ABSTRACT

Bacillus subtilis, a microorganism which is known to excrete itoic acid, a siderophore, and coproporphyrin when grown in culture media deficient in iron, was treated with 1-methyl-3-nitro-1-nitrosoguanidine (NTG) in order to obtain a constitutive mutant that would excrete itoic acid regardless of iron availability. Instead of such a mutant, the treatment produced many itoic acid defective mutants that lost ability to excrete itoic acid, as well as coproporphyrin, under iron deficient conditions. This finding supports the idea that the excretion of itoic acid is genetically controlled by an iron operon whose activities are regulated by iron concentration in the bacterial cell. It also suggests that the excretion of itoic acid and coproporphyrin is regulated at a common genetic locus in the iron operon. One itoic acid defective mutant examined seemingly gained the ability to excrete a yellow pigment that appeared to be similar to a pteridine-containing siderochrome found in Pseudomonas frisellum. Some of the other itoic acid mutants were morphological mutants that could have been defective in the genetic mechanism of cell wall formation. The appearance of the mutants having a variety of phenotypic characteristics in this study supports the fact that there is a wide range of mutations possible with the use of NTG.

BACILLUS SUBTILIS IRON OPERON

MUTANTS PRODUCED BY TREATMENT

WITH 1-METHYL-3-NITRO-1-NITROSOGUANIDINE

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Master of Science in Biology

by

Mark Steven Nelson

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INTRODUCTION

All living things have difficulty absorbing the iron necessary for their survival. The reason for this difficulty lies in the basic chemistry of iron. Iron commonly exists in two oxidation states, ferrous and ferric. Fe(II) is rapidly oxidized in air to Fe(III) at a neutral pH. Fe(III) is extremely insoluble in water, and the solubility product of ferric hydroxide (approximately 10^{-38}) attests to this fact. Through evolution, microbial cells developed the ability to produce siderophores (iron bearers), which are small organic molecules that have a very high affinity for Fe(III). Thus, siderophores function as solubilizing agents of this extremely insoluble form of iron, making it available for transport into microbial cells (Neilands, 1974; Emery, 1982).

The first siderophore isolated and chemically identified was itoic acid (Iron Transporting Orthophenolic Acid, or 2,3-dihydroxybenzoyl-glycine). Together with itoic acid, coproporphyrin was found to accumulate in an iron-deficient culture medium of Bacillus subtilis (Ito and Neiland, 1958). Itoic acid was also found to facilitate iron transport into bacterial cells (Peters and Warren, 1968).

The genetic hypothesis postulated by Neilands (1973, 1982) for the formation of siderophores inferred that repressor-iron binding would prevent the production of itoic acid. It implied that a modification of the hypothetical repressor gene could yield a constitutive mutant which would produce itoic acid regardless of iron availability to the organism.

In order to test this hypothesis, attempts were made to produce an itoic acid constitutive mutant. A widely-used experimental mutagen, 1-methyl-3-nitro-1-nitrosoguanidine (NTG), was selected for this purpose

because of its highly mutagenic properties, with mutants produced having
good survival rates (Adelburg et al., 1965). It was planned to identify
the mutant on a solid-growth medium containing high concentration of iron
by selecting colonies that would either produce a blue color around them
or would fluoresce under ultraviolet light. The methods of identification
were based on the knowledge that itoic acid (or phenolic compounds in
general) complexes with Fe(III) to form an intense blue color, and that
itoic acid produces intense yellow fluorescence under ultraviolet light
(254 nm). After repeated attempts to identify such colonies failed, it
was decided to investigate colonies that had interesting morphology and
that were more colored than others. The rationale behind the decision to
look for the colored colonies was that coproporphyrin, which is produced
together with itoic acid by Bacillus subtilis under iron-deficient conditions,
it a highly colored (pink) compound with an absorption peak of about 400 nm.

Three types of mutants were isolated which were particularly
interesting. Almost all of the isolates examined were itoic acid
defective; that is, they did not excrete itoic acid (nor coproporphyrin).
Some of these isolates were also cell wall defective and produced white,
irregularly shaped aggregates of cells in liquid media. Another itoic
acid defective isolate was a yellow pigmented mutant that produced
pteridine-like pigment(s) similar to the siderochrome-peptides found in
Pseudomonas frisellum (Frisell, 1971; Fletcher et al., 1983).

LITERATURE REVIEW

In general, siderophores are excreted into the surrounding environment by microorganisms only under iron-deficient conditions. They function as iron-transporting agents, dissolving insoluble Fe(III) (Neilands, 1973, 1974). Two types of siderophores are known; namely 2,3-dihydroxybenzoic acid (2,3-DOB) derivatives and secondary hydroxamates. Both of these are weak acids (pK_a9), and only oxygen atoms are in the coordination sphere of the bound metal ion (Neilands, 1974). Although the secondary hydroxamates are important, the emphasis of this research was on itoic acid, a phenolate type, and only literature about phenolate siderophores was reviewed.

Phenolate siderophores are widely distributed in nature and seem to be secreted mostly by bacteria (Neilands, 1973, 1974, 1981). A phenolate siderophore, itoic acid, was isolated in 1958 by Ito and Neilands from iron-deficient cultures of Bacillus subtilis in amounts of approximately 50 milligrams per liter, and was identified as 2,3-dihydroxybenzoyl-glycine. It was the first siderophore ever chemically identified. Because of its possible iron transporting function, Neilands coined the name "itoic acid", that is, Iron Transporting Orthophenolic Acid. The name occasionally appeared in subsequent biochemical and microbiological literature (Peters and Warren, 1968; Neilands, 1973). Itoic acid indeed stimulated iron uptake in Bacillus subtilis (Peters and Warren, 1968). The discovery of itoic acid was followed by the isolation of the serine analogue, 2,3-dihydroxy-N-benzoyl-L-serine (Brot et al., 1966), the bis-lysine derivative, 2-N, 6-N-di-(2,3-dihydroxybenzoyl)-L-lysine

(Corbin and Bulen, 1969), and enterobactin (Pollack and Neilands, 1970), which was an identical compound as enterochelin (O'Brien and Gibson, 1970), from bacterial cultures.

The original supposition that siderophores transported iron was based upon the observation that Fe(III) complexes were extremely stable (formation constant in the range of 10^{30} to 10^{50}), while Fe(II) complexes were relatively weak. This provided a possible mechanism for making an insoluble form of iron available for cellular uptake and use involving reduction of the ferric form to the ferrous form in the cells before becoming available for metabolic utilization, while at the same time ruling out the possibility of an electron transfer role for the phenolate compounds. Another line of evidence for siderophores being iron-transporting agents came from the fact that soil and dung inhabiting bacterial species required siderophores for growth (Neilands, 1973).

The biosynthetic pathways of certain phenolates, such as enterobactin, were partially elucidated and relatively well understood at the enzyme level (Gibson and Pittard, 1968). According to present knowledge, during the biosynthesis of aromatic compounds a branch pathway begins after the formation of chorismate. This terminates in the formation of enterobactin, with the sole purpose of the branch being to produce the phenolate siderophores.

Excretion of siderophores by microorganisms into their environment was considered (Neilands, 1974) as an evolutionary adaptation arising from the change in the earth's atmosphere from anaerobic to aerobic conditions. The fact that ferric hydroxide (formed by ferric ions and water) has an extremely low solubility product indicates that the

appearance of O_2 in the atmosphere was devastating to living organisms since it made absorption of iron extremely difficult.

