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Motility of Bacterial Pathogens in Minimal Media Containing Human Serum

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Authors' contributions

This work was carried out in collaboration between all authors. Authors APF and MAA designed the study, and guided the author NSM who performed the laboratory analysis and wrote the protocol. Author MAA wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To investigate the effect of human serum on growth pattern, cellular morphology, and motility of potentially pathogenic bacteria.
Study Design: This was an analytic experimental study.
Place and Duration of Study: Institute of Exact Sciences and Technology (ICET), Federal University of Amazonas (UFAM), Mycology Laboratory, between August 2012 and July 2013.
Methodology: Growth of *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis* and *Bacillus cereus* was examined on hard agar (1.5%) solid medium containing 0, 20, or 40 percent pooled human serum. Dilutions of each species were point-inoculated at the center of the plate. Cultivation was carried out aerobically for up to 20 days at 37°C in sealed humidified boxes. Spreading growth was examined by measuring colony diameters, analyzing macro- and micromorphology, and measuring the fractal dimension of colonies.
Results: *E. coli* and *S. typhi* strains grew better in Davis and Mingioli agar, whereas *B. subtilis* and *B. cereus* grew better in Fujikawa agar. *B. cereus* and *S. typhi* developed a white halo of proteolysis around the colony in the medium supplemented with serum. The addition of human serum to minimal hard-agar medium induced a cellular phenotypic change and a colony morphological change, especially in *B. cereus* and *S. typhi*. *B. cereus*

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and *S. typhi* developed elongated cells on the colony edge in the presence of human serum, showing cells in raft-like association. Generally, colonies of bacteria grown in the absence of human serum presented smaller fractal and growth dimensions and more-branched spreading, presumably in a sliding translocation.
Conclusion: Cells of "temperate swarmer" species translocated more efficiently on hard agar supplemented with human serum, by sliding and possibly by swarming. The presence of human blood or serum, despite the inhibitory activity of antibodies, may allow pathogenic bacterial cells to overcome the difficulties of low levels of nutrients and hard surfaces with little available water, and may facilitate translocation to other sites. Further investigations of the influence of human serum on swarming and sliding are warranted.

Keywords: Bacterial growth; motility; swarming; sliding; fractal; human serum.

1. INTRODUCTION

Bacteria were long viewed as simple unicellular organisms of limited capabilities. This began to change as we have gained an increasing awareness of the role of biofilms, communities of sessile organisms that secrete an extracellular matrix and aggregate as multicellular groups [1]. It is now known that bacteria compete or cooperate in response to various signals for available nutrients, optimal oxygen and when exposed to harsh environmental conditions such as starvation, hard surfaces, extreme heat, and hazardous chemicals in the environment [2]. To coordinate cooperative efforts, bacteria have developed methods for cell-cell signaling, including direct physical interactions, with extracellular secretion of lubricating materials, biochemical communication through quorum-sensing molecules and chemotactic signaling [3]. Bacteria can also detect a multitude of host defense or communication factors and develop specific adaptive responses [4]. Most of these factors include proteins and peptides (hormones, globulins, lipoproteins and enzymes) that can be found in human serum [5].

Multicellular cooperative behavior of bacteria is most often associated with surfaces, and surface-associated communities have advantages over individual cells, such as protection against unfavorable environmental conditions, including predation, the presence of antimicrobials and the host immune response [6,7]. Motility is one of the most extensively studied bacterial cellular processes. Most studies focus on swimming in aqueous environments, but other, clearly cooperative motility behaviors are observed when bacteria are propagated outside an aqueous setting [8]. Bacterial motility is a complex process that employs mechanisms such as swimming, swarming, twitching, gliding and sliding [9,10].

As reviewed by Patrick and Kearns [11], sliding motility is a passive form of surface-spreading that does not require an active motile force but instead relies on surfactants to reduce surface tension; swimming motility is a mode of bacterial movement that is powered by rotating flagella, but unlike swarming motility, swimming proceeds as individual cells move in liquid environments. Swarming is defined as a coordinated, flagella-dependent multicellular activity during which swarmer cells maintain lengthwise contact with each other. There are three basic requirements for bacterial swarming motility: (1) cells are motile and have functional flagella; (2) cells are in contact with or in close proximity to surfaces; and (3) cells are in contact with other motile cells [9].

According to Partridge and Harshey [12], swarming bacteria may be divided into two categories: robust swarmers, which can navigate across a hard agar surface (1.5% agar and above), and temperate swarmers, which can swarm only on a softer agar surface (0.5 to 0.8% agar). Robust swarmers include polarly flagellated bacteria that induce peritrichous flagellation upon surface contact, such as *Azospirillum*, *Rhodospirillum*, and *Vibrio* species, as well as the peritrichously flagellated *Proteus* species. These bacteria display hyperflagellated and hyperelongated swarm-cell morphology, which is dramatically different from their broth-grown (swimming) counterparts. Temperate swarmers include *Escherichia coli*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, and *Yersinia* species. Among these, *Bacillus subtilis* displays increased flagellar numbers and cell length, but this morphology is not as dramatic as that seen in robust swarmers [13].

