

Pathogenomics of *Pasteurella multocida*

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Abstract The first complete genome sequence of the *P. multocida* avian isolate Pm70 was reported in 2001. Analysis of the genome identified many predicted virulence genes, including two encoding homologues of the *Bordetella pertussis* filamentous haemagglutinins, and genes involved in iron transport and metabolism. Availability of the genome sequence allowed for a range of whole-genome transcriptomic and proteomic analyses and these have helped us understand how *P. multocida* responds to growth in the presence of antibiotics, under low iron conditions and in the host. Unfortunately, no new *P. multocida* genome sequences were determined during the rest of the decade, limiting any possible comparative genomic analyses until recently, when several new genome sequences have become available. Here we use the available data to identify a number of important similarities and differences between the strains and determine their phylogenetic relationships. Interestingly, based on the current data there is no clear correlation between phylogenetic relatedness and host predilection or disease.

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1 Sequenced *P. multocida* Genomes

There are currently two fully sequenced and annotated *P. multocida* genomes available: *P. multocida* strain Pm70 (accession number AE004439) (May et al. 2001) and *P. multocida* strain 36950 (accession number CP003022) (Michael et al. 2012b). Strain Pm70 is an avian isolate and while it was initially reported as a capsular type A (hyaluronan capsule), Heddlestone serovar 3 (A:3) strain (May et al. 2001) analysis of the capsule biosynthesis locus indicates that it produces a type F (chondroitin) capsule, as it lacks genes for hyaluronan synthesis (Townsend et al. 2001), but encodes a chondroitin synthase. Strain 36950 is an A:3 strain isolated from a calf with bovine respiratory disease (BRD). This strain is resistant to a range of antibiotics, including tetracyclines, chloramphenicol, sulphonamides, spectinomycin, streptomycin, enrofloxacin, florfenicol, tilmicosin and tulathromycin (Michael et al. 2012b). Three other *P. multocida* genomes are also publically available, but they are incomplete, limiting the range of analyses that can be undertaken. These are strain P3480, isolated from a pneumonic pig lung, strain Anand1 from a goat (Anand1G) and *P. multocida* subsp. *gallicida* strain Anand1 from poultry (Anand1P; Ahir et al. 2011). In addition, we have recently determined incomplete genome sequences (using Illumina GAIIX technology) of the two fowl cholera isolates X73 and VP161 (both A:1), the bovine haemorrhagic septicaemia isolate M1404 (B:2) and the swine isolate P903 (D:11).

All of the currently available *P. multocida* genomes are between 2 and 2.4 Mbp in length and comprise a single circular genome with a G+C content of between 40 and 41% (Table 1). Analysis of the Pm70 genome identified more than 100 genes predicted to be involved in virulence; of particular note were two very large genes (7.8 and 11.8 Kb in length) encoding predicted homologues of the *Bordetella pertussis* filamentous haemagglutinins (May et al. 2001). These have since been defined as important *P. multocida* virulence factors (Tatum et al. 2005). The Pm70 genome also contains the complete set of genes required for the following pathways: TCA cycle, glycolysis, glyconeogenesis, oxidative pentose phosphate and Entner–Doudoroff (May et al. 2001).

Table 1 Genome features of sequenced *P. multocida* strains

Strain	Complete	Host/tissue	Genome size (Mbp)	G+C content	CDS ^a
Pm70	Yes	Chicken	2.26	40.40	2,089
36950	Yes	Bovine/lung	2.35	40.44	2,202
Anand1G	No	Caprine	2.29	40.52	2,319
Anand1P	No	Avian/liver	2.02	40.22	2,337
P3480	No	Porcine/lung	2.08	40.15	1,933
X73	No	Avian	2.31	40.31	2,130
VP161	No	Avian	2.26	40.21	2,085
P903	No	Swine	2.35	40.14	2,184
M1404	No	Bovine	2.30	40.27	2,136

^a CDS = predicted number of coding sequences

2 Comparison of the Complete Genomes of Pm70 and 36950

Currently only the Pm70 and 36950 genomes are closed and complete. Both genomes contain six rRNA operons comprising 20 and 19 genes, respectively. Comparison of the sequences shows that they are highly colinear and contain nine large, locally colinear blocks (LCB) (Fig. 1). However, there are areas of novel sequence identified in each genome. Strain 36950 contains a large integrative conjugative element designated ICE*Pmul*, which is not found in Pm70 (approximately region 270–360 Kb; Fig. 1). This 82 Kb element contains approximately 88 genes and is capable of conjugative movement into *P. multocida*, *Mannheimia haemolytica* and *Escherichia coli*, as it contains the genes required for integration/excision and conjugation (Michael et al. 2012b). The element also contains genes encoding resistance to streptomycin, spectinomycin, tetracycline, chloramphenicol/florfenicol, sulphonamides, tilmicosin/clindamycin and tulathromycin, which explains the broad antibiotic resistances exhibited by this strain. Strain 36950 also contains point mutations outside the ICE*Pmul* element within *gyrA* and *parC*, potentially altering the target sites of quinolone/fluoroquinolone action (Michael et al. 2012a). The 36950 strain also contains genes for uptake/metabolism of mannose, trehalose and xylose, which are not present in Pm70.

