Evaluation and Method Development for the Biosynthesis of Microbial Lipopeptides by *Bacillus* Species

Dissertation to obtain the doctoral degree of

Natural Sciences (Dr. rer. nat.)

Faculty of Natural Sciences

University of Hohenheim

Institute of Food Science and Biotechnology

submitted by

Maliheh Vahidinasab

from Tehran, Iran

2023

Dean: Prof. Dr. Jan Frank

1st referee / examiner: Prof. Dr. Rudolf Hausmann

2nd referee / examiner: Prof. Dr. Fabian Commichau

3rd referee / examiner: Prof. Dr. Ralf Vögele

Submitted on: 8.5.2023

Oral examination on: 11.10.2023

“It is not only the question,
but the way you try to solve it”

Dr. Maryam Mirzakhani

Acknowledgements

I would like to express my sincere gratitude to **Prof. Dr. Rudolf Hausmann** for the trust and the support he gave me throughout my research. Your unwavering support has given me the confidence to tackle difficult challenges and has motivated me to strive for excellence.

I would also like to thank **Dr. Lars Lilge**, **Dr. Kambiz Morabbi Heravi** and **Prof. Dr. Marius Henkel** for their advice that helped me to continue my work. Their scientific competence allowed me to refine my ideas and improve my research methodology.

I am very grateful to the jury who judges this work. Thank you to **Prof. Dr. Fabian Commichau** and **Prof. Dr. Ralf Vögele** for having accepted to be an examiner of the thesis and participate in the evaluation jury of my work. Thanks to **Prof. Dr. Yanyan Zhang** for being present as a representative of the doctoral committee in my oral examination.

This thesis would not have been possible without the support of **my dear colleagues**. Thank you for the friendly and family-like atmosphere which guaranteed a great working time together: Thank you to my alumni and current colleagues: Eike Grunwald, Anja Sander, Claudia Harnau, Dr. Mareen Hoffmann, Chantal Treinen, Manuel Merkel, Peter Klausmann, Till Hennecke, Dirk Kiefer, Dr. Philipp Noll, Stephen Akyntayo, Lennart Biermann, Jakob Grether, Andre Fahriz Perdana Harahap, Seyyedeh Zahra Hashemi, Holger Dietmann, Eric Hiller, Dr. Elvio Henrique Benatto Perino and Katharina Kunz.

I also thank **all my motivated students** who contributed to the progress of my work by completing a bachelor thesis, master thesis or a project work: Relja Stepanović, Aline Reinfurt, Astrid Ney, Waranya Wataniyakun, Chanthiya Kuppusamy Nesamani, Shankar Shrestha, Pauel Grießhaber, Isabel Adiek, Philipp Becker, Blanca Camacho Page, Bahar Abrishamchi, Mohammad Salikandi, Erik Weber Nunes and Sandra Moneta.

I am very thankful of all the people who collaborated in this research, more particularly to Prof. **Dr. Ralf Vögele** and **Behnoush Hosseini** from the Department of Phytopathology of Uni Hohenheim, for giving me the opportunity to perform antifungal analysis experiments, to **Dr. Jens Pfannstiel** from Core-facility of Uni Hohenheim for his collaboration in the mass spectrometry analyses.

A big thank you to all the members of **Food security center of Hohenheim (FSC)**, with a special thought for **Dr. Heinrich Hagel** and **Daniela Gomez** for their friendly support and organizing excellent activities during four years funding of this thesis by FSC.

I would like to take this opportunity to thank my master thesis supervisor **Prof. Dr. Masoud Ahmadzadeh** and my mentor at the time, **Prof. Dr. Seyyed Hossein Goldansaz** from University of Tehran. Your encouragement and guidance have motivated me to pursue further education in the field that truly interests me.

Finally, I would like to express my deepest thanks to my friends and family. This thesis would not have been possible without them either.

All my **fantastic friends in Stuttgart and Tehran**, for being always available during the happy and difficult moments.

To **my friends in PREM**, especially **Sarah** for giving me the confidence and encouragement to be active in politics of University.

To my fiancé **Felix**, for his patience and care. Thank you for spending so many hours discussing my research with me. You were always there to listen, offer words of encouragement, and celebrate my milestones with me. I would also like to extend my deepest gratitude to the **Horlamus family** for their great love and support.

My greatest thanks go to **my family in Tehran**. I want to thank **my parents**, for teaching me to believe in myself. I also want to thank **my sister**, my fantastic cousins, and my dear aunt for their continuous love and support from a long distance. This accomplishment would not have been possible without all of you.

Publications & congress contributions

Peer reviewed original publications

Vahidinasab M., Ahmadzadeh M., Henkel M., Hausmann R., Morabbi Heravi K. (2019). *Bacillus velezensis* UTB96 is an antifungal soil isolate with a reduced genome size compared to that of *Bacillus velezensis* FZB42.

In: Microbiology Resource Announcements. Sep. 19; 8(38): e00667-19.
DOI:10.1128/MRA.00667-19

Vahidinasab M., Lilge L., Reinfurt A., Pfannstiel J., Henkel M., Morabbi Heravi K., Hausmann R. (2020).
Construction and description of a constitutive plipastatin mono-producing *Bacillus subtilis*.

In: Microbial Cell Factories. Nov. 10; 19(1): 1-12.
DOI: 10.1186/s12934-020-01468-0

Lilge L.*, Vahidinasab M.*, Adiek I., Becker P., Kuppusamy Nesamani C., Treinen C., Hoffmann M., Morabbi Heravi K., Henkel M., Hausmann R. (2021).
Expression of *degQ* gene and its effect on lipopeptide production as well as formation of secretory proteases in *Bacillus subtilis* strains.

In: MicrobiologyOpen. Oct. 14; 10(5): e1241.

DOI: 10.1002/mbo3.1241

*Co-first authorship

Vahidinasab M., Adiek I., Hosseini B., Akintayo S. O., Abrishamchi B., Pfannstiel J., Henkel M., Lilge L., Voegele R. T., Hausmann R. (2022).
Characterization of *B. velezensis* UTB96, demonstrating improved lipopeptide production compared to the strain *B. velezensis* FZB42.

In: Microorganisms, Nov. 10; 10(11): 2225
DOI: 10.3390/microorganisms10112225

Akintayo, S. O., Treinen, C., Vahidinasab M., Pfannstiel, J., Bertsche, U., Fadahunsi, I., Oellig, C., Granvogl, M., Henkel, M., Lilge, L. Hausmann, R. (2022).
Exploration of surfactin production by newly isolated *Bacillus* and *Lysinibacillus* strains from food related sources.

In: Letters in Applied Microbiology. April 29; 75(2): 378-387
DOI: 10.1111/lam.13731

Conference talk

Lilge L., Klausmann P., Hoffmann M., Treinen C., Vahidinasab M., Pfannstiel J., Henkel M., Hausmann R. (2022).
Strategies for improved surfactin production using *Bacillus subtilis*.

21st International Conference on Bacilli and Gram-Positive Bacteria.
June 14 - 17, 2022. Prague, Czech Republic.

Vahidinasab M., Hausmann R. (2022).
Developing microbial lipopeptide production to combat plant pathogenic fungi in agriculture.

World Food Day Colloquium 2022.
October 18, 2022. Hohenheim, Germany.

Poster presentations

Vahidinasab M., Lilge L., Akintayo S., Treinen C., Henkel M., Hausmann1 R. (2022). Microbial lipopeptides produced by *Bacillus* strains: a future solution against plant pathogenic fungi.

21st International Conference on Bacilli and Gram-Positive Bacteria.
June 14 - 17, 2022. Prague, Czech Republic.

Vahidinasab M., Lilge L., Treinen C., Akintayo S., Henkel M., Hausmann1 R. (2022). Bioproduction of microbial lipopeptides by *Bacillus subtilis* and *B. velezensis*: a future solution against plant pathogenic fungi.

Biosurfactants International Conference.
September 28 - 30, 2022. Stuttgart, Germany.

Vahidinasab M., Henkel M., Morabbi Heravi K., Lilge L., Hausmann1 R. (2019).
Bacillus velezensis UTB96 as a model for lipopeptide production in bioreactor systems.

Biosurfactants International Conference.
September 25 - 27, 2019. Stuttgart, Germany

Author's contribution statement

The publications included in this thesis have been published previously with the knowledge and approval of the Supervisor Prof. Dr.-Ing. Rudolf Hausmann. All further co-authors read and approved the manuscripts.

1. **Vahidinasab M.**, Ahmadzadeh M., Henkel M., Hausmann R., Morabbi Heravi K. (**2019**). *Bacillus velezensis* UTB96 is an antifungal soil isolate with a reduced genome size compared to that of *Bacillus velezensis* FZB42. Microbiology Resource Announcements. Sep. 19; 8(38): e00667-19.

MV designed, planned, executed the DNA sequence analysis, interpreted the data, and drafted the manuscript. **KM** significantly contributed to analysis of the whole genome sequencing. **MH** supported the interpretation of the results. **MA** and **RH** substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of the manuscript.

2. **Vahidinasab M.**, Lilge L., Reinfurt A., Pfannstiel J., Henkel M., Morabbi Heravi K., Hausmann R. **(2020)**. Construction and description of a constitutive plipastatin mono-producing *Bacillus subtilis*. Microbial Cell Factories. Nov. 10; 19(1): 1-12.

MV designed, planned and executed the experiments, collected data, created the graphs and drafted the manuscript. **AR** was involved in strain engineering and **JP** performed the mass spectrometry analysis. **MH** and **KM** supported the interpretation of results. **LL** significantly contributed to conception and design of the study and interpretation of the experiments. **RH** substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

3. Lilge L., **Vahidinasab M.**, Adiek I., Becker P., Kuppusamy Nesamani C., Treinen C., Hoffmann M., Morabbi Heravi K., Henkel M., Hausmann R. **(2021)** Expression of *degQ* gene and its effect on lipopeptide production as well as formation of secretory proteases in *Bacillus subtilis* strains. MicrobiologyOpen. Oct. 14; 10(5): e1241.

LL and **MV** contributed equally to this work. **MV** designed, planned and executed the experiments, collected and interpreted the data, created the graphs and drafted the manuscript. **LL** significantly contributed in design of experiments. **MV**, **IA**, **PB** and **CK** were involved in strain engineering and performing shake flask cultivations. **MHe, KM, MHo** and **CT** supported the interpretation of results. **RH** substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

4. **Vahidinasab M.**, Adiek I., Hosseini B., Akintayo S. O., Abrishamchi B., Pfannstiel J., Henkel M., Lilge L., Voegele R., Hausmann R. (**2022**). Characterization of *B. velezensis* UTB96, demonstrating improved lipopeptide production and fungicidal properties compared to the Gram-positive model strain *B. velezensis* FZB42. Microorganisms, Nov. 10; 10(11): 2225.

MV designed, planned and executed the experiments, collected and interpreted the data, created the graphs and drafted the manuscript. **LL** significantly contributed to design of experiments. **BH** was involved in performing antifungal tests. **IA** and **BA** were involved in performing shake flask cultivations. **JP** performed the mass spectrometry analysis. **SA** and **MH** and **RV** supported the interpretation of results. **RH** substantially contributed to conception and design of the conducted experiments and manuscript completion. **All** authors read and approved the final manuscript.

Abstract

Microbial lipopeptides are secondary metabolites produced by bacteria and single-celled microorganisms. They are amphiphilic in nature, composed of a cyclic or linear peptide chain linked to a lipid residue. Due to their high-foaming biosurfactant properties, they have various industrial applications such as in detergents, food emulsifiers, bioremediation, and enhanced oil recovery. Additionally, they possess other functional properties such as antifungal activity, making them an environmentally friendly alternative to synthetic fertilizers and fungicides. *Bacillus* species produce cyclic lipopeptides known for their potent antifungal activity, which makes them a potential source of bio-fungicides in agriculture. However, the production titer of wild-type *Bacillus* species does not meet industrial needs. Thereby, genetic modification of producer strains and bioprocess engineering can help increase the production of lipopeptides. Nevertheless, the regulation and basis of biosynthesis for *Bacillus* lipopeptides are still not completely understood, and ongoing research aims to enhance their production. In general, three main lipopeptide families, including surfactins, iturins, and fengycins are produced by different *Bacillus* species. Among these, surfactin as the strong biosurfactant is the most extensively studied lipopeptide produced by *Bacillus* species.

The focus of this doctoral thesis was mainly to evaluate the biosynthesis of iturin and fengycin families, which are strong antimicrobial lipopeptides produced by *Bacillus subtilis* and *Bacillus velezensis*. This involved developing strains through genetic engineering and enhancing the lipopeptide titer by evaluating the cultivation medium. Initially, the entire genome of the bacteria used in this thesis was examined in terms of lipopeptide

biosynthesis, and the structure and yield of the different produced lipopeptides were analyzed. Regarding the lipopeptide producer derivatives of the domesticated laboratory model strain *B. subtilis* 168 and *B. subtilis* 3NA, a spore deficient strain appropriate for bioreactor cultivation, surfactin is the lipopeptide with the highest yield, while plipastatin which is a member of fengycin family, is produced in lower quantities. In the present thesis, the biosynthesis of plipastatin by *B. subtilis* BMV9 as the lipopeptide producer derivative of strain 3NA was evaluated. The study aimed to convert BMV9 to a constitutive plipastatin mono-producer strain. In this sense, overexpressing plipastatin biosynthesis operon using the stronger constitutive P_{veg} promoter led to a five-fold increase in plipastatin production. Interestingly, it was observed that deletion of *srfAA-AD* operon in BMV9 and the constructed constitutive plipastatin producer strain has not improved plipastatin production. Therefore, it can be stated that presumably the biosynthesis of plipastatin may be positively influenced in a post-transcriptional manner by the surfactin synthetase or some of its subunits. However, the regulatory mechanism behind this effect remained unknown and requires further research. Another attempt to enhance the plipastatin biosynthesis in strain BMV9 was repairing the *degQ* expression. One main genome characterization of strains with *B. subtilis* 168 and 3NA background is that the pleiotropic *degQ* gene expression, which is known to have a positive effect on plipastatin biosynthesis, is silenced due to a mutation in the promoter area. However, while repair of *degQ* expression in BMV9 increased the plipastatin production, combination of both repaired *degQ* expression and promoter exchange (P_{pps}::P_{veg}) has not significantly increased the plipastatin yield.

To further evaluate the impact of *degQ* expression on surfactin and plipastatin biosynthesis, two strains of *B. subtilis* were selected: JABs24, a lipopeptide producer derived from the 168 strain, and DSM10^T, the wild-type strain expressing native *degQ*. The findings demonstrated that surfactin biosynthesis is negatively affected by DegQ-associated DegU regulation, while increased plipastatin biosynthesis is achieved in the presence of native *degQ* expression. In addition to production of lipopeptides, the DegU regulatory system also plays a role in the formation of secretory proteases. A comparison of extracellular protease activities between JABs24 and DSM10^T showed that *degQ* expression led to

DSM10^T having five times higher protease activity than JABs24. Interestingly, production of extracellular proteases has not affected the stability of both plipastatin and surfactin during cultivation, suggesting that lipopeptides are less targeted by extracellular proteases. In general, this section of the thesis has provided quantitative evidence of the impact of *degQ* expression on extracellular protease production and lipopeptide biosynthesis in *B. subtilis*. The results highlight the distinct mechanisms by which DegQ regulates surfactin and plipastatin biosynthesis.

The identification of proficient wild-type strains is critical to the advancement of bio-fungicide in agriculture. Therefore, the subsequent approach of this thesis centered on the production of microbial lipopeptide by wild-type *B. velezensis* strains. Here, the lipopeptide productivity and antifungal ability of *B. velezensis* UTB96 was higher than *B. velezensis* FZB42, as a well-established strain for biocontrol of plant pathogens in agriculture. Furthermore, addition of certain amino acids stimulated lipopeptide production, and using a bioreactor system resulted in enhancement of lipopeptide production, especially iturin A by UTB96. These results suggest that *B. velezensis* UTB96 is a promising candidate for further research to be used as an antifungal agent in agriculture.

Overall, the doctoral thesis evaluates the biosynthesis of antimicrobial lipopeptides produced by *B. subtilis* and *B. velezensis*. The study involves genetic engineering such as promoter exchange, deletion of genes involved in competing biosynthetic pathways and cultivation medium development with amino acid supplementation to enhance the lipopeptide titer. The thesis also identifies *B. velezensis* UTB96 as a promising candidate for further research to be used as a wild-type antifungal agent in agriculture.

Zusammenfassung

Mikrobielle Lipopeptide sind Sekundärmetabolite, die von Bakterien und einzelligen Mikroorganismen produziert werden. Sie sind von Natur aus amphiphil und bestehen aus einer zyklischen oder linearen Peptidkette, die an einen Lipidrest gebunden ist. Aufgrund der stark schäumenden Eigenschaften von Biotensiden, werden diese in der Industrie in verschiedenen Bereichen eingesetzt, wie z. B. in Reinigungsmitteln, Lebensmittelemulgatoren, bei der Bioremediation und der verbesserten Ölgewinnung. Darüber hinaus besitzen sie weitere funktionelle Eigenschaften, wie z. B. eine antimykotische Wirkung, was sie zu einer sicheren und umweltfreundlichen Alternative zu synthetischen Düngemitteln und Pestiziden macht. *Bacillus*-Arten produzieren zyklische Lipopeptide, die für ihre starke antimykotische Wirkung gegen Phytopathogene bekannt sind, was sie zu einer potenziellen Quelle für Bio-Fungizide in der Landwirtschaft macht. Der Produktionstiter von Wildtyp-*Bacillus*-Arten entspricht jedoch nicht dem industriellen Bedarf. Durch genetische Veränderung und Bioverfahrenstechnik kann die Produktion von Lipopeptiden gesteigert werden. Dennoch sind die Regulierung und die Grundlagen der Biosynthese von *Bacillus*-Lipopeptiden noch nicht vollständig geklärt, und die laufende Forschung zielt auf die Optimierung ihrer Produktion ab. Es ist bekannt, dass *Bacillus*-Arten drei Hauptlipopeptidfamilien produzieren, darunter Surfactin, Iturin und Fengycin. Unter diesen ist Surfactin das am besten untersuchte Biotensid.

Im Mittelpunkt dieser Doktorarbeit stand die Untersuchung der Biosynthese der Iturin- und Fengycin-Familien. Diese stark antimikrobiellen Lipopeptide werden von *Bacillus subtilis* und *Bacillus velezensis* produziert. Dazu wurden gentechnisch modifizierte

Stämme entwickelt und der Lipopeptid-Titer durch Verbesserung des Kultivierungsmediums erhöht. Zunächst wurde das gesamte Genom der in dieser Arbeit verwendeten Bakterien im Hinblick auf die Lipopeptidbiosynthese untersucht, und die Struktur und Ausbeute der verschiedenen produzierten Lipopeptide wurden analysiert. Bei den lipopeptidproduzierenden Derivaten des domestizierten Labormodellstammes *B. subtilis* 168 und *B. subtilis* 3NA, einem für die Bioreaktorkultivierung geeigneten nicht sporulierenden Stamm, ist Surfactin das Lipopeptid mit der höchsten Ausbeute. Im Gegensatz dazu wird Plipastatin, ein Mitglied der Fengycinfamilie, in geringeren Mengen produziert. In der vorliegenden Arbeit wurde die Biosynthese von Plipastatin durch *B. subtilis* BMV9, dem Lipopeptidproduzenten-Derivat des Stammes 3NA, untersucht. Ziel der Studie war die Umwandlung von BMV9 in einen konstitutiven Plipastatin-Monoproduzentenstamm. In diesem Sinne führte die Überexpression des Plipastatin-Biosynthese-Operons unter Verwendung des stärkeren konstitutiven P_{veg}-Promotors zu einer fünffachen Steigerung der Plipastatinproduktion. Zusätzlich wurde festgestellt, dass die Deletion des *srfAA-AD*-Operons in BMV9 und dem konstruierten konstitutiven Plipastatin-Produzentenstamm die Plipastatin-Produktion nicht verbessert hat. Daraus lässt sich schließen, dass die Biosynthese von Plipastatin vermutlich posttranskriptional durch die Surfactin-Synthetase oder einige ihrer Untereinheiten positiv beeinflusst werden kann. Der Regulationsmechanismus, der diesem Effekt zugrunde liegt, ist jedoch noch unerforscht und bedarf weiterer Forschung. Ein weiterer Versuch, die Plipastatin-Biosynthese im Stamm BMV9 zu verbessern, war die Reparatur der *degQ*-Expression. Eine wesentliche Genomcharakterisierung von Stämmen mit *B. subtilis* 168 und 3NA-Hintergrund besteht darin, dass die pleiotrope *degQ*-Genexpression, von der bekannt ist, dass sie sich positiv auf die Plipastatinbiosynthese auswirkt, aufgrund einer Mutation im Bereich des Promotors inaktiv ist. Während jedoch die Reparatur der *degQ*-Expression in BMV9 die Plipastatinproduktion erhöhte, konnte die Kombination aus reparierter *degQ*-Expression und Promotoraustausch (P_{pps}::P_{veg}) die Plipastatinausbeute nicht signifikant steigern.

Um die Auswirkungen der *degQ*-Expression auf die Surfactin- und Plipastatin-Biosynthese weiter zu untersuchen, wurden zwei *B. subtilis*-Stämme ausgewählt: JABs24, ein von Stamm 168 abgeleiteter Lipopeptidproduzent, und DSM10T, der Wildtyp-Stamm, der natives *degQ* exprimiert. Die Ergebnisse zeigten, dass die Surfactin-Biosynthese durch die DegQ-assoziierte DegU-Regulierung negativ beeinflusst wird, während die Plipastatin-Biosynthese bei Vorhandensein einer nativen DegQ-Expression gesteigert wird. Neben der Produktion von Lipopeptiden spielt das DegU-Regulationssystem ebenfalls eine Rolle bei der Bildung von sekretorischen Proteasen. Ein Vergleich der extrazellulären Proteaseaktivitäten zwischen JABs24 und DSM10T zeigte, dass die *degQ*-Expression bei DSM10^{T} zu einer fünfmal höheren Proteaseaktivität führte als bei JABs24. Interessanterweise hat die Produktion von extrazellulären Proteasen die Stabilität von Plipastatin und Surfactin während der Kultivierung nicht beeinträchtigt, was darauf hindeutet, dass Lipopeptide kaum von extrazellulären Proteasen angegriffen werden. Im Allgemeinen hat dieser Abschnitt der Arbeit quantitative Daten für die Auswirkungen der *degQ*-Expression auf die extrazelluläre Proteaseproduktion und die Lipopeptidbiosynthese in *B. subtilis* geliefert. Die Ergebnisse unterstreichen die unterschiedlichen Mechanismen, durch die DegQ die Surfactin- und Plipastatin-Biosynthese reguliert.

Die Identifizierung tauglicher Wildtyp-Stämme ist für die Weiterentwicklung von Biofungiziden in der Landwirtschaft von entscheidender Bedeutung. Daher konzentrierte sich der weitere Ansatz dieser Arbeit auf die Produktion von mikrobiellen Lipopeptiden durch Wildtyp-*B. velezensis*-Stämme. Dabei waren die Lipopeptidproduktivität und die antimykotische Fähigkeit von *B. velezensis* UTB96 höher als die von *B. velezensis* FZB42, einem etablierten Stamm zur Biokontrolle von Pflanzenpathogenen in der Landwirtschaft. Außerdem stimulierte die Zugabe bestimmter Aminosäuren die Lipopeptidproduktion, und die Verwendung eines Bioreaktorsystems führte zu einer Steigerung der Lipopeptidproduktion, insbesondere von Iturin A durch UTB96. Diese Ergebnisse deuten darauf hin, dass *B. velezensis* UTB96 ein vielversprechender Kandidat für weitere Forschungen ist, um als Antipilzmittel in der Landwirtschaft eingesetzt zu werden.

Zusammenfassend wird in der Dissertation die Biosynthese antimikrobieller Lipopeptide untersucht, die von *B. subtilis* und *B. velezensis* produziert werden. Die Studie umfasst gentechnische Eingriffe wie den Austausch von Promotoren, die Deletion von Genen, die an konkurrierenden Biosynthesewegen beteiligt sind, und die Entwicklung von Kultivierungsmedien mit Aminosäuresupplementierung zur Steigerung des Lipopeptid-Titers. In der Dissertation wird *B. velezensis* UTB96 als vielversprechender Kandidat für weitere Forschungen identifiziert, um als Wildtyp-Antimykotikum in der Landwirtschaft eingesetzt zu werden.

Table of contents

Part I
Introduction

1.1 Microbial lipopeptides

Microbial lipopeptides are secondary metabolites synthesized by various bacteria and single-celled microorganisms (Desai and Banat 1997). They are known as high-foaming biosurfactants with emulsifying properties, which lend to application in many industries, including cleaning products and detergents, food emulsifiers, bioremediation and enhanced oil recovery (Banat et al. 2010; Pacwa-Płociniczak et al. 2011). Nevertheless, the properties of microbial lipopeptides are not limited to their biosurfactant activity. During the last years, some of these compounds have attracted interest because of their functional properties like ability to interact with fungi and break the cell walls (Raaijmakers et al. 2010; Singh and Cameotra 2004). Moreover, microbial lipopeptides are biodegradable, safe and may help preserve the environment by reducing the need for agrochemicals and non-renewable energy. Therefore, they can be exploited as bio-fungicides for agricultural applications (Pérez-García et al. 2011; Sachdev and Cameotra 2013).

The discovery of new microbial lipopeptides has increased over time. Currently, more than 40 lipopeptide families have been identified in bacteria, which are listed in Table 1. This information is derived from a search of the annotation "lipopeptide" in NORINE database (Caboche et al. 2007; Flissi et al. 2020). Several Gram-negative bacteria can produce a variety of lipopeptides, most of which have antimicrobial activity (Ghequire and Mot 2014). In particular, these include 14 distinct families from *Pseudomonas* species that are recently classified by Geudens and Martins (2018) and lipopeptide families from *Burkholderia* (Thongkongkaew et al. 2018), *Halomonas* (Martinez et al. 2003), *Marinobacter* (Xu et al. 2002), *Janthinobacterium* (Schlosser et al. 2021) and *Serratia* species (Clements et al. 2019). *Pseudomonas* lipopeptides exhibit a variety of biological properties, such as antifungal (Jourdan et al. 2003), antibacterial (Gerard et al. 1997), antiviral (Groupé et al. 1951) and even insecticidal properties (Jang et al. 2013), in addition to their biosurfactant properties. A summary of further lipopeptides produced by Gram-negative bacteria is given by Xue et al. (2018).

Gram-positive bacteria that produce lipopeptides mostly belong to *Bacillales* and *Actinobacteria* (Aleti et al. 2015; Zhao et al. 2018a). *Actinobacteria* are a phylum of bacteria with high guanine and cytosine content in their DNA and include one of the largest bacterial genera, Streptomyces (Yadav et al. 2018). Daptomycin is the most studied lipopeptide produced by *Streptomyces* species and is used extensively in the field of medicine due to the antibacterial effect against human gram-positive bacteria (Fowler Jr et al. 2006).

Bacillus species are the major lipopeptide producers from the order *Bacillales* and can produce more significant quantities of lipopeptides with stronger biological properties, such as antifungal activity, compared to other lipopeptide-producing bacteria (Coutte et al. 2017). For example, while the lipopeptides produced by *Pseudomonas* or *Streptomyces* species are no more than a few milligrams per liter, some wild-type *Bacillus* strains can produce about a gram of lipopeptides (Liu et al. 2022; Yu et al. 2011). This is, of course, still far from a suitable application volume concentration as a bio-fungicide in agriculture, but there is room for improvement. Therefore, the number of studies on the optimization of *Bacillus* lipopeptides is increasing rapidly. Although, up to date, neither the regulation nor the basis biosynthesis condition of *Bacillus* lipopeptides are sufficiently known (Banat et al. 2010).

Table 1: Overview of lipopeptide families and their main activities produced by bacteria.

Bacteria	Lipopeptide families	Main activities	Reference
Gram-negative			
Pseudomonas	Orfamide, amphisin, viscosin, tolaasin, corrugatin, entolysin putisolvin, bananamide, pseudofactin, xantholysin, fuscopeptin, syringopeptin, syringafactin, syringomycin	Biosurfactant, antimicrobial	(Geudens and Martins 2018)
Burkholderia	Burriogladin, haereogladin, holrhizin		(Chen et al. 2020)
Halomonas	Aquachelin	Siderophore, biosurfactant	(Martinez et al. 2003)
Janthinobacterium	Jagaricin	Antimicrobial	(Graupner et al. 2012)
Marinobacter	Marinobactin	Siderophore, biosurfactant	(Xu et al. 2002)
Serratia	Serrawettin	Biosurfactant	(Clements et al. 2019)
Gram-positive			
Streptomyces	Daptomycin, friulimicin, Calcium dependent antibiotic, A54145, enduracidin	Antimicrobial	(Zhang et al. 2023)
Actinoplanes	Friulimicin, ramoplanin	Antimicrobial	(Muller et al. 2007)
Arthrobacter	Amphisin	Biosurfactant, antimicrobial	(Morikawa et al. 1993)
Corynebacterium	Coryxin	Antimicrobial	(Dalili et al. 2015)
Bacillus	Surfactin, iturin, fengycin, polymyxin, kurstakin, locillomycin	Biosurfactant, antimicrobial	(Geissler et al. 2019)
Paenibacillus	Polymyxin	Biosurfactant, antimicrobial	(Shaheen et al. 2011)
Aneurinibacillus	Aneurinifactin	Biosurfactant, antimicrobial	(Balan et al. 2017)
Staphylococcus	Unnamed lipopeptide	Biosurfactant, antimicrobial	(Eddouaoud a et al. 2012)
Rhodococcus	Rhodofactin	Biosurfactant	(Peng et al. 2008)

1.2 Current perspective of utilization of microbial lipopeptides in sustainable agriculture

Agriculture is considered the most important modality of natural resource management for sustaining human life. For thousands of years, the goal of agriculture has been to produce enough food and feed to fulfill society's needs (Velten et al. 2015). In recent decades, great emphasis has been placed on sustainable agriculture to preserve the environment and the natural resources of the earth. Nowadays it is apparent to everyone that depending on several factors such as the dose and duration of exposure, some of the common compounds used to control plant disease may persist in soil and water and therefore have long-term toxic effects on the environment. Moreover, since they are often non-biodegradable, they threaten our planet's health (Ferraro et al. 2020).

In view of the growing population and the impact of climate change on food security, the demand for biodegradable products is rising all over the world. This high demand underscores the need to replace applications of chemical fertilizers and pesticides with the use of environmentally friendly alternatives to improve the nutrient content of plants and their protection against pathogens and pests (Anderson et al. 2016; Ervin et al. 2010). The utilization of biological fungicide produced by different microorganisms may potentially represent a safe and environmentally friendly method for crop protection in farming. (Bhargava et al. 2017; Hader et al. 2022).

In recent years, biotechnology has contributed to sustainable agriculture in various ways. For instance, the genome of plants, animals, or microorganisms has been modified by scientists to make them resistant to certain pathogens, pests, or weeds, or even to tolerate adverse environmental conditions such as high temperatures (Stone 2010). However, there is a comprehensive framework for the authorization of the use of genetically modified organisms (GMOs) as pesticides in the European Union (EU). Indeed, there are currently no GMOs approved for use as pesticides in the EU. In fact, the use of GMOs in agriculture

in the EU is highly restricted (Friedrichs et al. 2019). Moreover, scientists and environmental groups have identified a potential threat to human health associated with GMOs in agriculture (Paparini and Romano-Spica 2004). Thus, the use of beneficial microorganisms at different stages of cultivation, such as seed preparation to postharvest disease control, could greatly benefit the crop. However, it is highly unlikely that the concentration of antimicrobial lipopeptides produced by microorganisms in their natural environment will be sufficient for antibiosis (Ongena and Jacques 2008). As an alternative to the use of live microorganisms, biotechnology can help to produce antimicrobial lipopeptides on a larger scale and prepare the purified lipopeptides for application (Foley et al. 2005; Hua et al. 2022). Whether these purified lipopeptides can be approved as pesticides in the EU requires further clarification.

In this dissertation, the lipopeptide compounds with antifungal properties produced by the beneficial *Bacillus* species have been studied and the methods for their biosynthesis process has been evaluated and improved. The development of methods to improve the bioproduction of antifungal lipopeptides may potentially increase the use of lipopeptides as bio-fungicides instead of non-biodegradable fungicides. Such a change could contribute to a path towards greater sustainability in agriculture.

1.3 Biosynthesis of microbial lipopeptides of *Bacillus* species

Lipopeptides are not synthesized ribosomally, but rather by stepwise reactions initiated by complex non-ribosomal peptide synthetases (NRPSs) or by non-ribosomal polyketide peptide hybrid synthetases (PKS - NRPSs) (Roongsawang et al. 2010). Activation of NRPS depends on the 4-phosphopantetheinyl transferase Sfp which converts the apoforms of lipopeptide synthases to the active holoforms (Lambalot 1996). The holoenzyme NRPSs are huge enzymes composed of several subunits. Each subunit consists of multi-domain modules catalyzing the binding of amino acids together for the elongation of the peptide chain. The total number of modules equals the number of amino acids in the peptide ring. Specifically, a module is a part of the NRPS that incorporates a certain amino acid into a peptide scaffold. The three main domains of each module are adenylation domain (A), peptidyl-carrier protein domain (PCP), also known as the thiolation domain (T); and condensation domain (C). After the amino acids are recognized and activated by A domain, PCP domain binds amino acid residues to peptide chain in the direction of the template of lipopeptide (Linne and Marahiel 2000). The C domain is a condensation domain which has an acceptor and donor site and is responsible for transferring the peptide between different modules. The C starter domain at the beginning of initiation module is selective to linkage of a CoA-activated 3-hydroxy or 3-amino fatty acid chain via its acceptor site. Moreover, additional domains such as the epimerization domain (E) can be present in the domain lines of an NRPS that are responsible for catalyzing the modification of the D- or L- configuration of amino acids. The final step is the release of lipopeptide and occurs after several peptide extensions by the terminal thioesterase domain (TE). TE is the last domain of the elongation modules and often performs internal cyclization of the oligopeptide chain upon release of the lipopeptide. In lipopeptides belonging to the iturin family, domains related to polyketide synthesase are present upstream of the first module of NRPS. These domains are associated with the elongation of the fatty acid chain in the final steps before transfer to the first amino acid of the peptide unit (Ongena and Jacques 2008).

The multienzyme complexes of NRPS and PKS are formed by large operons consisting of three or more open reading frames (ORFs) (Samel et al., 2006). As shown in Fig. 1 a and c, surfactin and fengycin NRPS are encoded by the *srfAA-AD* (26,073 bp) and *fenA-E* (37,578 bp) operons, respectively (Jacques 2011). In addition, the detailed domain lineages of the PKS-NRPS hybrid complex encoded by *ituA-D* (37,095 bp) responsible for producing iturin A as a member of the iturin family is depicted in Fig. 1b.

Beside the NRPS and PKSs genes, there are regulatory genes that affect the production of lipopeptide in a direct or indirect way. The following sections provide a deeper insight into the structure, regulation of biosynthesis, and potential applications of lipopeptides belonging to the surfactin, iturin, and fengycin families. The knowledge gained through a better understanding of genetic regulation could be used to construct high-yielding recombinant strains to be used in industrial applications.

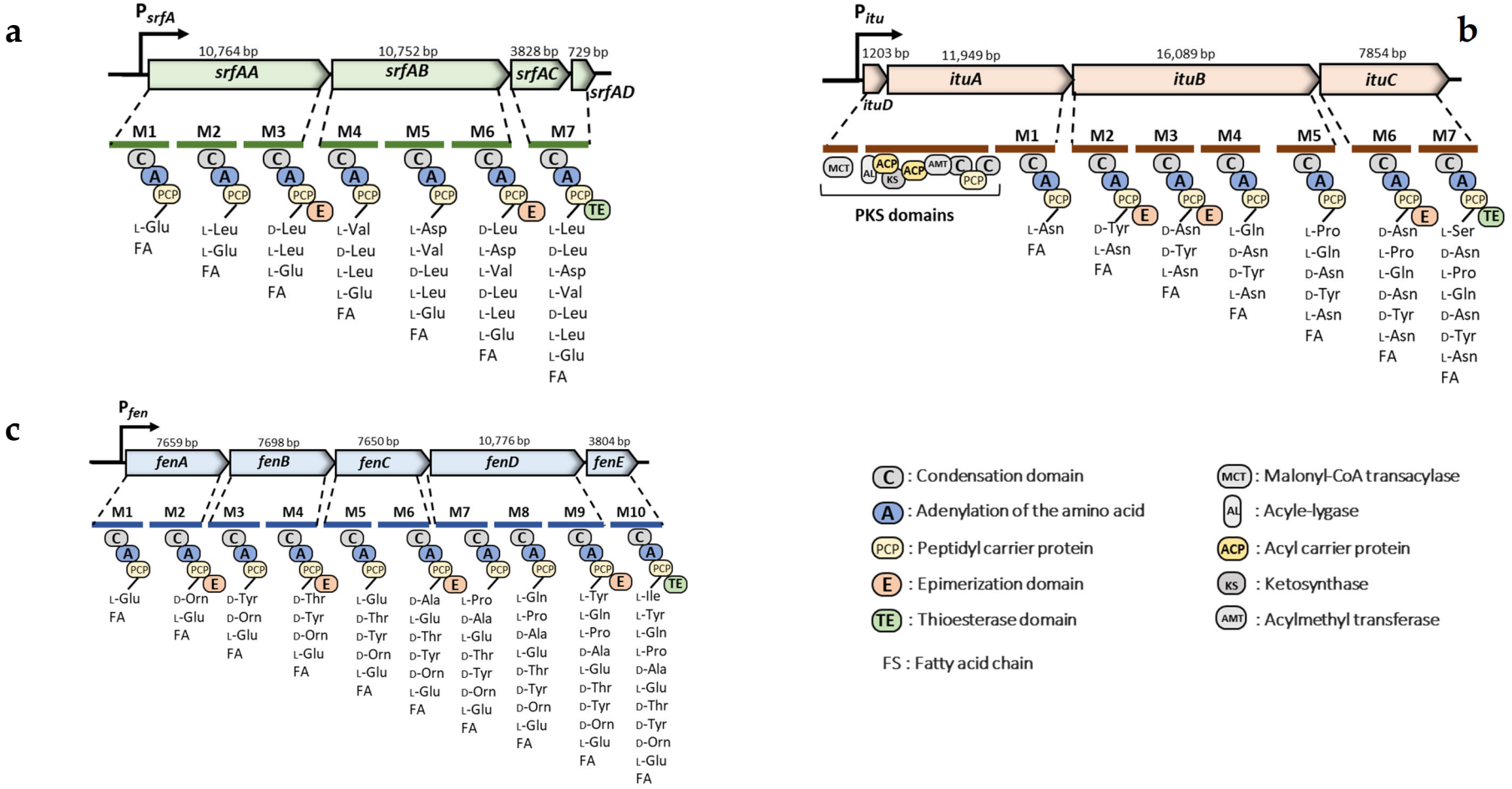


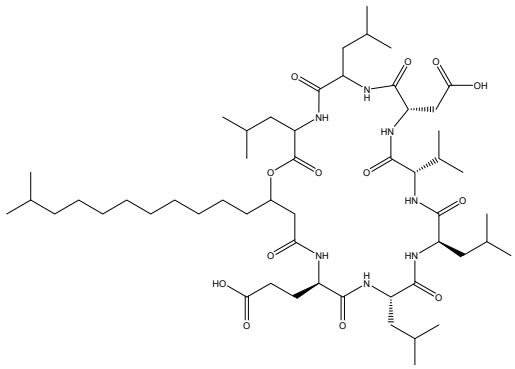
Figure 1: Genomic organization and modulation of the NRPSs producing surfactin, iturin A and fengycin in *Bacillus velezensis*; Surfactin operon contains of three ORFs of *srfAA*, *srfAB*, *srfAC* and *srfAD* (26,073 bp) (a), Iturin A contains four ORFs of *ituD*, *ituA*, *ituB* and *ituC* (37,095 bp) (b), fengycin operon contains four ORFs of *fenA*, *fenB*, *fenC* and *fenD* (37,576 bp) (c).

