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EXPERIMENTAL ARTICLES

Decrease of Phosphate Concentration in the Medium by *Brevibacterium casei* Cells

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Abstract—Brevibacteria able to decrease phosphate concentration in the medium are of interest for the study of the role of bacteria in the phosphorus cycle and for development of biotechnology of phosphate removal from waste. *Brevibacterium casei, Brevibacterium linens*, and *Brevibacterium epidermidis* grown in media with initial phosphorus concentrations of 1–11 mM were shown to decrease its concentration by 90%. The composition of the incubation medium required for *B. casei* to carry out this process was established. This process occurs in the absence of glucose but requires the presence of Mg^{2+}, NH_4^+, and α-ketoglutarate. The latter two components may be replaced by amino acids metabolized to NH_4^+ and α-ketoglutarate: histidine, arginine, glutamine, proline, or glutamic acid. No formation of insoluble phosphate salts was observed when the media were incubated under the same conditions with heat-inactivated cells or without cells at pH 7–8.5.

Key words: Brevibacterium, phosphate, uptake, magnesium ions, ammonium ions, amino acids, EBPR, α-ketoglutarate.

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Microorganisms play a substantial part in phosphate cycling in the biosphere [1]; they are an important component of activated sludge in treatment facilities providing removal of excessive phosphate from technogeneous waste [2–5]. The processes of phosphate transport into microbial cells, particularly those able to assimilate considerable amounts of phosphate from the environment, are therefore of great interest. The highest ability for phosphate uptake has been shown for bacteria isolated from the activated sludge of technological plants for wastewater purification, where the process of Enhanced Biological Phosphorus Removal (EBPR) takes place [6]. Cultures of *Acinetobacter johnsonii* [7], *Microlunatus phosphovorus* [8], and *Rhodocyclus* sp. [9] from activated sludge accumulated up to 0.6–1.0 mmol phosphate per gram of wet biomass. The main problem in the study of phosphate uptake by bacterial cells inhabiting activated sludge is the difficulty of obtaining pure cultures. As a result, phosphate transport in such bacteria has been little studied as yet. Quite recently, it has been shown that some microorganisms growing well in the pure culture are no less efficient in phosphate removal from a medium than the bacteria isolated from EBPR systems. These are cyanobacteria [10], halophilic archaea [11], and the halotolerant bacterium *Brevibacterium antiquum* [12].

The search for new microorganisms capable of phosphate removal from the environment is of interest for the study of the mechanism of this process and for development of the biotechnology for wastewater purification from technogeneous phosphorus pollution. In the present work, we have shown that other species of *Brevibacterium* are able to decrease phosphate content in the medium and determined the medium components required for this process.

MATERIALS AND METHODS

Object of research and growth conditions. Bacterial strains *Brevibacterium linens* VKM Ac-2112, *Brevibacterium epidermidis* VKM Ac-2108, and *Brevibacterium casei* VKM Ac-2114 from the All-Russian Collection of Microorganisms were the object of research.

Liquid nutrient medium contained (g l^{-1}): $MgSO_4 \times 7H_2O$, 20; glucose, 5; yeast extract, 3; and peptone, 5. Cultivation was carried out in 500-ml flasks with 200 ml of the medium in a shaker (200 rpm) at 29°C. KH_2PO_4 (10 mM) was added to study the effect of enhanced phosphate concentration (phosphate concentration of 1 mM was provided by this component present in yeast extract). In order to determine the effect of elevated salinity, the liquid medium was supplemented with NaCl (5–15%) as indicated in the figure

