Transcriptional regulation of the L-lactate permease gene lutP by the LutR repressor of Bacillus subtilis RO-NN-1

Kuo-Chin Chiu, Chen-Jyun Lin and Gwo-Chyuan Shaw

The Bacillus subtilis lutABC operon encodes three iron–sulfur-containing proteins required for L-lactate utilization and involved in biofilm formation. The transcriptional regulator LutR of the GntR family negatively controls lutABC expression. The lutP gene, which is situated immediately upstream of lutR, encodes an L-lactate permease. Here, we show that lutP expression can be strongly induced by L-lactate and is subject to partial catabolite repression by glucose. Disruption of the lutR gene led to a strong derepression of lutP and no further induction by L-lactate, suggesting that the LutR repressor can also negatively control lutP expression. Electrophoretic mobility shift assay revealed a LutR-binding site located downstream of the promoter of lutA or lutP and containing a consensus inverted repeat sequence 5′-TCATC-N₉-GATGA-3′. Reporter gene analysis showed that deletion of each LutR-binding site caused a strong derepression of lutA or lutP. These results indicated that these two LutR-binding sites can function as operators in vivo. Moreover, deletion analysis identified a DNA segment upstream of the lutP promoter to be important for lutP expression. In contrast to the truncated LutR of laboratory strains 168 and PY79, the full-length LutR of the undomesticated strain RO-NN-1, and probably many other B. subtilis strains, can directly and negatively regulate lutP transcription. The absence or presence of the N-terminal 21 aa of the full-length LutR, which encompass a small part of the predicted winged helix–turn–helix DNA-binding motif, may probably alter the DNA-binding specificity or affinity of LutR.

INTRODUCTION

L-Lactate is reductively converted from pyruvate—a metabolite generated from glucose by glycolysis. A wide variety of bacteria can utilize L-lactate as a sole carbon and energy source (Dong et al., 1993; Gibello et al., 1999; Goffin et al., 2004; Stansen et al., 2005; Chai et al., 2009; Pinchuk et al., 2009; Thomas et al., 2011; Gao et al., 2012b). L-Lactate uptake and utilization are important not only for energy production, but also for multicellular behaviours and bacterium–host interactions. L-Lactate acquisition is known to promote successful colonization of Neisseria gonorrhoeae in the murine genital tract (Exley et al., 2007). In N. meningitidis, L-lactate uptake can promote the production of certain determinants of pathogenicity (Exley et al., 2005). Acquisition of L-lactate is also known to be required for Haemophilus influenzae to cause bacteremia (Herbert et al., 2002). In Bacillus subtilis, L-lactate uptake and utilization are important for biofilm formation (Chai et al., 2009).

The lldPRD operon of Escherichia coli K-12 encodes the L-lactate permease LldP, the regulator LldR and the L-lactate dehydrogenase LldD (Dong et al., 1993). The L-lactate-responsive regulator LldR negatively regulates expression of the lldPRD operon through binding to two operator sites containing inverted repeats with the putative consensus sequence 5′-AATTGGCA-N₉-TGCCAATT-3′ (Aguilera et al., 2008). The lldPDE operon of Pseudomonas aeruginosa XMG comprises genes encoding the lactate permease LldP, the L-lactate dehydrogenase LldD and the D-lactate dehydrogenase LldE (Kemp, 1972; Gao et al., 2012a). The lldPDE operon is located adjacent to the lldR gene, which codes for a GntR-type regulator LldR. Both L-lactate and D-lactate can interfere with the binding of the LldR repressor to its operator, which contains an inverted repeat sequence 5′-TGTT-N₉-ACCA-3′ (Gao et al., 2012a). In the non-pathogenic Gram-positive bacterium Corynebacterium glutamicum ATCC 13032, the L-lactate utilization operon lldPD comprises the L-lactate permease gene lldP and the L-lactate dehydrogenase gene lldD (Stansen et al., 2005).
The GntR-type regulator LdLR negatively regulates expression of the \( \text{l}-\text{lactate-inducible} \) \( \text{l}-\text{lactate} \) utilization operon. An imperfect inverted repeat sequence 5′-TNGT-N3-ACNA-3′ can serve as an operator for the LdLR repressor (Gao et al., 2008; Georgi et al., 2008). The \( \text{lutABC} \) operon of the undomesticated strain NCIB 3610 of \( B. \text{subtilis} \) encodes three iron–sulfur-containing proteins responsible for \( \text{l}-\text{lactate} \) utilization (Chai et al., 2009). The expression of the \( \text{lutABC} \) operon is under the negative control of the LutR repressor and is induced strongly by \( \text{l}-\text{lactate} \), but not by \( \text{D}-\text{lactate} \) (Chai et al., 2009). The \( \text{lutR} \) gene is separated from the \( \text{lutABC} \) operon in the \( B. \text{subtilis} \) chromosome by ~15 kb (Chai et al., 2009). The \( \text{lutP} \) gene, which is situated immediately upstream of and divergently transcribed from \( \text{lutR} \), encodes an \( \text{l}-\text{lactate} \) permease (Chai et al., 2009). However, it is unknown whether LutR can regulate \( \text{lutP} \) expression. A recent report showed that the LutR protein of \( B. \text{subtilis} \) laboratory strain PY79 is a global regulator that can positively or negatively control expression of >150 genes (Irigul-Sonmez et al., 2014). A highly TC-rich sequence 5’-TTCCCTCCTTTTTTTTTT-3’ is proposed to be the consensus binding sequence for LutR (Irigul-Sonmez et al., 2014). However, no regulation of expression of \( \text{lutABC} \) and \( \text{lutP} \) by LutR was observed in this strain (Irigul-Sonmez et al., 2014). Here, we show that the LutR protein of the undomesticated strain RO-NN-1 of \( B. \text{subtilis} \) could efficiently negatively regulate \( \text{lutP} \) transcription.

