Magnesium-limited Growth of *Bacillus subtilis*, in Pure and Mixed Cultures, in a Chemostat

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SUMMARY

The influence of Mg^{2+} -limitation on the growth of a typical Gram-positive organism—*Bacillus subtilis*—was investigated and the data compared with that obtained with *Aerobacter aerogenes* grown under similar conditions. The magnesium contents of both organisms varied with growth rate but were very similar at corresponding growth rates. With Mg^{2+} -limited chemostat cultures of each organism, uptake of Mg^{2+} was almost complete at specific growth rates less than $0.7 \times$ maximum. Cellular Mg^{2+} was tightly bound, none being removed by suspension of the organisms at 20° in 0.85% (w/v) NaCl. When Mg^{2+} -limited organisms were suspended in environments containing Mg^{2+} , this ion was rapidly adsorbed; the amount adsorbed varied with both the initial extracellular Mg^{2+} concentration and the composition of the diluent. *B. subtilis* had a greater capacity for Mg^{2+} adsorption than *A. aerogenes* but its affinity for this ion was less. The latter difference correlated with the ability of *A. aerogenes* to outgrow *B. subtilis* rapidly in Mg^{2+} -limited chemostat cultures containing both organisms. The significance of these results is discussed in relation to the reports from other laboratories concerning differences between Gram-positive and Gram-negative bacteria in Mg^{2+} content, uptake of Mg^{2+} and ability to grow in media of low Mg^{2+} content.

INTRODUCTION

It has been reported by Webb (1949) and by Rouf (1964) that the magnesium contents of Gram-positive bacilli (*Bacillus subtilis*, *B. cereus*) are much greater than those of Gram-negative organisms (*Aerobacter aerogenes*, *Escherichia coli*). Furthermore, Webb (1966) concluded that, unlike the Gram-negative organisms, bacilli (*B. megaterium* and *B. mesentericus*, as well as *B. subtilis*) would not grow in simple salts media containing less than $1\text{--}2\ \mu\text{g}$. Mg^{2+} /ml. Recently the magnesium content of *A. aerogenes* has been found to be a function of the growth rate and to be related to the bacterial RNA content (Tempest & Strange, 1966; Tempest, Dicks & Hunter, 1966; Dicks & Tempest, 1966). Since the RNA contents of *A. aerogenes* and *B. megaterium* do not differ significantly when grown under similar conditions (Herbert, 1958), the apparent gross difference in magnesium requirement, and content, is of considerable interest and possible importance. It follows from the observations of Tempest & Strange (1966), and of Dicks & Tempest (1966), that a meaningful comparison of the magnesium contents of Gram-positive and Gram-negative organisms can be made only if they are grown under nearly identical conditions—particularly with regard to temperature and growth rate. Furthermore, because a variable amount of magnesium may be loosely bound to the walls of bacteria grown in media con-

taining an excess of this cation, it is preferable to compare organisms that have been grown in a Mg^{2+} -limited environment since these contain no detectable adsorbed magnesium (Tempest & Strange, 1966). Alternatively, adsorbed magnesium can be removed by washing organisms in 0.85 % (w/v) NaCl (Strange & Shon, 1964). This paper compares the magnesium contents of Mg^{2+} -limited *B. subtilis* and *A. aerogenes* grown at corresponding rates, at 35°; no significant differences were found. However, differences were observed in the abilities of the organisms to adsorb Mg^{2+} when suspended in various environments, and these findings correlated with the behaviour of the organisms in Mg^{2+} -limited mixed cultures. It is concluded that the saturation constants (K_s values) for Mg^{2+} of *B. subtilis* and *A. aerogenes* organisms differ substantially. A preliminary report on our findings has been published (Tempest, Dicks & Meers, 1967).

METHODS

Organisms: *Bacillus subtilis* var. *niger* (obtained from Fort Detrick, Md., U.S.A., originally called *Bacillus globigii*; strain ATCC9372 is considered to be from the same source) was maintained by monthly subculture on tryptic meat digest agar slopes containing 0.2 % (w/v) glucose; *B. megaterium* KM (obtained from Dr J. F. Wilkinson) was maintained on tryptic meat digest agar containing 0.2 (w/v) mannitol; *Aerobacter aerogenes* (NCTC418) was maintained on tryptic meat digest agar slopes; *Torula utilis* (NCYC321) was maintained on yeast extract, peptone, glucose agar slopes.

