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Title ISOLATION OF AUXOTHROPHIC MUTANTS OF BACILLUS CEREUS

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Bacillus cereus excretes large amounts of penicillinase into growth media during incubation, hence the widely-used penicillin selection technique for isolating biochemical mutants of bacteria is not applicable to this species. Genetic transfer in B. cereus has not been reported; this is partly due to the lack of an effective isolation method for selection of nutritionally deficient mutants in this organism.

The original purpose of this research was to work out an effective auxotroph isolation procedure which would be applicable to B. cereus; it was intended to take advantage of the difference in thermolability of spores and vegetative cells. Spores in which mutation has been induced by ultraviolet light irradiation do not germinate as readily as the normal spores in a germination medium; therefore, mutant spores can be concentrated selectively by heat inactivation to kill most germinated prototrophs.

Spore suspensions were irradiated with UV light at a distance of 40.5 cm for three to four minutes which resulted in 99 percent kill. The germinating broth medium contained adenosine and alanine in a pH 7.4 sodium phosphate buffer. Normally, germination was carried out in a 37°C water bath for 30 minutes. After heat-shocking at 65°C for 60 minutes, the culture was plated at appropriate dilutions onto minimal medium containing 0.2% nutrient broth-yeast extract medium (doubly-enriched minimal medium).

Mutant cells formed only minute colonies on this medium in contrast to the normal cells which formed large colonies. The minute colonies were picked and characterized for nutritional requirements by replica plating onto various minimal media supplemented with different nutrients. The requirements of the mutants were later confirmed by growing the mutant cells in the presence and absence of the suspected nutrients.

Results of this procedure indicated that nutritional mutants could not be readily obtained; the mutants isolated were found to be unstable in that they frequently reverted back to normal. Consequently, a second procedure, which was originally intended as a comparative method and involved using a chemical mutagen, was developed for selecting auxotrophs. Diethylsulfate (DES) in various concentrations was incorporated into a doubly-enriched minimal medium.

The plates were dried at 25°C for four hours, and 1,000 spores were plated directly onto this medium. The sectored colonies were picked and assayed for mutants.

DES in a final concentration of 4% (v/v) gave the following mutation frequencies (number of auxotrophs per number of total surviving colonies): 6464 cured, $4x10^{-3}$; 569R, $3x10^{-3}$; 6464A, $2x10^{-3}$; 569S, $1x10^{-3}$; 9139, $7x10^{-4}$; and 6464D, $1x10^{-4}$. In the presence of 6% DES, however, $2x10^{-3}$ auxotrophs were obtained with 6464D.

It was found that the mutagenic property of DES could be varied by changing the drying temperature of DES-containing agar plates. When the plates spread with 6464 cured were dried at 24°C for four hours, the auxotrophic frequency was $4x10^{-3}$; plates dried at 30°C and 37°C yielded mutation frequencies of $2x10^{-3}$ and $1x10^{-3}$ respectively. When different kinds of colonies picked were compared, the sectored colonies showed $4x10^{-3}$ auxotrophs in contrast to none obtained from small colonies at 24°C drying temperature.

The mutagenic effect of DES on vegetative cells was also investigated. Results indicated that DES could induce mutations readily in cells which were in the exponential growth phase. Although mutations also occurred in cells in the stationary phase, the auxotrophic frequencies were much lower than that of cells growing exponentially. It was also found that the

mutants obtained by DES treatment were far more stable than those obtained by UV irradiation. More than 70% of the mutants of 9139, 6464 cured and 569R were stable.

Approximately 50% of all the mutants isolated were characterized. The majority of the mutants required one of the following amino acids: arginine, cysteine, glycine, histidine, leucine, methionine, phenylalanine and serine. Some others were found to require nicotinamide, adenine, hypoxanthine, thiamine or uracil. Interestingly, mutants deficient in sulfur-containing amino acids (methionine, methionine and/or cysteine) were found to be more common than those requiring other nutrients.

ISOLATION OF AUXOTROPHIC MUTANTS OF *BACILLUS CEREUS*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Tables -- Continued

ISOLATION OF AUXOTROPHIC MUTANTS OF *BACILLUS CEREUS*

INTRODUCTION

Bacillus cereus excretes large amounts of penicillinase into growth media during incubation, hence the widely-used penicillin selection technique for isolating biochemical mutants of bacteria is not applicable to this species. Genetic transfer in *B. cereus* has not been reported; this is partly due to the lack of an effective isolation method for selection of nutritionally deficient mutants in this organism.

The original purpose of this research was to work out an effective auxotroph isolation procedure which would be applicable to *B. cereus*. It was intended to take advantage of the difference in thermolability of spores and vegetative cells. Spores in which mutation has been induced by ultraviolet light irradiation do not germinate as readily as the normal spores in a germination medium; therefore, mutant spores can be concentrated selectively by heat-shocking to kill most of the germinated prototrophs. The subsequent spreading of the heat-shocked population on enriched minimal medium shows that mutant cells form only minute colonies in contrast to the normal cells which form large colonies. The minute colonies are picked and characterized for

nutritional requirement by replica plating onto various media. Early in the present work it was discovered that nutritional mutants could not be readily obtained by this procedure. The mutants isolated were found to be unstable in that they frequently reverted back to normal. Because of this failure, a second procedure which was originally intended as a comparative method and involved use of a chemical mutagen, was developed for selecting auxotrophs; this thesis reports results obtained with the method developed.

HISTORICAL REVIEW

Mutation which is the result of a sudden stable change in gene structure takes place regularly in all species of organisms. Mutation can occur spontaneously (6, 11) or be induced by exposure to radiation (UV light and X-ray) or to certain chemicals. In 1926, Muller reported the production of mutations in Drosophila by X-rays (14, 192-195). Attempts during World War II by Auerbach and Robson (1, 302) to introduce mutations by chemical means were successful but this information was not published in declassified form until 1946. Since then the list of mutagenic chemicals has grown quite long and presently includes such diverse compounds as formaldehyde, urethane, various acridine dyes, β-propiolactone, manganous chloride, nitrous acid, mustard gas, various nitrogen mustards, epoxides, ethylene imines and methane sulfonic acids esters (4, 119-136).

The molecular basis of mutation is alteration of the nucleotide sequence in DNA. Modification or deletion of a base in the nucleotide sequence will result in mutation. The mechanism by which radiation or chemical mutagens produce their effects is still obscure. X-rays and similar types of radiation release large amounts of energy upon passage through living matter; when this happens in or near a chromosome it may result in the

physical or chemical alteration of gene. Mutagenic chemicals may react with the genes directly or indirectly through reactions with the cytoplasm (5, 185-212).

Auxotrophs, which are mutants lacking the ability to synthesize some essential metabolites, have played an important role in microbial genetics. After exposing a microorganism to a mutagenic agent, different kinds of auxotrophs can be isolated. By the selection of suitable biochemical mutants, it often is possible to determine a biosynthetic pathway for a particular nutrient. Studies on the biosynthesis of arginine in Neurospora crassa (19, 129-139) was one of the most classical examples of metabolic studies during which auxotrophs were used to great advantage.

The effective auxotroph selection methods which have contributed to the rapid advancement of microbial genetics were devised by a number of investigators. In 1940, Lederberg and Tatum reported (7, 381-382) the delayed enrichment technique to detect biochemical mutants of microorganisms. Three years later Davis (3, 4267) and Lederberg and Zinder (8, 4267-4268) reported simultaneously the ingenious penicillin selection method; by adding penicillin to minimal medium, auxotrophs could be concentrated by virtue of the fact that the antibiotic would kill only growing cells. This experimental technique combined with the replica plating technique

developed later by Lederberg and Lederberg (6, 399-406) has provided a most effective method for isolation of auxotrophs of many microorganisms.

