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26 Feb 2026
202620148 1

NOVEL TEMPERATURE-OPTIMIZED BACILLI

ABSTRACT

The present invention relates to new strains of *Bacillus paralicheniformis* with improved growth rates and plant growth promoting properties and new strains of *Bacillus paralicheniformis* with improved growth rates when compared to their parental strains from where they are derived.

2026201481 26 Feb 2026

NOVEL TEMPERATURE-OPTIMIZED BACILLI

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a divisional application of AU 2020339732, which is the Australian National Phase application of PCT/EP2020/074126 which claims priority to EP19194343.0, filed on 29 August 2019. The entire content of each of these applications is hereby incorporated herein by reference.

DESCRIPTION OF THE SEQUENCE LISTING SUBMITTED ELECTRONICALLY

Preceding applications contained a Sequence Listing which was originally submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. The present application contains a sequence listing which has been submitted electronically as an XML document in the ST.26 format and is hereby incorporated by reference in its entirety. Said XML copy, created on 23 December 2022, is named SQ.xml and is 12,089 bytes in size.

SUMMARY OF THE INVENTION

The present invention relates to a composition comprising *Bacilli* and *Bacillus paralicheniformis* with increased growth rates and higher biomass yields at different temperatures, to its use, to a process for its preparation, to the use of *Bacilli* and *Bacillus paralicheniformis* with increased growth rates and higher biomass yields at different temperatures for controlling, combating and/or conferring specific resistance to plant pests. Particularly, the invention relates to strains of *Bacilli* and *B. paralicheniformis* with altered functionality of the proteins encoded by one or more of the genes BioF and HrcA.

The inventors of present invention have generated strains that show increased growth rates and higher biomass yields at a range of different temperatures.

Further, selected new strains of present invention is shown to induce a slight improvement in plant growth experiments, as compared to the original strain in the model plant organism *Arabidopsis thaliana*.

2026201481 26 Feb 2026

Genotypic variations associated with the phenotypic changes are described. Derivative strains have been tested in plant experimental systems and proven to promote increased plant growth as compared to the original strain. Further, two distinct mechanisms explaining the impact of the genetic modifications disclosed and discussed herein.

FIELD OF THE INVENTION

In the current context of a modern and ecologic society, which is concerned with preserving the environment, biological control is considered an attractive alternative or supplement to conventional methods of control. Biological control is the use of one organism (predator, parasite or pathogen) that attacks another organism which is causing economic

[TEXT CONTINUES ON PAGE 2]

2026201481 26 Feb 2026

damage to crops. This is a very common strategy in agroecological systems, as well as in conventional agriculture which relies on the Integrated Pest Management (IPM).

Although the biological control brings positive effects in the reduction or withdrawal of pesticide use and improving farmers' income, an analysis of the set of experiments worldwide, shows that the results are still concentrated in only a few crops and in select geographies with climates supporting the growth rates of Bacilli and in particular plant growth-promoting rhizobacteria as *B. paralicheniformis*. There is still much to develop in areas of control of pests and diseases.

There has been a great emphasis on research on biological control with the use of bacteria colonizing the roots of plants, called rhizobacteria. The beneficial rhizobacteria for promoting growth and/or acting in the biological control of plant pathogenic bacteria are called plant growth-promoting rhizobacteria or PGPR.

One of the key factors for successful biological control by PGPR is successful colonization of the habitat e.g. by growth rates and biomass. Hence successful biofilm formation may increase the protective effect of the PGPR.

In summary, cold adapted derivative *B. paralicheniformis* DSM33110 strains were generated following an adaptive laboratory evolution campaign. 14 improved derivatives were selected, and their genomes sequenced to identify the acquired genotypic changes. Derivative strains were characterized physiologically and tested for performance in plant growth experiments. Based on results herein we propose mechanisms to explain the observed phenotypic differences with the parental strain. In addition, these evolution experiments have contributed with a new *Bacillus* strain showing improved properties in plant growth promotion.

STATE OF THE ART

The pressure of society to replace the chemicals with environmentally acceptable products or ecological techniques has encouraged the search for

2026201481 26 Feb 2026

alternative methods to promote plant health. In this context, biological control has been considered one of the alternatives within an integrated approach, in which one seeks to ensure sustainable development of agriculture.

The risks to humans and environments presented by using synthetic pesticides emphasize the need for tools such as biological control in optimizing sustainable agricultural systems.

Based on the idea that improved growth rates and biomass formation at different temperatures may improve the bioprotective effect of *Bacilli*, the inventors of present invention have selected derivative strains of *Bacilli* that show an increase in growth rates and biomass formation at different temperatures.

To the best of our knowledge specific genetic features linked to increased growth rates and biomass formation at different temperatures and associated mode of actions have never been described for *Bacilli* and in particular not for *B. paralicheniformis* strains.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 – **ALE generation of cold adapted strains: experimental approach. A.** ALE experimental approach to select for cold adapted DSM33110 derivatives was followed for 12 weeks. 5-10% dilution to fresh medium of 12 independent overnight cultures was done daily (6 independent cultures were evolved in two different growth media). OD (600 nm) measurements were taken daily and number of generations per week calculated. Samples from each tube were kept frozen as glycerol stocks weekly. Temperature was gradually decreased from 25°C to 18°C throughout the ALE progression. **B.** After 12 weeks of adaptive laboratory evolution, serial dilutions from each culture were plated, and 2x960 independent clones were selected, grown in LB broth, and kept frozen as glycerol stocks in 96-well microtiter plates. Individual clone growth profiles were compared first in a Growth Profiler 960 at 21°C. The 14 faster growers were selected and their growth patterns analyzed in different growth media and temperatures.

26 Feb 2026 2026201481

Figure 2 – **Growth profiles of the selected cold adapted derivatives vs parental strain DSM33110.** A. Cold adapted derivatives evolved in MSgg growth medium were grown in MSgg medium at 21°C and compared with the parental strain DSM33110. B. Cold adapted derivatives evolved in MS-Rex growth medium were grown in MS-Rex medium at 21°C and compared with the parental strain DSM33110. Maximum biomass yields reached by the different strains were determined.

Figure 3 – **Maximum growth rates (μmax) calculated for each derivative strain and parental strain DSM33110.** A. μmax values reached in MSgg medium at 21°C. B. μmax values reached in MS-Rex at 21°C.

Figure 4 – **Growth profiles for cold adapted derivatives in different growth media at 30°C temperature.** Growth profiles for selected cold adapted derivatives vs parental strain DSM33110. **(A-B)** Cold adapted derivatives were grown in LB medium at 30°C and compared with the parental strain DSM33110. **(C-D)** Cold adapted derivatives were grown in BHI medium at 30°C and compared with the parental strain DSM33110. X axes correspond to time scale of the experiment in hours. Maximum yields reached by the different strains was determined. A-C panels correspond to derivatives evolved in MSgg growth medium. B-D panels correspond to derivatives evolved in MS-Rex growth medium.

Figure 5 – **Plant growth promotion experiments.** Plant growth and fitness promotion quantification results from DSM33110 and 14 cold-adapted improved derivative strains CA1-CA7, and CAREX1-CAREX7 corresponding to CHCC32528-CHCC32529-CHCC36494-CHCC36497, and CHCC36751-36757, respectively. Results correspond to experiments done in an in vitro agar system. Left panel corresponds to the total leaf area measurements average calculated per plant (48 plants per strain). Central panel corresponds to the average fresh weight per plant. Right panel corresponds to chlorophyll quantification results normalized by fresh weight. Error bars correspond to standard deviation between samples.

Figure 6 **- A. Primary amino acid sequence alignment of BioF homologues** (*E. coli* K12, *B. subtilis* 168 and *B. paralicheniformis* DSM33110, as indicated).

26 Feb 2026 2026201481

Positions labelled with asterisks correspond to residues were identified SNPs result in an amino acid change. Squared-labelled positions correspond to catalytic residues. **B.** 3D structural model of $BioF_{DSM33110}$ protein. Red-circle labels the position of the catalytic residues. Green boxes correspond to position of amino acid changes identified.

Figure 7 - **Scheme representing the HrcA regulon described in *B. subtilis***. HrcA acts as transcriptional repressor of two operons, the heptacistronic *dnaK* operon (*hrcA-grpE-dnaK-dnaJ-3xorfs*), and the bicistronic *groE (groES-groEL)*. Analysis or the homologous genomic regions present in DSM33110 confirm conservation of these operons' organization.

Figure 8. **Plant growth promotion by cold adapted derivatives compared in *A. thaliana* grown in potting soil.** Plant growth and fitness promotion quantification results from parental strain DSM33110 and 2 cold adapted improved derivative strains (CA4 and CA6 corresponding to CHCC36494-CHCC36496, respectively). **A-B.** Plant growth and fitness promotion quantification results from parental strain (DSM33110) and two combinations of cold adapted derivatives CA4 and CA6 with *B. subtilis* strains DSM33015 and DSM32938, respectively. *A. thaliana* seedlings pre-grown in potting soil for 7 days were inoculated by root dipping on bacterial cultures resuspended in 10 mM $MgSO_4$ buffer (OD_{600} 0.01). Plantlets were allowed to grow for 14 additional days before data collection. Error bars correspond to standard deviation between replicates and statistical significance was determined by performing a t-test ($p<0.05$) assuming equal variance in the two samples. **A.** Plant growth experiments were grown in plant growth chamber at 22°C. **B.** Plant growth experiments were grown in plant growth chamber at 15°C.

2026201481 26 Feb 2026

DETAILED DESCRIPTION OF THE INVENTION

Rhizobacteria

The soils are home to a complex biological community, of which micro-organisms, prokaryotes and eukaryotes form a majority, both in number and in diversity. Some prokaryotes have ecological niches as the rhizosphere, and/or the rhizoplane of plants, where they multiply, survive and protect themselves from the rest of the antagonistic action of soil microflora. These organisms have been generically called rhizobacteria.

In association with plants, rhizobacteria may have a deleterious effect, no effect or a beneficial effect. Those who exercise a beneficial effect - growth promotion and biological control of disease - are called PGPR ("Plant Growth-Promoting Rhizobacteria). It is estimated that only 1% to 2% of rhizobacteria have some beneficial effect for the plant with which they are associated.

PGPR as biocontrol agents

PGPR have been used for biological control of plant diseases and thereby increase the productivity of crops. How and why this biological control is exercised, is still a topic that needs complementary studies.

In some situations, it is possible that biological control occurs by direct antagonism exerted by PGPR against the pathogen, with involvement of the known mechanisms of antibiosis: production of antimicrobial substances, direct parasitism, competition for nutrients and ecological niches.
Research has shown that certain PGPR appear to act as elicitor of ISR (induced systemic resistance), in the sense that the plant becomes systemically protected against more than one pathogen, unlike the classical biological control, which aims to implement the control more specifically.

A significant parameter affecting the PGPR ability to infect and colonize the plant surface is the ability of the PGPR to grow under the conditions to which it is exposed. The inventors of present disclosure therefore seek to improve the growth rates and biomass formation at different, and most preferably decreased

2026201481 26 Feb 2026

temperatures and thereby improve the plant growth promoting capabilities the *Bacilli* disclosed herein.

The rhizosphere environment

One of the most convenient methods of introducing a rhizobacteria in the root environment is through the application on the seeds before sowing. The process of seed germination releases carbohydrates and amino acids in abundance in the form of seed exudates. Thus, these organisms introduced with the seeds in the soil utilize exudates as a source of nutrition and colonize the roots as they emerge. Rhizobacteria isolates that have greater ability to utilize root exudates of seeds at different temperatures may have selective advantage in colonization of the roots.

