MOTILITY IN *BACILLUS SUBTILIS* DRIVEN BY AN ARTIFICIAL PROTONMOTIVE FORCE

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1. Introduction

Peritrichously flagellated bacteria swim by rotating their flagella as a bundle [1,2]. A motor-like model for the flagellar rotation was proposed by Berg [3], in which a flagellum is nearly a rigid propeller and is attached to a motor, namely the basal structure of flagella. When bacterial cells were tethered on microscope slides by means of antibodies specific for flagella, the cell bodies did rotate [4]. This finding gave a strong support to the above model. Then, what is the energy source for the rotation of the motor?

Larsen et al. [5] reported that the intermediate form in oxydative phosphorylation is the energy source for the motility in *Escherichia coli*, but not ATP directly. The recent studies on the mechanism of oxydative phosphorylation support the chemiosmotic hypothesis of Mitchell [6,7], in which the true nature of the intermediate form was supposed to be the protonmotive force (electrochemical potential of proton). A strong and direct support was obtained from the finding that an artificial protonmotive force drove ATP synthesis in mitochondria and also in bacterial cells [8–10]. According to this view, the protonmotive force is expected to be the energy source for the bacterial motility [8,11].

In this paper, we present the results showing that an artificial protonmotive force, which was created by diffusion potential of K^+ brought about by a K^+ ionophore (valinomycin) or by pH drop, or both, induced the transient motility in the starved and nonmotile cells of *B. subtilis*.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide

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2. Materials and methods

2.1. *Cell growth and starvation*

Cells of *Bacillus subtilis* BC26 (*phe*12, *arg*A, *ery*) were grown at 35°C in tryptone broth (1% bactotryptone–0.5% NaCl) to a mid-log phase. Cells (1–2 ml) were collected on Millipore membrane filters (HA, 0.45 μm) by filtration and washed at room temperature with 5 ml starvation buffer consisting of 10 mM sodium arsenate buffer (pH 7.5), 0.1 mM $CaCl_2$ and 0.1 mM EDTA. After resuspending in 5 ml same buffer, cells were starved by incubating at 35°C.

2.2. *Motility measurement*

A drop of cell suspension was placed on a microscope slide and the cells swimming at 30°C were recorded by a video tape recording system as described previously [12]. The motile fraction as expressed by the percentage of the motile cells among the total at a given time was measured as follows; some cells in a still TV picture were marked and then, the video tape was played on for several seconds to distinguish whether the marked cells were motile or not. This procedure was repeated 10–15 times to count a total number of cells more than 100. In this paper, motile cells were defined as those which showed translational swimming.

2.3. *ATP assay*

ATP content of the cells was measured by the method of Cole et al. [13].

3. Results and discussion

3.1. *ATP content and motility of* B. subtilis *cells*

The cells of *B. subtilis* were incubated in the starvation buffer without any supplement of an energy source. As shown in fig.1, the ATP level of the cell quickly dropped, accompanied by a gradual decrease in the motile fraction. After 30 min incubation, the ATP level became below the detectable limit (0.1 nmol/mg dry wt) and the cells became non-motile. The addition of an energy source, L-glutamate alone at this point caused an increase in the motile fraction. The ATP level, on the other hand, did not show any detectable increase. Thus, ATP is ruled out to be a direct energy source for motility in *B. subtilis*. It is noteworthy that the starvation of cells for 30 min was enough to deplete all of the endogenous energy source, because the addition of 50 mM phosphate did not cause any increase in the ATP level. However, the addition of phosphate together with L-glutamate resulted in a significant increase in the ATP level (fig.1b).

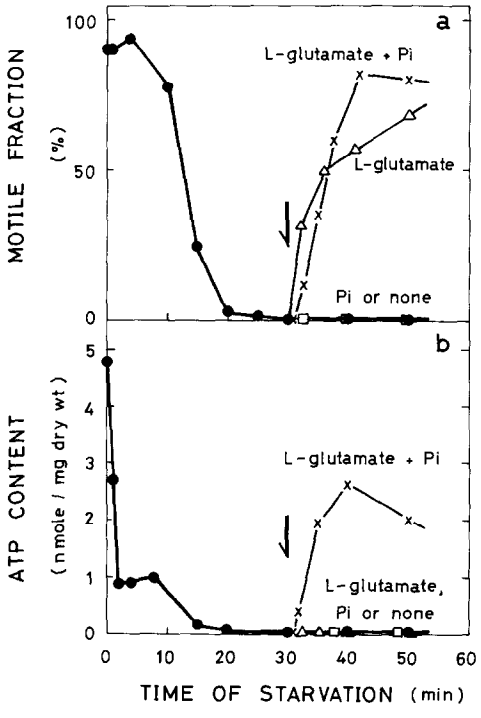

Fig.1. Effect of starvation and re-addition of an energy source on motility and ATP content of *B. subtilis* cells. Starvation was carried out as described in Materials and methods. After 30 min starvation, 10 mM of L-glutamate and/or 50 mM sodium phosphate buffer (pH 7.5) was added as indicated by arrows. (a) Motile fraction. (b) ATP content.