Understanding the varied requirements for initiating movement in different bacterial species is important for anticipating the sorts of challenges that a solid surface might present for bacterial movement, and how bacteria cope with these challenges [12]. Given that many swarmers are adapted for a pathogenic state, this knowledge is not only important for understanding biofilm formation, but will eventually find applications in controlling the successful establishment and spread of bacterial surface communities. Few investigators have examined the behavior of colony growth under the influence of human serum [14]. We conducted preliminary growth tests with potentially pathogenic flagellated bacteria that are considered temperate swarmers, under adverse conditions of low nutrient levels, a hard surface, and the presence or absence of pooled normal human serum (NHS).

2. MATERIALS AND METHODS

2.1 Test Organisms and Growth Conditions

The strains used were *E. coli* K-12 MG1655 (ATCC 700926), *E. coli* ATCC 25922, *Salmonella typhi* ATCC 6539, *B. subtilis* ATCC 19659 and *Bacillus cereus* ATCC 11778. Clinical isolates were previously identified by the API 20E test. Two culture media were used: 1) the Fujikawa medium, containing 5.0 g NaCl, 5.0 g K_2HPO_4, 1.0 g peptone, distilled H_2O 1000 mL, pH 7.1, with 1.5% agar (HiMedia Laboratories, Mumbai, India) as the solidifying agent [15],[16]; and 2) the minimal medium of Davis and Mingioli [17], with the following composition: 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.5 g Na_3-citrate x $3H_2O$, 0.1 g $MgSO_4$x $7H_2O$, 1.0 g $(NH_4)_2SO_4$, 2.0 g glucose (autoclaved separately), distilled H_2O 1000 mL, pH 7.0, also with 1.5% agar (HiMedia) as the solidifying agent. The medium mixture was autoclaved at 121ºC and 1.1 atm for 15 min and then allowed to cool at room temperature, and 20 mL of medium was added to Petri dishes of 88 mm diameter. The plates were allowed to stand closed (but not sealed) at room temperature for 24 h, until the weight of the plates decreased due to water evaporation. According to Ben-Jacob et al. [18], accurate and uniform thickness of agar and strict protocol are essential to obtain reproducible growth patterns, as colony development is very sensitive to small changes in growth conditions.

In growth assays, 10 µL of a suspension of fresh cells of each strain, adjusted to optical density (OD) 650nm = 1.0, was inoculated at the center of the Petri dishes. The plates were incubated at 37ºC for 15 to 20 days in a humid chamber [3].

Because all bacterial species used here may be associated with blood-borne infections, and to determine the influence of human serum on the growth pattern, they were also cultured on plates with the above-described culture media supplemented with 20% or 40% pooled NHS

from different individuals, for comparison, or with distilled water instead of serum. Other parameters such as temperature were kept constant. Blood from different healthy donors was obtained from the Public Clinical Analysis Laboratory of the municipality of Itacoatiara, Amazonas. After separation from the clot, the sera were mixed, filter sterilized through a 0.22 µm pore-size filter and immediately added in the respective concentrations (vol/vol) to the medium that was cooling after autoclaving. The serum contained no preservatives and had not been heat-treated. All experiments were performed in triplicate.

2.2 Microscopic Analysis of Motility Fronts

According to Henrichsen [19], a spreading zone is a broad or narrow film of one or at most a few layers of cells extending from the edge of a colony or an area of confluent growth. The macroscopic appearance of a spreading zone will sometimes be so characteristic that it directly reveals how the zone was produced. This can, however, in all cases be decisively established by examining the spreading zone under the microscope. Therefore, the cell length and the spreading zone were assessed by microscopic observations (Leica DM500, 1000X magnification) of Gram-stained preparations, to analyze the motility fronts and to determine the onset of motility [12]. Bacterial samples were taken by slide overlay of single agar blocks (5x5mm) that contained different colony portions [20].

2.3 Colony Expansion Rates

Colony expansion (mm d^{-1}) was monitored by measuring, with a 30-cm ruler, the maximum length of two perpendicular cross sections after 96, 120, 216, 240 and 360 h of incubation (modified from [21]). This procedure was used to measure the size of the colonies in the culture medium with added human serum (20% and 40%) or diluted with water (20% and 40%) instead of serum. Data were collected from 3-4 replicates of each strain tested.

2.4 Determination of Fractal Dimension (D) of Colonies by the Box-Counting Method

According to Golinski et al. [21], one of the most important limitations of D2 assays, such as colony measurements, is that similar values do not imply similar colony shapes, although measurement of fractal dimension does provide a relative ordering of the complexity of objects. Other work showed that the sensitivity to treatment effects is a major benefit of using fractal dimension, as fractal analysis provides quantitative indexes of morphology [22].

