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Integrated Regulation of Acetoin Fermentation by Quorum Sensing and pH in *Serratia plymuthica* RVH1▽

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During fermentation of sugars, a number of bacterial species are able to switch from mixed acid production to acetoin and 2,3-butanediol production in order to avoid lethal acidification of their environment, although the regulation of this switch is only poorly understood. In this study, we report the identification of the *budAB* structural operon, involved in acetoin production in *Serratia plymuthica* RVH1, and its activation by a LysR-type regulator encoded by *budR*, immediately upstream of this operon. In addition, the regulation of *budR* transcription was elucidated and found to be subject to negative control by BudR itself and to positive control by external stimuli such as *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) quorum sensing signaling molecules and acetate. Interestingly, however, we observed that induction of *budR* transcription by OHHL or acetate did not require BudR, indicating the involvement of additional regulatory factors in relaying these environmental signals to the *budR* promoter.

A wide variety of strains are able to switch to acetoin and 2,3-butanediol production during fermentative growth to counteract the lethal acidification that typically accompanies mixed acid production (3, 17, 21). Butanediol is produced by first converting two molecules of pyruvate into α-acetolactate by α-acetolactate synthase, after which α-acetolactate is processed to acetoin by α-acetolactate decarboxylase. Finally, acetoin can be converted reversibly to 2,3-butanediol through the action of acetoin reductase (25). Often, the structural genes for 2,3-butanediol production are organized into an operon that is controlled by a neighboring and divergently transcribed LysR homologue (6, 12, 15). Nevertheless, the regulatory aspects, and especially the integration of environmental cues into the control of this pathway, remain largely unexplored.

In this article, we report on the identification and regulation of the acetoin operon in *Serratia plymuthica* RVH1 and on its activation at the molecular level by signals of acidification and quorum sensing (QS).

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. plymuthica* RVH1 strains were cultured at 30°C in Luria-Bertani (LB) broth or agar (1.5% agar) or in M9 minimal medium (14). Where necessary, LB medium was acidified using 1 M HCl or buffered with 100 mM phosphate buffer at pH 7.0. As described previously (24), growth was monitored using a Bioscreen C microbiology reader (Thermo Life Sciences, Brussels, Belgium), while acetoin production and medium acidification were determined by the Voges-Proskauer and methyl red assays, respectively.

The following concentrations of sugar and/or antibiotics (Applichem, Darmstadt, Germany) were used when appropriate: glucose, 0.5%; ampicillin, 100 μg/ml; carbenicillin, 300 μg/ml; kanamycin, 50 μg/ml; and gentamicin, 20 μg/ml. The synthetic *N*-acyl-L-homoserine lactone (AHL) used in this study, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (5 μM solution in water), was purchased from Sigma (Bornem, Belgium).

Identification and sequencing of the RVH1 *bud* locus. A random knockout library of *S. plymuthica* RVH1 was constructed using an EZ-Tn5 KAN-2 Tnp transposome kit as described by the manufacturer (Epicentre, BiozymTC BV, Landgraaf, Netherlands). Circa 4,000 independent clones were purified, grown in LB-glucose, and screened for acetoin production by use of the Voges-Proskauer test and for acidification by use of the methyl red assay. One mutant that exhibited a yellow coloration in the former and a red coloration in the latter test, indicative of a loss of acetoin production and increased acidification of the growth medium, was selected. Genomic DNA of this clone was isolated using a Qiagen 100/G genomic tip according to the protocol supplied by the manufacturer. Subsequently, the insertion site was determined by sequencing (VIB Sequencing Facility, Antwerp, Belgium) the flanking site of the transposon, using KAN-2 RP-1 from an EZ-Tn5 KAN-2 Tnp transposome kit. Comparison of the obtained sequence with the GenBank nucleotide sequence database by use of BLASTX (1) revealed that the transposon was located in a gene homologous to an α-acetolactate synthase gene. In addition, part of the neighboring α-acetolactate decarboxylase gene sequence was also obtained.

To fully obtain the RVH1 sequences of these two genes and the neighboring *lysR* homologue (whose presence was suspected through comparison with the sequence of *Serratia proteamaculans* 568 [18]), primers budR_FW and budB_Rev were designed based on the nucleotide sequence of *S. proteamaculans* 568 and were combined with *S. plymuthica* RVH1-based primers budA_Rev and budA_FW, respectively. The resulting PCR products were sequenced using these four primers and the additional primers budR_gap, bud_intergen, and budB_final in order to close the gaps. The three genes were named *budR*, *budA*, and *budB*, among which *budA* and *budB* form an operon separated by 382 bp from *budR*, which is divergently transcribed (GenBank accession number HQ602654). All primers used in this study are summarized in Table 2.

