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Ionophore resistance in *Staphylococcus aureus*

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Abstract

Ionophores are molecules that bind ions and help them cross biological membranes. They are often used as antibiotics in livestock and poultry in order to prevent the parasitic disease coccidiosis and to act as growth promoters, by improving energy utilization of feedstuffs. The widespread use of ionophores may contribute to the general problem of antibiotic resistance.

This study aimed to get more information on the development of resistance to ionophores, by exposing *Staphylococcus aureus* to the ionophore monensin, selecting resistant mutants and characterizing them.

A fluctuation assay was performed in order to determine the mutation rate, broth microdilution and bioscreen assays were performed to determine the level of resistance and fitness of selected mutants. E-tests were used to detect cross-resistance to other antibiotics and whole genome sequencing was performed to identify underlying mutations.

It was possible to find resistant mutants and cross-resistance was found towards chloramphenicol, erythromycin, gentamicin and tetracycline. This indicates that ionophores have a potential to contribute to medically relevant antibiotic resistance, and further studies are needed to determine how big of an impact the widespread use of ionophores in animal production has on the evolution of antibiotic resistance.

Popular Science Summary

Antibiotic resistance has become a widespread phenomenon and is a challenge to health systems worldwide. One of the driving factors of antibiotic resistance is the excessive use of these drugs in animal production, where the constant exposure trains bacteria to adapt. Many countries have started to react and put restrictions on farmers regarding when, how much and which antibiotics to use. But ionophores have been left out, because these drugs are not used to treat human infections. Ionophores are used all over the world in livestock and poultry because they prevent the diarrheal disease coccidiosis and because animals grow faster when ionophores are included in their diet. But does the use of these drugs really not affect the emergence of antibiotic resistance?

So far there have not been a lot of studies on antibiotic resistance to ionophores, and the ones that do exist often come to contradicting conclusions. In addition, many of the scientists claiming that the use of ionophores is harmless, work for companies that sell animal feed with the drug, raising concerns about the objectivity of the studies.

We used *Staphylococcus aureus* as a model bacterium and grew it on plates that contain monensin, which is one of the ionophore antibiotics. Then we counted how often individual bacteria were able to grow despite of the drug and used that number to calculate the mutation rate. This serves as an indicator of how easy it is for bacteria to develop resistance to a specific drug. We then selected some of the mutant colonies that we found and did more tests on them. We let them grow with many different concentrations of monensin in order to find out how much of the drug is needed to prevent them from growing. We also looked at how fast they could grow using a machine that measures the number of bacteria present in the culture medium.

We were able to find several bacteria that became more resistant to monensin and grew faster than the original *S. aureus*. Then we tested how resistant they are to other antibiotics that are

commonly used in human medicine. One mutant in particular was very interesting because it was very resistant to the antibiotics erythromycin and chloramphenicol. Several of the other mutants showed some increase in resistance towards gentamicin and tetracycline. These results are very concerning, because they show that exposure to the ionophore monensin can result in resistances against other antibiotics. Since ionophores are used in many farms all over the world, they might make the situation regarding antibiotic resistance worse. More studies are needed, but these results suggest that ionophores pose a bigger danger than previously thought.

Abbreviations

Cfu: colony forming unit
CL: Chloramphenicol
DPC: Daptomycin
EE: Eagle Effect
EM: Erythromycin
GM: Gentamicin
LZ: Linezolid
MH2: Mueller-Hinton 2
MIC: Minimal inhibitory concentration
OD: Optic density
RI: Rifampicin
TC: Tetracycline

Introduction

Antibiotic resistance is one of the most important medical issues facing humans today. Many standard techniques in modern medicine, like cancer treatments or surgery, rely on the use of antibiotics to prevent infections and can be too risky to perform if a patient is colonized with drug resistant bacteria.[1] Furthermore, common infections that are treatable today may become deadly again in the future. The study of all aspects of antibiotic resistance is therefore urgently needed in order to find ways to alleviate the problem. When considering the scope of the antibiotic resistance problem it is imperative to look at it from a One Health perspective. Human health cannot be ensued without regarding animal health and the environment. Antibiotic resistance in livestock is important both for the sake of animal health, but also because it may be a factor in driving resistance evolution that affects humans as well.

Ionophores are a class of drugs commonly used in animal agriculture in order to prevent infections, but also as growth promotors. Ionophores are fermentative by-products of the soil bacteria *Acinetomycetes* that can carry ions across membranes. In general, it is a challenge to transport ions across membranes into cells or organelles. Because of their charge, they cannot cross the hydrophobic membrane by diffusion like neutral gases like O_2 and CO_2. There are three common mechanisms to solve this problem; Ion channels, ion pumps and ionophores. Ion pumps require energy and can transport ions in one direction through a membrane. Ion channels are large membrane-spanning molecules that form a hydrophilic path for diffusion. In contrast, ionophores are special carrier molecules that wrap around metal ions and help them cross a membrane by diffusion.[2]

There are many known ionophore structures, often including polyether rings, which can bind cations. These polyether, or carboxylic ionophores can be subdivided based on their binding preferences to either monovalent cations like K^+ and Na^+ or divalent cations Ca^{2+} and Mg^{2+}. Ionophores shield the charge of the ion through hydrogen bond formation with oxygen or nitrogen functional groups at the center of the molecule. The hydrophobic portion of the molecules lie toward the exterior and can interact with the lipid interior of the membrane. Therefore, the ionophore is able to "dissolve" and diffuse across the membrane and can thereby transport ions across it.[3]

