Antibiotic Resistance: Why You Need To Start Caring

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Abstract

Antibiotic resistance is a rapidly growing problem affecting every one of us, both in terms of health care and socioeconomics. Alternatives desperately need to be found. This research focuses on bacteriocins, which are very small proteins that are produced by bacteria when they are under threat, perhaps from other microorganisms competing for resources. These bacteriocins kill certain species of other bacteria very effectively and efficiently. It is hoped this line of research could lead to the development of therapeutic bacteriocins, which could eventually become a replacement for current antibiotics. We looked at the spectrum of killing of bacteriocins produced by *Streptococcus pneumoniae*, the most common cause of pneumonia. We also looked at different methods of potentially increasing the production of bacteriocins by artificially creating a threat.

To do this, we adopted a commonly used experiment to see which bacterial species could kill which others, called an overlay assay. To simulate threat, we added an antibiotic and another chemical. The results showed that *S. pneumoniae* had a larger spectrum of killing than originally thought and the methods we used did not increase production of bacteriocins.

Introduction

Bacterial infections are commonplace in every society across the world. There is, however, a massive disparity in how these often life-threatening infections are treated, based upon geographical location. In the developed West, a simple chest infection is nothing to worry about. In less economically developed countries it can be a death sentence. The difference between these two extremes is the widespread availability of antibiotics. In many Western countries, these life-saving drugs can be bought over the counter, while in poorer countries a mother will need to scrape together her life-savings to procure a single course for her sick child.

Antibiotics are chemical compounds that kill bacteria. In 1928, Alexander Fleming noted that penicillin, the first antibiotic to be isolated, was exceptionally good at killing the bacteria associated with many of the horrific ailments of the day

(Grossman, 2008). Mass production of this exciting new compound led to widespread clinical use by 1942 and revolutionised medicine. Gonorrhoea, syphilis, skin infections and many more could now not only be treated, but cured. This impacted the world in terms of both health care and economics. Those stricken with syphilis and other infections now lived longer, which meant that they could work longer and ultimately produce more for the economy.

The way in which Fleming discovered penicillin also proved to be very important. He correctly observed that the new bacteria-killing drug was produced by the *Penicillium* fungus. Its success prompted scientists to probe into the world of microorganisms in search of new chemicals to kill even more bacteria and cure a vast array of maladies. In a few short years, the number of antibiotics exploded and led to the US Surgeon General announcing 'The time has come to close the book on infectious disease. We have basically wiped out infection in the United States' (Upshur, 2008).

Antibiotic Resistance

The US Surgeon General had spoken too soon. In 1940, two years before the first clinical use of penicillin, researchers isolated a compound from certain bacteria. This compound was penicillinase, a chemical that bacteria were producing in order to protect themselves from the 'miracle-drug' (Abraham & Newton, 1956). This was the beginning of a worrying trend of growing bacterial resistance to antibiotics. As each newly discovered drug came to market, bacteria developed resistance to render it almost useless. In 1959, methicillin, a chemically altered version of penicillin, was synthesised specifically to combat the rise of multi-drug resistant *Staphylococcus aureus*, a very common pathogen. Two years later, methicillin-resistant *S. aureus* (MRSA) was isolated and spread to become the notorious hospital-associated infective agent.

MRSA is not the only problem. Many drugs that were once able to save lives are now unable to kill many species of bacteria. Fortunately, over the decades we have continually found new ways to modify antibiotics and to discover new ones and this has kept the growing threat of multi-drug resistant bacteria at bay. Now however, this supply of antibiotics is beginning to dry up and experts predict we could be without antibiotics in as little as 30 years (Price, 2014).

Resistance becomes more of a problem when we look at the uses of antibiotics. Far from just being used for simple infections, they are commonly used during surgery and childbirth to improve the outcomes for patients and mothers. Cancer chemotherapy and organ transplantation desperately need antibiotics as the other medications required for these treatments weaken the immune system, so patients cannot fight off infection on their own (ACS, 2015). It is not just health care that uses antibiotics. Certain drugs are given to farm animals to keep them healthy and some are even given to make cows gain muscle mass so that they produce more meat. Loss of antibiotics would create huge problems for suppliers of these products.

How Does Resistance Develop?

