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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Acanthamoeba release compounds which promote growth of *Listeria monocytogenes* and other bacteria

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Abstract *Listeria monocytogenes* can grow as a saphrophyte in diverse habitats, e.g., soil, rivers, lakes, and on decaying plant material. In these environments, the bacteria are frequently exposed to predatory protozoa such as *Acanthamoeba*. Although *L. monocytogenes* is a facultative intracellular pathogen it does not infect or survive intracellular in *Acanthamoeba castellanii*, unlike several other facultative intracellular bacteria. Instead, motile *L. monocytogenes* can form large aggregates on amoebal cells and are effectively phagocytosed and eventually digested by *Acanthamoeba*. Here, we demonstrate that non-motile *L. monocytogenes* represent a less preferred prey in co-cultures with *A. castellanii*. Moreover, we found that the presence of *Acanthamoeba* strongly promotes growth of the bacteria in non-nutrient saline, by releasing nutrients or other growth promoters. Thus, the lack of motility and ability to utilize amoebal metabolites may aid to avoid eradication by amoebal predation in low-nutrient environments.

Keywords *Listeria monocytogenes* · *Acanthamoeba castellanii* · Co-culture · Growth promotion · Secreted metabolites

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Introduction

The genus *Listeria* is comprised of Gram-positive, rod-shaped, non-spore-forming bacteria currently separated into ten species (Bertsch et al. 2013; Lang Halter et al. 2013). *Listeria monocytogenes* is pathogenic for humans and the causative agent of listeriosis, a disease which can lead to meningitis, meningoencephalitis, or septicemia. Together with *Listeria ivanovii*, an animal pathogen, *L. monocytogenes* is the only representative that exhibits a facultative intracellular life style. Both organisms seem to be highly adapted to a facultative intracellular lifestyle in mammals, birds, and possibly other animals. The hallmarks of *Listeria* pathogenicity and virulence have been intensively studied, and are reviewed and summarized elsewhere (Cossart 2011).

Listeria spp. can be ubiquitously found in natural environments where they exhibit a saprophytic lifestyle. They can be isolated from soil, vegetation, decaying plant material, rivers, lakes, ponds, and sewage (Freitag et al. 2009). Saprophytic growth properties, long-term survival, interaction with potential host organisms, and the ecology of free living *Listeria* are not yet fully understood. However, genome analyses provided valuable insights into biochemical pathways and features. Many strains of *L. monocytogenes* feature genes for uptake and utilization of various carbohydrates including fructose, rhamnulose, rhamnose, glucose, mannose, sucrose, cellulose, pullulan, trehalose, tagatose, maltose, and maltodextrin. Moreover, a vast set of diverse regulatory proteins indicates its ability to adapt to diverse environmental conditions (Glaser et al. 2001). An additional factor which could contribute to saprophytic growth of *L. monocytogenes* might be its ability to hydrolyze chitin (Leisner et al. 2008), a major source of carbon and nitrogen in nature. Moreover, *Listeria* tolerate a wide pH range (4.5–9.0), can grow at low temperatures, and effectively combat osmotic stress (Schmid et al. 2009).

In moisture-rich natural environments, saprophytically growing *Listeria* are also exposed to predatory protozoa such as *Acanthamoeba* (Zhou et al. 2007; Gourabathini et al. 2008; Huws et al. 2008; Anacarso et al. 2012). Amoebae are unicellular eukaryotes, which can exist as vegetative trophozoites, or persist as metabolically inactive dormant cysts. The trophozoites are motile, exhibit a characteristic amoeboid movement on surfaces, and are organized in a polar fashion with two distinct cell poles. The front (anterior) pole features the pseudopodia, and the rear (posterior) pole is termed uroid.

