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Metabolic Properties of Some L Forms Derived From Gram-Positive and Gram-Negative Bacteria

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ABSTRACT

WEIBULL CLAES (Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden), AND HANS GYLLANG. Metabolic properties of some L forms derived from gram-positive and gram-negative bacteria. J. Bacteriol. **89**:1443–1447. 1965.—L forms of two gram-positive bacteria, a staphylococcus and a diphtheroid, were found to be devoid of catalase and cytochromes, whereas the normal bacteria from which these L forms were derived contained large amounts of these enzymes. On the other hand, L forms of a gram-negative bacterium, *Proteus mirabilis*, contained the same cytochromes as normal *Proteus* bacteria. (Previous investigations showed that normal cells and L forms of *P. mirabilis* contain approximately the same amounts of catalase.) The respiratory quotients (Q_{O_2}) of all L forms studied were much lower than those of the corresponding normal bacteria. The conversion of the normal organisms into L forms did not markedly affect their growth rate, measured as the time required for doubling the bacterial mass during the exponential-growth phase.

Not very much is known about metabolic changes connected with the conversion of normal bacteria into L forms. It has been shown that L forms of *Proteus mirabilis* respire considerably less vigorously than normal *Proteus* bacteria (Kandler and Kandler, 1955; Mandel and Terranova, 1956). Mandel and Terranova (1956) also showed that normal *Proteus* cells produce more lactic acid than does the corresponding L form. Panos (1962) concluded from determinations of metabolic rates that there exists a major difference between the normal streptococcus and its L form in the metabolism of glucosamine and *N*-acetylglucosamine. The L form, moreover, displayed an adaptive response to glucose. Edwards and Panos (1962) found differences in the content of some nucleotides connected with cell-wall synthesis in the normal cells and L forms of the same streptococcus. Lynn (1962) found that a streptococcal L form contained less alkaline phosphatase but greater pyrophosphatase activity than did the streptococcus from which it was derived.

The differences mentioned above are of a quantitative nature. To our knowledge, the only qualitative difference in the enzyme content of a normal bacterium and its L form reported so far is the absence of proteinase activity in a *Proteus* L form derived from normal cells rich in this enzyme (Kandler and Kandler, 1955). The present report deals with some differences of a qualitative nature between normal cells and L forms of two gram-positive bacteria. Similar differences were not noted between normal cells and L forms of the gram-negative organism *P. mirabilis*.

MATERIALS AND METHODS

Organisms, growth conditions, and harvesting of bacterial crops. The organisms used, *P. mirabilis* strains VI, 9, 18, D52, the *Proteus* L forms L VI, L 9, L 18, L D52, *Staphylococcus aureus* ATCC 6538P, an L form derived from it, and a *Corynebacterium* sp. (strain NM1) with its L form, have been described previously (Dienes and Sharp, 1956; Weibull and Hammarberg, 1962; Weibull, 1963). The *Proteus* L form and the normal *Proteus* bacteria were grown at 30 C in Abrams (1955) liquid serum-free medium. No penicillin was, however, included in this medium. Unless otherwise stated, the staphylococcus, the diphtheroid, and the L forms derived from them were grown at 37 C in Brucella Broth (Albimi Laboratories, Inc., Flushing, N.Y.) supplemented with 1.5% NaCl and 0.2% $Na_2HPO_4 \cdot 12H_2O$ (final concentrations). No serum was added to this medium, the *p*H of which was adjusted to 7.5.

The organisms were usually grown in 200-ml Erlenmeyer flasks, each containing 50 ml of medium. The flasks were incubated on a rotary shaker

(60 rev/min). For studies of the bacterial cytochromes, which required large amounts of bacterial material, the L forms were grown in 3,000-ml Erlenmeyer flasks containing 2,000 ml of medium.

The bacterial crops were harvested by centrifugation at 6,500 × *g* (1.5 cm wide centrifuge tubes) or at 14,000 × *g* (6-cm tubes) for 30 min. Turbidity measurements on the supernatant fluids showed that practically all L bodies (and normal cells) were sedimented by this procedure.

Analytical methods. Catalase was determined by the method of Bonnichsen, Chance, and Theorell (1947) as carried out by Weibull and Hammarberg (1962).

The presence of cytochromes in bacterial cells was studied with a Hartridge reversion spectroscope. Bacterial suspensions containing 15 to 50 mg (dry weight)/ml were poured in 0.5- or 1.0-cm cuvettes, and the absorption bands were studied at room temperature with slit widths of 100 to 400 μ.

