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Actinomyces produce defensin-like bacteriocins (actifensins) with a highly degenerate structure and broad antimicrobial activity

Running title: Defensin-like bacteriocin production in *Actinomyces*

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Keywords

Actinomyces, bacteriocin, defensin, antimicrobial peptide, actifensin

Abstract

We identified a strain of *Actinomyces ruminicola* which produces a potent bacteriocin with activity against a broad range of Gram-positive bacteria – many of which are pathogenic to animals and humans. The bacteriocin was purified and found to have a mass of 4091+/-1 Da with a sequence of GFGCNLITSNPYQCSNHCKSVGYRGGYCKLRTVCTCY containing 3 disulphide bridges. Surprisingly, near relatives of actifensin were found to be a series of related eukaryotic defensins displaying greater than 50% identity to the bacteriocin. A pangenomic screen further revealed that production of actifensin-related bacteriocins is a common trait within the genus with 47 being present in 161 genomes. Furthermore, these bacteriocins displayed a remarkable level of diversity with a mean amino acid identity of only 52% between strains/species. This level of redundancy suggests that this new class of bacteriocins may provide a very broad structural basis on which to deliver and design new broad-spectrum antimicrobials for treatment of animal and human infections.

Importance

Bacteriocins (ribosomally produced antimicrobial peptides) are potential alternatives to current antimicrobials given the global challenge of antimicrobial resistance. We identified a novel bacteriocin from *Actinomyces ruminicola* with no previously characterised antimicrobial activity. Using publicly available genomic data we found a highly conserved yet divergent family of previously unidentified homologous peptide sequences within the genus *Actinomyces* with striking similarity to eukaryotic defensins. These actifensins may provide a potent line of antimicrobial defence/offence and the machinery to produce them could be used for design of new antimicrobials given the degeneracy that exists naturally in their structure.

Keywords

Actinomyces, bacteriocin, defensin, antimicrobial peptide, actifensin

Introduction

Novel antimicrobial compounds are increasingly important in the food, agriculture and medical fields due to decreasing efficacies of current antimicrobial treatments. Bacteriocins are ribosomally-synthesised antimicrobial peptides produced by bacteria which can target another bacterium of the same species (narrow spectrum) or bacteria of other species/genera (broad spectrum) (1). Bacteriocin producers are self-protected through the production of specific immunity proteins, and as bacteriocins are gene encoded, they can be genetically modified. Bacteriocins produced by Gram positive bacteria have been grouped according to their primary structure into class I (post-translationally modified bacteriocins) and class II (unmodified or cyclic bacteriocins) (2). Class II is split into several subgroups, including the class IId bacteriocins, which are a heterogenous group of linear, unmodified, non-pediocin like peptides (3).

. Defensins are antimicrobial peptides ubiquitous among eukaryotes which play a role in innate immunity but have also been found to act as signalling peptides, toxins, enzyme inhibitors, abiotic stress responders, and to have anti-cancer properties. Defensins are small (<10 kDa), cysteine rich (forming three to six disulphide bonds) peptides with low amino acid identity and the two superfamilies are thought to have evolved convergently (4). Only two expressed defensin-like bacteriocins have been described; the laterosporulins have been previously identified among prokaryotes and contain disulphide bonds in positions homologous to eukaryotic defensins (5, 6). Other disulphide bond-containing bacteriocins, such as bactofencin have been compared with eukaryotic defensins due to their highly cationic nature (7, 8). Laterosporulin, and its homolog Laterosporulin10 are class IId bacteriocins produced by *Brevibacillus* spp. which have been described as broad-spectrum antimicrobials against both Gram negative and Gram positive bacteria. The two peptides are 5.6 kDa and 6.0 kDa and share only 57.6 % amino acid sequence identity but have conserved cysteines which are characteristic of eukaryotic defensins (6).

Actinomyces spp. are a heterogenous group of high GC content, Gram positive non spore-forming facultative or obligate anaerobes that belong to the *Actinomycetaceae* family within the phylum Actinobacteria (9). In humans, a number of species are known colonisers of hard surfaces in the oral cavity where they play a key role in plaque biofilm formation (10, 11). They have been identified as core members of the oral bacteriome, present in moderate abundance (>0.1 - >2.0%) among geographically-diverse populations (10, 12-15). *Actinomyces* spp. have been implicated in oral health as being associated in greater abundance in individuals with dental caries, one of the most prevalent chronic oral diseases worldwide (14, 15). Most characterised strains are clinical isolates of human origin, while some opportunistically pathogenic species such as *Actinomyces israelii* and *Actinomyces gerecseriae* are known to cause the uncommon infectious disease actinomycosis (16). Though *Actinomyces* spp. are abundant in the oral cavity, little is known about their presence in the gut, probably due to their low abundance (<0.1%) (10). Many *Actinomyces* spp. have been isolated from faecal material and from the gastrointestinal tract of different animals, indicating a propensity for gastric transit survival and their presence has also been noted in the urogenital tract (17-24). Here, we identify a new group of bacteriocins using a pan-genomic *in silico* approach paired with functional screening. Many *in silico* genome mining tools have been developed for the successful detection of novel antimicrobial producing operons (25, 26). Obviously, these methods rely on relationships with previously known genes and therefore functional screening is crucial for the identification of unrelated antimicrobials. In this study we isolated a potent bacteriocin producing strain of *Actinomyces ruminicola* from sheep faeces – the bacteriocin produced resembled eukaryotic defensins - having 3 characteristic disulphide bridges. A subsequent pan genus *Actinomyces* analysis revealed that such bacteriocins are highly distributed in these bacteria albeit with a highly variable structure.

Results

Identification of a novel bacteriocin producing *Actinomyces* sp.

Actinomyces ruminicola DPC 7226 was isolated from sheep faeces. During an initial screen of >10,000 colonies for bacteriocin producers, this strain was found to produce a large zone of inhibition when overlaid with an acid tolerant indicator species *Lb. delbrueckii* ssp. *bulgaricus* LMG 6901 (Fig. 1a). The neutralised cell-free supernatant (CFS) was also found to produce a zone of inhibition against *Lb. delbrueckii* ssp. *bulgaricus* LMG 6901, indicating production of a soluble antimicrobial molecule (Fig. 1b). This activity was eliminated when the supernatant was treated with 20 mg ml^{-1} Proteinase K, demonstrating that the antimicrobial is a peptide (data not shown).

Antimicrobial activity was purified from pelleted bacterial cells (C18 SPE, Reversed phase HPLC) and CFS (Amberlite XAD, C18 SPE, Reversed phase HPLC) and MALDI-TOF MS of active peaks detected a mass of 4091±1 Da (Fig. 2a, Fig. 2b). The mass could also be detected by colony MS (Fig. 2c). The activity of the HPLC purified fraction from CFS was assayed against *Lb.delbrueckii* ssp. *bulgaricus* LMG 6901and found to be active at <1 μgml^{-1} (Fig. 2d). The antimicrobial was found to be heat stable, retaining almost all activity after 30 mins at 100 °C, but was completely lost after treatment at 121°C for 15 mins.

Spectrum of inhibition

A range of indicator organisms were tested against the purified antimicrobial to determine the spectrum of inhibition. The antimicrobial was active against a broad range of genera with 22 of the 27 strains screened inhibited to varying degrees, including species of the genera *Lactococcus, Enterococcus, Lactobacillus, Streptococcus, Pediococcus, Bacillus, Staphylococcus,* other *Actinomyces* sp. and *Clostridium* spp (Fig. 3). No inhibition was observed against the Gram negative species *Salmonella enterica* or *Escherichia coli*. *Listeria* spp., and *Bacillus* spp. were inhibited weakly or not at all (Fig. 3). Inhibition was found against other *Actinomyces* sp. and activity was particularly strong against *Staphylococcus aureus* and *Clostridium difficile.*

MICs were determined against *E. faecium* APC1031, *E. faecium* NCDO0942, *S. aureus* R693, *S. agalctiae* APC1055 and *C. difficile* DPC6534 (Supplementary Figure 1.) Enterococci were inhibited at 3.05 – 6.1 µM. *S. aureus* was inhibited at 3.05 µM. *S. agalactiae* and *C. difficile* were inhibited at 0.76 µM (Supplementary Figure 1.).

