Construction of *Salmonella* vaccines

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A thesis Submitted for the degree of Doctor of Philosophy.

June, 1988.

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Abstract

In this thesis the molecular cloning of the *gal* operon of *Salmonella typhimurium* LT2, the localisation of the *gal* promoter, and the *gal* genes: *galE, galT*, and *galK*, are described. The order of the genes and the direction of transcription was the same as occurs in *E. coli* K12 (promoter, *galE, galT, galK*). A restriction enzyme map of the operon was obtained and the termini of the three genes were precisely located by means of transposon insertion mutagenesis and minicell analysis of the *gal* proteins expressed by various plasmids.

A plasmid which contained a defined 0.4 kilobase deletion in the *galE* gene, but which still expressed *galT* and *galK* activities from the *gal* promoter was constructed. The *galE* deletion was recombined into the chromosomal *gal* operons of *S. typhimurium* and *Salmonella typhi* Ty2. The resulting strains were vigorous, non-reverting *galE* mutants which were sensitive to galactose–induced lysis at 0.06mM galactose. The *S. typhimurium galE* derivatives were avirulent and protective in mice whereas a *galE* mutant of *S. typhi* Ty2 (Ty2H1) was only partially attenuated in mice.

A rifampicin-resistant, Vi-negative (*via*) derivative of Ty2H1, strain EX462, was selected. Compared with the Ty2 parent strain, EX462 was serum–sensitive and highly attenuated in the mouse mucin virulence assay. When four human volunteers ingested 7×10^8 viable EX462, two became ill and developed a typhoid-like disease with fever and bacteremia. Blood isolates from these individuals were indistinguishable from the vaccine strain using a variety of criteria. We concluded that, even in a *via* background, the *galE* mutation was not attenuating for *S. typhi* in man.

A system was developed, whereby heterologous DNA encoding a protective antigen from an enteropathogen may be recombined into the chromosome of attenuated *Salmonella* carrier strains. This system involved: i) integration of a *hisOG* deletion mutation into the chromosome; ii) replacement of the *hisOG* deletion by the complete *hisOG* region and the segment of heterologous DNA

which encodes the antigen of interest. Recombinants were selected *his*$^+$. This system was used to integrate the pilin–encoding K88 genes of porcine enterotoxigenic *Escherichia coli* into the chromosome of a *galE* mutant of *S. typhimurium* (LT2H1). Recombinants were detected at a frequency of between 1.0×10^{-3} and 1.5×10^{-3}. A variety of tests confirmed that the K88 genes were integrated into the chromosome of LT2H1 and were expressed. The stability of the recombinant was tested both *in vivo* and *in vitro*. When administered orally to mice, the recombinant elicited a serum antibody response to K88, and retained the vaccine potential of the vector strain.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of the my knowledge and belief this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to the thesis being made available for photocopying and loan, if applicable, and if accepted for the award of the degree.

For ethical reasons the testing of mutant strain EX462 in humans (Chapter 5), was performed in collaboration with Dr. B. Forrest (MB BS, Adelaide), and the Department of Medicine of the Royal Adelaide Hospital.

David Hone

Acknowledgements

I would like to extend my sincere appreciation and thanks to Dr. J. Hackett for his encouragement and guiding supervision throughout the duration of this work. I also thank Dr. Steven Attridge for many useful discussions and his help with the animal experimentation performed in this thesis. My thanks must also go to Professor Peter Reeves for his supervision in the early stages of this work, to Dr R. Morona for many technical and theoretical discussions, to Ms D. James, Mr J. Mackrill, and Ms L. van den Bosch for their technical assistance, and to Dr. P. Ey for donating ELISA reagents for the experiments performed in Chapter 6. I would like to thank the members of the Department of Medicine of the Royal Adelaide Hospital for their assistance with the work performed in Chapter 5. Finally my thanks go to Dr. C. Thomas for his assistance in the preparation of this thesis.

Papers submitted in conjunction with this thesis:

Hone, D.M., Morona, R., Attridge, S., and Hackett, J., 1987. Construction of defined *galE* mutants of *Salmonella* for use as vaccines. J. Infect. Dis. **156**:167.

Hone, D.M., Attridge, S.R., Forrest, B., Morona, R., Daniels, D., LaBrooy, J.T., Bartholomeusz, R.C.A., Shearman, D.J.C., and Hackett, J., 1988. A *galE*, *via* (Vi–negative) mutant of *Salmonella typhi* Ty2 retains virulence in man. Infect. Immun. **56**:13.

Hone, D.M., Attridge, S.R., van den Bosch, L., and Hackett, J., 1988. A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strains. Microbial Path. (in press).

This thesis is dedicated to my parents who have supported me in many ways throughout the course of my education.

Chapter 1

Introduction

Enteric bacterial pathogens such as *Vibrio cholerae, Salmonella typhi, Shigella spp., Yersinia enterocolitica, Escherichia coli* and *Campylobacter jejuni*, are a significant public health burden in undeveloped countries (Levine *et al.*, 1983b). This group of pathogens also present a problem to travellers from developed countries visiting such areas. Furthermore, enteric bacteria such as *Salmonella spp., E. coli* and *Campylobacter spp.* are major causes of diarrhea amongst domestic animals, resulting in financial losses by primary producers. It is therefore desirable to develop a means to elicit protective immunity against these pathogens. At present, development of a means to induce protective antibody and/or cell mediated immunity (CMI) at the portal of entry of these infectious agents has raised much interest. This approach to vaccine development was adopted as an alternative to the many parenteral vaccines currently available because they have not always effected good immunity at the gut mucosal surface.

This review will first discuss the mechanisms of immunity which provide protection against enteric bacterial infections. The second part of this review will elaborate on some of the earlier attempts to elicit immunity against enteric bacteria and discuss the reasons for success/failure of these earlier attempts. This discussion will lead into the modern recombinant DNA approach for vaccine construction and demonstrate the effect these methods have had on vaccine construction.

1.1 Humoral responses of the mucosa

As a result of studies by Besredka (1919, 1927 – cited by Pollitzer, 1959) on oral infections by enteric bacteria, the concept of systemic antibodies in protection against cholera infection changed and the possibility of local immunity to the disease was proposed. Further support of Besredka's idea of intestinal immunity was provided when Davies (1922) demonstrated agglutinins in the feces of patients suffering from bacterial–induced dysentery. These fecal–associated agglutinins, which became known as coproantibodies, were shown to be important for immunity in guinea pigs as their appearance correlated with the development of protective immunity (Burrows *et al.*, 1947; Burrows and Havens, 1948). No such correlation was found in the case of serum antibody (Burrows *et al.*, 1947).

1.1.1 Secretory IgA: its synthesis and transport into the intestinal lumen

When Heremans and co–workers (1959) described a new isotype of antibody, IgA, and the subsequent demonstration that this isotype formed the major immunoglobulin component of human and animal exocrine secretions such as saliva, colostrum, bronchial fluid and intestinal juices (Tomasi and Zigelbaum, 1963; Cebra and Robbins, 1966; Heremans and Vaerman, 1971), the concept of local immunity became established. Immunochemical analysis of antigenic differences between serum IgA and secretory IgA (sIgA) led to the subsequent discovery of an additional polypeptide chain, associated with IgA, called the secretory component (SC) (Hanson, 1961; Tomasi *et al.*, 1965). The SC was described as a glycoprotein produced by epithelial cells lining the gastrointestinal and respiratory tracts.

The presence of the SC on the sIgA molecule was shown to confer resistance to proteolysis, a characteristic conveying fundamental advantages in the environment in which sIgA imparts its activity (Brown *et al.*, 1970; Shuster, 1971; Lindh, 1975). The SC was also shown to play an important role in trans-

port of dimeric IgA to the external surface of the intestine (Tomasi *et al.*, 1965; South *et al.*, 1966). While investigating the role of serum IgA, Butler and fellow researchers (1967) demonstrated that intravenously–injected radio–labelled monomeric IgA could not be found in significant amounts in nasal secretions. They concluded that circulating monomeric IgA did not contribute to the predominantly polymeric IgA found in nasal secretions. Support for Butler and his colleagues was provided by immunochemical studies in humans (Bienenstock and Straus, 1970) and rabbits (Lawton and Maage, 1969) which provided evidence that polymeric IgA was assembled from monomeric IgA within subepithelial IgA-secreting cells. Polymeric IgA then bound to the SC which acted as a receptor on the surface of the epithelial cell. Following this step the sIgA was shown to be transported into the external secretions (Brandzaeg, 1985). Thus the sIgA response was seen to involve both IgA–secreting plasma cells and SC–producing epithelial cells.

1.1.2 The follicle–associated epithelium of Peyer's patches — antigen uptake and processing

The Peyer's patches are structurally similar to the peripheral lymph node insofar as they are organized into B cell areas (called follicles) and T cell areas (called parafollicles; Parrott, 1976). The follicular region was designated secondary when germinal centers were present. They have in addition a third region, the follicle–associated epithelium (FAE; Owen and Jones, 1974)

Owen and Jones (1974) showed that within the FAE there was a reduced number of goblet cells with respect to the surrounding epithelium, and also noted the presence of specially modified cells called M cells. M cells differed from the surrounding columnar epithelial cells in that they lacked fully developed microvilli, had a thin atypical cytoplasm, and had very few lysosomal vesicles in the cytoplasm (Owen, 1982). The function of this highly modified epithelial tissue was thought to be one of antigen uptake from the intestinal lumen. Several

lines of evidence supported this idea. Firstly, the reduced number of mucous–secreting goblet cells reduced the mucous covering of the FAE, which in turn increased its accessibility to antigen (Owen and Jones, 1974). Owen and co–workers (1986) demonstrated that M cells were able to take up and transport *V. cholerae* across the epithelium. This was consistent with the earlier finding that M cells transported soluble antigen such as horse–radish peroxidase in an energy–dependent fashion (Owen, 1977). However, the *E. coli* strain RDEC-1 adhered to, but was not transported by, M cells (Inman and Cantey, 1983).

The parafollicular region interacts with the FAE

Other cells present in or in contact with the FAE are the T cells and macrophages (Owen, 1982; Mayrhofer *et al.*, 1983; Spalding *et al.*,1983). In one study, T cells of the helper phenotype were found to greatly outnumber those with a cytotoxic/suppressor phenotype in the parafollicular region of mice (Carlson *et al.*, 1985). This area was also seen to contain Ia^+ dendritic cells (Sminia *et al.*, 1982; Mayrhofer *et al.*, 1983). Furthermore macrophages and dendritic cells isolated from murine Peyer's patches were found to function as antigen–processing and –presenting cells *in vitro* (Inaba *et al.*, 1983; Richman *et al.*, 1983; Kapsenberg *et al.*, 1986).

Hence, this *prima faciae* evidence suggested that the primary function of the FAE and the parafollicular regions was antigen uptake (FAE), and antigen processing and presentation (parafollicular).

1.1.3 The Peyer's patches are an enriched source of IgA precursor cells

Antigen induced differentiation of IgA precursor cells

The mechanism by which IgA-secreting plasma cells were derived, and the primary location of precursor cells, was the subject of many investigations. By transfer of Peyer's patch lymphocytes to an irradiated host, lymphocytes that

expressed the L-chain allotype of the donor were found to repopulate the Peyer's patches and the intestinal mucosa (Jacobson *et al.*, 1961; Craig and Cebra, 1971). The majority of cells in the germinal centres of secondary follicles in Peyer's patches, bear surface IgA (Mowat, 1987). This contrasts with those in the germinal centres of peripheral lymph nodes which bear surface IgG or IgM (Kraal *et al.*, 1982). Interestingly, T cells with a T helper phenotype isolated from murine Peyer's patches were found to induce B cells from Peyer's patches to switch from expression of surface IgM to surface IgA (Kawanishi *et al.*, 1982, 1983a). This contrasted with a switch from surface IgM to surface IgG by splenic B cells in the presence of spleen derived T cell clones (Kawanishi *et al.*, 1983a). This specific switching of B cells in the Peyer's patches underlined their involvement in the IgA response.

These investigators put forward the proposal that the Peyer's patches were an enriched source of precursors for sIgA-producing plasma cells which had the potential of populating mucosal tissue during an immune response. This proposal was supported by evidence obtained using two isolated rabbit ileal loops, one with, and the other without a Peyer's patch, while keeping the flow of blood and lymph intact. The loop devoid of any Peyer's patches was unable to induce a sIgA response in either loop after antigenic stimulation. In contrast, the loop with a Peyer's patch was able to direct an sIgA response in either loop after antigenic stimulation (Robertson and Cebra, 1976). In contrast, Hamilton and co–workers (1981) and Heatly and co–workers (1981) demonstrated that removal of Peyer's patches diminished only slightly the intestinal immune response. Thus, although the Peyer's patches are probably the major location of precursor cells capable of proliferation, differentiation, migration,and repopulation of mucosal surface with sIgA-secreting plasma cells, other areas of the gut associated lymphoid tissue (GALT) may also be able to provide this response after local antigenic stimulation.

Migration and terminal differentiation of IgA$^+$ plasma cells

The follicles and germinal centres of the Peyer's patches were seen to be subject to constant depletion of cells that migrated to other sites in the secretory immune system (Reynolds and Pabst, 1984). Steer (1980) demonstrated that efferent lymph from Peyer's patches of rats contained approximately 75% immunoglobulin–negative cells (presumptive T cells) and 25% B cells, indicating that the Peyer's patches might be engaged in traffic of both subsets. The terminal differentiation of IgA$^+$ plasma cell precursors, derived in the Peyer's patches, was believed to occur in the mesenteric lymph node (Kawanishi *et al.*, 1983b).

In sumary then, the predominance of IgA precursor plasma cells, and the virtual absence of mature plasma cells, provided evidence that Peyer's patches were the site of antigenic stimulation of the GALT.

1.1.4 The common mucosal system

Subsequent investigations of immunity at other mucosal surfaces led to the identification of the bronchial–associated lymphoid tissue (BALT) (Rudzik *et al.*, 1977) as another source of precursor cells. While the majority of precursor cells, upon antigenic stimulation of the BALT or the GALT, returned to the site of antigenic stimulation, a minority of sIgA$^+$ plasma cells repopulated the remote secretory surfaces such as the mammary gland, parotid gland, lacrimal gland and cervical gland of the uterus (Husband and Gowans, 1978 ; McDermott and Bienenstock, 1979; Jackson *et al.*, 1981; Montgomery *et al.*, 1983). On the basis of these observations it was proposed that a common mucosal system was operational in both animals (McDermott and Bienenstock, 1979) and humans (Mestecky *et al.*, 1985).

1.2 The function of secretory IgA

A number of studies demonstrated that the biological function of sIgA was to prevent initiation of infection by organisms that use the mucosal surface as their primary location of attachment during development of disease. Early studies found that sIgA was neither opsonic nor did it have bacteriocidal activities (Steele *et al.*, 1977; Rowley, 1977). Other investigators demonstrated that sIgA could efficiently cross–link particles, prevent adhesion, and inactivate toxins (reviewed below; Ishizaka *et al.*, 1965; Williams and Gibbons, 1972; Svennerholm *et al.*, 1982).

1.2.1 Inhibition of adherence prevents colonisation

The anti–adherence activity of sIgA may prevent colonisation of the epithelium by enteropathogens. Anti–adherence activity was first demonstrated by Williams and Gibbons (1972), who showed that purified sIgA inhibited adhesion of oral *Streptococci* to buccal epithelial cells *in vitro*. Further evidence of anti–adhesion activity was provided by Fubara and Freter (1973). They used isolated intestinal loops of mice to demonstrate that *V. cholerae* colonised significantly less in mice that had been previously immunised orally with *V. cholerae*. This reduction was thought to be a result of increased levels of anti–*V. cholerae* sIgA in the intestine, which presumably excluded the vibrios from the mucosal surface (Fubara and Freter, 1973). Other investigators demonstrated that rabbits orally immunised with purified CFA/I fimbriae of ETEC were protected against challenge with virulent ETEC expressing homologous fimbriae, as judged by the RITARD model. This protection correlated with increased anti–CFA/I sIgA levels. This response was highly specific since protection was not provided against challenge (ETEC) expressing heterologous fimbriae (de la Cabada *et al.*, 1981; Evans *et al.*, 1982). Secretory IgA was also shown to prevent adhesion of *E. coli* to the human urinary tract epithelium (Svanborg–Eden and Svennerholm,

1978).

Further studies have shown that adherence of *Salmonella spp.* to the luminal wall of the intestine may be inhibited by sIgA (Magnusson *et al.*, 1979; Mangusson and Stjernstorm, 1982). In this case sIgA was thought to exert anti–adhesion activity by causing an increased surface hydrophilicity. This changed physical property, it was argued, led to increased affinity of the invading *Salmonella* pathogen for the mucous layer and resulted in its entrapment and removal by the natural cleansing action of the intestine (Magnusson *et al.*, 1979; Mangusson and Stjernstorm, 1982).

To test whether intestinal microflora amplified the immune effects of sIgA, both conventional and mono–associated mice were orally immunised with inactivated *V. cholerae*. Conventional animals were found to have 10– to 30–fold less vibrios in their intestine, after challenge with *V. cholerae*, than mono–associated animals. The slight inhibition of colonisation caused by the immune response, therefore, allowed the microflora to effectively exclude the challenge organisms (Sack and Miller, 1969; Miller *et al.*, 1972). Miller and colleagues (1972) have proposed that this mechanism might provide the selective advantage for serotype conversion often seen with intestinal pathogens.

1.2.2 Antitoxin activity prevents enterotoxins binding with their receptors

Much evidence has been provided to establish that sIgA has antitoxin activity. A close correlation was observed between emergence of antitoxin sIgA in the intestine and protection against experimental cholera in mice, after oral immunisation with cholera toxin (CT; Svennerholm *et al.*, 1978). The antitoxin activity of sIgA seen here was thought to act by blocking the adhesion of CT to its receptor, ganglioside GM_1, which is expressed by the intestinal enterocytes (Wu and Walker, 1976). Other investigations demonstrated that oral immunisation with ETEC heat–labile toxin (LT) or the B subunit of LT (LT-B) stimulated

synthesis of specific sIgA (Klipstein *et al.*, 1982a). These antitoxin antibodies were shown to be protective (Klipstein *et al.*, 1982a, 1982b, 1983). Furthermore, oral immunisation with LT-B was shown to provide a protective antitoxin sIgA response against LT or CT, whereas oral immunisation with the B subunit of CT (CT-B) induced weaker cross–protection (Klipstein *et al.*, 1984). In another study the number of antitoxin–producing cells in the intestine of mice were shown to correlate ($P<0.001$) with protection against intestinal challenge with CT (Lange *et al*, 1979).

1.2.3 Antibacterial versus antitoxin immunity

As discussed above experimental evidence suggests that sIgA has both antibacterial and antitoxic activity. Much speculation has ensued, therefore, as to whether immunity against the surface of bacterial pathogens or their toxins would be more effective in preventing disease. Neoh and Rowley (1972) investigated the protective potential, on a weight basis, of serum antibody specific for surface antigens of *V. cholerae* or CT, in preventing cholera in the infant mouse model. They found that antibacterial antibody was 10–fold more effective than antitoxin antibody in providing protection. Cash and co–workers (1974a) provided the best evidence that antibacterial mechanisms in the gut were more important than antitoxin mechanisms. They demonstrated that immunity elicited by natural choleraic disease (both antitoxin and antibacterial antibody) provided protection only against homologous challenge with virulent *V. cholerae*. This result suggested that antitoxin immunity was of little relevance to protection. Porcine ETEC infections also elicit a similar serotype–specific protective immune response (Morgan *et al.*, 1978). Recently Levine and his colleagues (1988) provided further evidence that antitoxin immunity was not an absolute prerequisite of protective immunity against cholera in humans. They found that oral immunisation of humans with a CT–deletion mutant of *V. cholerae* (expressing neither A nor B subunit of CT) provided significant protection against challenge with virulent *V. cholerae*.

1.2.4 The synergistic affects of anti–adherence and anti-toxin responses

Other workers believe that antibacterial and antitoxin immunity combine synergistically to provide high levels of protective immunity (Svennerholm and Holmgren, 1976). In one study passive antibody was used to demonstrate the synergistic effects of anti–LT antibody in combination with anti–CFA/I or anti–CFA/II antibody in rabbits (Ahren and Svennerholm, 1982). The combination of antiserum against CFA/I or CFA/II with anti–LT provided protection that equalled the product of the protective effects obtained with each antiserum alone, that is: the antisera cooperated synergistically (Ahren and Svennerholm, 1982).

1.3 The cell mediated immune response

It has been proposed that cell mediated immunity (CMI) might be divided into either systemic CMI, largely mediated by the liver and spleen (Mackaness, 1971) or intestinal CMI, mediated by the intestine (Sprinz *et al*, 1966; Newby, 1984). Very little is known about the latter, since early experimental models involving *S. typhimurium, S. enteriditis* or *Listeria monocytogenes* infections in mice were based primarily on parenteral vaccination. These early experimental models did, however, contribute to our understanding of the importance of CMI in protection against invasive organisms such as *Salmonella spp.*.

1.3.1 The involvement of macrophages in CMI

The involvement of the phagocytic cell in immunity to bacterial infections was first described by Metchnikoff (1893). He suggested that the phagocytic cell played a central role in host defence and that functional modification of such cells was necessary for the expression of resistance to certain bacteria.

In 1942, Lurie demonstrated that macrophages which contained ingested tubercle bacilli, harvested from rabbits vaccinated with *Mycobacterium bovis*

strain Bacille Calmette Guerin (BCG), when cultured in the anterior chamber of the eye of normal rabbits, were able to inhibit the growth of the ingested tubercle bacilli. Macrophages from normal rabbits containing ingested tubercle bacilli could not inhibit the growth under the same conditions. By performing this experiment, Lurie provided the first direct evidence that resistance to intracellular bacterial parasites depended on alteration of the properties of the phagocytic cells. Investigations by Suter (1953), Mackaness (1954, 1962 and 1968), Hobson, (1957), Elberg (1960), and Saito and co–workers (1962), using either *Brucella, Salmonella, Listeria* or a variety of *Mycobacteria*, established the importance of cellular factors in the immune response for the development of immunity against intracellular parasites. In particular, Mackaness (1962) correlated the onset of delayed type hypersensitivity (DTH) with resistance to challenge by *L. monocytogenes*. He was not able to transfer immunity passively with the serum and so attributed protection to the histological changes observed in macrophages.

Further support for the idea that macrophages were responsible for immunity to intracellular parasites came from results obtained using experimental *S. typhimurium* infections of mice. In a series of experiments mice were infected intravenously (iv) with sub–lethal doses of *S. typhimurium*. Challenge doses of *S. typhimurium* on day 8, 11, 14, or 18 after the primary infection was effectively cleared. This immunity was not transferred in the serum (Blanden *et al.*, 1966; Mackaness, *et al.*, 1966). These investigators concluded that the antibacterial mechanism which developed in immunity to *Salmonella* infection was due to macrophages with enhanced bactericidal activity. These results were confirmed by Collins and Mackaness (1968) and Collins (1969a and 1969b).

The changes that have been observed in activated macrophages include: increased phagocytosis and intracellular killing activity (Blanden *et al.*, 1966), increased specific activity of digestive enzymes (Auzins and Rowley, 1962; Damenberg, 1968; Hard, 1971), and transient expression of Ia^+ phenotype (Schwartz *et al.*, 1976; Lu *et al.*, 1979; Steinman *et al.*, 1980a; Barclay, 1981).

1.3.2 The involvement of T cells in CMI

Many investigators have studied the role of antigen–specific T cells in immunity to intracellular parasites. McGregor and fellow researchers (1973) demonstrated that thymectomised and irradiated rats mobilised their cellular defences against *L. monocytogenes* infection more effectively when they were reconstituted with thoracic duct lymphocytes. They argued that thymus–dependent lymphocytes influenced the development of cellular resistance to infection. Investigations with *Salmonella, Listeria*, and *Mycobacteria* established the concept that antigen–specific T cells play a critical role during the development of immunity to intracellular parasites (Mackaness, 1971; Lefford, 1975, Lefford and McGregor, 1978; North, 1975, 1981). T cells from immune mice were shown to release lymphokines which increased the number and bactericidal action of tissue macrophages (Collins, 1979; North, 1981). Furthermore, macrophages were shown to express Ia antigen in response to treatment with such T cell derived factors (Beller *et al.*, 1980; Steinman *et al.*, 1980a). Other researchers found that immune T cells were responsible for the longevity of immunity (immunological memory), which remained long after the transient state of macrophage activation (Collins, 1979; North, 1981).

Adoptive transfer systems and *in vitro* culture techniques were used to determine the nature of the T cell involved in DTH (Kaufmann and Hahn, 1982; Kaufmann, 1983; Attridge and Kotlarski, 1985a, 1985b). Such *in vitro* investigations demonstrated that T cells generated by *Listeria* infection were pluripotential. Kaufmann and Hahn (1982) isolated antigen–specific clones which proliferated and released lymphokines when cultured with specific antigen. The same T cell clones mediated DTH and protection *in vivo* (Kaufmann and Hahn, 1982). The phenotype of the T cell clones was $Lyt1^{+}2^{-}$ (L3T4) and their activity was restricted by the I-A subregion of the major histocompatibility complex (Kaufmann and Hahn, 1982; Kaufmann, 1983). Mediators of DTH induced in mice by infection with *S. enteriditis* strain 11RX were also found to be T cells with the surface phenotype of $Lyt1^{+}2^{-}$ (L3T4). T cells of this type were used

in an adoptive transfer system which required compatibility at the I-A subregion of the major histocompatibility region (Attridge and Kotlarski, 1985a). Attridge and Kotlarski (1985b) also found that these antigen–specific immune T cells, when cultured *in vitro* with specific antigen, secreted interleukin–2 and macrophage activating factor, revealing the pleuripotential role antigen–specific immune T cells play in immunity.

1.3.3 DTH as a measure of CMI

Much debate has arisen as to whether DTH was a good measure of CMI. Mackaness (1969) found that the onset of a DTH response correlated with protection. Other investigators have also found that immunity to *S. typhimurium* correlated with DTH (Ahmed *et al.*, 1978; Attridge and Kotlarski, 1985a). However, recent reports have suggested that DTH may not be a good indication of protective immunity. Eisenstein and co–workers (1984) showed that an *S. typhimurium aroA* strain induced protective immunity against virulent *S. typhimurium* challenge in hypersusceptible mouse strains C3H/HeJ and C3HeB/FeJ (Eisenstein *et al.*, 1984; Killar and Eisenstein, 1985). This immunity was found to be CMI and in particular macrophage activation as judged by the capacity of immunisation to induce immunity that was cross–protective against both virulent *S. typhimurium* challenge and *L. monocytogenes* challenge. Furthermore, immune spleen cells were able to adoptively transfer this immunity (Killar and Eisenstein, 1985). However, C3H/HeJ and C3HeB/FeJ mice never mounted significant DTH responses following immunisation with doses that provided protection against 1300 median lethal (LD_{50}) doses of *S. typhimurium*. In agreement with this finding, was the observation the that naturally resistant mouse strains, C3H/HeNCrlBR, displayed DTH in a dose dependent manner yet a dose could be found that resulted in increased resistance to infection but without concomitant elicitation of DTH (Killar and Eisenstein, 1986). It was therefore concluded that DTH might not be a reliable measure of immunity to *S. typhimurium* infection in mice.

1.3.4 Involvement of antibody in CMI

While the involvement of macrophages and T cells in immunity to intracellular parasites became an accepted concept much controversy arose over the role of antibody. If activated macrophages were non–specific then at the height of resistance any intracellular parasite would be phagocytosed and removed at a rate equal to that of the parasite to which the immunity was raised. Total non–specific killing of intracellular parasites was put under some doubt when Coppel and Youmans (1969) demonstrated that mice immunised and challenged with either *Listeria* or tubercle bacilli, eliminated the homologous challenge more efficiently than the heterologous challenge.

Earlier Jenkin (1963) had shown that specific opsonins both enhanced phagocytosis and influenced the fate of the ingested organism. McIntyre and co–workers (1967) used an *in vitro* experimental system which examined the interaction of mouse peritoneal macrophages with *S. typhimurium* or *L. monocytogenes* at a single cell level. In this system the presence of specific antibody affected the rate of phagocytosis as well as the rate of intracellular killing. Furthermore, the presence of specific antibody induced by immunisation with an alcohol–killed vaccine significantly reduced the size of an orally introduced challenge and delayed the dissemination of the organism to the liver and spleen (Collins and Carter, 1972). Further support for the involvement of antibody in CMI was provided by Rowley and his colleagues (1964) who demonstrated the presence of cytophilic antibody on the immune macrophage. This antibody, when eluted, transferred immunity to normal animals.

In general, the concept that macrophages, T cells and antibody play an important role in CMI against intracellular bacterial parasites has gained acceptance (Cooper *et al.*, 1983).

1.3.5 Local CMI of the intestine

As mentioned earlier, the significance of local CMI mechanisms in the intestinal tract has received little attention. However, some investigators have provided evidence that suggested that local CMI responses occur. Oral immunisation of domestic pigs with protein antigen (Huntley *et al.*, 1979) or enteric viral infection (Frederich and Bohl, 1976; Brundage *et al.*, 1980) significantly increased lymphokine release by T cells in the lamina propria. The possibility that this lymphokine release may activate macrophages has not yet been fully investigated. Sprinz and fellow researchers (1966) found an accumulation of mono–nuclear cells and a granulomatous response in the lamina propria of the intestine following experimental infection with typhoid bacilli. This mononuclear response preceded septicemia and the appearance of systemic antibody which suggested that it was part of a local intestinal response (Sprinz *et al.*, 1966). Furthermore, mice immune to *L. monocytogenes* cleared this organism rapidly upon secondary infection by the oral route. The site of clearance was found to be in the Peyer's patches which became heavily infiltrated with activated macrophages during the protective response (MacDonald and Carter, 1980). This intestinal response may occur very rapidly as cellular changes were noted two days after infection (Huntley *et al.*, 1979; Asherson *et al.*, 1977).

1.3.6 Other mechanisms of intestinal CMI

The possibility that local intestinal CMI might be mediated by mechanisms other than macrophage activation has also been investigated. Ferguson and MacDonald (1977) noted that T cell activation and lymphokine release increased the rate of production and the rate of loss of enterocytes. They argued that this increased loss of enterocytes might interfere with organisms colonising these cells and thus provide immunity. Some indirect evidence exists to support this proposal: crypt hyperplasia and villous atrophy are seen following infection of mice with *Trichinella spiralis*. The appearance of this T cell phenomenon

coincides with the expulsion of the worm (Manson-Smith *et al.*, 1976). However no evidence exists to demonstrate that this type of immunity occurs during enteric bacterial infections.

In addition cells have been identified in the lamina propria and epithelium that have functional characteristics of T cells such as cytotoxicity (Davies and Parrott, 1980; McDermott *et al.*, 1980), mediation of delayed hypersensitivity (Frederich and Bohl, 1976; Huntley *et al.*, 1979), or cells with natural killer activity (Kaminski and Jakobisiak, 1980) and cells capable of antibody–dependent cell–mediated cytotoxicity (ADCC; Arnaud–Battandier, 1978). The role these cells play in the local immune response to enteric infections is not clear although recently ADCC–capable cells were found in the peripheral blood of humans immunised with the live oral typhoid vaccine, Ty21a (Tagliabue *et al.*, 1985). The activity of ADCC–capable cells induced by immunisation with Ty21a, required antigen–specific IgA (found concomitantly in the serum) for their activity (Tagliabue *et al.*, 1985). Whether these ADCC–capable cells found in the peripheral blood are a result of local immunity or systemic CMI has not yet been resolved.

1.3.7 The functional role of the diffuse lymphoidal compartment in intestinal immunity

The diffuse mucosal lymphoid compartment consists primarily of the enormous number of lymphoid cells which are scattered in the mucosa throughout the length of the small intestine (Mowat, 1987). This compartment can be divided into two regions based on the phenotypic differences of lymphocyte populations. These regions are (i) the lamina propria, and (ii) the epithelial layer.

The lamina propria is enriched with effector cells

The first of these regions, the lamina propria, contains the cellular elements typical of a chronic inflammatory exudate — namely lymphocytes, plasma cells,

macrophages, and eosinophils. Although the numbers of these cells are influenced by establishment of a conventional gut flora, these lymphoid cells are present in segments of the intestine that are bacteria– and antigen–free (Ferguson and Parrott, 1972a; Ferguson and Parrott, 1972b). Mayrhofer *et al.* (1983) demonstrated that lymphoid cells are also present in the lamina propria of fetal rats. These observations implied that the lamina propria has some intrinsic characteristics that are conducive to the establishment of residence therein by lymphoid cells.

Functionally, the lamina propria appears to be the effector site of both humoral and cellular immunity. This was supported by the following lines of evidence. The majority of B cells (80-90%) were IgA–secreting plasma cells (Lyscom and Brueton, 1982; Tseng, 1982). Furthermore, lamina propria lymphocytes synthesized IgA spontaneously in culture and actively secreted immunoglobulin *in situ*, suggesting that the B cell population of the lamina propria was already terminally differentiated before arriving in the intestine (Tseng, 1982; Drew *et al.*, 1984). The number of lamina propria plasma cells was greatly enhanced by the establishment of an enteric flora (Ferguson and Parrotta, 1972a; Crabbe *et al.*, 1986). However some plasma cells have been shown to arise by local cell division and differentiation in the lamina propria suggesting antigenic stimulation and lymphoid cell differentiation can occur in this tissue (Husband and Gowans, 1978; Mayrhofer and Fisher, 1979; Husband, 1982, Pierce and Cray, 1982). The extent of this role in the humoral response is not clear.

Recent analysis of lymphocyte populations in the lamina propria showed that 30% are T cells (Lyscom and Brueton, 1982; Parrott *et al.*, 1982). T cells of a helper phenotype were shown to outnumber the suppressor/cytotoxic phenotype by 2:1 (Selby *et al.*, 1981, 1983). The T cells of the lamina propria respond only moderately to mitogenic stimulation implying that these cells are also terminally differentiated (Mowat, 1987).

Macrophages of the intestinal lamina propria have been observed to phagocytose invading bacteria in mice infected with *S. typhimurium* (Takeuchi

and Sprinz, 1967; Takeuchi, 1971) and in chickens infected with *Campylobacter fetus* (Ruiz-Palacios, 1981). Phagocytosis of invading *Shigella flexneri* was also observed in the colonic mucosa of monkeys (Takeuchi *et al.*, 1968; Takeuchi 1971). A high proportion of macrophages of the lamina propria were Ia^+, underlining their state of activation (Mayrhofer *et al.*, 1983; Wilders, 1984). However, only a minority of macrophages in normal rats (Barclay, 1981) and normal mice (Schwartz *et al.*, 1976; Lu *et al.*, 1979) expressed Ia antigens, and virtually no macrophages in newborn mice were Ia^+ (Barclay, 1981). T cell–derived factors were used to induce expression of the Ia antigen by lamina propria macrophages but expression was only transient as it was lost when cells were transferred to tissue culture (Beller *et al.*, 1980; Steinman *et al.*, 1980a, 1980b). Lamina propria macrophages have also been shown to extend cytoplasmic protrusions into the epithelial layer which might provide an additional method of antigen sampling (Mayrhofer *et al.*, 1983).

The intraepithelial lymphoid cells interact directly with the intestinal lumen

The second region of the diffuse mucosal lymphoid compartment is the epithelial layer. This layer is in closest proximity to antigen so it is not surprising that 15% of the epithelial cell population of mammalian species are lymphocytes (Mowat, 1987). The intraepithelial lymphocytes (IEL) are a heterogeneous population of cells whose origin and nature is of some controversy. Their numbers were greatly reduced in athymic animals (Fichtelius *et al.*, 1968, Ferguson and Parrott, 1972a; Mayrhofer, 1980) and results from human studies suggested that most IEL were T cells (Janossy *et al.*, 1980; Selby *et al.*, 1981). These findings were confirmed by fluorescent antibody analysis of IEL isolated from human gut (Greenwood *et al.*, 1983). Most IEL express suppressor/cytotoxic phenotype (70%) whereas only small numbers of lymphocyte helper phenotypes were found (Lyscon and Brueton, 1982; Parrott *et al.*, 1982; Greenwood *et al.*, 1983; Cerf–Bensussan *et al.*, 1984; Dillon and MacDonald, 1984). No other lymphoid organ shared

this unusual feature of large numbers of suppressor/cytotoxic T cells and low proportion of helper cells. Movement of IEL has been observed in both directions across the basement membrane and it seemed probable that most reentered the lamina propria. The possibility that some IEL are shed into the gut lumen cannot be ruled out (Toner *et al.*, 1971).

IEL have been shown to give rise to long term T cell lines in culture (Petit *et al.*, 1983; Schrader *et al.*, 1983). These T cells were found to be plurifunctional in that they had cytotoxic T cell activity (Davies and Perrott, 1981; Mowat *et al.*, 1983), produced lymphokines, proliferated in response to T cell mitogens and proliferated in mixed lymphocyte reactions (Arnand-Battandier and Nelson, 1982; Greenwood *et al.*, 1983; Dillon and MacDonald, 1984, Cerf-Bensussan *et al.*, 1984). In the above studies IEL responses *in vitro* were either potentiated by or absolutely required accessory cells. Therefore, although these findings suggest that IEL have the ability to provide many T cell functions it is not yet known whether these are active within the epithelium since accessory cells are lacking from this site.

1.4 Elicitation of Immunity by Vaccination

1.4.1 Parenteral vaccines

The systemic immune system and the local intestinal immune system appear to be compartmentalised (Orgra *et al.*, 1968; Heremans, 1974). Parenteral vaccines generally did not stimulate a sIgA response in the GALT since vaccines given by this route induced systemic humoral immunity, manifested primarily by IgM and IgG. Furthermore, numerous attempts using experimental *S. typhimurium* and *S. enteriditis* infections in mice found that inactivated parenteral vaccines failed to elicit significant CMI (Holland and Pickett, 1956; Ushiba *et al.*, 1959; Mackaness *et al.*, 1966; Collins, 1969a, 1969b; Collins and Carter, 1972).

