

Genomic insights on fighting bacterial wilt by a novel *Bacillus amyloliquefaciens* strain Cas02

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Summary

Bacterial wilt, caused by the *Ralstonia solanacearum*, can infect several economically important crops. However, the management strategies available to control this disease are limited. Plant growth-promoting rhizobacteria (PGPR) have been considered promising biocontrol agents. In this study, *Bacillus amyloliquefaciens* strain Cas02 was isolated from the rhizosphere soil of healthy tobacco plants and evaluated for its effect on plant growth promotion and bacterial wilt suppression. Strain Cas02 exhibited several growth-promoting-related features including siderophore production, cellulase activity, protease activity, ammonia production and catalase activity. Moreover, strain Cas02 showed a significant inhibitory growth effect on *R. solanacearum*, and its active substances were separated and identified to be macrolactin A and macrolactin W by HPLC-DAD-ESI-MS/MS. Both greenhouse and field experiments demonstrated a

good performance of Cas02 in plant growth promotion and bacterial wilt suppression. To explore the underlying genetic mechanisms, complete genome sequencing was performed and the gene clusters responsible for antibacterial metabolites expression were identified. Overall, these findings suggest that the strain Cas02 could be a potential biocontrol agent in bacterial wilt management and a source of antimicrobial compounds for further exploitation.

Introduction

Soil-borne pathogens pose a serious threat to global agricultural production (Gu *et al.*, 2020). Bacterial wilt, caused by the betaproteobacterium *Ralstonia solanacearum*, is one of the most overwhelming soil-borne diseases that can infect hundreds of plant species, including economically important crops such as tomato, potato and tobacco crops (Jiang *et al.*, 2017). *Ralstonia solanacearum* enters the plant roots through wounds, root tips or cracks, colonizes the cortex and invades the xylem vessels. It spreads systemically and blocks water transport, resulting in plant wilting and eventual death. Only for potato crops, \$1 billion are estimated to be lost annually due to this pathogen worldwide (Kwak *et al.*, 2018). Despite the inflicted damage, limited management strategies are available to control this pathogen. As the use of pesticides and agrochemicals poses a health and environmental risk, wherein toxic residues accumulate in the soil and enter the food supply (Cao *et al.*, 2018), a better method to control these pathogens is urgently needed to ensure crop production and reduce economic loss.

Biocontrol of bacterial wilt has gained importance as an environment-friendly disease management strategy in recent years (Wang *et al.*, 2019; Batista and Singh, 2021). Plant growth-promoting rhizobacteria (PGPR) are considered promising biocontrol agents. Principally, the biological control mechanisms include antibiotics, competition for nutrients, plant growth induction and plant resistance (Berlanga-Clavero *et al.*, 2020; Zhang *et al.*, 2021). The most studied microbes against *R. solanacearum* have been derived from the genera of *Pseudomonas* spp., *Streptomyces* spp., *Bacillus* spp. and phages (Álvarez and Biosca, 2017; Jiang *et al.*, 2017; Cao *et al.*, 2018; Le *et al.*, 2020). *Bacillus* species are gram-positive, spore-forming bacteria that are widely distributed in the

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environment (Han *et al.*, 2016). *Bacillus* species, such as *B. subtilis*, *B. amyloliquefaciens*, *B. brevis* and *B. cereus*, have been demonstrated to exert beneficial effects on plant growth and disease suppression in tomato, cotton, cucumber, tobacco and lettuce (Chowdhury *et al.*, 2015a, b; Saravanakumar *et al.*, 2019). In particular, the *B. amyloliquefaciens* strain PMB05 was reported to intensify plant immune responses to confer resistance against bacterial wilt in tomato (Ho *et al.*, 2020). *B. amyloliquefaciens* strain FJAT-2349 can produce non-ribosomally synthesized lipopeptides (e.g. surfactin, iturin and fengcin) with strong antimicrobial activity (Chen *et al.*, 2019a–c). Additionally, *Bacillus* spp. offer better stability and higher acid tolerance during heat processing and low-temperature storage (Elshagabee *et al.*, 2017). Therefore, *Bacillus* species are good candidates for biological control purposes.

Although some biocontrol studies have been conducted for *R. solanacearum*, limited performance efficiency in the field has hindered the commercialization of the studied biological control agents (Jiang *et al.*, 2017). It is, thus, necessary to explore new biological control strains with strong and stable control effects on bacterial wilt. Therefore, the objectives of the present study were to fully characterize a new isolated bacterial strain, Cas02, based on physiological assays and antagonism against *R. solanacearum* both *in vitro* and *in vivo*. Furthermore, the complete genome sequence of Cas02 was analysed to reveal the biocontrol mechanisms and to provide a scientific basis for the optimization of the field applications of this strain.

Results and discussion

Antagonistic activity against bacterial pathogens

To obtain the candidate antagonistic bacterial strains from the rhizosphere soils, 35 soil samples were used for bacterial isolation, and 246 bacterial isolates that showed antibacterial activity were selected for further analysis. Of these, isolate Cas02 showed the highest antibacterial activity against *R. solanacearum*, with a strong inhibitory zone (30 mm) (Fig. 1). In addition to the *R. solanacearum*, strain Cas02 also exhibited strong antagonistic activity against *Escherichia coli* FM3, *Pectobacterium carotovorum* EC101, *Burkholderia glumae* 291, *Pseudomonas syringae* B301D and *Xanthomonas campestris* MSCT1, with inhibition zones all greater than 20 mm (Fig. 1). An important biocontrol mechanism of biological control agents (BCAs) involves the secretion of antibacterial substances and inhibition of pathogen growth (Zhou *et al.*, 2021). Among the top 10 bacterial species listed based on their scientific and economic importance in plant diseases, *Pseudomonas syringae* pathovars and *Ralstonia solanacearum* were ranked as the first and second most important bacterial pathogens

respectively (Mansfield *et al.*, 2012). In this study, strain Cas02 exhibited strong antibacterial activity against these pathogens, indicating its broad-spectrum antibacterial abilities. It also provides a new microbial resources for preventing and controlling plant diseases.

Biocatalytic activity

The plant growth-promoting potential of Cas02 was characterized *in vitro* using enzyme assays. Cas02 was confirmed to produce siderophores, as indicated by colour change from blue to orange on the CAS agar plate (Fig. S1A). Siderophores produced by PGPR may reduce iron deficiency in plants (Saha *et al.*, 2016), and sequester iron from phytopathogens that require iron for growth and establishment (Gu *et al.*, 2020), thereby achieving the purpose of plant disease protection. Cas02 also showed a significant ability to produce lytic enzymes such as cellulases and proteases (Fig. S1B,C), which could be linked to its ability to degrade the cell wall of phytopathogens (Durairaj *et al.*, 2018). Ammonia production was observed for Cas02, as indicated by a brown/yellow colour compared with the control (Fig. S1D). A previous study reported that the ammonium produced by bacteria could induce the synthesis of various plant polyphenols such as flavonoids, phenylpropanoids and lignins to protect plants from pathogen attacks (Babalola, 2010). Catalase enzyme production was also observed for Cas02, as indicated by oxygen release upon adding hydrogen peroxide to the bacterial growth (Fig. S1E). This enzyme can help the bacterium to protect itself from the overproduction of reactive oxygen species (ROS) under stress conditions, thereby indirectly promoting plant growth (Bumunang and Babalola, 2014). Furthermore, β -1,3-glucanase activity and phosphate solubilization were also tested; however, neither activity was observed for Cas02.

