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Genomic analysis and secondary metabolite production in *Bacillus amyloliquefaciens* AS 43.3: A biocontrol antagonist of Fusarium head blight

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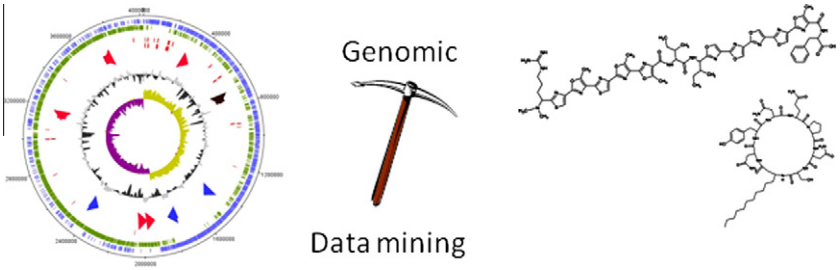
HIGHLIGHTS

- The genome of biocontrol antagonist *Bacillus amyloliquefaciens* AS 43.3 was sequenced.
- Data mining identified nine secondary metabolite gene clusters.
- Chromatography and tandem mass spectroscopy confirmed metabolites in culture.

GRAPHICAL ABSTRACT



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ABSTRACT

The complete genome of the biocontrol antagonist *Bacillus amyloliquefaciens* AS 43.3 is reported. *B. amyloliquefaciens* AS 43.3 has previously been shown to be effective in reducing Fusarium head blight in wheat. The 3.9 Mbp genome was sequenced, assembled, and annotated. Genomic analysis of the strain identified 9 biosynthetic gene clusters encoding secondary metabolites associated with biocontrol activity. The analysis identified five non-ribosomal peptide synthetase clusters encoding three lipopeptides (surfactin, iturin, and fengycin), a siderophore (bacillibactin), and the antibiotic dipeptide bacilysin. In addition, three polyketide synthetase clusters were identified which encoded for the antibacterials: bacillaene, difficidin, and macrolactin. In addition to the non-ribosomal mediated biosynthetic clusters discovered, we identified a ribosomally encoded biosynthetic cluster that produces the antibiotic plantazolicin. To confirm the gene clusters were functional, cell-free culture supernatant was analyzed using LC–MS/MS. The technique confirmed the presence of all nine metabolites or their derivatives. The study suggests the strain is most likely a member of the *B. amyloliquefaciens* subsp. *plantarium* clade. Comparative genomics of eight completed genomes of *B. amyloliquefaciens* identify the core and pan-genomes for the species, including identifying genes unique to the biocontrol strains. This study demonstrates the growing importance of applying genomic-based studies to biocontrol organisms of plant pathogens which can enable the rapid identification of bioactive metabolites produced by a prospective biological control organism. In addition, this work provides a foundation for a mechanistic understanding of the *B. amyloliquefaciens* AS 43.3/Fusarium head blight biocontrol interaction.

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1. Introduction

The desire to reduce the use of potential harmful chemical fungicides to control plant diseases has grown substantially over the past few decades. This desire, in turn, prompted the growth of research on the use of microbial agents to control plant diseases. Recently, the availability of next-generation sequencing has also spurred the growth of research into microbial biocontrol strains by greatly expanding the number of publicly available completely sequenced genomes. In turn, the availability of completely sequenced genomes of microbial biocontrol strains has started to

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revolutionize the way biocontrol strains are characterized, resulting in better insight into how they interact in biocontrol pathosystems. For instance, the genomes of some important *Bacillus amyloliquefaciens* biocontrol strains have recently been sequenced (Blom et al., 2012; Chen et al., 2007; Hao et al., 2012) and this information provides a means for assessing the genomic determinants of the biocontrol activities of these *B. amyloliquefaciens* strains. All the completed and annotated genomes available in Genbank for *B. amyloliquefaciens* were from strains isolated from the soil or the rhizosphere of plants, while strains isolated from the phyllosphere are not represented. Biocontrol applications of *B. amyloliquefaciens* on phyllosphere targets are promising and represent a unique ecological niche (Pérez-García et al., 2011).

Bacillus sp. AS 43.3 is an antagonist of *Gibberella zeae* (anamorph: *Fusarium graminearum*), the primary causal agent of Fusarium head blight (FHB) in the United States (Khan et al., 2001). This strain was originally isolated from a wheat head and was subsequently shown to be effective in reducing FHB incidence and severity in wheat (Khan et al., 2001, 2004; Schisler et al., 2002a,b).

The goal of the current study was to sequence, assemble, and annotate the genome of *Bacillus* sp. AS 43.3 and subsequently determine its potential to produce secondary metabolites that impart desirable biocontrol activities (e.g., the production of anti-fungal compounds). Once putative biosynthetic gene clusters of secondary metabolites were identified, liquid chromatography and tandem mass spectroscopy (LC–MS/MS) was used to confirm gene functionality and product production in liquid culture. A secondary goal was to compare the genomes of completed *B. amyloliquefaciens* strains to determine the core-genome and pan-genome of this group of strains. In addition, our goal was to compare the *B. amyloliquefaciens* strains with biocontrol applications to those that are used in industrial applications to determine the genetic features specific to biocontrol strains.

2. Material and methods

2.1. Strain culturing

Bacillus sp. strain AS 43.3 was obtained from the ARS Culture Collection (http://nrrl.ncaur.usda.gov) as NRRL B-30210. The strain was subsequently produced in a semi-defined liquid culture medium (SDLC) (Slininger et al., 2007) for all assays. Samples of bacterial cultures frozen at −80 °C in 10% glycerol were streaked for purity onto one-fifth strength tryptic soy broth agar, pH 6.8 (Difco Laboratories, Detroit, MI). After 24 h incubation at 28 °C, cells were removed from the surface of colonized plates using sterile cotton swabs and utilized to initiate liquid starter cultures of the strain. Ten milliliters of SDLC in 50 ml Erlenmeyer flasks were inoculated to an optical density of approximately 0.2 at 620 nm wavelength light (A_{620}) for liquid starter cultures. The starter cultures were then incubated in a shaker incubator (Inova 4230, New Brunswick Scientific, Edison, NJ) at 25 °C with a throw of 2.5 cm and 250 rpm for 24 h. Afterwards, the starter cultures were used to inoculate liquid test cultures composed of 50 ml of SDLC in 250 ml Erlenmeyer flasks to an optical density of 0.1(A_{620}). Test cultures were incubated as described for liquid starter cultures and harvested at 12, 24, and 48 h of culture growth as needed for conducting analyses and bioassays.

