Transcriptional profiling of *Mycoplasma hyopneumoniae* during iron depletion using microarrays

Melissa L. Madsen,¹ Dan Nettleton,² Eileen L. Thacker¹
and F. Chris Minion¹

Correspondence

F. Chris Minion

fcminion@iastate.edu

Departments of Veterinary Microbiology and Preventive Medicine¹ and Statistics², Iowa State University, Ames, IA, USA

Mycoplasma hyopneumoniae, the causative agent of swine enzootic pneumonia and a major component of the porcine respiratory disease complex, continues to confound swine producers despite control programmes worldwide. The disease is chronic and self-limiting, but the host is subject to immunopathological changes that potentiate respiratory disease associated with other pathogens. The response of *M. hyopneumoniae* to environmental stress is of interest because of its relevance to virulence mechanisms in other bacterial pathogens. One of these stressors, iron deprivation, is a prominent feature of the host innate immune response, and most certainly impacts growth of mycoplasmas *in vivo*. To study this, microarray technology was applied to the transcriptome analysis of *M. hyopneumoniae* during iron deprivation. An array consisting of 632 of the 698 ORFs in the genome was used to compare the mRNA isolated from organisms grown under normal laboratory conditions with that from organisms subjected to iron deprivation with the chelator 2,2'-dipyridyl. This analysis identified 27 genes that were either up- or down-regulated in response to low-iron growth conditions ($P < 0.01$), with an estimated false discovery rate below 10%. These included genes encoding transport proteins, enzymes involved in energy metabolism, and components of the translation process. Ten of the 27 identified genes had no assigned function. These studies indicate that *M. hyopneumoniae* can respond to changes in environmental conditions, but the mechanism employed remains unknown.

Received 8 November 2005

Revised 5 January 2006

Accepted 11 January 2006

INTRODUCTION

Mycoplasma hyopneumoniae is the causative agent of swine enzootic pneumonia (Ross, 1993) and contributes significantly to porcine respiratory disease complex (Thacker *et al.*, 1999). It is ubiquitous throughout swine herds worldwide and is transmitted via aerosol from pig to pig. Despite aggressive vaccination programmes, *M. hyopneumoniae* continues to induce lesions in as many as 80% of the pig population (Ross, 1992). It colonizes the respiratory epithelia, where it attaches exclusively to the cilia (Mebus & Underdahl, 1977), causing ciliostasis and cell death (DeBey & Ross, 1994; Livingston *et al.*, 1972).

Like all mycoplasmas, *M. hyopneumoniae* must acquire essential nutrients from its host because it lacks biosynthetic pathways due to the limited coding capacity of its genome (Minion *et al.*, 2004). One of these nutrients, iron, is an essential metal for almost all living systems. The one known exception is the agent of Lyme disease, *Borrelia burgdorferi* (Posey & Gherardini, 2000). Iron serves as a cofactor or as a prosthetic group for essential enzymes that are involved in basic cellular functions (O'Halloran, 1993). Free iron,

however, does not exist in useful concentrations under neutral pH conditions since it is readily oxidized forming the hydroxide $\text{Fe}(\text{OH})_2^+$, which is soluble only at 1.4×10^{-9} M at pH 7 (Andrews *et al.*, 2003). Free iron catalyses the formation of free oxygen radicals and as such is highly toxic to cells. Therefore, iron is normally bound to specific proteins in the host such as transferrin and lactoferrin to prevent its precipitation in aqueous systems and to protect the cell from damaging UV light and lipid peroxidation reactions. Transferrin, lactoferrin and lactotransferrin have iron-binding constants in the range of $\sim 10^{20}$ (Aisen & Listowsky, 1980; Baker *et al.*, 1994), and transferrin is never fully saturated under normal conditions, giving the host additional iron-sequestration capabilities during bacterial infections. In this way, iron is both sequestered for use by the host and use by invading pathogens is prevented as part of the innate immune defence.

Most bacteria require iron concentrations in the range of 10^{-6} to 10^{-7} M for their metabolic processes (Weinberg, 1978). Bacteria have evolved mechanisms for iron acquisition that are tightly controlled by the level and availability of

iron in the environment (Ratledge & Dover, 2000), since considerable energy investment is needed by the bacteria to express competitive iron-acquisition systems (Crosa, 1989). These mechanisms involve transcriptional regulators such as Fur and cognate DNA-binding sites (Fur-binding sites) in genes involved in iron uptake (Weinberg, 1995). The function of these genes involves binding of host transferrin or lactoferrin and their reduction and uptake, siderophore production, secretion or transport systems (Ratledge & Dover, 2000). The Fur-regulated genes are also thought to contribute to virulence in many pathogens, because the existence of multiple iron-acquisition systems allows for alternative means of iron acquisition and virulence under changing environmental conditions within the host during disease. The regulation of iron-acquisition genes linked with virulence factors allows for cell invasion, and possibly cell death, increasing the iron available for uptake by the bacteria (Salyers & Whitt, 2002). Sequence analysis of the *M. hyopneumoniae* genome has not identified any putative fur genes or their regulators (Minion *et al.*, 2004); it was therefore of interest to determine if *M. hyopneumoniae* would respond to an environment in which iron was sequestered by a chelator other than transferrin or lactoferrin.

