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Induction and Propagation of a *Bacillus subtilis* L Form in Natural and Synthetic Media

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The L form of *Bacillus subtilis* NRRL B-3275 was induced in a 7% NaCl broth medium and subsequently propagated in natural and synthetic media. The L form grew readily in tryptone broth supplemented with glucose, NaCl, and phosphate buffer, and in a synthetic medium containing only glucose and biotin, in addition to the required salts. Successive transfers from the bacillus inoculum and subsequent large bodies in the tryptone broth with 7% NaCl resulted in gradual selection or transition from the bacillary form to a stable L form without the addition of an antibiotic. The number of viable granules attained in the broth culture exceeded 9×10^7 per ml, and numerous large bodies were always present in rapidly growing cultures.

Protoplasmic granules associated with bacterial cultures were first reported by Klieneberger (10), and their derivation from bacteria was demonstrated by Dienes (7). Since their discovery, numerous L forms have been produced and propagated, although the L-form growth of most bacterial species requires complex media and nearly always an agar support. Moreover, successful initiation of growth in broth usually requires the incorporation of agar blocks containing L-form colonies. However, mutants of *Proteus* that lack the subsurface components of typical 3B type L-form colonies on agar have been shown to grow readily on direct transfer to broth (2).

Development of synthetic media for the study of bacterial L forms other than those of *Proteus* has evolved slowly. A semidefined broth medium was compounded by Abrams (1), and a completely synthetic broth was described by Medill and O'Kane (14) for the *Proteus* L forms. In addition, a *Staphylococcus aureus* L form (5) and the 3B type L form of *Bacillus subtilis* (15) have been cultured on semidefined and defined media, respectively. The *B. subtilis* L form required an agar support.

Transition of bacteria to L forms previously has been observed only on media solidified with agar (9), although commitment can occur in liquid penicillin media in the absence of cell division (11).

B. subtilis NRRL B-3275 did not require agar for L-form induction, transfer, or propagation. The L form was induced with 7% NaCl in a tryptone broth. Several transfers of the bacillus and subsequent large bodies provided L-form cultures that could be readily propagated in broth or on agar media. This report describes the methods of induction, propagation, growth rates, and some morphological features of large bodies of this L form.

MATERIALS AND METHODS

Culture identification. A bacillus was isolated from moist corn and became the subject of study during characterization because large, spheroplast-like bodies, as well as bacilli, were always observed in test media containing 7% NaCl. The motile, gram-positive bacillus averaged 0.7 μ in diameter and 2.0 μ in length. Endospores of the organism were formed in abundance on Trypticase Soy Agar (BBL). The spores were about 0.6 μ in diameter and 1.5 μ in length. Colonies from heat-shocked spores grew readily on glucose agar, soybean agar, and in tryptone broth containing 7% NaCl. Starch, casein, and gelatin were hydrolyzed, and the *p*H of 0.3% tryptone broth declined from 7.2 to about 6.0 after 7 days of incubation at 28 C. Reduction of nitrate to nitrite, however, was not shown. The isolation was tentatively identified as *B. subtilis* and was given the number NRRL B-3275. Additional characteristics of this bacterium will be given in another section of this paper.

Media. The L form was propagated in three media, each medium containing the listed ingredients per liter, and resulted in *p*H values from 6.8 to 7.0. T-medium consisted of tryptone, 3.0 g; glucose, 1.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 3.0 g; and NaCl, 17.0 g. The glucose-salts medium (GS) contained glucose, 5.0 g; KH_2PO_4, 2.0 g; K_2HPO_4, 4.0 g; $(NH_4)SO_4$, 0.5 g; NaCl, 17.0 g; $MgCl_2 \cdot 6H_2O$, 0.25 g; $CaCl_2$, 50 μg; $CuSO_4 \cdot 5H_2O$, 40 μg; $FeCl_3 \cdot$

$6H_2O$, 100 μg; $MnSO_4 \cdot H_2O$, 100 μg; $ZnSO_4 \cdot 7H_2O$, 400 μg; and biotin, 20 μg. The third medium was Yeast Nitrogen Base (YNB; Difco) supplemented with K_2HPO_4, 3.0 g; glucose, 5.0 g; and NaCl, 17.0 g. The glucose and the $MgCl_2$ were autoclaved separately, and YNB required filtering to obtain a clear broth.