Construction of *budR* mutant. To construct a *budR*-deficient mutant, primers budR_FW and budA_Fus were used to amplify the *budR* locus, after which this PCR product was ligated with pUC18 digested with SmaI (26). A unique MunI restriction site in the center of the *budR* gene was used to digest the resulting construct. After the ends were blunted, a chloramphenicol resistance cassette obtained from λ NK1324 (7) by BamHI cleavage and blunting was cloned in this location. PCR amplification of the resulting *budR*::*cat* allele by use of primers pUC47 and pUC24bis, followed by ligation with an EcoRV-digested pSF100 suicide plasmid (13), resulted in plasmid pSF100-*budR*::*cat*. *E. coli* S17-1 λ*pir*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference
Strains		
E. coli strains		
MG1655	F^- *rph-1*	5
S17-1 λ*pir*	*recA thi pro hsd*(r^- m^+) RP4:2-Tc:Mu:Km Tn*7* λ*pir* Tp^r Sm^r	16
S. plymuthica strains		
RVH1	Wild type; natural isolate	20, 23
RVH1 *splI*	*splI*::*aacC1* Gm^r	22
RVH1 *budB*	*budB*::EZ-Tn*5* Km^r	This study
RVH1 *budR*	*budR*::*cat* Cm^r	This study
RVH1 *budA*::*lacZ*	RVH1 with chromosomal *lacZ* reporter fusion to *budAB* promoter; Km^r	This study
RVH1 *splI budA*::*lacZ*	RVH1 *splI* with chromosomal *lacZ* reporter fusion to *budAB* promoter; Gm^r Km^r	This study
RVH1 *budR budA*::*lacZ*	RVH1 *budR* with chromosomal *lacZ* reporter fusion to *budAB* promoter; Cm^r Km^r	This study
Plasmids		
pTRC99a	Cloning vector carrying IPTG-inducible *trc* promoter (P_{trc}); Ap^r	2
pTRC99a-P_{trc}-*budAB*	pTRC99a carrying the *budAB* operon fused downstream of P_{trc}	This study
pFPV25	Cloning vector carrying promoterless *gfp*; Ap^r	19
pFPV25-P_{budR}-*gfp*	pFPV25 carrying the *budR* promoter fused upstream of *gfp*	This study
pUC18	Cloning vector; Ap^r	26
pUC18-*budA*::*lacZ-kan*	pUC18 carrying the *budAB* promoter fused to *lacZ*	This study
pSF100	Pir-dependent replicon; Ap^r Km^r	13
pSF100-*budR*::*cat*	pSF100 carrying *budR*::*cat*	This study
pSF100-*budA*::*lacZ-kan*	pSF100 carrying the *lacZ* reporter cloned into the *budA* locus	This study

(16) carrying this plasmid was conjugated with *S. plymuthica* RVH1, and exconjugants were selected on M9 minimal medium containing chloramphenicol. Colonies in which double homologous recombination had occurred were selected by loss of ampicillin or carbenicillin resistance. All primers used in this study are summarized in Table 2.

Construction of a chromosomal *budAB* promoter reporter fusion to *lacZ*. A *budAB* promoter reporter fusion to *lacZ* was constructed by PCR amplification of the intergenic region between *budR* and *budA* together with the first ca. 400 bp of *budA*, using the up-bud-SalI and down-bud-SalI primers, after which the obtained PCR product was digested with SalI and ligated with a SalI-digested pUC18 plasmid (26). PCR amplification of this construct was performed using the primers up-bud-XhoI and down-bud-XhoI, after which the resulting fragment was ligated to a phosphorylated kanamycin resistance gene obtained through PCR amplification of a pKD4 plasmid (4) by use of the pKD3/4-P1-Fw and pKD3/4-P2-Rev primers. From the resulting plasmid, an amplicon was prepared with primers down-bud-XhoI and pKD3/4-P1-Fw and was ligated to a phosphorylated amplicon of the *lacZ* gene that was obtained through PCR amplification of genomic DNA of *E. coli* with primers lacZ-Fw and lacZ-Rev.

