BACTERIAL CELL SURFACES AND PATHOGENESIS

Collected Publications 1975 - 1998

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PREFACE

In support of my candidature for the degree of Doctor of Science of the University of Adelaide I submit a thesis comprising one hundred and seventy one published works on the general theme of **Bacterial Cell Surfaces and Pathogenesis**. In general, the papers are grouped according to the bacteria which are being studied and presented in chronological order within each section. Subject to the **Statement on Authorship and Contribution**, the publications report original work undertaken by myself, alone or in collaboration, or work carried out under my supervision in my laboratory at the University of Adelaide.

This collection of papers represents my research interests over a period of more than 20 years. In this work I have sought to address important areas facing medical research by the application of bacterial genetics and molecular biology.

My research career began in 1974 as a Ph.D. student, and later as a post-doctoral fellow in 1977, by studying bacterial conjugation, a phenomenon involving the interaction of bacterial surfaces. It is the main mechanism by which multiple antibiotic resistance is being spread in Gram-negative bacteria creating bacteria that are refractory to standard therapies.

Since establishing in 1980 my own research group, which subsequently became the **Microbial Pathogenesis Unit** in 1993, a variety of bacteria have been studied. These include "old" diseases such as cholera where the causative agent was identified by Robert Koch more than a century ago but is still a serious problem, especially in developing countries, and recently emerging pathogens such as O111 Shiga-toxin producing *Escherichia coli* associated with haemorrhagic uraemic syndrome. These studies have addressed such diverse themes as gene regulation, antigenic variation, vaccine design, and the evolution of lipopolysaccharide

O-antigens. The common goal was to provide a better understanding of the mechanisms of pathogenesis of the diseases and how these pathogens have evolved such that this information might be applied to vaccine design and diagnosis.

DECLARATION

This thesis contains no material submitted for another degree or diploma in any University except where due reference is made in the **Statement on Authorship and Contribution**.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

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In addition, I would like to express especial thanks to my Ph.D. supervisor Professor Peter Reeves and to my postdoctoral collaborators Dr. Mark Achtman and Professor Kenneth Timmis. In conclusion, I would like to gratefully acknowledge Professor Derrick Rowley, who encouraged me to return to Adelaide and begin my research into *Vibrio cholerae*, and Professor Thomas F. Meyer with whom I shared a Max Planck Forschungspreis.

LIST OF PUBLICATIONS

I. Studies on *Escherichia coli* K-12.

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SUMMARY OF WORK

The work presented in this thesis encompasses two major inter-related themes, namely the study of Bacterial Cell Surfaces and the study of Pathogenesis of a number of bacterial infections. In order to put each of the papers into perspective, they will be grouped according to the organism or class thereof.

I. Studies on *Escherichia coli* K-12.

Recipient ability in Bacterial Conjugation

At the commencement of my PhD candidature the state of both functional and genetic knowledge of the outer membrane proteins of *Escherichia coli* was limited. However, a collection of bacteriophage- and colicin-resistant mutants of *Escherichia coli* K-12 was available within the laboratory of Dr. (now Professor) Peter Reeves where I was undertaking my research, but the degree of characterization of these mutants was quite variable. These strains provided a resource to begin examining the nature of the mutations, in particular, their effect on the ability of the mutant bacterium to act as a recipient in bacterial conjugation, the process by which multiple antibiotic resistance is transferred from cell to cell in Gram negative bacteria. Examination of these mutants with a variety of conjugal donor strains (F'*lac*, Hfr, R100-1 and R64-11) enabled the differentiation of the cell surface interactions with the various donors (1,6). The data also highlighted that *con* mutants had the most significant defect as recipients in conjugation with F-plasmid donors. These mutations were subsequently shown to be affecting the same gene as *tolG* and *tut* mutations described in other laboratories. This gene was shown to be the structural gene for a major outer membrane protein of *E. coli* K-12 (3,4,5). Consequently, the gene was designated *ompA* (3). These

studies also characterized the OmpA protein (also referred to as protein 3A, protein II or II*) electrophoretically providing evidence to differentiate it from other outer membrane proteins. It is worth noting, that at this time there was no generally accepted system for examining the outer membrane proteins and it was only with the advent of slab polyacrylamide gels that it became possible to unify the nomenclature of the proteins described in different laboratories (7).

Studies of proteins from the various bacteriophage-resistant mutants under different electrophoretic conditions also revealed a correlation between the presence of a particular outer membrane protein and receptor activity for bacteriophage T6 and colicin K (2). The structural gene for this protein was designated *tsx*. The Tsx protein could be purified and shown to have the receptor or neutralizing capacity for both agents (9).

As a consequence of this work, it became possible to start analyzing the actual physiological role of the major outer membrane proteins of *E. coli* K-12 (8). In particular, the panel of *ompA* mutants, which had been isolated (4), could be used to demonstrate that the presence of OmpA was critical for a variety of growth functions.

My initial work as a post-doctoral fellow in Dr. Mark Achtman's laboratory was a continuation of the studies of different classes of conjugation-defective mutants of *E. coli* K-12 (10). It was concluded that *ompA* mutants were defective in conjugation and could not form stable mating aggregates and could be used to define one of the stages of the conjugation process (151).

Donor-associated functions in conjugation

As a result of experience with outer membrane functions of *E. coli*, it was decided to initially focus on the conjugal phenomenon of surface exclusion, which is responsible for the

poor recipient-ability of conjugal donors with donors harbouring the same or related sex factor. This also prevents unnecessary conjugation between donor cells in a pure culture.

At that time, the *tra* region of the F plasmid encoding the genes involved in conjugal DNA **tra**nsfer, F-pilus biosynthesis, surface exclusion and other donor functions required for conjugation, had only recently been cloned. Analysis of the cloned genes in *E. coli* mini-cells provided a means of identifying most of the proteins encoded within the *tra* region and also procedures were developed to fractionate the mini-cells so that the actual cellular location of the proteins could be determined (12).

Complementation analysis of F'*lac* point mutants and subclones of the *tra* region of the F plasmid defined the genes, *traS* and *traT*, to be solely responsible for the phenomenon of surface exclusion (13). However, F plasmids with mutations in *traJ*, a *tra* region positive transcriptional regulator, were also defective indicating that TraJ plays a role in expression of the promoter distal cistrons *traS* and *traT*. This posed somewhat of a quandary when RNA polymerase binding studies and promoter analyses were performed. Electron microscopic visualization of *E. coli* RNA polymerase complexed to *tra* region DNA identified several strong binding sites internal to the *tra* operon (24). This implied the existence of promoters in addition to that regulated by *traJ* at the beginning of *tra* region. In particular, both *traS* and *traT* appeared to have their own promoters. Nucleotide sequence analysis of *traS* and *traT* together with analysis of this region in promoter detection vectors helped clarify matters (see below; 26). Both were shown to have constitutive promoters as well as being co-regulated by TraJ. The properties of various strains, including F-minus phenocopies, donor strains grown under conditions such that they behave like recipients, led to the conclusion that TraS is probably rapidly turned over in the cell. In addition, for TraT to function in surface exclusion, it may need to be newly synthesized, and presumably associated with zones of adhesion between the cytoplasmic and outer membranes.

Two new chromosomal genes also were shown to affect surface exclusion. The products of *sfrA* and *sfrB* are both required for surface exclusion, but function at the level of regulation (18). The product of *sfrA* is needed for efficient transcription of *traJ* whereas the *sfrB* product has a novel regulatory mechanism affecting the synthesis of a variety of different cell surface-associated components including outer membrane proteins, lipopolysaccharide and flagella besides the F pilus.

The promoter-distal region of the *tra* region was subjected to extensive genetic analysis by isolating both deletion- and insertion-derivatives of pRS31 containing this region cloned in pSC101. These were mapped by both restriction analysis and by DNA heteroduplex formation using the electron microscope. Together with identifying the plasmid-encoded proteins, this resulted in a detailed physical and functional map of the last third of the F sex factor *tra* region (11,19). Cloned regions of the closely-related conjugative plasmid R100 became available and so it was of particular interest to perform a similar analysis in order to make comparisons because they belong to different surface exclusion classes, that is, they do not exclude one another. At the same time DNA homology of the *tra* regions of both F and R100 was examined electron microscopically by DNA-DNA heteroduplexes. These studies demonstrated a high degree of conservation with the exception of the *traS*, which was shown to be responsible for the plasmid specificity of surface exclusion (20,21).

In line with predictions from the earlier studies (12,14) nucleotide sequence analysis revealed that TraS and TraT were inner and outer membrane proteins, respectively (26). TraT was also predicted to be a lipoprotein, which together with its association with peptidoglycan and high degree of surface exposure, as determined by its ability to be readily iodinated on intact cells by lactoperoxidase (14), indicates that it is a trans-outer membrane protein. The

reduced ability of OmpA to be labeled in the presence of TraT also inferred that the mechanism of action of TraT in surface exclusion was to occlude access to OmpA.

At the same time as these studies were being conducted, colleagues in Dr. (now Professor) Ken Timmis' laboratory had begun an analysis of plasmid R6-5-encoded serum resistance. Plasmid R6-5 belongs to the same incompatibility group as plasmids F and R100, and so I provided Dr. Timmis with a variety of subclones and mutants which had been constructed in the above studies (19,20). The results these studies implied that these latter plasmids also encoded serum resistance and that it was TraT that was responsible for the phenomenon. Consequently, this directed the experimental approach to R6-5 and led to the demonstration that the R6-5 TraT protein was responsible for plasmid-determined resistance to serum bactericidal activity (15,17). A series of mutants in R6-5 *traT* were isolated by hydroxylamine mutagenesis in order to begin a structure-function analysis of TraT (22). Surface-exposed TraT was shown to be essential for mediating both serum resistance and surface exclusion.

