Molecular and Physiological Logics of the Pyruvate-Induced Response of a Novel Transporter in *Bacillus subtilis*

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**ABSTRACT** At the heart of central carbon metabolism, pyruvate is a pivotal metabolite in all living cells. *Bacillus subtilis* is able to excrete pyruvate as well as to use it as the sole carbon source. We herein reveal that *ysbAB* (renamed *pftAB*), the only operon specifically induced in pyruvate-grown *B. subtilis* cells, encodes a heterooligomeric membrane complex which operates as a facilitated transport system specific for pyruvate, thereby defining a novel class of transporter. We demonstrate that the LytST two-component system is responsible for the induction of *pftAB* in the presence of pyruvate by binding of the LytT response regulator to a palindromic region upstream of *pftAB*. We show that both glucose and malate, the preferred carbon sources for *B. subtilis*, trigger the binding of CcpA upstream of *pftAB*, which results in its catabolite repression. However, an additional CcpA-independent mechanism represses *pftAB* in the presence of malate. Screening a genome-wide transposon mutant library, we find that an active malic enzyme replenishing the pyruvate pool is required for this repression. We next reveal that the higher the influx of pyruvate, the stronger the CcpA-independent repression of *pftAB*, which suggests that intracellular pyruvate retroinhibits *pftAB* induction via LytST. Such a retroinhibition challenges the rational design of novel nature-inspired sensors and synthetic switches but undoubtedly offers new possibilities for the development of integrated sensor/controller circuitry. Overall, we provide evidence for a complete system of sensors, feed-forward and feedback controllers that play a major role in environmental growth of *B. subtilis*.

**IMPORTANCE** Pyruvate is a small-molecule metabolite ubiquitous in living cells. Several species also use it as a carbon source as well as excrete it into the environment. The bacterial systems for pyruvate import/export have yet to be discovered. Here, we identified in the model bacterium *Bacillus subtilis* the first import/export system specific for pyruvate, PftAB, which defines a novel class of transporter. In this bacterium, extracellular pyruvate acts as the signal molecule for the LytST two-component system (TCS), which in turn induces expression of PftAB. However, when the pyruvate influx is high, LytST activity is drastically retroinhibited. Such a retroinhibition challenges the rational design of novel nature-inspired sensors and synthetic switches but undoubtedly offers new possibilities for the development of integrated sensor/controller circuitry. Overall, we provide evidence for a complete system of sensors, feed-forward and feedback controllers that play a major role in environmental growth of *B. subtilis*.

**KEYWORDS** *Bacillus subtilis*, LytST, PftA PftB, YsbA YsbB, catabolite repression, malate, pyruvate transport, two-component regulatory systems

Several carboxylic acids are substantially secreted by plant roots into the rhizosphere (1). Root exudates are composed of carboxylic acids, such as malate, citrate, and pyruvate, and vary across environments, specifically in response to the presence of phytotoxic compounds (2). Root exudates and released carboxylic acids also enable...
recruiting beneficial bacteria, such as the Gram-positive model bacterium *Bacillus subtilis*, that help to reduce susceptibility to plant pathogen attack (3–5). The assimilation of carboxylic acids by *B. subtilis* mainly relies on active transport systems, whose expression is induced via two-component systems (e.g., maeN by Malk/R [6]) by the transported carbon source (7). Besides carboxylic acids, *B. subtilis* is capable of utilizing a wide variety of carbon sources, including plant materials such as pectin, galactan, polygalacturonan, and rhamnogalacturonan (8–10). Indeed, *B. subtilis* has recently been suggested to be an epiphyte (11). The coassimilation of carbon sources in bacteria is strictly controlled by carbon catabolite repression (CCR). In *B. subtilis*, glucose and malate are the two preferred carbon sources and therefore impose a strict hierarchy for the use of alternative carbon sources (12). At the transcriptional level, the glucose-mediated CCR operates via the master regulator of carbon metabolism CcpA and its cofactors (HPr and Crh) to repress transcription of several targets, among which are the genes encoding the transporters of alternative carbon sources (13). Malate also represses the cutilization of alternative, glycolytic substrates by hijacking the usual glucose-mediated CcpA-dependent catabolite repression (12, 13). Repression occurs upon binding of CcpA in complex with the serine-phosphorylated HPr (P-Ser-HPr) or Crh (P-Ser-Crh) to regions in promoters called catabolite responsive elements (cre sites). The carbon-specific gene regulatory networks together with the global mechanism of catabolite repression can be viewed as sensors, feed-forward and feedback controllers that tightly adapt the overall metabolism to changing environments.

Pyruvate is the simplest of the alpha-keto acids and a key metabolite for living cells as the end product of glycolysis, a major substrate for oxidative metabolism, and a branching point for glucose, lactate, acetate, fatty acid, and amino acid syntheses. Because it is at the junction of several essential pathways in both eukaryotic and prokaryotic cells, tight control of its homeostasis and fate is crucial to ensure cell structural stability and robustness to changing environmental growth conditions. In eukaryotes, the mitochondrial enzymes that metabolize pyruvate are physically separated from the cytosolic pyruvate pool and rely on a transport system to shuttle pyruvate across the inner mitochondrial membrane. This transport system consists of a hetero-oligomeric complex composed of carriers MPC1 and MPC2 (14, 15). In prokaryotes, knowledge about pyruvate uptake systems is scarce. To date, two monocarboxylate transport systems with low affinity for pyruvate have been identified and characterized, MctC in *Corynebacterium glutamicum* (16) and MctP in *Rhizobium leguminosarum* (17). For these two systems, the uptake of pyruvate is driven by the electrochemical proton potential, as opposed to a facilitated diffusion, where the energy is provided by the concentration gradient of the substance transported. Although *B. subtilis* is able to grow on pyruvate as the sole carbon source (7), no clear homolog of any pyruvate transporter was found in its genome (18). Recently, the *ysbA* and *lytS* genes were shown to be essential for pyruvate utilization in *B. subtilis* (19). YsBA and LytS present homology to an antiholin-like protein and to a two-component system (TCS) sensor kinase, respectively. The *ysbA* gene is induced in the presence of extracellular pyruvate and transcribed in an operon with the *ysbR* (encoding a putative holin-like protein) gene (20). Immediately upstream from *ysbAB* on the *B. subtilis* chromosome, the *lytS* and *lytT* genes (encoding a putative TCS response regulator) are constitutively transcribed as an operon. van den Esker et al. showed that the deletion of *lytS* abolishes the expression of *ysbA*, which indicates a direct or indirect regulatory role of the putative TCS LytST (19). In addition, the level of induction of *ysbA* in the presence of both pyruvate and glucose is significantly reduced (19).

*B. subtilis* is a long-time model organism (21), and a highly detailed genome sequence, along with transcriptome- and proteome-wide responses to a hundred environmental conditions have been determined (18, 20, 22). Gene essentiality has also been investigated, and libraries of nonessential genes and intervals are freely available (23–25). However, about one-fourth of its genome codes for poorly characterized or completely unknown functions. In the present work, we identified the first bacterial transporter specific for pyruvate, which is a hetero-oligomeric membrane complex
composed of YsbA and YsbB and operating as a facilitated transporter. For the sake of clarity, we renamed YsbAB pftAB for pyruvate-facilitated transporter. We examined the regulation of pftAB by LytST and CcpA in depth and revealed that malate represses pftAB by an additional, CcpA-independent mechanism. Although the extracellular pyruvate activates the LytST TCS, we discovered that when the pyruvate influx is high, LytST activity is drastically retroinhibited.

