Global transcriptional analysis of the stringent response in *Enterococcus faecalis*

Anthony O. Gaca, Jacqueline Abranches, Jessica K. Kajfasz and José A. Lemos

Correspondence

José A. Lemos

jose_lemos@urmc.rochester.edu

Center for Oral Biology and Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA

In *Enterococcus faecalis*, production of guanosine tetraphosphate/guanosine pentaphosphate [(p)ppGpp], the effector molecule of the stringent response, is controlled by the bifunctional synthetase/hydrolase RelA and the monofunctional synthetase RelQ. Previously, the (p)ppGpp profiles of strains lacking *relA*, *relQ* or both genes indicated that RelA is the primary enzyme responsible for (p)ppGpp synthesis under stress conditions, while the contributions of RelQ to the stringent response and cell homeostasis remained elusive. Here, survival within the mouse-derived macrophage cell line J774A.1 and killing of *Galleria mellonella* supported initial evidence that virulence was attenuated in the (p)ppGpp⁰ $\Delta relA\Delta relQ$ strain but not in the $\Delta relA$ or $\Delta relQ$ strains. We performed, for the first time to our knowledge, global transcriptome analysis in a documented (p)ppGpp⁰ Gram-positive bacterium and provided the first insights into the role of a Gram-positive monofunctional (p)ppGpp synthetase in transcriptional regulation. Transcription profiling after mupirocin treatment confirmed that RelA is the major enzyme responsible for the (p)ppGpp-mediated transcriptional repression of genes associated with macromolecular biosynthesis, but also revealed that RelQ is required for full and timely stringent response induction. The delayed transcriptional response of $\Delta relQ$ could not be correlated with reduced or slower production of (p)ppGpp, in part because RelA-dependent (p)ppGpp accumulation occurred very rapidly. Comparisons of the transcriptional responses of $\Delta relA$ or $\Delta relA\Delta relQ$ strains with the parent strain under starvation conditions revealed upregulation of operons involved in energy metabolism in the (p)ppGpp⁰ strain. Thus, while $\Delta relA$ and $\Delta relA\Delta relQ$ cannot use (p)ppGpp to sense and respond to stresses, fitness of $\Delta relA\Delta relQ$ may be further impaired due to an unbalanced metabolism.

Received 24 April 2012

Revised 23 May 2012

Accepted 25 May 2012

INTRODUCTION

While often considered a second-rate pathogen, the Gram-positive bacterium *Enterococcus faecalis* ranks among the leading causes of complicated nosocomial infections (Arias & Murray, 2012; Boucher *et al.*, 2009). A major trait associated with the virulence of this organism is its ability to survive under conditions inhospitable to most bacteria. For example, *Ent. faecalis* is inherently resistant to commonly used antibiotics and is also known for its capacity to rapidly acquire and disseminate determinants of antimicrobial

resistance (Arias & Murray, 2012). In addition to antibiotic tolerance, *Ent. faecalis* is a hardy and versatile organism able to grow at a wide range of temperatures (10 to >45 °C) and pHs (4.6–9.6), at high salt concentrations, and at high concentrations of detergents, ethanol and reactive oxygen species, and it is able to survive for prolonged periods in the complete absence of nutrients (Hartke *et al.*, 1998; Murray, 1990).

The stringent response is a global stress response mechanism controlling broad metabolic alterations necessary for bacterial adaptation to adverse conditions (Potrykus & Cashel, 2008). This response is mediated by the accumulation of two modified guanine nucleotides, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp. In *Escherichia coli*, (p)ppGpp is synthesized by the strong (p)ppGpp synthetase RelA and the bifunctional SpoT, which exerts weak synthetase and strong hydrolase activities (Potrykus & Cashel, 2008). In Gram-positive bacteria, the bifunctional RelA (also known as Rsh for Rel Spo homologue) is a strong (p)ppGpp synthetase, like the *E. coli* RelA, that also has (p)ppGpp-hydrolase activity

Abbreviations: BCAA, branched-chain amino acid; PFGRC, Pathogen Functional Genomics Resource Center; ppGpp, guanosine tetraphosphate; pppGpp, guanosine pentaphosphate; qRT-PCR, quantitative RT-PCR.

The microarray data discussed in this paper are available from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE34561.

Four supplementary tables are available with the online version of this paper.

characteristic of SpoT (Mechold *et al.*, 1996, 2002). In addition to RelA, the genomes of Gram-positive bacteria encode one or two monofunctional (p)ppGpp synthetases (Abranches *et al.*, 2009; Battesti & Bouveret, 2009; Lemos *et al.*, 2007; Nanamiya *et al.*, 2008; Yan *et al.*, 2009). Unlike the bifunctional RelA, the contributions of these Gram-positive monofunctional enzymes to cell homeostasis remain poorly understood.

The accumulation of (p)ppGpp induces large-scale transcriptional alterations that shift cellular resources toward adaptation to a non-growth state. In *E. coli*, the classic transcriptional response to (p)ppGpp accumulation is a strong repression of stable RNAs, such as rRNA, along with genes encoding proteins that belong to the transcriptional and DNA replication machineries (Cashel *et al.*, 1996; Durfee *et al.*, 2008; Traxler *et al.*, 2008). Concurrently, there is increased transcription of genes necessary for adaptation to unfavourable growth conditions, including amino acid biosynthesis, alternate carbon transport and utilization, alternate sigma factors and general stress response pathways (Durfee *et al.*, 2008; Traxler *et al.*, 2008). In the Gram-positive model organism *Bacillus subtilis*, a similar pattern of (p)ppGpp-dependent control has been observed at both the transcriptional and translational levels (Eymann *et al.*, 2002).

Previous studies identified and characterized the two enzymes responsible for the production of (p)ppGpp in *Ent. faecalis*, the bifunctional RelA and the monofunctional synthetase RelQ (Abranches *et al.*, 2009). The (p)ppGpp profile of $\Delta relA$, $\Delta relQ$ and double $\Delta relA\Delta relQ$ strains revealed that the rapid accumulation of (p)ppGpp during stress conditions was entirely dependent on the RelA enzyme. Physiological characterization of the Δrel strains linked elevated background (p)ppGpp levels during unstressed growth, characteristic of $\Delta relA$, to enhanced tolerance toward antibiotics that inhibit cell wall synthesis and to a reduced ability to grow under several physiologically relevant stress conditions (Abranches *et al.*, 2009). Around the same time, Yan and colleagues showed that complete or partial deletion of the *relA* gene in *Ent. faecalis* V583 resulted in multiple stress-sensitive phenotypes (Yan *et al.*, 2009). Complete lack of (p)ppGpp, as seen in a double $\Delta relA\Delta relQ$ strain, alleviated most of the stress-related phenotypes of $\Delta relA$ but led to overall decreased antibiotic tolerance and attenuated virulence in the *Caenorhabditis elegans* model (Abranches *et al.*, 2009).

To assess the scope of (p)ppGpp regulation in *Ent. faecalis*, we used microarrays to analyse the global responses of the wild-type strain OG1RF and its respective Δrel mutants ($\Delta relA$, $\Delta relQ$ and $\Delta relA\Delta relQ$) to mupirocin. To our knowledge, this is the first time gene expression profiling has been performed on a documented (p)ppGpp⁰ Gram-positive bacterium.

