Antimicrobial production in nontoxigenic *Clostridium botulinum*

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Abstract

With the widespread emergence of drug-resistant pathogens, attention has been drawn to unknown and unexplored antimicrobial agents like bacteriocins. Bacteriocins are proteins or peptides generated by ribosomes that have antibacterial action against a small range of bacteria. Most gram-positive bacteria (such as *Clostridium, Staphylococci, Streptococci, Listeria, Bacilli, and Enterococci* species) produce a wide range of bacteriocins. However, the vast majority remain undiscovered. The importance of bacteriocins as an alternative to antibiotics cannot be denied anymore, due to their potential applications in food preservation and pharmaceutical products. Bacteriocins are valued for their selectivity and low toxicity. Furthermore, due to post-translational modification, bacteriocins provide a substantial reservoir of antimicrobial compounds with a wide range of structures and modes of action. This article examines the antimicrobial potency of three nontoxigenic *Clostridium botulinum* strains (1985, HA1, and ZO42). The antimicrobial activity of compounds produced by *C. botulinum* can be studied based on whole genome sequence analysis. All three producer strains displayed antimicrobial activity against three or more indicator strains. For HA1, two sactipeptides and one ranthipeptide cluster were identified. The biosynthetic gene cluster found in 1985 encodes a biosynthetic RiPP-like protein. In ZO42 a sactipeptide cluster was found. To characterize the compound, heat, cold, UV, proteinase K, and β-mercaptoethanol treatments were done. For 1985 a heat and UV-resistant compound was encountered. The compounds of both 1985 and HA1 were resistant to β-mercaptoethanol, indicating disulfide bonds are not present. No antimicrobial activity was observed when proteinase K was added to the supernatant, establishing their proteinaceous nature. To determine the potency of the identified antimicrobial compounds, MID assays were used. A very potent compound was discovered for 1985. To study the mode of action, light and time-lapse microscopy are performed. Both HA1 and 1985 produce bactericidal, lysogenic compounds.

Introduction

Clostridium botulinum is a gram-positive, strictly anaerobic, spore-forming bacteria that produces a deadly neurotoxin that is responsible for botulism. Seven serologically distinguishable neurotoxins have been discovered and labeled with the letters A-G. Neurotoxins A, B, E, and F present the greatest threats to humans. The bacteria are categorized into four groups (I-IV) based on their physiological and biochemical properties. Group I and II are associated with human botulism (Eklund *et al.*, 2004).

Botulism occurs primarily when the toxin is ingested through contaminated food (foodborne botulism). Bacterial growth and toxin production occur when low acid canned or bottled foods are not adequately heat-treated, or when minimally heated, chilled foods are not stored at low temperatures (Peck M.W., 2014). Other types of botulism are intestinal toxemia botulism, which develops due to ingestion of bacteria and/or spores, and wound botulism as a consequence of outgrowth of spores in a wound (Anses, 2010).

While the causes and methods to prevent foodborne botulism have been well studied, other aspects of *C. botulinum* bacteria such as their ability to produce antimicrobial agents have received much less attention. The emergence of antibiotic resistance has sparked major efforts to find alternative anti-microbials for treating drug-resistant pathogens (Yang *et al.*, 2014). Recent advances in whole genome sequencing (WGS) and *in silico* mining have enhanced the exploration of novel antimicrobial biosynthetic gene clusters (BGC) which encode the production of a wide range of structurally and functionally diverse compounds.

An important group of such compounds are bacteriocins, most of which are produced by gram-positive bacteria such as *Staphylococci*, *Streptococci*, *Listeria*, *Bacilli*, *and Enterococci* species (Meade *et al.*, 2020). These are ribosomally produced proteins or peptides with antimicrobial activity against a small group of bacteria. The bacteria are usually but not necessarily closely related to the producer strain. Some examples include sactipeptides and lanthipeptides, which both belong to the ribosomally synthesized and post-translationally modified peptides (RiPP). Generally, the BGC responsible for RiPP production consists of a set of genes that encodes for the precursor peptide, modification enzymes, transporters, and a protease. The precursor peptide consists of a core peptide and a leader peptide, which serves as a binding site for the modification enzymes, and which is removed by the protease after modification of the peptide. The active modified peptide is then exported by a transporter (Yanyan and Rebuffat, 2020). These posttranslational modifications establish a vast diversity of RiPP products.

While both bacteriocins and antibiotics display antimicrobial activity, a key difference is that bacteriocins have a narrow spectrum (Samanthi, 2019). As antibiotics are widely used, antimicrobial resistance has emerged. Furthermore, it has been shown that administration of antibiotics disturbs the microbiomes in the gut and other parts of the body due to their broad spectrum of activity. These microbiomes do not always return to their original state, and this may have a negative impact on the patient's health. Bacteriocins, in contrast, are appreciated for their selectivity and safety profile. Moreover, bacteriocins constitute an enormous reservoir of antimicrobial compounds with an enormous diversity in structure and mode of action, owing to the post-translational modification (Syngulon, 2018).