Antibiotic resistance is governed by DNA and genes. To kill bacteria, antibiotics need to be able to get into the cell to interfere with its normal processes. If bacteria gain an antibiotic resistance gene it allows them to protect themselves from this in a number of ways. The cell could produce a chemical that breaks down the antibiotic, such as penicillinase which works by breaking down a crucial structure in penicillin and related antibiotics before they get near the cell (Abraham & Newton, 1956). Another method is to change the target for the antibiotic so that it can no longer bind to its specific receptor. This means that the drug cannot start the process of killing the cell as it can no longer be internalised, a method used by *S. aureus* among others (Li *et al.*, 2015). The final method is to produce proteins that actively export the antibiotic from the cell after it has entered. These proteins, called efflux pumps, can export many different antibiotics and detergents and are used by *Pseudomonas aeruginosa*, a common cause of chest infections (Dorotkiewicz-Jach *et al.*, 2015). A summary of methods of antibiotic resistance is shown in figure 1.

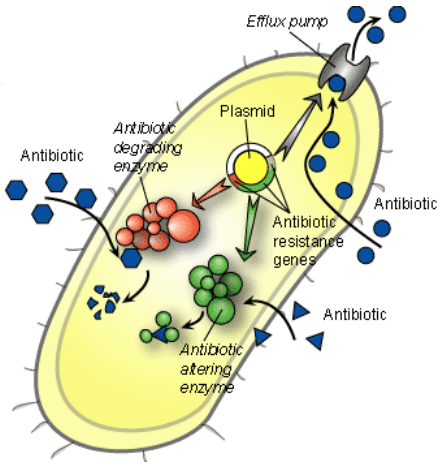


Figure 1 *- Diagram showing methods of antibiotic resistance. Taken from Kenneth Todar*

These resistance genes are not present in all bacteria and are often only acquired while the bacterial colony is in the presence of a threatening drug. There are two ways of acquiring them: either by random mutation of the genome (the sum total of all the genetic information in a cell) resulting in a change to the host cell's protein; or by taking up genes from the environment and other bacteria (known as horizontal transfer). Horizontal transfer is the most common method.

Horizontal transfer can occur in three ways. The first is by taking DNA from the environment and incorporating it into its own genetic make-up, which is called transformation. The second is direct interaction between bacteria, resulting in circular pieces of DNA, called plasmids, being transferred from one to the other. A single bacterium can contain many plasmids and it is a very common way for resistance genes to be acquired. The third method is transduction, where a virus containing resistance genes from another bacterium infects the host cell and passes on the stolen genes.

Evolution is very important in this process. Horizontal and vertical transfer are entirely random, so it is impossible to say which cells will acquire which genes. Natural selection ensures that if a horizontally acquired mutation is beneficial to a bacterium, for example making it drug-resistant, it will survive to reproduce and pass on the gene that facilitated its survival. If the acquired gene is harmful, the cell will not survive.

Every gene that is acquired has a fitness cost. This concept means that even though there may be an advantage to having a particular gene, there may also be a disadvantage. In resistance, acquired genes will often have the fitness cost of reduced nutrient uptake. This means that once the threat posed by the antibiotic is removed, the resistance gene's benefits no longer outweigh its cost and it will therefore be lost.

Is it our Fault?

The terrifying decline in the effectiveness of antibiotics would have happened eventually due to natural selection. Moreover, the actions of health care professionals have certainly exacerbated and accelerated this process by creating a selective pressure on bacteria.

There are three main ways that doctors and patients have sped up the development of resistance. The first is overuse of antibiotics. When they were first used, doctors marvelled at the effects of these ‘magic bullets’ (Breedveld, 2000). As a result, they were given out for almost every ailment, a habit which has continued to this day. It also led to patients wanting antibiotics for every infection, whether bacterial or not. The problem with this is overexposing the normal bacterial flora of the human body to these drugs and giving them the ability to develop resistance. Even if the overuse does not affect the individual or their bacteria, the antibiotic is excreted in urine. This means that bacteria in the sewage system are exposed to the selective pressure. It does not take much for these to get back into human circulation. Using antimicrobials in farming has a similar effect, which becomes a problem when animal bacteria gain the ability to infect humans.

The second problem is the misuse of antibiotics. If prescribed antibiotics, it is usually for a week. This is to give time for the drug to kill the vast majority of bacteria. Any cells that are left are destroyed by the immune system. However, the infected individual will often start to feel better in four days, because the number of bacteria has been supressed. A problem arises when the patient, feeling as if they are cured, stops taking the antibiotic after four days. Most of the bacteria will have been killed, but there will still be too many for the immune system to remove. This allows the remaining bacteria, some of which are likely to have developed resistance due to exposure, to re-populate. You now have an infection composed of resistant organisms which can cause disease and spread the resistance genes.