Monensin is an ionophore produced by *Streptomyces cinnamonensis*,[4] with preference for monovalent cations. The ionophore properties result in a mechanism of action different from those of most antibiotics used in human medicine, which tend to target enzymes, the ribosome or the cell wall. Monensin inserts itself into the cell membrane and acts as a metal/proton anti-porter; driven by the import of H^+, Na^+ is exported from cell, while K^+ is imported into the cell when H^+ gets exported (Figure 1). This results in a disruption of the ion gradients necessary for the transport of nutrients and to maintain the proton-motive force needed for energy production. In response to the drug, the cell exports H^+ using the reversible ATPase/ATP synthase and activates ATP dependent pumps to export Na^+ and import K^+. As a result, there is not enough ATP for the cell to fulfill its regular functions during the cell cycle and growth is inhibited.[5]

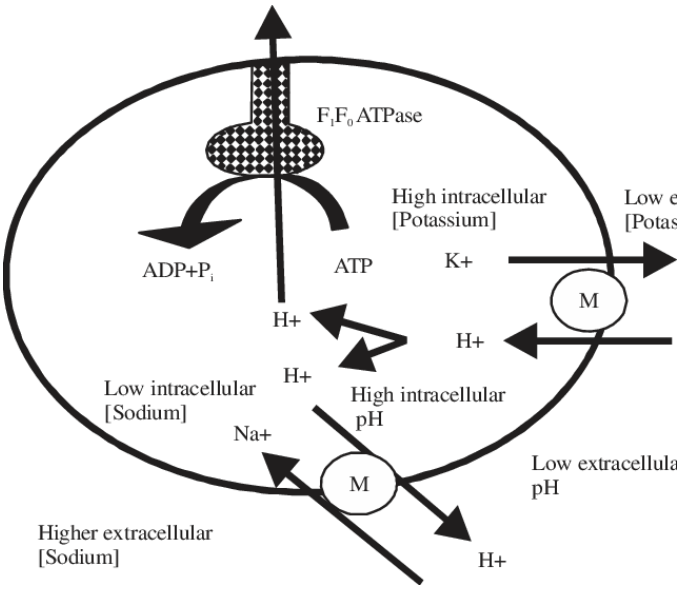

Figure 1: Gradient dissipating mechanism of monensin.[5]

Monensin, as well as other ionophores like Lasalocid and Laidlomycin, are frequently used in livestock, especially in cattle and poultry. In Europe, the use of monensin, along with all other antibiotics as feed additives was banned in 2006.[6] However in 2013, monensin was reapproved as a prescription-only drug under the name of Kexxtone®. In contrast, the use of antibiotics in animal feed is a common phenomenon in most of the rest of the world. Although the USA has started to limit the use of some antibiotics, monensin and other ionophores are not affected by these restrictions, as they are currently not considered to be medically relevant to humans due to their toxicity.[7] In the year 2015, ionophores represented 30 % of all domestic sales of antibiotics approved for the use in food producing animals.[8]

Ionophores are used in livestock for two main reasons; they prevent coccidiosis and increase energy utilization of feedstuffs. Coccidiosis is a parasitic disease, which often causes disease in

younger calves and chickens. The primary symptom is diarrhea and the disease is associated with a substantial economic loss. The disease is most commonly caused by parasites of the genus Eimeria, which are part of the Apicomplexa family, together with malaria and toxoplasma. They are unicellular, spore forming obligate endoparasites, which have an organelle called apicoplast as a distinguishing common feature.[9]

Another important reason for the use of ionophores in cattle is their function as growth promotors. Monensin changes the microbial content of the rumen to reduce the presence of Gram-positive bacteria and thereby increase the proportion of Gram-negative bacteria. These bacteria have different fermentation processes of sugars, which results in a shift of primary fermentation products in the rumen; the amount of acetic acid produced decreases, while the production of propionic acid increases. During the fermentation of glucose to two molecules of acetic acid, two carbon atoms are lost in the form of carbon dioxide or methane. This uses the energy available in the feed to a lesser degree than glucose fermentation resulting in propionic acids, where no carbon dioxide or methane is produced. This results in a better energy utilization of the available glucose; 680 kcal/mol glucose compared to 419 kcal/mol with acetic acid as the primary fermentation product and 734 kcal/mol with propionic acid as the intermediate. This improved energy utilization results in an increase in the average daily weight gain of the animals, and therefore increases production for the farmer.[10]

While ionophores are tolerated by cattle in appropriate dosage, they generally are toxic compounds. Side-effects include lethargy, cyanosis, depression, pulmonary edema, myocardial degeneration, and death.[6] Different animals are affected to varying degrees by the drug. Horses especially are strongly affected, with the dosage required to kill 50% of animals (LD50) being $1/100^{th}$ that of ruminants. Toxicity in humans has not been thoroughly studied, however there are cases of individuals dying from monensin poisoning.[6] Because of this, monensin is not currently used in human medicine, however, with antibiotic resistance becoming an ever increasing concern, it may be necessary in the future to fall back on less ideal antibiotics and develop them for use in humans. However, although there are *in vitro* studies suggesting that monensin may be useful to target MRSA, there is a lack of *in vivo* studies to make any claims about that.[11] Furthermore, ionophores including monensin show activity against *Plasmodium falciparum* and have potential to be repurposed as an antimalarial drug in the future.[12]

