

## AUXOTROPHIC MUTATION ASSOCIATED WITH LOW STREPTOMYCIN RESISTANCE AND SLOW GROWTH IN *BACILLUS SUBTILIS*

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**Summary.** Nutrient agar plates containing 100–200  $\mu\text{g}$  streptomycin/ml were heavily inoculated with *Bacillus subtilis* strain 168 ind<sup>-</sup>. Small or medium sized colonies developed in a proportion of  $10^{-6}$  after a few days incubation at 37°C. About one fourth of the isolates produced minute colonies when transferred to blood agar. Isolates showing normal colony size on blood agar exhibited certain new nutritional requirements allowing the classification of the mutants into 5 groups. The majority of the mutants multiplied well on complete media as yeast peptone agar but failed to grow under defined nutritional conditions. Approximately one tenth of the mutants showed a deficiency in the pathway of tetraporphyrin (protohaem) synthesis. A few isolates with a dependence on aromatic amino acids were also encountered. Although the isolates were obtained at high streptomycin concentrations, they exhibited a low tolerance to this antibiotic.

Slow growth was another feature of the mutants. Auxotrophy, slow growth and moderately increased streptomycin tolerance apparently resulted from mutation at one single locus since reversion of auxotrophy to prototrophy restored their sensitivity and normal growth. It has been concluded that a direct action of streptomycin on chromosomal replication might be involved in the genesis of these peculiar mutants.

ANDERSON and IVÁNOVICS [1] have recently observed that various effects, including treatment with copper sulphate or nitrosoguanidine, induced haemin dependent mutants in *Bacillus subtilis*. In preliminary experiments on streptomycin-containing medium they obtained  $\delta$ -aminolaevulinic acid (ALA) dependent isolates. In examining these mutants we were able to study an unfrequent type of streptomycin resistance characterized by low antibiotic tolerance, increased nutritional requirement and slow growth. The increased nutritional requirement was characterized by a deficiency in protohaem synthesis. Since all these three features were associated in both appearance and reversion, it seemed justified to regard these mutants as a separate group of auxotrophs.

### Materials and methods

**Bacterial strains.** *B. subtilis* strain 168 and a haemin dependent isolate [1] were used. The genotype of the latter (RI 5, ALA dependent) was expressed as *hem Al*.

**Culture media.** Medium GGM containing glutamate and glycerol was prepared as described previously [1]. If necessary, the medium was supplemented with tryptophan (20  $\mu\text{g}$ /ml). Yeast extract peptone medium (YP) was described in reference [2.] Medium YT: yeast extract (Oxoid), 2.5 g; Trypton (Oxoid), 10 g;  $\text{K}_2\text{HPO}_4$ , 1 g; NaCl, 5 g; tap water, 1000 ml; pH 7.5.

Medium *HAC*: GGM was supplemented with haemin (2.5–5 µg/ml), cysteine (20 µg/ml) and fraction *V* bovine serum albumin (1–2 mg/ml). *Blood agar*: to YP agar 5% citrated human blood was added.

*Inoculum*. Precultivation was performed overnight at 37°C in YP broth without shaking. Then the culture was diluted 1 : 20 with the same medium and shaken gently at 37°C until an optical density of 0.5–1.0 had been reached. Density measurements were carried out in a Bausch and Lomb Spectronic "20" spectrophotometer. The culture was centrifuged and the bacteria were resuspended in 1/10 volume. This suspension was seeded undiluted or after dilution in 0.1 ml aliquots onto streptomycin-containing agar plates. The number of inoculated colony formers varied between  $10^7$  and  $10^8$ .

Ultraviolet-irradiated inoculum was prepared as described in reference [1].

*Determination of catalase activity*. The bacteria were grown on YP or heated blood agar. After 20 hours incubation the cells were harvested in phosphate buffer (0.1 M, pH 6.8) and the suspension was adjusted to o. d. = 0.5. To 2 ml suspensions, 5 ml 0.015–0.017 M  $H_2O_2$  were added. After 5 minutes standing at 37°C the amount of decomposed  $H_2O_2$  was determined iodometrically [3].

*Minimal nutritional requirement*. The isolates were first grown on blood agar then transferred to YP and blood agar. If the isolate seemed to be porphyrin dependent, it was inoculated onto GGM–ALA (2 µg/ml) and HAC media.

If the isolate grew on YP agar but not on GGM agar, its specific requirement was tested for all amino acids, nucleic bases and 11 water-soluble vitamins.

