The ComQXPA Quorum Sensing System May Play an Important Role in the Synthesis of Bacillomycin D in *Bacillus Amyloliquefaciens* Q-426

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Abstract

Background: *Bacillus amyloliquefaciens* Q-426 can secrete numerous cyclic lipopeptides that have antifungal and antitumor activities. ComQXPA is a common quorum sensing (QS) system in *Bacillus* species. Most *B. amyloliquefaciens* strains are encoding the QS gene cluster *comQXPA*, however, the biological function of the ComQXPA system in *B. amyloliquefaciens* has not been well studied. In this study, we identified the *comQXPA* gene locus and the chemical structure of ComX_{Q-426} in *B. amyloliquefaciens* Q-426, and explored the function of ComX_{Q-426} in regulating lipopeptide production.

Results: We identified and analyzed the *comQXPA* locus in Q-426. The full length of the *comQXPA* gene cluster was 4,014 bp, including 912 bp of *comQ*, 165 bp of *comX*, 2292 bp of *comP*, and 645 bp of *comA*. The *comQXPA* locus belongs to group B, as *comQ* and *comX* overlap by only one base pair. ComX_{Q-426} consists of six amino acids (GGDWKY) that contain a modified tryptophan residue. The antifungal activity of *Q426ΔcomX* was significantly affected, and almost no antifungal activity was observed, while the antifungal activity of strain *Q426ΔcomX/comQX* was restored to the same level as that of the wild-type strain. When the ComX_{Q-426} was added to the culture medium at a final concentration of 8 μg/L at the early stage of the log-phase, the antifungal activity of the wild-type strain Q-426 was significantly improved. Knocking out the *comX* gene did not affect the growth of the bacteria, however, the strain *Q426ΔcomX* lost its swimming ability, was unable to form colonies when spread on a solid surface, and could not form biofilms on the interface between the gas and liquid medium.

Conclusions: Disruption of the ComPA signaling pathway in the Q-426 strain resulted in significant effects on bacillomycin D production, morphology, and motility.

Background

Bacillus amyloliquefaciens, a Gram-positive and soil-dwelling bacterium, is a species in the genus *Bacillus* with high similarity to *Bacillus subtilis*. During growth, *B. amyloliquefaciens* can produce a wide range of secondary metabolites (surfactins, iturins, and fengycins) that have antagonistic effects on fungal pathogens such as *Fusarium graminearum* and *Rhizopus stolonifera* [1–3]. Therefore, *B. amyloliquefaciens* is considered a good candidate for biocontrol [4–6].

Bacteria coordinate their group behavior through quorum sensing (QS), whereby cells secrete diffusible signal molecules called autoinducers that activate a cognate receptor to control a wide array of quorum sensing responses in a density-dependent manner. In most Gram-positive bacteria, quorum responses are controlled by signaling peptides that are typically produced from oligopeptide precursors. In *B. subtilis*, quorum responses contribute to antibiotics production, the induction of competence development, sporulation, and a multitude of social (multi-cellular) behaviors, including swarming and sliding motility, exoprotease production, and biofilm formation [7–10].

The ComQXPA QS system of *B. subtilis* is a typical QS system of *Bacillus* bacteria; the system controls the expression of nearly 200 genes, including both extracellular and intracellular factors [11]. This QS system consist of an isoprenyl transferase (ComQ), an autoinducer (ComX), a histidine kinase (ComP), and a response regulator (ComA). The autoinducer ComX is initially synthesized as a 47–73 residue pro-peptide (pre-ComX) and then processed and modified by ComQ. Extracellular accumulation of the modified ComX leads to phosphorylation of ComA, and then the phosphorylated ComA directly modulates the expression of various genes, including the *srfA* operon required for surfactin biosynthesis [12–16].

Several studies have indicated that the *comQXPA* gene cluster is widespread in *Bacillus* and that it exhibits a remarkable degree of intraspecific diversity [14, 15, 17, 18]. These studies have shown that the locus encoding the ComQXPA system is highly polymorphic, especially the coding regions for *comQ*, *comX*, and the 5'-end of *comP*, which are poorly conserved. However, the conservation of the genes followed the same order: ComA > ComP > ComQ > ComX. The genomes of many bacteria contain substantial fractions of overlapping co-directional gene pairs (resulting in overlapping reading frames), and gene overlaps are known to play an important role in their evolution and biological function [19, 20]. Dogsa *et al.* performed comparative experiments with *comQXPA*-like gene arrangements in 2620 complete and 6970 draft prokaryotic genomes. Their results demonstrated that in addition to *B. subtilis* and close relatives, other bacteria species have *comQXPA*-like loci and characteristic gene-overlap patterns as in the *comQXPA* loci. Dogsa and co-workers divided the types of gene-overlaps into five groups based on the number of overlaps in the reading frames and the number of amino acids (Fig. S1). Group A exhibits no overlapping reading frames between genes of *comQ*, *comX*, *comA*, and *comP*; group B exhibits an overlapping reading frame between *comQ* and *comX*; group C exhibits a continuous overlapping reading frame between *comQ*, *comX*, and *comP*; group D exhibits an overlapping reading frame between *comP* and *comA*, and group E exhibits a continuous overlapping reading frame between *comX*, *comP*, and *comA*. The overlap types are dominated by an apparent mutation in the *comQ* stop codon that results in a 13–18 amino acid long C-terminal extension [19].