Members of the genus Bacillus are gram positive, aerobic, spore forming bacteria. When penicillin is present during incubation, these organisms excrete large amounts of inducible penicillinase into the surrounding medium (17, 269-271). Therefore the penicillin selection method is not suitable for use in selecting mutants of organisms in this genus. Attempts to overcome this limitation were made by Bott and Lundgren (2, 281-283) who used staphcillin in place of penicillin in effort to select mutants of B. cereus. Auxotrophs were obtained; however, the kinds of mutants were limited to those requiring sulfur-containing amino acids.

The various problems associated with germination of bacilli have provoked many investigations. It has been shown that manganese, L-alanine, adenosine, heat and glucose are stimulating agents for germination of B. megaterium (9, 368-374) and B. subtilis (22, 384-389). O'Brien and Campbell (16, 522-525) reported that spores of B. cereus var. terminalis required isolencine, leucine, valine and methionine for outgrowth and the addition of L-alanine and adenosine greatly accelerated the rate of germination at 35°C. Thorley and Wolf (20, 1-13) investigated the optimum pH and temperature for

germination of different strains of B. cereus when L-alanine, adenosisne and inosine were used as stimulating chemicals. They found that maximum germination occurred at 30°-37°C at pH 6.2 to 8.3 for most strains.

By taking advantage of the difference in thermolability of spores and vegetative cells, Lyer (10, 309-310) developed a germination and heat-shocking method for selecting mutants of B. subtilis. Mutated spores induced with UV light irradiation did not germinate as readily as the prototrophs in a minimal medium, therefore the auxotrophs could be concentrated selectively by heat inactivation to kill most of the germinated prototrophs. Auxotrophs were then selected either by the replica plating method or the delayed double enrichment technique.

The mutational synergism of UV light and caffein in Escherichia coli was reported by Shankel (18, 410-415). The number of streptomycin resistant mutants increased after the irradiation of cells with non-lethal dosage of UV light followed by the incubation of culture on medium which contained caffein (500 μg/ml).

MATERIALS AND METHODS

Cultures

Bacillus cereus strains 6464A, 6464D, 569R (rough form), 569S (smooth form), 6464 cured, 9139, and 7064 were obtained from Dr. C. B. Thorne. Auxotrophic mutants derived from these strains were isolated by the diethyl-sulfate (DES) method except for a few which were isolated following ultraviolet (UV) light irradiation and germination. Both of these procedures are described below. The auxotrophic mutants and their requirements, where known, are shown in Table 1 and Table 16.

Media

Nutrient broth-yeast extract (NBY) medium was composed of 8 g of Difco dehydrated nutrient broth and 3 g of Difco yeast extract per liter of distilled water.

Potato medium (21, 106-111) was prepared as follows: Diced potatoes (200 g) were boiled for five minutes in one liter of water, and the material was filtered through Whatman No. 1 filter paper supported on a Buchner funnel. Two grams of Difco yeast extract and 20 g of N-Z case peptone were added. The pH was adjusted to 7.2 with HCl or NaOH, and the solution was diluted to 2 liters. Potato agar was made by adding 20 g of agar per liter of potato broth medium.

Minimal I medium (21, 106-111) was composed of 2 g

of $(NH_4)_2SO_4$, 6 g of KH_2PO_4, 1 g of sodium citrate, 5 g of glucose (autoclaved separately), 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 0.04 g of $FeCl_3 \cdot 6\ H_2O$, 0.00025 g of $MnSO_4 \cdot H_2O$, and 1 g of L-glutamic acid per liter. The pH of the medium was adjusted to 6.9-7.0 with HCl or NaOH. Enriched Minimal I medium (MNBY) was made in the same manner but in addition had 1 ml (single strength) or 2 ml (double strength) of NBY broth added per 100 ml of medium.

Phage assay broth (PAB) (21, 106-111) was composed of 8 g of Difco nutrient broth, 5 g of NaCl, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 0.05 g of $MnSO_4 \cdot 2H_2O$, and 0.15 g of $CaCl_2 \cdot 2\ H_2O$ per liter. The pH was adjusted to 5.9-6.0 with HCl.

Minimal I-casamino acid (MCA) medium was Minimal I medium with 3 g of vitamin-free casamino acids (Difco) added per liter. Minimal I-yeast extract (MY) medium was 3 g of yeast extract added to 1 liter of Minimal I medium.

Sterile agar medium was stored in prescription bottles and sterile broth medium in 250 ml flasks until ready for use. Sterilization consisted of autoclaving for 20 minutes at 121°C.

Cultural conditions

Unless otherwise stated, cultures were grown on a

rotary shaker in 250 ml Erlenmeyer flasks containing 50 ml of medium. All cultures were incubated at 37°C.

Preparation of spore suspensions

Stock spore suspensions were prepared by two methods:

(a) Potato agar slopes in screw cap tubes (6-inches) were inoculated with the appropriate culture and incubated at 37°C for about five days or until most of the cells had sporulated. Five ml of sterile distilled water were added to each tube to suspend the growth. The suspensions from each tube were pooled, mixed thoroughly and heat-shocked at 65°C for 60 minutes.

(b) The appropriate culture was inoculated into a 250 ml flask containing 50 ml of PAB and the culture was incubated on a rotary shaker at 37°C for about five days or until most of the cells had sporulated. The culture was centrifuged at 3090 x g for 15 minutes. The supernatent was decanted, and the pellet was resuspended in 50 ml of sterile distilled water. The resuspended growth was centrifuged at 3090 x g for 15 minutes. The pellet was finally suspended in 20 ml of sterile distilled water, and the suspension was heated at 65°C for 60 minutes to kill the vegetative cells.

Exponential growth determination

A spore suspension (0.1 ml) was inoculated into a 250 ml flask, containing 50 ml of NBY, and incubated on

a rotary shaker at 37°C. At varying time intervals, total and spore counts were determined by plating appropriate dilutions on NBY agar plates. The growth curve which was used to determine the length of time needed to achieve cells in the exponential growth phase is shown in Figure 1.

Isolation of auxotrophic mutants

Auxotrophic mutants were isolated by two methods:

(a) Germination method: A spore suspension (5 ml at 10^9 spores/ml) was irradiated with UV light for a predetermined time in order to achieve 99% killing. Two 15-watt General Electric germicidal tubes were used as the light source. The spores were irradiated in an open flat bottom petri dish placed on a rotary platform 16 inches from the light source.

Vitamin free casamino acids (containing no tryptophan) were incorporated into Minimal I medium. Initially this germinating broth was intended for isolation of tryptophan requiring mutants. Theoretically, prototrophs and all auxotrophs other than those dependent on tryptophan or vitamins would germinate during incubation. The 5 ml irradiated spore suspensions were transferred to a 250 ml flask containing 50 ml of Minimal I medium supplemented with 0.3% vitamin free casamino acids. The culture was incubated on a rotary shaker at 37°C for eight hours. The resulting culture was held at 65°C for

one hour to kill all the germinated spores. The spores were than plated on single enrichment Minimal I agar at a dilution that gave 200-300 colonies per plate. Any tiny colonies that came up among the large prototrophic colonies after appropriate incubation were picked and tested to see whether they were auxotrophs. By the replica plating technique (6, 399-406) colonies which did not show growth on either minimal agar and MCA agar plates were presumed to be tryptophan or vitamin dependent. Colonies showing no growth on minimal medium were regarded as auxotrophs for an unidentified nutrient.