Unlike other bacteria, mycoplasmas have minimal gene coding capacity (Hutchison & Montague, 2002) and lack cell walls, so it is thought that their interactions with the host are less complex, including the acquisition of essential nutrients. Pathogenic mycoplasmas pose interesting questions concerning iron metabolism because they lack a host-free lifestyle, they have a single membrane and few studies have been performed on iron requirements. While it has not been unequivocally shown that iron is essential for growth, Bauminger *et al.* (1980) showed that iron was stored in *Mycoplasma capricolum* and Tryon & Baseman (1987) have shown that *Mycoplasma pneumoniae* can take up human lactoferrin. Mycoplasmas also contain enzymes that have been shown in other systems to contain iron, such as dehydrogenases (Earhart, 1996).

Whether mycoplasmas contain complex iron-acquisition mechanisms is not known. Iron deprivation in other bacterial systems not only triggers the genes required for its acquisition but also in many pathogens is a signal for controlling virulence factor expression (Lamont *et al.*, 2002; Rodriguez & Smith, 2003; Schaible & Kaufmann, 2004; Szczebara *et al.*, 1999). Thus, virulence factors can sometimes be identified by their response to low-iron growth conditions. In these studies, 2,2'-dipyridyl was used to chelate iron in growing *M. hyopneumoniae* cultures, and experiments using microarrays showed that iron deprivation *in vitro* leads to transcriptional changes. Both up- and down-regulated genes were identified.

METHODS

Mycoplasma strains and culture conditions. Pathogenic *M. hyopneumoniae* strain 232, a derivative of strain 11, was used in this study (Mare & Switzer, 1965). Cultures were passed fewer than 15

times *in vitro* in Friis media as previously described (Friis, 1975). Twelve 250 ml flasks containing 125 ml cultures were grown at 37 °C to early exponential phase as determined by medium colour change and optical density. To six flasks, 2,2'-dipyridyl (Sigma) was added to a final concentration of 1 mg ml⁻¹. Six flasks were left untreated, and all flasks were incubated for 2 h at 37 °C. Cells were pelleted by centrifugation at 24 000 g, and 500 µl RNAlater (Ambion) was added to the pellet. Pellets were stored at -70 °C until the total RNA was isolated.

To assess the effect that various iron chelators would have on mycoplasma growth, *Mycoplasma gallisepticum* (ATCC 19610) and *Mycoplasma pulmonis* UAB6510 (Davis *et al.*, 1980) were grown in modified Hayflick's medium as described except that the medium was supplemented with 25 µg ml⁻¹ cefoperazone (Pfizer) instead of thallium acetate and benzylpenicillin (Freundt, 1983). Hayflick's medium was supplemented with 2,2'-dipyridyl (0.2 and 1 mg ml⁻¹), sodium citrate (1, 2, 5 and 7.5 mM) and deferoxamine mesylate (desferal) (0.2 and 1 mg ml⁻¹), and growth was determined by plating 10-fold serial dilutions of these broth cultures at different time points onto Hayflick's agar medium lacking chelators. Colony-forming units (c.f.u.) were determined after incubation at 37 °C for 4–5 days.

Microarray. The *M. hyopneumoniae* microarray consists of PCR products (probes) spotted to Corning UltraGAPS glass substrates. Ninety-one per cent (632/698) of the *M. hyopneumoniae* ORFs are represented on the array as PCR products 125–350 bp in length. Each product is a unique sequence even within paralogous families as described by Minion *et al.* (2004). The absence of 66 ORFs is due to an inability to design suitable primers (12 ORFs) or failed PCR reactions due to incorrect product size, multiple bands or no band (54 ORFs). Only ORFs larger than 125 bp are represented on the array and no tRNAs or rRNAs were included. The primer design, array construction and validation have been described (Madsen *et al.*, 2006).

Experimental design. Six independent RNA samples from iron-depleted cells were paired with six independent RNA samples from control cells for hybridization on six two-colour microarrays. For three of the six arrays, the control sample was labelled with Alexa 555 dye and compared to the iron-depleted sample labelled with Alexa 647 dye (Molecular Probes). The dye assignment to control and treated samples was reversed for the other three arrays. The three slides were hybridized under identical conditions as described below.

