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Altered chemotaxis of *Bacillus sphaericus* L-ethionine-resistant sporulation mutant

A probable link between chemotaxis and sporulation

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Abstract

A UV irradiation-induced mutant of *Bacillus sphaericus* 2362 whose sporulation was inhibited neither by natural amino acids nor by L-ethionine was selected. The mutant (A61) grew slowly in rich amino acid medium and contained increased concentrations of heat-resistant spores throughout the growth. Slow growth of A61 was related to continuous presence of aging and sporulating cells even when the medium was rich in nutrients. Ability of the mutant to sense nutrient presence in the environment and to relate this information to systems regulating the switch from vegetative growth to sporulation seem to be damaged. A61 also demonstrated impaired chemotaxis. In contrast to the parent strain, only few amino acids elicited chemotactic response in A61. Methylation of the A61 methyl-accepting chemotaxis protein(s) was lower than that of the parent strain by one order of magnitude. Spontaneous fast-growing phenotypic revertants of A61 displayed sporulation behavior characteristic of *B. sphaericus* 2362. Their chemotaxis to amino acids was considerably improved. To some amino acids, it proved to be even stronger than in the original strain, *B. sphaericus* 2362. It is suggested, that methyl transfer events originating in the chemotactic system are involved in the triggering of sporulation, the A61 mutation being located in this signalling pathway.

Key words: Chemotaxis; Sporulation; Regulation; Mutant; Revertant; L-Ethionine; Methyl-accepting chemotaxis protein; *Bacillus sphaericus*

1. Introduction

Early events in sporulation such as processing of nutrient depletion signals alerting sporulation initiation are yet obscure [1]. It was suggested that methyl-accepting chemotaxis proteins (MCPs) are mediators of these environmental signals [2]. A methodical study of chemotaxis and sporulation in *B. sphaericus* provided experimental evidence that these pathways are able to communicate [3,4]. It seems that this communication involves methyl group exchange on the MCP. Thus, methylation of *B. sphaericus* MCP (P53) was equally high during mid- and late-logarithmic stage of growth of a batch culture, while chemotaxis significantly decreased during the latter [3]. This paradox indicated, in our opinion, an additional regulatory function for MCP transmethylation, besides mediation of chemotactic signals. Indeed, the transition from the mid- to the late-logarithmic stage of growth coincided not only with the loss of sensory component of chemotactic response but also with the commitment of *B. sphaericus* cells to undergo sporulation [4]. 4-Aminophenylboronic acid (APBA), a known inhibitor of sporulation in *Bacilli*, as well as L-ethionine, a known inhibitor of bacterial chemotaxis inhibited sporulation, chemotaxis and methyl group turnover on the MCP of *B. sphaericus* [4]. A mutual effect of inhibitors upon chemotaxis and sporulation was attributed to a common regulatory pathway involving protein transmethylation. However, the pleiotropic, non-specific character of both inhibitors made it difficult to rule out the possibility of coincidental inhibition of both regulatory pathways by unrelated mechanisms. It was, therefore, of interest to obtain a sporulation mutant defective in chemotaxis and to show, that the reversion to the wild type (*B. sphaericus* 2362) in terms of sporulation, involves modification of the mutant chemotactic phenotype.

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 [3,4]. For some experiments, glutamate-based medium [4] or Casamino acids medium (g/l): K_2HPO_4 3, Na_2HPO_4 4, yeast extract 5, $CaCl_2$ 0.1, $MnSO_4 \cdot H_2O$ 8.45×10^{-3}, $MgCl_2 \cdot 6H_2O$ 0.2, Casamino acids (Difco) 12, and EDTA 0.1 mM at pH 7.0, were used.

Mutagenesis and screening: *B. sphaericus* 2362 was grown in the NYSM medium for 24 h whereas sporulation of more than 98% was achieved. Culture samples (0.3 ml) were washed with phosphate buffer

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; MCP, methyl-accepting chemotaxis protein; APBA, 4-aminophenylboronic acid; CFU, colony-forming units.

