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Exploring Iron Scavenging as an Under-Explored Mode for Pathogen Elimination by *Bacillus*-Based Probiotics

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Abstract

Iron is a vital nutrient for aerobic organisms, but its (bio-)availability is limited in the lower intestine, leading to competition among microbial species. To address this scarcity, microbes produce iron- scavenging compounds such as siderophores. Certain gut pathogens are more iron-dependent than beneficial gut microbes, and iron promotes their replication and virulence. Bacillus species have antimicrobial effects against these pathogens through the production of secondary metabolites with direct inhibitory effects. The current study aimed to explore the less-studied effect of siderophores from a novel Bacillus licheniformis G3 in the fight against Salmonella. Earlier research has shown that the Bacillus licheniformis G3 reduced the prevalence of Salmonella in salmonellosis-infected broiler birds. The genomic mining of the Bacillus licheniformis G3 revealed the presence of several siderophore gene clusters. The in vitro iron binding capacity of the Bacilli was confirmed by chrome azurol S assay, while E. coli and Salmonella enterica demonstrated poor binding capacity. Further, the preferential iron binding capacity of the cell-free supernatant (CFS) of the Bacilli strain was tested by incubating it with Salmonella enterica in a medium spiked with ferric salt. Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) analysis of the spent media revealed decreased iron concentration in the group supplemented with CFS, indicating reduced iron availability for Salmonella growth, possibly due to iron chelation by the CFS. These findings suggest the positive role of siderophores in combating Salmonella's iron-dependent pathogenicity. Further exploration of Bacilli-based siderophores in regulating host iron homeostasis against other enteric pathogens may unveil their pivotal role in conferring an advantage over pathogens.

Keywords: Iron Scavenging, *Bacillus licheniformis G3*, Siderophores, *Salmonella*.

Introduction

Iron is one of the most abundant elements in the Earth's crust and is essential for the survival of almost all aerobic microorganisms. Iron plays a crucial role in metabolism including oxygen transport, cellular respiration, and DNA synthesis, due to its ability to form coordination bonds with electronegative atoms and transition between the ferrous (Fe (II)) and ferric (Fe (III)) oxidation states, making iron an important redox catalyst [1]. However, higher concentrations of free iron may also produce reactive oxygen species (ROS). Hence, the bioavailability of iron is tightly regulated in the host. Most of the iron in the host is insoluble and bound to transferrin, with only a small amount being free. This regulation occurs during absorption, transport, and storage to prevent iron toxicity and oxidative stress, mainly in the upper intestine [2]. In the lower intestine, the bioavailability of iron is minimal and this constraint creates a competitive niche for microbial species. Hence, microbes evolved a range of iron acquisition systems focused on siderophores, iron transporters, and host iron recovery (E.g., metabolizing heme-bound iron) [3, 4]. Siderophores are small molecules with high affinity to iron and they bind ferric iron after secretion into the extracellular environment [5]. After reuptake, the iron can be released for microbial consumption through enzymatic degradation of the siderophore or via reduction of ferric to ferrous iron, which

destabilizes the iron-siderophore complex. Besides the bioavailability of iron and the means of utilizing this limited resource, not all organisms have the same iron requirements. Some of the common gut enteric pathogens such as *Salmonella, E coli, Shigella and Clostridium* are more iron-dependent than beneficial gut microbes such as *Lactobacilli* [6, 7]. Iron promotes replication and virulence in these pathogens. The antimicrobial effects of *Bacilli* strains against these pathogens have been well reported, majorly by the production of secondary metabolites with direct inhibitory effects [8-10]. The current study aimed to explore the less-studied effect of siderophores from a novel *Bacillus licheniformis G3* which has previously been shown to reduce the relative abundance of *Salmonella* in the intestine of boiler chickens during an in *vivo* trial (unpublished internal data).

Materials and Methods

Gene Cluster Identification

The whole genome of *Bacillus licheniformis G3* (ATCC PTA-127113) was sequenced using Illumina HiSeq paired-end short-read sequencing at the Institute for Life Science Entrepreneurship (Union, NJ, USA). The secondary metabolite gene clusters were identified using bacterial antiSMASH versions 6 and 7 [11, 12].

