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# FACTORS AFFECTING GROWTH OF *STAPHYLOCOCCUS AUREUS* L FORMS ON SEMIDEFINED MEDIUM

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## ABSTRACT

BANVILLE, ROBERT R. (The Catholic University of America, Washington, D.C.). Factors affecting growth of *Staphylococcus aureus* L forms on semidefined medium. J. Bacteriol. **87**:1192-1197. 1964.—A semidefined agar medium was found suitable for production and cultivation of the L form of *Staphylococcus aureus*. In semidefined liquid medium, growth of the L form took place in the form of a sediment containing large masses of cells, but heavy and diffuse growth occurred in the same medium with 0.05% agar. The optimal pH for L-colony formation on solid medium was 6.5. More L colonies developed on 0.75% agar than at higher agar concentrations. L colonies developed in greater numbers on pour plates than on streak plates, and in some cases more L colonies appeared under anaerobic incubation. L-colony formation appeared to be inhibited by sodium citrate. The vitamin requirements of the L forms studied were similar to those of the classical form.

The L forms of most species of bacteria do not grow well on ordinary culture media, and for many years they were customarily cultivated on media containing serum or other body fluids. Medill and O'Kane (1954) showed that a chemically defined amino acid medium, similar to that which supported optimal growth of the classical form, supported more abundant growth of *Proteus* L forms than did the complex media previously used. They suggested that the usual peptone, meat extract, or meat infusion media contained substances toxic to the L form, and that serum in complex media neutralized these substances. Lederberg and St. Clair (1958) reported the use of a synthetic medium for the growth of L forms of *Escherichia coli*. However, most studies on the L forms of other species, especially gram-positive species, have been conducted with complex media.

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Staphylococcal L forms were first observed, but not successfully subcultured, by Dienes and Sharp (1956). Later, Schonfeld (1959), Prozorovskii (1959), Marston (1961*a*), and Kagan, Molander, and Weinberger (1962) produced staphylococcal L forms which grew when transplanted to fresh medium. These investigators used complex media containing horse serum or ascitic fluid together with penicillin or other antibiotics. Mattman, Tunstall, and Rossmoore (1961) reported that staphylococcal L colonies developed in the absence of penicillin or any other inducing agent on a synthetic medium to which no vitamins had been added. Since this medium was suitable for the development of the classical form only when several B vitamins were added, it appeared that the L form had lower vitamin requirements than the classical form. There has been no information published, however, as to whether this is also true of L forms induced by penicillin. The present work was designed to provide a better understanding of the effects of the constituents of the medium and other environmental factors on the growth of penicillin-induced staphylococcal L forms. Particular emphasis was placed on the development of a relatively simple medium well suited to growth of such L forms, as it was felt that a medium of this type would be useful for future biochemical and serological studies.

## MATERIALS AND METHODS

*Strains.* All strains used were isolated in the laboratories of Providence Hospital, Washington, D.C., within the last 2 years, and were identified as *Staphylococcus aureus*. They were maintained on nutrient agar slants. Although only strains which appeared penicillin-sensitive by the antibiotic disc method were selected for use in these studies, preliminary experiments using the medium of Marston (1961*a*) showed that some of these strains gave rise to classical colonies on medium containing 1,000 units of penicillin per

ml. Only strains which showed no tendency to grow in the classical form at this penicillin concentration were used in the experiments described below. Most of the work was done with two strains, 696 and 467-B, which had been shown in preliminary experiments to give rise frequently to L colonies, but several other strains were also used to test the generality of the findings.

*Media.* The components of the semidefined solid medium developed in the course of this work are given in Table 1. The medium is chemically defined except for the presence of Vitamin Free Casamino Acids as an amino acid source. For growth in fluid medium, The same components were used without agar. The pH of both solid and liquid media was adjusted to 6.5 with HCl. Penicillin and vitamin solutions were prepared separately, sterilized by filtration, and added to the medium after autoclaving. In plates which were to be incubated anaerobically, the medium was prepared with 1 mg of uracil per ml, since staphylococci have been reported to require uracil for anaerobic growth (Richardson, 1936).

*Inocula.* Tryptose Phosphate Broth cultures of each strain were incubated for 18 hr at 37 C. The cells were then packed by centrifugation and washed three times with 0.85% saline. The washed-cell suspension was diluted with saline to 80% transmittance at 530 mμ in a Bausch & Lomb Spectronic-20 colorimeter. A 0.1-ml portion of this suspension was placed on the surface of each agar plate and spread over the surface of the medium with a glass rod.