- **METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The oligonucleotide primers are listed in Table S1. \( E. \text{coli} \) strain DH5\( \alpha \) was used as a host for cloning purposes: \( E. \text{coli} \) strain JM109 was used as a host for the heterologous production of His-tagged LutR from a pQE80-based vector. \( E. \text{coli} \) and \( B. \text{subtilis} \) cells were grown in Luria–Bertani (LB) medium, which contained tryptone (10 g l\(^{-1}\)), yeast extract (5 g l\(^{-1}\)) and NaCl (5 g l\(^{-1}\)), or in modified M9 minimal medium, which consisted of 0.05% NaCl, 0.05% MgSO\(_4\), 0.1% NH\(_4\)Cl, 0.0001% FeSO\(_4\), 0.0002% MnSO\(_4\), 0.002% CaCl\(_2\), 0.3% KH\(_2\)PO\(_4\) and 1.7% NaHPO\(_4\)·12H\(_2\)O, supplemented with 0.4% glycerol as the carbon and energy source. In the case of analysis of catabolite repression, 0.4% sodium citrate was used instead of 0.4% glycerol. The antibiotics used were ampicillin (100 μg ml\(^{-1}\)), chloramphenicol (5 μg ml\(^{-1}\)), erthyromycin (1 μg ml\(^{-1}\)) and kanamycin (10 μg ml\(^{-1}\)).

**Construction of plasmids.** To construct plasmid pGS2471 for the disruption of \( \text{lutR} \), a DNA fragment (+46 to +352 relative to the translational start site of \( \text{lutP} \)) was amplified by PCR with the primer pair A961 plus A968, and then cloned between HindIII and BamHI sites of the thermosensitive plasmid pRN5101 (Fedhila et al., 2002). To construct plasmid pGS2614 for the disruption of \( \text{lutP} \), a DNA fragment (+6 to +352 relative to the translational start site of \( \text{lutP} \)) was amplified by PCR with the primer pair B098 plus B099, and then cloned between HindIII and BamHI sites of the thermosensitive plasmid pRN5101.

To construct plasmids pGS2530, pGS2552 and pGS2554, which carried transcriptional fusions of distinct lengths of the regulatory region of \( \text{lutA} \) (~405 to ~218, ~390 to ~53 and ~380 to ~21, respectively) to \( \text{laczZ} \), DNA fragments were amplified by PCR with primer pairs A997 plus B019, B011 plus B102 and B101 plus B111, respectively, cleaved with EcoRI plus BamHI, and inserted individually into the integrative promoter probe vector pDG1661 (Guérout-Fleury et al., 1996). To construct plasmid pGS2700, which carried a transcriptional fusion of both the regulatory region and the coding region of \( \text{lutA} \) (~380 to ~771) to \( \text{laczZ} \), a DNA fragment was amplified by PCR with primer pairs B011 plus B341, cleaved with EcoRI plus BamHI, and inserted into pDG1661. To construct plasmids pGS2539, pGS2587, pGS2588, pGS2610, pGS2611, pGS2628 and pGS2673, which carried transcriptional fusions of various lengths of the regulatory region of \( \text{lutA} \) to \( \text{laczZ} \), DNA fragments were amplified by PCR with primer pairs B087 plus A955, B087 plus B154, B087 plus B155, B202 plus B155, B203 plus B155, B242 plus B155 and B243 plus B155, respectively, and then inserted between EcoRI and BamHI sites of pDG1661. To construct plasmid pGS2617, which carried a transcriptional fusion of the regulatory region and a part of the coding region of \( \text{lutR} \) (~227 to ~151) to \( \text{laczZ} \), a DNA fragment was amplified by PCR with primer pair B208 plus B207, and then inserted between EcoRI and BamHI sites of pDG1661.