2.2. Genome sequencing

Bacillus sp. strain AS 43.3 was cultured as described above to early stationary phase (~24 h) and harvested by centrifugation. DNA extraction was performed on the pelleted bacterial biomass using the Epicentre Masterpure DNA Purification kit (Illumina Inc, Madison, WI). The total genomic DNA extraction was subsequently fragmented to 200 bp and size-selected using an E-gel apparatus (Life Technologies Inc, Grand Island, NY). Sequencing adapters were ligated using an Ion Express™ Plus Fragment Library kit (Life Technologies Inc, Grand Island, NY). Emulsion PCR to incorporate the DNA fragment library to the sequencing beads was performed using the Ion OneTouch™ instrument with an Ion OneTouch™ System Template kit (Life Technologies Inc, Grand Island, NY). The library sample was finally sequenced on an Ion Torrent Personal Genome Machine using an Ion 316 chip and the Ion PGM™ 200 sequencing kit (Life Technologies Inc, Grand Island, NY) following the manufacturer's suggested protocols. The resulting reads were quality trimmed to the Q20 confidence level. The genome was assembled by SeqMan NGen® (DNAstar Inc, Madison, WI) using default parameters. The gaps were closed using primer walking with standard PCR. The sequence was deposited in NCBI Genbank under accession number CP003838.

2.3. Culture production and sample preparation of secondary metabolites

Bacillus sp. strain AS 43.3 was cultured as described above. The culture supernatant was collected during the exponential growth phase (12 h), early stationary phase (24 h) and late stationary phase (48 h). The cells were removed by centrifugation, and the cell-free supernatant was partitioned into fractions using a C_{18} syringe cartridge (Maxi-clean SPE 300 mg C_{18}, Grace Inc, Deerfield, IL). Four milliliters of cell-free supernatant was loaded onto the cartridge and washed with 5 mL of water, then five fractions were eluted using 1 mL of 20%, 40%, 60%, 80%, and 100% methanol.

2.4. Reverse-phase liquid chromatography tandem mass spectrometry (RP-LC–MS/MS)

The C_{18} fractionated supernatant mixtures (20% MeOH; 40% MeOH; 60% MeOH; 80% MeOH; and 100% MeOH) from three phases of growth were analyzed by RP-LC–MS/MS (20 µl injections; Agilent 1100 HPLC, equipped with a PDA monitoring at λ 280 nm) through a C_{18} column (3.0 mm × 15 cm, 3 µm particle size; Inertsil, GL Sciences, Inc., Torrance, CA) running a gradient elution of 95% A:5% B (buffer A 0.1% formic acid, buffer B 100% acetonitrile) to 5% A:95% B over 60 min at a flow rate of 250 µl/min, followed by a 5 min B washout and 15 min re-equilibration, while maintaining a constant column temperature of 30 °C. Electrospray positive mode ionization data were collected with a Q-oTOF (quadrupole-orthogonal time-of-flight) mass spectrometer (Applied Biosystems/MDS Sciex Qstar/Elite) via turbospray ionization in the positive mode. Fragmentation data was attained using collision energy (CAD = 5, CE = 45). Fragmentation energies were held at a level that maintained a large percentage of the parent ion peak in order to minimize the generation of secondary fragments. Tandem mass spectra were collected isolating the 1^+ charge state, as these were the most prevalent ions.

Due to the complexity and diversity of the potential antibiotic components of *B. amyloliquefaciens* and the potential interferences of media components a sub fractionation and LC–MS method was used to identify antibiotic products. The elution gradient was designed to be broad so that all analytes of interest could be separated and detected. Q-oTOF detection was chosen for its mass accuracy (around 5 ppm), where identification of lipopeptide products typically require only MS^2 spectra to identify components. Additionally, Q-oTOF detection has high sensitivity that was beneficial for identification of antimicrobial metabolites from uninduced cultures where they may not be present at biological significant levels. Initial metabolite identity was established by creating extracted ion chromatograms based on the calculated

mass of the parent ion of the anticipated product(s) and corroborated the correct peak using isotope distribution to confirm its empirical formula, MS/MS fragmentation data, and relative HPLC retention time.

2.5. Bionformatics

The determination of open reading frames (ORFs) and annotation was carried out using both RAST server and NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP). The annotations were manually refined by direct comparison to closely related completed genomes. The gene names and the functionality for the metabolite cluster comes from previous studies: surfactin (Lee et al., 2007), iturin (Tsuge et al., 2001), fengycin (Wu et al., 2007), bacillibactin (May et al., 2001), bacilysin (Steinborn et al., 2005), macrolactin (Chen et al., 2007) bacillaene and difficidin (Chen et al., 2006) and plantazolicin (Scholz et al., 2011). The genome alignments were made using progressiveMauve (Darling et al., 2010). The core-genome and pan-genome were determined using progressiveMauve and summed directly from output file (*.backbone).

3. Results and discussion

3.1. Genome sequencing

Bacillus sp. strain AS 43.3 was sequenced to an average coverage depth of 43×. Sequencing generated 1,256,467 reads covering 200.8 Mbp, and the resultant contigs yielded one scaffold with a length of 3,961,420 bp. A summary of the results is provided in Table 1. The genome size, gene complement, and GC content is consistent with those of previously published *B. amyloliquefaciens* genomes (Blom et al., 2012; Chen et al., 2007; Hao et al., 2012; Yang et al., 2011). Potential secondary metabolite clusters were screened using an automated genome mining algorithm (Li et al., 2009) or direct searching with BLAST for gene clusters known to be present in *B. amyloliquefaciens* strains. The automated genome mining algorithm was useful in identifying the two common types of secondary metabolite gene clusters, non-ribosomal peptide synthetases and polyketides synthetases. A summary of the gene clusters identified is provided in Table 2.

Table 1
Genome statistics of *B. amyloliquefaciens* AS 43.3.

Size	3961291 bp
Number of genes	4037
Number of proteins	3919
tRNAs	89
rRNAs	29
GC%	46.60%
Average coverage	43×

3.2. Non-ribosomal peptide synthetase clusters

The genome of *B. amyloliquefaciens* strain AS 43.3 contains five non-ribosomal peptide synthetase (NRPS) gene clusters (Table 2). In *Bacillus* sp. strain AS 43.3 these clusters were comprised of genes that encode three predicted lipopeptides: surfactin, iturin and fengycin. These three lipopeptide products encoded by the clusters were previously characterized in our laboratory (Dunlap et al., 2011). The remaining two clusters possessed genes that were predicted to encode the antibacterial dipeptide bacilysin and the siderophore bacillibactin. All of the predicted compounds from all five clusters were found in culture supernatants using the previously described LC–MS/MS methods, thus confirming that the clusters are functional and metabolically active.

One complicating feature of the lipopeptide antibiotic classes is the potential heterogeneity of the products through either modification of the hydrophilic tail or a sequence variation of the peptide macrocycle. Changes to the peptide structure are often determined by changes to the overall mass of the antibiotic (e.g. asp to asn has a −1 dalton mass shift). These mass shifts are discernible by many forms of mass spectrometry (e.g. electrospray-MS or MALDI-MS); however some classes of lipopeptide exist as isomers that require chromatography, tandem MS, or both to identify. For example, the iturin family lipopeptides iturin A and mycosubtilin only differ by two of the peptide amino acids that are transposed in their respective gene sequences, which makes it impossible to resolve them based on molecular mass alone, whereas MS/MS allows for the isomers to be resolved based on their respective fragmentation patterns (Yu et al., 2002).

