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Motility and chemotaxis in *Bacillus sphaericus*

Dependence upon stage of growth

J. Andreev, P.A. Dibrov**, S. Braun*

Department of Biological Chemistry, A. Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

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Abstract

Chemotaxis and motility of *Bacillus sphaericus* 2362 were monitored as a function of the batch culture age. It was found that both functions changed independently during growth of the culture. Motility was low until the late logarithmic stage ensued, whereafter it increased sharply. The ability of cells to respond to chemo-effectors peaked at the mid-logarithmic phase. A major methyl-accepting chemotaxis protein (P53, M_r = 53 kDa) was identified. The extent of label incorporation in this protein from L-[*methyl*-^{3}H]methionine was maximal in mid- and late-logarithmic phases of the growth. Cells in stationary cultures incorporated very low amounts of the label. At any stage, the labeling was maximal in starved cells; it was almost abolished in cells pre-incubated with amino acids. Although extents of P53 labeling in mid- and late logarithmic cells were similar, late logarithmic cells demonstrated a considerably impaired chemotaxis. Supermotile sporulating cells were practically insensitive to environmental stimuli. The difference in development of sensory and locomotive functions may be interpreted as an adaptive response. A well developed sensory apparatus would allow vegetative cells to adapt efficiently to fluctuating attractant gradients. Insensitive sporulating cells would tend to disperse randomly from the nutrient-exhausted area. Thus, spore formation would occur in larger volume of the habitat, increasing the chance of microbial population to survive.

Key words: Chemotaxis; Motility; Methyl-accepting chemotaxis protein; Regulation; Culture age; *Bacillus sphaericus*

1. Introduction

Bacillus sphaericus is a Gram-positive spore-forming bacterium that produces a potent mosquito larvicide at the onset of sporulation. This organism does not utilize carbohydrates for the lack of key enzymes of the Embden–Myerhof–Parnas, the Entner–Doudoroff, and hexose monophosphate pathways [1]. Amino acids are the best source of both nitrogen and carbon for this bacterium [2]. The simplified metabolism, as well as the industrial interest, makes *B. sphaericus* an attractive model for studying chemotaxis regulation during the life span of a sporogenic culture.

Regulation of chemotaxis and motility during the life cycle of spore-forming bacteria has not been thoroughly investigated and very few observations have been made. In various *Bacilli*, sporulating cells are becoming hypermotile [3]. This upsurge in motility of *B. subtilis* has been related to the σ^D-dependent transcription of the flagellin gene [4]. It has been concluded from the DNA sequence analysis, that in *B. subtilis* as well as in *E. coli* and *Salmonella typhimurium*, chemotactic and flagellar genes are organized in a regulon containing promoters controlled by alternative sigma factors [5,6–9]. The *sigD* gene is clustered in one regulon with motility, flagellin and chemotaxis genes [10]. The transcription from *sigD*-controlled sequences reaches its peak during the transition from logarithmic to stationary phase [7,11]. Furthermore, *B. subtilis* mutants, possessing either a disrupted *sigD* gene or a low level of σ^D protein, have an impaired expression of methyl-accepting chemotaxis proteins (MCPs) and, concurrently, a low chemotactic activity [10]. It would be reasonable to expect that in consistence with these data, the functions of motility and chemotaxis should be expressed synchronously.

In the following report, motility and chemotaxis in *B. sphaericus* 2362 were monitored at different growth stages. The methyl-accepting chemotaxis protein (P53) of this organism was identified, and its in vivo methylation during the life cycle was determined. Data presented in this study suggest a complex interplay between the motility and chemotaxis in *B. sphaericus*. In contrast to the expected simultaneous development of sensory and locomotive functions, these two constituents of chemotactic response make different contributions to the integral behavioral response of population at various stages of bacterial life cycle.

2. Materials and methods

B. sphaericus 2362 was generously supplied by Dr. H. de Barjac (Pasteur Institute, Paris). Stock cultures of the organism were maintained on Difco nutrient agar slants at 4°C for one month. For chemo-

* Corresponding author. Fax: (972) (2) 519 597.

** *Present address:* Division of Microbial and Molecular Ecology, Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel.

Abbreviations: EDTA, ethylenediamine tetraacetic acid; MCP, methyl-accepting chemotaxis protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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taxis experiments, bacteria were inoculated into NYSM [12] medium (50 ml) supplemented with glycerol (2.5 g/l). The culture in Erlenmeyer flask (250 ml) was incubated at 30°C in a New Brunswick rotatory shaker operated at 240 rpm until the desired density of the population was reached. Growth was measured as the absorbance of suspension at 650 nm (A_{650}).