The Pm70 genome also contains a small number of regions not found in 36950. Most of the Pm70 region encoding the genes *pm1935–pm1949* (approximately region 2,172–2,187 Kb; Fig. 1) is not present in strain 36950. This section of DNA has a low G+C content (37.6%) and *pm1949* encodes a predicted site-specific recombinase, suggesting that this region of the chromosome may have been laterally acquired. Pm70 also contains two genes (*pm0698–pm0699*) encoding components of a predicted restriction/modification system which is not present in strain 36950 and two unique phage-like genes (*pm0202* and *pm0203*).

A comparison of the strain 36950 genome with Pm70 shows that strain 36950 contains more than 30 non-functional genes (pseudogenes). These include *pfbB1*, encoding one of the two Pm70 filamentous haemagglutinins, *hsf_1*, encoding one of the two predicted autotransporter surface adhesins, four genes (*pfrR*, *pm0745*, and

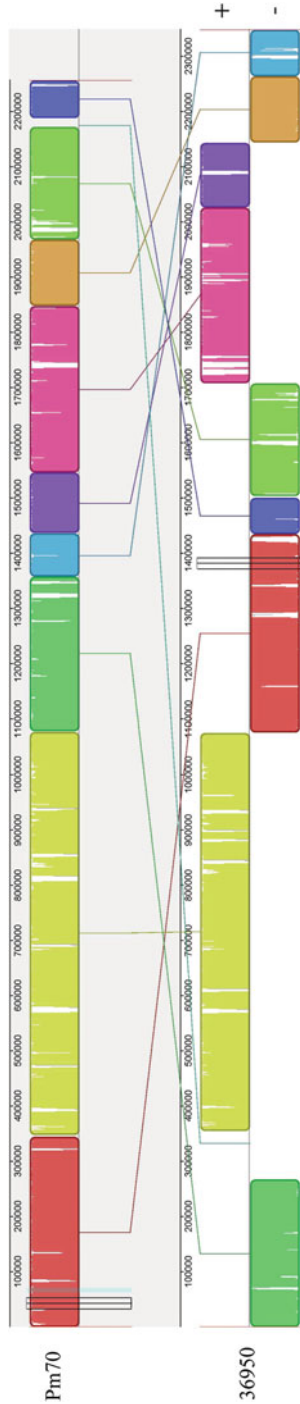


Fig. 1 Comparison of the Pm70 and 36950 closed genome sequences using progressiveMauve alignment (Darling et al. 2010). Rectangles of similar colour show colinear blocks of genes. In the 36950 panel, the upper coloured blocks labelled “+” indicate genes oriented in the same direction as in the Pm70 genome while blocks labelled “-” indicate genes oriented in the reverse direction to the Pm70 genome. Areas of low identity within colinear blocks are shown by reduced height of the shading

two homologues of *pm1622*) encoding predicted Ton-B dependent outer membrane receptors and *plpE*, encoding a protective outer membrane lipoprotein. In addition, Pm70 expresses three highly related, predicted extracellular solute iron binding proteins (Afu1, Afu2 and Afu3) and although the corresponding genes are present in strain 36950, both *afu2* and *afu3* are present as pseudogenes. As a number of these pseudogenes have putative functional paralogues (PfhB1 and PfhB2, Hsf1 and Hsf2), and there is likely significant redundancy in the expressed iron transporters, it is possible that the loss of these functional genes has little impact on the phenotype of this strain.

3 Analysis of the Incomplete Genome Sequences of Strains X73, VP161, Anand1P, Anand1G, P903, M1404 and P3480

Comparison of the Pm70 and 36950 genomes with the incomplete genome sequences of strains X73, VP161, Anand1P, Anand1G, P903, M1404 and P3480 using BLAST, identified a number of regions that were absent, or highly divergent, in all of the comparison strains (Fig. 2). The *ICEPmu1* element is not present in any of the sequenced strains except 36950; this absence correlates with the low level of antibiotic resistance observed in the other strains. Similarly, the Pm70 genes *pm1935–pm1949* are not present in any of the other genomes. These comparisons also suggest that the sequence coverage of P3480 is low, as there are two very large regions of the genome that are missing from the sequence data obtained for this strain (Fig. 2a; 1400–1500 Kb and 1760–1970 Kb on the Pm70 map).

