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Nutritional Regulation of Sulfonamide Antibiotic Biodegradation by Microbacterium sp. Strain C448

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ABSTRACT

Sulfonamide antibiotics are frequently released into the environment as the result of their widespread use in livestock production. The presence of sulfonamides in the environment represents a potential selection pressure for the development and dissemination of sulfonamide resistance. Recently, a sulfonamide-degrading bacterium *Microbacterium* sp. Strain C448 was discovered, whose activity has the potential to be used as a method of sulfonamide removal from agricultural soils. This research aims to gain insight into the sulfonamide biodegradation pathway by 1) evaluating the nutritional regulation of sulfamethazine biodegradation and 2) comparing the proteomes of cells grown in the presence and the absence of sulfamethazine. I found that sulfamethazine degradation was suppressed by methionine, but stimulated by sucrose, NH_4Cl and glutamate. Additionally, several candidate proteins potentially involved in the degradation pathway were also identified. An understanding of how nutrient availability influences sulfonamide degradation and can help to maximize bioremediation potential of *Microbacterium* C448.

KEY WORDS: *Microbacterium,* sulfonamide antibiotics, biodegradation, antimicrobial resistance, sulfamethazine, bioremediation

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm
ADMP	2-amino-4,6-dimethylpyrimidine
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance genes
C448	*Microbacterium* C448
C544	*Microbacterium* C544
CAM	Chloramphenicol
DHPS	Dihydropteroate synthase
HPLC	High performance liquid chromatography
LB	Lysogeny broth
LC-MS	Liquid chromatography – mass spectrometry
MS	Minimal salts medium
OD_{600}	Optical density at 600 nm
PABA	*p*-Aminobenzoic acid
SA	Sulfanilic acid
SDZ	Sulfadiazine
SMZ	Sulfamethazine
SMX	Sulfamethoxazole
UspA	Universal stress protein A
WHO	World Health Organization

1 INTRODUCTION AND BACKGROUND

1.1 The golden era of antibiotic discovery

The discovery of the first antimicrobial compounds during the first half of the 20th Century marked a crucial turning point in the history of human medicine. It all began with the targeted development of the arsenic-based drug Salvarsan in 1909 by researchers in Paul Ehrlich's lab (Valent *et al.*, 2016). Salvarsan was one of 606 compounds that were synthesized in hopes of creating an antimicrobial agent that would be safe for human consumption (Valent *et al.*, 2016). Of all the compounds generated, Salvarsan was found to be effective against the syphilis causing bacterium *Treponema pallidum* and was on the market by 1910 (Valent *et al.*, 2016). Following this, research aimed at generating antibiotics from compounds used in synthetic dyes lead to the development the drug Prontosil in 1932 by Gerhard Domagk and Josef Klarer (Aminov, 2010). Prontosil was eventually removed from the market during the 1960's, and is considered the first sulfonamide antibiotic to be produced (Aminov, 2010).

Several years later, Alexander Fleming made the next major antibiotic discovery when he noticed that the secretion from an unexpected mould growing on his culture plates was responsible for killing the staphylococcus cultures he was growing at the time (Sengupta, 2013). Analysis of this secretion revealed that a compound, now known as penicillin, was effective against a wide range of bacteria (Sengupta, 2013). Eventually, penicillin was successfully mass-produced and placed on the market where it was widely used during World War II (Quinn, 2013). It was also at this time that the rate of antibiotic discovery began to rapidly increase, with peak discovery happening from 1950 to 1970, a period of time often referred to as the 'golden era of antibiotic discovery' (Gould, 2016; Aminov, 2010). Many of the drugs discovered during this era are still used in human medicine today, however the rate of antibiotic discovery has dropped dramatically since then (Davies, 2006; Aminov, 2010).

Currently, antibiotics are among the most commonly prescribed pharmaceutical products worldwide, with an estimated 177 million prescriptions being filled in the US and Canada annually (CDC, 2016; PHAC, 2016), with a 36% increase in consumption seen between 2000 and 2010 (Van Boeckel *et al.*, 2014). Commonly used for the treatments of common infectious diseases such as syphilis, urinary tract infections, and strep throat, antibiotics have undoubtedly become an invaluable form of human medicine (Piddock, 2012). Their eventual integration into human medical practices resulted in an increase human life expectancy from an average of approximately 54 years to around 80 years by greatly reducing the number of deaths resulting from bacterial infections (Piddock, 2012). This widespread antibiotic use is especially beneficial for people who are more susceptible to disease such as the young, the elderly, or those with weakened immune systems (Ventola, 2015). Furthermore, the use of antibiotics as an adjunct to surgery has greatly improved the success rates of these procedures by reducing the risk of infection both during and after surgical procedures (Piddock, 2012).

1.2 From healthcare to agriculture

In addition to their success in treating human disease, many antibiotics are also an effective mode of treatment against diseases in animals (Gustafson and Bowen 1997). Antibiotic treatment was thought to be a an especially beneficial component in rearing food-producing animals due to the fact that it reduced the number of animals lost to disease (Hao *et al.,* 2014; Krausse and Shubert, 2010), controlled spread of disease throughout the herd or flock (Elder *et al.,* 2002; Landers *et al.,* 2012), prevented the transmission of zoonotic pathogens from animals to humans (Nagaraja and Taylor, 1987), and were also effective against some protozoan and parasitic infections (Hao *et al.*, 2014; Hemaparasanth *et al.*, 2012). Additionally, it was found that animals whose diets were supplemented with antibiotics throughout their lives grew to a larger adult weight than their unsupplimented counterparts (Moore *et al.,* 1946; Stokstad *et al.,* 1949; Carpenter 1951; Cromwell *et al.,* 1984). This combination of more and larger animals surviving into adulthood has helped increase food-animal production to meet the demands of a growing population.

