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Transcriptional Responses of *Mycoplasma gallisepticum* Strain R in Association with Eukaryotic Cells▽

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Mycoplasma gallisepticum **is an etiologic agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys. Other than proteins important for cytadherence, few *M. gallisepticum* factors or pathways contributing to host cell interactions have been identified. In this study, an oligonucleotide-based microarray was utilized to investigate transcriptional changes in *M. gallisepticum* strain R_{low} upon exposure to eukaryotic cells. Fifty-eight genes were either up- or downregulated upon exposure to MRC-5 lung fibroblasts grown in vitro, including genes encoding transport-, metabolism-, and translation-associated proteins. Twenty of the 58 regulated genes have no assigned function. These results indicate that *M. gallisepticum* regulates gene expression upon exposure to eukaryotic cells, revealing genes and pathways likely to be important for host-bacterium interaction.**

Mycoplasma gallisepticum represents a major threat to the poultry industry, acting as an important pathogen in chronic respiratory disease and resulting in reduced weight gain and egg production in infected birds. *M. gallisepticum* is also pathogenic in other species, causing infectious sinusitis in turkeys and conjunctivitis in house finches (4, 15). With the exception of attachment proteins GapA and CrmA (10), and to a lesser extent dihydrolipoamide dehydrogenase (Lpd) (3), little is known about factors responsible for survival or persistence in the host.

Mycoplasmas lack obvious homologues of conventional elements of transcriptional regulation, including sigma factors, signaling factors, and transcription factors. This absence has led to the supposition that differences in gene expression in mycoplasma species are due to population selection and heterogeneity rather than more traditional mechanisms. Although several basic investigations into transcriptional responses have shown differences due to heat shock (5, 17) and iron depletion (6), changes that are not attributable to population selection, no study has thus far examined the whole transcriptomic response of mycoplasmas upon exposure to eukaryotic cells.

The availability of the genome sequence of *M. gallisepticum* strain R (11) enables a method of screening for transcriptomic changes; namely, an oligonucleotide-based microarray has been developed representing all known open reading frames (ORFs) based on this sequence. Utilizing this microarray, we investigated transcriptional changes when *M. gallisepticum* was incubated with a cell culture monolayer of human lung fibroblasts. In the absence of an established chicken trachea epithelial cell line, MRC-5 human lung fibroblasts have been used in previous studies as an in vitro model for *M. gallisepticum* interaction with host cells (9, 10, 12). This approach identified 25 upregulated and 33 downregulated transcripts that were differentially expressed upon incubation with MRC-5 cells and thus provide evidence of their function, suggesting a potential role in the interaction of *M. gallisepticum* with host cells in vivo.

MATERIALS AND METHODS

Microarray design. An oligonucleotide-based microarray specific for *M. gallisepticum* strain R_{low} was developed by MWG Biotech (Raleigh, NC). Oligonucleotides, each 50 nucleotides in length, were selected to represent each of the 756 putative ORFs. Thirty-six blank and eight *Arabidopsis* control features were included as negative controls. All features were spotted twice on glass slides, representing the genome in duplicate on each slide. Based on BLAST analysis (1), 21 features predicted to cross-hybridize with more than one genetic locus were excluded from further analysis.

Culture conditions and experimental design. *M. gallisepticum* strain R_{low} (passage 14) was cultured at 37°C in Hayflick's complete medium (2) until mid-log phase, as determined by color change and optical density. MRC-5 human lung fibroblasts (ATCC, Manassas, VA) were cultured to 95% confluence in 150-cm^2 flasks (Fisher Scientific, Pittsburgh, PA) in minimal essential medium with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids at 37°C with 5% CO_2. MRC-5 cell monolayers were washed three times in phosphate-buffered saline prior to exposure to mycoplasmas. Mid-log-phase R_{low} cultures were pelleted by centrifugation at 10,000 × *g* for 10 minutes, resuspended in 10 ml of Hayflick's complete medium, and incubated with washed MRC-5 cells for 1 hour at 37°C. Mid-log-phase R_{low} cultures, incubated 1 hour at 37°C, were used as reference samples for microarray and reverse transcriptase PCR (RT-PCR) analysis. Prior to RNA extraction, mycoplasma-fibroblast cocultures were washed three times with phosphate-buffered saline.