PGPR of the genus *Bacillus* have been associated with nematode control. Sikora, R.A. (Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. Medicine Faculty Landbouww Rijksuniv Gent, Landbouww, v.53, n.2b, p. 867-878, 1988) observed reductions in infection of *Meloidogyne arenaria*, *M. incognita* and *Rotylenchulus reniformis* around 60-65% with treatment of seeds of various crops with a strain of *Bacillus*.

Advantages of rhizobacteria for commercial application

The rhizobacteria have several advantages over chemical pesticides or even on other biological control agents: they are easy to mass-produce, they are easy to store and are adaptable to the formulation technology.

The rhizobacteria can be applied by treating the substrate, immersing the seedling root systems in bacterial suspensions, watering the plant with bacterial suspension by dipping/coating the seeds in suspension of rhizobacteria or by applying PGPR with the pelleting of seeds.

Bacteria of the genus *Bacillus*

The *Bacillus* species are Gram-positive bacteria characterized by having thick cell walls and the absence of outer membranes, which differs from the Gram-

2026201481 26 Feb 2026

negative bacteria. Much of the cell wall of Gram-positive bacteria is composed of peptidoglycan.

Gram-positive species are divided into groups according to their morphological and biochemical characteristics. The genus *Bacillus* is belonging to the group of sporulating bacteria. Bacterial spores are one of the most resilient cell types; they resist many environmental changes, withstand dry heat and certain chemical disinfectants and may persist for years on dry land.

The beneficial effect of *Bacilli* such as e.g. *B. paralicheniformis*, when applied near the seed or the soil, may not be solely due to the antagonism afforded to pathogens. The PGPR has a positive influence on germination, development and crop yield due also to the production of substances which promote growth and improvement in plant nutrition by solubilization of phosphorus.

In the context of the present invention, a biofertilizer is a mixture of living microorganisms that when applied to seeds, plants or soil, promote the increase of nutrient supply, such as NH_4^+, SO_4^{2-} , K^+ or PO_4^{3-} (Vessey, 2003).

In the context of the present invention, a plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content. By extension, plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms (du Jardin, 2015).

In the context of the present invention, plant growth promoting agent or plant growth promoting microorganism is a microorganism with the ability to colonize roots and/or inner plant tissues and promote plant growth and health by either acting as a biofertilizer, biostimulant or via biological control of plant disease. Said agent or microorganism is a soil and rhizosphere-inhabiting microorganism that can colonize plant roots in significant numbers (10^5–10^7 CFU per gram of fresh root) and influence plant growth in a positive manner (Spaepen et al. 2009; Antoun and Prevost 2005).

2026201481 26 Feb 2026

Thus, the first aspect of the invention relates to the herein described novel strains or mutants thereof.

The composition of the present invention may, besides the active components, contain agrochemical acceptable excipients and/or vehicles thereof. The composition of the invention further comprises agrochemically acceptable carriers, vehicles and/or adjuvants.

Among the main crops of plants are sugar cane, coffee, soybeans, cotton, corn, potatoes, tomatoes, tobacco, banana, rice, wheat, avocado, pineapple, squash, cacao, coconut, oats, onion, lettuce, beet, carrot, cassava, beans, sunflower, pepper, turnip, apple, strawberry, okra, radish and onion.

For fruticulture: citrus, grape, guava, papaya, fig, peach, plum and nespereira are of particular relevance and with regard to horticulture: eggplant and cruciferous.

For floriculture: rose, chrysanthemum, lisianthus, gerbera, amaryllis, begonia and celosia.

The composition of present invention may be coated on the plant seed and can include an amount of *Bacillus*, such as e.g. *B. paralicheniformis* spores from about 1.0×10^2 CFU/seed to about 1.0×10^9 CFU/seed.

The plant seed can include, but is not limited to, the seed of monocots, dicots, Cereals, Corn, Sweet Corn, Popcorn, Seed Corn, Silage Corn, Field Corn, Rice, Wheat, Barley, Sorghum, Brassica Vegetables, Broccoli, Cabbage, Cauliflower, Brussels Sprouts, Collards, Kale, Mustard Greens, Kohlrabi, Bulb Vegetables, Onion, Garlic, Shallots, Fruiting Vegetables, Pepper, Tomato, Eggplant, Ground Cherry, Tomatillo, Okra, Grape, Herbs/Spices, Cucurbit Vegetables, Cucumber, Cantaloupe, Melon, Muskmelon, Squash, Watermelon, Pumpkin, Eggplant, Leafy Vegetables, Lettuce, Celery, Spinach, Parsley, Radicchio, Legumes/Vegetables (succulent and dried beans and peas), Beans, Green beans, Snap beans, Shell beans, Soybeans, Dry Beans, Garbanzo beans, Lima beans, Peas, Chick peas, Split peas, Lentils, Oil Seed Crops, Canola, Castor, Cotton, Flax, Peanut, Rapeseed, Safflower, Sesame, Sunflower, Soybean,

2026201481 26 Feb 2026

Root/Tuber and Corm Vegetables, Carrot, Potato, Sweet Potato, Beets, Ginger, Horseradish, Radish, Ginseng, Turnip, sugarcane, sugarbeet, Grass, or Turf grass.

Further, the plant seed can include seed of a drybean, a corn, a wheat, a soybean, a canola, a rice, a cucumber, a pepper, a tomato, a squash, a cotton, a grass, and a turf grass.

In an alternative embodiment, the *Bacillus* or composition of present invention may be added to: soil or growth medium surrounding the plant; soil or growth medium before sowing seed of the plant in the soil or growth medium; or soil or growth medium before planting the plant, the plant cutting, the plant graft, or the plant callus tissue in the soil or growth medium.

In one or more embodiments, the plant can include soybean, bean, snap bean, wheat, cotton, corn, pepper, tomato, potato, cassava, grape, strawberry, banana, peanut, squash, pumpkin, eggplant, and cucumber.

In the compositions and methods of the present invention, the pathogenic infection can be caused by a wide variety of plant pathogens including, for example, but not limited to, a plant fungal pathogen, a plant bacterial pathogen, a rust fungus, a Botrytis spp., a Botrytis cinerea, a Botrytis squamosa, an Erwinia spp., an Erwinia carotovora, an Erwinia amylovora, a Dickeya spp., a Dickeya dadantii, a Dickeya solani, an Agrobacterium spp., a Agrobacterium tumefaciens, a Xanthomonas spp., a Xanthomonas axonopodis, a Xanthomonas campestris pv. carotae, a Xanthomonas pruni, a Xanthomonas arboricola, a Xanthomonas oryzae pv. oryzae, a Xylella spp., a Xylella fastidiosa, a Candidatus spp., a Candidatus liberibacter, a Fusarium spp., a Fusarium culmorum, a Fusarium graminearum, a Fusarium oxysporum, a Fusarium oxysporum f. sp. Cubense, a Fusarium oxysporum f. sp. Lycopersici, a Fusarium virguliforme, a Sclerotinia spp., a Sclerotinia sclerotiorum, a Sclerotinia minor, Sclerotinia homeocarpa, a Cercospora/Cercosporidium spp., an Uncinula spp., an Uncinula necator (Powdery Mildew), a Podosphaera spp. (Powdery Mildew), a Podosphaera leucotricha, a Podosphaera clandestine, a Phomopsis spp., a Phomopsis viticola, an Alternaria spp., an Alternaria tenuissima, an Alternaria porri, an Alternaria alternate, an

2026201481 26 Feb 2026

Alternaria solani, an Alternaria tenuis, a Pseudomonas spp., a Pseudomonas syringae pv. Tomato, a Phytophthora spp., a Phytophthora infestans, a Phytophthora parasitica, a Phytophthora sojae, a Phytophthora capsici, a Phytophthora cinnamon, a Phytophthora fragariae, a Phytophthora spp., a Phytophthora ramorum, a Phytophthora palmivara, a Phytophthora nicotianae, a Phakopsora spp., a Phakopsora pachyrhizi, a Phakopsora meibomiae an Aspergillus spp., an Aspergillus flavus, an Aspergillus niger, a Uromyces spp., a Uromyces appendiculatus, a Cladosporium spp., a Cladosporium herbarum, a Rhizopus spp., a Rhizopus arrhizus, a Penicillium spp., a Rhizoctonia spp., a Rhizoctonia solani, a Rhizoctonia zeae, a Rhizoctonia oryzae, a Rhizoctonia caritae, a Rhizoctonia cerealis, a Rhizoctonia crocorum, a Rhizoctonia fragariae, a Rhizoctonia ramicola, a Rhizoctonia rubi, a Rhizoctonia leguminicola, a Macrophomina phaseolina, a Magnaorthe oryzae, a Mycosphaerella spp., Mycosphaerella graminocola, a Mycosphaerella fijiensis (Black sigatoga), a Mycosphaerella pomi, a Mycosphaerella citri, a Magnaporthe spp., a Magnaporthe grisea, a Monilinia spp., a Monilinia fruticola, a Monilinia vacciniicorymbosi, a Monilinia laxa, a Colletotrichum spp., a Colletotrichum gloeosporiodes, a Colletotrichum acutatum, a Colletotrichum Candidum, a Diaporthe spp., a Diaporthe citri, a Corynespora spp., a Corynespora Cassiicola, a Gymnosporangium spp., a Gymnosporangium juniperi-virginianae, a Schizothyrium spp., a Schizothyrium pomi, a Gloeodes spp., a Gloeodes pomigena, a Botryosphaeria spp., a Botryosphaeria dothidea, a Neofabraea spp., a Wilsonomyces spp., a Wilsonomyces carpophilus, a Sphaerotheca spp., a Sphaerotheca macularis, a Sphaerotheca pannosa, a Erysiphe spp., a Stagonospora spp., a Stagonospora nodorum, a Pythium spp., a Pythium ultimum, a Pythium aphanidermatum, a Pythium irregularum, a Pythium ulosum, a Pythium lutriarium, a Pythium sylvatium, a Venturia spp, a Venturia inaequalis, a Verticillium spp., a Ustilago spp., a Ustilago nuda, a Ustilago maydis, a Ustilago scitaminea, a Claviceps spp., a Claviceps puprrea, a Tilletia spp., a Tilletia tritici, a Tilletia laevis, a Tilletia horrid, a Tilletia controversa, a Phoma spp., a Phoma glycinicola, a Phoma exigua, a Phoma lingam, a Cocliobolus sativus, a Gaeumanomyces gaminis, a Colleototricum spp., a Rhychosporium spp., Rhychosporium secalis, a Biopolaris spp., a Helminthosporium spp., a

2026201481 26 Feb 2026

Helminthosporium secalis, a Helminthosporium maydis, a Helminthosporium solai, and a Helminthosporium tritici-repentis, or combinations thereof.