The final problem is incorrect prescribing of antibiotics by doctors, mostly GPs. 90% of GPs feel pressured by patients to give antibiotics, even if the problem is not bacterial (Price, 2014). It can be very difficult to stand up to patients, but giving antibiotics for viral infections only exposes normal flora to selective pressure without any benefit to the patient.

What Can We Do to Stop it?

Thankfully, there are things that can be done to stem the flow of resistance progression. As previously discussed, fitness cost means that if antibiotics are no

longer placing pressure on organisms, they are likely to lose their resistance genes over time. The first thing to do is to stop overusing and misusing antibiotics. We also need to research alternatives or find new classes of antibiotic that work on different targets.

Finding new antibiotics is not easy, mainly because all the obvious ones have already been found. A lot of time and money is being spent on exploring new environments trying to find previously unidentified bacteria that may produce chemicals that can kill pathogens. Unfortunately, pharmaceutical companies are often reluctant to fund the search for new medicines because antibiotics are not profitable long term. The methods currently being used are also very inefficient and produce few results. Added to which, finding new antibiotics only solves half the problem, as it is very likely resistance to these new drugs will develop very quickly.

Alternative therapies look the most promising. Potential solutions currently being researched are predatory bacteria, which are injected to hunt down the disease causing pathogen, antibodies which are very specific immune system molecules that can inactivate parts of bacteria (Irani *et al.*, 2015) and some research has even been looking into ancient texts and translating their recipes for medicines, which are proving to be effective in some instances (Harrison *et al.*, 2015). Research is also looking into antimicrobial peptides, small molecules produced by many animals including frogs and sharks, which have an anti-bacterial effect. The much publicised CRISPR-Cas system could also be used to cut a bacterial cell's DNA resulting in its death (Severinov *et al.*, 2016). My research however, focused on bacteriocins.

My Research: A Potential Solution

In order to tackle antibiotic resistance, this work started looking into bacteriocins. Bacteriocins are small proteins produced by bacteria that kill other closely-related bacteria. These chemicals have been heavily researched in certain bacteria and could potentially be a replacement for antibiotics in the future. Bacteriocins produced by *Streptococcus pneumoniae*, known as pneumocins, have not garnered as much attention thus far and could provide vital information about the action and spectrum of other bacteriocins (Dawid *et al.*, 2007).

The aim of my research was to examine the range of bacteriocins produced by different strains of *S. pneumoniae*, and to test the effects of artificially stimulating the SOS response (a bacterial response to potential threats) and the competence system on the production of bacteriocins, as discussed below. Given the wider context of rapidly progressing antimicrobial drug resistance, it is crucial that we work on as many potential replacements as possible. If these research paths prove fruitful, it is also important that we can maximise the efficiency of production of these chemicals. This is the rationale behind looking into the SOS response and competence.

Streptococcus pneumoniae

Streptococcus pneumoniae, also known as the pneumococcus, is the leading cause of community acquired pneumonia, as well as a common cause of ear infections, sinusitis and more serious invasive diseases, such as meningitis, bone infection and sepsis. It has over 90 distinct types, 23 of which cause the majority of serious disease (Scmitt, 2010).

The pneumococcus is a major cause of infectious disease globally, with the most affected being children under two years of age and the elderly. In 2000, there were around 14.5 million cases of pneumococcal infection which resulted in around 826,000 deaths of children (O'Brien *et al.*, 2009). Growing resistance of these organisms to antibiotics has led to development of vaccines to protect vulnerable groups and a need for novel therapies to treat infections when they occur (WHO, 2011).

Why Bacteriocins?

The biggest advantage of bacteriocins over conventional antibiotics and other novel replacements is that they only kill a very limited number of other bacteria, and they kill very efficiently (Dawid *et al.*, 2007). This specificity and potency means that bacterial resistance to bacteriocins is far less likely to develop, and at least if it does, it will take much longer to do so.

Bacteriocins are often produced as a result of stress (Anastasiou *et al.*, 2015). When *S. pneumoniae* detects an oncoming assault on its cell, often by DNA damage, it triggers an SOS response. The SOS response is common to many

bacteria; it allows the organism to change the way it uses its genetic information in order to produce new molecules or processes that will help it survive the attack (Ghazaryan *et al.*, 2014). Pneumocins are produced to help kill the potential invader, allowing *S. pneumonia* to remain living in its environmental niche. It is therefore likely that bacteriocins evolved in order to give a competitive advantage to producing organisms. This also explains why the spectrum of killing is so narrow, because the organism only has to fight off the few other organisms that want to compete for the unique space the producing organism currently colonises.

