# AUSTRALIAN POULTRY CRC

## FINAL REPORT

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# Confirmation of virulence genes and pathogenetic mechanisms of *Pasteurella multocida*

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**Confirmation of virulence genes and pathogenetic mechanisms of *Pasteurella multocida***
*Project No. 03-13*



**Researcher contact details**

Ian Wilkie
School of Veterinary Science
University of Queensland
St Lucia, Qld. 4072

Phone: +61 7 3365 2066
Fax: +61 7 3365 1255
Email: i.wilkie@uq.edu.au

Prof. Ben Adler
Director, Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University VIC 3800
Tel: +61 3 9905 4815
Fax: +61 3 9905 4811
Email: Ben.Adler@med.monash.edu.au

In submitting this report, the researcher has agreed to the Australian Poultry CRC publishing this material in its edited form.

**Australian Poultry CRC contact details**
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: info@poultrycrc.com.au.
Website: http://www.poultrycrc.com.au

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## Executive Summary

The research supported by this project formed part of an ongoing programme of research into the precise sequence of molecular events that lead to the development of the disease known as Avian Cholera. This major disease of poultry worldwide has resisted all previous efforts to make truly efficacious vaccines against it. The basic research we have undertaken is helping to build a more complete understanding of the pathophysiology of the disease, with the ultimate aim of identifying bacterial components which can be used for an effective vaccine. Furthermore, understanding the critical points in establishment and multiplication in the host could lead to novel forms of control, minimising or eliminating the need for current style antimicrobials.

### Objectives

An important objective of this project was to participate in a co-operative project to identify genes of the bacterium *Pasteurella multocida* which contribute to its ability to colonise and cause disease in chickens. The products of these genes will then be characterised and tested for their vaccine potential.

A second major part of the project was to monitor the levels of specific cytokines (chemical signals produced by cells which help to regulate the immune responses of animals) in chickens during the first 18 hours of infection by highly virulent disease-causing strains of *P. multocida* and comparing with the responses to strains of low virulence.

A third area of participation was a study of chicken immunological responses to 105 recombinant proteins which have been identified by proteomics analysis to be secreted or capsule-associated.

## Results

The first and third objectives have been achieved within the context of this project. Approximately 12 genes have been identified as being essential to disease-causing ability (virulence), and one gene has been characterised and its effects on virulence found to be due to a minor change in the lipopolysaccharide capsule of the bacterium. A paper reporting the findings is now in press (Harper et al. Inf. Immun.).

Findings of the chicken immunological responses to recombinant *P. multocida* proteins have been published (Al-Hasani et al, Microbial Cell Factories. 2007, 6:3).

The study of chicken cytokine responses remains incomplete due to technical difficulties with the methodologies used and late appointment of a postgraduate student. However, the study will be completed within the next few months and the CRC will receive due acknowledgement for its support when findings are published.

There will be at least one more publication arising from the virulence gene identification, and this work will also form part of a PhD thesis. Again, all due acknowledgement will be given to the CRC for its support.

This research has made a number of valuable contributions to the understanding of a very complex disease which has frustrated attempts at control and elimination from poultry flocks for more than a hundred years. We are grateful to the CRC for funding this work, even though the dividends may not be fully realised for many years to come. Financial support is duly acknowledged in all scientific papers arising from this project.

# Contents

## Introduction

*Pasteurella multocida* is a very important pathogen which causes several distinct disease syndromes in livestock throughout the world. Certain strains are responsible for an acute septicaemic disease of poultry known as 'Avian cholera' as well as more chronic problems in older birds, such as arthritis. For reasons not completely understood, the acute disease is more frequent, and more severe, in free-ranging birds. Intensively-reared birds are not immune from acute disease, and outbreaks do occur, particularly in turkeys. However, low levels of chronic disease also have a significant impact on productivity, particularly in parent breeder stock, for example.

Although vaccines against the recognised diseases caused by this organism have been made and used for more than a hundred years, none is very effective. If currently-available vaccines give any protection at all, it is only against a single strain, and lasts for a very brief period. Live vaccines are the most protective, but carry a risk that the bacteria revert to virulence, with occasional disasterous effects. It has long been known that animals surviving virulent infections develop broader and much longer-lasting immunity than those 'artificially' vaccinated, implying that there are substances produced by the bacteria *in vivo* that are powerfully immunogenic. A large part of our research efforts for the last 12 years have been directed at identifying and characterising these substances.