Colony images were captured after 15-20 days of incubation with a Canon PowerShot Camera, from a height of approximately 15 cm, with natural illumination. Images were processed with the software Adobe Photoshop and PhotoScape CS5.1 v3.6.3, filtering all unwanted material and correcting optical errors, making it suitable for estimating the fractal dimension, on binary images (grayscale, 8-bit images) by counting the number of black pixels, with a box size determined in 64 divisions, using the box-counting method in 1.46r ImageJ (Image Processing and Analysis Program in Java), available in the public domain of the National Institutes of Health, USA. In this method, when the colony is covered by a grid of equal side length, the number of boxes overlapped by the colony can be counted. Prior to the determination of fractal dimensions, the Image J 1.46r program was validated by analysis of ten previously published images [15,16,23,24,25,18] with their respective fractal dimensions, with good results (data not shown).

3. RESULTS AND DISCUSSION

Movement over surfaces may enable pathogenic bacteria to migrate over, adhere to and disperse from sites of infection. Swarming may protect pathogens from macrophages, as swarm cells were shown to have enhanced resistance to engulfment [1]. Toxin secretion is often co-regulated with swarming motility [1]. Furthermore, several species of bacteria seem to become resistant to a broad range of antibiotics when swarming [1]. Uropathogenic strains of *Proteus mirabilis* produce higher levels of specific virulence factors during their swarm-cell state, and swarming is used to ascend the host's urinary tract to colonize the kidney [20].*Salmonella* may also utilize swarming to ascend the biliary tract in a similar fashion [8]. Copeland and Weibe [9] noted that a fundamental unanswered question in this area is the limitations on the types of surfaces that support swarming.

Here, we tested the growth behavior of potentially pathogenic species in minimal culture media that were previously employed in motility studies [24,26], supplemented with pooled NHS or diluted with water. The strains *E. coli* K-12MG1655, *E. coli* ATCC 25922 and *S. typhi* ATCC 6539 grew better in Davis and Mingioli agar. The strains *B. subtilis* ATCC 19659 and *B. cereus* ATCC 11778 grew better in Fujikawa agar. After 96 h of incubation, *B. cereus* ATCC 11778 developed a white halo of proteolysis around the colony. The colonies of this strain did not show a fractal structure in Fujikawa medium with 20% (Fig. 1D) or 40% serum (Fig. 2A), but did develop it in the diluted medium, with a highly branched appearance extending beyond the inoculation zone (Figs 1A and 2B). *S. typhi* ATCC 6539 formed larger fractal colonies than those of other bacteria, reaching 7.5 x 7.1 cm in Davis and Mingioli medium with serum (Figs 3A). *S. typhi* ATCC 6539 (D = 1.914 in serum and 1.924 in water) (Figs 3A and 3B) and *E. coli* ATCC 25922 (D = 1.875 in serum and 1.898 in water) formed spreading colonies in Davis and Mingioli medium with serum or with distilled water.

E. coli K-12MG1655 formed fractal colonies in Davis and Mingioli medium, but the central area of the colony showed a less-compacted mass. A similar less-compacted central area was observed in fractal colonies of *B. subtilis* ATCC 19659 (D = 1.891) in Fujikawa medium supplemented with serum. In Fujikawa medium with water instead of serum, this strain did not show fractal structure (data not shown).

The fractal dimension, as explained above, is not indicative of colony size or the degree of growth of the microorganism. Instead, it reveals the degree of complexity or tortuosity of the image (colony). Accurate quantification of fractal dimensions from experimental data is difficult because of limitations on resolution and scale [27]. However, it is a useful comparative measure, and in this study it was used to characterize the nature of the observed patterns and the effects of different growth conditions. The present results concord with other observations such as those of Roy et al. [2], that the culture period or the amount of nutrients, while allowing colony growth, do not necessarily make it more complex.