RNA extraction. Total RNA was extracted from pelleted broth-grown R_{low}, mycoplasma–MRC-5 cocultures, and MRC-5 monolayers using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was treated with DNase (Sigma, St. Louis, MO) and purified using phenol-chloroform–isoamyl alcohol (Fisher Scientific), and concentration was determined based on absorbance at the 260-nm wavelength. Eukaryotic ribosomal and polyadenylated RNAs were removed from samples derived from infected monolayers using the MICROBEnrich kit according to the manufacturer's instructions (Ambion, Austin, TX). RNA extracted from broth-grown R_{low} cultures was also treated once according to the protocol of the MICROBEnrich kit as a control. Each RNA sample was viewed in a 0.8% agarose gel to confirm RNA integrity.

Microarray hybridization. Fifty micrograms of total RNA from each condition was reverse transcribed using the Amino Allyl cDNA labeling kit (Ambion) according to the manufacturer's instructions. Samples were labeled with either Cy3 or Cy5 (Amersham Biosciences, Buckinghamshire, United Kingdom), excess

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TABLE 1. Transcripts upregulated when R_{low} is exposed to MRC-5 cells

ORF product identity	ORF	Mean relative increase in expression	SD of relative increase in expression	*P* value	Validation by RT-PCR[a]
Unique hypothetical	MGA_122	1.81	0.19	0.023	ND
Unique hypothetical	MGA_184	2.12	0.13	0.007	Yes
SrmB ATP-dependent helicase	MGA_206	3.03	0.27	0.004	ND
OppD oligopeptide transport ATP-binding protein	MGA_232	2.49	0.07	0.013	ND
RpsB ribosomal protein S2	MGA_242	2.08	0.02	0.048	ND
50S ribosomal protein I28	MGA_253	3.62	0.16	0.003	ND
RpsG ribosomal protein S7	MGA_261	1.78	0.04	0.022	ND
Unique hypothetical	MGA_267	6.63	1.59	0.000	Yes
Unique hypothetical	MGA_324	1.61	0.02	0.045	ND
Conserved hypothetical	MGA_340	1.87	0.04	0.046	ND
Hypothetical macrophage-activating protein, p47	MGA_398	2.95	0.54	0.013	ND
RpsR ribosomal protein S18	MGA_421	1.89	0.02	0.024	ND
RpsP ribosomal protein S16	MGA_440	1.77	0.07	0.006	ND
RnpA RNase P protein component	MGA_630	2.01	0.38	0.030	ND
RpsI ribosomal protein S10	MGA_705	2.06	0.04	0.027	ND
RpsT ribosomal protein S20	MGA_844	2.88	0.14	0.014	ND
RplJ ribosomal protein L10	MGA_872	1.86	0.10	0.025	Yes
Unique hypothetical	MGA_875	3.80	0.31	0.008	ND
VlhA 4.01 pMGA family protein	MGA_966	2.41	0.39	0.024	ND
Conserved hypothetical lipoprotein	MGA_993	3.56	0.22	0.002	ND
Conserved hypothetical	MGA_1107	3.63	0.33	0.008	Yes
ABC-type transport system, permease	MGA_1140	3.08	0.87	0.034	ND
RplM ribosomal protein L13	MGA_1154	1.98	0.06	0.012	ND
RplU ribosomal protein L21	MGA_1290	2.63	0.19	0.016	ND
RpmA ribosomal protein L27	MGA_1292	3.21	0.08	0.003	ND

[a] ND, not done.

dye was removed using the Nuc-Away spin columns provided in the Amino Allyl cDNA labeling kit, and labeled cDNA was resuspended in hybridization buffer (MWG Biotech). Microarray slides were blocked in blocking buffer (1% bovine serum albumin and 2% sodium dodecyl sulfate in 1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) for 1 hour at 42°C and washed three times in 0.1× SSC buffer prior to hybridization. Labeled cDNA was resuspended in hybridization buffer, heated briefly at 95°C, cooled on ice, hybridized to the blocked slide at 42°C for 18 hours, and then washed three times with decreasing concentrations of SSC buffer (2× SSC with 0.1% sodium dodecyl sulfate, 1× SSC, and 0.1× SSC). Separate RNA extractions and hybridizations were repeated five times, switching sample dyes for two of the five experiments.