This CRC project was a continuation of a series of cooperative research projects on Pasteurellosis which has been highly productive since they began in 1994. This broad-based co-operative approach has been maintained through numerous projects funded by a number of different bodies. Researchers at Monash Medical Microbiology (under the direction of prof. Ben Adler), and a small group directed by prof. Alan Frost at the University of Queensland Veterinary School, first co-operated on an ACIAR-funded project to improve the quality and efficacy of vaccines for both avian and mammalian forms of *Pasteurella*-induced diseases important to Australia and South-East Asia. A significant part of the research effort was directed at basic research, with the eventual goal of developing novel vaccines which would invoke a truly protective response based on antigens shown to be crucial to immunity. That project continued for 6 years, and resulted in more than 25 journal publications, many of which were contained in a single issue of 'Veterinary Microbiology' (Vet Microbiol. 2000, 72(1-2). Vaccines for Avian Cholera and Haemorrhagic septicaemia were improved, but these vaccines were based on the existing types and, with the exception of a live vaccine developed from an Australian field strain, were only marginally more effective than existing vaccines, and still suffered from the shortcomings of a narrow spectrum of activity and brief duration of protection. The live vaccine is not suited to large-scale use, and is inappropriate for use in developing countries because of the problems with transport, storage and proper administration. It also suffers from the same defect as any other live vaccine in that it may, under unpredictable circumstances, express elements of virulence, particularly in ducks.

Identifying the precise antigens elaborated *in vivo* which invoke protective immunity proved to be more difficult than anyone anticipated, and our research efforts have since evolved into very detailed molecular studies of the organism. By understanding the mechanisms by which *P. multocida* infects and develops in its hosts, and how it causes disease, (that is, the 'molecular pathogenesis'), we hope to reveal critical steps which are most amenable to immune attack. Knowing the precise immunogenic molecules involved will lead naturally to vaccines which contain only those antigens crucial to solid and durable immunity.

During the last 10 years, our group has adopted and adapted new techniques for identifying and characterising genes essential to pathogenesis, and were the first to publish a study of whole-genome expression of a pathogen *in vivo* (Boyce et al, 2002). We have also adapted and made

extensive use of the technique of Signature-Tagged Mutagenesis (STM) in which transposons are used to insert labelled sequences into the bacterial genome and disrupt single genes (Harper et al, 2003). We are fortunate in having also developed a very reliable *in vivo* disease model which faithfully replicates field disease. This allows us to test the virulence of modified bacteria in a very precise manner.

Data obtained using a whole-genome microarray indicated a very large number of genes are either up- or down-regulated *in viv*o compared with *in vitro* growth (Boyce et al 2002,2004), with around 30 genes that were most likely to be directly involved in pathogenesis. STM screening of a large library of signature-tagged mutants has allowed us to confirm the importance of a small number of genes and investigate their contributions to virulence in more detail.

## Objectives

Our stated objectives were:

1. To screen a large library of transposon mutants
2. To characterise the products of genes which appear to be related to altered virulence, and define the *in vivo* function of those products.
3. (Added during project period) To screen a number of gene products for vaccine potential.

(1 to 3 were co-operative efforts, mostly funded by my Monash partners.)

4. To investigate the early immune response of infected chickens by employing a microarray for chicken inflammatory cytokines.

## Methodology

Experiments to achieve objectives 1 to 3 were all undertaken in conjunction with prof. Adler's group at Monash. Methods used included:

1(a). Contruction of a library of signature-tagged mutants (done by a PhD student (Mr. Jason Steen) at Monash. The method used has been developed at Monash and is described in several publications (Harper et al, 2003).

1(b). The *in vivo* screening was done in Brisbane as a joint undertaking. The method is a refinement of the published method (Harper et al, 2003) but the esentials remain unchanged. Briefly, birds are dosed simultaneously with a number of tagged mutants (45). At a set time after dosing, a blood sample is taken. Culture of the sample yields a sample of mixed bacteria from which RNA is extracted. It can then be established which mutants have survived and proliferated in the host by RT-PCR comparison with RNA extracted from the input dose. Any mutants which appear to be missing or in very low numbers are then checked by comparison in an *in vivo* competition assay with the wild-type.