1.4 Main lipopeptide families of *Bacillus* species

1.4.1 Surfactin family

Lipopeptides belonging to the surfactin family are considered promising biosurfactants due to their exceptionally strong surface activity (Jacques 2011). Since the first discovery of surfactin in 1968, surfactin is the most studied lipopeptide produced by *Bacillus* species (Arima et al. 1968). All members of surfactin family consist of a heptapeptide ring connected to a 3-hydroxy-13-methyltetradecanoic fatty acid chain that could be linear, iso or anteiso branches with 12 to 16 carbons (Fig. 2, a) (Ongena and Jacques 2008). Up to now, over 40 variants of surfactin has been identified (Geissler et al. 2019). Important members of the surfactin family are surfactin, lichenysin, and pumilacidin (Fig. 2, b). Lichenysin is a natural analog of surfactin produced by *B. licheniformis* and pumilacidin is produced by *B. pumilus.*

a



b

	1	2	3	4	5	6	7
Surfactin	^{L}Glu –	$^{L}\mathbf{XS_2}$ –	^{D}Leu –	$^{L}\mathbf{XS_4}$ –	^{L}Asp –	^{D}Leu –	$^{L}\mathbf{XS_7}$
Lichenycin	$^{L}\mathbf{XL_1}$ –	$^{L}\mathbf{XL_2}$ –	^{D}Leu –	$^{L}\mathbf{XL_4}$ –	^{L}Asp –	^{D}Leu –	$^{L}\mathbf{XL_7}$
Pumilacidin	^{L}Glu –	^{L}**Leu** –	^{D}Leu –	^{L}**Leu** –	^{L}Asp –	^{D}Leu –	$^{L}\mathbf{XP_7}$

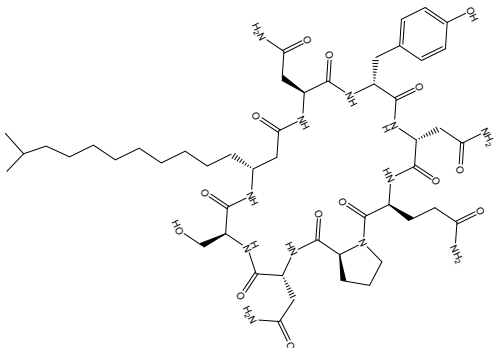
XS_2: Val, Leu or Ile, **XS_4:** Ala, Val, Leu or Ile, **XS_7:** Val, Leu or Ile
XL_1: Gln or Glu, **XL_2:** Leu or Ile, **XL_4** and **XL_7:** Val or Ile
XP_7: Val or Ile

Figure 2: Chemical structure of surfactin (a); amino acid sequence of peptide moiety in different member of surfactin family (b).

1.4.2 Iturin family

Iturins are potent bioactive lipopeptide derived from several *Bacillus* strains (Jacques 2011). Mycosubtilin is a member of iturin family and is the first identified iturin compound found in 1949 (Walton and Woodruff 1949). A year later in 1950 a similar compound was found from a soil sample in a region called Ituri in Congo (Delcambe 1950) and was named iturin. Iturin and mycosubtilin were first identified as strong antifungal metabolites and later other properties of them were discovered in several studies. All iturins consist of a cyclic peptide of 7 amino acids linked to a mixture of 3-amino-12-methyltridecanoic acid fatty acid often of iso- or anteiso-type of variable length from 14 to 17 carbons (Maget-Dana and Peypoux 1994; Peypoux et al. 1978) (Fig. 3, a). Differences in amino acid and fatty acid residue lead to various homologs of iturins. There are six main members in iturin family; Iturin A and C have a common amino acid sequence and different fatty acid residue. Other members are bacillomycin D, F, L and mycosubtilin. The peptide chain of the iturin lipopeptides is shown in Fig. 3, b (Dunlap et al. 2019).

a



b

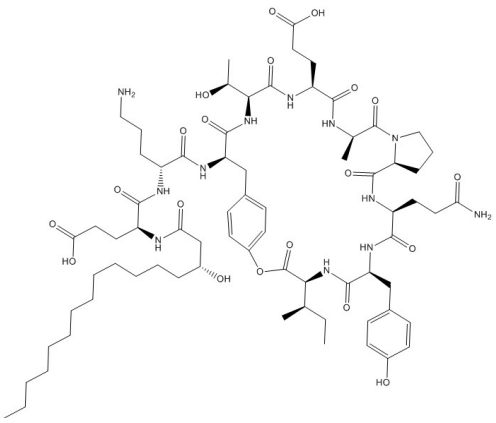
	1	2	3	4	5	6	7
Iturin A, C	^{L}Asn	^{D}Tyr	^{D}Asn	^{L}Gln	^{L}Pro	^{D}Asn	^{L}Ser
Mycosubtilin	^{L}Asn	^{D}Tyr	^{D}Asn	^{L}Gln	^{L}Pro	^{D}Ser	^{L}Asn
Bacillomycin L	^{L}Asn	^{D}Tyr	^{D}Asn	^{L}Ser	^{L}Glu	^{D}Ser	^{L}Thr
Bacillomycin D	^{L}Asn	^{D}Tyr	^{D}Asn	^{L}Pro	^{L}Glu	^{D}Ser	^{L}Thr
Bacillomycin F	^{L}Asn	^{D}Tyr	^{D}Asn	^{L}Gln	^{L}Pro	^{D}Asn	^{L}Thr
Mojavensin	^{L}Asn	^{D}Tyr	^{D}Asn	^{L}Gln	^{L}Pro	^{D}Asn	^{L}Asn

Figure 3. Chemical structure of Iturin A (a); amino acid sequence of peptide moiety in different member of iturin family (b).

1.4.3 Fengycin family

Fengycin was first identified simultaneously by two different German and Japanese researchers in 1986. The structures of fengycin and plipastatin are almost identical and both consist of a decapeptide chain, 8 amino acids of which form a peptide ring. The peptide chain is linked to a 3-hydroxyl fatty acid that may be saturated or unsaturated with 12 to 18 carbon atoms (Wu et al. 2007) (Fig. 4, a). In plipastatin, the enantiomers of the amino acids in positions 3 and 9 (Tyr3 and Tyr9) are in the L and D form, respectively; while in fengycin, they are in the reverse configuration (Fig. 4, b) (Inès and Dhouha 2015). Since fengycin and plipastatin belong to the same conger family, some authors do not distinguish these lipopeptides, although the enantiomers of the amino acids in position 3 and 9 are different. (Honma et al. 2012; Stein 2005).

a



b

	1	2	3	4	5	6	7	8	9	10
Fengycin A	^{L}Glu	^{D}Orn	^{D}Tyr	^{D}Thr	^{L}Glu	^{D}**Ala**	^{L}Pro	^{L}Gln	^{L}**Tyr**	^{L}Ile
Fengycin B	^{L}Glu	^{D}Orn	^{D}Tyr	^{D}Thr	^{L}Glu	^{D}**Val**	^{L}Pro	^{L}Gln	^{L}**Tyr**	^{L}Ile
Fengycin C	^{L}Glu	^{D}Orn	^{D}Tyr	^{D}Thr	^{L}Glu	^{D}**Val**	^{L}Pro	^{L}Gln	^{L}**Thr**	^{L}Ile
Fengycin S	^{L}Glu	^{D}Orn	^{D}Tyr	^{D}**Ser**	^{L}Glu	^{D}**Val**	^{L}Pro	^{L}Gln	^{L}**Thr**	^{L}Ile
Plipastatin A	^{L}Glu	^{D}Orn	^{L}**Tyr**	^{D}Thr	^{L}Glu	^{D}**Ala**	^{L}Pro	^{L}Gln	^{D}**Tyr**	^{L}Ile
Plipastatin B	^{L}Glu	^{D}Orn	^{L}**Tyr**	^{D}Thr	^{L}Glu	^{D}**Val**	^{L}Pro	^{L}Gln	^{D}**Tyr**	^{L}Ile

Figure 4. Chemical structure of fengycin (a); amino acid sequence of peptide moiety in different member of fengycin family (b).

1.5 Intricate regulation of lipopeptide biosynthesis

The biosynthesis of lipopeptides produced by *Bacillus* strains is regulated by complex regulatory networks in a growth-phase dependent manner (Raaijmakers et al. 2010). In the lipopeptide biosynthesis process, in addition to the NRPS operon and the *sfp* gene (Reuter et al. 1999), quorum sensing (QS) systems and global regulators such as Spo0A, AbrB and DegU play essential roles (López and Kolter 2010). The following is a summary of the known regulation mechanism of surfactin, iturin, and fengycin biosynthesis.

1.5.1 Regulation of surfactin

Among lipopeptides, regulation of surfactin operon has been studied relatively well at molecular level. Recently, the molecular interactions including all the identified activator and repressors proteins related to surfactin biosynthesis has been summarized (Rahman et al. 2021). In general, ComA, PerR and PhoP are positive regulator of *srfA* operon; In contrast, transcriptional pleiotropic regulators CodY and Spx and AbrB are the main repressor of *srfA* operon (Kobayashi 2019; Miras and Dubnau 2016).

During the exponential growth, the transition state regulator AbrB and its homolog Abh repress the expression of surfactin operon (Chumsakul et al. 2011; Klausmann et al. 2021). Along with increasing bacterial cell densities and during the transition from exponential to stationary growth, phosphorylated ComA in cytoplasm activates the expression of *srfA* operon by binding to the P_{srfA} (Nakano and Zuber 1991). ComA is a response regulator protein in the two component ComA-ComP system. Upon accumulation of a short autoinducer pheromone called ComX from out of the cell, ComP, as a membrane histidine-kinase protein, is autophosphorylated and later phosphorylates ComA in the cytoplasm. (Magnuson et al. 1994). For the modification and transport of ComX from inside the cell to the extracellular area, ComQ is required (Ansaldi et al. 2002). Hence, the phosphorylated form of ComA regulates the expression of surfactin operon and activates the expression of

many other genes (Comella and Grossman 2005; Hamoen et al. 2003; López and Kolter 2010) through promoting the expression of pleiotropic regulator *degQ* (Msadek et al. 1991; Spacapan et al. 2018). DegQ is a member of DegU regulon. Similar to ComA, DegU is a response regulator in the two component DegS-DegU system. The membrane histidine-kinase DegS, phosphorylates the DegU (Dahl et al. 1991). The function of DegQ in this process is contribution in the autophosphorylation of DegS and phosphorylation of DegU (Kobayashi 2007). The phosphorylated DegU regulates not only the expression of various genes responsible for the production and secretion of extracellular proteases and several other secreted enzymes, (Msadek et al. 1991; Murray et al. 2009), but also the biosynthesis of lipopeptides including surfactin are affected (Miras and Dubnau 2016; Tsuge et al. 1999). In case of surfactin biosynthesis, there are some evidence that the phosphorylated form of DegU has an inhibitory effect on P_{srfA} (Miras and Dubnau 2016). In addition, as mentioned above, CodY and Spx are known direct repressors of P_{srfA} (Nakano et al. 2003; Serror and Sonenshein 1996). Through an indirect pathway, the phosphatase regulator, Phr proteins and their cognate response regulator aspartyl phosphatases Rap proteins, also repress the expression of *srfA* operon by causing dephosphorylation of ComA (Shank and Kolter 2011). A recent review by Kalamara et al. (2018) has been summarized the actions of multiple Rap-Phr systems and their physiological function regulated in *Bacillus subtilis*.

1.5.2 Regulation of iturin

Lipopeptides belonging to iturin and fengycin families are generated during stationary phase and its under direct or indirect influence of main regulatory system such as Qs system (Raaijmakers et al. 2010). Before the bacteria enter the stationary phase, AbrB inhibits the expression of the *srfA* operon, as well as the expression of at least forty other genes, including *itu* and *fen* operons (Chumsakul et al. 2011; Phillips and Strauch 2002; Xu et al. 2020). ComA-ComP two-component signal transduction system, is the main QS system in *Bacillus* specious (Kalamara et al. 2018). Therefore, it is not surprising that this important regulatory system, controls expression of a variety of genes including biosynthesis of secondary metabolites (Duitman et al. 2007). Among the lipopeptides belonging to the

iturin family, bacillomycin D regulation has been studied more than other members. For instance, it has been shown that the promoter of bacillomycin D (P_{bam}) in *B. velezensis* FZB42, previously known as *B. amyloliquefaciens* FZB42, is positively regulated by the direct action of phosphorylated DegU and the indirect regulatory effects of DegQ and ComA. In other words, phosphorylated DegU can bind to the promoter region of P_{bam} and activate it; Whereas, ComA by activating *degQ* expression is indirectly involved in the regulation of bacillomycin D biosynthesis (Koumoutsi et al. 2007). Moreover, in another study, it has been shown that overexpression of *spo0A*, enhanced the bacillomycin D production in *B. amyloliquefaciens* fmbJ (Sun et al. 2021).

1.5.3 Regulation of fengycin

There is limited information on the regulation of fengycin lipopeptide biosynthesis, although it has been proven that the expression of the *pps* operon in *B. subtilis* 168 is repressed by AbrB during the exponential phase (Chumsakul et al. 2011), whereas the expression of *degQ* has a positive effect on the synthesis of plipastatin (Tsuge et al. 2007), indicating the possible involvement of the DegS-DegU two-component system (Chumsakul et al. 2011; Phillips and Strauch 2002).

1.6 Biological activity and related application potential in agriculture and food industry

Lipopeptides produced by the *Bacillus* species have biological properties due to their unique structure (Ongena and Jacques 2008). In nature, lipopeptides produced by bacteria have beneficial effects for the producing bacteria. Three of the most important of these positive effects are antagonistic properties against different pathogens, help in bacterial mobility, and help in biofilm formation (Mahapatra et al. 2022; Raaijmakers et al. 2010). To date, numerous studies have been conducted on the biological properties of lipopeptides. The antibacterial, antiviral and especially antifungal properties of lipopeptides have been reported several times (Chowdhury et al. 2015; Huang et al. 2006; Lilge et al. 2022; Yin et al. 2013). Members of the iturin family are the most potent lipopeptides with antifungal properties. Iturin acts on a broad spectrum of plant pathogenic fungi in a variety of plant hosts (Cawoy et al. 2015; Zhao et al. 2017). This is followed by fengycin family, with antifungal properties mostly limited to filamentous fungi (Koumoutsi et al. 2004). Surfactin alone, on the other hand, has no antifungal activity, but when produced together with iturin or fengycin, it can enhance antifungal activity (Théatre et al. 2021). Iturin and fengycin have direct antagonistic activity and can destroy the fungal cell membrane through a variety of mechanisms (Zhao et al. 2017). To study the bioactivity of individual lipopeptides in several studies, a collection of deletion mutants with different combinations of lipopeptide production have been constructed. For instance, (Romero et al. 2007) have constructed strains with different combinations of lipopeptide production in order to evaluate the biocontrol ability of the different mutants against growth of *Podosphaera fusca* causes powdery mildew disease in cucurbits. They have figured out that iturin and fengycin have a major role in the antagonism of *B. subtilis* toward *P. fusca*. Their derivative mutants deficient in bacillomycin D or iturin A, lost completely their ability to suppress powdery mildew. Due to such a vast array of biological activities of lipopeptides, they have the potential to been applied in agriculture and food industry as bio-fungicide. A bio-fungicide

is made from natural materials, such as plant extracts, bacteria, fungi, or other microorganisms that can either directly attack the fungus or stimulate the plant's natural defenses against it. Unlike synthetic fungicide, which may have negative impacts on the environment and human health, bio-fungicides are generally considered to be safer and more sustainable alternatives. They may also have additional benefits, such as promoting plant growth and enhancing soil health (Kiss 2003). Accordingly, iturin and fengycin can be exploited as bio-fungicides for agricultural use and by reducing the level of non-biodegradable synthetic chemical needs, maintain environmental health and reduces non-renewable energy. Furthermore, these antimicrobial lipopeptides have a great potential to be used as a source of functional food additive in order to enhance the abundance of probiotic foods or to control the yeast contamination in food and drinks (Shi et al. 2018). Moreover, lipopeptides from *Bacillus* species have also the potential to be used in the field of medicine due to anti-tumor activity and inhibition of cancer cells (Cheng et al. 2016). Although, the anti-cancer potential of all three lipopeptide families has been studied, (Zhao et al. 2018b) have found that the major fractional lipopeptide for inhibition of leukemia cancer cell K562, was identified as an iturin.

In general, lipopeptides produced by *Bacillus* species have been found to possess various biological activities, making them attractive for use in a range of fields, including agriculture as a bio-fungicide. However, despite their potential benefits, the limited production of these lipopeptides has hindered their practical application. Therefore, further research and development are needed to enhance their volume of production.

1.7 Challenges and prospects of biotechnological production of *Bacillus* lipopeptides

Referring back to earlier in this thesis, *Bacillus* lipopeptides have potential for being used instead of synthetic surfactants and conventional chemical antifungals in agriculture (Ongena and Jacques 2008). In wild-type *Bacillus* species, surfactin represent the dominant produced lipopeptides, while iturin and fengycin are produced only in lesser amounts. Presumably this is the reason why there are much less studies on large scale production of iturin and fengycin in comparison to surfactin despite their well-documented pronounced bioactivity. In case of surfactin, a few companies such as a Japanese chemical company named Kaneka, have produced surfactin on comparatively large scale for a few times in a research capacity (Kaneka 2022). Nevertheless, none of the *Bacillus* lipopeptides are commercially available in mass quantities for several reasons. For instance, the initial produced titer by wild-type *Bacillus* species is not fulfilling the industrial demand and large-scale production of these metabolites is combined with several difficulties such as controlling the foam during fermentation. However, these limitations have not caused research in this area to be abandoned. In fact, the use of biodegradable products as bio-fungicide in agriculture is increasing due to growing concerns about biodiversity loss and chemical pollution. Biodegradable products are a considered more sustainable alternative to chemical pesticides, and their use can help reduce greenhouse gas emissions and minimize environmental pollution.

An ongoing area of lipopeptide research is the genetic modification of the producer strains with the aim of boosting their productivity and to identify the regulation pathway in the producer strain (Gaur et al. 2022). Genetic engineering for the improvement of lipopeptide production by *Bacillus* species has previously concentrated on random mutagenesis with incrementally increased production quantities (Das et al. 2008). This proceeding generates undirected mutations and does not correspond to the state of the art in science and technology. Lately, lipopeptide biosynthesis enhancement has been approached by

different genetic engineering techniques. Nowadays, having access to the whole genome sequence of the targeted producer strain may provide useful information to study lipopeptide regulation and biosynthesis. In the meanwhile, discovery of potentially new lipopeptides from a wide variety of bacteria will open many perspectives for further diversification of lipopeptide biosynthesis. Another approach to increase the overall titer of lipopeptide production is to optimize the cultivation medium as well as to set up suitable physicochemical parameters of cultivation.

To conclude, prospects for biotechnological production of *Bacillus* lipopeptides are promising due to advancements in genetic engineering and fermentation technology. These advancements have enabled the development of new *Bacillus* strains with enhanced production capabilities and the optimization of fermentation conditions to improve yields. The demand for a "green" future places emphasis on ongoing research in this area to achieve the goal of replacing hazardous chemicals in industry with green-based biodegradable compounds. The long road to this goal has already been taken and much more needs to be done.

Part II
Publications

2.1 1st Publication:

Bacillus velezensis UTB96 is an antifungal soil isolate with a reduced genome size compared to that of *Bacillus velezensis* FZB42

Vahidinasab M., Ahmadzadeh M., Henkel M., Hausmann R., Morabbi Heravi K. **(2019)**.

In: Microbiology Resource Announcements. Sep. 19; 8(38): e00667-19.

DOI:10.1128/MRA.00667-19

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Resource Announcements

GENOME SEQUENCES

Bacillus velezensis UTB96 Is an Antifungal Soil Isolate with a Reduced Genome Size Compared to That of *Bacillus velezensis* FZB42

Maliheh Vahidinasab,[a] Masoud Ahmadzadeh,[b] Marius Henkel,[a] Rudolf Hausmann,[a] Kambiz Morabbi Heravi[a]

[a]Institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150k), University of Hohenheim, Stuttgart, Germany
[b]Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

ABSTRACT *Bacillus velezensis* UTB96 was isolated from soil based on its antifungal activity. Whole-genome sequencing of strain UTB96 provided further information about its secondary metabolite gene clusters. Compared to the well-known strain FZB42, UTB96 lacks an IS*3* element and a type I restriction endonuclease.

Bacterial strain *Bacillus velezensis* UTB96 was isolated from the soil of the pistachio tree in an extensive screening of the plant probiotic bacterial community in Kerman, Iran (1). Out of thousands of isolated strains, UTB96 had the highest antifungal activity against *Aspergillus flavus*. Likewise, strain UTB96 was able to degrade the aflatoxin produced by *Aspergillus flavus* on pistachio nuts (1, 2). Also, UTB96 showed antagonistic activity against important soilborne fungal phytopathogens, for example, *Fusarium graminearum* (M. Ahmadzadeh, unpublished data) or *Phytophthora drechsleri* (3). Such antifungal activity could be a result of the production of three lipopeptide families (surfactin, iturin, and fengycin) by strain UTB96 (1, 2, 4).

The previous identification of strain UTB96 was performed using biochemical tests and 16S rRNA gene sequencing (NCBI accession number KY992857), which showed that UTB96 is *Bacillus amyloliquefaciens* (2). To characterize the genetic background and identify the genes responsible for biosynthesis of lipopeptide and antifungal metabolites, whole-genome sequencing of strain UTB96 was performed. In detail, a single colony of UTB96 was used to inoculate LB medium, which was then incubated overnight at 37°C with shaking at 120 rpm. Afterwards, chromosomal DNA was extracted using an innuPrep bacterial DNA kit (Analytik Jena, Jena, Germany). The library preparation and whole-genome sequencing were carried out by Eurofins Genomics (Ebersberg, Germany). Genome sequencing was obtained using an Illumina HiSeq 4000 instrument and library prepared with the 2 × 150-bp paired-end read length, including DNA fragmentation, adapter ligation, amplification, and size selection. The quality of the final library was assessed by determination of size distribution and quantification. All of the steps were performed according to Eurofins Genomics protocols, resulting in a total 3,270,666 reads. Raw data analysis and sequencing assembly were carried out using Geneious software ver. 11.1.5 (Biomatters Ltd.). Unless otherwise specified, default software settings were used for all analyses. In a preliminary approach, after pairing the reads, the quality of paired-end reads was controlled by quality trimming. The adapter sequences and the sequences with low quality were trimmed with a quality limit of 0.05. The paired-end reads were merged, and chimeric reads were removed. DNA assembly was performed by *de novo* assembly. A total of 24 contigs were generated, including a 981,939-bp contig as the largest contig and an N_{50} contig size of 496,461 bp. A nucleotide BLAST search of this contig showed the highest similarity to *B. velezensis*. Based on this result and together with the nucleotide BLAST search of DNA topoisomerase-encoding genes, it was clear that UTB96 is a *B. velezensis*

Citation Vahidinasab M, Ahmadzadeh M, Henkel M, Hausmann R, Morabbi Heravi K. 2019. *Bacillus velezensis* UTB96 is an antifungal soil isolate with a reduced genome size compared to that of *Bacillus velezensis* FZB42. Microbiol Resour Announc 8:e00667-19. https://doi.org/10.1128/MRA.00667-19.

Editor Frank J. Stewart, Georgia Institute of Technology

Address correspondence to Kambiz Morabbi Heravi, k.morabbi@uni-hohenheim.de.

Received 4 June 2019
Accepted 16 August 2019
Published 19 September 2019

strain. Therefore, in the final and main approach, the genome sequence of strain *B. velezensis* FZB42 (GenBank accession number NC_009725) was used as a reference map for genome assembly of UTB96 using the raw sequencing data files. Comparative BLAST analysis of UTB96 by NCBI indicated its similarity to strain FZB42 (query coverage, 99%; identity, ≥97.99%).

The genome of strain UTB96 consists of a circular sequence of a 3,715,675-bp chromosome with a G+C content of 46.9%. *B. velezensis* strains are known to produce various secondary antimicrobial metabolites, such as lipopeptides and polyketides (5–7). Due to production of these metabolites, *B. velezensis* has the ability to exert an antimicrobial effect against many phytopathogenic microorganisms (8–10). Production of secondary metabolites by UTB96 was analyzed using the antiSMASH tool with the default setting (11). Genomic clusters responsible for the biosynthesis of antimicrobial secondary metabolites were observed. These clusters involve genes encoding lipopeptides of surfactin, bacillomycin D, fengycin, and bacillibactin and polyketides of bacillaene, difficidin, and macrolactin. Likewise, *B. velezensis* is considered a plant growth-promoting bacterium (PGPB) and is a promising candidate for replacing chemicals in sustainable agriculture and biofarming systems (12, 13). Unlike that of *B. velezensis* FZB42, the genome of strain UTB96 did not have the IS*3* element or a type I restriction endonuclease.

Data availability. The complete genome sequence of *B. velezensis* UTB96 has been deposited in the NCBI database under the GenBank accession number CP036527. The raw read data are available at the NCBI Sequence Read Archive under SRA accession number SRX5559694.

ACKNOWLEDGMENT

This work was supported by a grant from the Food Security Center (FSC), an excellence center for exchange and development at the University of Hohenheim (funding reference DAAD 57160040).

REFERENCES

1. Afsharmanesh H, Ahmadzadeh M. 2016. The iturin-like lipopeptide as key factor in antagonism of *Bacillus subtilis* UTB96 toward *Aspergillus flavus* R5. Biol Control Pests Plant Dis 5:79–95. https://doi.org/10.22059/jbioc.2016.58619.
2. Bagheri N, Ahmadzadeh M, Ghasemi S, Vahidinasab M, Ghoreshi SS. 2018. *Bacillus amyloliquefaciens* UTB96, a superior plant probiotic and aflatoxin-degrading bacterium. J BioControl Plant Prot 6:1–17.
3. Ghasemi S, Ahmadzadeh M. 2013. Optimisation of a cost-effective culture medium for the large-scale production of *Bacillus subtilis* UTB96. Arch Phytopathol Plant Prot 46:1552–1563. https://doi.org/10.1080/03235408.2013.771469.
4. Keshavarzi S, Ahmad Zadeh M, Mirzaei S, Behboudi K, Bandehpour M. 2018. Enhancing surfactant production in *Bacillus subtilis* UTB96 by fermentation optimization. J BioControl Plant Prot 5:13–26. https://doi.org/10.22092/BCPP.2018.117885.
5. Ruiz-Garcia C, Bejar V, Martinez-Checa F, Llamas I, Quesada E. 2005. *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain. Int J Syst Evol Microbiol 55:191–195. https://doi.org/10.1099/ijs.0.63310-0.
6. Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C, Mahillon J. 2019. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front Microbiol 10. https://doi.org/10.3389/fmicb.2019.00302.
7. Stein T. 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56:845–857. https://doi.org/10.1111/j.1365-2958.2005.04587.x.
8. Abdelhamid AG, Hussein WE, Gerst MM, Yousef AE. 2019. Draft genome sequence of *Bacillus velezensis* OSY-GA1, which encodes multiple antimicrobial metabolites and expresses antimicrobial activity against foodborne pathogens. Microbiol Resour Announc 8:e01725-18. https://doi.org/10.1128/MRA.01725-18.
9. Cao Y, Pi H, Chandrangsu P, Li Y, Wang Y, Zhou H, Xiong H, Helmann JD, Cai Y. 2018. Antagonism of two plant-growth promoting *Bacillus velezensis* isolates against *Ralstonia solanacearum* and *Fusarium oxysporum*. Sci Rep 8:4360. https://doi.org/10.1038/s41598-018-22782-z.
10. Zhao J, Liu H, Liu K, Li H, Peng Y, Liu J, Han X, Liu X, Yao L, Hou Q, Wang C, Ding Y, Du B. 2019. Complete genome sequence of *Bacillus velezensis* DSYZ, a plant growth-promoting rhizobacterium with antifungal properties. Microbiol Resour Announc 8:e01217-18. https://doi.org/10.1128/MRA.01217-18.
11. Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, Medema MH, Weber T. 2019. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res. https://doi.org/10.1093/nar/gkz310.
12. Glick BR. 2012. Plant growth-promoting bacteria: mechanisms and applications. Scientifica 2012:1. https://doi.org/10.6064/2012/963401.
13. Zhang Y, Gao X, Wang S, Zhu C, Li R, Shen Q. 2018. Application of *Bacillus velezensis* NJAU-Z9 enhanced plant growth associated with efficient rhizospheric colonization monitored by qPCR with primers designed from the whole genome sequence. Curr Microbiol 75:1574–1583. https://doi.org/10.1007/s00284-018-1563-4.

2.2 2nd Publication:

Construction and description of a constitutive plipastatin mono-producing *Bacillus subtilis*

Vahidinasab M., Lilge L., Reinfurt A., Pfannstiel J., Henkel M., Morabbi Heravi K., Hausmann R. **(2020)**.

In: Microbial Cell Factories. Nov. 10; 19(1): 1-12.

DOI: 10.1186/s12934-020-01468-0

Additional material associated with this publication can be accessed at::

https://microbialcellfactories.biomedcentral.com/articles/10.1186/s12934-020-01468-0#Sec17

Vahidinasab *et al. Microb Cell Fact* (2020) 19:205
https://doi.org/10.1186/s12934-020-01468-0

Microbial Cell Factories

RESEARCH Open Access

Construction and description of a constitutive plipastatin mono-producing *Bacillus subtilis*

Maliheh Vahidinasab[1], Lars Lilge[1*], Aline Reinfurt[1], Jens Pfannstiel[2], Marius Henkel[1], Kambiz Morabbi Heravi[1] and Rudolf Hausmann[1]

Abstract

Background: Plipastatin is a potent *Bacillus* antimicrobial lipopeptide with the prospect to replace conventional antifungal chemicals for controlling plant pathogens. However, the application of this lipopeptide has so far been investigated in a few cases, principally because of the yield in low concentration and unknown regulation of biosynthesis pathways. *B. subtilis* synthesizes plipastatin by a non-ribosomal peptide synthetase encoded by the *ppsABCDE* operon. In this study, *B. subtilis* 3NA (a non-sporulation strain) was engineered to gain more insights about plipastatin mono-production.

Results: The 4-phosphopantetheinyl transferase Sfp posttranslationally converts non-ribosomal peptide synthetases from inactive apoforms into their active holoforms. In case of 3NA strain, *sfp* gene is inactive. Accordingly, the first step was an integration of a repaired *sfp* version in 3NA to construct strain BMV9. Subsequently, plipastatin production was doubled after integration of a fully expressed *degQ* version from *B. subtilis* DSM10^{T} strain (strain BMV10), ensuring stimulation of DegU-P regulatory pathway that positively controls the *ppsABSDE* operon. Moreover, markerless substitution of the comparably weak native plipastatin promoter (P_{pps}) against the strong constitutive promoter P_{veg} led to approximately fivefold enhancement of plipastatin production in BMV11 compared to BMV9. Intriguingly, combination of both repaired *degQ* expression and promoter exchange (P_{pps}::P_{veg}) did not increase the plipastatin yield. Afterwards, deletion of surfactin (*srfAA-AD*) operon by the retaining the regulatory *comS* which is located within *srfAB* and is involved in natural competence development, resulted in the loss of plipastatin production in BMV9 and significantly decreased the plipastatin production of BMV11. We also observed that supplementation of ornithine as a precursor for plipastatin formation caused higher production of plipastatin in mono-producer strains, albeit with a modified pattern of plipastatin composition.

Conclusions: This study provides evidence that *degQ* stimulates the native plipastatin production. Moreover, a full plipastatin production requires surfactin synthetase or some of its components. Furthermore, as another conclusion of this study, results point towards ornithine provision being an indispensable constituent for a plipastatin mono-producer *B. subtilis* strain. Therefore, targeting the ornithine metabolic flux might be a promising strategy to further investigate and enhance plipastatin production by *B. subtilis* plipastatin mono-producer strains.

*Correspondence: lars.lilge@uni-hohenheim.de
[1] Institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150K), University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany
Full list of author information is available at the end of the article

Keywords: *Bacillus subtilis*, Lipopeptide, Surfactin, Fengycin, Biosurfactant, Promoter exchange, Strain engineering, Bottlenecks, Ornithine, Fungicide

Background

The preservation of food security is a global concern, especially with regard to increasing population. At the same time, there is a growing demand for organic agriculture products in both developed and developing countries. This high demand emphasizes the need for an effective, environmentally friendly alternative to chemical fertilizers, fungicides, insecticides, etc. *Bacillus* strains e.g. *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis* are among the beneficial microorganisms known as effective cell factories that can produce many different secondary antimicrobial metabolites, including lipopeptides [1–3]. Plipastatin is a bioactive lipopeptide produced by *Bacillus subtilis.* In general, the main lipopeptides produced by *Bacillus* strains are classified in the three families of surfactin, iturin and fengycin [4, 5], including plipastatin as a member of the fengycin family [6]. Lipopeptides mostly display additional biological activities besides their amphiphilic properties. It is reported that the fengycin group exhibits a broad antagonistic effect on various soil-borne and post-harvest fungal phytopathogens, specifically on filamentous fungi [7, 8]. Besides antifungal activity, various authors [6–10] have frequently reported the antibacterial, antiviral and anticancer properties of fengycins. Additionally, these lipopeptides act also as the elicitor of induced systemic resistance in plants [11]. However, in contrast to the very well investigated applicability of surfactin, the application potential of fengycins have so far been investigated only in a few cases, principally based on poor bacterial productivity of fengycins and therefore, laborious and ineffective production approaches.

The structure of lipopeptides comprises a fatty acid connected to a peptide moiety [12]. The composition of amino acids in the circular peptide chain and length of the fatty acid residue provides a unique property for every lipopeptide [13]. Plipastatin consists of a decapeptide chain (L-Glu – D-Orn – L-Tyr – D-Thr – L-Glu – D-Ala – L-Pro –L-Gln – D-Tyr – L-Ile), 8 amino acids of which form a peptide ring and is linked to a 3-hydroxy fatty acid with 14–19 carbon atoms that may be saturated or unsaturated [14, 15]. Although, the structure of fengycin is almost identical to plipastatin, the enantiomers of the amino acids in positions 3 and 9 (Tyr3 and Tyr9) are present in the L- and D-form in plipastatin, respectively, while in fengycin, they are in the reverse configuration [6, 13, 16]. Plipastatin and other lipopeptides are formed by step-by-step reactions of specific non-ribosomal peptide synthetases (NRPSs) [17]. The lipopeptide biosynthesis depends on the 4-phosphopantetheinyl transferase Sfp which converts the inactive apoforms of NRPSs to the active holoforms [18]. In case of *B. subtilis* 168, the genome has two large operons of *srfAA-AD* and *ppsABCDE,* which encode the subunits of NRPSs for surfactin and plipastatin production, respectively. However, due to a single base duplication in *sfp* gene, *B. subtilis* 168 is incapable to synthesize these lipopeptides [19–21]. Nevertheless, after repairing the *sfp* mutation, its lipopeptide production is restored [22, 23].

Usually, *Bacillus* spp. encode for more than one lipopeptide synthetase, which are not synthesized simultaneously in the same growth phase showing the involvement of different regulators. More specifically, in the genome of bacteria that have the *ppsABCDE* operon, the *srfAA-AD* operon is always present as well [24, 25]. However, while surfactin is being produced in the late exponential phase, the biosynthesis of plipastatin has been characterized in the stationary phase [25–27]. Lipopeptide synthetases are usually regulated by complex regulatory networks in a growth-phase dependent manner. In the case of plipastatin, the *ppsABCDE* operon is repressed by AbrB, the transition state regulator, during exponential growth phase [28]. Moreover, expression of the pleiotropic regulator *degQ* gene has a positive effect on the synthesis of plipastatin showing the possible involvement of DegSU two-component system [20, 21, 29]. In *B. subtilis* 168, the −10 promoter region of *degQ* has a single base mutation that leads to low gene expression. Nevertheless, substitution of this mutation enables overexpression of the *degQ* gene and results in increased plipastatin biosynthesis [23, 30, 31].

In this study, we used *B. subtilis* 3NA as a model strain for genetic engineering of plipastatin production in order to construct a plipastatin mono-producer. This strain was previously described as a hybrid strain encoding genetic features from *B. subtilis* W23 and 168 [21]. In detail, the surfactin (*srfAA-AD*) operon was deleted in a way that their competence formation remained intact. Subsequently, the plipastatin was increased by promoter exchange and manipulation of the *degQ* expression. Finally, the effect of supplementing potentially critical amino acids on plipastatin formation was evaluated.

Results

Plipastatin production in different *B. subtilis* strains

To construct a plipastatin mono-producer, two *B. subtilis* strains were compared for their potential in biosynthesis of plipastatin. This included the derivatives of the well-known laboratory model strain *B. subtilis* 168 and strain 3NA. Plipastatin operon and its promoter region are identical in these two strains [21]. Nevertheless, strain 3NA is a sporulation-deficient strain caused by a frame shift mutation in *spo0A* which makes this strain suitable for fermentation [21]. These domesticated *B. subtilis* strains are known to have a mutation in their *sfp* gene disabling them to produce lipopeptides, such as surfactin and plipastatin [20, 21]. Therefore, the *sfp* + derivates of strain 168 (JABs24) and strain 3NA (BMV9) were used to ensure lipopeptide production. Both strains were cultivated in mineral salt medium. Figure 1 shows the lipopeptide production, glucose consumption and optical density (OD_{600}) over 72 h of cultivation at 30 °C. The comparison of growth rates showed a faster cell growth of BMV9 strain compared to JABs24. However, optical density of BMV9 was drastically decreased without any stationary phase after glucose depletion. In contrast, JABs24 exhibited an entry into the stationary phase when 20% of glucose were metabolized. Both strains revealed surfactin production from the beginning of cultivation through to the early stage of stationary phase. Thus, surfactin quantities gradually increased until a peak was reached in the late exponential phase. In case of plipastatin, no detectable amounts could be measured up to the middle of exponential phase. The highest amount of plipastatin was produced by BMV9 in the stationary phase and was about 17 mg/L. Conversely, the production of plipastatin by JABs24 was lower than BMV9 (10 mg/L after 72 h). Based on these results strain BMV9 was considered for further strain engineering.