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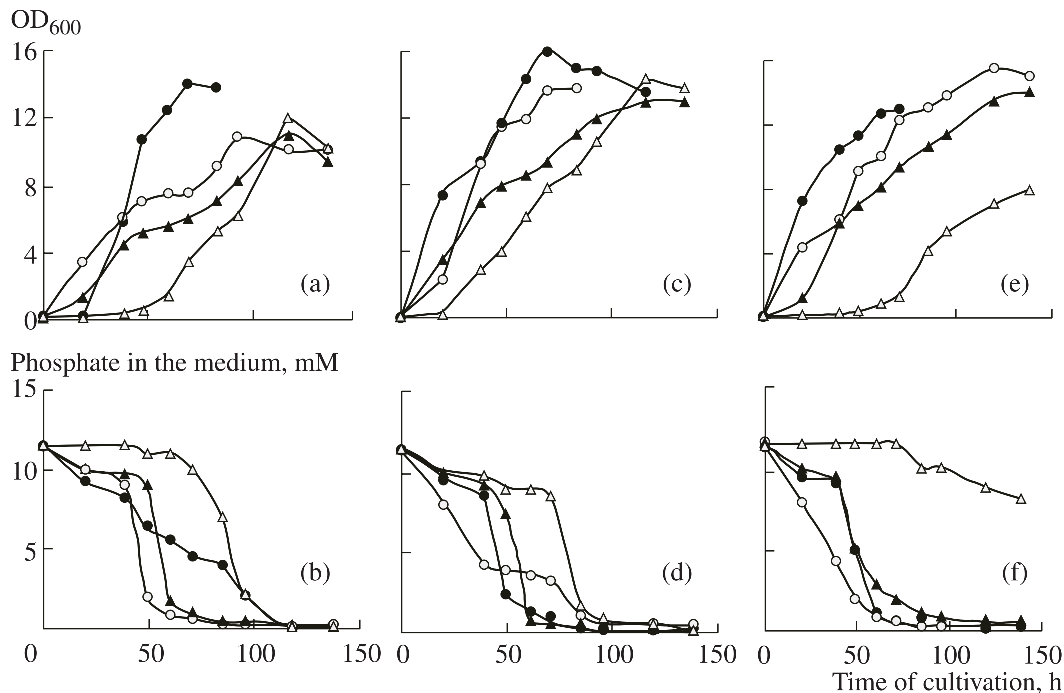

Fig. 1. Culture growth and phosphate content in the medium: *Brevibacterium linens* (a, b), *Brevibacterium epidermidis* (c, d), and *Brevibacterium casei* (e, f) at different NaCl concentrations. Symbols: –●– without NaCl, –○– 5% NaCl, –▲– 10% NaCl, –Δ– 15% NaCl (initial phosphate concentration: 11.5 mM; $MgSO_4$: 80 mM).

captions. The cultures were maintained on the same medium with the addition of 2% agar. Phosphate content in culture liquid was determined in the course of growth [13].

The effect of the medium components on decrease of phosphate concentration. The effect of medium components on phosphate concentration decrease in the presence of *B. casei* cells was studied in bacteria from the stationary growth phase (50 h). The cells were harvested by centrifugation at 5000 *g* for 30 min, washed twice with distilled water, and incubated in liquid medium in a shaker (200 rpm) at 29°C. In these experiments, the culture density was the same as in the stationary growth phase (16 g of wet biomass l^{-1}). The concentrations of all medium components are given in the legends to figures and tables. If necessary, pH of the medium was adjusted to the required value with KOH. After incubation, the cells were removed by centrifugation and phosphate content in the supernatant was determined [13].

RESULTS

Decrease of phosphate concentration in the medium during the growth of three *Brevibacterium* species. Decrease of phosphate concentration in the medium has been studied during the growth of *B. linens, B. epidermidis*, and *B. casei* at 1 mM (not illustrated) and 11 mM (Fig. 1) of this component in the medium. The growth curves of the cultures and the biomass quantity in the stationary growth phase did not differ on the two media with different initial phosphate concentrations; thus, phosphate concemtration of 1 mM was sufficient for growth of the cultures under study. This component completely disappeared from the medium after 30 and 60 h of cultivation when initial phosphate concentrations were 1 and 11 mM, respectively. When phosphate was present in excess, most of it was removed from the medium during the stationary growth phase.

The effect of NaCl on growth and decrease of phosphate concentration in the medium. The cultures grew at NaCl concentrations of up to 15%. Growth was absent at 20% NaCl. The decrease in the growth rate caused by salt depended on its concentration (Figs. 1a, c, e). This slowdown was not the same for the three species investigated, i.e. the cultures differed in halotolerance. The NaCl concentrations used in our experiments had the most pronounced inhibitory effect on the growth of *B. casei* (Fig. 1e).