Induction of the L form. Colonies that were to be induced to the L form were grown on Nutrient Agar (Difco) inoculated with spores heat-shocked for 30 min at 70 C. A portion of a 36-hr colony was transferred to 10 ml of T-medium with 7% NaCl. Transfers (0.5 ml) were continued in this medium until only viscid strands of large, spherical bodies were microscopically visible; four to eight transfers were generally required. L forms so induced were successively transferred to T-medium and T-medium with 1.2% agar (15) a minimum of five times to observe their stability in the reduced salt concentration. In addition, the cultures were plated onto T-medium with the NaCl deleted to test their osmotic fragility. Transfers were made at 2-day intervals with an incubation temperature of 28 C.

Proof of osmotic fragility. In this study, the criteria for functional impairment or absence of a cell wall were based on those of Lederberg and St. Clair (13), and they include (i) osmotic fragility, and (ii) loss of rigidity resulting in spherical or amoeboid forms. The inability of the organism to grow on transfer to media with less than 0.1 M NaCl but growing in the same medium with 0.3 M NaCl was considered proof of osmotic fragility. Our working definition of a stable L form was taken from Landman and Halle (12) and refers to L forms induced by a mass conversion process, but they continued to multiply in the L stage for successive generations after the inducing agent had been withdrawn.

Physiological tests. Carbon assimilation tests were made in GS medium with the carbon source to be tested replacing glucose, and a biotin requirement was shown by successively transferring 0.1 ml of culture in GS medium from which the biotin was omitted. The remainder of the tests utilized T-medium and T-medium with 1.2% agar as the basal substrate.

Growth measurements. Three flasks of each growth medium, 50 ml in a 300-ml Erlenmeyer flask, were inoculated with 0.5 ml of a culture grown in the same medium and of the following ages. The T-medium inoculum was from a culture transferred at 2-day intervals; it was incubated in stationary culture at 28 C; and inoculum for GS and YNB was incubated for 3 days. Optical densities of L-form growth were determined at a wavelength setting of 450 mμ in a Bausch & Lomb Spectronic-20 colorimeter. Direct counts of large bodies were made in a Petroff-Hausser bacteria counting chamber; L-form numbers were also estimated by most probable number (MPN) in T-medium with a three-tube method (3).

RESULTS AND DISCUSSION

Induction and stability of the L form. Ready induction of the L form from B-3275 and the simplicity of propagation allow one freely to initiate L-form cultures. In a composite of two tests, 14 of 22 colonies were induced to multiply in a stable, spherical form, after eight successive transfers in T-medium with 7% NaCl. Several of the L forms were cultured in T-medium for more than 40 transfers without reversion. The ease of induction and propagation of *Proteus* L forms have been attributed to their comparatively tough membranes (2), and a similar feature may account for the stability of this *B. subtilis* strain in the L-form state. Unlike L forms described previously, agar support is not essential for induction and propagation of this *B. subtilis* L form. It is apparent that a high salt concentration provides specific inhibitory effects concerning cell-wall synthesis and the physical condition for reproduction of this osmotically fragile cell.

Evidence for the derivation of the L form from B. subtilis B-3275. The L form has a high degree of similarity to *B. subtilis* B-3275 in its physiological characters (Table 1). Eleven of the thirteen compounds tested as sole carbon sources resulted in similar growth responses for the L form and the bacillus. Rhamnose and sodium citrate were not satisfactory carbon sources for the L form, but the bacillus grew adequately upon repeated transfer in the salts of GS medium with these carbon sources. The bacillus differed from the L form in its ability to hydrolyze gelatin. In addition, starch was readily hydrolyzed by the bacillus, whereas only a faint zone of hydrolysis was evident beneath the smear of L-form growth.

A biotin requirement was discovered in the bacillus, and the induced L form, likewise, required only this vitamin. Both bacterial forms are sensitive to aflatoxin. The significance of this test is questionable, but only species of the genus *Bacillus* (6) and members of the Actinomycetales (4) have been shown to be inhibited by 30 μg per ml of this mycotoxin. When one considers the physiological similarities of the two bacterial forms and the initiation of the L form from heat-shocked spores, the probability for the derivation of the spherical form from the bacillus is extremely good.