The first lipopeptide cluster in the genome encodes for the lipopeptide surfactin. Surfactin production is widespread across most *B. subtilis* and *B. amyloliquefaciens* strains (Hofemeister et al., 2004) and is closely connected to the competence development pathway (Hamoen et al., 2003). The competence development pathway is an adaptive process, which allows DNA transfer between microorganisms. In addition, surfactin has been shown to be required for another adaptive strategy: biofilm development (Hofemeister et al., 2004). Surfactin may also synergistically impact the anti-fungal activity of other lipopeptides, such as iturin (Hiraoka et al., 1992). It has also been shown to play an important role in the swarming process of *B. subtilis* (Julkowska et al., 2005; Kearns and Losick, 2003). These multiple roles for surfactin suggest it plays a critical part in the life cycle of the strain. In culture, the surfactin class is represented by the presence of strong signals indicating the presence of each expected chain length on the lipophilic tail (C_{12}–C_{16}; m/z 994.8, 1008.8, 1022.8, 1036.8, 1050.8), with tandem mass spectra consistent with the surfactin macrocyclic sequence.

The second lipopeptide cluster encodes the lipopeptide iturin (Fig. 1a). Within the iturin class, three ions were observed (m/z 1043.5, 1057.5, and 1071.5) in the culture corresponding to either the iturin A or mycosubtilin; each ion was present as a single sharp

Table 2
Secondary metabolite synthetase clusters in *B. amyloliquefaciens* AS 43.3.

Compound	Synthetase type	Genes	Size (kb)	Location
Surfactin	NRPS	srfAA,AB,AC,AD	25.4	334466...360623
Iturin	NRPS	ituD,A,B,C	37.2	1932736...1969969
Fengycin	NRPS	fenA,B,C,D,E	37.7	1994150...2031818
Bacillibactin	NRPS	besA,dhbA,C,E,B,F	12.7	3054020...3066760
Bacilysin/chlorotetaine	NRPS	bacA,B,C,D,E,ywfH	5.9	3629064...3634979
Macrolactin	PKS	mlnA,B,C,D,E,F,G,H,L	53.2	1454237...1507486
Bacillaene	PKS	baeB,C,D,E,acpK baeG,H,I,J,L,M,N,R,S	72.4	1760169...1832637
Difficidin	PKS	dfnA,Y,X,B,C,D,E,F,G,H,I,J,K,M,L	69.5	2333952...2403478
Plantazolicin	TOMM	pznA,B,C,D,E,F,G,H,I,J,K,L	9.9	716178...726067

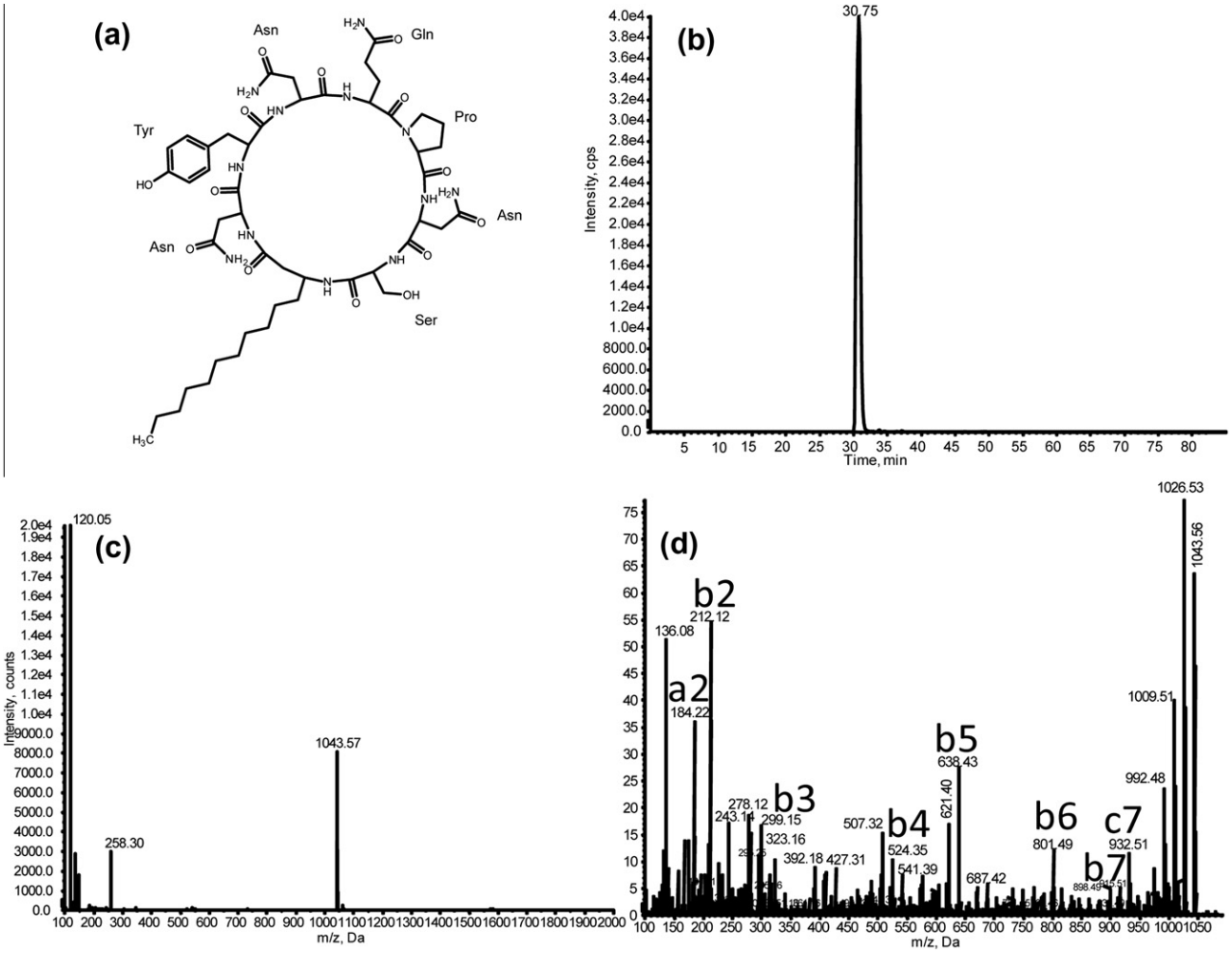

Fig. 1. (a) Structure of Iturin A C14. (b) Extracted ion chromatogram of *m*/*z* 1043.5 ion from 48 h incubation 100% methanol fraction. (c) Mass spectrum of EIC peak at 30.75 min. (d) Tandem mass spectrum of 1043.5 ion showing Iturin A sequence features.

peak indicating that it is likely a single isomeric product. The *m*/*z* 1043.5 ion corresponding to either mycosubtilin or iturin A with a 14 carbon tail is present as a single peak in the extracted ion chromatogram (RT 30.75 min, Fig. 1(b and c)). The difference between the two classes is the transposition of two amino acids within the macrocycle structure (i.e. -Asn-Ser- vs. -Ser-Asn-). The absence of mycosubtilin was determined by tandem mass spectral data. The tandem MS spectrum of *m*/*z* 1043.5 is dominated by b-type ions from the peptide portion of the molecule, using standard peptide fragmentation nomenclature (Roepstorff and Fohlman, 1984). The presence of a2 (*m*/*z* 184.22) and b2 (*m*/*z* 212.12) fragment ions in Fig. 1d corresponding to a Pro-Asn-Ser sequence confirms the presence of iturin A (Yu et al., 2002). If mycosubtilin had been present as a co-eluting analyte a2 (*m*/*z* 157) and b2 (*m*/*z* 185) fragment ions would have been observed. Due to the identical sequences from that point on, all other fragment ions are the same beginning with the b3 ion (*m*/*z* 299).

