Chapter 11
My Memories of *Pseudomonas* in the Twentieth Century

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The palest ink is better than the best memory (Chinese proverb)

Abstract The genus *Pseudomonas* is highly significant for health sciences, agriculture, microbiology, biotechnology and environmental science. This was not always so. An historical and personal perspective or this rise from microbiological obscurity in the mid twentieth century is provided by a microbial geneticist who spent fifty years working on this genus. When microbial science began to expand in the late 1940's, *Pseudomonas* had only one well defined species, *P. aeruginosa*, and a confused taxonomy of other isolates assigned to the genus. The extraordinary metabolic variation of these isolates had been recognised, but there was little biochemical knowledge by way of explanation. This situation had the virtue of attracting scientists of many disciplines who in the next ten to twenty years demonstrated novel biochemical pathways, constructed a sound taxonomic basis for the genus and established a system of genetic analysis. In turn, this groundwork enabled the potential of *Pseudomonas* in human disease, plant disease, biotechnology and aspects of the environment to be realised and exploited. The importance of *P. aeruginosa* in cystic fibrosis has stimulated an understanding of pathogenicity and virulence for human bacterial pathogens in general. All aspects of *Pseudomonas* research have been greatly enhanced following the introduction of genomic techniques in more recent years.

Keywords Historical perspective · *Pseudomonas* genetics · *Pseudomonas* taxonomy · *Pseudomonas* variability · *Pseudomonas* plasmids · *Pseudomonas* patents · Cystic Fibrosis · Pre-genomic era · Genomic era · R68.45

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In the Beginning…….

When I decided to work on the genetics of *Pseudomonas* in 1953, I was aware that *P. aeruginosa* infected wounds and other species of *Pseudomonas* were associated with plant disease. I was not to know that by the end of the century, the genus *Pseudomonas* would be recognised as one of the most widespread living organisms on the planet and would be declared one of the six "top priority, dangerous microbes" (Talbot et al. 2006). When I started to study the genetics of bacteria, it was not known how genetic information was stored in living organisms. DNA was known to be involved but it seemed too simple a molecule to store the amount of information needed and there was a view that proteins played a part in this essential biological function. In 1953 Watson and Crick published their classic paper on DNA structure, but it took time for biologists to appreciate the many implications of this discovery. Microbial genetics would play a major role in defining the molecular basis of biological information.

During the twentieth century a number of issues drove research on *Pseudomonas*. Its biological variation, unusual substrate use and novel biochemistry were always and continue to be great attractions. The rise of microbial genetics provided new tools and insights to investigate bacterial function. The importance of bacteriophages and extrachromosomal elements such as plasmids added to the genetic complexity and enabled new genetic concepts and techniques to be developed. Finally, the disease aspect of *P. aeruginosa*, particularly its importance in cystic fibrosis has been a major factor. Interest in phytopathogenic pseudomonads has become more widespread. The genomic era of biology would totally change how all these issues would be studied and my involvement covered both the pre-genomic and genomic periods. As John W. Gardner remarked "History never looks like history when you are living through it".

My Introduction to *Pseudomonas*

I completed my Ph.D. at the California Institute of Technology in October 1952. My supervisor was Nobel Laureate George Beadle and the thesis topic I had selected was "Heterocaryosis in *Neurospora crassa*". As it turned out, I was Beadle's last graduate student and I learnt a lot about genetics at Caltech from exciting people. In addition to Beadle, another two of my teachers at Caltech would go on to win Nobel Prizes—Edward Lewis and Max Delbrück. I returned to Australia to take up an offer of a Research Fellowship in Microbial Genetics by Frank Fenner, Professor of Microbiology at the newly established Australian National University in Canberra. I was the only bacteriologist and the only geneticist in a department of virologists. Frank gave me complete freedom to choose my own research topic provided it did not involve *N. crassa!*

While passing through New York on my way home from Pasadena, I had heard the first public seminar by Norton Zinder on transduction in *Salmonella typhimurium* (Zinder and Lederberg 1952) and this stimulated my interest in studying the genetics of bacteria. While there had been little interest in the genetics of bacteria while I was at Caltech, Max Delbruck's phage group had demonstrated the value of studying genetics in organisms other than those then in vogue namely *Drosophila melanogaster*, *N. crassa* and *Zea mays*. In 1945 Edward Tatum and Joshua Lederberg had published ground breaking papers on conjugation in *Escherichia coli* and the significance of this work was beginning to be widely recognised. I appreciated the difficulties of competing with the larger and well-funded bacterial genetic groups working on *E. coli* in the USA, so I looked for an alternative bacterium. To begin with I studied the role of bacteriophage in toxin production in *Corynebacterium diphtheria* which had just been discovered by Neil Groman at the University of Washington, but I made little progress so I decided to work with another bacterium. I prepared a list of essential characteristics which included ready availability of strains, the ability to obtain auxotrophic mutants, the presence of bacteriophages, low pathogenicity and given my work location, of medical interest. I searched through the major microbiological tome of the time, Topley and Wilson's Principles of Bacteriology and Immunity published in 1946 for a suitable candidate and *P. aeruginosa* fitted all these requirements. As well, to my knowledge at that time (incorrect as it turned out) no one else was working on the genetics of this organism.

John Loutit had commenced his doctoral studies at the University of Adelaide in 1947 under the supervision of Nancy Atkinson, well known for her work on *Salmonella*. His thesis topic was genetic recombination in *P. aeruginosa.* Using one strain, he isolated a range of auxotrophic mutants, being the first to do so for this organism (Loutit 1952). By growing mixtures of different auxotrophs of another strain, Loutit found that prototrophic derivatives occurred, suggesting that some sort of genetic exchange was occurring at low frequency (Loutit 1955).

It is possible that another search for gene exchange mechanisms in *Pseudomonas* was initiated by André Lwoff at the Pasteur Institute in 1950. François Jacob in his Nobel Laureate address (Jacob 1965) wrote:

> Having come to prepare a doctoral thesis with Andre Lwoff, I was assigned the study of lysogeny in *Pseudomonas pyocyanea*. Thus I conscientiously set out to irradiate this organism. However, it soon became apparent that the problem of lysogeny was primarily that of the relationship between the bacterium and bacteriophage, in other words, a matter of genetics.

There is no evidence to suggest that Jacob actively sought to find a genetic system in *P. aeruginosa* but he did publish a series of papers on lysogeny and bacteriocins in that organism. Most microbial research at the Pasteur Institute at that time was with *E. coli*, so perhaps Lwoff was seeking to extend the knowledge of genetics to other bacteria.

In the second half of 1953 I obtained strains of *P. aeruginosa* from Melbourne, Adelaide and South Africa. I made auxotrophs of two strains which I called Strain 1 (later to be PAO1) and Strain 2 (later to be PAT2) using manganous chloride

as the mutagen. I isolated a range of auxotrophs for both strains in the very first experiment. Influenced by my work with *N. crassa* which requires two strains to be mated, I mated auxotrophs of strains 1 and 2, using the technique pioneered by Lederberg and Tatum which selects for prototrophic recombinants. The first mating produced prototrophs at frequencies similar to that found in *Escherichia coli* K-12. Further matings, which included auxotrophs of two other strains, showed linkage of unselected markers and all the data was consistent with a system of conjugation in *P. aeruginosa* (Holloway 1955).