Even though ionophores are currently only used in veterinary medicine, possible resistance development may still be a concern for human health. Resistant subpopulations could spread from animals to their owners and then further into the general population. Furthermore, many resistance mechanisms in other antibiotics confer resistance to several antibiotics. Considering that ionophores are used in poultry and livestock in most parts of the world, this might be a driving factor in resistance evolution. However, not a lot is currently known about the development of resistance to ionophores or about potential cross-resistance to antibiotics, relevant in human medicine. There have been few studies looking at this issue, many of which have been done by companies selling ionophore-containing feed. While McConville *et al.* claim that no resistance to ionophores appears at all[13], others found resistance to varying degrees. For example, in the study performed by Simjee *et al*. resistance appeared but was unstable[3] and Houlihan and Russel found resistance, but no cross-resistance to other antibiotics.[14] In contrast Nilsson *et al*. found evidence for a plasmid-borne ABC transport that increases the minimum inhibitory concentration of narasin in *Enterococcus faecium* in Swedish broilers.[15]

This study aimed to get more information on the development of resistance to ionophores, by exposing *Staphylococcus aureus* to monensin, determining the mutation rate and selecting resistant mutants. Furthermore, some of these mutants were characterized based on their minimal inhibitory concentration to monensin, growth rates, cross-resistance to other antibiotics and genome sequences.

Material and Methods

Culturing conditions

For all experiments, bacteria were grown in Mueller-Hinton 2 (MH2) broth or agar and incubated at 37°C.

Fluctuation assay

Mutation rates were determined with a fluctuation assay. A 1 mL starter culture from a single colony of *Staphylococcus aureus* (DA28823) was grown over night. The culture was stepwise diluted to contain approximately 10^3 cfu/mL and grown overnight as 30 independent cultures. The next day, 100 µl of each culture was plated on MH2 plates containing 0.5, 1, 2 and 4 µg/mL monensin. The plates were incubated over 3 days or in the case of the 4 µg/mL concentrations over a 6-day period. Additionally, three cultures were diluted to 10^{-6} and plated on agar without monensin in order to determine the cell density of the original culture. The mutants were counted and used to calculate the mutation rate using the bz-rates mutation rate calculator.[16]

Stock preparation

Selected mutants were streaked on MH2 plates containing the same monensin concentration as the plate they were picked from. Single colonies were then used to prepare stock cultures, which were stored at -80°C, after addition of 10% dimethylsulfoxid (DMSO).

Broth microdilution

Broth microdilution MIC tests were performed in a total volume of 100 µl MH2 containing monensin concentrations of either 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 31, 64 or 128 µg/ml. After diluting the overnight cultures to 10^{-3}, each well was inoculated with 2 µl to reach a final concentration of approximately $2x10^5$ cfu/ml. Incubation took place for 18.5 hours at 37°C. The results were either evaluated by eye or using a plate reader to measure the optic density.

Cross resistance

Cross resistance to other antibiotics was tested by plating 100 µl of a 1:10 dilution of a dense overnight culture and applying E-test strips (bioMérieux) of selected antibiotics. After 18-20 hours of incubation, the minimum inhibitory concentration was read off the strip.

Growth rates

Overnight cultures of three single colonies were grown and diluted 1:1000. 350 µl culture were added into four wells of a microtiter plate and grown for 16 hours in a Bioscreen C plate reader. Optic density measurements were taken every four minutes at 600 nm. The resulting data was analyzed using an online bioscreen analysis tool.[17]

Growth rates in the presence of monensin were measured by adding 10 µl of 1:100 diluted overnight cultures to 1 mL media containing either 0, 0.125, 0.25, 0.5, 2 or 8 µg/mL monensin. Two technical replicates were used and a final volume of 350 µl was analyzed. This was done with cultures from two single colonies in order to get two biological replicates. The resulting data was analyzed using the same online tool.[17] The results were normalized by calculating the growth rate as the average double time in the cultures without monensin, divided by the double time of each well containing monensin.

DNA extraction and sequencing

In order to perform whole genome sequencing, genomic DNA was extracted from each mutant using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre). For better yield, Lysostaphin from *Staphylococcus simulans* (Sigma) was used instead of the lysozyme delivered with the kit. The extracted DNA was sequenced with MiSeq (Illumina) according to the standard protocol. The sequence data was analyzed using CLC workbench version 11. The sequenced fragments were assembled to match the wild type sequence available from previous experiments and mutants were analyzed for differences compared to the wild type.

Results

Mutation rates to monensin resistance

To assess the ability of *Staphylococcus aureus* to become resistant to monensin, a fluctuation assay was performed. For this, *S. aureus* was grown on plates containing different monensin concentrations and resistant mutants were counted to determine the mutation rate. Additionally, some of them were selected for further analysis. All plates showed some background growth, but only colonies that stood out clearly from the background were counted as mutants. The plates had between 0 and 20 mutants on them. Table 1 shows the number of mutant colonies on each plate. Three control plates without monensin were used to determine the number of cells present in the cultures before plating, $6.5x10^9$ cells. The mutant count and the total number of cells plated were used to calculate the mutation rates using an online mutation rate calculator.[16] The mutation rate per cell per division for 2 and 4 µg/mL was $3.7x10^{-10}$ and $3.6x10^{-10}$ respectively. The mutation rate for 1 µg/mL was higher at $1.1x10^{-9}$ (Table 2).

Table 1. Number of mutant colonies from the 30 independent *S. aureus* cultures (SA1-30). Cultures were plated on MH2 containing 0.5, 1, 2 and 4 µg/mL monensin.

