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# An insect gut environment reveals the induction of a new sugar-phosphate sensor system in *Bacillus cereus*

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**Bacteria survive under various conditions by sensing stimuli triggering specific adaptive physiological responses, which are often based on membrane-integrated sensors connected to a cytoplasmic regulator. Recent studies reveal that mucus glycans may act as signal molecules for two-component systems involved in intestinal colonization. *Bacillus cereus,* a human and insect opportunistic pathogen was used to identify bacterial factors expressed in an insect gut infection model. The screen revealed a promoter involved in the expression of a gene with so far unknown functions. A search for gut-related compounds, inducing its transcription, identified glucose-6-phosphate as an activation signal. The gene is part of a five-gene cluster, including a two-component system. Interestingly such five gene loci are conserved in the pathogenic *Bacillus* group as well as in various *Clostridia* bacteria and are with analogy to other multi-component sensor systems in enteropathogenic bacteria, such as *E. coli*. Thus our results provide insights into the function of two-component and auxiliary sensor systems in host-microbe interactions and opens up possible investigations of such systems in other gut associated bacteria.**

**Keywords:** two component system, *Bacillus cereus,* glucose-6-phosphate, gut environment, auxiliary sensor, insect, *Galleria mellonella*, sugar phosphate uptake

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## Introduction

The interplay between the gut, the normal microbiota and gut pathogens has received particular attention in recent years (for review see refs. 1-3), however, many aspects still need to be investigated. For instance, it is of interest to identify bacterial genes, which are specifically expressed in the gut environment and which might explain how and why the bacteria can survive there. Among bacterial systems known to sense environmental factors, are membrane-integrated sensors. Such systems can be composed of several components, but the main actors are two-component systems (TCS) often composed of a membrane spanning histidine-kinase (HK) and a cytosolic response regulator (RR) activated by HK. In addition recent studies provide evidence for that other co-sensors, notably substrate binding proteins or transporters, which are functional actors as well (for a review see ref. 4). Some systems are involved in nutrient uptake and others will help the bacteria to avoid toxic compounds and host defense mechanisms. Almost 10 years ago it was highlighted that the prominent human gut symbiont, *Bacteroides thetaiotaomicron* genome contained a large number of ECF (extracytoplasmic sigma-factors) and TCS sensing systems which might be linked to glycan processing[5] and recently a study published in Nature Letter, reported that fucose sensing in *E. coli* regulates bacterial intestinal colonization via a TCS systems,[6] also indicating the importance of "sugar" compounds in gut homeostasis.

The here described work is dealing with results obtained with *Bacillus cereus,* an opportunistic human and insect pathogen, which is often associated with mild intestinal affections as well as more serious systemic infections in human.[7] *B.cereus sensu stricto*

is part of the *B.cereus* group which includes the insect pathogen *B. thuringiensis* and *B. anthracis* the causative agent of Anthrax which are all spore-forming Gram-positive bacteria (for a review see refs. 8-11). These bacteria are found in many environments, notably in the soil and on plants where they can be associated with invertebrates[8,12] or unicellular organisms.[13,14] They can also reach the digestive tract of higher animals and humans during food uptake (**Fig. 1**), where they can interfere with the host by expressing various bacterial effectors such as TCS-signaling pathways and virulence factors.

In this addendum we are referring to results published in The FASEB Journal, by Song et al.[15] which deals with a new sugar phosphate sensor and uptake system in *B. cereus* specifically expressed in an insect larva gut. Interestingly, homologous gene-clusters are found in a certain number of other gut associated Gram-positive bacteria of which functional orthologs in Gram negative bacteria are described as well. Then, we emphasize that such systems might be relevant for the adaptation to one or more environments encountered during the life cycle of these bacteria, as they are adapted to survive both inside and outside various eukaryotic cells, notably in the gut environment.