Using PAO1, as it will be referred to, a series of multiple marker derivatives were isolated and an infectious sex factor, FP2, isolated from Strain 2. Conjugation in *E. coli* had been greatly helped by the use of high frequency donor strains known as Hfr variants, in which the F factor had become inserted into a site on the chromosome and chromosome transfer from the donor Hfr strain to a recipient or F- strain commenced at that site. No such Hfr strains were ever found with FP despite efforts to do so over many years.

The discovery of transduction in *S. typhimurium* stimulated me to study bacteriophages in *P. aeruginosa*. It was already known that lysogeny in *P. aeruginosa* was common and I had no difficulty in isolating bacteriophages from my strains. Indeed one strain, Strain 3, was lysogenic for four different bacteriophages (Holloway et al. 1960) and one of these, B3, was found to be transducing (Holloway and Monk 1959). I later isolated phage F116 which proved to be more efficient in transduction and has been widely used for fine structure mapping in PAO1. With Phage D3 we found that when PAO1 was made lysogenic for this strain the bacterial surface antigenic structure was changed, the first example of lysogenic conversion in *P. aeruginosa* (Holloway and Cooper 1960). Prophages have since been shown to be a significant component of the *P. aeruginosa* genome and be important for virulence properties of this organism. By contrast, they are less common in *P. putida* and fluorescent pseudomonads but do occur in some phytopathogenic pseudomonads.

In early 1957 I moved to the University of Melbourne to take up the offer of a tenured Senior Lectureship offered by Sydney Rubbo, Professor of Bacteriology in the Medical School. This gave me the opportunity to recruit graduate students to the *Pseudomonas* project. In 1958 at the invitation of Syd Rubbo, Joshua and Esther Lederberg spent about 4 months in the Department of Bacteriology. It was an amazing experience for me to work with them on both teaching and research. What better way to learn about *E. coli* genetics?

A Greater Research Profile for *Pseudomonas*

As part of the major expansion of scientific research which occurred in the United States after the Second World War, more scientists worked on genetics and the use of a wider range of experimental organisms enabled advances in the biochemical implications of genetic function. *N. crassa* and *E. coli* became more commonly

used. Both organisms had a significant experimental advantage compared to organisms such as fruit flies, corn and mice in that they were haploid, which meant that mutations were expressed phenotypically without further genetic manipulation. *E. coli* already had an established reputation as a genetics research tool through the work of Emory Ellis who studied bacteriophages at Caltech from 1936. Max Delbrück met Ellis in 1938 and was intrigued by the possibilities of phage biology. Further collaboration with Salvador Luria and Alfred Hershey led to The Phage Group using *E. coli* B as the host. *E. coli* had been a popular and effective subject for metabolic studies since the mid nineteen thirties. It was easy to grow in a chemically defined medium and did not present any safety issues for biochemists untrained in microbiological techniques. The isolation of auxotrophic mutants was first achieved by R. Roepke and his colleagues in 1944 (Roepke et al. 1944). Shortly afterwards Tatum confirmed these results which enabled he and Lederberg to demonstrate conjugation in *E. coli* K-12 (Tatum and Lederberg 1947).

By contrast, *Pseudomonas* had a more diffuse history. While the type species, *P. aeruginosa,* had been isolated and studied extensively during the early part of the twentieth century, this was principally due to its importance as a human pathogen. Other species of *Pseudomonas* were isolated from a wide variety of environmental locations, but the taxonomy was imprecise so that there was no single species or strain which was accepted and identified for study by microbiologists in different laboratories as had been the case with *E. coli*.

During the 1940's and early 1950's a group of highly distinguished microbiologists joined the Department of Bacteriology at the University of California in Berkeley. Michael Doudoroff arrived in 1940, followed after the war by Roger Stanier, Edward Adelberg, Germaine Cohen-Bazire, Norberto Palleroni and Bill Sistrom. This group would have a profound effect on the understanding of the metabolism of *Pseudomonas* and the taxonomy of this genus. This period has been well documented by Roger Stanier (Stanier 1980) and Patricia Clarke (Clarke 1986). During the 1950's and 1960's, research interest in *Pseudomonas* increased particularly in the USA. A range of different Pseudomonads isolated from different environments were shown to have a remarkable capacity to use a wide range of carbon and nitrogen sources, a range unparalleled by any other bacterial genus. The taxonomic and biochemical implications were apparent immediately.

In 1950, Roger Stanier graphically described the current state of knowledge of bacterial metabolism (Stanier 1950):

> …if one is hardy enough to attempt its construction, (bacterial metabolism) resembles a seventeenth century geographer's map of North America. The shape of the continent is roughly outlined, some small local areas already penetrated by the pioneers are correctly shown, but vast regions are either simply left bland or filled in as fancy dictates.

Two major issues dominated *Pseudomonas* research at this time. First there was the question of identity. What was a *Pseudomonas*? How was a species of *Pseudomonas* defined? The second was the ability of many isolates to grow on a wide range of chemical substrates. The goal of bacterial physiologists at this time was to identify as many pathways of assimilation and dissimilation as possible and to determine

their regulation. However, some of the essential biochemical techniques were yet to be introduced. Fragmentation of cells for the extraction of enzymes was a problem, as the French Press and the Raytheon oscillator were yet to be commonly found in microbiological laboratories. However, major discoveries began to emerge.

Doudoroff investigated sucrose utilisation by *P. saccharophila (*now reclassified as *Pelomonas saccharophila* but his results are valid for other species of *Pseudomonas*), leading to the discovery of a previously unknown major pathway of glucose degradation in bacteria, the Entner-Doudoroff pathway (Entner and Doudoroff 1952). Stanier discovered that fluorescent *Pseudomonas* cells grown on aromatic substrates oxidised a range of other similar substrates much quicker than cells grown on non-aromatic compounds, identifying the principle of simultaneous adaptation which is not found in most other bacteria. This became a major tool in elucidating the pathways by which aromatic compounds were metabolised. It took more than 10 years to identify all those pathways and the enzymes involved. For the first time, genetics contributed to this work with the isolation of mutants lacking specific enzymatic function which were used to identify metabolic pathways, emphasising the value of genetics to the solution of biochemical problems.

From the early days of this work, particular interest was focussed on how *Pseudomonas* metabolised aromatic compounds—a property not found in *E. coli.* Beginning in Stanier's laboratory in the early 1960's, a cascade of work on the β-ketoadipate pathway has resulted in a broader understanding of gene arrangement, gene regulation, evolutionary mechanisms, enzymatic structure and function which has extended beyond *Pseudomonas* to other species of bacteria and even to Eucaryotes (Ornston 2010; Harwood and Parales 1996).

The work at Berkeley resulted in the classic paper by Stanier et al. (1966) in which 267 strains were examined and 146 compounds were screened as carbon and nitrogen sources. This was an approach first developed by den Dooren de Jong (1926), but his results had been unavailable to microbiologists generally because it was published as a doctoral thesis written in Dutch. As it happened, Roger Stanier had a copy of this paper. The Berkeley paper provided a firm basis in *Pseudomonas* taxonomy available to the world community of microbiologists. Working both independently and in collaboration with Stanier, Irwin Gunsalus at the University of Illinois made a significant contribution to characterising the enzymology and regulation of *Pseudomonas* metabolic pathways. He has written a very modest account of his long term, major contributions to *Pseudomonas* biochemistry and genetics (Gunsalus 1996).