2. This objective was the sole responsibility of the Monash team.

3. Details as set out in Al-Hasani et al, 2007. Briefly, 105 recombinant products predicted to be secretory or cell membrane associated, were evaluated for their ability to induce an immune response in chickens. Groups of 4 birds vaccinated twice with approximately 120 micrograms of protein either alone, or in mixtures of four different products, (mixed with a commercial adjuvant (Alhydrogel)) were bled 2 weeks after the second vaccination. Sera of these birds were tested for imunoreactivity to the specific products bt Western blotting. All groups were then challenged with virulent *P. multocida* and onset & severity of illness compared with unvaccinated control groups.

4. The methods proposed to study chicken leucocyte cytokine responses in early infection, i.e. using a 'cytokine' microarray recently developed at the Animal Health Laboratories, Geelong, proved to be impractical due to the amount of RNA which we were able extract from chicken blood samples in pilot trials. We therefore decided on an alternative method, targeting specific cytokines by RT-PCR, which requires far less RNA.

The experimental design aims to compare cytokine responses to a virulent strain with responses to either a very low-virulence strain, and no infection. RNA is extracted from leucocytes obtained from blood samples at fixed time intervals from 1 hour post-infection to around 18 hours post infection. We hope also to obtain useful RNA from te infection site and the liver. The relative proportions of specific cytokines will be established by RT-PCR on the extracted RNA.

## Results

1. Screening of the transposon mutant library was completed in mid 2006. Of aproximately a dozen genes identified as having a major effect on virulence, two were chosen for more detailed study. This work is part of the PhD project of an ongoing student and is being prepared for publication, but has not yet been submitted. It will be submitted by the second half of 2007. Many of the data obtained will not be suitable for journal publication, but will be available in the thesis once the degree has been awarded.
2. One of the genes which had a profound effect on virulence has been further characterised. This gene codes for a heptosyltranferase which results in an altered polysaccharide, which abolishes virulence in the affected bacterium. Details are included in Harper et al, (in press, Infect. & Immun.)
3. A preliminary account of the recombinant product screening has been published, (Al-Hasani et al, 2007) and is attached as Appendix I.
4. This part of the project is not yet completed. At this time, we have refined methods to extract suitable amounts of leucocyte RNA from blood samples of 5ml or more. We have constructed and tested primers for the major acute-phase cytokines which we believe will show the most differences in the initial phases of infection, but are yet to conduct the final experiments. We are waiting for suitable animal housing to become available in order to complete the study. We would hope that at least one, possibly two, journal papers will be submitted after completion.

## Discussion of Results

The most significant result so far of the research completed during the project has been demonstration of the effect of a single gene, the deletion of which causes a seemingly minor alteration in the lipopolysaccharide coat of *P. multocida* and completely abrogates virulence. It has long been known that biochemically and immunologically identical strains of bacteria could have widely variant pathogenicities (Wilkie et al, 2000), but we were at a loss to explain this difference until now. At this point, it is not possible to exploit this finding in terms of vaccine development, but as the effects of the ten or so genes remaining to be characterised are elucidated, a much clearer explanation of pathogenetic mechanisms should result. It is already clear from preliminary vaccine trials that the protein products of these remaining genes are not capable of stimulating protective immunity, (at least not in the combinations so far used). As has been adequately demonstrated, birds mount a strong antibody response to numerous components of the bacterium, but we are still do not know which proteins stimulate protective immunity.

We have also clearly demonstrated that a number of genes appear to be central to pathogenicity. Final results from the mass screening for virulence genes, and the cytokine responses of birds infected with virulent and avirulent will add to the mounting store of information on the natural history of this perplexing organism, which, in the short to medium term, could lead to methods to manipulate *P. multocida in vitro* to produce highly immunogenic products which will give protection that is much more solid and durable than any vaccines currently in use.

## Implications

The immediate technical implications of the research supported in in part by this grant are discussed in the relevant published papers attached as Apendices I & II. All objectives of the project as originally stated have been achieved or exceeded, and a considerable amount of further basic information on the molecular pathogenesis of Avian Cholera has been added to the published record. We have identified a large number of apparent virulence factors, and research will proceed to characterise more of these, as resources are available. Exploitation of these findings in terms of vaccines and disease control is still at least several years away, and will require much more effort to complete the details of the pathophysiology of this disease.