As of mid-2017, the total global population was estimated to be 7.6 billion, and is projected to reach a population of 9.7 billion by 2050 (United Nations, 2017). It is projected that the demand for meat and dairy products will increase by over 200 million tonnes by the year 2050, resulting in a larger amount of antibiotics being used in animal husbandry (FAO, 2009; Thornton, 2010; Van Boeckel *et al.,* 2015). Currently, it is estimated that 18 – 126 million pounds of antibiotics are used in the treatment of animals each year, with a projected increase of 67% by the year 2030 (Landers *et al.,* 2012; Van Boeckel *et al.,* 2015). These values account for a large proportion of the total antibiotic use worldwide, with many of these antibiotics also being important for human medicine (Landers *et al.,* 2012; Van Boeckel *et al.,* 2015).

1.3 Environmental fate of antibiotics

One drawback to the use of antibiotics in livestock production is that these drugs are poorly metabolised by medicated animals, with approximately 40% – 90% of ingested antimicrobials being excreted intact in feces and/or urine (Haller *et al.*, 2002; Hamscher *et al.*, 2012). Additionally, conjugates of some antibiotics are converted back into the parent compound once excreted in the animal wastes (Jjemba 2002). Animal manures are a commonly used form of plant fertilizer, and spreading these manures onto fields entrains antibiotic residues into the environment (Kumar *et al.*, 2005a; Kemper, 2008). Once in the environment, many of these compounds tend to spread to different areas, often ending up in adjacent water resources (Jjemba 2002). A wide variety of antibiotics including sulfamethazine, penicillin, tylosin, chlortetracycline, and oxytetracycline have been found in different environmental reservoirs including ground water, surface water and agricultural soils (Kumar *et al.*, 2005b; Accinelli *et al.*, 2007; Kemper *et al.*, 2008; Srinivasan and Sarmah, 2014). Although the fate of each antibiotic is largely dependent on the properties of the drug, there are several governing factors that influence both the persistence and stability of these compounds once they are released into the environment (Kümmerer, 2009; Domínguez *et al.*, 2014).

1.3.1 Factors influencing antibiotic persistence in the environment

Some antibiotics are susceptible to degradation by environmental (abiotic) factors such as the amount of light exposure, water content, or pH of the surrounding environment (Jjemba, 2008; Loftin *et al.*, 2008). Tetracyclines (Samuelsen 1989; Oka *et al.*, 1989), tylosin (Werner *et al*, 2007) and some fluoroquinolones (Thiele-Bruhn 2003;

Batchu *et al.,* 2014) are examples of antibiotics that are broken down by light. This process is highly dependent on the absorption spectrum of the antibiotic compound, and is more prevalent in clearer waters where exposure to light energy is increased (Kümmerer, 2009). Another factor influencing antibiotic persistence is the pH of the environmental matrix. Antibiotics belonging to the β-lactam (Gilbertson et al., 1990; Kheirolomoom *et al.,* 1999) and tetracycline (Kühne *et al.,* 2000) classes disappeared more readily from soil and manure samples that had a lower pH (Gilbertson *et al.,* 1990; Jjemba, 2008). On the other hand, macrolide antibiotics are the most stable at a neutral pH, but prone to degradation in both acidic and basic conditions (Sarmah *et al.,* 2006; Jjemba, 2008). β-Lactams such as ampicillin, cefalotin and cefoxitin are also susceptible to hydrolysis under neutral conditions (Mitchell *et al.*, 2014).

It is also possible for antibiotics to be degraded by microorganisms they encounter within the environment (i.e., biodegradation). Some fluoroquinolones are susceptible to degradation by environmental fungal species such as *Gloeophyllum straitum* (Martens *et al.,* 1996; Wetzstein *et al.,* 1999), and *Phanerochaete chrysosporium* (Martens *et al.,* 1996). Certain macrolides (Loke *et al.*, 2000), β-lactams (Gilbertson *et al.*, 1990; Braschi *et al.*, 2013) and tetracyclines (Kühne *et al.,* 2000) are degraded by microorganisms present in animal waste products.

1.3.2 Factors influencing antibiotic mobility in the environment

The chemical structure of each antibiotic determines how mobile it will be within the environment. Characteristics such as the isoelectric point and the nature of the functional groups influence how strongly an antibiotic will bind to soils, while the overall polarity of the antibiotic will affect how water soluble it will be (Tolls, 2001; Jjemba, 2008). Fluoroquinolones and tetracyclines are less mobile in the environment due to the fact that they adhere strongly to soils and manures (Tolls, 2001; Hamscher *et al.,* 2002; Jjemba, 2002), whereas macrolides do not bind as readily to soils and are more easily spread (Westergard *et al.,* 2001). However, the strength of these interactions is dependent on soil type. Certain antibiotics tend to show lower absorbance to sandy and silty soils, and higher adsorbance in clays and loamy soils (Tolls, 2001; Boxall *et al.,* 2005; Chander *et al.,* 2005). Furthermore, the amount of organic matter in the soil has also been shown to increase tetracycline and macrolide absorbance (Thiele – Brun, 2003; Chander *et al.,* 2005).

1.4 Antimicrobial resistance: a growing cause for concern

Defined as the acquired ability of microorganisms to survive in the presence of an antimicrobial agent, antimicrobial resistance (AMR) has been identified as one of the largest threats to global health by the World Health Organization (WHO) (WHO, 2016a). This is largely due to the fact that the frequency of reported AMR cases is steadily increasing in every region across the world (WHO, 2016a; CDC, 2015). Furthermore, it is predicted that an estimated 10 million people will succumb to infections involving resistant bacteria by the year 2050 (O'Neil, 2014). This rapid spread of resistant bacteria is potentially ushering us into the 'post antibiotic era' where we will once again succumb to diseases that are currently easily treated by antibiotics (WHO, 2014).

