Fis Is Essential for Capsule Production in Pasteurella multocida and Regulates Expression of Other Important Virulence Factors

Jason A. Steen¹, Jennifer A. Steen¹, Paul Harrison², Torsten Seemann², Ian Wilkie³, Marina Harper¹, Ben Adler¹², John D. Boyce²⁴*

¹Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, Victoria, Australia, ²Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia, ³Veterinary Pathology and Anatomy, University of Queensland, St Lucia, Queensland, Australia, ⁴Department of Microbiology, Monash University, Clayton, Victoria, Australia

Abstract

P. multocida is the causative agent of a wide range of diseases of animals, including fowl cholera in poultry and wild birds. Fowl cholera isolates of P. multocida generally express a capsular polysaccharide composed of hyaluronic acid. There have been reports of spontaneous capsule loss in P. multocida, but the mechanism by which this occurs has not been determined. In this study, we identified three independent strains that had spontaneously lost the ability to produce capsular polysaccharide. Quantitative RT-PCR showed that these strains had significantly reduced transcription of the capsule biosynthetic genes, but DNA sequence analysis identified no mutations within the capsule biosynthetic locus. However, whole-genome sequencing of paired capsulated and acapsular strains identified a single point mutation within the fis gene in the acapsular strain. Sequencing of fis from two independently derived spontaneous acapsular strains showed that each contained a mutation within fis. Complementation of these strains with an intact copy of fis, predicted to encode a transcriptional regulator, returned capsule expression to all strains. Therefore, expression of a functional Fis protein is essential for capsule expression in P. multocida. DNA microarray analysis of one of the spontaneous fis mutants identified approximately 30 genes as down-regulated in the mutant, including pfhB_2, which encodes a filamentous hemagglutinin, a known P. multocida virulence factor, and plpE, which encodes the cross protective surface antigen PlpE. Therefore these experiments define for the first time a mechanism for spontaneous capsule loss in P. multocida and identify Fis as a critical regulator of capsule expression. Furthermore, Fis is involved in the regulation of a range of other P. multocida genes including important virulence factors.

Introduction

Pasteurella multocida is an important veterinary pathogen of worldwide economic significance; it is the causative agent of a range of diseases, including fowl cholera in poultry, hemorrhagic septicemia in ungulates and atrophic rhinitis in swine. P. multocida is a heterogeneous species, and is generally classified into five capsular serogroups (A, B, D, E and F) and 16 somatic LPS serotypes (1–16) [1]. Each serogroup produces a distinct capsular polysaccharide, with serogroups A, D and F producing capsules composed of hyaluronic acid (HA) [2], heparin and chondroitin [3], respectively. The structures of the serogroup B and E capsules are not known, although preliminary compositional analysis suggests that these capsules have a more complex structure than those produced by serogroups A, D and F [4]. The genes involved in biosynthesis, export and surface attachment of the capsular polysaccharide have been identified for all capsule types [5–7]. For strains which express HA, the capsule biosynthetic locus (cap) consists of 10 genes; phyA and phyB are predicted to encode proteins responsible for lipidation of the polysaccharide, hyaE, hyaD, hyaC and hyaB encode proteins required for polysaccharide biosynthesis and hexD, hexC, hexB and hexA encode proteins responsible for transport of the polysaccharide to the bacterial surface [6].

The P. multocida capsule is a major virulence determinant in both serogroup A and B strains. In the serogroup A strain X-73 (A:1), inactivation of the capsule transport gene hexA resulted in a mutant strain that was highly attenuated in both mice and chickens, and was more sensitive to the bactericidal activity of chicken serum [8]. Similarly, mutation of the hexA orthologue, cexA, in the serogroup B strain M1404 also resulted in significant attenuation; the M1404 cexA mutant was 4–6 times more sensitive than the parent to phagocytosis by murine macrophages [9].

Spontaneous capsule loss during in vitro sub-culture has been described in P. multocida [10,11]. In one study, acapsular variants were derived from capsulated parent strains by repeated laboratory passage (>30 sub-cultures) [12]. Sequence analysis of the cap locus in one of these acapsular variants identified two nucleotide changes...
Author Summary

*Pasteurella multocida* is an animal pathogen of worldwide economic significance. It causes fowl cholera in wild birds and poultry, hemorrhagic septicaemia in ungulates, and atrophic rhinitis in swine. The major virulence factor in fowl cholera-causing isolates is the polysaccharide capsule, which is composed of hyaluronic acid. Although there have been reports of spontaneous capsule loss in some strains, to date there has been no systematic investigation into the molecular mechanisms of this phenomenon. In this study, we characterize for the first time the underlying transcriptional mechanisms required for the expression of capsule in *P. multocida*, and identify a transcriptional regulator required for capsule production.

near the putative promoter region, but the authors did not determine whether these changes were responsible for the observed acapsular phenotype. No further work has been published on the genetic mechanisms of regulation of *P. multocida* capsule production.

Fis is a growth phase-dependent, nucleoid-associated protein which plays a role in the transcriptional regulation of a number of genes in diverse bacterial species (reviewed in [13]). In *Escherichia coli*, Fis is expressed at high levels in actively growing cells (>50 000 molecules per cell in early exponential growth phase), and expression drops to very low levels during stationary phase [14,15]. In addition to growth phase regulation, levels of Fis are negatively regulated by the stringent response during nutrient starvation [16]. Fis can act as both a positive or negative regulator of transcription and it has both direct and indirect effects on gene transcription. In *E. coli* and *Salmonella*, Fis binds to a degenerate 15-bp consensus sequence GNtYAaWWWtTRaNC, inducing DNA bending, but only a few of the sequences fitting this consensus are high affinity binding sites [17,18]. Fis is involved in the regulation of genes encoding a wide range of functions, including quorum sensing in *Vibrio cholerae* [19], and certain virulence factors in *Erwinia chrysanthemi* [20], pathogenic *E. coli* [21,22] and *Salmonella* [23].

In this study, we have characterized three independently isolated spontaneous acapsular derivatives of the *P. multocida* A1 strain VP161. Whole genome sequencing and DNA microarrays were used to show that the global regulator Fis not only controls the expression of capsule biosynthesis genes in *P. multocida*, but also regulates a number of other genes, including known and putative virulence factors.

Results

Identification of spontaneous acapsular *P. multocida* strains

Spontaneous capsule loss has been reported previously in *P. multocida*, and is generally associated with long term *in vitro* passage on laboratory media [10–12]. During routine strain maintenance of a signature-tagged mutagenesis library, we identified three independent *P. multocida* strains that presented with both large mucoid and small non-mucoid colonies after recovery from short term (<1 year) −80°C glycerol storage. Re-isolation of either colony type resulted in stable populations with colony morphologies identical to those of their parents, such that AL609, AL622 and AL620 gave rise to the small non-mucoid variants AL1114, AL1162 and AL1396 and to the large mucoid variants AL1115, AL1163 and AL1397, respectively (Table 1). Quantitative HA assays confirmed that all three small, non-mucoid colony variants (AL1114, AL1162 and AL1396) expressed significantly less capsule material than their paired large, mucoid colony variants (AL1115, AL1163 and AL1397) and the parental VP161 strain (Fig. 1). Indeed, the small colony variants expressed similar levels of HA to that expressed by a defined (hyaB) acapsular polysaccharide biosynthesis mutant generated by single crossover allelic exchange (AL919; Table 1) (Fig. 1). As each of the paired strains was derived from transposon mutants with different transposon insertion sites (AL609, AL622 and AL620; Table 1), and the transposon was still present at identical positions in both the paired mucoid and non-mucoid derivatives of each type, we concluded that the acapsular phenotype was independent of the initial transposition event.

Decreased capsule production in the acapsular strains results from decreased transcription of the *cap* biosynthetic locus

The genes responsible for HA capsule polysaccharide biosynthesis and transport have been identified previously and are located in a single region of the *P. multocida* chromosome [6] (Fig. 2). In order to investigate whether the loss of capsule production in these spontaneously arising acapsular variants was due to a mutation in the *cap* biosynthetic locus, the nucleotide sequence of the entire 14,935 bp locus of AL114 and the wild-type parent VP161 was determined. Comparison of the sequences revealed that they were identical; indicating that the acapsular phenotype observed in AL1114 was not due to mutation within the *cap* locus.

As there were no sequence changes observed between these two strains, we investigated transcription across the *cap* locus by quantitative real-time RT-PCR (qRT-PCR). Transcription of the *P. multocida* capsule biosynthetic genes is predicted to initiate from divergent promoters located in the intergenic region between the divergent *phaA* and *hyaE* genes (Fig. 2, [6]). Transcription of *phaA* and *hyaE* was significantly reduced in the acapsular variant AL1114 as compared to both the paired capsulated strain AL1115 and the parent strain VP161 (Fig. 3). Furthermore, a directed *P. multocida* *hyaB* mutant (AL919, Table 1), showed high levels of transcription across both genes showing that transcription across the locus is not affected by the level of capsule polysaccharide on the cell surface. These data show that the reduced capsule production in the acapsular variant AL1114 was likely a result of reduced transcription across the biosynthetic locus.