METHODS

Bacterial strains and growth conditions. *Ent. faecalis* OG1RF and its derivatives JAL1 ($\Delta relA$), JAL2 ($\Delta relQ$) and JAL3 ($\Delta relA\Delta relQ$)

strains have been previously described (Abranches *et al.*, 2009). For macrophage survival and killing of *Galleria mellonella* experiments, cells were grown overnight in brain heart infusion (BHI) medium. For microarray analysis, cells were grown at 37 °C in the chemically defined medium FMC containing 10 mM glucose (FMCG). The FMC medium was originally developed to support the growth of oral streptococci and contains all amino acids, eight different vitamins, three nucleobases (adenine, guanine and uracil) and salts (Terleckyj *et al.*, 1975). All strains were allowed to grow in FMCG to OD₆₀₀ 0.3 and the cultures divided into two aliquots. To one aliquot, 50 µg mupirocin ml⁻¹ was added and the cells were incubated at 37 °C for 15 and 30 min (mupirocin-treated cells), while the other aliquot was collected by centrifugation and immediately frozen (control cells).

Ent. faecalis-macrophage co-culture. The murine macrophage cell line J774.A1 (ATCC) was grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with GlutaMAX, 10% fetal bovine serum, 50 U penicillin G ml⁻¹ and 50 µg streptomycin ml⁻¹ at 37 °C under a 5% CO₂ atmosphere. The J774.A1 cells were seeded onto 24-well tissue culture plates at a density of 5 × 10⁵ macrophages per well 24 h prior to use. Overnight cultures of *Ent. faecalis* OG1RF and Δrel strains were resuspended in Hank's Buffered Salt Solution (HBSS) and diluted to 5 × 10⁷ c.f.u. ml⁻¹ in supplemented DMEM lacking antibiotics. To the macrophage monolayers, 1 ml diluted culture was added, yielding an approximate m.o.i. of 100:1. Bacteria-to-macrophage contact was induced by centrifugation at 500 g for 5 min followed by further incubation for 25 min. Extracellular bacteria were killed by the addition of 300 µg gentamicin ml⁻¹ and 50 µg penicillin G ml⁻¹ followed by a 1.5 h incubation. Macrophage cells were lysed in deionized water, serially diluted and plated on trypticase soy agar (TSA) for enumeration of viable internalized bacterial cells.

G. mellonella infection. For the *G. mellonella* killing assays, insects in the final larval stage were purchased from Vanderhorst. Groups of 20 larvae, ranging from 200 to 300 mg in weight, were randomly chosen and used for subsequent infection. A Hamilton syringe was used to inject 5 µl aliquots of bacterial inoculum (5 × 10⁵ c.f.u.) into the haemocoel of each larva via the last left proleg. Groups injected with heat-inactivated *Ent. faecalis* (20 min at 75 °C) were used as a control. After injection, larvae were kept at 37 °C, and survival was recorded at selected intervals. Kaplan-Meier killing curves were plotted and estimation of differences in survival compared by using the Mantel-Cox log-rank test. All data were analysed with GraphPad Prism 4.0 software. Experiments were performed independently three times with similar results.

RNA extraction. To isolate RNA from *Ent. faecalis*, cells were harvested by centrifugation at 4 °C and then treated with RNeasy Protect reagent (Qiagen). Total RNA was isolated from homogenized *Ent. faecalis* cells by the hot acid-phenol method as described previously (Abranches *et al.*, 2006). RNA pellets were resuspended in nuclease-free H₂O and treated with DNase I (Ambion) at 37 °C for 30 min, and RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The RNA was purified again using the RNeasy Mini kit (Qiagen), including a second on-column DNase treatment that was performed as recommended by the supplier.

Microarray experiments. Transcriptome analysis was performed using the *Ent. faecalis* microarrays provided by the J. Craig Venter Institute Pathogen Functional Genomics Resource Center (PFGR) (<http://pfgrc.jvri.org/index.php/microarray>). The microarray slides consist of 6600 70-mer DNA probes encompassing all unique ORFs from seven sequenced *Ent. faecalis* strains, OG1RF, ATCC 29200, TUSoD Ef11, HH22, TX0104, TX1322 and V583, including its three

plasmids, printed in duplicate. Additional details regarding the arrays can be found at http://pfgrc.jcvi.org/index.php/microarray/array_description/enterococcus_faecalis/version1.html. Given that the *Ent. faecalis* V583 genome annotation (Paulsen *et al.*, 2003) has been widely used in the literature, we adopted the V583 gene designation throughout the text and tables. However, differentially expressed genes present in the OG1RF genome but absent in V583 are presented with the original OG1RF designation (Bourgogne *et al.*, 2008).

A reference RNA prepared from a large-scale culture of *Ent. faecalis* OG1RF cells that had been grown in BHI broth to OD₆₀₀ 0.5 was used in every experiment. A description of the advantages of using a reference RNA in microarray studies is presented elsewhere (Shi *et al.*, 2006). Four individual cDNA samples originating from four independent cultures of each strain grown under control (FMCG) and experimental conditions (FMCG containing mupirocin) were hybridized to the arrays along with reference cDNA. Test cDNA was coupled with Cy3-dUTP, while reference cDNA was coupled with Cy5-dUTP (Amersham Biosciences). The slides were hybridized to a mixture containing equal amounts of test and reference cDNA for 16 h at 42 °C in a MicroArray User Interface (MAUI) hybridization chamber (BioMicro Systems). Hybridized slides were washed and scanned using a GenePix scanner (Axon Instruments). Data were analysed using software available at the J. Craig Venter Institute PFGR website. Single-channel images of the slides were loaded into Spotfinder and overlaid. MIDAS software was used to normalize the spot intensity data using locally weighted scatterplot smoothing (LOWESS) and iterative log mean centring with default setting,

followed by in-slide replicate analysis. Statistical analysis was carried out with Biometric Research Branch (BRB) array tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) with a cut-off *P* value of 0.001. Additional details regarding array protocols are available at <http://pfgrc.jcvi.org/index.php/microarray/protocols.html>.

Real-time quantitative PCR. A subset of genes (Table 1) was selected to validate the microarray analysis by real-time quantitative RT-PCR (qRT-PCR). Reverse transcription and real-time RT-PCR were carried out according to protocols described previously (Abranches *et al.*, 2006). Student's *t* test was performed to verify the significance of the real-time PCR quantifications.

Detection of (p)ppGpp accumulation patterns. For (p)ppGpp measurements, cells were grown in FMCG as previously described (Abranches *et al.*, 2009). Exponentially grown cultures (OD₆₀₀ ~0.15) were pre-labelled with 50 µCi (1.85 MBq) carrier-free [³²P]orthophosphate (Amersham Biosciences) for one generation, followed by the addition of different concentrations of mupirocin (ranging from 0.05 to 50 µg ml⁻¹) to experimental aliquots. The control sample consisted of an aliquot that was not treated with mupirocin, but was labelled with ³²P for the duration of the experiment. Nucleotide pools were extracted by adding an equal volume of 13 M formic acid, followed by two freeze-thaw cycles. Acid extracts were centrifuged briefly, and the supernatant fluids were spotted onto polyethyleneimine-cellulose plates (Selecto Scientific) for separation by TLC in 1.5 M KH₂PO₄ (pH 3.4).

