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RESEARCH ARTICLE

Insights into a Pyruvate Sensing and Uptake System in *Vibrio campbellii* and Its Importance for Virulence

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ABSTRACT Pyruvate is a key metabolite in living cells and has been shown to play a crucial role in the virulence of several bacterial pathogens. The bioluminescent *Vibrio campbellii*, a severe infectious burden for marine aquaculture, excretes extraordinarily large amounts of pyruvate during growth and rapidly retrieves it by an as-yet-unknown mechanism. We have now identified the responsible pyruvate transporter, here named BtsU, and our results show that it is the only pyruvate transporter in *V. campbellii*. Expression of *btsU* is tightly regulated by the membrane-integrated LytS-type histidine kinase BtsS, a sensor for extracellular pyruvate, and the LytTR-type response regulator BtsR. Cells lacking either the pyruvate transporter or sensing system show no chemotactic response toward pyruvate, indicating that intracellular pyruvate is required to activate the chemotaxis system. Moreover, pyruvate sensing and uptake were found to be important for the resuscitation of *V. campbellii* from the viable but nonculturable state and the bacterium's virulence against brine shrimp larvae.

IMPORTANCE Bacterial infections are a serious threat to marine aquaculture, one of the fastest growing food sectors on earth. Therefore, it is extremely important to learn more about the pathogens responsible, one of which is *Vibrio campbellii*. This study sheds light on the importance of pyruvate sensing and uptake for *V. campbellii*, and reveals that the bacterium possesses only one pyruvate transporter, which is activated by a pyruvate-responsive histidine kinase/response regulator system. Without the ability to sense or take up pyruvate, the virulence of *V. campbellii* toward gnotobiotic brine shrimp larvae is strongly reduced.

KEYWORDS LytTR, pyruvate transport, chemotaxis, histidine kinase, overflow metabolism, viable but nonculturable cells

Pyruvate is one of the most important molecules in both pro- and eukaryotic cells. Being the end product of glycolysis and—converted to acetyl coenzyme A—the starting compound of the citric acid cycle and fatty acid synthesis, as well as a substrate for fermentation in the case of oxygen limitation, pyruvate acts as the hub between aerobic and anaerobic metabolism (1). It can also be converted into carbohydrates via gluconeogenesis and be used to produce amino acids like alanine. Moreover, pyruvate serves as a scavenger for reactive oxygen species (ROS) such as H_2O_2, since it inactivates them by a nonenzymatic oxidative decarboxylation reaction (2–6).

For a wide variety of microbial pathogens, it has been demonstrated that pyruvate and its metabolism are crucial for fitness and virulence, including *Borrelia burgdorferi*, *Leptospira interrogans*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Yersinia pseudotuberculosis*, *Staphylococcus aureus*, and uropathogenic *Escherichia coli* (7–12). Furthermore, pyruvate has been shown to play a role in the resuscitation of viable but nonculturable (VBNC) bacteria (13–15). Many different species can enter this dormant state of low metabolic activity, which enables them to withstand stressful environmental conditions. By this means, they can for instance survive antibiotic treatments without being detected by

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standard cultivation methods (16, 17). In *E. coli*, pyruvate was also found to be important for the formation of persister cells, as well as for protein overproduction (18). Beyond that, pyruvate has become a focus of attention in the context of metabolic engineering for industrial applications (19, 20). In eukaryotes, two mitochondrial pyruvate carriers, MCP1 and MCP2, were identified which transport pyruvate across the mitochondrial membrane (21). Moreover, pyruvate was found to be important for cancer cells to cope with hypoxia (22) and for activation of human intestinal immune cells (23).

Vibrio harveyi ATCC BAA-116, a model organism for quorum sensing that was reclassified as *V. campbellii* in 2010 (24), is a marine, motile, luminous gammaproteobacterium and an opportunistic pathogen for fish, shrimps, squids, and other marine invertebrates (25–27). Aquaculture is one of the fastest growing food-producing sectors on earth and now accounts for 50% of the world's fish consumed (28). To prevent the loss of entire aquaculture populations owing to infections, antimicrobial treatments are often unavoidable, although these measures can lead to severe problems such as antimicrobial resistances (29). This underlines the need to investigate pathogens such as *V. campbellii*, including their virulence and metabolism, in more detail. It has been known since the 1970s that bacteria of the genus *Vibrio* excrete large amounts (up to 3 mM) of pyruvate during exponential growth and rapidly take it up again, whereas other genera excrete much smaller amounts, usually in the micromolar range (30, 31). The physiological function of this excretion and reuptake of pyruvate is not fully understood, but it presumably represents a form of overflow metabolism to avoid excessive accumulation of pyruvate in the cells and rebalance intracellular pyruvate levels—a typical adaptation phenomenon during shifts from aerobic to anaerobic growth (32–34). However, why *Vibrio*, in contrast to other genera, should excrete such extraordinary amounts of pyruvate remains unclear.

In the model organism *E. coli*, two different histidine kinase/response regulator systems are known to be responsible for pyruvate sensing. One consists of the histidine kinase BtsS (formerly known as YehU) and the response regulator BtsR (formerly known as YehT) (31, 35), the other comprises the histidine kinase YpdA and the response regulator YpdB. These LytS/LytTR systems are found in many bacterial phyla, especially in plant and human pathogens, but most species harbor only one of the two systems (31). In *E. coli*, BtsS has been shown to sense pyruvate at very low concentrations, and BtsR then activates transcription of *btsT* (formerly known as *yjiY*), which codes for the high-affinity pyruvate/H^+ symporter BtsT, a member of the CstA transporter family with 18 predicted transmembrane domains (36, 37). In addition to BtsT, *E. coli* possesses at least one other pyruvate transporter (38–40).

V. campbellii can grow on pyruvate as a sole carbon source, which indicates that it must be able to take up the compound by a hitherto-unknown means. Work by Behr et al. (31) has shown that the species possesses homologs of the *btsS*/*btsR* system of *E. coli* (VIBHAR_RS04665 and VIBHAR_RS04660) and of the pyruvate transporter gene *btsT* (VIBHAR_RS04670), but to date nothing is known about this gene cluster nor about pyruvate sensing or transport in *V. campbellii*. Since bacteria of the genus *Vibrio* excrete and take up large amounts of pyruvate (30, 31), we hypothesized that the system must play an important role for the cells. This study reports first insights into pyruvate sensing and uptake in *V. campbellii* and its relevance for this pathogen. Deletion mutants of the respective homologous genes were generated and investigated in terms of several phenotypes to reveal gene functions and relevance.

Our results show that BtsU, the transporter protein encoded by the ortholog of the *E. coli btsT* gene, is the only pyruvate transporter in *V. campbellii*. In the presence of pyruvate, its expression is activated by the sensor kinase BtsS via the response regulator BtsR. Further characterization of the deletion mutants demonstrates the importance of the system for chemotaxis, resuscitation from the viable but nonculturable (VBNC) state, and virulence.

RESULTS AND DISCUSSION

Characterization of the homologs of the *E. coli* genes *btsS*, *btsR*, and *btsT* in *V. campbellii*. Pyruvate sensing by the two-component system BtsS/BtsR and uptake by the transporter BtsT in *E. coli* has been described in detail in recent years (15, 18, 31, 35–37, 41).

