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Possible Role of N-Acetylglucosamine for Induction of Polyhydroxybutyrate Depolymerase in Streptomyces sp. SFB5A

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Streptomyces **sp. SFB5A is a filamentous, Gram-positive bacterium that makes a polyhydroxybutyrate (PHB) depolymerase to degrade PHB for energy usage. Its life cycle begins as spores germinate to form vegetative mycelia. Starvation prompts formation of aerial mycelia and ultimately spores after 5-7 days. During starvation,** ***Streptomyces*** **may use peptidoglycan hydrolases to degrade its own cell walls, releasing N-Acetylglucosamine (NAG). PHA depolymerase activity is detected during growth of strain SFB5A on NAG as the sole carbon source, suggesting that NAG induces PHA depolymerase synthesis. To test this hypothesis, strain SFB5A was grown for 4 days on PHB, and a colorimetric assay for NAG was performed on culture supernatants from samples taken every 12 h. NAG concentration peaked at about 24-36 h, when cells might be expected to autolyze. However, absorbance readings were only slightly above the hypothetical limit of detection, most likely due to very low NAG concentrations. Increasing the final concentration of borate buffer in the assay increased the assay sensitivity 2-fold, and suggested that culture supernatants could be concentrated at least 2-fold to increase detection. To detect peptidoglycan hydrolase secreted during autolysis, samples of culture supernatants were spotted onto agarose gels containing heat-killed cells. However, no clearing zones appeared after 6 h incubation. Instead, cells grew in the spotted areas after 24 h, probably because of residual cells in the supernatants. Concentrating supernatants by freeze drying might increase sensitivity and filtration of supernatants would remove residual cells.**

Introduction

Streptomyces is described as a Gram-positive, filamentous soil organism, known for having biologically active secondary metabolic compounds and its uniquely complex life cycle (NIH-Signals and regulators). The genus is a common soil inhabitant and contains a PHB depolymerase, which degrades PHB for energy usage. Beginning as a spore and germinating to form a mycelium, the genus eventually generates spores after 5-7 days (Fig. 1).

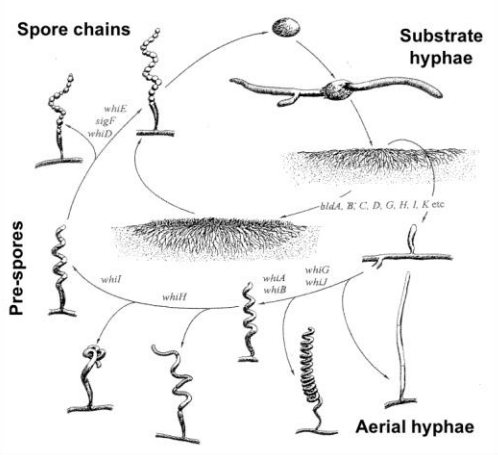


Figure 1. During its vegetative state, after germination of a spore, *Streptomyces* grows down into the medium, forming substrate hyphae. When it is starved, it forms aerial hyphae, which eventually segment to form spores [Image from Keiser 1].

During starvation, *Streptomyces* diverts its resources from a growing vegetative state to this spore-forming state. It is hypothesized that, in times of starvation, *Streptomyces* degrades its own cell walls, using peptidoglycan hydrolases. This degradation releases N-Acetylglucosamine (NAG) molecules, which can be used for metabolism and eventual production of spores, which would enhance the species' survival chances in times of starvation. As NAG is released during autolysis, it is internalized by the cell, phosphorylated through the phosphotransferase system, and converted to glucosamine-6-phosphate (NAG-6P) [2]. NAG-6P acts as an allosteric effector of a global regulator, called DasR, that inhibits its DNA binding and indirectly activates previously repressed genes [2].

High concentrations of NAG have also shown to be a "major checkpoint for the onset of secondary metabolism," including the production of antibiotics in *Streptomyces* [2]. Specifically, research has suggested that the production of antibiotics in these species is activated during the planned autolysis and formation of spores [2]. As previously stated, when NAG is internalized and phosphorylated, the DasR regulator is indirectly activated, resulting in the activation of genes that produce antibiotics in the species. While NAG has been shown to cause developmental arrest in *Streptomyces* when grown on nutrient-rich media, it activates antibiotic production and sporulation when grown on nutrient-poor media [2]. Therefore, NAG is seen as a signaling molecule for the DasR-mediated nutrient sensing system. Under famine, the accumulation of NAG promotes sporulation and antibiotic production.

