Isolation and Characterisation of a Novel Bacteriocin Active Against Clinically Relevant Strains of *Clostridioides difficile*

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Bacteriocin Introduction

What is a bacteriocin?

Bacteriocins are ribosomally synthesized peptides that are thought to be produced by bacteria as a natural defense against competing bacteria and are predicted to be produced by 99% of bacteria (Klaenhammer, 1988, Riley and Wertz, 2002, Gillor *et al*, 2008). Bacteriocins have received positive publicity and interest, a result of them being generally harmless to humans and the environment due to their sensitivity to proteolytic enzymes.

What are the current antimicrobial treatments?

The current treatment for CDI are the antibiotics, oral metronidazole, fidaxomicin and vancomycin, for more severe cases (Kelly, 2012). Metronidazole is an inexpensive standard for non-severe cases, however almost all of the drug is absorbed in the gut and there are increasing reports of treatment failure. Fidaxomicin is a more expensive drug and not as readily used by clinics and due to the risk of horizontal gene transfer of vancomycin resistance to enterococcus, vancomycin is a last resort drug. Therefore we need new treatments.

Bacteriocins vs antibiotics as a treatment

Bacteriocins

- Ribosomally synthesised
- Generally harmless to humans
- Potential Generally Regarded As Safe (GRAS) producers
- Good publicity
- Commercially viable examples

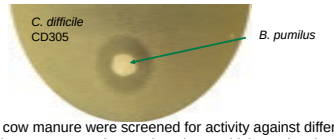
Antibiotics

- Usually secondary metabolite
- Antibiotics as additives in human food illegal
- Bad publicity apart from medical use
- Commercially viable examples

Bacteriocins against *C. difficile*

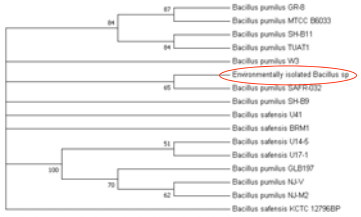
Bacteriocins	Host		Reference
Lacticin 3147	*L. lactis*		Ryan *et al* (1996) & Rea *et al* (2007)
Nisin A & Z	*L. lactis*	Commercially available	Le Lay *et al* (2016)
Thuricin CD	*B. thuringiensis*		Rea *et al* (2010)
LFF571	*Planobispora rosea*	Completed phase II clinical trials	Mullane *et al* (2015)
Actagardine A	*Actinoplanes liguriea*		Boakes *et al* (2012)

Environmental isolate active against *C. difficile* - *Bacillus pumilus*



Environmental strains from cow manure were screened for activity against different strains of *C. difficile*. Once zones of clearance were observed against multiple strains the identity of the bacteria was determined via 16s PCR, and then whole genome sequencing. One strain isolated was determined to be *B. pumilus*, which has previously been used in a commercial probiotic known as Biosubtyl[NG], and is known to produce bacteriocins.

Bootstrapped phylogenetic tree of 16s gene of the environmental isolate vs *B. pumilus* and *B. safensis* strains

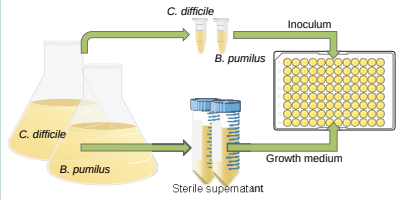


A strain of *Bacillus pumilus* was found to be active against 15 strains of *C. difficile* of varying PCR Ribotype. The level of activity differs between each strain however there stable antibacterial activity.

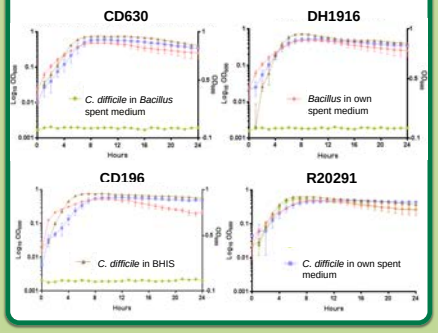
C. difficile strains	PCR Ribotype	Activity
CD305	023	+++
CD196	027	++
LIV29	001	++
LIV22	106	++
EK28	078	+++
CF5	017	+/-
R20291	027	+
CD630	012	++
DH1916	027	++
TL178	002	++
CD196	027	+
BI-9	001	++
BI-1	027	++
M68	017	+
M120	078	+++
TL176	014	++

+++ is a large zone >0.5cm, ++ medium zone <0.5cm, + small zone of 1-2mm, +/- inconsistent or hazy zone

Activity in the soluble fraction



To determine if the active agent was secreted, *C. difficile* was grown in sterile *B. pumilus* supernatant. There was no growth of *C. difficile* DH1916, CD630, and CD196 in *B. pumilus* spent supernatant however, there was growth of R20291.



Enriched Protein Activity

Current Method

Grown until OD_{600} 0.9 → Ammonium Sulphate 40% saturation → Pellet, resuspend and dialysed

Results

Overlay of supernatant precipitated with 40% or 80% ammonium sulphate (fig 1). The active fraction is only in the 40% precipitant, which showed a zone of clearance against *C. difficile* CD630

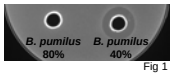
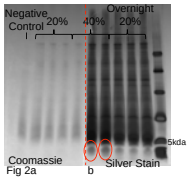
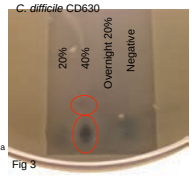


Fig 1





Different fractions were run on an SDS-page gel and stained with coomassie blue (fig 2a), silver stain (fig 2b) or overlaid with soft-top agar inoculated with *C. difficile* CD630 (fig 3). The larger zone of clearance corresponds to a band ~2kda seen on the silver stained gel half. There is a smaller zone of clearance corresponding to a 5kda smeared area as well. This demonstrates that the activity seen is proteinaceous and possibly a bacteriocin.

In silico Identification

Predicted Bacteriocin

Whole Genome sequencing → Bacteriocin database analysis → Potential bacteriocins

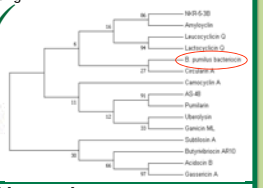
One predicted bacteriocin, a cyclical uberolysin-like bacteriocin:

Requirements of pfam class:

- Alpha-helical bundle of 4 or 5 alpha helices creating Saposin-like fold ✓
- No cysteines ✓

Predicted model of protein covering ~60% of amino acids

Bootstrapped phylogenetic tree of known cyclical bacteriocins



Self-immunity gene

Upstream of the potential bacteriocin gene, is a microcin C resistance peptidase gene. A self-immunity gene for *E. coli* with microcin C, a class I bacteriocin. This gene, *mccF*, is a S66 serine peptidases and cleaves a C-N bond detoxifying the bacteriocin. *mccF* homologs have been found as a stand alone gene in other bacteria e.g. *B. anthracis*, where it demonstrates the same detoxifying action.

B. pumilus MccF			
Versus	QC (%)	E-value	ID (%)
B. anthracis MccF	98	1e-166	63
E. coli MccF	98	9e-127	52

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