Environmental Conditions Impact the Chirality of Branching *Bacillus* Strains.

Jenna Conner
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Marine Biological Laboratory

Abstract

Bacillus mycoides forms rhizoid colonies, with branches that curve clockwise or counter-clockwise depending on the strain. The mechanisms regulating the chirality in this organism are unknown. In this study, it was demonstrated that the presence of sodium selenite, which this organism reduces into elemental selenium, causes the clockwise-curving morphotype to switch to a counter-clockwise pattern. Furthermore, it was shown that this response is not caused solely by the changes in ionic strength, osmolarity, or oxidative stress that are associated with increased selenite concentrations.

Introduction

Chiral growth patterns can be observed in various systems in nature, such as microbial communities and gastropod shells. Among organisms that form chiral patterns, mutants with inverted chirality are often observed. One such example is *Bacillus mycoides*, a Gram-positive bacterium that commonly grows in soils. Two distinct chiral morphotypes of this organism exist: the dextral (DX) form, which grows in clockwise-turning branches when viewed from the bottom of a petri dish, and the sinistral (SIN) form, which grows counter-clockwise.

Although the DX and SIN morphotypes were first characterized nearly a century ago, little is known about the regulation of chiral colony growth in this organism. Di Franco et al. (2002) identified several mutants of *B. mycoides* that lost chirality with repeated passaging, but none that inverted.[1] Czirok and Vicsek noted that branching *Bacillus subtilis* strains could convert to a sinistral chiral growth pattern on soft (0.75%) agar medium, but dextral growth was not observed in the range of tested conditions.[2]

In this study, sodium selenite was shown to invert chirality in the DX morphotype, but not the SIN morphotype. Furthermore, it was shown that sulfite, ionic strength, osmolarity, and oxidative stress affect the chirality of branches in both morphotypes, though none of these conditions was solely responsible for the inversion.

Materials and Methods

Media Construction

5YE medium was prepared as follows:

- Per L ddH_2O: add 5 g Yeast Extract, as well as 15 g agar for plates

Additional compounds were added in the following concentrations:

- Sodium selenite ($Na_2SeO_3^{2-}$): 2 mM, 4 mM, 10 mM
- Sodium sulfite (Na_2SO_3): 2 mM, 10 mM
- Potassium tellurite (K_2TeO_3): 2 mM, 4 mM, 10 mM
- Sodium chloride (NaCl): 4 mM, 8 mM, 50 mM

- Potassium chloride (KCl): 4 mM, 8 mM, 20 mM, 50 mM
- Lactose ($C_{12}H_{22}O_{11}$): 4 mM, 8 mM, 20 mM
- Sucrose ($C_{12}H_{22}O_{11}$): 20 mM
- Hydrogen peroxide (H_2O_2): 300 μm, 500 μm

Quantification of Chirality

SIN and DX cells were spotted into the center of plates containing 5YE + added compounds, and then imaged with a Zeiss dissecting microscope after 18-24 hours of growth. N (roughly 20) branches from each "spot colony" were counted; each clockwise (CW) branch was assigned a value of +1, and each counter-clockwise (CCW) branch was assigned a value of -1. Totals were calculated for CW and CCW, and then divided by the value of N to obtain a percentage (in the case of CCW/N, this percentage was a negative value). Then, the percentages for CW/N and CCW/N were added, and the resulting value was plotted in a dose-response graph. At least three (and up to six) biological replicates were evaluated for DX and SIN under each condition; roughly 2,200 branches were counted in total.

Results and Discussion

Figure 1. Sampling site at Pie in the Sky, Woods Hole, MA.

DX and SIN isolates are morphologically similar to *Bacillus mycoides*.

A sample of soil and dried fern leaves was collected from the front courtyard of Pie in the Sky in Woods Hole, MA (Fig. 1). The sample was used to inoculate a test tube containing 5 ml of 5YE medium and boiled at 100°C for ten minutes, and then the supernatant was serially diluted and plated onto solid 5YE medium. Within 24 hours, a colony with chiral, branching growth was observed on the 1:100 dilution plate. When observed from the bottom of the petri dish, all branches of the colony curve in a clockwise direction (Fig. 2); this isolate was deemed the DX (dextral) morphotype. A fellow MD student isolated an endospore-former that forms counter-clockwise branches - this isolate was deemed the SIN (sinistral) morphotype (Fig 2). Several re-streaks were performed to obtain pure colonies of both strains.

