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Clostridioides difficile Motility in Defined Culture Media and its Response to Nutrients

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CLOSTRIDIOIDES DIFFICILE MOTILITY IN DEFINED CULTURE MEDIA AND ITS RESPONSE TO NUTRIENTS

by

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A thesis submitted in partial fulfillment of the requirements
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ABSTRACT

In recent years, *Clostridiodes difficile* has caused a sharp increase in hospital-acquired infections. Patients on multiple courses of antibiotics experience a general clearing of normal gut microbiota, leading to dysbiosis and an opportunity for *C. difficile* to colonize the colon. Understanding which nutrients produce optimal respiratory metabolism in *C. difficile* will help to determine the reasoning behind whether the microbe invests its energy into various cellular processes, such as motility. Different *C. difficile* strains were first cultured in BHIS broth (brain-heart infusion, supplemented) and CDMM broth (*C. difficile* minimal media) before being used to inoculate soft agar wells. The culture growth in the wells was observed to record the motility of each strain in each condition. Initial analysis demonstrated that motility was observed to a greater extent in less viscous, minimal media. This trend pointed to the bioenergetic need of *C. difficile* to expend limited energy resources in exhibiting a chemotactic response towards more distant nutrient sources after metabolizing nearby nutrient stores. Additional studies were performed to elucidate the motility patterns of various mutants in both rich and minimal media in the least viscous agar condition. In this study, the motility of two wild type strains, R20291 and JIR8094, were examined along with several mutant strains that lack selenoproteins. Motility was observed in JIR8094 in defined minimal medium after several days of incubation, and this result was surprising based on the published literature. Some reduction in motility was observed in a *selD* mutant in both rich and defined minimal medium. These results suggest that energy derived from selenoenzymes may contribute to motility. Additional research could be conducted to test this hypothesis.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS/ABBREVIATIONS

ATP – adenosine triphosphate

selD – selenophosphate synthetase gene

I. INTRODUCTION

1.1 *Clostridiodes difficile*

Clostridiodes difficile has been implicated in a growing number of hospital-acquired intestinal infections in recent years, particularly in patients taking a rigorous course of antibiotics, which clears out commensal microbiota.[1-6] It is an opportunistic organism that releases toxins into the large intestine, causing inflammation, diarrhea, and death in severe cases.[2, 3, 7] To understand the mechanisms of its colonization of the human gut, an investigation into its motility is warranted. *C. difficile* possesses a whip-like structure known as a flagellum, as many other motile bacteria do.[2, 8-10] The flagellum in bacteria is powered by a proton motive force (PMF), using energy in the form of ATP.[9-12] This process uses a considerable amount of energy produced by the cell that may have been used for other purposes of cell growth and maintenance. Thus, a cell must have good reason to engage in motility, such as swimming towards nutrients.

1.2 Chemotaxis

Many other organisms have been shown to exhibit motility as a chemotactic response towards nutrients essential for optimal metabolic function, including carbohydrates such as glucose.[11, 13-16] For example, *H. pylori* exhibits motility towards zinc and away from nickel.[17] Furthermore, *E. coli* has long been shown to demonstrate chemotactic motility in response to various sugars and amino acids.[11, 13, 14, 16] However, it has also been shown that flagellar mutants arise when bacteria are exposed to nutrient-rich media.[11, 16] While the exact reasons have not yet been fully described, it is thought that bacteria will mutate to no longer have a flagellum in nutrient-rich environments. This saves energy for the cell to use for faster growth and metabolism since it is already near the nutrients it needs and no longer needs to be motile.

1.3 Toxin Production

The genetic sequences that produce toxins and flagella in *C. difficile* must be alternately switched on and off, depending on the environmental conditions.[18-23] Thus, investigations of *C. difficile* toxin production have revealed the environmental conditions in which the microbe will select between toxin production and flagellar production via genetic switches.[12, 18-20, 22, 24, 25] Pathogenesis of *C. difficile* is determined by how much motility it exhibits compared to the amount of toxin that is produced.[6, 9, 10, 18] The ability of the bacteria to metabolize certain nutrients affects its motility.[24, 26-28] Therefore, further investigations into the metabolism of the microbe are warranted.

1.4 Metabolism and Motility

Experiments conducted using minimal media have allowed for a better understanding of chemotactic motility.[25, 29-31] In the host organism, *C. difficile* is exposed to nutrients present in the colon, such as succinate and sorbitol produced by the host, which it can metabolize.[32] However, in minimal media, only the nutrients that the organism cannot synthesize and cannot survive without are present, forcing the bacteria to become motile from the region of inoculation to consume enough nutrients for growth and metabolism.[33, 34] *C. difficile* is unable to survive without proline, so growth studies involving this organism must contain proline or any related compounds, such as hydroxyproline.[27, 35, 36] Depending on the presence of proline in the growth medium, *C. difficile* can switch between utilizing the Wood-Ljungdahl pathway (WLP) when proline is absent or Stickland reactions when proline is present to metabolize proline.[37-44] Since proline is essential for the growth of *C. difficile*, it has been hypothesized that this organism may respire on proline.[35, 36]

Mutants have been generated from the wild-type strain JIR8094 to observe its growth in the absence of functional proline reductase and glycine reductase, two vital enzymes in the metabolic pathway of proline. The mutants relevant to this study were the following: LB-CD4, LB-CD7, LB-CD8, and LB-CD12. Both LB-CD4 and LB-CD8 lack the ability to produce a fully functional proline reductase enzyme.[36] LB-CD7 lacks the ability to incorporate selenium into proline reductase, while LB-CD12 lacks the ability to produce the enzyme glycine reductase.[36] The inclusion of these strains into this study was essential to understanding the potential roles that proline and glycine reductase play in facilitating motility.

Respiration through the Stickland pathway produces more energy in the form of ATP for the cell to use during motility.[38, 45] However, fermentation through WLP reactions does not yield as much energy for growth, toxin production, and motility.[43, 44] The KNM6 *selD* mutant of the wild-type R20291 clinical isolate is unable to incorporate selenium into the enzyme proline reductase, which is essential for proline metabolism in Stickland reactions.[38, 42, 46-48] It is unknown whether this mutant can produce enough energy without metabolizing proline to engage in motility. For this reason, wild-type and mutant strains were tested in both rich and minimal media to observe motility under various metabolic conditions. The clinical isolate R20291 and the reverted strain KNM9 are believed to be capable of anaerobic respiration using proline, while the *selD* mutant is not. If that is the case, the wild-type and reverted *C. difficile* strains should display motility and metabolic byproducts in both rich and minimal media containing proline and related compounds, while the *selD* mutant should not.

II. METHODS AND MATERIALS

2.1 Research Objectives

The experiment aimed to determine if wild-type strains exhibited differences in motility based on the type of inoculation media, namely rich (BHIS) versus defined (CDMM) media. The preliminary motility study was conducted in BHIS tubes of varying agar concentrations to understand the role of media viscosity in motility. In the first experiment, agar viscosity was varied across both types of media to determine the effects on motility of two strains, R20291 and JIR8094. In a follow-up experiment, eight strains were inoculated into 0.25% soft agar of both BHIS and CDMM. These strains included R20291 and JIR8094, as well as the KNM6 *selD* mutant of R20291 and its recombinant, KNM9. Four mutants of JIR8094 were also included, which are LB-CD4, LB-CD7, LB-CD8, and LB-CD12. Radial motility from the inoculation line was measured in millimeters on the bottom of the well plates to allow for accurate observations of motility and to compare between each of the strains in both types of media.

2.2 Preliminary Data for Motility in Varied Agar Concentrations

C. difficile strains R20291, KNM6, KNM9, JIR8094, and VPI 10463 were grown overnight on BHIS-DCS250 agar plates. Single colonies of each strain were used to inoculate 1 mL of BHIS broth for overnight cultures. Cultures were stab inoculated with thin wooden inoculation sticks into individual tubes containing either 0.25%, 0.3%, 0.35%, 0.9%, or 1.5% agar concentrations of BHIS tubes. BHIS media contained 37 g/L brain-heart infusion, 5 g/L yeast, and 0.1% cysteine. The experiment was done in triplicates for each of the five strains on each of the five concentrations of agar, for a total of seventy-five tubes. The tubes were incubated at 37°C in the anaerobic chamber. Motility in each tube was observed over a period of five days.

2.3 Motility in Varied Agar Concentrations

C. difficile strains R20291 and JIR8094 were grown overnight on BHIS-DCS250 agar plates. Single colonies of each strain were used to inoculate 5 mL of complete CDMM broth and 5 mL of BHIS broth for overnight cultures. Cultures were stab inoculated with toothpicks (one per each set of triplicates) into complete CDMM wells and BHIS wells with the following agar concentrations: 0.25%, 0.5%, 0.75%, 1%, 1.25%, and 1.5%. BHIS media contained 37 g/L brain-heart infusion, 5 g/L yeast, and 0.1% cysteine. Complete CDMM media contained the following amino acids and their respective concentrations: tryptophan (100 mg/L), valine (100 mg/L), isoleucine (100 mg/L), leucine (1,000 mg/L), cysteine (500 mg/L), proline (800 mg/L), arginine (100 mg/L), histidine (100 mg/L), methionine (100 mg/L), glycine (100 mg/L), and threonine (100 mg/L). Complete CDMM media also contained a carbohydrate source of glucose at a concentration of 2,000 mg/L, as well as vitamin sources of calcium-D-pantothenate (B_5) (1 mg/L), pyridoxine (B_6) (0.1 mg/L), and biotin (B_7) (0.01 mg/L). The following minerals were included in complete CDMM: potassium phosphate monobasic (KH_2PO_4) (300 mg/L), sodium phosphate dibasic (Na_2HPO_4) (1,500 mg/L), sodium chloride (NaCl) (900 mg/L), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) (26 mg/L), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (20 mg/L), manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (10 mg/L), ammonium sulfate ($[NH_4]2SO_4$) (40 mg/L), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (4 mg/L), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) (1 mg/L), sodium bicarbonate ($NaHCO_3$) (5,000 mg/L), sodium selenite (Na_2SeO_3) (1 µM), sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) (1 µM), and sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$) (1 µM).

Each well contained 4 mL of media, which filled about 80% of the well. The experiment was done in triplicates for each strain in the twelve conditions, for a total of six 12-well plates.

The plates were incubated at 37°C in the anaerobic chamber. Motility in each well was observed from the top of the well plates and recorded in millimeters at the bottom of the well plates from the line of inoculation to the point of average motility over a period of ten days.

