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Low Temperature (23 °C) Increases Expression of Biofilm-, Cold-Shock- and RpoS-Dependent Genes in *Escherichia coli* K-12

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Low temperature (23 °C) increases expression of biofilm-, cold-shock- and RpoS-dependent genes in *Escherichia coli* K-12

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Temperature serves as a cue to regulate gene expression in *Escherichia coli* and other bacteria. Using DNA microarrays, we identified 297 genes whose expression is increased at 23 °C compared to 37 °C in *E. coli* K-12. Of these genes, 122 are RpoS-controlled, confirming genome-wide the model that low temperature serves as a primary cue to trigger the general stress response. Several genes expressed at 23 °C overlap with the cold-shock response, suggesting that strategies used to adapt to sudden shifts in temperature also mediate long-term growth at 23 °C. Another category of genes more highly expressed at 23 °C are associated with biofilm development, implicating temperature as an important cue influencing this developmental pathway. In a candidate set of genes tested, the biofilm genes (*adrA*, *bolA*, *mlrA*, *nhaR*, *csgA*, *yceP*/*bssS)* and cold-shock genes (*otsA*, *yceP*/*bssS)* were found to be RpoS- and DsrA-dependent for their transcription at 23 °C. In contrast, transcription of three genes (*ycgZ*, *dps* and *ymgB*) was either partially or fully independent of these regulators, signifying there is an alternative thermoregulatory mechanism(s) that increases gene expression at 23 °C. Increased expression at 23 °C compared to 37 °C is retained in various media tested for most of the genes, supporting the relative importance of this cue in adaptation to changing environments. Both the RpoS-dependent gene *otsA* and the RpoS-independent gene *ymgB* demonstrated increased expression levels within 1 h after a shift from 37 to 23 °C, indicating a rapid response to this environmental cue. Despite changes in gene expression for many RpoS-dependent genes, experiments assessing growth rate at 23 °C and viability at 4 °C did not demonstrate significant impairment in *rpoS*::Tn*10* or *dsrA*::*cat* mutant strains in comparison to the wild-type strain. Biofilm formation was favoured at low temperature and is moderately impaired in both the *rpoS*::Tn*10* and *dsrA*::*cat* mutants at 23 °C, suggesting genes controlled by these regulators play a role necessary for optimal biofilm formation at 23 °C. Taken together, our data demonstrate that a large number of genes are increased in expression at 23 °C to globally respond to this environmental change and that at least two thermoregulatory pathways are involved in co-ordinating this response – the RpoS/DsrA pathway and an alternative thermoregulatory pathway, independent of these regulators.

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INTRODUCTION

In the course of its existence, *Escherichia coli* may transit through many different locations, both in the external environment and within an animal host. Bacteria have the ability to sense a multitude of environmental stimuli and use these cues to regulate gene expression to adapt cellular activities to these changing surroundings (reviewed by Cotter & Miller, 1998; Mahan *et al.*, 1996; Mekalanos, 1992).

Bacteria have the ability to respond to temperature as a primary cue to regulate gene expression. For mesophilic organisms, the most well studied are the reactions to temperatures at the limit of growth for these bacteria – the heat- (42 °C) and cold-shock (15 °C) responses (reviewed by Gross, 1996; Phadtare *et al.*, 2000; Yura *et al.*, 2000). Recently, microarray studies from our laboratory and others have focused on genes that are preferentially expressed at 37 °C, identifying genes regulated by this host cue that may ensure more efficient colonization (Brooks

Abbreviations: COGs, clusters of orthologous groups of proteins; qRT-PCR, quantitative real-time RT-PCR.

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et al., 2003; Han *et al.*, 2004; Motin *et al.*, 2004; Revel *et al.*, 2002; Smoot *et al.*, 2001; White-Ziegler *et al.*, 2007). In these studies, the comparison is made to growth at lower temperatures. Adaptation to growth at the lower temperature is particularly relevant in *Yersinia pestis* and *Borrelia burgdorferi* as it mimics the environment of an insect host (Brooks *et al.*, 2003; Han *et al.*, 2004; Motin *et al.*, 2004; Revel *et al.*, 2002). During its life cycle, *E. coli* is likely to encounter shifts to lower temperatures, either long-term or transient, that are similar to ambient indoor room settings (18–23 °C). An understanding of how *E. coli* adapts to this temperature is particularly important in medical and food industry settings where prevention of bacterial contamination is imperative.

One well understood model of gene regulation by low temperature (25 °C) is through the action of the general stress response sigma factor RpoS and the small regulatory RNA DsrA. Sledjeski *et al.* (1996) demonstrated that low temperature causes the increased expression of RpoS under low-temperature conditions during exponential phase in both rich (LB) and minimal (M63) medium through the action of the small regulatory RNA DsrA. Transcription of *dsrA* is increased at low temperature and DsrA interacts with the *rpoS* mRNA to alter its secondary structure to allow more efficient translation of the *rpoS* mRNA (reviewed by Lease & Belfort, 2000; Repoila *et al.*, 2003). Thus, transcription of RpoS-dependent genes is expected to be induced at low temperature in exponential phase, although this model has only been directly proven for a few genes, including *csgB*, *csgA*, *csgD* (Brown *et al.*, 2001; Olsen *et al.*, 1993b) and *dsrB* (Sledjeski *et al.*, 1996).

In this study, microarray studies using *E. coli* K-12 MC4100 were completed and demonstrate that 297 genes, approximately 7 % of the genome, are more highly expressed at 23 °C compared to growth at 37 °C. Approximately 40 % of the genes preferentially expressed at 23 °C are RpoS-controlled genes, broadly supporting and expanding the model that low temperature is a primary environmental cue that triggers the general stress response. Of the genes with increased transcription at 23 °C, two categories of genes were specifically noted – those associated with cold shock and biofilm development. The former set indicate that similar mechanisms used to adapt to a sudden decrease in temperature (>15 °C) are also used for long-term adaptation to growth at ambient conditions, whereas the latter set would suggest that temperature is an environmental cue that might impact biofilm development. In this study, the effect of low temperature was investigated for its effect on gene expression as well as its physiological effects on growth, cold-shock viability and biofilm formation. Given the number of genes controlled by RpoS that are expressed at 23 °C, the impact of temperature was tested in the wild-type and in *rpoS* and *dsrA* mutant strains to assess the contribution of these regulatory factors to the overall thermoregulatory response.

METHODS

Strains and media. The strains and bacteriophage used in this study are shown in Table 1. Luria–Bertani (LB) and M9 minimal media and antibiotics were prepared as described by Miller (1972) and Silhavy *et al.* (1984). *rpoS*::Tn*10* (from RH90) and the *dsrA*::*cat* (from SG12067) mutations were introduced into DL1504 by P1 transduction (Silhavy *et al.*, 1984) to create DL3106 and CWZ458, respectively (Table 1).

Bacterial growth conditions. For microarray analyses, bacterial cultures were inoculated and grown in M9 glycerol (M9 minimal liquid medium containing 2.45 μM ferric citrate, 30 μM thiamine, 100 μM calcium chloride, 1 mM magnesium sulfate and 0.2 % glycerol as a carbon source, pH 7) with aeration as described previously (White-Ziegler *et al.*, 2000, 2007). An inoculum from a 37 °C-grown single colony was used to initiate parallel 37 °C and 23 °C cultures. The cells from these cultures were harvested at equivalent optical densities after approximately 9–11 generations of growth in early mid-exponential phase (OD_{600}=0.2–0.6). Cell pellets were subsequently frozen and stored at −80 °C for RNA isolation. Identical methods were used for cells grown in LB or M9 glucose media.

For temperature shift experiments in M9 glycerol, an initial culture was inoculated as described above and grown at 37 °C to

Table 1. Bacterial strains and bacteriophages used in this study

Strain/bacteriophage	Description	Reference or source*
E. coli		
MC4100	F⁻ λ⁻ *araD139* Δ(*argF-lac*)*U169 e14-flhD5301* Δ(*fruK-yeiR*)*725*(*fruA25*) *relA1 rpsL150*(*strR*) *rbsR22* Δ(*fimB-fimE*)*632*(::*IS1*) *deoC1*	Casadaban (1976); Peters *et al.* (2003)
DL1504	MC4100 (λ354 lysogen *papBA-lacZYA* operon fusion)	Braaten *et al.* (1994)
RH90	MC4100 *rpoS359*::Tn*10*	Lange & Hengge-Aronis (1991)
DL3106	DL1504 containing *rpoS*::Tn*10*	D. A. Low
SG12067	MC4100 *dsrA*::*cat*	S. G. Gottesman
CWZ458	DL1504 containing *dsrA*::*cat*	This study
Bacteriophage		
P1L4	Virulent phage P1	D. A. Low

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mid-exponential phase. The culture was diluted 1 : 1 in fresh M9 glycerol and grown for an additional 45 min at 37 °C. The culture was then shifted to 23 °C and samples were collected at 0, 1, 2, 5, 7 and 11 h. For time points of 2 h and longer, the starter culture was additionally diluted in M9 glycerol to ensure the collection of cells within the exponential phase.

For growth curve analyses, an initial culture was inoculated as described above and grown at 37 °C to early exponential phase. The culture was subsequently shifted to 23 °C and spectrophotometer readings were taken at time points after the shift. The generation time for each strain was determined as described by Miller (1972).

