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Heterologous production of a cyanobacterial bacteriocin with potent antibacterial activity

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ABSTRACT

With regard to the emerging health threat by antibiotic resistant bacteria, bacteriocins are considered as a promising way to overcome this urgent problem. In this work, a hitherto unknown bacteriocin from the terrestrial cyanobacterium *Chroococcidiopsis cubana* was heterologously expressed in *Escherichia coli*, purified to homogeneity and tested for activity against several bacteria and one yeast species. The compound showed potent bacteriolytic activity against several Gram-positive bacteria and slight activity against one Gram-negative strain. The bacteriocin had no cytotoxic impact on mouse neuroblastoma N2a cells, indicating its potential for treatment against Gram-positive bacterial pathogens in human diseases.

Introduction

Cyanobacteria are a rich source of different bioactive substances and valuable secondary metabolites (Carroll et al., 2019; Lakatos & Strieth, 2017; Singh et al., 2020). On the one hand there are the widely known cyanobacterial toxins as Anatoxin A from *Anabaena* (Mahmood & Carmichael, 1987) and Microcystin from *Microcystis* (Konst et al., 1965), which are very harmful for mammals and water organisms. On the other hand, cyanobacteria produce numerous valuable secondary metabolites such as pigments, long-chain fatty acids, proteins and polysaccharides, which can be, and partly already are used in food industry (Borowitzka, 1995; Burja et al., 2007). Additionally, cyanobacteria also produce a wide range of bioactive substances with potent activity against other bacteria (Ghasemi et al., 2004; Volk & Furkert, 2006), fungi (Shishido et al., 2015), viruses (Carpine & Sieber, 2021) and also tumour cells (Silva-Stenico et al., 2011). Many of these metabolites are produced by nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), or PKS-NRPS hybrids, large multienzyme complexes which assemble and modify single peptides to one bioactive molecule (Jones et al., 2009; Welker et al., 2012). However, next to this mode of complex molecule synthesis, many cyanobacteria also generate ribosomal peptides with very promising biological activities – the bacteriocins. These small molecules are produced by a wide range of organisms, but especially lactobacterial bacteriocins were in focus of research for a long time (Gradisteanu Pircalabioru et al., 2021). The most prominent representative nisin, produced by *Lactococcus lactis*, is a widely applied antibiotic peptide for the prevention of bacterial growth in the food industry (Delves-Broughton et al., 1996). Most bacteriocins belong to the heterogeneous class of ribosomally-synthesized and post-translationally-modified peptides (RiPPs), with the exception of class II lactic acid bacterial bacteriocins, which are unmodified or cyclic. The superordinate term RiPPs comprises a large number of diverse subclasses which are produced from many different prokaryotic and eukaryotic organisms and undergo various posttranslational modifications (Arnison et al., 2013). However, the mode of biosynthesis is a commonality that all RiPPs share. The ribosomally synthesized precursor peptide contains the core peptide, which is N-terminally flanked by a leader peptide and sometimes also C-terminally by a recognition sequence. The latter can be important for further processing of the core peptide. Eukaryotic precursor peptides often contain an additional signal sequence at the N-terminus of the leader peptide. During the maturation process the core peptide gets enzymatically cleaved from the leader peptide und can undergo further modifications. Whereas the leader regions of all RiPPs show significant sequence similarities, the core peptides are extremely variable (Oman and van der Donk, 2010).

Since the discovery of nisin in 1933 (Whitehead, 1933), Gram-positive bacteria and especially lactobacteria were in focus of bacteriocin research. However, also Gram-negative bacteria receive attention as bacteriocin producing organisms. Well-known examples are the colicins and microcins from *E. coli* and some other *Enterobacteriaceae* (Luria & Suit, 1987). More recently, the group of cyanobactins was

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described, which contains small cyclic peptides that possess highly diverse bioactivities and only occur in cyanobacteria (Sivonen et al., 2010; Welker & Von Döhren, 2006). A major breakthrough in the identification of novel putative bacteriocins became possible by the massive advances of the *in silico* techniques. The number of sequenced genomes is quickly growing, enabling a rapid search for conserved motifs of already known bacteriocin gene clusters. By this, a double-glycin-type motif was identified as a conserved amino acid sequence of bacteriocins in Gram-negative bacteria, among them also several cyanobacterial genera such as *Synechococcus*, *Synechocystis*, *Nostoc* and *Prochlorococcus* (Dirix et al., 2004). As subclasses of these double-glycine-type precursor peptides, the nitrile hydratase leader (NHLP) family and the N11P family, which is annotated as Nif11 nitrogen-fixing protein, were identified in cyanobacteria (Haft et al., 2010).