As energy sources and carbon storages, many organisms, including

some *Streptomyces* species, produce polyesters called polyhydroxyalkanoates (PHA). These polyesters are of interest because they can be used to make plastic-like material objects that degrade over time, serving importance in terms of alternative energy and renewable resources. One of the most common polymers of PHA is polyhydroxybutyrate (PHB), which is also produced by many microbes and is degraded by PHB depolymerases, which break PHB into R-3-hydroxybutanoic acid monomers (3HB). To do this, many microbes use extracellular PHB depolymerases to degrade PHB to 3HB, transport the monomers into the cell, and catabolize them.

The soil bacterium used in Dr. Baron's laboratory, *Streptomyces* sp. SFB5A, produces a PHB depolymerase and degrades PHB during growth on PHB. The PHB depolymerase has been purified and characterized. It degrades PHB in vitro to 3HB monomers, with lower amounts of dimers and trimers, as determined by electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS) [3]. Interestingly, ESI-TOF-MS assay data also reveals that low but significant PHB depolymerase activity (2.4% of the activity with PHB) is detected in supernatants from cultures grown on NAG as the sole carbon source [HM Blevins and SF Baron, unpublished data]. It is hypothesized that NAG, in addition to promoting sporulation and antibiotic production, may be an inducer for PHB depolymerase synthesis. During starvation, as the organism prepares to sporulate, it might autolyse, releasing NAG and PHB depolymerase, which could degrade either exogenous from other bacteria or endogenous PHB stores with strain SFB5A itself (although endogenous PHB has not yet been detected in strain SFB5A). The degradation of PHB might provide energy for subsequent sporulation. This hypothesis could explain why NAG might serve as an initial inducer for PHB depolymerase synthesis.

The overall goal of the project was to explore the relationship between NAG and PHB depolymerase activation. Questions to be addressed included: 1. Does the organism release NAG from its cell wall during a) starvation (no carbon source) or b) growth on PHB and 2. Does the organism make a peptidoglycan hydrolase during a) starvation or b) growth on PHB.

Materials and Methods

Media and Culture Conditions

Nutrient broth (NB) was used for routine culturing of *Streptomyces* sp. SFB5A. A mineral salts medium, Schlegel's Nitrogen Complete (SNC) [4] was used for autolysis studies. The medium was supplemented with 0.2% PHB as desired and with 1.5% agar for plate media.

Cell Culture for N-Acetylglucosamine Assay. One colony of *Streptomyces* sp. SFB5A was inoculated into 4 mL of NB

and incubated at 30℃ with 200 rpm shaking for 16-24 h. Gram stains were performed on the cultures to ensure purity. The culture was centrifuged and resuspended in 4 mL of SNC. The resuspended cells were transferred into 100 mL of SNC with or without 0.2% PHB as the sole carbon source. Two samples of 1.8 mL were pipetted from the flasks at 0, 12, 24, 36, 48, 72, and 96 hours. Each sample was centrifuged for 1 minute, and the supernatant was removed and transferred into another centrifuge tube for later use in enzyme activity and assays. The supernatant and cell pellets were stored at -80℃ for later use.

N-Acetylglucosamine Assay NAG concentrations were determined using an adaptation of a colorimetric assay that converts NAG in an alkaline borate buffer (pH 9.0) to a red product that absorbs light at 585 nm [5]. Two versions of this assay were used: A and B. For both versions, 50 µg/mL NAG was prepared as a working stock and appropriately diluted to obtain the final concentrations indicated in Table 1.

Table 1. N-Acetylglucosamine standard concentrations.

