EFFECTS OF COPPER ON *BACILLUS SUBTILIS*[1]

LAWRENCE L. WEED

Department of Microbiology, School of Medicine, Western Reserve University, Cleveland, Ohio

ABSTRACT

WEED, LAWRENCE L. (Western Reserve University, Cleveland, Ohio). Effects of copper on *Bacillus subtilis*. J. Bacteriol. **85**:1003–1010. 1963.—Variants have been isolated from liquid cultures of *Bacillus subtilis* 168 after exposure to copper. The variations manifested are in terms of loss of capacity to be transformed from tryptophan auxotrophy to prototrophy as shown by variant NTCu, and in terms of colony size and altered base composition of the deoxyribonucleic acid as shown by variant SC-22. In addition to the data on altered morphology and chemical composition of the variants, detailed studies on the reversion of one of the variants to *B. subtilis* are presented.

Previous work has shown that a stable small-colony variant of *Escherichia coli* appears in a population of organisms exposed to copper (Weed and Longfellow, 1954). Similar findings were reported by Clowes and Rowley (1955) and Hirsch (1961). Associated with the changes in colonial morphology are differences in metabolism, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content, and resistance to ultraviolet irradiation. The present study deals with variants isolated from cultures of a transformable strain of *Bacillus subtilis* exposed to copper; the principal aim has been to compare the variants with the original organism in terms of both transformability (Spizizen, 1958) and the base composition of the DNA.

The extensive investigations of the Russian school on microbial variation (Timakov, 1959) and, more specifically, the findings of Spirin et al. (1958) have suggested that large changes in base composition of DNA occur in microorganisms. The latter workers have reported alterations of the guanine-cytosine (G-C) content of an "acid-producing" strain of *E. coli* from 52.2 to 41.6% in a "neutral form" and 67.1% in an "alkali-producing" form. There would be important implications, including taxonomic considerations, if it could be demonstrated conclusively that large changes in the chemical composition and biological activity of the DNA of an organism can be brought about by environmental influences in the same sense that small changes in DNA have been brought about by ultraviolet irradiation and the chemical mutagens. The maximal chemical alteration in DNA consistent with survival of an organism has not yet been defined.

Data will be presented on two variants which appeared in cultures of a tryptophan-requiring auxotroph of *B. subtilis* 168 exposed to copper. One variant (designated NTCu) is generally similar to the original strain in its morphological and colonial characteristics but differs principally in that it is no longer transformable to prototrophy by wild-type DNA. The other variant is a small-colony form (designated SC-22). The DNA of this variant differs in both biological activity and chemical composition from the DNA of the parent organism. These variants are stable after many transfers in the absence of copper.

MATERIALS AND METHODS

Strains. *B. subtilis* 168 and 23 were first isolated by Burkholder and Giles (1947).

Media. The minimal medium (Spizizen, 1958) employed contained, in addition to the salts, 0.5% glucose, 0.02% casein hydrolysate, and 0.005% L-tryptophan (designated as supplemented minimal medium). When agar plates containing minimal medium were made, the casein hydrolysate and tryptophan were omitted.

Potato agar plates were prepared from a broth consisting of 200 g of potatoes, 1,000 ml of tap water, and 5 mg of $MnSO_4$, all brought to a pH of 6.8. For plating, 15 g of agar were added.

Tryptose Blood Agar Base (TBAB) plates were prepared from the standard Difco preparation.

The medium (referred to as F.J. medium) first described by Fraser and Jerrel (1953) was modi-

1003

fied slightly and prepared as follows: Na₂HPO₄, 10.5 g; KH₂PO₄, 4.5 g; NH₄Cl, 2 g; MgSO₄, 0.3 g; CaCl₂ (1 M solution), 0.3 ml; gelatin (1% solution in 0.5% NaCl), 1 ml; and Casamino Acids, 2.5 g (all dissolved in 1 liter of distilled water).

Isolation of DNA from B. subtilis. The DNA of *B. subtilis* was obtained by the method of Anagnostopoulos and Spizizen (1961).

Isolation of nucleic acids from SC-22. A significant portion of SC-22 organisms lyse in water, permitting direct isolation of the DNA by alcohol precipitation.