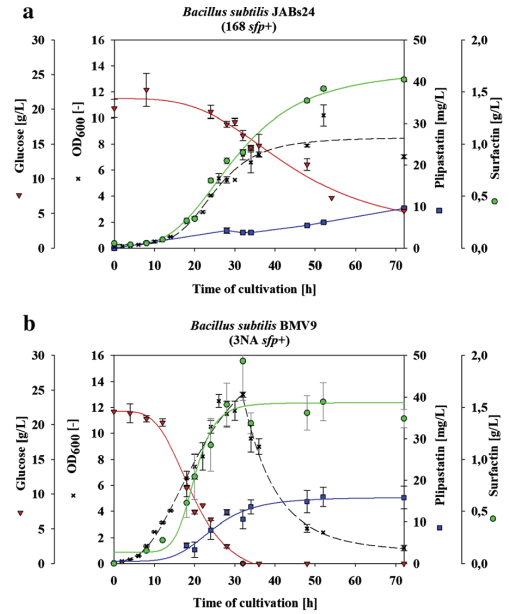


Fig. 1 Comparison of cultivation parameters for **a** *B. subtilis* JABs24 (168 *sfp* +) and **b** *B. subtilis* BMV9 (3NA *sfp* +); The shake flask cultivations were performed in mineral salt medium containing 20 g/L glucose and 50 mM urea as carbon and nitrogen sources at 30 °C and 120 rpm. Besides optical density (OD_{600}), glucose consumption, plipastatin and surfactin production were detected

Verification of different putative bottlenecks concerning plipastatin production

As mentioned before, *B. subtilis* 3NA is a hybrid strain that exhibits genetic features from both strains W23 and 168 [21]. Likewise, the *degQ* gene and upstream promoter region are identical to that of strain 168. Therefore, a single base mutation in the *degQ* promoter region drastically decreases the corresponding gene expression [32]. Previous studies demonstrated that this circumstance has a negative effect on plipastatin production [31]. We hypothesized that a repair of *degQ* expression combined with the deletion of the competitive surfactin operon as well as an exchange of a weak plipastatin promoter against a constitutively active promoter lead to a highly efficient plipastatin mono-producer strain. However, before constructing the mutant strain that exhibits these three characteristics, BMV9 strain (3NA *sfp* +) was used to construct BMV10, BMV11 and BMV12, showing repaired *degQ* expression, plipastatin promoter exchange and surfactin elimination, respectively. Based on the observation that plipastatin level reached a plateau after 40 h of cultivation (Fig. 1), the production outcomes of all following mutant strains were measured after 48 h of cultivation (Fig. 2).

As shown in Fig. 2, BMV10 which holds a repaired *degQ* gene, showed a twofold higher plipastatin production but a decrease in surfactin formation compared to the control strain (BMV9). Furthermore, the promoter exchange of native plipastatin promoter against the constitutive promoter region of the *veg* gene resulting in BMV11 strain, increased the plipastatin titer from 15 mg/L in control strain BMV9 to 70 mg/L. Interestingly, the surfactin formation was unaffected by the enhanced plipastatin production. In order to eliminate surfactin synthesis, the entire *srfAA-AD* operon was deleted and the regulatory *comS* gene which is co-expressed with *srfAA-AD* operon restored back to the genome with the native P_{srfA} promoter. Consequently, the strains BMV12 (Δ*srfAA-srfAD* with native P_{pps} promoter) and BMV13

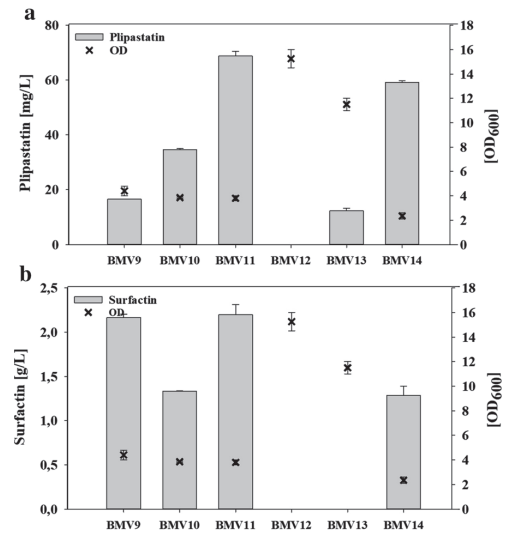


Fig. 2 Overview about production of plipastatin (**a**) and surfactin (**b**) in respect to reached optical densities (OD_{600}) of different engineered mutant strains after 48 h cultivation in mineral salt medium; BMV9, control strain (3NA *sfp* +); BMV10, repaired *degQ* expression; BMV11, promoter exchange of *pps* operon; BMV12, deletion of *srfA* operon; BMV13, combination of promoter exchange and *srfA* operon deletion; BMV14, combination of promoter exchange and repaired *degQ* expression

(Δ*srfAA-srfAD* with P_{pps}::P_{veg} promoter exchange) were constructed. It was observed that even though the deletion of the surfactin operon caused enhanced cell growth in both BMV12 and BMV13 strains, plipastatin production reduced (Fig. 2a). Plipastatin production was significantly decreased not only under native expression of plipastatin operon (BMV12) but also when P_{pps} was exchanged against constitutive P_{veg} promoter (BMV13).

In order to see the effect of combining the features of promoter exchange and repairing *degQ* gene expression, the strain BMV14 was constructed. Comparably to strain BMV9, the expression of *degQ* had a negative effect on surfactin production (Fig. 2b). Interestingly, no additive effect was observed on plipastatin production by BMV14 (Fig. 2a). In summary, we concluded that under the same conditions, BMV11 was able to produce the highest amounts of plipastatin compared to all other mutant strains constructed.

Impact of amino acid supplementation on plipastatin production

To verify the impact of amino acid precursors on plipastatin and surfactin formation (Additional file 1), seven different amino acids including glutamic acid, glutamine, isoleucine, alanine, threonine, proline and ornithine with a concentration of 30 mM were additionally supplemented in mineral salt medium. BMV9 (3NA *sfp* +) and BMV12 (3NA *sfp* + plipastatin mono-producer) were selected to evaluate the produced plipastatin under control of native P_{pps} promoter, in the presence and absence of the surfactin operon.

Table 1 Plipastatin titers achieved of *B. subtilis* BMV9 (3NA *sfp* +) and *B. subtilis* BMV12 (3NA *sfp* + plipastatin mono-producer)

Supplementation	*B. subtilis* BMV9 Plipastatin (mg/L)	*B. subtilis* BMV12 Plipastatin (mg/L)
Control	19.1 ± 0.1	n. d.*
Glutamic acid	7.8 ± 0.2	n. d
Glutamine	6.6 ± 0.9	n. d
Isoleucine	10.5 ± 2.6	n. d
Alanine	14.3 ± 0.9	n. d
Threonine	11.3 ± 0.8	n. d
Proline	14.4 ± 1.3	n. d
Ornithine	18.4 ± 2.4	6.3 ± 0.5

Plipastatin titers measured after 48 h cultivation in mineral salt medium supplemented with 30 mM of the indicated amino acids compared to control cultivations (without any amino acid supplementation).

*Not detectable

As it is shown in Table 1, except for ornithine, a decrease in plipastatin titer was observed when supplementing BMV9 strain with the other six amino acids. Interestingly, in the plipastatin mono-producer BMV12 strain, supplementation of the ornithine led to a detectable plipastatin titer. Neither in control cultures (without amino acid supplementation) nor in the presence of other amino acids, no detectable production was observed. Subsequently, it was observed that cultivation of BMV13 (constitutive plipastatin mono-producer) in supplemented medium with ornithine let to enhance in plipastatin titer (about 10%). Furthermore, it is noteworthy to mention that the observed plipastatin chromatogram on silica HPTLC plate of BMV12 (plipastatin mono-producer) in the presence of ornithine exhibited a modified pattern compared to the plipastatin standard (*B. subtilis* plipastatin/fengycin standard, Lipofabrik france) and parental BMV9 strain (Fig. 3A). Although the detected signals in the chromatogram showed similar Rf values compared to the standard, not all standard peaks could be observed. Therefore, HPLC–MS analysis was performed for identification of plipastatin variants or homologs.

(See figure on next page.)

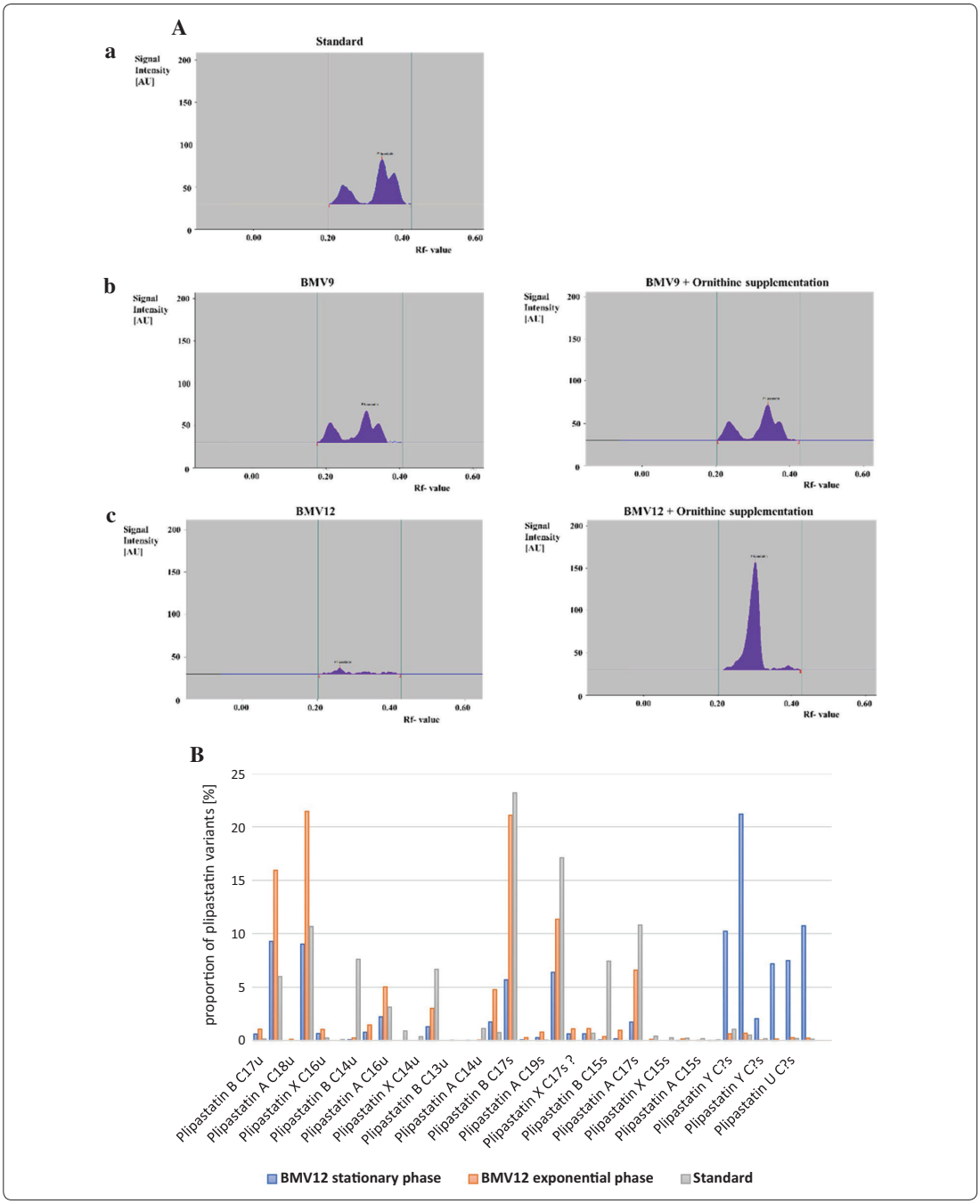
Fig. 3 **A** Detected HPTLC chromatograms of the standard plipastatin in comparison with plipastatin produced by BMV9 (3NA *sfp*+) and BMV12 (3NA plipastatin mono-producer) with and without ornithine supplementation. Plipastatin standard produced by *B. subtilis* (Lipofabrik, France) [a], plipastatin produced by BMV9 strain after 48 h cultivation in mineral salt medium without any amino acid and with ornithine supplementation [b]; plipastatin produced by BMV12 strain after 48 h cultivation in mineral salt medium without amino acid and with ornithine supplementation [c]. **B** Comparison of plipastatin variants ratios in plipastatin standard and mono-producer BMV12 strain during exponential and stationary phase. The BMV12 was cultivated in mineral salt medium supplemented with 30 mM of ornithine. The samples were taken after 24 h (exponential phase) and 48 h (stationary phase). Peak areas were determined by extracted ion chromatograms for each plipastatin variant using their precise m/z values. Ratios of the peak areas of plipastatin variants were calculated within the standard and the two sample points

Mass spectrometry analyses of plipastatin variants produced by BMV12 with supplementation of ornithine

Plipastatin produced by *B. subtilis* is a mixture of several homologs based on the length of fatty acid chain and variants within peptide moiety [33]. The patterns of the HPTLC chromatograms of produced plipastatin are comparable between the *B. subtilis* plipastatin standard and the sample extracts of the parental BMV9 strain. In both cases, plipastatin splits into several peaks. This might indicate that several plipastatin variants were produced (Fig. 3A [a, b]). Subsequently, plipastatin pattern of mono-producer strain BMV12 was analyzed by HPTLC and it appeared in one peak in the same Rf values (Fig. 3A [c]). Thus, it was assumed that deletion of *srfA* operon resulted in an accumulation of specific plipastatin variants whereas other subtypes were diminished. To get a perception about the different plipastatin variants present in *B. subtilis*, the high-purified plipastatin standard was analyzed by mass spectrometry (HPLC–ESI–MS). The results showed that a large variety of plipastatin subtypes with saturated and unsaturated fatty acid chains is present in the plipastatin standard (Additional file 2A). Mainly, A and B type plipastatin were detected in the LC–ESI–MS analysis, but also other plipastatin variants were observed. Some of them could be assigned to plipastatin C, D or S types [34] based on their diagnostic marker ions. In addition, other plipastatin variants were detected that were not described in previous studies in *B. subtilis* so far. Therefore, they were named in this study as W, U and Y. To get an overview about the differences in plipastatin variants produced by BMV12, shake flask cultivation was performed in mineral salt medium supplemented with ornithine. Samples were then harvested from the exponential and stationary phase (plipastatin HPTLC chromatograms were comparable in both growth phases) and also analyzed by LC–ESI–MS. Afterwards, the peak areas of identified plipastatin variants were used to determine ratios in between BMV12 samples and were compared to that of the plipastatin standard (Fig. 3B). Variants of the subtypes plipastatin A and plipastatin B were detected in both BMV12 samples and the standard, even though especially plipastatin B C_{14}u (unsaturated fatty acid chain with 14 carbon atoms) and plipastatin B C_{15}s (saturated fatty acid chain with 15 carbon atoms) showed higher relative abundance in the standard in contrast to BMV12 samples. In contrast, slightly higher abundances of plipastatin B C_{16}u and plipastatin B C_{18}s were detected in both samples of BMV12. Taken together, no major differences were detected between standard and BMV12 plipastatin extracts. Hence, the reason for the variation of the plipastatin pattern based on the deletion of surfactin operon could not be explained. Another interesting observation was the accumulation of different plipastatin variants (named in this study plipastatin Y, W and U) during the stationary phase. Due to the cultivation with mineral salt medium, a limitation of the amino acids during the stationary phase is very likely. Insofar, the accumulation of plipastatin Y, W and U could be due to the incorporation of different amino acids into the peptide residue of plipastatin.

Discussion

In this study, *B. subtilis* 3NA was engineered to construct a plipastatin mono-producer strain and to gain more insights about plipastatin production. Previously, it was shown that DegQ positively regulates plipastatin production [30]. In fact, DegQ stimulates autophosphorylation of DegS sensor kinase resulting in enhanced phosphotransfer to DegU response regulator [29, 35]. As a result, the phosphorylated and activated DegU-P version causes higher expression of *ppsABCDE* operon and increases the plipastatin production [36]. In this study, repair of *degQ* expression (strain BMV10) ensured DegU-P activation, which doubled the production of plipastatin compared to parental BMV9 strain (3NA *sfp*+). In this context, previous results from Tsuge et al. [30] showed a tenfold higher plipastatin production when *ppsABCDE*, *degQ* and *sfp* were combined in a *B. subtilis* plasmid expression system. Furthermore, Wang et al [31]. described a decrease of plipastatin after in-frame mutagenesis of *degQ* in *B. subtilis* NCD-2. Afterwards, with respect to the relatively low expression level of *ppsABCDE* operon, an approximately fivefold higher plipastatin formation was achieved by the exchange of the native P_{pps} promoter against constitutive P_{veg} promoter (strain BMV11). Previous to this study, the effect of promoter exchange on the amount of plipastatin


A
a
Standard
Signal Intensity [AU]
Rf- value
b
BMV9
BMV9 + Ornithine supplementation
c
BMV12
BMV12 + Ornithine supplementation
B
proportion of plipastatin variants [%]
Plipastatin B C17u
Plipastatin A C18u
Plipastatin X C16u
Plipastatin B C14u
Plipastatin A C16u
Plipastatin X C14u
Plipastatin B C13u
Plipastatin A C14u
Plipastatin B C17s
Plipastatin A C19s
Plipastatin X C17s ?
Plipastatin B C15s
Plipastatin A C17s
Plipastatin X C15s
Plipastatin A C15s
Plipastatin Y C?s
Plipastatin Y C?s
Plipastatin U C?s
BMV12 stationary phase
BMV12 exponential phase
Standard

produced was reported only on a few cases. For instance, promoter exchange of native P_{pps} promoter against native P_{fen} from *B. subtilis* 21332 caused no plipastatin overproduction. In contrast, a tenfold higher plipastatin production was obtained when P_{fen} from strain BBG21 (a spontaneous mutant of *B. subtilis* ATCC 21332) was integrated [37]. In the following step, it was observed that the combination of both repair of *degQ* expression and the P_{pps} promoter exchange (BMV14), had no additional effect on the plipastatin titer compared to BMV11 (constitutive plipastatin producer). This is in contrast to the comparison of parental BMV9 and BMV10 when functional *degQ* expression increases plipastatin production about twofold. An explanation could be addressed by the DegQ mediated activation of DegU-P regulon, which causes in general the increase of secretory proteases [38, 39]. These proteases could target plipastatin for degradation. In sum, this negative effect could be more noticeable in a constitutive *pps* operon expression (P_{veg}) which make a higher amount of DegQ not benefical.

Another aim of this study was to construct a strain that ensures constitutive plipastatin mono-production. In a recent work, it was shown that deletion of *srfAA* significantly reduced the plipastatin production and on the other hand deletion of *srfAC* showed no effect [25]. The authors argued that probably *srfAA* has a regulatory effect on plipastatin production. Furthermore, they also observed another significant decrease in plipastatin production when *pnp* gene, which is responsible for biosynthesis of multifunctional polynucleotide phosphorylase (PNPase) [40] was deleted. The regulation of PNPase on plipastatin biosynthesis can happen through effect on *comS* expression. Subsequently, it was hypothesized that *comS* expression in an unknown complex pathway positively regulates plipastatin formation. In this study, we have deleted the whole *srfAA-AD* operon and retained *comS* with the native P_{srfA} promoter (BMV12). In BMV12, even though *comS* was retained, plipastatin titer decreased to a non-detectable concentration confirming previously described study [25] that *srfAA* or in general, the expression of surfactin synthetase has a positive effect on plipastatin production. This observation was consistent after exchange of native P_{pps} against P_{veg} (BMV11 compared to BMV13). Likewise, in another study P_{pps} promoter was exchanged against a strong P_{amyQ} promoter from *Bacillus amyloliquefaciens* resulting in an increase in plipastatin production. However, after elimination of surfactin synthetase by deletion of *srfAB*, *srfAC* and *srfAD* genes, plipastatin production did not change [11]. Hence, in respect to our results and previously described studies [11, 25] we conclude that, subunits of the surfactin synthetase have different impacts on plipastatin production.

Another rational factor in increasing the plipastatin production is the presence of a sufficient quantity of precursors in the cultivation medium. Therefore, we have attempted to improve plipastatin production by supply of seven amino acids of the plipastatin peptide chain in the mineral salt medium. Previously, these seven amino acids as the only nitrogen source were used in the mineral salt medium and compared with other nitrogen sources such as urea and ammonium carbonate [41]. Accordingly, urea was introduced as the best nitrogen source and alanine, followed by glutamic acid, were the best sources of nitrogen among the other amino acids. In this study, approximately the same concentration of amino acids were added to the medium besides urea as the main nitrogen source. Since, surfactin synthetase, assumably has a regulatory effect on plipastatin production, the supplementation of amino acids was examined in two strains of BMV9 (3NA *sfp* +) and BMV12 (3NA *sfp* + plipastatin mono-producer). Among all the amino acids used, ornithine was the only amino acid induced a detectable plipastatin production in mono-producer (BMV12), while it has not been measured in the other cultivations of BMV12. Interestingly, in the strain BMV9, the supplementation of different amino acids had different effects on final plipastatin titer. As it was shown in Fig. 1, the optical density of BMV9 directly decreased after glucose consumption whereas the plipastatin titer remained stable over time. Therefore, a comparison of the plipastatin production per biomass after 48 h cultivation is not reasonable. Accordingly, the results show that expect for ornithine, the additional supplementation of amino acids reduced in general the plipastatin titer in BMV9. This can be explained due to (de-) activation of stringent response in *B. subtilis* which occurs in amino acid limitation. Activation of stringent response results in enhanced provision of branched-chain amino acids [42, 43]. Conversely, by addition of amino acids in the cultivation medium, the positive side effect of stringent response will be reduced. However, supplementation of ornithine in the medium had no negative effect and it was the only amino acid that enhanced the plipastatin produced by BMV12 (plipastatin mono-producer) to a detectable level. Therefore, it is concluded that ornithine is an indispensable constituent for a plipastatin mono-producer *B. subtilis* strain.

Conclusions

This study provides evidence that *degQ* stimulates the native plipastatin production. A significant decrease in plipastatin productivity after deletion of the surfactin operon in a constitutive plipastatin producer strain suggested that full plipastatin production requires the surfactin synthetase or some of its components. Nevertheless, the impact of surfactin synthetase existence on

Table 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference
Strains		
Escherichia coli		
JM109	*mcrA recA1 supE44 endA1 hsdR17* ($r_K^-m_K^+$) *gyrA96 relA1 thi Δ(lac-proAB) F′* [*traD36 proAB*$^+$ *lacI*^{q} *lacZ ΔM15*]	[48]
Bacillus subtilis		
JABs24	*B. subtilis* 168 Δ*manPA*; *trp*+; *sfp*+;	[49]
3NA	*spo0A3*;	[50]
JABs32	*spo0A3*; Δ*manPA::erm*; *sfp*+;	J. Altenbuchner (unpublished)
BMV9	*spo0A3*; Δ*manPA*; *sfp*+;	This study
BMV10	*spo0A3*; Δ*manPA*; *sfp*+; Δ*amyE*:: *degQ* (from *B. subtilis* DSM10^T)	This study
BMV11	*spo0A3*; Δ*manPA*; *sfp*+; P_{pps}-*ppsA-E*:: P_{veg}–*ppsA-E*	This study
BMV12	*spo0A3*; Δ*manPA*; *sfp*+; Δ*srfAA-AD*:: *comS -erm*	This study
BMV13	*spo0A3* Δ*manPA*; *sfp*+; Δ*srfAA-AD*:: *comS -erm*; P_{pps}-*ppsA-E*::P_{veg}–*ppsABCDE*	This study
BMV14	*spo0A3*; Δ*manPA*; *sfp*+; P_{pps}-*ppsABCDE*::P_{veg}–*ppsABCDE*; Δ*amyE*::*degQ* (from *B. subtilis* DSM10^T)	This study
Plasmids		
pJOE6743.1	ori_{pUC18}, *bla*, *spc*, *manP*, *ter-lacI-lacZa-ter*	[51]
pJOE7644.2	ori_{pUC18}, *bla*,P_{manP}-*manP*, *spc*, *manR-ctaO*	[46]
pJOE4786.1	ori_{pUC18}, *bla*, *ter-′lacI-lacZa-ter*	[52]
pKAM312	ori_{pBR322}, *rop*, *ermC*, *bla*, *amyE′*-[*ter*-P_{glcR}-*lacZ-spcR*]- *′amyE*	[46]
pMAV3	pJOE4786.1 containig P_{veg}::P_{pps} exchange fragment (integrated by *Sma* I)	This study
pMAV4	pJOE6743.1 containig P_{veg}::P_{pps} exchange fragment (integrated by *Hind* III)	This study
pMAV5	pKAM312 containing *degQ* (*B. subtilis* DSM10^T) (integrated by *Hind* III)	This study

plipastatin formation is still unknown. In order to construct a plipastatin mono-producer strain suitable for cultivation in large quantities in a bioreactor, understanding the mutual impact between surfactin and plipastatin syntheses might help to increase the final plipastatin production. Furthermore, as another conclusion of this study, results point towards ornithine provision being an indispensable constituent for a plipastatin mono-producer *B. subtilis* strain. Therefore, targeting the ornithine metabolic flux might be a promising strategy to further investigate and enhance plipastatin production by *B. subtilis* plipastatin mono-producer strains.

Materials and methods

Chemicals, materials and standard procedures

All chemicals were acquired from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) if not, mentioned otherwise. Standard molecular techniques were carried out as described by Sambrook and Russell [44]. The desired DNA fragments were amplified in polymerase chain reactions using DNA Polymerase (Phusion High-Fidelity #M0530S, New England BioLabs, Frankfurt am Main, Germany). The PCRs were carried out on a PCR thermal cycler (prqSTAR 96X VWR GmbH, Darmstadt, Germany). Chromosomal DNA was purified with a ready to use kit (innuPREP Bacteria DNA Kit) and plasmid DNA was extracted with innuPREP Plasmid Mini Kit (Analytik Jena AG, Jena, Germany). After PCR reactions, amplified DNA fragments were extracted after agarose-based gel electrophoresis with QIAquick PCR & Gel Cleanup Kit, according to the manufactures' instruction. Restriction enzymes and alkaline phosphatase (#M0290) was purchased from New England BioLabs (Frankfurt am Main, Germany) and T4 DNA ligase were purchased from Thermo Fisher Scientific (Karlsruhe, Germany). All ligation reactions were performed overnight at 4 °C. For better efficiency of ligation, a PEG 8000 solution was added. Oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany).

Strains, plasmids and transformation method

All strains and plasmids used in this study are shown in Table 2. Oligonucleotides used for construction of strains and plasmids are listed in Table 3. *Escherichia coli* JM109 was used for plasmid propagation and cloning. Transformation of *E. coli* strains were carried out according to the standard heat-shock method [45]. *B. subtilis* JABs32 strain, a *sfp*+derivate of *B. subtilis* 3NA, was used for mannose counterselection. Therefore, erythromycin resistance gene (*erm*) for *manPA* deletion was removed

Table 3 Oligonucleotides used in this study

Name	Sequence 5′–3′	Purpose
s1009	CTGCCGTTATTCGCTGGATT	Integration of *degQ* gene (*B. subtilis* DSM10^T) (+510 bp) in *amyE* locus underlined sequences highlight the *Nde* I and *EcoR* I restriction site
s1410	ATTATTAA**CATATG**CGGCGTACCTCATACGGATACAC	
s1409	ATTATTAA**GAATTC**CTCCTTGATCCGGACAGAATC	
s1010	AGAGAACCGCTTAAGCCCGA	
s1221	GGAAAGTGAAAAAAGGAGAAGG	Construction of P_{veg}::P_{pps} promoter exchange
s1222	CCTATGCAGGTTTTCAACTGTTATTGATTTGCCAAAATGACAG	
s1223	CAGTTGAAAACCTGCATAGG	
s1224	TGCATCCACCTCACTACAT	
s1225	ATGTAGTGAGGTGGATGCATTGAGCGAACATACTTATTCTTTAAC	
s1226	CATTTAAAGAGATTCCATCCATTATGATATG	
s1162	CATGATTTTCAGGTCTGCAAGAAC	Construction of *srfAA-AD*:: *comS-erm*
s1163	GTTCAAACGTCTGCTCCTCCTTAATCTTTATAAGCAGTGAACATGTGC	
s1164	AGGAGGAGCAGACGTTTGAAC	
s1165	CTTCTCCCTCCAGCAGAAGTAC	
s1166	CTTCTGCTGGAGGGAGAAGTAGGTATAAATTTAAC-GATCACTCATCA TGTTC	
s1167	GACCGATAGATTTTGAATTTAGGTGTC	
s1168	CACCTAAATTCAAAATCTATCGGTCGAATGCCAAT-TTCTGCATGGTATAATAG	
s1169	GGCAACCTGATGGATAAAGAAATTG	

by the use of plasmid pJOE7644.2 resulting in BMV9 [46]. Strain BMV9 was used as parental strain for construction of further mutant strains. Transformation of natural competent *B. subtilis* strains was performed according to the "Paris method" [47]. Depending on the selection marker, the transformants were selected on LB agar supplemented with ampicillin (100 µg/mL), spectinomycin (100 µg/mL) or erythromycin (10 µg/mL for *E. coli* and 5 µg/mL for *B. subtilis*). All plates were incubated at 37 °C.

Construction of plasmids for strain engineering

For markerless promoter exchange, LFH-PCR method was used [53]. Accordingly, upstream and downstream wild-type sequence of *pps* promoter region was fused with *veg* promoter. After ligation into *Sma* I digested pJOE4786.1 resulting in pMAV3, target sequence was isolated by *Hind* III digestion and was subsequently integrated into pJOE6743.1 (results in pMAV4). Afterwards, plasmid pMAV4 was transformed into strain BMV9 followed by the protocol described before [51].

For the integration of the *degQ* gene including promoter region (+510 bp) and terminator structure from *B. subtilis* DSM10^T, the primer s1410 and s1409, containing *Nde* I and *EcoR* I restriction sites, were used. After restriction digestion, the *degQ* fragment was ligated into pKAM312 [46] resulting in pMAV5. After transformation of pMAV5 into parental strain BMV9 and other mutant strains, transformants were selected on LB agar plates containing spectinomycin. To ensure the correctness of plasmids and mutant strains, all constructs were confirmed by sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany).

Deletion of surfactin operon and retain of *comS* gene

The principle of LFH-PCR was utilized to design a DNA fragment for deletion of *srfA* operon and simultaneously retain of *comS* gene. A fusion of upstream region of *srfA* operon with *comS* gene ensured a wild-type expression. For a simple strain selection, *comS* gene was additionally linked with erythromycin resistance cassette (*erm*) of pKAM312. An uncoupled *erm* gene expression was ensured by maintaining the natural P_{erm} promoter region from pKAM312. Figure 4 illustrates the described strategy.

Cultivation in mineral salt medium

The mineral salt medium used was based on the fermentation medium of Willenbacher et al. [54] with slight changes. The composition of the final medium was: 20 g/L glucose, 4.0×10^{-6} M $Na_2EDTA \times 2\ H_2O$, 7.0×10^{-6} M $CaCl_2$, 4.0×10^{-6} M $FeSO_4 \times 7\ H_2O$, 1.0×10^{-6} M $MnSO_4 \times H_2O$, 50 mM Urea, 0.03 M KH_2PO_4, 0.04 M $Na_2HPO_4 \times 2\ H_2O$ and 8.0×10^{-4} M $MgSO_4 \times 7\ H_2O$.

For the first preculture, 10 mL LB medium (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) was inoculated with 10 µL glycerol stock solution in a 100 mL

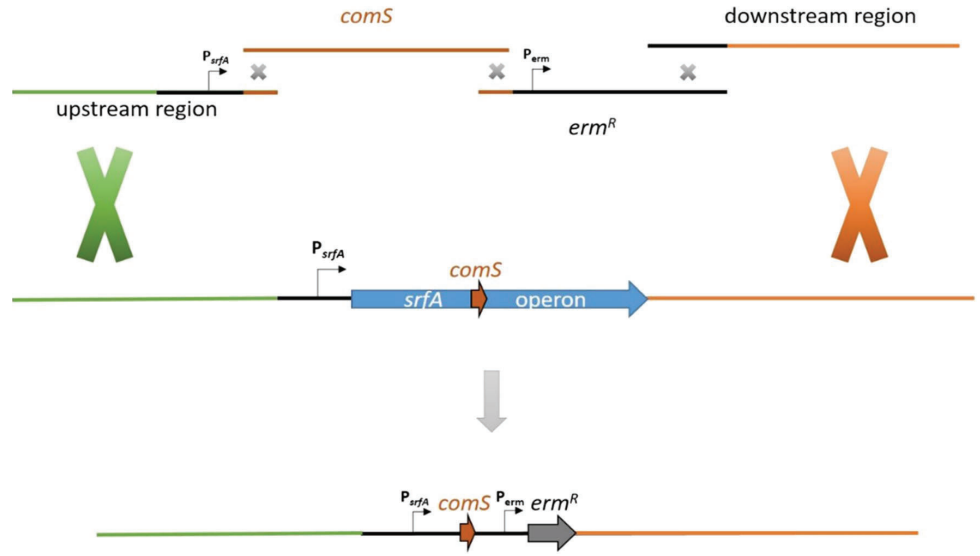


Fig. 4 Schematic illustration of the construction of PCR fragment in order to delete surfactin operon and retain back *comS* with the native P_{srfA} promoter using LFH-PCR method

baffled shake flask. After 8 h of cultivation, the cells were transferred to 10 mL mineral salt medium with an initial OD_{600} of 0.1 as a second preculture. This preculture was incubated overnight and after reaching an OD_{600} between 2–4 the main culture was inoculated. The main cultivations took place in 1 L Erlenmeyer baffled flasks with the final volume of 100 mL and initial OD_{600} of 0.1. All cultivation had three biological replicates and were performed at 30 °C and 120 rpm in an incubation shaker (Innova 44®R, Eppendorf AG, Hamburg, Germany).

Additionally, the influence of potentially critical amino acids including glutamic acid, glutamine, isoleucine, alanine, proline and ornithine on plipastatin production was tested using mineral salt medium complemented with 30 mM of each amino acids.

Extraction of lipopeptides and HPTLC analysis

The cell-free supernatants were obtained by centrifugation at 4700 rpm and 4 °C and were used for extraction of lipopeptides following the method described before with slight changes [55]. In detail, 2 mL of cell-free supernatant was mixed 3 times with 1 mL 1-butanol 95% (*v/v*) by vortexing for 1 min, followed by 5 min centrifugation at 3000 rpm to separate organic phase. After complete evaporation of butanol phases (RVC2-25 Cdplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 10 mbar and 60 °C, the remaining residues were dissolved in 2 mL methanol. To quantify surfactin and plipastatin production, these methanolic fractions were separated by HPTLC (CAMAG, Muttenz, Switzerland) as described previously [56].

Structural analysis of plipastatin variants by Mass spectrometry

LC–MS analysis of plipastatin was performed on a 1290 UHPLC system (Agilent, Waldbronn, Germany) coupled to a Q-Exactive Plus Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Bremen, Germany). Analyte separation was achieved by a Waters ACQUITY CSH C18 column (1.7 µm, 2.1 µm × 150 mm). The column temperature was maintained at 40 °C. Samples were dissolved in methanol and 5 µl of each sample was injected. Mobile phase A was 0.1% formic acid in water (v/v), and mobile phase B 0.1% formic acid in acetonitrile (v/v). A constant flow rate of 0.3 mL/min was used and the gradient elution was performed as follows: 0 – 15% B from 0 to 15 min, 15–75% B from 15 to 29 min, 75–100% B from 29 to 32 min, isocratic at 100% B from 32 to 36 min, the system was returned to initial conditions from 100% B to 0% B from 36 to 37 min. The HESI source was operated both in positive and negative mode, with a capillary voltage of 3.90 kV and an ion transfer capillary temperature of 350 °C. The sweep

gas and auxiliary pressure rates were set to 35 and 10, respectively. The S-Lens RF level was 50%, and the auxiliary gas heater temperature was 150 °C. The temperature of ion transfer capillary, spray voltage, sheath gas flow rate, auxiliary gas flow rate and S-lens RF level were set to 325 °C, 3.5 kV, 60, 30 and 55, respectively. The Q-Exactive Plus mass spectrometer was calibrated externally in positive and negative ion mode using the manufacturer's calibration solutions (Pierce/Thermo Fisher, Germany). Mass spectra were acquired in MS mode within the mass range of 600–1800 m/z at a resolution of 70,000 FWHM using an Automatic Gain Control (AGC) target of 1.0×10^6 of and 100 ms maximum ion injection time. Data dependent MS/MS spectra in a mass range of 200–2000 m/z were generated for the five most abundant precursor ions with a resolution of 17,500 FWHM using an Automatic Gain Control (AGC) target of 5.0×10^4 of and 64 ms maximum ion injection time and a stepped collision energy of 20, 60 and 150. Xcalibur™ software version 4.0.27 (Thermo Fisher Scientific, San Jose, USA) was used for data acquisition and data analysis.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-020-01468-0.

Additional file 1. Overview about production of surfactin and corresponding optical densities (OD_{600}) of *B. subtilis* BMV9 (3NA *sfp*+) after 48 h cultivation in mineral salt medium supplemented with 30 mM of different amino acids.

Additional file 2. A: Plipastatin variants detected by MS analysis in *B. subtilis* standard. B: Detailed mass spectrometry data of extracted-ion chromatograms regarding to plipastatin standard produced by *B. subtilis* and the sample extracts of BMV12 strain from exponential phase and stationary phase.

Acknowledgments
We are very grateful to Dr. Josef Altenbuchner for providing strain *B. subtilis* JABs32.

Authors' contributions
MV, AR and JP performed the practical work for this study. All authors were involved for interpretation of the results. MV and LL structured the work packages of this project. MV, LL, JP, MH, KMH and RH were involved in writing and editing the manuscript. All authors read and approved the final manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL.. This work was supported by a grant from the Food Security Center (FSC), an excellence center for exchange and development at the University of Hohenheim (funding reference DAAD 57160040).