Table 1. qRT-PCR primers used to validate the microarray results

Primer	Sequence (5'→3')	Locus	Gene symbol
Pr013	ATCGCATTACAGATGATATTC	EF1741	<i>ccpA</i>
Pr014	ATATTAACGCTACCGACTT	EF1741	<i>ccpA</i>
Pr017	CCACAACGCTTGATGAAT	EF2355	<i>clpB</i>
Pr018	TGTATCTTCTACTGTTGGTTCT	EF2355	<i>clpB</i>
Pr019	GCGTATATTATTAGCAGTTCA	EF1645	<i>codY</i>
Pr020	TATTGGCTTCGGTTACTT	EF1645	<i>codY</i>
Pr021	TTGATGCCAGTGATGCTA	EF2378	<i>polC</i>
Pr022	ATAATCCGTTAATTGTTCCCTCTG	EF1645	<i>polC</i>
Pr025	ATTGTTGATAATGAGTCTG	EF3057	
Pr026	AACTAACGGAGATAAGAA	EF3057	
Pr027	TTGGTGTCATTGGCTATC	EF2178	
Pr028	ACTATAAACTATCGGCATCC	EF2178	
Pr029	AAGATGGTGAACAATTAG	EF0914	<i>infC</i>
Pr030	TTCCATAATCCATAATTCG	EF0914	<i>infC</i>
Pr031	GGATTATCTCAGTTGTTT	EF1681	<i>msrA</i>
Pr032	CACTAATTGTTTCGTAAGA	EF1681	<i>msrA</i>
Pr037	GTTTCTCTTACTTACATTTATGGT	EF0231	<i>rpsM</i>
Pr038	TATCAATTTCGCACGAAT	EF0231	<i>rpsM</i>
Pr095	CGCTTCACAAGCCATTAACGCTCA	EF0440	<i>dtpT</i>
Pr096	ACAGCTACAAGTCCGACAATCCCA	EF0440	<i>dtpT</i>
Pr103	ATTAAACGCCGTGGTGCTGGAATC	EF1898	<i>rplS</i>
Pr104	AGCAACACGTGGTGTGTGTAATGG	EF1898	<i>rplS</i>
Pr105	GATGCAGCCATTTCAGCACGATGT	EF2899	<i>trxB</i>
Pr106	GCGCCGTTACCAATCGCAAAGATA	EF2899	<i>trxB</i>
Pr107	AAGTTCGGTCGCTACCAAGGATGT	EF3015	<i>ygjU</i>
Pr108	GCCACCGAATGAACACCAAACCT	EF3015	<i>ygjU</i>
Pr049	AATCGCCATCGTCGGTATTGTCT	EF1685	<i>hlyIII</i>
Pr050	CGCTGTTCTGTAAACCAAGAGA	EF1685	<i>hlyIII</i>

RESULTS

In vitro macrophage survival and killing of *G. mellonella* further confirm the attenuated virulence of the $\Delta relA\Delta relQ$ strain

Previously, we showed that the virulence of the double mutant $\Delta relA\Delta relQ$, but not $\Delta relA$ or $\Delta relQ$, was attenuated in the *C. elegans* model (Abranches *et al.*, 2009). However, *C. elegans* lacks cell-mediated immunity and requires suboptimal growth temperatures for modelling human infection. Here, we used an *in vitro* macrophage survival assay and the *G. mellonella* infection model to bolster our previous observation in two models that more closely resemble mammalian infections. In co-culture with the mouse-derived macrophage J774A.1 cell line, the wild-type OG1RF, $\Delta relA$ and $\Delta relQ$ strains showed minimal death during the course of the experiment (72 h), whereas the $\Delta relA\Delta relQ$ strain showed significant loss of cell viability compared with OG1RF ($P=0.042$) after the first 24 h (Fig. 1a). No differences were observed in the c.f.u. counts for each strain 2 h post-infection, showing that all strains were internalized equally well. Killing of the larvae of *G. mellonella* has been routinely used as a model to assess *Ent. faecalis* virulence (de Oliveira *et al.*, 2011; Lebreton *et al.*, 2009; Yan *et al.*, 2009; Zhao *et al.*, 2010). As previously seen using the *C. elegans* model (Abranches *et al.*, 2009), virulence of $\Delta relA\Delta relQ$, but not of the $\Delta relA$ and $\Delta relQ$ strains, was significantly ($P=0.0039$) attenuated in *G. mellonella* (Fig. 1b).

Mupirocin treatment triggers a classic stringent response in a (p)ppGpp-dependent manner

Microarrays were used to obtain the transcriptional profile of OG1RF and Δrel strains before and after mupirocin treatment. Although the $\Delta relA$ strain grew slightly slower

than the parent strain prior to mupirocin treatment, all strains showed modest but similar increases in OD₆₀₀ after the addition of mupirocin. As a result, all strains were at nearly identical growth phases at the 15 and 30 min sampling points (data not shown). Using a twofold cut-off at an assigned P value of ≤ 0.001 to verify changes in the transcriptome of mupirocin-treated cultures, a large number of genes were found to be differentially expressed at the two time points evaluated (see Table S1 available with the online version of this paper). In the wild-type strain, 206 and 581 genes were differentially expressed after 15 and 30 min of mupirocin treatment, respectively. Among the genes differentially transcribed in OG1RF at 15 min, 57.8% ($n=119$) were downregulated and 42.2% ($n=87$) were upregulated. Similar trends were observed at the 30 min time point, with 56.6% ($n=329$) downregulated and 43.4% ($n=252$) upregulated. The transcriptional profile of $\Delta relQ$ followed the same trends observed in the parental strain, albeit with fewer differentially expressed genes (75 genes at 15 min and 463 at 30 min). In the $\Delta relA$ and $\Delta relA\Delta relQ$ strains there were, respectively, 86 and 43 genes differentially expressed after 15 min of mupirocin exposure, and 159 and 129 after 30 min of mupirocin exposure. When compared with OG1RF and $\Delta relQ$, the reduced number of genes with altered expression in the RelA-negative strains, $\Delta relA$ and $\Delta relA\Delta relQ$, aligns well with the expected regulatory contribution of a RelA-mediated (p)ppGpp accumulation (Fig. 2). While there was a significant overlap in the number of genes with mutually altered expression in the wild-type and $\Delta relQ$ strains at 30 min (54.4% shared genes), the percentage of genes shared by these two strains was considerably smaller (17.9% shared genes) at the 15 min time point. This difference was largely due to the reduced number of genes with altered expression in $\Delta relQ$ at 15 min (75 genes) when compared with OG1RF (206 genes).

Fig. 1. The $\Delta relA\Delta relQ$ mutant shows reduced survival in macrophages and reduced virulence in *G. mellonella*. (a) Intracellular survival of OG1RF and its derivatives within murine macrophage cell line J774.A1 (m.o.i. 100:1). The curves shown are means $\pm$ sds of the results from two independent experiments ($n=6$). A Student's t test indicated that the differences observed between $\Delta relA\Delta relQ$ and the other three strains at 72 h were statistically significant ($P=0.042$). (b) Kaplan–Meier plots of larvae that received injections of *Ent. faecalis* OG1RF and its Δrel derivatives. The experiments were repeated three times, and the results are representative of a typical experiment. Compared with the wild-type strain, $\Delta relA\Delta relQ$ showed attenuated virulence ($P=0.0039$).

Fig. 2. Venn diagrams of the differentially expressed genes (upregulated in white type, downregulated in black type) of OG1RF and Δrel strains treated with mupirocin for 15 and 30 min compared with control (non-stressed) samples of each strain. (a) Comparisons of OG1RF and $\Delta relQ$ strains. (b) Comparisons of OG1RF, $\Delta relA$ and $\Delta relA\Delta relQ$ strains. All genes were subjected to a twofold differential expression cut-off at $P \leq 0.001$.

