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Transcriptional response of *Mycoplasma genitalium* to osmotic stress

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Mycoplasma genitalium is the causative agent of non-gonococcal, chlamydia-negative urethritis in men and has been linked to reproductive tract disease syndromes in women. As with other mycoplasmas, *M. genitalium* lacks many regulatory genes because of its streamlined genome and total dependence on a parasitic existence. Therefore, it is important to understand how gene regulation occurs in *M. genitalium*, particularly in response to environmental signals likely to be encountered *in vivo*. In this study, we developed an oligonucleotide-based microarray to investigate transcriptional changes in *M. genitalium* following osmotic shock. Using a physiologically relevant osmolarity condition (0.3 M sodium chloride), we identified 39 upregulated and 72 downregulated genes. Of the upregulated genes, 21 were of unknown function and 15 encoded membrane-associated proteins. The majority of downregulated genes encoded enzymes involved in energy metabolism and components of the protein translation process. These data provide insights into the *in vivo* response of *M. genitalium* to hyperosmolarity conditions and identify candidate genes that may contribute to mycoplasma survival in the urogenital tract.

INTRODUCTION

Mycoplasma genitalium is an important sexually transmitted pathogen that causes non-gonococcal, chlamydia-negative urethritis in men and reproductive tract disease syndromes in women (Baseman & Tully, 1997; Jensen, 2004). It is considered to be a parasite of the urogenital tract that colonizes the surface of epithelial cells through multiple adhesin-mediated cytadherence mechanisms (Alvarez *et al.*, 2003; Baseman, 1993). Although *M. genitalium* is often regarded as an extracellular pathogen, it has been reported to invade host cells and establish long-term persistence (Baseman *et al.*, 1995; Blaylock *et al.*, 2004; Ueno *et al.*, 2008). This observation may account for the chronic infections associated with *M. genitalium*, despite extensive antibiotic therapies and an intact and functional host immune system.

M. genitalium represents the smallest known self-replicating micro-organism that can be passaged in artificial medium. Also, *M. genitalium* has a streamlined genome (0.58 Mbp) containing only 482 protein-encoding genes, which approximates the minimal gene set essential to sustain independent life (Fraser *et al.*, 1995; Glass *et al.*, 2006). Despite the limitation of genome content, *M. genitalium* must be able to adapt to changing *in vivo* environmental conditions given its intimate interaction with host cells. Unfortunately, little information is available concerning the mechanisms by which *M. genitalium* regulates gene expression. Transcriptional control in mycoplasmas appears unique, as these prokaryotes possess relatively few regulatory factors compared with other bacteria, possibly due to reduced genome size (Himmelreich *et al.*, 1997) and their successful adaptation to host environments (Weiner *et al.*, 2000). The availability of DNA microarrays facilitates the study of transcriptional regulation in mycoplasmas by examining global transcriptional changes. The first evidence of differential expression of genes in pathogenic mycoplasmas was revealed using microarrays in *Mycoplasma pneumoniae* during heat shock (Weiner *et al.*, 2003). Later, additional *Mycoplasma* species were tested under conditions of heat shock (Madsen *et al.*, 2006a), oxidative stress (Schafer *et al.*, 2007), depletion of iron (Madsen *et al.*, 2006b), association with eukaryotic cells (Cecchini *et al.*, 2007) or during infection (Madsen *et al.*, 2008). To date, only a subset of conserved heat-shock genes of *M. genitalium* has been shown by macroarray to be differentially expressed in response to elevated temperature (Musatovova *et al.*, 2006) and no global transcriptional analysis has been performed on this smallest self-replicating prokaryote.

Abbreviations: *Q* value, false discovery rate; RT, reverse transcriptase.

Microarray data have been submitted to the Gene Expression Omnibus database with the accession number GSE22661.

A supplementary figure, showing an expression map of *M. genitalium* in the presence of 0.3 M NaCl, and a supplementary table, listing *M. genitalium* genes preferentially upregulated in the presence of 0.2 M NaCl, are available with the online version of this paper.

In this study, we developed an oligonucleotide-based microarray specific for *M. genitalium*, which represents all known ORFs annotated in the genome. Utilizing this microarray, we examined the transcriptional changes of *M. genitalium* in response to osmotic stress, an environmental change that *M. genitalium* encounters in the human urinary tract where osmotic pressure fluctuations are created by passing and residual urine. With this approach, we identified a set of genes that displayed differential expression under osmotic stress.