To characterize further the transcriptional regulation of the *cap* locus, we identified the position of the *hyaE* transcriptional start site by fluorescent primer extension (Fig. 2). A primer extension product was generated from *P. multocida* VP161 RNA using the primer BAP5476 (Table 2). A 190 bp extension fragment was detected, (Fig. 2B), which identified the transcript start site for *hyaE* as 37 bp upstream of the *hyaE* start codon (Fig. 2). The −10 and −35 regions of the *hyaE* promoter were predicted based on the identified transcript start site (Fig. 2). Repeated attempts failed to detect a primer extension product corresponding to *phaA*.

To determine the activity of the *phaA* and *hyaE* promoters in both encapsulated and non-encapsulated strains, the intergenic region between *phaA* and *hyaE* (Fig. 2) was cloned in both orientations into the *P. multocida* promoter-detecting vector pMKΩ (Table 1; [24]) to generate pAL596 and pAL597. In pAL597 the pMK2 kanamycin resistance gene is under the control of the *hyaE* promoter while in pAL596 it is under the control of the putative *phaA* promoter. These plasmids were then tested for their ability to confer kanamycin resistance on the wild-type *P. multocida* strain VP161, the encapsulated strain AL1115 and the acapsular variant AL1114 (Table 1). Both plasmids conferred higher levels of kanamycin resistance to the wild-type strain VP161 and the encapsulated strain AL1115 than when present in the acapsular
Table 1. Strains and plasmids used in this study.

| Strains/plasmid | Genotype and Description | Reference/source |
|-----------------|--------------------------|-----------------|
| **E. coli**     |                          |                 |
| Sm10 λ. pir     | Strain for propagation of pUA826 and its derivatives. | [69] |
| AL912           | Sm10 λ. pir containing pAL499 | This study |
| **P. multocida**|                          |                 |
| VP161           | Wild type P. multocida serotype A:1 strain. Capsulated and virulent | [70] |
| AL435           | VP161 carrying a Tn916 insertion in pm1417. Capsulated and virulent. TetR | [57] |
| AL609           | VP161 carrying a Tn916 insertion in viaA | This study |
| AL620           | VP161 carrying a Tn916 insertion between galR and mglB | This study |
| AL622           | VP161 carrying a Tn916 insertion between thiM and pm1263 | This study |
| AL919           | Single cross-over hyaB mutant of AL435. Expresses no polysaccharide capsule | This study |
| AL1114          | Small non-mucoid colony variant of AL609. Expresses no polysaccharide capsule | This study |
| AL1115          | Large mucoid colony variant of AL609. Expresses polysaccharide capsule | This study |
| AL1162          | Small non-mucoid colony variant of AL622. Expresses no polysaccharide capsule | This study |
| AL1163          | Large mucoid colony variant of AL622. Expresses polysaccharide capsule | This study |
| AL1164          | VP161 carrying a Tn916 insertion in the capsule biosynthesis gene phyB. Contains the plasmid pAL597 | This study |
| AL1396          | Small non-mucoid colony type derivative of AL620. Expresses no polysaccharide capsule | This study |
| AL1397          | Large mucoid colony type derivative of AL620. Expresses polysaccharide capsule | This study |
| AL1398          | AL1114 containing vector pPBA1100 | This study |
| AL1399          | AL1114 containing pAL727 | This study |
| AL1400          | AL1162 containing vector pPBA1100 | This study |
| AL1401          | AL1162 containing pAL727 | This study |
| AL1402          | AL1396 containing vector pPBA1100 | This study |
| AL1403          | AL1396 containing pAL727 | This study |
| AL1404          | VP161 marker-free fis TargeTron mutant generated using pAL706 | This study |
| AL1405          | VP161 marker-free fis TargeTron mutant generated using pAL708 | This study |
| AL1571          | VP161 containing pAL795 | This study |
| AL1572          | AL1114 containing pAL795 | This study |
| AL1573          | AL1115 containing pAL795 | This study |
| AL1574          | VP161 containing pAL796 | This study |
| AL1575          | AL1114 containing pAL796 | This study |
| AL1576          | AL1115 containing pAL796 | This study |
| AL1577          | VP161 containing pAL797 | This study |
| AL1578          | AL1114 containing pAL797 | This study |
| AL1579          | AL1115 containing pAL797 | This study |
| AL1580          | VP161 containing pAL798 | This study |
| AL1581          | AL1114 containing pAL798 | This study |
| AL1582          | AL1115 containing pAL798 | This study |
| AL1583          | VP161 containing pAL799 | This study |
| AL1584          | AL1114 containing pAL799 | This study |
| AL1585          | AL1115 containing pAL799 | This study |
| AL1586          | VP161 containing pMKΩ | This study |
| AL1587          | AL1114 containing pMKΩ | This study |
| AL1588          | AL1115 containing pMKΩ | This study |
| AL1654          | VP161 containing pAL597 | This study |
| AL1655          | AL1114 containing pAL597 | This study |
| AL1656          | AL1115 containing pAL597 | This study |
| AL1657          | VP161 containing pAL596 | This study |
| AL1658          | AL1114 containing pAL596 | This study |
| AL1659          | AL1115 containing pAL596 | This study |
strain AL114 (Fig. 4). In contrast, the acapsular strain AL114, harboring either pAL596 or pAL597 remained kanamycin sensitive, indicating that the phyA and hyaE promoters are inactive in this strain. These data show that both the phyA and hyaE promoters are active only when present in the encapsulated strains VP161 and AL1115, indicating that the activity of these promoters is regulated by a trans-acting transcriptional regulator which is inactive in the acapsular strain AL114.

Identification of the transcriptional regulator of *P. multocida* capsule expression

In order to identify the trans-acting transcriptional regulator responsible for the regulation of capsule expression, we sequenced the entire genomes of the acapsular variant AL114 and its paired capsular strain AL1115, using high-throughput short read sequencing. Reference assemblies were generated for both strains using the fully annotated *P. multocida* PM70 genome [25] as a scaffold. These assemblies were then used to determine nucleotide differences between AL1114 and AL1115, and thus identify changes unique to AL1114. Three nucleotide changes were identified as unique to AL1114 when compared to AL1115 (Table 3); of these, the mutations within *asd* and *bnaA* were silent and did not result in amino acid substitutions. However, the observed nucleotide T to C transition within an ORF annotated as encoding Fis, would result in a non-conservative L28S amino acid substitution within the Fis protein. The deduced *P. multocida* Fis protein is 99 amino acids in length and shares 80% identity with the 98 amino acid *E. coli* Fis (NCBI GeneID 947697; http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=947697) which has been shown to be a nucleoid-associated transcriptional regulator.

To confirm that the mutation observed in *fis* was associated with loss of capsule expression, the nucleotide sequence of *fis* was determined by Sanger dideoxy sequencing in all three acapsular variants (AL1114, AL1162 and AL1396) and their paired capsulated strains (AL1115, AL1163 and AL1397). Analysis of these sequence data confirmed the L28S mutation in Fis in AL1114 compared to AL1115. In AL1162, a G to T transversion at nucleotide 3 resulted in a change from the methionine start codon to the non-start codon isoleucine (ATT), stopping translation of Fis. In AL1396, a C to T substitution at nucleotide 222 resulted in a nonsense mutation (Q75*) and termination of the Fis protein at amino acid 74. These data are consistent with the hypothesis that mutation of *fis* was correlated with loss of capsule expression in all three spontaneous acapsular *P. multocida* strains.

Functional Fis is required for capsule expression

In order to confirm Fis as the transcriptional regulator of capsule expression, we complemented each of the spontaneous acapsular variants with an intact copy of Fis in trans. In *E. coli*, fis is auto-regulated, and is expressed as part of a two gene operon together with the upstream gene *yhdG*, predicted to encode a tRNA-dihydrouridine synthase [14,16,26]; the same organization is observed in *P. multocida*. We initially attempted to clone *fis* by itself but were unable to successfully make this construct. Therefore, we cloned *fis*, the overlapping upstream gene *pm1087* and the predicted native promoter, into the *P. multocida* shuttle vector pPBA1100, generating the plasmid pAL727 (Table 1). The plasmid pAL727 and the vector only (pPBA1100) were separately introduced into each of the acapsular variants AL1114, AL1162 and AL1396, respectively. All three variants containing the plasmid pAL727 expressed capsules, and all three variants containing only the vector remained acapsular. This confirmed the regulatory role of Fis in capsule expression.

