

## Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* Species Results in Coiling Phagocytosis, Endamebic Bacterial Survival, and Escape

TIMOTHY J. J. INGLIS,<sup>1,2\*</sup> PAUL RIGBY,<sup>3</sup> TERRY A. ROBERTSON,<sup>4</sup> NICHOLE S. DUTTON,<sup>4</sup>  
MANDY HENDERSON,<sup>1</sup> AND BARBARA J. CHANG<sup>2</sup>

Division of Microbiology and Infectious Diseases, Western Australian Centre for Pathology and Medical Research, Nedlands, Western Australia 6009<sup>1</sup> and Department of Microbiology,<sup>2</sup> Biomedical Confocal Microscopy Research Centre, Department of Pharmacology,<sup>3</sup> and Electron Microscopy Unit, Department of Pathology,<sup>4</sup> University of Western Australia, Nedlands, Western Australia 6907, Australia

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*Burkholderia pseudomallei* causes melioidosis, a potentially fatal disease whose clinical outcomes include rapid-onset septicemia and relapsing and delayed-onset infections. Like other facultative intracellular bacterial pathogens, *B. pseudomallei* is capable of survival in human phagocytic cells, but unlike mycobacteria, *Listeria monocytogenes*, and *Salmonella* serovar Typhimurium, the species has not been reported to survive as an endosymbiont in free-living amoebae. We investigated the consequences of exposing *Acanthamoeba astronyxis*, *A. castellani*, and *A. polyphaga* to *B. pseudomallei* NCTC 10276 in a series of coculture experiments. Bacterial endocytosis was observed in all three *Acanthamoeba* species. A more extensive range of cellular interactions including bacterial adhesion, incorporation into amoebic vacuoles, and separation was observed with *A. astronyxis* in timed coculture experiments. Amoebic trophozoites containing motile intravacuolar bacilli were found throughout 72 h of coculture. Confocal microscopy was used to confirm the intracellular location of endamebic *B. pseudomallei* cells. Transmission electron microscopy of coculture preparations revealed clusters of intact bacilli in membrane-lined vesicles inside the trophozoite cytoplasm;  $5 \times 10^2$  CFU of bacteria per ml were recovered from lysed amoebic trophozoites after 60 min of coculture. Demonstration of an interaction between *B. pseudomallei* and free-living amoebae in vitro raises the possibility that a similar interaction in vivo might affect environmental survival of *B. pseudomallei* and subsequent human exposure. Endamebic passage of *B. pseudomallei* warrants further investigation as a potential in vitro model of intracellular *B. pseudomallei* infection.

*Burkholderia pseudomallei* causes melioidosis, a potentially fatal infection with septicemic, subacute, and chronic forms that is endemic in northern Australia and Southeast Asia. The acute septicemic form has a mortality rate of over 30% (31) and may present after a disease-free interval of several decades (27). Survival of *B. pseudomallei* within macrophages has been demonstrated in vitro and is proposed as an explanation for delayed-onset disease (17, 24). Survival of *B. pseudomallei* for extended periods in the absence of nutrients and under acid conditions may allow persistence in a hostile intracellular habitat (18, 32) but does not explain how *B. pseudomallei* enters or escapes from professional phagocytic cells. A potential explanation is that *B. pseudomallei* has developed the capacity to invade and survive within free-living amoebae in the moist soil and surface water environment where the bacterial species is normally found (5, 15, 29) and amoebae are also likely to be present (25).

Several facultative bacterial intracellular pathogens, notably *Legionella pneumophila*, *Mycobacterium avium*, and *Listeria monocytogenes*, have been shown to survive as endosymbionts in free-living amoebae such as *Acanthamoeba*, *Hartmannella*, and *Naegleria* (8, 20, 26). It has been suggested that growth in an amoebic intracellular environment might assist these bacteria to adapt to survival in mammalian phagocytic cells (3, 7, 11,

26). Moreover, incorporation of bacteria in amoebic cysts has also been shown to confer resistance to adverse environmental conditions such as exposure to biocidal agents (1). In addition, amoebic endosymbiosis is known to augment the virulence of *L. pneumophila* and *M. avium* (6–8). The endocytosis of *L. pneumophila* by both *Acanthamoeba* and macrophages has common features including an unusual, coiling form of phagocytosis (3, 14). Although evidence for the direct involvement of amoebic endosymbionts in the pathogenesis of human infection has yet to be found, molecular methods and animal models indicate a possible role for free-living amoebae as determinants of bacterial virulence (4, 6–8, 11).