Comparison of the genomes identified large sections of colinear genes in all genomes, with the largest colinear block approximately 250 Kb in length and containing more than 200 genes (Pm70 region *pm0506–pm0712*). Progressive Mauve alignment (Darling et al. 2010) was used to determine colinear orthologues between genomes at a 60% identity level with greater than 70% gene coverage. Across all nine genomes there is a shared set of 1,100 genes and each of the strains contains between 73 (Pm70) and 638 (Anand1P) unique genes (Fig. 3a). It is likely that the number of core genes identified by this analysis is artificially low due to the low coverage of the P3480 sequence and the high number of incomplete and broken gene sequences in the Anand1P and Anand1G genomes. The number of unique genes may also be overestimated in the incomplete genomes due to broken gene sequences. Therefore, we repeated the orthologue analysis with only the high quality and high coverage genomes, Pm70, 36950, X73, P903 and M1404. Across this set of five strains, there is a shared set of more than 1,780 genes, or 88% of the Pm70 gene content (Fig. 3b) and the pan genome, or total gene set, is more than 2,800 genes. Pm70 contains approximately 90 unique genes; strain 36950, 170 genes; X73, 177 genes; P903, 261 genes and M1404, 215 genes. Of the 90 unique Pm70 genes, more than 80% encode hypothetical proteins with no, or poorly defined, predicted function. This very high percentage of hypothetical proteins in the unique gene set is also observed in the other strains. For 36950, more than 80

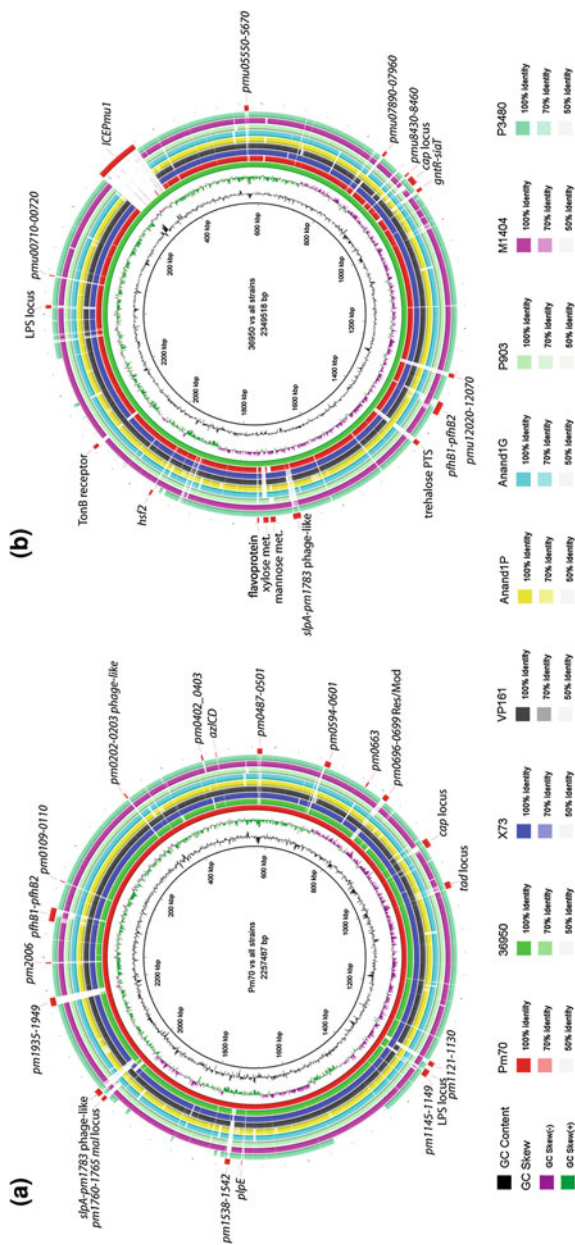


Fig. 2 Comparison of the genomes of 36950, X73, VP161, AnandIP, AnandIG, P903, M1404 and P3480 with the genome of Pm70 (a) and Pm70, X73, VP161, AnandIP, AnandIG, P903, M1404 and P3480 with the genome of 36950 (b). The three *inner rings* show the DNA size, GC content and GC skew of the reference genome (Pm70 in a and 36950 in b). The nine *outer rings* show regions of the comparison genomes which match the reference genome (shown as the inside *solid coloured ring*). *Red arcs* on the outside identify particular regions that are absent, or show reduced identity, in some or all of the comparison genomes. Figures were drawn using BLAST ring image generator (Alikhan et al. 2011)

