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Global transcriptional analysis of *Mycoplasma hyopneumoniae* following exposure to norepinephrine

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Mycoplasma hyopneumoniae, a component of the porcine respiratory disease complex, colonizes the respiratory tract of swine by binding to the cilia of the bronchial epithelial cells. Mechanisms of pathogenesis are poorly understood for *M. hyopneumoniae*, but previous work has indicated that it responds to the environmental stressors heat shock, iron deprivation and oxidative compounds. For successful infection, *M. hyopneumoniae* must effectively resist host responses to the colonization of the respiratory tract. Among these are changes in hormonal levels in the mucosal secretions. Recent work in the stress responses of other bacteria has included the response to the catecholamine norepinephrine. The idea that *M. hyopneumoniae* can respond to a host hormone, however, is novel and has not previously been demonstrated. To test this, organisms in the early exponential phase of growth were exposed to 100 μM norepinephrine for 4 h, and RNA samples from these cultures were collected and compared to RNA samples from control cultures using two-colour PCR-based *M. hyopneumoniae* microarrays. The *M. hyopneumoniae* response included slowed growth and changes in mRNA transcript levels of 84 genes, 53 of which were upregulated in response to norepinephrine. A larger proportion of the genes upregulated than those downregulated were involved with transcription and translation. The downregulated genes were mostly involved with metabolism, which correlated with the reduction in growth of the mycoplasma. Approximately 51 % of the genes were hypothetical with no known function. Thus, in response to norepinephrine, *M. hyopneumoniae* appears to upregulate protein expression while downregulating general metabolism.

INTRODUCTION

Mycoplasma hyopneumoniae causes enzootic pneumonia in swine and affects the immune response to other bacterial and viral agents (Thacker *et al.*, 1999b, 2001). The mechanisms involved in colonization and disease are not well understood. In experimental systems, pathogen-free pigs infected with *M. hyopneumoniae* present a progressive lung disease until about 28 days, at which time the disease slowly resolves, reaching a chronic state of infection. Depending upon the strain, lesions can involve up to 30 % of the lung at the height of disease, and in many animals, the organism is never completely eliminated. In more conventional piggeries, an important effect of *M. hyopneumoniae* in the respiratory tract is to increase the severity of disease caused by other pathogens such as *Pasteurella multocida*, PRRSV (Thacker *et al.*, 1999a) and PCV2 (Opriessnig *et al.*, 2004). To achieve this, *M. hyopneumoniae* must circumvent the host immune response, both the innate and adapted phases.

Adherence mechanisms have been studied (Djordjevic *et al.*, 2004; Hsu *et al.*, 1997; Jenkins *et al.*, 2006) and the genome sequence is now available (Minion *et al.*, 2004), but the fundamental processes used by *M. hyopneumoniae* to effect changes to the host immune response have not been elucidated. In previous studies, transcriptional responses of *M. hyopneumoniae* to heat shock, iron deprivation and oxidative stress, all environments encountered *in vivo*, have been analysed using microarrays (Madsen *et al.*, 2006a, b; Schafer *et al.*, 2007).

Recently, the responses of bacterial pathogens to the host stress factors epinephrine and norepinephrine have been studied with surprising results (Waldor & Sperandio, 2007). Originally it was thought that bacteria would not

Abbreviation: qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.

The Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) accession number for microarray data for this paper is GSE8494.

A supplementary table with details of genes differentially expressed during exposure to 100 μM norepinephrine and a supplementary figure showing the locations of significantly regulated genes on the *M. hyopneumoniae* chromosome are available with the online version of this paper.

respond to mammalian signalling molecules such as catecholamine neuroendocrine hormones, but this has not been the case. Several different bacterial species have been shown to respond, notably by increasing growth rates and inducing virulence factors. Freestone *et al.* (2007) showed that *Escherichia coli* O157 : H7 and *Yersinia enterocolitica* increased their growth rate when exposed to epinephrine and norepinephrine. *E. coli* O157 : H7 demonstrated a twofold increase not only in growth rate but also in shiga toxin production when stimulated with norepinephrine (Voigt *et al.*, 2006). In addition, enhancement in attaching and effacing lesions occurs as a consequence of AI-3 and norepinephrine binding (Reading *et al.*, 2007) resulting in increased binding to murine caecal mucosa (Chen *et al.*, 2003). It is also interesting to note that the AI-3 receptor QseC serves as the epinephrine/norepinephrine receptor in *E. coli* O157 : H7 (Clarke *et al.*, 2006). Bansal *et al.* (2007) also showed that norepinephrine affected *E. coli* O157 : H7 chemotaxis and adherence to HeLa S3 cells. Another recent study of *E. coli* transcription levels showed that several virulence factor transcripts were upregulated in response to norepinephrine as well as iron-acquisition systems and SOS-response genes (Dowd, 2007).