An iron operon was postulated (Neilands, 1973, 1982) that controls the production of siderophores in microorganisms. According to the postulate, this operon is controlled by the internal concentration of Fe(II). As long as the concentration of the iron is adequate, iron binds with the repressor protein forming a repressor-iron complex which subsequently binds to the operator locus, preventing transcription of the structural genes that control the production of the siderophore. However, when the concentration of the iron drops below a certain level there is no binding at the operator locus because the low iron concentration prevents the formation of the repressor-iron complex. This allows transcription of the structural genes, which in turn allows for the production of the siderophore which can be transported out of the cells to chelate with the environmental Fe(III). The ferric-siderophore complex molecules are transported into the cells through specific binding proteins on the cell membrane. Once inside the cells, the iron is reduced to the ferrous form, thus decreasing affinity of the iron for the siderophore molecules. The reduced iron is then released for metabolic utilization, as well as for the formation of the repressor-iron complex.

Among many chemical mutagens (Singer and Kusmierek, 1982) 1-methyl-3-nitro-1-nitrosoguanidine (NTG), a methylating agent, has been widely used experimentally to produce bacterial mutants. The use of NTG as an experimental chemical mutagen was first described by Mandel and Greenberg in 1960. The fact that NTG is a mutagen was confirmed by demonstrating the presence of auxotrophic mutants that failed to grow on glucose-salts

medium when replica plating was conducted on the complete medium after NTG treatment (Mandel and Greenberg, 1960). NTG was also shown to be carcinogenic (Schoental, 1966; Sugimura et al., 1966; Druckrey et al., 1966), but at low levels it served as an inhibitor of cancerous growth (Leiter and Schneiderman, 1959; Goldin et al., 1959; Skinner et al., 1960). It induced primarily base transition mutations at the replicating fork of the DNA, as opposed to nonreplicating native regions (Cerda-Olmado et al., 1968). They were of the GC $\rightarrow$ AT type, although AT $\rightarrow$ GC transitions, transversions, and frameshifts appeared to arise at low frequencies. These types of mutations took place via the methylation due to nucleophilic substitution of the DNA bases guanine and adenine (Lawley, 1968). Guanine was methylated at the 7N position to 7-methylguanine (Craddock, 1968; Lawley, 1968). Adenine was methylated at the 3N position to 3-methyladenine (Lawley, 1968; Craddock, 1969) and, to a much lesser extent, at the 1N position to 1-methyladenine (Craddock, 1969). Evidence that NTG can react directly with DNA was shown by its inactivation of transforming DNA in vitro (Terawaki and Greenberg, 1965), even though the extent of the reaction of NTG with DNA was small.

By the use of a relatively simple procedure, NTG produced large numbers of mutants with over 50 percent survival of the treated cells (Adelberg et al., 1965).

MATERIALS AND METHODS

ORGANISM: Bacillus subtilis (NRRL B1471; ATCC 15933) was used for
this study because it is known to excrete itoic acid and coproporphyrin
under iron-deficient conditions.

GROWTH MEDIA: The liquid iron-deficient growth medium consisted of
the following chemical per liter: potassium sulfate, 1.0 g; dibasic
potassium phosphate, 3.0 g; ammonium acetate, 3.0 g; sucrose, 20 g;
citric acid, 1.05 g; thiamine hydrochloride, 2.2 mg; copper sulfate
$\cdot 5H_2O$, 0.02 mg; manganese chloride $\cdot 4H_2O$, 0.13 mg; zinc chloride, 4.2 mg;
magnesium sulfate, 0.81 g; pH 6.8 with HCl (Ito and Neilands, 1958).
The iron-supplemented growth medium was prepared by adding 1 mg
$FeCl_3 \cdot 6H_2O$ to 1 liter of the iron-deficient growth medium. The solid-
growth medium was Trypticase Soy Agar (TSA) at a concentration of
30 g/liter. The overlay used in some experiments consisted of 0.5 percent
agar containing 2 g $FeCl_3 \cdot 6H_2O$ per liter, pH 7.0.

MUTAGEN: The mutagen used was 1-methyl-3-nitro-1-nitrosoguanidine
(NTG) at 200 μg/ml, filtered sterile using a 0.12 micron disposable
millipore filter unit.

DETERMINATION OF KILLING CURVE: The killing curve represents the
relationship between the NTG dose (time of the exposure) and the logarithm
of the number of surviving cells. It was determined by the method
describe by Miller (1972). A standing culture of Bacillus subtilis was
grown overnight (10-18 hours) at $37^{o}C$ in the previously described
iron-deficient medium. From this culture, 1 ml was inoculated into 50 ml
of fresh iron-deficient medium and was shaken at 100 cycles per minute

for about six hours at 37°C. At that time, a 3 ml aliquot was taken and tested for growth at 520 nm to make sure the culture yielded a density of $3\sim5 \times 10^8$ cells/ml. The rest of the culture was centrifuged at 8,000 rpm in a Sorvall RC2-B centrifuge for ten minutes and then suspended in 50 ml of NTG (200 μg/ml), and 4 ml aliquots were taken at 0, 10, 20, 30, and 40 minutes. Each aliquot was filtered, using a 0.12 micron disposable millipore filter unit, and was washed once, using 5 ml of physiological saline (0.85 percent). The cells on the filter were then resuspended in 5 ml of physiological saline and 10^0 to 10^{-4} serial dilutions were made from each in duplicate. From each dilution, 1.0 ml was inoculated into petri plates containing Trypticase Soy Agar and was incubated at 37°C until colonies appeared. Colonies were then counted and divided by the dilution factor to give colony-forming units (cfu) per ml. These numbers were averaged for each time interval and the log of the cfu/ml was plotted against each time interval to arrive at the killing curve for NTG on Bacillus subtilis.

PREPARATION OF MUTANTS BY NTG TREATMENT: The cells were grown over-night (10-15 hours) in the iron deficient medium at 37°C without shaking. After the growth, 1 ml of the standing culture was inoculated into 25 ml fresh iron-deficient growth medium and was shaken at 100 cycles/minute on a gyratory shaker for 1 - 3 hours at 37°C. This was done so that, prior to treatment with the mutagen, the cells would be in the logarithmic phase of growth. The culture was checked for growth using McFarland Standard and, when the density of the cells reached $3\sim5 \times 10^8$ cells/ml, they were centrifuged at 8,000 rpm for 10 minutes. The cells were suspended in 20 ml of NTG (200 μg/ml) and incubated for 10 minutes at

$37^{\circ}C$ without shaking. After incubation, the cells were centrifuged at 8,000 rpm for 10 minutes and then resuspended in 25 ml of fresh iron-deficient growth medium. Serial dilutions of 10^{-4} were made, and 5 ml was plated on large plates (8" X 11") of Trypticase Soy Agar. The plates were incubated overnight at $37^{\circ}C$ and then overlayed with sterile 0.5 percent agar containing 2 g $FeCl_3 \cdot 6H_2O$ per liter, pH 7.0. The plates were then incubated for two days at room temperature.

SELECTION OF MUTANTS AND TESTS OF THE MUTANTS FOR ITOIC ACID PRODUCTION AND FOR OTHER CHARACTERISTICS: The colonies which appeared colored (red and yellow), or fluoresced under the ultraviolet light (254 nm) derived from cells surviving the NTG treatment were transferred from the overlayed plates into 10 ml of sterile (0.85 percent) physiological saline. Quadruplicate flasks containing 25 ml of iron-supplemented and iron-deficient growth medium were inoculated with 0.5 ml of the suspension. The flasks were incubated for 72 hours at $37^{\circ}C$ with shaking to give adequate time for the production of itoic acid and coproporphyrin. Cultures were centrifuged at 8,000 rpm for 10 minutes, and absorbance of the supernatants was read at 310 nm and 400 nm for the measure of itoic acid and coproporphyrin, respectively, using a Beckman Model DB spectrophotometer. The pellets from some of these suspensions that showed some altered morphological characteristics (aggregates) were resuspended in physiological saline and later light and transmission electron photomicrographs were made. Absorption spectrum was run on a Gilford Spectrophotometer of the supernatant of the yellow pigmented mutant grown in the iron-deficient growth medium. The highly purified pigment from Pseudomonas frisellum used for comparison to the yellow

pigmented mutants' supernatant was a gift from the laboratory of
Dr. Wilhelm Frisell. All mutants were also Gram-stained to insure that
they were the test organism and not some environmental contaminant.