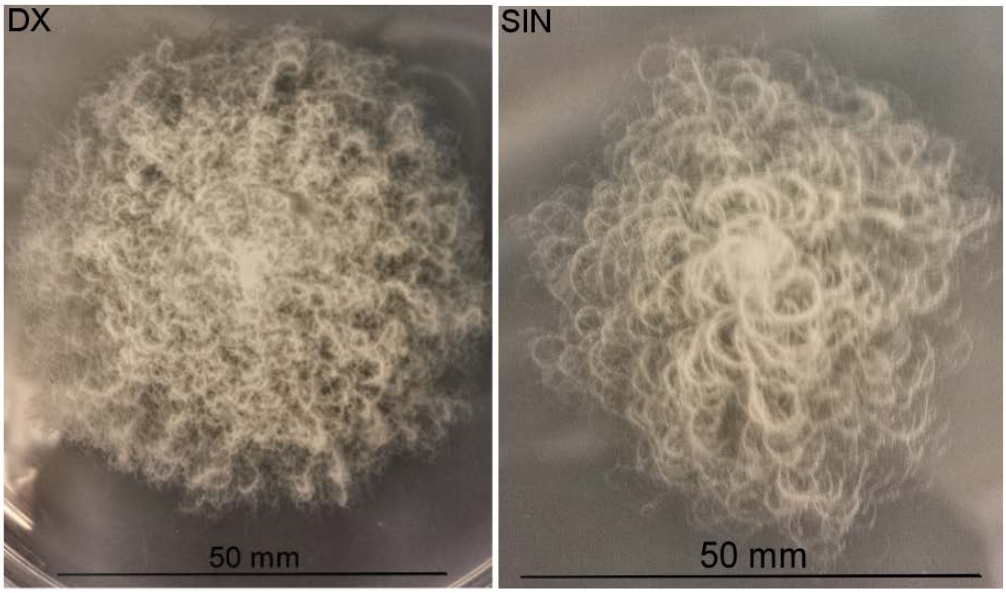

Figure 2. DX and SIN colony morphologies on 5YE medium after 3 days of growth.

The cells (Fig. 3), endospores (Fig. 4), and colonies (Fig. 2) formed by the DX and SIN isolates resemble those of *Bacillus mycoides*, however 16S rRNA sequences could not be obtained to confirm this classification.

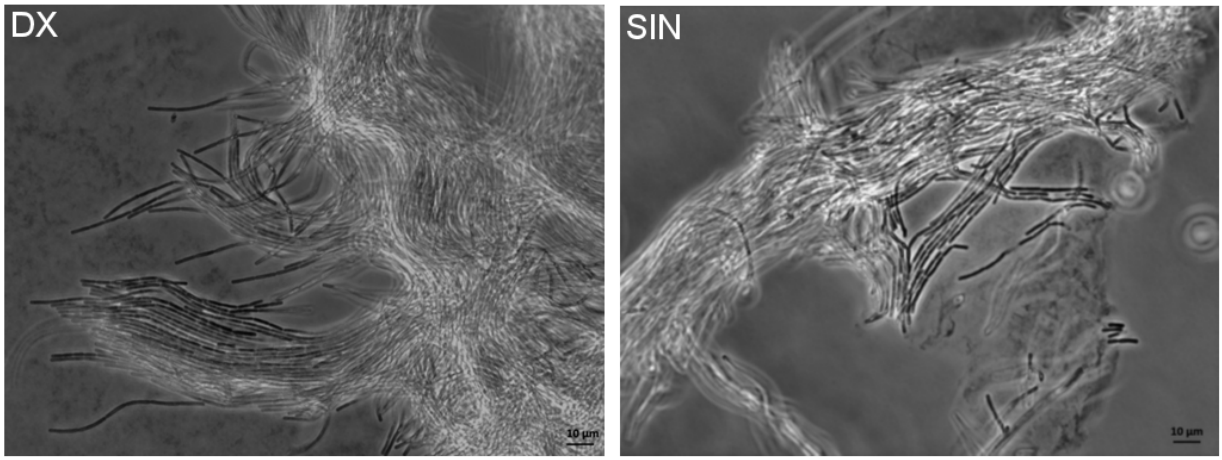

Figure 3. Filamentous growths of DX and SIN.

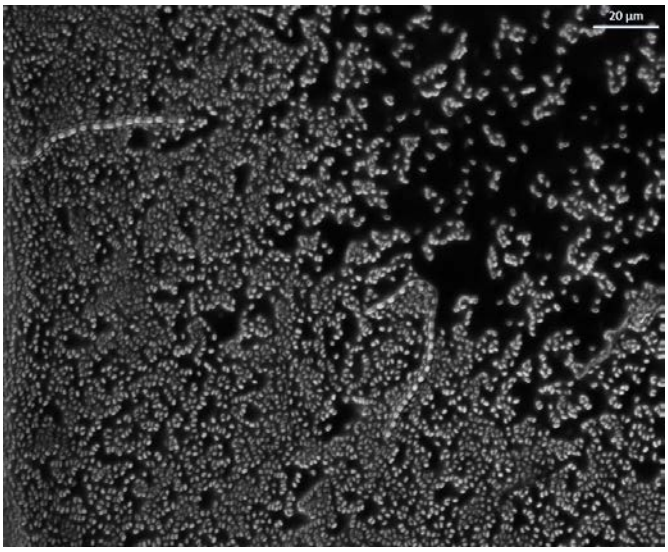

Figure 4. Spore formation by DX after 1 day of drying on a glass slide.