For the cold-shock assay, an initial culture was inoculated as described above and grown at 37 °C to early exponential phase. A tenfold dilution series of each culture was performed in microtitre plates in M9 glycerol salts and 5 µl of each dilution was plated on M9 glycerol plates for determination of the number of c.f.u. ml^{-1}. A set of plates was incubated at 23 °C for immediate colony outgrowth ($t=0$). The remaining plates were incubated at 4 °C in a humidified chamber from 1 to 6 days and subsequently incubated at 23 °C for colony outgrowth.

RNA isolation. For microarray analyses, RNA was isolated by phenol/chloroform extraction as described previously (White-Ziegler *et al.*, 2007). For quantitative real-time RT-PCR (qRT-PCR) experiments, RNA isolation was done using Qiagen RNeasy Mini columns as described previously (White-Ziegler *et al.*, 2007). RNA concentrations and purity were determined by spectrophotometer readings. Isolated RNAs were stored at −80 °C until used.

cDNA synthesis, labelling and hybridization. Synthesis, labelling of cDNA with Cy3/Cy5 and hybridization was performed using the 3DNA Array 350 RP Expression Array Detection kit as described previously (White-Ziegler *et al.*, 2007). cDNA for each condition was prepared from 2 µg total RNA. cDNA from cultures grown at both temperatures (37 and 23 °C) was co-hybridized to slides containing full-length PCR products from all 4290 annotated ORFs in *E. coli* MG1655. Slides were produced by the University of Wisconsin-Gene Expression Center (www.biotech.wisc.edu/GEC/) and obtained at a reduced cost through the Genome Consortium for Active Teaching (www.bio.davidson.edu/projects/gcat/gcat.html).

Microarray data analysis. Five slides were used in the analysis with cDNAs, representing three independent growth experiments and two technical replicates. Hybridized slides were scanned and the data were analysed as described previously (White-Ziegler *et al.*, 2007). Significance analysis was completed as a one-class response using Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001) with $\Delta=0.65$ and a median false discovery rate of 1 %. An ORF was considered temperature-regulated if it demonstrated a statistically significant change in expression greater than 1.7-fold.

qRT-PCR. Reactions were completed using the SYBR Green One Step qRT-PCR kit (Invitrogen) as described previously (White-Ziegler *et al.*, 2007). All reactions were performed in triplicate, with no reverse transcriptase and no RNA controls run for each RNA sample to detect DNA contamination and reagent contamination, respectively. All reactions were normalized by using the same amount of total RNA (50 ng) in each reaction. Relative levels of gene expression and error analysis were calculated as described previously (Livak & Schmittgen, 2001; White-Ziegler *et al.*, 2007)

Biofilm formation assays. The biofilm assay was modified from a method described by O'Toole & Kolter (1998) with the following changes. Wells of 96-well polystyrene microtitre plates were inoculated with 200 µl bacterial culture that was diluted to a calculated starting OD_{600} of 0.003 (approx. 1 : 1000 dilution of an overnight culture) in fresh M9 glycerol medium. The plates were incubated in a humidified chamber for varying times at 37 or 23 °C. At a given time point, 100 µl of the culture was removed to a separate plate and the OD_{600} was recorded as a measure of planktonic growth. To the remaining 100 µl in the original microtitre plate, 100 µl M9 salts and 25 µl 1 % crystal violet was added and allowed to stain for 15 min. The plates were subsequently rinsed vigorously with water. To quantify biofilm formation, 250 µl 95 % ethanol was pipetted into each well and 125 µl was removed to a separate plate where the OD_{600} was recorded as a measure of biofilm formation. Differences in biofilm formation were determined to be statistically significant ($P<0.05$) using two-way analysis of variance (ANOVA) using STATA SEM software (StataCorp). Data represent the mean -fold change determined from at least three independent experiments.

RESULTS AND DISCUSSION

Microarray design to identify temperature-regulated genes in *E. coli* K-12

We wanted to determine on a genome-wide scale which genes were modulated in adapting to changes in temperature in *E. coli*, both ambient room temperature (23 °C) and human body temperature (37 °C). Parallel cultures grown at 23 and 37 °C in M9 minimal glycerol medium were harvested in exponential phase ($OD_{600}=0.2$–0.6) at 9–11 generations of growth after inoculation. Thus, the results presented here reflect the adapted state and signify genes whose expression is differentially maintained over long-term growth at a given temperature. Overall, 297 genes were more highly expressed at 23 °C (Table 2) whereas 126 genes were found to be more highly expressed at 37 °C (White-Ziegler *et al.*, 2007).

A large number of genes from all functional groups demonstrate increased expression at 23 °C

The 297 genes with increased expression at low temperature (23 °C) were categorized using the clusters of orthologous groups of proteins (COGs) database (Tatusov *et al.*, 1997). The genes span the different COG functional categories with none of the known functional classes particularly dominating the response. For each functional COG category, the number of genes in each category ranges between 0 and 8 % of the total genes (297) identified. The largest subset of genes (36 %, 107/297) are not categorized in a particular COG functional group. Strikingly, approximately half of the genes more highly expressed at low temperature are hypothetical or of unknown function. Thus, there are a significant number of uncharacterized genes whose expression is increased in response to low temperature and are likely to be important in adaptation to this environmental change.

Low temperature increases expression of RpoS-controlled genes

Comparison of our gene list with other published sources showed that 122 genes with increased expression at 23 °C

Table 2. Genes demonstrating increased expression at 23 °C

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
Amino acid transport and metabolism						
ddpA	b1487	Putative ABC transport system periplasmic binding protein	2.0			
ddpF	b1483	Putative ABC transport system ATP-binding protein	1.8			
gabP	b2663	γ-Aminobutyrate transport protein, RpoS-dependent (APC family)	2.3	Weber *et al.* (2005); Metzner *et al.* (2004)		
gadA	b3517	Glutamate decarboxylase A, isozyme, PLP-dependent	14.0	Weber *et al.* (2005)		
gadB	b1493	Glutamate decarboxylase isozyme	22.0	Weber *et al.* (2005)		
gadC	b1492	Acid sensitivity protein, putative transporter	21.5	Weber *et al.* (2005); this study		
ggt	b3447	γ-Glutamyltranspeptidase	4.1		Hashimoto *et al.* (1997)	
metA	b4013	Homoserine transsuccinylase	1.9			
metF	b3941	5,10-Methylenetetrahydrofolate reductase	2.6			
oppB	b1244	Oligopeptide transport permease protein	2.3			
poxB	b0871	Pyruvate dehydrogenase/oxidase: FAD- and thiamine PPi-binding	3.9	Weber *et al.* (2005)	Phadtare & Inouye (2004)	
prlC	b3498	Oligopeptidase A	1.9			
proV	b2677	ATP-binding component of transport system for glycine, betaine and proline	2.3	Rajkumari & Gowrishankar (2001)	Rajkumari & Gowrishankar (2001)	
ybaS	b0485	Putative glutaminase	14.8	Weber *et al.* (2005)		
ybaT	b0486	Putative amino acid/amine transport protein	10.1			
ybdL	b0600	Putative PLP-dependent aminotransferase	3.1			
ybdR	b0608	Putative dehydrogenase, NAD(P)-binding	2.4			
ydcV	b1443	Putative ABC transporter permease protein	5.8			
ydjL	b1776	Hypothetical zinc-type alcohol-dehydrogenase-like protein	1.9			
yehX	b2129	Putative ATP-binding component of a transport system	1.7	Checroun & Gutierrez (2004)		
yehY	b2130	Putative glycine/betaine/choline transport protein, osmoprotection	2.5	Checroun & Gutierrez (2004)		
yfdZ	b2379	Putative PLP-dependent aminotransferase	4.0			
ypdF	b2385	Putative peptidase	2.2			
Carbohydrate transport and metabolism						
amyA	b1927	Cytoplasmic α-amylase	6.1	Weber *et al.* (2005)		
aqpZ	b0875	Transmembrane water channel, aquaporin Z	2.9	Soupene *et al.* (2002)		
gcd	b0124	Glucose dehydrogenase	3.0			
glgP	b3428	Glycogen phosphorylase	1.8			
manX	b1817	PTS enzyme IIAB, mannose-specific	2.1			
otsA	b1896	Trehalose-6-phosphate synthase	2.9	Kandror *et al.* (2002); Weber *et al.* (2005)	Phadtare & Inouye (2004)	
otsB	b1897	Trehalose-6-phosphate phophatase, biosynthetic	3.5	Kandror *et al.* (2002); Weber *et al.* (2005)	Phadtare & Inouye (2004)	
proP	b4111	Low-affinity transport system, proline permease II	3.0	Mellies *et al.* (1995); Rajkumari & Gowrishankar (2001)		
talA	b2464	Transaldolase A	3.6	Weber *et al.* (2005)		
tktB	b2465	Transketolase 2 isozyme	3.7	Weber *et al.* (2005)		
treA	b1197	Trehalase, periplasmic	5.1	Weber *et al.* (2005)		
ugpB	b3453	sn-Glycerol 3-phosphate transport protein (ABC superfamily, peri_bind)	2.3	Weber *et al.* (2005)		
yedA	b1959	Putative transmembrane protein	2.5		Phadtare & Inouye (2004)	