The cyanobacterial genus *Chroococcidiopsis* is known to include extremely desiccation and radiation tolerant strains and was therefore even treated as a possible candidate for terraforming Mars (Imre Friedmann and Ocampo-Friedmann, 1995; Billi et al., 2011). *Chroococcidiopis* appears as single coccoid cells which duplicate through binary fission (Rippka et al., 1979). Strain *C. cubana* SAG 39.79 was isolated from a soil sample (Pinar del Rio, Cuba) in 1966 (Komárek & Hindak, 1975). In antimicrobial screenings prior to this work, *C. cubana* showed hints for producing growth inhibiting substances effective against *Micrococcus luteus* (data not shown). Therefore, this terrestrial cyanobacterium was further investigated by bioinformatic- and molecular biological attempts.

Material and methods

Generation of expression strains

The DNA sequence of *b135CC* was amplified from *C. cubana* gDNA by PCR (Phusion DNA Polymerase, Thermo Scientific™, Waltham, USA), using the primers 5′-AAAAGGATCCATGAAAGCAAGTACT AACTTTAC-3′ and 5′-AAAACTCGAGGCTAACTATCCCTGTCACCG-3′ (restriction sites underlined). The gDNA was extracted with the NucleoSpin Tissue Mini Kit (Macherey-Nagel, Düren, Germany). The amplified DNA was purified by agarose gel electrophoresis (1 % w/v) and retrieved with the GeneJet Gel Extraction Kit (Thermo Scientific™, Waltham, USA). *b135CC* was cloned into the vector pJET1.2/blunt (CloneJET PCR Cloning Kit, Thermo Scientific™, Waltham, USA) and competent *E. coli* NEB 5-alpha cells (New England Biolabs, Ipswich, USA) were transformed with the resulting recombinant plasmid. Correctly transformed cells were identified on LB agar (1.5 % w/v) with ampicillin (100 μg ml^{-1}) and by analytical PCR. The insert was cut from pJET1.2/blunt using the restriction enzymes *Bam*H I and *Xho* I (Thermo Scientific™, Waltham, USA) and subsequently cloned into the digested expression vector pET23a (Merck, Darmstadt, Germany). The plasmid was used for transformation of competent *E. coli* LEMO21 (DE3) cells (New England Biolabs, Ipswich, USA). Correctly transformed cells were identified on LB agar (1.5 % w/v) with ampicillin (100 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}), as well as by analytical PCR. All DNA ligation steps were carried out with T4 DNA ligase (Thermo Scientific™, Waltham, USA). Isolation of plasmids was done with the NucleoSpin Plasmid Mini Kit (Macherey-Nagel, Düren, Germany). The correct orientation and sequence of the DNA insert was checked by sequencing (LGC Genomics GmbH, Berlin, Germany). All procedures were carried out according to the manufacturers' recommendations.

Protein expression

Main cultures of the expression strain were inoculated with 2 % of an overnight-grown preculture (30 °C, 200 rpm, TR-125, Infors AG, Bottmingen, Switzerland). LB supplemented with 100 μg ml^{-1} ampicillin, 25 μg ml^{-1} chloramphenicol and 1 mM L-rhamnose was used as cultivation medium. The expression was induced at OD_{600} 0.8 with 400 μM IPTG. The main culture was cultivated at 37 °C and 200 rpm for 20 h.

Cell harvest and cell disruption

Cell harvest was performed for 15 min at 2360 x *g* (Rotanta 460 R, Hettich Zentrifugen, Germany), followed by a wash step with 0.9 % (w/v) sterile saline. Cells were disrupted via ultrasonic probe (Bandelin HD 70, UW 70, Bandelin, Berlin, Germany; 5 × 30 s, 100 % power; 1 min cooling on ice between each cycle).

Protein purification

The cell extract was clarified by centrifugation at 2360 x *g* for 100 min (Rotanta 460 R, Hettich Zentrifugen, Tuttlingen, Germany). Purification of the bacteriocin was achieved by affinity chromatography using a HisTrap™ HP 5 ml column and an Äkta™ start FPLC system (Cytiva Europe GmbH, Freiburg Germany). Binding buffer: 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 5 mM imidazole, pH 7.4; elution buffer: 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 0.5 M imidazole, pH 7.4. The bacteriocin was desalted with a HiTrap™ 5 ml desalting column (Cytiva Europe GmbH, Freiburg, Germany), using 20 mM NaH_2PO_4/Na_2HPO_4 buffer. The protein concentration was determined by Bradford assay.