METHODS

Mycoplasma strain and growth. *M. genitalium* type strain G37 was routinely grown in Spiroplasma (SP)-4 medium at 37 °C. To assess the growth of *M. genitalium* in the presence of various concentrations of NaCl, we measured the incorporation of ^{14}C-labelled lysine into protein over time. Equal numbers of strain G37 cells (1×10^6 colour change units) were inoculated into three 12-well plates (1 ml SP-4 medium per well). After incubation at 37 °C for 60 h, NaCl was added to final concentrations of 0.1, 0.2 and 0.3 M to triplicate wells of each plate. The remaining three wells were untreated and served as controls. Then, 0.1 μCi (0.37 kBq) L-[U-^{14}C]-lysine (Amersham) was added to each well, and cultures were reincubated at 37 °C. At each time point (72, 96 and 120 h), one plate was processed by aspirating the spent medium, washing wells once with PBS, lysing mycoplasma cells with 0.5 ml 10 % SDS, and determining radioactivity values by scintillation counting.

Microarray design. An oligonucleotide-based microarray specific for *M. genitalium* was designed and synthesized by Qiagen Operon. Oligonucleotides (70-mers) representing 482 putative ORFs of type strain G37 (ATCC 33530) were printed on slides by Microarrays Inc. In addition, 24 empty spots were included as negative controls. All spots were printed in triplicate on one slide to improve the reproducibility of array data.

Experimental design. To examine the effects of hyperosmolarity on *M. genitalium* transcription, four 50 ml cultures of strain G37 in 75 cm^2 tissue culture flasks (Corning) were grown to exponential phase, as determined by medium colour change and colony density. Then, NaCl was added to three flasks to achieve final concentrations of 0.1, 0.2 and 0.3 M. Parallel cultures of *M. genitalium* in the absence of NaCl served as controls. All cultures were incubated for 1 h at 37 °C prior to RNA extraction. Experiments were repeated six times, which produced six independent RNA sample pairs from NaCl-treated cultures and control cultures for each NaCl condition. Dye swap was performed on three of six RNA pairs to minimize effects caused by biased labelling efficiencies.

RNA manipulation. Tri Reagent (Sigma) was used to extract total *M. genitalium* RNA according to manufacturer's guidelines. Briefly, surface-attached *M. genitalium* cells were washed twice with ice-cold sterile PBS, and Tri Reagent (8 ml per 75 cm^2 flask surface) was added to lyse cells. For microarray analysis, total RNA preparations were further processed with an RNeasy mini kit (Qiagen) to remove RNAs less than 200 nt. To stabilize RNA samples, 200 units RNase inhibitor RNaseOUT (Invitrogen) was added to each RNA preparation. For real-time PCR analysis, total RNA preparations were further treated with amplification-grade DNase I (Invitrogen) to remove residual genomic DNA.

Microarray hybridization. For probe preparation, hybridization and slide washing, we followed microarray protocols (SOP no. M007 and M008) from the Pathogen Functional Genomics Resource Center of the J. Craig Venter Institute with slight modification (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). Hybridization probes were generated with a set of 482 gene-specific primers (designed and synthesized by Operon Biotechnologies) by reverse transcription of 4 μg total *M. genitalium* RNA in the presence of aminoallyl-labelled dUTP (2 : 1 ratio of aa-dUTP : dTTP). After clean-up, the aminoallyl-labelled cDNA preparations were coupled to either cyanine (Cy)3 or Cy5 fluorescent molecules (Amersham Biosciences). Hybridization and slide washing followed protocol SOP no. M008.

Data acquisition and analysis. Microarray slides were scanned with a GenePix 4000B microarray scanner (Axon Instruments) at 10 μm resolution, and laser power was kept constant for Cy3/Cy5 scans of all slides. Photomultiplier tube (PMT) voltages were adjusted using GenePix Pro 6.0 (Axon Instruments) to obtain maximal signal intensities of each feature with a saturation of <0.005 % and count ratio close to 1.0. The resulting images were gridded and processed using GenePix Pro 6.0 to obtain the signal intensity for each spot. The data were further imported into and analysed by Acuity 4.0 (Axon Instruments). The fluorescence levels for each feature of individual slides were first normalized using the ratio-based method and a dataset was created. The relative expression level of each ORF was calculated, and the *P*-values were determined across the dataset using Student's *t* test. Then, the 482 *P*-values from the *t* test were imported into *QVALUE* (Storey & Tibshirani, 2003) and false discovery rate (*Q*) values were computed at an estimated false discovery rate of 5 %. Along with *Q* values, differentially expressed genes were identified by filtering features that were up- or downregulated more than twofold ($P<0.01$).