Other studies have noted changes by pathogens in response to norepinephrine. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Shigella somnei* and *Staphylococcus aureus* showed increased growth rates when exposed to norepinephrine, which seemed to be due to iron availability (O'Donnell *et al.*, 2006). In *Vibrio parahaemolyticus*, the modulation of virulence factors was observed (Nakano *et al.*, 2007). Norepinephrine was shown to bind and upregulate the adherence protein OspA in *Borrelia burgdorferi* (Scheckelhoff *et al.*, 2007). There was an increase in the pathogenic potential of *Campylobacter jejuni* (Cogan *et al.*, 2006), and norepinephrine upregulated the gene for the ferric enterobactin outer-membrane receptor BfeA in *Bordetella bronchiseptica* and *Bordetella pertussis*, thereby enhancing growth (Anderson & Armstrong, 2006). Thus, many pathogens respond to host catecholamines by enhancing their growth and upregulating genes needed for colonization and survival in the host environment.

Norepinephrine release is one of the stress responses in lung infections, and thus it was of interest to better understand how *M. hyopneumoniae* might respond to a clinical level in broth culture. For these studies, microarrays were used to assess the transcriptional responses of *M. hyopneumoniae* following supplementation of growth media with norepinephrine. Interestingly, our results showed that *M. hyopneumoniae* demonstrated reduced growth and significant changes in transcript levels in response to norepinephrine exposure.

METHODS

Mycoplasma strain and culture conditions. Pathogenic *M. hyopneumoniae* strain 232 was used in this study. Cultures were initially inoculated with lung inoculum, and passaged as previously described (Madsen *et al.*, 2006a). Mycoplasma growth in the presence or absence of 50 or 100 μM norepinephrine (Sigma) was measured by colour-changing units (DeBey & Ross, 1994). To confirm the colour-changing unit results, DNA real-time PCR was performed on a time-course experiment measuring increases in mycoplasma DNA in the presence of 100 μM norepinephrine. Samples were collected at 0, 4, 24 and 48 h following addition of norepinephrine. Two hundred microlitres of culture was removed and DNA was extracted using the QIAamp DNA mini kit (Qiagen). The DNA was carefully eluted in 100 μl elution buffer. Real-time assays contained 2.5 μl template DNA, 50 nM forward primer (5′-CCAGAACCAAATTCCTTCGCTG-3′), 900 nM reverse primer (5′-ACTGGCTGAACTTCATCTGGGCTA-3′) and 50 nM probe (5′-6_FAM-AGCAGATCTTAGTCAAAGTGC-CCGTG-BHQ_1) with the Universal PCR master mix (Applied Biosystems) in a total volume of 25 μl. Primers were obtained from Integrated DNA Technologies. The following cycling parameters were used: 95 °C, 10 min; 40 cycles of 95 °C, 15 s; then 60 °C, 1 min. All real-time assays were performed on a Rotor-Gene RG-3000 (Corbett Research). Data were analysed according to Gallup & Ackermann (2006).

For microarray studies, twelve 250 ml flasks containing 150 ml culture were grown to early exponential phase as determined by colour change of phenol red. Norepinephrine from a 5 mM stock solution in water was added to six flasks to a final concentration of 100 μM, and all twelve flasks were incubated at 37 °C for 4 h. Cells were pelleted and stored at −70 °C in RNAlater (Ambion) as previously described (Madsen *et al.*, 2006a).

Microarray. The *M. hyopneumoniae* microarray consists of 618 PCR-amplified *M. hyopneumoniae* ORF gene products 125–350 bp in length. These products encompass 89 % (618/698) of the ORFs in the genome. The construction and validation of these arrays have been previously described (Madsen *et al.*, 2006a).

Experimental design. Six independent RNA samples from norepinephrine-exposed cultures were paired with six independent RNA samples from control cultures for hybridization to six two-colour microarrays. For three arrays, the control RNA sample was labelled with Cy3 dye and the experimental RNA sample was labelled with Cy5 dye; the dyes were reversed for the other three arrays to account for any dye bias.

RNA isolation. RNA was isolated from frozen cell pellets using the Versagene RNA purification System (Gentra Systems). The manufacturer's protocol was followed with the exception of the DNase treatment, which was extended to 40 min. The filter cutoff of 150 bp prevented small RNA fragments from interfering in subsequent manipulations.

