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Impact of Iron Availability on *Bacillus amyloliquefaciens* Growth

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Abstract

***Bacillus amyloliquefaciens* is a biocontrol agent whose genome has been sequenced. Within the genome of *B. amyloliquefaciens* are genes associated with iron chelation, but these genes are not found within all sequenced strains. The impact of iron availability on the *B. amyloliquefaciens* physiology was examined in this study. *B. amyloliquefaciens* ATCC 23843 was cultured under iron-replete and iron-deplete conditions for 48 hours, at 37˚C. Final growth yields were dependent on iron concentration. Cultures grown in the absence of detectable iron were restricted in growth, but reached their highest yields at 48 hours. Iron restriction was confirmed by the presence of iron chelators in the filtrates. In contrast, *B. amyloliquefaciens* ATCC 23843 cultures grown with ferric ammonium citrate as the iron source research reached the highest yields at 24 hours. Iron chelator production was not detected in the ferric ammonium samples. A significant decrease in turbidity was observed for these cultures, which coincided with elevated spore production in *B. amyloliquefaciens* ATCC 23843. A decrease in turbidity was also observed on blood agar, where hemolysis was readily evident. We propose that iron impacts numerous physiological responses and further studies will elucidate the complex regulatory mechanisms governed by iron availability.**

Keywords

Bacillus, Catechol, Hemolysis, Iron

1. Introduction

The importance of iron in microbial growth and virulence has been extensively explored in *Bacillus* species.

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Iron is an essential element for almost all living things, however, the amount of iron readily available within an environment is limited. *Bacillus* species utilize a variety of iron acquisition mechanisms that allow them to survive in diverse backgrounds, from the soil to a human host. One of the most effective and widely examined is the production of iron chelators, or siderophores [1].

Siderophores are low molecular weight, ferric specific chelators that have a high affinity for iron. These chelators are released under iron limiting conditions, and thus, are tightly regulated by iron concentrations. There are two main groups of chelators based on their iron binding motiety: catechols and hydroxamates. While both types of siderophores have been identified in Gram-negative bacteria, only catecholate siderophores have been identified in *Bacillus* species [2].

Bacillus amyloliquefaciens is a rhizobacterium known for producing numerous plant promoting factors [3]. This microbe is genetically similar to *Bacillus subtilis*, a ubiquitous organism demonstrated to produce bacillibactin under iron-limiting conditions [4]. Bacillibactin is a 2,3-dihydroxybenzoic acid (2,3-dhb) containing molecule encoded by the *dhb* operon and is similar to the 2,3-dhb containing siderophore produced by Gram-negative bacteria, enterobactin [5]. Both the simple catechol and the complex siderophore are produced, although the siderophore is typically observed during the latter stages of growth [6]. Production of these catechols is tightly regulated by the environmental conditions, including iron concentration and salinity [4] [7].

There are few studies that have explored the genetic, biochemical and physiological impacts of iron on *B. amyloliquefaciens*. The *dhb* operon has been detected in the genome of several *B. amyloliquefaciens* strains [8] [9], although the operon encoding bacillibactin is not found in at least one strain [10]. Siderophore production in the soil isolate *B. amyloliquefaciens* NAR38.1 was regulated by metal ions, with production being maximum under iron restricted conditions [11]. Because of the diversity observed within the genomes, we questioned how iron availability would impact *B. amyloliquefaciens* ATCC 23843.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

B. amyloliquefaciens ATCC 23843 was purchased from the American Type Culture Collection (ATCC). Cultures were maintained as spores on sporulation agar (23 g nutrient agar, 0.5 g yeast extract, 6.0 mg MnCl2, 95.0 mg MgCl2, 78.0 mg CaCl2 per liter) at 4˚C. For routine growth, bacteria were transferred from the spore stocks to brain-heart infusion agar (BHIA) slants for overnight incubation at 37˚C. Cells were removed from the slants with Chelex (Bio-Rad®) treated MM9 medium, hereafter referred to as Chelex-treated medium or CTM, with no added iron [12]. All cultures were incubated with aeration (200 rpm) at 37˚C. Trace metal contamination was decreased on all glassware by rinsing with 6 N hydrochloric acid and de-ionized water.

2.2. Iron Chelator Detection

To detect excretion of iron chelators, aliquots of filtrate were removed from cultures. Samples were centrifuged for 2 min at 14,000 g to remove cells. One mL samples were removed and mixed with 25 µL of ferric chloride (1%). Chelator production is indicated by a color change to green, purple or red, depending on the nature of the chelator.

