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Characteristics of a New Strain of *Bacillus popilliae* Sporogenic In Vitro

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Conditions are described that led to the isolation of NRRL B-2309M, a strain of *Bacillus popilliae* which sporulates regularly in laboratory culture. Colonies grown on a medium formulated with yeast extract and the ingredients of Mueller-Hinton with phosphate, trehalose, and agar, produced 20% spores in 10 to 12 days. The quantity and kind of yeast extract determine the extent of sporulation, although there are other requirements for optimal growth and sporulation. Spore inocula free of viable vegetative cells are necessary to maintain sporogenicity since asporogenic substrains arise spontaneously on solid and in liquid media. One such substrain, NRRL B-2309N, is also asporogenic in larvae, but lethal, owing to vigorous vegetative growth. Strain B-2309M is infective when vegetative cells or spores are injected into Japanese beetle larvae but fewer spores are formed in vivo than when infections are caused by NRRL B-2309. The characteristics of four related strains of *B. popilliae* are tabulated.

Biological control of larvae of the Japanese beetle *Popillia japonica* Newman has been amply demonstrated by using spores of the milky disease pathogen *Bacillus popilliae* Dutky (1; S. R. Dutky, U.S. Patent 2,258,319, 1940). Production of spores by artificially infecting captive larvae is not economical for this purpose. Widespread application of milky disease as a control measure will come only when infective spores can be produced inexpensively in fermentation equipment.

Conditions have been developed for vegetative proliferation of *B. popilliae* in shake flasks and in fermentors (8). Spores have appeared in liquid cultures containing activated carbon, but yields have been low (2). Steinkraus and Tashiro (11) reported that growth from a nutrient agar sporulated when transferred to a starvation agar medium, although they make no mention of the quantity of spores produced. Steinkraus and Provvidenti (10) later concluded that sporulation of *B. popilliae* on solid medium was inconsistent. Spores produced in vitro would not continue to generate vegetative cells that would sporulate in turn.

Rhodes et al. (4) isolated a sporogenic strain of *B. popilliae*, designated NRRL B-2309S; this strain first appeared as smooth colonies when the parent, NRRL B-2309, was grown on solid medium containing acetate. B-2309S sporulated on acetate agar only if there were fewer than 30 colonies per plate. From 0.1 to 0.3% spores occurred after incubation for 4 weeks. The present study began as an effort to improve the extent of sporulation of strain B-2309S on agar and to induce sporulation in liquid medium.

MATERIALS AND METHODS

The JB medium previously used for propagation of milky disease organisms (3, 7) contained 1.5% yeast extract (Difco), 0.5% tryptone (Difco), and 0.2% glucose, and was buffered with either 0.3% or 0.6% K_2HPO_4. The same medium minus tryptone was designated MD. For liquid culture, 100 ml of media was dispensed in 500-ml Erlenmeyer flasks. Solid versions contained 2% agar (Difco); glucose was always autoclaved separately.

The MYPT liquid medium, developed during this study, contained 1% Mueller-Hinton (MH) broth medium solids (Difco), 1% yeast extract (Difco), 0.3% K_2HPO_4, and from 0.05 to 0.1% trehalose. These constituents were filter sterilized together and aseptically dispensed into sterile flasks.

Solid MYPT medium was sterilized in two stages as follows: 8 g of agar was autoclaved for 30 min in 200 ml of distilled water in a 16-oz bottle fitted with a 100-ml Seitz pressure filter. The sterile agar was cooled to 50 C, and nutrient ingredients sufficient for 400 ml final volume (dissolved in 200 ml distilled water) were filter sterilized into the bottle (final agar concentration, 2%). After all ingredients were mixed, the bottles were either refrigerated for future use or were adjusted to 50 C for immediate pouring

into plates. Each plate contained 30 ml (agar depth, 6 to 7 mm).

Viability of vegetative cells and spores was determined by standard colony counting techniques. A Petroff-Hauser bacteria counter was used for microscopic counts.

