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A Widely Conserved Gene Cluster Required for Lactate Utilization in *Bacillus subtilis* and Its Involvement in Biofilm Formation▽

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We report that catabolism of L-lactate in *Bacillus subtilis* depends on the previously uncharacterized *yvfV-yvfW-yvbY* (herein renamed *lutABC*) operon, which is inferred to encode three iron-sulfur-containing proteins. The operon is under the dual control of a GntR-type repressor (LutR, formerly YvfI) and the master regulator for biofilm formation SinR and is induced during growth in response to L-lactate. Operons with high similarity to *lutABC* are present in the genomes of a variety of gram-positive and gram-negative bacteria, raising the possibility that LutABC is a widely conserved and previously unrecognized pathway for the utilization of L-lactate or related metabolites.

The spore-forming bacterium *Bacillus subtilis* is capable of forming complex multicellular communities on surfaces (8, 25, 30, 40). These communities, known as biofilms, consist of long chains of cells that are held together by an extracellular matrix consisting of protein and polysaccharide (9, 31, 36, 37). Production of the matrix is governed by a complex regulatory network, at the heart of which are two parallel pathways of repression and antirepression (4, 10–12, 27). One pathway involves the repressor AbrB, which controls the expression of many different kinds of genes, including genes involved in biofilm formation, during the transition from exponential growth to stationary phase (4, 12, 24). Relief from AbrB-mediated repression is brought about in part by the antirepressor AbbA, which binds to and inactivates the repressor (4). The other pathway, consisting of the repressor SinR and its antirepressor SinI, is dedicated largely to genes involved in biofilm formation (10–12, 27). The principal targets of the SinR repressor are the 18 genes of the *epsA*-to-*O* and *yqxM-sipW-tasA* operons, which are responsible for the production of the matrix (7, 11, 27, 32). Several other genes and operons that are not directly required for matrix production are also under the control of the SinI-SinR pathway (11), one of which, the *yvfV-yvfW-yvbY* operon, is the subject of this report.

The initial goal of the current investigation was to elucidate the role, if any, of the *yvfV-yvfW-yvbY* operon, whose protein products were of unknown function, in biofilm formation. As we report herein, a clue as to the function of the operon came from comparative genomics, which led to the discovery that the *yvfV-yvfW-yvbY* operon specifies a pathway for the utilization of L-lactate. Here we demonstrate that the operon is required for growth on L-lactate as a sole carbon source; that it is subject to dual regulation, which allows it to be induced during both growth in liquid culture and biofilm formation; and that the operon influences the architectural complexity of biofilms formed in the presence of L-lactate. We therefore rename *yvfV*, *yvfW*, and *yvbY* as *lutA*, *lutB*, and *lutC*, respectively (for *l*actate *ut*ilization). Interestingly, homologous operons of *lutABC* are found in the genomes of many different bacteria, including some only distantly related to *B. subtilis*. These observations suggest that LutA, LutB, and LutC represent a previously unrecognized and widely conserved pathway for the utilization of L-lactate.

MATERIALS AND METHODS

Strains and media. *Bacillus subtilis* strains were grown in Luria-Bertani (LB) medium at 37°C for general purposes and grown in MSgg and modified MSgg at 23°C for assays related to biofilm formation. The recipe for MSgg was described previously (27). *Escherichia coli* strains were grown in LB medium at 37°C. In assays testing the L-lactate auxotroph phenotypes of *B. subtilis* and *E. coli* strains, the following minimal medium was used: 50 mM phosphate buffer (pH 7), 100 mM MOPS buffer (pH 7), 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 1 μM $ZnCl_2$, 50 μM $FeCl_3$, 2 μM thiamine, 50 μg ml^{-1} phenylalanine, 50 μg ml^{-1} tryptophan, 50 μg ml^{-1} threonine, 0.5% L-lactate, and 0.5% NH_4Cl_2. Bacto agar (1.5%) was added when making solid minimal medium. Minimal media used as controls were prepared similarly, except that 0.5% glucose or pyruvate was used as the sole carbon source.

The strains and primers used in this work are summarized in Table 1 and Table 2, respectively. L-Lactate, D-lactate, and pyruvate were purchased from Sigma. Antibiotics were added to the media at the following concentrations: 10 μg ml^{-1} of tetracycline, 100 μg ml^{-1} of spectinomycin, 10 μg ml^{-1} of kanamycin, 5 μg ml^{-1} of chloramphenicol, and 1 μg ml^{-1} of erythromycin. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at a final concentration of 40 μg ml^{-1}.

Strain construction. To construct the P_{lutA}-*lacZ* fusion, an ~300-bp DNA sequence upstream of the *lutA* gene was amplified by PCR using primers P_{lutA}-F and P_{lutA}-R and chromosomal DNA from strain 3610, an undomesticated *B. subtilis* strain, as the template. The PCR products were then cloned into the EcoRI and BamHI sites of the vector pDG268 (1), which carries a chloramphenicol resistance marker and a polylinker upstream of the *lacZ* gene between two arms of the *amyE* gene. The recombinant plasmid was introduced into *B. subtilis* PY79 by transformation, and transformants were selected for double-crossover recombination of P_{lutA}-*lacZ* into the *amyE* locus on the chromosome. The P_{lutA}-*lacZ* fusion was then transferred from the PY79 background into 3610 and its derivatives by SPP1 phage-mediated transduction (11), resulting in strains YC125, YC126, YC143, and YC289. An *epsH* mutation was also introduced into the above strains to prevent cell aggregation during growth (27).

To construct insertional knockout strains of the genes studied in this work, we applied long-flanking PCR mutagenesis (41). Plasmids pAH49 and pAH54 (containing kanamycin and spectinomycin drug resistance genes, respectively; kindly provided by A. Camp in the lab) were used as templates for marker replacement. All primers used in this work are summarized in Table 2.