2.4 Motility of Mutants in 0.25% Soft Agar

C. difficile strains R20291, KNM6, KNM9, JIR8094, LB-CD4, LB-CD7, LB-CD8, and LB-CD12 were grown overnight on BHIS-DCS250 agar plates. Single colonies of each strain were used to inoculate 5 mL of complete CDMM broth and 5 mL of BHIS broth for overnight cultures. Cultures were stab inoculated with toothpicks (one per each set of triplicates) into complete CDMM wells and BHIS wells each containing a 0.25% agar concentration. Each type of media was made with their respective compounds and concentrations as mentioned in the previous experiment. Each well contained 4 mL of media to fill about 80% of the well. The experiment was done in triplicates for each strain in the two conditions, for a total of four 12-well plates. The plates were incubated at 37°C in the anaerobic chamber. Motility in each well was observed from the top of the well plates and was recorded in millimeters at the bottom of the well plates from the line of inoculation to the point of average motility over a period of three days. The experiment was repeated twice to confirm the results. The motility observation period in the repetition of this experiment was conducted over a period of five days.

III. RESULTS

3.1 Greater Gas Production by R20291 in Viscous Rich Media

A preliminary experiment was conducted in BHIS tubes of various agar concentrations to study the effects of media viscosity on motility. The control condition was the standard 1.5% agar concentration, while the experimental conditions were 0.25%, 0.3%, 0.35%, and 0.9% agar concentrations. Across all five strains, tubes with lower agar concentrations (0.25%, 0.3%, and 0.35%) appeared to display lower turbidity levels compared to tubes with higher agar concentrations (0.9% and 1.5%), which may point to less motility exhibited by the cells in more viscous media conditions. However, this experiment may need to be repeated in CDMM to control for any confounding variables in the nutrient concentrations of BHIS media.

The wild-type R20291 demonstrated high levels of gas production in the higher agar concentrations (0.9% and 1.5%) (Figure 1). This is a surprising finding that may indicate alternative metabolic processes, such as fermentation, that may only be undertaken in viscous rich media. Further analysis should be conducted to determine the type of gas produced to elucidate which type of metabolic process has taken place. However, such analysis may be difficult to conduct due to the confounding presence of carbon dioxide and hydrogen gas in the anaerobic chamber.

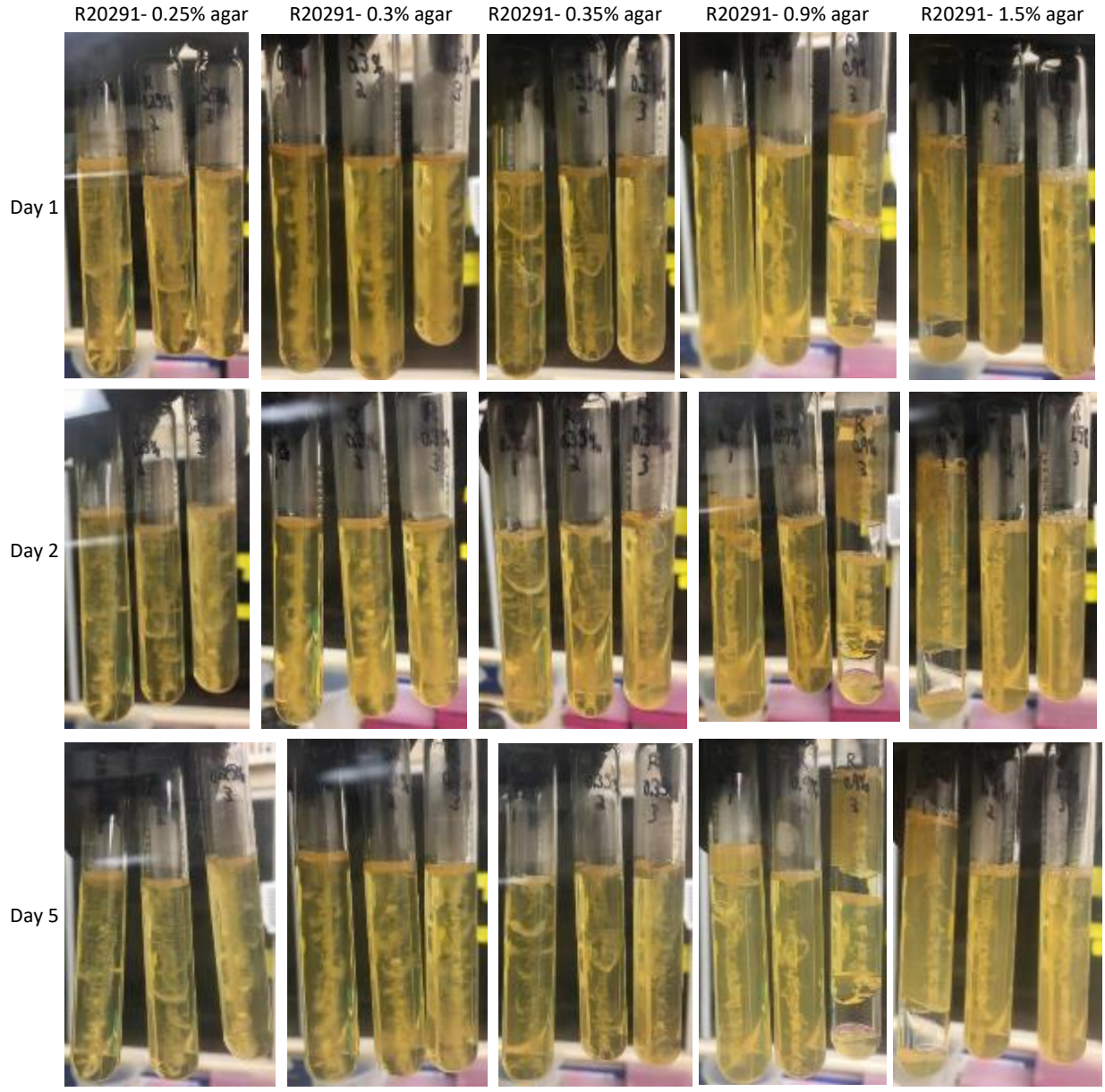


Figure 1. Turbidity is greater in lower agar concentrations while gas production is greater in higher agar concentrations. Wild-type *C. difficile* strain R20291 was grown at 37°C in rich (BHIS) media at varying agar concentrations (0.25%, 0.3%, 0.35%, 0.9%, and 1.5%).

3.2 No Significant Gas Production by KNM6

The KNM6 *selD* mutant did not demonstrate a dramatic increase in gas production in higher agar concentrations, which differed from the trapped gas bubbles observed under the same conditions in the tubes that were inoculated with R20291 (Figure 2). Some gas bubbles were still observed on the top of the agar in the 1.5% agar tubes, as well as in the middle of the tubes in the 0.35% agar concentration. The 0.9% and 1.5% agar tubes were slightly less turbid compared to the other three agar concentrations, which is consistent with the findings for the wild-type R20291 strain. This may indicate that increasing agar viscosity limits the extent of motility, as the turbidity of the tubes around the line of inoculation is used as an indication of the overall motility of *C. difficile*.

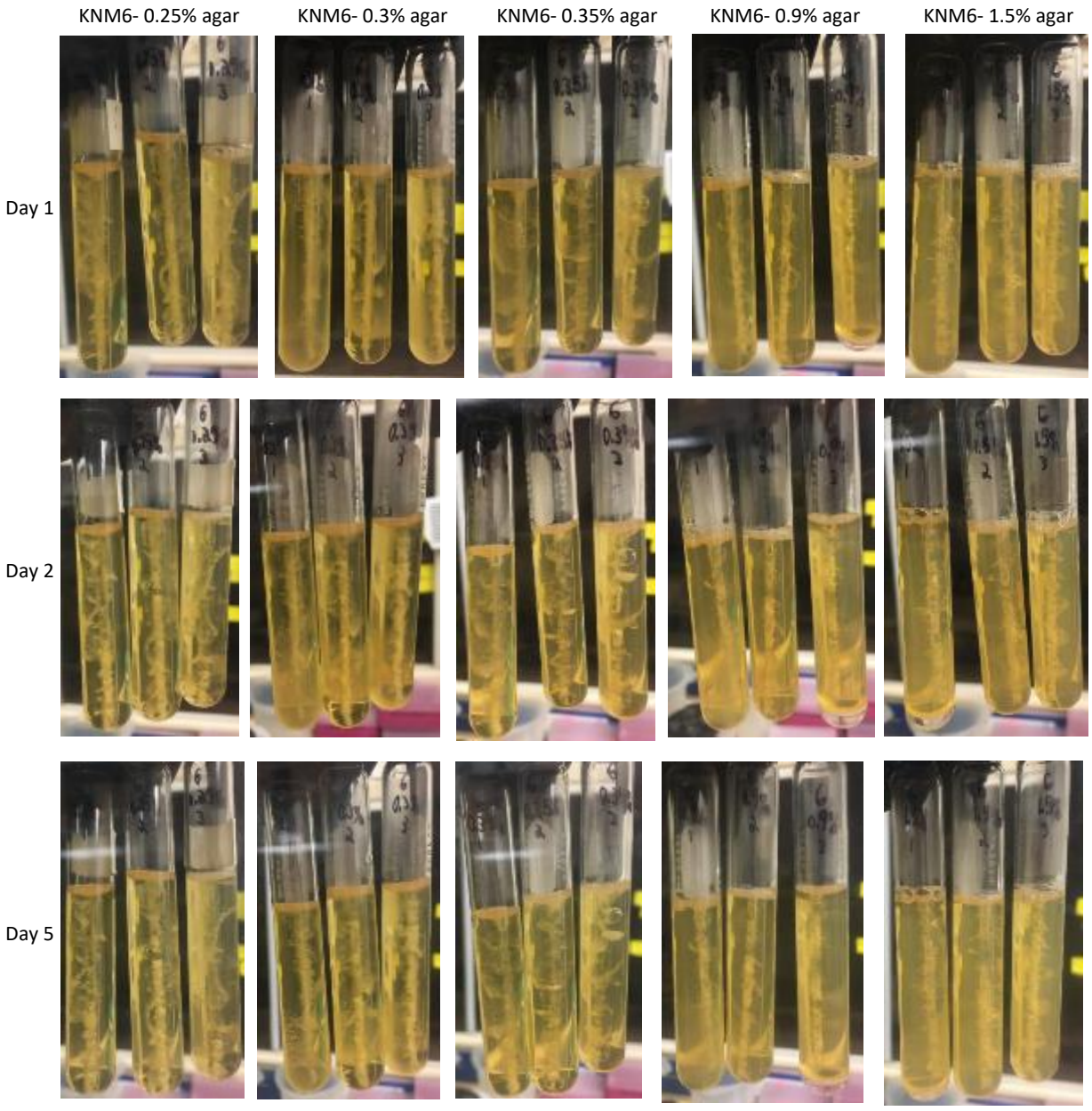


Figure 2. KNM6 exhibits less gas production than R20291 in higher agar concentrations. The KNM6 *selD* mutant was grown at 37°C in rich (BHIS) media at varying agar concentrations (0.25%, 0.3%, 0.35%, 0.9%, and 1.5%).