Strain Cas02 identification

The 16S rRNA gene is frequently used for phylogenetic classification of microorganism species (Woo *et al.*, 2008; Wang *et al.*, 2016). However, the resolving power of rDNA gene is often limited, particularly at the species level within *Bacillus* genus. A multi-locus sequence typing (MLST) scheme using different housekeeping genes is a more suitable method to study the phylogenetic relationships between *Bacillus* strains (Kamada *et al.*, 2015; Le *et al.*, 2019). In this study, MLST based on the sequences of internal fragments of seven housekeeping genes (glpF, ilvD, ptA, purH, pycA, rpoD and tpiA) was selected for MLST analysis (www.pubmlst.org/bsubtilis). The obtained sequences were concatenated into one sequence for each strain using the GENEIOUS PRIME (2019.2.1). A neighbour-joining phylogenetic tree based

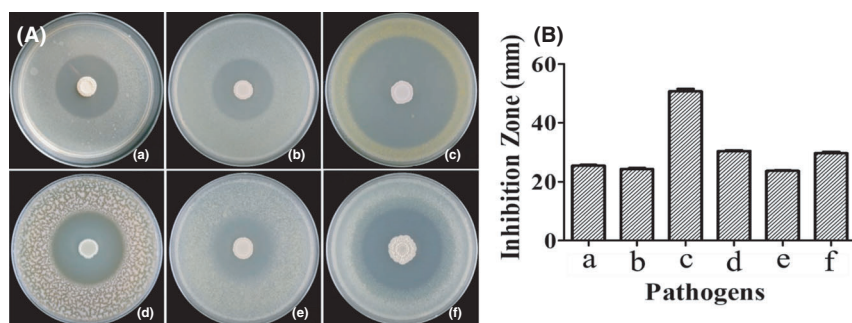


Fig. 1. Antagonistic effect of *Bacillus amyloliquefaciens* Cas02 on pathogens (a. *Escherichia coli*; b. *Pectobacterium carotovorum*; c. *Xanthomonas campestris*; d. *Ralstonia solanacearum*; e. *Pseudomonas syringae* pv. *tabaci*; f. *Colletotrichum acutatum*).

on concatenated sequences (Fig. 2) was constructed using MEGA X software. MLST analysis showed that strain Cas02, *B. amyloliquefaciens* subsp. *plantarum* FZB42 and *B. amyloliquefaciens* ALB69 were tightly clustered on the same branch, which indicated that strain Cas02 was a member of the species of *B. amyloliquefaciens*.

Bacillus amyloliquefaciens Cas02 could promote the plant growth and confer protection against bacterial wilt

To investigate the plant growth-promoting effect of strain Cas02, pepper seedlings were grown under the set environmental conditions, and the agronomic parameters including stem height, leaf number, maximal leaf length, maximal leaf width and biomass dry weight were measured. As shown in Fig. 3A and B, except for the leaf number, other agronomic parameters were significantly increased after the application of strain Cas02 compared with the control ($P < 0.05$). *Bacillus* species have been shown to promote plant growth (Chen *et al.*, 2019a–c).

In many cases, the plant growth-promoting activity is linked with the ability to suppress soil-borne plant pathogens via production of antimicrobial substances such as siderophores, lytic enzymes, cellulase, protease and catalase (Chowdhury *et al.*, 2015a,b). In addition to its plant growth-promoting activity, Cas02 can significantly reduce the severity of bacterial wilt caused by *R. solanacearum* in pepper plants. Pepper seedlings treated with strain Cas02 exhibited fewer disease symptoms (Fig. 3 C), and the control efficacy was 67.86% after 7 days of inoculation with *R. solanacearum*. The incidence and disease indices were significantly decreased compared with those in the control (Fig. 3D). These results indicate that strain Cas02 has potential use as a biological control agent for controlling bacterial wilt caused by *R. solanacearum*.

The field pot assay was conducted in Linyi City, Shandong Province. The agronomic traits of tobacco plants were measured 30 days after the final treatment with Cas02, when tobacco was in the rapid growth phase. The agronomic characteristics indicated that the strain

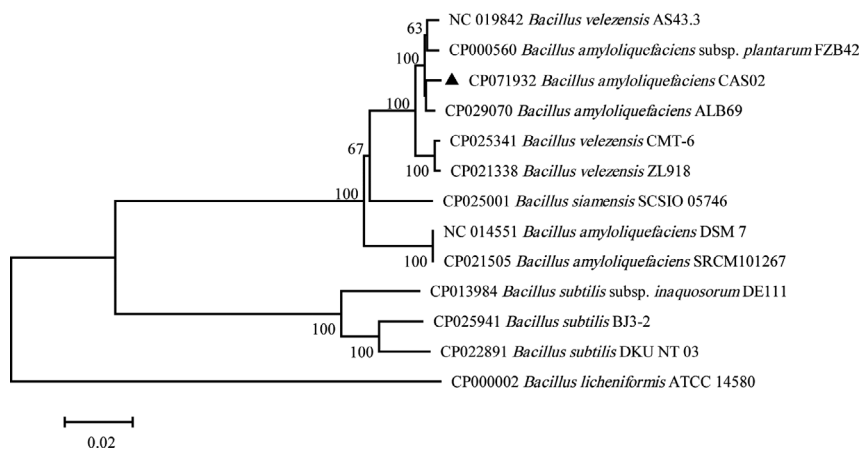


Fig. 2. Neighbour-joining phylogenetic tree based on concatenated MLST DNA sequences of strain Cas02 and representative *Bacillus* reference strains. The bootstrap consensus tree, inferred from 1000 replicates, was constructed using neighbour-joining method, and GenBank accession numbers were indicated.

