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Full Length Research Paper

Growth responses of *Escherichia coli* and *Myxococcus xanthus* on agar gel substrates with different levels of stiffness

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Bacteria colonize surfaces responding to the physicochemical properties of substrates. A systematic study was carried out with growing single bacterial colonies on the surface of agar media to decipher the interaction between bacterial growth and substrate stiffness. We investigated the growth kinetics of wild-type *Escherichia coli*, non-motile *E. coli*, and *Myxococcus xanthus*, cultured on semi-solid agar substrates containing different amounts of nutrient and agar. We found that substrate stiffness, which was controlled by agar concentration, modulates the growth of motile bacteria, such as wild-type *E. coli* and *M. xanthus*, independently of the nutrient level, but does not affect the growth response of non-motile *E. coli*. Interestingly, growth of *M. xanthus* moving with type IV pili correlates negatively with the substrate stiffness in contrast to wild-type *E. coli* propelled by flagella. The present study demonstrates that the type of surface motility is a key determinant of the growth response of bacteria to substrate stiffness, and has potential application to the design of surfaces that prevent or promote biofilm formation.

Key words: Bacterial colonization, substrate stiffness, surface motility, swarming, *Escherichia coli, Myxococcus xanthus*.

INTRODUCTION

Although there have been tremendous efforts to regulate bacterial growth through biochemical approach, eradication of bacteria is yet to be achieved because of the solidity of bacterial colony protected under the biofilm. Biocides also cause environmental pollution and harmful effect on human health. In this study, we explore an echo-friendly new method to control bacterial growth through mechanical approach particularly using stiffness variation of agar gel substrate. Agar gel is generally used to induce bacterial growth in the field of microbiology, and its stiffness can be controlled easily by adjusting agar content. We made use of the agar gel as a stiffness-variable substrate.

After bacteria attach to a surface, they make biofilms by secreting high molecular weight compounds. Biofilms protect them from the surrounding environment and promote their survival rate (Ophir and Gutnick, 1994; Hall-Stoodley et al., 2004). Bacteria in the colony detect environmental signals and respond to the physicochemical properties of substrates with change in their physiology and morphology, which can affect their motility and

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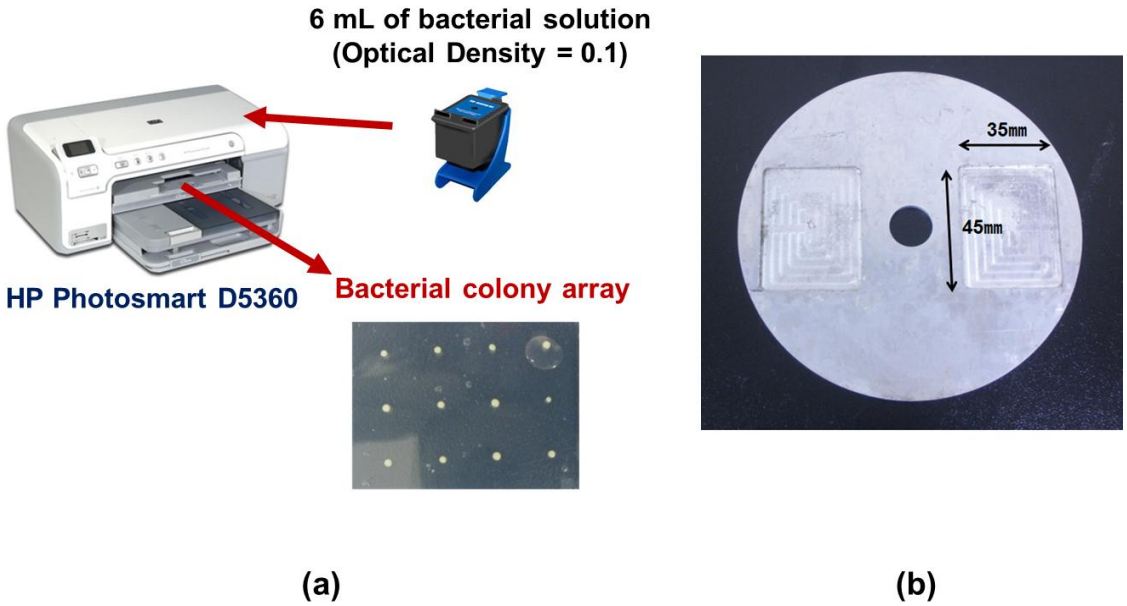

