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# Biochemical Markers of the Penicillin-Induced L Phase of a Group B, Type III *Streptococcus* sp.

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The penicillin-induced L phase of growth of the group B, type III streptococcal strain 76-043 was examined for biochemical properties used for the identification of group B streptococci. After numerous serial subcultures in the cell wall-defective state, this stable L phase continued to produce hemolysin, hippuricase, and CAMP factor in addition to the group- and type-specific antigens. Hemolysin production by the L-phase cells was observed on solid and in liquid media containing sheep erythrocytes. Washed whole L-phase cells hydrolyzed hippuric acid. CAMP factor was detected by the characteristic hemolysis produced on blood agar by L-phase cells or filtered culture supernatants. CAMP factor activity was quantitated by an agar well diffusion system and a macrotube assay with partially purified preparations of CAMP factor and staphylococcal beta-hemolysin. Hyperimmune cow serum neutralized the CAMP activity of the L phase and parent bacterial phase to the same degree, suggesting identity of the CAMP factors. Production of hemolysin, hippuricase, and CAMP factor confirmed the bacterial origin of this L phase. Assay for these biological markers could be used to identify L-phase organisms derived from group B streptococci.

In the L phase, i.e., the cell wall-defective replicating state of growth (18), streptococci may or may not retain all the markers of the parent bacterial phase. A summary of some of the properties retained or lost by group A streptococci after conversion to the L phase was made by Maxted (19) and updated by Hryniewicz (12). Among those markers kept by the cell wall-defective state of this streptococcal group are production of M protein (10, 21), DNase (10), hemolysin (5, 10), and bacteriocin (13). Of particular importance among the markers lost by the L phase of group A streptococci (10, 21) and also of group D streptococci (11, 14) is the group-specific antigen. The group antigen could not be detected in cell extracts as well as in the culture media from L-phase organisms of these two streptococcal groups.

Recently we have reported the penicillin-induced conversion of group B streptococci to the L phase of growth. The L phase of the type III strain 76-043, isolated originally from cerebrospinal fluid, was studied in more detail and was found to produce both the group- and the type-specific polysaccharide antigens even after numerous subcultures in the cell wall-defective state (9). To characterize more extensively the L phase of this streptococcal strain, additional studies have been undertaken.

Data are presented here on the examination of the stable L phase of strain 76-043 for biochemical markers characteristic of group B streptococci and useful in the identification of this streptococcal group. Methods for the detection of these markers are described in detail.

## MATERIALS AND METHODS

**Bacterial strains.** The penicillin-induced L phase of the group B, type III streptococcal strain 76-043 was maintained by continuous serial subculture or was kept frozen at −70°C (9). Samples of the parent bacterial phase of strain 76-043 were kept stored at −20°C. Cultures of the bacterial phase of the group B type Ib strain 74-608, type III strain 74-360, group A wild-type strain 81-388, and *Staphylococcus aureus* 71-889 were kept frozen similarly. Since this staphylococcal strain produces a good quantity of sphingomyelinase C (beta-hemolysin), it had been used previously in our laboratory to study the group B streptococcal Christie-Atkins-Munch-Peterson (CAMP) factor (3).

**Growth media.** The basic L-phase medium consisted of Todd-Hewitt broth supplemented with 0.7 M sucrose and 0.5% PPLO serum fraction. For some experiments, penicillin G was included to a final concentration of 1,000 U/ml. To prepare solid medium, 1.2% Noble agar was added. Bacterial-phase media were Todd-Hewitt broth or L-phase medium without penicillin. Except for the sucrose (Spectrum Chemical Manufacturing Corp., Redondo Beach, Calif.) and penicillin (Sigma Chemical Co., St. Louis, Mo.), all medium components were from Difco Laboratories, Detroit, Mich.

**Subculture.** The L phase was subcultured as described previously (9). Subculture was also made on repeated occasions onto plain sheep blood agar to ensure that there were no revertants. This L phase has been subcultured serially 255 times in the presence or absence of penicillin without spontaneous reversion to the bacterial phase of growth.