Unlike several other facultative intracellular bacteria, *L. monocytogenes* cannot infect *Acanthamoeba* or survive phagocytosis (Akya et al. 2009a). Instead, the normally motile cells can be trapped by the amoebae employing a unique mechanism, resulting in rapid formation of large aggregates termed "backpacks" at the uroid, followed by phagocytosis (Doyscher et al. 2013). In contrast, non-motile *L. monocytogenes* remain free. Notably, this process is not specific for *Listeria*, other motile bacteria can also be trapped by the amoebae the same way (Doyscher et al. 2013). It seems clear now that *L. monocytogenes* is unable to escape from the amoebal phagolysosome and therefore cannot enter the cytosol.

The aim of this study was to better understand the interaction and microbial ecology of *Acanthamoeba* and *Listeria*, using defined co-cultivation conditions. Besides the observation that non-motile *Listeria* are less preferentially phagocytosed, we found that growth of the extracellular bacteria is strongly promoted by the presence of the amoebae under co-cultivation conditions which is likely due to diffusible amoebal metabolites used as nutrient source by the bacteria.

Materials and methods

Growth conditions

All bacterial strains were cultured in half-strength Brain Heart Infusion (1/2 BHI) broth. Incubation at 30 °C was performed for *Listeria monocytogenes* EGDe ATCC BAA-679 and *Listeria monocytogenes* ScottA ATCC 49594, *Listeria grayii* ATCC 19120, *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC 19119, *Listeria seeligeri* ATCC 35967, *Listeria welshimeri* ATCC 35897, *Bacillus subtilis* DSM 675, *Brevibacterium epidermis* DSM 20660, *Brochothrix thermosphacta* DSM 20171, and *Corynebacterium ammoniagenes* DSM 20306. *Cronobacter sakazakii* ATCC 29544, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* DSM 1117, *Staphylococcus aureus* DSM 346, *Staphylococcus carnosus* DSM 20501, and *Staphylococcus epidermidis* DSM 1798 were cultivated at 37 °C. Viable counts were determined by plating appropriate dilutions on agar plates.

Acanthamoeba castellanii strain Neff was provided by Matthias Horn (University of Vienna, Austria). Cultures were maintained axenic as monolayers in 25-cm^2 culture flasks (Nunc, Roskilde, Denmark), in peptone yeast glucose (PYG) broth (20 g/L protease peptone, 18 g/L glucose, 2 g/L yeast extract, 1 g/L sodium citrate $2H_2O$, 980 mg/L $MgSO_4 \cdot 7H_2O$, 452 mg/L $Na_2PO_4 \cdot 12H_2O$, 340 mg/L KH_2PO_4, and 20 mg/L $Fe(NH_4)_2(SO_4)_2$ $6H_2O$), at room temperature. *Acanthamoeba castellanii* was subcultured in fresh medium once a week, and cell counts were determined using a Neubauer improved counting chamber as previously described (Doyscher et al. 2013).

Co-culture conditions

Prior to co-cultivation of amoebae and bacteria, *A. castellanii* was harvested from a PYG culture and washed three times with a non-nutrient saline buffer (PAS: 0.12 g/L NaCl, 2.5 mg/L $MgSO_4 \cdot 2H_2O$, 4 mg/L $CaCl_2 \cdot 2H_2O$, 358 mg/L $Na_2PO_4 \cdot 12H_2O$, and 136 mg/L KH_2PO_4), by gentle centrifugation at 1,000×*g* for 10 min. No additional sources of carbon or nitrogen were added to the PAS. For co-cultivation, 1×10^5 trophozoites of *A. castellanii* were seeded in 24-well plates (Nunc, Roskilde, Denmark), followed by the addition of 1×10^4 to 5×10^6 colony forming unites (cfu) of the bacteria. The cultures were incubated at 24 °C or 37 °C, for 3 to 5 days. Sterile filtered supernatants were obtained by centrifugation (1,000×*g*, 5 min) of cultures and subsequent filtration using Filtropur S 0.2 μm pressure filtration units (Sarstedt, Nümbrecht, Germany). In order to obtain supernatant from heat-inactivated co-cultures the cultures were incubated for 10 min at 100 °C prior to centrifugation.

