Full Paper

Switching of the rotational direction of rhizoidal colonies in a newly isolated *Bacillus mycoides* strain Ko01

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Bacillus mycoides are known to form rhizoidal colonies on solid medium. In this study, a new strain of *B. mycoides*, strain Ko01, was isolated from soil. Genetic and growth patterns indicated that this strain belongs to subgroup II of the *B. cereus* group. Strain Ko01 forms extensive rhizoidal colonies with predictable directions of rotation. The concentration of the agar, and not the chemical composition, altered the direction of the colony rotation, switching from counterclockwise to clockwise. Agar concentration-dependent switching of rotation direction was unique to strain Ko01 and was not seen in colonies of other *B. mycoides* strains that were tested. Factors affecting colony chirality patterns appeared to be variable among *B. mycoides* strains. This feature can be used for the classification of *B. mycoides* strains.

Key Words: *Bacillus mycoides*; chirality; colony morphology; spreading

Introduction

Bacillus mycoides is a group of *Bacillus* strains that forms rhizoidal colony morphologies on solid media (Bergey and Holt, 2009). The bacterial spreading is responsible for the formation of rhizoidal colony shapes (Kearns, 2010). Spreading does not require flagellum and it is therefore considered as a passive movement. The spreading is a distinctive mode of locomotion compared to swimming or swarming, which requires flagellum and high water availability on solid surfaces (Harshey, 2003). Although it has been widely accepted that spreading depends on the expansion pressure of the growing bacterial population, the exact mechanism for the spreading, as well

as for the formation of the rhizoidal colony, remains unknown.

The spreading rhizoidal colonies appear fractal with rotating branches that often exhibit a specific chirality (Di Franco et al., 2002). Under a given condition, a colony exhibits a clockwise or counterclockwise rotation. Previous studies have found that environmental factors greatly influence the spreading pattern. The chemical composition of the agar (e.g. salt concentration) or the physical forces applied to the media can affect the colony morphology (Stratford et al., 2013; Uomi et al., 2012).

B. mycoides strains are commonly found members of soil ecosystems (Bergey and Holt, 2009). Phylogenetically, they belong to the *Bacillus cereus* group, comprising *B. mycoides*, *B. cereus*, *B. thuringiensis*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. anthracis*, and *B. cytotoxicus* (Soufiane and Cote, 2013). *B. cereus* group is further classified into subgroups I, II and III. A common property of strains belonging to group II is psychrotolerance. Such strains can grow at 7°C, and in some cases at 4°C. By contrast, strains belonging to group I and III are mesophilic. Signature sequences of housekeeping genes such as 16S rRNA, *cspA* and *glpF* can also be used to differentiate these subgroups (Pruss et al., 1999; Soufiane and Cote, 2013).

Phylogeny of *Bacillus cereus* groups are complex. Despite *B. mycoides* strains sharing a common phenotypic trait of forming rhizoidal colonies, *B. mycoides* strains are not clustered into a single phylogenetic lineage (Soufiane and Cote, 2013). For example, *B. mycoides* strains BGSC 6A14, BGSC 6A68, and BGSC 6A19, are classified into subgroups I, II and III of *B. cereus* group, respectively.

We have isolated a novel *B. mycoides* strain from the soil outside the Indiana University Kokomo campus. The strain Ko01 exhibits extensive spreading patterns on solid medium containing more than 1.5% agar. We examined the effects of agar concentrations, as well as other chemi-

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Table 1. Colony morphology of *Bacillus* strains used in the study.

Strains	Colony morphology
Ko01	Rhizoidal
<i>B. mycoides</i> BGSC 6A12	Non-Rhizoidal
<i>B. mycoides</i> BGSC 6A13	Rhizoidal
<i>B. mycoides</i> BGSC 6A14	Rhizoidal
<i>B. mycoides</i> BGSC 6A19	Rhizoidal
<i>B. mycoides</i> BGSC 6A20	Rhizoidal
<i>B. mycoides</i> BGSC 6A47	Rhizoidal
<i>B. mycoides</i> BGSC 6A68	Rhizoidal
<i>B. mycoides</i> Flugge	Rhizoidal (Stratford et al., 2013)
<i>B. weihenstephanensis</i> BGSC 6A24	Non-Rhizoidal
<i>B. weihenstephanensis</i> BGSC 6A50	Non-Rhizoidal
<i>B. cereus</i> BGSC 6A36	Non-Rhizoidal

cal compositions, on spreading. We found that the agar concentration is the sole determinant of the rotational direction of rhizoidal colonies in strain Ko01. Other factors, such as nutrients, salts, and temperature, did not affect the direction of rotation. We further examined the effects of agar concentration on chirality switching in other *B. mycoides* strains. Shifting the chirality of the spreading colony in response to the changing agar concentration was not observed in other bacteria. The ability to switch colony spreading patterns on varying agar concentrations can be used to differentiate *B. mycoides* strains, and strain Ko01 could be used as a model organism to study the effects of the physical properties of solid media on spreading patterns.

