ruoslahti, 1988) shows one monomer of the fibronectin dimer with the positions of the p70, p30, p45, and CIII (III<sub>1</sub>-C) fragments indicated.

The HN001 binding site within the fibronectin molecule was determined by examining HN001 adhesion to plasma fibronectin, cellular fibronectin, super fibronectin, three different proteolytic fragments of fibronectin (p70, p45 and p30) and the C-terminal part of the first type three repeat in fibronectin (III<sub>1</sub>-C). The III<sub>1</sub>-C fragment is of interest as it is used to cause polymerisation of plasma fibronectin into super fibronectin (Morla *et al.*, 1994). The binding assay was performed as outlined in Sections 2.16.1 – 2.16.3. BSA was included as a negative control protein. HN001 was found to adhere to the CIII fragment to a similar degree as to plasma fibronectin. HN001 also bound to the p30 fragment to a lesser degree as to plasma fibronectin. HN001 binding to p70 and p45 fragments was not significantly different to BSA binding levels (Figure 16).



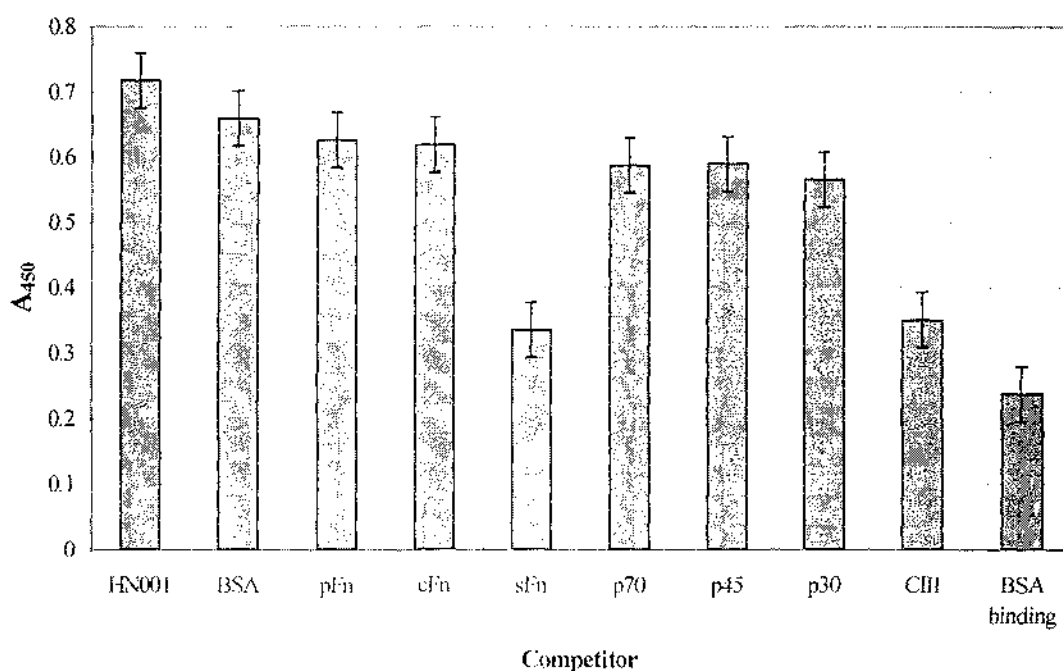
**Figure 16 HN001 binds CIII and p30 fragments of fibronectin**

HN001 binding to fragments of fibronectin in the solid-phase was investigated by incubating  $5 \times 10^7$  cfu HN001 in wells coated with various proteins. Bound HN001 cells were detected by ELISA. Each data point is the mean of 3 samples (each assayed in two separate experiments). Error bars indicate  $\pm 2$  standard error of the mean. pFn = plasma fibronectin, cFn = cellular fibronectin, sFn = super fibronectin, p70 = 70KDa Heparin I + Collagen binding domain, p45 = 45KDa Collagen binding domain, p30 = 30KDa Heparin I binding fragment and CIII = III<sub>1</sub>-C fragment (Figure 15).

### **3.2.7 Specificity of interaction between HN001 and fibronectin**

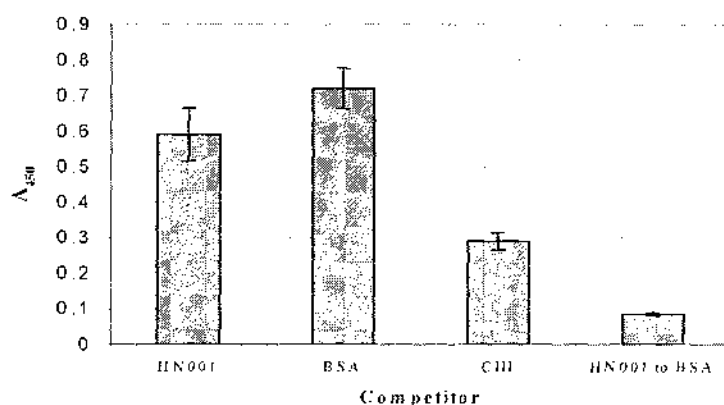
The specificity of the interaction between HN001 and immobilised fibronectin was investigated by competition with various types and fragments of fibronectin. The binding assay was performed as outlined in Sections 2.16.1 – 2.16.3 except that HN001 cells were preincubated with a 400µg/ml solution of the competitor protein for 1 hr at 37°C before adding to protein-coated wells. BSA was used at the same concentration as the fibronectin fragments as a non-competing negative control. BSA-coated wells were used to show the background ‘non-binding’ level of the assay. Only super fibronectin and the CIII fragment were able to disrupt HN001 adhesion to immobilised plasma fibronectin (Figure 17). Plasma fibronectin in liquid-phase did not compete with HN001 binding to immobilised plasma fibronectin. This has been observed with other fibronectin-binding bacteria (Section 4.1) and is presumably due to possible conformational changes when plasma fibronectin is immobilised on a surface. This conformation may be similar to the conformation of super fibronectin, which is supported by the fact that super fibronectin disrupts HN001 binding to immobilised plasma fibronectin. CIII disrupted HN001 binding to immobilised plasma fibronectin; therefore, HN001 may bind specifically to the CIII domain (or a similar domain) in the fibronectin molecule and it may be that this domain is exposed in immobilised plasma fibronectin.

The role of CIII in fibronectin binding was further investigated by preincubating HN001 with CIII before adding to wells with immobilised super fibronectin and vice versa as outlined above. CIII was found to disrupt super fibronectin binding to a greater extent than super fibronectin disrupted CIII binding (Figures 18 and 19). The results indicate that HN001 binds to CIII with greater affinity than to super fibronectin and that the CIII fragment has a role in super fibronectin binding.



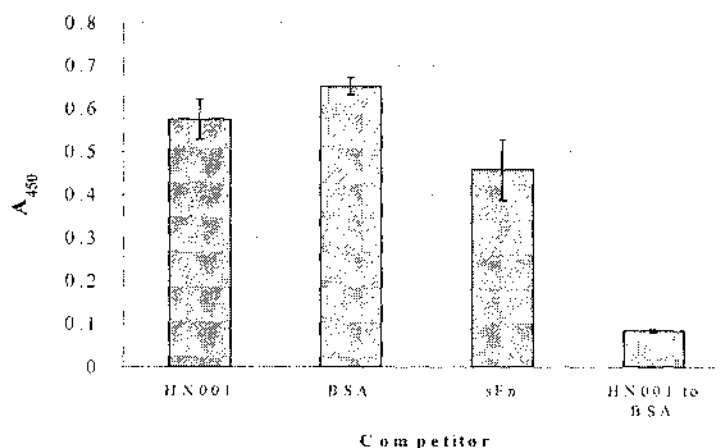
**Figure 17 Super fibronectin and CIII disrupt HN001 binding to immobilised plasma fibronectin**

Competition of HN001 binding to plasma fibronectin was investigated by incubating  $5 \times 10^7$  cfu HN001 with a protein solution before adding to wells coated with plasma fibronectin. Bound HN001 cells were detected by ELISA. Each data point is the mean of 3 samples (each assayed in duplicate). Error bars indicate  $\pm 2$  standard error of the mean. The sample labelled HN001 is HN001 binding to plasma fibronectin-coated wells with no competition. BSA, pFn (plasma fibronectin), cFn (cellular fibronectin), sFn (super fibronectin), p70, p45, p30 and CIII are HN001 binding to plasma fibronectin-coated wells with protein competition (named). The BSA binding sample is HN001 binding to BSA-coated wells with no competition.



**Figure 18 CIII disrupts HN001 binding to super fibronectin (sFn)**

Competition of HN001 binding to sFn was investigated by incubating  $5 \times 10^7$  cfu HN001 with a protein solution before adding to wells coated with sFn. Bound HN001 cells were detected by ELISA. Each data point is the mean of 3 samples (once with triplicate samples). Error bars indicate  $\pm 2$  standard error of the mean. The sample labelled HN001 is HN001 binding to sFn-coated wells with no competition. BSA and CIII are HN001 binding to sFn-coated wells with protein competition (named). The HN001 to BSA sample is HN001 binding to BSA-coated wells with no competition.



**Figure 19 Super fibronectin has small effect on HN001 binding to CIII**

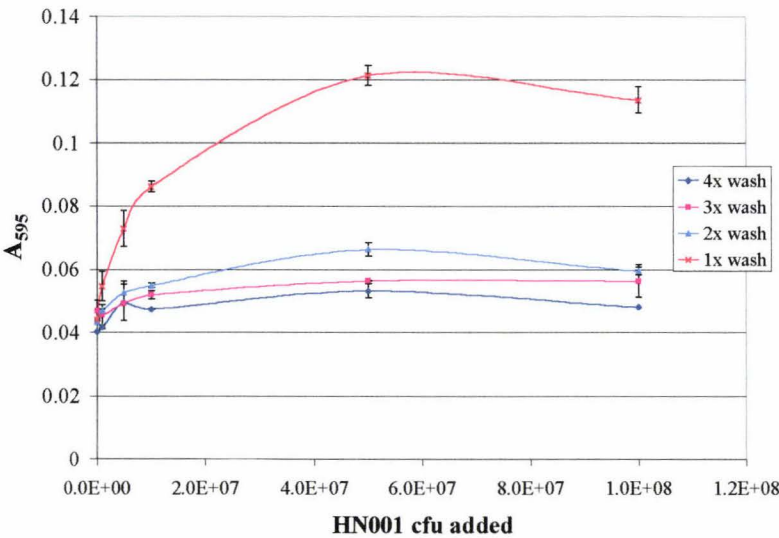
Competition of HN001 binding to CIII was investigated by incubating  $5 \times 10^7$  cfu HN001 with a protein solution before adding to wells coated with CIII. Bound HN001 cells were detected by ELISA detection. Each data point is the mean of 3 samples (once with triplicate samples). Error bars indicate  $\pm 2$  standard errors of the mean. The sample labelled HN001 is HN001 binding to CIII-coated wells with no competition. BSA and sFn (super fibronectin) are HN001 binding to CIII-coated wells with protein competition (named). The HN001 to BSA sample is HN001 binding to BSA-coated wells with no competition.

**3.2.8 Evaluation of different methods to detect HN001 cells**

As quantitation of HN001 cells by ELISA relies on binding of primary antibodies to HN001 cells, ELISA could not reliably quantitate the effect of treatments that may alter the surface. Thus, a method independent of ELISA detection was needed. The other attempts to develop assays for HN001 binding investigated in this study are detailed in Sections 3.2.8.1 – 3.2.8.5.

**3.2.8.1 Crystal violet detection**

Crystal violet staining of HN001 cells, based on a method by Mongodin et al. (2002), was performed as outlined in Section 2.16.5. Staining of various amounts of cells (0,  $1 \times 10^6$ ,  $5 \times 10^6$ ,  $1 \times 10^7$ ,  $5 \times 10^7$  and  $1 \times 10^8$  cfu added per well) was investigated. There was a relationship between absorbance at 595nm and HN001 cells added but only if washing of wells was restricted to one wash (Figure 20). When the washes were increased the assay was not linear. There was a lot of residual crystal violet stain left in the wells after one wash which might reduce the accuracy of this detection method. Therefore, this method was not considered to be sufficiently accurate and reliable.



**Figure 20 Crystal violet staining not suitable for detecting HN001 cells**

Detection of HN001 cells by crystal violet staining was investigated by coating microtitre wells with various amounts of HN001 cells (0,  $1 \times 10^6$ ,  $5 \times 10^6$ ,  $1 \times 10^7$ ,  $5 \times 10^7$  and  $1 \times 10^8$  cfu per well). Bound cells were fixed, stained, lysed and absorbance at 595nm measured. Each data point is the mean of 3 samples. Error bars indicate +/- 2 standard error of the mean.

### **3.2.8.2 OD<sub>403</sub> detection**

The detection of HN001 cells by OD<sub>403</sub>, based on a method by Roos and Jonsson (2002), was performed as outlined in Section 2.16.7. Detection of various amounts of cells (0,  $1 \times 10^6$ ,  $5 \times 10^6$ ,  $1 \times 10^7$ ,  $5 \times 10^7$  and  $1 \times 10^8$  cfu) was investigated. There was no difference in OD<sub>403</sub> seen between 0 and  $1 \times 10^8$  HN001 cfu even with only one wash. Thus this method was not suitable for detection of HN001 cells.

### **3.2.8.3 Phase-contrast microscopy detection**

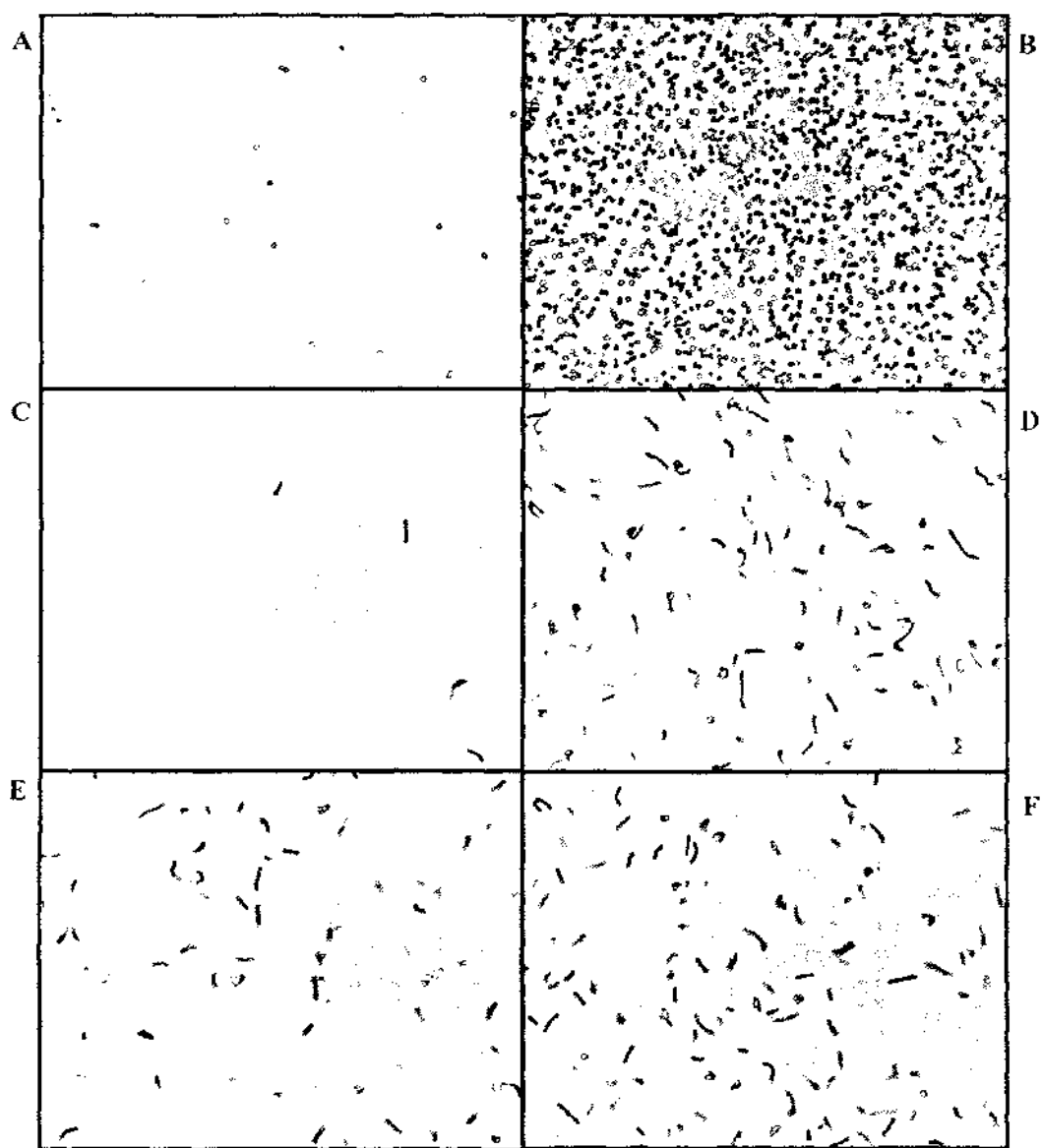
Detection of HN001 by phase-contrast microscopy was performed as outlined in Section 2.16.6. Detection of various amounts of cells (0,  $1 \times 10^6$ ,  $5 \times 10^6$ ,  $1 \times 10^7$ ,  $5 \times 10^7$  and  $1 \times 10^8$  cfu) was investigated. More HN001 cells bound around the edge of the microtitre wells than in the centre making quantitation by visual counting of cells in random microscopic fields impossible. Thus this method was not suitable for detection of HN001 cells.

### **3.2.8.4 Slide-binding assays**

Bacterial adhesion to protein-coated glass slides, based on a method by Kukkonen et al. (1993), was performed as outlined in Section 2.16.8. The positive control for fibronectin binding, *Staphylococcus aureus* ATCC 25923 (Proctor *et al.*, 1982), bound slides coated with fibronectin with more affinity than slides coated with BSA. HN001 had variable binding to fibronectin coated slides (Figure 21). Also, quantitation of binding was time-consuming and difficult. Thus this method was not considered suitable for assaying HN001 adhesion to fibronectin.

### **3.2.8.5 Radioisotope labelling**

The radiolabelling of HN001, based on the method described by Cohen and Laux (1995), was performed as outlined in Section 2.16.4. HN001 cells were labelled with <sup>3</sup>H-uracil and <sup>3</sup>H-adenine typically to a specific activity of approximately 0.02 cpm/cfu. Since this method of detection of HN001 cells was reliable and not affected by treatment of HN001 cell surfaces it was used to assay the effect of protease and periodate treatment of HN001 cell surfaces on fibronectin binding.



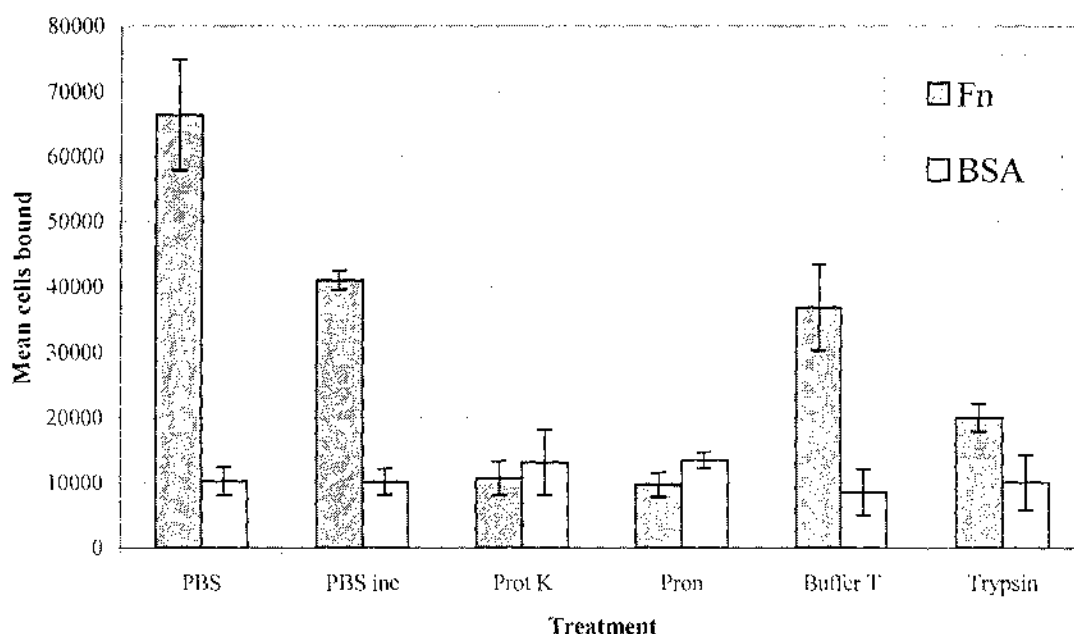
**Figure 21 *Staphylococcus aureus* ATCC 25923 binds fibronectin-coated glass slides but HN001 binding is variable**

Protein-coated glass slides were incubated with bacteria and examined under phase contrast microscopy. Each photograph is one field of view from a slide. **A** is *S. aureus* ATCC 25923 binding to BSA-coated slides. **B** is *S. aureus* ATCC 25923 binding to fibronectin-coated slides. **C** and **E** are HN001 binding to BSA-coated slides. **D** and **F** are HN001 binding to fibronectin-coated slides.