Then, the loss of phosphate from the media with different NaCl concentrations and excessive phosphate (11 mM) was determined during the growth of these three cultures (Figs. 1b, d, f). In the presence of NaCl, the loss of phosphate from the medium as well as bacterial growth was slower. For example, the growth of *B. casei* at 15% NaCl was approximately half of the control value, and the decrease of phosphate concentration was also twice less than in the absence of NaCl (Figs. 1e, f). Comparison of the data from Figure 1

shows that increasing salinity of the medium results in concurrent inhibition both of culture growth and phosphate removal from the medium. The obtained results demonstrate that the ability to decrease phosphate concentration is not associated with halotolerance and the above three cultures insignificantly differ in this respect.

The effect of medium components on decrease of phosphate concentration at incubation with *B. casei* in the absence of growth. To determine the effect of medium components on phosphate concentration decrease, *B. casei* cells grown to the stationary phase were incubated in liquid medium at the culture density typical of this stage (16 g of wet biomass l^{-1}). Under these conditions, biomass did not increase during the experiment, even when the incubation system was the culture medium described in Methods. Thus, we succeeded to exclude the indirect effect of medium components on the process under study, which could be due to their effect on growth. Microscopy showed no cell lysis under the above-described conditions. Irrespective of the incubation medium composition (its different variants are given below), phosphate-containing precipitate was not formed in the absence of cells at pH 5–8.6.

Figure 2 shows the dependence of phosphate removal from the medium on time, when cells are incubated under the above-described conditions in the medium used for their cultivation. Since the culture density was equal to that of the stationary growth phase, phosphate decrease was expected to start from the moment of addition of the cells to the incubation medium. However, the process began only after eight hours. This delay was not caused by induction of the synthesis of the transport system components, because the addition of chloramphenicol together with the cells did not change the dynamics of phosphate decrease (not illustrated).

The effect of individual medium components on phosphate removal from the medium after 15 h of incubation with *B. casei* cells was then studied. This process requires the presence of Mg^{2+} ions: phosphate content did not change in their absence (Table 1). A 5 mM concentration of $MgSO_4$ was sufficient to remove 5 mM phosphate from the medium. Under these conditions, increasing Mg^{2+} concentration to 40 and 80 mM had no effect on phosphate removal. In all the subsequent experiments, 5 mM $MgSO_4$ was introduced into incubation medium. Exclusion of glucose had no effect on phosphate removal from the medium during 15 h of incubation (Table 1, Fig. 2). Without yeast extract and peptone, no decrease of phosphate concentration was observed both in the absence and in the presence of glucose (Table 1).

It is important to note that the cells of *Brevibacterium antiquum* cultivated under phosphate excess in the medium were shown to contain a poorly soluble salt $(NH_4)MgPO_4$, which could be extracted from the cells only after their destruction by extrusion from the frozen

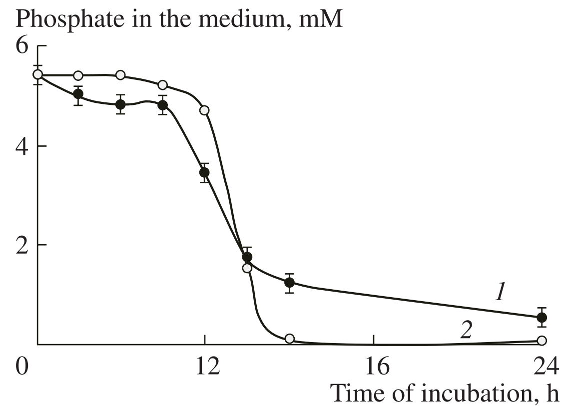

Fig. 2. Decrease of phosphate content in the medium at the incubation of *Brevibacterium casei* cells (16 g of wet biomass l^{-1}) (see Methods): incubation in complete cultivation medium (*1*); incubation in a medium containing the amino acid mixture, 5 g l^{-1}, KH_2PO_4 and $MgSO_4$, 5 mM (*2*).

state [12]. It is not improbable that *B. casei* cells form the same salt in the course of growth or incubation under appropriate conditions. Hence, further experiments were performed to compare the effects of different nitrogen sources on phosphate concentration decrease. The data from Table 1 show that the presence of peptone or yeast extract is sufficient for this process. It proceeds as effectively as at the addition of the amino acid mixture obtained by enzymatic hydrolysis of casein (casamino acids), which contains no vitamins or nucleotides (Table 1, Fig. 2). Moreover, it slightly slows down in the presence of glucose (Fig. 2).