The third lipopeptide cluster encodes for the lipopeptide fengycin. Ions corresponding to the presence of fengycin products were also observed (*m*/*z* 1435.9, 1449.9, 1463.9, 1477.9, 1491.7, 1505.7), in many cases as two resolved chromatographic peaks indicating the presence of isomers in accordance with previous reports (Bie et al., 2009; Pecci et al., 2010). The two fengycin classes are divided by the incorporation of an alanine (Feng A), residue mass 71 Da, or valine (Fen B), residue mass 99 Da, in the macrocycle at position 6. The two methylene difference in amino acid sequence causes an identical mass for a fengycin B and a fengycin A with a two methylene (+28 Da) extension on the lipophilic tail. Using chromatography, two fengycin peaks with *m*/*z* 1463.9 can be identified from the extracted ion chromatograms at retention times 31.1 and 32.3 min. These two peaks have differing tandem mass spectra where fragments corresponding to the characteristic fengycin A fragments (*m*/*z* 966.45; 1080.55 corresponding to an Ala present on the ring at RT 32.3 min) where the corresponding fengycin B product ion fragments (*m*/*z* 994.46; 1108.58) had a two methylene (+28 Da) mass shift corresponding to the presence of the Val in the macrocyclic structure (at retention time 31.1 min). The importance of these antibiotics in biological control systems is well established and studied (Jacobsen et al., 2004). Two recent studies have identified fengycin as the prominent lipopeptide in *B. subtilis* strains which antagonize *G. zeae* (Ramarathnam et al., 2007; Wang et al., 2007).

In addition to the lipopeptide products, AS 43.3 uses non-ribosomal peptide synthetases to produce the antibacterial dipeptide bacilysin and the siderophore bacillibactin. Bacilysin is encoded on a 5.9 kb cluster composed of eight genes. Bacilysin was originally identified from a *Bacillus subtilis* strain more than 60 yrs ago (Newton, 1949). Bacilysin is a pro-drug and requires the action of a peptidase to become active. Bacilysin is a dipeptide and removal of the N-terminal alanine releases the active component L-anticapsin (Kenig et al., 1976).

When we screened the cell-free culture supernatant, we were unable to definitively identify bacilysin in the sample due to the presence of multiple ions with *m*/*z* signals similar to bacilysin. However, we identified chlorotetaine, a chloronated derivative of bacilysin. Chlorotetaine as shown in Fig. 2 a was differentiated from multiple *m*/*z* 289.1 peaks due to the natural isotopic abundance of chlorine (^{35}Cl 76%, ^{37}Cl 24%) providing the expected isotopic distribution (*m*/*z* 289.1 and 291.1) in the mass spectrum (Fig. 2). Interestingly, the same observation was recently made with the closely related *B. amyloliquefaciens* strain GA1 (Arguelles-Arias et al., 2009). Another strain, *Bacillus* sp. strain CS93 was also reported to make chlorotetaine (Phister et al., 2004). A subsequent study on the strain indentified the bacilysin cluster by PCR, but could not find bacilysin or chlorotetaine in the culture media (Moran et al., 2010). Chlorotetaine was originally isolated from a *B. subtilis* strain that co-produced bacilsyin, and the authors noted while bacilysin production was widespread in their *B. subtilis* collection only one also made chlorotetaine (Rapp et al., 1988). A member of the same group later reported bromo- and chlorotetaine from *B. amyloliquefaciens* (Katzer, 1991). These observations suggest chlorotetaine is primarily observed in *B. amyloliquefaciens* strains. A BLAST search of the gene cluster (bacABCDE, ywfG) from AS 43.3 showed 95–99% sequence similarity with other *B. amyloliquefaciens* strains in the NCBI GenBank, while the similarity to *B. subtilis* strains averaged 81%. All of the *B. amyloliquefaciens* genomes in GenBank share the same flanking genes in this gene cluster, suggesting that chlorotetaine may be a trait specific to *B. amyloliquefaciens* strains.

The final non-ribosomal peptide synthetase cluster produces the siderophore bacillibactin (Fig. 3a). Bacillibactin is encoded by a 12.7 kb cluster containing five genes and was originally isolated from a *B. subtilis* strain as a product of the *dhb* operon (May et al., 2001). Siderophores selectively bind iron with high affinity making the iron unavailable to other organisms. In many microbial environments, iron availability is far below the optimum needed for growth. This competition for available iron had been recognized as an important trait for many biocontrol antagonists (Miethke and Marahiel, 2007). While the role of siderophores has been demonstrated in biocontrol systems, studies with the siderophore bacillibactin are limited. Woo and Kim recently isolated bacillibactin from *B. subtilis* AH18 and demonstrated the purified bacillibactin exhibited suppressive activity against *Phytophthora capsici* (Woo and Kim, 2008). We are not aware of any studies that demonstrate the specific importance of bacillibactin to the biocontrol activity of *B. amyloliquefaciens* strains.

The structure of bacillibactin consists of a cyclic tri-threonine (Thr) ester core covalently bonded through the sidechains. The amino groups of each threonine are extended with glycine (Gly)-dihydroxybenzoic acid (DHB) units (Fig. 3a). Bacillibactin was identified as a single peak from the extracted ion chromatogram of the calculated *m*/*z* for the singly charged ion at 883.25 Da (Fig. 3b and c). The tandem mass spectral fragmentation pattern of the 883.27 ion (Fig. 3d) is dominated by fragments ions of DHB, DHB-glycine, Thr-Thr, and DHB-Gly-Thr. In addition, ions consistent with the losses of all or part of single or multiple branches were observed (e.g. M-DHB; M-DHB-Gly; etc.), thus confirming bacillibactin as a secondary metabolite. As an aside, we were able to detect low levels of bacillibactin in the cell-free culture supernatant without the need for inducing production with an iron starving media preparation.

3.3. Polyketide synthetase gene clusters

Bacillus sp. strain AS 43.3 harbors three polyketide synthetase (PKS) gene clusters. These clusters encode the antibacterial polyketides: bacillaene, difficidin and macrolactin. PKSs are a large family of enzymes that catalyze the synthesis of a broad range of secondary metabolites. The functions and organization of PKS clusters have been reviewed extensively (Fischbach and Walsh, 2006; Staunton and Weissman, 2001). In general, their biosynthetic pathways follow an assembly line-like organization, where different enzyme functionalities are ordered in a modular fashion to create unique products. *Bacillus* species are known to produce a wide range of PKS clusters (Albertini et al., 1995; Chen et al., 2006; Emmert et al., 2004; Reddick et al., 2007).