Bioactivity test

The growth assays of the indicator bacteria were performed in 96 well plates (Nunc™ MicroWell™ 96-Well Microplates, Thermo Scientific™, Waltham, USA). Per well, a volume of 50 μl bacterial suspension (OD_{600} 0.2) was mixed with 50 μl prediluted bacteriocin suspension. Final B135CC concentrations of respectively 150, 100 and 50 μg ml^{-1} were used for *M. luteus*, *M. phlei* and *S. auricularis*; reduced concentrations of 25, 12.5, 6.25 and 5 μg ml^{-1} were applied for *S. auricularis*. All used bacteria were cultivated in Müller-Hinton medium and harvested in the exponential growth phase. The bacterial suspensions were diluted with Müller-Hinton medium, the bacteriocin dilutions were done with 20 mM NaH_2PO_4/Na_2HPO_4 buffer.

Protein leakage test

Samples of *S. auricularis* (OD_{600} 1.0) were mixed with different concentrations of B135CC, followed by an incubation period of 6 h at 30 °C. Samples were taken every two hours, the protein concentrations were determined by Lowry-Assay (Pierce™ Modified Lowry Protein Assay Kit, Thermo Scientific, Massachusetts, USA). The respective share of total *S. auricularis* cell protein was determined through comparison with cells disrupted by ultrasound (Bandelin HD 70, UW 70, Bandelin, Berlin, Germany; 5 × 30 s, 100 % power; 1 min cooling on ice between each cycle).

Cytotoxicity assay

The cytotoxicity of B135CC was tested by Lactate Dehydrogenase (LDH) activity test. The assay was performed with the Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), as specified by the manufacturer. The bacteriocin was diluted with N2a medium (DMEM high glucose, 1 % penicillin/streptomycin, 1 % sodium pyruvate, 1 % L-glutamine, 1 % nonessential amino acids (all from Gibco, Thermo Fisher Scientific, Massachusetts, USA), and 10 % FBS (PAN Biotech, Aidenbach, Germany) to a final volume of 100 μl and added in different concentrations to the N2a cells in a 96-well plate, followed by an overnight incubation at 37 °C and 5 % CO_2. Addition of 100 μl 10 mM HEPES

buffer (vehicle of the protein) to the N2a cells (neuroblastoma mouse cells) was used as a control. Further tests for positive and negative control were carried out the following day using lysis buffer for maximum LDH release and distilled H_2O to measure spontaneous LDH activity, respectively.

Results and discussion

As the genome of *C. cubana* was sequenced for phylogenetic analyses in 2019 (Will et al., 2019), an *in silico* scan for biosynthetic gene clusters could be performed using the open source online platform *antiSMASH* (Blin et al., 2019). Besides several NRPS and PKS domains, four bacteriocin precursor encoding gene clusters (locus tags ctg7_159, ctg38_34, ctg42_31/32 and ctg135_5) were identified. All four genetic sequences were amplified by PCR and used to generate *E. coli* expression strains, of which only strain *E. coli* LEMO21 pET23a::*ctg135_5* produced a significant amount of recombinant protein. The associated putative bacteriocin was designated as B135CC. The corresponding gene was identified by *antiSMASH* as an RiPP-like protein encoding sequence, which contains an NHLP family leader peptide domain (TIGR03793). Upstream of *ctg135_5* a short-chain dehydrogenase was identified as a putative additional biosynthetic gene (Fig. 1a). In a sequence alignment with four other cyanobacterial NHLP-like proteins from *Nostoc* sp. PCC 7120 (WP_010996185), *Anabaena variabilis* (WP_011317978), *Nostoc punctiforme* (WP_012409643) and *Nostoc* sp. PCC 7120 (WP_010996188), the high degree of sequence homology in the leader peptide region became evident (Fig. 1B, N-terminal sequence area up to peptidase cleavage site).