### 3.2.9 Binding of protease and periodate treated cells to fibronectin

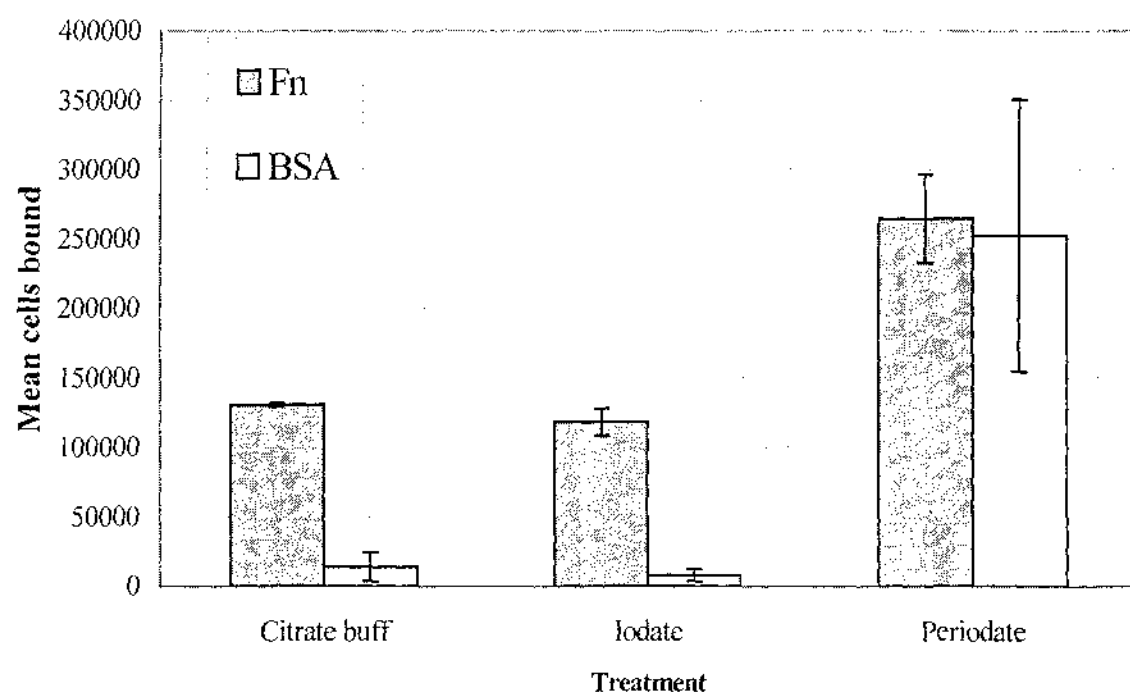
HN001 cells were treated with three different proteases (proteinase K, pronase and trypsin) to assess the role of proteins on HN001 cell surface in fibronectin binding. Treatment of cells was performed as outlined in Section 2.18.1 and the binding assay was performed as outlined in Section 2.16.4. All three proteases were found to decrease fibronectin binding indicating that HN001 cell surface proteins are involved in fibronectin binding (Figure 22).



**Figure 22 Protease treatment of HN001 cells decreases binding to fibronectin**

The role of HN001 cell surface proteins was investigated by treating  $^3\text{H}$ -labelled HN001 with proteases and incubating  $5 \times 10^7$  cfu HN001 in wells coated with plasma fibronectin. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (each assayed in duplicate). Error bars indicate  $\pm 2$  standard error of the mean. The PBS sample is the positive control for the binding assay with no pretreatment of HN001 cells. PBS inc. is the control for the proteinase K and pronase samples. Buffer T is the control for trypsin.

HN001 cells were treated with sodium periodate to assess the role of carbohydrate on HN001 cell surface in fibronectin binding. Sodium periodate is an oxidising agent which oxidises the carbohydrate on bacterial cell surfaces. Treatment of cells was performed as outlined in Section 2.18.2 and the binding assay was performed as outlined in Section 2.16.4. Sodium iodate, which does not affect bacterial cell surface carbohydrate, was used as a negative control for the oxidation treatment. Periodate treatment of HN001 cell surface increased both fibronectin and BSA binding, which indicated that non-specific binding had been increased (Figure 23). No conclusions could be drawn about the role of HN001 cell surface carbohydrate in fibronectin binding.



**Figure 23 Periodate oxidation of HN001 cell surfaces increases non-specific binding**

The role of HN001 cell surface proteins was investigated by treating  $^3\text{H}$ -labelled HN001 with periodate and incubating  $5 \times 10^7$  cfu HN001 in wells coated with plasma fibronectin. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (each assayed in duplicate). Error bars indicate  $\pm 2$  standard error of the mean. Citrate buff is the control for Iodate and Periodate. Iodate is a control for Periodate.

### 3.3 Caco-2 adhesion assays

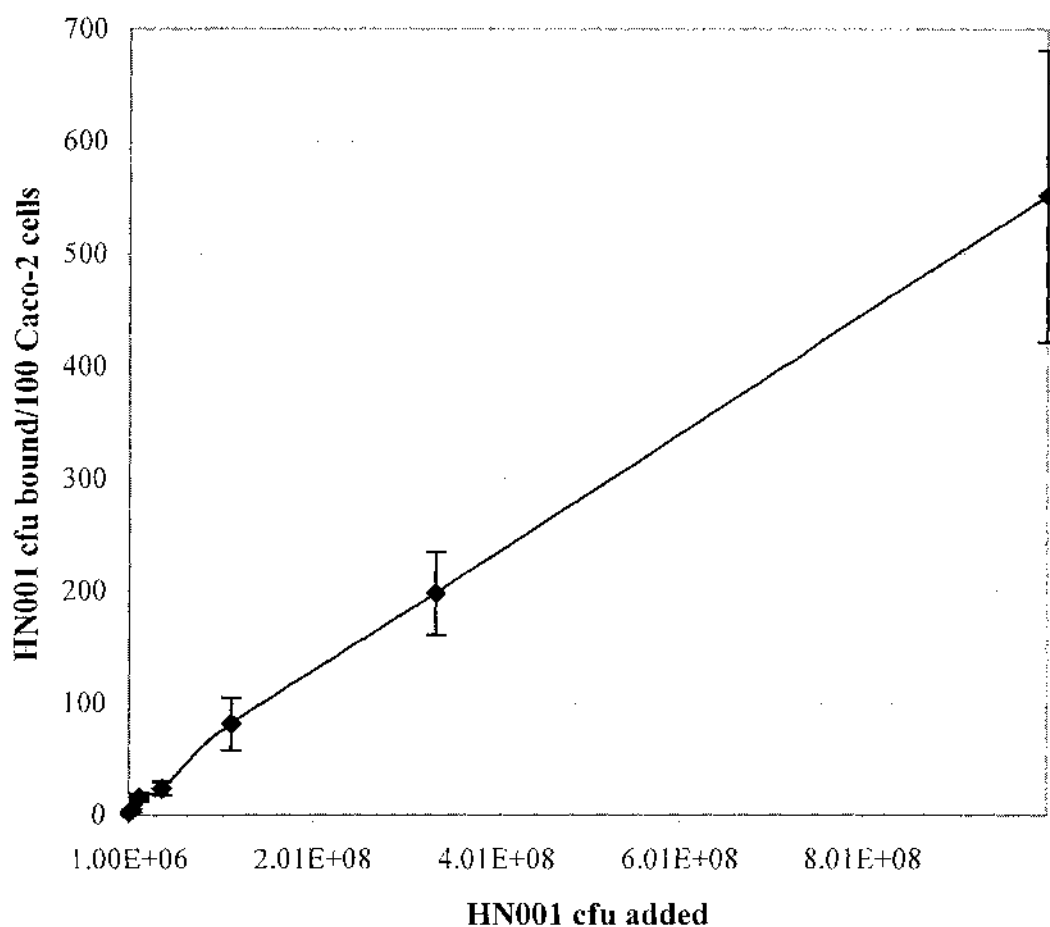
The intestinal cell line Caco-2 is a commonly used model of human intestinal epithelial cells (Section 1.3.4). HN001 binding to Caco-2 cells was investigated as described in Sections 3.3.2 – 3.3.3 and 3.3.5 – 3.3.7. Unless stated all binding assays were performed at least twice with triplicate samples in two different Caco-2 cell passages.

#### 3.3.1 There are approximately $7 \times 10^6$ Caco-2 cells per adhesion assay

The number of Caco-2 cells per well was estimated as outlined in Section 2.17.2, so that the HN001 cfu bound per 100 Caco-2 cells in binding assays could be calculated. The average of the calculated number of Caco-2 cells per well from 7 fields of view (from two different wells) was  $7 \times 10^6$  Caco-2 cells per well. See Appendix 4 for a sample calculation and photo.

#### 3.3.2 Dose-dependent adhesion of HN001 to Caco-2 cells

Dose-dependent adhesion of HN001 to Caco-2 cells was investigated to determine an appropriate amount of HN001 cells to add to subsequent assays. The binding assay was performed as outlined in Sections 2.17.3 except the amount of HN001 added ranged from  $1 \times 10^6$  to  $1 \times 10^9$  cfu. A linear relationship was observed between HN001 cells added and HN001 cells adhering to Caco-2 cells (Figure 24). This indicated that binding assay was not saturated in the range of cell concentrations assayed.  $1 \times 10^8$  cfu HN001 was chosen for subsequent assays. Presumably it would be possible to saturate Caco-2 cells with HN001, but saturating levels were not reached at the highest cell concentration tested.

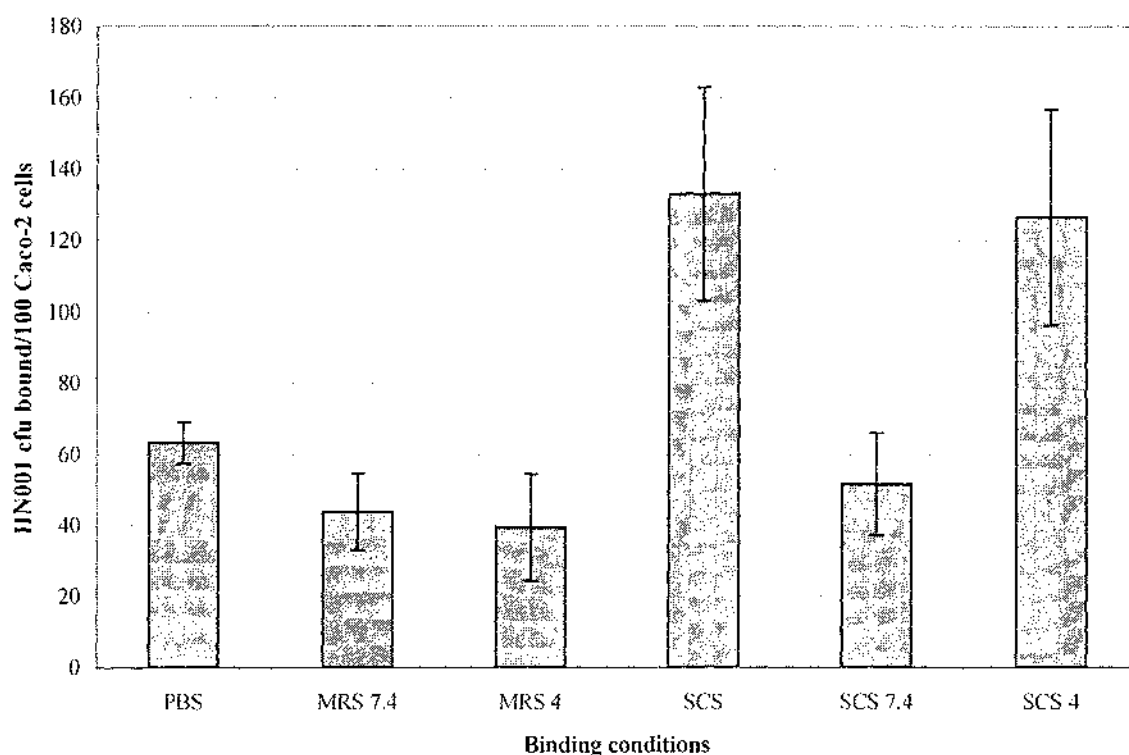


**Figure 24 Dose-dependent adhesion of HN001 to Caco-2 cells**

Dose-dependent adhesion of HN001 to Caco-2 cells was investigated by incubating from  $1 \times 10^6$  to  $1 \times 10^9$  cfu  $^3\text{H}$ -labelled HN001 with Caco-2 monolayers. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (assay performed once). Error bars indicate  $\pm 2$  standard error of the mean.

### **3.3.3 A factor in HN001 spent culture supernatant (SCS) promotes HN001 adhesion to Caco-2 cells**

Several bacteria have been found to secrete a factor into the growth medium that promotes adhesion to intestinal cell lines (Section 4.2). The possible role of a secreted factor in HN001 spent culture supernatant in HN001 adhesion to Caco-2 cells was investigated as outlined in Section 2.17.3 except that HN001 cells were resuspended in HN001 spent culture supernatant (Section 2.3.4), MRS medium (Section 2.2.1) or 1×PBS, pH 7.4 (Section 2.2.2). HN001 SCS is naturally at pH 4.0 after 18 hr of growth in MRS medium. The SCS 7.4 sample is SCS adjusted to pH 7.4 with NaOH. The SCS at pH 7.4 was adjusted back down to pH 4.0 with the addition of HCl to test the possibility of the addition of salt (when adjusting pH) affecting the binding reaction (SCS 4.0 sample). Addition of HN001 SCS to the adhesion assay dramatically increased HN001 adhesion to Caco-2 cells (Figure 25). This effect was found to be due to a factor present only in the SCS and not in the MRS media (included as a control). The effect of the factor in HN001 SCS was influenced by pH, with a positive effect on binding at pH 4.0 and no discernible effect at pH 7.4. Since the binding of HN001 increases again after adjustment of SCS pH 7.4 to pH 4.0, the effect of the factor on binding is not due to salt concentration. Despite the large error in some of the data points the experiment was found to be very reproducible.

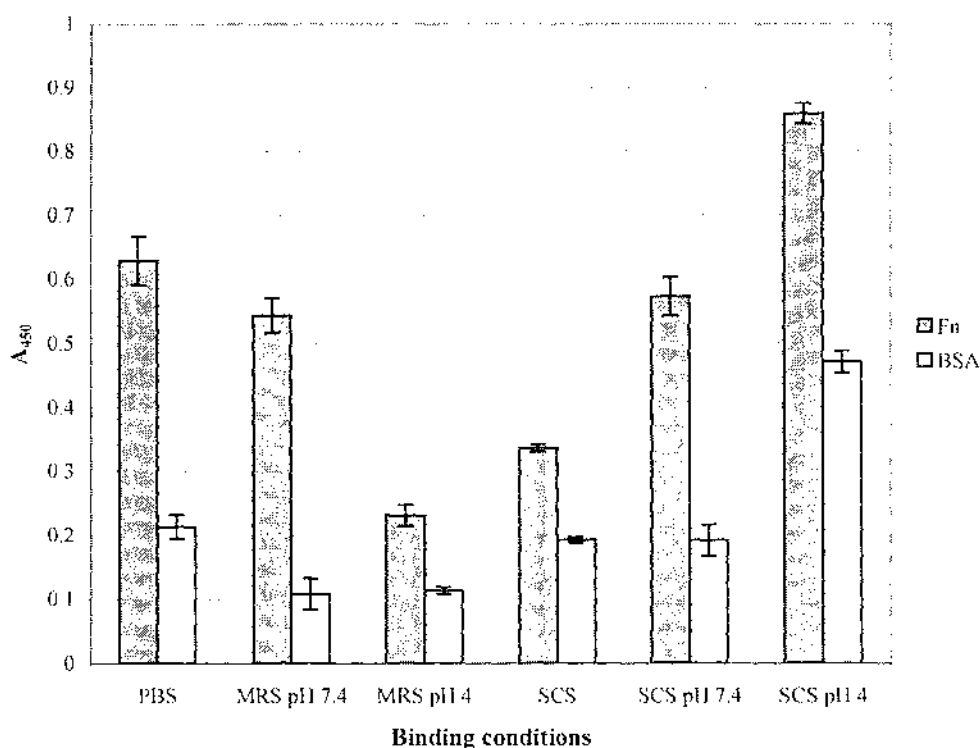


**Figure 25 A factor in HN001 spent culture supernatant promotes adhesion of HN001 to Caco-2 cells in a pH dependent manner**

The role of HN001 SCS in adhesion to Caco-2 cells was investigated by incubating  $1 \times 10^8$  cfu HN001 resuspended in either PBS, MRS or HN001 SCS with Caco-2 monolayers. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (each assayed in two separate experiments). Error bars indicate  $\pm 2$  standard error of the mean. PBS is the positive control for the binding experiment. MRS 7.4 and 4 are samples of MRS media adjusted to pH 7.4 and 4 respectively. SCS is HN001 spent culture supernatant (naturally at pH 4.0). SCS 7.4 is SCS adjusted to pH 7.4. SCS 4 is SCS 7.4 adjusted to pH 4.0.

### 3.3.4 Role of HN001 spent culture supernatant in fibronectin binding

The discovery of a factor in HN001 spent culture supernatant that promotes binding of HN001 to Caco-2 cells (Section 3.3.3) prompted the investigation of its role in fibronectin binding. The binding assay was performed as outlined in Sections 2.16.1 – 2.16.3 except that HN001 cells were resuspended in HN001 spent culture supernatant (Section 2.3.4), MRS medium (Section 2.2.1) or 1×PBS, pH 7.4 (Section 2.2.2). No effect specific to HN001 spent culture supernatant compared to the MRS controls was observed (Figure 26). The increased binding in the SCS pH 4 sample could be due to the increased salt concentration (as a result of adjusting the pH). Refer to Section 3.3.3 for a description of controls used.