3.3 KNM9 Gas Production Resembles that of KNM6

The recombinant strain KNM9 demonstrated turbidity consistent with previously observed trends (Figure 3). This indicates similar overall patterns of motility in rich media tubes across these three strains. However, it did not exhibit high gas production in greater agar concentrations. Since KNM9 is the recombinant strain, it is expected to resemble the growth patterns of R20291. Instead, it appeared to resemble the amount of gas production demonstrated by the KNM6 strain. This may indicate reversion to the mutation, but additional studies should be conducted to confirm this hypothesis.

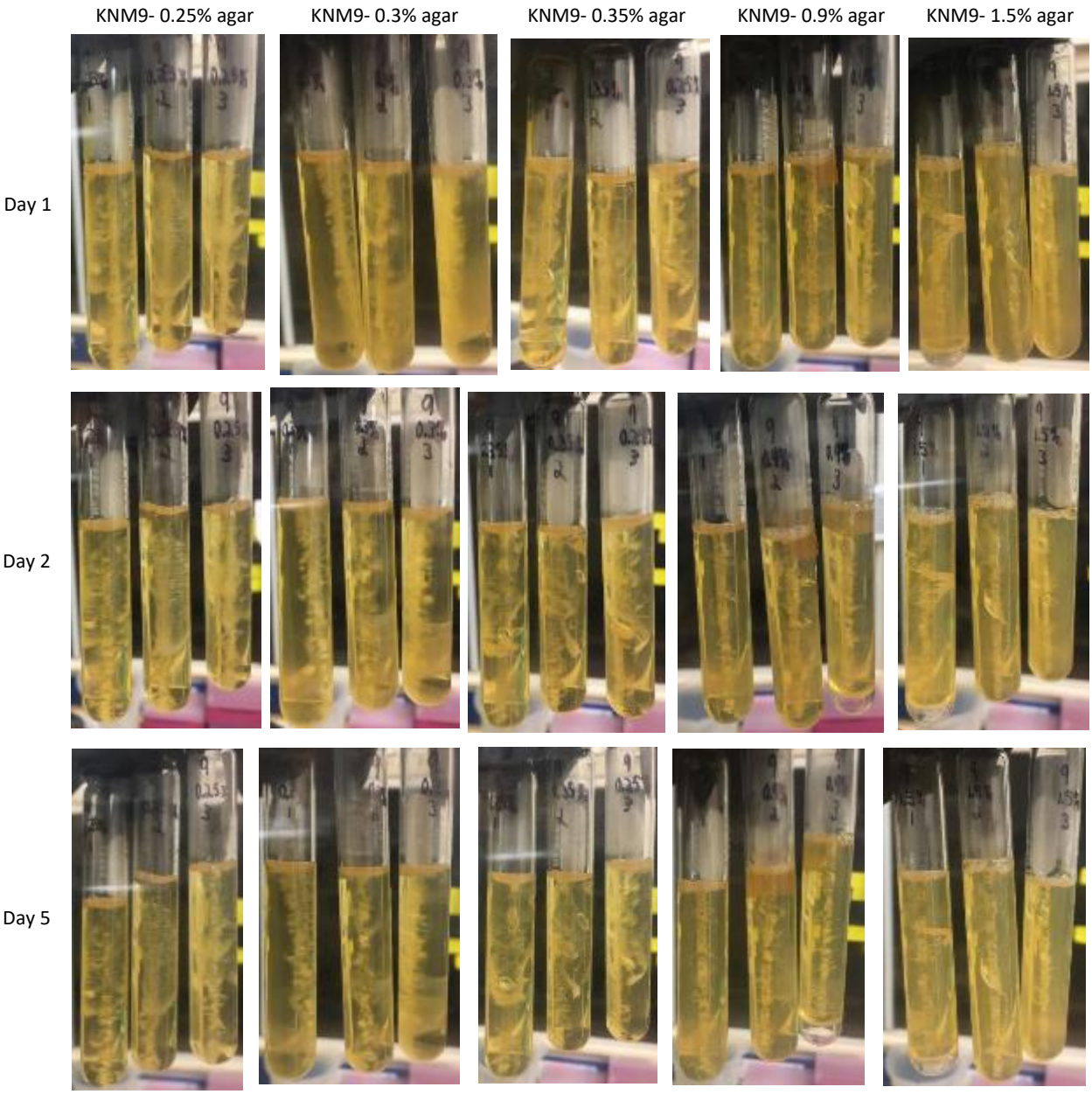


Figure 3. KNM9 does not resemble the gas production exhibited by R20291 in higher agar concentrations. The KNM9 recombinant strain was grown at 37°C in rich (BHIS) media at varying agar concentrations (0.25%, 0.3%, 0.35%, 0.9%, and 1.5%).

3.4 JIR8094 Gas Production Resembles that of R20291

The wild-type JIR8094 strain exhibited similar patterns of motility to the previous three strains (Figure 4). The more viscous agar concentrations of 0.9% agar and 1.5% agar do not appear to exhibit as much turbidity as the lower agar concentrations. This is an interesting finding, given that JIR8094 is not known to be motile. Therefore, it was originally included in the study as a negative control against which the motility of other strains would be compared. However, these findings indicate that JIR8094 can exhibit motility under certain conditions, although the exact mechanisms underlying this observed phenotype are not known. It also exhibited gas production in the higher agar concentrations in a similar manner to the wild-type R20291 strain. This is expected since they are both clinical isolates that lack significant genetic mutations. Therefore, they are expected to display similar phenotypes.

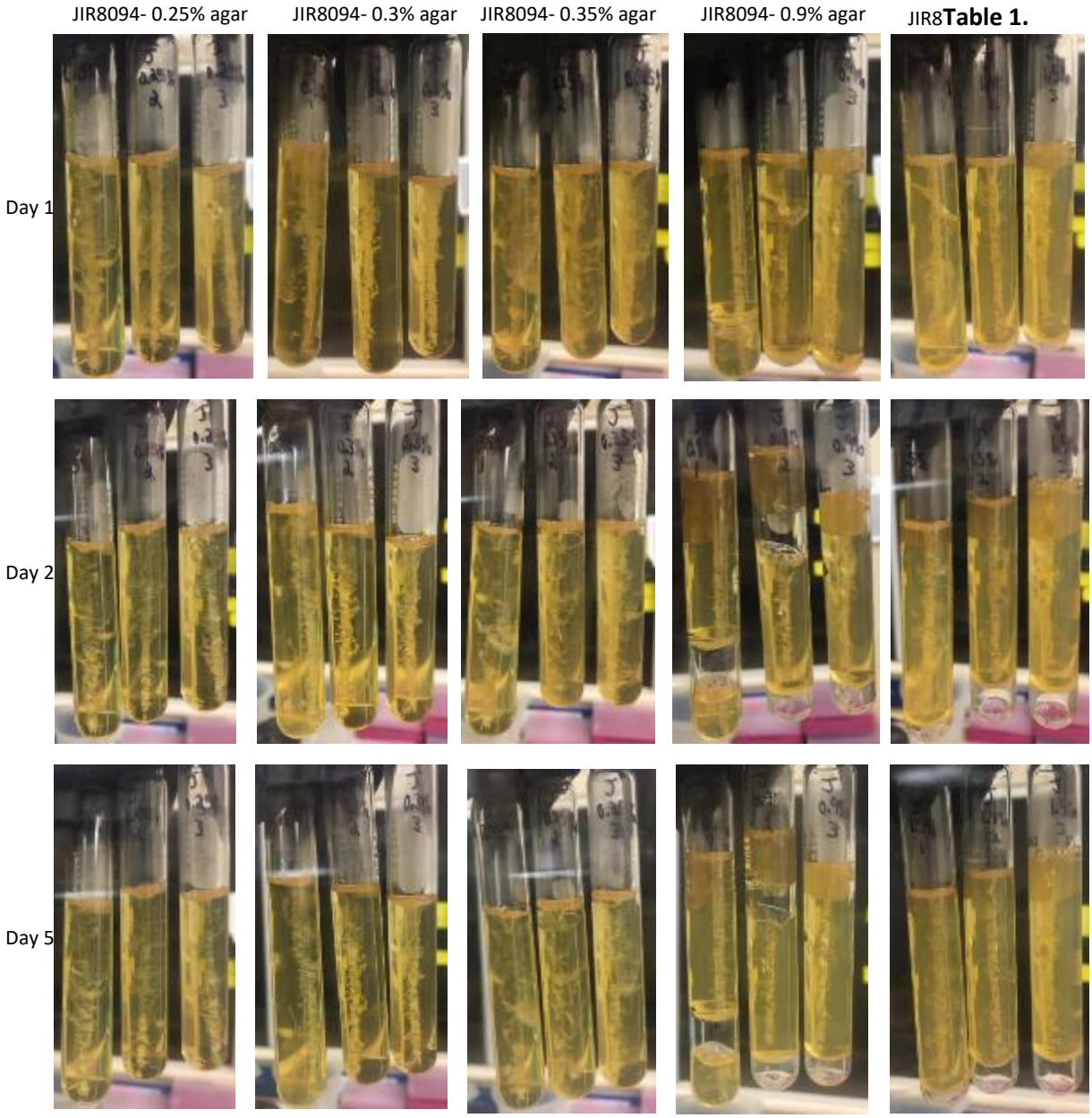


Figure 4. JIR8094 resembles the gas production exhibited by R20291 in higher agar concentrations. The wild-type JIR8094 strain was grown at 37°C in rich (BHIS) media at varying agar concentrations (0.25%, 0.3%, 0.35%, 0.9%, and 1.5%).

3.5 VPI 10463 Gas Production Resembles that of Both R20291 and JIR8094

The strain VPI 10463 displayed similar patterns of turbidity to the previous four strains (Figure 5). This apparent homogeneity may have been confounded due to the use of tall, narrow tubes, which does not allow for sufficient room *C. difficile* to swim significantly from the line of inoculation. Therefore, the findings of this preliminary experiment informed the future direction of the study. This formed the basis of the decision to transition into using flatter, wider wells to better visualize the extent of motility of various strains under different media conditions.

