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Poster Presentation

Genomic and proteomics approaches for vaccine development in *Pasteurella multocida*

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Background

P. multocida is a Gram-negative pathogen responsible for causing disease in animals of economic significance to livestock industries throughout the world. It is the causative agent of numerous diseases in animals including fowl cholera in avian species, hemorrhagic septicaemia in ungulates and atrophic rhinitis in swine. Current vaccines include bacterins, which only provide limited protection against homologous serotypes and live attenuated strains, which have been observed to revert to virulence. Therefore there is a need for more effective vaccines to control diseases caused by *P. multocida*.

As a step towards developing protective vaccines against fowl cholera, a genomics based approach was applied to the identification of putative vaccine antigens. This approach utilised a bioinformatics analysis of the *P. multocida* genome sequence using several types of algorithms (eg. PSORTB) to select candidate genes according to their vaccine potential, based on their predicted sub-cellular location (outer membrane or secreted) and their similarity to other proteins with putative or confirmed experimental roles in infection and immunity as well as expression analysis based on microarray studies. The bioinformatics-based predictions were complemented by a comprehensive proteomics analysis of the *P. multocida* outer membrane to identify highly expressed membrane associated proteins.

Results

Over 130 *P. multocida* proteins were predicted to be surface exposed and/or secreted extracellularly, thus necessitating the adoption of a high-throughput cloning strategy. A GatewayTM (Invitrogen) cloning and expression system was used to clone PCR-amplified *P. multocida* ORFs. The forward primers were designed to include the sequence 5'-CACCATG required for directional topoisomerase cloning, as well as the addition of an ATG initiation codon. PCR primers were designed so as to amplify the region that encompasses the entire coding sequence for the subunit but does not include the sequence encoding the signal peptide. As the genes were to be expressed in frame with a C-terminal tag the native stop codon was removed when designing the reverse primers. The PCR products were cloned into the Gateway entry vector pENTR/SD/D-TOPO® (Invitrogen). A sample of the PCR products is shown in Figure 1, panel A.

After the target genes were verified by sequencing and restriction digestion analysis, they were transferred by recombination (LR Clonase kit – Invitrogen) from the entry clone to the Invitrogen destination vector pBAD-DEST49TM to allow subsequent expression of C-terminal fusions of the expressed proteins. A sample of an expression screening of 14 clones is shown in Figure 1, panel B. 80% of target clones showed positive expression (target clones are fused to an N-terminal Thioredoxin His-Patch as well as a C-terminal His-tag).

