THE EFFECT OF AMINOACIDS ON GROWTH, SPORULATION AND CRYSTAL FORMATION IN *BACILLUS THURINGIENSIS* VAR. *THURINGIENSIS**

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ABSTRACT

Histidine, threonine, valine, tyrosine, leucine, isoleucine, serine, lysine and B vitamins added to Glucose–mineral salts medium did not promote growth of Bacillus thuringiensis. *Aspartic acid, arginine, glycine, proline, asparagine, methionine and glutamine supported growth of the organism with a longer lag period as compared to cystine which stimulated exponential growth in 9 hr after inoculation. The total growth increased in relation to the concentration of cystine in the medium. Presence of excess cystine along with glucose inhibited sporulation and consequent parasporal crystal formation.*

Key words: Aminoacids, growth, sporulation, *Bacillus thuringiensis*.

1. INTRODUCTION

Bacillus thuringiensis produces a proteinaceous crystalline inclusion during sporulation which is known to be toxic to lepidopteran larvae.[1] In view of its use in the biological control of some agricultural pests, interest has centered round studies involving this microorganism and its crystal formation.

Vinter[2] had reported that cystine inhibited spore formation in several species of *Bacilli*. In *B. thuringiensis* the development of spore and the (insecticidal) proteinaceous crystalline inclusion body occur simultaneously. In view of this and the following reported observations that (*a*) the crystalline proteinaceous inclusion contains a small quantity of cystine/cysteine and that (*b*) sulphydryl groups are required for the biological activity of the toxic subunit,[3] we undertook these studies and report here the effect

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of vitamins and aminoacids with particular reference to cystine on the growth, sporulation and crystal formation in this important *Bacillus*.

2. Materials and Methods

Conditions of culture: *Bacillus thuringiensis* var. *thuringiensis* serotype I was obtained from Professor H. Barjac, Institute Pasteur, Paris. The culture was maintained routinely on nutrient agar slants.

The growth medium to study the effect of aminoacids consisted of phosphates to give pH 7·2 10·0 g; $MgSO_4 \cdot 7H_2O$ 2·0 g; $FeCl_3 \cdot 6H_2O$ 0·007 g; $CaCl_2 \cdot 2H_2O$ 0·9 g; $(NH_4)_2SO_4$ 0·05 g; micronutrient solution I ml, H_2O 1 L, glucose 20·0 g. The micronutrient solution consisted of $ZnSO_4 \cdot 7H_2O$ 1·0 g, $MnSO_4 \cdot 4H_2O$ 5·0 g, H_3BO_3 0·05 g, $Na_2MoO_4 \cdot 2H_2O$ 2·0 g, $CuSO_4 \cdot 5H_2O$ 0·007 g, water 1 L. Glucose and salts were sterilized separately and mixed aseptically with phosphate buffer.

In all experiments (*a*) 200 ml medium was placed in 500 ml Erlenmeyer flasks and incubated on a rotary shaker (250 rpm) at 30° C, (*b*) phosphate buffer at pH 7·2 was used and (*c*) the growth was measured as absorbance in a 'spectronic 20' colorimeter (Bauch and Lomb) at 600 nm after appropriate dilution where necessary.

The inoculum for growth studies was prepared as follows: Two hundred ml of glucose-salt medium (without growth factor) was inoculated with 1 ml of twice-washed suspension of O.D. 3·5 of vegetative cells from a 14 hr growth on nutrient agar. The culture grew after a lag period of more than 70 hr. These cells were collected in the exponential phase and washed twice by centrifugation with 0·05 M buffer and the same volume of O.D. 3·5 used as inoculum for various growth studies.

All the aminoacids, except cystine as well as the vitamin mixture were autoclaved with glucose-salts mixture. Cystine was autoclaved along with the phosphate buffer. Unless otherwise mentioned, 0·05% W/v amino acids were used with 2% glucose-mineral salts.

The vitamin mixture consisted of 10 μg each of thiamin, riboflavin, nicotinic acid, pantothenic acid, cyanocobalamin, folic acid, biotin and PABA (per 100 ml medium).

Cystine was estimated in the growth medium by descending paper chromatography followed by colorimetric estimation.[4]

L or DL aminoacids were purchased from reputed commercial sources. The other chemicals used were of reagent grade.

3. Results

Bacillus thuringiensis var. *thuringiensis* does not grow in a glucose-salts medium without the addition of certain aminoacids as growth factors.