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TABLE 1. Strains used in this study

Strain	Genotype/description	Source or reference
E. coli		
DH5α	F′ ϕ80d*lacZ*ΔM15 Δ(*lacIZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1*	Invitrogen
BW25113	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB3*) λ^- *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	3
JW0300	Insertional knockout of *ykgF* in BW25113, Kan^r	3
B. subtilis		
3610	Undomesticated wild strain capable of forming robust biofilms	8
PY79	An SPβ-cured prototroph strain used as a host for transformation	42
RL3852	Δ*epsH*::*tet* in 3610	27
RL3856	Δ*epsH*::*tet* and Δ*sinR*::*spec* in 3610	27
YC125	*amyE*::P_{lutA}-*lacZ* Cm^r Δ*epsH*::*tet*	This work
YC126	*amyE*::P_{lutA}-*lacZ* Cm^r Δ*epsH*::*tet* Δ*sinR*::*spec*	This work
YC141	Δ*lutABC*::*spec*	This work
YC142	Δ*lutABC*::*spec* complemented with *B. s*ubtilis operon, Kan^r	This work
YC143	*amyE*::P_{lutA}-*lacZ* Cm^r Δ*epsH*::*tet* Δ*lutR*::*spec*	This work
YC150	Δ*lutP*::*kan*	This work
YC151	Δ*lctP*::*kan*	This work
YC232	Δ*lctE*::*kan*	This work
YC235	Δ*lutP*::*spec* Δ*lctP*::*kan*	This work
YC261	Δ*lutABC*::*spec* complemented with *E. coli* operon, Cm^r	This work
YC276	Δ*lutABC*::*spec* complemented with *B. coli* operon, Kan^r	This work
YC275	Δ*lutABC*::*spec* complemented with *E. faecalis* operon, Kan^r	This work
YC289	*amyE*::P_{lutA}-*lacZ* Cm^r Δ*epsH*::*tet* Δ*lutR*::*spec* Δ*sinR*::*kan*	This work
YC547	In-frame deletion in the *lutA* gene in 3610	This work
YC548	In-frame deletion in the *lutB* gene in 3610	This work
YC549	Δ*lutC*::*spec* in 3610	This work

To construct in-frame deletion mutants of *lutA* and *lutB*, we used PCR to amplify ~700 bp of DNA flanking the boundaries of the intended deletion (codons +4 to +712 in the case of *lutA* and codons +4 to +1420 in the case of *lutB*). Next, the two amplified DNAs were cloned sequentially into the BamHI and SalI and the EcoRI and NcoI restriction sites of a temperature-sensitive suicide vector, pMAD (2). The resulting recombinant plasmid was then introduced into PY79 by transformation. Transformants with the plasmid integrated into the chromosomal locus via Campbell integration were selected at a nonpermissive temperature (37°C) on LB agar plates (+ macrolides-lincosamides-streptogramin B [Mls] + X-Gal). The integrated plasmid was then transferred from the PY79 background into 3610 by SPP1 phage-mediated transduction. Mls-resistant blue colonies were picked and grown at a permissive temperature (30°C) in LB liquid medium to stationary phase to allow integrated plasmid to excise from the chromosome. Cells were then diluted 1,000-fold to fresh LB liquid medium and grown at a nonpermissive temperature (37°C) for 4 h. Cells were then diluted serially and plated on LB agar plates (+ X-Gal). The next day white colonies were picked from the plates and checked for loss of Mls drug resistance. The presence of in-frame deletions was verified by PCR (the deletion mutants occurred in ~50% of the white colonies).

To construct complementation *B. subtilis* Δ*lutABC* strains, homologous operons of *B. subtilis* 3610, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ZK1475, and *Escherichia coli* K-12 (*B. cereus* and *E. faecalis* strains were kindly provided by A. Earl, Harvard Medical School) were amplified by PCR using primers listed in Table 2. The PCR products were cloned into the restriction sites (indicated in the primers) of the vector pDP111 (17), which contains an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *hyper-spank* promoter and *lacI* flanked by two arms of the *amyE* gene. Each forward primer also contains a ribosome binding site optimized for protein translation in *B. subtilis*. The recombinant plasmids were then introduced into *B. subtilis* PY79 by transformation and were selected for double-crossover recombination at the *amyE* locus of the chromosome. The fusions at the *amyE* locus were then introduced into the *B. subtilis* Δ*lutABC* strain (YC141) from the PY79 background by SPP1 phage-mediated transduction (11).

β-Galactosidase assays. Cells were incubated in MSgg at 37°C in a water bath with shaking. One milliliter of culture was collected at each time point. Cells were spun down, and pellets were resuspended in 1 ml Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM KCl, and 38 mM β-mercaptoethanol) supplemented with 200 μg ml^{-1} freshly made lysozyme. Resuspensions were incubated at 30°C for 15 min. Reactions were started by adding 200 μl of 4 mg ml^{-1} ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and stopped by adding 500 μl of 1 M Na_2CO_3. Samples were briefly spun down. The soluble fractions were transferred to cuvettes (VWR), and optical density at 420 nm (OD_{420}) values of the samples were recorded using a Pharmacia ultraspectrometer 2000. The β-galactosidase specific activity was calculated according to the formula [OD_{420}/time × OD_{600}] × dilution factor × 1,000. Assays were conducted at least in duplicate.

