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In *Bacillus subtilis* LutR is part of the global complex regulatory network governing the adaptation to the transition from exponential growth to stationary phase

Öykü İrigül-Sönmez,[1]† Türkan E. Köroğlu,[1]† Büşra Öztürk,[1] Ákos T. Kovács,[2]‡ Oscar P. Kuipers[2] and Ayten Yazgan-Karataş[1]

Correspondence
Ayten Yazgan-Karataş
karatasay@itu.edu.tr

[1]Molecular Biology, Biotechnology and Genetics Research Center (MOBGAM) and Molecular Biology and Genetics Department, 34469, Istanbul Technical University, Istanbul, Turkey

[2]Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

The *lutR* gene, encoding a product resembling a GntR-family transcriptional regulator, has previously been identified as a gene required for the production of the dipeptide antibiotic bacilysin in *Bacillus subtilis*. To understand the broader regulatory roles of LutR in *B. subtilis*, we studied the genome-wide effects of a *lutR* null mutation by combining transcriptional profiling studies using DNA microarrays, reverse transcription quantitative PCR, *lacZ* fusion analyses and gel mobility shift assays. We report that 65 transcriptional units corresponding to 23 monocistronic units and 42 operons show altered expression levels in *lutR* mutant cells, as compared with *lutR*$^+$ wild-type cells in early stationary phase. Among these, 11 single genes and 25 operons are likely to be under direct control of LutR. The products of these genes are involved in a variety of physiological processes associated with the onset of stationary phase in *B. subtilis*, including degradative enzyme production, antibiotic production and resistance, carbohydrate utilization and transport, nitrogen metabolism, phosphate uptake, fatty acid and phospholipid biosynthesis, protein synthesis and translocation, cell-wall metabolism, energy production, transfer of mobile genetic elements, induction of phage-related genes, sporulation, delay of sporulation and cannibalism, and biofilm formation. Furthermore, an electrophoretic mobility shift assay performed in the presence of both SinR and LutR revealed a close overlap between the LutR and SinR targets. Our data also revealed a significant overlap with the AbrB regulon. Together, these findings reveal that LutR is part of the global complex, interconnected regulatory systems governing adaptation of bacteria to the transition from exponential growth to stationary phase.

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INTRODUCTION

The GntR family of transcriptional regulators, which comprises more than 2000 members distributed over a diverse group of bacteria, directs the regulation of genes involved in a wide variety of biological processes. These regulators have been shown to act as environmental sensors for controlling genes involved in responding to external stimuli (Rigali *et al.*, 2002). They are typically composed of an N-terminal DNA-binding winged helix–turn–helix (wHTH) domain followed by a C-terminal effector-binding domain, which may bind diverse ligands (Rigali *et al.*, 2002; Haydon & Guest, 1991; Rosinski & Atchley, 1999; van Aalten *et al.*, 2000; Lee *et al.*, 2000; Aravind & Anantharaman, 2003; Gorelik *et al.*, 2006).

In the first *Bacillus subtilis* genome project, *lutR* (formerly *yvfI*) was identified as a gene that potentially encodes an unknown protein belonging to the GntR family of transcriptional regulators (Kunst *et al.*, 1997). A Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2005) search with the LutR protein has revealed the presence of a FadR C-terminal ligand-binding (FCD) domain (PFAM

†These authors contributed equally to this work.

‡Present address: Terrestrial Biofilms Group, Institute of Microbiology, Friedrich Schiller University of Jena, Jena, Germany.

Abbreviations: EMSA, electrophoretic mobility shift assay; MLS, macrolide–lincosamide–streptogramin B; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

The microarray data from this study have been submitted to the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE 34414.

Supplementary methods, one supplementary table and three supplementary figures are available with the online version of this paper.

07729). Additionally, a second UniProt entry (O07007; EMBL accession number CAB08003) for *B. subtilis lutR* extends the sequence as annotated by Kunst *et al.* (1997) by 44 amino acids at the N terminus. This extended LutR protein reveals an incomplete but significant match to the GntR wHTH domain (PF00392). Inspection of the LutR sequence showed that this protein displays high homology with FadR-like proteins as a GntR subfamily group (Marchler-Bauer *et al.*, 2005). FadR-like proteins function in the regulation of many pathways, e.g. those involved in amino acid metabolism, L-lactate and sugar utilization, fatty acid transport and degradation, as well as in many metabolic pathways including those for aspartate, pyruvate, glycolate and galactonate metabolism (Rigali *et al.*, 2002; DiRusso *et al.*, 1992; Black & DiRusso, 1994).

The regulatory role of LutR in *B. subtilis* has previously been identified as being required for the production of the dipeptide antibiotic bacilysin (Köroğlu *et al.*, 2008). In *B. subtilis*, bacilysin is a non-ribosomally synthesized dipeptide antibiotic composed of L-alanine and L-anticapsin. A poly-cistronic operon, *ywfBCDEFG*, and a mono-cistronic gene, *ywfH*, are responsible for bacilysin biosynthesis in *B. subtilis* 168 (Inaoka *et al.*, 2003). Genes *ywfBCDEF* were found to contain the biosynthetic core functions and were renamed *bacABCDE* (Inaoka *et al.*, 2003; Steinborn *et al.*, 2005; Tabata *et al.*, 2005). Very recently, LutR has been reported to repress the *lutABC (formerly yvfV-yvfW-yvbY)* operon, which is required for lactate utilization (Chai *et al.*, 2009). It was demonstrated that the *lutABC* operon is under dual control of LutR and SinR, the master regulator of biofilm formation, and is induced during both growth in liquid culture and biofilm formation in response to L-lactate. The *lutABC* operon was also shown to influence the architectural complexity of biofilms formed in the presence of L-lactate (Chai *et al.*, 2009).

In the present study, we focus on understanding the broader regulatory role of LutR in *B. subtilis*. We show that the LutR regulon is very pleiotropic and that LutR participates in the regulation of numerous physiological processes associated with the onset of stationary phase in *B. subtilis*, such as degradative enzyme production, antibiotic production and resistance, transfer of mobile genetic elements, induction of phage-related genes, sporulation, delay of sporulation and cannibalism, and biofilm formation. Furthermore, we report a close interaction between LutR and SinR regulators in addition to a significant overlap with the AbrB regulon.

METHODS

Bacterial strains, media and culture conditions. *B. subtilis* strains used in this study are listed in Table 1. All are isogenic derivatives of the wild-type *B. subtilis* strain PY79(ICEBs1+) (ICE*Bs1*-carrying variant of PY79 based on diagnostic PCR amplification and sequence analyses as described in the supplementary information available in *Microbiology* Online) unless indicated otherwise. *Escherichia coli* Top10F′ was used for routine cloning experiments and *E. coli* and *B. subtilis* strains were normally grown at 37 °C in Luria–Bertani (LB) medium, with the following antibiotics when necessary: erythromycin (1 μg ml^{-1}), lincomycin (25 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), chloramphenicol (5 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). A macrolide–lincosamide–streptogramin B-resistant phenotype (MLS^R) was selected as previously described (Nakano *et al.*, 1988). Sporulation of *B. subtilis* was attained in Difco sporulation medium (DSM). Perry and Abraham (PA) medium, which includes sucrose as the carbon source and glutamate as nitrogen source (Perry & Abraham, 1979), was used to grow *B. subtilis* strains overnight at 37 °C. Then, they were used to inoculate PA medium to an initial optical density of about 0.1 at 600 nm (OD_{600} 0.1). The cultures were grown at 37 °C and 200 r.p.m. for further assays.

Strain construction. For the construction of *lutR*::*lacZ* and *rapI*::*lacZ* fusion strains, plasmid pMutinT3 (Vagner *et al.*, 1998) was integrated into the *lutR* and *rapI* genes on the chromosome via a single-crossover event as follows. A 488 bp *lutR* gene fragment was amplified by PCR with the following specific primers: 5′-GCCAAGCTTATGAAACAGGGAGAAGGC-3′ and 5′-CGGGGATCCAATATCCCGAAAGCACAT-3′. A 389 bp *rapI* gene fragment was amplified by PCR with the following specific primers: RapI F (5′-GCCAAGCTTTTGCGG GGTGTTTTCTTA-3′) and RapI R (5′-CGGGGATCCTTCAGCTATTCGATAAGC-3′). The primer sets contained *Hin*dIII and *Bam*HI sites, respectively, at the 5′ ends of the primers (underlined residues). The PCR products were digested with *Hin*dIII and *Bam*HI and ligated into the corresponding restriction sites in pMutinT3. The resulting recombinant plasmid containing a transcriptional *lutR–lacZ* fusion was used to transform *B. subtilis* PY79 to MLS resistance. The *lutR–lacZ* fusion is thus based on a pMutin-derived plasmid integrated into the *lutR* gene on the chromosome via a single-crossover event; so the *lutR* gene was inactivated, which is expected to influence its own expression. Therefore, the levels of *lutR* transcript in parent strain PY79 and *lutR–lacZ* fusion strain TEK7 were compared by employing reverse transcription quantitative PCR (RT-qPCR) at various growth intervals. There was no significant difference in the amount of *lutR* transcript between wild-type and TEK7 strain (data not shown) and thus the constructed TEK7 strain was used for further transcriptional analysis. The recombinant plasmid carrying a transcriptional *rapI–lacZ* fusion was used to transform not only *B. subtilis* wild-type strain but also the *lutR*::Tn*10*::*spc* (TEK1) strain to MLS resistance. The correct genomic insertions were confirmed by PCR analysis. In order to construct a *spoIIE*::*lacZ* expression cassette in wild-type and in *lutR* mutant cells, competent cells of both wild-type and the TEK1 strain were transformed with the chromosomal DNA of the *spoIIE-lacZ*-bearing strain ML105 (*spoIIE-lacZ*::*cat*). OGU1LR (*bacA*::*lacR*::*erm lutR*::Tn*10*::*spc*), NAO1LR (*ywfH*::*lacR*::*erm lutR*::Tn*10*::*spc*) and NCIB3610LR (*lutR*::Tn*10*::*spc*) strains were constructed by transforming the competent cells of OGU1, NAO1 and NCIB3610 with chromosomal DNA of TEK1.