Figure 1. (a) Schematic of process of single colony inoculation using inkjet printer and picture of colony array. (b) Photograph of a custom-made aluminum disk containing two chambers.

growth (Harshey, 2003; Park et al., 2003; Hochbaum and Aizenberg, 2010). Bacteria colonize surfaces and proliferate themselves in various growth patterns according to the substrate bioavailability and nutrient level (Takhistov and George, 2004; Steager et al., 2008).

Recently, eukaryotic cells have provided further evidence of response to the physical characteristics of environment. For example, migrating 3T3 cell generates stronger traction force on stiffer substrate, thus movement of the cell could be controlled by the rigidity of the substrate (Lo et al., 2000). Matrix elasticity and mechanical stress modulate the decision of stem cell specialization (Engler et al., 2006; Chowdhury et al., 2010). Although those studies demonstrated that eukaryotic cells react to the mechanical properties of substrates, bacteria are also likely to respond to contact surface presumably because some of the structural elements in a bacterial cell are known to have functions analogous to those in a eukaryotic cell. Many proteins of bacteria function similarly to eukaryotic cytoskeletal proteins (Dye and Shapiro, 2007; Kim et al., 2006). Although most experimental studies have shown that bacterial reactions are more in the form of chemical or quorum sensing of the environment, such as the concentrations of nutrients or proteins, rather than mechanical sensing, we investigated how bacteria react to the stiffness of substrate.

Henrichsen (1972) identified six different types of bacterial surface translocation, such as swimming, swarming, gliding, twitching, sliding, and darting. The driving forces of swarming motility and gliding motility are the rotation of flagella and the retraction of polar type IV pili, respectively. Flagella and pili have been thought to play important roles in the development of these motilities, although the exact mechanisms underlying their responses to the substrate are unknown. Focusing on two types of motility, swarming and gliding, we investigated the growth kinetics of swarming wild-type *Escherichia coli* (*E. coli*), non-motile *E. coli*, and gliding *Myxococcus Xanthus* (*M. xanthus*), grown on soft or hard agar substrates controlled with different amounts of nutrient and agar.

MATERIALS AND METHODS

Bacteria culture

Glycerol stocks of motile wild-type *E. coli* (ATCC 25922) and non-motile *E. coli* (ATCC 26) were incubated in Tryptic Soy Broth (TSB) at 37°C, 180 rpm. A glycerol stock of motile *M. xanthus* (ATCC 25232) was incubated in Casitone medium at 32°C, 180 rpm.

Preparation of agar substrate

In order to produce substrates having different mechanical properties, three kinds of agar solution were made with agar concentrations of 0.5, 1.5, and 2.5% (g/L). And then the various amounts of broth powder were added into the agar solutions. The amount of standard nutrient of TSA medium is 30 g of TSB in 1 L of agar solution and in the case of Casitone agar medium the amount of standard nutrient is 20 g of Casitone in 1 L of agar solution. The amount of nutrient is the basis for standard nutrient (100%) and 50 and 10% of nutrient media were also made. These agar solutions were sterilized by autoclaving at 120°C for 15 min. After autoclaving, each of the agar solution was poured into chambers ($35 \times 45 \times 1.7$ mm^3) in a custom-made aluminum disk (Figure 1). After cooling them, substrates with controlled concentrations of agar and nutrient were completed.