The Foundations of Pseudomonad Taxonomy

Once the Berkeley group had decided to focus on understanding the metabolic behaviour of *Pseudomonas* the identity of independent isolates of apparently the same species became important, if only for comparison and validation of results. Establishing a taxonomic framework for the genus *Pseudomonas* was always going to be

a major challenge. Norberto Palleroni, a major participant in the taming of *Pseudomonas* taxonomy has written the definitive history from the inside perspective of a journey that has taken over 50 years. (Palleroni 2003, 2010). Palleroni came to the Department of Bacteriology at the University of California at Berkeley in 1953 to work with Mike Doudoroff. Over the next 20 years, with the active collaboration of Roger Stanier and others in the Department of Bacteriology—The *Pseudomonas* Group as it came to be known (Palleroni 1978)—the biology, metabolic activities and variation of hundreds of *Pseudomonas* isolates were studied. This work clearly established the basis for a definitive approach to the taxonomy of this genus.

It soon became clear that with such a biologically diverse group of organisms, multiple taxonomic criteria would be necessary. Palleroni recognised the value of DNA/DNA hybridisation techniques which had become available but at first this approach did not provide any additional taxonomic criteria. Independently, a study of ribosomal RNA had demonstrated high sequence conservation in bacteria and this provided a new and accurate means by which *Pseudomonas* isolates could be distinguished (Palleroni et al. 1973). This was the first time that RNA sequence homology had been used for taxonomic purposes in bacteria and it soon became widely used for other genera. Palleroni and his colleagues (Palleroni et al. 1973) showed that by using ribosomal ribonucleic acid homologies, the genus *Pseudomonas* consisted of five distinct groups. In 1992, Yabuuchi and his co-workers (Yabuuchi et al. 1992) in Osaka suggested a new genus, *Burkholderia,* to differentiate some *Pseudomonas* isolates. This was followed in 1995 (Yabuuchi et al. 1995) with a further proposal to include some *Burkholderia* species in a new genus, *Ralstonia*. There has been an ongoing reassignment of *Pseudomonas* species to these and other new genera as additional taxonomic criteria have become available. Much remains to be done to classify the hundreds of isolates in the literature named *Pseudomonas*. Given the rate of technologically development in determining genome sequences, it is likely that genomic analysis, combined with bioinformatics will provide the ultimate taxonomic criteria for pseudomonads.

Regulation of Microbial Metabolism

The characterisation of the lactose operon in *E. coli* by the group at the Pasteur Institute in Paris led by François Jacob was the first correlation of genetic organisation and enzyme regulation in any organism. The was made possible solely by the ability to identify and precisely map genes in *E. coli*, assay the relevant enzymes and relate the role bacteriophages play to these activities (Jacob et al. 1960; Ames and Martin 1964). A further example of an operon was discovered in *Salmonella typhimurium*. In 1959, a transduction analysis by Milislav Demerec and Phil Hartman at Cold Spring Harbor showed that genes of the biosynthetic pathway of histidine were clustered and probably contiguous (Demerec and Hartman 1959). As well, the coordinate expression of histidine biosynthetic enzymes was demonstrated by Bruce Ames and his colleagues (Ames and Garry 1959).

For those working with fluorescent and other Pseudomonads, this work would be a stimulus to find the genetic basis of enzymatic regulation in those organisms. The necessary equivalent tools would not become available for some years to come for the fluorescent Pseudomonads. Developments in the genetics of *P. aeruginosa* did enable a comparison of this species with enteric bacteria. The first mapping data for *P. aeruginosa* had shown that not all the genes for the tryptophan biosynthetic pathway are closely linked, in contrast to the situation in *E. coli* where they are all contiguous. In 1960, Barbara Fargie and I, followed independently by others, extended this type of gene arrangement to include nine biosynthetic pathways (Holloway and Fargie 1960; Holloway 1969).

In 1962, I spent a year with Salvador Luria at the Massachusetts Institute of Technology. During this time, I learnt a lot about science in general and microbial genetics in particular. He proposed that I work on one of his projects involving *E. coli* and lambda phage, but soon after I started, I did an experiment which demonstrated that the theory underlying the project was questionable. Surprisingly to me, Salva was pleased with this outcome, I found he was always more interested in data than his own theories. By this time, I had established interactions with other MIT staff and Salva suggested that I collaborate with them. This gave me experience in isolating and characterising DNA as well as in electron microscopy. I used *Pseudomonas* phages for this work so it had ongoing value to our overall *Pseudomonas* program (Davison et al. 1964; Slayter et al. 1964).

During my time at MIT, I attended the 1963 Cold Spring Harbor Symposium, a memorable experience. While there I met Gunny Gunsalus for the first time, and this was the beginning of a long and productive collaboration. During the 1960's I went to Urbana, Illinois several times to work with Gunny's group which at that time included Al Chakrabarty. Shortly after returning to Melbourne, I had my first overseas visitor—Ned Shrigley from Indiana University, which I had arranged during my time at MIT. He was interested in "autoplaque" formation in *P. aeruginosa* (Berk 1963). When some strains of this organism are grown on solid media, they develop what looks like phage plaques, but no phage can be isolated from these manifestations and the plaques are a manifestation of autolysis. We had hoped that a genetic approach to this problem might be successful but that did not happen. D'Argenio et al. (2002) suggested that extracellular signalling may be involved in this phenomenon but there is still no satisfactory explanation of this autolysis despite the best efforts of many workers over the years.

Pseudomonad Genetics in the Pre-Genomic Era

Genetic analysis for other species of *Pseudomonas* became possible in the nineteen sixties. Using a transduction system in *P. putida*, Gunsalus and his colleagues demonstrated a similar gene arrangement for biosynthetic pathways to that found in *P. aeruginosa* (Chakrabarty et al. 1968). They went on to study gene arrangement in the mandelate pathway and found a mixture of closely linked and unlinked

structural genes (Gunsalus et al. 1968). George Hegeman and his co-workers at Berkeley studied the genetics and regulation of the β-keto adipate pathway, a pathway that was to figure largely in the understanding of gene regulation in pseudomonads (Hegeman 1975). Overall, the results showed that *P. aeruginosa* and *P. putida* were similar in the distribution of genes for most of the pathways studied.

The regulation of many biosynthetic pathway genes in *P. aeruginosa* was shown to be quite different for the same enzyme in enteric bacteria. While coordinate repression is the predominant mode for *E. coli,* this is not the case in *P. aeruginosa*. In this organism, if such genes are clustered they show repression, but if unlinked they are constitutive in expression. This was also found to be the case for *P. putida*. Hegeman demonstrated the coordinate induction of enzymes in *P. putida* converting D-mandelate to benzoate and subsequently Nicholas Ornston demonstrated the supra-operonic genetic control of genes in the aromatic pathway in *P. putida* (reviewed in Holloway 1969).

As indicated above, much of the seminal work on bacterial metabolism and genetics was done using *E. coli* K-12 and its bacteriophages. There were good logistical reasons for this but it did lead to a proprietorial attitude by some *E. coli* workers as described in Nick Ornston's highly entertaining autobiographical article describing his work at both Berkeley and Yale (Ornston 2010). He relates that microbiologists who were not working with *E. coli* were asked "*Why* aren't you working with *E. coli*?" Millard Sussman's 1970 review entitled "General Bacterial Genetics" (Sussman 1970) was devoted entirely to *E. coli* genetics. Jacques Monod (Friedman 2004) wrote that "Anything found to be true for *E. coli* must also be true for elephants". However, the genetics and biochemistry of pseudomonads have demonstrated significant differences which are important for microbiology and biochemistry generally.

