KINETICS OF SPORE GERMINATION OF *BACILLUS SUBTILIS* IN LOW CONCENTRATIONS OF L-ALANINE

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The kinetics of L-alanine-induced spore germination of *Bacillus subtilis* was studied in a very low range ($<6 \mu\text{M}$) of concentration of L-alanine (ALA), where departure from Michaelis-Menten kinetics is frequently observed. For this purpose, a very sensitive method for measuring germination was devised based on the fact that a DNA repair-deficient mutant, UVSSP42-1, produces spores extremely sensitive to ultraviolet (UV) radiation, and the spores acquire normal UV resistance upon germination. After UV irradiation at an appropriate fluence, germination percentage was calculated from the survival of spores. Germination as low as 0.1% could be measured with sufficient accuracy for kinetic analysis. In a range of ALA concentration of 1.75 to $6 \mu\text{M}$, a double logarithmic plot of germination rate against ALA concentration gave a straight line with a slope of about 3. It may be assumed that binding of three molecules of ALA per receptor is required to trigger germination.

Spores of many bacilli are triggered to germinate by a single compound, L-alanine (ALA). Although the nature of the trigger reaction is not yet known, models based on interaction of ALA and a specific receptor site (ALA receptor) have been presented (1, 2).

In kinetic analysis of ALA-triggered germination, data are frequently fitted to the Michaelis-Menten equation, but in some cases departures from Michaelis-Menten kinetics are observed, being especially marked at lower ALA concentrations (e.g. 10^{-5} M , 3). Kinetic analysis of germination at these low concentrations of ALA seems especially valuable in elucidating the mode of interaction of ALA and the ALA receptor, since at very low concentrations most of the germinating spores will receive the minimum number of ALA molecules necessary for triggering germination. However, such analysis has been hampered by the lack of a suitable method for estimating low rates of germination. In usual practice, germina-

tion has been measured by germination-specific changes such as loss of resistance to heat and chemical agents, loss of refractility, gain of stainability, etc. (4). Although some of these methods are frequently employed in kinetic studies, they are not suited for estimation or detection of germination occurring in a small fraction (e.g. a few per cent) of a total spore population.

We have devised a method by which germination of less than 1% can be measured. This method is based on the fact that a DNA repair-deficient mutant of *Bacillus subtilis*, UVSSP42-1 (5), produces spores highly sensitive to ultraviolet (UV) radiation, and the spores become much more resistant to UV upon germination (6, 7). In this paper we describe this method and its use to investigate the kinetics of spore germination of strain UVSSP42-1 at low ($<6 \mu\text{M}$) ALA concentrations.

MATERIALS AND METHODS

Bacterial strain. *Bacillus subtilis* UVSSP42-1 (*uvrA42ssp-1*, 5) which originates from *B. subtilis* 168 (*thy trp*) was kindly supplied by Dr. Nobuo Munakata.

Spores. Spores were produced, cleaned and stored as described previously (8).

Germination. After being heat-shocked at 65° for 120 min, spores were suspended in 0.1 M potassium phosphate buffer (pH 6.6) containing 1 mM MgSO_4 at a density of $0.5\text{--}5 \times 10^5$ spores/ml, and germinated at 37° with various amounts of ALA.

UV survival. Before UV irradiation, the spore suspension was quickly cooled to 0° to arrest germination. A sample (4 ml) was irradiated in a Pyrex watch glass (90 mm in diameter) with gentle shaking under a 15 W germicidal lamp covered with a perforated cardboard to lower the fluence rate. The fluence rate was $0.25 \text{ J}/(\text{m}^2 \cdot \text{sec})$, as measured by a Topcon MRA-85 UV-radiometer (Tokyo Kagaku Kikai Co., Tokyo).

Survival of spores was determined as described previously (8) except that 0.1 M potassium phosphate buffer (pH 6.6) was used as diluent.

RESULTS

Estimation of germination from UV survival

Strain UVSSP42-1 produces extremely UV sensitive spores (5). The spores acquire normal UV resistance upon germination (6, 7). Therefore, germination can be estimated from survival after UV irradiation that kills almost all of the non-germinated spores and allows a majority of the germinated spores to survive.