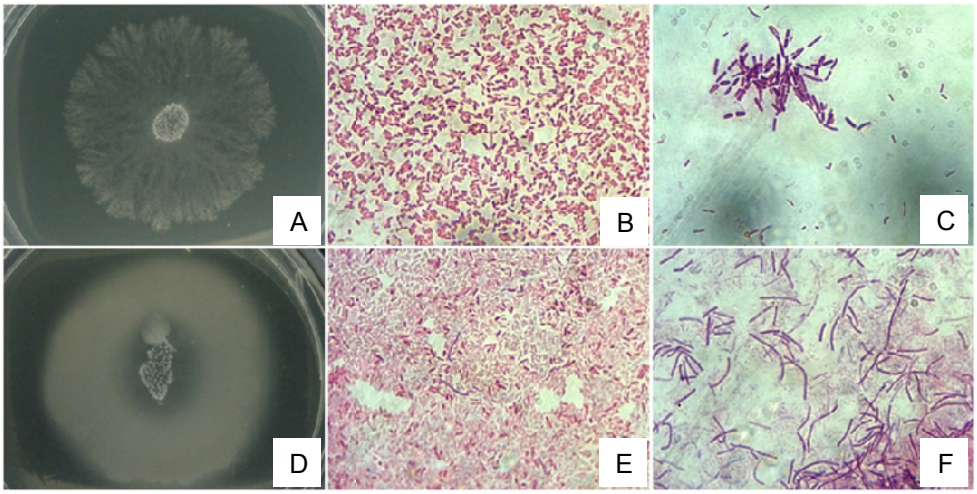

Fig. 1. Macro- and micromorphology of *Bacillus cereus* ATCC 11778 in media with 20% distilled water or pooled human serum. [A] Fractal colony in Fujikawa medium diluted with 20% distilled water (D = 1.828), [B] Gram staining of central zone of colony in Fujikawa medium diluted with 20% distilled water, [C] Gram staining of colony edge in Fujikawa medium diluted with 20% distilled water, [D] Colony in Fujikawa medium plus 20% human serum (D = 1.946); [E] Gram staining of central zone of colony in Fujikawa medium plus 20% pooled human serum, [F] Gram staining of edge of growth in Fujikawa medium plus 20% pooled human serum, showing elongated cells. Results are representative of three experiments

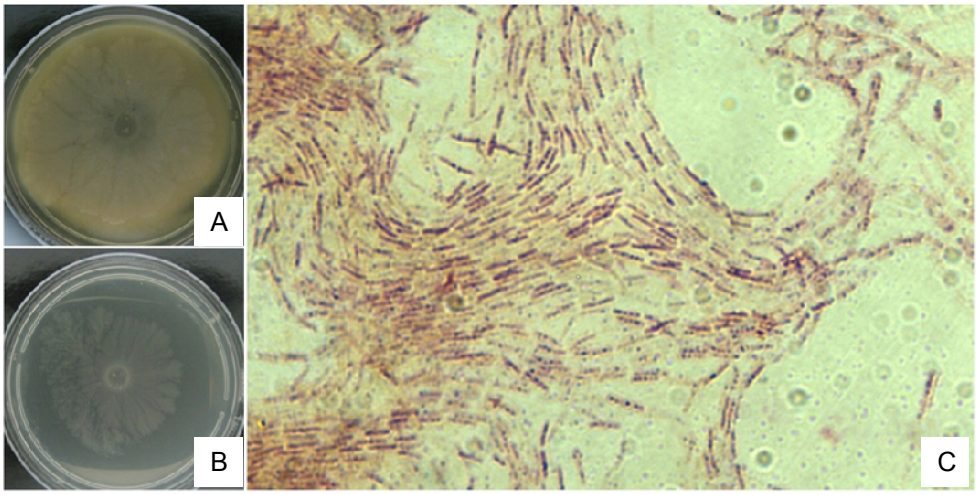

Fig. 2. Macro- and micromorphology of *Bacillus cereus* ATCC 11778 in media with 40% distilled water or pooled human serum. [A] Colony in Fujikawa medium plus 40% human serum, showing proteolysis area (D = 1.749) [B] Fractal colony in Fujikawa medium diluted with 40% distilled water (D = 1.787), [C] Gram staining of colony edge in Fujikawa medium plus 40% pooled human serum, showing cells in raft-like association. Results are representative of three experiments