In conjunction with these studies several other plasmid-encoded functions were analyzed. It was possible to identify the F plasmid TraG protein and establish its role in conjugation (16). TraG (and TraN) was shown to have a comparable role for the donor to that of OmpA in the recipient. In particular, the cytoplasmic membrane-associated TraG protein was required for the formation of stable mating aggregates; however, it was also involved in F pilus biosynthesis. It was suggested that TraG may be part of a basal organelle essential for such functions as F pilus retraction that is necessary for converting the initial F pilus contacts into stable donor-recipient wall to wall contacts. A subsequent stage in conjugation is the DNA transfer, which has been shown to involve TraD (151). Nucleotide sequence analysis of *traD* predicted that TraD was a cytoplasmic membrane protein with features that imply it functions as the actual channel for conjugal DNA transfer (27).

Incidental to the studies on plasmid R6-5, another plasmid-encoded function was identified and characterized (25). It was apparent that R6-5 inhibited the induction of the SOS response and the induction of prophage λ. The responsible genes, designated *psiA* and *psiB*, were characterized and the sizes of their products determined.

Finally, collaborative studies with Dr. Reeves when I returned to Adelaide led to the characterization of another *E. coli* K-12 outer membrane protein associated with sensitivity to colicins (23). The TolC protein was found to form aggregates in the outer membrane, however, unlike the porin proteins, OmpC and OmpF, it did not appear to be peptidoglycan-associated.

II. Studies on enteric pathogens.

II.1. *Vibrio cholerae*

My return to the University of Adelaide in 1980 was the result of gentle persuasion by Professor Derrick Rowley to commence a molecular analysis of *Vibrio cholerae* with an aim to genetically define the protective antigens, which could be then manipulated for vaccine development (30). However, this study expanded into a long term undertaking to understand the pathogenesis of cholera. The timing was quite opportune, as little molecular work on *Vibrio cholerae* had been performed to that point, and recombinant DNA technology was just starting to be applied to studies of bacterial pathogenesis. This was occurring probably because of the awareness of rapidly increasing multiple drug resistance in a wide range of bacterial diseases, and also the perceived potential of this technology to quickly make advances in vaccine development and precisely manipulate bacterial strains. An overview of the situation at this time and how this work developed has been reviewed

(167). Initially, one of the major aims of this work was to develop a bivalent cholera/typhoid vaccine using attenuated *Salmonellae* to express protective antigens of *V. cholerae* (157). However, as studies progressed other vaccine strategies became apparent (156,160) and the mechanism of pathogenesis of cholera infections and the biosynthesis and variation of its virulence determinants became more intriguing resulting in the broadening of the themes of the research.

Cholera is an ancient disease and although the causative agent, *Vibrio cholerae*, was identified in the 19th century and named by Robert Koch in the 1880s, over 100 years of research has still not enabled us to control the disease in many parts of the world (156,158,171). In order to begin a detailed analysis of its virulence determinants and to facilitate these studies it was considered necessary to establish appropriate genetic tools.

Genetic tools in *Vibrio cholerae*

The basic means of genetic manipulations of bacteria involve transformation, conjugation and transduction. Thus, it was important to determine which of these systems could be applied to study *V. cholerae* (154, 155). Transformation involves the uptake of DNA in solution and can be limited by the presence of DNA restriction and modification systems and secreted DNases. It was possible to show that there were biotype-specific restriction and modification systems (29) which meant that even if transformation were feasible, it would be primarily between strains of the same biotype. In addition, it was possible to identify the presence of extracellular DNases (45,64,65). The structural gene, designated *dns*, for one of the DNases was cloned. The Dnase was found periplasmically trapped in *E. coli* suggesting that it needs accessory machinery in order to be secreted (45). A series of fusions between Dns and β-lactamase demonstrated that the periplasmic location was a normal step in the pathway of secretion in *V. cholerae* (65). However, the fusion

proteins could not be secreted intact but were proteolytically processed into the separate domains. Dns possesses six Cys residues that are essential and form disulphide bonds in order to produce activity. Sequential mutation of the two *V. cholerae* Dnases to produce a Dnase-less strain demonstrated that these enzymes posed a barrier to transformation (64).

As mentioned in **section I**, conjugation is mediated by plasmids and *V. cholerae* was known to possess the conjugative plasmid known as the P sex factor. The plasmids of a P factor containing strain were characterized, mapped and cloned and their distribution in other *Vibrio* isolates examined (48,49). The P plasmid was seen to be uncommon and this may have been due to its attenuating effect on strains that possessed it (61). This attenuation was shown to be due to the suppression of the critical colonization factor TCP (see below). Transposon-tagged conjugation-proficient derivatives of P were able to be isolated (48). These plasmids, such as P::Tn*3* and P::Tn*10*, were able to mobilize chromosomal genes in much the same way as Hfr strains in *E. coli* K-12 and could be similarly used for mapping (55).

Bacteriophage CP-T1 had been reported by others to mediate generalized transduction but also to be a temperate phage. Based on these proposed features a detailed analysis of the phage was undertaken (31). CP-T1 packages its DNA by a head-full mechanism and its genome is terminally redundant, but the phage could not be shown to form lysogens or be present in purported lysogens. In fact, it behaved like a virulent phage and could readily be used to isolate resistant mutants, which had LPS defects (39). Purified LPS could be shown to function as the cell surface receptor for CP-T1 and was implied as the receptor for a number of the other typing phages based upon cross-resistance patterns. The phage *pac* site at which packaging of its genome begins during phage maturation was localized and characterized (50). This has been used to construct vectors that can be

transduced at high frequency as well as a transposon derivative Tn*pac* that can be used for chromosomal mapping by producing a gradient of transduction (155).

In order to be able to perform genetic complementation experiments, it was desirable to have *recA* mutants that were defective in homologous recombination (75). The mutants were constructed by allelic exchange using the cloned *recA* gene, which was also sequenced. The mutants behaved like the wild type parent strain in the infant mouse cholera model and were thus suitable for complementation studies.

Cell envelope proteins of *Vibrio cholerae*

At the commencement of this work little was known about the composition of the cell envelope of *Vibrio cholerae* other than that it seemed to be typical for what was known about Gram negative bacteria. The presence of lipopolysaccharide was known and immunological studies implied the presence of surface proteins that were protective antigens, however, the presence of fimbriae was disputed. In addition although various haemagglutinins, presumed adhesins, had been defined, none were identified at the protein level (153).

A survey of *V. cholerae* strains revealed the presence of a number of major outer membrane proteins with the properties usually associated with porins, the non-specific diffusion channels for small hydrophobic molecules, and also identified a common 25 kDa outer membrane protein (28). This protein could be shown to be highly immunogenic and surface exposed (32) and these observations led to an interest in characterizing it at a more detailed level. Consequently, the 25-kDa protein was purified, antibodies were raised and used to isolate *E. coli* clones expressing the protein (35,53). The protein was the first *V. cholerae* outer membrane protein to be cloned and was designated OmpV. Although it was highly expressed in *V. cholerae*, it was only weakly detected in western blots of *E. coli*

harbouring the cloned gene. Nucleotide sequence analysis clearly identified features consistent with its outer membrane location, and the presence of an extensive stem-loop structure encompassing the ribosome-binding site also suggested why its expression might be poor. Indeed, preliminary studies suggested that translation was limited in *E. coli* (54). A series of fusion proteins comprising a portion of MS2 replicase fused in-frame with a set of progressively larger deletions of *ompV* was used for epitope analysis to define the location of the major B-cell epitopes within OmpV (43,54). Two major antigenic determinants were identified, one present only in the purified, and presumably denatured, form and the other in the membrane-associated form. They were distinct from one another and mutually exclusive. Although this protein was initially considered a potential component of a vaccine (46), it was subsequently dropped since it appeared to be primarily a structural protein rather than being critical for pathogenesis (157).

Whilst screening gene libraries of *V. cholerae* DNA cloned in *E. coli*, with protective sera, clones expressing a 22 kDa outer membrane protein were repeatedly isolated (34). The gene encoding this protein was subsequently sequenced and designated *ompW* (60). The protein is surface-exposed and has recently been shown to be homologous to a pilin first identified in non-O1 *V. cholerae*.

V. cholerae also contains a major structural protein similar to the OmpA protein of *E. coli* K-12 (44). This could be inferred from a series of analyses of the electrophoretic behavior, trypsin sensitivity, cross-reactivity with an anti-serum to *E. coli* OmpA, and by using a probe to the C-terminus of the *Serratia marcescens ompA* gene.

El Tor haemolysin and associated genes

V. cholerae O1 exists as two biotypes designated classical and El Tor based upon a number of biochemical differences, including the ability of El Tor strains only to produce a

soluble haemolysin for sheep erythrocytes. It was this genetic variability and the potential for the haemolysin to be an accessory virulence determinant that prompted investigation. An El Tor chromosomal fragment expressing the haemolytic activity was cloned in *E. coli* and shown to encode the structural gene, *hlyA*, for the haemolysin along with two accessory proteins, *hlyB* and *hlyC*, which appeared to be also involved in the activity (33). The haemolysin was exported to the periplasm but could not be secreted from *E. coli,* except in the periplasmic-leaky *tolA* or *tolB* mutants that could mimic the secretion process (37,52).