TEST OF DETECTION METHOD FOR ITOIC ACID PRODUCING COLONIES: As
described in INTRODUCTION, the itoic acid producing mutant colonies would
have been detected by selecting those colonies that would produce an
intense blue color or would fluoresce under ultraviolet light on a
solid-growth medium containing a high iron concentration. A model
experiment was performed to test how sensitive the method of detection
was. TSA plates were prepared, and six plugs were removed from each
plate by a cork borer. Each plug was refilled with agar containing
itoic acid or 2,3-DOB at differing concentrations, i.e. 1 mg/ml, 0.5 mg/ml,
0.2 mg/ml, 0.1 mg/ml, and control. After the plates solidified, they were
overlayed with 0.5 percent agar containing 1 g $FeCl_3 \cdot 6H_2O$ per liter
pH 6.8 and were incubated 72 hours until expected blue zones were formed
between the plugs and the overlay.

RESULTS

A killing curve experiment showed that exposure of a log phase
culture of <u>Bacillus</u> <u>subtilis</u> for approximately 10 minutes with 200 µg/ml
NTG produced approximately 70 percent lethality (Fig. 1). This condition
was used for all subsequent NTG treatments in preparation of mutants.

In the experiment testing the sensitivity of the detection method
of itoic acid producing colonies, no clearly discernible blue zone was
observed near any of the plugs after 72 hours of incubation. However,
some discoloration was detected near the plugs of higher concentrations.

Colonies grown from the NTG treated cells were examined for ability
to excrete itoic acid and coproporphyrin. In three different NTG treat-
ments, most colonies examined showed no ability to accumulate itoic acid
and coproporphyrin (Table 1). Two colonies examined from Experiment #1
showed decreased production of itoic acid, while one produced as much
as the wild type. All other colonies examined from two other treatments
showed decreased production of these two compounds.

From Experiment #2, the cells not only showed decreased production
of itoic acid and coproporphyrin, but a morphological mutation was also
observed. A large, white aggregate appeared in the iron-deficient and
iron-supplemented liquid culture medium inoculated with colonies #2 and
#3 (Plate 1). Light microscopic examination of the Gran-stained particles
from a crushed aggregate showed large irregularly-shaped Gram positive
bacilli sticking together end-to-end and side-to-side (Plate 2). Electron
micrographs made by using Bright Field Optics (Plate 3) showed that the
cells were unusually large and irregular in shape. There were varying

numbers of white vacuoles in each mutated cell. The characteristic
bacillus shape was also lost in the mutant, whereas it was maintained
in other types of mutants (Plate 4).

When a yellow colony (Colony #1) from Experiment #3 was grown in
the iron-deficient growth medium, neither coproporphyrin nor itoic acid
was detected in the culture medium. The cells of this yellow mutant were
removed by centrifugation, and the yellow supernatant was dialyzed over-
night. The dialyzed supernatant (about 10 ml) lost its color upon addition
of a few drops of 6 N hydrochloric acid, and a precipitate was formed.
Upon addition of 6 N sodium hydroxide, the yellow color of the dialyzed
supernatant remained, but intensified. Upon addition of a drop of 1 %
$FeCl_3 \cdot 6H_2O$ to the dialyzed supernatant, the color turned to brown.

Absorption spectrum of the dialyzed supernatant showed a peak at
around 400 nm (Fig. 2). Absorption spectrum of a highly purified
pteridine-containing siderochrome of Pseudomonas frisellum (Frisell,
1971; Fletcher et al., 1983) was determined for comparison, and it
also showed a peak at near 400 nm (Fig 3).

DISCUSSION

With the NTG dose (200 µg/ml for 10 minutes) giving 70 percent
lethality, the number of survivors was approximately 2 million cfu/ml,
certainly enough for examination for mutants (Fig. 1).

There are at least two possibilities that can explain the failure
to detect the itoic acid excreting constitutive mutants on the iron-
overlayed agar plates. One is that the detection method originally
proposed for the itoic acid forming colonies on the plates was not
sensitive enough to identify them. Another is that no such constitutive
mutants were formed upon treatment with NTG. The latter is a good
possibility, since biosynthesis of itoic acid from chorismate would
require a number of structural genes in the iron operon, and perhaps in
other operons, and since a damage upon one part of any of these genes
would cause a potential termination of the biosynthesis. This could
be the reason that many colonies examined after NTG treatments showed no
ability to excrete itoic acid under iron-deficient conditions.

It seems evident from this research that a wide range of mutations
is possible with the use of NTG. NTG produces multisite mutants; there-
fore, each mutation may have a combination of mutational effects. However,
in general, the majority of mutant types resulting from the treatment with
NTG cannot be detected because of the lack of specific selection methods
which can identify the mutant types against a large background of other
cells (Carlton and Brown, 1981). This appears to be the case in this
study, too. For obtaining an itoic acid constitutive mutant, a simple
and sensitive method must be found.

The finding of the itoic acid defective mutants does tend to support Neilands' hypothesis that the production of siderophores is genetically controlled (1973, 1982). The gene in Neilands' postulated iron operon, targeted by NTG in the present study, was the repressor gene which ultimately controls the assimilation of Fe(III). It is possible that at least some of the structural genes in the operon were altered so that the enzymes required to form itoic acid were not produced, or so that some enzymes ceased to be functional.

All the itoic acid defective mutants were also unable to excrete coproporphyrin. This suggests that the formation of itoic acid and coproporphyrin in this organism is controlled at a common regulatory locus in the iron operon.

The morphological mutation observed upon the NTG treatment may be related to defects in the genetic mechanism controlling the production of the peptidoglycan constituents of the cell wall. The large size of the mutant cell and the loss of the characteristic bacillus shape suggested overproduction of the peptidoglycan. The large vacuoles were possibly large masses of the peptidoglycan constituents. No further investigation of these mutants was possible because, upon trying to maintain the cells in the mutant form, reversion to the original cell shape occurred. It is likely that the reversion was the result of the increased methyltransferase activity in Bacillus subtilis. Adaptation of Bacillus subtilis to NTG results in an approximately 10-fold increase in methyltransferase activity and in an increase in resistance to both killing and mutagenesis (Hadden et al., 1983).

The yellow pigment in the dialyzed supernatant from the yellow mutant cells grown in the liquid growth medium seemed a relatively large molecule, since it was not dialyzable. It had characteristics of siderophores. For example, it changed color upon addition of Fe(III), thus indicating formation of a complex between the pigment and Fe(III). The comparison of the visible spectrum of this pigment with that of the pteridine-containing siderochrome of Pseudomonas frisellum indicated their similar chemical nature (Frisell, 1971; Fletcher et al., 1983). Slight differences in absorption spectra observed between these pigments might be due to the fact that the pigment from the yellow mutant was not as purified. Whether the yellow pigment of this mutant is indeed a pteridine is a matter for further investigation.

However, if the pigment produced by the yellow mutant is indeed a pteridine-type siderophore, an interesting question arises. Could it be possible that the NTG treatment activated the genes for the production of the pteridine siderophore pigment while at the same time destroying the genes that control the production of itoic acid and coproporphyrin?