In the search for new bacteriocins, a combination of techniques is used. The halo inhibition assay is performed to test whether bacterial strains exhibit antimicrobial activity toward other strains. Additionally, several bioinformatics tools are used to

explore genome sequences for novel biosynthetic gene clusters responsible for bacteriocin production.

This project aims to identify novel antimicrobial compounds in *C. botulinum* strains and to further analyze their antimicrobial activity. Specific characteristics of the bacteriocins are elucidated and their effects on sensitive target cells are examined by light and time-lapse microscopy. The results from halo assays are combined with bioinformatics analysis to identify which BGC could be responsible for bacteriocin production in the producer strains.

Materials and methods

Materials. Ten indicator strains, *C. botulinum* CM2, KI2, ZS3, WHH32, ZS6, ZBS1, EV32, SDE2.2, MEL, and *C. sordellii* ZKN33, and three producer strains, *C. botulinum* 1985, ZO42, and HA1 were obtained from the Lab of Food Microbiology strain collection**.** These strains had been previously isolated from food and environmental samples and subjected to whole genome sequencing. The enzyme proteinase K was purchased from ThermoFisher. β-mercaptoethanol was purchased from Merk. The bacterial strains were grown in Oxoid™ Reinforced Clostridial Medium (RCM) and plated on bacteriological (European type) No. 1 agar, purchased from Neogen. LSL-LE 8200 agarose, purchased from Lonza, was used to make agar plates for the microscopy experiment.

Determination of antimicrobial activity. The antimicrobial activity of the bacterial strains was determined by the halo assay method. 200 µl of an overnight culture of the indicator was spotted on an agar plate and spread out using sterile glass beads. Subsequently, 10 µl of an overnight culture of each producer strain was spotted in one of the quadrants of the plate, leaving one quadrant vacant as a negative control.

Bioinformatics analysis. *In silico* mining for potential bacteriocin biosynthetic gene clusters was performed by analyzing the WGS of three producer strains in BAGEL4 and the bacterial version of antiSMASH (University of Groningen, s.d.; Blin K. *et al*., 2021). BAGEL4 uses known genes and biosynthetic gene clusters from its databases to search for potential clusters in the examined genome whilst antiSMASH uses a rule-based approach (University of Groningen, s.d.; Blin *et al.*, 2021). This is why both programs give different outputs. The relevant clusters (which encode for a leader peptide, core peptide, modification enzymes, transporters, and a protease) from antiSMASH were then compared to those from BAGEL4 using MultiGeneBlast. For each strain, a database of all the relevant clusters from BAGEL4 was created to test whether they matched the clusters identified in antiSMASH. If both clusters were similar, a cluster given by both programs under different names, only one of the duplicates was used for further analysis. In the last step of the analysis, all the identified clusters were tested against a database containing 23 strains. Hence, the indicator strains were analyzed to see whether they contain a particular cluster. A high similarity between the input cluster and the database strain indicates the occurrence of the cluster in that strain. The results of the bioinformatics analysis are then compared to the findings of the halo assay experiments. For antimicrobial activity, the cluster must be present in the producer strain and not in the indicator strain. Consequently, the clusters that appeared in the indicator strains were discarded. Figure 1 depicts a schematic overview of the bioinformatics analysis. SignalP is used to detect the cleavage site within the identified clusters, which is needed to determine the potential precursor peptide (Teufel *et al.,* 2022).

Characterization of antimicrobial compounds. The sensitivity of the antimicrobial compounds produced by 1985 and HA1 to heat, cold, UV exposure, proteinase K, and β-mercaptoethanol was evaluated. Cells from an overnight culture of strains 1985 and HA1 were removed by two consecutive centrifugations at 4°C and 8000 g for 10 minutes. The supernatant was subjected to different treatments. Heat treatment was done at 40, 50, 70, and 96 °C for 30 minutes in a Thermo Shaker Incubator. Cold treatment was for three days at -20°C. UV sensitivity was evaluated by placing a petri dish with 100 µl supernatant in a UV Exposure Chamber at a UV energy of 0.2 and 0.5 J/m^2. To check whether the bacteriocins are of proteinaceous nature, the samples were incubated with 2 mg/ml proteinase K for 2 h in a Thermo Shaker Incubator at 400 rpm at 65°C (optimal activity temperature of proteinase K). Proteinase K was thereafter inactivated by incubation at 90°C for 10 minutes. A negative control, a mixture of water and proteinase K at a final concentration of 2 mg/ml, was incubated at 90°C for 14 minutes. To test for the presence of disulfide bridges, the samples were treated with 5 mmol/ml β-mercaptoethanol. Negative control was made by combining water with β-mercaptoethanol at a final concentration of 5 mmol/ml. The treated supernatants were then assayed for antimicrobial activity using a spot assay, and with untreated supernatant as a positive control.