Assays of growth in L-lactate minimal medium. To test growth on solid L-lactate minimal medium and other control minimal media (with either glucose or pyruvate as the sole carbon source), cells were first streaked out on LB agar medium and grown at 37°C. The next day, cells from single colonies on LB agar medium were picked and streaked onto solid agar plates containing minimal medium. IPTG was added at a final concentration of 500 μM to the minimal medium, when appropriate. Agar plates were then incubated at 37°C for 24 or 48 h. Pictures of the plates were taken using a Nikon CoolPix 950 digital camera.

To test bacterial growth on liquid L-lactate minimal medium, cells were first grown in LB broth until log phase, then washed with L-lactate minimal medium, and diluted into 20 ml L-lactate minimal medium. Cells were then grown at 37°C with shaking. At various time points, cell density (OD_{600}) of the cultures was recorded using a Pharmacia ultraspectrometer 2000. Growth rate was determined by calculating the increase of cell density during a certain period of time.

Colony morphology analysis. For assays of colony architecture on solid agar medium, cells were first grown in LB broth to exponential phase. A total of 3 μl of cells was then applied to minimal medium solidified with 1.5% Bacto agar. Plates were incubated at 23°C for 3 days. Images of the colonies on the plates were taken using a Nikon CoolPix 950 digital camera.

RESULTS

SinR controls the expression of a widely conserved but uncharacterized operon. We previously identified SinR as a master regulator for biofilm formation in *B. subtilis* (27). We

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)
P_{lutA}-F	GTCGAATTCGACAACAAAAGACATTACGCTGGAG
P_{lutA}-R	GTCGGATCCGACTTTCATGATGAACCCCTCTCTC
lutA-P1	GTGCCATCACCATTTCTCAATTTG
lutA-P2	CAATTCGCCCTATAGTGAGTCGTGATGAACCCCTCTCTCATCC
lutC-P1	GATCCGCCGTCACATATCGTAGCG
lutC-P2	CAATTCGCCCTATAGTGAGTCGTCACTGCTCCTCCTTCGTGCG
lutC-P3	CCAGCTTTTGTTCCCTTTAGTGAGGAACTCAGGAAGCCCGGCAG
lutC-P4	CAACATAAGAAAACGTCATCGAG
lutR-P1	TGCGAAATCTCCATCGCCGGAATC
lutR-P2	CAATTCGCCCTATAGTGAGTCGTTTCGACAAGCCCCATCGCTT
lutR-P3	CCAGCTTTTGTTCCCTTTAGTGAGAACCAACTCATTTCCCGGGC
lutR-P4	TGATACTCACAATGCTTACCCTGT
lutP-P1	GCGGAATTGCCCGGGAAATGAG
lutP-P2	CAATTCGCCCTATAGTGAGTCGTCCTTGAAACATAAACTGGCG
lutP-P3	CCAGCTTTTGTTCCCTTTAGTGAGTTCAGCTGGATGATTCCTTA
lutP-P4	GTCGTTAGAACCGGCTGGAATCG
lctE-P1	GCCTGAGGAACTCTCGAACCCG
lctE-P2	CAATTCGCCCTATAGTGAGTCGTTAATCATCCTTGCAGGGTATG
lctE-P3	CCAGCTTTTGTTCCCTTTAGTGAGACCGCAACTTTAGAGTAAAGG
lctE-P4	AGCGCTTTGAAGGCAGGAAGG
lctP-P1	GTAAATAAAGTAGCTTTAATCG
lctP-P2	CAATTCGCCCTATAGTGAGTCGTTGACAATCAGCCCTTTACTC
lctP-P3	CCAGCTTTTGTTCCCTTTAGTGAGCTTTTCCCATAAATAAAATG
lctP-P4	GATTACCTTAGTCGTATCCATTG
lutA-IFD-P1	GTACGGATCCTATGACAGTCATCGGACAAG
lutA-IFD-P2	GTACGTCGACCATGATGAACCCCTCTCTCA
lutA-IFD-P3	GTACGAATTCGATAAAACTGGATTCAGAGG
lutA-IFD-P4	GTACCCATGGCCTTCATTGGTCACAAGGCT
lutB-IFD-P1	GTACGGATCCCAACGGTTGAGCTGCTGGAG
lutB-IFD-P2	GTACGTCGACCATGATCCTTTCCCCCTCTG
lutB-IFD-P3	GTACGAATTCGCACGAAGGAGGAGCAGTGA
lutB-IFD-P4	GTACCCATGGGCTGCCTTTACTGGTCCATG
ykgEFG-F(*Ec*)	GATAAGCTTAAGGAGGAACTACTATGAATGTCAATTTCTTTGTC
ykgEFG-R(*Ec*)	GATGAGCTCGCTAGCTCAACAATCCTCAATAATCAG
ykgEFG-F(*Ef*)	GATGTCGACAAGGAGGAACTACTGTGAAAGTGAGTATATTTTCG
ykgEFG-R(*Ef*)	GATGCTAGCTTAGCGATCCATCACAACCAC
ykgEFG-F(*Bc*)	GATGTCGACAAGGAGGAACTACTATGAAAGTTACTTTATTTGTT
ykgEFG-R(*Bc*)	GATGCTAGCTTATACGATGAAATATACTGC
ykgEFG-F(*Bs*)	GATGTCGACAAGGAGGAACTACTATGAAAGTCTCACTTTTTGTC
ykgEFG-R(*Bs*)	GATGCTAGCTCAGCGGTCAGAGACGAGAAT

showed that SinR directly controls the *yqxM-sipW-tasA* and *epsA*-to-*O* operons, which are responsible for the production of the extracellular matrix (7, 11, 27), and *slr*, a regulatory gene that stimulates transcription of the *yqxM-sipW-tasA* operon (12, 28). Transcriptional profiling and bioinformatic analyses additionally identified two previously uncharacterized genes, *lutA* and *lutB* (formerly *yvfV* and *yvfW*) (11), that were likely to be under the direct negative control of SinR. Further analysis revealed that *lutA* and *lutB* are in an operon with a third gene, *lutC* (formerly *yvbY*) (Fig. 1A). The predicted products of all three genes contain putative iron-sulfur clusters and domains that resemble oxidoreductases (29).