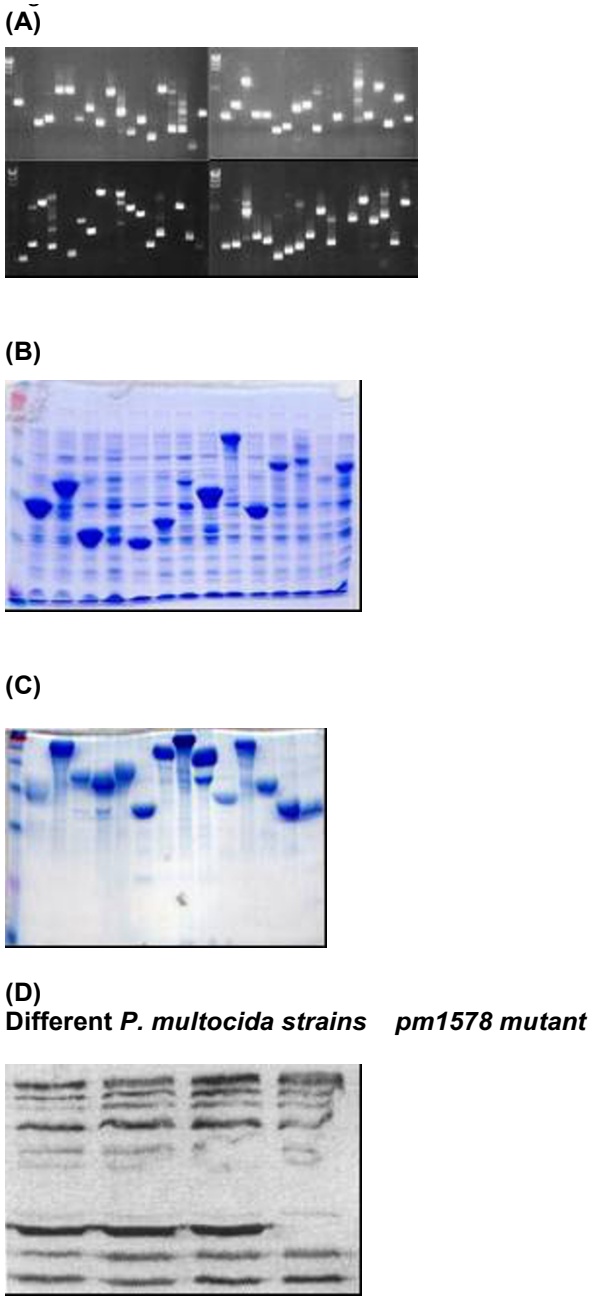

Figure 1
Different *P.* multocida strains pm1578 mutant. Shown are (**A**) PCR amplification of *P. multocida* vaccine target ORFs for Gateway cloning. (**B**) SDS-PAGE and Coomassie Blue staining of crude lysates expressing P. multocida X-73 recombinant proteins in *E. coli* DH5α. All cultures were induced with arabinose at mid-log phase (**C**) Isolation of target proteins from inclusion bodies. Coomassie Blue staining of 14 recombinant proteins solubilised in 8 M Urea. (**D**) Proteins in lysates were separated by electrophoresis and the immunoblot was probed with mouse antiserum raised against PM1578.

Greater than 95% of the fusion proteins were found to be insoluble. Expression at different temperatures as well as the transfer of 20 targets into an N-terminal GST vector did not enhance solubility. These expressed proteins are currently being purified as insoluble inclusion bodies (see Figure 1, panel C). The induced cultures were chemically lysed by incubation of the culture with PopCulture (Novagen), benzoase and lysozyme for 20 min at 20°C 1 ml of the cell lysate was then added to a 96-well filter plate and the solution was drawn through the filter under vacuum. The insoluble inclusion bodies were retained while soluble proteins passed through the filter. The retained inclusion bodies were washed once to remove any remaining soluble proteins. The inclusion bodies were then denatured by the addition of 200 μl of 8 M urea to each corresponding well, incubated for 16 h at 4°C and collected under vacuum.

Approximately 25 μg of each antigen were injected subcutaneously into 5 mice followed by a booster injection using aluminium hydroxide as an adjuvant. Antisera were raised against 18 antigens. Immunoblot analysis was performed with cell lysates prepared from different strains of *P. multocida* and probed with polyclonal antiserum specific for PM1578, which recognised the native *P. multocida* protein. PM1578 was absent in strains harbouring a mutation in *pm1578* (see Figure 1, panel D). Antisera raised against 8 of the 18 antigens injected into mice recognised the corresponding wild-type proteins (data not shown).

To evaluate expression of Gateway cloned antigens from *P. multocida* X-73 during infection in the chicken host, convalescent-phase sera from three infected chickens were tested for reactivity against all the recombinant proteins using Western blot analysis. The results demonstrated that immune sera generated during *P. multocida* infection recognised a total of 23 recombinant proteins (data not shown).

Conclusion and future development

Gateway cloning system provides a highly efficient method to generate plasmids harbouring genes from *P. mutlocida* X-73 and for the generation of expression clones.

In silico analysis identified 130 membrane associated and secreted proteins, 114 of which have been successfully cloned into a Gateway entry vector. Out of these 114 entry clones, 92 were transferred to a Gateway expression vector. Of these, 80% were shown to express measurable levels of protein in *E. coli*.

Because of the relatively poor efficiency of recovering soluble proteins tagged with the thioredoxin tag, efforts are underway to express the target proteins in different expres-

sion systems. To address this hurdle and as means of preferably obtaining correctly folded proteins for vaccine and functional studies, preliminary attempts to express genes as recombinant proteins in NusA fusion expression constructs resulted in significant improvement.