C-Protein α-Antigen Modulates the Lantibiotic Thusin Resistance in *Streptococcus Agalactiae*

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Abstract

Screening for producers of potent antimicrobial peptides, resulted in the isolation of *Bacillus cereus* BGNM1 with strong antimicrobial activity against *Listeria monocytogenes*. Genome sequence analysis revealed that BGNM1 contains the gene cluster associated with the production of the lantibiotic, thusin, previously identified in *B. thuringiensis*. Purification of the antimicrobial activity confirmed that strain BGMN1 produces thusin. Both thusin sensitive and resistant strains were detected among clinical isolates of *Streptococcus agalactiae*. Random mutagenesis of a thusin sensitive strain, *S. agalactiae* B782, was performed in an attempt to identify the receptor protein for thusin. Three independent thusin resistant mutants were selected and their complete genomes sequenced. Comparative sequence analysis of these mutants with the WT strain revealed that duplication of a region encoding a 79 amino acids repeat in a C-protein a-antigen was a common difference, suggesting it to be responsible for increased resistance to thusin. Since induced thusin resistant mutants showed higher level of resistance than the naturally resistant B761 strain, complete genome sequencing of strain B761 was performed to check the integrity of the C-protein a-antigen-encoding gene. This analysis revealed that this gene is deleted in B761, providing further evidence that this protein promotes interaction of the thusin with receptor.

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides of bacterial origin that are considered promising novel, potent antimicrobials (Cotter et al. 2013). This divergent group includes post-translationally modified, unmodified or even cyclic molecules with bactericidal or bacteriostatic effects (Cotter et al. 2013). The characteristics of bacteriocins, including the presence of modifications together with their mode of action, are used as the basis for a number of classification schemes, with lanthionine containing antibiotics (lantibiotics) being included with other extensively modified bacteriocins in Class I (Alvarez-Sieiro et al. 2016). The lantibiotics have been further subclassified on the basis of their biosynthetic machinery and the amino acid sequence of their structural peptide (Rea et al. 2011).

Lantibiotics are considered the best-characterized bacteriocins that exhibit broad-spectrum antimicrobial activity against Gram-positive clinically relevant pathogens (Cotter et al. 2013; Willey and van der Donk 2007; Dischinger et al. 2014; Sandiford 2014). Lantibiotic-producing strains usually contain a gene cluster that encodes the structural lantibiotic peptide as well as the proteins responsible for its modification, transport across the cell membrane and immunity (self-protection) (Willey and van der Donk 2007; Singh et al. 2014). Lantibiotics generally show specificity for components of the cell envelope of Gram-positive bacteria and mechanisms of action include, but are not limited to, binding to lipid II or other peptidoglycan (PG) precursors and/or pore formation leading to membrane leakage (Héchard and Sahl 2002; Bierbaum and Sahl 2009).

The best characterized lantibiotic is nisin A (subclass 1), which has been used in the dairy and food industries for decades. It targets the cell wall precursor, lipid II, and exerts inhibitory activity through inhibition of cell wall biosynthesis and stable pore formation in the target membrane (Willey and van der

Donk 2007; Islam et al. 2012). The lantibiotics nukacin (bacteriostatic) and mersacidin also use lipid II as a docking molecule, but can only inhibit cell wall biosynthesis and do not form pores (Asaduzzaman et al. 2009; Hasper et al. 2006). In the case of two-peptide lantibiotics, such as lacticin 3147, the mechanism of action is based on interaction of the α-peptide with lipid II, followed by interaction with the β-peptide to form an active complex that causes inhibition of cell wall biosynthesis, pore formation and potassium efflux (Islam et al. 2012; Morgan et al. 2005). Despite structural differences between the two peptides of haloduracin and those of lacticin 3147, these two bacteriocins show similarities in their mode of action, in that, both bind to a target on the surface of Gram-positive bacteria, though haloduracin inhibits cell wall biosynthesis without pore formation (Islam et al. 2012; Morgan et al. 2005).

Mechanisms of lantibiotic resistance include, but are not limited to, innate systems that sense cationic proteins or those that are based on sensing cell wall damage, and are not considered to be lantibiotic specific (Draper et al. 2015). Other mechanisms of resistance include spore or biofilm formation, as well as specific mechanisms such as the production of the nisin lytic protein, nisinase - an enzyme that degrades the lantibiotic nisin, and the phenomenon of immune mimicry (Draper et al. 2015).

Bacillus strains are a rich reservoir of antimicrobials including antibiotics, lipopeptides, and bacteriocins, including numerous lantibiotics from different subclasses. The in-built heat and protease stability and low levels of resistance associated with many of these lantibiotics increases their potential as natural food preservatives in the food industry and as antibiotic alternatives in human and animal health (Abriouel et al. 2011). Examples of lantibiotics produced by *Bacillus* strains include the single peptide lantibiotics subtilin, ericin A and ericin S, mersacidin, paenibacillin and sublancin 168 (Abriouel et al. 2011; Acedo et al. 2018) while two-peptide lantibiotics include haloduracin, lichenicidin and formicin Dischinger et al. 2009; Oman and van der Donk 2009; Shenkarev et al. 2010; Collins et al. 2016; Xin et al. 2016). The recently discovered a two-peptide lantibiotic thusin, produced by *Bacillus thuringiensis* (Xin et al. 2016), is active against a broad spectrum of Gram-positive pathogens and even inhibits the outgrowth of *B. cereus* spores, though it is inactive against Gram-negative pathogens (Xin et al. 2016).