To construct plasmids pGS2639, pGS2674 and pGS2675, which carried a 4 bp mutation (TATA to ATAT) in the ~10 boxes of the predicted \( \sigma^\text{d} \) promoters of \( \text{lutP} \), \( \text{lutA} \) and \( \text{lutR} \), respectively, a two-step PCR method (Higuchi et al., 1988) using primers A955, B087, B257 and B258 (for pGS2639), primers B019, B101, B307 and B308 (for pGS2674), and primers B207, B208, B309 and B310 (for pGS2675), respectively, was employed for site-directed mutagenesis. The PCR-amplified DNA fragments were then restricted with EcoRI plus BamHI, and cloned individually into pDG1661. To construct plasmids pGS2697, pGS2702 and pGS2704, which carried a 3 bp mutation (TGG to AAC) for pGS2697, TGG to AAC for pGS2702 and TTTT to AAAT for pGS2704, respectively) in the ~35 boxes of the predicted \( \sigma^\text{d} \) promoters of \( \text{lutP} \), \( \text{lutA} \) and \( \text{lutR} \), respectively, a two-step PCR method using primers A955, B087, B332 and B333 (for pGS2697), primers B019, B101, B334 and B335 (for pGS2702), and primers B207, B208, B354 and B355 (for pGS2704), respectively, was employed for site-directed mutagenesis. The PCR-amplified DNA fragments were then cleaved with EcoRI plus BamHI, and cloned individually into pDG1661.

To construct plasmid pGS2706, which carried a 2 bp mutation (CG to GC) in the highly conserved central CG of a putative catabolite-responsive element (CRE) sequence in the regulatory region of \( \text{lutP} \) in plasmid pGS2539, a two-step PCR method using primers A955, B087, B357 and B358 was employed for site-directed mutagenesis. The PCR-amplified DNA fragments were then cleaved with EcoRI plus BamHI, and cloned into pDG1661.

To construct plasmid pGS2576 for overproduction of the His-tagged full-length LutR of \( B. \text{subtilis} \) RO-NN-1 in \( E. \text{coli} \), a DNA fragment carrying the coding sequence of \( \text{lutR} \) was amplified by PCR using the primer pair B074 plus B040, and ligated into BamHI/HindIII-cleaved pQE80 (Qiagen). To construct plasmid pGS2699 for overproduction of the His-tagged truncated LutR in \( E. \text{coli} \), a DNA fragment was amplified by PCR using the primer pair B342 plus B343, and ligated into NcoI/BamHI-cleaved pQE80 (Qiagen). To construct plasmid pGS2701 for expression of the His-tagged full-length LutR from the
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
<td>DH5a</td>
<td>F− Δ (lacZYA−argF) recA1 gyrA endA1 relA1 supE44 hsdR17 lacZD153 M15 traD36 proAB+ lacIq lacZD153 M15</td>
<td>Laboratory stock</td>
</tr>
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<td>Takara</td>
</tr>
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<td><strong>B. subtilis strains</strong></td>
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<td>RO-NN-1</td>
<td>WT</td>
<td>Earl <em>et al.</em> (2012); Bacillus Genetic Stock Center (Columbus, OH, USA)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pDG1661</td>
<td>Promoter probe vector carrying a lacZ reporter gene for ectopic integration at the anyE locus; Ap&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Guéroult-Fleury <em>et al.</em> (1996)</td>
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<tr>
<td>pGS1051</td>
<td>Expression vector carrying the IPTG-inducible P&lt;sub&gt;pol&lt;/sub&gt; promoter; Ap&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lin <em>et al.</em> (2012)</td>
</tr>
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<td>pQE60</td>
<td>IPTG-inducible expression vector for producing C-terminally His-tagged proteins in <em>E. coli</em>; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Qiagen</td>
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<tr>
<td>pQE80</td>
<td>IPTG-inducible expression vector for producing N-terminally His-tagged proteins in <em>E. coli</em>; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Qiagen</td>
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<tr>
<td>pRN5101</td>
<td>Vector used for gene disruption; Ap&lt;sup&gt;R&lt;/sup&gt; Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Fedhila <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>pGS2471</td>
<td>pRN5101 carrying an internal region close to the N terminus of <em>lutR</em>; lutR disruption plasmid</td>
<td>This work</td>
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<td>pGS2530</td>
<td>pDG1661 carrying the regulatory region of <em>lutA</em> (−405 to −18 relative to the translational start site)</td>
<td>This work</td>
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<tr>
<td>pGS2539</td>
<td>pDG1661 carrying the regulatory region of <em>lutP</em> (−250 to −1)</td>
<td>This work</td>
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<tr>
<td>pGS2552</td>
<td>pDG1661 carrying a part of the regulatory region of <em>lutA</em> (−380 to −33)</td>
<td>This work</td>
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<td>pGS2554</td>
<td>pDG1661 carrying a part of the regulatory region of <em>lutA</em> (−380 to −21)</td>
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<td>pGS2576</td>
<td>pQE80 carrying the full-length <em>lutR</em> gene</td>
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<tr>
<td>pGS2587</td>
<td>pDG1661 carrying a part of the regulatory region of <em>lutP</em> (−250 to −51)</td>
<td>This work</td>
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<td>pGS2588</td>
<td>pDG1661 carrying a part of the regulatory region of <em>lutP</em> (−250 to −75)</td>
<td>This work</td>
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<td>pGS2610</td>
<td>pDG1661 carrying a part of the regulatory region of <em>lutP</em> (−380 to −75)</td>
<td>This work</td>
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</table>
IPTG-inducible expression vector pGS1051 (Lin et al., 2012) in B. subtilis for complementation analysis, a DNA fragment was amplified by PCR using plasmid pGS2576 as template and primer B073 plus B338. After digestion with HindIII and KpnI, the DNA fragment was ligated into pGS1051.