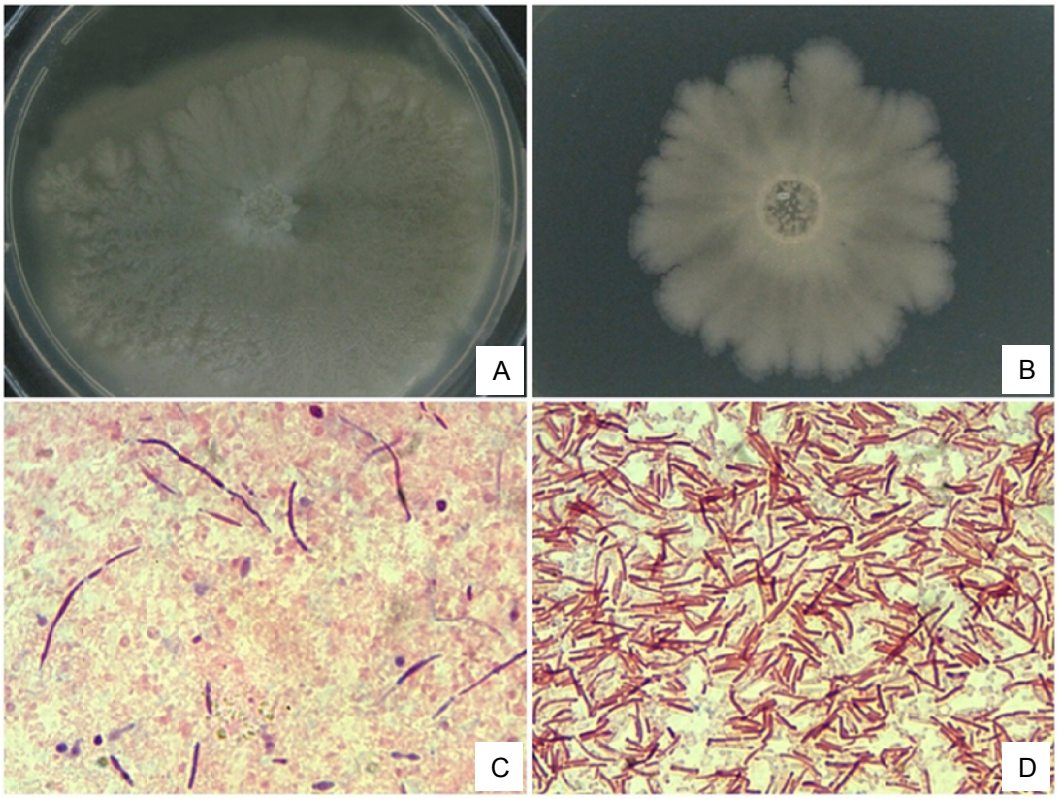

Fig. 3. Macro- and micromorphology of *Salmonella typhi* ATCC 6539 in media with 20% distilled water or pooled human serum. [A] Colony in Davis and Mingioli medium plus 20% human serum (D = 1.914), [B] Colony in Davis and Mingioli medium diluted with 20% distilled water (D = 1.924); [C] Gram staining of colony edge in Davis and Mingioli medium plus 20% human serum; [D] Gram staining of colony edge in Davis and Mingioli medium diluted with 20% distilled water. Results are representative of three experiments

In contrast to other differentiation processes in bacteria, swarming is not a starvation response [20]. The human serum may provide nutrients to power the flagella. Despite the idea of swarming as a rapid multicellular movement across a surface [1], in some cases, the migration of cells in the formation of swarming-type colonies can be seen only after 12 days, as with *Stenotrophomonas maltophilia* [28].

Motility varies significantly depending on the agar concentration and type of agar used. The agar concentration of 1.5% prevents, in principle, the swarming motion of all temperate swarmers, including the species tested here. Agar concentrations of more than 0.8% render the substrate semi-solid at room temperature and prevent the bacteria from swimming inside it [13]. Clemmer et al. [10] demonstrated that the use of Difco agar resulted in a branching, tentacle-like motility (probably a fractal colony), whereas Eiken agar resulted in a dramatically different pattern of motility. Different agars appear to provide different amounts of free water to cells, and water is the most critical element for swarming. In hard agar (>1.5%), water seems to be trapped within the agar gel, with no free water at the gel surface. Gram-negative bacteria appear to use osmolytes, polysaccharides, lipopolysaccharides

(LPS), and enterobacterial common antigen (ECA) to draw water toward the cells [12]. Here we used only one agar brand (HiMedia), and did not vary the type of solidifying agent, but future studies could include such variations. Zhang et al. [29] reported that the ability of cells of *E. coli* to swarm over an agar surface depends on the structure of the agar: Eiken agar works well but Difco agar does not, presumably because Eiken agar is more wettable.

The Gram staining was performed with *B. cereus* ATCC 11778 cells in the inoculation zone and with cells outside this area (edge of the colony). In the inoculation zone, in the medium diluted with water, short bacilli were observed, mostly Gram-negative (Fig. 1B). In the inoculation zone, in the medium with human serum, a small number of slightly elongated bacilli were seen, also Gram-negative (Fig. 1E). Outside the inoculation zone of water-diluted medium, many more-elongated bacilli were observed in binary division, mostly Gram-positive (Fig. 1C). Some of the cells had become elongated as straight rods with rounded ends. Cells outside the inoculation zone of medium with serum were elongated. Practically all rods were markedly larger and many were split, mostly Gram-positive (Fig. 1F). According to Fazlani et al. [30], young cultures of *B. cereus* stain Gram-positive and old cultures stain Gram-negative. Similarly variable Gram-staining behavior was observed here. Harshey and Matsuyama [31] also reported filamentous (5-20μm long) cells near the periphery of the colony, while in the internal regions of the colony the cells were shorter. The long cells were multinucleate and generally devoid of the external constrictions that mark the presence of cell septa, which seems to agree with our observations.

