GENETICS OF

SPORULATION

IN BACILLUS SUBTILIS

AN INVESTIGATION ON THE GENETICS OF SPORULATION IN BACILLUS SUBTILIS

by

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SCOPE AND CONTENTS: The investigation was carried out in order to eliminate a hypothetical sporulation factor from wild-type strains of Bacillus subtilis using agents known to remove episomes. The subsequent investigations produced no mutants with properties predicted for strains missing the sporulation factor. When cells were exposed to UV light prior to treatment with acridine dyes there was, however, a significant increase in the production of normal sporulation mutants.

PREFACE

This thesis describes studies carried out in the Research Unit for Biochemistry, Biophysics and Molecular Biology, McMaster University, from June 1964 to August 1965. Except where others are specifically mentioned, it consists entirely of my own original work. No similar thesis has been submitted at any other university.

My grateful thanks are due to my supervisor, Dr. I. Takahashi for advice and encouragement throughout the work, and also for the donation of the strains of Bacillus subtilis upon which this work was founded.

I am also deeply indebted to the Government of Ontario for the award of an Ontario Graduate Fellowship covering the period of the research reported herein.

Research Unit for Biochemistry,
Biophysics and Molecular Biology,
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CHAPTER I

INTRODUCTION

Although asporogenous mutants in Bacillus species have been known for many years (Pasteur, 1881, Roux, 1890 and Eisenberg, 1912), no genetic studies have been carried out until recently. In their first attempt to study the genetics of sporulation using the transformation technique (Spizizen, 1958, 1959), Schaeffer and his co-workers used two transformable sporogenic strains of Bacillus subtilis, the one being a wild-type Marburg strain, the other requiring indole for growth (Schaeffer et al, 1959). Both strains formed brown colonies on nutrient agar after sporulation. One colony in 10,000, however, was white and did not contain spores. They isolated two types of white, asporogenous mutants; those which occasionally produced a sporogenic clone--called reversible mutants--and those which never gave rise to sporogenic clones--called irreversible mutants.

Both kinds of mutants could be transformed to the sporogenic condition using DNA from the wild-type strain. They envisioned from their experimental results a similarity between a factor for sporulation and prophage. Both factors can be removed from the cell in their entirety, giving rise in the case of the sporulation factor to irreversible sporulation mutants or they can both be subject to mutation causing reversible asporogenous strains in Bacillus.

Later, working with the same strains of B. subtilis, they more clearly defined the types of mutants they had isolated (Schaeffer and Ionesco, 1960). The term asporogenous, which was previously general, was now reserved for those mutants which had a rate of reversion to the sporogenic state of less than 10^{-8}. Mutants which exhibited a greater rate of reversion (higher than 10^{-6}) were called oligosporogenous. In addition there were white mutants called albinos which were in fact sporogenous but which were unable to produce the brown pigment on nutrient agar. In their transformation experiments they used strains which were double mutants, both asporogenous and unable to synthesize tryptophan. Carrying out reciprocal transformations between four of the double mutants and a wild-type strain, they came to the following conclusions:

1) If two mutants are defective at the same genetic site, they will not be able to transform one another to the sporogenous condition.
2) Transformation of a mutant to sporogeny occurs with the same frequency whether DNA from another mutant or from the wild-type is used.
3) One strain, although readily transformable to prototrophy was not transformable to sporogeny even when DNA from sporogenous bacteria was used.

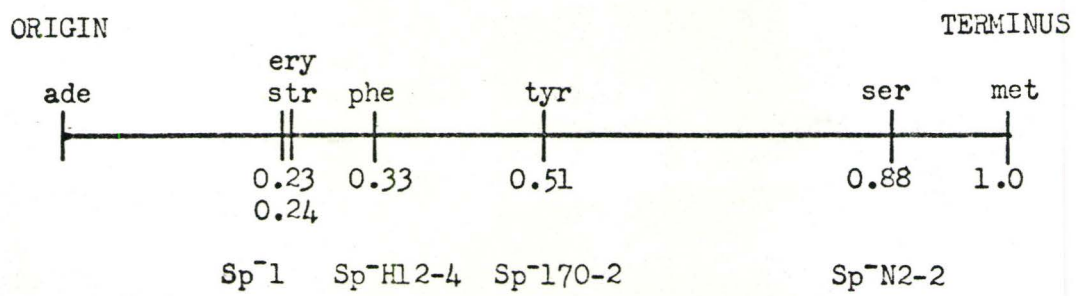
As a method of genetic transfer, transduction had first been discovered in Salmonella (Zinder and Lederberg, 1952). Takahashi (1961) demonstrated that sporogenesis as well as nutritional characteristics could be transduced in B. subtilis by the bacteriophage PBS 1. An

irreversible asporogenous strain, B. subtilis $Sp^{-}1$, derived from a wild-type Marburg strain, was able to produce sporogenous clones after transduction using lysates of a wild-type strain SB19. This same mutant could not, however, be transformed to sporogeny using DNA derived from the same sporulating wild-type.

Transduction studies (Takahashi, 1965a) using bacteriophage PBS 1 (Takahashi, 1961) produced results similar to those obtained by Schaeffer and his colleagues working with transformation. This supported the previous evidence that the genes are many at which a mutation can affect sporulation and that they are not linked with one another. Spizizen, on the other hand, reported all of his mutational sites linked to a group of genes which control the synthesis of a proteolytic enzyme and a wall-lytic enzyme (Spizizen, Reilly and Dahl, 1963).

A high degree of genetic linkage between auxotrophic markers or antibiotic markers and some sporulation markers was obtained (Takahashi, 1965a). One strain, B. subtilis $Sp^{-}1$, could be jointly transduced to sporogeny and to resistance to antibiotics such as streptomycin, erythromycin and neomycin. Other mutants, $Sp^{-}N2$-2 (asporogenous and requiring serine), $Sp^{-}170$-2 (asporogenous and requiring tyrosine), and $Sp^{-}H12$-4 (asporogenous and requiring phenylalanine) could be jointly transduced to sporogeny and prototrophy. Schaeffer (1961) failed to observe any auxotrophic markers which were associated with his spore markers. This may have been due, in part, to the fact that all of his sporulation mutants were derived from one strain, 168 (Takahashi, 1965a). The observed close linkage between sporulation markers and other genetic markers indicate that the sporulation markers are not clustered in a small region of the chromosome.

Assuming that the replication of a bacterial chromosome was polar or oriented and that it proceeds at a uniform rate, it was possible to construct a genetic map giving the location of several known auxotrophic markers for B. subtilis (Yoshikawa and Sueoka, 1963). Once replication has been initiated, those genes at the origin will appear in any sample of DNA, twice as frequently as those genes at the terminus. In an exponentially growing population, therefore, there should be a predictable difference in the frequency of genes depending on their position on the chromosome. In a stationary-phase culture the numbers of a gene at the origin should equal the numbers of a gene at the terminus since the chromosome is not replicating. By comparing the ratio, for different genes, of the number of transformants produced from DNA isolated from logarithmic-phase cells to the number of transformants produced from DNA isolated from stationary-phase cells, Yoshikawa and Sueoka were able to give the relative positions of these genes on the chromosome. Furthermore by the use of Yoshikawa and Sueoka's technique (1963), Takahashi (1965b) succeeded in locating the sporulation markers on the chromosome by determining the position of the auxotrophic or antibiotic resistance markers to which the sporulation markers were closely linked. The following is the proposed chromosome map:



NOTE:

1) ade - adenine

str - streptomycin resistance

ery - erythromycin resistance

phe - phenylalanine

tyr - tyrosine

ser - serine

met - methionine

This clearly indicated that genes controlling the formation of spores were not clustered in a small region of the chromosome but were located far apart. One can deduce from this that sporulation may be controlled by a number of operons and that a mutation at any one of them would prevent the formation of mature spores.

Six intermediary stages were observed cytologically in the process of sporulation (Ryter, et al, 1961, and Schaeffer, et al, 1963). All mutants examined were blocked at one of these morphological stages. When these sporulation mutants were grown in a suitable medium, sporulation proceeded up to the point at which they were blocked genetically. Sporulation, then, seems to follow a logical sequence of biochemical events from initiation of sporulation to the formation of mature spores.

Two types of genes for sporulation were envisioned, those regulating each sequence of biochemical events, called regulator genes and those called structural genes, which determine the amino acid sequence of proteins (Schaeffer, et al, 1963). Sporulation genes are, like other genes, present in all stages of the life cycle but they are repressed during vegetative growth. Once sporulation is initiated these

genes may be derepressed by a sequential induction (Schaeffer, et al, 1963).

Wild-type strains of Bacillus species produced an antibiotic (Ab) active on Staphylococcus aureus whose action could be observed on nutrient agar plates (Balassa et al, 1963 and Spizizen, 1965). Some sporulation mutants, however, were not able to synthesize the antibiotic. When these asporogenous mutants were transformed or transduced to sporogeny they always acquired the ability to form the antibiotic. Because no linkage could be invoked between sporulation and production of the antibiotic, it was concluded that there was a physiological relationship between the two, the antibiotic arising from a sporulation-specific reaction (Balassa, Ionesco and Schaeffer, 1963). By examining these two types morphologically, it was discovered that those mutants unable to produce the antibiotic were blocked at a very early stage in the sporulation cycle; they were not able to progress to the stage which first exhibits a morphological change discernible by electronmicroscopy (Ryter, Schaeffer and Ionesco, 1966). Thus by their action on Staphylococcus they could easily distinguish mutants which were blocked at some point earlier than the first stage from those which were blocked later.

Using B. licheniformis, Bernlohr discovered that the antibiotic was produced only when conditions suitable for sporulation existed in the culture medium (Bernlohr and Novelli, 1960a and 1960b). There appeared to be some competition between the sporulation process and the production of antibiotic since optimum conditions for the formation of spores gave little antibiotic and conditions of minimum spore formation gave a much higher yield of the antibiotic.

A protease is also produced by wild-type strains and by all sporulation mutants blocked after the first stage. Some mutants blocked before the first stage and therefore unable to produce the antibiotic can produce the protease. Results similar to those revealing a competition between sporulation and antibiotic production were obtained with the protease (Bernlohr and Novelli, 1964). Glucose, a known inhibitor for spore formation, also inhibited the production of the protease. It was suggested that protease was a product of presporulating events in the cell and that it could be used for the degradation of vegetative cellular material for later use in sporulation. The antibiotic could conceivably inhibit those genes which transcribe for the vegetative state (Halvorson, 1965). Functioning in this way both the antibiotic and the protease would be required in the early stages of sporulation.