Validation of microarray data by real-time PCR. Real-time PCR analysis was performed with the ABI Prism 7900 sequence detection system and SYBR green chemistry (Applied Biosystems). Primers were designed using Primer Express 2.0 (Applied Biosystems; Table 1). All primers were tested to be specific for individual genes, as indicated by a single peak after dissociation of each amplification product and a single band after agarose gel electrophoresis. Efficiencies of the target amplification and reference (endogenous control) amplification were confirmed to be equal ($R^2 > 0.99$) using five serial 10-fold dilutions of

Table 1. Primers used in real-time PCR analysis

Primer	Sequence (5′–3′)
MG_003F	TGCTGGTGGCACTGCTAAAA
MG_003R	CAACGTTTAAAATCTTTCCTCTTAAGG
MG_074F	CCTCTTAGTCTTTGTCTTGCTTTTCTT
MG_074R	GCAAACAAGCAGTGTAGGAAAATACT
MG_149F	TGAAAGAAAAAATATGAGTGGTTCAACTAG
MG_149R	AAGAGAGCTTACGTTCCTCTTTATGTTC
MG_151F	CACCGCTTTCAGGGTTCTG
MG_151R	AAAAACACGCTGCGCACTACT
MG_274F	TCTTCAGCTACCGGCAAGGT
MG_274R	CTCCTCTTCTTGTTTGGTTCTGTAGA
MG_275F	CACTTGCTGTTAGTGGTGTTGTTAAA
MG_275R	GTTAGCGCCCATCTGTTTCAACT
MG_278F	TGGCATGAAAACCAGAAACG
MG_278R	CCATGTTCCATTCAACTAGTGATAATG
MG_451F	AAACGTCACTATGCCCATGTTG
MG_451R	TGCAGCACCTGTGATCATATTTT
MG_454F	TTGCACAAACTGAAACTGGCA
MG_454R	TGAGAAAAACAACTTGCATAAGCAG

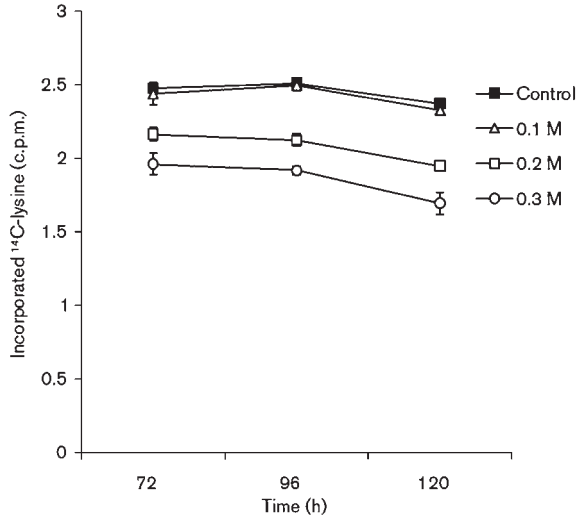

Fig. 1. Growth of *M. genitalium* in the presence of NaCl. A semi-logarithmic plot based upon the incorporation of ^{14}C-lysine (c.p.m.) at 72, 96 and 120 h is presented. NaCl and ^{14}C-lysine were added to cultures after 60 h of growth. Thereafter, at the indicated times, mycoplasma cells were washed and lysed for scintillation counting. Error bars represent standard deviations of values obtained from three independent replicates.

M. genitalium genomic DNA (10^8–10^4 copies per reaction) as templates according to User Bulletin No.2 (Applied Biosystems). To prepare the cDNA template, 1 μg DNase I-treated RNA was reverse transcribed with SuperScript Reverse Transcriptase (RT) II (Invitrogen). Then, cDNA templates were diluted and mixed with SYBR green PCR master mix and corresponding primers. Also, RT-negative RNAs were included as templates to estimate the residual contamination of genomic DNA. Amplifications were carried out under default conditions provided by the manufacturer. We applied the comparative threshold cycle method to compare amounts of transcripts under different experimental conditions. The relative levels of transcripts were expressed as fold changes (*n*-fold) compared with control values, and calculations were completed using RQ Manager 1.2 (Applied Biosystems).

Microarray data accession number. Microarray data were submitted to the Gene Expression Omnibus database under the accession number GSE22661.

RESULTS

Transcriptional profiling of *M. genitalium* treated with hyperosmolarity conditions

To examine the transcriptomes of *M. genitalium* in response to osmotic shock, we added NaCl to exponential-phase cultures of *M. genitalium* to final concentrations of 0.1, 0.2 and 0.3 M and continued incubation for 1 h. The addition of NaCl raised the osmotic pressure of SP-4 medium from 394 mOsm kg^{-1} to 584, 771 and 930 mOsm kg^{-1}, respectively, which are within the physiological range of human urine osmolarity (50–1400 mOsm kg^{-1}). The growth of *M. genitalium* was not affected in the presence of 0.1 M NaCl, as indicated by the rate of protein synthesis (Fig. 1). At high NaCl concentrations (0.2 and 0.3 M NaCl), although *M. genitalium* protein synthesis was reduced, the viability of *M. genitalium* was not significantly affected based upon similar numbers of c.f.u. before and after each NaCl treatment (data not shown).