TABLE 2. Primers used in this study

Primer	Sequence
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG
budR_FW	TCACTCCCAGCTCGGGGGCT
budB_Rev	TTAAATCATCTGGCTGAA
budA_Rev	CGTGGTCGAGCTGATAATCC
budA_FW	AACCGACCTTTGCCTTTCTC
budR_gap	CGCCCGCTATGTTGAAC
bud_intergen	GTTCAACATAGCGGGCG
budB_final	GATCTCCAACGGCCAGCAG
budA_Fus	CCCCTTAGAAACCCTGCACCAAATG
pUC47	CGCCAGGGTTTTCCCAGTCACGAC
pUC24bis	GAGTCAGTGAGCGAGGAAGGC
budAB_start	ATGAACGAAAAGCAGGGTGG
budR_Fus	CCCCTTAGGCGTCATTCATCTTTGC
Up-bud-XhoI	CCCCCTCGAGCATTACGTACTCCTCCACCAG
Down-bud-XhoI	CCGGCTCGAGAAGCAGGGTTGTTCCTGTGCG
pKD3/4-P1-FW	GTGTAGGCTGGAGCTGCTTC
pKD3/4-P2-Rev	CATATGAATATCCTCCTTAG
lacZ-FW_XhoI	TCGGCTCGAGACCATGATTACGGATTCACT
lacZ-RV_XhoI	TCCGCTCGAGTTTTTGACACCAGACCAACT
Up-bud-SalI	GGGGGTCGACGGGGCCTACGGACGGTAAGAG
Down-bud-SalI	GCGCGTCGACCGCAGAACAGGTTCGGTGAAG

The resulting plasmid had *lacZ* and the kanamycin resistance gene inserted at the 5′ end of the *budA* gene. After restriction analysis to confirm the correct orientation of the *lacZ* gene with regard to the *budAB* promoter, the plasmid was designated pUC18-*budA*::*lacZ*-Km. Next, the entire fragment, containing the *budA* promoter fusion coupled to *lacZ* and the kanamycin resistance gene, was amplified by a PCR using the up-bud-SalI and down-bud-SalI primers and was ligated to a SalI-digested pSF100 plasmid (13) from which the kanamycin resistance gene was previously removed by SalI digestion. Using *E. coli* S17-1 λ*pir* (16), the resulting pSF100-*budA*::*lacZ*-Km plasmid was conjugated to the *S. plymuthica* RVH1 wild type and its *splI* and *budR* derivatives. Only transconjugants resistant to kanamycin and sensitive to carbenicillin were retained, as in these clones double homologous recombination via the ca. 300- to 400-bp flanking regions mediated genomic insertion of the *lacZ* reporter and the kanamycin resistance marker into the *budA* gene (referred to as *budA*::*lacZ*). Proper genomic insertion of the *budA*::*lacZ* fusion was further confirmed by PCR analysis and by loss of acetoin production in RVH1.

Construction of pTRC99a-P_{trc}-*budAB* and pFPV25-P_{budR}-*gfp*. To construct pTRC99a-P_{trc}-*budAB*, a plasmid containing an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *budAB* operon, the *budAB* genes were PCR amplified using the budAB_start and budB_Rev primers. Next, this product was ligated to a SmaI-digested pTRC99a plasmid (2). Sequence analysis confirmed complete and correct insertion of the *budAB* operon. Heterologous expression of the *budAB* genes from this plasmid in *E. coli* MG1655 (5), which does not naturally produce acetoin, imposed acetoin production, as confirmed by the Voges-Proskauer assay.

A *budR* promoter reporter fusion to *gfp* (i.e., P_{budR}-*gfp*) was constructed by PCR amplification of the intergenic region between *budR* and *budA*, using the budA_Fus and budR_Fus primers, after which the obtained PCR product was ligated into a SmaI-digested pFPV25 plasmid (19) which contained a promoterless *gfp* gene. Sequence analysis confirmed correct insertion of the fragment. All primers used in this study are summarized in Table 2.