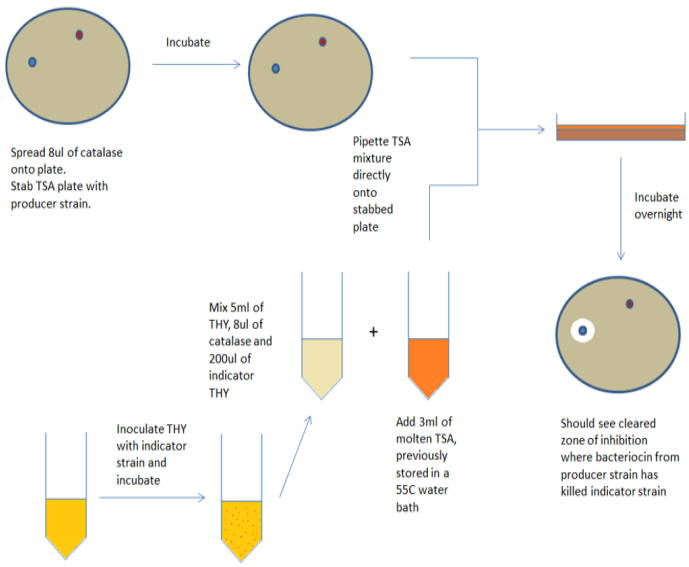
Another part of the SOS response is competence. Competence is the ability of bacteria to take up foreign genetic material, in the hope that it will gain a survival advantage (Ahn *et al.*, 2014). *S. pneumoniae* is among the most naturally competent bacteria, so it is important to examine how bacteriocins affect this vital aspect of the organism's life cycle. It is regulated by a complex cascade of different chemicals which are triggered by similar signals to those which stimulate bacteriocin production. The competence cascade also has a similar end goal: fighting off invaders or anything else that threatens the bacterial community's existence (Claverys *et al.*, 2006; Pozzi *et al.*, 1996). Because of these common start and end points, we hypothesised that competence and bacteriocin production may be related in some way.

Also important for bacteriocin production is a high density of bacteriocin-producer cells, as this leads to activation of the quorum-sensing (QS) system (Leung *et al.*, 2015). This system allows bacteria in a colony or community to communicate with each other via small signalling molecules, once a threshold concentration of bacteria (quorum) is reached. QS controls a wide variety of bacterial functions, including genetic competence and bacteriocin production.

Methods

To test the spectrum of pneumocins, we used an overlay assay. Overlay assays are qualitative experiments that have been extensively used to test the killing power of organisms or chemicals (Maricic & Dawid, 2014). In this instance, it involved two layers of agar (bacteria food); the bottom one for the organism we thought would produce bacteriocins, and the top for the organism we thought would be killed

(also known as the indicator strain). Figure 2 shows a summary of the method used.



All the organisms had to be cultured first with optimum growth conditions. Indicator strains were cultured in a liquid nutrient broth until they were placed into the top agar layer (Table 1). Bacteriocin-producing strains were stabbed into the bottom layer of agar and allowed to grow overnight, to

Figure 2 - Performing the overlay assay*. TSA and THY are growth media for the bacterial species used. Catalase was added to break down any hydrogen peroxide that might be produced.*

provide an adequate number of bacterial cells. Multiple stabs were performed on each petri dish to allow for repeats and controls.

Table 1: Growth Times of Organisms Used as Indicator Strains

Organism	Strain	Time incubated	Dilution	Time incubated after dilution
S. pneumoniae	ST306	6 hr	N/A	N/A
	TIGR4	Overnight	1/10	6hr
S. oralis		Overnight	1/10	6hr
S. mutans		Overnight	1/10	6hr
S. mitis		Overnight	1/10	6hr
S. sanguinaris		Overnight	1/10	6hr
S. salivarius		Overnight	1/10	6hr
Pseudomonas aeruginosa	PA01	6hr	N/A	N/A

To simulate conditions that would lead to an SOS response, we used an antibiotic called Mitomycin C (MMC), which works by damaging the DNA of the target bacterial cell. This is the same as many stress-inducing compounds (Tomasz, 1995). We added MMC as DNA damage is known to stimulate the SOS response. This allowed us to monitor the effects on bacteriocin production. We added 20µl to the agar containing the producing strain. To test whether competence has an effect on bacteriocin production, we added 0.1µl of competence stimulating peptide (CSP)

to a liquid culture of the main producing strain, S. *pneumoniae* ST180, then after 6 hours stabbed the liquid into the agar. This was then incubated as normal.