The first PKS cluster in the subject strain directs the synthesis of bacillaene. Bacillaene is an antibiotic that is active against a broad spectrum of bacteria and originally isolated from the fermentation broth of a *B. subtilis* strain (Patel et al., 1995). The 72.4 kb gene cluster consists of 14 genes and was originally characterized from a *B. amyloliquefaciens* strain (Chen et al., 2006). Recently the cluster has been identified in a number of newly published genomes (Arguelles-Arias et al., 2009; Chen et al., 2007; Deng et al., 2011). A BLAST search of the gene cluster finds it is located in 15 other strains (eight from *B. amyloliquefaciens*, six from *B. subtilis*, and one from *B. atrophaeus*) in the NCBI GenBank. While most of these strains are biocontrol strains, there are only a limited number of studies have been performed that directly correlate the presence of bacillaene with a biocontrol activity. This result is due to the fact that these strains make multiple antibiotics, which obscures the effect of individual compounds. The presence of bacillaene was confirmed in LC–MS/MS by the presence and fragmentation of the molecular ion at *m*/*z* 583.4 and doubly charged *m*/*z* 292.2 ions as a distinct peak at 40.5 min in the 60% methanol fraction collected during the exponential growth phase (12 h timepoint).

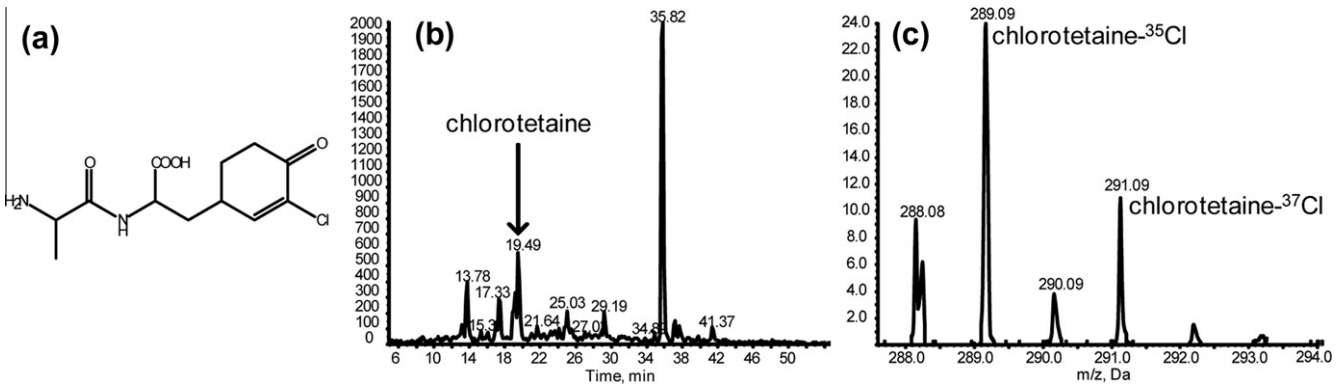

Fig. 2. (a) Structure of chlorotetaine. (b) Extracted ion chromatogram of *m*/*z* 289.1 ion from 48 h incubation 40% methanol fraction. (c) Mass spectrum of EIC peak at 19.49 min showing the isotopic distribution of chlorotetaine.

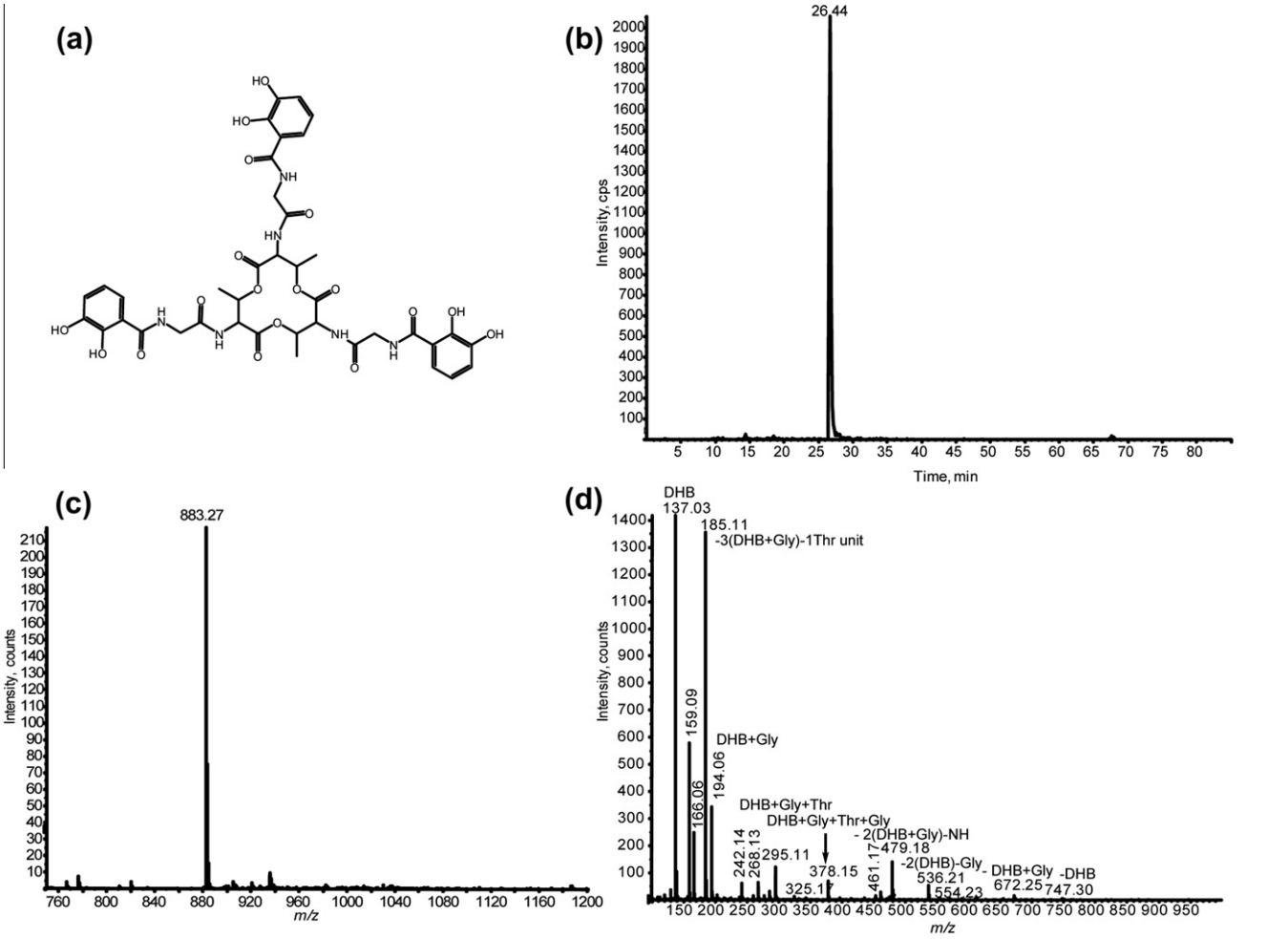

Fig. 3. (a) Structure of bacillibactin. (b) Extracted ion chromatogram of *m*/*z* 883.27 ion from 48 h incubation 20% methanol fraction. (c) Mass spectrum of EIC peak at 26.44 min. (d) Tandem mass spectrum of 883.27 ion showing bacillibactin fragmentation pattern.