The correctness of sequences of PCR-amplified DNA fragments was verified by DNA sequencing.

Construction of the lutR or lutP disruption mutant. Disruption of the chromosomal lutR or lutP gene by integration of plasmid pGS2471 or pGS2614 through a Campbell-like single-crossover recombination was performed as described previously (Fedhila et al., 2002). The integration plasmid was introduced into competent B. subtilis cells by the method described previously (Conteste & Dubnau, 1979). The correctness of integrants was verified by PCR.

Construction of strains with a lacZ fusion integrated at the amyE locus. Competent cells of B. subtilis were prepared and transformed by pDG1661-derived integrative plasmids as described previously (Conteste & Dubnau, 1979). Transformants were selected by growth on chloramphenicol-containing LB agar plates. Correct integration was verified by PCR.

Overproduction and purification of His-tagged LutR. Overproduction of His-tagged full-length LutR and His-tagged truncated LutR in E. coli and purification by affinity chromatography on a Ni²⁺-nitrilotriacetic acid agarose column were carried out exactly as described previously (Lin et al., 2014).

Electrophoretic mobility shift assay (EMSA). A DNA fragment spanning positions −211 to +50 (relative to the translational start site of lutP and containing the inverted repeat) and a DNA fragment spanning positions −211 to −75 (with deletion of the inverted repeat) were amplified by PCR using primer pairs B111 plus B150 and B102 plus B150, respectively. After digestion by HindIII and KpnI, these probes in the binding solution (final volume 30 μl) containing 40 mM Tris/HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.01% BSA and 0.1 μg poly(dI–dC)–poly(dI–dC). After incubation at room temperature for 20 min, the mixtures were run on a 6% native polyacrylamide gel. Bands were visualized by using a Molecular Dynamics PhosphorImager. The phosphorimage was analysed with ImageQuant software (GE Healthcare).

Other methods. Activity of β-galactosidase (LacZ) was measured as described previously (Miller, 1972). Protein concentrations were determined by the bicinchoninic acid protein assay according to assay kit manufacturer’s instructions (Pierce Biotechnology) with BSA as the standard.

RESULTS AND DISCUSSION

Analysis of lutR genes from various B. subtilis strains

The lutR genes from various B. subtilis strains, including NCIB 3610 (GenBank accession number KJ580506), ROMN-1 (Earl et al., 2012), BAB-1 (GenBank accession number YP_007664070), BEST195 (Nishito et al., 2010), BSN5 (Deng et al., 2011), MB73/2 (GenBank accession number EME08645), W23 (Zeigler, 2011) and XF-1 (Guo et al., 2013), encode a transcriptional regulator of 240 aa belonging to the GntR family [National Center for Biotechnology Information (NCBI) annotation]. The regulators of the GntR family contain a DNA-binding domain at the N terminus of the protein and an effector-binding domain at

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Table 1. cont.

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<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<td>pGS2701</td>
<td>pDG1661 carrying an lutR allele expressing the His-tagged full-length LutR</td>
<td>This work</td>
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<td>pGS2702</td>
<td>pGS2530 carrying a mutation in the −35 hexamer of the lutA promoter</td>
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<td>pGS2704</td>
<td>pGS2539 carrying a mutation in the −35 hexamer of the lutP promoter</td>
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<td>pGS2706</td>
<td>pGS2539 carrying a mutation in the CRE of the regulatory region of lutP</td>
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<td>pGS2510 carrying a mutation in the −35 hexamer of the lutA promoter</td>
<td>This work</td>
</tr>
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<td>pGS2700</td>
<td>pGS2510 carrying a mutation in the −35 hexamer of the lutP promoter</td>
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</tr>
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<td>pGS2704</td>
<td>pGS2539 carrying a mutation in the −35 hexamer of the lutP promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pGS2706</td>
<td>pGS2539 carrying a mutation in the CRE of the regulatory region of lutP</td>
<td>This work</td>
</tr>
<tr>
<td>pGS2701</td>
<td>pGS1051 carrying an lutR allele expressing the His-tagged full-length LutR</td>
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<td>pGS2704</td>
<td>pGS2539 carrying a mutation in the −35 hexamer of the lutP promoter</td>
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**Analysis of lutR genes from various B. subtilis strains**