Ultrafiltration of co-cultures of *Acanthamoeba* and *Listeria* was performed by centrifugation in ultrafiltration tubes with cut-off values between 5 and 100 kDa (Vivaspin 500, Sartorius, Göttingen, Germany) according to the manufacturer.

In a different experimental setup, transwell inserts (cell culture grade, 0.4 μm pore-size membrane) were used in 24-well plates (Nunc, Roskilde, Denmark), in order to physically separate the bacteria from *A. castellanii*. In this modified assay, the bacteria were inoculated into the insert, while *A. castellanii* trophozoites were seeded into the bottom of the wells.

All experiments were performed in triplicates, and independently repeated three times. Mean values and standard deviations of cell counts were calculated using Microsoft Excel software.

Spectrophotometry

To determine changes in adsorption spectra of *Acanthamoeba* and *Listeria* PAS supernatants, respectively, without further

sample purification we used a Nanodrop 1000 spectrophotometer (Thermo Scientific).

Results

Co-culture of *A. castellanii* with non-motile *L. monocytogenes* promotes bacterial growth

A. castellanii preys on motile bacteria employing a unique trapping mechanism (Doyscher et al. 2013). Here, we monitored the growth response of untrapped extracellular *L. monocytogenes* during co-culture with *A. castellanii* in non-nutrient saline buffer. In *L. monocytogenes*, flagellation and motility are temperature dependent. Below 37 °C, *Listeria* cells feature peritrichous flagella and are highly motile. However, at temperatures of 37 °C or higher, the regulatory factor MogR represses expression of the *fla* operon encoding the structural components of the flagellum (Shen and Higgins 2006). Figure 1 depicts that *L. monocytogenes* cells resuspended in non-nutrient PAS only were unable to grow, and viable counts actually declined over the time course of the experiment. In contrast, bacteria showed significant growth response and increase in viable counts by 2–3 logs after 4 days of co-incubation with *A. castellanii*. We also found that non-motile *L. monocytogenes*, i.e., either wild-type cells incubated at 37 °C, or *ΔflaA* mutants multiplied even faster compared to flagellated and motile wild-type bacteria. It should be noted that trophozoites of *A. castellanii* remained stable during co-culture with *Listeria*, and did not develop into cysts until the end of the experiment (data not shown).

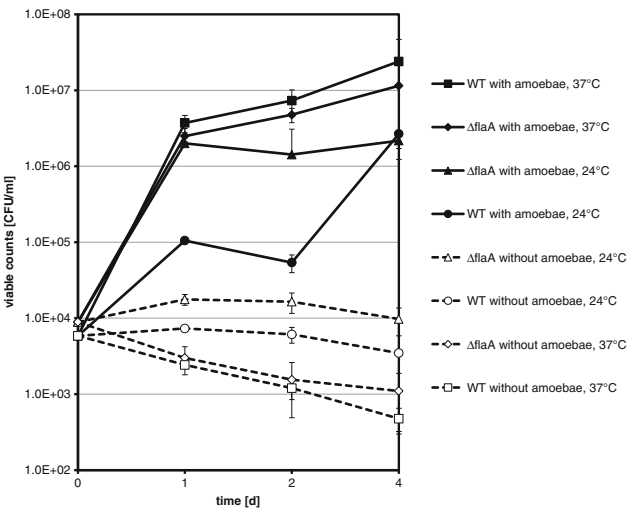

Fig. 1 Growth response of *L. monocytogenes* wild-type (*WT*) and flagella-deficient (*ΔflaA*) mutant bacteria in the presence (*solid lines*) and absence (*dashed lines*) of *A. castellanii* in co-culture, at 24 and 37 °C, respectively. Colony forming units (*CFU*) represent viable counts of extracellular bacteria. As a control, PAS buffer alone was used

Compounds released from *A. castellanii* are responsible for bacterial growth promotion