Distribution of genes encoding bacteriocins in the genus *Actinomyces*

As the active mass could not be matched to any previously known antimicrobial peptide, and no antimicrobial compounds have previously been described within the species, the genome of *A. ruminicola* DPC 7226 was sequenced**.** Following genome annotation, the draft genome was analysed using BAGEL4 to search for potential antimicrobial encoding operons. Gene clusters were identified containing putative genes for thiopeptide production (data not shown) but the masses predicted, 2195.4 Da and 1152.5 Da, did not correlate with the mass detected in the antimicrobial HPLC fraction.

In conjunction with screening the genome of *A. ruminicola* DPC 7226, we also set out to characterise the antimicrobial potential of the genus. One hundred and sixty one *Actinomyces* spp. genomes in various stages of assembly were screened using BAGEL4

(Supplementary Table 1). The isolates were obtained from humans (78.2%), other animals (16.1%), or unknown origin (4.9%), while one was an environmental isolate (0.6%). One hundred and six areas of interest were revealed in 76 strains, covering 18 species. Ninety areas of interest contained complete operons for antimicrobial production. Twenty nine were predicted to be class I bacteriocins, including 7 LanBC modified lantibiotics, Sixteen LanM modified lantibiotics, one single-peptide sactibiotic, three lasso peptides, and two thiopeptide producing operons were also detected. Thirteen operons were predicted to encode class II d bacteriocins and a further 48 operons were predicted to encode bacteriolysins. A phylogenetic tree was generated from the 16S rRNA sequences of 142 *Actinomyces* with *Bacteroides fragilis* ATCC 25285 as the root, and overlaid with operon type and strain source (Fig. 4). Bacteriocin production was widely distributed across the *Actinomyces* pangenome, though bacteriolysin production was found exclusively among human isolates (Fig. 4).

Genetic and molecular characterization of the actifensin determinant

To identify the gene encoding the 4091±1 Da peptide within the genome of *A. ruminicola* DPC 7226, pure peptide was subjected to N-terminal sequencing which revealed a primary sequence consisting of Gly-Phe-Gly-X-Asn-Leu-Ile-Thr-Ser-Asn-Pro-Tyr-Glu-X-Ser, with blanks at residue positions 4 and 14 denoted as probable cysteines (Fig. 5a). This 15 amino acid sequence could be matched to a 69 residue small open reading frame in the draft genome, capable of encoding a 37 amino acid mature peptide (hereafter referred to as actifensin) with a predicted mass of 4097.7 Da preceded by a 32 residue leader sequence (Fig. 5a).

The genetic locus encoding actifensin is shown in Fig. 5b, where *afnA* encodes actifensin. Within an approximately 6.5 kbp upstream region of *afnA*, genes encoding an ABC transporter permease (*afnJ*), an ATP binding ABC transporter (*afnK*) and another ABC transporter permease (*afnL*) were identified as being present. Downstream of *afnA* three hypothetical genes of unknown function (*afnG* - *afnI*) were found, followed by genes encoding another ATP binding ABC transporter (*afnF*), a predicted α/β hydrolase superfamily protein (*afnE*) another protein of unknown function, a subtilisin like protease and a LuxR family transcription factor (*afnD, afnC,* and *afnB* respectively). Within *afnE* is a predicted RHO-independent transcription terminator, and upstream of the structural gene are four predicted promoters. A putative ribosome binding site was also identified nine base pairs upstream of the ATG start codon for the peptide consisting of a purine rich sequence 5' – GAAAGG – 3' (Fig. 5a).

The leaderless structural peptide was found to have a predicted mass of 4097.7 Da. This mass was 6 Da higher than detected by MALDI-TOF MS. The difference between predicted and observed masses most likely corresponds to the loss of 6 hydrogen atoms during the formation of disulphide bonds between the six cysteines. Short peptides with numerous disulphides in specific positions are characteristic of the defensin peptide families (4). To confirm the presence of disulphide bonds in actifensin, pure peptide was reduced and alkylated to break open the disulphide bonds and then subjected to trypsin digestion and peptide mass fingerprint analysis by MALDI-TOF MS. Reduction and alkylation of actifensin resulted in a 4440 Da mass which correlates with the expected increase in mass of 58 Da for each cysteine. MALDI TOF MS analysis of the subsequent trypsin digest detected a mass of 2257.02 Da which corresponds to the first 19 amino acids of the peptide (Gly-1 to Lys-19) containing three alkylated cysteine residues. Three other predicted masses, Ser-20 to Arg-24, Gly-25 to Arg-31, and Thr-32 to Tyr-37 (predicted mass and alkylated masses 581.30 Da, 584.25 Da, and 803.31 Da respectively) were not detected.

Discovery of actifensin homologs

BLASTp analysis with AfnA found homologous ORFs within the fungal genera *Blastomyces*, *Emmonsia*, and *Emergomyces*, *Helicocarpus griseus,* and a defensin from the mollusc species *Rhuditapes philippinarum* (58%, 58%, 55%, 52%, and 61% identity, respectively, Supplementary Figure 2)**.** Characteristic conserved cysteines were noted though low sequence identity was observed between the mature actifensin peptide and eukaryotic defensins. The same was found when AfnA was compared with known previously characterised arthropod, ascomycete and mollusc defensins (Fig. 6a) which conserved secondary structures (Fig. 6b). BLASTp analysis using the 69 residue AfnA sequence identified 37 homologous structural genes within the genus *Actinomyces* and one homolog from a *Corynebacterium* sp. sequence (Fig. 6a). Further analysis indicated that the homologs were present in 15 operons from 14 strains, in addition to conserved genes for transport, transcription regulation, and proteolytic activity (Fig. 6b). *Actinomyces* sp. 2119, *A. oris* S64C, *A. succiniciruminis* AM4, *A. oris* CCUG34286, *Actinomyces* sp. F0337, *Actinomyces* sp. HMSC075C01, and *A. oris* MMRCO6-1 had at least two actifensin homologs, while *Actinomyces* sp. F0337 containing an operon with seven copies, the most observed within one genome, (Fig. 7b). The genome of *A. oris* MMRCO6-1 contained six encoded actifensin homologs detectable over two contigs but only one (contig 50) contained the other conserved ORFs (*afnB-I*, *J-K*) present in the actifensin operon. Twelve of 14 operons had a highly conserved arrangement of *afnB-I*, all of which also had ABC transporter genes directly upstream of the bacteriocin ORF. The mean amino acid identity between all structural genes was 52%. The highest identity observed between actifensin and a homolog was 77% identity with *afnA* in *Actinomyces* sp. CTC72, though higher identities were observed between other peptides (Supplementary Fig. 3). We proceeded to characterise ten predicted cysteine-stabilised αβ (CSαβ) peptides predicted by Dash *et al*. (2019). The peptides are present in five *Actinomyces* genomes bringing the total number of peptides to 47 homologous structural genes in 19 strains *Actinomyces oris* S24-V, *Actinomyces denticolens* PA, *Actinmoyces* sp. Chiba-101, *Actinomyces johnsonii* F0542 and *Actinomyces* sp. F0330, have genes which were not identified using BLASTp and the actifensin propeptide sequence (27). S24-V, PA, and Chiba-101 display the conserved *afnB* to *afnI* ORFs following *afnA,* which is absent in strains F0330 and F0542 (Fig. 7b).

The propeptide contains a conserved G-X-E motif prior to the start of the mature peptide (Fig. 7a). In 36 of the peptides, an alanine residue is present after the glycine which may be involved in secretion and cleavage. This putative GA cleavage signal is replaced by a TS motif in eight of the 49 peptides (*A. oris* S64C *afnA5*, *A. oris* CCUG34286 *afnA7*, *A. oris* MMRCO6-1 contig 75 *afnA2*, *Actinomyces* sp. F0337 *afnA4*, *Actinomyces* sp. HMSC075C01 *afnA4*, *A. oris* MMRCO6-1 contig 50 *afnA4, afnA3* and *A. oris* S24V *afnA5*). A conserved Pro residue was noted following the first conserved Cys in addition to a conserved G-Y-X-G-G-X-C sequence at positions 56-62 of the propeptide (22-28 in the active peptide, Fig. 7a).