A second germination broth which contained substances stimulating germination was used for isolation of auxotrophs. The irradiated spores were added to a screw cap tube which contained the following concentration of germination materials: (1) Single strength---adenosine 2.5 μmoles/ml; alanine 5.0 μmoles/ml and 0.03 M Na_2HPO_4 buffer at pH 7.4. (2) Double strength--- adenosine 5.0 μmoles/ml; alanine 10.0 μmoles/ml; and 0.01 M Na_2HPO_4 buffer. The germination was carried out in a 37°C water bath until 99% germination was obtained. The isolating and testing procedures were the same as described above. The irradiated pregermination spores were used as a control.

(b) Diethylsulfate method: One thousand spores or cells which were in the logarithmic growth phase were

plated directly onto DES plates (4% v/v DES) which were immediately dried at 24°C for four hours. The plates, which contained 25 ml of double enriched MNBY agar, were incubated for two days at 37°C in order to allow mutated cells to develop completely. The tiny and sectored colonies were picked and tested by replica plating onto Minimal I agar plates. The stability of auxotrophs was further confirmed by repeating the picking and replication.

Characterization of auxotrophic mutants

The nutritional requirements of the auxotrophs were determined by replica plating onto plates of Minimal I agar supplemented with appropriate nutrients. Stock solutions of the nutrients were made in distilled water at a concentration of 2 mg of each constituent per ml and used in a final concentration of 20 µg per ml (vitamins were used at a final concentration of 5 µg per ml). Pools of these substances were set up as shown in Table 2. For example, if an auxotroph only grew on pools 3 and 8 it was presumed to require tryptophan. If an auxotroph failed to grow on any of the pools, it was believed to require more than one substance or other growth factors not included in the pools. Diagnoses were confirmed by spreading cells of the auxotrophs on a minimal agar plate and placing on the agar a filter paper disc saturated with a solution of the suspected compound.

If the diagnosis was correct, the cells would grow near the disc but not elsewhere on the plate.

Unidentified auxotrophs were further diagnosed by subdividing them into two categories: (1) those which grew both on MCA and MY medium agar had multiple requirements for amino acids only, and (2) those which grew on MY medium agar required both amino acids and vitamins or nitrogenous bases of nucleic acids. The Group 1 mutants were tentatively diagnosed by replicating them onto minimal medium pools supplemented with various amino acids. Group 2 mutants were characterized in the same manner by using vitamins, purine, and pyrimidine derivatives incorporated into minimal medium pools. Stock solutions were prepared and used in the same manner as in the preceding diagnosis. Pools of these constituents were set up as in Table 3 and Table 4 respectively.

RESULTS AND DISCUSSION

UV death curve

When UV light was used as mutagenic agent, the dosage which caused 99% lethality of spores showed the greatest inducing effect. Therefore a UV light killing curve was needed to determine the length of irradiation time which gave 99% destruction. Two strains used were 6464D (8.4×10^8 spores/ml) and 569 (3×10^9 spores/ml). Spore suspension (5.0 ml) was irradiated with UV light at 40.5 cm for 0.5, 1.0, 2.0, 4.0 and 6.0 minutes. The survival counts at each time were made by plating the appropriate dilutions of the suspensions on NBY agar. The resulting death curves are shown in Figure 2. Times of three minutes and four minutes irradiation, which caused 97% kill of 6464D and 99% kill of 569 respectively, were chosen as the optimum UV light dosage in subsequent auxotroph isolation experiments. The longer irradiation time needed for 569 suggested that this strain was more resistant to UV light than 6464D.

Attempts to isolate tryptophan requiring mutants with MCA medium

Originally the medium and procedure used was intended for the isolation of tryptophan requiring mutants. When MCA medium (containing no tryptophan or vitamins) was used as the germinating broth, prototrophs

and auxotrophs, other than those dependent on tryptophan or vitamins, would germinate readily and were killed by heat-inactivation. The surviving spores were then plated onto singly enriched minimal agar to carry out the mutant isolation procedure. Those colonies which failed to show growth on MCA agar were assumed to be tryptophan mutants. Unexpectedly, only one apparent tryptophan mutant appeared among 629 colonies picked from 6464A and none were obtained from 815 6464D tiny colonies. The assumption that cells which failed to grow on MCA medium were tryptophan mutants was therefore proved wrong; furthermore, the supposed tryptophan mutant of 6464A did not show any growth around a filter paper disc saturated with tryptophan solution. Since no vitamins were present in the casamino acids, seven minimal pools with nicotinamide, riboflavin, thymine, para-aminobenzoic acid, calcium pantothenate, cytosine, inositol, uracil, hypoxanthine, pyridoxine and xanthine were used to diagnose its nutritional requirements. It was found that the auxotroph showed growth on the pools which contained either hypoxanthine or xanthine. When a confirmed test was carried out, the auxotroph showed growth around either hypoxanthine or xanthine filter paper discs and no growth appeared between these two discs. Therefore the diagnosis indicated that the assumed tryptophan requiring mutant was really a hypoxanthine or xanthine singly

dependent mutant. This suggested that the metabolic pathway of purine synthesis could be blocked for either hypoxanthine or xanthine in this mutant. The relative position of hypoxanthine and xanthine in this pathway could not be determined, although the growth on hypoxanthine plates was better than that on minimal medium supplemented with xanthine.

These results indicated that MCA medium was not a favorable medium for isolating tryptophan requiring mutants since mutants deficient in vitamins and nucleic acids also could be produced. The low auxotrophic frequencies in both the preceding and following experiments also revealed that some medium other than MCA medium was needed for auxotroph isolation.

Following these experiments attempts were made to enlarge the types of mutants selected by using the heat-inactivated stock culture described above; the same procedure and media were used, except the master plate, which was planted with tiny colonies, was replicated onto both minimal agar and MCA agar plates. Cells failing to grow only on minimal agar would be deficient for amino acids. A large number (278) of colonies was picked from 6464A and only two turned out to be auxotrophs (both unidentified); 214 colonies were picked from 6464D and only three assayed as auxotrophs (two requiring serine and one unidentified). The auxotrophic frequencies,

$1x10^{-4}$ for 6464A and $2x10^{-4}$ for 6464D, were quite low. These low frequencies probably resulted from a predominance of prototrophic spores in the population which remained in the MCA medium after heat inactivation. This predominance of prototrophs could result from the resporulation of germinated spores during incubation.

Attempts to isolate auxotrophs using germination stimulants

Next an attempt was made to utilize some chemicals which are known to have a stimulating effect on germination of spores. Design of the following experiment was based on the assumption that adenosine and alanine, both of which stimulate the germination of spores, would only stimulate the germination of wild type spores in minimal medium. At first, single strength adenosine and alanine were used in the germination broth. Irradiated spores (2.0 ml) were added to a screw-cap tube which contained 0.4 ml of adenosine (stock solution 25 μmoles/ml), 0.4 ml of alanine (stock solution 50 μmoles/ml), and 1.2 ml of pH 7.4 sodium phosphate buffer (stock solution 0.03 M). The resulting mixture was incubated in a 37°C water bath for 30 minutes to allow germination of the spores. After heat-shocking the culture at 65°C for one hour, the routine isolation procedure was carried out. The results shown in Table 7 indicate that no auxotrophs

were isolated from either pregermination or post-germination spores. The failure to obtain any auxotrophs could be due to the low germination rate (less than 70%). Two possibilities could explain the low percentage of germination: (1) insufficient concentration of adenosine and alanine; (2) the resistance of the strains to germination in the germinating broth.