Recent investigations on the effectivness of inactivated parenteral vaccines

were performed using mouse strain C3H/HeJ. Earlier work demonstrated that mouse strain C3H/HeJ was hypersensitive to *Salmonella* infection (O'Brien *et al.*, 1982). This increased susceptibility was thought to be a result of a genetic defect (lps^d), which rendered the mice hyporesponsive to the lipid A portion of lipopolysaccharide (Sultzer, 1968, 1969, 1976; Watson *et al.*, 1978). Eisenstein and Sultzer (1983) demonstrated that C3H/HeJ mice were poorly immunised by parenteral administration of inactivated *Salmonella* vaccines. They were able to demonstrate a humoral response after such immunisation but could not measure CMI. In contrast, Eisenstein and her colleagues (1984) were able to induce high levels of protective immunity against challenge doses as high as 9000 LD_{50} values of virulent *S. typhimurium* in mouse strain C3H/HeJ. This immunity was provided by parenteral immunisation with live bacilli of strain SL3235 (aro^-), an avirulent derivative of *S. typhimurium* (Eisenstein *et al.*, 1984). They found that this immunity was a result of macrophage activation and occurred without correction of the lps^d defect (Eisenstein *et al.*, 1984). This provided evidence that live vaccines provided superior protection than that provided by inactivated parenteral vaccines in mice.

Controlled field trials with parenteral vaccines

Despite evidence which suggested that parenteral typhoid vaccines did little to induce protective CMI, inactivated preparations of *S. typhi*, given parenterally, were able to confer significant protection against typhoid fever in humans, as demonstrated by a series of controlled field trials (Ashcroft *et al.*, 1964; Yugoslav typhoid committee, 1964; Polish typhoid committee, 1965). These trials demonstrated that an acetone–inactivated *S. typhi* vaccine provided between 79-93% protection. Phenol–inactivated preparations of *S. typhi* were not as effective at providing immunity, suggesting that maintenance of the integrity of antigenic determinants might be important for protection. Of the vaccinees receiving inactivated typhoid vaccines, 25% developed severe systemic and local reactions (Ashcroft *et al.*, 1964; Yugoslav typhoid committee, 1964; Polish

typhoid committee, 1965). Similar results were obtained in controlled field trials with parenterally–administered inactivated cholera vaccines (Azurin *et al.*, 1967; Mosley *et al.*, 1968). This was unexpected, as, results obtained from experimental animal models had demonstrated that analogous vaccination did little to induce local sIgA responses (Orgra *et al.*, 1968; Heremans, 1974). In contrast, inactivated *Shigella* preparations given parenterally to humans stimulated high titres of circulating antibody but provided little if any protection (Formal and Levine, 1984).

Parenteral vaccination boosts preexisting immunity

The mechanism by which parenteral vaccines afforded high levels of protective efficacy has been the subject of some debate. An investigation by Dham and Thompson (1982) demonstrated that parenteral typhoid/paratyphoid vaccine (TAB) stimulated both humoral and cellular immunity. This confirmed the results of an earlier investigation by Kumar and co–workers (1974).

Other investigators argued that the outcome of parenteral vaccination was strongly influenced by prior exposure to the antigen being administered (Ashcroft *et al.*, 1964; Hornick *et al.*, 1970b). This proposal was supported by experimental evidence in humans and mice. Swedish women who were not previously exposed to *V. cholerae*, when immunised parenterally with inactivated cholera vaccine, did not secrete sIgA antibodies (Svennerholm *et al.*, 1980). In contrast, when the same immunisation protocol was used on Pakistani women, all of whom were previously exposed to *V. cholerae*, a significant sIgA response was observed (Svennerholm *et al.*, 1980). Further evidence was provided by Bloom and Rowley (1979), who found a significant increase in sIgA when using a protocol of oral vaccination, followed by parenteral vaccination, with a cholera vaccine in mice. Furthermore, Hornick and Woodward (1966) found parenteral typhoid vaccines, given to North American volunteers, to be significantly less protective against typhoid challenge than appeared to be the case when the vaccines were used in typhoid–endemic regions.

Future prospects of parenteral vaccines

Parenteral vaccines might be improved by the use of adjuvants or improved methods of inactivation which preserve protective antigens (Wong *et al.*, 1974; Robbins and Robbins, 1984; Tacket *et al.*, 1986). Other investigators suggested that covalent linkage of T-independent antigens to a protein carrier might convert such antigens to T-dependence as demonstrated with *Haemophilus influenzae* type b polysaccharide/protein conjugates (Schaeerson *et al.*, 1980). Therefore, although there is still interest in inactivated parenteral vaccines, until methods can be devised which enable such vaccines to elicit CMI and/or sIgA responses, these vaccines will remain of limited use.

1.5 Vaccination by peroral administration

Since it was established that the local secretory immune response was important in protection against enteric infections, and that parenteral vaccines did little to stimulate either this type of response or CMI, many groups have turned their attention towards the development of oral vaccines. Oral vaccines have been given as either inactivated preparations or suspensions of live attenuated organisms.

1.5.1 Inactivated oral vacccines

As a general rule, inactivated oral vaccines elicited relatively poor immune responses and moreover, maximisation of the local immune response required very large doses of antigen. Laboratory rodents manifested local immune responses when repeated doses of about 1×10^{10} inactivated bacteria were given orally (Waldman *et al.*, 1972; Michalek *et al.*, 1978; Stokes *et al.*, 1979), while in larger animals such as pigs and man the number of inactivated bacteria required to induce an immune response was of the order of 1×10^{11} (Giannella *et al.*, 1973; DuPont *et al.*, 1971a, 1971b; Evans *et al.*, 1981; Newby *et al.*, 1981; Dziaba *et*

al., 1980). Coproantibodies were detected by Freter and Gangarosa (1963) after oral administration of inactivated *V. cholerae* (150 mg dry wt.) given daily for 4 successive 7 day periods. Such vaccines appeared best able to boost immunity induced by parenteral vaccination. Thus, it has been shown that when rats were immunised with LT-B of porcine ETEC either orally, parenterally or in combination, the highest levels of protection were seen in animals immunised by the parenteral–oral route combination (Klipstein and Engert, 1981).

In a human volunteer study, inactivated oral cholera vaccines did not afford protection as high as that observed after natural *V. cholerae* infection (Cash *et al.*, 1974a). Moreover, parenteral immunisation provided higher levels of protection than was seen after oral administration of 1.6×10^{10} inactivated vibrios, given daily for 10 days or, in the same schedule, with a follow–up booster of 5 extra daily doses (Cash *et al.*, 1974a).

Combination cholera vaccines

Higher levels of protection were obtained using combination vaccines which stimulated both antitoxic and antibacterial immunity. These two immune components were shown to act synergistically to provide high levels of protection (Svennerholm and Holmgren, 1976; Ahren and Svennerholm, 1982). One such combination vaccine consisted of heat/formalin inactivated *V. cholerae* of Classical and El Tor biotypes, and both Ogawa and Inaba serotypes (total of 1×10^{11} vibrios/dose) in combination with procholeragenoid (50μg for the first two doses and 200μg for the third dose). Although a vaccine efficacy of only 27% was observed there was significant reduction in the severity of the disease, indicating that some immunity was elicited (Levine *et al.*, 1983b). Another combination vaccine given orally to human volunteers consisted of alcohol–inactivated *V. cholerae* (El Tor, Inaba; 5×10^{10}/dose) in combination with glutaraldehyde–treated cholera toxin (2.0 mg/dose). Volunteers received 2 mg of toxoid weekly for 4 weeks followed by a dose of inactivated vibrios twice weekly for 4 weeks. Challenge of the vaccinees and controls 1 month later with 1×10^{6} El Tor Inaba

vibrios showed that this vaccine provided 67% protection, as well as a clearly attenuated illness in the two vaccinees that became ill (Levine *et al.*, 1983b).

More recently a CT-B–whole cell vaccine (B-WCV) against cholera has been extensively evaluated. Svennerholm and fellow researchers (1984) demonstrated that two doses 28 days apart of B-WCV (2.5×10^{10} Classical Inaba, 2.5×10^{10} Classical Ogawa, with purified CT-B (0.25 mg for the first dose and 0.5 mg for the second dose)), induced intestinal and serum antibody responses in healthy Bangladeshi women. Moreover the immune response seen after such vaccination closely resembled the immune response which was seen in patients with naturally acquired cholera disease. In a subsequent study Swedish volunteers who had previously been immunised with B-WCV (five years earlier) were shown to manifest a 10–fold higher serum response (both IgG and IgA) than controls when re–immunised with B-WCV. Thus, immunological memory to *V. cholerae* antigens was sustained in these Swedish volunteers for up to five years following vaccination with B-WCV (Jertborn *et al.*, 1988). Evidence demonstrating the relevance of this long term immunity to long–term protection was not provided as the qualitative nature of this memory response was not investigated. Nonetheless it was clear that B-WCV was highly immunogenic and that it elicited long term immunological memory.

The efficacy of B-WCV was evaluated in North American volunteers (Black *et al.*, 1987a). The volunteers received 3 doses consisting of 2×10^{11} inactivated vibrios (a mixture of Classical Inaba, Classical Ogawa, and El Tor Inaba) in combination with 5 mg of purified CT-B. In this study oral administration of B-WCV afforded 64% protection against challenge with virulent *V. cholerae* and the WC component when given alone afforded 56% protection (Black *et al.*, 1987a).

The efficacy of B-WCV has also been evaluated in a large–scale field trial conducted in Bangladesh (Clemens *et al.*, 1986b). Here the WC component consisted of 1×10^{11} inactivated vibrios (4 different preparations of 2.5×10^{10} cells: i] heat inactivated *V. cholerae* 01 Classical Ogawa; ii] heat inactivated *V. cholerae*

01 Classical Inaba; iii] formalin–inactivated *V. cholerae* 01 El Tor Inaba; iv] formalin–inactivated *V. cholerae* 01 Classical Ogawa) in combination with CT-B (1mg/dose). Each vaccinee received 3 doses, given orally, of either B-WCV or WCV alone. The B-WCV was initially found to provide 85% protection whereas the WCV gave only moderate protection (53%). The level of protection was influenced by the force of infection such that the protection afforded by B-WCV dropped to as low as 53% when an epidemic spread over the test area (Clemens *et al.*, 1986b).

After a one year surveillence period the protective efficacy of the WCV was 53% and the protective efficacy of the B-WCV was 62% (Clemens *et al.*, 1987). The B-WCV was seen to give better protection for the first 8 month period after which the protection provided by B-WCV was the same as that given by the WCV alone. Furthermore, protection was greater in volunteers aged $\geq$6 years; protection in younger volunteers lasted for a shorter period (Clemens *et al.*, 1987). This suggested that a booster may be required in younger children to prolong protection.

Future improvement of B-WCV

The pathogenesis of *V. cholerae* O1 appears to be dependent on adhesion of this organism to epithelial cells of the intestinal lumen (Freter, 1980a, 1980b; Jones, 1980). The expression of fimbriae had long been suggested as the mediators of adhesion of *V. cholerae* (Tweedy *et al.*, 1968; Faris *et al.*, 1982; Ehara *et al.*, 1986), however, it was not until recently that the control of expression of fimbriae has become understood (Taylor *et al.*, 1987). Taylor and co–workers (1987) have also demonstrated that the gene product of the *tcpA* gene was the major fimbrial subunit of *V. cholerae*. The expression of the *tcpA* gene was found to be strigently controlled by the gene product of *toxR* (Taylor *et al.*, 1987). *V. cholerae* that lacked expession of the TcpA protein were unable to adhere to epithelial cells (Taylor *et al.*, 1987). It was proposed, therefore, that augmentation of fimbrial expression during preparation of inactivated B-WCV

might improve the immunogenicity of such vaccines, by increasing their ability to adhere to the luminal wall of the intestine (Taylor *et al.*, 1988).

Inactivated oral typhoid vaccines

In contrast to the success achieved with inactivated combination cholera vaccines, little or no efficacy was observed when inactivated preparations of *S.typhi* bacilli were given orally to humans. When 6 tablets (1×10^{11} acetone–inactivated *S. typhi* Ty2/tablet) were given to volunteers, no protective efficacy was detected. However, a reduction in attack rate (controls and vaccinees were compared) was seen when the dose was increased to 12 tablets. This suggested that some antibacterial immunity had been induced (Hornick *et al.*, 1970b; DuPont *et al.*, 1971a, 1971b).

This vaccine was further evaluated in a series of field trials conducted in India. However, no significant protection was noted even when tablets containing as many as 4×10^{11} acetone–inactivated *S. typhi* bacilli were given orally (Chuttani *et al.*, 1976). The conclusions derived from these field trials were questioned (Germanier, 1984), because vaccine efficacy was judged by unconventional methods. The incidence of typhoid in a vaccinated group before and after vaccination was compared, as was the incidence of typhoid in a vaccinated group living in one area to the incidence in a non-vaccinated group living in another area (Chuttani *et al.*, 1976).

Factors which affect the success of inactivated oral vaccines

Many factors might influence the effectiveness of orally administered inactivated vaccines. Pigs which lacked the receptor for the K88 adhesin of porcine ETEC did not mount a significant response to K88–bearing *E. coli*. It was presumed that in the intestine of such pigs *E. coli* bearing K88 were unable to adhere and therefore, could not interact with the GALT (Sellwood, 1981). This result suggested that contact with the luminal wall of the intestine was of importance in the induction of the secretory immune response. Levine and co–workers (1986)

found that direct administration of CFA/II fimbriae (CS1 and CS3 antigens) into the duodenum resulted in elicitation of immune responses in four out of five volunteers, as compared to negligible immunogenicity when the same preparation was given orally. This result suggested that passage of this antigen though the stomach affected immunogenicity. Thus it would seem that the gastric acidity presents a formidable barrier to immunogenicity (Levine *et al.*, 1986). Other investigators have demonstrated that other natural host defences such as the mucous coat of the luminal wall (Walker and Bloch 1983), intestinal proteolysis (Udall *et al.*, 1984), indigenous microbial flora (Udall *et al.*, 1981), and peristalsis (Udall *et al.*, 1981) affect attachment and absorption of macromolecules in the intestine and therefore, probably influence the interaction of inactivated vaccines with the GALT.

The danger that inactivated oral antigen might induce oral tolerance was investigated. Mice fed inactivated *E. coli* bearing K88 fimbriae produced significantly less serum antibody on subsequent parenteral immunisation than controls (Stokes *et al.*, 1979). These investigators suggested that the lack of response was probably a result of immunological tolerance.

To summarise, it would seem that oral administration of inactivated vaccines can invoke protective immune responses against non–invasive pathogens but that several doses of large amounts of antigen would be required. Whether such vaccines can provide immunity against invasive pathogens such as *S. typhi* remaines an open question.

1.5.2 Live oral vaccines

Germanier (1972) found that oral inoculation of mice with sublethal doses of virulent *S. typhimurium* provided high levels of immunity against subsequent homologous challenge. Parenteral vaccination on the other hand provided little or no protection against similar challenge (Germanier, 1972). In humans, epidemilogical data suggested that prior exposure to *S. typhi* infection provided significant protection against further infection by *S. typhi* (Ashcroft, 1964). However

typhoid relapses are known to occur at a reasonable frequency (Hornick *et al.*, 1970b). Marmion *et al.* (1953) found that relapse may be the result of incomplete immunity. In this investigation they demonstrated that secondary infection was with *S. typhi* of a different phage type, and that in some cases higher inocula were involved (Marmion *et al.*, 1953). Hornick *et al.* (1970b) suggested that antibiotic intervention in typhoid fever might also cause elicitation of incomplete immunity. Futher evidence that natural infection induced protective immunity came from studies with experimentally induced cholera (Cash *et al.*, 1974a; Levine *et al.*, 1981). Immunity induced by *V. cholerae* infection was found to provided high levels of protection against homologous challenge (Cash *et al.*, 1974a), and immunity against cholera after such infection lasted up to three years (Levine *et al.*, 1981).

Investigators developing prophylactic measures against enteric pathogens have often sought to find a vaccine that would provide long–lasting protective immunity after a single dose. A live oral attenuated organism may be able to provide a level of immunity that is normally seen after infection with virulent organisms.

Live oral vaccines must comply with three important criteria. Firstly, attenuation must be sufficient to prevent symptomatic disease. Secondly, the attenuation must not be so severe as to prohibit effective colonisation of the intestine and interaction with the GALT, as this capacity correlates with vaccine potential (Mackaness *et al.*, 1966; Germanier and Furer, 1971; Carter and Collins, 1974b; Hohman *et al.*, 1978; Srisart *et al.*, 1985). Finally, live oral vaccines must be so stably attenuated that reversion to wild type (and virulence) is negligible.

Investigators seeking to construct live oral vaccines have used a variety of techniques which included selection of spontaneous mutants of enteric pathogens, non-specific mutagenesis of enteric pathogens, and hybrid strain construction. More recently modern genetic techniques have been used to construct strains with well–defined mutations. Many early attempts to construct live oral vac-

cines were unsuccessful because the criteria: hypoattenuation–hyperattenuation balance and genetic stability, were not met for a number of reasons.

Spontaneously derived attenuated variants

i) Spontaneous mutants of *Shigella*: A spontaneous invasion–negative mutant, strain T32, was isolated by serially passaging virulent *S. flexneri* 2a on 2% nutrient agar slants 32 times (Istrati, 1961). This strain when given orally to animals and humans was found to be safe and to provide significant protection. However multiple doses containing about 10^{10} viable organisms were required to induce protective immunity (Istrati, 1961; Istrati *et al.*, 1965, 1967). Thus, this vaccine strain had a potency similar to that of killed oral vaccine. LaBrec and colleagues (1964) also recognised the importance of invasion in the pathogenesis of shigellosis. They derived from *S. flexneri* 2a, strain 2457T, a spontaneous mutant (strain 2457O), which was unable to invade the epithelium of guinea pigs or monkeys. However, despite an inability to invade strain 2457O was highly immunogenic in monkeys (Formal *et al.*, 1965a). When strain 2457O ($\geq 10^{10}$ viable bacteria) was given orally to humans a large proportion of the volunteers exhibited symptoms typical of shigellosis such as diarrhea, dysentery and fever (DuPont *et al.*, 1972a). Bacteria isolated from coprocultures taken from patients who displayed such symptoms were tested for their capacity to invade epithelial cells. Such isolates were found to be revertants that had reacquired the invasive phenotype of parent strain 2457T. These investigators concluded that the spontaneous invasion–negative strain 24570 was reactogenic by virtue of its ability to revert to invasion–positive *in vivo* (DuPont *et al.*, 1972a).

Another class of spontaneously derived candidate live oral vaccines, the streptomycin–dependent (SmD) mutants, were avirulent by virtue of their inability to proliferate to any great extent in the absence of an exogenous source of streptomycin. The safety and protective capacity of spontaneous SmD mutants of *Shigella* was tested in humans by Mel and co–workers (1965a, 1965b, 1965c, 1968, 1971, 1974). Typically, five doses containing between 2×10^{10}–4×10^{10} vi-

able SmD bacilli given orally provided high levels of protection against homologous *Shigella* strains. Protection elicited by SmD shigellosis vaccine did not apear to be long lived and therefore required a booster dose every 12 months (Mel *et al.*, 1974). Moreover, oral administraton of these SmD bacilli caused emesis and diarrhea in a significant proportion of the volunteers (3–6%), although this reactogenicity did not accompany reversion to streptomycin independence (Mel *et al.*, 1965b, 1971). The illness caused by the SmD vaccine did not persist for longer than 48 hours (Mel *et al.*, 1965b). A small–scale volunteer trial was conducted in the United States with Mel's SmD vaccine strains (DuPont *et al.*, 1972b). These researchers noted similar reactogenicity when $>10^{10}$ organisms were given to volunteers. More to the point, the protection provided by these SmD vaccines was effective only in the face of low challenge doses. When volunteers were challenged with about 10^2 virulent *S. flexneri* 2a, 60% protection was provided. This dropped, however, to 49% when the volunteers were challenged with about 10^4 virulent *S. flexneri* 2a. This observation explained the lack of efficacy seen when SmD shigellosis vaccines were used in custodial institutions, where the force of infection was high (Levine *et al.*, 1974, 1975). Furthermore, Levine and co–workers (1975) provided evidence that a SmD mutant of *S. sonnei* reverted *in vivo* to streptomycin–independence. Therefore, although SmD vaccines against shigellosis provided measurable protection against homologous challenge, their value was limited because of the need for multiple doses and a boost every 12 months, and the occurrence of genetic instability in vaccine lots of one serotype.

ii) Spontaneously derived SmD mutants of *S. typhi*: Reitman (1967) isolated a SmD mutant (strain 27V) from *S. typhi* 19V, which required approximately 10 μg/ml of streptomycin for growth *in vitro*. To investigate the effect of streptomycin on virulence, the LD_{50} value of this SmD derivative was measured, in mice, with and without concomitant streptomycin administration. Strain 19V, injected intraperitoneally (ip) suspended in 5% hog gastric mucin (HGM) had an LD_{50} value of 4×10^3 whereas under the same conditions the LD_{50}

value of strain 27V was 7×10^7. This contrasted with an LD_{50} value of 8×10^4 for strain 27V when streptomycin was given subcutaneously concomitantly with the ip challenge. This demonstrated that streptomycin–dependence *per se* caused the avirulence of 27V in mice. Furthermore, no revertants to streptomycin–independence were isolated suggesting that strain 27V was inherently stable (Reitman, 1967). Strain 27V provided high levels of protection (efficacy 78%) when freshly grown bacteria (3×10^{10} organisms/dose) and streptomycin (1.0g) were given orally to human volunteers twice weekly for 4 weeks (Levine *et al.*, 1976). Slightly less protection was provided (efficacy 66%) if the schedule was changed to 5 doses (3×10^{10}–10^{11}/dose) with concomitant streptomycin on the fourth and fifth doses. Unfortunately, minimal protection (efficacy $\leq$ 28%) was afforded if strain 27V was given as a lyophilised preparation with or without concurrent streptomycin (Levine *et al.*, 1976). This lack of efficacy of lyophilised preparations of strain 27V was difficult to explain in the face of colonisation data which suggested that lyophilised preparations colonised to the same extent as non–lyophilised preparations. This strain was not evaluated further, presumably because of the failure of lyophilised preparations to provide protection.

iii) Spontaneously derived mutants of *V. cholerae*: Spontaneous toxin–negative derivatives of *V. cholerae* were assessed as candidate live oral vaccines (Bhaskaran and Sinha, 1967; Mukerjee, 1963; Cash *et al.*, 1974b; Levine *et al.*, 1982). Cash and co–workers (1974b) fed two non–toxigenic environmental isolates to human volunteers and found them to be avirulent. However, only one volunteer out of 27 had vibrio–positive coproculture and very few volunteers seroconverted. Furthermore, challenge with virulent *V. cholerae* demonstrated that no protection was provided by immunisation with one such environmental isolate (Cash *et al.*, 1974b). Similar results were reported by Levine and fellow researchers (1982) when they tested a non–toxigenic isolate from Brazilian sewage. These investigators concluded that in order to develop a live oral vaccine against cholera the candidate vaccine strain should have the capacity to colonise the small intestine (Levine *et al.*, 1982).

It would seem, therefore, that spontaneously derived avirulent derivatives of enteropathogens failed to gain acceptance either because of lack of protective efficacy, or because of genetic instability causing reversion to the virulence of the wild type strain.

Hybrid derivatives of *Shigella*

The genetic homology which exists between *E. coli* and *Shigella spp.* has led to the construction of hybrid *Shigella* strains in which *E. coli* chromosomal DNA was introduced into the chromosome of virulent *Shigella*.

i) Hybrid derivatives of virulent *Shigella*: Formal and co–workers (1965a) found that substitution of the *xyl-rha* chromosomal region of *Shigella* with the *E. coli xyl-rha* chromosomal region produced a hybrid strain with reduced capacity to multiply in the intestinal mucosa but which was not significantly impaired in its ability to invade epithelial cells (Formal *et al.*, 1965b). When fed to starved guinea pigs this hybrid strain was significantly impaired in its ability to grow in the intestine compared to its virulent parent. Furthermore, oral administration of this candidate vaccine to monkeys demonstrated that such a hybrid strain was significantly attenuated and that protection was provided after a single dose (Formal *et al.*, 1965a, 1966a, 1966b). It is worth noting, however, that monkeys were found to be about 10^6–fold more resistant to shigella infection than humans (Formal *et al.*, 1965a). When 1.0×10^8 hybrid organisms were given to human volunteers 37% displayed severe reactions such as fever, emesis, and watery diarrhea (DuPont *et al.*, 1972a). This reactogenicity limited further use of this strain as a live oral vaccine.

ii) Hybrids derived from mutant *Shigella* strains: Other hybrid strains were derived from spontaneous mutant *Shigella* strains which were avirulent. One such mutant–hybrid strain was constructed by introducing the *E. coli xyl-rha* chromosomal region into a non–invasive derivative of *S. flexneri* 2a, strain 2457O. This mutant–hybrid strain which caused minimal side reactions in humans was not, however, very immunogenic, as only 8% of the volunteers

seroconverted after receiving four oral doses of 5×10^{10} organisms (DuPont *et al.*, 1972a).

A mutant-hybrid strain of *S. dysenteriae* 1 was also described. In this case the starting strain was 43A, a spontaneous non–invasive derivative of *S. dysenteriae* 1 (Levine *et al.*, 1973). Strain 43A was further attenuated by replacing the *xyl–rha* chromosomal region with the analogous *E. coli* region. When fed to humans the mutant–hybrid (strain 482) underwent extended periods of replication in the intestine as judged by positive coprocultures. One volunteer excreted the mutant-hybrid for 27 days. Unfortunately strain 482 reverted to invasion–positive in one volunteer (out of a total of 80 volunteers) who received 5×10^{10} organisms. The volunteer subsequently developed classical dysentery (Levine *et al.*, 1973). This genetic instability of strain 482 prevented its further evaluation in humans.

In summary, while vaccines developed using this approach showed promise the undefined nature of the attenuating lesions led to unpredictable genetic stability. Further development of live oral shigellosis vaccines will require a better understanding of the molecular basis of the virulence of *Shigella* (Mills *et al.*, 1988).

Live vaccines derived by non–specific mutagenesis

In the 1970s mutagenesis with chemicals such as N-methyl-N′-nitro-N-nitrosoguanidine (NG) or by ultra–violet light irradiation, were used in the derivation of avirulent mutants. Such techniques were used by Honda and Finkelstein (1979) and Germanier and Furer (1975) to isolate avirulent mutants of *V. cholerae* and *S. typhi* respectively.

i) Toxin–negative derivative of *V. cholerae*: Strain Texas-Star was derived (NG mutagenesis) from *V. cholerae* El Tor Ogawa 3083. The strain produced CT-B but could not produce the A-subunit of cholera toxin (CT-A; phenotype: A^-, B^+; Honda and Finkelstein, 1979). The parent of Texas-Star, strain 3083, was previously shown to avidly colonise the small intestine (Nelson

et al., 1976). Strain Texas–Star–SR (TS-R), a streptomycin–resistant derivative of Texas–Star, colonised the rabbit intestine poorly compared to its parent strain 3083 (Tokunaga *et al.*, 1984). This poor colonisation was thought to be a result of unrecognised defects generated by NG mutagenesis (Tokunaga *et al.*, 1984). However, TS-R induced protective antibacterial immunity in experimental rabbits (Boesman–Finkelstein and Finkelstein, 1982).

When TS-R was given orally to human volunteers protective immunity was induced against both homologous challenge (59% efficacy) and heterologous challenge (62% efficacy; Levine *et al.*, 1983a, 1984). Evidence that colonisation by TS-R of the human intestine (thought to be important in the development of protection) occurred, was inferred by the observation that serum vibriocidal antibody levels were similar in a group that received 10^5 organisms compared to volunteers that received higher inocula (Levine *et al.*, 1984). Of the coprocultures taken after TS-R–immunised volunteers were challenged with virulent *V. cholerae*, 90% contained live *V. cholerae* as against 37% after similar challenge of volunteers that had recovered from experimentally induced cholera (Levine *et al.*, 1984). This suggested that the antibacterial immunity provided by TS-R was not as effective as that provided by infection by virulent *V. cholerae*.

Strain TS-R caused mild diarrhea in 25% of volunteers who were given this strain (Levine *et al.*, 1984). This reactogenicity was attributed to either the presence of other (non–CT) toxins in the strain, the act of colonisation, or a combination of both these factors. Further use of TS-R as a vaccine was not considered feasible because of the possibility that the virulence of this strain might be enhanced in malnourished children living in cholera–endemic areas (Levine *et al.*, 1984).

ii) NG derived mutants of *S. typhi*: Germanier (1970, 1972) and Germanier and Furer (1971) investigated the vaccine potential of several rough mutants of *S. typhimurium* in mice. They demonstrated that *galE* mutants afforded protection close to that obtained after sublethal infection with virulent *S. typhimurium* whereas other rough mutants provided little or no protection.

The capacity of *galE* mutants to induce protective immunity was accredited to their ability to make smooth lipopolysaccharide (LPS) in the presence of exogenous galactose. It was argued that galactose was available in sufficient quantities *in vivo* to support synthesis of smooth LPS and to effect galactose–induced lysis (Germanier and Furer, 1971).

NG mutagenesis of *S. typhi* Ty2 was subsequently used to generate a *galE* mutant, strain Ty21a (Germanier and Furer, 1975). The LD_{50} value of Ty21a when injected ip into mice suspended in 5% HGM or 0.85% NaC1 (saline) was $>10^8$ as contrasted with the LD_{50} values of Ty2 (6.3×10^3 when suspended in 5% HGM; 10^6–10^7 when suspended in saline; Germanier and Furer, 1975). This attenuation of Ty21a was reflected in the fact that its persistence in the liver and spleen of mice was reduced when compared to an equivalent dose of parent Ty2. Futhermore, strain Ty21a was sensitive to galactose–induced lysis. This appeared to be a result of accumulation of precursors of galactose catabolism such as galactose-1-phosphate and uridinediphosphate galactose (Germanier and Furer, 1975). Nevertheless, strain Ty21a could utilize limiting amounts of exogenous galactose to synthesize smooth LPS which was thought to be immunologically important (Germanier and Furer, 1983).

A small scale trial was conducted to test the safety and immunogenicity of strain Ty21a in humans (Gilman *et al.*, 1977). No untoward reactions were noted when 155 volunteers received 5-8 doses ($3–10\times10^{10}$ organisms/dose). The number of viable organisms isolated in coprocultures was low, and most isolates occurred on day 1 post–vaccination. No gal^+ revertants were detected throughout the trial. These volunteers were subsequently challenged with 10^5 virulent *S. typhi* (Quailes strain) suspended in 45ml milk. Strain Ty21a when grown in the presence of exogenous galactose prior to inoculation (smooth–grown Ty21a) provided high levels of protection (87%), whereas no significant protection was seen if Ty21a was grown without galactose (Gilman *et al.*, 1977). The percentage of positive coprocultures noted, after challenge with virulent *S. typhi*, from vaccinees who received smooth–grown Ty21a was not significantly different

for the first three days post–challenge when compared to unvaccinated controls, whereas there was a significant difference (controls excreted more bacteria) from day 4-30 (Gilman *et al.*, 1977). This probably reflected a delay in the expression of antibacterial immunity in the intestinal lumen of the vaccinees after challenge. Strain Ty21a when grown in the absence of exogenous galactose did not provide significant antibacterial immunity, as judged by the lack of a significant difference (when compared with non–vaccinated controls) in the numbers of positive coprocultures at any time post–challenge (Gilman *et al.*, 1977).

Further evidence that demonstrated the safety and immunogenicity of Ty21a was provided by several independent investigators. Cancelliere and Fara (1985) demonstrated anti–LPS and anti–flagella IgA in human feces after immunisation with Ty21a. This observation was confirmed by Bartholameuz and co–workers (1986), who demonstrated significant increases in intestinal anti–*S. typhi* LPS IgA and IgM after vaccination with Ty21a. This increased level of specific intestinal antibody was sustained for up to a year (Bartholameuz *et al.*, 1986). These investigators also found that serum responses to *S. typhi* LPS were of a lower magnitude than the intestinal responses. Kantele and co–workers (1986) supplied further evidence that supported the immunising capacity of Ty21a. They found a significant increase of *S. typhi*–specific IgA secreting cells in the peripheral blood of volunteers after vaccination with Ty21a. The numbers of such cells peaked on day 7 post–vaccination, were undetectable by day 14 post–vaccination, and were specific for *S. typhi* antigens (Kantele *et al.*, 1986). They argued that these cells were part of the gut–derived lymphocyte traffic which would eventually home back to the site of antigen stimulation. It was of some disappointment therefore that these investigators could not demonstrate an anti–*S. typhi* sIgA response in the feces of such Ty21a–vaccinated volunteers (Kantele *et al.*, 1986).

Other investigators demonstrated CMI responses in volunteers, following vaccination with Ty21a. Sarasombath and colleagues (1987) demonstrated that peripheral blood cells taken from volunteers following Ty21a vaccination,

when stimulated with *S. typhi* derived antigens, displayed an increased capacity to inhibit leucocyte migration. More notably, Tagliabue and co–workers (1985) demonstrated that immunisation of humans with Ty21a elicited peripheral blood cells with an increased ability to inhibit or inactivate bacterial growth (*S. typhi*–specific). Interestingly, the mechanism of this antigen–specific antibacterial activity was shown to be the result of ADCC–capable cells and was dependent on antigen–specific serum IgA (Tagliabue *et al.*, 1986).

However, strain Ty21a did not always provide consistent results. Hirschel and fellow researchers (1985) studied a group of Ty21a– immunised Swiss travellers that visited typhoid–endemic areas. They could not demonstrate that any protection against typhoid was afforded by immunisation with Ty21a. It was proposed that this lack of efficacy was a result of either the formulation of the vaccine, the stability of the vaccine when stored at room temperature, or the schedule of immunisation that was used in the study (Hirschel *et al.*, 1985).

Two large scale trials have been performed to test the efficacy of Ty21a in areas where typhoid was considered endemic. The first of these was performed in Alexandria, Egypt, with a total of 16,486 children in the vaccinated group, 15,902 children in the placebo group, and an unvaccinated group of 25,628 children. The vaccinees received 1–3 doses (14,735 received 3 doses) which contained between $1–8\times10^9$ viable organisms, given at two day intervals (Wahdan *et al.*, 1980). Minimal untoward reactions were observed and no evidence of the spread of the vaccine strain to unvaccinated children was found. On the basis of the 3 year results the vaccine efficacy was estimated to be 95% (Wahdan *et al.*, 1982; Woodward and Woodward, 1982).

The second large field trial of Ty21a was performed at Santiago, Chile, to test different vaccine formulations and immunisation schedules (Black *et al.*, 1983; Levine *et al.*, 1987a). The vaccine was given as one of two formulations: enteric coated capsules containing 10^9 lyophilised bacteria, or plain gelatine capsules given with a bicarbonate capsule to neutralize gastric juices. The schedules employed were either a long interval schedule – 3 doses at 21 day intervals, or

a short interval schedule – 3 doses given at 1–2 day intervals (Black *et al.*, 1983). Levine and his colleagues (1987a) found that strain Ty21a provided 67% protective efficacy for up to 3 years (as compared to placebo controls) when 3 doses of enteric–coated capsules were given at short intervals. The protective efficacy dropped to 49% when Ty21a in the enteric–coated capsules was given at long intervals. No significant protective efficacy was provided by Ty21a given in gelatin–coated capsules at either short or long intervals. Interestingly, immunisation with Ty21a also provided a small amount of protection against *S. paratyphi* B (Edelman and Levine, 1986) which correlated with similar cross–reactive bacterial inhibition against *S. paratyphi* B activity noted in peripheral blood cells taken from volunteers after immunisation with Ty21a (Tagliabue *et al.*, 1986). The difference in efficacy provided by Ty21a in the Chilean trial as compared to the Egyptian trial might be a result of either the different formulations and schedules used, or the greater force of natural typhoid infection in the Chilean trial area (Levine *et al.*, 1987a).

The method of construction of strain Ty21a may contribute to the shortcomings seen in its performance as a live oral vaccine. Strain Ty21a was made by non–specific mutagenesis in a manner similar to the derivation of Texas–Star (Germanier and Furer, 1975). Other mutations were introduced into Ty21a by this non–specific technique. These include i) a *via* mutation blocking Vi antigen synthesis (Germanier and Furer, 1983), ii) one or more mutations giving Ty21a a growth rate half that of its parent Ty2 (Germanier and Furer, 1975), iii) mutation(s) causing a requirement for isoleucine and valine (R. Morona, unpublished observation), and iv) an inability to produce H_2S (Germanier and Furer, 1983). Other mutations might also exist which have not yet been detected. Furthermore, it is not unfeasible that Ty21a could revert to gal^+. Some isolated reports of the isolation of gal^+ revertants of Ty21a have appeared, but no evidence exists that such revertants are more virulent in humans than Ty21a (Silva–Salianas *et al.*, 1985; Silva *et al.*, 1987).

Vaccines constructed by modern genetic techniques

Strains TS-R and Ty21a served as useful prototype vaccines. However, modern recombinant DNA techniques have made it possible to construct strains of a more defined nature and which are therefore expected to be free of some of the shortcomings of earlier strains. Such techniques have been used to delete either a gene or genes, resulting in partial or complete loss of virulence in the mutant strain. Such deletions were genetically stable, but (as shall be discussed below) live oral vaccine strains displaying the required hypo/hyper–attenuation balance have yet to be constructed.

i) Mutants of *V. cholerae* derived by recombinant DNA techniques: Strains JBK70 (phenotype: CT-A^-, CT-B^-) and CVD101 (phenotype: CT-A^-, CT-B^+) were derived from virulent *V. cholerae* El Tor Inaba N16961, and *V. cholerae* Classical Ogawa 395, respectively, by recombinant DNA techniques (Kaper *et al.*, 1984; Levine *et al.*, 1988). When either JBK70 (single dose of 10^6–10^{10}) or CVD101 (single dose of 10^4–10^8) were given orally to human volunteers, 53% of the vaccinees developed mild diarrhea. Positive coprocultures, and the development of prominent serum vibriocidal activity, demonstrated that both JBK70 and CVD101 were avid colonisers of the human intestine. In agreement with the CT phenotype of these two strains there was a significant rise in serum antitoxin antibodies after immunisation with CVD101, but not after immunisation by JBK70. It was notable, therefore, that the antibacterial immunity elicited by JBK70 provided 89% protection against homologous challenge, as this suggested that protection against cholera challenge does not require antitoxin immunity (Levine *et al.*, 1988). Evidence that immunisation of volunteers with JBK70 elicited antibacterial immunity was provided by the finding that coprocultures taken from vaccinees after challenge showed reduced levels of challenge organisms (compared to controls), implying that vaccination affected the growth of the challenge strain *in vivo* (Levine *et al.*, 1988).