Discussion

We describe a novel group of bacteriocins with broad spectrum inhibitory activity within the *Actinomyces* genus. Actifensin is the first such bacteriocin to be discovered which is produced by a strain of *Actinomyces ruminicola*.

Actifensin inhibited a broad range of Gram-positive species including notable pathogens such as vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus*. Given the global challenge of the increase in antibiotic resistance there is an urgent need for new classes of antimicrobials. Bacteriocins have been suggested as an alternative to conventional antibiotics due to their effectiveness at low concentrations and their potential to be genetically modified [2]. Class II bacteriocins are diverse in sequence and structure, whose mechanism of action is through interaction with the cell membrane, causing permeabilization, pore formation and dissipating membrane potential [31]. The defensin-like bacteriocin laterosporulin10 has been found to act on the cell membrane of *S. aureus* Mtb H37Rv, disrupting cellular homeostasis [7]. Plectasin and eurocin, fungal C6 defensins are known to bind lipid II, inhibiting bacterial cell wall biosynthesis [32, 33]. Actifensin possesses an N-terminal loop extension which in other defensin peptides has been implicated in membrane disruptive capability (28). The loop consists of nine residues between Cys-4 and Cys-14 beginning with an Asn. In most of the other peptide sequences identified, the N-loop is six residues long, beginning with a Pro, (excepting afnA from *Actinomyces* sp. F0588, and *A. naeslundii* S44D which have an eight residue N-loop with a serine or arginine in the first position respectively, followed by a Pro. (Fig. 7a).

Actifensin also inhibited the growth of *C. difficile* and *C. sporogenes*. Clostridia are known colonizers of the rumen [37, 38], and as *A. ruminicola* DPC7226 was isolated from the faeces of a ruminant, actifensin production may provide a competitive advantage in the gut microbiome. *Actinomyces neuii* and *Actinomyces radingae* were both inhibited by actifensin, however, it would be interesting to see if cross resistance between actifensin and other actifensin-like producers exists.

A pan genus *in silico* screen revealed that the genus *Actinomyces* (Fig. 4) are a rich source of antimicrobials and have genes for bacteriolysin and lantibiotic production (48/90, and 29/90 operons respectively). Thirteen class II bacteriocins were predicted by BAGEL, but neither the actifensin operon, nor its homologs were detected due to lack of similarlarity with known systems. One previous study described odontolycin, a bacteriocin produced by an *Actinomyces odontolyticus* dental plaque isolate, though no further research on the peptide was reported (29). Interestingly in our study no operons for bacteriocin production were found among five *A. odontolyticus* genomes screened (Fig. 4).

The actifensin structural gene comprises of a 37 amino acid mature peptide preceded by a 32 amino acid leader sequence (Fig. 5). A GA motif at positions -3 and -2 was identified, which is a known cleavage signal used in ABC transporter mediated secretion [29]. Indeed, there are a number of predicted ABC transporter genes within the actifensin operon. ABC transporter genes could also play a role in self-immunity to the actifensin peptide. Unusually, an additional glutamic acid residue is present at -1 before the mature peptide. As the purified peptide was subjected to N-terminal sequencing, we can be certain that the mature peptide begins with a glycine residue, therefore the additional glutamic acid residue at -1 is most likely subject to exopeptidase cleavage prior to activity, and indeed there are genes present with predicted protease activities (Fig. 5).

The GA cleavage motif is present in 36 of the homolog structural genes, with TS replacing the motif in eight instances, GT and GG in two cases, and GS SA, and DA in one each (Fig. 7a). A double glycine is the most commonly found motif for ABC transporter mediated cleavage among bacteriocins, though GA and GS have also been observed [29]. It will be interesting to see if the peptides bearing other residues at this location are indeed subject to ABC-mediated transport. We note that each operon containing a gene with a non-traditional TS/GT/SA/DA signal contains at least one more structural gene than those with a GG/GA sequence. This could indicate potential diversification of a repertoire of bacteriocins enabling improved ability to combat multiple competitors. It was also surprising that an actifiensin homolg was found in a distantly related *Corynebacterium* sp., though many of the conserved genes in the *Actinomyces* spp. operons were not present (Fig. 7b). As such this may be non-functional as ABC transporter related genes are missing upstream of the structural gene and the conserved *afnB* - *afnI* pattern is absent. The genera *Corynebacterium* and *Actinomyces* are distantly related members within the phylum Actinobacteria and some species are known members of plaque biofilms, providing an opportunity for horizontal gene transfer [16], though given the dissimilarity of the operons, they may have been acquired independently at some stage.

As stated above, the laterosporulins produced by *Brevibacillus* sp. are two structurally defensin-like bacteriocins with broad spectrum inhibitory activity [6, 7]. They are 57.6% similar in amino acid sequence to each other which is comparable to actifensin and its predicted homologs but share the conserved cysteine residues which form disulphide bridges. Conserved disulphides are characteristic of defensins and are present in vertebrate, invertebrate, plant, fungal defensins, and defensin like peptides [4]. Actifensin has a predicted mass of 4097.7 Da but the actual mass is 4091±1 Da by MALDI-TOF MS. The same discrepancy in predicted and observed mass was noted with laterosporulin, where six hydrogen atoms are lost in the formation of disulphide bonds. We hypothesize that bonds in actifensin likely form in the 1-4, 2-5, 3-6 formation similar to ascomycete and arthropodC6 defensins (Fig. 6), as the amino acid motifs (C-X[5-12]-C-X[3]-C-X[9-10]-C-X[4-5]-C-X-C) are conserved [5] The structure of laterosporulin10 has been determined to be architecturally similar in structure to human α-defensin though its disulphide connectivity is homologous to that of β-defensins (Fig. 8) [30]. The overall architecture and disulphide connectivity of actifensin is likely to be homologous to that of C6 defensins consisting of an N-terminal α-helix followed by a two-stranded antiparallel beta sheet-stabilized by disulphide bridges (Fig. 8). Interestingly an actifensin homolog we identify as afnA from *Actinomyces* sp. oral taxon 171 str F0337 has had its 3D structure determined and is publicly available from an online database. The peptide labelled actinomycesin is strikingly similar to C6 fungal and arthropod defensins which have also been characterised (Fig. 6), however no published material is available regarding its activity, antmicrobial or otherwise. Indeed, two antiparallel beta-sheets stabilised by disulphide bonds with an interposed short turn region, previously described as the γ-core motif, are a ubiquitous feature of antimicrobial peptides (30). Actifensin exhibits the highly conserved GXC (positions 26-28 in the mature peptide) as do all of its homologs.

CSαβ peptides comprise one of the most widespread families of defensins, and defensin-like peptides. A recent publication identified a number of CSαβ sequences in bacterial genomes with potential for antimicrobial, toxin, or signalling activity (27). Of 58 peptides identified within the phylum *Actinobacteria* by Dash *et al*. (2019), 34 were of the genus *Actinomyces*, 24 of which we identified using BLAST with the actifensin propeptide sequence (Supplementary Table 2). A further 113 bacterial peptide sequences identified by Dash *et al.* remain to be characterised from a functional perspective and could be a potent source for future antimicrobials. Interestingly a bacterial defensin-like peptide AdDLP identified *in silico* was synthesised and recombinantly expressed, and the peptide was found to have anti-*Plasmodium* activity (31). The bacterial CSαβ peptides could be an untapped source of potential applications, and have been proposed as the ancestral evolutionary origin of eukaryotic defensins (32).