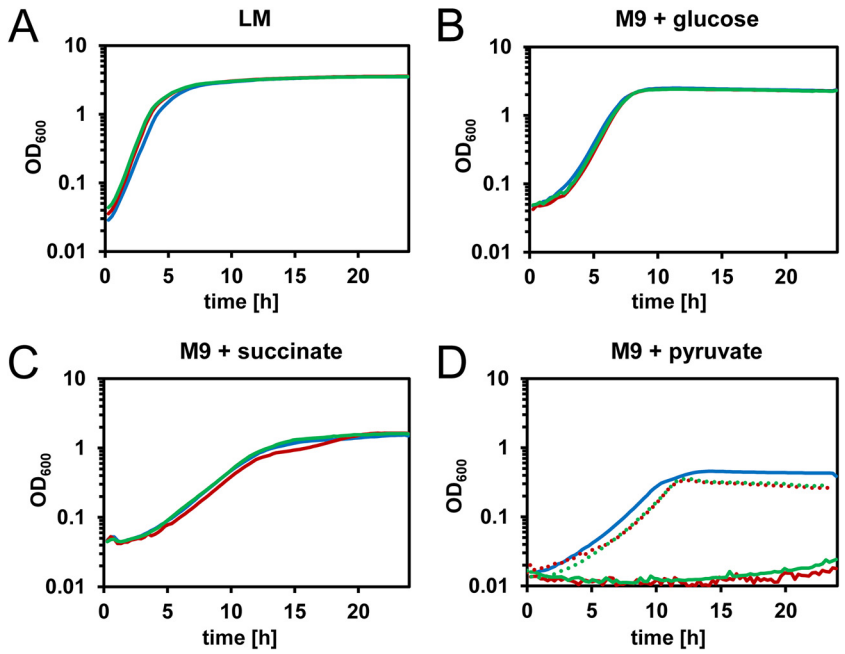

FIG 1 *V. campbellii* Δ*btsU* and Δ*btsSR* cells are unable to grow on pyruvate. *V. campbellii* wild-type (blue line), Δ*btsU* (red line) and Δ*btsSR* (green line) cells were grown in a plate reader (Tecan) at 30°C in LM medium (A) or in M9 minimal medium (2% [wt/vol] NaCl) with 20 mM glucose (B), 20 mM sodium succinate (C), or 20 mM sodium pyruvate (D). The *V. campbellii* Δ*btsU* and Δ*btsSR* strains were complemented with full-length *btsU* and *btsSR*, respectively, at the native loci via double homologous recombination. Complemented Δ*btsU* (red dotted line) and Δ*btsSR* (green dotted line) cells were grown in M9 minimal medium (2% [wt/vol] NaCl) with 20 mM sodium pyruvate.

The full-length amino acid sequences of these proteins were used to identify homologs of the *E. coli* genes *btsT*, *btsS*, and *btsR* in *V. campbellii* by a local alignment search against the *V. campbellii* ATCC BAA-1116 genome, using Protein BLAST (42). In contrast to their counterparts in *E. coli*, all three genes are located adjacent to each other in the *V. campbellii* genome: VIBHAR_RS04670 (the homolog of *E. coli*'s *btsT*, old locus tag VIBHAR_00986), VIBHAR_RS04665 (the homolog of *E. coli*'s *btsS*, old locus tag VIBHAR_00985), and VIBHAR_RS04660 (the homolog of *E. coli*'s *btsR*, old locus tag VIBHAR_00984). Using the online tool Clustal Omega (43), we compared the corresponding amino acid sequences with the sequences of the *E. coli* proteins and found the following identity values: 19% for BtsT (coverage 66.2%), 57% for BtsS (coverage 98.9%), and 50% for BtsR (coverage 99.6%).

VIBHAR_RS04670 codes for an uncharacterized transporter protein, parts of which are assigned by Pfam analysis to CstA, a member of the carbon starvation family (44). A topology prediction for the 53-kDa protein (449 amino acids) with the online tool TMPred predicted 12 transmembrane domains (45), whereas the *E. coli* BtsT, also a member of the CstA family, has 18 predicted transmembrane domains (37). Therefore, we propose to rename VIBHAR_RS04670 as *btsU* (transporter BtsU) to reflect the low similarity of its predicted product to BtsT. VIBHAR_RS04665 codes for a LytS-type sensor histidine kinase of 556 amino acids (61 kDa), and VIBHAR_RS04660 encodes a LytTR-type response regulator of 242 amino acids (27 kDa). These two genes are named *btsS* and *btsR* here, in accordance with their respective homologs in *E. coli*, coding for the proteins BtsS and BtsR, respectively.

***V. campbellii* cells lacking *btsU* or *btsSR* are unable to grow on pyruvate.** We constructed in-frame deletion mutants for the transporter gene (Δ*btsU*), as well as for the genes of the two-component system (Δ*btsS* and Δ*btsR* as single gene deletions and Δ*btsSR* as a double deletion of both genes) to learn more about their functions in *V. campbellii*. The deletion mutants were tested for several phenotypes.

All strains grew equally well in LM medium, and in minimal medium supplemented with glucose or succinate (Fig. 1A to C). However, in minimal medium with pyruvate as

sole carbon source, only the wild type was able to grow (Fig. 1D). Mutants lacking either the transporter (Δ*btsU*) or the two-component system (Δ*btsSR*) did not grow on pyruvate at all. In complemented *V. campbellii* deletion mutants, growth on pyruvate was restored to the wild-type level (Fig. 1D). Thus, we conclude that BtsU is the only pyruvate transporter in *V. campbellii*, and we suggest that its transcription might be activated (as in *E. coli*) by the two-component system BtsSR upon detection of external pyruvate. These results stand in clear contrast to phenotypes of the *E. coli* Δ*btsT* and Δ*btsSR* strains, which are able to grow on pyruvate, as expected in light of the presence of alternative pyruvate transporters (35, 37). This also highlights the much greater importance of this pyruvate sensing and uptake system for *V. campbellii*, since there are no substitutes.

We also assessed swimming motility, cell aggregation, pH changes during growth in Luria marine (LM) medium, and the amount of excreted indole, and found no significant differences between the deletion mutants of *V. campbellii* and the wild type (see Fig. S1 in the supplemental material). The same was true for bioluminescence and macrocolony formation, indicating that the system does not play any role in processes that are regulated by quorum sensing (see Fig. S1).

***V. campbellii btsU* expression is activated by the two-component system BtsSR in the presence of pyruvate.** Growth defects of both mutant strains on pyruvate as sole carbon source implied that not only the transporter BtsU but also the two-component system BtsSR is crucial for pyruvate uptake and is thus presumably necessary for the expression of *btsU*. To determine whether the two-component system fulfills the same function in *V. campbellii* as in *E. coli*, namely, activation of the transporter gene *btsU* when pyruvate is present to be taken up (36), we monitored *btsU* expression in LM medium in mutants lacking either one or both of the genes *btsS* and *btsR*. Using a reporter construct of the *btsU* promoter fused to the *mcherry* gene, chromosomally integrated upstream of the native locus, *btsU* expression could be monitored over time at the transcriptional level in both wild-type and mutant cells.