TABLE 1. *Composition of basic semidefined medium*

| Component | Amt |
|---|---|
| Glucose | 1.0 g |
| NaCl | 5.0 g |
| Sodium lactate | 2.0 g |
| Noble agar | 750 mg |
| Vitamin Free Casamino Acids (Difco) | 500 mg |
| L-Cystine | 10 mg |
| L-Tryptophan | 10 mg |
| $K_2HPO_4$ | 500 mg |
| $MgSO_4 \cdot 7H_2O$ | 40 mg |
| $FeSO_4 \cdot 4H_2O$ | 2 mg |
| $MnCl_2 \cdot 4H_2O$ | 5 mg |
| Biotin | 0.1 μg |
| Nicotinic acid | 500 μg |
| Thiamine hydrochloride | 100 μg |
| Penicillin | 100,000 units |
| Distilled water | 100 ml |

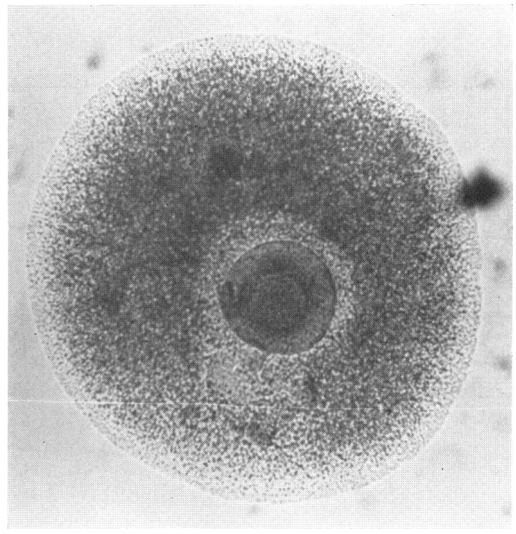

FIG. 1. *Typical Staphylococcus aureus L colony on semidefined medium.* × *100.*

*Incubation.* All plates were incubated at 37 C. Plates incubated aerobically were sealed with tape or paraffin to prevent loss of moisture. Anaerobic incubation was performed in a Brewer anaerobic jar.

## RESULTS AND DISCUSSION

A medium containing the three vitamins known to be required by most strains of *S. aureus* for optimal growth, niacin, thiamine, and biotin (Knight, 1937; Gretler et al., 1955), was found to be satisfactory for production of the L form. Numerous L colonies of typical appearance (Fig. 1) developed from all strains inoculated on this medium (Table 1), although there were considerable variations between different strains in the numbers of L colonies produced. L colonies were formed more abundantly by all strains on this medium than on the Brain Heart Infusion Agar medium recommended by Marston (1961*a*). The production of L colonies was found to depend on several factors as discussed below.

*Effect of vitamin content of medium.* Because the work of Mattman et al. (1961) indicated that staphylococcal L forms might not require a

TABLE 2. *Effect of agar concentration on number of L colonies developing from standard inoculum**

| Agar concn | L colonies per plate | |
|---|---|---|
| | Strain 467-B | Strain 696 |
| % | | |
| 0.75 | 55 | 97 |
| 1.0 | 3 | 8 |
| 1.25 | 0 | 1 |
| 1.5 | 0 | 0 |

* Standard inoculum: 0.1 ml of 1:10 dilution of washed-cell suspension standardized to 80% transmittance. Each figure represents the average of three trials.

medium supplemented with B vitamins, tests were performed in which the medium in Table 1 was prepared with penicillin but without the addition of vitamins. Control plates were prepared containing medium with niacin and thiamine, and with niacin, thiamine, and biotin. On the medium without vitamins, cells of strain 696 gave rise to very minute, irregular colonies embedded in the agar; in the presence of niacin and thiamine, conventional L colonies were produced but these had scant surface growth and did not grow well on subculture; only on medium containing all three vitamins were well-developed L colonies formed which gave rise to numerous new colonies on subculture. Cells of strain 467-B on the vitamin-free medium gave rise to colonies which were embedded in the agar, without surface growth; these did not grow on subculture. On the medium with niacin and thiamine, typical L colonies developed, but the zones of surface growth were much wider on medium with all three vitamins; colonies grew on subculture on both these media. The numbers of L colonies did not appear to vary significantly among the different media with either strain.

It appears, then, that L forms isolated by the action of penicillin have nutritional requirements similar to those of the parent form, requiring at least niacin and thiamine, and sometimes biotin, for satisfactory growth. The limited growth which did occur in the absence of added vitamins may have been due to vitamins released by the breaking down of other cells in the inoculum, since only a small fraction of the cells inoculated formed L colonies. It appears that, if L colonies are also formed on vitamin-free medium, such L forms probably are significantly different physiologically from those produced by the action of penicillin.