The *hly* genes appeared to be conserved in both O1 and non-O1 *V. cholerae* including the non-haemolytic classical strains (38,52). Nucleotide sequence analysis of the *hly* region from both El Tor strain O17 and classical strain 569B revealed that 569B had an 11 bp deletion within the structural gene, *hlyA*, for the haemolysin leading to a truncated non-haemolytic protein referred to as HlyA* (51). HlyA was synthesized as an 82 kDa precursor with an N-terminal signal sequence, and after secretion was cleaved into two peptides of 65 kDa and 15 kDa, whereas HlyA* remained as 27 kDa. The 65-kDa protein is haemolytically active. A probe was developed based upon the nucleotide sequence data and essentially covered the region deleted within the 569B *hlyA* gene (58). A wide variety of classical and El Tor along with a range of other strains were examined with the probe. The results suggested that the particular deletion was a common feature of classical strains, and implied that the probe could be used to differentiate the two biotypes.

A number of genes linked to *hlyA* have also been characterized. Immediately downstream lies *hlyB,* which was shown to affect the production of HlyA at certain stages of growth (59). It is now known to encode a chemotactic transducer and may indirectly affect expression of *hlyA* since it is complex and sensitive to various environmental influences (63,73,162). Studies on the expression of *hlyA* revealed that the mRNA contained a long 5'-untranslated region placing the promoter 430 nt upstream of the initiation codon (63).

Cloning of this region in promoter-detection vectors revealed that it was not functional in *E. coli* and implied the presence of a regulatory protein in *V. cholerae*. This provided a strategy for cloning the regulator and yielded *hlyU*, which was shown to encode a transcriptional activator (63). HlyU is a small helix-turn-helix containing regulatory protein, which belongs to a family of regulators that interact with heavy metals. Introduction of *hlyU* into *V. cholerae* on a high copy number plasmid led to massive up-regulation of *hlyA* and also facilitated the identification of another gene also under HlyU control and designated *hcp* (for **h**aemolysin **c**o-regulated **p**rotein) encoding a novel secreted protein (84). Fortuitously, adjacent to *hlyU* is another regulatory gene designated *nhaR* based upon its similarity to the regulator (NhaR) of a sodium/proton antiporter, NhaA, in *E. coli* K-12 (97). The *V. cholerae nhaR* can functionally replace its homologue from *E. coli*.

The role of the El Tor haemolysin in pathogenesis was examined by constructing defined mutants in *hlyA*. These were evaluated in the infant mouse cholera model and in ligated rabbit ileal loops together with the effect of culture supernatants on cultured cell lines (62). Comparison of the effects of HlyA and HlyA* suggested that the haemolytic activity resided in the C-terminus. In addition, *hlyA* mutants were attenuated and HlyA appeared responsible for at least part of the residual diarrhea seen in toxin-deleted strains. When *hlyU* mutants were examined in the infant mouse model, they were found to be more attenuated than *hlyA* mutants suggesting that additional factors associated with virulence were also under HlyU control (73). However, this was not due to Hcp since mutant strains, in which both copies of *hcp* had been inactivated, displayed no reduction in virulence or colonization, implying that additional unidentified factor(s) was involved (84).

Re-examination of the region encoding the proposed *hlyC* revealed a sequencing error that, when corrected, produced an open reading frame that extended out of the cloned DNA (94). By chromosomal walking, via inverse PCR, the region downstream was cloned

and the additional open reading frames characterized. This revealed three genes designated *lipA* (corresponding in part to *hlyC*), *lipB* and *prtV*. *lipA* and *lipB* comprise an operon encoding an extracellular lipase and its activator, respectively. *prtV* encodes an extracellular metallo-protease. The genes are arranged end to end such that they share a common transcriptional terminator, and together with the other genes, the whole region is suggestive of a pathogenicity island encoding proteins capable of damaging eukaryotic cells and/or extracellular proteins involved in nutrient acquisition. However, mutants with mutations in these genes were unaffected in virulence possibly because of the multiplicity of these extracellular enzymes (unpublished data).

Lipopolysaccharide (LPS) and O-antigen biosynthesis

V. cholerae exists as more than 150 serotypes and it is the O1 serotype that has been associated with epidemic cholera. These serotypes correspond to different cell surface polysaccharides and in most cases this is the O-antigen of the lipopolysaccharide (LPS). The O1 serotype can be subdivided into Inaba, Ogawa and Hikojima based upon the relative expression of three epitopes A, B and C. Inaba express A and C, Ogawa express A, B and small amounts of C, and Hikojima express A, B and higher levels of C. The regions of *the V. cholerae* chromosome encoding the Inaba and Ogawa serotypes were cloned from the respective *V. cholerae* strains, effectively converting *E. coli* to the corresponding serotype (41). This was the first demonstration of cloning the genes for an entire *rfb* region, encoding O-antigen biosynthesis, into a heterologous host. In addition, since the O-antigen was the only *V. cholerae* antigen expressed in the recombinant *E. coli*, this provided a basis for evaluating its capacity as a protective antigen. It was unequivocally demonstrated that antibodies to the Inaba and Ogawa O-antigens were protective and could cross-protect, inferring their utility in vaccine production (46). To assess this, a set of vectors based upon

the *thyA* gene of *E. coli* were constructed and used for generating hybrid strains expressing the *V. cholerae* O-antigens suitable for use as vaccines (66).

Comparison of the cloned *rfb* DNA showed that essentially the same region was cloned from both Inaba and Ogawa suggesting that the differences, which accounted for serotype specificity, were subtle. This was confirmed by detailed restriction analysis and constructing a physical map of the *rfb* regions (encoding O-antigen biosynthesis) from the Inaba and Ogawa cloned DNA (47). It was also possible to define that the minimal required region was contained within a 20 kb *Sac*I (=*Sst*I) fragment.

By simultaneously selecting for resistance to either bacteriophage VcII or CP-T1, both of which use the O-antigen of the LPS as receptor, and transposon-encoded antibiotic resistance, it was possible to isolate transposon insertions in the *rfb* region (55). This enabled the isolation of Tn*5* and Tn*2680* insertion mutations which could be mapped to the *rfb* DNA, and to the chromosomal locus previously described as *oag* associated with serotype specificity.

The *V. cholerae* O-antigen was assembled on the *E. coli* K-12 lipid A + core oligosaccharide (41). Thus, by using a set of *E. coli* K-12 strains with sequential defects in the core oligosaccharide, it was possible to define the minimal requirements for linking the *V. cholerae* O-antigen to the *E. coli* LPS (67). This demonstrated that only sugars from the outer core could be eliminated without affecting the ligation reaction. A heptose-less LPS remained totally unsubstituted. It was assumed that this reflected a similar requirement within *V. cholerae* for attachment of the O-antigen to the homologous LPS core oligosaccharide.

The nucleotide sequences of the entire 20 kb *Sac*I fragments from both Inaba and Ogawa strains were determined and compared (68). Very little sequence variation was detected even though the strains, from which the cloned DNA came, were isolated more than

30 years apart and were of different biotype as well as serotype. One change that came under suspicion was a substitution in *rfbT* leading to a truncated protein in the Inaba sequence. Further examination of other *rfbT* genes and constructing a specific mutation in *rfbT* confirmed that its product was essential for Ogawa-specificity and that Inaba strains have defects in *rfbT* leading to a non-functional protein. This provided an explanation for inter-conversion between the Inaba and Ogawa serotypes in *V. cholerae* O1; Inaba strains can be obtained by a variety of possible mutations in *rfbT*, however, the converse event requires precise reversion of the specific mutation.

Analysis of the nucleotide sequence revealed that the *rfaD* gene, associated with synthesis of the first heptose within the LPS core oligosaccharide, was linked to the *rfb* genes (78). However, unlike *E. coli*, it was not possible to isolate mutations in *rfaD* suggesting that it is an essential function.

The *V. cholerae rfb* region could be subdivided into clusters of genes corresponding to particular functional pathways (161). At the promoter proximal end is a set of four genes, *rfbA*, *rfbB*, *rfbD* and *rfbE*, predicted to encode the biosynthesis of perosamine which makes up the backbone of the O-antigen (81). The pathway is based upon the similarity of the encoded proteins to those involved in alginate biosynthesis and to an enzyme involved in the synthesis of a related molecule, desosamine. Located centrally in the gene cluster is a pair of genes, *rfbH* and *rfbI*, encoding the proteins involved in O-antigen transport across the cytoplasmic membrane and corresponding to the transport channel and its ATP-binding energizer, respectively (79). At the promoter distal end are the genes encoding the pathway for synthesis of 3-deoxy-L-*glycero*-tetronic acid and its coupling onto perosamine to complete the O-antigen subunit (80). The product of the *rfbN* gene is particularly interesting as it shows marked similarity to LuxC and LuxE involved in bioluminescence in *Vibrio*

harveyi. RfbN would appear to be the product of fusion of the genes at the opposite ends of the *lux* operon after deletion of the intervening genes.