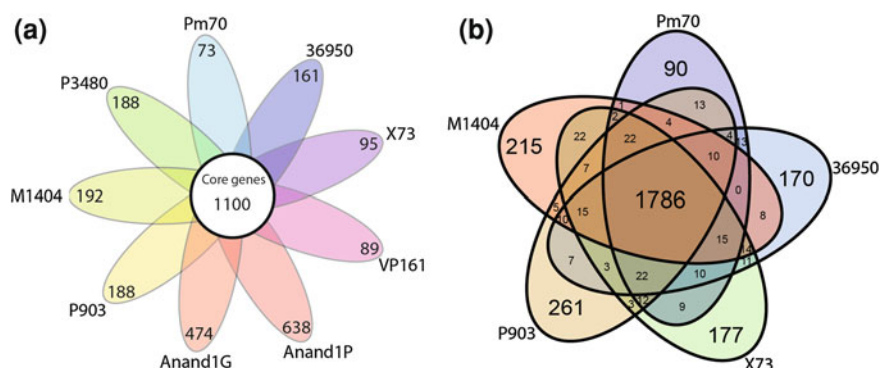


Fig. 3 Flowerplot of the unique genes in each of the *P. multocida* strain Pm70, 36950, X73, VP161, Anand1P, Anand1G, P903, M1404 and P3480 genomes (a) and a five set Venn diagram showing unique genes and all combinations of shared genes in the strains Pm70, 36950, X73, P903 and M1404. Orthologues were identified using ProgressiveMauve alignment (Darling et al. 2010) and have not been manually curated

of the 170 unique genes are within the ICE*Pmul* element, whereas for M1404 approximately 60 of the 215 unique genes are on DNA sections predicted to be within temperate phage or phage-derived elements.

The phylogenetic relationship between the nine strains was predicted by comparison of all single nucleotide polymorphisms at conserved genome positions. For the overall phylogenetic analysis, the three related species, *Gallibacterium anatis*, *Mannheimia haemolytica* and *P. dagmatis* were included as outliers (Fig. 4a). This tree, based on 7,931 SNPs at shared positions in all 12 strains, shows that *P. dagmatis* is the most closely related species and all of the *P. multocida* strains are very closely related to each other. The finer phylogenetic relatedness of the nine *P. multocida* strains shows that there appears to be little or no correlation between the phylogenetic relatedness of the strains and the country of isolation, the serogroup or serovar, the disease or the host predilection (Fig. 4b). It will be of interest to discover whether this trend continues as more *P. multocida* genomes are sequenced. A similar phylogeny was produced using the nucleotide sequences of the seven multi-locus sequencing typing genes described previously by Subaaharan et al. (2010) (data not shown).

4 Plasmids Carried by *P. multocida* Strains

While Pm70 and 36950 do not contain plasmids, some *P. multocida* strains carry multiple plasmids which may be either cryptic or carry antibiotic resistance genes. Initial studies on a *P. multocida* type D strain, isolated from a pig with atrophic rhinitis, identified the presence of four plasmids ranging in size from 1.5 to 5.4 Kb (Wright et al. 1997). The largest of these plasmids, designated pIG1, could replicate in both *P. multocida* and *E. coli* and carried genes for sulphonamide and streptomycin resistance. This plasmid was further identified in 11 of 12 Australian *P. multocida* pig isolates. The replication region of pIG1 was similar to the