Inoculation by inkjet printing

We used a micropipette and an inkjet printer to inoculate the agar substrate with a suspension of bacteria. In order to make a bacterial colony array, *M. xanthus* and *E. coli* were inoculated on agar substrates by using a modified inkjet printer (Photosmart D5360, HP). The printer cartridge was washed with alcohol and distilled water to remove the ink. The cartridge was filled with 6 mL of bacterial culture medium (optical density = 0.1 at 600 nm). Locations for the inoculation of bacterial cells were specified by a template prepared with the Microsoft PowerPoint. After ejection of bacterial solution on agar substrates, the substrates were incubated at 37°C. To observe the dynamics of colony formed by the bacteria of high density and large volume, the center of each agar substrate was inoculated with 5 μL of a bacterial suspension with optical density of 1.7 manually by using a micropipette, and then the substrates were incubated at 37°C.

Quantitative analysis of bacterial colony growth

Colony growth can be surveyed by the count of viable cells. A single bacterial colony was dissolved in 1 mL of distilled water, and then the optical density of the bacterial suspension was measured by a spectrophotometer (Ultrospec 10, Amersham Biosciences). The number of viable cells in the colony was linearly proportional to the optical density. Optical densities of single colonies selected from the array of *E. coli* colonies were measured at 20, 40 and 60 h after incubating the agar substrates, and the same procedure was conducted for the array of *M. xanthus* colonies at 40, 80, and 110 h after incubation.

Measurement of diffusion rate of nutrient in agar substrate

To estimate the diffusion rate of the nutrient in an agar substrate, we used a fiberoptic-based fluorescence recovery after photobleaching (Fiberoptic-based FRAP) system modified from our previous version (Lee et al., 2010). Small pieces of agar substrates with 0.5, 1.5, and 2.5% (g/L) agar concentrations were immersed in 3 kDa FITC-Dextran solution for 48 h. In the FRAP method, a small volume of sample full of fluorophores is bleached briefly by a high-intensity laser beam, and then the bleached region is filled by diffusion of unbleached fluorophores, which contribute to fluorescence recovery. From the fluorescence recovery signal, the diffusion rate of biomolecules can be deduced. We used a neutral density (ND) filter to control the output power of a blue diode laser with 473 nm wavelength. After photobleaching the sample by using a ~20 mW laser for 500 ms, we detected the fluorescence recovery signal by using a photomultiplier tube (PMT) at 0.01~0.1 mW of laser power. A program made with the LabVIEW software (National Instruments, TX, USA) was used for operating the FRAP experiment and acquiring the data from the PMT. A fractional fluorescence recovery was calculated as $f(t) = [F(t) - F(0)]/[F(\infty) - F(0)]$, where $F(\infty)$ and $F(0)$ represent the fluorescence intensity before and after photobleaching, respectively. We obtained half recovery time ($t_{1/2}$) from the fluorescence recovery curve, and evaluated relative diffusion rate as $D_{gel}/D_{solution} = t_{1/2,\ solution} / t_{1/2,\ gel}$.

Measurement of agar substrate stiffness

In elastic region, if external force is applied perpendicular to substrates, the stiffness of substrates can be measured by length variation (Pelham and Wang, 1997). Three kinds of agar solution, 0.5, 1.5, and 2.5% (g/L), were put into separate bottles. These agar solutions were sterilized by autoclaving at 120°C for 15 min. After autoclaving, each of the agar solution was poured into a cylindrical tube of 40 mm diameter and cooled at room temperature. The cylindrical agar gels were pulled out of the tubes and their initial heights were measured. After 75.34 and 191.41 g weights were put on the top of an agar gel one after another, the heights of the agar gel were checked. The elastic modulus (E) of an agar substrate was calculated as $E = \sigma/\varepsilon = (F/A) / (\Delta L/L_0)$, where σ and ε denote the stress and strain, respectively.