Method I. Variant SC-22 was grown for 24 hr in 2,500-ml volumes of F.J. medium in 4-liter flasks placed on a rotary shaker at 37 C. The cells were harvested by centrifugation and were resuspended in distilled water (1:50 to 1:100 of the original volume). After 2 to 4 hr of mixing at 4 C, the suspension became viscous. The mixture was then centrifuged at 12,000 × *g* for 40 min, and the supernatant fluid was poured into 5 volumes of cold 95% alcohol without agitation. After several hours, a fibrous precipitate containing the DNA rose to the surface.

SC-22 organisms, which had been streaked on TBAB plates and incubated for 48 hr at 37 C and then at room temperature an additional 1 to 4 weeks, were treated as follows. Approximately 2 ml of distilled water were placed on each plate, and colonies were gently scraped off, forming a highly viscous solution of organisms undergoing lysis. The contents of 30 to 40 plates, suspended in 60 to 80 ml of water, were centrifuged at 12,000 × *g* for 40 min. The supernatant fluid was treated with alcohol, as described above, or by high speed centrifugation, as in method II.

Method II. SC-22 organisms grown in liquid medium and organisms grown on plates were harvested in a "ribosome buffer" consisting of K₂HPO₄, 279 mg; KH₂PO₄, 54.4 mg; MgSO₄·7H₂O, 1,232 mg; all dissolved in 1 liter of distilled water (Gillchrist and Bock, 1958). After centrifugation at 12,000 × *g* for 40 min, the supernatant fluid was separated and again centrifuged at 12,000 × *g*. The supernatant fluid was then centrifuged at 100,000 × *g* in a size 40 rotor of a model L Spinco ultracentrifuge for 3 hr, resulting in a supernatant fluid which was saved for analysis and a pellet in the bottom of the tube which was treated in the following manner. "Ribosome buffer" (1 to 2 ml) was added gently to the side of each tube. The tube was then rotated gently one to three times, causing a portion of the pellet to separate and

float freely in the buffer. This fraction was then poured off, and another 1 to 2 ml of "ribosome buffer" were added to the remaining pellet. This was done until all the visible material had been removed from the centrifuge tube and until no more ultraviolet absorbing material was obtained. Each portion of the total pellet was then analyzed for RNA, DNA, and protein.

Transformation procedures. These studies were performed as described by Anagnostopoulos and Spizizen (1961).

Chemical determinations. DNA was determined by the colorimetric diphenylamine method of Burton (1956), using a thymus DNA preparation as a standard. RNA was determined by the phloroglucinol method of Dische and Borenfreund (1957), using yeast RNA as a standard. Protein was determined by the method of Lowry et al. (1951), using crystalline serum albumin as a standard.

Studies on the stability of the small colony trait of SC-22. Tubes (inner diameter: 17 mm) containing minimal media (0.5% glucose, 0.0005% L-tryptophan, 0.01% casein hydrolysate) in 0.8 volumes were prepared. Inocula consisted of either 0.04 ml of a suspension of just visible turbidity of SC-22 organisms from TBAB plates, or 0.04 ml of a solution of DNA (400 μg/ml) containing 200 to 400 SC-22 organisms per ml. The inocula were always plated to assure the absence of any large colonies of *B. subtilis*, and the studies were performed in a dust-free room separated from the laboratory where *B. subtilis* was used daily. In some of the experiments in which DNA was added to the inoculum, deoxyribonuclease was added to a final concentration of 50 μg/ml. The tubes were incubated for 24 hr at 37 C on a reciprocal shaker.

Method of purine and pyrimidine analyses. Degradation of the DNA was performed in a sealed tube with concentrated formic acid at 170 C for 30 min (Vischer and Chargaff, 1948). Purines and pyrimidines were separated by ascending chromatography in the isopropanol-water-HCl solvent of Wyatt (1955), using Whatman no. 1 filter paper. The extinction coefficients employed were adenine, 13,100 at 260 mμ (Beaven, Holiday, and Johnson, 1955); guanine, 11,400 at 248 mμ (Beaven et al., 1955); thymine, 7,890 at 264.5 mμ (Shugar and Fox, 1952); and cytosine, 10,000 at 274 mμ (Shugar and Fox, 1952).

Morphology. Gram stains were done on all the organisms described, and organisms from all the

liquid cultures were examined under a phase microscope.