	0.5 µg/mL	**1 µg/mL**	**2 µg/mL**	**4 µg/mL**
SA1	0	6	0	0
SA2	1	2	0	0
SA3	0	9	0	0
SA4	0	1	1	1
SA5	1	0	1	4
SA6	1	1	0	0
SA7	1	1	0	0
SA8	0	2	2	1
SA9	0	4	1	1
SA10	1	1	0	0

SA11	5	5	0	0
SA12	1	0	0	0
SA13	1	1	0	0
SA14	3	3	1	4
SA15	3	1	1	0
SA16	21	7	0	1
SA17	5	4	3	0
SA18	0	6	1	0
SA19	0	1	0	1
SA20	7	3	3	0
SA21	0	0	3	15
SA22	1	21	1	15
SA23	6	4	0	1
SA24	3	8	0	15
SA25	6	8	0	0
SA26	0	2	0	1
SA27	0	0	1	10
SA28	0	0	0	2
SA29	1	3	1	0
SA30	4	5	7	0

Table 2. Mutation rates at the four different monensin concentrations determined from the fluctuation assay.

	0.5 µg/mL	**1 µg/mL**	**2 µg/mL**	**4 µg/mL**
Mutation rate	$6.0x10^{-10}$	$1.1x10^{-9}$	$3.7x10^{-10}$	$3.6x10^{-10}$

Selection of mutants for further study

All colonies that stood out clearly over the limited background growth were counted as monensin-resistant mutant colonies. Approximately 120 mutants were selected for stock preparation from the plates from different monensin concentrations, and from the independent cultures. Additionally, mutants were selected to represent different sizes and colors. Of these, a subset was selected for further testing. Table 3 shows the DA number of the selected mutants, the culture number and the monensin concentration from which it was picked.

Table 3. Mutants selected for further studies.

DA number	**Monensin**	**Culture**
DA28823	-	Wild type
DA65169	64 µg/ml	preliminary testing
DA65170	4 µg/ml	SA8
DA65171	4 µg/ml	SA9
DA65172	4 µg/ml	SA16
DA65173	4 µg/ml	SA17
DA65174	4 µg/ml	SA22
DA65175	4 µg/ml	SA26
DA65176	4 µg/ml	SA28

DA65177	2 µg/ml	SA1
DA65178	2 µg/ml	SA8
DA65179	2 µg/ml	SA14
DA65180	1 µg/ml	SA11
DA65181	1 µg/ml	SA11
DA65182	1 µg/ml	SA13
DA65183	1 µg/ml	SA27
DA65184	1 µg/ml	SA29
DA65185	1 µg/ml	SA30

Minimum inhibitory concentrations

In order to determine the degree of resistance towards monensin, a broth microdilution test was performed. An estimate of the growth can be seen in the summary Table 6. However, these results were difficult to interpret by eye, due to precipitation of monensin at higher concentrations. It was therefore hard to determine what constitutes growth and what is precipitate of the drug. Furthermore, many mutants show a paradoxical growth pattern, which is known as the eagle effect (EE).[18] For these mutants, growth was inhibited at moderate concentrations, but the bacteria were able to grow in high concentrations, as was for example the case for mutant DA65173. In order to better represent the data, the optical densities were measured, and the data was plotted in the graph shown in Figure 2. In this experiment, the wild type (DA28823) is represented with the red line and shows a MIC of 4. Some of the mutant strains have a similar MIC, while others show the eagle effect and DA65171 shows some growth in all of the wells. These results indicate that it is possible to find monensin resistant mutants by exposing the bacteria to the drug.