An unusual strain had been isolated which was derived spontaneously from B. subtilis H12 designated Sp⁻H12-3 (asporogenous and requires phenylalanine) (Takahashi, 1965a). This mutant never produced any revertants to sporogeny under any conditions. It could act as donor to all other asporogenous mutants and produced recombinants but it could not itself be transduced to the wild-type even when a wild-type strain was used as donor. It appeared to be physiologically competent in transduction since it could produce erythromycin-resistant or prototrophic transductants at a normal frequency. From the behaviour of Sp⁻H12-3, one might suspect that it has mutations at many sites on the chromosome or a deletion in the sporulation genome. A far more interesting

hypothesis is the episome factor for sporulation proposed by Schaeffer, Ionesco and Jacob (1959). They conceived that there were two types of mutants; those which could revert with a low frequency, having a chromosomal mutation causing asporogeny and those which were irreversibly asporogenous, having lost as a whole some extra-chromosomal factor.

In 1958, Jacob and Wollman proposed the term "episome" to describe accessory extra-chromosomal genetic elements which could exist either autonomously or integrated with the chromosome (Jacob and Wollman, 1958). Later, they provided the criteria for the recognition of characters determined by genetic structures having the properties of episomes (Jacob and Wollman, 1961). Such characters must be dispensible since episomes are not necessary constituents of the cell-sporulation is not necessary for the survival of the cell. Such characters may be lost irreversibly since episomes themselves can be lost irreversibly--at least one irreversible asporogenous strain, Sp^{-}H12-3 is known to us. An alternation of phenotype may occur corresponding to the alternation of the episome from the integrated to the autonomous state--the alternation from the vegetative cell to the spore is a process in which there is a marked difference in phenotype. Fulfilling these criteria does not necessarily mean that a given system has episomic control of a genetic character within it. Deletions, multisite mutations, purely cytoplasmic structures, epistasis or environmentally controlled regulation mechanisms may determine one or several of these characteristics or criteria.

The artificial elimination of episomes, notibly the F-factor or sex factor of E. coli could be carried out by treating the cells

with various substances of both organic and inorganic origin. The salts of heavy metals such as cobalt or nickel have been effective in converting male strains of E. coli to female strains by irreversibly removing the episomal F-factor (Hirota, 1956). Acridine dyes were more effective as curing agents for the F-factor. Strains which were stable to metal treatment were easily converted to female cells by the acridine treatment (Hirota and Iijima, 1957). Later it was observed that only F^+ male strains could be cured of the F-factor and not the high frequency recombination (Hfr) male strains (Hirota, 1960). Maleness in Hfr cells is inherited chromosomally as a gene linked to certain markers, as determined by genetic recombination. The transfer of the F-factor apparently unlinked to the host chromosome and the non-segregational uniform inheritance of F-factors corresponds to cytoplasmic transfer of the male determinant. These facts supported the postulate that acridines eliminated episomal factors in the autonomous state and not those which were integrated with the chromosome.

Transition of an episome from the integrated form to the autonomous state may be induced by a variety of chemical and physical agents. Lwoff and his co-workers (1950) noted that treatment of certain lysogenic bacteria with ultraviolet light induced the development of mature phages (autonomous state) from the prophage (integrated state). This induction has subsequently been shown to be elicited by a variety of physical and chemical agents, as well as by manipulations of metabolic balance (Driskell-Zamenoff, 1964).

Other attempts have been made to eliminate the bacterial episome. Using the hypothesis that episomes were in some way associated with the cell membrane, Landman (Kawakami and Landman, 1965) treated cells with reagents which damaged the membrane expecting to remove or impair in some way the extranuclear episomes. Drug resistance factor (R-factor) was eliminated from E. coli and Salmonella by treating the cells with penicillin, which was known to attack primarily the cell membrane. The tendency to be cured seemed to depend on the special characteristics of both the episome and its host bacterium.

In the same year, the colicin factors which could not be removed from the cell by acridine treatment were eliminated from thymine-less mutants under conditions of thymine-limited growth. Other extra-chromosomal elements were eliminated with thymine deprivation but there was a striking difference in the susceptibility to elimination shown by various autonomous episomes (Clowes, Moody and Pritchard, 1965).

Encouraging evidence that cytoplasmic factors were possibly involved in the genetics of sporulation was uncovered when white colonies, usually typical of the asporogenous phenotype, where isolated after treatment of B. subtilis with acriflavine (Rogolsky and Slepecky, 1964). Cells treated during early log phase were most affected by the dye. These mutants, as yet, have not been categorized into sporulation mutant types and it is not known whether truly irreversibly asporogenous bacteria were isolated. Bott and Davidoff-Abelson (1966) using acridine orange in their experiments were able to isolate sporulation mutants. All of the mutants proved, however, to be oligosporogenous.

The isolation of mutant Sp⁻H12-3 and such evidences in the past which have indicated the possibility of an extrachromosomal determinant for sporulation have lead to the problem investigated and reported in this thesis. The problem is twofold: to produce irreversible asporogenous mutants similar to strain Sp⁻H12-3 by agents known to eliminate episomes and to study their genetic behaviour as sporulation mutants.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Symbols

Throughout this work the following symbols were used:

sp^{+} - sporogenous

sp^{-} - asporogenous

str^{r} - resistant to streptomycin 1000 ug/ml

ery^{r} - resistant to erythromycin 1 ug/ml

ind - indole

ser - serine

tyr - tyrosine

phe - phenylalanine

leu - leucine

met - methionine

arg - arginine

glu - glutamic acid

UV - ultraviolet light

prot - prototrophic

Strains

Three wild-type strains were used throughout this work, *Bacillus subtilis* strains W, SB19 and SB19E. Strain W was originally isolated

by C. Anagnostopoulos (1961). Strain SB19 (*str*^{r}, *prot*) was isolated by transformation of 168 (*ind*) to prototrophy using DNA derived from 23 (*thr*) (Nester and Lederberg, 1961). Strains 168 and 23 originally came from the collection of Burkholder and Giles (1947). Strain SB19E (*str*^{r}, *ery*^{r}, *prot*) was derived from SB19 (Takahashi, 1965a).

The mutants utilized were as follows:

Strain	Origin	Characteristics
168	Marburg (UV) (Burkholder and Giles, 1947)	*sp*$^+$ *ind*
N2	W (UV) (Takahashi, 1965b)	*sp*$^+$ *ser*
170	W (*str*^{r} - 100 ug/ml) (Takahashi, 1965b)	*sp*$^+$ *tyr*
H12	W (UV) (Takahashi, 1965b)	*sp*$^+$ *phe*
A	Mu8u5u6 (*ade*, *leu*, *met*) (Yoshikawa and Sueoka, 1963) (transformation and UV) (Takahashi, 1965b)	*sp*$^+$ *ade*
M	Mu8u5u6 (*ade*, *leu*, *met*) (Yoshikawa and Sueoka, 1963) (transformation and UV) (Takahashi, 1965b)	*sp*$^+$ *met*
B33	W (UV) (Takahashi)	*sp*$^+$ *arg*

B37	W (UV) (Takahashi)	sp^+ glu
Sp^-1	W (UV) (Takahashi, 1965a)	sp^-, requires casein hydrolysate
Sp^-N2-2	N2 (UV) (Takahashi, 1965a)	sp^- ser
Sp^-170-2	170 (UV) (Takahashi, 1965a)	sp^- tyr
Sp^-H12-3	H12 (spontaneous) (Takahashi, 1965a)	sp^- phe
Sp^-H12-4	H12 (UV) (Takahashi, 1965a)	sp^- phe

The wild-type strains and all of the mutants were supplied by Dr. I. Takahashi. Dr. R.A. Slepecky (Syracuse University) kindly donated for several experiments a culture of his W strain designated W (s). A culture of Staphylococcus aureus Oxford strain was obtained from the Hamilton General Hospital for use in antibiotic assays.

Culture Media

A variety of culture media was used during the course of the investigation. As these media were often modified by the addition of various supplements, only a description of their basic components will be given here. Modifications are described as they are utilized throughout the text. Unless stated otherwise, all media were autoclaved at 120°C for 15 min.

1- Spizizen's Minimal Medium (SMM) - (Spizizen, 1958)

$(NH_4)_2SO_2$	2.0 gm
K_2HOP_2	14.0 gm
KH_2PO_4	6.0 gm
Sodium citrate	1.0 gm
$MgSO_4.7H_2O$	0.2 gm
distilled water	1.0 l

The pH was adjusted to 7.0

To prepare minimal agar, 15 gm of Difco Agar were added.

Before use 5 ml of 10% glucose was added to 100 ml of the medium.

Where biochemical supplements were used in the medium, they were autoclaved separately and added to a final concentration of 50 ug/ml unless otherwise stated.

NOTE: For SMM pH 7.6 the following were used instead:

K_2HPO_4	19.0 gm
KH_2PO_4	2.0 gm

and in addition:

0.02% peptone (Difco)

For sporulation minimal agar the followirg were added after autoclaving separately:

$MnSO_4$ (1.0%)	0.5 ml/l
$FeCl_3$ (0.5%)	0.5 ml/l

In some cases, the SMM which contained one-half the amount of the salts was used as diluent. This was designated as 1/2 SMM.

2- Sporulation Medium (Schaeffer, 1961)

This medium provides the essential requirements for good sporulation in Bacillus subtilis. On agar one can easily distinguish between the brown sporogenous colonies and the white asporogenous colonies.

Nutrient broth (Difco)	8.0 gm
$MgSO_4.7H_2O$	0.25 gm
KCl	1.0 gm
$MnCl_2$ (1.9%)	0.1 ml
distilled water	1.0 l

For Citrate Sporulation agar (CS agar) 11.76 gm Sodium citrate was added.

For Citrate Yeast Extract Sporulation Agar (CYS agar) the following were added:

Sodium citrate	11.76 gm
Yeast extract	0.5 gm

The pH was adjusted to 7.0

To prepare sporulation agar 15.0 gm of Difco Agar were added.

Before use the following sterile solutions were added:

$FeSO_4.7H_2O$ (0.27%)	0.1 ml
$Ca(NO_3)_2.4H_2O$ (2.3%)	10.0 ml

3- Saline Sodium Citrate (SSC)

This medium is used mainly for the extraction of DNA.