The significant reactogenicity of strains JBK70 and CVD101 might have been effected by other (non–CT) toxins produced by *V. cholerae* and/or the

act of colonisation. To investigate whether colonisation *per se* caused the mild diarrhea that resulted from ingestion of these two strains, strain CVD102 (a spontaneous thymine dependent mutant (*thy*) of CVD101), was obtained. The thymine requirement of CVD102 was introduced to reduce intestinal colonisation by this strain. Large doses of CVD102 given orally to volunteers did not cause any notable untoward reactions. Strain CVD102, however, was poorly immunogenic, indicating that its ability to colonise was excessively handicapped by the *thy* mutation (Levine *et al.*, 1988).

To investigate the contribution of a gene that might confer a toxigenic phenotype on the CT-A$^-$ strains, *hly* (Hemolysin) genes were deleted from JBK70 and CVD101 using recombinant DNA techniques. However, the *hly*$^-$ derivatives (CVD104 and CVD105 respectively) were still diarrheagenic, implying that the *hly*$^+$ genotype of JBK70 and CVD101 did not have a bearing on the reactogenicity of these candidate vaccine strains.

Some strains of *V. cholerae* were shown to produce a shiga–like toxin (SLT; O'Brien *et al.*, 1984). This was not the case with *V. cholerae* Classical Inaba strain 569B. A CT-A$^-$, CT-B$^+$ derivative of 569B, CVD103, caused diarrhea in only 11% of volunteers who ingested 10^8 organisms (Levine *et al.*, 1988). This suggested that SLT might be a major factor in the reactogenicity of the earlier vaccine candidates (JBK70 and CVD101). However, the parent (strain 569B) was a notoriously poor coloniser of the intestine, and because CVD103 was probably capable only of similar colonisation this factor alone might have influenced the outcome of such experiments (Taylor *et al.*, 1988). On this basis it was argued that a better understanding of the colonisation defect in 569B might enable construction of strains with a more predictable outcome in the field (Taylor *et al.*, 1988). Furthermore, genetic reduction of the expression of fimbriae by strains such as JBK70 and CVD101, might eliminate the reactogenicity of these strains (Taylor *et al.*, 1988)

Hence, recombinant DNA techniques have enabled provision of extensive data on the role of a variety of genetic determinants in the virulence of, and im-

munity of humans to, *V. cholerae*. Whether the construction of non–reactogenic attenuated strains of *V. cholerae* is possible is still an open question.

ii) Modern genetic approach to constrution of live oral typhoid vaccines: Second generation typhoid vaccines have been prepared by modern genetic techniques (Hoiseth and Stocker, 1981; Stocker *et al.*, 1983). These investigators introduced two non–reverting deletions from *S. typhimurium*, one in the *aroA* gene and the other in the *purA* gene, into wild type *S. typhi* strain CDC10-80 (phage–type A) by bacteriophage P22–mediated transduction. Much experimental evidence earlier sugggested that either *aroA* or *purA* alone were attenuating mutations.

The *aroA* mutation caused dependence on five metabolites for growth: tryptophan, tyrosine, phenylalanine, *p*–aminobenzoic acid (PABA) and 2,3–dihydroxybenzoic acid (DHB). Two of these metabolites (PABA and DHB) are not found in the interstitial fluid or the cytoplasm of vertebrate hosts (Hoiseth and Stocker, 1981; Stocker *et al.*, 1983). Bacon and co–workers (1950a, 1950b, 1951) had previously demonstrated that a PABA–dependent mutant of *S. typhimurium* was attenuated in mice. This observation was confirmed recently by Brown and Stocker (1987) who used the same PABA–dependent mutant in studies with mice. Strains of *S. typhimurium* which carry *aroA* defects were highly attenuated and immunogenic in a variety of animal experimental models (Smith *et al.*, 1984; Robertson *et al.*, 1983; Stocker *et al.*, 1983; Nnalue and Stocker, 1987; Dougan *et al.*, 1987b; Eisenstein *et al.*, 1984).

The *purA* mutation in *S. typhimurium* caused a requirement for adenine (or adenosine) to support growth on minimal medium *in vitro*. This defect was proposed to result in poor growth in mammalian tissue (Bacon *et al.*, 1950b, 1951; Batson *et al.*, 1949; Formal *et al.*, 1954). McFarland and Stocker (1987), whilst investigating a variety of purine auxotrophs, found that *S. typhimurium* (FIRN and WRAY biotypes) and *S. dublin* were highly attenuated in mice by either a *purA* or a *purB* mutation. The protein products of these genes, adenylosuccinate synthetase and adenylosuccinate lyase respectively, catalyse the con-

vertion of inosine monophosphate to adenosine monophosphate, two distal reactions of *de novo* purine biosynthesis. Interestingly, mutants with defects in the proximal end of *de novo* purine biosynthetic pathway (conversion of phosphoribosyl pyrophosphate to inosine monophosphate), were not as severely attenuated as those carrying *purA* or *purB* defects (McFarland and Stocker, 1987).

The *purA, aroA* double mutant of strain CDC10-80 was further derivatised by the introduction of a *hisG* mutation. This *aroA, purA, hisG* derivative, strain 541Ty, was the parent of a spontaneous Vi–negative mutant, strain 543Ty.

The *his* mutation was included in strains 541Ty and 543Ty to assist with biochemical identification, and was thought not to contribute to avirulence (Levine *et al.*, 1987b). However, a recent investigation demonstrated that his^+ selection was favoured *in vivo* (Wright and Rosenberg, 1986). Therfore it is not clear whether the *his* mutation affects the outcome of immunisation with strains 541Ty and 543Ty.

Recently the safety, infectivity, immunogenicity and *in vivo* stability of strains 541Ty and 543Ty were tested in humans. Neither strain caused detectable untoward clinical reactions even when doses as high as 2×10^{10} organisms (541Ty) were given orally (Levine *et al.*, 1987b). As many as 80% of the volunteers that ingested the candidate vaccine strains gave positive coprocultures on the first day after vaccination but no vaccinee excreted organisms longer than 96 hours. These investigators concluded that both 541Ty and 543Ty were safely attenuated in humans.

The capacity of strains 541Ty and 543Ty to elicit either humoral or cell–mediated immune responses was investigated (Levine *et al.*, 1987b). Significant serum and intestinal antibody responses occurred in only a small number of volunteers. In contrast 100% of the peripheral blood samples taken from these vaccinees manifested cell–mediated responses as judged by antibody–dependent mononuclear cell inhibition of *S. typhi* growth. Similar antigen–specific cellular immunity was found in peripheral blood of volunteers taken after immunisation with Ty21a (Tagliabue *et al.*, 1985, 1986). The relevance of this measure of

immunity to protection was not clear, however, inhibition of *S. typhi* growth by peripheral cells was not detected after oral immunisation with killed typhoid vaccine (Tagliabue *et al.*, 1986).

Thymidine uptake by peripheral blood lymphocytes when cultured in the presence of inactivated *S. typhi*, or purified *S.typhi* O-antigen, was shown to be a good measure of prior contact with *S. typhi* (Murphy *et al.*, 1987). Furthermore, of peripheral blood lymphocytes taken from human volunteers that were given Ty21a orally, 100% exhibited an increased thymidine uptake in the presence of inactivated *S.typhi* (Murphy *et al.*, 1987). Of the peripheral blood lymphocytes taken from the vaccinees that received $\geq 10^9$ 541Ty or 543Ty bacilli, 89% incorporated increased levels of thymidine in such lymphocyte replication assays.

Although volunteers vaccinated with either 541Ty or 543Ty manifested detectable cellular responses, the meagre humoral responses may indicate that both of these strains were hyperattenuated. Currently a *S. typhi* derivative that carries two unlinked chromosomal mutations in the *aroA* and *aroD* genes, is being assessed for use as a live oral typhoid vaccine (Stocker, 1988). Such a double mutant would be expected to have genetic stability similar to that of strains with two mutations in independent biosynthetic pathways. However, since both of these genetic defects affect the same aromatic biosynthetic pathway, the degree of attenuation that might result from such mutations may not be as debilitating *in vivo* as that seen with strains 541Ty and 543Ty (Stocker, 1988).

1.5.3 Construction of bivalent live oral vaccines

Many investigators have proposed that immunisation with an attenuated strain which carries a gene encoding a protective antigen of another enteric pathogen might induce protective immunity against both the carrier organism and the enteric pathogen from which the heterologous DNA was cloned (Formal *et al.*, 1981; Clemens and El Morshidy, 1984; Yamamoto *et al.*, 1985; Brown *et al.*, 1987; Maskell *et al.*, 1987). Success with such hybrid vaccines (bivalent vaccines)

will depend primarily on two factors: i) the stability of the introduced antigen, ii) selection of a carrier strain that elicits the required immune response. Other factors which might affect the outcome of immunisation with such vaccines are: the choice of protective antigen, and the immunogenicity of the antigen in the carrier strain. These latter two topics will not be discussed here.

Stability of the heterologous antigen

Stability of the heterologous antigen during *in vitro* and *in vivo* growth is an important consideration. All bivalent vaccine strains developed to date harboured recombinant plasmids which maintained the foreign genes. The stability of such recombinant plasmids was quite variable (Maskell *et al.*, 1987) and some bacterial strains such as *S. typhi* may be inherently poor at maintaining plasmid vectors (Murray *et al.*, 1985).

Furthermore, plasmids which are components of hybrid vaccines must be non–conjugative and non–mobilisable (Guidelines for research involving recombinant DNA molecules, 1982). Although plasmid pBR325 was demonstrated to be highly contained *in vivo* (Levine *et al.*, 1983c), three problems may be experienced in the administration to humans of hybrid strains carrying recombinant plasmids. Firstly, the possibility of plasmid transfer from the hybrid to the human bacterial commensals or environmental bacteria must be addressed. Considerable experimentation may be required to demonstrate that such transfer is negligible. Secondly, the introduction of a recombinant plasmid into the carrier strain (typically by transformation) usually requires selection for an antibiotic–resistance marker of the plasmid. Such markers are undesirable in strains intended for release outside the laboratory (Guidelines for research involving recombinant DNA molecules, 1982). Finally, even when the hybrid strain carries a plasmid specifying antibiotic–resistance, it will be difficult to maintain selection for the plasmid *in vivo*. If the plasmid is unstable in the absence of selection, the efficacy of the live vaccine will be adversely affected.

Hence the success of bivalent vaccines will depend on the development

of stably inherited plasmid replicons or of another method of maintenance of stability of the heterologous DNA.

Choice of bacterial carrier strain

The choice of the bacterial carrier strain has not hitherto received much attention because only a limited number of suitably attenuated strains were available for assessment.

Non–pathogenic strains of *E. coli* have been used to present *Shigella* antigens to the immune system (Formal *et al.*, 1970, 1984). One such hybrid strain was constructed by conjugal transfer of the *his* and *met* chromosomal regions of *S. flexneri* 2a into *E. coli* strain RJ90 (Formal *et al.*, 1970). The hybrid expressed *S. flexneri* type II antigens and O–group antigens 3 and 4 as surface antigens (Formal *et al.*, 1970; Levine *et al.*, 1977). However, no protection (against *S. flexneri* 2a challenge) was provided when the vaccine was given orally to human volunteers, even when the challenge dose was as low as 10^2 organisms (Levine *et al.*, 1977). Since coprocultures indicated good colonisation had occurred the lack of efficacy provided by the *E. coli–Shigella* hybrid was attributed to the lack of invasion potential of the hybrid strain (Levine *et al.*, 1977). More recently an *E. coli–S. flexneri* hybrid which expresses an invasive phenotype was constructed (Formal *et al.*, 1984). This hybrid was constructed by transferring *S. flexneri* 2a chromosomal regions *his* (group antigen 3,4) and *pro* (type I antigen) as well as *S. flexneri* 2a plasmid pWR110 (which confers an invasion–positive phenotype) into the *E. coli* host. Furthermore, this hybrid strain, EC104, was found to confer high levels of protection in experimental monkeys (Formal *et al.*, 1984). To date however, there have been no reports of the safety and immunogenicity of this hybrid in humans.

Attenuated *Salmonella* strains have received the most attention as prospective carriers of heterologous antigens. The capacity of attenuated *Salmonella* strains to colonise the Peyer's patches and invoke both local and systemic immune responses (Hohmann *et al.*, 1978; Moser *et al.*, 1980; Srisart

et al., 1985) implied that these strains may potentially be exploited to provide immunity against a wide range of pathogens including enteric bacterial pathogens, viral pathogens, and protozoan and metazoan parasites (Maskell *et al.*, 1987; Stocker, 1988; Clemens and El Morshidy, 1984; Formal *et al.*, 1981; Taylor *et al.*, 1988).

Clemens and fellow researchers (1986a) transformed a *S. enteriditis aroA* mutant with a recombinant plasmid that encoded genetic information necessary for expression of *E. coli* LT-B. The transformant, strain EL23, secreted LT-B that was antigenically and electrophoretically identical to that expressed by the *E. coli* parent strain from which the genes were cloned. Mice immunised orally with EL23 manifested both local intestinal sIgA and serum IgG antitoxin responses (Clemens *et al.*, 1986a). Other investigators using *S. typhimurium aroA* derivatives, which expressed LT-B from plasmid encoded genes, found similar responses in the intestine and serum (Maskell *et al.*, 1987; Dougan *et al.*, 1987).

In humans, strain Ty21a, a *galE* derivative of *S. typhi* Ty2, has been investigated as a potential carrier of heterologous antigens. A hybrid Ty21a–based strain carrying a recombinant plasmid which encoded and expressed LT-B, was safe and immunogenic in mice and guinea pigs (Clemens and El Morshidy, 1984). To date there has been no report of the efficacy of this hybrid in humans. Another hybrid, based on Ty21a which expressed *V. cholerae* O–antigen, was constructed and shown to be safe and immunogenic in human volunteers (Forrest *et al.*, 1987).

As mentioned earlier, strain Ty21a was also exploited as a carrier of the form 1 antigen of *S. sonnei* (Formal *et al.*, 1981; Seid *et al.*,1984). Recently Black and co–workers (1987b) reported on the safety, immunogenicity and protective efficacy of such a hybrid when given to human volunteers. No untoward reactions were noted when doses as high as 8×10^9 were given. When the vaccinees were challenged with virulent *S. sonnei* only modest levels of protection (protective efficacy *ca.* 47%) were afforded by vaccination. Furthermore, lot to

lot variation of vaccine efficacy was detected (Black *et al.*, 1987b). The protection observed in this volunteer trial correlated with the level of anti–*S. sonnei* LPS serum IgA and IgG before challenge (Black *et al.*, 1987b)

The current development of attenuated *E. coli* (Kaper and Levine, 1988) and *V. cholerae* strains (Levine *et al.*, 1988) may provide new carriers of foreign antigens. However, this approach might be limited to induction of a local secretory immune response and therefore applicable only against pathogens whose infection is prevented by this arm of the immune response. The development of auxotrophic derivatives of *Shigella* have recently been reported (Lindberg *et al.*, 1988). Such attenuated strains might be worth considering as carriers of heterologous antigens. *Shigellae* as carrier organisms have one added advantage – they are highly resistant to gastric acidity (Levine *et al.*, 1973) and therefore small doses may give effective immunisation.

1.6 Aims of this thesis

At present the only live oral vaccine in widespread use is Ty21a. This strain has the shortcoming that it was made by non–specific mutagenesis and as a result is poorly defined genetically. For this reason, it is difficult to assess the contribution of the *galE* mutation to avirulence.

Therefore, the first aim of this thesis was to clone the *gal* operon of *S. typhimurium*, with a view to the introduction of a defined deletion into the cloned *galE* gene, and recombination of the modified *gal* operon into the chromosome of *Salmonella* strains of human and veterinary importance. Such strains would possess defined, non–revertible *galE* mutations, and would otherwise be almost isogenic with the virulent parent strains. This should allow evaluation of the contribution of a *galE* mutation to avirulence, and if successful should provide a variety of candidate *Salmonella* vaccine strains. In Chapter 3, the requisite cloning work is described. In Chapter 4, strains of *S. typhimurium* and *S. typhi* carrying defined deletions are constructed. In Chapter 5, the safety and immunogenicity of a *via* (Vi–negative) rifampicin–resistant derivative of a *S. typhi* *galE* mutant, is tested in humans.

There is much interest in the construction of bivalent vaccines, but, as mentioned earlier, no method has yet been developed by which introduced DNA may be stably maintained in the carrier strain. The second aim of this thesis, then, was to construct a chromosomal integration system. Such a system would enable the integration of heterologous genes into the chromosome of a *Salmonella* carrier strain thereby providing a new means of stable incorporation of such heterologous DNA. This work is detailed in Chapter 6.

Chapter 2

Materials and Methods

2.1 Materials

The following chemicals were purchased from Sigma: *Salmonella typhi* LPS (Catalog no. L6386), D(+)galactose (Catalog no. G0750), D(+)glucose (Catalog no. G5000), tris(hydroxymethyl)aminomethane (Tris; Catalog no. T8524), ethylenediaminetetraacetic acid (EDTA; Catalog no. E5134), calf thymus DNA (Catalog no. D1501), ethidium bromide (Catalog no. E8751), adenosine triphosphate (ATP; Catalog no. A9139), dithiothreitol (DTT; Catalog no. D9779), Phosphatase substrate pellets (Catalog no. 104105), ampicillin (Catalog no. A9393), spectinomycin (Catalog no. S9007), rifampicin (Catalog no. R3501), chloramphenicol (Catalog no. C0378), and polyvinylpyrrolidine (Catalog no. PVP360). The following chemicals were purchased from Boehringer–Mannheim: isopropyl–β–D–thiogalactopyranoside (IPTG; Catalog no. 724815), ribonuclease A (RNaseA; Catalog no. 109169), deoxyadenosine–triphosphate (dATP; Catalog no. 103977), deoxyguanidine–triphosphate (dGTP; Catalog no. 104078), deoxycytidine–triphosphate (dCTP; Catalog no. 104264), and deoxythymidine–triphosphate (dTTP; Catalog no. 104264). Difco (Detroit, Michigan) was the source of media for bacterial growth. Nutrient agar was Blood agar base^R (Catalog no. 0045), nutrient broth was Catalog no. 003, Bacto methionine assay medium was Catalog no. 09901501, Bacto histidine assay medium was Catalog

no. 0992153 and Bacto agar^R was Catalog no. 014001. Restriction enzymes *Eag*1 (Catalog no. 505) and *Aat*II (Catalog no. 117) were purchased from New England Biolabs. All other restriction endonucleases, sodium dextran sulphate (Catalog no. 17034002), T4 DNA polymerase (Catalog no. 27091802), Ficoll^R 400 (Catalog no. 17040002), Sepharose^R CL–6B (Catalog no. 17016001), and T4 DNA ligase (Catalog no. 2708702) were purchased from Pharmacia. CsCl (Catalog no. 958) for purification of plasmid DNA was from Ajax chemicals. The radioactive nucleotide used for DNA labelling was α–[^{32}P]–deoxycytosine triphosphate (BRESA; Catalog no. ADC1). Radiolabelling was performed with BRESA oligolabelling kits (Catalog no. OLKC). Xylene cyanol was purchased from BioRad (Catalog no. 1610423), Coomassie^R brilliant blue R250 was from Serva (Catalog no. 35051); alkaline phosphatase conjugated with either goat anti–human IgG (Catalog no. 151002), goat anti–rabbit IgG (Catalog no. 011506), or goat anti–human IgA (Catalog no. 151001) were purchased from KPL. Sheep anti–rabbit IgG conjugated with alkaline phosphtase was purchased from Sara–Lab, Catalog no. SDL2507, Sussex, UK; Rabbit anti–mouse IgG was kindly donated by Dr. P. Ey (University of Adelaide, Adelaide, Australia); specially pure sodium dodecyl sulphate (SDS (Catalog no. 44244)) and bromophenol blue (Catalog no. 20015) were obtained from BDH; Millex^R–HA 0.45μm filters were from Millipore (Catalog no. SLHA0250S); L–[^{35}S]–methionine (>800 Ci/mmol) was obtained from Amersham (Catalog no. SJ204), and hog gastric mucin (Koch–Light Catalog no. 4065h, Batch no. 44022) was a generous gift from Dr. D. M^{c}Kay (Australian National University, Canberra, Australia). Low melting point agarose was from SeaPlaque^R (Catalog no. 50102) and high gelling temperature agarose was from SeaKem^R. ELISA trays were purchased from Costar (Catalog no. 2595). Acrylamide, ammonium persulphate and N,N′–methylenebisacrylamide were ultra pure chemicals from BDH. Triton X–100 was from Ajax chemicals (Catalog no. 1552) and bovine serum albumin (fraction 5 purity) was from Flow laboratories (Catalog no. B451). Nitrocellulose filters (Catalog no. S401196) and nitrocellulose disks (Catalog no. S401116), used

for Southern tranfers and colony transfers respectively were supplied by Schleicher and Schuell. All other chemicals were analytical reagent grade. Microbact 12E biochemical identification trays were purchased from Disposable Products, Adelaide, Australia.

2.2 Methods

2.2.1 Media

Shaking liquid cultures were grown for 16hr at 37^0C in NBG (1.6%(w/v) nutrient broth, 0.5%(w/v) NaCl and 25 mM glucose), or in NBGG (NBG with 6mM galactose). Nutrient agar (NA: 1.6%(w/v)), was normally supplemented with glucose (25mM). M9 minimal medium is described elsewhere (Miller, 1972), however, sodium citrate was omitted, and glucose was added to a final concentration of 25mM. Minimal galactose medium was the same modified M9 medium with galactose (6mM) substituted for glucose as the sole carbon source. Indicator plates that were used to identify gal^+ colonies were NA supplemented with 6mM galactose instead of glucose, and with neutral red (0.03%(w/v)) as the pH indicator. For the growth of *S.typhi* Ty2 and its derivatives M9 medium was supplemented with tryptophan (20μg/ml) and cystine (20μg/ml) (Germanier and Furer, 1983). Ty21a required isoleucine (20μg/ml) and valine (20μg/ml) for growth in minimal medium. M9 agar was M9 minimal medium with 1.5% (w/v) Bacto agar^R. Histidine–auxotroph selection medium was M9 minimal medium supplemented with histidine assay medium (0.2% (w/v)). To explore the phenotype of *his* mutants, histidinol (10μg/ml) was sometimes added to M9 agar. Selection and growth of rifampicin-resistant mutants was effected on nutrient agar plates supplemented with 100μg/ml rifampicin (Sigma, Catalog No.R3501) (Miller, 1972). The concentrations of other antibiotics used were: ampicillin (Ap: 200μg/ml), spectinomycin (Sp: 120μg/ml)and chloramphenicol (Cm: 50μg/ml).

2.2.2 Bacteria and bacteriophage

The bacterial strains used here are listed (Table 2.1). All experiments were performed with freshly grown bacteria. Bacteriophages P22 and Felix-O were a gift of Professor P. Reeves, Department of Microbiology, University of Sydney, Sydney, Australia. Bacteriophage ViII (propogated here on *S.typhi* Ty2) was a gift from Professor B. Stocker, Department of Medical Microbiology, Stanford University, California, USA. Bacteriophage propagation and storage techniques are described elsewhere (Miller, 1972).

2.2.3 Plasmid vectors

The plasmid vectors used in this study were as follows: pJRD158B (Ap^r; Davison *et al.*, 1984), pUC9 (Ap^r; Vieira and Messing, 1982), pKO1 (Ap^r; McKenney *et al.*, 1981), pGB2 (Sp^r; Churchward *et al.*, 1984).

2.2.4 Maintenance of bacterial strains

All strains were maintained as lyophilised cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to a 10ml bottle of NBG and grown with shaking at 37^0C for 16h. The other half was streaked onto two nutrient agar plates and incubated at 37^0C for 16h. Antibiotics were added to the media when appropiate. If the colony form was uniform, single colonies were selected and picked off plates for subsequent storage or use. Short–term storage of strains in routine use was acheived by suspension of freshly grown bacteria in glycerol (32% (v/v)) and peptone (0.6% (w/v)) at -70^0C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar plate (with of without antibiotic) followed by incubation at 37^0C for 16h just prior to use.

Bacterial strains were prepared for long–term storage by suspension of several colonies in a small volume of sterile skimmed milk. In the case of *galE*

Table 2.1: Bacterial strains used in this report

Strain	Genotype	Source/Reference*
Salmonella typhimurium:		
C5	wild type	IMVS
C5H1	*galE*-H1	This thesis
LT2	wild type	IMVS
LT2H1	*galE*-H1	This thesis
J706	*galE*-H1, del *hisOG*	This thesis
J754	*galE*-H1, K88$^+$	This thesis
J770-772	*galE*-H1, K88$^+$	This thesis
P9032	del(*flaA*-*rfbO*) 658	Nikaido *et al.*, 1968
LB5010	*galE*, *leu*, *ilv*452, *pro*, $hsdR_{LT}$, $hsdR_{SA}$, $hsdR_{SB}$, *metA*22, *metE*551, *trpD*2	Bullas and Ryn, 1976
SL1654	*hsdA*, *hsdC*, *trpB*2, *nml*, H1(b), *flaA*66, H2(e,n,x), *rspL*, *xylT*404, *ilvE*452, *metE*551, *metA*22	P. Reeves
Salmonella typhi:		
Ty21a[1]	*ile*, *val*, *galE*, *via*	R. Germanier
Ty2	*cys*, *trp* (wild type)	IMVS
R2	*cys*, *trp*, rough, Vi-negative	IMVS
Ty2H1	*cys*, *trp*, *galE*-H1	This thesis
Ty2Vi	*cys*, *trp*, *via*[2]	This thesis
Ty2H1Vi	*cys*, *trp*, *galE*-H1, *via*	This thesis
EX462	*cys*, *trp*, *galE*-H1, *via*, *rif*[2]	This thesis
J669	*cys*, *trp*, *galE*-H1, *via*, *rif*	This thesis
J670	*cys*, *trp*, *galE*-H1, *via*, *rif*	This thesis
J671	*cys*, *trp*, *galE*-H1, *via*, *rif*	This thesis
J672	*cys*, *trp*, *galE*-H1, *via*, *rif*	This thesis
EX590	*cys*, *trp*, *gal*$^+$	This thesis
EX592	*cys*, *trp*, *gal*$^+$, *via*, *rif*	This thesis

1. Strain Ty21a may have other uncharacterized mutations.
2. *via* denotes that the genetic locus affected by mutation is likely to be *viaA* or *viaB*; *rif* denotes a mutation that produces a rifampicin-resistant phenotype.

Table 2.1: Bacterial strains used in this report (continued)

Strain	Genotype	Source/Reference*
Escherichia coli:		
M30	wild type enterotoxigenic *E. coli* ($K88_{ab}$), O-non-typable	S. Tzipori
DH1	*gyrA*96, *recA*1, *relA*1, *endA*, *thi*-1, *hsdR*17, *supE*44, lambda$^-$	Maniatis *et al.*, 1982
DH1(pFM205)	Same as DH1 except $K88^+$ and Ap^r	Mooi *et al.*, 1984
DH1(pGB2)	Same as DH1 except Sp^r	Churchward *et al.*, 1984
P2905	*tonA*2, *lacY*1, *tsx*-1, *supE*44, *trp*31, *hisG*1, *argG*6, *rpsL*104, *malA*1, *xyl*-7, *mtl*-2, *metB*1, *hsdR*, *gal*-6	P. Reeves
TT2619	*hisG*9651::Tn*5*-7	P. Reeves
Δ4	Δ4 (*galK*-*attL*), *recA*, *trpC*	Ahmed (1984)
KA56	*galE*45, HfrH, *relA*1, *spoT*1, *thi*-1, λ^-	Davey *et al.* (1984)
CA13	*galT*118, HfrH, *relA*1, *spoT*1, *thi*-1, λ^-	Davey *et al.* (1984)
P1700	*galK*2, *thr*-1, *leu*-6, *proA*2, *lacY*1, *trp*(UAG), *his*-4, *non*-9, *rpsL*31, *xyl*-5, *mtl*-1, *argE*3, *thi*-1, *ara*-14	P. Reeves
DS410	*minA*, *minB*, *rpsL*	Achtman *et al.*, 1975
RU2901	R_{ts}1::Tn*1725*	R. Schmidt
RM11	Not known	R. Maurer

*IMVS: Institute of Medical and Veterinary Science, Adelaide, Australia. Dr S. Tzipori is at the Royal Children's Hospital, Melbourne, Australia. Dr P. Reeves is at the University of Sydney, Australia. Dr R. Schmidt's address is Lehrstuhl für Genetik, Universitat Regensburg, D-8400 Regensburg, Federal Republic of Germany. Dr R. Maurer is at the Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, Ohio, U.S.A., 44106.

strains, NB supplemented with 5% glucose was used instead of skimmed milk. Approximately 0.2ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25in.×4in. freeze drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilised in a freeze drier. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constriction was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns and then sealed at the constriction without releasing the vacuum. Finally the ampoules were labelled and stored at 4^0C.

2.2.5 Selection of bacteria spontaneoulsy cured of a plasmid specifying Sp–resistance

Colonies grown on nutrient agar plates without Sp selection were picked into pre–heated (37^0C) NBG (10ml), and grown with aeration at 37^0C to a cell density of about 2×10^8 bacteria/ml. Sp was added to a concentration of 120μg/ml and the culture shaken at 37^0C for 15 min. Ampicillin (100μg/ml) was then added and the cultures shaken further until lysis of the bulk of the bacterial suspension occurred (about 2h). As Ap lysed only growing cells, cells surviving this treatment were highly enriched for Sp-sensitive bacteria. The suspension was then pelleted and washed in 0.9% (w/v) sodium chloride (saline) to remove the residual antibiotic and the survivors examined further.

2.2.6 Selection of histidine auxotrophic mutants by Ap enrichment

Ap selection of auxotrophic mutants was performed as previously described (Miller, 1972). Colonies grown on nutrient agar plates with Sp selection were picked into pre–heated (37^0C) M9 minimal medium supplemented with histidine (20μg/ml; 10ml) and grown with aeration at 37^0C to a cell density of about 2×10^8 bacteria/ml. The resulting culture was pelleted by centrifugation

and resuspended in histidine auxotroph selection medium with Ap (200μg/ml). The culture was further incubated (37^{0}C) with aeration for about 2h. The surviving bacteria were grown in M9 minimal medium supplemented with histidine as before and the procedure was repeated. After the second round of enrichment for *his*$^-$ bacteria appropriate dilutions of the bacterial suspension were plated onto either nutrient agar plates or minimal agar plates.

2.2.7 Selection of spontaneous *via* mutants

The *via* mutants of Ty2 and Ty2H1 (Ty2Vi and Ty2H1Vi respectively) were selected as follows. An overnight liquid culture (NBG) was divided into 0.1ml aliquots (*ca.* 5×10^8 cfu) which were mixed with 0.1ml of ViII phage (*ca.* 5×10^9 pfu). After absorption (standing 37°C, 10min) the mixture was spread evenly on a nutrient agar plate. Typically, about 50 to 100 colonies were detected after overnight incubation at 37^{0}C. Phage-resistant mutants that showed no agglutinating activity with anti-Vi serum, and increased sensitivity to the bactericidal action of serum, were stored.

2.2.8 Selection of bacteriophage Felix-O–resistant mutants

Bacteriophage Felix-O selects for core deficient mutants of *Salmonella* amongst which should be *galE* mutants. Bacteria, cured of Sp–resistant plasmid, were mixed with 0.1 ml of phage Felix-O (the concentration of the phage was adjusted to give an moi of about 10). This mixture was incubated at 37^{0}C for 10 min then spread onto a NA plate supplemented with 6mM galactose, and incubated for 16h at 37^{0}C.

2.2.9 Selection of rifampicin–resistant (*rif*) mutants

To assist with monitoring of the candidate typhoid vaccine strain in humans a rifampicin-resistance marker was introduced into Ty2H1Vi as previously de-

scribed (Miller, 1972). Care was taken to check that the rifampicin-resistant strain (EX462) had a growth rate, in NBG, comparable to that of Ty2H1Vi.

2.2.10 Genetic stability of *gal* mutants

To test the reversion frequency of *galE* mutants to gal^+, large (20 x 20 cm) plates of minimal galactose agar were spread with about 10^{12} bacteria of the test strain, suspended in 5ml saline, and incubated at 37°C for 3d.

2.2.11 Sensitivity of strains to galactose–induced lysis

Two methods were used to test for sensitivity to galactose-induced lysis:

i) Plate method. Varying concentrations of galactose (from 6mM to 60nM) were added to nutrient agar (without glucose). Bacteria were grown in NBG to stationary phase and then swabbed across the galactose-containing plates. After 16hr at 37°C, sensitivity to a given galactose concentration was detected by inhibition of growth at that concentration. A nutrient agar plate supplemented with both glucose (25mM) and galactose (6mM) served as a positive control.

ii) Liquid culture method. Aliquots (10ml) of nutrient broth (without glucose) were inoculated with single colonies of strains under test and shaken at 37°C to a cell density of about 2 x 10 ^{8}/ml (OD_{650nm} was 0.2). Galactose (the stock was a 1.1M solution in water) was added to various concentrations and growth at 37°C with shaking was continued. A strain described here as galactose–sensitive showed a decrease in OD_{650} reading from about 0.2 at the time of galactose addition to less than 0.02 within 1 hour after galactose addition.

2.2.12 Determination of Leloir pathway enzyme levels

The enzymes of the Leloir pathway catabolize galactose to UDP–glucose (Kalckar *et al.*, 1959). Strains were grown in shaking liquid culture (NBG) at 37°C overnight. The overnight culture was then subcultured 1 in 100 in nu-

trient broth with galactose (6μM) and incubated at 37^{0}C, with aeration, to late log phase. Enzyme extracts were prepared by the method of Saito *et al* (1967). Galactokinase (EC2.7.1.6, *galK*) was assayed as described elsewhere (Saito *et al.*,1967).The Galactose–1–phosphate uridylyltransferase (EC2.7.7.9, *galT*) and UDP–glucose–4–epimerase (EC5.1.3.2, *galE*) assays are also described elsewhere (Kalckar *et al.*, 1959).

2.2.13 Determination of the *in vitro* stability of K88 expression

Bacteria were seeded in NBG to give an initial viable count of 1–5 per ml and shaken at 37^0 for 11h which usually resulted in 8.0×10^8–1.0×10^9 bacteria per ml (*ca.* 27 generations). A fresh broth was seeded with these bacteria, and grown as before. This process was repeated. Samples of the culture were taken at each transfer (0, 27, 54, and 81 generations). Dilutions of the samples taken during the experiment were spread onto NA plates and incubated for 16h at 37^{0}C. Resulting colonies were then transferred to nitrocellulose and immunoblotted.

2.2.14 Preparation of K88 antigen

Strain DH1(pFM205), grown on NA, was resupended in 0.6 l of 0.1M phosphate buffer, pH 6.5 (PB) (*ca.* 2×10^9 bacteria/ml). Fimbriae were sheared from the suspension by treatment in a Sorvall^R Omnimixer at half speed for 2 min. After centrifugation at 8000g for 20 min (4^{0}C), the supernatant was dialysed against 0.1M acetate buffer, pH 3.9, for 16h at 4^{0}C, and the precipitate collected by centrifugation. The pellet, resuspended in 12ml PB, was layered onto sucrose gradients (4×10ml; 10–50% (w/v) sucrose in 10mM Tris, pH 7.5), and centrifuged (1h, 4^{0}C) at 1.5×10^{5}g in a Beckman SW41 rotor. Visible bands were removed, and dialysed against PB. The dialysate had 4mg/ml protein, with less than 10% (w/w) contamination by lipopolysaccharide. Over 95% of the protein ran as a single band in SDS–PAGE.

2.2.15 Lambda 1059 methods

A gene bank of *S. typhimurium* LT2 DNA in the lambda cloning vector λ 1059 (Karn *et al.*, 1980) was the kind gift of Dr R. Maurer. Here, *Sau*3A-cut *S. typhimurium* DNA was cloned into *Bam*HI-cut vector. To probe the bank for phage carrying *gal* fragments, the recombinant phage were propagated on strain RM11 on nutrient agar plates. DNA from recombinant λ 1059 plaques was transferred to nitrocellulose (Maniatis *et al.*, 1982) and probed with [^{32}P]–labelled plasmid pKO1. DNA from positive clones was prepared from large plate lysates of the clones (Maniatis *et al.*, 1982). Hybrid phage clones which contained a functional *gal* operon were detected by lytic complementation of the *gal* defect in strain Δ4, when grown on minimal galactose plates (Davis *et al.*, 1980).

2.2.16 Tests for bacteriophage sensitivity

Bacteria were grown to stationary phase in NBG, then swabbed across a nutrient agar plate supplemented with 25mM glucose or onto the same medium additionally supplemented with 6mM galactose. Bacteriophage P22 and Felix-O were spotted (*ca.* 10^6 pfu in 5μl NBG) onto the bacteria and the plates were incubated at 37^{0}C for 16hr. Strains were scored bacteriophage-sensitive if a clear zone of lysis appeared. Clearing of *S. typhi* by P22 probably occurred as a result of killing by phage infection without lysis, since P22 does not grow in *S.typhi*. To determine the minimum galactose concetration required for bacteriophage sensitivity, the supplementary galactose was diluted in 10 fold steps from 6mM to 60nM. The minimum galactose concentration was the lowest concentration in the presence of which the bacteriophage could effect complete lysis.

2.2.17 Phage P22 mediated transduction and bacterial transformation

Bacteriophage P22 was propagated on SL1654 and the lysate used to transduce *galE*-H1 strains (grown in NBGG) to *gal*$^+$ by selection on minimal galactose. All

transductants were tested for sensitivity to P22 to ensure no lysogens had been selected (Miller, 1972). Bacterial transformation was performed as described elsewhere (Silhavy *et al.*, 1984).