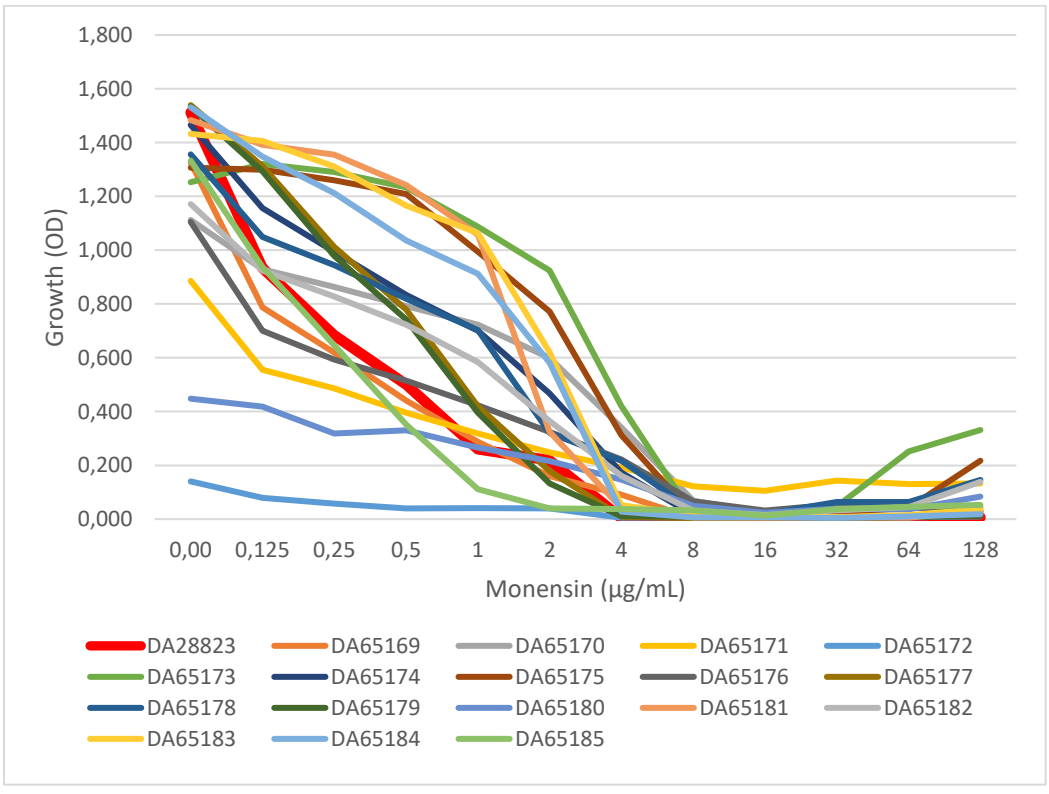

Figure 2. Broth microdilution experiment. Optical densities measured after overnight growth of the mutant strains in presence of different monensin concentrations. Average of three replicates.

Growth rates

In order to further characterize the selected mutants, growth rates were measured both without and with monensin. The growth rates without monensin are presented in the summary Table 6. Some mutant strains had growth rates similar to the wild type (e.g. mutants DA65169 and DA65177) whereas other mutants grew slower (e.g. DA65171 and DA65172). Thus, the fitness costs varied extensively between the different mutants.

Figure 3 shows the graph with the growth rates in presence of different monensin concentrations. The wild type DA28823 shows a steady decrease of growth with increasing monensin. Many of the mutants have a similar trajectory to the wild type. However, several mutants were less affected by the drug and showed higher growth rates than the wild type. In particular, the strains DA65172, DA65180 and DA65185 had growth rates that were higher than the wild type strain at, for example, 8 µg/mL monensin. Thus, these strains showed higher resistance towards monensin compared with the wild type.

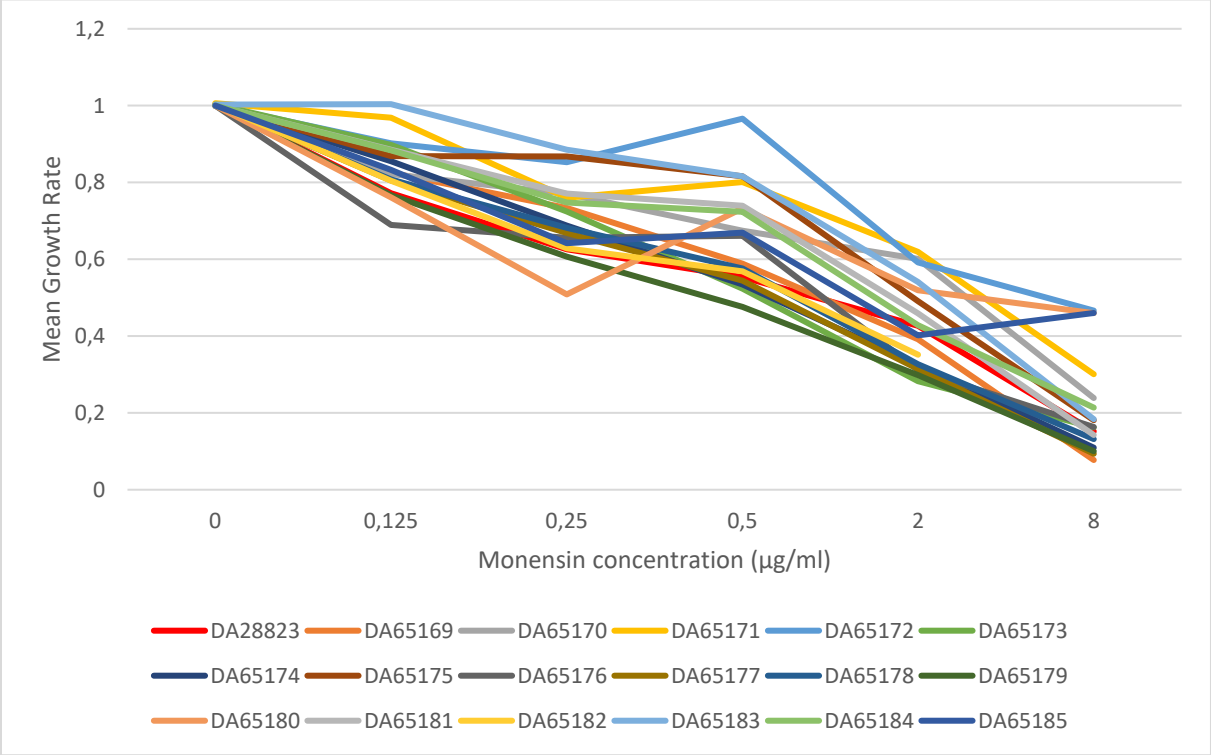

Figure 3. Mean growth rates of the different strains in the presence of different concentrations of monensin. The growth rates were normalized for better comparison.

The growth rates at 8 µg/mL monensin were divided by the growth rate of the wild type and represented in the summary Table 6. This ratio gives an indication of how much better or worse the mutants grow at this concentration compared to the wild type. The fast-growing mutants DA65172, DA65180 and DA65185 grew around 3 times as fast, while DA65169 grew only half as fast as the wild type.