In order to find out which amino acids stimulated the decrease of phosphate concentration, individual amino acids (5 mM) were added to the incubation medium (Table 1). In all these experiments, pH value was adjusted to 6.5 prior to incubation. In the presence of histidine, arginine, and glutamine, the process was nearly as effective as in the presence of the complete amino acid mixture. Figure 3 shows the dependence of phosphate removal after 15 h of incubation with *B. casei* cells on histidine concentration. The process was less intensive in the presence of proline and glutamic acid. With methionine, serine, threonine, lysine, or isoleucine (5–10 mM), phosphate content in the medium did not change throughout the experiment (Table 1).

Arginine, histidine, glutamine, and proline are characterized by the common catabolic pathway, which results in the formation of α-ketoglutarate through glutamate and deamination [14]. Phosphate content in the medium was shown to decrease at joint addition of α-ketoglutarate and $(NH_4)_2SO_4$ but did not change in the control variant with $(NH_4)_2SO_4$ without α-ketoglutarate (Table 1, Fig. 4a). Although the rate of phosphate removal from the medium was higher in the presence of the amino acid mixturethan in the presence of these two components, phosphate content in these two variants after 25 h of incubation was close; this is particularly

Table 1. The effect of medium components on phosphate content decrease at incubation of *B. casei* cells in the absence of growth (16 g of wet biomass l^{-1}) at pH 7.0. Concentrations, g l^{-1}: glucose, 5.0; yeast extract, 3.0; peptone, 5.0; amino acid mixture, 5.0; mM: $(NH_4)_2SO_4$, 5; KH_2PO_4, 5; $MgSO_4$, 5; and amino acids, 5

Components of the incubation medium	Phosphate content in the medium after 15 h of incubation, % of initial value
KH_2PO_4, $MgSO_4$, glucose, yeast extract, peptone	14.8
KH_2PO_4, glucose, yeast extract, peptone	79.6
KH_2PO_4, Mg^{2+}, glucose	96.3
KH_2PO_4, Mg^{2+}	100
KH_2PO_4, Mg^{2+}, yeast extract, peptone	1.9
KH_2PO_4, Mg^{2+}, yeast extract	5.5
KH_2PO_4, Mg^{2+}, peptone	1.9
KH_2PO_4, Mg^{2+}, amino acid mixture (Casaminoacids, Difco)	4.6
KH_2PO_4, Mg^{2+}, L-histidine	6.5
KH_2PO_4, Mg^{2+}, L-arginine	5.9
KH_2PO_4, Mg^{2+}, L-glutamine	14.8
KH_2PO_4, Mg^{2+}, L-glutamic acid	37.1
KH_2PO_4, Mg^{2+}, L-proline	54.8
KH_2PO_4, Mg^{2+}, DL-methionine	97.4
KH_2PO_4, Mg^{2+}, DL-serine	100
KH_2PO_4, Mg^{2+}, DL-threonine	100
KH_2PO_4, Mg^{2+}, L-lysine	96.8
KH_2PO_4, Mg^{2+}, L-isoleucine	91.0
KH_2PO_4, Mg^{2+}, $(NH_4)_2SO_4$	100
KH_2PO_4, Mg^{2+}, $(NH_4)_2SO_4$, α-ketoglutarate	25.4

true for the initial pH value of 7.0 (Fig. 4a). It is probable that the amino groups cleaved from these amino acids in the course of catabolism serve as a source of NH_4^+ ions and are used for $(NH_4)MgPO_4$ formation.

The effect of pH of the medium on phosphate concentration decrease at incubation with *B. casei* cells. It was shown in the course of the work that at lower pH values of the incubation medium the complete removal of phosphate required more time. The effect of pH on phosphate removal was determined by adjusting the initial pH values to 5.5 and 7.0. Under these conditions, phosphate removal from the medium in the presence of either the amino acid mixture or $(NH_4)_2SO_4$ together with α-ketoglutarate was compared (Fig. 4a). At an initial pH of 7.0, the decrease of phosphate concentration in both cases commenced at the very beginning of incubation; at initial pH 5.5, this process started after ~5 h of incubation. Under cell incubation, the pH of the medium shifted to alkaline values (Fig. 4b). At initial pH 5.5, its value became ~7 after 5 h of incubation. During this same period of time, phosphate concentration started to decrease. Thus, the process occurred at pH close to neutral or higher. The prolonged lag period under more acidic initial pH values is explained by the fact that *B. casei* cells alkalize the incubation medium during this period.