Data acquisition and analysis. Microarray slides were scanned using a GenePix 4000B scanner (Axon Instruments, Sunnyvale, CA), and data were acquired with the GenePix 4.0 (Axon Instruments). Features were individually analyzed and normalized using the Bayesian Analysis of Gene Expression Level (BAGEL) model (8) (University of California, Berkeley, CA). Briefly, fluorescence levels for each feature in each data set are normalized, and then a relative expression level and a *P* value are determined across all data sets through the application of a specific algorithm by the BAGEL model. Up- or downregulated genes were selected by averaging values across duplicate features and filtering reproducible features that were significantly up- or downregulated more than 1.5-fold ($P < 0.05$). The relative expression level for each transcript was averaged across duplicate features.

Validation of microarray data. Relative transcription levels of differentially regulated transcripts were investigated by RT-PCR. RNA was generated in three separate extractions from R_{low} grown in broth culture and mycoplasma–MRC-5 cocultures as described above with the addition of RNALater (Ambion) according to the manufacturer's instructions. All primers (MWG Biotech) were used at a final concentration of 400 nM and checked for cross-hybridization with MRC-5 RNA. Reactions were performed using 100 ng and 10 ng of RNA and the SuperScript RT-PCR kit (Invitrogen). Cycling conditions for RT-PCRs were as follows: 50°C reverse transcription for 25 min, 94°C denaturation for 2 minutes, amplification for 35 cycles using 94°C denaturation for 30 seconds, 50°C annealing for 30 seconds, and 72°C extension for 45 seconds, followed by a final 72°C extension for 10 min. Contaminating DNA was detected by replacing the SuperScript Platinum *Taq* in the reaction mix with AmpliTaq (Applied Biosystems, Foster City, CA). Reactions were run in a 0.8% agarose gel with 0.01% ethidium bromide. Pictures of each gel were taken under UV light and scanned at 300 dpi. Bands of the same dilution were densitometrically compared using Adobe Photoshop 6.0 by measuring mean pixel intensity, subtracting background, and averaging the triplicate results for each condition.

Microarray data accession number. Microarray data were submitted to the Gene Expression Omnibus database under the accession number GSE6717.

RESULTS

Transcriptional profiling of *M. gallisepticum* strain R interacting with host cells. Transcriptomic comparisons of R_{low} cultures grown in broth in relation to cultures incubated with MRC-5 cell monolayers were investigated by microarray analysis a total of five times. Duplicate features that reproducibly showed a significant ($P < 0.05$) increase or decrease in signal greater than 1.5-fold were included in the data set. A total of 25 upregulated (Table 1) and 33 downregulated (Table 2) transcripts met these criteria.

Validation of microarray data. Sixteen transcripts identified as differentially regulated by the microarray were chosen randomly for validation by RT-PCR, representing 27% of the total data set. Specific primers were designed for each transcript (Table 3), and transcripts were compared between R_{low} grown in broth culture and R_{low} incubated with MRC-5 cells. Equal amounts of RNA were reverse transcribed, and band intensities of the resulting products were compared by measuring mean pixel intensity using Adobe Photoshop 6.0. This comparison was performed on three separate extractions of RNA for each condition. Fourteen of the 16 transcripts tested (88%) showed an upregulation or downregulation consistent with the