In some embodiments, the pathogenic infection can be caused by one or a combination of: Soybean rust fungi (Phakopsora pachyrhizi, Phakopsora meibomiae) and the plant comprises soybean; Botrytis cinerea (Botrytis Blight) and the plant comprises grape; Botrytis cinerea (Botrytis Blight) and the plant comprises strawberry; Botrytis cinerea (Botrytis Blight) and the plant comprises tomato; Alternaria spp. (e.g. A. solani) and the plant comprises tomato; Alternaria spp. (e.g. A. solani) and the plant comprises potato; Bean Rust (Uromyces appendiculatus) and the plant comprises common bean; Microsphaera diffusa (Soybean Powdery Mildew) and the plant comprises soybean; Mycosphaerella fijiensis (Black sigatoga) or Fusarium oxysporum f. sp. cubense (Panama disease) and the plant comprises banana; Xanthomonas spp. or Xanthomonas oryzae pv. oryzae and the plant comprises rice; Xanthomonas axonopodis and the plant comprises cassava; Xanthomonas campestris and the plant comprises tomato; Botrytis cinerea (Pepper Botrytis Blight) and the plant comprises pepper; Powdery mildew and the plant comprises a cucurbit; Sclerotinia sclerotiorum (white mold) and the plant comprises snap bean; Sclerotinia sclerotiorum (white mold) and the plant comprises potato; Sclerotinia homeocarpa (dollar spot) and the plant comprises turfgrass; Southern White Mold and the plant comprises peanut; Leaf spot (Cercospora/Cercosporidium) and the plant comprises peanut; Fusarium graminearum (Wheat Head Scab) and the plant comprises wheat; Mycosphaerella graminicola (Septoria tritici blotch) and the plant comprises wheat; Stagonospora nodorum (glume blotch and septoria nodorum blotch), and the plant compromises wheat; Erwinia amylovora, and the plant compromises apple, pear and other pome fruits; Venturia inaequalis, and the plant compromises apple, pear and other pome fruits; or Rhizoctonia solani and the plant comprises wheat, rice, turfgrass, soybean, corn, legumes and vegetable crops. The compositions including the bacilli as described herein strain can be in the form of a liquid, an oil dispersion, a dust, a dry wettable powder, a spreadable granule, or a dry wettable granule. More specifically the composition may for example be an emulsion concentrate (EC), a suspension concentrate (SC), a suspo-emulsion (SE), a capsule suspension

2026201481 26 Feb 2026

(CS), a water dispersible granule (WG), an emulsifiable granule (EG), a water in oil emulsion (EO), an oil in water emulsion (EW), a micro-emulsion (ME), an oil dispersion (OD), an oil miscible flowable (OF), an oil miscible liquid (OL), a soluble concentrate (SL), an ultra-low volume suspension (SU), an ultra-low volume liquid (UL), a dispersible concentrate (DC), a wettable powder (WP) or any technically feasible formulation in combination with agriculturally acceptable adjuvants.

Hence, the present invention relates to a composition comprising *Bacillus paralicheniformis* DSM 33238, DSM 33239, DSM 33240, DSM 33241, DSM 33242, DSM 33243, DSM 33244 or mutants thereof or a mutant thereof, and to a kit comprising the composition, or prepared by the process of preparing the composition, as well as instructions and a suitable recipient. Accordingly, the present invention also relates to a process for preparing a composition comprising *Bacillus paralicheniformis* DSM 33238, DSM 33239, DSM 33240, DSM 33241, DSM 33242, DSM 33243, DSM 33244, or a mutant thereof together with agrochemically acceptable carriers, vehicles and/or adjuvants, and use of said composition for controlling, combating and/or conferring specific resistance to phytonematodes are also given.

In addition, the invention refers to the use of effective amounts of *Bacillus paralicheniformis* DSM 33238, DSM 33239, DSM 33240, DSM 33241, DSM 33242, DSM 33243, DSM 33244 or a mutant thereof, in the manufacture of an agrochemical composition with plant growth promoting effect in a plant culture, as well as processes for promoting plant health.

In a preferred aspect the invention relates to a *Bacillus* having a mutation in the *bioF* gene, wherein the mutation changes the enzyme kinetics of the protein encoded by *bioF*, when compared to the parental strain of the *Bacillus* having a mutation in the *bioF* gene.

In yet a preferred aspect the invention relates to a *Bacillus* having a mutation in the *hrcA* gene, wherein the mutation renders the protein encoded by *hrcA* dysfunctional such loss of function, when compared to its parental strain.

2026201481 26 Feb 2026

Further, the present invention relates to the following aspects:

Aspect 1. A *Bacillus* having mutation in the *bioF* and/or *hrcA* gene.

Aspect 2. A *Bacillus* having a mutation in the *hrcA* and/or *bioF* gene when compared to the corresponding ortholog genes of *hrcA* and *bioF* in *B. paralicheniformis* deposited as DSM33110.

Aspect 3. A *Bacillus* having a mutation in the *hrcA* gene when compared to SEQ ID NO:1 and/or a mutation in the *bioF* gene when compared to the SEQ ID NO:3.

Aspect 4. A *Bacillus* selected from a list consisting of the strains deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen with accession No's. DSM 33238, DSM 33239, DSM 33240, DSM 33241, DSM 33242, DSM 33243, DSM 33244.

Aspect 5. A *Bacillus* according to any of the preceding aspects, wherein the closest ortholog of the *bioF* gene of said *Bacillus* share less than 100% such as e.g. less than 99%, less than 98% sequence identity with SEQ ID NO:1.

Aspect 6. A *Bacillus* according to any of the preceding aspects, wherein the closest ortholog of the *bioF* gene of said *Bacillus* share at least 95% such as e.g. at least 96%, at least 97, at least 98, at least 99% sequence identity with SEQ ID NO:1.

Aspect 7. A *Bacillus* according to any of the preceding aspects, wherein the closest ortholog of the *hrcA* gene of said *B. paralicheniformis* share less than 100% such as e.g. less than 99%, less than 98% sequence identity with SEQ ID NO:3.

Aspect 8. A *Bacillus* according to any of the preceding aspects, wherein the closest ortholog of the *hrcA* gene of said *Bacillus* share at least 95% such as e.g. at least 96%, at least 97 sequence identity with SEQ ID NO:3.

2026201481 26 Feb 2026

Aspect 9. A *Bacillus* according to any of the preceding aspects, wherein the mutation is a deletion, substitution or insertion.

Aspect 10. A *Bacillus* according to any of the preceding aspects, wherein the mutation causes a frameshift, introduces a stop codon or impacts the kinetics of the encoded protein.

Aspect 11. A *Bacillus* according to any of the preceding aspects, wherein the protein encoded by one or more of the genes *hrcA and/or bioF* or one or more of their closest orthologs is structurally impacted by the mutation.

Aspect 12. A *Bacillus* according to any of the preceding aspects, wherein the genome of the strain is at least 99%, such as e.g. at least 99.5%, such as e.g. at least 99.8%, such as e.g. at least 99.9% identical to the genome of the strain deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen with accession No. DSM33110.

Aspect 13. A *Bacillus* according to any of the preceding aspects, wherein the *Bacillus* is selected from: *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus paralicheniformis*, *Bacillus cereus*, *Bacillus velenzensis*, *Bacillus megaterium*.

Aspect 14. A *Bacillus* according to any of the preceding aspects, wherein the strain is derived from the strain deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen with accession No. DSM33110 or a strain sharing pheno- or genotypical characteristics with the strain deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen with accession No. DSM33110

Aspect 15. A *Bacillus* according to any of the preceding aspects wherein the *Bacillus* has the pheno- or genotypical characteristics of one or more of the *Bacillus paralicheniformis* strains deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen with accession No's. DSM

2026201481 26 Feb 2026

33238, DSM 33239, DSM 33240, DSM 33241, DSM 33242, DSM 33243, DSM 33244.

Aspect 16. A *Bacillus* according to any of the preceding aspects showing increased growth rates when compared with the *Bacillus paralicheniformis* deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen with accession no. DSM33110, when grown at 21°C in MSgg and/or MS-rex medium.

Aspect 17. A *Bacillus* according to aspect 16 wherein the growth rate is determined as described in Example 2 herein.

Aspect 18. A *Bacillus* according to any of aspects 16 or 17 where the growth rate exceeds the growth rate of the parental strain by at least 20%, such as at least 40%, such as at least 60%, such as at least 80%, such as at least 100%.

Aspect 19. A composition comprising a *Bacillus* according to any of the preceding aspects.

Aspect 20. A composition comprising a *Bacillus* according to any of the preceding aspects and agrochemically acceptable excipients and/or carriers thereof.

Aspect 21. The composition of any of aspects 19 or 20, further comprising one or a combination of a microbial, a biological, or a chemical insecticide, fungicide, nematicide, bactericide, herbicide, plant extract, plant growth regulator, or fertilizer present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant, a carrier, a surfactant, a dispersant, or a yeast extract.

Aspect 22. Use of a composition according to any of aspects 19 to 21 or a *Bacillus* according to any of aspects 1 to 18 as a biostimulant and/or bionematicide.

2026201481 26 Feb 2026

Aspect 23. Use of a composition, according to any of aspects 19 to 21, or a *Bacillus* according to any of aspects 1 to 18 for controlling, combating and/or conferring specific resistance to phytonematodes.

Aspect 24. Use according to any of aspects 22 or 23, wherein the phytonematodes are selected from the group consisting of *Meloidogyne, Pratylenchus, Heterodera, Globodera, Ditylenchus, Tylenchulus, Xiphinema, Radopholus, Rotylenchulus, Helicotylenchus* and *Belonolaimus*.

Aspect 25. Use according to any of aspects 22 to 24, wherein the phytonematode is selected from the group consisting of *Meloidogyne incognita, Meloidogyne javanica, Meloidogyne exigua, Meloidogyne paranaensis, Heterordera glycines* and *Pratylenchus zeae*.

Aspect 26. Use according to any of aspects 22 to 25 wherein the composition according to any of aspects 19 to 21 or the *Bacillus* according to any of aspects 1 to 18 is applied on a plant, a seed or in the habitat of a plant

Aspect 27. Use according to aspect 26 wherein the plant is selected from the group consisting of corn, rice, sugar cane, soybean, potato, carrot, coffee and banana.

Aspect 28. Process for conferring improved resistance to phytonematodes, comprising applying an effective amount of a *Bacillus* of any of aspects 1 to 18 or a composition according to any of aspects 19 to 21 on plants and/or their habitat.

Aspect 29. Kit, comprising the composition as defined in any one of aspects 19 to 21, instructions for use and a suitable container.

Aspect 30. A plant seed coated with a composition according to any of aspects 19 to 21 present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant.

2026201481 26 Feb 2026

Aspect 31. The plant seed of aspect 30, wherein the composition comprises an amount of spores of the *Bacillus* of any of aspects 1 to 18 from about 1.0×10^2 CFU/seed to about 1.0×10^9 CFU/seed.

Aspect 32. The plant seed of aspect 30 or 312, wherein the composition further comprises one or a combination of a microbial, a biological, or a chemical insecticide, fungicide, nematicide, bactericide, or plant growth regulator present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant.

Aspect 33. A *Bacillus* according to any of aspects 1 to 18 wherein BioF is encoded by the DNA sequence of SEQ ID NO:1 or amino acid sequence of SEQ ID NO: 2 or homologs thereof and/or HrcA is encoded by the DNA sequence of SEQ ID NO:3 or amino acid sequence homologs thereof.

Item 1. A *Bacillus paralicheniformis* having a mutation in the *hrcA* and/or *bioF* gene when compared to the corresponding ortholog genes of *hrcA* and/or *bioF* in *B. paralicheniformis* deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with accession no. DSM33110.

Item 2. A *Bacillus paralicheniformis* having a mutation in the *hrcA* and/or *bioF* gene when compared to the corresponding ortholog genes of *hrcA* encoded by SEQ ID NO: 1 and/or *bioF* encoded by SEQ ID NO: 3 in e.g. *B. paralicheniformis* deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as DSM33110.

Item 3. A *Bacillus paralicheniformis* according to any of items 1 or 2, wherein the *Bacillus paralicheniformis* is a mutant derived from *B. paralicheniformis* deposited as DSM33110.

Item 4. A *Bacillus paralicheniformis* deposited at Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7B, D-

2026201481 26 Feb 2026

38124 Braunschweig (DSMZ) as DSM 33238, DSM 33239, DSM 33240, DSM 33241, DSM 33242, DSM 33243 or DSM 33244.

Item 5. A *Bacillus paralicheniformis* according to any of items 1 to 4 wherein the mutation in the *bioF* gene causes a genotypic change imposing an amino acid change from Pro314 to Ser, Ala244 to Val, Asp300 to Gly and/or Arg37 to Trp.

Item 6. A *Bacillus paralicheniformis* according to any of items 1 to 5 wherein the mutation in the *hrcA* gene causes a genotypic change imposing a stop codon, rendering an inactive HrcA repressor.

