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Chemotaxis, sporulation, and larvicide production in *Bacillus sphaericus* 2362

The influence of L-ethionine, and of aminophenylboronic acid

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Abstract

4-Aminophenylboronic acid (APBA), a known inhibitor of sporulation in *Bacilli*, as well as L-ethionine, a known inhibitor of chemotaxis in *Enterobacteria*, inhibited both sporulation and chemotactic behavior but not growth of *Bacillus sphaericus*. Both compounds also inhibited the methyl group turnover on the methyl-accepting chemotaxis protein (P53) in this microorganism. Sporulation of *B. sphaericus* was inhibited only when APBA was added to the growing culture before the late logarithmic stage. It was previously demonstrated that the ability of *B. sphaericus* to respond to chemoattractants sharply declines at the same age of the culture. Thus, it seems plausible that the action of both inhibitors upon sporulation may be attributed to the inhibition of some regulatory pathway common to chemotaxis and sporulation and involving protein methylation. Possible exchange of the nutrient depletion-related sensory information between chemotaxis and sporulation systems at the level of methyl group transfer is discussed.

Key words: Chemotaxis; Sporulation; Inhibitor; Boronic acid; Ethionine; Methyl-accepting chemotaxis protein; Regulation; *Bacillus sphaericus*

1. Introduction

Significant progress has been achieved during the last decade in understanding the intracellular mechanisms of sporulation in *Bacilli*. However, environmental and cell cycle signals initiating the sporulation process are yet to be identified [1]. It is generally accepted that sporulation in *Bacilli* is triggered by nutrient depletion. The biochemical nature of the system registering nutrient depletion and generating primary signals for the initiation of sporulation is unknown. The system responsive to the fluctuation of temporary nutrient gradient, namely, the chemotactic system has been studied in detail [2]. It was suggested by Sherman [3] that the chemotaxis system may gather the information concerning nutrient concentrations and transmit signals to sporulation control mechanisms. Indication of a possible link between chemotaxis and sporulation may be found in the observation that some *che* mutants of *B. subtilis* are oligosporogenic [4]. Protein transmethylation seems to be a shared regulation principle for both regulatory systems [2,5,6]. In *B. subtilis* [7,8] as well as in *B. sphaericus* [9], attractants cause a transient increase in the methyl group turnover on methyl-accepting chemotaxis proteins (MCPs). Methylation of a membrane protein (P40) unrelated to MCPs was postulated to be a part of nutrient sensing system triggering sporulation in *Bacilli* [5,6]. In *B. subtilis* and in *B. licheniformis*, the addition of a nitrogen source or phosphate to starved vegetative cells results in methylation of this protein (P40). Some *spoO* mutants of *B. subtilis* possess an altered nutrient-dependent methylation of both P40 and MCPs [10].

Chemotaxis in *Bacilli* is more complicated than in enterobacteria. Although all key components of the enterobacterial chemotactic system are conserved in *Bacilli* [2], the methyl group is not removed from MCPs to water but is transferred to an intermediate methyl acceptor termed by Ordal 'X' [2,11]. The function of this protein is unknown. We would like to hypothesize that this function is triggering the sporulation in *Bacilli*, when the concentration of nutrients (attractants) in the medium is persistently dropping.

We shall demonstrate here that the chemotaxis inhibitor, L-ethionine [12] and the sporulation inhibitor, 4-aminophenylboronic (APBA) acid [13] affect both chemotaxis and sporulation in *B. sphaericus*. We believe, that these inhibitors may serve as a convenient tool for the dissection of a complicated regulatory network covering both chemotaxis and sporulation.

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Abbreviations: APBA, 4-aminophenylboronic acid; CFU, colony-forming units; MCP, methyl-accepting chemotaxis protein; SAM, *S*-adenosylmethionine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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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 and bacteria grown in the NYSM medium as described [9]. For some experiments, bacteria were grown in a glutamate-based medium containing (g/l): K_2HPO_4 3, Na_2HPO_4 4, yeast extract 0.5, glycerol 5, $CaCl_2$ 0.025, $MgSO_4$ 1, monosodium glutamate 5.44, and trace elements ($FeSO_4$ 0.1 mM, $MnSO_4$ 2.5 μM, $ZnSO_4$ 15 μM, $CoCl_2$ 20 μM, Na_2MoO_4 8 μM, $CuCl_2$ 8 μM, H_3BO_3 8 μM) at pH 7.0.

Stock solutions of various additives, such as amino acids, APBA, and L-ethionine, were sterilized separately by filtration and added to media before inoculation. Heat-resistant spore content was determined in fermentation broth samples preheated at 80°C for 10 min. The samples were plated on nutrient agar at various dilutions, and colonies formed after incubation for 24 h at 30°C were counted. The heating was omitted for the determination of total cell concentration in colony-forming units (CFU).

