

Effects of Chelating Reagents on Colonial Appearance of *Paenibacillus alvei* Isolated from Canine Oral Cavity

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ABSTRACT. A bacterial strain isolated from the oral cavity of a healthy dog revealed an unusual colony formation in nebular appearance on agar plates. The isolated bacterial strain was Gram-positive, spore-forming rod with peritrichous flagella, and grown under aerobic conditions, but unable to grow at 45°C. The strain was tentatively classified as *Paenibacillus alvei* according to the biochemical properties and the 16S rRNA gene sequence. The isolate exhibits collective locomotion on solid agar plates. The bacterial motility was inhibited with EDTA and was restored by adding magnesium. We concluded that magnesium ion is essential for collective locomotion of *P. alvei*. This suggests that EDTA is useful for inhibition of biofilm formation.

KEY WORDS: biofilm, EDTA, magnesium, *Paenibacillus alvei*, swarming.

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Some species among the *Bacillaceae* have been shown to produce a variety of complex patterns during colony development [8, 10, 11]. Various and sometimes contradictory hypotheses have been proposed to explain why pattern formation occurs, i.e. adaptive morphogenesis, the need for a colony to alter its shape to survive in adverse environments [9], or humidity response, the ability of the bacteria to respond to other environmental factors, such as local humidity [31], regardless of environmental adversity.

The genus *Bacillus*, a systematically diverse taxon [16], has been divided into at least 10 phylogenetic groups by the 16S rRNA gene sequence analyses [4, 5, 20, 35, 40, 45, 46, 48]. The rRNA group 1 includes the type species *Bacillus subtilis*, and the rRNA group 3 was proposed for the new genus *Paenibacillus* by Ash *et al.* in 1991 [5]. The genus *Paenibacillus* currently encompasses more than 50 species, and *Paenibacillus polymyxa* is assigned as the type species of the genus [4]. Members of the genus *Paenibacillus* are facultatively anaerobic or strictly aerobic, rod-shaped, produce ellipsoidal spores in swollen sporangia and show G+C contents ranging from 45 to 54 mol% [4]. *Paenibacillus* strains have been detected in a variety of environments such as soil [12], water, rhizosphere [44], vegetable matter, foods, tree roots, forage and insect larvae [17], and clinical materials in hospital [14].

In the present study, we examined a bacterial strain which produces a distinguished colonial appearance on agar plates, isolated from oral swabs of a healthy dog. On the basis of the phenotypic and phylogenetic findings presented here, we tentatively identified it as *Paenibacillus alvei*. We also found magnesium ions to be essential for collective locomotion of the isolated strain on the agar plates. Flagella con-

tribute not only to motility but also to adhesion [27, 38] and colonization implying biofilm formation. This bacterial strain is considered to benefit the model of flagellation and biofilm formation.

MATERIALS AND METHODS

Bacterial isolation: A bacterial strain isolated on tryptic-soy agar plates from the oral cavity of an apparently healthy dog. Isolated strain, designated as AUG6, was a spore-forming, Gram-positive rod, and grown under aerobic conditions, but unable to grow at 45°C. The strain showed a fractal pattern of colony formation on solid medium and was grown in liquid medium as well.

Flagella staining: Flagella staining was performed on the bacterial cells grown on the LB agar plates prepared by using Luria Broth (SIGMA-ALDRICH, Inc., Tokyo, Japan) with or without 1.5% Difco Agar Noble (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) at 37°C for 20 hr, as described by Leifson [28]. Bacterial cells were picked up from the edge of spreading colonies and were subjected to flagella staining.

Biochemical examination: Acid production from 49 carbon sources was examined at 37°C under aerobic conditions by using an API 50CH kit combined with API 50CHB/E medium (bioMérieux, Lyon, France). Reading was done at 24 and 48 hr after inoculation.

