Metabolome Profiling Reveals Metabolic Cooperation between *Bacillus megaterium* and *Ketogulonicigenium vulgare* during Induced Swarm Motility\(^\dagger^\)

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The metabolic cooperation in the ecosystem of *Bacillus megaterium* and *Ketogulonicigenium vulgare* was investigated by cultivating them spatially on a soft agar plate. We found that *B. megaterium* swarmed in a direction along the trace of *K. vulgare* on the agar plate. Metabolomics based on gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF-MS) was employed to analyze the interaction mechanism between the two microorganisms. We found that the microorganisms interact by exchanging a number of metabolites. Both intracellular metabolism and cell-cell communication via metabolic cooperation were essential in determining the population dynamics of the ecosystem. The contents of amino acids and other nutritional compounds in *K. vulgare* were rather low in comparison to those in *B. megaterium*, but the levels of these compounds in the medium surrounding *K. vulgare* were fairly high, even higher than in fresh medium. Erythrose, erythritol, guanine, and inositol accumulated around *B. megaterium* were consumed by *K. vulgare* upon its migration. The oxidation products of *K. vulgare*, including 2-keto-gulonic acids (2KGA), were sharply increased. Upon coculturing of *B. megaterium* and *K. vulgare*, 2,6-dipicolinic acid (the biomarker of sporulation of *B. megaterium*), was remarkably increased compared with those in the monocultures. Therefore, the interactions between *B. megaterium* and *K. vulgare* were a synergistic combination of mutualism and antagonism. This paper is the first to systematically identify a symbiotic interaction mechanism via metabolites in the ecosystem established by two isolated colonies of *B. megaterium* and *K. vulgare*.

The biosphere is dominated by microorganisms. Microorganisms usually live together with other organisms and form various ecosystems, such as predator-prey, mutualism, and symbiotic interaction (8). An understanding of symbiotic interaction provides fundamental insights into the screening and production of new natural chemical compounds (26). Symbiotic interaction could be achieved by several strategies, among which metabolic cooperation is one of the most common. Metabolic cooperation is usually achieved by diverse means, including transferring intermediate metabolites, removing limiting by-products, and performing different functions to complete the energy cycling (25).

To investigate metabolic cooperation in the ecosystem, the isotope tracer technique, chromatographic separation, and high-throughput chemical analysis techniques were also applied (1, 5, 10, 11, 14, 28, 29). For example, the elemental-isotope technique has been used to study the nitrogen transfer pathway (11), phosphorus metabolism in legume-*Rhizobium tropici* symbiosis (1), and *Bacillus* detoxification of indole-based inhibitors of *Bacillus* to enhance the growth of *Symbio bacterium thermophilum* (29). Recently, new techniques have advanced the study of metabolic cooperation at the system level. Metagenomic approaches were applied to study the function and interaction mechanisms of complex ecosystems, such as the human intestinal microbiota and marine microorganisms (5, 10). Metaproteomics were used to identify new proteins that dictated metabolic cooperation in ecosystems. Understanding microbial community composition through 16S rRNA gene sequence analysis has also been helpful (14, 28). However, all of these techniques were not enough to provide a comprehensive understanding of metabolic interactions in ecosystems.

The ecosystem consisting of *Bacillus megaterium* and *Keto gulonicigenium vulgare* has been used to synthesize 2-keto-gulonic acids (2KGA), the precursor of vitamin C (32). However, the interaction mechanism in this ecosystem remains vague. Systematic understanding of the symbiotic interactions in the ecosystem would be of great significance for the optimal production of 2KGA. To this end, we cultured *B. megaterium* and *K. vulgare* on soft agar by seeding them in different areas of the agar plate and allowing them to migrate and interact, which facilitated systematic elucidation of the symbiotic interaction mechanism. To systematically analyze cellular interaction via metabolites at the system level, metabolomics based on gas chromatography coupled with time-of-flight mass spectrometry...
Two spectra were recorded per second in the mass range of 50 to 800 m/z with dynamic range extension (DRE) function.