In 1992, an epidemic of cholera commenced in the vicinity of the Bay of Bengal but the causative agent was a non-O1 *V. cholerae* assigned the new serotype of O139. Initial analyses demonstrated that the LPS was different in its electrophoretic behavior compared to O1 LPS and that all of the *rfb* genes were deleted with the exception of a region corresponding to the insertion sequence IS*1358* (83,88,161). However, whereas O1 strains contain a defective form of IS*1358* in which the transposase gene, *tnpA,* has accumulated a variety of mutations, O139 strains have an intact *tnpA*. Transposon insertion mutagenesis confirmed that the genes responsible for the new serotype are linked to IS*1358* (83). Nucleotide sequence analysis of the region around IS*1358* in O139 reveals a number of direct and inverted repeats suggestive of the remnants of transposition and recombination events that were involved in generating the new serotype (88).

The novel DNA encoding the O139 serotype specificity was cloned and sequenced in several laboratories, and this laboratory was responsible for generating a physical map of the 35 kb region and for determining the sequence of about half of the region (88,92). This confirmed that the O139 DNA was homologous to genes associated with LPS or capsular polysaccharide biosynthesis, however, unlike the O1 *rfb* region, the genes were not present in functional clusters but rather scattered within the region. Such an arrangement is suggestive of genes from a variety of sources being brought together by a series of recombination events. The data also revealed that the chromosomal location of the *rfb* genes, namely linkage to the *rfaD* gene, was conserved. That is, the O139 serotype was generated essentially by the precise replacement of the genes determining the synthesis of the surface polysaccharide.

As a consequence of characterizing the end points of the O139 *rfb* DNA, a re-evaluation of the O1 *rfb* region was performed (91). This demonstrated that an additional region of O1 DNA beyond that contained within the 20 kb *Sac*I fragment (see above) was not present in O139. This led to the identification of additional genes that were required for O-antigen biosynthesis in *V. cholerae* O1 but which could be compensated for in a heterologous host such as *E. coli* K-12.

In various studies, mutants with different defects in O-antigen biosynthesis have been isolated (39,55,68,83). These mutants were severely attenuated in the infant mouse cholera model both in LD_{50} and also in competition experiments (96,159). This reduction in virulence could be attributed to a number of phenotypic effects, which are the result of a defective LPS. The mutants showed an altered major outer membrane protein composition corresponding to changes in the relative amounts of the porin proteins. In addition, they showed reduced motility and did not have TCP on their surface but accumulated TcpA subunits intracellularly, indicating a defect in pilus assembly. This was different to the defect seen with *tcpT* mutants (90,93, see below). In particular, TcpA subunits were processed and had taken up their mature configuration but were unable to be translocated to the cell surface. Preliminary studies suggested that the defect might be an indirect effect on either of the outer membrane proteins TcpC or TcpF.

The toxin co-regulated pilus (TCP)

Earlier studies had shown that *V. cholerae* possessed non-LPS protective antigens. Thus, in order to clone the genes for these antigens, a highly absorbed protective antiserum was used to screen a gene library (56). This was accomplished by constructing the library, in *E. coli* using a mobilizable plasmid vector, that was then conjugated into a *V. cholerae* strain lacking this protective activity, and screening with the antiserum in a colony

hybridization. The advantage of this approach is that if the genes or the protective antigen required *V. cholerae* specific regulatory proteins, then they would then be available to ensure gene expression. By this means it was possible to isolate clones expressing the *tcp* gene cluster encoding the toxin co-regulated pilus or TCP (56). Studies with these clones provided a means to demonstrate unequivocally that TCP was able to enhance the virulence of a TCP-negative strain and that it is a protective antigen.

Nucleotide sequence analysis of the *tcp* region revealed that the major structural subunit of TCP, TcpA, belonged to the type 4 family of pilins found in a number of other pathogenic bacteria (57,159). Since all of the genes required for TCP are clustered, it has been argued that it provides a good model for studying type-4 pilus biogenesis (163,168,169). The genes within the *tcp* cluster are present as several transcriptional units but there are complex interactions between their promoters and the *toxRS* regulon (71,82,86). In addition, a further regulator, ToxT (initially called TcpN, 69) is encoded within this region. ToxT is a member of the AraC family of transcriptional regulatory proteins and is required for activation of *tcp* transcription. However, this is complicated by the observations that the product of *tcpP* is also able to activate transcription of its own promoter and that of *tcpA* (82). Thus, the production of TCP would appear to be mediated via a cascade of regulatory events.

The functions of few of the Tcp proteins have been defined, but TcpC has the features of a surface-anchored outer membrane lipoprotein and is proposed to be part of the basal structure that holds the pilus on the cell surface (70). Based upon a variety of computer predictions, TcpF is thought to form an outer membrane channel, possibly the means by which the TcpA subunits are extruded to form the pilus (71,163,169).

Comparison of the *tcp* region from classical and El Tor biotypes revealed a high degree of conservation with the exception of the regulatory sequences between *tcpI* and *tcpP*, and between *tcpH* and *tcpA*, and in the sequence of *tcpA* (86). The differences in the

regulatory sequences probably account for the difference in regulation of TCP in classical and El Tor strains (82). Comparison of *tcpA* from a broader range of strains showed that the predicted amino acid sequences of TcpA were conserved within the biotypes but were significantly different from each other at the C-termini (76). Since this part of the protein has been proposed by others to constitute the major epitopes recognized in vivo, this implied that TcpA mediated antibody protection would also be biotype-specific.

Although it was clear that TCP is a critical colonization factor for classical strains (159) there was some dispute over the role of TCP in the pathogenesis of El Tor cholera. This was in part due to the differences in regulation of the *tcp* region between classical and El Tor strains, and the inability to demonstrate TCP expression in El Tor strains (74,86). The *tcpA* gene was inactivated, and with it TCP expression, by allelic exchange with the insertion of a kanamycin-resistance cartridge in classical and El Tor strains. The strains were evaluated in the infant mouse cholera model and confirmed that TCP is a critical virulence factor for both biotypes (74). The mutations in these strains could have polarity on other genes within the *tcpA-tcpT* operon; consequently in-frame deletion mutants in *tcpA* were constructed (85). Comparison of these mutants with their isogenic parent strains in *in vivo* competition experiments indicated that the deletion in *tcpA* led to a defect in colonization in both biotypes. El Tor TcpA-specific antibodies were also shown to passively protect against cholera in the infant mouse model but this protection was specific for TCP of the homologous biotype. Thus, the differences at the amino acid sequence level (76,86) reflect the biotype-specific protective epitopes indicating that both pilin types would need to be present in a vaccine to provide a broad spectrum immunity to cholera (85). These studies also confirmed that TCP in O139 strains was of the El Tor biotype.

At the same time as these studies were being conducted there was controversy over the role of the mannose-sensitive haemagglutinin pili (MSHA) in El Tor pathogenesis.

Others had proposed that MSHA provided a comparable function for El Tor strains in colonization as TCP in classical strains. Thus, to resolve this issue mutants in *mshA,* the major structural subunit of MSHA, were isolated in both O1 El Tor and O139 strains and compared with *tcpA* mutants in the infant mouse cholera model (87). Not only were the *mshA* mutants unaffected in their virulence, but antibodies to MSHA were not protective whereas antibodies to El Tor TCP were highly protective for both El Tor and O139 strains.

The TcpT protein was chosen to begin a study of the roles of the individual Tcp proteins in TCP biogenesis. TcpT is proposed to be an ATP-binding protein that energizes the translocation or assembly process (163,169). Mutants in *tcpT* are unable to assemble TCP but the defect is at an earlier stage than that characterized in *rfb* (O-antigen biosynthesis) mutants. TcpT is required for the translocation of TcpA out of the cytoplasmic membrane into pre-assembly complexes. Thus, TcpA is processed by TcpJ but has not taken up its mature configuration, clearly differentiating this stage in biogenesis to that affected by *rfb* mutants.

The mannose-fucose resistant haemagglutinin and linked genes

In order to identify potential adhesins of *V. cholerae*, a gene library in *E. coli* was screened for the ability of the clones to mediate haemagglutination (159). A clone expressing mannose-fucose resistant haemagglutination of mouse erythrocytes was obtained and the region of DNA encoding the responsible genes, *mrhA,B*, was defined by deletion mutagenesis (40). This region was sequenced and a mutant was constructed by allelic exchange (72). This mutant showed a marked defect in virulence in the infant mouse model and in competition experiments suggesting that it plays a role in colonization, however, the exact roles of *mrhA* and *mrhB* have yet to be fully elucidated. MrhA does not have any homologues in the

databases whereas MrhB is homologous to DNA-methylases suggesting that it may have a global regulatory function.

Further sequencing of the recombinant plasmid encoding the *mrhA,B* region revealed an unusual arrangement of the genes (77). Each of the genes, or in some cases pairs of genes, is flanked by a copy of a 124-bp direct repeat sequence, designated VCR for *V. cholerae* repeat. By Southern hybridization, the VCR-linked genes were localized to a single region of the genome and quantitative densitometry indicates that there are 60-100 copies of VCR. A closer analysis of the features of the region suggests that it is part of a large chromosomal integron serving as a site for the acquisition of new genes by *V. cholerae* (95). Unlike previously described integrons, the VCR region is chromosomal rather than plasmid localized, it has genes other than those encoding antibiotic resistance, contains more than 10-times as many genes and it contains multiple copies of some genes. One of such genes is *vlpA*, which encodes a lipocalin belonging to the eukaryotic α_2-microglobulin superfamily (89). VlpA is a surface-anchored lipoprotein proposed to be involved in the transport of a small hydrophobic substrate. The number of copies of *vlpA* varies between 2 and 5 in different strains as shown by Southern analysis.