In the search for novel antimicrobials for application in health and food, genomic and pangenomic approaches are becoming increasingly common [3, 4]. These approaches are advantageous in that large amounts of genetic data can be analysed to identify novel antimicrobials/bacteriocins, and can even allow one to ‘reincarnate’ otherwise ‘dormant’ genes [5]. However, such analyses are dependent on the ability of programs to predict based on databases of previously identified sequences, and so peptides with novel structures and operons may not be detected. Though a number of bacteriocin operons were found in the *Actinomyces* spp. genomes using BAGEL, actifensin was not identified by genome sequence alone, which highlights the importance of functional screening for antimicrobial compounds in addition to *in silico* screening. Using BLAST thirty-seven structural genes with homology to actifensin were found in *Actinomyces* spp., and a single structural gene from a

Corynebacterium sp. As some CSαβ peptides function as toxins future applications will require any potential cytotoxic effects to be assayed. We propose that actifensins and the laterosporulins may constitute a new subgroup of class II bacteriocins; the defensin-like bacteriocins. These bacteriocins share only moderate identity to each other but do contain highly conserved cysteine residues and are structurally related to eukaryotic defensins.

Conclusions

A series of novel defensin-like bacteriocins within the genus *Actinomyces* were identified using an *in silico* pan-genomic approach coupled with a functional screen, many of which are ubiquitous members of the oral microbiome. The bacteriocins represent a potential new class of antimicrobial peptides, defensin-like bacteriocins which may have widespread applications as antimicrobials in food and human health.

Experimental Procedures

Isolation of bacteria and identification of bacteriocin production

Samples of raw milk, unpasteurised cheeses, sheep faeces and honey were serially diluted in maximum recovery diluent (Oxoid) and plated on several media types for the isolation of bacteriocin producing bacteria; *Streptococcus thermophilus* selective agar (tryptone 10.0 gL^{-1}, sucrose 10.0 gL^{-1}, yeast extract 5.0 gL^{-1}, K_2HPO_4 2.0 gL^{-1}, bromocresol purple 0.03 gL^{-1}, agar 15.0 gL^{-1}) incubated aerobically at 42 °C; M17 (Merck) supplemented with 10% w/v lactose incubated at 30 °C aerobically; de Man, Rogosa, and Sharpe (MRS, Difco) agar supplemented with 30 μgmL^{-1} L-vancomycin hydrochloride incubated at 37 °C; MRS adjusted to pH 5.4 incubated at 42°C anaerobically; *Lactobacillus* selective agar (LBS, supplier) incubated at 30 °C anaerobically; and TOS (Transgalactosylated oligosaccharide) agar supplemented with 50 µgmL^{-1} lithium mupirocin incubated at 37 °C anaerobically.

Isolates were subject to an initial bacteriocin production screen by overlaying with 10 mL 'sloppy' MRS agar (7.5 gL^{-1} agar) tempered to 50 °C and seeded with an overnight culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG6901 (0.25% v/v). Cultures which were found to produce distinct zones of inhibition in the agar overlay were cultured in broth for well diffusion assays. For well diffusion assays, 20 mL of 'sloppy' MRS agar seeded with *L. bulgaricus* LMG6901 as before, was poured and allowed to set in which 6 mm wide wells were then bored. 50 µL of cell-free supernatant was added to each well and plates were incubated at 37 °C overnight. Zones of inhibition were indicative of antimicrobial activity.

Bacterial Strains, media, reagents

Strains used in this study and their incubation conditions are listed in Supplementary Table 3. *A. ruminicola* DPC 7226 was routinely maintained on BHI (Oxoid) anaerobically at 37 °C. Media reagents were sourced from Sigma-Aldrich (Wicklow, Ireland) unless stated otherwise.

Purification of actifensin

A. ruminicola DPC 7226 was grown anaerobically, statically at 37 °C in 500 mL volumes of BHI broth for 48 h. Following centrifugation, cell-free supernatant was applied to an Econo column containing 30 g Amberlite XAD beads prewashed with Milli Q water. The column was washed with 300 mL 30% ethanol and 300 mL 2-propanol 0.1% TFA (IPA). IPA was removed by rotary evaporation and the sample was applied to a 60 mL, 10 g Strata–E

C18 SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 60 mL 25% ethanol and then 60 mL IPA.

Centrifuged cells were combined with 100 mL IPA and stirred at room temperature for 3-4 h. The resulting suspension was centrifuged and the cell extract and purified CFS were assayed by MALDI TOF mass spectrometry to determine the molecular mass of antimicrobial compounds (Axima TOF^2 MALDI-TOF mass spectrometer, Shimadzu Biotech, Manchester, UK). A MALDI target plate was precoated with CHCA matrix solution, 0.5 µl of the supernatant from the cell extract was then placed on the target and a final layer of matrix solution was added. Positive-ion linear or reflectron mode was used to detect peptide masses.

Actifensin characterisation

Characterisation was performed using purified bacteriocin. To test protease susceptibility 100 µL aliquots of 50 µgmL^{-1} were subjected to treatment with 10 mgmL^{-1} proteinase K (Sigma-Aldrich) and α-chymotrypsin (Sigma-Aldrich) at 37 °C for 3h, followed by a 10 min incubation at 100 °C to denature the enzymes. 50 µL aliquots were assayed on *L. delbrueckii* ssp. *bulgaricus* LMG6901 indicator plates. Heat stability was determined by 30 min incubations at 60, 70, 80, 90, 100 °C and by autoclaving at 121 °C for 15 min.

For spectrum of activity, a well diffusion assay was carried out as described above with the strains in in the appropriate medium. 50 µL of purified bacteriocin at a concentration of 50 µgmL^{-1} was added to a well. Following overnight incubation under the appropriate conditions zones of activity were measured and categorised as no inhibition, weak inhibition (0.5 mm – 2 mm), strong inhibition(2.5 mm – 5 mm), and very strong inhibition (>5 mm) (Table 1). Minimum inhibitory concentration against selected pathogens was assayed as above, starting at 100 µgmL^{-1} peptide solution serially diluted 1:2 to 0.78 µgmL^{-1}.

Draft genome sequencing

DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma) and prepared for sequencing using a Nextera XT kit (Illumina) for library preparation. DNA was quantified using a Qubit 2.0 fluorometer. Sequencing was carried out using an Illumina MiSeq platform with paired-end 2 x 300 base pair reads by the Teagasc Sequencing Centre, Teagasc Food Research Centre Moorepark. Assembly was performed using tools available on the public server from usegalaxy.org (33). Assembly was performed *de novo* using SPADES (version 3.0.0) and resulted in 116 contigs. Contigs were aligned to a reference genome using Mauve (version 20150226 build 10), followed by annotation with RAST (version 2.0). The annotated genome was analysed for predicted bacteriocin and secondary metabolite production clusters using BAGEL4 (34), and any further annotation was carried out using Artemis genome browser (version 16.0.0). Genomic data are available from GenBank/EMBL under accession no. SPKK00000000.

BAGEL screen and phylogenetic analysis of *Actinomyces* species

Genbank and fasta assemblies of the genus *Actinomyces* were acquired from the NCBI assembly database and screened using BAGEL4 (35). Where available corresponding 16S rRNA sequences were acquired from the RDP database (36) and where unavailable *Actinomyces* spp. genomes were subject to analysis using RNAmmer (37). 16S rRNA sequences were aligned using MUSCLE (38, 39) and a phylogram was generated using iTOL (40). The phylogram was then overlaid with the BAGEL screen data.

Reverse bacteriocin identification, peptide and structure prediction and homology

Two hundred µg freeze-dried purified peptide was sent for N-terminal amino acid sequencing (AltaBioscience, UK). The resulting 15 residue sequence, GFGXNLITSNPYQXS, was used to search for a bacteriocin structural gene with Artemis genome browser. Following identification of the structural gene, other genomes were searched for genes homologous to the active and pro-peptide using BLASTp, genes on contigs consisting of less than 5 kbp were excluded. Additional actifensin homologs were identified from Dash *et al.* (2019) among 147 non-redundant bacterial CSαβ peptide sequences (27). Alignments were generated using Clustal Omega (41), and visualised with Jalview (42). Structural modelling was performed using SWISSMODEL (43) online software and structural images were generated using PyMOL (44).

Availability of data and material

Genomic data analysed in this study are publicly available from the NCBI database at https://www.ncbi.nlm.nih.gov/.