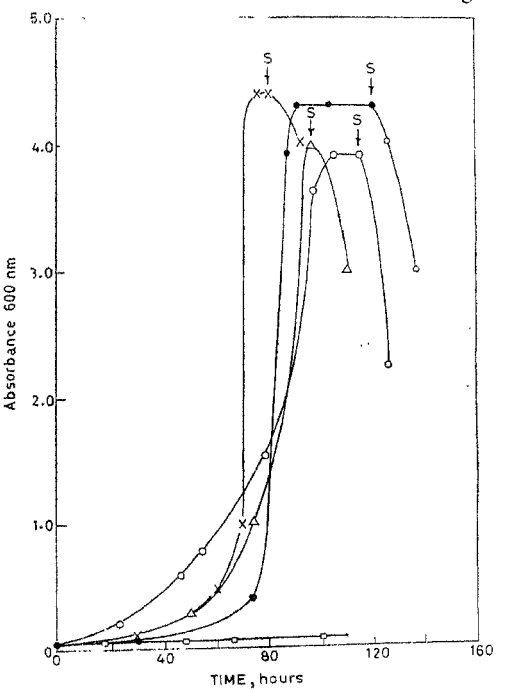

Fig. 1. Stimulation and inhibition of growth of *B. thuringiensis* by aminoacids. (□——□) leucine, isoleucine, serine, lysine, histidine, tyrosine, threonine, valine and control without amino-acid; (× ——×)—glutamine; (● ——●)—glycine; (○ ——○)—proline; (△ △)———methio-nine. S-indicates spore formation.

Histidine, threonine, tyrosine, valine, isoleucine, leucine, serine, lysine and B vitamins did not support growth of the organism, while arginine, aspartic acid, glycine, proline, asparagine, methionine and glutamine supported growth with a longer lag period compared to cystine which stimulated exponential growth in 9 hr after inoculation (Figs. 1 and 2).

Relation between sporulation and cystine concentration :

Growth was seen to be enhanced by increasing the cystine concentration in the medium. This was confirmed by studying the effect of cystine

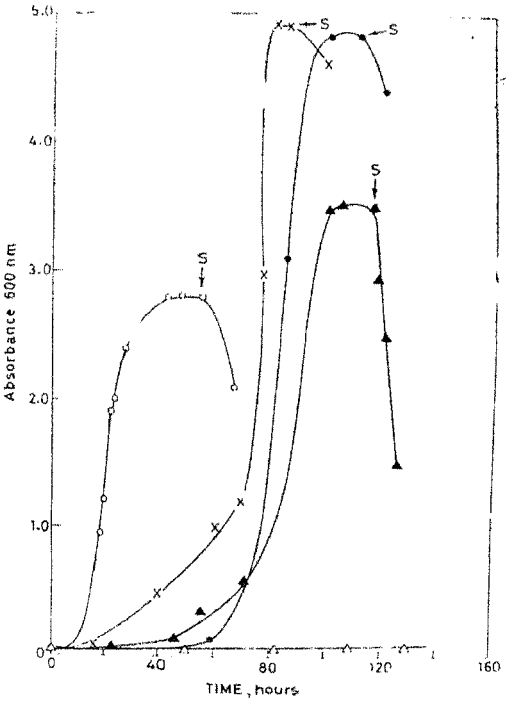

FIG. 2. Stimulation of growth of *B. thuringiensis* by aminoacids. (△——△)—Control without aminoacid; (●——●)—aspartic acid; (○——○)—cystine; (×——×)—arginine; (▲——▲)—asparagine.

concentration in the medium on growth and sporulation (Fig. 3). It was further shown that cystine concentration in the growth medium decreased to zero level at the time when sporulation began (Fig. 4). Addition of excess cystine along with other nutrients during growth completely inhibited sporulation in *B. thuringiensis* and vegetative growth continued (Fig. 5). In this experiment observations were stopped when an O.D. of 12·0 was reached. Microscopic examination by staining of the culture at the highest O.D. showed complete absence of spores.

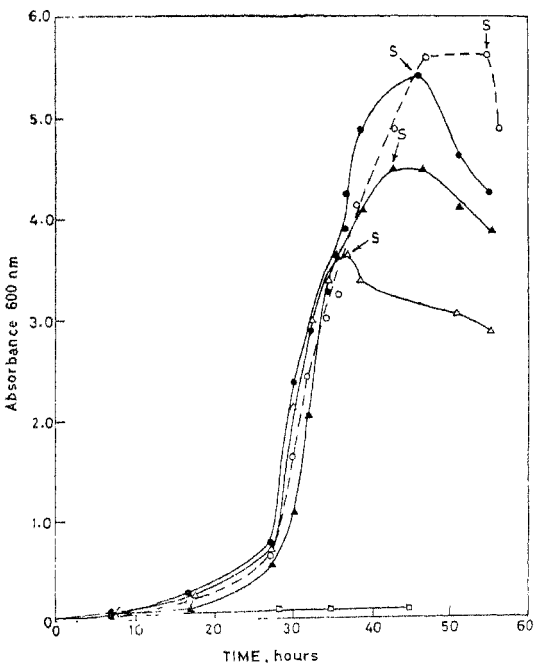

FIG. 3. Effect of increasing concentrations of cystine on growth of *B. thuringiensis*. (□——□)—nil addition; (△——△)—0·01% w/v; (▲——▲)—0·02%; (●——●)—0·03%; (○——○)—0·04%.