RNA isolation. RNA was isolated from frozen cell pellets using the Versagene RNA Purification System (Gentra Systems) according to the manufacturer's protocol. The optional step of DNase treatment was performed on column according to the manufacturer's recommendation. With a cut-off of 150 bp, 5S rRNA and tRNAs were removed from the samples, limiting interference in downstream manipulations. Samples were quantified and checked for purity using a Nanodrop ND-1000 spectrophotometer. If necessary, samples were concentrated using Microcon YM-30 micro-concentrators (Millipore) for optimal cDNA generation.

Target generation and hybridization. Fluorescently labelled cDNA targets were generated and purified using the SuperScript Indirect cDNA Labelling System (Invitrogen) with a set of 129 ORF-specific hexamer oligonucleotide primers designed as previously described (Madsen *et al.*, 2006). Following purification of the labelled cDNA, samples were dried in a vacuum centrifuge and then resuspended in 10 µl Pronto! cDNA/long oligo hybridization solution (Corning). Targets were denatured at 95 °C for 5 min and centrifuged at 13 000 r.p.m. for 2 min at room temperature. Targets from a control and an iron-deprived culture were then combined, pipetted onto an array, and covered with a 22 × 22 mm HybriSlip

(Schleicher & Schuell). Slides were placed in a Corning hybridization chamber and incubated in a 42 °C water bath for 12–16 h. Slides were washed according to Corning's UltraGAPS protocol and dried by centrifugation.

Data acquisition and normalization. Each array was scanned with each dye channel using a ScanArray Express laser scanner (Applied BioSystems) three times under varying laser power and photomultiplier tube gain settings to increase the dynamic range of expression measurement (Dudley *et al.*, 2002). Images were quantified using the softWorRx Tracker analysis software package (Applied Precision). Spot-specific mean signals were corrected for local background by subtracting spot-specific median background intensities. The natural logarithms of the background-corrected signals from a single scan were adjusted by an additive constant so that all scans of the same array-by-dye combination would have a common median. The median of these adjusted-log-background-corrected signals across multiple scans was then computed for each spot to obtain one value for each combination of spot, array and dye channel. These data for the two dye channels on any given array were normalized using locally weighted scatterplot smoother (LOWESS) normalization to adjust for intensity-dependent dye bias (Dudoit *et al.*, 2000; Yang *et al.*, 2002). Following LOWESS adjustment, the data from each channel were adjusted by an additive constant so that the median for any combination of array and dye would be the same for all array-by-dye combinations. The normalized values for triplicate spots were averaged within each array to produce one normalized measure of expression for each of the 632 probe sequences and each of the 12 RNA samples.

Data analysis. A separate mixed linear model analysis was conducted for each probe sequence using the normalized data (Wolfinger *et al.*, 2001). Each mixed model included fixed effects for treatment (iron depletion versus control), slide region (upper versus lower) and dye (Alexa 555 versus 647) as well as random effects for slide and slide-by-region interaction. A *t*-test for differential expression across treatments was conducted for each probe as part of our mixed linear model analyses. The 632 *P*-values from these *t*-tests were converted to *q*-values using the method of Storey & Tibshirani (2003). These *q*-values can be used to obtain approximate control of the false discovery rate at a specified value. For example, declaring probes with *q*-values less than or equal to 0.05 to be differentially expressed produces a list of significant results for which the false discovery rate is estimated to be approximately 5%. Along with *q*-values, estimates of fold change were computed for each probe by taking the inverse natural log of the mean treatment difference estimated as part of our mixed linear model analyses.

Confirmation of microarray data. To confirm significant transcriptional differences between genes, semi-quantitative RT-PCR analysis was performed on a subset of genes that showed significant up- or down-regulation in the microarray analysis. The PCR was performed in 96-well plates in an MJ Research Dyad Thermal Cycler (Bio-Rad) in 20 µl reactions. The six cDNA preparations for each control and iron-depleted sample were used with previously described conditions (Madsen *et al.*, 2006). The reactions were analysed by agarose gel electrophoresis, stained and digitized, and each band density was measured with FluorChem 8000 version 2.0 software (Alpha Innotech). Analysis of variance was performed to determine differences between band density values of iron-depleted vs control samples. Band densities were background corrected by subtracting the background density within the reaction lane from the sample band density. Estimated differences were considered significant if the *P*-value of a *t*-test was <0.05.