**Sera.** The serum (31 May 1972) of cow 144 was used since it contained a high titer of neutralizing antibody to CAMP factor which developed after experimental group B streptococcal mastitis (3). This hyperimmune cow serum prepared in our laboratory and normal rabbit serum were kept at −20°C.

**Hemolysin.** Solid and liquid L-phase media containing 6% sheep blood were used to test for hemolysin production. Pour plates were made with the L-phase cells with liquified solid medium at 48°C. Once solidified, the plates were incubated at 35°C and were examined daily under a microscope at ×24 for the appearance of hemolysis. Liquid cultures were examined visually for hemolysis. The bacterial-phase cultures were tested similarly in L-phase liquid and solid media without penicillin. The filtered supernatant of liquid cultures without added blood was tested for hemolytic activity by spotting on blood agar plates. L-phase cells were also tested for extractable cell-bound hemolysin by the method of Ferrieri (6). The cells were grown in the usual L-phase medium, harvested in the early stationary growth phase, washed with 0.1 M $MgCl_2$ and then extracted with a phosphate-buffered saline (PBS)–glucose–Tween 40–$MgCl_2$ extraction mixture. The test supernatant was filtered and assayed subsequently for extractable hemolytic activity. The last subculture tested for hemolysis was the 183rd serial passage.

**Hippuricase.** The method of Ferrieri et al. (8) was followed to test for hippuricase, the enzyme which hydrolyzes hippuric acid to benzoic acid and glycine. Bacterial-phase and L-phase media were inoculated with bacterial-phase and L-phase cells, respectively. Bacterial-phase cells were harvested at 20 h, the 212th serial subculture of the L phase at 27 h. The cells were washed with normal saline and suspended in 0.01 M Tris (Fisher Scientific Co., Fair Lawn, N.J.; pH 7.1) buffer containing 0.003 M each of $MgSO_4$ (Spectrum) and $CaCl_2$ (J. T. Baker Chemical Co., Phillisburg, N.J.). Sodium hippurate (Difco) was added, and the tubes were incubated at 35°C for 24 h. After the addition of $FeCl_3$ (J. T. Baker), formation of the heavy brown insoluble ferric benzoate precipitate was read as a positive reaction. A negative reaction was characterized by a fine orange precipitate. Two group B wild-type strains were used as positive controls, and a group A wild-type strain was the negative control.

**CAMP factor.** The 219th serial subculture of the L phase was examined for production of the diffusible CAMP factor (4) on solid and in liquid media.

To test for CAMP factor production on solid medium, modified sheep blood agar plates were prepared with Todd-Hewitt broth, 1.2% agar, 3.0% NaCl, 0.5% serum fraction, and 5% thrice-washed sheep erythrocytes. The beta-hemolysin-producing *S. aureus* 71-889 was streaked across the diameter of the plate. The L phase was streaked starting at 5 mm from the staphylococcal culture and at a 40° angle from it. The plate was incubated at 35°C overnight. The point where the two cultures were the closest was examined subsequently for the flame-shaped area of glassy clear hemolysis characteristic of the CAMP reaction. The parent bacterial culture and the control group A and group B cultures were tested similarly.

The L-phase and bacterial-phase cultures were grown in L-phase liquid medium without penicillin to study CAMP factor production by broth cultures. After 27 h (19 h for bacterial-phase cultures) of incubation at 35°C, the L-phase culture was centrifuged, and the supernatant was filtered through an 0.22-μm filter. The supernatant was tested directly or the CAMP factor was precipitated by the method of Brown et al. (3). The pH was adjusted to 7.4 and $(NH_4)SO_4$ was added to 50% saturation. The precipitate was suspended in 1/10th of the original volume of 0.9% NaCl. It was then dialyzed against multiple changes of 0.01 M PBS (pH 7.4). The material was concentrated further with Carbowax (polyethylene glycol compound 20-M; Union Carbide Corporation, New York, N.Y.) and then was dialyzed briefly.