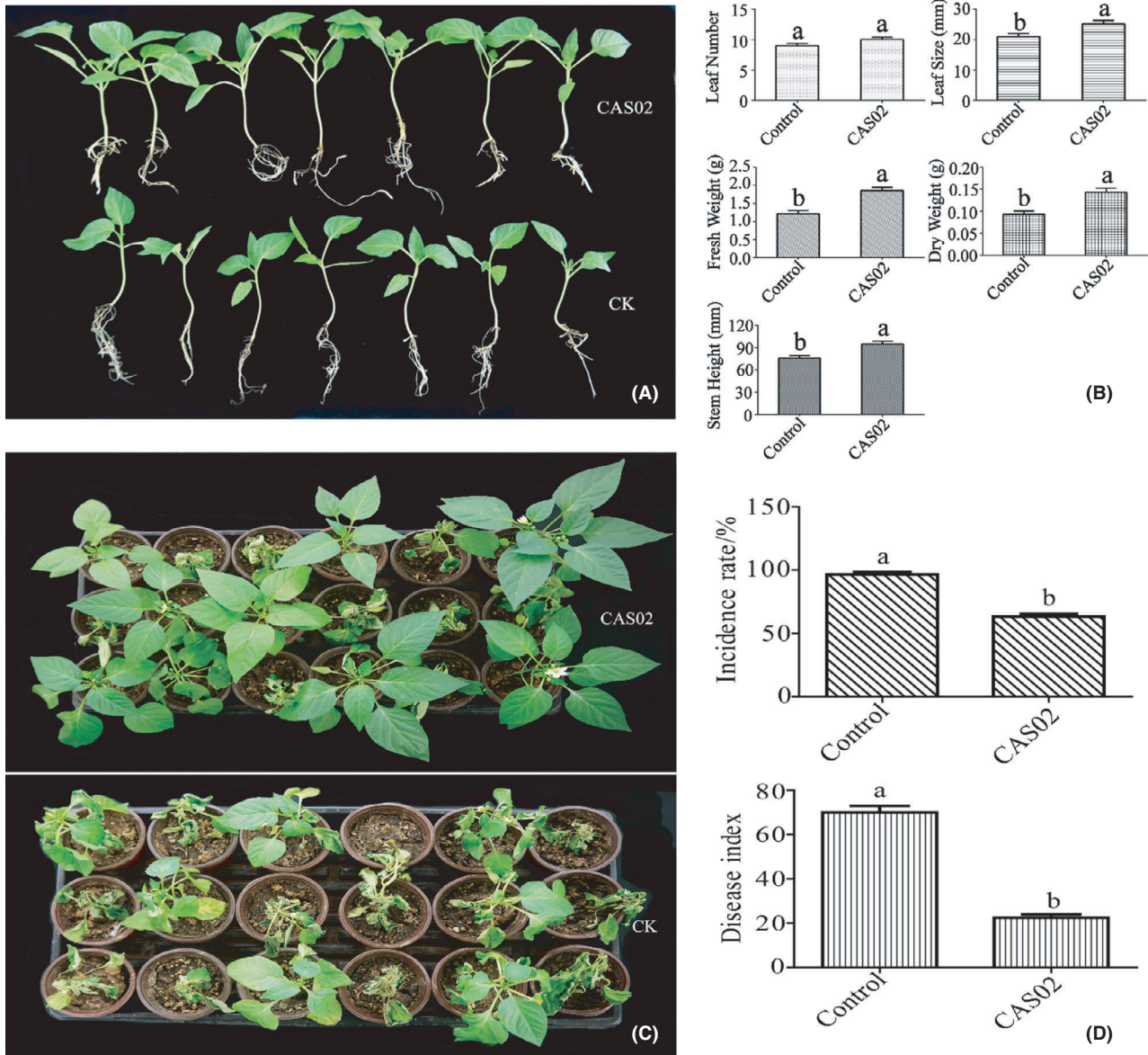


Fig. 3. Pepper growth promotion and biocontrol effect of strain Cas02 in green house condition. A. The images of pepper seedling growth with or without Cas02 treatment. B. The leaf number, leaf size, stem height, fresh and dry weight and chlorophyll of the pepper seedlings with or without Cas02 treatment. C. The images of pepper plant wilt disease symptoms with or without Cas02 treatment. D. The disease rate and disease index of pepper plant with or without Cas02 treatment.

Cas02 had promoting effect on tobacco growth under field conditions, with plant height, leaf number and maximum leaf length increased significantly compared with those in the control (Fig. 4). The greenhouse and field pot assays indicated that strain Cas02 has good potential to promote plant growth and suppress plant disease.

Soil physical and chemical analyses indicated that application of strain Cas02 can alter the physical and chemical properties of rhizosphere soil. Soil enzymes are mainly derived from rhizosphere microbes and are

the most active part of the soil organic components (Zhao *et al.*, 2020). As an important indicator for the conversion of soil nutrients, soil enzyme activity can reflect the circulation and health of soil nutrients (Baćmaga *et al.*, 2021). Catalase (CAT), NH_4^+ , available phosphorous and soil alkaline phosphatase activities were significantly increased compared with the control ($P > 0.05$) (Fig. 5). CAT can enhance the soil nitrogen cycle and reduce the toxic effects of hydrogen peroxide on organisms. The increase in available phosphorus and soil

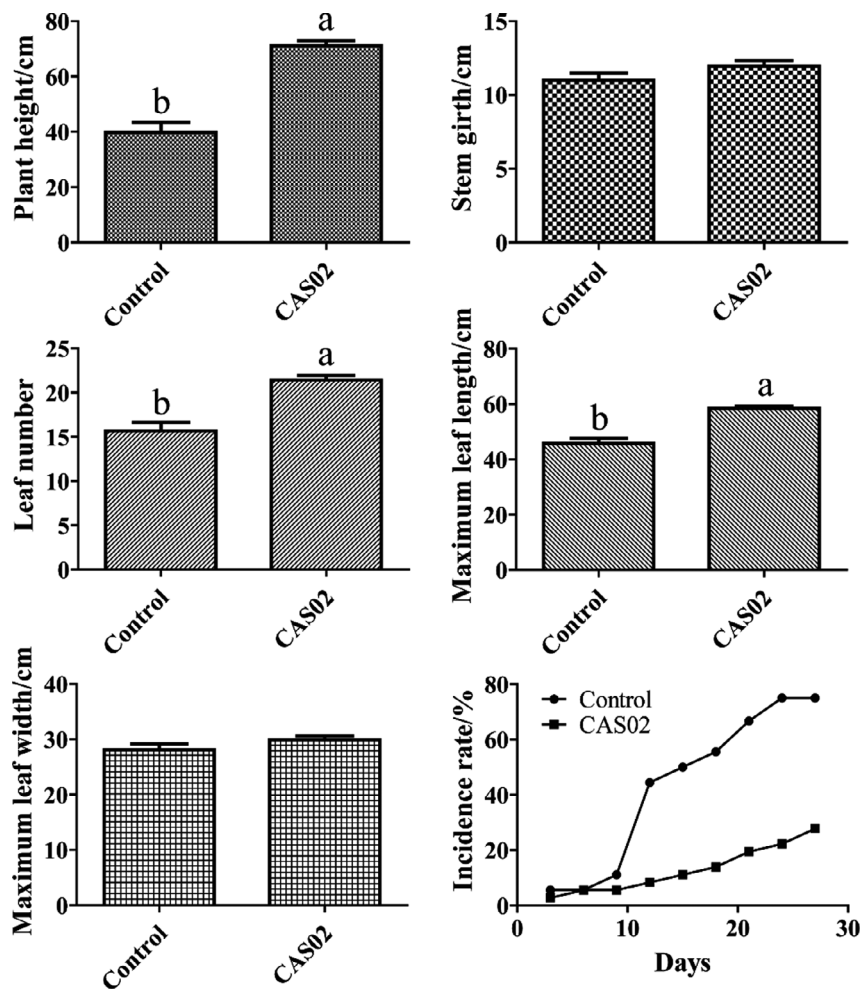


Fig. 4. Agronomic traits (plant height, stem girth, leaf number, maximum leaf length and maximum leaf width) and disease incidence of tobacco in the field condition. Two groups were included: control, normal operation only; Cas02, normal operation + strain Cas02 treatment.

alkaline phosphatase has a direct promoting effect on plant nutrient absorption and soil fertility improvement (Oliveira Filho *et al.*, 2021). The results indicated that the strain Cas02 could be able to improve the rhizosphere soil enzyme activity and nutrient metabolism.

Root colonization of strain Cas02

The ability to colonize plant roots is essential for rhizobacteria to be effective for plant growth promotion and disease suppression. As shown in Fig. 6A, laser confocal microscopic images showed high fluorescence signals on the tobacco root, suggesting effective colonization by the Cas02-GFP strain. Cell recovery counting also demonstrated that Cas02 successfully colonized the roots of tobacco seedlings, and that the populations were stable within 13 days (Fig. 6B). However, after 20 days of inoculation, the population density of Cas02 began to decrease, possibly because of

competition with indigenous microorganisms (Han *et al.*, 2016). Therefore, it is necessary to strengthen its effect with an additional inoculation of the strain after 20 days to maintain a certain population for antagonism.