RESULTS

Iron depletion studies

Preliminary growth studies were performed with *M. gallisepticum* and *M. pulmonis* to determine optimal concentrations of 2,2'-dipyridyl, sodium citrate or desferal to use in studies with *M. hyopneumoniae*. The rationale for using these mycoplasmas to study the effects of iron deprivation on growth is that *M. hyopneumoniae* growth is difficult to quantitate due to its poor growth on agar surfaces and forms pinpoint-sized colonies only rarely. Both *M. gallisepticum* and *M. pulmonis* grow well in comparison to *M. hyopneumoniae*, readily form colonies on agar surfaces, and similar to *M. hyopneumoniae* are respiratory pathogens with a host-adapted life style. It was hypothesized that *M. hyopneumoniae* would react in a similar fashion to them under iron-limiting conditions since all three species grow in an environment replete with transferrin and lactoferrin. Since little is known about iron-sequestering mechanisms in mycoplasmas, three chelators with different iron-binding mechanisms and affinities were used in the preliminary studies. Our goal was to identify a chelator that could slow or stop the growth of *M. gallisepticum* and *M. pulmonis* while growing in serum-rich medium. Fig. 1 shows the results of supplementing Hayflick's growth medium with 2,2'-dipyridyl at 0.2 and 1 mg ml⁻¹. This demonstrates that 2,2'-dipyridyl consistently slowed growth but only at the highest concentration (1 mg ml⁻¹). At no time was growth completely stopped. Neither sodium citrate nor desferal had an effect on growth at any concentration tested (data not shown). Assuming that 2,2'-dipyridyl has the same effect on *M. hyopneumoniae* growth, subsequent iron-limiting studies with *M. hyopneumoniae* were performed using a concentration of 1 mg ml⁻¹.

Microarray studies

To assess the effect of iron deprivation on *M. hyopneumoniae* global transcriptional profiles, broth-grown cultures were supplemented with 2,2'-dipyridyl and microarray analysis was performed on these and control cultures. Total RNA was extracted from six replicate control and six 2,2'-dipyridyl-supplemented cultures, and cDNA reactions were performed independently on each of the twelve RNA preparations. Total RNA concentrations post-purification were approximately 15–20 µg total RNA, and cDNA concentrations ranged from 2 to 6 µg total cDNA. Control and iron-depleted samples were paired and equal concentrations of total RNA were added to cDNA reactions. The label incorporation of fluorescent dyes ranged from 20 to 65 bp per dye molecule, which is well within the recommended range of label incorporation (<http://probes.invitrogen.com/resources/calc/basederatio.html>). To account for variation due to dye incorporation, the experimental design included a dye swap with the three remaining sample pairs.

Data from each of the six replicates were used in the statistical analysis. Statistical analysis indicated that 27 genes

Fig. 1. Effect of 2,2'-dipyridyl on growth of *M. pulmonis* (a) and *M. gallisepticum* (b). Overnight cultures of both mycoplasmas were diluted 1:20 in fresh culture medium containing 0.1 mg ml⁻¹ (○), 0.2 mg ml⁻¹ (●) and 1 mg ml⁻¹ (□) 2,2'-dipyridyl and incubated at 37 °C for the indicated time. Samples were removed, serially diluted and plated on mycoplasma agar lacking chelator for c.f.u. counts at the times indicated. Data represent the means of triplicate platings.

had significant transcriptional differences with a *P*-value < 0.01. The results are presented in Table 1 and Fig. 2. Nine genes demonstrated a significant increase in transcriptional activity under iron-limiting conditions compared to control

cells grown under normal *in vitro* conditions. Eighteen genes, however, were down-regulated under these conditions. Fig. 2 displays the results of the 632 genes as a volcano plot, with up- and down-regulated genes marked. The data

Table 1. Differentially expressed genes of *M. hyopneumoniae* during growth under iron-limiting conditions

Gene ID	Gene*	Description	<i>P</i> -value	<i>q</i> -value	FC†
Upregulated					
mhp140	UH	Unique hypothetical, potential lipoprotein	0.0083	0.0957	1.29
mhp151	<i>iolB</i>	<i>myo</i> -Inositol catabolism	0.0069	0.0957	2.35
mhp152	<i>iolC</i>	<i>myo</i> -Inositol catabolism	0.0083	0.0957	2.15
mhp275	CH	Hypothetical protein, P102 paralogue	0.0076	0.0957	1.68
mhp317	<i>glpQ</i>	Glycerophosphoryl diester phosphodiesterase	0.0024	0.0957	1.42
mhp319	<i>mgIA</i>	ABC transporter ATP-binding subunit	0.0013	0.0633	1.59
mhp505	<i>ackA</i>	Acetate kinase	0.0049	0.0957	1.95
mhp510	UH	Unique hypothetical	0.0041	0.0957	1.34
mhp558	<i>potB</i>	Spermidine/putrescine transport permease	0.0007	0.0588	1.33
Downregulated					
mhp030	<i>gatB</i>	Glu-tRNA amidotransferase, subunit B	0.0063	0.0957	1.63
mhp081	UH	Unique hypothetical	0.0055	0.0957	1.23
mhp083	<i>fusA</i>	GTP-binding chain-elongation factor EF-G	0.0012	0.0633	1.63
mhp085	<i>rpsL</i>	30S ribosomal protein S12	0.0002	0.0347	1.34
mhp087	CH	Conserved hypothetical	0.0031	0.0957	1.35
mhp117	<i>hpt</i>	Hypoxanthine phosphoribosyl transferase	0.0097	0.0957	1.53
mhp167	<i>oppD</i>	Oligopeptide transport system permease	0.0042	0.0957	2.41
mhp168	<i>oppC</i>	Oligopeptide transport system permease	0.0062	0.0957	2.12
mhp169	<i>oppB</i>	Oligopeptide transport system permease	0.0050	0.0957	1.50
mhp176	CH	Conserved hypothetical, ATP-binding site motif, PP-loop	0.0009	0.0631	1.50
mhp190	<i>rpl2</i>	50S ribosomal protein L2	0.0100	0.0957	1.76
mhp337	UH	Unique hypothetical	0.0062	0.0957	1.17
mhp411	CH	Conserved hypothetical	0.0079	0.0957	1.13
mhp450	<i>metK</i>	S-Adenosylmethionine synthetase	0.0100	0.0957	1.36
mhp637	<i>rplL</i>	50S ribosomal protein L7/L12	0.0028	0.0957	1.46
mhp639	CH	Conserved hypothetical	0.0001	0.0333	1.60
mhp651	<i>ushA</i>	5'-Nucleotidase, putative lipoprotein	0.0003	0.0347	2.46
mhp666	CH	Conserved hypothetical, possible DHH phosphoesterase	0.0047	0.0957	1.31