NAG Concentration (µg/mL)	(µL) 0.05 mg/mL NAG Stock	µL SNC
0	0	133.0
2.5	6.65	126.4
5.0	13.3	119.7
10.0	26.6	106.4
20.0	53.2	79.8
40.0	106.4	26.6
50.0	133	0

For Version A, reaction mixtures were prepared by combining 133 µL of standards or culture supernatants with 134 µL of 80 mM sodium borate in 1.7 mL screw cap microfuge tubes. Each tube was vortexed briefly and incubated in a 100℃ water bath for 10 minutes. After cooling for 5-10 minutes in a room temperature water bath, 773 µL of 10 mg/mL dimethylaminobenzaldehyde (DMAB) reagent [5] was added to each tube and incubated at 37℃ for 20 minutes. Microfuge tubes were cooled for 5 minutes in a 20℃ water bath. The absorbance of each tube was recorded at 585 nm versus a deionized water blank to determine the NAG concentration of each standard. Parallel standard curves prepared using SNC instead of water to prepare the standards were done to test if SNC medium affected color production in the assay. The final concentrations of borate and DMAB in this version were 10.7 mM and 7.33 mg/mL, respectively.

Version B was similar to version A, except that 30 µL of 800 mM borate was combined with 133 µL of standards or culture supernatants, and 900 µL of DMAB reagent was added after the boiling step. The final concentrations of borate and DMAB in this version were 22.2 mM and 8.33 mg/mL, respectively.

Cell Sample Collection for Peptidoglycan Hydrolase Assays. One colony of *Streptomyces* sp. SFB5A from a PHB plate was inoculated into a sterile vial of 4 mL of nutrient broth and incubated overnight at 30℃ with shaking at 200 rpm. Gram stains were performed on the cultures in order to detect any contamination. The culture was combined into a 1 L flask with 200 mL of nutrient broth and incubated overnight at 30℃ with shaking at 200 rpm. The culture was centrifuged at 5500 rpm for 10-12

minutes to a pellet. The pellet was resuspended in 100 mL of 2X phosphate and 80 mL of mineral mix. The culture was distributed (90 mL each) into 2 500 mL flasks, one with 10 mL of sterile water and one with 5 mL of 4% PHB as a carbon source and 5 mL of sterile water. 5.5 mL of samples from each culture were taken at 0, 12, 24, 36, 48, 72, and 96 hours. Each sample was centrifuged for 4-8 minutes, as needed, at speed 5 before the supernatant fluid and pellet were separated. From the supernatant of each sample, 625 µL was transferred into a 2 mL screwcap microfuge tube for later use. Samples were stored at -80℃ for later analysis.

Zymographic Assay for Peptidoglycan Hydrolase A zymographic assay was used to detect peptidoglycan hydrolase [6]. The method involved spotting of culture supernatants onto test plates containing killed cells of strain SFB5A. To prepare the test plates, cells of SFB5A were grown overnight at 30 °C with shaking (200 rpm) in 1 L of NB in a 2L Erlenmeyer flask, then harvested by centrifugation. Cells were killed by resuspending and incubating the cell pellet in 20% w/v trichloroacetic acid for 10 min at 100°C, followed by washing 3 times each with buffer and deionized water. The killed cells were included at 4% (w/v) into molten agarose gels poured into petri plates, resulting in an opaque suspension after hardening of the gel.

Test plates were spotted using a grid pattern with 2 µL samples of culture supernatants from cultures with or without PHB. As a positive control, a plate was spotted with 20 µg samples of lysozyme, a known peptidoglycan hydrolase. Plates were incubated at 37°C, and observed for clearing zones (A).

Ammonium sulfate precipitation was used to concentrate culture supernatants to increase sensitivity of the zymographic assay. To ~625 µL of culture supernatant was added 1.38 mL of 100% saturation $(NH_4)_2SO_4$ in RB (25 mM Tris-Cl pH 7.5 containing 1 mM $CaCl_2$). After incubation on ice for 30 min, the tubes were centrifuged for 15 min at 20,000 x *g.* All traces of the supernatant fluids were discarded, and the pellets were each redissolved in 25 µL of RB before spotting onto the test plate.