The analysis of the genes differentially expressed after mupirocin treatment in the two RelA-positive strains (OG1RF and $\Delta relQ$) showed a strong repression of genes associated with the translation apparatus, RNA synthesis and DNA replication, a hallmark of the stringent response (Fig. 3, Tables S2 and S3). Specifically, mupirocin treatment resulted in global repression of genes encoding several ribosomal proteins and translation factors in the OG1RF and $\Delta relQ$ strains; very few genes involved in macromolecular biosynthesis were repressed in the mupirocin treated $\Delta relA$ and $\Delta relA\Delta relQ$ strains (see Table S1). A closer analysis of the genes coding for ribosomal proteins was strongly suggestive of a delayed stringent response induction in the $\Delta relQ$ strain (Fig. 3). For example, at the 15 min time point, 18 genes encoding ribosomal proteins were repressed in OG1RF, whereas only three genes were repressed in $\Delta relQ$. At 30 min, the number of repressed ribosomal protein genes increased to 45 in the parent strain and 32 in $\Delta relQ$, suggesting that RelQ is necessary for a rapid and fully efficient repression of genes encoding ribosomal proteins. In addition to genes associated with the translational apparatus, expression of a number of genes involved in DNA replication (*dnaI*, *parE*, *polC*, *topA* and *topB-1*), RNA synthesis and modification (*nusA*, *rpoA*, *rnhB*, *trmB* and *trmE*), and menaquinone biosynthesis, were significantly repressed in OG1RF and $\Delta relQ$ strains. In all cases, a delayed response was observed in $\Delta relQ$ and a complete lack of stringent control was observed in the $\Delta relA$ and $\Delta relA\Delta relQ$ strains.

Fig. 3. Heat map of genes involved in transcription, translation and DNA replication repressed by mupirocin treatment in OG1RF and $\Delta relQ$. Column titles indicate comparisons between mupirocin-treated cells (15 or 30 min) and control cells for each strain (e.g. OG1RF mupirocin 15 min versus OG1RF control). Colours represent fold repression: pink, $2 \leq x < 5$; magenta, $5 \leq x < 10$; red, ≥ 10 .

A subset of the genes ($n=14$) identified in the microarrays was validated by qRT-PCR, and the results were highly consistent with the expression trends observed in the microarrays (Table 2).

Time-course analysis of (p)ppGpp levels after mupirocin treatment reveals that the RelA-dependent accumulation of (p)ppGpp occurs in less than one minute

The delayed transcriptional repression of genes involved in macromolecular biosynthesis and replication in the $\Delta relQ$ strain during the initial 15 min of mupirocin treatment strongly suggested that the (p)ppGpp synthetase activity of RelQ is required for full and timely stringent response induction. In a previous study, we assessed (p)ppGpp accumulation in the parent OG1RF and Δrel strains in as little as 15 min after mupirocin addition and observed no obvious differences in (p)ppGpp levels between the parent and $\Delta relQ$ strains for up to 90 min post induction (Abranches *et al.*, 2009). Here, we asked whether the delayed stringent response of $\Delta relQ$ could be correlated with reduced (p)ppGpp production during the initial minutes of mupirocin challenge. Initially, we measured (p)ppGpp accumulation in OG1RF and $\Delta relQ$ strains every minute during the first 5 min after mupirocin addition

Table 2. qRT-PCR validation of microarray data

Locus	Gene symbol	Comparison	Microarray*	qRT-PCR*	P value
EF1741	<i>ccpA</i>	OG1 M30' vs OG1	0.33	0.113	$P \leq 0.001$
EF2355	<i>clpB</i>	OG1 M30' vs OG1	5.16	2.68	$P \leq 0.01$
EF1645	<i>codY</i>	OG1 M30' vs OG1	0.25	0.22	$P \leq 0.001$
EF1645	<i>codY</i>	relQ M30' vs relQ	0.3	0.22	$P \leq 0.001$
EF2378	<i>polC</i>	OG1 M30' vs OG1	0.19	0.46	$P \leq 0.001$
EF2378	<i>polC</i>	relQ M30' vs relQ	0.33	0.18	$P \leq 0.001$
EF3057	Hypothetical	OG1 M30' vs OG1	8.36	11.59	$P \leq 0.001$
EF3057	Hypothetical	relQ M30' vs relQ	8.5	9.07	$P \leq 0.01$
EF2178	Hypothetical	OG1 M30' vs OG1	0.2	0.71	$P \geq 0.01$
EF2178	Hypothetical	relQ M30' vs relQ	0.31	0.3	$P \leq 0.001$
EF0914	<i>infC</i>	OG1 M30' vs OG1	0.08	0.12	$P \leq 0.001$
EF0914	<i>infC</i>	relQ M30' vs relQ	0.11	0.08	$P \leq 0.001$
EF1681	<i>msrA</i>	OG1 M30' vs OG1	5.12	4.91	$P \leq 0.01$
EF0231	<i>rpsM</i>	OG1 M30' vs OG1	0.15	0.63	$P \geq 0.01$
EF0231	<i>rpsM</i>	relQ M30' vs relQ	0.17	0.07	$P \leq 0.01$
EF0440	<i>dtpT</i>	OG1 M15' vs OG1	0.09	0.08	$P \leq 0.001$
EF1898	<i>rplS</i>	OG1 M15' vs OG1	0.13	0.05	$P \leq 0.001$
EF2899	<i>trxB</i>	OG1 M15' vs OG1	0.12	0.09	$P \leq 0.001$
EF3015	<i>ygjU</i>	OG1 M15' vs OG1	4.44	0.09	$P \leq 0.01$
EF1685	<i>hlyIII</i>	OG1 M15' vs OG1	4.69	10.1	$P \leq 0.001$

*Fold changes result from comparison of mupirocin-treated with untreated samples. All microarray genes listed were subjected to a twofold differential expression cut-off at $P \leq 0.001$. qRT-PCR values represent the mean fold change derived from at least three independent replicates assayed in triplicate. Statistical significance ($P \leq 0.001$) of qRT-PCR data was determined using Student's *t* test.

(50 $\mu\text{g ml}^{-1}$). We were also able to consistently assess (p)ppGpp accumulation in cells exposed to mupirocin for as little as 30 s, but shorter incubation periods were not feasible due to the minimum required time for sample processing. Remarkably, we found that (p)ppGpp production was almost instantaneous in both strains, an indication that the RelA enzyme is primed for synthesis and that (p)ppGpp begins to accumulate in less than one minute (Fig. 4). We next tested reduced concentrations of mupirocin (ranging from 10 to as low as 0.05 $\mu\text{g ml}^{-1}$) in an attempt to slow down (p)ppGpp production. Despite being able to delay (p)ppGpp accumulation with a reduced dose of mupirocin (5 $\mu\text{g ml}^{-1}$), again we did not observe obvious differences in (p)ppGpp levels between OG1RF and ΔrelQ (data not shown). Therefore, by our methods of detection, the delayed transcriptional response of ΔrelQ could not be correlated with reduced or slower production of (p)ppGpp.