Cross-resistance

The mutants were tested for cross-resistance to other antibiotics. Table 4 shows the MICs measured using E-tests. Especially interesting is mutant DA65172 which showed resistance against both erythromycin and chloramphenicol at levels above the clinical breakpoint for resistance. Furthermore, strains DA65178, DA65180 and DA65182 had a three to four-fold

increase in MIC towards gentamicin compared to the wild type and DA65177 showed increased resistance to tetracycline. No increased resistance was observed against linezolid, daptomycin and rifampicin. These results suggest that exposure to monensin can select for mutants that show cross-resistance against some other classes of antibiotics that are used in human and veterinary medicine Interestingly, many mutants were one order of magnitude more susceptible against daptomycin.

Table 4. E-tests for erythromycin (EM), gentamicin (GM), linezolid (LZ), chloramphenicol (CL), tetracycline (TC), daptomycin (DPC) and rifampicin (RI). When different inhibition zones were present, the one with higher resistance is indicated in parenthesis. The clinical break point (EUCAST 2019) is also shown.

Mutant	**EM**		**GM**		**LZ**		**CL**		**TC**		**DPC**		**RI**	
DA28823	0.25	0.25	0.25	0.25	1.5 (4)	1 (4)	3 (8)	3 (12)	0.19	0.38 (1.5)	0.5	0.75	0.023	0.016
DA28823	0.25		0.19		1 (4)		3 (8)		0.38		0.75		0.012	
DA65169	0.19	0.25	0.25	0.19	0.75 (3)	0.75 (3)	2 (6)	3 (12)	0.5	0.38 (1.5)	0.5	0.75	0.016	0.016
DA65170	0.19	0.125	0.75	0.75	0.5 (1)	0.25 (0.75)	0.75 (1)	0.75 (1)	0.19	0.38	0.047	0.047	0.008	0.008
DA65171	0.125	0.125	0.5	0.75	1 (1.5)	0.75 (1)	0.75 (1)	1 (1.5)	0.5	0.38	0.064	0.047	0.008	0.006
DA65172	8	4	0.19	0.19	0.75 (1.5)	0.75 (3)	12 (24)	12 (24)	0.5	0.38	0.094	0.064	0.012	0.012
DA65173	0.19	0.19	0.5	0.75	0.5 (1)	1 (2)	2 (4)	1,5 (3)	0.38	0.5	0.75	0.25	0.006	0.004
DA65174	0.25	0.38	0.25	0.19	1.5 (4)	1.5 (4)	6 (16)	4 (12)	0.5	0.75	0.75	0.5	0.012	0.012
DA65175	0.19	0.19	0.25	0.19	2 (4)	1.5 (4)	4 (6)	4 (8)	0.75	0.5	0.5	0.38	0.012	0.023
DA65176	0.38	0.38	0.125	0.19	1.5 (2)	1 (1.5)	1.5 (2)	1.5 (2)	0.5	0.75	0.094	0.047	0.008	0.012
DA65177	0.25	0.25	0.19	0.25	2 (4)	1.5 (3)	4 (8)	3 (8)	1	1	0.75	0.38	0.023	0.023
DA65178	0.125	0.125	0.75	1	0.75	0.75 (1.5)	1	1.5	0.38	0.5	0.016	0.032	0.006	0.008
DA65179	0.19	0.19	1*	0.25	2 (4)	2 (4)	4 (8)	3 (6)	0.75	0.75 (2)	0.75	0.38	0.023	0.047
DA65180	0.19	0.125	0.75	1	0.5	0.5 (1)	1	1 (2)	0.38	0.38 (1)	0.064	0.064	0.008	0.012
DA65181	0.25	0.25	0.38	0.5	2	(2) 4	3	6 (12)	0.5	0.5 (1)	0.5	0.5	0.016	0.023
DA65182	0.125	0.125	0.75	1	0.75	(0.75) 2	1.5	2	0.38	0.5 (1)	*	0.94	0.006	0.016
DA65183	0.38	0.38	0.38	0.75	1.5 (3)	1.5 (4)	4 (8)	4 (8)	0.38	0.5	0.75	0.5	0.012	0.012
DA65184	0.25	0.25	0.5	0.5	2 (4)	(2) 4	4 (8)	4 (8)	0.5	0.38 (1)	0.5	0.5	0.012	0.016
DA65185	0.125	0.125	0.38	0.38	0.75	1.5	1.5	1.5	0.25	0.5 (1)	0.047	0.057	0.006	0.012
Clinical Break-point[19]	R>2		R>1		R>4		R>8		R>2		R>1		R>0,5	

*distinct colonies within inhibition zone

DNA sequencing

In order to identify the mutations that result in monensin resistance, DNA was extracted from the selected mutants and analyzed using MiSeq whole genome sequencing. However, it proved more difficult to extract the DNA of some of the mutants, and not all sequences are available

at this time. Table 5 shows the affected genes, the identified mutations and the protein or RNA they encode. The gene which was mutated most frequently is *apt*. *Apt* encodes an adenine phosphoribosyl transferase, which catalyzes the first step in an AMP salvage reaction.[20] Secondly, three mutations were found in *mnh* genes, which encode subunits of a Na^+/H^+ antiporter.[21] Other mutations occurred in different *rpo* genes, which encode RNA polymerase subunits (Table 5).

Table 5. List of genes affected by mutations, type of mutation and gene product.