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the C terminus (Rigali et al., 2002; Hoskisson & Rigali, 2009). The LutR protein of B. subtilis RO-NN-1 shows 28.9% overall amino acid sequence identity and 48.0% similarity with LldR (231 aa) of C. glutamicum ATCC 13032. Both LutR and LldR possess a winged helix–turn–helix (wHTH) DNA-binding motif (Gajiwala & Burley, 2000; Gajiwala et al., 2000) at the N terminus (NCBI annotation). The wHTH motif in LutR of 240 aa is predicted to span aa 10–75 (NCBI annotation). The lutR genes from these B. subtilis strains are preceded by a putative Shine–Dalgarno sequence (Shine & Dalgarno, 1974) 5′-GGGGTGA-3′ with an appropriate spacing from the translational start site of lutR (Fig. 1a). However, the lutR genes of the laboratory strains 168 (Barbe et al., 2009) and PY79 (Schroeder & Simmons, 2013) contain a single-nucleotide deletion of an A at codon 11 (Fig. 1a). This deletion causes a shift in the reading frame of lutR. Translation of lutR thus prematurely stops after codon 14. A smaller ORF is located downstream of the mutation site and potentially encodes a truncated form of LutR (219 aa) that corresponds to aa 22–240 of the full-length LutR (240 aa). Thus, the truncated LutR lacks the N-terminal 21 aa of the full-length LutR, but a large part of the wHTH DNA-binding motif is still present at the extreme N terminus of the truncated LutR. The ORF encoding the truncated LutR is preceded by an atypical putative Shine–Dalgarno sequence 5′-AGCGGATGC-3′, which may not be favourable for expression of this ORF. Both the full-length LutR and the master regulator SinR can negatively control expression of the lutABC operon in B. subtilis strain NCIB 3610 (Chai et al., 2009). However, it has not been demonstrated whether the full-length LutR and SinR control lutABC expression in a direct manner. The truncated LutR of strain PY79 cannot regulate expression of lutABC and lutP (Irigul-Sonmez et al., 2014), but it becomes capable of positively and negatively, albeit mostly in a modest manner, regulating expression of numerous genes involved in a wide range of cellular processes (Irigul-Sonmez et al., 2014). To ascertain whether the full-length LutR can directly regulate lutP expression and to identify the consensus binding sequence for the full-length LutR, we used the undomesticated B. subtilis strain RO-NN-1, whose genomic sequence is now available (Earl et al., 2012), in the following studies.

lutP expression is strongly induced by l-lactate in a dose-dependent manner

To examine the effect of L-lactate on lutP expression, we constructed a transcriptional fusion of the regulatory region of lutP to the reporter gene lacZ and integrated it at the amyE locus of WT B. subtilis. Cells were grown in modified M9 minimal medium supplemented with glycerol as the carbon and energy source in combination with or without L-lactate. It was found that expression of the lutP-lacZ fusion was strongly induced by the addition of L-lactate to the culture medium and this induction was in a dose-dependent manner (Fig. 2a).

Expression of lutP is subject to partial catabolite repression by glucose

Given that lutP expression is L-lactate-inducible, we then tested whether lutP expression was subject to catabolite repression (Hueck & Hillen, 1995; Stülke & Hillen, 2000) by glucose. B. subtilis cells harbouring the lutP-lacZ fusion were grown in modified M9 minimal medium supplemented with citrate as the carbon and energy source in
LutR regulates the L-lactate permease gene lutP

Fig. 2. Effects of L-lactate and glucose on expression of lutP–lacZ and lutA–lacZ. (a) Induction of expression of the lutP–lacZ fusion by L-lactate. Cells bearing the lutP–lacZ fusion (BM2258) were grown in modified M9 minimal medium supplemented with 0.4% glycerol as the carbon source in combination with or without various concentrations (w/v) of L-lactate to OD$_{600}$ 1.0 before harvesting. The values are the mean from two independent experiments. Individual values did not differ by >15% from the mean. (b) Effects of L-lactate and glucose on expression of the lutP–lacZ fusion containing the WT regulatory region of lutP (BM2258) or the lutP–lacZ fusion containing a mutation in the CRE sequence (BM2504). Cells were grown in modified M9 minimal medium supplemented with 0.4% citrate as the carbon source in combination with or without 1% L-lactate or 1% L-lactate plus 2% glucose. It was found that glucose could partially repress the inducing effect of L-lactate (Fig. 2b). Inspection of the nucleotide sequence of the regulatory region of lutP in the lutP–lacZ fusion revealed a 14 nt sequence (5’-AGAAACCGCTTACA-3’) (Fig. 1a) that was located downstream of the lutP promoter (see below) and conformed to the consensus sequence CRE, 5’-(T/A)GAA(A/C/G)CGN(T/A)/(T/A)NCA-3’ (Weickert & Chambliss, 1990; Hueck et al., 1994). Mutation of this putative CRE in the lutP–lacZ fusion by altering the highly conserved central CG to GC caused a nearly total loss of glucose catabolite repression of lutP–lacZ (Fig. 2b), indicating that this CRE sequence was responsible for the catabolite repression of lutP. It should be mentioned that each value of Miller units of the strain with a mutation in CRE (BM2504) was significantly higher than each corresponding value of the strain without the mutation (BM2258) (Fig. 2b). It is possible that this mutation might have also altered the sequence of a partially overlapping binding site for an as-yet unidentified negative transcriptional regulator, leading to a partial derepression of lutP–lacZ expression.