Expression of genes responsible for guanine nucleotide pools is affected by (p)ppGpp levels

Based on the (p)ppGpp profiles of OG1RF and Δrel mutants (Abranches *et al.*, 2009), two distinct types of (p)ppGpp accumulation pattern emerged following mupirocin treatment. The parent and ΔrelQ strains accumulated high levels of (p)ppGpp, a pattern that was completely absent in the ΔrelA and $\Delta\text{relA}\Delta\text{relQ}$ strains (Abranches *et al.*, 2009). Considering these two patterns, a small subset of genes was identified as showing opposite (p)ppGpp-dependent

responses (e.g. activated in OG1RF and ΔrelQ and repressed in ΔrelA and $\Delta\text{relA}\Delta\text{relQ}$, or vice-versa). Of particular interest, genes responsible for controlling guanine nucleotide pools, *guaD* and *guaC*, were repressed in OG1RF and ΔrelQ and induced in ΔrelA and $\Delta\text{relA}\Delta\text{relQ}$ after mupirocin

Fig. 4. Time-course accumulation of (p)ppGpp in *Ent. faecalis* after mupirocin treatment. Mid-exponential phase cultures were labelled with [^{32}P]orthophosphate and were treated with 50 $\mu\text{g mupirocin ml}^{-1}$ (Mup) or untreated (Control) for the time indicated. Nucleotide acid extracts were spotted onto polyethyleneimine-cellulose plates for TLC in 1.5 M KH_2PO_4 .

treatment. The opposing regulatory pattern of these genes in OG1RF and $\Delta relQ$ in comparison with $\Delta relA$ and $\Delta relA\Delta relQ$ is likely associated with differences in GTP/GDP pools that serve as a substrate for (p)ppGpp synthesis and therefore undergo a sharp RelA-mediated decrease during the stringent response.

Stringent response-defective mutants show unique gene expression profiles in response to mupirocin treatment

As we previously reported (Abranches *et al.*, 2009), $\Delta relA$ and $\Delta relA\Delta relQ$ mutants often show divergent phenotypes. To dissect the response of each strain to starvation, we carried out transcriptional comparisons between $\Delta relA$ or $\Delta relA\Delta relQ$ mutants and the parent strain following 30 min of mupirocin treatment. As expected, both *relA*-deficient mutants showed considerable overlap in their transcriptional profiles corresponding to an activation of genes normally repressed by the stringent response in $RelA^+$ strains (Table S1). However, $\Delta relA$ had 315 unique differentially expressed genes, while $\Delta relA\Delta relQ$ had 228 uniquely expressed genes when each mutant was compared with the correspondingly treated parent strain (Table S4). The $\Delta relA\Delta relQ$ (p)ppGpp⁰ mutant displayed a strong activation of three operons involved in energy metabolism, the pyruvate formate lyase complex (*pflA* and *pflB*), the citrate catabolism pathway (*citC*, *D*, *E*, *F*, *M* and *X*) and the serine dehydratase complex (*sdhA-1* and *sdhB-1*). Moreover, 16 genes encoding the sugar phosphotransferase system (PTS) were upregulated in $\Delta relA\Delta relQ$ (Table S4). The $\Delta relA$ single mutant showed an activation of genes responsible of the biosynthesis of both purines (*purD*, *F* and *N*) and, more so, pyrimidines (*pyrB*, *C*, *DB*, *DII*, *F*, *E* and *R*) (Table S4). This enhanced transcription of nucleotide synthesis genes likely stems from the altered guanine nucleotide pools, since $\Delta relA$ is unable to effectively regulate (p)ppGpp:GTP ratios as it lacks (p)ppGpp hydrolytic function, associated with RelA, but still retains the synthetic capacity of RelQ.

The (p)ppGpp-independent response to mupirocin

Genes that were differentially expressed upon mupirocin treatment in all four strains were considered to be under (p)ppGpp-independent control. As previously observed in *E. coli* and *B. subtilis* (Blumenthal *et al.*, 1976; Eymann *et al.*, 2002), five genes encoding aminoacyl-tRNA synthetases (aaRSs) responsible for charging the tRNA with a specific amino acid during protein synthesis were repressed in a (p)ppGpp-independent manner. The exception was the strong transcriptional activation of isoleucyl tRNA synthetase (*ileS*) in all strains, because mupirocin specifically inhibits charging of tRNA^{Ile}. The repression of aaRSs genes was expected, as these genes are regulated by the T box riboswitch regulatory mechanism (Green *et al.*, 2010), which operates independently of (p)ppGpp (Geiger *et al.*, 2010; Nascimento *et al.*, 2008; Reiss *et al.*, 2012). Interestingly, an operon encoding four glycolytic enzymes (*gap-2*, *pgk*, *tpiA*

and *eno*) was also repressed in a (p)ppGpp-independent manner.

Transport and stress-related genes are under positive (p)ppGpp control

Another hallmark of the stringent response is the induction of amino acid biosynthesis, transporters and stress survival genes (Potrykus & Cashel, 2008). By focusing on genes that appeared to be upregulated at one or more time points after mupirocin treatment in the wild-type or $\Delta relQ$ strains, but not in the $\Delta relA$ and $\Delta relA\Delta relQ$ strains, we identified 123 genes that are under apparent (p)ppGpp-positive control. Near half of these genes encoded uncharacterized hypothetical proteins ($n=56$), suggesting that a significant proportion of the genes positively controlled by (p)ppGpp remain unknown. A number of stress-related genes ($n=18$) were positively regulated in OG1RF and/or $\Delta relQ$, including genes encoding the CtsR transcriptional repressor and its target ClpB, ClpC and ClpE ATPases, the universal/general stress proteins UspA, GlsB24 and GlsB, the ribosomal stress protein RplY, and the oxidative stress regulators Spx and PerR. Additionally, genes encoding detoxifying enzymes, including two putative lactoylglutathione lyase genes (*gloA* and *lgl*), thioredoxin (*trx*), catalase (*kata*), NADH peroxidase (*npr*), DNA-binding protein (*dps*) and methionine sulfoxide reductase (*msrA* and *msrB*) were also induced in a (p)ppGpp-dependent manner (Table 3). Notably, independent studies have implicated the products of *clpB*, *gls24*, *msrA-msrB*, *perR* and *spx* in *Ent. faecalis* virulence (Choudhury *et al.*, 2011; de Oliveira *et al.*, 2011; Kajfasz *et al.*, 2012; Verneuil *et al.*, 2005; Zhao *et al.*, 2010). Although only three genes specifically involved in amino acid biosynthesis (aspartate kinase, cysteine synthase and aspartate aminotransferase) showed a (p)ppGpp-dependent positive regulation pattern, several genes encoding products involved in transport and binding processes were upregulated in OG1RF and/or $\Delta relQ$, including genes encoding subunits of ABC transporters predicted to participate in oligopeptide and amino acid uptake (Table 3). Among the genes highly induced upon mupirocin treatment (up to 25-fold induction) were two apparently co-transcribed genes, encoding a metal-transporting P-type ATPase and the MgtC family magnesium transporter (EF0758 and EF0759). Finally, a gene predicted to encode haemolysin III (*hlyIII*), a possible virulence factor, was also induced during (p)ppGpp accumulation.

DISCUSSION

Previous transcriptome profiling and proteomic studies of the stringent response in Gram-positive bacteria have relied on wild-type and $\Delta relA$ single mutants (Brockmann-Gretza & Kalinowski, 2006; Eymann *et al.*, 2002; Nascimento *et al.*, 2008), which possess residual (p)ppGpp amounts due to the presence of monofunctional (p)ppGpp synthetases (Lemos *et al.*, 2007; Nanamiya *et al.*, 2008). To our knowledge, this

Table 3. Amino acid biosynthesis, transport, stress and virulence-associated genes positively regulated by (p)ppGpp in OG1RF (wild-type) and/or $\Delta relQ$ strains after mupirocin treatment

Fold changes result from comparison of mupirocin-treated samples (15 or 30 min) with untreated samples within each strain.