NaCl	8.5 gm
Sodium citrate	4.4 gm
distilled water	1.0 l

4- Brain Heart Sporulation Agar (BS agar)

This medium was used for growing fastidious $CoCl_2$ treated organisms to detect sporulation. It contained

Brain Heart Infusion Broth (Difco)	37.0 gm
$MnCl_2$ (1.9%)	0.1 ml
$MgSO_4.7H_2O$	0.25 gm
Agar (Difco)	15 gm
distilled water	1.0 l

The pH was adjusted to 7.0

Before use the following sterile solutions were added:

$FeSO_4.7H_2O$ (0.27%)	0.1 ml
$Ca(NO_3)_2.4H_2O$ (2.3%)	10.0 ml

5- Adsorption Medium (Ad)

This medium was used for the assay of phage.

NaCl	4.0 gm
K_2SO_4	5.0 gm
KH_2PO_4	1.5 gm
Na_2HPO_4	3.0 gm
$MgSO_4.7H_2O$	0.12 gm
$CaCl_2$ (1.0%)	1.0 ml
$FeCl_3$ (0.5%)	2.0 ml
Yeast extract (Difco)	1.0 gm
distilled water	1.0 l

6- Preserving Medium

Because asporogenous mutants cannot form spores, they were stored at -35°C in the frozen state in Penassay Broth which contained 15% glycerol.

7- Transforming Medium

Except for strain 168, which requires a special transformation technique, all transformations were carried out in the following medium:

SMM	100 ml
Glucose (10%)	5.0 ml
Casamino acids (1%) (Difco)	1.0 ml
Yeast extract (1%) (Difco)	1.0 ml

8- Peptone-Glucose Medium (PG)

Peptone (Difco)	10.0 gm
Glucose	2.0 gm
distilled water	1.0 l

Two acridine dyes were used for the acridine treatment of cells. Neutral acriflavine was purchased from Allied Chemical Company and acridine orange from Fisher Scientific Company.

METHODS

Methods which apply generally to all phases of the work are reported in this section. Special techniques or modifications of these general methods are described as they occur. Broth cultures were obtained by inoculating P broth with cells grown on TB agar overnight and incubating

with shaking for 4 hr. Unless otherwise stated all incubations were at 37°C, all cultures were plated using glass spreaders, all shaking was done on a reciprocal shaker at a frequency of 120 strokes per min. and all centrifugations were at 5000 rpm (3020 g) for 15 min. in a Sorvall refrigerated centrifuge using an SS34 rotor.

1- Isolation of Crude DNA for Transformation

A small inoculum of cells grown overnight on TB agar was placed in 30 ml of P broth and the flask was incubated with shaking for 4 hr. The 30 ml of culture was poured into a flask containing 500 ml P broth and the latter was shaken on a reciprocal shaker with incubating for 90 min. The cells were spun down, the supernatant liquid discarded and the cells were resuspended and collected in a small volume of SSC (2-3 ml). If it was so desired, the suspension was stored at this point by freezing.

Lysozyme was added to the cell suspension at a final concentration of 100 ug/ml and incubated without shaking for 30 min. Cell lysis was completed by the addition of 2 ml of 5% sodium lauryl sulphate. Two volumes of 95% ethanol were added slowly and the crude DNA was collected by carefully stirring the solution in one direction with a glass rod. The fibre wound on the glass rod was washed with 95% ethanol. The fibre was then dissolved by shaking for 1 hr. at room temperature in a solution which contained 25.0 ml of SSC and 2.5 ml of 5% sodium lauryl sulphate. The shaking was continued for additional 3 hr. Proteins were precipitated by the addition of solid NaCl to a final concentration of 1 M and removed

by centrifugation at 10,000 rpm (12,100 g) for 30 min. using an SS34 rotor. The DNA was precipitated and collected again by adding two volumes of 95% ethanol and stirring with a glass rod. The crude DNA fibre was stored and sterilized in 70% ethanol. The DNA fibre was dissolved in 5 ml of sterile SSC and used in transformation experiments.

2- Transformation Technique (Spizizen, 1958)

To obtain competent recipient cultures a loopful of cells was inoculated into 5 ml of P broth and incubated with shaking for 4 hr. The culture was diluted 10 times (to 5×10^7 cells/ml) in the transforming medium and shaken for 90 min. at 37°C at which time the cells became highly transformable. For transformation, 0.1 ml of DNA solution was added to 3 ml of recipient culture and incubated with shaking for 30 min. As controls, 0.1 ml of SSC was added to another flask containing the same culture. In the present study a DNA concentration of more than 1 ug/ml which is a saturation concentration for transformation was used. The treated cultures (0.1 ml samples) were spread on minimal agar or sporulation agar to score transformants.

Strain 168 was transformed by the above technique with some modifications. The first culture medium was SMM supplemented with casamino acids (0.02%) and dl tryptophan (50 ug/ml) and competent cultures were prepared in SMM supplemented with casamino acids (0.01%) and dl tryptophan (5 ug/ml).

3- Preparation of Phage Lysates

Bacteriophage PBS 1 (Takahashi, 1961) was used exclusively for transduction experiments throughout this study.

To prepare phage lysates 4 hr. cultures were diluted 10 times (to 5 x 10^7 cells/ml) in P broth and infected with PBS 1 to give a multiplicity of infection (moi) of about 1. The infected cultures were incubated with shaking for 60 min. and incubation was continued overnight without shaking. The bacterial cells and cell debris in the lysate were removed by centrifugation and cell-free lysates were obtained by filtering the supernatant liquid through a Millipore filter (0.45 micron).

4- Transduction Technique (Takahashi, 1961)

To 1.0 ml of a 4 hr.-old culture 0.25 ml of lysate was added and shaken for 30 min. at 37°C. For controls, 0.25 ml of P broth was added to the culture instead of lysate. The cultures were then plated on appropriate selective media to score transductants.

5- Ultraviolet Irradiation

Five ml of culture were placed in a glass petri dish and exposed to UV light from a Mineralight UVS-12 lamp with constant swirling. The exposure was at a distance of 20 cm for 2 min. in the dark. This produced a 95-98% killing dose. The irradiated culture was then diluted and 0.1 ml was spread on agar to detect mutants. On occasion, cultures to be irradiated were centrifuged and cells were resuspended in the same volume of 1/2 SMM or physiological saline (0.85%).

6- Isolation of Sporulation Mutants

On sporulation agar, those colonies which were brown after 2 days incubation produced spores. White colonies, on the other hand, were either sporogenous albinos or asporogenous. The white colonies were picked up

individually, streaked on sporulation agar and the plates were incubated for 2 days. The isolates were then examined with a phase contrast microscope for the presence of spores. Those which showed no sign of sporulation under the microscope were preserved in the frozen state. They were also examined for their nutritional requirements to determine whether they retained the requirements of the parent strain. If the mutant passed these first three tests (white colony formation, microscopic examination and amino acid requirement) its rate of reversion to sporogeny was then determined. In some later work, an actual reversion rate was not determined as only those which showed no reversion at all were selected.

7- Determination of Reversion Rates

Two sporulation agar plates were heavily inoculated with young cells by means of an inoculating loop and were incubated for 2 days. Cells were then collected and resuspended in 1 ml of 1/2 SMM. Some strains, A and M for example, formed crusty colonies on sporulation agar and a slightly different technique had to be used. A thick cell suspension in 5 ml of sporulation broth was poured into TB agar plates and these were incubated for 2 days to allow sporulation to take place. From this point the same method was used for removing the cells from the agar surface.

Viable cell counts were made, after appropriate dilutions, by plating 0.1 ml samples on TB agar. The number of spores was determined by heating the cell suspensions at 85°C for 10 min. and plating on sporulation agar. The reversion rates were then calculated from viable cell counts and spore counts.

8- Detection of Spores by the Replica-plating Technique

This method was used when only the detection of spores was of interest since it was not designed to determine the true reversion rate. The cell suspensions (0.1 ml) were plated on sporulation agar and these were incubated for two days. For the detection of spores produced in transduction or transformation, the treated cultures were similarily plated on sporulation agar and incubated. Twice the agar surface was sprayed with chloroform using a chromatography solvent sprayer to kill vegetative cells and the plates were left upright at room temperature for 4 hr. Each plate was then replica-plated on sporulation agar and the latter were incubated for 2 days to detect spore-forming brown colonies.

9- The Assay of Phage Lysates

An appropriate dilution series of phage lysates was prepared using Ad medium. In a test tube, 1.4 ml of Ad medium, 0.1 ml of fully-grown indicator bacteria in P broth and 3 ml of diluted phage were mixed. After 5 min., at which time most bacteriophages were adsorbed on host bacteria, 4.5 ml of 1% TB agar melted and kept in a 50°C water bath were pipetted into the test tube and mixed. Using the same pipette, 8 ml were removed and the freshly-made TB agar plates were each overlayed with 3 ml of the soft agar mixture. The plates were incubated overnight at 28°C in an upright position. In this method, the number of plaques produced on a plate represents the number of plaque-forming units per ml of diluted phage lysates.

10- Test for the Production of an Antibiotic for Staphylococcus

The strains to be tested and S. aureus were grown overnight on TB agar at 37°C. A thin suspension of S. aureus or the B. subtilis strains was made in 1/2 SMM. A drop of the B. subtilis suspension was placed on TB agar plates which were inoculated with S. aureus by spreading 0.1 ml of cell suspension and the plates were incubated overnight. The presence of a clear zone of lysis around the B. subtilis colony indicated the production of an antibiotic active on S. aureus.

11- Test for the Adsorption of Bacteriophage PBS 1 by B. subtilis strains

The stock lysate was diluted to 5×10^7/ml and 0.8 ml of this was added to 0.8 ml of bacterial culture and 2.4 ml of Ad medium. This resulted in final concentrations of cells and phage particles of 10^8/ml and 10^7/ml respectively. The mixture was allowed to stand for 5 min. at room temperature for the phage to adsorb and the cells were removed by centrifugation. The supernatant liquid was diluted to 10^{-3} and 10^{-5} and the number of unadsorbed phages was determined by the assay method described earlier.

CHAPTER III

ATTEMPTS TO ELIMINATE THE SPORULATION FACTOR FROM SPOROGENOUS STRAINS BY ACRIDINES OR ULTRAVIOLET LIGHT

It has been reported that sporulation mutants could be produced in *B. subtilis* by the addition of acriflavine (Rogolsky and Slepecky, 1964). The frequencies of the *sp*⁻ mutants produced varied according to the stage of growth cycle at which the dye was added.