Maximal inhibitory dilution (MID) determination. The maximal inhibitory dilution of the bacteriocins produced by 1985 and HA1 was determined by assaying, according to the halo method, a two-fold dilution series of the supernatant against indicator strains KI2 and ZO42 respectively. Undiluted supernatant was also assayed as a control. The highest dilution that inhibited the growth of the indicator after overnight incubation was considered the MID.

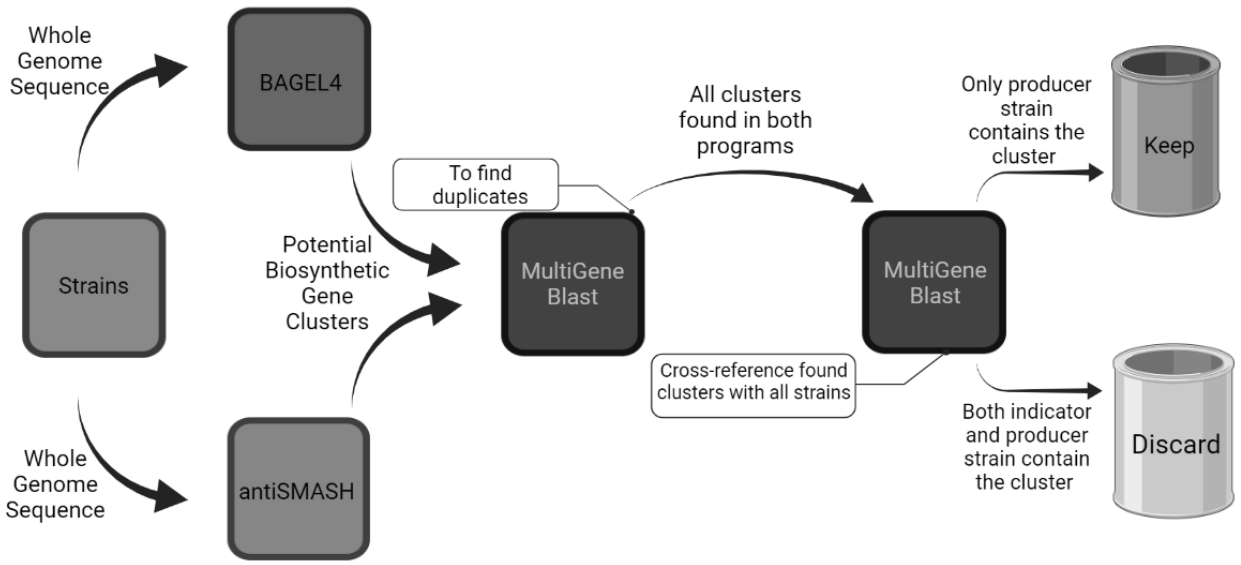

Figure 1: Schematic overview of the bioinformatics analysis (made with BioRender, 2022)

MID determination was also performed using a 96-well assay. The dilutions of supernatants of 1985 and HA1 were added to different wells. The wells were inoculated with 1:1000 dilution of overnight cultures of the indicator strains KI2 and ZO42 respectively. One well was filled with only medium as a negative control, and another well was filled with living cells and medium as a positive control. The contents of the wells were then incubated overnight. From this, three dilutions (1:10, 1:100, and 1:1000) were made and spotted on a plate. Growth was visually evaluated.

The producer cultures were centrifuged to ensure that the supernatant was clear of living cells. There should be no growth of producer cells when the supernatant is assayed. If contamination occurred, the cultures were recentrifuged. Additionally, the tubes with supernatant were left open to allow oxygen diffusion to kill the remaining living cells.

The minimal inhibitory concentration (MIC), the lowest concentration of a compound that prevents the growth of bacteria was calculated as:

$$arbitrary\ units\ \left(\frac{AU}{ml}\right) = \frac{Reciprocal\ of\ the\ highest\ dilution}{Amount\ of\ bacteriocin} * 1000$$

(Formula 1)

Microscopy. Diluted stationary cell cultures from ZO42 (1:100) and KI2 (1:1000) were mixed with different dilutions of the HA1 (1:2 & 1:4) and 1985 (1:2 & 1:16) supernatant, respectively. Exponential cells (OD_{600} value of 0.3) were handled in a similar manner. To make the microscopic slides, 2 µl of each sample was spotted on an RCM agarose pad. For control, liquid RCM was used. A coat of nail polish was used to prevent oxygen diffusion into the microscopic plates. The plates were analyzed using the light microscope 100x at different time points (at t = 0h, 2h, 12h, and 24h).