Identification of the active compounds from strain Cas02 against bacterial wilt

To identify the antibacterial metabolites of Cas02, a crude fermentation extract was purified using macroporous adsorption resins, which are frequently employed for isolating various natural products (Tang *et al.*, 2021). The XAD-16 resin was used in this work because of its ideal physical properties to adsorb large amounts of uncharged compounds (Yuan *et al.*, 2012). The results (Fig. 7) demonstrated that washing with water followed by a 40% methanol solution excluded most impurities without the loss of active compounds. The purified metabolites were then eluted using 100% methanol, and

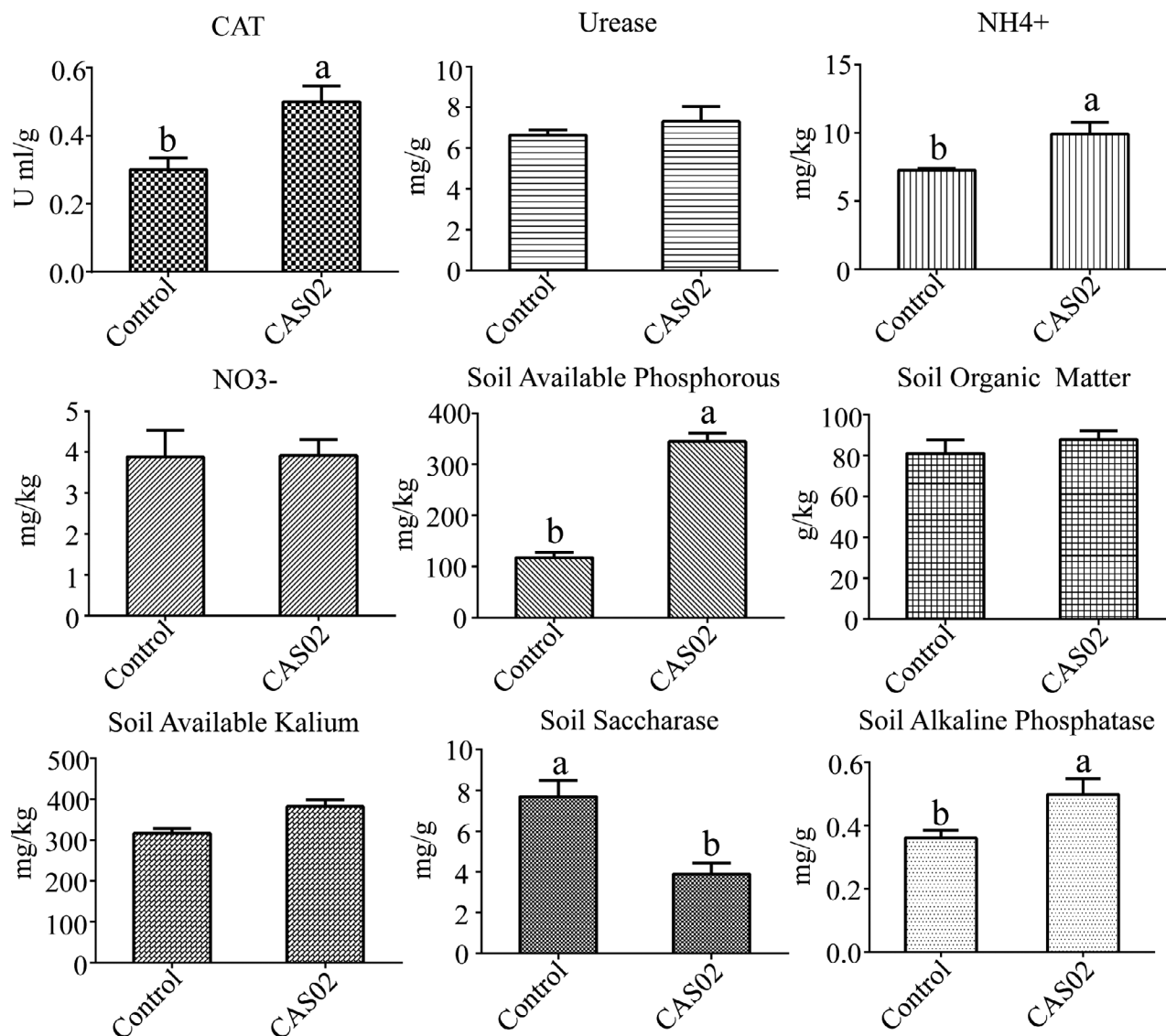


Fig. 5. Effect of strain Cas02 on soil properties. Two groups were included: control, normal operation only; Cas02, normal operation + strain Cas02 treatment.

showed significantly higher inhibitory activity against *R. solanacearum* compared with the original crude extract. Five peaks with retention times of 17.29, 20.01, 24.09, 25.70 and 39.56 min were observed in the HPLC spectrum of 100% methanol washing elutes (Fig. 7D), which were potentially responsible for the antibacterial effects against *R. solanacearum*. Among these, peaks 1, 3 and 4 showed similar UV spectra with a λ_{max} of 277 nm, whereas peaks 2 and 5 showed UV spectra with a λ_{max} of 228 nm and 264 nm respectively (Table S1).

The molecular weights and molecular formulae of the five compounds were analysed by ESI-MS/MS, and the results are shown in Fig. 8. The spectrum of peak 1 showed molecular ions of 1043.56 Da $[M + H]^+$ and

1065.54 Da $[M + Na]^+$, and was identified as iturin A (C-14) with a molecular weight of 1042 Da by comparing with published studies (Yuan *et al.*, 2011). The spectrum of peak 2 exhibited a molecular ion of 687.30 Da $[M + Na]^+$, which was identified as $C_{34}H_{48}O_{13}$. In addition, fragment ions with m/z ratios of 647.31 Da $[M - H_2O + H]^+$, 551.25 Da $[M - \text{succinic acid} - H_2O + Na]^+$, 529.27 Da $[M - \text{succinic acid} - H_2O + H]^+$, 511.26 Da $[M - \text{succinic acid} - 2H_2O + H]^+$ and 367.23 Da $[M - C_6H_{12}O_6 - \text{succinic acid} + H]^+$ were found and helped to identify the peak 2 as macrolactin W (Table S1) (Mojid Mondol *et al.*, 2011). The mass spectra of peaks 3 and 4 exhibited the same molecular ions of 1057.57 Da $[M + H]^+$ and 1079.55 Da $[M + Na]^+$, both of which were assigned

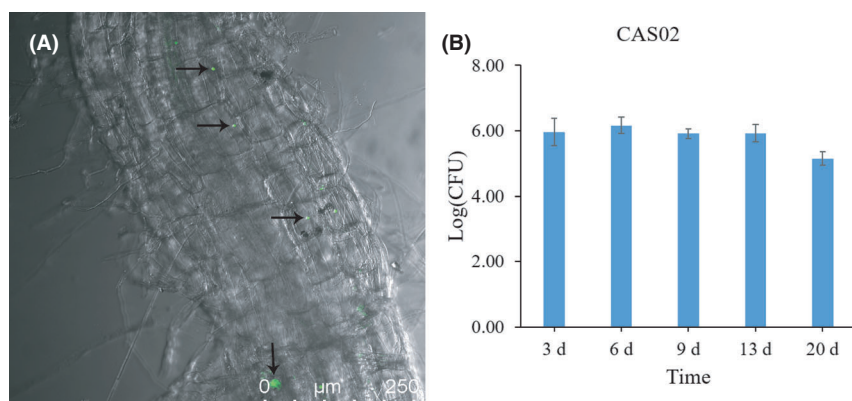


Fig. 6. Root colonization of strain Cas02.

A. LSCM image of colonization by Cas02-GFP on the 4th day after inoculation.

B. The colonization of Cas02 cells on tobacco root determined by counting colony-forming unit (cfu) per gram root.

a molecular formula of $C_{49}H_{76}N_{12}O_{14}$. The MS/MS spectra of the ion products of these two compounds also had the same patterns (Fig. S2A–D), indicating that peaks 3 and 4 could be the isomers of iturin A (C-15) with similar structures. The possible structures of the two compounds are shown in Fig. S2E. The spectrum of peak 5 revealed molecular ion peaks at m/z 425.23 Da $[M + Na]^+$, 385 Da $[M - H_2O + H]^+$ and 367.23 Da $[M - 2H_2O + H]^+$. Based on these data, the molecular formula of this compound was inferred to be $C_{24}H_{34}O_5$, and was identified as macrolactin A, according to previous literatures (Kim *et al.*, 2011; Chen *et al.*, 2019a–c; Salazar *et al.*, 2020).