RESULTS

Population changes in a culture of B. subtilis exposed to copper. A very turbid suspension of *B. subtilis* 168 (tryptophan auxotroph) from an overnight growth on TBAB was prepared, and 0.5 ml was then inoculated into two 5-ml portions of supplemented minimal medium. One culture was made 4×10^{-4} M with respect to $CuSO_4$. Both tubes were allowed to stand at 37 C. Once during each 24-hr period, the tubes were shaken, and a sample was removed for a viable count, as determined on TBAB plates using the appropriate dilution. In the first 24 hr, the control culture showed lysis and a loss of 85% of the viable organisms. In the same period, 98% of the organisms exposed to copper appeared to be nonviable. By the third day, the viable count of the control culture rose slowly to 62% of the original value and then fell by the fourth day to 7% and remained low. The culture exposed to copper slowly rose to 25% of the original viable count and remained at that level for the duration of the experiment. In earlier work (Weed and Longfellow, 1954; Hirsch, 1961), data on the dynamics of growth of *E. coli* in the presence of copper were presented.

A study of colony types during the course of the above counting procedures showed characteristic changes. Although colony size was not significantly different until approximately the seventh day, the surface appearance began to change as early as the third day. The colony became more raised and had sharper edges and a smoother surface. By the seventh day, there were much smaller colonies; eventually a smooth, small colony developed, which, when studied under low magnification, had a granular appearance with sharply defined edges. The early changes were reproducible and appeared regularly in a high proportion of the population in the presence of copper at 37 C and above. The small-colony variants arose infrequently from *B. subtilis* 168 on the initial plating from the liquid medium containing copper. However, on repeated subculture of the smaller colonies on TBAB plates, a variety of small-colony types was attained.

The recent work of Eichhorn (1962), demonstrating the in vitro effectiveness of copper in lowering the "melting-out curve" of DNA, prompted us to repeat some of our earlier experiments using incubation temperatures of 39, 43,

and 45 C, as well as 37 C. A predominance of small colonies did result from routine plating from four tubes incubated at 43 C for 7 days in the presence of copper, whereas small colonies did not appear in control tubes containing no copper, and tubes containing no inoculum remained sterile. The tubes incubated at 37 and 40 C showed only an occasional change in colony type, whereas one of the tubes incubated at 45 C showed the same high frequency of altered colonies as was noted at 43 C. These preliminary results suggest that temperature may play an important role. Copper appears necessary under the conditions employed in our experiments, but it may be that under other conditions the changes described here may occur, at least on occasion, in the absence of copper.

Isolation of variant NTCu. A heavy suspension of *B. subtilis* 168 from an overnight growth on TBAB was prepared; 0.5 ml was then inoculated into two 5-ml portions of supplemented minimal medium. One culture was made 4×10^{-4} M with respect to $CuSO_4$. Both tubes were allowed to stand at 37 C for a 13-day period. At regular intervals, an inoculum from each tube was placed on a TBAB plate and allowed to incubate for 12 hr at 37 C in the absence of copper. These cultures were used in replating, and the resulting organisms were then used in a typical transformation study. Beginning on the fourth day of exposure to copper, there was a steady decline in the transformability of the organisms (Table 1). Organisms made nontransformable in this manner maintained this characteristic after 13 daily transfers on TBAB in the absence of copper. After the thirteenth transfer, they were placed on potato agar, incubated for 48 hr at 37 C, and then stored in a cold room for several weeks. At the end of this period, the NTCu organisms were still nontransformable.

TABLE 1. *Per cent transformation of a tryptophan-requiring mutant of Bacillus subtilis after increasing periods of exposure to 4×10^{-4} M copper sulfate in minimal medium at 37 C*

Day	Control*	Copper-treated organisms
2	2.3	1.8
4	1.2	0.08
6	1.1	0.04
10	1.2	5.00×10^{-4}
13	2.0	1.00×10^{-5}

* Control cultures contained no copper sulfate.

The colony formed by the nontransformable organism resembled that of the original *B. subtilis* in size but had a sharper edge and a somewhat smoother appearance. This difference was sufficiently apparent so that, with a concentration of copper one-half that described above, two types of colonies could be detected over the 13-day exposure period; one type yielded nontransformable organisms and the other yielded organisms which were transformed to the same degree as the parent strain. Over the 13-day period, there was a gradual shift in the population to a predominance of the nontransformable type. The dynamics of the shift in populations have not been elucidated. Variant NTCu can be obtained with considerable regularity.