According to serological typing, based on serogroup-specific polysaccharides, *Streptococcus agalactiae* is classified as a group B streptococcus (GBS), and represents an important pathogen that causes infections in humans, most notably in neonates, and mastitis in cattle (Lancefield 1934). Surface proteins of *S. agalactiae* are likely to play important roles in virulence and various stages of infection and are promising components of vaccines against this pathogen (Lindahl et al. 2005; Maeland et al. 2015). Nearly all *S. agalactiae* strains express alpha-like proteins (Alps) such as Cα, Alp1, Alp2, Alp3, Rib, and Alp4. Alps are high-molecular-mass proteins with a signal peptide of 50 amino acids (aa), an N-terminus of 170 to 180 aa, a C-terminal domain of 40 to 50 aa with a cell wall-anchoring motif, which probably provides binding to the cell wall, and a region of tandem repeats of about 80 aa consisting mostly of 8 to 10 repeats in clinical isolates (Lindahl et al. 2005; Maeland et al. 2015; Michel et al. 1992). The first surface protein antigen identified in *S. agalactiae* was the C protein, expressed by most clinical isolates of capsular types Ia, Ib, and II and thought to play a role in both virulence and immunity (Wilkinson and Eagon 1971).

In this study, we screened different, underexplored habitats for new cultivable bacterial isolates capable of producing natural antimicrobial molecules effective against human multidrug resistant pathogens. We found *B. cereus* BGNM1, a natural soil isolate that produces the potent two-peptide lantibiotic, thusin, from soil. Additionally, by applying random mutagenesis to a thusin-susceptible strain of *S. agalactiae*, we identified an apparent target molecule for thusin on the cell surface. All resistant mutants of *S. agalactiae*, both spontaneously generated and those with existing resistance, had changes in the C-protein α-antigen highlighting the crucial role of this protein in facilitating the activity of the two-peptide lantibiotic, thusin.

Materials And Methods

Bacterial strains and culture conditions

Strains used in this study are listed in Table 1. *Acinetobacter, Burkholderia, Erwinia, Escherichia, Klebsiella, Pseudomonas, Salmonella* and *Staphylococcus* strains were grown in Luria Bertani (LB) medium at 37°C with aeration, while *Bacillus, Ralstonia* and *Xanthomonas* strains were grown in the same medium at 30°C with aeration. *Lactococcus* and *Listeria* strains were grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% w/v) (GM17) at 30°C. *Streptococcus* strains were grown in Brain Heart Infusion (BHI) medium (Oxoid, Basingstoke, Hampshire, England) at 37°C and an atmosphere of 5% CO_2. Solid media and soft agar were made by adding 1.5% or 0.7% (w/v) agar (Torlak, Belgrade, Serbia) to the liquid media, respectively. Bacterial strains producing antimicrobials were isolated on solid media (HiMedia, Mumbai, India).

Table 1
Bacterial strains used in this study

Strains	Culture conditions	Source or reference
Acinetobacter baumanii 6077/12	37°C, with aeration	(Novovic et al. 2015)
Bacillus cereus 11778	30°C, with aeration	ATCC
Burkholderia cenocepacia ST856	37°C, with aeration	(Vasiljevic et al. 2016)
Erwinia amylovora	30°C, with aeration	LMM collection
Escherichia coli 25922	37°C, with aeration	ATCC
Klebsiella pneumoniae Ni9	37°C, with aeration	(Novovic et al. 2017)
Lactococcus lactis BGMN1-596	30°C, no aeration	(Kojic et al. 2006)
Listeria monocytogenes 19111	37°C, no aeration	ATCC
Pseudomonas aeruginosa MMA83	37°C, with aeration	(Jovcic et al. 2011)
Ralstonia solanacearum	37°C, with aeration	LMM collection
Salmonella Enteritidis 13076	37°C, with aeration	ATCC
Staphylococcus aureus 25923	37°C, with aeration	ATCC
Streptococcus agalactiae B782WT	37°C, 5% CO_2	Pasteur laboratory, Belgrade
Streptococcus agalactiae B761	37°C, 5% CO_2	Pasteur laboratory, Belgrade
Streptococcus agalactiae B782 R1, R2, R3	37°C, 5% CO_2	This work
Xanthomonas oryzae	37°C, with aeration	(Beric et al. 2012)

ATCC-American Type Culture Collection

LMM collection – Collection of Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia

Sampling, isolation and selection of bacteria

Soil (5 samples), sewage water (2 samples) and fermented milk products (6 samples) were collected to isolate bacterial strains that were subsequently screened for the production of antimicrobials active against pathogenic bacteria. Five grams or milliliters of each sample was suspended in 45 ml of saline solution (8.9 g/l NaCl) and incubated at room temperature in an orbital shaker at 200 rpm for 30 min. Mixtures were allowed to settle and 10-fold serial dilutions, up to 10^{-5}, were prepared using sterile saline solution. Bacteria were isolated from these mixtures by spreading aliquots of serial dilutions of each sample on four different growth media (LB, BHI, GM17 and MRS) in quadruplicate and incubating the plates at 30 and 37°C for 48 hours under aerobial and anaerobical atmosphere. The Petri dishes

containing colonies (50-150) were overlaid with each of the four different indicator strains (two Gram negative pathogens: *Klebsiella pneumoniae* Ni9, *Pseudomonas aeruginosa* MMA83 and two Gram positive pathogens: *Staphylococcus aureus* ATCC25923, *Listeria monocytogenes* ATCC19111) suspended in soft agar and plates were incubated overnight at 37°C. Colonies producing antimicrobial activity were detected by the appearance of a zone of inhibition around them. Purification of antimicrobial producers was achieved by repeated streaking of single colonies from the center of inhibition zones on the medium from which it was originally selected.

Identification of isolates with antimicrobial activity

Taxonomic determination of isolates was performed by 16S *rRNA* gene sequencing using universal primers 16S – Fw (GAATCTTCCACAATGGACG) and 16S – Rev (TGACGGGCGGTGTGTACAAG). Platinum™ *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific, Waltham, MA, USA) was used to amplify the gene for 16S rRNA using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions (94°C/30 sec, 55°C/30 sec, 72°C/60 sec/[30 cycles]). PCR products were checked by agarose gel electrophoresis (1% agarose; constant voltage of 80 V) and purified using a Thermo Scientific PCR Purification Kit (Thermo Scientific, Lithuania) according to the manufacturer's protocol. The PCR products were sequenced by the Macrogen Sequencing Service (Macrogen, Europe, Amsterdam, The Netherlands) and analyzed by BLAST algorithm.