Author's contributions

CS, CH, and RPR were involved in study design, guidance with experiments and interpretation of the results. IS performed the *in silico* screen, isolated the bacteriocin producer, characterised the spectrum of inhibition, whole genome sequencing, genetic and stability characterisation of actifensin, and identified and characterised actifensin homologs and prepared the manuscript. POC performed MALDI-TOF MS, bacteriocin purification and alkylation of the peptide. All authors took part in reviewing the manuscript and approved the final manuscript.

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Figure 1: Antimicrobial activity of *Actinomyces ruminicola* DPC 7226 from (a) colonies overlaid with *L. delbrueckii* ssp. *bulgaricus* LMG6901in sloppy MRS (b) and in well diffusion with neutralised CFS.

Figure 2: Detection of actifensin 4091 Da +/_ 1 Da (indicated by arrows), by MALDI-TOF MS from (a) cell free supernatant, (b) cell extract, (c) colonies on a plate. (d) The 4091±1 compound when purified was active to <1 µgmL^{-1}, indicator *L. bulgaricus* LMG 6901.

Figure 3: Inhibition of actifensin against a broad spectrum of indicator species. Weak inhibition, 0.5 - 3 mm zone, strong inhibition, 3 – 5 mm zone, very strong inhibition > 5 mm zone. *Vancomycin-resistant *Enterococcus*, +Methicillin-resistant *Staphylococcus aureus.*

Figure 4: Phylogram of *Actinomyces* genomes using 16Sssequences overlaid with BAGEL4 predictions, strain source and presence of actifensin or predicted homolog operon.

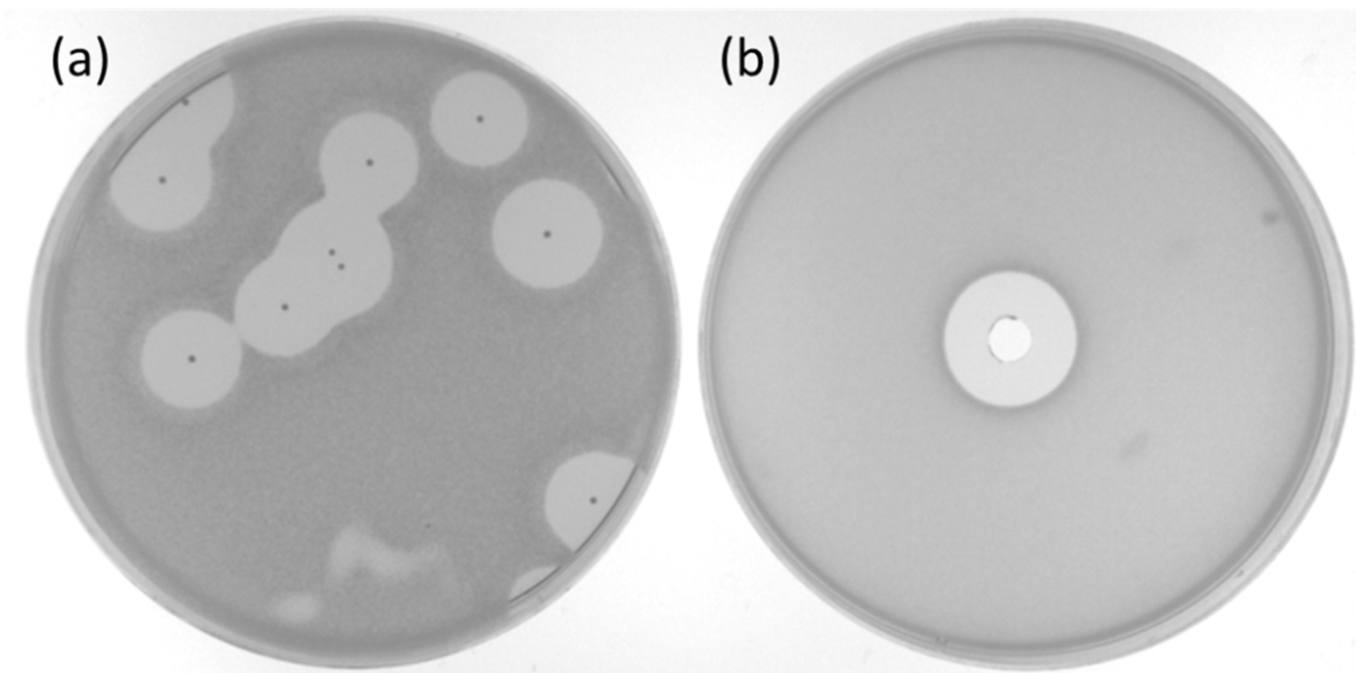
Figure 5: (a) 69 residue pro-peptide identified following genome analysis using the 15 amino acid sequence (underlined) determined by N-terminal amino acid sequencing, Putative ribosome binding site highlighted 8 base pairs upstream of start codon. (b) Genetic vicinity of structural gene containing nearby genes for transport, hypothetical, proteolytic proteins, and a transcription factor.

Figure 6: (a) Mature peptide sequence alignment of afnA with characterised defensin family peptides from different phyla. Known disulphide connectivities outlined above highlighted cysteine residues. (b)Available 3-D structures of above sequences. Alpha-helices coloured red, beta-sheets shown in blue. Protein data bank accession numbers shown below in brackets.

Figure 7: (a) Sequence alignment of actifensin propeptide sequence (boxed) with structural genes predicted *Actinomyces* spp. peptides. Amino acids with greater than 80% conservation

are coloured, leader sequence and mature active peptide are highlighted above. Putative disulphide connectivity between conserved cysteines of the mature peptide are indicated below, right and putative cleavage site is indicated below, centre. (b) Diagram of actifensin homolog production operons. Multiple bacteriocin genes within one operon are denoted *afnA1 – afnA7* where present.