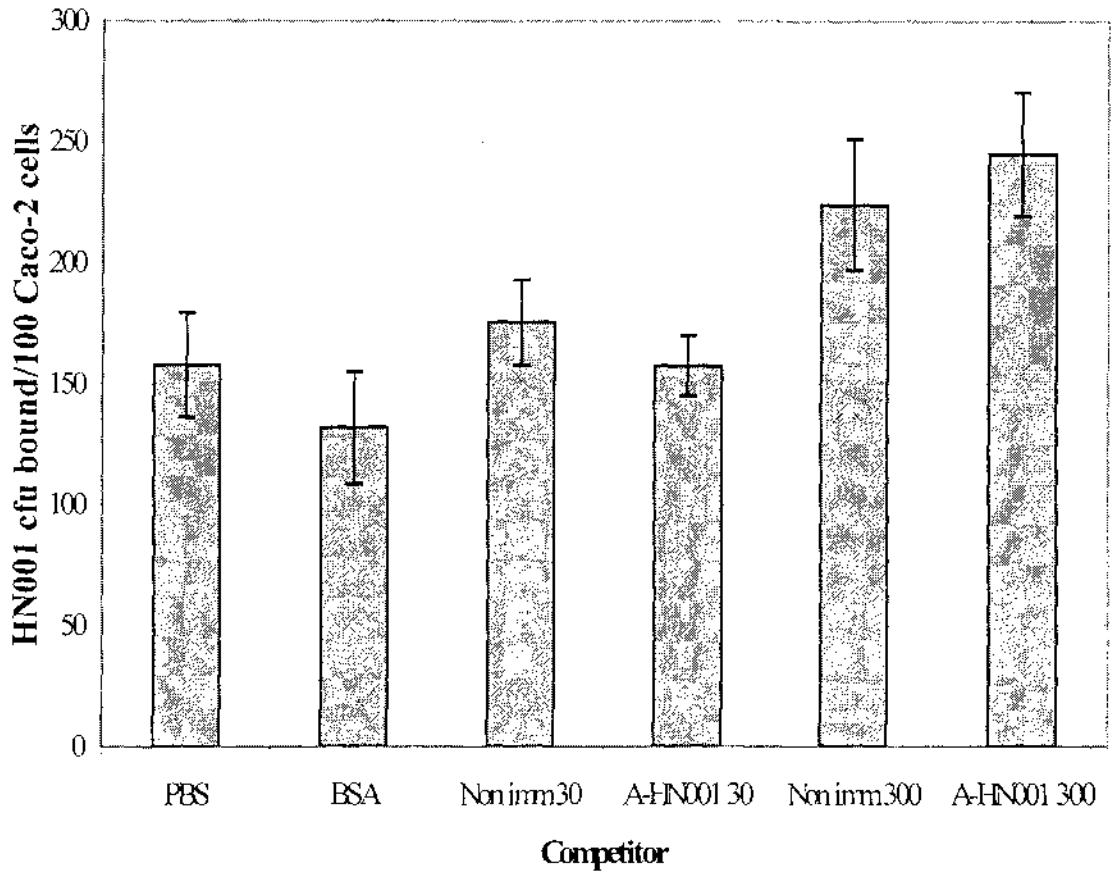
**Figure 26 HN001 spent culture supernatant does not have a positive influence on fibronectin binding**

The role of HN001 SCS in fibronectin binding was investigated by incubating  $5 \times 10^7$  cfu HN001 resuspended in either PBS, MRS or HN001 SCS in wells coated with fibronectin or BSA. Bound HN001 cells were detected by ELISA detection. Each data point is the mean of 3 samples (each assayed in two separate experiments). Error bars indicate  $\pm$  2 standard error of the mean.

### 3.3.5 Competition of HN001 adhesion to Caco-2 with IgG

Rabbit anti-HN001 cell envelope IgG (Section 2.14) was used to assess the effect of antiserum to HN001 cell surface components on adhesion to Caco-2 cells. If the antibodies bind to structures involved in adhesion to Caco-2 cells they will interfere with HN001 adhesion to Caco-2 cells. The binding assay was performed as outlined in Section 2.17.3 except  $5 \times 10^8$  cfu HN001 were added per well and HN001 cells were preincubated for 1hr at 37°C with either 30µg/ml rabbit anti-HN001 cell envelope IgG, 300µg/ml rabbit anti-HN001 cell envelope IgG, 30µg/ml rabbit non-immunised IgG, 300µg/ml rabbit non-immunised IgG in 20mg BSA/ml PBS or BSA alone as a control. HN001 cells were used at  $5 \times 10^8$  cfu instead of the usual  $1 \times 10^8$  cfu, in an attempt to reduce error. Rabbit non-immunised IgG was IgG purified from serum of rabbits not immunised with HN001 cell envelope. Both anti-HN001 cell envelope IgG and rabbit non-immunised IgG increased adhesion of HN001 to Caco-2 cells (Figure 27). While unexpected, this data does not rule out the possibility that HN001 surface components are involved in adhesion of HN001 to Caco-2 cells. Any loss of binding caused by antibodies binding and blocking an adhesin on the HN001 cell surface may have been masked by other adhesive interactions between HN001, IgG and Caco-2 cells. One explanation for the stimulation of adhesion by both non-immunised IgG and anti-HN001 cell envelope IgG is that HN001 is able to bind IgG specifically or non-specifically. It is also likely that non-immunised IgG binds epitopes generally common to Gram-positive organisms and hence binds HN001. The bound IgG may then aggregate HN001 cells and perhaps promote binding of HN001 cells to the Caco-2 cells thereby increasing the apparent binding of HN001 to Caco-2 cells and masking any loss in binding to Caco-2 cells (Section 4.2).



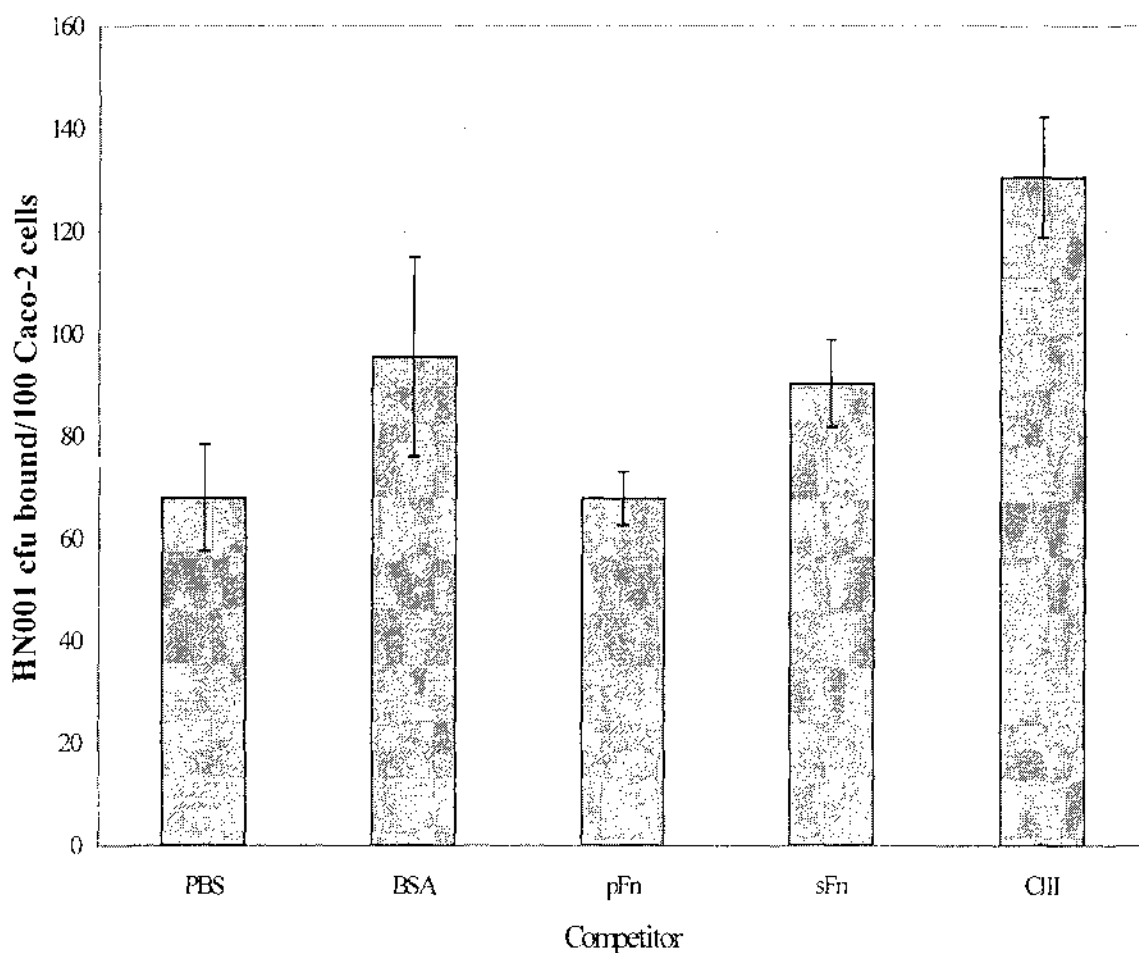


**Figure 27 IgG appears to increase HN001 adhesion to Caco-2 cells**

The role of HN001 cell surface proteins in adhesion to Caco-2 cells was investigated by pretreating HN001 with rabbit anti-HN001 cell surface IgG or rabbit pre-immune IgG at either 30µg/ml or 300µg/ml.  $1 \times 10^8$  cfu HN001 was incubated with Caco-2 monolayers. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (each assayed in two separate experiments). Error bars indicate  $\pm$  2 standard error of the mean. PBS is the positive control for the binding assay. BSA is the control for the samples containing antibodies. Non imm 30 is HN001 cells preincubated with 30µg/ml rabbit non-immune IgG. A-HN001 30 is HN001 preincubated with 30µg/ml rabbit anti-HN001 cell envelope IgG. Non imm 300 and A-HN001 300 are as above but HN001 cells were preincubated with 300µg/ml IgG.

### 3.3.6 Competition of HN001 adhesion to Caco-2 by fibronectin

HN001 cells were preincubated with a saturating amount of either plasma fibronectin, super fibronectin, CIII fragment or BSA (negative control) to assess the role of fibronectin in HN001 adhesion to Caco-2 cells. The binding assay was performed as outlined in Section 2.17.3 except HN001 cells were incubated in a 20µg/ml protein solution in 20mg BSA/ml PBS for 1hr at 37°C before binding to the Caco-2 cells. The amount of fibronectin needed to saturate  $1 \times 10^8$  cfu HN001 was determined in liquid-phase assays described in Section 3.1.2. It was assumed that the same amount of super fibronectin and CIII fragment would saturate  $1 \times 10^8$  cfu HN001. Preincubation with the two forms of fibronectin and CIII did not inhibit HN001 adhesion to Caco-2 cells any more than the no-competition control (BSA) (Figure 28). The apparent positive effect of CIII on HN001 adhesion to Caco-2 was not found to be reproducible in other experiments. The result of this experiment does not support fibronectin having a significant role in HN001 adhesion to the surface of Caco-2 cells. However, this result can not be extrapolated to HN001 adhesion to intestinal surfaces, as more fibronectin may be exposed to HN001 cells *in vivo* than on Caco-2 cell monolayers.



**Figure 28 No support for role of fibronectin in HN001 adhesion to Caco-2 cells**

The role of fibronectin binding in adhesion to Caco-2 cells was investigated by pretreating HN001 with plasma and super fibronectin and CIII.  $1 \times 10^8$  cfu HN001 was incubated with Caco-2 monolayers. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (each assayed in two separate experiments except sFn, which was performed once). Error bars indicate  $\pm 2$  standard error of the mean. PBS is the positive control for the binding assay. BSA, pFn (plasma fibronectin), sFn (super fibronectin), and CIII are HN001 adhering to Caco-2 cells with protein competition (named).

### **3.3.7 Adhesion of surface-treated HN001 cells to Caco-2 cells**

HN001 cells were treated with three different proteases (proteinase K, pronase and trypsin) to assess the role of HN001 cell surface proteins in adhesion to Caco-2 cells as outlined in Section 2.18.1. The binding assay was performed as outlined in Section 2.17.3. However, no firm conclusions could be drawn due to the large variation in results and the large error in two separate experiments (data not shown).

HN001 cells were treated with sodium periodate to assess the role of carbohydrate on HN001 cell surface in fibronectin binding as outlined in Section 2.18.2. The binding assay was performed as outlined in Section 2.17.3. Sodium iodate was used as a control for the oxidation treatment. Again, no conclusions could be drawn due to the large variation in results and the large error in two separate experiments (data not shown). The failure of these experiments illustrates the short comings of the Caco-2 adhesion assays, which is discussed further in Section 4.2.

### 3.4 Characterisation of *fbl*

Since fibronectin-binding was identified as a possible mechanism for adherence of HN001 to intestinal tissues, HN001 genome DNA sequence was examined for genes encoding putative fibronectin-binding proteins (Section 3.4.1). The most likely candidate gene was *fbl* (fibronectin-binding like). The sequence of *fbl* was verified and extended and the putative gene product was characterised *in silico* (Sections 3.4.2 – 3.4.3). The function of *fbl* was investigated by both biochemical and genetic approaches. The *fbl* gene product (Fbl) was expressed, purified and used in competitive binding experiments of HN001 binding to fibronectin (Section 3.4.4). An insertional mutant of the *fbl* gene was created and its phenotype was investigated in binding assays (Section 3.4.5).

#### 3.4.1 Identification of Fbl as a putative fibronectin-binding protein

A database of genes previously constructed by BLASTX of draft HN001 genome sequence was searched using key terms (before this study). A search for fibronectin-binding proteins revealed Fbl as the most likely candidate.

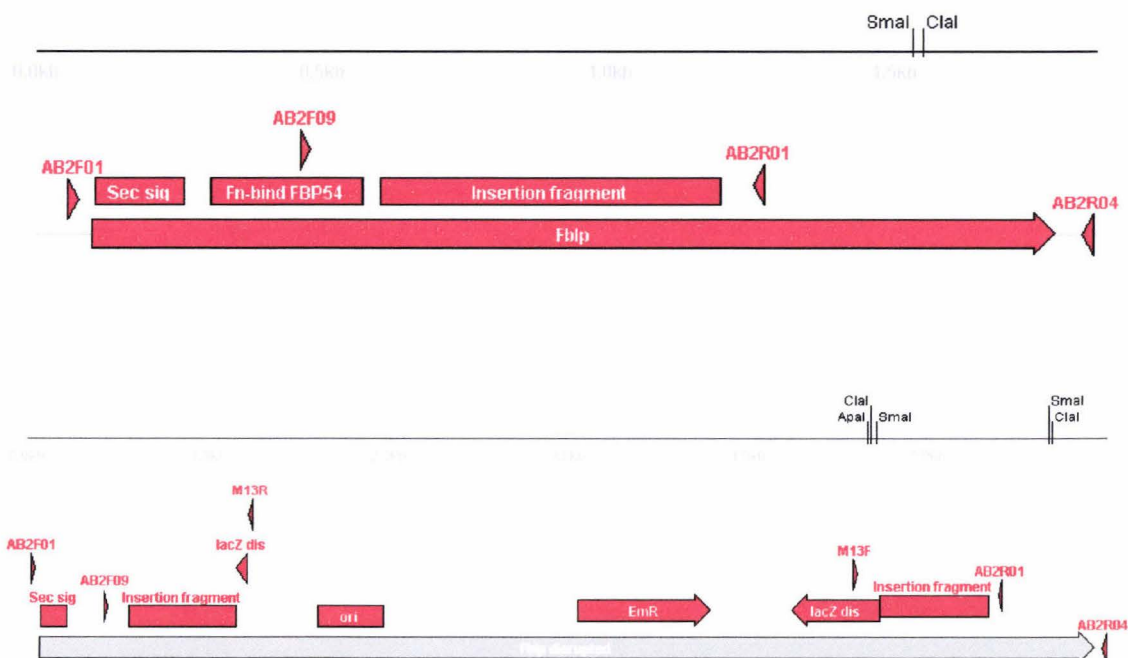
#### 3.4.2 Sequencing of *fbl*

The sequence of *fbl* contained on HN001 genome contigs was confirmed by sequencing both strands of the *fbl* gene. PCR products were sequenced (Section 2.5.4) to give 2-fold coverage of *fbl*. The *fbl* sequence encoding the N-terminal region of Fbl was extended by approximately 100 base pairs using genomic sequencing as outlined in Section 2.4.8. The verified sequence of *fbl* and a sequence assembly diagram is included in Appendix 3. The promoter region of *fbl* was not identified due to difficulties with genomic sequencing in this region. A map of the *fbl* genomic region is shown in Figure 29.

#### 3.4.3 *In silico* characterisation of *fbl*

##### 3.4.3.1 Use of BLASTP and sequence alignments

The computer program Omega was used to predict the amino acid sequence of the open reading frame within the *fbl* gene sequence as outlined in Section 2.6.1. A single open reading frame with a predicted 557 amino acid sequence was identified (Figure 30).