VPI 10463 also exhibited similar amounts of gas production as the wild-type strains R20291 and JIR8094 displayed in higher agar concentrations. This observation may be due to the inability of these strains to become highly motile in viscous media. Thus, the bacteria may be metabolizing most of the nutrients in the immediate area, forcing them to switch to using different metabolic pathways in the absence of the ability to exhibit motility due to environmental pressures. However, this hypothesis should be tested further to determine its validity. Such extensive production of gas warrants a future investigation into the metabolic processes that make this observation possible.

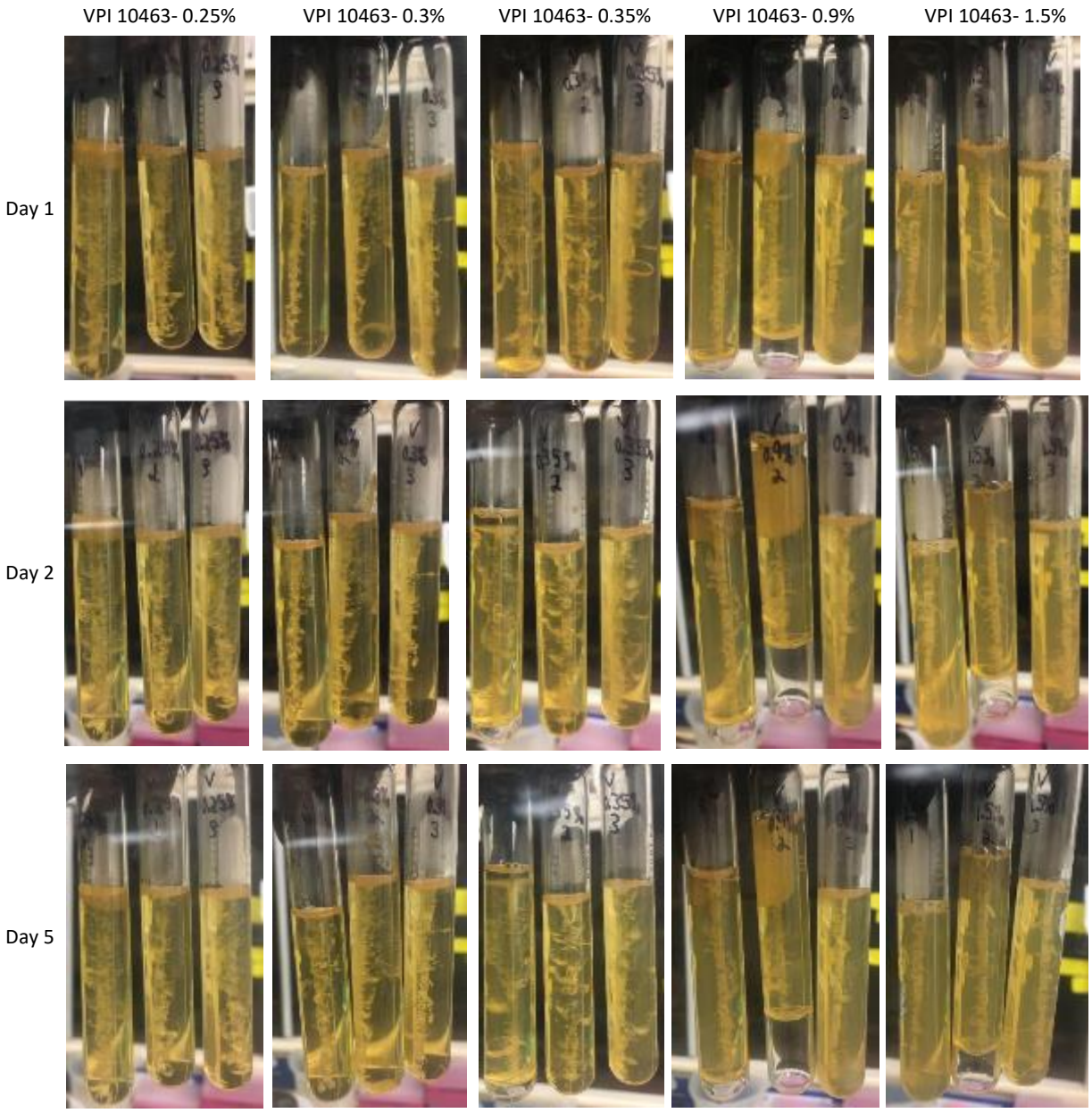
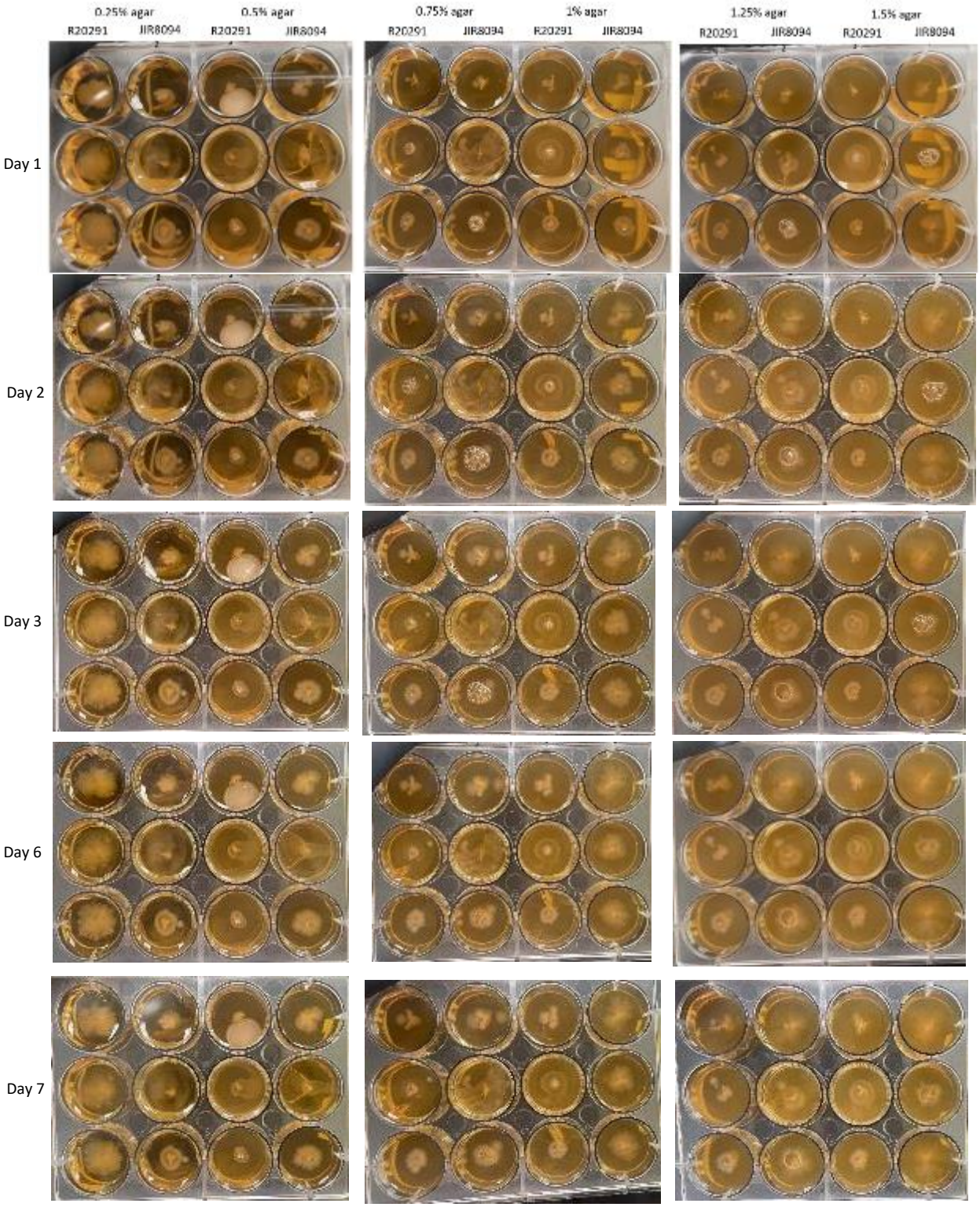


Figure 5. VPI 10463 resembles the gas production exhibited by R20291 in higher agar concentrations. VPI 10463 was grown at 37℃ in rich (BHIS) media at varying agar concentrations (0.25%, 0.3%, 0.35%, 0.9%, and 1.5%).

3.6 R20291 Exhibits Greatest Motility in 0.25% Agar in BHIS

In BHIS, the wild-type strain R20291 demonstrated greater motility than JIR8094 (Figure 6). The only agar concentration that allowed for quick motility in both CDMM and, to a greater extent, BHIS is 0.25% agar. Some bubbles indicating gas production were observed, which suggests alternative metabolic processes, such as fermentation, may have taken place. Most growth, especially in BHIS, was confined to the line of inoculation. However, some turbidity was observed around the line of inoculation. Furthermore, some ring formations were observed in some wells. This may be due to growth along the top of the agar, sporadic mutations during motility, or periodic phases of motility. Future studies would need to be conducted to confirm these hypotheses.

The motility observations were taken from the top of the well plates, which were not as clear as possible due to growth confined to the top of the agar. This growth may have been due to humidity, but it does not appear to affect the actual motility within the wells. These observations would have been better taken from the bottom of the well plates to minimize any ambiguity. For this reason, the radial motility measurements were taken from the bottom of the well plates. The measurements were taken in millimeters from the line of inoculation to the point of average turbidity in each well.