DX and SIN reduce selenite and form particles of elemental selenium.

When grown on plates supplemented with sodium selenite, DX and SIN produced a bright red color (Fig. 5). Scanning electron microscopy and energy dispersive spectroscopy were used to verify that the red coloration results from the production of elemental selenium (Se^0) via selenite reduction. DX and SIN colonies were removed from agar and fixed for 4 hours with 2.5% paraformaldehyde + 2.5% glutaraldehyde in 1X PBS buffer. EDS revealed that the sections from both morphotypes contained round selenium particles that varied between 0.1 and 5 µm in size (Figs. 6 & 7).

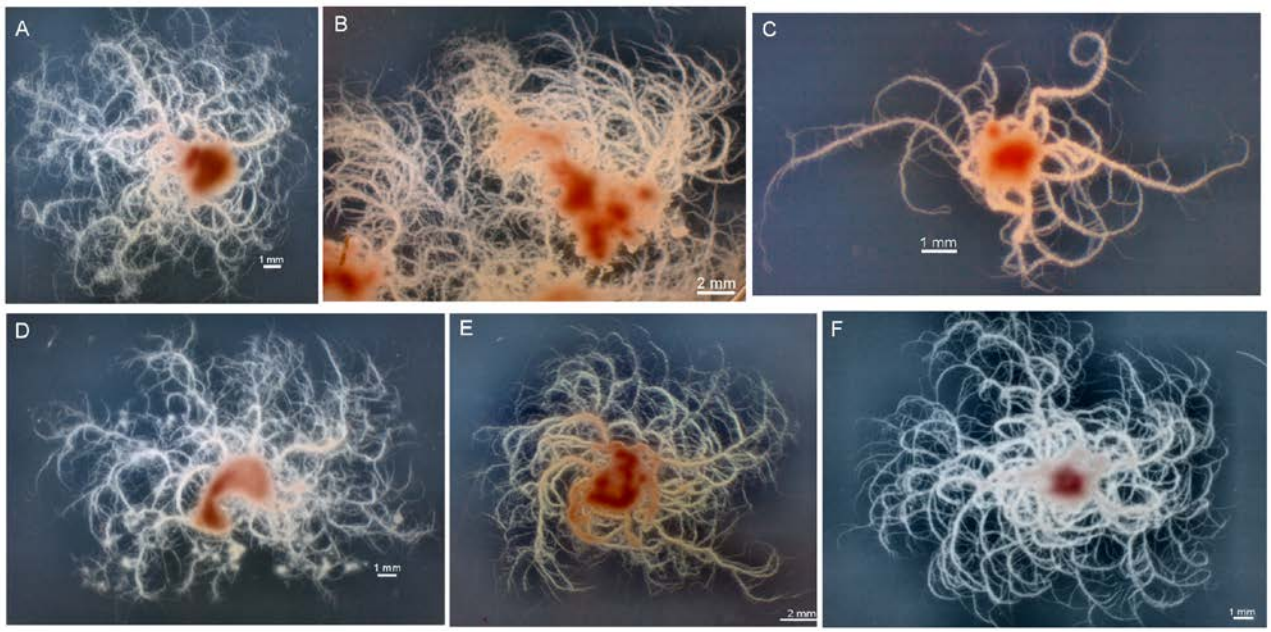

Figure 5. A & B) DX on 5YE + 2 mM sodium selenite. C) DX on 5YE + 4 mM sodium selenite. D & E) SIN on 5YE + 2 mM sodium selenite. F) SIN on 5YE + 4 mM sodium selenite.