The best evidence in support of a putative endosymbiosis involving *B. pseudomallei* and free-living protozoa is circumstantial: *Ralstonia* (previously *Burkholderia*) *pickettii*, a close phylogenetic relative of *B. pseudomallei*, has been found in *Acanthamoeba* species from a hospital environment (23). More recently, intra-amoebic survival of *Burkholderia cepacia* has been demonstrated in *Acanthamoeba* strains in vitro (21). However, there has been no report of any interaction between *B. pseudomallei* and free-living amoebae to date, nor has there been any report of simultaneous recovery of *B. pseudomallei* and *Acanthamoeba* species from the same environmental location. During investigations into a recent cluster of acute melioidosis cases in Western Australia, the strain of *B. pseudomallei* responsible for the outbreak was isolated from potable water in the affected community (15). Several *Acanthamoeba* species were also recovered from water specimens collected during the initial outbreak and in further environmental investigations over the following year, prompting the

\* Corresponding author. Mailing address: Division of Microbiology and Infectious Diseases, PathCentre, Locked Bag 2009, Nedlands, WA 6009, Australia. Phone: 618 9 346 3461. Fax: 618 9381 7139. E-mail: tim.inglis@health.wa.gov.au.

TABLE 1. Cellular features observed during timed coculture of *B. pseudomallei* and *A. astronyxis*

Time (min)	Adherent bacilli <sup>b</sup>	No. of trophozoites <sup>a</sup>				
		Rotating bacilli <sup>c</sup>	Single-bacillus vacuoles <sup>d</sup>	Multibacillary vacuoles <sup>e</sup>	Bacillary tufts <sup>f</sup>	Bacillary tangles <sup>g</sup>
0	4.3 ± 1.5	1.7 ± 0.6	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20	12.3 ± 3.8	6.0 ± 2.6	1.7 ± 1.5	2.0 ± 3.5	0.0 ± 0.0	0.0 ± 0.0
40	13.7 ± 3.0	6.0 ± 3.0	3.7 ± 2.1	5.7 ± 5.1	0.3 ± 0.6	0.0 ± 0.0
60	12.0 ± 2.6	3.0 ± 2.0	4.0 ± 1.7	10.3 ± 3.5	1.0 ± 1.0	0.0 ± 0.0
1,440	20.0 ± 0.0	17.7 ± 3.2	3.3 ± 1.5	12.3 ± 0.6	16.3 ± 1.5	0.7 ± 0.6

<sup>a</sup> At each time interval, 20 trophozoites were examined for the presence of each indicated feature. Values shown are the mean ± standard deviation of the number of trophozoites exhibiting each feature from three consecutive replicate experiments.

<sup>b</sup> Bacilli adherent to external trophozoite surface.

<sup>c</sup> One or more adherent bacilli in high-frequency rotation around point on external trophozoite surface.

<sup>d</sup> One or more trophozoite vacuoles containing single bacilli.

<sup>e</sup> One or more trophozoite vacuoles containing more than one bacillus.

<sup>f</sup> Five or more bacilli in single tuft on external trophozoite surface.

<sup>g</sup> Bacilli in tangled web of chains external to trophozoite or cyst surface.

present study into possible interactions between *Acanthamoeba* species and *B. pseudomallei*.

#### MATERIALS AND METHODS

**Organisms.** The three amebic species used were *Acanthamoeba castellanii* CCAP 1534/1, *A. polyphaga* CCAP 1501/3A, and *A. astronyxis* CCAP 1534/1. These were obtained as axenic strains and maintained in axenic culture media (PYG broth; Excel Laboratory Products, Bentley, Western Australia, Australia) in tissue culture flasks incubated at 30°C. Culture media were changed weekly. The supernatant fluid was kept in a sterile plastic container at 20°C and used within 1 week of harvesting. Amebic suspensions were examined by bright-field and phase-contrast microscopy immediately prior to use. Viable trophozoite counts were obtained with a Fuchs-Rosenthal hemocytometer and trypan blue vital stain.

*B. pseudomallei* NCTC 10276 was used in all coculture experiments. NCTC 10276 was kept in 20% glycerol broth at -70°C and subcultured via 5% horse blood agar to Trypticase soy broth (Excel Laboratory Products), in which it was grown at 37°C for 18 h. In previous experiments, it had been found that the logarithmic phase of bacterial growth commenced 3 to 4 h after inoculation of the growth medium. The pellicle was lifted aside with a sterile loop and a 1.0-ml aliquot of bacterial suspension transferred to 9.0 ml of fresh Trypticase soy broth. The mixture was incubated at 37°C for 1 h to obtain rapidly motile bacteria in mid-log phase and then for 2 and 24 h. Bacterial motility was checked by examination of bacteria at ×400 magnification under phase-contrast illumination. All aerosol-generating procedures were carried out in a biological safety cabinet.