Probiotic Culture Preparation

One mL of the bacterial strain *Bacillus licheniformis G3* (ATCC PTA-127113) was inoculated in 100 mL of TSB (tryptic soy broth) from a glycerol stock of the culture stored at -80°C. The culture was incubated at 37°C overnight at 120 rpm in a shaking incubator. The culture was later passaged one more time before its usage. For passaging, again 2 mL of the culture was inoculated into 100 mL of TSB and incubated for 24 h at 37°C in a shaking incubator at 120 rpm.

Cell-Free Supernatant (CFS) Preparation

10 mL of each probiotic culture was taken in sterile 15 mL centrifuge tubes and centrifuged at 20°C at 10621 x g for 10 min. The supernatants were filter sterilized using 0.22 µm sterile syringe filters.

Chrome Azurol S (CAS) Colorimetric Assay

The cell-free supernatant (CFS) of a freshly grown culture of *Bacillus licheniformis G3* was incubated with an equal volume of chrome azurol sulfonate (CAS) reagent for 20 mins, and optical density was measured at 630 nm and compared to a media control. Two industrially relevant strains, *E. coli* ATCC 25922 and *Salmonella enterica* ATCC 13576 were used as controls [13].

Iron Binding Estimation

Overnight grown culture of *Salmonella enterica* ATCC 13706 (approx. 10^8 CFU/ml) was incubated with CFS of *Bacilli* strain, spiked with known concentrations of ferric iron salt, in a 24-well plate at optimal growth conditions. CFS and media (tryptic soy broth) controls were also incubated. The supernatant of the *Salmonella enterica* culture with and without CFS was tested for the total iron content using inductively coupled optical emission spectroscopy (ICP-OES). The growth of *Salmonella enterica* was monitored by measuring the optical density at 600nm in a microplate reader.

Results and Discussion

Genome Sequencing and Secondary Metabolite Gene Cluster Identification

Bacillus licheniformis G3 (ATCC PTA-127113) was genome sequenced using Illumina HiSeq equipment (2x 150bp, paired end). The resulting coverage was >400-fold and the assembly resolved the genome into a total of 141 contigs with an estimated total genome size of 4.46 megabases. The max length of the contigs was 1000770 bases, while 14 contigs were over 10.000 bases long. This indicates that the assembly of the genome was not ideal, however, the quality scores of ≥Q20 and ≥Q30 were 99.72% and 93.58% respectively and this was deemed sufficient for the purpose of the current study.

Next, the genome was submitted to bacterial antiSMASH versions 6, where a total of 12 seconday metabolite gene clusters were identified over 9 different contigs. However, the total number of secondary metabolite gene clusters found in *Bacillus licheniformis G3* is 10. Two separate clusters of siderophores were identified: one utilising the hydroxamate iron binding moety, while the other siderophore is of the catecholate-type.

Siderophore Detection by CAS Assay

The production of siderophores by *Bacillus licheniformis G3*, under lab conditions was detected using the CAS assay, a laboratory colometric method detecting iron binding through a color change in the ferric indicator complex. Quantitatively, 38% iron-binding was observed in the CFS of the *Bacillus* strain. When subjected to the same conditions, this was higher than the observed iron binding in the industrially relevant standard strains of *E. coli* (6%) and *Salmonella enterica* (0%). This is especially remarkable as both *E. coli* and *Salmonella* are known to produce the strong siderophore enterobactin. Most, but not all *Salmonella* can produce enterobactin, but is notably generally capable of uptaking the enterobactin, even when not producing it, increasing the necessity of reducing both the *Salmonella* and the *E. coli* abundance in the gut [14, 15].