Swarming is a group behavior that requires the cells to reach a certain cell number before the process is initiated, which is termed swarming lag [6]. In this study, a clearly round central area and a rim that delimited this area and cells that migrated from this zone were observed (Fig. 2B and 3B). In the serum plates, *B. cereus* may tried to translocate by swarming, since there was no formation of fractal colonies and cells became elongated (Fig. 1D and 1F). Senesi et al. [20] demonstrated that *B. cereus* is capable of swarming differentiation: individual swimmer cells are short oligoflagellated rods, and the differentiated swarm cells are 3 – 4 times longer than the swimmer cells and hyperflagellated. The differentiated cells do not divide but possess the unique ability to migrate away from the colony in organized groups of tightly bound cells, which constitute an advancing-front movement at the rim of growing colonies, as seen on our serum plates. The swarming response of *B. cereus* was induced by surface sensing; but differently from other swarming bacteria, occurred over a wide range of medium viscosity (0.4-2% agar) and temperatures (25-38ºC). *B. cereus* swarm colonies never exhibited macroscopically layered consolidation phases due to regularly spaced cycles of swarming migration and consolidation, as seen in *P. mirabilis* [20]. However, it is commonly thought that swarming cells suppress cell division and that cell elongation is either a requirement for or an indicator of swarming motility. Different bacterial species were found to have subpopulations of long cells at the leading edge of a swarm. However, according to Kearns [1], it is still unclear whether elongated cells are required for swarming or whether they simply accumulate at the swarm edge. Kaiser [32] noted that an enhancer of cell flow that is found with all types of engines is that swarm cells are all long and they are flexible.

Despite the importance of elongation in the theory of swarming motility, no mechanistic or regulatory connection has been elucidated at the molecular level for the control of cell division during swarming. Furthermore, substantial cell elongation is neither a requirement for nor co-regulated with swarming motility in many bacteria [1].

When *B. cereus* grows in soil, it changes from a single-cell to a multicellular phenotype, which allows it to translocate. This morphogenic phase is analogous to *B. cereus* swarming on agar media. Clusters of elongated bacillary forms of *B. cereus* were found in Gram stains of histological sections of hemorrhagic brain tissue [33]. Bottone [33] noted that, when grown under aerobic conditions on 5% sheep blood agar at 37ºC, *B. cereus* colonies are dull gray and opaque with a rough matted surface. Colony perimeters are irregular and, according to Bottone, represent the configuration of swarming from the site of initial inoculation, perhaps due to *B. cereus* swarming motility. Zones of beta-hemolysis surround and conform to the colony morphology. Bottone [33] also observed that smears prepared from the distal and frontal (spreading) advancing perimeters of a mature colony may show two distinct morphologies. Smears prepared from the distal edge show uniform bacillar forms with prominent, centrally situated spores mixed with chains of Gram-positive bacilli, while smears from the advancing edge are comprised predominantly of masses of entangled bacillar chains and a marked absence of spore-containing bacilli. This was also seen here (Fig. 2). Bottone [33] hypothesized that, as the colony spreads from the initial inoculum site it leaves behind a trail of metabolic end products, which alters the pH and oxygen content of the growth environment, thereby inducing spore formation.

Human serum is an aqueous solution (about 95% water) containing more than 4200 compounds [5]. As reviewed by Lesouhaitier et al. [34], during the establishment of the infectious process, bacteria are exposed and can detect eukaryotic products. A number of pathogenic bacteria have receptors for plasma proteins and for different extracellular matrix proteins. Serum metabolites may change quorum sensing, virulence [34], bacterial morphology and the swarming phenotype, as roughly observed in this study. Mijouin et al. [35] observed that a skin neuropeptide (substance P) increases biofilm formation in the model organism *B. cereus*. Kalai Chelvam et al. [36] did not observe any significant difference in surface swarming between blood- and stool-borne strains of *Salmonella typhi*, but found a significant difference between these strains in swimming motility. According to Kalai Chelvam et al. [36], for *Salmonella*, for example, differences in motility raise the possibility that strains with high motility may be more capable of swimming through the intestinal mucus and replicate within macrophages and infected phagocytes.

Partridge and Harshey [12] reported that *Salmonella* cultured on non swarm (hard) agar displayed a mixture of short and long cells; they calculated the average length as the mean of swimming and swarm cell lengths. These cells showed a mixture of phenotypes: shorter cells had a single nucleoid and longer ones had two nucleoids. They concluded that cell lengths increase on both swarm and non swarm agar but that cell lengths are more uniform on swarm agar. In our serum plates (Fig. 3C), *Salmonella* cells seemed to be uniformly elongated, in contrast with cells in water-diluted medium (Fig. 3D). Analysis of multiple parameters including cell length, flagella, hooks, and nucleoids showed that *Salmonella* swarm cells only appear to be hyperflagellated; in fact, the number of flagella per cell does not increase. The doubling of cell length is due to suppression of cell division, as evidenced by two nucleoids per swarming cell, with no apparent cell-wall constriction at the mid-cell division site. Verstraeten et al. [6], in transcriptome studies in *Salmonella*, observed no changes in expression of cell-division genes, which is reasonable given the modest increase in swarm cell length. Nontyphoidal salmonellae have a variety of virulence factors, and Pilsczek et al. [37] found that exposure to NHS induced *S. montevideo,* but not *S. typhimurium,* to shed its flagella. With or without exposure to NHS, a wild-type *S. typhimurium* strain remained motile [37].