2.2.18 Preparation of formalin–inactivated cells

Stationary phase broth cultures (10ml; NBG) were subcultured (1 in 10)into NBG and grown, with aeration at 37°C, to approximately 2×10^9 cells/ml. The bacteria were harvested by centrifugation, washed once in saline, and resuspended to 10^{10} cells/ml. A sample was kept for a viable count and formaldehyde (1% v/v) was added to the remaining cells. The mixture was incubated at 37°C for 60 min with occasional shaking. The cells were then centrifuged, washed 3 to 4 times with saline, and a sample plated to check the proportion of viable cells remaining. The samples were measured by microscopic count and the inactivated suspensions were adjusted to 10^{10} cells/ml.

2.2.19 Transposition with Tn*1725*

Tn*1725* (Cm^r) transpositon to plasmid DNA was performed in the following manner: $R_{ts}1$::Tn*1725* was transferred into an *E. coli* K-12 derivative harbouring the target plasmid by the mating for 3h at 30°C in standing culture of 0.1 ml of a stationary phase culture of RU2901, 0.9 ml of a stationary phase culture of Δ4(pADE107), diluted 1:1 with NBG. Following plating of 0.1 ml of mating mix on NA containing Cm and Ap, independent exconjugants were purified, and single colonies grown for 16h at 42°C with selection for the transposon (Cm^r) and the plasmid (Ap^r). Small scale DNA preparations from 10 ml cultures were used to transform Δ4, again with selection for both plasmid (Ap^r) and the transposon (Cm^r). Both gal^+ and gal^- transformants were chosen for further analysis.

2.2.20 Minicell Procedures

Minicells were purified and the plasmid–encoded proteins labelled with [^{35}S]–methionine as described by Kennedy and co–workers (1978) and modified by Achtman and co–workers (1979). Bacteria were grown in 500 ml cultures of NBGG at 37^{0}C for 16h. The cells were harvested by centrifugation and re-suspended in 5 ml of BSG (0.85% (w/v) NaCl, 0.03% (w/v) KH_2PO_4, 0.06% (w/v) Na_2HPO_4, 100 μg/ml gelatin). Separation of minicells from whole cells was acheived by centrifugation of the cells through two successive sucrose gradients. The purified minicells were then pulse–labelled with L–[^{35}S]–methionine. To induce synthesis of the enzymes encoded by the galactose operon, pulse–labelling was performed in minimal galactose medium supplemented with 0.2% (w/v) methionine assay medium. IPTG (to 1mM) was added in some instances to induce the *lac* promotor of pUC9. After labelling, the minicells were pelleted by centrifugation, washed twice in saline, resuspended in 100 ul of 1× sample buffer (Lugtenberg *et al.*, 1975) and solubilized by heating at 100^{0}C for 10 min.

2.2.21 Lipopolysaccharide preparation prior to SDS–PAGE

For analysis of LPS by SDS–PAGE followed by LPS–specific silver staining, 1 ml (*ca.* 5×10^9 cells/ml) of a stationary phase liquid broth culture (in either NBG or NBGG) was pelleted in an Eppendorf tube. The pellet was resuspended in 50 μl of lysing buffer (2% (w/v) SDS, 4% (v/v) β-mercapto-ethanol, 10% (v/v) glycerol, 1M Tris-HCl pH 7.6, 0.1% (w/v) Bromophenol Blue), and boiled for 10 min. Proteinase K (10μl of a 2.5 mg/ml solution in lysing buffer) was added and the resulting mixture was incubated at 60^{0}C for 2h. Samples (10-20μl) were boiled at 100^{0}C for 3 min prior to SDS–PAGE.

2.2.22 Deoxyribonucleic acid (DNA) methods

DNA enzyme reactions

Restriction endonuclease digests were usually performed in one of the three buffers decribed by Davis *et al* (1980). The exceptions were *Eag*1 and *Aat* II. Digestions with these two enzymes were performed in the buffers specified by the supplier (New England BioLabs). Restriction endonuclease digestions were usually incubated at 37^{0}C for 2h. Alkaline phosphatase treatment of restriction endonuclease generated ends was performed as described elsewhere (Maniatis *et al.*, 1982).

Protruding ends created by cleavage with enzymes that generated 5′ cohesive ends were infilled with the Klenow fragment of *E. coli* K12 DNA Polymerase 1. Typically, 1μg of restriction enzyme–digested DNA, 1 μl of 10× nick-translation buffer (Maniatis *et al.*, 1982), 1 μl of each dNTP (*viz*: 50μM dATP, 50μM dGTP, 50μM dCTP, and 50μM dTTP), 1 unit Klenow fragment and water (to 10 μl) were mixed and incubated at 37^0 for 30 min. Reactions were stopped by heating at 60^{0}C for 10 min, followed by purification of the DNA from the reaction mixture on a Sepharose CL-6B mini–column.

Plasmid DNA cleaved with restriction enzymes that generated 3′ cohesive ends were end–filled with T4 DNA polymerase in a final volume of 25 μl containing 2 μg DNA, 2 units T4 DNA polymerase, 1 μl of each dNTP (50μM) and 1 μl 10× DNA polymerase 1 buffer (Maniatis *et al.*, 1982). After a 5 min incubation at 37^{0}C the reaction was stopped by heating at 70^{0}C for 10 min. Salt, unincorporated nucleotides, and enzyme were removed by passing the reaction mixture through a Sepharose CL-6B mini–column.

Ligation reactions with T4 DNA ligase were performed in 1×ligase buffer (20mM Tris–HCl pH7.5, 10mM $MgCl_2$, 0.6mM ATP, 10mM DTT, and BSA (100μg/ml)) and incubated at 10^{0}C for 6–8hrs. Restriction enzymes were heat–inactivated at 65^0 for 15 min prior to ligation. Phosphorylated synthetic linkers were ligated to flush DNA ends generated by T4 DNA polymerase or Klenow

fragment on restriction enzyme–generated protruding ends. Some 1 μg of plasmid DNA was ligated with approximately 3 μg of linker DNA, using 4 units T4 DNA ligase, in a final volume of 10 μl of 1× ligase buffer for 16h at 4^0C.

DNA preparation

Small–scale (usually 10 ml cultures) DNA preparations were performed by the method of Ish–Horowicz and Bourke (1981). Large scale preparations of DNA (usually from 1 litre cultures) using CsCl isopycnic density gradients are described elsewhere (Silhavy *et al.*, 1984). Analysis of the cryptic plasmid of *Salmonella* were performed as described elsewhere (Hackett *et al.*, 1986).

Agarose gel electrophoresis (AGE)

AGE was performed as described by Maniatis *et al.* (1982). For analytical gels the agarose concentration was 0.8% (w/v) and electrophoresis was performed at 80–100V in TBE buffer (67 mM Tris, 22 mM boric acid, and 2 mM EDTA, final pH 8.8). Analytical gels were 13 cm long by 13 cm wide and 0.5 cm thick whereas mini–gels for rapid analysis were 5cm long, 10 cm wide and 2-3 mm thick. Sample wells were 0.5 mm or 1.0 mm wide and 5 mm long. Samples were loaded in 1× running buffer (1.5% (w/v) Ficoll^R 400, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol and RNase (10μg/ml)).

For preparative gels, Sea Plaque low gelling temperature agarose at a concentration of 0.6% (w/v) was used for the separation of restriction fragments. DNA bands were excised and the agarose melted at 65^0C. Five volumes of 20 mM Tris-HC1, 1 mM EDTA pH 8.0 buffer was added, and the agarose extracted with phenol:water and then phenol:chloroform (Maniatis *et al.*, 1982). Residual phenol was removed with chloroform and the DNA precipitated by addition of two volumes of ethanol and one tenth volume of 3 M sodium acetate pH 5.0.

The sizes of restriction fragments were determined by comparing their relative mobilities on gels with those of DNA molecules of known size. The standards used were λ DNA cleaved with *Hind*III (Philippsen *et al.*, 1978), and

Bacillus subtilis phage SPP1 cut with *Eco*RI (Ratcliff *et al.*, 1979). Molecular weights of large fragments were calculated from the sum of the molecular weights of their sub–fragments obtained after digestion with a second enzyme.

DNA labelling, Southern transfers, and DNA blotting

DNA probes were labelled with α–[^{32}P]–dCTP as described by Feinberg and Vogelstein (1983). Southern transfers, DNA–DNA hybridization and autoradiography were performed as described elsewhere (Silhavy *et al*, 1984).

DNA quantitation

DNA concentration in solution was determined by measurement of absorption at 260 nm assuming a A_{260} of 1.0 was equivalent to 50 μg DNA/ml (Miller, 1972).

2.2.23 Electrophoresis and analysis of proteins and LPS

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed on either 11–20% (w/v) SDS–PAGE gradient gels (protein) or uniform 20% (w/v) or 25% (w/v) polyacrylamide gels (for lipopolysaccharides), using a modification of the procedure of Lugtenberg *et al.*, (1975) as described previously by Achtman *et al.* (1978). Samples were denatured by heating at 100^{0}C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 5 hr (11-20% gradient gels) or 10 mA constant current for 16 hr (25% PAGE gels).

Protein staining

Protein staining was achieved by incubation of the gel with gentle agitation for 60 min with 0.5% (w/v) Coomassie^R brilliant blue R250 in 50% (v/v) methanol, 10% (v/v) acetic acid. Destaining was with several changes of 7.5% (v/v) acetic acid, 10% (v/v) methanol, 10% (v/v) ethanol with gentle agitation for 24 hr.

Protein size determination

Molecular weight markers (Bio-Rad) were phosphorylase B (Mr 92500), bovine serum albumin (Mr 66200), ovalbumin (Mr 45000), carbonic anhydrase (Mr 31000), soybean trypsin inhibitor (Mr 21500) and lysozyme (Mr 14400).

Lipopolysaccharide–specific silver staining

Silver staining of LPS was performed using a modification of the method described by Tsai and Frasch (1982). The following procedure was used: SDS–PAGE gels were fixed for 16h in 40% (v/v) ethanol, 10% (v/v) acetic acid, then oxidized for 5 min with 0.7% (v/v) periodic acid in 40% (v/v) ethanol, 10% (v/v) acetic acid. After oxidation the gels were washed 4 times (30 min each) with water and then stained (10 min) in a solution composed of 28 ml sodium hydroxide (0.1 M), 2 ml ammonium hydroxide (30% (w/v)) and 5 ml of silver nitrate (20% (w/v)). The stained gels were developed in a solution of 50μg/ml citric acid and 0.05% (w/v) formaldehyde.

Western transfer

After SDS–PAGE the separated proteins were tranferred to nitrocellulose in a Bio–Rad apparatus as described elsewhere (Towbin *et al.*, 1979).

Colony transfer

Colony transfers were achieved by placing a circular nitrocellulose filter onto a nutrient agar plate which had been seeded with *ca.* 100–200 colonies derived from the strain of interest and incubated (37^{0}C, 16hr). After 2 min the filter was removed and the excess cellular material removed by washing with saline.

Immunoblotting

Nitrocellulose filters (colony transfer or Western transfer) were immunoblotted to detect the presence of K88 antigen. Primary antibody was supplied by addi-

tion of rabbit anti–K88 serum. The nitrocellulose sheet was incubated for 20-30 min in Blotto (5% (w/v) skim milk powder suspended in 0.05% (w/v) Tween 20, 20 mM Tris-HCl and 0.9% (w/v) NaCl (TTBS)). Primary antibody was diluted 1/1000 in TTBS with 0.02% (w/v) skim milk powder, and incubated with the nitrocellulose filter for 2-16h with gentle shaking. The nitrocellulose sheet was then washed three times in TTBS (10 min/wash). The filter was then incubated for 2h with goat anti–rabbit IgG coupled with horseradish peroxidase, diluted 1/500 in TTBS with 0.02% w/v skim milk powder. The nitrocellulose filter was then washed twice (5 min/wash) in TTBS, and then four times (5 min/wash) in TBS (20 mM Tris-HCI, 0.9% (w/v) NaCl). Staining solution (0.5mg/ml 4-chloro-1-napthol,30% (v/v) methanol,0.1% (w/v) H_2O_2 in TBS) was then added and the filter was stained for 10-15 min with gentle agitation at room temperature, as described by Hawkes *et al.* (1982).

2.2.24 Preparation of serum

Rabbit anti–sera were raised by intramuscular injection of formalin–inactivated bacteria (Neoh and Rowley, 1972). Rabbit anti–K88 was raised using formalin–inactivated DH1(pFM205) as the eliciting antigen. This serum was absorbed with *E.coli* strain DH1 and *S. typhimurium* before use. Rabbit anti–Vi was raised using formalin inactivated *S.typhi* as the eliciting antigen. This serum was absorbed with *S. typhi* Ty2Vi before use. Rabbit anti–O 9,12 was raised using formalin–inactivated Ty2Vi as the eliciting antigen and was absorbed with *S. typhi* R2 (a rough Vi–negative variant of Ty2) before use.

Absorption of serum with bacteria

Exponential phase bacteria were washed three times in cold saline and then resuspended in serum at a concentration of approximately 10^{10} organisms/ml. After standing for 16h at 4^0C, the bacteria were deposited by centrifugation and the serum was removed with a Pasteur pipette. The absorbed serum was then sterilised by filtration through a 0.45 μm Millex^R–HA filter and either used

immediately or stored at 4^0C.

2.2.25 Hemagglutination (HA) inhibition assay

These assays were generally performed using suspensions of formalin killed bacteria to test the expression of O–antigen by *galE*–H1 strains when grown either smooth or rough. Bacterial strains were grown in either NBG or NBGG for 16h with aeration at 37^0C. They were then subcultured 1 in 10 into fresh medium of the same type and incubated at 37^0C with aeration until the cultures reached late exponential phase. The bacterial culture was then formalin–killed as decribed above. The HA inhibition system involved the agglutination of sheep erythrocytes, which had been sensitized with *S.typhi* LPS (Crumpton *et al.*, 1958), by limiting concentrations (4HA units/ml) of a rabbit antiserum prepared against Ty2Vi bacteria, a *via* derivative of *S.typhi* Ty2. Two-fold falling dilutions of the suspensions of inactivated bacteria were tested for their capacities to inhibit HA, the endpoints being expressed as the number of wells showing clear inhibition (control wells received saline diluent). Formalin-killed Ty2Vi was used as a bacterial standard, and a suspension of *S.typhi* LPS indicated that the level of detection of O-antigen was typically 0.1μg/ml.

2.2.26 Enzyme Linked Immunosorbent Assay (ELISA)

Human immune responses to *S. typhi*

To determine the serum and intestinal humoral immune responses after peroral immunization of humans with EX462, ELISAs were performed (Bartholomeusz *et al.*, 1986). ELISA trays were sensitized with *S.typhi* LPS. Goat anti-human IgA and IgG, each conjugated with alkaline phosphatase were used as the secondary antibody. Color development after 2hr at 37°C was read (at 405nm) using a Titertek Multiskan. ELISA values were expressed as absorption units per mg of total immunoglobulin of the given class.

Mouse immune responses to K88 bearing strains

Mouse sera were titrated for IgG antibodies to K88 using an enzyme–linked immunosorbent assay (ELISA). The K88 antigen was diluted to 5μg/ml in TSA buffer (25mM Tris, pH 7.5, 132mM NaCl, 0.05% (w/v) sodium azide) and used to sensitize 96–well vinyl ELISA trays for 16h at 4^{0}C (100μl/well). After washing off unbound protein, the trays were blocked using a solution containing BSA (200μg/ml) and Tween 20 (0.05% (w/v)) for 2h at 37^{0}C, and then washed five times with TSA. Duplicate four–fold dilutions were made from each test serum, and the trays were then incubated for 4h at 37^{0}C in a humidified chamber; control wells received diluent (the BSA–Tween blocking solution) only. After washing, an alkaline phosphatase–conjugated anti–mouse IgG secondary antibody was added (1:15,000 dilution, 100μl/well) and the trays incubated for 16h at 4^{0}C. After washing, 0.1ml of substrate solution (Sigma phosphatase substrate pellets at 1mg/ml in 1M diethanolamine–HCl, pH9.8) was added to each well and colour development read after 3h at 37^{0}C using a Titertek Multiscan (OD_{405}nm). ELISA titres were calculated by interpolation using an OD of 0.15 as the endpoint.

2.2.27 ELISA inhibition assay (EIA)

K88 expression was quantitated using an ELISA–inhibition assay (EIA), in which dilutions of bacterial suspensions or K88–containing suspensions were tested for their capacity to inhibit the binding of a limiting concentration of rabbit (IgG) anti–K88 to K88–sensitized trays. Equal volumes (50μl) of inhibitor and antibody dilution were incubated in K88–sensitized wells for 4h at 37^{0}C. Each sample was titrated in duplicate and purified K88 was used to obtain a standard inhibition curve; negative controls for inhibition contained antibody diluent instead of inhibitor. After washing, an alkaline phosphatase–conjugated sheep anti–rabbit IgG was added (1:6,000 dilution) and the ELISA completed as above. Inhibition curves were plotted for each test sample and the endpoint

determined by interpolation as that dilution acheiving 50% reduction in colour development, which in the absence of inhibitor was *ca.* 0.5 OD units. The K88 standard curve showed the sensitivity of the assay to be *ca.* 0.05μg/ml, and this figure, together with the dilution endpoint, was used to calculate the K88 expression for each sample.

2.2.28 Bactericidal assay

Four–fold serial dilutions of heat inactivated (56°C for 30min) sera (from the recipients of the candidate typhoid vaccine) were prepared in 0.1%(w/v) peptone in saline, and mixed with equal volumes (0.4ml) of a suspension of the indicator bacteria, Ty2Vi (*ca.* 4×10^3/ml), in the same diluent containing 20%(v/v) guinea pig serum as a complement source. After 60 minutes at 37°C the tubes were transferred to an ice water bath and 0.1ml aliquots were spread on nutrient agar plates to determine residual viability. By plotting the viability as a function of reciprocal serum dilutions, bactericidal titres were obtained by interpolation, and expressed as the reciprocal serum dilution killing 50% of the added bacteria.

2.2.29 Serum sensitivity of various strains

Normal human serum, obtained from workers in our laboratories that had no history of prior exposure to *S. typhi* or to vaccination against *S.typhi*, was adsorbed before filtration and use. Bacteria were grown as described in HA inhibition assay; they were diluted to *ca.* 2500 cfu/ml in saline and 0.1ml aliquots were mixed with 0.9ml of varying concentrations (90%, 30%, 10%, 3%, and 1% (v/v)) of the adsorbed human serum, in saline. The mixtures were incubated at 37^{0}C for 1hr and plated on nutrient agar for determination of residual viability. From a plot of viability as a function of serum concentration, the serum resistance of a bacterial strain is expressed as the concentration of serum which reduced viability by 50% (relative to viability in saline control tubes).

2.2.30 Animal Experiments

Preparation of bacteria for injection

Live preparations of various bacteria were required for immunization and challenge of mice. They were prepared by inoculation of a 10ml NBGG broth with 1.0ml of a stationary phase broth culture of the strain required. Each broth was incubated with aeration at 37^0 for 3 hours, to give about 2×10^9 bacteria/ml. Bacteria were harvested by centrifugation, washed in saline, and resuspended in saline. Bacterial concentrations were estimated by applying typical viability indices to total microscopic counts. Cultures were then appropriately diluted with cold saline.

Mice

Female LACA mice of an average weight of 25g were used in this study. They were bred under specific-pathogen-free conditions at the Waite Agricultural Institute, Adelaide and were allowed to acclimatize in our conventional animal house for 24–96hr with free access to food and water.

Bacterial virulence by the intraperitoneal (ip) or oral route

Bacteria given orally or ip were suspended in saline and injected (0.2 ml/dose) using a blunt 19 gauge (g) needle, or a 23 g needle respectively. Oral inoculation of mice was preceded by administration of 0.2 ml of 50% saturated $NaHCO_3$. LD_{50} values were calculated by the method of Reed and Muench (1938).

Mouse mucin virulence assay

After appropriate initial dilutions of the bacterial culture in saline, each suspension was diluted 1 in 40 into iron-supplemented hog gastric mucin (5%(w/v)). Doses (0.5ml) of this final suspension were injected intraperitoneally (ip) into mice and LD_{50} values, based on the number of survivors after 72hr, were calculated by the method of Reed and Muench (1938).

To prepare mucin, a 5%(w/v) suspension of hog gastric mucin (in saline) was heated at 56^{0}C for 1hr with occasional shaking, and boiled for 6min (no contaminants were detected after such treatment). After boiling, the mucin was supplemented with ammonium ferric citrate (BDH Catalog no.27163) so that each mouse eventually received 5mg of Fe^{+++} per kg body weight (Powell *et al.*, 1980). Finally, the mucin was neutralized (pH7.0) with 10M NaOH.

Comparison of vaccine potential

Five fold serial dilutions of the strain of interest were prepared in saline and used to immunize (ip) groups of 8 mice. Control mice received 0.5ml saline only. Fourteen days later all mice were challenged with Ty2 (*ca.* 12,000 cfu) in Fe^{+++}–supplemented mucin, as described above. Survival data at 72hr were used to calculate the number of organisms that constituted an effective immunizing dose for 50% of the mice (EID_{50}). This value was obtained by interpolation from a plot of cumulative percentage mortality (calculated according to Reed and Muench (1938)) versus immunizing dose. The LD_{50} value obtained for Ty2 in this experiment was inexplicably high; survival of the control mice indicated that in this experiment the challenge dose represented 40 LD_{50}s.

Enumeration of bacteria in infected animals

The fate of a bacterial inoculum, used to experimentally infect mice, was studied by determination of the number of viable organisms in various organs and tissues of the infected animals. Results were expressed as the geometric means (plus or minus the standard error) of the individual values obtained. Briefly, the methods involved were:

(a) Peritoneal Cavity: mice were killed by cervical dislocation at various times after injection of the bacterial strain being tested and swabbed with ethanol (70% v/v). The abdominal skin was reflected and the peritoneal cavity rinsed out with 2.0ml of sterile saline. The abdomen was massaged vigorously during the washout to ensure maximum recovery of bacteria. The washout

fluid was diluted appropriately and plated in duplicate on nutrient agar plates followed by incubation at 37^0C for 16h.

(b) Spleens: spleens were removed aseptically and placed in separate sterile bottles containing 5.0ml of sterile saline. After homogenisation the suspensions were diluted appropriately in sterile saline. Duplicate 0.1ml samples were plated on nutrient agar plates and incubated at 37^0C for 16h.

(c) Livers: the technique was essentially as above (spleens), but with the use of 10ml of sterile saline for homogenisation.

(d) Peyer's patches: typically groups of 5 mice were sacrificed. The small intestine and cecum were removed, separated, and freed from the surrounding mesentery. Saline (10 ml) was passed through each small intestine as a routine preliminary washing procedure. The Peyer's patch tissue was excised from the small intestine and placed into 5ml of saline. This was then homogenised and undiluted homogenate or dilutions of the homogenate (10–fold) were spread onto nutrient agar plates and incubated at 37^0C for 16h.

2.2.31 Volunteer trial

Volunteers

With the approval of the Ethics Committee of the Royal Adelaide Hospital, four volunteers, 24–34 years of age, gave their informed consent and took part in a phase 1 trial of the candidate typhoid vaccine described here, which was designed to examine its safety and immunogenicity. Each volunteer was assessed as being normal on physical examination with no contra-indication to participation being suggested by their past medical history. Hematological indices and serum biochemistry were within normal limits. As part of the information provided to the volunteers before they consented for the study, they were asked to contact one of the clinical investigators if they noticed any untoward symptoms such as fever or disturbance in bowel habit.

Administration of the vaccine

Each volunteer fasted overnight and the next morning ingested 1g $NaHCO_3$, in 50ml water, followed within 10min by 7.0×10^8 cfu of the vaccine organism, EX462, in saline. The bacteria were harvested from an exponential phase culture in NBGG, and hence, were phenotypically smooth.

Sampling of serum and intestinal fluid

In the week preceding the trial, and on d22 after vaccine administration, samples of serum and intestinal fluid were obtained from the volunteers in order to determine the antibody response to the vaccine (Bartholomeusz *et al.*, 1986). Intestinal fluid was aspirated through an intestinal tube positioned just before the ligament of Treitz, its position being confirmed by fluoroscopy. Only samples of intestinal fluid with a pH >6.5 were retained. Samples were stored on ice until collection was complete, centrifuged at 10^4g for 30min at 4^0C, and the supernatants were stored as aliquots at -70^0C until assayed.

Stool samples

Stool samples were collected daily from each volunteer from 7 days before until 10 days after they had ingested the vaccine.

Blood cultures

Blood samples from the febrile volunteers (20ml) were mixed with an equal volume of medium (0.2%(w/v) glucose, 0.5%(w/v) Difco peptone, 1.5%(w/v) Difco tryptone, 0.5%(w/v) Difco yeast extract, 0.4%(w/v) NaCl, 0.25%(w/v) NaH_2PO_4 and p–aminobenzoic acid (50ug/ml)) and incubated standing at 37^0C for 16h, after which time culture aliquots were Gram–stained or spread on nutrient agar plates with 25mM glucose for colony isolation.

Chapter 3

Cloning of the *gal* operon of *Salmonella typhimurium*

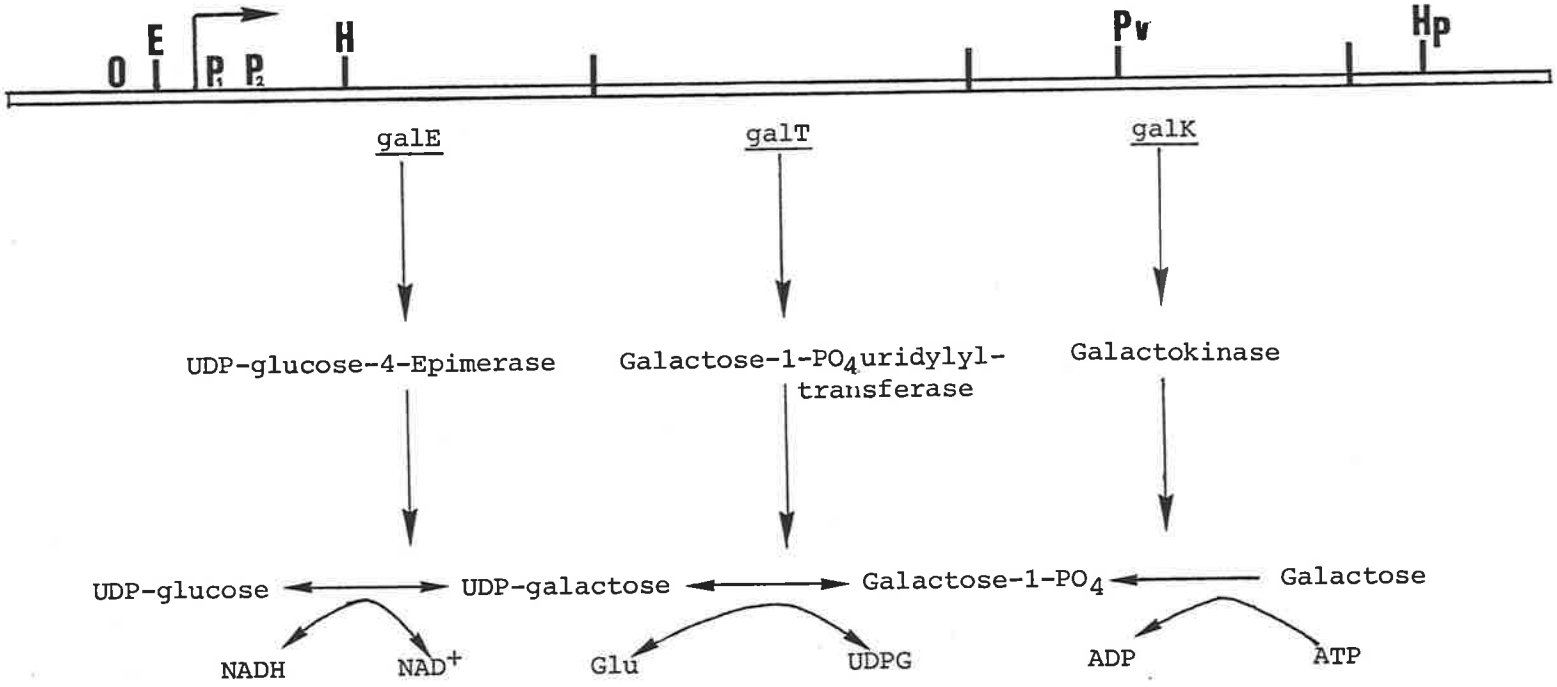
3.1 Introduction

Little attention has been focused on the structure of the *gal* operon of *Salmonella* whereas many detailed investigations have been carried out on the *gal* operon of *E. coli* K12. The galactose operon of *E. coli* K12 was found to be composed of three genes: *galE, galT,* and *galK* (Adhya and Shapiro, 1969). The gene products of the *galK*, *galT*, and *galE* genes, galactokinase, galactose–1–phosphate uridylyltransferase, and UDP–glucose–4–epimerase respectively, synthesize UDP–glucose from galactose (Fig. 3.1). The nature of this operon is amphibolic owing to the reversibility of the reactions catalysed by UDP–glucose–4–epimerase and galactose–1–phosphate uridylyltransferase (Saito *et al*, 1967; Wilson and Hogness, 1969a; 1969b). Hence, in the absence of exogenous galactose, UDP–galactose, which is an important precursor of polysaccharide synthesis, can be synthesised. In the presence of exogenous galactose the *gal* operon enables utilisation of this hexose as a carbon source as well as for the synthesis of LPS and other galactose–containing polysaccharides.

The order of genes in the *E. coli* K12 *gal* operon: (promoter) *galE*, *galT*, *galK* (Fig. 3.1), has been described (Adler and Kaiser, 1963; Adler and

Figure 3.1

The *gal* operon of *Escherichia coli* K12: The genetic organization of the *gal* operon of *E. coli* K12, its gene products, and the reactions they catalyse are shown (see text for details). Some notable restriction endonuclease digestion sites have been included for comparison with the *Salmonella gal* operon (see text). **O**: *gal* operator site. $\mathbf{P_1}$: Promoter 1 of the *gal* operon. $\mathbf{P_2}$: Promoter 2 of the *gal* operon.
E: *Eco*RI; **H**: *Hind*III; **Pv**: *Pvu*II; **Hp**: *Hpa*I
UDP:Uridine diphosphate; Glu: Glucose; UDPG: UDP–glucose;
ADP: Adenosine diphosphate; ATP: Adenosine triphosphate;
NAD^+: Nicotinamide adenine dinucleotide (oxidized form)
NADH: Nicotinamide adenine dinucleotide (reduced form).


O
E
P1 P2
H
Pv
Hp
galE
galT
galK
UDP-glucose-4-Epimerase
Galactose-1-PO4uridylyl-transferase
Galactokinase
UDP-glucose
UDP-galactose
Galactose-1-PO4
Galactose
NADH
NAD+
Glu
UDPG
ADP
ATP

Templeton, 1963; Shapiro and Adhya, 1965). Galactose–transducing variants of phage lambda were subsequently described (Feiss *et al.*, 1972),and one of these tranducing phage was the source of *gal* DNA for the cloning (in plasmid pBR322) of the *gal* operon of *E. coli* K12 (Busby *et al.*, 1982). The DNA sequences of the *gal* promoter and the *galK* gene have been described (Debouck *et al.*, 1985). The *E. coli gal* operon has two notable internal restriction endonuclease cleavage sites, a *Hind*III site in the *galE* gene and a *Pvu*II site in the *galK* gene, and is flanked by an *Eco*R1 site (*gal* promoter end) and a *Hpa*1 site (*gal* terminator end; Busby *et al.*, 1982) (Fig. 3.1).

The operator–promoter region of the cloned *E. coli* K12 *gal* DNA was found to carry two promoters and two operators within a 0.1 kb DNA segment (Adhya and Miller, 1979; Dilauro *et al.*, 1979; Aida *et al.*, 1981; Busby *et al.*, 1982; Irani *et al.*, 1983). The operator sites interact with the *gal* repressor protein (*galR* product). The two promoters of *gal* are both inactivated by GalRp. The mechanism by which GalRp inhibits RNA–polymerase association with, and initiation of transcription from, either of the promoters has not been completely resolved. The *gal* operon is also subject to control by cyclic adenosine monophosphate–catobolic repressor protein complex (cAMP–CRP). One of the two promoters is activated by cAMP–CRP complex whereas the other is inhibited by this complex (Adhya and Miller, 1979). The need for two promoters which produce discoordinate expression of the *gal* genes was thought to reflect the amphibolic nature of this operon (Adhya and Miller, 1979). The presence of Rho–dependent terminators at both intracistronic locations and at the *galK* end of the operon also demonstrated the necessity of discoordinate expression of the *gal* encoded genes (deCrombrugghe *et al.*, 1973).

Some investigators have reported the approximate subunit sizes of the *gal* encoded proteins. Earlier studies with the purified *E. coli* enzymes indicated that all the *gal*–encoded proteins were of subunit Mr about 40 kDal (Saito *et al.*, 1967; Wilson and Hogness, 1969a, 1969b). However, a recent investigation using 2–dimensional–PAGE established that the Mr values of the *gal* proteins

of *E. coli* K12 were: 32 kDal (GalEp), 39 kDal (GalTp) and 40 kDal (GalKp) (Merril *et al.*, 1981). The GalEp and GalTp function as dimers whereas GalKp functions as a monomer.

In this chapter, the cloning and partial characterisation of the *gal* operon of *S. typhimurium* LT2 is described. The promoter analysis vector pKO1, which harboured the *galK* gene of *E. coli* K12, was used as a probe to identify recombinant λ1059 phage which harbored *S. typhimurium gal* DNA insertions. DNA which conferred a gal^+ phenotype upon a Δgal strain of *E. coli* K12 was subcloned from recombinant phage and analysed by transposon mutagenesis and minicell analysis.

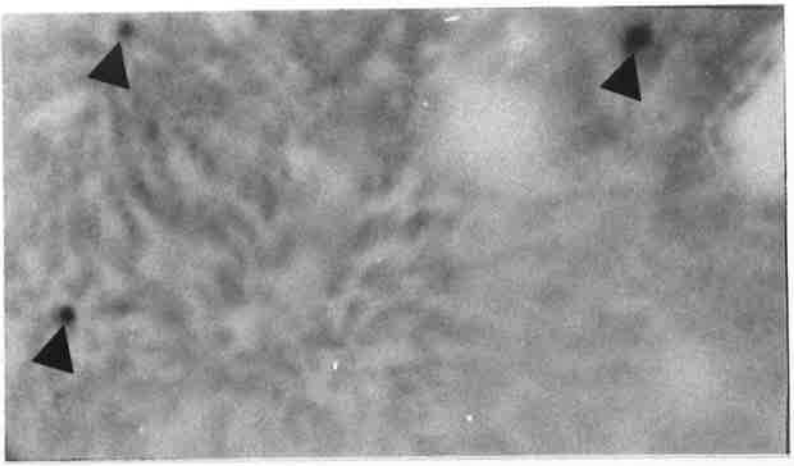
3.2 Results

3.2.1 Isolation of clones of the *gal* operon

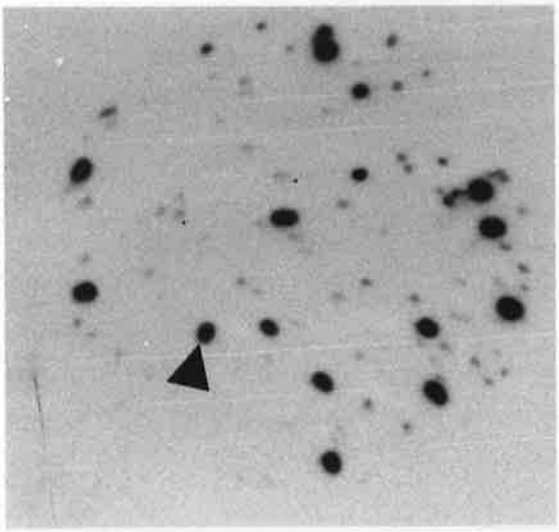
A lambda bank of *S. typhimurium* LT2 DNA, in the vector λ1059 (a gift from R. Maurer), was probed with nick–translated plasmid pKO1 (McKenney *et al.*, 1981). Of about 2×10^6 plaques screened, three reacted with the probe (Fig. 3.2). The gal^+ recombinant phage were purified by eluting phage from single plaques and re–probing until 100% purity was achieved (Fig. 3.2). Restriction enzyme analysis (Fig. 3.3) showed that each of the three phage contained similar cloned DNA. Analysis using lytic complementation of the *E. coli* strain Δ4 implied that λB1.2 (Fig. 3.3) contained the entire *gal* operon, while the other two lambda clones did not. Furthermore, DNA probing using labelled pKO1 as probe, showed that DNA homologous to the *E. coli* K12 *galK* gene lay on the 5.6kb *Hind*III fragment of λF6.1 (Fig. 3.3, DNA probing not shown). Taken together, the results of the lytic complementation test, and the DNA probing implied that the entire *gal* operon of *S. typhimurium* might lie in the DNA at or near the left–hand end of cloned DNA in λB1.2 (Fig. 3.3).

Figure 3.2

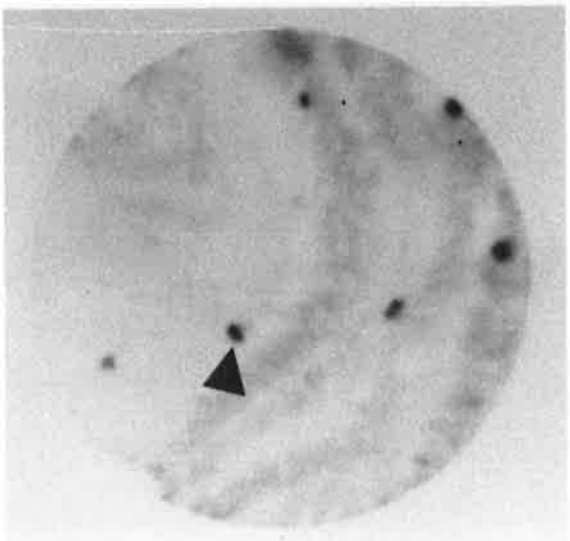
Probing a *Salmonella typhimurium* λ1059 gene bank with pKO1: A lambda gene bank (in λ 1059) of *S. typhimurium* DNA was probed with labeled pKOI DNA (encoding the *galK* gene of *E. coli* K12; McKenney *et al.*, 1981). Three gal^+ recombinants were detected (**1**). The phage were subsequently purified (**2** and **3**). Arrows indicate the location of gal^+ plaques. See text for details.