Material and Methods

Strains and media. The *Bacillus mycoides* strain Ko01 was isolated from the soil outside the Indiana University Kokomo campus using tryptic soy agar (TSA) media. The additional *Bacillus* strains used in the study were purchased from the *Bacillus* Genetic Stock Center (BGSC) and are listed in Table 1. Unless otherwise noted, TSA consisted of 7.5 g/l tryptic soy medium (Neogen, MI) supplemented with varying amounts of Bacto agar (Becton, Dickinson and Company, NJ). For the growth assay, strains 6A13, 6A14, 6A19, 6A24, 6A50, and 6A36 were grown on TSA, while strains 6A12, 6A20, 6A47, and 6A68 were grown on LB medium. The poured plates were allowed to solidify and left at room temperature for exactly 20 minutes prior to inoculation. For inoculation, a small amount of cells were first resuspended in 1 ml of TSA broth. 2 μ l cell suspensions were spotted to 3 separate locations on the agar plate. The plates were incubated at 25°C or at a designated temperature for 48 hours or 7 days. The shapes of the colony were observed and recorded from directly above the agar plates.

CaCl₂, KCl, MnCl₂, ZnCl₂, MgCl₂, Zn₂SO₄, KH₂PO₄, Fe(SO₄)₂, Cu(SO₄)₂, CdCl₂, NaNO₃ or (NH₄)₂SO₄ were separately added to the medium to study the possible effects of chemical contaminants of agar on bacterial spreading. A chemical was added to the nutrient medium containing 1.5% Bacto agar so that the final concentration of calcium, chloride, copper, iron, magnesium, manganese, nitrate, phosphate, potassium, sulfate, and zinc was 0.537%, 0.063%, 0.003%, 0.006%, 0.204%, 0.003%,

0.015%, 0.015%, 0.363%, 5.334%, and 0.003%, respectively. These concentrations correspond to the concentrations of these chemicals that would be present in a medium containing 4.5% Bacto agar (Becton, Dickinson and Company, NJ).

DNA manipulation. Chromosomal DNA of *B. mycoides* strain Ko01 was extracted using the phenol/chloroform method (Sambrook et al., 1989). The entire genome was sequenced using Illumina platform (Masuda, unpublished result). DNA sequence of 16S rRNA gene and housekeeping genes (*cspA*, *glpF*, *gmk*, *purH*, and *tpi*) were concatenated and used to construct a phylogenetic tree using MEGA (Tamura et al., 2013).

Results

Phylogenetic analysis of conserved gene sequence

The concatenated sequences of 16S rRNA and housekeeping genes of the strain Ko01 showed the highest similarity to the strains belonging to the subgroup II of the *B. cereus* group (Fig. 1). We further analyzed the small region of *cspA*, *glpF*, *gmk*, *purH*, and *tpi* genes with a known SNP that are unique to each subgroup of *B. cereus* group strains (Lechner et al., 1998; Soufiane and Cote, 2013). All of the SNP sequences of Ko01 were identical to the consensus sequences of the group II strains (Table 2), supporting our notion that the Ko01 strain belongs to the group II *B. cereus* group. The DNA sequences of the conserved genes for the strain Ko01 were deposited into GenBank with accession No. KT698098, KT698099, KT698100, KT698101, KT698102, and KT698103.

Strain Ko01 is psychrotolerant

Since members of the subgroup II are psychrotolerant, we examined whether Ko01 was also able to grow at low temperatures. Ko01 strains grew well at 4°C and 7°C, indicating that it is indeed psychrotolerant. Strain Ko01 also grew at ambient temperatures up to 35°C. No growth was observed above 37°C.

Rhizoidal growth of Ko01

Strain Ko01 exhibited extensive rhizoidal growth patterns when it was grown on LB media or TSA media that are routinely used in microbiology laboratories. Such media contain 1.5% agar. Most of the bacteria including *E. coli* form concentric colonies at this agar concentration. Moreover, this agar concentration does not allow bacterial swimming or swarming (Kearns, 2010). The formation of rhizoidal colonies by Ko01 was observed even at higher concentrations of agar (6%), which was the maximum amount of dissolved agar to make consistent solid surfaces.