While the phenomenon of AMR allows bacteria to survive antibiotic treatment, the mechanisms used by microorganisms to do so are quite varied. These mechanisms fall into five broad categories: the modification of the antibiotic gene targets, altering the permeability of the cell membrane, removal of the antibiotic via efflux pumps, mechanisms designed to modify and inactivate the drug molecules, and the emergence of antibiotic degradation pathways (Alekshun and Levy, 2007; Sherrard *et al.,* 2014). In some cases, the end products of these degradation pathways can also serve as an alternate source of energy for bacteria grown in nutrient limited conditions (Dantas *et al.*, 2008). Regardless of the mechanism acquired, evidence suggests that the presence of antibiotic residues in the environment plays a role in the propagation of AMR (Witte, 2000).

1.4.1 AMR as a result of antibiotic use in livestock

Prolonged exposure of microorganisms to antibiotics is one of the main driving forces behind the propagation of AMR (Cox and Wright, 2013; Ventola, 2015). When a bacterial community is exposed an antibiotic, susceptible bacteria are killed off while any bacteria harbouring beneficial mutations will survive and reproduce, giving rise to a new colony of resistant strains. The spreading of manures taken from medicated animals onto fields introduces these antibiotic residues into the environment, creating a favourable environment for the resistance to develop (Kemper, 2008). The application of these manures to soils is often coupled with an increase in resistant bacteria compared to untreated soils (Heuer *et al.,* 2011; Marti *et al.,* 2013; Udikovich-Kolic *et al.,* 2014; Liu *et al.*, 2017). Additionally, environmental exposure to one type of antibiotic can drive the development of resistance to other unrelated antibiotics (Sengeløv *et al.,* 2003; Hao, 2014). This process is known as co-selection, and one example of this is the increase in abundance of ampicillin and penicillin resistant *Escherichia coli* in response to soils being treated with manure containing sulfamethazine (Alexander *et al.*, 2010).

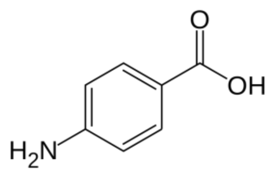
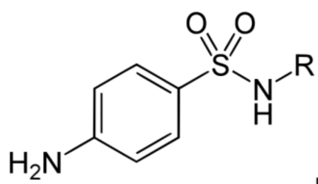
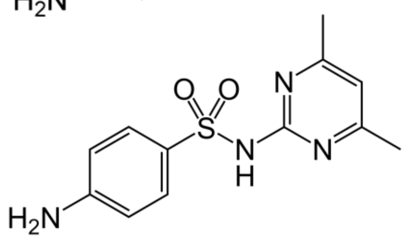
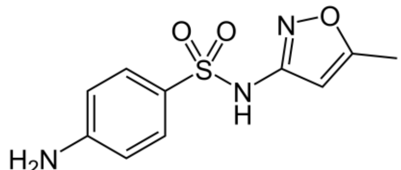
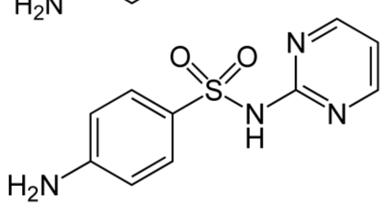
Furthermore, numerous antibiotic resistance genes (ARGs) have been identified, many of which are associated with mobile genetic elements, often resulting in the transmission of these genes between bacteria in a process known as horizontal gene transfer (Roberts, 2005; Robiscek *et al.*, 2006; Hanssen and Ericson, 2006; Wellington *et al.*, 2013). Although many of these genes are naturally present in soils, their abundance is increased by the treatment of manure from antibiotic treated livestock (Poirel *et al.*, 2012; Marti *et al.*, 2013; Perry and Wright, 2013; Liu *et al.*, 2017).

1.5 Sulfonamide antibiotics

Classified as a 'highly important antimicrobial', sulfonamide antibiotics were among the earliest classes of antibiotics to be discovered (WHO, 2016b). Characterized by the presence of a sulfonamide functional group, drugs in this class are structural homologues of *p*-aminobenzoic acid (PABA), a compound involved in the folic acid biosynthesis pathway (Table 1). Sulfonamides disrupt this pathway by acting as competitive inhibitors of the bacterial dihydropteroate synthase (DHPS) enzyme, inhibiting bacterial growth and proliferation. Sulfonamides are effective against both gram-positive and gram-negative bacteria, and are used in the treatment of bacterial infections including urinary tract infections, bronchitis, and ear infections in humans. These drugs are often used in conjunction with other antimicrobials, such as trimethoprim, to further improve their efficacy (Manyando *et al*, 2013).

Sulfonamides are also among the most commonly used classes of veterinary antibiotics, accounting for roughly 20% of the antibiotics used (by mass) in livestock production due to their low costs and relative ease of production (Sarmah *et al.,* 2006; Kools *et al.,* 2008). However, the widespread use sulfonamides is problematic due to the fact that approximately 40 – 50% percent of these drugs are metabolized by the animals and large amounts of unmetabolized drug product are excreted in the animal wastes (Lanshöft *et al,* 2007; Gutiérrez, 2010). Furthermore, an additional 20% of ingested sulfonamides are excreted as acetylated conjugates of the parent compound (Lanshöft *et al.,* 2007). During manure storage, these conjugates often convert back into the parent compound, resulting in a total of 60% of consumed sulfonamides being released into the environment (Lanshöft *et al.*, 2007). Sulfonamides have been detected at concentrations from the ng kg^{-1} to mg kg^{-1} ranges in manures, with lower concentrations generally being detected in manures that have been stored the longest (Haller *et al.,* 2002; Kemper *et al.,* 2008; Karcī and Balcioglu 2009; Wu *et al.,* 2011). Sulfonamides have also been quantified in soils treated with sulfonamide containing manures (Thorsten *et al.,* 2003; Karcī and Balcioglu*,* 2009), as well as in surface and ground waters adjacent to sites receiving the manures at concentrations ranging from the ng L^{-1} to μg L^{-1} range (Boxall *et al.,* 2002; Chrisian *et al.,* 2003; Förster *et al.,* 2009; Garcia-Galen *et al.,* 2011).