*Streptomycin resistance*. In orientation experiments a slightly opalescent suspension was prepared from the colony tested, then agar plates containing streptomycin at various concentrations were seeded with loopful amounts of bacteria ( $10^4$ – $10^5$  colony formers). In order to obtain more precise data, in quantitative experiments  $1$ – $2 \times 10^3$  colony formers were streaked on a series of blood agar plates with gradually increasing streptomycin concentrations. The degree of tolerance was expressed as the decrease in the number of colony formers. Resistance to the bactericidal effect of streptomycin will be presented in the experimental part of this paper.

## Results

*Isolation and nutritional requirement of mutants*. As in our previous experiments, Hartley agar plates containing streptomycin (200 µg/ml) were seeded with 10 to  $100 \times 10^6$  colony formers and the media were incubated at 37°C. After 48 hours few colonies were visible. Their number increased on further incubation. After 96 hours, varying with the size of inocula and with individual experiments, 10 to 200 colonies were observed per plate. Similar numbers of colonies were obtained on other complete media (YP, YT or blood agar). On GGM or HAC medium containing streptomycin (200 µg/ml) there was no growth in several repeated experiments. Irradiation of the inoculum did not affect the results obtained with inoculum without ultraviolet irradiation.

After 96 hours incubation on streptomycin agar the size of colonies varied considerably. About 3/4 of colonies was small (0.5–1 mm). Large colonies, 3 mm or more in diameter, occurred in about 10%. The size of the remaining colonies varied between these values.

The following results reflect findings obtained in 5 different experiments. Colonies grown on antibiotic agar plates were transferred to blood agar. About 1/5 of the isolates failed to develop into colonies, i.e. they consisted of dead bacteria. On blood agar 72 out of the 266 transferred isolates formed colonies 0.5 mm or less in diameter. These "minute" colonies were highly unstable in

further subcultures: they often lost viability or segregated colonies resembling those of the parent cultures. In phenotype (nutritional requirement) 85 out of 194 isolates obtained on blood agar were identical with the parent strain. Part of these isolates had probably originated from a contamination of the inoculum. More than one hundred colonies showed an alteration in nutritional requirements; 57 isolates were examined in detail. A deficiency in porphyrin synthesis was observed in 14 cultures. Eight isolates required aromatic amino acids. Further 19 isolates failed to grow on GGM agar even when the medium had been supplemented with all amino acids, nucleic acid bases and water-soluble vitamins. All of them grew, however, abundantly on YP and on other complete media. The remaining 6 isolates multiplied only on blood agar.

In subsequent experiments a decrease in the concentration of streptomycin (lowest value, 50  $\mu\text{g/ml}$ ) resulted in an increase in the number of colonies. At low concentrations mainly "minute" colonies or auxotrophs with undefinable requirement developed. In the presence of 200  $\mu\text{g}$  streptomycin/ml the proportion of colony formers was  $0.2 \times 10^{-6} - 10^{-6}$ . At lower concentrations this value increased to  $10^{-5}$ .

In this study about one thousand isolates were examined for defined characters. Undefinable auxotrophs occurred frequently; these were preceded in number only by "minute" mutants. The proportion of mutants deficient in porphyrin synthesis varied greatly in the individual experiments. Sometimes 20% of the colony formers required haemin or ALA, while in other experiments

Table I

Relevant phenotypes of mutants isolated from strain 168 ind<sup>-</sup>

| Class of mutants                   | Growth on different media |                        |                  |                        |    |            |
|------------------------------------|---------------------------|------------------------|------------------|------------------------|----|------------|
|                                    | GGM                       | GGM + ALA <sup>1</sup> | HAC <sup>2</sup> | GGM + aro <sup>3</sup> | YP | YP + blood |
| Parent strain 168 ind <sup>-</sup> | +                         | +                      | +                | +                      | +  | +          |
| "Minute"                           | —                         | —                      | —                | —                      | ±  | +          |
| nda                                | —                         | —                      | —                | —                      | +  | +          |
| nda-b                              | —                         | —                      | —                | —                      | —  | +          |
| aro                                | —                         | —                      | —                | +                      | +  | +          |
| hemA                               | —                         | +                      | +                | —                      | —  | +          |
| hem                                | —                         | —                      | +                | —                      | —  | +          |

<sup>1</sup> GGM with  $\delta$ -aminolaevulinic acid (2  $\mu\text{g/ml}$ )

<sup>2</sup> See methods

<sup>3</sup> GGM with tyrosine and phenylalanine (10  $\mu\text{g/ml}$  each)

Remarks to the designation of isolates. The nomenclature of DEMEREC *et al.* [4] was adopted. Mutants lacking  $\delta$ -aminolaevulinic acid synthetase are denoted as *hemA*. Haemin dependent mutants are designated by their phenotypes as *hem* isolates.

these mutants were entirely absent. The lowest frequency was found with *aro* mutants; in two individual experiments only 8 isolates were found.