Capillary assays were carried out as described [9]. 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. The random motility was measured as the number of cells accumulating in a capillary in the absence of attractant.

Methyl group turnover on *B. sphaericus* methyl-accepting chemotaxis protein (P53) was determined and quantified as described previously [9].

B. sphaericus larvicide was determined according to the standard bioassay procedure [14] using 2nd instar larvae of *Culex pipiens*. Samples of larvicide were prepared by harvesting the insoluble material from the fermentation broth as described [15].

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). Glycerol was from Frutarom, Israel. Yeast extract was supplied by Biolife Italiana. Aminophenylboronic acid (APBA) and L-ethionine were from Sigma. L-[*methyl*-^{3}H]Methionine was from Amersham, Inc.

3. Results and discussion

S-Adenosylmethionine (SAM) required for chemotaxis of enteric bacteria can be depleted by incubation with L-ethionine, the methionine analog [12]. SAM is a donor of a methyl group for the methylation of MCPs in *B. subtilis* [2,4] as well. In contrast to *E. coli* and *S. typhimurium*, this bacterium can convert L-ethionine into *S*-adenosylethionine [16]. In *B. sphaericus*, L-ethionine (10 mM) markedly reduced the chemotaxis of mid-logarithmic cells (Fig. 1). L-Ethionine, added to the growth medium of *B. sphaericus* 2362 at inoculation, also suppressed sporulation but not the growth, of this microorganism (Fig. 2, upper panel). Methionine or other amino acids present in the medium competed for the ethionine effect. Thus, lower concentrations of L-ethionine were needed to suppress sporulation in the poor glutamate medium than in the rich NYSM medium.

Production of binary mosquito larvicide by *B. sphaericus* is associated with early stages of sporulation [17]. Ethionine, added to the growth medium at inoculation inhibited the larvicide production in 24 h cultures: the effect was significant at low (1 mM) concentrations of ethionine (LC_{50} = 0.015 mg/l), and dramatic at higher (20 mM) concentrations (LC_{50} = 0.1 mg/l). Culture grown without inhibitors yielded LC_{50} = 0.0055 mg/l.

The capability of L-ethionine to modify sporulation behavior is not entirely a new finding. Addition of L-ethionine (1 mM) to the culture of *B. subtilis* ethionine-tolerant strain *eth*A1 in the presence of glucose increases sporulation frequency [16]. Although the biochemical basis of the opposite effects of ethionine on sporulation of *B. sphaericus* and of *B. subtilis eth*A1 is not yet clear, the ability of L-ethionine both to support and to inhibit sporulation argues that it affects a specific pathway, rather than simply mimics a nutritional amino acid. Thus, Allen et al. proposed [16] that ethionine may induce sporulation in *B. subtilis eth*A1 by preventing SAM-dependent methylation of some molecule distinct from DNA.

While L-ethionine, the known inhibitor of chemotaxis inhibited sporulation of *B. sphaericus*, it was logical to expect that some known sporulation inhibitors would affect chemotaxis. Such an inhibitor was, indeed, found. Aromatic boronic acids are potent inhibitors of sporulation in *B. subtilis* [13]. These compounds have been studied as transition state analogs forming tetrahedral adduct with the active site serine of serine proteases [18] such as subtilisin. They also inhibit triacylglycerol lipase [19]. The mechanism of action of these compounds on sporulation was attributed to the inhibition of intracellular serine proteases [13]. This is, probably, incorrect, since mutants lacking all major serine proteases sporulate efficiently [20].

4-Aminophenylboronic acid (APBA) inhibited the sporulation (Fig. 2, lower panel) at concentrations not limiting growth. APBA (5 mM) also decreased larvicide production in 24 h cultures yielding only LC_{50} = 0.1 mg/l. Mid-logarithmic *B. sphaericus* cells, exposed to APBA for one hour at concentrations preventing sporulation, demonstrated significantly reduced (Fig. 1) chemotaxis. The response to all attractants was reduced regardless of whether APBA was present or absent during the chemotaxis measurements, or even when cells were washed several times with chemotaxis buffer before the assay.

In contrast, preincubation of cells with L-ethionine

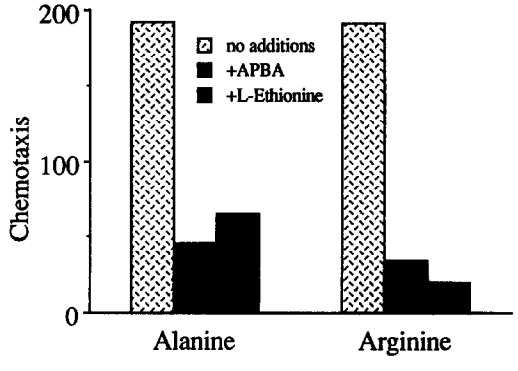

Fig. 1. Effect of L-ethionine (10 mM) and APBA (5 mM) on chemotaxis toward alanine and arginine of mid-logarithmic *B. sphaericus* 2362 cells. Chemotaxis was measured as described in [9].