Growth conditions. Continuous cultures of organisms were maintained in 0.5 l. chemostats of the type described by Herbert, Phipps & Tempest (1965). The temperature was controlled at 35°; the pH value was regulated at 6.5 and 7.0, respectively, for the *Aerobacter aerogenes* and *Bacillus subtilis* cultures. The growth medium, which was limited with respect to its Mg^{2+} content, had the following composition: Na_2HPO_4 , $5.0 \times 10^{-3} M$; $NH_4H_2PO_4$, $4.5 \times 10^{-2} M$; $(NH_4)_2SO_4$, $2.5 \times 10^{-2} M$; K_2SO_4 , $3.0 \times 10^{-3} M$; citric acid, $1.0 \times 10^{-3} M$; $MgCl_2$, $2.5 \times 10^{-4} M$; $CaCl_2$ and $FeCl_3$, each $1.0 \times 10^{-4} M$; $MnCl_2$ and $ZnCl_2$, each $2.5 \times 10^{-5} M$; $CuCl_2$, $CoCl_2$ and Na_2MoO_4 each $5 \times 10^{-6} M$; a sterile glucose solution, in water, was added to a final concentration of 30 g. glucose/l. after sterilization (121°, 30 min.) of the bulk medium. Batch cultures were grown in a similar medium, differing only in the Mg^{2+} concentration.

Analytical procedures. Culture bacterial concentrations (equiv. mg. dried bacteria/ml. culture) and the macromolecular compositions of organisms were determined by methods described previously (Tempest, Hunter & Sykes, 1965). Bacterial magnesium contents were determined by atomic absorption (using an EEL Model 140 Spectrophotometer) on $HClO_4$ extracts, prepared as described by Tempest & Strange (1966).

Mixed culture experiments. Cultures of *Bacillus subtilis*, *Aerobacter aerogenes* and *Torula utilis* were grown in 0.25 l. chemostats (designed by Dr D. Herbert) without pH control. The growth medium was as follows: Na_2HPO_4 , $2.5 \times 10^{-2} M$; $NH_4H_2PO_4$, $3.75 \times 10^{-2} M$; K_2SO_4 , $2.0 \times 10^{-3} M$; citric acid, $5.0 \times 10^{-4} M$; $MgCl_2$, $3.75 \times 10^{-5} M$; $CaCl_2$ and $FeCl_3$, each $2.5 \times 10^{-5} M$; trace amounts of Mn^{2+} , Cu^{2+} , Co^{2+} , Zn^{2+} and Na_2MoO_4 ; glucose to a final concentration of 10 g./l. The pH value was adjusted to 6.4 before sterilization; after sterilization (autoclaving at 121° for 30 min.) the pH value was 6.5–6.6. Continuous cultures of each organism were established, in separate chemostats, at a dilution rate of 0.3 hr^{-1} and with the temperature regulated at 33°. The steady-state pH values were found to be 6.4, 6.3 and 5.5, respectively, for

cultures of *B. subtilis*, *T. utilis* and *A. aerogenes*. Mixed cultures were produced by pipetting small volumes (1.0 ml. unless stated otherwise) from one chemostat culture into another chemostat culture. The flow rates were maintained at 0.3 hr^{-1} and samples taken at convenient times thereafter for determination of microbial content and distribution. Total numbers of bacteria and yeast were determined by direct observation and counting, using a Thoma chamber of 0.02 mm depth, and phase-contrast microscopy. The proportions of *A. aerogenes* organisms in mixed cultures containing *B. subtilis* were assessed by microscopic examination of Gram-stained preparations.

RESULTS

*Influence of dilution rate on the yield value and macromolecular composition of Mg^{2+} -limited *Bacillus subtilis**

With Mg^{2+} -limited chemostat cultures of *Aerobacter aerogenes*, the yield (g. bacteria formed/g. Mg^{2+} consumed) varied with the dilution rate (Tempest, Hunter & Sykes, 1965). This indicated a change in cellular Mg^{2+} content with growth rate which

Fig. 1

Fig. 2

Fig. 1. Steady-state concentrations of *Bacillus subtilis* and extracellular Mg^{2+} as functions of dilution rate (35° ; pH 7.0) in a Mg^{2+} -limited chemostat culture. Values at each dilution rate were determined over a 2- to 3-day period, after initial equilibration of the culture for a period of 2 days. ●, mg. dry wt bacteria/ml. culture; ○, $\mu\text{g.}$ extracellular Mg^{2+} /ml. culture.