Table 1. Cont.

| Strains/plasmid | Genotype and Description | Reference/source |
|-----------------|-------------------------|-----------------|
| Plasmids        |                         |                 |
| pJIR750ai       | Commercial TargeTron vector | Sigma-Aldrich   |
| pAL99           | *P. multocida*-E. coli expression vector. Cloned fragments are expressed from the *P. multocida* tpiA promoter [71] |
| pAL499          | pUA826 containing a 720 bp internal fragment of *hyaB*. For single-crossover insertional mutagenesis. This study |
| pAL596          | pMK2 containing the 738 bp intergenic region between *phyA* and *hyaE*. Has the *phyA* promoter directing expression of the kanamycin resistance gene. This study |
| pAL597          | pMK2 containing the 738 bp intergenic region between *phyA* and *hyaE*. Has the *hyaE* promoter directing expression of the kanamycin resistance gene. This study |
| pAL692          | *P. multocida*-specific TargeTron vector. Strep®Spec® |
| pAL705          | *P. multocida*-specific TargeTron vector. Kan® |
| pAL706          | pAL705 retargeted to *fis* using BAPS932, 5933, 5934 This study |
| pAL708          | pAL692 retargeted to *fis* using BAPS932, 5933, 5934 This study |
| pAL727          | Fis complementation construct containing a 1415 bp BamHI/Sall fragment containing *fis* and the upstream overlapping ORF *pm1087* cloned in pPBA1100 This study |
| pAL795          | pMK2 containing the 206 bp region upstream of *pgaA* This study |
| pAL796          | pMK2 containing the 466 bp region upstream of *pm0998* This study |
| pAL797          | pMK2 containing the 411 bp region upstream of *pm1078* This study |
| pAL798          | pMK2 containing the 1049 bp region upstream of *lspB_2* This study |
| pAL799          | pMK2 containing the 400 bp region upstream of *pm1818* This study |
| pPBA1100        | *P. multocida*-E. coli shuttle vector [72] |
| pMK2           | *P. multocida* promoter detecting vector [24] |
| pUA826          | λ, pir dependent conjugative plasmid for single-crossover mutagenesis in *P. multocida*. A modified derivative of pUA826 containing the *P. multocida* tpiA promoter which allows for the transcription of downstream genes after insertional mutagenesis. Strep®, Spec® [57] |

doi:10.1371/journal.ppat.1000750.t001
Identification of *P. multocida* genes regulated by Fis

Fis is known to be a global regulator in a number of bacterial species [15,20,27]. We therefore used whole genome DNA microarrays to compare the transcriptome of the acapsular variant AL1114 (expressing Fis L28S) with the encapsulated paired strain AL1115 (expressing wild-type Fis). Custom Combinatrix 12k microarrays were designed and used as described in Materials and Methods. Thirty one genes were identified as significantly down-regulated and eleven genes as significantly up-regulated in the acapsular variant AL1114 (Table 4). Seven of the ten capsule biosynthesis genes were identified as down-regulated in AL1114 compared to AL1115, supporting the qRT-PCR data showing reduced transcription of *phylA* and *hyaE*. Expression of the known *P. multocida* virulence gene *phylB_2* and its predicted secretion partner *lpB_2* was reduced 3- to 4-fold in AL1114. In addition, the gene encoding the outer membrane lipoprotein *PpE*, which is a surface exposed lipoprotein that can stimulate cross-serotype protective immunity against *P. multocida* infection [28], was also down-regulated in the acapsular strain AL1114. Other down-regulated genes included *pglA*, encoding a cryptic heparosan synthase; *exhB*, an iron-regulated virulence factor; *pm1042*, encoding a predicted LPS-specific phosphoethanolamine transferase; *hha*, encoding a heat shock protein; *fruB*, encoding the fructose operon repressor; *glmS* encoding a fructose-6-phosphate aminotransferase, and a range of genes encoding proteins of unknown function. Eleven genes were identified as up-regulated in the acapsular strain. These included *pm1819* and *pm1820*, which encode proteins with similarity to the *Salmonella* SrIB and SrIC proteins, both putative virulence factors also controlled by Fis [23], *tadC*, a gene predicted to be involved in an outer membrane secretion system, and seven genes encoding proteins of unknown function. Several of the genes identified as differentially expressed were physically linked on the chromosome and given their similar expression patterns we predict that these are expressed as operons (e.g., *plpE*, *pm1516* and *pm1515*, *pm0996*, *pm0997* and *pm0998*, *pm1819*, *pm1820* and *pm1821*; Table 4).

Confirmation of the microarray data

In order to confirm the differential expression of some of the genes identified by the microarray analyses, two different methods were used. Firstly, the putative promoter regions of each of the down-regulated genes *pglA*, *pm0998*, *pm1078*, *lpB_2* and the region upstream of *pm1818* containing the putative promoter for the operon containing the up-regulated genes *pm1819*, *pm1820* and *pm1821*, were cloned into the *P. multocida* promoter probe vector pMK2 (Table 1), generating the recombinant plasmids pAL795, pAL796, pAL797, pAL798 and pAL799, respectively (Table 1). The recombinant plasmids were transformed into the acapsular strain AL1114 (expressing Fis L28S), the capsulated paired strain AL1115 (expressing wild-type Fis) and the wild-type parent strain VP161 (Table 1). With the exception of pAL799, each of the recombinant pMK2 derivatives conferred higher levels of kanamycin resistance to AL1115 and the wild-type VP161 than to the acapsular variant AL1114 (Fig. 4). These data support the microarray results and show that the activity of the promoters for *pglA*, *pm0998*, *pm1078* and *lpB_2* are significantly reduced in the absence of wild-type Fis. Each of the strains harboring pAL799 showed similar levels of kanamycin resistance regardless of the capsule phenotype, suggesting that the cloned fragment in this construct does not contain a Fis regulated promoter, or that the fragment does not contain all the necessary Fis binding sites required for repression of this promoter.

As a second method of confirmation, qRT-PCR and western immunoblot analyses were undertaken to confirm the reduced
expression of \textit{plpE} in AL1114. \textit{PlpE} is a predicted outer membrane lipoprotein which can stimulate cross-serotype protective immunity against \textit{P. multocida} [28]. Microarray analysis indicated that the \textit{plpE} transcription was reduced by approximately 3.8-fold in the spontaneously arising acapsular strain (Table 4). Transcriptional analysis of \textit{plpE} by qRT-PCR confirmed that the transcription of this gene was significantly reduced (data not shown). Western immunoblots using antiserum generated against recombinant \textit{PlpE} (Fig. 5) confirmed that \textit{PlpE} expression was significantly reduced in the spontaneously arising acapsular variants AL1114 and AL1162, as well as a directed \textit{plpE} mutant AL1172 (Table 1), but not in the directed \textit{phyB} acapsular strain AL1164. Therefore, \textit{PlpE} mRNA levels are positively regulated by \textit{Fis}.

**Discussion**

The expression of a polysaccharide capsule is critical for the virulence of \textit{P. multocida} [8,9]. While there is evidence that the level of capsule expression in \textit{P. multocida} responds to certain environmental conditions (such as growth in the presence of antibiotics, low iron or specific iron sources such as hemoglobin [29–32], there is no information on the mechanism of capsule regulation. There have been numerous reports of spontaneous loss
of capsule expression in P. multocida strains following in vitro passage
[10,11], but the mechanism by which this occurs has not been
determined. Previous work with laboratory-derived, spontaneous
acapsular strains has indicated that loss of capsule expression was
associated with reduced transcription of genes within the capsule
biosynthesis locus [12]. In this study, we identified three
independent spontaneous acapsular strains and showed that loss of
capsule expression in these strains was also due to reduced
transcription of the cap locus. Furthermore, we showed that this
reduced transcription was due to point mutations within the gene
encoding the global transcriptional regulator Fis. Thus, Fis is
essential for capsule expression and these experiments define a
mechanism by which spontaneous capsule loss can occur and
identify for the first time a transcriptional regulator required for
capsule expression in P. multocida. Importantly, while Fis has been
shown to be involved in the regulation of a large number of genes
in a range of bacterial species, to our knowledge this is the first
report showing a role for Fis in the regulation of capsule
biosynthesis. Furthermore, this is the first report that a functional
Fis protein is expressed in P. multocida and that it acts as a regulator
of gene expression.

Fis was initially identified as the factor for DNA inversion in the
Hin and Gin family of DNA recombinases [33,34]. Subsequently,
diverse roles for Fis have been described, including both positive
and negative regulation of gene expression. Fis has also been
identified in other members of the Pasteurellaceae, including
Haemophilus influenzae Rd. While the H. influenzae Fis shared 81%
identity with the E. coli Fis, it did not display identical activity [35].
Interestingly, P. multocida Fis shares 92% identity with the H.
influenzae Fis but only 80% identity with E. coli Fis.