It should be noted that addition of serine, threonine, lysine, and isoleucine also increased the medium pH from 5.0–6.0 to 7.0–7.3; however, phosphate concentration in the presence of these amino acids was unchanged even at the initial pH value of 7.5. Consequently, the neutral pH value of the medium is a significant but insufficient factor for the process under study. It is known that phosphate uptake from the liquid phase increases in activated sludge under alkaline conditions [15]. It should be noted that the observed change of pH is a result of bacterial metabolism and does not occur in any of the used variants of incubation media in the absence of cells.

Control experiments demonstrating that decrease in phosphate concentration in the medium is the result of *B. casei* metabolism. The data from Table 2 show that phosphate concentration decreases in the course of incubation with living *B. casei* cells independent of the initial pH value (7 or 8.5). No decrease of phosphate concentration was observed when the medium was incubated without cells or with cells exposed to 20-min boiling. If the cells that had accumulated phosphate were resuspended in distilled water and then pH was brought to 6.0 by adding HCl, no more

Table 2. Decrease of phosphate content in the medium at incubation with the living *B. casei* cells and the cells killed by boiling (16 g of wet biomass l^{-1})

Components of the incubation medium	Initial pH	pH value after 15 h	Phosphate content in the medium after 15 h of incubation, % of initial value
Living *B. casei* cells	7.0/8.5	8.6/8.6	7.2/8.1
Killed *B. casei* cells	7.0/8.5	8.1/8.1	93.7/89.0
Cell-free medium	7.0/8.5	7.0/8.6	96/100

Note: The incubation medium contained amino acid mixture (casamino acids), 5.0 g l^{-1}; KH_2PO_4, 5 mM; and $MgSO_4$, 5 mM. The values corresponding to initial pH values of 7.0 and 8.5 are divided by a slash.

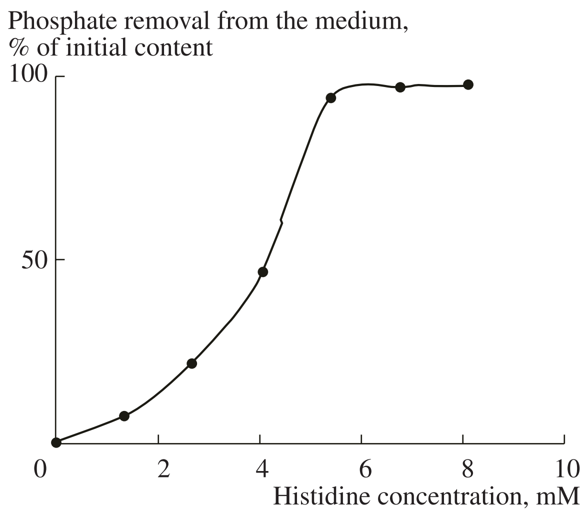

Fig. 3. Dependence of the decrease of phosphate concentration in the medium with *Brevibacterium casei* cells (16 g of wet biomass l^{-1}) on histidine concentration. Concentrations of KH_2PO_4 and $MgSO_4$ are 5 mM.