The second PKS cluster encodes the synthesis of difficidin and its hydroxylated derivative oxydifficidin. Difficidin and oxydifficidin are antibiotics that are active against a broad spectrum of bacteria and were originally isolated from the fermentation broth of a *B. subtilis* strain (Zimmerman et al., 1987). It was recently reported that difficidin producers are found in a plant associated lineage of *B. amyloliquefaciens* strains (Borriss et al., 2011). The results of a BLAST search of the 69.5 kb gene cluster showed that it is located in three other strains (all from *B. amyloliquefaciens*) ; FZB42 (Chen et al., 2007), CAU B946 (Blom et al., 2012), and YAU B9601-Y2 (Hao et al., 2012) in the NCBI GenBank. This suggests that difficidin may be specific to *B. amyloliquefaciens*. The search for difficidin in the cell-free culture supernatant only yielded its hydroxylated derivative oxydifficidin shown in Fig. 4a. LC–MS/MS data identify a strong extracted ion peak for *m*/*z* 561.26 at 25.2 min (Fig. 4b and c), with a tandem mass spectrum consistent with an oxydifficidin structure (Fig. 4d). There have been no detailed studies of the biosynthesis of difficidin and oxydifficidin. It remains unclear what enzyme catalyzes the hydroxylation of oxydifficidin, since it does not appear to be an uncatalyzed air oxidation based on earlier reports (Suphantharika et al., 1994). Interestingly, a closely related species *B. amyloliquefaciens* GA1 was reported to only produce oxydifficidin (Arguelles-Arias et al., 2009), while several other *B. amyloliquefaciens* strains were reported to produce difficidin (Borriss et al., 2011). The role of difficidin in biocontrol applications has only recently begun to be explored. However, it was recently shown to control the fire blight causal agent, *Erwinia amylovara* (Chen et al., 2009). Additional studies are needed to clarify the role of difficidin/oxydifficidin in biocontrol systems.

The third PKS cluster encodes the synthesis of macrolactin. Macrolactins are a class of antibiotics originally isolated from a deep sea marine bacterium (Gustafson et al., 1989) and have subsequently been isolated from several *Bacillus* sp. strains (Han et al., 2005; Jaruchoktaweechai et al., 2000; Romero-Tabarez et al., 2006; Schneider et al., 2007). These antibiotic compounds have been shown to be effective in controlling common scab, a disease of potato caused by *Streptomyces scabei* (Han et al., 2005). Recently, purified macrolactin A, 7-O-malonyl macrolactin A, and 7-O-succinyl macrolactin A were shown to inhibit the soil plant pathogenic bacteria *Ralstonia solanacearum* (Yuan et al., 2012). The results of a BLAST search of the 53.3 kb gene cluster show that it is located in four other strains (three from *B. amyloliquefaciens*) FZB42 (Chen et al., 2007), CAU B946 (Blom et al., 2012), and YAU B9601-Y2 (Hao et al., 2012) and one from *B. subtilis* B11 (accession No. DQ844599). To confirm that the gene cluster was metabolically functional, we screened fermentation broth for macrolactin. Using LC–MS/MS to screen the broth, we located a mass to charge ion consistent with macrolactin, 403.5 *m*/*z* $[M+H]^+$ and additional ions consistent with malonyl- (*m*/*z* 489.4) and succinyl- (*m*/*z* 503.4) derivatives of macrolactin. Assignments of products were made by the combination of LC retention time (to rule out or include isomers), mass spectrometry, and targeted tandem mass spectrometry of expected low abundance products.

