



# Pleomorphic mammalian tumor-derived bacteria self-organize as multicellular mammalian eukaryotic-like organisms: morphogenetic properties in vitro, possible origins, and possible roles in mammalian 'tumor ecologies'

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**Summary** Highly pleomorphic bacteria have regularly been isolated from mammalian tumors and leukemic bloods. Here, it is shown that highly pleomorphic, cell-wall deficient bacteria derived from a mammalian tumor self-organize in vitro into mammalian tissue-like morphogenetic patterns consisting of multicellular tissue-like sheets and capillary-like networks. It is proposed that these pleomorphic mammalian tumor-derived (MTD) bacteria, during morphogenesis, express mammalian tissue morphogenesis-related genes that were acquired through eukaryote-to-prokaryote DNA transfer. Similar pleomorphic MTD bacteria might play important roles as symbiotic multicellular mammalian eukaryotic-like organisms in mammalian 'tumor ecologies' that include malignant and nonmalignant mammalian eukaryotic cells. From a mammalian tumor ecology perspective, eradication of tumors in some mammalian hosts may depend upon the elimination of pleomorphic MTD bacteria self-organized as symbiotic multicellular mammalian eukaryotic-like organisms. Further investigations of the extraordinary mammalian eukaryotic-like multicellularity of these bacteria may yield fundamental insights into the evolution of multicellularity and multicellular development and may challenge basic assumptions regarding cellular evolution.

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## Introduction

Highly pleomorphic bacteria have been regularly isolated from a variety of mammalian tumors and leukemic bloods [1,2]. The extreme pleomorphism exhibited by some mammalian tumor-derived

(MTD) bacteria includes a panoply of forms that are variably large and small, coccoid, rod-like, filamentous, branching, and globoidal, among others. Some pleomorphic MTD bacteria have been observed to exhibit fungus-like morphologies [3].

Experiments in our laboratory using in vitro cell culture and in silico sequence-based comparative genomics methods indicate that some highly pleomorphic bacteria express mammalian eukaryote-derived genes acquired through a process of

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eukaryote-to-prokaryote DNA transfer that is driven by changes in the oxygen environment [4]. These experimental findings echo other studies using sequence-based comparative genomics which indicate that, historically, some bacterial species appear to have acquired genes from their eukaryotic hosts through horizontal gene transfer [5,6]. To date, however, there appears to be no literature describing efforts to sequence MTD bacterial genomes to determine whether or not they contain mammalian eukaryote-derived DNA. As the initial step in determining whether or not the genomes of pleomorphic MTD bacteria contain mammalian eukaryote-derived genes, bacteria were to be isolated from mammalian tumor specimens. The genomes of MTD bacteria that exhibited high degrees of pleomorphism, as a morphologic indicator that their genomes might contain mammalian eukaryote-derived genes, would then be sequenced and analyzed in silico using sequence-based comparative genomics methods.

## Observations

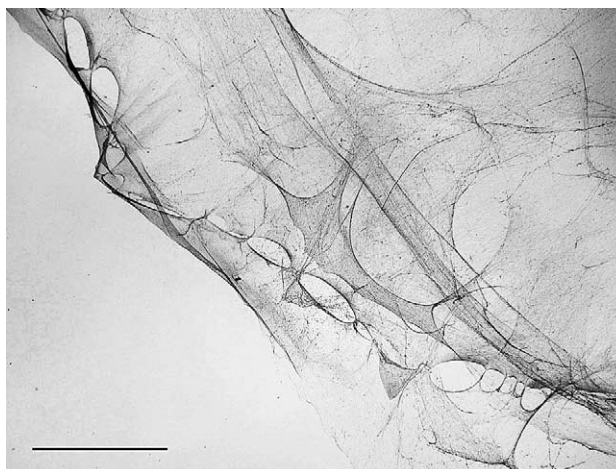
Bacteria were isolated from canine lymphoma specimens using a culture method that involves subjecting eukaryotic cells to an environmental pressure of alternating anaerobic and aerobic atmospheres [4,7]. Strict sterile procedures were employed during isolations and included the use of a laminar flow hood to prevent environmental contamination of specimens and cell cultures. After isolation and culture on solid media, bacteria were identified taxonomically using biochemical

methods and bacterial 16S ribosomal RNA (rRNA) gene sequence analysis [8]. All bacteria isolated were Gram-positive. Observing that several bacterial isolates appeared to self-organize in vitro, one, identified as the Gram-positive facultative anaerobe *Staphylococcus epidermidis* and designated as *MH*, was selected for a study of its morphogenetic properties.