Native polyacrylamide gel assay for peptidoglycan hydrolase. An electrophoretic method was also used to detect peptidoglycan hydrolase in culture supernatants. Native polyacrylamide gel electrophoresis (Native-PAGE) was performed on gels of 7.5%T using the Laemmli system [7] without sodium dodecylsulfate or 2-mercaptoethanol. Proteins from 625 µL of culture supernatants were concentrated with ammonium sulfate as described above. To 22 µL of each sample was added 3.83 µL of native gel electrophoresis dye [7]. Samples were loaded into individual wells of the gel. Proteins were separated at 20 mA constant current. The running buffer was chilled in an ice bath and kept

stirring with a magnetic stirrer. After completion of native-PAGE, the gel was overlaid with a 1% w/v agarose gel made up in RB and containing 4% v/v killed cells. The gel sandwich was incubated at 25°C overnight and analyzed for bands of clearing in the killed cell-agarose overlay.

Results and Discussion.

It was hypothesized that during starvation, *Streptomyces* might autolyse, releasing NAG. NAG might then serve as an initial inducer for synthesis of PHB depolymerase synthesis, which could degrade either exogenous or endogenous PHB.

Therefore, the purpose of the experiment was to detect NAG in culture supernatants or peptidoglycan hydrolases, both of which would indicate autolysing occurring.

Our first approach to testing this hypothesis was to perform the NAG assay with only authentic NAG standards to determine the effectiveness of the assay. After various trials with different NAG stocks, the NAG stock concentration of 50 µg/mL made in both deionized water and SNC medium gave sufficient results for the standards. The NAG concentrations of the standards were plotted versus the

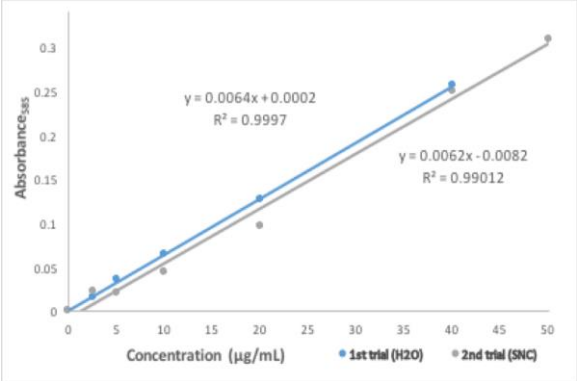


Figure 2. Absorbances at 585 nm for authentic NAG standards prepared in water versus SNC. Version A of the NAG assay was used. Data points are individual readings of absorbances.

absorbance at 585 nm. The standard curves showed substitution of SNC for water in the NAG standards did not significantly affect the color production in the standards, as shown in Figure 2.

Accordingly, two sets of culture supernatants were assayed for NAG concentration. The first set was uninterpretable so only the second set was analyzed. Because absorbance values were so low, any slight variation could have dramatically influenced the data trend. In the second set, NAG concentration peaked at about 24-36 hours, when cells might be expected to autolyze (Fig. 3). A linear regression was run to determine the limit of detection (LOD), limit of

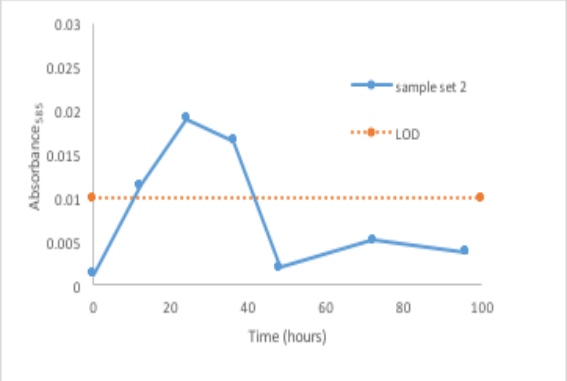


Figure 3. NAG concentration versus time for culture supernatants of *Streptomyces* 5A grown in SNC-PHB. Samples were removed at time intervals, processed, and assayed for NAG as described in Materials and Methods. Version A of the assay was used. Data points are individual readings of absorbances.

quantitation (LOQ), and signal to noise ratio value (SNR) were calculated to determine the significance of the data. The limit of detection refers to the absorbance value at which NAG is detected but not quantitated. The LOD was determined to be an A_{585} of 0.01, and the LOQ, 0.06. As previously mentioned, there were 3 data points that peaked above the LOD around 24-26 hours, indicating NAG was detected. No data points exceeded the LOQ, which would have allowed quantification of NAG in the samples. However, the linear regression also revealed that the SNR value was below 3. This may have been partly due to the calibration curve of the standards not being the best fit, having an R^2 value of only 0.990 and the samples having a very small amount of NAG, if any, initially. Therefore, the data was insignificant and conclusions of whether NAG was present or not could not be made.