The ComX pheromone is a post-translationally modified oligopeptide. The modification is catalyzed by ComQ and is essential for the function of the pheromone. Previous studies have demonstrated that striking polymorphism occurs in the amino acid sequence of the ComX pheromone in certain *Bacillus* species, but each ComX pheromone possesses a tryptophan residue at either the 3rd or 4th residue from the C-terminus. Okada *et al.* noted that the tryptophan residue is modified with either a geranyl group or a farnesyl group at its γ -position, resulting in the formation of a tricyclic structure that includes a newly-formed proline-like five-membered ring [17, 21–28]. Thus far, the chemical structures of only seven ComX pheromones have been identified.

In previous studies, we reported that *B. amyloliquefaciens* Q-426 isolated from compost exhibits strong antifungal activity and produces various lipopeptides, including bacilomycin D, fengycin A, and fengycin B [29–31]. Whether the production of those lipopeptides is directly regulated by ComQXPA QS system and is a critical function for cell survival of *B. amyloliquefaciens* Q-426 remains unclear. Here, we

identified and analyzed the *comQXPA* locus of *B. amyloliquefaciens* Q-426. Moreover, we identified the chemical structure of ComX_{Q-426} and investigated its function in regulating lipopeptide production and its biological characteristics. We found that the production of lipopeptide-like antifungal compounds is controlled by the ComPA signaling pathway in *B. amyloliquefaciens* Q-426, and we demonstrate that a lack of production of lipopeptides affects the swarming ability and biofilm formation of Q-426.

Results

Gene overlapping in the *comQXPA* locus of *B. amyloliquefaciens* Q-426

The entire *comQXPA* gene cluster was amplified from the chromosomal DNA of *B. amyloliquefaciens* Q-426. The full length of the *comQXPA* gene cluster was 4,014 bp, including 912 bp of *comQ*, 165 bp of *comX*, 2292 bp of *comP*, and 645 bp of *comA*. The chromosomal arrangement of the *comQXPA* locus in strain Q-426 is shown schematically in Fig. 1A.

In *Bacillus*, each gene in the *comQXPA* locus is differentially conserved and overlapped. Approximately half of the *comQXPA* loci exhibit no overlapping gene pairs belonging to group A; 31.6% of the loci contain overlapping gene pairs belonging to group B and only a few loci belonging to groups C, D and E [19]. The *comQXPA* locus in strain Q-426 belongs to group B, because only one base pair overlapped between *comQ* and *comX*.

We analyzed 40 *comQXPA* sequences from completely annotated *Bacillus* genomes that had at least one lipopeptide synthesis gene cluster, for example iturins, surfactins, and fengycins. Some of the sequences were from important commercial strains (*B. velezensis* QST713, *B. velezensis* FZB42). A phylogenetic tree was constructed based on the *comQ* gene sequences, as the *comQ* gene shows high diversity in the *comQXPA* locus. The gene overlapping types are marked in different colors in the Fig. 2. As shown in Fig. 2, most of the *comQXPA* loci belong to group B, and the number of overlapping base pairs varied from 1 to 49 base pair overlaps. There was no corresponding relationship between species and overlapping type, and the relationship between the number of overlaps and the function was unclear.

Many of *B. amyloliquefaciens* strains were registered as biological control agents and are commercially available because they can produce numerous antifungal metabolites with well-established activity *in vitro* such as bacillomycin, fengycin, and surfactin [32, 33]. *B. amyloliquefaciens* is one member of the *B. subtilis* group that includes *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*. However, these species were frequently misidentified for many years due to the lack of distinguishing phenotypes, and they are poorly resolved by 16S rRNA sequencing. In recent years, the taxonomy of the *B. subtilis* species group has been updated and clarified based on their whole genome sequences. Some of the strains that were previously registered as *B. subtilis* or *B. amyloliquefaciens* are actually strains of *B. velezensis* [34, 35]. For example, some important commercial strains, *B. subtilis* QST713 and *B. amyloliquefaciens* subsp. *plantarum* FZB42, were reidentified as *B. velezensis* QST713 and *B. velezensis* FZB42 based on phylogenetic analysis [36, 37]. We also found that many strains belonging to the *B.*

subtilis group were not consistent with the current taxonomy (Table S1). In this study, it was noticed that strain Q-426 was in the same clade with *B. velezensis* GB03, *B. velezensis* YJ11-1-4 and *B. velezensis* IT-45 (Fig. 2). Furthermore, strains in the same clade with Q-426 on the phylogenetic tree reported in a previous paper [30] have been updated to *B. velezensis*. Therefore, we need to perform whole-genome sequencing of Q-426 in the future to ensure its taxonomic status.