**Light microscopy.** After it was ensured that amebic cells were present in all three identifiable forms (rounded trophozoites, trophozoites with acanthopodia, and thick-walled cysts) and no bacterial contamination had occurred, a 10-μl drop of bacterial suspension was combined with a 10-μl drop of amebic suspension. The interaction was followed under phase contrast at ×400 magnification for 120 min. Separate aliquots were kept in sterile containers at 20°C for 24 h and then reexamined under phase-contrast conditions. Each bacterial-amebic challenge was repeated on at least three occasions with each of the *Acanthamoeba* spp. The interaction between *B. pseudomallei* and *A. astronyxis* was repeated on three further consecutive occasions, when aliquots were taken at time (*t*) = 0, 20 min, 40 min, 60 min, and 24 h. On each occasion, 20 intact trophozoites were examined for the presence of surface-adherent bacilli, rapidly rotating bacilli, vacuoles containing single bacilli, multibacillary vacuoles (>2 bacilli per vacuole), polar tufts of bacilli (at least five), and extracellular bacterial tangles.

The interaction was followed using a confocal laser scanning microscope with simultaneous differential interference contrast microscopy (Bio-Rad, Hemel-Hempstead, United Kingdom). The vital stains SYTO 9 (1 μl of 3.34 mM in dimethyl sulfoxide; Molecular Probes, Eugene, Oreg.) and propidium iodide (1 μl of 20 mM in dimethyl sulfoxide; Molecular Probes) were added to 1 ml of microbial suspension and incubated for 5 min to assess bacterial viability in unfixed, wet-mounted preparations. Viable cells fluoresced bright green, and nonviable cells fluoresced red. Bacterial suspensions were centrifuged for 5 min at 1,000 × *g* and resuspended in sterile 0.89% NaCl solution; 24-h-old coinoculated preparations were stained by direct addition of 1 μl of 3.34 mM SYTO 9 to 10 μl of cell suspension without a centrifugation or washing step. The internal structure of amebic cells was examined with the microscope's optical sectioning facility to confirm the intracellular location of bacteria. Time-lapse confocal microscopy was used to confirm movement of bacteria within amebic vacuoles.

**Transmission electron microscopy.** After confirmation of phagocytosis by phase-contrast microscopy, suspensions of *B. pseudomallei* cocultured with *A. astronyxis* were centrifuged at 300 × *g* for 10 min. The supernatant was removed by pipette and replaced with fresh 2.5% glutaraldehyde. Cell suspensions were

fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 24 h, washed several times in 0.2 M cacodylate buffer (pH 7.4), resuspended in 10% albumin solution, placed in a microcentrifuge tube, and centrifuged; the resultant sediment of cells was fixed in 2.5% glutaraldehyde for a further 24 h. The tip of the tube was cut with a single-edged blade, and the block containing cells was postfixed in 1% osmium tetroxide, dehydrated in graded solutions of ethanol, infiltrated with Araldite, and embedded in polyethylene capsules. After retrimming of each block face, 50-nm ultrathin sections were cut, mounted on 200-mesh thin-bar copper grids, double stained in uranyl acetate and lead citrate, and examined in a Philips 410LS transmission electron microscope at an accelerating voltage of 80 kV.

**Recovery of intracellular bacteria.** Intracellular bacteria were recovered by a kanamycin-trophozoite lysis procedure as follows. A 5.0-ml coculture volume was set up with a suspension of 10<sup>5</sup> of trophozoites per ml in 0.9 N NaCl and a 1-h preparation of rapidly motile *B. pseudomallei* at 10<sup>7</sup> CFU/ml. Aliquots of 1.0 ml were removed at *t* = 0 and *t* = 1 h. These were centrifuged at 300 × *g* for 5 min, and the pellet was resuspended in 0.9 N NaCl with 100 μg of kanamycin sulfate (Sigma, St. Louis, Mo.) per ml. After 2 h of incubation at 20°C, the aliquot was centrifuged again at 300 × *g* for 5 min and resuspended in sterile, demineralized water. The sealed container was incubated in a waterbath at 37°C for 30 min and then vortex mixed for 30 s and sonicated for 15 min. The suspension was then centrifuged at 1,000 × *g* for 5 min and resuspended in 0.9 N NaCl. This suspension was spread in 50-μl aliquots on plate count agar in triplicate, using a spiral plating device. The remaining 850 μl was spread on a further plate count agar plate to detect very low bacterial counts. The same plating procedure was used with aliquots of the uninoculated amebic stock suspension, sterile demineralized water, and 0.9 N NaCl as negative controls. Plates were incubated in air at 37°C for 48 h before counting using a standard template (Don Whitley). Viable counts were obtained using the spiral plater interpretive tables. Viable counts were also performed at *t* = 0 and *t* = 60 min after inoculation of 0.9% NaCl with a 1-h *B. pseudomallei* preparation to determine whether bacterial growth occurred in the coculture medium during this period.