Figure 8; Conserved structures of the defensin peptide superfamily and defensin-like bacteriocins, laterosporulin and actifensin. β-sheets coloured blue, α-helices coloured red, disulphide bonds shown in yellow.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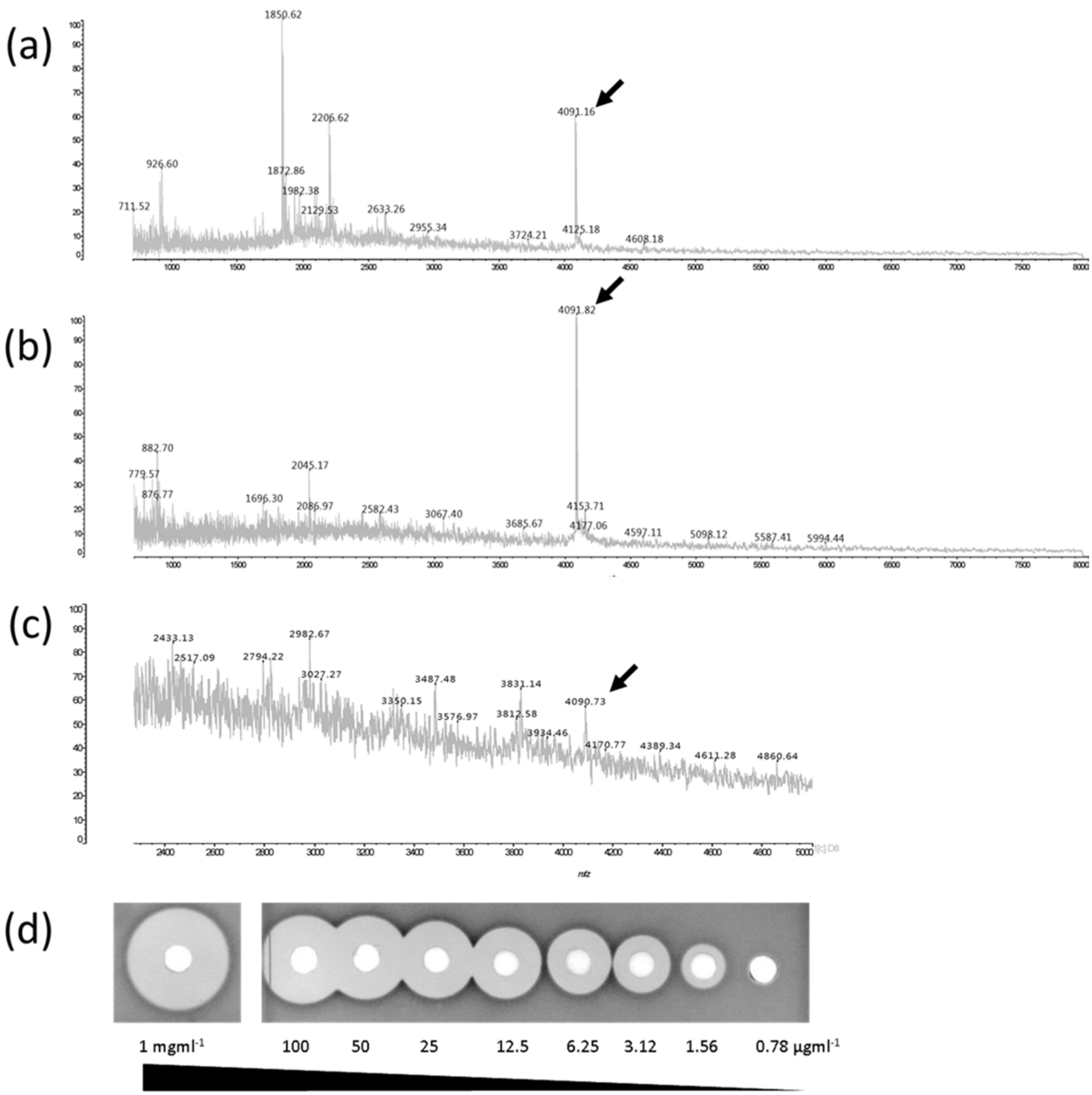
(b)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4125.18
4608.18
(b)
4091.82
882.70
779.57
2045.17
876.77
1696.30
2086.97
2582.43
3067.40
4153.71
3685.67
4177.06
4597.11
5098.12
5587.41
5994.44
(c)
2433.13
2517.09
2794.22
2982.67
3027.27
3487.48
3831.14
3350.15
3576.97
3812.58
4090.73
3934.46
4170.77
4389.34
4611.28
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m/z
(d)
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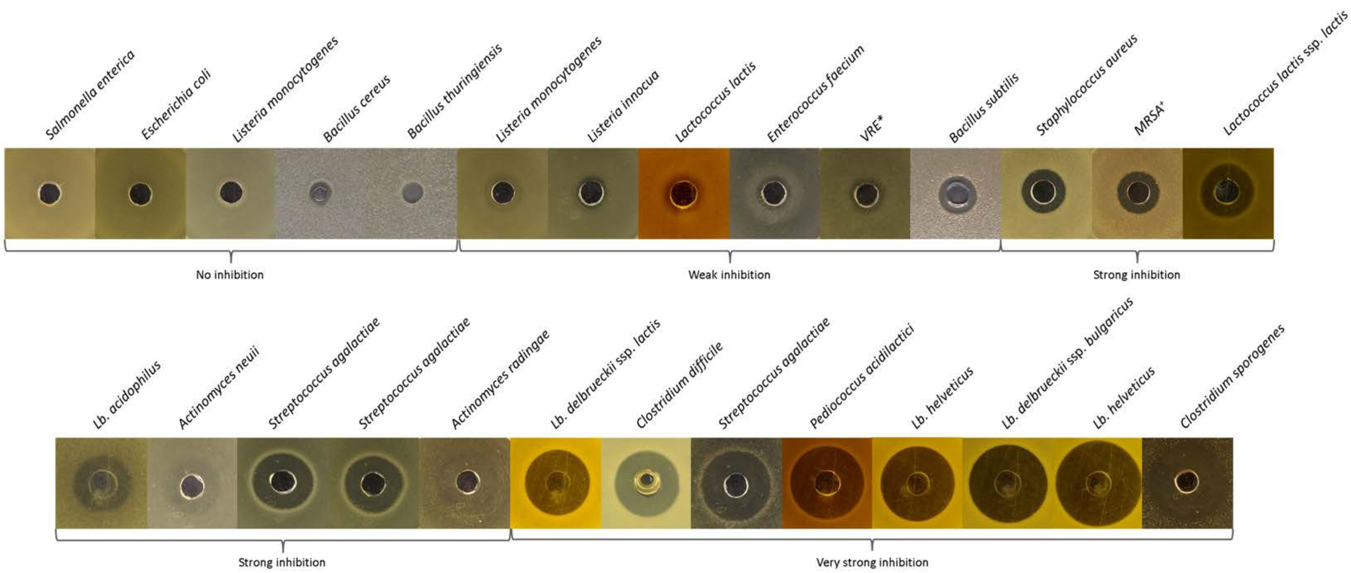
25
12.5
6.25
3.12
1.56
0.78 µgml-1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Bacillus cereus
Bacillus thuringiensis
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Listeria innocua
Lactococcus lactis
Enterococcus faecium
VRE*
Bacillus subtilis
Staphylococcus aureus
MRSA⁺
Lactococcus lactis ssp. lactis
No inhibition
Weak inhibition
Strong inhibition
Lb. acidophilus
Actinomyces neuii
Streptococcus agalactiae
Streptococcus agalactiae
Actinomyces radingae
Lb. delbrueckii ssp. lactis
Clostridium difficile
Streptococcus agalactiae
Pediococcus acidilactici
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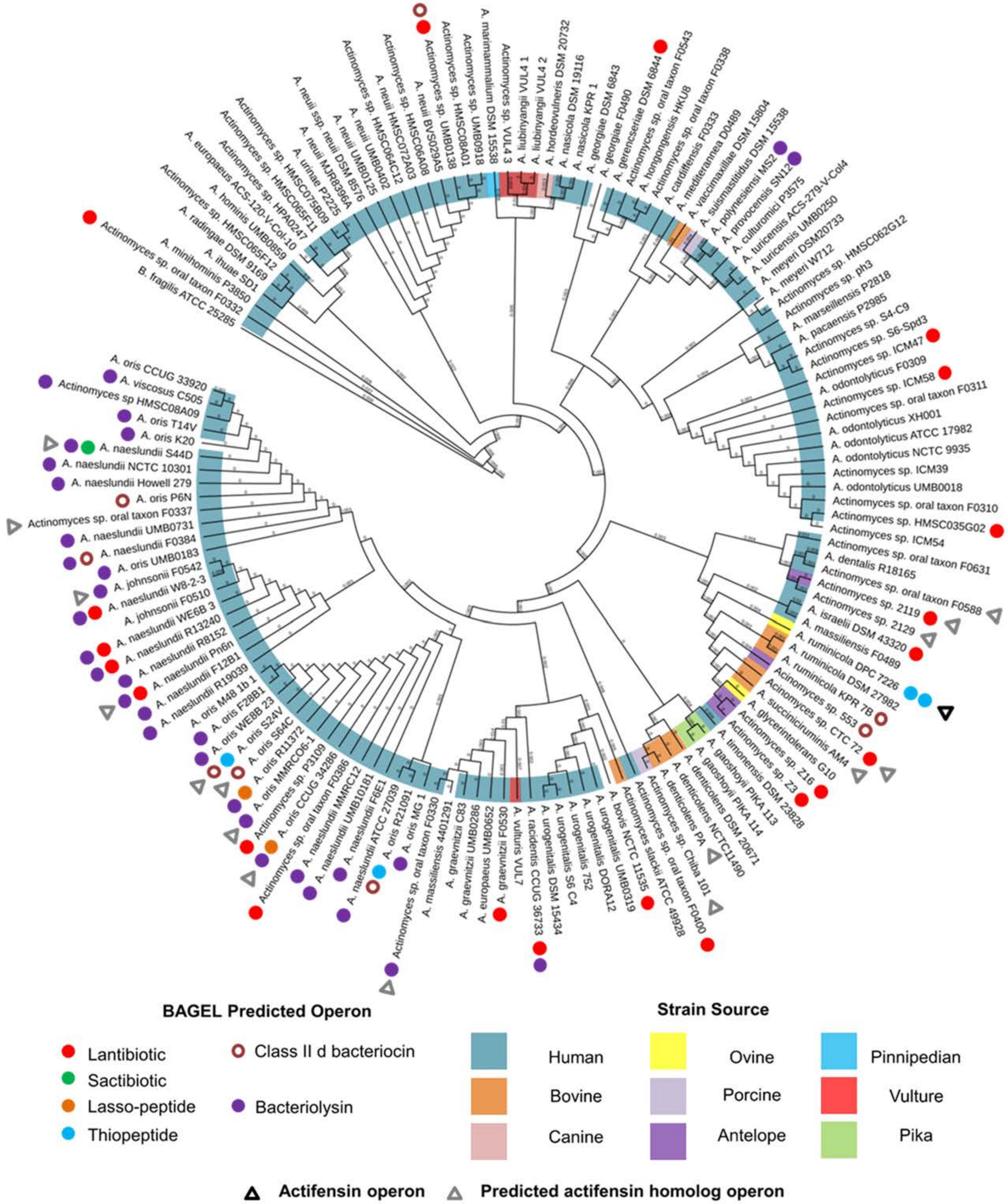
Lb. delbrueckii ssp. bulgaricus
Lb. helveticus
Clostridium sporogenes
Strong inhibition
Very strong inhibition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Actinomyces sp. HMSC065F11
Actinomyces sp. HMSC075B09
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A. neuii ssp. neuii DSM 8576
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A. neuii UMB0402
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A. neuii BVS029A5
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Actinomyces sp. HMSC08A01
Actinomyces sp. UMB0918
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Actinomyces sp. VUL4 3
A. liubinyangii VUL4 1
A. liubinyangii VUL4 2
A. hordeovulneris DSM 20732
A. nasicola DSM 19116
A. nasicola KPR 1
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A. georgiae F0490
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Actinomyces sp. Z3
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A. oris S24V
A. oris WE8B 23
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A. oris M48 1b 1
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A. naeslundii S44D
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A. viscosus C505
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BAGEL Predicted Operon
Lantibiotic
Sactibiotic
Lasso-peptide
Thiopeptide
Class II d bacteriocin
Bacteriolysin
Strain Source
Human
Bovine
Canine
Ovine
Porcine
Antelope
Pinnipedian
Vulture
Pika
Actifensin operon
Predicted actifensin homolog operon