Table 1. Names and structures of *p*-amino benzoic acid, the sulfonamide functional group, and three sulfonamide antibiotics

Chemical structure	Compound name
	*p*-Aminobenzoic acid (PABA)
	Sulfonamide functional group
	Sulfamethazine (SMZ)
	Sulfamethoxazole (SMX)
	Sulfadiazine (SDZ)

1.5.1 Environmental fate of sulfonamide antibiotics

Sulfonamide antibiotics tend to be fairly mobile within the environment due to the fact that they adhere poorly to soil particles (Boxall *et al.,* 2002; Thiele-Bruhn and Aust, 2004; Wang *et al.*, 2015). In general, sulfonamides have low adsorption coefficients (K_d) with values typically ranging from 0.88 – 3.5 L kg^{-1} (Boxall *et al.*, 2002; Thiele-Bruhn and Aust, 2004; Accinelli *et al.*, 2007). However, the strength of these interactions are influenced by the composition of the soil, with stronger interactions being observed in finely-textured soils, soils with higher clay contents, and soils rich in organic matter (Thiele-Bruhn *et al.,* 2004; Gao and Pedersen, 2005; Accinelli *et al.,* 2007). The pH of the soil environment also plays a role in the strength of the interaction between sulfonamides and soil particles with more sorption being associated with a lower pH (Boxall *et al.*, 2002; Wang *et al.*, 2015, Zhang *et al.*, 2014). This is due to the fact that sulfonamides are weak acids, with isoelectric points that fall within a pH range of 4-5, and will predominantly exist in its anionic form in alkaline conditions (Avisar *et al.*, 2009; Park and Huwe, 2016). Not only are sulfonamides more water soluble in their ionic form, but they are also more mobile in soils due to the fact that soil particles generally carry an overall negative charge (Laak *et al.*, 2005; Park and Huwe, 2016). This is illustrated by the fact the mobility of sulfonamides tend to increase in manure treated soils due to the fact that the alkalinity of manures temporarily increases the pH of the soils they are applied to (Boxall *et al.*, 2002; Karcī and Balcioglu, 2009; Srinivasan and Sarmah, 2014; Zhang *et al.*, 2014). As a result, sulfonamides are more likely to leach into surface and ground waters surrounding the application sites (Zhang *et al.*, 2014; Srinivasan and Sarmah, 2014).

Another factor influencing the environmental fate of sulfonamide antibiotics are their chemical structures. The high stabilizing resonance energy of their characteristic ringed structures make them highly resistant to degradation because of the large amount of oxidative power required to provide the energy needed to open the aromatic rings (Thiele-Bruhn and Aust, 2003). Sulfonamides are fairly resistant to both hydrolysis and aerobic degradation, but are eventually degraded under aerobic conditions, with reported half-lives in soils and sediments ranging from 18.6 - 54 days (Accinelli *et al.,* 2007; Yang *et al.,* 2008).

1.5.2 Sulfonamide resistance

Resistance to sulfonamide antibiotics is widespread and usually associated with *sul1, sul2* and *sul3* genes, which are mutated versions of the drug target, DPHS (Sköld, 2000; Perreten and Boerlin, 2003). These alternative versions of the DHPS still effectively bind PABA but have a lower binding affinity for sulfonamides, allowing folate biosynthesis to occur in the presence of the drugs (Swedberg and Sköld, 1980). *Sul1* genes are commonly associated with class 1 integrons, while *sul2* and *sul3* are found on plasmids (Sköld, 2000; Enne *et al.,* 2001; Perreten and Boerlin, 2003). Much like other veterinary antibiotics, the application of manure onto agricultural soils has been linked to an increased abundance of both sulfonamide resistant bacteria and the associated sulfonamide resistance genes in the environment (Heuer and Smalla, 2007; Byrne-Bailey *et al.,* 2009; Kopmann *et al.,* 2013; Hsu *et al.,* 2014; Wang, 2014).

1.5.3 Strategies for removing sulfonamide antibiotics from the environment

While it is important to reduce the amount of sulfonamide antibiotics in the environment, large-scale methods capable of doing so in agricultural soils are scarce. Currently, most methods of environmental sulfonamide removal are only effective in water and require materials such as activated carbon (Adams *et al.*, 2003), micelle- clay systems (Polubesova *et al.*, 2006), activated sludge (Sheng-Fu *et al.*, 2006), or involve the use of a combination of gamma radiation and Fe^{2+} ions (Liu *et al.*, 2014). Ding *et al.* reported that treatment with laccase enzymes removed several sulfonamide antibiotics from soils (2016); however, producing the large amounts of enzyme required for large-scale drug removal is costly (Margot *et al.*, 2013). A more cost-effective alternative strategy would be to introduce sulfonamide degrading bacteria, such as the recently discovered *Microbacterium* sp. C448, to contaminated soils as a means of bioremediation (Topp *et al.*, 2013).

1.6 *Microbacterium* C448 other sulfonamide biodegrading species

In 2012 an unknown, gram- positive species of *Microbacterium* (designated Strain C448), capable of degrading sulfonamide antibiotics was discovered in London, Ontario (Topp *et al*, 2013). This discovery was the result of a long-term field experiment designed to observe the effect of annual spring applications of commonly used veterinary antibiotics on soil microbial communities (Topp *et al.*, 2013). Sulfamethazine is taken up by the bacterium and cleaved into two main constituents: 2-amino-4,6-dimethylpyrimidine (ADMP) and the hypothesized sulfanilic acid moiety (SA) (Figure 1). 2-amino-4,6-dimethylpyrimidine is released from the cell, while carbon from the remaining portion of the drug is further mineralized into CO_2 (Topp *et al,* 2013). Although other strains of sulfonamide-degrading *Microbacterium* species have now been isolated (Boujou *et al.,* 2012; Tappe *et al,* 2013), the exact method of degradation remains elusive. Recent work on another sulfonamide degrading species of *Microbacterium* (Strain BR1) postulated that the pathway is initiated with a hydroxylation reaction happening at the ipso-position of the molecule, which is followed by a series of redox reactions to facilitate the cleavage of the aromatic ring in the metabolized portion of the molecule (Ricken *et al.*, 2013; Ricken *et al.*, 2015).