Isolation of SC-22. SC-22, the small-colony variant (Fig. 1) similar to that described in *E. coli*, was obtained after 7 days of incubation of *B. subtilis* 168 in minimal medium containing copper, under conditions similar to those described above for the isolation of NTCu. Growth inhibition of *B. subtilis* by copper and yield of the variant were extremely variable. On repeated occasions, small-colony variants were observed but, in many instances, a pure culture was not achieved. Such variation has been noted in the production of small-colony variants of *E. coli*, and the recent work of Hirsch (1961) dealt with the causes of the variability in that system. SC-22 was sent to the Public Health Diagnostic Laboratories in Atlanta, Georgia. They reported it as an "unidentified" gram-positive rod showing many pleomorphic forms.

The morphology and staining properties of SC-22 were extremely variable and dependent upon the conditions under which the organisms were grown. For example, after 5 hr on potato agar, they appeared as gram-positive rods show-

FIG. 1. *Photograph of colonies of Bacillus subtilis 168 (left) and SC-22 (right) taken after 48 hr of growth on Tryptose Blood Agar Base.*

ing considerable variation in size. After several days on potato agar, only gram-positive cocci were present. When transferred from potato agar to TBAB, the rodlike forms reappeared.

When SC-22 was allowed to remain on TBAB plates for 1 to 4 weeks at room temperature after an initial incubation at 37 C for 2 days, the colonies acquired a more mucoid appearance. There was not the spreading and lysis commonly seen with *B. subtilis* 168. These organisms were coccoid in appearance and appeared approximately one-fourth the size of the original organism. When viable counts were done on suspensions of SC-22 organisms grown on TBAB plates and compared with viable counts of SC-22 organisms grown in liquid medium, the count was approximately four times as high for a given density in the case of organisms grown on TBAB. This would appear to demonstrate that the organisms remain viable after long periods on TBAB plates, a fact which adds further interest to the RNA-DNA relationships described below.

Nucleic acid composition of SC-22. The ratio of RNA to DNA is extremely variable, being as high as 10.3:1 in organisms grown in liquid medium and as low as 0.7:1 in organisms harvested from TBAB plates. That these organisms grown on TBAB plates are still viable was shown as stated above.

Isolation of DNA from SC-22. As described earlier, the nucleic acids in water lysates of SC-22 grown in either liquid F.J. medium or on TBAB plates could be isolated either by precipitation in alcohol (method I) or by high-speed centrifugation (method II). When the fibrous precipitate that rose to the surface of the alcohol (method I) was analyzed, it contained as much as 75% RNA and 25% DNA; this RNA-DNA relationship was maintained after four reprecipitations, whereas the bulk of the protein was lost during the repeated alcohol reprecipitations and resuspensions in 2 M NaCl. If the fibrous precipitate of RNA and DNA was dissolved in water instead of 2 M NaCl before reprecipitating in alcohol, most of the RNA became a fine precipitate at the bottom of the alcohol, whereas most of the DNA continued to rise to the surface in a fibrous form. Final removal of all the protein and RNA from the DNA was accomplished by treatment with deoxycholate and ribonuclease, respectively, as described by Spizizen (1958).

The fibrous precipitate obtained from organisms grown on TBAB plates instead of F.J.
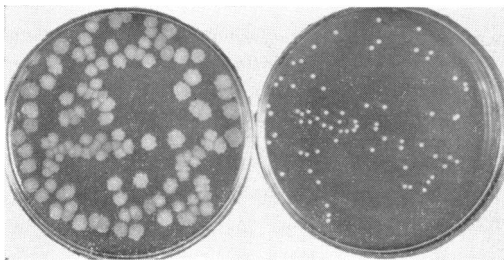
medium contained relatively less RNA than DNA.

If the water lysate of organisms was subjected to high-speed centrifugation (method II) instead of alcohol precipitation, it was possible to recover the DNA from the water lysate, and, in the case of the organisms grown on TBAB plates, the DNA could be recovered almost completely free from RNA by this physical procedure alone.

The percentage recovery of the nucleic acid from the organisms by water extraction was variable and depended upon the conditions of growth and upon the morphological stage of the organism at the time lysis of the organism was attempted in a hypotonic environment.