Antimicrobial activity assay

Antimicrobial activity was detected by an agar-well diffusion assay. Each indicator strain was inoculated into soft agar of the appropriate medium (10^5 cfu/mL), and wells (diameter 5 mm) were made in the plate. The wells were filled with 50 µl of sample from a putative producer from an overnight culture or CFS (0.22 µm, Thermo Fisher Scientific) and plates were incubated under appropriate conditions for the respective indicator strain. A spot-on-the-lawn inhibition assay was used for testing antimicrobial activity of purified antimicrobial peptide. The presence of inhibition zones was examined after 24 h of incubation at the appropriate temperature (30 or 37°C) for indicator strains. A clear zone of inhibition was taken as evidence of antimicrobial production. The antimicrobial activity assay was performed in at least two independent repetitions. The proteinaceous nature of the antimicrobial molecule was determined by placing a crystal of the the proteolytic enzyme, pronase E (Merck, Germany) on the agar surface near the well and a reduction of the clearing/inhibition zone around the crystal was taken as evidence that the antimicrobial activity derives from molecule(s) of a protein nature.

Purification of antimicrobial peptides from *Bacillus cereus* BGNM1

Bacillus cereus BGNM1 was grown in 2 litres of LB broth, aerobically, at 30°C. Cells were separated from supernatant by centrifugation at 11,000 x g for 20 minutes at 10°C. The cell free supernatant (CFS) was passed through a column containing 30 g Amberlite XAD16N beads to allow binding of the antimicrobial molecule. The column was washed with 35% ethanol and the antimicrobial molecule eluted with 70% 2-propan-ol 0.1% TFA (IPA). The IPA was removed from the XAD IPA eluent and the remaining sample

applied to a 10 g, 60 ml Strata® C18-E SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 30% ethanol (30 E) and antimicrobial activity eluted with IPA. IPA was removed from the C18 SPE IPA eluent and the remaining sample was applied to a semi preparative Jupiter Proteo RP-HPLC column (10 x 250 mm, 90Å, 4µ) (Phenomenex, Cheshire, UK) running a 30–46% acetonitrile 0.1% TFA gradient where buffer A is 0.1% TFA and buffer B is 90% acetonitrile 0.1% TFA. Eluent was monitored at 214 nm and fractions were collected at 30 second intervals. Fractions were assayed on *L. lactis* BGMN1-596 indicator plates. Active fractions were assayed for molecular mass by MALDI TOF mass spectrometry in positive ion linear mode.

Selection of *Streptococcus agalactiae* thusin-resistant mutants

Two groups of *Streptococcus agalactiae* isolates, thusin sensitive and resistant, were selected from the laboratory collection of clinical isolates. One isolate (B782) from thusin sensitive *S. agalactiae* strains was selected for the isolation of thusin resistant mutants. Random mutagenesis using N-methyl-N-nitro-N'-nitrosoguanidine (MNNG) as mutagen and selection of *S. agalactiae* B782 mutants resistant to different concentrations of thusin were performed.

Determination of MICs

The MIC values for different strains of *S. agalactiae* to the thusin were determined using the broth microdilution method proposed by Steinberg et al. (1997) and by spot-on-the-lawn inhibition assay on Petri dish. Microdilution testing with in-house-prepared panels was performed following the methods of the Clinical and Laboratory Standards Institute. The microdilution testing assay used a mixture of the indicator strains and increasing concentrations of the thusin. Indicator strains were diluted to a 0.5 McFarland standard, from which 20 µl was distributed to the wells of a clear 96-well flat-bottom microtiter plate. Thusin (6 µM) was 2-fold serially diluted to give a dilution series with concentrations ranging from 2.95 µM to 0.023 µM. The microtiter plates were incubated under appropriate conditions for 24 h, and the optical density at 595 nm (OD595) was recorded at 30-min intervals (Infinite M200pro; Tecan, Switzerland). The values obtained were used to illustrate the antimicrobial activity of the lantibiotic thusin. Control wells contained appropriate medium (negative) and untreated cultures (positive). All experiments were done in triplicate. For determination of MIC on Petri dish spot-on-the-lawn inhibition assay was used in which 5 microliters of different concentrations of thusin were spotted on the surface of top agar inoculated with tested strains. Petri dishes were incubated under optimal conditions for *S. agalactiae* and the level of inhibition of different concentrations of thusin was compared. To determine whether nisin and thusin lantibiotics use the same mechanism of activity, MIC values to nisin were determined for thusin-sensitive and resistant strains of *S. agalactiae* using the same methods as for tushin: microdilution and spot-on-the-lawn inhibition assay. Commercial nisin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used to determine the MIC by serial double dilution of concentrations from 1000–1 IU/ml.

DNA sequencing and sequence analysis

Amplified DNA fragments and constructs were sequenced by the Macrogen sequencing service (Macrogen Europe, Amsterdam, Netherlands). Sequence annotation and a database search for sequence similarities were completed using the BLAST program of the National Center for Biotechnology Information – NCBI.

Genome sequencing and analysis

Genomic DNA was extracted by the method described by Hopwood et al. (1985) with minor modifications: logarithmic phase cells were pre-treated with lysozyme (4 mg/ml, for 15 min at 37°C) prior to treatment with 2% SDS. The genome sequences of *B. cereus* BGNM1, *S. agalactiae* B782WT, *S. agalactiae* B782R1, *S. agalactiae* B782R2, B782R3 and *S. agalactiae* B761 were determined using Illumina HiSeq using the MicrobesNG service (MicrobesNG, IMI-School of Biosciences, University of Birmingham, Birmingham, UK). The genomic sequence of *B. cereus* BGNM1 was analysed for MLST using the PubMLST scheme for *Bacillus cereus* (https://pubmlst.org/organisms/bacillus-cereus).