SUMMARY

Bacillus subtilis, a microorganism which is known to excrete itoic acid, a siderophore, and coproporphyrin when grown in culture media deficient in iron, was treated with 1-methyl-3-nitro-1-nitrosoguanidine (NTG) in order to obtain a constitutive mutant that would excrete itoic acid regardless of iron availability. Instead of such a mutant, the treatment produced many itoic acid defective mutants that lost ability to excrete itoic acid as well as coproporphyrin under iron deficient conditions. This finding supports the idea that the excretion of itoic acid is genetically controlled by an iron operon whose activities are regulated by iron concentration in the bacterial cell. It also suggests that the excretion of itoic acid and coproporphyrin is regulated at a common genetic locus in the iron operon. One itoic acid defective mutant examined seemingly gained the ability to excrete a yellow pigment that appeared to be similar to a pteridine-containing siderochrome found in Pseudomonas frisellum. Some of the other itoic acid mutants were morphological mutants that could have been defective in the genetic mechanism of cell wall formation. The appearance of the mutants having a variety of phenotypic characteristics in this study supports the fact that there is a wide range of mutations possible with the use of NTG.

TABLE, FIGURES, AND PLATES

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EXTENT OF ITOIC ACID AND COPROPORPHYRIN FORMATION BY

BACILLUS SUBTILIS MUTANTS PRODUCED BY NTG TREATMENT

The NTG dose was 200 µg/ml for 10 minutes. Each number represents the mean absorbance of four determinations, at 310 nm and 400 nm, the absorbance maxima for itoic acid and coproporphyrin respectively. The ranges are given in parentheses. See text for other details.

*These flasks contained the large white aggregates which showed evidence of being cell wall defective mutants in later examinations. (See Plates 1, 2, and 3.)

ABSORBANCE

	310 nm		400 nm	

IRON DEFICIENT MEDIUM

Experiment #1

Colony #1	6.06	(4.00-7.98)	1.69	(1.30-2.30)
Colony #2	3.24	(1.20-4.40)	0.740	(0.320-1.20)
Colony #3	2.97	(1.52-4.45)	0.880	(0.550-1.40)
Wild Type	5.25	(4.90-5.40)	0.890	(0.650-1.35)

Experiment #2

Colony #1	0.670	(0.610-0.820)	0.350	(0.280-0.470)
Colony #2*	0.988	(0.835-1.07)	0.365	(0.260-0.450)
Colony #3*	0.780	(0.710-0.850)	0.200	(0.150-0.300)
Colony #4	0.670	(0.590-0.900)	0.350	(0.250-0.580)
Wild Type	4.90	(4.10-6.10)	1.40	(0.800-2.40)

Experiment #3

Colony #1	0.293	(0.260-0.310)	0.073	(0.066-0.080)
Colony #2	0.384	(0.352-0.398)	0.097	(0.081-0.108)
Wild Type	3.11	(2.15-5.53)	0.968	(0.660-1.74)

IRON SUPPLEMENTED MEDIUM

Experiment #1

Colony #1	1.36	(1.27-1.42)	0.440	(0.400-0.470)
Colony #2	1.28	(1.08-1.46)	0.330	(0.210-0.490)
Colony #3	1.28	(1.21-1.36)	0.350	(0.200-0.490)
Wild Type	1.40	(1.26-1.44)	0.410	(0.380-0.480)

Experiment #2

Colony #1	0.680	(0.610-0.770)	0.460	(0.370-0.590)
Colony #2*	0.840	(0.620-1.43)	0.220	(0.200-0.350)
Colony #3*	0.793	(0.620-1.04)	0.170	(0.130-0.260)
Colony #4	0.750	(0.535-0.945)	0.280	(0.150-0.470)
Wild Type	1.35	(1.30-1.40)	0.320	(0.290-0.370)

Experiment #3

Colony #1	0.391	(0.387-0.398)	0.097	(0.097-0.097)
Colony #2	0.381	(0.357-0.409)	0.071	(0.066-0.081)
Wild Type	1.76	(1.67-1.83)	0.402	(0.377-0.444)

FIGURE 1

KILLING CURVE OF <u>BACILLUS</u> <u>SUBTILIS</u> TREATED WITH NTG

A log phase culture was treated with 200 ʌg/ml

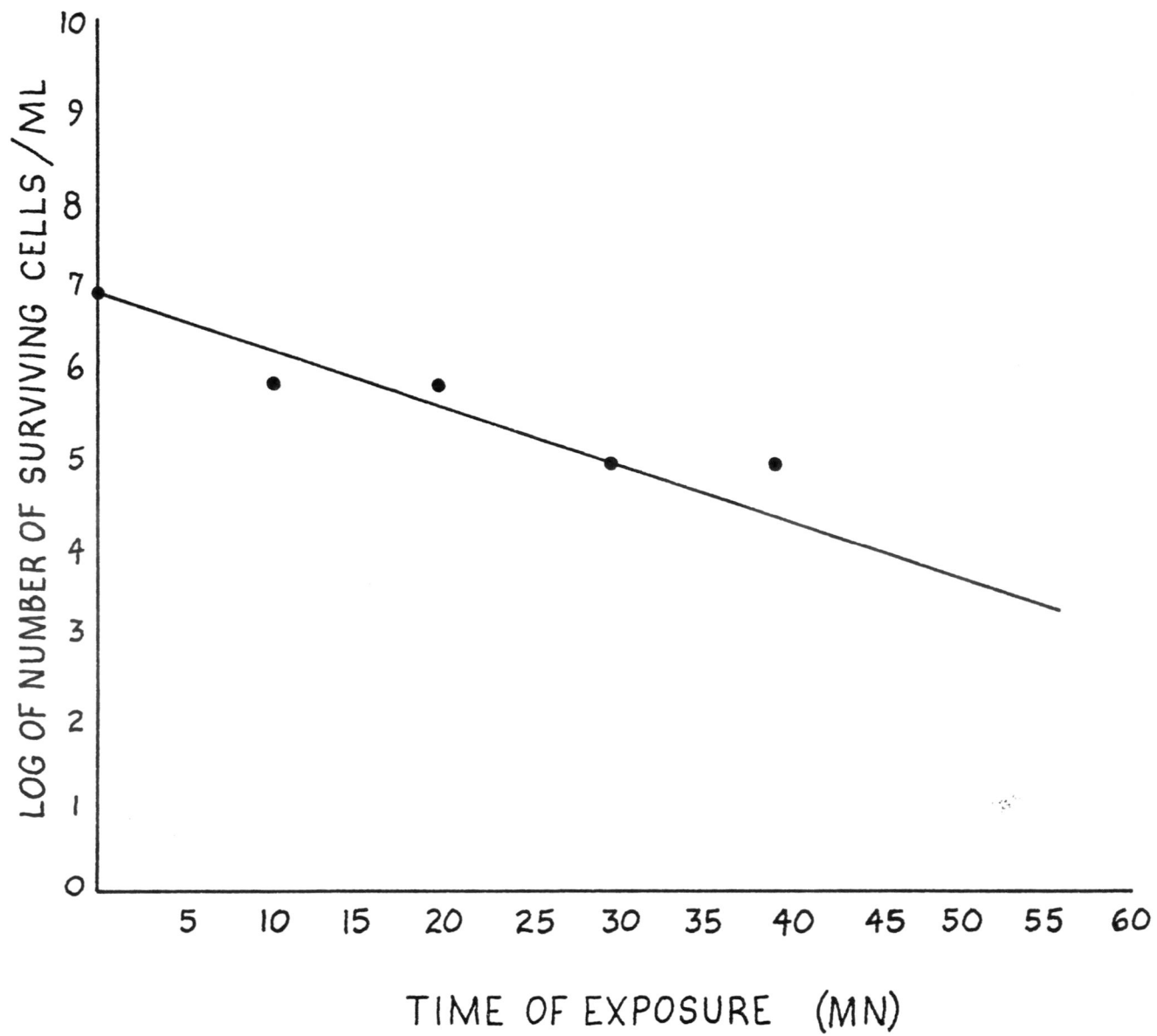NTG for the times indicated in the abscissa.