The results showed that the *btsU* promoter was activated only in the presence of BtsSR (Fig. 2A), demonstrating that this two-component system is crucial for expression of *btsU* in *V. campbellii*. Sensing of pyruvate thus serves as a precondition for pyruvate uptake. In the Δ*btsU* deletion mutant, the reporter gene fused to the *btsU* promoter was upregulated 10-fold relative to its expression in the wild type (Fig. 2A). *V. campbellii* seems to register the absence of its sole pyruvate transporter, which leads to an even stronger attempt to produce it to take up the metabolite. It is also reasonable that in wild-type cells the presence of the transporter BtsU causes negative feedback of its expression. The exact mechanism of this feedback regulation requires further analysis, and transcriptional or posttranscriptional regulatory mechanisms are conceivable.

When wild-type cells harboring the reporter construct for the *btsU* promoter were grown in M9 minimal medium supplemented with different carbon sources, the strongest transcriptional induction of *btsU* was observed in the presence of pyruvate (Fig. 2B). This result supports the assumption that expression of *btsU* is activated specifically by pyruvate via BtsSR in order to enable the sensed metabolite to be transported into the cells. To further determine this dependence of *btsU* expression on pyruvate, wild-type cells were grown in M9 minimal medium with different pyruvate concentrations and a constant basic level of succinate (20 mM) as the carbon source. Expression levels attributable to the presence of succinate, as well as the autofluorescence of the cells, were subtracted. Transcriptional activation of *btsU* increased in accordance with the concentration of pyruvate, with a threshold concentration of 500 μM being required for induction (Fig. 2C). The pyruvate concentration resulting in half-maximal *btsU* expression was estimated to be 3 ± 0.5 mM. Based on these data, we conclude that transcription of the pyruvate transporter gene *btsU* is activated by the two-component system BtsSR in a pyruvate-concentration dependent manner.

***V. campbellii* excretes large amounts of pyruvate during growth, and BtsU is required for reuptake of the compound from the medium.** To further characterize the relevance of pyruvate sensing and uptake in *V. campbellii*, different phenotypes of

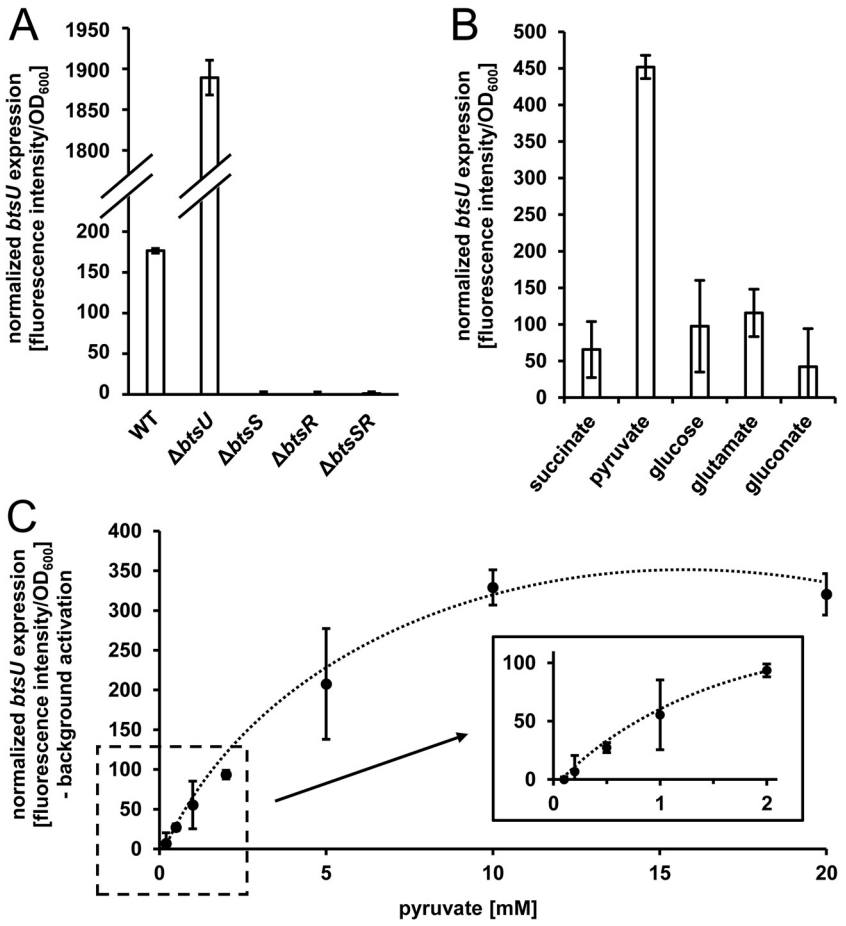

FIG 2 *V. campbellii btsU* expression is activated by the two-component system BtsSR in the presence of pyruvate. *V. campbellii* wild-type, Δ*btsU*, Δ*btsS*, Δ*btsR*, and Δ*btsSR* cells carrying a chromosomally integrated reporter comprising the promoter of *btsU* fused to *mcherry* (P_{btsU}-*mcherry*) were grown in a plate reader (Tecan) at 30°C in different media. Activation of the *btsU* promoter was monitored by measuring the intensity of mCherry fluorescence, normalized to an OD_{600} of 1. (A) Promoter activation of *btsU* in *V. campbellii* wild type and the indicated deletion mutants in LM medium. (B) Promoter activation of *btsU* in *V. campbellii* wild type by different carbon sources. Cells were grown in M9 minimal medium (2% [wt/vol] NaCl) supplemented with 20 mM sodium succinate, sodium pyruvate, glucose, sodium glutamate or sodium gluconate. (C) Promoter activation of *btsU* in *V. campbellii* wild type as a function of pyruvate concentration. Cells were grown in M9 minimal medium (2% [wt/vol] NaCl) with 20 mM sodium succinate as carbon source and different concentrations of sodium pyruvate. Baseline promoter activation by sodium succinate was subtracted (see panel B). All experiments were performed in triplicate, and error bars represent the standard deviations of the mean.

the deletion mutants were analyzed. It was shown previously that bacteria of the genus *Vibrio* excrete high levels of pyruvate during growth and then take it up again (30, 31). Measurements of external pyruvate concentrations in LM medium confirmed that *V. campbellii* excretes large amounts of pyruvate during exponential growth (Fig. 3). When the cell density had reached an optical density at 600 nm (OD_{600}) of ~2, the external pyruvate concentration was determined to be higher than 3 mM. In wild-type cultures, this peak was followed by a rapid decrease which reduced the concentration of the compound to the initial value. This finding shows that wild-type cells could rapidly and completely "reclaim" the pyruvate from the medium. Cells lacking the transporter protein BtsU or the sensing system BtsSR were unable to do so, and the external pyruvate concentration remained essentially unchanged after reaching its peak.

Thus, pyruvate reuptake from the medium after its excessive excretion depends entirely on the pyruvate transporter BtsU, transcription of which is activated after sensing of the excreted pyruvate by BtsSR. Other bacterial species do not excrete pyruvate

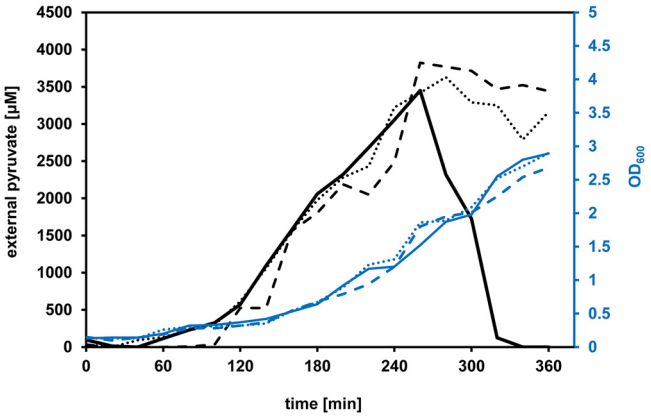

FIG 3 *V. campbellii* excretes large amounts of pyruvate and requires BtsU for its reuptake. *V. campbellii* wild-type (solid lines), Δ*btsU* (dotted lines), and Δ*btsSR* (dashed lines) cells were grown in LM medium at 30°C. Growth was monitored, and supernatant samples were collected to determine external pyruvate concentrations.

to such an extent (31). Therefore, pyruvate sensing and uptake presumably play an especially important role for *V. campbellii* in comparison to other microbes.