The genome sequence of *B. cereus* BGNM1 was additionaly analysed using the online bioinformatics tools AntiSMASH -Antibiotics & Secondary Metabolite Analysis Shell (Medema et al. 2011) (https://antismash.secondarymetabolites.org/#!/start) and BAGEL4 - identification of genes encoding bacteriocins and non-bactericidal post-translationally modified peptides (van Heel et al. 2018) (http://bagel4.molgenrug.nl/) with a view to detect genes encoding potential antimicrobial compounds.

Genome alignment of *S. agalactiae* B782WT and resistant mutants was performed using progressive Mauve (Darling et al. 2004).

Results And Discussion

Isolation and identification of the antimicrobial producing strain

Approximately one thousand colonies were isolated from 13 samples grown on different media. From these, seven colonies were chosen as potential producers of antimicrobial molecules active against at least one of the tested pathogenic strains. Following purification of the isolates and confirmation of antimicrobial production, it was found that one isolate (BGNM1), that originated from soil, showed a markedly larger zone of inhibition against the Gram-positive indicator strain *Listeria monocytogenes* ATCC19111, (20 mm zone) (Fig. 1) and was chosen for further analysis. 16S rRNA gene sequence (BLAST) analysis of the antimicrobial producing isolate revealed that *Bacillus cereus* BGNM1 showed 100% identity with many *Bacillus cereus* strains including A1, M13, FDAARGOS_798, WPySW2, DLOU. According to the PubMLST scheme, *B. cereus* BGNM1 belongs to ST1465, clonal complex ST-142. Representatives of *B. cereus* ST1465 were isolated from primary cutaneous anthrax-like infection in newborn infants in India. However, their role in the development of cutaneous lesions has been considered arguable since strains belonging to ST-142 clonal complex are generally regarded as foodborne with potential to cause foodborne illness (Saikia et al. 2019). In this study, the number of antimicrobial producing colonies was very low, which is in contrast to other surveys where up to 68% of

tested bacterial isolates produced antimicrobial substances (Ahmed Sheikh 2010; Motta et al. 2004). The choice of indicator strains, especially significant human pathogens that are not closely related to the producers of antimicrobial molecules, may have impacted the positivity rate.

Bacteria from the genus *Bacillus* and related genera produce many substances with antimicrobial activity, including non-ribosomally synthesized lipopeptides and peptides (Fischbach and Walsh 2006; Marahiel and Essen 2009), polyketide compounds (Weissman and Leadlay 2005) and bacteriocins (Le Marrec 2000; Kiss 2008). To determine the nature of the antimicrobial activity produced by BGNM1, it was treated with a crystal of the proteolytic enzyme, pronase E. Following incubation, no antimicrobial activity was detected in the vicinity of the crystal on all tested indicator strains, thereby confirming its proteinaceous nature (Fig. 1).

Spectrum of activity of *Bacillus cereus* BGNM1 cell free supernatant

The antimicrobial activity of *B. cereus* BGNM1 CFS was tested against various Gram-positive and Gram-negative spoilage and pathogenic bacteria (Table 2). The CFS of strain BGNM1 inhibited the growth of most Gram positive tested, and just three Gram negative bacteria (*Ralstonia solanacearum, Xanthomonas oryzae* and *Erwinia amylovora*) (Table 2). During testing, it was noticed that some isolates of *S. agalactiae* species were sensitive while others were resistant to the CFS of *Bacillus cereus* BGNM1 (Table 2).

Table 2
Antimicrobial activity of BGNM1 CFS

Indicator strains	Activity
Staphylococcus aureus ATCC25923	+
Listeria monocytogenes ATCC19111	+
Streptococcus agalactiae B782	+
Streptococcus agalactiae B761	-
Lactococcus lactis BGMN1-596	+
Bacillus cereus ATCC11778	+
Bacillus subtilis subsp. *subtilis* ATCC23857	+
Ralstonia solanacearum	+
Xanthomonas oryzae	+
Erwinia amylovora	+
Acinetobacter baumanii 6077/12	-
Burkholderia cenocepacia ST856	-
Salmonella Enteritidis ATCC13076	-
Escherichia coli ATCC 25922	-
Klebsiella pneumoniae Ni9	-
Pseudomonas aeruginosa MMA83	-

Genome sequence analysis shows that *Bacillus cereus* BGNM1 strain encodes, the two-peptide lantibiotic, thusin

The genome of *B. cereus* BGNM1 was sequenced and summary statistics for genome assemblies is presented in Supplementary Table S1. The draft genome sequence of isolate BGNM1 was deposited in the NCBI GenBank database under accession number GCA_013303115.

AntiSMASH and BAGEL4 analyses of BGNM1 genome sequence revealed the presence of a ~ 10 kb gene cluster that is 100% identical to the gene cluster responsible for the production of two-peptide lantibiotic thusin in *Bacillus thuringiensis* BGSC 4BT1 (Xin et al. 2016) (GenBank Accession number KT454399.1). There was no evidence of additional genetic determinants in BGNM1 genome that were likely to encode other antimicrobial compounds, suggesting that the antimicrobial activity of the BGNM1 strain could be entirely attributable to the production of thusin.