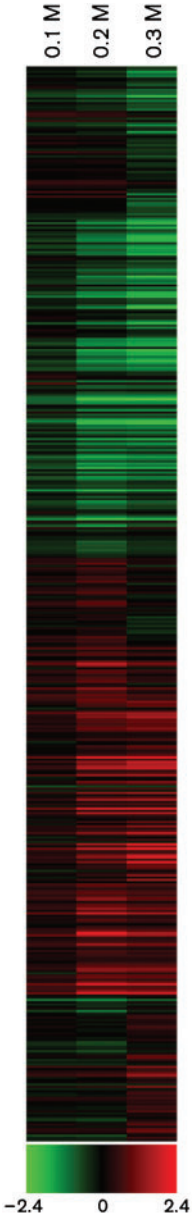

Fig. 2. Hierarchical clustering analysis of *M. genitalium* genes upon exposure to NaCl for 1 h. Genes were grouped on the basis of similarity of expression patterns. Each gene is represented by a single row of coloured lines (red, induced; green, repressed). The colour scale ranges from saturated green for log ratios −2.4 and below to saturated red for log ratios 2.4 and above.

Table 2. *M. genitalium* genes upregulated in the presence of 0.3 M NaCl

Gene ID	Gene	Description or product	Fold change	*P*-value	*Q* value
MG_003	*gyrB*	DNA gyrase, B subunit	5.21	0.00004	0.00136
MG_004	*gyrA*	DNA gyrase, A subunit	3.39	0.00010	0.00136
MG_005	*serS*	Seryl-tRNA synthetase	2.23	0.00388	0.00190
MG_011		Conserved hypothetical protein	2.15	0.00424	0.00198
MG_032		Conserved hypothetical protein	2.68	0.00071	0.00136
MG_064*		ABC transporter, permease protein, putative	3.01	0.00000	0.00136
MG_067*		Lipoprotein, putative	3.25	0.00008	0.00136
MG_068*		Lipoprotein, putative	3.05	0.00001	0.00136
MG_074*		Conserved hypothetical protein	6.88	0.00003	0.00136
MG_075*		116 kDa surface antigen	2.16	0.00372	0.00187
MG_097		Uracil-DNA glycosylase, putative	3.68	0.00002	0.00136
MG_098		Glutamyl-tRNA/aspartyl-tRNA amidotransferase, C subunit	3.60	0.00014	0.00136
MG_099		Glutamyl-tRNA/aspartyl-tRNA amidotransferase, A subunit	2.53	0.00589	0.00246
MG_149*		Lipoprotein, putative	9.98	0.00001	0.00136
MG_478*		Conserved hypothetical protein, previously MG_149.1	5.30	0.00001	0.00136
MG_240		Conserved hypothetical protein	2.87	0.00219	0.00156
MG_248		Conserved hypothetical protein	2.38	0.00011	0.00136
MG_249	*rpoD*	RNA polymerase sigma factor RpoD	2.42	0.00065	0.00136
MG_278	*relA*	GTP pyrophosphokinase	2.90	0.00001	0.00136
MG_280*		Conserved hypothetical protein	4.17	0.00001	0.00136
MG_281*		Conserved hypothetical protein	2.59	0.00437	0.00202
MG_283	*proS*	Prolyl-tRNA synthetase	2.27	0.00782	0.00302
MG_288		Protein of unknown function	3.00	0.00004	0.00136
MG_289*		Phosphonate ABC transporter, substrate binding protein, putative	2.29	0.00064	0.00136
MG_517		Glycosyltransferase, group 2 family protein, previously MG_335.2	2.79	0.00028	0.00136
MG_341	*rpoB*	DNA-directed RNA polymerase, beta subunit	3.11	0.00003	0.00136
MG_342		NADPH-dependent FMN reductase domain protein	2.66	0.00171	0.00147
MG_346		RNA methyltransferase, TrmH family, group 2	2.39	0.00186	0.00150
MG_369		DAK2 phosphatase domain protein	4.70	0.00001	0.00136
MG_525*		Conserved hypothetical protein, previous MG_414	2.85	0.00002	0.00136
MG_415*		Conserved hypothetical protein	2.54	0.00744	0.00292
MG_425		ATP-dependent RNA helicase, DEAD/DEAH box family	3.16	0.00005	0.00136
MG_426	*rpmB*	Ribosomal protein L28	2.40	0.00087	0.00136
MG_428		LuxR bacterial regulatory protein, putative	3.11	0.00008	0.00136
MG_439*		Lipoprotein, putative	4.27	0.00002	0.00136
MG_440*		Lipoprotein, putative	3.89	0.00005	0.00136
MG_457*	*ftsH*	ATP-dependent metalloprotease	3.04	0.00002	0.00136
MG_469		Chromosomal replication initiator protein DnaA	2.34	0.00107	0.00137
MG_470		CobQ/CobB/MinD/ParA nucleotide binding domain	3.55	0.00020	0.00136

*Genes encoding membrane proteins or membrane-associated proteins indicated by the presence of a transmembrane domain(s) in the primary amino acid sequence.