0.25% agar
0.5% agar
0.75% agar
1% agar
1.25% agar
1.5% agar
R20291
JIR8094
Day 1
Day 2
Day 3
Day 6
Day 7

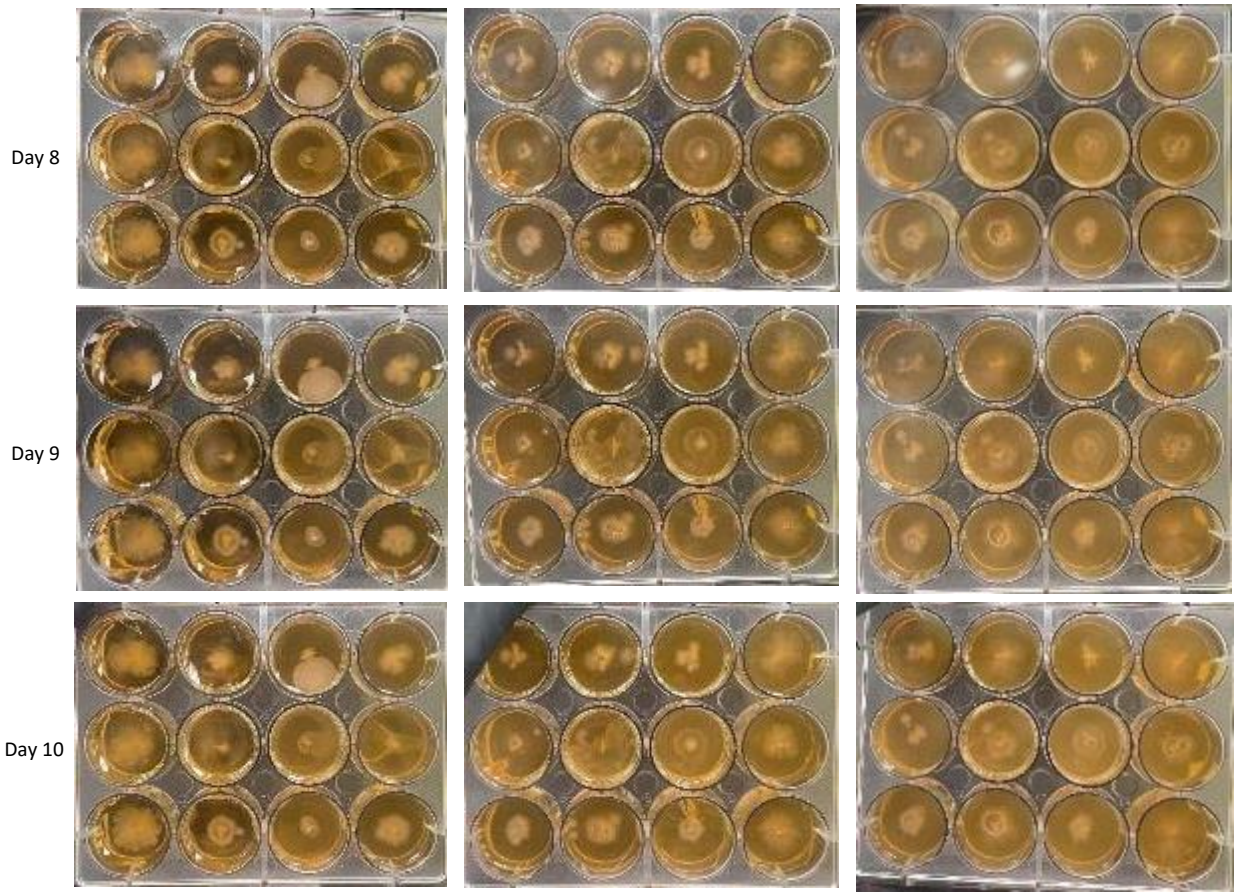


Figure 6. R20291 exhibits greater motility than JIR8094 in BHIS, especially in 0.25% agar. The wild-type *C. difficile* strains R20291 and JIR8094 were grown at 37°C in rich (BHIS) media at varying agar concentrations (0.25%, 0.5%, 0.75%, 1%, 1.25%, and 1.5%).

3.7 R20291 Motility Occurs in 0.25% Agar BHIS

R20291 was the only strain that demonstrated significant motility from the inoculation line, and this only occurred in 0.25% agar (Table 1). Its motility steadily increased from the time of inoculation until day 3, when it reached the maximum. Afterwards, the turbidity in the wells started to fade, which was reflected in the decreasing radial measurements. This decreased turbidity may indicate sporulation or cell death, but further analysis would be required to confirm these hypotheses. JIR8094 showed minimal motility only in 0.5% agar, which may indicate some genetic changes.

Table 1. R20291 exhibits motility in 0.25% agar BHIS. Motility in each well was observed and recorded in millimeters from the line of inoculation to the point of average motility over a period of ten days.

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 1	R20291	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 2	R20291	7	6	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 3	R20291	8	7	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 6	R20291	7	6	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 7	R20291	7	5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

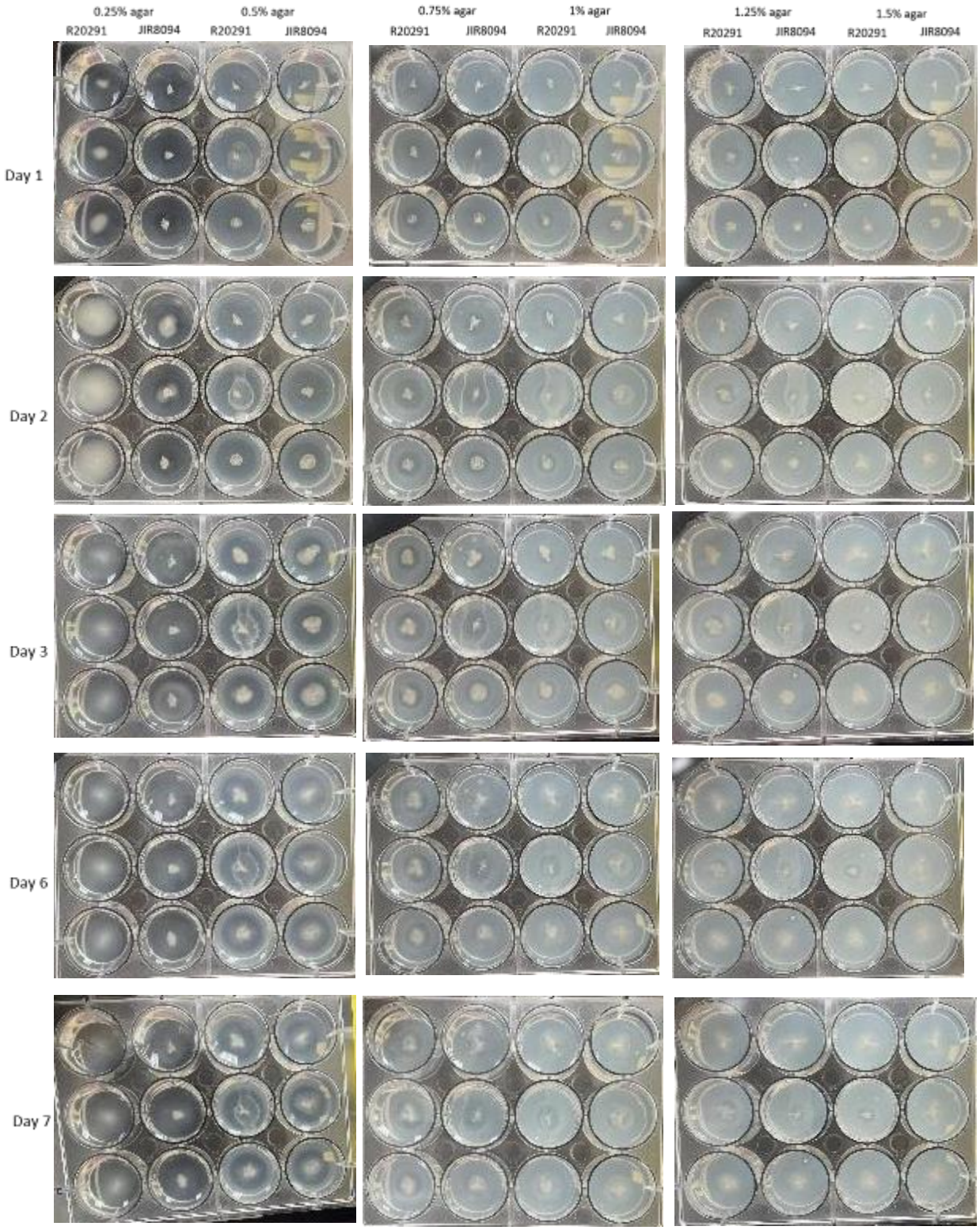
	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 8	R20291	7	6	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 9	R20291	6	6	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	BHIS	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 10	R20291	6	5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

3.8 Greatest Motility in CDMM is Achieved by R20291 in 0.25% Agar

In CDMM, motility was observed later for both strains in all agar concentrations (Figure 7). These finding may indicate that *C. difficile* is exhibiting chemotaxis toward nutrients, which is not as essential in rich media like BHIS. In BHIS, there is a high concentration of nutrients in the immediate vicinity of the bacteria at the site of inoculation. Therefore, *C. difficile* does not need to exhibit motility to acquire nutrients to produce energy. However, motility is required in CDMM since there is a low concentration of nutrients at the site of inoculation. In minimal media, *C. difficile* quickly metabolizes nearby stores of nutrients and thus is required to exhibit motility to access nutrients that are farther away. JIR8094 started to show some motility but only in 0.25% agar CDMM, which is interesting since it is not expected to show motility.


0.25% agar
R20291
JIR8094
0.5% agar
R20291
JIR8094
0.75% agar
R20291
JIR8094
1% agar
R20291
JIR8094
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R20291
JIR8094
1.5% agar
R20291
JIR8094
Day 1
Day 2
Day 3
Day 6
Day 7