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
yfbH	b2256	Hypothetical protein	2.2			
yliI	b0837	Putative dehydrogenase	2.2			
ypdH	b2387	Putative PTS family enzyme IIB component	2.3			
Cell cycle control, mitosis and meiosis						
fic	b3361	Induced in stationary phase, recognized by *rpoS*, affects cell division	2.5	Weber *et al.* (2005)		
Cell wall/membrane biogenesis						
arnT	b2257	4-Amino-4-deoxy-L-arabinose transferase (lipid A modification)	2.0			
blc	b4149	Outer-membrane lipoprotein (lipocalin)	2.7	Weber *et al.* (2005)		
cfa	b1661	Cyclopropane fatty acyl phospholipid synthase	4.7	Eichel *et al.* (1999); Wang & Cronan (1994)	Phadtare & Inouye (2004)	
csgG	b1037	Curli production assembly/transport component, 2nd curli operon	12.5	Brown *et al.* (2001); Olsen *et al.* (1993b)		Prigent-Combaret *et al.* (2001); Vidal *et al.* (1998); Olsen *et al.* (1993a); Cookson *et al.* (2002); Romling *et al.* (1998)
galU	b1236	Glucose-1-phosphate uridylyltransferase	1.8			
mscS	b2924	Component of the MscS mechanosensitive channel	2.8	Stokes *et al.* (2003)		
nlpD	b2742	Lipoprotein	1.7			
ompC	b2215	Outer-membrane pore protein 1b (Ib, c)	7.0		Phadtare & Inouye (2004)	
ompX	b0814	Outer-membrane protease, receptor for phage OX2	2.8			Otto & Hermansson (2004)
ugd	b2028	UDP-glucose 6-dehydrogenase	1.7			
yehZ	b2131	Putative glycine/betaine/choline transport protein, osmoprotection	2.5	Checroun & Gutierrez (2004)		
yhiU	b3513	Multidrug resistance protein (lipoprotein)	8.0	Hirakawa *et al.* (2006)		
yhjG	b3524	Hypothetical protein	3.1	Weber *et al.* (2005)		
ynaI	b1330	Putative transmembrane protein	4.7			
Coenzyme transport and metabolism						
menB	b2262	Dihydroxynaphthoic acid synthetase	1.7			
metK	b2942	Methionine adenosyltransferase 1 (AdoMet synthetase)	2.1			Ren *et al.* (2004)
Defence mechanisms						
yadG	b0127	Putative ATP-binding component of a transport system	1.7			
yhiV	b3514	Multidrug transport protein, RpoS-dependent (RND family)	7.6	Hirakawa *et al.* (2006)		
Energy production and conversion						
acnA	b1276	Aconitate hydrase 1	2.1			
glcA	b2975	Glycolate permease (LctP family)	2.0			
hyaA	b0972	Hydrogenase-1 small subunit	2.1			
hyaB	b0973	Hydrogenase-1 large subunit	2.2			
narV	b1465	Cryptic nitrate reductase 2 γ subunit	3.3			
narW	b1466	Cryptic nitrate reductase 2 δ subunit	4.6			
narY	b1467	Cryptic nitrate reductase 2 β subunit	4.2	Weber *et al.* (2005)		
narZ	b1468	Cryptic nitrate reductase 2 α subunit	2.4			
qor	b4051	Quinone oxidoreductase	2.1	Weber *et al.* (2005)		
tas	b2834	Putative reductase, NAD(P)-linked	1.9			
yccX	b0968	Putative phosphohydrolase	1.8			

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
yceJ	b1057	Cytochrome b561 homologue 2	2.4			
ydcW	b1444	Putative aldehyde dehydrogenase	6.1			
Inorganic ion transport and metabolism						
apaG	b0050	Hypothetical protein	1.8			
chaA	b1216	Sodium–calcium/proton antiporter	2.0			
chaC	b1218	Cation transport regulator	2.9			
corA	b3816	Mg^{2+} transport, system I	2.2			
dps	b0812	Global regulator, starvation conditions	11.8	This study; Weber *et al.* (2005)	Phadtare & Inouye (2004)	
kch	b1250	Putative potassium channel protein	3.0			
kdpC	b0696	P-type ATPase, high-affinity potassium transport system, C chain	1.8			
narU	b1469	Nitrate extrusion protein (MFS family)	3.5	Weber *et al.* (2005)		
nhaA	b0019	Na^{+}/H antiporter, pH-dependent	2.4			
pstS	b3728	High-affinity phosphate transport protein (ABC superfamily, peri_bind)	2.4			
sodC	b1646	Superoxide dismutase precursor (Cu–Zn)	2.6	Lacour & Landini (2004)		
yeaR	b1797	Putative tellurite resistance protein	2.7			
yfdC	b2347	Putative transport protein	2.9			
Lipid transport and metabolism						
atoB	b2224	Acetyl-CoA acetyltransferase	1.7			
ybhO	b0789	Cardiolipin (CL) synthase 2	2.8			
yciA	b1253	Putative enzyme	1.9			
ymdC	b1046	Putative synthase	2.2			
Nucleotide transport and metabolism						
pyrB	b4245	Aspartate carbamoyltransferase, catalytic subunit	1.8			
Post-translational modification, protein turnover, chaperones						
cbpA	b1000	Curved DNA-binding protein, co-chaperone of DnaK (Hsp40 family)	3.2	Weber *et al.* (2005)		
dnaK	b0014	Chaperone Hsp70 in DNA biosynthesis and cell division	2.5			
groS	b4142	GroES, 10 K_d chaperone binds to Hsp60	2.4			
ibpA	b3687	Heat-shock protein	2.9			
msrA	b4219	Peptide methionine sulfoxide reductase	1.9			
osmC	b1482	Osmotically inducible protein	5.4	Weber *et al.* (2005)		
yfcF	b2301	Putative glutathione *S*-transferase	2.6	Weber *et al.* (2005)		
yfcG	b2302	Putative *S*-transferase	2.8			
yqjG	b3102	Putative enzyme with *S*-transferase domain	1.8	Weber *et al.* (2005)		
Replication, recombination and repair						
dbpA	b1343	ATP-dependent RNA helicase	2.0			
topA	b1274	DNA topoisomerase type I, omega protein	1.9			
Secondary metabolites biosynthesis, transport and catabolism						
folM	b1606	Putative oxidoreductase	2.4			
ycaC	b0897	Putative cysteine hydrolase	5.1	Weber *et al.* (2005)		
yghA	b3003	Putative oxidoreductase	2.1	Weber *et al.* (2005)		
yohF	b2137	Putative oxidoreductase	2.3	Weber *et al.* (2005)		
Signal transduction mechanisms						
arcA	b4401	Negative response regulator of genes in aerobic pathways (sensors, ArcB and CpxA)	2.1			
glnL	b3869	Histidine protein kinase sensor for GlnG regulator (nitrogen regulator II, NRII)	2.4			
hnr/rssB	b1235	Hnr protein	2.0	Weber *et al.* (2005)		
luxS	b2687	Quorum-sensing protein, produces autoinducer molecules	2.1			

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
adrA/yaiC	b0385	Putative membrane protein	4.4	This study		Romling *et al.* (2000); Zogaj *et al.* (2001)
ycgF	b1163	Hypothetical protein	2.1		Polissi *et al.* (2003)	
yciR	b1285	RNase II modulator	2.5	Weber *et al.* (2005)		
ydaM	b1341	Hypothetical protein	3.4	Weber *et al.* (2005)		
yddV	b1490	Hypothetical protein	3.2			
ydeH	b1535	Hypothetical protein	2.2			
yeaG	b1783	Hypothetical protein	8.4	Weber *et al.* (2005)		
yhjB	b3520	Putative regulator	2.1			
yiiT	b3923	Putative regulator	2.0			
Transcription						
cadC	b4133	Transcriptional activator of *cad* operon	2.2			
csgD	b1040	Putative 2-component transcriptional regulator for 2nd curli operon	12.5	Brown *et al.* (2001); Olsen *et al.* (1993b)	Brown *et al.* (2001); Olsen *et al.* (1993b)	Cookson *et al.* (2002); Olsen *et al.* (1993a); Prigent-Combaret *et al.* (2001); Romling *et al.* (1998); Vidal *et al.* (1998)
csiE	b2535	Stationary-phase inducible protein	3.0	Marschall & Hengge-Aronis (1995)		
cspI	b1552	Qin prophage; cold-shock-like protein	3.9		Phadtare & Inouye (2004); Polissi *et al.* (2003)	
gadE	b3512	Putative regulator	22.7	Weber *et al.* (2005)		
gadW	b3515	Putative ARAC-type regulatory protein	6.1	Weber *et al.* (2005)		
gadX	b3516	Putative ARAC-type regulatory protein	3.7	Weber *et al.* (2005)		
hipB	b1508	Persistence to inhibition of murein or DNA biosynthesis, regulatory protein	1.7			
iscR	b2531	Fe–S cluster-containing transcription factor	2.4			
metR	b3828	Regulator for *metE* and *metH*	3.0			
mlrA	b2127	Putative transcriptional regulator	4.2	Brown *et al.* (2001); this study		Brown *et al.* (2001)
nhaR	b0020	Transcriptional activator of cation transport (LysR family)	2.5	Toesca *et al.* (2001); this study		
rcsA	b1951	Positive regulator for *ctr* capsule biosynthesis, positive transcription factor	4.5			
rpoS	b2741	RNA polymerase, sigma S (sigma 38) factor	2.3			
rsd	b3995	Regulator of sigma D	2.1			
ycgE	b1162	Putative transcriptional regulator	3.5			
yeiI	b2160	Putative (sugar/sugar-nucleotide) kinase	2.0			
yfjR	b2634	CP4-57 prophage, putative transcriptional repressor (DeoR family)	2.4			
yhcO	b3239	Hypothetical protein	2.6			
yhiF	b3507	Putative transcriptional regulator (LuxR/UhpA family)	13.2			
yiaG	b3555	Putative transcriptional regulator	6.9	Weber *et al.* (2005)		
yidL	b3680	Putative ARAC-type regulatory protein	2.1			
Translation						
arnA	b2255	Putative formyltransferase	2.1			
dusB	b3260	Conserved protein, FMN-linked	1.8			
miaA	b4171	Δ(2)-Isopentenylpyrophosphate tRNA-adenosine transferase	1.7			