RESULTS
Deletion of pftA and pftB drastically reduces growth on pyruvate. The pftA gene (formerly known as ysbA) is known to be essential for growth on pyruvate (19). In order to check whether pftB (formerly ysbB), the gene downstream of pftA within the same operon, is essential for growth of B. subtilis on pyruvate, we constructed single and double knockout (KO) mutant strains of pftA and pftB (Table 1). To circumvent a polar effect of the pftA KO mutation on the expression of pftB, we reinserted upstream of pftB the native PpftA promoter sequence (Table 1). The deletion of either pftA, pftB, or pftAB resulted in similar, severely impaired growth on pyruvate (M9 medium [12, 13] with pyruvate [M9P]) (Fig. 1A). These strains did not show any phenotype different from that of the wild-type (WT) strain when grown on other substrates (e.g., glucose [M9 medium with glucose [M9G]], malate [M9 medium with malate [M9M]], and glutamate and succinate [M9 medium with glutamate and succinate [M9SE]]) [see Table S1 in the supplemental material]. Complementation by an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pftAB cassette (PpftAB) inserted at the amyE locus restored growth on M9P (Fig. 1A; Table S1) to the WT level (0.25 h⁻¹). Although we did not observe a strict essentiality of pftA for cell growth on pyruvate, our findings are consistent with that of van den Esker et al. (19) and additionally demonstrated that pftB is necessary for growth on pyruvate. This suggested that pftA and pftB gene products may operate in concert.

PftA and PftB form a membrane protein complex. Both PftA and PftB were predicted to be membrane proteins (Fig. S1A) (19). To validate their localization, we constructed B. subtilis cells expressing C-terminal sequential peptide affinity (SPA)-tagged PftA (PftA-SPA) and SPA-tagged PftB (PftB-SPA) and analyzed samples corresponding to the cytosolic and membrane fractions by Western blotting using anti-FLAG antibodies. The results showed that both PftA-SPA and PftB-SPA were mainly, if not exclusively, present in the membrane fraction (Fig. 1B). We next asked whether PftA and PftB form a protein complex in the membrane. We performed tandem affinity purification (TAP) using strains expressing the SPA-tagged PftA and PftB proteins and appropriate controls. Strains were grown in M9P, and the purified (PftA-SPA and PftB-SPA) and copurified proteins were identified using mass spectrometry after tryptic or tryptic/chymotryptic digestions. PftB was specifically detected in the sample containing SPA-tagged PftA (Fig. S1B), which suggests that PftA and PftB form a complex in the membrane. However, mass spectrometry failed to detect PftA in the tandem affinity-purified sample containing SPA-tagged PftB due to the fact that the trypsin and trypsin/chymotrypsin digestions of PftA generated only poorly detected peptides.

To fully demonstrate that PftA and PftB form a membrane complex, a new construct was made which allowed easy detection of PftA. A N-terminal SPA-tagged PftA along with a C-terminal His-tagged version of PftB were assembled. The SPA-pftA and pftB-His₆ synthetic genes were inserted at the amyE locus under the control of the xylose-inducible promoter, Pₓᵥ. The expression of SPA-PftA and PftB-His₆ was induced for 3 h during exponential growth. The membrane fraction was first loaded onto a Ni²⁺ column to capture PftB-His₆. Western blotting using anti-FLAG antibodies revealed the presence of copurified SPA-PftA (Fig. 1C, Ni²⁺ column, lane E). The eluate was consecutively loaded onto a CaM column to capture SPA-PftA. A Western blot using anti-His antibodies was performed, and copurified PftB-His₆ was detected (Fig. 1C, CaM column, lane E). In addition, the flowthrough fractions of the first and second columns did not show the presence of any of the two partner proteins, indicating that the complex is stable. The two-step purification experiment was also conducted swapping the order of
| Strain | Relevant genotype | Reference(s), source, or construction |
|--------|------------------|-------------------------------------|
| **B. subtilis strains** | | |
| BSB168 | Wild type (prototroph) | 20, 22 |
| GM2924 | Δchr::aphA3 | 13 |
| GM2933 | ptsH1::cat | 13 |
| GM1619 | Δmae::pMUTIN2 (erm) maeA::aphA3 trpC2 | Laboratory collection |
| GM2907 | ccppA::Tn917Δ(erm lacZ); phleo | Laboratory collection |
| GM1626 | ywk8::pMUTIN (erm) trpC2 | Laboratory collection |
| TC01 | maeA::[pMUTIN2 Δ(lacZ-ery);kan] | GM1619 → BSB168 |
| TC03 | ywk8::pMUTIN2 (erm) | GM1626 → BSB168 |
| TC28 | maeA::[pMUTIN2 Δ(lacZ-ery);kan] PmaI5/cm | pDR111::maI5 → TC01 |
| TC29 | PmaI5/cm | pDR111::maI5 → BSB168 |
| TC35 | PpftB-gfpmut3/spec | pBSB::pPftB → BSB168 |
| TC36 | ccppA::Tn617Δ(term lacZ); phleo PpftAB-gfpmut3/spec | pBSB::pPftAB → GM2907 |
| TC58 | ΔltyST::cm | pDG1661::PpftAB → BSB168 |
| TC59 | ΔltyST::cm PpftAB::gfpmut3/spec | TC63 → GM2907 |
| TC60 | ΔpftAB::cm | pDG1664::PpftAB → TC60 |
| TC61 | ΔpftB::cm | pDG1664::PpftAB → TC61 |
| TC62 | ΔpftA::cm PpftA::pftB | pDG1664::PpftAB → TC62 |
| TC63 | PpftAB-lacZ/cm | TC63 → GM2907 |
| TC64 | ccppA::Tn917Δ erm lacZ); phleo PpftAB-lacZ/cm | pDG1661::PpftAB → BSB168 |
| TC73 | ltyST::cm | pMUTIN4 LystST/erm |
| TC74 | ΔpftAB::cm amyePpftAB::lacZ | pDR111::pftAB → TC60 |
| TC75 | ΔpftB::cm amyePpftAB::lacZ | pDR111::pftAB → TC61 |
| TC76 | ΔpftA::cm PpftA::pftAB | pDR111::pftAB → TC62 |
| TC86 | ccppA::Tn917Δ erm lacZ); phleo maeA::[pMUTIN2 Δ(lacZ-ery);kan] | TC01 → GM2907 |
| TC87 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) | TC01 → GM2907 |
| TC88 | maeA::[pMUTIN2 Δ(lacZ-ery);kan] PmaI5/gfpmut3/spec | TC01 → TC35 |
| TC89 | ywk8::pMUTIN2 (erm) PpftAB::gfpmut3/spec | TC01 → TC35 |
| TC90 | ccppA::Tn917Δ erm lacZ); phleo maeA::[pMUTIN2 Δ(lacZ-ery);kan] PpftAB-gfpmut3/spec | TC01 → TC36 |
| TC91 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC92 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC93 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC94 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC95 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC96 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC97 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC98 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC99 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC100 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC101 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC102 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC103 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC104 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC105 | ccppA::Tn917Δ erm lacZ); phleo ywk8::pMUTIN2 (erm) PpftAB-gfpmut3/spec | TC03 → TC36 |
| TC112 | ΔpftAB::cm PpftAB::gfpmut3/spec | TC35 → TC60 |
| TC113 | ΔpftB::cm PpftB::gfpmut3/spec | TC35 → TC61 |
| TC114 | ΔpftA::PpftA::lacZ | TC35 → TC62 |
| TC122 | PpftA::SPA/erm | pPftA::SPA → BSB168 |
| TC124 | PpftB::SPA/erm | pPftB::SPA → BSB168 |
| TC125 | PpftA::Tn917Δ ltyST::gfpmut3/spec | pBSB::pPftA::ltyST → BSB168 |
| TC126 | PpftB::Tn917Δ ltyST::gfpmut3/spec | pBSB::pPftB::ltyST → BSB168 |
| TC132 | amyePpftA::spa | pDR111::pftA → BSB168 |
| TC136 | amyePpftB::spa | pBSB::pPftB::ltyST → BSB168 |
| TC149 | PpftA::Tn917Δ ltyST::gfpmut3/spec | pBSB::pPftB::ltyST → BSB168 |
| TC150 | ΔpftB::cm PpftB::gfpmut3/spec | TC60 → TC149 |
| TC151 | ΔpftA::cm PpftA::gfpmut3/spec | TC60 → TC126 |
| TC152 | ΔpftA::cm PpftA::gfpmut3/spec | TC60 → TC125 |
| TC153 | ΔpftA::cm PpftA::gfpmut3/spec | TC60 → TC125 |
| TC163 | ccppA::Tn917Δ erm lacZ); phleo PpftAB::cm | TC60 → GM2907 |
| TC164 | ccppA::Tn917Δ erm lacZ); phleo PpftAB::cm PpftAB::gfpmut3/spec | TC60 → TC133 |
| TC165 | ccppA::Tn917Δ erm lacZ); phleo PpftAB::cm PpftAB::gfpmut3/spec | TC60 → TC147 |
| TC175 | PpftA::gfpmut3/spec | pDR111::pPftA → GM2907 |
| TC178 | ccppA::Tn917Δ erm lacZ); phleo PpftA::gfpmut3/spec | GM2907 → TC175 |
| TC199 | amyePpftA::SPA/ptfB::His::spec | pSG-SPA-NTER → BSB168 |
| **C. glutamicum strains** | | |
| ATCC 130TC | Wild type | 16 |
| Cg0953 | ΔmctC | 16 |
| TC200 | Wild type | pXmJ19 → ATCC 13032 |