On the basis of phenotype the mutants were classified into groups, as shown in Table I.

Among other results the following are worth mentioning. We were unable to characterize the nutritional requirement of *nda* (non-defined auxotrophs). In the presence of a great number of various metabolites some of them exhibited a minimal growth on GGM agar. Apart from this difference in phenotype all isolates seemed to be uniform; occasional traces of growth were attributed to the "leaky" character of the culture. Mutants *nda-b* (*nda* with blood requirement) grew poorly on GGM blood agar but abundantly on YP blood agar. Members of group *aro* required aromatic amino acids (tyrosine and phenylalanine). These isolates were probably deficient in the synthesis of prephenic acid or its precursor.

Some strains representing all kinds of isolates were examined for catalase activity. On the average, 2 ml suspension of the parent strain decomposed 3 ml 0.01 M  $H_2O_2$ . The reaction was inhibited by sodium azide. Within the limits of experimental error, identical results were given by all representatives in that mutation was never associated with an alteration in catalase activity. Bacteria deficient in the haematin pathway were, of course, cultured in the presence of exogenous porphyrin.

Mutants with changed nutritional requirements were obtained not only with inocula containing vegetative bacteria, but also with spores.

**Streptomycin tolerance of the isolates.** Isolates obtained on plates containing 100–200  $\mu$ g streptomycin/ml showed a very low tolerance to the antibiotic. For example, in one experiment 49 colonies were examined by preparing slightly opalescent suspensions from blood agar subcultures and transferring  $10^4$ – $10^5$  bacteria to blood agar containing streptomycin. Table II shows the number of isolates giving confluent growth in the presence of streptomycin at different concentrations.

Quantitative streptomycin tolerance tests for defined mutants are presented in Table III.

Table II

*Antibiotic tolerance of colonies selected at random from growth on YP agar with 200  $\mu$ g streptomycin/ml*

| Streptomycin, $\mu$ g/ml | 5 | 20 | 50 | 100 | "Dependent" |
|--------------------------|---|----|----|-----|-------------|
| No. of isolates          | 9 | 26 | 4  | 5   | 5           |

In nutritional requirement 15 out of the 49 isolates tested were identical with the parent strain. These isolates tolerated 100  $\mu$ g streptomycin/ml. The remaining isolates appeared to be *nda* in phenotype. "Dependent" isolates tolerated streptomycin at low concentrations (20  $\mu$ g/ml or less) and grew somewhat better in the presence of the antibiotic.

Table III

Percentage of colony formers of individual isolates with defined nutritional requirements on blood agar plates containing streptomycin at different concentrations

| Strain               | Size of inoculum | Per cent of survivors at $\mu\text{g}$ streptomycin/ml |     |     |     |     |
|----------------------|------------------|--------------------------------------------------------|-----|-----|-----|-----|
|                      |                  | 5                                                      | 10  | 20  | 50  | 100 |
| 168 ind <sup>-</sup> | 1200             | 90                                                     | 7   | —   | —   | —   |
| <i>hemA1</i>         | 1300             | 95                                                     | 100 | 100 | —   | —   |
| <i>hemA3</i>         | 1350             | 100                                                    | 38  | 2   | —   | —   |
| <i>hemA5</i>         | 1500             | 95                                                     | 97  | 100 | 100 | —   |
| <i>hem-8</i>         | 2300             | 100                                                    | 90  | 80  | 40  | —   |
| <i>hem-10</i>        | 2100             | 96                                                     | 72  | 90  | 80  | —   |
| <i>hem-11</i>        | 2500             | 90                                                     | 55  | 50  | 5   | —   |
| <i>hem-12</i>        | 2500             | 95                                                     | 80  | 62  | 9   | —   |
| <i>hem-13</i>        | 2800             | 50                                                     | 50  | 60  | 35  | —   |
| <i>hem-15</i>        | 2400             | 70                                                     | 40  | 46  | —   | —   |
| <i>aro-1</i>         | 1800             | 100                                                    | 96  | 90  | —   | —   |
| <i>aro-2</i>         | 2400             | 2                                                      | —   | —   | —   | —   |
| <i>aro-8</i>         | 4000             | 34                                                     | 32  | 10  | —   | —   |

Streptomycin sensitivity of strain 168 ind<sup>-</sup> was tested also at very low concentrations. All bacteria of the inoculum consisting of 1500 colony formers produced colonies at 2, 3 and 4  $\mu\text{g}$  streptomycin/ml. Survival rate was 90% at 5  $\mu\text{g}/\text{ml}$ , 15% at 8  $\mu\text{g}/\text{ml}$ , and 4% at 10  $\mu\text{g}/\text{ml}$ .