Data analysis. Masslynx software (version 4.1; Waters Corp.) was applied for mass spectral peak identification and quantification. Automatic peak detection and deconvolution were performed using a peak width of 2.0 s. For quantification, peaks with signal/noise values lower than 10 were rejected. Automatic assignments of unique fragment ions for each metabolite were taken as the default and manually corrected when necessary. Compound identification was performed by comparing the mass spectra with a commercially available standard library, the National Institute of Standards and Technology mass spectral library (NIST 2005). After normalizing and mean centering, the data for the metabolite concentration were analyzed. Multivariate data analysis was performed by PCA, using Matlab 7.0 to distinguish the samples.

RESULTS

Metabolomic profiling could distinguish B. megaterium, K. vulgare, and their coculture. We used a metabolomics approach based on GC-TOF-MS to investigate the metabolites that were released by individual B. megaterium and K. vulgare bacteria and their metabolic cooperation in the ecosystem. A total of 242 peaks were identified in all the samples, among which 127 metabolites were unique. Ninety-two metabolites were found in the extracts of agar, which included the metabolites released by the bacteria: 45, 91, and 105 metabolites were found in the intracellular samples of B. megaterium, K. vulgare, and their coculture, respectively. The main classes of these compounds included sugars, amino acids, organic acids, alcohols, and amines. Over 50 metabolites were determined to be related to the amino acid biosynthetic pathway, central carbon metabolism, and substrate oxidation of K. vulgare.

Multivariate data analysis was performed by PCA, which had been successfully applied to identify biomarkers responsible for distinguishing different samples, to analyze these metabolomics data (21). The metabolite profiles of the monoculture and the coculture were analyzed by PCA (Fig. 1). The score plot showed that samples were clearly separated (Fig. 1a), which indicated that the cultured samples differed greatly from each other. In these samples, the monocultures of B. megaterium and K. vulgare at different time points were clustered together, but the cocultures at 48 h and 72 h were clustered together at a short distance from the coculture at 24 h, which indicated that the physiological state of the coculture differed with time. The loading plot suggested that many compounds contributed more significantly to distinguishing different samples, which could be regarded as potential biomarkers (Fig. 1b), including amino acids, sugars, amines, and some nucleotide derivatives. Their levels differed greatly in these samples. For example, erythrose, erythritol, guanine, and most amino acids are the major discriminators of the monoculture of B. megaterium from the monoculture of K. vulgare and their coculture. Glycerate and putrescine accumulated in K. vulgare. Xylose and adenosine were responsible for distinguishing the coculture from the pure monocultures.

We analyzed the levels and types of the intracellular metabolites in the individual monoculture of B. megaterium and K. vulgare. As the basic nutritional components of a cell, amino acids played many significant roles in primary and secondary metabolism. Therefore, amino acids were significant biomarkers of the nutritional states of cells. We compared the amino acid contents in B. megaterium and K. vulgare. As shown in Fig. 2.
2, the concentrations of most amino acids in *B. megaterium* were significantly higher than those in *K. vulgare*.

Most primary metabolites related to central carbon metabolism and nucleotide metabolism showed similar trends in *B. megaterium* and *K. vulgare*, with the exception of pyruvic acid, xylulose, and gluconate. The independent growth rate of *K. vulgare* is fairly low, which indicates that *K. vulgare* might have a significant deficiency in primary metabolism. Indeed, we found that pyruvic acid, an important intermediate in glycolysis and the tricarboxylic acid (TCA) cycle, was usually maintained at a high level in *K. vulgare* while other metabolites, such as glucose, succinic acid, and fumaric acid, were not detected.

**Migration dynamics of *B. megaterium* and *K. vulgare* in solid coculture.** Coculturing bacterial ecosystem in the solid phase and studying their interaction dynamics facilitates a systematic elucidation of the symbiotic interaction mechanism via exchanging metabolites (25). To this end, we cocultured *B. megaterium* and *K. vulgare* on soft agar by seeding them in different locations on the agar plate and allowing them to migrate and interact. As shown in Fig. 3, *B. megaterium* swarmed along the trace of *K. vulgare* on the agar plate (1.7% [wt/wt] agar density), and such swarming occurred mainly from the edge of the colony. The migration of the two microbes stopped after 72 h of coculturing. Many factors (including the cell density, nutrient content, and viscosity of the medium) can affect bacterial swarming. We therefore inoculated approximately equal amounts of the bacteria on the agar, and the inoculation distance between *B. megaterium* and *K. vulgare* was varied. We found *B. megaterium* would not swarm toward *K. vulgare* when they were seeded 5 mm apart initially. This is due to the restraint of nutrient diffusion in the agar. When the initial isolation distance was shortened to 0.5 mm, swarming of *K. vulgare* toward *B. megaterium* started to occur. In comparison to the directional migration of *B. megaterium* along the trace of *K. vulgare*, swarming of *B. megaterium* in other directions was not obvious.