Attempts were therefore made to duplicate such results and to isolate mutants similar to Sp⁻H12-3. Two methods were used which were based on Rogolsky and Slepecky's results. The two differed basically in the manner of determining the various stages in the growth cycle at which the acridines were added. In method A, time was used to determine the stage and in method B, turbidity of the culture. A third method, C, similar to that proposed by Hirota (1960) to eliminate the F-factor in *E. coli*, was used on strain H12 for a limited number of experiments.

At the outset of this study ultraviolet light was used on various strains of *B. subtilis* in an effort to produce sporulation mutants and although data are not available on the percentage of these mutants isolated, a list is given of those which did not revert to sporogeny. All asporogenous strains unable to revert to sporogeny produced throughout the present study were also tested for the production of an antibiotic active on *Staphylococcus*.

METHOD A

Broth cultures were diluted 10^{-2} (to 5×10^6 cells/ml) in a flask containing 5 ml of SMM pH 7.6. The flask was shaken at 45°C and at various times from 0 to 240 min. Acridine orange (AO) or neutral acriflavine (AF) was added to a final concentration varying from 0.03 to 6.0 ug/ml. Shaking at 45°C was continued for a further 24 hr. After making appropriate dilutions in 1/2 SMM, 0.1 ml portions were plated on several spore agar plates and incubated for 2 days. White colonies produced were then tested for the production of spores.

METHOD B

Broth cultures were diluted 10^{-2} (to 5×10^6 cells/ml) in 5.0 ml of SMM pH 7.6, and the flasks were shaken at 45°C. At various Klett unit values as determined by a Klett-Summerson Photoelectric Colorimeter ranging from 4 to 108 (wavelength = 500-570 millimicrons), AF was added to a final concentration varying from 1.0 to 6.0 ug/ml and shaking was continued at 45°C for an additional 24 hours. After diluting the treated cultures in 1/2 SMM, 0.1 ml samples were plated on several sporulation agar plates and incubated for 2 days.

METHOD C

After P broth cultures were diluted 10^{-4} (to 5×10^4 cells/ml) in SMM medium pH 7.6, AF was added to a final concentration of 5 to 10 ug/ml and the cultures were shaken overnight at 37°C. After diluting in 1/2 SMM, 0.1 ml samples were plated on sporulation agar and incubated for 2 days.

Growth curves of strains HL2 and W were constructed using both viable counts and Klett units in order to estimate the number of cells present and the stage in the growth cycle at which the acridines were added.

Results

Although no mutants of any type were produced in strain W using method A (Table I), there appears to be some correlation between the concentration of dye used, the time of its addition and the number of viable cells at the end of the experiments. A certain number of viable cells could be obtained when 5 ug/ml of AF was added to cultures during any part of the growth cycle. On the other hand, practically the entire population of cells was killed by the addition of 6 ug/ml of AF.

Under the same conditions strain H12 would not grow at all if AF was added before early log-phase (Table II). Only a small number of surviving cells were observed when AF at a concentration of 6 ug/ml was added at the middle of log-phase. Oligosporogenous mutants were isolated when AF was added at the middle of log-phase but no asporogenous mutants were ever found.

All cells of SB19E were killed by AF at a concentration of 1 to 3 ug/ml if added at early log-phase indicating that this strain can tolerate only a very low level of AF in the culture medium.

No mutants were obtained from 29,830 colonies examined when strain W (s) was treated with AF by method B (Table III). Strain W (s) grows in the presence of AF at concentrations ranging from 1 to 6 ug/ml regardless of the time of addition. The initial growth of the cells in 1/2 B broth in method B may be responsible for the resistance of the cells to the early addition of 6 ug/ml of AF which was not observed using method A. The number of survivors increases 50-fold if the bacteria are allowed to proceed to middle of log-phase of the growth cycle before AF is added.

The important point to note here is that neither method A nor method B produced any sporulation mutants from strain W, even when the very W strain and sample of AF used by Rogolsky and Slepecky were employed.

There is no correlation between the concentration of acriflavine or the time of its addition and growth in strain H12 treated by method B (Table IV). No asporogenous strains similar to Sp⁻H12-3 were produced. Many oligosporogenous strains were produced with different concentrations at various times of addition and two asporogenous strains Sp⁻H12-30 and Sp⁻H12-31 were obtained from cultures treated with 6 ug/ml AF during middle of log-phase. Strain Sp⁻H12-30, having a reversion rate of 8.3×10^{-8}, is very close to the selection point of 10^{-7} which arbitrarily distinguishes asporogenous strains from oligosporogenous strains. Strain Sp⁻H12-31 is unable to produce spores (less than 8.3×10^{-10}), but is readily transducible to sporogeny.

It was postulated at this point that the oligosporogenous mutants obtained from one acridine treatment may have lost only part of the extra-chromosomal determinants for sporulation. Therefore, one of these oligosporogenous mutants, H12-1, which was resistant to AF (1 ug/ml) was further treated at various stages in the growth cycle with 6 ug/ml AF to produce sporulation mutants. No mutants out of 18,260 colonies examined were found (Table V).

Further serial treatment was continued by selecting strain H12-1-2 from amongst H12-1 treated cells. Strain H12-1-2 was oligosporogenous and resistant to AF (1 ug/ml) but after exposure to AF did not produce any asporogenous mutants (Table VI).

Strain 168 underwent treatment with 6 ug/ml AF (Method B) at different stages in the growth cycle but no asporogenous mutants were isolated (Table VII). This strain grew normally when the dye was added at later growth stages and albino mutants were produced which proved to be sporogenous.

Method C (Table VIII) was used only with strain H12. No surviving cells were obtained when the cells were treated with 10 ug/ml of AO. Out of 123,200 colonies obtained from cells treated with lower concentrations of AO (5-7.5 ug/ml), 3 mutants were isolated, 2 oligosporogenous and one asporogenous (Sp^-H12-36) which showed a reversion rate of 3×10^{-8}.

Table IX summarizes the number of colonies examined for each strain and method of treatment in use. Three asporogenous strains were isolated all of which were derived from strain H12 and only one of these would not revert to sporogeny.

A list is given in Table X of a number of asporogenous mutants incapable of reverting to sp^+ isolated from several different parent strains after exposure to UV light and AF. Also included in the table is the maximum reversion rate and the ability of the mutant to produce the antibiotic against Staphylococcus which indicates whether it is blocked before or after stage I in the sporulation cycle. Strain Sp^-H12-3 is presumably blocked at a very early stage, since it does not produce the antibiotic.

All of the mutants listed, with the exception of two, produced sporogenous clones after transduction. This at first leads one to believe that these two, Sp^-A-6 and Sp^-A-11, are behaving similarly to

Sp⁻H12-3. Upon further experimentation it was discovered that they were not transducible to prototrophy either. Investigation of the reason for this lack of transduction led to the finding that the bacteriophage could not adsorb to the bacterium in these two cases. Compared with 99.9% adsorbtion in strain SB19E, Sp⁻A-6 and Sp⁻A-11 were able to adsorb only 0.6% and 0.1% of the phage, respectively. Nothing meaningful therefore, can be stated about the fact that they are not transducible to the sporogenous state.

Figure 1

Growth Curve of B. subtilis H12 in SMM at pH 7.6 at 45°C.

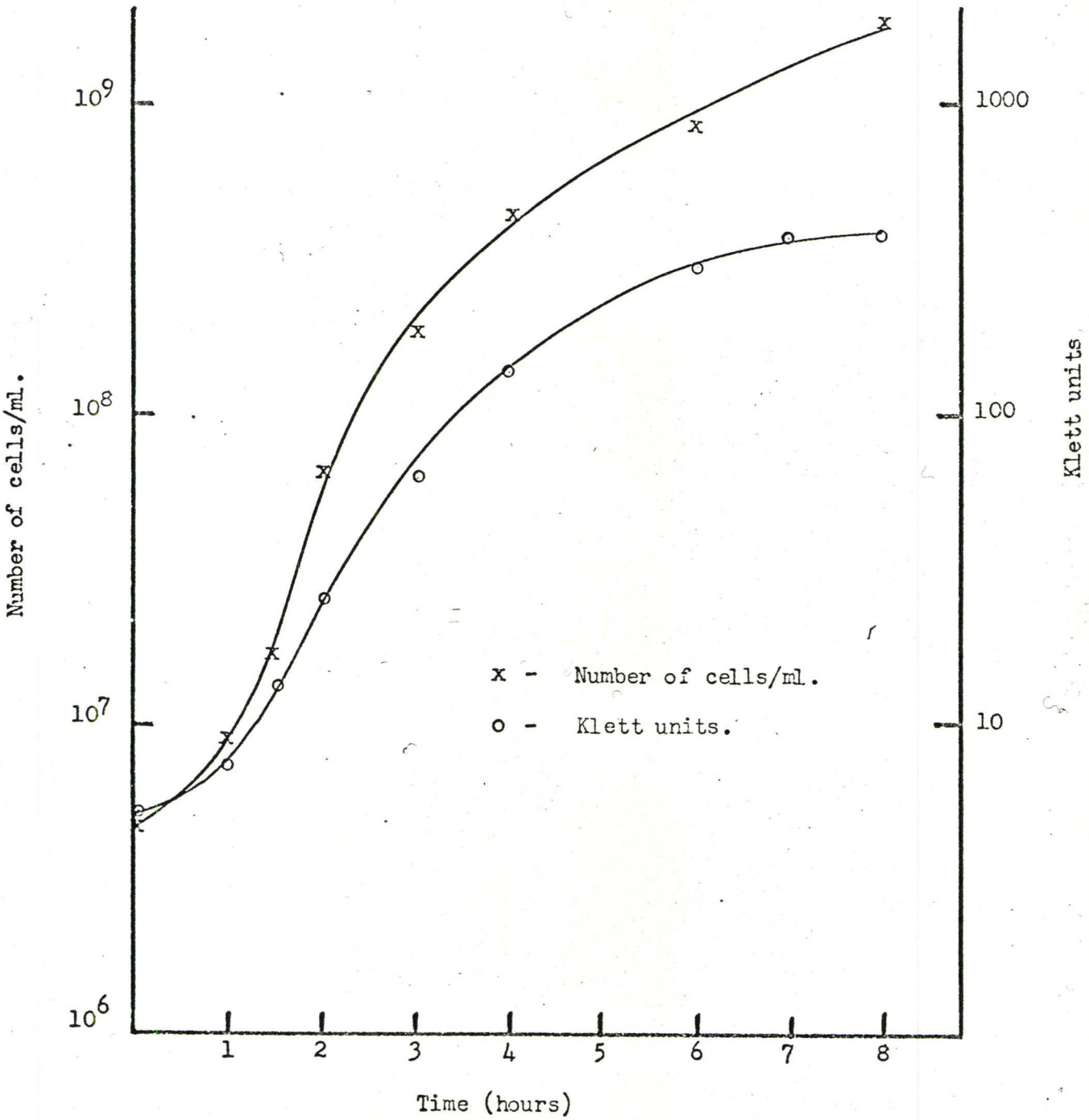


Figure 2

Growth Curve of B. subtilis W in SMM pH 7.6 at 45°C.