(Continued on next page)
the columns, and identical results were obtained (not shown). These results indicated
that PftA and PftB form a hetero-oligomeric membrane protein complex.

The PftAB complex operates as a pyruvate transporter. We asked whether pftAB
encodes a pyruvate uptake system. During growth of C. glutamicum on pyruvate,
pyruvate is taken up by the monocarboxylate transporter MctC, and as a result, a mctC
mutant is unable to grow on pyruvate as the sole carbon source (16). We therefore
tested for functional complementation of the C. glutamicum mctC mutant using an
IPTG-inducible pftAB expression plasmid and appropriate controls. Since the mctC
mutant cannot grow on pyruvate, we precultured the strains in rich medium, washed
the cells, and inoculated a minimal medium plus pyruvate at an optical density of 600

![FIG 1](image_url)

**FIG 1** Role and localization of pftAB. (A) Growth of the WT, ΔpftA PpftABpftB, ΔpftB, ΔpftAB, and ΔpftAΔpftB
PpftAB strains on M9P. (B) Cytoplasmic (C) versus membrane (M) localization of PftA-SPA (24 kDa) and
PftB-SPA (32 kDa). Cells were grown in M9SE + P. Western blotting was performed using an anti-FLAG
monoclonal antibody as the primary antibody and horseradish peroxidase-conjugated anti-mouse
antibody as the secondary antibody. The positions of molecular mass markers (in kilodaltons) are
indicated to the left of the gel. (C) Copurification of a N-terminal SPA-tagged PftA and of a C-terminal
His-tagged version of PftB. The membrane fraction was first loaded onto a Ni²⁺ column to capture
PftB-His, and a Western blot using anti-FLAG antibodies revealed the presence of copurified SPA-PftA (F
and E stand for flowthrough and eluate, respectively). The eluate was next loaded onto a CaM column
to capture SPA-PftA, and a Western blot using anti-His antibodies revealed the presence of copurified
PftB-His. A representative experiment is presented in each panel.

| Strain  | Relevant genotype | Reference(s), source, or construction |
|---------|-------------------|---------------------------------------|
| TC201   | ΔmctC             | pxmj19 → Cg0953                        |
| TC202   | P_tac-pftAB/cm    | pxmj.pftAB → ATCC 13032                |
| TC203   | ΔmctC P_tac-pftAB/cm | pxmj.pftAB → Cg0953        |

*Arrows indicate construction by transformation.
The expression of \( \text{pftAB} \) was first fully induced using a high IPTG concentration (1 mM). As shown in Fig. 2A, the \( \text{mctC} \) mutant transformed with the empty pXMJ19 plasmid (\( \text{P}_{\text{tac-empty}} \)) or pXMJ19-pftAB plasmid (\( \text{P}_{\text{tac-pftAB}} \), IPTG-inducible) in the presence of 1 mM IPTG. The 95% confidence intervals are shown by the shaded areas. (B) Biomass of the \( \Delta \text{mctC} \text{P}_{\text{tac-pftAB}} \) strain upon entry into stationary phase after growth in MM1+P in the presence of different IPTG concentrations (0, 50, 100, 150, and 1,000 \( \mu \text{M} \)). (C) Growth rate of the \( \Delta \text{mctC} \text{P}_{\text{tac-pftAB}} \) strain grown in MM1+P in the presence or absence of IPTG. In panels B and C, mean values ± standard deviations (error bars) from at least six independent experiments are presented. Data were fit with a Michaelis-Menten equation; the 95% confidence intervals of the fits are shown in gray. The data corresponding to the control strains are shown in Fig. S2 in the supplemental material.

FIG 2  Functional complementation of the pyruvate uptake-deficient \( \Delta \text{mctC} \) mutant of \( \text{C. glutamicum} \) by PftAB. (A) Growth in MM1 medium plus pyruvate (MM1+P) of the WT and \( \Delta \text{mctC} \text{C. glutamicum} \) strains transformed with the empty pXMJ19 plasmid (\( \text{P}_{\text{tac-empty}} \)) or pXMJ19-pftAB plasmid (\( \text{P}_{\text{tac-pftAB}} \), IPTG-inducible) in the presence of 1 mM IPTG. The 95% confidence intervals are shown by the shaded areas. (B) Biomass of the \( \Delta \text{mctC} \text{P}_{\text{tac-pftAB}} \) strain upon entry into stationary phase after growth in MM1+P in the presence of different IPTG concentrations (0, 50, 100, 150, and 1,000 \( \mu \text{M} \)). (C) Growth rate of the \( \Delta \text{mctC} \text{P}_{\text{tac-pftAB}} \) strain grown in MM1+P in the presence or absence of IPTG. In panels B and C, mean values ± standard deviations (error bars) from at least six independent experiments are presented. Data were fit with a Michaelis-Menten equation; the 95% confidence intervals of the fits are shown in gray. The data corresponding to the control strains are shown in Fig. S2 in the supplemental material.