Item 7. A *Bacillus paralicheniformis* according to any of the preceding items showing increased maximum growth rates when compared with the *Bacillus paralicheniformis* deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with accession no. DSM33110, when grown at 21°C in MSgg.

Item 8. A *Bacillus paralicheniformis* according to item 7 wherein the maximum growth rate is determined as described in Example 2 herein.

Item 9. A *Bacillus paralicheniformis* according to any of the preceding items wherein the growth rate of the *Bacillus paralicheniformis* strain according to any of the preceding items exceeds the growth rate of the *Bacillus paralicheniformis* deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under accession no. DSM33110 by at least 20%, such as at least 40%, such as at least 60%, such as at least 80%, such as at least 100%.

Item 10. A *Bacillus paralicheniformis* according to any of the preceding claims which when applied to a *Arabidopsis thaliana Col-0* plants, in a gnotobiotic plant system and grown for 22°C and/or 15°C is able to increase the total leaf area, the fresh weight or the chlorophyll content in said A. thaliana.

2026201481 26 Feb 2026

Item 11. A composition comprising a *Bacillus paralicheniformis* according to any of the preceding items and agrochemically acceptable excipients and/or carriers thereof.

Item 12. A composition of item 11, further comprising one, or a combination of a microbial, a biological, or a chemical insecticide, fungicide, nematicide, bactericide, herbicide, plant extract, plant growth regulator, or fertilizer present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant, a carrier, a surfactant, a dispersant, or a yeast extract.

Item 13. Use of a composition according to any of items 11 to 12 or a *Bacillus paralicheniformis* according to any of items 1 to 9 as a biostimulant, growth promoter and/or bionematicide.

Item 14. Use according to item 13, wherein the phytonematodes are selected from the group consisting of *Meloidogyne, Pratylenchus, Heterodera, Globodera, Ditylenchus, Tylenchulus, Xiphinema, Radopholus, Rotylenchulus, Helicotylenchus* and *Belonolaimus such as e.g. Meloidogyne incognita, Meloidogyne javanica, Meloidogyne exigua, Meloidogyne paranaensis, Heterordera glycines* and *Pratylenchus zeae*.

Item 15. Use according to any of items 13 to 14 wherein the composition according to any of items 11 to 12 or the *Bacillus licheniformis* according to any of items 1 to 10 is applied on a plant, a seed or in the habitat of a plant, such as in the soil.

Item 16. Use according to any of items 13 to 15 wherein the plant is selected from the group consisting of corn, rice, sugar cane, soybean, potato, carrot, coffee and banana.

Item 17. A plant seed coated with a composition according to any of items 11 to 12 present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant.

2026201481 26 Feb 2026

Item 18. The plant seed of item 17, wherein the composition comprises an amount of spores of the *Bacillus* of any of items 1 to 10 from about 1.0×10^2 CFU/seed to about 1.0×10^9 CFU/seed.

Item 19. The plant seed of item 18 or 18, wherein the composition further comprises one or a combination of a microbial, a biological, or a chemical insecticide, fungicide, nematicide, bactericide, or plant growth regulator present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant.

The illustrative examples presented below serve to better describe the present invention. However, the formulations described merely refer to some means to some embodiments of the present invention and should not be taken as limiting the scope thereof.

As used in present disclosure the strain descriptors are used interchangeably according to the table below:

CHCC32528	CA1
CHCC32529	CA2
CHCC32530	CA3
CHCC36494	CA4
CHCC36495	CA5
CHCC36496	CA6
CHCC36497	CA7
CHCC36751	CAREX1
CHCC36752	CAREX2
CHCC36753	CAREX3

2026201481 26 Feb 2026

CHCC36754	CAREX4
CHCC36755	CAREX5
CHCC36756	CAREX6
CHCC36757	CAREX7

EXAMPLES

Growth media compositions:

MSgg medium (pH 7) (filter sterilized):

MOPS solution 1x:

100 mM morpholinepropanesulfonic acid (MOPS) (pH 7)

5 mM KH_2PO_4/K_2HPO_4

1x Trace elements solution:

50 µM $MnCl_2$

1 µM $ZnCl_2$

100 µM $FeCl_3$

2 mM $MgCl_2$

700 µM $CaCl_2$

2 µM thiamine

Aminoacid supplements

50 µg/ml threonine, tryptophan and/or phenylalanine

26 Feb 2026 2026201481

Carbon sources

0.5% glutamate

0.5% glycerol

MS-REX (pH 7) filter sterilized

Murashige and Skoog (MS) medium is a plant growth medium used in the laboratories for cultivation of plants or plant cell cultures. MS was supplemented with corn root exudates (1X) collected from hydroponic maize plants cultures. Root exudates had been collected in water, and liophylized to obtain a concentrate of the root exudates (x25).

MS composition

Major salts (macronutrients)/ 1L

Ammonium nitrate (NH_4NO_3) 1,650 mg/l

Calcium chloride ($CaCl_2 \cdot 2H_2O$) 440 mg/l

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) 370 mg/l

Monopotassium phosphate (KH_2PO_4) 170 mg/l

Potassium nitrate (KNO_3) 1,900 mg/l.

Minor salts (micronutrients)/ 1L

Boric acid (H_3BO_3) 6. 2 mg/l

Cobalt chloride ($CoCl_2 \cdot 6H_2O$) 0.025 mg/l

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) 27.8 mg/l

2026201481 26 Feb 2026

Manganese(II) sulfate ($MnSO_4 \cdot 4H_2O$) 22.3 mg/l

Potassium iodide (KI) 0.83 mg/l

Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) 0.25 mg/l

Zinc sulfate ($ZnSO_4 \cdot 7H_2O$) 8.6 mg/l

Ethylenediaminetetraacetic acid ferric sodium (NaFe-EDTA) constituting 5 ml/l of a stock solution containing 5.57 g $FeSO_4.7H_2O$ and 7.45 g Na2-EDTA per litre of water.

Copper sulfate ($CuSO_4 \cdot 5H_2O$) 0.025 mg/l

Vitamins and organic compounds/ 1L

Myo-Inositol 100 mg/l

Nicotinic Acid 0.5 mg/l

Pyridoxine · HCl 0.5 mg/l

Thiamine · HCl 1.0 mg/l

Glycine 2 mg/l

Example 1: Adaptive laboratory evolution (ALE)

We performed an adaptive laboratory evolution (ALE) campaign to develop derivatives of *B. paralicheniformis* DSM33110 with improved growth rates at cold temperatures. Two different growth media were selected to carry on the evolution process in parallel. 12 independent cultures (6 per growth medium) were grown over-night and diluted every day to fresh medium (see Figure 1). Initially, MSgg or MS-Rex growth media were inoculated from a stock of *Bacillus paralicheniformis* DSM33110 cell culture (OD600 1). Optical density of DSM33110 subcultures was monitored daily and subsequently diluted to fresh growth medium. Two glycerol stocks were prepared per tube weekly. One was used for inoculation of a new

2026201481 26 Feb 2026

round of growth passages, while the second one was kept frozen. Temperature was gradually decreased from 25°C to 18°C throughout the 12 weeks of ALE campaign. Total number of generations reached was counted, and after 12 weeks of adaptive evolution, cultures were grown for 120-140 generations. Final samples from the evolution experiment were serially diluted and plated on LB agar plates to obtain isolated colonies. 2x960 clones were picked with a colony picker robot and grown in 96 well plates to generate individual glycerol stocks.

Example 2: Growth rate analysis

To compare growth profiles and growth rates between adapted derivatives and the parental strain, 2x958 clones (plus parental strain DSM33110) were inoculated into 96-deep-well plates containing either MSgg or MS-Rex medium. Growth experiments were performed in a Growth Profiler at 21°C. Data obtained allowed identification of the faster growing derivatives and selection of best 30 individual clones. Growth curves were then determined in triplicates to ensure reproducibility. Growth rates were calculated applying the slope function to OD600 recorded values in exponential phase. Umax values correspond to change in OD600 unit per hour (h^{-1}) **Figure 2** shows average growth data from the seven best derivatives developed from each growth media. **Figure 3** shows calculated maximum growth rates (μmax) for derivatives and mother strain DSM33110.

Example 3 - Genome sequencing and SNP analysis of improved derivatives

Final selected clones (2x7) were deposited in the CHCC collection and genome sequenced. Single nucleotide polymorphism (SNP) analysis was performed by comparing the genome sequences of the selected cold adapted strains to the DSM33110 genome sequence.

CHCC number	Strain name	SNPs analysis	Frequency
CHCC32528	CA1	8-amino-7-oxononanoate synthase (EC2.3.1.47):p.Pro314Ser (BioF) YbgE homologue-Branched-chain amino acid aminotransferase (EC2.6.1.42):p.Asp3fs	8 out of 14 (57%) 2 out of 14 (14%)
CHCC32529	CA2	8-amino-7-oxononanoate synthase (EC2.3.1.47):p.Ala64Val (BioF) Flagellar biosynthesis protein FliP:p.Lys156Glu Smc-superfamily protein:p.Ser163fs	8 out of 14 (57%) 1 out of 14 1 out of 14

2026201481 26 Feb 2026

CHCC32530	CA3	Heat-inducible transcription repressor HrcA:p.Gln6* SNP in region upstream of BioWAFDB operon. Maybe affecting the promoter region	5 out of 14 2 out of 14 (10/14)
CHCC36494	CA4	8-amino-7-oxononanoate synthase (EC2.3.1.47):p.Arg37Trp (BioF) Flagellar biosynthesis protein FlhA:p.Ala128Val	8 out of 14 (57%) 3 out of 14 (21%)
CHCC36495	CA5	SNP in region upstream of BioWAFDB operon. Maybe affecting the promoter region	2 out of 14 (9/14)
CHCC36496	CA6	8-amino-7-oxononanoate synthase (EC 2.3.1.47):p.Pro314Ser (BioF) YbgE homologue-Branched-chain amino acid aminotransferase (EC 2.6.1.42):p.Phe195fs Flagellar biosynthesis protein FlhB:p.Glu16fs	8 out of 14 (57%) 2 out of 14 (14%) 1 out of 14 (7/14)
CHCC36497	CA7	Heat-inducible transcription repressor HrcA:p.Pro314Thr ABC transporter permease protein:p.Lys624fs	5 out of 14 1 out of 14
CHCC36751	CAREX1	Glycogen synthase, ADP-glucose transglucosylase (EC 2.4.1.21):p.Lys150fs Heat-inducible transcription repressor HrcA:p.Gln11*	1 out of 14 5 out of 14
CHCC36752	CAREX2	8-amino-7-oxononanoate synthase (EC 2.3.1.47):p.Ala244Val (BioF) Flagellar biosynthesis protein FlhA:p.Ala128Val	8 out of 14 (57%) 3 out of 14 (21%)
CHCC36753	CAREX3	Heat-inducible transcription repressor HrcA:p.Gln11*	5 out of 14
CHCC36754	CAREX4	8-amino-7-oxononanoate synthase (EC2.3.1.47):p.Pro314Ser (BioF) SNP in promoter region of YpfA homologue, receptor of c-di-GMP: T to C Flagellar basal-body rod protein FlgG:p.Gln152* Hypothetical protein in operon with YteA, a RsbR paralogue: :p.Phe5fs.	8 out of 14 (57%) 2 out of 14 2 out of 14 1 out of 14
CHCC36755	CAREX5	8-amino-7-oxononanoate synthase (EC 2.3.1.47):p.Asp309Gly (BioF) Transcriptional regulator GabR of GABA utilization:p.Arg30Leu SNP in promoter region of YpfA homologue, receptor of c-di-GMP: T to C Flagellar basal-body rod protein FlgG:p.Gln152*	8 out of 14 (57%) 1 out of 14 2 out of 14 2 out of 14
CHCC36756	CAREX6	8-amino-7-oxononanoate synthase (EC 2.3.1.47):p.Arg37Trp Flagellar biosynthesis protein FlhA:p.Ile182fs and several other SNPs	8 out of 14 (57%) 3 out of 14 (21%)
CHCC36757	CAREX7	Heat-inducible transcription repressor HrcA:p.Gln11*	5 out of 14

Table 1. SNP analysis and ORF target identification. Table contains information linking cold adapted derivatives ID/name with the corresponding CHCC number. SNPs identity and frequency with which they are found are summarized.