Target generation and hybridization. Fluorescently labelled cDNA targets were generated with a 129 oligo-specific hexamer primer set used in previous studies (Madsen *et al.*, 2006a). Fluorescently labelled cDNA was prepared by the incorporation of aminoallyl dUTP (Fermentas) during reverse transcription. Reverse transcription reactions contained 10 μg total RNA; 11.6 μg hexamer primers (Integrated DNA Technologies); 380 U Superscript III reverse transcriptase (Invitrogen); 1 mM each of dATP, dCTP, and dGTP; 200 μM dTTP; 400 μM aminoallyl dUTP; 10 mM dithiothreitol; and 1 × RT buffer (Invitrogen). The reaction was allowed to proceed overnight at 42 °C. RNA was hydrolysed with 10 mM EDTA and 10 mM sodium hydroxide (final concentrations) for 10 min at 65 °C. The solution was neutralized with 500 mM HEPES pH 7.0, and the aminoallyl-labelled cDNA was then purified using the UltraClean PCR Clean-Up kit (Mo Bio Laboratories).

The subsequent coupling of Cy3 and Cy5 dyes (GE Healthcare) to the purified aminoallyl-labelled cDNA was performed in 17.5 μl reaction

including 10 μl nuclease-free water, 1.5 μl 100 mM sodium bicarbonate (pH 9.0) solution (90 mM final concentration) and 6 μl dye. To prepare the dye, one vial of Cy3 (product PA23001, GE Healthcare) or Cy5 (product PA25001) was first dissolved in 72 μl DMSO, from which 6 μl aliquots were made and stored in the dark at −70 °C. The reaction was incubated at room temperature in the dark for a minimum of 2 h. The dye-labelled cDNA was then purified using the UltraClean PCR Clean-Up kit and dye incorporation evaluated by spectrophotometry (model ND-1000 NanoDrop, NanoDrop Techologies). After the purification of the labelled cDNA targets, they were hybridized to the array using the previously described protocol (Madsen *et al.*, 2006a). Slides were washed using the Corning UltraGAPS slide wash protocol and dried by centrifugation at 1500 ***g*** for 2 min.

Data acquisition, normalization and data analysis. After hybridization and washing, the arrays were scanned and analysed as described previously (Madsen *et al.*, 2006a, b). Spot signals were corrected for background, transformed, and adjusted to a common median for each scan of the same array–dye combination. Locally weighted scatterplot smoother (LOWESS) normalization was applied and triplicate spots were averaged together to produce one normalized measurement for each probe on the array. A separate mixed linear model analysis was conducted for each probe sequence using the normalized data (Wolfinger *et al.*, 2001). *t*-tests for differential expression between treatments were conducted for each probe in the mixed linear model analyses. *P*-values from these tests were converted to *q*-values using the method of Storey & Tibshirani (2003). These *q*-values were used to approximate the false discovery rate (FDR) for any given *P*-value as described by Benjamini & Hochberg (2000). Fold changes of expression between treatments were also estimated for each probe by taking the inverse natural log of the estimated mean treatment difference from our mixed linear model analyses.

Validation of microarray data. Significant transcriptional differences between the treatment and control cultures were verified by qRT-PCR. Four upregulated (mhp008, mhp092, mhp549 and mhp684) and two downregulated genes (mhp034 and mhp078) were chosen for confirmation based on the magnitude of transcriptional change or their presumed importance in proliferation and virulence. The gene mhp345 was used as a control for RT reactions because it has not shown significant change in previous work (Madsen *et al.*, 2006a, b) or this study. The qRT-PCR was performed using the Brilliant SYBR Green qRT-PCR kit (Stratagene), following the manufacturer's protocol. The primers used are listed in Table 1. Cycling was performed on the MyIQ Single-Colour Real-time-PCR Detection System (Bio-Rad). Data were analysed according to Gallup & Ackermann (2006).