Fig. 2. The magnesium content of Mg^{2+} -limited *Bacillus subtilis* and *Aerobacter aerogenes* as a function of growth rate. Mean magnesium content (% of bacterial dry weight) of *B. subtilis* (solid line) and the corresponding magnesium content of Mg^{2+} -limited *A. aerogenes* (broken line).

paralleled the change in RNA content. Figure 1 shows the effect of dilution rate on the steady-state concentration of *Bacillus subtilis* organisms in a Mg^{2+} -limited chemostat culture. Like the Mg^{2+} -limited *A. aerogenes* culture, bacterial concentration varied with growth rate; an increase in dilution rate caused a decrease in yield. Over 90 % of the Mg^{2+} added to the culture was present in the organisms at all dilution rates below 0.5 hr^{-1} (Fig. 1); thus, the magnesium content of *B. subtilis* varied with the growth rate (Fig. 2). Also plotted in Fig. 2 are data on the variation in magnesium content of *A. aerogenes* (Tempest & Strange, 1966); the two sets of data are strikingly similar.

The macromolecular composition of Mg^{2+} -limited *Bacillus subtilis* at various growth rates is shown in Table 1. There was a progressive increase in cellular RNA

and carbohydrate contents with increasing growth rates, whereas the DNA and protein contents decreased or varied irregularly. An interrelationship between RNA and magnesium was suggested by the relatively constant RNA/Mg²⁺ ratio (Table 1); this was quantitatively similar to that reported with *Aerobacter aerogenes* (Tempest *et al.* 1966) and with *Pseudomonas fluorescens* (Sykes & Tempest, 1965).

Binding of Mg²⁺ to Bacillus subtilis

It was reported by Strange & Shon (1964) that *Aerobacter aerogenes* organisms which had been separated from cultures containing Mg²⁺ in excess of the growth requirement, and which had been washed in distilled water, had an amount of Mg²⁺

Table 1. *Macromolecular composition of Mg²⁺-limited Bacillus subtilis grown at different dilution rates*

All the data in this table are average values, obtained from at least two samples (collected and processed on different days) grown at each rate.

Diln rate (hr ⁻¹)	Dry wt bacteria (mg./ml. culture)	g./100 g. dry bacteria				Molar ratio RNA/Mg ²⁺
		Protein	Carbohydrate	DNA	RNA	
0.10	4.35	62.1	12.5	1.9	9.4	4.6
0.21	3.40	53.1	12.5	1.9	12.2	4.6
0.41	2.56	56.7	13.4	1.8	13.9	4.2
0.56	0.93	55.3	15.0	1.8	15.5	4.5

Table 2. *Comparison of magnesium contents of water- and saline-washed Bacillus subtilis organisms, grown at various dilution rates (35°, pH 7.1)*

Samples of bacteria, separated from chemostat cultures, were washed with distilled water or 0.85 % (w/v) NaCl and extracted with N-HClO₄ for Mg²⁺ assay (see Methods).

Diln rate (hr ⁻¹)	g. Mg ²⁺ /100 g. dry bacteria		Ratio water-washed: NaCl-washed
	Water-washed	NaCl-washed	
0.10	0.140	0.140	1.00
0.21	0.169	0.169	1.00
0.21	0.184	0.188	0.98
0.40	0.238	0.233	1.02
0.41	0.212	0.216	0.98
0.57	0.247	0.237	1.04
0.57	0.257	0.252	1.02

bound loosely at their surface; this adsorbed Mg²⁺ could be removed by suspension of the organisms in 0.85 % (w/v) NaCl solution. Mg²⁺-limited *A. aerogenes* organisms were devoid of surface-bound Mg²⁺ (Tempest & Strange, 1966); similarly, no significant difference was found between the magnesium contents of water- and saline-washed Mg²⁺-limited *Bacillus subtilis* organisms (Table 2). However, both Mg²⁺-limited *A. aerogenes* and *B. subtilis* organisms would take up Mg²⁺ when exposed to dilute solutions of MgCl₂ for brief periods of time (2 min. or less). Over 80 % of this Mg²⁺ could be recovered from the organisms by subsequent treatment with 0.85 % saline, suggesting that it was largely adsorbed. *B. subtilis* had a greater capacity for Mg²⁺ adsorption than *A. aerogenes*. Thus, with organisms grown at a dilution rate of 0.2 hr⁻¹ and washed once, in 2.5 mM-MgCl₂ solution, *B. subtilis* adsorbed 5.6 µg.