Out of 14 cold adapted DSM33110 derivatives sequenced, 8 showed mutations targeting the *bioF* orf. *bioF* encodes an 8-amino-7-oxononanoate synthase (EC2.3.1.47), enzyme in the biotin cofactor biosynthetic pathway. See below for physiological interpretation of the SNPs identified.

2026201481 26 Feb 2026

Genome sequencing results revealed the presence of several SNPs (found in 5 out of 14 derivatives) within the orf *hrcA*, encoding the heat-inducible transcriptional repressor HrcA. In most cases the identified mutations correspond to stop codons, and only in one of the mutant strains we identified an amino acid change Pro314 to Thr. Most likely all those mutations render an inactive repressor.

Example 4 – Growth advantage specificity analysis

To assess whether the growth improvement phenotype was restricted to a specific growth medium composition or low temperatures, we compare the growth profiles of cold-adapted derivatives with the parental strain DSM33110 in complex media (LB and BHI) at 30°C temperature.

Higher biomass yields and faster growth rates were observed for the cold adapted derivatives, suggesting that the mutations do not only help the strains to grow at low temperatures. During the adaptive evolution process we have selected for fast growers and derivatives that reach higher yields.

Example 5 - Plant growth promotion experiments

Plant growth promotion efficiency was compared between the derivative strains at 22°C and 15°C using *A. thaliana Col-0* plants, in a gnotobiotic system based on the use of 24-well plates filled with plant growth medium solidified with agar. Based on the results from three independent experiments, both in soil and agar systems, derivative strains CA4, CA6 and CAREX1 were selected (CHCC36494, CHCC36496, and CHCC36751, respectively) as the best cold adapted DSM33110 derivatives. Results shown in figure 5.

To further evaluate the plant fitness promotion effect of cold adapted selected derivatives, a series of experiments were setup by the application team. Plant growth promotion efficiency was compared between derivative strains using *A. thaliana* plants in a soil system. Experiments were performed in plant growth chambers. Two different strain combinations containing cold adapted improved derivatives CA4 and CA6 were compared with the parental strain in their ability to promote *A. thaliana* plant growth. Strains *B. paralicheniformis* CHCC36494 (CA4) and CHCC36496 (CA6) were co-inoculated with 2 different *Bacillus subtilis* strains,

2026201481 26 Feb 2026

as indicated in figure 19. *A. thaliana* seedlings were germinated and pre-grown in potting soil for 7 days prior inoculation. Bacterial cultures were grown over-night the day before inoculation. In the morning of inoculation day, bacterial cultures were diluted and grown to OD_{600} 1 in LB broth. Bacterial cells were then washed and resuspended into 10 mM $MgSO_4$ buffer at OD_{600} 0.01. Plants were inoculated with 1e6 CFUs (8 plant replicates per strain), comparing the results from control non-inoculated plants with the parental strain and cold adapted derivatives. Plants grew in growth chambers for 2 weeks, with 16h light/8h dark photoperiod at two different temperatures (22°C and 15°C) to evaluate the performance of these strains under lower, suboptimal Tª conditions. Shoot fresh weight quantification was done 2 weeks post-inoculation. Results obtained showed an increase in plant growth for all treatments compared to non-inoculated plants, with a more relevant increase being observed for the two combinations containing the cold adapted improved derivative CA6 (19%-40% and 36%-36% increase, at 22°C and 15°C temperature, respectively). Both at 22°C and 15°C, strain combinations containing derivative CA6 (CHCC36496) returned the best results. Nevertheless, only one strain combination showed statistically significant results at 15°C compared to non-inoculated plants. Therefore, strain combination CA6 (CHCC36496) plus *B. subtilis* DSM33015 was selected to carry on additional testing in crop plants and field trials.

Example 6 - Physiological interpretation of MoA associated with identified SNPs

BioF - Biotin (also known as vitamin H) is a covalently bound enzyme cofactor required by all forms of life. In 5 out the 8 mutated sequences, the genotypic change renders a protein with an amino acid change from Pro 314 to Ser. In the other 3 mutated sequences the amino acid changes are different (Ala244 to Val, Asp300 to Gly and Arg37 to Trp). Homology searches, ClustalW alignments, and protein 3D structural modelling of the BioF DSM33110 amino acid sequence suggest that selected SNPs result in amino acid changes that most likely are not part of the catalytic site (1) (Figure 6). How those SNPs affect the activity of the BioF enzyme and intracellular levels of biotin is currently under investigation.

Biotin is an essential cofactor required for diverse key metabolic enzymes that carry out carboxylation and decarboxylation reactions in fatty acid synthesis,

2026201481 26 Feb 2026

amino acid metabolism and gluconeogenesis (2). In *B. cereus*, limiting amounts of biotin were reported to restrict growth and alter the cell membrane fatty acid composition (3). In *Bacillus spp*., the first intermediate in fatty acid and phospholipid synthesis is malonyl-CoA. Malonyl-CoA is synthesized from acetyl-CoA by the acetyl-CoA carboxylase, encoded on the *accBC* genes. The *accBC* operon from *Bacillus* codes for two subunits of acetyl-CoA carboxylase, biotin carboxyl-carrier and biotin carboxylase. In addition, a direct correlation between the levels of transcription of the fatty acid *accBC* genes and the rate of cellular growth was reported in *B. subtilis* (4)

HrcA - HrcA has previously been described in *B. subtilis* as the transcriptional repressor of the class I heat-shock genes, the HrcA regulon (5). The HrcA regulon consists of just two operons, the heptacistronic dnaK operon and the bicistronic *groE* (Figure 7). Both operons encode for molecular chaperons and respond to heat stress. HrcA binds to the inverted repeats located in both operons and known as CIRCE (Controlling inverted repeat of chaperon expression) element. DnaK, DnaJ, GrpE and GroES-GroEL are chaperons over-expressed in response to both cold and high temperature treatments (Schumann, W. 2003). A B. subtilis *hrcA* knockout causes high constitutive expression of both class I heat-shock operons in the absence of heat-shock. In addition, deletion of *hrcA* in B. subtilis helps the strain to resume growth faster after a temperature change (heat or cold shock). Accordingly, HrcA over-expression has the opposite effect and cells are less capable to respond to temperature shifts (6).

Genome analysis of cold adapted derivatives identified mutations affecting the *hrcA* orf. Those SNPs corresponded in most cases to stop codons, and only in one of the mutant strains we identified an amino acid change Pro314 to Thr. Most likely all those mutations render an inactive repressor, which will consequently allow overexpression of the two operons in the regulon.

2026201481 26 Feb 2026

Conclusion

To summarize, strains developed from *B. paralicheniformis* (DSM33110) with increased growth rates and higher biomass yields at different temperatures were developed following an adaptive laboratory evolution campaign. 14 different derivatives were selected, and their genomes sequenced to identify the acquired genotypic changes. Derivative strains were characterized physiologically and tested for performance in plant growth experiments.

Based on our results, specific mechanisms to explain the observed phenotypic differences with the parental strain is presented.

In addition, these evolution experiments have supplied/contributed with new *Bacillus* strains showing improved properties in plant growth promotion.

In a preferred aspect, the term *bioF* gene as used herein is intended to mean the BioF region 8-amino-7-oxononanoate synthase (EC_2.3.1.47).
In yet a preferred aspect, the term *hrcA* gene as used herein is intended to mean the Heat-inducible transcription repressor HrcA.

DEPOSITS and EXPERT SOLUTION

The applicant requests that a sample of the deposited microorganisms stated below may only be made available to an expert, until the date on which the patent is granted.

The applicant deposited *Bacillus paralicheniformis* parent strain was deposited as DSM33110 on May 8th, 2019 at Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7B, D-38124 Braunschweig.

The applicant deposited the strains derived from the parent strain on August 14, 2019 at Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7B, D-38124 Braunschweig as:
Bacillus paralicheniformis CHCC32530 = DSM 33238
Bacillus paralicheniformis CHCC36494 = DSM 33239
Bacillus paralicheniformis CHCC36496 = DSM 33240

2026201481 26 Feb 2026

Bacillus paralicheniformis CHCC36751 = DSM 33241
Bacillus paralicheniformis CHCC36753 = DSM 33242
Bacillus paralicheniformis CHCC36754 = DSM 33243
Bacillus paralicheniformis CHCC36755 = DSM 33244

The deposits were made according to the Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure.

SEQUENCES

Forming part of present description is the sequence listing attached hereto.
As specified therein, the sequences
SEQ ID NO:1 defines the *hrcA* nucleotide sequence
SEQ ID NO:2 defines the HrcA protein sequence
SEQ ID NO:3 defines the *bioF* nucleotide sequence
SEQ ID NO:4 defines the BioF protein sequence.

2026201481 26 Feb 2026

CLAIMS

1. An isolated nucleic acid having at least 95% nucleotide sequence identity to the hrcA nucleotide sequence set forth in SEQ ID NO:1, said isolated nucleic acid comprising a loss-of-function mutation that renders the encoded HrcA protein non-functional as a transcriptional repressor of the dnaK and groE operons.

2. The isolated nucleic acid of claim 1, wherein said loss-of-function mutation is a nonsense mutation that introduces a premature stop codon into SEQ ID NO:1.

3. An isolated nucleic acid having at least 95% nucleotide sequence identity to the bioF nucleotide sequence set forth in SEQ ID NO:3, said isolated nucleic acid comprising a missense mutation relative to SEQ ID NO: 3.

4. The isolated nucleic acid of claim 3, wherein said missense mutation causes an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp.

5. An isolated polypeptide comprising a HrcA polypeptide derived from *Bacillus paralicheniformis* DSM33110, wherein the polypeptide is truncated as a result of a premature stop codon in the hrcA coding sequence relative to the full-length HrcA amino acid sequence set forth in SEQ ID NO:2.

6. An isolated polypeptide comprising a BioF polypeptide derived from *Bacillus paralicheniformis* DSM33110, wherein the polypeptide comprises an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp relative to the BioF amino acid sequence set forth in SEQ ID NO:4.

7. A method of identifying a temperature-optimized *Bacillus paralicheniformis* strain, the method comprising:
(a) obtaining a nucleic acid sample from a *Bacillus paralicheniformis* test strain;

2026201481 26 Feb 2026

(b) analyzing the nucleic acid sample to determine whether the test strain comprises:

(i) a loss-of-function mutation in the closest ortholog of the hrcA gene of DSM33110, optionally a nonsense mutation that introduces a premature stop codon relative to SEQ ID NO:1; and/or

(ii) a missense mutation in the closest ortholog of the bioF gene of DSM33110, optionally a missense mutation that causes an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp relative to SEQ ID NO:4; and

(c) identifying the test strain as a temperature-optimized strain when the mutation of step (b)(i) and/or step (b)(ii) is detected.

8. The method of claim 7, wherein analyzing the nucleic acid sample comprises:

(b)(a) performing polymerase chain reaction (PCR) using primers that hybridize to regions flanking the mutation in the hrcA and/or bioF gene; and

(b)(b) detecting the mutation by sequencing, restriction fragment analysis or allele-specific PCR.

9. The method of claim 7 or claim 8, further comprising:

(d) cultivating the identified temperature-optimized strain in MSgg medium at 21°C;

(e) measuring optical density at 600 nm over time; and

(f) calculating the maximum growth rate $\mu_{\max}$ as the change in OD600 per hour,

wherein the $\mu_{\max}$ of the temperature-optimized strain at 21°C exceeds that of *Bacillus paralicheniformis* DSM33110 grown under the same conditions.