Microarray analysis was performed to compare the global transcriptome profiles of mycoplasma cultures grown in the presence of NaCl with control cultures. Genes exhibiting similar expression patterns under osmotic stress were grouped by hierarchical clustering analysis (Fig. 2). We observed that the expression of genes was not significantly changed when *M. genitalium* was treated with 0.1 M NaCl compared with controls. In contrast, we observed significant differential expression of genes when mycoplasma cells were exposed to 0.2 M and 0.3 M NaCl. Although some genes were preferentially expressed under 0.2 M NaCl treatment (see Supplementary Table S1, available with the online version of this paper), more genes tended to be differentially expressed under 0.3 M NaCl treatment. Subsequently, differentially expressed genes under 0.3 M NaCl treatment were identified by the criteria of increases or decreases in signal greater than twofold, *P*-value less than 0.01 and an estimated false discovery rate of 5 %. A total of 39 upregulated genes (Table 2) and 72 downregulated genes (Table 3) met the criteria. The location of these genes and their status of expression are presented in Supplementary Fig. S1 (available with the online version of this paper). To verify that these genes were differentially expressed due to

Table 3. *M. genitalium* genes downregulated in the presence of 0.3 M NaCl

Gene ID	Gene	Description or product	Fold change	*P*-value	*Q* value
MG_022		DNA-directed RNA polymerase, delta subunit	−2.76	0.00002	0.00136
MG_023	*fba*	Fructose-1,6-bisphosphate aldolase, class II	−2.38	0.00002	0.00136
MG_040		Lipoprotein, putative	−2.80	0.00018	0.00136
MG_061		Mycoplasma MFS transporter	−2.30	0.00029	0.00136
MG_062	*fruA*	PTS system, fructose-specific IIABC component	−2.41	0.00013	0.00136
MG_069	*ptsG*	PTS system, glucose-specific IIABC component	−4.09	0.00002	0.00136
MG_081	*rplK*	Ribosomal protein L11	−3.18	0.00005	0.00136
MG_082	*rplA*	Ribosomal protein L1	−2.60	0.00002	0.00136
MG_111	*pgi*	Glucose-6-phosphate isomerase	−4.16	0.00002	0.00136
MG_112	*rpe*	Ribulose-phosphate 3-epimerase	−3.55	0.00002	0.00136
MG_124	*trx*	Thioredoxin	−3.23	0.00359	0.00136
MG_125		Cof-like hydrolase, putative	−2.80	0.00004	0.00136
MG_139		Metallo-beta-lactamase superfamily protein	−2.04	0.00475	0.00136
MG_187		ABC transporter, ATP-binding protein	−3.14	0.00412	0.00136
MG_188		ABC transporter, permease protein	−2.10	0.00274	0.00136
MG_189		ABC transporter, permease protein	−4.29	0.00008	0.00136
MG_190		Phosphoesterase, DHH subfamily 1	−2.54	0.00004	0.00136
MG_196	*infC*	Translation initiation factor IF-3	−2.72	0.00056	0.00136
MG_207		Ser/Thr protein phosphatase family protein	−2.45	0.00740	0.00136
MG_227	*thyA*	Thymidylate synthase	−2.06	0.00069	0.00136
MG_228	*dhfR*	Dihydrofolate reductase	−2.33	0.00011	0.00136
MG_229	*nrdF*	Ribonucleoside-diphosphate reductase, beta chain	−2.63	0.00002	0.00136
MG_230	*nrdI*	NrdI protein	−2.80	0.00002	0.00136
MG_231	*nrdE*	Ribonucleoside-diphosphate reductase, alpha chain	−3.33	0.00001	0.00136
MG_255		Conserved hypothetical protein	−2.82	0.00079	0.00136
MG_270		Lipoyltransferase/lipoate-protein ligase, putative	−2.32	0.00002	0.00136
MG_273	*pdhB*	Pyruvate dehydrogenase component E1, beta subunit	−2.11	0.00082	0.00136
MG_274	*pdhA*	Pyruvate dehydrogenase component E1, alpha subunit	−2.80	0.00001	0.00136
MG_275	*nox*	NADH oxidase	−4.24	0.00001	0.00136
MG_299	*pta*	Phosphate acetyltransferase	−3.51	0.00001	0.00136
MG_300	*pgk*	Phosphoglycerate kinase	−3.20	0.00001	0.00136
MG_301	*gap*	Glyceraldehyde-3-phosphate dehydrogenase, type I	−2.85	0.00001	0.00136
MG_305	*dnaK*	Chaperone protein DnaK	−2.56	0.00002	0.00136
MG_311	*rpsD*	Ribosomal protein S4	−2.39	0.00002	0.00136
MG_312	*hmw1*	HMW1 cytadherence accessory protein	−2.55	0.00022	0.00136
MG_326		DegV family protein	−2.08	0.00169	0.00136
MG_332		Expressed protein of unknown function	−2.47	0.00003	0.00136
MG_333		Acyl carrier protein phosphodiesterase, putative	−4.21	0.00001	0.00136
MG_348		Lipoprotein, putative	−2.80	0.00002	0.00136
MG_353		DNA-binding protein HU, putative	−3.47	0.00001	0.00136
MG_354		Conserved hypothetical protein	−2.16	0.00006	0.00136
MG_357	*ackA*	Acetate kinase	−3.22	0.000001	0.00136
MG_361		Ribosomal protein L10	−3.19	0.00003	0.00136
MG_362	*rplL*	Ribosomal protein L7/L12	−2.74	0.00003	0.00136
MG_363	*rpmF*	Ribosomal protein L32	−2.19	0.00064	0.00136
MG_386	*p200*	P200 protein	−2.38	0.00001	0.00136
MG_396	*rpiB*	Ribose 5-phosphate isomerase B	−2.08	0.00040	0.00136
MG_398	*atpC*	ATP synthase F1, epsilon subunit	−2.52	0.00013	0.00136
MG_399	*atpD*	ATP synthase F1, beta subunit	−2.88	0.00001	0.00136
MG_400	*atpG*	ATP synthase F1, gamma subunit	−2.47	0.00001	0.00136
MG_401	*atpA*	ATP synthase F1, alpha subunit	−2.71	0.00001	0.00136
MG_402	*atpH*	ATP synthase F1, delta subunit	−2.14	0.00001	0.00136
MG_403	*atpF*	ATP synthase F0, B subunit	−2.97	0.00001	0.00136
MG_404	*atpE*	ATP synthase F0, C subunit	−3.79	0.00001	0.00136
MG_405	*atpB*	ATP synthase F0, A subunit	−3.02	0.00001	0.00136
MG_407	*eno*	Enolase	−2.29	0.00002	0.00136