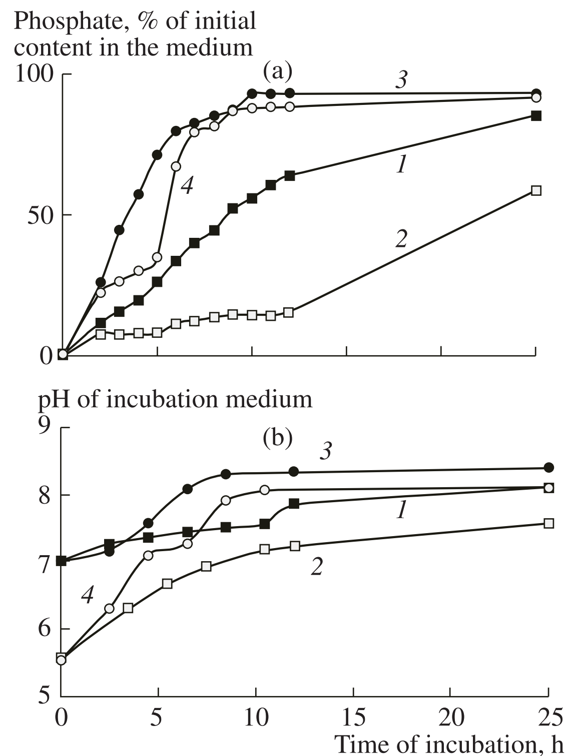

Fig. 4. Dependence of the decrease of phosphate concentration in the medium with 5 mM KH_2PO_4 and $MgSO_4$ in the presence of *Brevibacterium casei* cells (16 g of wet biomass l^{-1}) on time (a) and pH change during phosphate uptake (b). Additional components of the incubation medium and initial pH: 5 mM $(NH_4)_2SO_4$ and α-ketoglutarate, initial pH 7.0 (*1*); 5 mM $(NH_4)_2SO_4$ and α-ketoglutarate, initial pH 5.5 (*2*); amino acid mixture 5 g l^{-1}, initial pH 7.0 (*3*); amino acid mixture 5 g l^{-1}, initial pH 5.5 (*4*).

than 40% phosphate was dissolved. Moreover, the same amount of phosphate was recovered after treatment of such cells with 1 N HCl or $HClO_4$. The remaining 60% of phosphate were bound with the biomass. In the experiments performed previously with *B. antiquum*, NH_4MgPO_4 could be extracted from the biomass only after destruction of the cells by extrusion [12]. It may be suggested that some part of phosphate removed from the medium is adsorbed on the surface of *B. casei* cells, while the other part is localized inside the cells.

In the absence of cells, the NH_4MgPO_4 precipitation until complete removal of phosphate from the supernatant occurred at pH 10 in the medium with 5 mM $(NH_4)_2SO_4$, 5 mM KH_2PO_4, and 5 mM $MgSO_4$. The formed precipitate completely dissolved at pH 6.8. Thus, the process of decrease of phosphate concentration in the presence of *B. casei* living cells differs from chemical precipitation of phosphate.

DISCUSSION

Thus, phosphate removal from the medium as a result of *B. casei* metabolism requires the presence of Mg^{2+}, NH_4^+, and α-ketoglutarate (or amino acids which are catabolized with its formation and release of NH_4^+). It is important to note that phosphate removal as a result of bacterial metabolism occurs at pH values when chemical precipitation of phosphate is still not observed. This is in good agreement with the data on the composition of reserve phosphorous compound NH_4MgPO_4 in a bacterium belonging to the same genus, *B. antiquum* [12].

The data suggest that at least some part of phosphate can be transported into the cells. It should be taken into account that phosphate transport in many bacteria occurs concurrently with the transport of bivalent cations [15–17]. Since glucose, the main energy source for the transport processes under the cultivation conditions used, does not affect the process under study, the question arises how the transport across the membrane could be realized. As regards ammonium uptake by bacterial cells, different bacteria were shown to have both energy-dependent transport systems and channels working independently of both ATP and the electrochemical potential [18, 19]. The interrelation of ammonia absorption systems and glutamate synthase should be taken into account [20] and, consequently, metabolism of the amino acids which stimulate the process under study. Besides, ketoglutarate (both of external origin and produced via amino acid catabolism) may be the energy source for transport. It is known that the ketoglutarate transport into the cells of some bacteria is achieved via facilitated diffusion; further oxidation of this substrate provides the cells with ATP for the formation of the proton electrochemical gradient on the plasma membrane, which can be used for the work of secondary transport systems [19]. Thus, it can be sug-

gested that ketoglutarate, as well as the amino acids forming it in the course of catabolism, are probably an energy source also for the transport of ammonium and magnesium ions and phosphate. Without cell growth, all these three components are stored as a complex salt.

Application of *Brevibacterium* as an experimental model allowed us for the first time to determine the factors necessary for phosphate removal from a medium by bacteria comparable with that for bacteria obtained from the plants for Enhanced Biological Phosphorus Removal (EBPR). This ability makes brevibacteria promising for further research into the mechanism of this process and is of interest for practical application in the development of systems for wastewater purification from phosphate.

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