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(10 mM, pH 7.5) containing EDTA (0.1 mM), centrifuged, diluted in the same buffer (3 ml) and heated at 80°C for 10 min to kill vegetative cells. The spore solution (10^8 spores/ml) was placed in a sterile glass Petri dish and irradiated for 1.5 min under a 15-W bactericidal UV lamp. The dose of irradiation was set to give 25% survival of spores. The selection of mutants able to sporulate either in the presence of amino acids or in the presence of a sporulation inhibitor (L-ethionine) was carried out as follows. UV-irradiated spores were inoculated into the rich Casamino acids medium (50 ml) in an Erlenmeyer flask (250 ml) and grown for 8 h (mid-logarithmic growth). The culture was then heated at 80°C for 10 min. The particulate material was harvested, transferred into glutamate-based medium [4] containing L-ethionine (10 mM), grown for 24 h and heated at 80°C for 10 min. After repeating the selection cycle, this time with inoculation rates of 0.5% between the transfers, the spores were placed on nutrient agar (Difco). Small colonies formed after incubation for 48 h at 30°C were picked up, and mutant strains were re-isolated.

Stock solutions of various additives, such as amino acids, and L-ethionine, were freshly prepared, 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). Cell count at growth stages where aggregation was observed was done in a hemocytometer after diluting the culture with NaOH (5 M) to achieve disaggregation.

Capillary assays were carried out as described [3]. 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. Methyl group turnover on *B. sphaericus* methyl-accepting chemotaxis protein (P53) was determined by labelling with L-[*methyl*-^{3}H]methionine and quantified as described previously [3].

B. sphaericus larvicide toxicity was determined using 2nd instar larvae of *Culex pipiens* [4].

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

The strategy for mutant selection was based upon the postulated common regulatory event linking chemotaxis and sporulation. The desired mutant phenotype was of an organism that misinterprets environmental stimuli and, thus, enters sporulation in a nutrient-rich medium. Such mutant (A61) was, indeed, isolated following the procedure described in section 2. The main steps of the selection were (a) selection for heat-resistant spores formed during short (8 h) growth in the rich Casamino acids medium from UV-irradiated *B. sphaericus* 2362; (b) inoculation of these spores in medium containing the L-ethionine (24 h) and harvesting heat-resistant spores formed despite the presence of the inhibitor.

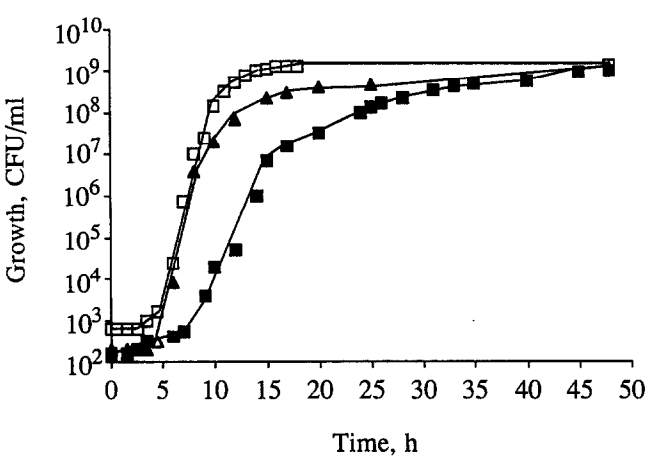

Fig. 1. Growth curve (30°C) of *B. sphaericus* 2362 (□), mutant A61 (■) and of the revertant A61 R1 (▲). The growth curve of the 2nd revertant, A61 R2, similar to that of A61 R1 is not shown.