FIGURE 2

ABSORPTION SPECTRUM OF DIALYZED SUPERNATANT OF YELLOW

MUTANT PRODUCED DURING EXPERIMENT #3

Pigment was dialyzed overnight before the spectrum
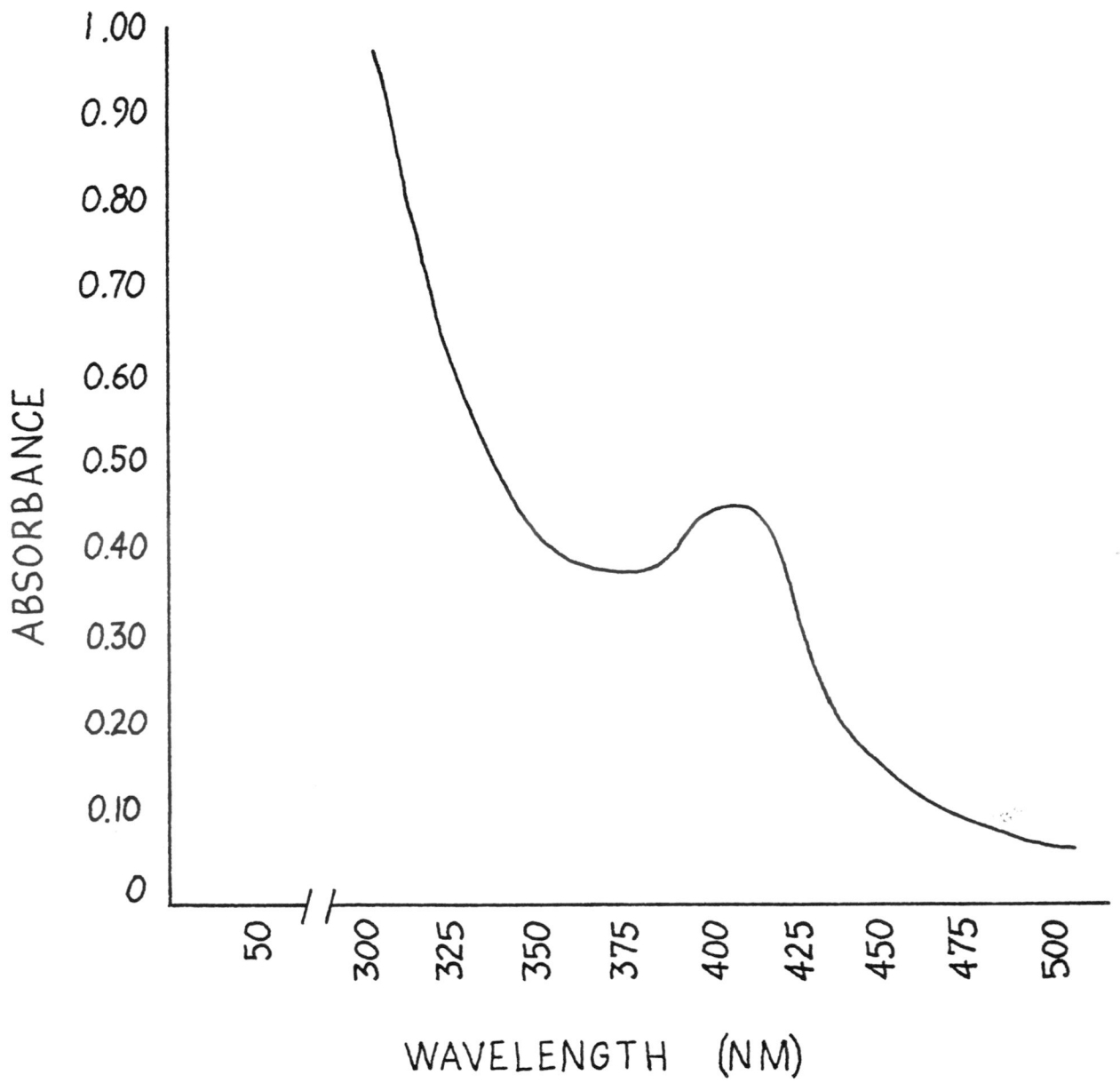
was determined.

FIGURE 3

ABSORPTION SPECTRUM OF PTERIDINE PIGMENT

Absorption spectrum of pteridine pigment

(2 mg/ml water) produced when Pseudomonas

frisellum was grown in sarcosine as a sole

source of carbon. The purified pigment was a

gift from the laboratory of Dr. Wilhelm R. Frisell.
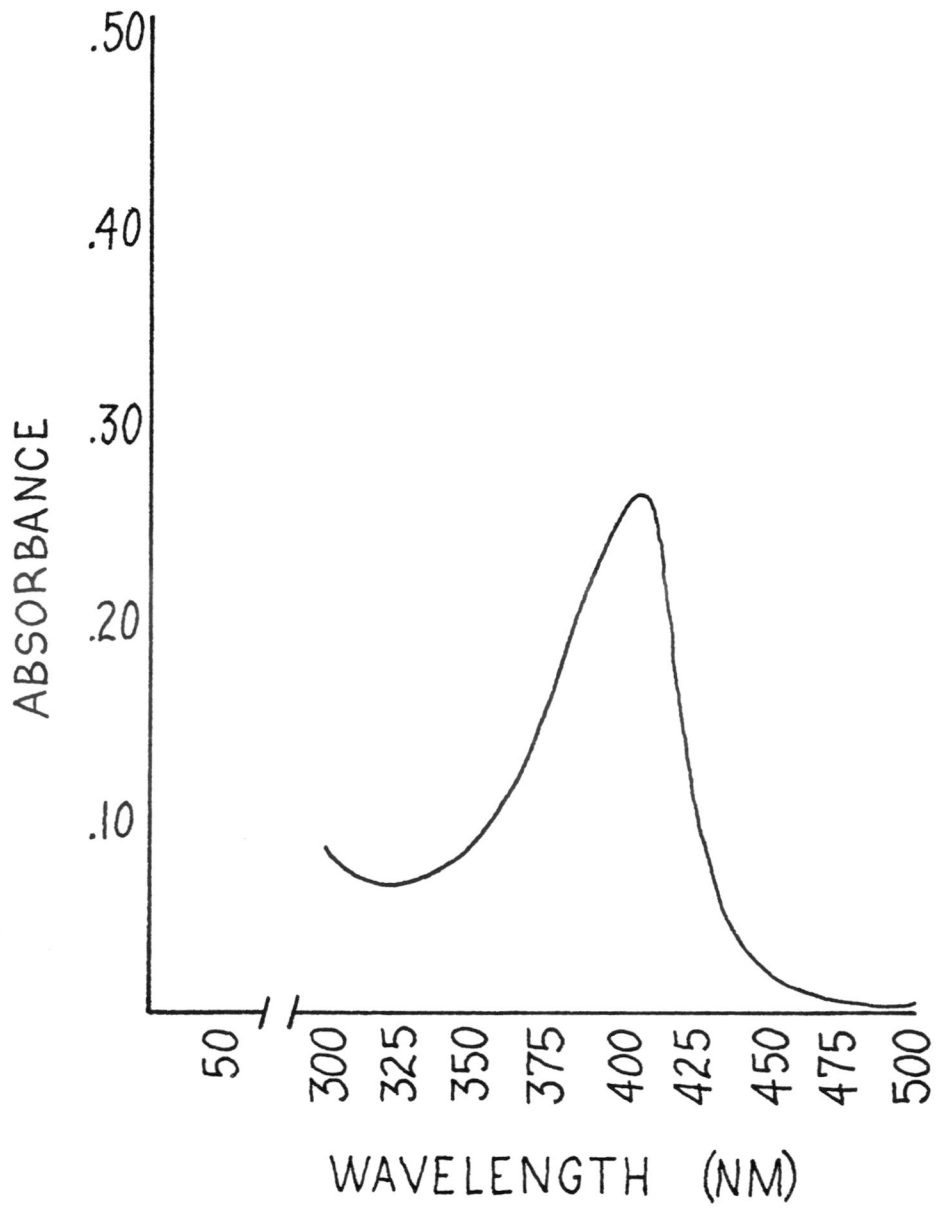
PLATE 1.   Cell wall defective mutant. Cells appearing as

large, white aggregates resulting from NTG

treatment in Experiment #2.