lutA expression is not subject to catabolite repression

Given that lutA expression is known to be L-lactate-inducible (Chai et al., 2009), we also attempted to determine whether lutA expression would be subject to catabolite repression by glucose. An integrative lutA–lacZ fusion, which carries both the regulatory region and the coding region of lutA (–380 to +771 relative to the translational start site of lutA), was constructed (pGS2700). No catabolite repression of the lutA–lacZ fusion was observed (Fig. 2c). This is in agreement with the observation that no potential CRE sequence was present in the regulatory region and the coding region of lutA. Partial catabolite repression of lutP and no catabolite repression of lutA may have allowed cells to co-utilize glucose and L-lactate, consistent with the notion that L-lactate utilization is not only required for energy production, but also for other cellular behaviours such as biofilm formation in B. subtilis (Chai et al., 2009). This is not unprecedented as co-utilization of exogenous glucose and L-lactate has previously been observed in C. glutamicum (Stansen et al., 2005).

lutP expression is negatively controlled by the LutR repressor

To investigate whether LutR was a regulator for lutP expression, we constructed a lutR disruption mutant as described in Methods and then integrated the lutP–lacZ fusion at the amyE locus of the lutR mutant to generate strain BM2290. It was found that expression of the lutP–lacZ fusion was much higher in the lutR mutant than in the WT (Fig. 3a). No further induction by 1% L-lactate was observed in the lutR mutant (Fig. 3a), suggesting that the LutR repressor could negatively control lutP expression.

We also performed a genetic complementation analysis to further confirm the role of LutR in lutP expression. As His-tagged LutR has been shown to be able to interact with the regulatory region of lutP in vitro (see below), we ectopically
leading to an alteration in the DNA-binding specificity or affinity of LutR.

It is of note that, in the above-mentioned lutR mutant where the His-tagged full-length LutR was ectopically expressed from a multi-copy P_{lac} promoter-driven expression plasmid, 1% L-lactate could still strongly induce expression of the lutP–lacZ fusion (Fig. 3b). This result suggested that L-lactate or its metabolite could inhibit the binding of His-tagged LutR to its operator DNA in vivo and excluded the possibility that the His-tag might somehow have altered the structure of the LutR protein, rendering the in vitro binding of LutR to DNA insensitive to L-lactate (see below).

Mutational analyses of putative promoters of lutP, lutR and lutA

Inspection of nucleotide sequences of the regulatory regions of lutP, lutR and lutA revealed a putative σ^A promoter for each gene (Fig. 1a, b). To determine whether these putative promoters were functional in vivo, we used site-directed mutagenesis to alter the −10 hexamer of each putative promoter within each promoter region–lacZ fusion (TTA to ATAT), which was integrated individually at the anyE locus of the lutR mutant to avoid the possible repressive effect of LutR. It was found that mutation of the predicted promoter of lutP or lutR totally abolished expression of the lutP–lacZ or lutR–lacZ fusion, respectively (Fig. S1a, b, available in the online Supplementary Material). However, the mutated lutR–lacZ fusion still exhibited a residual expression (Fig. S1c), implying that either the mutated lutA promoter still had some residual activity or another as-yet-unidentified promoter(s) had a minor contribution to lutA expression.

To further confirm the correctness of the predicted promoters of lutP, lutA and lutR, we also mutated the −35 box of each predicted promoter within each promoter region–lacZ fusion (TTG to AAC for lutP and lutA, TTAT to AAAT for lutR). It was found that mutation of the −35 box of the predicted promoter of lutP or lutR almost abolished expression of the lutP–lacZ or lutR–lacZ fusion, respectively (Fig. S1a, b), whereas expression of the mutated lutA–lacZ fusion was only partially reduced (Fig. S1c). Altogether, these results suggested that these three predicted promoters were functional in vivo.

In vitro identification of a LutR-binding site downstream of the lutP promoter

Inspection of the nucleotide sequence of the regulatory region of the lutP gene of B. subtilis revealed an inverted repeat (5′-TCATC-N_4-GATGA-3′) located downstream of the σ^A promoter of lutP (Fig. 1a). This inverted repeat sequence was also present downstream of the putative σ^A promoter of lutP of B. amyloliquefaciens, B. coagulans, B. licheniformis, B. megaterium, Brevibacillus brevis, Geobacillus kauustophilus and Geobacillus thermoglucosidasius (Fig. S2a). To examine in vitro whether this inverted

![Graph showing lacZ activity](image-url)
repeat could serve as a LutR-binding site, we constructed plasmid pGS2576, which was able to overproduce His-tagged LutR of *B. subtilis* RO-NN-1 in *E. coli*, and then purified His-tagged LutR by affinity chromatography on a Ni²⁺-nitrilotriacetic acid agarose column. A ³²P-labelled DNA fragment containing the inverted repeat and a ³²P-labelled DNA fragment with deletion of the inverted repeat were used as probes in EMSAs. The result showed that purified His-tagged LutR could retard the DNA containing the inverted repeat, but could not retard the DNA with deletion of the inverted repeat at the corresponding assay concentrations (Fig. 4a). This result indicated that the inverted repeat was required for LutR binding.