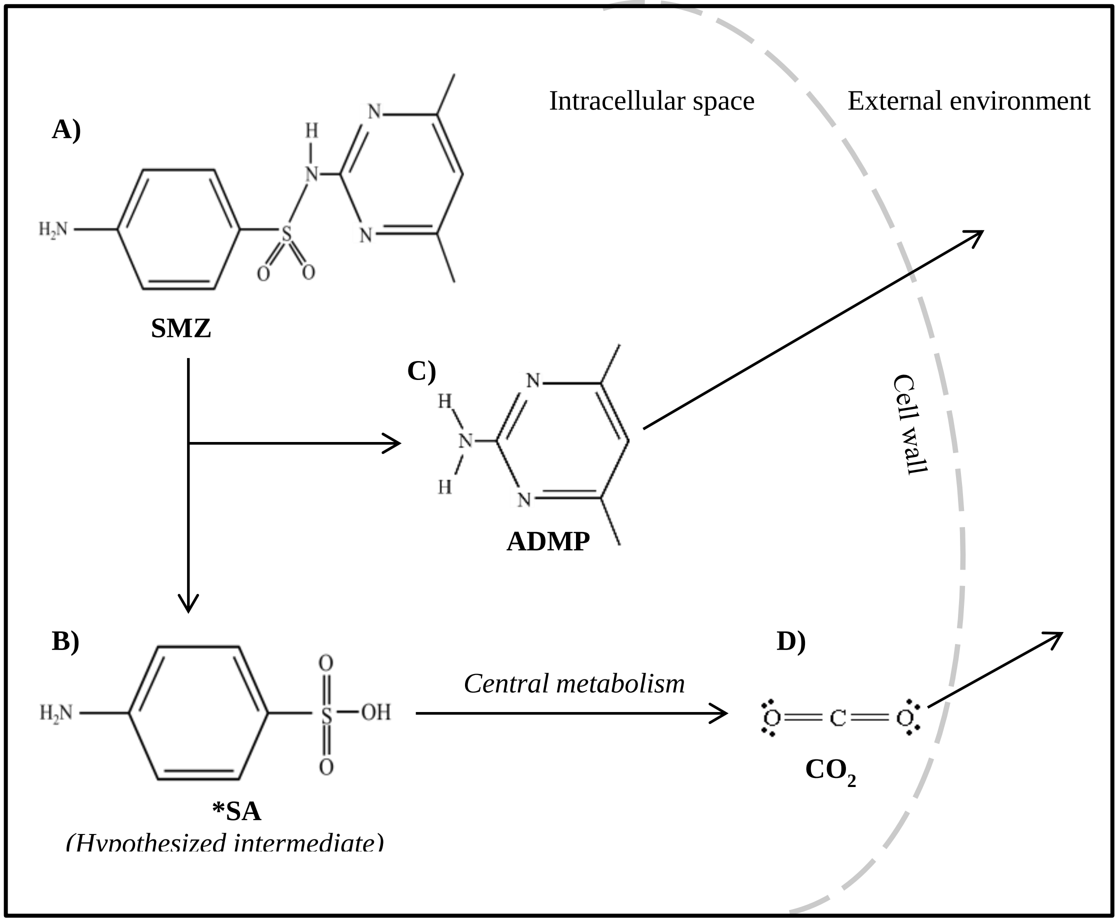


Figure 1. Chemical structures of the known and hypothesized products of sulfamethazine biodegradation. Sulfamethazine (A) gets taken into the bacterial cell where it is split into sulfanilic acid (hypothesized intermediate) (B), and 2-amino-4,6-dimethylpyrimidine (C). The carbon in the sulfanilic acid moiety enters central metabolism where it is eventually mineralized to CO_2 (D), while ADMP is secreted into the culture medium as an end product of metabolism.

1.7 Hypothesis and research objectives

The nutrient content of a given environment can influence the efficiency of microbial metabolism (D'Annibale *et al.,* 2005; Hamdi *et al.,* 2007). Therefore, the main objective of this research was to evaluate how the nutrient composition of the growth medium influences SMZ degradation by C448, with the hypothesis that sulfur-containing nutrient sources will act as negative regulators to the sulfonamide degradation pathway. To test this hypothesis, differences in SMZ degradation were evaluated in C448 cultures grown in the presence of varying amounts and sources of sulfur (Na_2SO_4), nitrogen (NH_4Cl), carbon (sucrose) and amino acids (methionine, glutamate). Additionally, a comparative proteomic analysis of cells grown in the presence and absence of SMZ was carried out in order to identify proteins that may play a role in the sulfonamide biodegradation pathway.

2 MATERIALS AND METHODS

2.1 Culture preparation

A culture of *Microbacterium* sp Strain C448 (abbreviated C448) was obtained from the Topp Lab (Agriculture and Agri-Food Canada, London, ON) and maintained through weekly plating onto fresh Lysogeny-broth (LB) agar and incubated at 30 °C (Topp *et al*, 2013). Following each weekly incubation, a small, round, yellow colony was selected to be used in the next week's incubation. Seed cultures for each experiment were grown in a complete minimal salts (MS) medium which was prepared as follows: The main components of the medium were combined in the proportions listed in Table 2A, and supplemented with 1 mL/L of the trace element stock solution (Table 2B). Following this, the media was then autoclaved for 15 minutes at 120 °C**,** cooled, and then further supplemented with 1 mL/L of filter-sterilized (0.22 μm pore size cellulose acetate filter; SteriCup, Millipore, Mississauga, ON) additive components stock solution (Table 2C) and 500 mg/L SMZ. Sulfamethazine stock solutions were prepared at a concentration of 250 g/L, filter sterilized, and stored in the dark at room temperature for up to 2 weeks.

