

# Partial Characterization of the Factor Responsible For Tryptophanless Death in *Bacillus subtilis*

SERGIO BARLATI,<sup>1</sup> AND IRENE MAJERFELD

Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

Received for publication 10 November 1969

The decline in colony-forming ability observed during tryptophan starvation of *Bacillus subtilis* auxotrophs is a concentration-dependent phenomenon. It does not manifest itself when the initial cell concentration is  $10^6$  cells/ml or lower. This property has been used to test the killing activity of different fractions of the dying cells. Most of the activity recovered is found in the supernatant fluid of the starved culture. Sensitive and resistant strains can be identified. Active supernatant fluids can only be isolated from tryptophan auxotrophs sensitive to tryptophanless death. Resistant cells neither produce nor respond to the factor, and sensitive cells respond only when deprived of tryptophan. The killing activity is continuously produced and released into the medium at least up to 4 hr after removal of tryptophan from the culture. The killing activity is deoxyribonuclease-, ribonuclease-, and heat-resistant.

When actively growing cultures of *Bacillus subtilis* strain SB57 are starved of tryptophan, they undergo a decline in colony-forming ability at a rate of about 50% of the population per hour (5). After 6 hr of starvation, the viable count levels off at a value that is 0.5 to 5% of the original number of cells.

In the preceding paper, we demonstrated that tryptophanless death (Trp-LD) is a reversible phenomenon (5). Viability can be restored not only by addition of tryptophan or indole, a precursor that can be converted into tryptophan, but also by tryptophan analogues like 5-hydroxytryptophan (5-Htrp) and 5-methyl-tryptophan, compounds that are incorporated into the proteins of *B. subtilis* during tryptophan starvation (1).

Both Trp-LD and recovery require protein synthesis, which seems to be dependent on the presence of stable ribonucleic acid (RNA) molecules.

In the present paper, we describe another form of recovery which is independent of protein synthesis. Attempts for a partial characterization and purification of the killing factor are also reported.

## MATERIALS AND METHODS

**Strains.** The following strains, all derivatives of Marburg indole-requiring *B. subtilis* 168M, were used: SB57 ( $arg^- trp_2^-$ ); SB202 ( $tyr^-$ ,  $trp_2^-$ ,  $his^-$ ,  $aro_2^-$ ); and SB25 ( $his_2^- trp_2^-$ ).

<sup>1</sup> Present address: Istituto di Genetica, Università di Pavia, 27100 Pavia, Italy.

Media and conditions for growth and amino acid starvation are described in the preceding paper (5). Occasionally the medium was enriched with 0.5% casein hydrolysate or with a 20  $\mu$ g/ml amino acid mixture.

**Preparation of supernatant fraction and extract.** After 90 min of tryptophan starvation, the culture was centrifuged for 5 min at 7,000 rev/min. The upper part of the supernatant fraction was removed and stored frozen. The cells were washed with Spizizen minimal medium (9) supplemented with 0.5% glucose and  $10^{-2}$  M glutamic acid (S medium) containing 20% sucrose (20% SuS); they were then resuspended in 1% of the original volume of the same medium. Lysozyme dissolved in 20% SuS was added to a final concentration of 500  $\mu$ g/ml, and the cells were incubated at 37 C. When most of the cells were transformed to protoplasts, they were collected by centrifugation, rinsed twice with 2 ml of 20% SuS, and resuspended in one-tenth the original volume of S medium with 5  $\mu$ g of deoxyribonuclease and ribonuclease. The lysate was clarified by centrifugation (5 min at 8,000 rev/min) and stored frozen.

## RESULTS

**Dilution effect on Trp-LD.** It was observed that the decrease in colony-forming ability that occurs during tryptophan starvation is dependent on the initial cell concentration.

The maximal loss of viability undergone by SB57 takes place when the initial cell concentration is between  $10^7$  to  $10^8$  cells/ml (Table 1).