Availability of data and materials
All raw data and biological material are saved in the institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150 k), University of Hohenheim, Fruwirthstraße 12, Stuttgart 70599, Germany. In case of requirement, please contact the corresponding author for any detailed question.

Ethics approval and consent to participate
Not applicable.

Consent for publication
We agree to publish the manuscript and supplemental data.

Competing interests
Not applicable.

Author details
[1] Institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150K), University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany. [2] Core Facility Hohenheim, Mass Spectrometry Unit, University of Hohenheim, August-von-Hartmann-Str. 3, 70599 Stuttgart, Germany.

Received: 26 June 2020 Accepted: 29 October 2020
Published online: 10 November 2020

References

1. van Dijl J, Hecker M. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. Microb Cell Fact. 2013;12:3.
2. Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C, Mahillon J. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front Microbiol. 2019;10:302.
3. Vahidinasab M, Ahmadzadeh M, Henkel M, Hausmann R, Heravi KM. *Bacillus velezensis* UTB96 Is an Antifungal Soil Isolate with a Reduced Genome Size Compared to That of *Bacillus velezensis* FZB42. Microbiol Res Announc. 2019;8:e00667-e719.
4. Cochrane SA, Vederas JC. Lipopeptides from *Bacillus* and *Paenibacillus* spp.: a gold mine of antibiotic candidates. Med Res Rev. 2016;36:4–31.
5. Geissler M, Heravi KM, Henkel M, Hausmann R. Lipopeptide biosurfactants from *Bacillus* species. In: Hayes D, Solaiman D, Ashby R, editors. Biobased surfactants. New York: Elsevier; 2019. p. 205–40.
6. Raaijmakers JM, de Bruijn I, Nybroe O, Ongena M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev. 2010;34:1037–62.
7. Jacques P. Surfactin and other lipopeptides from *Bacillus* spp. In: Soberon-Chavez G, editor. Biosurfactants. Berlin: Springer; 2011. p. 57–91.
8. Cawoy H, Debois D, Franzil L, de Pauw E, Thonart P, Ongena M. Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. Microb Biotechnol. 2015;8:281–95.
9. Huang X, Lu Z, Zhao H, Bie X, Lü F, Yang S. Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against pseudorabies virus, porcine parvovirus, newcastle disease virus and infectious bursal disease virus in vitro. Int J Pept Res Ther. 2006;12:373–7.
10. Yin H, Guo C, Wang Y, Liu D, Lv Y, Lv F, Lu Z. Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. Anticancer Drugs. 2013;24:587–98.
11. Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, et al. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol. 2007;9:1084–90.
12 Hamdache A, Azarken R, Lamarti A, Aleu J, Collado IG. Comparative genome analysis of *Bacillus* spp. and its relationship with bioactive nonribosomal peptide production. Phytochem Rev. 2013;12:685–716.
13. Inès M, Dhouha G. Lipopeptide surfactants: production, recovery and pore forming capacity. Peptides. 2015;71:100–12.
14. Wu C-Y, Chen C-L, Lee Y-H, Cheng Y-C, Wu Y-C, Shu H-Y, et al. Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. J Biol Chem. 2007;282:5608–16.
15. Gao L, Guo J, Fan Y, Ma Z, Lu Z, Zhang C, et al. Module and individual domain deletions of NRPS to produce plipastatin derivatives in *Bacillus subtilis*. Microb Cell Fact. 2018;17:1–13.
16. Hussein W. Fengycin or plipastatin? A confusing question in *Bacilli*. BioTechnologia. 2019;100:47–55.
17. Samel SA, Wagner B, Marahiel MA, Essen L-O. The thioesterase domain of the fengycin biosynthesis cluster: a structural base for the macrocyclization of a non-ribosomal lipopeptide. J Mol Biol. 2006;359:876–89.

18. Lambalot RH, Gehring AM, Flugel RS, Zuber P, LaCelle M, Marahiel MA, et al. A new enzyme superfamily—the phosphopantetheinyl transferases. Chem Biol. 1996;3:923–36.
19. Zeigler DR, Prágai Z, Rodriguez S, Chevreux B, Muffler A, Albert T, et al. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. J Bacteriol. 2008;190:6983–95.
20 Kunst F, Ogasawara N, Moszer I, Albertini A, Alloni GO, Azevedo V, et al. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature. 1997;390:249–56.
21 Reuß DR, Schuldes J, Daniel R, Altenbuchner J. Complete genome sequence of *Bacillus subtilis* subsp. subtilis strain 3NA. Genome Announc. 2015;3:e00084-15.
22. Nakano MM, Corbell N, Besson J, Zuber P. Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. Mol General Genet MGG. 1992;232:313–21.
23. Tsuge K, Ano T, Hirai M, Nakamura Y, Shoda M. The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrob Agents Chemother. 1999;43:2183–92.
24. Kim PI, Ryu J, Kim YH, Chi Y-T. Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. J Microbiol Biotechnol. 2010;20:138–45.
25. Yaseen Y, Diop A, Gancel F, Béchet M, Jacques P, Drider D. Polynucleotide phosphorylase is involved in the control of lipopeptide fengycin production in *Bacillus subtilis*. Arch Microbiol. 2018;200:783–91.
26 Karata AY, Çetin S, Özcengiz G. The effects of insertional mutations in *comQ*, *comP*, *srfA*, *spo0H*, *spo0A* and *abrB* genes on bacilysin biosynthesis in *Bacillus subtilis*. Biochimica et Biophysica Acta BBA. 2003;1626:51–6.
27. Zeriouh H, de Vicente A, Pérez-García A, Romero D. Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. Environ Microbiol. 2014;16:2196–211.
28. Chumsakul O, Takahashi H, Oshima T, Hishimoto T, Kanaya S, Ogasawara N, Ishikawa S. Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and its homolog Abh reveals their interactive role in transcriptional regulation. Nucleic Acids Res. 2011;39:414–28.
29. Do T-H, Suzuki Y, Abe N, Kaneko J, Itoh Y, Kimura K. Mutations suppressing the loss of DegQ function in *Bacillus subtilis* (natto) poly-γ-glutamate synthesis. Appl Environ Microbiol. 2011;77:8249–58.
30. Tsuge K, Matsui K, Itaya M. Production of the non-ribosomal peptide plipastatin in *Bacillus subtilis* regulated by three relevant gene blocks assembled in a single movable DNA segment. J Biotechnol. 2007;129:592–603.
31. Wang P, Guo Q, Ma Y, Li S, Lu X, Zhang X, Ma P. DegQ regulates the production of fengycins and biofilm formation of the biocontrol agent *Bacillus subtilis* NCD-2. Microbiol Res. 2015;178:42–50.
32. Stanley NR, Lazazzera BA. Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-DL-glutamic acid production and biofilm formation. Mol Microbiol. 2005;57:1143–58.
33. Kaki AA, Smargiasso N, Ongena M, Ali MK, Moula N, de Pauw E, Chaouche NK. Characterization of New Fengycin Cyclic Lipopeptide variants produced by *Bacillus amyloliquefaciens* (ET) originating from a Salt Lake of Eastern Algeria. Curr Microbiol. 2020;77:443–51.
34. Li X-Y, Mao Z-C, Wang Y-H, Wu Y-X, He Y-Q, Long C-L. ESI LC-MS and MS/MS characterization of antifungal cyclic lipopeptides produced by *Bacillus subtilis* XF-1. J Mol Microbiol Biotechnol. 2012;22:83–93.
35. Miras M, Dubnau D. A DegU-P and DegQ-dependent regulatory pathway for the K-state in *Bacillus subtilis*. Frontiers in microbiology. 2016;7:1868.
36. Jeong D-E, So Y, Lim H, Park S-H, Choi S-K. Scarless genomic point mutation to construct a *Bacillus subtilis* strain displaying increased antibiotic plipastatin production. J Microbiol Biotechnol. 2018;28:1030–6.
37. Yaseen Y, Gancel F, Drider D, Béchet M, Jacques P. Influence of promoters on the production of fengycin in *Bacillus* spp. Res Microbiol. 2016;167:272–81.
38. Mäder U, Antelmann H, Buder T, Dahl M, Hecker M, Homuth G. *Bacillus subtilis* functional genomics: genome-wide analysis of the DegS-DegU regulon by transcriptomics and proteomics. Mol Genet Genomics. 2002;268:455–67.
39. López D, Kolter R. Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. FEMS Microbiol Rev. 2010;34:134–49.
40. Briani F, Carzaniga T, Dehò G. Regulation and functions of bacterial PNPase. Wiley Interdiscipl Rev RNA. 2016;7:241–58.
41. Yaseen Y, Gancel F, Béchet M, Drider D, Jacques P. Study of the correlation between fengycin promoter expression and its production by *Bacillus subtilis* under different culture conditions and the impact on surfactin production. Arch Microbiol. 2017;199:1371–82.
42. Geiger T, Wolz C. Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. Int J Med Microbiol. 2014;304:150–5.
43. Steinchen W, Bange G. The magic dance of the alarmones (p) ppGpp. Mol Microbiol. 2016;101:531–44.
44 Sambrook J, Russell DW, Sambrook J. The condensed protocols: from molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006.
45. Chung CT, Niemela SL, Miller RH. One-step preparation of competent *Escherichia coli:* transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci. 1989;86:2172–5.
46. Heravi KM, Altenbuchner J. Cross talk among transporters of the phosphoenolpyruvate-dependent phosphotransferase system in *Bacillus subtilis*. J Bacteriol. 2018;200:e00213-e218.
47. Harwood CR, Cutting SM. Molecular biological methods for *Bacillus*. Hoboken, NJ: Wiley; 1990.
48. Yanisch-Perron C, Vieira J, Messing J, Chambers SP, Prior SE, Barstow DA, et al. Improved M13 phage cloning vectors and host strains: nucleotide. Gene. 1985;33:103–19.
49. Geissler M, Kühle I, Heravi KM, Altenbuchner J, Henkel M, Hausmann R. Evaluation of surfactin synthesis in a genome reduced *Bacillus subtilis* strain. AMB Express. 2019;9:1–14.
50. Michel JF, Millet J. Physiological Studies on Early-blocked Sporulation Mutants of *Bacillus subtilis*. J Appl Bacteriol. 1970;33:220–7.
51. Wenzel M, Altenbuchner J. Development of a markerless gene deletion system for *Bacillus subtilis* based on the mannose phosphoenolpyruvate-dependent phosphotransferase system. Microbiology. 2015;161:1942–9.
52. Altenbuchner J, Viell P, Pelletier I. [40] Positive selection vectors based on palindromic DNA sequences. Methods Enzymol. 1992;216:457–66.
53 Wach A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast. 1996;12:259–65.
54. Willenbacher J, Zwick M, Mohr T, Schmid F, Syldatk C, Hausmann R. Evaluation of different *Bacillus* strains in respect of their ability to produce Surfactin in a model fermentation process with integrated foam fractionation. Appl Microbiol Biotechnol. 2014;98:9623–32.
55 Yazgan A, Özcengiz G, Marahiel MA. Tn10 insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin. Biochem Biophys Acta. 2001;1518:87–94.
56. Geissler M, Oellig C, Moss K, Schwack W, Henkel M, Hausmann R. High-performance thin-layer chromatography (HPTLC) for the simultaneous quantification of the cyclic lipopeptides surfactin, iturin A and fengycin in culture samples of *Bacillus* species. J Chromatogr B. 2017;1044:214–24.

Publisher's Note

2.3 3rd Publication:

Expression of *degQ* gene and its effect on lipopeptide production as well as formation of secretory proteases in *Bacillus subtilis* strains.

Lilge L.*, **Vahidinasab M.***, Adiek I., Becker P., Kuppusamy Nesamani C., Treinen C., Hoffmann M., Morabbi Heravi K., Henkel M., Hausmann R. (2021).

In: MicrobiologyOpen. Oct. 14; 10(5): e1241

DOI: 10.1002/mbo3.1241

*: Co-first authorship

Received: 8 April 2021 | Accepted: 16 September 2021

DOI: 10.1002/mbo3.1241

ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

Expression of *degQ* gene and its effect on lipopeptide production as well as formation of secretory proteases in *Bacillus subtilis* strains

Lars Lilge | **Maliheh Vahidinasab** | **Isabel Adiek** | **Philipp Becker** | **Chanthiya Kuppusamy Nesamani** | **Chantal Treinen** | **Mareen Hoffmann** | **Kambiz Morabbi Heravi** | **Marius Henkel** | **Rudolf Hausmann**

Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology (150), University of Hohenheim, Stuttgart, Germany

Correspondence
Lars Lilge, Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology (150), University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany.
Email: lars.lilge@uni-hohenheim.de

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: 398354917; Food Security Center, Grant/Award Number: 57160040

Abstract

Bacillus subtilis is described as a promising production strain for lipopeptides. In the case of *B. subtilis* strains JABs24 and DSM10^T, surfactin and plipastatin are produced. Lipopeptide formation is controlled, among others, by the DegU response regulator. The activating phospho-transfer by the DegS sensor kinase is stimulated by the pleiotropic regulator DegQ, resulting in enhanced DegU activation. In *B. subtilis* 168, a point mutation in the *degQ* promoter region leads to a reduction in gene expression. Corresponding reporter strains showed a 14-fold reduced expression. This effect on *degQ* expression and the associated impact on lipopeptide formation was examined for *B. subtilis* JABs24, a lipopeptide-producing derivative of strain 168, and *B. subtilis* wild-type strain DSM10^T, which has a native *degQ* expression. Based on the stimulatory effects of the DegU regulator on secretory protease formation, the impact of *degQ* expression on extracellular protease activity was additionally investigated. To follow the impact of *degQ*, a deletion mutant was constructed for DSM10^T, while a natively expressed *degQ* version was integrated into strain JABs24. This allowed strain-specific quantification of the stimulatory effect of *degQ* expression on plipastatin and the negative effect on surfactin production in strains JABs24 and DSM10^T. While an unaffected *degQ* expression reduced surfactin production in JABs24 by about 25%, a sixfold increase in plipastatin was observed. In contrast, *degQ* deletion in DSM10^T increased surfactin titer by threefold but decreased plipastatin production by fivefold. In addition, although significant differences in extracellular protease activity were detected, no decrease in plipastatin and surfactin produced during cultivation was observed.

KEYWORDS
Bacillus subtilis, *degQ*, lipopeptide, plipastatin, secretory proteases, surfactin

Lars Lilge and Maliheh Vahidinasab contributed equally to this work.

1 | INTRODUCTION

B. subtilis is one of the best characterized gram-positive bacteria and serves as a model organism for fundamental and applied research. The knowledge about the physiology of *B. subtilis* made this strain an important microbial host in biotechnology (Stein, 2005). In this context, *B. subtilis* is used as a super-secreting cell factory due to benefits such as excellent fermentation properties, high product yields in gram per liter range, and the lack of toxic by-products (van Dijl & Hecker, 2013). In addition to the production of industrially relevant enzymes and vitamins (Cui et al., 2018), *B. subtilis* natively forms a variety of secondary metabolites. Among these compounds, three lipopeptide families, in particular, namely surfactin, iturin, and fengycin, are reported to have broad bioactivity based on a common amphipathic structure comprising a fatty acid linked to a peptide moiety (Geissler et al., 2019; Marvasi et al., 2010). Different amino acid sequences in the circular peptide and variable fatty acid chain lengths give each lipopeptide unique properties (Zhao et al., 2017). In the genome of *Bacillus* spp., bacteria encoding for fengycin biosynthesis also show the ability to produce surfactin (Kim et al., 2010; Yaseen et al., 2018). In this context, regulatory crosstalk of nonribosomal peptide synthetases (NRPSs) is conceivable (Vahidinasab et al., 2020; Yaseen et al., 2018). Surfactin is described as one of the most powerful microbially produced biosurfactants and has great potential to be used in many industrial sectors such as cosmetics, pharmaceuticals, as well as food (Henkel et al., 2017; Hoffmann et al., 2021). The benefits of surfactin are not limited to emulsifying activity, as some studies reported antimicrobial and anticancer properties (Béven & Wroblewski, 1997; Kameda et al., 1974). Fengycins, including plipastatin as a member of this group, have been shown to have several antagonistic effects for soil-borne fungal phytopathogens and may act as elicitors for systemic plant resistance (Cawoy et al., 2015). Moreover, fengycin has been described to have antiviral, antibacterial, and anticancer properties (Huang et al., 2006; Ongena et al., 2007; Raaijmakers et al., 2010; Yin et al., 2013). Due to these characteristics, fengycin has great potential for future agricultural applications.

Structurally, lipopeptides consist of a cyclic peptide and a fatty acid chain. In the case of surfactin and plipastatin, a hepta- or decapeptide moiety, respectively, is linked to a β-hydroxy fatty acid chain of varying length (Cochrane & Vederas, 2016; Gao et al., 2018). The production of lipopeptides depends on NRPSs expressed by the *srfAA-AD* operon for surfactin and by the *ppsA-E* operon for plipastatin (Nakano et al., 1988; Tosato et al., 1997). Posttranslationally, NRPSs need to be activated by the 4-phosphopantetheinyl transferase Sfp (Nakano et al., 1992; Quadri et al., 1998). In addition, superordinated stimuli such as quorum sensing and nutrient availability influence lipopeptide biosynthesis. Specifically, several global regulators including Spo0A, AbrB, CodY, and DegU are involved in the control of NRPS expression (Nakano et al., 1992; Serror & Sonenshein, 1996; Sun et al., 2021; Vahidinasab et al., 2020).

Different physiological adaptations are associated with the DegU regulon, including the formation of extracellular enzymes, genetic competence, and biofilm formation (Dahl et al., 1992; Kobayashi, 2007; Mäder et al., 2002; Msadek et al., 1990; Shimotsu & Henner, 1986). Moreover, also surfactin and plipastatin production are affected by DegU regulation (Miras & Dubnau, 2016; Tsuge et al., 1999). As a response regulator, DegU is part of the two-component DegS-DegU system. After activating phospho-transfer from histidine kinase DegS to the response regulator DegU, the phosphorylated DegU version (DegU-P) can regulate the expression of various genes (Murray et al., 2009). In addition to this process, DegQ, a small protein of 46 amino acids, stimulates the autophosphorylation of DegS and is important for the complete activation of DegU by phosphorylation (Do et al., 2011; Yang et al., 1986). In the case of the domesticated laboratory model strain *B. subtilis* 168, a single base mutation in the −10 box silences *degQ* gene expression (Stanley & Lazazzera, 2005). As a result, phospho-transfer for DegU activation is reduced.

In this study, the lipopeptide-producing *B. subtilis* strain JABs24, an *sfp*$^+$ derivative of *B. subtilis* 168, and the wild-type strain DSM10^T, which exhibits a native *degQ* expression, were used to analyze the effect of *degQ* expression on lipopeptide production and formation of secretory proteases.

2 | MATERIALS AND METHODS

2.1 | Chemicals, materials, and standard procedures

All chemicals were purchased from Carl Roth GmbH & Co. KG, if not otherwise mentioned. Standard molecular techniques were performed as described by Sambrook and Russell (2006). PCRs were carried out on a PCR thermal cycler (peqSTAR 96X VWR GmbH) using DNA polymerase (Phusion High-Fidelity #M0530S, New England BioLabs). PCR reactions were purified after agarose-based gel electrophoresis using QIAquick PCR & Gel Cleanup Kit (Qiagen). Plasmid DNA was extracted with innuPREP Plasmid Mini Kit (Analytik Jena AG), and chromosomal DNA was purified using the ready-to-use innuPREP Bacteria DNA Kit (Analytik Jena AG) according to the manufacture's instruction.

2.2 | Strain construction, plasmids, and transformation method

All strains and plasmids used in this study are summarized in Table 1. The oligonucleotides used to construct the strains and plasmids are listed in Table 2. *Escherichia coli* JM109 was used for plasmid propagation and cloning. Transformation of naturally competent *B. subtilis* strains was performed according to the "Paris method" (Harwood & Cutting, 1990). Depending on the selection marker, transformants were selected on Lysogeny Broth agar supplemented with ampicillin (100 μg/ml), spectinomycin (100 μg/ml), or erythromycin (10 μg/ml for *E. coli* and 5 μg/ml for *B. subtilis*). All plates were incubated at 37°C.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Origin or Genotype	References
Strains		
Escherichia coli		
JM109	*mcrA recA1 supE44 endA1 hsdR17* ($r_K^- m_K^+$) *gyrA96 relA1 thi* Δ(*lac-proAB*) *F´*[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*]	Yanisch-Perron et al. (1985)
Bacillus subtilis		
JABs24	*trp+*; Δ*manPA*; *sfp+*	Geissler et al. (2019)
DSM10^T	wild-type strain	German Collection of Microorganisms and Cell Cultures GmbH
BCKN1	*trp+*; Δ*manPA*; *sfp+*; Δ*amyE*::+510 bp-*degQ*	This study
BCKN2	DSM10^T; Δ*degQ*::*erm*	This study
BKE31720	*trpC2*; Δ*degQ*::*erm*	Bacillus Genetic Stock Center
BMV15	DSM10^T wild-type; *amyE*::[P_{degQ}-*lacZ*, *spcR*] (*degQ* promoter region for *lacZ* fusion was derived from *B. subtilis* DSM10^T)	This study
BMV16	*trp+*; Δ*manPA*; *sfp+*; *amyE*::[P_{degQ}-*lacZ*, *spcR*] (*degQ* promoter region for *lacZ* fusion was derived from *B. subtilis* DSM10^T)	This study
BMV17	DSM10^T wild-type; *amyE*::[P_{degQ}-*lacZ*, *spcR*] (*degQ* promoter region for *lacZ* fusion was derived from *B. subtilis* JABs24)	This study
BMV18	*trp+*; Δ*manPA*; *sfp+*; *amyE*::[P_{degQ}-*lacZ*, *spcR*] (*degQ* promoter region for *lacZ* fusion was derived from *B. subtilis* JABs24)	This study
Plasmids		
pKAM446	ori_{pUC18}, *bla*, *rop*, *ermC*, *amyE´*-[*ter*-P_{srfAA}-*lacZ*, *spcR*]- *´amyE*	Hoffmann et al. (2021)
pMAV5	ori_{pBR322}, *rop*, *ermC*, *bla*, *amyE´*-[*ter*-P_{glcR} - +510 bp-*degQ*-*spcR*]-*´amyE*	Vahidinasab et al. (2020)
pMAV14	ori_{pUC18}, *bla*, *rop*, *ermC*, *amyE´*-[*ter*-P_{degQ}-*lacZ*, *spcR*]- *´amyE* (*degQ* promoter sequence derived from *B. subtilis* JABs24)	This study
pMAV15	ori_{pUC18}, *bla*, *rop*, *ermC*, *amyE´*-[*ter*-P_{degQ}-*lacZ*, *spcR*]- *´amyE* (*degQ* promoter sequence derived from *B. subtilis* DSM10^T)	This study

For the construction of BCKN1, the *degQ* gene of *B. subtilis* DSM10^T including native promoter region (+ 510 bp) and terminator structure was integrated into the *amyE* locus of *B. subtilis* JABs24 using plasmid pMAV5 (Vahidinasab et al., 2020). BCKN2 was created by integrating the deletion of *degQ* gene in *B. subtilis* DSM10^T using chromosomal DNA of *Bacillus* knockout erythromycin (BKE) strain

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5´→ 3´)	Purpose
S1411	GATTAAAGACCGTATCCACTTC	Amplification of
S1412	GGCGCTTAAGATATAAGTAAATCAG	Δ*degQ*::*erm* locus from BKE31720 strain
S1079	TCGGTGAAAAATGAGCC	Verification of Δ*degQ*::*erm* integration into DSM10^T strain
S1080	GCTCAATAACGACTTCC	
S1009	CTGCCGTTATTCGCTGGATT	Verification of +510 bp-*degQ* integration into *amyE* locus
S1010	AGAGAACCGCTTAAGCCCGA	
S1699	TGGATCCGGCGCCCACGTGGCTCG-CAAAAAAGGATGTTTCTATATG	Construction of P_{degQ} reporter plasmids
S1700	AGTGAATCCGTAATCATGGTCATCG-TTTCCACACTCCTTT	

BKE31720 carrying the deletion of the *degQ* gene (Δ*degQ*::*erm*) (Koo et al., 2017). The plasmids for the construction of the P_{degQ} reporter strains were cloned using Gibson Assembly (New England BioLabs). Therefore, the pKAM446 plasmid was digested with *Nhe*I and *Nde*I before integrating amplified *degQ* promoter regions from JABs24 and DSM10^T, respectively. The correctness of all mutant strains was ensured by sequencing (Eurofins Genomics Germany GmbH).

2.3 | Cultivation and growth conditions

The composition of the mineral salt medium used in this study was based on the fermentation medium containing 8 g/L glucose of Willenbacher et al., (2014) with slight modifications: 4.0×10^{-6} M $Na_2EDTA \times 2\ H_2O$, 7.0×10^{-6} M $CaCl_2$, 4.0×10^{-6} M $FeSO_4 \times 7\ H_2O$, 1.0×10^{-6} M $MnSO_4 \times H_2O$, 50 mM $(NH_4)_2SO_4$, 0.03 M KH_2PO_4, 0.04 M $Na_2HPO_4 \times 2\ H_2O$ and 8.0×10^{-4} M $MgSO_4 \times 7\ H_2O$. An overnight culture in Lysogeny Broth medium (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) was used for the first preculture. The second preculture using a mineral salt medium was inoculated with exponentially growing cells from the first preculture. When the cell culture reached the exponential phase, the main culture was inoculated into 1 L Erlenmeyer baffled flasks with a final volume of 100 ml and an initial OD_{600} of 0.1. All cultivations had three biological replicates and were performed at 37°C and 0.4 *g* in an incubation shaker (Innova 44®R, Eppendorf AG). Samples were taken regularly every four hours to measure optical density (OD_{600}) using a spectrophotometer (Biochrom WPA CO8000, Biochrom Ltd.), glucose concentration using HPTLC measurement (Geissler et al., 2019), β-galactosidase activity (Miller Assay) described by Hoffmann et al., (2020), and (endo)-protease activity. Surfactin and plipastatin concentration were measured as previously described by Geissler et al., (2017). Specifically, 2 ml of cell-free supernatant was extracted three times with chloroform/methanol (2:1). The pooled solvent layers were dried using a rotary evaporator at 10 mbar and 40°C. Dried samples were resolved in 2 ml methanol and applied in 6 mm bands on a silica HPTLC plate. As a mobile phase, chloroform/methanol/water (65:25:4) was used with a migration distance over 60 mm. Surfactin standard from Sigma Aldrich and plipastatin standard from Lipofabrik were used for quantification.

2.4 | Data analysis

For the conversion of OD_{600} into cell dry weight (CDW), the correlation factor of 3.762 was determined in a preliminary experiment described by Willenbacher et al. (2014). The product yield of biomass $Y_{P/X}$ [g g^{-1}] was calculated using Equation 1. For the calculation, the mean values of the total mass of the product ($m_{Surfactin}$, $m_{Plipastatin}$) and CDW (m_{CDW}) from the beginning of cultivation to the time point at the end of the exponential phase were used.

$$Y_{P/X} = \frac{\Delta m_{\text{surfactin or plipastatin}}}{m_{CDW}} \left[g \bullet g^{-1}\right] \quad (1)$$

2.5 | Assay for extracellular protease activity

The total activity of the degrading proteins in the cultivation medium was analyzed by azocasein assay. The measurement method was adapted from Charney and Tomarell (1947) and applied by Baur et al., 2015. In detail, 100 µl of cell-free supernatant was mixed with an equal volume of a pre-warmed (40°C for 5 min) azocasein solution (5 g/L, pH 7, dissolved in H_2O_{dd}) and subsequently incubated for 1 h at 37°C and 1.07 *g*. The reaction was stopped by adding 20 µl trichloroacetic acid (2 M). Precipitated azocasein was removed by centrifugation at 1715 *g* for 10 min at 4°C. Subsequently, 150 µl of the supernatant was transferred to a microtiter plate and mixed with 50 µl NaOH (1 M). The absorbance was measured in a microtiter plate spectrophotometer (MULTISKAN GO, Thermo Scientific) at 450 nm. A blank for the measurement was performed with the cell-free supernatant after the addition of trichloroacetic acid. A calculation of the protease activity is summarized in Equations (2) and (3).

$$\Delta A = \frac{\sum A}{n} - A_{Blank}[-] \quad (2)$$

$$EA = \frac{\Delta A \bullet F \bullet V}{t \bullet v} \bullet 10 \left[\Delta A \bullet h^{-1} \bullet mL^{-1}\right] \quad (3)$$

The calculation of the absorption difference (ΔA) is described in Equation (2). The following volumetric peptidase activity is defined in Equation (3) and is determined by the absorption difference (ΔA), a dilution factor (F), the total measurement volume (V) [µl], the incubation time (t) [h], and the volume of the cell-free supernatant used for the assay (v) [µl].

3 | RESULTS

3.1 | Expression of *degQ* gene in *B. subtilis* strains JABs24 and DSM10^T

As a derivative of *B. subtilis* 168, the surfactin-forming strain JABs24 exhibits a point mutation within the −10 box of the *degQ* gene compared to *B. subtilis* wild-type strains such as *B. subtilis* DSM10^T (Figure 1). This base substitution (T::C) in the promoter region of *degQ* was previously described by Stanley and Lazazzera (2005) and leads to significantly reduced gene expression of *degQ* in strain 168. This point mutation is also prominent when comparing the genome sequences of *B. subtilis* strains 168 and DSM10^T (Kunst et al., 1997; Lilge et al., 2021).

To analyze the effect of promoter point mutation on *degQ* gene expression, reporter strains with chromosomally integrated P_{degQ}-*lacZ* fusions were constructed. Accordingly, time-resolved expression patterns were measured for both *degQ* promoter versions in JABs24 (BMV16 and BMV18) and DSM10^T (BMV15 and BMV17). The corresponding β-galactosidase activity showed that the P_{degQ} promoter region of DSM10^T exhibited a significantly higher expression level compared with that of JABs24 (Figure 2). In the transition between the exponential and stationary phase, approx. 14-fold higher Miller Units were detected for the *lacZ* fusion with *degQ* promoter from DSM10^T.

In addition to the confirmation that *degQ* expression is nearly silenced in strain JABs24, the results have shown that both *degQ* promoter versions were more active in the DSM10^T background. Thus, threefold increased promoter activity was detected for P_{degQ}^{DSM10T} and sixfold higher Miller Units were calculated for P_{degQ}^{JABs24} in the transition phase.

3.2 | Effect of *degQ* expression on the formation of lipopeptides and secretory proteases

It is already known that DegQ acts as a stimulator for autophosphorylation of DegS signal kinase leading to enhanced activation of DegU response regulator (Do et al., 2011). In the active state, DegU-P controls a variety of genes encoding secretory proteases, flagellin proteins, and non-ribosomal peptide synthetases for the biosynthesis of plipastatin and surfactin (Hsueh et al., 2011; Mäder et al., 2002; Miras & Dubnau, 2016; Tsuge et al., 1999; Wang et al., 2015). To get an overview of the influence of different *degQ* gene expressions on biotechnologically relevant production of plipastatin and surfactin as well as secretory proteases, production strains with different *degQ* expression capabilities were analyzed. For this purpose, the wild-type strains JABs24, DSM10^T, and their *degQ* mutant strains BCKN1 and BCKN2 were examined. While BCKN1 represents strain JABs24 with native *degQ* gene expression, BCKN2 is the DSM10^T strain with *degQ* deletion.

Using 8 g/L glucose, cell dry weights reduced drastically after complete glucose depletion during the cultivation, resulting in detectable cell lysis without any stationary phase (Willenbacher et al., 2015). However, as previously shown by Vahidinasab et al., (2020), the concentrations of surfactin and plipastatin are not negatively affected by the reduction of cell dry weight (CDW) during glucose

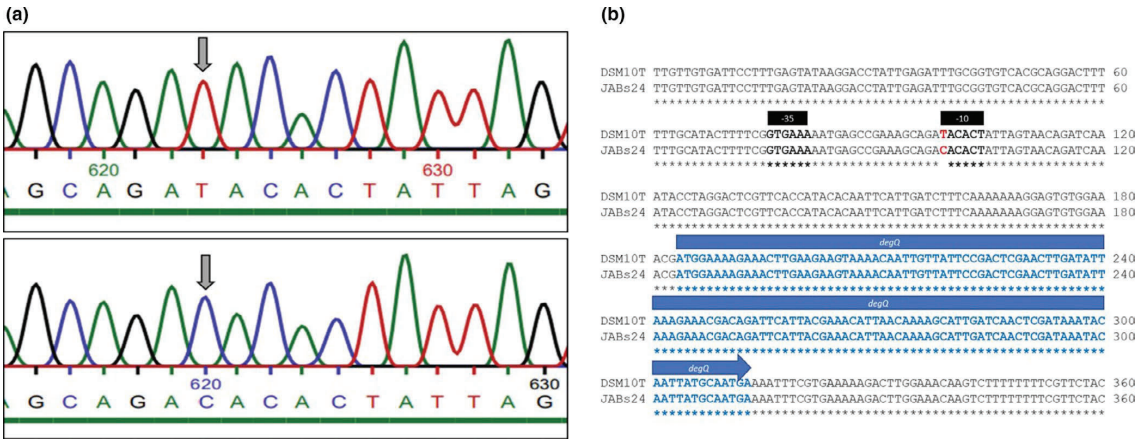


FIGURE 1 Comparison of *degQ* locus between JABs24 (168 *sfp+*) and DSM10^T strain. (a) The chromatograms obtained after the sequencing process show the base-pair substitution (T::C) in the −10 promoter region of *degQ*. The extended *degQ* regions of *B. subtilis* strains DSM10^T (top) and JABs24 (bottom) were amplified and sequenced by Eurofins Genomics (Ebersberg, Germany). (b) Obtained sequences were compared and identical nucleotides were marked by stars (*). Information about the annotation of the *degQ* promoter region was used from Stanley and Lazazzera (2005)

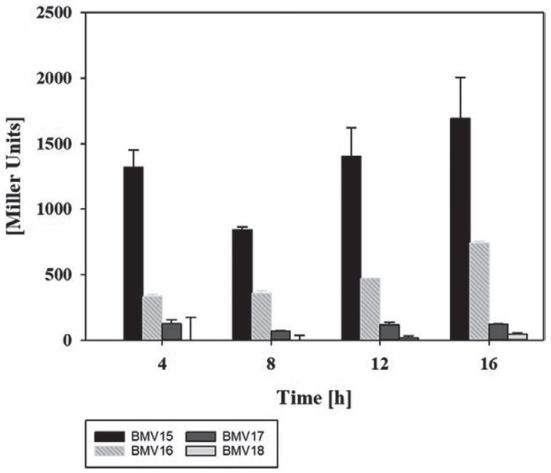


FIGURE 2 Comparison of *degQ* gene expression under the control of native and point-mutated *degQ* promoter during 16-hour shake flask cultivation with 8 g/L glucose. The *lacZ* fusion construct with native *degQ* promoter was chromosomally integrated into *B. subtilis* DSM10^T and JABs24, resulting in strains BMV15 and BMV16, respectively. Similarly, strains BMV17 and BMV18 are the reporter strains with point-mutated *degQ* promoter for DSM10^T and JABs24. Data points represent a mean of three biological replicates. The error bars show the standard deviation of the calculated values

limitation. Specifically, the wild-type strains JABs24 and DSM10^T showed contrary productivities with respect to surfactin and plipastatin formation (Figure 3a,b). While JABs24 produced a maximum surfactin concentration of 1007 mg/L, only 0.6 mg/L of plipastatin was detected (LOD and LOQ for plipastatin is 27 and 82 ng/zone, Geissler et al., 2017). In contrast, DSM10^T produced a comparatively low surfactin titer of 473 mg/L but 27 mg/L of plipastatin. In comparison, the corresponding *degQ* mutant strains showed altered lipopeptide productivities (Figure 3c,d). In the case of BCKN1, the strain JABs24 with native *degQ* expression, a reduced surfactin concentration of 753 mg/L was measured, whereas the plipastatin titer was increased to 4.1 mg/L. In contrast, BCKN2, the *degQ* deletion mutant of DSM10^T, showed a promising increase in surfactin production up to 1520 mg/L, whereas a 5.2-fold decrease in plipastatin production to 5.2 mg/L was determined.

Since *degQ* expression is also directly associated with secretory protease production, JABs24 and DSM10^T wild-type strains as well as their *degQ* mutants were examined for their ability to form extracellular proteases. Therefore, endopeptidase activity was measured in cell-free supernatants using an azocasein assay. In detail, JABs24 showed a basal activity for extracellular proteases during cultivation with a comparatively small increase to 8.7 ΔA/(h·mL) during the late exponential phase (Figure 3a). In contrast, strain DSM10^T showed the highest protease activity of up to 42.8 ΔA/(h·mL), which reached a plateau after 12 h of cultivation (Figure 3b). In respect of the stability of lipopeptides, no reduction of surfactin and plipastatin concentration was detected for both JABs24 and DSM10^T suggesting that secretion of proteases has an inferior impact on lipopeptide production.

In comparison, integration of a natively expressed *degQ* version from the DSM10^T strain into JABs24 increased secretory protease activity 2 times (17.6 ΔA/(h·mL) after 24 h) compared to JABs24 (Figure 3c). A comparably great effect was observed for BCKN2, resulting in a continuous basal level of up to 6.2 ΔA/(h·mL) at the end of cultivation (Figure 3d). In this way, deletion of the *degQ* gene in DSM10^T reduced extracellular protease activity sevenfold.

Table 3 gives an overall summary of the effect of *degQ* gene expression on the lipopeptide productivity and secretory protease formation of the wild-type strains JABs24 and DSM10^T and their *degQ* mutant strains.

4 | DISCUSSION

Due to the point mutation within the *degQ* promoter region, *B. subtilis* JABs24, the lipopeptide-forming derivative of *B. subtilis* 168, shows a drastically reduced *degQ* gene expression. This circumstance was already described by Stanley and Lazazzera (2005) and confirmed by using *lacZ* reporter strains for a time-resolved comparison of the expression of the two *degQ* promoter versions until the transient growth phase (Figure 2). In this process, the wild-type strain DSM10^T showed much higher P_{degQ} promoter activity compared to JABs24. Since DegQ is directly involved in the activation of the DegU response regulator, it is reasonable to assume that DSM10^T also displays a more stimulated DegU regulation. The positive feedback regulation of DegU-P on *degQ* gene expression amplifies the difference between JABs24 and DSM10^T in terms of P_{degQ} promoter activity. The varying DegQ-mediated activation of the DegU regulon was also observed by the detection of the lipopeptides surfactin and plipastatin as well as the formation of secretory proteases. Accordingly, a natively expressed *degQ* version reduced surfactin but increased plipastatin production, while a significantly higher extracellular protease activity was detected in the presence of the non-mutated *degQ* promoter version.