	Gene	**Mutation**	**Product**
DA65173 DA65174 DA65181 DA65183	*apt*	Phe74Tyr Ile159fs Val134Asn Ala57Gly	Adenine phosphoribosyltransferase
DA65173 DA65175 DA65183	*mnhF* *mnhA*, *mnhF*	Leu19_Ala20insLeu Gly402Ser Met46Arg	Na^+/H^+ antiporter
DA65169 DA65174 DA65175	*rpoB*, *rpoE*, *rpoF*	Pro963Ser Pro93fs Asn73fs	RNA polymerase
DA65177 DA65179	*purR*	Lys120fs Gly68Ser	Pur operon repressor PurR
DA65178 DA65185	*rrsC*	several mutations	16S rRNA
DA65178 DA65185	*SAUSA300_1822*	several mutations	tRNA-Met
DA65169	*pknB*	Asn277fs	Protein kinase
DA65173	*rsbU*	Leu245del	Protein phosphorylation
DA65185	*SAUSA300_1232*	Glu107fs	Catalase KatA
DA65169	*SAUSA300_0137*	Gln186*	Transcriptional regulator, GntR family
DA65179	*SAUSA300_0012*	Ser161Leu	Putative homoserine O-acetyltransferase
DA65183	*argJ*		Arginine biosynthesis
DA65185	*SAUSA300_1066*	several mutations	tRNA-Arg

Fs: frame shift, ins: insertion, del: deletion, *: stop codon

Summary of results

Each mutant was tested regarding the MIC, growth rates, cross-resistance and mutations and Table 6 gives a summary of the results. Among the most interesting mutants are DA65171, which had the highest MIC as determined by the broth microdilution test and showed a growth rate twice as fast as the wild type in presence of 8 µg/mL monensin, as well as increased resistance to gentamicin. Mutant DA65172, had a low MIC according to broth microdilution, however, the growth rate with monensin is three times as high as the wild type and it possesses cross-resistance above the clinical breakpoint to both erythromycin and chloramphenicol. For both of these cases, the underlying mutations are not yet identified. The two mutants which have somewhat increased resistance to tetracycline DA65177 and DA65179, both seem to have

lost their resistance to monensin, with MICs of 4 µg/mL and growth rates below the wild type. Interestingly, they both have a mutation in a repressor of the *pur* operon, *purR.*

Table 6. Summary of collected data on each mutant. The MIC is given as an estimation by eye in a broth micro-dilution assay. Growth with monensin is the ratio between the growth rate of the wild type and mutant in presence of 8 µg/mL monensin.

Mutant	MIC	Growth rate	Growth with monensin	Cross-resistance	Mutations
DA28823	4	1.00	1.00	Wild type	Wild type
DA65169	8	0.95	0.51	none	*SAUSA300_0137, rpoB, pknB*
DA65170	16-64	0.47	1.57	3x GM	
DA65171	>128	0.67	1.98	2-3x GM	
DA65172	4	0.41	3.07	16-32x EM, 3-4x CL	
DA65173	8, EE	0.89	1.07	2-3x GM	*mnhF, apt, rsbU*
DA65174	8	1.18	0.72	none	*apt, rpoE*
DA65175	8, EE	1.26	1.19	none	*mnhA, rpoF*
DA65176	16- 128	0.52	1.07	none	
DA65177	4	1.19	0.62	2,5x Tet	*purR*
DA65178	8-16, EE	0.59	0.87	3-4x GM	*tRNA: SAUSA300_1822, rrsC- rRNA*
DA65179	4	1.17	0.66	2x Tet	*SAUSA300_0012, purR*
DA65180	8-16, EE	0.45	3.02	3-4x GM	
DA65181	4 to 8	1.23	0.94	none	*apt*
DA65182	8-16, EE	0.68		3-4x GM	
DA65183	8	1.10	1.21	none	*argJ, mnhF, apt*
DA65184	4 to 8	1.14	1.41	none	3 mutations in parts of the genome without annotations
DA65185	16, EE	0.54	3.02	none	*tRNA: SAUSA300_1822, tRNA: SAUSA300_1066, rrsC- rRNA, SAUSA300_1232*

Discussion

Monensin is widely used in livestock and poultry all over the world. Resistance to monensin might therefore be an important contributor to the problem of antibiotic resistance. One indicator regarding the risk of resistance evolution is the mutation rate. The fluctuation assay used in this study to calculate the mutation rate is based on the work by Luria and Delbrück in 1943.[22] It uses multiple cultures grown in parallel under identical conditions. Importantly, the cultures are started with a small inoculum. Therefore, all mutations arise independently and based on the distribution of mutants in the cultures one can calculate the rates at which mutations arise per cell per cell division. Our experiments show that *S. aureus* has a mutation rate of 3.6×10^{-10} to monensin resistance. This suggests that more than one mutation may be needed to confer monensin resistance, or that several genes need to be mutated to confer

resistance. Similar mutation rates have been observed against other antibiotics. For example, Wang *et al.* determined a mutation rate of $8x10^{-10}$ in *H. pylori* against the macrolide clarithromycin.[23]