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
rmf	b0953	Ribosome modulation factor	2.4			
yjgH	b4248	Putative translation factor	3.6	Weber *et al.* (2005)		
General function prediction only						
chaB	b1217	Cation transport regulator	2.9	Weber *et al.* (2005)		
cof	b0446	Putative hydrolase, contains phophatase-like domain	1.9			
elaA	b2267	Putative transferase	2.0			
gloB	b0212	Probable hydroxyacylglutathione hydrolase	2.0			
osmY	b4376	Hyperosmotically inducible periplasmic protein	9.1	Weber *et al.* (2005); this study		
rhtA	b0813	Putative membrane protein	1.9			
tam	b1519	Trans-aconitate 2-methyltransferase	2.6	Weber *et al.* (2005)		
wrbA	b1004	Flavodoxin-like protein, *trp* repressor-binding protein	3.3	Weber *et al.* (2005)	Phadtare & Inouye (2004)	
ybeM	b0626	Putative NAD(P)-binding amidase-type enzyme (C–N hydrolase family)	1.9			
ybhP	b0790	Putative DNase	2.4			
yeaE	b1781	Putative aldehyde reductase	2.2			
yedJ	b1962	Hypothetical protein	1.7			
yedU	b1967	Hypothetical protein	2.5	Weber *et al.* (2005)		
yhbO	b3153	Putative intracellular proteinase with catalase domain	3.1			
yieF	b3713	Putative oxidoreductase, flavoprotein	1.9			
yjdJ	b4127	Putative acyltransferase domain	4.2	Weber *et al.* (2005)		
yjgR	b4263	Putative enzyme with P-loop containing NTP hydrolase domain	2.0	Weber *et al.* (2005)		
yjjU	b4377	Putative transcriptional regulator	3.0			
ymdB	b1045	Putative polyprotein	3.2			
Function unknown						
elaB	b2266	Unknown CDS	5.8	Weber *et al.* (2005)		
hdeD	b3511	Putative membrane protein	17.1	Weber *et al.* (2005)		
iscA	b2528	Involved in Fe–S biosynthesis	1.6			
phnB	b4107	Hypothetical protein	4.1	Weber *et al.* (2005)		
ybdK	b0581	Hypothetical protein	2.4	Weber *et al.* (2005)		
ybgA	b0707	Hypothetical protein	3.2	Weber *et al.* (2005)		
yccT	b0964	Hypothetical protein	3.9			
ycgB	b1188	Putative sporulation protein	6.6	Weber *et al.* (2005)		
yeaH	b1784	Hypothetical protein	7.6	Weber *et al.* (2005)		
yeaQ	b1795	Hypothetical protein	3.2			
yeeA	b2008	Putative membrane protein, transport	2.1			
ygaU	b2665	Hypothetical protein	3.4	Weber *et al.* (2005)		
yggE	b2922	Putative actin	2.6	Weber *et al.* (2005)		
yhhQ	b3471	Putative integral membrane protein	1.9			
yhiD	b3508	Putative Mg^{2+} transport ATPase	9.7	Weber *et al.* (2005)		
yiiS	b3922	Hypothetical protein	2.3			
yjbJ	b4045	Unknown CDS	2.7	Weber *et al.* (2005)		
yjbQ	b4056	Hypothetical protein	2.3			
yjbR	b4057	Hypothetical protein	2.4			
yjdI	b4126	Hypothetical protein	2.9	Weber *et al.* (2005)		
yjiN	b4336	Putative transmembrane protein	2.6			
ymbA	b0952	Hypothetical protein	1.8			
ymgE	b1195	Transglycosylase-associated protein	2.7			
ynhG	b1678	Putative ATP synthase subunit	2.2	Weber *et al.* (2005)		
yniA	b1725	Conserved protein, protein kinase-like	2.4			
yqjD	b3098	Hypothetical protein	4.1	Weber *et al.* (2005)		
yqjE	b3099	Hypothetical protein	3.3	Weber *et al.* (2005)		

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
Not in COGS database						
adhP	b1478	Alcohol dehydrogenase	6.4	Weber *et al.* (2005)		
aidB	b4187	Putative acyl coenzyme A dehydrogenase	3.1	Weber *et al.* (2005)		
aldB	b3588	Aldehyde dehydrogenase B (lactaldehyde dehydrogenase)	5.4	Xu & Johnson (1995)		
asr	b1597	Acid-shock protein	1.9			
b0165	b0165	Unknown CDS	1.8			
b2999	b2999	Putative enzyme	2.8			
b3000	b3000	Putative enzyme with α/β-hydrolase domain	3.9			
bdm	b1481	Biofilm-dependent modulation protein	2.9			
bolA	b0435	Possible regulator of murein genes	3.5	Weber *et al.* (2005); this study		Vieira *et al.* (2004)
csgA	b1042	Curlin major subunit, coiled surface structures, cryptic	22.9	Brown *et al.* (2001); Olsen *et al.* (1993b); this study	Brown *et al.* (2001); Olsen *et al.* (1993b)	Cookson *et al.* (2002); Olsen *et al.* (1993a); Prigent-Combaret *et al.* (2001); Romling *et al.* (1998); Vidal *et al.* (1998)
csgB	b1041	Minor curlin subunit precursor, nucleator for assembly of adhesive surface organelles	20.4	Brown *et al.* (2001); Olsen *et al.* (1993b)	Brown *et al.* (2001); Olsen *et al.* (1993b)	Cookson *et al.* (2002; Olsen *et al.* (1993a); Prigent-Combaret *et al.* (2001); Romling *et al.* (1998); Vidal *et al.* (1998)
csgC	b1043	Putative curli production protein	13.8	Brown *et al.* (2001); Olsen *et al.* (1993b)		Cookson *et al.* (2002); Olsen *et al.* (1993a); Prigent-Combaret *et al.* (2001); Romling *et al.* (1998); Vidal *et al.* (1998)
csgE	b1039	Curli production assembly/transport component, 2nd curli operon	11.6	Brown *et al.* (2001); Olsen *et al.* (1993b)		Cookson *et al.* (2002); Olsen *et al.* (1993a); Prigent-Combaret *et al.* (2001); Romling *et al.* (1998); Vidal *et al.* (1998)
csgF	b1038	Curli production assembly/transport component, 2nd curli operon	17.6	Brown *et al.* (2001); Olsen *et al.* (1993b)		Cookson *et al.* (2002); Olsen *et al.* (1993a); Prigent-Combaret *et al.* (2001); Romling *et al.* (1998); Vidal *et al.* (1998)
ddg	b2378	Palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase, cold-induced	1.7			
dicC	b1569	Qin prophage, transcriptional repressor of cell division inhibition protein	3.5			
dkgV	b3012	2,5-Diketo-D-gluconate reductase A	2.9	Weber *et al.* (2005)		