The description of this new TCS, with its auxiliary partners, along with the important and rare identification of in vivo stimulator molecules, provides new insights into the impact of two-component signal transduction systems in host-pathogen interactions and might be of particular interest for both intracellular and extracellular gut associated bacteria. Thus, although our results (see the below section) are focused on molecular activation mechanisms and bacterial genetics in a single bacterial species, we intend in this addendum to highlight that the results might be relevant for bacteria other than from the *B. cereus* group. We present our findings and perspectives from both an ecological and functional point of view.

## Summary of Results

Following oral infection of the larval stage of the greater Wax moth, *Galleria mellonella*, with a *B. cereus* strain carrying an in vivo Expression Technology (IVET) system, we previously identified 20 promoters responsible for the transcription of various factors during the infection process.[16] In the FASEB paper, we are dealing with investigations of one of these genes, mapping within a five-gene cluster in the *B. cereus* ATCC 14579 strain. These genes encode a new sugar phosphate sensor (Sps) system composed of a two-component system (TCS) called SpsK and SpsR, a protein of unknown function (SpsA) a putative ABC transporter substrate-binding protein (SpsB) and a putative phospho-glycerate transporter protein (SpsC) (**Fig. 2**). We report here on the expression and the relative role of these genes in vitro and in vivo.

First, we determined the spatiotemporal expression of the promoter during the insect infection process. The results showed that the promoter activation occurred specifically from 3 to 5 h post ingestion (see Figs. 1 and 6 in FASEB paper). The homology of SpsC with other Hexo phosphate transporters and the specific transcription of *sps* genes in the gut, in contact with the "sugar rich" mucus structures (peritrophic matrix)[17,18] directed our search for the activating signal toward several sugar-phosphates. Finally, only glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) were found to activate *sps* transcription and thus production of SpsABC. Next we aimed at identifying whether the upstream located putative TCS system was also activated by these sugar-phosphates. Quantitative real-time RT-PCR performed on the 5 genes of the locus showed that only *spsA*, *spsB*, and *spsC* were upregulated in the presence of G6P, indicating that separate transcriptional units encode the SpsRK (TCS) and the SpsABC proteins. In addition, transcriptome studies in presence and absence of G6P showed that among the whole genome only *spsABC* and another hypothetical protein (BC2417) were overexpressed in response to G6P. These results suggest that the SpsABC components are particularly dedicated for responding to G6P and F6P. To investigate the possible sensor role of SpsRK, a plasmid carrying the *spsABC*-promoter'*lacZ* transcriptional fusion was introduced in a Δ*spsRK* mutant strain and β-galactosidase activity was assayed. No expression occurred, clearly indicating the SpsRK TCS dependency on *spsABC* expression. Next, we aimed at understanding the implication of the three *spsABC* gene products in the transcription of the *spsABC* operon. The deletion of *spsA* and s*psB* resulted in complete loss of transcription, while the deletion of *spsC* provoked increased and continuous expression and the deletion of all three *spsABC* showed low and G6P independent transcription (Fig. 4A in FASEB). These findings allowed concluding a clear dependency of the three compounds for balanced activation.

To elucidate whether SpsC had a role in G6P uptake, the concentration of G6P was measured in the growth medium of Δ*spsC* mutant and wild-type strains. A higher concentration of G6P was found in the medium of the mutant. The role of SpsC in the uptake function was further demonstrated by introducing a plasmid carrying *spsC* into the other *sps* mutant strains. This resulted in production of SpsC and thus in G6P uptake for all constructs. Finally, although the Sps system is only activated for a short period in the insect gut, we considered whether the various mutants were affected in their capacity to infect and kill *G. mellonella* larvae. The results showed that the deletion of the *sps* genes did not reduce or increase the virulence or the development of the bacteria in the insect larvae. Then, so far no clear role of SpsRK and SpsABC during the infection process has been found, apart from the role in G6P uptake. Meanwhile, in the below discussion we speculate on possible functions in particular steps during the bacterial life cycle in various host (**Fig. 1**) which might also be relevant for other bacteria with similar systems. We also propose a new (different from the one presented in the FASEB paper) and more interactive model (**Fig. 2**) reflecting the possible molecular mechanisms involved in the function of this SpsRKABC system.