To confirm that the *lutABC* operon is under the negative control of SinR, we fused the region just upstream of *lutA* (extending from +24 to −298 bp upstream of the *lutA* open reading frame) to the *lacZ* gene to create a transcriptional fusion, P_{lutA}-*lacZ*. The *lacZ* fusion was integrated into the chromosome at the *amyE* locus of *B. subtilis*. Expression of the P_{lutA}-*lacZ* fusion was about 10-fold higher in a *sinR* mutant than in the wild type (see Fig. 3B), a finding in confirmation of the expectation that the *lutABC* operon is under SinR control.

Interestingly, the *lutABC* operon is conserved among a wide variety of bacterial species, including gram-positive and gram-negative bacteria (Fig. 1B). For instance, the inferred products of the homologous operon from *E. coli* (*ykgEFG*) share about 54%, 57%, and 38% amino acid sequence identities with the corresponding *B. subtilis* proteins, respectively (unpublished observations) (6, 29). In contrast, *sinR* itself and other SinR-controlled genes are not widely conserved.

The wide conservation of the *lutABC* operon suggests that it plays an important but previously unknown role in bacterial physiology. Moreover, its regulation by SinR suggests that the operon also contributes in some unknown way to biofilm formation in *B. subtilis*.

Comparative genomics reveals that the *lutABC* operon is often found in close proximity to the gene for lactate permease. A comparison of genes in the vicinity of the *lutABC* operon in *B. subtilis* with those in the corresponding region of the genome of the closely related bacterium *Bacillus licheniformis* revealed a high degree of synteny, with the exception of a large (~15-kb) stretch of DNA just upstream of the *lutABC* operon in *B. subtilis* (Fig. 1A). In *B. subtilis*, this 15-kb interval separates the *lutABC* operon from the *lutR* (formerly *yvfI*), *lutP* (formerly *yvfH*), *sigL*, and *yvfG* genes and the *epsA*-to-*O*

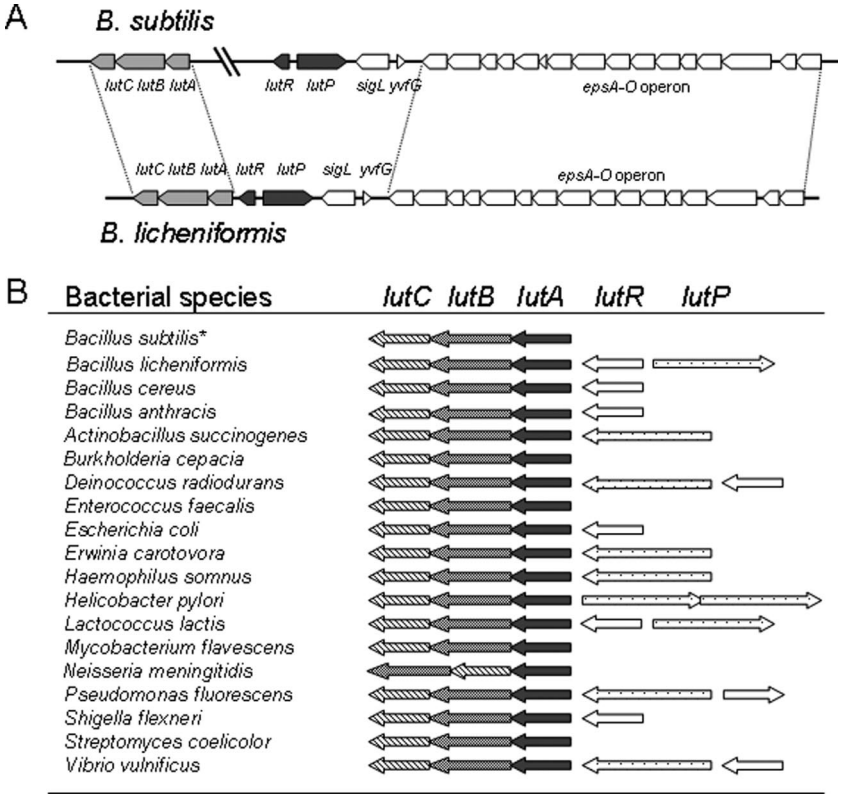

FIG. 1. Chromosomal regions containing the *lutABC* (formerly *yvfV-yvfW-yvbY*) operon. (A) An alignment of the chromosomal regions containing the *lutABC* and *epsA*-to-*O* operons of *B. subtilis* and *B. licheniformis*. In *B. subtilis*, the *lutABC* operon and the *lutR* (formerly *yvfI*) gene are separated by an ~15-kb sequence (indicated as double slashes) that is not present in *B. licheniformis*. (B) Alignments of chromosomal regions containing homologs of *lutABC* in a variety of bacteria. The asterisk indicates that in *B. subtilis*, the *lutP* (formerly *yvfH*) and *lutR* genes are located 15 kb away from the *lutABC* operon (panel A).

operon (Fig. 1A). That is, in *B. licheniformis*, the *lutABC* operon is immediately adjacent to *lutR*, which encodes a GntR-type repressor, and is followed by *lutP*, which is predicted to encode a lactate permease (Fig. 1A) (33, 39). We also noticed that the *lutABC* operon is located adjacent to homologs of the *lutR* and *lutP* genes in several other bacteria, including distantly related species (Fig. 1B). A simple interpretation of these observations is that an insertion in an ancestor of *B. subtilis* separated the *lutABC* operon from the *lutR*, *lutP*, *sigL*, *yvfG*, and *epsA*-to-*O* gene cluster. These observations led us to hypothesize that the function of the *lutABC* operon is related to that of one or more members of the cluster. Specifically, and given that *lutP* is predicted to encode a lactate permease, we hypothesized that the *lutABC* operon is involved in lactate metabolism.