Measurement of fluorescence and β-galactosidase. Fluorescence was measured by either a flow cytometer (Guava Easycyte Plus; Guava Technologies, Millipore, Brussels, Belgium) or a microplate reader (Fluoroskan Ascent FL apparatus; Thermo Life Sciences, Brussels, Belgium). For flow cytometry, cells were harvested and diluted in 10 mM phosphate buffer (pH 7.0) to obtain a concentration of 500 to 2,500 cells/μl. Subsequently, the mean green fluorescent protein (GFP) content of ca. 5,000 cells was determined using a Guava Easycyte Plus flow cytometer, using excitation and emission filters of 488 nm and 525 nm, respectively. For the microplate reader, 300 μl of a cell suspension was placed in a well of a 96-well microplate, and GFP fluorescence was recorded at 520 nm,

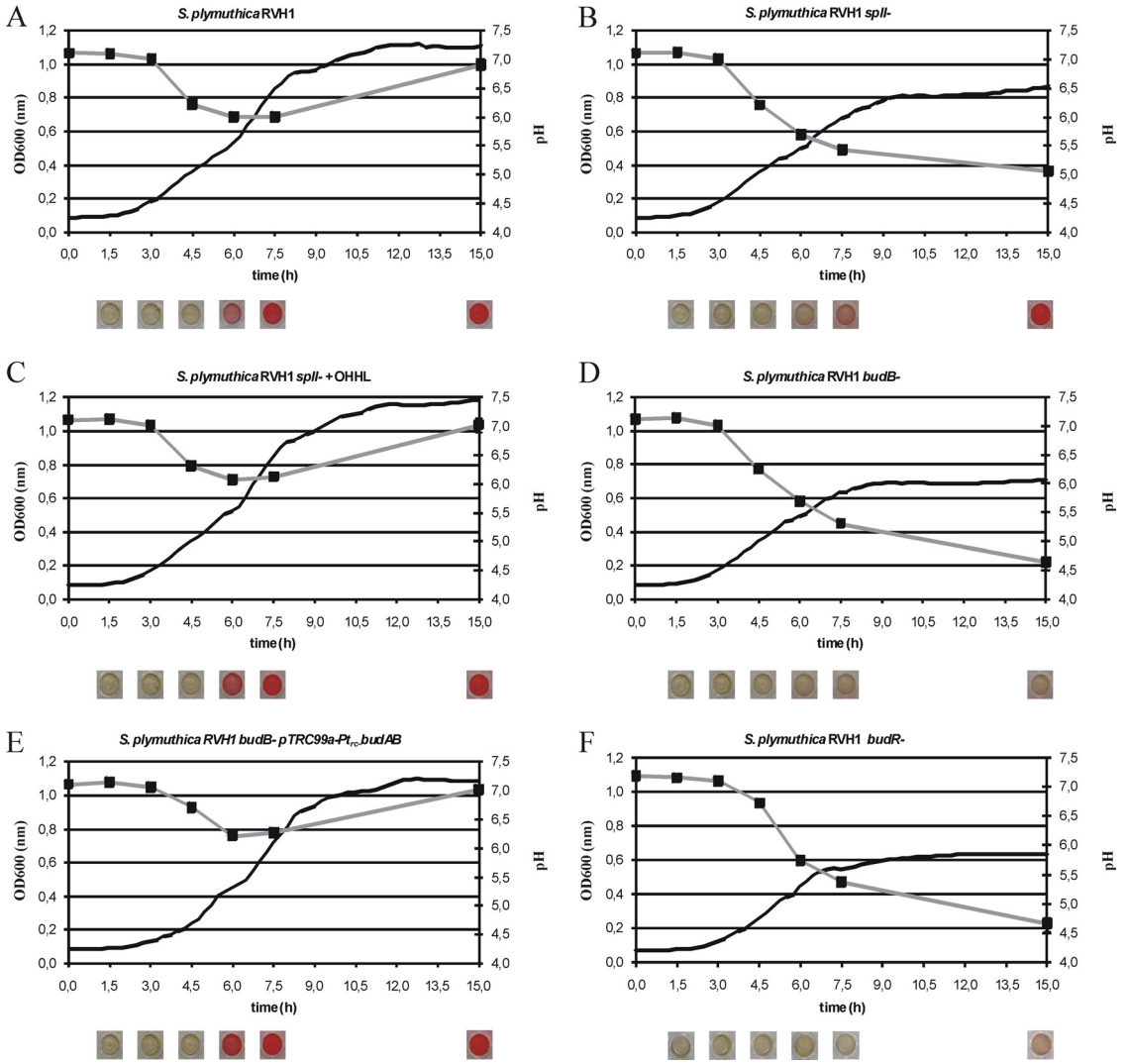

FIG. 1. Growth curves (black lines), pH profiles (gray lines; black boxes represent measurement points), and acetoin production (images below each graph) at indicated time points for different *S. plymuthica* RVH1 mutants. Representative results of three independent experiments are shown.

using an excitation wavelength of 480 nm. Finally, the production of β-galactosidase was measured using the chromogenic substrate *ortho*-nitrophenyl-β-D-galactosidase (ONPG) as described by Miller (11).