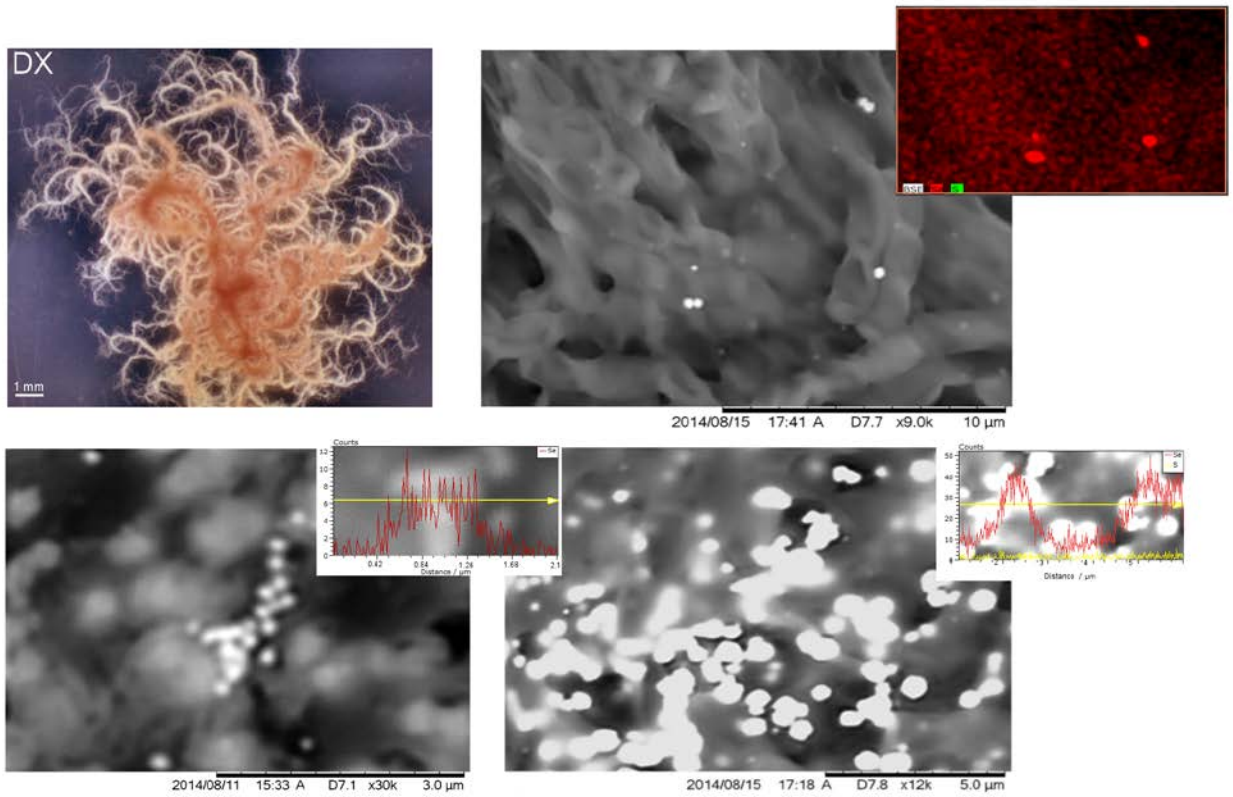

Figure 6. SEM and EDS analysis of a fixed section from a DX colony (shown in upper left).