Attempts then were made to determine the incubation time which gave 99% germination when the concentrations of adenosine and alanine were increased. A double strength germination broth mixture was used which contained 1 ml of irradiated spores, 0.4 ml each of adenosine and alanine, and 0.2 ml of phosphate buffer (same stock solutions used as above). Germination was carried out in a 37^{o}C water broth and at each designated time spore counts were made by plating appropriate dilutions of the heat inactivated germination mixture on NBY agar. The results shown in Table 6 indicate that the single strength germinating nutrients could not effectively stimulate the germination of spores even though the spores had been incubated for germination for 60 minutes. However, in the presence of double strength adenosine and alanine, 30 minutes of incubation gave rise to about a 2 logarithmic unit drop of spore count when compared to that of pregermination counts. The almost steady spore counts after 30 minutes of incubation could result

from an equilibrium between cell division and spore reformation. Therefore, it seemed that it might be possible to isolate auxotrophs with 30 minutes of incubation at 37°C in the presence of double strength adenosine and alanine. Such, however, was not the case. The results shown in Table 7 indicate that although the post germination spore count was reduced by two logarithms, no auxotrophs were isolated from either 569R or 569S. Some other substances such as casamino acids, yeast extract, glycine and threonine, and double strength adenosine and alanine with added glucose were also tested for possible stimulation of germination. The results indicated that double strength adenosine and alanine had the greatest effect on germination of spores of 569. Glucose added to double strength adenosine and alanine did not increase the germination. Since spores of auxotrophs could not germinate as readily as prototrophs in the minimal broth (except those requiring adenosine and alanine), failure to obtain auxotrophs by using germinating broth apparently was due to the presence of too few mutated spores to be detected in the germinating culture. This indicated that the B. cereus strains investigated were resistant to UV light mutation under the conditions used.

Mutagenic induction of spores by diethylsulfate

Originally the DES method was intended for use as a comparison with the germination method. However, due to the author's inability to obtain mutants with the germination method, use of the chemical mutagenic agent was tested. This method turned out to be very promising during preliminary testing and therefore it was studied in more detail.

With one exception (strain 6464D), use of 4%(v/v) DES and double strength MNBY medium resulted in the easy isolation of mutants from spores. Twenty-five ml of MNBY agar was used per plate to minimize the effect of volume of medium on the mutagenic strength of DES. Initially, the DES-MNBY plates were dried at room temperature (23^{o}-25^{o}C) for four hours before the spores were plated. However, all the DES plates used in later experiments were dried in a 25^{o}C incubator for the same time in order to strictly control the strength of DES. These two drying temperatures did not appear to influence the frequency of auxotrophs obtained. Usually 1,000 spores were plated onto DES plates except controls for spontaneous mutation test which consisted of plain MNBY plates spread with 500 spores. From Table 8 it may be seen that the frequencies of auxotrophs found for the different strains were: 6464 cured, $4x10^{-3}$; 569R, $3x10^{-3}$; 6464A, $2x10^{-3}$; 569S, $1x10^{-3}$; 9139, $7x10^{-4}$; and 6464D, $1x10^{-4}$.

The high mutation frequency of 6464 cured may be related to the removal of phage x, since 6464A and D are lysogenic for phage x. The rather low mutation frequency of 6464D in contrast to a relatively high frequency of 6464A could indicate a genotypic difference between these two types in response to mutation by 4% DES. One auxotroph which appeared on each of the plates of 6464A and 6464 cured perhaps resulted from spontaneous mutation. Those colonies picked (Table 10) were small or sectored and both were considered prospective auxotrophs. However, the results indicated (see below) that almost every auxotroph originated from sectored colonies instead of the tiny colonies. The difference in mutation frequency between 569R and 569S was due to the former having more sectored colonies than that of the latter, and the same case was also revealed in 6464A and 6464D. There was no evidence to relate mutation frequency to the percent survival. However, the survival percentage could be affected by such following factors: concentration of DES; concentration of enrichment; thickness of agar; drying period and drying temperature after spreading DES.

Failure to obtain auxotrophs from 6464D when 4% DES and double enrichment were used perhaps was due to insufficient concentration of DES or the fact that the double enrichment was too rich to differentiate between

colonies of wild types and auxotrophs. Therefore another experiment was run to test the effect of different concentrations of both DES and enrichment on the mutation frequencies. The results of this experiment are shown in Table 9. When single enrichment was used, there were no auxotrophs obtained with different concentrations of DES. The survival percentage of single enrichment was also much lower than that of double enrichment when the two corresponding DES concentrations were compared. This indicated that single enrichment would not support the growth of the mutants under the inhibiting effect of DES. However, when minimal medium was doubly enriched, the frequency of auxotrophs was 2×10^{-3} and 1×10^{-3} with 6% and 8% DES respectively. Therefore double enrichment was the appropriate concentration for growth of the mutated spores.

Based on the results of preceding experiments, DES revealed itself to be a prospective chemical mutagen for B. cereus, although the frequencies of mutation varied with respect to strains. The combination of 4% DES and double enrichment could be used for isolation of mutants of all strains except 6464D. However, when double enrichment was incorporated with 6% DES, more than 1.5×10^{-3} auxotrophs of 6464D were observed. Therefore double enrichment of media containing various concentrations of DES, depending on the strains investigated, provided

an effective method for isolating auxotrophs of B. cereus.

Effect of temperature of drying DES plates and kinds of colonies on auxotrophic frequencies

The following experiment was run to see whether the drying temperature of DES plates and kinds of colonies picked could influence the mutation frequencies. The strain used for this study was 6464 cured which showed a high percentage of auxotrophs ($4x10^{-3}$) and was expected to make the effect clearly shown. MNBY plates containing 4% DES each were dried at room temperature (22^{o}-24^{o}C), 30^{o}C and 37^{o}C for four hours, three hours and three hours respectively. Spores (1,000) were plated onto each plate which were incubated at 37^{o}C for two days. Small and sectored colonies were picked and assayed for auxotrophs as described under Methods and Materials. The results are shown in Table 10. Drying DES plates at room temperature (22^{o}-24^{o}C) gave $4x10^{-3}$ auxotrophs in contrast to a lower $2x10^{-3}$ at 30^{o}C and $1x10^{-3}$ at 37^{o}C when only sectored colonies were considered. The comparative frequencies of auxotrophs obtained from small colonies were 0, $3x10^{-4}$ and $1x10^{-4}$ for room temperature, 30^{o}C and 37^{o}C respectively. The higher frequency of auxotrophs from sectored colonies obviously indicated that the great majority of auxotrophs resulted from sectored colonies instead of small ones. The higher frequency of mutation resulting from lower drying temperature could be due to the lower rate

of evaporation of DES during drying and hence the stronger the inducing effect. The higher survival percentage at higher temperatures also revealed that the increased rate of evaporation contributed to the reduced inhibiting effect of DES on cells.

Effect of UV light and DES on spores

It has been shown (18, 410-415) that caffein and UV light exert a synergistic effect on the mutation of E. coli. It therefore seemed worth while to determine whether or not UV light plus DES would further increase the percentage of auxotrophs. Spores (1,000) irradiated with UV light for one minute were plated onto DES plates. Doubly-enriched MNBY medium and 4% DES were used for all strains of B. cereus investigated except 6% DES in the case of strain 6464D. All the sectored colonies appearing on the plates were picked and tested by the procedures given in the Methods and Materials section. The results shown in Table 11 indicate that the mutagens had no apparent significant synergistic effect on spores, although there was a slight increase in frequencies of auxotrophs in strains 6464A, 569R and 9139. The high spontaneous mutation rate of 6464A and 6464 cured on plain MNBY medium may indicate that both strains were more sensitive to induction by nutrient deficiency.