RESULTS AND DISCUSSION

Both quorum sensing and low pH regulate acetoin production in *S. plymuthica* RVH1. Following up on our previous discovery that an AHL-based QS system activates acetoin and 2,3-butanediol production in *Serratia plymuthica* RVH1 (24) (Fig. 1A to C), we observed that a mutant deficient in the production of QS signaling molecules (RVH1 *splI*) still exhibited residual, though delayed, acetoin (i.e., precursor of 2,3-butanediol) production (Fig. 1B), indicating that QS does not constitute the only regulation of acetoin production in RVH1. Since the RVH *splI* mutant strongly acidified the medium, and since low pH was previously observed to affect acetoin production in *Bacillus subtilis* and *Klebsiella terrigena* (10, 12), we compared the effects of acidic and neutral environments on acetoin production in the RVH1 *splI* mutant (Fig. 2). As such, we could clearly see that for cultures starting growth under acidic conditions (pH 5.5), acetoin became detectable earlier and rose to higher levels than those for cultures starting at a pH of 7.0 (buffered or unbuffered). Moreover, despite the more or less constant pH of ca. 5.5, the cell density even exceeded that for the same strain starting growth at a nonbuffered pH of 7.0. Finally, while clearly attenuated, the residual acetoin production of RVH1 *splI* grown in LB-glucose buffered at pH 7.0 most likely stemmed from the slight drop in pH experienced by the culture as the buffering capacity was exceeded.

Together, these results demonstrate that acetoin production in *S. plymuthica* RVH1 is responsive to both pH and QS, a feature that was previously also established for *Vibrio cholerae* (8). In the latter, however, QS regulation is mechanistically different from that in *S. plymuthica*, since it consists of an AI-2 and CAI-1 signal cascade instead of an AHL system. This

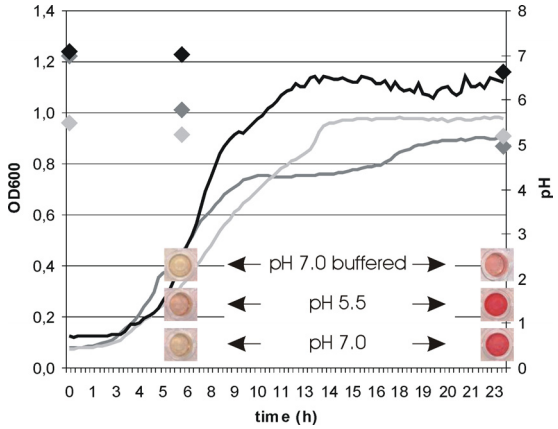

FIG. 2. Growth curves for an RVH1 *splI* mutant in LB containing 0.5% glucose with an initial pH of 7.0 (dark gray), 5.5 (light gray), or 7.0 buffered with 100 mM phosphate buffer (black). Measurements of pH (diamonds in corresponding colors) and acetoin production (images below each graph) are shown at the indicated time points. Representative results of three independent experiments are shown.

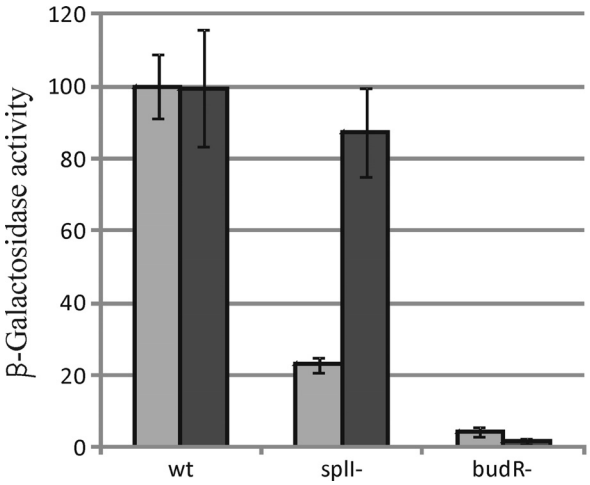

FIG. 3. Expression of a chromosomal *budA*::*lacZ* promoter fusion in wild-type RVH1 (wt), RVH1 *splI*, and RVH1 *budR* grown in LB without (light gray) or with (dark gray) added OHHL. Stationary-phase cultures were diluted 1/10 and exposed for 4 h to the indicated conditions. β-Galactosidase activity was calculated in Miller units and expressed relative to the activity in wild-type RVH1 without OHHL, which was arbitrarily set at 100%.