Locus	Description	OG1RF M15'	OG1RF M30'	$\Delta relQ$ M15'	$\Delta relQ$ M30'
EF0063	Oligopeptide ABC transporter		9.99		5.36
EF0079	General stress protein – <i>gls24</i>		4.16		3.47
EF0080	General stress protein – <i>glsB</i>		4.15		
EF0243	BCAA transport carrier protein – <i>brnQ</i>				3.04
EF0358	Lactoylglutathione lyase – <i>lgl</i>		6.04		5.26
EF0368	Aspartate kinase	4.24	5.46	3.79	4.44
EF0467	MgtC family magnesium transporter	3.74	3.48		
EF0674	ABC superfamily transporter		4		3.17
EF0675	ABC superfamily transporter		3.68		
EF0706	Clp ATPase – <i>clpE</i>		5.54		5.83
EF0758	Possible cadmium-exporting ATPase	13.49	25.99		20.67
EF0759	MgtC family magnesium transporter	15.12	22.54	7.52	18.16
EF0807	Oligopeptide ABC transporter	3.46	6.82		4.44
EF0820	Ribosomal stress protein – <i>rplY</i>		5.75		3.75
EF0867	Lactoylglutathione lyase – <i>gloA</i>	3.96	3.27		3.81
EF0891	Aspartate aminotransferase		2.94		3.02
EF0893	Amino acid ABC transporter	3.18	2.7		
EF0910	Oligopeptide ABC transporter				2.53
EF1117	Glutamine ABC transporter				7.72
EF1118	Glutamine ABC transporter		4.65		4.78
EF1119	Glutamine ABC transporter		4.8		
EF1120	Glutamine ABC transporter		3.47		
EF1211	NADH peroxidase – <i>npr</i>		3.47		
EF1405	Thioredoxin – <i>trx</i>	3.21	2.98		
EF1413	ABC superfamily transporter		7.03		8.17
EF1513	Oligopeptide ABC transporter		9.72		11.36
EF1584	Cysteine synthase A – <i>cysK</i>	3.28	5.25		3.44
EF1585	Fur family transcriptional regulator – <i>perR</i>	5.61	5.42		4.16
EF1597	Catalase/peroxidase – <i>kata</i>		3.33		
EF1672	Peptide ABC superfamily transporter		8.43		6.82
EF1681	Peptide methionine sulfoxide reductase A – <i>msrA</i>	2.92	5.12		
EF1685	Haemolysin III – <i>hlyIII</i>	4.69	4.04	2.92	3.9
EF1982	Universal stress protein – <i>uspA</i>		5.22		5.42
EF2075	ABC superfamily transporter				4.36
EF2076	ABC superfamily transporter		7		
EF2355	Clp ATPase – <i>clpB</i>	3.28	5.16		3.03
EF2509	BCAA exporter – <i>azlC</i>		3.94		4.75
EF2678	Transcriptional regulator – <i>spx</i>	5.63	3.93		4.24
EF3015	Serine/threonine transporter	4.44	6.49		5.79
EF3164	Methionine sulfoxide reductase B – <i>msrB</i>	8.63	17.35		8.21
EF3233	DNA-binding protein – <i>dps</i>		3.05		
EF3282	Clp ATPase – <i>clpC</i>		3.16		3.21
EF3283	Transcriptional regulator – <i>ctsR</i>		7.69	4.44	7.46

report describes the first transcriptome analysis of a bona fide (p)ppGpp⁰ strain in a Gram-positive organism. By using the $\Delta relA$ and $\Delta relA\Delta relQ$ strains, which were both unable to produce (p)ppGpp upon mupirocin challenge, and $\Delta relQ$, which accumulated wild-type levels of (p)ppGpp after mupirocin treatment, we provided the first insights into the role of the Gram-positive monofunctional (p)ppGpp synthetases. In agreement with the (p)ppGpp profile of the $\Delta relA$, $\Delta relQ$ and $\Delta relA\Delta relQ$ strains

(Abranches *et al.*, 2009), our microarray results confirmed that RelA is the major enzyme responsible for the transcriptional changes associated with the stringent response, e.g. repression of genes associated with cell growth and replication, and activation of genes involved in amino acid biosynthesis, nutrient transport and stress survival. It was interesting to note that while RelQ did not contribute to the rapid accumulation of (p)ppGpp in *Ent. faecalis* as judged by our (p)ppGpp quantifications, comparisons of the

transcriptome of mupirocin-treated $\Delta relQ$ with the wild-type strain revealed a delayed repression of genes involved in translation, DNA replication and RNA synthesis, indicating that RelQ plays an unappreciated role in the induction of the stringent response. At present, it is not clear how RelQ contributes to a timely stringent response. One possibility is that RelQ can synthesize additional regulatory molecules, which may take part in the stringent response process. Although the biological significance of pyrophosphorylated nucleotides other than (p)ppGpp remains unclear, few studies have showed that unusual nucleotides, other than pppGpp and ppGpp, accumulate during amino acid starvation. For example, inhibition of isoleucyl tRNA synthetase in *B. subtilis* leads to the accumulation of (p)ppGpp as well as pppApp and ppApp (Nishino *et al.*, 1979). In *Staphylococcus aureus*, mupirocin treatment triggers production of ppGpp and ppGp, but not of pppGpp (Crosse *et al.*, 2000). Finally, a recent study identified an additional catalytic activity for the monofunctional RelA enzyme of *E. coli* that was capable of synthesizing pGpp *in vitro* (Sajish *et al.*, 2009).

By comparing our results with previous studies with closely related low-GC Gram-positives, it can be noted that the downregulation of genes involved in translation and replication is highly conserved, closely resembling the responses observed in the bacterial paradigms *E. coli* and *B. subtilis* (Eymann *et al.*, 2002; Kazmierczak *et al.*, 2009; Nascimento *et al.*, 2008; Reiss *et al.*, 2012; Traxler *et al.*, 2008). A few other genes and pathways showed a conserved transcriptional response among different Gram-positive species examined, including the (p)ppGpp-independent regulation of the T box-regulated aaRSs genes, which are negatively regulated by the ratio of charged:uncharged tRNA species, and the (p)ppGpp-dependent induction of genes involved in amino acid biosynthesis, nutrient transport and stress responses. Notably, the genome of *Ent. faecalis* lacks most genes involved in the synthesis of branched-chain amino acids (BCAAs), which are strongly induced by (p)ppGpp in other organisms (Eymann *et al.*, 2002; Kazmierczak *et al.*, 2009; Nascimento *et al.*, 2008; Reiss *et al.*, 2012; Traxler *et al.*, 2008), providing a partial explanation for the small number of amino acid biosynthetic genes activated in *Ent. faecalis*. Among the (p)ppGpp-induced genes with an established or putative role in stress responses, the increased transcription of oxidative stress genes in *Ent. faecalis* has also been observed in streptococci and *S. aureus* (Kazmierczak *et al.*, 2009; Nascimento *et al.*, 2008; Reiss *et al.*, 2012), suggesting a link between the stringent response and oxidative stress responses. In fact, a direct association between the stringent response and oxidative stress has been recently established in Gram-negative bacteria (Nguyen *et al.*, 2011). In this study, wild-type and (p)ppGpp⁰ strains of *Pseudomonas aeruginosa* were used to demonstrate that the stringent response, not simply growth arrest, serves as a protective mechanism against bactericidal antibiotics that kill bacteria through a common oxidative damage mechanism (Kohanski *et al.*, 2007). Mechanistically, it appears that the stringent response

augments tolerance to oxidative damage by blocking the production of pro-oxidant molecules and by increasing production of antioxidant enzymes such as superoxide dismutase and catalase (Nguyen *et al.*, 2011).