The *lutABC* operon is required for growth on L-lactate as the sole carbon source. To test our hypothesis, we constructed a *B. subtilis* mutant lacking the *lutABC* operon (Δ*lutABC*). We then tested whether the mutant was impaired in growth on solid minimal medium with 0.5% L-lactate as the sole carbon source. As shown in Fig. 2A, the mutant (strain 2 in the figure) exhibited little growth compared to the wild type (strain 1). As a control, both the wild type and the mutant grew equally well on solid minimal medium with 0.5% glucose as the sole carbon source (Fig. 2C) or with a mixture of glucose and L-lactate (data not shown). Also, for comparison, neither the wild type nor the mutant grew appreciably on minimal medium with 0.5% D-lactate as the sole carbon source (data not shown). We conclude that the *lutABC* operon encodes a previously undiscovered pathway for L-lactate utilization.

To determine whether each gene in the operon was required for growth on L-lactate, we constructed in-frame deletions in *lutA* and *lutB* and an insertion in *lutC* (creating strains YC547, YC548, and YC549, respectively; see Materials and Methods). None of the mutants grew on minimal medium with L-lactate as the sole carbon source (data not shown), indicating that all three genes are indispensable for L-lactate utilization. Because all three genes in the *lutABC* operon contain domains that resemble oxidoreductases (29), we postulate that this pathway oxidatively converts L-lactate to pyruvate. Indeed, both the wild type and the mutant grew equally well in minimal medium with 0.5% pyruvate as the sole carbon source (data not shown). LutABC is evidently the only pathway for L-lactate utilization in *B. subtilis*, since the mutant exhibited little or no growth on L-lactate under the conditions tested (Fig. 2A). Further, BLAST analysis failed to reveal an ortholog of the *lldD* gene from *E. coli*, which encodes a lactate oxidase that also allows cells to utilize lactate (unpublished observations) (15).

B. subtilis does, however, contain *lctE*, which encodes an NAD-dependent L-lactate dehydrogenase (14). It has been reported that this gene plays an important role in production of L-lactate during fermentative growth of *B. subtilis* (14, 34). In

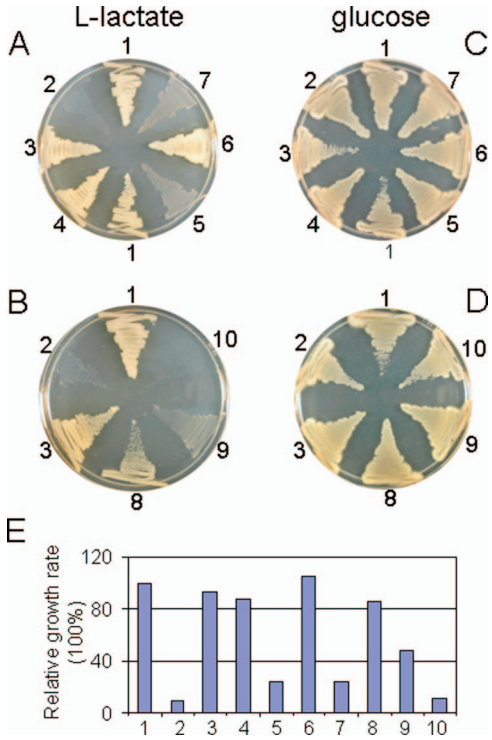

FIG. 2. The *lutABC* operon is required for growth on L-lactate. (A and B) the growth of wild-type and mutant strains of *B. subtilis* on solid minimal media containing 0.5% L-lactate as the sole carbon source. (C and D) The growth of the same set of strains (shown in panels A and B) on solid minimal media containing 0.5% glucose as the sole carbon source. (E) Comparison of the growth rates of *B. subtilis* wild-type and mutant strains (represented by numbers on the *x* axis) in liquid minimal medium with 0.5% L-lactate. The *y* axis is the growth rate relative to the wild type (strain 1) and is expressed as a percentage. *B. subtilis* strains shown in this figure are represented as follows: 1, 3610; 2, Δ*lutABC* mutant (YC141); 3, Δ*lutABC* mutant complemented with *lutABC* at *amyE* (YC142); 4, a Δ*lctE* mutant (YC232); 5, a Δ*lutP* mutant (YC150); 6, a Δ*lctP* mutant (YC151); 7, a Δ*lutP* Δ*lctP* double mutant (YC235); 8, Δ*lutABC* mutant complemented at *amyE* with the homologous operon from *B. cereus* (YC276); 9, Δ*lutABC* mutant complemented at *amyE* with the homologous operon from *E. faecalis* (YC275); 10, Δ*lutABC* mutant complemented at *amyE* with the homologous operon from *E. coli* (YC261).

some other bacteria, similar NAD-dependent L-lactate dehydrogenases were shown to be capable of reversibly interconnecting pyruvate and lactate (23). We therefore wondered whether in *B. subtilis*, the *lctE* gene also contributes to L-lactate utilization. A null mutant of the *lctE* gene was created in *B. subtilis*, and the mutant strain was tested for growth on L-lactate minimal medium. The results showed that the mutant was unimpaired in growth on L-lactate (Fig. 2A, strain 4). This observation suggests that in *B. subtilis* lactate dehydrogenase contributes only to the conversion of pyruvate to L-lactate, but not vice versa. Alternatively, the dehydrogenase does convert L-lactate to pyruvate but not under the conditions tested. For example, it has been reported that *lctE* is strongly induced under anaerobic growth (13, 38).