Purification and characterization of the ComX pheromone of *B. amyloliquefaciens* Q-426

The QS pheromone, ComX, is a strain-specific signaling oligopeptide that is modified from pro-ComX by the corresponding protein ComQ. The ComX pheromone has a unique modified tryptophan (W) residue with a geranyl group or a farnesyl group at the 3-position of its indole ring, resulting in the formation of a tricyclic structure. The structural variability of ComX also is related to the sequences of the peptide backbones [14, 15, 17, 22, 38]. As shown in Fig. 3A, conservation appears to be restricted to the N-terminus of the protein, whereas high diversity in the C-terminus marks divergence within the pheromone-forming region (in red letters). To date, the chemical structures of ComX pheromones have been identified for only a few *Bacillus* strains (Fig. 3A). No chemical structures have been identified for ComX from *B. amyloliquefaciens*.

To determine the chemical structure of ComX from *B. amyloliquefaciens* Q-426, we co-expressed ComQ_{Q426} and ComX_{Q426} in *E. coli* BL21(DE3) and purified the ComX_{Q426} by gradient reverse-phase HPLC (Fig. S2). MALDI-MS spectra of the fraction collected from a retention time of 34.0 min are shown in Fig. 3B and Fig. S3. This fraction contained the characteristic compound with a molecular ion at m/z 929.2068 ($[C_{49}H_{68}N_8O_{10}]^+$) and other characteristic fragment ions that matched the chemical structure of an oligopeptide with six amino acids and the 3rd residue from the C-terminus, a Trp residue, modified with a farnesyl group (Fig. 3C).

Deletion of *comX* decreased lipopeptide production in *B. amyloliquefaciens* Q-426

To investigate the effects of the quorum sensing system on lipopeptide production, *comX* was deleted in wild-type strain Q-426, and ComQX was overexpressed in *Q426ΔcomX*, resulting in the gene deletion strain *Q426ΔcomX* and overexpression strain *Q426ΔcomX/comQX*. The antifungal activity of *Q426ΔcomX* was significantly affected by the deletion, and almost no antifungal activity was observed (Fig. 4A-a), while the antifungal activity of strain *Q426ΔcomX/comQX* was restored to the same level as that of the wild-type strain (Fig. 4A-c, 4B-c). When the purified ComX_{Q-426} pheromone was added to the culture medium at a final concentration of 8 μg/L at the early stage of the log-phase, the antifungal activity of the wild-type strain Q-426 was significantly improved (Fig. 4A-d). Also, it was observed that the antifungal activity was restored by adding extra ComX_{Q-426} (at a final concentration of 8 μg/L) during the culture of *Q426ΔcomX*. (Fig. 4A-e and 4B-e).

The previous results revealed that the Q-426 strain could produce a variety of cyclic lipopeptide compounds which were identified as C-14, C-15, C-16 bacillomycin D, C-15, C-16, C-17 fengycin A, and c-

17 fengycin B [30]. Peak 1 and peak 2 in Fig. 4B were confirmed to be C-15 bacillomycin D, and C-16 bacillomycin D, respectively. As shown in Fig. 4B, after the addition of the purified ComX_{Q-426} pheromone, the yield of lipopeptide production, especially, the yields of C-15 bacillomycin D (peak 1) and C-16 bacillomycin D (peak 2), were significantly increased (Fig. 4B-d and 4B-e), while there were no obvious peaks at the retention time corresponding to C-15 bacillomycin D or C-16 bacillomycin D in strain *Q426ΔcomX* (Fig. 4B-a). The above results showed that the lipopeptide synthesis is related to the quorum sensing system in strain Q-426. Collectively, these results suggested that ComX might positively affect lipopeptide synthesis, and deletion of *comX* contributed to the decrease of lipopeptide production.

Effects Of Comx Deficiency On Morphology And Motility

The wild-type strains were able to grow normally on all five media; the colony surface was rough and wrinkled; the edges were rough, and the color and size of the colonies changed according to the different media. For example, in FM medium, the colony color was dark yellow, and the folds were more obvious. In the MSgg and NB media, the colony color was white, the frill was lighter, and the edge was relatively neat (Fig. 5A and 5C). The mutant strain only formed colonies at the initial inoculation site on all the tested media and could not expand to the periphery. Except for growth on FM medium, the colonies were smooth with neat edges (Fig. 5B and 5D). The swarming ability of strain *Q426ΔcomX* was significantly reduced. It was difficult to expand colonies on the surface of solid medium, because the biosynthesis of lipopeptides was downregulated. As shown in Fig. 6A, when cells were grown in FM medium with a reduced agar concentration, the whole plate (bottom, wild-type strain) was quickly covered with colonies. The mutant could not move within the agar plates at any concentration of agar, and there was no significant change in the colony size. We also investigated the effects of the *comX* gene deletion on biofilm formation. As shown in Fig. 6B, in both of FM and LB media, the mutant could not form biofilms, and the bacterial cells were deposited at the bottom of the wells. Figure 7 shows the growth curves of the wild-type strain and mutant strains. As shown from the growth curve, the lag-phase of the mutant was approximately 0–3 h and then the cell soon entered the log-phase, while the lag-phase of the wild-type strain was 0–5 h. However, the wild-type strain had a longer log-phase than the mutant, and the final cell density of the wild-type strain was higher than that of the mutant. Therefore, the deletion of *comX* had a certain effect on the growth, but the effect was not significant.