Results

The results of some of the initial experiments are shown below in figures 3 and 4. In an overlay assay, the bottom bacteria often kill the top bacteria, leaving a visible zone of inhibition, or halo, on the petri dish. As illustrated, halos appeared around some of the initial producer stabs. These halos are created by the producer organism killing the indicator organism in the top layer of agar.

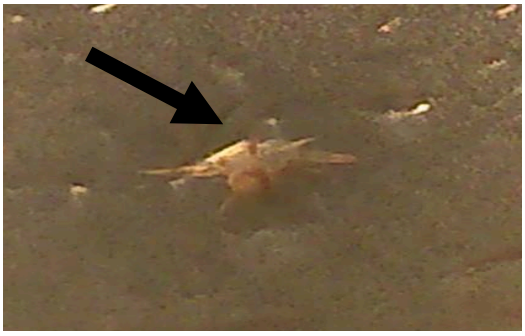


***Figure 4** - ST180 as a producer stab, TIGR4 as the indicator strain. ST180 incubated for 6 hours, TIGR4 incubated in THY overnight, then throughout the day until overlay. Overlay incubated overnight. Inner halo equals 4mm diameter.*

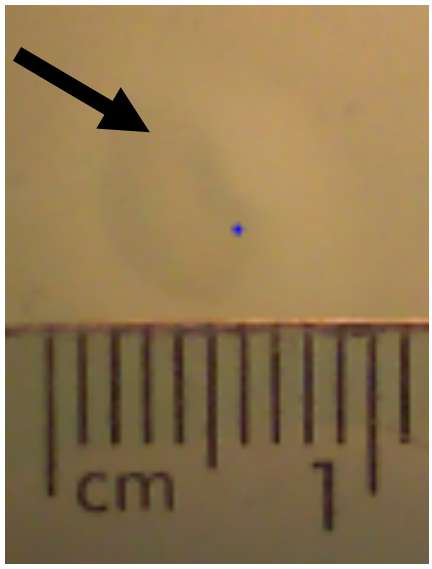


***Figure 3** - ST180 as a producer stab, PA01 as the indicator strain. Both incubated for 6 hours, then overnight after overlay performed. Photograph taken after overnight incubation. Outer halo equals 1cm, inner halo equals 5mm.*

The results of stimulating the SOS response (Figure 6 & 7) and competence system are shown below (Figure 5). We added MMC and CSP to do this and then observed the change in the halo compared to a control. No halos appeared in the MMC experiments despite halos appearing in experiments without MMC. The original ST180 colony could also not be seen. In CSP assays, the original producing ST180 strain was visible and no halo was produced, even where one had been produced before, in assays without CSP.

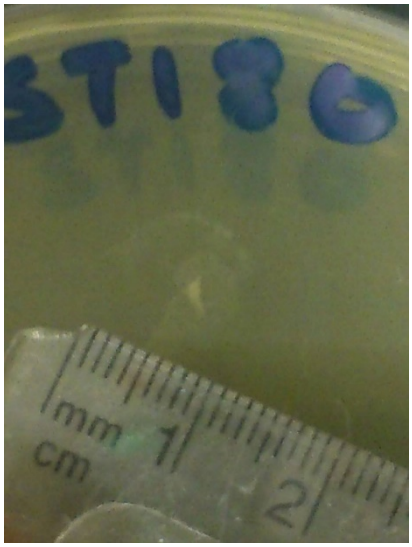


Figure 5 *- ST180 inoculated with CSP as producer, TIGR4 as indicator. Incubated overnight. No halo visible.*