Percentage sporulation was calculated from microscopic counts of vegetative cells, prespores, and spores. Sporulation in colonies was determined from a 0.1% tryptone suspension of the growth contained in three to five colonies. After correcting the counts for dilution and number of colonies used, calculations were made as follows: per cent spores per colony = (number of spores)/(number of spores + prespores + vegetative cells) × 100. Microscopic counts did not change in diluent suspensions that were left standing for at least 3 hr.

True vegetative cells of *B. popilliae* are killed by drying. Thus, inocula containing viable spores and dead vegetative cells were obtained by drying selected sporulating colonies in a vacuum desiccator containing a solid desiccant (Drierite) and concentrated sulfuric acid. From 2 to 4 ml of a 0.1% tryptone suspension of cells and spores was placed in a sterile 50-ml beaker covered with two thicknesses of milk filter material and dried in a vacuum of 26 inches of mercury for 2 or 3 days. Dried inoculum was aged for 30 days or longer under room conditions to minimize asporogenicity.

RESULTS

Conditions contributing to maximal growth in conventional bench-size fermentation vessels have been described (5, 8). Microscopic evidence of prespore development was reported in 2- to 3-week-old cultures of *B. popilliae* NRRL B-2309S grown in filter-sterilized MD medium.

Derivation of the M strain. A few mature spores appeared in flask cultures of strain B-2309S after incubation for 3-weeks in a filter-sterilized medium formulated with MH solids combined with yeast extract, phosphate, and trehalose. Sporulation was inconsistent and depended on some undetermined balance of aeration, *p*H, and proximity of cells. Cultures were incubated for 3 days on a rotary shaker and then held as still cultures for 10 to 15 days before spores appeared in a layer of cells settled to the bottom. A culture containing microscopically visible spores was vacuum dried and used to inoculate both liquid and solid MYPT media. Vegetative outgrowth became visible in about 48 hr in both media. The resultant substrain, unique in that it was derived from *B. popilliae* spores produced in liquid medium, was designated NRRL B-2309M.

Sporulation in liquid MYPT became consistent, but was only slightly increased by repeated growth-sporulation-germination cycles. Variations of the medium or of fermentation conditions did not improve sporulation. About 1.5×10^6 mature spores of strain B-2309M can be produced per ml of liquid culture, as determined by microscopic counts. Maximum viability of 800×10^6 vegetative cells per ml occurs in these cultures at 18 hr. Microscopic spore counts were made after 20 days when total viability had dropped to about 5×10^6 proliferating units per ml and when, after vacuum drying, 1.3×10^4 proliferating units were found per ml.

Sporulation on solid medium. Moderate numbers of spores were also formed in colonies of B-2309M on solid MYPT medium. Variation of the proportion of medium constituents changed the rate of sporulation and the relative numbers of cells, prespore forms, and mature spores. Initially, the MYPT medium contained 0.1% trehalose, and about 5% spores appeared after 12 to 13 days. When trehalose was reduced to 0.05%, some spores appeared in 6 to 7 days and sporulation increased to 10% in relatively smaller colonies. Concentrations of trehalose greater than 0.1% yielded large colonies and few or no spores.

When either yeast extract or MH solids were omitted from the medium, sporulation was reduced to 1 to 3% and we found that both ingredients were required at the 1% level for optimal growth and sporulation. The type, as well as quantity, of yeast extract influences growth and, to a much greater extent, sporulation. Most, but not all, commercial yeast extracts promote growth of *B. popilliae* on solid media. Those that support reasonable growth usually sustain limited sporulation. However, different manufacturers' lots of the same yeast extract vary markedly in their ability to support sporulation. Only Difco lot no. 492496 will regularly yield maximal numbers of spores. All other lots of Difco yeast extract that were studied were inferior. Apparently, there is a factor or a balance of nutrients necessary for sporulation that is not consistently satisfied.

Choice sporulating colonies to be used for inoculum were vacuum dried. After several cycles of drying, germination, outgrowth, and sporulation, the spores in colonies increased to about 20%.