To determine whether the effect is independent from direct contact of the bacteria and protozoa, we designed experiments to physically separate the organisms while allowing access of the bacteria to diffusible substrates released from the amoebae. For this purpose, transwell microplate filter inserts were used to expose *L. monocytogenes* to *A. castellanii* trophozoites in PAS co-culture assays. Figure 2 illustrates that *L. monocytogenes* grew and multiplied well, indicating that direct contact between *L. monocytogenes* and the amoebae is not required to enhance growth of the bacteria. In controls without transwell inserts, total viable counts of *L. monocytogenes* remained lower, which is due to continuous phagocytosis of the bacteria by the amoebae. Again, *L. monocytogenes* was unable to grow in PAS only. In conclusion, these findings indicated that *A. castellanii* releases metabolites or other compounds, which could be efficiently used as nutrients by *L. monocytogenes* enabling growth and multiplication by up to 2.5 logs.

To exclude the possible carry-over of nutrients or other compounds from the PYG medium used for axenic amoebal cultures, we conducted an additional experiment where *L. monocytogenes* was added to starving *A. castellanii* trophozoites (Fig. 3). For this purpose, trophozoites were carefully washed and incubated for 24 h in PAS without supplementation with carbon or nitrogen sources, prior to the addition of *L. monocytogenes* cells. Although under these conditions the onset of growth of *L. monocytogenes* was retarded, i.e., revealed an increased lag-phase, the bacteria eventually multiplied by 1.6 logs after 4 days of co-incubation. This demonstrated that growth of *L. monocytogenes* is solely based upon compounds released by the amoebae, and not due to leftovers from the protozoan culture medium.

In an attempt to preliminarily characterize the compounds/metabolites released by the amoebae, spectrophotometry was employed for spectral analysis of PAS supernatants from *Acanthamoeba* and *Listeria* cultures, respectively. A prominent absorption peak at 210–215 nm from axenic *Acanthamoeba*-PAS supernatants was absent from axenic *Listeria*-PAS supernatants. To further characterize the nature of the growth-promoting compound native supernatant from a pure PAS culture of *A. castellanii* without *Listeria*, as well as supernatant from a heat-treated (10 min, 100 °C) pure PAS culture of *A. castellanii* was applied for growth of *L. monocytogenes* cells resuspended in nutrient-free PAS. Determination of CFU/ml for *L. monocytogenes* after 24 h revealed that both supernatants provided the same growth-supporting effect as the native supernatant from co-cultures of *L. monocytogenes* and *A. castellanii*. *Listeria monocytogenes* in PAS without addition of supernatant showed no growth. To obtain information about the size of the active compounds supernatants of co-cultures of *Acanthamoeba* and *Listeria*

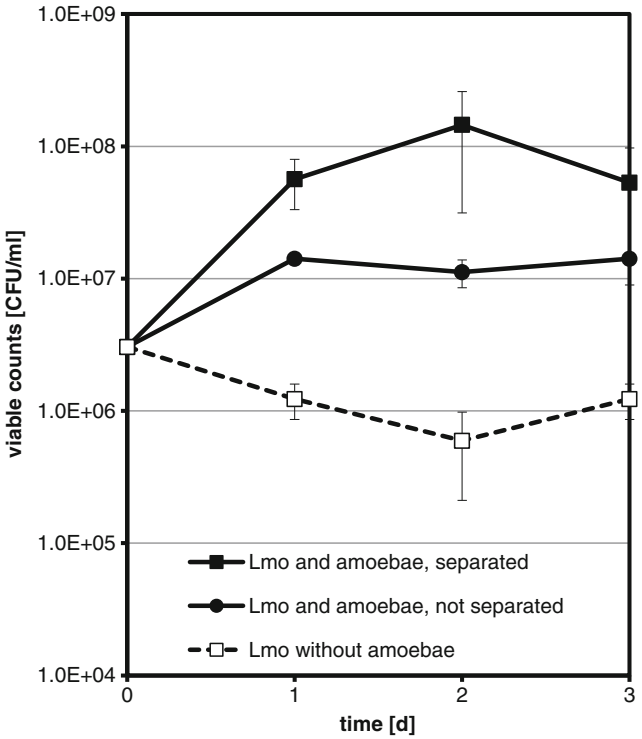

Fig. 2 Growth response of *L. monocytogenes* (*Lmo*) in direct (*not separated*) and indirect (*separated*) co-culture with *A. castellanii*. To physically separate the bacteria from the amoebae cell culture, filter-based transwell inserts were used (see "Materials and methods"). As a control, PAS buffer alone was used