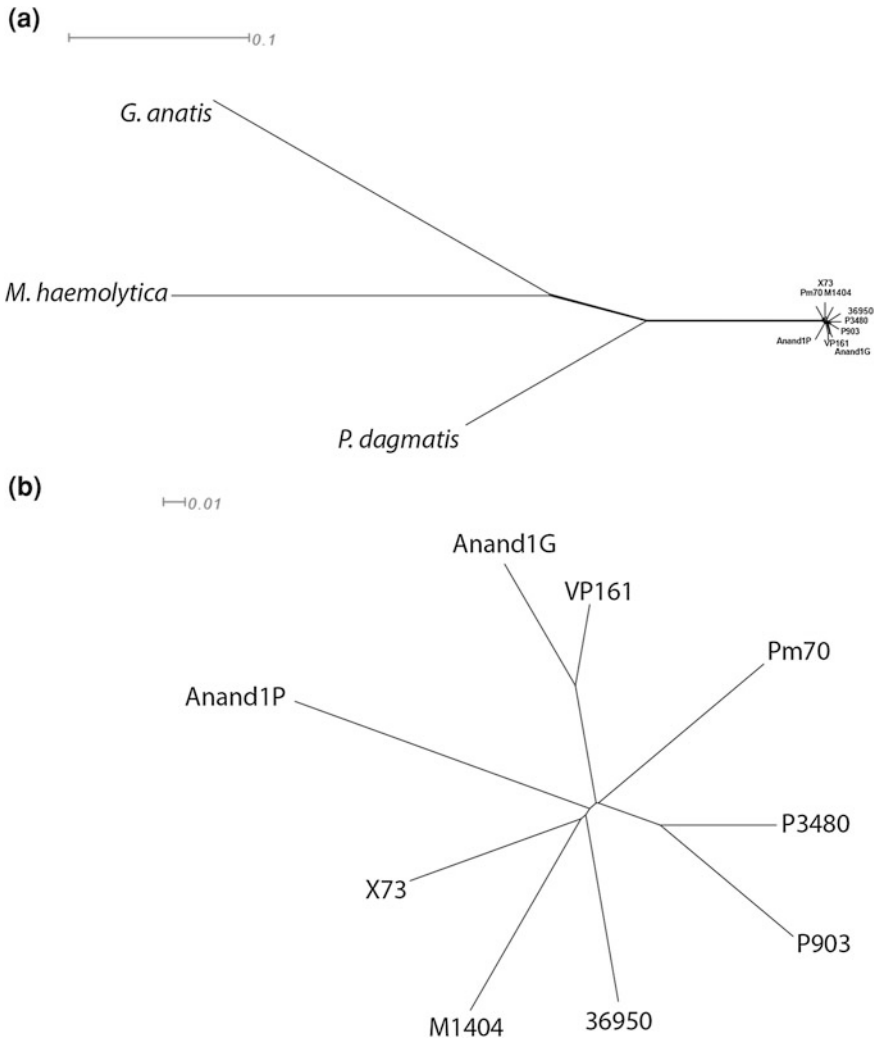


Fig. 4 Unrooted neighbour-joining tree showing the phylogenetic relationship between *Gallibacterium anatis*, *Mannheimia haemolytica*, *Pasteurella dagmatis* and the *P. multocida* strains Pm70, 36950, X73, VP161, Anand1P, Anand1G, P903, M1404 and P3480 (a) and only the *P. multocida* strains Pm70, 36950, X73, VP161, Anand1P, Anand1G, P903, M1404 and P3480 (b). Phylogenetic relatedness was determined by analysis of only the single nucleotide polymorphisms (SNPs) found at conserved positions in all genomes of the comparison set. The tree in (a) is based on analysis of 7,931 SNPs while the tree in (b) is based on analysis of 54,415 SNPs. The tree was rendered using SplitsTree4 (Huson and Bryant 2006)

corresponding region in the *Haemophilus ducreyi* plasmid, pLS88, and the *Mannheimia [P.] haemolytica* plasmid, pYFC1. Mobilisation of pIG1 into *E. coli* SM10 was dependent on three genes with shared identity to the *E. coli* ColE1 plasmid mobilisation genes *mbeA*, *mbeB* and *mbeC* (Wright et al. 1997).

A number of *P. multocida* plasmids have now been isolated that carry multiple resistance genes. The plasmid pVM111, isolated from a fowl cholera-causing strain, carries genes for sulphonamide, streptomycin and tetracycline resistance (Kehrenberg et al. 2003), while the mobilisable plasmid pCCK381 that was isolated from a serogroup A bovine respiratory disease isolate, carries genes for florfenicol, chloramphenicol and sulphonamide resistance. Interestingly, the plasmid replication and mobilisation genes on pCCK381 were highly similar to those on the *Dichelobacter nodosus* plasmid pDN1, whereas other sections were nearly identical to the *E. coli* plasmid pMBSF1 and the *Vibrio salmonicida* plasmid pRVS1. These data suggest that pCCK381 is the result of interplasmid recombination which may have been selected during florfenicol treatment of infected livestock (Kehrenberg and Schwarz 2005).

Recently it was shown that some antibiotic resistant *P. multocida* strains carry multiple plasmids, each with one or two antibiotic resistance genes (San Millan et al. 2009). While studying ampicillin resistant and multiple antibiotic resistant *P. multocida* isolates from pigs, a range of highly related plasmids was identified; pB1000 which was a ColE1-type plasmid, 4,613 bp in length and carried the ampicillin resistance gene *bla*_{ROB-1}, pB1005 encoding streptomycin and sulphonamide resistance and pB1006, pB1001 and p9956 that carried the tetracycline resistance genes *tet*(O), *tet*(B) or *tet*(H), respectively. Some strains contained more than one of these plasmids; ampicillin, streptomycin and sulphonamide resistant strains generally contained both pB1000 and pB1005, whereas some ampicillin, streptomycin, sulphonamide and tetracycline resistant strains contained pB1000, pB1005 and pB1006. The plasmids all contained highly related mobilisation and *oriV* regions. The *oriV* region is predicted to be responsible for plasmid replication and copy number control. Although the *oriV* regions of the different plasmids were closely related, they were not identical; it is assumed that these differences are sufficient to allow stable coexistence of up to at least three of these plasmids in the same cell (San Millan et al. 2009).

5 Bacteriophages of *P. multocida*

The genome sequence of Pm70 contains very few phage-like sequences or insertion elements and does not appear to contain any complete temperate phage genomes. However, the region between *slpA* and *pm1783* contains a number of hypothetical genes, two of which encode proteins with similarity to phage proteins: a predicted phage integrase (*SlpA*) and a predicted subunit of a phage terminase (Pm1777). This set of genes is also present in strain 36950, but not in X73, VP161, P903 or M1404 (Fig. 3). The Pm70 genes *pm0282* and *pm0283* are present only in Pm70 and encode proteins with similarity to the *E. coli* phage lambda proteins *ea59* and *ea31*, respectively.