16S rRNA gene sequence: The 16S rRNA gene of AUG6 strain was amplified with universal primers 8FPL (5'-AGTTTGATCCTGGCTCAG-3'), 806R (5'-GGACTACAGGGTATCTAAT-3'), 515FPL (5'-TGCCAGCAGCCGCGTAA-3'), and 1492RPL (5'-GGTTACCTTGTTACGACTT-3') by PCR [41]. Briefly, a single colony was first picked from the plate and suspended in 100 µl of water and incubated at 95°C for 3 min. Bacterial suspension (5 µl) of the AUG6 strain was added into 45 µl of the PCR master

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mixture consisting of 5 μ l of 10X PCR buffer, 8 μ l of 2.5 mM deoxynucleoside triphosphates, 10 pmol each primer (combinations of 8FPL and 806R, or 515FPL and 1492RPL), 100 nmol $MgCl_2$ and 2 units of AmpliTaq Gold DNA polymerase LD (Applied Biosystems, Tokyo, Japan). PCR was performed with an initial denaturation at 94°C for 30 sec, followed by 30 cycles for 30 sec at 94°C, 2 min at 55°C, and 1 min at 72°C as described previously [21]. Amplified products were sent to a reference laboratory (TaKaRa Custom Services, Shiga, Japan) for DNA sequencing. Nucleotide sequences were combined without overlap. The nucleotide sequence was deposited to the international DNA databases under the accession number AB377108, and was evaluated by checking against existing sequences by the BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>).

Agar concentration: To observe the colonial formation on different agar concentration, Difco Agar Noble (Becton, Dickinson and company) was added to Luria Broth (SIGMA-ALDRICH). Agar concentrations examined varied at 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. After agar was autoclaved, 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) and 24 μ g of isopropyl- β -D-thiogalactopyranoside (IPTG) per ml were added. Bacterial strain was inoculated in the middle of 90 mm agar plates in Petri dish. Plates were incubated at 37°C and observed after 24 hr.

Chelating reagents: Chelating reagents, disodium EDTA (Nacalai, Kyoto, Japan) and disodium EGTA (Nacalai), were used at the following concentrations, 0.25 and 0.5 mM. Each chelating reagent was added to Luria Broth supplemented with 0.5% or 1.5% Difco Agar Noble. Bacterial strain was inoculated in the middle of the agar plates of 90 mm diameter. Plates were incubated at 37°C and observed after 24, 48 and 72 hr after inoculation.

Magnesium sulfate: 100 μ l of 100 mM magnesium sulfate heptahydrate (Nacalai) solution was applied on 1.5% agar plates prepared with Luria Broth supplemented with 0.25 or 0.5 mM EDTA and 1.5% Difco Agar Noble. Magnesium sulfate solution was spread uniformly on the surface of plates by using Conradi rods.

RESULTS

Flagellum staining: Peritrichous flagella were observed on bacterial cells obtained from micro-colonies moving on 1.5% agar plates (Fig. 1), but not apparent on bacterial cells from broth cultures (data not shown).

Biochemical examination: After 24 hr incubation, acid was produced from glycerol, ribose, adonitol, galactose, glucose, *N*-acetylglucosamine, esculin, cellobiose, maltose, trehalose and starch. Acid was also produced from α -methyl-D-glucoside, arbutin, salicin, melibiose, sucrose, raffinose, glycogen, gentiobiose, and 5-ketogluconate after 48 hr incubation. Acid was not produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, β methyl-D-xyloside, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, β -methyl-D-mannoside,

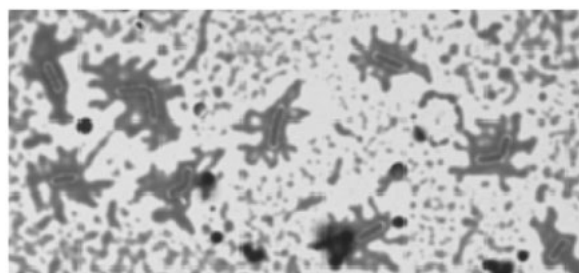


Fig. 1. Flagella of bacterial cells grown at 37°C for 20 hr on 1.5% agar plates prepared with Luria Broth were stained by the method described by Leifson.

amygdalin, lactose, inulin, melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, or 2-ketogluconate.

16S rRNA gene sequence: PCR-amplified nucleotide sequences were combined into a single nucleotide sequence of 1484 bp, covering over the entire region of the 16S rRNA gene of the isolate. This sequence showed 1465/1480 (98%) to 1458/1472 (99%) nucleotide similarities to *Paenibacillus alvei* strains of the accession numbers with AJ320491 and AB073200, respectively.

Agar concentration: *Paenibacillus alvei* strain AUG6 did not form isolated colonies on the agar plates of less than 1.0% concentration of agar and grew entire area on the plates (Fig. 2). After 24 hr, colonies were observed entire area on the 1.5% and 2.0% agar plates. On 2.5% agar plates, colonies were observed about 70 mm in diameter and about 20 mm in diameter on 3.0% agar plates. We observed two types of bacterial motility by microscopic observation. On the plates at low concentration of agar, bacterial cells moved individually but at high concentration of agar, bacterial cells formed micro-colonies and swarmed (Fig. 3).