Iturins are a class of lipopeptides composed of C14 to C17 β -amino fatty acids and heptapeptides. They often contain several structural homologs with identical amino acid sequences of heptapeptides and different fatty acid chains (C₁₄, C₁₅ or C₁₆) (Zhou *et al.*, 2020). In this work, it is likely that two C-15 iturin A molecules with different fatty acid chains were isolated from the fermentation extracts as they showed the same MS/MS patterns. Iturins are well known for their antifungal activity and exhibit limited antibacterial activity according to previous literature records (Yuan *et al.*, 2011; Chowdhury *et al.*, 2015a,b; Zhou *et al.*, 2020). The inhibitory effect of iturin A against *R. solanacearum* was, thus, tested using an authentic reference standard (Sigma-Aldrich Co., St. Louis, MO, USA). The results (Fig. 7E) indicated that iturin A showed no activity against *R. solanacearum*. Furthermore, the retention times of the reference standard were 17.29, 24.09 and 25.70 min, which were identical to the retention times in the 100% methanol washing elutes, thus verifying the presence of iturin A in the metabolites. Macrolactins, an important group of 24 membered macrolides, have been described as one of the most producing clinical antibiotic resources (Li *et al.*, 2016). Macrolactins have been reported to exhibit antibacterial activity against human clinical pathogens, cytotoxicity

against B16-F-10 murine melanoma cancer cells and antiviral activities against human immunodeficiency virus (HIV) (Li *et al.*, 2016; Salazar *et al.*, 2020). In recent years, the antibacterial activity of macrolactins against plant pathogenic bacteria has also been studied. Chen *et al.* (2021) revealed that macrolactins contribute to the biocontrol activity of crown gall disease caused by *Agrobacterium tumefaciens*. Yuan *et al.* (2012) demonstrated that macrolactin exerts an efficient antagonistic effect on *R. solanacearum*. Therefore, the activity against *R. solanacearum* observed in this work is most likely attributed to the macrolactins. However, further work is necessary to isolate each compound and identify its activities.

Genomic analysis of strain Cas02

To better understand the genetic background of strain Cas02, we further analysed its complete genome (Fig. 9). The complete genome of Cas02 was 3.93 Mb with an average GC content of 47.33% in the chromosome. In total, 3957 CDSs were predicted. The Cas02 genome contained 27 rRNA, 86 tRNA and 31sRNA genes. The general features are shown in Table S2. Furthermore, one 1.4 kb plasmid was found when assembling the genome sequence of this strain.

According to the results of COG annotation, 2923 proteins were classified into 25 COG families. The largest group of genes was involved in amino acid transport and metabolism (310 genes, 10.61%). In total, KEGG orthologues were found for 2556 proteins using BLAST. Furthermore, the GO annotation results revealed that the largest group of genes was involved in the biological process domain. Among these, the genes related to the metabolic process category formed the largest subgroup.

The gene clusters related to secondary metabolite synthesis were annotated using the antiSMASH 6.0 database.

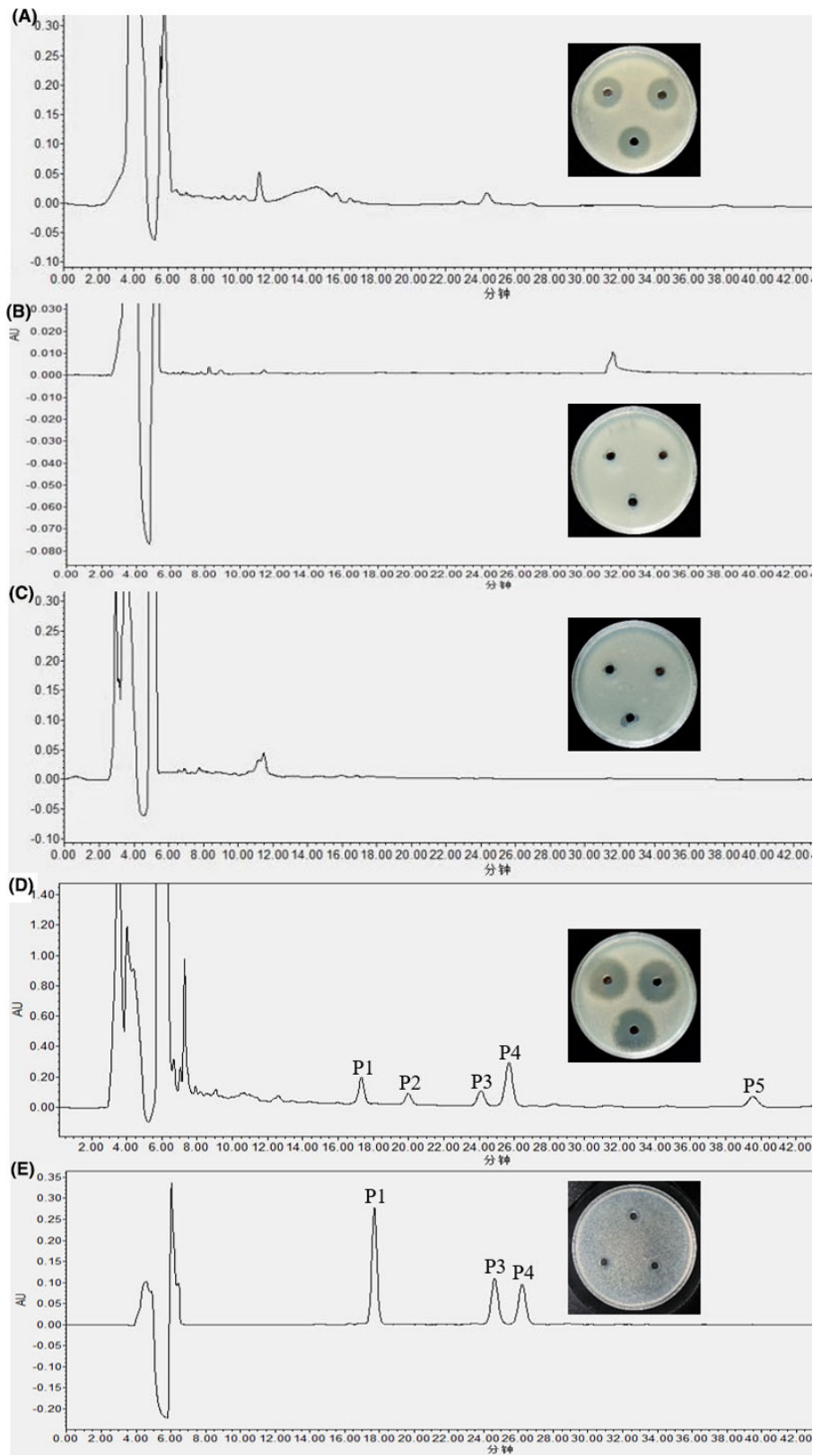


Fig. 7. HPLC profiles and antagonistic effect of (A) crude extracts; (B) water washing elutes; (C) 40% methanol washing elutes; (D) 100% methanol washing elutes; (E) authentic reference standard of iturin A.