Physical conditions also affect sporulation. Optimal spore production by strain B-2309M occurs when only 10 to 12 colonies are carefully distributed equidistant from one another on a plate. Because of poor germination, spore suspensions were used as inoculum and carefully placed with a platinum loop at 10 evenly spaced points on each plate. Consequently, mature colonies resulted from the fusion of several microcolonies. Each colony requires about 10% of the area and volume of available medium for maximal sporulation per colony and the highest spore yield per plate.

Colonies are larger when fewer than 10 develop on a plate, but the yield of spores is below the maximal value of about 2×10^9 spores per plate. Nutrients required for growth are present in excess because colonies continue to expand by peripheral vegetative growth after sporulation occurs. A free exchange of gases is also required for sporulation. Either the use of tight-fitting

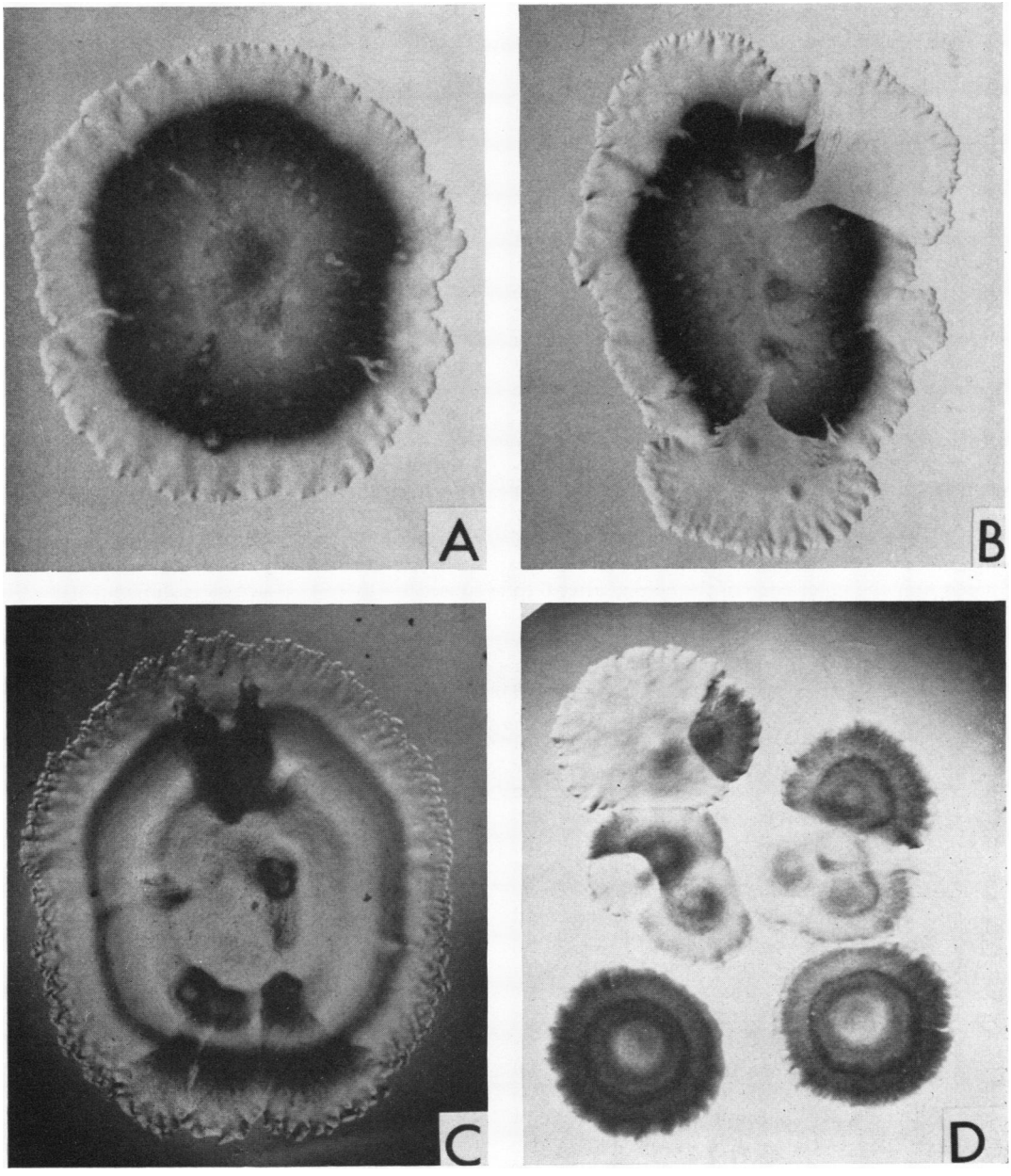

FIG. 1. *Sporulating colonies of B. popilliae* (×9). (*A*) *Mature sporulating colony. Spores appear in the upper cell layers of the dark peripheral ring.* (*B*) *Sporulation ring broken by nonsporulating, clear fan-shaped sectors.* (*C*) *Mature colony resulting from vegetative inoculum after 20 transfers in liquid medium; spores occur only in the dark sectors.* (*D*) *Colonies resulting from standard spread plating technique of vegetative inoculum after four transfers in liquid medium. Clear nonsporulating colony and sporulating sector, poor sporulation in colonies with hazy ring, and 5 to 10% sporulation in two darkest colonies.*

plates or storage of inoculated plates in plastic bags (to minimize evaporation) interferes with sporulation.

Distribution of spores within the colony. In a mature sporulating colony, spores were concentrated in a concentric ring near the outer edge of the colony, and only the upper 8 to 10 cell layers contained mature spores (Fig. 1A). Viable vegetative cells and prespore forms predominated in the outer fringe area of the colony, whereas the area enclosed by the sporulating ring contained cellular debris, granular vegetative cells, and an occasional spore.

Asporogenisis. Asporogenic substrains of B-2309M appeared as nonsporulating sectors of many colonies. These areas of vegetative, nonsporulating growth caused a break in the sporulation ring (Fig. 1B). The outer boundary of most sectors formed a protrusion in the periphery of the colony, indicating that nonsporogenic vegetative growth continued while the regular cells were forming spores.