In a typical experiment, using a single extraction in water, 18% of the DNA and 40% of the RNA were recovered from a 24-hr growth of SC-22 in F.J. medium.

Base composition of the DNA. The purine and pyrimidine base compositions of the DNA of *B. subtilis* and SC-22 were determined (Table 2). The gross differences are apparent. The values reported for SC-22 are essentially the same irrespective of whether the DNA was isolated by method I or II and irrespective of whether the organisms were grown in liquid medium or on TBAB plates.

The data on the base composition of the DNA of SC-22 are based on analyses of DNA from the water extracts of the organisms. Data on DNA isolated by means other than water extraction from both SC-22 and other variants have been obtained. These data and physical studies on the DNA of all the variants will be presented elsewhere.

Stability of SC-22. Over a period of 1.5 years, SC-22 was transferred on TBAB plates every 48 hr, and only a rare reversion to a large *B. subtilis* type colony was noted. When inocula of SC-22 from TBAB plates were transferred to liquid media as described earlier, large-colony type organisms appeared in 1 or 2 of 45 tubes. This was observed repeatedly in spite of precautions taken against contamination. When the DNA solutions, which were prepared after high-speed centrifugation, and which contained small numbers of SC-22 and no large-colony forms, were used as the inoculum in the presence of deoxyribonuclease, the frequency of the appearance of large-colony forms increased significantly.

A total of 32 large-colony types were isolated from 324 cultures of SC-22 grown under the con-

TABLE 2. *Base composition of the deoxyribonucleic acid of Bacillus subtilis and SC-22*

Base	B. subtilis*	B. subtilis 168	SC-22
Thymine	28.7	29.3	17.9
Adenine	28.9	28.8	17.5
Cytosine	21.4	21.5	32.4
Guanine	21.0	20.4	32.4

* Values for this strain are those of Belozersky and Spirin (1960).

ditions described above; of these, 18 are prototrophs and 14 are tryptophan auxotrophs. Although the work of Briggs et al. (1957) describing the conversion of gram-positive cocci to gram-negative bacilli has not yet been confirmed (Paine and Daniel, 1959; Hilson and Elek, 1959; Briggs et al., 1959), it is of interest that a similar variety of organisms was described by them when the gram-negative bacilli reverted to gram-positive cocci. Among the revertant organisms were those identical to the original *Staphylococcus* and those with changed properties; e.g., some had identical phage-typing patterns and others were nontypable.

Transformation studies were performed using DNA derived from the "revertant" forms isolated from cultures of SC-22 during the studies on the stability of the small-colony trait of SC-22. Table 3 shows the results obtained by using one of the tryptophan-requiring auxotrophs (no. 8) both as a recipient and as a source of DNA to act as a donor.

When *B. subtilis* wild-type 23 was crossed with auxotroph no. 8, the efficiency of transformation was approximately the same as when wild-type 23 was crossed with strain 168. When strain 168, the original auxotroph used in these studies, was crossed with auxotroph no. 8, the per cent transformation was less than when wild-type 23 was used as a donor but was still highly significant. The difference is to be expected when neither of the two organisms being crossed is the wild type and both are blocked in the same given biosynthetic pathway. It has also been shown that two auxotrophs isolated from cultures of SC-22 are capable of transforming one another to prototrophy.

Several of the tryptophan auxotrophs isolated were not transformed to prototrophy by DNA derived from the original auxotroph, *B. subtilis* 168. Table 3 shows, in addition, that good trans-

TABLE 3. *Transformation studies using deoxyribonucleic acid (DNA) derived from the large-colony-forming organisms (no. 8 and 9) isolated from cultures of SC-22 and from the two strains of Bacillus subtilis, 168 and 23*

Recipient organism	Nutritional requirement	Donor DNA	Nutritional requirement	Per cent transformation*
168	Tryptophan	23	None	1.2
168	Tryptophan	No. 8	Tryptophan	0.06
No. 8	Tryptophan	23	None	1.0
No. 8	Tryptophan	168	Tryptophan	0.04
168	Tryptophan	No. 9	None	0.23
168	Tryptophan	168	Tryptophan	$<10^{-5}$
No. 8	Tryptophan	No. 8	Tryptophan	$<10^{-5}$

* In all cases where transformation was obtained, deoxyribonuclease control studies were done to insure that it was a case of true transformation. Control studies in which no DNA was added did not show reversions which could account for these results.

formation results were achieved when the donor DNA was derived from a revertant large-colony prototroph (no. 9) isolated from a growing culture of SC-22.