RESULTS

Inoculation using an inkjet printer provided more quantitative and sophisticated control of the bacterial colony array than using a micropipette. A droplet having a volume of approximately hundreds of picoliters was ejected from the inkjet printer, and bacterial colonies were formed at regular intervals on agar gel substrates (Figure 1). Figure 2 shows the optical densities of a gliding motile *M. xanthus* colony (Figure 2(a-c)), a swarming motile *E. coli* colony (Figure 2(d-f)) and a non-motile *E. coli* colony (Figure 2(g-i)), describing how colonial growths of different bacterial species change according to the variation of agar concentration and nutrient grade on a substrate. Culture substrates were made to contain three levels of agar concentration, 0.5, 1.5, and 2.5% (g/L), and three levels of nutrient concentration, 10, 50, and 100% of standard quantity. *M. xanthus*, which uses the retraction of type IV pili for gliding, exhibits that there are more viable cells on substrates of lower agar concentration (Figure 2(a-c)). Likewise, more viable cells are detected on the substrate of lower agar concentration at the initial phase of motile *E. coli* colony growth (Figure 2(d)), but this tendency is reversed over time (Figure 2(e, f)). In the case of non-motile *E. coli* colony, the number of viable cells is almost constant independently of agar concentration (Figure 2(g-i)).

Generally, regardless of bacterial species, there are fewer viable cells on substrates with lower amount of nutrients. The variation of the cell number with agar concentration shows a similar trend on substrates of 50 and 100% nutrients. But the growth of bacterial colony on substrates of 10% nutrient shows little dependence on the agar concentration and thus the number of viable cells is kept at a low value. One microliter of high-density wild-type *E. coli* cell suspension was inoculated on 0.5 and 2.5% agar substrates, respectively, to induce rapid growth of a single bacterial colony. Table 1 and Figure 3 show the variations of the diameters of bacterial colonies with respect to agar concentration and elapsed time after inoculation. At 22 h, colonies on the 2.5% agar substrate are already bigger than those on the 0.5% agar.

It is known that the molecular weights of approximately 90% of the ingredients of TSB and Casitone are less than 2 kDa and those of 99% are less than 5 kDa. We measured a half recovery time for 3 kDa FITC-Dextran in an agar gel by using FRAP technique to estimate the effect of agar gel concentration on the diffusion rate of nutrients. Table 2 displays the relative diffusion coefficients in agar gels procured from the ratios of the half reco-