Asporogenicity occurred spontaneously throughout colony development. The apex of certain large sectors extended to the point of origin of the colony, indicating that asporogenicity surely commenced at or soon after germination of inoculum spores. Other, smaller sectors originated near the outer edge of the colony after 8 to 10 days of regular vegetative growth.

The incidence of large asporogenic sectors depended upon the inoculum used. When fresh vacuum-dried spores were applied to produce 10 evenly spaced colonies, an asporogenic sector occurred in about one of every five colonies. When dried spores were aged for 30 days or longer, asporogenicity was minimized and the large sectors appeared at a frequency of less than one per plate.

When vegetative cells from young colonies (started from dried spores) were applied similarly as inoculum, approximately one sector appeared per colony. When vegetative cells from liquid cultures (started from dried spores) were so applied, more than one clear sector occurred per colony (Fig. 1B). Clear sectors constituted at least 50% of the area of colonies resulting from inoculum taken after five transfers in liquid. Inoculum from a culture carried in liquid for 20 transfers produced colonies that were almost devoid of spores (Fig. 1C). All of the above colonies resulted from the fused outgrowth of several spores or vegetative cells applied with a small loop to 10 spaced points on the agar surface.

To ensure that asporogenicity was not somehow generated by the merging of several microclones to form one mature colony, dried in vitro spores were diluted and spread on a plate to give isolated spores. Nonsporulating clear segments appeared in about 1% of the resulting colonies. However, all colonies formed spores. When liquid-grown cells were diluted and spread on plates, a few colonies appeared that were devoid of spores and the incidence of such colonies increased with the number of transfers of the inoculum in liquid medium. Vegetative inoculum taken after three or four transfers in liquid produced about 13% spore-free colonies when plated by spreading (Fig. 1D). After 20 transfers in liquid, spread vegetative inoculum produced colonies that sporulated poorly or not at all.

When vegetative cells from a clear asporogenic sector were transferred to plates of fresh MYPT medium, clear colonies grew without a ring of sporulation and without any spores or pigment. Colony morphology remained the same through repeated transfer on plates. This asporogenic substrain of B-2309M has been designated NRRL B-2309N and has been preserved by lyophilization.