RBS

(a)

```
gaa caa ccgaaaggta aac acc atg aag aag ttc att cgc cgc agc agc agc ctc gcc gcc gcc agc
                           M   K   K   F   I   R   R   S   S   S   L   A   A   A   S
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 F   E   Q   A   F   Q   S   E   T   Q   V   P   L   E   G   A   E   G   F   G   C   N
ctc atc acc tcg aac ccc tac cag tgc agc aac cac tgc aag agc gtc ggc tac cgg ggc ggc tac
 L   I   T   S   N   P   Y   Q   C   S   N   H   C   K   S   V   G   Y   R   G   G   Y
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 C   K   L   R   T   V   C   T   C   Y   *
```
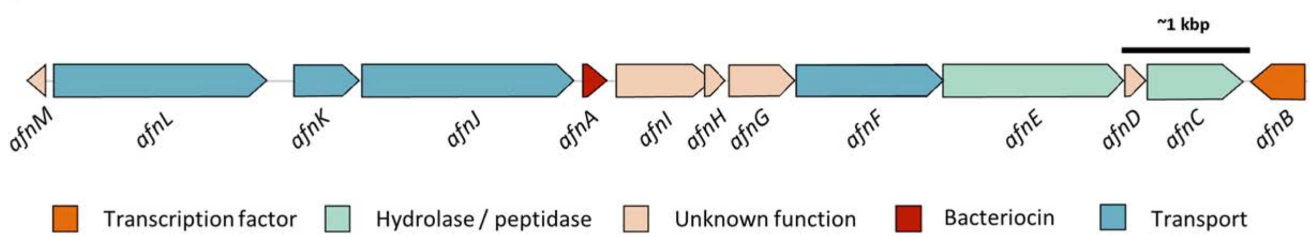
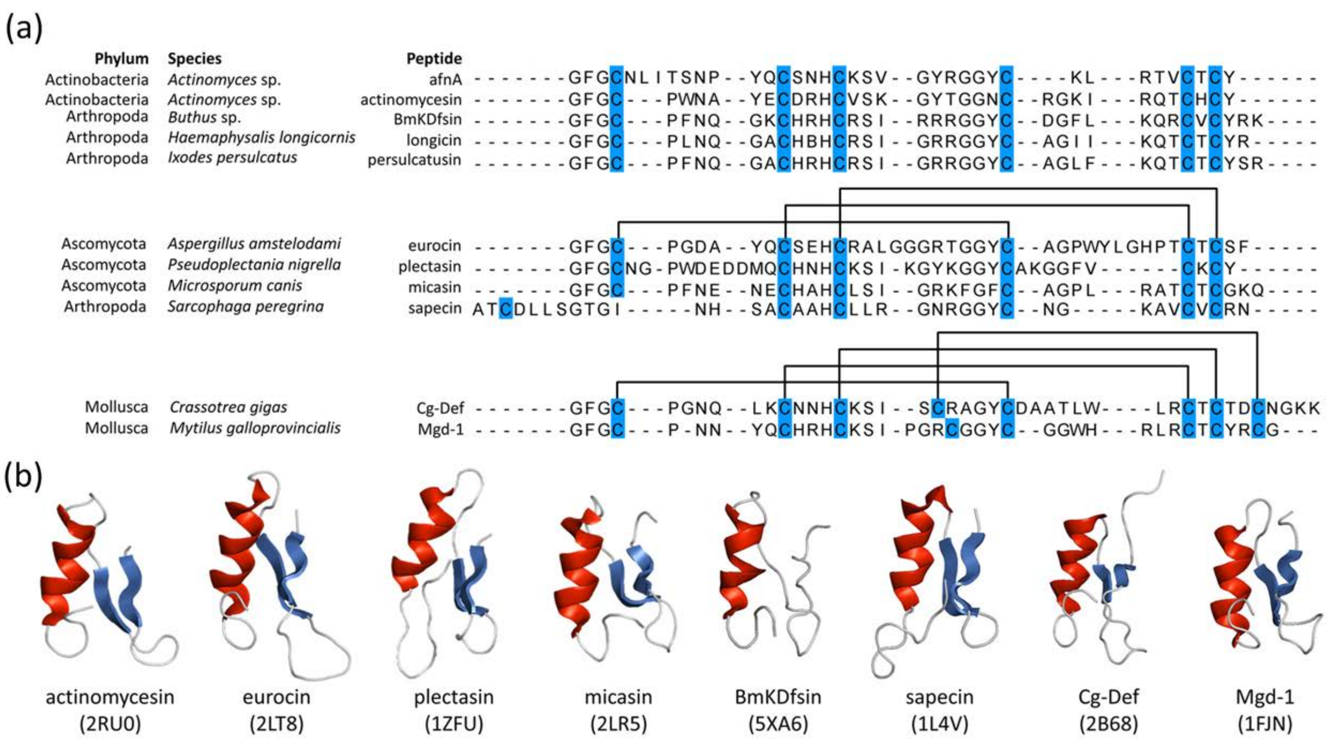

(b)