PLATE 2.   Light micrograph, using Bright Field optics, of

cell wall defective mutant compared with wild

type at 2,900X.   Upper, cell wall defective
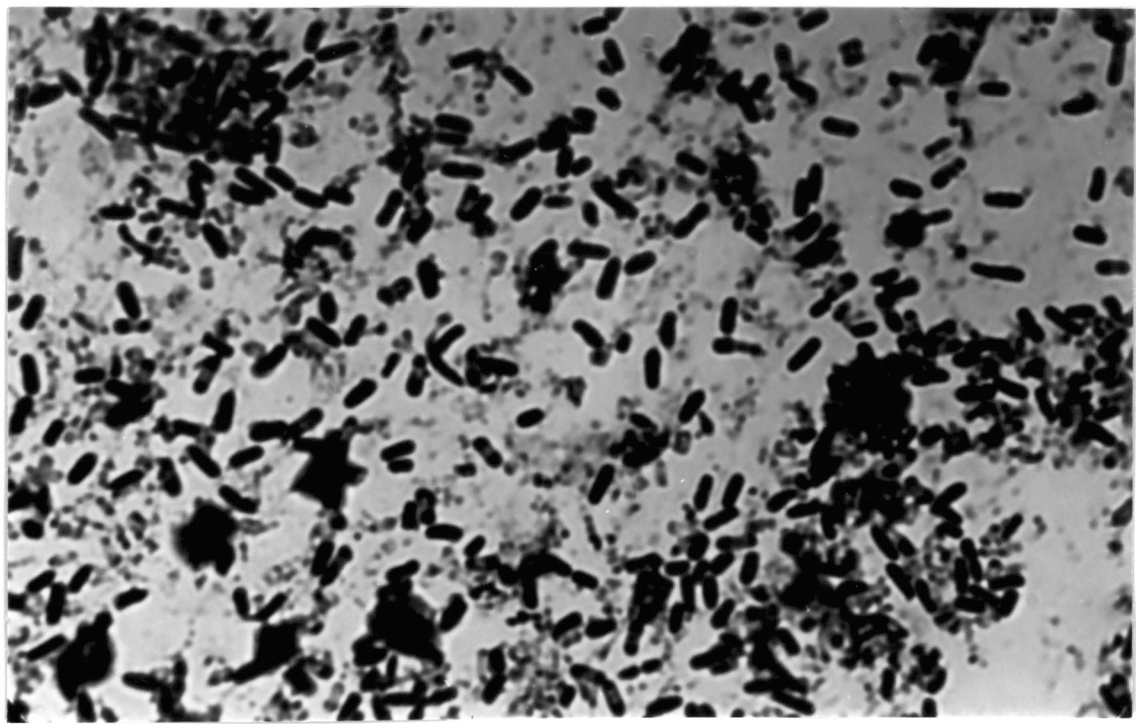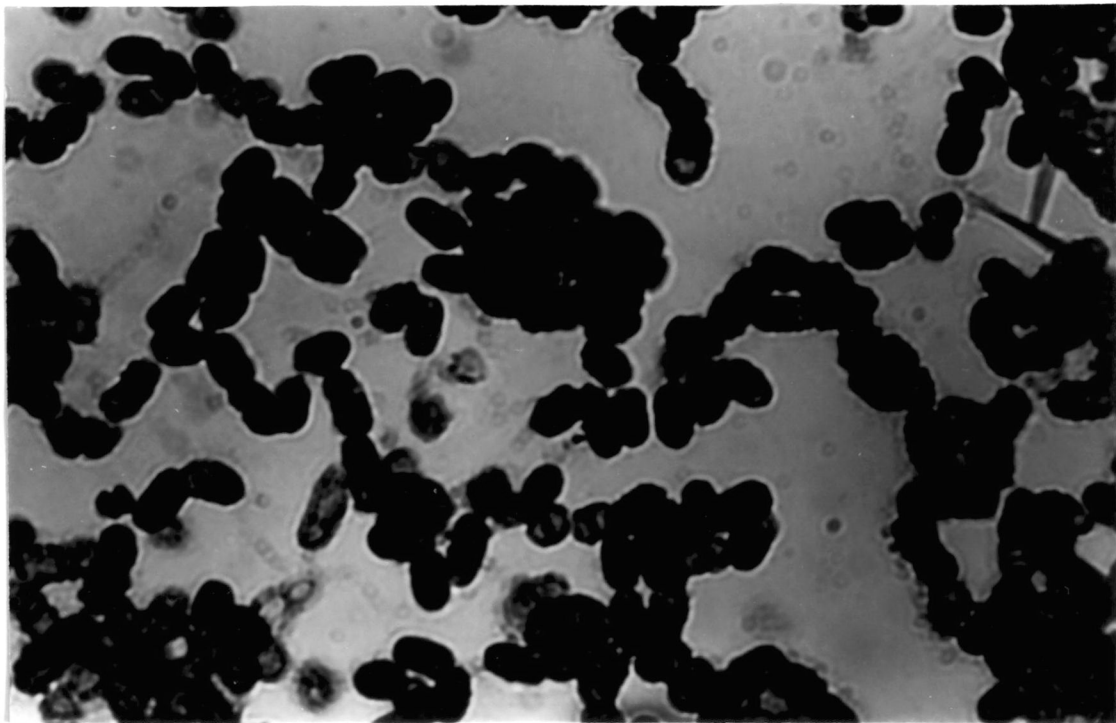
mutant;   Lower, wild type.