Interestingly, the conserved region containing the typical double glycine peptide cleavage site (Fig. 1B, red box) differs from the other sequences, as the second glycine (N to C terminus) is replaced by a histidine. However, it is known that only the first glycine is constantly conserved in this region, while the second glycine can be substituted by other amino acids (Havarstein et al., 1994). Although leader peptide sequences of double-glycine-type motif bacteriocins tend to be highly conserved, the core peptides are hypervariable (Oman and van der Donk, 2010). That can also be noticed in the depicted sequence alignment (Fig. 1B). However, the core peptide of B135CC is significantly longer than those of the compared precursor proteins. In case of most other bacteriocin gene clusters found in cyanobacteria, several other domains involved in bacteriocin production, maturation and transport were found in close proximity to the bacteriocin core gene(s) (Wang et al., 2011). As already elucidated in 1995, bacterial bacteriocins are transported across the cytoplasmic cell membrane by ATP-binding cassette (ABC) transporters, which contain a C39 peptidase domain concomitantly cleaving the leader peptide off the core peptide. The associated genes are mostly situated next to the precursor peptide encoding genes. NHLP like bacteriocin precursors were identified in association with thiazole/oxazole-modified microcins (TOMM) (Haft et al., 2010). These posttranslational modifications are performed by the combined action of a cyclohydratase, a dehydrogenase and a docking/scaffolding protein, which work on serine, threonine and cysteine residues in the core peptide to form the according heterocycles. The associated genes are generally located next to the precursors. B135CC also contains an NHLP leader peptide domain and ten threonine and seven serine residues, which could be converted to oxazoles and methyloxazoles, respectively. However, there is no evidence for this assumption in case of B135CC.

The bacteriocin B135CC was heterologously produced with *E. coli* Lemo21 (New England Biolabs GmbH) and purified by affinity chromatography taking advantage of the C-terminal poly His-tag conferred by the used medium-copy expression vector pET23a (Fig. 2).

The result of the SDS-PAGE (Coomassie staining) showed a full size protein (of approx. 29.3 kDa) that was not cleaved by an *E. coli* peptidase, although *E. coli* generally possesses several ABC transporters containing a C39 peptidase domain. The purified protein was subsequently tested for antimicrobial activity by microtiter plate growth assays against the Gram-positive bacteria *Bacillus subtilis*, *Micrococcus luteus*, *Mycobacterium phlei* and *Staphylococcus auricularis*, the Gram-negative strains *Acinetobacter baylyi*, *Pseudomonas fluorescens*.

E. coli, as well as against the yeast *Candida auris* (Fig. 3, only results of sensitive strains shown).

The bacteriocin B135CC from the terrestrial cyanobacterium *C. cubana* showed a slight inhibitory impact on the growth of *P. fluorescens* and significant effects on *M. luteus* (150 µg ml^{-1}), *M. phlei* (100 and 150 µg ml^{-1}) and *S. auricularis* (5, 6.25, 12.5 and