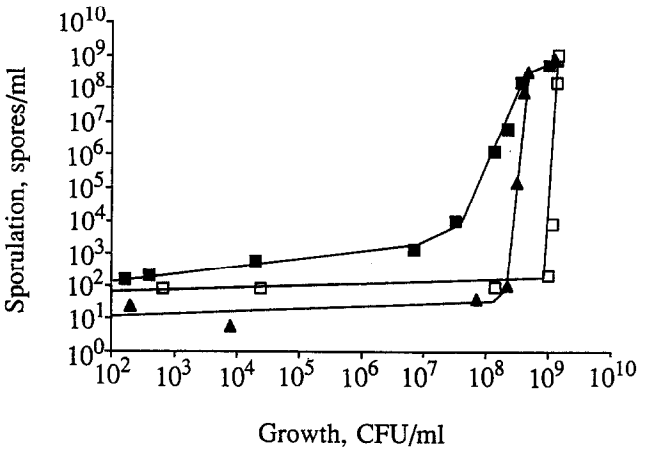

Fig. 2. Sporulation of *B. sphaericus* 2362 (□), mutant A61 (■) and of the revertant A61 R1 (▲) at various cell densities during culture growth. Data for A61 R2, similar to that of A61 R1 are not shown.

Several mutant clones were obtained by repeating the isolation protocol. One of the mutants, A61, was thoroughly investigated. On nutrient agar plates, A61 formed colonies morphologically identical to the parent strain but much smaller. It also grew much slower than the parent strain in NYSM medium (Fig. 1), though the maximal cell concentration of the mutant and of the parent after 48 h was identical. The cell morphology and motility of these two strains at various stages of growth differed considerably. The growth curve of A61 featured a significant lag followed by the logarithmic growth characterized by short hypermotile cells corresponding in appearance to late logarithmic cells of the parent strain. The growth was accompanied by aggregation, that become extensive at the late logarithmic stage, again reminiscent of stationary cultures of the parent. At the end of the late logarithmic stage (above the concentration of 10^8 cell/ml) hypermotile cells disappeared, the motility decreased by an order of magnitude and remained low thereafter (data not shown).

Morphological characteristics as well as the markedly higher proportion of spores in exponentially growing population indicated abnormal aging of the culture. Throughout the batch culture growth, the mutant contained significantly elevated concentrations of heat-resistant spores (Fig. 2). A difference of two orders of magni-

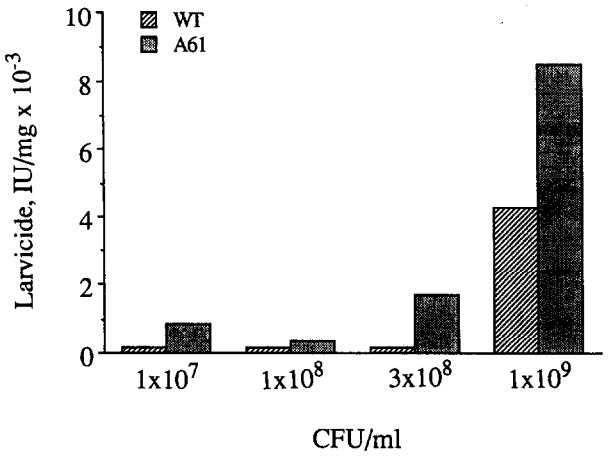

Fig. 3. Larvicide production on various stages of growth by *B. sphaericus* 2362 and by the mutant A61. Larvicide toxicity in International Units per mg of *B. sphaericus* dry biomass was determined in comparison to the Pasteur Institute (Paris) standard SPH 88. Prior to larvicide determination, cells were collected at the concentrations indicated in the figure, washed and freeze-dried.

tude was observed already at $3 \cdot 10^7$ cell/ml rising to 6 orders of magnitude at $3 \cdot 10^8$ cell/ml. Increased sporulation at the early stages of growth was not dependent upon unsynchronized germination of the mutant spores. In fact, spores of A61 were found to germinate earlier than those of the parent strain (data not shown). Binary larvicide production, an early marker of sporulation [6], was also significantly enhanced compared to the parent strain at the same cell densities and growth stages (Fig. 3).