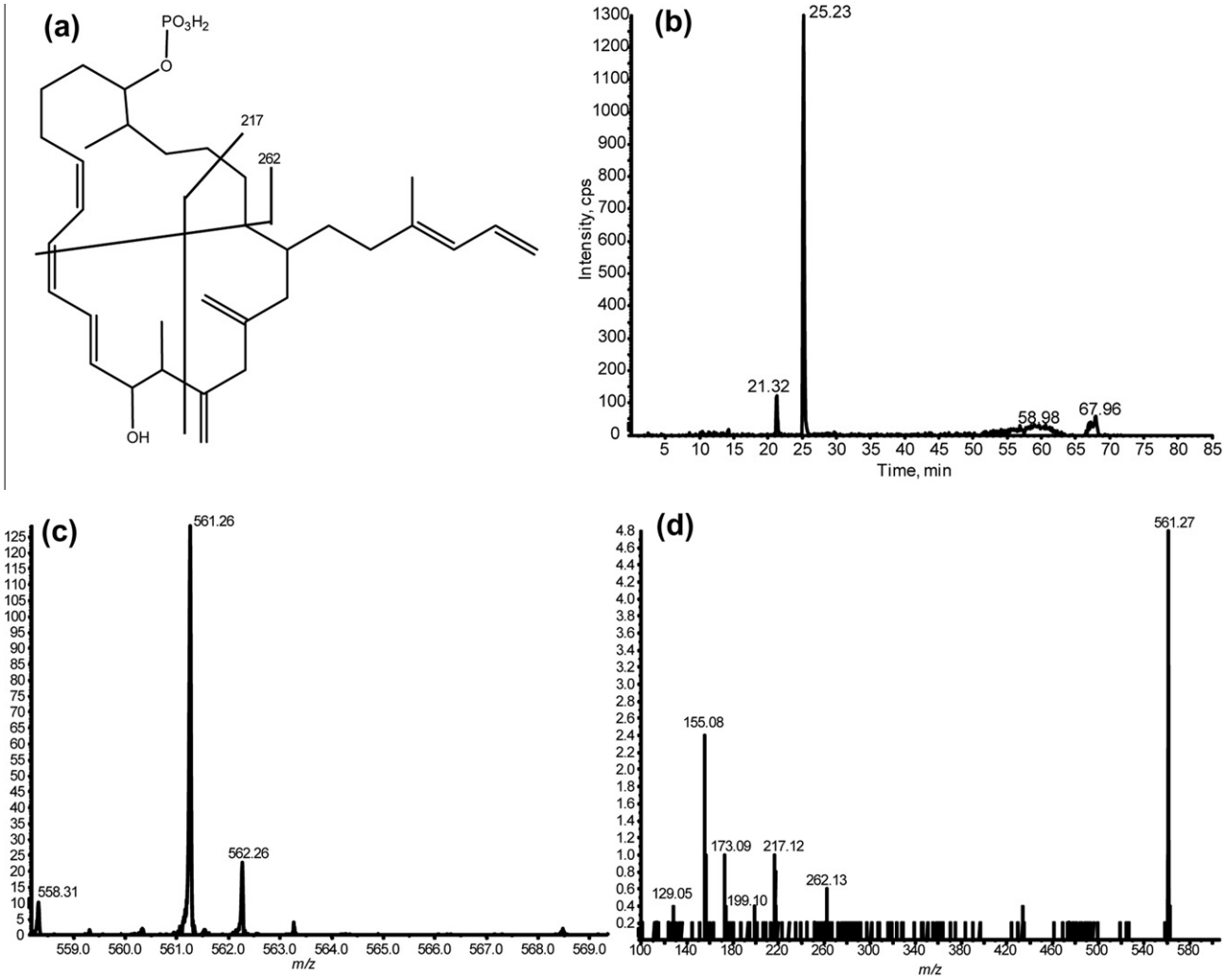

Fig. 4. (a) Structure of oxydifficidin. (b) Extracted ion chromatogram of *m*/*z* 561.26 ion from 48 h incubation 20% methanol fraction. (c) Mass spectrum of EIC peak at 25.23 min. (d) Tandem mass spectrum of 561.3 ion.

3.4. Plantazolicin

In addition to the non-ribosomal mediated biosynthetic clusters discovered, we have identified a ribosomally encoded biosynthetic cluster. This cluster produces the antibiotic plantazolicin. Plantazolicin was recently discovered and isolated from *B. amyloliquefaciens* FZB42 (Scholz et al., 2011) and belongs to a group of compounds classified as thiazole/oxazole-modified microcins (TOMMs) (Haft et al., 2010). Plantazolicin has been shown to inhibit several closely related *Bacillus* and *Paenibacillus* strains (Scholz et al., 2011). The results of a BLAST search of the 9.9 kb gene cluster show that it is found in two other strains (both from *B. amyloliquefaciens*) FZB42 (Chen et al., 2007) and CAU B946 (Blom et al., 2012) in NCBI GenBank. The unique structure of plantazolicin was recently elucidated (Kalyon et al., 2011). In culture, plantozolicin was identified by retention time separation and mass spectrometry only, as the low abundance signal did not adequately provide fragment ions for structural confirmation. The role of plantazolicin in biocontrol systems has yet to be established.

3.5. Strain classification

This strain was originally classified as a *Bacillus subtilis*/*amyloliquefaciens* strain (Khan et al., 2001). This identification was based on identification by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany, based on 16S rDNA sequence homologies and biochemical and physiological tests of taxonomic utility (Khan et al., 2001). After sequencing the genome and identifying the synthetic gene clusters in the current study, the data suggested it is more appropriately identified as a *B. amyloliquefaciens* strain. To confirm this hypothesis, we performed a BLAST search on the sequence of the *recA* and *recN* gene of the subject strain based on previous work which used these genes to differentiate *B. subtilis* and *B. amyloliquefaciens* from each other (Arguelles-Arias et al., 2009). This BLAST search identified *B. amyloliquefaciens* FZB42 as having greater than 99% sequence similarity to *Bacillus* sp. strain AS 43.3 for both genes. These results suggest that the latter may be classified as a *B. amyloliquefaciens* strain. A recent study has proposed that *B. amyloliquefaciens* should be separated in two subspecies based on phenotypic and genomic traits (Borriss et al., 2011): the plant-associated *B. amyloliquefaciens* subsp. *plantarum* and the non-plant-associated *B. amyloliquefaciens* subsp. *amyloliquefaciens*. The defining features between the two subspecies are differences in carbohydrate degrading enzymes and non-ribosomal secondary metabolites. The presence of macrolactin and difficidin in the subject strain is consistent with the plant-associated subspecies. In addition, the presence of *amyE*, *BglC* and *XynA* carbohydrate degrading genes and the lack of the *amyA* gene in the subject strain are consistent with the plant-associated subspecies. Therefore, it is

proposed the subject strain be reclassified as a *B. amyloliquefaciens* subsp. *plantarum* strain.

3.6. Genomic comparison with closely related strains

Because the genomes of several *B. amyloliquefaciens* strains have been sequenced, a comparison of the strains was now possible. As mentioned in the previous section, the species can be separated into two subspecies which are plant-associated and a non-plant-associated. Conveniently, these classifications also describe the use of these strains with the plant-associated strains all being used as biocontrol strains and the non-plant-associated strains all being used or proposed to being used for industrial applications (enzyme or metabolite production). Fig. 5 shows the alignment of completed *B. amyloliquefaciens* genomes using progressiveMauve (Darling et al., 2010). The alignment shows the four biocontrol strains are very similar, with *B. amyloliquefaciens* strain AS43 most similar to *B. amyloliquefaciens* strain FZB42. The colored blocks represent conserved regions free from intra-region rearrangements, while the white areas within a block represent regions unique to that genome. The few unique regions in *B. amyloliquefaciens* strain AS 43.3 are primarily phage proteins or hypothetical proteins with unknown function. Colored blocks below the centerline of a plot are coded in the opposite direction, which are associated with genomic rearrangements. Comparing the biocontrol strains with the industrial strains, industrial strains TA208 and XH7 show a major genomic rearrangement with a 1.2 Mbp region (~1,100,000–2,300,000) in the center of their genomes reversed. The nine metabolite clusters discussed in this study, if present in another strain, are found in the same relative physical location in the genome and with the same collinear gene order. This suggests the gene clusters are homologous and share the same ancestry.

The progressiveMauve alignment also provides the means of calculating the core-genome and pan-genome for the strains (Darling et al., 2010). The core-genome is the conserved genes found in all the strains of a group, while the pan-genome is a superset of all the genes present in the group. The values of the core-genome and pan-genome are reported in Table 3. For *B. amyloliquefaciens* strain AS 43.3, the genes in the core-genome make up 83.7% of the genome. To better understand what genes in *B. amyloliquefaciens* strains are important to biocontrol applications, the subset of genes unique to the biocontrol strains also were determined (Table 3). The unique genes are the difference between the core-genome of the four biocontrol strains and the core-genome of all eight strains. The unique genes cover 263690 bp widely scattered across the biocontrol genomes. The bulk of these genes make up the macrolactin, difficidin and parts of the fengycin clusters. After these clusters, the next biggest region is a 5884 bp (@ location 1911885 in AS 43.3) region coding genes (*kdgK*, *yjmF*, *uxuA*, *kdgA*) associated with D-galacturonate and D-glucuronate utilization. It is unclear the role these genes in a biocontrol system and may be related to their survival on plant ecological niches. The three large synthetic clusters found in the core-genome of the biocontrol strains are also specific to the plant-associated *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al., 2011), described in the previous section. Part of the fengycin cluster (*fenD*,*E*) is found in one of the industrial strains (DSM-7), but it is not functional since it lacks some of the enzymes needed (*fenA–C*) (Borriss et al., 2011). Interestingly, the four genomes associated with industrial applications don't share any conserved genes that are not also found in the biocontrol strains.