While surfactin production is negatively affected by DegQ-associated DegU regulation, increased plipastatin biosynthesis is achieved in the presence of native *degQ* expression (Miras & Dubnau, 2016; Vahidinasab et al., 2020). This opposing regulatory mechanism was transferable to both JABs24 and DSM10^T. Accordingly, after integration of a natively expressed *degQ* version in JABs24, the resulted strain BCKN1 produced only approx. 75% of surfactin but eightfold increased plipastatin titers, while the elimination of *degQ* in DSM10^T (strain BCKN2) showed a threefold increase in surfactin concentration and a fivefold reduction in plipastatin formation. In summary, DegQ can be considered as a regulatory decision point for DegU-mediated production of either surfactin or plipastatin. Accordingly, lipopeptide-producing derivative strains of *B. subtilis* 168, encoding silenced *degQ* expression, appear to be predestinated for surfactin formation, whereas DSM10^T and other *B. subtilis* wild-type strains show more effective plipastatin or fengycin production.

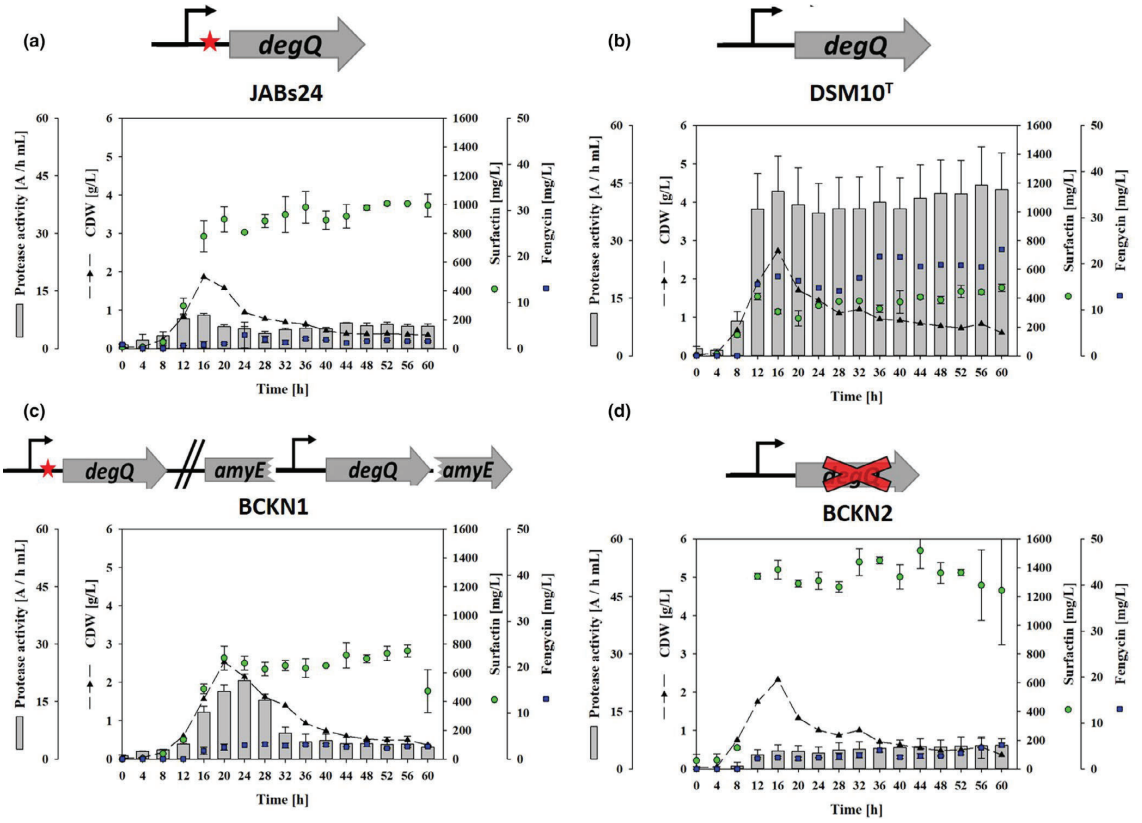


FIGURE 3 Comparison of lipopeptide production and extracellular protease activity during the time course of shake flask cultivation with 8 g/L glucose. Production parameters were determined for (a) JABs24 (168 *sfp*+), (b) DSM10^T, (c) BCKN1 (JABs24 *amyE*::P_{degQ}-*degQ* from DSM10^T), and (d) BCKN2 (DSM10^T *degQ*::*erm*). Gray bars indicate the extracellular protease activity, dashed lines represent the cell dry weight (CDW) and green dots display the surfactin, blue dots represent the plipastatin concentration over cultivation time. The data points represent a mean of at least two biological replicates. The error bars show the standard deviation of calculated values

TABLE 3 Summary of parameters of cultivation with JABs24 and DSM10^T wild-type strains and their inversed *degQ* mutant strains BCKN1 and BCKN2.

	End of exponential phase						
***B. subtilis* strains**	**Cultivation time [h]**	**CDW [g/L]**	**surfactin conc. [mg/L]**	**$Y_{P/X,\ surfactin}$ [mg/g]**	**plipastatin conc. [mg/L]**	**$Y_{P/X,\ plipastatin}$ [mg/g]**	**secretory protease activity [ΔA/h·mL]**
JABs24	20	1.58	898.7	568.8	0.5	0.32	5.7
DSM10^T	16	2.73	306.9	112.4	18.6	6.81	42.8
BCKN1	16	1.56	488.0	312.8	4.2	2.69	9.8
BCKN2	20	1.33	1290.0	969.9	3.2	2.41	4.7

Besides the biotechnologically useful production of lipopeptides, another aspect is the DegU-associated formation of secretory proteases. In this study, the comparison of extracellular protease activities between JABs24 and DSM10^T showed the effect of silenced *degQ* gene expression. Thus, DSM10^T was found to have fivefold higher protease activity compared to JABs24. Notably, both surfactin and plipastatin showed no decrease in their concentrations during the cultivation process, although secretory protease activity differed significantly between both strains, suggesting that lipopeptides are less targeted by native extracellular proteases. Subsequent integration of a natively expressed *degQ* version in JABs24 (strain BCKN1) increased extracellular protease production twofold, whereas a sevenfold decrease was observed after deletion of *degQ* in DSM10^T (BCKN2). Altogether, evidence for a quantitative effect of *degQ* expression on the production of extracellular proteases is documented.

5 | CONCLUSIONS

The *degQ* loci of the lipopeptide-producing strains DSM10^T and JABs24 differ by a single point mutation that leads to a drastic reduction of *degQ* gene expression in JABs24. Based on opposing regulatory mechanisms related to the DegU regulator, the presented strains show beneficial yields in surfactin or plipastatin production, which was confirmed by constructed *degQ* mutant strains. An additional negative effect of silenced *degQ* expression in JABs24 was furthermore quantitatively examined on the formation of extracellular proteases. Although a lipopeptide degradation cannot be excluded, different signal strengths of the protease activities measured during the cultivation processes did not lead to a decrease in lipopeptide concentration.

ETHICS STATEMENT

None required.

ACKNOWLEDGMENTS

We thank Jens Pfannstiel and Philipp Hubel for their critical reading of the manuscript and fruitful discussions. The study was financially supported by the German Research Foundation (DFG), grant number 398354917, and Maliheh Vahidinasab was financially supported by a personal grant from the Food Security Center (FSC), an excellence center for exchange and development at the University of Hohenheim (grant number DAAD 57160040).

CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

Lars Lilge: Conceptualization—Lead, Project administration—Lead, Investigation—Equal, Supervision—Lead, Data curation—Equal, Formal analysis—Equal, Methodology—Equal, Writing-original draft—Equal, Writing-review & editing—Equal. Maliheh Vahidinasab: Visualization—Leading, Data curation—Equal, Formal analysis—Equal, Methodology—Equal, Writing-original draft—Equal, Writing-review & editing—Equal. Isabel Adiek, Philipp Becker and Chanthiya Kuppusamy Nesamani: Methodology—Equal, Data curation—Equal, Formal analysis—Equal. Chantal Treinen: Formal analysis—Equal, Methodology—Equal, Writing-review & editing—Equal. Mareen Hoffmann and Kambiz Morabbi Heravi: Formal analysis—Equal, Writing-review & editing—Equal. Marius Henkel: Formal analysis—Equal, Data curation—Equal, Writing-review & editing—Equal. Rudolf Hausmann: Funding acquisition—Leading, Formal analysis—Equal, Writing-review & editing—Equal.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. An overview of the collected data is available in Zenodo at https://doi.org/10.5281/zenodo.5511929

ORCID

Lars Lilge https://orcid.org/0000-0002-7693-477X

REFERENCES

Baur, C., Krewinkel, M., Kutzli, I., Kranz, B., Neubeck, M. V., & Huptas, C. (2015). Isolation and characterization of a heat-resistant peptidase from *Pseudomonas panacis* withstanding general UHT processes. *International Dairy Journal*, *49*, 46–55.

Béven, L., & Wroblewski, H. (1997). Effect of natural amphipathic peptides on viability, membrane potential, cell shape and motility of mollicutes. *Research in Microbiology*, *148*(2), 163–175. https://doi.org/10.1016/S0923-2508(97)87647-4.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P., & Ongena, M. (2015). Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. *Microbial Biotechnology*, *8*(2), 281–295.

Charney, J., & Tomarelli, R. M. (1947). A colorimetric method for the determination of the proteolytic activity of duodenal juice. *Journal of Biological Chemistry*, *171*(2), 501–505. https://doi.org/10.1016/S0021-9258(17)41059-3.

Cochrane, S. A., & Vederas, J. C. (2016). Lipopeptides from *Bacillus* and *Paenibacillus* spp.: A gold mine of antibiotic candidates. *Medicinal Research Reviews*, *36*(1), 4–31.

Cui, W., Han, L., Suo, F., Liu, Z., Zhou, L., & Zhou, Z. (2018). Exploitation of *Bacillus subtilis* as a robust workhorse for production of heterologous proteins and beyond. *World Journal of Microbiology and Biotechnology*, *34*(10), 145. https://doi.org/10.1007/s11274-018-2531-7.

Dahl, M. K., Msadek, T., Kunst, F., & Rapoport, G. (1992). The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in *Bacillus subtilis*. *Journal of Biological Chemistry*, *267*(20), 14509–14514. https://doi.org/10.1016/S0021-9258(19)49742-1.

Do, T.-H., Suzuki, Y., Abe, N., Kaneko, J., Itoh, Y., & Kimura, K. (2011). Mutations suppressing the loss of DegQ function in *Bacillus subtilis* (natto) poly-γ-glutamate synthesis. *Applied and Environmental Microbiology*, *77*(23), 8249–8258. https://doi.org/10.1128/AEM.05827-11.

Gao, L., Guo, J., Fan, Y., Ma, Z., Lu, Z., Zhang, C., Zhao, H., & Bie, X. (2018). Module and individual domain deletions of NRPS to produce plipastatin derivatives in *Bacillus subtilis*. *Microbial Cell Factories*, *17*(1), 84. https://doi.org/10.1186/s12934-018-0929-4.

Geissler, M., Heravi, K. M., Henkel, M., & Hausmann, R. (2019). Lipopeptide biosurfactants from *Bacillus* species. In *Biobased surfactants* (pp. 205–240). AOCS Press.

Geissler, M., Kühle, I., Heravi, K. M., Altenbuchner, J., Henkel, M., & Hausmann, R. (2019). Evaluation of surfactin synthesis in a genome reduced *Bacillus subtilis* strain. *AMB Express*, *9*(1), 1–14. https://doi.org/10.1186/s13568-019-0806-5.

Geissler, M., Oellig, C., Moss, K., Schwack, W., Henkel, M., & Hausmann, R. (2017). High-performance thin-layer chromatography (HPTLC) for the simultaneous quantification of the cyclic lipopeptides surfactin, iturin A and fengycin in culture samples of *Bacillus* species. *Journal of Chromatography B*, *1044*, 214–224. https://doi.org/10.1016/j.jchromb.2016.11.013.

Harwood, C. R., & Cutting, S. M. (1990) Molecular biological methods for *Bacillus: Wiley*.

Henkel, M., Geissler, M., Weggenmann, F., & Hausmann, R. (2017). Production of microbial biosurfactants: Status quo of rhamnolipid and surfactin towards large-scale production. *Biotechnology Journal*, *12*(7), 1600561. https://doi.org/10.1002/biot.201600561.

Hoffmann, M., Braig, A., Fernandez Cano Luna, D. S., Rief, K., Becker, P., Treinen, C., Klausmann, P., Morabbi Heravi, K., Henkel, M., Lilge, L., & Hausmann, R. (2021). Evaluation of an oxygen-dependent self-inducible surfactin synthesis in B. subtilis by substitution of native promoter P_{srfA} by anaerobically active P_{narG} and P_{nasD}. *AMB Express*, *11*(1), 1–13. https://doi.org/10.1186/s13568-021-01218-4.

Hoffmann, M., Fernandez Cano Luna, D. S., Xiao, S., Stegemüller, L., Rief, K., Heravi, K. M., Lilge, L., Henkel, M., & Hausmann, R. (2020). Towards the anaerobic production of surfactin using *Bacillus subtilis*. *Frontiers in Bioengineering and Biotechnology*, *8*, 554903. https://doi.org/10.3389/fbioe.2020.554903.

Hoffmann, M., Mück, D., Grossmann, L., Greiner, L., Klausmann, P., Henkel, M., Lilge, L., Weiss, J., & Hausmann, R. (2021). Surfactin from *Bacillus subtilis* displays promising characteristics as O/W-emulsifier for food formulations. *Colloids and Surfaces B: Biointerfaces*, *203*, 111749. https://doi.org/10.1016/j.colsurfb.2021.111749.

Hsueh, Y.-H., Cozy, L. M., Sham, L.-T., Calvo, R. A., Gutu, A. D., Winkler, M. E., & Kearns, D. B. (2011). DegU-phosphate activates expression of the anti-sigma factor FlgM in *Bacillus subtilis*. *Molecular Microbiology*, *81*(4), 1092–1108. https://doi.org/10.1111/j.1365-2958.2011.07755.x.

Huang, X., Lu, Z., Zhao, H., Bie, X., Lü, F., & Yang, S. (2006). Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against pseudorabies virus, porcine parvovirus, newcastle disease virus and infectious bursal disease virus *in vitro*. *International Journal of Peptide Research and Therapeutics*, *12*, 373–377. https://doi.org/10.1007/s10989-006-9041-4.

Kameda, Y., Ouhira, S., Matsui, K., Kanatomo, S., Hase, T., & Atsusaka, T. (1974). Antitumor activity of *Bacillus natto*. V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. *Chemical and Pharmaceutical Bulletin*, *22*(4), 938–944.

Kim, P. I., Ryu, J., Kim, Y. H., & Chi, Y.-T. (2010). Production of biosurfactant lipopeptides iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. *Journal of Microbiology and Biotechnology*, *20*, 138–145.

Kobayashi, K. (2007). Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Molecular Microbiology*, *66*(2), 395–409.

Koo, B.-M., Kritikos, G., Farelli, J. D., Todor, H., Tong, K., Kimsey, H., Wapinski, I., Galardini, M., Cabal, A., Peters, J. M., Hachmann, A.-B., Rudner, D. Z., Allen, K. N., Typas, A., & Gross, C. A. (2017). Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell Systems*, *4*(3), 291–305. e7. https://doi.org/10.1016/j.cels.2016.12.013.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., … Danchin, A. (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature*, *390*(6657), 249–256. https://doi.org/10.1038/36786.

Lilge, L., Hertel, R., Morabbi Heravi, K., Henkel, M., Commichau, F. M., & Hausmann, R. (2021). Draft genome sequence of the type strain *Bacillus subtilis* subsp. *subtilis* DSM10. *Microbiology Resource Announcements*, *10*(10):e00158–21.

Mäder, U., Antelmann, H., Buder, T., Dahl, M., Hecker, M., & Homuth, G. (2002). *Bacillus subtilis* functional genomics: genome-wide analysis of the DegS-DegU regulon by transcriptomics and proteomics. *Molecular Genetics and Genomics*, *268*(4), 455–467. https://doi.org/10.1007/s00438-002-0774-2.

Marvasi, M., Visscher, P. T., & Casillas Martinez, L. (2010). Exopolymeric substances (EPS) from *Bacillus subtilis*: Polymers and genes encoding their synthesis. *FEMS Microbiology Letters*, *313*(1), 1–9.

Miras, M., & Dubnau, D. (2016). A DegU-P and DegQ-dependent regulatory pathway for the K-state in *Bacillus subtilis*. *Frontiers in Microbiology*, *7*, 1868. https://doi.org/10.3389/fmicb.2016.01868.

Msadek, T., Kunst, F., Henner, D., Klier, A., Rapoport, G., & Dedonder, R. (1990). Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in *degS* and *degU*. *Journal of Bacteriology*, *172*(2), 824–834. https://doi.org/10.1128/jb.172.2.824-834.1990.

Murray, E. J., Kiley, T. B., & Stanley-Wall, N. R. (2009). A pivotal role for the response regulator DegU in controlling multicellular behaviour. *Microbiology*, *155*(1), 1–8. https://doi.org/10.1099/mic.0.023903-0.

Nakano, M. M., Corbell, N., Besson, J., & Zuber, P. (1992). Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Molecular and General Genetics MGG*, *232*(2), 313–321. https://doi.org/10.1007/BF00280011.

Nakano, M. M., Marahiel, M. A., & Zuber, P. (1988). Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *Journal of Bacteriology*, *170*(12), 5662–5668. https://doi.org/10.1128/jb.170.12.5662-5668.1988.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J.-L., & Thonart, P. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental Microbiology*, *9*, 1084–1090. https://doi.org/10.1111/j.1462-2920.2006.01202.x.

Quadri, L. E. N., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., & Walsh, C. T. (1998). Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. *Biochemistry*, *37*(6), 1585–1595.

Raaijmakers, J. M., de Bruijn, I., Nybroe, O., & Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiology Reviews*, *34*, 1037–1062.

Sambrook, J., & Russell, D. W. (2006) The condensed protocols from molecular cloning: a laboratory manual.

Serror, P., & Sonenshein, A. L. (1996). CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. *Journal of Bacteriology*, *178*(20), 5910–5915. https://doi.org/10.1128/jb.178.20.5910-5915.1996.

Shimotsu, H., & Henner, D. J. (1986). Modulation of *Bacillus subtilis* levansucrase gene expression by sucrose and regulation of the steady-state mRNA level by *sacU* and *sacQ* genes. *Journal of Bacteriology*, *168*(1), 380–388. https://doi.org/10.1128/jb.168.1.380-388.1986.

Stanley, N. R., & Lazazzera, B. A. (2005). Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-DL-glutamic acid production and biofilm formation. *Molecular Microbiology*, *57*(4), 1143–1158.

Stein, T. (2005). *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Molecular Microbiology*, *56*(4), 845–857. https://doi.org/10.1111/j.1365-2958.2005.04587.x.

Sun, J., Liu, Y., Lin, F., Lu, Z., & Lu, Y. (2021). CodY, ComA, DegU and Spo0A controlling lipopeptides biosynthesis in *Bacillus amyloliquefaciens* fmbJ. *Journal of Applied Microbiology*, *131*(3), 1289-1304. https://doi.org/10.1111/jam.15007.

Tosato, V., Albertini, A. M., Zotti, M., Sonda, S., & Bruschi, C. V. (1997). Sequence completion, identification and definition of the fengycin operon in *Bacillus subtilis* 168. *Microbiology (Reading)*, *143*(Pt11), 3443–3450. https://doi.org/10.1099/00221287-143-11-3443.

Tsuge, K., Ano, T., Hirai, M., Nakamura, Y., & Shoda, M. (1999). The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. *Antimicrobial Agents and Chemotherapy*, *43*(9), 2183–2192.

Vahidinasab, M., Lilge, L., Reinfurt, A., Pfannstiel, J., Henkel, M., Morabbi Heravi, K., & Hausmann, R. (2020). Construction and description of a constitutive plipastatin mono-producing *Bacillus subtilis*. *Microbial Cell Factories*, *19*(1), 1–12. https://doi.org/10.1186/s12934-020-01468-0.

van Dijl, J. M., & Hecker, M. (2013). *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microbial Cell Factories*, *12*(3). https://doi.org/10.1186/1475-2859-12-3.

Wang, P., Guo, Q., Ma, Y., Li, S., Lu, X., Zhang, X., & Ma, P. (2015). DegQ regulates the production of fengycins and biofilm formation of the biocontrol agent *Bacillus subtilis* NSD-2. *Microbiology Research*, *178*, 42–50.

Willenbacher, J., Yeremchuk, W., Mohr, T., Syldatk, C., & Hausmann, R. (2015). Enhancement of Surfactin yield by improving the medium composition and fermentation process. *AMB Express*, *5*, 57. https://doi.org/10.1186/s13568-015-0145-0.

Willenbacher, J., Zwick, M., Mohr, T., Schmid, F., Syldatk, C., & Hausmann, R. (2014). Evaluation of different *Bacillus* strains in respect of their ability to produce Surfactin in a model fermentation process with integrated foam fractionation. *Applied Microbiology and Biotechnology*, *98*(23), 9623–9632. https://doi.org/10.1007/s00253-014-6010-2.

Yang, M., Ferrari, E., Chen, E., & Henner, D. J. (1986). Identification of the pleiotropic *sacQ* gene of *Bacillus subtilis*. *Journal of Bacteriology*, *166*(1), 113–119. https://doi.org/10.1128/jb.166.1.113-119.1986.

Yanisch-Perron, C., Vieira, J., & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, *33*(1), 103–119.

Yaseen, Y., Diop, A., Gancel, F., Béchet, M., Jacques, P., & Drider, D. (2018). Polynucleotide phosphorylase is involved in the control of lipopeptide fengycin production in *Bacillus subtilis*. *Archives of Microbiology*, *200*, 783–791.

Yin, H., Guo, C., Wang, Y., Liu, D., Lv, Y., Lv, F., & Lu, Z. (2013). Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. *Anti-Cancer Drugs*, *24*, 587–598. https://doi.org/10.1097/CAD.0b013e3283611395.

Zhao, H., Shao, D., Jiang, C., Shi, J., Li, Q. I., Huang, Q., Rajoka, M. S. R., Yang, H., & Jin, M. (2017). Biological activity of lipopeptides from *Bacillus*. *Applied Microbiology and Biotechnology*, *101*(15), 5951–5960. https://doi.org/10.1007/s00253-017-8396-0.

How to cite this article: Lilge, L., Vahidinasab, M., Adiek, I., Becker, P., Kuppusamy Nesamani, C., Treinen, C., Hoffmann, M., Morabbi Heravi, K., Henkel, M., & Hausmann, R. (2021). Expression of *degQ* gene and its effect on lipopeptide production as well as formation of secretory proteases in *Bacillus subtilis* strains. *MicrobiologyOpen*, 10, e1241. https://doi.org/10.1002/mbo3.1241

2.4 4th Publication:

Characterization of *Bacillus velezensis* UTB96, Demonstrating Improved Lipopeptide Production Compared to the Strain *B. velezensis* FZB42.

Vahidinasab M., Adiek I., Hosseini B., Akintayo S. O., Abrishamchi B., Pfannstiel J., Henkel M., Lilge L., Voegele R. T., Hausmann R. (2022).

In: Microorganisms, Nov. 10; 10(11): 2225

DOI: 10.3390/microorganisms10112225

Additional material associated with this publication can be accessed at:

https://www.mdpi.com/article/10.3390/microorganisms10112225/s1

microorganisms

MDPI

Article

Characterization of *Bacillus velezensis* UTB96, Demonstrating Improved Lipopeptide Production Compared to the Strain *B. velezensis* FZB42

Maliheh Vahidinasab [1,*], Isabel Adiek [1], Behnoush Hosseini [2], Stephen Olusanmi Akintayo [1], Bahar Abrishamchi [1], Jens Pfannstiel [3], Marius Henkel [4], Lars Lilge [1], Ralf T. Voegele [2] and Rudolf Hausmann [1]

[1] Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany
[2] Department of Phytopathology (360a), Institute of Phytomedicine, Faculty of Agricultural Sciences, University of Hohenheim, Otto-Sander-Str. 5, 70599 Stuttgart, Germany
[3] Core Facility Hohenheim, Mass Spectrometry Unit, University of Hohenheim, August-von-Hartmann-Str. 3, 70599 Stuttgart, Germany
[4] Cellular Agriculture, TUM School of Life Science, Technical University of Munich, Gregor-Mendel-Str. 4, 85354 Freising, Germany
* Correspondence: vahidin@uni-hohenheim.de; Tel.: +49-711-459-24726; Fax: +49-711-459-24722

Abstract: *Bacillus* strains can produce various lipopeptides, known for their antifungal properties. This makes them attractive metabolites for applications in agriculture. Therefore, identification of productive wild-type strains is essential for the development of biopesticides. *Bacillus velezensis* FZB42 is a well-established strain for biocontrol of plant pathogens in agriculture. Here, we characterized an alternative strain, *B. velezensis* UTB96, that can produce higher amounts of all three major lipopeptide families, namely surfactin, fengycin, and iturin. UTB96 produces iturin A. Furthermore, UTB96 showed superior antifungal activity towards the soybean fungal pathogen *Diaporthe longicolla* compared to FZB42. Moreover, the additional provision of different amino acids for lipopeptide production in UTB96 was investigated. Lysine and alanine had stimulatory effects on the production of all three lipopeptide families, while supplementation of leucine, valine and isoleucine decreased the lipopeptide bioproduction. Using a 45-litre bioreactor system for upscaling in batch culture, lipopeptide titers of about 140 mg/L surfactin, 620 mg/L iturin A, and 45 mg/L fengycin were achieved. In conclusion, it becomes clear that *B. velezensis* UTB96 is a promising strain for further research application in the field of agricultural biological controls of fungal diseases.

Keywords: *Bacillus*; surfactin; fengycin; iturin A; bacillomycin D; lipopeptide; secondary metabolite; antimicrobial; biosurfactant; fungicide

Citation: Vahidinasab, M.; Adiek, I.; Hosseini, B.; Akintayo, S.O.; Abrishamchi, B.; Pfannstiel, J.; Henkel, M.; Lilge, L.; Voegele, R.T.; Hausmann, R. Characterization of *Bacillus velezensis* UTB96, Demonstrating Improved Lipopeptide Production Compared to the Strain *B. velezensis* FZB42. *Microorganisms* **2022**, *10*, 2225. https://doi.org/10.3390/microorganisms10112225

Academic Editor: Francois Coutte

Received: 17 October 2022
Accepted: 9 November 2022
Published: 10 November 2022

1. Introduction

Bacillus velezensis is known to be associated with plant roots. It is indigenous to the rhizosphere and provides many benefits to the plants [1,2]. Its value for agronomic applications lies primarily in the production of a variety of secondary metabolites that can benefit the plant by acting directly as antagonists against fungal pathogens or indirectly as triggers for systemic plant resistance, and by retaining nutrients such as nitrogen, phosphate, and iron that can promote plant growth. Therefore, *Bacillus velezensis* is recognized as a plant-growth-promoting rhizobacterium (PGPR) [3–8].

Among the *Bacillus* secondary metabolites, one of the most important groups are lipopeptides, represented by surfactin, iturin, and fengycin [9]. Notably, *Bacillus velezensis* wild-type strains can produce all three types of lipopeptides [10]. Lipopeptides are synthetized by complex non-ribosomal peptide synthetases (NRPSs), hybrid polyketide synthases (PKSs) or a combination of both enzymes [11,12]. These multi enzyme complexes

are encoded in huge operons and work as a ribosomal-independent "machine" for the biosynthesis of respective lipopeptides. The structure of lipopeptides consists of a cyclic peptide chain of 7 to 10 amino acids linked to a β-amino or β-hydroxy fatty acid residue containing 14 to 19 carbons. The surfactin family consists of a hepta-peptide and the fengycin family consists of a deca-peptide attached to a β-hydroxy fatty acid chain with variable chain length [13,14]. All iturins consist of a hepta-peptide linked to a ß-amino fatty acid often of *iso*- or *anteiso*-type and of variable length (C_{14}-C_{18}) [15] (Figure 1). Moreover, differences in amino acids and fatty acid residues lead to various homologs [16].

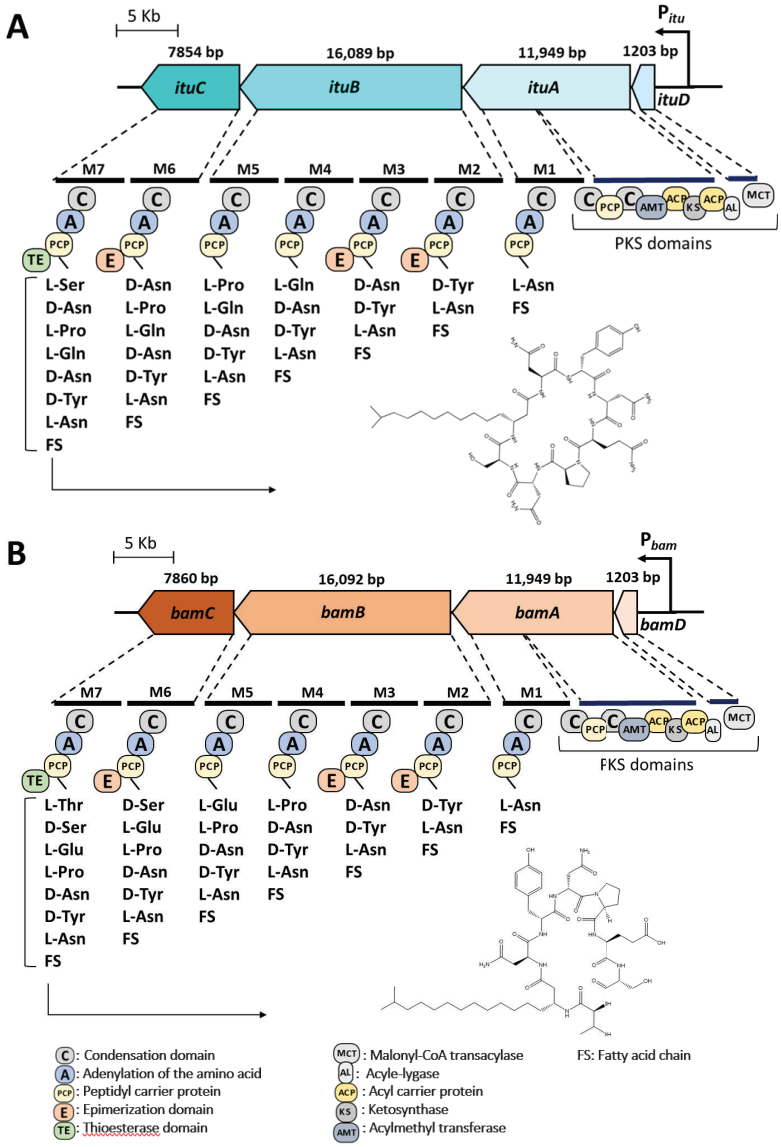


Figure 1. Genomic organization and modulation of the NRPSs producing bacillomycin D and iturin A. (**A**) Iturin A operon in *B. velezensis* UTB96 contains four ORFs of *ituD*, *ituA*, *ituB*, and *ituC* (37,246 bp), (**B**) Bacillomycin D operon in *B. velezensis* FZB42 contains four ORFs of *bamD*, *bamA*, *bamB*, and *bamC* (37,251 bp) [17]. The organization of the modules is comparable in both operons, although modules 4–7 catalyze different amino acids for the integration into the peptide moiety.

Several significant biological functions have been reported for these three lipopeptide families, such as antifungal, antibacterial, antiviral, antioxidant, and antitumor activities [18–21]. Nevertheless, there is the suspicion that many of the described effects become effective only through the interaction of the lipopeptides with other bioactive metabolites [22]. Regardless, lipopeptides have the potential to find application in a variety of areas [19,23]. In more detail, surfactins are better known as biosurfactant metabolites and iturins reveal the strongest antifungal activity followed by fengycins [9,24]. Many studies have shown that the antifungal activity of fengycins is mostly limited to filamentous fungi [25], while iturins have a broader range and even stronger antifungal activity. Iturins have direct antagonistic activity and can destroy the fungal cell membrane through a variety of mechanisms [23]. Based on the unique structure of each lipopeptide, the respective molecule exhibits a special structure-based property. Interestingly, even within the variants of one lipopeptide, there could be a different antifungal activity. It has been already shown that iturins with longer fatty acid chains have stronger antimicrobial activity [26–28]. However, although the bioactivity of lipopeptides, especially the iturin and fengycin families, is beneficial, it has not yet been possible to carry out targeted application trials with the isolated substance in agriculture or other disciplines. This is mainly due to the low lipopeptide titers synthesized by native wild-type producer strains, and thus costly and ineffective production. Most of biocontrol agents currently in use are based on living microorganisms, mostly as liquid suspensions and dried formulations prepared from durable spores [17]. As the native lipopeptide production capabilities seem to be insufficient, improved bacterial production strains and processes need to be developed. One approach for increased lipopeptide production is the enhanced availability of precursors, such as amino acids [29–32].

In the present study, to compare the wild-type strain *B. velezensis* UTB96 [33–35] with the established *B. velezensis* model strain FZB42 [36,37], the lipopeptide production was characterized. Differences in the operons encoding the lipopeptide-producing NRPSs were identified by bioinformatic analyses and the lipopeptide production validated by mass spectrometry analyses. In addition, the potential of UTB96 as a strain with antifungal properties was highlighted by comparative batch cultivation processes and antifungal approaches. In addition, strain UTB96 was applied in upscaling with 45-litre bioreactor systems and the lipopeptide production was analyzed, showing the potential of this strain for future studies in agriculture. Finally, to identify bottlenecks in the lipopeptide biosynthesis of UTB96, the influence of the availability of different amino acids on lipopeptide production was analyzed.

2. Materials and Methods

2.1. Bacterial and Fungal Strains

Bacillus velezensis strains UTB96 and FZB42 were used for characterization of lipopeptide production and their potential for antifungal activity. UTB96 was previously isolated from the soil around a pistachio tree in Iran [33] and is deposited at the Leibniz Institute German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) under the accession number DSM 114406. *Bacillus velezensis* FZB42 (DSM 23117) is a model strain for Gram-positive PGPR and its antifungal activity has frequently been reported [20].

The fungal pathogen *Diaporthe longicolla* is described as a seedborne pathogen of soybean [38]. In this study, *D. longicolla* DPC_HOH20 causing pod and stem blight on soybean plants was kindly provided by the Institute of Phytomedicine of the University of Hohenheim [39].

2.2. Primers and DNA Sequencing

The list of primers is shown in the Supplementary Material S1. Chromosomal DNA was purified with the ready to use kit innuPREP Bacteria DNA Kit (Analytik Jena AG, Jena, Germany) and used as a template for amplifying DNA fragments (Phusion High-Fidelity Polymerase #M0530S, New England BioLabs, Frankfurt am Main, Germany) using a PCR thermal cycler (prqSTAR 96X VWR GmbH, Darmstadt, Germany). The amplified

DNA fragments were purified using the QIAquick PCR and Gel Cleanup Kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions. All PCR fragments were sequenced by Eurofins Genomics Germany GmbH (Ebersberg, Germany). Finally, the DNA sequence was analyzed using the AntiSMASH database version 6 [40] and was blasted using the National Center for Biotechnology Information Blast database (Rockville Pike Bethesda, MD, USA).

2.3. LC-MS/MS Analyses of Lipopeptides

The LC-MS/MS analysis of lipopeptides was performed on a 1290 UHPLC system (Agilent, Waldbronn, Germany) coupled to a Q-Exactive Plus Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Bremen, Germany). Analyte separation was achieved by an ACQUITY CSH C18 column (1.7 µm, 2.1 µm × 150 mm, Waters, Eschborn, Germany). The column temperature was maintained at 40 °C. Samples were dissolved in methanol and 10 µL of each sample was injected. Mobile phase A was 0.2% formic acid in water, and mobile phase B 0.2% formic acid in acetonitrile. A constant flow rate of 0.3 mL/min was used and the gradient elution was performed as follows: 40–70% B from 0 to 12 min, 70–95% B from 12 to 20 min, isocratic at 95% B from 20 to 24 min, the system was returned to initial conditions from 95% B to 40% B from 24 to 26 min.

The HESI source was operated in the positive ion mode with a spray voltage of 4.20 kV and an ion transfer capillary temperature of 360 °C. The sweep gas and auxiliary pressure rates were set to 60 and 20, respectively. The S-Lens RF level was 50%, and the auxiliary gas heater temperature was 150 °C. The Q-Exactive Plus mass spectrometer was calibrated externally in positive ion mode using the manufacturers calibration solutions (Pierce/Thermo Fisher, Germany). Mass spectra were acquired within the mass range of 500 to 1600 m/z at a resolution of 70,000 FWHM using an Automatic Gain Control (AGC) target of 1.0 × 10E6 of and 100 ms maximum ion injection time. Data dependent MS/MS spectra in a mass range of 50 to 1600 m/z were generated for the five most abundant precursor ions with a resolution of 17,500 FWHM using an AGC target of 3.0 × 10E6 and 100 ms maximum ion injection time and a stepped collision energy of 15, 30 and 45. The m/z values of iturin A, bacillomycin D, fengycin and surfactin lipopeptides were predefined in an inclusion list to ensure that MS/MS spectra of corresponding precursors were acquired. Xcalibur™ software version 4.3.73.11 (Thermo Fisher Scientific, San Jose, USA) was used for data acquisition and data analysis. Peak areas of individual lipopeptides were calculated based on extracted ion chromatograms (XICs) of the corresponding precursor ions. Samples were analyzed in triplicate. Assignment of individual lipopeptides was based on the precise m/z value of the precursor ion, manual inspection of corresponding MS/MS spectra and comparison with available MS/MS spectra from literature [41–44].

2.4. Media and Cultivation Procedure

2.4.1. Shake Flask Cultivations

A mineral salt medium based on the fermentation medium of Vahidinasab et al. [45] was used for cultivation in shake flasks. The initial pH of the medium was set as 7 and it consisted of 4.0×10^{-6} M $Na_2EDTA \times 2\ H_2O$, 7.0×10^{-6} M $CaCl_2$, 4.0×10^{-6} M $FeSO_4 \times 7\ H_2O$, 1.0×10^{-6} M $MnSO_4 \times H_2O$, 50 mM Urea, 30 mM KH_2PO_4, 40 mM $Na_2HPO_4 \times 2\ H_2O$ and 8.0×10^{-4} M $MgSO_4 \times 7\ H_2O$. In addition, glucose was used as the sole carbon source at concentrations of 8 g/L, 20 g/L, or 40 g/L (m/v). Furthermore, to verify the impact of amino acid supplementation on lipopeptide formation, 0.5 mM of a single amino acid was optionally added to the cultivation process.