The transition from vegetatively growing culture to sporulation in the parent strain coincided with the cessation of growth. In the mutant, increased rate of sporulation appeared much earlier than growth arrest caused by the exhaustion of nutrients (Figs. 1 and 2). The slow growth rate of A61, thus, resulted from the presence throughout the growth of a mixed microbial population containing vegetative as well as aging and sporulating cells. All these observations support the conclusion that A61 is a sporulation mutant. The mutant phenotype featured an unusual ability to sporulate spontaneously in the presence of excess growth substrate. Granted, the content of sporulating cells increased with the increased age of the culture. Thus, the mutant seems to be either partially sensitive to nutrient depletion stimuli, or to respond by increased sporulation to alternative signals, such as external pH, accumulation of secondary metabolites, or to the cell internal metabolic status.

The chemotaxis of A61 was also impaired. Only four amino acids (alanine, arginine and cysteine) retained their chemotactic activity; chemotaxis to other amino acids was lost or diminished (Table 1). The MCP methylation (P53 labeling) of logarithmic A61 cells was hardly detectable; it was at least one order of magnitude lower than in the parent strain (data not shown). Thus, A61 was, undoubtedly, a chemotaxis mutant, as well.

Although a low dose of UV irradiation was used during mutagenesis to prevent numerous mutations, the rigorous selection could favor coincidental double mutations in both sporulation and chemotaxis loci. The probability of such double mutations is low, however, finite. One would then expect revertants either to the parent chemotaxis phenotype or to the original sporulation phenotype. Without rigorous selection, the probability of double revertants is negligible.

Small colony size on NYSM agar plates was one of the characteristics of A61 mutant phenotype stemming from its lower growth rate. Spontaneous revertants of this phenotype forming large colonies were frequently observed among colonies formed by cells taken from A61 stationary batch cultures and plated on the nutrient agar. Two of such phenotypic revertants (A61 R1 and A61 R2) were randomly selected and investigated.

Growth curves, morphology and sporulation of revertant strains A61 R1 and A61 R2 resembled *B. sphaericus* 2362 more than A61 (Figs. 1 and 2). The sharp transition from the vegetative growth to sporulation was restored (Fig. 2). The chemotaxis toward amino acids in both revertants was different from either *B. sphaericus* 2362 or A61 (Table 1). The trend was clearly compensatory. In both revertants chemotaxis to most amino acids was higher than in A61. Chemotaxis to glutamate and arginine was notably higher than in the parent strain. Thus, two randomly selected revertants demonstrated a phenotype approaching this of the original strain both in increased sensitivity to nutrient depletion and in increased chemotaxis to amino acids. These observations strongly support the notion that a single mutation is responsible for impaired chemotaxis and for the altered sporulation of A61. As mentioned above, accidental phenotypic res-

Table 1
Chemotaxis toward amino acids in *B. sphaericus* 2362, A61 mutant and two phenotypic revertants (R1 and R2)

Amino acid	*B. sphaericus* strains			
	2362	A61	A61 R1	A61 R2
Ala	67	67	67	67
Ile	60	5	9	15
Thr	59	5	11	17
Cys	57	52	84	34
Gln	42	1	8	1
Glu	40	5	142	67
Arg	40	36	115	125
Asp	32	5	1	17
Pro	26	1	13	6
Val	17	1	25	6

Chemotaxis toward various amino acids (10 mM) was determined by the capillary assay as described [3]. Cells of all the strains were collected at the logarithmic stage (10^7 cell/ml).

toration of both defects is hardly possible. Genetic analysis of the A61 mutant will allow for the identification of the mutated gene. Insufficiently developed genetics of *B. sphaericus* hinders the progress of this investigation. However, lack of sugar metabolism [7] makes *B. sphaericus* uniquely suitable for physiological analysis of sensory networks involving processing of environmental signals. Attempts are now made to detect and identify proteins involved in gathering of long-term sensory information related to nutrient presence in the environment and in information transfer to regulatory systems governing sporulation initiation. Strongly reduced methylation of MCPs in the mutant implies involvement of methyl transfer events in the communication between these pathways.

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