II.2 Pathogenic *Escherichia coli* : ETEC

Enterotoxigenic *Escherichia coli* (ETEC) produce a diarrhoeal disease similar to that of cholera, however, the bacteria are thought to be simpler in terms of the virulence determinants produced. They express fimbrial adhesins and toxins, either heat-stable (ST) or a cholera-toxin like heat labile toxin (LT). It has been shown that fimbriae are critical determinants for colonization of porcine ETEC. Consequently, it was the cloned genes for the K88 porcine ETEC fimbriae that were used to construct a hybrid strain to evaluate attenuated Salmonellae as potential vaccine strains (98). This construct was evaluated and

shown to mediate high levels of antibodies suggesting that this approach would have merit. As a result, the cloning of the genes for fimbriae of human ETEC was addressed.

Human ETEC belonging to the CFA/II class produce two of three adhesins, either CS1 and CS3 or CS2 and CS3. Thus, the genes for the common fimbrial type, CS3, were cloned and restriction analysis of the set of overlapping clones enabled a partial physical map of the large ETEC plasmid to be constructed and implied that CS3 synthesis was encoded on a 4.7 kb *Hin*dIII fragment (99). Transposon insertion and deletion mutagenesis of the cloned region implied that almost this entire *Hin*dIII fragment was required for CS3 expression and that at least 4 proteins were involved. This fragment has been sequenced and the genes subjected to a variety of site-directed mutagenesis techniques in order to confirm the genetic organization and aid in predicting the functions of the encoded-proteins (105). A novel mechanism of gene regulation was identified in which the presence of an internal amber (stop) codon controls the level of expression of at least one of the proteins. This stop codon must be read through in order for CS3 synthesis to occur. In addition, it appears that translational reinitiation occurs within some of the open reading frames to produce further proteins which consequently totally overlap with larger ones.

The genetic organization of the *cstA-H* genes encoding CS3 biosynthesis is quite compact and can be divided into two parts – the fimbrial subunit and the assembly genes. This has facilitated using CS3 as a vector for expressing foreign antigenic epitopes with potential for vaccine development (110). Epitope analysis of the major subunit of CS3, CstH, has been performed and defined several exposed domains that have been evaluated for their permissiveness for insertion. These domains have been used to express epitopes from LT and the whole of mature ST. These epitopes are presented on CstH and the hybrid proteins can be assembled into fimbriae. These epitopes are immunogenic and provide a basis for vaccination against ETEC in humans.

A survey was conducted of ETEC amongst aborigines with acute watery diarrhoea presenting at Alice Springs Hospital in Central Australia (101). During the survey period two major outbreaks of particular ETEC occurred as well as sporadic cases due to a variety of strains. One of these outbreaks was due to an O126 serotype producing CFA/I fimbriae. The other was due to an O115:H40 strain producing what was initially thought to be a new colonization factor but was subsequently shown to be PCF8775 (now referred to as CFA/IV) (100). Like CFA/II, CFA/IV strains produce more than one fimbrial type corresponding to combinations of CS4, CS5 and CS6. CS5 fimbriae were purified from the O115:H40 strain and used for amino-terminal sequencing and also raising a specific antiserum, which was then used for selecting *E. coli* K-12 clones expressing CS5 (104). The N-terminal sequence data was used to predict the DNA sequence and oligodeoxyribonucleic acid probes were synthesized and used in Southern hybridization to localize the fragments containing the structural gene for the major subunit. The nucleotide sequence of this region has been determined and the major subunit shows considerable similarity to that of F41 porcine ETEC fimbriae.

Studies with animal ETEC were being performed as it was anticipated that an ETEC vaccine could be commercially viable. Whilst cloning the genes for K99 from a strain of the O101 serotype, it was possible to identify a clone efficiently expressing the O101 O-antigen (102). A physical map of the cloned *rfb* region was constructed and deletion and insertion mutants enabled the minimum-coding region to be defined. It could also be shown that the region appeared to be highly conserved in other O101 isolates. The product of one of the genes in this region encoded an 80 kDa protein which, although not required for O-antigen biosynthesis *per se*, was needed for an O-antigen of the correct serotype specificity and electrophoretic mobility (109). The nature of this function has not been elucidated, but is novel for O-antigen biosynthesis.

Subclones of part of the DNA either contained in, or immediately adjacent to, the O101 *rfb* region seemed to be highly conserved and were used to assess their ability to differentiate strains that were thought to be clonally related (103). A set of O2 and O18 pathogenic *E. coli* strains were compared for restriction fragment length polymorphisms (RFLP). The results revealed that the strains could be readily and reliably grouped into the same classification as had been proposed by the laboratory of Dr. Mark Achtman using a considerably more extensive study involving outer membrane profiles, LPS patterns, and a battery of multi-locus isoenzyme analyses. As an aside, the *E. coli* O2 *rfb* region was also cloned from one of the strains and characterized so that additional comparisons could be made (107).

The Central Australian survey of ETEC isolates identified several serotypes and patterns of haemagglutination that had not been previously reported (101). An LT+ O9:H$^-$ strain was shown, on CFA agar, to produce luxuriant fimbriae, which could be purified and had the unusually large subunit size of 27 kDa. N-terminal sequencing showed the subunit to have sequence similarity to other known fimbrial subunits, but it was clearly unique. The fimbriae were designated PCFO9 (**p**utative **c**olonization **f**actor from serotype **O9**) and the genes coding for their biosynthesis were cloned.

II.3 Pathogenic *Escherichia coli* : STEC

Shiga-**t**oxigenic ***Escherichia coli*** (STEC) also referred to as **V**ero-**t**oxigenic ***E. coli*** (VTEC), **S**higa-**l**ike **t**oxin-producing ***E. coli*** (SLTEC) or **e**nterohaemorrhagic ***E. coli*** (EHEC) associated with diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP) produce cytotoxins related to Shiga toxin of *Shigella dysenteriae*. As part of a general survey on the incidence of STEC, a strain of the OX3:H21 serotype was isolated from a case of SIDS (sudden infant death syndrome) (111).

Using specific DNA probes to the Shiga-toxin variant genes, it was possible to identify a clone expressing Vero cell cytotoxicity. Nucleotide sequence analysis showed that the sequence of the Shiga-like toxin was related to SLT-II but distinct from previously reported sequences.

Faecal isolates of *E. coli* from a child with HUS were screened for the presence of SLT-encoding genes (112). This identified a strain belonging to the O111:H- serotype, however, unlike previous SLT-I and SLT-II producing strains, it was not possible to induce a phage encoding the genes. But when the genes were cloned from the chromosome and sequenced, the genes were found linked to both an insertion sequence (95% homologous to IS*629* of *Shigella sonnei*) and bacteriophage λ-related DNA. This suggested that the genes were indeed phage-encoded, but that the phage was defective perhaps due to the insertion sequence. These SLT genes were very closely related to Shiga toxin (SH) of *Shigella dysenteriae* type 1.

Both the OX3:H21 and O111:H- strains described above were shown by Southern hybridization to have a second set of SLT-related genes (115). Consequently, it was decided to isolate the additional genes from these strains. Nucleotide sequence analysis showed the genes to be SLT-II variants and identified further variations compared to the published sequences. This variation provoked a comparison of the relative toxicity of the toxins encoded by different SLT-II operons as well as hybrids generated by combining heterologous A- and B-subunit coding regions (117). These studies revealed marked differences in toxicity and virulence in the combinations. In particular, two residues within the A–subunit were necessary for maximal oral virulence in the streptomycin-treated mouse model (118).

The availability of these various SLT gene sequences provided a basis for developing a polymerase chain reaction (PCR) detection method for the rapid identification of SLT genes directly from primary faecal cultures (116). This method was able to identify less than 10

organisms per ml of culture, and served as a crucial basis for screening isolates of a food-borne outbreak due to *E. coli* O111 STEC (Paton, A.W., Ratcliff, R., Doyle, R.M., Seymour-Murray, J, Davos, D., Lanser, J.A. and Paton, J.C.: Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry-fermented sausage contaminated with Shiga-like toxin producing *Escherichia coli*. Journal of Clinical Microbiology 34, 1622-1627, 1996).

Considerable emphasis has been given to the toxins produced by STEC; however, much less information was available on other pathogenic determinants. The above studies provided a useful set of strains for making comparisons in order to identify common features amongst pathogenic isolates versus the co-incidental isolates (119). Analysis of adherence of the bacteria to the Henle 407 intestinal cell line demonstrated that those isolates associated with diarrhoea adhered to higher levels than animal STEC or STEC that occurred in the meat but were not isolated from patients. Confocal microscopy revealed different patterns of adherence to the Henle 407 cells. For example, enteropathogenic O111 *E. coli* (non-STEC, EPEC) showed a localized pattern suggestive of co-operative binding, whereas the diarrhoea/HUS associated STEC strains usually appeared as single adherent cells. Considerable deposition of actin was detected underlying the sites of adherence of the EPEC strain whereas no obvious actin polymerization was observed below the STEC.

All patients in the outbreak mentioned above also produced a strong antibody response to the LPS (120,121). Antibodies to O111 and O157 LPS were evaluated for their ability to inhibit adherence of O111 and O157 STEC to Henle 407 cells. The antibodies inhibited adherence of STEC strains of the homologous but not heterologous serotype, however, pre-incubation with purified LPS itself did not, suggesting that LPS is not involved in adhesion but that the antibodies are effecting a steric inhibition. In any case, these results imply that antibodies to homologous LPS may be therapeutic.