Hemin solutions for growth-factor studies were prepared by dissolving hemin in four equivalents of dilute NaOH. The solutions were used immediately after preparation.

Respiratory quotients (Q_{O_2}) were determined by the conventional Warburg technique, with KOH in the central wells of the cups. To obtain accurate determinations of the oxygen consumption of the L forms, cultures of these organisms were concentrated 10-fold by centrifugation. The sedimented cells were suspended in one part of the supernatant fluid and two parts of fresh growth medium.

Growth curves were obtained by inoculating 10 volumes of fresh growth medium with 1 volume of a bacterial culture incubated overnight, and following the turbidity changes at suitable time intervals. The turbidities, measured at 700 mμ, were then substituted for dry-weight values, obtained from separate determinations. The content of bacterial substance (dry weight) in cultures of L forms was determined according to the formula given by Weibull and Beckman (1960).

TABLE 1. *Catalase activities of normal cells of a staphylococcus and a diphtheroid and of the corresponding L forms*

Organism	Activity*
Staphylococcus aureus ATCC 6538P normal cells	13.3
S. aureus ATCC 6538P L form	<0.012
Corynebacterium sp. (strain NM1) normal cells	61.6
Corynebacterium sp. (strain NM1) L form	<0.012

* The activities are expressed as micromoles of H_2O_2 decomposed per milligram (dry weight) of bacterial material per minute. At least two independently grown cultures of each organism were assayed, samples being taken during the exponential-growth phase.

Immunological techniques. To obtain immune sera, aqueous suspensions of L forms treated with formalin (0.2% final concentration) were injected into rabbits three times weekly in increasing doses, until 50 to 100 mg of bacterial material (dry weight) were given to each animal. Conventional agar diffusion and complement-fixation tests were carried out with these sera and suspensions or extracts of normal bacteria.

RESULTS

The *Staphylococcus* and *Corynebacterium* L forms used during the present investigation retained the specific morphological properties of the original L-form cultures (L. Dienes, *personal communication*). To establish the relatedness of these L forms with the parent normal *S. aureus* strain 6538P and the *Corynebacterium* strain NM1, agar diffusion and complement-fixation tests were carried out with suspensions or extracts of the normal organisms and immune sera prepared against the L forms. Complement-fixation titers were low and nonspecific, and no precipitation lines were observed in the diffusion tests. The *Proteus* L form L 9 was also studied with the diffusion technique, and gave precipitation lines with endotoxin prepared from the normal *Proteus* strain 9.

Previous investigations (Weibull and Hammarberg, 1962) indicated that normal cells of a gram-negative bacterium, *P. mirabilis*, and the corresponding L forms contain approximately the same amount of catalase per milligram of bacterial material (dry weight). According to the results of the present determinations, on the other hand, two gram-positive organisms, a staphylococcus and a diphtheroid, lose their content of this enzyme when the normal cells are converted into L forms (Table 1).

Some experiments were carried out to ascertain whether the addition of hemin to the growth medium (final hemin concentration, 10^{-4} M) would induce catalase production in the L forms. The results were, however, negative. The fact that the L forms grew as a single coherent mass of cells in the presence of hemin complicated detailed growth-factor studies.

Since the catalase assays indicated that the gram-positive bacteria studied completely lost a heme-containing enzyme upon their conversion to L forms, it was deemed of interest to ascertain whether the cytochrome spectra of normal cells and L forms of these organisms differed in some respects. In these experiments, no cytochrome bands could be detected in the L forms obtained from gram-positive bacteria, whereas the spectra of normal cells and L forms of *P. mirabilis* were identical (Table 2). It should be pointed out that

TABLE 2. *Cytochrome spectra of normal cells and L forms of some gram-negative and gram-positive bacteria**

Organism	Position of cytochrome peaks (mμ)				
Proteus mirabilis strains VI, 9, 18, D52 (normal cells)	632		561		530
P. mirabilis strains L VI, L 9, L 18, L D52 (L forms)	632		561		530
Staphylococcus aureus ATCC 6538P normal cells		604	561		529
S. aureus ATCC 6538P L form	No absorption bands				
Corynebacterium sp., strain NM1, normal cells	634	600	563	555	528
Corynebacterium sp., strain NM1, L form	No absorption bands				

* A very weak band at 595 mμ was sometimes observed in the spectrum given by *P. mirabilis*. The cultures were harvested during the exponential or early stationary phase of growth.

the cytochrome peaks shown by the various *Proteus* bacteria were not only located at the same wavelengths but also had similar relative intensities. The concentrated bacterial suspensions used for the cytochrome studies were all strongly brown in color with the exception of suspensions of *Staphylococcus* and *Corynebacterium* L forms, which were very faintly yellowish.