indicates that this dual regulation arose more than once during evolution, which advocates the apparent benefit of both cues providing input in the regulation of 2,3-butanediol production. Most likely, QS allows cells to anticipate and circumvent the potentially lethal acidification of a progressively densifying culture engaged in mixed acid fermentation by switching in a timely manner to 2,3-butanediol production.

Identification of the *bud* locus in *S. plymuthica* RVH1. In order to further dissect the regulation of acetoin production at the genetic level, we searched for the corresponding structural genes by screening a random transposon knockout library of RVH1 for mutants unable to produce acetoin and counteract acidification during the fermentation of glucose. As such, a clone was obtained whose transposon insertion site was mapped to a gene homologous to an α-acetolactate synthase gene (designated *budB*), which allowed the subsequent identification of a neighboring LysR gene homologue (designated *budR*) and an α-acetolactate decarboxylase gene (designated *budA*). More specifically, the *budA* and *budB* genes form an operon separated by 382 bp from the upstream and divergently transcribed *budR* gene. The predicted gene products show a high degree of homology with the corresponding proteins of *S. proteamaculans* 568: 74% for BudR (LysR), 92% for BudA, and 92% for BudB.

Whereas growth of the RVH1 wild type on glucose was characterized by an initial pH decrease (from 7.1 to 6.0) followed by an increase (from 6.0 to 7.0) (Fig. 1A), the RVH1 *budB* mutant showed a continuous pH decrease (from 7.1 to 4.5) and concomitantly displayed hampered growth (Fig. 1D). Furthermore, while acetoin was already clearly detectable in the wild-type strain after 6 h of growth, its production was completely abolished in the *budB* mutant, which indicates that the isolated *bud* locus is absolutely required for acetoin production in RVH1. Finally, it should also be noted that acidification, loss of acetoin production, and impaired growth of the *budB* mutant could be reversed completely by constitutive expression of the *budAB* genes from a plasmid (Fig. 1E).

Interestingly, a *de novo*-constructed RVH1 *budR* mutant growing on glucose was phenotypically indistinguishable from a *budB* mutant and likewise failed to produce any acetoin (Fig. 1F). To confirm this at the genetic level, a chromosomal *budAB-lacZ* promoter fusion was constructed and validated to be responsive to quorum sensing (Fig. 3). Moreover, knocking out *budR* decreased expression levels of a chromosomal *budAB-lacZ* promoter fusion to circa 4% of those observed in the RVH1 wild type (Fig. 3). Together, these results indicate that BudR is an essential activator of the *budAB* operon, which is in agreement with BudR functionality in *Bacillus subtilis*, *Klebsiella terrigena*, and *Vibrio cholerae* (8, 10, 12).

Regulation of *budR* transcription in *S. plymuthica* RVH1. The abolition of acetoin production and *budAB* expression in the *budR* knockout strain also implied that the regulation of *budAB* by both QS and pH must be mediated by BudR. Therefore, we decided to construct a plasmid-borne *budR* promoter fusion to the *gfp* gene (i.e., P_{budR}-*gfp*) to examine the regulation of *budR* transcription more closely. As such, we observed that P_{budR} activity in an RVH1 *splI* mutant was considerably attenuated compared to that in the wild-type strain but could be rescued by the addition of OHHL signaling molecules (Fig. 4A), clearly demonstrating that QS control of 2,3-butanediol production is genetically integrated at the P_{budR} promoter.