Purification of thusin produced by *Bacillus cereus* BGNM1

The antimicrobial molecule(s) produced by BGNM1 was purified by Amberlite XAD16N, C18 SPE and RP-HPLC and the molecular mass of the active peptides confirmed by MALDI-TOF mass spectrometry. HPLC fractions collected at 30 second intervals were assayed against *Lactococcus lactis* BGMN1-596 and activity detected in fractions 75–77 (Fig. 2A) corresponding to a single peak eluting at 37.5 minutes on the HPLC chromatogram (Fig. 2A). MALDI TOF mass spectrometry analysis revealed that fraction 76 contained masses of 3926 Da and 2908 Da (Fig. 2B), which correspond with the theoretical masses of the individual thusin peptides, Thsα (3928 Da) and Thsβ (2908 Da), respectively (Xin et al. 2016). This suggests that the peptides were co-eluting. Further attempts to separate the peptides using an analytical RP-HPLC column and shallower acetonitrile gradients were unsuccessful. However, the successful purification of thusin from BGNM1 CFS and the lack of evidence of production of other antimicrobials is in agreement with the genome sequence data.

Selected thusin-resistant mutants of the thusin-sensitive strain *Streptococcus agalactiae* B782 show higher level thusin resistance than naturally resistant strains

The spectrum of activity of BGNM1 CFS showed that some *Streptococcus agalactiae* strains were resistant to thusin while others were naturally sensitive, suggesting differences in the cell envelope of natural isolates. To identify the target molecule/structure for thusin on the cell membrane, random mutagenesis was used to generate thusin resistant mutants from an *S. agalactiae* sensitive strain, with a view to comparing induced mutations in thusin resistant derivatives with naturally occurring resistant isolates. MNNG (200 µg/ml) was used to generate random mutations in wild-type *Streptococcus agalactiae* B782, a strain naturally sensitive to thusin, with a survival rate of about 2%. Thusin-resistant mutants were selected on BHI agar plates containing 2x concentrated cell-free culture supernatant of the thusin-producing strain, BGNM1. Twenty-six colonies grew on the selective plates after incubation for 48 h at 37°C in an atmosphere of 5% CO_2. All mutants showed a thusin-resistant phenotype in repeated antimicrobial tests. Comparative analysis of MIC values, obtained by broth microdilution method, of the thusin-resistant mutants, named B782R1, B782R2 and B782R3 (0.368 µM), with the thusin sensitive parental strain, *S. agalactiae* B782 (0.046 µM), showed that the resistant mutants have eight times higher MIC values. Furthermore, comparison with *S. agalactiae* B761, the naturally resistant strain, showed that mutants express higher level of resistance to thusin (0.368 µM) comparing to naturally resistant isolates (0.184 µM). Similar results of MIC determination were obtained by spot-on-the-lawn inhibition assay on Petri dish (Fig. 3). Since different lantibiotics, both single-peptide and two-peptides, have been found to target the same receptor on the cell membrane (Islam et al. 2012; Morgan et al. 2005), thusin resistant *S. agalactiae* mutants were tested for nisin sensitivity. A wide range of nisin concentrations of double dilutions were used to determine MIC values for thusin sensitive and resistant derivatives. Nisin sensitivity results showed that there is no difference in nisin sensitivity between thushin sensitive and resistant derivatives (250 IU/ml) indicating that there is no cross resistance for these two lantibiotics, ie that these two lantibiotics use different receptors to interact with sensitive cells and achieve antimicrobial effect.

Comparative analysis of the genome sequences of *Streptococcus agalactiae* B782 mutants reveals that the C-protein α antigen is involved in thusin resistance

A genome sequencing approach was used to identify gene(s) involved in the thusin resistance phenotype. Specifically, the genomes of three of the 26 thusin-resistant mutants (*S. agalactiae* B782R1, B782R2 and B782R3), and one naturally thusin-resistant clinical isolate, *S. agalactiae* B761, were sequenced and compared to *S. agalactiae* B782WT, the wild type sensitive strain. Genome alignment of *S. agalactiae* B782WT and resistant mutants is presented in Fig. 4.

Sequence analysis showed that all three newly generated thusin-resistant mutants (B782R1-B782R3) possessed a common duplication of a region encoding 79 amino acids of the repeat domains in the C-protein α antigen compared to those of the sensitive WT strain, *S. agalactiae* B782WT. No other common gene mutations were identified. This suggests that an intact C-protein α antigen, which is an important cell surface protein contributing to the virulence and immunity of *S. agalactiae* (Lindahl et al. 2005; Maeland et al. 2015; Michel et al. 1991), is necessary for the activity of thusin against *S. agalactiae*. Specifically, genome analysis of *S. agalactiae* B782WT showed that it possesses two genes annotated as the C-protein α antigen; one is a truncated gene encoding a 109 amino acid protein and the second complete, encoding a protein with 79 amino acid repeat regions. Thusin induced resistant mutants *S. agalactiae* B782R1, B782R2 and B782R3 also have two such genes, with the truncated gene unchanged, but the second containing additional duplications of the region encoding 79 amino acids potentially resulting in thusin resistance. Genome analysis of the naturally resistant *S. agalactiae* B761 strain, revealed deletions within both genes encoding the C-protein α antigen containing only the C-terminal part of C protein II, thus confirming its involvement in thusin resistance. Taken together, the results strongly suggest that the C-protein α antigen is critical to the activity of thusin against *S. agalactiae* and that resistance evolves through multiplication of the region encoding repeatable 79 aa domains, most likely changing the 3D structure and preventing direct interaction, or deletion mutations of the gene. It is interesting that both types of changes in the C-protein α antigen, through either prolongation of the protein or its deletion, lead to the resistance to thusin (Fig. 3), indicating that in both cases the ability of thusin to inhibit is reduced. Species other than *S. agalactiae* are also sensitive to thusin, but do not have C-protein α antigen or similar protein. In addition, structural changes in C-protein α antigen lead to an increase in MIC values, only. Thus C protein α antigen is probably not a receptor protein for thusin, but is definitely involved in modulation of the thusin-receptor interaction. Variations in the number of repeating domains within cell-surface proteins of streptococci have previously been shown to impact significantly on their biological functions. More specifically, changes in the number of tandem repeats within the C-antigen proteins of streptococci have been shown to affect their immune evasion and pathogenicity in immunized hosts, where proteins with lower number of repeats were probably hidden by capsular polysaccharide and other cell surface proteins resulting in decrease in antigen size (Gravekamp et al. 1996). Furthermore, changes in a number of R28 protein tandem repeats, among alpha-like proteins of streptococci, resulted in changes in transcriptional levels of the gene encoding the given protein and, in turn, alternations of virulence and global transcriptomic changes (Eraso et al. 2020). Variability in Rib (resistance to proteases, immunity, group B) domain number of cell-surface proteins has also been