Mg²⁺/mg. equiv. dry bacteria whereas *A. aerogenes* adsorbed 3.6 µg. Mg²⁺/mg. equiv. dry bacteria (3.05×10^{-15} g. Mg²⁺/organism and 1.29×10^{-15} g. Mg²⁺/organism, respectively).

The greater capacity for Mg²⁺ adsorption of *Bacillus subtilis*, compared with *Aerobacter aerogenes*, indicated a greater number of cation binding sites per organism (about 8×10^7 , compared with about 3×10^7); it provided no information on strength of binding (Mg²⁺-affinity) or ion specificity. This was sought by studying the adsorption of Mg²⁺ in the presence of a competing ion. Figure 4 shows the results of an experiment in which Mg²⁺-limited *A. aerogenes* and *B. subtilis* organisms were suspended (for 2 min.) in saline containing graded concentrations of Mg²⁺, then washed with water and analysed for Mg²⁺. Although *B. subtilis* had the greater Mg²⁺ binding capacity at high Mg²⁺ concentrations, at low Mg²⁺ concentrations (in the presence of saline) these organisms adsorbed less Mg²⁺ than *A. aerogenes* organisms (Fig. 3); this indicated a difference in affinity for this cation. Similarly, the presence of growth-medium constituents, or of EDTA, markedly influenced the uptake of Mg²⁺ by *B. subtilis*, whereas they had substantially less effect on Mg²⁺ adsorption by *A. aerogenes* organisms (Fig. 3).

Growth of Bacillus subtilis in Mg²⁺-limited mixed cultures

Webb (1966) concluded that Gram-positive bacilli, unlike Gram-negative organisms, were unable to utilize Mg²⁺ when the external concentration of this cation was low. Whereas it is clear (Fig. 1) that *Bacillus subtilis* would grow in a chemostat under conditions where the extracellular Mg²⁺ concentration was 10%, or less, of the minimum value reported to support the growth of other bacilli in a batch culture (see Webb (1966), his table 1), the differences in Mg²⁺ adsorption observed between *B. subtilis* and *Aerobacter aerogenes* accorded, qualitatively, with Webb's conclusion. However, the ability of organisms to assimilate Mg²⁺ may not be related directly to their surface adsorption properties. Therefore mixed population experiments were carried out, in Mg²⁺-limited chemostat cultures, to assess the relative efficiencies of Mg²⁺ utilization (i.e. to compare the saturation constants for Mg²⁺ of the various organisms).

Mixed culture experiments were carried out as described in the Methods section and the results of these are contained in Figs 4 and 5. In each of three experiments in which Mg²⁺-limited *Aerobacter aerogenes* organisms were added to growing Mg²⁺-limited *Bacillus subtilis* cultures (initial *B. subtilis* concentration, 4.5×10^8 organisms/ml.; initial *A. aerogenes* concentration, less than 2×10^6 organisms/ml.), the Gram-negative organism rapidly outgrew the bacillus; after 24 hr the *A. aerogenes* concentration had increased to $6.7\text{--}8.5 \times 10^8$ organisms/ml. and the *B. subtilis* concentration had diminished to less than 6×10^6 organisms/ml. (Fig. 4).