BtsU actively transports radiolabeled pyruvate, driven by the proton motive force. To ensure that the phenotypes of the deletion strains were indeed due to a defect in pyruvate uptake and to further characterize BtsU function, we directly monitored the transport of radiolabeled pyruvate by wild-type *V. campbellii* cells in comparison to Δ*btsU* and Δ*btsSR* cells. Experiments were done at 18°C to slow down the metabolization of pyruvate in the cells. The results clearly show that wild-type cells transported pyruvate with an uptake rate of 8 nmol of pyruvate per mg of total protein per min, whereas for Δ*btsU* and Δ*btsSR* cells no transport of pyruvate could be detected (Fig. 4A). These data support the conclusions drawn from the mutant phenotypes related to growth and the uptake of external pyruvate, i.e., that BtsU is the sole pyruvate transporter in *V. campbellii*, and that BtsSR serves as a pyruvate sensing system crucial for *btsU* expression.

To identify the driving force for pyruvate transport by BtsU in *V. campbellii*, various protonophores and ionophores were tested for their effect. Uptake of radiolabeled pyruvate was abolished by the addition of the hydrophobic protonophores carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and 2,4-dinitrophenol (DNP), whereas the ionophores valinomycin (selective for K^+), nigericin (selective for K^+/H^+), and nonactin, which forms complexes with K^+, Na^+, NH_4^+, and other cations, had no obvious or specific effect on pyruvate transport (Fig. 4B). This indicates that pyruvate uptake by BtsU in *V. campbellii* is driven by the proton motive force.

Pyruvate sensing and uptake are required for chemotaxis toward pyruvate. As stated above, swimming motility of *V. campbellii* was not affected by the deletion of *btsU* or *btsSR* (see Fig. S1 in the supplemental material). Motile bacteria make use of a chemotaxis network system to perform directed movement along a chemical gradient, for instance toward nutrients and favorable environments, by changing the direction of rotation of their flagellum (46, 47). Chemotaxis has not yet been investigated in *V. campbellii*, unlike in its relatives *V. harveyi* and *V. cholerae* (47, 48). The *V. campbellii* deletion strains were tested for chemotaxis toward several compounds by using a plug-in-pond assay, in which cells are mixed with warm soft agar (0.3% [wt/vol] agar) and poured over hard agar plugs (1.5% [wt/vol] agar) containing the test compounds (Fig. 5). After incubation, movement of cells toward the test compounds is observed, and this serves as an indicator for chemotaxis.

We found that wild-type cells could swim along the gradient of pyruvate created by diffusion from the hard agar plugs and form circles of cell density around them, with the circle size increasing with the pyruvate concentration in the plug. Both Δ*btsU* and Δ*btsSR* strains were unable to migrate toward pyruvate (Fig. 5). In contrast, all strains

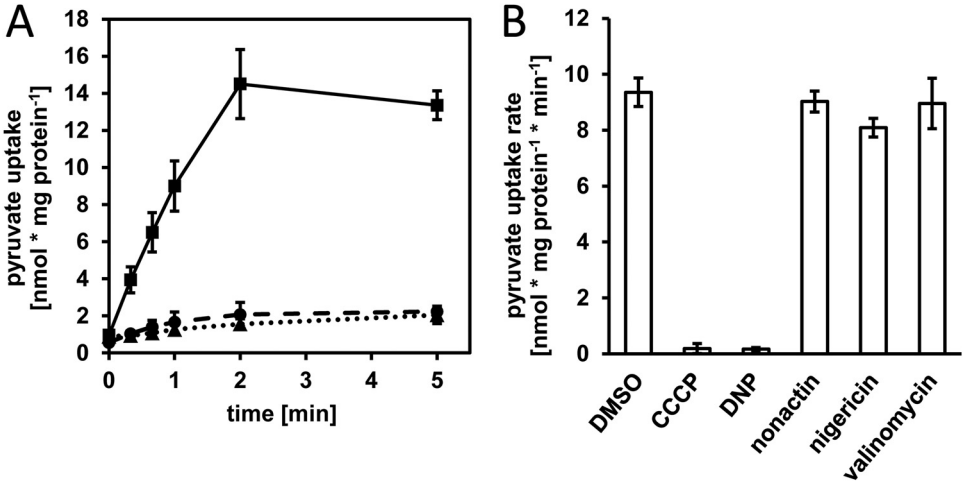

FIG 4 Pyruvate transport by BtsU in *V. campbellii*. (A) Uptake of [^{14}C]pyruvate by *V. campbellii* wild-type (solid line), Δ*btsU* (dotted line), and Δ*btsSR* (dashed) cells monitored over time at a final pyruvate concentration of 10 μM at 18°C. (B) The impact of different protonophores and ionophores on pyruvate transport was determined after preincubation of the cells with the indicated compounds at 25°C for 30 min. DMSO was used as a control. All experiments were performed in triplicate, and error bars represent standard deviations of the mean.

showed chemotaxis toward succinate (Fig. 5) and other compounds tested (see Fig. S3). Hence, the defect is specific for pyruvate. Being unable to detect and follow a gradient of pyruvate can be a severe disadvantage when the bacterium needs this important molecule, either as energy source or as a scavenger of ROS.

Since both cells lacking the transporter and cells lacking the two-component system were unable to perform chemotaxis toward pyruvate, transport of the compound into the cells must itself be crucial for functional chemotaxis toward pyruvate. We therefore suggest that pyruvate is in some way sensed intracellularly as an attractant by the chemotaxis system. It has been shown in *E. coli* that cytoplasmic pyruvate is sensed by the phosphotransferase (PTS) system—presumably based on the ratio of pyruvate to phosphoenolpyruvate—and that this signal is transmitted linearly to the chemotaxis pathway (49, 50). The exact mechanism of this signaling network still needs to be investigated, but an increase in intracellular pyruvate levels detected via the PTS system could also activate chemotaxis of *V. campbellii*.

Resuscitation of VBNC *V. campbellii* cells by pyruvate is impaired in mutants lacking BtsU or BtsSR. Earlier studies have demonstrated that pyruvate is an important factor for the resuscitation of VBNC bacteria owing to its function as a scavenger of ROS and the fact that it is a C-source that can easily be metabolized without prior phosphorylation (13–15). To test whether this also applies to *V. campbellii*, the VBNC state was induced in wild-type, Δ*btsU*, and Δ*btsSR* cells by long-term storage in the cold under nutrient limitation. Actively growing cells were adjusted to the same optical density in M9 minimal medium, with a higher salt concentration (2% [wt/vol] NaCl), but without any carbon source, and stored at 4°C. Periodic plating on LM agar plates showed a steady decrease in culturable cells over time (Fig. 6A). After 163 days, no colonies could be detected on the plates, indicating that the remaining living cells had entered the VBNC state. A characteristic change in cell morphology to a very small and rounded shape was observed for both wild-type and mutant cells (Fig. 6C), similar to that previously reported for VBNC bacteria, including members of the genus *Vibrio* (51–53).