shown to result in differential projection of key host-colonization domains affecting immune evasion of streptococci (Whelan et al. 2019). With this in mind, we speculate that the increase in 79 amino acid repeats within C-protein of group B streptococci could be an adaptive trait that enables survival in the presence of thusin as a selective agent. By multiplication of the 79 amino acids domain in C-protein α antigen *S. agalactiae* achieves a higher level of resistance to thusin, which indicates that in this way C-protein α antigen masks the thusin binding site on the receptor. Since *S. agalactiae* modulates the number of 79 aa repeats of the C-protein α antigen as an adaptation to the host immune response, this modulation could be successfully used to influence the thusin-receptor interaction, indicating an even more significant role of this surface protein in survival and infection. Further research is needed to elucidate all relevant participants for thusin activity, mutual positions of C-protein α antigen and membrane receptor as well as whether direct interaction between C-protein α antigen and thusin occurs and, if so, which domains are involved.

Declarations

Declarations of interest: none.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Author contributions

The study was conceptualized by MK and NM. Laboratory work was done by NM, MO, POC and MK. Whole-genome analysis was carried out by BF and MK. Supervision, Project administration and Funding were carried out by MK. The manuscript was drafted by NM, MO and POC, reviewed by BJ and edited by MK, POC and PC. All authors have read and approved the final version of the manuscript.

Ethical statement

This study does not describe any experimental work related to human or animals.

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Figures

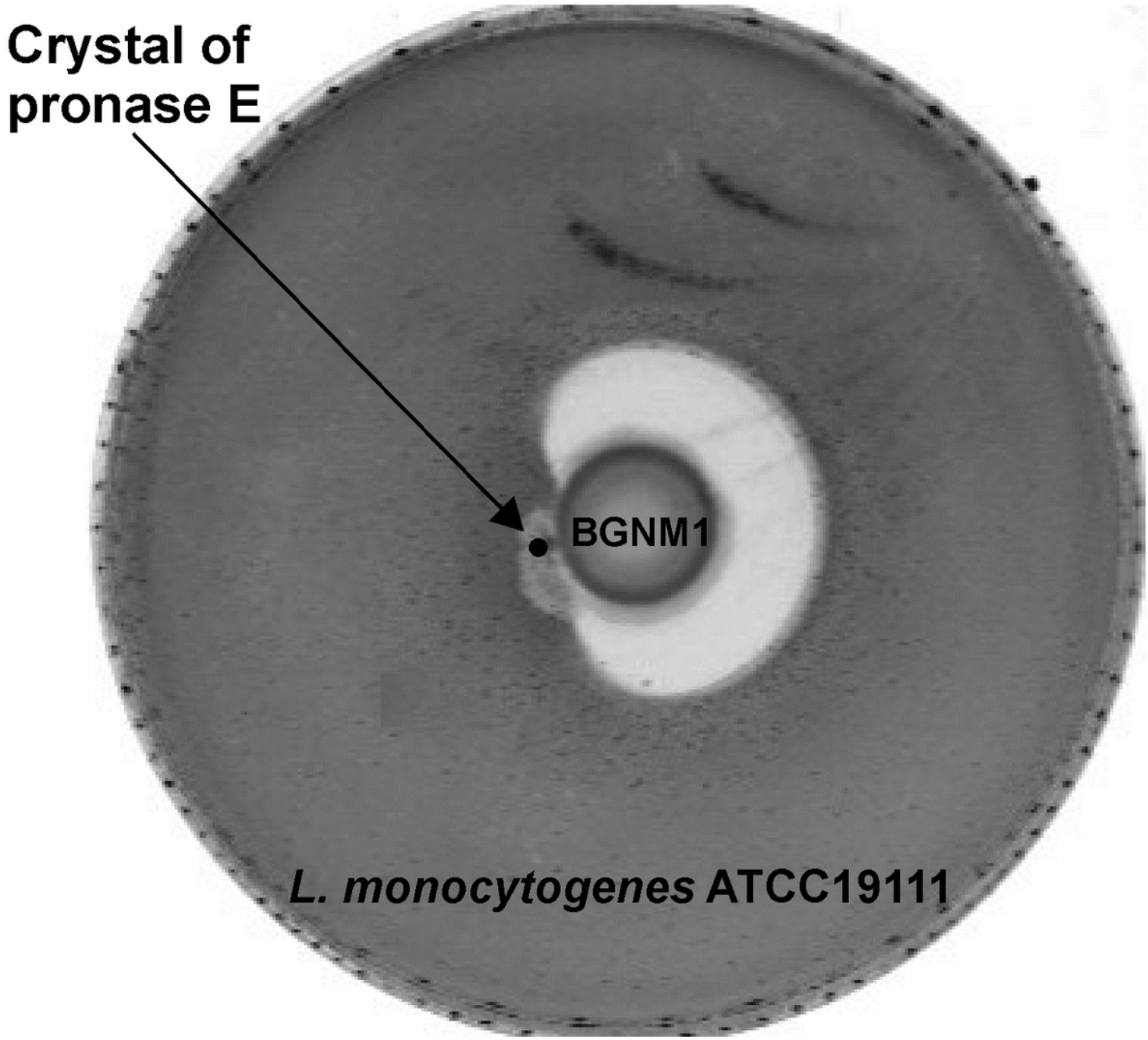

Figure 1

Antimicrobial activity of Bacillus cereus BGNM1 against Listeria monocytogenes ATCC19111. The position of added pronase E crystal is indicated by a dot. Typical inhibition of antimicrobial activity in the vicinity of the pronase E crystal was obtained confirming the protein nature of the inhibitory molecule.