## Recommendations

There has been an increasing tendency by researchers to exaggerate the imminence of major breakthroughs, or suggest that potent vaccines for stubborn diseases involve only a matter of months of high-tech research, in order to impress granting bodies and maintain funding, when this is clearly not the case. Basic research is inherently a 'low-yield' process, which is nevertheless vital to underpin research applied to solve immediate problems. This is probably most pertinent in the case of diseases which have refused to yield to 'standard' measures of prevention over a hundred years or more of research. What all investigators continually discover is that most diseases are much more complex than anyone imagined even 25 years ago, as the molecular era began to gain momentum. It is important that basic research into the modus operandi of common and important pathogens continue until we have real understanding of the pathogenesis of the diseases they cause.

Furthermore, as production methods change, and current antibacterials lose their efficacy (not to mention social pressure to avoid their use altogether in production animals) we must develop novel strategies to prevent and control potentially devastating diseases such as Avian Cholera. Although the mechanisms of individual diseases vary widely, there are also common pathogenetic pathways, and factors elucidated for one can often help provide answers for others.

The complexity of disease-causing organisms and their effects in the hosts requires broad input from generalists to specialists, and this project has helped to maintain what has been, and continues to be, a very productive partnership. Even though the information gained may not be applicable in chicken sheds for many years to come, some of will be vital to the design of future vaccines and control methods. I thank the CRC for supporting our research in a manner which has allowed our partnership to continue.

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## Plain English Compendium Summary

| **Project Title:** | **Confirmation of virulence genes and pathogenetic mechanisms of *Pasteurella multocida*** |
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| Poultry CRC Project No.: | 03-13 |
| Researcher: | Ian Wilkie |
| Organisation: | University of Queensland Veterinary School |
| Phone: | (07) 3365 2066 |
| Fax: | (07) 3365 1255 |
| Email: | i.wilkie@uq.edu.au |
| **Project Overview** | This project has supported ongoing basic research into the fundamental mechanisms by which the bacterium *Pasteurella multocida* causes a major disease of poultry known as 'Avian Cholera'.<br>In this project, we identified genes which initiate specific properties of the bacterium, known as 'virulence attributes'. These are the factors which allow it to colonise birds and establish the conditions which we recognise as disease . Using methods (developed in previous projects) which disable single genes, we have identified a number of genes which appear to have a direct bearing on virulence. When these genes are disabled, it greatly reduces the ability of the organism to infect birds, and/or cause disease, without affecting their ability to grow under normal laboratory conditions. These genes appear to be necessary only for establishment, growth, or reproduction within the bird hosts.<br><br>Vaccine trials using the products a number of other genes identified by protein analysis as being likely antibody targets were undertaken. The results were encouraging, but none was sufficiently protective to warrant further development as putative vaccines.<br><br>A second line of research, which was intended to parallel the work on bacterial virulence attributes, involved investigating the chemical signals (known as cytokines) by which the immune system regulates its responses to infection. This has reached an advanced stage, but is presently incomplete because we do not have access to animal housing of suitable containment level. As soon as this becomes available this work will be finalised and results published.<br>To date, we have published one paper, and a second is in press, in which results supported by this grant are reported. At least two further journal publications are expected to be produced from this work by mid 2007. |
| **Background** | *Pasteurella multocida* is a very important pathogen which causes many distinct disease syndromes in livestock throughout the world. Certain strains are responsible for an acute septicaemic disease of poultry known as 'Avian cholera' as well as more chronic problems such as arthritis. For reasons not completely understood, the acute disease is more frequent, and more severe in free-ranging birds. Intensively-reared birds are not immune from acute disease, particularly turkeys, but in intensively reared birds, low levels of chronic disease have a significant impact on productivity, particularly in parent breeder stock, for example. Although vaccines for the recognised diseases have been made and used for more than a hundred years, they are not very effective. Any protection afforded by these vaccines lasts for a very brief period, and is usually effective only against the exact same strain of bacteria from which the vaccine was made. However, it has long been known that animals surviving virulent infections develop broader and much longer-lasting |