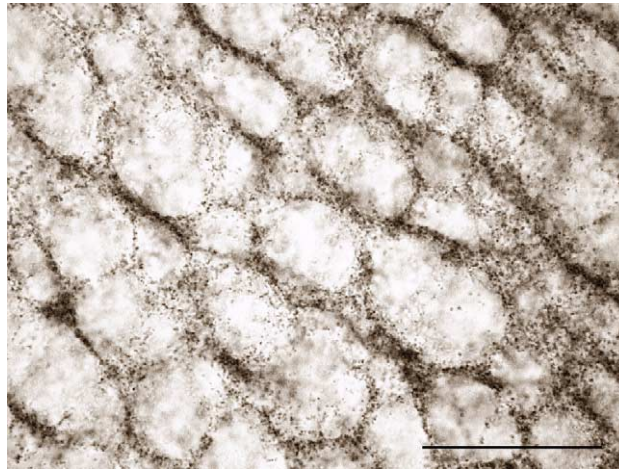
Within 72 h of transfer from solid media into liquid culture, *MH* forms creamy-white material at the bottom of plastic test tubes. Over the next several days and weeks, an expanding cell mass attaches to the bottom of test tubes and may grow to 0.25 cm or more in height. When aspirated with a pipette, this mass can be stretched to 2–3 cm in length, indicating the highly elastic nature of the cell material. This property persists despite repeated liquid–solid–liquid media transfers.

Microscopic examination of fresh cell preparations collected from the adherent, elastic cell mass and stained with the supravital dye new methylene blue N solution (Brecher formula) reveals tissue-like sheets that contain lacunae (Fig. 1). In some tissue-like sheets, reticular, honeycomb-like patterns are found in which the planar culture is tessellated with polygons with sides defined by strands of bacteria (Fig. 2). In addition, capillary-like networks composed of strands of bacteria are found attached to multicellular tissue-like sheets (Fig. 3) and floating free in the liquid medium. Bacterial strands in capillary-like networks recoil in turbulent medium, revealing their elastic properties.

Microscopic examination of Gram-stained tissue-like sheets at high magnification reveals a multicellular network of pleomorphic, cell-wall deficient bacteria (Fig. 4). Bacteria with a coccal



**Figure 1** *MH* self-organizes into multicellular tissue-like sheets that contain numerous lacunae (8-day old liquid culture; stained with the supravital dye new methylene blue N solution (Brecher formula) and examined by light microscopy). Scale bar = 500  $\mu$ m.



**Figure 2** *MH* self-organizes into reticular, honeycomb-like patterns (7-day old liquid culture; stained with the supravital dye new methylene blue N solution (Brecher formula) and examined by light microscopy). Scale bar = 40  $\mu\text{m}$ .

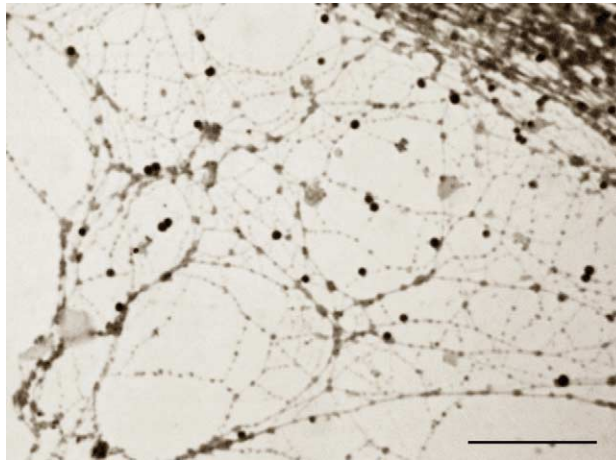


**Figure 3** *MH* self-organizes into a capillary-like network. The edge of a multicellular tissue-like sheet appears in the upper left corner (50-day old liquid culture; stained with the supravital dye new methylene blue N solution (Brecher formula) and examined by light microscopy). Scale bar = 250  $\mu\text{m}$ .