In order to evaluate if there are other surface-associated pathogenic determinants that are recognized by the immune system during the course of a HUS infection, convalescent phase sera were used to identify the corresponding reactive proteins (121). The sera were also used to identify recombinant clones expressing these protein antigens. One of the immuno-reactive proteins was sequenced and shown to correspond to intimin, a product of the *eaeA* gene, however, the O111 intimin showed significant divergence from that previously described for O157:H- STEC especially at the C-terminal end of the protein (122). This region appeared to contain important epitopes since antibodies, which recognized the O111 intimin, did not recognize the corresponding from O157 strains. This antigenic variability suggests that intimin would not be a good antigen in vaccines against STEC. Linked to the *eaeA* gene is the gene for its receptor that is actually transferred to the eukaryotic cell to which intimin binds. This gene is designated *tir* for translocated intimin receptor. Analysis of the deduced amino acid sequences of Tir from O26, O11 and O157 STEC strains revealed considerable heterogeneity. Tir reacts with the convalescent phase sera from HUS patients. It seems that there is co-evolution of intimin and its receptor and this may be a means whereby STEC can avoid host immune responses without compromising the adhesin-receptor interaction.

Certain pathogenic *E. coli* strains have been shown to produce haemolysis of erythrocytes due to the presence of entero-haemolysins (Ehly). This activity is most frequently expressed by STEC and a recombinant clone expressing Ehly1 was isolated from an O26 strain. This clone was extensively characterized, both genetically and also the encoded proteins, and the region responsible for the entero-haemolytic activity was localized (113). This activity was associated with a 60 kDa protein, however, nucleotide sequence of the cloned DNA revealed an open reading frame whose length was sufficient to encode a protein of only 29.6 kDa suggesting that it must dimerize to be active. Probing of a variety of

entero-haemolytic strains showed that only about 10% of strains hybridized with the probe, suggesting the existence of multiple entero-haemolysins.

The production of Ehly2 was found associated with temperate phages related to bacteriophage λ (114). Part of the Ehly2-encoding region that was able to be used as an Ehly2-specific DNA probe reacted with about half of the entero-haemolytic strains and differentiated between Ehly1- and Ehly2-producing strains.

II.4 Salmonella

The worldwide incidence of non-typhoid salmonellosis in humans has been rapidly increasing in recent years, disseminated by contaminated food. Analysis of plasmids in isolates associated with an outbreak mediated by *S. typhimurium* revealed the presence of a distinctive restriction and modification system in the outbreak strain along with a small plasmid designated pIMVS1 (123). This plasmid was shown to be a stable epidemiological marker. Nucleotide sequence analysis showed that pIMVS1 is essentially cryptic and homologous to plasmids such as p15A and has a mobilization origin similar to ColE1. These features probably account for its stability and make it potentially useful as a vector for acquiring genes within Salmonella and facilitating their dispersion.

S. sofia was showing a marked increase in incidence in poultry in Australia without a similar increase in humans, whereas it was being commonly isolated in both humans and poultry in Israel (124). This posed the question as to whether there is a difference in virulence for humans between the organisms in the two countries and whether there are any markers that can be used to differentiate the strains. A small plasmid was found in Australian isolates but not in those from Israel could be used as an epidemiological marker. However, random cosmid clones used in Southern hybridization for RFLP analyses provided an additional level of discrimination.

S. enteriditis strain 11RX has been used as a model for studying the role of T cells in immunity to intracellular bacterial parasites. These studies have identified a low molecular weight (14k-18k) protein as an important T-cell stimulating antigen. A 14-kDa antigen with these properties was purified from 11RX and N-terminal sequencing showed almost complete identity with the mature subunit of SEF14 pili (126). A panel of monoclonal antibodies to the 14-kDa antigen recognized SEF14 fimbrial subunits in a Western blot and also highlighted the pili in immuno-gold electron microscopy. These monoclonal antibodies were used to identify recombinant *E. coli* clones expressing SEF14 (127). The *sefA* gene was localized and sequenced and the information used to construct specific *sefA* mutants by allelic exchange of *S. enteriditis* 11RX and a highly virulent strain. These mutants were used to evaluate the role of SEF14 in colonization and invasion of the intestinal epithelium in mice. It was possible to demonstrate that SEF14 was important for colonization by 11RX, however, this effect was masked by other determinants in the highly virulent strain. 11RX was also more readily cleared from the livers and spleens of the mice, although their growth intracellularly was unaffected.

II.5 Shigella

Shigellae have no known reservoir other than humans and are responsible for bacillary dysentery in both developing and developed countries. Studies on immunity to shigellosis indicate that protection is serotype specific suggesting a role for the O-antigen of the LPS. As a consequence of successfully cloning *rfb* regions from *V. cholerae* and enterotoxigenic *E. coli*, it was decided to begin an analysis of *rfb* regions of *Shigella.*

The *rfb* region was cloned from a *Sh. flexneri* serotype 2a strain and shown to encode the Y serotype specificity (128). This was as predicted since it is thought that temperate phages encode the various serotype specific modifications. [The O-antigen subunit is a

tetrasaccharide composed of GlcNAc-Rha-Rha-Rha, and acetylation and/or glucosylation of these residues (165) generate the serotype specificities.] A physical map of the *rfb* region was constructed and comparison of different cosmid clones as well as deletion derivatives identified a function that controls O-antigen chain length and subsequently designated as Rol (**r**egulation of **O**-antigen chain **l**ength). It could also be shown by Southern hybridization that the *rfb* region was conserved in all *Sh. flexneri* serotypes with the exception of type 6. This could be further confirmed when the *rfb* region encoding the type 6 specificity was cloned (130). The type 6 *rfb* region is essentially unrelated to that found in the other serotypes.

Nucleotide sequence analysis of the *rfb* region showed that the genes were flanked by *galF* and the *his* operon (132). The genes encoding rhamnose (Rha) biosynthesis are highly homologous to those of *S. typhimurium* and form an operon at the beginning of the region. Another function within the *rfb* region is the O-antigen polymerase encoded by *rfc* (133). The deduced Rfc protein is predicted to be a hydrophobic protein with multiple trans-cytoplasmic membrane domains, however, even using powerful expression systems such as the phage T7 promoter/polymerase, it was unable to be visualized possibly reflecting an abundance of rare codons and lack of an identifiable ribosome binding site. The size of Rfc and these other features are common to Rfc proteins from other species, although there appears to be little direct sequence homology.

The *rol* gene was shown to lie at the 3' end of the *rfb* region in agreement with the physical map of the region generated by deletion analysis (128,134). The cellular location and topology of Rol has been examined both genetically and physically. Rol has two membrane spanning domains at its extremities and the bulk of the protein is periplasmically exposed. The mechanism of action of Rol is still to be elucidated but it has been proposed to work together with Rfc to determine the appropriate O-antigen chain length.

An intact LPS and the correct modal length of LPS molecules are important for full virulence as shown in the guinea pig keratoconjunctivitis model (137). It would appear that the effect on virulence is due to indirectly affecting the distribution and level of IcsA on the surface. IcsA is essential for intracellular motility and intercellular spread, both of which are mediated via the polar deposition and polymerization of F-actin. This can be readily examined by confocal microscopy (164). Mutants in *rfbD* and *rol* were both proficient at invasion and intracellular multiplication, but did not move normally within the cytoplasm of the infected cell. They were also unable to produce plaques on tissue culture monolayers indicating defects in both intracellular movement and intercellular spread.

Following polymerization of the O-antigen to the correct chain length and transfer to the lipid + core oligosaccharide to complete the LPS molecule, this molecule must be translocated to the bacterial cell surface. This is thought to involve a flippase and *rfbX* is proposed to encode this function (135). It is a hydrophobic cytoplasmic membrane protein like Rfc and is similar to other described RfbX proteins.

Sf6 is a temperate bacteriophage encoding O-acetylation of the Rha-linked to GlcNAc in the O-antigen subunit of the LPS (129,165). A physical map of the phage has been constructed and its mechanism of packaging been defined. The region of the genome encoding *oac*, the O-antigen acetylase, has been cloned and sequenced along with the 5' flanking gene that encodes the phage integrase. The *oac* gene has been confirmed to mediate serotype conversion.

Another phage, designated SfII because it encodes expression of the type II antigen, has been isolated from a *Sh. flexneri* serotype 2b strain (138). It was shown to encode O-antigen glucosylation, however, this process is genetically complex requiring at least two, and possibly three, genes. The first, *bgt*, is required for the transfer of glucose to the lipid carrier, bactoprenol, and *gtrII* is the glucose transferase which transfers the glucose to the O-antigen

subunit. These genes form an operon with another open reading frame (ORF2) and like Sf6 are linked to the phage integrase. The combination of ORF2, Bgt and GtrII leads to efficient serotype conversion.

Examination of the cloned *rfb* genes and various mutant derivatives in *E. coli* K-12 yielded LPS with properties different from that predicted (136). These results demonstrated the essentiality of several of the *Sh. flexneri rfb* genes for O-antigen biosynthesis in *E. coli* K-12. They also confirmed that the residual *E. coli* K-12 *rfb* genes were functional and that there are additional functions outside this region, which are also required.