**Figure 29 *fbl* genomic region**

Top diagram is the WT *fbl* gene locus showing the position of restriction endonuclease sites used in the Southern Blot predicted by analysis of the genome sequence data. The bottom diagram is the disrupted *fbl* gene locus (*fbl*::pBeryFbl1) showing the same restriction endonuclease sites. The positions of the PCR primers used to screen HN001 colonies for integration of pBeryFbl1 into the *fbl* gene locus are shown on the bottom diagram (AB2F09, M13R, M13F and AB2R01). Sec sig indicates the position of the predicted secretion signal. Insertion fragment indicates the position of the fragment cloned into pBery-1 to create pBeryFbl1. Fn-bind FBP54 indicates the region of *fbl* similar to the identified fibronectin-binding region of Fbp54 from *S. pyogenes* (Courtney *et al.*, 1994). LacZ dis, ori and EmR are features present in the pBery-1 DNA in the disrupted *fbl* gene locus.

```

MAQELNTTLS GGRVAKIQQP YENEIIITIR AGRKNHPLLL SANPOYARVQ 50
ITHIEFTNPD VPATFTMTLR KYFNAATLTE IHQVQNDRLV HFEFSTRDEL 100
GDELGLRLII EMMGRHSNIF LVSKRTGKII DLRHVSADQ NRYRPLMPGA 150
PYVEPPKQDK VDPFHDSERI YHELERQVTP SLSRAALLQQ HYQGLAKDSA 200
AELALRLNQG DAGWDSFEAA LATPEPTITT QGKKAVETAI PYQSLTGEQQ 250
HFPTLSAMLD AYYAQKAEHD RVLQQGGLNI HVIKNVIDKD RKKQRKLKRT 300
LEETEKADDY RIRGEILTTY LSQVKRGMTS IELPNFYADN EPIKITLSNQ 350
LTPSRNAQKY FAKYTKLRNA VAHVHQMQE NQEELDYLEG IMAQIDVASE 400
KDLVDIRLEL QQQGYLRKQK SGKKGNKRQK VSKPDQFYAS DGTKIWVGKN 450
NLQNDQLTLH TAKKTDIWLH VKDIPGSHVI IDSSDPSEKT LLEAAKLAAY 500
FSKARDSANV PVDWIEVKKI RKPNGAKPGF VIYEGQKTVS VTPDADLVAK 550
LRNPPTK 600

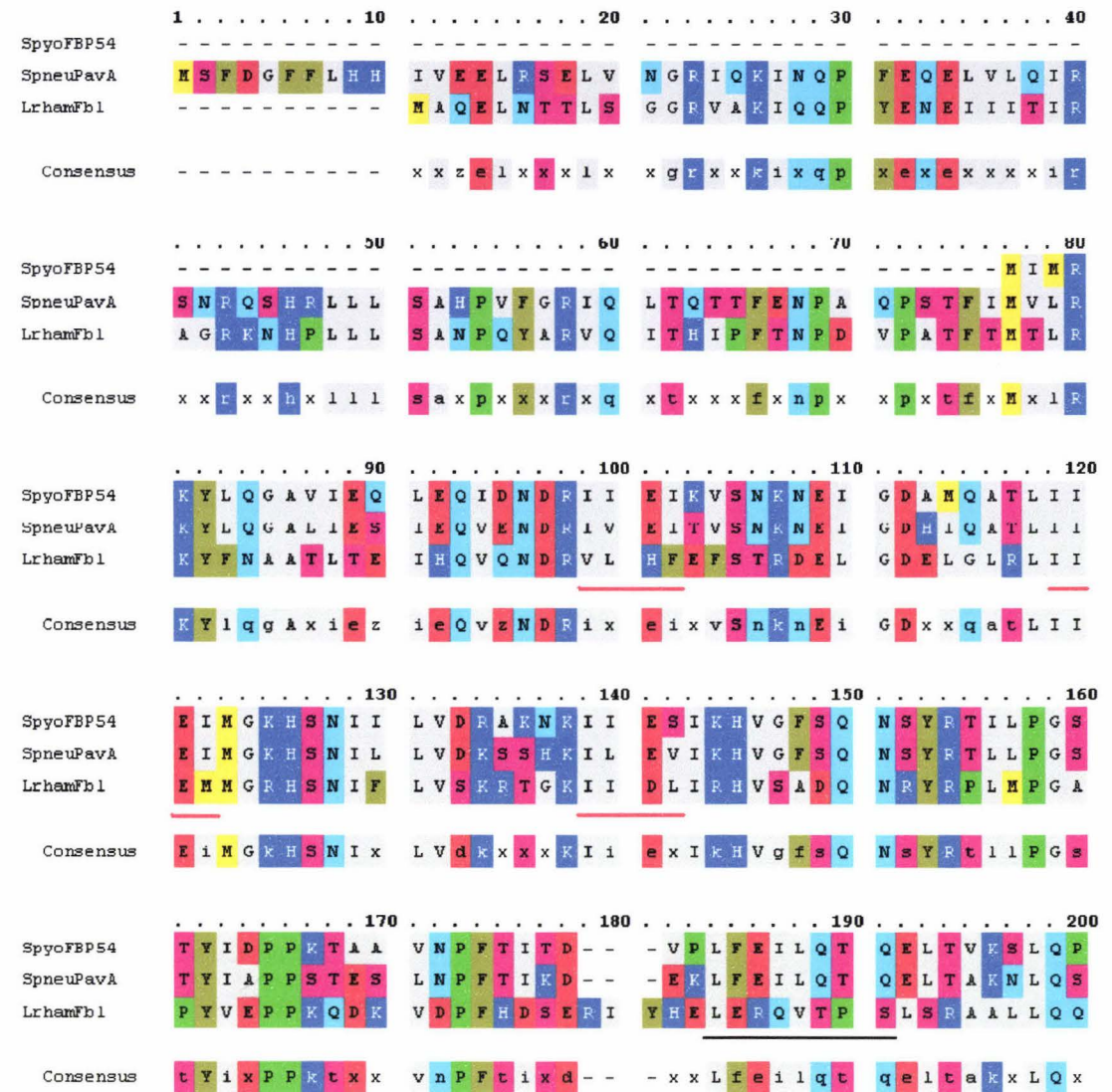
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**Figure 30 Predicted amino acid sequence of Fbl**

The BLASTP algorithm (Section 2.6.1) was used to assign a putative function to Fbl by correlating the function of gene products with similar amino acid sequence to the function of Fbl. Hits with BLASTP were considered to be significant if the Expect (E) value was lower than  $1e-10$ . Two of the significant hits in the BLASTP search were fibronectin-binding proteins with experimental evidence to support their annotation. The two proteins are PavA from *Streptococcus pneumoniae* (Holmes *et al.*, 2001) and Fbp54 from *Streptococcus pyogenes* (Courtney *et al.*, 1994). The other top scoring hits were all proteins that had been assigned a putative fibronectin-binding function through similarity to known fibronectin-binding proteins. See Appendix 3 for the BLASTP results.

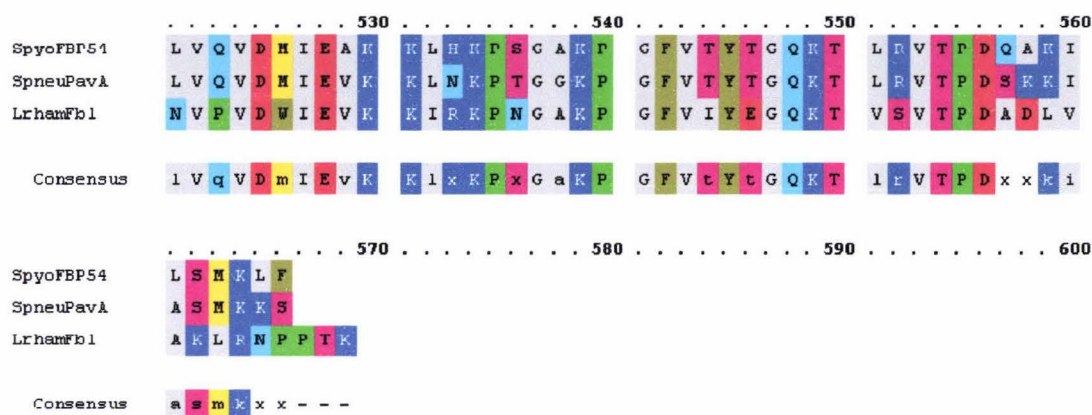
Multiple sequence alignments (Section 2.6.1) were used to determine if an entire fibronectin-binding protein was likely to be encoded in the *fbl* sequence assembled. The predicted amino acid sequence of the open reading frame of *fbl* was compared to the protein sequences of several similar proteins. The predicted N-terminus of the Fbl protein sequence started 10 amino acid residues after the N-terminus of PavA and three other proteins with high similarity. The N-terminus of Fbp54 started 70 amino acids residues after the predicted N-terminus of Fbl. It was possible that the whole gene encoding Fbp54 was not cloned as no signal peptide was found at the N-terminus

(Courtney *et al.*, 1994). All of the sequences aligned ended at a similar position. The results of the multiple sequence alignment indicated that it is probable that the whole *fbl* gene has been identified and that the 5' sequence may encode a signal peptide. See Figure 31 for a diagram of the multiple sequence alignment. A Dot Plot of the protein sequence of Fbl (Appendix 3) was used to determine if any repeat regions were present in the sequence. Repeat regions are of interest as many fibronectin-binding proteins have repeat regions (Joh *et al.*, 1998). A reasonably conserved repeat sequence of 8 amino acids was identified starting at residue 174 and residue 347 (shown in Figure 31). A Dot Plot of the DNA sequence of *fbl* was used to determine if the sequence contained a transcriptional terminator (a stem-loop structure). No evidence of a transcriptional terminator was identified at the C-terminus of the gene or beyond (data not shown). Dot Plots were performed as outlined in Section 2.6.1.





	210	220	230	240
SpyoFBP54	A F S P L R G E T P	K R I G E L L T T D	K L K R F R E F F A	R P T Q A N L T T A
SpneuPavA	L F Q G L G R D T A	N E L E R I L V S E	K L S A F R N F F N	Q E T K P C L T E T
LrhamFb1	H Y Q G L A K D S A	A E L A L R L N Q G	D A G W D S F F A A	L A T P E P T I T T
Consensus	x f q g L x x d t a	x e l x x x L x x x	k l x x f r x f f a	x x T x x x l t t t
	250	260	270	280
SpyoFBP54	S - - - - F A P V	L F S D S H - - - A	T F E T L S E M L D	H F Y Q D K A E R D
SpneuPavA	S - - - - F S P V	P F A N Q A G - - E	P F A N L S D L L D	T Y Y K N K A E R D
LrhamFb1	Q G K K A V r I A I	P Y Q S L I G E Q Q	H R F I L S A M L D	A Y Y A Q K A E R D
Consensus	s - - - - F x p v	p f x b x x g - - z	x F x t L S x m L D	x y Y x b K A E x D
	290	300	310	320
SpyoFBP54	R I N Q Q A S D L I	H R V Q T E L D K N	R N K L S K Q E A E	L L A T E N A E L F
SpneuPavA	R V K Q Q A S E L I	R E V E N E L Q K N	R H K L K K Q E R E	L L A T D N A E E F
LrhamFb1	R V L Q Q G G N L I	H V I K N V I D K D	R K K Q R K L K R T	L E E T E K A D D Y
Consensus	R v x Q Q a s b L I	h r v z n e l d K n	R x K l x K q e r e	L l a T e n A e x f
	330	340	350	360
SpyoFBP54	R Q K G E L L T T Y	L S L V P N N Q D S	V I L D N Y Y T G -	E K I E I A L D K A
SpneuPavA	R Q K G E L L T T F	L H Q V P N D Q D Q	V I L D N Y Y T N -	Q P I M I A L D K A
LrhamFb1	R I R G E I L T T Y	L S Q V K R G M T S	I E L P N F Y A D N	E P I K I T L S N Q
Consensus	R q k G E l l T T y	L s q v p n b q d s	v i l d n y y t b -	e p i x i a l d k a
	370	380	390	400
SpyoFBP54	L T P N Q N T Q R Y	F K R Y Q K L K E A	V K H L S G L I A D	T R Q S I T Y F E S
SpneuPavA	L T P N Q N A Q R Y	F K R Y Q K L K E A	V K Y L T D L I E E	T K A T I L Y L E S
LrhamFb1	L T P S R N A Q K Y	F A R Y T K L R N A	V A H V H Q Q M Q E	N Q E E L D Y L E G
Consensus	L T P n q N a Q r Y	F k k Y q K l k e A	V k h l x x l i z e	t k z x i x Y l e s
	410	420	430	440
SpyoFBP54	V D Y N L S Q A S I	D D I E D I R E E L	Y Q A G F L K S - -	- R Q R D K R H K
SpneuPavA	V E T V L N Q A G L	E E I A E I R E E L	I Q T G F I E R - -	- R Q R E K I Q K
LrhamFb1	I N A Q I D V A S P	K D L V D I R L E L	Q Q Q G Y L R R Q K	S G K K G N K R Q K
Consensus	v x x x l b q A s x	x d i x d I R e E L	x Q x G f l r x - -	- r q r b K r q K
	450	460	470	480
SpyoFBP54	R K K P E Q Y L A S	D G K T I L H V G R	N N L Q N E E L T F	K M A K K G E L U F
SpneuPavA	R K K L E Q Y L A S	D G K T I I Y V G R	N N L Q N E E L T F	K M A R K E E L U F
LrhamFb1	V S K P D Q F Y A S	D G - T K I V V G K	N N L Q N D Q L T L	H T A K K T D I U L
Consensus	r k K p e Q y l a s	D G k T i i x V G r	N N L Q N e e L T f	k m A k K x e l u f
	490	500	510	520
SpyoFBP54	H A K D I P G S H V	I I K D N L D P S D	E V K T D A A E L A	A Y Y S K A R L S N
SpneuPavA	H A K D I P G S H V	V I S G N L D P S D	A V K T D A A E L A	A Y F S Q G R L S N
LrhamFb1	H V R D I P G S H V	I I D S S D - P S E	R T L L E A A K L A	A Y F S K A R D S A
Consensus	H a K D I P G S H v	i I x x n i d P S d	x v k t d A A e L A	A Y f s k a R l s n



**Figure 31 Multiple sequence alignment of Fbl against PavA and Fbp54**

SpyoFBP54 is the Fbp54 protein from *Streptococcus pyogenes* (Courtney *et al.*, 1994). SpneuPavA is the PavA protein from *Streptococcus pneumoniae* (Holmes *et al.*, 2001). LrhamFbl is the Fbl protein from HN001. The IIXI motif is marked with a red line. The GELLT motif is marked with a blue line. The repeat sequence in *fbl* is marked in black.

### 3.4.3.2 Use of motif search engines

Both Prosite 15.0 and Pfam 7.6 were used to search for motifs in the Fbl protein sequence as outlined in Section 2.6.2. A common Gram-positive cell wall anchoring motif, LPXTG was not identified in the sequence of Fbl. However, it is possible that Fbl is still located on the surface of HN001 cells, as some Gram-positive surface-located proteins have no cell wall anchoring motifs, including Fbp54 and PavA (Courtney *et al.*, 1994; Holmes *et al.*, 2001). No significant motifs were found in the Fbl protein sequence by using Prosite and Pfam (data not shown).

A putative signal peptide from residue 1 to residue 51 of the Fbl protein sequence was identified using Signal P2.0 (Appendix 3) as described in Section 2.6.2. The signal peptide motif searched for was a positively charged N-terminus, a central hydrophobic core and a helix-breaking region. Signal peptides from Gram-positive organisms have been reported to be typically longer than signal peptides from Gram-negative organisms, with several examples being 50 amino acids long (von Heijne *et al.*, 1989).

Visual assessment of the predicted protein sequence of Fbl was used to identify a motif described for Fbp54 from *Streptococcus pyogenes* (Courtney *et al.*, 1994). A repeated motif, IIXI, was speculated to have a role in fibronectin-binding because it is present in



the fibronectin-binding region of Fbp54 and is conserved in several other fibronectin-binding proteins. This motif was also conserved in the Fbl protein sequence. Another motif described for Fbp54, GELLT, was not conserved in Fbl or PavA. The positions of the IIXI motif in Fbl and the GELLT motif in Fbp54 are illustrated in the multiple sequence alignment in Figure 31.

### 3.4.4 Biochemical characterisation of Fbl function

Fbl was expressed and purified in order to use the protein in liquid-phase binding assays to compete with HN001 binding to fibronectin.

#### 3.4.4.1 Expression of Fbl

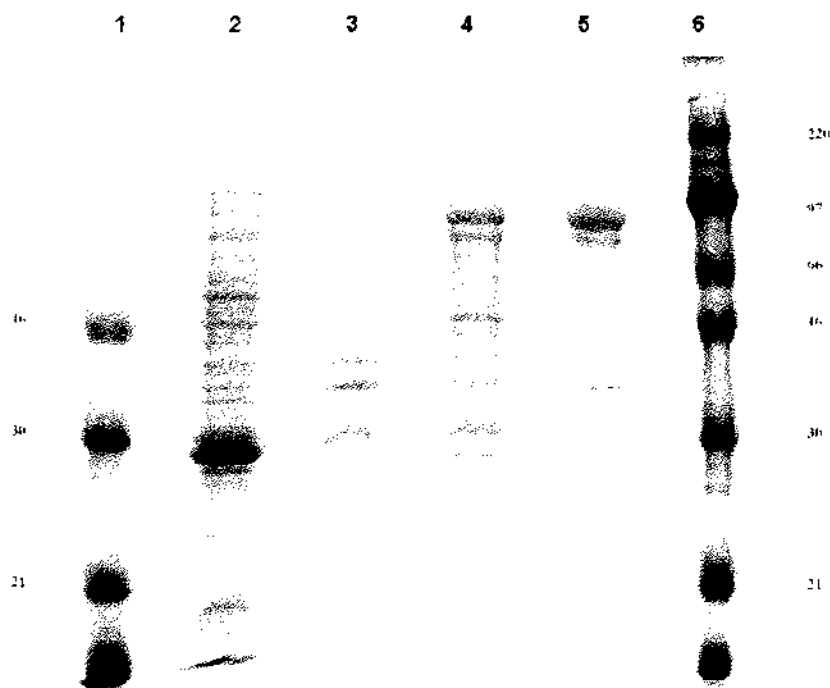
In order to express Fbl protein, the full-length gene as well as various truncations of *fbl* were cloned into pGex-6p-3 or pBAD/HisB expression vectors as outlined in Section 2.7. Descriptions of all the plasmids constructed are included in Table 2 and the maps of pFbl2 and pFbl4 (both express soluble proteins as described below) are shown in Figure 32.



**Figure 32 Plasmid maps of pFbl2 and pFbl4**

GST tag is the coding region for GST fused to *fbl*. *fbl* w/out sec sig is the full-length *fbl* gene without the putative signal peptide. AB2F06, AB2R05, AB2F10 and AB2R08 are the PCR primers used to amplify the *fbl* fragment cloned in the pGex-6p-3 vector. Truncated *fbl* is the N-terminal third of *fbl* including the putative signal peptide.

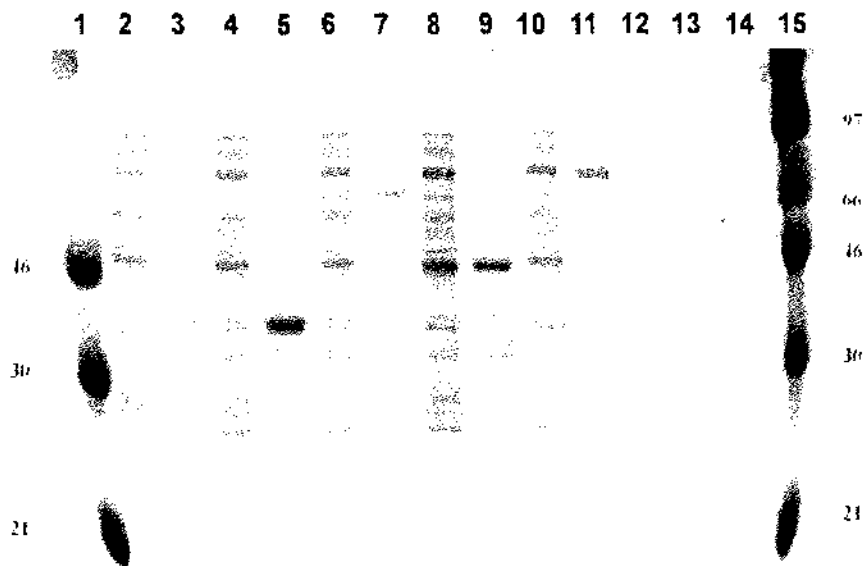
Proteins were expressed as outlined in Section 2.8. A reasonable amount of soluble Fbl2-GST (entire *fbl* gene without the putative signal peptide fused to GST) was expressed when induction was performed with 0.1mM IPTG for 7 hr at 20°C (Figure 33). The expected size of Fbl2-GST (92KDa) and GST (29KDa) was observed on the gel. Purification of Fbl2-GST is outlined in Section 3.4.4.2.



**Figure 33 Expression of soluble Fbl2-GST**

Induction was performed with 0.1mM IPTG for 7 hr at 20°C. Lanes are: 1: Rainbow low molecular weight marker (sizes in kDa indicated), 2: GST control – soluble, 3: GST control – insoluble, 4: Fbl2-GST – soluble, 5: Fbl2-GST – insoluble and 6: Rainbow high molecular weight marker (sizes in kDa indicated).

A low level of soluble Fbl4-GST (N-terminal third of *fbl* gene fused to GST) was expressed when induction was performed with 1mM IPTG for 6 hr at 20°C (Figure 34). The expected size of Fbl4-GST (50KDa) was observed on the gel but a strong band corresponding to GST (29KDa) was not seen. Fbl4-GST was then purified as outlined in Section 3.4.4.2.



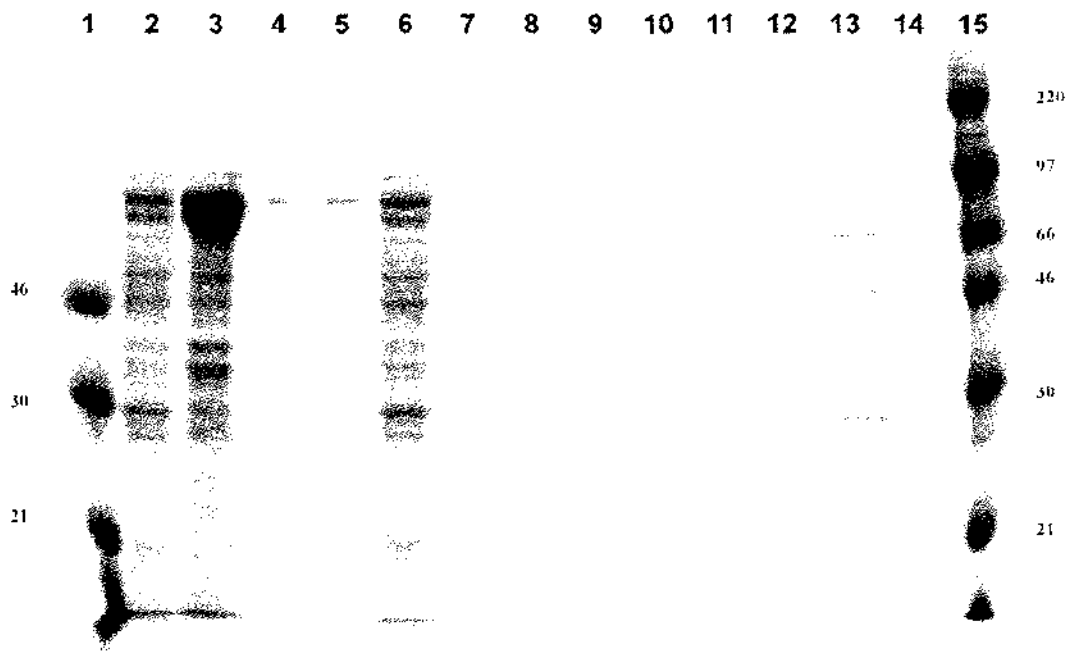
**Figure 34 Expression of soluble Fbl4-GST**

Induction was performed with 1mM IPTG for 6 hr at 20°C. Lanes are: 1: Rainbow low molecular weight marker (sizes in kDa indicated), 2: GST control – soluble, 3: GST control – insoluble, 8: Fbl4-GST – soluble, 9: Fbl4-GST – insoluble, and 15: Rainbow high molecular weight marker (sizes in kDa indicated).

Several other GST-Fbl fusion proteins were expressed but were found to be insoluble (data not shown). The pBAD/HisB expression system yielded very low quantities of Fbl fusion proteins. An attempt to optimise the expression conditions by varying the amount of inducer added did not increase the protein expression (data not shown). The levels of expression obtained were considered too low to attempt purification of the His-tagged Fbl fusion proteins.

**3.4.4.2 Purification of Fbl fusion proteins**

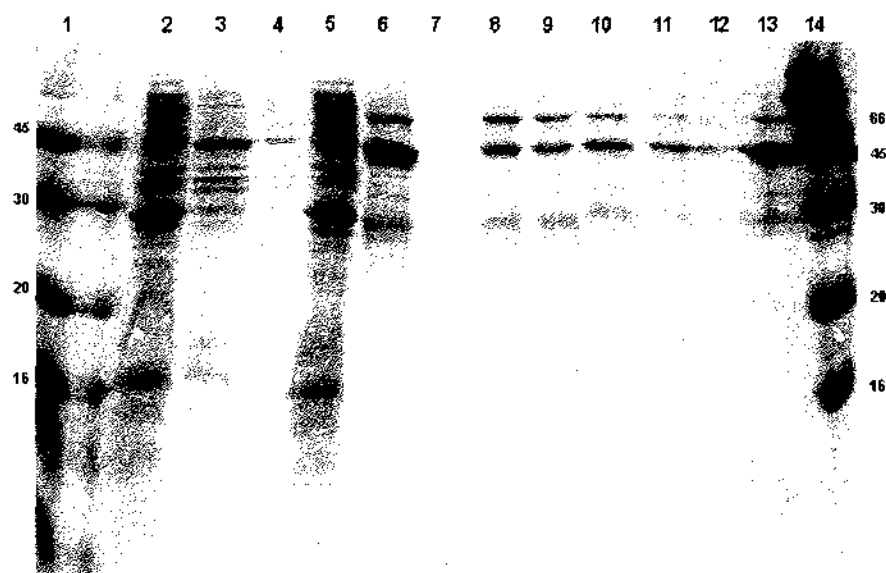
Attempts were made to purify Fbl2 (expressed using the pGex-6p-3 expression system) by cleaving the GST tag off Fbl2 bound to glutathione sepharose with PreScission protease as outlined in Section 2.9. Cleavage of Fbl2 from the Fbl2-GST fusion protein was not successful (Figure 35). This may have been due to tertiary folding of Fbl2 blocking the cleavage site of the protease.



**Figure 35 Purification of Fbl2 by cleavage**

SDS-PAGE gel of purification of Fbl2 by cleavage. Lanes are: 1: Rainbow low molecular weight marker (sizes in kDa indicated), 2: lysate, 3: pellet, 5: matrix with protein bound, 6: non-binding proteins, 8: cleavage reaction, 9 and 10: washes, 13: the matrix after the cleavage and 15: Rainbow high molecular weight marker (sizes in kDa indicated).