The staphylococcal beta-hemolysin was prepared as described by Wilkinson (23). After growth of the *S. aureus* culture in Todd-Hewitt broth (pH 7.4) for 24 h, the supernatant was filtered, and the beta-hemolysin was precipitated with 2 volumes of acetone at −20°C. The precipitate was dissolved in 0.01 M PBS with 0.001 M $MgCl_2$ (pH 7.4) and was dialyzed overnight against the same buffer.

An agar well diffusion test was used to estimate CAMP factor activity. Sheep blood agar plates were prepared with tryptose blood agar base and 5% thrice-washed sheep erythrocytes. A row of wells 6 mm in diameter was punched into the agar. At 10 mm from them, a parallel row of wells was made. To find the best concentration of beta-hemolysin for this test, twofold dilutions of crude or partially purified staphylococcal beta-hemolysin were prepared. These were placed in one row of wells and allowed to diffuse overnight at room temperature against the crude L-phase or bacterial-phase filtered culture supernatants in the parallel row. The dilution of beta-hemolysin which produced a darkened halo 26 mm in diameter in the agar and with which both the bacterial-phase and the L-phase supernatants produced a positive CAMP reaction was used subsequently to titrate the CAMP factor of the L phase and its parent bacterial phase. Serial twofold dilutions were made of the streptococcal supernatants or of the partially purified CAMP factor preparations. These were placed in one row of wells and allowed to diffuse as described previously against the chosen dilution of the staphylococcal beta-hemolysin. The endpoint was considered to be the highest dilution of the streptococcal CAMP factor producing beta-hemolysis characteristic of the CAMP reaction.

A macrotube assay was used to quantitate CAMP factor activity. To do this, the hemolytic activity of the partially purified staphylococcal beta-hemolysin was determined as described by Bernheimer et al. (1) with slight modifications. The buffer used was 0.01 M PBS with 0.001 M $MgCl_2$ (pH 7.4). The suspension of washed sheep erythrocytes was adjusted to approximately 0.8% by using both 100 and 50% hemolysis controls to give absorbance readings at 540 nm of 0.80 and 0.40 (±0.015), respectively. One hemolytic unit (HU) was the amount of beta-hemolysin which produced 50% lysis of erythrocytes in the test system.

The activities of the partially purified CAMP factors from the L phase and parent bacterial phase were assayed by the method of Bernheimer et al. (2). However, the 0.8% sheep erythrocyte suspension was prepared as described above, and serial twofold dilutions of the streptococcal preparations were made in 0.01 M PBS. To 0.5 ml of CAMP factor dilution was added an equal volume of a dilution of staphylococcal beta-hemolysin containing approximately 10 HU/ml. One milliliter of the sheep erythrocyte suspension was added, and the test mixture was incubated in a 30°C water bath for 30 min. The tubes were centrifuged at 960 × *g* for 10 min, and the amount of hemoglobin released was read at 540 nm. From these readings, the number of HU per milliliter was calculated. One HU was considered to be the amount of CAMP factor producing 50% hemolysis in the described test system.

Neutralization of CAMP activity was investigated by a modification of the macrotube assay. Dilutions of hyperimmune cow serum or of normal rabbit serum were preincubated for 15 min at 37°C with bacterial-phase or L-phase partially purified CAMP factor preparations of comparable activity. To these serum-CAMP factor mixtures, beta-hemolysin, as well as sheep erythrocytes, was added, and the test was continued as described above. The percentage of HU neutralized was determined by comparing the CAMP activity remaining in systems preincubated with the various dilutions of hyperimmune or control serum to the units of activity in test systems without serum. The beta-hemolysin and CAMP factor dilutions used were assayed just before setting up the neutralization test to verify their hemolytic activity.

**DNase.** The sixth serial subculture of the L phase and parent bacterial phase of growth of strain 76-043, as well as the bacterial cultures of strains 74-369 and 74-608, were tested for DNase production by the microtitration method of Nelson et al. (20) and the well diffusion technique of Ferrieri et al. (7).