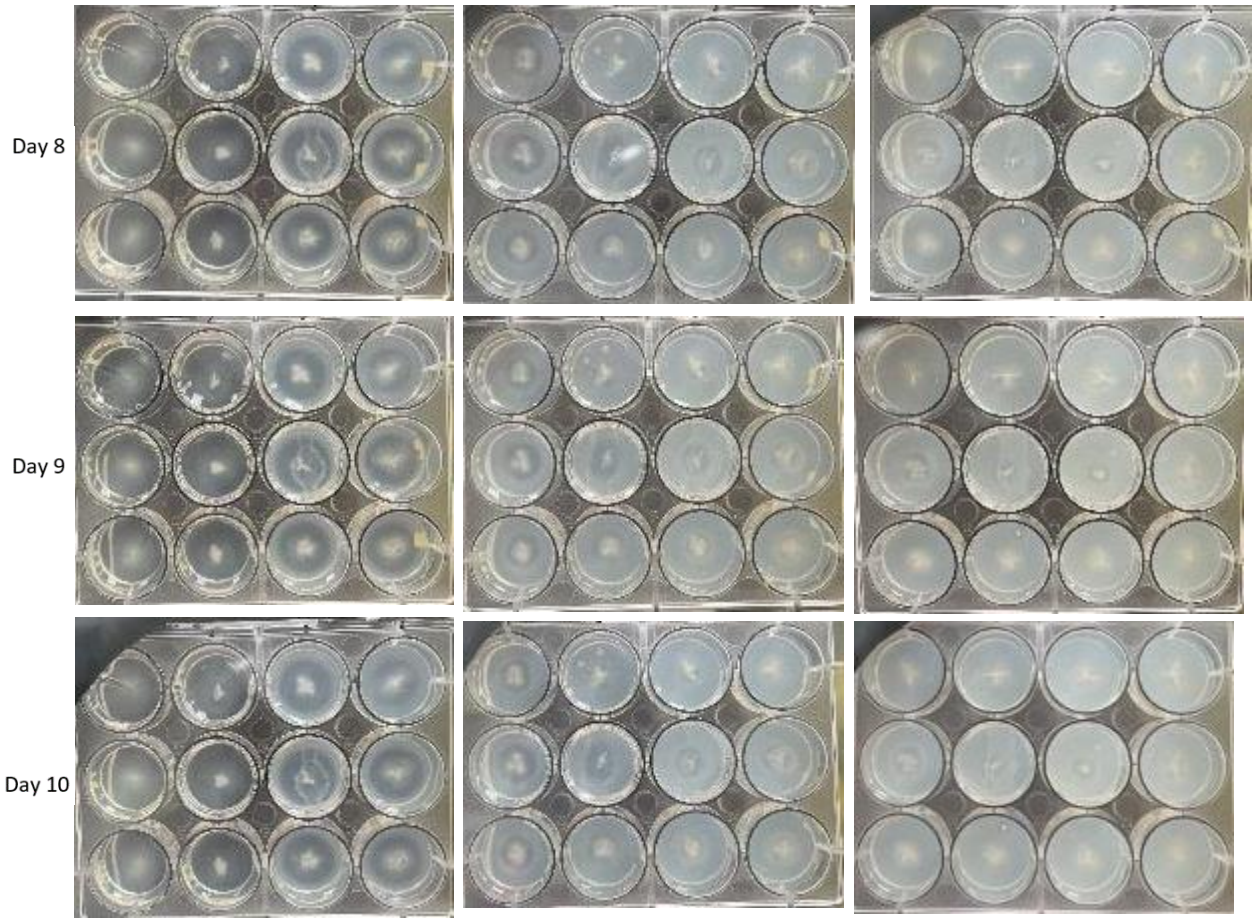

Figure 7. R20291 exhibits greater motility than JIR8094 in CDMM, especially in 0.25% agar. The wild-type C. difficile strains R20291 and JIR8094 were grown at 37℃ in minimal (CDMM) media at varying agar concentrations (0.25%, 0.5%, 0.75%, 1%, 1.25%, and 1.5%).

3.9 Wild-Type Strains Exhibit Motility in All Agar Concentrations

In CDMM, the first motility observed was by R20291 in 0.25% agar (Table 2). This observation can be explained by the fact that R20291 is a wild-type strain that is known to be motile. Also, 0.25% agar offers the least resistance to motility, which allows *C. difficile* to demonstrate chemotaxis more easily. The radial motility of R20291 in millimeters increased to its maximum by day 3, and JIR8094 also began to demonstrate motility. Both strains displayed motility across all agar concentrations to some extent. However, radial motility from the inoculation line consistently remained greatest for R20291 in 0.25% agar.

Table 2. Wild-type strains unevenly exhibit motility across all agar concentrations. Motility in each well was observed and recorded in millimeters from the line of inoculation to the point of average motility over a period of ten days.

	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 1	R20291	3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 2	R20291	8	9	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	JIR8094	1	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 3	R20291	9	9	9	3	0	2	2	2	2	2	0	2	3	2	2	2	0	2
	JIR8094	7	8	8	4	3	4	3	0	2	3	0	2	2	0	0	1	0	0
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 6	R20291	5	5	4	2	0	3	3	3	3	4	0	3	4	3	3	3	0	3
	JIR8094	2	1	0	4	5	4	3	0	2	5	5	5	2	0	3	2	2	1
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 7	R20291	5	5	4	3	0	3	5	6	4	2	0	2	4	4	3	3	0	3
	JIR8094	2	2	0	2	2	1	4	0	2	4	5	4	3	0	3	3	3	3
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 8	R20291	5	4	5	2	0	3	3	3	3	2	0	1	3	0	2	3	0	2
	JIR8094	2	3	0	0	0	0	2	0	0	2	0	1	2	0	1	3	0	2
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 9	R20291	4	4	5	3	0	3	4	5	4	3	0	2	3	3	3	3	0	3
	JIR8094	1	1	0	3	3	2	3	0	1	1	2	1	2	0	2	3	2	2
	CDMM	0.25%			0.5%			0.75%			1%			1.25%			1.5%		
Day 10	R20291	4	5	4	3	0	3	4	5	4	3	0	2	3	3	2	3	0	3
	JIR8094	2	2	0	3	0	2	3	0	0	2	0	1	2	0	3	2	0	0

3.10 R20291, KNM6, and KNM9 Exhibit Motility

In BHIS, only strains R20291, KNM6, and KNM9 showed motility when measured from the bottom of the well plates (Figure 8). Some growth appeared on the top of the wells from the inoculation site, but this growth did not extend downwards throughout the rest of the well. Therefore, it was not counted as motility. Motility was thus measured more accurately from the bottom of the well plate to avoid mistaking top growth for motility. Observations from experiments conducted in well plates indicate that a better course of action would have been to record observations from the bottom of the well plates to keep motility observations consistent with the radial measurements presented in the tables. This would have allowed for better comparison between different presentations of the results to confirm the validity of the observations.

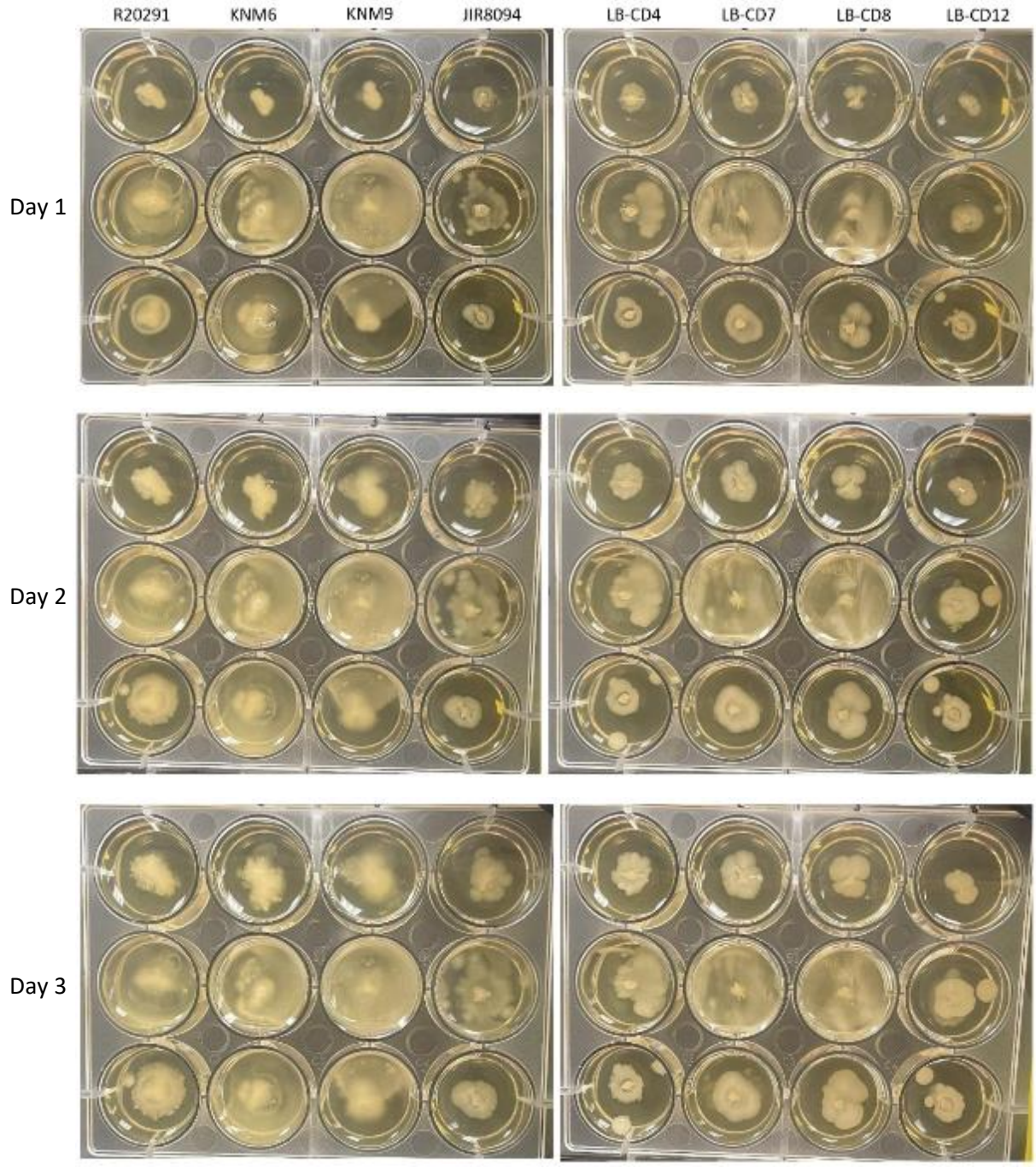

Figure 8. R20291, KNM6, and KNM9 exhibit motility in 0.25% agar BHIS. The following *C. difficile* strains- R20291, KNM6, KNM9, JIR8094, LB-CD4, LB-CD7, LB-CD8, and LB-CD12- were grown at 37°C in rich (BHIS) media at an agar concentration of 0.25%.

3.11 All Strains Display Motility in 0.25% Agar CDMM

In this study, the LB mutants showed growth far from the inoculation line (Figure 9). It is unclear whether these observations indicate contamination of the wells or an alternate demonstration of motility. These observations are further made ambiguous by the fact that they were not replicated in the repetition of the experiment, which does not allow for any definitive conclusions to be made concerning them. Furthermore, the R20291, KNM6, and KNM9 strains exhibited greater motility in CDMM compared to in BHIS. JIR8094 motility was also observed, which is unexpected since it was originally included as the non-motile negative control strain. Some bubbles indicating gas production were observed, indicating that CDMM appears to be more useful for observing unique phenotypes.