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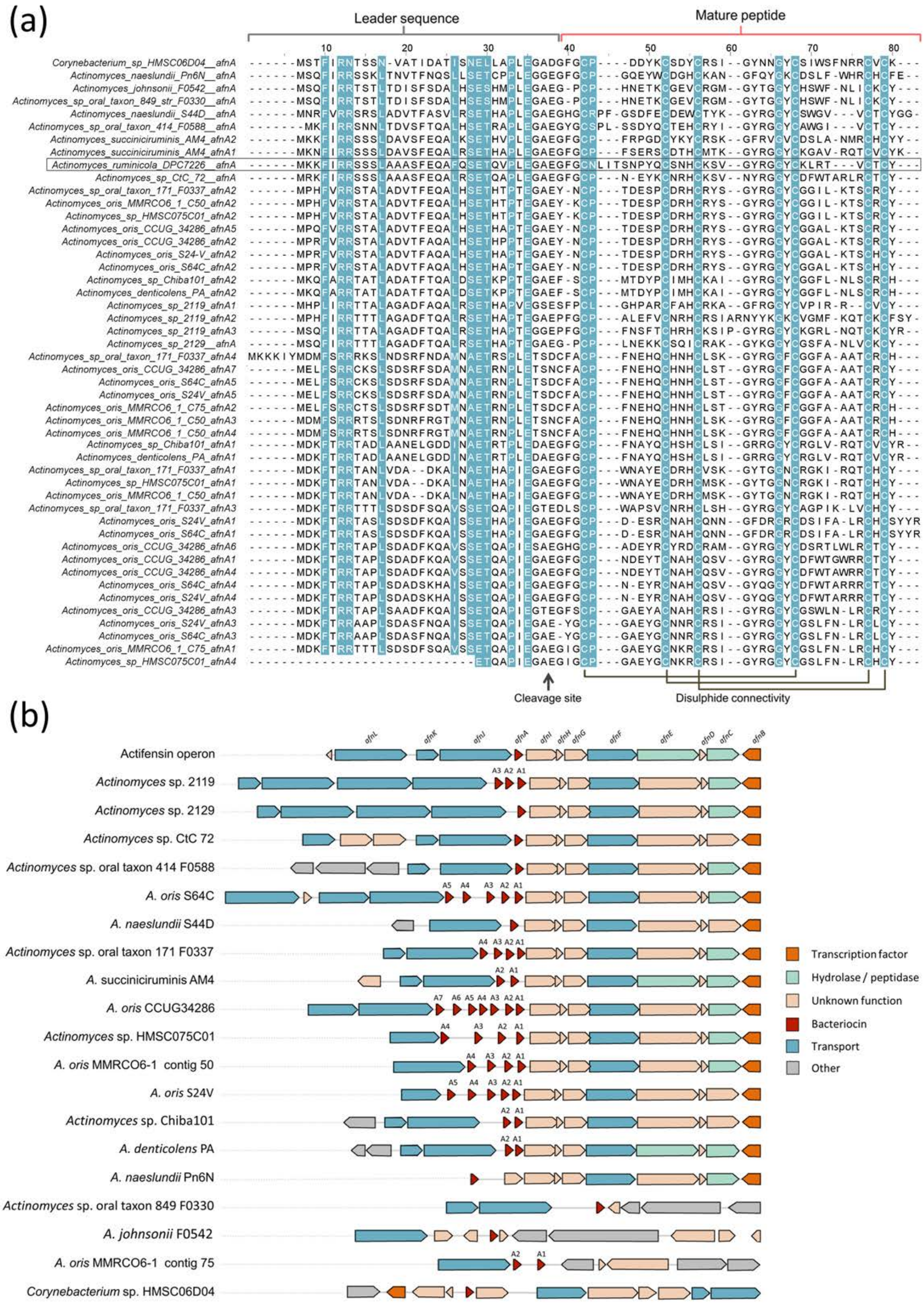
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Actinomyces_oris_S64C_afnA2
Actinomyces_sp_Chiba101_afnA2
Actinomyces_denticolens_PA_afnA2
Actinomyces_sp_2119_afnA1
Actinomyces_sp_2119_afnA2
Actinomyces_sp_2119_afnA3
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Actinomyces_oris_S24V_afnA5
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Actinomyces_oris_MMRCO6_1_C50_afnA3
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Actinomyces_oris_S64C_afnA3
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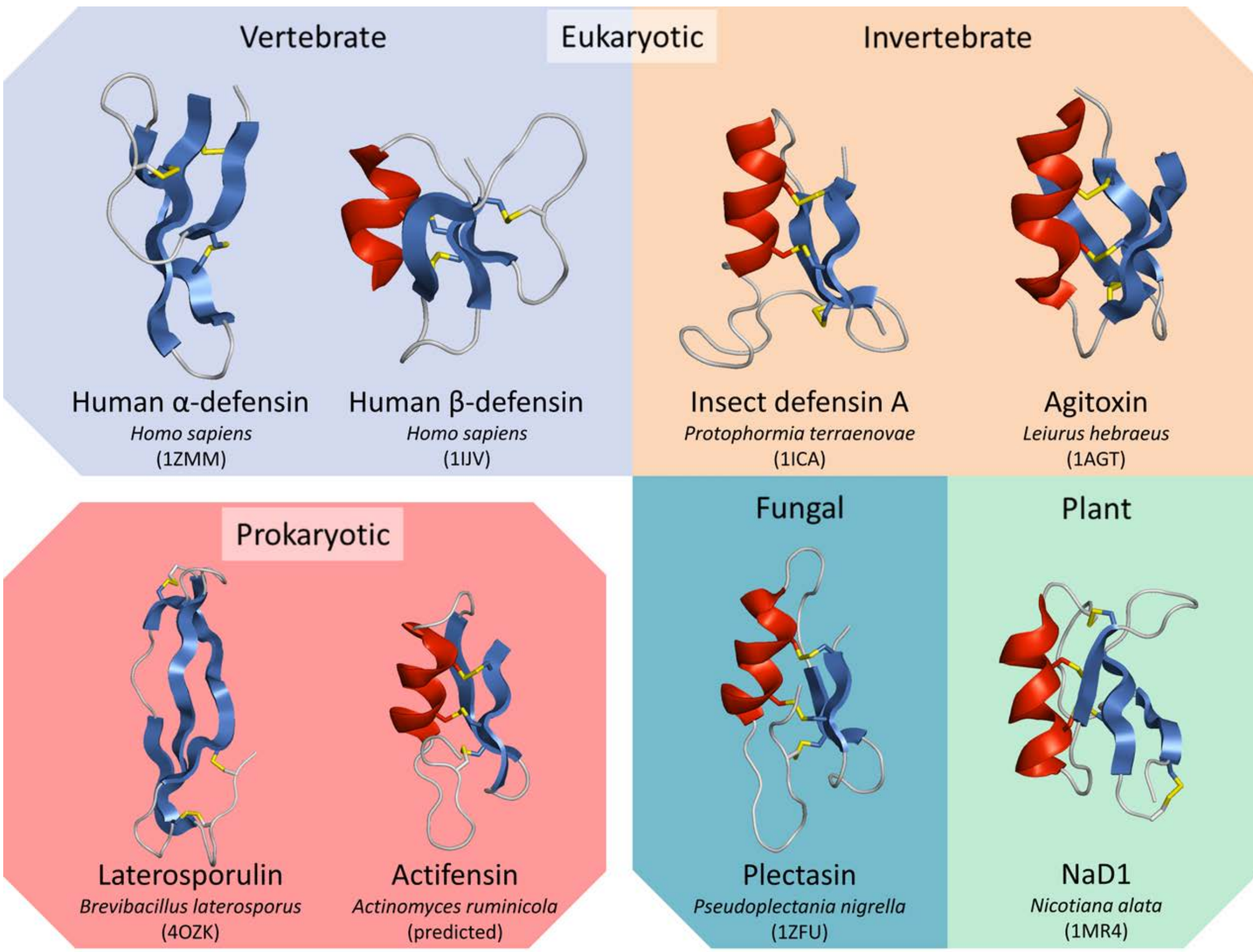
Actinomyces_sp_HMSC075C01_afnA4
Cleavage site
Disulphide connectivity
(b)
Actifensin operon
afnL
afnK
afnJ
afnA
afnI
afnH
afnG
afnF
afnE
afnD
afnC
afnB
Actinomyces sp. 2119
Actinomyces sp. 2129
Actinomyces sp. CtC 72
Actinomyces sp. oral taxon 414 F0588
A. oris S64C
A. naeslundii S44D
Actinomyces sp. oral taxon 171 F0337
A. succiniciruminis AM4
A. oris CCUG34286
Actinomyces sp. HMSC075C01
A. oris MMRCO6-1 contig 50
A. oris S24V
Actinomyces sp. Chiba101
A. denticolens PA
A. naeslundii Pn6N
Actinomyces sp. oral taxon 849 F0330
A. johnsonii F0542
A. oris MMRCO6-1 contig 75
Corynebacterium sp. HMSC06D04
Transcription factor
Hydrolase / peptidase
Unknown function
Bacteriocin
Transport
Other



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