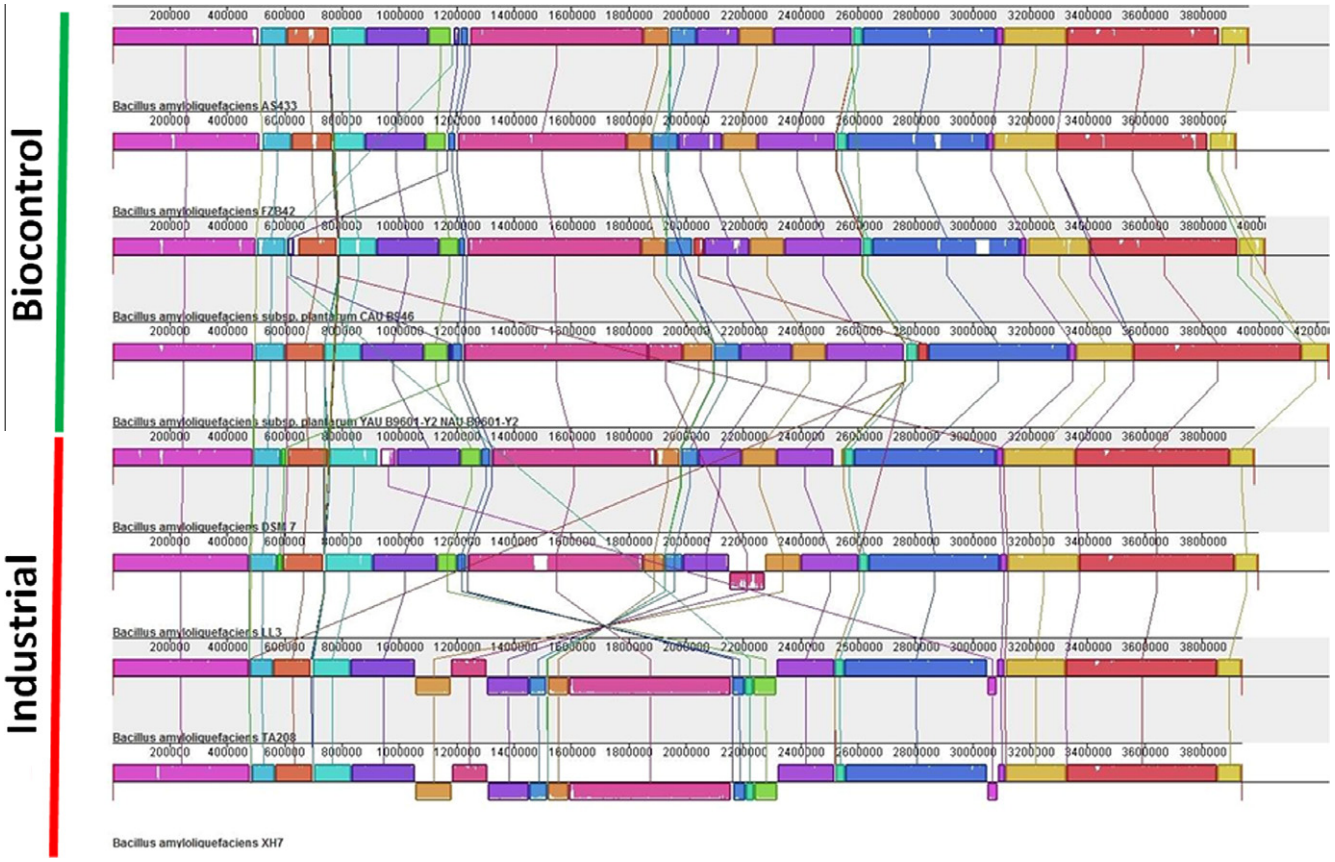

Fig. 5. Genome alignment of *B. amyloliquefaciens* strains using progressiveMauve. The colored blocks represent conserved regions free from intra-region rearrangements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Genomic comparison of eight *B. amyloliquefaciens* strains.

Core-genome	3316600 bp
Pan-genome	5529004 bp
[a]Unique to all biocontrol strains	263690 bp
[a]Unique to all industrial strains	0 bp

[a] The difference between the core-genome of the biocontrol (or industrial) strains and the core-genome of all eight strains.

3.7. Impact on controlling Fusarium head blight

The genome of this Fusarium antagonist sheds new light on the possible mechanisms of action for controlling Fusarium head blight by *B. amyloliquefaciens* strain AS 43.3. It has long been known that *Bacillus* sp. strains produced lipopeptides from the iturin and fengycin classes that demonstrated strong antifungal activity. After previously identifying these lipopeptides in *B. amyloliquefaciens* strain AS 43.3, we assumed this was the mode-of-action, direct antibiosis via lipopeptides. The genome has now offered the possibility of additional modes-of-action in this pathosystem. Specifically, the presence of the siderophore, bacillibactin suggests the competition for iron could also contribute to the strain's biocontrol effect. An additional mode-of-action could be based on the presence of the antibacterials macrolactin and (oxy)difficidin. These antibacterials could alter the bacterial community on the wheat head, which impacts the survival of the fungal pathogen in the case of FHB. It is also possible that changes in the bacterial community could benefit the fungal pathogen. It remains unclear how the presence of these other metabolites (bacillibactin and the antibacterials) impact the biocontrol efficacy of *B. amyloliquefaciens* strain AS 43.3 in the FHB pathosystem. How the quantity and ratio of all of the aforementioned metabolites produced by strain AS 43.3 is impacted when the strain is subjected to a multitude of stresses including nutrient availability, UV light, moisture fluctuation, and microbial competition on wheat heads also remains unknown. Unraveling these factors and their interactions should greatly improve our understanding of this biocontrol system.

4. Conclusions

In this study we report the completed genome of *B. amyloliquefaciens* strain AS 43.3. We have identified and confirmed the presence of eight antimicrobial secondary metabolites and one siderophore. This work adds to the growing database of *B. amyloliquefaciens* genomes, while providing detailed information of the secondary metabolites they produce. By providing accurate sequencing information coupled with accurate metabolite determination the results will speed the ability to accurately predict gene function of these important biocontrol metabolites. This study provides the first complete genome of a *B. amyloliquefaciens* strain isolated from the phyllosphere. This study also provides the second report of a microorganism that can produce the antimicrobial, plantazolicin. In addition, the analysis supports the division of *B. amyloliquefaciens* into subspecies representing plant associated and plant non-associated strains. This study identifies the core genome and pan genome of eight *B. amyloliquefaciens* strains. The genomic comparisons allowed us to identify a subset of genes shared and specific to *B. amyloliquefaciens* strains isolated for biocontrol applications. The study highlights the continued growing importance of genomics in understanding biocontrol pathosystems in general and specifically, in the present study, in rapidly characterizing the metabolite potential of biocontrol strains.

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