Time-lapse microscopy was performed for HA1 and 1985 supernatant against ZO42 and KI2 respectively. The microscope takes an image every ten minutes to track the indicator's growth and its reaction to the antimicrobial activity in the supernatant. As a control for normal growth, one slide of indicator cells without producer supernatant was used.

Results

Determination of the antimicrobial activity of 1985, ZO42, and HA1. Three producer strains were tested for antimicrobial activity against a total of ten indicator strains. The results of the halo assays are scored on an arbitrary unit scale based on the diameter and clarity of the halo, ranging from - (no activity) to +++ (highest activity) (Table 1).

Table 1: Antimicrobial activity scored with arbitrary units. Vertical: Indicator strains. Horizontal: Producer strains. Superscript indicates the classification (Group I or II).

I/P	1985[2]	ZO42[1]	HA1[1]
CM2[2]	++	-	-
KI2[2]	+++	-	-
ZS6[2]	-	+++	+++
ZKN33[1]	-	-	+
ZS3[1]	-	+++	++
WHH32[1]	-	-	+
ZBS1[1]	+	+++	+++
EV32[1]	-	-	+++
SDE2.2[1]	-	-	+++
MEL[1]	-	-	++

Bioinformatics analysis. All the potential clusters contain a precursor peptide, modification enzymes, transporters, proteases, and regulators. For HA1, three different potential clusters were identified. Two sactipeptides and one ranthipeptide. Cluster 1.16-3 (BAGEL4) encodes a hypothetical sactipeptide protein. This potential precursor peptide was identified with SignalP. It also encodes a radical *S*-Adenosyl methionine (rSAM) and an rSAM peptide maturase. Contig 13.9-2 (BAGEL4) encodes a putative sactipeptide bacteriocin protein, as well as an rSAM. Cluster 13.2 (AntiSMASH) encodes a biosynthetic ranthipeptide. For ZO42, two different potential clusters were identified. One sactipeptide and one RRE-containing cyclo-lactone autoinducer (CLA). Cluster 2.1 (BAGEL4) encodes a hypothetical sactipeptide protein. The potential precursor peptide was identified with SignalP. Cluster 2.2 (AntiSMASH) encodes a biosynthetic CLA/RRE-containing protein as well as an rSAM. For 1985, one potential cluster was identified. Cluster 13 (AntiSMASH) encodes a bacteriocin RiPP-like protein and a bacteriocin-type signal sequence.

Figure 2 shows the clusters of HA1, 1985 and ZO42. For ZO42 cluster RRE/CLA there are 3 possible precursor peptides.

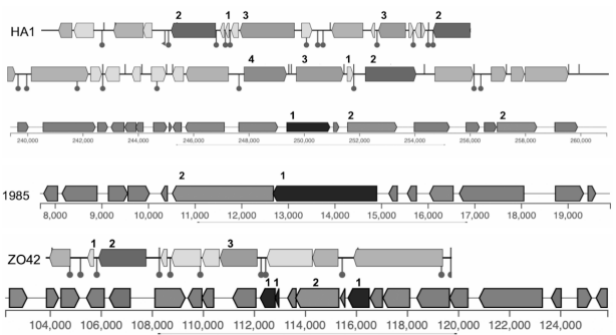

Figure 2: Potential clusters of HA1, 1985, and ZO42 identified in BAGEL4 or AntiSmash. 1: Precursor peptide. 2: Transporter. 3: Modification enzyme. 4: Protease.

Initial characterization of identified bacteriocins. Producer strain 1985 was plated out against indicator strains ZKN33 and KI2. Strain HA1 was plated out against ZO42, WHH32, MEL, ZKN33, and ZBS1.

The bacteriocins produced by strain 1985 display the same activity after temperature and UV treatment as the untreated supernatant (control) (Table 2). They are not sensitive to β-mercaptoethanol. These treatments did not affect their activity. However, proteinase K treatment interfered with the microbial activity of the bacteriocins. The antimicrobial activity is scored on an arbitrary unit scale based on the diameter and clarity of the halo, ranging from - (no activity) to +++ (highest activity). 1985 supernatant was active against KI2 but not ZKN33.

The bacteriocins produced by HA1 exhibit a lower activity after temperature treatment compared to the untreated super-natant (control) (Table 2). They lose all activity after UV treatment at 0.2 Joules or higher. They are not sensitive to β-mercaptoethanol, and proteinase K treatment inhibits their microbial activity. HA1 supernatant was active against ZO42, ZKN33, and WHH32, but not ZBS1 and MEL.

Table 2: Effects of temperature, UV, proteinase K, and β-Mercaptoethanol against 1985 and HA1 bacteriocin activity.