In our previous work (Abranches *et al.*, 2009), we demonstrated that the $\Delta relA$ and $\Delta relA\Delta relQ$ strains often display different, sometimes divergent, phenotypes (Abranches *et al.*, 2009). Furthermore, the virulence of the $\Delta relA\Delta relQ$ strain, but not of $\Delta relA$, was significantly attenuated in *C. elegans* (Abranches *et al.*, 2009). Here, we expanded the characterization of the Δrel strains and showed, using two models more closely resembling systemic infection in mammals, that survival within macrophages and virulence in *G. mellonella* were only impaired in the $\Delta relA\Delta relQ$ strain. Thus, despite the fact that both strains were unable to mount a stringent response, it appears that the complete lack of (p)ppGpp, as in $\Delta relA\Delta relQ$, plays a fundamental role in processes associated with *Ent. faecalis* virulence. Notably, comparisons of the transcriptome of the $\Delta relA\Delta relQ$ and parent strains under starvation conditions revealed upregulation of operons involved in energy metabolism in the (p)ppGpp⁰ strain, suggesting that low levels of (p)ppGpp may be important for balancing cell metabolism. Thus, while the $\Delta relA$ and $\Delta relA\Delta relQ$ strains cannot use (p)ppGpp to sense and respond to stresses, the fitness of the $\Delta relA\Delta relQ$ may be further impaired due to an unbalanced metabolism.

Several studies have demonstrated a correlation between (p)ppGpp levels and CodY activity (Bennett *et al.*, 2007; Geiger *et al.*, 2010; Lemos *et al.*, 2008; Sonenshein, 2007). CodY is a global metabolic regulator of low-GC Gram-positive bacteria that is activated by the presence of BCAAs, and in *B. subtilis*, also by intracellular GTP pools (Ratnayake-Lecamwasam *et al.*, 2001). While CodY is mostly involved in the repression of genes associated with amino acid biosynthesis and virulence (Sonenshein, 2005, 2007), it has also been implicated in the transcriptional activation of selected genes (Lemos *et al.*, 2008; Shivers *et al.*, 2006). We used the xBASE2 bioinformatic search tool (<http://www.xbase.ac.uk>) and a maximum allowance of three mismatches from the CodY-binding consensus sequence (den Hengst *et al.*, 2005) to search for CodY-binding motifs in the upstream region of genes under positive (p)ppGpp control with an established or putative role in amino acid biosynthesis, amino acid/oligopeptide transport, stress tolerance or virulence. This approach identified potential CodY-binding motifs upstream of the transcriptional start sites of *ctsR*–*cplC*, *hlyIII*, *spx*, *trx*, *uspA* and two putative amino acid/oligopeptide transporters (EF0063 and EF3015). Notably, the presence of potential CodY regulatory sequences upstream of the *Spx* and *CtsR* stress regulators integrates the stringent response and CodY with other prominent stress regulons in which (p)ppGpp accumulation may be an important step in the activation of a regulatory cascade used to optimize and integrate a sophisticated stress response network.

In summary, we showed that mupirocin provokes a classic stringent response in *Ent. faecalis* and that the bifunctional

RelA is the major enzyme mediating this response. RelQ was found to be important for a timely activation of the stringent response, providing the first insights into the biological relevance of a monofunctional (p)ppGpp synthetase of a Gram-positive organism. Additionally, we showed that complete lack of (p)ppGpp leads to an apparent metabolic imbalance and activation of energy generation pathways during starvation. Although not experimentally confirmed, *in silico* analysis revealed that a subset of the genes activated by (p)ppGpp are likely members of the CodY regulon. We are currently using a combination of genetic, physiological and biochemical approaches to further dissect the role of RelQ in the stringent response and to characterize the interactions between (p)ppGpp metabolism and CodY in *Ent. faecalis*.

ACKNOWLEDGEMENTS

We thank the J. Craig Venter Institute for supplying the *Ent. faecalis* microarrays. We also thank Mike Cashel for helpful discussions. A.O.G. was supported by the National Institute of Dental and Craniofacial Research (NIDCR) training program in oral science grant 1T90DE021985-01.

REFERENCES

- Abranches, J., Candella, M. M., Wen, Z. T., Baker, H. V. & Burne, R. A. (2006). Different roles of EIIAB^{Man} and EII^{Glc} in regulation of energy metabolism, biofilm development, and competence in *Streptococcus mutans*. *J Bacteriol* **188**, 3748–3756.
- Abranches, J., Martinez, A. R., Kajfasz, J. K., Chávez, V., Garsin, D. A. & Lemos, J. A. (2009). The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*. *J Bacteriol* **191**, 2248–2256.
- Arias, C. A. & Murray, B. E. (2012). The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol* **10**, 266–278.
- Battesti, A. & Bouveret, E. (2009). Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein–SpoT interaction. *J Bacteriol* **191**, 616–624.
- Bennett, H. J., Pearce, D. M., Glenn, S., Taylor, C. M., Kuhn, M., Sonenshein, A. L., Andrew, P. W. & Roberts, I. S. (2007). Characterization of *relA* and *codY* mutants of *Listeria monocytogenes*: identification of the CodY regulon and its role in virulence. *Mol Microbiol* **63**, 1453–1467.
- Blumenthal, R. M., Lemaux, P. G., Neidhardt, F. C. & Dennis, P. P. (1976). The effects of the *relA* gene on the synthesis of aminoacyl-tRNA synthetases and other transcription and translation proteins in *Escherichia coli* A. *Mol Gen Genet* **149**, 291–296.
- Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. & Bartlett, J. (2009). Bad bugs, no drugs: no ESCAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* **48**, 1–12.
- Bourgogne, A., Garsin, D. A., Qin, X., Singh, K. V., Sillanpää, J., Yerrapragada, S., Ding, Y., Dugan-Rocha, S., Buhay, C. & other authors (2008). Large scale variation in *Enterococcus faecalis* illustrated by the genome analysis of strain OG1RF. *Genome Biol* **9**, R110.
- Brockmann-Gretza, O. & Kalinowski, J. (2006). Global gene expression during stringent response in *Corynebacterium glutamicum* in presence and absence of the *rel* gene encoding (p)ppGpp synthase. *BMC Genomics* **7**, 230.
- Cashel, M., Gentry, D. R., Hernandez, V. J. & Vinella, D. (1996). The stringent response. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, vol. 1, pp. 1458–1496. Edited by F. C. Neidhardt, R. C., III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & A. E. Umbarger. Washington, DC: American Society for Microbiology.
- Choudhury, T., Singh, K. V., Sillanpää, J., Nallapareddy, S. R. & Murray, B. E. (2011). Importance of two *Enterococcus faecium* loci encoding Gls-like proteins for *in vitro* bile salts stress response and virulence. *J Infect Dis* **203**, 1147–1154.
- Crosse, A. M., Greenway, D. L. & England, R. R. (2000). Accumulation of ppGpp and ppGp in *Staphylococcus aureus* 8325-4 following nutrient starvation. *Lett Appl Microbiol* **31**, 332–337.
- de Oliveira, N. E., Abranches, J., Gaca, A. O., Laport, M. S., Damaso, C. R., Bastos, M. C., Lemos, J. A. & Giambiagi-deMarval, M. (2011). *clpB*, a class III heat-shock gene regulated by CtsR, is involved in thermotolerance and virulence of *Enterococcus faecalis*. *Microbiology* **157**, 656–665.
- den Hengst, C. D., van Hijum, S. A., Geurts, J. M., Nauta, A., Kok, J. & Kuipers, O. P. (2005). The *Lactococcus lactis* CodY regulon: identification of a conserved *cis*-regulatory element. *J Biol Chem* **280**, 34332–34342.
- Durfee, T., Hansen, A. M., Zhi, H., Blattner, F. R. & Jin, D. J. (2008). Transcription profiling of the stringent response in *Escherichia coli*. *J Bacteriol* **190**, 1084–1096.
- Eymann, C., Homuth, G., Scharf, C. & Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J Bacteriol* **184**, 2500–2520.
- Geiger, T., Goerke, C., Fritz, M., Schäfer, T., Ohlsen, K., Liebeke, M., Lalk, M. & Wolz, C. (2010). Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of *Staphylococcus aureus*. *Infect Immun* **78**, 1873–1883.
- Green, N. J., Grundy, F. J. & Henkin, T. M. (2010). The T box mechanism: tRNA as a regulatory molecule. *FEBS Lett* **584**, 318–324.
- Hartke, A., Giard, J. C., Laplace, J. M. & Auffray, Y. (1998). Survival of *Enterococcus faecalis* in an oligotrophic microcosm: changes in morphology, development of general stress resistance, and analysis of protein synthesis. *Appl Environ Microbiol* **64**, 4238–4245.
- Kajfasz, J. K., Mendoza, J. E., Gaca, A. O., Miller, J. H., Koselny, K. A., Giambiagi-Demarval, M., Wellington, M., Abranches, J. & Lemos, J. A. (2012). The Spx regulator modulates stress responses and virulence in *Enterococcus faecalis*. *Infect Immun*. (In press)
- Kazmierczak, K. M., Wayne, K. J., Rechtsteiner, A. & Winkler, M. E. (2009). Roles of *relS_{pm}* in stringent response, global regulation and virulence of serotype 2 *Streptococcus pneumoniae* D39. *Mol Microbiol* **72**, 590–611.
- Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**, 797–810.
- Lebreton, F., Riboulet-Bisson, E., Serror, P., Sanguinetti, M., Posteraro, B., Torelli, R., Hartke, A., Auffray, Y. & Giard, J. C. (2009). *ace*, Which encodes an adhesin in *Enterococcus faecalis*, is regulated by Ers and is involved in virulence. *Infect Immun* **77**, 2832–2839.
- Lemos, J. A., Lin, V. K., Nascimento, M. M., Abranches, J. & Burne, R. A. (2007). Three gene products govern (p)ppGpp production by *Streptococcus mutans*. *Mol Microbiol* **65**, 1568–1581.
- Lemos, J. A., Nascimento, M. M., Lin, V. K., Abranches, J. & Burne, R. A. (2008). Global regulation by (p)ppGpp and CodY in *Streptococcus mutans*. *J Bacteriol* **190**, 5291–5299.