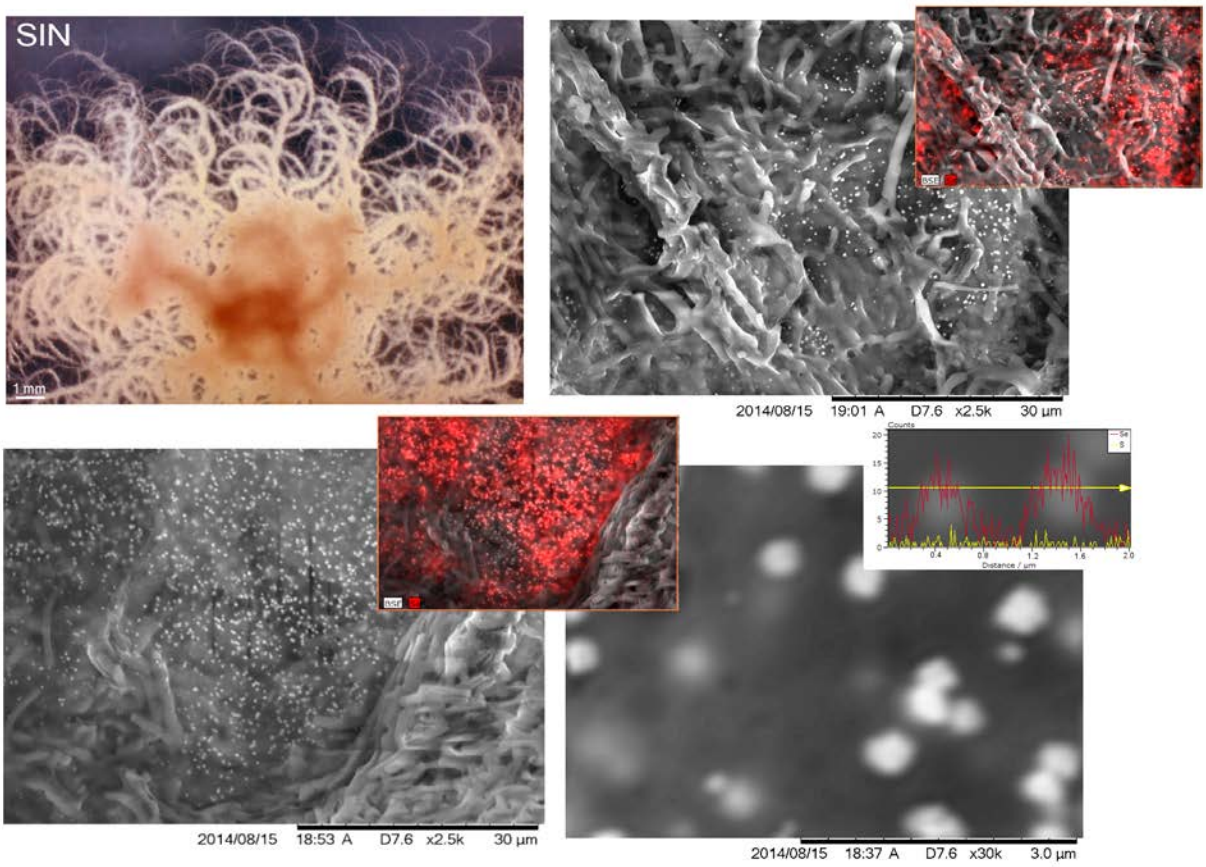

Figure 7. SEM and EDS analysis of a fixed section from a SIN colony (shown in upper left).

Sodium selenite inverts the chirality of DX colonies.

After approximately 18 hours of incubation on 5YE plates supplemented with 2 mM sodium selenite, DX displayed inverted, counter-clockwise morphology. The same phenomenon was observed with higher concentrations of sodium selenite (Fig. 5), though the response did not increase linearly with higher concentration (Fig. 8). Conversely, the SIN morphotype retained its counter-clockwise chirality at all concentrations of sodium selenite (Figs. 5 & 8).

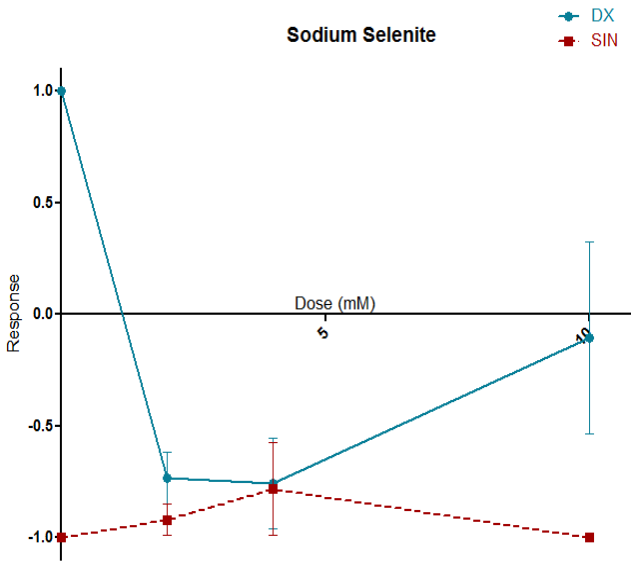

Figure 8. Effect of sodium selenite on chirality of DX and SIN .

To test whether chemically similar compounds would cause the same response, DX and SIN were plated onto 5YE containing varying concentrations of potassium tellurite and sodium sulfite. All tested concentrations of tellurite (2, 4, and 10 mM) were lethal to cells. A sodium sulfite dose-response plot (Fig. 9) reveals that the chirality of both strains is altered, but not inverted.