The PftAB complex is a pyruvate-specific facilitated transporter. We next asked whether PftAB operates as an active or passive pyruvate transport system. A straightforward approach to experimentally address this issue is to evaluate whether PftAB transports pyruvate along or against the concentration gradient of pyruvate. The concentration of intracellular pyruvate in \( \text{B. subtilis} \) cells grown in minimal medium containing glucose is about 1 mM (26). We therefore determined whether PftAB is specifically able to export intracellular pyruvate into minimal medium containing glucose (Fig. 3A). We quantified extracellular pyruvate during growth in M9G (glucose) of the WT, \( \Delta \text{pftAB} \), and \( \text{P}_{\text{n-pftAB}} \) strains. The three strains exhibited an identical growth phenotype (Fig. 3B, top panel). The extracellular pyruvate measured in the growth medium of the WT and \( \Delta \text{pftAB} \) strains steadily increased until the late exponential phase to about 0.03 g · liter\(^{-1} \) (~0.34 mM) with a specific production rate (\( q_{\text{Pyr}} \)) of ~0.5 mmol · h\(^{-1} \) · g of cells\(^{-1} \) (dry weight). Afterward, pyruvate concentration dropped to zero, which suggests that cells rapidly used the pyruvate before entering stationary

nm (OD\(_{600}\)) of ~0.02. The expression of \( \text{pftAB} \) was first fully induced using a high IPTG concentration (1 mM). As shown in Fig. 2A, the \( \text{mctC} \) mutant transformed with the empty plasmid was unable to grow on pyruvate as the sole carbon source. Conversely, the strong induction of \( \text{pftAB} \) restored growth of the \( \text{mctC} \) mutant. The biomass reached an OD\(_{600}\) of ~0.9 and the growth rate was ~0.08 h\(^{-1}\) (in comparison with an OD\(_{600}\) of ~1 and a growth rate of ~0.16 h\(^{-1}\) for the control strain), which indicated that PftAB functionally replaced MctC and enabled pyruvate uptake. In order to check whether the pyruvate uptake, and consequently growth restoration, was limited by the level of expression of PftAB, the experiment was repeated using IPTG at low (50 \( \mu \text{M} \)), intermediate (100 and 150 \( \mu \text{M} \)), and high (1 mM) concentrations. At low and intermediate levels of \( \text{pftAB} \) induction, the biomass reached an OD\(_{600}\) of up to ~0.6 unit (Fig. 2B), and the growth rates were between 0.04 and 0.06 h\(^{-1}\) (Fig. 2C and Fig. S2), which indicated that cell growth was IPTG dependent. Altogether, these results indicated that \( \text{pftAB} \) encodes a pyruvate import system.
growth phase. This result is consistent with the well-known phenomenon of excretion of pyruvate by *B. subtilis* prior to its (re)assimilation when glucose is depleted (7, 22). With the strain overexpressing pftAB (P_{hsp}pftAB), pyruvate culminated at ~0.09 g · liter$^{-1}$ (~1.02 mM) after 7 h of culture (Fig. 3B, bottom panel). The specific production rate of pyruvate ($q_{Pyr}$) peaked at ~5 mmol · h$^{-1}$ · g cells$^{-1}$ (dry weight) after 4 h of culture and quickly decreased thereafter. The specific glucose consumption and specific acetate and citrate production rates did not show any differences between the three strains (Fig. S3A to E). These results indicated that PftAB can specifically export pyruvate and also that there exists at least another pyruvate transport system allowing the import and export of pyruvate (consistent with the residual growth on pyruvate of the ΔpftAB mutant). Altogether, our findings prompted us to conclude that the gradient of pyruvate drove the PftAB-mediated transport of pyruvate.

To reinforce this conclusion, we cultivated the same strains in M9G supplemented with 0.15 g · liter$^{-1}$ (~1.70 mM) pyruvate. In this experiment, the pyruvate gradient is reversed so that if PftAB operates as a facilitated transporter, we expect to monitor an import of pyruvate (Fig. 3C). As shown in Fig. 3D, the concentration of extracellular pyruvate measured for the WT strain showed a slight increase until 10 h of culture, followed by a drop as soon as cells entered stationary phase. Interestingly, this drop coincided with the induction of pftAB when glucose was depleted (see next paragraphs) (19). Consistently, in the ΔpftAB strain, the consumption of pyruvate was delayed and strongly reduced. Conversely, the extracellular concentration of pyruvate measured with the strain overexpressing pftAB dropped from the beginning of the culture to reach zero before entry into stationary phase. There were no differences in the specific glucose consumption and specific acetate and citrate production rates in the three strains (Fig. S3F to J). Taken together, these results demonstrated that the gradient of pyruvate drove the PftAB-mediated transport of pyruvate across the cell membrane, which indicated that PftAB operates as a pyruvate-specific facilitated transporter. Making use of a simple model of facilitated transport (Text S1 and Table S2), we estimated that the PftAB complex had a maximum rate ($V_{max}$) of approximately 10.0 ± 1.0 mmol · h$^{-1}$ · g of cells$^{-1}$ (dry weight) and an apparent affinity constant for pyruvate ($K_m$) of approximately 1.0 ± 0.1 mmol · liter$^{-1}$.

**Pyruvate induces expression of pftAB by binding of LytT upstream of the pftAB promoter.** The *lytST* operon, which is located immediately upstream of *pftAB*, was...
recently shown to be involved in the induction of pftA upon the entry of Luria-Bertani (LB)-grown \textit{B. subtilis} cells in stationary phase and essential during growth on minimal medium plus pyruvate (19). van den Esker et al. (19) proposed that LytST directly induces pftAB based on the chromosomal proximity of these two operons. We tested this assumption \textit{in vitro} by performing electrophoretic mobility shift assay (EMSA) using a DNA fragment of 257 bp containing the intergenic region of the \textit{lytST} and \textit{pftAB} operons (Fig. 4A). As shown in Fig. 4B, increasing amounts of the response regulator His-LytT resulted in a shift in migration of the labeled DNA band, which indicated LytT DNA binding.

In silico comparative-genomic analysis recently predicted putative binding sites for a series of two-component systems (TCSs) of unknown functions (27). Hence, LytT may bind a DNA sequence within the 88-bp above-mentioned region, which is composed of two boxes of 13 nucleotides separated by 8 nucleotides (Box 1 and Box 2 [Fig. 4A]). We therefore tested this hypothesis using synthetic sequences mutated for these two boxes either independently (P_{pftAB-\Delta lytT1} and P_{pftAB-\Delta lytT2}) or together (P_{pftAB-\Delta lytT1-2}) (Fig. 4C). LytT shifted the nonmutated DNA fragment but none of the three synthetic fragments (Fig. 4D), which strongly suggested that each of the boxes is required for LytT binding.

In order to \textit{in vivo} validate the LytT binding site, gfp transcriptional fusions reporting the promoter activity of pftAB using either the wild-type sequence (P_{pftABgfp}) or the above-mentioned synthetic sequences (P_{pftAB-\Delta lytT1}, P_{pftAB-\Delta lytT2}, and P_{pftAB-\Delta lytT1-2}) were inserted at the pftAB locus in the WT strain and in a \Delta lytST mutant strain (Fig. 4C and Table 1). As expected, the growth of the \Delta lytST strain was drastically reduced on pyruvate, while no growth defect was observed when replacing pyruvate by other substrates (Table S1). Because of this growth defect, cells were grown in M9SE with or without pyruvate. The green fluorescent protein (GFP) abundance was ~4.5 units per OD_{600} unit (U \cdot OD_{600}^{-1}) for WT cells grown in M9SE with pyruvate (M9SE+P) and barely detectable in M9SE (Table 2). In contrast, the GFP abundance was ~0.5 U \cdot OD_{600}^{-1} for the \Delta lytST strain under both conditions. The GFP abundance from the P_{pftAB-\Delta lytT1}, P_{pftAB-\Delta lytT2}, and P_{pftAB-\Delta lytT1-2} synthetic reporter strains grown on M9SE+P was similar to that in the \Delta lytST strain (Table 2). Altogether, these results prompted us to conclude that each of the two predicted boxes is essential for the induction of pftAB by the LytST TCS. A genome-wide sequence homology search for other putative LytT binding motifs did not give any significant hits (not shown). Consistently, a comparative analysis performed using two recently published transcriptome data sets, M9P and M9E (7), revealed that pftA and pftB were the only genes specifically induced in cells grown in M9P compared to cells grown in M9SE (Fig. S4). Altogether, these results established that pyruvate induced expression of only pftAB by binding the LytT response regulator upstream of the pftAB promoter.