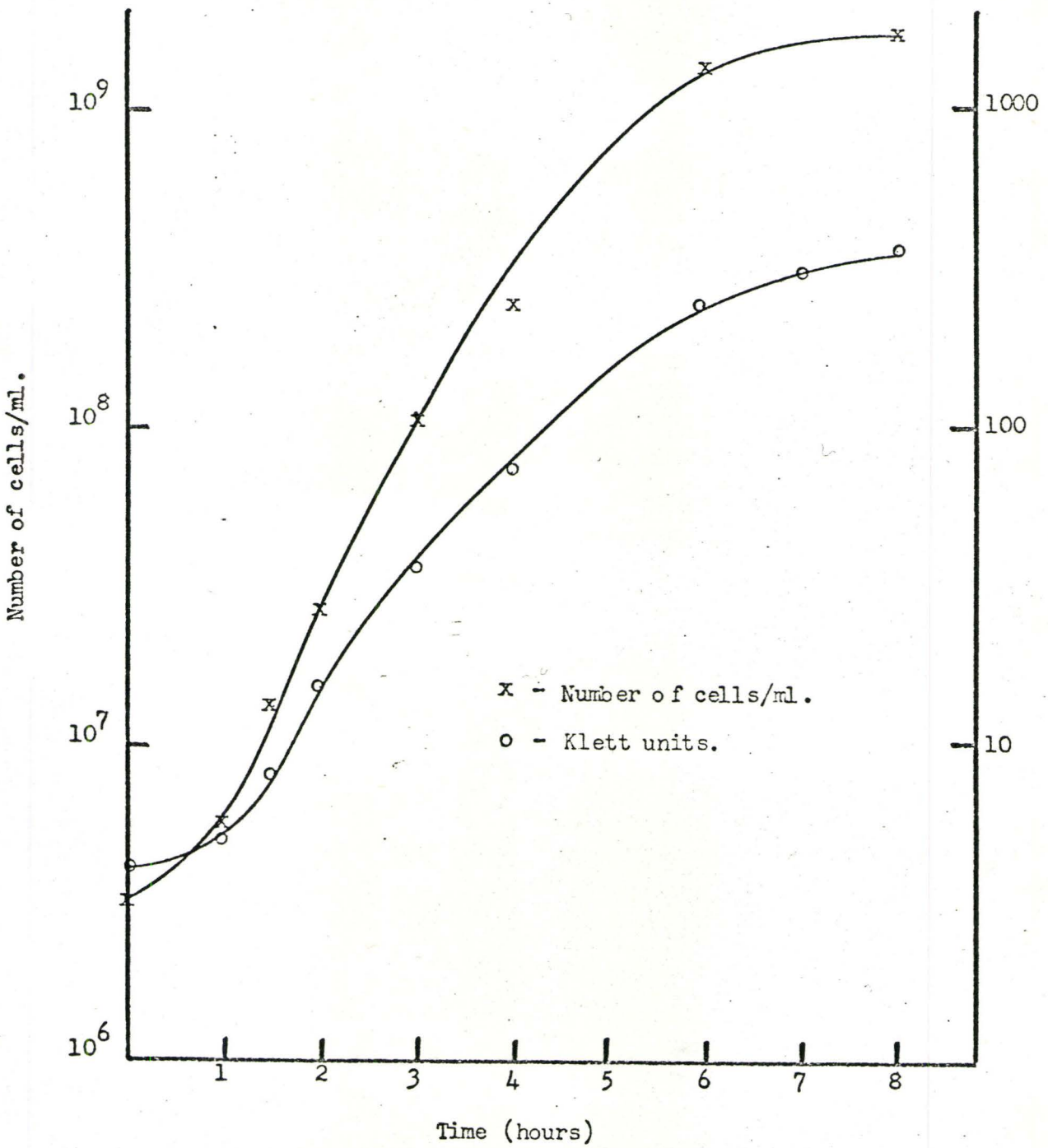


Table I

The Effect of Acridines on Strain W - Method A

Time (min.)	Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
✱ 0	5	3.75×10^8	4015	0
0	2.5	No counts made		0
30	5	8300	6397	0
30	6	0	0	
60	5	5800	4470	0
60	6	0	0	
120	0.03	2.5×10^8	4180	0
120	0.1	2.9×10^8	3520	0
120	0.3	3.2×10^8	3190	0
120	1	3.8×10^8	2750	0
120	2.5	No counts made		0
120	6	0	0	
180	6	120	120	0

more than 28632 colonies examined

✱ After 3 hours growth in 5 ug/ml AO the number of viable cells was reduced 3.5 times. All other experiments recorded in Table I were performed using Neutral Acriflavine.

Table II

The Effect of Neutral Acriflavine on Strain H12 - Method A

Time (min.)	Concentration (ug/ml)	Viable Cells/ml	Colonies Examined	Type of Mutants
30	6	0	0	
60	1	0	0	
60	3	0	0	
60	6	0	0	
120	1	4.4×10^7	4840	0
120	3	0	0	
120	6	0	0	
180	1	8.2×10^7	9020	0
180	1	2.8×10^7	3080	Oligosporogenous
180	6	450	450	Oligosporogenous
210	1	2.9×10^7	3190	0
240	1	4.8×10^7	5280	0
		Total	25860	

Table III

The Effect of Neutral Acriflavine on Strain W (s) - Method B

Klett Units	Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
✱ 4)	1	1330	1330	0
8)	1	790	790	0
12)	1	1020	1020	0
15	6	660	660	0
15	6	530	530	0
16	6	720	720	0
32	1	3.4×10^5	3410	0
32	6	3.6×10^5	3575	0
57	1	2.3×10^5	2255	0
58	6	4.5×10^5	4455	0
76	1	5.2×10^5	5225	0
81	6	5.8×10^5	5830	0
		Total	29830	

✱ All colonies tested were resistant to 1 ug/ml of Neutral Acriflavine.

Table IV

The Effect of Neutral Acriflavine on Strain H12 - Method B

Klett Units	Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
✱ 9)	1	1180	1180	Oligosporogenous
9)	1	1210	1210	Oligosporogenous
13)	1	820	820	Oligosporogenous
15	6	100	100	0
31.5	1	2270	2270	Oligosporogenous
32	6	1.2×10^8	13653	0
45	1	2.1×10^8	23430	0
45	6	2.5×10^5	2750	Oligosporogenous
59	6	6.9×10^7	6759	0
75	6	2.8×10^8	1540	Oligosporogenous
79	6	1090	1090	1 (Sp^-H12-30)
88	6	1330	1330	1 (Sp^-H12-31)
88	6	910	910	0
100	1	1.2×10^8	12980	0
107	6	3.3×10^8	1815	0
		Total	71537	

✱ All colonies tested were resistant to 1 ug/ml Neutral Acriflavine.

Table V

The Effect of Neutral Acriflavine on Strain H12-1 - Method B

Klett Units	Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
31	6	1.1×10^5	6270	0
* 46.5	6	8.8×10^4	4840	0
57	6	1.3×10^5	7150	0
		Total	18260	

* H12-1-2 (Sp$^+$, oligosporogenous, AF^r) was isolated for further treatment with acriflavine.

Table VI

The Effect of Neutral Acriflavine on Strain H12-1-2 - Method B

Klett Units	Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
16.5	1	1.7×10^5	1650	0
27	1	9.3×10^4	930	2 Oligosporogenous
		Total	2580	

Table VII

The Effect of Neutral Acriflavine on Strain 168 - Method B

Klett Units	Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
46	6	5.5×10^3	27	0
70	6	3.2×10^8	1760	Albinos
108	6	3.3×10^8	1815	Albinos
		Total	3602	

Table VIII

The Effect of Acridine Orange on Strain H12 - Method C

Concentration (ug/ml)	Viable cells/ml	Colonies Examined	Type of Mutants
5	4×10^8	44000	2 Oligosporogenous
5	4.5×10^8	39600	1 (Sp^- H12-36)
7.5	4.5×10^8	39600	0
10	0	0	
	Total	123200	

Table IX

Total Colonies Examined After Treatment with Acridines

Strain	Method	Colonies Examined	Mutants
W	A	28632	0
W (s)	B	29830	0
168	B	3602	Albinos
H12	A	25860	Oligosporogenous
H12	B	71537	2
H12	C	123200	1
H12-1	B	18260	0
H12-1-2	B	2580	Oligosporogenous
	Total	303500	

Table X

Mutants Showing No Reversion to sp^t

	Mutant	Agent	Maximum Reversion Rate	Antibiotic Production
✱	Sp^-A-6	UV	5.6×10^{-10}	-
	Sp^-A-9	UV	5.9×10^{-10}	+
✱	Sp^-A-11	UV	4.6×10^{-10}	-
	Sp^-B37-2	UV	2.2×10^{-9}	+
	Sp^-B37-3	UV	5.6×10^{-10}	-
	Sp^-B37-4	UV	2.5×10^{-9}	+
	Sp^-B37-6	UV	2.2×10^{-9}	+
	Sp^-B33-6	UV	3.0×10^{-9}	-
	Sp^-B33-7	UV	2.2×10^{-8}	-
	Sp^-H12-11	UV	4.8×10^{-9}	+
	Sp^-H12-14	UV	1.2×10^{-9}	-
	Sp^-H12-15	UV	5.3×10^{-10}	-
	Sp^-H12-17	UV	1.5×10^{-9}	-
	Sp^-H12-21	UV	1.6×10^{-9}	+
	Sp^-H12-22	UV	1.5×10^{-9}	-
	Sp^-H12-23	UV	1.0×10^{-9}	+
	Sp^-H12-31	AF (6 ug/ml)	8.3×10^{-10}	+

✱ Strains Sp^-A-6 and Sp^-A-11 are not competent for transduction.