## Discussion

### Ecology related aspects

This study has unraveled the presence of a new sugar-phosphate sensor system

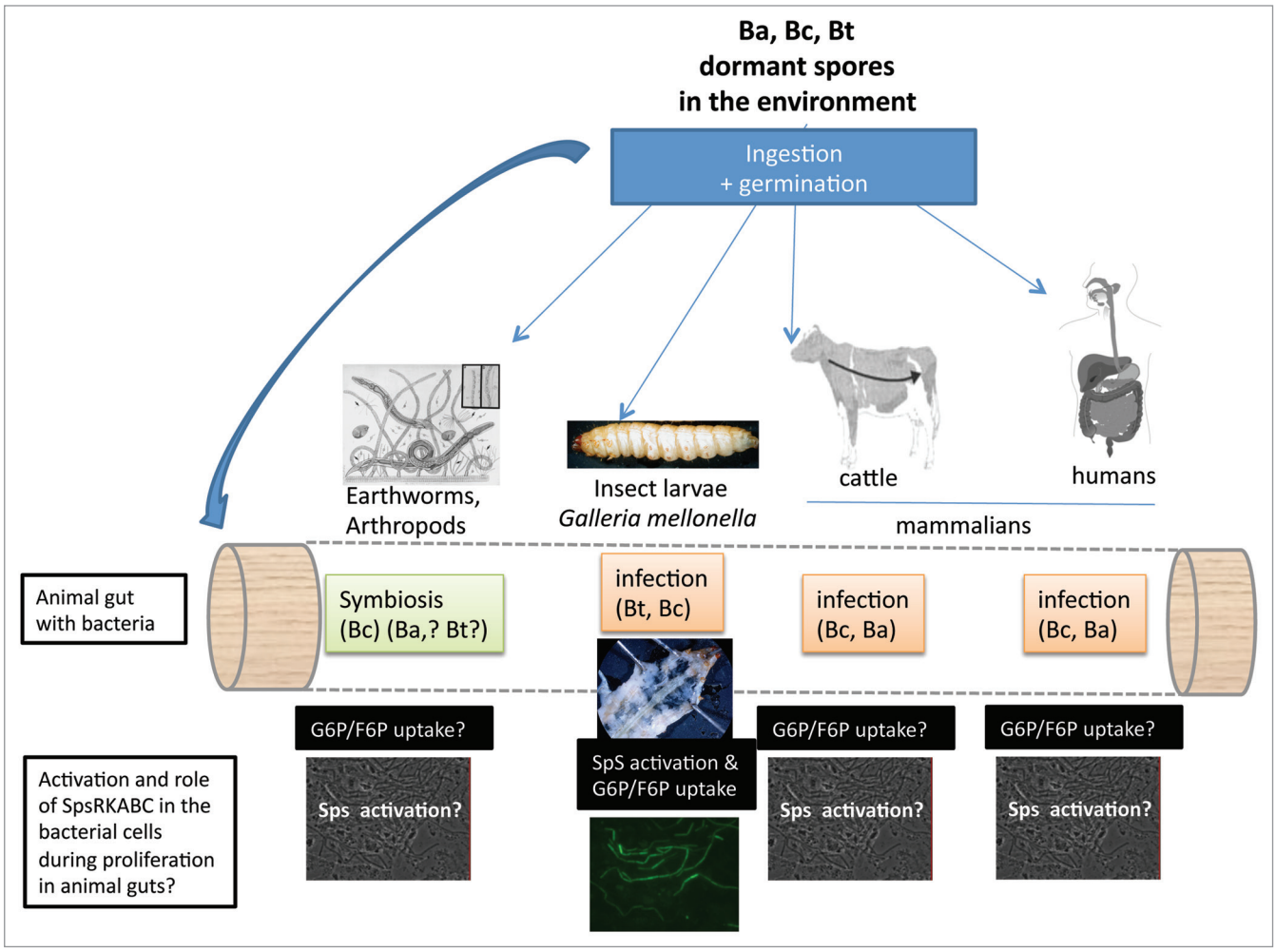
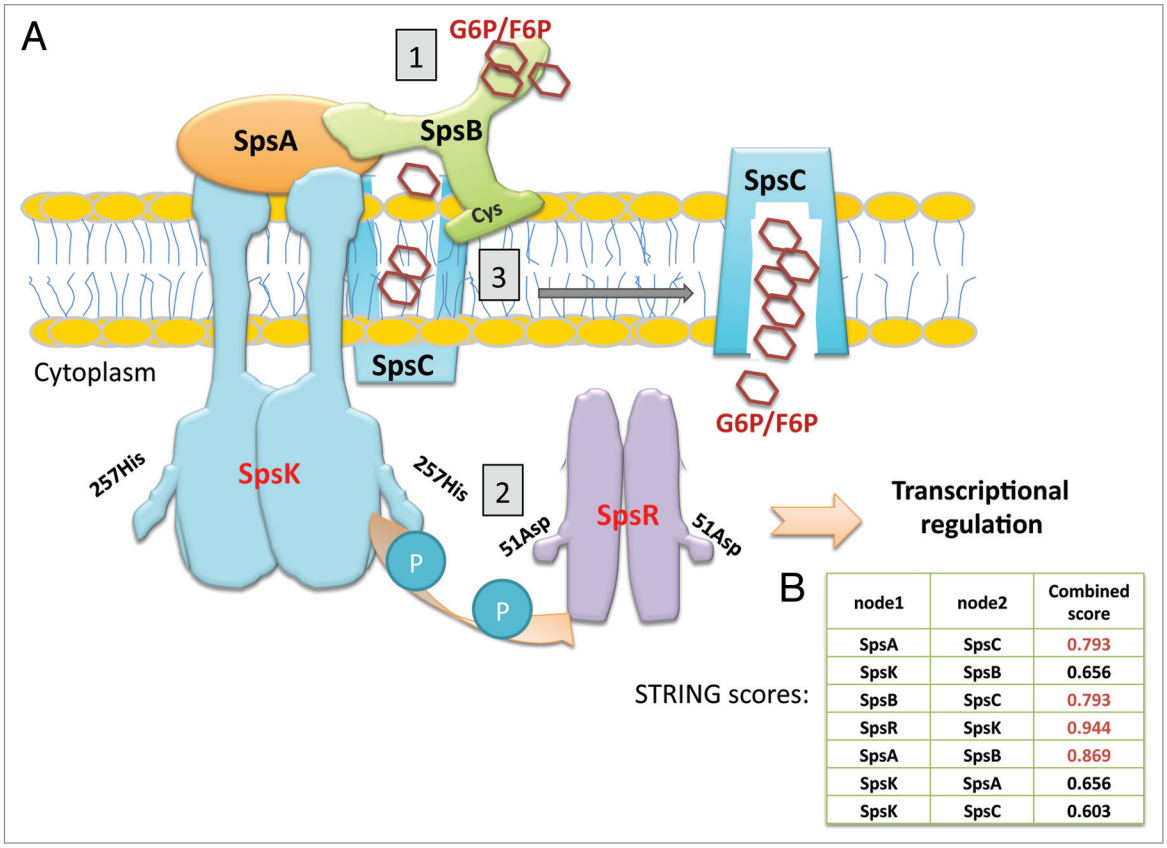