Discussions

In this study, we identified and characterized the *comQXPA* gene locus of *B. amyloliquefaciens* Q-426 and found that the stop codon of comQ and the start codon of comX overlapped by one base pair. Furthermore, we demonstrated that most of the *comQXPA* loci from the *Bacillus* genus belong to group B in the variations of gene overlap. Disruption of the ComPA signaling pathway in the Q-426 strain resulted in significant effects on lipopeptide production, morphology, and motility.

Recent studies have demonstrated that the ComQXPA QS system not only exists in *Bacillus*-related species but also in other Gram-positive species such as *Lysinibacillus*, *Geobacillus*, and *Anoxybacillus*

[19]. In *Bacillus*, the ComQXPA QS system exhibits striking polymorphism and is coupled to the bacterial phenotypes, which are defined as groups of bacteria that are able to communicate through comX pheromones [14, 15, 17, 38, 39]. The evolutionary history of the *comQXPA* locus and the interrelation between the structural characteristics of the *comQXPA* locus and its ecological functions are still unclear. In this study, we analyzed 40 *comQXPA* sequences from completely annotated *Bacillus* genomes. Most of the *comQXPA* loci from the *Bacillus* genus belong to group B, in which the *comQ* and *comX* genes overlap, and the number of overlapping base pairs varied from 1 to 49 base pairs. From these results, although we cannot see any corresponding relation between species with overlapping type, the number of overlaps was the same within the strains located in the same clade. This suggests that overlapping reading frames in the *comQXPA* locus may result in more efficient or individual transcriptional control and may be associated with the evolution of specific biological functions.

Bacillomycin D, one member of the iturinic lipopeptide family, has strong antifungal activity, especially against filamentous fungi. Iturinic lipopeptides are mainly produced by members of the *B. subtilis* group through non-ribosomal synthetases machinery, and they exhibit strong diversity within the *B. subtilis* species group; some lipopeptides are only produced by one species, whereas certain others can be produced by up to three species [40]. The chemical principles for the biosynthesis of iturinic lipopeptide are also largely characterized [41, 42], but little is known about the regulatory mechanisms that control the expression of the iturinic lipopeptide. To date, the biosynthesis mechanisms and expression regulation mechanisms of lipopeptides except surfactin have not been extensively studied. Surfactin biosynthesis is regulated by the ComQXPA QS system in *B. subtilis*. Upon reaching a threshold concentration, ComX activates ComP autophosphorylation, and then ComP~P transfers the phosphoryl group to ComA [25], which controls the expression of *srfA* encoding a very large protein complex for surfactin production that contributes to swarming motility and biofilm formation [13, 35, 38, 43–48]. Spacapan *et al.* also found that exoproteases required for biofilm formation are regulated by the ComQXPA system through ComX induction in *B. subtilis* [9]. In *B. amyloliquefaciens* FZB42, it has been shown that bacillomycin D is under direct transcriptional control of DegU, and DegQ and ComA also are required for the full transcriptional activation [49].

In our previous studies, we demonstrated that *B. amyloliquefaciens* Q-426 can produce seven lipopeptides (C-14, C-15, and C-16 bacillomycin D; C-15, C-16, and C-17 fengycin A; C-17 fengycin B), among which the levels of C-15 and C-16 bacillomycin D were higher than others [29, 30, 50]. To investigate whether the ComQXPA QS system is involved in regulating the lipopeptide synthesis in *B. amyloliquefaciens* Q-426, we disrupted the ComQXPA QS system through constructing the *Q426ΔcomX* mutant. Our results revealed that the ComQXPA QS system might be involved in regulating bacillomycin D production in *B. amyloliquefaciens* Q-426. Interestingly, knocking out the *comX* gene does not affect the growth of the bacteria, but the growth capacity of the *Q426ΔcomX* mutant in liquid medium was slightly lower than that of the wild-type strain. However, the *Q426ΔcomX* lost its swimming ability, was unable to form colonies when spread on a solid surface, and could not form biofilms on the interface between the gas and liquid medium. Unfortunately, we did not confirm the direct effects of the ComQXPA QS system or ComA on lipopeptide synthesis, and this is our immediate goal. It is widely known that

lipopeptides exhibit strong antifungal activities, but their other physiological roles in *Bacillus* cells are still unclear. We speculate that the ComQXPA QS system of strain Q-426 modulates some physiological and biochemical properties by regulating the biosynthesis of lipopeptides. These lipopeptide compounds themselves may also be signal molecules, and each lipopeptide has its own specific biological regulatory circuit. In a recent study, Xu and co-researchers pointed out that bacillomycin D is a signal that promotes biofilm development of *B. velezensis* SQR9 [51].