![Diagram showing the principal-component analysis results for different samples of intracellular metabolites, including monoculture of *B. megaterium* (*B.m*), monoculture of *K. vulgare* (*K.v*), and coculture, at different times. (a) Score plot. The samples of a monoculture of *B. megaterium* are in red, the samples of a monoculture of *K. vulgare* are in blue, and cocultured samples are in green. Samples in different time spots also have different symbols: 24 h, cross; 48 h, circle; and 72 h, asterisk. (b) Loading plot. The horizontal axis in both figures was defined as the 1st principal component, and the vertical axis was defined as the 2nd principal component.](http://aem.asm.org/)

FIG. 1. Principal-component analysis results for different samples of intracellular metabolites, including monoculture of *B. megaterium* (*B.m*) monoculture of *K. vulgare* (*K.v*), and coculture, at different times. (a) Score plot. The samples of a monoculture of *B. megaterium* are in red, the samples of a monoculture of *K. vulgare* are in blue, and cocultured samples are in green. Samples in different time spots also have different symbols: 24 h, cross; 48 h, circle; and 72 h, asterisk. (b) Loading plot. The horizontal axis in both figures was defined as the 1st principal component, and the vertical axis was defined as the 2nd principal component.
Metabolic cooperation induces the migration dynamics of the ecosystem. We further used the metabolomics approach to investigate how metabolic cooperation determines the migration dynamics of the ecosystem. We found that the chemical composition of the agar surrounding *K. vulgare* played a significant role in the swarming of *B. megaterium* toward *K. vulgare*. As shown in Fig. 4a, we compared the metabolites in different agar extracts and found that the amino acids and intermediates involved in central carbon metabolism were in similar patterns. We also found the contents of these amino acids in the agar extract surrounding *K. vulgare* were significantly higher than those in the agar extract of *B. megaterium* and the pure medium. High levels of amino acids released by *K. vulgare* were consumed by *B. megaterium* (Fig. 4b).

To form a mutually synergistic ecosystem, *B. megaterium* also provided several metabolites for the growth of *K. vulgare* in exchange. As shown in Fig. 5, *B. megaterium* released high levels of erythrose, erythritol, guanine, and inositol, which were quickly consumed by *K. vulgare*. The time series of erythrose, erythritol, guanine, and inositol in Fig. 5 also showed that the levels of these metabolites released by *B. megaterium* increased during its growth, which remained at a low level after the migration of *B. megaterium*.

*B. megaterium* assists *K. vulgare* via metabolite exchange. The substrate (sugars and alcohols) oxidation products, including 2KGA, gluconate, 2-keto-glucorionate, and glucarate, were found to be sharply increased in agar extracts during the migration of *B. megaterium* in the agar. It was found that *K. vulgare* shared many similar features with the *Gluconobacter* strains, which could incompletely oxidize alcohols and sugars to keto acids or aldonic acids (6, 12). In our ecosystem, *K. vulgare* mainly oxidized the sugars and alcohols using sorbose/sorbosone dehydrogenase (S/SNDH), which is a dehydrogenase coupled with the respiratory chain of *K. vulgare* for energy generation (2). Therefore, the accumulation of these oxidation products functions as an index of the substrate oxidization.

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**FIG. 2.** Comparison of important primary metabolites in *B. megaterium* (solid bars) to those in *K. vulgare* (hatched bars) at 24 h. Relative abundance was calculated by normalization of the peak area of each metabolite to the internal standard and to the dry weight. **, *P < 0.01; ***, *P < 0.001. The error bars represent standard deviations.