**In vitro identification of a LutR-binding site downstream of the lutA promoter**

Given that the aforementioned inverted repeat sequence is also present downstream of the $\sigma^A$ promoter of the lutA gene of *B. subtilis* (Fig. 1b), as well as some other species (Fig. 2b), we next used EMSAs to examine whether this inverted repeat could also act as a LutR-binding site. The result showed that purified His-tagged LutR at a concentration of ~0.12 $\mu$M could completely retard the DNA containing the inverted repeat, but could not retard the DNA with deletion of the inverted repeat (Fig. 4b). This result indicated that this inverted repeat was also required for LutR binding. This is in contrast with a previous study which showed that 2 $\mu$M His-tagged truncated LutR of *B. subtilis* PY79 exhibited no significant binding to the regulatory region of lutA (Irigul-Sonmez et al., 2014).

**EMSA analysis of effects of L-lactate and some of its metabolites on interaction of LutR with its binding site**

We also tested effects of L-lactate, L-alanine, pyruvate or acetyl-CoA on *in vitro* interaction of LutR with its binding site in the regulatory region of lutP by EMSA. It was found that none of them at a concentration of 20 mM could inhibit significantly the binding of LutR (Fig. S3). It is possible that an as-yet unidentified metabolite of L-lactate might be the direct inducer for L-lactate-inducible expression of lutP. As the above-mentioned result indicated that L-lactate or its metabolite could inhibit the binding of His-tagged LutR to its operator DNA *in vivo*, the alternative possibility that the His-tag might somehow alter the structure of the LutR protein, rendering its binding to DNA insensitive to L-lactate, was thus excluded.

**Effect of deletion of the inverted repeat on expression of the lutP promoter region–lacZ fusion in vivo**

To examine the effect of deletion of the inverted repeat downstream of the lutP promoter on expression of the lutP–lacZ fusion *in vivo*, PCR-amplified DNA fragments containing the lutP promoter region with or without the inverted repeat were transcriptionally fused to lacZ and integrated at the amyE locus of WT *B. subtilis*. It was found that 1% L-lactate could strongly induce expression of the lutP–lacZ fusion containing the inverted repeat (Fig. 5a). After deletion of the inverted repeat, a strong derepression of the lutP–lacZ fusion was observed and 1% L-lactate could not further enhance its expression (Fig. 5a). This result was consistent with the notion that this inverted repeat acted as an operator for the L-lactate-responsive repressor LutR to negatively control lutP expression *in vivo*.

It is noteworthy that, in the absence or presence of 1% L-lactate, the expression level of the lutP–lacZ fusion in BM2317 (Fig. 5a) was much higher than the corresponding...
expression level in BM2258 (Fig. 2a). The regulatory region of the lutP–lacZ fusion in BM2258 (Fig. 2a) spans positions −250 to −1 (relative to the translational start site of lutP), whereas the regulatory region of the lutP–lacZ fusion in BM2317 (Fig. 5a) spans positions −250 to −51, which lacks the above-mentioned CRE sequence and the possible binding site for an as-yet unidentified negative transcriptional regulator (see above). Deletion of these two negative cis-elements might thus abolish glycerol repression of lutP–lacZ expression, leading to a strong derepression of lutP.

**Effect of deletion of the inverted repeat on expression of the lutA promoter region–lacZ fusion in vivo**

We also examined effect of deletion of the inverted repeat downstream of the lutA promoter on expression of the lutA–lacZ fusion in vivo. PCR-amplified DNA fragments containing the lutA promoter region with or without the inverted repeat were transcriptionally fused to lacZ and integrated at the amyE locus of WT B. subtilis to generate strains BM2270 and BM2260, respectively. We also integrated these two lutA–lacZ fusions individually at the amyE locus of the lutR mutant to generate strains BM2271 and BM2272. The results showed that deletion of the inverted repeat caused a strong depression of lutA–lacZ expression in the WT cells, whereas no difference in expression of these two lutA–lacZ fusions in the lutR mutant was observed (Fig. 5b). This result was in good agreement with the idea that the inverted repeat constitutes a LutR-binding site and contributes to negative control of lutA expression in vivo. On the basis of the in vitro and in vivo evidence, we propose that the conserved inverted repeat sequence 5′-TCATC-N1-GATGA-3′ is an operator for the full-length LutR in B. subtilis RO-NN-1 and probably other B. subtilis strains as well as some Bacillus-related species, as mentioned above. This is completely distinct from the highly TC-rich sequence 5′-TTCCCTCTTT-TNTTT-3′ proposed previously to be the consensus binding sequence for LutR of B. subtilis PY79 (Irigul-Sonmez et al., 2014). Our data have also demonstrated that the full-length LutR directly controls expression of lutA and lutP.

**Computer search for potential target genes of LutR**

To identify other potential target genes of the full-length LutR, we used the consensus binding sequence of LutR (5′-TCATC-N1-GATGA-3′) as the search pattern to search the SubtiList database (http://genolist.pasteur.fr/SubtiList/). The search region was set to be the regulatory region and within 300 bp upstream of a predicted gene. The result showed that only lutA and lutP were found as possible target genes, implying that the targets of the full-length LutR may be limited to a few genes and that the full-length LutR is probably not a global regulator.