TABLE 2. Transcripts downregulated when R_{low} is exposed to MRC-5 cells

ORF product identity	ORF	Mean relative decrease in expression	SD of relative decrease in expression	*P* value	Validation by RT-PCR[a]
PepB leucyl aminopeptidase	MGA_114	1.62	0.03	0.030	ND
Conserved hypothetical	MGA_123	2.09	0.02	0.002	ND
GroEL heat shock protein	MGA_152	1.67	0.08	0.040	ND
Lpd dihydrolipoamide dehydrogenase	MGA_161	2.28	0.27	0.005	Yes
AceF dihydrolipoamide acetyltransferase	MGA_162	2.22	0.10	0.024	Yes
Hypothetical methyltransferase	MGA_249	1.93	0.08	0.011	ND
Hlp1 cytoskeletal protein (HMW1-like protein)	MGA_306	2.21	0.18	0.005	Yes
CpsG phosphomannomutase	MGA_358	1.64	0.07	0.031	ND
DeoD purine-nucleoside phosphorylase	MGA_364	2.61	0.06	0.007	ND
GatB glutamyl/aspartyl tRNA amidotransferase	MGA_412	1.52	0.02	0.045	ND
TopA DNA topoisomerase I	MGA_454	1.74	0.07	0.037	ND
Rpe pentose-5-phosphate-3-epimerase	MGA_455	2.41	0.43	0.020	ND
Unique hypothetical	MGA_482	2.12	0.32	0.016	ND
Conserved hypothetical	MGA_484	1.94	0.12	0.033	ND
Unique hypothetical	MGA_487	2.14	0.13	0.024	ND
HsdS restriction endonuclease S subunit	MGA_539	1.53	0.17	0.021	ND
Unique hypothetical	MGA_573	2.56	0.18	0.000	ND
SerS seryl-tRNA synthetase	MGA_608	3.33	0.31	0.001	ND
Dihydroxyacetone kinase	MGA_661	3.44	0.26	0.002	Yes
Hypothetical nuclease	MGA_676	1.92	0.32	0.027	Yes
ABC transporter ATP-binding protein	MGA_677	2.36	0.22	0.010	ND
Mdh lactate dehydrogenase	MGA_746	3.50	0.59	0.004	Yes
Unique hypothetical	MGA_867	2.30	0.46	0.004	ND
Hlp3 cytoskeletal protein (HMW3-like protein)	MGA_928	1.81	0.14	0.022	No
Conserved hypothetical	MGA_1011	2.44	0.22	0.003	ND
Exo 5′–3′ exonuclease	MGA_1052	1.84	0.24	0.026	ND
AtpB ATP synthase *a* subunit	MGA_1164	3.38	1.14	0.015	Yes
AtpF ATP synthase *b* subunit	MGA_1168	2.97	0.98	0.009	Yes
AtpG ATP synthase *g* subunit	MGA_1174	3.93	2.32	0.012	ND
AtpD ATP synthase *b* subunit	MGA_1177	3.03	0.72	0.011	Yes
PlpA fibronectin-binding protein	MGA_1199	2.29	0.23	0.003	Yes
Unique hypothetical	MGA_1224	2.90	1.20	0.037	No
GrpE heat shock protein	MGA_1232	2.23	0.11	0.020	ND

[a] ND, not done.

microarray data. Figure 1 shows an example comparison of one replicate for both conditions at two different dilutions, displaying a clear downregulation of AtpB in R_{low} associated with MRC-5 cells compared to the broth-grown culture. Densitometric analysis of the reaction shown indicated a 2.5-fold downregulation of this gene in R_{low} incubated with MRC-5 cells compared with R_{low} grown in broth culture. RT-PCR was performed on 23S rRNA and *dnaK* as housekeeping genes, as these genes would not be expected to be differentially regulated and were not predicted to be differentially regulated by