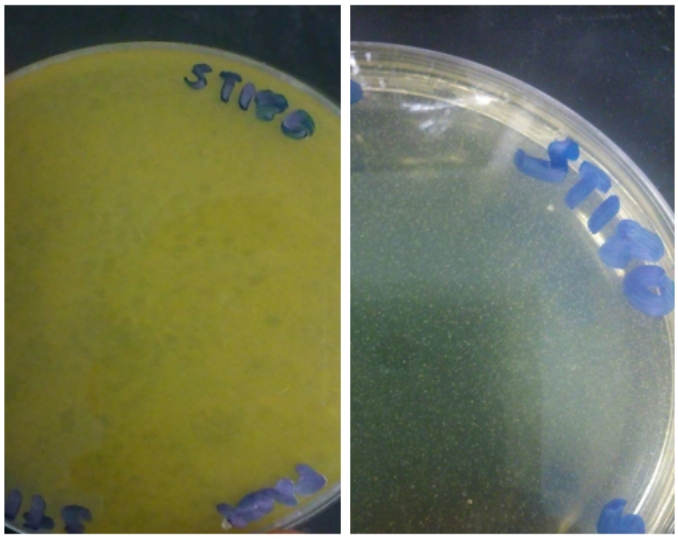


Figure 6 & 7 *- Producer strains and control stabbed into agar inoculated with mitomycin C. Incubated overnight. No halo visible, producer colonies gone.*

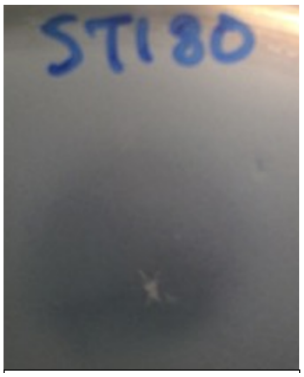


Figure 8 *- ST180 as the producer, S. oralis as the indicator strain. A large halo is visible, although this was unable to be measured due to time contraints.*

We also tested the effects of pneumocins produced by S. *pneumoniae* ST180 on oral species of Streptococci that are common competition rivals in a human host (Figure 8). Of the five species tested, only one (*S. oralis*) was visibly inhibited by pneumocins.

Conclusions

Although it is not possible to prove the exact cause of bacterial inhibition in these experiments, it is clear that inhibition of many strains and species took place. The known bacteriocin producer, *S. penumoniae* ST180 clearly and repeatedly inhibited the TIGR4 strain of *S. pneumoniae*.

These strains often colonise similar environments and therefore it is not surprising that one evolved a strategy to inhibit the other to gain a competitive advantage. It is surprising however, that ST180 was able to kill *Pseudomonas aeruginosa* PA01 because these organisms, although living in the same environment, are very different to each other. Bacteriocins typically only act on similar species, so it is unexpected that inhibition took place (Dawid *et al.*, 2009).

Also surprising is the lack of activity on oral Streptococci apart from S. *oralis*. Given that there is relatively little difference between these species, it is hard to say exactly why this is. All of these organisms live in the mouth alongside ST180, yet only S. *oralis* is susceptible to the pneumocins. Due to the time constraints of my project however, it was not possible to perform adequate repeats, so this may be an aberration. If this is the case, it is surprising that S. *oralis* was killed so effectively.

When we added MMC to the plates, we thought it may increase bacteriocin production due to stimulation of the SOS response. However, no halos appeared where there had been some before, and the producer colonies appeared to disappear also. It is possible, therefore, that the stress we induced was too great in magnitude for ST180 to survive. It is, after all, an antibiotic. Similarly, no halos were seen on plates with CSP. Given that without CSP, a halo was produced, it is possible that stimulating the competence system actually decreased the production of bacteriocins, which was previously unknown.

Impact and Future Work

In the context of wider research into antimicrobial resistance, the discoveries made in these experiments give new information about the range of bacteriocins. Perhaps they are not as narrow-spectrum as previously thought, at least in S. *pneumoniae*. This work also raises questions about the role of competence in bacteriocin production. This information could help in the development of bacteriocins into replacements for the conventional antibiotics that are running out.

In future, the method of bacterial killing seen in these assays would have to be confirmed to be pneumocin-mediated. This is to validate the results. This would be possible by creating engineered mutant bacteria that don't contain the DNA for bacteriocin production. These could then be used as a comparison to the wild-type, bacteriocin producing species used in this project. Further research into the action of pneumocins may also cast some light on the difference in killing of the oral Streptococci. It would also be interesting to look deeper into the mechanism behind the competence system dampening down the bacteriocin production response.

This project, along with other projects looking into the use of bacteriocins clinically to provide therapeutic benefit and as an alternative to antibiotics, are very much in their early days. The future of this area of enquiry does look promising however and, combined with other avenues of research, could really make a significant difference in the fight against multi-drug resistant infections.

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