1



2



3

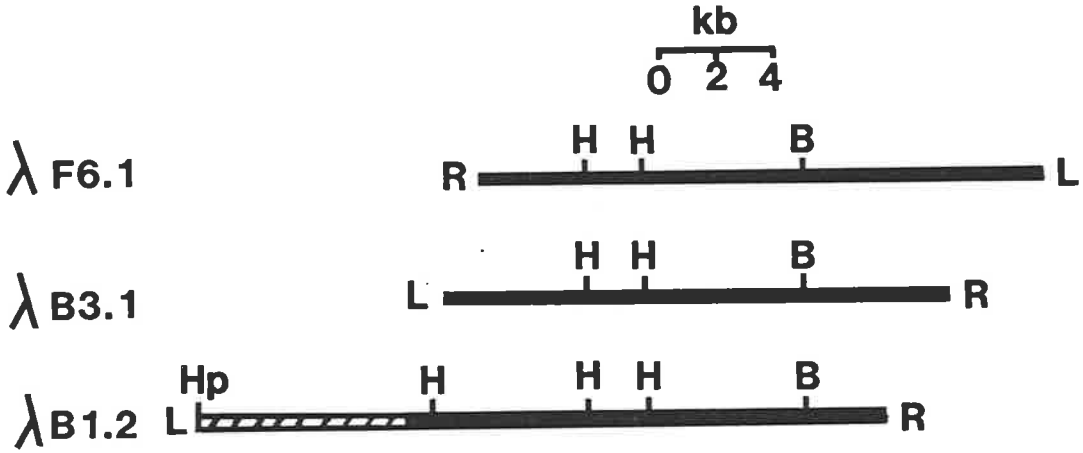
Figure 3.3

Analysis of DNA extracted from recombinant λ1059:DNA was prepared from the *gal*$^+$ clones and restriction enzyme digests showed that the three clones had insert DNA in common — the inserts are drawn to scale above and aligned in a manner which emphasies their homology. The junction points between lambda DNA and insert DNA were defined by *Hind*III and *Hpa*I–*Hind*III digests of the clones. The lambda right arm has a *Hind*III site 2.0 kb from the junction point, while the lambda left arm has a HpaI site 6.9 kb from the junction point. This *Hpa*I site is shown only for λ B1.2, as it was used in a plasmid cloning step which resulted in pADE100 (Figure 3.4).

L: left arm of λ 1059; **R**: right arm of λ 1059; **B**: *Bam*HI site;

H: *Hind*III site; **Hp**: *Hpa*I site.

▬▬: *S. typhimurium* DNA.


kb
0 2 4
λ F6.1
H H B
R L
λ B3.1
H H B
L R
λ B1.2
Hp H H H B
L R

3.2.2 Subcloning of the *gal* DNA from λB1.2

DNA isolated from λ B1.2 was digested with *Hpa*I and ligated with *Bam*HI linkers. The DNA was then digested with *Bam*HI and ligated with phosphatase–treated *Bam*HI–digested pJRD158B DNA. The ligation mix was transformed into *E. coli* K12 strain Δ4 and plated on galactose containing indicator plates with selection for Ap-resistance. A single gal^+ transformant was obtained. Plasmid DNA (pADE100) was prepared from this strain and a partial restriction enzyme map of the insert DNA (20.5kb) was obtained (Fig. 3.4). The restriction sites for the enzyme *Hind*III were as expected if the cloned DNA in pADE100 had come from the analogous region of λB1.2 (Fig. 3.4). Since the insert DNA in pADE100 was 20.5kb in size, a *Hind*III subclone (pADE105) of the clone was prepared (Fig. 3.4). Plasmid pADE105 was examined for its ability to complement the *gal* deletion in strain Δ4. Plasmid pADE105, and an *Eco*R1 cutdown of pADE105 (pADE107; Fig. 3.4), were effective in this complementation. Plasmid pADE107 harboured only 4.7kb of cloned DNA, and was used for further study. A restriction map of the *S. typhimurium* DNA inserted into plasmid pADE107 was obtained (Fig. 3.5).

3.2.3 Localisation of the *gal* genes in the cloned DNA of pADE107

Transposon mutagenesis

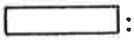
Transposon Tn*1725* insertions into the *S. typhimurium* DNA in pADE107 were obtained and mapped (Fig. 3.5). Each of the plasmids carrying a Tn*1725* insertion in the cloned DNA were transformed into each of *E. coli* K12 *galE, galT*, and *galK* strains, and the ability of the plasmids to complement the chromosomal *gal* mutation was screened on indicator plates (Table 3.1).

Of a total of 12 Tn*1725* plasmids with insertion mutations to be discussed here, one (pADE133) did not affect the *gal* phenotype of the plasmid pADE107 (Table 3.1; Fig. 3.5). Four plasmids with insertion mutations (pADE134,

Figure 3.4

Subcloning of the *gal* DNA of *S. typhimurium* LT2: DNA of λ B1.2 was cut with *Hpa*I and *Bam*HI linkers were added. Subsequently, the DNA was cut with *Bam*HI and ligated with *Bam*HI-cut pJRD158B DNA to give pADE100. Plasmid pADE105 is a *Hind*III subclone of pADE100 in pUC9. An *Eco*RI cutdown of pADE105 was constructed which resulted in plasmid pADE107. Plasmids pADE105 and pADE107 both contain the entire *gal* operon.

B: *Bam*HI site; **R:** *Eco*RI site; **H:** *Hind*III site; **Hp:** *Hpa*I site.


:cloned *S. typhimurium* DNA;  : λ 1059 DNA;

: pJRD158B DNA;  : pUC9 DNA.

A scale (kb) appears below.

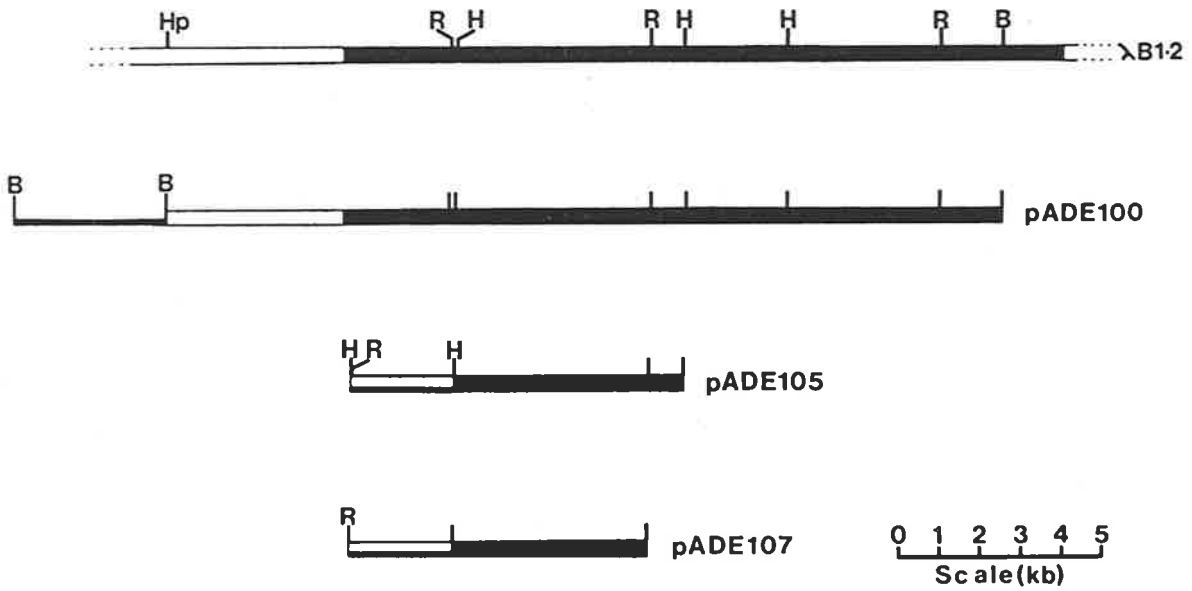
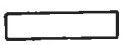

Hp
R H
R H
H
R
B
λB1-2
B
B
pADE100
H R
H
pADE105
R
pADE107
0 1 2 3 4 5
Scale (kb)

Figure 3.5

Characterization of pADE107: Restriction enzyme map of pADE107 and localization of transposon insertion mutations and the *galE, galT*, and *galK* genes. Plasmid pADE107 contains a 4.7kb fragment of *S. typhimurium* DNA in pUC9 and expresses all *gal* genes (see Text). A restriction map of the cloned DNA was obtained. In addition to the sites shown the following enzymes did not have sites in the insert: *Bam*HI, *Dra*I, *Kpn*I, *Pvu*II, *Sac*I and *Xho*I. Transposon *Tn*1725 insertion mutations were constructed in the cloned DNA; the plasmid numbers shown are those of the insertion–bearing plasmids. Δ :Insertion not affecting gal phenotype; ▲ :insertion affecting *gal* phenotype. The transposon insertion mutants were examined for expression of *galE*, *galT*, and *galK* genes, and this data, together with minicell data on protein sizes, allowed the location of the *gal* genes to be defined.

A, *Acc*I site; **Bg**, *Bgl*II site; **C** , *Cla*I site; **H**, *Hind*III site;
M, *Mlu*I site; **N**, *Nae*I site; **P**, *Pst*I site; **R**, *Eco*RI site.

 , *galE* gene;  , *galT* gene;  , *galK* gene;

 , pUC9 DNA;  , cloned non–*gal* DNA.

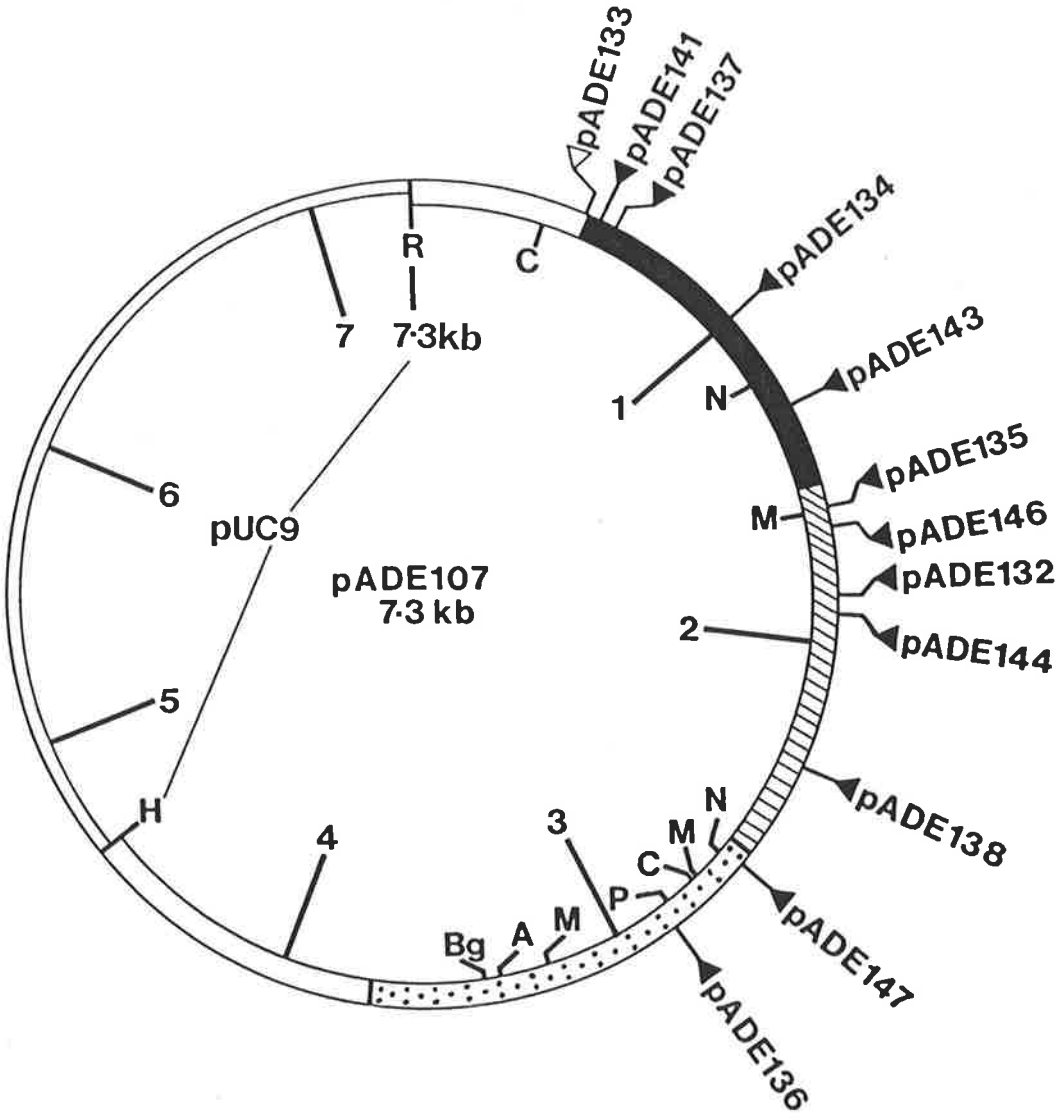

pADE133
pADE141
pADE137
pADE134
pADE143
pADE135
pADE146
pADE132
pADE144
pADE138
pADE147
pADE136
R
C
7
7·3kb
1
N
M
2
6
pUC9
pADE107
7·3 kb
5
H
4
3
N
M
C
P
Bg
A
M

Table 3.1: The ability of transposon Tn1725 insertion mutants of the gal$^+$ plasmid pADE107 to complement galE, galT, and galK strains of E. coli K12

Plasmid	Complementation[1]		
	E	T	K
pADE107	+	+	+
pADE133	+	+	+
pADE137	−	−	−
pADE141	−	−	−
pADE134	−	+	+
pADE143	−	+	+
pADE132	+	−	+
pADE146	+	−	+
pADE138	+	−	+
pADE144	+	−	+
pADE146	+	−	−
pADE147	+	+	−
pADE136	+	+	−

1. Transposon Tn1725 insertion mutants were obtained in the cloned DNA of pADE107 (gal$^+$). The mutated plasmids were transformed into each of galE, galT, and galK strains of E. coli K12, and the ability of the plasmids to complement the chromosomal gal lesions screened on indicator plates.

   E: KA56(galE) T: CA13(galT) K: P1700(galK)

+: complementation; −: no complementation. The points of transposon insertion in the various mutant plasmids are shown in Figure 3.5.

pADE137, pADE143 and pADE141) gave a *galE*$^-$ phenotype (Table 3.1; Fig. 3.5)). In two of the plasmids (pADE137 and pADE141), the expression of the *galT* and *galK* genes was also eliminated. The insertion mutations in the other two plasmids (pADE134 and pADE143) did not result in elimination of expression of the *galT* and *galK* genes (Table 3.1). These results implied that the start of the *galE* gene lay between the transposon insertion point in pADE133 and the insertion point in pADE141, and that the *galE* gene extended to or past the insertion point in pADE143 (Fig. 3.5).

Of the remaining plasmids carrying insertion mutations to be discussed, all had a *galE*$^+$ phenotype, and all mutations lay downstream of the mutation in pADE143 (Fig. 3.5). Five had a *galT*$^-$ phenotype (pADE132, pADE135, pADE138, pADE144, pADE146) and of these, four were *galK*$^+$ (Table 3.1). It appeared, then, that the *galT* gene extended from a point at or upstream of the insertion in pADE135, but downstream of the insertion in pADE143, to a point at or downstream of the insertion in pADE138 (Fig. 3.5).

The last two plasmids carried insertions that caused a *galE*$^+$, *galT*$^+$ phenotype, but were *galK*$^-$ (pADE136, pADE147: Table 3.1; Fig. 3.5). This suggested that the *galK* gene extended from a point which lay between the insertion in pADE138 and the insertion in pADE147.

Minicell analysis of the genes encoded by pADE107

Various plasmids were transformed into the minicell–producing strain DS410, and plasmid–encoded proteins were labelled with [^{35}S]–methionine and, subjected to SDS–PAGE followed by autoradiography (Fig. 3.6). Of the several protein bands that were labelled with [^{35}S]–methionine (Fig. 3.6), four are encoded by either the vector pUC9 (bands W and X) or the transposon Tn*1725* (bands Y and Z). Bands W and X were seen in a strain DS410(pUC9) (Fig. 3.4, tracks 1 and 2). Band Z was Cm-transacetylase enzyme derived from Tn*1725* (data not shown; Manning *et al.*, 1985), while band Y was encoded by the Tn*1725* (data not shown). The remaining four bands, labelled E, T, K and E′

Figure 3.6

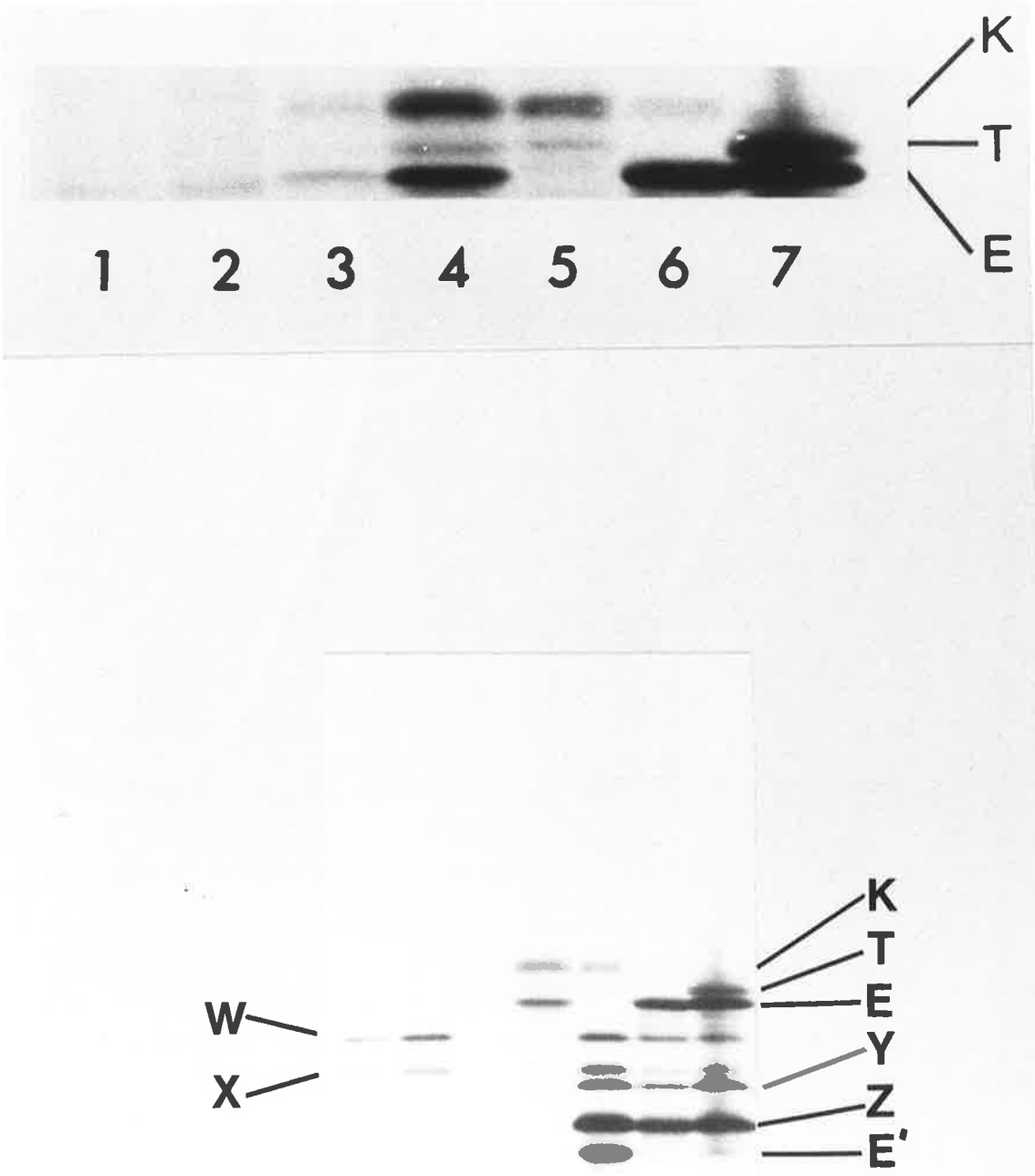
Minicell analysis of the proteins encoded by various plasmids containing *S. typhimurium gal* DNA: Various plasmids were transformed into the minicell–producing strain DS410, and plasmid-encoded proteins viewed by autoradiography of an SDS-PAGE gel of total minicell proteins after [^{35}S]-methionine labelling of minicells purified from the various strains. The main body of the Figure is a 72h autoradiograph, the insert is an enlargement to show the *gal* encoded protein bands more clearly. Bands **W** and **X** are encoded by pUC9 (see the text), while bands **Y** and **Z** are encoded by transposon *Tn*1725 (see the text). Bands **E**, **T**, **K**, and **E′** represent the products of the *galE, galT* and *galK* genes, and a truncated *galE* protein, respectively, as explained in the text.

1: DS410(pUC9); **2**: DS410(pUC9), IPTG-induced;

3: DS410(pADE107); **4**: DS410(pADE107), IPTG-induced;

5: DS410(pADE134); **6**: DS410(pADE132);

7: DS410(pADE136).


K
T
E
1 2 3 4 5 6 7
W
X
K
T
E
Y
Z
E'
1 2 3 4 5 6 7

(Fig. 3.6), are explained below.

Strain DS410(pADE107) expressed three extra proteins which when run on SDS–PAGE correspond to E, T, and K proteins (Fig. 3.6, tracks 3 and 4), the levels of which were higher if IPTG induction was employed (Fig. 3.6, track 4). The augmented expression of the *gal* gene products by strain DS410(pADE107) when grown in the presence of IPTG (Fig.3.5; track 4), was probably the result of either IPTG induced transcription from the *lac* promoter of pUC9 or increased expression of methyl–β–D–thiogalactoside permease (*lacY* gene product) by strain DS410, resulting in a greater rate of transport of galactose into the cells (Adhya and Echols, 1966).

Band E was not visualized in DS410(pADE134) (Fig. 3.6, track 5), but a new band (E′) appeared. Since the transposon insertion in pADE134 (Fig. 3.5) was in the *galE* gene (Table 3.1), band E′ was thought to be a truncated GalE protein. The fact that the transposon insertion in pADE134 inactivated the *galE* gene, but not *galT* and *galK* (Fig. 3.5; Table 3.1), taken together with the absence of band E expression implied that band E was the *galE* gene product. The lack of polarity of transposon insertion made it possible to visualize band T and K (Fig. 3.6, track 5), however, further analysis was required to differentiate the genetic origin of these two proteins.

Plasmid pADE132 ($galE^+$, $galT^-$, $galK^+$(Table 3.1) expressed bands E and K when harbored by DS410 (Fig. 3.6, track 6). The level of band E was higher than the level seen in DS410(pADE107), while the level of band K produced was lower. Band T was not obvious. This implied that band T was the *galT* product.

Finally, DS410(pADE136) ($galE^+$, $galT^+$, $galK^-$ (Table 3.1)) produced bands E and T in large amounts (Fig. 3.6, track 7), yet band K was not detected. Thus it was inferred that band K was the gene product of *galK*.

3.3 Discussion

Transposon insertion mutagenesis established that the gene order in the *S. typhimurium gal* DNA was *galE*, *galT*, *galK*, and that the genes appeared to constitute an operon or part of an operon, with the promoter region lying before the *galE* gene (Fig. 3.5). The arrangement of the genes in *S. typhimurium* was therefore seen to be the same as that seen in *E. coli* K12.

The transposon insertion in pADE133 did not inactivate the *gal* genes (Table 3.1) whereas the transposon insertion in pADE141 inactivated expression of the *gal* genes (Table 3.1). Since these two transposons were mapped 0.1kb apart, it was likely that the promoter was located in the intervening region. The production of band E′ by DS410(pADE134) provided indirect confirmation of this location. The RNA transcript from which this protein was translated probably started at the *gal* promoter and translation of this mRNA was subsequently terminated at or near the site of disruption of its coding sequence by Tn*1725* insertion. The Mr of the protein which corresponded to band E′ was about 20 kDal (not shown). If the translation termination point were at the site of transposon insertion in pADE134, the translation start point should lie about 0.6 kb from this insertion point. If the *gal* promoter was located close to the start of translation, as is the case in *E. coli* K12, then it should also lie in the region 0.6kb upstream of the insertion in pADE134. This region (0.6kb upstream of pADE134) is located in the region between the insertions in pADE133 and pADE141. Since two of the transposon insertions generated *galE* mutations which were polar on *galT* and *galK*, it also seemed that these two genes did not possess their own promoters, and that the single promoter responsible for the expression of the *galE*, *galT* and *galK* genes in pADE107 lay near the insertion point of the transposon in pADE133 as described above.

While transposon insertion mutations are generally though to be polar, the Tn*1725* appears to contain a promoter which reads from the Tn*1725* out through an end of the transposon (Focareta and Manning, 1985). All of

the non–polar transposon insertion mutations described in this section had the transposon inserted in the same orientation such that genes downstream of the insertion point were still expressed. All polar insertions were found to be in the opposite orientation to that of the non–polar orientation.

Minicell analysis enabled the identification of the *gal* encoded proteins. Bands E, T and K (Fig. 3.6) were shown to be the products of the *galE*, *galT*, and *galK* genes, respectively, by a process of elimination. The Mr values of these proteins were respectively 37 kDal, 40 kDal, and 42.5 kDal (not shown), and the DNA coding capacities required for proteins of such sizes would be about 1.1, 1.2 and 1.3 kb respectively. These values were used to show the probable termini of the genes in Fig. 3.5. The level of GalTp was lower than that of GalEp and GalKp except when expression of GalKp was blocked by transposon insertion (Fig. 3.6). This result implied that GalKp was regulating the expression of GalEp and GalTp. The *galK* gene produce of *E. coli* K12 was shown to to affect expression of *gal* (Kalckar and Sundararajan, 1961). Here the absence of galactokinase activity caused constituitive expression of the *gal* operon since internal galactose levels were maintained at a level that was sufficient to induce *gal* (Kalckar and Sundararajan, 1961). The expression of GalKp by DS410(pADE132) was from the transposon–derived promoter. This probably resulted in the lower level of expression of GalKp seen (Fig. 3.6, track 6).

The cloning and analysis of the *gal* operon of *S. typhimurium*, and the identification of the proteins encoded by the *gal* operon in terms of their Mr values, invites comparison with the well–studied *gal* operon of *E. coli* K12. As mentioned earlier the order of *gal* genes was the same in *S. typhimurium* as previously found in *E. coli*. The protein molecular weights also correspond to the analogous *E. coli* proteins. However, restriction endonuclease cleavage sites for *Hind*III, *Pvu*II, and *Hpa*I that were previously mapped in *E. coli* were not in the same location in the *S. typhimurium gal* operon (compare Fig. 3.1 with Fig. 3.5). This probably reflects more extensive underlying DNA sequence differences

but the extent of such differences can be resolved only by sequencing the two *gal* operons.

3.4 Conclusion

The purpose of this initial study was to clone and map the position of the *galE* gene of *S. typhimurium*. As shown (Fig. 3.5) the *galE* gene extended from a position downstream of the transposon insertion in pADE133 but upstream of the insertion in pADE141 to a position downstream of the insertion in pADE134 but upstream of the insertion in pADE135. Minicell analysis confirmed this location.

Chapter 4

Introduction of defined *galE* deletions into *Salmonella* strains, to construct strains for use as live oral vaccines

4.1 Introduction

In the absence of galactose, UDP-glucose is isomerised to produce UDP-galactose, a reaction catalysed by galactose-4-epimerase, the gene product of *galE*. This reaction is reversible. Consequently, when more UDP-galactose is synthesised than is required it is isomerised to UDP-glucose and used for other cellular purposes. Inactivation of the *galE* of *S. typhimurium* generated mutant strains which became sensitive to galactose–induced lysis *in vitro* (Fukasawa and Nikaido, 1961a, 1961b; Nikaido, 1961). Lysis was thought to be a result of accumulation of galactose-1-phosphate and UDP-galactose, two intermediates of galactose metabolism (Nikaido, 1961). Glucose inhibited galactose–induced lysis of *galE* mutants primarily by inhibiting the transport of galactose into the cell.

The same mutational change also caused strains of *Salmonella* to become avirulent. Strain G30, a *galE* derivative of *S. typhimurium* LT2 (Osborn *et al.*, 1972), was avirulent in mice when administered either orally or ip, but protected mice against subsequent challenge with virulent *S. typhimurium* (Germanier, 1970; Moser *et al.*, 1980).Other rough mutants of *S. typhimurium* were found to be avirulent but did not afford the same level of protection that was seen with *galE* mutants. The capacity of *galE* mutants to provide protective immunity, therefore, was thought to be due to their ability to synthesize smooth LPS in the presence of exogenous galactose (Germanier, 1970, 1971).

By NG mutagenesis, Germanier and Furer (1975) isolated a stable *galE* derivative of *S. typhi* Ty2, a causative agent of typhoid fever in man. This strain (Ty2la) was sensitive to galactose–induced lysis *in vitro*. When administered ip to mice, Ty2la was markedly avirulent and protected the mice against subsequent challenge with virulent Ty2 (Germanier and Furer, 1975). The protective immunogenicity of strain Ty2la was attributed to the ability of the strain to synthesise immunologically important cell wall LPS in the presence of low levels of galactose (Germanier and Furer, 1971), while the inability of the strain to kill mice was believed to be due to cell lysis after accumulation of intermediates of galactose metabolism (Germanier and Furer, 1975). These properties led the authors to propose the use of Ty2la as a live oral vaccine against typhoid infection in man (Germanier and Furer, 1975). Field trials using Ty2la have been conducted in Egypt (Wahdan *et al* 1982; Woodward and Woodward, 1982), where the strain was shown to be most effective (95%) in protecting children against typhoid. Here, the vaccine was administered orally as a lyophilised preparation reconstituted in 20–30 ml of liquid (Wahdan *et al.*, 1982). A further trial (in Chile) employed enteric–coated capsules of lyophilised bacteria. When children received 3 capsules within 7 days, 60% protection (over 3 years) was observed (Levine *et al.*, 1986).

While Ty2la therefore showed promise as a vaccine against typhoid, it seems possible that other *galE* strains of *S. typhi* might also be worth investiga-

tion. Strain Ty2la grows poorly (Germanier and Furer, 1975), and, in our hands, viability is significantly reduced by lyophilisation. The *galE* mutation in Ty2la was not defined in molecular terms, and while revertants to *galE* have not been observed (Germanier and Furer, 1975) the possibility of such reversion cannot yet be excluded (Silva–Salinas *et al.*, 1985; Silva *et al.*, 1987). If derivatives of *S. typhi* were available which possessed stable defined *galE* mutations and which were also vigourous in growth and tolerant of lyophilisation, such strains would also be vaccine candidates. Furthermore the use of *galE* strains as vaccines need not be confined to *S. typhi* (Attridge *et al.*, 1988).

As described earlier, one of the aims of this work was to construct defined deletions in the *galE* gene of the cloned DNA, and to introduce this defined *galE* mutation into the chromosomes of *Salmonella* strains of human or veterinary importance. Preliminary results using Southern hybridisation indicated excellent homology between the cloned DNA in pADE107 on the one hand and chromosomal DNA from a variety of *Salmonella* strains on the other (data not shown). Such vaccine strains should have the advantages of the *S. typhi* derivative Ty21a, insofar as ability to synthesise immunologically functional LPS, and galactose–sensitivity, are concerned, and have the possible additional benefits of vigourous growth and tolerance to lyophilisation. The *galE* mutation in these strains, being a deletion, would be non–revertible.

This chapter describes the construction of a plasmid bearing such a *galE* mutation and the subsequent introduction of the mutation into the chromosomes of three *Salmonella* strains.

4.2 Results

4.2.1 Construction of a plasmid bearing the *galE*-H1 mutation

Plasmid pADE161 carries DNA of the *gal* operon in which 0.4 kb of *galE* has been deleted. This plasmid was constructed in two steps. First, the 3.7 kb *Eco*RI–*Hind*III fragment of pADE134 was subcloned into pGB2 (Fig. 4.1). The resulting plasmid, pADE160, was then linearised with *Eco*RI and ligated to the 0.6 kb *Eco*RI fragment of pADE137 (Fig. 4.1). Because the Tn*1725* insertions in pADE134 and pADE137 were located 0.4 kb apart in *galE*, the intervening DNA has not been included in pADE161 (Fig. 3.5). The orientation of the 0.6 kb *Eco*RI fragment was checked by reference to the asymmetrical *Cla*I sites in the 0.6 kb *Eco*R1 fragment (Fig. 3.5). The modified *galE* gene of pADE161 was designated *galE*-H1.

4.2.2 Integrating *galE*-H1 into the chromosome of *Salmonella* strains

Plasmid pADE161 was initially transformed into *S. typhimurium* LT2. After growth in medium without Sp, a small proportion of the bacteria may not only have incorporated the *galE*-H1 gene into the chromosomal *gal* operon by recombination but also may have spontaneously lost the pADE161 plasmid. The steps described below were designed to enrich for such bacteria.

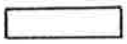
The bacterial suspension (grown without Sp) was subjected to enrichment for Sp–susceptible cells (see Materials and Methods). After this procedure, cells were pelleted by centrifugation, resuspended in 0.1 ml of nutrient broth, and spread with phage Felix-0 (moi *ca.* 10). Phage Felix-0 selected for core–defective mutants, among which should be the *galE* mutants (galactose is a *S. typhimurium* core constituent). Felix-0-resistant colonies were then checked for susceptibility to Sp (to ensure that pADE161 had been cured) and for suscep-

Figure 4.1

Construction of pADE161, a plasmid that harbors *galE*–H1: This figure shows the construction of a plasmid with the *gal* promoter and the *galT* and *galK* genes of *S. typhimurium*, but with a 0.4kb deletion in the *galE* gene. The details are described in the text.

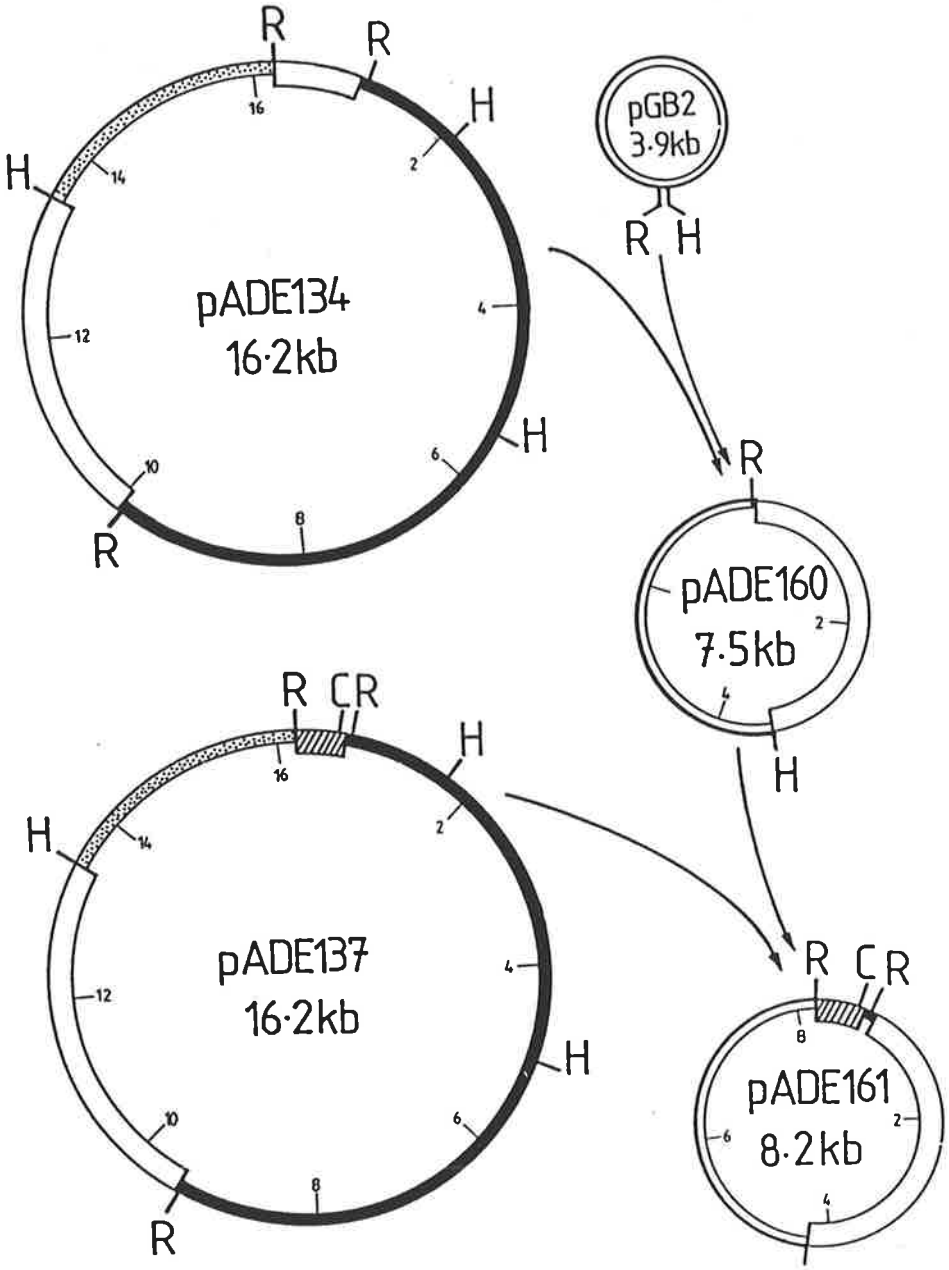
C: *Cla*I site; **H**: *Hind*III site; **R**: *Eco*RI site.