The first preculture was prepared by inoculating 10 mL of LB medium (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) with 10 µL of a glycerol stock solution in a 100 mL baffled shake flask. After 8 h of cultivation, the first preculture was used to inoculate 10 mL mineral salt medium with an initial optical density (OD_{600}) of 0.1 as the second preculture. The second preculture was incubated for 10 to 12 h. Exponentially growing cells from the

second preculture were washed and used for inoculation of the main culture with a final volume of 100 mL and an initial OD_{600} of 0.1 in a 1 L baffled shake flask. All cultivations had three biological replicates and were performed at 30 °C and 120 rpm in an incubation shaker (Innova 44®R, Eppendorf AG, Hamburg, Germany).

2.4.2. Bioreactor Cultivations

Batch-bioreactor cultivations were carried out with the two biological replicates in 42 L custom-built bioreactors (ZETA GmbH, Graz/Lieboch, Switzerland) with a filling volume of 20 kg. The media used for the fermentation processes were described by Willenbacher et al. [46].

The bioreactors are equipped with pH (EasyFerm Bio HB Arc 120, Hamilton Bonaduz AG, Bonaduz, Switzerland) and pO_2 probes (VisiFerm DO ARC 120 H0, Hamilton Bonaduz AG), a temperature sensor and three Rushton turbines. The temperature was fixed at 30 °C, and the pH was regulated to a value of 7.0 by the addition of 4 M NaOH or 4 M H_3PO_4. At the beginning of the fermentation process, the stirrer was adjusted to constant 300 rpm; afterwards, the stirrer was regulated by the online control of the dissolved oxygen that was set to a minimum of 20%. The airflow was adjusted to 0.07 vvm. Foam fractionation methods are described by Klausmann et al. [47] including the use of a foam centrifuge as well as the antifoam agent Contraspum A4050 (Zschimmer and Schwarz GmbH, Lahnstein, Germany). Moreover, a foam trap was installed in front of the exhaust gas filter to collect the potentially over-foaming medium.

2.5. Lipopeptide Extraction and Quantitative Analysis

Cell-free supernatants were obtained after 10 minutes centrifugation at 4700 rpm and were used for extraction of lipopeptides according to the method described by Yazgan et al. [48]. Specifically, a volume of 2 mL of the cell-free supernatant was mixed three times with 1 mL of 1-butanol 95% (*v*/*v*) by vortexing for 1 min, followed by 5 min centrifugation at 3000 rpm. The organic phases were pooled and used for evaporation of butanol phases (RVC2-25 Cdplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 10 mbar and 60 °C. The remaining residues were dissolved in 2 mL methanol. To quantify the total amounts of lipopeptides, purified fengycin was purchased from Lipofabrik (Lesquin, France) and surfactin and iturin A standards were ordered from Sigma–Aldrich (Seelze, Germany). High-performance thin-layer chromatography (HPTLC) was performed for quantification of lipopeptides. All HPTLC instruments and chambers were from CAMAG (Muttenz, Switzerland) and instruments were controlled by winCATS Software 1.4.7 as described previously [49].

2.6. Evaluation of Antifungal Activity

Antagonism of *B. velezensis* UTB96 and *B. velezensis* FZB42, respectively, against *Diaporthe longicolla* DPC_HOH20 was determined using a dual-culture assay according to a method previously described by Johnson et al. [50]. Specifically, a 0.6 cm mycelial plug from the margins of an actively growing 5-days-old culture of *D. longicolla* was placed in the corner of the plate with 150 mm distance from the edge of the Petri dish containing potato dextrose agar (PDA) and LB agar (1:1) medium.

In a first approach, sterile filter paper (MN 617 G) with the thickness of 0.22 mm and diameter of 0.5 cm was soaked in a cell suspension of fresh bacterial overnight culture in LB medium with an OD_{600} of 2.5. The soaked filter paper was placed 150 mm from the edge of the Petri dish.

In a second approach, instead of inoculating the plate with bacterial cells, filter sterilized cell-free supernatant from *B. velezensis* UTB96 and *B. velezensis* FZB42, respectively, was used. The supernatant was taken from a cell suspension cultivated for 48 h in mineral salt medium with 40 g/L glucose. Finally, a volume of 200 µL of the cell-free supernatant was transferred to an 8 mm diameter well, which was 150 mm in distance from the edge of the Petri dish.

In the control treatment, a mycelial plug of *D. longicolla* was placed in the plate without bacterial strains. All plates were incubated in the dark at room temperature for 5 days. To measure the percentage of inhibition radial growth (PIRG), the following formula was used:

$$\text{PIRG (\%)} = [(R1 - R2)/R1] \times 100$$

where R1 represents the radius of the control fungus and R2 is the radius of the fungi in treatment with the bacteria. Each treatment was replicated three times and the experiment was repeated thrice.

2.7. Data Analysis

The growth rate μ, specific productivity ($q_{P/X}$), product per substrate ($Y_{P/S}$) and product per biomass ($Y_{P/X}$) for each lipopeptide, as well as the yield of biomass per substrate ($Y_{X/S}$), were determined using the equations shown in [13]. Variable $Y_{X/S}$was quantified at the maximum cell dry weight (CDW_{max}), while $Y_{P/S}$, $Y_{P/X}$ and $q_{P/X}$ were quantified at the maximum lipopeptide concentrations. The statistical analyses, such as One-way ANOVA, were performed using SigmaPlot (version 13) software. In all the graphs, Error bars indicate the standard deviation between different sample replicates.

3. Results

3.1. Bioinformatic Analyses of Lipopeptide Biosynthesis

Bacillus velezensis has been described extensively for its bioproduction of lipopeptides and can be used in agriculture as a biofungicide [51]. Therefore, in this study, the capability of *B. velezensis* UTB96 for lipopeptide production was first analyzed by bioinformatic approaches, using the genome of the model strain *B. velezensis* FZB42 (accession number: NC_009725.2) as reference. Using the AntiSMASH tool version 6 software [40,52,53], three operons encoding nonribosomal peptide synthetases (NRPSs) were identified for both strains. Besides the commonly known NRPS for biosynthesis of surfactin and fengycin, another gene cluster was identified, which showed only moderate comparability to the *bam* operon of the FZB42 strain encoding for the biosynthesis of bacillomycin D (Table 1).

Table 1. Comparison of the gene clusters encoding NRPS for biosynthesis of the main lipopeptides, surfactin, iturin, and fengycin, in the genomes of *Bacillus velezensis* UTB96 and the reference strain *Bacillus velezensis* FZB42.

		Similarity with the Reference Strain *Bacillus velezensis* FZB42	
Lipopeptide	**Chromosomal Localization (from–to)**	**Similarity in Nucleotide Sequence**	**Similarity in Amino Acid Sequence**
Surfactin	301,941 nt–366,339 nt	97.53%	91.67%
Iturin A	1,762,934 nt–1,800,180 nt	97.02%	67.94%
Fengycin	1,823,096 nt–1,860,765 nt	96.50%	97.08%

To determine the type of iturin produced by *B. velezensis* UTB96, the nucleotide sequence of the whole iturin operon was analyzed. The sequencing results revealed an operon length of 37,246 bp including four open reading frames (ORFs) encoding for iturin A biosynthesis in UTB96 (accession number: OK274217.1). A subsequent AntiSMASH analysis revealed the modulation of the NRPS (Figure 1A). In this way, seven amino acid-activating modules responsible for the biosynthesis of the peptide ring, as well as the modules for fatty acid maturation were identified. Compared to the bacillomycin D biosynthesis of FZB42 (Figure 1B), both iturin versions share the first three amino acids in the circular peptide ring (L-Asn, D-Tyr, L-Asn) linked to a β-amino fatty acid, while the next four amino acids are different.

3.2. Comparative Structure-Based Iturin A and Bacillomycin D Analysis by Mass Spectrometry

To further characterize the congener composition of the two types of iturins, as well as the surfactin and fengycin variants throughout the cultivation process, samples were taken after 48 h of cultivation and analyzed by liquid chromatography electrospray mass spectrometry (LC-ESI-MS/MS). The relative abundance of congeners was calculated using the corresponding peak areas. Both strains, FZB42 and UTB96, produced all three major classes of lipopeptides, namely iturin, fengycin, and surfactin. While *B. velezensis* FZB42 exclusively produced bacillomycin D, *B. velezensis* UTB96 exclusively produced iturin A lipopeptides (Figure 2). In *B. velezensis* FZB42, the peaks within the range of m/z = 989.488 to 1073.582 were assigned to protonated ion species $[M+H]^+$ of bacillomycin D based on precise m/z values and the corresponding MS/MS spectra (data not shown). The C15 congener of bacillomycin D was the most abundant (approx. 45%) through the cultivation, followed by C14 (~35%). In contrast, C16 (~15%) and C17 congeners (~5%) were produced less during the cultivation period. In comparison, *B. velezensis* UTB96 produced iturin A congeners of C14, C15 and C16 at similar proportions (approx. 34, 33 and 30%, respectively) throughout the cultivation process, while C17 (~3%) was significantly underrepresented. The mass spectra of iturin A congeners were in the mass range of m/z = 1015.516 to 1085.594.

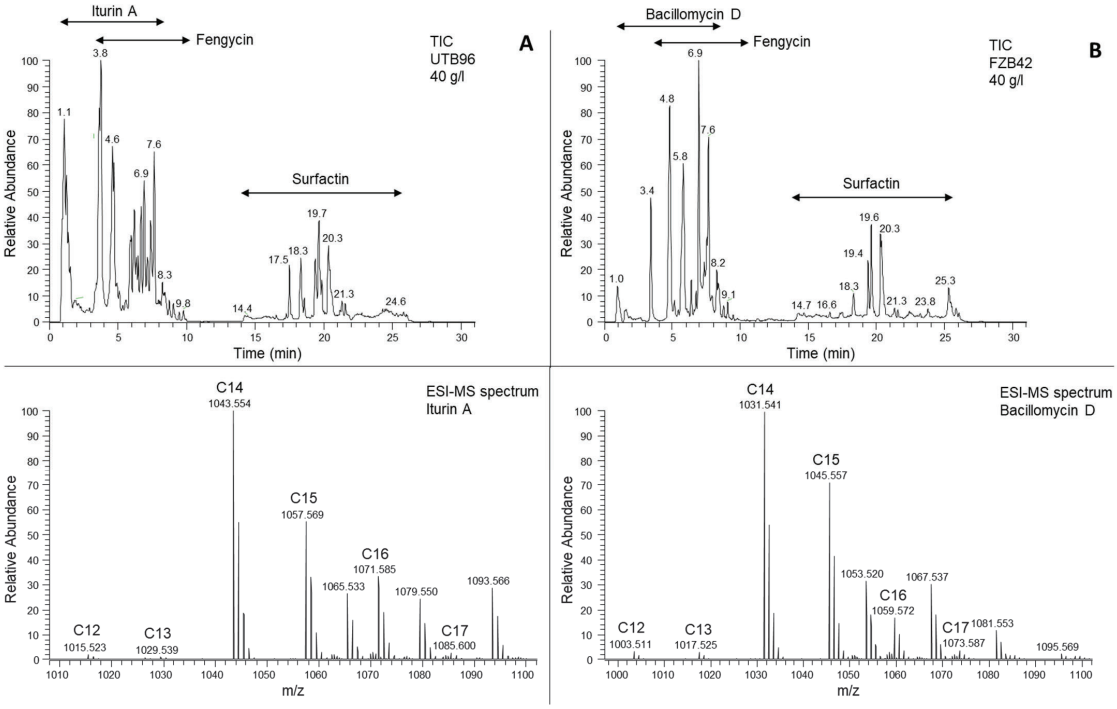


Figure 2. LC-ESI-MS analysis of the lipopeptide compounds produced by the *B. velezensis* UTB96 and *B. velezensis* FZB42 strains. (**A**) Total ion chromatogram (TIC, upper panel) and ESI-MS spectrum (lower panel) of the extracted iturin A lipopeptides from *B. velezensis* UTB96. The ESI-MS spectrum shows m/z values of protonated iturin A lipopeptides $[M+H]^+$ eluted in the time interval from 0.8–8 min. Fatty acid chain length of different iturin A lipopeptides is indicated. (**B**) Total ion chromatogram (TIC, upper panel) and ESI-MS spectrum (lower panel) of the extracted bacillomycin D lipopeptides *B. velezensis* FZB42. The ESI-MS spectrum shows m/z values of protonated bacillomycin D lipopeptides $[M+H]^+$ eluted in the time interval from 0.8–8 min. Fatty acid chain length of different bacillomycin D lipopeptides is indicated.

Fengycin produced by *B. velezensis* UTB96 and FZB42 is a mixture of several homologs based on the length of saturated or unsaturated fatty acid chain and variants within peptide moiety. For both strains, fengycin peaks with saturated fatty acid chain at m/z = 1435.766 to 1519.858 and for unsaturated fatty acid chain at m/z = 1433.787 to 1489.850 were observed. Mainly, fengycin A and B with saturated or unsaturated fatty acid chain with 14 to 18 carbon atoms for both strains were detected, with the saturated fatty acid chain variants being more abundant in both strains. In addition, several fengycin variants with substitutions in the peptide moiety were detected as described by Pathak et al. [44]. Both strains produced Fengycin A (Ala6, Ile10), Fengycin B (Val6, Ile10) as well as Fengycin A2 (Ala6, Val10) and Fengycin B2 (Val6, Val10).

Surfactin lipopeptides comprised a range of different surfactins congeners with saturated fatty acid chain between 12 to 17 carbon atoms that were detected in both strains at m/z = 994.636 to 1064.715. In the addition, several amino acid substitutions within the peptide sequence were observed as described by Kecskeméti et al. [42]. While the most abundant variant in both strains had the peptide sequence E-I/L-I/L-V-D-I/L- I/L [Sur], amino acid substitutions at positions 2, 4 and 7 (Val2, Val7, Ala4) as well as an aspartic acid 4-methyl ester at position 5 (AME5) were also detected (see extracted-ion chromatograms in Supplementary File S2).

3.3. Lipopeptide Production of B. velezensis UTB96 and FZB42 under Varying Substrate Availability

To get an overview of the production capability of *B. velezensis* UTB96, the extracellular accumulation of surfactin, fengycin and iturin was quantitatively analyzed and compared with the bioproduction of the model strain *B. velezensis* FZB42. Therefore, both strains were cultivated in shake flasks using mineral salt medium with different initial glucose concentrations of 8, 20 and 40 g/L. Figure 3 summarizes the surfactin, fengycin, and iturin A or bacillomycin D production, as well as the cell dry weight (CDW) and glucose consumption during a cultivation period of 72 h at 30 °C.

The comparison of growth behavior showed similar growth rates for both strains UTB96 and FZB42. However, a faster glucose consumption was detected for the FZB42 strain, resulting in slightly accelerated biomass formation compared to UTB96. After complete glucose depletion, a decline in CDW was detected for both *B. velezensis* strains.

With respect to the lipopeptide production, model strain FZB42 was able to synthesize stabilized titers of surfactin (~50 mg/L), fengycin (~30 mg/L) and bacillomycin D (~11 mg/L) using 8 g/L glucose. In comparison, the strain UTB96 produced clearly higher amounts of fengycin (~95 mg/L) and iturin A (~65 mg/L), while a maximum surfactin titer of 166 mg/L was reached during the exponential growth phase before a decline was detected and a final stabilized concentration of ~40 mg/L was maintained.

In cultivation with 20 g/L glucose, a higher availability of carbon source did not result in higher concentrations of fengycin and surfactin in FZB42 (~20 mg/L surfactin, ~30 mg/L fengycin) and UTB96 (~50 mg/L surfactin, ~90 mg/L fengycin), respectively, while for both iturin congeners, bacillomycin D and iturin A, increases of about two folds (22 mg/L) and three folds (190 mg/L) were observed. The highest initial glucose concentration used was 40 g/L. As a result, lipopeptide concentrations of 56.5 mg/L surfactin, 50.5 mg/L fengycin and 49 mg/L bacillomycin D were detected after 34 h of cultivation for FZB42 before a decline was observed for all lipopeptides. In contrast, relatively stable concentrations could be detected for UTB96 with the highest titers of 55 mg/L surfactin, 90 mg/L fengycin, and 100 mg/L iturin A in the stationary phase. However, a decline of surfactin and fengycin was detectable also in UTB96 at the end of cultivation. Overall, the UTB96 strain showed superior production values for all lipopeptide types (Table 2), especially for iturin and fengycin, which are more important for microbial antifungal activity [17].

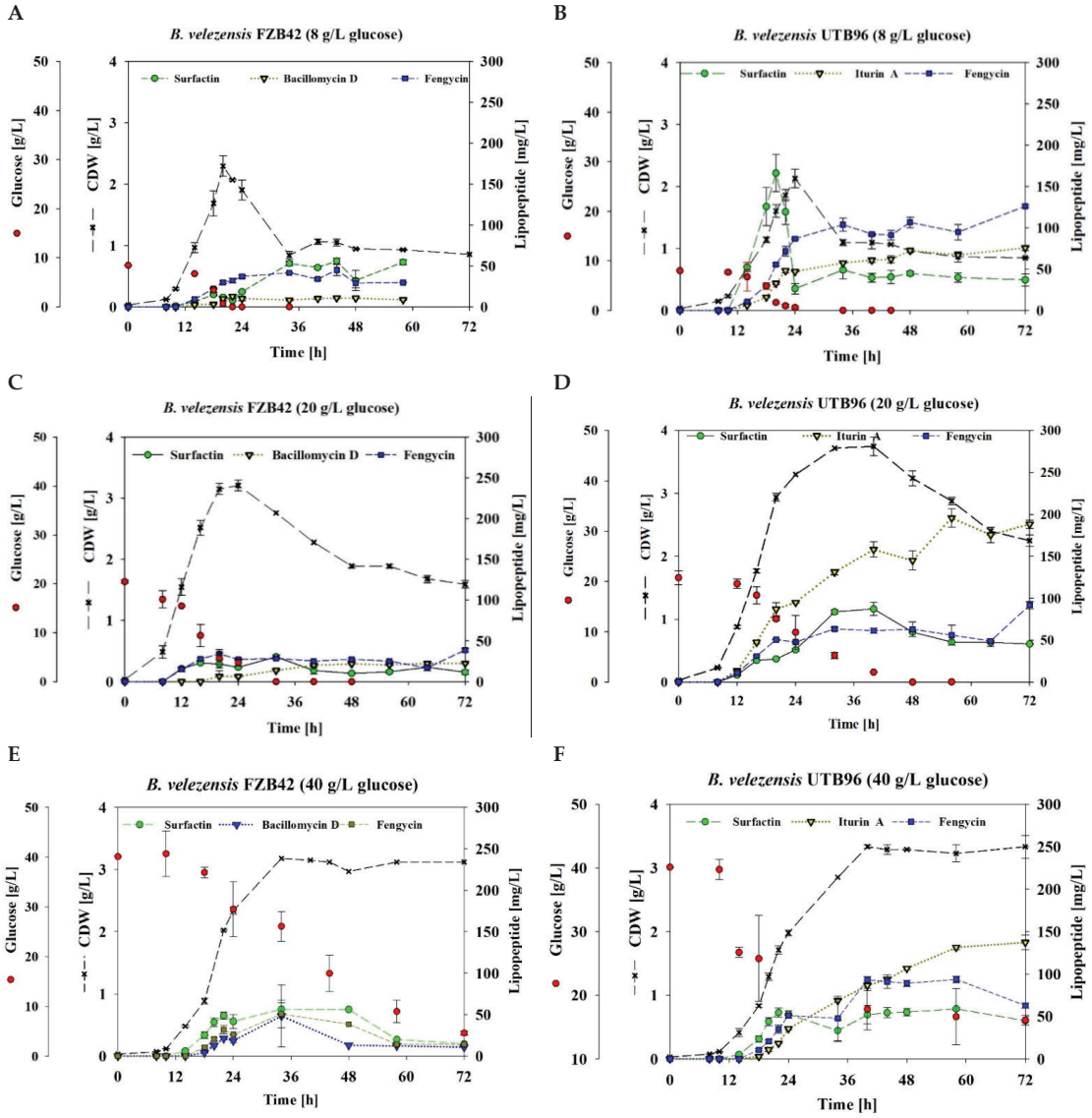


Figure 3. Overview of the time course of lipopeptide production by *B. velezensis* FZB42 and UTB96. The shake flask cultivations were conducted as biological triplicates in mineral salt medium containing different glucose concentration of 8 g/L (**A**,**B**), 20 g/L (**C**,**D**) and 40 g/L (**E**,**F**) at 30 °C, 120 rpm and initial pH of 7.

3.4. Antifungal Activity

To characterize the antifungal properties of *B. velezensis* UTB96, inhibition assays of the cell suspension and the corresponding cell-free supernatant were analyzed and compared with the previously established strain FZB42 (Figure 4). For this purpose, a dual-culture assay was used to determine the growth inhibitory effect on the soybean fungal pathogen *Diaporthe longicolla* DPC_HOH20 as an indicator strain. In this way, distinct zones of inhibition were formed between the *D. longicolla* DPC_HOH20 and the bacterial strains, and the width of the inhibition zone remained unaffected for at least one month. In more detail, UTB96 was shown to have comparable antifungal activity in both

cell-free supernatant and grown cells compared to the FZB42 reference strain. Specifically, UTB96 revealed approx. 28% (cell-mediated inhibition) and 9% (supernatant-mediated inhibition) larger zones of inhibition (Figure 4). However, both strains showed similar growth-inhibiting properties against *D. longicolla*, confirming the potential of strain UTB96 for agricultural applications.

Table 2. Overview of the lipopeptide production parameters including surfactin, iturin A or bacillomycin D and fengycin by *B. velezensis* UTB96 and *B. velezensis* FZB42 and in shake flask cultivations with initial glucose concentrations of 8 g/L, 20 g/L and 40 g/L and in a batch bioreactor using 40 g/L of glucose.

				Surfactin			Iturin A in UTB96 or Bacillomycin D in FZB42			Fengycin		
B. velezensis	**Initial Glucose [g/L]**	**$Y_{X/S}$ [g/g]**	**Growth Rate μ [1/h]**	**$Y_{P/S}$ [mg/g]**	**$Y_{P/X}$ [mg/g]**	**q [mg/g.h]**	**$Y_{P/S}$ [mg/g]**	**$Y_{P/X}$ [mg/g]**	**q [mg/g.h]**	**$Y_{P/S}$ [mg/g]**	**$Y_{P/X}$ [mg/g]**	**q [mg/g.h]**
UTB96	**8 (SF)**	0.2 ± 0.01	0.2 ± 0.0	12.9 ± 1.16	27.4 ± 1.59	2.3 ± 0.13	5.8 ± 0.25	168.3 ± 8.76	1.2 ± 0.06	9.6 ± 0.13	281.5 ± 6.35	2.0 ± 0.04
	20 (SF)	0.6 ± 0.02	0.1 ± 0.0	3.6 ± 0.08	46.3 ± 5.93	0.6 ± 0.07	7.0 ± 0.86	134.4 ± 10.33	1.2 ± 0.09	3.4 ± 0.08	82.6 ± 1.41	0.7 ± 0.01
	40 (SF)	0.1 ± 0.01	0.1 ± 0.0	1.7 ± 0.1	62.4 ± 4.08	1.4 ± 0.09	3.5 ± 0.30	81.9 ± 9.51	0.6 ± 0.06	2.5 ± 0.13	55.4 ± 1.57	0,7 ± 0.01
	40 (BR)	0.6 ± 0.0	0.2 ± 0.0	8.4 ± 0.2	43.8 ± 3.8	0.9 ± 0.1	16.3 ± 0.8	249.4 ± 15.3	1.8 ± 0.0	1.2 ± 0.0	17.4 ±0.3	0.1 ± 0.0
FZB42	**8 (SF)**	0,2 ± 0.021	0.2 ± 0.00	5.0 ±0.24	120.6 ± 1.75	1.8 ± 0.00	1.4 ± 0	12.0 ± 2.04	0.3 ± 0.04	3.7 ± 0.59	81.9 ± 8.09	0.9 ±0.03
	20 (SF)	0.2 0.01	0.2 ± 0.01	1.2 ± 0.11	21.7 ± 1.68	0.3 ± 0.00	0.9 ± 0.01	35.2 ± 3.11	0.2 ± 0.02	1.3 ± 0.06	54.4 ± 6.23	0,3 ± 0.04
	40 (SF)	0.1 ± 0.0	0.1 ± 0.0	2.1 ± 0.04	39.6 ± 5.01	0.5 ± 0.06	0.4 ± 0.00	7.5 ± 0.53	0.1 ± 0.0	1.9 ± 0.4	32.1 ± 9.63	0.5 ± 0.14

SF—shake flask; BR—bioreactor.

3.5. Batch Bioreactor Cultivation

As *B. velezensis* UTB96 appeared superior in the bioproduction of lipopeptides and showed slightly superior antifungal activity against the phytopathogen *D. longicolla*, a first attempt of upscaling with a batch culture to produce lipopeptides was made (Figure 5). For this purpose, a custom-built 42-L bioreactor with a filling volume of 20 kg medium was used. After inoculation, a lag phase of about 10 h occurred before an exponential growth phase started for the next 12 h. Afterwards, the cell culture entered the stationary phase and reached a maximum CDW of 6.3 g/L. The initial glucose concentration of 40 g/L was depleted after 62 h. In this way, a growth rate of 0.22 1/h and a biomass yield of 0.81 g/g was reached. Regarding lipopeptide production, surfactin was shown to be produced first after about 12 h and reached a maximum of 140 mg/L after 38 h. In contrast, an accumulation of iturin A started after about 22 h of cultivation. However, compared to surfactin and fengycin, a steady increase in the amount of iturin A was observed until the end of cultivation, with a maximum concentration of 620 mg/L. Finally, after 24 h of cultivation, fengycin production was detected, reaching a maximum titer of 42 mg/L, which remained constant until the end of the cultivation. Overall, improved production of surfactin (2.5-fold) and iturin A (3.2-fold) was observed, while the productivity of fengycin (2.2-fold) was reduced compared to previous shake flask cultivations. An overview of the lipopeptide production rates is provided in Table 2.

3.6. Effect of Amino Acid Availability on the Lipopeptide Production of B. velezensis UTB96

Bacillus velezensis UTB96 proved to be the comparatively more productive strain for lipopeptides and was therefore used for further studies on possible bottlenecks in amino acid precursor availability. To identify potential bottlenecks in the provision of amino acids as precursor molecules in the bioproduction of lipopeptides using the *B. velezensis* strain UTB96, a defined concentration of 0.5 mM of 21 different amino acids was supplemented to the previously described shake flask cultivation process with 40 g/L glucose. Because a stabilized lipopeptide concentration was previously ensured (Figure 3F), the effect of

specific amino acid supplementation on the production of surfactin, fengycin and iturin A was analyzed after 48 h of cultivation.

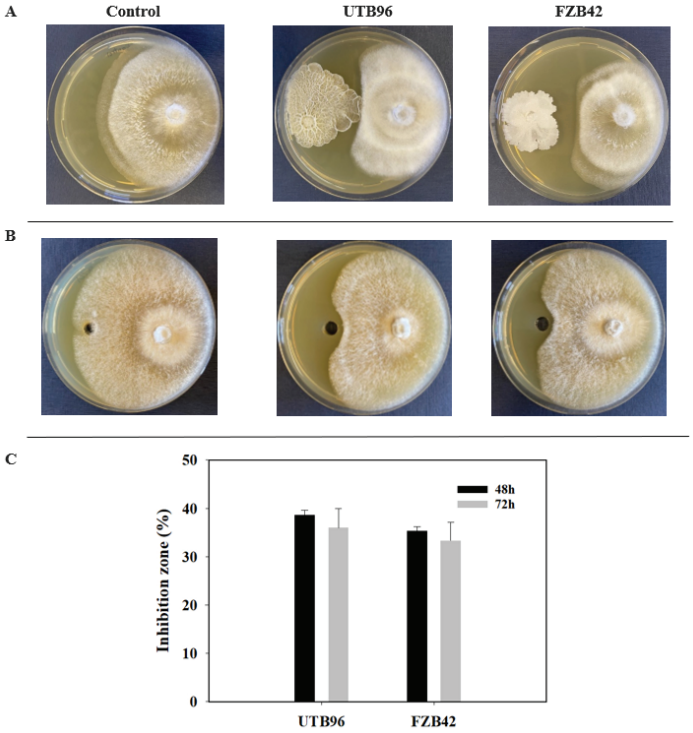


Figure 4. Comparison of antifungal activity; (**A**) cell suspension and (**B**) cell free supernatant taken after 48 and 72 h of *B. velezensis* strains UTB96 and FZB42 were each co-incubated with the soybean fungal pathogen *D. longicolla* strain DPC_HOH20 for 5 days. (**C**) The growth inhibitory effect of UTB96 and FZB42 against *D. longicolla* are summarized in bar graphs. Growth of *D. longicolla* DPC_HOH20 without the co-incubated *B. velezensis* strain was used as a control. Each treatment was repeated three times, while the approach was repeated thrice.

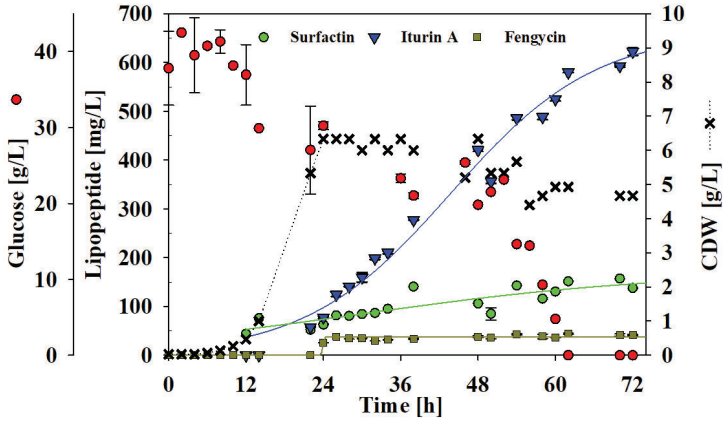


Figure 5. Time course of the cell dry mass (CDW) of the glucose and lipopeptide concentrations during a batch bioreactor fermentation with strain UTB96, glucose (red dots), CDW (black crosses), and the lipopeptide concentration of surfactin (green dots), iturin A (blue inverted triangles) and fengycin (brown squares).

In the reference process without amino acid supplementation, strain UTB96 achieved a CDW of 3.65 g/L and concentrations of ~55 mg/L surfactin, ~107 mg/L iturin A, and ~89 mg/L fengycin. However, when histidine (−52%), aspartic acid (−52%), glutamic acid (−49%), glycine (−47%), arginine (−45%), methionine (−45%), lysine (−43%), glutamine (−41%), or alanine (−36%) were added, a drastically reduced biomass formation was observed. Nevertheless, the lipopeptide production was only slightly affected. Conversely, although the CDW was unaffected when leucine, valine or isoleucine were added, significantly reduced concentrations for all three lipopeptide families were detected. Thus, the addition of leucine reduced the amount of surfactin by 17% and of fengycin by 63%, while valine showed a negative effect on surfactin by 80% and on fengycin by 54%, and for isoleucine a reduction in surfactin by 74%, in iturin A by 41% and in fengycin by 65% was observed. Further amino acids that have a negative effect on surfactin were glutamic acid (−41%), phenylalanine and tryptophane (−51%). In contrast, supplementation of lysine showed a remarkable positive effect on the production of surfactin by 43% and iturin A by 65% and slightly increased the fengycin production by 27%. Furthermore, the addition of alanine allowed an improvement in the production of surfactin by 31%, of iturin A by 77%, and of fengycin by 47%. In addition, surfactin production was improved by the addition of proline and ornithine (~30%). Interestingly, except for isoleucine, an improved iturin A production was observed with all amino acids, suggesting that the provision of a nitrogen source may have a positive effect on iturin A bioproduction. Specifically, phenylalanine and tryptophan followed by tyrosine were the best-influenced amino acids on production of iturin A.

4. Discussion

Bacillus velezensis has been reported several times for its antifungal activity and thus for its potential for the use in agriculture [33–35]. In this context, most *B. velezensis* strains can synthesize all three types of lipopeptides, namely surfactin, fengycin, and iturin. Fengycin and iturin in particular are associated with bioactivity against fungi [17]. In this study, the strain *B. velezensis* UTB96 was compared with the reference strain FZB42. Although only marginal differences were found in the nucleotide sequences of the operons encoding the corresponding NRPSs, the protein sequences of the iturin synthetases of UTB96 and FZB42 differ drastically (Table 1). Thus, strain UTB96 can synthesize iturin A, while FZB42 produces bacillomycin D. As these molecules differ in the peptide structure (Figure 1), different antifungal properties could be assumed. Iturin A was reported to cause cell wall disappearance, membrane degeneration and hyphal fragmentation [54]. This makes strain UTB96 a promising candidate for future studies as a potent natural iturin-A producer. A subsequent comparison of lipopeptide productivity showed that UTB96 is clearly more productive than the reference strain FZB42 (Figure 3). In particular, the production of fengycin and iturin was superior, which makes the UTB96 strain interesting for further strain engineering work, as the natural productivity of the antifungal lipopeptides iturin and fengycin is relatively high. Future comparative analyses of the proteome and the metabolome may reveal the reason for the better lipopeptide production of UTB96 compared to FZB42, although both strains are closely related in genome [33]. As *B. velezensis* UTB96 has superior lipopeptide production, it was reasonable to observe enhanced antifungal activity against *D. longicolla* compared to FZB42 (Figure 4). Previous studies have demonstrated that *Bacillus velezensis* strains have a broad antagonism activity against several fungal phytopathogens such as soil-borne pathogens *Fusarium graminearum*, *F. solani*, *F. oxysporum*, *Rhizoctonia solani*, *Ralstonia solanacearum*, *Rosellinia necatrix* [55–58] and the common postharvest pathogens like *Botrytis cinerea*, *Penicillium digitatum* and *Monilinia fructicola* [59–61]. More specifically, it is reported that the lipopeptides bacillomycin D and fengycin produced by FZB42 contribute significantly to antifungal activity [62,63]. Similarly, purified Iturin A is reported to be capable of suppressing *Fusarium* sp. at relatively low concentrations [64].

In the subsequent upscaling approach using a custom-built bioreactor system, *B. velezensis* UTB96 showed promising productivities (Figure 5). In particular, iturin A production

did not show any plateau after the end of the cultivation process of 72 h. Consequently, UTB96 appears to be a potent strain for iturin A production reaching 620 mg/L iturin A at the end of batch bioreactor cultivation. To increase the iturin A production fed-batch cultivations with the appropriate feeding strategy is an obvious solution. Similar experiments on the time course and kinetics of iturin A production have already been performed by Jin et al. [65]. They applied a two-step glucose feeding strategy and were thus able to maintain glucose levels at a low concentration and a desirable ratio of spores to total cells. As a result, they were able to achieve twice the production of iturin-A (1.12 g/L) compared to batch fermentation.

To gain further insights into possible bottlenecks in the bioproduction of lipopeptides in *B. velezensis* UTB96, different amino acids were added to the cultivation process (Figure 6). In this study, it was shown that the addition of the branched-chain amino acids valine, leucine, and isoleucine had a negative effect on the production of lipopeptides in the UTB96 strain. Wu et al. [30] reported that the production of iturin A by *Bacillus amyloliquefaciens* BPD1 was slightly increased by the addition of proline and asparagine, while the addition of serine significantly increased the yield of iturin A. These results contrast with a previous study, which reported that serine had no significant effect on iturin A production while asparagine had the best effect on the iturin A yield among other amino acids [32]. In the current study, the effect of serine supplementation on iturin A production by UTB96 was also not significant. This points to the possibility that each strain may need different types of amino acids as precursors for a specific lipopeptide production. The branched-chain amino acids valine, leucine, and isoleucine are essential amino acids [66]. Another explanation could be the action of global transcriptional regulator CodY, which is active in the presence of branched-chain amino acids. In this context, CodY acts as a repressor for the *srfA* operon expression [67], while a stimulatory effect has been described for the iturin operon (bacillomycin D) in the *B. amyloliquefaciens* fmbJ [68]; however, the possible effect of CodY on fengycin operon is unknown. Regarding the fengycin production, we have found that alanine is the most effective amino acid. Likewise, in the study by Yaseen et al. [31], alanine was shown to be the best nitrogen source among other amino acids to produce fengycin by *B. subtilis*. Indeed, in this study, we found that lysine and alanine had a stimulatory effect on the production of all three types of lipopeptides by UTB96. To determine the beneficial cellular adaptation in presence of these amino acids, future studies should focus on molecular adaptation in terms of the proteome and metabolome. For a deeper understanding, the omics profile of several bacterial strains can be compared. Notably, in this study, iturin A concentration increased independently of the amino acid supplemented, suggesting that higher availability of nitrogen sources stimulates iturin A bioproduction. All these findings should be taken into account in further studies, in which lipopeptide concentrations as well as spore number and quality should be simultaneously optimized in fed-batch bioreactor cultivations of *B. velezensis* UTB96, and the applicability of spore preparations as antifungal agents should be further elucidated in further application trials.

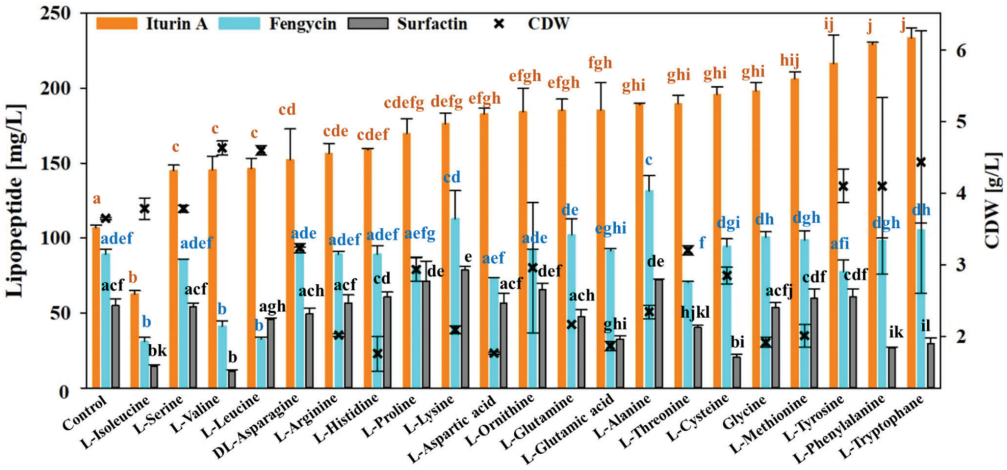


Figure 6. Lipopeptide concentrations and cell dry weight (CDW) achieved in dependence of different amino acid supplements (0.5 mM) by *B. velezensis* UTB96 after 48 h of cultivation. All tests were performed by On-Way ANOVA based on Holm–Sidak method. Bars with the same superscript letter are not significantly different ($p < 0.05$).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10112225/s1, Additional file S1: List of primers used in this study for amplification of iturin A operon in *Bacillus velezensis* UTB96. Additional file S2: Extracted Ion Chromatograms for iturin A, surfactin and fengycin families produced by *B. velezensis* UTB96 as well as for bacillomycin D, surfactin and fengycin produced by *B. velezensis* FZB42. Assignments of lipopeptides were based on precise m/z values and manual inspection of the corresponding MS/MS spectra.