Five further isolates with *aro* character were also included in these tests; all but one (*aro-5*) exhibited a high sensitivity to streptomycin similar to that of *aro-2*. Dash indicates no growth.

It is seen that all isolates examined were heterogeneous in streptomycin sensitivity. The parent strain (168 ind<sup>-</sup>) was highly sensitive. Mutants *hemA* and *hem* differed slightly but significantly from the parent culture. Only 3 *aro* isolates were slightly resistant, the remaining 4 strains behaved like the parent culture. Similar experiments will be presented later in this paper.

It should be noted that, corresponding to the original technique, media containing 100–200  $\mu\text{g}$  streptomycin/ml were seeded with large inocula ( $10^7$  bacteria) of some isolates. The applied complete media were supplemented with metabolites (e.g. ALA) when necessary. Depending on the streptomycin concentration and composition of the medium sometimes a thin film of confluent growth developed. Mostly, however, even these large inocula gave rise to few colonies.

*Stability of isolates.* Mutants *hem* and *hemA* showed a remarkably weak sporulating capacity. When subcultured frequently, *hem* mutants split off revertants. Mutants *hemA* and *aro* were highly stable. Isolates *nda* and *nda-b* were unstable and had therefore to be maintained by freeze-drying.

Isolates showing nutritional requirements different from those of the parent strain reverted spontaneously to the original phenotype. In order to

Table IV

Change in streptomycin tolerance associated with reversion of auxotrophy to the parent phenotype  
(The inoculum varied between 970 to 2400 bacteria)

| Isolate and its revertant             | Per cent of survivors at $\mu\text{g}$ streptomycin/ml |     |     |     |    |     |
|---------------------------------------|--------------------------------------------------------|-----|-----|-----|----|-----|
|                                       | 5                                                      | 10  | 15  | 20  | 50 | 100 |
| <i>hemA6</i> <sup>1</sup>             | 100                                                    | 85  | 50  | 45  | 15 | 0   |
| <i>hemA6</i> revertant                | 0                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>hemA6</i> <sup>1</sup>             | 60                                                     | 30  | 30  | 30  | 0  | 0   |
| <i>hemA6</i> revertant                | 5                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>hemA11</i>                         | 45                                                     | 2   | 1   | 0   | 0  | 0   |
| <i>hemA11</i> revertant               | 0                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>hem-8</i>                          | 50                                                     | 50  | 30  | 30  | 0  | 0   |
| <i>hem-8</i> revertant                | 5                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>hem-10</i>                         | 30                                                     | 5   | 2   | 0   | 0  | 0   |
| <i>hem-10</i> revertant               | 0                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>hem-13</i>                         | 80                                                     | 9   | 3   | 0   | 0  | 0   |
| <i>hem-13</i> revertant               | 40                                                     | 0   | 0   | 0   | 0  | 0   |
| <i>hem-14</i>                         | 100                                                    | 10  | 1   | 1   | 0  | 0   |
| <i>hem-14</i> revertant               | 3                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>hem-15</i>                         | 100                                                    | 100 | 100 | 100 | 0  | 0   |
| <i>hem-15</i> revertant               | 20                                                     | 0   | 0   | 0   | 0  | 0   |
| <i>hem-16</i>                         | 100                                                    | 100 | 80  | 80  | 0  | 0   |
| <i>hem-16</i> revertant               | 60                                                     | 0   | 0   | 0   | 0  | 0   |
| <i>aro-2</i> <sup>2</sup>             | 5                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>aro-2</i> revertant                | 2                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>aro-5</i>                          | 100                                                    | 100 | 100 | 90  | 0  | 0   |
| <i>aro-5</i> revertant                | 90                                                     | 1   | 0   | 0   | 0  | 0   |
| <i>nda-3</i>                          | 100                                                    | 100 | 100 | 100 | 90 | 0   |
| <i>nda-3</i> revertant                | 5                                                      | 1   | 1   | 1   | 0  | 0   |
| <i>nda-15</i>                         | 100                                                    | 100 | 100 | 90  | 0  | 0   |
| <i>nda-15</i> revertant               | 13                                                     | 0   | 0   | 0   | 0  | 0   |
| <i>nda-17</i>                         | 100                                                    | 29  | 20  | 5   | 1  | 0   |
| <i>nda-17</i> revertant               | 7                                                      | 0   | 0   | 0   | 0  | 0   |
| <i>nda-b-6</i>                        | 100                                                    | 100 | 100 | 100 | 50 | 1   |
| <i>nda-b-6</i> revertant <sup>3</sup> | 55                                                     | 5   | 0   | 0   | 0  | 0   |

<sup>1</sup> Individual experiments with two revertants isolated independently.