Parameter	Value	Activity	
		1985	**HA1**
Control		+++	+++
Temperature (°C)	40	+++	+
	50	+++	+
	70	+++	+
	96	+++	+
	-20	+++	-
UV (J)	0.2	+++	-
	0.5	+++	-
Proteinase K		-	-
β-mercaptoethanol		+++	+++

MID. A MID value of 1:4 was obtained for the HA1 supernatant plated on ZO42 (Figure 2, no halo at dilution 1:8). According to formula 1, this gives a MIC of 80 AU/ml. A MID value of 1:64 was obtained for the 1985 supernatant plated on KI2 (Figure 3, no halo at dilution 1:128). According to formula 1, this gives a MIC of 1280 AU/ml. For HA1, the results of the well assay did indicate an identical MID value of 1:4. For 1985, no workable results were obtained using the well assay. A dilution up to 1:16 was used, and all dilutions inhibited growth.

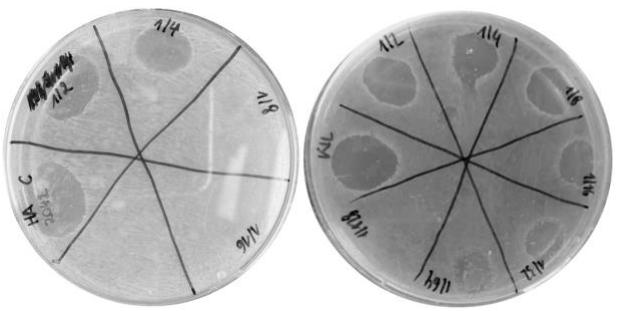

Figure 3: 1: MID assay twofold dilution up to 1:16 of HA1 supernatant on ZO42. 2: MID assay twofold dilution up to 1:128 of 1985 supernatant on KI2.

Microscopy. In light microscopy of stationary and exponential phase ZO42 with HA1 supernatant, the control shows normal growth. For the stationary phase, some cells began to lyse 12 hours after a 1:2 dilution of HA1 supernatant was introduced. Long cells were seen after 12 hours in the 1:4 dilution. After 12 hours of the exponential phase, some cells began to lyse after being exposed to a 1:2 dilution of HA1 supernatant. In the 1:4 dilution, long cells were visible after 12 hours. Both lysed and long cells are observed at t=26h (Figure 4). Control indicates normal growth in light microscopy of stationary and ex-ponential phase KI2 with 1985 supernatant. All the cells of the

stationary phase were lysed after 26 hours of 1:16 dilution (Figure 5).

In time-lapse microscopy of the exponential phase of ZO42, the control indicates normal growth (Figure 6). When a 1:2 dilution of HA1 is introduced, cells begin to lyse. It takes approximately three hours for the first cell to lyse (Figure 7). When a 1:4 dilution of HA1 is introduced, cells begin to swell. After one hour, the first cell will burst and lyse (Figure 8). Time-lapse microscopy for 1985 did not give workable results.

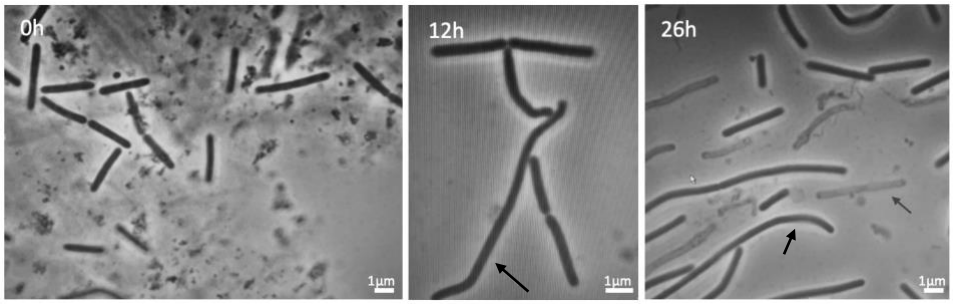

Figure 4: Light microscopy of ZO42 with 1:4 dilution of HA1 supernatant. 1: t=0. 2: t=12h. 3: t=26h. The arrows point to either an elongated cell or a lysed cell (light grey).

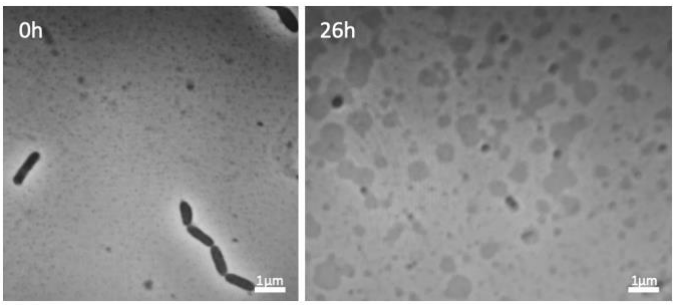

Figure. 5: Light microscopy of KI2 with a 1:16 dilution of 1985 supernatant. 1: t=0. 2: t=26h.