Author Contributions: M.V. designed, planned, and executed the experiments, collected, and interpreted the data, created the graphs, and drafted the manuscript. L.L. significantly contributed to the design of experiments. B.H. was involved in performing antifungal tests. I.A. and B.A. were involved in performing shake flask cultivations. J.P. performed the mass spectrometry analysis. S.O.A., M.H. and R.T.V. supported the interpretation of results. R.H. substantially contributed to conception and design of the conducted experiments and manuscript completion. All authors have read and agreed to the published version of the manuscript.

Funding: M.V. was supported by a grant from the Food Security Center (FSC), an excellence center for exchange and development at the University of Hohenheim (funding reference DAAD 57160040).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All raw data and biological material are saved in the institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150 k), University of Hohenheim, Fruwirthstraße 12, Stuttgart 70599, Germany. In case of requirement, please contact the corresponding author with any detailed questions.

Acknowledgments: We are very grateful to Elvio Henrique Benatto Perino for his assistance in the statistical analysis of this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rabbee, M.F.; Ali, M.S.; Choi, J.; Hwang, B.S.; Jeong, S.C.; Baek, K.-h. *Bacillus velezensis*: A valuable member of bioactive molecules within plant microbiomes. *Molecules* **2019**, *24*, 1046. [CrossRef] [PubMed]
2. Ye, M.; Tang, X.; Yang, R.; Zhang, H.; Li, F.; Tao, F.; Li, F.; Wang, Z. Characteristics and application of a novel species of *Bacillus*: *Bacillus velezensis*. *ACS Chem. Biol.* **2018**, *13*, 500–505. [CrossRef]

3. Wu, G.; Liu, Y.; Xu, Y.; Zhang, G.; Shen, Q.; Zhang, R. Exploring elicitors of the beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 to induce plant systemic resistance and their interactions with plant signaling pathways. *Mol. Plant-Microbe Interact.* **2018**, *31*, 560–567. [CrossRef]
4. Borriss, R.; Wu, H.; Gao, X. Secondary metabolites of the plant growth promoting model rhizobacterium *Bacillus velezensis* FZB42 are involved in direct suppression of plant pathogens and in stimulation of plant-induced systemic resistance. In *Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms*; Springer: Berlin/Heidelberg, Germany, 2019; pp. 147–168.
5. Wu, L.; Wu, H.-J.; Qiao, J.; Gao, X.; Borriss, R. Novel routes for improving biocontrol activity of *Bacillus* based bioinoculants. *Front. Microbiol.* **2015**, *6*, 1395. [CrossRef]
6. Chen, L.; Wang, X.; Ma, Q.; Bian, L.; Liu, X.; Xu, Y.; Zhang, H.; Shao, J.; Liu, Y. *Bacillus velezensis* CLA178-induced systemic resistance of *Rosa multiflora* against crown gall disease. *Front. Microbiol.* **2020**, *11*, 587667. [CrossRef] [PubMed]
7. Cawoy, H.; Debois, D.; Franzil, L.; de Pauw, E.; Thonart, P.; Ongena, M. Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. *Microb. Biotechnol.* **2015**, *8*, 281–295. [CrossRef] [PubMed]
8. Lugtenberg, B.; Kamilova, F. Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* **2009**, *63*, 541–556. [CrossRef]
9. Théatre, A.; Cano-Prieto, C.; Bartolini, M.; Laurin, Y.; Deleu, M.; Niehren, J.; Fida, T.; Gerbinet, S.; Alanjary, M.; Medema, M.H.; et al. The surfactin-like lipopeptides from *Bacillus* spp.: Natural biodiversity and synthetic biology for a broader application range. *Front. Bioeng. Biotechnol.* **2021**, *9*, 623701. [CrossRef]
10. Caulier, S.; Nannan, C.; Gillis, A.; Licciardi, F.; Bragard, C.; Mahillon, J. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. *Front. Microbiol.* **2019**, *10*, 302. [CrossRef]
11. Stein, T. *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Mol. Microbiol.* **2005**, *56*, 845–857. [CrossRef]
12. Finking, R.; Marahiel, M.A. Biosynthesis of nonribosomal peptides. *Annu. Rev. Microbiol.* **2004**, *58*, 453. [CrossRef] [PubMed]
13. Geissler, M.; Heravi, K.M.; Henkel, M.; Hausmann, R. Lipopeptide biosurfactants from *Bacillus* species. In *Biobased Surfactants*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 205–240.
14. Dunlap, C.A.; Bowman, M.J.; Rooney, A.P. Iturinic lipopeptide diversity in the *Bacillus subtilis* species group–important antifungals for plant disease biocontrol applications. *Front. Microbiol.* **2019**, *10*, 1794. [CrossRef] [PubMed]
15. Maget-Dana, R.; Peypoux, F. Iturins, a special class of pore-forming lipopeptides: Biological and physicochemical properties. *Toxicology* **1994**, *87*, 151–174. [CrossRef]
16. Cochrane, S.A.; Vederas, J.C. Lipopeptides from *Bacillus* and *Paenibacillus* spp.: A gold mine of antibiotic candidates. *Med. Res. Rev.* **2016**, *36*, 4–31. [CrossRef] [PubMed]
17. Ongena, M.; Jacques, P. *Bacillus* lipopeptides: Versatile weapons for plant disease biocontrol. *Trends Microbiol.* **2008**, *16*, 115–125. [CrossRef] [PubMed]
18. Dussert, E.; Tourret, M.; Dupuis, C.; Noblecourt, A.; Behra-Miellet, J.; Flahaut, C.; Ravallec, R.; Coutte, F. Evaluation of antiradical and antioxidant activities of lipopeptides produced by *Bacillus subtilis* strains. *Front. Microbiol* **2022**, *13*, 914713. [CrossRef]
19. Raaijmakers, J.M.; De Bruijn, I.; Nybroe, O.; Ongena, M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: More than surfactants and antibiotics. *FEMS Microbiol. Rev.* **2010**, *34*, 1037–1062. [CrossRef]
20. Chowdhury, S.P.; Hartmann, A.; Gao, X.; Borriss, R. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42–a review. *Front. Microbiol.* **2015**, *6*, 780. [CrossRef]
21. Jiang, J.; Zhang, H.; Zhang, C.; Han, M.; Du, J.; Yang, X.; Li, W. Production, purification and characterization of 'Iturin A-2'a lipopeptide with antitumor activity from Chinese Sauerkraut Bacterium *Bacillus velezensis* T701. *Int. J. Pept. Res. Ther.* **2021**, *27*, 2135–2147. [CrossRef]
22. Lilge, L.; Ersig, N.; Hubel, P.; Aschern, M.; Pillai, E.; Klausmann, P.; Pfannstiel, J.; Henkel, M.; Morabbi Heravi, K.; Hausmann, R. Surfactin shows relatively low antimicrobial activity against *Bacillus subtilis* and other bacterial model organisms in the absence of synergistic metabolites. *Microorganisms* **2022**, *10*, 779. [CrossRef]
23. Zhao, H.; Shao, D.; Jiang, C.; Shi, J.; Li, Q.; Huang, Q.; Rajoka, M.S.R.; Yang, H.; Jin, M. Biological activity of lipopeptides from *Bacillus*. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 5951–5960. [CrossRef] [PubMed]
24. Desai, J.D.; Banat, I.M. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 47–64. [PubMed]
25. Koumoutsi, A.; Chen, X.H.; Henne, A.; Liesegang, H.; Hitzeroth, G.; Franke, P.; Vater, J.; Borriss, R. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42 2004. *Am. Soc. Microbiol.* **2004**, *186*, 1084–1096.
26. Moreno-Velandia, C.A.; Ongena, M.; Kloepper, J.W.; Cotes, A.M. Biosynthesis of cyclic lipopeptides by *Bacillus velezensis* Bs006 and its antagonistic activity are modulated by the temperature and culture media conditions. *Curr. Microbiol.* **2021**, *78*, 3505–3515. [CrossRef]
27. Kaspar, F.; Neubauer, P.; Gimpel, M. Bioactive secondary metabolites from *Bacillus subtilis*: A comprehensive review. *J. Nat. Prod.* **2019**, *82*, 2038–2053. [CrossRef]
28. Leconte, A.; Tournant, L.; Muchembled, J.; Paucellier, J.; Héquet, A.; Deracinois, B.; Deweer, C.; Krier, F.; Deleu, M.; Oste, S.; et al. Assessment of Lipopeptide Mixtures Produced by *Bacillus subtilis* as biocontrol products against apple scab (*Venturia inaequalis*). *Microorganisms* **2022**, *10*, 1810. [CrossRef]
29. Qian, S.; Sun, J.; Lu, H.; Lu, F.; Bie, X.; Lu, Z. L-glutamine efficiently stimulates biosynthesis of bacillomycin D in *Bacillus subtilis* fmbJ. *Process Biochem.* **2017**, *58*, 224–229. [CrossRef]

30. Wu, J.-Y.; Liao, J.-H.; Shieh, C.-J.; Hsieh, F.-C.; Liu, Y.-C. Kinetic analysis on precursors for iturin A production from *Bacillus amyloliquefaciens* BPD1. *J. Biosci. Bioeng.* **2018**, *126*, 630–635. [CrossRef]
31. Yaseen, Y.; Gancel, F.; Béchet, M.; Drider, D.; Jacques, P. Study of the correlation between fengycin promoter expression and its production by *Bacillus subtilis* under different culture conditions and the impact on surfactin production. *Arch. Microbiol.* **2017**, *199*, 1371–1382. [CrossRef]
32. Besson, F.; Hourdou, M.L. Effect of amino acids on the biosynthesis of β-amino acids, constituents of bacillomycins F. *J. Antibiot.* **1987**, *40*, 221–223. [CrossRef]
33. Vahidinasab, M.; Ahmadzadeh, M.; Henkel, M.; Hausmann, R.; Morabbi Heravi, K. *Bacillus velezensis* UTB96 is an antifungal soil isolate with a reduced genome size compared to that of Bacillus velezensis FZB42. *Microbiol. Resour. Announc.* **2019**, *8*, e00667-19. [CrossRef] [PubMed]
34. Bagheri, N.; Ahmadzadeh, M.; Mariotte, P.; Jouzani, G.S. Behavior and interactions of the plant growth-promoting bacteria *Azospirillum oryzae* NBT506 and *Bacillus velezensis* UTB96 in a co-culture system. *World J. Microbiol. Biotechnol.* **2022**, *38*, 101. [CrossRef] [PubMed]
35. Afsharmanesh, H.; Ahmadzadeh, M. The Iturin lipopeptides as key compounds in antagonism of *Bacillus subtilis* UTB96 toward *Aspergillus flavus*. *Biol. Control Pests Plant Dis.* **2016**, *5*, 79–95.
36. Fan, B.; Wang, C.; Song, X.; Ding, X.; Wu, L.; Wu, H.; Gao, X.; Borriss, R. *Bacillus velezensis* FZB42 in 2018: The gram-positive model strain for plant growth promotion and biocontrol. *Front. Microbiol.* **2018**, *9*, 2491. [CrossRef] [PubMed]
37. Krebs, B.; Höding, B.; Kübart, S.; Workie, M.A.; Junge, H.; Schmiedeknecht, G.; Grosch, R.; Bochow, H.; Hevesi, M. Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterization of *Bacillus subtilis* strains. *J. Plant Dis. Prot.* **1998**, *105*, 181–197.
38. Santos, J.M.; Vrandečić, K.; Ćosić, J.; Duvnjak, T.; Phillips, A.J.L. Resolving the *Diaporthe* species occurring on soybean in Croatia. *Pers. -Mol. Phylogeny Evol. Fungi* **2011**, *27*, 9–19. [CrossRef]
39. Hosseini, B.; El-Hasan, A.; Link, T.; Voegele, R.T. Analysis of the species spectrum of the *Diaporthe/Phomopsis* complex in European soybean seeds. *Mycol. Prog.* **2020**, *19*, 455–469. [CrossRef]
40. Blin, K.; Shaw, S.; Kloosterman, A.M.; Charlop-Powers, Z.; van Wezel, G.P.; Medema, M.H.; Weber, T. antiSMASH 6.0: Improving cluster detection and comparison capabilities. *Nucleic Acids Res.* **2021**, *49*, W29–W35. [CrossRef]
41. Bóka, B.; Manczinger, L.; Kecskeméti, A.; Chandrasekaran, M.; Kadaikunnan, S.; Alharbi, N.S.; Vágvölgyi, C.; Szekeres, A. Ion trap mass spectrometry of surfactins produced by *Bacillus subtilis* SZMC 6179J reveals novel fragmentation features of cyclic lipopeptides. *Rapid Commun. Mass Spectrom.* **2016**, *30*, 1581–1590. [CrossRef]
42. Kecskeméti, A.; Bartal, A.; Bóka, B.; Kredics, L.; Manczinger, L.; Shine, K.; Alharby, N.S.; Khaled, J.M.; Varga, M.; Vágvölgyi, C.; et al. High-frequency occurrence of surfactin monomethyl isoforms in the ferment broth of a *Bacillus subtilis* strain revealed by ion trap mass spectrometry. *Molecules* **2018**, *23*, 2224. [CrossRef]
43. Lin, L.Z.; Zheng, Q.W.; Wei, T.; Zhang, Z.Q.; Zhao, C.F.; Zhong, H.; Xu, Q.Y.; Lin, J.F.; Guo, L.Q. Isolation and characterization of fengycins produced by *Bacillus amyloliquefaciens* JFL21 and its broad-spectrum antimicrobial potential against multidrug-resistant foodborne pathogens. *Front. Microbiol.* **2020**, *11*, 579621. [CrossRef] [PubMed]
44. Pathak, K.V.; Keharia, H.; Gupta, K.; Thakur, S.S.; Balaram, P. Lipopeptides from the banyan endophyte, *Bacillus subtilis* K1: Mass spectrometric characterization of a library of fengycins. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 1716–1728. [CrossRef] [PubMed]
45. Vahidinasab, M.; Lilge, L.; Reinfurt, A.; Pfannstiel, J.; Henkel, M.; Morabbi Heravi, K.; Hausmann, R. Construction and description of a constitutive plipastatin mono-producing *Bacillus subtilis*. *Microb. Cell Factories* **2020**, *19*, 205. [CrossRef] [PubMed]
46. Willenbacher, J.; Rau, J.-T.; Rogalla, J.; Syldatk, C.; Hausmann, R. Foam-free production of Surfactin via anaerobic fermentation of *Bacillus subtilis* DSM 10T. *Amb Express* **2015**, *5*, 21. [CrossRef] [PubMed]
47. Klausmann, P.; Hennemann, K.; Hoffmann, M.; Treinen, C.; Aschern, M.; Lilge, L.; Morabbi Heravi, K.; Henkel, M.; Hausmann, R. *Bacillus subtilis* high cell density fermentation using a sporulation-deficient strain for the production of surfactin. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 4141–4151. [CrossRef]
48. Yazgan, A.; Özcengiz, G.; Marahiel, M.A. Tn10 insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin. *Biochim. Et Biophys. Acta (BBA)-Gene Struct. Expr.* **2001**, *1518*, 87–94. [CrossRef]
49. Geissler, M.; Oellig, C.; Moss, K.; Schwack, W.; Henkel, M.; Hausmann, R. High-performance thin-layer chromatography (HPTLC) for the simultaneous quantification of the cyclic lipopeptides Surfactin, Iturin A and Fengycin in culture samples of *Bacillus* species. *J. Chromatogr. B* **2017**, *1044*, 214–224. [CrossRef]
50. Johnson, L.F.; Curl, E.A. Methods for research on the ecology of soil-borne plant pathogens. In *Methods for Research on the Ecology of Soil-Borne Plant Pathogens*; Burgess Publishing Company: Minneapolis, MN, USA, 1972.
51. Alenezi, F.N.; Slama, H.B.; Bouket, A.C.; Cherif-Silini, H.; Silini, A.; Luptakova, L.; Nowakowska, J.A.; Oszako, T.; Belbahri, L. *Bacillus velezensis*: A treasure house of bioactive compounds of medicinal, biocontrol and environmental importance. *Forests* **2021**, *12*, 1714. [CrossRef]
52. Li, X.; Munir, S.; Xu, Y.; Wang, Y.; He, Y. Combined mass spectrometry-guided genome mining and virtual screening for acaricidal activity in secondary metabolites of *Bacillus velezensis* W1. *RSC Adv.* **2021**, *11*, 25441–25449. [CrossRef]
53. Pupin, M.; Flissi, A.; Jacques, P.; Leclère, V. Bioinformatics tools for the discovery of new lipopeptides with biocontrol applications. *Eur. J. Plant Pathol.* **2018**, *152*, 993–1001. [CrossRef]

54. Xiao, J.; Guo, X.; Qiao, X.; Zhang, X.; Chen, X.; Zhang, D. Activity of fengycin and iturin A isolated from *Bacillus subtilis* Z-14 on *Gaeumannomyces graminis* var. *tritici* and soil microbial diversity. *Front. Microbiol.* **2021**, 1602. [CrossRef]
55. Cao, Y.; Pi, H.; Chandrangsu, P.; Li, Y.; Wang, Y.; Zhou, H.; Xiong, H.; Helmann, J.D.; Cai, Y. Antagonism of two plant-growth promoting *Bacillus velezensis* isolates against *Ralstonia solanacearum* and *Fusarium oxysporum*. *Sci. Rep.* **2018**, *8*, 4360. [CrossRef]
56. Sawant, S.S.; Song, J.; Seo, H.-J. Characterization of *Bacillus velezensis* RDA1 as a Biological Control Agent against White Root Rot Disease Caused by *Rosellinia necatrix*. *Plants* **2022**, *11*, 2486. [CrossRef]
57. Anith, K.N.; Nysanth, N.S.; Natarajan, C. Novel and rapid agar plate methods for in vitro assessment of bacterial biocontrol isolates' antagonism against multiple fungal phytopathogens. *Lett. Appl. Microbiol.* **2021**, *73*, 229–236. [CrossRef]
58. Chowdhury, S.P.; Uhl, J.; Grosch, R.; Alquéres, S.; Pittroff, S.; Dietel, K.; Schmitt-Kopplin, P.; Borriss, R.; Hartmann, A. Cyclic lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* colonizing the lettuce rhizosphere enhance plant defense responses toward the bottom rot pathogen *Rhizoctonia solani*. *Mol. Plant-Microbe Interact.* **2015**, *28*, 984–995. [CrossRef] [PubMed]
59. Calvo, H.; Mendiara, I.; Arias, E.; Blanco, D.; Venturini, M.E. The role of iturin A from *B. amyloliquefaciens* BUZ-14 in the inhibition of the most common postharvest fruit rots. *Food Microbiol.* **2019**, *82*, 62–69. [CrossRef]
60. Kim, Y.S.; Lee, Y.; Cheon, W.; Park, J.; Kwon, H.T.; Balaraju, K.; Kim, J.; Yoon, Y.J.; Jeon, Y. Characterization of *Bacillus velezensis* AK-0 as a biocontrol agent against apple bitter rot caused by *Colletotrichum gloeosporioides*. *Sci. Rep.* **2021**, *11*, 626. [CrossRef] [PubMed]
61. Jin, Q.; Jiang, Q.; Zhao, L.; Su, C.; Li, S.; Si, F.; Li, S.; Zhou, C.; Mu, Y.; Xiao, M. Complete genome sequence of *Bacillus velezensis* S3-1, a potential biological pesticide with plant pathogen inhibiting and plant promoting capabilities. *J. Biotechnol.* **2017**, *259*, 199–203. [CrossRef] [PubMed]
62. Gu, Q.; Yang, Y.; Yuan, Q.; Shi, G.; Wu, L.; Lou, Z.; Huo, R.; Wu, H.; Borriss, R.; Gao, X. Bacillomycin D produced by *Bacillus amyloliquefaciens* is involved in the antagonistic interaction with the plant-pathogenic fungus *Fusarium graminearum*. *Appl. Environ. Microbiol.* **2017**, *83*, e01075-17. [CrossRef] [PubMed]
63. Chen, X.H.; Vater, J.; Piel, J.; Franke, P.; Scholz, R.; Schneider, K.; Koumoutsi, A.; Hitzeroth, G.; Grammel, N.; Strittmatter, A.W.; et al. Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* **2006**, *188*, 4024–4036. [CrossRef]
64. Yokota, K.; Hayakawa, H. Impact of antimicrobial lipopeptides from *Bacillus* sp. on suppression of *Fusarium yellows* of tatsoi. *Microbes Environ.* **2015**, ME15062.
65. Jin, H.; Li, K.; Niu, Y.; Guo, M.; Hu, C.; Chen, S.; Huang, F. Continuous enhancement of iturin A production by *Bacillus subtilis* with a stepwise two-stage glucose feeding strategy. *BMC Biotechnol.* **2015**, *15*, 53. [CrossRef] [PubMed]
66. Belitsky, B.R. Role of branched-chain amino acid transport in *Bacillus subtilis* CodY activity. *J. Bacteriol.* **2015**, *197*, 1330–1338. [CrossRef] [PubMed]
67. Serror, P.; Sonenshein, A.L. CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. *J. Bacteriol.* **1996**, *178*, 5910–5915. [CrossRef]
68. Sun, J.; Liu, Y.; Lin, F.; Lu, Z.; Lu, Y. CodY, ComA, DegU and Spo0A controlling lipopeptides biosynthesis in *Bacillus amyloliquefaciens* fmbJ. *J. Appl. Microbiol.* **2021**, *131*, 1289–1304. [CrossRef] [PubMed]

Part III
Discussion

3.1 General discussion

The lipopeptides produced by *Bacillus* species exhibit excellent antifungal properties that make them a promising agent for use against phytopathogens in agriculture. However, there are several challenges associated with large-scale production of lipopeptides by *Bacillus*, including the complexity of the production mechanisms and inadequate ability of wild-type lipopeptide producer strains. Indeed, there is a need to improve bacterial production strains and develop bioprocess methods of scaling up production to increase efficiency and reduce costs.

This doctoral thesis focused on the evaluation of the biosynthesis of microbial lipopeptides including surfactin, iturin and fengycin (plipastatin) produced by *B. subtilis* and *B. velezensis*; specifically, strain development by genetic engineering and enhancement of lipopeptide titer by development of the cultivation medium. In the initial approach, the whole genome of the bacteria in terms of lipopeptide biosynthesis were analyzed using beneficial bioinformatics tools such as AntiSMASH (**publication 1**). Next to this analysis, the structure and yield of different produced lipopeptides were compared (**publication 2-4**). In the genome of *Bacillus* species, mainly two lipopeptide operons are present. In the case of domesticated laboratory model strain *B. subtilis* 168, the operons of surfactin and plipastatin are present in the genome. However, surfactin is the dominant produced lipopeptide and plipastatin is produced in the lower amount and therefore got less attention by researchers (Théatre et al. 2021). In this sense, the current thesis focused at further analysis on production of plipastatin by *B. subtilis* 168 and *B. subtilis* 3NA as a spore deficient strain suitable for bioreactor cultivation (**publication 2**). Moreover, based on the literature, the *sfp*$^+$ derivative of *B. subtilis* 168 is a better producer of surfactin than wild-type *B. subtilis* strains such as *B. subtilis* DSM10^T. One main genome characterization of *B. subtilis* 168 is that the pleiotropic *degQ* gene is due to a mutation in the −10 box of promoter is silenced (Stanley and Lazazzera 2005). Thereby, the effect of *degQ* expression on surfactin and plipastatin production was examined using a *sfp*$^+$ derivative of *B. subtilis* 168, and the wild-type *B. subtilis* DSM10^T, which expresses native *degQ* (**publication 2 and 3**). The next

approach of this thesis focused on bioproduction of microbial lipopeptides by *B. velezensis*. Identification of productive wild-type strains is crucial to the development of biopesticides. Here, the lipopeptide structure, productivity, and antifungal ability of *B. velezensis* UTB96 was compared with *B. velezensis* FZB42, as a well-established strain for biocontrol of phytopathogens in agriculture (**publication 4**).

In this chapter, throughout the following sections, the results obtained in this doctoral thesis are discussed exclusively to provide a deeper understanding of *Bacillus* lipopeptide biosynthesis by wild-type and genetically modified strains.

3.2 Whole-genome sequencing analysis as a comprehensive first overview of microbial lipopeptide gene clusters

Whole-genome sequencing (WGS) of different microorganisms has become more accessible and affordable in recent years (Quainoo et al. 2017). Analysis of the complete bacterial genome via WGS could replace routine conventional methods for the characterization of the lipopeptide production such as oil spreading and emulsification assays (Akintayo et al. 2022). The characterization of the genome of newly discovered wild-type antimicrobial lipopeptide producers may provide new information on biosynthesis and clusters of lipopeptides and lead to the introduction of more effective strains as fungicides (Teixeira et al. 2021). In this thesis, *B. velezensis* UTB96 as a wild-type Plant Growth Promoting Bacteria (PGPB) isolated from rhizosphere area of pistachio tree in Iran was selected for evaluation of its capability of lipopeptide production. Previous studies have demonstrated the pronounced antifungal activity of UTB96 against various fungal phytopathogens (Afsharmanesh and Ahmadzadeh 2016). In this sense, the whole genome sequencing of *B. velezensis* UTB96 was performed and submitted in the NCBI database under the GenBank accession number CP036527. As a next step, genome mining with tools like "antibiotics and secondary metabolite analysis shell, antiSMASH" used to identify natural secondary metabolites produced by *B. velezensis* UTB96. The AntiSMASH tool was initially released in 2011 and since then, it has become an established part of many workflows for discovering natural secondary metabolites produced by bacteria and fungi (Blin et al. 2021). In this thesis, to get a better comparison of the lipopeptide produced by *B. velezensis* UTB96, the genome of the model strain *B. velezensis* FZB42 used as the reference. Surfactin, bacillomycin D and fengycin are three main NRPS gene clusters in *B. velezensis* FZB42 (Fan et al. 2018). In the case of *B. velezensis* UTB96, additionally to the commonly known NRPS gene cluster for surfactin and fengycin biosynthesis, another cluster showed only moderate comparison with the bacillomycin D operon of the FZB42 strain. Further analysis showed that unlike strain FZB42, UTB96 harbors the iturin A operon. Considering the different peptide

structures of these molecules (Figure 3 in chapter 1), different antifungal properties could be expected. Therefore, further evaluation has been made to compare the lipopeptide productivity, antifungal activity and medium improvement for the production of lipopeptides by *B. velezensis* FZB42 and UTB96 as will be discussed later in sections 3.3 and 3.4. The other strains used in this thesis are the domesticated laboratory model *B. subtilis* 168, the spore deficient *B. subtilis* 3NA and the wild-type *B. subtilis* DSM10^{T}. The whole genome of these strains is already deposited in the NCBI database under the accession number of GCF_000009045.1, CP010314.1 and JAEPVU000000000.1 respectively (Lilge et al. 2021; Reuß et al. 2015). The genome map of these strains has guided us for further comparison of the regulatory genes such as *degQ* for lipopeptide biosynthesis that are discussed in the following section.

3.3 Evaluation of putative regulatory bottlenecks concerning lipopeptide production in *B. subtilis*

Regulatory bottlenecks in lipopeptide production in *B. subtilis* refer to the barriers that limit the biosynthesis of lipopeptides. The evaluation of potential regulatory bottlenecks involves identifying the various steps in lipopeptide biosynthesis and the regulatory mechanisms controlling these steps, the environmental factors affect lipopeptide production, and exploring the significance of post-transcriptional regulation of lipopeptide biosynthesis (Das et al. 2008). By assessing these regulatory bottlenecks, researchers can develop strategies to improve production and optimize its use in various application fields. In this thesis, a *sfp*$^{+}$ derivative of *B. subtilis* 3NA, named *B. subtilis* BMV9 was used for genetic engineering of plipastatin production in order to gain more insights on constitutive plipastatin mono-producer strains (publication 2). *B. subtilis* 3NA is a hybrid of the model strain *B. subtilis* 168 and strain W23 (Reuß et al. 2015). Strain 3NA is not able to produce spore due to a frameshift mutation within *spo0A* which makes it suitable for high cell

density fermentations. Despite the deletion of *spo0A*, *B. subtilis* 3NA shows natural competence development due to an elongation in the *abrB*.

Subsequently, the effect of pleiotropic *degQ* gene expression on production of plipastatin and surfactin was analyzed (publication 2 and 3). The following subsections discuss these results, that are presented in publication number 2 and 3.

3.3.1 Novel insights into the mono-production of plipastatin by *B. subtilis*

Biosynthesis of a certain metabolite in *B. subtilis* may compete with other biosynthetic pathways, leading to lower yields or undesired side products (Iqbal et al. 2023). Several genetic engineering approaches have been used to employ bacteria for mono-production of a single metabolite, such as deletion of genes involved in competing biosynthetic pathways and redirect metabolic flux towards the desired pathway (Tolibia et al. 2023). In this thesis, *B. subtilis* BMV9 was engineered to construct a strain that would exclusively produce plipastatin. To achieve this, the *srfAA-AD* operon was deleted from the genome of *B. subtilis* BMV9, while still maintaining the competence formation of the strain. However, deletion of *srfAA-AD* operon, did not have a positive effect on biosynthesis of plipastatin. Indeed, the decrease in plipastatin production, supports the hypothesis that *srfAA-AD* operon expression may stimulate plipastatin biosynthesis. Partially deletion of *srfAA-AD* operon has already done by other researchers. For instance, a Δ*srfAA* mutation in a *sfp*+ derivative of *B. subtilis* 168 reduced plipastatin production, while disruption of *srfAC* had no significant effect (Yaseen et al. 2018). Similarly, a recent study determined that deletion of *srfAB* and *srfAC* had no adverse effect on plipastatin production (Zou et al. 2020).

Another strategy for construction of a high lipopeptide mono-producer strain is to overexpress the genes encoding the enzymes involved in the biosynthesis pathway of the desired metabolite. This can be done by using strong constitutive promoters to drive the expression of these genes, or by introducing multiple copies of the genes into the bacterial genome. One reason for the comparably very low plipastatin formation in *B. subtilis* can

likely be attributed to the genetic regulation of plipastatin biosynthesis. The P_{pps} promoter is considered "weak" because it has relatively low transcriptional activity compared to stronger promoters (Yaseen et al. 2016). However, its activity is sufficient to drive the expression of the genes involved in plipastatin biosynthesis in its native *Bacillus* host. Weak native promoters in *B. subtilis* can be a challenge for gene expression studies (Liu et al. 2018). As this is still unknown, more fundamental studies need to be done in order to examine the regulation of native P_{pps} promoter mechanism. Nevertheless, the focus of this thesis was not on the expression of the *ppsA-E* operon by the native promoter. Therefore, an approximately five-fold enhancement of plipastatin production was achieved after the promoter exchange of native plipastatin promoter against the constitutive promoter region of the *veg* gene. In the next step, deleting the entire *srfAA-AD* operon and retaining *comS* in the genome through the P_{srfA} promoter was also performed in constitutive plipastatin production strain. Interestingly, unlike what it was expected, disruption of *srfAA-AD* operon had a negative effect not only when plipastatin is expressed by native P_{pps} promoter but also under expression of P_{veg}. In 2007, in a similar study after disruption of the surfactin synthetase by eliminating the *srfAB*, *srfAC*, and *srfAD* genes in a constructed constitutive plipastatin producer strain, the production of plipastatin did not affected (Ongena et al. 2007). To conclude, it can be assumed that the surfactin synthetase, or one of its subunits most likely *srfAA*, positively stimulates plipastatin production. Therefore, overexpression of the subunit may result in a plipastatin mono-producer without decreasing, if not increasing, plipastatin titer. However, the regulatory mechanism behind this effect remains to be elucidated.

3.3.2 Significant effect of DegU regulon on lipopeptide biosynthesis

The DegU regulon is a global regulatory system in *B. subtilis* that is involved in controlling various physiological processes, including sporulation, motility, and competence (Murray et al. 2009). It consists of the DegU response regulator and the DegS histidine kinase, which function together to sense environmental signals and activate downstream genes (Miras

and Dubnau 2016). It has been demonstrated that the expression of genes involved in production of secretory proteases and lipopeptide biosynthesis, including the the *bamA-D* operon for bacillomycin D biosynthesis, is under the control of the DegU regulon (Koumoutsi et al. 2007; Tsuge et al. 1999). Specifically, when the DegU is phosphorylated by the histidine kinase DegS, can directly bind to the promoter regions of *bamA-D* operons and activate the transcription (Koumoutsi et al. 2007; Miras and Dubnau 2016). The pleiotropic DegQ protein, has been shown to enhance the phosphorylation of DegU by the DegS histidine kinase, leading to increased activation of DegU (Kobayashi 2007). The *degQ* gene in the laboratory strain 168 is silenced due to a mutation in its promoter area, as mentioned earlier (Stanley and Lazazzera 2005). In this thesis, the effect of *degQ* gene expression on surfactin and plipastatin biosynthesis and secretion of proteases in two strains of *B. subtilis*: JABs24, a lipopeptide-producing strain derived from *B. subtilis* 168 that carries the *sfp*+ gene, and *B. subtilis* DSM10^T, a wild-type strain with native expression of *degQ*, were compared to each other. The *lacZ* fusion construct with native and point mutated *degQ* promoter was chromosomally integrated into *B. subtilis* DSM10^T and JABs24 and different *lacZ* reporter strains were constructed and compared. Thereby, we confirmed this reduction in *degQ* expression in JABs24 is due to inactivity of mutated *degQ* promoter, while native *degQ* promoter expression was significantly higher. These findings demonstrate that surfactin production is negatively affected by DegQ-associated DegU regulation, while increased plipastatin biosynthesis is achieved in the presence of native *degQ* expression. This opposing regulatory mechanism has been transferable to both JABs24 and DSM10^T highlighting its broad applicability. Thus, integration of a natively expressed *degQ* version in JABs24 resulted in the production of only approximately 75% of surfactin but 8-fold increased plipastatin titers, while elimination of *degQ* in DSM10^T showed a 3-fold increase in surfactin concentration and a 5-fold reduction in plipastatin formation. In addition to the production of lipopeptides, another important aspect of DegU-associated regulation is the formation of secretory proteases. The comparison of extracellular protease activities between JABs24 and DSM10^T revealed the effect of *degQ* gene expression, with DSM10^T showing 5-fold higher protease activity compared to JABs24. Interestingly, both surfactin and plipastatin showed no decrease in their concentrations during cultivation,

suggesting that lipopeptides are less targeted by native extracellular proteases. Further experiments involving the integration of a natively expressed *degQ* version in JABs24 and the deletion of *degQ* in DSM10^T resulted in an enhancement and reduction in extracellular protease production, respectively.

In conclusion, this section of the thesis has demonstrated for the first time a quantitative effect of *degQ* expression on the production of extracellular proteases and lipopeptide biosynthesis in *B. subtilis*. Moreover, the results underscore the contrasting mechanisms by which DegQ regulates surfactin and plipastatin biosynthesis. Understanding the current results may have applications in improvement of the biosynthesis of other bioactive secondary metabolites in other bacterial species and the development of novel biotechnological applications. Additionally, this research may have implications for understanding the broader role of DegQ in bacterial gene regulation and protein quality control.

3.4 *Bacillus velezensis* UTB96 as a promising strain for agriculture

Bacillus velezensis has shown great application potential in agriculture due to its ability to produce a range of bioactive compounds that can enhance plant growth and health while providing protection against plant diseases (Rabbee et al. 2019). One of the key benefits of *Bacillus velezensis* is its ability to produce antimicrobial lipopeptide that can protect plants against a range of phytopathogens. These compounds include iturin, surfactin, and fengycin lipopeptides, which have been shown to be effective against a range of fungal and bacterial pathogens that can cause significant damage to crop. In particular, fengycin and iturin lipopeptides have strong antifungal properties (Teixeira et al. 2021). Additionally, *Bacillus velezensis* produces enzymes that can break down plant debris in the soil, releasing valuable nutrients that can be taken up by plants. In addition to its ability to protect plants against disease, *Bacillus velezensis* is also known for its plant growth-promoting properties. The bacterium produces a range of plant hormones, which can stimulate root growth and

increase plant biomass. This leads to healthier, more robust plants that are better able to withstand environmental stresses (Ngalimat et al. 2021). This section aimed to evaluate a comparative study between wild-type *Bacillus velezensis* UTB96 and the model strain FZB42, with a focus on their antifungal lipopeptide biosynthesis capacity. As previously mentioned in section 3.1, although both strains have the operon for fengycin and surfactin, they produce different types of iturin. Specifically, *B. velezensis* UTB96 produces iturin A, while FZB42 produces bacillomycin D. Comparison of antimicrobial lipopeptide production showed that UTB96 is a better producer of iturin A and fengycin. The UTB96 strain also exhibited enhanced antifungal activity against the soybean fungal pathogen *Diaporthe longicolla* compared to FZB42. In addition, in the upscaling approach, *B. velezensis* UTB96 demonstrated promising lipopeptide production using a custom-built bioreactor system. Notably, the biosynthesis of iturin A continued until the end of cultivation, resulting in an increase from around 135 mg/L in shake flask to 620 mg/L under stable bioreactor conditions.

Overall, optimization of lipopeptide production by different *Bacillus velezensis* strains and comparison of their lipopeptide titer and antimicrobial activity are important steps towards the development of efficient and sustainable processes for lipopeptide production with potential applications. Nevertheless, in addition to the lipopeptide titer and antimicrobial activity, other factors such as cost, safety, and regulatory requirements should be considered when selecting the most suitable *Bacillus velezensis* strain for agriculture application and industrial production. To conclude, the finding of this section adds to the growing body of research on the potential use of *Bacillus velezensis* as a plant probiotic, particularly in the control of fungal pathogens. Furthermore, the study highlights the importance of strain selection in agricultural applications, as even closely related strains can differ significantly in their ability to produce specific antimicrobial compounds.

3.5 Medium optimization: a true challenge for enhancement of lipopeptide biosynthesis

The production of lipopeptides is influenced by several factors, including the composition of the cultivation medium (Xu et al. 2020). In recent years, there has been increasing interest in the optimization of lipopeptide production by supplementing the cultivation medium with amino acids. Amino acids play a crucial role in the biosynthesis of lipopeptides, as they serve as precursors for the synthesis of the peptide and lipid moieties (Wu et al. 2018). For instance, the addition of L-glutamine in the cultivation medium of *Bacillus subtilis* fmbJ stimulates biosynthesis of bacillomycin D (Qian et al. 2017). Nevertheless, as it is discussed in publication 2 and 4, amino acid supplementation does not necessarily increase the lipopeptide biosynthesis. In this thesis, to improve plipastatin production, *B. subtilis* BMV9 and the constructed *B. subtilis* BMV12, a plipastatin mono-producer strain, were supplemented with the amino acids present in the structure of plipastatin. Ornithine was the only amino acid that induced detectable levels of plipastatin in the plipastatin mono-producer BMV12 strain. Interestingly, the addition of other amino acids, except for ornithine, resulted in a reduction of plipastatin titer in BMV9. The observed phenomenon can be attributed to the activation or deactivation of the stringent response in *B. subtilis*, which occurs in response to amino acid limitation. When the stringent response is activated, the provision of branched-chain amino acids is increased. Conversely, the addition of amino acids to the growth medium reduces the beneficial effects of the stringent response. However, the addition of ornithine to the medium did not have a negative effect, and in fact, it was the only amino acid that increased the production of plipastatin to detectable levels in the BMV12 strain.

