Sarcobium lyticum gen. nov., sp. nov., an Obligate Intracellular Bacterial Parasite of Small Free-Living Amoebae†

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A new genus and species of obligate intracellular bacterial parasite of small free-living amoebae is described. This bacterium causes fatal infections in amoebae belonging to the Acanthamoeba-Naegleria group. It does not grow on any artificial substrate deprived of living amoeba cells. The entry of the bacterium into a host occurs by phagocytosis, but growth occurs in the cytoplasm, not in phagosomes. This parasite is readily distinguished from other kinds of previously recognized bacteria that live within amoeba cells on the basis of its host cell lytic activity. The bacterium is a gram-negative, short rod with tapered ends. It multiplies intracellularly by a transverse central pinching-off process. Cells are motile by means of a polar tuft of flagella. The bacterium is surrounded by a distinct multilayered cell envelope with a chemotype Aγ peptidoglycan. The peptidoglycan is unique in its high content of glucosamine residues with free amino groups. The DNA base composition of this organism is 43 mol% guanine plus cytosine. The name Sarcobium lyticum gen. nov., sp. nov. is proposed for this bacterium. The type strain of S. lyticum is strain L2 (= PCM 2298).

More than 30 years ago a new obligate intracellular bacterial parasite (OIBP) which causes fatal infections of hosts was found in a raw culture of amoebae that were freshly isolated from soil (7). This OIBP was isolated from soil from the Lublin area, Poland. In previous reports I have described the infection processes for Acanthamoeba castellanii, Hartmannella rhysodes, Hartmannella astronyxis, Mayorella palestinensis, Didasculus thorntoni, Schizopyrenus ruselli, and Naegleria gruberii, as well as 41 other unclassified amoebae isolated from soil and water reservoirs (8, 10). The OIBP did not grow on any medium when living amoebae were not present. Even a thick suspension of disrupted amoeba cells was ineffective. It has been shown that the OIBP cannot multiply if after infection the mixed culture is heated at 43°C for 10 min; this treatment is sufficient to kill trophic forms of the amoeba, but the bacterium survives. Microscopy has shown (8, 11), that the parasites are readily phagocytized by amoebae and that their immediate place of residence is within phagosomes. The neighboring phagosomes fuse extensively with parasite-containing vacuoles; however, even dead bacteria evade lysosomal attack because the cell wall is resistant to the host's bacteriolytic enzymes. Later, living bacteria escape from phagosomes into the cytoplasm, where multiplication occurs. The final effect of the infection is total lysis of the host cells. Although the morphology of the OIBP and the presence of peptidoglycan in its cell wall suggest that this organism is a bacterium, it has not been possible to classify it previously because of a lack of nutritional tests; the OIBP does not correspond to any bacterium described in Bergey's Manual of Determinative Bacteriology (3). Most of the many kinds of previously recognized bacteria that live within amoeba cells behave as temperate endosymbionts, and some appear to provide benefit to the hosts (1, 4, 15–18, 23, 25). To my knowledge, the only exception is the OIBP, which causes lysis of the amoeba cells. This organism was recognized as being unusual, and therefore I decided to attempt to classify it.

MATERIALS AND METHODS

Organisms and isolation procedure. The OIBP, the causative organism of fatal infections of small free-living amoebae belonging to the Acanthamoeba-Naegleria group, was found in a raw culture of amoebae that were isolated from soil from the Lublin area, Poland (7), by using the enrichment method. Crumbs of soil were placed onto the surface of a non-nutrient agar plate covered with a thin film of a thick suspension of Aerobacter aerogenes and incubated at room temperature for 8 to 10 days. In this way the growth of all microorganisms except those that feed on bacteria or parasitize bacterium feeders was checked. When this procedure was used, several different isolates which could infect amoebae were obtained. The isolate described in this paper was designated strain L2T (T = type strain). Strain L2T was purified from contaminating bacteria by allowing infected amoebae to migrate on agar surface as described previously (8). A two-member (host-parasite) culture was established by growing infected amoebae in PYG medium (8).