In 1968 I accepted an offer by the recently established Monash University, located in the suburbs of Melbourne, to be Foundation Professor of Genetics and as was the custom in those days, I was also Chairman of the Department of Genetics until 1992. Monash had taken its first students in 1961 so this was a rare opportunity to start a Department of Genetics from the ground up. I expanded the microbial genetics group, but I also appointed staff experienced in other aspects of genetics. I brought Jill Isaac and Vilma Stanisich with me from Melbourne University and I appointed Viji Krishnapillai and Tony Morgan. We attracted more graduate students and our grant funding increased. Jill Isaac and I continued to study regulation of biosynthetic pathways in *P. aeruginosa* focussing on the pyrimidine biosynthetic pathway (Isaac and Holloway 1968). We found a completely different pattern of regulation for genes in this pathway to that shown in *E. coli*.

A major research goal was to expand the *P. aeruginosa* chromosomal map. Vilma Stanisich and I published the first substantial paper on conjugation mapping in *P. aeruginosa* in 1969 (Stanisich and Holloway 1969). We tried to find Hfr versions of FP2 without success and looked for other fertility factors. We looked for other FP plasmids and found FP39, which had a different site of origin to FP2 but we were not able to demonstrate circularity of the *P. aeruginosa* chromosome at this time.

In 1972 spent 6 months with Patricia Clarke at University College, London. Pat had introduced an entirely new system for studying genetic regulation of enzyme function in *P. aeruginosa* using the aliphatic amidases. This work has contributed extensively to this area of *Pseudomonas* biology and has enabled a closer comparison to the situation in enteric bacteria (Clarke 1980).

The Genomic Era Begins

Since the mid 1970's, the ability to analyse and manipulate genetic material has totally changed the overall approach to biological and medical research. The two key technologies involved were the use of restriction enzymes for cloning and the sequencing of DNA bases. Bacterial genetics provided the research base for the first of these and again, the interaction of bacteria and bacteriophage was an important part of the experimental history. It was shown in the early 1950's that the ability of a bacteriophage to replicate in a given bacterial strain was dependent on the previous strain in which it was propagated. Two enzymes were shown to be involved—a restriction endonuclease and a modification methylase (Boyer 1971). Further work revealed multiple restriction endonucleases which cut double stranded DNA at a variety of base sequence recognition sites. Combined with the characterisation of plasmids, this led to the technique of cloning DNA (Morrow et al. 1974). While *E. coli* cloning vectors were widely used for many years, subsequently vectors better suited to *Pseudomonas* were constructed (Bagdasarian and Timmis 1982)

The biology and genetics of restriction and modification were studied in a variety of bacteria including *P. aeruginosa* (Holloway 1968). The features of these characteristics were similar to those found in *E. coli* with one exception. If *P. aeruginosa* was grown at 43 °C, the restriction and modification phenotypes were suppressed, so that foreign DNA could enter these bacterial cells without being degraded. This change in phenotype persisted for about 60 generations when bacteria previously grown at 43 °C were subsequently grown at 37 °C after which they reacquired the original restriction and modification phenotypes (Holloway 1965). This curious type of inheritance was named "the 43° effect" but a satisfactory explanation has never been found. However it became a valuable experimental tool in allowing foreign DNA to enter and function in *P. aeruginosa*. This was particularly useful when the wide host range Inc P1 plasmids became vectors of foreign DNA. I now think it possible that the 43° effect is due to epigenetic inheritance, made more likely as it involves changes in DNA methylation, but this hypothesis has never been tested.

The second key technique of the genomic era was DNA sequencing (Sanger et al. 1977). The mechanisms involved have advanced in ease, affordability, cost and analysis of the resulting data to the point where now it is a major source of biological information with even greater future potential.

More Genetic Complexity

Research in other aspects of medical microbiology indicated that the bacterial genome had other, novel ways of acquiring additional genetic information in nature. The discovery and use of antibiotics enabled the successful treatment of infections previously considered intransigent. An unexpected outcome was the development of infectious bacterial strains resistant to the widely used antibiotics, and from the early days of bacterial genetics, this has always been an important research topic. It needed to be established whether bacterial resistance mutants arise independently or is their formation a result of the presence of the antibiotic in the environment? While in today's idiom, this may seem like a no brainer, there were vigorous proponents for each view at the time but eventually the former view was established experimentally.

The genetics of antibiotic resistance became even more interesting in the light of clinical experience in Japan after the war. From 1945 onwards, *Shigella* strains with high resistance to sulphonamide began to appear in isolates from patients. However, from 1957 *Shigella* strains with multiple antibiotic resistance appeared, the antibiotics involved being sulphonamide, chloramphenicol, tetracycline and streptomycin. A few years later, it was demonstrated that these multiple resistances could be transferred from *Shigella* to *E. coli* in mixed culture by genetic elements similar to the fertility factor F of *E. coli*. These elements were christened plasmids, episomes or resistance transfer factors (RTF) (Watanabe 1963). They are now a significant problem in the treatment of infectious disease worldwide. The characterisation of plasmids carrying antibiotic resistance genes became a major area of research. This area attracted major funding from governmental agencies and the pharmaceutical industry because these plasmids had the potential for rendering antibiotics ineffective with major impacts on how infections were treated clinically.

Antibiotic Resistance in Pseudomonas

Antibiotic treatment of *P. aeruginosa* infections has always been difficult, given the high innate resistance of this organism to most of the commonly used antibiotics. Edward Lowbury, a surgeon in Birmingham, England was the first to isolate drug resistance plasmids in *P. aeruginosa* and which conferred resistance to a number of antibiotics including carbenicillin. More important, these plasmids could be transferred to other gram negative bacteria (Lowbury et al. 1969). This landmark discovery of wide host range plasmids created difficult clinical issues but provided a singular genetic opportunity.

Vilma Stanisich and I obtained some of these plasmids from Lowbury and tested them for their ability to transfer chromosome in *P. aeruginosa*. The first experiments were highly successful (Stanisich and Holloway 1972). This was the origin of

the work which resulted in the development of the chromosome mobilising plasmid R68.45 which would have a substantial impact on the chromosomal mapping of *P. aeruginosa* and other gram-negative bacteria.

I looked for plasmid variants that were more efficient in chromosomal transfer. I argued that as recombinants, usually selected as prototrophic recombinants were rare, these recombinants themselves might carry the plasmid variant that had caused their formation. I chose the Inc P1 plasmid R68, and set up crosses to select for recombinants prototrophic for one marker, but still carrying another auxotrophic marker so that I could test such variants in a mating to detect chromosomal transfer ability. My prediction turned out to be true and one such variant, R68.45, produced chromosomal recombinants at a frequency a thousand fold greater than that obtained with FP2 in *P. aeruginosa*.