B. amyloliquefaciens Q-426 produces an oligopeptide pheromone, ComX_{Q-426}, that inducts its QS system. ComX_{Q-426} consists of six amino acids that contain a modified tryptophan residue located at the third position from the C-terminus, resulting in the formation of a tricyclic scaffold with a pyrrolidine ring (Fig. 3C). ComX pheromones from different *Bacillus* strains exhibit unique amino acid sequences and residues, indicating that each bacterium has its own oligopeptide language for activating QS signaling. The linear oligopeptide (GGDWKY) without modification of the tryptophan residue with farnesyl did not activate QS signaling in the *Q426ΔcomX* strain to produce lipopeptides such as bacillomycin D (Fig. S4). This result was in close agreement with other research in that the tricyclic scaffold of the tryptophan residues in the ComX pheromone is critical to activating kinase activity of the receptor protein ComP, but there is no effect on the recognition of the receptor with pheromone molecules [17, 21, 23–25]. Further research should clarify how the sequence of amino acids of ComX_{Q-426} contributes to the specific recognition of ComX_{Q-426} by the receptor protein ComQ_{Q-426}.

For *Bacillus* strains, it is now estimated that at least 5%-10% of the genome is devoted to the production of antimicrobial compounds [45, 52]. These compounds are mainly antimicrobial cyclic peptides containing D-amino acids and hydrophobic residues. At present, studies of lipopeptides mainly focus on their physicochemical properties and biological activities, but further studies of their biological functions and the regulatory mechanisms of their biosynthesis are needed. The understanding of such biological functions and the molecular regulatory mechanism of biosynthesis will provide a theoretical basis for constructing a high-yielding lipopeptide-producing strain. This will help us to better understand the molecular mechanisms of secondary metabolism in *Bacillus* and its environmental adaptations.

Conclusion

In this study, we identified and characterized the *comQXPA* gene locus of *B. amyloliquefaciens* Q-426 and analysed 40 *comQXPA* sequences from completely annotated *Bacillus* genomes. Most of the *comQXPA* loci from the *Bacillus* genus belong to group B, in which the *comQ* and *comX* genes overlap and the number of overlapping base pairs varied from 1 to 49 base pair overlaps. We identified the chemical structure of ComX_{Q-426} that consists of six amino acids that contain a modified tryptophan residue located at the third position from the C-terminus, resulting in the formation of a tricyclic scaffold with a pyrrolidine ring. We found that strain *Q426ΔcomX* exhibited significantly reduced antifungal ability, almost no antifungal activity was observed, while the antifungal activity of strain *Q426/comX* could be restored to the same level as that of the wild-type strain. Moreover, the antifungal activity of *B.*

amyloliquefaciens Q-426 was significantly improved by adding the ComX_{Q-426} pheromone to the culture medium. Interestingly, knocking out the *comX* gene did not affect bacterial growth. However, the Q-426 $\Delta comX$ mutant lost swimming ability, was unable to form colonies when spread in on solid surface, and could not form biofilms on the interface between the gas and liquid medium. These funding suggest that the production of lipopeptides is controlled by the ComPA signalling pathway and relate to the swarming ability and biofilm formation in *B. amyloliquefaciens* Q-426.

Methods

Bacterial strains, plasmids, media and culture conditions

B. amyloliquefaciens Q-426 (CCTCC M2010237) was isolated from compost samples in Dalian, China. *Escherichia coli* DH5 α and *E. coli* BL 21(DE3) were used in genetic experiments. Luria-Bertani (LB) medium comprised (g/L) peptone (10.0), yeast extract (5.0) and NaCl (5.0). FM medium comprised (g/L) tryptone (12.4), glucose (20), NaCl (5.0), K₂HPO₄ (1.5), MnSO₄·2H₂O (0.04), FeSO₄·7H₂O (1.7), and MgCl₂·6H₂O (1.2), pH 7.0. NB medium comprised (g/L) tryptone (10), beef powder (3.0), and NaCl (5.0), pH 7.2. MSgg medium comprised 5 mM potassium phosphate (pH 7.0), 100 mM morpholinepropane sulfonic acid (pH 7.0), 2 mM MgCl₂, 700 μ M CaCl₂, 50 μ M MnCl₂, 50 μ M FeCl₃, 1 μ M ZnCl₂, 2 μ M thiamine, 0.5% glycerol, 0.5% glutamate, tryptophan (50 μ g/mL), and phenylalanine (50 μ g/mL). BGM medium comprised LB medium plus 0.15 M ammonium sulfate, 100 mM potassium phosphate (pH 7.0), 34 mM sodium citrate, 1 mM MgSO₄, and 0.1% glucose. M9 medium comprised (g/L) glucose (5.0), yeast extract (4.0), NH₄NO₃ (1.0), NaCl (0.5), K₂HPO₄ (1.5), KH₂PO₄ (0.5), and MgSO₄ (0.2). Solid medium was obtained by adding 15 g/L agar to the liquid medium. All strains were grown at 37 °C. Unless otherwise indicated, the final concentrations of antibiotics were as follows (mg/L): ampicillin (Amp), 50 and kanamycin (Kan), 30.