Acanthamoeba castellanii, which was obtained from W. Balamuth, Department of Zoology, University of California, was used as a host for maintenance of the OIBP.

Propagation of the OIBP. The OIBP was propagated by transferring a small amount of lysate from an infected culture into a 2-day-old culture of amoebae grown on PYG medium as described previously (8, 24). Bacteria were also propagated on amoeba cells harvested from an axenic culture at the exponential phase of growth and suspended in saline prepared as described by Band (2). The OIBP propagated on amoebae suspended in saline produced more uniform cells and survived for a longer period of time than the OIBP propagated on amoebae in PYG medium (9).

Microscopic observations. Observations on the course of infection were made with living materials by using phase-contrast microscopy and fixed preparations stained by the methods of Macchiavello (5) and Gimenez (15). The stains used for bacteria liberated from their hosts were the stains

† Dedicated to W. J. H. Kunicki-Goldfinger on the occasion of his 70th birthday in friendship and admiration.
described by Norris and Swain (22). For transmission electron microscopy, infected amoebae were fixed in amoeba saline supplemented with 1.5% glutaraldehyde and postfixed in Veronal-acetate-buffered OsO₄. Following dehydration in ethanol, pellets packed by centrifugation were embedded in Vestopal W (Serva). Thin sections of embedded specimens were cut with a Tesla model BS490A ultramicrotome and stained with uranyl acetate and Reynold lead citrate. Samples for negative staining with sodium phosphotungstate were prepared as described previously (12). Grids were examined with Zeiss model DZ and Tesla model BS613 electron microscopes that were operated at 50 or 80 kV.

**Oxygen consumption measurements.** A conventional Warburg respirometer was used for oxygen consumption measurements. Each flask usually received $4 \times 10^{10}$ OIBP cells suspended in saline and substrate to bring the total volume to 2.0 ml. The bacteria were prepared by the procedure described below. A 2-day-old axenic culture of Acanthamoeba castellanii, which had reached a concentration of $3 \times 10^6$ cells per ml, was infected with the OIBP. The ratio of bacteria to amoebae at the time of infection was approximately 7:1. The infected amoebae were incubated on a shaker at 28°C. Bacteria liberated from the infected amoebae were pelleted and freed from cysts and unbroken host cells by fractional centrifugation at 120 and 3,000 x g, resuspended in saline, and starved for 2 h on a shaking water bath. Respiration measurements were carried out at pH 7.4 and 25°C for 2 h with a gas phase of air. CO₂ was trapped with 0.2 ml of 20% KOH.

**DNA extraction and estimation of base composition.** DNA was extracted from bacteria frozen in saline (0.15 M NaCl, 0.1 M EDTA, pH 8.0) after disruption of the cells in 2% sodium dodecyl sulfate at 60°C and then purified by using the method of Marmur (20). The purified DNA samples were dissolved in SSC (0.15 M NaCl plus 0.015 M sodium citrate) and stored at 2°C over chloroform. Base composition was determined by performing a melting point analysis, using the
RESULTS

Microscopic observations. (i) Microscopy of infected amoebae. Typical Acanthamoeba castellanii cells which were infected with the OIBP are shown in Fig. 1 and 2. At 2 h after infection the cells remained remarkably intact. When phase-contrast microscopy was used, differentiation of the cytoplasm into ecto- and endoplasmic layers was clearly observed. Also, the nuclei and other cell structures appeared to be physically undamaged. However, the food vacuoles bearing bacteria became enlarged, yet they were not filled with OIBP cells. Within 6 to 9 h after infection, although the bacteria were present in almost all of the food vacuoles, no distinct morbid changes in host cells were observed. The differentiation of ectoplasm and endoplasm was still maintained, and the host nuclei were prominent and appeared to be physiologically intact. The amoebae moved normally, collected food, and even reproduced; however, the invading bacteria had increased in numbers and occupied a large area of the host endoplasm. In the later stages of infection some bacteria remained in the parasitophorous vacuoles, but most of them were found in the cytoplasm, where lacunae filled with parasites were formed. In heavily infected cells the organelles were displaced by bacteria. In the last stage of infection the host cells took on a ball-shaped form and were filled with rapidly moving bacteria. The rupture of each plasma membrane liberated about $2 \times 10^3$ bacteria into the environment.