Effects of chelating reagents: Bacterial motility was not always affected by EGTA added into agar plates. In case of 0.5 mM EGTA-contained 1.5% agar plates, a little time lag delayed in spread of bacterial colonies was observed. On the other hand, addition of EDTA into agar plates reduced bacterial motility at 0.25 mM (Fig. 4), and suppressed the bacterial growth on the agar plates at 0.5 mM. After 24 hr of incubation, a bacterial colony enlarged to about 50 mm in diameter on 0.5% agar plates and to about 20 mm in diameter on 1.5% agar plates containing 0.25 mM EDTA. Bacterial cells on 1.5% agar plates containing 0.25 mM EDTA were elongated-filamentous and shrunk under microscopic observation (Fig. 5).

Effect of magnesium sulfate: Bacterial motility on agar plates containing 0.25 mM EDTA was markedly restored by addition of magnesium sulfate, and bacterial cells spread over the entire area on the plates after 24 hr. On the other hand, a time lag delayed in spread of bacterial cells on agar plates containing 0.5 mM EDTA was observed as compared to those on the 1.5% plain LB agar plates. It took around five days to spread over the entire area on the plates of 90 mm in diameter.

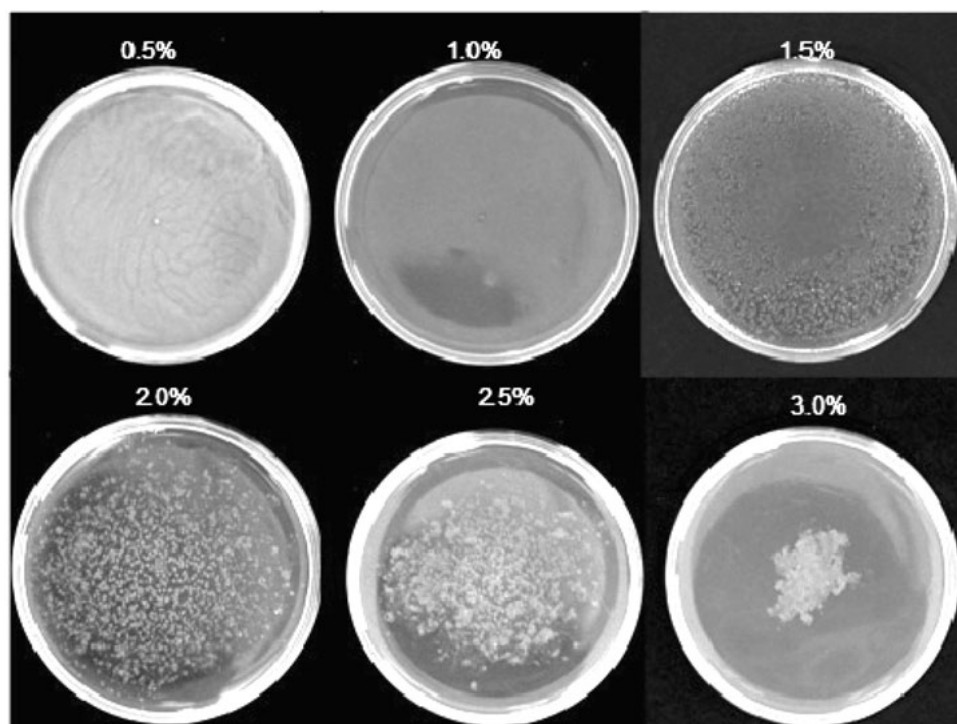


Fig. 2. Colonial appearance on plates at several agar concentrations. Colonies were observed after 24 hr of incubation at 37°C.

DISCUSSION

It is known that polar flagella contribute for swimming and lateral flagella for swarming, and increased viscosity of the medium inhibits polar flagella motility and induces the lateral flagella expression. In the present study, our results were compatible with these properties of flagellum activity. On the plates at low concentration of agar, bacterial cells moved individually but at high concentration of agar, bacterial cells formed micro-colonies and swarmed (Fig. 3).