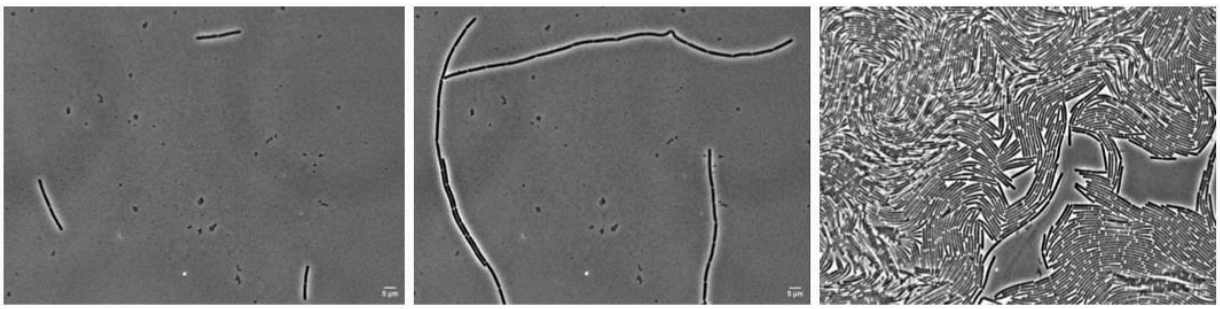

Figure 6: Time-lapse microscopy of exponential phase ZO42 without HA1 supernatant. 1: t=10 min 2: t=3h20 3: t=10 h.

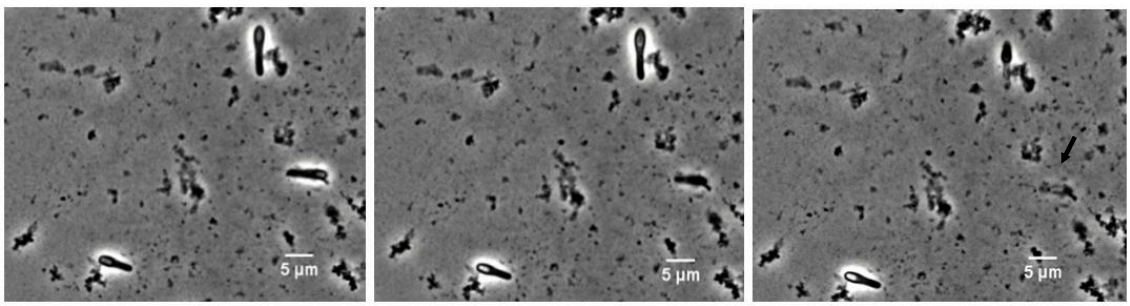

Figure 7: Time-lapse microscopy of exponential phase ZO42 with a 1:2 dilution of HA1 supernatant. 1: t=10 min 2: t=3h 20 3: t=6h 40. The arrow points to a lysed cell.

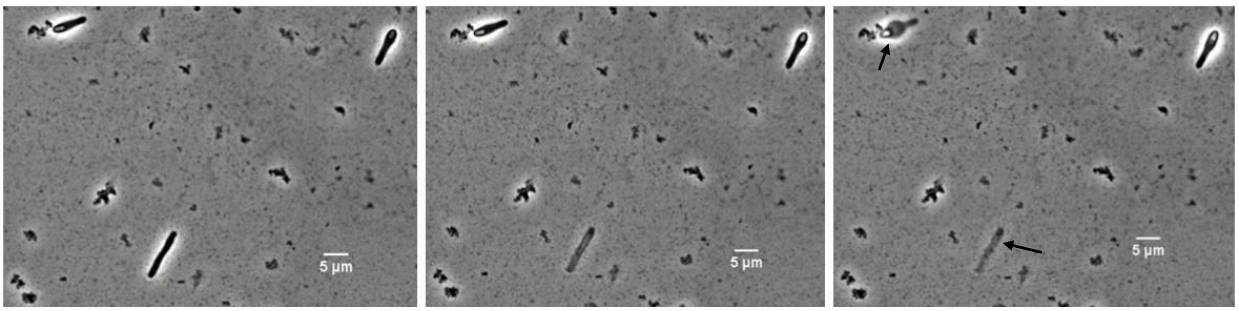

Figure 8: Time-lapse microscopy of exponential phase ZO42 with a 1:4 dilution of HA1 supernatant. 1: t=10 min 2: t=1h30 3: t= 6h40. The arrows point to either the swelling or a lysed cell (light grey).