*UH, unique hypothetical; CH, conserved hypothetical.

†FC, fold change.

Fig. 2. Volcano plot of transcriptional differences in *M. hyopneumoniae* during low-iron growth conditions. Data represent individual gene responses plotted as $-\log_{10}(P\text{-value})$ vs $\log_2(\text{fold change})$. Points above $-\log_{10}(P\text{-value})=2$ are significantly up- or down-regulated at $P<0.01$.

for selected genes are shown in Fig. 3. These data are shown as normalized data, and each line in the graph connects the two conditions, iron depletion and control, for one array. The data for all six arrays used in the statistical analysis are plotted. The quality of the data is reflected by the similarity in the slopes of the lines for each gene.

Validation of microarray data

Semi-quantitative RT-PCR was performed on five genes to validate the microarray results. The primers used for the RT-PCR reactions are given in Table 2. A 16S RNA control reaction was included. RT-PCR reactions were performed using the 12 RNA samples from the control and low-iron studies. The reactions contained equal RNA concentrations and were performed at the same time to reduce experimental error. Reaction products were separated on an agarose gel, stained, and the band intensities quantified. The twelve experimental replicates for each gene (six control and six low-iron samples) were analysed for significance. The RT-PCR product signal intensities for genes mhp140, mhp151 and mhp558 from low-iron conditions were significantly different from the control at $P<0.01$ (Fig. 4). Genes mhp275 and mhp639 were not significantly different at $P=0.01$.

DISCUSSION

The goal of this study was to determine the steady-state levels of individual mRNA species on a global level during growth of *M. hyopneumoniae* under normal laboratory conditions and compare them with the levels in organisms grown under iron-limiting conditions. This would allow the definition of iron-regulated genes in *M. hyopneumoniae* as a first step towards identifying their mechanism of regulation and possible role in virulence. The medium for *M. hyopneumoniae* is supplemented with 20 % swine serum,

Fig. 3. Gene-specific examples of normalized data. These are examples of significantly differentially expressed genes. Each line represents the normalized data for the two treatments on one array. The circles and squares represent Alexa 555 and 647 dyes, respectively. The plots exhibit relatively consistent expression differences between conditions across multiple slides. This can be seen by the similarity of the slopes of the six lines.

which is at the higher level normally used to culture mycoplasmas, and is a rich source of iron-binding transferrin. Under *in vivo* conditions, *M. hyopneumoniae* is exposed to

Table 2. RT-PCR primer sequences

Gene ID	Primer name	Primer sequence (5'–3')
rRNA	RNA-F	AGACGATGATGTTTAGCGGGG
	RNA-R	TGCTGCTCTTTGTAGTAGCCATG
mhp182	182-F	TAAAAACCGTGATTGAGGGC
	182-R	GCTGTTCAAATGCTTGTCCTC
mhp275	275-F	TGATATTGTTGATCTAGTCGACGG
	275-R	GGGCTTACACCTTCTTTGGC
mhp374	374-F	TTTGGTTATTCTAATTGGCG
	374-R	AACCACTTGGTTTTAGTTCGAC
mhp558	558-F	GAAGCCGGGACTGATTTAGG
	558-R	AGGGTCACCAAAACAAGCG
mhp639	639-F	AGGTCATCGAAAACGGGC
	639-R	ATAGTTTCAAGGGCTTGGCG
mhp677	677-F	CCAGAATATGTTTGGTGGGG
	677-R	TTGATCAACTCGGGACATCG