Resuscitation experiments were performed by temperature upshift and addition of different nutrients. To exclude regrowth of any putatively remaining culturable cell, experiments were first done after 1 week of daily plating during which no colonies were detectable on the plates, and dilutions of the VNBC cell suspensions were used as suggested before (54, 55). Moreover, ampicillin was added to prevent growth of any

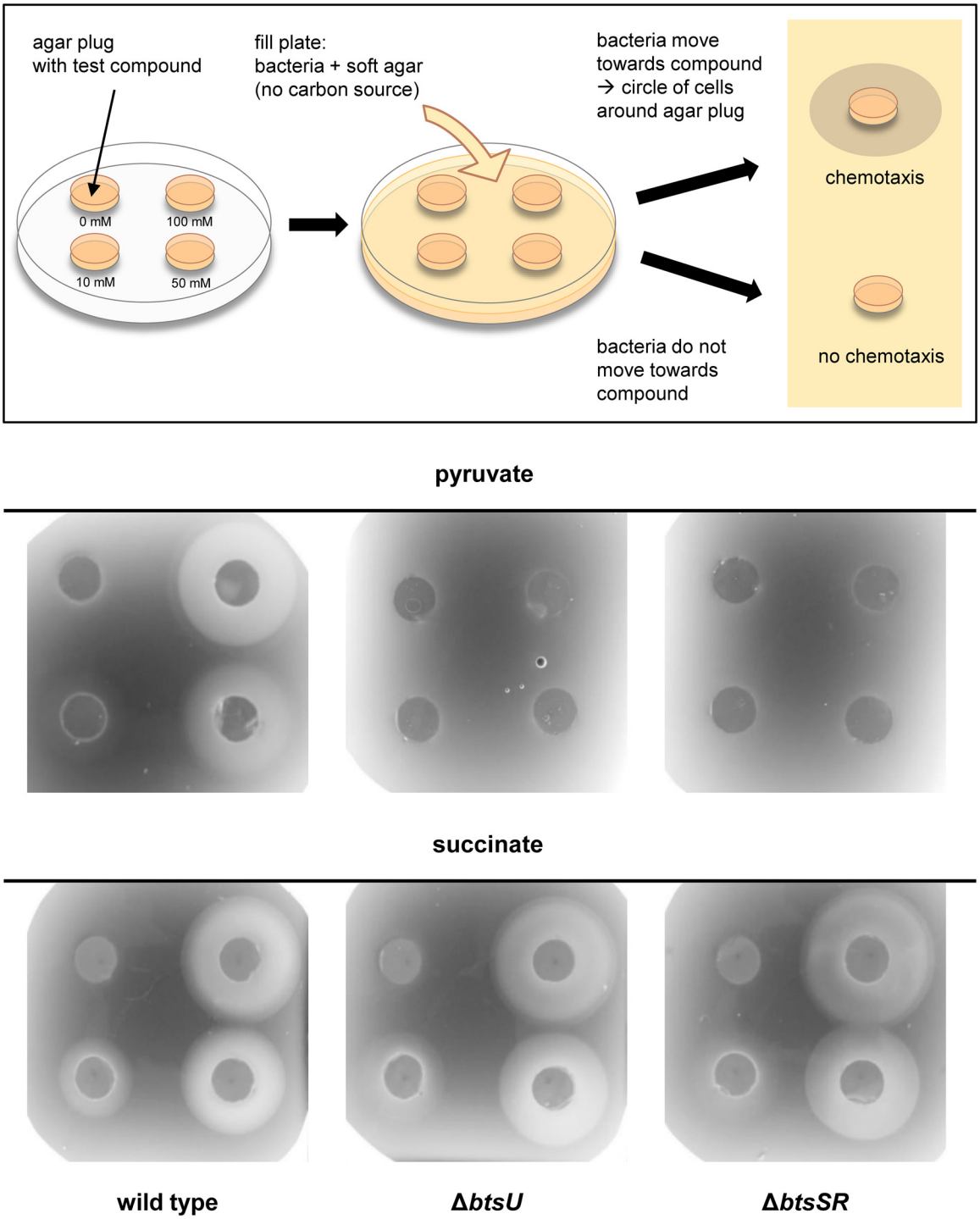

FIG 5 *V. campbellii* Δ*btsU* and Δ*btsSR* cells lost chemotactic response toward pyruvate. *V. campbellii* wild-type, Δ*btsU*, and Δ*btsSR* cells were tested for chemotaxis toward sodium pyruvate and sodium succinate in a plug-in-pond assay, which is schematically illustrated. Cells were mixed with soft agar (0.3% [wt/vol] agar) and poured over agar plugs (1.5% [wt/vol] agar) containing either sodium pyruvate or sodium succinate at concentrations of 0, 10, 50, and 100 mM (counterclockwise). Plates were incubated for 3 h at 30°C, and the images of cell accumulations are representative of three independent experiments.

contaminating bacteria, as *V. campbellii* is resistant to this antibiotic. VBNC *V. campbellii* cells were barely resuscitated by temperature upshift alone, but they did respond to the addition of nutrients (Fig. 6B). After 12 h of incubation, first colonies could be detected on plates, indicating the return of the cells to the culturable state. This could