morphology, single and in pairs, are stained purple with the Gram-stain procedure and, consequently, are Gram-positive by Gram-stain criteria [9], biochemical methods, and bacterial 16S rRNA gene sequence analysis. The pleomorphic bacteria comprising the capillary-like multicellular network are stained red with the Gram-stain procedure and, consequently, are "Gram-negative" by Gram-stain criteria although Gram-positive by biochemical methods and bacterial 16S rRNA gene sequence analysis. This finding indicates that their cell-walls are deficient in peptidoglycan [9]. The largest bacteria in the multicellular network have amorphous, undulating cell-walls; the smallest appear as beads on a necklace and are a fraction of the size of a coccus with cell-wall intact ( $\sim 1 \mu\text{m}$ ). Some polygonal areas circumscribed by this irreg-

ularly tessellated bacterial network are only 3–4  $\mu\text{m}^2$ . If present in the tumor microenvironment, networks of cell-wall deficient bacteria of these dimensions might be difficult to detect using conventional histopathology techniques.

The multicellular self-organization in vitro of *MH* into tissue-like sheets and capillary-like networks shows similarities to morphogenetic patterns observed in in vitro models of mammalian vascular network formation [10–13]. However, this multicellular bacterial self-organization in vitro exhibits an autonomy and complexity of biological pattern formation not observed in these in vitro models of mammalian vascular network formation. Notably, while these models require extracellular matrix (ECM)-coated culture vessels and, in some cases, serum-factor supplements to support endothelial



**Figure 4** *MH* self-organizes into multicellular tissue-like sheets and capillary-like networks. The largest pleomorphic, cell wall-deficient bacteria have amorphous, undulating cell walls; the smallest appear as beads on a necklace and are a fraction of the size of a coccus with cell wall intact ( $\sim 1 \mu\text{m}$ ). In fresh preparations, these bead-like bacterial forms stain with the supravital dye new methylene blue N solution (Brecher formula). In this black-and-white microphotograph, pleomorphic, cell wall-deficient bacteria appear gray while cocci with cell walls intact appear black (7-day old liquid culture). Scale bar =  $20 \mu\text{m}$ .

cell self-organization, *MH* self-organizes in vitro in simple trypticase soy broth nonadherently and without the use of exogenous ECM or serum-factor supplements. The consistent observation of these morphogenetic patterns despite the use of two different staining techniques, one using methylene blue to stain fresh, i.e., live, cell preparations and the other using the Gram method to stain heat-fixed cell preparations, indicates that the morphogenetic patterns observed were not the result of artifacts produced by a particular staining technique.

In the first 2–3 passages after isolation, another bacterium, isolated from a canine lymphoma specimen and identified taxonomically as *Staphylococcus intermedius*, self-organized in vitro into morphogenetic patterns similar to morphogenetic patterns formed in vitro by *MH*. However, in later passages, this MTD bacterial isolate's morphogenetic properties dissipated, so that only occasionally were strands of bacteria observed to form in liquid media.

### To be or not to be – a multicellular eukaryotic-like organism

Multicellularity represents one of the major transitions in the evolution of biologic complexity [14]. Bacterial species such as *Myxococcus xanthus* exhibit characteristics of multicellular organisms including cooperative behavior among individuals and coordinated cell–cell attachment [15]. The

extraordinary multicellularity of *MH* appears to be unlike any bacterial multicellularity previously reported; an exhaustive review of the literature indicates that no other bacteria (including, of course, species of the eubacterial genus *Staphylococcus*) have been reported to self-organize into mammalian tissue-like morphogenetic patterns consisting of multicellular tissue-like sheets and capillary-like networks.