The disappearance of Trp-LD at a higher cell concentration seems to be due mainly to poor aeration. In fact, at high cell concentration, the

TABLE 1. *Effect of cell concentration on survival during tryptophan starvation*

Initial cell concn <sup>a</sup>	Viable count after 300 min of tryptophan starvation
$2.2 \times 10^8$	$1.5 \times 10^8$
$2.2 \times 10^7$	$3.5 \times 10^6$
$2.2 \times 10^6$	$1.1 \times 10^6$
$2.2 \times 10^5$	$3.2 \times 10^5$
$2.2 \times 10^4$	$8.4 \times 10^4$

<sup>a</sup> Logarithmic-phase SB57 cells were washed, resuspended in medium without tryptophan, and diluted to the concentrations shown. Viable counts were followed as a function of the time of incubation at 37 C.

rate of Trp-LD is dependent on the size of the incubation flask.

A  $10^2$  to  $10^5$  times dilution of the cells in S medium with or without arginine at any time during tryptophan starvation brings full recovery of the apparently dead cells in a way similar to that produced by tryptophan analogues (Fig. 1).

Recovery in the presence of tryptophan analogues in a concentrated culture requires protein synthesis (5). Since viability is regained by dilution even in the absence of arginine, it could be argued that the two forms of recovery are due to different processes.

It seems that during Trp-LD the cells elaborate a compound whose effect can be inactivated or diluted in liquid medium but not on solid medium.

Recovery after dilution is reminiscent of the liquid holding recovery observed in irradiated cells of *Escherichia coli* K-12 and B (3, 4, 6). In both cases, there is a gradual restoration of viability that can occur in the absence of protein synthesis; but to recover from Trp-LD, cells have to be diluted at least 100 times.

**Interactions between sensitive and resistant strains.** By using tryptophan auxotrophs carrying other genetic markers and thus permitting independent scoring of viability, it was possible to test the possibility of release of the killing agent into the medium and the interactions between various strains. A series of stocks were tested, and it was possible to identify two classes of tryptophan auxotrophs that were defined as sensitive or resistant, depending upon whether or not loss of viability was observed when incubated in the absence of tryptophan. Table 2 summarizes the results obtained when concentrated cultures of sensitive and resistant strains were mixed with diluted and concentrated cultures of sensitive and resistant strains. A sensitive strain like SB25 induces Trp-LD in another sensitive strain like SB57 in conditions in which the latter

would not spontaneously die, but it cannot kill resistant strains like 168M or SB202. A resistant strain, such as SB202, does not prevent the killing of a dying culture and does not induce Trp-LD in a sensitive strain in conditions in which the sensitive strain would not spontaneously die. This suggests that a resistant strain fails to produce the killing factor (and is resistant to it), nor does it make a neutralizing compound.

**Killing activity in the supernatant fraction of a dying culture.** To better characterize the killing factor present in a dying culture, an assay for its activity was needed. Standard tests to determine antibiotic activity failed to give positive results. An assay was devised which takes advantage of the fact that dilution of a culture undergoing Trp-LD in fresh medium prevents further death or allows recovery. Alternatively, when the cells are diluted in a medium containing the killing activity, they continue to die. It should be emphasized that cells have to be preincubated in the