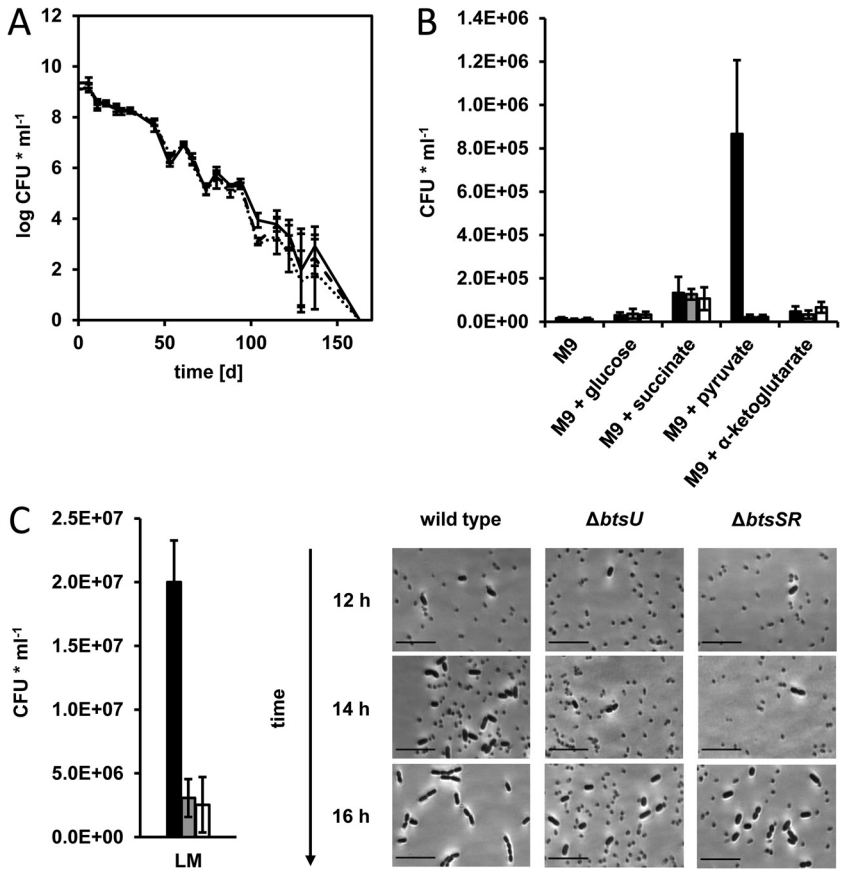

FIG 6 Induction of the VBNC state in *V. campbellii* and resuscitation by different nutrients. (A) The VBNC state was induced in *V. campbellii* wild-type (solid line), Δ*btsU* (dotted line), and Δ*btsSR* (dashed line) cells by long-term storage at 4°C under nutrient starvation in M9 minimal medium (2% [wt/vol] NaCl) without a carbon source. CFU on LM agar plates were determined periodically. (B) Resuscitation of wild-type (black), Δ*btsU* (gray), and Δ*btsSR* (white) VBNC cells upon temperature upshift to 30°C and addition of the indicated nutrients to M9 minimal medium (2% [wt/vol] NaCl). CFU on LM agar plates were determined after 14 h. (C) Resuscitation of wild-type (black), Δ*btsU* (gray), and Δ*btsSR* (white) VBNC cells in LM medium at 30°C. CFU on LM agar plates were determined after 14 h. Micrographs of wild-type, Δ*btsU*, and Δ*btsSR* cells during resuscitation in LM medium after the indicated time points are also shown. There is a mixture of small (VBNC) and large (growing) cells in the mutant cultures at 16 h. Scale bars, 10 μm.

also be seen under the microscope, as first cells were elongated and regained their normal shape (Fig. 6C). Numbers of colonies on plates were compared after 14 h of incubation in the presence of different compounds: the addition of succinate resuscitated more cells than the addition of glucose or α-ketoglutarate, which has been shown to promote resuscitation of other bacterial species (14). Addition of pyruvate led to the highest number of culturable cells—but only for the wild type, as the deletion mutants are unable to take up pyruvate. This demonstrates once again the importance of pyruvate and of the BtsSRU system for *V. campbellii*, which promotes efficient resuscitation from the VBNC state.

Addition of LM medium to the VBNC cells also restored culturability to a large extent (Fig. 6C). Interestingly, Δ*btsU* and Δ*btsSR* cells were impaired in resuscitation also in LM medium, which could also be followed under the microscope with less regularly shaped cells for the mutants compared to the wild type (Fig. 6C). LM medium contains at least 200 μM pyruvate (31). Thus, we conclude that the resuscitating effect of pyruvate is also the key factor in the resuscitation of VBNC *V. campbellii* cells in LM medium. Mutants unable to sense or take up pyruvate can enter the VBNC state and survive unfavorable conditions, but they are impaired in returning to the culturable state upon provision of pyruvate. Loss of the BtsSRU system thus puts dormant *V. campbellii*

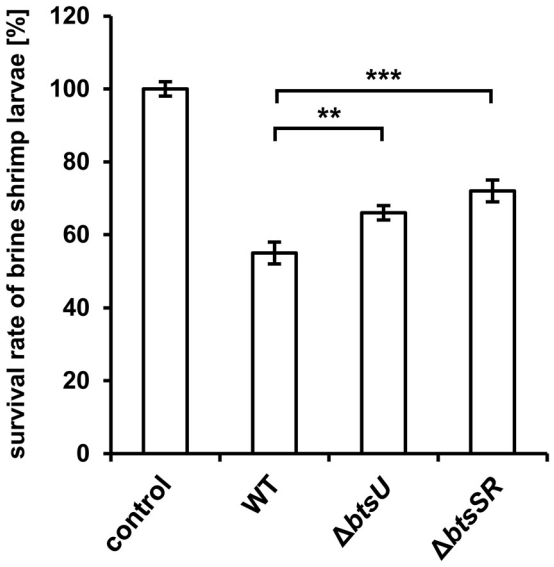

FIG 7 Reduced virulence of *V. campbellii* Δ*btsU* and Δ*btsSR* cells toward gnotobiotic brine shrimp larvae. Axenic *Artemia franciscana* larvae in sterile seawater (1 animal ml^{-1}) were challenged with *V. campbellii* wild-type, Δ*btsU*, and Δ*btsSR* cells at 10^7 cells ml^{-1}. Unchallenged animals were used as a control. After 48 h of incubation at 28°C, the survival of the larvae was determined. Experiments were performed in triplicate, and error bars represent standard deviations of the mean. *t* tests were performed to compare the treatments. Significant differences are indicated by asterisks (**, $P < 0.01$; ***, $P < 0.001$).

cells at a severe disadvantage, and pyruvate therefore plays an important role in this context too.

Virulence of *V. campbellii* toward gnotobiotic brine shrimp larvae is reduced in the absence of pyruvate sensing or uptake. It was shown previously for several bacterial pathogens that pyruvate is important for virulence and infection (7–12). Since *V. campbellii* is an important marine pathogen, we were interested in the relevance of the pyruvate sensing and uptake system described here for the virulence of the cells *in vivo*. To this end, we performed a standardized challenge test with gnotobiotic brine shrimp (*Artemia franciscana*) larvae to determine the ability of the bacteria to infect and kill their host. Wild-type *V. campbellii*, as well as Δ*btsU* and Δ*btsSR* cells, was added to sterile *Artemia* larvae cultures at 10^7 cells per ml. After 2 days of incubation at 28°C, the surviving brine shrimp larvae were counted. This number was then normalized to the number of live brine shrimp larvae in the control group, to which the pathogen was not added.

In the samples of *Artemia* larvae that were challenged with wild-type *V. campbellii* cells, the numbers of surviving larvae were almost 50% lower than in the control group, in which nearly all animals were still alive (Fig. 7). This illustrates how effectively the pathogen can infect and kill its host. In contrast, the relative survival of *Artemia* challenged with *V. campbellii* Δ*btsU* or Δ*btsSR* cells was significantly higher: In the group of animals exposed to Δ*btsU* cells, 66% survived the challenge, and in the group of animals treated with Δ*btsSR* cells even 72% survived (Fig. 7). We conclude that without pyruvate sensing and uptake, the virulence of *V. campbellii* toward gnotobiotic brine shrimp larvae is significantly reduced. The BtsSRU system, and hence pyruvate sensing and uptake, seem to be important for full virulence of the pathogen.

The expression of virulence factors in *V. campbellii* is regulated by quorum sensing (56). Here, we found no evidence that deletion of elements of the BtsSRU system affects quorum-sensing regulated processes, although we observed a clear effect on virulence. Thus, we suggest that pyruvate is linked to virulence in *V. campbellii* by a mechanism that is not connected with the quorum-sensing system. This might involve the ability of pyruvate to scavenge ROS. Zebrafish infected with *V. alginolyticus* were shown to produce high levels of ROS (57). Relating this to our study, with respect to

their ability to establish an infection, *V. campbellii* mutants that cannot take up pyruvate as an antioxidant against ROS may well be at a disadvantage in comparison to wild-type bacteria.