Previous observations on the growth of a contaminant bacillus (probably *Bacillus subtilis*) in K⁺- and Mg²⁺-limited chemostat cultures of *Torula utilis* suggested that bacilli may not be able to outgrow *T. utilis* in environments where growth rate is limited by the availability of Mg²⁺. Therefore experiments were carried out to study the growth of *B. subtilis* in mixed Mg²⁺-limited cultures containing *T. utilis*. When 10 ml. of a Mg²⁺-limited *B. subtilis* culture (4.5×10^8 organisms/ml.) were added to 90 ml. of a similarly limited culture of *T. utilis*, containing 6×10^7 organisms/ml., the bacterial concentration in the mixed chemostat culture progressively diminished over

24 hr from 5×10^7 organisms/ml. to 1×10^6 organisms/ml. (Fig. 5). However, contrary to our expectations when small amounts of *T. utilis* (equivalent, after mixing, to about 6×10^6 organisms/ml.) were added to a Mg^{2+} -limited *B. subtilis* culture (4.5×10^8 bacteria/ml.) the yeast failed to grow. Even *T. utilis* organisms at an initial concentration of 2.8×10^7 organisms/ml. (obtained by adding 50 ml. of yeast culture to 50 ml. of the bacterial culture) failed to outgrow the bacillus (Fig. 5). This suggested the presence, in the Mg^{2+} -limited *B. subtilis* culture, of either a mycostatic substance or a bacterial product which enhanced Mg^{2+} uptake by the bacillus. Since *T. utilis* would grow, without lag and at an undiminished rate, in a bacteria-free Mg^{2+} -limited *B. subtilis* culture fluid (supplemented with Mg^{2+}) it seemed unlikely that a mycostatic substance was synthesized by *B. subtilis*. Therefore the possible involvement of extracellular bacterial products in Mg^{2+} uptake by the bacillus was investigated.

Fig. 3

Fig. 3. Adsorption of Mg^{2+} by *Bacillus subtilis* and *Aerobacter aerogenes* organisms. The organisms were grown at a dilution rate of 0.2 hr^{-1} (35° ; pH 7.0 and 6.5, respectively), centrifuged and resuspended in various solutions containing 0.05–0.50 mM- $MgCl_2$. Suspensions were centrifuged (within 2 min. of mixing), the supernatant solutions discarded and the bacteria washed once with distilled water. The bacterial magnesium was extracted with 1N- $HClO_4$ (Tempest & Strange, 1966) and determined by atomic adsorption spectrophotometry. Adsorbed Mg^{2+} was calculated as that in excess of magnesium present in untreated organisms. ■, □, Mg^{2+} adsorbed from solutions containing 0.85% (w/v) NaCl; ●, ○, Mg^{2+} adsorbed from solutions containing 0.85% (w/v) NaCl plus 1.5 mM-EDTA; ▲, △, Mg^{2+} adsorbed from the growth medium salts solution (each containing the graded concentrations of $MgCl_2$ as detailed above). The closed symbols refer to adsorption by *B. subtilis*, and the open symbols to adsorption by *A. aerogenes*. The broken line indicates Mg^{2+} adsorption by each organism in the absence of competing ions.

Webb (1966) had shown that addition of amino-acid mixtures to a simple basal medium increased Mg^{2+} uptake by *Bacillus megaterium* and decreased the value of the minimum Mg^{2+} concentration necessary for initiation of growth. When the experiment detailed in Fig. 5 was repeated using a medium modified by the addition of 1% (w/v) Casamino Acids (Difco Laboratories, Detroit, U.S.A.) plus tryptophan

(0.02 %, w/v), the bacillus now outgrew the yeast. In subsequent experiments the initial *B. subtilis* concentration was decreased to 2×10^6 organisms/ml.; the bacilli still outgrew the yeast, though requiring 48 hr to replace the yeast completely (cf. Fig. 4).

Fig. 4

Fig. 5

Fig. 4. Growth of *Bacillus subtilis* (●) and *Aerobacter aerogenes* (○) in a Mg^{2+} -limited simple salts medium in a chemostat. The initial concentration of *B. subtilis* was 4.5×10^8 organisms/ml. and of *A. aerogenes*, 2×10^6 organisms/ml. (99.5 % and 0.5 %, respectively, of the initial population). The dilution rate was 0.3 hr^{-1} and the temperature 33° ; the initial pH value was 6.4 but decreased to 5.5 with the progressive increase in the concentration of *A. aerogenes* organisms. Identical results were obtained when 1 % (w/v) Casamino Acids was present in the medium.