Bacterial cells capable of swarming produce highly organized communities, consisting of vegetative cells, the swimmer cells, which undergo a coordinated differentiation process characterized by the production of hyperflagellated, elongated, multinucleate cells like the swarming cells [19, 24–26]. 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. Collective swarming motility simultaneously stops, and swarming cells dedifferentiate in unison, reverting to the short, oligo-flagellated, actively growing swimmer cells [2, 24–26]. Alternate cycles of swarming and consolidation are peculiar to the developmental behavior of swarming colonies, which may exhibit macroscopic layered consolidation phases, forming regularly spaced concentric rings such as those produced by *Proteus mirabilis* [25].

Swarming is thought to be a successful strategy devel-

oped by flagellated bacteria to ensure their rapid expansion in the natural environment, where microbial activities are often associated with biofilm formation on solid surfaces. Swarming motility was also proven to play a role in the colonization of host mucosal surfaces by infectious agents [2, 7]. Moreover, the finding that some bacteria, such as uropathogenic strains of *Proteus mirabilis*, produce higher levels of specific virulence factors during swarming [1, 3], is of intrinsic interest as well as of great medical relevance.

On the other hand, elongation of the bacterial cells treated with 0.25 mM EDTA (Fig. 5) was considered to be due to inhibition of cell division. EDTA is known to block GTP hydrolysis, because GTPases require the presence of a divalent cation (usually Mg^{2+}) coordinating the phosphates of the GTP for efficient catalysis to proceed [30]. Monomers of FtsZ protein, an essential cell division protein, localize to the mid-cell [13] and associate *in vitro* in a GTP-dependent fashion into linear, un-branched, polymeric fibers, shortening and bending of the polymers whose dynamics results in the hydrolysis of GTP [29, 43]. FtsZ is highly conserved among prokaryotes with only one phylum of archaeobacteria except for *Ureaplasma urealyticum*, *Prostheco bacter dejongii*, and *Pirellula sp.* [22, 47]. FtsZ polymer dynamics depends on multiple factors, such as pH, concentrations of magnesium, potassium, calcium, competing nucleotide triphosphates, macromolecular crowding and the presence of FtsZ interacting proteins [23, 32–34, 42, 50]. Extended cripate cells are assumed to be due to magnesium chelating