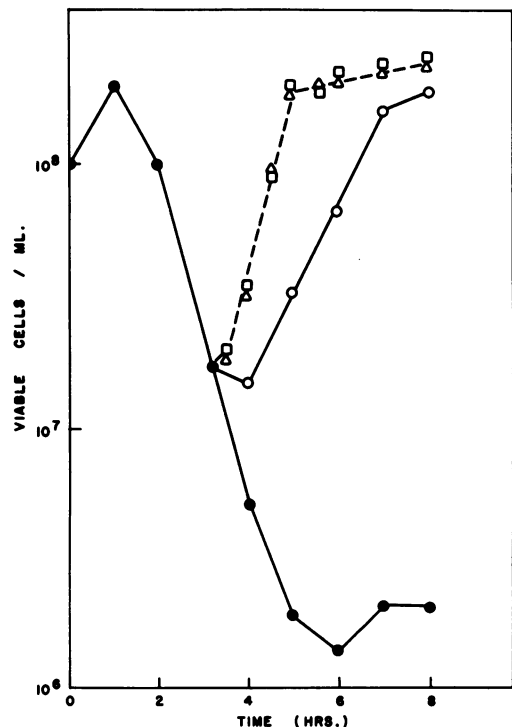


FIG. 1. Recovery from Trp-LD by dilution or by addition of analogues. After 210 min of tryptophan starvation,  $5 \mu\text{g}$  of 5-methyl-tryptophan per ml was added to a sample of the culture (○). Another sample was diluted  $10^6$  times in fresh medium of the same composition in the absence (△) or in the presence (□) of 5-methyl-tryptophan. In the dilution experiment the cell concentrations were corrected for the dilution factor. Trp-LD (●).

TABLE 2. Response of Trp-LD-resistant and -sensitive strains in mixed incubation<sup>a</sup>

Killing of	Incubation in presence of			
	SB57 (high cell concn) <sup>b</sup>	SB202 (high cell concn)	SB25 (high cell concn)	None
SB57 (low concn) <sup>b</sup>		- <sup>c</sup>	+ <sup>c</sup>	-
SB57 (high concn)		+	+	+
SB202 (low concn)	-		-	-
168M (low concn)	-	-	-	-
SB25 (high concn)	+		+	+

<sup>a</sup> The cultures were mixed together at the time when tryptophan starvation was initiated. Additional genetic markers permitted independent scoring of strain viability.

<sup>b</sup> High cell concentration:  $2 \times 10^7$  to  $8 \times 10^7$  cells/ml; low cell concentration:  $2 \times 10^6$  to  $8 \times 10^6$  cells/ml.

<sup>c</sup> (-) No loss in viability, (+) loss in viability during tryptophan starvation of the cultures listed in the left column in presence of those listed at the top of the table.

absence of tryptophan to be able to respond to the killing activity present in the medium.

Most of the recovered activity is found in the supernatant fraction of a culture undergoing Trp-LD. Only about 10% of the activity found in the supernatant fraction was recovered in the extract, whereas none was found in the other cellular fractions. The supernatant fluid and extract prepared from SB57 cells simultaneously starved for arginine and tryptophan, conditions that prevent Trp-LD, have no killing activity.

The supernatant fluid and the extract of a resistant strain culture (like 168M), obtained in conditions of tryptophan starvation, are not active on any sensitive or resistant culture, as would be predicted from the results of the mixed culture experiments. Likewise, 168M cells do not die when diluted in a supernatant fluid that has killing activity on SB57 cells (Fig. 2).

The supernatant activity is identified as that responsible for Trp-LD, since 5-Htrp added together with the supernatant fluid prevents the killing (5), and its addition at a later time produces a rapid recovery of the lost viability (Fig. 3).

Supernatant fluids obtained from the dying cultures can be stored frozen. They remain active at least for several months.

Figure 4 shows the kinetics of release of the killing activity into the supernatant fraction.

Supernatant fluids were prepared at different times during tryptophan starvation and assayed as previously described. After 5 to 15 min of starvation, activity accumulated in the supernatant fraction was sufficient to be detected. After 30 min, no further increase in detectable activity was observed.

When the cells are resuspended in fresh medium after 90 min of tryptophan starvation, they continue to die, and the new supernatant fraction isolated at a later time will be as active as the first one. This implies that the killing activity can be continuously released during tryptophan starvation.

**Characterization of the killing activity.** Pretreatment of the supernatant fraction with 5  $\mu$ g of ribonuclease or deoxyribonuclease per ml for 10 min at 37 C does not destroy the killing activity. Heating for 10 min at 100 C also failed to inactivate the supernatant fraction, but 1 hr of incubation at 37 C removed most of the activity.