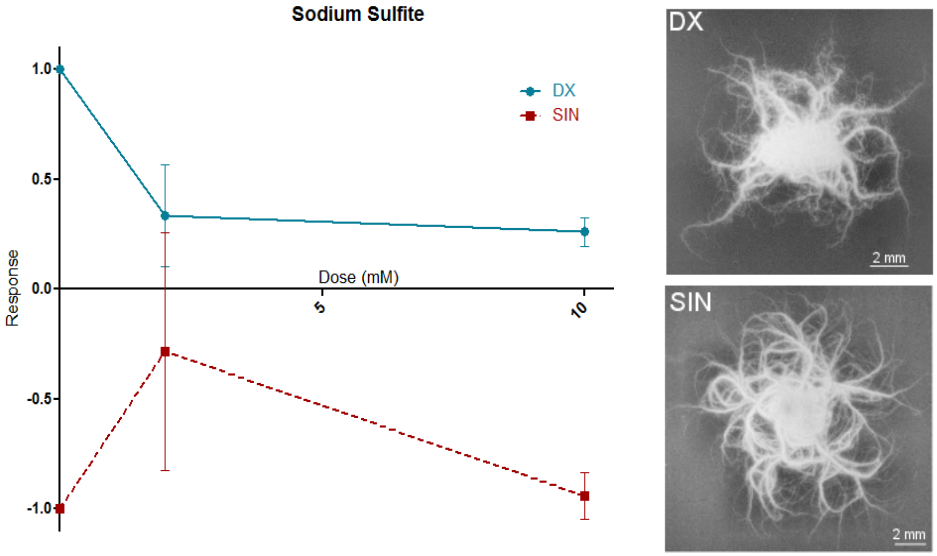

Figure 9. Effect of sodium sulfite on chirality of DX and SIN. At right: representative images of DX and SIN grown on 5YE + 2 mM sodium sulfite.

Addition of sodium selenite to the medium increases its ionic strength. To test whether this was responsible for the inversion in chirality, DX and SIN were plated on 5YE plates supplemented with sodium chloride and potassium chloride. Dose-response plots reveal that while both compounds impacted the chirality of DX and SIN, neither caused a complete inversion.

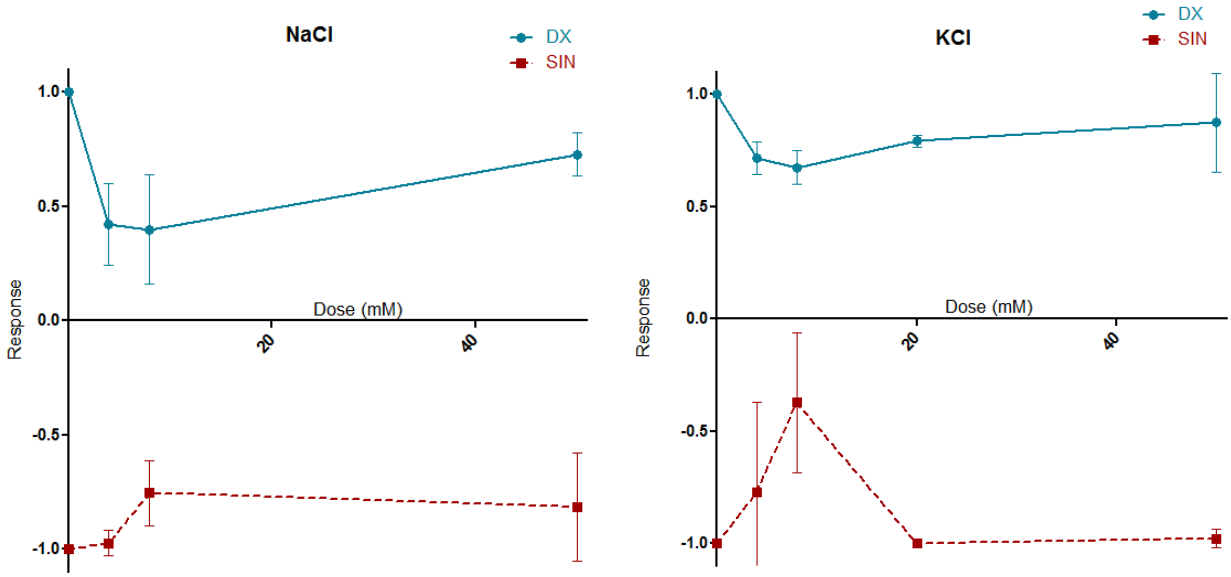

Figure 10. Effect of NaCl and KCl on chirality of DX and SIN.

To test whether the change in osmolarity of the medium was responsible for the inversion in chirality, DX and SIN were plated on 5YE plates supplemented with lactose. At a concentration of 20 mM lactose, colonies completely lost chirality and developed fuzzy, round colonies.