10. A kit for identifying temperature-optimized *Bacillus paralicheniformis* strains, the kit comprising:

(a) at least one oligonucleotide primer or probe that specifically hybridizes to a region encompassing a mutation that introduces a loss-of-function

2026201481 26 Feb 2026

mutation, optionally a nonsense mutation that introduces a premature stop codon, in the closest ortholog of the hrcA gene of DSM33110; and/or (b) at least one oligonucleotide primer or probe that specifically hybridizes to a region encompassing a missense mutation, optionally a missense mutation that causes an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp, in the closest ortholog of the bioF gene of DSM31110.

11. The kit of claim 10, wherein the closest ortholog of the hrcA gene of DSM33110 has at least 95% nucleotide sequence identity with SEQ ID NO: 1.

12. The kit of claim 10, wherein the closest ortholog of the bioF gene of DSM33110 has at least 95% nucleotide sequence identity with SEQ ID NO: 3.

13. A method of producing a temperature-adapted *Bacillus* strain, the method comprising:
(a) cultivating a *Bacillus* strain in MSgg medium at a temperature gradually decreased from about 25°C to about 18°C over a period of at least 8 weeks, with serial passaging into fresh medium at intervals of about 24 hours;
(b) after at least about 120 generations, isolating individual colonies from the evolved cultures;
(c) determining the growth profiles of the isolated colonies at 21°C in MSgg medium by monitoring optical density at 600 nm over time; and
(d) selecting at least one colony whose maximum growth rate $\mu_{\max}$ at 21°C exceeds that of the *Bacillus* strain by at least 20%.

14. The method of claim 13, further comprising:
(e) determining whether the select colony comprises a loss-of-function mutation, optionally a nonsense mutation that introduces a premature stop codon, in the closest ortholog of the hrcA gene of DSM33110.

2026201481 26 Feb 2026

15. The method of claim 13, further comprising:
(e) determining whether the select colony comprises a missense mutation, optionally a missense mutation that causes an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp, in the closest ortholog of the bioF gene of DSM33110.

16. The method of any one of claims 13-15, wherein the serial passaging is carried out in MSgg medium or MS-Rex medium as defined in the specification.

17. A method of selecting a bacterial strain for use as a plant growth-promoting agent at reduced temperature, the method comprising:
(a) providing a plurality of *Bacillus paralicheniformis* candidate strains;
(b) for each candidate strain, detecting the presence or absence of:
(i) a mutation in the closest ortholog of the hrcA gene of DSM33110, optionally a loss-of-function mutation, optionally a nonsense mutation that introduces a premature stop codon, in the closest ortholog of the hrcA gene of DSM33110; and/or
(ii) a mutation in the closest ortholog of the bioF gene of DSM33110, optionally a misssense mutation that causes an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp relative to SEQ ID NO:4, in the closest ortholog of the bioF gene of DSM33110; and
(c) selecting, as a plant growth-promoting candidate for use at growth temperatures of 15–22°C, at least one strain comprising the mutation of step (b)(i) and/or step (b)(ii).

18. The method of claim 18, wherein the detection in step (b) is performed using the kit of claim 11.

19. A method of characterizing the mechanism of temperature adaptation in *Bacillus paralicheniformis*, the method comprising:
(a) obtaining a nucleic acid sample from a cold-adapted derivative of *Bacillus paralicheniformis* DSM33110 generated by adaptive laboratory evolution at 18–21°C;

2026201481 26 Feb 2026

(b) sequencing at least the hrcA gene and the bioF gene of the derivative; and

(c) identifying, in the derivative,

(i) a mutation in the hrcA gene, optionally a loss-of-function mutation, optionally a nonsense mutation that introduces a premature stop codon relative to SEQ ID NO:1, and/or

(ii) a mutation in the bioF gene, optionally a missense mutation that results in an amino acid substitution selected from Pro314Ser, Ala244Val, Asp300Gly and Arg37Trp relative to SEQ ID NO:4.

20. Use of the nucleic acid of any one of claims 1 to 4, for detecting or confirming temperature-optimized *Bacillus paralicheniformis* strains generated by adaptive laboratory evolution from DSM33110.

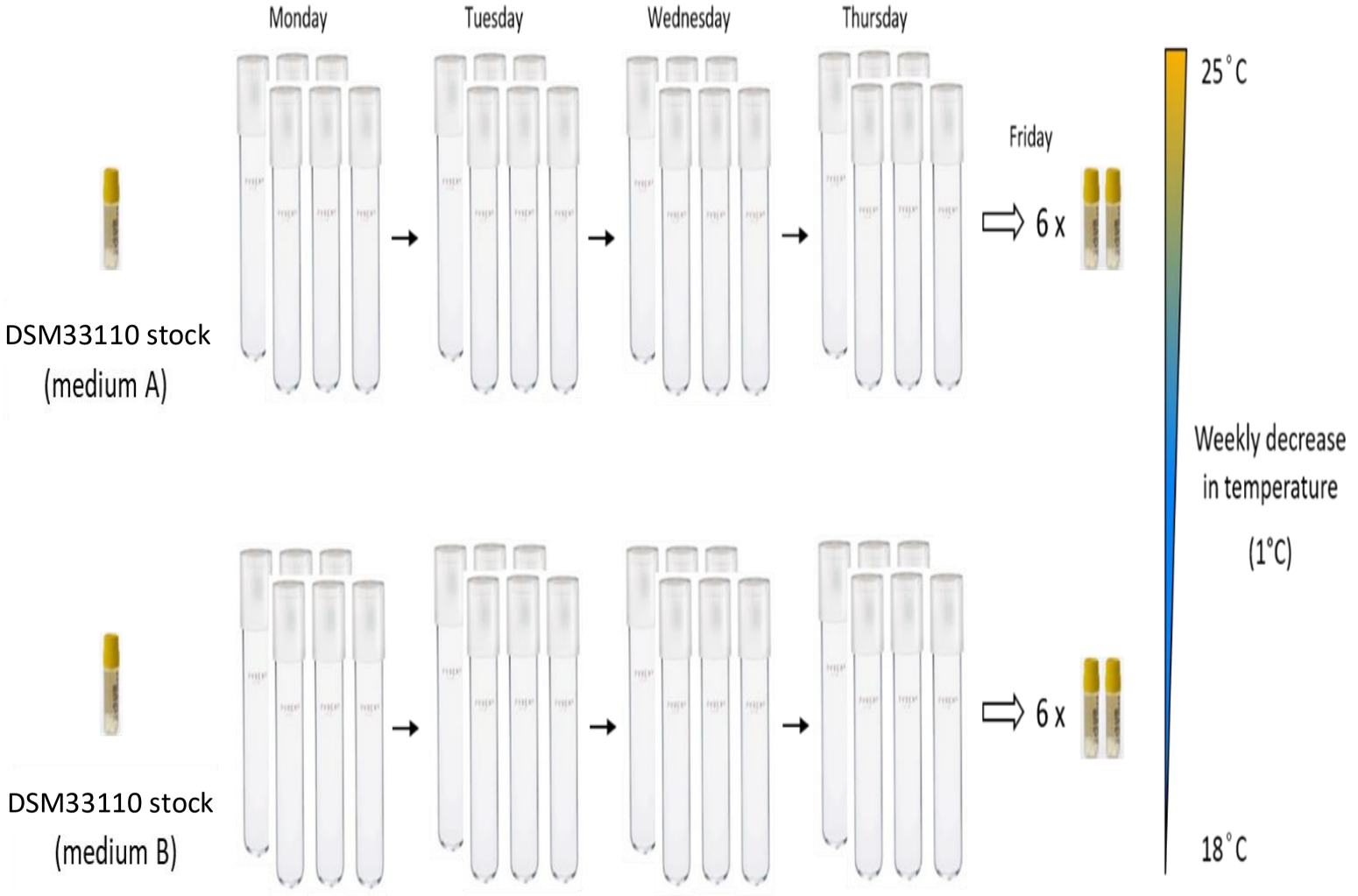
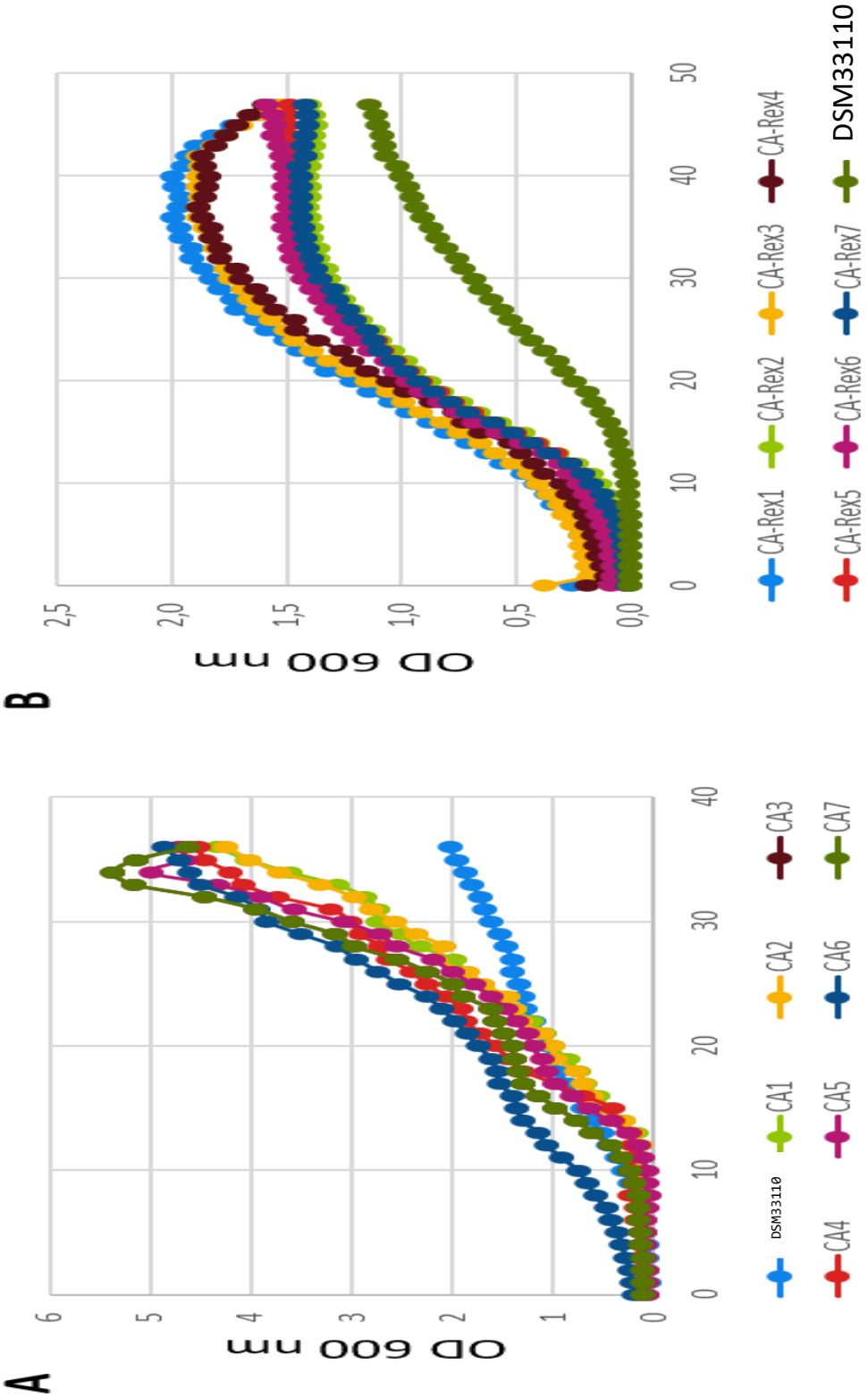