Mutagenic induction of vegetative cells by DES

Since the DES method worked very well with spores, it was thought that the same method could also be applied to vegetative cells. Spores (0.1 ml) were inoculated into each of two 250 ml flasks containing 50 ml of NBY broth and incubated on the 37°C rotary shaker for appropriate times (determined from Figure 1) for the cells of one flask to be in exponential growth and cells of the other in stationary growth. Cells (1,000) from the respective cultures were plated onto DES plates and the sectored colonies were picked and assayed by the procedure described in the Methods and Materials section. The results of both experiments shown in Tables 12 and 13 revealed that a higher frequency of auxotrophs were obtained from cells in the exponential growth phase than that from cells in the stationary growth phase. The average frequency of auxotrophs with vegetative cells was higher than in the case of the spores, and fewer sectored colonies appeared in the former. The lower survival percentage of cells when compared with that of spores indicated a stronger inhibiting effect on cells, and resulted in the appearance of fewer sectored colonies. That one of the two sectored colonies appearing on control MNBY medium turned out to be an auxotroph confirmed the observation that the 6464 cured culture contained some spontaneous mutants. The lower mutant

frequency of cells in the stationary growth phase apparently was due to the lesser susceptibility of older cells to DES induction. It seems likely that spores may have already germinated before DES acted.

Characterization of auxotrophs

The nutritional requirements of mutants were determined by replica plating the master plates onto plates of minimal medium supplemented with appropriate nutrients. The procedures are given in the Methods and Materials section and the nutritional pools for characterization are shown in Tables 2, 3 and 4.

From the data it may be concluded that DES is a better mutagen than UV light. It was also found that the mutants obtained by DES treatment were more stable than those obtained by UV irradiation. Stability of the auxotrophs obtained from B. cereus 9139, 6464 cured and 569R, based on the results shown in Table 14, was greater than 70 percent.

The results shown in Table 15 indicate that use of the method devised provided characterization of approximately 50 percent of all the mutants isolated. The majority of the mutants characterized required one of the following amino acids: arginine, cysteine, glycine, histidine, leucine, methionine, phenylalanine or serine. Interestingly, the requirement for sulfur-containing amino acids (methionine, methionine and/or cysteine) was much more common than other requirements. Nicotinamide

was the only requirement found in 6464 cured. A few other mutants were found to require adenine, hypoxanthine, thiamine and uracil respectively. Those mutants which could not be characterized are believed to be multiple mutants which, by their growth patterns, could be subdivided into two distinct groups: (1) those which have multiple requirements for amino acids only and (2) those which appear to require both amino acids and vitamins or nitrogenous bases of nucleic acids.

It was evident that the DES method was much better than the staphcillin method when both frequency and mutant types were compared. By using the drug staphcillin only sulfur-containing amino acid deficient mutants were reported in B. cereus 4343, however, other varieties of mutant requirements were found with the DES method. The explanation for the common deficiency in sulfur-containing amino acids is not clear, although it is known that other investigators working with different organisms have noticed that mutants deficient in sulfur-containing amino acids are very common (2, 281-283).

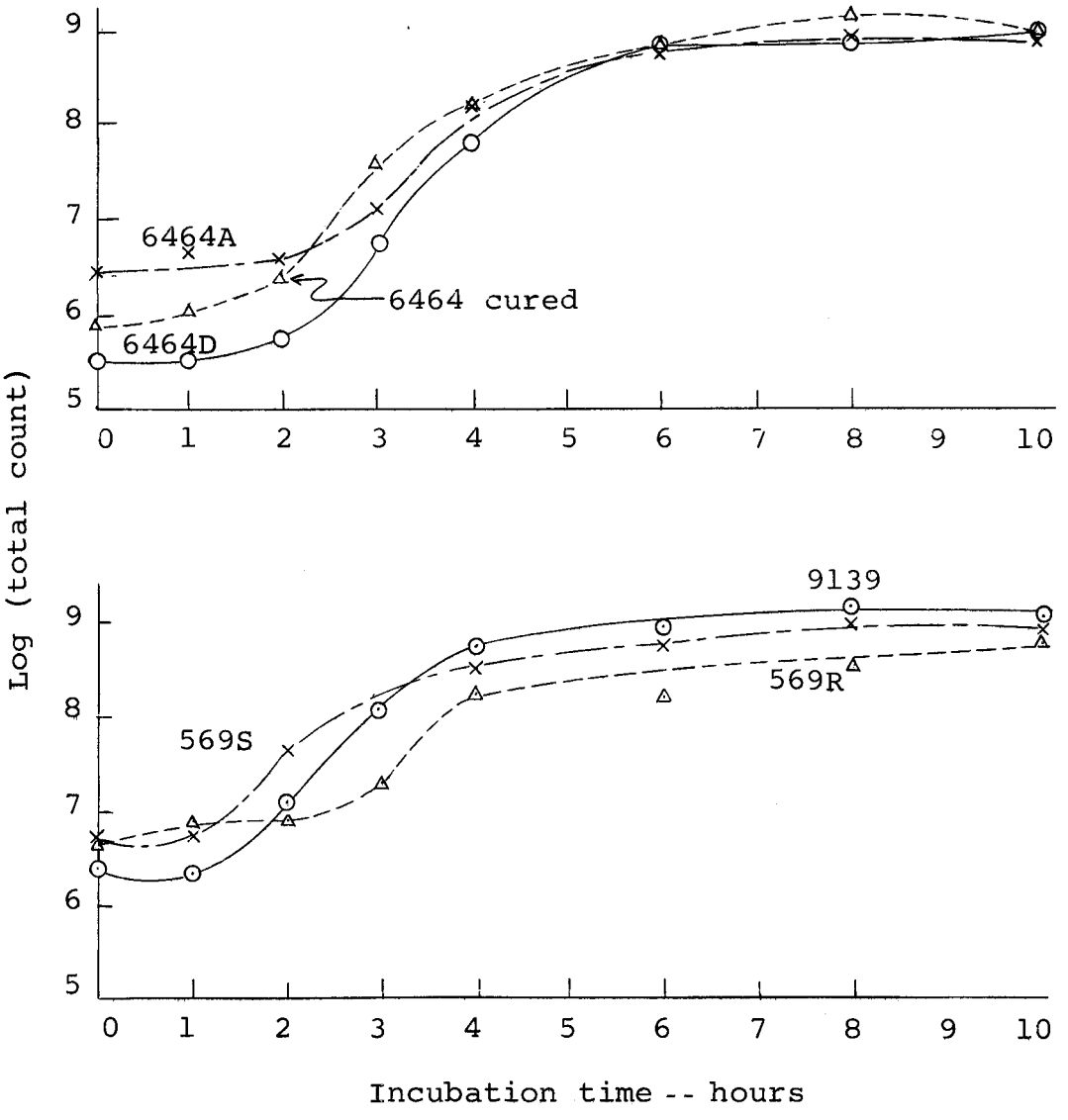

Figure 1. Growth curve of different strains of B. cereus determined by growing 0.1 ml spores in 50 ml NBY broth culture at 37°C.