**Figure 1.** Ecology: Questioning on the role of SpsRK-ABC multicomponent sensing system in gut of various hosts. Spores of *Bacillus cereus* (Bc) *B. anthracis* (Ba), and *B. thuringiensis* (Bt) are widespread in soils and plants. Occasionally, the spores enter the digestive tract of various eukaryotic hosts where they germinate and may begin a vegetative cycle. The SpsRKABC is activated in response to G6P/F6P: it may allow the utilization of these nutrient sources and favor Ba/Bc/Bt growth despite a competitive microflora. Ba/Bc/Bt can display either symbiosis or infective stages with the eukaryotic hosts. Our study has only been dealing with activation in an invertebrate (the larval stage of the lepidopteran insect *Galleria mellonella*). Expression was observed when the bacteria are in the gut (see insets of dissected larvae [whole digestive tract] and green fluorescent bacteria isolated from the gut lumen). For the other possible hosts, and for Ba and Bt, further investigations are needed. Pictures courtesy of Wikimedia Commons (By Mariana Ruiz Villarreal [public domain]) (human gut), Jensen et al.,[20] (Cow), and Margulis et al.[17] (Arthropodes).

which is conserved in all completely sequenced members of the *B. cereus* group bacteria. As this group of bacteria is occupying similar biotopes one could speculate on ecological correlation. The soil environment is considered as the main reservoir for spores of the *B. cereus* group bacteria. However, their multiplication is believed to occur mostly in association to various eukaryotic hosts: arthropods including insects, earthworms, and several mammalians.[8,11,19-21] In all these animals, the gut is the first environment where *B. cereus* group bacteria encounter favorable conditions to allow germination of spores followed by vegetative growth. Recently, interactions with unicellular eukaryotes (i.e., amoeba) have also been shown to favor *B. cereus* group bacteria growth.[13,14,22]. Once in the gut, *B. cereus* group bacteria may display a symbiotic relation with their eukaryotic partners, as it is believed to occur in soil arthropods for instance.[19] Although a recent publication indicates that these supposed *B. cereus* are actually Lachnospiraceae.[21] They may also proliferate in detriment to their hosts, in a pathogenic relationship.[20] During these critical steps for the *B. cereus* group bacteria lifecycle, the SpsRKABC system is activated in response to G6P/F6P possibly allowing utilization of such nutrient sources to favor growth despite the presence of a competitive microflora or intestinal protozoa. Therefore, the SpsRKABC system might play a role during persistence and initial multiplication in the gut, a critical step leading to the infection process.

Interestingly Sps orthologs are also found in several other Gram-positive pathogens, including some *Clostridia*: *C. novyi* has two copies and *C. perfringens* has one copy of SpsRKABC; and two *C. botulinum* strains carry two different Sps-like systems, SpsRKABC and SpsRKAB (**Fig. S2** in FASEB paper). To extend the search for similar systems to other




| node1 | node2 | Combined score |
|---|---|---|
| SpsA | SpsC | 0.793 |
| SpsK | SpsB | 0.656 |
| SpsB | SpsC | 0.793 |
| SpsR | SpsK | 0.944 |
| SpsA | SpsB | 0.869 |
| SpsK | SpsA | 0.656 |
| SpsK | SpsC | 0.603 |

**Figure 2.** Model of interaction of Sps proteins in *B. cereus* in three steps: (**A**) G6P is sensed by SpsB to form the SpsB-G6P complex, which needs to interact with SpsA in order to bind to SpsK (1). This interaction causes a conformational change of SpsK, leading to histidine autophosphorylation of its cytoplasmic transmitter domain. Following phosphoryl transfer (2) the affinity of the response regulator SpsR for the *spsABC* promoter is enhanced, such that *spsABC* transcription is induced (2). SpsC might be located close to the SpsAB complex promoting the transfer of G6P from SpsB to SpsC resulting in G6P import and its release into the cytosol (3), due to the pivoting antiporter mechanism of SpsC. (**B**) String interaction modeling (http://string-db.org/). The highest combined score (0.944) is between SpsR and SpsK which indicate that they are functional partners. The combined score between SpsA and SpsB is 0.869 also suggesting a strong interaction. However it is only 0.656 between SpsA or SpsB and SpsK respectively. Thus we speculate that the SpsA-SpsB complex interacts with SpsK and then senses G6P or F6P. The lowest score 0.603 is found for SpsC and SpsK indicating no or low interaction.