A well isolated single colony taken from the LB agar plates was then added to a 50 mL Erlenmeyer flask containing 15 mL MS medium and incubated (New Brunswick Scientific, Enfield, CT) at 30 °C with agitation (220 rpm) for up to 5 days. Following this, 3 mL of this culture was used to inoculate a 125 mL Erlenmeyer flask containing 27 mL of MS medium. Once again the cells were incubated at 30 °C with agitation (220 rpm) for up to 5 days. This process was repeated weekly for the duration of the induction and nutritional analyses (*Sections 2.2- 2.4*).

Table 2. Composition of the mineral salts (MS) medium

A) Main components of the MS medium

Salts and Sucrose	Concentration (g/L)	MS 'Complete'	S-free	C- free	N-free
K_2HPO_4	1.35	+	+	+	+
KH_2PO_4	0.39	+	+	+	+
NaCl	0.43	+	+	+	+
NH_4Cl	0.09	+	+	+	-
Sucrose	0.29	+	+	-	+

B) Composition of the trace element stock solution

Trace elements	Concentration (mg/L)	MS 'Complete'	S-free	C- free	N-free
H_3BO_3	2.00	+	+	+	+
$MnSO_4 \bullet H_2O$	1.80	+	-	+	+
$MnCl_3 \bullet H_2O$	2.00	-	+	-	-
$ZnSO_4$	0.20	+	-	+	+
$ZnCl_2$	0.20	-	+	-	-
$CuSO_4$	0.10	+	-	+	+
$CuCl_2$	0.10	-	+	-	-
$NaMoO_4$	0.25	+	+	+	+

C) Composition of the supplementary stock solution

Additive Components	Concentration (mg/L)	MS 'Complete'	S-free	C- free	N-free
$MgSO_4 \bullet 7H_2O$	160.00	+	-	+	+
$MgCl_2 \bullet 6H_2O$	162.00	-	+	-	-
$CaCl_2 \bullet 2H_2O$	25.00	+	+	+	+
$FeSO_4 \bullet 7H_2O$	10.00	+	-	+	+
$FeCl_3 \bullet 6H_2O$	9.70	-	+	-	-
Biotin	0.10	+	+	+	+
Thiamine	0.04	+	+	+	+

2.2 Inducibility of the sulfonamide biodegradation pathway

To study the activation of SMZ biodegradation, whole cell suspensions were cultured in MS 'complete' medium (Table 2) in either the presence or absence of SMZ (500 mg/L) and incubated at 30 °C with agitation (220 rpm) for approximately 20-22 hours until late log phase. At this point, 1.5 mL of these cultures were transferred to sterile 2 mL Eppendorf tubes and the cells were harvested by centrifugation at 13,500 x g for 2 minutes at 4 °C (PrismR, Montreal Biotech Inc., Dorval, QC). The supernatant was discarded, and the cell pellets were washed 2x with SMZ-free MS medium (Table 2A). Following this, the pellets were resuspended in 1.5 mL fresh MS medium containing a combination of SMZ (500 mg/L) and chloramphenicol (CAM) (20 mg/L). These cell suspensions were added to 50 mL Erlenmeyer flasks containing 13.5 mL of the same MS/SMZ/CAM combination resulting in a final culture volume of 15 mL. Treatment was prepared in triplicate.

At the start of the experiment, 500 μL portions of each culture were obtained, dispensed into sterile 2 mL Eppendorf tubes and centrifuged at 13,500 x g for 2 minutes at 4 °C. Supernatants were collected, filter sterilized, and stored at 4 °C until ready to be analyzed for residual SMZ. Cell growth was monitored spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) by dispensing 100 μL of each sample into disposable cuvettes and measuring the optical density at 600 nm (OD_{600}). Sampling was continued at 2-hour intervals for a total of 8 hours.

A portion of each supernatant was diluted 10-fold in SMZ-free MS media and analysed using the Epoch2 Microplate Reader and the Gen5.1 software (Biotek, Winooski, VT) to visually estimate the extent of SMZ degradation at each time point by measuring the absorbance at the λmax of SMZ (260 nm). The resulting absorbance spectra were imported into Microsoft Excel 2010 (Microsoft Corp., Mississauga, ON) for further analysis. The residual concentration of SMZ at each time point was calculated by fitting the absorbance at 260 nm (A_{260}) values of each sample to the equation generated by the calibration curve that was generated by plotting the A260 values of SMZ standards against their corresponding concentrations.

The remaining culture supernatants were diluted 4-fold in Milli-Q H_2O and used for HPLC- UV analysis to quantify residual SMZ. The UV detector was set to detect analytes at both 260 and 280 nm and samples were resolved on an Agilent Eclipse XDB C-18 column (4.6 x250 mm, 5μm pore size; Santa Clara, CA) with an Agilent Eclipse XDB-C18 guard column (4.6 x 12.5 mm, 5μm pore size, Santa Clara, CA). The mobile phase consisted of H_2O + 0.05% formic acid: acetonitrile (60:40), with an injection volume of 2 μL, a flow rate of 0.250 mL min^{-1} and a column temperature of 25 °C. The retention times of 2-amino-4,6-dimethylpyrimidine (ADMP), SMZ and CAM were 3.6, 6.0, and 7.7 minutes respectively. A control experiment was also run in the absence of CAM, using the same methods outlined above. Once again, each experiment was carried out in triplicate.