Growth and growth measurements. In stationary broth cultures of the L form, an opaqueness developed throughout the medium and a film of cells collected on the bottom of the culture medium. When swirled, the growth could be observed as a mixture of white and transparent, viscid strands. On a moist agar surface of T-medium, the L-form colonies grew to a diameter of 2 to 3 mm in 4 days; the white viscid growth became dull yellow in about 1 week. A drop of broth on a cover slip allows it to be placed on the surface, with only slight disruption of the colony, to be microscopically examined. Large, pliable cells could be observed at the edges of large colonies

(Fig. 1). Large bodies in rapidly growing cultures were generally phase dark with a light central area in one orientation (Fig. 2); these features were also observed in lysozyme protoplasts. As the large body developed, the lighter area near the cell center increased in size and smaller phase dark bodies became evident at the periphery (Fig. 3). A typical log-phase culture contained numerous phase dark large bodies some joined by viscid connecting strands (Fig. 4). Growth rates

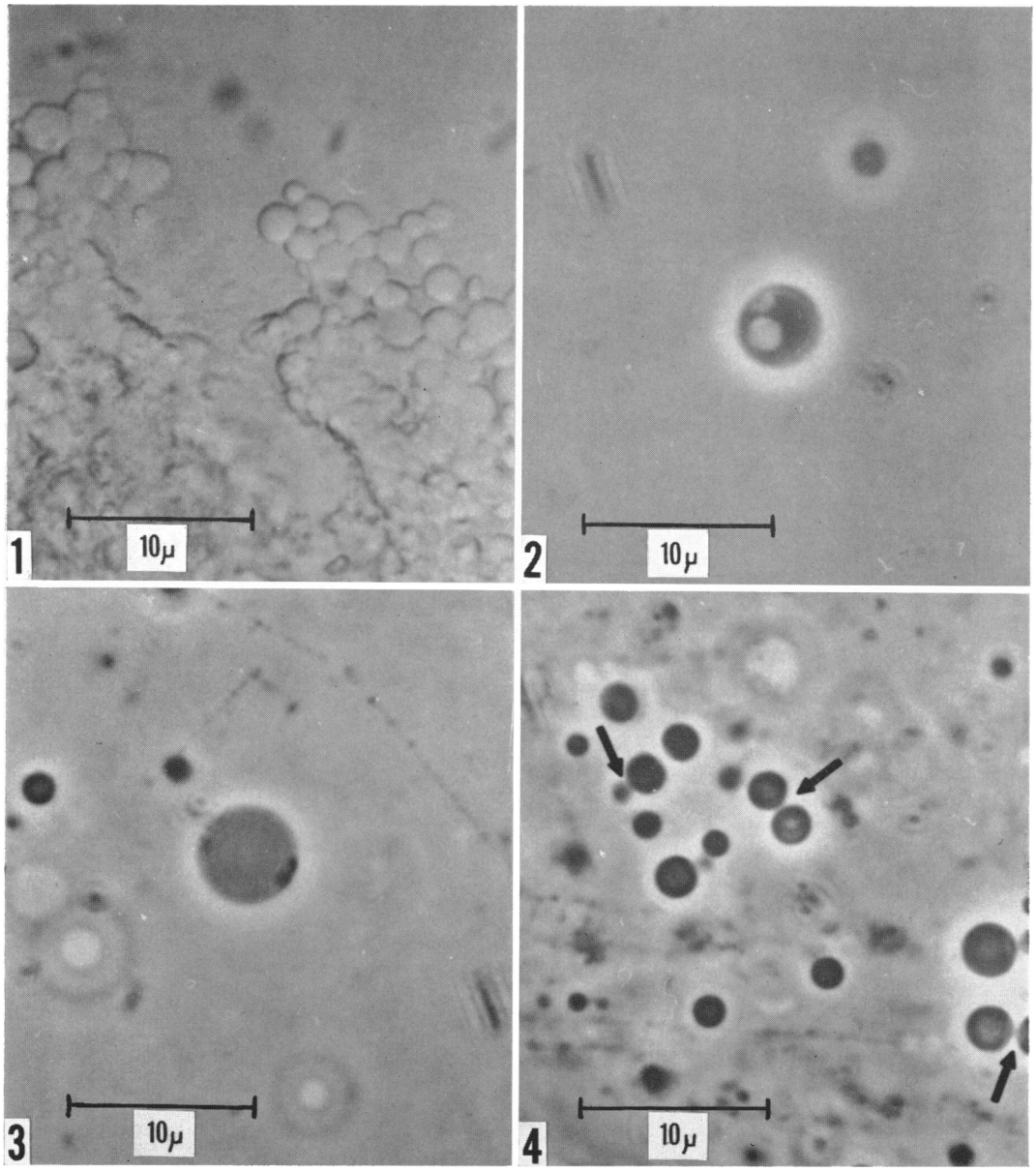

FIG. 1. *Periphery of a large L-form colony of Bacillus subtilis propagated on T-medium agar. This picture was taken with an incandescent light source passing through a layer of T-medium agar and a plastic petri dish bottom.*