The composition of the medium did not seem to influence the cytochrome spectra given by the normal gram-positive bacteria. Thus, cells grown in broth prepared from fresh meat and containing no supplementary NaCl gave the same spectra as cells grown in Brucella Broth supplemented with 1.5 to 3% NaCl.

Some experiments were carried out to compare the general metabolic properties of the L forms with those of their parent bacteria. All L forms respired less vigorously than the corresponding normal bacteria (Table 3). The data indicate, however, that the respiration was more depressed in the L forms of the gram-positive organisms than in the *Proteus* L form.

In terms of maximal yield of cell mass, all L forms grew less vigorously than their parent bacteria. Moreover, the L forms showed a tendency to lyse shortly after the end of the exponential-growth phase. However, the L forms and the normal bacteria exhibited approximately the same growth rate during the exponential-growth phase. Typical growth curves of normal cells and L forms of *S. aureus* and *Corynebacterium* sp. are shown in Fig. 1. Growth curves characteristic of *P. mirabilis* (normal cells and L form) were given by Weibull and Beckman (1960).

TABLE 3. *Respiratory quotients of L forms and the corresponding normal cells of some gram-positive and gram-negative bacteria**

Organism	Q_{O_2}	Ratio Q_{O_2}, L form / Q_{O_2}, normal cells
Staphylococcus aureus L form	119 ± 19	0.20
S. aureus normal cells	606 ± 71	
Corynebacterium sp. L form	63 ± 10	0.18
Corynebacterium sp. normal cells	360 ± 58	
Proteus mirabilis L form	371 ± 60	0.32
P. mirabilis normal cells	1,148 ± 161	

* The ± signs indicate 95% confidence limits. At least three independently grown cultures of each strain were assayed, samples being taken during the exponential-growth phase.

DISCUSSION

The serological tests performed by us showed that the *Proteus* L form L 9 was related to the normal *Proteus* strain 9, but they failed to prove the relatedness of the *Staphylococcus* and *Corynebacterium* L forms to the normal bacterial strains used for the production of these forms. However, it has been shown that L forms of gram-positive bacteria may lack completely certain immunologically active substances present in their parent bacteria (Sharp, Hijmans, and Dienes, 1957; Freimer, Krause, and McCarty, 1959). Moreover, Dienes, Weinberger, and Madoff (1950) were unable to establish a serological relationship between one *Proteus* L form and its parent bacterium (two other *Proteus* L forms showed such a relationship).

By means of the assay method used in the present study, it was shown (Weibull and Hammarberg, 1962) that several strains of pleuropneumonia-like organisms (PPLO) contained, on an average, less than 0.06 molecules of catalase per organism, each organism having a diameter of approximately 0.4 μ. This implies that the PPLO studied did not contain any catalase at all unless the populations were enzymatically highly heterogeneous.

On account of the extremely heterogeneous size distribution characteristic for populations of L bodies, exactly the same conclusion cannot be