2.3 Effects of exogenous sulfur, carbon and nitrogen on SMZ biodegradation

A series of four experiments were set up with each treatment in triplicate, each designed to evaluate how levels of exogenous inorganic sulfur, nitrogen, or carbon, in the growth medium would influence SMZ degradation. For each experiment, 1.5 mL aliquots of late-log phase cells collected from the seed culture were transferred to 50 mL

Erlenmeyer flasks containing 13.5 mL of MS medium supplemented with either 1x, 10x, or 50x the molar amount of sulfur (1.7 mM), nitrogen (1.7 mM), or carbon (10.8 mM) found in the sulfanilic acid portion of 500 mg/L SMZ (Tables 2 and 3; Figure 1B).

2.3.1 Sulfur

To determine if increasing amounts of exogenous inorganic sulfur in the growth medium would have an effect on SMZ degradation, 3 mL of C448 seed cultures were added to a 125 mL Erlenmeyer flask containing 27 mL 'S-free' MS medium supplemented with 500 mg/L SMZ (Table 2). These cells were incubated at 30 °C, with agitation (220 rpm) until they reached late-log phase (20-22 hours). Following this, the cells were harvested by centrifugation at 13,500 x g for 2 minutes at 4 °C, washed twice using sterile SMZ-free MS medium, and resuspended in 30 mL 'S-free' MS. Once again, 1.5 mL aliquots of this cell suspension were added to 50 mL Erlenmeyer flasks containing 1.5 mL S-free MS medium supplemented with either Na_2SO_4 or methionine at concentrations representative of either 1x (1.7 mM), 10x (17.0 mM), or 50x (85 mM) the molar amount of sulfur present in the metabolized portion of SMZ (Table 3). NaCl was used to normalize the molarity of Na across the Na_2SO_4 treatment groups (Table 3A). An additional 0x treatment was also prepared in which the 500 mg/L SMZ present in the solution served as the sole source of sulfur for the bacterium and a parallel experiment was also run in the absence of SMZ to determine whether or not increasing amounts of methionine on its own would be toxic to C448. Both culture growth and SMZ degradation were monitored over the course of 24 hours using the same protocol outlined in Section 2.2, with the exception of the method used for the HPLC-UV analysis.

In this experiment, the remaining culture supernatants were diluted 500-fold in Milli-Q H_2O and the mobile phase was made up of MeOH: 40mM ammonium acetate (30:70) with a flow rate of 1 mL min $^{-1}$ and an injection volume of 50 μL. Retention times of SMZ and ADMP were 3.0 and 4.6 minutes respectively.

Table 3. Media composition for the sulfur experiments

A)

Treatment Group	**Volume seed culture (mL)**	**Volume S$^-$ AMS (mL)**	**Volume Na_2SO_4 stock[a] (µL)**	**Volume NaCl[b] stock (µL)**
0x Na_2SO_4	1.5	12.7	0	800
1x Na_2SO_4	1.5	12.7	16	784
10x Na_2SO_4	1.5	12.7	160	640
50x Na_2SO_4	1.5	12.7	800	0

B)

Treatment Group	**Volume seed culture (mL)**	**Volume S$^-$ AMS (mL)**	**Volume Methionine stock[c] (mL)**
0x Methionine	1.5	13.5	0
1x Methionine	1.5	13.43	0.07
10x Methionine	1.5	12.85	0.65
50x Methionine	1.5	10.2	3.3

[a] [Na_2SO_4] stock = 225 mg/mL
[b] [NaCl] stock = 184.9 mg/ mL
[c] [Methionine] stock = 55.6 mg/mL dissolved in ‘S-free’ MS

2.3.2 Nitrogen and glutamate

To determine whether or not increasing amounts of exogenous nitrogen has an effect on the biodegradation pathway, C448 cultures were grown in 'N-free' MS medium (Table 2A) supplemented with 500 mg/L SMZ, and NH_4Cl acting as a source of inorganic nitrogen (Table 4A). To distinguish between the effects of nitrogen and carbon on SMZ degradation, a parallel experiment was also run using sodium glutamate as a combined source of carbon and nitrogen (Table 4B). NaCl was used to normalize the molarity of Na^+ across the glutamate treatment groups (Table 4B). Bacterial growth and degradation were tracked over the course of 7 days for the NH_4Cl experiment, and 24 hours for the glutamate experiment. These experiments were carried out using the same protocol outlined in Section 2.3.1.

Table 4. Media composition for nitrogen experiments.

A)

Treatment Group	Volume seed culture (mL)	Volume S-free AMS (mL)	Volume NH_4Cl stock[a] (µL)	Volume H_2O (µL)
0x NH_4Cl	1.5	12.7	0	800
1x NH_4Cl	1.5	12.7	16	784
10x NH_4Cl	1.5	12.7	160	640
50x NH_4Cl	1.5	12.7	800	0

B)

Treatment Group	Volume seed culture (mL)	Volume N-free MS (mL)	Volume glutamate stock[b] (mL)	Volume NaCl stock[c] (mL)
0x Glutamate	1.5	11.15	0	2.35
1x Glutamate	1.5	11.15	0.05	2.3
10x Glutamate	1.5	11.15	0.47	1.88
50x Glutamate	1.5	11.15	2.35	0

2.3.3 Carbon

To determine whether or not increasing amounts of exogenous carbon on its owwn has an effect on the biodegradation pathway, C448 cultures were grown in 'C-free' MS medium supplemented with 500 mg/L SMZ, and sucrose acting as a source of carbon (Table 5), These experiments were carried out using the same protocol outlined in Section 2.3.1.