In subsequently examining the effect of low pH on *budR* transcription, no significant difference in P_{budR} activity could be observed between wild-type RVH1 exposed to LB at pH 5.5 or pH 7.0 (Fig. 4B), indicating that it was not the effect of low pH itself that was conveyed to the *budR* promoter. However, when acetate was examined as a possible trigger, it emerged that P_{budR} activity could indeed be increased considerably in the presence of 15 mM acetate. However, this induction occurred only at a low pH (pH

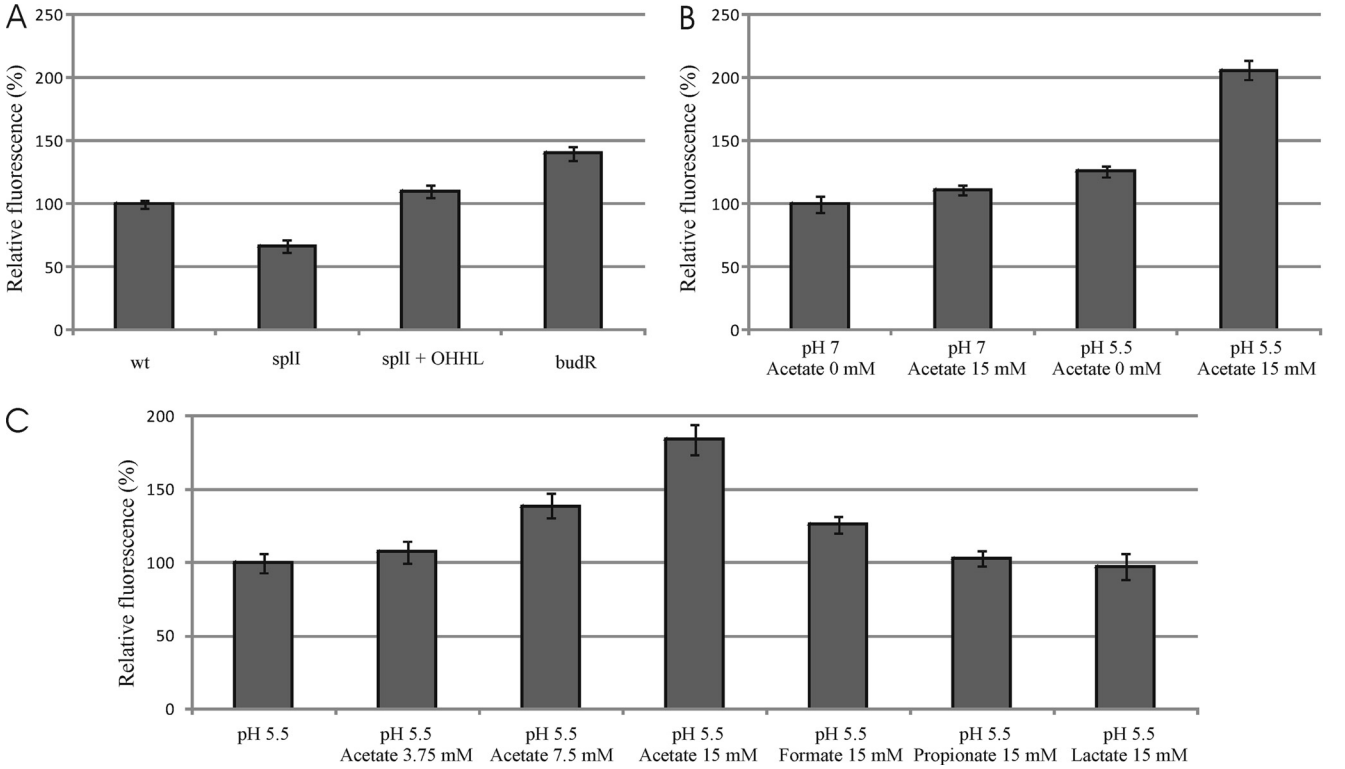

FIG. 4. Relative expression of plasmid-borne P_{budR}-*gfp* in wild-type RVH1, RVH1 *splI*, RVH1 *splI* complemented with OHHL, and RVH1 *budR* grown in LB at pH 7.0 (A); in wild-type RVH1 grown in LB medium at pH 5.5 or 7.0 in the presence or absence of 15 mM acetate (B); and in RVH1 *splI* grown in LB medium at pH 5.5 in the presence of 0, 3.75, 7.5, or 15 mM acetate or in the presence of 15 mM formate, propionate, or lactate (C). Stationary-phase cultures were diluted 1/100 and exposed for 4 h to the indicated conditions before GFP expression was measured. In each graph, GFP expression of the control strain (i.e., first bar) was arbitrarily set at 100%. Average values and standard deviations for three independent experiments are shown.

5.5) and completely disappeared at neutral pH, suggesting that *budR* transcription is responsive to acetate only once its protonated and neutral form can enter the cell. Finally, while a clear dose-response relationship between acetate concentration and *budR* transcription could be established (Fig. 4C), limited or no induction could be obtained with formate, propionate, or lactate at pH 5.5 (Fig. 4C), although it should be noted that the dissociation constants of these molecules differ.