Structurally, Fis folds into four α-helices (A–D) and a β-hairpin
[36]. Helices A and B provide the contacts between Fis monomers,
facilitating dimer formation, whereas the C and D helices form a
helix-turn-helix motif that is essential for DNA binding [37–39].
We identified three spontaneous acapsular strains with point
mutations within fis. The Fis mutation L28S is predicted to result in
a highly unstable protein, as a mutation in the equivalent
position in the E. coli Fis (L27R) resulted in an unstable protein
with no discernible activity [36]. However, as the P. multocida Fis
shares only 80% amino acid identity with the E. coli Fis, we can not
rule out the possibility that the P. multocida L28S Fis is a partially
functional protein that is impaired in only some specific functions
of WT Fis. Computational models also suggest a requirement for
hydrophobic residues at this position [40] and the substitution of
leucine by the hydrophilic residue serine would significantly
reduce the hydrophobicity at this position. The second spontaneous
acapsular variant, AL1162, contained a mutation within the fis
start codon, which would result in complete abrogation of Fis expression.
Finally, the fis gene in AL1396 contained a nonsense mutation at nucleotide 222, terminating protein translation at
amino acid 74. This mutant Fis protein would lack the last 16
amino acids, including the helix-turn-helix motif that is essential
for DNA binding [39].

As Fis is known to regulate a number of genes in other species,
we used DNA microarrays to compare the transcriptome of wild-
type P. multocida and the Fis L28S mutant (AL1114) during
aerobic growth. Comparison of the fis mutant strain with wild-
type P. multocida identified 31 genes (representing at least 20
predicted operons) as positively regulated by Fis, and 11 genes
( representing at least nine operons) as negatively regulated by Fis.
In E. coli, two transcriptional studies have been conducted
comparing gene expression in wild-type and Fis mutant strains.
The first study identified more than 200 genes that were regulated
by Fis at various growth phases (＞2 fold change, p＜0.05) [15].
The second study identified ＞900 genes (21% of the E. coli
genome) that were significantly differentially regulated in the fis
mutant (false discovery rate ＜1%), although only 17 of these were
＞2 fold differentially regulated [27]. Interestingly, approximately
70% of the 900 genes shown to be differentially regulated in the
second study showed no Fis binding as determined by chromatin
immuno precipitation coupled with high resolution whole genome
arrays [27]. In P. multocida we identified 42 genes as
differentially regulated by Fis during the exponential growth
phase. During this growth phase the regulation of P. multocida
genes by Fis was skewed towards the up-regulation of genes by
Fis (70% of operons), indicating that Fis generally acts as a
transcriptional activator, a finding consistent with previous studies
[15,27].

Both P. multocida and E. coli Fis share significant similarity across
the C-terminal DNA binding region, suggesting that they may
recognize similar sequences. However, using the available E. coli
position specific weight matrix [15], we were unable to identify
conserved sites upstream of all of the Fis regulated genes identified
in the DNA microarray experiments. This is not entirely unexpected,
| Oligo | Sequence (5'-3') | Description |
|-------|-----------------|-------------|
| BAP169 | GTAAGGATTGCGATTGC | Reverse primer flanking hyaB |
| BAP276 | GAAATTTAATGTTGGTG | Forward primer flanking hyaB |
| BAP2782 | GCCCTACAAATTGGGAGA | pUA826 specific primer |
| BAP4399 | GAAGAGCGTCGACTTCATACGGCATT |.hyA internal forward primer for mutagenesis. |
| BAP4995 | CGACTCGAGCATCTGAGGATA | qRT-PCR of the VP161 hyaE |
| BAP4996 | GTCAAGGATTGCCAATATCTAAT | qRT-PCR of the VP161 hyaE |
| BAP4997 | ATGCCGAGATCATCTGGGATA | qRT-PCR of the VP161 phyA |
| BAP4998 | TGCACATCTCACTGCGGTTTGGAG | qRT-PCR of the VP161 phyA |
| BAP5091 | CATGTGGATCTTTCATCAAGACAAATGGAAGAG | Forward primer for amplification of the intergenic region between phyA and hyaE |
| BAP5092 | TCGTTGGATCTTTCATCAAGACAAATGGAAGAG | Forward primer for amplification of the intergenic region between phyA and hyaE |
| BAP5476 | GGTCAAGGTTAGCCCAATAATCTAAT | 5'-6-FAM labelled primer for fluorescent primer extension of hyaE promoter |
| BAP5358 | CGACTTCGACATCTGGGATA | For amplification of the pAL99 vector excluding the kanamycin resistance gene |
| BAP5359 | CGTGAGATCATCTGGGATA | For amplification of the pAL99 vector excluding the kanamycin resistance gene |
| BAP5360 | CGAGATATCGCAACACTTTCCTACTAC | For amplification of the aadA gene from pUA826 |
| BAP5361 | AATTGCTATCGGATCATCTGGGATA | For amplification of the aadA gene from pUA826 |
| BAP5433 | CTGGGATATCGCCTTGAAGGTTGGAGAAAG | For amplification of the kanamycin resistance gene from pAL99 |
| BAP5434 | CCCAGATATCGCAACACTTTCCTACTAC | For amplification of the kanamycin resistance gene from pAL99 |
| BAP5540 | CGTTGAGATCATCTGGGATA | qRT-PCR of the VP161 pipE |
| BAP5541 | AAGAAATCATACAGGCGGTTTATGT | qRT-PCR of the VP161 pipE |
| BAP5826 | GACGTGATCCCGCACCAATGTCACGAC | Reverse primer for amplification of predicted pm1078 promoter region |
| BAP5827 | CCACAGGATATCCACTTCAAACTATAGCAAC | Reverse primer for amplification of predicted pm1078 promoter region |
| BAP5828 | TACCCGATATGCTCTAAAGAGCCACTC | Forward Primer for amplification of predicted pm0998 promoter region |
| BAP5829 | TATTTGAGATCCAAACCTGTAATAAAATGAAATTG | Reverse primer for amplification of predicted pm0998 promoter region |
| BAP5832 | ACAAAGGTAGCCGCTAAAGTGCGTTAATAC | Reverse primer for amplification of predicted lsp8_2 promoter region |
| BAP5833 | AGTAGCGATCACAATAAATAATTTCAATCT | Reverse primer for amplification of predicted lsp8_2 promoter region |
| BAP5834 | ACATTTGAGATCCCGAATTAACGACCGCTGTG | Forward primer for amplification of predicted pg14 promoter region |
| BAP5835 | CATGTCAGATCGAGATTATTGGATCATCTTTTGGTATTTTGG | Reverse primer for amplification of predicted pg14 promoter region |
| BAP5838 | TGCGTGAGATCGTCCTCCTCATACATCAGCTAG | Reverse primer for amplification of predicted pm1818 promoter region |
| BAP5839 | CAATTGAGATCCCACTTCGCCCCAATGTC | Forward Primer for amplification of predicted pm1818 promoter region |
| BAP5932 | AAAAAAGCTTATAATTACCTTACTAACCAGTGATACGACCCGATGAGGTTG | Fis - 3203|3204s-IBS. |
| BAP5933 | CAGATTTGACAATGTTGGATACGATAAGCTGATACGATATACCTACCTTCTTTGTTT | Fis - 3203|3204s-EBS1d |
| BAP5934 | TGAACAGATTCTTTTCAGGTTTTTCTGATTGCTGATCGAGGAAGGTGTT | Fis - 3203|3204s-EBS2 |
| BAP5967 | ATGAAGTCCAGCGACGGAAAATCTAAGTCCGC | Reverse primer for amplification of pm1087/fis operon |
| BAP5968 | TTGCTGAGATCTTACAGGAAATTCAACCTAATC | Primer within fis |
| BAP5969 | GCTAAATGTATCTATAGCGTCCTCTATCG | Primer for amplification of pm1087/fis operon |

*restriction sites are underlined.
doi:10.1371/journal.ppat.1000750.t002
as Fis has been shown to bind a variety of AT-rich sequences more or less non-specifically [13]. Indeed, the ability of Fis to induce DNA bending is probably the most important factor in its ability to control transcription [41]. The relatively low number of *P. multocida* genes regulated by Fis in the exponential growth phase is somewhat surprising. However, as Fis expression in other bacteria has been shown to be growth phase dependent [15,42,43], it is likely that other *P. multocida* genes will be differentially regulated by Fis during different growth phases. While an equivalent L27R mutation in *E. coli* Fis results in an unstable protein, we can not rule out the possibility that the L28S mutation in *P. multocida* Fis results in a protein that retains some, but not all, Fis regulatory functions. Thus, it is possible that more genes might be observed as differentially regulated in a Fis null mutant strain and we are currently investigating this possibility.