Monday
Tuesday
Wednesday
Thursday
Friday
6 x
DSM33110 stock
(medium A)
6 x
DSM33110 stock
(medium B)
25˚C
Weekly decrease
in temperature
(1°C)
18˚C

Figure 1

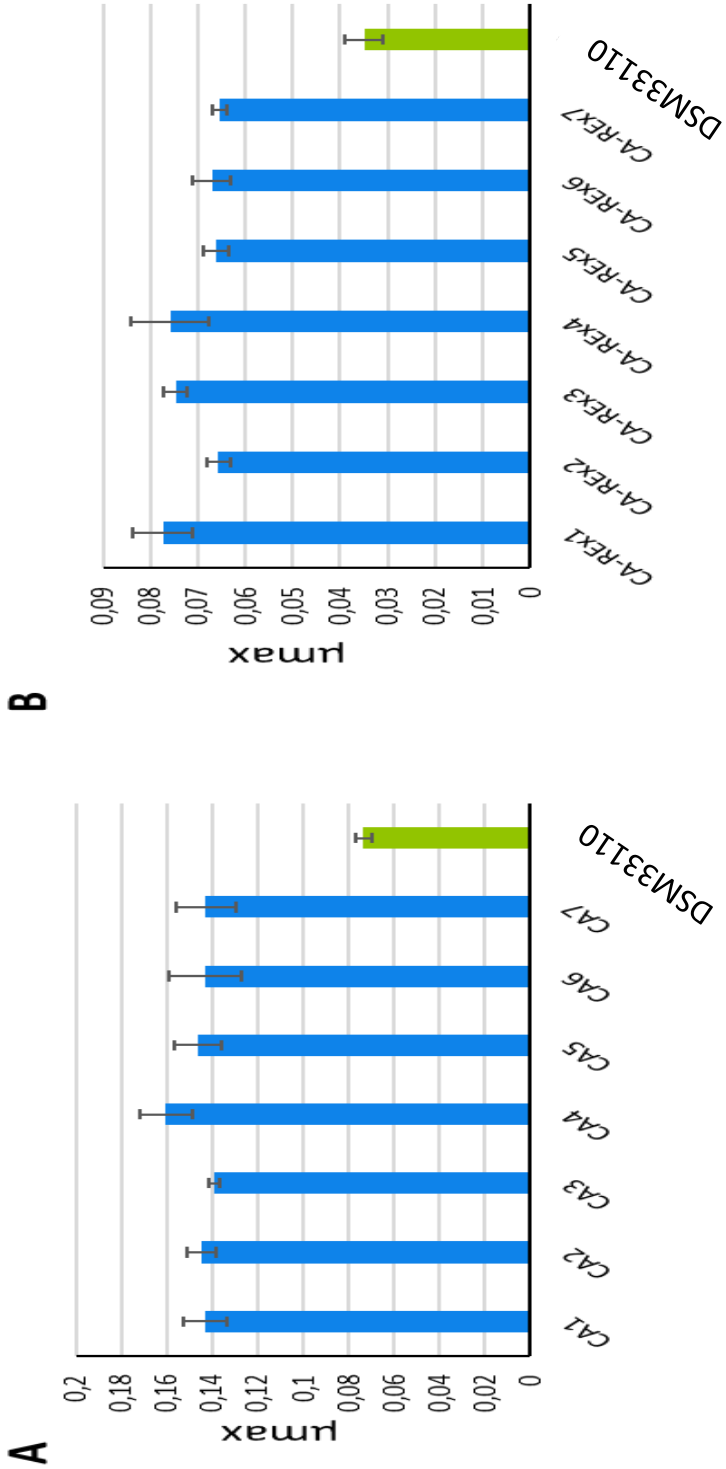
2026201481 26 Feb 2026

Figure 2



2026201481 26 Feb 2026

Figure 3



2026201481 26 Feb 2026

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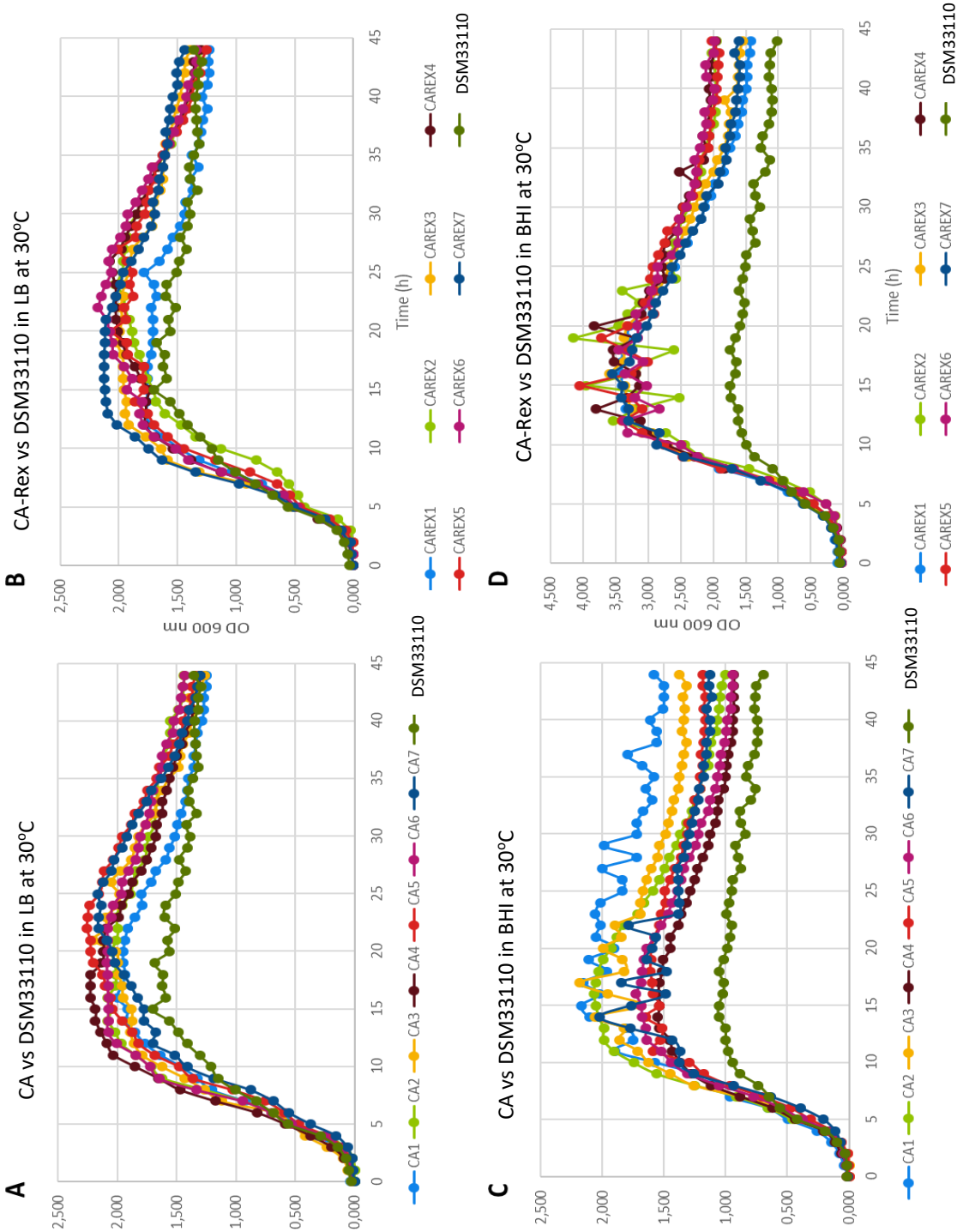

2026201481 26 Feb 2026

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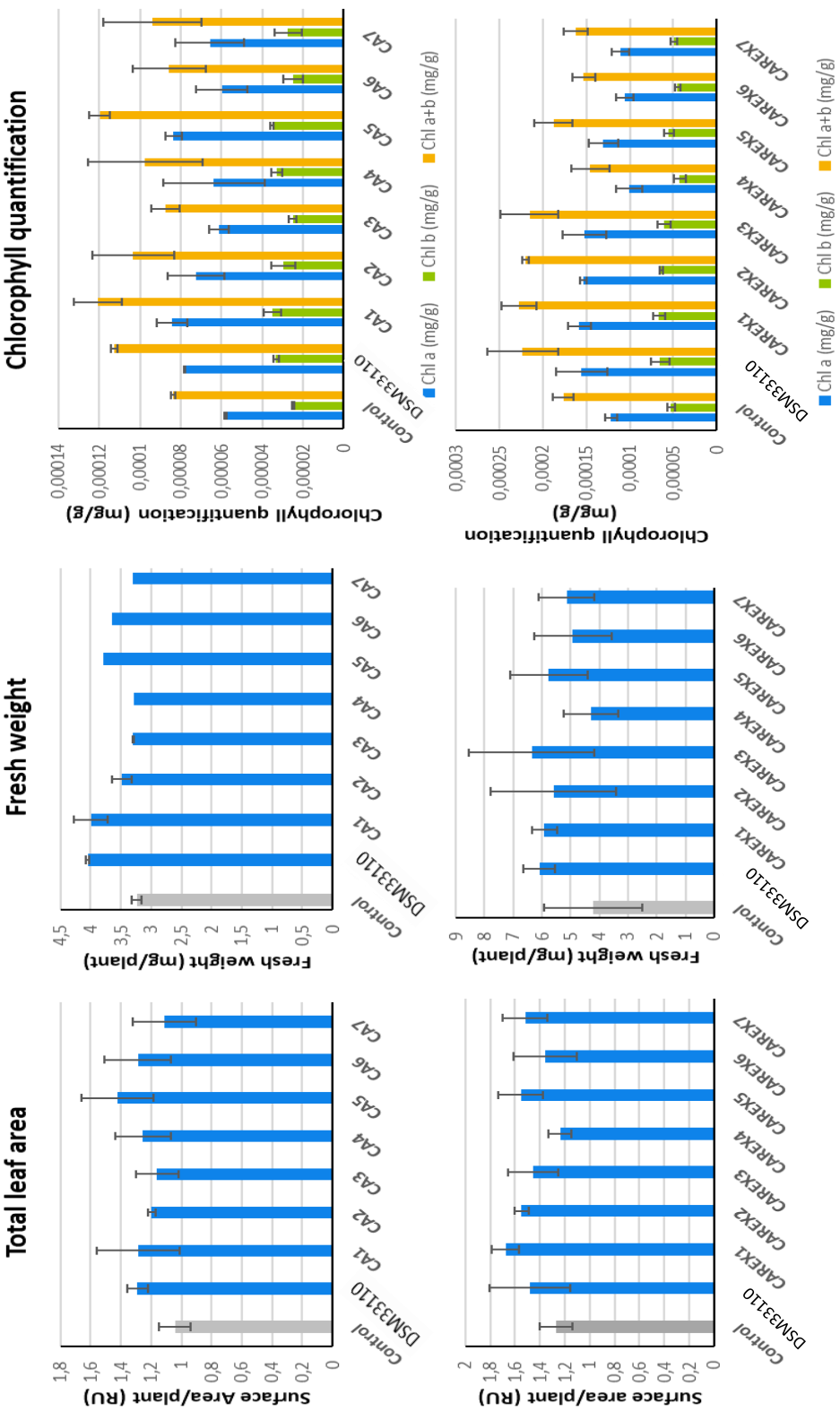

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BioF_2   DSM33110         MPIDEWLSSRLARTKAAGLYRSLKP-PQA-------VAEAKRTNRASNDY
                          *  :. :.  *   : *.  *    .  :.          :    :**:*

BioF                      LGLSHHPQIIRAWQQGAEQFGIGSGGSGHVSGYSVVHQALEEELAEWLGY
BioF_1                    LGLASDRRLIDAAQTALQQFGTGSSGSRLTTGNSVWHEKLEKKIASFKLT
BioF_2                    LSLANDKRLIHAAETALRRFGAGSTGSRLTSGNTEWHEKLERKIAGFKQT
                          *.*: . ::* * : . .:** ** **  .:* :  *: **.::* :

BioF                      SRALLFISGFAANQAVIAAMMAKEDRIAADRLSHASLLEAASLSPSQLRR
BioF_1                    EAALLFSSGYLANVGVLSSLPEKEDVILSDQLNHASMIDGCRLSKADTVV
BioF_2                    EAALLFSSGYLANIGVLSSLPEKGDVILSDQLNHASIIDGCRLSKADTVV
                          . **** **: ** .*::::  * * * :*:*.***::::. ** ::

BioF                      FAHNDVTHLARLLA-SPCPGQQMVVTEGVFSMDGDSAPLAEIQQVTQQHN
BioF_1                    YRHIDMNDLENKLNETQRYQRRFIVTDGVFSMDGTIAPLDQIISLAKRYH
BioF_2                    YRHTDMNDLEEKLRTAQSRARCFIVTDGVFSMDGTIAPLDEIMLLAKRYR
                          : * *:..* . *  :    : ::**:*******  *** :*  :::::.