Table 1. Primers used in qRT-PCR

Gene	Direction	Sequence (5′–3′)
mhp345	Forward	TGAAGCGCTTATGCTACTGAG
	Reverse	ATTGCGGTTGTACGAGCGACCTTA
mhp008	Forward	TGGATGGTACAAGCAAAGGTGGGA
	Reverse	CATCGCTTCGCCAAGTCCAACTAA
mhp092	Forward	TTTGCGGGCTTATGCTGACTTCTG
	Reverse	AAATTTGCAACAAGGGCGGCGT
mhp549	Forward	CGAAATTCCGCCTCCAAGTGCTTT
	Reverse	CAATTGCAACAGCGGGAATCTGGA
mhp684	Forward	AACAAGTTCCCAAGGCCAAGTTCC
	Reverse	TGATTTGAGGCCTCTGCTCCTTGA
mhp034	Forward	ACGGGCAATTCCAGATGTCAGAGA
	Reverse	TGGGCAAGTCTGACAAGAGCATCA
mhp078	Forward	GTAAATCGACTTTGGCCGAC
	Reverse	TGCCTGAATCCCTTGACTTG

RESULTS AND DISCUSSION

Growth effects

Cultures of *M. hyopneumoniae* were treated with 50 and 100 μM norepinephrine and the growth was monitored by colour-changing units. These concentrations of catecholamine were chosen based on studies with other bacterial pathogens (Anderson & Armstrong, 2006; Dowd, 2007; Freestone *et al.*, 2007; Nakano *et al.*, 2007). Surprisingly, norepinephrine slowed growth of *M. hyopneumoniae* cultures by approximately twofold (data not shown). Cultures with 100 μM norepinephrine took twice as long as control cultures to reach exponential phase as measured by colour changes. Cultures with 50 μM norepinephrine exhibited similar slowed growth but not as dramatic. To confirm this, a DNA real-time PCR assay was used to measure changes in mycoplasma DNA concentration in cultures treated with 100 μM norepinephrine compared to controls. The results of this analysis are shown in Fig. 1, which clearly shows a decrease in growth based on DNA concentration in cultures exposed to 100 μM norepinephrine. At 24 h, difference between control and norepinephrine-treated samples was significant at $P<0.05$. Based on these results, 100 μM norepinephrine was added to the treated group in the microarray studies.

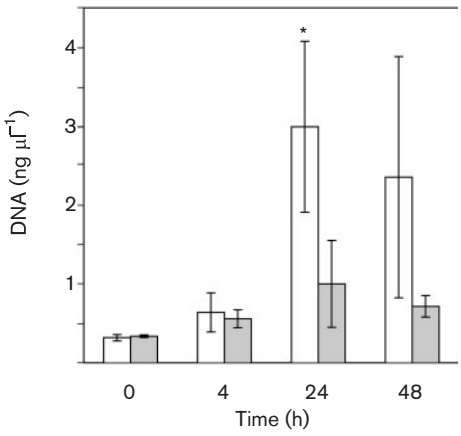

Fig. 1. Analysis of *M. hyopneumoniae* growth in the presence of 100 μM norepinephrine. Norepinephrine was added to freshly diluted mid-exponential-phase cultures (grey bars), and 200 μl samples were removed at 0, 4, 24 and 48 h. Control cultures (white bars) had no added norepinephrine. The DNA was purified, and the concentration of *M. hyopneumoniae* DNA was determined by DNA real-time PCR at each time point. Data represent mean ng DNA μl^{-1} ± two times the standard error of the mean of duplicate samples of three biological replicates. The asterisk indicates statistical significance at $P<0.05$.

Table 2. Significant differentially regulated genes in *M. hyopneumoniae* following exposure to norepinephrine