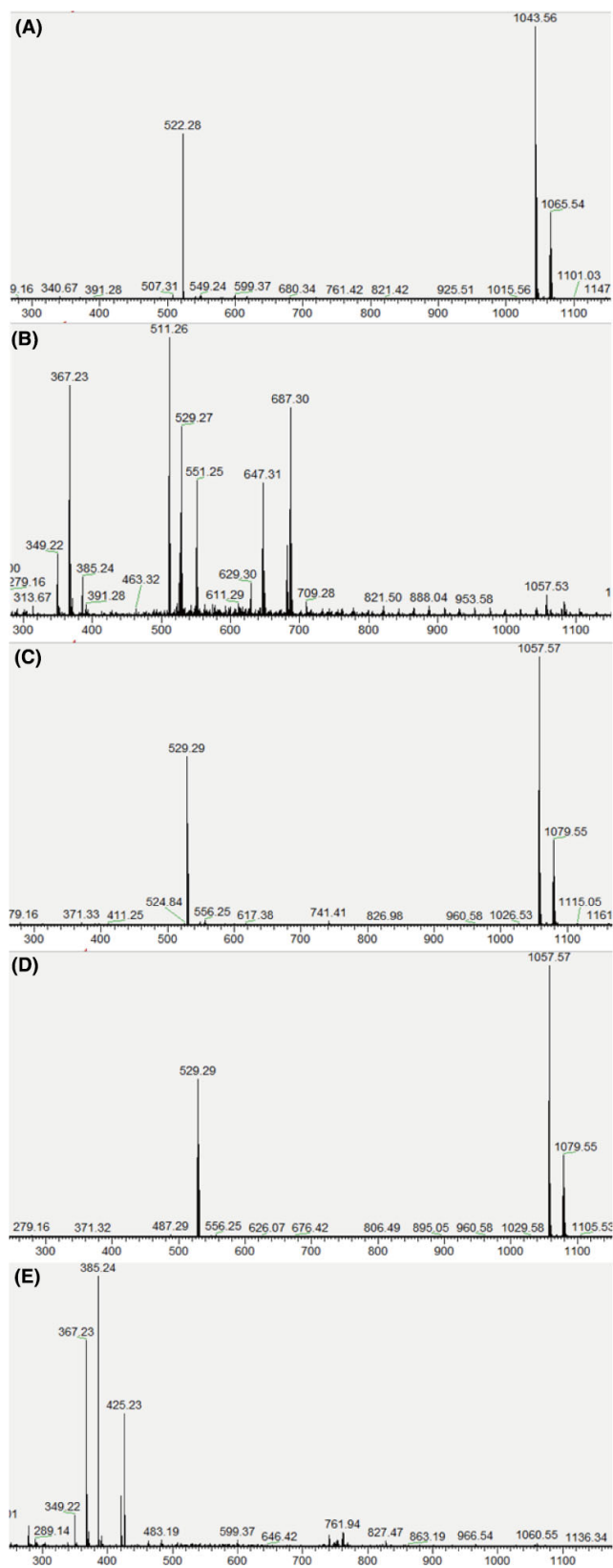


Fig. 8. MS spectrums of (A) peak 1; (B) peak 2; (C) peak 3; (D) peak 4; (E) peak 5.

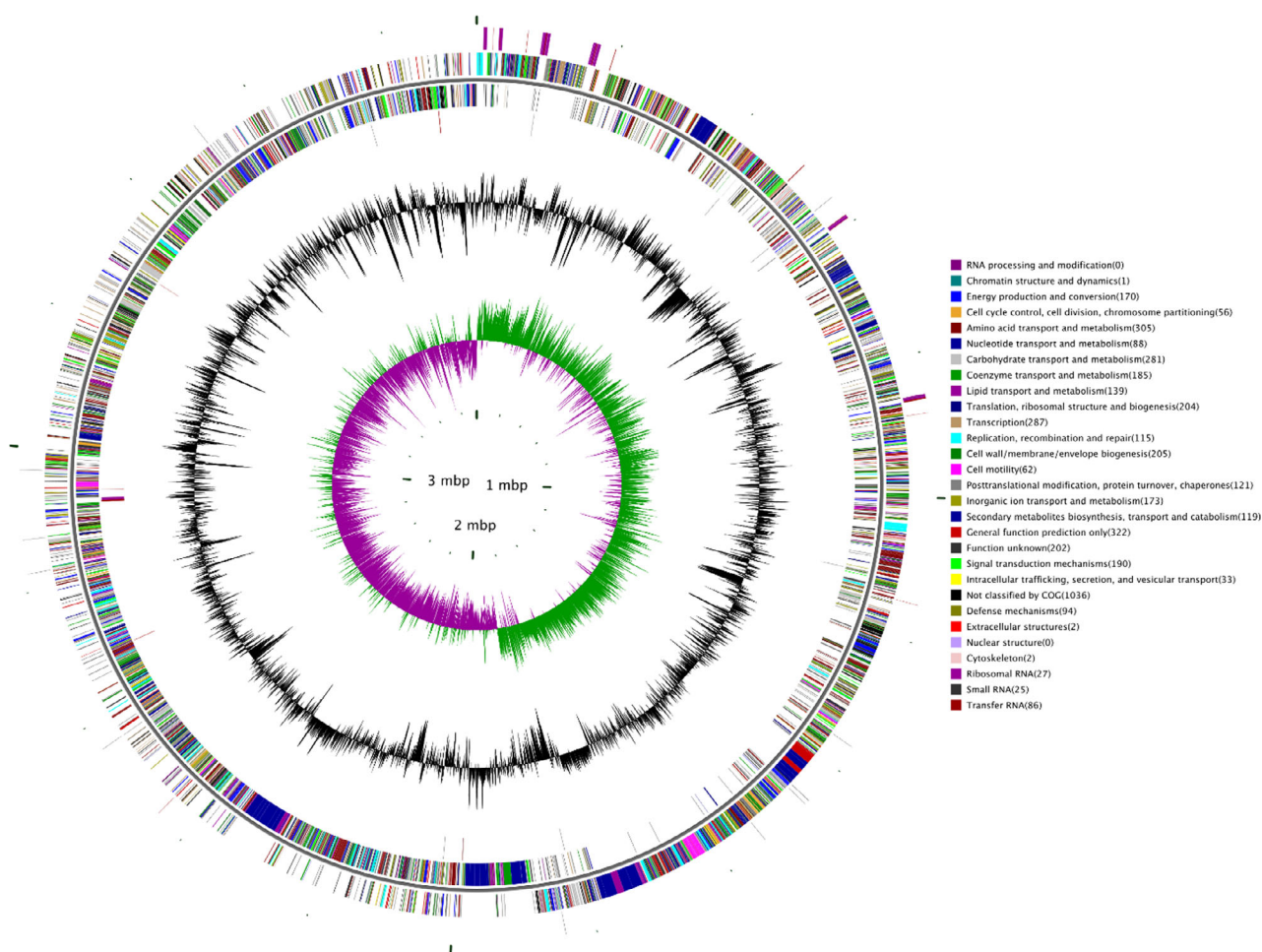


Fig. 9. Circular map of the genome of the *B. amyloliquefaciens* strain Cas02. The distribution of the circle from the outside to the inside indicates the forward tRNA, rRNA and sRNA, (second circle) forward COG, (third circle) reverse COG, (fourth circle) reverse tRNA, rRNA and sRNA, (fifth) GC ratio (black, taking the average GC as the baseline, the outward protruding means higher than the average and the inward protruding means lower than the average) and (sixth circle) GC skew (purple means less than 0, green means more than 0 respectively).

A total of 12 gene clusters related to secondary metabolites were identified. Among these, 6 clusters showed 100% similarity with reported secondary metabolite biosynthesis gene clusters (Table 1), which were related to the biosynthesis of compounds including macrolactin, bacillaene, fengycin, diffidin, bacillibactin and bacilysin.