Table 3. cont.

Gene ID	Gene	Description or product	Fold change	*P*-value	*Q* value
MG_408	*msrA*	Methionine-*S*-sulfoxide reductase	−3.09	0.00003	0.00136
MG_430	*gpmI*	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	−2.58	0.00007	0.00136
MG_431	*tpiA*	Triosephosphate isomerase	−2.47	0.00024	0.00136
MG_432		Membrane protein, putative	−2.54	0.00158	0.00136
MG_433	*tsf*	Translation elongation factor Ts	−3.32	0.00001	0.00136
MG_434	*pyrH*	Uridylate kinase	−2.13	0.00006	0.00136
MG_444	*rplS*	Ribosomal protein L19	−2.16	0.00001	0.00136
MG_445	*trmD*	tRNA (guanine-N1)-methyltransferase	−3.96	0.00001	0.00136
MG_446	*rpsP*	Ribosomal protein S16	−3.60	0.00001	0.00136
MG_451	*tuf*	Translation elongation factor Tu	−3.84	0.00001	0.00136
MG_452		Membrane protein, putative	−2.56	0.00002	0.00136
MG_453	*galU*	UTP-glucose-1-phosphate uridylyltransferase	−2.36	0.00163	0.00136
MG_454		OsmC-like protein	−5.09	0.00001	0.00136
MG_455	*tyrS*	Tyrosyl-tRNA synthetase	−2.43	0.00020	0.00136
MG_460	*ldh*	L-Lactate dehydrogenase/malate dehydrogenase	−5.72	0.00001	0.00136
MG_468.1		ABC transporter, ATP-binding protein	−2.08	0.00204	0.00136

osmotic upshift rather than from salt-specific effects, sucrose was used to increase osmolarity, and similar results were obtained (data not shown).

Validation of microarray data

Real-time PCR was employed and eight genes, which exhibited highly differential expression by microarray and represented important physiological roles, were chosen to confirm microarray results. MG_151, which encodes a ribosomal protein exhibiting relatively stable expression under osmotic shock, was used as the endogenous control. The primers selected for these genes are listed in Table 1. RNA samples from two biological replicates were analysed. As shown in Fig. 3, the expression patterns of all eight genes were consistent with the microarray results, although the fold changes of these osmotically upregulated genes were lower than those observed by microarray analysis. This discrepancy could be due to variations in the expression of MG_151 expression upon osmotic shock.