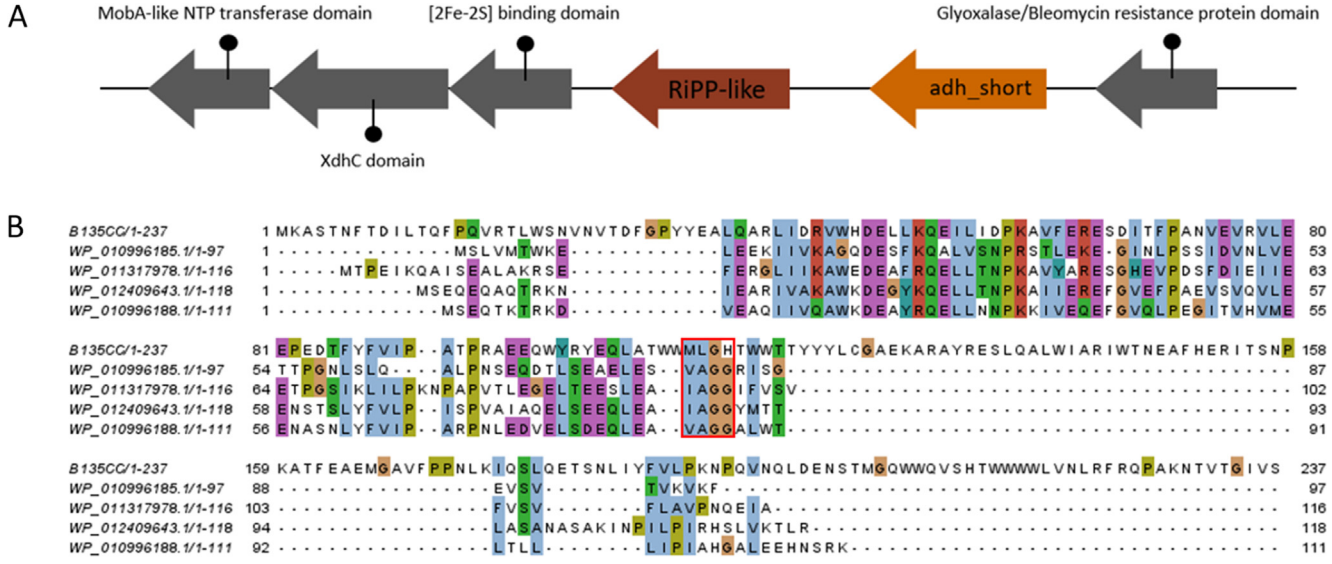

Fig. 1. A, Relevant genomic region of *b135CC*, putative gene products and protein domains identified by *antiSMASH*. Black pins indicate predicted protein domains. B, Sequence alignment of B135CC (locus tag ctg135_5) with four cyanobacterial NHLP-like proteins from *Nostoc* sp. PCC 7120 (WP_010996185), *Anabaena variabilis* (WP_011317978), *Nostoc punctiforme* (WP_012409643) and *Nostoc* sp. PCC 7120 (WP_010996188). Sequence alignment done with the online tool *MUSCLE* (Madeira et al., 2019), image created with *Jalview* (Waterhouse et al., 2009). Red box indicates the putative double glycine leader peptide cleavage site.

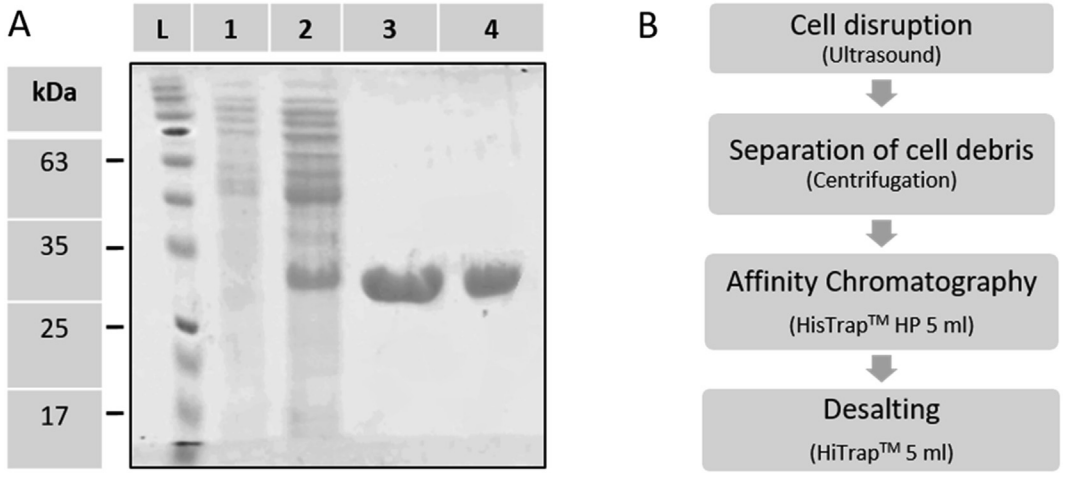

Fig. 2. A, Purification of the His-tagged bacteriocin B135CC. Protein expression was performed in *E. coli* LEMO21 (DE3) pET23a::*b135cc* (New England Biolabs GmbH). L, protein ladder (SERVA Triple Color Proteinstandard III); 1, cell-free extract, empty vector control; 2, cell-free extract; 3, HisTrap elution fraction; 4, desalted HisTrap elution fraction. Stained with Quick Coomassie Stain, Serva, Heidelberg, Germany). B, Purification steps.

25 μg ml^{-1}). Growth of the latter strain was strongly inhibited by B135CC, pre-tests using concentrations of 50–150 μg ml^{-1} completely prevented *S. auricularis* growth, therefore dilutions up to 5 μg ml^{-1} were tested for determination of the minimum inhibitory concentration (MIC). Until a bacteriocin concentration of 6.25 μg ml^{-1}, *S. auricularis* growth was completely inhibited. When the B135CC concentration was decreased to 5 μg ml^{-1}, the bacterium could grow to an OD_{595} of 20 %, compared to the negative control. As a conse-