Infectivity. *B. popilliae* B-2309S causes milky disease when the vegetative cells are injected into Japanese beetle larvae (4). Strain B-2309M also is pathogenic via injection of either vegetative cells or spores formed in vitro. However, the disease caused by these two strains is different from the typical infection; larvae perish during early stages of the disease and, as a consequence, fewer spores accumulate. The hemolymph of 36 milky larvae infected with B-2309M was pooled and found by microscopic count to contain 61% mature spores, 24% vegetative cells, and 15% prespore forms.

Strain B-2309N resembles the parent B-2309, in that they do not sporulate in vitro. To investigate the possibility that B-2309N might be a reversion of B-2309M to B-2309, B-2309N vegetative cells were injected into Japanese beetle larvae. Unlike B-2309 and other strains, B-2309N did not sporulate in the larvae and thus can be considered a new substrain. Although asporogenic both in vitro and in vivo, it does make rapid and extensive vegetative growth in larval hemolymph, and most larvae of an injected series become moribund and perish within 4 or 5 days, at which time the hemolymph contained an average of 44×10^9 granular vegetative cells per ml.

The sporogenic capability of *B. popilliae* B-2309M is readily preserved in lyophilized vegetative cells only one or two transfers from germinated spores. Air- and vacuum-dried spores produced in vivo and in vitro also remain viable, and sporogenicity is retained for 3 years and probably indefinitely. However, a small fraction of the viability remaining after drying is transitory and

gradually disappears in about a month of aging at room temperature and pressure. Since we have found that drying is sufficient to kill all vegetative cells in young liquid cultures and young colonies, we believe this transient viability in dried sporulating colonies is due to some intermediate form between vegetative cell and spore that has a short-lived resistance to drying. When spore inoculum from colonies is subjected to a sequence of three or four heat treatments at 50 C or above, with intermittant outgrowth and sporulation, the number of typical spores is reduced and the number of aberrant refractile bodies formed is increased in the final colonies (Fig. 2A).

Nevertheless, resistance both to drying and to heat treatment is proof of spore status, and spores of B-2309M formed in vitro react to such tests much the same as B-2309 spores from diseased larvae. For example, an average sporulating colony of B-2309M at 25 days contained 1,065 $\times$ 10^6 vegetative cells, 325 $\times$ 10^6 prespore forms, and 265 $\times$ 10^6 mature spores, as measured by microscopic count. Most vegetative cells were nonviable as indicated by granulation and confirmed by an initial plate count of 8.8 $\times$ 10^6. After vacuum drying, 2.5 $\times$ 10^6 viable forms survive; this amount is considered to be the basic viable spore count. At the same time, viable counts were 15.2 $\times$ 10^6 and 0.4 $\times$ 10^6 spores per colony after moist-heat treatment for 15 min at 50 and 80 C, respectively. These data indicate that most vegetative cells in a mature sporulating colony cannot proliferate, that most mature spores do not germinate, and that in vitro spores are activated by mild heat but are inactivated at higher temperatures. In general, these results agree with data obtained with in vivo *B. popilliae* spores (6, 9).

Infectivity by feeding. Strain B-2309M is infective when injected into Japanese beetle larvae, but no larvae were infected in a standard feeding trial where 30 $\times$ 10^6 spores from laboratory cultures were used to inoculate each gram of soil. A second