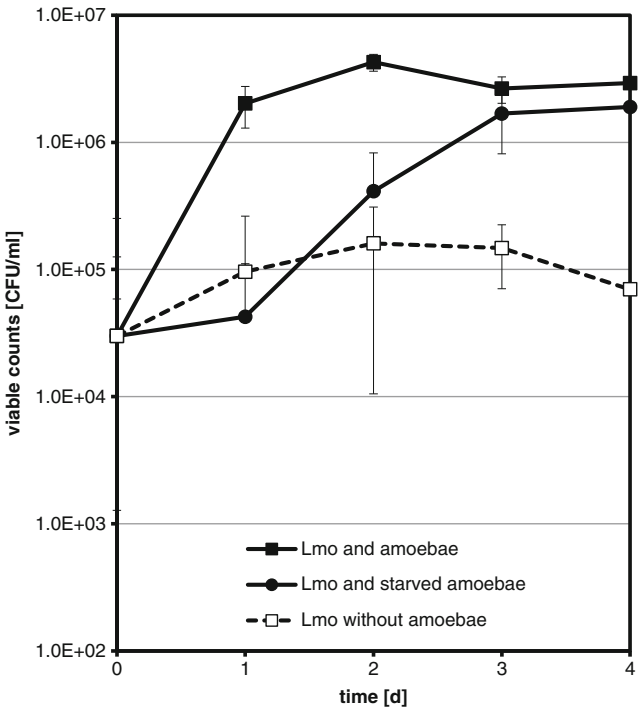

Fig. 3 Growth of *L. monocytogenes* (*Lmo*) in different supernatants from co-culture and single cultures of *A. castellanii* and *L. monocytogenes*, respectively. As a control, PAS buffer alone was used

were treated by ultrafiltration using tubes of various cut-off values from 5 to 100 kDa. It turned out that even the filtrate from the smallest ultrafiltration unit (5 kDa) supported growth of *L. monocytogenes* ScottA to more than three orders of magnitude after 24 h. These results indicated that the growth-promoting compounds are a matter of heat-stable molecules with a molecular weight below 5 kDa.

Growth-promoting effect is not specific for *Listeria*

From co-cultures of *A. castellanii* with other bacteria, it became obvious that utilization of the secreted amoebal metabolites was not restricted to *L. monocytogenes* or other *Listeria* species, which exhibited similar growth-stimulating properties in co-culture with *A. castellanii*. In brief, *Listeria ivanovii*, *L. grayi*, and *L. welshimeri* multiplied by approximately 1.0 log, *L. seeligeri* by 1.7 logs, and *L. innocua* by 2.7 logs (data not shown). Additional experiments demonstrated that also other bacteria were able to utilize the compounds released by *A. castellanii*. While *E. coli* multiplied by approximately 1 log after 4 days of co-incubation, *B. thermosphacta, C. sakazakii* and *S. aureus* showed a strong response, i.e., more than 3 logs increase in viable counts after 4 days of incubation (Fig. 4). In contrast, *Bacillus subtilis*, *B. epidermidis*, *C. ammoniagenes*, *P. aeruginosa*, *S. carnosus*, and *S. epidermidis* did not show any growth enhancement in co-culture with *A. castellanii* (data not shown).