***lutP* is also required for growth on L-lactate.** The clue to the discovery that the *lutABC* operon specifies a new L-lactate utilization pathway was the presence of nearby *lutP* (formerly *yvfH*), which is predicted to encode a lactate permease (29). We therefore sought to determine whether *lutP* was indeed required for growth on L-lactate. We found that a null mutant (Δ*lutP*) was markedly impaired (but not totally blocked) in growth on L-lactate minimal medium (Fig. 2A and E, strain 5) but not impaired in growth on glucose minimal medium (Fig. 2C). These observations are consistent with the idea that the *lutP* gene encodes the principal permease for the uptake of L-lactate.

That the Δ*lutP* mutant cells could still grow to some extent on L-lactate raised the possibility that there is an additional L-lactate permease(s) in *B. subtilis*. A candidate is *lctP*, which is also predicted to encode an L-lactate permease (14). We wondered whether this *lctP* gene contributed to the slow growth of the Δ*lutP* mutant cells in L-lactate minimal medium. Accordingly, we created a Δ*lctP* Δ*lutP* double mutant. However, the double knockout mutant grew no more slowly than did the Δ*lutP* single mutant (Fig. 2A and E, strain 7). Also, a Δ*lctP* single mutant showed no measurable growth defect in L-lactate minimal medium under the conditions tested (Fig. 2A and E, strain 6).

In toto, these results suggest that (i) *lutP* encodes the principal permease for import of L-lactate; (ii) *lctP* either was not expressed under our conditions or participates only in L-lactate export—in fact, it has been reported that a *lctP* mutant is impaired in the accumulation of L-lactate in the medium (14); and (iii) an additional unrecognized permease, perhaps one that is not specific for L-lactate, also contributes to a limited extent to L-lactate uptake. It is also possible that limited L-lactate uptake is facilitated by Na^+ or K^+ symporters, as there are a number of such symporters present in *B. subtilis* that are predicted from the genome sequence (29).

LutR and SinR act cooperatively to repress the *lutABC* operon. Next, we turned our attention to *lutR* (formerly *yvfI*), which encodes a GntR-type repressor and which is immediately adjacent to the *lutABC* operon in a variety of bacterial genomes (Fig. 1A and B). We therefore hypothesized that LutR is involved in regulating the *lutABC* operon. To test this idea, we examined the effect of a *lutR* null mutation on the activity of a P_{lutA}-*lacZ* fusion in *B. subtilis*. The results showed that the activity of P_{lutA}-*lacZ* was significantly higher in a *lutR* mutant than in the wild type (Fig. 3B). These observations are consistent with the idea that the *lutABC* operon is subject to repression by LutR as well as by SinR. Given that the activity of GntR-type repressors is often governed by a cognate ligand whose presence causes derepression of target genes (38), we hypothesized that the ligand of LutR would be L-lactate and hence that L-lactate would induce the operon. Consistent with these ideas, addition of L-lactate (but not D-lactate; data not shown) to the medium strongly induced the P_{lutA}-*lacZ* fusion and did so in a dose-dependent manner (Fig. 3A). Moreover, the *lutR* mutant was similarly derepressed in the presence and absence of L-lactate (Fig. 3B). These findings reinforce the conclusion that the *lutABC* operon is a metabolic operon for L-lactate utilization.

In brief, we have demonstrated that the *lutABC* operon is under the dual control of LutR and SinR. Moreover, LutR and SinR seem to act cooperatively, as the absence of either repressor alone resulted in derepression of the operon. Also, expression of P_{lutA}-*lacZ* was only modestly higher in the Δ*sinR*

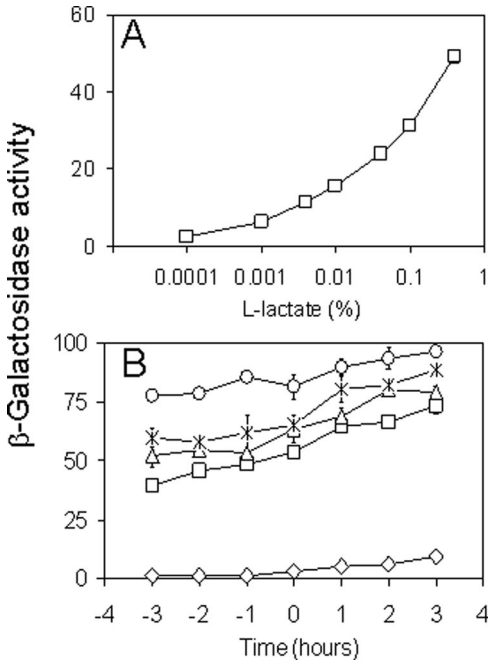

FIG. 3. Regulation of the *lutABC* operon. (A) The induction of P_{lutA}-*lacZ* (in strain YC125) caused by addition of L-lactate to the medium ranged from 0.0001% to 0.4% (wt/vol). (B) Comparison of the levels of expression of P_{lutA}-*lacZ* in the wild type (YC125) (diamonds), the Δ*sinR* mutant (YC126) (stars), the Δ*lutR* mutant (YC143) (squares), the Δ*lutR* mutant grown in MSgg supplemented with 0.4% L-lactate (YC143) (triangles), and the Δ*lutR* Δ*sinR* double mutant (YC289) (circles).