Gram-positive or Gram-negative bacteria, a larger tblast search was run on the base of the presence of TCS being in the vicinity of *sps* like genes and having homology to SpsC and or other G6P transporters. The search was performed against all genomes available in the NCBI microbial genome database except for the *B. cereus* group and *Clostridia* (2139 genomes on 01/02/2013). A cluster of *spsRKABC* orthologous genes was identified in two other organisms: *Brevibacillus laterosporus* LMG 15441 and *Paenibacillus dendritiformis* C454 (E-value 0.0) which are sporeforming bacteria found associated with invertebrate guts.[23] In many organisms, including Gram-negative bacteria such as the gut bacteria *Vibrio cholerae*, *spsC*-orthologs were found. In contrast, *spsB*-orthologs were rare and *spsA*-orthologs were only marginally identified. Some sequence homology was often observed for *spsRK* orthologs, because of the conserved structure of the TCS domains involved in signal transduction. Also, in other bacteria, functional analogs are described, such as the *Listeria monocytogenes* hexose-phosphate transporter Hpt,[24] but they do not display sequence similarity to the *B. cereus sps* multicomponent system. In Gram-negative bacteria like *E. coli* the well-described UhpABCT system is also a sugar-phosphate sensor.[4,25,26] Thus, although complete *spsRKABC* loci are mainly found in Gram-positive spore-forming bacteria, these systems might be functionally close and it is worth underlining that all mentioned bacteria are able to develop in gut environments. This highlights that G6P sensing and uptake systems are conserved functions for Gram-negative and Gram-positive bacteria in such habitat, but the specific contexts and roles still need to be elucidated for most of them.

**Perspectives: the functional mechanisms of the SpsRKABC multi component sensor system**

Many TCSs play roles in adaptation, survival or virulence of bacterial cells in hosts.[27] Various TCSs have been found to require auxiliary proteins to sense environmental stimuli[28] and for instance the recently studied *B. thetaiotaomicron* starch utilization system (Sus) involves membrane spanning Hybrid-TCS,[29] which is different from classical TCS, such as the here reported Sps. We demonstrate that Sps activity requires SpsA and SpsB, which may act as auxiliary proteins for

SpsRK-dependent sugar phosphate sensing in *B. cereus*. According to conserved domain analysis, SpsA is an exported protein and SpsB is an extracellular solute-binding protein. The solute-binding proteins in Gram-negative bacteria and the homologous lipoproteins in Gram-positive bacteria were reported to serve as receptors initiating signal transduction pathways.[30] A cysteine residue, which may function as an N-terminal lipid anchor[31] was found just downstream from the signal peptide of SpsB. It suggests that SpsB is bound to the membrane and probably serves as receptor to trigger Sps activity via the SpsK kinase. The *spsC* mutant failed to remove G6P from the environment, suggesting that SpsC is also an auxiliary protein as it takes part in the balance between extra and intra-cellular G6P.