Some descriptions of swarming state that, as observed by microscopy, swarming cells have more flagella than swimming cells. In fact, few if any publications on swarming have counted flagella in swimming and swarming cells, because flagella are surprisingly difficult to count. Experimentally, common light microscopy techniques to stain flagella use mordants that cause flagella to adhere to one another, and high-resolution electron microscopy is technically challenging, requires small sample sizes, and is no less susceptible to counting problems [11]. According to Kaiser [32], the similarities between a swarm produced by cells rotating flagella and one produced by cells lacking flagella through the other two swarm-propulsive engines currently known, type IV pili and slime secretion, suggest that the strategy of swarming transcends the mechanics of the engines that happen to be employed. Kaiser also suggested that perhaps the "twitching motility" and "gliding motility" of Henrichsen [19] is swarming propelled by type IV pili. Type IV pili seem to be an important swarming mechanism. For example, it seems likely that the spread of *Neisseria meningitidis* and *Neisseria gonorrhoeae* in diseased tissue depends on these pili [38]. Recently, Anderson et al. [39] demonstrated that exposure of *N. gonorrhoeae* to seminal plasma alters bacterial motility, producing high-velocity translocation. Kuchma et al. [40] demonstrated that, for *Pseudomonas aeruginosa*, minor pilins, PilW and PilX of the type IV pilus system participate in negative regulation of swarming, indicating that the participation of pili in swarming is only beginning to be elucidated.

According to Copeland and Weibel [9], when cells are grown on the surface of agar gels (>2% w/vol) that typically do not support swarming motility, the cells grow into non-motile colonies and retain their planktonic morphology and phenotype. Conversely, cells that are introduced on the surface of nutrient-limited hard agar grow slowly and form a colony that resembles a fractal. In our experiments, the use of nutrient-limited hard agar supported fractal colony growth, but with the addition of human serum, cells and the whole colony seemed to change their morphology, possibly in an attempt to move by swarming. Kearns [1] noted that it is conceivable that the standard 1.5% agar used to solidify media in the laboratory was specifically chosen to inhibit swarming. However, common culture medium is normally enriched in nutrients. The media used here are minimal media, but the addition of human serum seemed to help the cells to translocate in agar. It must be taken into account that, on prolonged incubation, such as here, the colony diameter of a non-swarming strain may increase owing to the contribution of sliding motility. Kearns [1] warned that sliding is easily mistaken for swarming and can occur when flagella are disrupted in bacteria that would normally swarm. The human serum in the culture medium may have inhibitory activity of immunoglobulins and many other factors including siderophore-like molecules, ions or communication molecules that can influence bacterial growth, motility, virulence and biofilm formation; and bacterial motility seems to be required for both biofilm formation and pathogenesis in bacteria [36]. Also, the serum nutrients may be exploited to increase microbial growth [5]. The growth of strains in serum-containing medium was significantly different from the growth in medium with water ($P < 0.05$ - Student T test - for all four cases – Fig. 4). As a consequence of swarming, bacteria are able to continue to grow after their cells have begun to compete with each other for access to nutrients.

Gram-negative temperate swarmers do not show a significantly altered swarmer cell morphology, consistent with the absence of changes in flagellar gene regulation. This distinction is important, because it clarifies the general confusion regarding whether or not these bacteria "differentiate" during swarming. When cells differentiate into the swarming phenotype, they generally elongate into multinucleate filaments and the density of flagella on their surface increases 3-50 x per unit area of cell surface. However, Partridge and Harshey

[41] stated that this is not always the case, as some swarming genera, such as species of *Bacillus* and *Pseudomonas*, according to these authors, do not elongate.