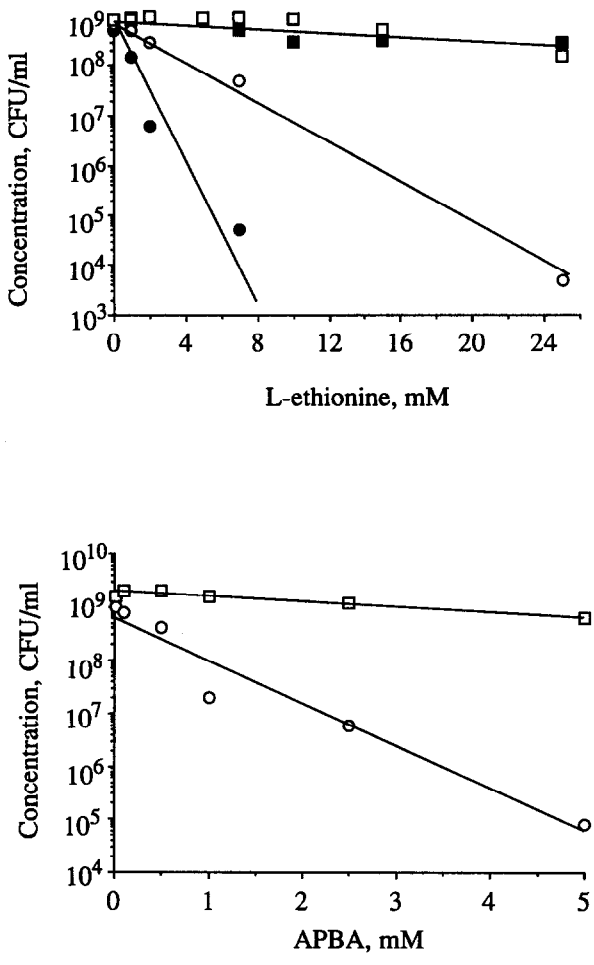

Fig. 2. Effect of L-ethionine and APBA on growth and sporulation of *B. sphaericus* 2362. *B. sphaericus* 2362 was grown in the presence of L-ethionine (upper panel) or APBA (lower panel) in NYSM (open symbols) or glutamate-based medium (filled symbols) for 24 h. Total concentrations of cells (squares) and spores (circles) were determined as described in section 2.

had no effect upon the chemotaxis, if the inhibitor was removed by washing the cells with chemotaxis buffer. L-Ethionine added to the chemotaxis buffer without preincubation, however, strongly inhibited chemotaxis. It seems, therefore, that APBA is an irreversible inhibitor of chemotaxis in a manner similar to its action on serine proteases, while L-ethionine probably competes with methionine for methionine adenosyltransferase.

Since L-ethionine acts on chemotaxis as the inhibitor of the SAM-dependent MCP methylation, it was interesting to find out whether APBA also affects methylation of proteins involved in the chemotaxis of *B. sphaericus* (P53, [9]). Indeed, mid-logarithmic cells preincubated (20 min) with either 10 mM L-ethionine (Fig. 3, upper panel) or 5 mM APBA (Fig. 3, lower panel), lost their ability to methylate P53. Both inhibitors caused rapid demethylation of P53 when added 1.5 min after the addition of L-[*methyl*-^{3}H]methionine. Secondary accumulation of the label was obvious after the addition of APBA indicating two phases of the effect: fast demethylation and slow methylation probably due to the inactivation of a methyl transferring enzyme or inhibition of methionine synthesis that causes dilution of the label.

Thus, both inhibitors of sporulation and chemotaxis in *B. sphaericus* affect methylation of P53. It can now be suggested that methylation of P53 or a consequent methyl group transfer to another methyl acceptor are regulatory events shared by both signal-transducing pathways, sporulation and chemotaxis.