Discussion

HA1 displayed antimicrobial activity against ZS6, a group II strain, as well as ZKN33, ZS3, WHH32, ZBS1, EV32, SDE2.2, and MEL, all group I strains. The antimicrobial spectrum of HA1, which is a group I strain, covers both group I and group II strains. The halo assays with living HA1 cells and the halo assays with the supernatant gives contradictory results. Living HA1 cells displayed antimicrobial activity against ZBS1 and MEL. When the supernatant of the producer strain was assayed, there was no antimicrobial activity against the same indicator strains. As HA1 supernatant has antimicrobial activity against ZO42, the hypothesis that the supernatant does not retain any antimicrobial agents can be dismissed. A possible explanation for this phenomenon is that HA1 produces two different antimicrobial compounds. One compound is secreted in the supernatant and acts against ZO42. The other one is not secreted and acts against ZBS1 and MEL. One possible reason is that this compound requires a type of competition (e.g., the presence of indicator strain) to be produced. It could also be that the conditions in the liquid medium are not optimal for compound production.

The bioinformatics results are in line with the results from the antimicrobial halo assays. At least two different BGCs were expected, to produce a compound that is secreted in the supernatant and one that is not secreted. Two sactipeptides were identified and one ranthipeptide. With the available data, it is not possible to determine which cluster is responsible for which compound. Further analysis is needed. An enzymatic protein treatment can be used to selectively break the thioether bonds in sactipeptides and ranthipeptide. Sactipeptides have a characteristic thioether bond between the sulfur atom of cysteine residue and the α-carbon of another amino acid. The ranthipeptides have a thioether binding on the β or γ carbon-atom (Chen *et al.*, 2021). Additionally, a series of gene knockout experiments can be performed on each cluster. If antimicrobial activity is observed when a cluster is perturbed, bacteriocin production cannot be assigned to this cluster.

The HA1 agents showed reduced activity after heat exposure which is not consistent with other experiments as the antimicrobial compounds usually do not show any decrease in activity (Chopra *et al.*, 2014). Cold and UV treatment inhibited all activity. UV stability is not easily compared to other experiments as different approaches were used. The antimicrobial activity of the compounds produced by HA1 was lost after treatment with proteinase K. This treatment was carried out at 65°C, after which the enzyme was inactivated at 90°C. Because the compounds are not heat-stable, it is impossible to attest their proteinaceous nature. For future experiments treatment with proteinase K should be carried out at a lower temperature, but for a longer period of time. Treatment with β-mercaptoethanol did not affect the antimicrobial agents, indicating the peptides do not have disulfide bridges. This is consistent with the results of other experiments (Chopra *et al.*, 2014).

For HA1, a MID value of 1:4 was obtained in the experiments with halo assays. In the HA1 well assay, there was contamination which could be explained by a lack of sterility or the presence of living cells in the supernatant. However, they indicate an identical MID value of 1:4 with a corresponding MIC value of 80 AU/ml. For future experiments, extra centrifugation is recommended. Additionally, treatment with chloroform can be performed, and the tubes can be left open to allow oxygen diffusion to eliminate living cells. The focus of these experiments was on identifying antimicrobial compounds and qualitatively analyzing their effect. There was less attention to quantitative analysis. In future experiments, a MIC value in µg/ml should be obtained to compare the identified compound to other antimicrobials.

In the time-lapse microscopy experiment, sporulating ZO42 cells were observed in the 1:2 and 1:4 dilutions of the HA1 supernatant. These sporulating cells were not observed in the control. This could be a response to the antimicrobial compound. However, sporulating cells were not observed in the live microscopy experiments, in which ZO42 was also treated with HA1 supernatant. Sporulation could also be due to contamination as the microscopic slides with HA1 supernatant show a lot of debris. Since this is not observed in the control, it is possible that the HA1 supernatant used in the time-lapse microscopy was contaminated. Contamination may cause stress conditions, inducing sporulation.

In both the 1:2 and 1:4 dilution, a swelling phenotype was observed. Swelling of a cell is caused by an uncontrollable in and outflow of ions and water. When the pressure inside the cell becomes too high, the membrane collapses (Haschek *et al.*, 2010). Maculatin 1.1, an antimicrobial compound that acts against *Escherichia coli*, shows cell membrane disruption and leakage of bacterial content (Benfield and Henriques, 2020). HA1 probably works in a similar manner, which can be confirmed through leakage assays. The lysis of sporulating cells (Stage V: coat synthesis) demonstrated that the antimicrobial

agents produced by HA1 are able to kill cells during sporulation. Sporulating cells were more resistant to the compounds than regular cells since they lysed after a longer period of time. Other bacteriocins (e.g., nisin and enterocin produced by *Enterococcus faecalis*) have also been found to have sporicidal effects against spore-forming *C. botulinum* cells (Meade *et al*., 2020). The produced compounds have a bactericidal effect on the indicator strains, as shown by time-lapse and light microscopy.