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
dsrB	b1952	Hypothetical protein	3.2	Sledjeski *et al.* (1996)	Sledjeski *et al.* (1996)	
hdeA	b3510	Hypothetical protein	16.0	Weber *et al.* (2005)		
hdeB	b3509	Hypothetical protein	17.4	Weber *et al.* (2005)		
iscS	b2530	Cysteine desulfurase	1.7			
katE	b1732	Catalase, hydroperoxidase HPII (III), RpoS-dependent	5.2	Weber *et al.* (2005)		
kptA	b4331	2′-Phosphotransferase	1.9			
marR	b1530	Multiple antibiotic resistance protein, repressor of *mar* operon	1.8			
mmuP	b0260	CP4-6 prophage; putative *S*-methylmethionine transport protein (APC family)	2.1			
msyB	b1051	Acidic protein suppresses mutants lacking function of protein export	4.9	Weber *et al.* (2005)		
osmB	b1283	Lipoprotein, osmotically inducible	2.7	Weber *et al.* (2005)		
osmE	b1739	Activator of *ntrL* gene	3.4	Conter *et al.* (1997)		
phoA	b0383	Alkaline phosphatase	1.7			
psiF	b0384	Induced by phosphate starvation	5.9	Weber *et al.* (2005)		
pspD	b1307	Phage shock protein	1.7			
pyrL	b4246	*pyrBI* operon leader peptide	2.4			
rpsV	b1480	30S ribosomal subunit protein S22	6.5	Weber *et al.* (2005)		
slp	b3506	Outer-membrane protein induced after carbon starvation	12.9	Weber *et al.* (2005)		
tfaS	b2353	CPS-53 (KpLE1) prophage	2.0			
uspB	b3494	Universal stress protein B	2.5	Weber *et al.* (2005)		
wecD	b3790	Putative acyltransferase, lipopolysaccharide biosynthesis protein	2.1			
yabQ	b0057	Unknown CDS	1.8			
yacH	b0117	Putative membrane protein	2.2			
yaiB	b0382	Hypothetical protein	1.9			
ybaA	b0456	Hypothetical protein	2.5			
ybaQ	b0483	Conserved protein, DNA-binding domain	1.8			
ybaY	b0453	Glycoprotein/polysaccharide metabolism	3.2	Weber *et al.* (2005)		
ybeH	b0625	Unknown CDS	1.9			
ybfC	b0704	Unknown CDS	1.9			
ybgS	b0753	Putative homeobox protein	3.2	Weber *et al.* (2005)		
ybiU	b0821	Hypothetical protein	3.9			
ybjP	b0865	Putative lipoprotein	2.1	Weber *et al.* (2005)		
ycbW	b0946	Hypothetical protein	1.8			
yccD	b0999	Hypothetical protein	3.2			
yccJ	b1003	Unknown CDS	3.1	Weber *et al.* (2005)		
ycdF	b1005	Unknown CDS	5.5			
yceK	b1050	Unknown CDS	3.9	Weber *et al.* (2005)		
yceP	b1060	Hypothetical protein	2.8	This study	Polissi *et al.* (2003)	Domka *et al.* (2006); Ren *et al.* (2004)
ycgZ	b1164	Unknown CDS	9.9	Weber *et al.* (2005)	Polissi *et al.* (2003)	
yciG	b1259	Hypothetical protein	5.0	Weber *et al.* (2005)		
yciU	b1248	Hypothetical protein	1.8			
ycjJ	b1296	Putative amino acid/amine transport protein	2.2			
ycjK	b1297	Putative glutamine synthetase	2.4		Polissi *et al.* (2003)	
ydcK	b1428	Putative LpxA-like enzyme	2.2			
ydcL	b1431	Hypothetical protein	2.7			
yddJ	b1470	Unknown CDS	3.0			
ydgT	b1625	Hypothetical protein	2.0			
ydiH	b1685	Unknown CDS	2.2			
ydiZ	b1724	Hypothetical protein	4.0			

Table 2. cont.

Gene*	Blattner no.	Product†	23/37 °C‡	RpoS§	Cold shock‖	Biofilm¶
yebF	b1847	Hypothetical protein	2.4	Weber *et al.* (2005)		
yebV	b1836	Unknown CDS	4.8			
yecF	b1915	Unknown CDS	2.8			
yegP	b2080	Hypothetical protein	4.2	Weber *et al.* (2005)		
yehE	b2112	Hypothetical protein	3.5			
ygaM	b2672	Hypothetical protein	5.2	Weber *et al.* (2005)		
ygbA	b2732	Hypothetical protein	2.0	Weber *et al.* (2005)		
ygcW	b2774	Putative deoxygluconate dehydrogenase	2.8			
ygdI	b2809	Unknown CDS	4.6			
ygjG	b3073	Probable ornithine aminotransferase	5.8	Weber *et al.* (2005)		
yhdJ	b3262	Putative methyltransferase	1.7			
yhfG	b3362	Hypothetical protein	4.2	Weber *et al.* (2005)		
yhhT	b3474	Putative permease (PerM family)	2.9	Weber *et al.* (2005)		
yhiM	b3491	Putative transport protein	7.6	This study		
yhjS	b3536	Hypothetical protein	2.1			
yhjT	b3537	Unknown CDS	1.9			
yhjU	b3538	Putative membrane protein	2.1			
yhjY	b3548	Putative lipase	2.1			
yibI	b3598	Unknown CDS	2.8			
yjbO	b4050	Hypothetical protein	1.9			
yjfO	b4189	Hypothetical protein	2.7			
yjgB	b4269	Putative alcohol dehydrogenase	3.4	Weber *et al.* (2005)		
yjgG	b4247	Unknown CDS	3.3	Weber *et al.* (2005)		
yjhT	b4310	Putative enzyme contains galactose-oxidase-like domain	2.2	Weber *et al.* (2005)		
yjjY	b4402	Unknown CDS	2.4			
ymdA	b1044	Hypothetical protein	13.4	This study		
ymgA	b1165	Unknown CDS	8.6	Weber *et al.* (2005)		
ymgB	b1166	Unknown CDS	9.5			
ymgC	b1167	Unknown CDS	4.8			
yoaC	b1810	Hypothetical protein	3.0			
yodC	b1957	Unknown CDS	3.0	Weber *et al.* (2005)		
yodD	b1953	Unknown CDS	4.3	Weber *et al.* (2005)		
yohC	b2135	Putative transport protein	4.4			
yphA	b2543	Putative transmembrane protein	2.4	Weber *et al.* (2005)		
yqgD	b2941	Unknown CDS	2.6			
yqiJ	b3050	Putative oxidoreductase	3.4			
yqjC	b3097	Hypothetical protein	3.3	Weber *et al.* (2005)		
yqjK	b3100	Hypothetical protein	3.5	Weber *et al.* (2005)		
yrbL	b3207	Hypothetical protein	2.6			
yrdA	b3279	Putative acyl transferase, ferripyochelin-binding	1.7			
yrfG	b3399	Putative hydrolase, contains a phophatase-like domain	1.9			
ytfK	b4217	Unknown CDS	4.2			

*Genes included are those that show increased levels of expression at 23 °C in comparison to growth at 37 °C in M9 glycerol medium as described in Methods.

†Product descriptions and functional categories are based upon the *E. coli* K-12 COGS categorization. Descriptions were shortened in some instances.

‡The -fold change is indicated as the mean ratio of medians (23/37 °C). All genes included showed a statistically significant increase at 23 °C as described in Methods.

§References provided indicate RpoS dependence under one or more environmental conditions.

‖References provided indicate expression under cold-shock or low-temperature conditions.

¶References provided indicate expression during biofilm development.

are known to be RpoS-controlled in response to at least one other environmental stress (Table 2), with the majority (92 genes) overlapping a core set of genes known to be induced by three different environmental stresses (high osmolarity, low pH and stationary-phase growth) (Weber *et al.*, 2005). Included within our dataset are four genes previously shown to specifically be induced by low temperature in an RpoS-dependent manner – *csgB*, *csgA*, *csgD* and *dsrB* (Brown *et al.*, 2001; Olsen *et al.*, 1993b; Sledjeski *et al.*, 1996). These data thus confirm and demonstrate, genome-wide, that low temperature serves as a primary environmental stimulus that causes the co-ordinated expression of a large set of RpoS-dependent genes and specifically defines those RpoS-controlled genes activated in response to this cue. At the same time, it is important to note that for the remaining 175 genes, it is unknown what thermoregulatory mechanism controls their increased expression at 23 °C.

Genes expressed at 23 °C overlap the cold-shock response

In mesophiles, the cold-shock response is characterized by the transient, increased production of cold-shock proteins after a temperature decrease (generally >10 °C) that subsequently facilitates adaptation to prolonged growth at low temperature (Phadtare *et al.*, 2000). Of the cold-shock-inducible (Csp) proteins (CspA, CspB, CspG and CspI), *cspI* expression showed a 3.9-fold increase during growth at 23 °C compared to 37 °C. CspI, a *cspA* homologue and thought to act as a chaperone that denatures RNA for more efficient translation at low temperature, was shown previously to have increased transcription and mRNA stability upon a shift to 15 °C (Wang *et al.*, 1999). Several other genes from our microarray overlapped with genes that show increased expression in two other microarray studies in which cells were exposed to cold-shock conditions, either transiently (*cfa*, *otsA*, *otsB*, *poxB*, *dps*, *ycjK*, *ycgF*, *ycgZ*, *yceP*/*bssS*, *yedA*) or after prolonged (*ompC*, *wrbA*) growth at 15–16 °C (Phadtare & Inouye, 2004; Polissi *et al.*, 2003).

Of particular interest are the genes proven to be important for viability under cold-shock conditions. In our study, *otsA* and *otsB*, genes required for the synthesis of trehalose, an osmoprotectant which increases cell viability when cells undergo cold shock at 4 °C (Kandror *et al.*, 2002), showed increased mRNA levels (2.9- and 3.5-fold, respectively) at 23 °C, similar to previous studies in which these genes were induced in an RpoS-dependent manner at 16 °C (Kandror *et al.*, 2002). Our results show that *cfa* transcription is increased 4.7-fold at 23 °C compared to 37 °C. Cyclopropane fatty acyl phospholipid synthase (Cfa) modifies membrane phospholipids, converting the fatty acid moieties of these lipids from the unsaturated to the cyclopropane form. It is hypothesized that this modification of fatty acids by Cfa might decrease membrane fluidity to allow adaptation to stressful conditions, supported by the fact that *cfa* mutants have an increased sensitivity to freeze–thaw cycles (Grogan & Cronan, 1984, 1997; Zhao *et al.*, 2003). *proP* and *proV* also demonstrated increased expression at 23 °C (3.0- and 2.3-fold, respectively) consistent with previous results implicating low temperature (10 °C) as an inducing signal for production of this osmoprotectant transporter (Rajkumari & Gowrishankar, 2001, 2002). Given that the microarray results described here represent cells adapted to 23 °C for approximately 10 generations, these data suggest that strategies used to protect the cell upon dramatic shifts in temperature are also likely to be valuable for long-term adaptation to growth at ambient temperature.