Dna Manipulation Techniques

Oligonucleotide synthesis and DNA sequencing were performed by Sangon Biotech Co., Ltd. (Shanghai, China). The isolation and manipulation of recombinant DNA were carried out as described previously [53]. All enzymes were commercial preparations. Phusion DNA high-fidelity polymerase was purchased from NEB (Shanghai, China). *B. amyloliquefaciens* transformations were performed by artificially inducing genetic competence [54].

Cloning and sequence analysis of QS-related genes (*comQXPA*)

Genomic DNA of strain Q-426 was isolated from the bacterial culture using the Bacterial DNA Isolation Kit (Foregen, Beijing, China). To amplify the whole gene of the *comQXPA* locus of *B. amyloliquefaciens* Q-426, the upstream and downstream primers (*com_F* and *com_R*; the location is indicated in Fig. 1A in red) were designed from the *degQ* and *luxR* gene sequences, which are located upstream of *comQ* and

downstream of *comA*, respectively. PCR amplification was employed in a 50 μ L final volume using the following program: at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min; final extension at 72 °C for 10 min. PCR products were detected by 1% w/v agarose gel electrophoresis at 100 V for 30 min. The nucleotide sequences of amplified PCR fragments were determined by Sangon Biotech Co., Ltd. using an ABI DNA Sequencer (3730XL, USA) and then submitted to NCBI GenBank for BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST>, GenBank accession no. MF579444).

The nucleotide sequence of the amplified PCR fragment was then compared with the corresponding gene clusters from strains with homologous genes from other *Bacilli*, which were obtained from the NCBI nucleotide/protein database. Alignments for the phylogenetic tree were made using ClustalX-1.81 software. Phylogenetic analyses were conducted using MEGA version 7.0.20 for Neighbor-Joining and ME analyses using the Kimura 2-parameter model. Measures of bootstrap support for internal branches were obtained from 1000 pseudoreplicates.

Deletion of the *comX* gene in *B. amyloliquefaciens* strain Q-426

The left flanking (LF) region (85 bp), kan region (813 bp), and right flanking (RF) region (80 bp) were amplified from the genomic DNA of Q-426 and pET-28-(a+) using the primer pairs of *comX*_F/*comX*_R, *kan*_F/*kan*_R and *comX* Π _F/*comX* Π _R (Table 1, Fig. 1B), respectively. These three fragments were fused using overlap PCR in the order of LF, KAN, and RF. The resulting 1.0-kb *comX* deletion amplicon (1 μ g) was directly transformed (25 μ F, 600 Ω) into strain Q-426, and 53 colonies were obtained from LB plates containing Kanamycin (30 μ g·mL⁻¹). Three colonies confirmed by PCR were cultivated to an OD₆₀₀ of 1.0 without Kan, and a 100- μ L aliquot of a 10-fold dilution of the cultures (approximately 10⁵ cells) was plated on LB-Kan medium. Mutants growing on LB-Kan were further confirmed by PCR and DNA sequencing. Mutants selected were retreated on LB-Kan plates for 20 times to confirm their stability.

Table 1
Plasmids and strains used in this study

Plasmids and strains	Descriptions	Origin
Q-426	<i>B. amyloliquefaciens</i> wild-type strain	Lab stock
<i>Escherichia coli</i> DH5α	F ⁻ Φ80lacZΔM15Δ(lacZYA-arg ^F) U169 <i>recA1 endA1 hsdR17</i> (rK ⁻ , mK ⁺) <i>phoA supE44 λ- thi-1 gyrA96 relA1</i>	Sangon Biotech, Shanghai, China
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Lab stock
<i>Q426ΔcomX</i>	Q-426 derivate, Δ <i>comX</i>	This study
pET-28a (+)	N-terminal His•Tag®/thrombin/T7•Tag®, optional C-terminal His•Tag	Lab stock
pET-28a (+)-Kan-ComX _{Q426}	pET-28a (+) containing the gene <i>comX</i>	This study
pET-28a (+)-Amp-ComQ _{Q426}	pET-28a (+) containing the gene <i>comQ</i> , the Kan-resistant gene replaced to Amp-resistant gene	This study
pHY300PLK	<i>E. coli</i> - <i>B. amyloliquefaciens</i> shuttle vector, Apr(<i>E. coli</i>), Tcr (<i>E. coli</i> and <i>B. amyloliquefaciens</i>)	BioVector NTCC Inc., China
pHY- <i>comQX</i>	pHY300PLK containing the gene <i>comQX</i>	This study