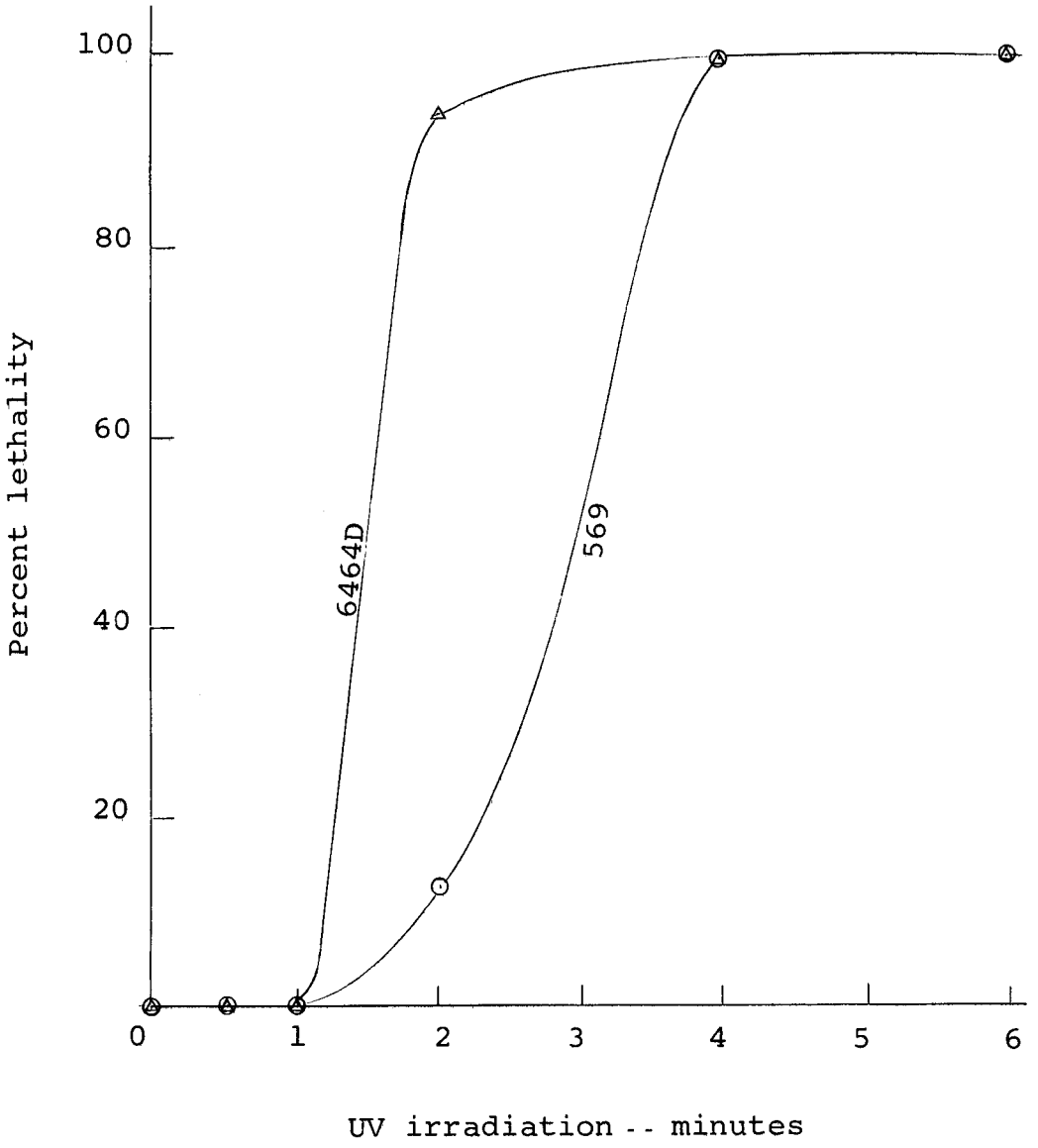

Figure 2. UV irradiation lethality curve

Table 1

Auxotrophic mutants with their identified requirements

Mutant	Requirement
6464A - K1	Hypoxanthine (G)*
K5	Adenine
K6	Methionine and/or cysteine
K7	Adenine and/or hypoxanthine
K8	Histidine
K13	Methionine
6464D - K1	Serine (G)
K4	Methionine
K5	Histidine
K7	Methionine and/or cysteine
K8	Trytophan
K14	Leucine
569R - K1	Methionine and/or cysteine
K2	Tryptophan
K3	Phenylalanine
K16	Arginine
K18	Methionine
K24	Leucine
K37	Glycine
K48	Thiamine
569S - K1	Uracil
K3	Leucine
K4	Methionine
K13	Arginine
9139 - K1	Methionine
K2	Arginine
6464 cured - K1	Nicotinamide

*G: Obtained by germination method

Table 2

Nutrient pools for characterization of auxotrophic mutants

Pool number	1	2	3	4	5
6	adenine	guanine	cysteine	methionine	uracil
7	histidine	leucine	aspartic acid	valine	lysine
8	phenylalanine	glycine	tryptophan	threonine	proline
9	hypoxanthine	serine	alanine	cytosine	arginine
10	tyrosine	thiamine	thymine	riboflavine	pyridoxine

Table 3

Nutrient pools for characterization of amino acid dependent auxotrophs

Pool number	1	2	3	4
5	DL-amino butyric acid	DL-phenyl alanine	D-ribose	L-asparagine
6	DL-trytophane	γ -amino butyric acid	DL-aspartic acid	DL-alanine
7	hydroxy-L-proline	oleic acid	DL-threonine	L-ornithine
8	β -alanine	L-glutanine	DL-homocystine	DL-isoleucine

L-isoleucine tested separately.

Table 4

Nutritional pools for characterization of vitamin and/or nucleic acid dependent auxotrophs

Pool number	1	2	3	4	5
6	adenosine	p-amino benzoic acid	folic acid	xanthosine	purine
7	choline	guanosine	Ca-pantho-thenate	pyrimidine	inosine
8	xanthine	acetyl-choline	thymidine	nicotinamide	pyri-doxamine
9	pyridoxal	cytidine	nicotinic acid	uridine	biotin
10	inositol				

Vitamins: 5 µg/ml
Other constituents: 20 µg/ml

Table 5 - UV Induction

Frequency of auxotrophs when MCA medium used as germination broth

Strain	Total surviving colonies(A)	Auxotrophs(B)	Frequencies($\frac{B}{A}$)
6464A	4.2×10^4	1	$*3 \times 10^{-5}$
6464D	4.5×10^4	0	*0
6464A	1.8×10^4	2	1×10^{-4}
6464D	1.7×10^4	3	2×10^{-4}

Five ml irradiated spore suspension was transferred into a 250 ml flask containing 50 ml of MCA medium broth and incubated on the shaker at 37°C for eight hours. The resulting culture was heated at 65°C for 60 minutes and appropriate dilutions were plated onto single enriched Minimal I agar. The tiny colonies were picked and assayed as described in the Method and Materials section.

* Only triptophan mutant screened.

Table 6

Germination of spores with germinating substances

Incubation time(min.)	Spores/ml			
	Single strength		Double strength	
	569R	569S	569R	569S
0	5.7×10^8	9.6×10^8	5.7×10^8	9.6×10^8
10	3.7×10^8	8.4×10^8	8.4×10^7	2.3×10^8
20	3.0×10^8	7.6×10^8	1.2×10^7	5.4×10^7
30	3.7×10^8	4.6×10^8	5.6×10^6	8.9×10^6
40	2.8×10^8	6.3×10^8	5.7×10^6	8.8×10^6
50	3.2×10^8	4.3×10^8	5.4×10^6	8.7×10^6
60	2.5×10^8	4.4×10^8	5.8×10^6	9.2×10^6

Two ml of irradiated spores were added to each of two screw-cap tubes which contained single strength and double strength of adenosine and alainine respectively. The germination was carried out in a 37°C water bath. At the designated times, the spore counts were made by plating appropriate dilutions onto NBY agar medium. Values are average of duplicate plates.