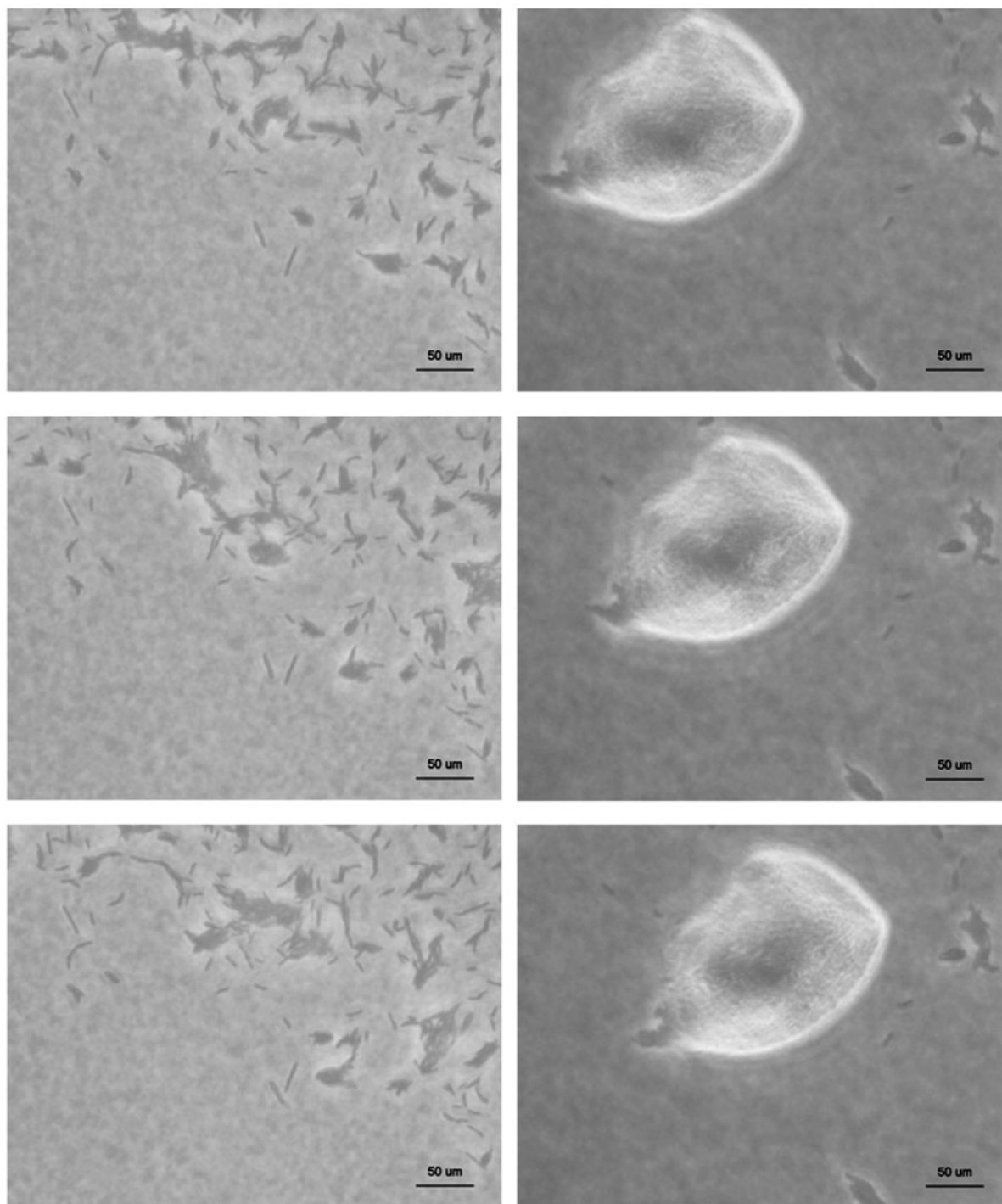


Fig. 3. Bacterial motility on agar plates. Left pictures show individual motility on 1.0% agar plates, and right pictures show social motility on 1.5% agar plates. Three sequential pictures were taken every ten sec.

effect inhibited by FtsZ. FtsZ and eukaryotic tubulin share the same structural fold and form similar proto-filaments [36, 37]. But, in our experiments, addition of tubulin polymerize inhibitors (1.5 mM of colchicine, 15 μ M of nocodazole, 15 μ M of vinblastine, and 50 μ M of vincristine) caused

nothing to change in bacterial morphology and motility (data not shown).

Magnesium ion is known to promote flagellation of *Vibrio fischeri* [39]. Flagella contribute not only to motility but also to adhesion [27, 38] and colonization implying bio-

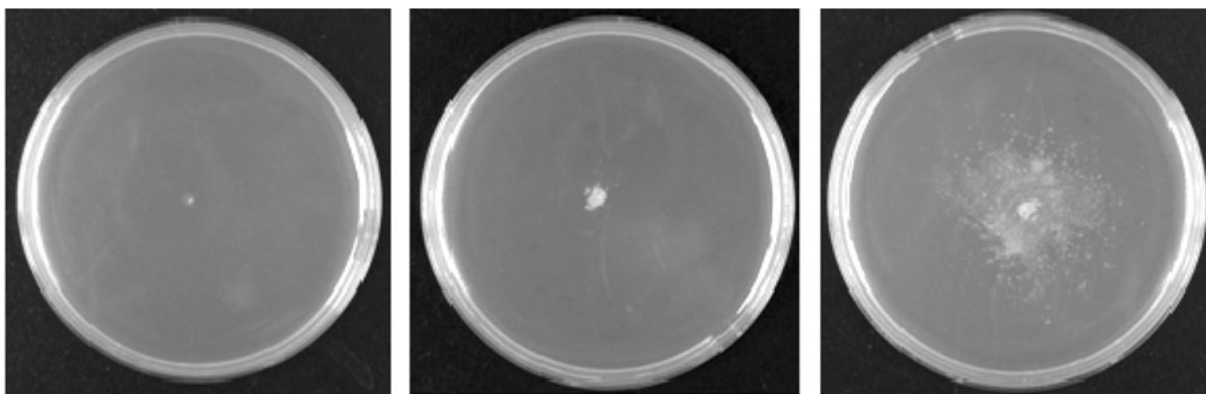


Fig. 4. Colony formation on 0.25 mM EDTA added Luria Broth plates with 1.5% Difco™ Agar Noble. Pictures were taken after 24, 48, and 72 hr of inoculation.