DISCUSSION

There were substantial and constant differences between *B. subtilis* 168 and variants isolated from a culture of strain 168 when the latter was exposed to copper. It is recognized that the outstanding question in the present work, as well as that of Spirin et al. (1958), is whether contaminating organisms, parasitism, or associated bacteriophage have been ruled out. That this has been done in these systems with absolute certainty is always open to question, but the current interest in the findings of the Russian workers and the provocative nature of the experimental findings described here have led to this communication at the present time.

In regard to the origin of variant NTCu, its morphological and colonial appearance, the persistence of its auxotrophic state in regard to tryptophan, and the consistency with which it has appeared leave little doubt about its relationship to the original *B. subtilis* 168. Whether the change in its transformability brought about by copper is the result of a small chemical change in the DNA, or whether another element such as the presence or absence of a phage is the controlling factor and is subject to the actions of copper, is not clear. In this regard, Romig (*personal communication*) recently interpreted electron microscopic studies on the transformable strains of *B. subtilis* as indicative of the presence of bacteriophage.

Evidence that copper has an important biological role has come from many sources. A recent study of Kolodziej and Slepecky (1962) on *Bacillus megaterium* showed a highly specific effect of copper on the sporulation process over a relatively narrow concentration range. Also, much data suggesting that copper may have an important role in metabolism (McElroy and Glass, 1950) and in problems of abnormal growth (Howell, 1958) has accumulated.

There is nothing in the present work that proves a specific or direct action of copper on the nucleic acids of the cell. However, the work of Frieden and Alles (1958) demonstrated the effectiveness of cupric ion chelation with respect to nucleic acids and nucleic acid components. They demonstrated that the deoxyribose derivatives are uniformly stronger cupric ion chelators than are the other components. Frieden and Alles (1958) stated that the intensity of these reactions is such that they "may be germane to the biological function of nucleic acids in tissue." Recently, Eichhorn (1962) demonstrated that, in the presence of cupric ion, there is a very significant shift of the "melting-out curve" of DNA and much lower temperatures are required to produce the single-stranded state. This is in contrast to the stabilizing effect of other divalent cations such as magnesium.

It is recognized that the large changes in base composition described here are not easily comprehended within the present framework of our knowledge concerning the biosynthesis of DNA. That the present framework may require extension, however, is suggested by the facts that it cannot be stated with certainty whether there is

or is not "nonsense" DNA, whether a single or many DNA polymerases are active within a cell, whether single or multiple primers are utilized, and whether the naturally occurring enzymes capable of making adenine-thymine polymers (Schachman et al., 1960) have any active biological role. If a complex of enzymes and primers prove to be involved in the synthesis of DNA, the relative activities and structures of both enzymes and primers may be significantly altered as the temperature and ionic characteristics of the environment vary. Possible action of degradative enzymes in the findings presented was not explored, but the possibility is recognized that they may prove to be playing a significant role.

Data on significant alterations in morphology, in the chemical composition of the mucopeptide of the cell wall, and in the resistance to ultraviolet light of SC-22 and other variants will be presented elsewhere.

ACKNOWLEDGMENT

The author is grateful to John Spizizen, who kindly made possible the initiation of this work, and to Lester Krampitz, who has provided continuous encouragement and support.

LITERATURE CITED

ANAGNOSTOPOULOS, C., AND J. SPIZIZEN. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:740–746.

BEAVEN, G. H., E. R. HOLIDAY, AND E. A. JOHNSON. 1955. *In* E. Chargaff and J. N. Davidson [ed.], The nucleic acids, vol. 1. Academic Press, Inc., New York.

BELOZERSKY, A. N., AND A. S. SPIRIN. 1960. Chemistry of the nucleic acids of microorganisms, p. 147–185. *In* E. Chargaff and J. N. Davidson [ed.], The nucleic acids, vol. 3. Academic Press, Inc., New York.

BRIGGS, S., K. CRAWFORD, E. P. ABRAHAM, AND G. P. GLADSTONE. 1957. Some properties of gram-negative bacilli obtained from a strain of *Staphylococcus aureus* in the presence of benzyl penicillin. J. Gen. Microbiol. **16**:614–627.