Low temperature increases expression of genes associated with biofilm development

Notably, several genes with increased expression at 23 °C in our microarrays are implicated in biofilm development (Table 2). Multiple studies have described a role for curli in adherence, important for biofilm formation (Cookson *et al.*, 2002; Olsen *et al.*, 1993a; Prigent-Combaret *et al.*, 2001; Romling *et al.*, 1998; Vidal *et al.*, 1998). In our microarray, all of the curli genes show dramatic increased expression at 23 °C. YaiC, the homologue to *Salmonella* AdrA, increases cellulose biosynthesis involved in biofilm development in *E. coli* and *Salmonella enterica* serovar Typhimurium (Romling *et al.*, 2000; Zogaj *et al.*, 2001) and its mRNA levels were increased 4.4-fold in our microarray experiments. *mlrA*, which encodes a positive regulator of curli (*csgD* and *csgBA*) operons in avian-pathogenic *E. coli* (Brown *et al.*, 2001) and of *adrA* expression in *S. enterica* serovar Typhimurium (Garcia *et al.*, 2004), is increased 4.2-fold at low temperature in our microarray. *bolA* has been shown to be important for biofilm formation in *E. coli* MC1061 at 37 °C in minimal medium (Vieira *et al.*, 2004) and its transcription is induced in response to a variety of stresses (acid, heat, osmotic) (Santos *et al.*, 1999); our microarrays indicate that low temperature increases *bolA* expression 3.5-fold. YceP (BssS), implicated in biofilm formation and quorum sensing (Domka *et al.*, 2006), is increased 2.8-fold at 23 °C in our study. NhaR activates the biofilm adhesin poly-β-1,6-*N*-acetyl-D-glucosamine (Goller *et al.*, 2006) and its transcription is increased 2.5-fold at low temperature. Together, these data suggest that low temperature is an important environmental cue used to increase expression of several biofilm genes.

Both RpoS/DsrA-dependent and -independent thermoregulatory mechanisms mediate increased gene expression at 23 °C

We chose a set of genes associated with biofilm development and the cold-shock response to determine if their low-temperature transcription was fully RpoS- and DsrA-dependent under our growth conditions (M9 glycerol medium) or whether other response regulators might be involved in the low-temperature induction of

these genes. Five genes associated with biofilm formation were tested – *adrA*/*yaiC*, *csgA*, *mlrA*, *bolA* and *nhaR*, whereas three genes that overlapped the cold-shock response were assessed – *otsA*, *dps* and *ycgZ*. Expression of *yceP*/*bssS* was also measured which has been shown to be associated with both biofilm development and cold shock. In addition, we also assessed the expression of three other genes for which there was no information on their function or regulation – *ymdA*, *ymgB* and *yhiM* – which showed high levels of transcription at 23 °C in the microarrays (13.4-, 9.5- and 7.6-fold, respectively).

All of the genes associated with biofilm development were dependent upon both RpoS and DsrA for transcription at 23 °C in M9 glycerol medium. Confirming the microarray results, all of the genes demonstrated increased mRNA levels at 23 °C compared to 37 °C in the wild-type strain (Table 3). In the *rpoS*::Tn*10* and *dsrA*::*cat* mutant strains, transcription of all of the biofilm-associated genes at 23 °C was reduced to levels similar to those measured at 37 °C in the wild-type strain, demonstrating the induction at 23 °C is dependent upon RpoS and DsrA. Similarly, expression at 23 °C of the cold-shock genes (*otsA*, *yceP*/*bssS*) and genes of unknown function (*ymdA* and *yhiM)* was also fully dependent upon these two regulators (Table 3). The novel finding that *ymdA* and *yhiM* are within the RpoS regulon demonstrate that there may be additional targets of RpoS/DsrA within our list of genes whose increased expression at low temperature is dependent upon these regulators. These genes had not been previously identified as being RpoS-dependent, despite genome-wide comparisons under other environmental conditions (Weber *et al.*, 2005).

Increased expression of *ycgZ* and *dps* at 23 °C was only partially dependent upon RpoS and/or DsrA, thus indicating that other thermoregulatory mechanisms contribute to the expression of these genes at 23 °C (Table 3). For *ycgZ*, reduced mRNA levels were observed in the *rpoS*::Tn*10* mutant compared to the wild-type strain, indicating that maximal expression at 23 °C is an additive effect of RpoS-dependent and -independent mechanisms. Notably, this effect did not apply to the *dsrA*::*cat* mutant where *ycgZ* expression levels were equivalent to those measured in the wild-type strain at 23 °C. *dps* expression at 23 °C was fully dependent upon RpoS, but only partially dependent upon DsrA as evidenced by an intermediate level of expression of this gene in the *dsrA*::*cat* mutant at 23 °C.

The transcription of *ymgB* was fully independent of RpoS and DsrA, identifying this gene as one whose increased expression at low temperature is fully mediated by an alternative thermoregulatory mechanism (Table 3). High-level expression of *ymgB* at 23 °C is retained in the *rpoS*::Tn*10* and *dsrA*::*cat* mutant strains, similar to or greater than levels expressed in the wild-type strain. It is currently unknown how *ymgB* expression is increased at low temperature. During cold shock, increased mRNA stability has been identified as a major factor in the increase of certain transcripts (reviewed by Gualerzi *et al.*, 2003) and it is possible that this may play a role during prolonged growth at 23 °C. Alternatively, other thermoregulatory factors, either protein or RNA in nature, may be critical in directly activating transcription of this gene.

Interestingly, *ymgB* is located within a region of the genome that contains six genes that are temperature-regulated based

Table 3. mRNA levels at 37 and 23 °C in wild-type, *rpoS*::Tn*10* and *dsrA*::*cat* strains

Gene expression levels were measured by qRT-PCR. For each given gene, the mean expression levels are shown in bold and are relative to levels measured at 37 °C in the wild-type strain DL1504. Numbers shown in parentheses indicate the SD based on the results from three independent experiments.

Gene	Wild-type		*rpoS*::Tn*10*		*dsrA*::*cat*	
	37 °C	23 °C	37 °C	23 °C	37 °C	23 °C
Biofilm						
adrA/*yaiC*	**1.0** (0.6–1.6)	**6.6** (2.5–17.6)	**1.5** (1.1–2.0)	**0.9** (0.8–1.1)	**0.8** (0.7–1.1)	**0.5** (0.4–0.6)
bolA	**1.0** (0.8–1.2)	**3.0** (2.3–3.9)	**0.7** (0.5–1.0)	**0.7** (0.6–0.8)	**0.7** (0.4–1.2)	**0.7** (0.5–1.2)
csgA	**1.0** (0.7–1.4)	**14.3** (10.7–19.1)	**0.6** (0.3–1.1)	**0.8** (0.6–1.1)	**0.5** (0.4–0.7)	**0.6** (0.4–1.2)
mlrA	**1.0** (0.8–1.3)	**5.9** (4.6–7.4)	**0.6** (0.5–0.6)	**0.4** (0.2–0.6)	**0.5** (0.4–0.8)	**0.7** (0.5–1.0)
nhaR	**1.0** (1.0–1.1)	**3.7** (1.9–7.1)	**1.2** (1.1–1.3)	**1.3** (1.1–1.5)	**0.7** (0.6–0.8)	**0.7** (0.4–1.3)
Cold shock						
dps	**1.0** (0.9–1.1)	**4.0** (3.6–4.4)	**0.5** (0.4–0.7)	**0.8** (0.6–1.1)	**0.7** (0.6–0.9)	**2.2** (1.7–2.9)
otsA	**1.0** (0.8–1.2)	**5.1** (4.6–5.6)	**0.4** (0.3–0.5)	**0.1** (0.1–0.2)	**0.5** (0.3–0.8)	**0.5** (0.2–0.9)
yceP/*bssS*	**1.0** (0.9–1.1)	**5.8** (4.2–8.1)	**1.5** (1.1–2.2)	**0.9** (1.2–5.2)	**0.8** (0.3–1.8)	**0.8** (0.2–3.1))
ycgZ	**1.0** (0.6–1.7)	**20.4** (10.2–41.0)	**1.7** (1.5–2.0)	**10.9** (8.6–13.8)	**0.9** (0.6–1.4)	**25.6** (22.2–29.5)
Unknown						
ymdA	**1.0** (0.3–3.9)	**32.3** (31.8–32.9)	**1.6** (0.7–3.8)	**1.8** (1.2–2.6)	**2.9** (0.5–6.3)	**1.1** (0.5–2.2)
ymgB	**1.0** (0.9–1.1)	**20.7** (16.7–25.7)	**2.5** (1.6–3.9)	**17.3** (14.6–20.5)	**1.1** (0.7–1.5)	**46.7** (34.2–63.6)
yhiM	**1.0** (0.8–1.3)	**13.6** (6.1–30.4)	**1.1** (1.0–1.3)	**0.6** (0.5–0.8)	**0.7** (0.5–0.9)	**0.5** (0.4–0.7)

on our results and two of the genes, *ycgZ* and *ycgF*, are expressed in cold-shocked cells (Polissi *et al.*, 2003). *ycgZ-ymgA-ymgB-ymgC* are encoded on one strand on the genome, whereas *ycgF-ycgE* are located divergently on the opposite strand. Their localization suggests the possibility that these two sets of genes may be organized in operons. However, combining our studies and those of others (Weber *et al.*, 2005), transcription of *ycgZ* and *ymgA* are impacted by RpoS whereas *ymgB* is fully independent of RpoS, arguing that these genes are driven by different promoters. Further studies are required to determine the transcriptional units for these genes, their function and relevance for growth at 23 °C.