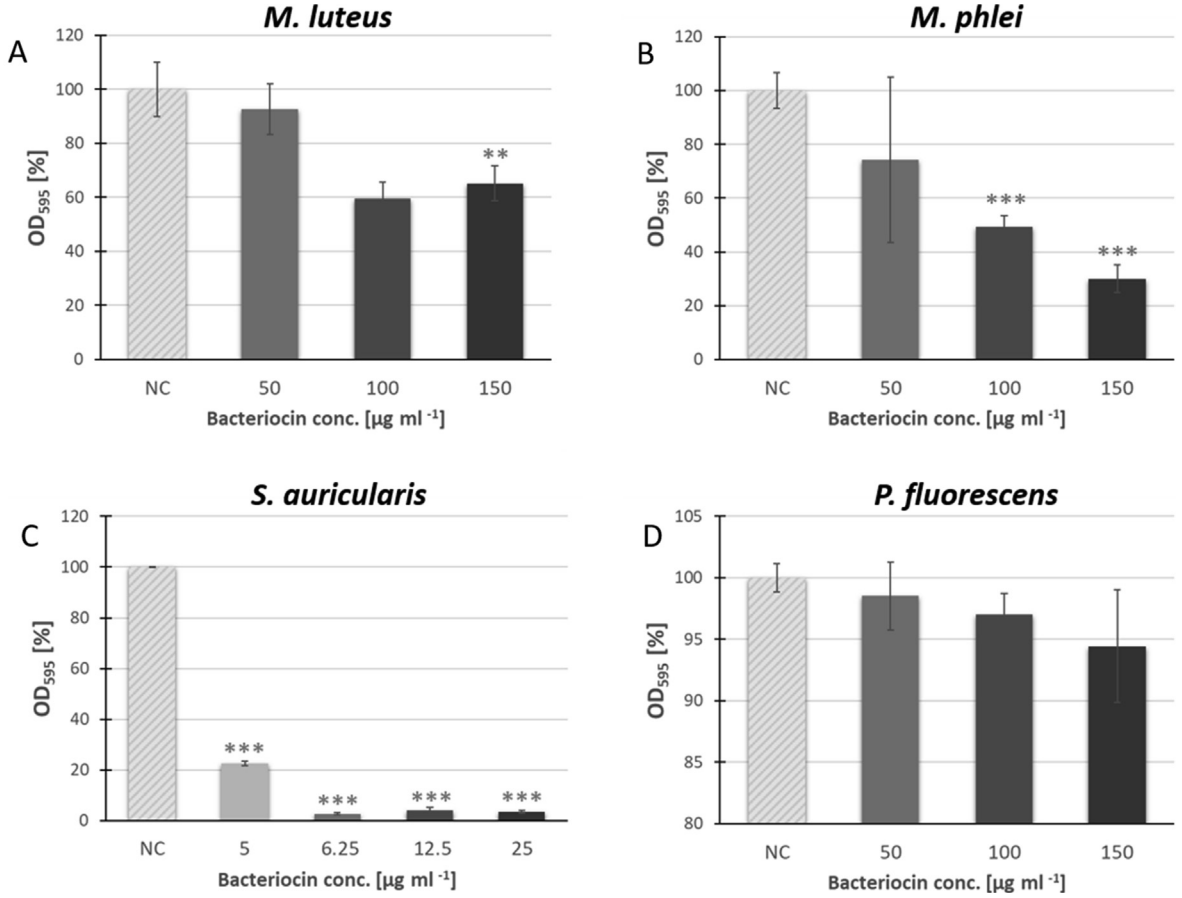

Fig. 3. Bioactivity (microtiter plate) test of B135CC against A, *Micrococcus luteus*; B, *Mycobacterium phlei*; C, *Staphylococcus auricularis* and D, *Pseudomonas fluorescens*. The bars depict the relative OD_{595} of the indicated bacterial strains after growth for 15 h at 30 °C and low agitation on a microtiter plate reader (BioTek EL808, BioTek Instruments Inc., Winooski, USA) with initial addition of different B135CC concentrations. The negative control (NC, 20 mM NaH_2PO_4/Na_2HPO_4 buffer) served as reference. Error bars indicate standard deviation, n = 3; Asterisks indicate significant differences compared to the control (*, $p \leq 0.01$; ***, $p \leq 0.001$), determined by a two-tailed homoscedastic Student's *t*-test.

quence, a bacteriocin concentration of 6.25 μg ml^{-1} was determined as the MIC for *S. auricularis*. The results of the bioactivity test show a strong antimicrobial activity of B135CC towards the Gram-positive strain *S. auricularis*, whereas a significantly lower inhibition was observed against *M. luteus*. Consequently, the bacteriocin does not act as a general antibiotic compound against Gram-positive bacteria, but seems to possess specificity for *S. auricularis* cells. Antibiotics like lyostaphin, a bacteriolytic enzyme produced by *Staphylococcus simulans*, specifically binds to *Staphylococcus aureus* cells, due to a cell wall targeting C-terminal sequence (Baba & Schneewind, 1996). Lyostaphin acts as peptidoglycan hydrolase and thus damages the cell wall of *S. aureus*. Although no evidence for such a cell wall targeting sequence could be found for B135CC, a similar mechanism could explain the potent activity against *S. auricularis*.

In order to illuminate the bacteriocin's mode of action, a protein leakage test was conducted with *S. auricularis* (Fig. 4), as the release of proteins after treatment with the bacteriocin can indicate a bacteriolytic activity (Devi et al., 2010). For this test, B135CC concentrations of 62.5–500 μg ml^{-1} were applied, since high concentrations lead to quick protein releases. A prolonged incubation phase could lead to falsified results because of protease activity.