The production and secretion of secondary metabolites is a specific characteristic of biocontrol bacteria (Rückert *et al.*, 2011). Approximately 18.92% of the *B. amyloliquefaciens* Cas02 genome encodes for genes involved in the production of secondary metabolites with antimicrobial properties. These antimicrobial compounds include bacillaene, fengycin, surfactin, bacilysin and macrolactin (Chen *et al.*, 2019a–c; Farzand *et al.*, 2019). LC-MS analysis revealed that strain Cas02 can produce macrolactin and iturin A. However, when using anti-SMASH to predict the gene clusters of secondary metabolites, we did not find the biosynthetic cluster of

iturins in the initial prediction results, which conflicted with the results of LC-MS. In-depth mining of the prediction results showed that the cluster responsible for 'fengycin' was also involved in the biosynthesis of the compound 'iturin' in *B. subtilis* and 'bacillomycin D' in *B. velezensis* FZB42, suggesting that similar genes (clusters) may have different annotations among different strains. Furthermore, sequence alignment of 'iturin' and 'macrolactin' core biosynthetic genes showed high sequence identity between strain Cas02 and strain FZB42, with sequence identities of 87.65% and 98.13% respectively (Tables S3 and S4). Therefore, Cas02 was considered to encode the genes encoding macrolactin and iturins.

Conclusion

As an important biocontrol resource, *Bacillus* spp. play an important role in the sustainable development of

Table 1. Putative gene clusters predicted in the genome of *Bacillus amyloliquefaciens* Cas02.

Type	Size (bp)	Most similar known cluster ^a		Similarity
NRPS	63 977	Surfactin	NRP: Lipopeptide	82%
PKS like	41 244	Butirosin A/butirosin B	Saccharide	7%
Terpene	17 408			
Lanthipeptide	28 888			
transAT-PKS	87 835	Macrolactin H	Polyketide	100%
transAT-PKS, T3PKS, transAT-PKS-like, NRPS	109 574	Bacillaene	Polyketide + NRP	100%
NRPS, transAT-PKS, betalactone	134 310	Fengycin	NRP	100%
Terpene	21 883			
T3PKS	41 100			
transAT-PKS-like, transAT-PKS	106 182	Difficidin	Polyketide + NRP	100%
NRPS, bacteriocin	51 791	Bacillibactin	NRP	100%
Other	41 418	Bacilysin	Other	100%

NRPS, non-ribosomal peptide synthetase cluster; PKS, polyketide synthetase.

a. Putative biosynthetic gene clusters were predicted by antiSMASH 6.0.

agriculture. *B. amyloliquefaciens* strain Cas02 isolated from the plant rhizosphere exhibited significant plant growth promotion activity and antimicrobial activity against *R. solanacearum* both *in vitro* and *in vivo*. Future work will focus on the effect of Cas02 on the plant rhizosphere microbial population, and the plant response in the simultaneous presence of strain Cas02 and the pathogen. Overall, the use of Cas02 as a biocontrol agent seems advantageous, and need to be confirmed through further studies.

Experimental procedures

Isolation and selection of bacteria

Bacterial isolates with potential biocontrol activity were isolated from the rhizosphere soils of healthy tobacco plants in Linyi, Shandong Province (E 118.64°, N 35.85°). To obtain candidate antagonistic bacteria, 1 g of the collected soil sample was weighed and placed in a test tube containing 9 ml of sterilized distilled water. The tube was then placed on a shaker at 28°C for 30 min at 180 rpm. Tenfold serial dilutions were made, and a 100 µl aliquot of the soil suspension was spread on nutrient agar (NA) plates. The plates were incubated at 28 ± 2°C for 24 h. Individual colonies formed on the NA plates were picked with a sterilized loop and transferred to fresh NA plates for further purification. The pure cultures were stored in 20% glycerol at -80°C for further study.

In vitro screening for antagonism

The antibacterial activity of the candidate strain was evaluated using a nutrient broth yeast (NBY) plate bioassay (Wang *et al.*, 2016). *Escherichia coli* FM3, *Pectobacterium carotovorum* EC101, *Burkholderia glumae* 291, *Pseudomonas syringae* B301D, *Xanthomonas campestris*

MSCT1 and *R. solanacearum* RS10 were used as indicator strains to test the antibacterial activity. The bacterial isolates were cultured in liquid NBY for 24 h, the cells were collected and then re-suspended in sterilized distilled water to an optical density at 420 nm of 0.3 (approximately 10⁸ CFU ml⁻¹). The centre of each NBY plate was inoculated with a 10 µl droplet of the bacterial suspension and incubated for 48 h at 28°C. The plates were then sprayed with a suspension of indicator bacteria (OD₄₂₀ = 0.3), and after 24 h, the inhibition zones were measured from the margins of the bacterial colonies. The plate bioassays were then performed in triplicate.

Analysis of biocatalytic activity

The biocatalytic activity of the candidate strain was characterized for cellulase activity, protease activity, β-1,3-glucanase, catalase activity, phosphate solubilization, siderophore production and ammonia production as described in a previous study (Puri *et al.*, 2020). Each experiment was performed in triplicate.

MLST analysis of strain Cas02

Genomic DNA of strain Cas02 was prepared with a Solarbio DNA extraction kit (Solarbio, Beijing, China). DNA concentration and purity were determined via Qubit Fluorometer and Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Primers for PCR amplification of the seven housekeeping genes (*glpF*, *ilvD*, *ptA*, *purH*, *pycA*, *rpoD* and *tpiA*) were described as Le (Le *et al.*, 2019). The sequences were then used for blast analysis and concatenated into one sequence for each strain using the GENEIOUS PRIME (2019.2.1). The bootstrap consensus tree was constructed using neighbour-joining method (Saitou and Nei,

1987). Evolutionary analyses were conducted in MEGA X software (Kumar *et al.*, 2018). Branch quality was assessed by the bootstrap test using 1000 replicates. Strain *B. licheniformis* ATCC 14580 was used as the outgroup.

Greenhouse experiment

Growth promotion effect of Cas02 on pepper seedlings. Pepper seedlings (*Capsicum annuum* L. cv. Chang Feng) were grown in a seedling substrate in a greenhouse at $25 \pm 3^\circ\text{C}$ and a relative humidity of 65% under a 16/8 h light/dark cycle. The culture of Cas02 ($\text{OD}_{600} = 0.3$) suspended in Hoagland solution was root irrigated with 20 ml per individual plant when the seedlings grew to two leaves; the sterilized Hoagland solution was used as a control. The seedlings were irrigated every 7 days for three times. Each treatment included three replicates of 15 plants per replicate. The leaf number, leaf size, stem height, fresh and dry weight and chlorophyll content of the pepper plants from each replicate were measured as described previously (Yuan *et al.*, 2020).

Evaluation of the biocontrol efficacy of Cas02 in a greenhouse. Pepper seedlings (Chang Feng) with four leaves were root irrigated with Cas02 three times, as described above. *Ralstonia solanacearum* cell suspensions were prepared and adjusted to a final concentration of 10^8 CFU ml^{-1} . The *R. solanacearum* culture was root irrigated with 20 ml per individual plant, 7 days after the final irrigation with Cas02. Wilt disease symptoms were recorded from the 7th day after inoculation with *R. solanacearum*. Each treatment included three replicates of 15 plants per replicate. The disease index was scored using the following scale (Hu *et al.*, 2021):

- Level 0: no diseased leaves;
- Level 1: less than one-half of the leaves withered;
- Level 3: one-half to two-thirds of the leaves withered;
- Level 5: more than two-thirds of the leaves withered;
- Level 7: all leaves withered;
- Level 9: the plant is basically dead.