<sup>2</sup> Similar experiments with three further *aro* isolates exhibited also a low rate of tolerance.

<sup>3</sup> To parent type strain. Intermediate revertant *nda* showed an increased tolerance to streptomycin.

determine the frequency of reversion, bacteria grown in sufficiently supplemented media were centrifuged and washed in GGM. The suspension was seeded on GGM and sometimes on YP plates. The proportion of reversion for 9 *hem* isolates was 0.5–2.8 per  $10^7$  bacteria. A lower frequency reversion was observed with *hemA* isolates: sometimes even  $2 \times 10^8$  organisms failed to yield revertants. Reversion was more frequent in *nda* isolates. Revertants always

occurred in populations of  $10^8$  bacteria; the frequency of reversion reached a maximum of  $100/10^7$  bacteria. Reversion of *nda-b* strains occurred in two steps: first *nda* (independent from blood) then the parent phenotype appeared. Unlike with *aro* isolates, in size and structure of colonies the revertants and the parent culture were identical. When revertants were sought on GGM medium two different kinds of colonies were observed: a small proportion of the colonies resembled to the parent culture, but the majority comprised slowly-growing small colonies (about 2 mm) even after 48 hours incubation. In the latter the reversion was probably due to a suppressor mutation.

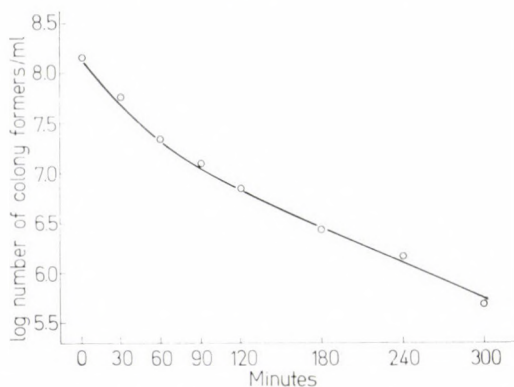


Fig. 1. The killing action of streptomycin on the parent strain (*B. subtilis* 168 ind<sup>-</sup>). A 1.9 ml sample of exponential phase culture in YP broth was mixed with 0.1 ml 1% streptomycin sulphate. The suspension was incubated as a thin layer at 37°C and bacterial counts were made at intervals

*Comparative examination of auxotrophic mutants and their revertants.*

Isolates reverted from mutants with new requirements showed a decreased streptomycin tolerance similar to the tolerance of the parent strain (Table IV).

It would appear that the revertants were somewhat more sensitive to streptomycin than was the parent strain (see Table III). Because of the low tolerance of *aro* cultures only *aro-5* could be compared to the corresponding auxotrophic mutant. Reversion of this mutant was also associated with a decrease in streptomycin tolerance.

Streptomycin at high concentrations was bactericidal to cells of the parent strain. The kinetics of this effect are presented in Fig. 1.

Table V shows the bactericidal effect of the antibiotic to several isolates and to their revertants.

The sensitivity of the parent strain varied within reasonable limits in different experiments. The auxotrophic isolates were more resistant than was the parent culture. Reversion decreased their resistance to the bactericidal action of streptomycin. Strain *aro-7* was an exception as after reversion it showed no change in sensitivity. It should be noted that this culture showed

Table V

*The killing action of 500 µg streptomycin/ml on bacteria after 1 hour exposure in broth*

| Strain                                | Survivors,<br>per cent |
|---------------------------------------|------------------------|
| Parent (168 ind <sup>-</sup> ) exp. 1 | 6.4                    |
| Parent (168 ind <sup>-</sup> ) exp. 2 | 12.8                   |
| Parent (168 ind <sup>-</sup> ) exp. 3 | 8.2                    |
| <i>hemA6</i>                          | 48.8                   |
| <i>hemA6</i> revertant                | 20.9                   |
| <i>hemA8</i>                          | 85.8                   |
| <i>hemA8</i> revertant                | 13.3                   |
| <i>hem-9</i>                          | 47.2                   |
| <i>hem-9</i> revertant                | 18.6                   |
| <i>aro-7</i>                          | 21.7                   |
| <i>aro-7</i> revertant                | 22.3                   |
| <i>nda-3</i>                          | 96.2                   |
| <i>nda-3</i> revertant                | 10.5                   |
| <i>nda-15</i>                         | 45.9                   |
| <i>nda-15</i> revertant               | 2.4                    |