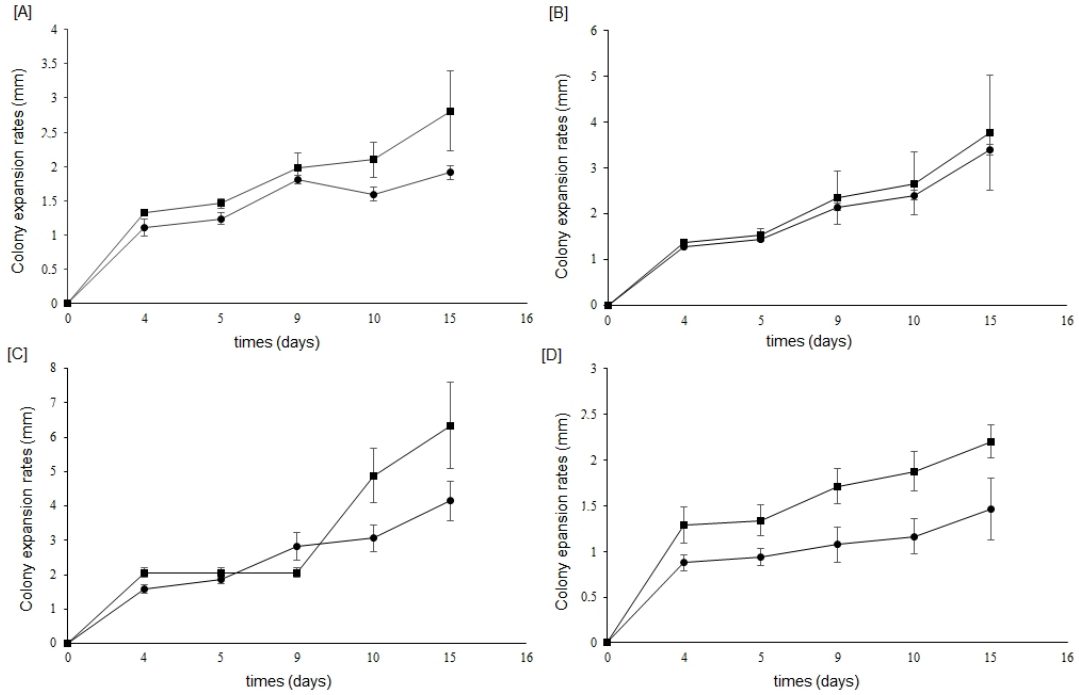

Fig. 4. Colony expansion rates (mm) in minimal medium (Fujikawa and Davis and Mingioli) diluted with distilled water (circles) or with 20% normal human serum (squares) of strains: [A] *Escherichia coli* K-12MG1655; [B] *E. coli* ATCC 25922; [C] *Salmonella typhi* ATCC 6539 and [D] *Bacillus subtilis* ATCC 19659

Butler et al. [13] showed that adaptive resistance is a property of high cell densities within the swarming colony and not of swarming-specific physiology. Here, in plates supplemented with serum, the cell densities, particularly at the edge of the colonies, were higher than in plates without serum (Fig. 2C). Many of the virulence factors reported to be associated with the occurrence of swarming are exoenzymes, specifically extracellular proteases. While a strong link between swarming extracellular protease production and pathogenesis has been noted, only a few studies have focused on the requirement of proteases for swarm motility. Connelly and coworkers [42] hypothesized that total extracellular proteolytic activity plays a central role in swarming motility in *B. subtilis*. We observed here that the presence of human serum supports the proteolytic activity, which may contribute to the translocation of pathogens (Figs 1D, 2A and 3A).

Bacteria swim as individuals, but swarming bacteria move in side-by-side cell groups called rafts. Raft formation is dynamic: cells recruited to a raft move with the group, whereas cells lost from a raft quickly become non-motile [1]. Individual isolated swarming cells are non-motile on surfaces. In hard agar and in the presence of serum, despite the possible inhibitory action of the immune system, cells seem to generate rafts in order to translocate (Fig. 2C). Physical contact between swarming cells is important for surface migration, as individual cells from a colony do not move unless the agar is supplemented with a surfactant or has a

layer of liquid on the surface [9]. Henrichsen [19] observed that the size of the rafts is clearly dependent on the amount of available moisture, and the drier the agar, the larger the rafts. On insufficiently dried agar, individual cells may be seen to move, whereas rotating and wandering cells of *Bacillus* are best demonstrated on either very dry plates or on plates with a high concentration of agar.

Kearns [1] pointed out that although the perfect surface of a carefully dried agar plate is never found in the environment, swarming may occur on soft, nutrient-rich substrates such as hydrated soils, plant roots and animal tissues. Partridge and Harshey [12] also speculated that open surfaces of animal tissues or ripe fruits that exhibit agar-like gelatinous properties may be a fertile ground for swarming. In the wild, solid-liquid interfaces on organic or inorganic substrates could conceivably also support swarming. Given that chemotaxis is generally not required for outward migration, the purpose of swarming appears to be to acquire more territory and increase population size.

4. CONCLUSION

Addition of human serum to minimal hard-agar medium (1.5%) induced a cellular phenotypic change and a colony morphological change, especially in *B. cereus* and *S. typhi*. Cells of these “temperate swarmer” species translocated more efficiently in hard agar supplemented with human serum. It might be useful to better exploit the influence of human serum or blood on motility, particularly in swarming and sliding. The presence of human blood or serum, despite the inhibitory activity of antibodies, may allow pathogenic bacterial cells to overcome the adverse conditions of low levels of nutrients and hard surfaces with small amounts of available water, and facilitate translocation to other sites.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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