- Mechold, U., Cashel, M., Steiner, K., Gentry, D. & Malke, H. (1996). Functional analysis of a *relA/spoT* gene homolog from *Streptococcus equisimilis*. *J Bacteriol* **178**, 1401–1411.
- Mechold, U., Murphy, H., Brown, L. & Cashel, M. (2002). Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel_{Seq}, the Rel/Spo enzyme from *Streptococcus equisimilis*. *J Bacteriol* **184**, 2878–2888.
- Murray, B. E. (1990). The life and times of the *Enterococcus*. *Clin Microbiol Rev* **3**, 46–65.
- Nanamiya, H., Kasai, K., Nozawa, A., Yun, C. S., Narisawa, T., Murakami, K., Natori, Y., Kawamura, F. & Tozawa, Y. (2008). Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. *Mol Microbiol* **67**, 291–304.
- Nascimento, M. M., Lemos, J. A., Abranches, J., Lin, V. K. & Burne, R. A. (2008). Role of RelA of *Streptococcus mutans* in global control of gene expression. *J Bacteriol* **190**, 28–36.
- Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schaffhauser, J. & other authors (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* **334**, 982–986.
- Nishino, T., Gallant, J., Shalit, P., Palmer, L. & Wehr, T. (1979). Regulatory nucleotides involved in the Rel function of *Bacillus subtilis*. *J Bacteriol* **140**, 671–679.
- Paulsen, I. T., Banerjee, L., Myers, G. S., Nelson, K. E., Seshadri, R., Read, T. D., Fouts, D. E., Eisen, J. A., Gill, S. R. & other authors (2003). Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299**, 2071–2074.
- Potrykus, K. & Cashel, M. (2008). (p)ppGpp: still magical? *Annu Rev Microbiol* **62**, 35–51.
- Ratnayake-Lecamwasam, M., Serron, P., Wong, K. W. & Sonenshein, A. L. (2001). *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* **15**, 1093–1103.
- Reiss, S., Pané-Farré, J., Fuchs, S., François, P., Liebeke, M., Schrenzel, J., Lindequist, U., Lalk, M., Wolz, C. & other authors (2012). Global analysis of the *Staphylococcus aureus* response to mupirocin. *Antimicrob Agents Chemother* **56**, 787–804.
- Sajish, M., Kalayil, S., Verma, S. K., Nandicoori, V. K. & Prakash, B. (2009). The significance of EXDD and RXKD motif conservation in Rel proteins. *J Biol Chem* **284**, 9115–9123.
- Shi, L., Reid, L. H., Jones, W. D., Shippey, R., Warrington, J. A., Baker, S. C., Collins, P. J., de Longueville, F., Kawasaki, E. S. & other authors (2006). The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* **24**, 1151–1161.
- Shivers, R. P., Dineen, S. S. & Sonenshein, A. L. (2006). Positive regulation of *Bacillus subtilis* *ackA* by CodY and CcpA: establishing a potential hierarchy in carbon flow. *Mol Microbiol* **62**, 811–822.
- Sonenshein, A. L. (2005). CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. *Curr Opin Microbiol* **8**, 203–207.
- Sonenshein, A. L. (2007). Control of key metabolic intersections in *Bacillus subtilis*. *Nat Rev Microbiol* **5**, 917–927.
- Terleckyj, B., Willett, N. P. & Shockman, G. D. (1975). Growth of several cariogenic strains of oral streptococci in a chemically defined medium. *Infect Immun* **11**, 649–655.
- Traxler, M. F., Summers, S. M., Nguyen, H. T., Zacharia, V. M., Hightower, G. A., Smith, J. T. & Conway, T. (2008). The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol* **68**, 1128–1148.
- Verneuil, N., Rincé, A., Sanguinetti, M., Posteraro, B., Fadda, G., Auffray, Y., Hartke, A. & Giard, J. C. (2005). Contribution of a PerR-like regulator to the oxidative-stress response and virulence of *Enterococcus faecalis*. *Microbiology* **151**, 3997–4004.
- Yan, X., Zhao, C., Budin-Verneuil, A., Hartke, A., Rincé, A., Gilmore, M. S., Auffray, Y. & Pichereau, V. (2009). The (p)ppGpp synthetase RelA contributes to stress adaptation and virulence in *Enterococcus faecalis* V583. *Microbiology* **155**, 3226–3237.
- Zhao, C., Hartke, A., La Sorda, M., Posteraro, B., Laplace, J. M., Auffray, Y. & Sanguinetti, M. (2010). Role of methionine sulfoxide reductases A and B of *Enterococcus faecalis* in oxidative stress and virulence. *Infect Immun* **78**, 3889–3897.

Edited by: K. E. Weaver