To determine the optimal UV fluence, the UV sensitivity of non-germinated and germinated spores was compared. For this purpose, a sample of germinated spores was prepared by incubating the heat-shocked spores with a "saturating"

Fig. 1. Survival curves for non-germinated (○) and germinated (●) spores of *Bacillus subtilis* UVSSP42-1.

concentration (1.2 mM) of ALA at 37° for 30 min. At this concentration, germination was almost complete (>98% as measured by the loss of heat resistance) after 20 min of incubation.

UV survival curves for non-germinated (heat-shocked) and germinated spores are shown in Fig. 1. In agreement with MUNAKATA (6) and WANG and RUPERT (7), 99.9% of non-germinated spores were killed by a UV fluence of 10 J/m², whereas about 50% of germinated spores survived after irradiation at 15 J/m². A UV fluence of 15 J/m² (60 sec at a fluence rate of 0.25 J/(m²·sec)) was adopted for estimating germination.

Germination percentage was calculated as

$$\text{Germination \%} = \frac{S - S_0}{S_{100}} \times 100$$

where S is the survival of a sample after irradiation, S_0 the survival of non-germinated spores and S_{100} the survival of 100% (or nearly 100%) germinated spores. Since S_0 was about 0.02% relative to S_{100} , it was negligible when the germination percentage was sufficiently great (e.g., >0.5%). For S_{100} , the spores germinated in 1.2 mM ALA at 37° for 30 min were used.

Time course of germination

At saturating concentrations ALA induced almost 100% germination within 20 min, whereas germination was slow and limited in extent at low (e.g., $<10^{-5}$ M) concentrations. Figure 2 shows the time course of germination in $6\text{ }\mu\text{M}$ ALA. Germination was roughly linear against time up to 30 min and the rate of germination decreased thereafter. In $2\text{ }\mu\text{M}$ ALA, germination was much slower and linear against time up to at least 120 min (data not shown). We define in this paper the term "germination rate" as the percentage of spores germinated in 30 min of incubation. The germination rate so defined may be regarded as the rate of "commitment" (2), because essentially the same value was obtained when the germinating spores were allowed to stand for a further 10 min at 37° in the presence of 1 mM D-alanine, instead of being immediately chilled for irradiation.

Effect of ALA concentration on germination rate

A small fraction (about 0.07% in 30 min) of the spores germinated upon incubation at 37° without added ALA. This is not due to ALA or related substances that might be excreted from the spores because the germinating fraction was not decreased by washing the heat-shocked spores with distilled water or by addition of 1 mM D-alanine to the germination buffer. Therefore, this "spontaneously" germinating fraction was subtracted from germination data when the effect of ALA concentration on the germination rate was studied.

The germination rate increased rapidly and non-linearly with ALA concentration. A double logarithmic plot of germination rate against ALA concentra-

Fig. 2.

Fig. 2. Time course of germination of *Bacillus subtilis* UVSSP42-1 spores in $6\text{ }\mu\text{M}$ ALA.

Fig. 3.

Fig. 3. Double logarithmic plot of germination rate against ALA concentration.

tion (1.75 to 6 μM) resulted in a straight line with a slope of about 3 (Fig. 3). Thus, in this range, the germination rate increased roughly in proportion to the cube of ALA concentration.

DISCUSSION

Although the applicability of the method reported here is confined to the strains harboring *ssp* and *uvr* mutations, it has some unique advantages over the conventional methods. It is capable of measuring germination of 0.1% or less with considerable accuracy. Because spore suspensions of low spore density (e.g., $10^6/\text{ml}$) are used, the effect of components excreted from the spores or the inhibitory effect of spore alanine racemase (9), which may interfere with the kinetic studies, can be minimized.

Furthermore, the fact that spores of a *ssp uvr* strain become much more resistant to UV upon germination may offer a useful tool for selecting germinated spores. For example, it may be useful in genetical studies including enrichment and isolation of mutants (or revertants) that germinate under specified conditions.

The non-linearity of the germination rate against ALA concentration clearly indicates a departure from Michaelis-Menten kinetics, suggesting that the trigger reaction is a multimolecular process with respect to ALA. From the slope of the double logarithmic plot (Fig. 3), it may be assumed that binding of three molecules of ALA per receptor is required for triggering germination.

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