2.3. Calculation of Spore Production

Aliquots were removed from cultures and incubated for 30 minutes, at 65˚C to kill all vegetative cells. Following heat treatment, samples were serially diluted in CTM, spread on BHIA and incubated overnight at 37˚C. The plates were performed in duplicate and the number of colonies recorded.

3. Results

3.1. Iron Dependent Growth of *B. amyloliquefaciens* ATCC 23843

CTM was inoculated with cells grown overnight on agar slants at an approximately concentration of 1×10^3 cells per mL. Cultures were incubated with aeration for 48 hours at 37˚C and the OD taken. The average of three experiments and the standard deviation for the samples were calculated. At 24 hours, cell growth was more than 10× greater in the samples with 10 µM ferric ammonium citrate (FAC) as compared to the <0.1 µM, or low, iron

samples (**Figure 1**). At 48 hours, cell growth in the low iron cultures had increased. In contrast, the number of cells measured spectrophotometrically in the 10 µM FAC samples decreased significantly, when compared to the 24 hour iron samples (**Figure 1**). The final readings of these samples were also slightly lower than the 48 hour low iron cultures.

3.2. Iron Chelator Production in Response to Iron Limitations

Under iron limitation, *Bacillus* microbes secrete iron chelators [12]. It is predicted that *B. amyloliquefaciens* ATCC 23843 produces 2,3-dihydroxybenzoic acid and bacillibactin in response to iron limitations. In the presence of iron, these compounds form an iron-catechol complex that is purple, with the depth of the color associated with the amount of chelator present. Sterile culture filtrates were mixed with ferric chloride and observed for a color change. As indicated in **Table 1**, a positive reaction was identified in the <0.1 µM iron cultures at 48 hours, indicating that these cultures were iron restrictive. No color reaction was observed for the 10 µM FAC samples at either time point.

3.3. *B. amyloliquefaciens* Spore Formation

Because of the decrease in optical density identified for the 48 hour ferric ammonium samples, we questioned whether the loss of turbidity was associated with an increase in the number of spores produced. *B. amlyloliquefaciens* aliquots were heated to kill vegetative cells and plated on BHIA to measure the number of spores. At 24 hours, spores were observed in the ferric ammonium citrate samples (**Figure 2(a)**). By 48 hours, spores were isolated from both samples, with the greatest concentration observed in the 10 µM FAC (**Figure 2(b)**).

3.4. *B. amyloliquefaciens* Hemolytic Activity

A predicted component of iron acquisition is hemolysis, which releases the heme-iron complex. *B. amyloliquefaciens* activity has been documented in environmental strains [13]. When streaked onto blood agar plates, *B. amyloliquefaciens* ATCC23843 displayed hemolytic activity. Unlike *B. cereus* 14579 and *B. thuringiensis* 33679, the colonies appeared clear, as opposed to opaque (**Figure 3**). Cells isolated from this area were small, rods and Gram-variable (data not shown).

4. Discussion

The impact of iron availability has long been examined as a major regulatory trigger for microbes. Iron sequestration is an important biological defense mechanism that can limit the progression of certain microbes [14]. In response to the iron limitations of the host environment, microbes employ a variety of iron acquisition mechanisms

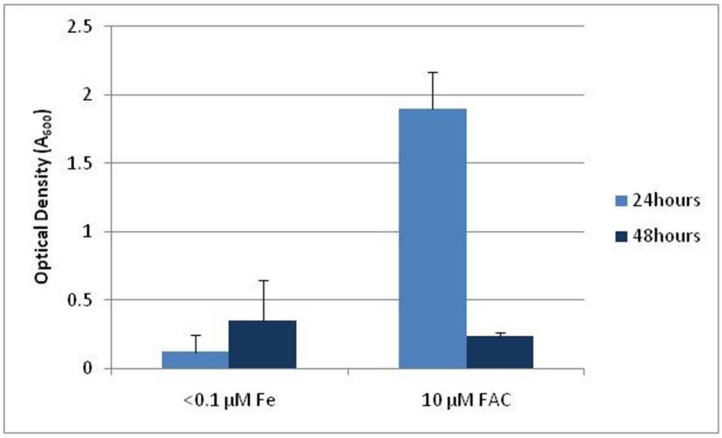
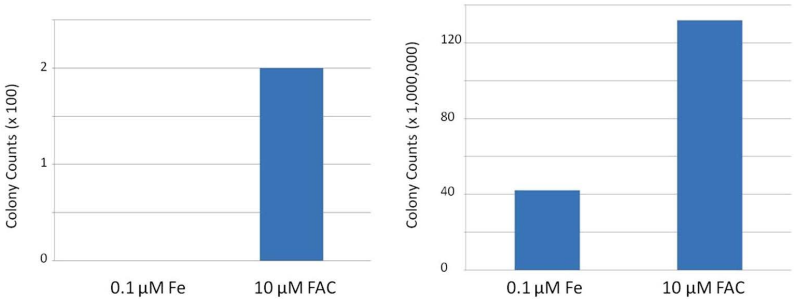