Since the addition of tryptophan allows for recovery of the apparently dead cells, the preferential liberation of this amino acid by Pronase

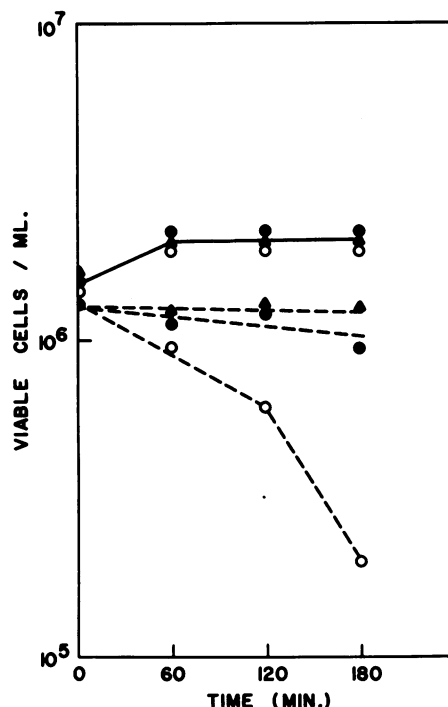


FIG. 2. Supernatant fraction activity of Trp-LD-sensitive and -resistant strains. 168M (—) or SB57 (---) cells starved of tryptophan for 90 min were diluted 200 times into fresh minimal medium lacking tryptophan (●), 168M supernatant fraction (▲), or SB57 supernatant fraction (○). Supernatant fractions were prepared as described.

makes this enzyme unsuitable to test the hypothesis that the killing factor is a protein. Indeed, cell growth was observed when such an experiment was performed.

The killing activity is lost by pretreatment of the supernatant fraction with chymotrypsin, but it was not possible to rule out that the enzyme exerts its action on the cells themselves used for the assay. Preincubation of the active supernatant fraction with trypsin and subsequent neutralization with pancreatic trypsin inhibitor does not destroy the killing activity present. This is the case also when trypsin is not inactivated with its inhibitor, indicating that the enzyme by itself does not damage the cells present in the assay. However, owing to the specificity of trypsin, this result does not exclude the possibility that the killing factor is a protein.

All attempts to purify the factor failed. The activity was lost by fractionation on a G25 Sephadex column; nor could it be recovered

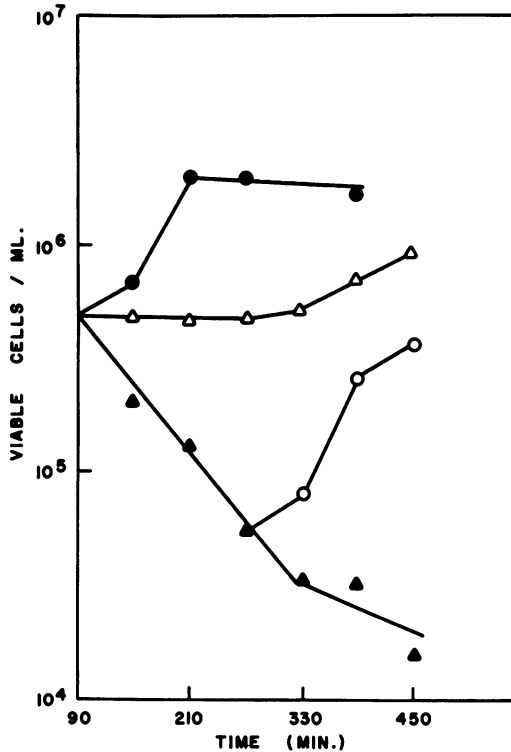


FIG. 3. Effect of 5-hydroxy-tryptophan on the killing activity of SB57 supernatant fraction. SB57 cells starved of tryptophan for 90 min were diluted 200 times into tryptophan-free medium ( $\bullet$ ) or previously prepared SB57 supernatant fraction ( $\blacktriangle$ ). At zero time ( $\triangle$ ) and at 180 min ( $\circ$ ), 20  $\mu\text{g/ml}$  of 5-hydroxy-tryptophan was added to portions of the cells diluted in SB57 supernatant fraction.