DISCUSSION

Bacteria respond to stressful environmental conditions by altering the expression of genes that enhance their survival. In the case of *M. genitalium*, a cell-wall-free bacterium that colonizes the genito-urinary tract, no information was available about its transcriptional response to osmotic stress, a condition likely to be encountered *in vivo*. By utilizing a whole-genomic microarray, we observed differential expression of genes when *M. genitalium* was exposed to 0.2 and 0.3 M NaCl for 1 h. In total, we identified 39 upregulated and 72 downregulated genes after 0.3 M NaCl treatment, which represents about 23 % (111/482) of predicted genes encoded by the genome. The microarray data were further confirmed by selecting eight genes for real-time PCR analysis, with all genes showing consistent patterns of expression. Of 39 genes upregulated by osmotic shock, 15 genes encode membrane or membrane-associated proteins, as suggested by the presence of a transmembrane domain(s) in their primary amino acid

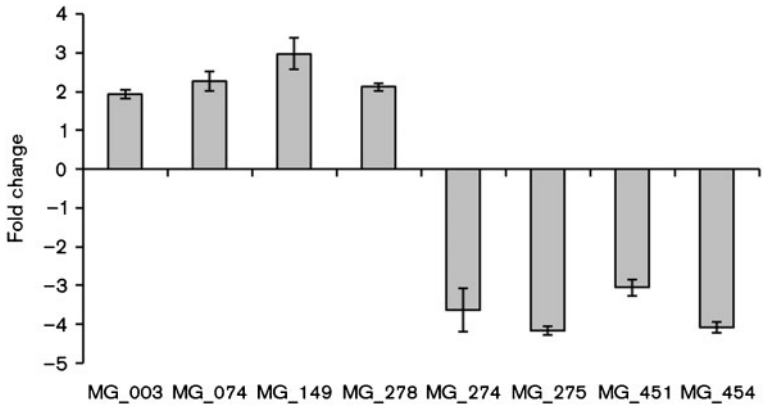

Fig. 3. Real-time PCR validation of selected *M. genitalium* genes differentially expressed under 0.3 M NaCl. MG_151, which encodes a ribosomal protein, was used as the normalizer. Data are presented as mean ± SD (error bars) from two biological replicates with each being performed in triplicate.

sequences (Table 2). It is noteworthy that five (MG_067, MG_068, MG_149, MG_439 and MG_440) of the 21 putative lipoprotein genes encoded by the genome were significantly induced, with MG_149 being the most highly upregulated. This is consistent with the idea that pathogenic bacteria adapt to various host environments by varying the synthesis of surface components (Mekalanos, 1992). Together, these results indicate that membrane remodelling may occur in *M. genitalium* in order to cope with high-osmolarity environments. Since the majority of these induced genes are annotated as being hypothetical, their upregulation under osmotic stress implies their functionally important role in maintaining mycoplasma cellular integrity during osmotically stressful conditions *in vivo*. An unexpected finding is that MG_454, which encodes an osmotically inducible OsmC-like protein that is upregulated in *Escherichia coli* and other bacteria (Atichartpongkul *et al.*, 2001), was downregulated (Table 3). The downregulation of MG_454 was validated by both real-time PCR (Fig. 3) and Northern blot analysis (data not shown). Although MG_454 shares sequence homology with OsmC, it is likely that transcription of MG_454 in *M. genitalium* is different from transcription in other bacteria given the paucity of factors involved in transcription regulation.

Cells typically accumulate osmotically active compounds referred to as compatible solutes, such as proline and glycine betaine, which are highly congruous with cellular functions that offset the deleterious effects of high osmolarity. Therefore, genes involved in the biosynthesis or uptake of compatible solutes are often induced by osmotic shock (Kempf & Bremer, 1998). As *M. genitalium* relies entirely on exogenous supplies of nutrients for growth, the pathways for synthesizing compatible solutes are not easily identifiable from the genome. Interestingly, we noticed that MG_064 and MG_289, two genes encoding putative ABC transporters, showed significant upregulation under osmotic shock (Table 2). MG_064 encodes a putative permease, which has been revealed to be indispensable under *in vitro* growth conditions (Glass *et al.*, 2006). MG_289 was annotated as a gene encoding a putative substrate binding protein of phosphonate ABC transporter, which shares sequence homology with p37 (recently designated Cypl) of *Mycoplasma hyorhinis*, an important factor involved in mycoplasma virulence, tumorigenic transformation and putative extracytoplasmic thiamine-binding (Sippel *et al.*, 2009). Since SP-4 medium is enriched with amino acids that could serve as compatible solutes, it is interesting to speculate that MG_064 and MG_289 might play a role in their uptake. It remains to be investigated which substrate(s) might be transported by MG_064 and MG_289 when *M. genitalium* encounters osmotic stress.