CHAPTER IV

THE FREQUENCY OF SPORULATION MUTANTS PRODUCED IN B. SUBTILIS BY THE COMBINED TREATMENT WITH ULTRAVIOLET LIGHT AND ACRIDINE ORANGE

From the results described in the previous chapter, it was postulated that if some episomal sporulation factor exists in B. subtilis, it must be tightly bound to the chromosome since acridines were unable to remove the factor. Acridine dyes can only eliminate factors in the autonomous state and those in the integrated state will not be affected. It was pointed out earlier, in the introduction, that UV light induces the transition of episomes from the integrated to the autonomous state. If the sporulation factor resides on the chromosome and if UV light can dissociate it from the chromosome, then acridine dyes might more efficiently eliminate the factor to produce truly asporogenous mutants of the Sp^{-}H12-3 type.

Here, in addition to attempting the elimination of a spore factor in B. subtilis, a comparison was made between the influence of UV, AO and UV plus AO on the numbers and types of sporulation mutants produced from various sporogenous strains.

Mutants were divided into 5 categories according to the following phenotypes. Group 1 contained all of those which produced white colonies when streaked on sporulation agar and therefore included albinos as well as asporogenous strains. In Group 2 were those mutants which exhibited no spores when examined under the phase contrast microscope--here the

albinos were eliminated. In Group 3 the oligosporogenous strains were not included since only those mutants with a reversion rate of less than 10^{-7} were retained. Group 4 included those which did not show any reversion to sporogeny at all under the experimental conditions, and were considered irreversible mutants. Those which belonged to Group 4 and were not transducible to sporogeny using a wild-type donor strain, and therefore presumably very similar to strain Sp^{-}H12-3, were grouped together into Group 5. Mutants which belong to Groups 4 and 5 are listed in Tables XVI and XVII, respectively.

Method

Cells of sp^{+} strains received the following three treatments. A portion of cells was exposed to UV light as described in Chapter II or treated with AO at concentrations varying from 1.0 to 100 ug/ml. The third sample was first irradiated with UV light and treated with AO. Treated cells in SMM pH 7.6 were incubated overnight with shaking then diluted and plated in sporulation agar to detect sp^{-} mutants.

Results

The first sp^{+} strain treated by the above method was H12 which spontaneously produced 27 Group 1 mutants and one Group 2 mutant out of 148,217 colonies examined. As shown in Table XI low concentrations (1 and 2 ug/ml) and high concentrations (60 and 100 ug/ml) were not effective in producing mutants of Group 4 when UV-irradiated cells were treated. No Group 5 mutants were produced in strain H12. The frequencies of mutants produced by the three treatments mentioned above varied markedly.

In Group 1 for instance, UV light produced 0.074% white colonies, AO alone produced 0.033% mutants but UV plus AO produced 0.23%. Even when the separate effects of UV and AO were added together the total came to only 0.107%, one-half of the number of mutants isolated by the combined treatment of UV and AO. The same tendency was also noted for other groups of mutants.

Frequencies of sporulation mutants obtained from strain W by the UV-AO treatment were significantly higher than those produced from other $\underline{sp^t}$ strains. The frequency of Group 1 mutants produced from strain W by the combined UV-AO treatment was four times higher than the sum of the frequencies observed with separate UV and AO treatments. Ten W mutants are listed which were not transducible to sporogeny.

The frequency of mutants isolated from strain SB19E is reported in Table XIII. Again the same pattern results; there are far more mutants produced by UV and AO acting together than separately. Although several irreversible strains were obtained, no mutants of the Group 5 type were isolated. Increased frequencies of sporulation mutants by the UV-AO treatment were discovered for strains N2 and 168 and these are reported in Table XIV.

The results of the whole series of experiments are summarized in Table XV. The ratios of the percentages of mutants produced by the combined treatment over the sum of those produced by UV and those by AO range from 2.7 for Group 1 to 4.6 for Group 4. This indicates that the action of UV light plus AO is of a synergistic nature rather than an additive one.

Table XVI is a list of irreversible mutants produced in this section of the work and included is the ability to produce the antibiotic. Maximum reversion rate refers to the highest possible rate of reversion to sporogeny within the limits of the method. Where the reversion rate is given as 0, a viable count of the suspension was not determined and it is therefore only known that there were no spores amongst a very large number of vegetative cells.

Several mutants which were not transducible for sp^+, were discovered amongst the isolates from strain W and one from strain N2. These, it turned out, were also unable to be transduced for an erythromycin-resistance marker; therefore, they may be incompetent in transduction. In order to understand why these strains were not transducible, two tests were carried out. First, it was determined if they were capable of adsorbing bacteriophage PBS 1 in the medium used for transduction. Unlike strains Sp^-A-6 and Sp^-A-11 which could hardly adsorb the phage, these mutants adsorb as much as 99.8% of the added phage (Table XVII). The reason for their inability to undergo transduction is not because they cannot adsorb the phage particles.

A second possibility is that as these mutants appear to be able to accept genetic material normally, they may be defective in the process of integrating the donor DNA into the chromosome. In E. coli, it has been found that recombination mutants (Rec^-) are more sensitive to UV light than wild-type strains (Clark and Margulies, 1965 and Howard-Flanders and Theriot, 1966). Earlier, it had been postulated that certain enzymes served in both genetic recombination and repair after irradiation (Howard-Flanders and Boyce, 1964). A similar observation had been made with

B. subtilis (Searashi and Strauss, 1965). Thus if one compares the UV-sensitivity of asporogenous mutants which are not transducible with that of the parent strain, an insight may be gained as to a possible defectiveness in the DNA repair mechanisms which may also be linked to the process of genetic recombination.

The above possibility was tested with strains Sp⁻W-4 and Sp⁻W-18 which exhibited the highest percentage of phage adsorption but were not transducible. Broth cultures were centrifuged and resuspended in an equal volume physiological saline (0.85%). A viable count was made before exposure to UV light and after 5.0 ml portions were exposed to UV for 30 and 60 seconds. Table XVIII shows the fraction of surviving cells after each irradiation. The mutant strains are less sensitive to UV irradiation than the parent strain W. It is unlikely, therefore, that Sp⁻W-4 and Sp⁻W-18 are recombination mutants.

Explanation of the Tables XI to XV

The number in the Group 1 column represents the percentage of white colonies found amongst the total number of colonies examined. The first number in the remaining four Group columns represents that Group's percentage of the total colonies examined and the number in brackets represents the number of colonies examined for that Group. The average percentage at the bottom of each Group column, where more than one concentration of AO was used, was determined by first summing the colonies examined for that Group and the total colonies examined. The average percentage for AO alone and UV alone were added together for comparison with that obtained by the combined UV-AO treatment.

Table XI

The Frequency of Sporulation Mutants Produced by UV Plus AO, AO Alone and UV Alone in B. subtilis H12

Conc. of AO (ug/ml)	Total Colonies Examined	Group 1	Group 2	Group 3	Group 4	Group 5
UV-AO Treatment						
1	8250	0.145	0.012 (12)	0.012 (1)	0 (1)	
2	20515	0.15	0.037 (24)	0.037 (6)	0 (6)	
5	251112	0.21	0.039 (339)	0.032 (63)	0.0093 (51)	0 (15)
10	92015	0.18	0.024 (60)	0.018 (8)	0.006 (6)	0 (2)
20	74805	0.41	0.13 (82)	0.09 (26)	0.025 (18)	0 (5)
30	28270	0.26	----------			
40	40810	0.36	0.21 (12)	0.21 (7)	0.059 (7)	0 (2)
50	58245	0.34	0.091 (30)	0.079 (8)	0.034 (7)	0 (3)
60	32450	0.08	0.013 (6)	0.013 (1)	0 (1)	
100	86570	0.16	0.02 (24)	0.02 (3)	0 (3)	

Table XI (Cont'd.)

Conc. of AO (ug/ml)	Total Colonies Examined	Group 1	Group 2	Group 3	Group 4	Group 5
AO Treatment Alone						
5	84590	0.033	0.006 (22)	0.0045 (4)	0.0015 (3)	0 (1)
10	27500	0.0073	0 (2)			
40	65	0				
50	34870	0.029	0.005 (6)	0.005 (1)	0 (1)	
100	50160	0.052	0.0087 (6)	0.0087 (1)	0 (1)	
A. Average of AO Treatments						
	197185	0.033	0.0051 (36)	0.0042 (6)	0.0008 (5)	
B. UV Treatments Alone						
	238084	0.074	0.0175 (89)	0.0133 (21)	0.0033 (16)	0 (4)
C. Average of UV-AO Treatments						
	693042	0.23	0.049 (589)	0.04 (123)	0.011 (100)	0 (27)
A. + B.	435269	0.107	0.0226(125)	0.0175 (27)	0.0041 (21)	0 (5)

Table XII

The Frequency of Sporulation Mutants Produced by UV Plus AO, AO Alone and UV Alone in B. subtilis W

Conc. of AO (ug/ml)	Total Colonies Examined	Group 1	Group 2	Group 3	Group 4	Group 5
UV-AO Treatment						
20	18200	1.97	0.48 (78)	0.41 (19)	0.35 (16)	0.25 (14) ✱
40	12700	2.4	0 (6)			
A. AO Treatment Alone						
20	31950	0.15	----------			
B. UV Treatment Alone						
	31540	0.39	----------			
C. Average of UV-AO Treatments						
	30900	2.1	0.49 (84)	0.41 (19)	0.35 (16)	0.25 (14)
A. + B.	63490	0.54	----------			

✱ Sporulation Mutants - $Sp^{-}W$-3, $Sp^{-}W$-4, $Sp^{-}W$-5, $Sp^{-}W$-7, $Sp^{-}W$-8, $Sp^{-}W$-10, $Sp^{-}W$-17, $Sp^{-}W$-18, $Sp^{-}W$-19, $Sp^{-}W$-20

Table XIII

The Frequency of Sporulation Mutants Produced by UV Plus AO, AO Alone and UV Alone in *B. subtilis* SB19E