Discussion

Following some controversy, recent studies demonstrated that the facultative intracellular pathogen *L. monocytogenes* is unable to infect and multiply in *Acanthamoeba* spp. (Akya et al. 2009a; Huws et al. 2008; Akya, Pointon and Thomas 2010). Instead, *L. monocytogenes* is phagocytized and digested by the amoebae (Huws et al. 2008; Akya et al. 2009a; Akya et al. 2010; Doyscher et al. 2013). *Acanthamoeba* exhibit a unique trapping strategy to feed on bacterial prey. At the posterior cell pole (the uroid), bacteria are bound in a motility-dependent manner and form large extracellular aggregates on the amoebal surface, termed backpacks (Doyscher et al. 2013). Soon thereafter, the amoebae start to phagocytose portions of the attached bacteria, diminishing the aggregate in a stepwise fashion. However, only flagellated and motile bacteria attach to the uroid during co-cultivation, while non-motile bacteria remain free. This observation was not restricted to *Listeria*, since other motile bacteria could also be trapped (Doyscher et al. 2013). Surprisingly, non-motile, extracellular cells were less prone to predation, and were found to not only persist, but even multiply in the presence of the amoebae. Growth of *L. monocytogenes* was also reported in co-culture with other protozoa such as *Tetrahymena*