Gene ID	Gene	Gene function	*P*-value	*q*-value	Fold change*	
					Array	qRT-PCR
Upregulated genes						
mhp002	*dnaN*	DNA polymerase III, β chain	0.0023	0.020	1.23	
mhp006	CH	Conserved hypothetical	0.0070	0.032	1.17	
mhp008	*ftsY*	Cell division protein	0.0015	0.017	1.24	3.64
mhp013	UH	Unique hypothetical	0.0010	0.014	1.41	
mhp022	CH	ABC transporter ATP-binding protein	0.0018	0.018	1.26	
mhp054	*atpG*	ATP synthase γ chain	0.0073	0.032	1.26	
mhp059	*tsf*	Elongation factor ts	0.0097	0.035	1.23	
mhp087	CH	Conserved hypothetical	0.0052	0.028	1.19	
mhp092	CH	Conserved hypothetical	0.0011	0.015	2.26	2.24
mhp097	*topA*	DNA topoisomerase I (omega-protein)	0.0014	0.017	1.25	
mhp110	*smpB*	SsrA-binding protein	0.0081	0.033	1.64	
mhp118	CH	Conserved hypothetical	0.0066	0.031	1.59	
mhp156	*nrdF*	Ribonucleoside-diphosphate reductase β chain	0.0066	0.031	1.73	
mhp170	UH	Unique hypothetical, putative lipoprotein	0.0003	0.010	1.19	
mhp171	CH	ABC transporter ATP-binding protein	0.0049	0.028	1.87	
mhp188	*rpl4*	50S ribosomal protein L4	0.0077	0.032	1.11	
mhp189	*rpl23*	50S ribosomal protein L23	0.0095	0.035	1.21	
mhp190	*rpl2*	50S ribosomal protein L2	0.0092	0.035	1.40	
mhp191	*rps19*	30S ribosomal protein S19	0.0010	0.014	1.58	
mhp198	*rpl14*	50S ribosomal protein L14	0.0026	0.020	1.71	
mhp202	*rps8*	30S ribosomal protein S8	0.0061	0.031	1.46	
mhp203	*rpl6*	50S ribosomal protein L6	0.0073	0.032	1.39	
mhp205	*rps5*	30S ribosomal protein S5	0.0048	0.028	1.22	
mhp208	*adk*	Adenylate kinase	0.0019	0.018	1.30	
mhp209	*map*	Methionine aminopeptidase	0.0023	0.020	1.21	
mhp213	*rpoA*	RNA polymerase α chain	0.0018	0.018	1.19	
mhp230	CH	Conserved hypothetical	0.0045	0.027	1.21	
mhp240	CH	Conserved hypothetical	0.0010	0.014	1.46	
mhp284	CH	Conserved hypothetical	0.0094	0.035	1.24	
mhp377	CH	Conserved hypothetical, lipoprotein	0.0091	0.035	1.25	
mhp401	CH	Conserved hypothetical	0.0081	0.033	1.16	
mhp429	UH	Unique hypothetical	0.0039	0.025	1.35	
mhp430	*efp*	Translation elongation factor	0.0010	0.014	1.18	
mhp431	*tktA*	Transketolase	0.0004	0.011	1.22	
mhp434	CH	Conserved hypothetical	0.0009	0.014	1.23	
mhp451	CH	Conserved hypothetical	0.0024	0.020	1.60	
mhp452	CH	Conserved hypothetical	0.0016	0.017	1.38	
mhp477	*atpA*	ATP synthase α chain	0.0075	0.032	1.56	
mhp478	CH	Conserved hypothetical	0.0062	0.031	1.56	
mhp479	CH	Conserved hypothetical	0.0062	0.031	1.53	
mhp480	CH	Conserved hypothetical	0.0003	0.010	2.09	
mhp481	CH	Conserved hypothetical	0.0001	0.006	2.08	
mhp482	CH	Conserved hypothetical	0.0008	0.014	1.76	
mhp499	*oppD*	Oligopeptide transport system permease	0.0057	0.031	1.37	
mhp501	*oppB*	Oligopeptide transport system permease	0.0075	0.032	1.34	
mhp526	CH	Conserved hypothetical	0.0039	0.025	1.47	
mhp527	CH	Conserved hypothetical	0.0069	0.031	1.42	
mhp533	CH	Conserved hypothetical	0.0049	0.028	1.42	
mhp549	*polC*	DNA polymerase III, α chain	0.0085	0.034	1.16	12.20
mhp634	CH	Conserved hypothetical	0.0002	0.010	1.38	
mhp635	*rpoC*	RNA polymerase β chain	0.0038	0.025	1.22	
mhp680	*pepP*	Xaa-Pro aminopeptidase	0.0016	0.017	1.18	
mhp684	*p146*	P146 adhesin like-protein, p97 paralogue	0.0034	0.023	1.17	3.71

Table 2. cont.