Glucose represses transcription of pftAB by binding of CcpA to the \textit{\textminus}35 region of the promoter. Since PftA can import and export pyruvate, its expression in cells grown on multiple carbon sources must be tightly regulated to ensure proper pyruvate homeostasis. The promoter of pftAB contains a putative cre site overlapping the \textit{\textminus}35 region, which suggests that pftAB expression may be under the control of CcpA, the master regulator of carbon catabolite repression (CCR). We tested this hypothesis \textit{in vitro} by conducting EMSAs with increasing amounts of purified CcpA-His, serine-phosphorylated HPr (P-Ser-HPr), and various restriction products of the previously described Cy5-labeled PCR fragment of 257 bp (Fig. 4A). As shown in Fig. 4E, increasing amounts of the CcpA/P-Ser-HPr complex resulted in a shift in migration of fragments of 170 bp for AluI, 116 bp for Ddel, and 189 bp for EcoRI. In contrast, no shift was observed after the fragment was treated by Afel, which cuts in the putative cre box, the binding
These results prompted us to conclude that CcpA binds within a 48-bp region bounded by the DdeI and EcoRI restriction sites.

In order to validate in vivo that pftAB expression is under the control of the CcpA-dependent catabolite repression, we quantified the PpftABgfp expression level in the WT, ΔccpA, ptsH1Δcrh, and ptsH1Δcrhstrains under inductive or repressive conditions (Table 3). The deletion of ccpA fully relieved the glucose-mediated repression for CcpA. These results prompted us to conclude that CcpA binds within a 48-bp region bounded by the Ddel and EcoRI restriction sites.

In order to validate in vivo that pftAB expression is under the control of the CcpA-dependent catabolite repression, we quantified the PpftABexpression level in the WT, ΔccpA, ptsH1, Δcrh, and ptsH1Δcrh strains under inductive or repressive conditions (Table 3). The deletion of ccpA fully relieved the glucose-mediated repression.
tion of pftAB in M9P with glucose (M9P+G). As expected, the glucose-mediated repression of pftAB was maintained in the ptsH1 and Δcrh single mutant strains (Table 3), which confirmed that the two cofactors can functionally replace each other (13). Consistently, the glucose-mediated pftAB repression was significantly reduced in the ptsH1 Δcrh double mutant. Hence, glucose repressed transcription of pftAB via CcpA and its cofactors by binding of the CcpA/P-Ser-HPr complex or the CcpA/P-Ser-Crh complex to the −35 region of the promoter.

**Table 2**: Induction of pftAB by the LytST TCS

| Strain | Genotype | P<sub>pftAB</sub> expression (U · OD<sub>600</sub>⁻¹) |
|--------|----------|-----------------------------------------------|
|        |          | M9SE                                     | M9SE+P                                      |
| TC35   | WT       | 0.2 ± 0.3                                 | 4.4 ± 0.2                                   |
| TC39   | ΔlytST   | 0.7 ± 0.5                                 | 0.3 ± 0.4                                   |
| TC149  | P<sub>pftAB-ΔlytT</sub><sup>b</sup> | 0.1 ± 0.2                                 | 0.3 ± 0.2                                   |
| TC125  | P<sub>pftAB-ΔlytT</sub><sup>b</sup> | 0.2 ± 0.3                                 | 0.4 ± 0.1                                   |
| TC126  | P<sub>pftAB-ΔlytT</sub><sup>b</sup> | 0.5 ± 0.7                                 | 0.4 ± 0.3                                   |

<sup>a</sup> More-complete relevant genotypes are given in Table 1.
<sup>b</sup> The deleted LytT binding sites are shown in Fig. 4C.

**Malate represses transcription of pftAB by a malic enzyme-dependent but CcpA-independent mechanism**. Malate was recently shown to hijack the usual CcpA-mediated catabolite repression (13). Consistently, the expression level of P<sub>pftABgfp</sub> was ~0.4 U · OD<sub>600</sub>⁻¹ for WT cells grown in M9P with malate (M9P+M), which is about 9% of the expression level in M9P (Table 3). However, the malate-mediated repression of pftAB was not relieved in the ΔcrrA mutants or in any of the ΔcrrA, ptsH1, Δcrh, and ptsH1 Δcrh mutants (M9P+M medium [Table 3]). These results prompted us to conclude that malate repressed pftAB transcription by at least one CcpA-independent mechanism.

To identify the key players of this CcpA-independent mechanism, a mini-Tn<sub>10</sub> insertion library was constructed from a B. subtilis pftAB reporter strain in a ΔcrrA background. This library was then screened on plates for pftAB derepression in the presence of malate. Thirteen positive clones from six independent pools of transposants were isolated and further characterized (Fig. S5). Most of the clones with strong pftAB derepression were mutated in the TCS malK/malI and its regulon involved in malate transport (maeA) and utilization (maeA) (6, 28). As maeA is the first gene of the mae operon, a possible role of ywkB (of unknown function) could not be excluded. We thus analyzed the effect of the deletion of each gene. The inactivation of maeA fully relieved pftAB expression in M9P+M, while the inactivation of ywkB had no significant effect (Table 3). These results showed that the CcpA-independent malate-dependent repression requires maeA.

**Table 3**: CcpA-dependent and independent catabolite repressions of pftAB

| Strain | Genotype<sup>a</sup> | P<sub>pftAB</sub> expression (U · OD<sub>600</sub>⁻¹)<sup>b</sup> |
|--------|-----------------------|-----------------------------------------------|
|        |                       | M9G  | M9M  | M9P  | M9P+G | M9P+M |
| TC35   | WT                    | <0.1 | 0.6 ± 0.1 | 4.5 ± 0.2 | 0.6 ± 0.2 | 0.4 ± 0.2 |
| TC36   | ΔcrrA                 | <0.1 | 0.3 ± 0.2 | 4.8 ± 0.2 | 3.5 ± 0.5 | 0.4 ± 0.1 |
| TC101  | ptsH1                 | <0.1 | 0.5 ± 0.2 | 4.9 ± 0.6 | <0.1 | 0.9 ± 0.2 |
| TC100  | Δcrh                  | 0.1 ± 0.1 | 0.4 ± 0.1 | 5.3 ± 0.6 | <0.1 | 1.0 ± 0.2 |
| TC102  | ptsH1 Δcrh            | <0.1 | 0.2 ± 0.1 | 4.8 ± 0.5 | 2.8 ± 0.2 | 0.3 ± 0.1 |
| TC90   | ΔcrrA maeA            | 4.9 ± 0.2 | – | – | 4.8 ± 0.5 |
| TC91   | ΔcrrA ywkB            | 4.9 ± 0.6 | – | – | 0.6 ± 0.1 |
| TC105  | ΔcrrA ΔmaeA           | 4.4 ± 0.4 | – | – | 3.7 ± 0.3 |
|        | P<sub>mae</sub>S      | – | – | 5.2 ± 0.4 | – | 0.4 ± 0.2 |

<sup>a</sup> More-complete genotypes are given in Table 1.
<sup>b</sup> -, not determined.
<sup>c</sup>, inducer. Overexpression of maS was carried out using 200 μM IPTG.
**B. subtilis** possesses four paralogous malic enzymes: YtsJ, a NADP-dependent malate dehydrogenase, plays the major role in malate utilization, whereas MaeA, MalS, and MleA are NAD dependent and are dispensable for growth on malate (29). The CcpA-independent repression of *pftAB* was dependent on the NAD-dependent activity, as the overexpression of *malS in a ΔmaeA strain fully restored the CcpA-independent catabolite repression (Table 3). Altogether, our results indicated that the NAD-dependent malic enzyme activity but not the MaeA protein per se is essential to drive the malate-mediated CcpA-independent repression of *pftAB*.