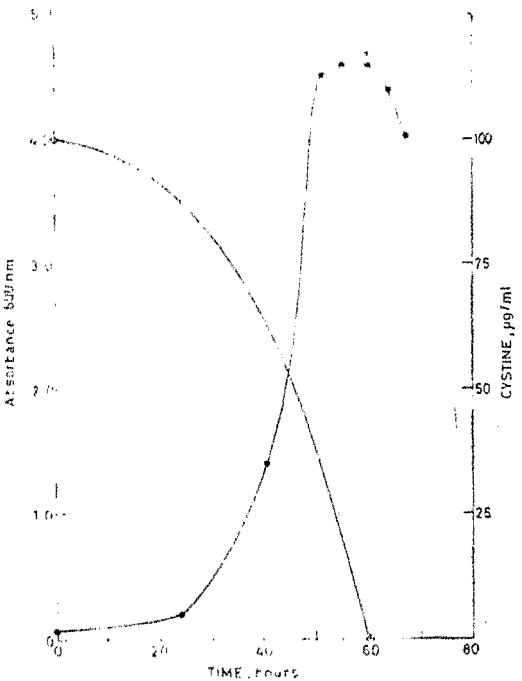

FIG. 4. Effect of cystine concentration in the medium on growth and sporulation in *B. thuringiensis*. (●——●)—growth; (○——○)—cystine concentration.

4. DISCUSSION

Nickerson and Bulla[6] had showed that citrate, aspartate and glutamate promoted good growth of 18 strains of *B. thuringiensis*, whereas malate, fumarate, succinate or oxalacetate did not promote growth of the strains. Few of the strains tested grew on malate. These workers had also reported that supplementation with vitamin mixture did not elicit any growth response in 17 out of the 18 strains tested. It was emphasized that *B. thurin-*

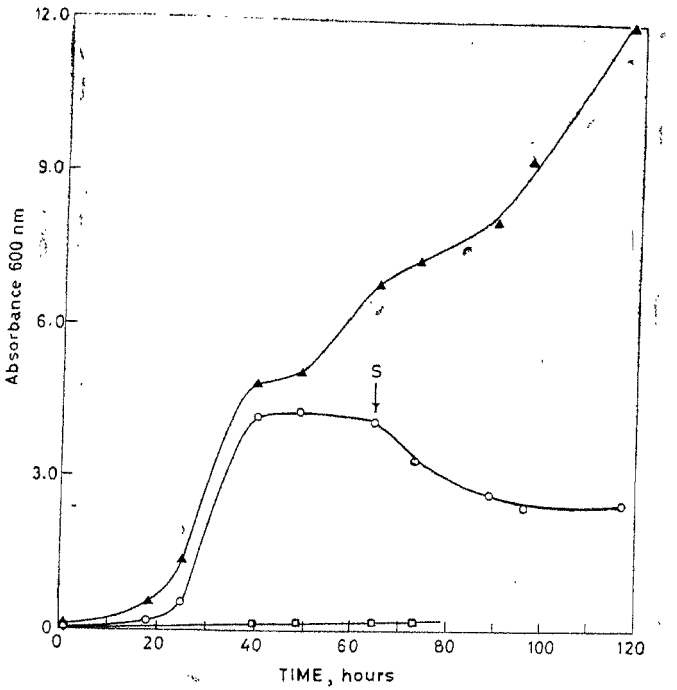

FIG. 5. Effect of addition of excess cystine in the medium on growth and sporulation in *B. thuringiensis*. (□——□)—control without cystine; (○——○)—medium containing 1% glucose-mineral salts and 0·05% cystine; (▲——▲)—medium containing 1% glucose-mineral salts with 0·05% cystine and additions of cystine and nutrients during growth as follows: 100 mg cystine suspended in 2·0 ml H_2O was sterilised and added at each turbidity reading. One gm glucose in 2 ml H_2O was added at each alternate point on the curve. Mineral salts mixture including ammonium sulphate was added after 49 hr growth (fourth point on the curve).

Note: 'S' in all the figures represents beginning of sporulation.

giensis does not grow in a glucose–mineral salt medium without addition of growth factors. Cohner and Hansen[6] had reported growth inhibition of *B. thuringiensis* by isoleucine, leucine and serine. The inhi-

bition of the latter could be overcome by methionine. Singer and Rogoff[7] have reported growth inhibition of *B. thuringiensis* by leucine, isoleucine, threonine and serine while Shieh and Rogoff[8] showed growth inhibition by histidine which could be reversed by glycine.

Our work confirms these observations. The finding of promotion of growth, inhibition of sporulation and crystal formation by cystine represents first report. From the applied point of view this finding could be used to devise an inexpensive growth medium for obtaining large quantities of spore-crystal mixture for the manufacture of commercial spray formulations. This could be done by adjusting the concentration of cystine to prolong vegetative growth.

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