Capillary assays were carried out as described [13] with minor modifications. The fresh culture (20 ml) was centrifuged for 7 min at 10,000 × *g*, washed twice with and resuspended in an appropriate volume of the chemotaxis buffer (potassium phosphate 10 mM, EDTA 0.1 mM, glycerol 0.5%, sodium lactate 5 mM, $CaCl_2$ 0.14 mM, $(NH_4)_2SO_4$ 0.3 mM) at pH 7.0. The standard chamber used in chemotaxis capillary assay [13] was not suitable for *B. sphaericus* (an obligative aerobe), since it does not permit sufficient oxygen diffusion for the maintenance of motility. Instead, the bacterial suspension (0.2 ml) was placed into a disposable spectrophotometric cuvettte (4 ml). Diffusion of oxygen from the surface of suspension was sufficient to provide for constant motility during at least one hour. The capillary tube (1 × 50 mm) was cleaned by squirting distilled water through it, sealed in a flame and filled with an attractant solution (10 mM, unless otherwise mentioned) or with the chemotaxis buffer as described [13]. The capillary was then fixed in a cork and inserted with the open end ca. 1 mm under the surface into the cuvette containing the bacterial suspension ($2 \cdot 10^6$ cells/ml) in the chemotaxis buffer. The bacteria were allowed to accumulate in the capillary for 45 min. Then the capillary was removed, its content was squirted on the camera of a haemacytometer. Trichloroacetic acid (50 mM, 5 μl) was added to paralyze the cells and the number of bacteria was counted under a phase-contrast microscope. All capillary assays were performed in triplicate or quadruplicate.

The chemotaxis of *Bacilli* was expressed as the ratio of the number of cells accumulating in a capillary in the presence of attractant to the number of cells accumulating in its absence. This parameter characterizes the ability of bacteria to respond to an external effector [10]. The random motility was measured as the number of cells accumulating in a capillary in the absence of attractant.

To measure in vivo methylation, cells were harvested at a desired growth stage by centrifugation at 12,000 × *g* for 7 min at 4°C, resuspended in potassium maleate solution (20 mM, pH 7), and sedimented again. The cells were suspended at $2 \cdot 10^8$ cells/ml in the protoplast preparation buffer (potassium maleate 20 mM, sucrose 0.5 M, EDTA 0.1 mM, sodium lactate 20 mM, $MgCl_2$ 20 mM, glycerol 0.5%, chloramphenicol 50 μg/ml) at pH 7. Aliquots (0.81 ml) were transferred into wells of a 24-well Co-Star plate and incubated at 42°C for 2–3 min in a water bath. Lysozyme (0.25 mg/ml) was added and the incubation continued for 30 min. The plate was then removed from the water bath and gently shaken for 5 min at the room temperature. L-[*methyl*-^{3}H]Methionine (16.2 μCi/ml) was added to samples and the labeling was allowed to go on for various periods of time. It was terminated by transferring the samples into Eppendorf tubes containing ethanol (0.5 ml) and phenylmethylsulfonyl fluoride (PMSF, 0.1 ml, 10 mM). After centrifugation at 14,000 × *g*, pellets were collected and mixed with Laemmli sample buffer (40 μl). Samples (10 μl) were heated for 10 min at 90°C and separated on SDS-PAGE (10%). After staining, the gels were soaked in Amplify fluorographic reagent (Amersham) and dried. A preflashed X-ray film (Fuji Photo Film Co, Ltd) was exposed to the gel for 9 days at −80°C. Autoradiographic exposures were quantitated with a soft laser densitometer (SL-TRFF, Biomed. Instruments, Inc.). The average density of labeled protein bands was expressed in arbitrary units (arb. un.).

Lysozyme (EC 3.2.1.17) from egg white was supplied by Sigma. All inorganic chemicals, sugars and amino acids were of analytical grade (Merck). L-Arginine, L-cysteine, and L-histidine were used as hydrochlorides; L-glutamate was added as sodium salt, the rest were free L-amino acids. Chemically pure glycerol was from Frutarom, Israel. Yeast extract was supplied by Biolife (Italy). L-[*methyl*-^{3}H]Methionine (80 Ci/mmol) was from Amersham.