The higher the pyruvate influx and/or the intracellular pyruvate concentration, the stronger the CcpA-independent repression of *pftAB*. The NAD-dependent malic enzyme MaeA catalyzes the transformation of malate into pyruvate during *B. subtilis* growth on malate (7). This prompted us to hypothesize that the end product of the reaction (i.e., intracellular pyruvate) is responsible for the CcpA-independent catabolite repression (Table 3). Altogether, our results indicated that the NAD-dependent malic enzyme activity but not the MaeA protein per se is essential to drive the malate-mediated CcpA-independent repression of *pftAB*.

**FIG 5** Pyruvate influx tightly controls *pftAB* expression. (A) Expression of *pftAB* in the WT (white), Δ*pftAB* (green), and *P_hspftAB* (red) strains grown in M9SE + P (at pyruvate concentrations ranging from 0.1 to 100 mM). The *P_hspftAB* strain was grown with 1 mM IPTG. The dashed line represents the estimated *K_m* of PftAB. (B) Genomic structure of the *P_n*-*P_pftAB*~*gfp* at the *amyE* locus. The red and blue boxes represent *P_n* and *P_pftAB* respectively. Black boxes indicate DNA binding sites: cre for CcpA and lacO for LacI. (C) Expression of *P_n*-*P_pftAB*~*gfp* in the WT and Δ*ccpA* strains grown in M9SE + P in the presence (+) or absence (−) of malate (M) or 200 μM IPTG (II). Expression was estimated in the exponential phase of growth; mean values ± standard deviations from at least six experiments are presented in panels A and C.

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although extracellular pyruvate triggered the induction of pftAB, this induction is strongly inhibited (14-fold between the ΔpftAB and PₚₚₜₐₜₐB strains) by the activity of PftAB, i.e., by pyruvate uptake. It is worth noting that this feedback regulation led to a repression of pftAB of ~90%, which is similar to the level of the malate-dependent, CcpA-independent repression. Hence, the malate-dependent, CcpA-independent repression of pftAB most probably results from the strong increase in pyruvate influx and/or concentration generated by the high malic enzyme activity due to the induction of maeA in the presence of malate.

The elevated pyruvate influx and/or concentration seems to alter the induction of pftAB by LytST. Although no DNA-binding regulatory protein other than MalR was identified by our transposon mutagenesis, the high malic enzyme flux in the presence of malate may activate a yet unidentified regulator responsible for the CcpA-independent repression of pftAB. Alternatively, the CcpA-independent repression of pftAB may be mediated by the LytST TCS itself, i.e., by reducing its activator activity. In order to test these assumptions, we constructed a synthetic fusion of the IPTG-inducible PₚₜₚₜₐₜₐB and the native PₚₚₜₐₜₐB upstream of the gfp gene (PₚₚₜₚₜₐₜₐB-gfp) (Fig. 5B). If the CcpA-independent repression of pftAB is mediated by an unknown DNA-binding repressor acting on or downstream of PₚₚₜₐₜₐB, the expression driven by the upstream PₚₜₚₜₐₜₐB will be altered by this repressor, somewhat acting as an artificial roadblock. In contrast, if the CcpA-independent repression of pftAB results from a lower activation of LytST, the expression from PₚₜₚₜₐₜₐB will be fully relieved in the presence of IPTG. As shown in Fig. 5C, the malate-dependent repression was maintained on the synthetic PₚₚₜₚₜₐₜₐB-gfp with or without IPTG. However, in the ΔccpA mutant, while the CcpA-independent repression by malate was maintained in the absence of IPTG, the repression was fully relieved in the presence of IPTG (Fig. 5C). This experiment revealed that CcpA repressed expression from the synthetic PₚₚₜₚₜₐₜₐB-gfp in the presence of malate by binding on the −35 region of PₚₚₜₐₜₐB (as in the presence of glucose) and by acting as a roadblock for the transcribing RNA polymerase recruited by PₚₜₚₜₐₜₐB (Fig. 5B). Altogether, these results suggested that there is no other DNA-binding protein that repressed PₚₚₜₐₜₐB in the presence of malate, which implies that the CcpA-independent pftAB repression actually resulted from a lower level of induction of pftAB by LytST.

DISCUSSION

In this study, we characterized the molecular and physiological logics of the pyruvate-induced response of a novel pyruvate transporter in B. subtilis. This novel bacterial transport system consists of a hetero-oligomeric complex of PftA and PftB which operates as a pyruvate-specific facilitated transporter. In the presence of extracellular pyruvate, the pftAB operon is induced by the TCS LytST. As for the transporters of alternative substrates, pftAB expression is repressed by the CcpA-dependent catabolite repression when a preferred carbon source, glucose or malate, is present in the medium. Unexpectedly, however, in the absence of preferred carbon source when the pyruvate influx is high, LytST activity is drastically retroinhibited (Fig. 6). Hence, LytST constitutes, together with the transporter PftAB, an original regulatory system ensuring proper adaptation to changing environments.

It was recently shown in B. subtilis forming biofilms that pftAB is induced in the presence of extracellular acetate (30). When we added acetate to the medium, we observed no induction of pftAB in exponentially growing cells. However, we observed a weak, dose-dependent induction when cells reached the stationary phase after growth in repressive conditions (data not shown). A reasonable hypothesis is that the reduction of the acetate export consequent to the presence of extracellular acetate led to an overflow metabolic shift toward an increase of pyruvate export. Indeed, it is well-known that when the intracellular pyruvate pool is high, the overflow metabolism, in particular pyruvate export, is stimulated (7). We showed that pyruvate export during exponential growth on glucose is independent from PfrAB which is kept under the control of the catabolite repression (Fig. 6). Hence, at the onset of the stationary phase
when the CcpA-dependent repression is relieved, the exported pyruvate triggered $pftAB$ induction in a dose-dependent manner.

The $pftA$ and $pftB$ genes were originally annotated as encoding homologs of the *Staphylococcus aureus* LrgA and LrgB membrane proteins (18). The molecular mechanisms controlling death and lysis during biofilm development of *S. aureus* are organized around the CidR-regulated $cidABC$ and LytSR-regulated $lrgAB$ operons. $cidA$ and $lrgA$ encode proteins that are believed to function as holin and antiholin, respectively, while $cidB$ and $lrgB$ are of unknown function (31). Although PftA and PftB share strong homologies with holin/antiholin systems, the finding that these proteins belong to a new class of effective bacterial transporters is consistent with the recent discovery of the chloroplastidic glycolate/glycerate transporter, PLGG1, that most likely evolved from a gene fusion of bacterial $lrgA$ ($pftA$) and $lrgB$ ($pftB$) homologs (32, 33). Also consistent with our findings in *B. subtilis*, an impaired ability to utilize pyruvate was observed in the lytST-like lytSR mutant of *Staphylococcus epidermidis* (34). In addition, it was recently shown in the evolutionarily distant bacterium *Escherichia coli* that the LytST-like YpdAB TCS is weakly activated by pyruvate (35, 36). Remarkably, the YpdB binding site is similar to the *B. subtilis* LytT binding site (Fig. 4C). However, it was proposed that in *S. aureus*, LytSR senses decrease in membrane potential and responds by inducing $lrgAB$ transcription. It was also proposed that a primary intermediate of overflow metabolism, acetyl phosphate (acetyl-P), already shown to act as a small phosphodonor to response regulators (37), directly activates LytR in an alternative signaling pathway (31). Our data, however, demonstrated that the higher the pyruvate influx, the lower the induction level of $pftAB$ (Fig. 5A). This finding argues in favor of a negative-feedback regulation by the level of intracellular pyruvate (Fig. 6). Besides, we observed no induction of $pftAB$ in either the WT or ΔccpA strain under conditions that are known to give rise to elevated concentrations of the intermediates of overflow metabolism and high acetate excretion rates (7, 22). Hence, if acetyl-P acts as a phosphodonor to LytT in *B. subtilis*, it does not result in its activation, as has been proposed for *S. aureus* LytR. Overall, there are undeniable similarities in gene sequences and signaling between the homologous staphylococcal LytSR, *E. coli* YpdAB, and *B. subtilis* LytST TCSs but also significant physiological and functional divergences, which are likely to be related to niche-specific evolutionary constraints.