BioF                      GWLMVDDAHGTGVIGEQGRGSCWLQKVKPELLVVTFGKGFGVSGAAVLCS
BioF_1                    AFVVVDDAHATGVLGDSGQGTSEYFGVCPDIVIGTLSKAVGAEGGFAAGS
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BioF                      STVADYLLQFARHLIYSTSMPPAQAQALRASLAVIRSDEGDARREKLAAL
BioF_1                    AVFIDFLLNHARTFIFQTAIPPASCAAAHEAFNIIEAS-R-EKRQLLFSY
BioF_2                    KALIDFLLNHARTFIFQTAVPPASCAAACRALDIIKDS-R-DKRRLLQSS
                           .. *:**:.** :*:.*::***.. *   :: :*. .    :*. * :

BioF                      ITRFRAGVQDLPFTLADSCSAIQPLIVGDNSRALQLAEKLRQQGCWVTAI
BioF_1                    ISMIRTSLKNMGYVVKGDHTPIIPVVIGDAHKTVLFAEKLQGKGIYAPAI
BioF_2                    VTTIKRGLEDIGFTVKGEDTPIIPVMIGDPQKAVRFANGLKEKGIEAPAI
                          :: :: .:::: :.: .. :.* *:::**  ::: :*: *: :*  ..**

BioF                      RPPTVPAGTARLRLTLTAAHEMQDIDRLLEVLHGNG-------
BioF_1                    RPPTVAPGESRIRITITSDHSMGDIDHLLQTFHSIGKELHII-
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                          *****. * :*:*:*:*: :.: **: **: ::  *
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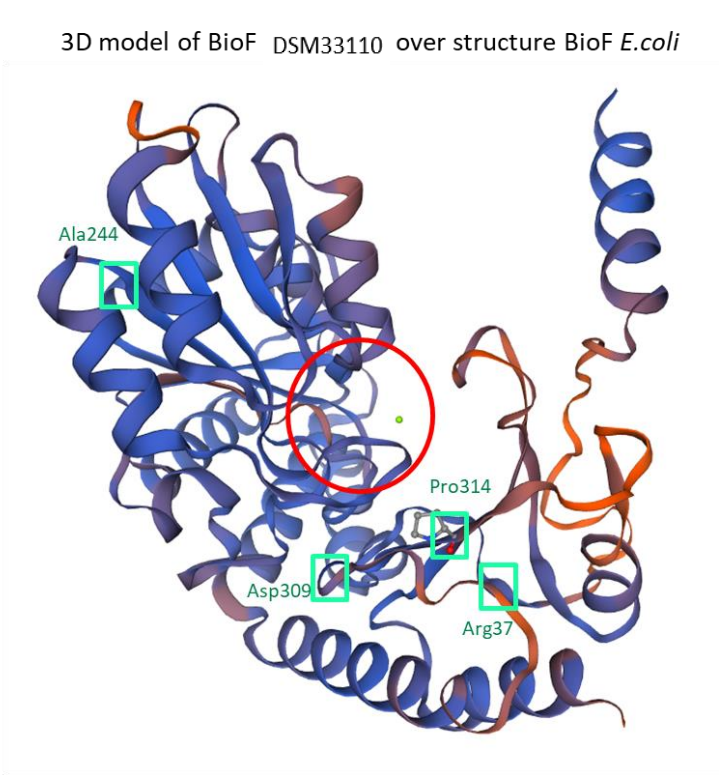


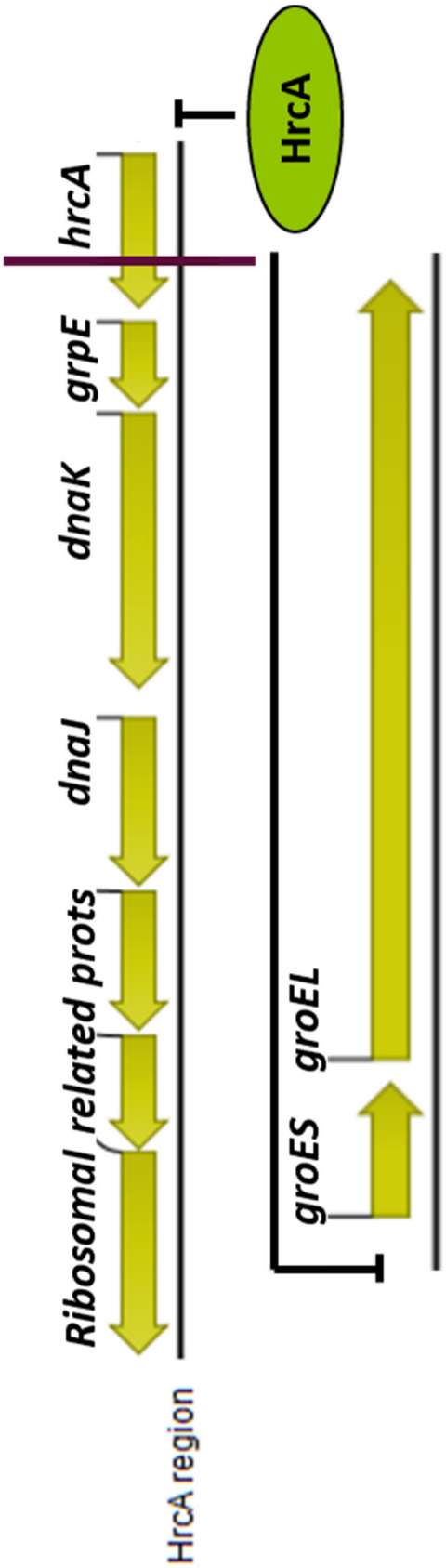
* SNP

☐ Catalytic residues

Figure 6

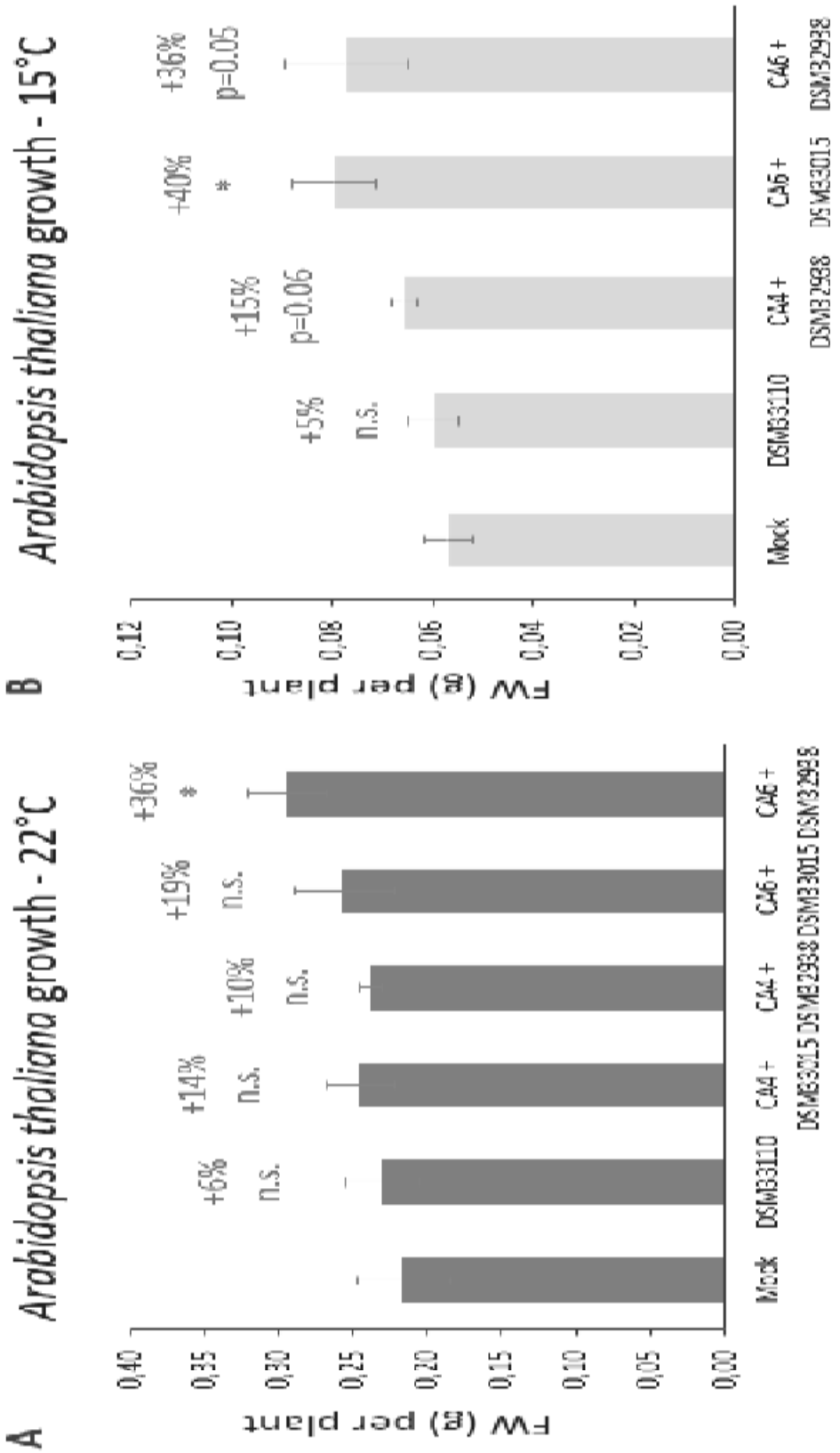
2026201481 26 Feb 2026

Figure 7



2026201481 26 Feb 2026

Figure 8



20262014 81 26 Feb 2026

Sequence Listing

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1-3	Software Name	WIPO Sequence
1-4	Software Version	2.3.0
1-5	Production Date	2026-02-12
1-6	Original free text language code	en
1-7	Non English free text language code	
2	**General Information**	
2-1	Current application: IP Office	US
2-2	Current application: Application number	17665-US-PCD
2-3	Current application: Filing date	2026-02-12
2-4	Current application: Applicant file reference	17665-US-PCD
2-5	Earliest priority application: IP Office	EP
2-6	Earliest priority application: Application number	19194343.0
2-7	Earliest priority application: Filing date	2019-08-29
2-8en	Applicant name	CHR HANSEN A/S
2-8	Applicant name: Name Latin	
2-9en	Inventor name	
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2-10en	Invention title	NOVEL TEMPERATURE OPTIMIZED BACILLI
2-11	Sequence Total Quantity	7

202620148 1 26 Feb 2026

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20262014 81 26 Feb 2026

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