Of the 42 differentially expressed genes identified in the *P. multocida* Fis mutant, a large number (16/42; 38%) encode proteins that are surface expressed or involved in the synthesis of surface exposed structures. Clearly Fis regulates the genes involved in the biosynthesis of, and surface presentation of, capsular polysaccharide, and expression of these genes is critical for virulence. Fis is also involved in the regulation of the surface exposed virulence factor PfhB_2 and its outer membrane secretion partner LspB_2 and the surface expressed lipoprotein PlpE. Both PlpE and PfhB_2 factor PfhB_2 and its outer membrane secretion partner LspB_2 are also involved in the regulation of the surface exposed virulence factor PfhB_2 and its outer membrane secretion partner LspB_2. PfhB_2 and PfhB_2, a gene located within the tight adherence locus that has been shown in *Aggregatibacter actinomycetemcomitans* to be responsible for non-specific attachment to surfaces and is required for full expression of the RcpABC outer membrane proteins [45]; *pm1078*, encoding a putative iron-specific ABC transporter component; and the genes encoding PM0998 [46], PM1050 and PM1819 [47] which have all been experimentally shown to be present in outer membrane sub-fractions of the *P. multocida* proteome. Fis also regulates the expression of ExbB, an inner membrane protein that interacts with TonB and is critical for iron uptake and virulence in *P. multocida* [48].

The *P. multocida* filamentous hemagglutinin PfhB_2 has been previously identified as a virulence factor [49,50] and recent work has shown that vaccination with recombinant PfhB_2 can induce protective immunity in turkeys [44]. In other species such as *Bordetella pertussis* the secretion of the filamentous hemagglutinin (FHA) into the extracellular medium is reliant on the outer membrane protein FlaC [51]. In both *Haemophilus influenzae* and *Bordetella*, expression of the outer membrane secretion component is controlled by the two-component signal transduction systems *ospE4* [52] and *bagA4* [53] respectively. It is clear from our work that in *P. multocida* Fis co-ordinately activates the expression of PfhB_2 and its upstream predicted secretion partner LspB_2. However, we can not exclude the possibility that expression of these genes is also dependent on other regulatory mechanisms such as a two component signal transduction system.

Of particular significance is the finding that Fis regulates *plpE*, which encodes the cross protective antigen PlpE [28].

**Figure 4. Sensitivity of *P. multocida* strains to kanamycin as determined by disc diffusion assays.** The putative promoter-containing regions upstream of hyaE, phyA, pgiA, pm0998, pm1078, lspB_2, and pm1818 were cloned into the *P. multocida* promoter detecting vector pMK1, and the ability of the recombinant constructs (pAL597, pAL596, pAL795, pAL796, pAL797, pAL798 and pAL799 respectively) to confer kanamycin resistance to strains AL1114 (spontaneous acapsular strain), AL1115 (paired capsulated strain) and VP161 (capsulated wild-type parent) assessed. Empty pMK1 vector was used as a negative control. The average relative expression was determined from three biological replicates and the values shown are the mean ±1 SEM. *** p < 0.001, ** p < 0.01.

doi:10.1371/journal.ppat.1000750.g004

**Table 3.** Sequence differences identified by whole-genome Illumina short read sequencing between the acapsular strain AL1114 and the paired capsulated strain AL1115.

| Pm70 Reference position | Reference base | Base call (frequency) AL1114 | Base call (frequency) AL1115 | Gene (strand) | Resulting change in AL1114 | P value |
|-------------------------|----------------|-----------------------------|-----------------------------|---------------|---------------------------|---------|
| 1282559                 | A              | A (180), T (1)              | G (230)                     | fis (−)       | L28S substitution          | 1 × 10^{-70} |
| 1843077                 | A              | A (138), G (12)            | G (180)                     | asd (−)       | silent                     | 7 × 10^{-35} |
| 2140869                 | C              | C (361), A (1)             | C (171), A (21)             | bioA (+)      | silent                     | 0.003   |

*All sequencing reads were aligned to the annotated *P. multocida* Pm70 genome sequence and the position of each mutation in relation to this sequence shown.

doi:10.1371/journal.ppat.1000750.t003
Table 4. Genes identified as differentially regulated between the *P. multocida* wild-type strain and a *fis* mutant strain as determined by DNA microarray analysis.

| GeneID\(^{a}\) | Locus | Predicted function | COG\(^{b}\) | Fold Change (log2) | Adjusted p Value |
|----------------|-------|--------------------|-------------|------------------|-----------------|
| **Genes down-regulated in AL1114** |
| 1244121 | hyaE | Capsule Biosynthesis | D | -2.52 | 0.001 |
| 1244123 | hyaC | Capsule Biosynthesis | M | -1.81 | 0.001 |
| 1244127 | hexB | Capsule Biosynthesis | G/M | -1.55\(^{c}\) | 0.008 |
| 1244125 | hexD | Capsule Biosynthesis | M | -1.49\(^{c}\) | 0.003 |
| 1244126 | hexC | Capsule Biosynthesis | M | -1.47 | 0.004 |
| 1244120 | phya | Capsule Biosynthesis | M | -1.36\(^{c}\) | 0.004 |
| 1244122 | hyaD | Capsule Biosynthesis | M | -1.04\(^{c}\) | 0.024 |
| 1244345 | PM0998 | Unknown | - | -1.15 | 0.030 |
| 1244344 | PM0997 | Putative membrane protein | M | -1.24 | 0.004 |
| 1244343 | PM0996 | Putative ABC transporter | V | -1.07 | 0.009 |
| 1244863 | PM1516 | Putative DNA methylase | L | -1.95\(^{c}\) | 0.001 |
| 1244864 | plpE | Protective lipoprotein | - | -1.94 | 0.001 |
| 1244662 | PM1515 | Unknown | - | -1.15 | 0.030 |
| 1243045 | lspB\(_2\) | Hemolysin accessory protein | U | -1.98 | 0.001 |
| 1243406 | pfhB\(_2\) | Filamentous haemagluttinin | S | -1.57 | 0.003 |
| 1245078 | glmS | Fructose-6-phosphate aminotransferase | M | -1.94\(^{c}\) | 0.004 |
| 1244389 | PM1042 | Putative Ptn transferase | R | -1.94\(^{c}\) | 0.001 |
| N/A | Unknown | Unknown | - | -1.69 | 0.003 |
| 1243932 | sohA | Putative protease | O/U | -1.49\(^{c}\) | 0.022 |
| 1243761 | pgIA | Cryptic heparosan synthase | M | -1.41\(^{c}\) | 0.010 |
| 1245040 | cysK | Cysteine synthase | E | -1.40\(^{c}\) | 0.043 |
| 1244425 | PM1078 | Hemin binding receptor | P | -1.40\(^{c}\) | 0.004 |
| 1243799 | PM0452 | Hypothetical periplasmic protein | P | -1.36\(^{c}\) | 0.050 |
| 1244215 | fruR | Fructose repressor | K | -1.36\(^{c}\) | 0.004 |
| 1244081 | htrA | Heat shock protein | O | -1.32\(^{c}\) | 0.010 |
| 1244442 | PM1095 | Unknown | - | -1.32\(^{c}\) | 0.031 |
| 1244533 | exbB | Iron regulated virulence protein | U | -1.24\(^{c}\) | 0.023 |
| 1244083 | dnaK | Predicted chaperone | K | -1.15\(^{c}\) | 0.043 |
| 1244505 | PM1158 | Putative methyltransferase | H | -1.11\(^{c}\) | 0.024 |
| 1244397 | pm1050 | Unknown | M | -1.06\(^{c}\) | 0.033 |
| 1244287 | nrdD | Ribonucleoside-triphosphate reductase | F | -0.79\(^{c}\) | 0.035 |
| **Genes upregulated in AL1114** |
| 1245166 | PM1819 | srIB, putative virulence factor | S | 0.92\(^{c}\) | 0.012 |
| 1245167 | PM1820 | srIF, putative virulence factor | S | 1.04\(^{c}\) | 0.013 |
| 1245168 | PM1821 | Unknown | - | 1.68\(^{c}\) | 0.003 |
| 1245664 | PM1217 | Unknown | - | 1.48 | 0.044 |
| 1244194 | tadC | Hypothetical protein | N/U | 1.09\(^{c}\) | 0.034 |
| 1244021 | PM0674 | Unknown | - | 1.85\(^{c}\) | 0.008 |
| 1244889 | pckA | Predicted phosphoenolpyruvate carboxykinase | C | 2.13\(^{c}\) | 0.044 |
| 1243866 | PM0519 | Unknown | S | 2.18 | 0.044 |
| 1245193 | ptsB | Sucrose-specific PTSII | G | 2.19 | 0.047 |
| 1244890 | PM1543 | Unknown | - | 2.39\(^{c}\) | 0.007 |
| 1244799 | PM1452 | Unknown | S | 2.70\(^{c}\) | 0.024 |

\(^{a}\)NCBI accession number of the identified gene.