The results of the protein leakage test (Fig. 4) show a significant release of *S. auricularis* proteins, after treatment with *C. cubana* bacteriocin B135CC. After six hours of incubation, all applied concentrations (62.5–500 μg ml^{-1}) led to a release of about 30–35 % of total cell protein. The negative control without B135CC caused only a minor protein release that climaxed after four hours. The obtained results emphasise that B135CC acts as a bacteriolytic bacteriocin.

For further characterisation of the bacteriocin, a temperature stability test was done with *S. auricularis* (Fig. 5).

Temperature stability tests confirmed a bacteriocin activity till 60 °C. From 70 °C on, activity was significantly diminished. Together with the obtained data about the bacteriocin's activity spectrum and its size, B135CC shows similarities to class III bacteriocins as lysostaphin from *Staphylococcus simulans,* helveticin J from *Lactobacillus helveticus* or the colicin-type family from *E. coli* and some other *Enterobacteriaceae*. These bacteriocins tend to be much larger than class I or II bacteriocins, are heat labile and mainly act on Gram-positive bacteria (Bastos et al., 2010). However, all class III bacteriocins possess a common gene cluster organisation, that includes a structural bacteriocin gene, a lysis gene and an immunity gene (Luria & Suit, 1987), which

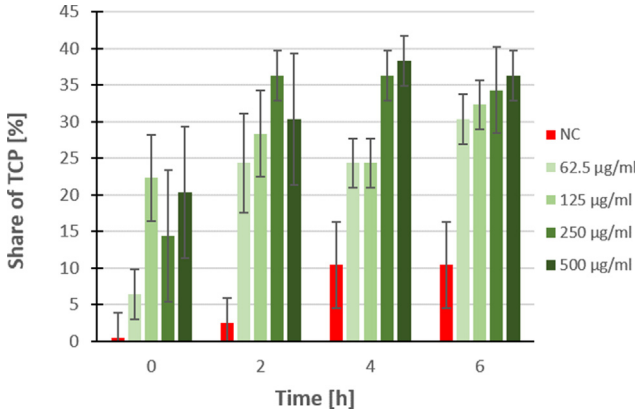

Fig. 4. Protein leakage test with *S. auricularis*. Samples of the indicator bacterium were mixed with different concentrations of B135CC and incubated at 30 °C. Samples were taken every two hours and the protein concentration was determined by Lowry-assay. NC, negative control, *S. auricularis* without addition of the bacteriocin. The share of total cell protein (TCP) was calculated on the basis of untreated *S. auricularis* cells, disrupted by ultrasound. Error bars indicate standard deviation, n = 3.

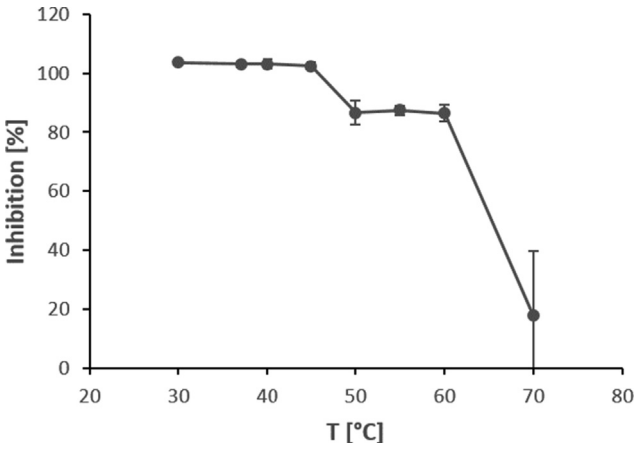

Fig. 5. Temperature stability test of B135CC. The bacteriocin was incubated at different temperatures for one hour and subsequently tested for activity. The inhibition test was done with *S. auricularis* in microtiter plates. Error bars indicate standard deviation, n = 3.

is not the case for B135CC. Moreover, most known class III bacteriocins do not exhibit a double glycine leader motif, except for colicin V, a less than 10 kDa protein which does possess a conserved double glycine peptide cleavage site (Havarstein et al., 1994). For evaluation of the applicability of the bacteriocin as food preservative or as a possible treatment for human bacterial infections, B135CC was tested for toxicity against mammalian N2a-cells (Fig. 6).