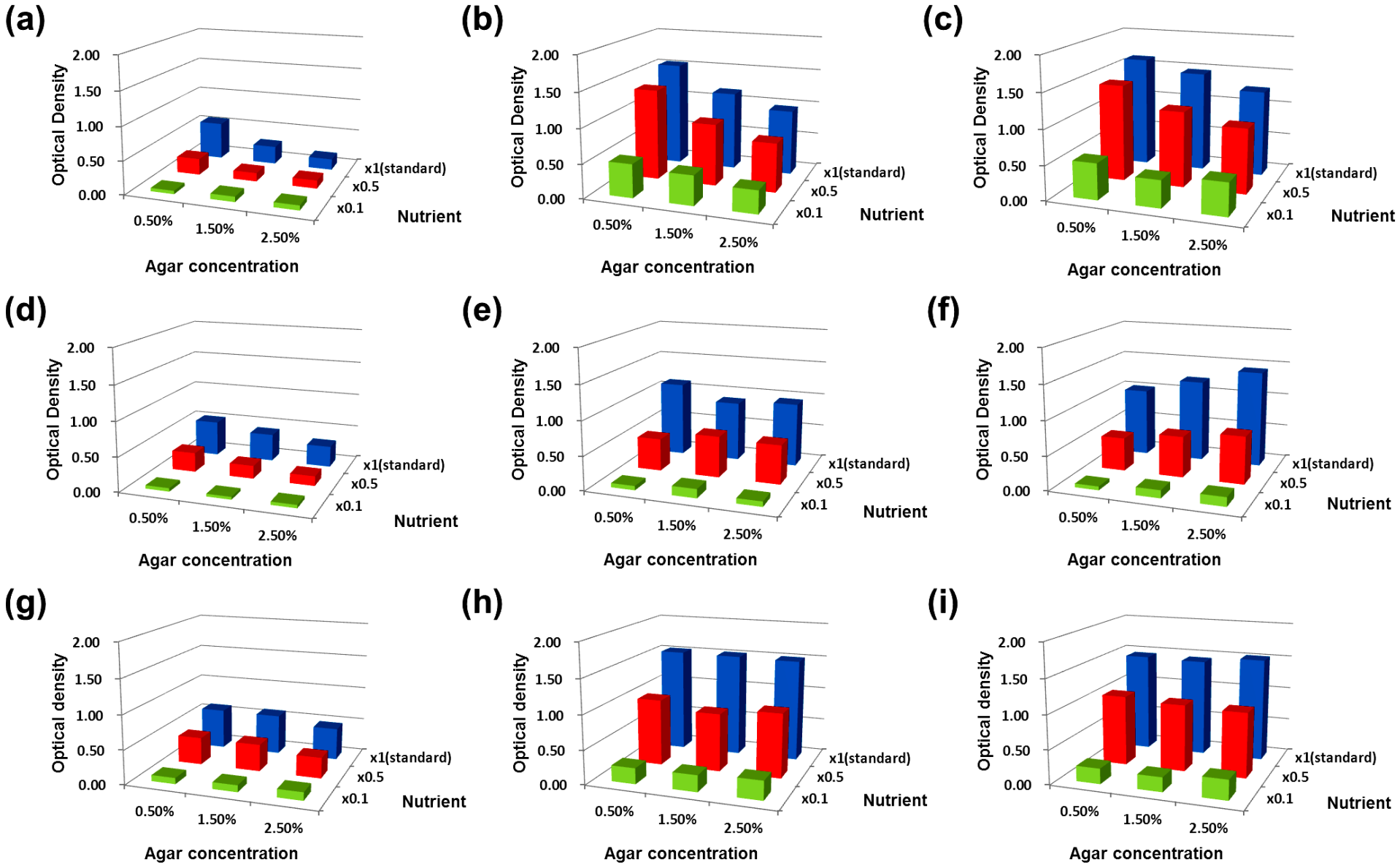

Figure 2. Change in optical density of growing single bacterial colony with variation of agar and nutrient levels on a substrate. (motile *M. xanthus* at (a) 40, (b) 80, and (c) 110 h after inoculation, motile *E. coli* at (d) 20, (e) 40, and (f) 60 h after inoculation, non-motile *E. coli* at (g) 20, (h) 40, and (i) 60 h after inoculation).

Table 1. Variation of the diameters of bacterial colonies with respect to agar gel concentration and elapsed time after inoculation.

Agar concentration (%)	Mean diameter of *E. coli* bacterial colonies (mm)			
	22 h	30 h	47 h	57 h
0.5	1.3	1.4	1.8	2.0
2.5	1.9	2.3	3.4	3.8

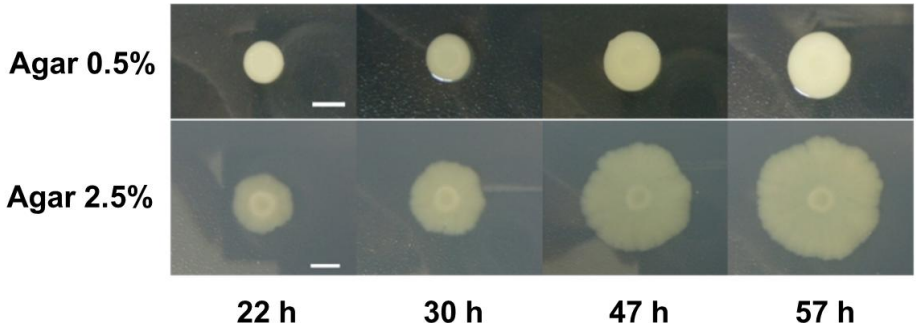

Figure 3. Swarming motility of wild-type *E. coli* cells in single colony (OD = 1.7) at 22, 30, 47 and 57 h after incubation (from left to right) on the tryptic soy agar gels of two different agar concentrations (top: 0.5% gel, bottom: 2.5% gel). Scale bar = 1 mm.

Table 2. Relative diffusion coefficients of FITC-Dextran (MW = 3 kDa) in distilled water and gels of different agar concentrations measured by fiberoptic-based fluorescence recovery after photobleaching technique.