Fig. 4. Semi-quantitative RT-PCR performed on the mRNA transcripts in control and iron-depleted samples. Lanes 1, 3, 5, 7, 9 and 11 are control samples from *M. hyopneumoniae* incubated in normal growth medium. Lanes 2, 4, 6, 8, 10 and 12 are samples from *M. hyopneumoniae* incubated in medium containing 2,2'-dipyridyl. The gene designation is given on the left and the *P*-value of the densitometry readings on the right.

mucosal secretions, a rich source of iron-binding transferrin and lactoferrin and similar to the *in vitro* growth conditions in terms of iron availability. If mycoplasmas are to respond to host iron-sequestration mechanisms during disease, they must contain mechanisms for binding and transporting iron across their membrane. Whether *M. hyopneumoniae* or any other mycoplasma requires iron for growth is not known, but it is likely that enzymes with iron-containing prosthetic groups are present in mycoplasmas. One study examined the interaction of *M. pneumoniae* and *Mycoplasma genitalium* with these proteins and found that *M. pneumoniae* binds lactoferrin but not transferrin (Tryon & Baseman, 1987). *M. genitalium* did not bind either protein. Iron storage occurs in *M. capricolum* (Bauminger *et al.*, 1980), but iron acquisition or iron requirements have not been studied further in the mycoplasmas.

The rationale for using chelators in this study was to expose the mycoplasma to conditions that prevent iron uptake through normal pathways. This should be reflected in slower or lack of growth under the iron-limiting conditions, assuming there is a requirement for iron. Our initial studies used *M. gallisepticum* and *M. pulmonis* with three different iron chelators to estimate the need for iron among the mycoplasmas and to identify a chelator and concentration that might provide a slower growth response. We examined a range of chelator concentrations from 0.1 to 1 mg ml⁻¹; the lower limit reflects concentrations used in other bacterial

systems (Delany *et al.*, 2001; Paustian *et al.*, 2001; Rosner *et al.*, 2002) and the upper limit was thought necessary to provide sufficient chelator for serum-supplemented medium. Since both species demonstrated reduced growth with 2,2'-dipyridyl at 1 mg ml⁻¹ (Fig. 1), this chelator and concentration were used in transcriptional profiling studies with *M. hyopneumoniae*. We also chose a single time point, 2 h, because it represents an approximate generation time for *M. hyopneumoniae* under the growth conditions employed.

Analysis of the microarray data indicates that nine genes were up-regulated during iron deprivation: *iolB* (mhp151), *iolC* (mhp152), *glpQ* (mhp317), *mgla* (mhp319), *ackA* (mhp505) and *potB* (mhp558), in addition to three hypothetical genes (mhp140, mhp275 and mhp510). One of the latter, mhp275, is a member of the P102 paralogue family (Minion *et al.*, 2004). The function of P102 or any of its paralogues is not known, but it is interesting to speculate that one of these might have a role in iron uptake. Some of the known genes (*mgla* and *potB*) are involved in transport, while others (*iolCB*, *iolC*, *glpQ* and *ackA*) are involved in metabolism. Interestingly, mhp319 (*mgla*) is the only member of the ATP transporter family (there are 13 homologues in the genome) that showed differential transcription during iron deprivation. Could this be a transporter involved in iron uptake? Possibly, but without additional information, this would be entirely speculative. One of the genes, mhp140, has a prokaryotic membrane lipid attachment site and is thought to be a lipoprotein (Minion *et al.*, 2004).

Eighteen genes were down-regulated ($P < 0.01$) during iron limiting conditions. Seven of these (mhp081, mhp087, mhp176, mhp337, mhp411, mhp639 and mhp666) are hypothetical genes with no identified function. The remaining genes are identified as *gatB* (mhp030), *fusA* (mhp083), *rpsL* (mhp085), *hpt* (mhp117), *oppB-D* (mhp167–169), *rpL2* (mhp190), *metK* (mhp450), *rplL* (mhp637) and *ushA* (mhp651). Resistance to fusidic acid in *fusA* mutants of *Salmonella enterica* serovar Typhimurium alters growth both *in vivo* and *in vitro* (Macvanin *et al.*, 2003). So, although the serovar Typhimurium *fusA* mutants have some antibiotic resistance, their ability to grow is also stunted. The down-regulated genes identified in this study have roles in translation and cell growth. The gene products of *oppB-D* are involved in oligopeptide uptake, and as such, their down-regulation fits well with the idea of a slowed metabolism. The lack of biosynthetic pathways makes amino acid uptake a requirement in mycoplasmas. Alternatively, down-regulation of *oppB-D* might indicate a need for the mycoplasma to replace normal activities of the membrane with iron-acquisition components, although this is entirely speculative. The regulation or role of *ushA* during transcription is not clearly defined; however, it is known to increase twofold as cells enter the stationary phase (Wanner, 1996). In *M. hyopneumoniae* this protein has a prokaryotic lipid attachment site and is thought to be a lipoprotein. The down-regulation of *ushA* is most likely a response to iron

depletion and not due to cells entering the stationary growth phase under our experimental conditions. *hpt* is involved in the salvage of guanine for guanine nucleotide synthesis (Zalkin & Nygaard, 1996). Since cellular metabolism and growth are slowed during iron deprivation, the need to synthesize nucleotides is expected to decrease as well.