The increased expression at 23 °C of *ymgB*, *adrA* and *otsA* is modulated by growth medium

To determine if the thermoregulatory response was retained in other growth media for both RpoS-dependent (*otsA*, *adrA*) and RpoS-independent genes (*ymgB*), the wild-type strain DL1504 was grown in either M9 glucose or LB at both 37 and 23 °C (Fig. 1). For all three genes, there is higher expression at 23 than at 37 °C in M9 glucose, indicating that a temperature differential is retained and identifying temperature as an important cue in regulating the expression of these genes. Glucose as a carbon source did not greatly alter transcription of any of the genes at 37 °C, but had differential effects on mRNA levels at 23 °C. *otsA* expression is increased 2.6-fold, whereas *ymgB* and *adrA* expression at 23 °C is decreased 1.4- and 3.5-fold, respectively, in M9 glucose compared to M9 glycerol. Thus, the temperature differential between expression at 37 and 23 °C in M9 glucose expands (*otsA*) or contracts (*ymgB*, *adrA*) for these genes compared to growth in M9 glycerol.

Growth in LB was stimulatory for *ymgB* transcription at both 37 and 23 °C compared to growth in M9 glycerol and a high temperature differential is retained, with levels 28.4-fold higher at 23 than at 37 °C (Fig. 1). In contrast, for *adrA* and *otsA*, growth in LB decreased expression for both genes at 23 °C such that differential expression based on temperature is either decreased (*otsA*) or abrogated (*adrA*). Overall, temperature serves as an important cue in regulating gene expression in various media and is a dominant cue for regulating *ymgB* expression, whereas *otsA* and *adrA* clearly demonstrate how *E. coli* can integrate nutritional cues that modify the thermoregulatory response.

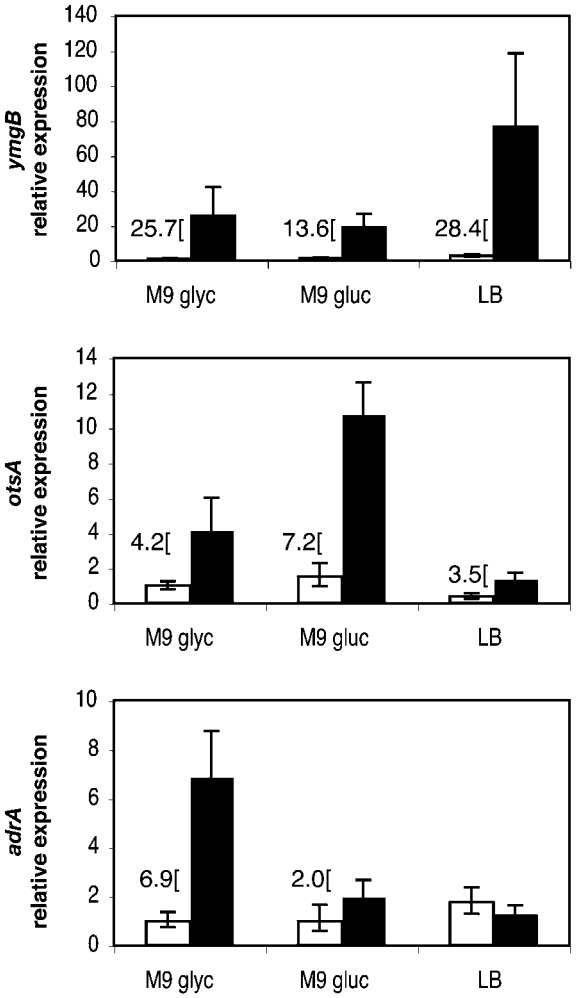

Fig. 1. The effect of growth medium on the thermoregulation of gene expression for *ymgB*, *otsA* and *adrA* at 23 (black bars) and 37 °C (white bars). Bars indicate relative levels of expression measured in the wild-type strain DL1504 in M9 glycerol medium (M9 glyc), M9 glucose medium (M9 gluc) and Luria–Bertani medium (LB). Relative levels of expression were measured by qRT-PCR and are shown in comparison to the wild-type strain grown in M9 glycerol at 37 °C. Bar height represents the mean level of expression based on three independent experiments. Errors are expressed as ±1 SD from the mean. A bracket indicates a statistically significant difference in expression levels based on temperature within the given condition and is accompanied by the ratio of expression (23/37 °C).

A shift to low temperature rapidly increases mRNA levels of temperature-regulated genes

To test how quickly cells respond to low temperature, an exponentially growing 37 °C culture was shifted to 23 °C and gene expression was measured at times after the shift. For the RpoS-dependent gene *otsA*, mRNA levels increased 7.7-fold by 1 h and peaked at 2 h after the shift with levels 21.2-fold higher than the initial amount at 37 °C (Fig. 2). Levels of *otsA* mRNA subsequently decreased by 5 h and reached levels at 11 h that approximate the steady-state levels measured by qRT-PCR (Table 3). For the RpoS-independent gene *ymgB*, mRNA levels showed a similar rapid increase in response to the shift in low temperature, peaking at 2 h with levels 86.9-fold greater than the initial 37 °C culture, but subsequently decreasing by 11 h to levels approximately twofold greater than that measured at

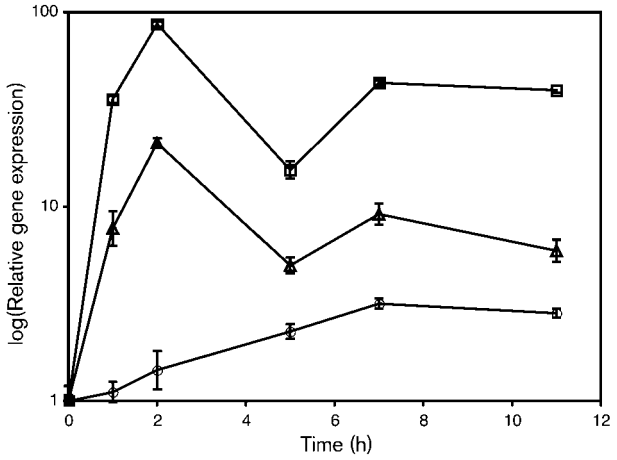

Fig. 2. The effect of a temperature shift from 37 to 23 °C on *otsA* (△), *ymgB* (□) and *adrA* (○) gene expression in the wild-type strain DL1504 in M9 glycerol medium. Relative levels of expression were measured by qRT-PCR and are shown relative to the amount in the starting 37 °C culture at $t=0$ grown in M9 glycerol medium. A representative experiment is shown. Errors are expressed as ±1 SD from the mean based on three measurements at each time point.

steady state (Table 3). For both these genes, there is a relatively rapid increase in mRNA levels, followed by a dramatic drop at 5 h before expression levels stabilize at the later time points, suggesting that these early time points represent the time period during which transcription is being fine-tuned to bring target gene expression to steady-state levels.

For *adrA*/*yaiC*, a more gradual increase in gene expression was evident, peaking at 7 h after the shift to 23 °C with levels 3.8-fold higher than the initial culture. Expression subsequently decreased by 11 h to levels 2.8-fold greater than the starting 37 °C culture (Fig. 1). The delayed timing of peak mRNA levels for *adrA*/*yaiC* may represent that it is an indirect, rather than direct target of RpoS. *adrA*/*yaiC* expression is stimulated by the transcriptional regulator CsgD (Brombacher *et al.*, 2003) such that the delay may be attributed to time needed for production of this regulator whose expression itself is RpoS-dependent at 23 °C (Brown *et al.*, 2001; Olsen *et al.*, 1993b). Overall, these data signify that bacteria can quickly respond to changing temperature by regulating gene expression through both RpoS-dependent and RpoS-independent thermoregulatory mechanisms.

The *rpoS*::Tn*10* and *dsrA*::*cat* mutations do not greatly alter growth at 23 °C or viability at 4 °C

Given the large number of RpoS-dependent genes expressed at 23 °C, particularly those related to cold shock, we hypothesized that *rpoS*::Tn*10* and *dsrA*::*cat* mutant strains might be impaired for growth at 23 °C or long-term viability at 4 °C in comparison to the wild-type strain. To assess growth at 23 °C, a 37 °C culture in early exponential phase was used to initiate two cultures (37 and 23 °C) in M9 minimal glycerol medium and optical density readings were taken at time points thereafter. At 37 °C, the growth rates were 2.27 ± 0.17, 1.74 ± 0.10 and 2.04 ± 0.03 h per generation, respectively, in the wild-type, *rpoS*::Tn*10* and *dsrA*::*cat* strains, whereas at 23 °C the growth rates were 5.12 ± 0.11, 5.00 ± 0.37 and 4.85 ± 0.07 h per generation, respectively. While the growth rates were significantly slowed at 23 °C compared to 37 °C for all strains, the generation times did not greatly change between the mutants and the wild-type strain at either temperature.

Similarly, the viability of the wild-type, *rpoS*::Tn*10* and *dsrA*::*cat* strains upon a shift to 4 °C did not differ greatly. Cells were grown to exponential phase, plated on M9 minimal agar containing glycerol, and incubated for varying times at 4 °C before transfer for outgrowth at 23 °C. All the strains behaved similarly with little loss in viability for up to 4 days incubation at 4 °C (data not shown). However, fewer than 10 % of the cells from all strains were viable after 6 days of incubation at 4 °C. These results indicate that the *rpoS*::Tn*10* and *dsrA*::*cat* mutant strains are not impaired in their response to cold-shock conditions compared to the wild-type strain.