The colony of Ko01 strain on 1.5% agar consistently exhibited counterclockwise rotations (Fig. 2). The changing concentrations of the agar altered the orientation of colony rotation. When Ko01 was grown on a solid medium containing between 1.5 and 4.5% agar, colonies exhibited branching with counterclockwise (CCW) rotations. By contrast, the direction of the branching changed to clockwise (CW) rotations when the agar concentration was increased to above 4.5%. Changing the source of nutri-

Fig. 1. Phylogenetic tree of selected *Bacillus* strains, constructed with the concatenated sequences of house-keeping genes (16S rRNA, *cspA*, *glpF*, *gmk*, *purH*, and *tpi*).

Bootstrap values are shown. Roman numerals (I, II, and III) represent subgroups of the *B. cereus* group.

Table 2. Nucleotide sequences variation of 16S rRNA, *cspA*, *glpF*, *gmk*, *purH*, and *tpi* gene in Ko01 and their Conesus Sequences for Group I, II, and III subgroups of the *Bacillus cereus* group.

Genes	16S rRNA	<i>cspA</i>	<i>glpF</i>	<i>gmk</i>		<i>purH</i>	<i>tpi</i>
SNP location	1008	4	250	90	531	1284	157
Groups							
Group I	CCTAGAGATAGG	GCAGTA	GGT/GGC	GGAT	TGAAA/TAAAA	CGGT	GCG/GCA
Group II	TCTAGAGATAGA	ACAGTT	ATG	AGAC	CGACC	AGGC/AGGT	AAG
Group III	CCTAGAGATAGG	GCAGTA	GGT/GGA	AGAT	TGAAC	TGGT	GCG
Strain							
Ko01	TCTAGAGATAGA	ACAGTT	ATG	AGAC	CGACC	AGGC	AAG

ents (i.e. LB vs. TSB) or the growth temperature (4°C to 32°C) did not change the orientation of rotation.

Changing the rotational direction of the colony is a phenotypic change

The switching of chirality did not require genetic change. When cells grown on a 1.5% agar plate were inoculated onto a medium containing 4.5% agar, chirality of the colony was switched to a clockwise rotation from the initial inoculation. When the cells from the 4.5% agar plate were transferred back to the 1.5% agar plate, cells switched the colony rotation again without a prolonged incubation period.

Ability to switch chiral rotation based on agar concentration can be used for strain classification

Next, we examined whether the above observations on chirality switching were also observed in other *B. mycoides* strains. All of the *B. mycoides* strains that we tested, except for the strain 6A12, exhibited rhizoidal growth on solid media containing greater than 1.5% agar (Table 1). We first noticed that the orientation of the chirality at given agar concentrations was not identical among strains. Some strains showed CCW rotation while others rotated CW on the 1.5% agar medium. Strain 6A20 had rhizoidal growth,

but its chirality could not be clearly determined.

While we noticed that increases in agar concentration generally had a positive effect on the radius of rotating branches and the width between branches, none of the strains exhibited the switching of chirality when the agar concentration was altered (Table 3). In strains 6A47 and 6A19, the chirality of the colony remained unchanged (Fig. 3). In strains 6A13, 6A14 and 6A68, the colonies lost preferential rotation of the branches at higher agar concentrations (Fig. 3).

The direction of rotation, as well as the mode of switching, with changing agar concentration is a highly variable feature among *B. mycoides* strains. Among seven rhizoidal colony forming strains, five distinctive patterns of how colony rotation changes in response to the variations in agar concentration were observed (Table 3). So far, the classification of *B. mycoides* has been mainly restricted to sequence-based phylogeny and growth temperature studies (Lechner et al., 1998; Soufiane and Cote, 2013). We propose that colony chirality patterns can be used as an additional tool to distinguish *B. mycoides* strains.

Switching orientation of rhizoidal growth is not due to the change in chemical compositions

What aspect of agar leads to the colony chirality switch-

Table 3. Chirality of *B. mycoides* colonies at various agar concentrations. The colony shapes were described using the following abbreviations: CCW (rhizoidal colony with counterclockwise rotation), CW (rhizoidal colony with clockwise rotation), Mix (mixture of CCW and CW rotation within a single colony), or No-rotation (branching colonies did not exhibit rotation but expanded in a linear fashion).

[Agar] g/L	Strains						
	Group I			Group II			Group III
	Ko01	6A13	6A14	6A20	6A47	6A68	6A19
15	CCW	CW	CW	Mix	CCW	CW	CW
30	CCW	CW	No rotation	Mix	CCW	CW	CW
45	CW	CW	No rotation	Mix	CCW	CW	CW
60	CW	Mix	No rotation	Mix	CCW	Mix	CW

Fig. 2. Colony morphology of the strain Ko01 on TSA containing 1.5% or 4.5% agar. The direction of the colony rotation is indicated by the arrow.