The complex multicellular self-organization of *MH* should not be confused with the “multicellular” organization of bacterial biofilms [16–20] including biofilms formed by *Staphylococcus epidermidis* [21,22]. A biofilm may be defined as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface [16]”. Unlike the relatively rudimentary “multicellular” characteristics of a biofilm, the complex multicellular self-organization of *MH* includes: (1) elastic, multicellular tissue-like sheets containing lacunae (Fig. 1); (2) reticular, honeycomb-like, mammalian vascular-like morphogenetic patterns (Fig. 2); (3) irregular tessellations of the planar culture with polygons of various shapes with sides defined by interconnected strands of bacteria forming patterns suggestive of capillary-like networks (Figs. 3 and 4); (4) cell-wall deficiency and extreme pleomorphism (Fig. 4).

Multicellularity may provide adaptive advantages to MTD bacteria such as *MH* by: (1) promoting more efficient proliferation through a cellular division of labor; (2) providing a critical cell mass that can better access nutrients; (3) forming a

collective defense against antagonists that eliminate individual cells; (4) optimizing population survival by differentiation into distinct cell types [23]. Because of its capacity for self-organizing into mammalian tissue-like morphogenetic patterns, *MH* might be considered to be a multicellular mammalian eukaryotic-like organism. In losing much of its collective, rigid bacterial cell-wall during morphogenesis, *MH* exhibits a mammalian eukaryotic-like feature, i.e., a flexible, eukaryotic-like cell 'membrane', which appears to be critical to the formation of the numerous intercellular connections (mammalian eukaryotic VE-cadherin-mediated? [24]) necessary to this mammalian tissue-like multicellularity [25]. Mammalian eukaryotic-like multicellularity may provide *MH* and similar MTD bacteria the advantages as noted above; in addition, this remarkable bacterial multicellularity potentially provides mammalian eukaryotic tumor cells a sophisticated bacterial support network (as will be discussed below). However, unlike a multicellular mammalian eukaryotic organism, *MH* has the evolutionary option of existing as a unicellular life form – a potential every man-for-himself survival strategy in tumor microenvironments bombarded with toxic agents from the cancer-treatment arsenal. This unusually plastic cellular pleomorphism may add a stealthy, dynamic evolutionary dimension to mammalian tumorigenesis that, once uncloaked, may represent a novel target for the treatment of mammalian cancers.

### The multicellularity of *MH*: a possible case of eukaryote-to-prokaryote DNA transfer

During its mammalian tissue-like morphogenesis, *MH* may express genes related to mammalian tissue morphogenesis that were acquired by way of eukaryote-to-prokaryote DNA transfer from canine eukaryotic tumor cells. Because some morphogenetic patterns formed by *MH* are similar to those formed by mammalian capillary-like networks, it is possible that *MH* specifically expresses mammalian eukaryote-derived genes related to mammalian angiogenesis [26]. As noted previously, sequence-based comparative genomics studies indicate that, historically, some bacterial species appear to have acquired genes from their eukaryotic hosts through horizontal gene transfer [5,6]. However, the current paradigm regarding the role of horizontal gene transfer in prokaryotic evolution (which is in considerable flux) indicates that: (1) the number of

genes transferred in any one event is small in relation to a recipient prokaryote's genome size; (2) transferred genes accumulate in any one recipient prokaryotic genome gradually; (3) selective barriers to gene transfer exist with regard to the degree of species relatedness between donor and recipient organisms [5,6,27–30]. It is difficult to imagine horizontal gene transfer, as portrayed by the current paradigm, to be the genome-shaping genetic mechanism underlying the execution of a multicellular mammalian eukaryotic-like developmental program by *MH*.