Sample	$t_{1/2}$ (s)	Relative diffusion coefficient
Solution	2.34 ± 0.218	1.00
0.5% agar gel	12.82 ± 1.033	0.18 ± 0.014
1.5% agar gel	14.68 ± 0.634	0.16 ± 0.007
2.5% agar gel	16.61 ± 0.801	0.14 ± 0.007

very time in solution to the half recovery times in gels. The relative diffusion coefficients in the substrates of 0.5, 1.5, and 2.5% agar concentrations are 0.18, 0.16, and 0.14, respectively, showing a narrow distribution.

After high pressure and high temperature sterilization, agar solution is gelated through an exothermic process. The stiffness of the agar substrate increases with agar concentration. Figure 4 represents the elastic moduli (kPa) of 0.5, 1.5, and 2.5% agar substrates. The elastic moduli of substrates could be represented as relative quantity to modulus of 2.5% agar substrate, because water in agar substrates evaporates over time leading to change in the absolute value of elastic modulus. The relative elastic moduli of 0.5 and 1.5% agar substrates to that of 2.5% are 0.04 and 0.32, respectively.

DISCUSSION

At the beginning of bacterial colonization, the change in viable cell numbers of both wild-type *E. coli* and *M. xanthus* followed the same trend - the number of cells was higher on the substrate of lower agar concentration - whereas the number of viable non-motile *E. coli* cells was independent of agar concentration (Figure 2). The tendency of wild-type *E. coli* growth was reversed over time. This indicates that agar concentration could affect bacterial surface motility because non-motile *E. coli*, genetically disabled from moving, did not react to agar concentration but the growth of motile bacteria correlated positively or negatively with agar concentration depending on the type of motility. After a certain time threshold, wild-type *E. coli* exhibited augmented growth with increased agar concentration. Especially, when the agar substrate was inoculated with a suspension of high density bacteria, the time threshold was advanced (Figure 3). During the growth of bacteria on the surface, the number of viable cells goes through a lag phase and then an exponential phase. The slope of exponential phase for wild-type *E. coli* cultured on 2.5% agar substrate was steeper than that of bacteria on 0.5% agar (Figure 5).

Bacterial motility is regulated by quorum sensing system based on the localized high- density of cells and also

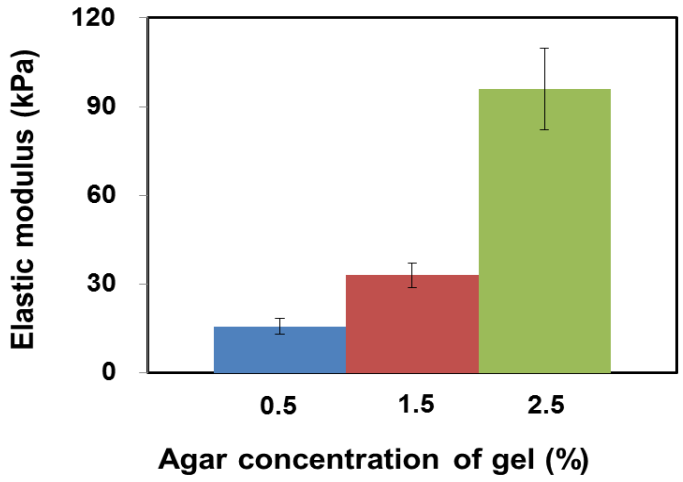

Figure 4. Elastic moduli (kPa) of substrates of 0.5, 1.5 and 2.5% (g/L) agar concentrations.

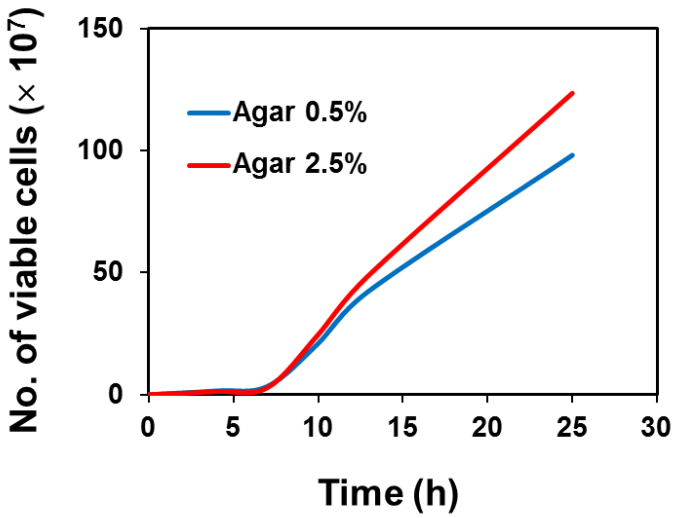

Figure 5. Number of viable wild-type *E. coli* cells on 0.5 and 2.5% of agar gel plates versus elapsed time after inoculation.