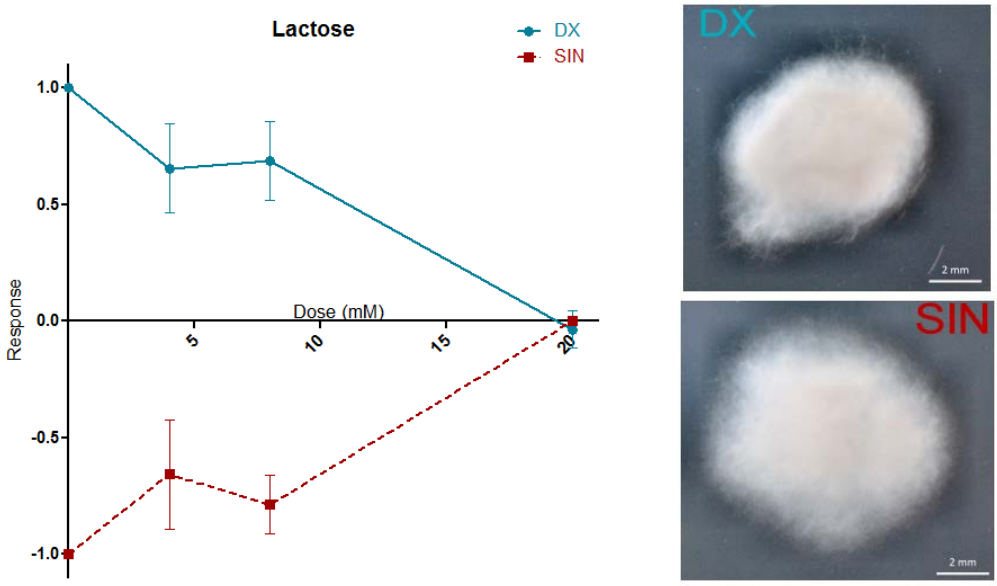

Figure 11. Effect of lactose on chirality of DX and SIN.

To determine whether sucrose caused the same response, DX and SIN were plated onto 5YE + 20 mM sucrose. The resulting colony morphology is similar to the morphology caused by 20 mM lactose. Microscopic analyses revealed rod-shaped cells that grow end-to-end, but do not form long branches (Fig. 12).

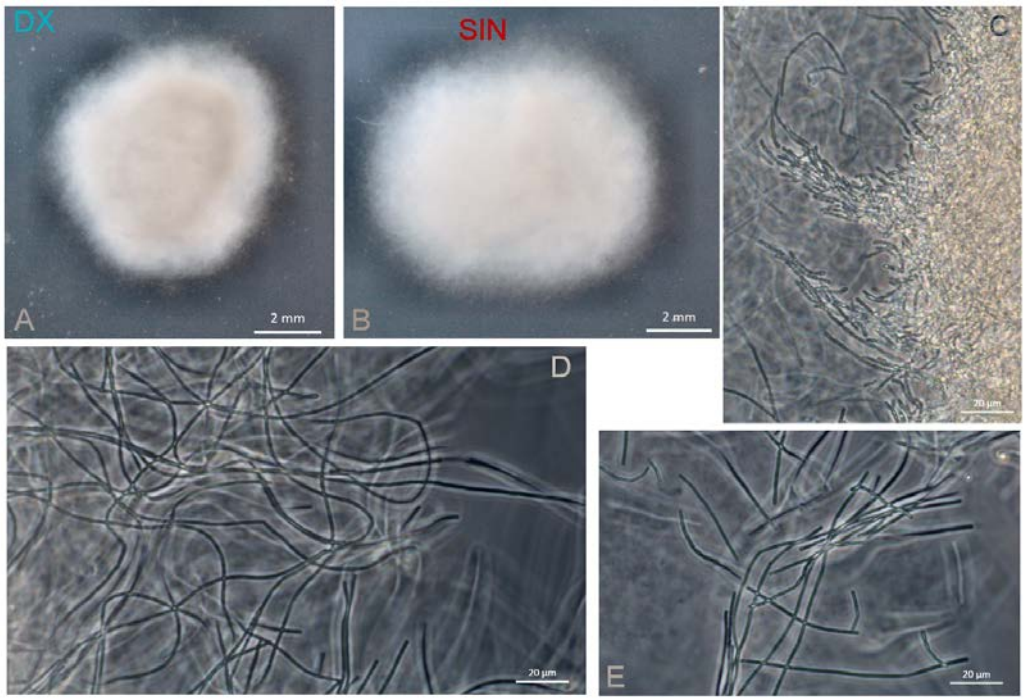

Figure 12. DX and SIN colony morphology (A & B) and DX cell morphology (C-E) in presence of 20 mM sucrose.

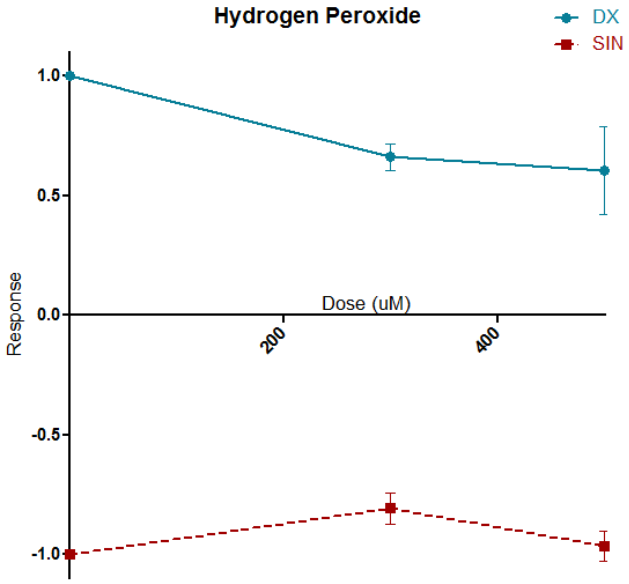

Figure 13. Effect of sodium selenite on chirality of DX and SIN.