Table 7 - UV-Induction
Frequencies of auxotrophs of B. cereus strains
using double and single strength germination substances

	Single strength			Double strength	
	6464D	569R	569S	569R	569S
Stock spore suspension (spores/ml)	1.3×10^8	3.2×10^9	3×10^9	2.0×10^9	2.5×10^9
Pregermination (spores/ml)	7.2×10^5	9.0×10^7	5.2×10^7	7.2×10^7	5.8×10^7
Postgermination (spores/ml)	1.2×10^5	3.2×10^7	2.7×10^7	9.0×10^5	1.1×10^5
Total surviving colonies (A)					
pregermination	1.4×10^4	1.5×10^4	1.4×10^4	1.0×10^4	1.2×10^4
postgermination	2.6×10^4	2.4×10^4	2.5×10^4	1.8×10^4	2.7×10^4
Auxotrophs (B)					
pregermination	0	0	0	0	0
postgermination	0	0	0	0	0
Frequencies $(\frac{B}{A})$					
pregermination	0	0	0	0	0
postgermination	0	0	0	0	0

Irradiated spores (2 ml) were mixed with single and double strength germinating substance in a screw-cap tube. The germination was performed in a 37°C water bath for 30 minutes. Non-germinated irradiated spores were used as the control. The tiny colonies appeared on the double enriched Minimal I agar, were picked and assayed as given in Methods and Materials. Spore counts are average of duplicate NBY plates.

Table 8

Frequency of mutation in various B. cereus strains using DES treatment of spores

Strain	Total surviving colonies (A)		Auxotrophs (B)		Auxotroph frequencies ($\frac{B}{A}$)		Survival %	
	DES	Control	DES	Control	DES	Control	DES	Control
6464A	$2x10^4$	$9.4x10^3$	31	1	$2x10^{-3}$	$1x10^{-4}$	50	47
6464D	$1.6x10^4$	$9.0x10^3$	1	0	$1x10^{-4}$	0	41	45
6464 cured	$0.9x10^4$	$9.8x10^3$	34	1	$4x10^{-3}$	$1x10^{-4}$	47	49
9139	$1.1x10^4$	$4.6x10^3$	8	0	$7x10^{-4}$	0	27	23
569R	$1.4x10^4$	$6.4x10^3$	160	0	$3x10^{-3}$	0	30	32
569S	$5.0x10^4$	$9.8x10^3$	53	0	$1x10^{-3}$	0	50	49

Spores (1,000) were plated onto 4% DES plates; 500 spores plated onto MNBY plates served as the control. After 48 hours of incubation at 37°C, both the tiny and sectored colonies were picked and assayed as given in Methods and Materials. Survival percents were calculated from average of 20 plates in DES treatment and 10 plates in the control by random selection in each experiment.

Table 9

Frequency of auxotrophs obtained by DES induction of spores of B. cereus 6464D

Exp.	DES conc. v/v	Total surviving colonies (A)	Auxotrophs (B)	Auxotroph frequencies ($\frac{B}{A}$)	Survival %
Single enrichment					
1	4%	3840	0	0	19.2
	6%	2580	0	0	8.6
	8%	420	0	0	2.1
2	4%	1600	0	0	8.0
	6%	840	0	0	2.8
	8%	100	0	0	0.5
Double enrichment					
1	4%	6380	0	0	31.9
	6%	4960	6	1×10^{-3}	24.8
	8%	3390	3	9×10^{-4}	11.3
2	4%	--	-	--	--
	6%	1960	3	2×10^{-3}	9.8
	8%	1770	2	1×10^{-3}	5.9

Spores (1,000) of 6464D were plated onto MNBY plates which contained different concentrations of DES. Tiny and sectored colonies were picked and assayed as given in the Methods and Materials. Survival percent was calculated from the average of 10 DES plates by random selection.

Table 10

Effect of drying temperature and kinds of colonies picked on auxotrophic frequencies of the 6464 cured strain of B. cereus.

Drying temperature	Total surviving colonies (A)	Auxotrophs (B)	Auxotroph frequencies ($\frac{B}{A}$)	Survival %
22°-24°C	6460			
sectored		26	4×10^{-3}	32.3
tiny		0	0	
30°C	6800			
sectored		10	2×10^{-3}	34.0
tiny		2	3×10^{-4}	
37°C	7960			
sectored		11	1×10^{-3}	39.8
tiny		1	1×10^{-4}	

Each of 60 double enrichment MNBY plates was spread with 4% DES. One set of 20 was dried at room temperature, 30°C and 37°C for four, three and three hours respectively. Spores (1,000) were plated onto each of 60 DES plates. The tiny and sectored colonies were picked and assayed separately as given in the Methods and Materials section.

Table 11

Effect of DES + UV on mutation frequencies and percent survival of various strains of *B. cereus*

Strain	Treatment	Total surviving colonies (A)	Survival %	Sectored colonies	Auxotroph (B)	Auxotroph frequencies ($\frac{B}{A}$)
6464A	control	3.0×10^3	29.5	8	1	3×10^{-4}
	DES	16.0×10^3	40.0	91	6	4×10^{-4}
	UV	3.0×10^3	30.1	6	0	0
	UV+DES	15.5×10^3	38.7	77	14	9×10^{-4}
6464D	control	3.5×10^3	34.5	0	0	0
	DES	6.4×10^3	15.9	16	6	9×10^{-4}
	UV	3.2×10^3	31.5	2	0	0
	UV+DES	0.1×10^3	10	0	0	0
6464 cured	control	3.5×10^3	35.1	9	1	3×10^{-4}
	DES	7.0×10^3	17.6	59	12	2×10^{-3}
	UV	9.2×10^3	23.1	7	2	3×10^{-4}
	UV+DES	7.3×10^3	18.3	122	12	2×10^{-3}
569R	control	3.3×10^3	32.8	21	0	0
	DES	14.5×10^3	36.2	458	55	4×10^{-3}
	UV	2.4×10^3	24.2	18	0	0
	UV+DES	9.4×10^3	23.6	556	70	8×10^{-3}
569S	control	4.2×10^3	42.1	47	0	0
	DES	14.8×10^3	37.1	183	20	1×10^{-3}
	UV	4.1×10^3	40.5	49	1	2×10^{-4}
	UV+DES	11.4×10^3	28.5	179	13	1×10^{-3}
9139	control	1.7×10^3	16.6	2	0	0
	DES	5.1×10^3	12.7	2	1	2×10^{-4}
	UV	3.5×10^3	34.6	0	0	0
	UV+DES	10.1×10^3	25.2	60	8	8×10^{-4}

Irradiated spores (1,000) were plated onto each of 40 DES plates (UV + DES). Another set of 40 DES plates was spread with 1,000 unirradiated spores (DES). The 500 spores, irradiated (UV) and non-irradiated (control) plated onto each of 10 MNBY plates made up the remaining sets. Sectored colonies were picked and assayed as given in Methods and Materials.

Table 12

DES induction of vegetative cells of *B. cereus* in exponential growth phase

Strain	Treatment	Total surviving colonies (A)	Survival %	Sectored colonies	Auxotroph (B)	Auxotroph frequencies ($\frac{B}{A}$)
6464A	control	400	8.0	0	0	0
	DES	175	0.7	6	3	2×10^{-2}
6464D	control	405	8.1	0	0	0
	DES	1300	5.2	17	4	3×10^{-3}
6464 cured	control	290	5.8	2	1	3×10^{-3}
	DES	375	1.5	8	5	1×10^{-2}
9139	control	20	0.4	0	0	0
	DES	25	0.1	0	0	0
569R	control	640	12.8	3	1	2×10^{-3}
	DES	300	1.2	33	16	5×10^{-2}
569S	control	1760	35.2	7	0	0
	DES	1150	4.6	18	4	4×10^{-3}

Cells (1,000) from NBY cultures in the exponential growth phase were plated onto 25 DES plates; 500 cells were plated on 5 MNBY plates for control purposes. Survival percent was calculated from the average of total plates in each experiment.