**Statistical methods.** Descriptive statistics and the unpaired *t* test were calculated with the assistance of statistical software (Prism, version 2.01; GraphPad Software, San Diego, Calif.).

#### RESULTS

**Early bacterium-ameba interactions.** When *B. pseudomallei* was added to a suspension of *A. astronyxis*, adhesion, endocytosis, and vacuole formation were all observed, in that order. The sequence of cellular events could be observed in individual amebic trophozoites and occurred in a 1-h time frame when a 1-h preparation of highly motile *B. pseudomallei* was added to acanthopodium-bearing *A. astronyxis* trophozoites. This sequence of events was quantitated in the timed coculture procedure where bacterial adhesion to the trophozoite surface and rapid bacillary rotation were followed by the appearance of vacuoles containing single bacilli, then multibacillary vacuoles, and finally formation of external bacillary tufts (Table 1). In the initial adhesion event, one end of a highly motile bacillus attached to the cytoplasmic membrane of the amebic trophozoite (Fig. 1a). In most instances, this was followed immediately by high-frequency helicopter blade rotation of the entire



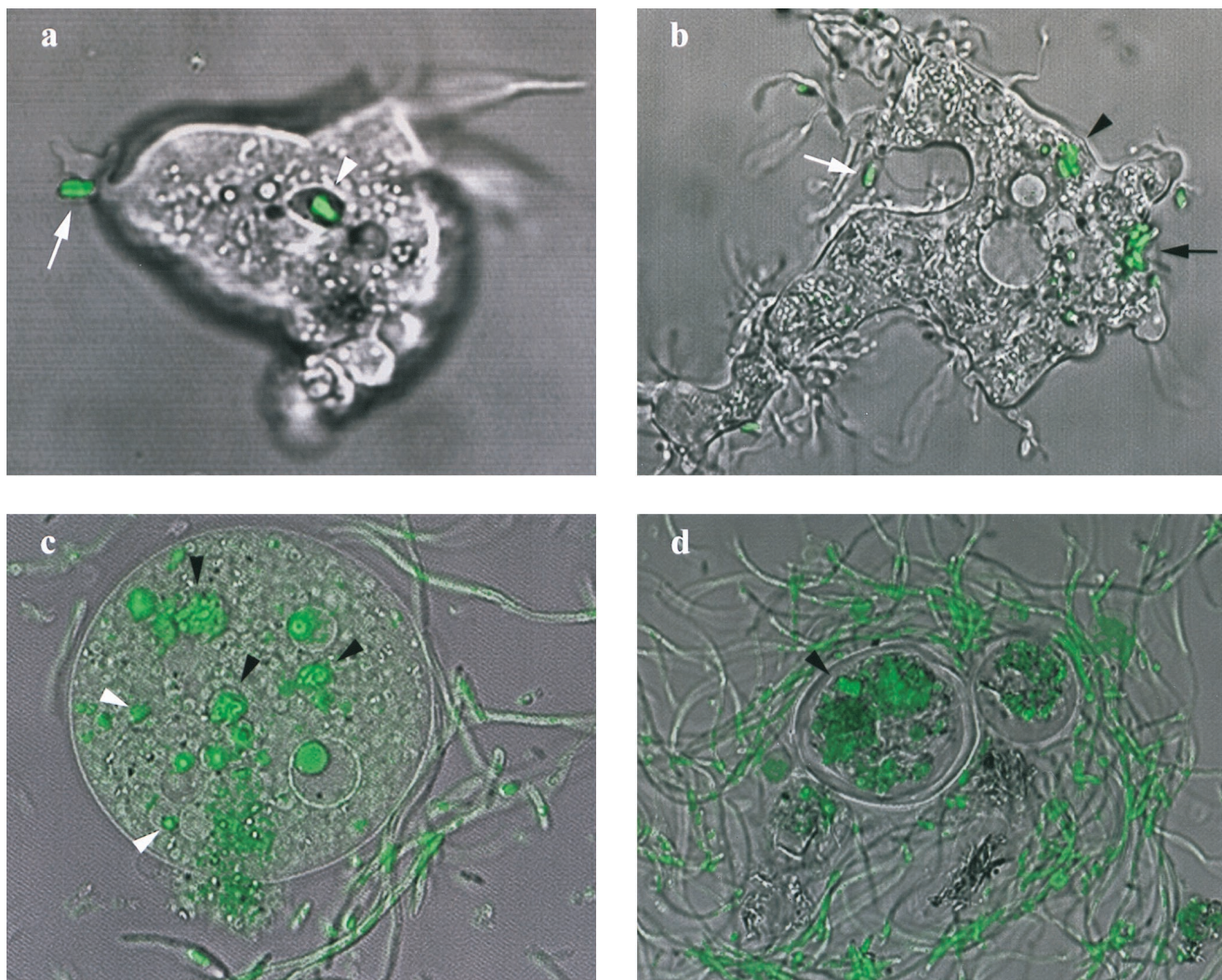


FIG. 1. Confocal laser scanning microscope images of *B. pseudomallei*-*A. astonyxis* coculture stained with epifluorescent dyes SYTO 9 and propidium iodide, superimposed on differential interference contrast images. (a) Adhesion of polar end of bacillus to trophozoite surface (white arrow) leading to inclusion in vacuole (white arrowhead) (magnification,  $\times 1,440$ ); (b) pseudopodia moving to enclose bacillus (white arrow), parasitophorous vacuoles in same trophozoite (black arrowhead), and external bacillary tuft (black arrow) ( $\times 1,220$ ); (c) amebic trophozoite with free cytoplasmic bacilli (white arrowheads) and intact parasitophorous vacuoles (black arrowheads) undergoing rupture of external cytoplasmic membrane ( $\times 1,130$ ); (d) after 24 h of cocubation, bacilli incorporated in amebic cyst (black arrowhead) and surrounded by bacillary tangle ( $\times 1,220$ ). Confocal microscope images were merged and processed using Confocal Assistant, version 4.02 (Bio-Rad) and Photoshop, version 5.0 (Adobe Systems Inc.).