by substrate sensing system based on substrate properties (Eberl et al., 1999). In our experiments, altered mechanical properties of the substrate affected bacterial surface migration. More specifically, the substrate with 2.5% agar concentration induced high cell density at a certain time after bacterial inoculation, and subsequently might affect cell-cell communication and bacterial motility. Although the exact mechanisms underlying these complicated interactions among cells, active molecules, and substrate are still unknown (Copeland and Weibel, 2009), the relation between substrate and bacterial surface migration would be significant for a better understanding of bacterial colonization.

The amount of agar broth changes the properties of an agar substrate, such as pore size (Maaloum et al., 1998), stiffness and surface roughness. We carried out extensive experiments to find which property of the substrate mainly controls bacterial growth. Availability of nutrients at the surface of agar substrate can be lowered by hampered diffusion of nutrients in an agar gel of small pore size. However, measured diffusion coefficients of 3 kDa FITC-Dextran, which are comparable in size to nutrients, in agar gels were not substantially changed by agar concentration (Table 2). Although both motile and non-motile bacteria preferred high-nutrient agar substrate, the growth of non-motile bacteria was independent of agar concentration (Figure 2). This finding suggests that the availability of nutrients was unchanged in the range of agar concentration used here. Thus, the pore size of the agar substrate is not one of the factors in the control of bacterial growth.

Substrate stiffness could have an effect on bacterial cell growth considering that measured elastic moduli of gels of 0.5, 1.5, and 2.5% (g/L) agar concentrations were different significantly (Figure 4). Prior studies reported that the stiffness of a substrate plays an important role in the behaviors of eukaryotic cells (Lo et al., 2000). Lichter et al. (2008) have shown that the adhesion of bacteria to a surface correlates positively with substrate mechanical stiffness in the range of 1 to 100 MPa. Average elongation rate of DRG neurite was higher for the low-stiffness substrate with a range of agarose concentration from 0.75 to 2.0% (g/L) (Balgude et al., 2001). Colonization of swarming bacteria has a cycle of cell differentiation and migration (Rauprich et al., 1996). Our experimental results imply that substrate stiffness influences the ability of motile bacterial cells to migrate on the surface, and consequently affects the number of viable cells in a colony.

E. coli cells move by rotating their flagella and interacting with neighboring cells. On the other hand, *M. xanthus* existing in soil can move using the type IV pili by attaching the pole of pili on the surface or the pole of other cells and then generate a traction force. Of the two motility systems contained in *M. xanthus*, S-motility is generated by the pilus and A-motility is powered by protein clusters (Mignot et al., 2007). *M. xanthus* cells sense a substrate and react to it by changing their motility system. It seems that they generated S-motility and sharply increase the portion of A-motility on the substrates of 0.5 and 2.5% agar concentrations, respectively (Figure 2). The growth responses of wild-type *E. coli* and *M. xanthus*, possessing different types of motility, observed at the present study showed opposing trend (Figure 2). Our results indicate that the type of surface motility is a key determinant of the growth response of bacteria to substrate stiffness.

In this study, we found that the growth of motile bacteria, but not that of non-motile bacteria, is modulated by substrate stiffness independently of the nutrient level. This finding enables us to assume that different species of bacteria have their own growth mechanisms responding to substrate mechanical properties. Although other

species or strains of bacteria should be investigated further considering additional physical parameters of substrates such as roughness, interaction energy or surface charge, the present work has potential application to the design of surfaces that prevent or promote biofilm formation.

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