, pUC9 DNA;

 , cloned DNA (including *gal* DNA) in pADE134;

 , DNA from pADE137 present in pADE161;

 , Tn*1725* DNA;  , pGB2 DNA.


R
R
H
H
16
14
2
12
4
pADE134
16·2kb
H
6
10
8
R
pGB2
3·9kb
R H
R
pADE160
7·5kb
2
4
H
R C R
H
16
14
2
12
4
pADE137
16·2kb
H
6
10
8
R
R C R
8
pADE161
8·2kb
6
2
4

tibility to galactose–induced lysis (a phenotype typical of *galE* mutants). The procedure was repeated with *S. typhimurium* C5 and *S. typhi* Ty2 and resulted in strains with a Sp–susceptible and galactose–sensitive phenotype. The strains derived from *S. typhimurium* LT2, *S. typhimurium* C5, and *S. typhi* Ty2 using this procedure were called LT2H1, C5H1, and Ty2H1, respectively.

4.2.3 Chromosomal DNA blotting

To determine if strains LT2H1, C5H1, and Ty2H1 carried the *galE*-H1 mutation from pADE161, chromosomal DNA was extracted, digested with *Eco*R1, and electrophoresed on a 1.0% agarose gel (Fig. 4.2). After electrophoresis, the DNA was transferred to nitrocellulose and probed with [^{32}P]–labelled pADE107. The chromosomes of *S. typhimurium* LT2 and *S. typhimurium* C5 both had 5.0 kb *Eco*R1 fragments that hybridized to pADE107. The *galE* derivatives, strains LT2H1 and C5H1, had two fragments (4.0 kb and 0.6 kb) that hybridized to pADE107. The sizes of these fragments implied that these two strains had the *galE*-H1 deletion from pADE161 integrated into the chromosome. Chromosomal DNA extracted from *Salmonella typhi* Ty2 and digested with *Eco*R1, produced a 9.2 kb *Eco*R1 fragment that hybridized to [^{32}P]–labelled pADE107 (Fig. 4.2). However, *Eco*R1–digested chromosomal DNA of the *galE*-H1 derivative of Ty2, Ty2H1, generated two fragments (8.2 kb and 0.6 kb) that hybridized to pADE107 (Fig. 4.2). Again, this showed that the *galE*-H1 deletion from pADE161 was integrated into the chromosome of Ty2H1. The *Eco*R1 fragment of chromosomal DNA from Ty21a that hybridized to [^{32}P]–labelled pADE107 was reduced in size, compared with that from Ty2 (from 9.2.kb to 8.7 kb; Fig. 4.2), suggesting that Ty21a carried a deletion on that fragment. The location of this deletion, however, remains to be determined (it may be located in *galE*).

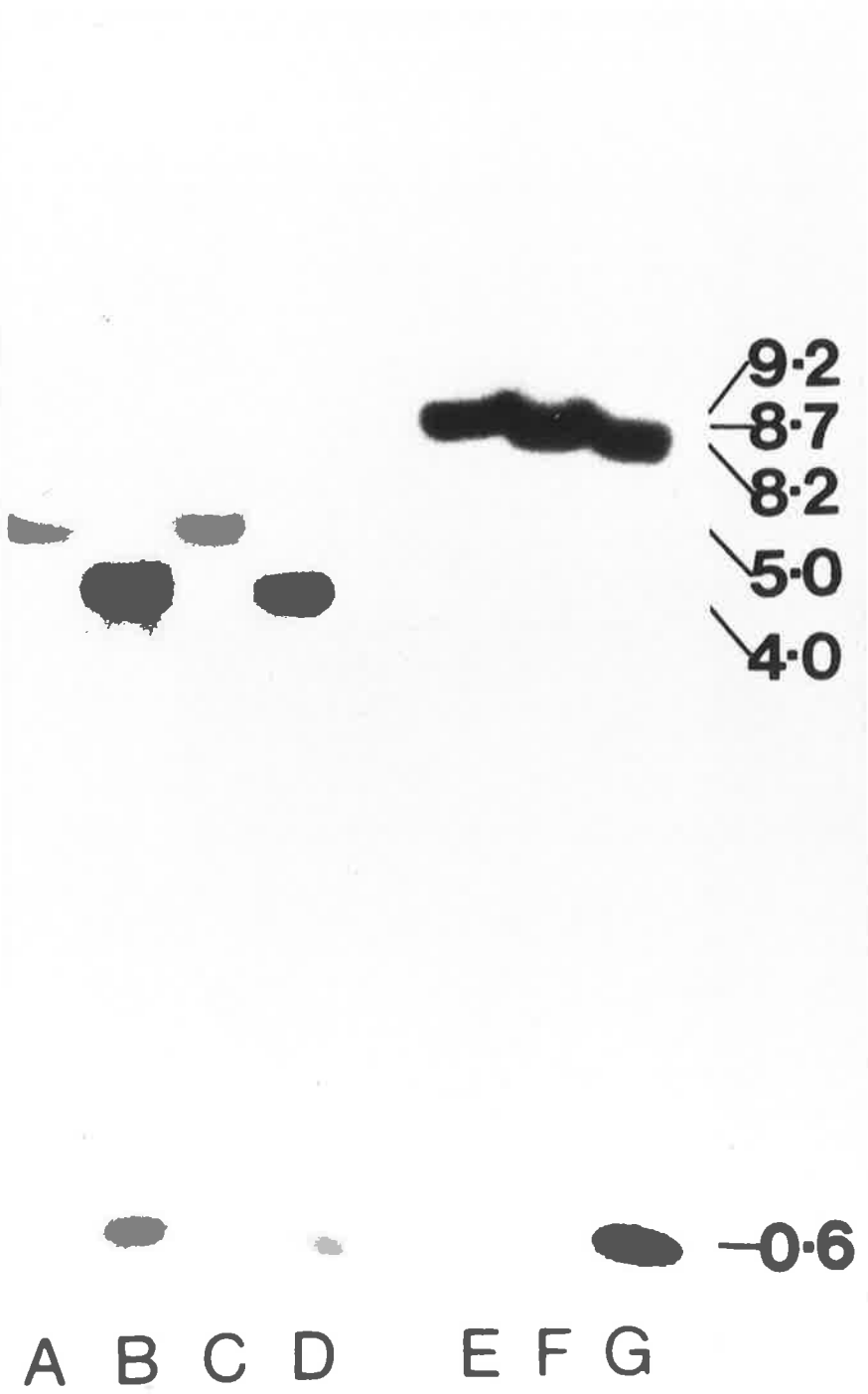
Figure 4.2

Integration of a *galE* deletion mutation into the chromosome of *Salmonella*: A *galE* deletion mutation in plasmid pADE161 was recombined into the chromosome of various *Salmonella* strains. Chromosomal DNA was prepared from these strains and control strains, digested with *Eco*RI and *Hind*III subjected to AGE, and probed with [^{32}P]–labeled pADE107. A 48h autoradiograph is shown. The numbers on the right are the sizes (in kb), of the bands seen.

A: *S. typhimurium* C5; **B**: *S. typhimurium* C5H1;

C: *S. typhimurium* LT2; **D**: *S. typhimurium* LT2H1;

E, *S. typhi* Ty2; **F**: *S. typhi* Ty21a; **G**: *S. typhi* Ty2H1.


9·2
8·7
8·2
5·0
4·0
0·6
A B C D E F G

4.2.4 Physical characteristics of the *galE* deletion strains.

Reversion of the *galE*-H1 mutation

Because candidate avirulent vaccine strains should not revert to virulence, the reversion rate of the *galE*-H1 strains to gal^+ was examined. C5H1, LT2H1, and Ty2H1 were grown for 16h at 37°C in NBG (1-liter cultures). The bacteria were pelleted, washed, resuspended in saline, and spread onto 20×20-cm plates of minimal galactose agar. The plates were incubated at 37°C for three days. No colonies appeared on the plates. The rate of reversion of the *galE*-H1 mutation to gal^+ was accordingly $<10^{-12}$.

Growth rates

The growth of the three *galE*-H1 strains was compared with the growth of their parent strains and Ty21a. In shaking NBG cultures at 37°C, Ty21a had a mean generation time of 65 min, whereas the other six strains had mean generation times of 25–30 min. Therefore, the *galE*-H1 mutation had no detectable effect on the growth properties of the strains under these conditions.

Resistance to lyophilisation

Various strains were lyophilised in nutrient broth in the presence or absence of 5% (w/v) glucose as cryoprotectant (Table 4.1). The recovery of Ty21a after lyophilisation was low (8% and 0.4% with or without cryoprotectant, respectively). The other strains examined (the three *galE*-H1 strains and their parent strains) had higher recoveries. Without cryoprotectant, the lowest recovery of the six strains was seen with Ty2H1 (3.7%). With cryoprotectant, the lowest recovery was seen with LT2H1 (94%). There were no significant differences between the survival of *galE*-H1 strains and the gal^+ parent stains.

Table 4.1: Tolerance of various strains to lyophilization

Strain	% recovery + S.E.	
	Nutrient broth	Nutrient broth + 5% (w/v) glucose
C5	6.3 ± 1.4	94 ± 3
C5H1	10.1 ± 2.7	107 ± 4
LT2	7.7 ± 1.4	96 ± 2
LT2H1	11.4 ± 3.4	94 ± 1
Ty2	5.0 ± 2.6	101 ± 3
Ty2H1	3.7 ± 0.8	98 ± 2
Ty21a	0.4 ± 0.1	8 ± 2

Various strains were streaked onto nutrient agar plates and after 16h at 37°C the bacteria were scraped into either nutrient broth, or nutrient broth with 5% (w/v) glucose, to give a concentration of 1.01-2.4 x 10^{11} /ml. Quantities (0.1ml) of these suspensions were lyophilized, and after 24h ampoules (3 for each strain freeze-dried in each medium) were opened and the contents resuspended in nutrient broth (10ml) for viable counts.

Synthesis of smooth LPS

The ability of Ty21a to effect the synthesis of smooth LPS when grown in low concentrations of galactose may be important for the immunogenicity of the strain *in vivo* (Germanier and Furer, 1971, 1975). Evidence was sought to determine if the *galE*-H1 strains were also able to effect such synthesis (Fig. 4.3). When grown in NBGG, all three *galE*-H1 strains produced long–chain LPS. The levels of LPS synthesised were approximately the same as those made by the parent strains (Fig. 4.3). The glucose was included in the galactose–containing medium because the lytic effect of galactose on *galE*-H1 strains was inhibited by glucose.

Presence of the 90kb virulence plasmid

A virulence plasmid of 90 kb, present in *S. typhimurium*, plays an important role in virulence (Hackett *et al.*, 1986; Curtiss *et al.*, 1987). The presence of the virulence plasmid, therefore, was checked in strains LT2H1 and C5H1. The virulence plasmid was present in all of the following strains: C5, LT2, C5H1, and LT2H1 (data not shown). No large plasmids were found in *S. typhi* Ty2 or strains Ty21a or Ty2H1 (data not shown).

4.2.5 Further characterisation of strains LT2H1 and C5H1

Susceptibility to galactose–induced lysis

The sensitivity of Ty21a to galactose–induced lysis *in vitro* (at >6mM galactose) may be important in its avirulence in humans (Germanier and Furer, 1971,1975). Therefore the effect of galactose on the growth of *galE*-H1 strains and their parent strains was examined. Strains C5H1 and LT2H1 were galactose–sensitive, as judged by the liquid culture method, whereas the parent strains were not galactose–sensitive under the same conditions. Although addition of 60 μM

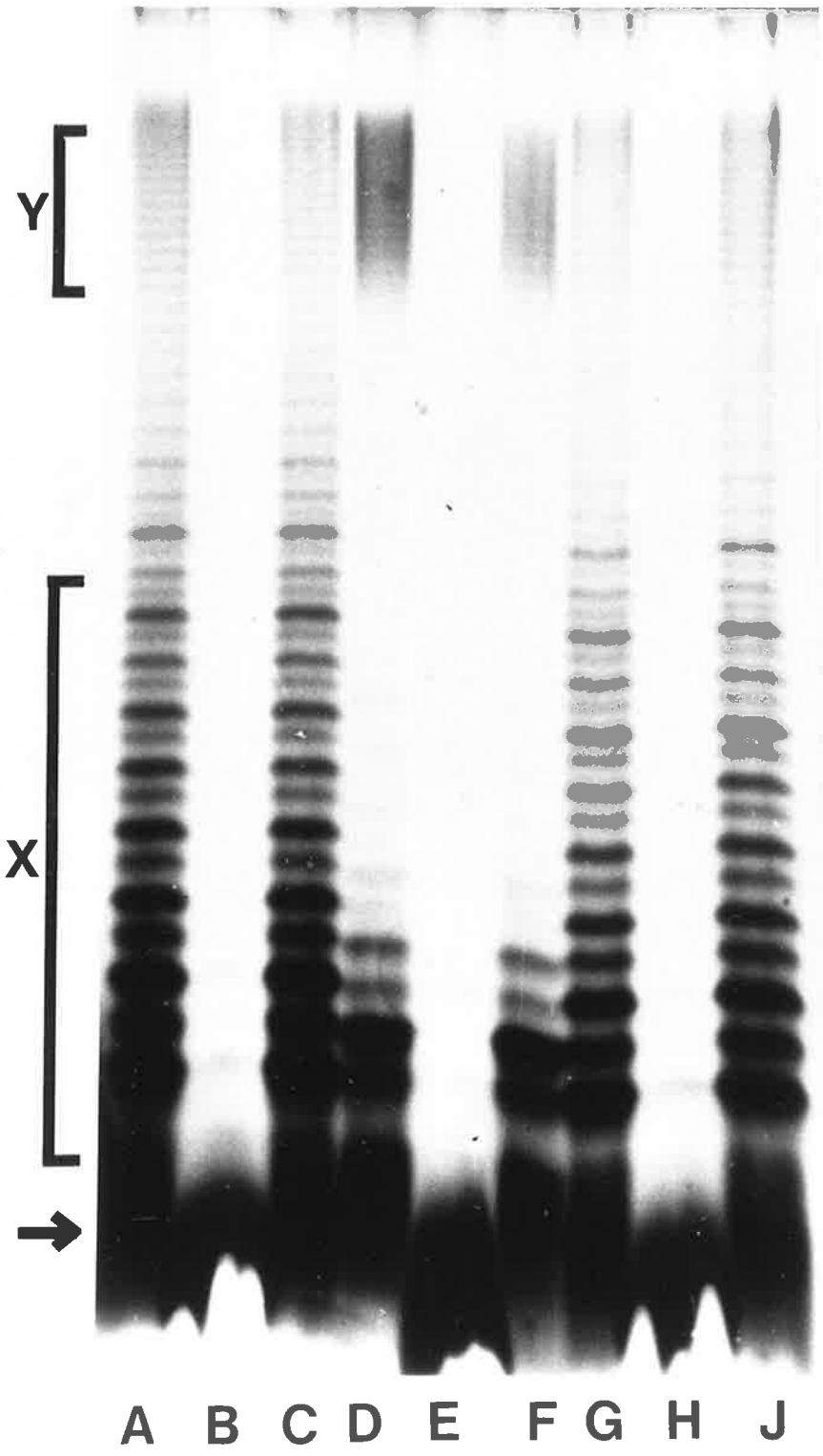
Figure 4.3

O–polysaccharide expression by various *galE* mutants grown with or without galactose: Various strains were grown to stationary phase either in NBG or in NBGG. The bacteria were pelleted, lysed, and treated with proteinase K. Samples from equal numbers of bacteria (*ca.* 3×10^8/track) were subjected to SDS-PAGE followed by lipopolysaccharide–specific silver staining.

GG, below, denotes growth in NBGG.

The arrow indicates the lipopolysaccharide uncapped core region, the bracket **X** indicates short O-polysaccharide chains attached to core, and the bracket **Y** indicates long, core-attached O-polysaccharide chains.

A: C5; **B**: C5H1; **C**: C5H1(GG); **D**: Ty2; **E**: Ty2H1; **F**: Ty2H1(GG); **G**: LT2; **H**: LT2H1; **J**: LT2H1(GG).


Y
X
A B C D E F G H J

galactose to NA plates was sufficient to cause lysis of the *galE*-H1 strains, galactose ($\leq$ 100 mM) did not affect the growth of the parent strains (Table 4.2).

Sensitivity to bacteriophage P22 and Felix-O

P22 and Felix-O are useful tools for determining the phenotypic characteristics of LPS defective mutants. Strains LT2H1 and C5H1 when grown in the absence of exogenous galactose were seen to be resistant to both P22 and Felix-O (Table 4.2). When strains LT2H1 and C5H1 were grown in the presence of $\geq$6.0μM galactose (and 25mM glucose to inhibit galactose–induced lysis) sensitivity to P22 and Felix-O was restored (Table 4.2). Strains LT2 and C5 were sensitive to phage P22 and Felix-O when grown with or without galactose (Table 4.2).

Sensitivity to killing by serum

Normal mouse serum adsorbed with *S. typhimurium* C5 was used to determine the susceptibility of strains LT2H1 and C5H1 to serum–induced lysis. When grown in the absence of galactose strains LT2H1 and C5H1 were lysed when cultured with 1% serum. However, they became resistant to killing by 100% serum when grown in the presence of galactose. Both parent strains (LT2 and C5) were resistant to killing by 100% serum when grown with or without galactose (Table 4.2).

4.2.6 Virulence, immunogenicity, and *in vivo* persistence of strains LT2H1 and C5H1 in mice

Virulence and immunogenicity of LT2H1 and C5H1 in mice

To evaluate the virulence of LT2H1 and C5H1, mice were inoculated ip or orally with various doses of the strains. In contrast to their parent strains, both LT2H1 and C5H1 were completely avirulent (Table 4.3).

To assess the efficacy of C5H1 and LT2H1 as live oral vaccines against *S. typhimurium* infection in mice, groups of mice were orally given the *galE*-

Table 4.2: Characteristics of galE-H1 derivatives of S. typhimurium

Bacterial strain	Galactose [1] sensitivity	Bacteriophage P22 and F-O sensitivitity [2]		Serum resistance [3]	
		Gal^-	Gal^+	Gal^-	Gal^+
LT2	R	S	S	>100	>100
LT2H1	S(0.06mM)	R	S	<1	>100
C5	R	S	S	>100	>100
C5H1	S(0.06mM)	R	S	<1	>100

1. Galactose sensitivity was judged by the plate method (see Materials and Methods).

2. S: Sensitive; R: Resistant.

3. Expressed as the % (v/v) serum that caused 50% killing of the bacterial strain relative to a saline control. Serum was adsorbed with S. typhimurium C5 prior to use.

Table 4.3: Virulence of S. typhimurium strains C5H1 and LT2H1 in mice

Strain	LD_{50}	
	Oral	Ip
C5	4.6×10^4	< 5
LT2	6.2×10^6	4.5×10^2
LT2H1	$> 10^9$	$> 10^7$
C5H1	$> 10^9$	$> 10^7$

NOTE. Mice received various oral or ip doses of the strains shown, and deaths to 35 days postchallenge were recorded. No deaths were seen with either C5H1 or LT2H1; the numbers in the LD_{50} columns are the highest doses administered. LD_{50} values were calculated by using the method of Reed and Muench (1938).

H1 strains and challenged orally with C5 14 days after administering the vaccine dose (Table 4.4). When mice received 2.1–2.5$\times 10^8$ organisms of C5H1 or LT2H1/mouse, the LD_{50} values of C5 in the immunized mice were $>5\times 10^9$ and $>5\times 10^8$, respectively, whereas the LD_{50} value of C5 in nonimmunised mice was 4.6×10^4 (Table 4.3).

In vivo persistence of LT2H1 and C5H1 in mice

The immunizing ability of strains C5H1 and LT2H1 was reflected in their ability to persist, after oral administration, in the Peyer's patches of the mouse intestine (Fig. 4.4). Although the virulent parent strains C5 and LT2 increased in numbers in the Peyer's patches until death of the mice, the *galE*-H1 strains C5H1 and LT2H1 increased in numbers only until day 3 postadministration and then declined. The establishment of such a limited infection in the Peyer's patches is important for generating immunity to *Salmonella* in mice (Srisart *et al.*, 1985). Consistent with these results, strain C5H1 was cleared within a few days of ip injection into mice; by day 6 after injection of 0.7×10^7 organisms/mouse, each of five mice examined had no detectable bacterial counts in the peritoneum, spleen, or liver. In contrast, six days after ip injection of 0.7×10^2 C5 organisms/mouse, 6–45$\times 10^6$ organisms were present in the peritoneum, liver, and spleen of each of five mice.

4.3 Discussion

The construction and subsequent integration of a defined *galE* mutation (*galE*-H1) into the chromosomes of *S. typhimurium* C5 and LT2 and *S.typhi* Ty2 was described in this chapter. DNA blots confirmed that the *galE*-H1 deletion (in which the *galE* gene lost 0.4 kb) had recombined into the chromosomes of the above strains, to give strains C5H1, LT2H1 and Ty2H1 respectively (Fig. 4.2).

The characteristics of C5H1, LT2H1, and Ty2H1 were assessed by a variety of techniques. These strains were found to be similar to their parent strains

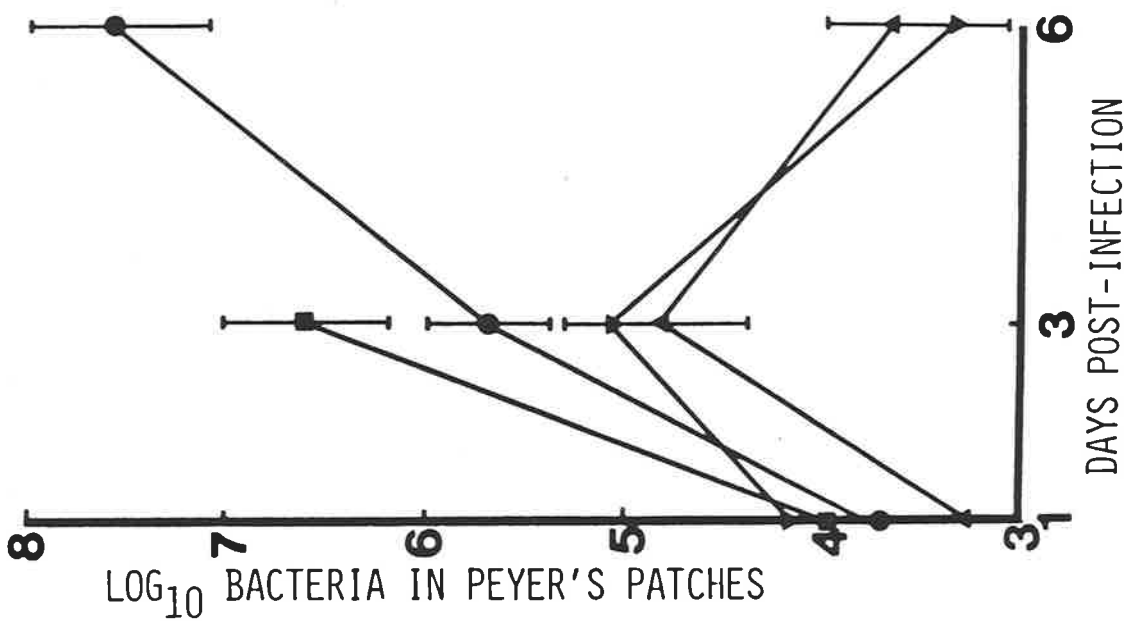
Table 4.4: Effectiveness of LT2H1 and C5H1 as live oral vaccines in mice

Challenge dose of C5	No. of deaths in mice immunized with	
	C5H1	LT2H1
5.4×10^9	3	ND
5.4×10^8	1	0
5.4×10^7	0	0
5.4×10^6	0	ND

NOTE. Groups of 10 mice were immunized orally with either C5H1 (2.5×10^8/mouse) or LT2H1 (2.1×10^8/mouse), and after 14 days they were challenged orally with C5 as shown above. Deaths to 30 days post-challenge were recorded. Control mice that were not immunized, but that were challenged with either 5.4×10^6 or 5.4×10^7 C5 at the time of challenge of the immunized group died within 12 days postchallenge. ND = not done.

Figure 4.4

Establishment of a limited infection in the Peyer's patches by various strains of *S. typhimurium*: Mice were fed with the strains shown (*ca.* 10^9 bacteria/mouse). Groups of five mice were killed at various times, and bacteria in the Peyer's patches were enumerated.
(■), C5 (all mice were dead by day 6 postinfection);
(●), LT2 (two of five mice were dead by day 6 postinfection);
(▼), C5H1; (▲), LT2H1.
Mean counts with standard error values are shown.


8
7
6
5
4
3
1
3
6
DAYS POST-INFECTION
LOG10 BACTERIA IN PEYER'S PATCHES

with respect to growth rate in NBG, tolerance to lyophilisation, and plasmid profiles. In the absence of exogenous galactose *galE*-H1 strains did not synthesise smooth LPS (Fig. 4.3). In the presence of exogenous galactose, however, these strains produced smooth LPS that seemed identical to that of their respective parent strains as judged by LPS–specific staining of SDS–PAGE gels. The *galE*-H1 strains were found to be sensitive to galactose–induced lysis at galactose concentrations $\leq 60\mu$M, whereas the parent strains did not display such sensitivity. The *galE*-H1 mutations were found to be genetically stable.

Strains C5H1 and LT2H1 were found to be highly attenuated for mice when given by both oral and ip routes (Table 4.3). Despite lack of virulence these strains retained the capacity to immunize and provided mice with protection against challenge with virulent *S. typhimurium* (Table 4.4). This immunogenicity was thought to be a result of the ability of these two strains to establish a limited infection *in vivo* (Fig. 4.4).

4.4 Conclusion

It was concluded, then, that although C5H1 and LT2H1 are vigourous, nonreverting strains that are tolerant of lyophilisation, they are both avirulent in mice and effective as live oral vaccines against mouse typhoid. Strain Ty2H1 was also shown to be vigourous, nonreverting, and tolerant of lyophilisation.

Chapter 5

A *galE*, *via* (Vi–negative) mutant of *Salmonella typhi* Ty2 retains virulence in man

5.1 Introduction

Although parenteral vaccines stimulate only a humoral response in mice (Collins and Carter, 1972; Carter and Collins, 1974b; Eisenstein *et al.*, 1984), killed *S.typhi* vaccines given parentally have nevertheless been able to confer significant protection against typhoid fever in humans, as demonstrated by a series of controlled field trials conducted by the World Health Organization in the 1960's (Ashcroft *et al.*, 1964; Yugoslav typhoid committee, 1964; Polish typhoid committee, 1965). However, the field trials were performed in areas where typhoid fever was endemic and it has been argued that such vaccines may be effective only in areas where vaccination serves to boost natural immunity acquired by periodic exposure to sub-clinical *S.typhi* infections (Germanier, 1984). Furthermore, 25% of the vaccinees receiving killed vaccines developed unacceptably severe systemic and local reactions (Ashcroft *et al.*, 1964; Yugoslav typhoid committee, 1964; Polish typhoid committee, 1965). Thus it is accepted that there is still a need to develop an effective, safe and non-irritating vaccine against typhoid, which

continues to present developing countries with a serious public health problem (Edelman and Levine, 1986). One way to achieve this is by the construction of attenuated live oral vaccines (Germanier, 1984).

Man is the only natural host of *S. typhi* (Gaines *et al.*, 1968a, 1968b; Carter and Collins, 1974a; O'Brien, 1982). Infection results from either drinking contaminated water or eating contaminated food (Hornick *et al.*, 1970a; Edelman and Levine, 1986). Other routes of infection, such as nasal or conjuctival routes, may be important modes of transfer of *S.typhi* but these routes have only been demonstrated in experimental animal systems (Moore, 1957; Tannock and Smith, 1972; Duguid *et al.*, 1976).

Usually infection occurs when one or more of the host's natural defences have been compromised or bypassed, or in the event of overwhelming inoculum (Hornick *et al.*, 1970a, 1970b). The fact that disease is the exception and not the rule emphasizes the role of the natural defence mechanisms in the overall *Salmonella*–host relationship.

The ID_{50} of *S. typhi* (Quailes strain) in man was *ca.* 10^7 organisms when fed orally (suspended in 30ml of milk; Hornick *et al.*, 1970a). However, Gilman and co–workers (1977) found 10^5 *S. typhi* Quailes strain, caused typhoid in 38–53% of the volunteers who ingested the organism. Such variations can be explained on the basis of statistical and biological variation (Levine, personal communication). When 3×10^6 viable *S.typhi* Ty2 were given orally to humans, 83% diplayed notable infection. However, only 33% of these infected volunteers required antibiotic intervention (Hornick *et al.*, 1970a). Of 19 volunteers who ingested $1.9times10^8$ *S. typhi* Ty2W (Vi–negative), 79% became ill; of these infected volunteers, 21% required antibiotic therapy. This suggested that in the absence of Vi–antigen, the virulence of *S. typhi* Ty2 in man was reduced (Hornick *et al.*, 1970a).

Once the typhoid bacilli reach the small intestine they adhere to, and invade, the mucosa (Woodward, 1963; Sprinz *et al.*, 1963; Gaines *et al.*, 1968a). The primary site of infection was shown to be the GALT (Huckstep, 1962; Sprinz

et al., 1963). However, Sprinz *et al.* (1966) while studying the histopathology of experimentally induced typhoid, found that *S. typhi* bacilli invaded the mucosal tissue along the length of the human small intestine. When fed orally to mice, *S. typhi* bacilli were found in the blood 30 seconds after inoculation, indicating that invasion of the mucosa may occur very rapidly (Gerichter, 1960). However the dynamics of this step of the infection in man and the bacterial factors involved have not been fully elucidated. After invasion *S.typhi* bacilli are thought to enter the mesenteric lymph nodes via the Peyer's patches. The bacilli undergo multiplication at these sites during the incubation period then enter the blood stream by way of the lymphatic drainage (Huckstep, 1962; Woodward, 1963; Gaines *et al.*, 1968a).

It is during this primary bacteremia phase that the typhoid bacilli enter other organs of the body including the liver, spleen and reticulo–endothelial system (Huckstep, 1962; Woodward, 1963). The typhoid bacilli that multiply in the liver pass through the liver capillaries into the bile cannulae and infect the gall bladder (Huckstep, 1962; Woodward, 1963). Once in the gall bladder the typhoid bacilli undergo further multiplication which produces a secondary invasion of the GALT through the infected bile (Huckstep, 1962; Woodward, 1963). This secondary invasion of the intestine is probably responsible for the intestinal lesion that occur during an uncontrolled typhoid infection(Huckstep, 1962; Woodward, 1963). The clinical manifestations of typhoid result from the rapid multiplication of *S. typhi* bacilli in the bile and the reticulo–endothelial system (Huckstep, 1962; Woodward, 1963; Gaines *et al.*, 1968a, 1968b).

Typhoid fever is a systemic disease and during the course of infection virtually every organ of the body can become infected (Huckstep, 1962). The Peyer's patches can become heavily infected and if the typhoid bacilli are allowed to continue multiplying without antibiotic intervention the Peyer's patches can undergo dramatic pathological changes. These changes can be divided into four stages: hyperplasia, necrosis, ulceration and perforation (Huckstep, 1962). The hyperplasia stage is due to massive infiltration of mononuclear cells and nu-

merous lymphocytes but very few polymorphonuclear cells ocurr. The necrosis stage, which usually occurs during the second week, is probably due to local toxaemia and surrounding cell death resulting from a block in the circulation due to the massive monocyte infiltration. The stage of ulceration follows the necrotic stage and is a result of sloughing of the necrotic tissue. This stage is potentially the most dangerous, usually occurring in the third week, as it often leads to the final stage, perforation. If perforation occurs, a state called peritonitis ensues and if uncontrolled will lead to death (Huckstep, 1962; Woodward, 1963).

Other regions that become infected are the respiratory system, the urinary tract, the heart, the skeletal system, the central nervous system and the dermis (Huckstep, 1962; Woodward, 1963). The genetic factors of *S.typhi* that confer upon it abilities to invade and multiply in such diverse locations within the body have not yet been elucidated. Some or all of these genes may be expressed only *in vivo*.

S. typhi infection of chimpanzees is similar to *S. typhi* infection of man (Gaines *et al.*, 1968a, 1968b). When *S. typhi* was given orally to chimpanzees, they were found to develop a disease with both clinical and histological features that resembled typhoid in man (Gaines *et al.*, 1968a, 1968b). The disease was, however, less severe, and shorter in duration; all *S. typhi* bacilli were usually cleared by day 85 post inoculation (Gaines *et al.*, 1968a). Thus, *S. typhi* is highly specific for its natural host, man.

S. typhi is not naturally infective in mice or guinea pigs, and in order to reach an LD_{50} high inocula of the typhoid bacilli must be injected parenterally (Nungester *et al.*, 1936; Carter and Collins, 1974a). Usually hog gastric mucin (HGM) was included to inhibit early phagocytosis of the inoculum when given ip (Nungester *et al.*, 1936). Under these conditions death usually occurs within 48–72hrs due to overwhelming peritonitis and endotoxaemia (Nungester *et al.*, 1936). Despite numerous studies of *S. typhi* infection in mice an unequivocal reason for the lack of virulence of this organism in mice hasnot been elucidated. The LD_{50} of *S.typhi* in mice was shown to be independent of mouse strain

and was not affected by pretreatment of the mice with silica (O'Brien, 1982). By supplying iron or an iron chelator with the inoculum, mice became more susceptible to *S. typhi* infection (O'Brien, 1982). These observations led to the suggestion that the lack of virulence of *S. typhi* in mice was in part caused by an inability of *S. typhi* to grow *in vivo* rather than due to rapid killing by monocytes (O'Brien, 1982). However, in this study all infections were by the ip route and not by the oral route. Since the ip infection with *S. typhi* may only measure an early rapid growth phase, this model may not be useful in accounting for the inability of *S. typhi* to kill mice by the oral route of inoculation.

The kinetics of *S. typhi* infection in mice are similar to that of attenuated strains of *S. typhimurium*. The typhoid bacilli undergo an early rapid clearing phase followed by a low level of persistence in the liver and spleen when given iv to mice (Carter and Collins, 1974a). Both Vi^- and rough strains grew to lesser extents under similar conditions inferring that these two somatic antigens were important for persistence of the typhoid organisms in mice (Carter and Collins, 1974a). *S. typhi*, when passaged up to 20 times through mice, showed no increased ability to kill mice, nor did fresh isolates from clinical cases of human typhoid (Carter and Collins, 1974a).

Although *S. typhi* infection in mice has not provided much information about the pathogenesis of typhoid fever in man it has been adapted as a model for both vaccine safety and vaccine efficacy (Powell *et al.*, 1980). The mouse potency test relies heavily on the ability of the typhoid bacilli to kill mice when given ip with HGM. The reproducibility of the LD_{50} under these conditions is dependant on the batch of HGM used, and in particular the iron content and the viscosity of the HGM suspension (Powell *et al.*, 1980; Brown and Stocker, 1987). However, the actual relevance of this model to typhoid infections in man has been questioned (Germanier, 1984).

Because of the lack of experimental animal models of *S. typhi* infection efforts towards attenuation relied heavily on knowledge gained from the experimental host–parasite relationships between *S.typhimurium* or *S.enteriditis* and

mice (Blanden *et al.*, 1966; Collins *et al.*, 1966; Germanier, 1970, 1972; Carter and Collins, 1974b; Hohman *et al.*, 1978). The progression of oral infections by these bacteria in mice to bacteremia, spleen involvement, and the development of the carrier state has obvious parallels with the human disease. However the applicability of such models to typhoid in man remains unclear.

An investigation by Nakano (1962) established that rough strains of *S. typhimurium* had a reduced virulence for mice. Germanier (1970) subsequently investigated the immunising capacity of different rough mutants of *S. typhimurium* in mice. He used mutants that expressed LPS with chemotypes Ra, Rb_1, Rb_3, Rc (*galE*), Rd, or Re. Strain G30 (Rc chemotype) when administered orally as a live preparation, was found to afford the highest level of protection. Moreover, the level of protection elicited by *galE* mutant, strain G30, was of the same magnitude as that which resulted from a sub–lethal infection of virulent *S. typhimurium* (Germanier, 1972). The capacity of *galE* mutants of *S. typhimurium* to elicit high levels of protection was thought to be due to expression of smooth LPS in the presence of low concentrations of exogenous galactose (Germanier and Furer, 1971). Strains with *galE* mutations in combination with either *galK* or *rfa* were not immunogenic in mice (Germanier and Furer, 1971). Germanier and Furer (1971) found that *galE* strains with reduced sensitivity to galactose–induced lysis displayed greater virulence in mice. Hence, it was argued that that sensitivity to galactose–induced lysis was an important attenuating factor of *S. typhimurium galE* mutants in mice.

Strain Ty21a, a *galE* derivative of *S.typhi* Ty2, has been extensively evaluated for its suitability as a live oral vaccine. Both small- and large-scale trials have shown that this strain stimulated moderate to good protection with minimal side reactions (Gilman *et al.*, 1977; Wahden *et al.*, 1982; Edelman and Levine, 1986; Levine *et al.*, 1987a). Strain Ty21a was made by extensive non-specific mutagenesis which induced mutations other than *galE* (see Introduction). Some of these mutations, and perhaps others yet to be detected, may contribute to the attenuation of Ty21a.

The construction of a genetically defined *galE* derivative of *S.typhi* Ty2, Ty2H1, was decribed in the previous Chapter. This strain lacks 0.4kb of the *galE* gene but is isogenic in all other respects to its parent *S.typhi* Ty2 (Chapter 4). An *S.typhimurium* strain carrying the same deletion as Ty2H1 was completely avirulent in mice and gave high levels of protection (Chapter 4).In this Chapter a rifampicin-resistant, *via* derivative of Ty2H1, EX462, is shown to be markedly attenuated when compared with its Ty2 parent strain in the mouse mucin virulence assay. Its safety and immunogenicity in humans was then assessed.

5.2 Results

5.2.1 Comparison of *S.typhi* with its *galE* and *via* derivatives

Derivation of strains Ty2Vi and EX462

Strains Ty2Vi and Ty2H1Vi were selected as spontaneous phage ViII–resistant mutants of Ty2 and Ty2H1 respectively. Rifampicin–resistant mutants of strain Ty2H1Vi were subsequently selected. One such rifampicin–resistant mutant, strain EX462, was chosen for this investigation.