The antimicrobial compounds in HA1 supernatant block cytokinesis in ZO42, demonstrated by the elongated cells in the light microscopy. Inhibition of the development of the FtsZ-ring, which is an important structure in cell division, is a potential mechanism of action. Inhibition of cell division in rod-shaped bacteria results in filamentous cells, which become less stable as they elongate and ultimately lyse. This phenomenon was also observed with light microscopy. An FtsZ GTPase Assay can be used to determine if the antimicrobial compounds inhibit the FtsZ protein (Stokes *et al*., 2005).

1985 displayed antimicrobial activity against ZBS1, a group I strain, as well as CM2 and KI2, both group II strains. The antimicrobial spectrum of 1985, which is a group II strain, covers both group I and group II strains. The results of the halo assay with living 1985 cells and the halo assays with supernatant provide consistent findings. For 1985, only one BGC was found. Cluster 13 encodes a biosynthetic RiPP-like protein. The RiPP-like protein is potentially responsible for the antimicrobial activity of this strain. Gene knockout experiments against this cluster could confirm this hypothesis.

The 1985 antimicrobial agents were found to be heat, cold, and UV stable. The results for the compound produced by 1985 are consistent with other experiments reporting the heat, cold, and β-mercaptoethanol stability, and proteinase K sensitivity of bacteriocins such as bacillocin 490 and MT93 bacteriocin (Chopra *et al*., 2014). UV stability is not easily compared to other experiments as different approaches were used. The antimicrobial activity of the bacteriocin produced by 1985 was lost after treatment with proteinase K, establishing its proteinaceous nature. Treatment with β-mercaptoethanol did not affect the bacteriocin, indicating the antimicrobial compound does not have disulfide bridges.

The MID value for 1985 is low, indicating a potent antimicrobial product, a corresponding MIC value of 1280 AU/ml was obtained. Solely the results of the halo assay were used to determine this value. For the well assay, only dilutions up to 1:16 were used, which wasn't diluted enough. The antimicrobial agents were still very active.

During the light microscopy experiment of the stationary phase, the control shows that KI2 culture still grows after 26h. However, when 1985 supernatant was added in both 1:2 and 1:16 dilution, no cells were observed after 26h, indicating bactericidal activity of the producer strain. The presence of cells at 0h indicates that cell lysis does not occur instantaneously. The microscopy of the exponential phase showed unexpected results. The KI2 control culture barely grew in two hours. This raises concerns about the validity of the experiment. No reliable conclusions can be made about this culture.

ZO42 displayed antimicrobial activity against ZS6, a group II strain, as well as ZS3 and ZBS1, both group I strains. The antimicrobial spectrum of ZO42, which is a group I strain, covers both group I and group II strains. In ZO42, two different clusters were identified. The first cluster encodes a biosynthetic CLA/RRE-containing protein. This protein has a role in quorum sensing and is not likely involved in antimicrobial activity (Schauder *et al*., 2001). The second BGC is a sactipeptide cluster. The activity and antimicrobial mechanisms of sactipeptides are diverse and their physiological function in the producer strains is not fully understood. Some compounds, such as those produced by *Bacillus subtilis*, are able to delay sporulation or even kill spores. Others, such as *Bacillus thuringiensis*, irreversibly disrupt the cell membrane of their target (Chen *et al*., 2021). To examine the mode of action of ZO42, microscopy experiments should be performed. However, this requires overcoming the challenge of extracting this compound from the cells as it is not secreted in the supernatant for similar reasons as HA1. To confirm this sactipetide is responsible for the antimicrobial activity of ZO42 against ZS6, ZS1, and ZBS1, gene knockout experiments should be performed.

Conclusion

Overall, the results imply that nontoxigenic *C. botulinum* can be used for the production of novel antimicrobial agents. All three producer strains, 1985, HA1, and ZO42, produce antimicrobial compounds against both group I and group II strains. Some surprising results were obtained in the experiments. HA1 probably produces at least two distinct antimicrobial agents. Three different clusters were obtained for HA1, two sactipeptides and one ranthipeptides. The supernatant compounds showed decreased activity after heat exposure. Cold and UV treatment inhibited these compounds. The compounds do not have any disulfide bridges. HA1 probably acts by inhibiting cytokinesis and inducing cell lysis in both normal and sporulating cells, indicating bactericidal activity. For 1985, a RiPP-like cluster was identified. Compound produced by 1985 was very potent. This protein was heat, cold, and UV-stable, and did not have any bridges. This compound displays bactericidal activity, but no reliable information was obtained concerning the precise mode of action. For ZO42, two different clusters were identified. The CLA/RRE-containing protein is not likely responsible for antimicrobial activity. The sactipeptide could be responsible. No characterization was performed on these compounds as they were not secreted in the supernatant. Their mode of action remains unknown. Further analyses, such as gene knockout experiments and enzymatic treatment, are required to discard the posed hypotheses or formulate conclusive answers about the potential cluster and compounds.

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