An examination of ultrathin sections of Acanthamoeba castellanii by transmission electron microscopy revealed that soon after infection bacteria engulfed by host cells were tightly surrounded by membranes of endocytic vacuoles. Later in infection the parasitophorous vacuoles increased to abnormal sizes because of the fusion of neighboring endocytic vacuoles containing bacteria with each other and with lysosomes (Fig. 3). At this stage of infection no division figures of bacteria were observed, and parasitophorous vacuoles were filled with membranelike structures scattered in an electron-translucent background, as well as with a few bacteria. Later in infection the membranes of bacterium-bearing parasitophorous vacuoles underwent lysis, and parasites escaped into the cytoplasm (Fig. 4 and 5). The electron-translucent space surrounding bacteria gradually underwent resorption, and as a consequence bacteria came into direct contact with the host cytoplasm, where they were clearly distinguished from other subcellular components (Fig. 6). Once the bacteria were inside the cytoplasm, multiplication started. In the last stage of infection lacunae filled with dividing bacteria increased in size, and the proliferating parasites disarranged the host cell structure, causing autolysis of mitochondria and disruption of the plasma membrane (Fig. 7). Division of the parasites was not observed outside the amoebae cells; however, the liberated bacteria remained alive for several months after being released from their hosts.

(ii) Morphology and fine structure of the OIBP. The bacteria liberated from host cells were straight rods with tapered ends. The mean size of the OIBP was calculated from

method of Marmur and Doty (21) and DNA from Escherichia coli (51 mol% guanine plus cytosine) as a reference.
organisms stained with phosphotungstic acid, was 0.6 μm wide by 1.9 μm long. Cells more than 5.0 μm long generally occurred in poorly aerated cultures. The surfaces of negatively stained bacteria were electron opaque and smooth. On cells immediately liberated from their hosts tufts of flagella on one pole were observed (Fig. 8). The maximum number of flagella observed at a single tuft was five. Phase-contrast microscopic observations of bacteria freshly liberated from hosts revealed the presence of motile rods. Motility occurred within the hosts and decreased rapidly after the bacteria were liberated into the ambient medium. The bacteria were gram negative. Neither capsules nor spores were observed. Thin sections showed that the OIBP was essentially a gram-negative bacterium with a trilaminar outer envelope and a cytoplasmic membrane (Fig. 9). With the technique used no definite structures were observed in the cytoplasm that might be interpreted as spores, beta-hydroxybutyric acid, or starch granules. The cytoplasm contained an obvious nucleoplasm which appeared to be a filamentous skein distributed along the entire length of the bacterium. The part of the cytoplasm that was not occupied by the nucleoplasm contained numerous small electron-dense granules that probably represented ribosomes.

Peptidoglycan. The procaryotic nature of this organism was established by the discovery in its cell wall of compounds that are regarded as specific to bacteria (e.g., the bag-shaped peptidoglycan composed of glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid in a molar ratio of 0.4:0.4:1.4:1:1)(12). The peptidoglycan was completely insensitive to lysozyme and bacteroiolytic \(N_O\)-diocetylmuramidase of amoebal origin. The resistance to the bacteriolytic enzymes was presumably due to the large content of glucosamine residues with free amino groups in the glucan strands of the peptidoglycan (13).