TABLE 3. Primers used in RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
23S rRNA	AGTCAAACCGTGAGGATTGG	CAAATCCGATAATGGGGATG
DnaK	GACCTTGGTGGTACGTT	CCACGTAATACCCCACCTTG
MGA_184	TGAACTCATCAAGGGTTGGTT	CCAGCGCGACAATTCATAA
MGA_267	CGGTTCTGCACAAGCTAACA	CTTTCATGCACCAGAAAGCA
RplJ	AGCTGGCATTGATGGATTTG	ACTGGAGCTTGAAGCACTGA
MGA_1107	CTATTGCGCAGCAAAATGAA	CGTTGGTAGTTATGCCTTCG
Lpd	GGGTGAATTGCTTGTTCT	GTGGTTTCTAAGATGCCA
AceF	CCAGTTGCGACTCCTTTAGC	GCTGGAATTTCTTCGTGAGC
Hlp1	TCGAAGTAACCAGTTCAAACTCA	AGGCACGGAATTTATTGTGG
MGA_661	GGGGATTTTGTTGATTCA	CTGCCATTAACTCTTGAG
MGA_676	CCAGCTGATAGCAAACCGTT	GCTGCAATCCCAATTGGTCTA
Mdh	TGTGACGTAATGGCTGGTGT	TAACTTCCATCCGTGCTTGC
Hlp3	TGATTACTACCCACCAGCTTATGA	CTTCGCACTCTTGGTTGTTG
AtpB	TTCCGACTGCTCATGTCTTG	TTTTCTCGCCCAATAGATCG
AtpF	CAAGCACGCGAGATTATCAA	ACATCAACGATCCGACCTTC
AtpD	CAACCGTTCTTTGTTGCTGA	CTTCATCGATCGAACCAACA
PlpA	AAAGAAGAGATCGACAGCTTGC	TAACGGTTATTGTAAGGGTC
MGA_1224	GGGAATAGTCGATGGATA	CGACTTGGGGTTCTTCTAGG

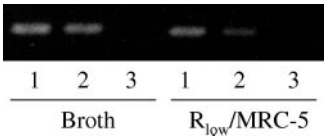

FIG. 1. RT-PCR amplification of AtpB transcript from *M. gallisepticum* R_{low} grown in broth culture and attached to MRC-5 cells. RT-PCR was performed on serially diluted RNA extracted from R_{low} grown in broth and from R_{low} exposed to MRC-5 cells. A representative one of three replicates is shown here including the following serial dilutions: lane 1, 100 ng; lane 2, 10 ng; lane 3, control for DNA contamination. Downregulation of AtpB, 1 of the 16 transcripts tested, in the presence of MRC-5 cells is shown here.

the microarray results. These genes did not show any difference by RT-PCR (data not shown).

DISCUSSION

This study presents, for the first time, evidence of transcriptional regulation in *M. gallisepticum* in response to environmental conditions and substantiates existing reports of transcriptional regulation in mycoplasmas (5, 6, 17). The 1-hour incubation time in these experiments was specifically chosen to be shorter than a generation time (approximately 2 hours) to eliminate the influence of population selection or outgrowth. Criteria, specifically a P of <0.05 and at least a 1.5-fold change, were selected in order to include genes that were significantly and clearly differentially regulated upon exposure to lung fibroblasts in culture. These genes included 25 upregulated and 33 downregulated transcripts, representing approximately 8% of the predicted *M. gallisepticum* transcriptome. When 16 of these transcripts were examined by RT-PCR, 14 (88%) demonstrated relative transcriptional differences that were consistent with the microarray results.

These experiments verified the upregulated expression of 10 ORFs annotated as being hypothetical, supporting their role as functional genes. Six of these ORFs are unique to *M. gallisepticum*, and three are conserved among other mycoplasmas. Additionally, one hypothetical protein (p47) was identified by Markham et al. as having sequence homology to a macrophage-activating lipoprotein but was determined to have no role in pathogenicity or colonization based on the behavior of an isogenic mutant in a tracheal explant model (7). Similarly, 10 hypothetical transcripts were downregulated, 5 of which are conserved primarily among other mycoplasmas and other related bacterial species. Two conserved hypothetical transcripts were further described to contain predicted functional domains based on BLAST analysis: MGA_676 contains a predicted nuclease domain, while MGA_249 contains a predicted methylase domain. This annotation does not assign specific functions to these transcripts, however.