**Group- and type-specific antigens.** Concentrated filtered culture supernatants of the bacterial phase and L phase were tested as described previously (9) by double diffusion and counterimmunoelectrophoresis for the presence of the group B and type III antigens. The most recent L-phase subculture tested for the presence of the group B and the type III antigens was the 254th serial passage.

## RESULTS

**Hemolysin.** Since studies have been carried out in our laboratory on the hemolysin of group B streptococci, this was one of the markers investigated in the L-phase culture. It was found that the L phase produced hemolysin in solid and in liquid media. The beta-hemolysis exhibited by the L phase in blood agar compared well with that of the parent bacterial phase (Fig. 1). The area of clearing extended well beyond the colony and was evident as soon as growth was visible. Hemolysis in liquid medium containing blood was detected easily when the culture was centrifuged. The presence of the osmotic stabilizer or of penicillin and the concentration of serum fraction in the medium did not affect hemolysin production by the L phase. However, hemolytic activity could not be detected in filtered culture supernatants, nor could it be extracted from the cells.

**Hippuricase.** The L phase exhibited hippuricase activity. Production of this enzyme by the L phase was not inhibited by the presence of penicillin or serum fraction or both in the medium (Table 1). The reactions of the bacterial phase and L phase of strain 76-043 were comparable. As would be expected, hippuricase was produced by the bacterial phase of the group B strains 76-360 and 74-608 but was not produced by the group A strain 81-388.

**CAMP factor.** The L phase was found to produce CAMP factor, the protein which acts synergistically with the sphingomyelinase C (also known as beta-hemolysin) from *S. aureus* to lyse sheep or ox erythrocytes. Even though the growth of the L phase was scant on sheep blood agar plates containing NaCl, the classical flame-shaped area of beta-hemolysis was observed at an angle between the staphylococcal and L-phase cultures after overnight incubation.

Filtered culture supernatants of the bacterial phase and L phase of strain 76-043 were found to contain CAMP factor. These CAMP factors and the staphylococcal beta-hemolysis were purified partially and then were tested in a well diffusion system in sheep blood agar plates (Fig. 2). After overnight incubation at room temperature, areas of beta-hemolysis were observed where the CAMP factor from the bacterial phase or that of the L phase interacted with the staphylococcal beta-hemolysin. Comparable areas of hemolysis were produced by the 1:64 dilution of the bacterial phase preparation and the 1:32 dilution of that of the L phase. No such areas were seen when a preparation from a CAMP factor-negative group B strain (74-360) was tested either undiluted or at a 1:2 dilution. By the macrotube assay method, it was determined that the partially purified CAMP factor preparations from the bacterial phase and L phase of strain 76-043 contained 48 and 25 HU/ml, respectively, and

TABLE 1. Examination of the bacterial phase and L phase of the group B, type III streptococcal strain 76-043 for hippuricase activity

| Medium composition | | Hippuricase activity | |
|---|---|---|---|
| Penicillin (1,000 U/ml) | PPLO serum fraction (0.5%) | Bacterial phase | L phase |
| + | + | NT[a] | + |
| + | − | NT | + |
| − | + | NT | + |
| − | − | + | + |

[a] NT, Not tested.