Unpredictable changing environments necessitate appropriate responses for successful bacterial adaptation. Appropriate growth strategies rely on sensing systems that globally adjust gene expression via transcription factor-mediated feed-forward and feedback regulations. In particular, bacterial TCSs combine a sensor (i.e., the sensor kinase) with a feed-forward controller (i.e., the response regulator) to induce expression of target genes involved in the adaptation process. The $pftAB$ expression levels in

![FIG 6 Roles of PftAB and LytST in pyruvate homeostasis. The products of the $pftAB$ operon form a hetero-oligomeric membrane complex encoding the major pyruvate import/export system in *B. subtilis*. The LytST TCS senses the extracellular pyruvate concentration and responds by inducing $pftAB$ transcription. The accumulation of intracellular pyruvate (or of an intermediate of overflow metabolism) reduces the level of induction of $pftAB$ via LytST. This accumulation results either from the uptake and metabolism of pyruvate or from the uptake of malate (by MaeN) and its consecutive transformation into pyruvate by the malic enzyme MaeA. Malate (and glucose) also triggers the catabolite repression of $pftAB$ via CcpA. There is at least one other pyruvate transporter yet to be identified (gray filled circle). P, phosphate.](mbio.asm.org/FIG_6.png)
response to different pyruvate concentrations (Fig. 5A) revealed the existence of an additional feedback control acting on LytST. To gain insight into the molecular mechanism that governed the opposed, feed-forward and feedback LytST-mediated regulation of pftAB by extracellular and intracellular pyruvate, we developed a simple model of gene expression and explored competitive, noncompetitive, and uncompetitive inhibitions of LytT activation by intracellular pyruvate (see Text S1 in the supplemental material). We assumed that the LytT regulatory protein binds to the pftAB promoter as a homodimer upon phosphorylation by LytS, which can be well represented by a Hill equation (38), but phosphorylation of LytT by LytS has not yet been demonstrated. The model simulations perfectly mimicked the induction of the pftAB promoter in the ΔpftAB strain (Fig. S6). However, the model could not account for pftAB induction in the other genetic backgrounds, in which cells are still capable of utilizing extracellular pyruvate (Text S1). Other molecular mechanisms may explain the feedback regulation of LytST by intracellular pyruvate, such as inhibition of LytS autophosphorylation or hindered recruitment of the RNA polymerase (Fig. 6) or a phosphorylation state-dependent proteolysis of LytT. To decide between these mechanisms and verify to which extent they apply to other TCSs, characterization of the transduction signal and of the pftAB regulation dependency on the LytT phosphorylation state should be performed. Indeed, such a retroinhibition challenges the rational design of novel nature-inspired sensors and synthetic switches but undoubtedly offers new possibilities for the development of integrated sensor/controller circuitry.

In conclusion, the influx and efflux of pyruvate from exponential to stationary growth phases result from the timely (re)routing of the central carbon metabolic fluxes and of the dynamics of the intermediate concentrations. Hence, the intracellular and extracellular sensor systems and the feed-forward and feedback regulations of pyruvate uptake ensured with respect to cell adaptation a tight management of pyruvate homeostasis.

MATERIALS AND METHODS

Media and bacterial strains. Escherichia coli DH5α and TG1 were used for plasmid construction and transformation using standard techniques (39). Bacillus subtilis and Corynebacterium glutamicum strains used in this study were verified by sequencing and are listed in Table 1. B. subtilis strains were derived from BSB168, a trpΔ derivative of B. subtilis 168 (20, 22). Luria-Bertani (LB) broth was used to grow E. coli, B. subtilis, and C. glutamicum for transformation procedures only. For other experiments, B. subtilis was grown in a modified M9 medium (40) and when necessary supplemented with 25 mg·liter⁻¹ isoleucine, 50 mg·liter⁻¹ leucine, 40 mg·liter⁻¹ valine, 20 mg·liter⁻¹ methionine, and 4 g·liter⁻¹ glutamate (41). C. glutamicum was grown in MM1 minimal medium (16). Carbon sources were used at concentrations of 3 g·liter⁻¹ glucose, 5 g·liter⁻¹ malate, 4 g·liter⁻¹ succinate plus 4 g·liter⁻¹ glutamate, or 6 g·liter⁻¹ pyruvate. When required, media were supplemented with antibiotics at the indicated concentrations: for E. coli, ampicillin (100 µg·ml⁻¹), spectinomycin (200 µg·ml⁻¹); for B. subtilis, spectinomycin (100 µg·ml⁻¹), kanamycin (5 µg·ml⁻¹), erythromycin (1 µg·ml⁻¹), phleomycin (1 µg·ml⁻¹), chloramphenicol (5 µg·ml⁻¹); for C. glutamicum, chloramphenicol (30 µg·ml⁻¹).

Gene deletion. Deletions of pftA (ysbA), pftB (ysbB), pftAB (ysbAB), and lyfST were performed by sequence replacement with a chloramphenicol resistance cassette (Cmr') expressed under the control of a constitutive promoter. The Cmr' cassette was PCR amplified from plasmid pDG1661 using primers containing either a ClaI, SmaI, or XbaI restriction site (see Table S3 in the supplemental material). PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) and then digested by the proper restriction enzymes. Regions containing pftA, pftB, pftAB, and lyfST were PCR amplified from genomic DNA using primers listed in Table S3. PCR products were purified and subcloned into the pGEM-T Easy vector according to the supplier’s protocol. Reverse PCR were performed on the resulting plasmids using primers containing either the ClaI, SmaI, or XbaI restriction site (Table S3). After purification and digestion, PCR products were ligated to the corresponding Cmr'-containing PCR product.

Inducible gene expression. Isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression of lyfST was performed by fusing lyfST to the Ppromoter from pMUTIN4 using BamHI and SacI restriction sites, followed by the single crossover integration of the resulting plasmid upstream pftAB in the ΔlysT strain. IPTG-inducible expression of masS and pftAB were carried out by fusing these genes to the Ppromoter using plasmids pDR111 (kind gift of David Rudner), pDG1661, and pDG1664 (42). Brieﬂy, PCR-ampliﬁed fragments (Table S3) of pftAB and masS were cloned into the pDR111 plasmid by SalI/Hind digestion leading to pDR111-PpftAB and by SalI/Sphi digestion leading to pDR111-PmasS. A PCR on pDG1664 was performed to introduce a NotI restriction site using appropriate primers (Table S3). The PpftAB from pDR111−PpftAB was then cloned into pDG1664-Not using BamHI and NotI restriction sites. The spectinomycin resistance cassette (Spec') of pDR111-PmasS was replaced by Cmr' from pDG1661 using EcoRI and SacI. The pDR111, pDG1661, and pDG1664 derivative plasmids were inserted
by double crossover at the amyE and thrB loci, respectively. IPTG-inducible expression of pftAB for complementation of the C. glutamicum mc4C mutant was performed by inserting the PCR-amplified sequence of pftAB (Table S3) in the pXn919 plasmid (kind gift of Gerd Selbold) using Smal and Sal restriction sites.