Conclusions. This study reveals first insights into pyruvate sensing and uptake in *V. campbellii* and its importance for this marine pathogen. Pyruvate is an indispensable metabolite for all living cells, since it not only functions as a central node of both aerobic and anaerobic metabolism but also protects cells against oxidative damage. *V. campbellii* excretes large amounts of pyruvate during growth, but nothing was previously known about the role of this compound for the pathogen. We demonstrate here that the sensor kinase BtsS senses pyruvate and activates expression of the only pyruvate transporter in this species, BtsU, via the response regulator BtsR. Inability to sense and thus to take up pyruvate affects many aspects of the normal behavior of this bacterium, including directed movement toward pyruvate, resuscitation from a dormant state and—most importantly—virulence. With regard to the increasing aquaculture production, to which *V. campbellii* presents a severe threat, this study is an important step toward a better understanding of the molecular mechanisms and factors that influence virulence. This study is not the first demonstrating the importance of pyruvate for fitness of and infection by pathogenic bacteria, indicating that this primary metabolite has a function that goes beyond its central role in metabolism and which makes it an extremely interesting and possibly so far underestimated molecule.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. *V. campbellii* and *E. coli* strains as well as plasmids used in this study are listed in Table 1. Oligonucleotide sequences are listed in Table S1 in the supplemental material. Clean in-frame deletions in *V. campbellii* were created by double homologous recombination using the pNPTS138-R6KT suicide plasmid (58). Upstream and downstream 800-bp regions of the respective gene were amplified from chromosomal DNA by PCR with appropriate oligonucleotides, retaining the first and last 15 bp of the coding sequence. The two DNA fragments were fused by overlapping PCR and cloned into the pNPTS138-R6KT plasmid following digestion with PstI and BamHI. Plasmids were then transferred into chemically competent *E. coli* DH5α λ*pir* cells (59). Plasmid sequences were confirmed by sequencing and transferred into *E. coli* WM3064 for conjugation with *V. campbellii*. Double homologous recombination was induced as previously described (58, 60). In short, mutants bearing single-crossover integrations were selected on LM agar plates containing kanamycin, then single clones were grown for 8 h in LM medium and selected for plasmid excision on LM agar plates containing 10% (wt/vol) sucrose. Kanamycin-sensitive clones were first checked for chromosomal in-frame gene deletions by colony PCR and finally confirmed by sequencing.

Complementation of *V. campbellii* Δ*btsU* and Δ*btsSR* strains with full-length genes inserted in the native locus was also done by double homologous recombination as described above, using the pNPTS138-R6KT plasmid (58) as the vector for amplified regions encompassing the respective full-length gene together with flanking up- and downstream regions. Correct complementation was confirmed by sequencing. Chromosomally integrated reporter constructs, with the *btsU* promoter fused to the *mcherry* gene upstream of the native locus, were assembled by single homologous recombination with the pNPTS138-R6KT plasmid as described previously (61). Briefly, a 500-bp region upstream of *V. campbellii btsU* was amplified by PCR and fused by overlapping PCR to the *mcherry* sequence, which was amplified by PCR from the pBAD-Cherry plasmid (62). This fragment was cloned into the pNPTS138-R6KT plasmid via restriction digestion using PstI and BamHI. For chromosomal integration of this reporter construct into the different *V. campbellii* strains, single homologous recombination was performed as described above.

Molecular biological methods. Molecular methods followed standard protocols (63) or were implemented according to manufacturer's instructions. Kits for the isolation of chromosomal DNA or plasmids and purification of PCR products were purchased from Südlabor. Enzymes were purchased from New England Biolabs. Chemicals were sourced from Roth or Merck.

Growth conditions. *V. campbellii* strains were grown overnight under agitation (200 rpm) at 30°C in Luria Marine (LM) medium (10 g liter^{-1} tryptone, 5 g liter^{-1} yeast extract, 20 g liter^{-1} NaCl) (64). Cells from the overnight culture were then transferred to the appropriate fresh medium. *E. coli* strains were grown under agitation (200 rpm) at 37°C in lysogeny broth (LB) medium (10 g liter^{-1} tryptone, 5 g liter^{-1} yeast extract, 10 g liter^{-1} NaCl) (65). The conjugation strain *E. coli* WM3064 was grown in the presence of 300 μM diaminopimelic acid. If necessary, media were supplemented with 50 μg ml^{-1} kanamycin sulfate and/or 100 μg ml^{-1} ampicillin sodium salt. To measure growth of *V. campbellii* strains on different carbon sources, cells were cultivated for 24 h at 30°C in M9 minimal medium (66) containing 2% (wt/vol) NaCl, supplemented with the carbon source to be tested (e.g., 20 mM sodium pyruvate). Growth was monitored by measuring the OD_{600} over time.

Analysis of *btsU* expression. Expression of *V. campbellii btsU* was determined by measuring fluorescence levels of the different *V. campbellii* reporter strains carrying a chromosomally integrated fusion of

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
V. campbellii		
ATCC BAA-116 (BB120)	Wild type	24
Δ*btsU* strain	In-frame deletion of VIBHAR_00986	This study
Δ*btsS* strain	In-frame deletion of VIBHAR_00985	This study
Δ*btsR* strain	In-frame deletion of VIBHAR_00984	This study
Δ*btsSR* strain	In-frame deletion of VIBHAR_00984-00985	This study
Δ*btsU*::*btsU* strain	Complemented Δ*btsU*	This study
Δ*btsSR*::*btsSR* strain	Complemented Δ*btsSR*	This study
Wild-type P_{btsU}-*mcherry* strain	Wild type with chromosomally integrated reporter construct for *btsU* upstream of the native promoter	This study
Δ*btsU* P_{btsU}-*mcherry* strain	Δ*btsU* with chromosomally integrated reporter construct for *btsU* upstream of the native promoter	This study
Δ*btsS* P_{btsU}-*mcherry* strain	Δ*btsS* with chromosomally integrated reporter construct for *btsU* upstream of the native promoter	This study
Δ*btsR* P_{btsU}-*mcherry* strain	Δ*btsR* with chromosomally integrated reporter construct for *btsU* upstream of the native promoter	This study
Δ*btsSR* P_{btsU}-*mcherry* strain	Δ*btsSR* with chromosomally integrated reporter construct for *btsU* upstream of the native promoter	This study
E. coli		
DH5α λ*pir*	*endA1 hsdR17 glnV44 thi-1 recA1 gyrA96 relA1* ϕ80′ *lac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) *U169 zdg*-232::Tn*10 uidA*::*pir*$^+$	72
WM3064	*thrB1004 pro thi rpsL hsdS lacZ* ΔM15 RP4-1360 Δ(*araBAD*)*567* Δ*dapA1341*::[*erm pir*]	W. Metcalf, University of Illinois
Plasmids		
pNPTS138-R6KT	Plasmid backbone for in-frame deletions; *mobRP4*$^+$ *sacB* Kan^r	58
pNPTS138-R6KT-Δ*btsU*	Plasmid for in-frame deletion of *btsU* in *V. campbellii*	This study
pNPTS138-R6KT-Δ*btsS*	Plasmid for in-frame deletion of *btsS* in *V. campbellii*	This study
pNPTS138-R6KT-Δ*btsR*	Plasmid for in-frame deletion of *btsR* in *V. campbellii*	This study
pNPTS138-R6KT-Δ*btsSR*	Plasmid for in-frame deletion of *btsSR* in *V. campbellii*	This study
pNPTS138-R6KT-P_{btsU}-*mcherry*	Plasmid used to create *V. campbellii* strains with a chromosomally integrated reporter construct (P_{btsU}-*mcherry*) upstream of the native locus	This study
pBAD-Cherry	*mcherry* in pBAD33	62

the *btsU* promoter with the *mcherry* gene upstream of the native locus. To this end, cells from overnight cultures were inoculated at a starting OD_{600} of 0.05 into various media in 96-well plates. Plates were then incubated under constant agitation at 30°C and mCherry fluorescence (excitation at 580 nm, emission at 610 nm) was measured at intervals of 10 min for 24 h in a Tecan Infinite M200 Pro plate reader. Fluorescence levels (normalized to 1 OD_{600}) were determined for each condition in exponential growth phase. Autofluorescence of the cells was subtracted.