Another GST fusion protein, Fbl4 (expressed using the pGex-6p-3 expression system), was purified by eluting Fbl4-GST off the glutathione sepharose beads as outlined in Section 2.9. This resulted in Fbl4-GST fusion protein (50KDa) at a concentration of 250ng/ul (Figure 36).

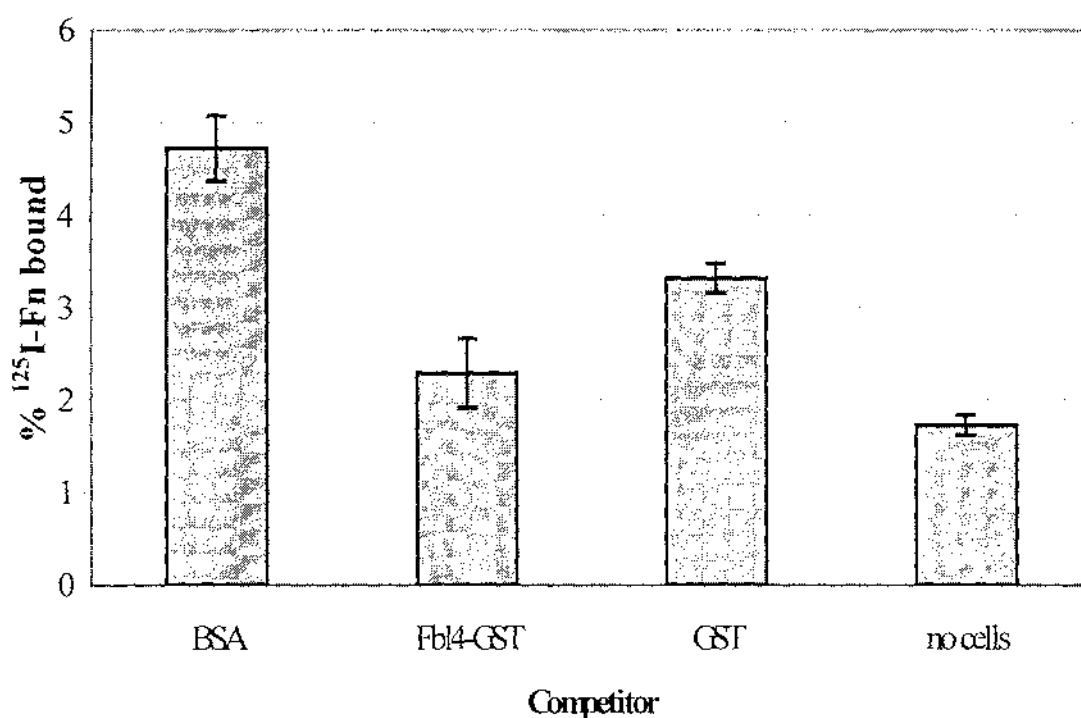


**Figure 36 Purification of Fbl4-GST by elution**

SDS-PAGE gel of purification of Fbl4-GST by elution. Lanes are: **1**: Rainbow low molecular weight marker (sizes in kDa indicated), **2**: lysate, **3**: pellet, **5**: matrix with protein bound, **6**: non-binding proteins, **8-11**: elution (4 lanes), **13**: the matrix after elution and **14**: Rainbow high molecular weight marker (sizes in kDa indicated).

#### 3.4.4.2 Competition of HN001 binding to $^{125}\text{I}$ -fibronectin with Fbl4-GST

The involvement of Fbl4 in fibronectin binding was investigated by determining if a 1000-fold excess of Fbl4-GST could disrupt  $^{125}\text{I}$ -Fn binding to HN001. The binding assay was performed according to the method outlined in Section 2.15.2 except 30  $\mu\text{g}$  of Fbl4-GST was added along with the  $^{125}\text{I}$ -Fn in the binding reaction. An equal amount of GST was added as a control for the GST present in the Fbl4-GST fusion protein. An equal amount of BSA was added as a negative (no competition) control. Fbl4-GST approximately halved  $^{125}\text{I}$ -Fn binding to HN001. However, GST also reduced binding to a similar extent (Figure 37). No conclusions could be drawn about the function of Fbl4 from this assay.



**Figure 37 Role of Fbl4 in fibronectin binding unclear**

A 1000-fold excess of unlabelled protein was added with 30 ng  $^{125}\text{I}$ -Fn to  $5 \times 10^8$  cfu HN001. HN001 was harvested by centrifugation and radioactivity associated with cell pellet was measured. Each data point is the mean of 3 samples (only performed once). Error bars indicate  $\pm 2 \times$  standard error of the mean. The no cells sample was included as the background level of labelled protein binding to the tube.

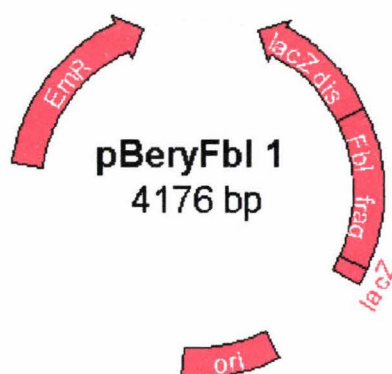


### 3.4.5 Genetic characterisation of Fbl function

During the biochemical characterisation of Fbl a method suitable for insertional disruption of HN001 genes was established. Given the difficulty expressing soluble Fbl and the inconclusive nature of the GST tagged protein function it was decided to attempt to construct an insertional mutant of *fbl*. In order to create an insertional mutant that would inactivate the *fbl* gene, an internal fragment from the gene was cloned into a suicide vector (Section 3.4.5.1). Electro-competent HN001 cells were transformed with the integration construct as outlined in Section 2.10.2 and HN001 transformants were screened for disruption of the *fbl* gene by HN001 colony PCR (Section 3.4.5.2). The disruption of the *fbl* gene was confirmed by Southern Blot (Section 3.4.5.3) and the phenotype of the insertional mutant was screened by binding assays (Section 3.4.5.4).

#### 3.4.5.1 Creation of integration construct

The vector used to create the integration construct (pBery-1) is described in Section 2.10.1. A 600bp fragment in the middle of *fbl* was cloned into pBery-1 as described in Section 2.7 (see Figure 29 for the position of *fbl* fragment cloned into pBery-1). A diagram of the plasmid created is included in Figure 38 and a diagram of the expected result of integration into the *fbl* gene locus is included in Figure 29.



**Figure 38 Plasmid map of pBeryFbl1**

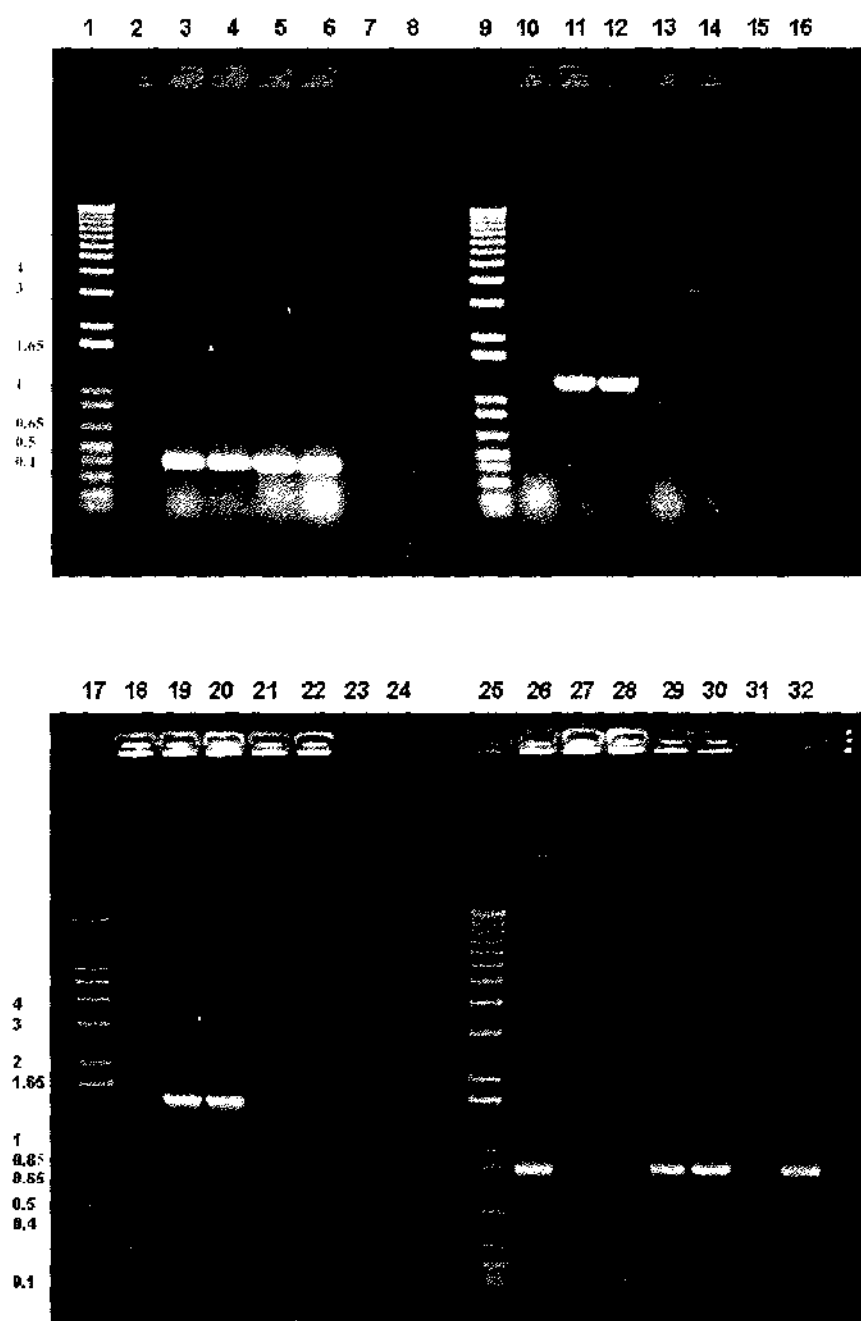
*EmR* is the erythromycin resistance gene of pBery-1. *ori* is the *E. coli* origin of replication. *Fbl frag* is the fragment of *fbl* cloned into pBery-1, which causes disruption of the *lacZ* gene (*lacZ* and *lacZ dis*).

#### 3.4.5.2 HN001 colony PCR

Transformants were screened for the presence of pBeryFbl1 in the *fbl* gene locus by PCR as outlined in Section 2.10.3. Colonies were screened with the primers ErmF01 and ErmR01 to indicate the presence of the erythromycin resistance gene (located within the integration constructs) in HN001 transformants. A primer specific to pBery-1 (M13F or M13R) and a primer corresponding to the beginning of *fbl* or at the end of *fbl* (not in cloned region) were used to amplify a diagnostic fragment that indicated that pBeryFbl1 had inserted into the *fbl* locus. Also, colonies were screened with a primer at the beginning of *fbl* and a primer at the end of *fbl*. If pBeryFbl1 had integrated into the correct locus then a much larger PCR product than the one amplified in the wild-type control would be expected. Figure 29 shows the location of the PCR primers used. Figure 39 shows PCR screening of five colonies, indicating that two had pBeryFbl1 integrated into the *fbl* gene locus.

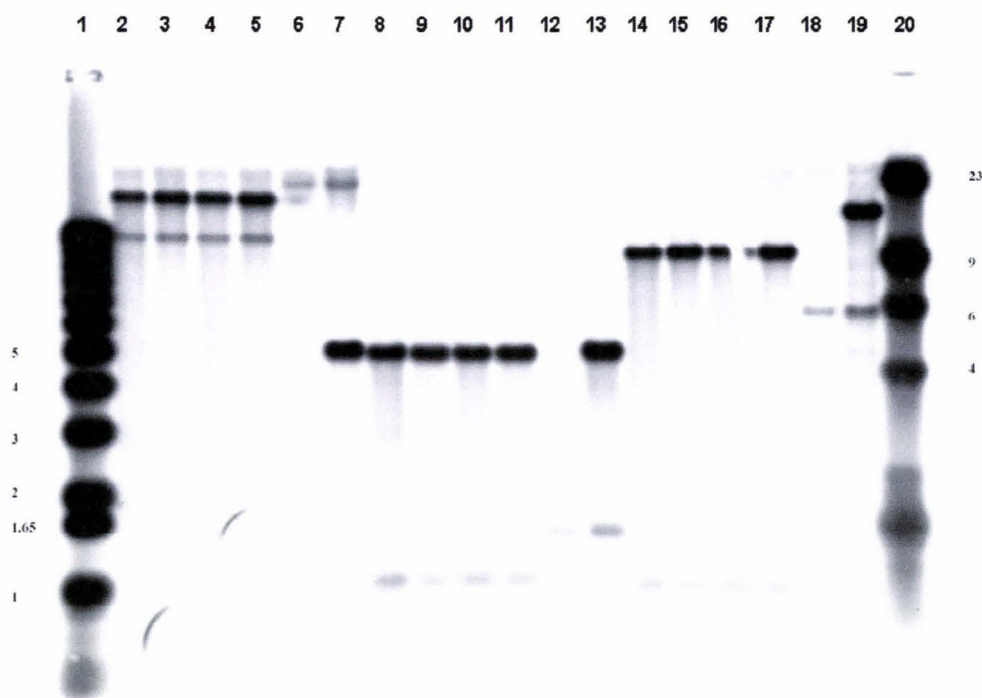
#### 3.4.5.3 Southern Blot

A Southern Blot was performed as outlined in Section 2.11 to confirm the integration of pBeryFbl1 into the *fbl* gene locus. Approximately 1µg of genomic DNA from wild-type HN001, a putative integrant in the *fbl* locus, and another integrant in an unrelated gene (control) were digested with three different restriction endonucleases chosen to give a pattern of DNA fragments that were characteristic of integration of pBeryFbl1 in the *fbl* locus. Linearised pBeryFbl1 was used as a probe to detect bands containing *fbl* or pBery-1 DNA. Probes to 1Kb+ and  $\lambda$ /Hind III ladders were used to detect molecular weight markers (Figure 40). The predicted cleavage positions of the restriction endonucleases used are included in Figure 29. Table 5 lists the expected sizes of the restriction endonuclease fragments detected in the Southern Blot and the actual sizes of the fragments detected in the Southern Blot. All three digests confirmed the integration of pBeryFbl1 in the *fbl* locus.



**Figure 39 Clones 2 and 3 have pBeryFbl1 integrated into the *fbl* gene locus - HN001 colony PCR**

Lanes 1-8, 9-16, 17-24 and 25-32 all correspond to 1Kb+ ladder (sizes in Kb indicated), Clones 1-5, PCR negative control and WT HN001 in that order. 1-8 PCR primers were EmF01 + EmR01, 9-16 PCR primers were M13R + AB2F01, 17-24 PCR primers were M13F + AB2R04 and 25-32 PCR primers were AB2F09 + AB2R01.



**Figure 40 Southern Blot of DNA from wild-type HN001, putative integrant (in *fbl* locus) and integrant (in unrelated gene)**

Genomic DNA was digested, separated on an agarose gel and blotted onto nitrocellulose membrane. DNA was then detected with linearised pBeryFbl1, 1Kb+ ladder and  $\lambda$ /Hind III ladder probes. Only a third of the 1Kb+ ladder and  $\lambda$ /Hind III ladder probes were hybridised to the Southern blot to limit the level of intensity of the ladders when detected. Lanes are: **1:** 1Kb+ ladder (sizes in kb indicated); Apal digest – **2:** clone 2 (grown with Em), **3:** clone 2 (grown without Em), **4:** clone 3 (grown with Em), **5:** clone 3 (grown without Em), **6:** WT, **7:** integrant (not in *fbl* locus); ClaI digest – **8:** clone 2 (grown with Em), **9:** clone 2 (grown without Em), **10:** clone 3 (grown with Em), **11:** clone 3 (grown without Em), **12:** WT, **13:** integrant (not in *fbl* locus); SmaI digest – **14:** clone 2 (grown with Em), **15:** clone 2 (grown without Em), **16:** clone 3 (grown with Em), **17:** clone 3 (grown without Em), **18:** WT, **19:** integrant (not in *fbl* locus); and **20:**  $\lambda$ /Hind III ladder (sizes in kb indicated).

**Table 5 Predicted and actual restriction fragments detected in Southern Blot**

<b>Restriction Endonuclease</b>	<b>DNA used</b>	<b>Predicted Bands</b>	<b>Detected Bands</b>
Apal	WT	#	~23Kb
	<i>fbl</i> integrant	?	>23Kb + between 9 and 23Kb
Clal	WT	#	1.7Kb
	<i>fbl</i> integrant	5.8Kb + 1Kb <sup>p</sup>	~5Kb + 1Kb
SmaI	WT	#	~6Kb
	<i>fbl</i> integrant	10Kb + 1Kb <sup>p</sup>	~9Kb + 1Kb

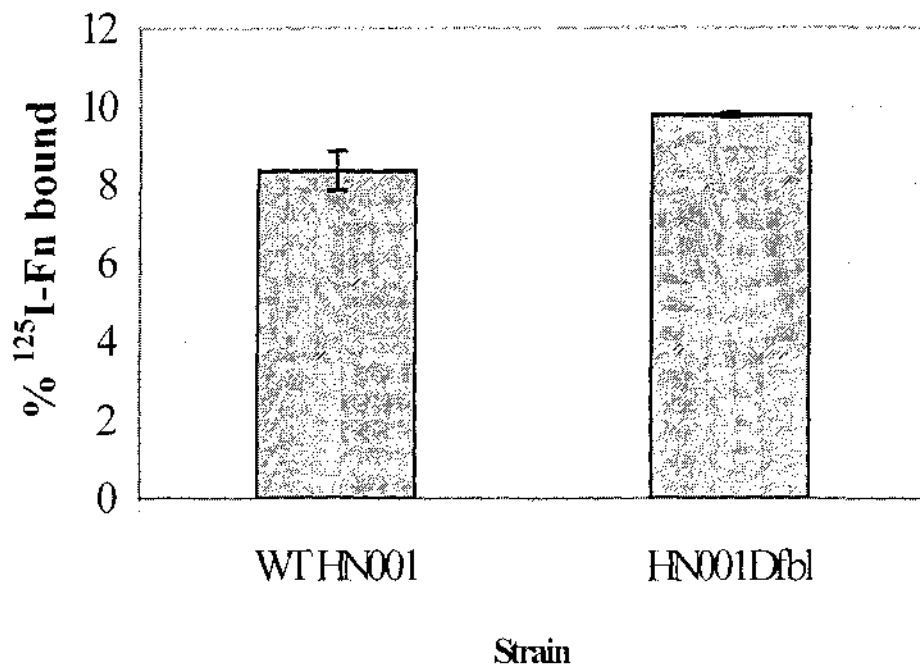
<sup>p</sup> Predicted from data from WT results and the size of pBeryFbII (4.1Kb)

# The product could not be predicted from the sequence data available

? The product could not be predicted from WT results

#### 3.4.5.4 Phenotype of HN001D*fbl*

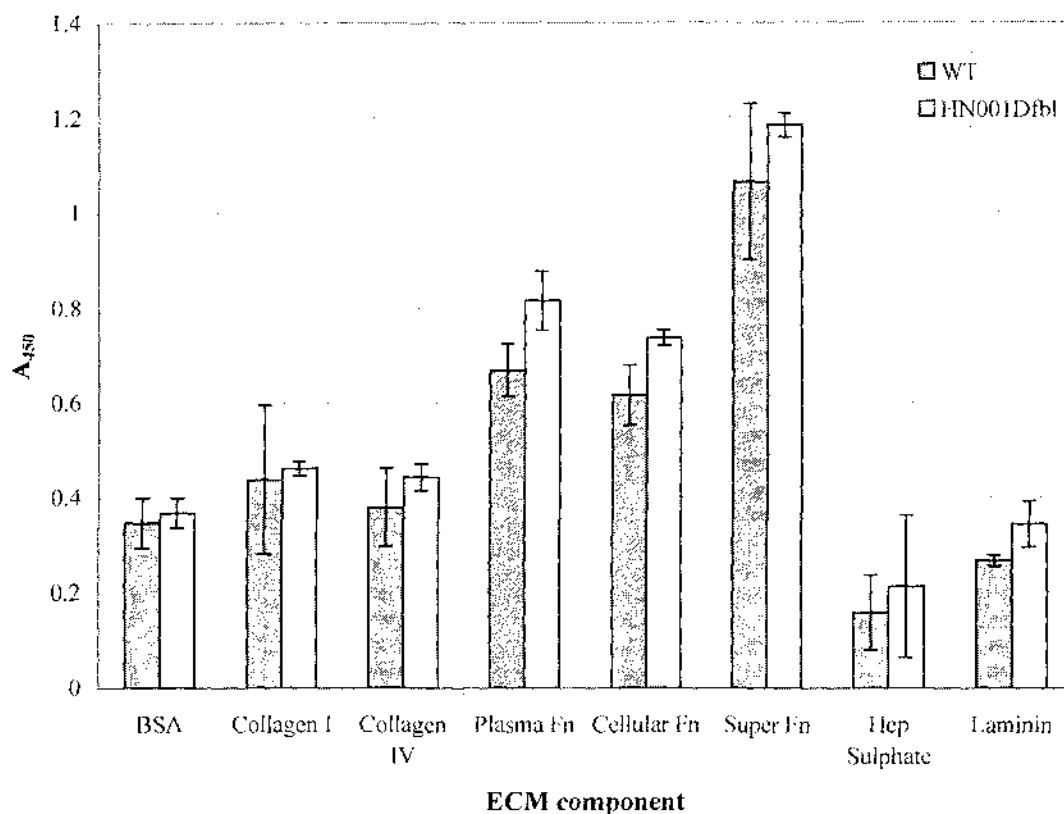
The phenotype of HN001D*fbl* was assayed in all three types of binding assays: liquid-phase, solid-phase and Caco-2 binding assays. HN001D*fbl* was compared to wild-type (WT) HN001 in liquid-phase binding to  $^{125}\text{I}$ -fibronectin as outlined in Section 2.15.2. Surprisingly, HN001D*fbl* was found to bind slightly more  $^{125}\text{I}$ -Fn than WT HN001 (Figure 41).



**Figure 41 Disruption of *fbl* did not decrease HN001 binding to  $^{125}\text{I}$ -Fn**

$5 \times 10^8$  cfu HN001D*fbl* and WT HN001 was incubated with 30ng  $^{125}\text{I}$ -Fn. HN001 was harvested by centrifugation and radioactivity associated with cell pellet was measured. Each data point is the mean of 3 samples (each assayed in two separate experiments). Error bars indicate  $\pm$  2 standard error of the mean.

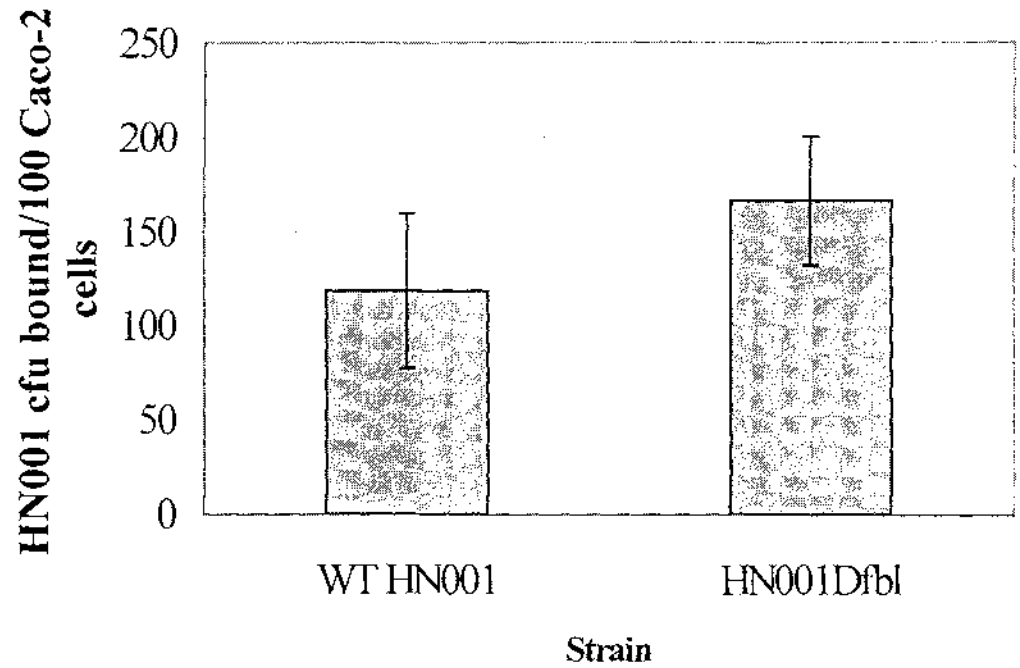
HN001D*fbl* was compared to WT HN001 in solid-phase binding to ECM components as outlined in Sections 2.16.1 – 2.16.3. HN001D*fbl* had either similar or slightly increased binding to all ECM components investigated compared to WT HN001 (Figure 42).



**Figure 42 HN001D*fbl* and WT HN001 have similar binding to intestinal molecules in the solid-phase**

The function of *Fbl* was investigated by incubating  $5 \times 10^7$  cfu HN001D*fbl* and WT HN001 in wells coated with various ECM components. Bound HN001 cells were detected using ELISA detection. Each data point is the mean of 3 samples (only performed once). Error bars indicate  $\pm 2$  standard error of the mean.

HN001D*fbl* was compared to WT HN001 in Caco-2 adhesion as outlined in Section 2.17.3. HN001D*fbl* had slightly increased adhesion to Caco-2 cells compared to WT HN001 (Figure 43). No conclusions could be drawn about Fbl function since a reduction in binding was not seen in all three assays performed. It is possible that HN001D*fbl* still produces functional Fbl since the integration of pBeryFbl1 into *fbl* leaves approximately 2/3 of the gene from the N-terminus intact, although the C-terminal cell wall attachment domain should have been disrupted. There may be several proteins involved in HN001 binding to extracellular matrix components and to Caco-2 cells. The slight increase in the binding of HN001D*fbl* compared to WT HN001 in the three assays is discussed in Section 4.



**Figure 43 Disruption of *fbl* does not decrease adhesion of HN001 to Caco-2 cells**

The function of Fbl was investigated by incubating  $1 \times 10^8$  cfu  $^3\text{H}$ -labelled HN001D*fbl* and WT HN001 with Caco-2 monolayers. Bound HN001 cells were lysed and detected by scintillation counting. Each data point is the mean of 3 samples (only performed once). Error bars indicate  $\pm 2$  standard error of the mean.



## CHAPTER FOUR: DISCUSSION

### 4.1 HN001 binds fibronectin

HN001 was found to bind immobilised fibronectin; a trait shared with several other *Lactobacillus* species (Lorca *et al.*, 2002). HN001 also bound immobilised cellular and super fibronectin. Fibronectin is one of the major components of the extracellular matrix (ECM), which is a structure that surrounds and underlies epithelial cells (Westerlund *et al.*, 1993). Fibronectin is also found on the surface of intestinal epithelial cell lines (Kapczynski *et al.*, 2000) and so is probably present on the surface of intestinal epithelial cells. See Section 1.3 for more information about the structure of the intestinal surface. Cellular fibronectin is a form of fibronectin produced by cells and super fibronectin is an *in vitro* fibril matrix form of fibronectin (Section 1.3.3.1). Therefore, HN001 may be able to bind to fibronectin on intestinal surfaces and in exposed extracellular matrix tissue. This may enable HN001 to colonise healthy or damaged intestinal surfaces, and may be a mechanism by which HN001 could exclude pathogen adherence by competitive exclusion (Styriak *et al.*, 2001) and/or by other probiotic effects (Section 1.2).

HN001 bound approximately 7% of the soluble  $^{125}\text{I}$ -fibronectin added. This is similar to the binding levels seen for a common inhabitant of the intestinal tracts of humans, *Lactobacillus crispatus* JCM 5810, which bound 7.1%  $^{125}\text{I}$ -Fn added. This level of binding was reported as not significant (Toba *et al.*, 1995). When binding of the human pathogen *Helicobacter pylori* to  $^{125}\text{I}$ -Fn was investigated, 11 of the 14 strains tested bound an average of 7%  $^{125}\text{I}$ -Fn added, which was above the 5% threshold considered to be significant by the investigators (Trust *et al.*, 1991). Whatever the case, HN001 only binds modest amounts of soluble fibronectin, and it is likely that the ability of HN001 to bind soluble fibronectin does not contribute significantly to its ability to act as a probiotic. Only a small amount of soluble fibronectin is likely to be available in the intestinal lumen, so soluble fibronectin binding probably is not relevant to bacteria in a healthy intestinal tract. However, when the intestinal surface is damaged, plasma fibronectin is probably released into the intestinal lumen, hence soluble fibronectin binding may become important in the ability of bacteria to attach to damaged surfaces.