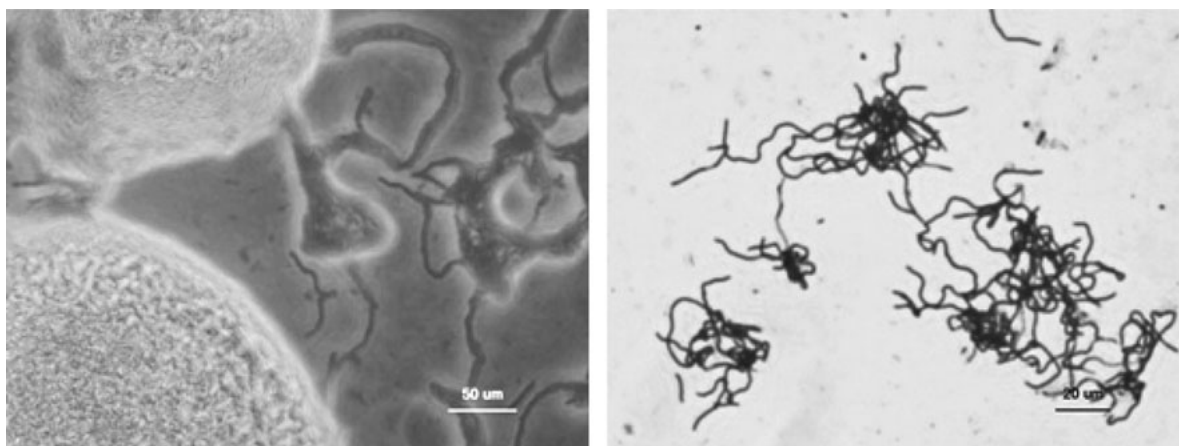


Fig. 5. Microscopic observation of bacterial cells grown on 0.25 mM EDTA added Luria Broth with 1.5% Difco™ Agar Noble, 24 hr after inoculated. Left picture was taken on agar plates, and right picture shows Gram stained bacterial cells.

film formation. Bacterial cells in biofilms are generally more resistant to host defences and antimicrobial agents, and also express more virulent factors as a result of gene activation by quorum sensing. In fact, biofilms of *Pseudomonas aeruginosa* are affected by high concentration of EDTA treatment and induced detachment and killing, which can be blocked by addition of magnesium, calcium, or iron. Similarly, EGTA is effective for bacterial detachment from the biofilm, but killing rate was five times lower than those treated with EDTA [6]. Thus, the addition of 0.25 mM EDTA may inhibit flagellation and has quorum quenching activity [18] against the multi-cellular behavior in bacterial motility. And, further experiments by supplying magnesium sulfate revealed that Mg^{2+} was requirement for *P. alvei* motility. However, supplying magnesium sulfate to 0.5 mM EDTA partly restored motility and cell division. It may mean just Mg^{2+} cannot account for all vital divalent cation. In fact, *P. alvei* did not grow on 10 mM EDTA agar plates containing magnesium sulfate.

Calcium ion is thought to be necessary for gliding motility in *M. xanthus* [49], and could not be replaced with Mg^{2+} , Ba^{2+} , or Sr^{2+} [15]. However, our results suggest that *P. alvei*

exerts swarming motility without calcium ion, because addition of EGTA which is said to present a more precise action on calcium ions than EDTA, made little effect on its motility. Even at 10 mM EGTA, *P. alvei* could proliferate and swarm though time lag delayed. It took a few days to grow over the whole surface of the 1.5% agar plates in 90 mm diameter containing 10 mM EGTA (data not shown).

In conclusion, gram positive rod isolated from the oral cavity of a healthy dog form atypical colonies on agar plates. The strain was tentatively classified as *Paenibacillus alvei* according to the biochemical properties and the 16S rRNA gene sequence. We observed bacterial swimming on soft agar and swarming on hard agar. This is explained by the increase in the media viscosity induced lateral flagella expression and advanced swarming. The addition of EDTA but not EGTA inhibited the bacterial motility and produced elongated-filamentous cells. Supplement of magnesium sulfate restored the bacterial motility, suggesting that Mg^{2+} was essential for nebular colony formation of *P. alvei* and necessary for cytokinesis and flagellation. Effects of EDTA on the bacterial motility were not fully understood, and await further investigation. This study may contribute to

sequel of inhibition of biofilm formation, and *P. alvei* AUG8 strain is considered to befit the model for flagellation and biofilm formation.

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