Some *P. multocida* strains do contain complete temperate phage genomes (Campoy et al. 2006; Nielsen and Rosdahl 1990; Pullinger et al. 2004). Importantly, the primary virulence factor for porcine atrophic rhinitis, the *P. multocida* PMT toxin,

is carried on a lysogenic bacteriophage (Pullinger et al. 2004). Three toxigenic serotype D strains (LFB3, P27 and G4) were treated with the DNA-damaging agent mitomycin C and all released isometric-headed bacteriophage of the *Siphoviridae* class; this class also includes lambda-like phage. The *toxA* gene, encoding the *P. multocida* PMT toxin, was present in the genomes of all phage examined. The presence of *toxA* on a lysogenic phage may be important for both the expression and release of the toxin during disease progression. PMT has no signal sequence and is not secreted from intact *P. multocida* cells (iDali et al. 1991; Pullinger et al. 2004). However, PMT must be released from the bacteria to allow it to interact with the cell surface receptors on host cells and it has been suggested that induction of the phage lytic cycle during host infection may result in bacterial lysis and PMT release. Furthermore, the amplification of the phage genome during lytic growth may increase the expression of the PMT under these conditions (Pullinger et al. 2004). The details of the mode of action of PMT are reported in Molecular Biology of *Pasteurella Multocida* toxin.

One temperate transducing phage (F108) has been isolated from a *P. multocida* serovar A strain and its genome completely sequenced (Campoy et al. 2006). The morphology of the F108 temperate phage is similar to phage belonging to the *Myoviridae* family, with a 50 nm hexagonal head and a 120 nm long tail. Its genome is approximately 30 Kb in length, predicted to have cohesive ends and contains more than 40 genes. Neither F108, nor the PMT-containing phages, have been identified in any of the currently sequenced *P. multocida* genomes.

Analysis of the genomes of 36950, X73, VP161, P903 and M1404 identified a small number of putative temperate phage, or temperate phage-derived genes. Both strains X73 and M1404 contain genes encoding proteins with identity to Mu-like bacteriophage proteins; in M1404 this section is greater than 25 Kb in length and contains more than 40 genes, while in X73 it is approximately 14 Kb in length and contains 14 genes. While both sections have identity with Mu-like phages, there is little shared identity, indicating that these sections are not the result of the acquisition of an identical phage. Strain M1404 also contains a novel 15.7 Kb region containing genes with identity to known bacteriophage genes. This section comprises 19 ORFS and is situated at the tRNA^{Met} downstream of the Pm70 gene *pm1654*; it is of note that the F108 phage specifically integrates into the t33tRNA^{leu} (Campoy et al. 2006) and the lysogenic phage which carries the PMT toxin preferentially integrates into the t3tRNA^{leu}.

6 Whole-Genome Transcriptomic Analyses

The completion of the Pm70 genome sequence (May et al. 2001) facilitated the construction of *P. multocida* DNA microarrays containing all 2,015 genes identified in this genome. These microarrays were essential for a range of new analyses into *P. multocida* pathogenic mechanisms.

Iron is an essential nutrient for most bacteria, but free iron is present at limiting concentrations in the host. Indeed, in mammalian and avian hosts iron is primarily

bound to carrier proteins, including transferrin, lactoferrin and ferritin (Paustian et al. 2002). Initial annotation of the Pm70 genome indicated that it contained at least 53 genes encoding proteins predicted to be involved in iron uptake or acquisition (May et al. 2001); current analysis suggests that this number is more than 60 genes. Thus, it is likely that the acquisition of iron is critical for *P. multocida* survival in vivo. The large number of genes involved in iron uptake also suggests that *P. multocida* has a number of redundant, or partially redundant, iron uptake systems.