Oxygen consumption. Purified suspensions of the OIBP exhibited metabolic activity, as shown by consumption of oxygen and production of carbon dioxide in the presence of disrupted amoeba cells or PYG medium. Freshly collected bacteria in buffered saline had an endogenous \(Q_o\) of \(24 ± 3 \mu l\) per \(10^{10}\) cells \(Q_o\) per \(10^{10}\) cells \(= \mu l\) of \(O_2\) taken up per \(1 \times 10^9\) cells per h). A homogenate of Acanthamoeba castellanii prepared from a suspension containing \(2 \times 10^7\) cells per ml did not consume \(O_2\) but stimulated respiration of the OIBP fivefold compared with endogenous respiration. Glucose and related sugars, as well as glutamate and amino acids synthesized by Acanthamoeba castellanii or included in the synthetic medium to support the growth of amoebae (6), were not metabolized by the OIBP. KCN at a final concentration of \(5 \times 10^{-4}\)M invariably inhibited all respiratory activities of the OIBP.

DNA base composition. The DNA of the OIBP contained 43 mol% guanine plus cytosine as determined by thermal melting point analysis.

**DISCUSSION**

Certain species of free-living amoebae harbor microorganisms. Many of these microorganisms are the same size as ordinary bacteria, and some have been identified as archaeobacteria. Unfortunately, because of their specialized ecological niche, none of these organisms can be cultivated apart from the eucaryotic cells which they inhabit. Consequently,
little information that permits assessment of the taxonomic affinities of these organisms has been obtained. However, the assignment of names to organisms which have not been cultivated in vitro is feasible partly because of our increasing knowledge of the bacteria living inside eucaryotic cells and partly because of the fact that the most reliable taxonomic characteristics are DNA base ratio and the chemical composition of cell walls. Most of the previously described bacteria which inhabit amoeba cells have one feature in common; namely, they behave as temperate parasites. Some even provide benefit to the host. The only exception to my knowledge is the OIBP described in this paper. The uncontrolled growth pattern of this organism in the cytoplasm of amoebae belonging to \textit{Acanthamoeba-Naegleria} group, its DNA base composition, and the chemical makeup of its cell wall peptidoglycan are sufficient to warrant placing it in a new genus. The proposed new genus \textit{Sarcobium} does not appear to belong to any of the currently recognized families of bacteria.

\textbf{Description of \textit{Sarcobium} gen. nov.} \textit{Sarcobium} (Sar. co' bi. um. Gr. n. sarkos, flesh; Gr. n. bios, life; M. L. neut. n. Sarcobium, that which lives in the sarcode or flesh [cytoplasm]). Rods that are 0.6 by 1.9 \textmu m. Gram negative. Motile by means of three to five polar flagella. Cell division occurs by a transverse central pinching-off process. The DNA base composition is 43 mol\% guanine plus cytosine, as determined by the thermal denaturation method. Chemotype A\textsubscript{2},\gamma peptidoglycan with a free amino group on the glucosamine residues. Obligate intracellular parasite of amoebae belonging to the \textit{Acanthamoeba-Naegleria} group and related protozoa (e.g., \textit{Dictiostelium discoideum}). The bacterium is able to escape from parasitophorous vacuoles into the cytoplasm, where it proliferates.

![FIG. 8. Electron micrograph of negatively stained OIBP with a tuft of flagella at one pole. Bar = 1 \textmu m.](image1)

FIG. 8. Electron micrograph of a section through cells of the OIBP. The ultrastructure of this bacterium resembles that of gram-negative bacteria. Bar = 1 \textmu m.

\textbf{Description of \textit{Sarcobium lyticum} sp. nov.} \textit{Sarcobium lyti- cum} (ly' ti. cum. M. L. neut. adj. lyticum, that which causes lysis). Description as for the genus. Type species of the genus \textit{Sarcobium}. The type strain of \textit{Sarcobium lyticum} is strain L2\textsuperscript{T}, which has been deposited in a two member culture with the Polish Culture Collection of Microorganisms as culture PCM 2298. The description of the type strain is the same as that given above for the genus and species, and, in the absence of cultivation in pure culture, this description forms the nomenclatural type Rule 18a of the \textit{International Code of Nomenclature of Bacteria} [19]).

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\textbf{REFERENCES}