FIG. 2. *Phase micrograph of a large body growing in T-medium for 48 hr at 28 C. The large bodies are morphologically similar to protoplasts.*

FIG. 3. *Large body of maximal size with distinct peripheral bodies. Phase-contrast micrograph.*

FIG. 4. *Large bodies from a 24-hr culture of the L form growing in T-medium at 28 C. Phase-contrast micrograph.*

TABLE 1. *Comparative physiological characteristics of Bacillus subtilis B-3275 and its L form*

Physiological test	B-3275	L form
Assimilation		
Glucose	+[a]	+
Sucrose	+	+
Maltose	+	+
Galactose	+	+
Lactose	−[a]	−
Mannose	+	+
Mannitol	+	+
Sorbitol	−	−
Xylose	+	+
Arabinose	+	+
Rhamnose	+	−
Na acetate	−	−
Na_2 citrate	+	−
Other tests		
Biotin	Required	Required
Catalase	+	+
Acetylmethylcarbinol	+	+
Growth in 7% NaCl	+	+
Growth at *p*H 6.0	+	+
Growth anaerobic	−	−
Growth at 55 C	−	−
Growth at 45 C	+	+
Casein hydrolysis	+	+
Gelatin hydrolysis	+	−
Starch hydrolysis	+	+
Nitrite from nitrate	−	−
Acid from xylose	+	+
Aflatoxin sensitivity, 30 μg/ml	+	+

[a] Symbols: +, growth or positive test; −, no growth or negative reaction.

of this organism were compared in three media (Fig. 5). Most rapid reproduction occurred in the T-medium, a maximum was obtained within 30 hr; growth in the synthetic media reached a maximum in about 3 days. The number of large bodies and the number of viable units in T-medium remained at a maximum for about 3 days. After the third day, there was a marked decrease in viable numbers and in the large body count. The number of viable units as determined by MPN exceeded the large body count by almost 1,000-fold at each sampling period (Table 2); this observation suggests that the L-form cultures are composed of particles not detectable by light microscopy or that the large bodies give rise to numerous viable granules. The latter observation is consistent with that of Dienes (8), who observed the development of viable granules inside large bodies of L forms derived from streptococci and and staphylococci.

This *B. subtilis* L form is unlike those derived from most other bacteria in the following ways.

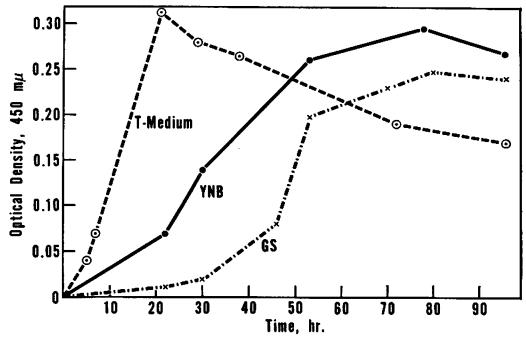

FIG. 5. *Comparative growth of Bacillus subtilis L form in a natural (T-medium) and two synthetic media (GS, YNB) in a stationary culture at 28 C.*

TABLE 2. *MPN, number of large bodies, and optical density (OD) of L form cultured in stationary T-medium broth at 28 C*

Time	MPN	Large bodies	OD
days			
0	9.3×10^5	—	0.02
1	9.3×10^7	1.8×10^5	0.26
2	9.3×10^7	1.3×10^5	0.25
3	9.3×10^7	1.0×10^5	0.24
4	2.3×10^6	3.3×10^4	0.21
5	2.7×10^4	4.0×10^4	0.23

(i) Agar or penicillin is not required for the transition of bacilli to L-colony forming cells, and a stable growth can be maintained in the absence of compounds known to inhibit cell-wall synthesis; and (ii) it can be propagated in synthetic media and grows readily in broth and on agar surfaces.

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