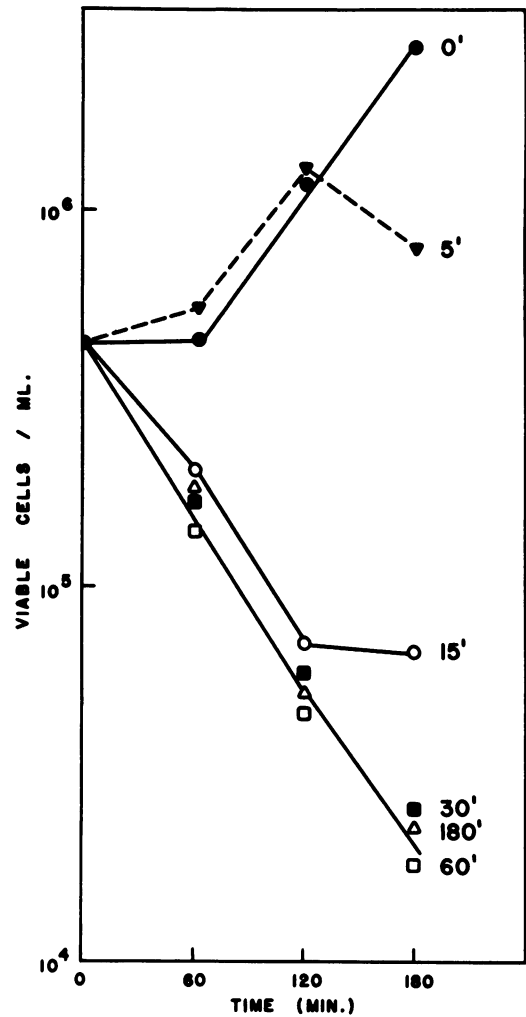


FIG. 4. Kinetics of release of the killing activity into the supernatant fraction. Supernatant fractions prepared from SB57 cultured after 0, 5, 15, 30, 60, and 180 min of tryptophan starvation were assayed on diluted SB57 as described in Fig. 2. The viability of the diluted cells in presence of the various supernatant fractions is plotted as a function of the time of incubation at 37 C.

from the filtrate of a dying culture or its supernatant fraction after filtration through Millipore (Millipore Corp., Bedford, Mass.) or Nucleopore (General Electric Co.) filters or dialysis.

The supernatant activity did not sediment after 2 hr of centrifugation at  $98,600 \times g$ .

Upon xylol or butanol extraction of the supernatant fraction, the activity was recovered in the water phase, thus indicating that it is not likely to be a lipid.

## DISCUSSION

We have shown that sensitive strains can be induced to undergo Trp-LD in conditions in which they would not die spontaneously. This can be produced by simultaneous incubation with a dying culture or by dilution of the cells in the previously prepared supernatant fraction of a culture undergoing Trp-LD. Tryptophan auxotrophs resistant to Trp-LD cannot be induced to die in the conditions mentioned above. Neither can the supernatant fractions prepared from resistant cultures prevent the killing of a sensitive strain, thus indicating that resistance is not provided by a neutralizing compound.

These results point to the existence of a killing factor that is released into the medium by the sensitive strain of a dying culture and that can exert its action on other sensitive cells but not on cells from a resistant strain. The resistant strain, on the other hand, does not seem to produce, or at least to release, the killing factor, since it does not die of Trp-LD and its supernatant fractions are not able to produce loss of viability on a sensitive strain in conditions in which it would not die spontaneously.

Schmitt and Freese (7) reported the isolation of several antibiotic activities from the medium of *B. subtilis* cultures undergoing sporulation. These compounds are extractable with butanol and cause cell lysis of growing *B. subtilis* cultures. These properties make them different from the killing factor produced during tryptophan starvation, since the latter cannot be extracted by butanol, is active only on tryptophan-starved cells, and does not cause lysis.

It is more difficult to rule out the possibility that the factor involved in Trp-LD is related to other peptide antibiotics produced by many species of bacilli (2). These antibiotics are not found free in the medium, and their synthesis is not inhibited by high concentrations of chloramphenicol or actinomycin. The factor under investigation here, on the other hand, is released into the medium and its production or activity is prevented by chloramphenicol.