Bacteria were grown in YP medium containing 2 µg ALA/ml or 2.5 µg haematin + 0.2% bovine albumin/ml. Simple YP broth was used for porphyrin independent mutants. When the o.d. value reached 0.5, 1.9 ml aliquots of the cultures were mixed with 0.1 ml 1% streptomycin solution and incubated in a water bath of 37°C for 60 minutes. Bacterial counts were made on blood agar plates.

no difference from the parent strain in the bacteriostatic effect of the antibiotic.

The results of an experiment carried out later with *aro-5* and its revertant has been omitted from Table V. In this experiment the degree of sensitivity of the parent strain (tested for comparison) was strikingly high. The proportion of survivors was only 1% for the parent culture, 15.5% for *aro-5*, and 3% for the revertant. The difference may be explained by the fact that in these experiments a new batch of medium was used.

The auxotrophs showed increased resistance to both the bacteriostatic and the bactericidal effect of streptomycin. The *aro* mutants behaved irregularly in this respect as well as in the type of reversion. In contrast to the finding of GORINI and KATAJA [5], who described some conditionally streptomycin dependent (CSD) *E. coli* strains, the new nutritional requirement of our typical auxotrophs could not be supplemented by the addition of streptomycin. Many of our isolates were examined as to whether their requirement for ALA, haemin or complete medium could be supplemented by streptomycin incorporated in minimal medium (GGM). In the range of 0.5–5 µg streptomycin/ml, however, no such effect was demonstrated.

Table VI

Colony size of isolates and their nutritional revertants on different media after 24 and 48 hours incubation at 37 °C

| Strain                   | Size of colonies in mm on media |     |                                   |      |            |     |                   |      |
|--------------------------|---------------------------------|-----|-----------------------------------|------|------------|-----|-------------------|------|
|                          | YP                              |     | YP + ALA or haematin <sup>1</sup> |      | Blood agar |     | Heated blood agar |      |
|                          | 24                              | 48  | 24                                | 48   | 24         | 48  | 24                | 48   |
| 168 ind <sup>-</sup>     | 5.9                             | 6.9 |                                   |      | 6.8        | 8.5 | 3.9               | 5.6  |
| <i>hemA1</i>             |                                 |     | 1.0                               | 2.8  | 1.2        | 6.0 | 1.8               | 5.0  |
| <i>hemA8</i>             |                                 |     | 0                                 | 2.0  | 0.6        | 5.0 | 1.0               | 5.1  |
| <i>hemA8</i> revertant   |                                 |     | 6.8                               | 7.6  | 6.0        | 8.0 | 4.6               | 7.3  |
| <i>hem-8</i>             |                                 |     | 0.5                               | 1.0  | 1.9        | 4.6 | 3.8               | 5.9  |
| <i>hem-8</i> revertant-1 |                                 |     | 5.3                               | 7.1  | 5.4        | 5.2 | 4.3               | 5.2  |
| <i>hem-8</i> revertant-2 |                                 |     | 10.6                              | 15.8 | 7.5        | 8.2 | 7.9               | 12.1 |
| <i>nda-3</i>             | 1.0                             | 2.9 |                                   |      | 1.0        | 1.9 |                   |      |
| <i>nda-3</i> revertant   | 4.6                             | 7.1 |                                   |      | 5.0        | 5.9 |                   |      |
| <i>nda-15</i>            | 1.8                             | 3.9 |                                   |      | 0.8        | 1.6 |                   |      |
| <i>nda-15</i> revertant  | 8.0                             | 9.9 |                                   |      | 5.0        | 5.0 |                   |      |
| <i>aro-2</i>             | 6.7                             | 9.1 |                                   |      | 4.9        | 5.6 |                   |      |
| <i>aro-2</i> revertant   | 6.5                             | 7.8 |                                   |      | 5.9        | 6.4 |                   |      |
| <i>aro-7</i>             | 3.1                             | 5.0 |                                   |      | 3.0        | 4.0 |                   |      |
| <i>aro-7</i> revertant   | 4.4                             | 6.2 |                                   |      | 4.2        | 5.3 |                   |      |

<sup>1</sup> Supplemented either with ALA for *hemA* isolates or with haematin + bovine albumin for *hem* mutants. 0 = Not visible by the naked eye.