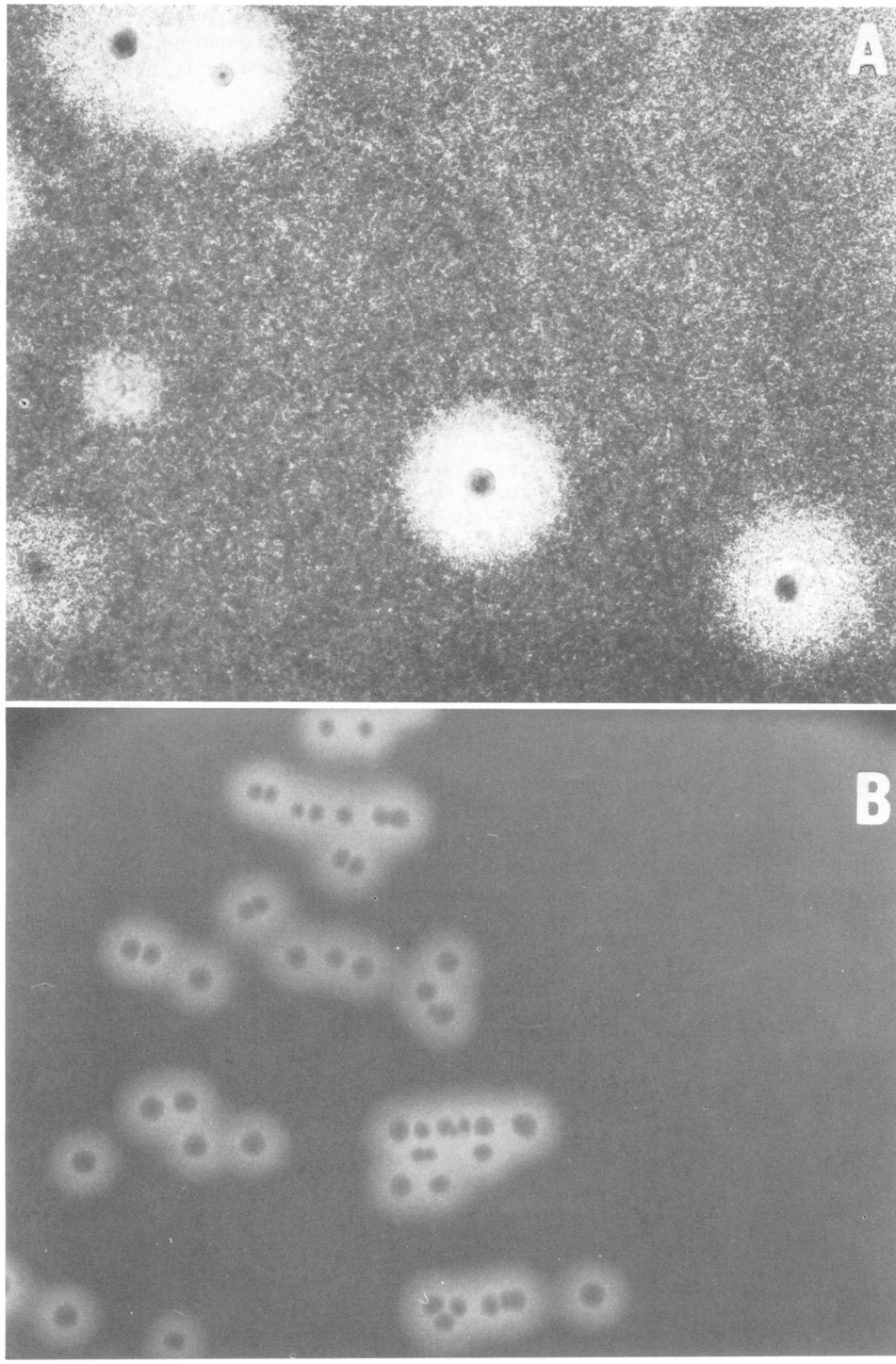


FIG. 1. Hemolysis produced by colonies of the L phase and parent bacterial phase of the group B, type III streptococcal strain 76-043 in sheep blood agar plates containing 0.7 M sucrose and 0.5% serum fraction. (A) L-phase colonies after 48 h of incubation at 35°C had dense centers and lacy peripheries and were surrounded by large halos of clear hemolysis. Magnification, ×29. (B) Bacterial-phase colonies and their zones of hemolysis after overnight incubation. Magnification, ×3.