We did not make a direct study of the effect of chloramphenicol or actinomycin on the production of the killing factor. However, we can make some inference from the effect of these agents on autointoxication of tryptophan-deprived cultures (5). The effect of the killing factor is blocked by chloramphenicol; about its production we can say nothing. It is produced in bacteria whose RNA synthesis is blocked by actinomycin. This requirement is analogous to that found for the tryptophan analogue-induced recovery reported in the previous paper (5).

In the previous paper (5) we have also discussed the unlikely possibility of phage induction (8, 10) being related to Trp-LD. It can be added that the killing activity is recovered in solution after centrifugation in conditions that would have sedimented phage particles (8).

Kinetic studies on the release of the killing factor in the supernatant fraction of a dying culture reveal that its maximal level in the medium is reached before the cells start showing any decrease in viability.

After tryptophan starvation, most of the cells can utilize this amino acid or its analogues in liquid medium but not on solid medium. This suggests that the killing factor is acting at the level of the utilization of tryptophan.

Assuming that the factor binds to cellular sites on the cell surface and that it is in equilibrium with the surrounding liquid phase, those cells with altered surfaces at the time of plating may not be able to form colonies on solid medium, because of their inability to utilize the required amino acid. When the dying cells are diluted and incubated in liquid medium (before plating), the killing factor may reach a new equilibrium with the medium. The low cell concentration may not permit attainment of a high level of killing factor in the medium so that recovery can take place. The resistance could be explained by assuming that the cellular site, to which the killing factor should bind, is modified. This would not explain the resistance of a sensitive strain to supernatant fractions from a resistant culture, unless one postulates that the resistant strain does not release or produce the factor. Since resistant colonies can be isolated fairly easily from a sensitive strain, it is difficult to believe that these characteristics are related with two independent changes. It seems more likely that a unique change might be responsible for both phenotypic expressions: resistance to supernatant fluids from sensitive cultures and absence of killing activity from supernatant fluids of resistant cultures.

## ACKNOWLEDGMENTS

We are grateful to Joshua Lederberg for support and discussion throughout this study and to Orio Ciferri for discussion and critical reading of the manuscript.

This investigation was supported by Public Health Service research grant AI-05610 from the National Institutes of Allergy and Infectious Diseases, and by the cooperative U.S.-Italy Science Program (GB 7785 of the National Science Foundation and 115 0308 04633 of the Consiglio Nazionale delle Ricerche).

## LITERATURE CITED

1. Barlati, S., and O. Ciferri. Incorporation of 5-methyl- and 5-hydroxytryptophan into the protein of *Bacillus subtilis*. *J. Bacteriol.* **191**:166-172.

2. Bodansky, M., and D. Perlman. 1969. Peptide antibiotics. *Science* **163**:352-363.
3. Charles, R. L., and L. N. Zimmerman. 1956. Dark reactivation in ultraviolet irradiated *Escherichia coli*. *J. Bacteriol.* **71**:611-616.
4. Ganesan, A. K., and K. C. Smith. 1968. Recovery of recombination deficient mutants of *Escherichia coli* K-12 from ultraviolet irradiation. Cold Spring Harbor Symp. Quant. Biol. **33**:235-242.
5. Majerfeld, I., S. Barlati, and O. Ciferri. Tryptophanless death in *Bacillus subtilis*. *J. Bacteriol.* **101**:350-354.
6. Roberts, R. B., and E. Aldous. 1949. Recovery from ultraviolet irradiation in *Escherichia coli*. *J. Bacteriol.* **57**:363-375.
7. Schmitt, R., and E. Freese. 1968. Curing of sporulation mutant and antibiotic activity of *Bacillus subtilis*. *J. Bacteriol.* **96**:1255-1265.
8. Seaman, E., E. Tarmy, and J. Marmur. 1964. Inducible phages of *Bacillus subtilis*. *Biochemistry* **3**:607-613.
9. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Nat. Acad. Sci. U.S.A.* **44**:1072-1078.
10. Subbaiah, T. W., C. D. Goldthwaite, and J. Marmur. 1965. Nature of bacteriophages induced in *Bacillus subtilis*, p. 435-446. In V. Bryson and H. J. Vogel (ed.), *Evolving genes and proteins*. Academic Press Inc., New York.