Fig. 5. Growth of *Bacillus subtilis* and *Torula utilis* in a Mg^{2+} -limited simple salts medium in a chemostat. In the experiment represented by the solid lines, the initial concentration of *B. subtilis* (●) was 4.5×10^7 organisms/ml. and of *T. utilis* (○) 5.4×10^7 organisms/ml., i.e. 45.5 % and 54.5 % of the initial population, respectively. Also recorded (broken lines) are the results of an experiment in which the initial concentration of *B. subtilis* was increased to 2.2×10^8 organisms/ml. (▲) and that of *T. utilis* reduced to 2.8×10^7 organisms/ml. (△), i.e. 89.5 % and 10.5 % of the initial population, respectively. In both experiments the dilution rate was 0.3 hr^{-1} and the temperature 33° . The pH value (6.4) did not vary during the course of each experiment.

DISCUSSION

Since the Mg^{2+} content of bacteria may vary with growth rate—which, in a batch culture, would depend on factors such as medium composition and temperature—then a meaningful comparison of the Mg^{2+} contents of various species would be possible only if the growth conditions were rigidly controlled and standardized. Clearly the differences in Mg^{2+} content between Gram-positive and Gram-negative organisms, reported by Webb (1949) and Rouf (1964), are of no significance, since widely different conditions were used in growing the various organisms.

The gross differences, reported by Webb (1966), between the abilities of Gram-positive bacilli and Gram-negative bacteria to concentrate Mg^{2+} (presumably intracellularly) from simple chemically defined media are not confirmed here. *Bacillus subtilis* var. *niger* grew at steady-state Mg^{2+} concentrations far below the minimum values reported to be necessary for the growth of other bacilli (*B. subtilis* F 3, *B. subtilis* MARBURG, *B. mesentericus*, *B. megaterium*). Whereas it is possible that products of

metabolism, excreted into the medium, may have stimulated Mg^{2+} uptake by Mg^{2+} -limited *B. subtilis* organisms, small numbers (about 5×10^6 organisms/ml., final concentration) of continuously grown Mg^{2+} -limited *B. subtilis* would readily grow when inoculated into a simple salts medium containing $0.3 \mu g.$ Mg^{2+} /ml.; after 24 hr, more than 95 % of the total Mg^{2+} could be recovered from the organisms whose concentration had increased to about 3×10^8 bacteria/ml.

It can be argued that, among the bacilli, *Bacillus subtilis* var. *niger* may be unique in requiring much lower concentrations of Mg^{2+} for growth. However, *B. megaterium* also would grow in a Mg^{2+} -limited chemostat culture and take up almost all the Mg^{2+} from the environment at dilution rates less than 0.3 hr^{-1} . We conclude, therefore, that the observations of Webb (1966) must reflect physiological factors other than the relative ability of bacilli and Gram-negative bacteria to take up from their environment, and utilize, low concentrations of Mg^{2+} . In this connexion the rapid death-rate of vegetative bacilli in aqueous suspension (Mr R. E. Strange, personal communication) and the known effects of Mg^{2+} on the survival of micro-organisms in aqueous environments (Postgate & Hunter, 1962, 1964; Tempest & Strange, 1966) may be factors contributing to Webb's findings.

Despite the quantitative differences between the results reported here and those of Webb (1966), the broad conclusions are similar. In the presence of competing ions *Bacillus subtilis* var. *niger* has a demonstrably lower affinity for Mg^{2+} than *Aerobacter aerogenes* (Fig. 3) and at low extracellular Mg^{2+} concentrations (imposed, in the chemostat, by maintaining the dilution rate at a value substantially less than the critical rate— D_c) the bacillus grows more slowly than the Gram-negative organism (Fig. 4). Furthermore, the rapid rate at which *A. aerogenes* outgrew *B. subtilis* in the Mg^{2+} -limited culture suggests that the respective saturation constants for Mg^{2+} are substantially different. In contrast, the rates of take-over of *B. subtilis* and *Torula utilis*, respectively, when inoculated together into a Mg^{2+} -limited simple salts medium with and without amino acids, indicates that the Mg^{2+} saturation constants of these organisms are less different. If, in our experiments, amino acids influenced Mg^{2+} uptake as Webb (1966) indicated, then it is clear that they had a greater effect on *B. subtilis* than on *T. utilis*, since they reversed the take-over patterns in mixed culture experiments. But addition of amino acids did not influence the rate at which *A. aerogenes* outgrew *B. subtilis* in a Mg^{2+} -limited mixed culture (Fig. 4), suggesting that these compounds have an equivalent effect on Mg^{2+} uptake by *A. aerogenes* and *B. subtilis*.

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