At this time, the possibility that *MH* acquired its capacity for multicellular mammalian eukaryotic-like development through a process of eukaryote-to-prokaryote DNA transfer that is rapid and vertical, i.e., related to eukaryote-to-prokaryote speciation, rather than gradual and horizontal, cannot be excluded. In contrast to horizontal gene transfer, speciation-related, vertical DNA transfer might rapidly produce a prokaryotic genome that contains the potentially numerous eukaryote-derived genes necessary to execute a multicellular mammalian angiogenesis-like developmental program [26]. As a cellular survival response of mammalian eukaryotic tumor cells, mammalian tumor-related eukaryote-to-prokaryote speciation can be viewed as either: (1) an evolutionary extrapolation of evolutionarily conserved, unicellular organism-derived SOS systems and mechanisms of replication activated in mammalian eukaryotic cells exposed to environmental stresses [31–38] or (2) the environmental stress-induced de novo evolution of prokaryotic organisms (which use sophisticated, environmentally stable mechanisms of RNA processing) from eukaryotic organisms (which use primitive, environmentally labile mechanisms of RNA processing) [4,39–42]. If mammalian tumorigenesis involves eukaryote-to-prokaryote speciation, then the bacteria so evolved will be unique to their ancestral mammalian eukaryotic tumor cell populations; if pleomorphic, such bacteria may display, on occasion, the monomorphic features of a particular *known* species of bacteria, but, at the level of the genome, may differ radically from that bacterial species' prototypical strain(s) banked in a cell repository [4]. (In considering a scenario of mammalian tumor-related eukaryote-to-prokaryote speciation, it is important to remember that the in vitro form of horizontal eukaryote-to-prokaryote DNA transfer known as 'gene cloning' is but a relatively unsophisticated biotechnology appropriated from Nature's lot of supremely sophisticated biological processes. In vivo, i.e., in nature, interorganismal DNA transfer processes are considerably more complex than in vitro 'gene cloning'

and might include the *vertical* DNA transfer associated with eukaryote-to-prokaryote speciation. Furthermore, the current body of scientific knowledge does not exclude the possibility of contemporaneous eukaryote-to-prokaryote speciation events.)

Regardless of any association with bacterial multicellularity or speciation events per se, mammalian tumor-related eukaryote-to-prokaryote DNA transfer may represent a mode of interorganismal DNA transfer fundamental to mammalian tumor biology. Observations regarding the *Staphylococcus intermedius* MTD bacterial isolate indicate that, after isolation and serial passage, the multicellularity of some MTD bacteria may dissipate in vitro; such morphogenetic degradation might be due to the convergence of unknown selective pressures and a genetically unstable mammalian eukaryote-derived developmental program. However, this MTD bacterial isolate and other pleomorphic MTD bacteria, though failing to maintain or exhibit ab initio features of multicellularity in vitro after isolation, may yet contain and express numerous mammalian eukaryote-derived genes that influence mammalian tumor development in vivo. Nonetheless, studies are underway to determine if eukaryote-to-prokaryote DNA transfer accounts for the mammalian eukaryotic-like multicellularity observed in *MH*.

### Possible roles in mammalian 'tumor ecologies'

As coevolved partners with animal eukaryotic cells, symbiotic bacteria have been shown to wield significant influence on animal development [43]. In symbiotic partnerships with mammalian eukaryotic tumor cells and mammalian capillary endothelial cells, *MH* and similar pleomorphic MTD bacteria may wield significant influence on mammalian tumor development. Self-organized as capillary-like works within the tumor microenvironment, they might stimulate tumor neovascularization (particularly if expressing mammalian eukaryote-derived proangiogenic factors), functioning as capillary-like 'roadmaps' that guide mammalian endothelial cell migration into anaerobic regions of the tumor microenvironment; the bacteria *Bartonella henselae* and *Agrobacterium tumefaciens* have been shown to trigger neovascularization in humans and plants, respectively [26,44,45]. Capillary-like networks of symbiotic multicellular MTD bacteria pervading the tumor microenvironment might also serve as a gene-flow 'highway', routing multiple,

multidirectional, simultaneous interorganismal DNA/gene transfers between eukaryotic cells and prokaryotic cells [4–6,46–48], so as to optimize overall tumor genetic adaptability.