The ComQX expression vector was constructed according to a previously reported method [55]. Briefly, the gene *comQX* was amplified by the corresponding primers (ComQX_F/R, Table 2). Then, the fragment was inserted into pHY300PLK, a shuttle vector for *E. coli* and *B. subtilis* (BioVector NTCC Inc., China), at the restriction enzyme sites BamHI/XbaI, resulting in the *comQX* expression vector named as pHY-*comQX*. The vectors pHY-*comQX* were first electroporated into *E. coli* DH 5α to confirm and prepare an efficient expression vector, and then electroporated into *Q426ΔcomX* competent cells. The recombinant strains were selected by tetracycline-resistance (25 μg·mL⁻¹) and named *Q-426ΔcomX /ComX*.

Table 2
The primers used in this study

Primers names	Sequence 5'→3'
com_F	GTTATCTGACTTGGCGATCAGC
com_R	GAGAAACAACCGACTCATTACG
comX_F	GGATCCATGCAGGAAATTGTAGGATACTTAACC
comXI_R	ATGGCTCATCTAACTCCGATCAGACTAGC
kan_F	CGGAGTTAGATGAGCCATATTCAACGGGAAAC
kan_R	GATCCTGGCTGCCGATTTTCGGCCTATTGG
comXII_F	AATCGGCAGCCAGGATCAAACAGATTGTG
comXII_R	GTCGACTCAATATTTCCAATCACCGCCGAAAG
RF-ComX_F	CATCATCATCATCATCACAGCAGCATGCAGGAAATTGTAGGATACTTAACC
RF-comX_R	GGCTTTGTTAGCAGCCGGATCTCAGTTAATATTTCCAATCACCGCCGAAAG
ComQ_F	<i>CGGGATCC</i> ATGTGTTTAGATGCCGAAACG
ComQ_R	<i>CGGTTCGACGCTAACTCCGATCAGACTAGC</i>
ComQX_F	<i>CGCGGATCC</i> ATGTGTTTAGATGCCGAAACG
ComQX_R	<i>CCGGAATTCTCAATATTTCCAATCACCGC</i>

Colony Morphology And Growth Curve Of Strain Q426Δcomx

Wild-type *B. amyloliquefaciens* Q-426 or the *Q426ΔcomX* mutants were grown overnight on LB agar plates, resuspended in PBS ($OD_{600} = 0.2$) and sonicated for 12 s with 1-s on/off pulses. Two microliters of each culture were then spotted onto the center of LB, NB, FM, BGM and MSgg agar plates and incubated at 30°C for 48 h.

For growth curve determination, a fresh single colony of each tested strain was inoculated into 200 mL FM liquid medium in a 1-L flask with 1% inoculation and incubated at 30°C and 180 rpm. A certain amount of liquid culture was taken from the flask, and the OD_{600} was measured every hour on the first day and then measured every 4 or 8 h after reaching a stable period. The average value was measured three times for each group of data.

Overexpression Of Comq And/or Comx

The recombinant plasmids pET-28a (+)-Kan-ComX and pET-28a (+)-Amp-ComQ were separately or simultaneously transformed into *E. coli* BL21(DE3) to express ComX, ComQ and ComX/ComQ, a protein with an His-tag at its N-terminal (Fig. S2). *E. coli* BL21(DE3) cells harboring pET-28a(+)-Kan-ComX or pET-28a(+)-Amp-ComQ or both pET-28a(+)-Kan-ComX and pET-28a(+)-Amp-ComQ were grown on LB medium (containing the indicated antibiotics) at 37 °C and 180 rpm until the OD₆₀₀ reached approximately 0.7, and gene expression was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. The expression of ComQ and ComX were confirmed by SDS-PAGE.

Western Blotting

Proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) by electroblotting for Western blot analysis. The mouse anti-His-tag antibody (Sangon Biotech Co., Ltd.) was used as the primary antibody at a 1:2000 dilution in blocking solution (2% no protein blocking solution, Sangon Biotech Co., Ltd.). The rabbit-anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Sangon Biotech Co., Ltd.) was used at a dilution of 1:3000. Protein bands were detected on photographic films using an enhanced chemiluminescent substrate. The EasySee® Western Marker (TRAN Co., Beijing, China) was used for calculating molecular weights of proteins after western blotting.