bacillus around a fixed point on the trophozoite surface. This lasted no more than a few seconds. Shortly after bacillary rotation ended, adjacent pseudopodia curled round to enclose the bacillus. Formation of a complete cytoplasmic bridge around the bacillus resulted in its inclusion within a membrane-lined vacuole. Pseudopod extension and curling were fast enough to observe in real time. These events occurred repeatedly in individual trophozoites, resulting in a collection of bacterium-containing vacuoles (Fig. 1b). No endocytosis by fully rounded trophozoites or cysts was observed. Using a 1-h motile bacterial preparation, intracellular bacilli were easily demonstrated by uptake of the fluorescent stain SYTO 9 (Fig. 1). Multiple vacuoles packed with bacilli were observed inside rounded intact trophozoites. No green fluorescent bacilli were observed moving freely in the cytoplasm of intact trophozoites. Some intravacuolar bacteria were seen to retain their motility for 1 to 2 h of continuous observation after initial vacuole formation.

When *B. pseudomallei* was incubated at  $37^{\circ}\text{C}$  for 2 h, either

no or very few bacteria exhibited rapid motility. Very few bacteria from these 2-h preparations attached to the trophozoite surface, and endocytosis was extremely rare. Stationary-phase bacteria obtained after 24 h of incubation at  $37^{\circ}\text{C}$  attached to the external trophozoite surface and rotated but only a few single bacteria vacuoles were formed.

On transmission electron microscopy, pseudopodia were found in contact with one end of each adherent bacillus minutes after the start of coculture (Fig. 2a). Membrane-lined vacuoles containing single bacilli were found inside amebic trophozoites. In some of these vacuoles, bacteria were surrounded by multilamellate membranes consistent with the pseudopodial coils or stacks seen in coiling phagocytosis (Fig. 2b). Membrane-lined vacuoles containing multiple bacilli were found inside amebic trophozoites after 30 min of coculture. Some bacterium-laden vacuoles were apposed to the external trophozoite surface (Fig. 2c), while in other trophozoites multiple bacterium-laden vacuoles could be seen (Fig. 2d).