Bébien et al. (2002) demonstrated that growth in the presence of selenite triggers expression of genes associated with oxidative stress in *E. coli*.[3] To test whether oxidative stress causes the switch in chirality, DX and SIN were plated onto 5YE with varying concentrations of hydrogen peroxide. The dose-response plot (Fig. 13) reveals that the tested concentrations of hydrogen peroxide do impact chirality, more so in DX than in SIN, but did not cause inversion.

Conclusions and Future Directions

The data shown in this report indicate that sodium selenite affects the chirality of SIN and DX strains of *Bacillus*, with a complete inversion in chirality for the latter. Sodium, ionic strength, osmolarity, and oxidative stress alter the chirality of both morphotypes, though the tested doses did not cause a complete switch in chirality for either strain. These results suggest the inversion may result from a combination of the above factors, or elemental selenium may trigger the switch in chirality, or another factor associated with selenite stress or the selenite reduction pathway is responsible. Future experiments will focus on testing the effects of more concentrations of the compounds used in this experiment, as well as tellurite and elemental selenium.

It has been proposed that chiral and non-chiral branching growth patterns evolved as a means for organisms to grow toward nutrients and away from stressors.[2,4] The result of this study support this theory, because they indicate that chirality is controlled by environmental regulation, not genotype alone. Though it is now apparent that chirality is subject to environmental regulation, much remains to be discovered about the mechanism controlling this growth pattern.

Levine and Ben-Jacob (2004) proposed that chiral pattern formation in *Paenibacillus dendritiformis* is controlled by the handedness of its flagellum and the development of a "lubricating fluid."[5] In this model, the lubricating fluid restricts the movement of the bacteria in a vertical direction, so the cells branch outward, and the rotation of the branch depends on the flagellar handedness of most of the cells within the branch. *B. mycoides* has never been shown to form flagella, and the end-to-end growth of this organism suggests that flagella are unlikely to be

responsible for chirality in this organism. However, it is possible that *B. mycoides* secretes a fluid or biofilm that restricts movement in a vertical direction, thus contributing to its rhizoid growth.

It has also been suggested that the dextral variant of *B. mycoides* produces more D-isomers in its biofilm relative to the sinistral form, and that DX produces an enzyme that specifically cleaves unnatural D-isomers.[6] If fluid/biofilm formation is important for chirality, then it is possible that the molecular chirality of molecules within biofilms could influence the curvature of a branch. Analyses of the *B. mycoides* secretome, including its specific rotation, could be a first step toward answering this question. Furthermore, development of genetic methods within this system is crucial - mutagenesis and comparison of DX and SIN genomes could provide valuable insight into the mechanisms controlling this organism's chiral growth pattern.

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"And now," cried Max, "let the wild rumpus start!"
— Maurice Sendak, Where the Wild Things Are

References

1. Franco C Di, Beccari E, Santini T, Pisaneschi G, Tecce G. Colony shape as a genetic trait in the pattern-forming Bacillus mycoides. *BMC Microbiol*. 2002;2(33).

2. Czirok A, Vicsek T. Cooperative Formation of Chiral Patterns during Growth of Bacterial Colonies. *Phys Rev Lett*. 1995;75(15):2899.

3. Bébien M, Lagniel G, Garin J, Touati D, Verméglio A, Labarre J. Involvement of Superoxide Dismutases in the Response of Escherichia coli to Selenium Oxides Involvement of Superoxide Dismutases in the Response of Escherichia coli to Selenium Oxides. *J Bacteriol*. 2002;184(6):1556–1564. doi:10.1128/JB.184.6.1556.

4. Ben-jacob E, Shmueli H, Shochet O, Tenenbaum A. Adaptive self-organization during growth of bacterial colonies. 1992;187:378–424.

5. Levine H, Ben-Jacob E. Physical schemata underlying biological pattern formation-examples, issues and strategies. *Phys Biol*. 2004;1(1-2):P14–22. doi:10.1088/1478-3967/1/2/P01.

6. Alpatov V V. Specific Action of Optical Isomers of Mepacrine Upon Dextral and Sinistral Strains of *Bacillus mycoides* Flügge. *Nature*. 1946:838.