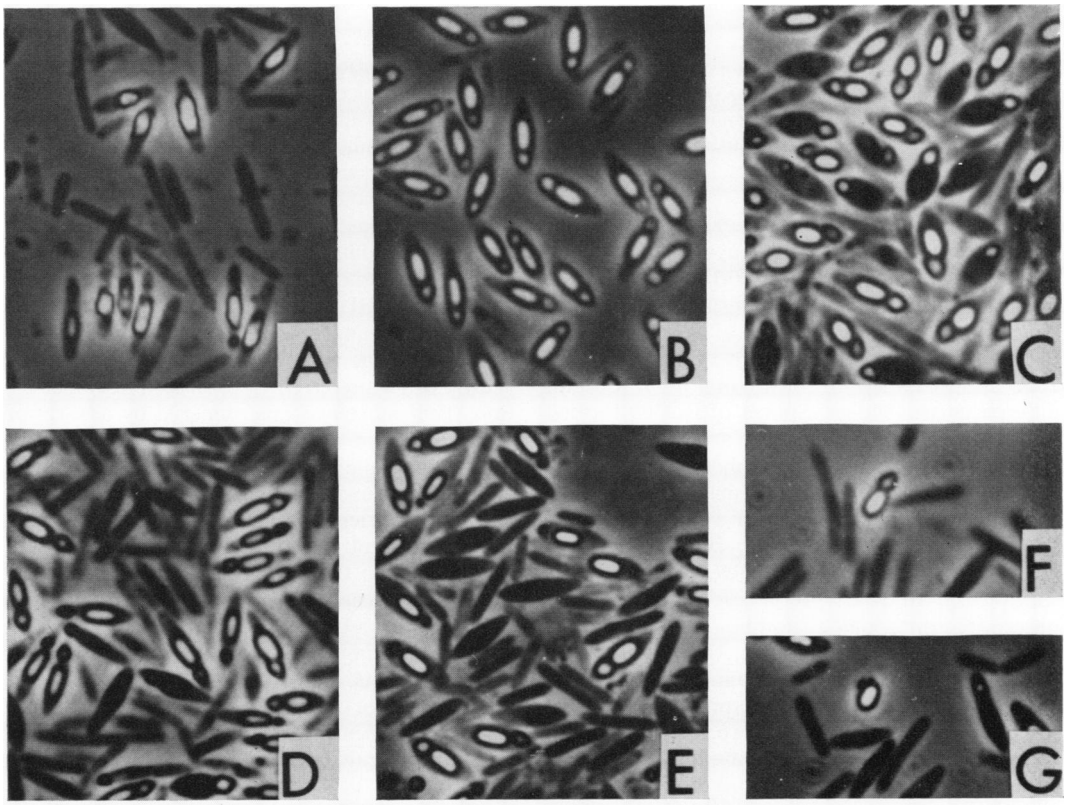

FIG. 2. *Microscopic appearance of B. popilliae spores (×2375, phase). (A) Aberrant forms of sporulating NRRL B-2309M from colonies; (B) spores of B-2309 formed in larvae; (C) spores of B-2309M formed in larvae; (D) spores of B-2309M from colonies; (E) early stage of sporulation of B-2309 in larvae; (F) spore and paraspore of B-2309M from colony without apparent sporangium; and (G) free spore of B-2309M from colony.*

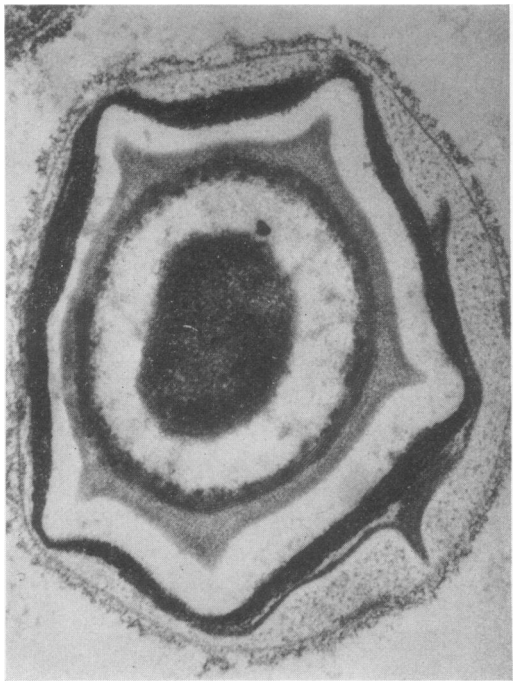

FIG. 3. *Cross-section of a B. popilliae spore, NRRL B-2309M, produced on solid medium. Photo courtesy of S. H. Black, Baylor University, Houston, Tex. ×48,000.*

trial with fresh larvae in the same inoculated soil also gave negative results. Apparently in vitro spores of *B. popilliae* B-2309M are unable to infect Japanese beetle larvae through the natural pathway, the insect gut.