3. Results and discussion

B. sphaericus grown on NYSM medium completed its growth cycle from spore germination to sporulation and cell lysis in about 24 h. For the purpose of this research, the standard growth curve (Fig. 1) was divided into five growth stages: (A) early logarithmic phase containing vegetative, filamentous, poorly motile cells; (B) mid-logarithmic phase characterized by vegetative, long, motile cells; (C) late logarithmic phase, with vegetative, short, rod-shape, hypermotile cells; (D) prestationary phase with short, swollen, hypermotile cells; and (E) early stationary phase with hypermotile cells containing refractile spores.

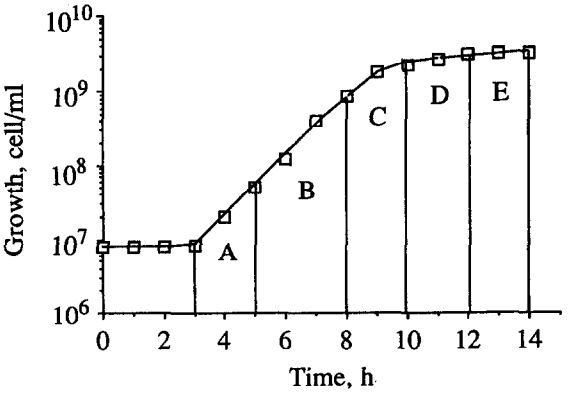

Fig. 1. A typical growth curve of *B. sphaericus* 2362 in batch culture. The following growth stages were arbitrarily discerned according to the cell density of the culture: A, early logarithmic; B, mid-logarithmic; C, late logarithmic; D, prestationary; and E, early stationary stage.

Accumulation of *B. sphaericus* in capillaries containing different concentrations of amino acids followed a pattern similar to that reported for *B. subtilis* [14]. Thus, for several amino acids, e.g. alanine, arginine and proline the accumulation of *Bacilli* in the capillary peaked at the attractant concentration of 10 mM. L-Glutamate at 10 mM was a relatively poor attractant; cell accumulation increased at higher concentrations. Hence, this concentration of attractant was selected for most of the experiments reported here. Matching dose–response profiles were observed at all growth stages (data not shown). As in *B. subtilis* [14], the number of *B. sphaericus* cells entering a capillary increased linearly while the density of bacterial suspension in the pond increased from 10^6 to 10^7 cells/ml. In the absence of attractant, the accumulation of the cells in the capillary was proportional to the cell concentration in the pond at all concentrations tested (up to $2 \cdot 10^8$ cells/ml).

B. sphaericus does not use sugars [1]. Indeed, glucose, lactose, sucrose, fructose, galactose, arabinose, maltose and xylose at concentrations 10^{-2}–10^{-8} M were not attractants at any stage of culture growth. Although glycerol was used as a carbon source [15], it failed to attract *B. sphaericus* cells. Attempts to induce chemotaxis to sugars or glycerol by including these compounds in the growth medium, or by incubating cells for one hour in the sugar-supplemented chemotaxis buffer followed by removal of the sugar, or both treatments, failed to induce

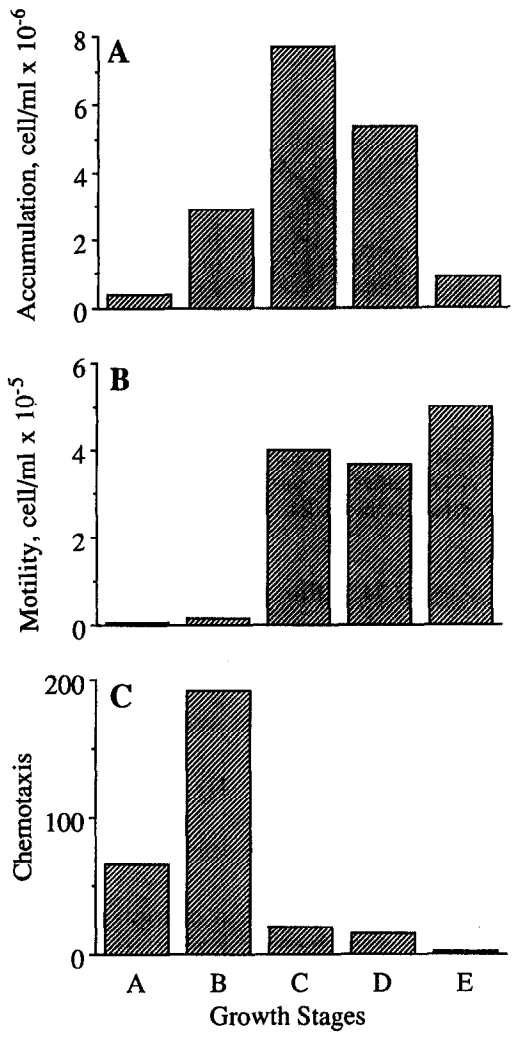

Fig. 2. Chemotactic response in *B. sphaericus* 2362: accumulation of cells in capillary (panel A), motility (panel B), and chemotactic activity (panel C) elicited by alanine (10 mM) at different stages of bacterial growth were determined as described in section 2.

chemotaxis. All natural amino acids were attractants for *B. sphaericus* 2362. At the early logarithmic stage, alanine was the most potent attractant, as it is for *B. subtilis* [14]. Lysine, glutamate, and glycine while good substrates [15] acted as poorer attractants.