DNA-microarray analysis. The *B. subtilis* PY79 and TEK1 mutant strains were grown in PA medium until the onset of stationary phase (OD_{600} 7) and samples were collected. Total RNA isolation, cDNA synthesis, hybridization, scanning and data normalization were performed as previously (Kovács & Kuipers, 2011) and are described in detail in Supplementary Methods. The microarray data from this study have been submitted to the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE 34414. Microarray analysis was performed using three independent biological replicates, resulting in six measurements per gene, since each slide contained two duplicate spots for all genes.

qPCR analysis. The parent strain *B. subtilis* PY79 and the *lutR* mutant TEK1 (*lutR*::T*n*10::*spc*) cells were grown to OD_{600} 7 in PA medium. Total RNA was isolated by using the RNeasy Mini RNA

Table 1. *B. subtilis* strains used in this study

Strain	Genotype	Source
B. subtilis PY79(ICE*Bs*1 +)	Wild-type, BSP-cured prototrophic derivative of *B. subtilis* 168	P. Youngman
MI105	*spoIIE*::*lacZ*::*kat*	A. D. Grossman
TEK1	*lutR*::Tn*10*::*spc*	This study
TEK7	*lutR*::*lacZ*::*erm*	This study
OYK2	*rapI*::*lacZ*::*erm*	This study
OYK2LR	*rapI*::*lacZ*::*erm lutR*::Tn*10*::*spc*	This study
OYK4	*spoIIE*::*lacZ*::*kat* in PY79	This study
OYK4LR	*spoIIE*::*lacZ*::*kat lutR*::Tn*10*::*spc*	This study
OGU1	*bacA*::*lacZ*::*erm*	Köroğlu *et al.* (2011)
OGU1LR	*bacA*::*lacZ*::*erm lutR*::Tn*10*::*spc*	This study
NAO1	*ywfH*::*lacZ*::*erm*	Laboratory stock
NAO1LR	*ywfH*::*lacZ*::*erm lutR*::Tn*10*::*spc*	This study
NCIB3610	Undomesticated wild-type strain biofilm producer	Bacillus Genetic Stock Center
NCIB3610LR	*lutR*::Tn*10*::*spc*	This study

Isolation kit (Qiagen) with cell aliquots at a concentration giving OD_{600} 1. Equal amounts (2 μg) of total RNAs were reverse transcribed by using a Transcriptor cDNA Synthesis kit (Roche) with random hexamer primers (60 μM) supplied with the kit. Amplification and detection of PCR products were performed with the SYBR Green Master Mix kit (Roche) and the LightCycler 480 (Roche) instrument. As recommended by the manufacturer, 2 μl cDNA synthesis reaction mixture was directly used as template in 20 μl real-time PCR mixture with 10 pmol of gene-specific primers listed in Supplementary Methods. Real-time PCRs were run at 52 °C annealing temperature, as all the primers used in this study were designed to work at this temperature to obtain comparable data. Melting curve analysis was used to monitor the specificity of the reaction (data not shown). The $2^{-\Delta\Delta C_t}$ method was used to calculate relative gene expressions (Pfaffl, 2004). The expression levels of the investigated genes were determined relative to the wild-type *B. subtilis* sample. The ratios ($2^{-\Delta\Delta C_t}$) were calculated and $\log_2$ transformed. Each qRT-PCR analysis was performed as three independent biological replicates. The target-gene expression was normalized to the unaffected reference gene expression. For this purpose *sigA, veg* and *qcrA* genes were used as internal controls, since the expression of those genes was constant under both control and mutant conditions in both microarray and real-time PCR experiments. The accurate normalization of RT-PCR data was obtained by geometric averaging of those three internal control genes, avoiding the erroneous normalization of the single internal control.

Gel mobility shift assay. We employed a fluorescence-based gel mobility shift assay (EMSA) in which detection was made by directly staining with SYBR Green as described previously (Köroğlu *et al.*, 2011). Predicted promoter regions of target genes, approximately 200–350 bp upstream of the start codon, were amplified by proof-reading PCR, using *B. subtilis* PY79 chromosomal DNA as template with specific primers given in the Supplementary Methods. EMSA analysis was performed in the presence of various amounts of purified LutR-His_6 or SinR-His_6 as indicated below. A reaction buffer [50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 8 % (v/v) glycerol in 10 mM Tris/HCl (pH 8)] was used, supplemented with competitor DNA poly(dI-dC) (1 μg μl^{-1}) and BSA (1 mg ml^{-1}). In each assay 45 ng of promoter region DNA was used and 25 μl of total reaction mixture was incubated at 37 °C for 15 min. Loading buffer [0.25 × TBE, 60 % (v/v); glycerol, 40 % (v/v); bromophenol blue, 0.2 % (w/v)] was added to each reaction and loaded on pre-run (4 °C, 180 V, 5 min) 4 % native polyacrylamide gels. Electrophoresis was performed in 1 × TGE running buffer at 250 V for 5 min and at 180 V for 25 min at 4 °C.

Purification of LutR-His_6 and SinR-His_6 proteins. The *lutR* and *sinR* genes were amplified by high-fidelity PCR using PY79 chromosomal DNA as template with oligonucleotide primers (for *lutR*, 5′-GCCCCATGGGTATGATCAAAAATGGCGAATTG-3′ and 5′-CGGGGATCCTTGCACATTTTCCTCGAAATA-3′; for *sinR*, 5′-CGGCCATGGGTTTGATTGGCCAGCGTATTAAA-3′ and 5′-GCCGGATCCCTCCTCTTTTTGGGATTTTCT-3′) containing restriction sites for *Nco*I and *Bam*HI (underlined residues). The PCR fragments were cloned into expression vector pQE60 (Qiagen) which is under the control of an isopropyl β-D-thiogalactoside (IPTG)-inducible promoter. Expression and purification of proteins were performed as described previously (Köroğlu *et al.*, 2011).

β-Galactosidase assay. *B. subtilis* cells were either induced to sporulate in Difco sporulation medium or grown in PA medium as specified by Nicholson & Setlow (1990). β-Galactosidase was assayed as described by Miller (1972) using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The specific activity was expressed in Miller units (Miller, 1972).

Colony and pellicle morphology analysis. In the biofilm experiments, a prototrophic natural isolate of *B. subtilis* NCIB3610 was used as wild-type organism. For colony morphology analysis on solid agar MSgg medium, overnight cultures of both wild-type NCIB3610 and its *lutR*-disrupted derivative strain, NCIB3610LR, were inoculated in fresh LB broth with 1 : 100 dilution and cells were grown at 37 °C with agitation until OD_{600} 1. Then, 3 μl of these cultures was spotted onto dried MSgg agar (Branda *et al.*, 2001) and the plates incubated at 30 °C for 72 h. For pellicle morphology analysis, NCIB3610 and NCIB3610LR were grown to mid-exponential phase in LB medium and 2.5 μl culture was used to inoculate 2.5 ml MSgg medium in one well of a 24-well plate. Each plate was then incubated at 30 °C for 72 h. For the RT-qPCR analysis, NCIB3610 and NCIB3610LR were grown on MSgg agar plates for 24 h at 30 °C. The colonies were then harvested by washing the plates with 0.85 % saline solution. Cell pellets were collected with centrifugation and stored at −20 °C. RNA isolation and RT-qPCR analysis were performed as described earlier.

RESULTS AND DISCUSSION

In order to identify the genes regulated by the transcriptional factor LutR, gene expression levels were first analysed on a genome-wide scale by comparing RNAs

from the *lutR*-disrupted mutant TEK1 (*lutR*::Tn*10*::*spc*) with RNAs from the *lutR*$^+$ parent strain PY79(ICEBs1+) taken at the onset of stationary phase (OD_{600} 7), since at that stage it was determined that the expression of *lutR* reaches a maximum level in cells grown in PA medium (Fig. 1). DNA microarrays used in this study contained probes for 4107 ORFs of *B. subtilis*. It is known that whole genome gene expression analyses such as DNA-microarray analyses can lead to some false-positive results (Murphy, 2002). Therefore, to confirm the microarray results, genes showing at least 2.71-fold difference (or $\log_2$ transformed expression ratios >1.44) with reproducible characteristics in three independent biological experiments [displaying an acceptable Bayes *P*-value ($P<0.01$)] were subjected to RT-qPCR analysis by focusing on the first or several genes of each transcriptional unit. In this manner, genes that showed a reproducible difference in RT-qPCR analysis as well as in the microarrays were identified as LutR-regulated genes (Table S1, available in the online Supplementary Material). Five genes (*yjcM, yqgA, yqxIJ, ybyB* and *yotH*) identified in the microarray experiment were not further validated but are listed in Table S1 as possible LutR-regulated genes. In six cases (*pyrP, trkA, yokD, yvcA, abh* and *rapI*), the Bayes *P* value was higher than acceptable (>0.01) but significant differential expression in the *lutR* mutant strain was confirmed by RT-qPCR, except for *rapI* which was confirmed by *lacZ* fusion analysis. Interestingly, the *lutABC* operon, which is known to be controlled by LutR, was not detected in our microarray analysis. The LutR-dependent expression of the *lutABC* genes was previously examined on biofilm minimal medium (MSgg) in the presence of lactate (Chai *et al.*, 2009). This locus might not be expressed significantly under the growth condition used in this study (i.e. lack of lactate in the medium). Thus we tested this possibility using RT-qPCR. Similar to our microarray analysis, no significant change could be detected in the transcriptional level of the *lutABC* operon ($\log_2$ transformed expression ratio, 0.14 ± 0.03). In addition, the effect of the *lutR* mutation on the expression of *czcD, citB, epsN, fabR, pbpE, sigW, spoIIE, yneN, bceA, bceB* and *yvcA* genes was detected with the RT-qPCR analysis employed, and the effect on the expression of *spoIIE*, *ywfH* and the *bacABCDE-ywfG* operon was detected with *lacZ* promoter fusion analysis, even though no significant change in the transcriptional level of those genes could be detected in our microarray analysis. Additionally, when most of the genes within an operon met the criteria, we included the other genes in the operon for comparison. Thus, in total, we found that 65 transcriptional units corresponding to 23 monocistronic units and 42 operons had altered expression levels in the *lutR* mutant compared with *lutR*$^+$ wild-type cells, as listed in Table S1.