\(^{b}\)Cluster of Orthologous Groups functional group (http://www.ncbi.nlm.nih.gov/COG/).

\(^{c}\)Only one of two probes for this gene was significantly differentially regulated.

doi:10.1371/journal.ppat.1000750.t004
Interestingly, previous studies on spontaneous acapsular strains have indicated that loss of a 39–40 kDa lipoprotein was correlated with the loss of capsular polysaccharide and it was hypothesized that the lack of expression of this protein on the surface was due to the physical loss of capsule [11,34–36]. We propose that the lipoprotein identified in the above studies is in fact PlpE and its expression is reduced in spontaneous acapsular strains because of the transcriptional down-regulation of the gene due to the absence of Fis.

In summary, we have characterized three independently derived, spontaneous, acapsular variants of *P. multocida*. In all three strains, loss of capsule production was due to a single nucleotide change in the gene encoding the nucleoid-associated protein Fis, identifying for the first time a mechanism for spontaneous capsule loss and a regulator critical for *P. multocida* capsule expression. Furthermore, analysis of gene expression in the *fis* mutant strain that produces no HA (AL1164). The filled arrow head represents the position of PlpE, whilst the unfilled arrowhead represents the background band used as a loading control for densitometry normalization. Each bar represents the average normalized densitometry readings from triplicate biological replicates ± SEM. ** p<0.01, * p<0.05.

doi:10.1371/journal.ppat.1000750.g005

Materials and Methods

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in 2YT broth and *P. multocida* in brain heart infusion (BHI) broth or nutrient broth (NB). Solid media were obtained by the addition of 1.5% agar. When required, the media were supplemented with streptomycin (50 μg/ml), spectinomycin (50 μg/ml), kanamycin (50 μg/ml) or tetracycline (2.5 μg/ml).

Molecular techniques

Restriction digests and ligations were performed according to the manufacturers’ instructions using enzymes obtained from NEB (Beverly, MA) or Roche Diagnostics GmbH (Mannheim, Germany). Plasmid DNA was prepared using Qiagen DNA miniprep spin columns (Qiagen, GmbH, Germany). *P. multocida* genomic DNA was isolated using the RBC genomic DNA purification kit (RBC, Taiwan). Amplification of DNA by PCR was performed using Taq DNA polymerase (Roche) and, when required, PCR amplified products were purified using the Qiagen PCR purification Kit (Qiagen, GmbH, Germany). Oligonucleotides used in this study (Table 2) were synthesized by Sigma, Australia. For transformation of plasmid DNA into *P. multocida*, electro-competent cells were prepared as described previously [8] and electroporated at 1800V, 600Ω, 29μF. DNA sequences obtained by Sanger sequencing were determined on an Applied Biosystems 3730S Genetic Analyser and sequence analysis was performed with Vector NTI Advance version 10 (Invitrogen, Carlsbad, CA).

Generation of a single cross-over mutant in *hyaB*

For inactivation of *hyaB* in *P. multocida* strain VP161 we used the previously described single-crossover insertional mutagenesis method which utilizes the λ *pir*-dependent plasmid pUA826 [57]. A 720 bp internal fragment of *hyaB* was amplified by PCR using oligonucleotides BAP4399 and BAP4400 (Table 2), and cloned into the SfiI site of pUA826pir, generating pAL499 (Table 1). This plasmid was then mobilized from *E. coli* SM10 λ *pir* into *P. multocida* strain AL435 by conjugation, and insertional mutants selected on BHI agar containing tetracycline, spectinomycin and streptomycin. Single cross-over insertion of the recombinant plasmid into *hyaB* was confirmed by PCR using either of the genomic flanking primers BAP276 or BAP169 (Table 1) together with BAP2702 located within the plasmid pUA826pir. One transconjugant with the correct insertion in *hyaB* was selected for further study, and designated AL919 (Table 1).

Disc diffusion assays

Disc diffusion assays were performed on soft agar overlays. Briefly, 100 μl of each *P. multocida* overnight culture was mixed with 5 ml of BHI containing 0.8% agar and immediately poured onto a BHI agar (1.5%) base. Kanamycin at a range of concentrations, was absorbed onto sterile Whatman paper discs, placed on the agar overlay containing *P. multocida*, and incubated for 18 h at 37°C. Inhibition of growth was determined as the diameter of the zone of clearing around the discs.

Quantitative HA assays

Crude capsular material was extracted as described previously [58] with the following modifications. One ml of a *P. multocida*
overnight culture was pelleted by centrifugation at 13 000 g, and washed once with 1 ml of PBS. Washed cells were resuspended in 1 ml of fresh PBS, incubated at 42°C for 1 h, then pelleted, and the supernatant, containing crude capsular polysaccharide, collected. HA content was determined as described previously [8].

Fluorescent primer extension

Fluorescent primer extension was performed as described previously [59], with the following modifications. First strand cDNA synthesis was performed with SuperScript III RT (Invitrogen) according to the manufacturer’s instructions. A typical reaction contained 10 μg of total RNA, 1 mM dNTPs and 6-FAM labeled primer at a final concentration of 100 nM. Fragment length analysis of FAM-labeled cDNAs was performed by the Australian Genome Research Facility (AGRF, Melbourne). cDNA fragments were separated on an AB3730 DNA analyzer (Applied Biosystems), and sizes determined using Genemapper V3.7 software (Applied Biosystems).

High-throughput genome sequencing and analysis

High-throughput sequencing was performed on an Illumina GA2 (Illumina, USA) by the Micromon Sequencing Facility (Monash University, Australia). Two lanes of 36-bp single end data were generated for both AL1114 and AL1115. Raw sequence data from both strains was aligned independently to the P. multocida PM70 genome sequence using SHRiMP [60] (average read depth ~200), which is able to produce alignments in the presence of single nucleotide polymorphisms (SNPs) and insertions and deletions (indels). These alignments were then used to compile, for each position in the reference, a contingency table of counts of observed bases in each of the two samples, and the significance of each different base call was determined using Fisher’s exact test. The determined significance values were corrected for multiple testing using the Bonferroni adjustment. Raw read data were also assembled de novo using VELVET [61] or CLC genomics workbench (CLC).

Complementation of spontaneous mutants and construction of plasmids for promoter analysis

For complementation of spontaneous acapsular strains, wild-type Fis and the overlapping upstream ORF pm1067, were amplified from P. multocida VP161 genomic DNA using oligonucleotides BAP5967 and BAP5969 (Table 2) containing SaI and BamHI sites respectively. The amplified fragment was digested and cloned into SaI- and BamHI-digested pBPA1100, generating pAL727 (Table 1). This plasmid was then used to transform the acapsular strains AL1114, AL1162 and AL1396, generating AL1399, AL1401 and AL1403. As a control, empty pBPA1100 was also used to transform each of these strains, generating AL1398, AL1400 and AL1402 (Table 1).

For analysis of promoter activity in P. multocida, predicted promoter containing fragments were amplified by PCR and cloned into the P. multocida promoter detecting vector pMKΩ, which contains a promoterless kanamycin resistance gene (Table 1, [24]). The genomic region containing the hypoA promoter and the predicted phyA promoter was amplified by PCR using the oligonucleotides BAP5691 and BAP5692 (Table 2) and cloned in both orientations into pMKΩ to generate pAL596 and pAL597. The predicted promoter containing fragments upstream of the genes pmgA, pm1098, pm1078, lbp12’ and pm1818 (the first gene in a putative operon containing pm1819, pm1820 and pm1821) were amplified by PCR using the oligonucleotide pairs BAP5834 and BAP5835 (hp51), BAP5828 and BAP5829 (pm1098), BAP5826 and BAP5827 (pm1078), BAP5832 and BAP5833 (hpB2) and BAP5830 and BAP5839 (pm1818) (Table 2) and each fragment cloned into pMKΩ to generate pAL795, pAL796, pAL797, pAL798 and pAL799, respectively (Table 1). Each of the recombinant plasmids was then transformed into the acapsular fis mutant strain AL1114 and its paired capsulated derivative AL1115. Promoter activity from the cloned fragment in pMKΩ was assessed semi-quantitatively by disc diffusion assays where a reduced zone of growth inhibition around kanamycin impregnated discs indicated a higher level of kanamycin resistance and therefore promoter activity from the cloned fragment.