Accumulation of *B. sphaericus* 2362 cells in the capillary filled with an amino acid solution (Fig. 2A) peaked at the late logarithmic stage (Stage C) and declined in older cultures. Measurement of random motility (Fig. 2B) indicated that different phenomena account for low cell accumulation in young and old cultures. In the early (Stage A) and mid-logarithmic (Stage B) cultures, low motility limited the total chemotactic response (Fig. 2A). In contrast, prestationary and stationary cells (Stages D and E) were as motile as the late logarithmic (Stage C) *Bacilli*. It is evident, that the decline in the total chemotactic response during the transition from stage C to stage E reflected the loss of a sensory component. Indeed, the chemotaxis of *B. sphaericus* was negligible at stage A, reached its maximum at stage B, and declined sharply in older cultures (Fig. 2C). It seems, that the ability of a bacterial cell to respond to chemoeffectors (Fig. 2C) and the random motility (Fig. 2B), are separately regulated. If the *che* and *mot* genes in *B. sphaericus*, as in *B. subtilis* [10], are located in a single regulon, some form of additional regulation is apparently involved. As it was mentioned above, transcription of both flagellin and MCPs genes in *B. subtilis* is controlled by σ^D [10]. Moreover, purified σ^D RNA polymerase was used to transcribe cloned MCP genes from *E. coli* [5]. Thus, such regulation seems to be universal in the eubacterial world. However, at least one *sigD*-independent *che* locus has been identified in *B. subtilis* [16].

Differential development of the sensory and locomotive component would make sense in terms of adaptation to environmental conditions. At early stages of growth, the highly developed sensory apparatus compensates for motility that is yet low, and enhances the ability of cells to respond efficiently to the attractant gradient. Relatively insensitive late logarithmic cells are able to react adequately to stimuli due to increased motility (Fig. 2A). Progressive loss of sensitivity to chemoeffectors culminates at the early stationary phase. Thus, swollen sporulating hypermotile cells would tend to disperse randomly from the nutrient-exhausted area. Therefore, spore formation would occur in a larger habitat, increasing the chance of survival of the population.

Chemotaxis in prokaryotes depends upon methylation of MCPs [17]. In *E. coli*, addition of attractant increases the extent of MCP methylation; the removal of attractant or the addition of repellent decreases it. In *B. subtilis*, the mechanism is thought to be more complex [17]. As in *E. coli*, MCPs of *Bacilli* are methylated by a methyltransferase. Methyl groups of MCPs are then transferred to an unidentified intermediate acceptor (X). At-

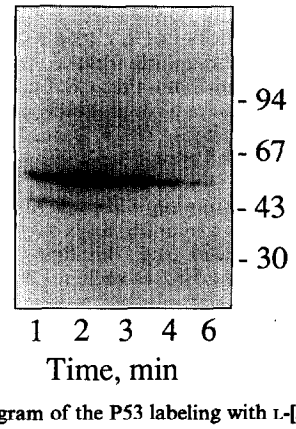

Fig. 3. Autoradiogram of the P53 labeling with L-[*methyl*-^{3}H]methionine in the mid-logarithmic cells. Prior to addition of L-[*methyl*-^{3}H]methionine cells were kept for 20 min in protoplast preparation buffer (starved cells). Autoradiogram was prepared as described in section 2. The column of numbers on the right shows molecular radii and positions of marker proteins.