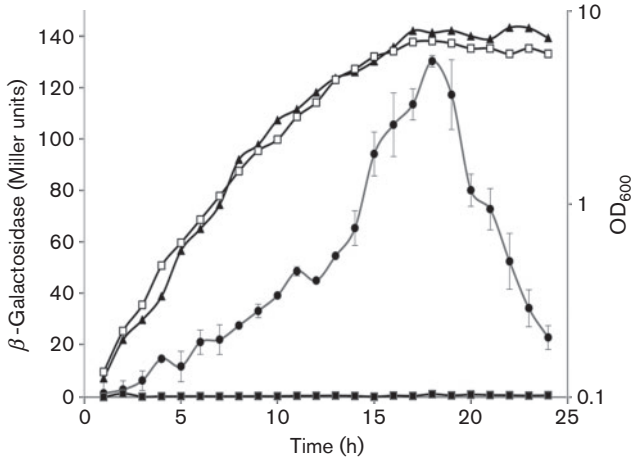

Fig. 1. Expression of transcriptional *lutR*::*lacZ* fusion. *B. subtilis* TEK7 (*lutR*::*lacZ*::*erm*) and wild-type PY79 cells which have no *lacZ* fusion were grown in PA medium and β-galactosidase activities were determined at the indicated times. Error bars indicate the standard deviation of the mean of three independent experiments ($n=3$). Triangles represent the growth profile and circles represent the β-galactosidase activity of strain TEK7. White squares represent the growth profile and black squares represent the β-galactosidase activity of the strain PY79.

Direct targets of LutR

To identify direct targets of LutR, we checked whether LutR binds to the putative upstream regulatory regions of the transcriptional units listed in Table 2. For this, EMSAs were performed with DNA fragments harbouring 200–350 bp upstream from the translational start codons of the first gene in each transcription unit and various concentrations of the purified C-terminal His_6-tagged LutR. LutR binding usually yielded a single shifted band and the degree of retardation increased with even modestly increased concentration of LutR, suggesting that each regulatory region contains a single binding site for LutR (Fig. 2). Observed DNA shifts were LutR-specific since LutR caused no retardation in electrophoretic mobility with the regulatory region of an unrelated gene, *ywbH* (Fig. 2), and all of the binding experiments were performed in the presence of a molar excess of poly(dI-dC). Each binding assay was repeated at least two times. As a result, LutR interacted with the upstream regions of 36 out of the 62 transcription units tested, indicating that 36 transcription units corresponding to 11 single-gene transcriptional units and 25 operons are likely to be under direct control of LutR (Figs 2 and S1). We also searched for a consensus binding site for LutR by performing a MEME search (Bailey *et al.*, 2009) on the upstream regulatory regions of directly affected genes. This search revealed a highly TC-rich DNA motif, i.e. TTCCTCCTTTTNTTT (Fig. S2), on the DNA fragments bound by LutR (regulatory regions of *acoA*, *aprE*, *czcD*, *cwlO*, *glnR*, *ispA*, *lip*, *msmR*, *pbpE*, *ppsA*, *pyrB*, *rapI*, *spoIIE*, *ybfO*, *ydjM*, *yhfE*, *yneN*, *tapA*, *bslA*, *yuaF*, *yvcA*, *liaI*, *bacA*, *ywfH* and *yydF* genes). A pyrimidine-rich motif such as this one strongly suggests that bending of this DNA region might play a role in the regulatory process (Gabdank *et al.*, 2009). Additional experiments will be

Table 2. Functional classification of genes that are regulated by LutR at the onset of stationary phase

Gene	Microarray*	qPCR†	EMSA‡	Function	Transcriptional organization§
Positively affected by LutR					
Fatty acid and lipid metabolism					
fabHA	1.87	1.00 (±0.12)	−	3-Oxoacyl-(acyl carrier protein) synthase III	$\overrightarrow{comZ}$ $\overleftarrow{yjzB}$ $\overrightarrow{fabHA}$ $\overrightarrow{fabF}$ ≠ $\overrightarrow{yjaZ}$
fabF	1.89			3-Oxoacyl-(acyl carrier protein) synthase II	
fabHB	3.00	1.98 (±0.05)	−	3-Oxoacyl-(acyl carrier protein) synthase III	$\overrightarrow{yhgE}$ ≠ $\overleftarrow{fabHB}$ $\overrightarrow{yhfC}$ ≠ $\overrightarrow{yhfD}$ $\overrightarrow{yhfE}$
fapR	1.36	1.16 (±0.09)	−	Fatty acid biosynthesis transcriptional factor	$\overrightarrow{recG}$ $\overrightarrow{fapR}$ $\overrightarrow{plsX}$ $\overrightarrow{fabD}$ $\overrightarrow{fabG}$
plsX	1.66			Putative glycerol-3-phosphate acyltransferase PlsX	
fabD	2.42		−	Malonyl-CoA-acyl carrier protein transacylase	$\overrightarrow{ylpC}$ $\overrightarrow{plsX}$ $\overrightarrow{fabD}$ $\overrightarrow{fabG}$ $\overrightarrow{acpA}$ ≠
fabG	2.28			*β*-Ketoacyl-acyl carrier protein reductase	$\overrightarrow{rnc}$ ≠ $\overrightarrow{smc}$ ≠ $\overrightarrow{ftsY}$
acpA	2.29		−	Acyl carrier protein	$\overrightarrow{fabG}$ $\overrightarrow{acpA}$ ≠ $\overrightarrow{rnc}$ ≠ $\overrightarrow{smc}$
yhdN	ND		−	Aldo/keto reductase	$\overrightarrow{yhdN}$ ≠ $\overrightarrow{plsC}$ ≠ $\overrightarrow{yhdP}$ $\overrightarrow{yhdQ}$ $\overrightarrow{yhdR}$ ≠
plsC	1.49	1.05 (±0.14)	−	1-Acyl-*sn*-glycerol-3-phosphate acyltransferase (lipid metabolism)	
Biotin biosynthesis					
bioW	1.99	1.46 (±0.06)	−	6-Carboxyhexanoate-CoA ligase	$\overleftarrow{bioI}$ ≠ $\overleftarrow{bioB}$ $\overleftarrow{bioD}$ $\overleftarrow{bioF}$ $\overleftarrow{bioA}$ $\overleftarrow{bioW}$ ≠
bioA	1.38			Adenosylmethionine-8-amino-7-oxononanoate transaminase	$\overleftarrow{ytaP}$ $\overrightarrow{msmR}$
bioF	1.45			8-Amino-7-oxononanoate synthase	
bioD	2.06			Dithiobiotin synthetase	
bioB	ND			Biotin synthase	
Energy metabolism					
atpI	1.19	0.56 (±0.03)	+	ATP synthase subunit I	≠ $\overleftarrow{atpC}$ $\overleftarrow{atpD}$ $\overleftarrow{atpG}$ $\overleftarrow{atpA}$ $\overleftarrow{atpH}$
atpB	1.93			ATP synthase subunit A	$\overleftarrow{atpF}$ $\overleftarrow{atpE}$ $\overleftarrow{atpB}$ $\overleftarrow{atpI}$ ≠
atpE	2.02	1.53 (±0.15)		ATP synthase subunit C	
atpF	1.99			ATP synthase subunit B	
atpH	1.93			ATP synthase subunit delta	
atpA	2.01	1.13 (±0.10)		ATP synthase subunit alpha	
atpG	1.91			ATP synthase subunit gamma	
Menaquinoine biosynthesis					
hepS	1.35	0.91 (±0.18)	+	Heptaprenyl diphosphate synthase component I	≠ $\overleftarrow{ndk}$ $\overleftarrow{hepT}$ $\overleftarrow{menH}$ $\overleftarrow{hepS}$ $\overleftarrow{mtrB}$ $\overleftarrow{mtrA}$ ≠
hepT	1.52			Heptaprenyl diphosphate synthase component II	$\overleftarrow{hbs}$ ≠ $\overleftarrow{spoIVA}$ ≠
ndk	1.37	1.01 (±0.24)		Nucleoside diphosphate kinase (purine nucleotide biosynthesis)	
Nitrogen metabolism					
pyrR	2.52	2.79 (±0.32)	+	Bifunctional pyrimidine regulatory protein	≠ $\overrightarrow{pyrR}$ ≠ $\overrightarrow{pyrP}$ ≠ $\overrightarrow{pyrB}$ $\overrightarrow{pyrC}$
pyrP‖	NR	3.48 (±0.18)		Uracil permease	

Table 2. cont.