Tests were also made in which the medium in Table 1 was supplemented with 100 $\mu$g of calcium D-pantothenate or pyridoxine hydrochloride per 100 ml of medium, since these vitamins were reported by Gretler et al. (1955) to stimulate the growth of some strains of staphylococci. No significant increases could be demonstrated in the numbers of L colonies formed on media containing these vitamins, but in some cases the colonies on medium containing panthothenate were larger and appeared to give better growth on subculture.

L colonies of one strain studied, even on medium with all vitamins, grew on subculture only in the areas around the transplanted agar blocks, suggesting that some substance which diffused outward from the block or the colony growing beneath it stimulated the growth of other L colonies. A similar effect was reported in L forms of other genera (Bandur and Dienes, 1963).

*Effect of agar concentration.* L-colony production was greatly enhanced on soft agar medium (Table 2). All strains studied gave rise to more L colonies on the lowest concentration of agar used, 0.75%, than on plates of any higher concentration tested. This may be due to greater ease of penetration of the bacterial cell into the agar, since growth of the colony begins beneath the surface. There is some evidence that the optimal agar concentration for L-colony production from some bacteria is lower with spread plates than with pour plates, in which penetration would not take place. For example, Lorkiewicz, Marciniak, and Zelozna (1956) reported that the best surface growth of *Proteus* L forms occurred at the lowest agar concentrations tested, 1.0 to 1.5%, but that L colonies were produced most abundantly in pour plates at 2.0%.

*Effect of pH.* When the standard medium was tested at pH values ranging from 4.5 to 8.0, the maximal numbers of L colonies developed at about pH 6.5 (Table 3). Very few colonies appeared on media with a pH above 7.5 or below 5.5. This low optimal pH value is in contrast to that of the classical form of *S. aureus*, which usually grows best at a slightly alkaline pH. Lederberg and St. Clair (1958) reported a similar optimal pH value, 6.3, for production of the L form of *E. coli*. However, Landman, Altenbern, and Ginoza (1958) reported that pH had little

effect on the numbers of *Proteus* and *Escherichia* L colonies formed in pour plates. These differing results may be due to different media; Mattman et al. (1960) reported that L-colony formation in the genus *Mycobacterium* occurred over a wider pH range when sucrose was used to maintain tonicity, rather than NaCl, which was used in this work.

*Effect of anaerobiosis.* When conditions were such that L colonies were produced abundantly on the surface of aerobic plates, similar results were usually obtained by incubation in an anaerobic jar. In some cases, however, when no L colonies, or only a few, were produced on aerobic plates, anaerobic incubation of similar plates resulted in the development of numerous L colonies. This effect may have been due to factors other than the absence of oxygen. Plates incubated in the anaerobic jar tended to be excessively moist; this moisture may have provided a better environment for the growth of the L form. Also, carbon dioxide produced in the reaction may have been absorbed by the medium, lowering the pH. Landman et al. (1958) reported that the yield of *Proteus* L colonies also was improved on certain media by anaerobic incubation, whereas on other media L-colony production was equally good under aerobic or anaerobic conditions.

*Effect of citrate.* Some media used for cultivation of staphylococci, such as that of Gretler et al. (1955), contain citrate rather than lactate. Such a medium was used for L-colony production in certain experiments, but it appeared that the citrate inhibited the formation of L colonies. When samples of the basic medium were prepared with 0.5% sodium citrate and with 0.5% sodium lactate, most strains produced significantly more L colonies on the lactate medium than on the citrate medium. In many cases, there were over 100 L colonies on plates containing sodium lactate but few or none on plates with sodium citrate. One strain, however, appeared to give rise to comparable numbers of L colonies on both media.

The inhibitory effect of citrate might be due either to a direct toxic effect or to a shortage of metallic ions available to the cell, resulting from complexes of these ions with the citrate. The physical state of the medium may have been involved also, since the citrate medium was perfectly clear, whereas the medium without citrate contained a fine precipitate. Since many of the media which have been developed for the cultivation of L forms of *Proteus* and other gram-negative bacteria contain sodium citrate, it would seem that the effect of citrate on the L forms of these organisms should be examined.

TABLE 3. *Effect of pH on number of L colonies developing from standard inoculum of staphylococci*

| pH | L colonics per plate | |
|---|---|---|
| | Strain 467-B | Strain 696 |
| 5.5 | 2 | 8 |
| 6.0 | 18 | 29 |
| 6.5 | 42 | 77 |
| 7.0 | 3 | 4 |
| 7.5 | 0 | 0 |

* Standard inoculum: 0.1 ml of 1:10 dilution of washed-cell suspension standardized to 80% transmittance. Each figure represents the average of three trials.