The microscopic appearance of spores of B-2309 and B-2309M is shown in Fig. 2. Certain differences are evident. Spores of B-2309 formed in larvae (Fig. 2B) are uniform and compact with a dense sporangium. The periphery of refractility of the spore is hazy and the shape of the paraspore is indefinite. Whereas many B-2309M spores formed in larvae (Fig. 2C) are indistinguishable from those of B-2309, in general the sporangium of B-2309M is less dense and the paraspore appears as a rhomboid crystal. Spores of B-2309M from colonies (Fig. 2D) are similar to those formed in larvae; in some forms the paraspore is separated from the spore and characteristically is of rhomboid shape. Compared to spores from larvae, the sporangium of in vitro spores generally is less dense and the periphery of refractility of the spore is sharply defined. The differences between spores of B-2309 and B-2309M may be due to a lack of maturity of the latter. Spores of B-2309 formed during early stages of larval infection (Fig. 2E) strongly resemble in vitro spores. A few spores from colonies have an attached paraspore but no visible sporangium (Fig. 2F). Free spores also occur in sporulating colonies of B-2309M (Fig. 2G); free spores are exceedingly rare in diseased larvae. Aside from cytological differences, preliminary data indicate that spores produced in vitro germinate more readily than do spores from larvae.

A cross-sectional view of an in vitro B-2309M spore is shown in the electron photomicrograph (Fig. 3). The fine structure of B-2309M in vitro spores is indistinguishable from that of B-2309 in vivo spores (S. H. Black, Baylor University, *personal communication*).

The growth and sporulation characteristics of four related strains of *B. popilliae* are summarized in Table 1.

DISCUSSION

The concentration of spores in a concentric ring near the periphery of a mature B-2309M colony is, insofar as we know, unique. An understanding of this phenomenon might explain the limited sporulation of *B. popilliae* on solid medium and perhaps provide a means to increase sporulation in liquid. As the sporulation ring begins to appear, interior volume of the colony shrinks to produce a concave surface inside the ring. The depressed portion contains granular cells and debris. It seems that sporulation proceeds in peripheral cells as older cells release some required substance. In elaboration of this assumption, we have preliminary evidence that filter-sterilized cytoplasm from disintegrated liquid-grown vegetative cells significantly increased sporulation when added to plates containing young colonies. A similar effect was produced by a substance found in the specific yeast extract preferred by B-2309M for sporulation. This substance was prepared by adsorption on, and elution from, activated carbon. Possibly, a factor contained in the yeast extract is taken up by rapidly growing cells of the young colony in amounts sufficient to commit them to sporulation, but insufficient to permit completion of the process. When these cells die and lyse, sufficient material is released to support spore formation by most of the viable cells in the upper part of the surrounding ring. We are continuing investigation of these aspects.

The presence of cellular debris in the interior of mature colonies indicates that some older cells have disintegrated and will not appear in microscopic counts; at the same time, limited vegetative cell division occurs in the periphery of the colony. Microscopic vegetative cell counts drop about 25% as colonies mature. However, the number of cells converted to prespores and spores can account for the decrease. There is apparently no