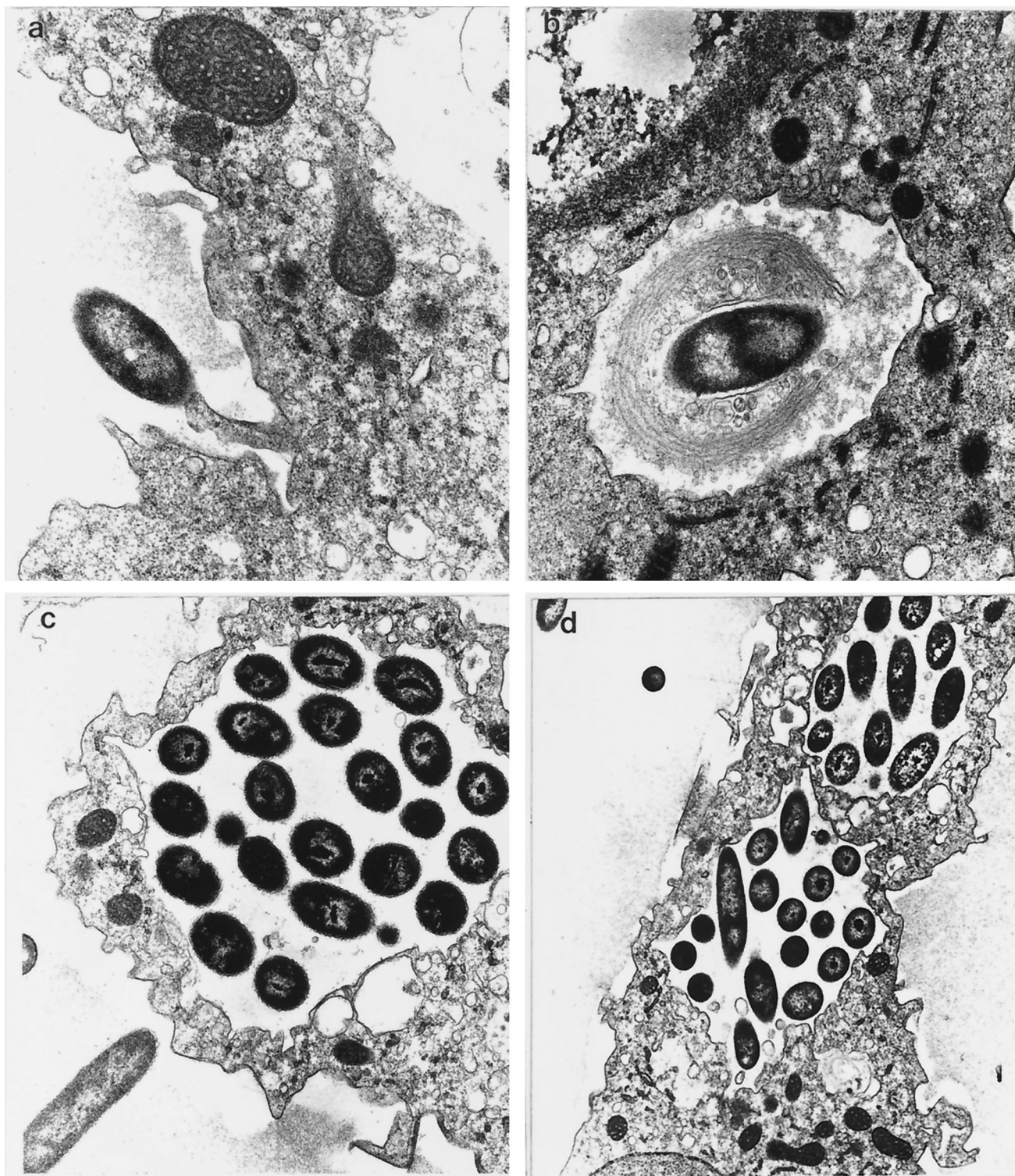


FIG. 2. Transmission electron micrographs of *B. pseudomallei*-*A. astronyxis* coculture. (a) Pseudopodium in contact with polar end of bacillus shortly after start of coculture (magnification,  $\times 18,700$ ); (b) membrane-lined vacuoles containing single bacillus inside amebic trophozoite, surrounded by multilamellate membrane ( $\times 18,700$ ); (c) membrane-lined vacuole containing multiple bacilli apposed to external trophozoite surface ( $\times 12,800$ ); (d) multiple bacterium-laden vacuoles formed after 60 min of coculture ( $\times 8,000$ ).