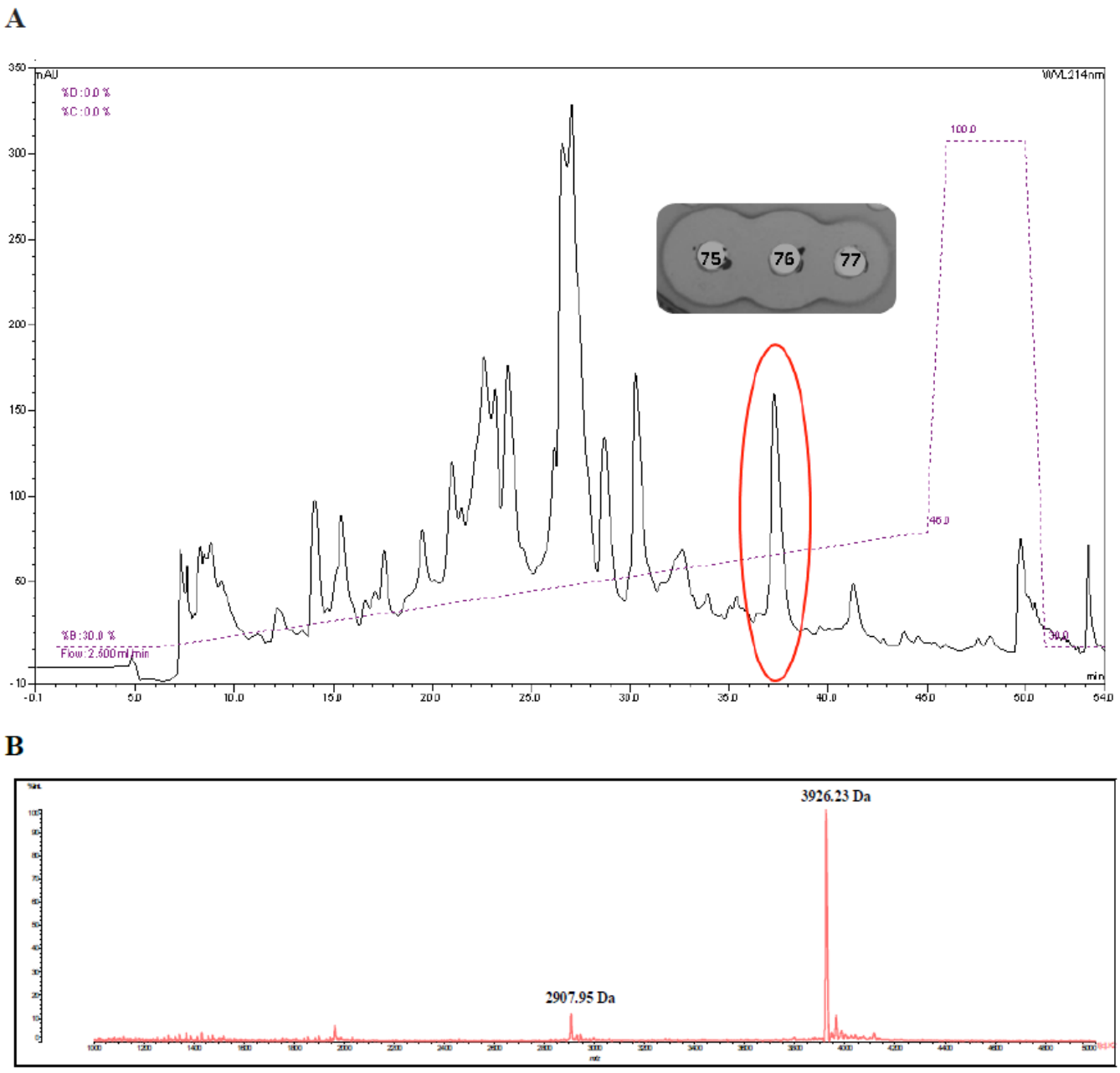

Figure 2

Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram of BGNM1 CFS with marked active peak (red ellipsoidal circle) and antimicrobial test of active fractions (75, 76 and 77) corresponding to active peak (A), and MALDI TOF mass spectrometry of central and most active fraction 76 (B).