Biochemical reactions

All strains (Ty2, Ty2H1, Ty2Vi and EX462) were tested in Microbact 12E biotyping trays. Their biochemical profiles were identical. When grown on M9 minimal media, Ty2 had growth requirements for cystine and tryptophan (Germanier and Furer, 1983),as had all its derivatives. No strain showed any additional growth requirements. The activities of the enzymes of the Leloir pathway (Galactokinase, Galactose–1–phosphate uridylyltransferase and UDP–glucose–4–epimerase) were assayed (Table 5.1), confirming that no detectable UDP–glucose–4–epimerase activity was found in the strains carrying the *galE*-H1 mutation (Ty2H1 and EX462). This result reflected the fact that no func-

Table 5.1: Characteristics of S. typhi Ty2 and its derivatives

Bacterial strain	Leloir	Enzyme	Activity[1]	Galactose sensitivity	Bacteriophage sensitivity Vi II (P22 & F-O)			HA inhibition[2]		Serum resistance[3]	
	galE	galT	galK			Gal$^-$	Gal$^+$	Gal$^-$	Gal$^+$	Gal$^-$	Gal$^+$
S. typhi Ty2	45.0	13.7	21.6	R	S	S	S	7	7	25	22
Ty2H1	U	5.6	11.5	0.06mM	S	R	S	<1	6	2	23
Ty2Vi	42.0	15.8	18.4	R	R	S	S	7	7	4	5
EX462	U	5.4	12.3	0.06mM	R	R	S	<1	7	<1	3
Ty21a	U	2.0	1.2	6mM	R	R	S^R	<1	7	<1	<1
EX590	ND	ND	ND	R	S	S	S	7	7	22	22
EX592	ND	ND	ND	R	R	S	S	7	7	3	5
J670	U	5.4	10.8	0.06mM	R	R	S	<1	6	<1	<1
J671	U	5.6	13.1	0.06mM	R	R	S	<1	6	<1	<1

1. Activities expressed as μmol of substrate converted, per mg of protein per hour. Galactose to a final concentration of 6μM was used to induce the gal operon. U = undetectable.

2. Expressed as the number of wells of HA inhibition relative to saline control. Two-fold serial dilutions of bacteria were used. Red cells were coated with S. typhi LPS.

3. Expressed as the % (v/v) serum that caused 50% killing of the bacterial strain relative to saline control.

F-O: Felix-O; S = Sensitive; R = Resistant; S^R = Partial Sensitivity; Gal$^+$ or Gal$^-$ = denotes presence or absence of galactose in the base medium. Glucose (25mM) was always present; ND: not done.

tional *galE* gene product could be made in these strains. Also, the levels of Galactokinase and Galactose–1–Phosphate uridylyltransferase, the gene products of *galK* and *galT* respectively, were lower in strains Ty2H1 and EX462 than in Ty2 and Ty2Vi. Strains Ty2H1 and EX462 exhibited identical sensitivities to galactose–induced lysis, which was seen at 0.06mM galactose, reflecting the similar expression levels of the *galT* and *galK* genes in these strains.

Outer-membrane properties

The ability of the *galE* and *via* mutants to synthesise smooth LPS in the presence or absence of exogenous galactose was examined. All strains showed sensitivity to bacteriophage specific for O-antigen (P22) and LPS-core (Felix-O) when grown on nutrient agar supplemented with 25mM glucose and 6mM galactose (Table 5.1). If galactose was omitted from the above medium, the *galE*-H1 strains, Ty2H1 and EX462, became resistant to both P22 and Felix-O. This confirmed that Ty2H1 and EX462 were able to make smooth LPS, but only when exogenous galactose was supplied, a phenotype typical of *galE* mutants (Fukasawa and Nikaido, 1961; Nikaido, 1961). The minimum level of galactose required for Ty2H1 and EX462 to become sensitive to P22 was 6μm. To quantitate the level of O-antigen expression, HA inhibition assays were performed. These data (Table 5.1), together with the phage resistance patterns, demonstrated clearly that the *galE*-H1 strains synthesised amounts of smooth LPS comparable to those made by the parent strain, Ty2, when exogenous galactose was supplied (Table 5.1).

Resistance to serum and *in vivo* killing

The bactericidal action of normal serum on Ty2 and its *galE* and *via* derivatives was determined following growth in the presence or absence of galactose . As previously described (Felix *et al.*, 1934, 1935; Felix and Pitt, 1934a, 1934b; Osawa and Muschel, 1964; Robbins and Robbins, 1984) the Vi antigen played an important role in conferring resistance to the killing effect of serum (Table

5.1). The *galE*-H1 mutation in both Ty2H1 and EX462 also reduced the ability to survive in serum but only if the organisms were cultured in the absence of exogenous galactose; in its presence, resistance equal to that of the gal^+ equivalents (Ty2 and Ty2Vi respectively) was obtained (Table 5.1).

To investigate *in vivo* survival each of the four strains (Ty2, Ty2H1, Ty2Vi and EX462) were injected ip into mice, and bacterial counts were enumerated in the peritoneal cavities and spleens to d15 post–injection (Fig. 5.1). The *in vivo* survival of these strains demonstrated that the two mutations (*galE*-H1 and *via)* acted synergistically to decrease the ability of *S.typhi* to persist *in vivo*. Ty2H1 and Ty2Vi survived in the spleen as did the parental strain, Ty2, but EX462 was never recovered from this organ. Strain Ty2H1 was rapidly cleared from the peritoneum; Ty2Vi persisted longer but was cleared more rapidly than Ty2. EX462, however, was rapidly cleared *in vivo*, and even on d1 post-injection no viable bacteria could be detected in the peritoneal washouts (Fig. 5.1). The behaviour of EX462 *in vivo* was comparable to that of Ty21a (data not shown).

5.2.2 Virulence and immunogenicity in mice

Virulence

Ty2 and the *galE*-H1, *via* derivative, EX462, were compared for virulence in mice when injected ip in 5%(w/v) mucin supplemented with iron (Table 5.2). The importance of Vi to the virulence of *S.typhi* in mice was consistent with results previously reported (Felix *et al.*, 1934, 1935; Felix and Pitt, 1934a, 1934b; Osawa and Muschel, 1964; Robbins and Robbins, 1984). By removing this component from Ty2 (Ty2Vi) the LD_{50} increased by a factor of 10^4 in the mouse mucin virulence assay. The *galE*-H1 mutation alone caused only a small increase (18-fold, Table 5.2) in LD_{50} value. However, there appeared to be a synergistic reduction when *S.typhi* Ty2 carried both the *galE*-H1 and *via* mutations. This synergy can be seen by comparing EX462 with its gal^+equivalent, Ty2Vi; here the *galE*-H1 mutation resulted in a 100 fold increase in the LD_{50} value. That

Figure 5.1

Persistence of Ty2, and derived strains, in the peritoneal cavities and spleens of mice after i.p. injection: Mice (groups of 25) were injected with bacteria, grown in NBGG, and suspended in saline, at the dose levels per mouse indicated by arrows on the left. At each time point shown, five mice per group were sacrificed and bacterial counts in the peritoneal cavities and spleens were enumerated. Geometric mean values, and standard error bars, are shown. The limit of detection in this assay was 25 bacteria ($10^{1.39}$). The levels of bacteria injected per mouse were as follows: Ty2, 6.1×10^4; Ty2H1, 5.0×10^4, Ty2Vi, 8.2×10^4, EX462, 4.7×10^6.

(A) Ty2; (B) Ty2H1; (C) Ty2Vi; (D) EX462.

Symbols: ● : peritoneal cavities; ■ : spleens.

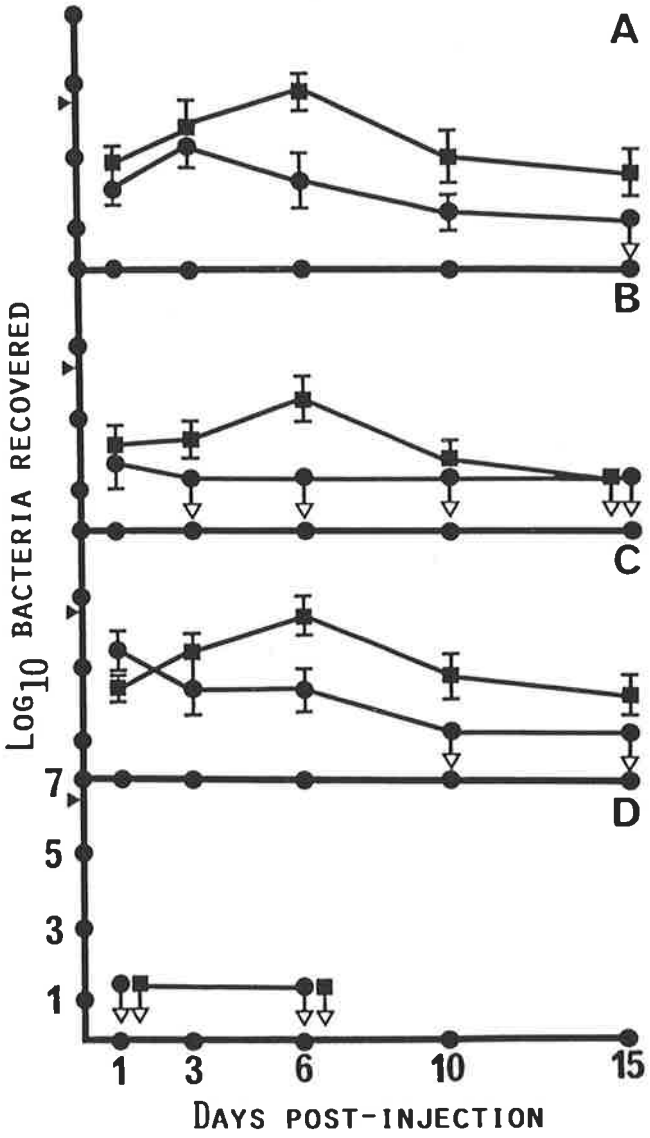

A
B
C
D
LOG10 BACTERIA RECOVERED
7
5
3
1
1
3
6
10
15
DAYS POST-INJECTION

Table 5.2: Virulence and vaccine potential of Ty2 and its derivatives in mice

Bacterial strain	LD_{50} [1]	EID_{50} [2]
S. typhi Ty2	15	$<1.2 \times 10^3$
Ty2H1	260	$<9.0 \times 10^3$
Ty2Vi	1.8×10^5	1.0×10^5
EX462	1.6×10^7	6.5×10^5
Ty21a	$>2.2 \times 10^7$	1.5×10^7
EX590	19	ND
EX592	3.4×10^5	ND
J670	1.6×10^7	ND
J671	2.8×10^7	ND

1. LD_{50} values were calculated by the method of Reed and Muench (1938) based on the number of survivors after 72 hrs.

2. The 50% effective immunizing dose (see Methods). The challenge dose was 40 LD_{50} values of Ty2.

ND = not done.

is, *galE* was five times more attenuating in mice when the Vi component was absent compared to when it was present. Strain Ty21a was commonly only 30% viable after growth in NBGG; consequently the upper limit that could be assigned to its LD_{50} value was restricted by the presence of non-viable bacteria which contributed to the total endotoxin load.

To determine if the *galE*-H1 mutation was the factor responsible for increasing the LD_{50} of strains Ty2H1 and EX462 we transduced the strains to gal^+ (EX590 and EX592 respectively). Both EX590 and EX592 reverted to the full virulence of the isogenic strains Ty2 and Ty2Vi respectively (Table 5.2), confirming that the *galE*-H1 mutation was the sole factor causing the LD_{50} differences seen between Ty2H1 and EX462 and their gal^+ counterparts Ty2 and Ty2Vi respectively.

Immunogenicity

To compare the vaccine potential of Ty2 and its derivatives, an experiment was performed to determine the 50% effective immunizing dose (EID_{50}, Table 5.2). The *via* derivative of Ty2, Ty2Vi, showed a significant decrease in its ability to immunize. This finding was consistent with previous reports that the Vi antigen contributes a significant proportion of the vaccine potential of *S.typhi* in mice (Robbins and Robbins, 1984). The EID_{50} of EX462 was 6.5 times higher than Ty2Vi but was about 20 times lower than that of Ty21a. Thus the vaccine potential of EX462 was high compared to Ty21a, a useful standard because of the known efficacy of Ty21a in humans (Edelman and Levine, 1986; Wahden *et al.*, 1982; Levine *et al.*, 1986).

5.2.3 Safety and immunogenicity in humans

Safety

Volunteers received a single dose of 7×10^8 viable organisms of the candidate vaccine strain, EX462 (suspended in saline and preceded by 1g of bicarbonate).

On a day in the week preceding vaccination, and to day 10 after, the excretion of viable organisms (EX462) in the feces was monitored. Three of the four volunteers produced at least one positive coproculture but no viable organisms were detected in stool samples after two days post–vaccination (Table 5.3). Two of the four volunteers developed an illness clinically indistinguishable from typhoid within a week of ingesting the candidate vaccine strain. On day 4, volunteer A, and on day 5, volunteer B, reported headaches which became increasingly severe along with nausea, anorexia, fever and rigors. Both volunteers were admitted to the Royal Adelaide Hospital. Blood cultures (volunteer A, 6 out of 7 cultures tested; volunteer B, 4 out of 6 cultures tested) were positive for a gram-negative bacillus identified as *S.typhi* by virtue of being positive for the O groups 9 and 12, when grown on nutrient agar with glucose (25mM) and galactose (6mM), and H1–d^+. The isolates were Vi^- (as judged by absence of agglutination with anti-Vi antisera), resistant to rifampicin, and did not grow on M9 minimal medium unless it was supplemented with cystine and tryptophan. Other investigations of the patients including leucocyte count, hemaglobin, platelet count, serum biochemistry, and urinary microscopy remained normal. Both patients were commenced on Amoxycillin intravenously (2g every 6 hours); the fever and other symptoms responded rapidly to treatment and on day 12 the two volunteers were discharged from hospital on oral Amoxycillin (a 10 day course of 0.5g 6 hourly). Neither volunteer C or D complained of any illness and on d9 physical examination was unremarkable. Twelve months later no illness has been noted in either volunteer.

Immunogenicity

Serum and small intestinal fluid were obtained from each of the four volunteers on d22. These and the corresponding pre–vaccination samples, were assayed for anti-*S.typhi* LPS antibodies by ELISA (serum and intestinal fluids) and for their bactericidal capacity against Ty2Vi (serum only, Table 5.3). Volunteers A and B showed marked rises in anti-*S.typhi* LPS and bactericidal activity. Subjects

Table 5.3: Human immunogenicity of EX462 given orally [1]

Volunteer	Stool counts [2]			Fever [3]	Serum bactericidal		Serum ELISA IgA		Serum ELISA IgG		Intestinal ELISA IgA	
	d1	d2	d3-d10		Pre	Post	Pre	Post	Pre	Post	Pre	Post
A	10^5	U	U	+(5)	760	34000	200	10350	42	126	60	767
B	10^6	10^3	U	+(6)	2300	32000	276	4080	71	300	87	575
C	10^6	10^4	U	-	630	9600	503	1760	17	161	320	1120
D	U	U	U	-	490	5900	5375	33125	33	71	200	6960
Mean Fold Rise [4]					18.4		11.3		2.3		10.0	

1. Volunteers received 1g of $NaHCO_3$ in 50ml water prior to ingestion of the 7×10^8 viable EX462; fluids for assay of immune responses were obtained 22d later. All assays were performed as described in Materials and Methods.
2. Figures show bacterial recoveries per g of stool. U: Undetectable. The limit of detection is 100 bacteria/g of stool.
3. + indicates that the volunteer became febrile after ingesting EX462. The bracketted number indicates the day post-vaccination on which the volunteer became ill.
4. The geometric means of the fold rises of individual volunteers.

C and D, neither of whom was ill, also showed anti-*S.typhi* LPS responses: the most marked rises were in serum IgA levels and bactericidal activity (Table 5.3). Volunteer D showed a 34.8-fold rise in intestinal anti-*S.typhi* LPS IgA (Table 5.3).

5.2.4 Characteristics of bacteria isolated from blood culture

As reported above, blood cultures from each febrile patient supported growth of *S.typhi*. Four such isolates (J669–J672, two from each patient), were purified on NA and stored at –70°C. Each had an identical serotype to the vaccine strain EX462. Furthermore, each displayed the same sensitivity to galactose-induced lysis (at $\geq$0.06mM) and displayed an unaltered bacteriophage P22 and Felix-O sensitivity pattern (Table 5.1). The minimum galactose concentration required to produce a smooth phenotype was also unaltered (6μM).The isolates were sent to the Microbiological Diagnostic Unit, Department of Microbiology, University of Melbourne, which confirmed that they were untypable in the Vi-phage typing system as was EX462, and hence providing further evidence that these strains had not reverted to *via*$^+$. Two of the isolates (J670 and J671, one from each volunteer) were characterized further. The activities of the Leloir enzymes, serum sensitivity (Table 5.1) and virulence in mice (Table 5.2) were found to be comparable to EX462, indicating the virulence of EX462 in humans could not be ascribed to a reversion to *gal*$^+$, nor to any other detectable phenotypic change.

5.3 Discussion

For ethical reasons knowledge of the pathogenesis of *S.typhi* and methods of its attenuation are heavily reliant on murine models of *Salmonella* infections (Germanier, 1984). These models have led to the belief that attenuation of *Salmonella* should not be so severe as to limit its ability to invade and undergo

limited growth in the gut associated lymphoid tissue, as this capacity correlates with vaccine potential (Germanier, 1970, 1972, 1984; Germanier and Furer, 1971; Collins and Carter, 1972; Carter and Collins, 1974b; Hohmann *et al.*, 1978; Srisart *et al.*, 1985). *S.typhimurium* carrying mutations in the *galE* gene evidently display the required level of attenuation in mice and other animals, being avirulent and yet protective (Germanier, 1970; Gilman *et al.*, 1977; Wray *et al.*, 1977; Clarke and Gyles, 1986), although a *galE* strain caused fatal infections when the mice were given cyclophosphamide (Wray *et al.*, 1977). This indicated that cellular immunity played an important role, by controlling the growth of *galE* strains *in vivo* (Wray *et al.*, 1977). Strain Ty21a, a *galE* mutant of *S.typhi* Ty2 made by extensive mutagenesis, was demonstrably safe and immunogenic in humans (Gilman *et al.*, 1977; Wahden *et al.*, 1982; Edelman and Levine, 1986; Levine *et al.*, 1986).

The previous Chapter reported the construction of a defined *galE* deletion in *S.typhi* Ty2, Ty2H1. This strain was only slightly attenuated in the mouse mucin virulence assay (Table 5.2). We sought to further attenuate Ty2H1 by removing the Vi antigen. The rifampicin–resistant derivative of this Vi–deficient strain, EX462, was highly attenuated in mice (Table 5.2).In the presence of the Vi capsule, the *galE*-H1 mutation increased the LD_{50} value only 18-fold (Table 5.2). However, if Vi was absent, the *galE*-H1 increased the LD_{50} value 100-fold (Table 5.2). Thus the *galE*-H1 mutation appeared to be more attenuating when the Vi antigen was absent, suggesting that the *galE*-H1 and *via* mutations acted in synergy to reduce virulence. The synergism of the double mutation, *galE*-H1; *via*, was also demonstrated by the inability of EX462 to persist *in vivo* in mice (Fig. 5.1). Strain EX462 was also highly sensitive to serum (Table 5.1).

Ty21a was demonstrably safe at doses as high as 10^{11} (Bartholemeusz *et al.*, 1976), and since EX462 was comparable to Ty21a by all the criteria we could apply (persistence and virulence in mice and serum sensitivity) we decided to assess its safety and immunogenicity in humans. Although only small numbers of volunteers were used, EX462 showed no detectable attenuation in

humans. The incubation period was similar to that of wild type *S.typhi* at a dose of 10^9/volunteer (Hornick *et al.*, 1970a), implying that growth of EX462 was not inhibited *in vivo* nor was it likely that it was being rapidly cleared. It seems unlikely that a rough Vi–negative strain could grow *in vivo* because of evidence (in mice) that suggests strains of this phenotype would be rapidly cleared (Felix *et al.*, 1934a; Germanier, 1970; Germanier and Furer, 1975). Smooth Vi negative strains have been previously shown to be moderately virulent in humans (Felix, 1934a; Hornick *et al.*, 1970b), leading us to the hypothesis that EX462 was able to scavenge sufficient exogenous galactose in human tissue to synthesise smooth LPS. The organisms isolated from the febrile volunteers were shown to be identical to EX462 with respect to phenotype and mouse virulence (Tables 5.1 and 5.2). We concluded that EX462 had not reverted *in vivo* and that mutations other than *galE* contributed to the attenuation of *S.typhi* Ty2 in strain Ty21a.

Strains of *Salmonella cholerae-suis* and *S.typhimurium* carrying the *galE* mutation have been shown to revert to virulence if they became resistant to galactose (Germanier and Furer, 1971; Nnalue and Stocker, 1986). However, the levels of the Leloir enzymes, and the sensitivities to galactose-induced lysis, were unaltered in the blood isolates from the febrile patients, as were the minimum levels of galactose required for the production of smooth LPS. This galactose sensitivity level was comparable to that effecting lysis of C5H1 and LT2H1, *galE*-H1 derivatives of *S.typhimurium* C5 and LT2 respectively, both of which were found to be avirulent and immunogenic in mice (Chapter 4).

The lack of galactose–induced lysis *in vivo* in man was attributed to either the absence of sufficient exogenous galactose to cause lysis of EX462 or the existence of sufficient exogenous catabolites (eg. glucose) that repressed the *gal* operon, and in the presence of which EX462 became galactose tolerant.

5.4 Conclusion

This Chapter demonstrated some important points. First, models of *Salmonella* infection in mice have their limitations when being used to assist vaccine development. Second, we have confirmed earlier observations that the Vi antigen does not play a critical role in the pathogenesis of *S.typhi* in man (Hornick *et al.*, 1970b). Finally, our observations here showed that the *galE* mutation effected no detectable reduction on the pathogenic potential of *S.typhi* in man.

Chapter 6

A chromosomal integration system for stabilisation of heterologous genes in *Salmonella* based vaccine strains

6.1 Introduction

Several groups have proposed that attenuated *Salmonella* strains carrying heterologous DNA encoding protective antigens of other enteropathogens might be suitable as bivalent live oral vaccines. It is envisaged that the *Salmonella* vector will generate protective immunity against subsequent *Salmonella* infection, while immune responses directed against the cloned determinant will confer resistance against the second pathogen of interest (Formal *et al.*, 1981; Clemens *et al.*, 1984; Stevenson and Manning, 1985; Dougan *et al.*, 1987). Existing candidate hybrid vaccines carry the heterologous DNA inserted into plasmids (Formal *et al.*, 1981; Clemens *et al.*, 1984; Stevenson and Manning, 1985). Plasmids which are components of hybrid vaccines must be non–conjugative and non–mobilisable (Guidelines for research involving recombinant DNA molecules, 1982). Although

plasmid pBR325 was demonstrated to be highly contained *in vivo* (Levine *et al.*, 1983), three problems may be experienced in the administration to humans of hybrid strains carrying recombinant plasmids. Firstly, the possibility of plasmid transfer from the hybrid to the human bacterial commensals, or environmental bacteria, must be addressed. Considerable experimentation may be required to demonstrate that such transfer is negligible. Secondly, the introduction of a recombinant plasmid into the carrier strain (typically by transformation) usually requires selection for an antibiotic–resistance marker of the plasmid. Such markers are undesirable in strains intended for release outside the laboratory (Guidelines for research involving recombinant DNA molecules, 1982). Finally, even when the hybrid strain carries a plasmid specifying antibiotic–resistance, it will be difficult to maintain selection for the plasmid *in vivo*. If the plasmid is unstable in the absence of selection, the efficacy of the live vaccine will be adversely affected.

There was a need then, to develop a system whereby heterologous DNA could be stably maintained in a carrier strain but in a manner which avoided the problems associated with plasmid vectors. To this end, a chromosomal integration system which enables recombination of heterologous DNA into the chromosome of a carrier strain has been developed. This Chapter shows the contruction of such a vector and demonstrates its use in the recombination of the K88 fimbrial genes of a pig enterotoxigenic *E. coli* strain (Mooi *et al.*, 1984) into an attenuated (for mice) *galE* derivative of *S. typhimurium* (LT2H1; Chapter 4).

6.2 Results

6.2.1 Plasmid construction

Overview of method

Firstly a plasmid carrying the *hisOGD* of *S. typhimurium* (pADE171) was obtained. Next, a deletion eliminating the *his* regulation region (*hisO*), and part of the *hisG* gene, was created in this plasmid to give plasmid pADE172. This deletion mutation was recombined into the chromosome of LT2H1, a *S. typhimurium galE* strain. The introduction of a *galE* deletion into *S. typhimurium* LT2 has been shown to completely attenuate the strain in mice (Chapter 4), yet to leave unaffected its capacity to function as a live oral vaccine in protection of mice from potentially lethal challenge with the highly virulent *S. typhimurium* C5. The *hisOG* delete strain (J706) was incapable of growth on M9 agar supplemented with histidinol, demonstrating that transcription through the *hisOG* deletion was not occuring. A 7kb DNA fragment encoding the K88 fimbrial genes of enterotoxigenic *E. coli* was inserted into plasmid pADE171 at a point upstream of *his*. This plasmid, pADE177, was transformed into strain J706, and recombination between homologous sequences in plasmid DNA (pADE177), and *S. typhimurium* chromosomal DNA in the *his* region, yielded J754, a his^+ strain with the K88 genes integrated into the chromosome upstream of *his*.

Cloning *his OGD* from *S. typhimurium*

The region upstream of the *his* operon was chosen as an integration site because sequence data on recombinant plasmids embracing this region was available (Barnes, 1978). Plasmid pWB91 carries *hisOGD* of *S. typhimurium* (Barnes, 1978). In this plasmid, the region upstream of the *his* regulator is undefined (a fusion between F factor DNA and *S. typhimurium* DNA; Barnes, 1978). Data obtained from DNA–blots of *S. typhimurium* chromosomal DNA probed with pWB91, was used to construct a restriction endonuclease map of the region up-

stream of *his* (data not shown). A 6.0 kb *Hind*III–*Eco*R1 fragment, containing DNA 3.0kb upstream of the *his* regulator region (the position of a *Hind*III cleavage site) to the start of the *hisC* gene (*Eco*R1 cleavage site), was cloned. To achieve this, *S. typhimurium* LT2 chromosomal DNA was digested with *Eco*R1 + *Hind*III and ligated to *Eco*R1 + *Hind*III digested pGB2 DNA. The ligation mix was used to transform strain P2905 and his^+ transformants were analysed by AGE of plasmid DNA digested with *Eco*R1 + *Hind*III. One his^+ transformant was found to harbor a plasmid (pADE171) of the expected restriction endonuclease pattern (Fig. 6.1). Plasmid pADE171 was found to complement the *hisG* mutation of TT2619 and enabled the *his* deletion strain P9032 to grow on M9 agar supplemented with histidinol (*hisD* expression; Winkler, 1987). This confirmed that plasmid pADE171 carried the *hisOGD* region and that the region was being expressed.

Construction of a *hisOG* deletion in pADE171

From available DNA sequence data (Barnes, 1978), restriction endonucleases *Eag*1 and *Aat*II were used to construct a deletion in pADE171 (Fig. 6.1). Plasmid pADE171 was digested with *Eag*1 and *Aat*II, endfilled, and circularised by ligation. The resulting plasmid, pADE172, carried a 0.9kb deletion spanning from 200bp upstream of the *his* regulator region to the middle of the *hisG* gene (Fig. 6.1; Barnes, 1978). The location of the deletion was confirmed by restriction endonuclease analysis with *Pst*1, *Eco*R1, *Hind*III, and *Eco*RV.

Cloning the K88 genes from pFM205 into pADE171

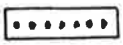
Plasmid pFM205, which carries the $K88_{ab}$ genes of an enterotoxigenic *E. coli* strain (Mooi *et al.*, 1984), was digested with *Pvu*1 and *Bam*H1. In parallel, plasmid pADE171 was digested with *Pst*1. Both plasmids were endfilled with T4 DNA polymerase. The resulting plasmid DNAs were mixed, ligated and used to transform strain LB5010 (Bullas and Ryu, 1983). Spectinomycin–resistant colonies were checked for expression of K88 fimbriae as judged by their ability

Figure 6.1

The chromosomal integration system: The plasmids used to integrate heterologous DNA into the chromosome of an attenuated strain of *S. typhimurium* are shown.

 : pGB2 DNA;

 : *S. typhimurium* DNA from the *his* region;

 : $K88_{ab}$–encoding DNA.

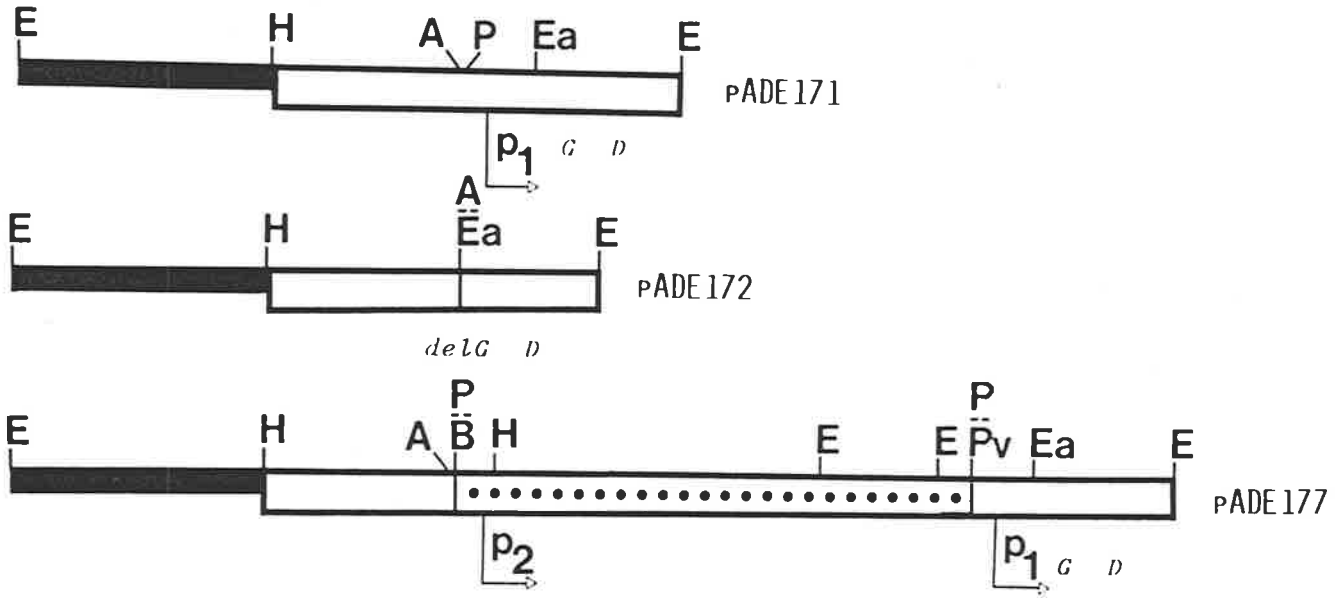
E: *Eco*RI site; **H**: *Hind*III site: **A**: *Aat*II site; **P**: *Pst*I site;

Ea: *Eag*I site; **P/B**: fused *Pst*I and *Bam*HI sites;

P/Pv: fused *Pst*I and *Pvu*I sites.

$\mathbf{P_1}$: *his* promoter; $\mathbf{P_2}$: pBR322 promoter active to transcribe the $K88_{ab}$ genes (Mooi *et al.*, 1983).

G: *hisG*; *D*: *hisD*; *del G*: deletion of *hisG*.


E
H
A
P
Ea
E
pADE171
p1
G
D
A
E
H
Ea
E
pADE172
delG
D
P
E
H
A
B
H
E
E
P
Pv
Ea
E
pADE177
p2
p1
G
D
1kb

to agglutinate with rabbit anti-K88 serum. One transformant, harbouring plasmid pADE177, was found to exhibit strong agglutination. Analysis of plasmid pADE177 with restriction endonucleases confirmed that it carried the *Pvu*1–*Bam*H1 fragment of pFM205 (Fig. 6.1).

6.2.2 Integration of the K88 genes into the chromosome of *Salmonella typhimurium*

Chromosomal integration of the K88 genes was achieved in two steps. Firstly the *hisG* deletion from pADE172 was recombined into the chromosome of LT2H1. Subsequent recovery of the his^+ phenotype by recombination with plasmid pADE177 simultaneously selected for the integration event (Fig. 6.2).

Step 1: Strain LT2H1 was transformed with plasmid pADE172. To select for *his* auxotrophs the transformant (LT2H1(pADE172)) was treated with ampicillin during exponential growth in M9 medium. This treatment was repeated and 0.1ml aliquots of dilutions of the resulting culture were spread onto NA. Screening for *his* auxotrophs was achieved by replication of colonies from NA onto NA and M9 agar. Auxotrophs of a *his* phenotype were found 0.8–1.0x10^3 fold more frequently when strain LT2H1 harboured plasmid pADE172 than when it did not. Six such *his* auxotrophs were cured of plasmid pADE172 (Chapter 2); chromosomal DNA was then prepared from the cured strains, digested with *Eco*R1 and *Hind*III , electrophoresed and transferred to nitrocellulose filters prior to probing with pADE171. The chromosomes of the six cured *his* auxotrophs were found to have a 0.9kb deletion in the *Eco*R1–*Hind*III fragment that spans the *his* region (Fig. 6.3). Chromosomal DNA from the *hisOG* deletion mutants did not hybridise to the *Eag*1–*Aat*II fragment of pADE171 (data not shown). It was concluded that the deletion on plasmid pADE172 had recombined into the chromosome of LT2H1 (Fig. 6.2; step 1). One such *his* deletion mutant was designated J706 (Fig. 6.3). Strain J706 did not grow on histidinol, implying that no upstream promoter was reading through the deleted

Figure 6.2

Integration of the K88 genes into the chromosome of *S. typhimurium* LT2H1: This two step process is described in the text.

▬▬: pGB2 DNA;

▭: *S. typhimurium* DNA from the *his* region;

▭ (dotted): $K88_{ab}$–encoding DNA, or ▬▬: chromosomal DNA.

E: *Eco*RI site; **H**: *Hind*III site: **A**: *Aat*II site; **P**: *Pst*I site;

Ea: *Eag*I site; **P/B**: fused *Pst*I and *Bam*HI sites;

P/Pv: fused *Pst*I and *Pvu*I sites.

$\mathbf{P_1}$: *his* promoter; $\mathbf{P_2}$: pBR322 promoter active to transcribe the $K88_{ab}$ genes (Mooi *et al.*, 1983).

G: *hisG*; *D*: *hisD*; *del G*: deletion of *hisG*.

1: Step 1; **2**: Step 2.

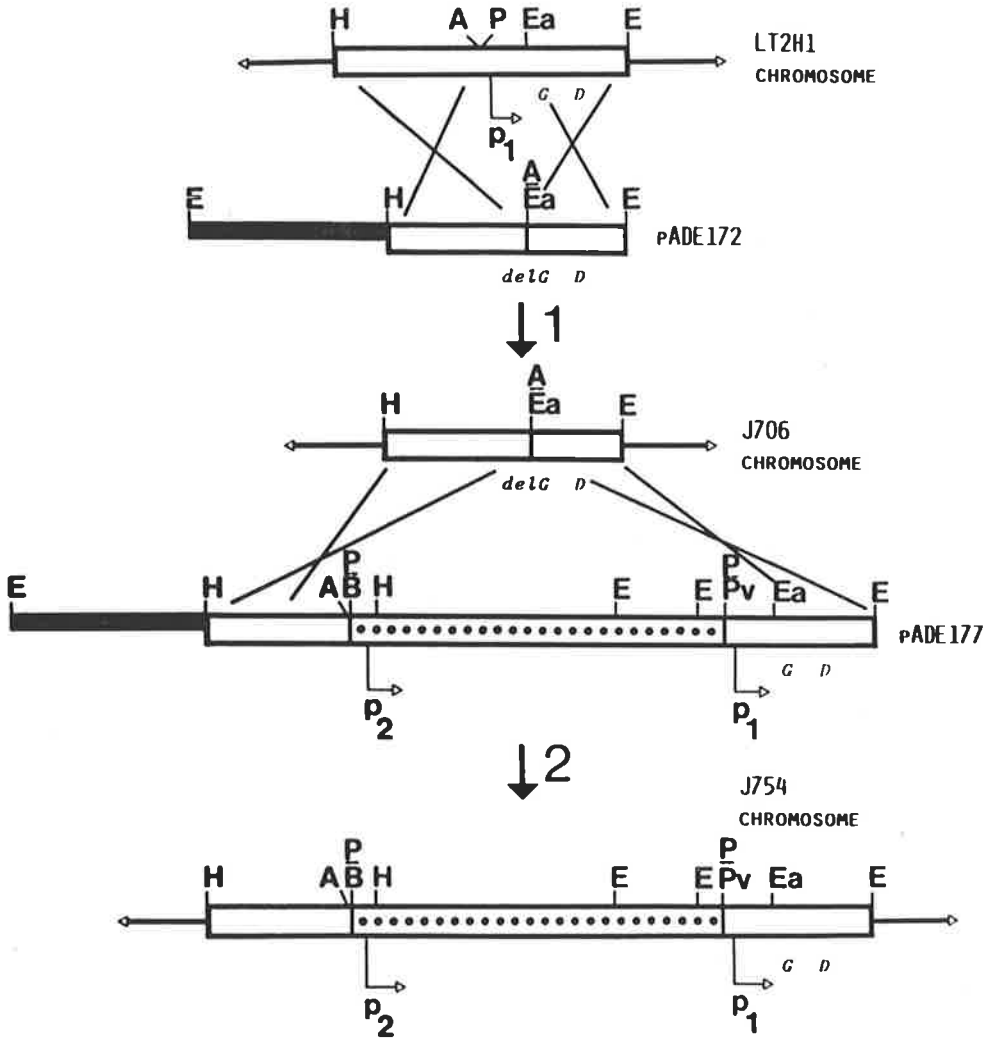
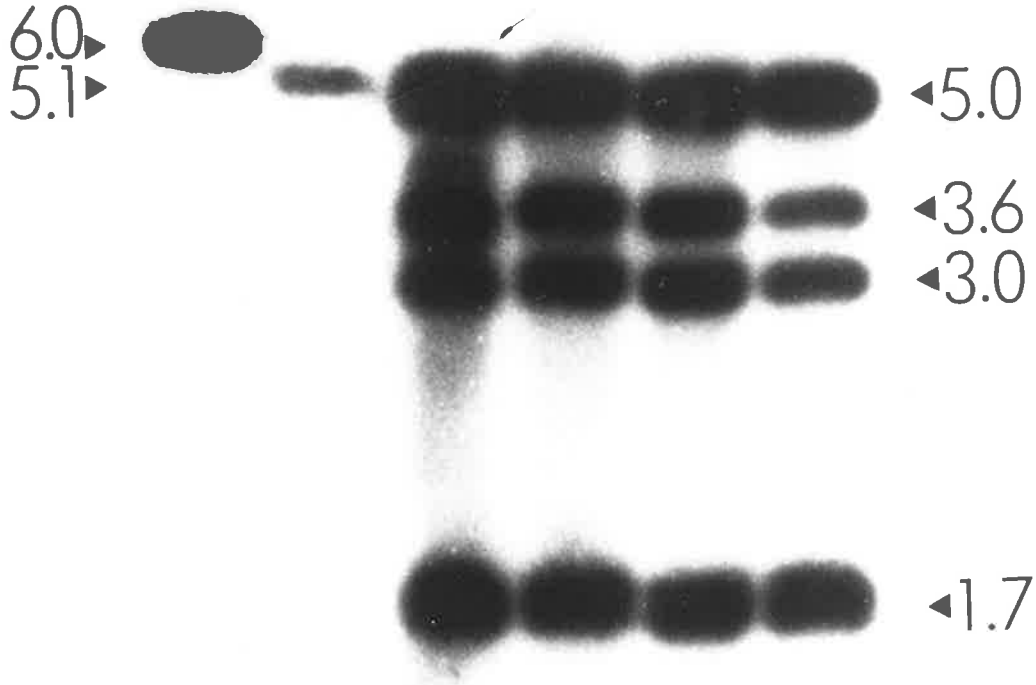

H
A P Ea
E
LT2H1
CHROMOSOME
G D
p1
A
Ea
E
H
E
pADE172
delG D
1
A
Ea
H
E
J706
CHROMOSOME
delG D
E
H
P
AB H
E
E
P
Pv
Ea
E
pADE177
G D
p2
p1
2
J754
CHROMOSOME
P
H
AB H
E
E
P
Pv
Ea
E
G D
p2
p1

Figure 6.3

The K88 genes are integrated into the chromosome of LT2H1.
Chromosomal DNA was prepared from various strains, digested with *Eco*RI and *Hind*III, subjected to AGE, transferred to nitrocellulose and probed with labelled pADE177 (Figure 6.1).
(A) LT2H1; **(B)** J706; **(C)** J754; **(D)** J770; **(E)** J771; **(F)** J772.
The numbers are the sizes, in kb, of the arrowed fragments.