If symbiotic multicellular MTD bacteria, i.e., symbiotic mammalian eukaryotic-like organisms, are essential to some mammalian tumors, then antitumor chemotherapeutic agents may work in some instances through antimicrobial mechanisms directed against them as well as through mechanisms directed against mammalian eukaryotic tumor cells [49–55]. This is none too surprising, given that the 'secondary-metabolite' antitumor antibiotics of the actinomycetes may have evolved primarily to provide a defense against microbial competitors [56]. In the case of mammalian tumor resistance to an antitumor antibiotic, a three-dimensional network or web of symbiotic multicellular MTD bacteria, surrounding mammalian eukaryotic tumor cells *and* resistant to that particular antitumor antibiotic because of, for example, the expression of a multidrug efflux pump, might metabolize and detoxify the antitumor antibiotic, shielding mammalian eukaryotic tumor cells from its toxic effects [57–61]. This scenario of antitumor antibiotic metabolism and detoxification by a symbiotic multicellular bacterial network pervading the tumor microenvironment reflects a distinct evolutionary advantage to mammalian eukaryotic tumor cells exposed to antitumor antibiotics.

In the realm of tumor immunology, a symbiotic multicellular MTD bacterial network surrounding eukaryotic tumor cells and expressing mammalian eukaryote-derived proteins, such as MHC Class I chain-related molecules, might deactivate natural killer (NK) cell immunity, cloaking mammalian eukaryotic tumor cells from NK cell-mediated cytotoxicity [62]. A symbiotic multicellular MTD bacterial network expressing mammalian eukaryote-derived  $\beta$ -2 microglobulin might inhibit the proliferation and function of dendritic cells important to host immune surveillance against tumors [63]. During progression to metastatic disease, symbiotic multicellular MTD bacteria may revert to a unicellular prokaryotic form to "metastasize" to tissue sites distant from the primary tumor, where, in an advance assault, they revert to a multicellular mammalian eukaryotic-like form, pervading and preparing the foreign microenvironment of the secondary site for invasion by mammalian eukaryotic tumor cells circulating within the vascular system [64,65]. In another possible 'ecological' interaction with malignant and nonmalignant mammalian eukaryotic cells, symbiotic multicellular MTD bacteria enjoy an optimized habitat with



assurances of an adequate nutrient supply and host immune tolerance [43,44]. (Particularly if evolved from host mammalian eukaryotic tumor cells, symbiotic multicellular MTD bacteria might be seen as 'self' by the host mammalian immune system.) Altogether, the complexities of interactions between host mammalian eukaryotic cells and networks of pleomorphic MTD bacteria, self-organized as symbiotic multicellular mammalian eukaryotic-like organisms, might be best entertained within the conceptual framework of a mammalian 'tumor ecology'.

Numerous other interactions between pleomorphic MTD bacteria and host eukaryotic cells can be envisioned when incorporating the concepts of mammalian tumor-related eukaryote-to-prokaryote DNA transfer, pleomorphic MTD bacteria self-organized as symbiotic multicellular mammalian eukaryotic-like organisms, and mammalian tumor ecology into the existing conceptual repertoire of mammalian tumor biology. From a mammalian tumor ecology perspective, eradication of tumors in some mammalian hosts may depend upon the elimination of pleomorphic MTD bacteria self-organized as symbiotic multicellular mammalian eukaryotic-like organisms. The use of antibacterial antibiotics as primary therapies to treat gastric and cutaneous MALT lymphomas and Hodgkin lymphoma is instructive in this regard [66–71].

## Conclusion

Further exploration of this extraordinary bacterial multicellularity will help determine if pleomorphic MTD bacteria self-organized as symbiotic multicellular mammalian eukaryotic-like organisms represent a fundamental feature of mammalian tumor biology that can be targeted with specific cancer therapies. Recognition of the more fundamental phenomenon of mammalian tumor-related eukaryote-to-prokaryote DNA transfer, which likely underlies the bacterial morphogenesis described, might be essential to developing more effective cancer therapies that involve a view of cancer as an evolutionary problem at the cellular, organismic, and 'ecological' levels. Accordingly, sequence-based comparative genomics studies of highly pleomorphic MTD bacteria are crucial. Finally, further investigations of the extraordinary mammalian eukaryotic-like multicellularity of these bacteria may yield fundamental insights into the evolution of multicellularity and multicellular development and may challenge basic assumptions regarding cellular evolution [5,6,12–14,27–30,72–76].

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