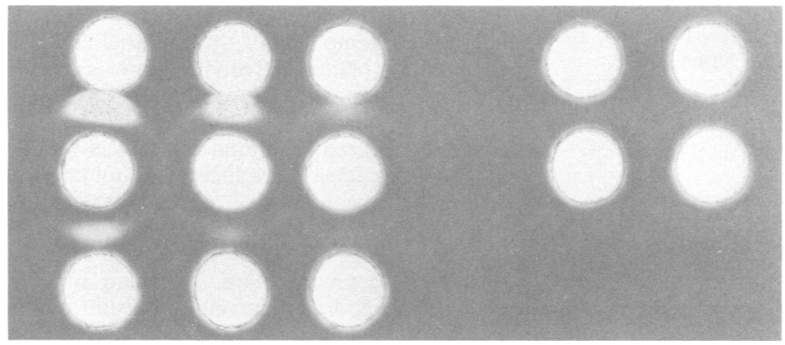

FIG. 2. Titration by a well diffusion system in sheep blood agar plates of the CAMP reaction produced by partially purified preparations of group B streptococcal CAMP factor and staphylococcal beta-hemolysin. The darkened halos produced by the beta-hemolysin can be observed in the background. Left: Serial twofold dilutions (1:16, 1:32, and 1:64) of the preparations from the parent bacterial phase (top row) and of the L phase (bottom row) were allowed to diffuse overnight at room temperature against a 1:8 dilution of the beta-hemolysin. Right: Undiluted and 1:2 diluted preparations from the CAMP factor-negative group B strain 74-360 in the top wells were tested against a 1:8 dilution of the beta-hemolysin in the bottom wells.

that the preparation of the bacterial strain 74-360 had less than 1 HU/ml (data not shown). Therefore, it was estimated that at comparable stages in the growth cycle, the L-phase supernatant contained approximately half as much CAMP factor as did the supernatant from the parent bacterial phase.

To compare the CAMP factors of the bacterial phase and L phase immunologically, neutralization experiments were carried out by a modification of the test tube method. The percentages of hemolytic activity remaining after preincubation of the bacterial-phase and L-phase partially purified CAMP factors with various dilutions of hyperimmune cow serum were almost identical (Fig. 3). Thus, the CAMP activity of the bacterial phase and L phase appeared to be neutralized to approximately the same degree by hyperimmune serum. Normal rabbit serum did not have any neutralizing effect on either system.

**DNase.** Tests of the L phase of strain 76-043 for DNase production gave negative results (Table 2). In addition, the parent bacterial phase was found to be a poor DNase producer.

**Group- and type-specific antigens.** The L phase continued to produce the group- and type-specific polysaccharide antigens (Table 2).

## DISCUSSION

These studies reveal that the stable L phase of the group B, type III strain 76-043 continued to produce biochemical markers characteristic of group B streptococci and useful in the identification of this streptococcal group. Despite numerous subcultures in the cell wall-defective state, the L phase continued to produce not only the group- and type-specific antigens (9) but also hemolysin, hippuricase, and CAMP factor. These findings imply that the genetic information, as well as the biosynthetic pathways and components for the production of these markers, were conserved and were operative in our serially subcultured L phase.

The incorporation of the osmotic stabilizer, serum fraction, or penicillin into the growth medium did not influence the sensitivity or specificity of the modified classical methods used in studying the biochemical markers. The L

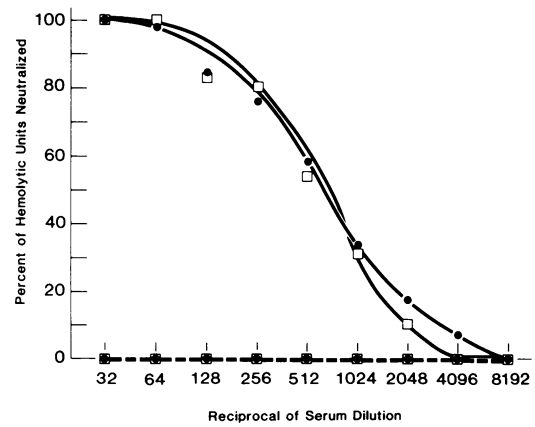


FIG. 3. Neutralization of the partially purified CAMP factors from the bacterial phase (●) and L phase (□) of the group B, type III streptococcal strain 76-043 by hyperimmune cow serum. Dilutions of the cow serum (——) or normal rabbit serum (------) were preincubated with a 1:32 dilution of the bacterial-phase CAMP factor or a 1:16 dilution of the L-phase CAMP factor before a macrotube assay of the remaining CAMP activity was performed.