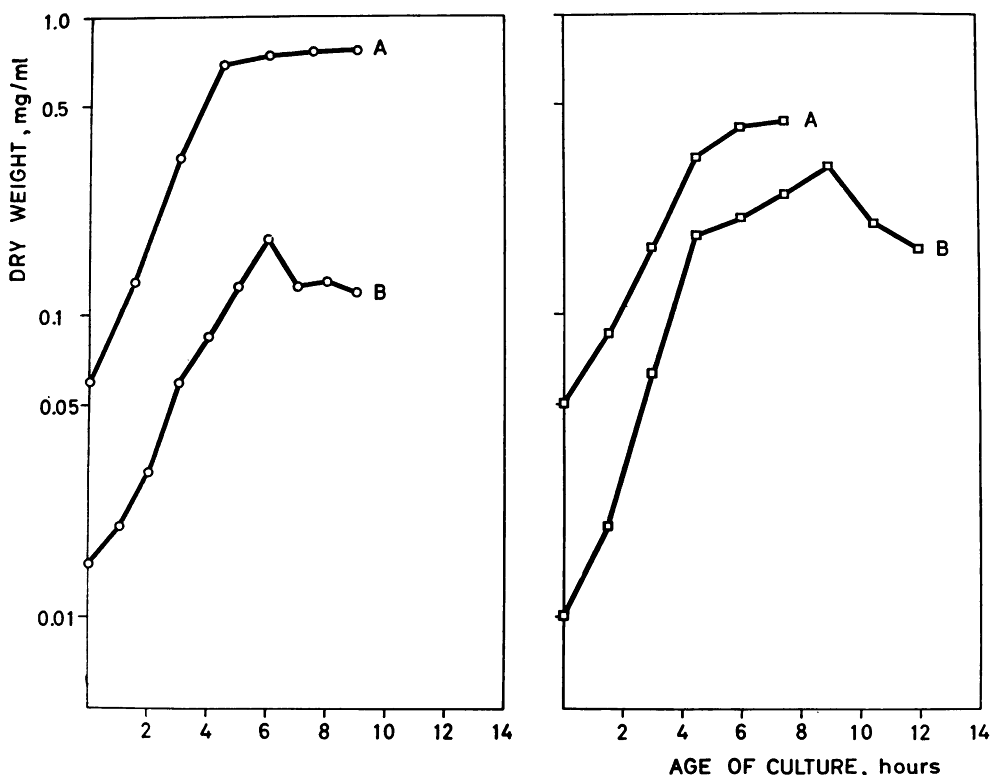

FIG. 1. *Growth curves of normal cells and L forms of Staphylococcus aureus ATCC 6538P (left) and Corynebacterium sp., strain NM1 (right). The values on the ordinate are plotted on a logarithmic scale. A, normal cells; B, L forms.*

drawn with respect to the *Staphylococcus* and *Corynebacterium* L forms. However, provided that the populations were enzymatically homogeneous, individual bodies of these L forms that had a diameter of about 1 μ could not contain more than one catalase molecule. According to separate investigations (Weibull, 1963), many *Staphylococcus* and *Corynebacterium* L bodies of this or a smaller size are found in cultures of these organisms.

Our measurements on the cytochrome bands present in normal bacteria agree reasonably well with earlier determinations (Smith, 1954). However, we observed only one band in the region of 550 to 570 mμ in spectra given by *S. aureus*, whereas Smith (1954) reported two separate bands. Fujita and Kodama (1934) did not find any band at 630 to 635 mμ in spectra given by *C. diphtheriae*.

The absence of cytochrome bands, as observed with the Hartridge spectroscope in spectra given by the *Staphylococcus* and *Corynebacterium* L forms, suggests that these forms do not contain any cytochromes. However, it should be borne in mind that the sensitivity of the spectroscopic method is far less than that of the catalase assays carried out by us.

Under the prevailing experimental conditions, the L forms derived from gram-positive bacteria had a respiratory quotient amounting to only about one-fifth of that of their parent bacteria. However, the respiratory rate of the L form of the gram-negative organism *P. mirabilis* was also rather low, about one-third of that displayed by normal *Proteus* bacteria. Thus, there is hardly any evidence indicating that the loss of catalase and cytochromes radically decreased the respiratory activity of the organisms concerned. Similarly, the loss of the heme-containing enzymes did not very much affect the growth rate of the *Staphylococcus* and *Corynebacterium* L forms. There was a marked effect on the total growth, which, however, also was noticeable in the case of L forms and normal cells of *P. mirabilis* (Weibull and Beckman, 1960). On the whole, more profound changes in the physiological and metabolic properties seemed to occur in bacteria deprived of their heme-containing enzymes by conven-

tional genetic mutations (Jensen and Thofern, 1953; Beljanski and Beljanski, 1957). In these mutations the catalase activity could be restored by adding heme to the growth medium. As mentioned, such a restoration was not noted in the L forms studied by us.

It seems hard to explain why certain changes of a qualitative nature occurred in the enzymatic composition of the gram-positive organisms upon their conversion to L forms, but not in the gram-negative organism, *P. mirabilis*. In any case, these changes may have a bearing on cell-wall biosynthesis and on the action of penicillin (Strominger, 1962), since L forms are more or less deprived of the bacterial wall and this loss is generally effected by penicillin treatment.

The present investigation shows that L forms from various bacteria may differ in important respects. Thus, *Proteus* L forms may roughly be regarded as cells more or less completely deprived of their rigid walls but otherwise similar to normal bacteria. Evidently, this view is not valid with respect to the streptococcal L forms studied by Panos (1962) and the *Staphylococcus* and *Corynebacterium* L forms investigated by us. Recently, Smith (1964) pointed out the importance of comparing the properties of L forms derived from different bacterial species.

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