In order to better understand the mutants detected in the fluctuation experiment, a subset of them was further analyzed regarding monensin resistance, fitness, cross-resistance and their underlying mutations. The determination of the MIC proved to be somewhat difficult, especially due to the paradoxical growth pattern demonstrated by several of the mutants. These mutants were inhibited by medium high concentrations of monensin but were able to grow at high concentration. This effect was first described for penicillin by Harry Eagle in 1948 and is known as the Eagle effect.[18] The reason for this growth pattern is unclear, however there are several hypotheses to explain this phenomenon. For instance, a resistance mechanism that requires a high concentration of the drug to be induced could result in the Eagle effect.[24] Another contributing factor could be the precipitation of the drug. Due to these problems, the observed MIC is not an ideal measure of the level of monensin resistance. A possible alternative to measure resistance is to use growth rates in the presence of monensin. Growth rates rely on the doubling time of the bacterial population and can easily be measured using a bioscreen. When using the growth rate of a mutant without monensin as a baseline of 100%, growth rates with monensin can indicate how much the drug slows down growth and therefore offers an alternative measure of how much a mutant is affected by it. However, the growth rate does not take into account how long the respective mutants are in exponential growth. This could result in an overestimation of growth in some mutants, which initially grow fast, but which consequently get inhibited by the drug. These differences could account for some of the discrepancies seen when comparing the level of resistance observed in the broth microdilution MIC test compared to the growth rates in presence of monensin. Nonetheless, both the MIC test and the growth rates indicate that some of the selected mutants acquired increased resistance to monensin. This is in agreement with several studies that have been able to identify some level of adaptation or resistance, including Simjee *et al*. However, it contradicts the work done by McConville *et al*, who claimed that it is impossible for resistance to arise against monensin.[13]

In addition to examine levels of monensin resistance, the selected mutants were also tested for cross-resistance to other antibiotics. Regarding cross-resistance, mutant DA65172 is the most interesting mutant, since it showed a 16-32 times increase in erythromycin, as well as a 3 to 4 times increase in chloramphenicol MIC. This strain is therefore resistant to both of the drugs above the clinical breakpoint as set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[19] Erythromycin is clinically used against a wide range of infections, mainly against Gram-positive ones.[25] Chloramphenicol is also a broad-spectrum antibiotic, however due to serious side effects, it is usually only used against more serious diseases like typhoid fever.[26] Both of these antibiotics target the 50S subunit of the ribosome. Additionally, several mutants showed increased resistance to gentamicin or tetracycline, although not to the level of clinical resistance. The fact that it was possible to select mutants that are cross-resistant to other, clinically important, antibiotics, contradicts the current literature, which claims that monensin does not cause cross-resistance.[14,27] This is a very significant result, because it implies that the emergence of resistance against monensin might have consequences for human health. The widespread use of the drug in animal farming is by many considered not to be a problem, because ionophores are not medically relevant to humans. However, these results show that they may contribute to the general resistance problem.

Sequencing results are at the moment still incomplete, which is unfortunate since sequences are missing for some of the mutants which have the most interesting characteristics. The results are, for example, missing for mutant DA65172, which shows clinical cross resistance to erythromycin and chloramphenicol, but also DA65171 and DA65180, which have a high resistance to monensin. It is possible that part of the reason why it was more difficult to extract DNA from these interesting mutants is their adaptation to monensin. Simjee *et al* have observed a thickening of the cell wall in monensin adapted strains.[3] Such a phenotypic change might have made extraction more difficult, because a thicker cell wall requires more Lysostaphin, or a longer incubation time to be broken down. Nonetheless, the available sequences already show some interesting results, especially the *mnh* genes which encode an Na^+/H^+ antiporter.[21] Considering that monensin acts by disrupting the sodium gradient in the cell, it is not surprising that changes in this gene could contribute to monensin resistance. Furthermore, most mutants have mutations in more than one gene. Whether all of them are always necessary for the observed phenotype remains to be determined, however, it is in line with the low mutation rate, which suggests that more than one mutation may be necessary. In order to determine exactly if and how *mnh,* and the other mutations contribute to monensin resistance, it is necessary to do further studies. One simple possibility would be to create knock-out mutants in these genes and to see if those result in the same phenotype.

In general, this project is still at the very beginning. There remain many additional experiments to be done in the future, which could help to better understand resistance evolution in Ionophores. For one, the selected mutants could be tested for cross-resistance to other ionophores, like lasalocid and salinomycin. This would indicate whether resistance to one ionophore results in resistance to the entire class, or if they require different resistance mechanisms. Furthermore, it would be interesting to test for cross-resistance to antimicrobial peptides like colistin and protamine.

Another aspect to consider is that the current experiments have only been done with *Staphylococcus aureus* and monensin. However, the question of ionophore resistance includes other ionophore drugs, as well as other bacteria species. It will for example be very interesting to perform the same experiments on *Enterococci* species. This would make it possible to better compare the results to some of the other literature, including the work from Simjee *et al.*, who have worked on *Enterococci* and *Clostridium perfringens*.[3] Another advantage would be that enterococci are part of the natural microbiome of cattle, and are therefore to a higher degree exposed to the drug, compared with *S. aureus*.

Another factor that could be considered in future studies is that so far, the mutants have been selected through direct plating. However, another approach is to passage bacteria in progressively increasing concentrations of monensin. This way would allow for an accumulation of resistances that could together result in highly resistant strains.

Overall, this study shows that exposing *S. aureus* to monensin can result in resistance. The identified mutants have different levels of resistance and fitness and they can also show cross-resistance to other antibiotics. With this in mind, the idea that the widespread use of ionophores as growth promotors or prophylaxis to parasitic infections are completely safe and bear no risk to human health has to be questioned. Further studies, both in laboratory settings, but also in the field, should be conducted in order to better evaluate the contribution of ionophores to resistance evolution. The results of such studies should be taken into consideration in legislature regarding the use of ionophores in animal agriculture.

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