3.2. *Motility induced by an artificial protonmotive force*

The starved and non-motile cells of *B. subtilis* in the starvation buffer (10 mM sodium arsenate (pH 7.5)–0.1 mM $CaCl_2$–0.1 mM EDTA) were exposed to 5 μM valinomycin instantaneously. As shown in fig.2, motile cells quickly appeared and the motile fraction reached the maximum level after 1 min. More than 30% of the cells showed translational swimming. Then, the motile fraction quickly decreased, and most of the cells returned to non-motile state after 3 min. The simultaneous addition of HCl with valinomycin in order to change the pH of the medium from 7.5–6.0 caused an increase in the maximum level of the motile fraction to over 50%. However, the appearance of the motile cells was

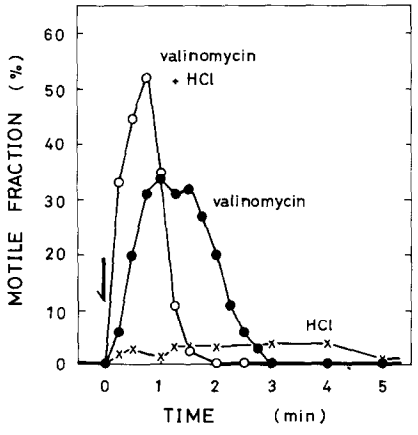

Fig.2. Time course of the transient motility in *B. subtilis* cells induced by an artificial protonmotive force. One ml of the starved and non-motile cells in the starvation buffer (pH 7.5) was mixed with 5 μM of valinomycin and/or HCl to drop the pH to 6.0. A drop of bacterial suspension was placed on a microscope slide and the motile cells at 30°C were counted.

accelerated and the time course of the increase and decrease in the motile fraction was shortened. The addition of HCl alone under the condition produced only a small fraction of the motile cells, and the swimming continued for about 5 min.

Since bacterial cells contain more than 200 mM of K^+ [9,14] and intracellular pH is about 7.5 [10,15], the results presented above are well explained by that the transient motility was induced by an artificial protonmotive force, which was created either by the diffusion potential of K^+ brought about by valinomycin (interior negative) or ΔpH due to pH drop (interior alkaline), or both. A low yield of the motile fraction by the pH drop alone may indicate that the ΔpH produced under the condition was not enough to support the translational swimming.

3.3. *Some properties of artificial motility*

Table 1 shows the effect of various compounds on the transient motility induced by an artificial protonmotive force. With increasing concentrations of KCl in the medium, the maximum levels of the motile fraction were lowered, and at 10 mM, the appearance of motile cells was almost inhibited. This inhibitory effect was K^+ specific, because the addition of 10 mM NaCl had no effect. Thus, the magnitude of the diffusion potential of K^+ strongly affected the yield of the motile cells.

CCP (1 μM) and nigericin (0.1 μM), which render bacterial membrane highly permeable to protons [16,17], caused complete inhibition of the transient motility. The result indicates that the protonmotive force is essential for the bacterial motility. The addition of DCCD, which is an inhibitor of ATP synthetase in bacteria [18], did not show any effect on the motility. This result confirms that ATP is not necessary for the motility.

Table 1
Effect of various compounds on the maximum level of the motile fraction

Additions		Motile fraction (%)
Control		50
+ KCl,	1 mM	22
	3 mM	13
	10 mM	5
+ NaCl	10 mM	53
+ CCCP	1 μM	0
+ Nigericin	0.1 μM	0
+ DCCD	100 μM	45

The transient motility was induced by the addition of valinomycin (5 μM) and HCl to drop the pH to 6.0. Chemicals were added 1 min before induction of motility

The simultaneous addition of HCl with valinomycin caused an increase not only in the motile fraction but also in the average speed of swimming. At 30°C, the average speed of valinomycin-driven swimming was less than 10 μm/s, while that of un-starved cells was 27 μm/s. The pH drop to 6.0 with the simultaneous addition of valinomycin caused the acceleration of the speed to about 18 μm/s. Thus, with the increasing magnitude of the protonmotive force, the swimming speed was increased. However, these results do not simply mean that the rotation of a flagellum requires a large protonmotive force. *Bacillus subtilis* has peritrichous flagella and a co-ordinated flagellar rotation as a bundle is necessary for the translational swimming. Therefore, a large protonmotive force might be required for the co-ordinated flagellar rotation. In fact, the addition of HCl alone induced only a small fraction of the translationally swimming cells but transiently induced a rotational motion of the cell bodies in many cells. This result suggests that under the condition, flagella can rotate but can not form a co-ordinated bundle. The use of the tethered cells may clarify this point.

In a recent review article [11], Skulachev cited the work of his laboratory and said that a rapid pH change in the starved cells of *Rhodospirillum rubrum* caused a transient motility. However, the detailed results have not yet been reported.

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