**Bacterium-ameba interactions after 24 h of coculture.** Twenty-four hours after the start of coculture preparations with bacteria from 2-h preparations, motile bacilli were observed inside endamebic vacuoles that contained three or

fewer bacilli. However, in cocultures using bacteria from 1-h preparations, amebae became enclosed in clumps or tangles of bacterial filaments not previously seen in any *B. pseudomallei* monoculture (Table 1; Fig. 1c and d). Many actively motile

intracellular bacilli were present and were observed up to 72 h after coculture began (the maximum period observed). Vortex mixing of coculture suspensions released amebic cysts from bacillary tangles. Bacilli were observed in only a small proportion of amebic cysts (Fig 1d). Some rounded trophozoites were present. These contained vacuoles packed with highly motile bacilli, restricted by the vacuolar membrane (Fig. 1c). Vacuoles packed with bacilli were often observed adjacent to the external plasma membrane. In some cases these protruded or were in the process of releasing bacilli to form an external tuft similar in appearance to the polar tuft formed during endocytosis. In a few trophozoites, single bacilli moved without restriction throughout the cytoplasm outside any vacuoles (Fig. 1c). Trophozoite lysis with spillage of cytoplasmic contents occurred shortly afterwards. These phenomena were not observed in cocultures containing 2-h bacterial preparations or lacking stellate, acanthopodium-bearing trophozoites.

**Coincubation with other *Acanthamoeba* species.** Some of the early features of bacterial endocytosis by *A. astronyxis* were demonstrated by repetition of the coculture procedure under optimal conditions using *A. castellani* and *A. polyphaga*. However, as vacuolation was less pronounced in these species than in *A. astronyxis*, the sequence of events was more difficult to deduce. No bacterium-containing vacuoles or external bacillary tufts were observed in either species. Initial phagocytosis and production of bacillary tangles were all demonstrated more clearly with *A. castellani* and *A. polyphaga* when challenged with *B. pseudomallei* at 37°C.

**Recovery of intracellular bacteria.** A mean bacterial count of 13 CFU/ml ( $\pm 6.7$ ) was obtained from three replicates from an aliquot taken immediately after commencement of coculture. After 60 min, the mean intracellular bacterial count from three replicates was 507 CFU/ml ( $\pm 76.9$ ) ( $t = 6.39$ ,  $df = 4$ ,  $P = 0.0031$ ). No growth was obtained from saline and sterile water controls. The mean viable count of *B. pseudomallei* in control coculture medium was  $3.6 \times 10^6$  ( $\pm 2.7 \times 10^5$ ) CFU/ml at  $t = 0$  and  $3.9 \times 10^6$  at  $t = 60$  min ( $\pm 1.1 \times 10^6$ ). There was no statistically significant difference between viable counts of controls at  $t = 0$  and  $t = 60$  min in coculture medium.

## DISCUSSION

An interaction between *B. pseudomallei* and *Acanthamoeba* spp. has not been described previously. In this investigation of a possible interaction between the two species, we observed coiling phagocytosis, the survival of *B. pseudomallei* in amebic vacuoles, and eventual bacterial escape from vacuoles into amebic cytoplasm and then into the surrounding medium. The optimal conditions for cellular interaction were dependent on the condition of both components of the coculture. A combination of rapidly motile bacilli and acanthopodium-bearing amebae was required to obtain a full range of cellular interactions.

The ability of *B. pseudomallei* to cause relapsing and late-onset infection up to 26 years after initial exposure has been attributed to an ability of this species to survive in macrophages (17, 24, 27). Intramacrophage survival may be helped by an ability to tolerate prolonged periods of nutrient deprivation and relatively acid conditions as is known to occur with *B. pseudomallei* (18, 32). It is therefore notable that we were able to recover viable bacteria from amebic trophozoites 1 h after endocytosis and were able to observe motile intracellular bacilli for up to 72 h after coculture began. Similar investigations into other facultative intracellular pathogens such as *L. pneumophila* have emphasized intracellular replication (2, 7). The formation of extracellular bacillary tufts and tangles provides a major methodological obstacle to using the conven-

tional approach to determining whether or not *B. pseudomallei* is capable of intraamebic replication. Moreover, the acidic conditions expected in amebic vacuoles may have rendered intracellular bacilli viable but not immediately culturable, as proposed recently (16), further confounding quantitative bacteriology. If late-onset endamebic replication of *B. pseudomallei* occurs, as has been shown recently with the related species *B. cepacia* (21), its demonstration will depend on advanced laboratory methods such as those developed recently for investigation of *M. avium-Acanthamoeba* interactions (28). Nevertheless, the current lack of evidence for endamebic replication of *B. pseudomallei* does not detract from the analogy between in vitro survival in endamebic vacuoles and survival in mammalian macrophages.