APBA effectively inhibited sporulation measured in the 24-h-old cultures only when added during the first 4 hours of growth (Fig. 4). Later on, the effect gradually diminished, so that no inhibition of sporulation occurred when APBA was added after 8 h of growth. Apparently, this inhibitor differentiates between two populations existing at the moment of APBA addition: (a) truly vegetative cells that could be prevented from entering sporulation, and (b) APBA-insensitive cells already committed to sporulation. Evidently, the transition between APBA-sensitive and insensitive metabolic states occurs in the mid logarithmic stage of growth. It seems hardly coinci-

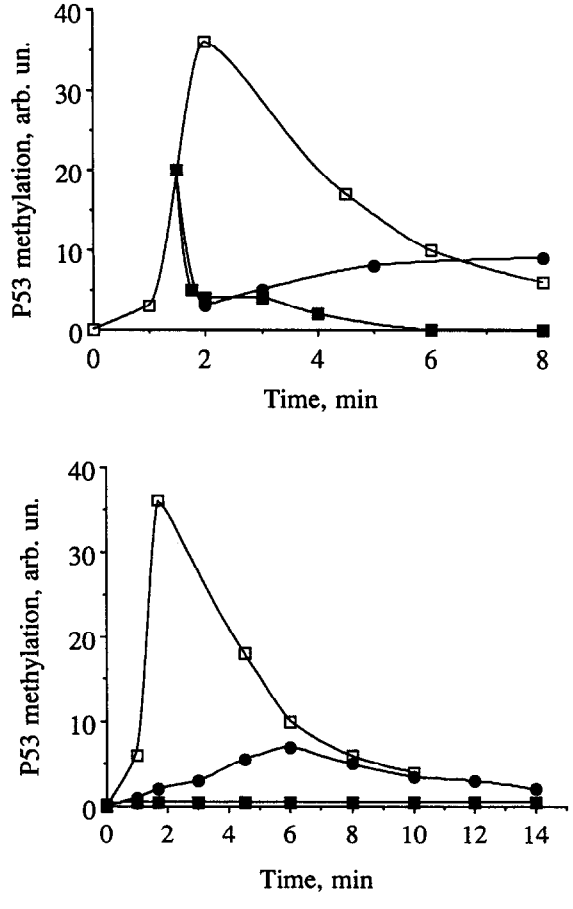

Fig. 3. Effect of L-ethionine (■) and APBA (●) on P53 methylation in mid-logarithmic cells. Upper panel, inhibitors were added 1.5 min after the addition of the label; lower panel, cells were preincubated with L-ethionine (10 mM) or APBA (5 mM) for 20 min at ambient temperature. (□), time-course of P53 methylation in the absence of effectors.

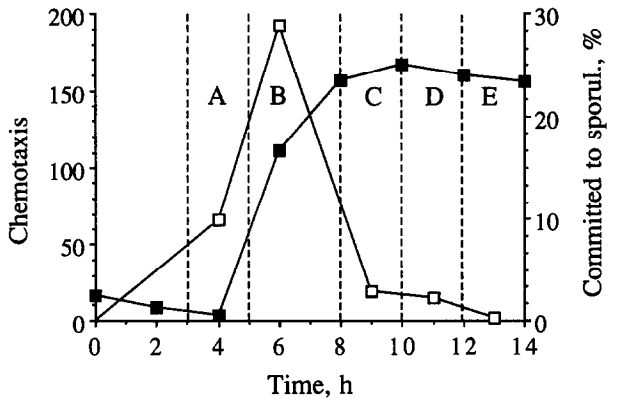

Fig. 4. Influence of APBA upon sporulation of *B. sphaericus* at various stages of growth: correlation with chemotaxis. *B. sphaericus* 2362 was grown in NYSM. APBA (5 mM) was added to cultures at different times, shown on the abscissa, and corresponding to different stages of growth [9]: A, early logarithmic; B, mid-logarithmic; C, late logarithmic; D, pre-stationary; and E, early stationary. At each stage, the population of *B. sphaericus* cells was supposed to consist of (i) cells committed to sporulation and, thus, able to form spores in the presence of APBA, and (ii) vegetative cells sensitive to APBA. The proportion of cells committed to sporulation, i.e. APBA-insensitive cells (■), was determined as the ratio of total cell concentration, measured at the time of APBA addition, to the spore concentration, reached in each culture 24 h after inoculation. Chemotaxis at each stage (□) is also shown (data taken from [9]).

dental, that the chemotactic activity of *B. sphaericus* cells (Fig. 4) peaks at the same stage. Simultaneous changes in chemotaxis and in sporulation argue, rather, strongly in favor of the common pathways involved in regulation of these functions.

It should be stressed that chemotaxis and sporulation differ in their time scale. Chemotactic response is a few minutes long, while sporulation initiation seems to require at least one cell cycle. A feasible information exchange may be based upon a chemotaxis-dependent methyl transfer that would occur at a rate much slower than in chemotaxis. It could be hypothesized that Ordal's intermediate methyl acceptor X [2] might serve as an integrating site transmitting chemotactic sensory input from MCPs to the sporulation triggering mechanisms. Slow changes in the methylation state of X during the culture growth would represent an integrated signal for nutrients presence or depletion in the environment. Experiments supporting this notion are currently under way.

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