Genes encoding ribosomal or translation-associated proteins were upregulated in the mycoplasmas associated with MRC-5 cells. These proteins included 10 ribosomal proteins as well as an RNA helicase (SrmB), which is involved in ribosome assembly, and RNase P (RnpA), which completes the development of tRNAs. A similar pattern was observed in *Campylobacter jejuni* cultured in rabbit ileal loops: the majority of ribosomal proteins were upregulated when attached to the intestinal epithelium (16). A number of ribosomal transcripts were previously found to be upregulated in *Mycoplasma pneumoniae* following heat shock (17), indicating that this response may be due to environmental stress; however, a similar response was not observed in a heat shock study on another mycoplasma species, *Mycoplasma hyopneumoniae* (5). Whether in response to the host cell specifically or a general stress response, this pattern may simply reflect a global increase in translation.

Eleven metabolism-associated transcripts are downregulated upon exposure to MRC-5 cells, including four of the eight ATP synthase subunits and two of the four components of the pyruvate dehydrogenase complex. One of these components, Lpd, had been shown previously to be a virulence-related determinant by virtue of the attenuation of an isogenic mutant (3). The observed downregulation in these experiments is not necessarily contradictory to the previous report, however, as the Lpd mutant is introduced to the host with this pyruvate dehydrogenase deficiency, affecting its survival. Wild-type R_{low}, conversely, will have a normal level of activity upon introduction to the host and may downregulate the pyruvate dehydrogenase complex subsequently, corresponding to the decrease observed here. Additional metabolism-related enzymes that are downregulated include a hypothetical protein possessing a dihydroxyacetone kinase domain (MGA_661), phosphomannomutase (CpsG), pentose-5-phosphate-3-epimerase (Rpe), purine-nucleoside phosphorylase (DeoD), and malate/lactate dehydrogenase (Mdh). With the exception of Rpe, all of these enzymes are in pathways involved in the metabolism of substrates other than glucose. Specifically, dihydroxyacetone kinase is involved in glycerol metabolism, DeoD is involved in nucleotide metabolism, and CpsG is involved in GDP-mannose metabolism (14). This pattern suggests that when *M. gallisepticum* is in association with the host cell, glucose may be available as a primary energy source, allowing alternative pathways to be downregulated.

As mentioned above, four of the eight ATP synthase subunits were downregulated when *M. gallisepticum* was in contact with host cells. In the majority of prokaryotes, the ATP synthase complex maintains a proton gradient through catabolism and hydrolysis of ATP; however, since mycoplasmas lack an electron transport chain necessary for further generation of ATP, the complex is believed to function primarily to maintain the electrochemical gradient. Published reports also suggest that the "reverse" ATP-generating function of this complex is active in mycoplasmas; namely, ATP may be generated by electron transfer to flavoproteins using oxygen as an intermediate rather than a terminator (flavin-terminated respiration) (13), and the *b* subunit of the complex, shown to be downregulated in this study, is likely surface exposed and thus potentially able to act in reverse (18). The observed transcriptional decrease of ATP synthase genes could, alternatively, reflect uptake of metabolic precursors from the host cell.

The data presented here are the first assessment of transcriptional responses of *M. gallisepticum* associated with a eukaryotic cell. The specific roles of the identified transcripts in vivo will have to be further investigated. In particular, the transcriptional responses presented here are specifically a result of an interaction with the host cell, but whether the attachment mechanism, the invasion of the host cells (19), or another component of the host cell is responsible remains to be

determined. The downregulation of metabolism-associated genes when mycoplasmas are in contact with a host cell could be described as a shift to parasitism with the uptake of substrates from the host cell compared to the noncompetitive environment of a nutrient-rich growth medium. This change is particularly supported by the streamlining of metabolic pathways to possibly focus on a greater availability of glucose (or more directly, ATP) in association with the eukaryotic cell. The hypothetical ORFs upregulated in the presence of eukaryotic cells may be involved in colonization of the host. These data provide insight into the responses of *M. gallisepticum* to interaction with the host cell and provide candidates for further investigation into their roles in *M. gallisepticum*-host interaction.

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