Gene	Microarray*	qPCR†	EMSA‡	Function	Transcriptional organization§
pyrB	1.37	4.34 (±0.01)	+	Aspartate carbamoyltransferase catalytic subunit	≠ $\overrightarrow{pyrR}$ ≠ $\overrightarrow{pyrP}$ ≠ $\overrightarrow{pyrB}$ $\overrightarrow{pyrC}$ $\overrightarrow{pyrAA}$ $\overrightarrow{pyrAB}$ $\overrightarrow{pyrK}$ $\overrightarrow{pyrD}$ $\overrightarrow{pyrF}$ $\overrightarrow{pyrE}$ ≠ $\overrightarrow{cysH}$
pyrC	1.89			Dihydroorotase	
pyrAA	2.31	5.85 (±0.20)		Carbamoyl-phosphate synthetase (glutaminase subunit)	
pyrAB	2.00			Carbamoyl-phosphate synthetase (catalytic subunit)	
pyrK	0.85			Dihydrooratate dehydrogenase	
pyrD	1.32			Dihydrooratate dehydrogenase (catalytic subunit)	
pyrF	1.21			Orotidine 5′-phosphate decarboxylase	
pyrE	1.08			Orotate phosphoribosyltransferase	
Degradative enzyme production					
aprX	1.57	2.98 (±0.17)	−	Serine protease	≠ $\overleftarrow{aprX}$ $\overrightarrow{ymaC}$ $\overrightarrow{ymaD}$ ≠ $\overleftarrow{ebrB}$
lip	2.38	2.65 (±0.09)	+	Secreted alkaliphilic lipase	≠ $\overleftarrow{lmrB}$ $\overleftarrow{lmrA}$ $\overrightarrow{yccC}$ ≠ $\overrightarrow{lip}$ ≠ $\overrightarrow{yczC}$ ≠
yhfE	1.63	3.65 (±0.04)	+	Putative endoglucanase	≠ $\overleftarrow{fabHB}$ $\overleftarrow{yhfC}$ ≠ $\overleftarrow{yhfD}$ $\overrightarrow{yhfE}$ $\overrightarrow{yhfF}$ ≠
yhfF	1.40	2.18 (±0.09)		Hypothetical protein	
cwlO	2.24	1.70 (±0.05)	+	Secreted cell wall D,L-endopeptidase	$\overleftarrow{yvcI}$ $\overleftarrow{trxB}$ $\overleftarrow{cwlO}$ ≠ $\overleftarrow{yvcD}$ $\overleftarrow{yvcC}$ ≠
Antibiotic production and resistance					
pksD	1.9	1.44 (±0.06)	−	Polyketide synthesis enzyme	$\overrightarrow{pksA}$ ≠ $\overrightarrow{pksB}$ $\overrightarrow{pksC}$ $\overrightarrow{pksD}$ $\overrightarrow{pksE}$ $\overrightarrow{acpK}$ $\overrightarrow{pksF}$ ≠ $\overrightarrow{pksG}$ $\overrightarrow{pksH}$ $\overrightarrow{pksI}$
pksE	1.59			Polyketide synthesis enzyme	$\overrightarrow{pksJ}$ $\overrightarrow{pksL}$ $\overrightarrow{pksM}$ $\overrightarrow{pksN}$ $\overrightarrow{pksR}$ ≠
acpK	1.77			Acyl-carrier protein	$\overleftarrow{pksS}$ $\overrightarrow{ymzB}$
pksG	2.10	1.42 (±0.18)	−	Acetyl-*S*-AcpK *β*-ketothioester polyketide intermediate transferase	
pksH	ND			Polyketide synthesis enzyme	
pksI‖	ND			Polyketide synthesis enzyme	
pksJ	2.73	4.14 (±0.01)		Polyketide synthase of type I	
pksL	3.95	6.19 (±0.06)		Polyketide synthase of type I	
pksM	2.17			Polyketide synthase	
pksN	2.96			Polyketide synthase of type I	
pksR	2.46			Polyketide synthase	
ppsA‖	NR		+	Plipastatin synthetase	≠ $\overleftarrow{ppsE}$ $\overleftarrow{ppsD}$ $\overleftarrow{ppsC}$ $\overleftarrow{ppsB}$ $\overleftarrow{ppsA}$ ≠
ppsB	1.1	1.09 (±0.18)		Plipastatin synthetase	
ppsC‖	NR			Plipastatin synthetase	
ppsD	1.24			Plipastatin synthetase	
ppsE	2.42			Plipastatin synthetase	
yokD	NR	2.65 (±0.25)	+	Aminoglycoside-*N*3′-acetyltransferase	≠ $\overleftarrow{yokF}$ $\overleftarrow{yokE}$ ≠ $\overleftarrow{yokD}$ $\overleftarrow{yokC}$

Table 2. cont.

Gene	Microarray*	qPCR†	EMSA‡	Function	Transcriptional organization§
yydF	ND		+	Biactive peptide eliciting cell envelope stress sensed by the LiaRS two-component system	≠ *yydJ yydI yydH yydG yydF fbp*
yydG	1.93	1.68 (±0.09)		Putative AdoMet radical enzyme	
yydH	0.62			Membrane-embedded protease	
yydI	0.51			YydIJ: an ATP-binding cassette (ABC) transporter	
yydJ	0.73			YydIJ: an ATP-binding cassette (ABC) transporter	
bacA¶	ND		+	Bacilysin biosynthesis protein, dehydratase	≠ *ywfA* ≠ *bacA bacB bacC bacD bacE*
bacB	ND			Isomerase component of bacilysin synthetase	*ywfG* ≠ *ywfH* ≠ *ywfI*
bacC‖	NR			Bacilysin biosynthesis oxidoreductase	
bacD	ND			Alanine-anticapsin ligase	
bacE‖	NR			Efflux protein for bacilysin excretion, self-protection against bacilysin	
ywfG‖	NR			Transaminase	
ywfH¶	ND		+	Carrier protein reductase of bacilysin biosynthesis	*ywfF ywfG* ≠ *ywfH* ≠ *ywfI*
Transport/binding proteins					
czcD‖	NR	2.25 (±0.22)	+	Potassium/proton-divalent cation antiporter	*yrdR yrdQ* ≠ *trkA czcD* ≠ *yrdN* ≠
trkA‖	1.76	1.02 (±0.06)		Potassium uptake oxidoreductase	
yybN	1.76	2.05 (±0.24)	+	Hypothetical protein	
yybM	ND		−	Integral inner-membrane protein	*yybN* ≠ *yybM yybL yybK yybJ yybI*
ybL	1.52	1.23 (±0.12)	−	Integral inner-membrane protein	*yybH yybG* ≠ *yybF*
yybK	1.73			Integral inner-membrane protein	
yybJ	1.52			ATP-binding cassette protein	
Protein translocation/synthesis/protein folding					
rnc	2.41	1.64 (±0.03)	−	RNase III	*acpA* ≠ *rnc smc* ≠ *ftsY* ≠ *ylqB*
smc	1.42			Chromosome condensation and segregation SMC ATPase	
ftsY	1.08			Signal recognition particle (SRP) receptor	*acpA* ≠ *rnc smc* ≠ *ftsY* ≠ *ylqB*
ylxM	1.32		+	Hypothetical, DNA-binding protein	*rnc* ≠ *smc ftsY* ≠ *ylqB ylxM ffh* ≠
ffh	1.51	2.91 (±0.04)	−	Signal recognition particle (SRP)-like GTPase	
rplJ	1.69	3.42 (±0.30)		50S ribosomal protein L10	*nusG rplK rplA* ≠ *rplJ rplL* ≠ *ybxB*
rplL	1.66			50S ribosomal protein L7/L12	
tig	1.68	1.11 (±0.01)	+	Trigger factor (prolyl isomerase), catalyses *in vitro* protein folding	≠ *lonA* ≠ *lonB clpX tig ysoA* ≠
tsf	1.69	1.31 (±0.53)	−	Ts elongation factor	*sigD ylxL rpsB* ≠ *tsf pyrH frr* ≠
pyrH	1.52	1.74 (±0.15)		Uridylate kinase	
frr	1.55	1.11 (±0.08)		Ribosome recycling factor	
Regulation of mobile genetic elements					
rapI‖¶	NR		+	Response regulator aspartate phosphatase	*yddK rapI phrI* ≠ *yddM* ≠ *yddN*
phrI‖	NR			Phosphatase RapI regulator	

Table 2. cont.

Gene	Microarray*	qPCR†	EMSA‡	Function	Transcriptional organization§
Activation of phage-related genes					
yukE	1.46	1.29 (±0.06)	+	Hypothetical protein	≠ *yueD yueC yueB yukA yukB yukC*
yukD	ND			Putative bacteriocin	*yukD yukE yukF* ≠
yukC	1.75			Hypothetical protein	
yukB	ND			Hypothetical protein	
yukA	1.48			Hypothetical protein	
yueB	0.6			Bacteriophage SPP1 adsorption protein	
Sporulation, cannibalism and sporulation delay					
ftsE	1.11	1.94 (±0.13)	+	Cell-division ABC transporter (ATP-binding protein)	*yvjB* ≠ *ftsX ftsE* ≠ *cccB yvjA* ≠
ftsX	1.68			Cell-division ABC transporter	
spoIIE¶	ND		+	Serine phosphatase	≠ *spoIIE yabS yabT yacA hprT*
sdpA	1.26	1.08 (±0.22)	+	Sporulation delay protein exporter of killing factor	*yvaV sdpA sdpB sdpC* ≠
sdpB	2.2	1.68 (±0.08)		Sporulation delay protein exporter of killing factor SpbC	
sdpC	ND			Killing factor SdpC	
Biofilm formation					
tapA	ND	2.65 (±0.06)	+	Lipoprotein for biofilm formation	*sinI sinR* ≠ ≠ *tasA sipW tapA yqzG yqzE*
sipW	2.12	1.42 (±0.14)		Type I signal peptidase	
tasA	1.50	2.65 (±0.17)		Major biofilm matrix component	
bslA	3.33	2.70 (±0.25)	+	Hypothetical protein	*yuac* ≠ *bslA* ≠ *yuaA yubG* ≠
epsD	2.21	2.63 (±0.05)	−	Extracellular matrix biosynthesis enzyme	≠ *sigL yvfG epsO epsN epsM epsL epsK*
epsE	2.08	1.34 (±0.31)		Glycosyltransferase	*epsJ epsI epsH epsG epsF epsE epsD*
epsK	1.49	1.10 (±0.21)	−	Extracellular matrix component exporter	*epsC epsB epsA slr*
epsN	ND	1.02 (±0.01)		Aminotransferase	
epsO	1.79	0.94 (±0.05)		Pyruvyltransferase	
yvcA‖	NR	1.56 (±0.01)	+	Lipoprotein	*yvcC* ≠ *yvzA yvcB yvcA* ≠ *hisI*
Cell cycle, cell division, cell wall synthesis					
murE	ND	2.02 (±0.05)	+	UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	≠ *spoVD murE mraY murD* ≠ *spoVE*
mraY	1.45	1.20 (±0.06)		Phospho-*N*-acetylmuramoyl-pentapeptide-transferase	
murD	1.53			UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase	
cwlO	2.24	1.70 (±0.05)	+	Secreted cell wall DL-endopeptidase	*yvcI trxB cwlO* ≠ *yvcD yvcC* ≠
gtaC	1.52	2.04 (±0.06)	−	Integral inner-membrane protein	≠ *galT galK gtaC ywcC ycwB*
Negatively affected by LutR					
Nitrogen metabolism					
glnR	−1.31	−0.73 (±0.10)	+	Transcriptional regulator (nitrogen metabolism)	*spoVG* ≠ *ynbA ynbB glnR glnA* ≠
glnA	−1.19			Glutamine synthase	
gltA	−1.31	−2.04 (±0)	+	Glutamate synthase (large subunit)	≠ *yofA yogA* ≠ *gltB gltA gltC* ≠
gltB	−1.45			Glutamate synthase small chain	*proJ proH*

Table 2. cont.