The coexistence of many intact trophozoites with dense clusters, tangles, or mats of *B. pseudomallei* shows that these species are capable of a nondestructive relationship. Furthermore, the small numbers of intracellular bacteria recovered after 60 min of coincubation with many times that number of bacteria implies a degree of control over bacterial access to the interior of the trophozoite. The observation of coiling phagocytosis, a process noted to occur during endocytosis of *L. pneumophila* by both *Acanthamoeba* and human monocytes (3), shows another possible connection between amebic endocytosis and mammalian phagocytosis of *B. pseudomallei*. The asymmetric, coiling extension of phagocytic pseudopodia by human mononuclear cells was one of the first pieces of evidence linking amebic endocytosis of *Legionella* to phagocytic events during *L. pneumophila* infection (14). Coiling phagocytosis of *B. pseudomallei* by mammalian macrophages has yet to be reported. If the analogy with *L. pneumophila* infection holds, the recent observation that prior intra-amebic bacterial infection enhances monocyte entry and virulence provides a possible experimental approach to the early stages of cellular interactions (6). The vacuolar and amebic escape of bacilli observed during the later stages of coculture further demonstrates the viability of intracellular bacteria. However, its relevance to human *B. pseudomallei* infection is speculative since the escape of sequestered bacilli from macrophages in vivo can only be inferred from bacterial replication that must occur in late onset or relapsing infections.

The cellular events that span the interaction between *B. pseudomallei* and amebic trophozoites bear some resemblance to phenomena previously observed in *Legionella-Acanthamoeba* cocultures (2, 3, 26). The ecological relationship between these two genera has been described as an endosymbiosis. A commensal relationship between *B. pseudomallei* and *Acanthamoeba* is possible in shared environmental habitats, but as we have yet to demonstrate their simultaneous occurrence in vivo or show a mutual codependency, it is not possible to describe the interaction as a true symbiosis. By analogy with *L. pneumophila* (1–3, 6, 7, 11, 26), it is possible that the capacity of *B. pseudomallei* to enter, survive within, and exit free-living amebae confers an ability to invade mammalian phagocytic cells, persist within, and escape from them as is thought to occur in relapsing and late-onset melioidosis. By the same token, it is also possible that there are key differences between the mechanisms used during amebic cytoinvasion compared with penetration of human macrophages (12, 13).

The observation of rapid rotation of the entire bacillus while anchored to a fixed point on the external trophozoite surface implies flagellar adhesion. It is of note that mutagenesis and animal model experiments highlight a possible role for the flagellum in the pathogenesis of *B. pseudomallei* infection (10). Other aspects of the initial interaction between *B. pseudomallei* and the amebic trophozoite that warrant further attention include expression of acid phosphatase, an enzyme produced by



*B. pseudomallei* under acid conditions and thought to be a virulence factor for intracellular bacterial pathogens (9). This enzyme has been identified as a tyrosine phosphatase (19). It is therefore of note that tyrosine phosphatase activity has been implicated recently in attachment of *L. pneumophila* to another free-living protozoan, *Hartmannella vermiformis* (30).

Exposure to *B. pseudomallei*-contaminated soil or water in the region where the organism is endemic is thought to be the principal means of exposure to infection (5, 15, 29). A detailed appraisal of the possible ecological significance of a *B. pseudomallei*-*Acanthamoeba* interaction in vivo is beyond the scope of this investigation. Nevertheless, the remarkable ability of *B. pseudomallei* to persist in hostile environmental niches could be explained in part by an ability to survive in free-living amoebae whose normal habitat lies within the vadosphere and in particular at air/water interfaces in the soil (25). It is possible that detailed study of the microbial ecology of the region where the organism is endemic may provide evidence for a putative role for free-living phagocytic protozoa in the pathogenesis of melioidosis. The observation that *Acanthamoeba* species such as *A. astronyxis* can be recovered from the human nasal mucosa (22) make inhalation of endamebic *B. pseudomallei* a little more plausible and provides a further avenue of investigation.

In conclusion, we have examined the interaction between cells of the facultative intracellular pathogen *B. pseudomallei* and several *Acanthamoeba* species. Nondestructive intracellular passage of bacteria and bacterial escape during trophozoite lysis were demonstrated. As yet it is unclear whether these are mutually exclusive or the extremes at either end of a range of outcomes. Whichever interpretation proves to be correct, these observations indicate that *B. pseudomallei* can adapt to a free-living intracellular habitat such as may be found in acanthamoebae. The successful demonstration of an interaction between *B. pseudomallei* and *A. astronyxis* that includes coiling phagocytosis and nondestructive bacterial passage via trophozoites may help attempts to demonstrate similar phenomena in mammalian phagocytic cells. Endamebic passage of *B. pseudomallei* deserves further attention as a possible in vitro model of intracellular *B. pseudomallei* infection.

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