Conc. of AO (ug/ml)	Total Colonies Examined	Group 1	Group 2		Group 3		Group 4		Group 5	
UV-AO Treatment										
20	119460	0.51	0.2	(66)	0.11	(26)	0.07	(14)	0	(9)
40	44550	0.4	0.2	(18)	0.022	(9)	0	(1)		
A. AO Treatment Alone										
20	58300	0.043	0	(12)						
B. UV Treatment Alone										
	63580	0.066	----------							
C. Average of UV-AO Treatments										
	164010	0.48	0.2	(84)	0.03	(35)	0.018	(15)	0	(9)
A. + B.	121880	0.109	0	(12)						

Table XIV

The Frequency of Sporulation Mutants Produced by AO Plus UV, AO Alone and UV Alone in B. subtilis 168 and N2

Conc. of AO (ug/ml)	Total Colonies Examined	Group 1	Group 2	Group 3	Group 4	Group 5
B. subtilis 168						
A. AO Treatment Alone						
5	19800	0.02	0.005 (4)	0.005 (1)	0 (1)	
B. UV Treatment Alone						
	23210	0.039	0.009 (9)	0 (2)		
C. UV-AO Treatment						
5	27830	0.11	0.02 (30)	0.0066 (6)	0 (2)	
A. + B.	43010	0.059	0.014 (13)	0.005 (3)	0 (1)	
B. subtilis N2						
A. AO Treatment Alone						
20	3003	0				
B. UV Treatment Alone						
	87560	0				
C. UV-AO Treatment						
20	55500	0.185	0.025 (30)	0.0185 (4)	0.012 (3)	0.006 (2) ✱
A. + B.	90563	0				

✱ Sporulation Mutant - $Sp^{-}N2\text{-}12$

Table XV

The Frequency of Sporulation Mutants Produced by UV Plus AO, AO Alone and UV Alone in all B. subtilis strains

	Colonies Examined	Group 1	Group 2	Group 3	Group 4	Group 5
A. AO Treatment Alone						
	310238	0.046	0.005 (64)	0.0043 (7)	0.0014 (6)	0 (2)
B. UV Treatment Alone						
	452974	0.077	0.018 (97)	0.013 (23)	0.0032 (16)	0 (4)
C. UV-AO Treatment						
	971282	0.33	0.076 (811)	0.056 (187)	0.021 (136)	0.0045 (52)
A. + B.	763212	0.123	0.023 (161)	0.0173 (30)	0.0046 (22)	0 (6)
C./A. + B.		2.7	3.3	3.2	4.6	

Table XVI

Irreversible Mutants (Group 4) Produced by UV Plus AO, UV Alone or AO Alone

Mutant		Agent (ug/ml)	Maximum Reversion Rate	Antibiotic Production
Sp⁻19-1		UV plus 20 AO	0	-
Sp⁻19-5		"	0	-
Sp⁻19-8		"	0	-
Sp⁻19-11		"	0	-
Sp⁻19-13		"	0	-
Sp⁻19-19		"	0	-
Sp⁻19-31		"	0	+
Sp⁻19-33		"	0	+
Sp⁻19-36		"	0	+
Sp⁻W-3	✱	UV plus 20 AO	0	+
Sp⁻W-4	✱	"	0	+
Sp⁻W-5	✱	"	0	+
Sp⁻W-7	✱	"	0	+
Sp⁻W-8	✱	"	0	+
Sp⁻W-10	✱	"	0	+
Sp⁻W-13		"	0	+
Sp⁻W-14		"	0	-
Sp⁻W-16		"	0	+
Sp⁻W-17	✱	"	0	+
Sp⁻W-18	✱	"	0	+
Sp⁻W-19	✱	"	0	+

Table XVI (Cont'd.)

Mutant	Agent (ug/ml)	Maximum Reversion Rate	Antibiotic Production
Sp⁻W-20 *	UV plus 20 AO	0	+
Sp⁻W-21	"	0	-
Sp⁻N2-12 *	"	0	-
Sp⁻N2-14	"	0	+
Sp⁻H12-52	UV plus 5 AO	5.0×10^{-10}	+
Sp⁻H12-53	"	1.7×10^{-10}	+
Sp⁻H12-56	"	2.8×10^{-10}	+
Sp⁻H12-58	"	2.0×10^{-10}	+
Sp⁻H12-59	"	7.7×10^{-11}	-
Sp⁻H12-62	"	1.2×10^{-10}	+
Sp⁻H12-69	"	2.0×10^{-10}	+
Sp⁻H12-70	"	3.1×10^{-10}	+
Sp⁻H12-80	"	1.2×10^{-10}	+
Sp⁻H12-89	"	1.4×10^{-10}	+
Sp⁻H12-90	"	1.3×10^{-10}	+
Sp⁻H12-92	"	1.9×10^{-9}	+
Sp⁻H12-94	"	2.4×10^{-10}	+
Sp⁻H12-97	"	1.8×10^{-10}	+
Sp⁻H12-102	"	2.9×10^{-10}	+
Sp⁻H12-105	UV	2.7×10^{-10}	+
Sp⁻H12-110	UV	2.4×10^{-10}	+
Sp⁻H12-121	UV plus 10 AO	2.6×10^{-10}	+

Table XVI (Cont'd.)

Mutant	Agent (ug/ml)	Maximum Reversion Rate	Antibiotic Production
Sp⁻H12-127	UV plus 5 AO	1.7×10^{-10}	+
Sp⁻H12-135	UV	3.1×10^{-10}	+
Sp⁻H12-145	UV plus 5 AO	3.8×10^{-10}	+
Sp⁻H12-148	UV plus 10 AO	4.8×10^{-10}	+
Sp⁻H12-149	UV plus 20 AO	4.7×10^{-10}	+
Sp⁻H12-160	UV plus 50 AO	1.1×10^{-9}	+
Sp⁻H12-162	UV plus 20 AO	6.7×10^{-11}	-
Sp⁻H12-163	"	1.2×10^{-10}	+
Sp⁻H12-166	UV plus 40 AO	2.5×10^{-10}	-
Sp⁻H12-169	"	6.7×10^{-11}	-
Sp⁻H12-171	"	2.3×10^{-10}	-
Sp⁻H12-173	UV plus 60 AO	5.6×10^{-11}	-
Sp⁻H12-174	50 AO	7.7×10^{-10}	-
Sp⁻H12-175	UV plus 50 AO	1.6×10^{-10}	+
Sp⁻H12-178	UV plus 50 AO	1.7×10^{-10}	+
Sp⁻H12-182	UV plus 100 AO	3.0×10^{-10}	+
Sp⁻H12-184	"	1.9×10^{-10}	+
Sp⁻H12-186	UV	3.6×10^{-10}	+
Sp⁻H12-193	UV plus 20 AO	0	+
Sp⁻H12-201	"	0	+

☆ These mutants were not competent for transduction.

Table XVII

Sporulation Mutants Incompetent in Transduction (Group 5)

Mutant	Antibiotic Production	Phage Adsorption (%)
Sp⁻N2-12	-	36.0
Sp⁻W-3	+	99.5
Sp⁻W-4	+	99.8
Sp⁻W-5	+	98.4
Sp⁻W-7	+	97.7
Sp⁻W-8	+	95.5
Sp⁻W-10	+	87.5
Sp⁻W-17	+	77.0
Sp⁻W-18	+	99.5
Sp⁻W-19	+	77.0
Sp⁻W-20	+	82.0

The mutants in this table were produced by the combined treatment of UV and AO (20 ug/ml).

Table XVIII

Sensitivity of B. subtilis strains to UV light

Strain	Dose of UV Light (sec.) 0	30	60
Sp⁻W-4	1.0	8.2×10^{-2}	3.5×10^{-3}
Sp⁻W-18	1.0	1.7×10^{-1}	4.2×10^{-3}
W	1.0	2.7×10^{-2}	1.0×10^{-3}

NOTE: The numbers in this table are the fraction of surviving cells.

CHAPTER V

THE EFFECTS OF COBALT ION ON THE SPORULATION FACTOR IN *B. SUBTILIS* STRAIN H12

The experiments reported in this chapter were based on the work of Hirota (1956) who was able to eliminate the F-factor by treating the male *E. coli* cells with cobalt ion (Co^{++}).

Two methods were used for exposing the cells to Co^{++} in an attempt to eliminate the sporulation factor from *B. subtilis*. In the first method, the resistance of the bacteria to Co^{++} was increased by serial transfer in the presence of cobalt ion, followed by plating and selecting sporulation mutants. In the second, the cells were exposed to a relatively high concentration of the ion overnight followed by plating on agar media containing citrate to chelate the cobalt ion.

Method A

A culture grown in PG broth for 4 hr. was diluted 10^{-2} in 5.0 ml of PG broth containing Co^{++} at concentrations from 0.1 to 0.3 mM. After shaking for a further 24 hr. at 37°C, the treated culture which showed visible growth in the presence of the highest concentration was diluted into 5.0 ml of PG broth containing still higher levels of Co^{++} and shaking was continued for 24 hr. This process of serial transfers to higher concentrations of Co^{++} was continued until the highest level was reached. The culture was then spread on sporulation agar to detect mutants after 2 days incubation.

Method B

Co^{++} was added to a culture grown in PG broth in the form of $CoCl_2$ to a final concentration of 1.0 to 20 mM. The cultures were further shaken with incubation overnight and plated (0.1 ml per plate), after appropriate dilution, on sporulation agar containing 40 mM of citrate ion (CS agar). At times the cells were also spread on CYS or BS agar.

Results

It was found that strain H12 was naturally resistant to 0.2 mM of Co^{++} and that by 4 or 5 serial transfers to higher concentrations it could grow in broth containing 1.5 mM of Co^{++}. When the resistant cultures were plated on CS agar, small dark brown colonies were found which were sporogenous and resistant to 1.5 mM of Co^{++}. No asporogenous strains, not even albinos, were isolated after an initial treatment by method A. The small dark brown colonies just mentioned were further treated by serial dilution in Co^{++} and their resistance was increased to 2.0 mM. Spreading on CS agar after this second treatment produced four types of colonies of strikingly different morphology. The first two were both star-shaped, one being small and transparent, the other, large, opaque and white. The second two types were smooth-edged. One was large and dark and the other was small, white and opaque. All four were sporogenous. When these strains were treated again with 2.0 mM of Co^{++} and replated, no asporogenous colonies were isolated.

After H12 was treated with Co^{++} by method B, many small brown sporogenous colonies were isolated. Their resistance had been increased to 1.0 mM of Co^{++}. These were then exposed by serial transfer to higher

concentrations of Co^{++} up to 2.0 mM and then plated on CS agar. As before, a diverse array of colonies was found. Three sporogenous strains were isolated which produced small dark, large dark and medium-sized, opaque colonies. A fourth strain was frequently isolated which formed a very small, white microcolony. These proved to be asporogenous but grew poorly under most conditions. A typical example of these microcolonies was isolated and designated as Sp^{-}H12-41.