SpsR belongs to the OmpR family of response regulators, which are associated with a wide range of functions.[27,32] Therefore, SpsA, SpsB, and SpsC, are expected to be auxiliary proteins of the extracellular Sugar Phosphate sensor SpsRK, which by itself is not activated by G6P. However, our results could also suggest that SpsRK and especially SpsAB are present to express SpsC to achieve an optimal regulation and thereby a balanced G6P or F6P uptake. Meanwhile the role and interaction between these proteins and SpsRK on signal transduction still needs to be addressed. We focus actually on the localization of SpsA and possible interaction with SpsB. As SpsA is a protein of so far unknown function, it is of particular interest to focus on that protein. Our preliminary results and unpublished data indicate that SpsA is located on the bacterial surface and that purified SpsA and SpsB proteins are able to interact in vitro, supporting our new model (**Fig. 2A**). In order to test for theoretical interactions between the five Sps proteins, a STRING (http://string-db.org/) interaction search was applied (**Fig. 2B**). The analysis suggests that all proteins have a more or less strong probability to interact. The highest score (0.944) is found for SpsK and SpsR, which is expected due to the typical histidine-kinase and response regulator structures of these molecules. Also the other two by two-partners have scores from 0.656 to 0.869, suggesting that they have chemically compatible structures. The analysis indicates that both SpsA and SpsB might interact with SpsK and SpsC, thus explaining why the presence of SpsC is important for both regulated transcription and G6P uptake (**Fig. 2A**). When SpsA and SpsB are expressed without SpsC the interaction with SpsK might be blocked in a position favoring a constant interaction with SpsR explaining the continuous and G6P independent transcription of the promoter as shown in Figure 4 in the FASEB paper. This interaction hypothesis needs to be tested both in vitro and in vivo by adapted biophysical approaches. Additional studies are aiming at elucidating the respective functions of the Sps components during the contact with a gut environment.

**Perspectives: Functions in a gut environment**

Our results describe a new mechanism involved in sugar phosphate sensing in bacteria during growth in the intestinal environment. The identification of the natural stimuli inducing TCS activation is a significant result: very few studies have successfully identified the exact stimuli for bacterial TCS in a gut environment except for *B. thetaiotaomicron,* a common inhabitant of the human intestine. Indeed, two different *B. thetaiotaomicron* TCSs sense fructose and α-mannosides, thereby controlling the fructan utilization and monosaccharide metabolism in the human gut, respectively[33,34] and other glycan sensing systems are also reported as the *E. coli* FusKR system[6] and the above mentioned *B. thetaiotaomicron* Sus systems.[29] Although we did not notice any strong role of the Sps system in virulence or adaptation in the insect *G. mellonella* larvae, it cannot be excluded that the G6P uptake system might be important for the bacteria in a particular step during infection, where G6P or F6P could be the only carbon sources available. Indeed, under in vitro (minimal medium) and carbon source restricted conditions (only G6P), we found that the SpsRK mutant was affected in growth compared with the wild-type strain, for which growth was almost as high with G6P as with D-Glucose (unpublished results). These results indicate that SpsRK and SpsABC might have a physiological role during infection. For instance, it has been shown that Hpt, the permease involved in G6P uptake in *L. monocytogenes*, had a strong influence on the intracellular growth of this bacteria in various cell lines,[24] for instance in liver cells. This is in agreement with the high concentration of the G6P in these cells where the turnover of glycogen through glycolysis results in increased G6P and F6P production. In enterobacteria like *Salmonella*, *Shigella*, and *E. coli,* the need for G6P uptake permeases were more pronounced in macrophages than in intestinal cell lines.[35] Thus, our investigations also suggest that the Sps system enables bacteria to rapidly import sugar phosphates in highly competitive environments, thereby facilitating its colonization and infection of the host intestinal cells or other cells (macrophages, protozoa, etc.) in different hosts as illustrated in **Figure 1**. For instance it was recently reported that G6P is involved in the regulation of the *B. cereus* virulence factor hemolysin HlyII.[36] Furthermore it would be interesting to investigate the role of the Sps system during infection of *B. cereus* in other host models and also in other human pathogens, such as *B. anthracis*, *Clostridium* species and in insect pathogens like *B. thuringiensis* or in *Paenibacillus*. Finally, we hypothesize that such multi-component bacterial substrate sensor systems are mechanisms involved in the complex interplay among bacterial communities and eukaryotic host cells in intestinal environments; and therefore could also be targets for functional studies of uncultivable bacteria resulting from gut microbiome investigations.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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