It is a bit perplexing that an RpoS response is induced in response to growth at low temperature to direct the expression of 122 genes, yet cells deficient in RpoS or DsrA do not appear to be significantly impacted with regard to low-temperature growth or viability at 4 °C. Unlike other stresses, the physiological role of RpoS in responding to non-optimal temperatures has not been fully elaborated. High temperature growth increases the expression of RpoS, primarily through decreased turnover (Muffler *et al.*, 1997), and there is decreased viability of *rpoS* mutant strains at elevated temperatures (48–55 °C) in *E. coli* K-12 (Berney *et al.*, 2006; Lange & Hengge-Aronis, 1991) and O157:H7 (Cheville *et al.*, 1996). Except for a mention by Sledjeski and colleagues of similar results to our own that *rpoS* and *dsrA* mutants did not impact growth rate (Sledjeski *et al.*, 1996), we have not found any experimental evidence in *E. coli* that a loss of *rpoS* decreases cell viability at low temperature. It is possible that under our *in vitro* conditions, where nutrients and moisture are present during incubation at 23 or 4 °C, the effect of their loss is not extremely detrimental, but it might be under other conditions. Indeed, in *S. enterica* serovar Typhimurium, *rpoS* mutant cells suspended in 0.85 or 6 % NaCl and incubated at 4.5 °C demonstrated decreased survival in comparison to a wild-type strain with the difference in survival between strains most pronounced after 10 days incubation (McMeechan *et al.*, 2007). Thus, a combination of other stress (e.g. nutrient starvation or hyperosmolarity) with low temperature may make RpoS more important for survival. However, the relevance of temperature alone to the decreased viability is a bit difficult to evaluate as decreased cell survival was also observed at 37 °C for the *rpoS* mutant strain.

An alternative hypothesis exists that may explain the lack of any difference in phenotype between the wild-type, *rpoS* and *dsrA* mutant strains at low temperature. Gene expression results presented here indicate there is at least one alternate thermoregulatory mechanism, independent of RpoS, that is responsible for increasing gene expression at 23 °C (e.g *ycgZ* and *ymgB*). While we cannot say if this represents single or multiple pathways, it could be that those genes critical for low-temperature growth utilize such a pathway, either alone or in addition to the RpoS pathway, that ensures their expression under changing temperature conditions. Such a system appears to exist in the thermal adaptation of *E. coli* shifted first to 42 °C prior to elevation to temperatures >50 °C (Lange & Hengge-Aronis, 1991). These cells do not demonstrate thermal sensitivity, even in the absence of RpoS, indicating there are alternative methods for adjusting to non-optimal higher temperatures (Lange & Hengge-Aronis, 1991). Also, the tolerance for a high level of *rpoS* mutants within *E. coli* populations (Ferenci, 2003) indicates that survival at low temperature is probably not mediated purely by the general stress response.

Low temperature increases biofilm formation and *rpoS*/*dsrA* mutations decrease biofilm formation at 23 °C

Overall, our microarray studies revealed that several biofilm-associated genes are expressed more highly at low temperature (Table 2) and our results show they are dependent upon RpoS and DsrA for transcription at 23 °C (Table 3). Thus, temperature, RpoS and DsrA were tested to determine their effect on biofilm development in M9 glycerol medium. We assessed biofilm formation at times where the planktonic growth in the well was similar at both temperatures such that any effect on biofilm formation could be primarily attributed to temperature and not to differences in growth. At the earlier time point (37 °C=7 h, 23 °C=10 h), planktonic growth was similar (Fig. 3a) and biofilm development was equivalent at both temperatures and in all strains, indicating that none of these factors (temperature, RpoS or DsrA) impacts biofilm formation at this point (Fig. 3b). However, at the later time point (37 °C=22.4 h, 23 °C=27 h), biofilm formation was a statistically significant 1.5-fold higher at 23 °C compared to 37 °C, indicating that low temperature favours biofilm formation. Biofilm formation in the *rpoS*::Tn*10* and *dsrA*::*cat* mutants showed a statistically significant reduction, in which biofilm levels were only 62 and 65 %, respectively, of those observed in the wild-type strain. Since growth of the wild-type strain and *rpoS*::Tn*10* mutant strain at 23 °C was identical at the later time point, these data indicate that RpoS is needed to attain maximal biofilm formation at 23 °C. In the *dsrA*::*cat* mutant, planktonic growth was slightly slower than for the wild-type and *rpoS*::Tn*10* strains and this may have contributed to decreased biofilm formation in this mutant. However, the requirement of RpoS for maximal biofilm formation at 23 °C and our data demonstrating that increased transcription of the biofilm-associated genes we tested at 23 °C is RpoS-dependent (Table 3) would suggest that the loss of DsrA may directly impact biofilm formation due to decreased levels of RpoS. In contrast, at 37 °C at the later time point, the *rpoS*::Tn*10* and *dsrA*::*cat* mutants were similarly efficient at biofilm formation and both showed statistically significant increased biofilm formation (1.2- and 1.5-fold, respectively) in comparison to the wild-type strain at 37 °C (Fig. 3b). Thus, from our experiments, both the *rpoS*::Tn*10* and *dsrA*::*cat* mutations are deleterious to

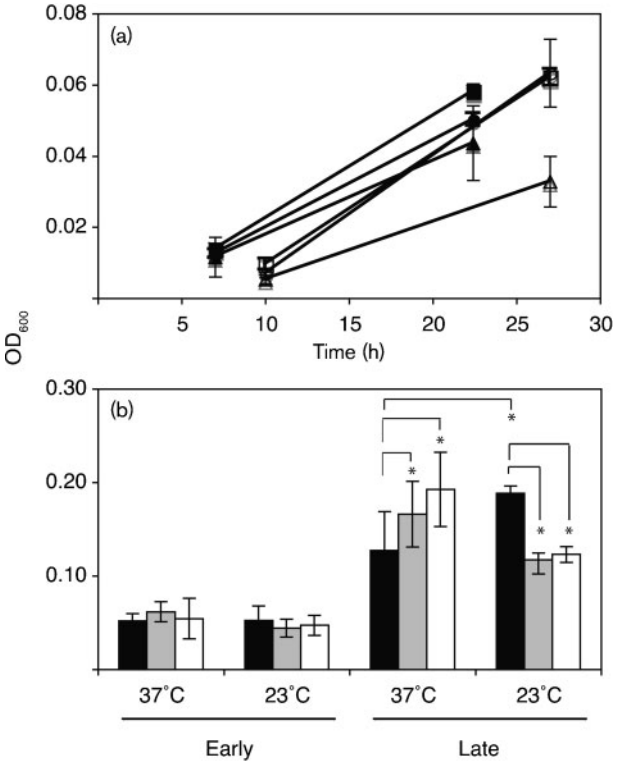

Fig. 3. Planktonic growth and biofilm formation at 37 (filled symbols) and 23 °C (open symbols) in wild-type (DL1504; squares), *rpoS*::Tn*10* (DL3106; circles) and *dsrA*::*cat* (CWZ458; triangles) strains. (a) Data points indicate planktonic growth measured as OD_{600} of a 100 µl sample removed at the times biofilm formation was measured (37 °C=7 and 22.4 h, 23 °C=10 and 27 h). Each data point represents the mean optical density from at least three independent experiments. Errors are expressed as ±1 SD from the mean. (b) Bars indicate biofilm formation as OD_{600} measured after crystal violet staining. The early time point displays biofilm formation measured for 37 °C cultures at 7 h and for 23 °C cultures at 10 h. The late time point displays biofilm formation measured for 37 °C cultures at 22.4 h and for 23 °C cultures at 27 h. Bar height represents the mean level of biofilm formation based on at least three independent experiments. Errors are expressed as ±1 SD from the mean. Statistically significant differences are indicated by an asterisk. Black bars, wild-type; grey bars, *rpoS*::Tn*10*; white bars, *dsrA*::*cat*.

biofilm formation at 23 °C in the later stages of development, but are advantageous at 37 °C.

These results could explain the conflicting evidence in the literature about the role of RpoS in *E. coli* biofilm formation. Our results are similar to a earlier report that an *rpoS* deletion leads to increased biofilm formation at 37 °C (Corona-Izquierdo & Membrillo-Hernandez, 2002). In contrast, data from Adams & McLean (1999) suggest that an *rpoS* deletion is deleterious to biofilm formation. These authors do not state at what temperature they conducted their studies, but if they were performed at low temperature their results would be in concordance with ours, indicating that an *rpoS* mutant has a deleterious effect on biofilm formation at 23 °C.

Conclusion

Overall, the studies here demonstrate that temperature has a dramatic effect on gene expression, signifying that adaptation to low temperature requires a co-ordinated, multifunctional response. These studies implicate RpoS and DsrA in the co-ordinated expression of a large subset of genes at 23 °C and are linked to expression of both cold-shock and biofilm development genes at 23 °C. However, there are clearly an even greater number of genes with increased expression at 23 °C for which the thermoregulatory mechanism is unknown. Evidence provided in this study of an additional thermoregulatory mechanism(s) responsible for increased gene expression at 23 °C raises the possibility that independent mechanisms exist to ensure the proper adaptation to low-temperature growth that is important for the bacterium as it encounters changing environmental conditions.

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