Gene ID	Gene	Gene function	*P*-value	*q*-value	Fold change*	
					Array	qRT-PCR
Downregulated genes						
mhp034	*parC*	Topoisomerase IV subunit A	<0.0001	0.003	1.59	11.11
mhp077	CH	Conserved hypothetical	0.0023	0.020	1.51	
mhp078	*lepA*	30 kDa GTP-binding protein LepA	0.0067	0.031	1.40	3.70
mhp128	*serS*	Seryl-tRNA synthetase	<0.0001	0.002	1.62	
mhp144	CH	Conserved hypothetical, putative dehydrogenase	0.0011	0.015	2.33	
mhp145	CH	Conserved hypothetical, putative D-ribose-binding protein	0.0095	0.035	2.52	
mhp146	*rbsC*	Ribose ABC transporter	0.0001	0.003	1.95	
mhp149	*iolD*	*myo*-Inositol catabolism	0.0029	0.021	2.21	
mhp150	CH	Conserved hypothetical	<0.0001	0.003	2.80	
mhp151	CH	Conserved hypothetical	0.0004	0.010	2.84	
mhp152	*iolC*	*myo*-Inositol catabolism	0.0025	0.020	1.55	
mhp153	*mmsA*	Methylmalonate-semialdehyde dehydrogenase	0.0020	0.018	1.69	
mhp181	CH	Conserved hypothetical	0.0005	0.011	1.11	
mhp224	CH	Conserved hypothetical	0.0061	0.031	1.12	
mhp226	*era*	GTP-binding protein Era homologue	0.0004	0.011	1.22	
mhp236	CH	Conserved hypothetical, chromate transport protein	0.0031	0.022	1.30	
mhp302	UH	Unique hypothetical	0.0065	0.031	1.50	
mhp318	*mglA*	ATP-binding protein	0.0046	0.027	1.25	
mhp325	CH	Conserved hypothetical	0.0096	0.035	1.77	
mhp326	UH	Unique hypothetical	0.0015	0.017	1.26	
mhp350	CH	Conserved hypothetical	0.0066	0.031	1.27	
mhp370	*glpK*	Glycerol kinase	0.0043	0.027	1.28	
mhp374	CH	Conserved hypothetical	0.0028	0.021	1.41	
mhp405	CH	Conserved hypothetical	0.0064	0.031	1.53	
mhp406	CH	Conserved hypothetical	0.0008	0.014	1.68	
mhp407	*ftsZ*	Cell division	<0.0001	0.003	1.81	
mhp461	UH	Unique hypothetical	0.0039	0.025	1.47	
mhp504	*pdhD*	Dihydrolipoamide dehydrogenase	0.0076	0.032	1.47	
mhp562	*ktrA*	Potassium uptake protein	0.0003	0.010	1.37	
mhp574	UH	Unique hypothetical	0.0018	0.018	1.47	
mhp590	*nagE*	PTS system, *N*-acetylglucosamine-specific enzyme II	0.0026	0.020	1.27	

*Fold changes by array or RT-PCR were correlated with up- or downregulation in all cases.

The fact that *M. hyopneumoniae* growth was slowed by norepinephrine was unexpected. The reason for this growth reduction was not clear since other bacteria react more positively to this hormone either by increasing growth rate or by inducing virulence factors. Whether the effect was due to a specific mechanism involving catecholamine receptors coupled with a signal transport system and gene regulatory components or was a toxic effect causing interference with the basic physiology of the organism could not be determined by these studies.

Microarray results

Microarrays were used to compare mRNA steady-state levels between control and norepinephrine-treated strain 232. Six microarrays representing six biological replicates of each treatment were analysed using a mixed linear model design. Estimated mean differences were significant for 84 genes at $P<0.01$ with a q-value <0.035 (Table 2, Fig. 2). Fifty-three of the 84 genes were upregulated (63 %) and 31 (37 %) were downregulated. Two of the upregulated genes, mhp170 and mhp377, are putative lipoprotein genes. Forty-three of the 84 genes (51 %) differentially transcribed are classified as hypothetical.

Confirmation of these microarray results was obtained by qRT-PCR using RNA from the samples used for microarray analysis and the primers described in Table 1. All six genes tested, four upregulated and two downregulated, demonstrated concordant transcript changes (Table 2).

Upregulated genes

A cursory examination comparing the genes upregulated with those downregulated demonstrates a larger propor-

tion involved with transcription and translation in the former group. One region of the genome, mhp188–mhp206, contains numerous ribosomal protein genes. Almost all of the genes within this region indicate an upregulation upon exposure to norepinephrine when using a cutoff of $P<0.05$ (Table 2; see also Supplementary Table S1, available with the online version of this paper). The positions of the regulated genes on the chromosome where $P<0.05$ are shown in Supplementary Fig. S1. Genes mhp478–mhp482 are conserved hypothetical genes, forming an apparent operon with unknown function. Another notable upregulated gene is *dnaN* (mhp002), the DNA polymerase β sliding clamp (Sutton, 2004). It is involved in SOS DNA repair, is a negative regulator of *dnaA*, and possibly functions to bind with other proteins such as MutS, Lig and GyrA for the broader repair of DNA (Wang & Crowley, 2005). FtsY (mhp008), the functional homologue of the mammalian signal recognition particle (SRP), is needed for the assembly of other proteins, and it binds to membranes and blocks trigger factor binding in the ribosome. Trigger factor is a ribosome-associated molecular chaperone involved in proline-limited protein folding (Scholz *et al.*, 1997). The alteration of trigger factor association with the ribosome could have dramatic effects on protein function in the cell independent of transcription. Upregulation of the RNA polymerase α chain protein, RpoA (mhp213), may affect transcription efficiency, as would upregulating the elongation factor ts (*tsf*, mhp059), which is in an operon with *rpsB* ($P=0.0118$), both of which are also upregulated and involved in translation. The upregulation of the SsrA-binding protein gene *smpB* (mhp110) may effect the trans-translation process to recycle ribosomes stalled on problematic mRNAs by interacting with tmRNA (Ivanova *et al.*, 2005). Trans-translation releases stalled ribosomes from truncated mRNAs and tags defective proteins for proteolytic degradation using tmRNA. The overall effect of an increased SmpB protein concentration in the cell would be to enhance the efficiency of translation without having to alter the concentration of ribosomes in the cell.