**No interaction of the full-length LutR with the regulatory regions of ispA and aprE**

To examine whether the full-length LutR could bind to the regulatory regions of target genes of the truncated LutR (219 aa), DNA fragments containing the predicted promoter region of ispA or aprE and spanning ~200 bp upstream from each translational start site, as described previously (Irigul-Sonmez et al., 2014), were used as probes in EMSAs. It was found that the full-length LutR could not bind to the regulatory region of ispA or aprE under the assay conditions where the full-length LutR could bind to the regulatory region of lutP (Fig. 5a). This result is in agreement with that from the bioinformatic analysis.

**No interaction of the truncated LutR with the regulatory regions of lutA and lutP**

We also used EMSA to investigate whether the truncated LutR could bind to the regulatory region of lutA or lutP.
The plasmid pGS2699, which was able to overproduce the truncated LutR in *E. coli*, was constructed in exactly the same way as described previously (Irigul-Sonnmez et al., 2014). It was found that the purified truncated LutR could not bind to the regulatory region of *lutA* or *lutP* under the assay conditions where the full-length LutR could (Fig. S4b). Taken together, these results supported the notion that the targets of the full-length LutR were distinct from those of the truncated LutR. The absence or presence of the N-terminal 21 aa of the full-length LutR protein may probably alter the DNA-binding specificity or affinity of LutR.

Deletion analysis of the upstream region of the *lutP* promoter

It is known that the UP element, comprised of an AT-rich sequence (5'-AWWWWWTITTTTTAAAAARNR-3') located generally from −57 to −38 relative to the transcriptional initiation sites of a wide variety of bacterial genes, can be recognized by the C-terminal domain of the α subunit of RNA polymerase (zCTD) (Ross et al., 1993; Blatter et al., 1994; Gourse et al., 2000). Inspection of the upstream region of the −35 hexamer of the *lutP* promoter revealed a 20 bp sequence (5'-GACTATTTGTCAAAAAGTC-3'), which was somewhat similar (15 out of 20 nt) to the consensus sequence of the UP element. To investigate whether this sequence contributed to *lutP* expression, we made a series of deletion constructs as shown in Fig. 6. These constructs excluded the LutR-binding site to avoid the repressive effect of LutR. It was found that deletion of part of the 20 bp sequence greatly reduced expression of the *lutP-lacZ* fusion (Fig. 6), indicating that this region was important for *lutP* expression. Further deletion of the −35 hexamer of the *lutP* promoter completely abolished *lutP-lacZ* expression (Fig. 6), confirming that this promoter was indeed functional *in vivo*. This 20 bp sequence may constitute a binding site for zCTD to facilitate activation of the *lutP* promoter. Nevertheless, given that this sequence also contains an imperfect inverted repeat, we cannot exclude the possibility that an as-yet unidentified transcription factor may bind to this region to activate the downstream σ^A^ promoter. Future studies would help clarify these possibilities.

*lutR* expression is not autoregulated, but *lutP* expression is still partially induced by L-lactate in the *lutP* mutant

We next examined whether *lutR* expression was subject to autoregulation. We constructed a transcriptional fusion of the regulatory region of *lutR* to *lacZ* and integrated it at the *amyE* locus of WT *B. subtilis* and the *lutR* mutant. It was found that there was no significant difference in expression of the *lutR-lacZ* fusion between the WT and *lutR* mutant (Fig. S5), suggesting that *lutR* expression was not subject to autoregulation. We also examined whether L-lactate could induce *lutP-lacZ* expression in the *lutP* mutant. We constructed a *lutP* mutant as described in Methods and integrated the *lutP-lacZ* fusion at the *amyE* locus of the *lutP* mutant to generate strain BM2359. It was found that expression of the *lutP-lacZ* fusion in the *lutP* mutant was still partially induced by the addition of 1 % L-lactate to the culture medium (Fig. 7). This is consistent with the previous proposal that LutP is a major permease for L-lactate import and other as-yet unrecognized permease(s) may also make a partial contribution to the uptake of L-lactate (Chai et al., 2009).

**CONCLUSIONS**

We conclude that, in contrast to the truncated LutR of *B. subtilis* laboratory strains 168 and PY79, the full-length LutR repressor of the undomesticated strain RO-NN-1, and probably many other *B. subtilis* strains, can directly and negatively regulate *lutP* transcription. We have identified two LutR-binding sites with a consensus inverted repeat sequence: one site acts as an operator for controlling *lutP* expression and the other site for controlling *lutABC* expression. The consensus binding sequence for LutR is likely to be applicable to many other *B. subtilis* strains and

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The diagram and figure captions are not transcribed here due to the limitations of the provided text format.
Fig. 7. Effect of l-lactate on expression of lutP–lacZ in the lutP mutant. Cells bearing the lutP–lacZ fusion in WT B. subtilis (BM2258) or the lutP mutant (BM2359) were grown in modified M9 minimal medium supplemented with 0.4% glycerol as the carbon source in combination with or without 1% l-lactate to OD<sub>600</sub> 1.0 before harvesting. The values shown are the mean ± SD of three independent experiments.

some Bacillus-related species. The absence or presence of the N-terminal 21 aa of the full-length LutR protein, which encompass a small part of the wHTH DNA-binding motif, may probably alter the DNA-binding specificity or affinity of LutR.

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2189