Dieter Haas, then working at the ETH in Zurich came to Monash in 1975 and we collaborated to characterise R68.45 (Haas and Holloway 1976, 1978). This plasmid was instrumental in expanding and refining the chromosome map of PAO1. As well, with its wide host range properties it was used by other workers to study conjugation and map the chromosomes in other organisms. The circular map of *Rhizobium* was constructed by Andy Johnstone at the John Innes Institute, Norwich using R68.45, an achievement reached even before we had shown a circular map for *P. aeruginosa*. R68.45 was subsequently shown to be able to promote chromosome transfer and enable genetic analysis in a range of gram negative bacteria including *Agrobacterium, Azospirillum, Erwinia, Escherichia, Klebsiella, Methylophilus, Rhizobium, Rhodopseudomonas* and *Zymomonas* (Holloway 1993).

The fact that R68.45 transfers the chromosome of PAO1 from multiple sites located in different chromosomal regions makes it a much better mapping tool than FP2 and it can be used to measure map distances in regions of the chromosome where other sex factors do not produce enough recombinants for accurate measurement. Used as a "large" generalised transducing phage, R68.45 proved to be valuable in the construction of PAO strains with desired phenotypes. We had many requests for the plasmid and we distributed it to all who asked. It was widely used in Japan as that country was slow in permitting *in vitro* recombinant DNA experiments in bacteria to be undertaken in laboratories. R68.45 was used for transfer of genetic material between unrelated bacteria as it met the Japanese Government regulatory requirement of natural DNA transfer. Subsequently, I isolated R prime plasmids with R68.45, analogous to the F prime plasmids of *E. coli*, which expanded the intergeneric transfer capabilities of this plasmid.

In 1981 we published the first circular chromosome map of *P. aeruginosa* PAO1 (Royle et al. 1981) and began to identify the molecular structure that made R68.45 so interesting, our first venture into the genomic era. We collaborated with Alf Puhler's group in Bielefeld, Germany to show that there was a tandem duplicated region on the R68.45 chromosome (Riess et al. 1980), and when Neil Willetts came from Edinburgh to spend a year at Monash, we demonstrated that this region was an insertion sequence, IS21 (Willetts et al. 1981). There was one copy of IS21 in R68 and two tandem copies in R68.45.

We regularly received many requests for our mutant strains of *P. aeruginosa* and this activity, along with the need to have a well-documented strain collection, resulted in the appointment of Elspeth Carey, as our Strain Collection Curator. She made a major contribution to *Pseudomonas* genetics by her meticulous attention to detail and techniques in maintaining stocks, checking their genotype and ensuring that all requests for strains by other laboratories were met. This also meant that other laboratories were willing to share their strains with us, so everyone benefited. When I retired in 1993 and support for the maintenance of this culture collection ceased at Monash, most of the collection was transferred to the Department of Microbiology at the University of East Carolina in Greenville, North Carolina, thanks to the enthusiasm and generosity of Paul Phibbs. This was one of the benefits from my long standing and rewarding association with Paul. He worked in my laboratory at Monash for a while and in 1986 I went to Greenville, North Carolina to work with him. After he retired, despite the continued efforts of other staff members in Greenville to maintain the collection and distribute strains on request to other laboratories, the Greenville collection ceased to exist except for some strains which were sent to the American Type Culture Collection. Sadly, this is the fate of a number of similar culture collections in the twenty first century.

Cystic Fibrosis and Pseudomonas Aeruginosa

Cystic fibrosis is of the most frequent genetic diseases in humans and *P. aeruginosa* infection is now a major factor affecting the longevity of patients with cystic fibrosis. It was not always like that. The increasing importance of cystic fibrosis has been one of the major drivers for research on *P. aeruginosa* which in turn has had a major impact on knowledge of other species of *Pseudomonas*. The first definitive clinical description cystic fibrosis was provided by Dorothy Anderson in 1938. Upper respiratory infections of these patients were always part of the disease syndrome but while they consisted predominantly of gram positive organisms, they were controlled clinically by antibiotic treatment. This resulted in a shift in the composition of the microbial flora in the lungs of cystic fibrosis patients so that now in the twenty first century, by their late teens most have *P. aeruginosa* infections and the overall treatment of these patients has become more difficult.

A significant development was the detection in 1964 of a colonial variant called "mucoid" in isolates of *P. aeruginosa* from cystic fibrosis patients (Doggett et al. 1964). Of particular interest is that this variant is hardly ever isolated from patients with other types of *P. aeruginosa* infections, for example burns or cancer cases. Appearance of the mucoid variant in the sputa of cystic fibrotics is associated with deterioration in the prognosis. There has been a sustained and expanded study of the causes of *P. aeruginosa* infections associated with cystic fibrosis and this has been largely funded by Cystic Fibrosis associations in various countries. A major source is the American Cystic Fibrosis Association which has funded many research projects unable to access US government sources of funding. It is now

known that mucoid strains result from the formation of alginate and the biosynthesis and regulation of alginate has been intensively studied. Alginate is not the sole virulence factor. Others include pyocyanin, the blue phenazine pigment so characteristic of *P. aeruginosa*, biofilm formation, the overarching regulatory mechanism of quorum sensing, small regulatory RNA molecules, exotoxins, and a variety of other genes including those for motility and attachment. Understanding the functions and regulation of such a broad genetic structure has been and remains a major challenge to *Pseudomonas* workers and the treatment of cystic fibrosis (Govan and Deretic 1996).

The ecology of *P. aeruginosa* in the lungs of cystic fibrosis patients is complex. The majority of bacterial cells are in a biofilm which is largely composed of a polysaccharide matrix. As well there are free living planktonic cells. The biofilms enhance the emergence of antibiotic resistant variants of *P. aeruginosa* which in turn complicates the clinical management of this disease. The understanding of the infectious process of *P. aeruginosa* in cystic fibrosis has served as an important model for other bacterial infections, particularly how antibiotic resistant variants arise in the host.

I had only a limited involvement in the microbial genetic aspects of cystic fibrosis. John Govan came to work in my laboratory at Monash for 6 months and that collaboration continued after he went back to Edinburgh. Later, the cosmid bank of *P. aeruginosa* we constructed (see below) was used in a very effective collaboration with Vojo Deretic then at San Antonio, Texas to map genes involving alginate formation.

Pseudomonas and the Environment

It was a reality of life in the 1950's that there was a general lack of scientific and public interest in what is now referred to as "the environment". The catalyst for what is now a global concern in the topic was the book "Silent Spring" written by Rachel Carson and published in 1962. The literature on multidisciplinary scientific studies of the environment as well as the general public and Government interest has escalated since the nineteen sixties and seventies.

Many studies have shown that *Pseudomonas* survives in a wide variety of predominantly aqueous environmental conditions and niches. Given the range of aromatic compounds which are now common pollutants, most of industrial origin, it was inevitable that *Pseudomonas* isolates would be of interest for bioremediation studies given the innate ability of this organism to degrade so many organic molecules. Chakrabarty's seminal work (see below) on genetically modified strains of this organism attracted workers to this topic. Since then, there have been extensive studies by Ken Timmis and his colleagues in Germany and Victor de Lorenzo's group in Spain which have maintained a wider interest. However, the widespread, commercially viable and effective use of genetically modified *Pseudomonas* or other microorganisms for bioremediation is still to be achieved (Cases and de Lorenzo 2005). Nevertheless, an active search for this goal continues.

Pseudomonas Meets Industry and the Law

The broad characteristics of the genetics and metabolism of pseudomonads attracted considerable commercial research interest during the 1970's and 1980's. In addition to the need to understand the biochemical diversity of this group of organisms, the possibility of commercial outcomes loomed larger as research organisations, funding agencies and corporate entities became more involved with the genetic renaissance of biotechnology.