A B C D E F

hisOG region to express *hisD*. The gene product of *hisD* is necessary for *S. typhimurium* to grow on histidinol (Winkler, 1987). The *hisG* deletion in strain J706 was not complemented by plasmid pADE171 (when grown on M9 agar), however, his^+ revertants were detected in strain J706(pADE171) at a frequency between $1.0x10^{-3}$ and $1.5x10^{-3}$. The his^+ revertants acquired in this manner were presumably recombinants in which the *hisOG* deletion in strain J706 had been replaced by the functional region from pADE171.

Step 2: Strain J706 was transformed with plasmid pADE177. The resulting transformant, J706(pADE177), was grown to log phase in NBG and 0.1ml aliquots of dilutions of this culture were spread onto either M9 agar or NA plates. Comparison of viable counts on the two media showed that reversion to his^+ occurred at a frequency between $1.0x10^{-3}$–$1.5x10^{-3}$. Reversion to his^+ was not detected when the parent strain, J706, was grown on M9 agar. Four his^+ colonies were cured of plasmid pADE177 and tested for their ability to agglutinate in the presence of anti–K88 serum. All four his^+ strains were found to exhibit agglutination. These four strains were designated J754, J770, J771 and J772. Chromosomal DNA was prepared from these strains, digested with *Eco*R1 and *Hind*III, electrophoresed, transferred to nitrocellulose and probed with plasmid pADE177. Four fragments were found to hybridise to plasmid pADE177 (Fig. 6.3). The sizes of these four fragments (5.0, 3.6, 3.0, and 1.7kb) were as expected (Fig. 6.2). Strain J754 was chosen for further analysis.

6.2.3 Expression and *in vitro* stability of chromosomally integrated K88 genes

Expression of chromosomal K88 genes

To check the level of expression, and the cellular location of the K88 fimbriae, bacteria were grown on NA and harvested after 16hrs at 37^0C into saline to give *ca.* $1.0x10^{11}$ bacteria/ml. Half of the suspension was formalin fixed and used in an ELISA inhibition assay. Purified K88 fimbriae from *E. coli* strain

DH1(pFM205) served as the standard (starting concentration 2.5 μg/ml; Table 6.1). The level of expression of K88 fimbriae by the recombinant, J754, was 3.1 μg per 10^{10} bacteria, about 22% that of either a K88$^+$ enterotoxigenic *E. coli* strain (M30) or the strain with the high copy–number recombinant K88$^+$ plasmid, LT2H1(pFM205).

The other half of the suspension was heated at 65°C for 30 min and centrifuged (Eppendorf centrifuge; 15 min). The supernatant was assayed for K88 fimbriae by ELISA inhibition assay (Table 6.1) or viewed on SDS–PAGE (Fig. 6.4). The relative expression levels were similar in the heat treatment preparations and the whole cell preparations (Table 6.1).

SDS–PAGE of supernatants derived from the heat treated bacteria showed that the recombinant, J754, expressed K88 fimbriae, of Mr 26,000, from chromosomally located genes (Fig. 6.4). Immunoblot with anti–K88 sera, after transfer of this material to nitrocellulose, confirmed the expression of K88 fimbriae by the recombinant J754 (Fig. 6.4).

In vitro stability

The *in vitro* loss of K88 expression was determined by growing strain J754 in NBG and subculturing every 11hr into fresh NBG. The rate of appearance of K88$^-$ colonies is shown (Table 6.2). The K88$^+$ phenotype of strain LT2H1(pFM205) was less stable than that of the recombinant J754 (Table 6.2).

6.2.4 Colonisation, stability, and immunogenicity of recombinant strains bearing K88 fimbriae in mice

Peyer's patch colonisation

To determine the colonisation ability of various strains *in vivo*, mice were given bacteria orally ($0.7\text{x}10^9$–$3.0\text{x}10^9$; day 0). At day 3 post–immunization bacterial counts were enumerated in the Peyer's patches of the small intestine. Isolated bacterial colonies were screened for expression of K88 fimbriae by immunoblot-

Table 6.1: Expression of K88 fimbriae by various strains

Strain	K88 fimbriae	
	$\mu g/10^{10}$ bacteria	μg fimbriae released from 10^{10} bacteria
LT2H1	< 0.05	< 0.05
M30	14	23
LT2H1(pFM205)	15	21.5
J754	3.1	5.7

Bacteria were grown on NA or NA with ampicillin (200µg/ml; LT2H1/pFM205 only) and harvested after 16h at 37°C into saline (1 ml per plate). Half the suspension (about 5×10^{10} bacteria) was formalin-fixed, diluted to 5×10^{10}/ml (by microscopic count), and used in E1A with purified K88 (2.5 µg/ml starting concentration) as standard (left column). The remaining suspension was heated at 65°C for 30 min, spun (Eppendorf centrifuge, 15 min at full speed) and the supernatant either assayed in E1A for K88 expression (right column) or viewed on SDS-PAGE (Figure 6.4).

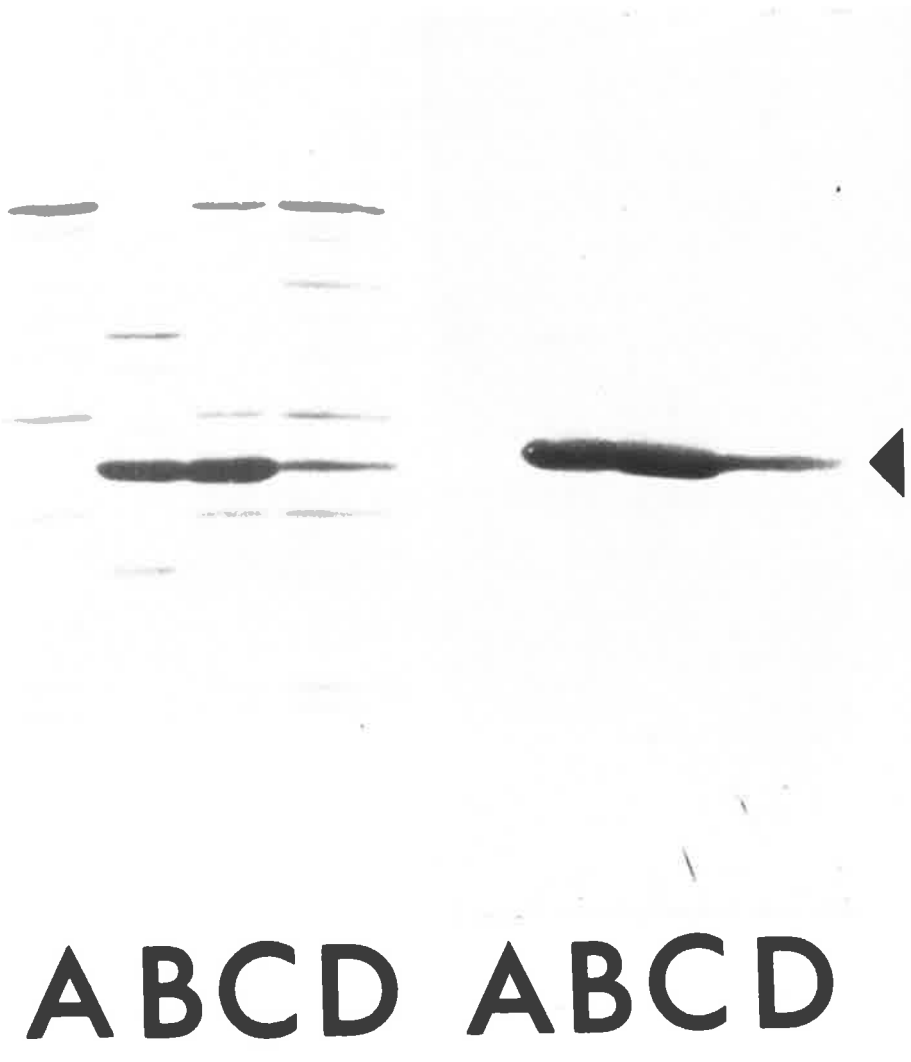
Table 6.2: Stability of K88 expression *in vitro*

Strain	Approximate generations of growth	$K88^-$ bacteria (%)
LT2H1(pFM205)	0	0
	27	17
	54	46
J754	0	0
	27	0
	54	10
	81	20

Strains were grown in NBG without ampicillin, samples of the growing culture at various times, and dilutions plated on NA. At each time, 200 colonies were transferred to nitrocellulose and immunoblotted with anti-K88 serum to give the values in the last column.

Figure 6.4

Expression of the K88 fimbrial polypeptide by various strains: Strains were grown in NBG and fimbriae prepared by heat-shock. Fimbriae from equal quantities of bacteria (about 10^8 bacteria/track) were subjected to SDS-PAGE with protein-specific staining (left panel), or to SDS-PAGE followed by Western transfer and immunoblotting with anti-K88 serum (right panel).
A: LT2H1; **B**: M30; **C**: LT2H1(pFM205); **D**: J754.
The arrow indicates the position of the K88 polypeptide.


A B C D A B C D

ting. The total number of bacteria in the Peyer's patches, and the retention of the capacity to express K88, is shown (Table 6.3). The pig enterotoxigenic *E. coli* strain M30 did not effectively colonise. The live LT2H1–based strains colonised successfully. Comparison of the colonization capacity of LT2H1(pFM205) and J754 was important since a *galE* derivative of *S. typhimurium* harboring plasmid pFM205 has previously proved immunogenic in mice and pigs (Stevenson and Manning, 1985, Attridge *et al.*, 1988). Both LT2H1(pFM205) and J754 retained their $K88^+$ status on colonisation (Table 6.3).

Stability of K88–bearing strains in mice

The stability of K88 expression *in vivo* was determined. Mice were immunised orally on day 0 with between $0.6–2.2\times10^9$ bacteria and groups of 5 mice were sacrificed on days 4, 11, and 18 post–immunisation. Bacterial numbers in the Peyer's patches were enumerated, and colony blots were used to determine the percentage of these bacteria that were $K88^+$ (Table 6.4). The data shown here clearly demonstrated that K88 expression by recombinant strain J754 was more stable than that of LT2H1(pFM205) *in vivo* (Table 6.4).

The J754–derived $K88^-$ strains, cultured from Peyer's patches, were divided into two classes, his^+ or his^-, as judged by their ability to grow on M9 agar. Six of the $K88^-$ mutants, three his^+ and three his^-, were analysed by DNA blotting (Fig. 6.5). No alteration was detected in the chromosomal restriction pattern of the his^+ strains. The lack of fimbrial expression in this class of $K88^-$ mutants was due to either a point mutation or a small deletion, either of which might be undetected in DNA blotting. In DNA blots of the second class of $K88^-$ mutants (his^-) only one fragment was found to hybridise. Loss of K88 fimbrial expression in this class was most likely due to a spontaneous deletion event.

Table 6.3: Colonization of mice by recombinant strains, and generation of anti-K88 immunity

Strain	Log_{10} bacterial counts in Peyer's patches (%$K88^+$)*	Anti-K88 serum IgG**
	d3	d20
LT2H1	4.92 ± 0.41	25
LT2H1(pFM205)	4.17 ± 0.32(100)	2505
J754	4.04 ± 0.40(97)	634
J754 (inactivated)	-	25
E. coli M30	1.25	2710

*Mice were fed bacteria ($0.7 - 3 - x\ 10^9$) on days 0, 7 and 14 and bacteria were enumerated in the Peyer's patches on days 3, 10 and 17. The figures in brackets are the % of bacteria isolated from the Peyer's patches which were $K88^+$.

**Reciprocals of ELISA endpoint titres.

Table 6.4: Stability of K88 expression *in vivo*

Strain	Log_{10} bacterial counts in Peyer's patches (%$K88^+$)*		
	d4	d11	d18
LT2H1	4.16 ± 0.28	1.3 ± 0.2	0.5 ± 0/1
LT2H1 (pFM205)	3.90 ± 0.43 (48)	0.8 ± 0.4 (22)	0.4 ± 0.2 (5)
J754	3.64 ± 0.27 (100)	1.7 ± 0.3 (84)	0.3 ± 0.1 (73)
E. coli M30	2.62 ± 1.40	-	-

*Mice were fed bacteria (0.6–1.0 x 10^9) on d0, and bacterial colonies from the Peyer's patches enumerated at the times shown. The colonization figures are the geometric mean values of counts obtained from 4–5 mice/group, with SE values. The figures in brackets are the % of bacterial colonies which were $K88^+$.

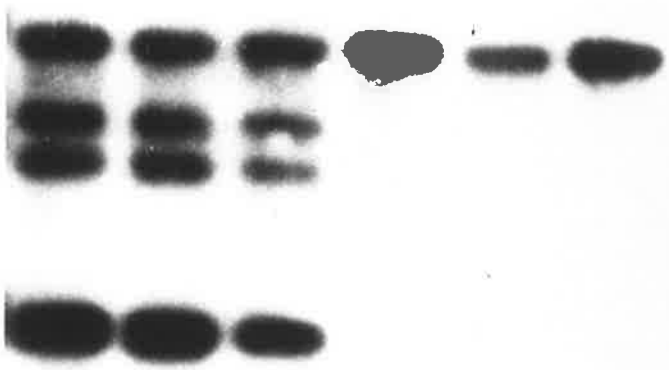
-: not done.

Figure 6.5

Some K88$^-$ revertants of the K88$^+$ recombinant, strain J754, show DNA rearrangements near *his*. Spontaneous K88$^-$ revertants of J754 were obtained *in vivo* following oral administration of the strain to mice (Table 6.4). Chromosomal DNA from these strains was digested with *Eco*RI and *Hind*III, subjected to AGE, transferred to nitrocellulose, and probed with labelled pADE177.

A-C: *his*$^+$, K88$^-$ revertants; **D-F**: *his*$^-$, K88$^-$ revertants.

The fragment sizes in tracks A-C are the same as the fragment sizes seen before (Figure 6.3, tracks C-F).



A B C D E F

Immunogenicity

Mice were immunised orally (0.7×10^9–3.0×10^9 bacteria/mouse) on days 0, 7 and 14 and serum was taken on day 20 for measurement, by ELISA, of serum IgG specific for K88 antigen. (Table 6.3). Mice immunised with LT2H1 or formalin killed J754 developed no detectable anti-K88 serum IgG response. Mice immunised with strain LT2H1(pFM205) or *E. coli* M30 developed the highest levels of serum IgG. The response to the recombinant when given orally to mice was 4-fold less than that to the plasmid–bearing strain, LT2H1(pFM205).

Vaccine potential of J754 against *Salmonella* challenge

Groups of 5–8 mice were immunised orally with 0.7×10^9–3.0×10^9 bacteria (strains M30, LT2H1, LT2H1(pFM205), or J754); 9 mice were kept aside as un–dosed controls. Twenty–eight days later all mice were challenged orally with 5×10^6 (*ca.* 100 LD_{50} values) viable *S. typhimurium* C5 bacilli. Survival was monitored for 28 days after administration of the challenge, at which time all control mice, and 5 of the 6 mice immunised with M30, had died. In contrast, no deaths were observed in the groups immunised with LT2H1 (n=5), LT2H1(pFM205) (n=7), or J754 (n=7).

6.3 Discussion

A two step system of chromosomal integration with positive selection for the integration of heterologous genes was described. The system is directed in that the site of integration is 200bp upstream of the *his* regulator region at 42 min on the *S. typhimurium* chromosome (Barnes, 1978). The first step was the construction of a deletion in the chromosomal *hisOG* locus and the second step (chromosomal integration) involved recovery of the chromosomal *hisOG* region and the simultaneous integration of the heterologous DNA fragment.

Expression of K88 fimbriae in M30 was under the control of the natural promoter whereas the expression of K88 fimbriae in the recombinant strain

J754 was controlled by a promoter derived from pBR322 (Mooi *et al.*, 1984). This promoter was included when the *Bam*H1–*Pvu*1 fragment of pFM205 was inserted into pADE171. The expression of K88 fimbriae by the recombinant strain was found to be lower than that of either of LT2H1(pFM205) or M30 (Table 6.1). SDS–PAGE confirmed that the level of expression of K88 fimbriae in the recombinant was lower than that in either M30 or LT2H1(pFM205) and immunoblotting confirmed the identity of putative K88 protein detected in this system (Fig. 6.4).

The stability of K88 expression in the recombinant (J754) was tested both *in vitro* and *in vivo*. Upon growth *in vitro* in the absence of selection for K88 plasmid, expression of K88 by LT2H1(pFM205) was inherently less stable than that observed with the recombinant strain J754 (Table 6.2). To examine whether improved stability of K88 expression by the recombinant could also be demonstrated *in vivo*, K88 expression by strains colonizing the Peyer's patches of mice was tested by comparison of the number of K88 expressing bacteria and the total number of bacteria. The initial colonization of LT2H1 was only marginally affected by the expression of K88 fimbriae (Tables 6.3 and 6.4). Some 97–100% of recombinants (J754) isolated from the Peyer's patches after the first immunization expressed K88 fimbriae. With increasing time after immunisation with $K88^+$ strains, larger numbers of $K88^-$ bacteria were isolated from the Peyer's patches of mice (Table 6.4). The proportion of recombinant bacteria (J754) that remained $K88^+$ with time was significantly greater than was found after administration of corresponding doses of the plasmid–bearing strain, LT2H1(pFM205) (Table 6.4). Recent experiments with a $K99^+$ enterotoxigenic *E. coli* strain in neonatal pigs have shown that $K99^-$ variants may be selected *in vivo* by intestinal anti–K99 immunity (Mainil *et al.*, 1987). The loss of expression of K99 fimbriae did not always result from the loss of the plasmid encoding the fimbriae (Mainil *et al.*, 1987). Mutants of recombinant strain J754 that lacked K88 expression were divided into two classes, based on their *his* phenotype. The $K88^-$, his^+ class probably arose either by point mutation or by small deletion

(<100bp). The K88$^-$, *his*$^-$ class probably arose by a deletion event, as indicated by DNA blotting (Fig. 6.5).

Porcine neonatal diarrhea was found to be the result of proliferation of certain serotypes of ETEC in the small intestine (Sojka, 1973). Such pathogenic ETEC were found to express K88 fimbriae (Sojka, 1973) or to a lesser extent other fimbrial adhesins (Gaastra and de Graaf, 1982). The adherent properties of ETEC bearing fimbriae conferred an ability upon the bacteria to proliferate in the intestine of neonatal pigs (Arbuckle, 1970; Drees and Waxler, 1970; Bertschinger *et al.*, 1972; Wilson and Hohmann, 1974). In 1978, Lingwood and co–workers demonstrated that passage of ETEC strains harboring the plasmid–encoded genes that expressed K88, in medium containing colostrum antibodies raised by vaccination of pigs with inactivated antigens (which included the K88 antigen), resulted in the loss of K88 plasmid *in vitro*. They suggested that piglets may acquire such colostral antibodies when feeding from a vaccinated sow. The presence of such passively acquired antibody in the intestine might provide protection against infection by ETEC to which the antisera was raised (Lingwood *et al.*, 1978). Smith (1972) produced evidence that supported the idea that colostral antibodies might protect neonatal pigs against ETEC–induced diarrhea. Oral administration of antisera raised against ETEC surface antigens was most effective in providing immunity against homologous ETEC infection in pigs (Smith, 1972). Neutralisation of the adhesive properties of K88 fimbriae by colostrum and milk from vaccinated sows, was shown to correlate with protection of neonatal pigs feeding on such vaccinated sows (Rutter *et al.*, 1976). These protective antibodies were found to be predominantly of the IgG class (Rutter *et al.*, 1976). However, antibodies of the IgA class have also been shown to play a role in protection (Attridge *et al.*, 1988). These investigators have demonstrated that oral immunisation of sows with live *S. typhimurium* strain G30 bearing K88 or K99 fimbriae provided neonatal pigs feeding on such vaccinated sows, with protection against homologous challenge with virulent ETEC (Attridge *et al.*, 1988).

The immunogenicity of the recombinant was tested by measuring its capacity to induce anti–K88 serum IgG in mice. Mice that had been immunised orally with strain J754 developed good serum IgG responses. Strain LT2H1(pFM205) when given orally to mice induced a better response. The enterotoxigenic *E. coli* strain M30 did not significantly colonise the Peyer's patches, however mice immunised with this strain developed anti–K88 IgG levels similar to those seen in mice immunised with strain LT2H1(pFM205). These results suggest that strain J754 might be a suitable candidate vaccine which would provide protection against K88–bearing ETEC infection in neonatal pigs.

Such recombinant strains have potential as vaccines both against K88–bearing ETEC and *Salmonellae*. We therefore sought to determine if the recombinant strain J754 retained its ability to elicit protective immunity, in mice, against subsequent challenge with virulent *S. typhimurium* C5. Mice immunised with either LT2H1, LT2H1(pFM205), or the recombinant J754 were found to be highly protected against 100 LD_{50} values, whereas unimmunised control mice or mice immunised with M30 were overcome. This result indicated that expression of K88 fimbriae by strains J754 and LT2H1(pFM205) had no detectable effect on the potential of these strains as *Salmonella* vaccines. We conclude that such strains would make suitable bivalent vaccines for control of K88–bearing ETEC and *Salmonella* infections in pigs.

It is intended that the generality of this chromosomal integration system will be improved. By replacement of the antibiotic resistance marker on pADE171 with a different resistance gene only one curing step would be required (the second plasmid would cure the first by incompatibility). A synthetic polylinker could be inserted into the *Pst*1 cleavage site of pADE171 to create multiple restriction insertion sites. The addition of a *mob* region to plasmid pADE171 would assist in the movement of large plasmids. Promoters that increase expression of the integrated genes may be inserted upstream of the integration site to improve expression of heterologous genes.

Other chromosomal integration systems have been developed. One

such system used tranposon *Tn*5 to integrate an endotoxin gene of *Bacillus thuringiensis* (deleterious to tobacco hornworms) into the chromosomes of strains of *Pseudomonas fluorescens* which naturally colonised the roots of corn (Obukowicz *et al.*, 1986). Another system was used to assay promoters once integrated into the chromosome (Vidal and Raibaund, 1985). In this system promoters were recombined into the chromosome upstream of the *malPQ* gene of *E. coli*. The strength of the promoter in a chromosomal environment was then assayed by measurement of the activity of amylomaltase, the product of the *malQ* gene (Vidal and Raibaund, 1985).

6.4 Conclusion

A system was described which is designed to integrate heterologous DNA into the chromosome of *Salmonella*, upstream of the *his* operon. The system was successfully used in the construction of a recombinant derivative of strain LT2H1 (a *galE* mutant of *S. typhimurium*). This recombinant strain (J754) stably expressed the introduced genes and was immunogenic when given orally to mice.

Chapter 7

Discussion

At this stage it is perhaps appropriate to restate both the aims and conclusions of this thesis. This will then be followed by a short discussion on the future prospects for live oral typhoid vaccines.

7.1 Aims of thesis

Strain Ty21a, a *galE* derivative of *S. typhi* Ty2, was attenuated and immunogenic when given to humans (Gilman *et al.*, 1977, Wahdan *et al.*, 1982; Levine *et al.*, 1987a). This strain was made by non–specific mutagenesis (with NG) and as a result several mutations other than *galE* were also introduced into this strain (see Introduction). Strain Ty21a was therefore difficult to grow, and viability after recovery from lyophilisation was notably reduced, as compared to Ty2. Furthermore, the undefined genetic characteristics of strain Ty21a did not allow definitive assessment of the contribution of its *galE* mutation towards attenuation in humans. The first aim of this thesis then, was to contruct defined *galE* mutants of *Salmonella* and to evaluate the effect of this mutation on virulence in animals and humans.

Modern recombinant DNA techniques have facilitated a rapid development in live oral vaccines. It has been proposed that these methods can be used to introduce heterologous DNA which encodes a protective antigen into an at-

tenuated carrier strain (Formal *et al.*, 1981; Clemens *et al.*, 1984). Such hybrid strains when given to mice can induce humoral and cellular immunity against the carrier and in addition against the pathogen from which the heterologous DNA was derived (Brown *et al.*, 1987). The stability of the foreign antigen in such carrier strains might have an important bearing on the effectiveness of this dual response (Dougan *et al.*, 1987). The possibility that plasmids might be transferred from the carrier strain into a new host has created the need for extensive characterisation of candidate vaccine strains before they can be released into the environment. Plasmid vectors which were hitherto used for the introduction of heterologous DNA into, and the maintenance of this DNA in, such carrier strains (Formal *et al.*, 1981; Clemens *et al.*, 1984; Yamamoto *et al.*, 1984; Dougan *et al.*, 1987) are known to be able to transfer to other bacteria, albeit at negligible frequencies in some cases (Murray *et al.*, 1986). Thus, the second aim of this thesis was to construct a system whereby heterologous DNA could be stably maintained in carrier strains but which avoided the use of plasmid replicons, thereby obviating the above–mentioned problems.

7.2 Conclusions

In Chapter 3 the *gal* operon of *S. typhimurium* LT2 was found to be encoded within a 4.7kb *Eco*R1–*Hind*III fragment. Transposon mutagenesis (with Tn*1725*) demonstrated that the *gal* genes were in the order *galE*—*galT*—*galK*. The promoter was located upstream of the *galE* gene and transcription proceeded in the direction of the *galK* gene (e.g.: promoter→*galE*→*galT*→*galK*). Minicell analysis facilitated the identification of the *gal* gene products and allowed calculation of their molecular weights. The molecular weight values obtained were: GalEp, 37 kDal; GalTp, 40 kDal; GalKp, 42.5 kDal. The arrangement of genes in the *gal* operon of *S. typhimurium* was seen to be similar to the genetic organisation of the *E. coli* K12 *gal* operon. The restriction endonuclease cleavage maps of *gal* DNA from *E. coli* K12 and *S. typhimurium* were not

identical, indicating absence of complete sequence homology.

In Chapter 4, a plasmid which carried a 0.4kb deletion in the *galE* gene, but which expressed gene products from the *galT* and *galK* genes, was constructed. This plasmid–borne *galE* deletion, designated *galE*-H1, was recombined into the chromosome of *S. typhimurium* strains C5 and LT2, and *S. typhi* Ty2, resulting in strains C5H1, LT2H1 and Ty2H1 respectively. These strains, when grown in the absence of exogenous galactose, were found to exhibit phenotypes characteristic of rough strains. They were resistant to O–specific phages P22 and Felix-O, sensitive to serum, and lacked smooth LPS when analysed by SDS–PAGE. These phenotypic characteristics reverted to those of the parents (serum resistant, sensitive to phage P22 and Felix-O, and demonstrative smooth LPS on SDS–PAGE), when these strains were grown in the presence of exogenous galactose. These *galE*-H1 strains were also sensitive to galactose–induced lysis at galactose concentrations as low as 60μM whereas the parent strains were unaffected by galactose concentrations as high as 100mM. Strains which carried the *galE*-H1 mutation were found to differ from strain Ty21a in that they were vigorous in growth and were tolerant to lyophilisation.

Strains C5H1 and LT2H1 were found to be avirulent in mice. Their LD_{50} values were $>10^7$ when administered by the ip route and $>10^9$ when administered by the oral route. Both of these strains were competent colonisers of the Peyer's patches of mice. Proliferation in this tissue, however, peaked on day 3 after infection, and bacterial numbers in this tissue declined thereafter. This ability to establish a limiting infection in the Peyer's patches was thought to confer upon C5H1 and LT2H1 the capacity to elicit protective immunity against challenge with virulent C5.

In Chapter 5, strain Ty2H1 was found to be only slightly attenuated when given to mice in 5% HGM. A *via* (Vi–negative) mutant of Ty2, strain Ty2Vi, was more attenuated than Ty2H1 in mice when administered under the same conditions but was not as attenuated as Ty21a. However, a *via* derivative of Ty2H1 which carried an additional rifampicin–resistance marker (for identifica-

tion purposes), strain EX462, was highly attenuated in mice when administered in 5% HGM. The two mutations in strain EX462, *galE* and *via*, were seen to act synergistically to attenuate this strain. This synergistic effect was also displayed in the poor persistence *in vivo* (in mice) and the marked serum–sensitivity, of strain EX462. Four volunteers took part in a trial to test the safety and immunogenicity of strain EX462. When volunteers ingested 7×10^8 viable organisms of strain EX462, preceded by 1g of bicarbonate, two became ill on days 4 and 5 post– immunisation. These two patients exhibited symptoms typical of typhoid fever. The humoral immune responses in the serum and intestinal juices of the four volunteers were measured. Strain EX462 had elicited high bactericidal titres and high intestinal IgA levels and thus was seen to be highly immunogenic.

The reactogenicity of strain EX462 demonstrated that a *galE, via* mutant of *S. typhi* was virulent in humans. This provided evidence that uncharacterised genetic defects might contribute to the attenuation of strain Ty21a in humans. Furthermore, the behaviour of the *S. typhi* mutants in a mouse model system was seen to be limited in predictive value for the behaviour of the strain in humans.

In Chapter 6, an integration system was constructed which facilitated the recombination of cloned fragments of DNA into the chromosome of *Salmonella* strains. This system was used to recombine the $K88_{ab}$ genes of porcine ETEC into the chromosome of strain LT2H1. The resulting recombinant strain J754 stably expressed K88 fimbriae both *in vivo* and *in vitro*. Moreover, the stability of K88 expression in J754 was significantly greater than that seen with plasmid–encoded K88 genes in strain LT2H1. Strain J754 was found to colonise Peyer's patches and to elicit serum anti–K88 IgG responses. This recombinant strain was both genetically stable and immunogenic, demonstrating that the integration system might be a useful tool in the construction of live oral bivalent vaccines.

7.3 Future development of live oral typhoid vaccines

Recombinant DNA techniques are rapidly becoming the accepted method for strain construction. This is because of two main reasons: i): strains derived by these methods are well defined genetically and ii): genetic defects constructed by these methods are more stable. Investigators who are engaged in the construction of live oral vaccines also consider the possibility that strains with a single attenuating defect might revert to virulence *in vivo* as a result of DNA transfer events. To limit the likelihood of this, strains with two chromosomally remote attenuating mutations are currently being investigated as candidate vaccine strains.

Strains 541Ty and 543Ty represented the first generation of strains with two chromosomally remote attenuating mutations (Hoiseth and Stocker, 1981; Stocker *et al.*, 1983). However, this double attenuation may have resulted in hyperattenuation of the strains, as reflected by poor anti–*S. typhi* LPS humoral immune responses manifested in volunteers who ingested these strains (Levine *et al.*, 1987b).

Curtiss and Kelly (1987) recently reported on strains of *S. typhimurium* which possessed two novel attenuating genetic defects. These mutations, Δcya and Δcrp, mapped at 83 min and 72 min on the *S. typhimurium* chromosome, respectively (Sanderson and Hurley, 1987). *In vivo* reversion of strains that possess these two mutations can only occur by Hfr transfer of large regions of chromosomal DNA (Curtiss and Kelly, 1987). Strains of *S. typhimurium* that possessed either a *cya* or a *crp* mutation, when given orally to mice, had LD_{50} values $>10^9$ (Curtiss and Kelly, 1987). When strains with the single mutations were given ip to mice, the LD_{50} value of the *cya* mutant was *ca.* 10^4, while the LD_{50} value of the *crp* mutant was 10^5–10^6. Strains with both *cya* and *crp* mutations had ip LD_{50} values in mice of 1.6×10^4–1.6×10^5 (Curtiss and Kelly, 1987). This value was increased further (to 10^5–10^6) if these mutations were

introduced into strains of *S. typhimurium* which were cured of the virulence plasmid (Curtiss and Kelly, 1987). Thus these mutants, given ip, were not as attenuated as *galE* mutants (Chapter 4).

The immunogenicity of both plasmid–containing and plasmid–cured strains, which also possessed the *cya, crp* double mutations, was investigated by assessment of the protective capacity of these strains (Curtiss and Kelly, 1987). Plasmid–containing strains with the Δcya Δcrp alleles were found to provide high levels of protection. Protection was reduced slightly when these deletion mutations were present in a plasmid–cured strain of *S. typhimurium* (Curtiss and Kelly, 1987).

Either the *cya* or the *crp* mutations alone reduced the *in vitro* growth rates of strains harbouring the mutations. The attenuating nature of these mutations was attributed to a proposed reduced capacity of strains carrying these mutations to proliferate *in vivo* (Curtiss and Kelly, 1987). However, suppressor mutations ocurred which resulted in partial restoration of virulence to *cya* mutants (Curtiss and Kelly, 1987). Curtiss and Kelly (1987) argued that the presence of the *crp* mutation, in a *cya* background, would obviate the possibility of strain reversion to increased virulence. Until the the basis of attenuation in these *S. typhimurium* strains has been further characterised it is unlikely that the *cya*, *crp* mutations will be used in the construction of attenuated strains of *S. typhi* for use in humans. The *S. typhimurium* strains, however, may become useful candidate vaccines and carrier strains for domestic animals.

As mentioned earlier, strains which possessed both *aroA* and *purA* mutations appeared to be hyperattenuated in humans (Levine *et al.*, 1987b). Stocker (1988) recommended the use of *aroA*, *aroD* double mutants of *S. typhi* as candidate vaccine strains. These two mutations are chomosomally remote but because they affect only a single biosynthetic pathway, the double mutant is expected to be not as disabled as strains 541Ty and 543Ty. The same may be said of *S. typhi* double mutants with both *purA* and *purB* mutations.

Recently O'Callaghan and co–workers (1988) reported on the effects of

either single *aroA* or *purA* mutations, or *aroA–purA* double mutations, on the virulence and immunogenicity of *S. typhimurium* in mice. When strains which possessed such mutations were given intravenously either *aroA* or *purA* were highly attenuating (O'Callaghan *et al.*, 1988). However, *aroA* mutants were more immunogenic than *purA* mutants. This capacity of *aroA* mutants to elicit high levels of protective immunity was thought to lie in their ability to undergo a limited period of proliferation in the liver and spleen (O'Callaghan *et al.*, 1988). Strains of *S. typhimurium* with *purA* mutations persisted for long periods in the liver and spleen of mice, but did not exhibit the same capacity to proliferate *in vivo* (O'Callaghan *et al.*, 1988). Thus it would seem that a strain of *S. typhi* carrying both *aroA* and *aroD* mutations is potentially a suitable candidate for a live oral anti–typhoid vaccine for humans.

The possibility that the ability of *aro* mutants to proliferate *in vivo* may be greater in humans than in mice must be considered. Retention of the capacity (of *S. typhi aro* mutants) to grow *in vivo* might result in systemic reactions in humans, and these may be potentially dangerous if they occur in malnourished children living in typhoid–endemic regions. Strains of *S. typhi* that possess *pur* mutations may be safer than *aro* strains in humans, and are therefore worth consideration as candidate live oral vaccines.

In summary, the only live oral typhoid vaccine in widespread use is strain Ty21a. However, attenuation of this strain can no longer be attributed to its *galE* mutation (Chapter 5). Further characterisation of this strain is required in order to provide unequivocal data on the nature of attenuation in this strain. Auxotrophic strains of *S. typhi* are currently being investigated for use as live oral vaccines. Strains of this type should preferably possess two chromosomally remote attenuating mutations, the effects of which are biochemically understood.

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