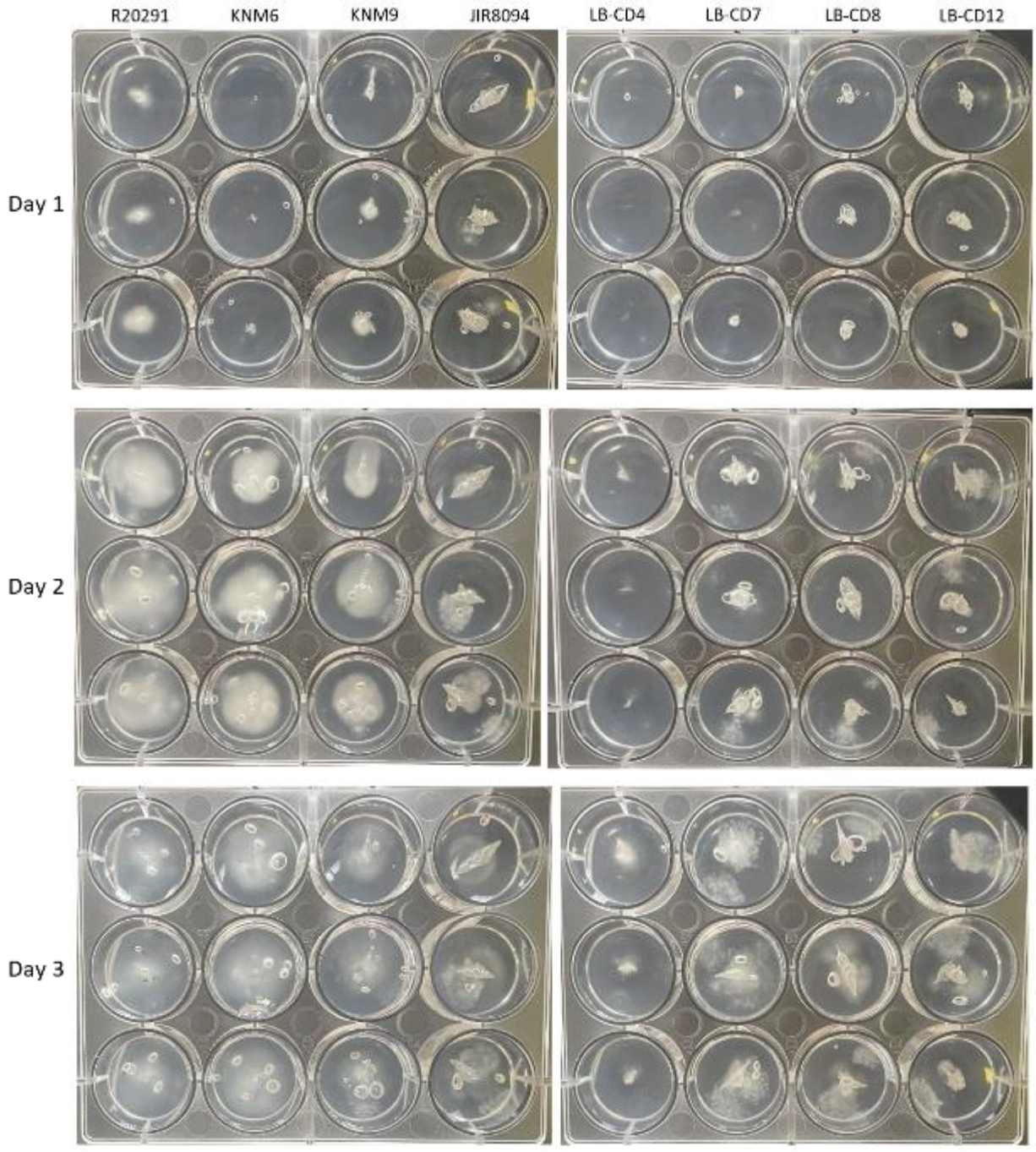

Figure 9. Motility in 0.25% agar CDMM increases across all strains. The following *C. difficile* strains- R20291, KNM6, KNM9, JIR8094, LB-CD4, LB-CD7, LB-CD8, and LB-CD12- were grown at 37℃ in minimal (CDMM) media at an agar concentration of 0.25%.

3.12 Motility Observed for All Strains in CDMM

R20291, KNM6, and KNM9 consistently displayed motility in both types of media (Table 3). Their motility appeared to be initially greater in BHIS compared to CDMM, but it became greater in CDMM by day 2 of the experiment. These observations indicate that rich media may cause more motility initially due to faster growth, but minimal media ensures that greater motility will occur consistently over a larger period. JIR8094 displayed motility in CDMM on day 1, which consistently increased as the experiment ran on. This is not typically observed in rich media, which demonstrates why JIR8094 has not been known to be motile. In CDMM, all strains eventually displayed at least some level of motility. This may indicate changing genotypes because of the environmental pressures posed by growth in minimal media. All measurements of radial motility from the inoculation line in millimeters were taken from the bottom of the well plates to ensure more accurate visualization. The measurements were recorded at the point of average turbidity in the wells, indicating the extent of motility.

Table 3. All strains display motility in 0.25% agar CDMM. Motility in each well was observed and recorded in millimeters from the line of inoculation to the point of average motility over a period of three days.

		R20291			KNM6			KNM9			JIR8094			LB-CD4			LB-CD7			LB-CD8			LB-CD12		
Day 1	BHIS	2	2	2	2	2	2	3	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	3	2	2	0	0	0	2	1	0	3	2	2	0	0	0	0	0	0	0	1	0	1	1	1
		R20291			KNM6			KNM9			JIR8094			LB-CD4			LB-CD7			LB-CD8			LB-CD12		
Day 2	BHIS	3	3	4	3	2	4	5	3	4	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	9	9	8	7	7	5	5	6	4	4	4	3	0	0	0	2	0	1	2	0	0	0	0	0
		R20291			KNM6			KNM9			JIR8094			LB-CD4			LB-CD7			LB-CD8			LB-CD12		
Day 3	BHIS	3	3	4	2	2	4	5	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	9	9	9	8	8	7	7	6	6	6	6	5	2	2	2	8	9	6	5	6	0	2	6	6

3.13 Motility Observed in R20291, KNM6, and KNM9

In BHIS, R20291 and KNM9 motility resembled each other, with KNM6 being slightly lower, which is expected since it is the *selD* mutant (Figure 10). The agar in some of the wells tended to come away from the well while measuring, which may have influenced some measurements. However, it is unknown whether these observations had any lasting impact on the actual motility of *C. difficile* within each well. Motility for R20291 and its mutants in BHIS had a mushroom-like shape, with more motility occurring towards the top of the well and narrowing towards the bottom. This could be an indication of uneven agar viscosity within the wells themselves, with less viscous agar near the top and more viscous agar near the bottom of the wells. However, since this hypothesis was not test in this experiment, its implications in the observed results cannot be definitively concluded.

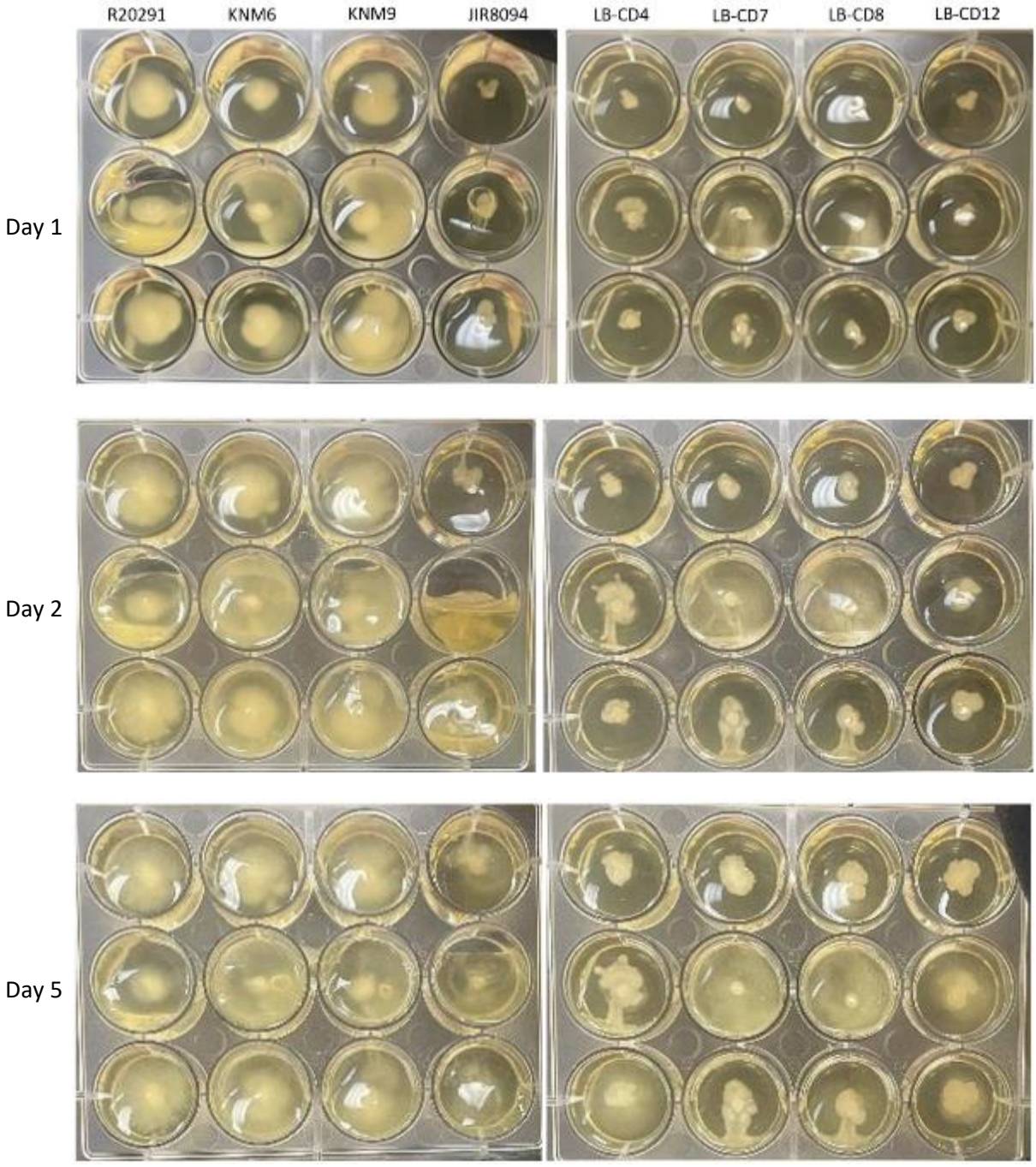

Figure 10. Motility was primarily observed in R20291, KNM6, and KNM9 in 0.25% BHIS. The following *C. difficile* strains- R20291, KNM6, KNM9, JIR8094, LB-CD4, LB-CD7, LB-CD8, and LB-CD12- were grown at 37℃ in rich (BHIS) media at an agar concentration of 0.25%.

3.14 All Strains Display Motility in CDMM

In CDMM, R20291 showed greater motility than KNM9, while KNM6 started to show similar motility to R20291 on day 5 (Figure 11). This may indicate reversal of the complementation and the mutation, but further studies should be conducted to validate this hypothesis. JIR8094 and its mutants started to show some motility on day 5, which is surprising given that it is not expected to display motility. All strains appeared to display similar levels of motility by day 5, which may be due to turbidity decreasing in the first three strains. This may have given enough time for JIR8094 and its mutants to exhibit motility to the extent displayed by the first three strains on the first two days of the experiment.