Construction of P. multocida directed fis mutants

The E. coli/P. multocida shuttle vector pAL90 (Table 1) was used to generate two TargetTrom vectors, pAL692 and pAL705, for the generation of marker-free fis mutants in P. multocida. The spectinomycin/streptomycin resistant TargetTrom vector pAL692 (Table 1) was constructed as follows: pAL90 was amplified by PCR using outward facing primers that flank the aph3 gene (BAP5358 and BAP5359) and digested with EcoRV. This fragment was ligated to an EcoRV-digested PCR fragment containing the aadA gene amplified from pUA826 using the primers BAP5360 and BAP5361. Following ligation the plasmid was digested with HindIII and FspI and ligated to a 4 kb HindIII/FspI-digested fragment of pJR750ai encoding the TargetTrom intron and inA gene (Table 1) such that transcription would be driven by the constitutive P. multocida tpiA promoter. For construction of the kanamycin resistant TargetTrom vector pAL705 (Table 1, the aph3 gene was amplified from pAL90 using the primers BAP5435 and BAP5434 then cloned into EcoRV-digested pAL692, thereby replacing the aadA gene.

Retargeting of the intron within each vector to nucleotide 48 of fis was performed as per the TargetTrom user manual (Sigma) using the oligonucleotides BAP5932-BAP5934 (Table 1). The retargeted mutagenesis plasmids, pAL706 (Kan®) and pAL708 (Strep+/Spec®) (Table 1) were used to transform P. multocida VP161 and antibiotic resistant transformants selected on either spectinomycin or kanamycin. Insertion of the intron into the P. multocida genome was detected using the fis-specific oligonucleotides BAP5967 and BAP5968 (Table 1); the presence of the intron resulted in a 0.9 kb increase in the size of the PCR product compared to the PCR product generated from the wild-type fis (data not shown). Mutants confirmed by PCR to have insertions in fis were cured of the replicating TargetTrom plasmid by a single overnight growth in NB broth without antibiotic selection, followed by patching of single colonies for either Strep® Spec® or Kan®. Strains cured of replicating plasmid were confirmed as fis mutants by additional PCR amplifications to show the presence of the intron and the absence of a copy of wild-type fis (data not shown). Finally, fis mutations were confirmed by direct genomic sequencing using intron-specific primers.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Bacteria were harvested from triplicate BHI cultures at late log phase (~5×10⁹ CFU/ml) by centrifugation at 13 000 g, and RNA was isolated using TRIzol reagent (Gibco/BRL) as described by the manufacturer. The purified total RNA was treated with DNase (15 U for 10 min at 37°C), and then further purified on RNeasy mini columns (Qiagen). Primers for qRT-PCR were designed using the Primer Express software (ABI) (BAP9995-BAP9998;(310,273),(497,284)). RT reactions were routinely performed in 20 μl volumes, containing 3 μg total RNA, 15 ng random hexamers, 0.5mM

PloS Pathogens | www.plospathogens.org
dNTPs and 300 U SuperScript III Reverse Transcriptase (Invitrogen) at 42°C for 2.5 h. The synthesized cDNA samples were diluted 50-fold prior to qRT-PCR, which was performed using an Eppendorf replex™ mastercycler with product accumulation quantified by incorporation of the fluorescent dye SYBR Green. Samples were assayed in triplicate using 2 μl of diluted cDNA with SYBR Green PCR master mix (ABI) and 50 nM concentrations of each gene-specific primer. The concentration of template in each reaction was determined by comparison with a gene-specific standard curve constructed from known concentrations of P. multocida strain VP161 genomic DNA. grE was used as the normalizer for all reactions. All RT-PCR amplifiers a single product as determined by melting curve analysis.

Microarray analysis
Custom Combinatrix 12k microarrays (Combinatrix, USA) were designed based on the published sequence of P. multocida PM70 [25], with the addition of probes specific for ORFs previously identified as unique to P. multocida strain VP161 [62]. cDNA for microarray hybridizations was prepared as for qRT-PCR, except that RNA contamination was removed from the cDNA by the addition of NaOH followed by column purification (Qiagen minElute, Qiagen). A total of 2 μg of purified cDNA was labeled using KREAtch Cy3-ULS (KREAtch, The Netherlands), and used in hybridizations with the Combinatrix 12k microarrays as per the manufacturer’s instructions. Triplicate hybridized arrays were scanned on a Genepix 4000b scanner, and spot intensities determined using Microarray Imagery v5.9.3 (Combinatrix, USA). After scanning, each array was immediately stripped and rescanned as per manufacturers’ instructions. Spot intensities of stripped arrays were used as background correction for the quantification of subsequent hybridizations. Spots from duplicate probes were averaged, and the averaged probe intensities analyzed using the LIMMA software package [63] as follows. Background correction was performed using the LIMMA “normexp” method [64], and the Log2 values calculated. Between-array quantile normalization [65] was then applied to the log transformed spot intensities. A moderated t-test on the normalized log intensities was performed to identify differentially expressed genes. Probes were sorted by significance, and the False Discovery Rate (FDR) [66] used to control for multiple testing. Probes showing ≥2-fold intensity change between AL1114 and AL1115, with a FDR of <0.05 were considered differentially expressed (Table 2). Two probes were designed for all genes over 500 bp in length; genes were classed as differentially expressed if one or both probes showed a differential expression of ≥2-fold. DNA microarray experiments were carried out according to MIAME guidelines and the complete experimental data can be obtained online from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) submission number GSE17666.

SDS-PAGE, western immunoblot and densitometry

SDS-PAGE was performed as described previously [67]. Proteins separated by SDS-PAGE were transferred to Immobilon-P membranes (Millipore). Western immunoblot analysis was performed using standard techniques [68], with chicken anti-recombinant PlpE, as the primary antibody and peroxidase-conjugated anti-chicken immunoglobulin (raised in donkeys) as the secondary antibody. Blots were visualized using CDP-Star (Roche), and imaged on a Fujifilm LAS-3000 chemiluminescent imager (Fujifilm). Densitometry was performed using Multi-gauge software v2.3 (Fujimilm).

Acknowledgments
We thank Marietta John for excellent technical assistance and Tamas Hatfaludi and Keith Al-Hasani for the generous donation of the anti-PlpE antiserum, the single cross-over insertion plpE mutant and complemented strain for use as controls.

Author Contributions
Conceived and designed the experiments: Jason Steen, Marina Harper, Ben Adler, John Boyce. Performed the experiments: Jason Steen, Jennifer Steen. Analyzed the data: Jason Steen, Jennifer Steen, Paul Harrison, Torsten Seemann, Ian Wilkie, Marina Harper, Ben Adler, John Boyce. Wrote the paper: Jason Steen, Jennifer Steen, Marina Harper, Ben Adler, John Boyce.

References
1. Carter GR, Chengappa MM. Recommendations for a standard system of designating serotypes of Pasteurella multocida. 1981. American Journal of Veterinary Laboratory Diagnostics, 37–42.
2. Cifonelli JA, Rebers PA, Heddleston KL (1970) The isolation and characterisation of hyaluronic acid from Pasteurella multocida. Carbohydr Res 14: 272–276.
3. DeAngelis PL, Gunay NS, Tooda T, Mao WJ, Linhardt RJ (2002) Identification of the capsular polysaccharides of Type D and F Pasteurella multocida as unmodified heparin and chondroitin, respectively. Carbohydr Res 337: 1347–1352.
4. Munialey N, Edgar J, Woolcock JB, Mukkur TKK. Virulence, purification, structure, and protective potential of the putative capsular polysaccharide of Pasteurella multocida type 6:B. In: Patten BE, Spencer TL, Johnson RB, DH, Lehan L, eds. Pasteurellosis in production animals; 1992. Bali, Indonesia, 47–53.
5. Boyce JD, Chung JY, Adler B (2000) Genetic organisation of the capsule biosynthetic locus of Pasteurella multocida capA-1. FEMS Microbiol Lett 166: 289–296.
6. Chung JY, Zhang YM, Adler B (1998) The capsule biosynthetic locus of Pasteurella multocida serogroup A. Infect Immun 66: 2487–2492.
7. Townsend KM, Boyle JD, Chung JY, Frost AJ, Adler B (2001) Genetic organization of Pasteurella multocida cap loci and development of a multiplex capsular PCR typing system. J Clin Microbiol 39: 904–909.
8. Chung JY, Wilkie I, Boyle JD, Townsend KM, Frost AJ, et al. (2001) Role of capsule in the pathogenesis of fowl cholera caused by Pasteurella multocida serogroup A. Infect Immun 69: 2487–2492.
9. Boyle JD, Adler B (2000) The capsule is a virulence determinant in the pathogenesis of Pasteurella multocida M1404 (B2). Infect Immun 68: 3463–3468.
10. Heddleston KL, Watko LP, Rebers PA (1964) Dissociation of a fowl cholera strain of Pasteurella multocida. Avian Dis 8: 649–657.
11. Champlin FR, Patterson CE, Austin FW, Ryals PE (1999) Derivation of extracellular polysaccharide-deficient variants from a serotype A strain of Pasteurella multocida. Curr Microbiol 38: 260–272.
12. Watt JM, Swiatlo E, Wade MM, Champlin FR (2003) Regulation of capsule biosynthesis in serotype A strains of Pasteurella multocida. FEMS Microbiol Lett 225: 9–14.
13. Grainger DC, Busby SJ (2008) Global regulators of transcription in Escherichia coli: mechanisms of action and methods for study. Adv Appl Microbiol 65: 93–113.
14. Ball CA, Osuna R, Ferguson KC, Johnson RC (1992) Dramatic changes in Fis levels upon nutrient upshift in Escherichia coli. J Bacteriol 174: 8043–8056.
15. Bradley MD, Beach MB, de Koning AP, Pratt TS, Osuna R (2007) Effects of Fis on Escherichia coli gene expression during different growth stages. Microbiol 153: 2922–2940.
16. Ninnenmann O, Koch C, Kahmann R (1992) The E. coli fis promoter is subject to stringent control and autoregulation. EMBO J 11: 1075–1083.
17. Pan CQ, Finkel SE, Crumpton SE, Feng JA, Sigman DS, et al. (1996) Variable structures of Fis-DNA complexes determined by flanking DNA-protein contacts. J Mol Biol 264: 675–695.
18. Shao Y, Feldman-Cohen LS, Osuna R (2008) Biochemical identification of base and phosphate contacts between Fis and a high-affinity DNA binding site. J Mol Biol 380: 327–339.
19. Lenz DH, Basler BL (2007) The small nucleoid protein Fis is involved in Vibrio cholerae quorum sensing. Mol Microbiol 63: 859–871.
20. Lauter T, Naser W (2007) The DNA nucleoid-associated protein Fis coordinates the expression of the main virulence genes in the phytopathogenic bacterium Bacillus atrophaeus. Mol Microbiol 66: 1474–1490.
21. Saldana Z, Xirohtsikos-Cartes J, Avellino F, Phillips AD, Kaper JB, et al. (2009) Synergistic role of curli and cellulose in cell adherence and biofilm formation.
and attaching and efacing *Escherichia coli* and identification of Fis as a negative regulator of curli. Environ Microb 11: 992–1006.