BRIGGS, S., K. CRAWFORD, E. P. ABRAHAM, AND G. P. GLADSTONE. 1959. Further observations on the relationships between gram-negative rods and staphylococci grown in the presence of penicillin. J. Gen. Microbiol. **21**:205–207.

BURKHOLDER, P. R., AND N. H. GILES, JR. 1947.

The induced biochemical mutations in *Bacillus subtilis*. Am. J. Botany **34**:345–348.

BURTON, K. 1956. A study of the condition and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.

CLOWES, R. C., AND D. ROWLEY. 1955. Genetic studies on small colony variants of *Escherichia coli* K-12. J. Gen. Microbiol. **13**:461–473.

DISCHE, Z., AND E. BORENFREUND. 1957. A new color reaction for the determination of aldopentose in presence of other saccharides. Biochem. Biophys. Acta **23**:639–642.

EICHHORN, G. L. 1962. Metal ions as stabilizers or destabilizers of the deoxyribonucleic acid structure. Nature **194**:474–475.

FRASER, D., AND E. A. JERREL. 1953. The amino acid composition of T2 bacteriophage. J. Biol. Chem. **205**:291–295.

FRIEDEN, E., AND J. ALLES. 1958. Subtle interactions of cupric ion with nucleic acid and components. J. Biol. Chem. **230**:797–804.

GILLCHRIST, W. C., AND R. M. BOCK. 1958. p. 1–10. *In* Microsomol particles and protein synthesis. Pergamon Press, New York.

HILSON, G. R. F., AND S. D. ELEK. 1959. An investigation into the development of gram-negative rods in penicillin-treated cultures of *Staphylococcus aureus*. J. Gen. Microbiol. **21**: 208–220.

HIRSCH, H. M. 1961. Small colony variants of *Escherichia coli*. Mode of action of copper in variant recovery and population dynamics of cultures containing variants. J. Bacteriol. **81**:448–458.

HOWELL, J. S. 1958. The effect of copper acetate on the *p*-dimethylamine-azobenzene carcinogenesis in the rat. Brit. J. Cancer **12**:594–608.

KOLODZIEJ, B. J., AND R. A. SLEPECKY. 1962. A copper requirement for the sporulation of *Bacillus megaterium*. Nature **194**:504–505.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

McELROY, W. D., AND B. GLASS. 1950. A symposium on copper metabolism. The Johns Hopkins Press, Baltimore.

PAINE, T. F., JR., AND R. R. DANIEL. 1959. Attempts to obtain gram-negative rods from staphylococci treated with penicillin. J. Gen. Microbiol. **21**:203–204.

SCHACHMAN, H. K., J. ADLER, C. M. RADDING, I. R. LEHMAN, AND A. KORNBERG. 1960. Enzymatic synthesis of deoxyribonucleic acid. VII. Synthesis of a polymer of deoxyadenylate and deoxythymidylate. J. Biol. Chem. **235**: 3242–3249.

SHUGAR, O., AND J. J. FOX. 1952. Spectrophotometric studies of nucleic acid derivatives and related compounds as a function of pH, I and II. Biochim. Biophys. Acta 9:199–218.

SPIRIN, A. S., A. N. BELOZERSKII, D. G. KUDLAI, A. G. SKAVRONSKIA, AND V. G. MITEREVA. 1958. Changes in the composition of nucleic acids in the formation of saccharolytic inert forms of intestinal bacteria. Biochemistry 23:147–155.

SPIZIZEN, J. 1958. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S. 44: 1072–1078.

TIMAKOV, F. C. 1959. Microbial variation. Pergamon Press, New York.

VISCHER, E., AND E. CHARGAFF. 1948. The composition of the pentose nucleic acids of yeast and pancreas. J. Biol. Chem. 176:715–734.

WEED, L. L., AND D. LONGFELLOW. 1954. Morphological and biochemical changes induced by copper in a population of Escherichia coli. J. Bacteriol. 67:27–33.

WYATT, G. R. 1955. Separation of nucleic acid components by chromatography on filter paper, p. 243–265. In E. Chargaff and J. N. Davidson [ed.], The nucleic acids, vol. 1. Academic Press, Inc., New York.