Once again, plasmids played an important role, as they had with developments in antibiotic resistance. Plasmids were found in strains of *Pseudomonas* isolated from particular environments which could enable these microorganisms to degrade specific hydrocarbons. The major plasmids were CAM (camphor), SAL (salicylates), NAH (naphthalene), OCT (n-alkanes), XYL (xylene) and TOL (xylene). While in most cases the strains of pseudomonads involved carried these plasmids as replicons separate from the chromosome, in other strains, the plasmids were integrated into the chromosome. This finding has special significance for studies on the evolution of the biochemical diversity of pseudomonads (Chakrabarty 1976).

In 1971, Ananda (Al) Chakrabarty started work at the General Electric Research and Development Center in Schenectady, New York. Shortly afterwards, he developed a variant of *P. putida* which was stable in culture, contained hydrocarbon degrading genes from four different plasmids and was effective in digesting organic compounds found in crude oil spills.

In 2005, Douglas Robinson and Nina Medlock wrote (Robinson and Medlock 2005):

> Chakrabarty is not well known outside the intellectual property community—the average person probably has never heard the name. Yet, Chakrabarty has affected the lives of virtually everyone in the United States, having contributed to a revolution in biotechnology that has resulted in the issuance of thousands of patents, the formation of hundreds of new companies and the development of thousands of bioengineered plants and food products

How did this come about? In 1972, Chakrabarty filed a patent application assigned to the General Electric Corporation claiming that he had constructed in the laboratory a genetically engineered bacterial strain that was man-made and possessed properties not found in naturally occurring microorganisms. The original patent was approved in part, but two claims relating to the bacterial strain were rejected on the grounds that microorganisms are products of nature and not patentable under US law.

Chakrabarty appealed this decision to the Patent Office Board of Appeals but the original decision was upheld. Next Chakrabarty took his case to the Court of Customs and Patent Appeals who reversed the previous decision declaring "…… the fact that microorganisms are alive is without legal significance". Sidney Diamond, the US Commissioner of Patents and Trademarks appealed this decision to the US Supreme Court. On June 16th, 1980 that Court, in a split 5–4 decision, upheld the view of The Court of Customs and Patent Appeals, confirming that genetically engineered microorganisms could be patented, creating the famous "Diamond v. Chakrabarty" case which has achieved worldwide recognition and acceptance (Diamond v. Chakrabarty 1980).

As a result, many biological inventions and discoveries, previously thought to be without legal protection, have been patented. This in turn has created a solid economic and legal basis for the international biotechnology industry and has added further distinction to the role that *Pseudomonas* has played in the history of microbiology. Of particular importance is the legal basis that this patent has provided to the patenting of DNA sequences.

Pseudomonas was to figure in another important legal and biotechnological event. Michael Vandenbergh, in an article on the regulation of the release of genetically modified microorganisms wrote (Vandenburgh 1986):

> When the Environmental Protection Agency (EPM) first approved a field test of a bioengineered microbe, one EPA official remarked: "We're not expecting this to the rutabaga that ate Pittsburgh".

The organism in question was *P. syringae*. Some bacteria have the ability of initiate ice formation and this was first demonstrated with *P. syringae* (Maki et al. 1974). Other species of *Pseudomonas* will also carry out this function as do some other bacterial genera (Gurion-Sherman and Lindow 1993). Such bacteria have a single protein—the "ice plus" protein on their cell surface which can provide a focus for ice crystals to form. While this is a very interesting interaction between biological and physical activities, it also has practical applications. The presence of such bacteria on the surface of plants can result in frost damage with significant economic effects on agricultural production.

Naturally occurring variants of *P. syringae* which lack the ability to catalyse ice formation were isolated. Working at Berkeley, Steve Lindow used recombinant DNA technology to create stable "ice minus" variants of *P. syringae* and sought to use them to minimise frost damage to potatoes and strawberries in California. Field tests for this purpose were given United States Government approval as was required with every release of genetically modified organisms. A legal challenge to the release was at first successful. However, the legal basis on which this challenge was based preventing field trials did not apply to commercial organisations. In 1983, Advanced Genetic Sciences obtained United States Government approval to conduct field trials using ice minus variants of *P. syringae* and *P. fluorescens* developed by Lindow. Further legal and public objections delayed final approval until 1987 when the release went ahead. Hence *P. syringae* achieved the distinction of being the first living genetically modified organism to be granted approval for release into the environment.

I have had two major interactions with industry. The first resulted from an error in microbial taxonomy. In the nineteen seventies, Imperial Chemical Industries (ICI) in the United Kingdom developed a new process for converting North Sea hydrocarbon gas into methanol and hence the need to find new markets for methanol. One was the use of microorganisms to convert methanol to biomass which could then be used as a dried animal feed called Pruteen. ICI purpose built a 6 million L stainless steel fermenter at Billingham in the North of England. They sought my advice on the genetics of the organism they had selected for this process and sent the strain to me in the belief that it was a species of *Pseudomonas*. We found, as

did other scientists at ICI, that it was not a pseudomonad, and was subsequently identified as *Methylophilus methylotrophus*, an obligate methylotroph. However, this did get my group working on the genetics of methylotrophs. ICI supported this work for some years and it was productive to transfer the genetic techniques we had developed in *Pseudomonas* to a completely different organism. The Pruteen project was closed down in the late 1980's when it became non-competitive with soybean protein.

My second industrial venture was with the Celanese Corporation in 1979. They proposed to manufacture specialty chemicals by bacterial fermentation from low cost substrates and had identified the β-ketoadipate pathway of pseudomonads and other organisms as a starting point. Together with Nick Ornston from Yale University and Howard Dalton from the University of Warwick in the United Kingdom, we provided a team of consultant advice to Celanese, and later a spin off biotechnology corporation, Celgene, in microbial applications to specialty chemical production from about 1980 to 1993. We made regular visits to the Corporation headquarters in New Jersey and they also funded research in each of our laboratories. In my case, this also involved sending members of the Monash team to New Jersey to pass on techniques to Celgene staff and in their staff coming to Monash for training. It was a highly productive association with the added benefit for me of working with two outstanding scientists as co-consultants. Friendships were established which have lasted long after the commercial activity ended.

Phytopathogenic *Pseudomonads*

Interest in bacterial phytopathogens generally became more pronounced in the period after World War II and the role of Pseudomonads in plant disease became to be more appreciated (Starr 1959). Issues of nomenclature and taxonomy became more important. The taxonomic criteria in vogue at the time did not fit well with the biological characteristics of the many organisms being isolated particularly if host range had to be considered. The phytopathogenic pseudomonads did not show any significant differences compared to saprophytic isolates but minor differences were used for differentiating host range. This was especially true for some species particularly *P. solanacearum* (Hayward 1991). In *P. syringae*, the problem was solved by use of the term pathovar, abbreviated to pv, to distinguish isolates causing disease on different hosts. In some isolates other criteria could be used to further differentiate pathovars (Hirano and Upper 1990).

For phytopathologists, the important question was how do bacteria cause disease and how do they differ from saprophytic bacteria? For Pseudomonads most of the work to address this problem has been done in two species, *P. solanacearum* and *P. syringae*. It has been shown that specific compounds produced by phytopathogenic bacteria play a role in pathogenicity, although other virulence factors are involved.