Gene	Microarray*	qPCR†	EMSA‡	Function	Transcriptional organization§
argC	−1.63	−1.98 (±0.22)	−	*N*-Acetyl-gamma-glutamyl-phosphate reductase	$\overrightarrow{yitY}$ $\overrightarrow{yitZ}$ ≠ $\overrightarrow{argC}$ $\overrightarrow{argJ}$ $\overrightarrow{argB}$ $\overrightarrow{argD}$
argJ	−1.89			Ornithine acetyltransferase/*N*-acetylglutamate synthase	
argB	−2.08			Acetylglutamate kinase	$\overrightarrow{carA}$ $\overrightarrow{carB}$ $\overrightarrow{argF}$ ≠ $\overrightarrow{yjzC}$
argD	−2.13			Acetylornithine aminotransferase	
carA	−2.22			Carbamoyl phosphate synthase small subunit	
carB	−2.15			Carbamoyl phosphate synthase large subunit	
argF	−2.16			Ornithine carbamoyltransferase	
argG	−1.68		+	Argininosuccinate synthase	≠ $\overrightarrow{ytzD}$ $\overrightarrow{argH}$ ≠ $\overrightarrow{argG}$ ≠ $\overrightarrow{moaB}$ ≠ $\overrightarrow{ackA}$ ≠
argH	−2.04			Argininosuccinate lyase	
trpE	−1.9	−1.26 (±0.05)	−	Anthranilate synthase component I	≠ $\overleftarrow{aroE}$ $\overleftarrow{tyrA}$ $\overleftarrow{hisC}$ $\overleftarrow{trpA}$ $\overleftarrow{trpB}$ $\overleftarrow{trpF}$ $\overleftarrow{trpC}$
trpD	−2.1			Anthranilate phosphoribosyltransferase	$\overleftarrow{trpD}$ $\overleftarrow{trpE}$ $\overleftarrow{aroH}$
trpC	−2.62			Indole-3-glycerol-phosphate synthase	
trpF	−2.22			*N*-(5′-phosphoribosyl) anthranilate isomerase	
trpB	−2.00			Tryptophan synthase subunit beta	
trpA	−1.04			Tryptophan synthase subunit alpha	
Carbohydrate metabolism					
acoA	−1.57	−3.07 (±0.22)	+	Acetoin dehydrogenase E1 component (alpha subunit)	≠ $\overrightarrow{fyjM}$ $\overrightarrow{fyjL}$ ≠ $\overrightarrow{acoA}$ $\overrightarrow{acoB}$ $\overrightarrow{acoC}$ $\overrightarrow{acoL}$ ≠
acoB	−1.54			Acetoin dehydrogenase E1 component (beta subunit)	$\overrightarrow{acoR}$ $\overrightarrow{sspH}$ ≠
acoC	−1.56			Branched-chain α-keto acid dehydrogenase subunit E2	
acoL	−1.36			Dihydrolipoamide dehydrogenase	
citA	−1.50	−2.20 (±0.30)	−	Citrate synthase 1	≠ $\overleftarrow{citR}$ $\overrightarrow{citA}$ $\overrightarrow{yhdF}$ ≠ $\overrightarrow{yhdG}$ ≠ $\overrightarrow{yhdH}$ ≠
citB	−1.38	−2.21 (±0.21)	−	Aconitate hydratase	≠ $\overleftarrow{cotM}$ $\overleftarrow{sspP}$ $\overleftarrow{sspO}$ $\overrightarrow{citB}$ ≠ $\overrightarrow{yneN}$
msmR	−1.48	−1.43 (±0.27)	+	Transcriptional regulator (LacI family)	$\overleftarrow{bioW}$ ≠ $\overrightarrow{ytaP}$ $\overrightarrow{msmR}$ $\overrightarrow{msmE}$ $\overrightarrow{amyD}$ $\overrightarrow{amyC}$
msmE	−1.51			Multiple sugar-binding lipoprotein	$\overrightarrow{melA}$ ≠
Degradative enzyme production					
aprE	−2.35	−3.07 (±0.91)	+	Extracellular alkaline serine protease (subtilisin E)	≠ $\overleftarrow{yhfM}$ $\overrightarrow{yhfN}$ ≠ $\overrightarrow{aprE}$ $\overrightarrow{yhfO}$ $\overrightarrow{yhfP}$ ≠
ispA	−2.01	−1.68 (±0.22)	+	Intracellular serine protease	≠ $\overleftarrow{metE}$ ≠ $\overrightarrow{ispA}$ $\overrightarrow{ykoB}$ ≠ $\overrightarrow{ykoC}$ $\overleftarrow{ykoD}$

Genes in an operon are grouped together.

NR, No reproducible data obtained.

ND, No differential expression observed.

*Numbers indicate the $\log_2$ transformed expression ratios (in the wild-type versus the TEK1 strain).

†Numbers indicate the $\log_2$ transformed expression ratios. The mean of a minimum of three independent replicate experiments is given and standard deviation of the mean is shown in parentheses.

‡A '+' indicates that gel retardation was observed.

§Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, '≠' indicates the termination sites.

||Bayes *P* value higher than acceptable value (>0.01).

¶Expression profiles were elucidated by *lacZ* fusion analysis.

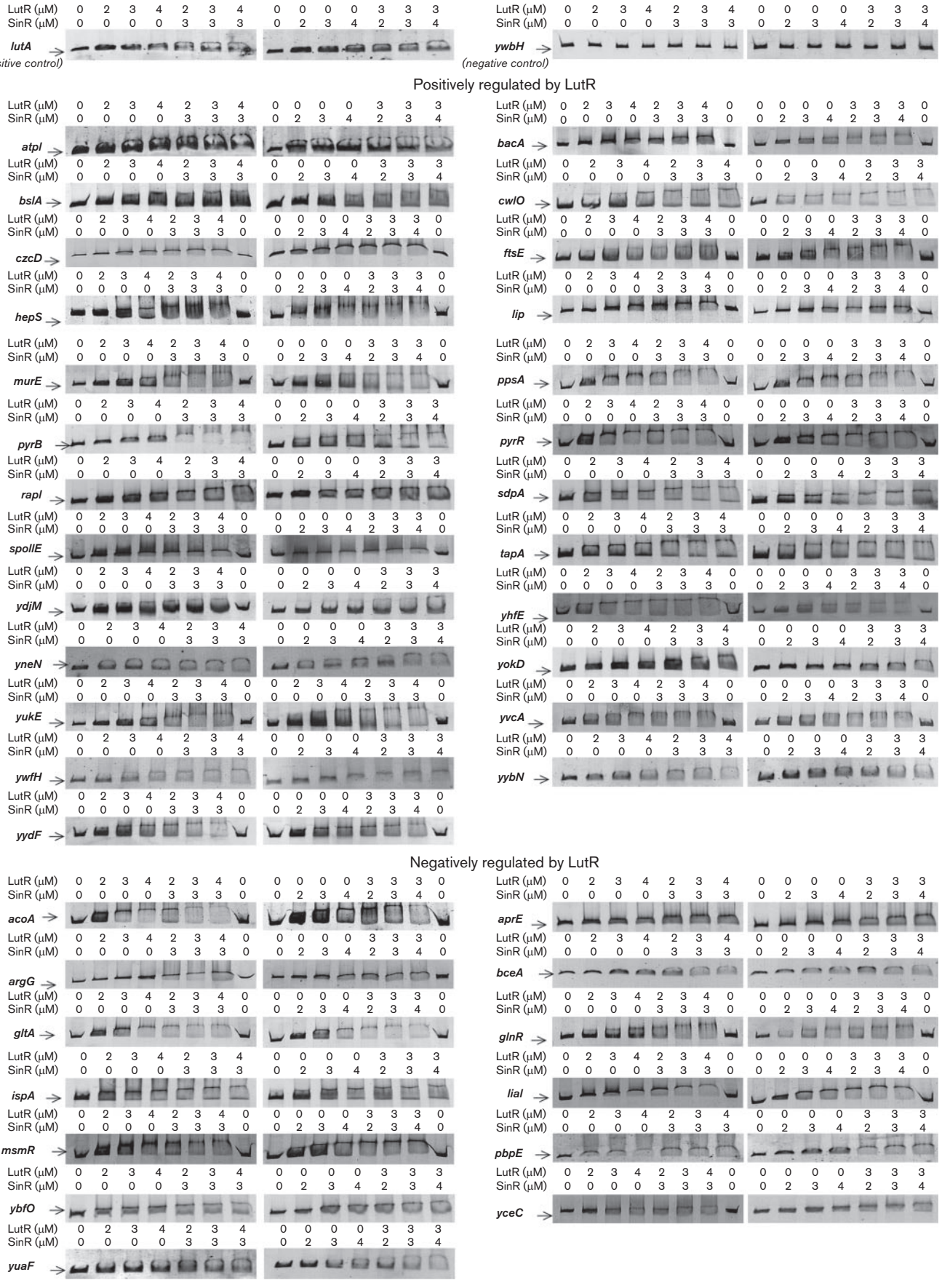
LutR (μM) 0 2 3 4 2 3 4 0 0 0 0 3 3 3
SinR (μM) 0 0 0 0 3 3 3 0 2 3 4 2 3 4
lutA
(positive control)
ywbH
(negative control)
Positively regulated by LutR
atpI
bacA
bslA
cwlO
czcD
ftsE
hepS
lip
murE
ppsA
pyrB
pyrR
rapI
sdpA
spoIIE
tapA
ydjM
yhfE
yneN
yokD
yukE
yvcA
ywfH
yybN
yydF
Negatively regulated by LutR
acoA
aprE
argG
bceA
gltA
glnR
ispA
lial
msmR
pbpE
ybfO
yceC
yuaF

Fig. 2. Regulatory regions of genes with observed gel-shift. EMSAs were performed with putative regulatory regions of target genes and various amounts of purified LutR-His_6 and SinR-His_6 alone or together, a fixed amount of one being mixed with increasing concentrations of the other as indicated. In each assay, 25 μl total reaction mixture was supplemented with competitor DNA poly(dI-dC) (1 μg μl^{-1}) and BSA (1 mg ml^{-1}). The promoter region of the unrelated gene *ywbH* was used as negative control. Positive and negative controls were run with each EMSA. For detection, gels were treated with SYBR Green I Nucleic Acid Gel Stain (1/10.000, v/v) (Roche) and visualized with a UV transilluminator. Each gel-shift assay was repeated at least two times.

required to validate the role of this putative DNA motif in binding of LutR.