TABLE 2. Biological markers of the bacterial phase and L phase of the group B, type III streptococcal strain 76-043

| Marker | Production by: | |
|---|---|---|
| | Bacterial phase | L phase |
| DNase | ± | − |
| Hemolysin | + | + |
| Hippuricase | + | + |
| CAMP factor | + | + |
| Group-specific antigen | + | + |
| Type-specific antigen | + | + |

phase produced hemolysin and CAMP factor in the presence of serum fraction concentrations ranging from 0 to 1%. Only in the case of CAMP factor production by cultures growing on solid medium was it necessary to substitute NaCl for sucrose. Although the growth of the L phase was not luxuriant on the medium osmotically stabilized with NaCl, good production of CAMP factor was obtained.

The reactions produced by the L phase in the test systems were very comparable to those of the parent bacterial phase under the same conditions. The hippuricase test reactions produced by the L phase were identical to those of the parent bacterial phase. Likewise, the partially purified CAMP factors of the L phase and its parent were neutralized to approximately the same degree by hyperimmune serum, and the beta-hemolysis produced by the L phase in sheep blood agar plates was like that of the parent. Similar observations have been made by Freimer et al. (10) on L forms of group A streptococci. These investigators found that the hemolysis produced was very much like that of the parent and that the DNase of the L forms reacted like that of the group A streptococcal parent with specific antisera.

The activities of the hemolysin and CAMP factor produced by the group B streptococcal L phase were easily distinguishable from each other. CAMP factor activity was expressed only in the presence of the staphylococcal beta-hemolysin. It was exhibited by L-phase colonies and could be detected easily in filtered culture supernatants also. In contrast, the activity of the L-phase hemolysin could be detected in whole cultures only. This last observation is in keeping with the studies of Ferrieri (6) as well as with those of Marchlewicz and Duncan (16). These investigators were unable to detect hemolysin in the culture supernatants of group B streptococcal bacterial-phase cultures. Since the hemolysin could not be extracted from the L-phase cells, it was not possible to characterize it further and compare it with the studies by Ferrieri (6) and by Marchlewicz and Duncan (17) on the hemolysin of the bacterial-phase culture.

Nuclease production was not exhibited by the L phase of strain 76-043. Even with the sensitive methods described by Ferrieri et al. (7), the parent bacterial strain was found to be a poor nuclease producer. It is not known whether poor production of this marker by the parent may have been advantageous for conversion to the L phase and for luxuriant growth in the cell wall-defective state. Landman and Spiegelman (15) reported that high concentrations of nucleases were detrimental to the survival of protoplasts of *Bacillus megaterium*. However, L forms of *S. aureus* have been found to produce DNase (22), and so have protoplasts of group A streptococci (10).

The L phase of strain 76-043, however, did continue to produce the group- and type-specific polysaccharide antigens, even after numerous serial subcultures in the cell wall-defective state. The continued production of the group antigen makes this L phase different from those of group A streptococci (21) and group D streptococci (11, 14), which have been found not to produce the group-specific antigen in the cell wall-defective state. However, like the L phase of group A streptococci which produces the type-specific M protein (10, 21), the L phase of the group B strain 76-043 did produce the type-specific polysaccharide antigen.

The continued production of hemolysin, hippuricase, and CAMP factor as well as the group B and type III streptococcal antigens confirmed the precise bacterial origin of the L phase of strain 76-043. The modified methods described were found to be specific, and therefore, they could be of practical use in the identification of unknown L-phase cultures.

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