The degree of transcriptional change that occurs during low-iron growth conditions may indicate that iron-acquisition systems in mycoplasmas are tightly controlled. The mechanism(s) employed to accomplish this remain unknown. The number of hypothetical genes affected by iron chelation in this study was consistent with the 44 % of hypothetical genes in the genome (10 of 27). Some of these may play important roles in *M. hyopneumoniae*'s response to iron stress. Since there is no clear indication of the functions of these genes, additional studies are required to determine their role in iron acquisition and/or cellular metabolism. When comparing the iron deprivation data with our previous heat shock data (Madsen *et al.*, 2006), three of the nine up-regulated genes, *mhp151*, *mhp152* and *mhp275*, are up-regulated under both conditions ($P < 0.01$). For those genes down-regulated during iron deprivation, three are also down-regulated during heat shock ($P < 0.01$) (*mhp030*, *mhp176* and *mhp337*). This suggests that these six genes might be considered 'stress response' genes.

The evaluation of samples taken from the lungs of swine at the height of infection may be informative in conjunction with the data found here. However, laboratory-grown organisms may be competing for iron in vastly different ways than those found in their host niche. How *M. hyopneumoniae* secures sufficient levels of iron for its metabolism and whether it is able to respond to low-iron conditions through transcriptional regulation is not yet understood, but studies on the regulation mechanism(s) of several genes identified during this study may begin to unravel some of the mystery.

ACKNOWLEDGEMENTS

We would like to thank Nancy Upchurch and Barb Erickson for assistance with mycoplasma cultures. Josh Pitzer performed the RT-PCR reactions. Monica Perez contributed to the construction of the microarray. Mike Paustian contributed to the array design and printing. Mike Carruthers assisted with spot finding. Funding for this project was provided in part by the Iowa Healthy Livestock Advisory Council.

REFERENCES

- Aisen, P. & Listowsky, I. (1980). Iron transport and storage proteins. *Annu Rev Biochem* **49**, 357–393.
- Andrews, S. C., Robinson, A. K. & Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**, 215–237.
- Baker, E. N., Anderson, B. F., Baker, H. M., Day, C. L., Haridas, M., Norris, G. E., Rumball, S. V., Smith, C. A. & Thomas, D. H. (1994). Three-dimensional structure of lactoferrin in various functional states. *Adv Exp Med Biol* **357**, 1–12.

- Bauminger, E. R., Cohen, S. G., Labenski-Dekanter, F., Levy, A., Aster, S., Kessel, M. & Rottem, S. (1980). Iron storage in *Mycoplasma capricolum*. *J Bacteriol* **141**, 378–381.
- Crosa, J. H. (1989). Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol Rev* **53**, 517–530.
- Davis, J. K., Delozier, K. M., Asa, D. K., Minion, F. C. & Cassell, G. H. (1980). Interactions between murine alveolar macrophages and *Mycoplasma pulmonis* in vitro. *Infect Immun* **29**, 590–599.
- DeBey, M. C. & Ross, R. F. (1994). Ciliostasis and loss of cilia induced by *Mycoplasma hyopneumoniae* in porcine tracheal organ cultures. *Infect Immun* **62**, 5312–5318.
- Delany, I., Spohn, G., Rappuoli, R. & Scarlato, V. (2001). The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. *Mol Microbiol* **42**, 1297–1309.
- Dudley, A. M., Aach, J., Steffen, M. A. & Church, G. M. (2002). Measuring absolute expression with microarrays with a calibrated reference sample and an extended signal intensity range. *Proc Natl Acad Sci U S A* **99**, 7554–7559.
- Dudoit, S., Yang, Y. H., Callow, M. J. & Speed, T. P. (2000). Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. <http://www.stat.berkeley.edu/users/terry/zarray/Html/papersindex.html>
- Earhart, C. F. (1996). Uptake and metabolism of iron and molybdenum. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 1075–1090. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Freundt, E. A. (1983). Culture media for classic mycoplasmas. In *Methods in Mycoplasmaology*, vol. 1, *Mycoplasma Characterization*, pp. 127–135. Edited by S. Razin & J. G. Tully. New York: Academic Press.
- Friis, N. F. (1975). Some recommendations concerning primary isolation of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*, a survey. *Nord Veterinaermed* **27**, 337–339.
- Hutchison, C. A., III & Montague, M. G. (2002). Mycoplasmas and the minimal genome concept. In *Molecular Biology and Pathogenicity of Mycoplasmas*, pp. 221–253. Edited by S. Razin & H. Herrmann. New York: Kluwer Academic/Plenum.
- Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I. & Vasil, M. L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **99**, 7072–7077.
- Livingston, C. W., Stair, E. L., Underdahl, N. R. & Mebus, C. A. (1972). Pathogenesis of mycoplasmal pneumonia in swine. *Am J Vet Res* **33**, 2249–2258.
- Macvanin, M., Bjorkman, J., Eriksson, S., Rhen, M., Andersson, D. I. & Hughes, D. (2003). Fusidic acid-resistant mutants of *Salmonella enterica* serovar Typhimurium with low fitness in vivo are defective in RpoS induction. *Antimicrob Agents Chemother* **47**, 3743–3749.
- Madsen, M. L., Nettleton, D., Thacker, E. L., Edwards, R. & Minion, F. C. (2006). Transcriptional profiling of *Mycoplasma hyopneumoniae* during heat shock using microarrays. *Infect Immun* **74**, 167–174.
- Mare, C. J. & Switzer, W. P. (1965). New species: *Mycoplasma hyopneumoniae*, a causative agent of virus pig pneumonia. *Vet Med Small Anim Clin* **60**, 841–846.
- Mebus, C. A. & Underdahl, N. R. (1977). Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with *Mycoplasma hyopneumoniae*. *Am J Vet Res* **43**, 1249–1254.
- Minion, F. C., Lefkowitz, E. L., Madsen, M. L., Cleary, B. J., Swartzell, S. M. & Mahairas, G. G. (2004). The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. *J Bacteriol* **186**, 7123–7133.