Genes and processes affected by LutR

When the LutR-affected genes were grouped according to their known or presumed functions, LutR appeared to participate in the regulation of genes involved in various metabolic and physiological processes associated with the post-exponential phase in *B. subtilis* (Table 2).

Metabolism. Regarding nitrogen metabolism, transcription of genes for glutamate synthase, *gltA* and *gltB*, nitrogen regulatory protein, *glnR*, and glutamine synthase, *glnA*, and transcription of the *argGH* operon, encoding the argininosuccinate synthase and argininosuccinate lyase proteins, respectively, were identified to be directly downregulated by LutR in early stationary phase cells. On the other hand, LutR appeared to directly induce the pyrimidine nucleotide biosynthetic (*pyr*) operon (*pyrB-pyrC-pyrAA-pyrAB-pyrK-pyrD-pyrF-pyrE*). As related with carbohydrate metabolism, the *acoABCL* operon, encoding the acetoin dehydrogenase complex required for acetoin utilization, was directly repressed by LutR. It is interesting to note that expression of this operon was activated during stationary growth phase. In addition, the *msmRE* operon encoding a putative regulatory protein belonging to the LacI family (*msmR*) and a putative binding protein for the transport of multiple sugars (*msmE*) was directly downregulated, suggesting that LutR also contributes to the regulation of carbohydrate transport and utilization systems.

Cell envelope-associated activities. LutR also seems to promote cell wall synthesis by directly acting on *murE* (UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase), *mraY* (phospho-*N*-acetylmuramoyl-pentapeptide transferase), *murD* (UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase), *cwlO* (peptidoglycan DL-endopeptidase) (Yamaguchi *et al.*, 2004; Bisicchia *et al.*, 2007), energy production by directly stimulating the expression of the ATP synthesis operon (*atpIBEFHAGDC*), and the menaquinone biosynthetic operon (*hepS-menH-hepT-ndk*) (menaquinone as a component of the respiratory chain in *B. subtilis* contributes to ATP production) in the early stationary phase cells.

Extra- and intracellular enzyme production. During the transition state, *B. subtilis* produces a wide variety of degradative enzymes to scavenge alternative nutrients. Consistent with this, we found four degradative enzyme genes that are likely to be involved in the adaptation to nutrients to be under the direct positive control of LutR. These are the *lip* gene, encoding an extracellular lipase (Eggert *et al.*, 2000), the *yhfEF* operon, encoding a putative endogluconase (Kunst *et al.*, 1997), and the *cwlO* gene, encoding a cell wall lytic enzyme, D,L-endopeptidase (Yamaguchi *et al.*, 2004). These findings highlight the involvement of LutR in adaptation to conditions of nutrient deficiency. As an endogluconase, YhfE could hydrolyse glucan in plant materials to provide alternative nutrients for cells. Extracellular lipase, Lip, could be used to degrade extracellular lipids that could also be used as a carbon and energy source. In addition to its role in cell wall synthesis (Yamaguchi *et al.*, 2004), the D,L-endopeptidase CwlO was very recently shown to be also involved in the degradation of poly-γ-glutamic acid (PGA) in *B. subtilis* (*natto*) (Mitsui *et al.*, 2011) and thus could degrade extracellular PGA or cell wall materials to generate nutrients.

Antimicrobials. In this study we found that LutR positively controls the non-ribosomally synthesized lipopeptide antibiotic fengycin (plipastatin) directly. Consistently, we previously showed that LutR activity is required for non-ribosomal biosynthesis of the dipeptide antibiotic bacilysin (Köroğlu *et al.*, 2008). In this study, *lacZ* fusion analysis indicated that *lutR* mutation affects the transcription of both the *bacABCDEF-ywfG* operon and the *ywfH* gene. Although *lutR* mutation significantly reduced the maximum transcription level of the *bacABCDE-ywfG* operon at the onset of stationary phase to about 57 % of wild-type level (Fig. 3a), it had a greater impact on the transcription of *ywfH* since there was no transition-state induction of *ywfH* expression (Fig. 3b). In agreement with this, EMSA suggested that LutR acts directly on both the *bacABCDE-ywfG* operon and the *ywfH* gene. At first glance, the effect of the *lutR* mutation on transcription of the known bacilysin biosynthetic genes seems to be quite modest to be a reason for the loss of bacilysin production in *lutR* mutant strains. On the other hand, a previous study performed by Inaoka *et al.* (2003) pointed out that a greater amount of the *ywfH* gene product is required for bacilysin production. Therefore, the loss of transition-state induction of *ywfH* expression in the *lutR* mutant together with the reduced expression level of the *bacABCDE-ywfG* operon could be the reason for the loss of bacilysin production in *lutR* mutant strains. However, we cannot rule out the possibility that LutR affects the transcription of at least one other gene required for production of bacilysin in addition to its role in the transcription of the known bacilysin biosynthetic genes.

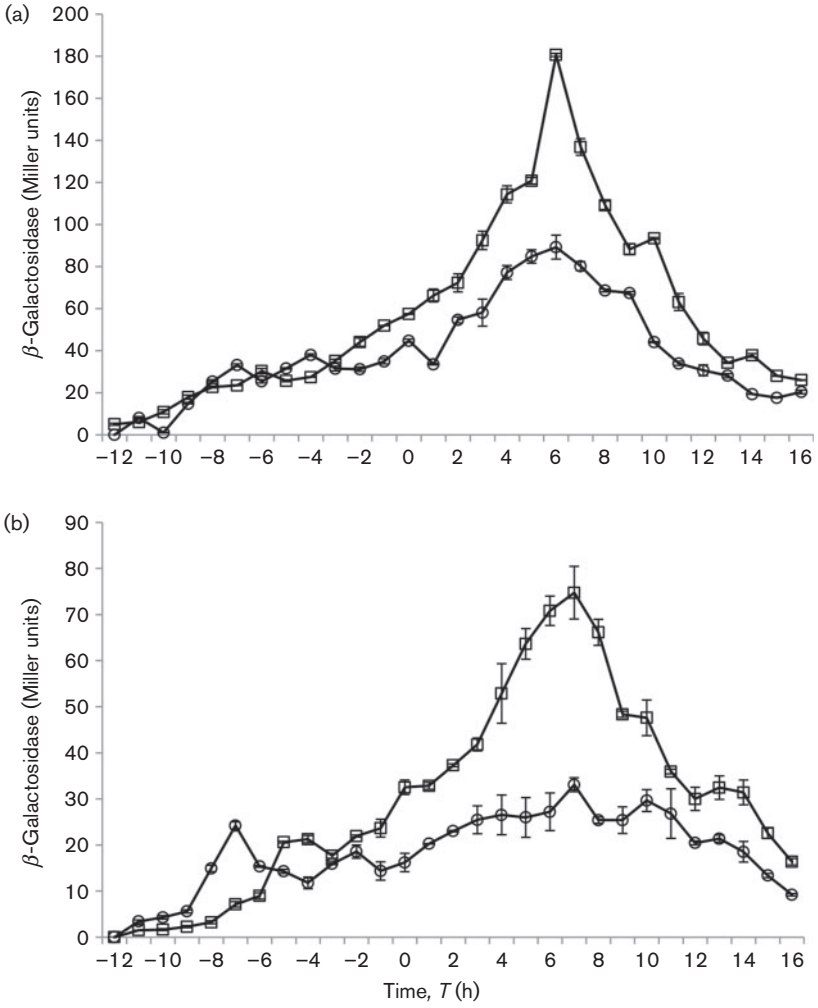

Fig. 3. Effect of the *lutR* mutation on the expression of the *bacABCDEF-ywfG* operon and *ywfH*. (a) β-Galactosidase activity of *bacA*::*lacZ* fusion (squares) and its isogenic derivative *lutR* mutant (*bacA*::*lacZ lutR*::Tn*10*::*spc*) (circles). (b) β-Galactosidase activity of the *ywfH*::*lacZ* fusion (squares) and its isogenic derivative *lutR* mutant (*ywfH*::*lacZ lutR*::Tn*10*::*spc*) (circles). *T*=0 denotes the end of exponential growth in PA medium. Error bars indicate the standard deviation of the mean of three independent experiments (*n*=3).