Appropriate dilutions of exponential growth phase liquid culture were plated. Inocula consisted of 15 to 20 colony formers. The values represent the average size of 30 to 50 colonies. Variation in the size of individual colonies was less than  $\pm 1$  mm.

Slow growth was another striking characteristic of our isolates. For technical reasons instead of determining the generation time, the time needed for the development of colonies was examined. As shown in Table VI there was a striking difference in the size of colonies between *hem*, *hemA* and *nda* isolates and the parent culture. The difference was especially great after 24 hours incubation but was still marked after 48 hours.

Reversion of the mutants restored the original rapid growth or resulted in an increased degree of multiplication. In this respect *aro* auxotrophs behaved irregularly. Their growth decreased slightly and reversion exerted little effect. Some *aro* revertants grew very slowly; these restored their original phenotype probably by suppressor mutation.

When heavy inocula of streptomycin sensitive bacteria of *B. subtilis* 168 were plated on nutrient agar media containing high concentration of streptomycin, the observations made could be summarized as follows. It was characteristic that about 80% of the colony formers differed in phenotype from the inoculated culture. The formed auxotrophs showed no wide range of vari-

ation: on examination of about 1000 colonies all isolates could be classified into 5 groups. Three out of the 5 groups were characterized by a deficiency in protohaem synthesis. The requirement of the most frequently isolated auxotroph (*nda*) could not be defined; it seemed to revert to its original phenotype in one step. It was characteristic that auxotrophy was accompanied by low streptomycin resistance and slow growth. Reversion from auxotrophy resulted in a loss of increased streptomycin tolerance and in regaining normal growth. The behaviour of some *aro* isolates was variable and less characteristic than that of the other mutants.

### Discussion

Colonies developing on nutrient agar plate containing aminoglycoside antibiotics may differ from the parent strain in colony morphology and minimal nutritional requirement. JENSEN and THOFERN [6] described streptomycin resistant mutants of *Staphylococcus aureus* the growth of which was highly enhanced by haemin. Haemin dependent mutants were isolated from *Escherichia coli* by serial passages on streptomycin-haemin medium [7]. On neomycin or streptomycin agar salmonellae were described to split off resistant mutants characterized by dwarf colonies [8]; these consisted partly of bacteria dependent on one or more metabolites (cysteine, serine, niacin). A well-characterized  $\delta$ -aminolaevulinic acid (ALA) deficiency in the porphyrin pathway was first demonstrated in a *B. subtilis* mutant obtained by the use of streptomycin. Shortly thereafter a similar mutant was isolated from *E. coli* with neomycin [9a]. YEGIAN *et al.* in 1959 [9b] isolated haemin dependent mutants from *Staph. aureus* with kanamycin. Recently, TIEN and WHITE [10a] applied kanamycin for isolating large numbers of mutants from the same organisms in order to map the genetic control of the biochemical pathway of protohaem synthesis. It was striking that the mutants failed to show kanamycin resistance.

The pleiotropic effect of streptomycin has long been known; here only some examples are mentioned. High streptomycin resistance in *Salmonella typhimurium* is accompanied by methionine (GOLDSCHMIDT *et al.* [10b]) or thiamine and niacin (DEMEREK and LAHR [10c]) requirement. WATANABE and WATANABE [11, 12] stressed the importance of the degree of streptomycin resistance. They showed that in *S. typhimurium* high ( $> 500 \mu\text{g/ml}$ ) and moderate streptomycin resistance is associated with different loci. Mutation to low resistance was characterized by slow growth and, in part of the isolates, associated with thiamine and niacin requirement. These mutants could be transduced in a single step to the wild-type phenotype; the transductant became streptomycin sensitive, grew rapidly and turned prototrophic in nutritional requirement. It has, therefore, been concluded that all these markers were controlled by one single locus.

In phenotype, our *B. subtilis* mutants seem to be similar to those described by WATANABE and WATANABE [11, 12]. The great variability in the properties of streptomycin resistant phenotypes justified their classification into well-defined groups. Accordingly, it is recommended that this peculiar type of streptomycin resistance should be designated with the symbol "ALSAS" (associated low streptomycin resistance, auxotrophy and slow growth).

In phenotype there are similarities between our mutants and the *E. coli* strains of GORINI and KATAJA [13] isolated from conditional streptomycin dependent mutants of *E. coli*. The phenotype of their revertants was associated with the appearance of a suppressor functioning also in the absence of streptomycin.