N2a cells, a mouse neuroblastoma cell line, is frequently used for the detection of neurotoxic compounds (Takser et al., 2016; Viallon et al., 2020). As many cyanobacteria tend to produce highly (neuro-) toxic compounds, it is especially important to exclude the production of such substances. The neurotoxicity tests with B135CC did not show any negative impact on N2a cells. All applied bacteriocin concentrations (6.25–150 μg ml^{-1}) caused a lower LDH activity than the negative control (10 mM HEPES).

Conclusion

The bacteriocin B135CC from the terrestrial cyanobacterium *C. cubana* shows promising narrow range antibacterial activity mainly against some Gram-positive strains, especially against *S. auricularis*. The latter was identified as an inhabitant of the human ear (Kloos & Schleifer, 1983) and can rarely cause infections and sepsis (Hoffman et al., 2007). Susceptibility of this bacterium to B135CC can particularly be seen as a hint for further applicability on related *Staphylococcus* strains as multi-resistant *Staphylococcus aureus*, which still causes thousands of dead every year (Navidinia, 2016). While all other sensitive strains had to be treated with concentrations of at least 50 μg ml^{-1} to observe a growth inhibition, B135CC almost completely prevented growth of *S. auricularis* in concentrations as low as 6.25 μg ml^{-1}. Moreover, the bacteriocin showed no cytotoxicity against mammalian N2a cells in concentrations up to 150 μg ml^{-1}. Together, our data suggest that B135CC could be of great use for the specific treatment of different Gram-positive pathogenic bacteria with highest activity against *Staphylococcus* species.

CRediT authorship contribution statement

Marco Witthohn: Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Supervision, Project administration. **Dorina Strieth:** Validation, Resources. **Simone Eggert:** Methodology, Validation, Investigation, Resources, Writing –

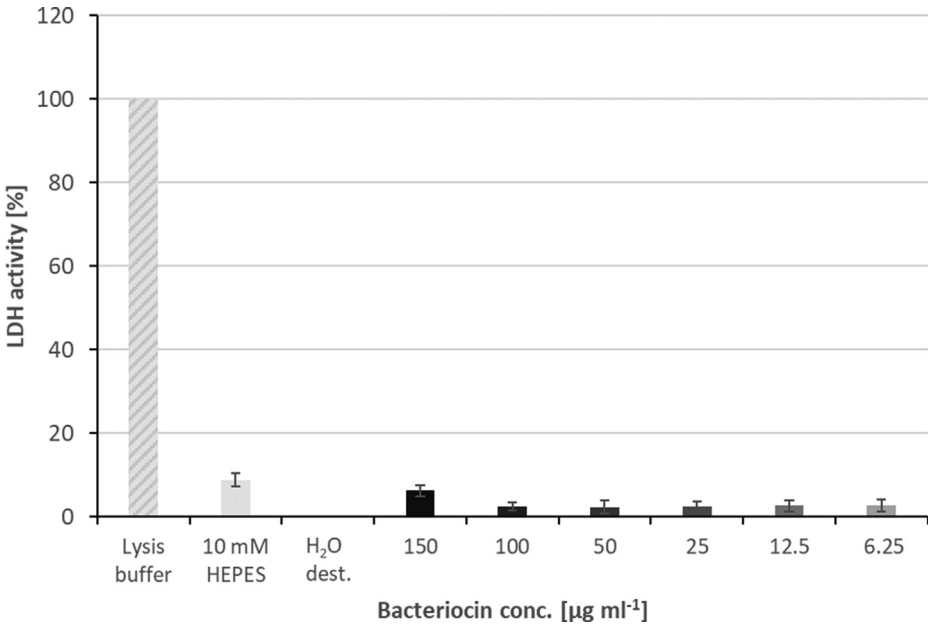

Fig. 6. LDH activity test for evaluation of B135CC-toxicity against mammalian N2a cells. For the toxicity tests, B135CC was eluted in 10 mM HEPES buffer in the desalting step after affinity chromatography. Subsequently it was diluted with N2a medium (6.25 – 150 µg ml^{-1} bacteriocin) to a final volume of 100 µl and added to the N2a cells in 96-well plates. The samples were incubated overnight at 37 °C and 5 % CO_2. The negative control with only 10 mM HEPES (without N2a medium) was incubated overnight. The following day, for maximal LDH activity control, N2a cells were incubated in 10 µl lysis buffer plus 90 µl N2a medium for 45 min. The spontaneous LDH activity was tested by incubation of the cells in 10 µl distilled water plus 90 µl N2a medium for 45 min. Error bars indicate standard deviation, n = 3.

review & editing, Funding acquisition. **Stefan Kins:** Resources, Writing – review & editing, Funding acquisition. **Roland Ulber:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Kai Muffler:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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