Of a total of 72 genes significantly downregulated by osmotic shock, many were metabolism-related, including those involved in glycolysis (*fba*, *pgi*, *pgk*, *gap*, *eno*, *gpml*, *ldh*, *pta*, *ackA*, *pdhA* and *pdhB*). Moreover, the expression of genes involved in protein translation was also repressed, including those encoding ribosomal proteins (*rpsP*, *rplL*, *rplS*, *rplK*, *rplA*, *rpsD* and *rpmF*), translation factors (*tuf* and *tsf*) and tRNA synthesis (*trmD*). The downregulation of these genes is consistent with the observed reduction in mycoplasma protein synthesis under osmotic shock (Fig. 1), similar to other bacteria (Weber & Jung, 2002). We noticed that eight genes (*atpA*, *atpB*, *atpC*, *aptD*, *atpE*, *atpF*, *atpG* and *atpH*), which encode proteins involved in the formation of F_1/F_0-ATP synthase complex, were repressed as well. In most prokaryotes, the function of the F_1/F_0-ATP synthase complex is to maintain a proton gradient through catabolism and hydrolysis of ATP. Since the cytochrome-dependent electron transport chain is absent in mycoplasmas, the function of the synthase complex is believed to be to chiefly maintain the electrochemical gradient in mycoplasmas. Decreased expression of genes encoding F_1/F_0-ATP synthase was observed in the early response of *E. coli* during osmotic shock, suggesting severe inhibition of respiration (Meury, 1994; Weber & Jung, 2002). Downregulation of four of the eight ATP synthase subunits has been observed in *Mycoplasma gallisepticum* when co-incubated with eukaryotic cells (Cecchini *et al.*, 2007). The functional implication of this downregulation of F_1/F_0-ATP synthase genes in *M. genitalium* under osmotic stress is unknown.

Presently, we cannot explain what mechanisms regulate the differential expression of genes under osmotic shock, given the presence of only a single σ factor in *M. genitalium*. However, the increased expression of genes encoding DNA gyrase (*gyrA* and *gyrB*) and GTP pyrophosphokinase (*relA*) during osmotic shock is of special interest (Table 2). DNA gyrase is involved in introducing negative supercoiling into DNA and maintaining the homeostasis of DNA topology of bacterial chromosomes together with DNA topoisomerase IV (Wang, 1996). The expression of gyrase is regulated by DNA supercoiling, which is often altered in response to environmental changes such as osmolarity and temperature, and has been identified as an important factor in globally influencing gene expression in many bacteria (Dorman, 1991). Previously, a microarray study identified a regulatory role of DNA supercoiling in controlling the osmotic stress response in *E. coli* (Cheung *et al.*, 2003). Therefore, the significant induction of *gyrB* and *gyrA* in *M. genitalium* under 0.3 M NaCl treatment (Table 2) suggests that DNA supercoiling of the chromosome is perturbed and may be essential in regulating genes under osmotic shock. Also, MG_278 (*relA*; Table 2) encodes a conserved enzyme responsible for the synthesis of ppGpp, which is an important signal molecule involved in stringent response. Interestingly, the overexpression of MPN_397, a homologue of MG_278 in *M. pneumoniae*, leads to transcriptional changes mainly related to stringent responses, indicating that the biological conservation of this signalling mechanism occurred despite genome reduction (Yus *et al.*, 2009). Induction of *relA* under osmotic shock has also been

demonstrated in *Lactococcus lactis*. Moreover, the disruption of *rel*, a homologue of *relA* in *Listeria monocytogenes*, reduced osmotolerance, suggesting that appropriate intracellular concentrations of ppGpp are essential for full osmotolerance (Okada *et al.*, 2002). Therefore, it appears that DNA gyrase and GTP pyrophosphokinase contribute to mechanisms of transcriptional regulation in *M. genitalium* during osmotic stress.

Another noteworthy feature of *M. genitalium* genes differentially expressed under osmotic shock is that many appear to be co-transcribed and comprise operons (Supplementary Fig. S1). For example, MG_003 and MG_004 encode two subunits of DNA gyrase (*gyrA* and *gyrB*) and both are induced under osmotic shock. A similar example among downregulated genes is the gene cluster encoding F_1/F_0-ATP synthase complex (MG_405 to MG_398). Operon structure remains unclear in *M. genitalium*, as genes are often clustered in the same orientation with small or no intergenic spaces. Since *M. genitalium* lacks rho factor, a rho-independent transcriptional terminator is predicted to operate; so far, few have been successfully identified (Kingsford *et al.*, 2007; Washio *et al.*, 1998). Consequently, genes of unrelated functions could be co-transcribed due to run-on expression (Benders *et al.*, 2005). A recent comprehensive analysis of *M. pneumoniae* transcriptomes describes the preferential use of hairpin formation to tightly regulate gene expression (Güell *et al.*, 2009). Furthermore, operon structure seems to be dynamic and modulated by environmental changes, with the expression of genes within an operon being linked to specific environmental conditions. Therefore, further analysis of gene clusters differentially regulated under osmotic shock in *M. genitalium* may help define operon structure and provide hints for assigning functional roles to hypothetical genes.

In conclusion, using a DNA microarray we provide the first assessment, to our knowledge, of global transcriptional responses of *M. genitalium* to osmotic stress. Our data offer insights as to how *M. genitalium* responds to physiologically relevant environmental cues. Moreover, genes differentially expressed under osmotic shock provide candidates for further investigation of mechanisms involved in transcription regulation in *M. genitalium*.

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