- O'Halloran, T. V. (1993).** Transition metals in control of gene expression. *Science* **261**, 715–725.
- Paustian, M. L., May, B. J. & Kapur, V. (2001).** *Pasteurella multocida* gene expression in response to iron limitation. *Infect Immun* **69**, 4109–4115.
- Posey, J. E. & Gherardini, F. C. (2000).** Lack of a role for iron in the Lyme disease pathogen. *Science* **288**, 1651–1653.
- Ratledge, C. & Dover, L. G. (2000).** Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**, 881–941.
- Rodriguez, G. M. & Smith, I. (2003).** Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. *Mol Microbiol* **47**, 1485–1494.
- Rosner, J. L., Dangi, B., Gronenborn, A. M. & Martin, R. G. (2002).** Posttranscriptional activation of the transcriptional activator Rob by dipyrrolyl in *Escherichia coli*. *J Bacteriol* **184**, 1407–1416.
- Ross, R. F. (1992).** Mycoplasmal disease. In *Diseases of Swine*, pp. 537–551. Edited by A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire & D. J. Taylor. Ames: Iowa State University Press.
- Ross, R. F. (1993).** Mycoplasma-animal pathogens. In *Rapid Diagnosis of Mycoplasmas*, pp. 69–109. Edited by I. Kahane & A. Adoni. New York: Plenum.
- Salyers, A. A. & Whitt, D. D. (2002).** *Bacterial Pathogenesis: a Molecular Approach*, 2nd edn. Washington, DC: American Society for Microbiology.
- Schaible, U. E. & Kaufmann, S. H. E. (2004).** Iron and microbial infection. *Nat Rev Microbiol* **2**, 946–953.
- Storey, J. D. & Tibshirani, R. (2003).** Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A* **100**, 9440–9445.
- Szczepara, F., Dhaenens, L., Armand, S. & Husson, M. O. (1999).** Regulation of the transcription of genes encoding different virulence factors in *Helicobacter pylori* by free iron. *FEMS Microbiol Lett* **175**, 165–170.
- Thacker, E. L., Halbur, P. G., Ross, R. F., Thanawongnuwech, R. & Thacker, B. J. (1999).** *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. *J Clin Microbiol* **37**, 620–627.
- Tryon, V. V. & Baseman, J. B. (1987).** The acquisition of human lactoferrin by *Mycoplasma pneumoniae*. *Microbial Pathog* **3**, 437–443.
- Wanner, B. L. (1996).** Phosphorus assimilation and control of the phosphate regulon. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 1362–1381. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Weinberg, E. D. (1978).** Iron and Infection. *Microbiol Rev* **42**, 45–66.
- Weinberg, E. D. (1995).** Acquisition of iron and other nutrients in vivo. In *Virulence Mechanisms of Bacterial Pathogens*, pp. 79–93. Edited by J. A. Roth and others. Washington, DC: American Society for Microbiology.
- Wolfinger, R. D., Gibson, G., Wolfinger, E. D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C. & Paules, R. S. (2001).** Assessing gene significance from cDNA microarray expression data via mixed models. *J Comput Biol* **8**, 625–637.
- Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J. & Speed, T. P. (2002).** Normalization for cDNA microarray data: a robust composite method for addressing single and multiple slide systematic variation. *Nucleic Acids Res* **30**, e15.
- Zalkin, H. & Nygaard, P. (1996).** *Biosynthesis of purine nucleotides*. Washington, DC: American Society for Microbiology.