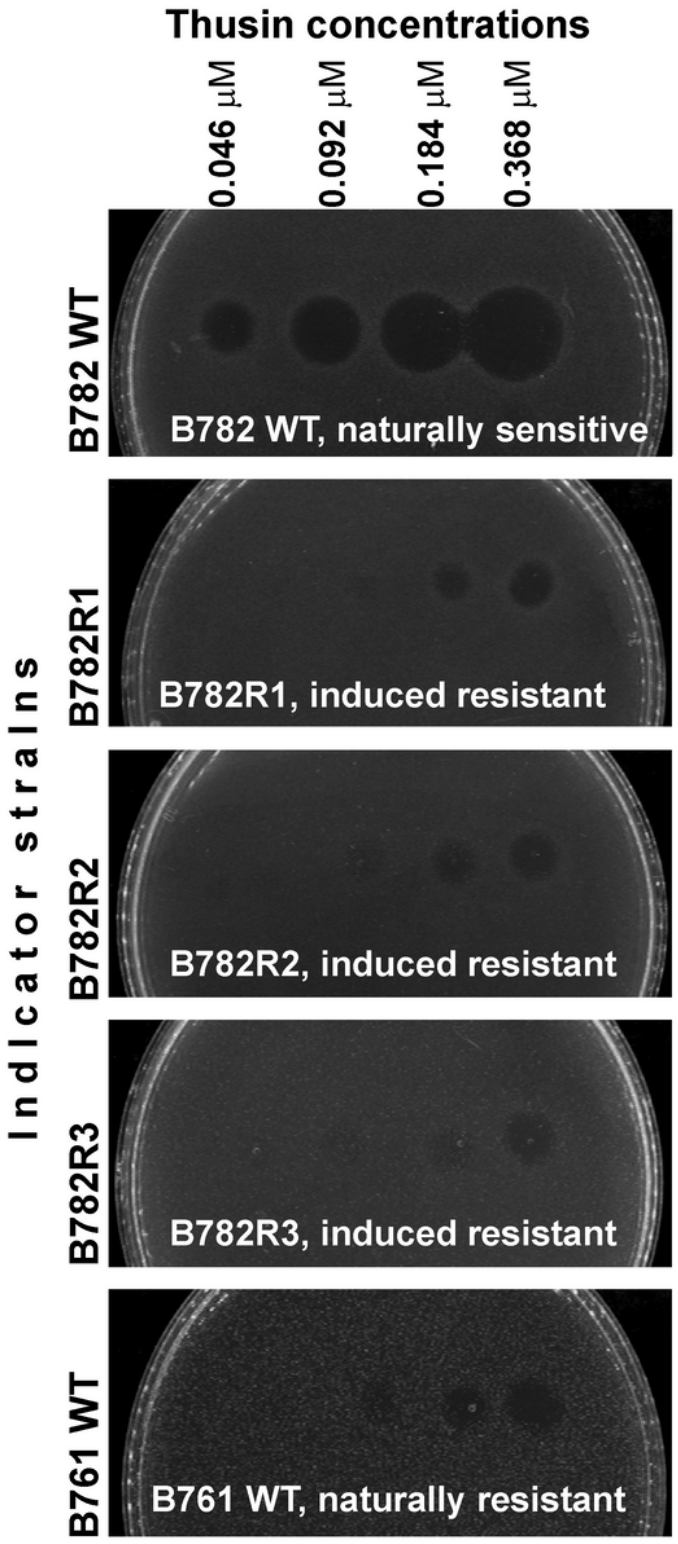

Figure 3

Spot on lawn antimicrobial assay of different concentrations of thusin on susceptible and resistant strains/mutants of Streptococcus agalactiae. 5 µl of different concentrations (0.046, 0.092, 0.184 and 0.368 µmol) of purified thusin were spotted on the surface of top agar inoculated with tested strains/mutants and incubated at 37°C in an atmosphere of 5% CO2.

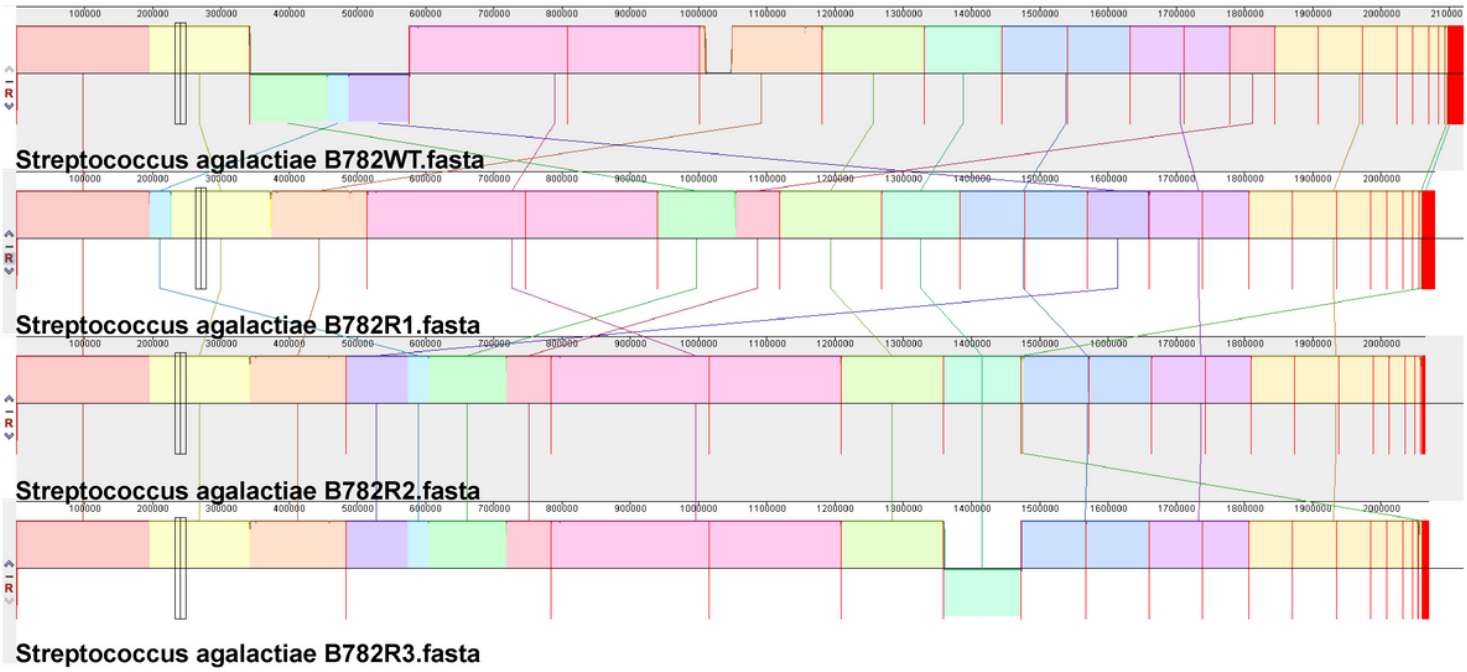

Figure 4

The progressive Mauve genome alignment of S. agalactiae strains. Bounded boxes indicate similar sequence composition among sequences.

Supplementary Files

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