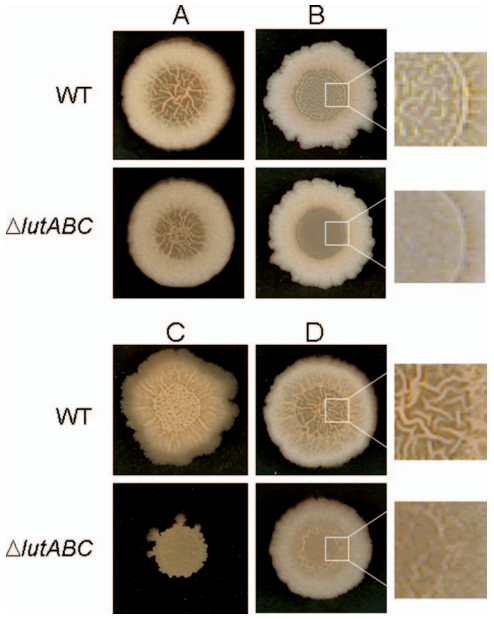

FIG. 4. Biofilm architecture is influenced by the *lutABC* operon. Shown are biofilms formed by the wild type (3610) and the Δ*lutABC* mutant (YC141) in MSgg (A), in modified MSgg in which 0.5% glycerol was replaced with 0.5% glucose (B) or replaced with 0.5% L-lactate (C), or in MSgg supplemented with 0.5% L-lactate (D). Enlargements of the indicated regions are shown to the right.

Δ*lutR* double mutant than in either single mutant (Fig. 3B). We speculate that LutR and SinR contact each other in the regulatory region for the *lutABC* operon and that both repressors depend on this cooperative interaction to bind efficiently to DNA.

A mutation of the *lutABC* operon is complemented by orthologous operons from other gram-positive bacteria. Next, we return to the observation that the *lutABC* operon is widely conserved (Fig. 1B). Are orthologs of the LutA, LutB, and LutC proteins responsible for lactate utilization in other bacterial species that contain the operon? We investigated this question by introducing into a *B. subtilis* mutant lacking the *lutABC* operon the orthologous operons from *Bacillus cereus* and *Enterococcus faecalis* (see Materials and Methods). The results showed that operons from the two gram-positive species complemented the growth defect of the mutant when tested on L-lactate minimal medium (Fig. 2B and E, strains 8 and 9). Both strains also grew well on glucose minimal medium (Fig. 2D). These results are consistent with the idea that orthologs of the *lutABC* operon mediate lactate utilization in other related, gram-positive species.

We failed, however, to observe complementation with the apparently orthologous operon (*ykgEFG*) from *E. coli* (Fig. 2B and E, strain 10). Moreover, an insertional mutant of the *E. coli* ortholog to *lutB* (*ykgF*) was unimpaired for growth on L-lactate minimal medium (data not shown). It is known that *E. coli* contains another operon (*lldP-lldR-lldD*) that mediates the conversion of L-lactate to pyruvate (the *lldD* gene encodes a lactate oxidase), and evidently the *lldP-lldR-lldD* operon suffices for growth on lactate (15).

The *lutABC* operon is involved in biofilm formation. The starting point for this investigation was the observations that the *lutABC* operon is under the control of SinR, the master regulator for biofilm formation in *B. subtilis*, and that the operon is located in close proximity in *B. licheniformis* to an operon (*epsA* to *-O*) that is required for production of exopolysaccharide during biofilm formation (27). It therefore seemed reasonable to expect that the *lutABC* operon is involved not only in lactate utilization during growth but also in biofilm formation.

Lactate is typically produced from glucose during glycolysis, and its generation (by reduction of pyruvate) serves to rebalance the NAD^+/NADH pool (13). In *B. subtilis*, lactate accumulates in the medium early in growth and is used as an energy source late in the growth cycle (14). Conceivably, therefore, lactate that has accumulated in the medium during growth serves as an energy source for biofilm formation as other nutrients are exhausted from the medium. We routinely study biofilm formation using MSgg, a medium that contains glycerol as a carbon source and glutamate as a nitrogen (and carbon) source (8), neither of which leads to substantial production of lactate. Indeed, a Δ*lutABC* mutant exhibited little or no defect in biofilm formation on MSgg medium (Fig. 4A and data not shown). In contrast, however, when we replaced glycerol with glucose in the medium, the surface of colonies of the operon mutant exhibited less architectural detail, especially in the central region of the biofilm (Fig. 4B). The most striking results were obtained when we replaced glycerol with L-lactate. Whereas the wild type formed robust biofilms (al-

though slightly different morphologically from the ones observed on MSgg medium), the Δ*lutABC* mutant formed small colonies that almost completely lacked surface architecture (Fig. 4C). Finally, we supplemented normal MSgg medium with L-lactate (that is, the medium contained both glycerol and lactate). Under these conditions, the wild type formed normal-looking biofilms, whereas the mutant colonies were less architecturally complex (Fig. 4D).

Thus, under all conditions in which the cells were expected to generate lactate or in which the medium contained lactate, biofilm formation as judged by architectural complexity on solid medium (or pellicle formation in liquid medium [data not shown]) was less robust in the absence of the lactate utilization operon than in its presence. In toto, the results lead us to the following view. The principal function of the *lutABC* operon in *B. subtilis* and at least in certain other gram-positive bacteria that contain the operon is to allow cells to grow with lactate as a carbon source. In *B. subtilis* and closely related species (e.g., *B. licheniformis*), however, the operon is additionally under the control of SinR, thereby allowing cells to utilize an alternative carbon source during biofilm formation.