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|---|---|
| | immunity than those 'artificially' vaccinated, implying that there are substances produced by the bacteria *in vivo* that are powerfully immunogenic.<br><br>Identifying the substances responsible for solid immunity proved to be more difficult than anyone anticipated (Gunawardana et al) and our research efforts have since evolved into more detailed molecular studies of the organism, in particular, the mechanisms it uses to establish and develop in its hosts, and how it causes disease, that is, its 'molecular pathogenesis'.<br><br>During the last 10 years, our group has adopted and adapted new techniques for identifying and characterising genes essential to pathogenesis. We have established a very reliable disease model which exactly mimics the natural disease, in which we can test the effects of deleting single *Pasteurella* genes. Early microarray data (Boyce et al, 2002, 2004) indicated a very large number of genes either increase or decrease their activity *in viv*o compared with *in vitro* growth, and we have since narrowed our focus to fewer than 25 genes which appear to be involved in disease production and |
| **Research** | The project has supported:<br>1. An M.Phil. student investigating the chicken cytokine responses.<br>2. Parts of a PhD project to identify the major virulence genes of Avian Cholera-causing strains.<br>2. Contributed to an investigation of immune recognition of surface-associated proteins of *P. multocida*<br>3. Contributed to investigation of the effects of deleting a gene associated with synthesis of part of the lipopolysaccharide capsule of *P. multocida* |
| **Project Progress** | All projected work has been completed exceot for the chicken cytokine study. (This work will be completed using other funds as soon as animal housing for the final experiments becomes available). |
| **Implications** | The work funded by this project is basic research into mechanisms of a disease. As such, no immediate applications were expected. However, the research so far has been productive, and has contributed significantly to two journal papers, one published, the other in press, with at least two more papers in preparation. This fundamental knowledge contributes to understanding of the natural history of the disease and will be crucial to formulating genuinely successful vaccines, or even totally novel methods of disease prevention and control. |
| **Publications** | **Identification of novel immunogens in *Pasteurella multocida***<br>Keith Al-Hasani1, John Boyce1, Victoria P McCarl1, Stephen Bottomley, Ian Wilkie and Ben Adler. Microbial Cell Factories. 2007, 6:3 ( http://www.microbialcellfactories.com/content/6/1/3)<br><br>***Pasteurella multocida* expresses two LPS glycoforms simultaneously but only a single form is required for virulence: identification of two acceptor specific heptosyl I transferases**<br>Marina Harper, John D. Boyce, Andrew D. Cox, Frank St. Michael, Ian W. Wilkie, P. J. Blackall and Ben Adler.( In press, Infect. & Immun.)<br><br>Two further papers in preparation are expected to be submitted in the second half of 2007 |

## Appendix I

**Microbial Cell Factories**
Research

**Identification of novel immunogens in *Pasteurella multocida***

Keith Al-Hasani1, John Boyce1, Victoria P McCarl1, Stephen Bottomley1, Ian Wilkie2 and Ben Adler*1

Address: 1Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Victoria 3800, Australia and 2School of Veterinary Science, University of Queensland, Queensland 4072, Australia

Email: Keith Al-Hasani - keith.Al-hasani@med.monash.edu.au; John Boyce - john.boyce@med.monash.edu.au; Victoria P McCarl - vicki.mcarl@sci.monash.edu.au; Stephen Bottomley - steve.bottomley@med.monash.edu.au; Ian Wilkie - i.wilkie@uq.edu.au; Ben Adler* - ben.adler@med.monash.edu.au

* Corresponding author

**Abstract**
*P. multocida* is a Gram-negative pathogen responsible for causing diseases in animals of economic significance to livestock industries throughout the world. Current vaccines include bacterins, which provide only limited protection against homologous serotypes. Therefore there is a need for more effective vaccines to control diseases caused by *P. multocida*. As a step towards developing vaccines against fowl cholera, a genomics based approach was applied for the identification of novel immunogens.

**Results:** Bioinformatics analysis of the *P. multocida* genome predicted 129 proteins as secreted, located in the outer membrane, or lipoproteins. 105 of the genes encoding these proteins were cloned and recombinant protein expressed in *Escherichia coli*. Polyclonal serum from *P. multocida*infected chickens reacted with a subset of these proteins.

**Conclusion:** These data show the range of bacterial immunogens recognized by the chicken immune system, including 6 novel immunoreactive proteins.

The full text of this article is attached to the hard copy of the report. It can also be accessed at: http://www.microbialcellfactories.com/content/6/1/3