PLATE 3. Transmission electron micrograph, using bright

Field optics, of cell wall defective mutant

compared with wild type at 23,000X.   Upper,
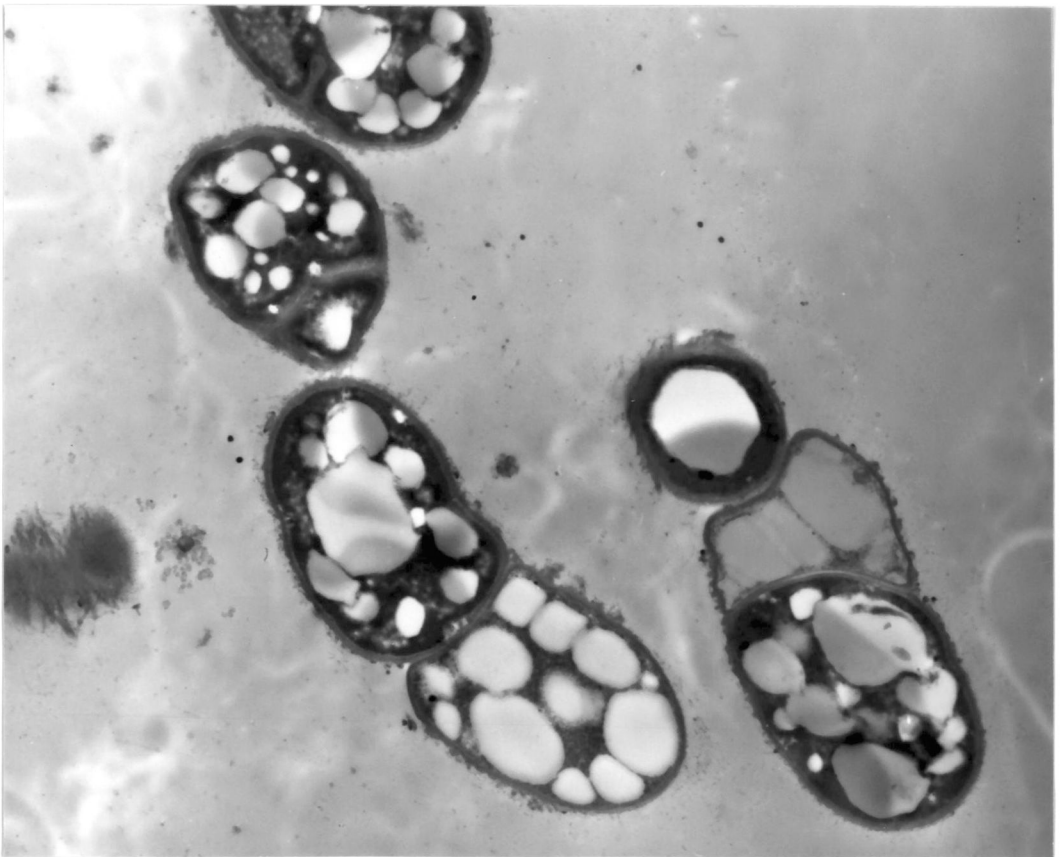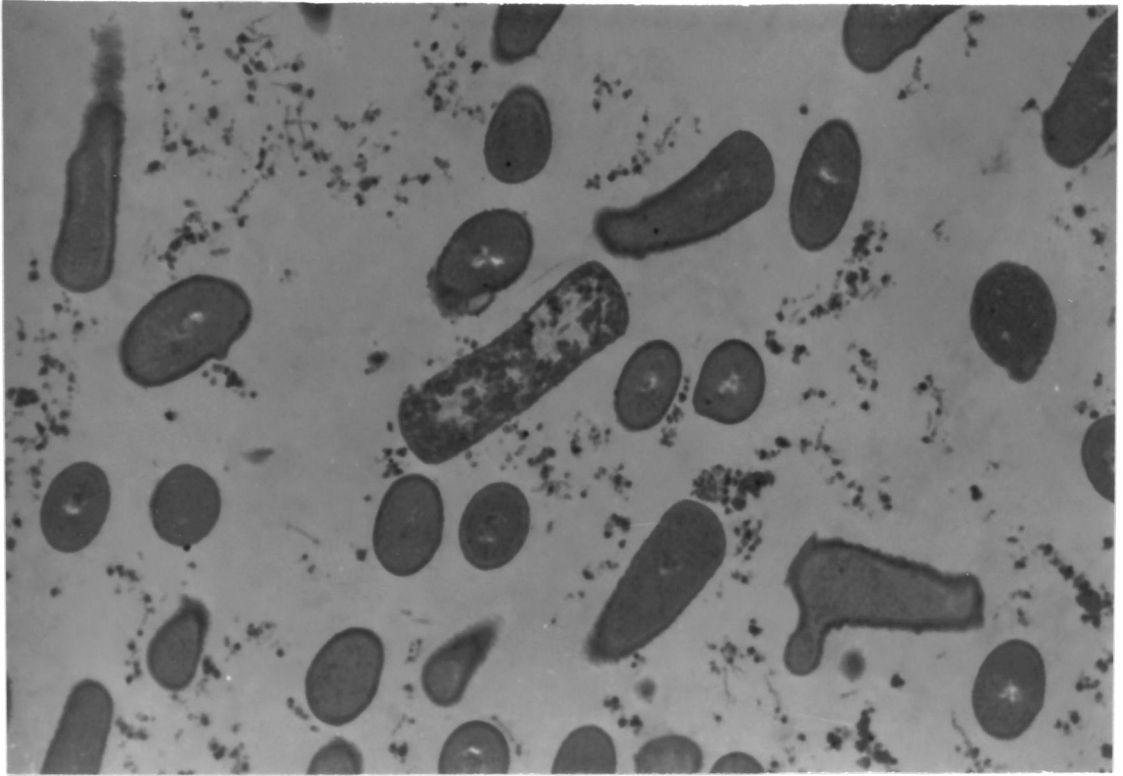
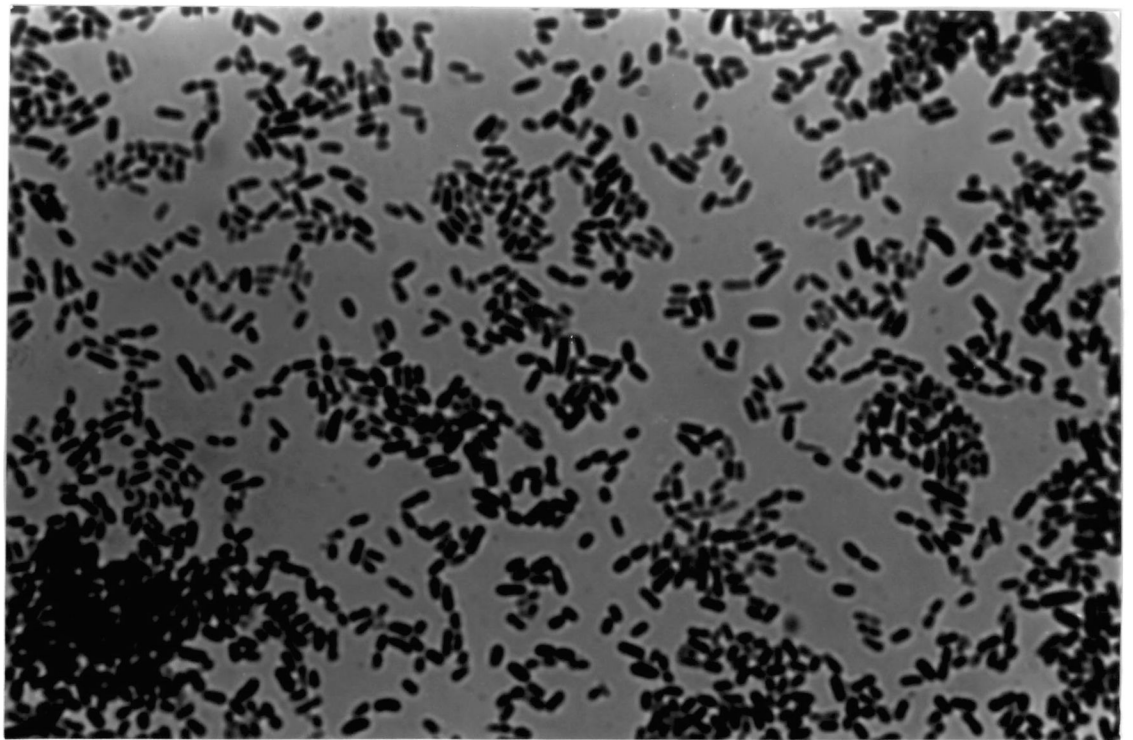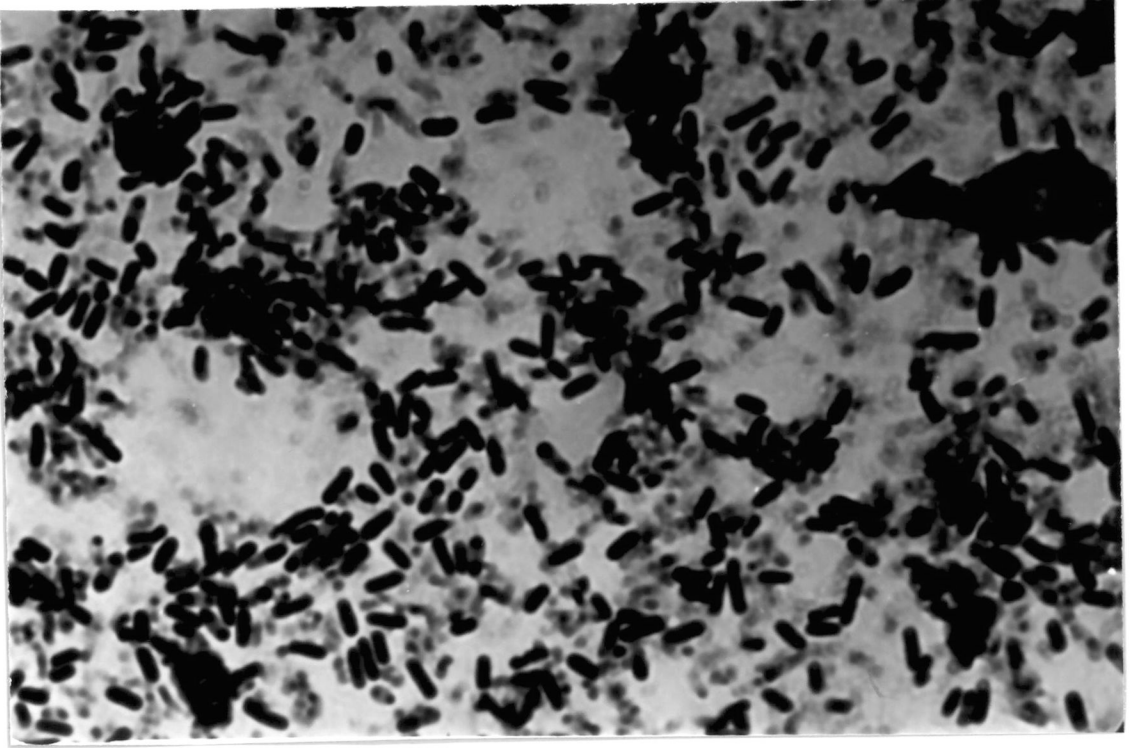wild type; Lower, cell wall defective mutant.