Various other genes were upregulated at a significance level of $P<0.01$ in response to norepinephrine, including 26 hypothetical genes. This represents approximately 49 % of the genes, a proportion equivalent to the entire genome (Minion *et al.*, 2004; Vasconcelos *et al.*, 2005). The gene for topoisomerase I, *topA* (mhp097), is activated during oxidative stress (Weinstein-Fischer & Altuvia, 2007) in addition to being responsive to norepinephrine. It has also been shown to be differentially regulated in *E. coli* in a Fis-dependent manner (Weinstein-Fischer & Altuvia, 2007). *polC*, the gene for DNA polymerase III α chain, is also upregulated, and both it and *topA* are involved in DNA replication. The oligopeptide transport system permease complex mhp498–mhp501 (*oppBCDF*) is upregulated ($P<0.05$); this has also been observed during heat shock in *M. hyopneumoniae* (Madsen *et al.*, 2006a). Perhaps this operon is part of a general stress response of the mycoplasma. Adenylate kinase (*adk*; mhp208), which is involved with maintaining adenine homeostasis, and methionine aminopeptidase (*map;* mhp209), which removes N-terminal methionines from proteins, are upregulated in response to norepinephrine. One of the P97 adhesin-binding homologue protein genes, *p146* (mhp684), is also upregulated (Adams *et al.*, 2005; Minion *et al.*, 2004). The function of P146 is unknown, but it could have an important role in tissue adherence and colonization. Finally, the upregulation of transketolase (*tktD*, mhp431), ribonucleoside-diphosphate reductase β chain (*nrdF*, mhp156) and *pepP* (mhp680), Xaa-Pro aminopeptidase, could serve to enhance metabolism in subtle, but undefined ways.

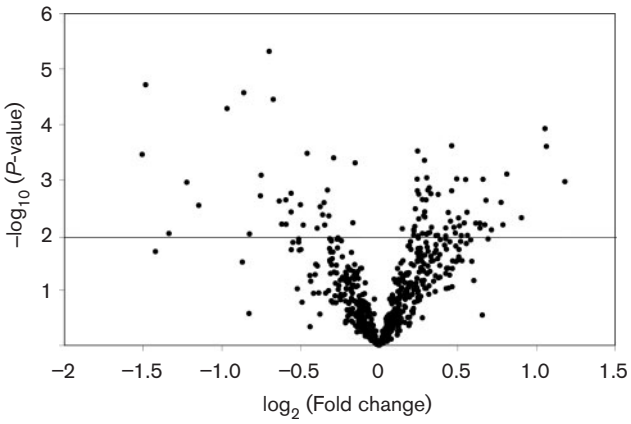

Fig. 2. Volcano plot of transcriptional responses in *M. hyopneumoniae* following exposure to 100 μM norepinephrine. Data represent individual gene responses plotted as $\log_2$(fold change) vs $-\log_{10}$(*P*-value). Points above $-\log_{10}$(*P*-value)=2.0 are significantly up- or downregulated relative to the control at $P<0.01$.