Promoter reporter fusions. Fusion of the P_{pftAB} promoter with lacZ (for transposon mutagenesis) was constructed using the pDG1661 plasmid and the PCR-amplified P_{pftAB} using appropriate primers (Table S3). The PCR fragment was inserted by HindIII/BamHI restriction/ligation. Fusion of P_{pftAB} with the gfpmut3 gene was carried out by ligation-independent cloning using the pBSBII plasmid (Table S3) as described previously (43). Reverse PCR on the pBSBII-P_{pftAB} plasmid using proper primers (Table S3) was used to substitute parts or all of the LytT binding site from the promoter region of pftAB by BamHI. The pBSBII derivative plasmids were inserted at the pftAB locus by single crossover. Fusion of the P_{pftAB} and P_{pftB} promoters upstream of gfpmut3 was performed by PCR amplification of the P_{pftAB}gfnp-containing region from the pBSBII-P_{pftAB} using appropriate primers (Table S3) and subsequent insertion downstream of P_{pftB} into pDR111. The resulting plasmid was integrated by double crossover at the amyE locus, which led to the P_{pftAB}gfnp transcriptional fusion.

Live-cell array and fluorescence analysis. Experiments and analyses were performed as previously described (22, 43). Cells were grown in 100 μl of medium in 96-well plates (Cellstar; Greiner Bio-One) and incubated at 37°C under constant shaking in a synergy II microplate reader (BioTek). The optical density at 600 nm (OD_{600}) and fluorescence were measured every 10 min. Each culture was performed in at least three technical replicates by two biological replicates (more than six values). The mean green fluorescent protein (GFP) concentration in exponentially growing cells was expressed as unit per OD_{600} with 1 unit being equivalent to 1 fmol fluorescein (Fig. S6).

Transposon mutagenesis. The mini-Tn10 delivery vector pIC333 was used for transposon mutagenesis as previously described (44, 45). This plasmid was introduced into B. subtilis strain TC64 (Table 1) at 25°C using erythromycin selection. Single transformants were used to inoculate independent cultures at 25°C in LB plus spectinomycin. In early temperature growth, the temperature was shifted to 40°C, and cultures were allowed to grow for 4 more hours. Appropriate culture dilutions were spread on solid M9 medium with pyruvate and malate (M9P) plus spectinomycin and 0.04% X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and incubated at 25°C to screen for clones with derepressed P_{pftAB}lacZ expression (blue colonies). Thirteen clones from independent pools of transposants were isolated in which the lacZ gene was expressed on M9P+M (Fig. S4). These clones were selected for backcross experiments, and genes inactivated by the transposon were identified by target rescue and sequencing.

PftA and PftB localization and quantitative pulldown assays. Fusion of the SPA (sequential peptide affinity) sequence at the 3' end of either pftA or pftB was performed using the pMUTIN-LICSPA plasmid, previously adapted from the pMUTIN-SPA plasmid for ligation-independent cloning to facilitate high-throughput (HTP) cloning (46) and appropriate primers (Table S3). Each SPA-tagged strain was precultured overnight in the presence of erythromycin at 37°C. The culture was initiated by a 500-fold dilution (to an OD_{600} of 0.001) in 2 liters of M9 medium with glutamate and succinate plus pyruvate (M9SE+P). When the OD_{600} reached 0.3, cultures were centrifuged at 3,000 x g for 10 min at 4°C. The pellet was resuspended in 50 ml of cold buffer (10 mM Tris-Cl [pH 8], 150 mM NaCl) and instantly frozen in liquid nitrogen. The cells were disrupted using a French press. Membrane and cytosolic fractions were separated by ultracentrifugation at 100,000 x g for 1 h. The membrane pellet was solubilized using buffer A (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA) supplemented with 1% DDM (n-dodecyl-β-D-maltoside). Protein concentrations were measured by the Bradford method, and identical protein amounts of the two fractions were loaded on 10% SDS-polyacrylamide gels for Western blotting using anti-FLAG antibodies. In addition, protein complexes from the membrane fraction were pulled down using a SPA purification method (47), then separated by native polyacrylamide gel electrophoresis (PAGE), and analyzed by nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) (Text S1).

Affinity capture of the SPA-PftA PftB-His complex. The pftAB operon was PCR amplified from the B. subtilis 168 chromosome with primers OOD141 and OOD142 (Table S3). The OOD142 primer allowed the PftB C-terminal fusion of six His residues. After digestion by Xhol and NotI, the PCR fragment was ligated to the pSG-SPA-NTER plasmid (48), enabling the PftA N-terminal fusion of SPA and placing the SPA-pftA pftB-His synthetic operon under the control of a xylose-dependent promoter P_{xyl}. The plasmid was used to engineer strain TC199 by double crossover at the amyE locus. Transformants were selected for resistance to spectinomycin and lack of amylase activity. Expression of SPA-pftA pftB-His was induced by 1% xylose (vol/vol) at mid-log growth and harvested 3 h later. Cell membranes were prepared after cell disruption by sonication in buffer A and centrifugation at 100,000 x g for 1 h. Membranes were solubilized using buffer A supplemented with 1% n-dodecyl-β-D-maltoside (DDM). The solution was either mixed with nickel-nitriolactic acid (Ni-NTA) agarose resin (Invitrogen) after the addition of 10 mM imidazole or mixed with the CaM Sepharose-4B resin (GE Healthcare) after the addition of 2 mM CaCl_{2} and left overnight at 4°C on a rotating wheel. For the Ni-NTA agarose column, washes were performed with buffer A supplemented with 20 mM imidazole, and the elution was performed with 250 mM imidazole. For the CaM Sepharose-4B column, washes were performed by buffer A supplemented with 0.1 mM CaCl_{2} and the elution was performed with buffer A supplemented with 3 mM EGTA. Both columns were also used sequentially, in both ways. Prior to the analyses by Western blotting, proteins were precipitated by the addition of 10% trichloroacetic acid (TCA).

Electrophoretic mobility shift assay. The LytT coding sequence was PCR amplified using primers enabling insertion of a His_{6} tag between the start codon and the coding sequence and cloning into the E. coli pJ411 expression vector (DNA2.0, Newark, CA), and His_{6}-LytT proteins were expressed in strain...
ER2566 (NEB). The His6-CcpA protein was expressed from a pQE30 derivative vector (kind gift of Anne Galnier). Cells were grown at 30°C in 1 liter LB medium supplemented with the required antibiotics, and expression was induced for 3 h by the addition of 500 μM IPTG when biomass reached an OD600 of 0.7. The cells were harvested by centrifugation, resuspended in 40 ml of 50 mM Tris-HCl (pH 8.0) and 1 M NaCl, sonicated, and centrifuged at 100,000 × g for 1 h at 4°C. Supernatants were loaded onto a preequilibrated Ni2+ affinity column (Ni-NTA agarose; Qiagen), and the His6-tagged proteins were purified by successive washing steps in 50 mM Tris-HCl (pH 8.0) and 1 M NaCl with increasing concentrations of imidazole (0 to 20 mM; Text S1). Samples eluted with 50 mM Tris-HCl (pH 8.0), 1 M NaCl, and 250 mM imidazole were dialyzed against a solution containing 50 mM Tris-HCl (pH 8.0), 0.4 M NaCl, 50% glycerol, 1 mM dithiothreitol (DTT). The dialyzed fraction containing the His6-LytT protein was further loaded onto a 1 ml Hitrap heparin column (GE) to remove contaminants (Text S1). The P

Acknowledgments

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