The availability of genetic approaches in bacteria has helped to define the basis of pathogenicity particularly through the use of mutants affecting disease producing

gene products. The use of rRNA homology groups has redefined the taxonomy of this group of organisms. This is particularly true in *P. solanacearum*, cause of Bacterial Wilt which affects major food crops including potato, tomato and banana. It is a particularly variable species for host range encompassing many species of 44 plant genera, ecological niches ranging over tropical, sub-tropical and temperate regions, and multiple disease characteristics. A system of races and biovars has been developed to catalogue this variability. It has been the subject of taxonomic revision, being first renamed *Burkholderia solanacearum* and its current denomination is *Ralstonia solanacearum*. Control of Bacterial Wilt is difficult given that it is a soil borne disease and the current major strategy is breeding for host resistance to disease.

In 1985 I was asked by the Australian Centre for International Agricultural Research (ACIAR) to undertake a research and training program on the genetics of *P. solanacearum,* working with developing country agricultural scientists on the application of molecular genetic techniques to this organism. Bacterial Wilt caused by this organism is a major problem in tropical and sub-tropical regions in which ACIAR has a major interest. I did not anticipate that this program would continue until 2002. I established collaborations with other research groups in Australia notably those led by at Chris Hayward at the University of Queensland and Jeremy Timmis at the University of Adelaide. The training program on molecular genetics involved selected agricultural scientists from developing countries coming to one or more of the three Australian institutions involved to obtain laboratory experience. We established procedures by which the home laboratories of those we trained were equipped with the necessary laboratory equipment for this work, and we sought to build up research groups so that the people we trained would not be working in isolation. One of the outcomes of this program was the development of a DNA probe for the specific identification of *P. solanacearum* which could be used in developing country institutions (Opina et al. 1997). I also started a program at Monash to develop the genetic analysis of *P. syringae* (Nordeen and Holloway 1990)

Pre-genomic genetic analysis of phytopathogenic pseudomonads had only limited success. Gene transfer systems, either or both transduction and conjugation were found for *P. glycinea* and *P. syringae* with all that work done since the early 1970's (Panopoulos and Peet 1985). It has been demonstrated that while single genes can decide the virulence of a bacterial strain for a particular host, in most cases disease production is the result of multigenic control. As well, there may be specific host—parasite gene interactions. Of particular interest was the demonstration that in some strains of *P. solanacearum* some virulence genes known as *hrp* genes were clustered on the DNA of a very large plasmid. It would be shown later (Salanoubat et al. 2002) by whole genome sequencing that the genome of this organism consists of a 3.7 Mbp chromosome and a megaplasmid of 2.1 Mbp, with the *hrp* genes located on the megaplasmid. Genomic techniques have subsequently created successful new opportunities for the understanding of phytopathogenic pseudomonads.

Bacterial Chromosome Mapping in the Genomic Era

In line with our long term aim to get the most detailed map of the *P. aeruginosa* chromosome, we had established circularity of the map in 1981 (Royle et al. 1981). This map had been constructed using a variety of sex factors and there was no certainty that the different plasmids mobilised chromosome at the same rate. Hence the measured distances between genes on the map were of questionable accuracy. What was needed was a circular chromosome map constructed using only one sex factor. This was achieved using two new tools. A mutant of the IncP 1 plasmid R68 temperature sensitive for replication was isolated by Carol Crowther. This was then loaded with a newly found transposon, Tn2521, which Martha Sinclair and I had isolated from clinical strains of *P. aeruginosa.* This hybrid plasmid, pMO514, was then transferred to selected PAO strains with appropriate auxotrophic markers. These were grown at 43 °C and selection made for streptomycin resistance, a marker encoded by Tn2521. It was found that pMO514 had integrated into the PAO chromosome at a range of different sites. When so integrated, it mobilized chromosome at high frequency from each site, thus creating donor strains comparable with the Hfr strains in *E. coli*. Time of entry data obtained from a range of crosses enabled the recalibration of the *P. aeruginosa* PAO1 chromosome map to 75 min, compared with the former value of 95 min (O'Hoy and Krishnapillai 1987).

Conventional genetic mapping in various species of *Pseudomonas* clearly demonstrates the frustrating labour intensive techniques involved, with limited data outputs which vary from species to species. The availability of DNA interactive techniques to create physical chromosome maps provided an approach which can be used for all bacteria. However, such maps do not eliminate the need to identify individual genes through mutation, complementation or other approaches.

The discovery and characterisation of restriction endonuclease enzymes provided the key to this novel mapping paradigm. Unlike cloning, where restriction enzymes having frequently occurring sites are required, physical mapping needs such enzymes which cut infrequently. This approach is combined with the techniques of pulsed field gel electrophoresis (PFGE) which permits the separation of DNA fragments of widely disparate sizes, even those having several megabases of double stranded DNA. Together with techniques which enable the order of the fragments to be determined, the size of an entire bacterial genome can be determined. (Smith and Condamine 1990; Fonstein and Haselkorn 1995).

The location of individual genes on such fragment maps can then be located using a combination of cloned material, DNA probing and complementation mapping (Holloway 1993). This combined approach was widely used in the 1990's for the construction of combined physical and genetic maps for a wide variety of microorganisms including *P. aeruginosa*, *P. putida*, *P. solanacearum*, *P. fluorescens* and *P. stutzeri*.

For more precise linkage analysis, we needed a vector that while retaining the wide host range of the P1 plasmids, transferred a smaller fragment of chromosome. While there were an increasing number of available cloning vehicles, for our work

we needed one with wide host range and low copy number. Other laboratories had similar needs and we were lucky that Ron Hanson's laboratory at the University of Minnesota developed a series of cosmids (a plasmid vector containing the *cos* site of lambda phage) that were derived from a P1 group plasmid. They generously let us have some of their strains and we found that one, pLA2917, was ideal for our purposes. It was to become a major tool for our genomic analysis of a number of bacteria including methylotrophs. We constructed combined physical and genetic maps of *P. aeruginosa*. For the genetic mapping we had the advantage of a lot of data from conjugation and transduction, so we knew the relative location of a lot of genes. In addition, we had a large collection of mutants for *P. aeruginosa*, some of them characterised for the metabolic function involved. While we had obtained some of this data ourselves, most of the gene function data had been obtained by making our mutants available to anyone who wanted them. We had sent mutants to many laboratories and in turn they had done the biochemical investigations on our strains. For the gene bank we adopted a new approach in that individual cosmids were selected for storage rather than the mixtures of cosmids which was the standard approach of the time. We stored fragments of *P. aeruginosa* chromosome in cosmids carried in individual *E. coli* cultures. A library of about 1000 individual cosmids was needed to be sure of covering the whole chromosome with some degree of duplication. By using the individual cosmid approach, we could identify and retain fragments that contained particular chromosomal regions identified by genes or restriction fragments and in this way, identify the genes carried by individual cosmids.

In summary, the essential components that we used to construct combined physical and genetic maps of the bacterial species that interested us were an individual cosmid library, data obtained by PFGE and restriction enzyme site mapping, complementation of known mutants, DNA probes used in Southern hybridisation and eventually DNA and protein sequence data bases. Genetic data obtained by pre-genomic approaches were an added bonus. A combined genetic and physical map of *P. aeruginosa* PAO1 was constructed (Ratnaningsih et al. 1990). Burkhardt Tümmler and his colleagues in Hanover had also constructed a physical map of *P. aeruginosa* PAO1 (Romling et al. 1989) and we collaborated to produce a more comprehensive physical and genetic map (Holloway et al. 1994).