Disease severity and the biocontrol efficacy of Cas02 were calculated as follows:

$$\text{Disease index} = \frac{\sum(\text{number of diseased plant} \times \text{corresponding level number})}{\text{total number of investigated plant} \times 9} \times 100$$

$$\text{Biocontrol efficacy} = \frac{\text{disease index of control group} - \text{disease index of treatment group}}{\text{disease index of control group}} \times 100\%$$

Field experiment

The field application of Cas02 as a biocontrol agent to suppress tobacco bacterial wilt caused by *R. solanacearum* was carried out in Linyi ($E 118.64^\circ$, $N 35.85^\circ$), Shandong Province, in 2020. The trial plot was being planted with tobacco for 10 years, and bacterial wilt disease had occurred significantly. The tobacco variety planted was NC102, a local conventional variety. The experiment adopted a single-factor randomized block design with two treatments, CK (normal operation) and Cas02 treatment (normal operation + Cas02). Each treatment contained 400 plants, with three replicates. The culture of Cas02 was root irrigated with 500 ml ($\text{OD}_{600} = 0.3$) per individual plant 7 days after the tobacco seedlings were transplanted to the field, and watered every 7 days for a total of three times. The control group was watered with the same amount of water. Plant height, stem circumference, number of leaves, maximum leaf length and maximum leaf area were measured at 30 days after the final treatment with Cas02, and 100 plants per plot were measured. After the onset of symptoms in the field, surveys were conducted every 3 days for 1 month.

The rhizosphere soil of plants was collected 30 days after the final treatment with Cas02 for testing the soil enzyme activity and soil physical and chemical properties. The rhizosphere soil samples of the plants were naturally air-dried and then passed through a 2 mm sieve. The available K (AK), available P (AP), NH_4^+ activity, CAT activity and alkaline phosphatase activity were determined using previously described methods (Zhang *et al.*, 2011; Zheng *et al.*, 2018).

Construction of Cas02-GFP strain

Escherichia coli JM109 was used as a host to maintain GFP-containing plasmid pIM1773-PRHIII-sfGFP-pNW33N. Strain Cas02 carrying pIM1773-PRHIII-sfGFP-pNW33N was obtained using electroporation and named Cas02-GFP (Blanchard *et al.*, 2014). The growth curve, colony morphology and antagonistic effect against *R. solanacearum* RS10 of both Cas02-GFP and original Cas02 strains were measured (Fig. S3B–D). The results indicated that the expression of green fluorescent protein did not adversely affect the characteristics of the original strain Cas02.

Root colonization

A total of 100 tobacco seedlings with 4–5 leaves (cultivar 'K326') were transplanted from the seedling tray to plastic pots (10 cm diameter × 11 cm height) containing 50 g vermiculite. The pots were incubated in a greenhouse at $25 \pm 3^\circ\text{C}$ and a relative humidity of 65% under a 16/8 h light/dark cycle. The pots were watered every 3 days. After 10 days of incubation, 20 ml of cell suspension containing freshly cultivated Cas02 cells ($\text{OD}_{600} = 0.3$) was used for root irrigation. Tobacco seedlings were randomly sampled at different times. At each sampling, five biological samples (replicates) were collected and rinsed with sterilized water. The root samples were then cut, weighed and stored in a sterile Eppendorf tube for laser confocal microscopic imaging using a Leica TCS SP8 confocal scanning microscope (Mannheim, Germany) and cell recovery counting.

Cell recovery counting: To the tube containing the pre-processed root, 1 ml of sterile water was added and vortexed for 10 min. The resulting suspension was serially diluted with distilled water. Cell suspensions of 100 μl from various dilutions (10^{-2} , 10^{-3} or 10^{-4}) were spread onto LB plates supplemented with 50 $\mu\text{g ml}^{-1}$ rifampicin. The plates were then incubated overnight at 28°C . The number of colony-forming units (cfu) per gram of roots was determined.

Purification and identification of active compounds

Purification of active compounds. Antagonistic substances were produced by fermenting the Cas02 strain in LB medium on a shaker at 37°C and 175 rpm for 48 h. The culture medium was centrifuged at 8000 rpm (4°C) for 10 min to separate the cells, and the supernatant was freeze-dried. The fermentation extract was purified using macroporous resin as described in a previous study (Yuan *et al.*, 2012). Briefly, 5 g of the extract was dissolved in 50 ml of water, which was then loaded onto a glass column (800 mm × 40 mm I.D.) wet-packed with XAD-16 macroporous adsorption resin, followed by successive elution with 600 ml of water, 40% methanol and 100% methanol. Each eluent was collected and concentrated using a rotary evaporator and dissolved in methanol at a concentration of 20 mg ml^{-1} .

Inhibitory effect of the methanol-dissolved extract on *R. solanacearum* was tested using an optimized spot-on-lawn assay. *R. solanacearum* was cultured overnight in fresh nutrient broth medium and sprayed evenly on an agar plate. After drying, a puncher was used to evenly punch three holes on the plate. Methanol-dissolved extract of 50 μL was added to each well, and methanol was used as the control. The plates were incubated at 28°C for 24 h to observe the growth of *R. solanacearum*.

Identification of active compounds by HPLC-DAD-ESI-MS/MS. HPLC was performed using an Agilent 1290 system equipped with a SunFire column (C18, 250×2.6 mm, Waters, USA). The mobile phase was 60% A [0.1% (v/v) aqueous acetic acid] and 40% B (acetonitrile) with a flow rate of 0.6 ml min^{-1} , column temperature 30°C and injection volume 10 μl .

The LC column was connected online to the standard ESI source of LTQ Orbitrap XL (Thermo Fisher Scientific) using a T-port valve. The spray voltage, capillary voltage and tube lens were 4.0 kV, 16 V and 35 V respectively. The capillary temperature was 300°C with a sheath gas flow rate of 40 l min^{-1} , and an auxiliary gas flow rate of 10 l min^{-1} . External calibration of the mass spectra routinely produced a mass accuracy of better than 3 ppm. Full mass spectra were acquired in the positive ionization mode at a resolution of 30 000 with a 100–1500 Da mass range, and followed by a data-dependent scan in the CID mode. Data acquisition and analysis were performed using XCALIBUR software version 4.1 (Thermo Fisher Scientific).

Complete genome sequencing of Cas02

The genomic DNA of strain Cas02 was extracted using a DNeasy[®] Blood & Tissue Kit from Qiagen (Hilden, Germany; Cat # 69506), according to the manufacturer's protocol. A NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) was used to ensure the DNA quality (≥ 10 μg , without degradation, $\text{OD}_{260}/\text{OD}_{280} \approx 1.8$ –2.0). Genome sequencing of Cas02 was completed using BGI (Qingdao, China) on the Oxford Nanopore sequencing platform. Assembly of the whole genome sequence, functional gene annotation and predictive analysis were performed in accordance with the standard procedures. The complete genome sequence of strain Cas02 was accessible from NCBI with GenBank accession numbers CP071932 and CP071933.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 19.0 and included analysis of variance (ANOVA) according to a random design. Duncan's test at $P < 0.05$ was applied for comparing significant differences between the measured values. Adobe Illustrator CS6 was used to draw the figures.

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Conflicts of interest

There are no conflicts of interest to declare.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Biocatalytic assay of Cas02. (A). Siderophores production; (B). Cellulase activity; (C). Protease activity; (D). Ammonia production; (E). Catalase activity.

Fig. S2. MS/MS spectrums of (A) product ion of 1057.57 of peak 3; (B) product ion of 529.29 of peak 3; (C) product ion of 1057.57 of peak 4; (B) product ion of 529.29 of peak 4.

Fig. S3. Construction of green fluorescent protein labelled Cas02-GFP strain. (A) Cas02-GFP showed luminescence

under the fluorescent microscope; (B) Growth curve of strain Cas02 and Cas02-GFP; (C) Colony morphology of strain Cas02 and Cas02-GFP; (D) Antagonism effect against *R. solanacearum* RS10 of strain Cas02 and Cas02-GFP.

Table S1. Identification of active compounds of strain CAS02 by HPLC-DAD-ESI-MS/MS.

Table S2. Chromosome statistics of *Bacillus amyloliquefaciens* CAS02.

Table S3. The core biosynthetic genes for iturin in *Bacillus amyloliquefaciens* strain FZB42 and Cas02.

Table S4. The core biosynthetic genes for macrolactin in *Bacillus amyloliquefaciens* strain FZB42 and Cas02.