PLATE 4. Light micrograph, using Bright Field optics, of

yellow mutant compared with wild type at 2,900X.

Upper, wild type; Lower, yellow mutant.

REFERENCES CITED

Adelberg, E. A., M. Mandell, and G. Chien-Ching-Chen. Optimal conditions for mutagenesis by nitrosoquanidine. Biochem. Biophys. Res. Commun. 18, 788-795 (1965).

Brot, N., J. Goodwin, and H. Fales. In Vivo and In Vitro formation of 2,3 dihydroxybenzoylserine by Escherichia coli Klz. Biochem. Biophys. Res. Commun., 25, 454-461 (1966).

Carlton, B. C., and B. J. Brown. Chapter 13 Gene Mutation. In Manual of Methods for General Bacteriology. ed. by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg and G. B. Phillips. American Society for Microbiology, Washington, D. C., pp. 222-242 (1981).

Cerda-Olmedo, E., P. C. Hanawatt. Diazomethane as the active agent in nitrosoguanidine mutagenesis and lethality. Molec. Genetics 101, 191-202 (1968).

Corbin, J. B., and W. A. Bulen. The Isolation and Identification of 2,3 dihydroxybenzoic acid and 2-N, 6-N-Di (2,3-dihydroxybenzoyl)-L-lysine formed by iron deficient Azotobacter vinelandii. Biochemistry 8, 757 (1969).

Craddock, V. M. The reaction of N-methyl-N'-nitro-N-nitrosoguanidine with deoxyribonucleic acid. Biochem. J 106, 921-922 (1968).

Craddock, V. M. Study of the methylation and lack of deamination of deoxyribonucleic acid by N-methyl-N'-nitro-n-nitrosoguanidine. Biochem. J 111, 615-619 (1969).

Druckrey, H., R. Preussmann, S. Ivankovic, B. T. So, C. H. Schmidt, J. J. Bucheler. Zur Erzenugung Subcutaner Sarkome an Ratten. Z. Krebsforsch, 68, 87-102 (1966).

Emery, T. Iron metabolism in humans and plants. American Scientist, 70, 626-632 (1982).

Fletcher, P. L., Jr., S. Debnath, and W. Frisell. Siderochrome-Peptides secreted by a pseudomonad cultured on single amino-acids. American Society of Biological Chemists Meeting, San Francisco, 1983.

Frisell, W. R. One-carbon metabolism in microorganisms. I. Oxidative demethylation in a sarcosine-utilizing bacterium. Arch. Biochem. Biophys. 142, 213-222 (1971).

Gibson, F. and J. Pittard. Pathways of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. Bact. Rev. 32, 465-492 (1968).

Goldin, A. J., M. Venditti, and I. Kline. Evaluation of antileukemia agents employing advanced leukemia L1210 in mice. Cancer Res. (Screening Data) 19, 249-266 (1959).

Hadden. C. T., R. S. Foote, and S. Mitra. Adaptive response of Bacillus subtilis to N-methyl-N'-nitro-N-nitrosoguanidine. J. Bacteriol. 153, 756-762 (1983).

Ito, T., and J. B. Neilands. Products of low iron fermentation with Bacillus subtilis: Isolation, characterization, and synthesis of 2,3-dihydroxybenzoyl glycine. J. Am. Chem. Soc. 80, 4645-4647 (1958).

Lawley, P. D. Methylation of DNA by N-methyl-n-nitrosourethane and N-methyl-N-nitroso-N'-nitrosoguanidine. Nature 218, 580-581 (1968).

Leiter, J., and M. A. Schneiderman. Screening data from the cancer Chemotherapy National Service Screening laboratories. Cancer Res. (Screening Data) 19, 31-279 (1959).

Mandell, J. D., and J. Greenberg. A new chemical mutagen for bacteria 1-methyl-3-nitro-1-nitrosoguanidine. Biochem. Biophys. Res. Commun. 3, 575-578 (1960).

Miller, J. H., Experiment 14: Nitrosoguanidine Mutagenesis. In Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 125-129 (1972).

Neilands, J. B. Microbial iron transport compounds (siderochromes), in Inorganic Biochemistry Vol. 1, ed. by Gunther L. Eichorn, Elsevier Scientific Publishing Company, Amsterdam. pp. 167-202 (1973).

Neilands, J. B. Iron and its role in microbial physiology. In Microbial Iron Metabolism, Academic Press, New York, ed. by J. B. Neilands, pp. 3-34 (1974).

Neilands, J. B. Microbial iron compounds. Ann. Rev. Biochem. 50, 715-731 (1981).

Neilands, J. B. Microbial envelope proteins related to iron. Ann. Rev. Microbio. 36, 285-309 (1982).

O'Brien, I. G., and F. Gibson. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from Escherichia Coli. Biochem. Biophys. Acta 215, 393-402 (1970).

Peters, W. J., and R. A. J. Warren. Itoic acid synthesis in Bacillus subtilis. J. Bacteriol. 95, 360-366 (1968).

Pollack, J. R. and J. B. Neilands. Enterobactin and iron transport compounds from Salmonella typhimurium. Biochem. Biophys. Res. Comm. 38, 989-991 (1970).

Schoental, R. Carcinogenic activity of N-methyl-N'-nitro-N-nitrosoguanidine. Nature 209, 726-727 (1966).

Singer, B. and J. B. Kusmierek. Chemical mutagenesis. Ann. Rev. Biochem. 51, 655-693 (1982).

Skinner, W. A., H. G. Gramm, N. O. Greene, J. Greenberg, and B. R. Baker. Potential anticancer agents. XXXI. The relationship of chemical structure to anti-leukemia activity with analogues of 1-methyl-3-nitro-1-nitrosoguanidine. J. Med. Pharm. Chem. 2, 299-333 (1960).

Sugimura, R., M. Nagao, and Y. Okada. Carcinogenic action of N-methyl N'-nitro-N-nitrosoguanidine. Nature 210, 962-963 (1966).

Terawaki, A. and J. Greenberg. Effect of some radiomimetic agents on deoxyribonueleic acid synthesis in Esherichia Coli and transformation in Bacillus subtilis. Biochem. Biophys. Acta 95, 170-173 (1965).