22. Goldberg MD, Johnson M, Hinton JC, Williams PH (2001) Role of the nucleoid-associated protein Fis in the regulation of virulence properties of *enteropathogenic* *Escherichia coli*. Mol Microbiol 41: 549–559.

23. Kelly A, Goldberg MD, Carroll RK, Danino V, Hinton JCD, et al. (2004) A global role for Fis in the transcriptional control of metabolism in *Salmonella enterica* serovar Typhimurium. Microbiol 150: 2037–2053.

24. Hunt ML, Boucher DJ, Boyce JD, Adler B. (2001) In vivo-expressed genes of *Pasteurella multocida*. Infect Immun 69: 3004–3012.

25. May BJ, Zhang Q, Li LL, Paustian ML, Whittam TS, et al. (2001) Complete genomic sequence of *Pasteurella multocida*, Path70. Proc Natl Acad Sci USA 98: 3460–3465.

26. Bihor AC, Xu J, Johnson RC, Schimmel P, Dargis M, Maloun F (1994) Modification of *Pasteurella multocida* capsular polysaccharide during growth under iron-restricted conditions and in vivo. Microbiol 140: 263–270.

27. Melkonov E, Schoenfeld C, Spehr V, Warrass R, Gunkel N, et al. (2007) A genomic-scale reconstruction of *P. multocida* identifies extended mobile *Pasteurellaceae* and confers by vaccination with recombinant *Pasteurella multocida* LpsA proteins by CprP. Infect Immun 75: 3420–3411.

28. Wu JR, Shien JH, Shieh HK, Chen CF, Chang PC (2007) Protective immunity conferred by recombinant *Pasteurella multocida* lipoprotein E (PpE). Vaccine 25: 4140–4148.

29. Jacques M, Belanger M, Diarra MS, Dargis M, Malouin F (1994) Modulation of *Pasteurella multocida* operon transcription in *Escherichia coli*. Mol Microbiol 14: 1593–1603.

30. Kuhn ML, Boyce JD, Cox AD, St Michael F, Wilkie JW, et al. (2007) *Pasteurella multocida* expression of the lipidopolysaccharide glycoforms simultaneously, but only single form is required for virulence: identification of two acceptor-specific heptosyl I transferases. Infect Immun 75: 3185–3189.

31. Gentry JM, Covert RE, Panacek RJ (1982) Expression of capsular material from *Pasteurellaceae* in vivo. Acta Pathol Microbiol Immunol Scand 140: 263–270.

32. Loyd AL, Marshall BJ, Bae BJ (2004) Identifying cloned Hbsbacter pholis promoters by primer extension using a FAM-labelled primer and GeneScan® analysis. J Microb Methods 60: 291–298.

33. Safo MK, Yang W-Z, Corselli L, Crampton SE, Yuan HS, et al. (1997) The *F. coli* CpxR response to iron limitation. Infect Immun 65: 4201–4206.

34. Rumble SM, Lacroute P, Dalca AV, Fiume M, Sidow A, et al. (2007) SHRiMP: accurate mapping of short colour-space reads. PLoS Comput Biol 5: e1000386. doi:10.1371/journal.pcbi.1000386.

35. Yamanaka S, Horiuchi S, Kamata Y, Kiyama E, Ikonomov M, et al. (2003) Capsule thickness and amounts of a 39 kDa capsular protein of avian *Pasteurella multocida* type A strains correlate with their pathogenicity for chickens. Vet Microbiol 97: 215–227.

36. Harper M, Boyce JD, Cox AD, St Michael F, Wilkie JW, et al. (2007) *Pasteurella multocida* expression of the lipidopolysaccharide glycoforms simultaneously, but only single form is required for virulence: identification of two acceptor-specific heptosyl I transferases. Infect Immun 75: 3185–3189.

37. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) Comparison of background correction methods for two-colour microarrays. Bioinformatics 19: 185–193.

38. Miehe VL, Mekalanos J (1989) A novel suicide vector and its use in construction of insertion mutations: oomorgulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires tonT. J Bacteriol 170: 2573–2583.

39.呵倉, R, Oda, A, Hidaka, T, et al. (2000) A comparison of background correction methods for two-colour microarrays. Bioinformatics 16: 167–174.

40. Johnson RC, Bruist MF, Simon MI (1986) Host protein requirements for *Haemophilus ducreyi* filamentous hemagglutinin. J Biol Chem 261: 3460–3465.

41. Wang J, Pomeroy SL, Arlett CF, Zhang J, Friend SH, et al. (2004) A comparison of background correction methods for two-colour microarrays. Bioinformatics 20: 185–193.

42. Kimberley BY, Sosada T, Kato K, Oyama K, Kawamoto E, et al. (2003) Capsule thickness and amounts of a 39 kDa capsular protein of avian *Pasteurella multocida* type A strains correlate with their pathogenicity for chickens. Vet Microbiol 97: 215–227.

43. Kimura M, Inoue M, Ikeda M, Kato K, Sato S, et al. (2001) Identification of the tRNA-dihydrouridine synthase family. J Biol Chem 276: 17465–17470.

44. Harper M, Boyce JD, Cox AD, St Michael F, Wilkie JW, et al. (2007) *Pasteurella multocida* expression of the lipidopolysaccharide glycoforms simultaneously, but only single form is required for virulence: identification of two acceptor-specific heptosyl I transferases. Infect Immun 75: 3185–3189.

45. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) Comparison of background correction methods for high density oligonucleotide array data based on bias and variance. Bioinformatics 19: 255–264.

46. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.

47. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) Comparison of background correction methods for high density oligonucleotide array data based on bias and variance. Bioinformatics 19: 255–264.

48. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) Comparison of background correction methods for high density oligonucleotide array data based on bias and variance. Bioinformatics 19: 255–264.

49. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.

50. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) Comparison of background correction methods for high density oligonucleotide array data based on bias and variance. Bioinformatics 19: 255–264.

51. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.

52. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) Comparison of background correction methods for high density oligonucleotide array data based on bias and variance. Bioinformatics 19: 255–264.

53. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.

54. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.

55. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 57: 289–300.
Author/s:
Steen, JA; Steen, JA; Harrison, P; Seemann, T; Wilkie, I; Harper, M; Adler, B; Boyce, JD

Title:
Fis Is Essential for Capsule Production in Pasteurella multocida and Regulates Expression of Other Important Virulence Factors

Date:
2010-02-01

Citation:
Steen, J. A., Steen, J. A., Harrison, P., Seemann, T., Wilkie, I., Harper, M., Adler, B. & Boyce, J. D. (2010). Fis Is Essential for Capsule Production in Pasteurella multocida and Regulates Expression of Other Important Virulence Factors. PLOS PATHOGENS, 6 (2), https://doi.org/10.1371/journal.ppat.1000750.

Persistent Link:
http://hdl.handle.net/11343/262190

File Description:
Published version

License:
CC BY