Table 13

DES induction of vegetative cells of B. cereus in stationary growth phase

Strain	Total surviving colonies (A)	Survival %	Sectored colonies	Auxotrophs (B)	Auxotroph frequencies ($\frac{B}{A}$)
6464A	4840	24.2	363	5	$1x10^{-3}$
569R	4060	20.3	259	30	$7x10^{-3}$
569S	3500	17.5	135	15	$4x10^{-3}$

Cells (1,000) from 7-hour NBY broth cultures were plated onto DES plates. Sectored colonies were picked and assayed as given in Methods and Materials. Survival percent was calculated from the average of total plates in each experiment.

Table 14

Stability of auxotrophs of B. cereus obtained with the DES method

Strain	No. of auxotrophs tested (A)	No. of stable auxotrophs (B)	% of stable auxotrophs ($\frac{B}{A}$)
6464A	31	20	65
6464 cured	34	26	77
569R	160	116	73
569S	53	31	59
9139	8	7	88

Stability of auxotrophs was the number of auxotrophs isolated with minimal medium divided by the number of auxotrophs which were stable during nutrient characterization.

Table 15

Percentage of auxotrophs identified as to nutritional requirement

Strain	No. of auxotrophs assayed (A)	No. of auxotrophs identified (B)	% of auxotrophs identified ($\frac{B}{A}$)
6464A	13	7	54
6464D	18	10	56
6464 cured	21	13	62
569R	56	28	50
569S	15	5	33
9139	3	3	100

Table 16

Description for nutritional mutants isolated during the present work along with their requirements where determined

Mutant	Requirement
6464A - K1	hypoxanthine (G)*
K2	unidentified (G)
K3	unidentified (G)
K4	unidentified
K5	adenine
K6	methionine and/or cysteine
K7	adenine and/or hypoxanthine
K8	histidine
K9	methionine and/or cysteine
K10	unidentified
K11	unidentified
K12	unidentified
K13	methionine
6464D - K1	serine (G)
K2	serine (G)
K3	unidentified (G)
K4	methionine
K5	histidine
K6	unidentified
K7	methionine and/or cysteine
K8	tryptophan
K9	methionine and/or cysteine
K10	unidentified
K11	methionine
K12	unidentified
K13	unidentified
K14	leucine
K15	unidentified
K16	tryptophan
K17	unidentified
K18	unidentified
569R - K1	methionine and/or cysteine
K2	tryptophan
K3	phenylalanine
K4	tryptophan
K5	methionine and/or cysteine
K6	methionine and/or cysteine
K7	methionine and/or cysteine
K8	tryptophan

Table 16 (continued)

Mutant	Requirement
569R - K9	unidentified
K10	unidentified
K11	unidentified
K12	tryptophan
K13	methionine and/or cysteine
K14	methionine and/or cysteine
K15	methionine and/or cysteine
K16	arginine
K17	unidentified
K18	methionine
K19	unidentified
K20	unidentified
K21	unidentified
K22	methionine
K23	methionine
K24	leucine
K25	arginine
K26	tryptophan
K27	methionine
K28	tryptophan
K29	unidentified
K30	arginine
K31	arginine
K32	tryptophan
K33	unidentified
K34	unidentified
K35	unidentified
K36	methionine
K37	glycine
K38	unidentified
K39	methionine
K40	leucine
K41	unidentified
K42	unidentified
K43	unidentified
K45	unidentified
K46	unidentified
K47	methionine and/or cysteine
K48	thiamine
K49	unidentified
K50	unidentified
K51	unidentified
K52	unidentified
K53	unidentified
K54	unidentified

Table 16 (continued)

Mutant		Requirement
569R	- K55	unidentified
	K56	unidentified
	K57	unidentified
569S	- K1	uracil
	K2	leucine
	K3	leucine
	K4	methionine
	K5	unidentified
	K6	unidentified
	K7	unidentified
	K8	unidentified
	K9	unidentified
	K10	unidentified
	K11	unidentified
	K12	unidentified
	K13	arginine
	K14	unidentified
	K15	unidentified
9139	- K1	methionine
	K2	arginine
	K3	methionine
6464 cured	K1	nicotinamide
	K2	nicotinamide
	K3	nicotinamide
	K4	nicotinamide
	K5	nicotinamide
	K6	unidentified
	K7	unidentified
	K8	unidentified
	K9	nicotinamide
	K10	nicotinamide
	K11	unidentified
	K12	nicotinamide
	K13	nicotinamide
	K14	nicotinamide
	K15	nicotinamide
	K16	unidentified
	K17	unidentified
	K18	unidentified
	K19	nicotinamide
	K20	unidentified
	K21	nicotinamide
	K22	unidentified

*G: Germination method

SUMMARY

The original purpose of this research was to work out an effective procedure for isolation of auxotrophic mutants of B. cereus; it was intended to take advantage of the difference in thermolability between spores and vegetative cells. Spores in which mutation has been induced by ultraviolet light irradiation do not germinate as readily as the normal spores in a germination medium; therefore, mutant spores can be concentrated selectively by heat-shocking to kill most germinated prototrophs.

Spore suspensions were irradiated with UV light at a distance of 40.5 cm for three to four minutes which resulted in 99% killing. The germinating broth medium contained adenosine and alanine in a pH 7.4 sodium phosphate buffer. Normally, germination was carried out in a 37°C water bath over 30 minutes. After heat-shocking at 65°C for 60 minutes the culture was plated at appropriate dilutions onto a doubly enriched minimal medium.

Mutant cells formed only minute colonies on this medium in contrast to the normal cells which formed large colonies. The minute colonies were picked and characterized for nutritional requirement by replica plating onto various minimal media supplemented with

different nutrients. The mutants were later definitely characterized by growing the mutant cells in the presence and absence of the suspected required nutrients.

Results of this procedure indicated that nutritional mutants could not be readily obtained. The mutants isolated were found to be unstable in that they frequently reverted.

Because of these negative results, a second procedure was developed for selecting auxotrophs involving the use of the chemical mutagen diethylsulfate; this chemical was incorporated into a minimal medium at various concentrations. After drying at 25°C for four hours, 1,000 spores were plated directly onto this medium. The sectored colonies which appeared after incubation were picked and assayed for mutation. DES in a final concentration of 4% (v/v) gave the best results.

It was found that the mutagenic property of DES could be varied by changing the drying temperature of DES Minimal I plates. When the plates were dried at 24°C for four hours the auxotrophic frequency was $4x10^{-3}$ while plates dried at 30°C and 37°C showed frequencies $2x10^{-3}$ and $1x10^{-3}$, respectively. Different kinds of colonies were compared for mutation frequency; sectored colonies showed 35.1% mutants while small colonies revealed none.

A study was made to evaluate synergistic effects of UV irradiation and DES on mutation rates of spores.

Results indicated that the mutagens had no significant synergistic effect.

The mutagenic effect of DES on vegetative cells was also investigated. Results indicated that DES could readily mutate cells in the exponential growth phase. Although the mutation also occurred with cells in the stationary phase, the auxotrophic frequencies were much lower than that of cells growing exponentially. Thus DES proved to be a better mutagen than UV light for both cells and spores of B. cereus. Also it was found that the mutants obtained by DES treatment were far more stable than those obtained by UV irradiation.

Approximately 50% of all the mutants isolated were characterized. The majority of the mutants characterized required one of the following amino acids: arginine, cysteine, glycine, histidine, leucine, methionine, phenylalanine and serine. Some others were found to require nicotinamide, adenine, hypoxanthine, thiamine or uracil. Interestingly, mutants deficient for sulfur-containing amino acids (methionine, methionine and/or cysteine) were found more common than other requirements.

Those mutants which could not be characterized were apparently multiple mutants which, by their growth patterns, could be subdivided into two distinct groups: (1) those which had a multiple requirement for amino

acids only, and (2) those which appeared to require both amino acids and vitamins or nitrogenous bases of nucleic acids. A method to identify these uncharacterized mutants has not yet been devised.

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