Similarly, LutR also appears to be involved in the production of a candidate small antimicrobial peptide YydF. Butcher *et al.* (2007) reported that the *yydFGHIJ* operon is responsible for the synthesis, modification, cleavage and export of a small modified antimicrobial peptide, YydF*, which elicits cell envelope stress sensed by the LiaRS two-component regulatory systems. In our study, in this operon, only the transcription of *yydG*, and not of *yydF* or *yydHIJ*, was found to be significantly affected by the *lutR* mutation. However, our EMSA indicated that the *yydFGHIJ* operon is directly regulated by LutR. The reading frame of *yydF* with 147 bp seems to be quite small for reliable detection on arrays (Britton *et al.*, 2002). Additionally, *yydHIJ* has been previously described to be under direct negative control of Rok (repressor of ComK) (Albano *et al.*, 2005). Most probably *yydHIJ* are not significantly expressed under the growth conditions used in this study.

Our observation that the expression of *yokD* is directly stimulated by LutR suggests that LutR might contribute to development of resistance to aminoglycoside antibiotics. A study performed by Hoffman *et al.* (2005) indicated that *yokD* encodes a putative aminoglycoside acetyltransferase which may be a component of the bacterial aminoglycoside resistance mechanism.

Mobile genetic elements. Auchtung *et al.* (2005) reported that excision and transfer of ICE*Bs1*, a 20 kb integrative and conjugative element found in the chromosome of *B. subtilis*, are regulated by two proteins encoded by ICE*Bs1*: a Rap protein, RapI, and a Phr peptide, PhrI. In agreement with our microarray study, *rapI–lacZ* transcriptional fusion analysis showed that the expression of *rapI* was highly derepressed in *lutR* mutant cells (Fig. 4). Futhermore, purified LutR-His_6 interacted directly with the promoter region of *rapI* (Fig. 2). Based on these findings, we concluded that LutR contributes to the regulation of the transfer of a mobile genetic element ICE*Bs1* in *B. subtilis*, as a novel direct negative regulator for *rapI*.

It was previously shown that the *yukE*, *yukD*, *yukC*, *yukAB* and *yueB* genes are organized as an operon in *B. subtilis* which is required for irreversible adsorption of SPP1 (São-José *et al.*, 2004). We found that *yukE*, *yukC* and *yukAB* genes were significantly stimulated by LutR via direct

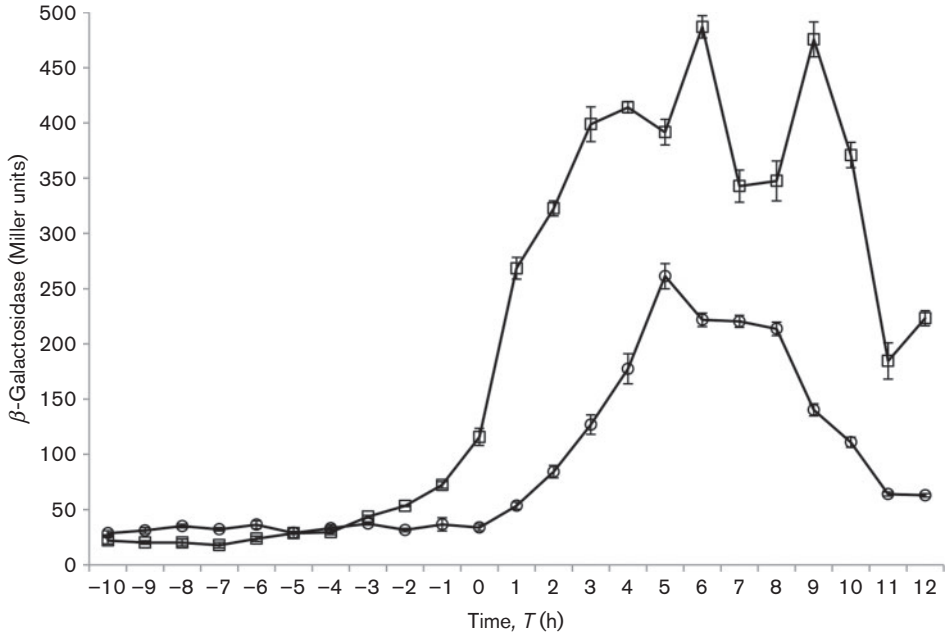

Fig. 4. Effect of *lutR* mutation on the expression of the *rapI* gene. β-Galactosidase activity of the *rapI*::*lacZ* fusion (squares) and its isogenic derivative *lutR* mutant (*rapI*::*lacZ lutR*::Tn*10*::*spc*) (circles). $T=0$ denotes the end of exponential growth in PA medium. Error bars indicate the standard deviation of the mean of three independent experiments ($n=3$).

binding. Very recently, Blom *et al.* (2011) reported that in *B. subtilis* cells, SigB regulon and phage-related genes covering the *yukE* operon are rapidly activated during the transition point in *B. subtilis* cells grown in rich medium that occurs as a stress response due to nutrient limitation at the end of the exponential growth phase. In agreement with its rapid induction profile at the transition phase, LutR seems to participate in the regulation of such a natural stress response.

Sporulation delay. The transcription of the *sdpABC* operon, also known as the 'sporulation delay operon' is affected directly by LutR in a positive manner. This operon is responsible for the production and export of the toxin protein SdpC, which participates in a killing process of non-sporulating Spo0A-inactive siblings termed cannibalism (González-Pastor *et al.*, 2003). It also behaves as a signalling molecule by inducing the transcription of the *sdpRI* immunity operon which protects the toxin-producing, Spo0A-active cells from being killed (Ellermeier *et al.*, 2006). Additionally, SdpR also delays sporulation in Spo0A-active cells, likely by activating lipid catabolism and ATP-producing enzymes, thereby increasing the energy production (González-Pastor *et al.*, 2003). Indeed, Spo0A was recently shown to link *de novo* fatty acid synthesis to sporulation and biofilm formation (Pedrido *et al.*, 2013). Consistent with this, the expression of the sporulation delay operon (*sdpABC*), the ATP synthesis operon and the menaquinone biosynthetic operon, together with the expression of the *lip* gene, encoding extracellular lipase, were upregulated by LutR in our study.

Finally, we found that in the absence of LutR, the transcription of the *ftsEX* operon, encoding an ABC transporter, was significantly downregulated and this operon was found to be a direct target of LutR. It has been shown that FtsEX deficiency delays induction of phosphorelay and, as a consequence, postpones Spo0A activation and thus delays sporulation (Garti-Levi *et al.*, 2008). Thus, to confirm the stimulatory effect of LutR on FtsEX, we investigated whether a *lutR* mutant has the same effect as an *ftsEX* mutant on the activation of the early sporulation gene *spoIIE* (Garti-Levi *et al.*, 2008), which is directly dependent on phosphorylated Spo0A (Errington, 2003). As shown in Fig. S3, similar to that in *ftsEX* mutant cells, activation of *spoIIE*-directed *lacZ* expression was delayed by 1 h in *lutR* mutant cells grown in DSM medium. Subsequently, EMSA analysis using the promoter region of *spoIIE* revealed that LutR might also directly affect *spoIIE* transcription (Fig. 2).

Biofilm formation. The main structural components of *B. subtilis* biofilm matrix are an exopolysaccharide polymer produced by the products of the 15-gene *epsA–O* operon and TasA amyloid fibres synthesized by the products of the *tapA-sipW-tasA* operon (Vlamakis *et al.*, 2013). Strikingly, based on our study, expression of the *tapA* operon is directly upregulated by the LutR protein while the expression of three *eps* genes, *epsD*, *epsE* and *epsK*, is indirectly upregulated by LutR. Consistently, the biofilm-related *lutABC* operon was previously shown to be under the dual control of LutR and SinR (Chai *et al.*, 2009). In addition, two DegU-regulated

genes required for complex colony architecture (Verhamme *et al.*, 2007; Kovács & Kuipers, 2011) are upregulated by LutR via direct binding. These are the *yvcA* gene, encoding a putative membrane-bound lipoprotein, and the *bslA* gene, encoding a small amphiphilic protein that forms a hydrophobic layer on the surface of biofilms (Kobayashi & Iwano, 2012; Hobley *et al.*, 2013), suggesting a protective function for BslA (Kovács *et al.*, 2012). Taken together, all these results strongly suggest that, as a regulator, LutR might affect the complex colony and/or pellicle architecture. To test this possibility, we constructed a *lutR* mutant NCIB3610 strain and then we monitored its complex colony and pellicle formations on MSgg medium (with glycerol as a carbon source). Although the *lutR* mutant exhibited a reduced colony size, there was no drastic defect in complex colony formation, but the colony architecture of the *lutR* mutant was significantly altered: the thickness of the wrinkled structures was considerably increased and the quantity of the wrinkled structures relatively reduced (Fig. 5). Consistently, *lutR* mutation did not affect the initiation of pellicle formation; however, the *lutR* mutant formed a very thick and smooth pellicle which lacks a distinctive macroscopic architecture (Fig. 5). Subsequently, by performing RT-qPCR analysis we confirmed that, as in the case of the domesticated laboratory strain, the expression of *bslA, yvcA* and the *tapA* operon is significantly reduced in the *lutR* mutant of the NCIB3610 strain grown on MSgg medium (data not shown), supporting the regulatory role of LutR in biofilm development.

Cell envelope stress. Interestingly, in our study, the expression of not only the gene for σ^W itself, but also many genes of the σ^W regulon (involved in the detoxification of and resistance to antibiotics and other agents eliciting cell envelope stress), such as *ybfO* (putative erythromycin esterase), *pbpE* (encoding a penicillin-binding protein, PBP4 endopeptidase), the *yuaF-floT-yuaI* operon (encoding a putative acetyltransferase) and the *yceCDEFGH* operon (encoding putative stress adaptation proteins similar to tellurium-resistance proteins), was found to be repressed by LutR via direct binding (Table 3). On the other hand, *sigW* was indirectly affected. The gene *sigW* itself, as well as some of the σ^W-dependent genes including *pbpE*, are known to be directly repressed by the transition-state regulator AbrB (Qian *et al.*, 2002; Huang *et al.*, 1999). Correspondingly, two AbrB-repressed loci, the *liaIHGFSR* operon and the subtilisin E-encoding gene, *aprE*, are directly repressed by LutR (Table 2). The *lia* operon is strongly induced in response to cell wall acting antibiotics such as vancomycin, bacitracin and nisin by the LiaRS two-component system (Mascher *et al.*, 2004) and contributes to nisin resistance in *B. subtilis* 168 (Hansen *et al.*, 2009). Taken together, these findings point to a significant overlap between the LutR and AbrB regulons.