In 1993, mandatory retirement at age 65 changed the nature of my relationship with Monash University. In 1994 I was appointed an Emeritus Professor for life and I continued as an Honorary Professorial Fellow until 2002 when my research funding ended.

During my time at Monash we were fortunate to attract a range of distinguished visitors from overseas. Given the geographical isolation of Australia it is more difficult and more expensive to do this than other regions. Some have been mentioned in the text above, others included Pat Clarke (England), Jack Leary (USA), Larry Bryan (Canada), Pieter van der Putte (Holland), Kyoshi Tagawa (Japan), Alexander Boronin (the then USSR), Norberto Palleroni (USA), Hideki Matsumoto (Japan), and Hans Schlagel (Germany). For our graduate students these visits were very stimulating and provided linkages that were important for their subsequent career

paths. For me they established and reinforced friendships which have continued over the years.

The success of the physical and genetic approach to mapping encouraged other workers to construct similar maps for other species of *Pseudomonas* using more sophisticated probing techniques. In 1996 Rainey and Bailey at Oxford University constructed a physical and genetic map of the *P. fluorescens* SBW25 chromosome (Rainey and Bailey 1996). This was followed 2 years later by Ramos-Diaz and Ramos in Granada, who constructed the same sort of map for *P. putida* (Ramos-Diaz and Ramos 1998). These maps enabled more detailed comparisons of the three species. There are many similarities, such as the lack of clustering of genes in biosynthetic pathways and similarities in the grouping of other genes. However the overall chromosome arrangement of all three species was different, reflecting the importance of chromosomal rearrangements and plasmid insertions to acquire genes. Over the years, a lot of our effort was put into mapping *P. aeruginosa* and establishing systems by which mapping could be achieved in other pseudomonads. This approach became largely redundant with the development of more efficient techniques for sequencing entire genomes. In 1995, the complete genomic sequence of a free living bacterium was first achieved with *Haemophilus influenza* Rd (Fleischmann et al. 1995). Sequences of other bacteria have followed with increasing frequency.

Sequencing the *Pseudomonas Aeruginosa* Genome

The first pseudomonad genome to be entirely sequenced was *P. aeruginosa* PAO1 in 2000 by a consortium led by the Pathogenesis Corporation in Seattle, Washington (Stover et al. 2000). The method used for sequencing was the same as that used for *H. influenza* by Venter and his group and details of the collaborative project and the strategy used are elegantly described by Pete Greenberg (Greenberg 2000). At 6.3 Mbp, more than the previous physical mapping had indicated, the genome of *P. aeruginosa* is large compared to all other bacteria. The sequence agrees in most aspects with the previously constructed physical map with the exception of an inversion comprising about 25 % of the entire genome. Subsequent studies have shown that such inversions occur in the propagation of *P. aeruginosa* on laboratory media and artificial storage. There is little gene duplication in the sequence from which it can be concluded genetic and functional diversity are a result of the genome being larger than usual and it is the basis for this organism's established ability to inhabit a wide variety of ecological niches. Knowledge of the whole sequence has given valuable clues as to the ability of *P. aeruginosa* to develop resistance to antibiotics and successfully and persistently inhabit such specific environments as the human lung. With easier and less expensive access to sequencing techniques, the knowledge of pseudomonad sequences will be a rich source of information for the development of strategies to combat current intransigent infections by this organism.

The Twenty-First Century

The last half of the twentieth century saw an amazing expansion of knowledge about *Pseudomonas*. This was driven by the intellectual curiosity of a cohort of top rank scientists in different countries and the need to seek a solution to infections associated with cystic fibrosis. Two driving forces that sustained that drive were the major increases in funding for research and tertiary education in most of the developed countries of the world and molecular genetics which enabled a greater understanding of how biological information is coded, stored and used.

Increased funding was at the heart of the enormous expansion of the biological knowledge base. For example, in 1950, the National Science Foundation was established in the United States. As well, there was a nine hundred fold increase in research funding for that nation's National Institutes of Health between 1950 and 2000 (Manton et al. 2009). Along with all other aspects of microbiology, *Pseudomonas* has benefited from this bonanza. Science funding in Europe has increased markedly since the formation of the European Economic Union.

The biochemical versatility of *Pseudomonas* which provided such an attractant for so many distinguished scientists to work on this organism in the 1950's should have resulted in major commercial biotechnological achievements by the turn of the century. However, it has taken another 10 years or more for that this expectation to be realised. Another goal is the control of Bacterial Wilt caused by *Ralstonia solanacearum* in tropical and sub-tropical developing countries where global food security has become a more urgent issue.

As with almost all current and future aspects of biological and medical research, it is apparent that genetics, combined with bioinformatics, has been a major factor in the development of knowledge and outcomes. In 1955 it was possible and commonplace for anyone working on bacterial genetics to have read the entire literature relating to conjugation, transduction, and transformation in all the bacterial species being studied. This probably amounted to about 200 publications. By comparison, the flood of articles on *Pseudomonas* in the present century is daunting and awesome. Personal computers, on line data bases, search engines and electronic publishing have entirely changed the way information is available and how it be found and used in science. For example, information about *Pseudomonas* genomes is available at http://www.pseudomonas.com/.

For *Pseudomonas* and other areas of biology, the availability of quicker and less expensive genome sequencing, combined with more sophisticated means of analysis of the data is leading to experimental approaches unforeseen even at the beginning of the current century. More species and strains of *Pseudomonas* are being sequenced (Silby et al. 2011) and it is estimated that the cost of sequencing an isolate of *Pseudomonas* will soon be less than US$10 and be accomplished in a day. In 1994 when a single chromosome of *Saccharomyces cerevesiae* was completely sequenced for the first time, it cost US$10 per base.

In 1954, the first information on the structure of the *Pseudomonas* genome was obtained by demonstrating the linkage in conjugation in *P. aeruginosa* PAO1 of a

streptomycin resistance gene to several but not all tryptophan biosynthesis genes. In the following years, the Monash *Pseudomonas* group and other *Pseudomonas* geneticists using pre-genomic techniques identified the location of more genes, insertion sequences, prophages, pyocin determinants, transposons and plasmids on the *P. aeruginosa* chromosome map. The genomic complexity of the pseudomonad genome determined by the sequencing of a number of *Pseudomonas* species could not have been predicted. There is a core genome comprising genes which are found in all isolates of *Pseudomonas* sequenced to date. In addition there is a pan or accessory genome which comprises the range of all the other genes and genetic elements found in all Pseudomonas isolates. There is a variety of different types of genetic elements which can be differentiated in the pan genome. These include integrative and conjugative elements, genomic islands, prophages, integrons, transposons and insertions sequences as well as other sequences which do not fit neatly into any of these elements. Genomic islands are rich in genes and prophages which are associated with virulence. Genomic analysis combined with other data gathering techniques such as proteomics will surely reveal why *Pseudomonas* has the environmental habitat range, substrate versatility, metabolic diversity and disease producing attributes which continue to mystify and excite. As the noted physicist Neils Bohr said "Prediction is very difficult, especially about the future".

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