A number of DNA microarray studies has analysed the whole-genome transcriptional response of *P. multocida* to growth under limiting concentrations of iron or in the presence of different sources of iron (May et al. 2001; Paustian et al. 2002, 2001). When *P. multocida* strain Pm70 was grown under iron limitation in an otherwise rich medium (brain heart infusion containing 200 mM 2,2'-dipyridyl), more than 150 genes showed significantly (more than two-fold) altered expression. In general, the response to iron limitation resulted in a decrease in expression of genes involved in energy metabolism and an increase in expression of genes involved in iron transport, amino acid metabolism and central intermediary metabolism (Paustian et al. 2001). The genes *eno*, *gapdh*, and *pgk*, which encode proteins of the glycolytic pathway, were all down regulated. As expected under iron-limited conditions, major increases in expression were observed for numerous genes encoding iron transport proteins. The genes *yfeABCD*, *fbpABC*, *fecBCD*, *tonB*, and *exbBD* all showed increased levels of expression of between 2.1- and 7.5-fold under conditions of iron limitation. In addition, the genes encoding the global stress response proteins RecX, HslV and HtrA showed increased expression under iron limitation, as did genes encoding the cell surface/membrane biogenesis proteins (GmhA, Skp, HexC, and PonB). Together, these data suggest that growth in the presence of reduced iron results in structural effects on the bacterial outer membrane. Interestingly, 27% of the genes showing altered expression encoded proteins with no characterised function (Paustian et al. 2001). Today, ten years after these initial studies were completed, the majority of these proteins remains uncharacterised. However, current analysis of the 27 hypothetical proteins identified as up-regulated showed that ten have domains now known or predicted to be associated with iron binding and/or transport.

The gene expression pattern of *P. multocida* in response to the sole iron sources haemoglobin, transferrin, ferric citrate and ferritin was also assessed by DNA microarrays (Paustian et al. 2002). Growth in the presence of transferrin had the most significant global effect on *P. multocida* gene transcription, with 10% of genes showing increased expression and 8% showing decreased expression, while growth in the presence of ferritin resulted in increased or reduced expression of only 5% of genes. Surprisingly, only two genes, namely *ptsN* and *sapD*, showed altered expression under all of the tested conditions and many known iron acquisition genes did not show altered transcription under any of the conditions. The ferric uptake regulator Fur, was expressed at elevated levels in all conditions except when haemoglobin was used as the sole iron source.

While the coordinated transcriptional response of *P. multocida* to either low iron or defined iron sources is likely to be important for both bacterial survival in

the host and disease pathogenesis, the true response to the host environment can only be assessed using animal studies. To this end, the Pm70 DNA microarrays have also been used to characterise the bacterial response to growth in host tissues; either in the blood or livers of infected chickens (Boyce et al. 2002, 2004). As *P. multocida* reaches very high numbers in both tissues late in infection ($>1 \times 10^9$ CFU/gram of tissue), it is possible to purify sufficient bacterial RNA for DNA microarray experiments. Indeed, as a consequence of the ability to recover sufficient RNA from these in vivo samples, the *P. multocida* experiments were among the first to characterise the transcriptional response of a pathogen while growing in its natural host. For these experiments, the virulent fowl cholera-causing strain X73 was used to infect chickens and bacteria recovered from either the blood or liver tissue of birds in the late stages of disease. The *P. multocida* transcriptional response under these in vivo conditions was then compared with the transcriptome of bacteria grown in vitro in rich medium (brain heart infusion) at the same temperature (41°C, the normal body temperature of chickens).

Analysis of the gene expression pattern of *P. multocida* recovered from the blood of three infected chickens, indicated that 17 genes were increased in expression and 23 genes were decreased in expression during growth in blood as compared to growth in vitro in rich medium (Boyce et al. 2002). A number of the up-regulated genes (*gdhA*, *asnA*, *aspC*, *ilvH*, *dppA*) encoded proteins predicted to be involved in the biosynthesis, transport and conversion of amino acids. Furthermore, the nitrate reductase (*napF*, *napA*, *napC* and *napB*) operon was also strongly expressed in vivo; this pathway may be used for nitrate scavenging and the biosynthesis of glutamate. These data strongly suggest that there is a limited supply of amino acids in the blood (at least during the late stages of infection) and that amino acid synthesis is critical for growth in this niche. The gene expression profile of *P. multocida* recovered from infected liver tissue was in general statistically similar to that observed in bacteria recovered from blood, although a significantly larger set (49 genes) was up-regulated during growth in liver (Boyce et al. 2002). There was a significant increase in expression of genes encoding proteins involved in anaerobic metabolism, suggesting that the liver environment is more anaerobic than the in vitro control growth conditions, and an increase in expression of a number of genes involved in carbohydrate transport and metabolism, suggesting a shift to different carbohydrate resources for energy production. Perhaps, unsurprisingly, most of the genes identified by the in vivo microarray analyses were involved in metabolic processes required directly for *P. multocida* survival in the in vivo niche. While it is possible that some of the uncharacterised proteins identified by these analyses may encode true virulence factors, experiments to confirm this have not been reported. Indeed, recovering bacteria from earlier in infection may be more useful for the identification of virulence factors; unfortunately, experimental limitations mean that whole-genome analyses at very early time points are not possible at present.