*Effect of pour plates.* Although most of the experiments in the present work were done with spread plates, on a few occasions pour plates were prepared by adding the inoculum to a tube of agar medium before pouring. In these cases, numerous small, round, nonpigmented colonies developed within the agar medium. At least two or three times as many colonies usually developed from the same inoculum on pour plates as on spread plates of the same medium.

*Growth in liquid and semisolid media.* Physiological and serological studies can be performed most conveniently when the bacteria studied can be grown in liquid medium, preferably evenly distributed. Therefore, a study was made of the ability of staphylococcal L forms to grow in a liquid medium having the same components as the solid medium in Table 1, except for agar. Freshly isolated strains of L forms reverted to cocci during the prolonged incubation periods required in liquid media. To avoid the addition of penicillin to the culture at intervals, three strains were used which had been stabilized in the L form by ten or more passages on medium with penicillin. These showed no tendency to revert. Al, strains behaved in approximately the same wayl although some produced heavier growth than others.

The first growth after inoculation of agar blocks containing L colonies appeared in 2 or 3 days in the form of a sediment composed of viscid,

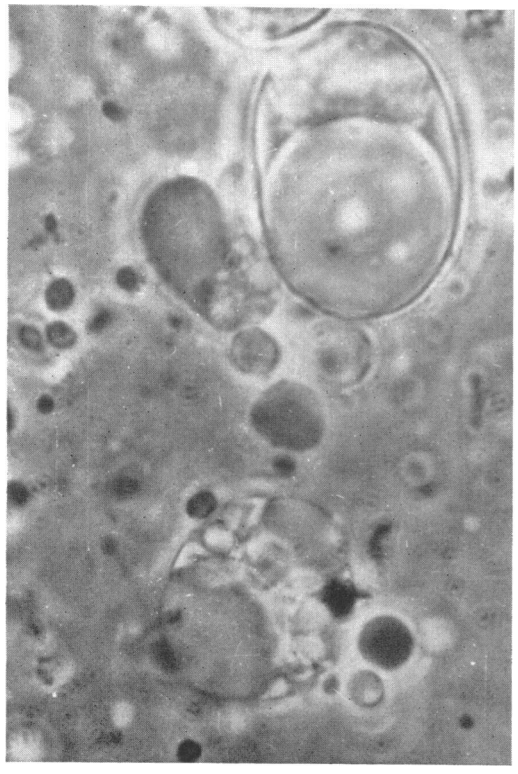

FIG. 2. *L forms from 0.05% agar medium. Phase contrast;* × *1,000.*

granular masses which tended to form long filaments extending out from the agar blocks. This was similar to the type of growth in the complex liquid medium described by Marston (1961*b*). After a period of 8 days to 2 weeks, a slight turbidity was noticeable throughout the flask. Microscopy showed the presence of large, pleomorphic cells throughout the flask. It is not certain whether these cells were able to multiply, or had broken away from the masses of L bodies in the sediment. The fact that growth could be established in fresh flasks only by transfer of the filamentous material suggests that the latter was the case.

When the same medium was prepared with as little as 0.05% Noble agar, inoculation of L colonies resulted in dense growth throughout the flask within a few days. Microscopy showed many pleomorphic bodies of various sizes, both singly and clustered within envelopes (Fig. 2). A medium of this sort might be of use in cases where it was desired to estimate the amount of growth by the turbidity of the medium.

When material from liquid or semiliquid cultures was inoculated on 0.75% agar plates, typical L colonies developed. Similar material inoculated on 1.5% agar plates gave rise to colonies which grew entirely or almost entirely on the surface of the agar with little or no embedded central area, although the whole colony adhered closely to the surface. One of the original strains, which tended to grow more readily than other strains in liquid medium, was observed to give rise to both types of L colony on 0.75% agar, and mainly to centerless colonies on 1.5% agar. It seems likely that strains tending to produce colonies with no embedded growth have a lesser requirement for the support of agar, and thus grow more easily in liquid medium. Altenbern and Landman (1960) reported similar types of L colonies from *P. mirabilis*. They suggested that the centerless variety was a mutant form having a greater resistance to the septum formation inhibiting effect of penicillin. Therefore, this form would have a greater ability to reproduce without the support of agar, the fibers of which they believed helped to cut off portions of the soft, protoplasmic growth of the L form, thus serving as a substitute for the normal division process. On soft agar plates, they found both types of colony could develop, but on hard agar medium cells could not easily penetrate to form embedded colonies, and only the centerless type appeared in large numbers. A similar phenomenon was reported by Bandur and Dienes (1963) to take place in *Serratia* L forms.

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