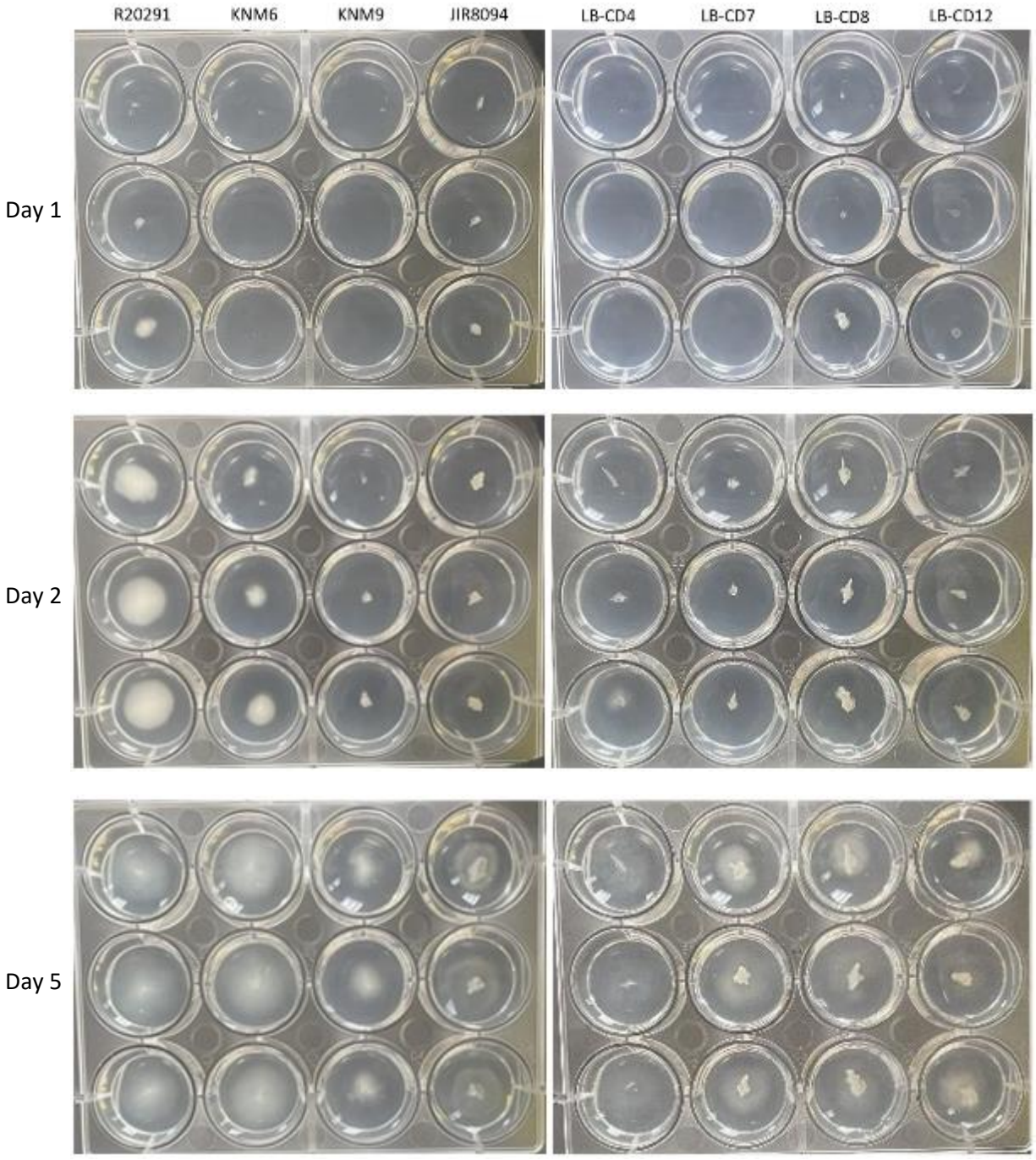

Figure 11. All strains display similar motility in 0.25% agar CDMM by day 5. The following *C. difficile* strains- R20291, KNM6, KNM9, JIR8094, LB-CD4, LB-CD7, LB-CD8, and LB-CD12- were grown at 37°C in minimal (CDMM) media at an agar concentration of 0.25%.

3.15 All Strains Exhibit Motility by Day 5 in 0.25% CDMM

All measurements were taken from the bottom of the well plates to facilitate accurate visualization (Table 4). Measurements were taken in millimeters from the line of inoculation to the point of average motility. On the first and second days of the experiment, motility was only observed in strains R20291, KNM6, and KNM9, primarily in BHIS. This is consistent with the first completion of this experiment. On the fifth day of this experiment, the motility measurements appeared to be more uniform across all strains for each type of media. This was starting to happen on day 3 of the last experiment. However, the effect became more pronounced by day 5 of this experiment due to leaving it to run for longer than the last experiment was allowed to continue.

Table 4. Motility becomes more uniform across all strains in 0.25% CDMM. Motility in each well was observed and recorded in millimeters from the line of inoculation to the point of average motility over a period of five days.

		R20291			KNM6			KNM9			JIR8094			LB-CD4			LB-CD7			LB-CD8			LB-CD12		
Day 1	BHIS	6	5	5	4	3	5	6	4	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		R20291			KNM6			KNM9			JIR8094			LB-CD4			LB-CD7			LB-CD8			LB-CD12		
Day 2	BHIS	4	6	8	9	7	8	9	6	8	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CDMM	6	5	5	4	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		R20291			KNM6			KNM9			JIR8094			LB-CD4			LB-CD7			LB-CD8			LB-CD12		
Day 5	BHIS	9	5	7	6	9	8	9	9	8	0	0	0	7	0	0	0	8	0	6	9	5	7	8	6
	CDMM	7	8	7	7	7	6	3	4	3	7	6	5	6	7	6	6	7	4	6	6	5	4	4	4

IV. DISCUSSION

4.1 Findings and Conclusions

The results of this study indicate that *C. difficile* exhibits greater motility in less viscous minimal media. Motility is not essential in rich media since no energy is needed to be expended if nutrients are already nearby. Therefore, increasing viscosity or nutrient availability may in effect limit its motility and growth.

The preliminary study demonstrated that gas production may be linked to a lesser extent of motility, especially in wild-type strains that were inoculated into higher agar concentrations. High media viscosity appeared to inhibit motility, which led *C. difficile* to utilize alternate metabolic pathways with gaseous by-products once immediate nutrient stores were metabolized. However, this hypothesis must be tested in future experiments to either prove or disprove its validity. The results of the preliminary experiment led to the development of the experiment in which wild-type strains were inoculated into rich and minimal media wells of varying agar concentrations.

R20291 demonstrated motility only in 0.25% agar in the BHIS wells, while both strains were eventually motile in all agar concentrations in CDMM. The results point to two main trends. First, motility is encouraged in less viscous media compared to more viscous media since there is less resistance in less viscous media. Second, motility is further encouraged in minimal media compared to rich media. As *C. difficile* metabolizes nearby nutrients, it is required to swim outward in the media to acquire more metabolites. Thus, minimal media encourages motility, even though it does not provide as many nutrients for high energy-consuming processes such as generating flagellar motion.

The last two experiments were conducted to isolate the effects of rich media compared to minimal media on the motility patterns of the two previous wild-type strains and their mutants. All media, both BHIS and CDMM, was prepared at 0.25% agar concentration. In this manner, motility was highly encouraged due to the lack of viscosity in the media. This lack of viscosity later presented some downsides, particularly when the agar came slightly apart from the wells during photography or measurement-taking. However, it is unknown whether this had any effect on the actual motility within the agar of each well.

Once the viscosity of the media was controlled for in these two experiments, the effects of rich media compared to minimal media were able to be clearly elucidated from the results. As in the previous experiment, R20291 began with the greatest motility. Its mutant strains, KNM6 and KNM9, also displayed significant motility. However, as time passed, all the strains began to exhibit motility in both types of media. Although these findings slightly confound the previously stated hypothesis, the results are still valuable in analyzing the motility patterns of each strain over time. Motility still tended to be the greatest for R20291 and its mutants in 0.25% agar, although it is unclear which type of media encouraged more motility.

The most interesting finding of these studies appears to be the fact that JIR8094 exhibited motility in CDMM. JIR8094 has not previously been known to display any motility. This may have been due to growth studies primarily taking place in rich media like BHIS, which did not allow JIR8094 to effectively display its motility. However, JIR8094 did exhibit some motility in BHIS in this study, especially in the preliminary experiment, so it is unknown why this effect has not yet been described. JIR8094 may have exhibited motility primarily in CDMM due to the environmental stresses of minimal media and possibly genetic mutations that arose as a result. However, this hypothesis would require independent studies to confirm its validity.

4.2 Future Research

This study could be expanded in the future to explore different directions of how *C. difficile* motility influences its ability to colonize the large intestine. The experiments could be repeated to confirm their results. More accurate growth measurements, such as measuring optical density as a function of time in liquid media, could be derived to generate more definitive data. Furthermore, more strains could be inoculated into various types of media at lower agar concentrations to elucidate their chemotactic response patterns. The study could also be repeated by inoculating LB mutants into varying agar concentrations in CDMM, which could confirm the trends observed in this study. Furthermore, since it is known that motility is related to toxin production, future studies could attempt to visualize the extent of toxin production in negative control strains like JIR8094 and its mutants using SDS-PAGE and western blot analysis. Utilizing fluorescent antibodies that bind to the toxins produced by *C. difficile* will allow for accurate visualization of the amount of toxin production that occurred. These procedures would have to be conducted after motility measurements have been taken, and the toxins would have to be extracted from the media before any analysis could be performed.

C. difficile has caused severe illness in many hospitalized patients taking rigorous courses of antibiotics. Its metabolic pathways illuminate how it uses the nutrients in its environment to compete against surrounding microbiota. Motility is a highly energy-consuming cellular process, so it is important to study the rationale behind it to aid in understanding how *C. difficile* causes disease in the colon. Motility can be understood in terms of chemotaxis toward certain nutrients. The amount of energy that each nutrient allows the cell to produce increases the likelihood that *C. difficile* will exhibit chemotaxis toward that specific nutrient. Therefore, understanding the energy demands of the cell and how it diverts energy stores to various cellular processes

illuminates its optimal conditions in causing disease, allowing for the development of more advanced and targeted treatments against *C. difficile* infections in hospital settings.

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