Soluble fibronectin binding is relevant for pathogens that can invade the epithelial layer and encounter plasma fibronectin. Non-pathogenic bacteria may bind soluble fibronectin in order to bind to intestinal surfaces, via fibronectin binding to integrins exposed on intestinal surfaces (Section 1.3.3.1). However, this mechanism of binding is probably not significant in comparison to binding to immobilised fibronectin. Also, coating in fibronectin might help the bacteria to be 'ignored' by the host immune system. Bacterial binding of soluble fibronectin may be a mechanism for aggregation of the bacteria. Finally, non-pathogenic bacteria might bind soluble fibronectin in order to 'soak up' excess fibronectin so it is not available for pathogens to bind.

In the case of pathogens, binding to extracellular matrix components may be a mechanism by which the bacteria can establish and maintain long-lasting deep tissue infection (Dubreuil *et al.*, 2002). Another mechanism by which pathogens can survive in the host is to invade non-phagocytic host cells (Joh *et al.*, 1999). Several studies have shown that fibronectin-binding is an important determinant in bacterial invasion of host cells (Joh *et al.*, 1999). Invasion of human colonic cells by *Campylobacter jejuni* is mediated by fibronectin-binding (Monteville *et al.*, 2002). Cellular invasion by *Staphylococcus aureus* involves the binding of the bacterium to fibronectin, which in turn binds integrins on host cells (mouse fibroblast line or human embryonic kidney cell line). This causes internalisation of *S. aureus* (Fowler *et al.*, 2000; Sinha *et al.*, 1999). The binding of soluble ECM components represents a mechanism for evading host immune defence mechanisms. Soluble fibronectin binding by *S. pyogenes* strains can recruit collagen to the bacterial cell surface. This has several effects including causing aggregation of *S. pyogenes* cells, promoting *S. pyogenes* colonisation of collagen fibres, and reducing *S. pyogenes* binding to polymorphonuclear cells. Thus, *S. pyogenes* may be protected from ingestion and killing by host phagocytes by binding soluble extracellular matrix components (Dinkla *et al.*, 2003).

Fibronectin is a large protein made up of several distinct domains (Section 1.3.3.1). Of the regions assayed, HN001 was found to bind to the C-terminal part of the first type III repeat (III<sub>1</sub>-C) in the fibronectin molecule to a similar degree as to the fibronectin molecule. A fragment corresponding to III<sub>1</sub>-C was used in this study because it can induce the formation of super fibronectin (an *in vitro* matrix form of fibronectin) from plasma fibronectin (Morla *et al.*, 1994). See Section 1.3.3.1 for more information about

the fibronectin type III repeat. Fibronectin type III repeats are found in a superfamily of animal proteins, which include extracellular proteins, membrane-spanning cytokine receptors, growth hormone receptors and adhesion molecules (Bork *et al.*, 1992). Therefore, HN001 may be able to bind to the cell-binding domain (integrin-binding) of fibronectin (which contains several type III repeats), and other animal proteins with exposed fibronectin type III repeats. HN001 also bound to the heparin I-binding domain (p30) of fibronectin, but to a lesser degree than to the fibronectin molecule. Binding of the heparin I-binding domain of fibronectin was also demonstrated for three vaginal *Lactobacillus* isolates (Nagy *et al.*, 1992). HN001 did not bind the heparin I/collagen-binding domain (p70) or the collagen-binding domain (p45) of fibronectin, although p70 contains the p30 heparin I-binding domain. This suggests that the rest of the p70 fragment either masks the p30 domain in p70, or the p30 fragment is in a different conformation in the p70 fragment. Unlike HN001, *Lactobacillus crispatus* JCM 5810 did not bind the heparin I-binding domain of fibronectin. JCM 5810 binds to the 120kDa cell-binding domain of fibronectin (not assayed for HN001) (Toba *et al.*, 1995). The cell-binding domain includes the III<sub>1</sub>-C fragment. Whether or not JCM 5810 binds this region of the cell-binding domain was not determined. Similar to HN001, the pathogen *Streptococcus pneumoniae* binds to the heparin I-binding domain of fibronectin and not to the collagen-binding domain. The *S. pneumoniae* strains tested also bind to the C-terminal heparin II-binding domain (not assayed for HN001) (van der Flier *et al.*, 1995). It has been reported that for many Gram-positive organisms, the primary binding region within the fibronectin molecule is the heparin I-binding domain (Joh *et al.*, 1998).

Only super fibronectin and III<sub>1</sub>-C were able to disrupt HN001 adhesion to immobilised fibronectin. When the interaction between HN001 and super fibronectin was further characterised, III<sub>1</sub>-C was found to disrupt HN001 adhesion to immobilised super fibronectin. These data indicated that HN001 binds to immobilised fibronectin in preference to soluble fibronectin and probably binds fibronectin via the III<sub>1</sub>-C repeat. Immobilised fibronectin may have a similar conformation to super fibronectin and may have more exposed III<sub>1</sub>-C domains than soluble fibronectin does. Preferential binding of immobilised fibronectin over soluble fibronectin has been seen for several other bacteria: *Lactobacillus acidophilus* CRL 639 (Lorca *et al.*, 2002), *L. crispatus* JCM 5810 (Toba *et al.*, 1995), and *Streptococcus pneumoniae* (van der Flier *et al.*, 1995).

van der Flier et al. (1995) also investigated whether the fragments that *S. pneumoniae* could bind to (described above), could compete binding to immobilised fibronectin. They found that the heparin I-binding domain didn't disrupt immobilised fibronectin binding (similar to HN001) but the C-terminal heparin II-binding domain (not assayed for HN001) did.

Fibronectin-binding of HN001 was found to be dependent on protease-sensitive cell surface components. Periodate oxidation of HN001 cell surface components increased both fibronectin and BSA binding, indicating that non-specific binding had been increased. However, no conclusions could be drawn about the role of carbohydrate in HN001 binding of fibronectin. The same observations for fibronectin binding were seen for three *Campylobacter jejuni* strains (Moser *et al.*, 1997), and *Lactobacillus acidophilus* CRL 639 (Lorca *et al.*, 2002), with protease treatment decreasing fibronectin-binding and periodate oxidation increasing fibronectin-binding. Numerous studies have demonstrated the role of a surface protein in fibronectin-binding of bacteria (Joh *et al.*, 1999). As for many bacterial strains, it appears that the fibronectin-binding activity of HN001 is most likely dependant on a cell surface protein.

The calculated number of fibronectin adhesins per HN001 cell (approximately 250) is similar to that reported for some other lactobacilli (<230 per cell) (Harty *et al.*, 1994). This is significantly lower than the number of adhesins per cell calculated for known pathogens. *Mycobacterium bovis* BCG has 8000-15000 adhesins per cell, *Mycobacterium paratuberculosis* has 1600 adhesins per cell, and *Porphyromonas gingivalis* has 3500 per cell (Joh *et al.*, 1999). However, it is hard to compare HN001 to these organisms, as they are significantly dissimilar to HN001. It is likely that the small numbers of adhesins on *Lactobacillus* cells are responsible for the modest amount of <sup>125</sup>I-fibronectin bound by HN001 and other lactobacilli.

The specificity of HN001 binding to soluble fibronectin (75% inhibition with addition of 1000-fold excess of unlabelled fibronectin) is similar to that seen for the enterotoxigenic strain *Escherichia coli* B34289c (94% inhibition with addition of 4000-fold excess of unlabelled fibronectin) (Froman *et al.*, 1984). Specific binding to soluble fibronectin was also demonstrated for three *Lactobacillus* strains (Nagy, 1992).

HN001 bound more soluble fibronectin in stationary-phase than in exponential-phase. A similar trend was seen in a comparative study of probiotic adhesion to Caco-2 cells in different growth conditions. Adhesion increased with the length of bacterial growth phase (more adhesion at stationary-phase) (Blum, 1999). However, capsule-producing *Lactobacillus* strains show the opposite trend of fibronectin binding. *L. acidophilus* CRL 639 has maximum fibronectin binding in exponential-phase and less binding in stationary-phase, which correlates with the formation of capsule (Lorca, 2002). The differences in binding fibronectin probably relate to differences in cell-surface components during the growth phase. It is possible that adhesins specific to binding soluble fibronectin are induced late in the growth phase of HN001 and/or cell-surface components that inhibit fibronectin-binding are degraded during the growth phase.

## 4.2 HN001 binds Caco-2 cells

The Caco-2 cell line was used in this study as a model of the human intestinal surface because it displays typical features of intestinal epithelial cell differentiation (Section 1.3.4). In this study, radiolabelled cells resuspended in 1× PBS were used to measure adhesion of HN001 to Caco-2 cells. Under these conditions, the number of HN001 cells adhering to 100 Caco-2 cells (adhesion index) varied from approximately 60 to 150. When HN001 was added with HN001 spent culture supernatant (SCS), HN001 had an adhesion index of approximately 130, which is twice as high as observed for the controls in 1× PBS and three times that for cells in fresh MRS at pH 4.0. The adhesion in 1× PBS seen in this study is comparable to the adhesion index of 76 observed for HN001 in 1× PBS in a previous study (Gopal *et al.*, 2001). In the previous study of HN001 adhesion, two independent methods were used to measure adhesion to Caco-2 cells: direct microscopic counting and counting of radiolabelled cells (Gopal *et al.*, 2001). The adhesion index of HN001 in SCS (130) is low compared to the adhesion index of HN001 in SCS in a previous study (219) (Gopal *et al.*, 2001). The adhesion indices of other *Lactobacillus* strains were: *L. leichmannii* 7830 and *L. delbrueckii* subsp. *lactis*, 231 and 153 respectively (Sarem *et al.*, 1996) and *L. acidophilus* LA1, 155 (but note four-fold higher numbers of cells were used compared to this study) (Bernet *et al.*, 1994). The adhesion indices of several bifidobacteria ranged from 28 to 210 (but note two-fold higher numbers of cells were used compared to this study)

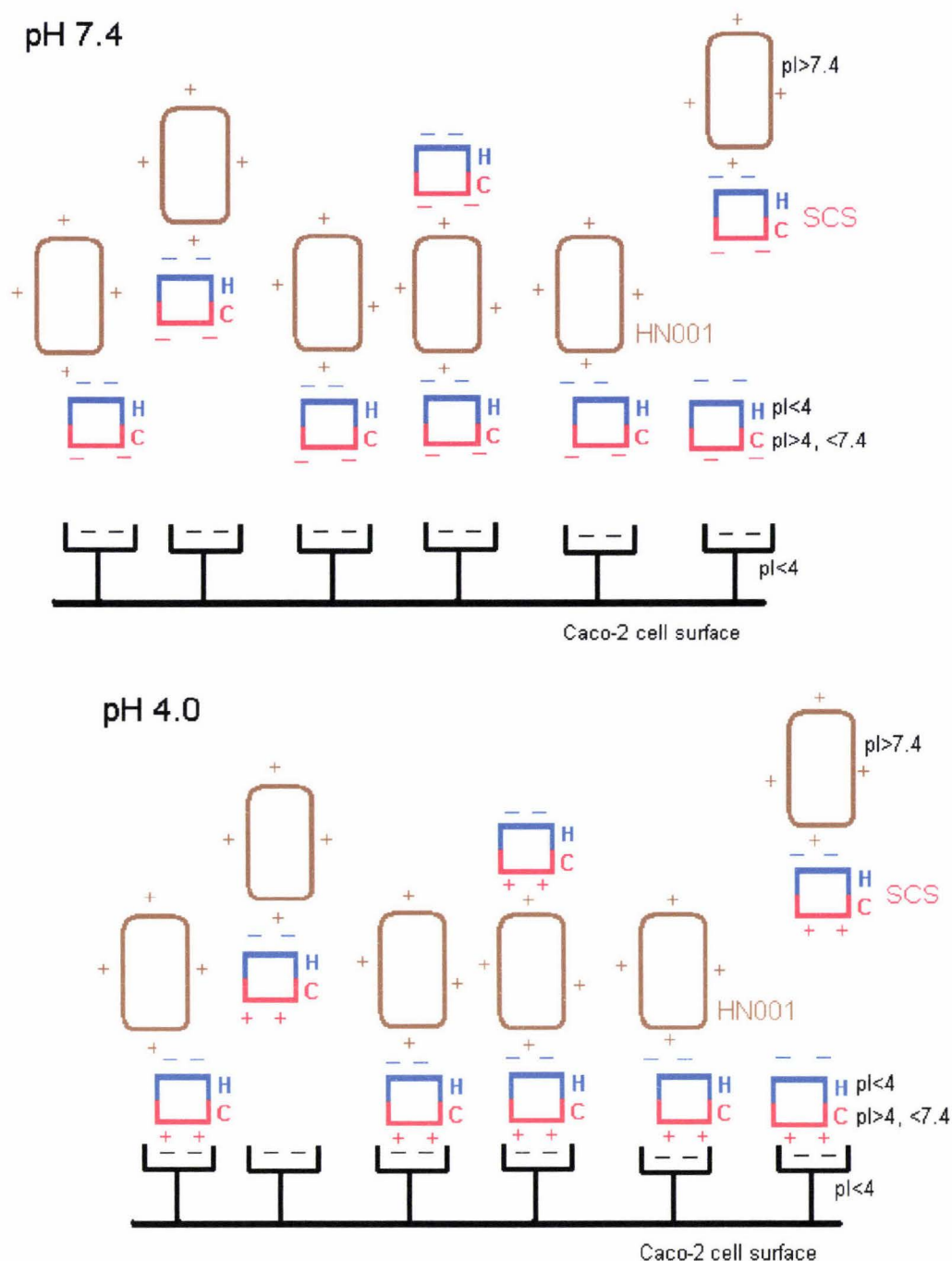
(Bernet *et al.*, 1993), suggesting that adhesion to Caco-2 cells is strain-specific. A recent study showed that there was considerable variation in adhesion between strains and no correlation between origin of the strain and its ability to adhere to Caco-2 cells (Jacobsen *et al.*, 1999).

As previously demonstrated (Gopal *et al.*, 2001), HN001 appears to be a relatively adhesive strain, and therefore may have the potential to adhere to intestinal epithelial cells *in vivo* and colonise the intestinal surface. The ability of HN001 to colonise intestinal surfaces has previously been inferred by feeding trials (Tannock *et al.*, 2000). It is believed that probiotic organisms can have maximum beneficial effect to the host if they are able to adhere to intestinal cells (Bezkorovainy, 2001), although conclusive proof is lacking. A recent study has demonstrated that adhesive *Lactobacillus* strains that persisted in the gastrointestinal tract for at least 24 hr, showed enhanced tetanus toxin (TTFC)-specific serum IgG and IgM antibody responses in comparison to a non-adhesive strain when these strains were added as an adjuvant with the toxin (Plant *et al.*, 2002). It has been demonstrated that HN001 can modulate the host immune system (Section 1.2.3) so perhaps it is through adhesion to intestinal surfaces that HN001 can exert this beneficial effect on the host.

In this study, the addition of HN001 SCS to the adhesion assay dramatically increased HN001 adhesion to Caco-2 cells. The factor in SCS was influenced by pH, with a positive effect on binding at pH 4.0 and no discernible effect at pH 7.4. The effect of salt due to pH adjustment, and a non-specific influence of pH on binding were discounted. A similar trend was observed for *Lactobacillus acidophilus* BG2FO4, where SCS demonstrated a pH-dependence on adhesion-promoting activity (Greene *et al.*, 1994). The addition of bacterial SCS has been found to increase binding of several strains of lactic acid bacteria and bifidobacteria including *Bifidobacterium breve* 4 (Bernet *et al.*, 1993), *L. acidophilus* LA1 (Bernet *et al.*, 1994), and *L. acidophilus* LB (Chauviere *et al.*, 1992). The SCS from *L. acidophilus* BG2FO4 was found to enhance the adhesion of the poorly adhering *L. casei* GG to Caco-2 cells (Coconnier *et al.*, 1992). The conclusion drawn from these studies was that there is a factor present in spent culture supernatant that enhances adhesion to Caco-2 cells. The conclusion from this study is the same. However, the addition of bacterial SCS does not always increase adhesion to intestinal cell lines. *L. casei* subsp. *rhamnosus* Lcr35 had similar adhesion