In case of *B. velezensis* UTB96, with the aim of improvement of all three lipopeptide families, the cultivation medium was supplemented with different amino acids. However, the addition of branched-chain amino acids, such as valine, leucine, and isoleucine, was found to have a negative impact on lipopeptide production. This may be due to the activation of the global transcriptional regulator CodY in the presence of these amino acids, which acts as a repressor for the expression of the *srfAA-AD* operon. While a stimulatory effect on the *bam* operon has been observed in the *B. amyloliquefaciens* fmbJ, the effects of CodY on the fengycin operon in UTB96 remain unknown. On the other hand, lysine and alanine were found to stimulate the biosynthesis of all three lipopeptides in UTB96. Interestingly, iturin A concentration increased regardless of the supplemented amino acid, indicating that a higher availability of nitrogen sources can boost iturin A production.

In summary, although amino acid supplementation is a promising strategy for optimizing lipopeptide production in *Bacillus* strains, the underlying mechanism is not yet fully elucidated. It is thought that amino acid supplementation may increase the availability of precursors for lipopeptide biosynthesis, thereby promoting biosynthesis. Additionally, amino acids may impact the expression of genes involved in lipopeptide biosynthesis, although further research is needed to investigate this possibility. It is worth noting that the optimal concentration of amino acids can vary depending on the strain and the specific lipopeptide of interest. A systematic approach such as response surface methodology (RSM), a statistical optimization technique that involves designing experiments based on a mathematical model, may be employed to identify the optimal conditions for maximum lipopeptide production. Furthermore, to gain insight into the beneficial cellular adaptations in response to amino acid supplementation, future studies could focus on examining changes in the proteome and metabolome during cultivations.

3.6 Concluding remarks

The research presented in this thesis provides promising insights into the improvement of lipopeptide production strains and bioprocess methods, including genetic engineering and development of cultivation medium. Indeed, the biosynthesis of metabolites in *B. subtilis* can be complex and compete with other biosynthetic pathways, leading to lower yields or undesired side products. Genetic engineering approaches may be used to construct mono-producer strains of desired metabolites. One strategy is to delete genes involved in competing biosynthetic pathways and redirect metabolic flux towards the desired pathway. However, deletion of the genes or operons belonging to biosynthesis side products may actually have a negative effect on production of target metabolite. This is what was observed in this thesis. Deletion of the surfactin operon had a negative effect on plipastatin production, suggesting that the surfactin synthetase, or one of its subunits, positively stimulates plipastatin biosynthesis. However, further fundamental studies are needed to elucidate the regulatory mechanisms behind these effects. Additionally, weak native promoters in *B. subtilis* can be a challenge for gene expression studies, but this may be overcome by replacing them with stronger promoters, as the constitutive P_{veg} promoter increased plipastatin production fivefold in this research. Overall, a high lipopeptide mono-producer strain can be constructed by modifying the biosynthesis pathway. In addition, genetic engineering can also be used to manipulate the regulatory networks that control metabolite production. For example, transcription factors that positively or negatively regulate the expression of genes involved in metabolite biosynthesis can be overexpressed or deleted, respectively, to increase the production of the desired metabolite. Once the strain has been created, it can be further optimized through fermentation process development to maximize metabolite production and yield. In this thesis, the effect of amino acid supplementation in the cultivation medium was analyzed. Another highlight of this study is the important role of the DegU regulon in regulating various physiological processes in *Bacillus subtilis*, including sporulation, motility, and competence. The study specifically focuses on the effect of *degQ* gene expression on the biosynthesis of extracellular

proteases and lipopeptides in two strains of *B. subtilis*. The findings suggest that DegQ-associated DegU regulation negatively affects surfactin production but enhances plipastatin biosynthesis. These opposing regulatory mechanisms may have broad applicability in improving biosynthesis of other bioactive secondary metabolites in other bacterial species and developing novel biotechnological applications. Further research is needed to fully understand the broader role of DegQ in bacterial gene regulation and protein quality control. Additionally, the thesis highlights the importance of identifying productive wild-type strains and the comparison of lipopeptide structure, productivity, and antifungal ability. As was mentioned earlier, the use of GMOs in agriculture in the EU is highly restricted. In this thesis, specifically, the potential of wild-type *B. velezensis* UTB96 as a potent strain for iturin A production was introduced. Nevertheless, further research is needed to explore its full antifungal potential against a wide spectrum of different pathogens and additional analysis of the proteome and metabolome may reveal the underlying reasons for the better lipopeptide production of *B. velezensis* UTB96 compared to the model *B. velezensis* FZB42.

The knowledge gained from this thesis could be important for research on other lipopeptide produced by Gram-positive, but possibly also in other bacteria. The findings may also facilitate further research on regulation and putative bottlenecks of lipopeptide biosynthesis and may help to improve the establishment of methods for development of industrial relevant lipopeptide producer strains. More specifically, in agricultural science production of microbial lipopeptide will enable applied research in the fields of biological control of phytopathogens.

List of references

Afsharmanesh H, Ahmadzadeh M (2016) The Iturin lipopeptides as key compounds in antagonism of *Bacillus subtilis* UTB96 toward *Aspergillus flavus*. Biological Control of Pests and Plant Diseases 5:79–95. doi:10.22059/jbioc.2016.58619.

Akintayo SO, Treinen C, Vahidinasab M, Pfannstiel J, Bertsche U, Fadahunsi I, Oellig C, Granvogl M, Henkel M, Lilge L (2022) Exploration of surfactin production by newly isolated *Bacillus* and *Lysinibacillus* strains from food-related sources. Letters in Applied Microbiology 75:378–387. doi: 10.1111/lam.13731.

Aleti G, Sessitsch A, Brader G (2015) Genome mining: prediction of lipopeptides and polyketides from *Bacillus* and related Firmicutes. Computational and Structural Biotechnology Journal 13:192–203. doi: 10.1016/j.csbj.2015.03.003.

Anderson JA, Gipmans M, Hurst S, Layton R, Nehra N, Pickett J, Shah DM, Souza TLP, Tripathi L (2016) Emerging agricultural biotechnologies for sustainable agriculture and food security. Journal of Agricultural and Food Chemistry 64:383–393. doi:10.1021/acs.jafc.5b04543.

Ansaldi M, Marolt D, Stebe T, Mandic-Mulec I, Dubnau D (2002) Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants. Molecular Microbiology 44:1561–1573. doi: 10.1046/j.1365-2958.2002.02977.x.

Arima K, Kakinuma A, Tamura G (1968) Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. Biochemical and Biophysical Research Communications 31:488–494. doi: 10.1016/0006-291x(68)90503-2.

Balan SS, Kumar CG, Jayalakshmi S (2017) Aneurinifactin, a new lipopeptide biosurfactant produced by a marine *Aneurinibacillus aneurinilyticus* SBP-11 isolated from Gulf of Mannar: Purification, characterization and its biological evaluation. Microbiological Research 194:1–9. doi: 10.1016/j.micres.2016.10.005.

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial biosurfactants production, applications and future potential. Applied Microbiology and Biotechnology 87:427–444. doi: 10.1007/s00253-010-2589-0.

Bhargava P, Singh AK, Goel R (2017) Microbes: bioresource in agriculture and environmental sustainability. In: Plant-microbe Interactions in Agro-ecological Perspectives. Springer, pp 361–376. doi: 10.1007/978-981-10-5813-4_18

Blin K, Shaw S, Kloosterman AM, Charlop-Powers Z, van Wezel GP, Medema MH, Weber T (2021) antiSMASH 6.0: improving cluster detection and comparison capabilities. Nucleic Acids Research 49:W29-W35. doi: 10.1093/nar/gkab335.

Caboche S, Pupin M, Leclere V, Fontaine A, Jacques P, Kucherov G (2007) NORINE: a database of nonribosomal peptides. Nucleic Acids Research 36:D326-D331. doi: 10.1093 /nar/gkm792.

Cawoy H, Debois D, Franzil L, Pauw E de, Thonart P, Ongena M (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis /amyloliquefaciens*. Microbial Biotechnology 8:281–295. doi: 10.1111/1751-7915.12238.

Chen H, Zhou H, Sun T, Xu J, Tu Q, Yang J, Zhang Y, Bian X (2020) Identification of holrhizins E–Q reveals the diversity of nonribosomal lipopeptides in *Paraburkholderia rhizoxinica*. Journal of Natural Products 83:537–541. doi: 10.1021/acs.jnatprod.9b00927.

Cheng W, Feng YQ, Ren J, Jing D, Wang C (2016) Anti-tumor role of *Bacillus subtilis* fmbJ-derived fengycin on human colon cancer HT29 cell line. Neoplasma 63:215–222. doi:10.4149/206_150518N270.

Chowdhury SP, Hartmann A, Gao X, Borriss R (2015) Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42–a review. Frontiers in Microbiology 6:780. doi: 10.3389/fmicb.2015.00780.

Chumsakul O, Takahashi H, Oshima T, Hishimoto T, Kanaya S, Ogasawara N, Ishikawa S (2011) Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and its homolog Abh reveals their interactive role in transcriptional regulation. Nucleic Acids Research 39:414–428. doi: 10.1093/nar/gkq780.

Clements T, Ndlovu T, Khan S, Khan W (2019) Biosurfactants produced by *Serratia* species: Classification, biosynthesis, production and application. Applied Microbiology and Biotechnology 103:589–602. doi: 10.1007/s00253-018-9520-5.

Comella N, Grossman AD (2005) Conservation of genes and processes controlled by the quorum response in bacteria: characterization of genes controlled by the quorum-sensing transcription factor ComA in *Bacillus subtilis*. Molecular Microbiology 57:1159–1174. doi: 10.1111/j.1365-2958.2005.04749.x

Coutte F, Lecouturier D, Dimitrov K, Guez J-S, Delvigne F, Dhulster P, Jacques P (2017) Microbial lipopeptide production and purification bioprocesses, current progress and future challenges. Biotechnology Journal 12:1600566. doi: 10.1002/biot.201600566

Dahl MK, Msadek T, Kunst F, Rapoport G (1991) Mutational analysis of the *Bacillus subtilis* DegU regulator and its phosphorylation by the DegS protein kinase. Journal of Bacteriology 173:2539–2547. doi: 10.1128/jb.173.8.2539-2547.1991.

Dalili D, Amini M, Faramarzi MA, Fazeli MR, Khoshayand MR, Samadi N (2015) Isolation and structural characterization of Coryxin, a novel cyclic lipopeptide from *Corynebacterium xerosis* NS5 having emulsifying and anti-biofilm activity. Colloids and Surfaces B: Biointerfaces 135:425–432. doi: 10.1016/j.colsurfb.2015.07.005.

Das P, Mukherjee S, Sen R (2008) Genetic regulations of the biosynthesis of microbial surfactants: an overview. Biotechnology and Genetic Engineering Reviews 25:165–186. doi: 10.5661/bger-25-165.

Delcambe L (1950) Iturine, new antibiotic produced by *Bacillus subtilis.* C. R. Seances Soc. Biol. Fil. 144:1431–1434.

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology Reviews 61:47–64. doi: 10.1128/mm br.61.1.47-64.1997.

Duitman EH, Wyczawski D, Boven LG, Venema G, Kuipers OP, Hamoen LW (2007) Novel methods for genetic transformation of natural *Bacillus subtilis* isolates used to study the regulation of the mycosubtilin and surfactin synthetases. Applied and Environmental Microbiology 73:3490–3496. doi: 10.1128/AEM.02751-06.

Dunlap CA, Bowman MJ, Rooney AP (2019) Iturinic lipopeptide diversity in the *Bacillus subtilis* species group–important antifungals for plant disease biocontrol applications. Frontiers in Microbiology 10:1794. doi: 10.3389/fmicb.2019.01794.

Eddouaouda K, Mnif S, Badis A, Younes SB, Cherif S, Ferhat S, Mhiri N, Chamkha M, Sayadi S (2012) Characterization of a novel biosurfactant produced by *Staphylococcus* sp. strain 1E with potential application on hydrocarbon bioremediation. Journal of Basic Microbiology 52:408–418. doi: 10.1002/jobm.201100268.

Ervin DE, Glenna LL, Jussaume RA (2010) Are biotechnology and sustainable agriculture compatible? Renewable Agriculture and Food Systems 25:143–157. doi: 10.1017/S1742 170510000189.

Fan B, Wang C, Song X, Ding X, Wu L, Wu H, Gao X, Borriss R (2018) *Bacillus velezensis* FZB42 in 2018: the gram-positive model strain for plant growth promotion and biocontrol. Frontiers in Microbiology 9:2491. doi: 10.3389/fmicb.2018.02491.

Ferraro DO, Ghersa F, Paula R de, Duarte Vera AC, Pessah S (2020) Historical trends of the ecotoxicological pesticide risk from the main grain crops in Rolling Pampa (Argentina). Plos One 15:e0238676. doi: 10.1371/journal.pone.0238676.

Flissi A, Ricart E, Campart C, Chevalier M, Dufresne Y, Michalik J, Jacques P, Flahaut C, Lisacek F, Leclère V (2020) Norine: Update of the nonribosomal peptide resource. Nucleic Acids Research 48:D465-D469. doi: 10.1093/nar/gkz1000.

Foley JA, DeFries R, Asner GP, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK (2005) Global consequences of land use. Science 309:570–574. doi: 10.1126/science.1111772.

Fowler Jr VG, Boucher HW, Corey GR, Abrutyn E, Karchmer AW, Rupp ME, Levine DP, Chambers HF, Tally FP, Vigliani GA (2006) Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. New England Journal of Medicine 355:653–665. doi: 10.1056/NEJMoa053783

Friedrichs S, Takasu Y, Kearns P, Dagallier B, Oshima R, Schofield J, Moreddu C (2019) An overview of regulatory approaches to genome editing in agriculture. Biotechnology Research and Innovation 3:208–220. doi: 10.1016/j.biori.2019.07.001.

G**aur VK, Sharma P, Gupta S, Varjani S, Srivastava JK, Wong JWC, Ngo HH (2022)** Opportunities and challenges in omics approaches for biosurfactant production and feasibility of site remediation: Strategies and advancements. Environmental Technology and Innovation 25:102132. doi: 10.1016/j.eti.2021.102132

Geissler M, Heravi KM, Henkel M, Hausmann R (2019) Lipopeptide biosurfactants from *Bacillus* species. In: Biobased surfactants. Elsevier, pp 205–240. doi: 10.1016/B978-0-12-812705-6.00006-X.

Gerard J, Lloyd R, Barsby T, Haden P, Kelly MT, Andersen RJ (1997) Massetolides A− H, antimycobacterial cyclic depsipeptides produced by two *pseudomonads* isolated from marine habitats. Journal of Natural Products 60:223–229. doi: 10.1021/np9606456.

Geudens N, Martins JC (2018) Cyclic lipodepsipeptides from *Pseudomonas* spp.–biological swiss-army knives. Frontiers in Microbiology 9:1867. doi: 10.3389/fmicb.2018.01867.

Ghequire MGK, Mot R de (2014) Ribosomally encoded antibacterial proteins and peptides from *Pseudomonas*. Federation of European Microbiological Societies Microbiology Reviews 38:523–568. doi: 10.1111/1574-6976.12079.

Graupner K, Scherlach K, Bretschneider T, Lackner G, Roth M, Gross H, Hertweck. C (2012) Imaging mass spectrometry and genome mining reveal highly antifungal virulence factor of mushroom soft rot pathogen. Angewandte Chemie International Edition 51:13173–13177. doi: 10.1002/anie.201206658.

Groupé V, Pugh LH, Weiss D, Kochi M (1951) Observations on antiviral activity of viscosin. Proceedings of the Society for Experimental Biology and Medicine 78:354–358. doi: 10.3181/00379727-78-19071.

Hader JD, Lane T, Boxall ABA, MacLeod M, Di Guardo A (2022) Enabling forecasts of environmental exposure to chemicals in European agriculture under global change. Science of The Total Environment:156478. doi: 10.1016/j.scitotenv.2022.156478.

Hamoen LW, Venema G, Kuipers OP (2003) Controlling competence in *Bacillus subtilis*: shared use of regulators. Microbiology 149:9–17. doi: 10.1099/mic.0.26003-0.

Honma M, Tanaka K, Konno K, Tsuge K, Okuno T, Hashimoto M (2012) Termination of the structural confusion between plipastatin A1 and fengycin IX. Bioorganic and Medicinal Chemistry 20:3793–3798. doi: 10.1016/j.bmc.2012.04.040.

Hua Y, Yuan Y, Qin Y, Zhang C, Wang X, Feng S, Lu Y (2022) Advances in the Agro-Environment migration of organic chemical pollutants and their biotransformation in Crops. Agronomy 12:3009. doi: 10.3390/agronomy12123009.

Huang X, Lu Z, Zhao H, Bie X, Lü F, Yang S (2006) Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against *pseudorabies* virus, porcine *parvovirus*, newcastle disease virus and infectious bursal disease virus in *vitro*. International Journal of peptide research and therapeutics 12:373–377. doi:10.1007/s10989-006-9041-4

Inès M, Dhouha G (2015) Lipopeptide surfactants: production, recovery and pore forming capacity. Peptides 71:100–112. doi: 10.1016/j.peptides.2015.07.006.

Iqbal S, Begum F, Rabaan AA, Aljeldah M, Al Shammari BR, Alawfi A, Alshengeti A, Sulaiman T, Khan A (2023) Classification and Multifaceted Potential of Secondary Metabolites Produced by *Bacillus subtilis* Group: A Comprehensive Review. Molecules 28:927. doi: 10.3390/molecules28030927.

Jacques P (2011) Surfactin and other lipopeptides from *Bacillus* spp. In: Biosurfactants. Springer, pp 57–91. doi: 10.1007/978-3-642-14490-5_3.

Jang JY, Yang SY, Kim YC, Lee CW, Park MS, Kim JC, Kim IS (2013) Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. Journal of Agricultural and Food Chemistry 61:6786–6791. doi: 10.1021/jf401218w.

Jourdan F, Lazzaroni S, Méndez BL, Lo Cantore P, Julio M de, Amodeo P, Iacobellis NS, Evidente A, Motta A (2003) A left-handed α-helix containing both L-and D-amino acids: The solution structure of the antimicrobial lipodepsipeptide tolaasin. Proteins: Structure, Function, and Bioinformatics 52:534–543. doi: 10.1002/prot.10418.

Kalamara M, Spacapan M, Mandic-Mulec I, Stanley-Wall NR (2018) Social behaviours by *Bacillus subtilis*: quorum sensing, kin discrimination and beyond. Molecular Microbiology 110:863–878. doi: 10.1111/mmi.14127

Kaneka (2022). Surfactin, URL: https://www.kaneka.co.jp/en/business/qualityoflife/nbd_002.html (visited on 01/04/2023).

Kiss L (2003) A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Management Science: Formerly Pesticide Science 59:475–483. doi: 10.1002/ps.689.

Klausmann P, Lilge L, Aschern M, Hennemann K, Henkel M, Hausmann R, Morabbi Heravi K (2021) Influence of *B. subtilis* 3NA mutations in *spo0A* and *abrB* on surfactin production in *B. subtilis* 168. Microbial Cell Factories 20:1–15. doi: 10.1186/s12934-021-01679-z

Kobayashi K (2007) Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. Molecular Microbiology 66:395–409. doi: 10.1111/j.1365-2958.2007.05923.x.

Kobayashi K (2019) Inactivation of cysL inhibits biofilm formation by activating the disulfide stress regulator Spx in *Bacillus subtilis*. Journal of Bacteriology 201:e00712-18. doi: 10.1128/JB.00712-18.

Koumoutsi A, Chen X-H, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R (2004) Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology 186:1084–1096. doi: 10.1128/JB.186.4.1084-1096.2004.

Koumoutsi A, Chen X-H, Vater J, Borriss R (2007) DegU and YczE positively regulate the synthesis of bacillomycin D by *Bacillus amyloliquefaciens* strain FZB42. Applied and Environmental Microbiology 73:6953–6964. doi: 10.1128/AEM.00565-07.

Lilge L, Hertel R, Morabbi Heravi K, Henkel M, Commichau FM, Hausmann R (2021) Draft genome sequence of the type strain *Bacillus subtilis* subsp. *subtilis* DSM10. Microbiology Resource Announcements 10:e00158-21. doi: /10.1128/MRA.00158-21.

Lilge L, Ersig N, Hubel P, Aschern M, Pillai E, Klausmann P, Pfannstiel J, Henkel M, Morabbi Heravi K, Hausmann R (2022) Surfactin shows relatively low antimicrobial activity against *Bacillus subtilis* and other bacterial model organisms in the absence of synergistic metabolites. Microorganisms 10:779. doi: 10.3390/microorganisms10040779.

Liu D, Mao Z, Guo J, Wei L, Ma H, Tang Y, Chen T, Wang Z, Zhao X (2018) Construction, model-based analysis, and characterization of a promoter library for fine-tuned gene expression in *Bacillus subtilis*. ACS Synthetic biology 7:1785–1797. doi: 10.1021/acssynbio.8b00115.

Liu S, Tang M-H, Cheng J-S (2022) Fermentation optimization of surfactin production of *Bacillus amyloliquefaciens* HM618. Biotechnology and Applied Biochemistry. doi: 10.1002/bab.2327.

López D, Kolter R (2010) Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. Federation of European Microbiological Societies Microbiology Reviews 34:134–149. doi: 10.1111/j.1574-6976.2009.00199.x.

Maget-Dana R, Peypoux F (1994) Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology 87:151–174. doi: 10.1016/0300-483X(94)90159-7.

Magnuson R, Solomon J, Grossman AD (1994) Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. Cell 77:207–216. doi: 10.1016/0092-8674(94)90313-1.

Mahapatra S, Yadav R, Ramakrishna W (2022) *Bacillus subtilis* impact on plant growth, soil health and environment: Dr. Jekyll and Mr. Hyde. Journal of Applied Microbiology 132:3543–3562. doi: 10.1111/jam.15480.

Martinez JS, Carter-Franklin JN, Mann EL, Martin JD, Haygood MG, Butler A (2003) Structure and membrane affinity of a suite of amphiphilic siderophores produced by a marine bacterium. Proceedings of the National Academy of Sciences 100:3754–3759. doi: 10.1073/pnas.0637444100.

Miras M, Dubnau D (2016) A DegU-P and DegQ-dependent regulatory pathway for the K-state in *Bacillus subtilis*. Frontiers in Microbiology 7:1868. doi: 10.3389/fmicb.2016.01868.

Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y, Imanaka T (1993) A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. Journal of Bacteriology 175:6459–6466. doi: 10.1128/jb.175.20.6459-6466.1993.

Msadek T, Kunst F, Klier A, Rapoport G (1991) DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. Journal of Bacteriology 173:2366–2377. doi: 10.1128/jb.173.7.2366-2377.1991.

Muller C, Nolden S, Gebhardt P, Heinzelmann E, Lange C, Puk O, Welzel K, Wohlleben W, Schwartz D (2007) Sequencing and analysis of the biosynthetic gene cluster of the lipopeptide antibiotic friulimicin in *Actinoplanes friuliensis*. Antimicrobial Agents and Chemotherapy 51:1028–1037. doi: 10.1128/AAC.00942-06.

Murray EJ, Kiley TB, Stanley-Wall NR (2009) A pivotal role for the response regulator DegU in controlling multicellular behaviour. Microbiology 155:1–8. doi: 10.1099/mic.0.023903-0.

Nakano S, Küster-Schöck E, Grossman AD, Zuber P (2003) Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis.* Proceedings of the National Academy of Sciences 100:13603–13608. doi: 10.1073/pnas.2235180100.

Nakano MM, Zuber P (1991) The primary role of *comA* in establishment of the competent state in *Bacillus subtilis* is to activate expression of *srfA*. Journal of Bacteriology 173:7269–7274. doi: 10.1128/jb.173.22.7269-7274.1991.

Ngalimat MS, Yahaya RSR, Baharudin MMA, Yaminudin SM, Karim M, Ahmad SA, Sabri S (2021) A review on the biotechnological applications of the operational group *Bacillus amyloliquefaciens*. Microorganisms 9:614. doi: 10.3390/microorganisms9030614.

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16:115–125. doi: 10.1016/j.tim.2007.12.009.

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny J-L, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environmental Microbiology 9:1084–1090. doi: 10.1111/j.1462-2920.2006.01202.x.

Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z, Cameotra SS (2011) Environmental applications of biosurfactants: recent advances. International Journal of Molecular Sciences 12:633–654. doi: 10.1007/s11356-014-2872-1.

Paparini A, Romano-Spica V (2004) Public health issues related with the consumption of food obtained from genetically modified organisms. Biotechnology Annual Review 10:85–122. doi: 10.1016/s1387-2656(04)10004-5.

Peng F, Wang Y, Sun F, Liu Z, Lai Q, Shao Z (2008) A novel lipopeptide produced by a Pacific Ocean deep-sea bacterium, *Rhodococcus* sp. TW53. Journal of Applied Microbiology 105:698–705. doi: 10.1111/j.1365-2672.2008.03816.x.

Pérez-García A, Romero D, Vicente A de (2011) Plant protection and growth stimulation by microorganisms: biotechnological applications of *Bacilli* in agriculture. Current Opinion in Biotechnology 22:187–193. doi: 10.1016/j.copbio.2010.12.003.

Peypoux F, Guinand M, Michel G, Delcambe L, Das BC, Lederer E (1978) Structure of iturine A, a peptidolipid antibiotic from *Bacillus subtilis*. Biochemistry 17:3992–3996. doi: 10.1021/bi00612a018.

Phillips ZE, Strauch MA (2002) *Bacillus subtilis* sporulation and stationary phase gene expression. Cellular and Molecular Life Sciences CMLS 59:392–402. doi: 10.1007/s00018-002-8431-9.

Qian S, Sun J, Lu H, Lu F, Bie X, Lu Z (2017) L-glutamine efficiently stimulates biosynthesis of bacillomycin D in *Bacillus subtilis* fmbJ. Process Biochemistry 58:224–229. doi: 10.1016/j.procbio.2017.04.026.

Quainoo S, Coolen JPM, van Hijum SA, Huynen MA, Melchers WJG, van Schaik W, Wertheim HFL (2017) Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis. Clinical Microbiology Reviews 30:1015–1063. doi: 10.1128/CMR.00016-17.

Raaijmakers JM, Bruijn I de, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics.

Federation of European Microbiological Societies Microbiology Reviews 34:1037–1062. doi: 10.1111/j.1574-6976.2010.00221.x.

Rabbee MF, Ali MS, Choi J, Hwang BS, Jeong SC, Baek K (2019) *Bacillus velezensis*: a valuable member of bioactive molecules within plant microbiomes. Molecules 24:1046. doi: 10.3390/molecules24061046.

Rahman FB, Sarkar B, Moni R, Rahman MS (2021) Molecular genetics of surfactin and its effects on different sub-populations of *Bacillus subtilis*. Biotechnology Reports 32:e00686. doi: 10.1016/j.btre.2021.e00686.

Reuß DR, Schuldes J, Daniel R, Altenbuchner J (2015) Complete genome sequence of *Bacillus subtilis* subsp. *subtilis* strain 3NA. Genome Announcements 3:e00084-15. doi:10 1128/genomeA.00084-15.

Reuter K, Mofid MR, Marahiel MA, Ficner R (1999) Crystal structure of the surfactin synthetase-activating enzyme *sfp*: a prototype of the 4′-phosphopantetheinyl transferase superfamily. The European Molecular Biology Organization Journal 18:6823–6831. doi: 10.1093/emboj/18.23.6823.

Romero D, Vicente A de, Rakotoaly RH, Dufour SE, Veening J-W, Arrebola E, Cazorla FM, Kuipers OP, Paquot M, Pérez-García A (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward Podosphaera fusca. Molecular Plant-Microbe Interactions 20:430–440. doi: 10.1094/MPMI-20-4-0430.

Roongsawang N, Washio K, Morikawa M (2010) Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. International Journal of Molecular Sciences 12:141–172. doi: 10.3390/ijms12010141.

Sachdev DP, Cameotra SS (2013) Biosurfactants in agriculture. Applied Microbiology and Biotechnology 97:1005–1016. doi: 10.1007/s00253-012-4641-8.

Schlosser N, Espino-Martínez J, Kloss F, Meyer F, Bardl B, Rosenbaum MA, Regestein L (2021) Host nutrition-based approach for biotechnological production of the antifungal cyclic lipopeptide jagaricin. Journal of Biotechnology 336:1–9. doi: 10.1016/j.jbiotec. 2021.06.015.

Serror P, Sonenshein AL (1996) CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. Journal of Bacteriology 178:5910–5915. doi:10.1128 /jb.178.20.5910-5915.1996.

Shaheen M, Li J, Ross AC, Vederas JC, Jensen SE (2011) *Paenibacillus polymyxa* PKB1 produces variants of polymyxin B-type antibiotics. Chemistry and Biology 18:1640–1648. doi: 10.1016/j.chembiol.2011.09.017.

Shank EA, Kolter R (2011) Extracellular signaling and multicellularity in *Bacillus subtilis*. Current Opinion in Microbiology 14:741–747. doi: 10.1016/j.mib.2011.09.016.

Shi J, Zhu X, Lu Y, Zhao H, Lu F, Lu Z (2018) Improving iturin A production of *Bacillus amyloliquefaciens* by genome shuffling and its inhibition against *Saccharomyces cerevisiae* in orange juice. Frontiers in Microbiology 9:2683. doi: 10.3389/fmicb.2018.02683.

Singh P, Cameotra SS (2004) Potential applications of microbial surfactants in biomedical sciences. Trends in Biotechnology 22:142–146. doi: 10.1016/j.tibtech.2004.01.010.

Spacapan M, Danevčič T, Mandic-Mulec I (2018) ComX-induced exoproteases degrade ComX in *Bacillus subtilis* PS-216. Frontiers in Microbiology 9:105. doi: 10.3389/fmicb. 2018.00105.

Stanley NR, Lazazzera BA (2005) Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-DL-glutamic acid production and biofilm formation. Molecular Microbiology 57:1143–1158. doi: 10.1111/j.1365-2958. 20 05.04746.x.

Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology 56:845–857. doi: 10.1111/j.1365-2958.2005.04587.x

Stone GD (2010) The anthropology of genetically modified crops. Annual Review of Anthropology 39:381–400. doi: 10.1146/annurev.anthro.012809.105058.

Sun J, Liu Y, Lin F, Lu Z, Lu Y (2021) CodY, ComA, DegU and Spo0A controlling lipopeptides biosynthesis in *Bacillus amyloliquefaciens* fmbJ. Journal of Applied Microbiology 131:1289–1304. doi: 10.1111/jam.15007.

Teixeira GM, Mosela M, Nicoletto MLA, Ribeiro RA, Hungria M, Youssef K, Higashi AY, Mian S, Ferreira AS, Gonçalves LSA (2021) Genomic insights into the antifungal activity and plant growth-promoting ability in *Bacillus velezensis* CMRP 4490. Frontiers in Microbiology 11:618415. doi: 10.3389/fmicb.2020.618415.

Théatre A, Cano-Prieto C, Bartolini M, Laurin Y, Deleu M, Niehren J, Fida T, Gerbinet S, Alanjary M, Medema MH (2021) The surfactin-like lipopeptides from *Bacillus* spp.: Natural biodiversity and synthetic biology for a broader application range. Frontiers in Bioengineering and Biotechnology 9:623701. doi: 10.3389/fbioe.2021.623701.

Thongkongkaew T, Ding W, Bratovanov E, Oueis E, García-Altares M, Zaburannyi N, Harmrolfs K, Zhang Y, Scherlach K, Müller R (2018) Two types of threonine-tagged lipopeptides synergize in host colonization by pathogenic *Burkholderia* species. American Chemical Society Chemical Biology 13:1370–1379. doi: 10.1021/acschembio. 8b00221.

Tolibia SEM, Pacheco AD, Balbuena SYG, Rocha J, López y López, Víctor Eric (2023) Engineering of global transcription factors in *Bacillus*, a genetic tool for increasing product yields: a bioprocess overview. World Journal of Microbiology and Biotechnology 39:12. doi: 10.1007/s11274-022-03460-9.

Tsuge K, Ano T, Hirai M, Nakamura Y, Shoda M (1999) The genes *degQ*, *pps*, and *lpa*-8 (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrobial Agents and Chemotherapy 43:2183–2192. doi: 10.1128/AAC.43.9.2183.

Tsuge K, Matsui K, Itaya M (2007) Production of the non-ribosomal peptide plipastatin in *Bacillus subtilis* regulated by three relevant gene blocks assembled in a single movable DNA segment. Journal of Biotechnology 129:592–603. doi: 10.1016/j.jbiotec.2007.01.033.

Velten S, Leventon J, Jager N, Newig J (2015) What is sustainable agriculture? A systematic review. Sustainability 7:7833–7865. doi: 10.3390/su7067833.

Walton RB, Woodruff HB (1949) A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth. The Journal of Clinical Investigation 28:924–926. doi: 10.1172/JCI102180.

Wu C-Y, Chen C-L, Lee Y-H, Cheng Y-C, Wu Y-C, Shu H-Y, Götz F, Liu S-T (2007) Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. Journal of Biological Chemistry 282:5608–5616. doi: 10.1074/jbc.M609726200.

Wu J-Y, Liao J-H, Shieh C-J, Hsieh F-C, Liu Y-C (2018) Kinetic analysis on precursors for iturin A production from *Bacillus amyloliquefaciens* BPD1. Journal of Bioscience and Bioengineering 126:630–635. doi: 10.1016/j.jbiosc.2018.05.002.

Xu G, Martinez JS, Groves JT, Butler A (2002) Membrane affinity of the amphiphilic marinobactin siderophores. Journal of the American Chemical Society 124:13408–13415. doi: 10.1021/ja026768w.

Xu Y, Cai D, Zhang H, Gao L, Yang Y, Gao J, Li Y, Yang C, Ji Z, Yu J (2020) Enhanced production of iturin A in *Bacillus amyloliquefaciens* by genetic engineering and medium optimization. Process Biochemistry 90:50–57. doi: 10.1016/j.procbio.2019.11.017.

Xue Y, Wang M, Zhao P, Quan C, Li X, Wang L, Gao W, Li J, Zu X, Fu D (2018) Gram-negative bacilli-derived peptide antibiotics developed since 2000. Biotechnology Letters 40:1271–1287. doi: 10.1007/s10529-018-2589-1.

Yadav AN, Verma P, Kumar S, Kumar V, Kumar M, Sugitha TCK, Singh BP, Saxena AK, Dhaliwal HS (2018) *Actinobacteria* from rhizosphere: molecular diversity, distributions, and potential biotechnological applications. In: New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier, pp 13–41. doi: 10.1016/B978-0-444-63994-3.00002-3.

Yaseen Y, Gancel F, Drider D, Béchet M, Jacques P (2016) Influence of promoters on the production of fengycin in *Bacillus* spp. Research in Microbiology 167:272–281. doi: 10.1016/j.resmic.2016.01.008.

Yaseen Y, Diop A, Gancel F, Béchet M, Jacques P, Drider D (2018) Polynucleotide phosphorylase is involved in the control of lipopeptide fengycin production in *Bacillus subtilis*. Archives of Microbiology 200:783–791. doi: 10.1007/s00203-018-1483-5.

Yin H, Guo C, Wang Y, Liu D, Lv Y, Lv F, Lu Z (2013) Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. Anti-cancer Drugs 24:587–598. doi: 10.1097/CAD. 0b013e3283611395.

Yu G, Jia X, Wen J, Wang G, Chen Y (2011) Enhancement of daptomycin production in *Streptomyces roseosporus* LC-51 by manipulation of cofactors concentration in the fermentation culture. World Journal of Microbiology and Biotechnology 27:1859–1868. doi: 10.1007/s11274-010-0644-8.

Zhang S, Chen Y, Zhu J, Lu Q, Cryle MJ, Zhang Y, Yan F (2023) Structural diversity, biosynthesis, and biological functions of lipopeptides from *Streptomyces*. Natural Product Reports 40:557-594. doi: 10.1039/D2NP00044J.

Zhao H, Shao D, Jiang C, Shi J, Li Q, Huang Q, Rajoka MSR, Yang H, Jin M (2017) Biological activity of lipopeptides from *Bacillus*. Applied Microbiology and Biotechnology 101:5951–5960. doi: 10.1007/s00253-017-8396-0.

Zhao P, Xue Y, Gao W, Li J, Zu X, Fu D, Feng S, Bai X, Zuo Y, Li P (2018a) *Actinobacteria*–derived peptide antibiotics since 2000. Peptides 103:48–59. doi: 10.1016/j.peptides. 2018.03.011.

Zhao H, Li J, Zhang Y, Lei S, Zhao X, Shao D, Jiang C, Shi J, Sun H (2018b) Potential of iturins as functional agents: safe, probiotic, and cytotoxic to cancer cells. Food and Function 9:5580–5587. doi: 10.1039/C8FO01523F.

Zou D, Maina SW, Zhang F, Yan Z, Ding L, Shao Y, Xin Z (2020) Mining new plipastatins and increasing the total yield using CRISPR/Cas9 in genome-modified *Bacillus subtilis* 1A751. Journal of Agricultural and Food Chemistry 68:11358–11367. doi: 10.22059/jbioc. 2016.58619.

Abbreviations and symbols

ATP	adenosine triphosphate
AntiSMASH	Antibiotics and secondary metabolite analysis shell
B. subtilis	*Bacillus subtilis*
B. velezensis	*Bacillus velezensis*
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms)
E. coli	*Escherichia coli*
EU	European Union
GMO	Genetically modified organism
ORF	Open reading frame
LP	Lipopeptide
UTB	University of Tehran Bacteria
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
n.d.	not determined
NRPS	Non-ribosomal peptide synthetases
OD	Optical density
PCR	Polymerase chain reaction
pO_2	Dissolved oxygen (%)
PGPB	Plant growth promoting bacteria
PKS	Polyketide peptide synthetases
QS	Quorum sensing system
RSM	Response surface methodology

Sfp	4-phosphopantetheinyl transferase
WGS	Whole-genome sequencing
μ	specific growth rate
g	gravitational acceleration
v/v	volume per volume (concentration)
q	Specific productivity
$Y_{P/S}$	Yield coefficient product to substrate
$Y_{P/X}$	Yield coefficient product to biomass
$Y_{X/S}$	biomass to substrate yield