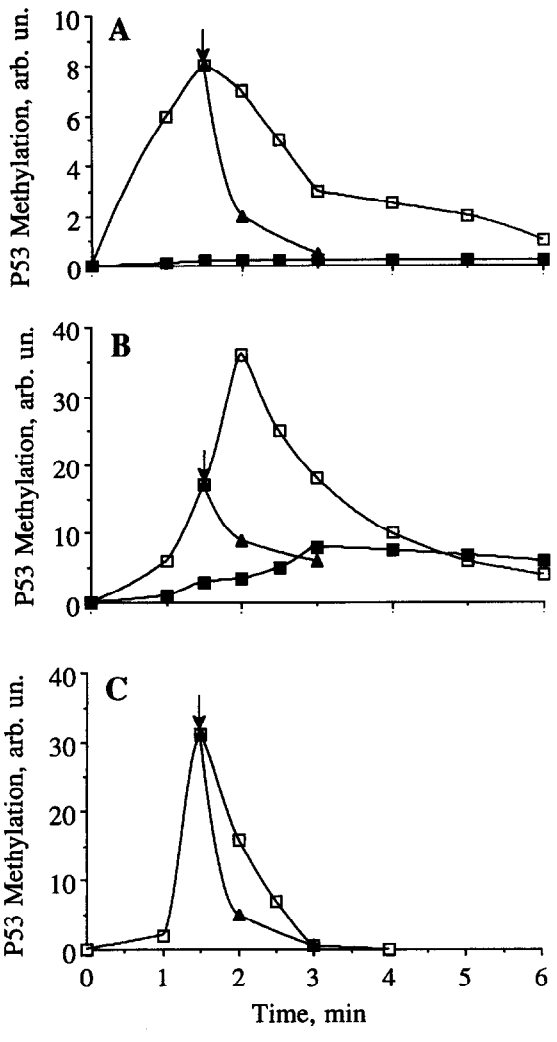

Fig. 4. Time course of the P53 labeling at different stages of growth. Panel A, early logarithmic culture (Stage A); panel B, mid-logarithmic culture (stage B); panel C, late logarithmic culture (stage C). Starved cells, □, were prepared as described in the legend for the Fig. 3. In some experiments (▲), L-glutamate (2.5 mM) was added to starved cells at times indicated by arrows. In other experiments, cells were preincubated for 20 min in the protoplast preparation buffer containing L-arginine (10 mM), ■.

tractants enhance the turnover of methyl groups on MCP. Both the rates of methylation and of methyl transfer swiftly reach a maximal level and then decline during the adaptation period [17]. These properties of bacillar MCPs were applied for the identification of MCP in *B. sphaericus*.

Prolonged incubation of mid-logarithmic cells with L-[*methyl*-^{3}H]methionine in the presence of chloramphenicol resulted in labeling of at least six proteins (not shown). However, only one major methyl acceptor (Fig. 3) with an apparent molecular size of 53 kDa (P53) demonstrated reversible methylation at a time fitting the time-scale of chemotactic response. The methylation of P53 reached a peak 2 min after the addition of labeled methionine (Fig. 4). All amino acids tested (L-Ala, L-Arg, L-Pro, L-Glu) enhanced demethylation of P53 in a concentration-dependent manner (data not shown). Thus, P53 conformed to two experimental criteria for bacillar MCP [17], namely, (a) transient labeling upon addition of L-[*methyl*-^{3}H]methionine and (b) acceleration of the methyl group turnover upon addition of an attractant.

P53 conformed to these criteria throughout the growth of *B. sphaericus* (Fig. 4). It was the major methyl group acceptor during the first ten minutes after addition of L-[*methyl*-^{3}H]methionine to cells collected at all stages of the culture. At any given stage, the extent of label incorporation was maximal in starved cells; the labeling was almost abolished in cells adapted to amino acids. However, markedly higher incorporation of the label was observed in middle and late-logarithmic cells (B and C), than in early logarithmic (A) cells. Cells collected in stationary (D and E) cultures incorporated very low amounts of the label (data not shown).

An additional labelled protein (P43) was sometimes observed (Fig. 3), especially at long exposures of the film. This protein, probably an MCP distinct of P53 or its proteolytic fragment, was not analyzed in this work due to its minority.

The study of motility and chemotaxis in *B. sphaericus* demonstrated physiologically different stages in the development of these functions in the batch culture. It seems probable that in the very rich initial medium corresponding to the early logarithmic stage, the expression of locomotive and sensory genes is catabolically repressed. Gradual depletion of the medium firstly leads to the formation of physiologically mature sensory apparatus (including MCP) and, later, to the increase of motility. During the late logarithmic stage, the sensory apparatus seems to deteriorate, while the motility is preserved even in sporulating cells. Discrepancy between high methylation of P53 and lowered chemotaxis in cells taken at stage C apparently deserves special attention. In our opinion, this observation indicates that besides playing the role in the mediation of chemotactic signals, methylation of MCP may have an additional function. We have recently obtained experimental evidence that methyl group transfer in *B. sphaericus* is, most likely, related to triggering of sporulation.

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