**FIG. 3.** Swarming pattern of the ecosystem via chemotaxis of species and exchange of metabolites. The photographs show monocultures of *B. megaterium* and *K. vulgare* and coculture after 48 h of growth at 30°C on soft agar.
ability of *K. vulgare* (Fig. 6). The accumulated 2KGA was produced from oxidation of L-sorbose. In industrial fermentation, the addition of *B. megaterium* to the mixture could sharply increase 2-keto-gulonate production (18). Our experiments also showed that the substrate oxidation ability of *K. vulgare* was enhanced with the assistance of *B. megaterium*.

On the other hand, with the nutrients consumed, it was found that 2,6-dipicolinic acid (DPA), a biomarker of sporo-
loration of Bacillus strains, accumulated in the cells. As illustrated in Fig. 7, the level of DPA in coculture was much higher than that in the monoculture of B. megaterium, especially at 72 h, when the migration of microbes stopped. We also observed a sharply decreased amino acid content (Fig. 8), which indicated that intracellular nutrition components were gradually exhausted. Compared to the level of DPA in the monocultured B. megaterium, this indicated that the sporulation of B. megaterium was induced by K. vulgare.

DISCUSSION

Metabolomic profiling provided valuable information on the dynamics of metabolic synergy. To comprehensively understand the metabolic cooperation within an ecosystem, metabolomics analysis had many advantages over traditional techniques, such as isotope labeling, column chromatography, thin-layer chromatography (TLC), and 16S rRNA gene sequencing. First, metabolomics analysis provided comprehensive information on most metabolites that participated in the mutual interactions in microbial ecosystems. In this study, 127 different metabolites were identified and measured based on GC-TOF-MS. Second, detecting the changes in the important metabolites at different time points could assist in elucidating the roles of these metabolites in the modulation dynamics of the ecosystem. Third, the chemical composition of the environment (i.e., agar) could also be monitored to reveal metabolic cooperation due to its role in metabolite exchanges (15, 24). Research on metagenomics and metaproteomics usually deduced the role of some organism in the ecosystem based on gene or protein analysis, but the metabolomics approach would provide a more intuitive understanding of how cells interact with each other via metabolites and the cellular responses to environments. The changes in the metabolite levels provide more precise information on metabolic cooperation.

From the multivariate data analysis, we found that the samples of monocultured and mixed cells could be distinguished based on their different intracellular metabolite levels (Fig. 1), and the mixed cells could be distinguished individually based on the time series, which indicated that the physiological states of the mixed cells changed with time during their migration. Metabolite profiles of K. vulgare and B. megaterium were analyzed to characterize their metabolism. Our results showed that the content of amino acids and sugars was much lower in K. vulgare than in B. megaterium. This result also led to a hypothesis that K. vulgare might be deficient in many primary metabolisms, including amino acid biosynthesis, carbon central metabolism, and nucleotide metabolism. This hypothesis was validated by the poor growth performance of K. vulgare in monoculture. Leduc et al. found that folic acid derivates, purines, and pyrimidines could serve as the major growth factors for the growth requirements of K. vulgare (17). However, pyruvic acid (an important intermediate in glycolysis and the
TCA cycle) was maintained at a high level in *K. vulgare*. Since other related metabolites such as glucose, succinic acid, and fumaric acid, were absent in *K. vulgare*, we speculated that this high level of pyruvic acid might be derived from the oxidation of lactate in the medium that accumulated in cells.

The migration dynamics of the ecosystem is via exchange of metabolites between *B. megaterium* and *K. vulgare*. In this study, we established an efficient approach to study the metabolic synergy mechanism in the microbial ecosystem formed by *B. megaterium* and *K. vulgare*. This approach allowed spatial segregation of the microorganisms, which facilitates revealing of the ecosystem formation and interactions within it. Thus, this experimental setup was of great help in elucidating the spatiotemporal interaction dynamics of microbial ecosystems. Our observation clearly showed that the swarming direction of *B. megaterium* was influenced by *K. vulgare* (Fig. 3). We further studied the dynamics of the metabolites surrounding *K. vulgare* for the swarming of *B. megaterium* and the formation of a symbiotic ecosystem (Fig. 4 to 6).