with addition of SCS or fresh media (Forestier *et al.*, 2001). Fibronectin-binding of HN001 was not influenced by the factor in HN001 SCS in the same manner as adhesion to Caco-2 cells was.

Figure 44 describes one possible model for the mode of action of an adhesion-promoting factor produced by HN001. The hypothetical factor has two regions of different pI. The region that interacts with HN001, labelled H in Figure 44, has a pI of less than 4, so is negatively charged at pH 4.0 and 7.4. This negatively charged region interacts with positively charged components on the surface of HN001. These positively charged surface components could be basic surface proteins that appear to be expressed on the HN001 surface (personal communication, Dr. Mike Collett). The second region of the hypothetical adhesion-promoting factor in HN001 SCS, labelled C in Figure 44, has a pI of between 4.0 and 7.4. At pH 7.4 this region is negatively charged and at pH 4.0 this region is positively charged. When positively charged (pH 4.0) the factor interacts with the negatively charged receptors on the Caco-2 surface. One type of receptor available for bacterial binding is a glycolipid containing sialic acid (gangliosides) (Ofek *et al.*, 1994), which are acidic and thus are negatively charged at pH 4.0 and above. In the proposed model there would be an interaction between positively charged components of the HN001 cell surface and the negatively charged receptors on the Caco-2 surface without the presence of HN001 SCS. Since HN001 adhesion to Caco-2 cells is greater in the presence of SCS it is proposed that the factor in SCS acts as a 'bridge' between HN001 and Caco-2 cells to provide a higher affinity interaction than the interaction without SCS. The model implies a non-specific promotion of adhesion. However, it is unknown whether the factor in HN001 SCS specifically promotes HN001 adhesion or whether it can promote the adhesion of other bacteria. In order for the factor in the SCS to promote adhesion of other bacteria non-specifically, they would have to have basic surface proteins, like HN001. If the factor in HN001 SCS promotes the adhesion of HN001 specifically, this implies that there are other interactions between HN001 and the factor, apart from charge interactions that occur to make the interaction specific.



**Figure 44 Proposed model of action of factor in HN001 SCS**

The top diagram demonstrates the binding of HN001 to Caco-2 cells in SCS at pH 7.4. Binding is not promoted by SCS since the charge interactions between the factor in SCS and receptors on the Caco-2 surface are not favourable. The bottom demonstrates binding of HN001 to Caco-2 cells in SCS at pH 4.0. Binding is promoted since there is a favourable interaction between the positively charged region (C) on the factor in SCS and the negatively charged receptors on the Caco-2 surface. The representation of HN001 and the molecules involved in adhesion to Caco-2 are not drawn to scale.



Concentrated spent culture supernatant (SCS) from HN001 reduces culturable numbers of *E. coli* O125:H7, and also reduces the adhesion and invasiveness of this pathogen to Caco-2 cells (Gopal *et al.*, 2001). These effects are possibly due to a synergistic action of lactic acid and proteinaceous substances (Gopal *et al.*, 2001). It is possible that the factor in SCS that increases adhesion of HN001 to Caco-2 cells (shown in this study), also acts to decrease adhesion of *E. coli* O125:H7 to Caco-2 cells.

Many high molecular weight proteins were detected by the anti-HN001 cell envelope antiserum in the immunoblot performed in this study. Typically Gram-positive adhesins are high molecular weight proteins located on cell surfaces (Clarke *et al.*, 2002; Jedrzejewski, 2001; Navarre *et al.*, 1999; Patti *et al.*, 1994). Therefore, the anti-HN001 cell envelope IgG would be expected to contain anti-adhesin IgG and therefore, should disrupt HN001 adhesion to Caco-2 cells. However, both anti-HN001 cell envelope IgG and a presumed non-specific IgG species (rabbit non-immunised IgG) increased adhesion to Caco-2 cells. This indicated that IgG was promoting HN001 adhesion to Caco-2 cells, rather than disrupting it. One explanation could be that HN001 is able to bind IgG non-specifically, perhaps through the Fc region of the antibody. Another explanation could be that the non-immunised IgG binds epitopes common to Gram-positive organisms due to previous exposure of the rabbit to Gram-positive organisms, and hence binds HN001. The bound IgG may then aggregate HN001 cells and/or bind to components of the Caco-2 cell surface that are common to both animal and bacterial cell walls (eg. carbohydrate complexes), increasing the apparent adhesion of HN001 to Caco-2 cells. Because BSA was added to the binding reactions, non-specific interactions between IgG and the Caco-2 cell surface are unlikely.

Since HN001 was found to bind fibronectin at significant levels the role of fibronectin-binding in adhesion to Caco-2 cells was investigated. The addition of two forms of fibronectin (plasma and super fibronectin) and III<sub>1</sub>-C to the binding reaction had no significant effect on HN001 adhesion to Caco-2 cells. If fibronectin is involved in HN001 adhesion to Caco-2 cells, super fibronectin and III<sub>1</sub>-C were both expected to disrupt HN001 adhesion to Caco-2 since they were able to disrupt HN001 adhesion to immobilised fibronectin. It is unclear whether fibronectin is exposed on Caco-2 cell surfaces, and therefore, whether fibronectin-binding is involved in adhesion to Caco-2 cells. Fibronectin does appear to have a role in adhesion to other intestinal cell lines.

*Lactobacillus acidophilus* 33199 and *L. agilis* 43564 bind to areas on the Intestinal 407 cell line where fibronectin is expressed (shown by fluorescent microscopy using an anti-fibronectin antibody). Also, pretreatment of bacteria with fibronectin decreases adhesion of *L. acidophilus* 33199 to Intestinal 407 cells from 14% to 5% indicating that fibronectin has a role in adhesion to Intestinal 407 cells (Kapczynski *et al.*, 2000). It is possible that small amounts of fibronectin that were likely to be present in serum used in the Caco-2 binding assays interfered with the assay. While fibronectin might not appear to have a role in HN001 adhesion to Caco-2 cells, it is possible that fibronectin-binding has a role in HN001 adhesion to intestinal surfaces *in vivo*, because fibronectin may be exposed to HN001 cells via epithelial cell shedding and damage, which are also key sites of pathogen adhesion.

Several problems were encountered with the use of the Caco-2 cell line in this study. The level of HN001 adhesion to Caco-2 showed considerable variation between experiments, ranging from 60 to 150 per 100 Caco-2 cells. The difference in adhesion could not be correlated to the passage number of the Caco-2 cell line. A possible source of variation between the experiments was the serum used in the binding assays and in the Caco-2 growth media. Several different batches of serum were used which may result in variable differentiation of the Caco-2 cells or variable adhesion of HN001. Also, the serum contained both fibronectin and IgG, which may have influenced some of the results obtained in this study. Another source of variation between experiments may have been due to variations in the Caco-2 monolayer. Clumps of cells were often seen after the trypsin treatment used to seed plates for experiments. This may have led to an uneven surface with differences in differentiation of the Caco-2 cells and the density of receptors exposed on the Caco-2 cell surface. Problems with the Caco-2 cell line model have been reported previously. *Lactobacillus acidophilus* LA1 was found to be non-adherent to Caco-2 cells in one study while previous studies showed that it adhered with high affinity to Caco-2 cells (Jacobsen *et al.*, 1999). A critical investigation of adhesion assays highlighted the problem of variability encountered with these studies. Differences in assay conditions were found to greatly influence the results obtained from *in vitro* adhesion assays, thus they concluded that conditions used for adhesion assays need to be standardised in order to compare results between labs (Blum *et al.*, 1999).

## 4.3 Characterisation of Fbl

### 4.3.1 Is Fbl likely to be a fibronectin-binding protein?

HN001 genome DNA sequence was examined for genes encoding putative fibronectin-binding proteins. The most likely candidate gene was *fbl* (fibronectin-binding like). *In silico* characterisation of Fbl indicates that it has most similarity to two known fibronectin-binding proteins, Fbp54 from *Streptococcus pyogenes* (Courtney *et al.*, 1994) and PavA from *Streptococcus pneumoniae* (Holmes *et al.*, 2001). The N-terminal region of Fbl contained a repeated IIXI motif, identified in Fbp54, adding confidence to a fibronectin-binding function for Fbl. The IIXI motif was speculated to have a role in fibronectin-binding because it is present in the fibronectin-binding regions of Fbp54 (determined by expressing N-terminally truncated Fbp54), and *S. aureus* fibronectin-binding proteins FnbpA and FnbpB (the D2-D3 repeat region) (Courtney *et al.*, 1994). However, the whole Fbp54 protein (474 amino acids), not just the N-terminal fibronectin-binding region, was needed to disrupt adhesion of streptococci to immobilised fibronectin by competition (Courtney *et al.*, 1994). The IIXI motif is also present in the N-terminal part of PavA, but it is the C-terminal part of PavA that is essential for fibronectin-binding (determined by expression of C-terminally truncated PavA and creation of a mutant expressing C-terminally truncated PavA) (Holmes *et al.*, 2001). For HN001, it is possible that Fbl may bind fibronectin through the N-terminal IIXI-like domain, and/or the C-terminal part of Fbl may mediate adhesion of HN001 cells to fibronectin (as for PavA of *S. pneumoniae*). Alternatively, HN001 Fbl may have a regulatory role in fibronectin binding. FbpA from *Streptococcus gordonii* has a high level of identity to both PavA and Fbp54, and plays a regulatory role in fibronectin-binding of *S. gordonii*. Although purified FbpA binds fibronectin and inactivation of the *fbpA* gene decreases fibronectin binding, it is the down-regulation of *csfA* expression, a known fibronectin-binding protein, in the *fbpA* mutant that causes the decreased fibronectin binding, indicating a regulatory role for FbpA (Christie *et al.*, 2002).

The presence of repeats is a common characteristic of fibronectin-binding proteins (Clarke *et al.*, 2002; Joh *et al.*, 1999). However, only two small repeats were identified in Fbl. It is possible that degenerate repeats like those described for Fbp54 (Courtney *et al.*, 1994) are present in Fbl but were not identified in the Dot Plot analysis.

A motif implicated in the fibronectin-binding activity of several fibronectin-binding proteins is EDT/S(X9-10)GG(X3-4)I/VDF (Joh *et al.*, 1999). This motif was not identified in Fbl or PavA (Holmes *et al.*, 2001). A non-repeated region present in PrtF1/2 of *S. pyogenes* has been implicated in fibronectin binding (Jaffe *et al.*, 1996; Ozeri *et al.*, 1996). This sequence is not found in PavA (Holmes *et al.*, 2001) or Fbl. It is probable that the presence of different motifs and/or binding regions of fibronectin-binding proteins relates to their binding of different domains within the fibronectin molecule or different conformations of fibronectin, such as the possible conformational changes that occur when soluble fibronectin is immobilised on a surface.

In order for Fbl to be involved in fibronectin-binding it should be exposed on the surface of HN001 cells. This requires a mechanism to allow it to traverse the cell wall and become anchored to the cell surface (Section 1.4.2.2). A putative signal peptide that may allow export of the polypeptide from the cell via the general secretion pathway (Izard *et al.*, 1994) was identified from residue 1 to 51. This is slightly longer than the signal peptides of fibronectin-binding proteins identified in other Gram-positive organisms (23-38 residues) (Joh *et al.*, 1994). However, there are several examples of signal peptides of surface-exposed proteins from Gram-positive organisms that are 50 amino acids long (von Heijne *et al.*, 1989) so it is reasonable to assume that the putative signal peptide identified in Fbl might be functional.

The mechanisms characterised to date for anchoring surface-exposed proteins in lactobacilli are: the S-layer protein anchor (Antikainen *et al.*, 2002), and the LPXTG motif (Gilbert *et al.*, 1996; Roos *et al.*, 2002). The LPXTG motif has been identified as part of the major mechanism by which surface proteins of Gram-positive organisms are anchored to the cell wall (Navarre *et al.*, 1999). A recent study has identified a possible novel cell-surface anchoring motif in Apf of *L. johnsonii* and *L. gasseri* (Jankovic *et al.*, 2002). Neither the S-layer, nor the LPXTG anchoring motifs were found in Fbl. Similarly, no signal peptide or LPXTG motif were identified in either PavA or Fbp54, despite strong evidence that both of these proteins are located on the bacterial cell surface (Courtney *et al.*, 1994; Holmes *et al.*, 2001). Other Gram-positive anchoring mechanisms like the transmembrane anchor, lipoprotein anchor, or the AcmA repeat