In an effort to find a medium on which Sp^{-}H12-41 would grow well, the following combinations of solid media and supplements were used: TB agar, TB agar plus 0.1 mM Co^{++}, TB agar plus 0.05% Yeast Extract (Difco), Sporulation agar, CS agar, CYS agar, Brain Heart agar and BS agar. It was discovered that this strain grew better on BS agar and on CYS agar. For this reason, these were used as well as sporulation agar in further experiments with Co^{++}.

Only microcolonies of the same type as Sp^{-}H12-41 were isolated when cells of H12 were irradiated with UV light prior to the Co^{++} treatment.

Strain Sp^{-}H12-41 had the following properties. In several reversion tests no revertants to sporogeny were observed. This may have been due in part to the fact that it would not grow well on solid media. In transduction experiments this strain was transduced to neither sporogeny nor prototrophy. Strain Sp^{-}H12-41 produces the antibiotic for Staphylococcus and is presumably blocked at a point later than stage I. Attempts to transform H12 for Co^{++} resistance by DNA from Sp^{-}H12-41 were unsuccessful.

Because of the inability to be transduced and to grow on ordinary culture media, no further study was carried out with this strain.

CHAPTER VI

DISCUSSION

Various attempts have been made to isolate sporulation mutants. Schaeffer (1961) and Takahashi (1961, 1965a) were able to produce stable asporogenous strains using UV light. Their mutants were, however, able to revert spontaneously to sp^+ and were transformable or transducible for the sporulation marker. In the present work, in addition to this type of mutant, two sp^- strains were isolated which could not revert spontaneously to sp^+ and which were not competent in transduction. Further tests showed that the two strains were unable to adsorb the bacteriophage PBS 1.

Other mutagenic agents used to produce sporulation mutants were the acridine dyes, AF and AO. These agents were known to eliminate episomes in the autonomous state from E. coli (Hirota and Iijima, 1957). Schaeffer and his co-workers (1959) proposed the existence of an episomal sporulation factor in B. subtilis, but they were unable to isolate sporulation mutants by acridine treatments (personal communication to I. Takahashi). To test this episome hypothesis, Rogolsky and Slepecky (1964) attempted to isolate sporulation mutants by treating cells with AF. They added AF to broth cultures at various stages of the growth cycle. Cells treated during the lag-phase produced the greatest number of mutants, almost all of which were oligosporogenous. On the other hand, significant numbers of asporogenous mutants were found only when the dye was added at early

log-phase. From these observations, Rogolsky and Slepecky postulated the presence of an autonomous genetic determinant for sporulation during the early stages of growth which becomes integrated in a later stage of growth.

According to the definition of episomes proposed by Jacob and Wollman (1961), when an episome is eliminated from the cells, for example by acridine treatment, the episome can be acquired only from an external source. Thus if the asporogenous mutants of Rogolsky and Slepecky are produced by the elimination of a sporulation episome, they should never revert to the sporogenous state. Since these authors reported no data on the rate of reversion, it is not possible to determine whether their mutants have really lost the sporulation episome.

Recently, Bott and Davidoff-Abelson (1966) have described similar experiments in which AO was used. Seven distinct types of sporulation mutants were produced and none of them was truly asporogenous. Glucose dehydrogenase, which is first detectable during very early sporulation stages, could not be detected in any mutants. They were, however, able to produce the protease and an antibiotic associated with early stages of sporulation.

Both AF and AO were used in the present study in an attempt to produce sporulation mutants. Treatment with AF produced many oligosporogenous mutants and only one strain in which $\underline{sp^+}$ revertants were not detectable. This strain, however, proved to be transducible to sporogeny. Acriflavine is known to be very bactericidal in its action; therefore, the conditions under which mutants are produced may be critical. It was

discovered that the cells were more resistant to AF during later stages in the growth cycle. This may be due to the fact that during early growth stages some metabolite may be produced which is particularly sensitive to AF. During later stages the cell's requirement for the metabolite or the concentration of the metabolite itself may be reduced, making the presence of AF less harmful. Alternatively, the differences in sensitivity of cells to AF may simply reflect a difference in the permeability to AF in cells at different growth stages.

Used alone, AO was less mutagenic than UV light and was not very effective in producing asporogenous strains. Several oligosporogenous mutants were examined but only one was isolated which showed no reversion to sporogeny. From the results of this and others' work acridines used alone do not appear to be effective in producing asporogenous mutants.

Episomes are known to exist in two states, integrated with the chromosome and autonomous or free in the cell. Hirota (1960) concluded that only episome factors in the autonomous state could be eliminated by acridines. Without abandoning the episome theory for sporulation, it is postulated that the sporulation factor may be in the integrated state and for this reason acridines are not effective in eliminating the episome and producing sporulation mutants. Transition of an episome from the integrated state to the autonomous state may be induced by UV light (Lwoff et al, 1950). A series of experiments were performed in this work in which cells were exposed to UV light prior to treatment with AO in an effort to eliminate the sporulation factor and produce sp$^-$ strains.

Five groups of mutants were isolated by the combined UV-AO treatment of cells. Albino mutants sporulated with a normal frequency but were unable to form the brown pigment associated with the production of spores. It is assumed, therefore, that they are not sporulation mutants but have a mutation affecting the synthesis of the pigment. Oligosporogenous mutants were able to produce spores with a frequency 10^3 to 10^6 times higher than that of asporogenous mutants. Since fairly large numbers of spores were produced, structural genes are probably not altered, but some regulatory function may be damaged. The third group included those which formed spores at a relatively low rate--similar to that expected for spontaneous mutation. These strains are most likely defective at a single site. Mutants in the fourth group showed no detectable $\underline{sp^+}$ revertants. These strains were transducible to $\underline{sp^+}$. The last group contained mutants which were similar to those of the fourth group but were not transducible to sporogeny. They were also unable to be transduced for other markers. Further tests showed that they were able to adsorb PBS 1 normally and that they were somehow unable to incorporate DNA into their genomes.

The most important finding made during the above experiments was that the frequency of asporogenous mutants produced by the combined UV-AO treatment was 2.7 to 4.6 times higher than the sum of the frequencies obtained by the single treatment of UV and AO (Table XV).

There are two hypotheses which may explain the increased frequency of sporulation mutants when cells are exposed to UV before treatment with AO. The first is a synergistic effect in which AO increases the

mutagenic activity of UV light. Shankel (1962) and Doneson et al (1964) reported a synergistic effect of caffeine or methylated purines on UV light. Witkin (1961) discovered that AF increased the mutagenic potency of low doses of UV light but showed no independent mutagenic activity on unirradiated bacteria itself. The interpretation was that UV light initiated mutagenesis by producing unstable changes in DNA and that the repair processes were blocked by the presence of AF. Acridines are known to combine with DNA probably by intercalation between the bases with extension of the backbone and consequently to cause a reversible change in the helical properties of the molecules (Lerman, 1961). Such modification of the DNA helix might well interfere with repair mechanisms.

The second hypothesis is that UV light causes the transition of the hypothetical sporulation factor from the integrated to the autonomous state and acridines eliminate the factor to produce sporulation mutants. In the integrated state, an episome appears to occupy a specific site on the bacterial chromosome (Lwoff, 1953). The critical process in induction is considered to be the uncoupling of the factor from chromosomal DNA. Once the sporulation factor is in the autonomous state it is subject to the effect of AF or AO and can be eliminated.

If elimination of a factor for sporulation is possible, one would expect the production of asporogenous mutants incapable of reverting to sp^+ spontaneously. These mutants should, however, be fully competent in transduction and should be able to act as donor strain in the transduction of normal sp^- strains to sp^+. One such strain, Sp^-H12-3, has been isolated and investigated (Takahashi, 1965a). In the present study it was also not

possible to detect any spores in strain Sp⁻H12-3. No sp⁺ clones could be obtained from this strain by transduction, transformation, cell to cell contact or treatment with cell extracts from a wild-type strain. An asporogenous strain possessing mutations at several sites would exhibit similar properties. Such a mutant should be able to transduce normal sporulating bacteria to asporogeny. No asporogenous clones were ever produced from strain 168 using Sp⁻H12-3 as donor in transduction and transformation experiments. By the same techniques, however, strain 168 readily formed prototrophic recombinants. It is most unlikely, in view of its inability to transfer asporogeny, that Sp⁻H12-3 has point mutations on the chromosome.

Strain Sp⁻H12-3 exhibits characteristics which might be attributed to a deletion on the chromosome. In transduction experiments, Sp⁻H12-3 was able to act successfully as donor for several sp⁻ mutants, each one of which was known to have its mutation located on the chromosome (Takahashi, 1965a). Therefore Sp⁻H12-3 does not appear to have a deletion of sporulation genes on the chromosome. It appears to have lost irreversibly some sporulation factor which cannot be replaced by any of the conventional techniques known to us.

Interesting hybrid competition experiments were performed by Yamagishi and Takahashi (in press). Strain Sp⁻H12-3 was the only mutant which failed to produce mRNA as early in the sporulation process as the end of log-phase. That it is a mutant blocked at a very early stage is confirmed by the inability to produce the antibiotic. The same competition experiments lead to the conclusion that the DNA of Sp⁻H12-3 was

missing a portion of the sporulation genome which transcribes for the early mRNA. Since this strain does not appear to have a chromosomal deletion, it may have lost an extrachromosomal determinant for sporulation. The inability to form spores after transduction may be due to the fact that the phage PBS 1 cannot incorporate DNA from the extrachromosomal determinant in wild-type strains. The inability to transfer the extrachromosomal determinant from wild-type strains to Sp⁻H12-3 may be peculiar to phage PBS 1.

In the present study the combined UV-AO treatment did not produce asporogenous mutants of the same type as strain Sp⁻H12-3. However, the fact that the production of mutants is enhanced by the combined UV-AO treatment encourages one to consider that it may be possible by improving the technique to isolate mutants similar to Sp⁻H12-3 and to study further their properties. Optimal conditions for the treatment of cells are still unknown. Concentrations and time of addition of the acridine dyes and UV doses to be used should be extensively investigated. In such an intricate system, small changes in procedure may be critical and produce vastly different results.

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