DISCUSSION

The pathway that generates lactate in bacteria is well understood. A single, widely conserved enzyme, NAD-dependent lactate dehydrogenase, converts pyruvate to lactate during glycolysis and at the same time oxidizes NADH back to NAD^+ (21). How most bacteria convert lactate to pyruvate, on the other hand, is less well understood. The *lldD* gene in *E. coli* encodes an L-lactate oxidase that allows cells to grow on minimal medium with L-lactate as the sole carbon source (15). L-Lactate oxidase catalyzes the oxidation of L-lactate using molecular oxygen, producing pyruvate and hydrogen peroxide as end products (16). However, *lldD* is not widely conserved. BLAST analysis revealed orthologs of *lldD* in certain gram-negative bacteria, such as *Salmonella* spp., *Pseudomonas* spp., and *Vibrio* spp. (unpublished observations). In some distantly related species, such as *Streptococcus* spp. and *Lactococcus* spp., orthologs of *lldD* (though with limited sequence similarity) were detected and shown to be associated with L-lactate oxidase activity (5, 22, 35). But only some of these species contain *lldD*. For instance, orthologs of *lldD* were found in only two out of eight species of *Streptococcus* examined (22). Also, in at least one species, *Neisseria meningitidis*, which does contain an ortholog of the gene, a null mutation of *lldD* did not impair growth on L-lactate (18).

The *lutABC* operon, in contrast, is highly conserved among a wide range of distantly related bacteria. Also, as we have shown, orthologous operons from other gram-positive species are capable of restoring lactate utilization to the *B. subtilis* mutant for the *lutABC* operon. On the other hand, *E. coli* relies on lactate oxidase for lactate utilization, even though it has a clear homolog of the *B. subtilis lutABC* operon (*ykgEFG*). Conceivably, the *ykgEFG* operon does contribute to lactate catabolism in *E. coli*, but under conditions other than those tested. Alternatively, the *ykgEFG* operon is responsible for the catabolism of a metabolite other than (but perhaps related to) lactate (e.g., malate) in *E. coli*. In any event, and given the striking conservation of the *lutABC* operon, it remains likely

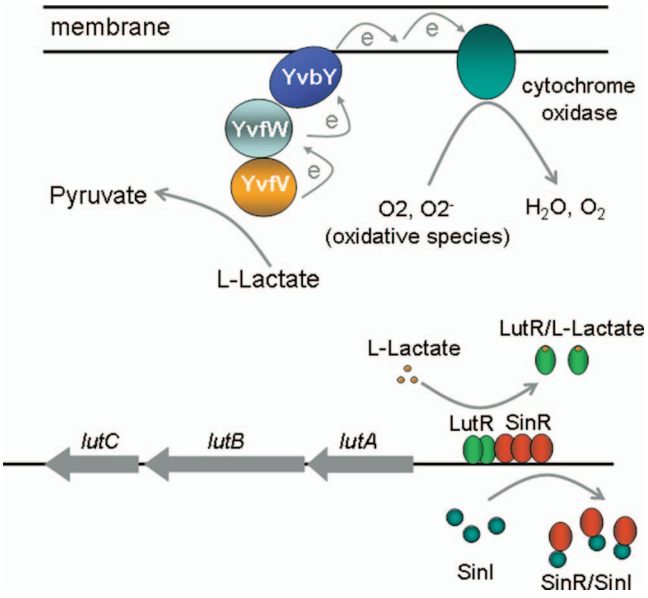

FIG. 5. Models for LutA-LutB-LutC-mediated oxidation of L-lactate and for regulation of the operon. The top cartoon depicts a proposed electron transport chain in the oxidation of L-lactate with electrons being transferred to oxygen. The order of iron-sulfur-containing proteins in the model is arbitrary. The bottom cartoon depicts a model for the regulation of the *lutABC* operon, in which the LutR and SinR repressors are hypothesized to interact with each other cooperatively in binding to DNA. L-lactate is depicted as binding to LutR, thereby derepressing the operon, which depends on both LutR and SinR to remain repressed.

that orthologs of LutA, LutB, and LutC mediate lactate utilization in gram-positive bacteria and perhaps in certain gram-negative species as well.

LutA, LutB, and LutC are inferred to contain iron-sulfur clusters. This suggests that oxidation of lactate occurs via a cytochrome-like electron transfer chain (Fig. 5). In this regard, it is interesting that a fourth gene encoding a putative iron-sulfur-containing oxidase is sometimes present in or near the *lutABC* operon (unpublished observations). Conceivably, this additional protein represents a fourth component in the hypothesized electron chain.

Lastly, we note that lactate utilization has also been reported to promote colonization of *Neisseria gonorrhoeae* in the genital tract (20). A mutant of the gene for the lactate permease, *lctP*, cannot take up exogenous lactate and is significantly attenuated in its ability to colonize and survive in the genital tract (20). Studies with *N. meningitidis* showed that lactate also promotes the production of certain determinants of pathogenicity, such as lipopolysaccharides (19). Finally, acquisition of lactate has been reported to be necessary for *Haemophilus influenzae* to cause bacteremia (26). Evidently, then, lactate utilization is important not only for metabolism but also for multicellularity and bacterium-host interactions in a variety of species.

In summary, we have shown that the previously uncharacterized *lutABC* operon is responsible for growth on L-lactate in *B. subtilis*. The operon is under the dual control of a GntR-type repressor, LutR, and the master regulator for biofilm formation, SinR, both of which are required to maintain repression and presumably act cooperatively (Fig. 5).

Repression is relieved either by L-lactate or by inactivation of SinR during biofilm formation. The high conservation of the *lutABC* operon suggests that it represents a widely distributed pathway for the conversion of lactate to pyruvate, and complementation experiments support the view that this is the case in related gram-positive bacteria. Alternatively, it may be responsible for the catabolism of metabolites related to L-lactate in certain bacteria. In *B. subtilis*, the operon has additionally come under the control of the regulatory circuit governing biofilm formation, under which conditions it contributes to the formation of architecturally complex communities when lactate is present.

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