Fig. 4. The effects of the agar structure on the colony morphology of the strain Ko01. (A) Strain Ko01 was grown at the edge of the agar plate. (B) Strain Ko01 was inoculated in close proximity to the cleaved agar edge.

Fig. 3. Colony morphology of *Bacillus mycoides* strain 6A47 and 6A14 on TSA containing 1.5% or 4.5% agar.

ing? To eliminate the possibility that changing the concentration of chemical contaminant(s) in the agar is responsible for chirality switching, we first tested 3 different types of agar. Growth properties were tested using Bacto Agar, noble agar (Becton, Dickinson and Company) and Agar purchased from Fisher Scientific. These agar particles had apparent differences in color and sizes, likely resulting from different chemical compositions. Despite these differences, altering the type of agar showed no effect on the colony chirality patterns of strain Ko01. We then separately added known contaminants of Difco agar into the prepared medium and observed its effects on the colony shapes. Contaminants were added to the 1.5% agar medium to contain the equivalent amount of contaminants that 4.5% agar would have. The list of inorganics tested were calcium, chloride, copper, iron, magnesium, manganese, nitrate, phosphate, potassium, sodium, sulfate, cadmium, ammonium, and zinc. None of these compounds affected the chirality pattern. A previous study demonstrated that a strain of *B. mycoides* changes colony chirality due to the change in

NaCl concentration (Uomi et al., 2012). In Ko01 strain, NaCl concentration did not alter the chirality of the colonies. Up to 5% NaCl was added while agar concentration was kept constant (1.5%). While a high NaCl had a negative effect on the growth rate, as seen by slower colony expansion rates, the chirality remained unchanged. The above results strongly suggest that switching colony chirality in the strain Ko01 is due to a change in the physical property of the solid medium as the agar concentration increases, rather than the increase in the concentration of certain chemicals present in the media.

Structure of the solid medium affects the movement

During our investigation, we noticed a variation of colony shapes in response to the structure of the solid medium. Solid agar medium naturally forms a concave surface adjacent to the wall of a petri dish during the solidification process. When a colony reached the edge of the agar medium, the colony lost chirality and expanded towards the edge of the solid medium in a straight manner (Fig. 4A). By contrast, when a convex shape was artificially introduced in the middle of the agar plate, the colony spiraled around the openings (Fig. 4B), reinforcing our conjecture that the spreading pattern of Ko01 is sensitive to the physical property of the medium.

Discussion

To the best of our knowledge, this is the first study demonstrating that a change in the physical property of the solid medium by altering agar concentrations has an impact on the direction of colony spreading. It remains unknown why the spreading pattern can be altered by a change in the physical properties of the medium. Since surface structure also had an impact on the Ko01 colony shapes, it is possible that the spreading of Ko01 is sensitive to the compressive and/or extensive forces on the agar surfaces. It is known that some bacteria orient themselves in response to the applied forces (elastictaxis or mechanotaxis) (Stanier, 1942; Stratford et al., 2013). In a study with mechanotaxis *B. mycoides* Flugge strain, curved surfaces had a similar influence on colony shapes as we have observed in strain Ko01 (Stratford et al., 2013), suggesting that strain Ko01 is also mechanotaxis.

Spreading is proposed to be a passive movement, in contrast to active locomotion such as swimming or swarming (Harshey, 2003). The complete understanding of the underlying principle that allows spreading on solid surfaces with such low water availability requires further study. Surfactants which alter the surface tension of fluids surrounding the colonies were shown to be important in the spreading of some bacteria (Angelini et al., 2009; Matsuyama et al., 1992). The effects of surfactant production on rhizoidal growth pattern and its involvement in chirality switching in Ko01 are currently under investigation.

Summary

Based on the phylogenetic analysis of 16S rRNA and other conserved genes, Ko01 belongs to the subgroup II

of the *B. cereus* group. Consistent with the other members of the subgroup II, Ko01 is psychrotolerant. Based on its ability to form rhizoidal colonies, we propose to name this newly isolated strain, *B. mycoides* strain Ko01. We have also observed that strain Ko01 switched direction of colony rotation, counterclockwise to clockwise, when agar concentrations were varied. We further demonstrated that the strain Ko01 alters colony shapes due to a change in the physical property of the media rather than a change in chemical composition. As many *B. mycoides* strains exhibited similar but distinctive colony patterns when the agar concentration was varied, this simple test can be used to distinguish *B. mycoides* strains from one another, in combination with genetic and temperature-dependent growth analysis.

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