A close regulatory interaction between LutR and SinR

It has been previously suggested that LutR and SinR act cooperatively to repress *lutABC* (Chai *et al.*, 2009). Based on this notion, we first checked whether the promoter of the *lutABC* operon (P_{lutA}) is the direct target of these regulators and/or whether they would stimulate each other's binding to P_{lutA}, which has not to our knowledge been examined before. For this, EMSAs were performed with purified SinR and LutR alone and together, in which a fixed amount of one protein was mixed with increasing concentrations of the other. The results demonstrate that SinR and LutR significantly stimulate each other's binding to P_{lutA}, and either SinR or LutR alone exhibits low affinity for binding to P_{lutA} (see Supplementary Methods) (Fig. 2). Besides the *lutABC* operon, the *tapA* operon and *aprE* were found to be under direct control of LutR. It has previously been published that these are directly regulated by SinR (Chu

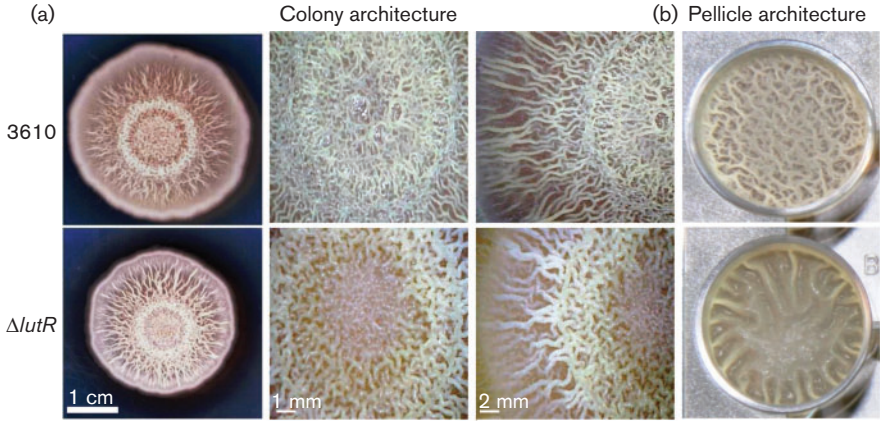

Fig. 5. Colony (a) and pellicle (b) architecture of wild-type NCIB3610 and its isogenic *lutR* mutant NCIB3610LR grown on MSgg medium. Images shown were taken after 72 h of growth at 30 °C. The experiments were repeated at least three times and only one of the sets is shown. Colonies were photographed with a digital camera.

Table 3. Functional classification of genes that are regulated by LutR at the onset of stationary phase and that display an overlap between σ^W and AbrB regulons

Gene	Microarray*	qPCR†	EMSA‡	Function	Transcriptional organization§
Detoxification					
ybfO	−1.76	−1.08 (±0.06)	+	Hypothetical protein; similar to erythromycin esterase	$\overrightarrow{psd}$ $\overrightarrow{ybfN}$ ≠ $\overrightarrow{ybfO}$ ≠ $\overrightarrow{ybfP}$ $\overrightarrow{ybfQ}$ ≠
yceC	−1.00	−0.88 (±0.06)	+	Putative stress adaptation protein	≠ $\overrightarrow{ycdI}$ $\overrightarrow{yceA}$ ≠ $\overrightarrow{yceB}$ $\overrightarrow{yceC}$ $\overrightarrow{yceD}$ $\overrightarrow{yceE}$ $\overrightarrow{yceF}$ $\overrightarrow{yceG}$ $\overrightarrow{yceH}$
yceD	−1.44			Putative stress adaptation protein	≠ $\overrightarrow{yceI}$
yceE	−1.39			Putative stress adaptation protein	
yceF	−1.73	−1.38 (±0.08)		Putative stress adaptation transporter	
yceH	−1.43			Putative stress adaptation protein	
bceA	ND	−2.68 (±0.32)	+	Bacitracin ABC efflux transporter ATP-binding protein	≠ $\overrightarrow{yttB}$ $\overrightarrow{yttA}$ ≠ $\overrightarrow{bceB}$ $\overrightarrow{bceA}$ $\overrightarrow{ytsB}$ $\overrightarrow{ytsA}$ ≠ $\overrightarrow{ytrF}$
bceB	ND	−3.77 (±0.06)		Bacitracin export permease protein BceB	
yuaF	−1.72	−1.12 (±0.09)	+	Membrane integrity integral inner-membrane protein	$\overrightarrow{yuaJ}$ ≠ $\overrightarrow{yuaI}$ $\overrightarrow{floT}$ $\overrightarrow{yuaF}$ $\overrightarrow{yuaE}$ ≠ $\overrightarrow{yuaD}$ ≠
floT	−1.46			Putative flotillin-like protein	
yuaI	−1.62			Putative acetyl-transferase	
liaI	−1.65	−1 (±0.05)	+	Putative transmembrane protein	$\overleftarrow{liaR}$ $\overleftarrow{liaS}$ $\overleftarrow{liaF}$ $\overleftarrow{liaG}$ $\overleftarrow{liaH}$ $\overleftarrow{liaI}$ ≠ $\overleftarrow{yvqJ}$ ≠ $\overleftarrow{yvqK}$
liaH	−1.78			Similar to phage-shock protein A (PspA) of *E. coli*	
liaG	−1.31			Putative membrane-anchored hypothetical protein	
liaF	−1.05			Membrane protein	
liaS	−1.02			LiaRS: two-component regulatory system	
liaR	−0.85				
Gene expression initiation and regulation					
abh‖	NR	−4.57 (±0.28)	−	Transcriptional regulator of transition state genes (AbrB-like)	≠ $\overrightarrow{ykpC}$ $\overrightarrow{mreBH}$ $\overrightarrow{abh}$ ≠ $\overrightarrow{kinC}$ $\overrightarrow{ykqA}$
sigW	ND	−4.31 (±0.01)	−	RNA polymerase σ factor σ^W	*trnSL-Gln2* ≠ $\overrightarrow{sigW}$ $\overrightarrow{ybbM}$ ≠ $\overrightarrow{ybbP}$
ybbM	ND			Hypothetical protein	
Sporulation					
pbpE	ND	−3.44 (±1.13)	+	Penicillin-binding protein 4‖ (spore cortex)	≠ $\overrightarrow{racX}$ $\overrightarrow{rbpE}$ $\overrightarrow{sacB}$ ≠ $\overrightarrow{yveB}$ ≠
racX	ND			Amino acid racemase	

Genes in an operon are grouped together.

NR, No reproducible data obtained.

ND, No differential expression observed.

*Numbers indicate the $\log_2$ transformed expression ratios (in the wild-type versus the TEK1 strain).

†Numbers indicate the $\log_2$ transformed expression ratios. The mean of a minimum of three independent replicate experiments is given and standard deviation of the mean is shown in parentheses.

‡A '+' indicates that gel retardation was observed.

§Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/; '≠' indicates the termination sites.

‖Bayes *P*-value higher than acceptable value (>0.01).

et al., 2006). Consequently, we wondered whether SinR would interact with the regulatory regions of all or only some LutR-target genes and/or they would affect each other's binding. For this, we applied EMSA analysis as described above with the regulatory regions of all of the LutR-target genes identified. To validate our EMSA analysis, each EMSA was repeated at least two times and the regulatory region of the unrelated *ywbH* was used as a negative control. Interestingly, the results of the EMSA analysis showed that SinR is capable of interacting with the regulatory regions of all of the LutR-target genes tested, but they exhibited variations in the nature of their interactions. (i) As in the case of P_{lutA}, they apparently stimulate each other's binding to the regulatory regions of *lip and bslA*. (ii) They exhibit additive or simultaneous binding to the regulatory regions of *acoA*, *argG*, *aprE*, *atpI*, *bacA*, *bceA*, *czcD*, *cwlO*, *ftsE*, *glnR*, *gltA*, *hepS*, *ispA*, *liaI*, *msmR*, *mraY*, *pbpE*, *ppsA*, *pyrB*, *pyrR*, *rapI*, *sdpA*, *tasA*, *ybfO*, *yceC*, *ydjM*, *yneN*, *yhfE*, *ywfH*, *yuaF*, *yukE*, *yvcA*, *yokD*, *yydF* and *yybN*. (iii) In the case of the *spoIIE* genes, only LutR apparently stimulates the binding capacity of SinR (see Supplementary Methods for more details) (Fig. 2). Conclusively, our overall data revealed a close relationship between the LutR and SinR regulators. Most likely they collectively fine-tune the level and timing of expression of genes involved in post-exponential phase processes as an important part of a complex interconnected regulatory system. Interestingly, SinR binding to the *tapA* promoter is sequestered by SlrR (Chai *et al.*, 2010), suggesting that DNA binding of SinR to specific promoter regions is modulated in different ways by distinct regulators.

CONCLUSIONS

Our work indicates that LutR is a pleiotropic regulator involved in the regulation of a wide variety of cellular processes associated with the onset of stationary phase, such as degradative enzyme production, antibiotic production and resistance, carbohydrate utilization and transport, transfer of mobile genetic elements, induction of phage-related genes, sporulation, sporulation delay and cannibalism, and biofilm formation, by acting either in a negative or in a positive manner. Our overall results gathered from transcriptional profiling studies and EMSA indicate a close target overlap between the LutR and SinR regulators besides a significant overlap with the AbrB regulon, which emphasize the important role of LutR within the complex interconnecting regulatory systems governing adaptation at the onset of stationary phase. LutR belongs to the GntR family, in which the binding of a cognate ligand by a small-molecule-binding domain results in a conformational change which influences the DNA-binding properties of the transcription factor, and subsequently results in activation or repression of transcription. Consequently, L-lactate could be a central signalling molecule in the LutR-mediated nutrient sensing system involved in the regulation of various cellular processes, and should be elucidated further.

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