anchor (Section 1.4.2.2) were not found in Fbl. This implies that Fbl might be surface-exposed via an as yet uncharacterised mechanism.

#### 4.3.2 Characterisation of Fbl function

Initially, Fbl was expressed using a GST-fusion expression system to characterise the function of Fbl. Due to the difficulties in expressing soluble Fbl fusion proteins, and the difficulty in cleaving Fbl off its GST tag (possibly due to blocking of the protease cleavage site by Fbl), the only Fbl fusion protein able to be purified was the N-terminal third of Fbl fused to GST (Fbl4-GST). Fbl4-GST was assessed for its ability to block HN001 binding of fibronectin. The rationale is if Fbl is able to disrupt binding of HN001 to fibronectin, then Fbl must bind the same binding site on fibronectin as HN001 or close enough to the HN001 binding site to sterically hinder binding of HN001. Unfortunately, the result of the competition of HN001 binding to fibronectin with Fbl4-GST was inconclusive and thus, no assessment of Fbl function could be made.

Due to the problems of expressing soluble Fbl and the availability of a system to create insertional mutants of genes in the HN001 genome, targeted disruption of *fbl* was used to characterise the function of the Fbl protein. A targeted disruption of the *fbl* gene was created using a homologous recombination strategy via a suicide vector with cloned target DNA. When the phenotype of a strain with a disrupted copy of *fbl* (HN001D*fbl*) was compared to WT HN001 in three different binding assays (liquid-phase fibronectin binding, solid-phase binding to several extracellular matrix components, and adhesion to Caco-2 cells) HN001D*fbl* did not show reduced binding to any of the ligands used. There are several possible reasons why disruption of the *fbl* gene in HN001 (HN001D*fbl*) did not produce a notable phenotype. Of course, it is possible that Fbl does not have a role in HN001 binding to the ligands assayed. It is also possible that there are other fibronectin-binding proteins present in HN001, thus Fbl has a redundant function. Alternatively, it is possible that *fbl* was not fully inactivated in HN001D*fbl*. The limitation of insertional mutagenesis of a target gene via a single crossover event (the only system currently available for HN001) is that the whole gene is still present in the genome, albeit in a disrupted form. Thus, it is possible that transcription of at least part of the gene occurs. The N-terminal 2/3 of *fbl* was intact in HN001D*fbl* and may be

transcribed. The C-terminal 2/3 of *fbl* is intact in HN001D*fbl* and may be transcribed if transcription is initiated in the integrated pBeryFbl1 DNA before the start of the cloned *fbl* fragment. The notable features in the N-terminal 2/3 of *fbl* are the putative signal peptide and the region similar to the fibronectin-binding region of Fbp54. This fragment is possibly transcribed, translated, transferred across the cell wall and may be attached to the cell wall in HN001D*fbl*. The C-terminal 2/3 of *fbl* is less likely to be transcribed, translated and transported to the cell surface in HN001D*fbl* but it is still possible. If only the N-terminal part of Fbl is required for fibronectin-binding function, then HN001D*fbl* might produce functional Fbl (hence no loss in binding ability).

Interestingly, HN001D*fbl* tended to bind the ligands assayed with more affinity than WT HN001. Two explanations are offered to clarify why this might happen. It is possible that the alteration of Fbl (perhaps decreased size or absence) on the surface of HN001 allowed other surface proteins, usually masked by Fbl, to bind ligands with more affinity than Fbl did. Also PCR was used to show that HN001D*fbl* lost the small endogenous HN001 plasmid (data not shown), possibly during the formation of electro-competent HN001 cells or during electroporation of the HN001 cells. This was confirmed by extracting the plasmids from HN001D*fbl* (personal communication, Ellen Hausman). It is possible that the loss of an endogenous plasmid may increase binding of HN001D*fbl* to the ligands assayed, compared to WT HN001 (which has the endogenous plasmids). The binding of an HN001 strain that has lost the small endogenous plasmid to various ligands needs to be investigated in order to prove/disprove this hypothesis.

## CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

### 4.1 Summary

HN001 binds both soluble and immobilised plasma fibronectin, with preference for immobilised fibronectin, and immobilised cellular and super fibronectin. Immobilised cellular and super fibronectin mimic the forms of fibronectin most likely available for HN001 to bind to on intestinal surface tissue. The binding site for HN001 within the fibronectin molecule appears to be the C-terminal part of the first type III repeat, which interestingly is involved in fibronectin matrix formation. HN001 also binds the N-terminal heparin I binding domain (a common binding site for Gram-positive organisms) with lower affinity. Fibronectin-binding of HN001 appears to be mediated through a cell-surface protein.

HN001 binds to the intestinal cell-line Caco-2 with similar affinity to that reported for adhesive *Lactobacillus* strains. The adhesion of HN001 is enhanced by a pH-sensitive factor present in HN001 spent culture supernatant. This factor was not found to enhance fibronectin-binding. Although HN001 binds to fibronectin with more affinity than to the other intestinal molecules assayed, no support could be given for fibronectin having a role in HN001 adhesion to Caco-2 cells.

The *fbl* (fibronectin-binding like) gene in HN001 was identified as a gene that may encode a fibronectin-binding protein. *In silico* characterisation revealed that Fbl has similarity to two known fibronectin-binding proteins. However, an expressed N-terminal fragment of Fbl was unable to disrupt HN001 adhesion to soluble fibronectin, and a targeted disruption of *fbl* failed to produce a phenotype differing from wild type HN001.

## 5.2 Future directions

While the results of this study are significant, this study is only a preliminary investigation into the molecular species involved in the adhesion of HN001 to intestinal surfaces *in vivo*.

Since HN001 adhesion to soluble fibronectin is probably not relevant to HN001 adhesion to intestinal tissues *in vivo*, further characterisation of binding to soluble fibronectin would not lead to further insights in the role of fibronectin binding in the probiotic activity of HN001. HN001 adhesion to immobilised components found in intestinal surfaces is probably of more relevance to HN001 adherence *in vivo*. Thus, HN001 adhesion to components including tenascin, heparin, entactin, and thrombospondin could be investigated. Also, commercially available extracellular matrix preparations, ECM gel (Sigma) and Matrigel™ (Becton Dickinson) could be used to model extracellular material. In order to further characterise HN001 adhesion to fibronectin, HN001 binding to the cell-binding domain, which contains the majority of the fibronectin type III repeats (including the first type III repeat) could be investigated. This, along with competition experiments with the C-terminal part of the first type III repeat, may demonstrate whether HN001 is binding specifically to the first type III repeat, or to type III repeats in general. It would also be interesting to investigate if HN001 can bind other proteins with fibronectin type III repeats. Exclusion of intestinal pathogen adherence to fibronectin by HN001 could be investigated, and may show if fibronectin-binding of HN001 is relevant to the ability of HN001 to act as a probiotic.

Although the factor in HN001 spent culture supernatant (SCS) was not found to enhance fibronectin-binding, it is possible that it enhances binding to other molecules present on intestinal surfaces. The effect of HN001 SCS on HN001 binding to molecules like mucin and heparan sulphate (both present on the epithelial cell surface) could be investigated. The specificity of the factor in HN001 SCS could be investigated by determining if it can increase the adhesion of non-binding *Lactobacillus* strains to Caco-2 cells. Also, the nature of the factor in HN001 SCS could be characterised by various chemical and physical treatments to determine if the activity is mediated by proteins or carbohydrate.



The role of fibronectin-binding in HN001 adhesion to intestinal surfaces could be further investigated using other intestinal cell lines (eg. HT-29 and Int 407). The location of fibronectin on Caco-2 cell surfaces could be investigated by visualising where fluorescent-labelled polyclonal anti-fibronectin antibodies bind to on the Caco-2 monolayer. The binding location of HN001 on Caco-2 cells could then be investigated to see if it was in the same region as fibronectin is located.

The function of Fbl might be determined by creating a mutant with the *fbl* gene fully deleted. A technique to achieve this in HN001 is currently being developed (personal communication, Dr. Mike Collett). The apparent increased binding of the mutant created in this study could be investigated by determining if the loss of the small endogenous plasmid impacts on the ability of HN001 to bind the extracellular matrix components assayed and Caco-2 cells. In order to identify proteins from HN001 involved in fibronectin binding, a total protein preparation from HN001 could be enriched for fibronectin-binding proteins by purifying on the basis of this function (ie. an affinity purification) as part of a purification scheme. Also, whole HN001 cell extracts could be incubated with  $^{125}\text{I}$ -fibronectin to identify fibronectin-binding proteins.

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## APPENDIX 1: FORMULAE USED

Molar ratio for ligation:

Mr of 10 nucleotides =  $6.6 \times 10^3$  g/mol (double stranded DNA)

$$\text{Mol} = \frac{\text{mass of DNA (g)}}{\text{Mr of DNA (g/mol)}}$$

Add a 3:1 mole ratio of insert DNA:vector DNA in the ligation reaction.

T<sub>m</sub> of oligonucleotide primers:

$$T_m (^{\circ}\text{C}) = 2(A + T) + 4(G + C)$$

Transformation frequency:

$$\frac{\text{cfu}}{\mu\text{g DNA}} \times \text{Dilution factor} = \text{Transformation frequency (cfu/}\mu\text{g)}$$

Standard error of the mean:

$$2 \text{ standard error of the mean} = 2(\text{SD}/\text{SQRT}(3))$$

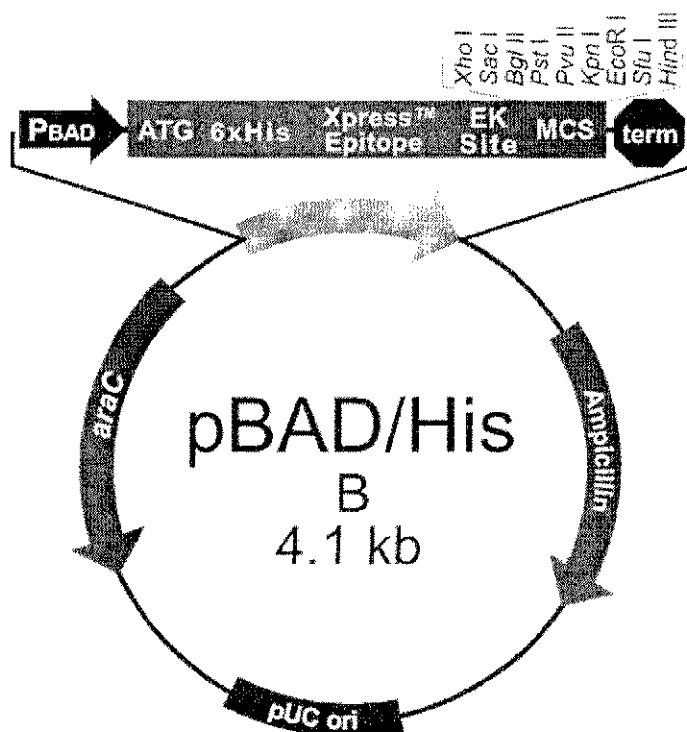
SD = standard deviation

SQRT (3) = square root of 3

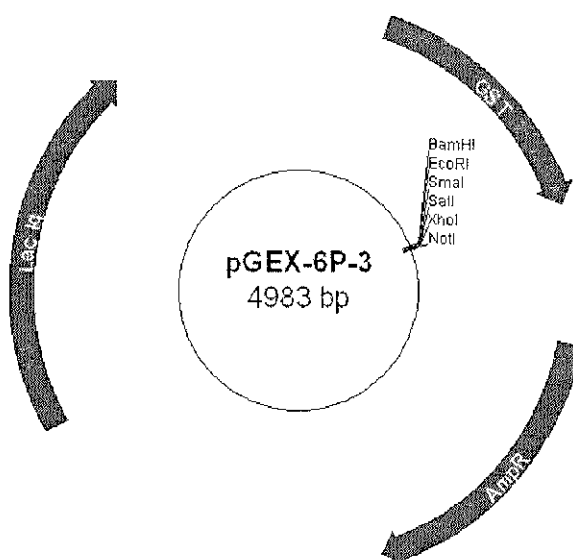
Quantitation of DNA by A<sub>260</sub>

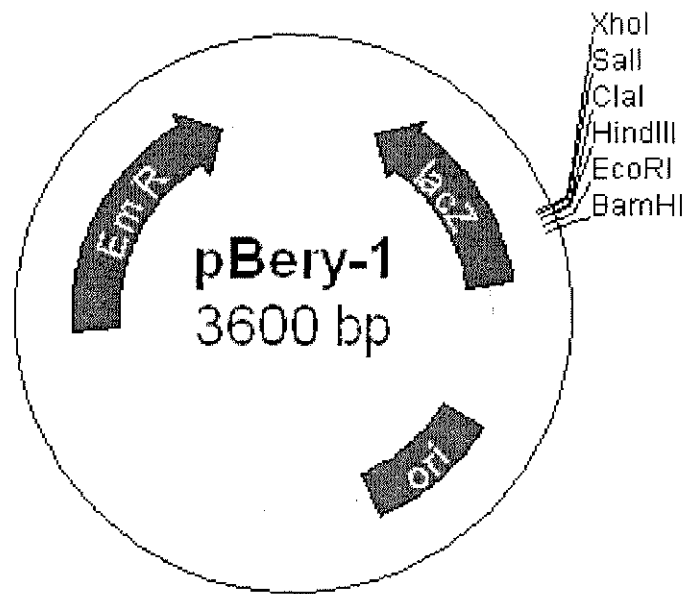
$$A_{260} \text{ value} \times \text{dilution factor} \times 50 \text{ (for dsDNA } A_{260} \text{ of 1} = 50\mu\text{g/ml)} = \chi \mu\text{g/ml (DNA concentration)}$$

## APPENDIX 2: MAPS OF VECTORS USED



pBAD/HisB diagram adapted from information obtained from Invitrogen

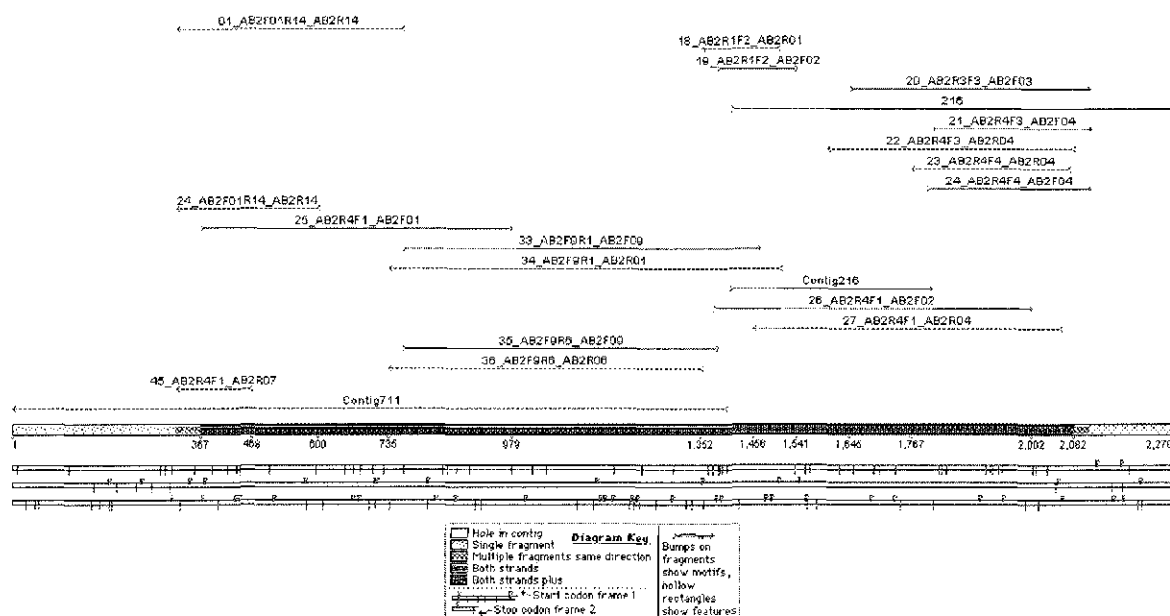




## APPENDIX 3: *IN SILICO* CHARACTERISATION

### Sequence assembly of *fbl*

Output from Sequencher™ (Version 4.0.5, Gene Codes Corporation)



### Prediction of a signal peptide

Output from Signal P2.0 (from [www.bionavigator.com](http://www.bionavigator.com))

SPScan of Fbl

July 1, 2001 21:35

Weight matrix: GenRunData:spgpos.dat

Minimum score for SPs (threshold): 3.0

Sequence: Fbl 1 to 557

1. 147

MPGAPYVEPPKQDKVDPFHDSEIRYHELERQVTPSLSRAALLQQHYQGLAKDSAAELALRLNQ  
GDAGW^DS 216

Score: 6.4

Probability: 8.721E-01

SP length: 68

Total hydropathy: 29.3

Maximum 8-residue hydropathy: 26.7, starting at 162

2. 328 MTSIELPNFYADNEPIKITLSNQLTPSRNA^QK 359

Score: 6.2

Probability: 5.467E-01

SP length: 30

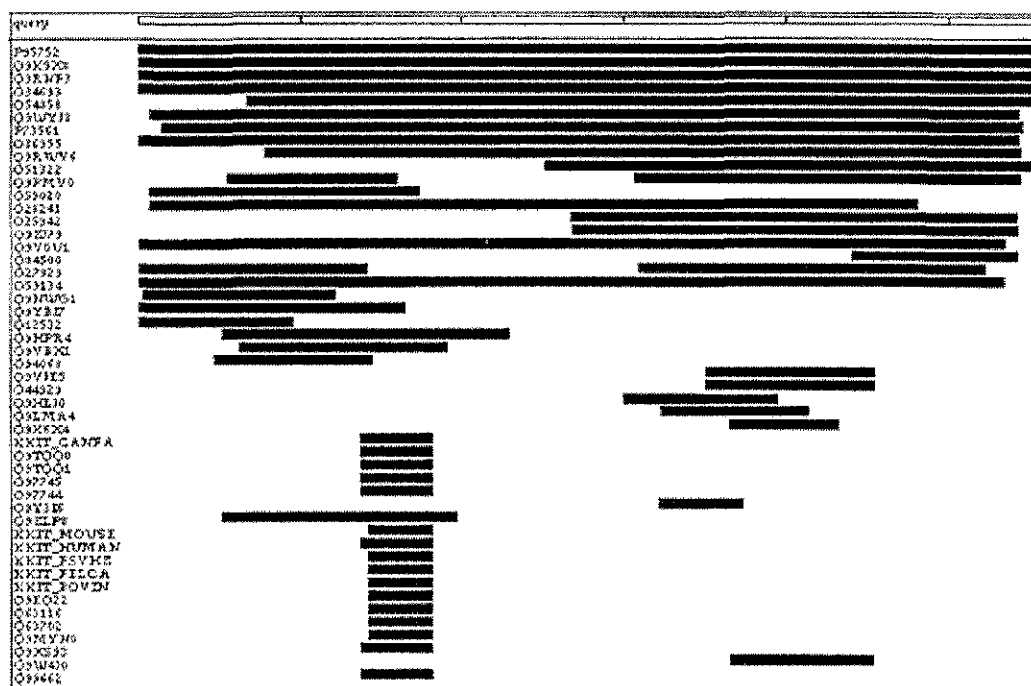
Total hydropathy: 65.3

Maximum 8-residue hydropathy: 35.5, starting at 341



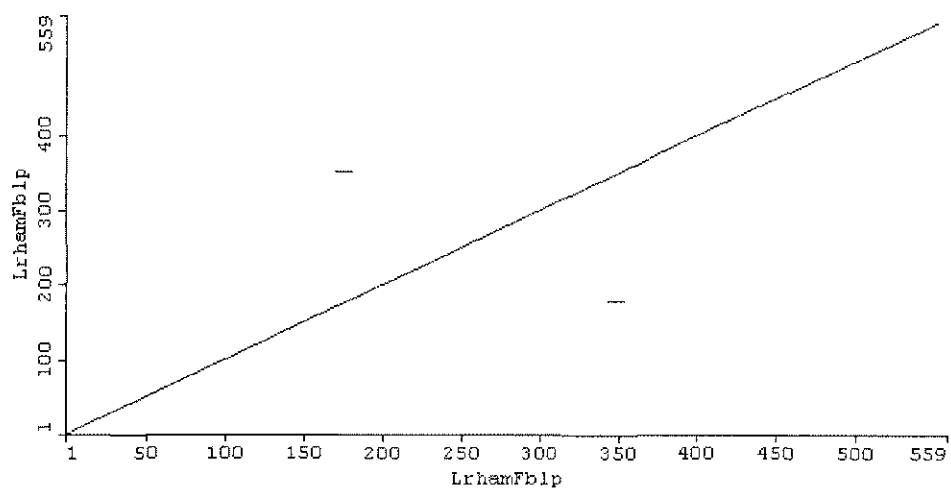
Maximum 8-residue hydropathy: 38.8, starting at 9

Maximum 8-residue hydropathy: 42.6, starting at 117



SwissProt entry	Begin-End	Description and organism	E value	Similarity *	Identity*
P95752	1-552	FlpA (putative) ( <i>Streptococcus gordonii</i> )	1e-118	61	43
Q9K9X8	1-552	Fn/Fgn-binding protein (putative) ( <i>Bacillus halodurans</i> )	1e-117	59	42
Q9RNF3	1-552	PavA ( <i>S. pneumoniae</i> )	1e-117	61	41
O34693	1-552	Fn-binding protein (putative) ( <i>B. subtilis</i> )	1e-114	57	41
Q54858	67-552	Fbp54 ( <i>S. pyogenes</i> )	1e-91	57	40

## Dot Plot results




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LrhamFb1p	:	176	LERQVTPS	183
LrhamFb1p	:	349	LSNQLTPS	356

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LrhamFb1p	:	349	LSNQLTPS	356
LrhamFb1p	:	176	LERQVTPS	183

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## APPENDIX 4: ENUMERATION OF CACO-2 CELLS

The cells in a field of view were counted, and the area of the field of view was calculated using a micrometer. This was used to estimate how many cells are present in a well. The area of each well in a 6-well plate is  $9.6\text{cm}^2$  ( $= 960\text{mm}^2$ ). A sample calculation and an example of a photo used for the calculation are included below:

$$\frac{\chi}{32 \text{ cells}} = \frac{960}{4.35 \times 10^{-3}}$$

$$\chi = 7.16 \times 10^6 \text{ cells/well}$$