In efforts to address the issue of low NAG concentrations, freeze drying was considered. Concentration of the supernatants by freeze drying might have increased NAG concentrations to more detectable levels. However, it would also concentrate SNC components, especially phosphate buffer (pH 7.4), which might acidify the borate buffer in the boiling step of the NAG assay and thus interfere with color development.

To address this issue, we increased the final concentration of borate buffer in the assay from 10.7 to 22.2 mM to minimize potential acidification during treatment with borate and increased the final DMAB concentration from 7.33 to 8.33 mg/mL (NAG assay Version B). Standard curves with authentic NAG prepared in water versus 2-fold (2X) SNC showed that the latter did not significantly affect color yield (Fig. 4).

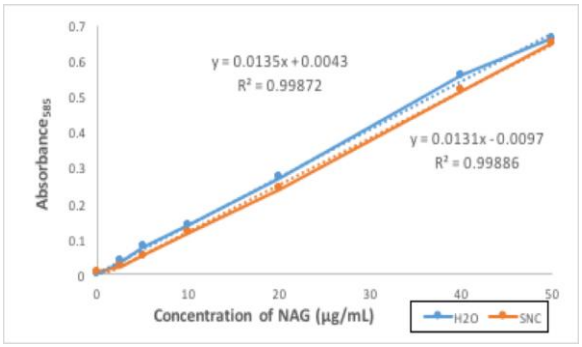
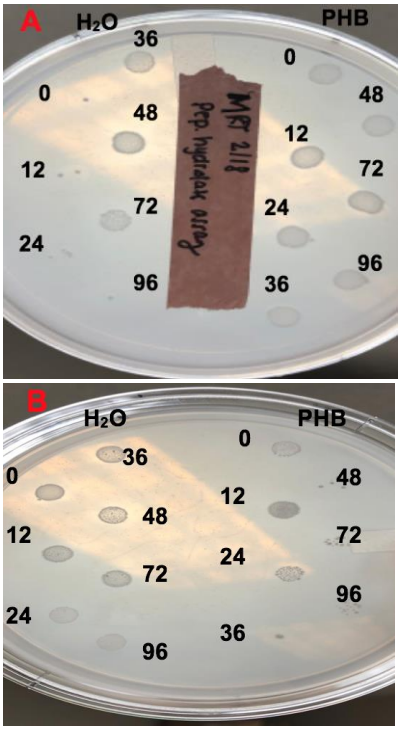


Figure 4. Absorbances at 585 nm for authentic NAG standards prepared in water versus 2XSNC medium. Version B of the NAG assay was used. Data points are individual readings of absorbances.

This indicated that culture supernatants could be concentrated at least 2-fold to increase sensitivity of the assay. In addition, comparison between Figure 3 and Figure 4 showed that increasing the

borate buffer and DMAB concentrations (Version B) increased the slope about 2-fold, thus improving the sensitivity of the assay. Due to time limitations, we were not able to try NAG assay version B on culture supernatants.

In order to detect the presence of a peptidoglycan hydrolase in strain SFB5A, which indicates autolysis, we evaluated a zymographic assay. SFB5A was grown in SNC with and without PHB. Supernatants were spotted onto agarose containing killed cells and observed for clearing zones (Figure 5A). However, no clearing zones appeared after 6 hours of incubation at 37℃. Instead, cells grew in the spotted areas after 24 hours, probably because of residual live cells in the supernatants.