Whole-genome DNA microarrays have also been used to analyse the response of *P. multocida* to the presence of antibiotics (Melnikow et al. 2008; Nanduri et al. 2009). The transcriptional response was measured following exposure to

novobiocin, tilmicosin, florfenicol, rifampin, trimethoprim, brodimoprim, cefquinome, enrofloxacin or thiazine (all at $1 \times$ the minimal inhibitory concentration (MIC)) and with enrofloxacin, amoxycillin and chlortetracycline (at $\frac{1}{4} \times$ MIC). When the bacteria were exposed to each antibiotic at $1 \times$ MIC, there was a major difference in the number of differentially regulated genes observed in response to the type of antibiotic used (Melnikow et al. 2008); bacteriostatic antibiotics resulted in large numbers of differentially expressed genes, whereas bactericidal antibiotics altered the regulation of very few genes. Indeed, addition of novobiocin resulted in altered expression of 39% of the genome whereas thiazine, enrofloxacin or cefquinome resulted in altered expression of less than 1.5% of the genome. Interestingly, the expression of many known virulence genes was reduced in the presence of antibiotics. Capsule biosynthesis genes showed reduced expression following all $1 \times$ MIC antibiotic treatments except when rifampin was used. Similarly, the iron transport genes *exbB*, *exbD* and *tonB* also showed reduced expression under almost all tested conditions. These data suggest that even without complete killing of a bacterial population many antibiotics may have effects through the suppression of *P. multocida* virulence gene expression.

7 Proteomic Analyses

The determination of the Pm70 genome sequence was also a prerequisite for complete proteomic studies, as the identification of proteins by mass spectrometry (peptide mass fingerprinting) requires knowledge of the sequence of all the proteins expressed by the strain. Initial proteomic studies of *P. multocida* characterised the protein components of the *P. multocida* outer membrane and determined how expression of these outer membrane proteins was altered under either low iron conditions or growth in the blood of infected chickens (Boyce et al. 2006). These analyses identified more than 30 proteins likely to be legitimate components of the *P. multocida* outer membrane, with the major outer membrane protein identified as Pm0786. Eleven of the outer membrane proteins were predicted lipoproteins, ten were predicted porins and half of these were predicted to be involved in iron uptake. One of these proteins, Pm0803, was only identified in the outer membrane fractions of bacteria grown under low iron conditions or recovered from the blood of infected chickens, indicating that it might be critical for in vivo survival. The gene encoding this protein was also identified as strongly up-regulated under low iron and in vivo growth conditions using DNA microarrays (Boyce et al. 2004; Paustian et al. 2001). However, a *pm0803* mutant is not attenuated for virulence (our unpublished data), consistent with functional redundancy in *P. multocida* iron transport systems.

Proteomic methods have also been applied to the analysis of the *P. multocida* response to sub-inhibitory ($\frac{1}{4} \times$ MIC) concentrations of antibiotic (Nanduri et al. 2006, 2008). Following antibiotic addition, *P. multocida* proteins were identified by two-dimensional liquid chromatography-electrospray ionisation tandem mass

spectrometry (2D-LC-ESI-MS/MS) using either unlabelled peptides, or peptides labelled with isotope-coded affinity tags (ICAT). Experiments using unlabelled peptides followed by 2D-LC-ESI-MS/MS identified more than 50% of the *P. multocida* Pm70 proteins (Nanduri et al. 2008) while the similar analysis using ICAT labelled peptides identified only 20% of the possible proteins (Nanduri et al. 2006). The more complete analysis (Nanduri et al. 2008) identified 147, 126 and 134 proteins as differentially expressed following treatment with amoxicillin, chlortetracycline or enrofloxacin, respectively. A strong shock response was detected with an increase in expression of the proteins GroES (following all treatments) and GroEL and DnaK (following amoxicillin treatment). Although a decrease in production of a small number of known virulence factors, including OmpA, was observed, the number of differentially expressed virulence factors was much less than was observed in similar transcriptional profiling experiments (see above).

8 Concluding Remarks

The publication and release of the first complete *P. multocida* genome in 2001 facilitated new approaches for studying *P. multocida* pathogenesis. Genomic analyses immediately identified the very large genetic repertoire of the organism for iron acquisition and identified the significant proportion of the genome used for production of the virulence factors, filamentous haemagglutinin and capsule. Furthermore, the use of whole-genome microarrays facilitated the dissection of how *P. multocida* adapts to survive under conditions of low free iron and within certain in vivo niches. However, the generation of multiple *P. multocida* genome sequences has lagged behind many other Gram-negative pathogens. Therefore, thorough comparative genomic analyses allowing for the identification of core and accessory *P. multocida* genes and/or disease-specific factors have not been possible. Only recently have new *P. multocida* genomes become available and it is hoped that once these are closed we can begin to define the genes responsible for *P. multocida* host specificity and identify those genes which make *P. multocida* the exquisitely adapted pathogen that it is.

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Pasteurella multocida

Molecular Biology, Toxins and Infection

Aktories, K.; Orth, J.H.C.; Adler, B. (Eds.)

2012, X, 150 p., Hardcover

ISBN: 978-3-642-31016-4