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

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*Mycobacteria 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.

INTRODUCTION

*Mycobacteria 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 Fe(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 ~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; Szczepanik 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 RNA later (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 labeled 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 Versagen 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, 35 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 93°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 analysis**

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 pooled 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/basedyeratio.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
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

![Graphs showing growth of M. pulmonis and M. gallisepticum](image)

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

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

*UH, unique hypothetical; CH, conserved hypothetical.
†FC, fold change.
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, 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...
mucosal secretions, a rich source of iron-binding transferrin and lactoferrin and similar to the mucosal secretions, a rich source of iron-binding transferrin and lactoferrin but not transferrin (Tryon & Baseman, 1987). Iron storage occurs in M. capricolum with these proteins and found 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), rplL (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 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 parologue 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).
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.

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REFERENCES