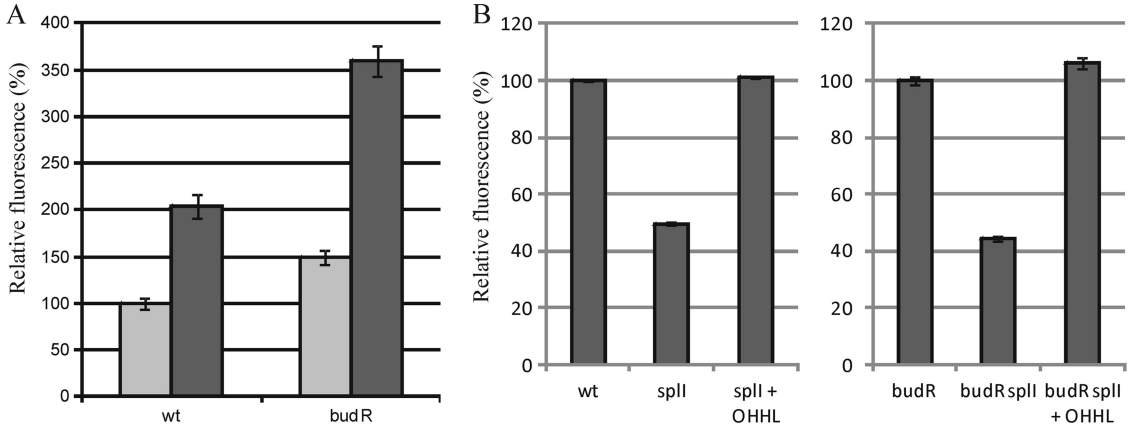

FIG. 5. (A) Relative expression of plasmid-borne P_{budR}-*gfp* in wild-type RVH1 and RVH1 *budR* in LB medium at pH 7.0 (light gray) or pH 5.5 in the presence of 15 mM acetate (dark gray). Stationary-phase cultures were diluted 1/100 and exposed for 4 h to the indicated conditions before GFP expression was measured. (B) Relative expression of plasmid-borne P_{budR}-*gfp* in wild-type RVH1, RVH1 *splI*, and RVH1 *splI* complemented with OHHL (left) and in RVH1 *budR*, RVH1 *splI budR*, and RVH1 *splI budR* complemented with OHHL (right). Stationary-phase cultures were grown under the indicated conditions before GFP expression was measured. In each graph, GFP expression of the control strain (i.e., first bar) was arbitrarily set at 100%. Average values and standard deviations for three independent experiments are shown.

Interestingly, when the P_{budR}-*gfp* reporter was transformed into an RVH1 *budR* mutant, we observed higher P_{budR} activities than those in the corresponding wild-type strain (Fig. 4A), suggesting a feedback mechanism in which BudR represses its own transcription. This is in contrast to its role as an activator of the *budAB* operon but in agreement with previous observations in *Klebsiella terrigena* (10). In general, most *lysR* homologues seem to interfere with their own expression (6, 15), since they typically bind the promoter regions of the genes they regulate and thus obstruct access to their own neighboring and divergently oriented promoters.

Surprisingly, however, we observed that in an RVH1 *budR* mutant, *budR* transcription was still similarly responsive to acetate (at pH 5.5) and OHHL as that in the wild-type parent (Fig. 5A and B), demonstrating that BudR itself is not required to convey these signals to the *budR* promoter. Accordingly, additional regulatory factors likely play an important role in directing *budR* transcription. This contrasts with currently forwarded hypotheses in which intracellular acetate (or one of its downstream metabolites) is assumed to associate with the BudR protein in order to activate it (8, 10, 12). Nevertheless, although many LysR homologues indeed appear to depend on inducer ligands (6, 9), direct binding of acetate to BudR has never been shown experimentally.

Conclusions. In this study, we report on the identification of structural (*budAB*) and regulatory (*budR*) genes responsible for acetoin production in *S. plymuthica* RVH1. Moreover, we demonstrate that dual regulation of acetoin production by QS and intracellular acetate seems to be integrated in the expression of the *budR* gene, encoding the activator of the *budAB* operon. However, while BudR was shown to be an autorepressor, activation of *budR* transcription by QS and acetate proceeded independently of BudR.

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