Pheromone Overproduction And Identification

The *E. coli* ComX-producing strain that co-expressed comX and comQ was grown overnight in completed M9 medium supplemented with a mixture of amino acids (Phe, Ser, His, Met, at 40 ug/mL; and Gln at 0.4 mg/mL), kanamycin (30 µg/mL), and ampicillin (50 µg/mL). At the stationary phase, 20 mL of the pre-culture was added to 1980 mL of the supplemented M9 medium to make a 2-L bacterial culture, which was then incubated at 37 °C and 110 rpm for 8 h. The expression of the *comQX* gene was induced with 0.1 mM IPTG at 37 °C and 110 rpm overnight. The culture broth was centrifuged for 10 min at 8000 × *g*. The supernatant was filtered through a 0.22 µm filter, and CH₃CN was added to a final concentration of 20%. The filtrate was loaded onto a column packed with HZ resin (Huazhen Tech, Shanghai, China) that was equilibrated with 10% CH₃CN and 0.1 TFA. The column was eluted with a step gradient of CH₃CN (20, 40, 50, 65, and 80% in 0.1% aqueous TFA). The 50% elute containing ComX was immediately neutralized with aqueous ammonia, concentrated with a rotary evaporator, and dried with a freeze dryer. The dried extract was analyzed through HPLC (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) equipped with a C18 column (Agilent Technologies, 5 µm × 4.6 × 150 mm) to detect the ComX pheromone. MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) was used to determine the mass and the amino acid sequences of the pheromone peptide present in the samples collected from HPLC.

Effects of the ComX pheromone and ComX gene deletion on lipopeptide production

The wild-type, *Q426ΔcomX*, and *Q426ΔcomX /ComX* strains were streaked on LB agar plates and then inoculated into 100 mL of FM liquid culture medium. *Q426ΔcomX* and *Q426ΔcomX /ComX* strains were transferred to the FM liquid culture medium containing corresponding antibiotics in a 1% inoculation volume. The fermentation process was conducted at 180 rpm for 72–76 h at 30°C. Lipopeptide productivity and antifungal activity assays were evaluated according to a previous study [31].

Declarations

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Authors' contributions

CQ, SF, WZ, and PZ designed the experiments. BG, JL, WZ, XS, and JL performed the experiments with assistance from RF. CQ, PZ, and LZ wrote the manuscript.

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Availability of data and materials

Sequence data is available in GenBank (GenBank accession no. MF579444). All datasets generated for this study are included in the article/Supplementary Material.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Figure 1

The *comQXPA* locus in *B. amyloliquefaciens* Q-426 and Q426Δ*comX*. (A) Gene diagram of *comQXPA* in wild-type strain. The location of primers (*com_F* and *com_R*) are indicated in red (B) Schematic overview of the *comX* gene disruption strategy. The primer pairs of *comX_F/comXI_R*, *kan_F/kan_R*, and *comXII_F/comXII_R* are labeled in different colors.

Figure 2

Neighbor-joining phylogenetic tree constructed from comQ gene. Different background color indicates different group. Green, Group A; Red, Group B, gradation of color shows variation of overlapping number of base pairs; Yellow, Group D; Pink, Group E.

Figure 3

Identification of ComX pheromone of *B. amyloliquefaciens* Q-426. (A) Comparison of amino acid sequences and the modified residue of ComX pheromones. Except for the Q-426 the pre-ComX sequences are originated from previous studies.^{17,24} The modified tryptophan residues (asterisk) are marked with blue box. The amino acid sequence of the mature ComX is shown in red. There are two types of posttranslational isoprenylations of ComX pheromones and the isoprenyl side chains are shown in purple box. (B) MALDI-MS spectra of ComXQ426 component obtained by HPLC. (C) The chemical structure of ComXQ426. The chemical bond breaking points are shown in red.

Figure 4

Effects of QS system on lipopeptide production in *B. amyloliquefaciens* Q-426. (A) Inhibitory activities of 100 µl concentrated CFS (cell-free supernatant) on PDA medium against *Curvularia lunata* (Walk) Boed. All strains were cultivated in FM liquid medium for 72 h at 30 °C and then the fermentation broth was condensed to 1/10 before detecting the antifungal activity. (B) HPLC detection of cyclic lipopeptide from fermentation broth in different strains. a, Q426ΔcomX; b, wild-type strain; c, Q426ΔcomX/comQXΔd, wild-

type strain + ComXQ-426. e, Q426 Δ comX+ ComXQ-426. Peak 1 and peak 2 in all samples were detected as C-15 bacillomycin D and C-16 bacillomycin D by HPLC-MS.

Figure 5

Colony morphology of Q426 Δ comX and its wild-type strain on different solid-media. (A) and (C), wild-type strain. (B) and (D), Q426 Δ comX. (C) and (D), Enlargement of colony image. Strains were grown in different solid media for 72 h at 30 °C

Figure 6

Biofilm formation and swarming motility of Q426 Δ comX. (A) Swarming motility. Strains were incubated on FM solid media with different concentration of agar for 72 h at 30 °C. (B) Biofilm formation ability. Strains were stationarity incubated in FM and LB liquid media for 72 h at 30 °C. Top, Q426 Δ comX; Bottom, wild-type strain.

Figure 7

Growth curve of mutant and wild strains. Solid cycle, wild-type strain; Squares, Q426 Δ comX

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