External pyruvate determination. Levels of excreted pyruvate were measured using a procedure adapted from O'Donell-Tormey et al. (3). *V. campbellii* strains were grown under agitation at 30°C in LM medium, and growth was monitored. At selected time points, 1-ml samples of supernatant were harvested by centrifugation at 4°C (10 min, 14,000 × *g*). Proteins were precipitated by the addition of 250 μl of ice-cold 2 M perchloric acid. After a 5-min incubation on ice, the samples were neutralized with 250 μl of 2.5 M potassium bicarbonate, and precipitates were removed by centrifugation (4°C, 10 min, 14,000 × *g*). Pyruvate concentrations of the clear supernatants, diluted 1:20 in 100 mM PIPES buffer (pH 7.5), were determined by using an enzymatic assay based on the conversion of pyruvate and NADH + H^+ to lactate by lactate dehydrogenase. The assay was performed as described before (39).

Pyruvate uptake measurement. To determine the uptake of pyruvate by *V. campbellii*, a transport assay was performed with radiolabeled pyruvate. Cells were grown under agitation at 30°C in LM medium and harvested in mid-log phase. Cells were pelleted at 4°C, washed twice, and resuspended in transport buffer (2.9 mM K_2HPO_4, 2.2 mM KH_2PO_4, 0.33 M NaCl, 30 mM $MgCl_2$, 6.8 M $CaCl_2$) to an absorbance of 5 at 420 nm, equivalent to a total protein concentration of 0.35 mg ml^{-1}. Uptake of [^{14}C]pyruvate (55 mCi $mmol^{-1}$; Biotrend) was measured at a total substrate concentration of 10 μM at 18°C. At various time intervals, transport was terminated by the addition of ice-cold stop buffer (100 mM potassium phosphate [pH 6.0], 100 mM LiCl), followed by rapid filtration through membrane filters (MN gf-5, 0.4-μm nitrocellulose; Macherey-Nagel). The filters were dissolved in 5 ml of scintillation fluid (MP Biomedicals), and radioactivity was determined in a liquid scintillation analyzer (Perkin-Elmer). The effects of protonophores and ionophores were tested after preincubation of cells in transport buffer supplemented with 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2 mM 2,4-dinitrophenol (DNP), 10 μM nonactin, 6 μM nigericin, 2 μM valinomycin, or dimethyl sulfoxide (DMSO; as control) at 25°C for 30 min.

Chemotaxis test. Chemotaxis of *V. campbellii* toward different compounds was tested using the plug-in-pond assay (67). Cells grown in LM medium were pelleted, resuspended to a final OD_{600} of 0.5 in M9 soft agar (M9 medium with 2% [wt/vol] NaCl and 0.3% [wt/vol] agar) without a carbon source, and poured into a petri dish, in which agar plugs (M9 medium with 2% [wt/vol] NaCl and 1.5% [wt/vol] agar) containing the test substances had been placed. Plates were incubated at 30°C for 3 h. Pictures were taken with a Canon EOS M50 camera.

Induction of the VBNC state. *V. campbellii* cells were grown under agitation at 30°C in LM medium, harvested by centrifugation in mid-log phase, and washed twice with sterile saline solution (2% [wt/vol] NaCl). Cells were resuspended in M9 minimal medium (2% [wt/vol] NaCl) without a carbon source to a final OD_{600} of 1 and stored at 4°C to induce long-term cold stress under nutrient limitation. Ampicillin sodium salt (100 μg ml^{-1}) was added to the medium to prevent contamination, since *V. campbellii* is naturally ampicillin resistant. Culturability was determined periodically by plating serial dilutions of samples on LM agar plates and counting CFU. When CFU could no longer be detected, cells were considered to be nonculturable.

Resuscitation from the VBNC state. VBNC cells were diluted 1:10 in different media and incubated under agitation at 30°C. At different time points, samples were taken, serial dilutions were plated on LM agar plates and CFU were counted. For microscopy, samples were centrifuged and resuspended in small volumes of sterile saline solution (2% [wt/vol] NaCl). Then, 3-μl drops were placed on agarose pads (2% [wt/vol] NaCl, 1% [wt/vol] agarose) and sealed with a cover slide. Microscopy was performed using a Leica DMI6000 B fluorescence microscope.

Axenic hatching of brine shrimp larvae. Samples (500 mg) of high-quality *Artemia franciscana* cysts (EG Type; INVE Aquaculture, Baasrode, Belgium) were hydrated in 45 ml of sterilized distilled water for 1 h. Sterile cysts were obtained by decapsulation based on the method described by Marques et al. (68). Briefly, 1.65 ml of NaOH (32% [wt/vol]) and 25 ml of NaOCl (50% available chlorine) were added to the hydrated cyst suspension to facilitate decapsulation. The process was stopped after 2 min by adding 35 ml of $Na_2S_2O_3$ (10 g liter^{-1}). Filtered (0.22 μm) aeration was provided during the reaction. The decapsulated cysts were washed with filtered (passed through a 0.45-μm membrane filter), autoclaved (moist heat at 121°C for 20 min) artificial seawater and then resuspended in 500 ml of filtered, autoclaved seawater and hatched for 28 h at 28°C with constant illumination (2000 lx). Air was bubbled into the suspension through a sterile glass tube extending to the bottom of the hatching vessel to ensure that all the cysts were kept in continuous motion (69). The axenity of cysts was verified by inoculating 1 ml of culture water into 9 ml of LM medium and incubating overnight at 28°C. After 28 h of hatching, batches of 30 larvae were counted and transferred to sterilized 50-ml glass tubes containing 30 ml of filtered and autoclaved seawater. Finally, the tubes were incubated on a rotor (4 rpm) and kept at 28°C. All manipulations were performed in a laminar-flow cabinet to maintain sterility of cysts and larvae.

Brine shrimp challenge test. The virulence of wild-type and mutant strains was determined in a standardized challenge test with gnotobiotic brine shrimp larvae. *V. campbellii* strains were grown to an OD_{600} of 1, and then cultures were washed with phosphate-buffered saline (pH 7.4) prior to inoculation of the brine shrimp samples at 10^5 CFU ml^{-1}. The challenge tests were performed as described by Defoirdt et al. (70) with some modifications. A suspension of autoclaved LVS3 bacteria (71) in filtered and autoclaved seawater was added at 10^7 cells ml^{-1} as feed to the culture water at the start of the challenge. Brine shrimp cultures to which only autoclaved LVS3 bacteria were added as feed were used as controls. The surviving larvae were counted 48 h after the addition of the pathogens. Each treatment was carried out in triplicate, and the experiment was repeated three times to verify reproducibility. At the end of the challenge, the sterility of the control treatments in each test was checked by adding 1 ml of rearing water to 9 ml of LM medium and incubating the mixture for 2 days at 28°C.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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