Sh. dysenteriae serotype 1 requires the presence of a small plasmid for the synthesis of a complete LPS molecule. The plasmid-encoded *rfp* determinant was localized and sequenced and two genes present as the *rfpA,B* operon were identified (131). The products of these genes are proposed to modify the LPS core oligosaccharide so that it is receptive to the addition of the polymerized O-antigen. RfpB was shown to have UDP-galactosyl transferase activity. Thus, in order to express the *Sh. dysenteriae* O-antigen in a heterologous strain, both these plasmid-encoded determinants and the chromosomal *rfb* region are required.

These studies provide a basis for Shigellosis vaccine development (165). The combination of the *Shigella rfb* regions with or without the phage-encoded modifications provides a basis for generating a number of serotype specificities in a heterologous vaccine strain.

III. Miscellaneous pathogens

A number of other pathogens were studied as parts of collaborative projects. These studies are all associated with evaluating factors, such as toxins, cytolysins and surface

proteins, which have been implicated in virulence in a variety of bacteria (and also in protozoa in the case of *Giardia intestinalis*) (152,166).

Streptococcus pneumoniae

The human pathogen *Streptococcus pneumoniae* is responsible for a variety of conditions ranging from life-threatening infections such as pneumonia and meningitis to the less severe but highly prevalent diseases such as otitis media and sinusitis. A major virulence determinant implicated in the disease states is pneumolysin, a pneumococcal toxin with cytolytic activity. As a prerequisite to construction of a recombinant toxoid, extensive gene libraries were constructed and screened for a recombinant clone expressing pneumolysin (139). The gene was cloned and the coding region localized. Expression and mutagenesis studies confirmed pneumolysin to be a 54-kDa protein.

Neisseria gonorrhoeae

Neisseria gonorrhoeae is a strict human pathogen responsible for the sexually-transmitted disease gonorrhoea. A feature of the organism is the phase and antigenic variation associated with many of its surface components, that are thought to provide a mechanism to evade the host immune response. One of these is the pilin subunit that can be found in a number of forms either as a component of the pili, as secreted pilin or as overlength pilin (L-pilin), which remains localized in the outer membrane. The loci associated with expression of several different L-pilins were cloned and compared with the silent pilin loci (140). Together with mRNA analyses, these data implied that the L-pilins are generated by means of an atypical recombination event. This event leads to more than one silent pilin gene being incorporated into the PilE expression locus and translational read-through produces the L-pilin.

Giardia intestinalis

Giardia is a small primitive parasitic unicellular protozoan that colonizes the intestine of vertebrates, causing severe diarrhoea. The genus is taxonomically poorly defined and appears to undergo antigenic variation of its surface antigens. In order to begin studies to address these areas, the gene encoding a 69-kDa surface protein, designated TSP11, was cloned from *Giardia intestinalis* from an expression library (144). Nucleotide sequence analysis confirmed the absence of introns and the deduced protein was Cys rich containing multiple copies of a Cys-X-X-Cys motif. A large portion of the deduced C-terminus of the protein was homologous to a surface antigen from an independent isolate and indicated the presence of a surface antigen gene family. Using a probe to part of *tsp*11, along with a probe to a repetitive DNA sequence, it was possible to distinguish the different *G. intestinalis* genetic groups by analysis of restriction fragment length polymorphisms (141).

Campylobacter hyointestinalis

Campylobacter hyointestinalis has been associated with proliferative enteritis in swine, and proctitis and diarrhoea in humans. Attempts to clone genes from *Campylobacter* species in *E. coli* K-12 have met with limited success and so it was proposed to develop systems within *Campylobacter* in order to facilitate genetic analysis (142). A survey of *C. hyointestinalis* strains revealed a small plasmid that seemed suitable as a basis for vector development (143). Analysis of the nucleotide sequence and protein analysis in minicells showed that the plasmid contained the minimum of information for replication, stable maintenance and the potential for mobilization (142). A kanamycin-resistant derivative of this plasmid containing the mobilization region (*mob* or *oriT*) of plasmid RP4 was constructed and used to isolate a restriction-less mutant of *C. hyointestinalis* (143). This

plasmid, an *E. coli* K-12 – *C. hyointestinalis* shuttle vector, and mutant combination provide a basis for introducing cloned genes back into this pathogen.

When the shuttle vector was mobilized from *E. coli* K-12 into *C. hyointestinalis* a smaller plasmid was isolated (145). Sequence analysis of this derivative suggested that creating a hybrid between oriT of RP4 and the putative mobilization origin of the shuttle vector generated it. This result indicated the way in which cleavage of DNA occurred during conjugation: conjugation is initiated by cleavage at *oriT* and the site of the second cleavage is determined by DNA homology.

Streptococcus pyogenes

Group A streptococci (*Streptococcus pyogenes*) are responsible for rheumatic fever which may lead to sequelae such as valvular heart disease. Rheumatic fever is a significant problem in the Australian aborigines but early diagnosis could facilitate preventing recurrence and reduce the severity of infection. Immunity to group A streptococcal infection is correlated with antibodies to the M-protein, a polymorphic filamentous surface protein. Thus, to develop a rapid diagnostic reagent, it was essential to determine which epitopes might be specific for rheumatic fever. This necessitated screening sera from aboriginal and non-aboriginal subjects with and without rheumatic fever (147). However, in order to do this a suitable immunoassay was developed (146). Using a set of overlapping peptides, it was possible to identify the linear epitopes associated with rheumatic fever and which could be used in a serological test.

Legionella

The genus *Legionella* contains at least 40 species with multiple serotypes, many of which are associated with the human disease legionellosis. Development of the disease is

thought to initiate by the inhalation of viable organisms in fine aerosols into the lung where they invade the alveolar macrophages and other phagocytic cells. The outer membrane protein Mip (**m**acrophage **i**nfectivity **p**otentiator) has been reported to have a significant role in virulence. The *mip* gene from *Legionella longbeachae* serogroup 1 was cloned and found to be highly conserved in other *L. longbeachae* serotypes (149). Mutants in *mip* were constructed by allelic exchange in the type strain and a recent pathogenic isolate and evaluated for their ability to infect *Acanthamoebae* and for virulence in the guinea pig model. The type strain was defective whereas the recent isolate showed reduced activity both for infection and virulence. However, the mutant strain was unable to kill guinea pigs after aerosol exposure clearly implicating Mip in virulence.

The *mip* genes from 35 *Legionella* species have been sequenced and the amino acid sequences compared (148). The data were used in conjunction with published 16S RNA sequences to establish the phylogenetic relationships between the species. No correlation could be found amongst the Mip proteins isolated from either disease or the environment. However, these data have led to the development of a classification scheme based upon the *mip* gene sequences (150).

STATEMENT ON AUTHORSHIP AND CONTRIBUTION

Papers 1-9

These papers describe the work carried out during my Ph.D. candidature (1974-1977) in the Department of Microbiology and Immunology at The University of Adelaide, under the supervision of Dr. P.R. Reeves who provided research direction and was involved in editing the manuscripts. Papers 1-8 were submitted with my Ph.D. thesis (The University of Adelaide, 1977) entitled **Characterization of conjugation defective mutants of *Escherichia coli* K-12.** I planned and carried out the majority (>80%) of the work with the exception of papers 4 and 8 as noted below. Paper 4 was a collaborative effort with another Ph.D. student, Mr. A. Puspurs, however, I was responsible for most (60%) of the work. Paper 8 was a joint effort (~40% each) with Dr. A.P. Pugsley who was a post-doctoral fellow in the laboratory. The writing of paper 9 was completed after the submission of my thesis but the work is mine (>80%) and essentially the contents on one of the thesis chapters.

Papers 10-27

These papers report the work primarily performed during my stay as a post-doctoral fellow at the Max-Planck Institut für Molekulare Genetik in Berlin during 1977 – 1980, although some work was completed after my return to Adelaide (papers 23, 26, 27). During this time I worked in the laboratory of Dr. M. Achtman but established collaboration with Dr. K.N. Timmis.

The papers 11,14,16,19,20,21,22,24,26 and 27 arose from work that I initiated and was in general carried out by myself or under my supervision, with the additional authors

providing particular advice or technical expertise. My average contribution was 60% except for paper 26, which was about 30%.

The remaining papers were collaborative efforts that were initiated in either Dr. Achtman's (10,13,18), Dr. Timmis' (15,17,22,25) or Dr. Reeves' (23) laboratories. I was involved in both the experimental work and the preparation of the manuscripts for submission with an average contribution of 30%.

Papers 28 – 150

These papers, with the exceptions of 101,111,112,115-125,131,139-141,144, and 146-150, are the result of work that was initiated and undertaken in my laboratory at the Department of Microbiology and Immunology at the University of Adelaide from 1980 until 1998. Within my laboratory, I maintained a policy of encouraging the various graduate students and research staff to prepare the first drafts of papers and consequently they were awarded the position of first author. With very few exceptions I have been the corresponding author and taken full responsibility for submission and publication.

The postgraduate- (Ph.D. and M.D) and B.Sc.(Hons.) students whose theses or reports include work described in my publications are listed below.

The Ph.D. students and the year in which they were awarded their degrees are: A. Guidolin (1985), H. Ward (1989), V. Franzon (1988), E. Bartowsky (1988), T. Focareta (1989), R. Alm (1990), M. Brown (1991), M. Jalajakumari (1992), S. Waterman (1992), A. Paton (1993), U. Stroeher (1993), A. Barker (1994), D. MacPherson (1995), B. Yakhchali (1996), A. Ogunniyi (1997), M. Ogierman (1997), J. Iredell (1997), and M. Mavris (1998). Other Ph.D. students who are yet to submit their theses and who work in my laboratory, under my direct supervision are : C. Clark, P. Kaewrakon, and L. Purins. In addition, I am co-

supervising R. Doyle and R. Ratcliffe, both of whom were located in the Institute of Medical and Veterinary Science.