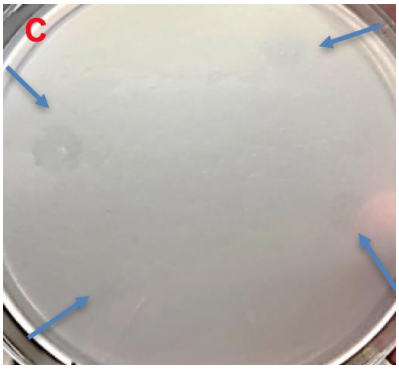


Figure 5. A) Zymographic peptidoglycan hydrolase assay of supernatants from culture of strain SFB5A grown with PHB or without (H_2O) with samples removed at time intervals (h) indicated by the numbers. The test plates were prepared and spotted as described in Materials and Methods and photographed after 24 h of incubation at 30°C. **B)** As in Panel A, except plates were spotted with ammonium sulfate precipitated culture supernatants. **C)** Test plate spotted with chicken egg white lysozyme (20 µg, 2 µL), providing a positive control. Clearing zones are indicated by blue arrows.

The lack of clearing zones could also be due to low sensitivity, especially given the low amounts of clearing in the positive controls (Fig. 5C).

In an attempt to increase sensitivity of the assay, we concentrated culture supernatants by ammonium sulfate precipitation before spotting on the test plates (Fig. 5B). However, results were the same as without this treatment (Fig. 5A). Concentrating larger volumes of supernatants by freeze drying might increase sensitivity. Additionally, filtration of supernatants would remove live cells that could overgrow in the spotting areas. However, we did not have time to test these possibilities.

An alternative electrophoretic method using Native-PAGE was used in another attempt to detect peptidoglycan hydrolase activity As opposed to an SDS-PAGE gel, which denatures proteins, a

native gel would preserve the activity of any enzymes such as peptidoglycan hydrolase. Thus when the completed protein gel was sandwiched together with an agarose gel containing killed cells, clearing zones would form if peptidoglycan were present. However, no clearing zones were observed (Fig. 6).

The reason for this is unknown as it was expected to see clearing due to proteins suspected to be present in the samples. Further, the positive control, lysozymes, did not show clearing zones either, indicating an issue within the gel.

A

B

Figure 6. A. Native 7.5% polyacrylamide gel loaded with ammonium sulfate precipitates of supernatants from a culture of strain SFB5A grown with PHB. After electrophoresis, the gel was overlaid with agarose containing killed cells. **B.** As in A, except that the gel was loaded with ammonium sulfate precipitates of supernatants from a culture of strain SFB5A incubated without PHB. Details of culture supernatants processing, gel electrophoresis, and peptidoglycan hydrolase activity detection are described in Materials and Methods.

Future Work

Future work would include performing the revised NAG assay on culture supernatants. A possible peak of NAG was seen at 24-36 hours in cultures, but data were unreliable because of low signal to noise ratios. The sensitivity of the NAG assay was improved by increasing the concentration of borate buffer and thus should be performed on culture supernatants in hopes of detecting NAG. Additionally, the peptidoglycan hydrolase assay was inconclusive due to poor sensitivity and overgrowth of cells. The assay should be repeated using freeze-dried and filtered culture supernatants. Future work could also include further research on how to absorb and concentrate NAG from solution using a chromatography column or analysis in-situ by NMR.

References

1. Kieser T, et al. (2000) Practical Streptomyces genetics. Norwich, UK: John Innes Centre.

2. Rigali, Sébastien et al. “Feast or Famine: The Global Regulator DasR Links Nutrient Stress toAntibiotic Production by Streptomyces.” EMBO Reports 9.7 (2008): 670–675. PMC. Web. 12 May 2018.

3. Blevins HM, Blue MD, Cobbs BD, Ricotilli TA, Kyler SL, Shuey CT, Thompson WD, Baron SF. 2018. Characterization of an Extracellular Polyhydroxyalkanoate Depolymerase from *Streptomyces* sp. SFB5A. J Bioremediat Biodegrad **9(5)**:425.

4. Schlegel, H.G., G. Gottschalk, and R. von Bartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria

(Hydrogenomonas). Nature (London). 191:463-465.

5. Reissig JL, Strominger JL, Leloir LF. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J Biol Chem 217:959-966.

6. Dufour, D. and Lévesque, C. M. (2013). Zymogram Assay for the Detection of Peptidoglycan Hydrolases in *Streptococcus mutans*. *Bio-protocol* 3(16): e855. DOI: 10.21769/BioProtoc.855.

7. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685. PMID: 5432063