The question arises as to how ALSAS mutants survive the high streptomycin concentration used for their isolation and how is it possible to obtain auxotrophs when the required metabolite is absent from the medium. For example, the ALA required for growth by *hemA* mutants is not contained by the usual nutrient media. The strict association between streptomycin tolerance and environment (constituents of the medium) is well known. According to the conclusion of GYÖRFFY and KÁLLAY [14] the survival of bacteria at a given streptomycin concentration "may be controlled either physiologically, *i.e.* by a favourable microenvironment or by temporary changes at the cellular level . . .; or the survival may be controlled genetically by heritable resistance". Components released from disintegrating bacteria of the heavy inoculum interfere with the effect of streptomycin only slightly, but yield metabolites lacking from the medium.

Mutants isolated by the use of aminoglycoside antibiotics behave peculiarly as they frequently show a deficiency in porphyrin synthesis. Streptomycin acts similarly on eukaryotes: it suspends chlorophyll synthesis irreversibly [15, 16]. As an effect of this antibiotic *Euglena gracilis* cells lose their pigmentation but retain their plastid structure [15, 18]. If ultraviolet irradiation affects in an isolated manner only the cytoplasm of *Euglena*, pigment production ceases. Irradiation of the nucleus fails to exert this effect [19, 20]. Of the papers published since 1962, that of RAY and HANAWALT [21] must be mentioned. These authors assumed that the chloroplast possesses its own DNA responsible for information on chlorophyll production and that this nucleic acid is different from that present in the nucleus. The chloroplast ribosomes are also specific and different from those of the nucleus [22].

According to the model of SCHIFF and ZELDIN [23] streptomycin induces a deficient DNA polymerase on plastid m-RNA and ribosome complex. This defective DNA polymerase would eventually lead to plastid loss associated with permanent bleaching of *Euglena*. Although this alteration cannot be considered a mutation in the usual sense, its result is identical with that of the latter. Despite the fact that several authors described the mutagenic effect of

streptomycin, this antibiotic has not been included in the list of mutagenic agents. Streptomycin has been shown to be mutagenic for the non-chromosomal determinant of *Chlamydomonas* [24], and also for the host range marker of T<sub>2</sub> phage [25]. The discoloration of *Euglena* induced by streptomycin has been regarded as mutation [26] as well as the disappearance of chlorophyll from the sprouting onion, where the antibiotic caused morphologically demonstrable chromosomal aberrations [27].

Streptomycin attacks primarily the cytoplasmic membrane [28]. LANDMAN and BURCHARD [29] assumed that the membrane contains an extranuclear factor taking part in membrane synthesis. Streptomycin-depleted dependent mutants fail to produce a normal cytochrome system [30]. Streptomycin resistant *Salmonella* L-forms do not yield bacterial revertants in the absence of the antibiotic. Streptomycin depletion causes a hereditary alteration in cell wall production [31]. Under physiological conditions, approximately 1/4 of all bacterial ribosomes integrate to the membrane where, bound to m-RNA, they exert a specific template function [32]. The model elaborated by JACOB *et al.* [33] reflects the association of bacterial chromosome and membrane. This association has been proven partly by electron microscopic studies [34] partly by biochemical examinations indicating that DNA replication took place in the membrane. The membrane fraction contained 1/4 of the DNA polymerase [35].

Does the bacterial membrane contain an integrated chromosomal segment more or less independent from nuclear DNA and does it supply information for the synthesis of the membrane and its enzymes? The question is far from being irrational. Part of the DNA is closely connected with the cell wall. BISHOP *et al.* [36] showed the presence of deoxyribonuclease resistant DNA in carefully isolated cell wall preparations of *B. subtilis*. It would be inconsistent with the economy of nature if enzymes associated with the membrane including the porphyrin-containing cytochrome system, were not produced locally. In *B. subtilis* there was a difference in frequency between membrane-associated and cellular DNA-transformable markers. The proportion of origin and terminal markers was higher in the membrane fraction than in cellular DNA [37]. This finding is in agreement with the fact that chromosomal replication begins in the membrane-associated DNA [38].

Streptomycin exerts its action first on the bacterial membrane where it causes an increase in the efflux of K ions and a transient stimulation of net RNA synthesis [39]. In the light of the above observation it is tempting to speculate on the possible mutagenic action of streptomycin. It seems conceivable that streptomycin, a cationic agent, may react with the strongly anionic DNA attached to the membrane. This reaction may result in failures in the replication of the corresponding segment of chromosome.

The mutants described in this report are being subjected to genetic investigation.

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