Downregulated genes

Fewer genes were downregulated in response to norepinephrine at a significance level of $P<0.01$. Among the 32 downregulated genes were 17 (53 %) hypothetical genes. *parC*, the catalytic centre for DNA cleavage and rejoining in topoisomerase IV is included among the genes with known functions. Interestingly, we demonstrated that *parC* (mhp034) is upregulated during heat shock in *M. hyopneumoniae* (Madsen *et al.*, 2006a), but it is downregulated in this study. How this gene may be differentially regulated in response to heat and catecholamines is unknown since little is known about the mechanisms of regulation of mycoplasma genes. Several genes directly involved in intermediate metabolism were downregulated in this study. These include *serS* (mhp128), seryl-tRNA synthetase, *rbsC* (mhp246), ribose ABC transporter, an ATP-binding transporter, *mglA* (mhp318), two GTP-binding proteins, *lepA* (mhp078) and *era* (mhp226), and glycerol kinase (*glpK*, mhp370). Several genes involved in

energy metabolism are downregulated, including *mmsA* (mhp153), a dehydrogenase, *pdhD* (mhp504), a dihydrolipoamide dehydrogenase, and *nagE* (mhp590), a member of the phosphotransferase (PTS) system. One protein involved in cell division, *ftsZ* (mhp407), was downregulated; this correlates with our observation that exposure of *M. hyopneumoniae* to norepinephrine slows growth, in contrast to what has been observed with other bacteria (Waldor & Sperandio, 2007).

An apparent operon containing mhp144–mhp153 is downregulated in response to norepinephrine. Among the genes in this operon are a putative dehydrogenase (mhp144), a putative ribose-binding protein (mhp145), a ribose transporter (mhp146), a ribose transport ATP-binding protein (mhp147), proteins involved in *myo*-inositol catabolism (*iolD*; mhp149 and *iolC*; mhp152), and methylmalonate-semialdehyde dehydrogenase (*mms*; mhp153). Three other genes in this operon are conserved hypothetical genes with unknown function. Many of these genes are upregulated under heat shock and low-iron conditions (Madsen *et al.*, 2006a, b).

Interpretation of the differential responses

When comparing the direction of regulation of differentially expressed genes in norepinephrine-exposed *M. hyopneumoniae* to those genes shown to be differentially expressed under heat shock (Madsen *et al.*, 2006a), iron deprivation (Madsen *et al.*, 2006b), oxidation conditions (Schafer *et al.*, 2007) and *in vivo* growth (Madsen *et al.*, 2008), an interesting correlation was observed. Genes upregulated during norepinephrine exposure were generally downregulated under the other conditions, if at all (Supplementary Table S1). Downregulated genes during norepinephrine exposure were generally upregulated under other conditions. These observations tend to support several hypotheses. First, *M. hyopneumoniae* has active gene regulatory mechanisms that have yet to be defined. It is well known that mycoplasmas have only a single sigma factor in their genomes and few known transcriptional regulators (Chang *et al.*, 2008), so how this regulation occurs and the components involved are still unknown. These regulatory mechanisms may not be as robust as in other organisms, resulting in smaller fold change values, but given the small size, slow growth, truncated energy-producing pathways and other limiting factors associated with a minimal genome, they obviously are sufficient for survival under the difficult environmental conditions encountered in the host. Second, at least two of the conditions studied, norepinephrine and iron, require an active sensor system for which no genes have been annotated. The genes are most likely in the large hypothetical group and may code for some of the 53 lipoproteins (Minion *et al.*, 2004). There is also a large group of transport-related proteins that might be involved and serve as receptors. Finally, given the diversity of genes regulated under different environmental conditions such as heat shock (Madsen *et al.*, 2006a), iron deprivation (Madsen *et al.*, 2006b), oxidative stress (Schafer *et al.*, 2007) and the pig lung (Madsen *et al.*, 2008), transcription in *M. hyopneumoniae* must involve multiple factors and mechanisms. We are only beginning to decipher those components and their mechanisms of action (Chang *et al.*, 2008).

In summary, our studies indicate that *M. hyopneumoniae* responds to the host catecholamine norepinephrine by differential expression of specific genes. An overall increase in transcription- and translation-related genes occurs, as does a decrease in several metabolically related genes. This is just one of the environmental changes expected to impact the overall physiology of *M. hyopneumoniae* as it colonizes and parasitizes the swine respiratory tract. Whether this growth effect due to norepinephrine occurs *in vivo* in the presence of other stressors is not clear since the levels of norepinephrine may fluctuate in response to many factors in the lung and the effects of multiple factors may be confounding on gene expression in *M. hyopneumoniae*. Interestingly, the set of genes identified by this study is different from those identified in other stress-related studies (Madsen *et al.*, 2006a, b; Schafer *et al.*, 2007). Perhaps a combination of stressors is necessary to induce the full complement of genes needed by *M. hyopneumoniae* to survive the host environment. Since so little is known about how mycoplasmas regulate their genes, more mechanistic studies will be needed to decipher the importance of single stressor signals in the overall pathogenesis of *M. hyopneumoniae*.

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