The B.Sc.(Hons.) students and the year in which they undertook their projects are: F. Imbesi (1981), A. Mercurio (1984), B. Miller (1985), B. Neal (1986), D. Beger (1987), G. Tsiolis (1988), R. Faast (1989), J. Beltrame (1989), C. Meaney (1990), A. Lech (1990), S. Thomas (1991), P. Shields (1991), D. Astill (1991), L. Varcoe (1992), L. Mourtzios (1992), S. Zabihi (1992), A. Fallerino (1992), K. Jedani (1994) and K. Dredge (1995).

In addition, the following students from Germany undertook experimental work towards degrees at their home universities in my laboratory. They are : J. Pohlner (1985, University of Heidelberg), T. Rieß (1998, University of Tübingen) and C. Kuehlewein (1998, University of Tübingen).

R. Norton (1998) was a M.D. student who I co-supervised and who was located in the Institute of Medical and Veterinary Science.

Research staff who worked in my laboratory under my direct supervision and whose names appear on my publications are listed below. The postdoctoral research staff are: Dr. M.W. Heuzenroeder, Dr. S.G. Williams, Dr. R. Morona, Dr. K.C. Cheah, Dr. S.R. Attridge, and Dr. E. Voss. The research assistants are: D.R. Haynes, C.A. Lagnado, G. Stevenson, L.E. Karageorgos, L. van den Bosch, C. Mavrangelos, and G. Parasivam.

The research described in these papers was carried out under research grants on which I was either sole or co- Chief Investigator from the following sources:

i. Queen Elizabeth II Fellowship from the Australian research Grants Scheme,

ii. the National Health and Medical Research Council,

iii. the World Health Organization,

iv. the Clive and Vera Ramaciotti Foundations,

v. the Australian Research Council,

vi. The University of Adelaide Research Grants Scheme,

vii. The National Biotechnology Program, and

viii. F.H. Faulding Pty. Ltd.

My sabbaticals in 1987-1988 in the Abteilung Infektionsbiologie at the Max-Planck Institut für Biologie in Tübingen, Germany and in 1991-1992 again in Tübingen and in the Bereich Mikrobiologie at the Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany, were supported by a Fellowship from the Alexander von Humboldt Stiftung.

Papers 28-97 (*Vibrio cholerae*)

These seventy papers represent a number of interrelated project areas that proceeded under my direct supervision and guidance with the exception of the following : 34,35,41,42,43,46,47,54,56,66 and 67.

Papers 34,35,41,46,47,66 and 67 were, in part, the result of a joint initiative with Enterovax Research which was a company that was established through the Department of Microbiology and Immunology, The University of Adelaide with funding from the National Biotechnology Program and F.H. Faulding Pty. Ltd. This was established initially with Prof. D. Rowley, Dr. P. Reeves and myself as the Chief Investigators. However, I was intimately involved in both the direction and execution of the work, and was responsible for the writing, submission and publication of all but 66 and 67, where I was only involved in revising the draft manuscripts. My overall contribution to these papers averaged about 25%.

J. Pohlner came as a visiting student to my laboratory from Dr. T.F. Meyer of the Abteilung Infektionsbiologie at the Max-Planck Institut für Biologie in Tübingen, Germany and worked under my supervision and direction. Papers 42 and 43 were a direct result of this

work and some of the work was also included in 46 and 54. The preparation of papers 42 and 43 was a joint effort whereas I was solely responsible for 46 and 54. My contribution was about 30%.

The work described in paper 56 was a joint initiative with Dr. S.R. Attridge at a time when Enterovax Research employed him but it was not part of their research program. I was actively involved in the supervision and direction of the work and assisted in the writing of the manuscript for publication. My contribution was about 20%.

Although the work in papers 62,83,87 and 97 was conceived in my laboratory and supervised by me, the success of the work was very dependent upon expertise and results provided by additional collaborators: 62, Drs. G. Mayrhofer and I. Kotlarski (Department of Microbiology and Immunology, The University of Adelaide); 83, Dr. M.J. Albert (International Centre for Diarrhoeal Diseases Research, Bangladesh); 87, Prof. J. Holmgren and Dr. G. Jonson (Department of Medical Microbiology and Immunology, University of Göteborg, Sweden); 97, Dr. O. Carmel-Harel (Division of Microbial and Molecular Ecology, Hebrew University of Jerusalem, Israel). My overall contribution was about 30%.

Papers 98-110 (Pathogenic *Escherichia coli* : ETEC)

Of these papers, 101 was performed completely in the Institute of Medical and Veterinary Science (IMVS) basically with my input into the supervision and direction of the work and providing strains to validate the system. I also was involved in co-writing the manuscript. My contribution was about 20%. Paper 100 was a joint effort with the IMVS but I was directly responsible for much of the experimental work and solely responsible for the preparation, and submission of the manuscript. My contribution was 50%. Paper107 arose out of work that initiated in my laboratory but was carried through to completion in the

laboratory of Prof. P. Reeves (Department of Microbiology, University of Sydney). My contribution was about 20%.

All of the other papers were conceived and executed in my laboratory under my guidance and direction with the exception of the N-terminal protein micro-sequencing performed by Dr. R. Halter (Abteilung Infektionsbiologie, Max-Planck Institut für Biologie, Tübingen, Germany) who is co-author on papers 104-106. In all cases I was responsible for the submission and publication off the manuscripts. My overall average contribution was 30%.

Papers 111-122 (Pathogenic *Escherichia coli* : STEC)

The work presented in these papers was undertaken as a collaboration with the laboratory of Dr. J. Paton at the Adelaide Women's and Children's Hospital and as Ph.D. supervisor for A. Paton. My contribution, about 20% in each case, was primarily in supervision, experimental design and direction and co-writing the papers but also included performing the confocal microscopy for paper 119.

Papers 123-127 (Salmonella)

The work reported in papers 123-125 was a collaboration with Drs. J. Lanser and M. Heuzenroeder in the Institute of Medical and Veterinary Science. Most of the work in papers 123 and 124 was performed in the IMVS and I assisted in their preparation. I took full responsibility for writing and submission of 125. My average contribution was 20%.

Papers 126 and 127 resulted from collaboration with Dr. I. Kotlarski (Department of Microbiology and Immunology, The University of Adelaide). Dr. Kotlarski has had a long-standing interest in immunity to Salmonella infections and discussions made it apparent that the work was amenable to a molecular approach. I supervised all the molecular aspects of the

work and took responsibility for the submission and publication of 127. My average contribution was 25%.

Papers 128-138 (Shigella)

With the exception of paper 131 these papers report work which I initiated, supervised and directed in my laboratory. Except for 129 and 130 the responsibility for preparation and submission of the manuscripts was shared with Dr. Morona. My average contribution was 20%.

Paper 131 was the result of work that I performed while on sabbatical with Prof. K.N. Timmis in the Bereich Mikrobiologie at the Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. I performed some of the experimental work and was shared the writing of the paper with Prof. Timmis. My overall contribution was about 20%.

Papers 139 – 150 (Miscellaneous Pathogens)

Papers 139, 141 and 144 were collaborations in which I provided expertise and my laboratory to establish recombinant DNA technology and construct gene libraries with Dr. Paton and Ey. My contribution averaged about 15%.

Paper 140 reports work that I undertook as part of a sabbatical supported by a Fellowship from the Alexander von Humboldt Stiftung, with Dr. T.F. Meyer in the Abteilung Infektionsbiologie at the Max-Planck Institut für Biologie in Tübingen, Germany. I performed part of the work and co-wrote the paper. My contribution was about 20%.

The work reported in papers 142, 143 and 145 commenced under the supervision of Dr. Hackett but Mr. Waterman transferred to my laboratory and the bulk of the work was undertaken there. I supervised the research and was involved in the preparation of the manuscripts for submission with an overall contribution of 20%.

Papers 146-150 report work that was initiated and undertaken in the Institute of Medical and Veterinary Science. My role was mainly supervisory and assisting in the writing of the papers. My average contribution was about 15%.

Papers 151 – 171

These papers are essentially reviews of the literature or of work that has been undertaken in my laboratory. Papers 153, 155-160, 162, 164, 166, 167, 169 and 171 were all solely my work. Review paper 151 was written by me in Berlin (Max-Planck Institut für Molekulare Genetik) in collaboration with Dr. M. Achtman and discusses bacterial conjugation. I wrote the review and took responsibility for publication and it was edited and revised by Dr. Achtman. My contribution was 70%.

Dr. K.N. Timmis wrote much of review paper 152 and handled the submission and publication process. My contribution was about 30%.

The reviews 154, 161, 163, 165 and 170 were co-written with postgraduate students and research staff in my laboratory on topics relating directly to the research programmes. In all cases I took responsibility for submission and publication. My contribution was about 50% in each case.

Publication 168 is the Preface to a special edition of the journal GENE that was edited by Dr. T.F. Meyer and myself. The issue was based upon the **Schloß Ringberg Workshop on Type 4 pili - biogenesis, adhesins, protein export and DNA import** (Tegernsee, Germany) which we jointly organized. Our contributions were equal.

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