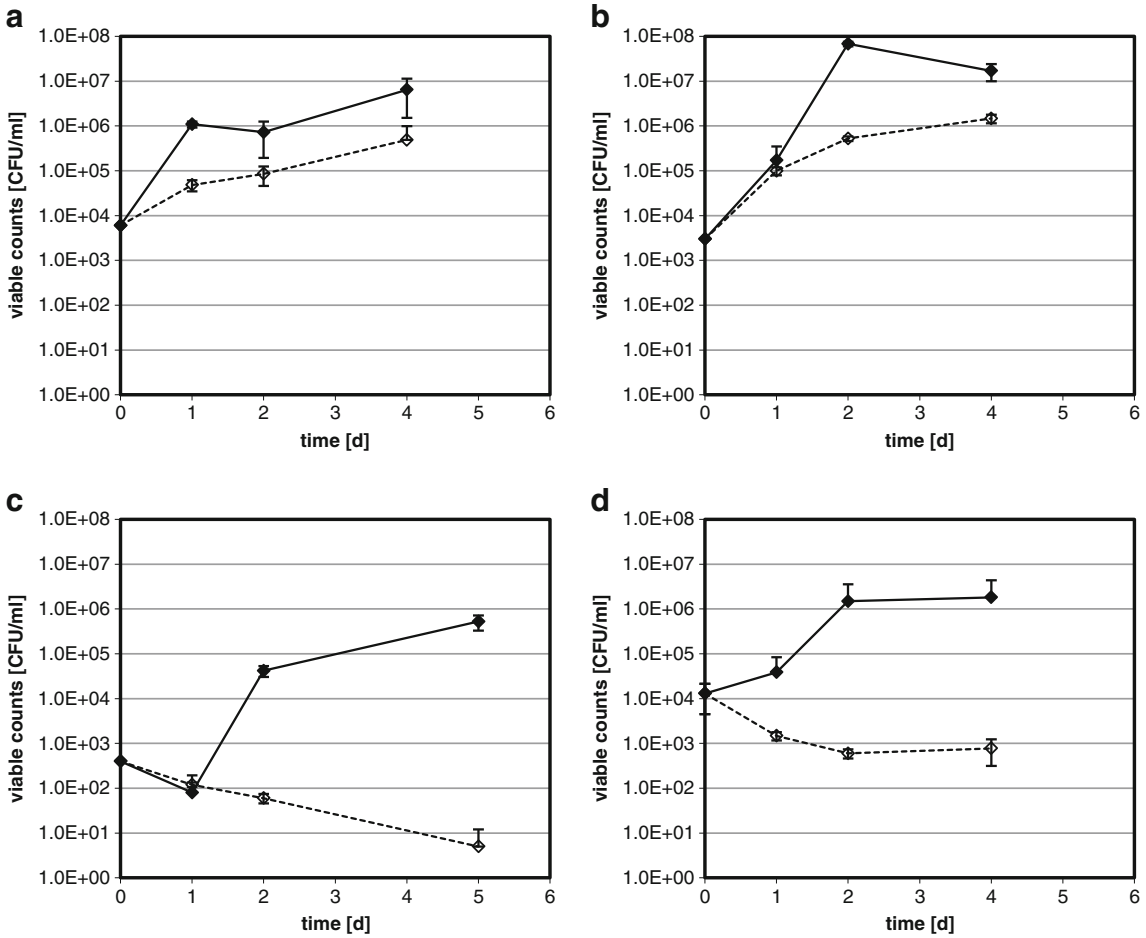

Fig. 4 Growth response of *E. coli* (**a**), *C. sakazakii* (**b**), *B. thermosphacta* (**c**), and *S. aureus* (**d**) in presence (*solid line*) and absence (*dashed line*) of *A. castellanii*, at 24 °C in PAS

pyriformis (Brandl et al. 2005), *Acanthamoeba palestinensis* (Gourabathini et al. 2008), *Acanthamoeba lenticulata*, and *Acanthamoeba polyphaga* (Akya et al. 2009b).

Physical separation of *Listeria* and amoebal cultures by membranes, or use of separately obtained amoebal culture supernatant indicated that *L. monocytogenes* utilize compounds secreted by *A. castellanii* for growth. Direct contact between the bacteria and the amoebae is therefore not required to trigger release or secretion of these compounds.

Utilization of amoebal substances is not specific for the genus *Listeria*, which agrees well with previous findings (Huws et al. 2008). However, the reason for the inability of several other bacteria tested here to use amoebal metabolites for growth is not known. Apparently, the ability to metabolize these compounds is not uniformly distributed among different bacterial genera.

Several strategies to prevent phagocytosis by predatory protozoa have evolved during the long-term coexistence of bacteria and protozoa (Matz and Kjelleberg 2005). Among them are the formation of enlarged bacterial cells that are difficult to phagocytose (Hahn and Hofle 1998), higher swimming speed to escape protozoan grazing (Matz and Jurgens 2005), surface masking to avoid recognition by the predator (Wildschutte et al. 2004), microcolony formation (Hahn and Hofle 2001), intra- or extracellular toxin release (Matz et al. 2004; Pushkareva and Ermolaeva 2010), resistance to phagolysosome conditions (Brown and Barker 1999), and intracellular survival or even infection and growth. Examples for these strategies are represented by numerous bacteria, e.g., *Chlamydia*, *Legionella*, *Salmonella*, *Shigella*, *Pseudomonas*, *Vibrio*, *Staphylococcus*, and *Mycobacterium*, which survive their encounter with free-living protozoa and establish a parasitic relationship (Thom et al. 1992; Michel et al. 1995; Essig et al. 1997; Steinert et al. 1998; Pickup et al. 2007; Abd et al. 2008; Bleasdale et al. 2009; Lau and Ashbolt 2009; Saeed et al. 2009; Douesnard-Malo and Daigle 2011; Hilbi et al. 2011; Lee et al. 2012; Valeru et al. 2012).

With respect to *Listeria*, loss of motility (perhaps transient) and the utilization of secreted compounds or metabolites from the amoebal cells might represent an additional strategy to minimize or overcome the deleterious effects of predation.

Non-motile bacteria are less preferentially phagocytosed by *Acanthamoeba*, and "atypical" non-motile or slightly motile wild-type *Listeria* strains have occasionally been isolated (Kathariou and Pine 1991; Lang Halter et al. 2013). In addition to escape, the ability to catabolize a wide range of substrates enables bacteria to overgrow a protozoan population. The growth-promoting compound could be characterized as small heat-stable molecules with a molecular weight below 5 kDa, but unfortunately, the detailed nature and composition of the substances released by the amoebae remains unknown. However, identification of these compounds could lead to the discovery of yet unknown factors and pathways involved in the bacterial metabolism able to stimulate a strong growth response at very low concentrations. From an ecological perspective, the ability to catabolize secreted amoebal compounds seems to be advantageous for saprophytic bacteria to prevent eradication by amoebal grazing in low-nutrient environments.

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