The elucidated metabolic synergy mechanism of the ecosystem. Mutualism between *K. vulgare* and *B. megaterium* was previously supposed to be based on metabolite exchanges (18). The directional migration of *B. megaterium* toward *K. vulgare* occurred via chemotaxis. We found that *K. vulgare* released high levels of amino acids, much higher than those in *B. megaterium* and in the medium. Based on the reported chemottractants (4, 20, 23), we speculated that the extracellular metabolites (e.g., amino acids and many intermediates of the central carbon metabolism) of *K. vulgare* were chemottractants for *B. megaterium*. In our experiments, we observed that these metabolites released by *K. vulgare* were quickly consumed by *B. megaterium* during its migration. On the other hand, *B. megaterium* released several metabolites for the growth of *K. vulgare*. *B. megaterium* released many metabolites into the environment, such as erythrose, erythritol, guanine, fructose, and inositol, which were in turn consumed by *K. vulgare* for its growth. Erythrose is an important metabolite in aromatic amino acid biosynthesis and pyridoxine metabolism (9, 16). With a low intracellular level of amino acids, erythrose might assist *K. vulgare* in synthesizing some amino acids. Erythrose is the precursor of pyridoxine, which could assist in the assimilation of amino acids and the improvement of the central carbon metabolism in *K. vulgare* (16).

The ability for glucose oxidation by *K. vulgare* was enhanced with the assistance of *B. megaterium* (Fig. 6), which was observed in industrial processes (32). Previous research suggested that the fermenting liquor of *B. megaterium* enhanced 2KGA production (19, 27). In this study, we showed that there were significantly different levels of keto acids and aldonic acids in mixed cells and monocultured *K. vulgare* (Fig. 6). DPA followed a trend similar to that of keto acids and aldonic acids (Fig. 7). Therefore, these compounds secreted during sporu-
lation of \textit{B. megaterium} might be essential for oxidation enhancement in \textit{K. vulgare}.

As shown in Fig. 7, the DPA content was much higher in the mixed culture than in the monoculture of \textit{B. megaterium}, which indicated that the physiological state of the ecosystem at 24 h was quite different from those at 48 h and 72 h (Fig. 1a). DPA-Ca$^{2+}$ played a key role in maintaining the heat resistance, UV resistance, and other features of the spores, and it was the basic component in the spore protoplast, cortex, and coat (3). However, DPA did not exist in the vegetative cells of \textit{Bacillus} strains; therefore, the detection of DPA was an indication of sporulation (3). Nutrient exhaustion could lead to the initiation of sporulation of \textit{B. megaterium} and other \textit{Bacillus} species (22). The intracellular sugars and amino acids were compared between \textit{B. megaterium} and the mixed culture at 48 h and 72 h. A sharply decreased amino acid content in the mixed-culture cells was shown, which indicated that intracellular nutrient components in the mixed-culture cells were gradually exhausted (Fig. 8). The decreasing level of nutrition resulted in the initiation of sporulation of \textit{B. megaterium}, so this result was evidence that the sporulation of \textit{B. megaterium} was induced by \textit{K. vulgare}.

In summary, we investigated the spatiotemporal interaction dynamics between \textit{B. megaterium} and \textit{K. vulgare}. The mechanism of symbiotic interaction between the two microbes via metabolites was analyzed using a metabolomics approach. Both mutualism and antagonism interactions existed in the interaction of \textit{B. megaterium} and \textit{K. vulgare}. Upon the migration of \textit{B. megaterium}, mutualistic interaction between \textit{B. megaterium} and \textit{K. vulgare} was initiated via metabolite exchanges. \textit{B. megaterium} was attracted by exogenous metabolites released by \textit{K. vulgare}. Meanwhile, \textit{K. vulgare} also benefited from erythrose, erythritol, and inositol secreted by \textit{B. megaterium}. The increased level of 2KGA and other sugar acids also suggested enhanced energy generation in \textit{K. vulgare}. As the migration of \textit{B. megaterium} proceeded, antagonism was launched in this ecosystem, as evidenced by the DPA level, which led to the sporulation of \textit{B. megaterium}.

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