Iron Binding by *Bacillus Licheniformis G3* Incubated with *Salmonella Enterica*

It has already been demonstrated that intestinal iron availability may be rate-limiting for the growth of bacteria [16]. Furthermore, many potential pathogens have higher iron requirements compared to beneficial microbes such as *Lactobacilli*. Since *Salmonella* is almost always present in the intestines of chickens, it is important to not just focus on the reduction of *Salmonella* prevalence but also on prevention of translocation to the host. The iron requirements of *Salmonella* are higher when it is in its pathogenic state compared to commensal growth. Therefore, CFS of *Bacillus licheniformis G3* was added when culturing *Salmonella enterica* in a medium containing different iron concentrations. The total iron content in the culture supernatant of Salmonella enterica with *Bacillus licheniformis G3* CFS was lower compared to the control without CFS, indicating iron scavenging by the siderophores. As seen in Figure 1, iron availability for *Salmonella enterica* reduced when *Bacillus licheniformis G3* CFS was added. Furthermore, there seems to be a linear reduction in iron content as the concentration of ferric salt in the media increased, indicating the competitive binding of iron. Additionally, the sensitivity of *Salmonella enterica* to iron present in the medium was tested. The growth of *Salmonella enterica* was

not affected, even in the highest concentrations of iron, confirming that the iron levels used are not toxic to *Salmonella enterica.*

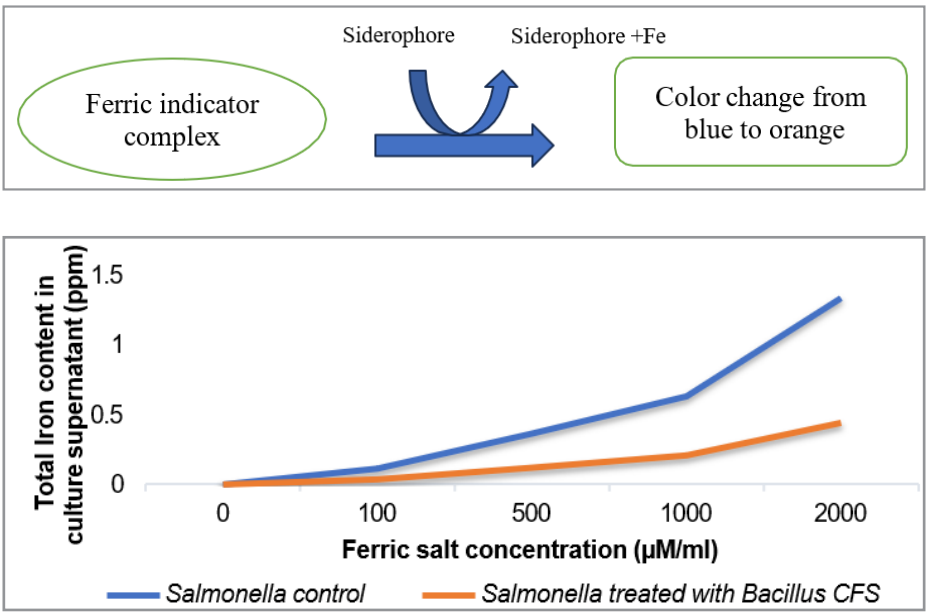

Figure 1: Estimation of iron binding by *Bacillus licheniformis* incubated with *Salmonella enterica* using ICP-OES

However, this does not yet indicate how the virulence of *Salmonella enterica* might be affected by reduced iron availability. qPCR of different virulence genes under the conditions tested above will provide information on the potential of *Salmonella enterica*to translocate in these conditions. Furthermore, enterobactin, which is commercially available, should be included as a separate control, as *Salmonella* can uptake iron using this siderophore. This will reduce the free iron in the medium, but not the availability of iron to *Salmonella*, informing if differential expression of virulence genes is indeed due to the iron availability or due to other effects that the *Bacillus licheniformis G3* CFS may have. These experiments can also be performed in the presence of a confluent layer of IPEC-J2 (intestinal porcine enterocytes), and monitoring transepithelial resistance (TEER) as an indicator of translocation [17].

Conclusions

The *Bacillus licheniformis G3* strain exhibited gene clusters of high-affinity iron-scavenging siderophores and production was confirmed by quantitative CAS assay. The iron scavenging ability of the siderophores was confirmed by the reduction in the iron content in the culture supernatant of *Salmonella enterica* treated with the CFS of the *Bacillus* strain. How the virulence is affected by the reduced availability of iron due to siderophore presence might be further substantiated in future experiments quantifying virulence gene expression or through cell culture studies. Further exploration of *Bacilli*-based siderophores in regulating host iron homeostasis against other enteric pathogens may unveil their pivotal role in conferring an advantage over pathogens.

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