TABLE 1. *Comparison of Bacillus popilliae NRRL B-2309M with related strains*

Growth medium or condition[a]	NRRL B-2309 parent strain	NRRL B-2309S derived from B-2309	NRRL B-2309M derived from B-2309S	NRRL B-2309N derived from B-2309M
MD or JB agar, autoclaved	Transparent colonies 1 to 3 mm diam; no spores	Transparent amber colonies 3 mm diam; when filter sterilized 5 mm diam; no spores	Opaque colonies 3 mm diam; when filter sterilized 5 mm diam; <0.1% spores	Colonies same as B-2309M; no spores
Acetate agar, autoclaved	Rough and smooth colonies 2 to 3 mm diam; <0.3% spores in smooth colonies; origin of B-2309S	B-2309 spores dried and replated yield B-2309S; 0.3% spores	Opaque colonies 3 mm diam; when filter sterilized 5 mm diam; <0.1% spores	Same as B-2309M; no spores
MYPT agar, filter sterilized	Transparent amber colonies 3 mm diam; refractile bodies; no spores	Transparent amber colonies 5 mm diam; refractile bodies; no spores	Opaque amber colonies 6 mm diam with ring of sporulation; 10 to 20% spores	Transparent colonies 6 to 7 mm diam; no spores; B-2309N arises from transfer of nonsporulating sectors of B-2309M
MYPT agar with bakers' or brewers' YE[b], filter sterilized	Opaque amber colonies 6 mm diam; few spores, <1%	Dark amber colonies 6 mm diam; refractile bodies; no spores	Dark amber colonies 6 to 7 mm diam; 1 to 3% spores	Transparent colonies 6 to 7 mm diam; no spores
MD or JB liquid, autoclaved	Vegetative growth, 8×10^8 viable cells/ml; no spores	Vegetative growth, 8×10^8 viable cells/ml; prespore forms; no spores	Vegetative growth, 10^9 viable cells/ml when filter sterilized; no spores	Same as B-2309M; no spores
MYPT liquid, filter sterilized	Vegetative growth, 8×10^8 viable cells/ml; no spores	Vegetative growth, 1.5×10^9 viable cells/ml; 1.5×10^3 spores/ml; origin of B-2309M	Vegetative growth, 2×10^9 viable cells/ml; 1.5×10^6 spores/ml—under special conditions	Vegetative growth same as B-2309M, except no spores
Performance in larvae	Infective by injection of vegetative cells or spores and by feeding of spores; 25% spores, 75% granular cells at 4 to 5 days; 80 to 90% spores by 14 to 21 days	Infective by injection of vegetative cells; infectivity by feeding unknown; sporulation less than B-2309	Infective by injection of vegetative cells or spores; apparently not infective by feeding; vigorous vegetative growth lethal to larvae; sporulation less than B-2309	Infective by injection of vegetative cells; vigorous vegetative cell growth lethal to larvae; no spores
Other	Motile, especially cells from solid media; malt extract increases motility	Motility extremely rare	Nonmotile; malt extract in solid medium increases hyphal type growth	Nonmotile

[a] MD medium contained 1.5% (Difco) yeast extract, 0.2% glucose, 0.3% K_2HPO_4, and 2% agar. JB medium contained ingredients of MD plus 0.5% tryptone. Acetate agar is MD medium with 0.14% sodium acetate substituted for 0.2% glucose. MYPT medium contained beef infusion, Casamino Acids, soluble starch (Mueller-Hinton ingredients at 1%), 1% yeast extract, 0.3% K_2HPO_4, and 0.5% trehalose.

[b] Extracts of bakers' or brewers' yeast prepared at NRRL by mechanical disruption of commercially grown yeast.

disintegration of cells when colonies are suspended in diluent for counting. Although viable counts decrease after about 1 hr, microscopic counts remain stable.

We recommend the vacuum drying and aging procedure as the best method of minimizing the persistence of asporogenicity in strain B-2309M. Heat treatment at temperatures as high as 80 C does not eliminate the subsequent appearance of asporogenic sectors, and it appears that a series of heat treatments is deleterious to the spores. Aging at room temperature and pressure eliminates those intermediate forms of the asporogenic substrain which resist vacuum drying but expire in a few weeks of storage. These phenomena associated with asporogenicity are being studied further.

We are concerned about the inability of B-2309M spores to infect larvae in feeding trials. Since the conclusion of our feeding tests with B-2309M, we have conducted extensive trials using in vivo spores of B-2309. Results with this parent strain indicate that only 11% infection occurred with 30×10^6 spores per g of soil. Possibly, B-2309M spores would be infective by feeding in soil if tested at a higher inoculum level. The sporangium of B-2309M, which appears less dense under phase microscopy than the sporangium of the parent strain, may not provide proper protection in the larval gut; alternatively, this could allow germination of B-2309M spores away from the site of invasion. However, the spores are infective after artificial injection into the hemocoel, and oral infectivity might be regained during growth and sporulation cycles in larvae.

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