Figure 1. *B. amyloliquefaciens* ATCC23843 growth in response to iron concentration was measured spectrophotometrically for 24 and 48 hours. Results indicate the average of three experiments and arrow bars indicate the mean standard deviation.

Table 1. Detection of iron *B. amyloliquefaciens* chelator production.

Iron Concentration	Iron Reactivity of Culture Filtration	
	24 hours	48 hours
<0.1 µM	ND	++
10 µM FAC	ND	ND

ND = not detected, ++ = purple color



(a) (b)

Figure 2. Spores were calculated for samples grown at <0.1 µM Fe and 10 µM FAC for 24 (a) and 48 (b) hours. Samples were heated for 30 minutes at 65˚C, serially diluted in CTM and plated on BHIA plates. Experiments were repeated 4 times, in duplicate, and a representative experiment is shown. Values indicate the average of the representative experiment.

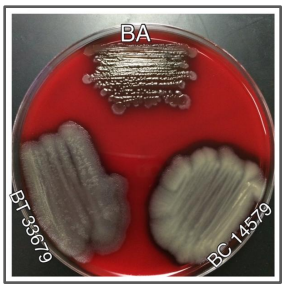


Figure 3. Microbes were streaked onto sheep red blood cell agar from BHIA slants and incubated overnight at 37˚C. The samples include *B. amyloliquefaciens* (BA), *B. cereus* (BC 14579) and *B. thuringiensis* (BT33679).

to survive. Expression of these mechanisms is tightly regulated via iron concentration [15]. Most of the work in iron acquisition has focused on the more pathogenic microbes, including *Bacillus anthracis* [16].

There has been limited research into the role of iron availability on the physiology of *B. amyloliquefaciens.* Genes for 2,3-dihydroxbenzoic acid and bacillibactin, catecholate iron chelators, have been identified in several *B. amyloliquefaciens* strains [9]. These compounds encoded by non-ribosomal peptide synthetases that are predicted to be important in the physiology of *B. amyloliquefaciens* [17]. In other *Bacillus* species, the production of catecholate chelators is regulated by environmental conditions, including oxygen and temperature [18] [19]. Iron availability, which controls catechol production via the ferric uptake regulation [20], has also been demonstrated to impact toxin production [21].

We observed that growth was diminished in *B. amyloliquefaciens* ATCC 23843 when iron was limited in the growth medium. While growth restriction has been documented in *Bacillus* species in response to iron restriction, the yields obtained within *B. amyloliquefaciens* ATCC 23843 were significantly lower at 24 hours of incubation [22] [23]. Catechol production was not detected in these cultures, which could be attributed to the low culture yields. Catechol production was identified at 48 hours by iron binding, and verified by the Arnow Assay (data not shown), which is specific for catecholates [14]. In the presence of 10 µM of iron, *B. amyloliquefaciens*

ATCC 23843 growth was maximum at 24 hours of incubation and the secretion of an iron chelator was not detected at any time interval. To our knowledge, this is the first report documenting the use of ferric ammonium citrate as an iron source by *B. amyloliquefaciens* ATCC 23843.

We observed that by 48 hours, more spores were present and there was a significant decrease in the culture turbidity. The decrease coincided with an increase in the number of spores isolated from the cultures. Similar results were observed when cells were grown on blood agar plates, with a loss of turbidity at 24 hours. A decrease in turbidity was observed for *B. amyloliquefaciens* LBM 5006 [24]. For these cultures, this also coincided with an increase in antimicrobial activity against the microbe *Paenibacillus larvae* [24]. Production of an iturin-like compound resulted in the lysis of *P. larvae* by *B. amyloliquefaciens* culture filtrates. It remains unclear whether the decrease is associated with cellular lysis, as observed against *P. larvae*, or cellular breakdown.

5. Conclusion

We have identified that *B. amyloliquefaciens* ATCC 23843 requires iron for optimal growth and spore production. Iron chelator production is tightly regulated by iron availability. Ferric ammonium citrate is an iron source that can be utilized by this organism to overcome iron limitation.

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