Table 5. Media composition for the carbon experiment

Treatment Group	Volume seed culture (mL)	Volume S^- AMS (mL)	Volume sucrose stock[a] (µL)	Volume H_2O (µL)
0x Sucrose	1.5	12.7	0	800
1x Sucrose	1.5	12.7	16	784
10x Sucrose	1.5	12.7	160	640
50x Sucrose	1.5	12.7	800	0

[a] [sucrose] stock = 268 mg/ mL

2.4 Isolation of free amino acids from dairy and swine manure

In order to isolate free amino acids from manure, 45 mL samples of liquid dairy or swine manure were centrifuged at 12,000 x g for 30 minutes at 4 °C. The resulting supernatants were carefully decanted into fresh 50 mL falcon tubes and this process was repeated until there was no longer any pellet formation. Following this, 300 μL aliquots of each supernatant were filter-sterilized (0.22 μm Millex syringe-driven filter unit, Millipore, Billerica, MA) and transferred to a clean 2 mL microfuge tube each containing 700 μL ethanol. The samples were then placed on a rotator and incubated at 4°C in the dark for 45 minutes. After the incubation period, the extracts were centrifuged at 14,000 x g for 10 minutes at 4 °C. The resulting supernatants were transferred to a fresh 2 mL microfuge tube containing 700 μL chloroform and vortexed. Following this, 300 μL of HPLC-grade H_2O was then added to each tube, and the samples were vortexed once again. These samples were then centrifuged for 15 minutes at 4 °C and the aqueous phase was recovered, ensuring that the volumes of each samples were equal between replicates. The samples were then dried in a vacufuge until the excess liquid had evaporated and handed over to the Marsolais lab where the amino acids present in each sample were quantified via HPLC (Jafari *et al*, 2016).

2.5 Comparative proteomic analysis

2.5.1. Sample preparation for LC-MS

Cultures of *Microbacterium* C448 were grown overnight in a carbon free-MS medium (Table 2) supplemented with 500 mg/ mL SMZ at 30°C with agitation (220 rpm) and allowed to reach late log phase (20-22 hours). At this point, 2 mL of this culture was transferred to a sterile 2 mL round bottom tube and spun at 8,000 x g for 3 minutes at 4 °C. The supernatant was discarded, the cells were resuspended using another 2 mL of culture and the process was repeated once more. The remaining pellet was resuspended in 50 mM cold, sterile ammonium bicarbonate buffer (pH 8) and transferred to a 1.5 mL Eppendorf tube where it was kept on ice until ready for lysis. This process was repeated with cells grown in MS medium containing sucrose as the sole carbon source, and with cells grown in MS medium containing a combination of sucrose and 500 mg/mL SMZ. A degradation-deficient mutant strain of *Microbacterium* C448 (designated strain C544) was also used in this analysis. C544 was generated by Dr. Calvin Lau via random UV mutagenesis and has lost the ability to degrade SMZ. In this experiment, C544 was grown in MS medium containing a combination of sucrose and 500 mg/mL SMZ prior to protein extraction. Four replications were prepared for each treatment.

To lyse the cells, each sample was sonicated on ice for 30 seconds, alternating with 30 seconds of rest for a total sonication time of 5 minutes per sample (Qsonica, Newton, CT; amplitude 50%). Following sonication, the lysates were spun at 16,000 x g for 15 minutes at 4 °C to remove cellular debris and 450 μL portions of each supernatant was transferred to sterile 2 mL round bottom microfuge tubes.

Next, 100 μL RapiGest surfactant (Waters, Milford, MA) was added to a 2 mL round bottom tube containing 100 μL of the protein extracts from each sample. Following this, each sample was heated at 99°C for 2 minutes (Eppendorf Thermomixer, Eppendorf, Hamberg, Germany), and then cooled at room temperature for 5 minutes. Once cool, 6.3 μL of 500 mM iodoacetamide (IAA) (Sigma-Aldrich, Missouri, USA) was added to each sample and incubated at room temperature in the dark for a total of 30 minutes. To digest the extracts, a combination of 2.1 μL of 100 mM $CaCl_2$ and 25 μL of a 0.1 μg /μL trypsin solution was added to each sample. These digests were subsequently incubated overnight at 33 °C, with agitation (300 rpm). The next day, enough trifluoroacetic acid was added to each sample to end up with a final 0.05% solution in order to precipitate the RapiGest. Each tube was incubated at 37 °C for 45 minutes with agitation (300 rpm), chilled on ice for 5 minutes, and spun at 4 °C at 16,000 x g for 10 minutes. Finally, 175 μL of each supernatant was removed, carefully avoiding the pellet, and transferred to a clean 2 mL round bottom tube. The samples were stored at -80°C until ready to be analysed by LC-MS.

2.5.2 Peptide identification, quantification and statistical analysis

The peptide digests were analyzed by Dr. Justin Renaud using an Easy-nLC 1000 nano-flow system with a 100 μm x 2 cm Acclaim C18 PepMap™ trap column and a 75 μm x 15 cm Acclaim C18 PepMap™ analytical column (Thermo Scientific) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The flow rate was 300 nL min^{-1} and 10 μL of the protein digest was injected. Peptides were eluted as follows: 97% mobile phase A (LC/MS Optima water, 0.1% formic acid) in B (LC/MS Optima acetonitrile 0.1% formic acid) was decreased to 90% over 4 minutes, followed by a linear gradient from 10-35% mobile phase B over 120 minutes, 35-90% over 4 minutes and maintained at 90% B for 16 minutes. The nanospray voltage was set at 2.0 kV, capillary temperature 275°C, and S-lens RF level 60. Samples were analyzed by a top 6 data-dependent acquisition experiments. The full scan (*m/z* 375-1800) was operated at 70,000 resolution, automatic gain control (AGC) of 1e6 and maximum injection time (IT) of 240 ms. The MS/MS scans were acquired at 17,500 resolution, AGC of 1e6, maximum IT of 110 ms, intensity threshold of 4.4×10^4, normalized collision energy of 27 and isolation window of 2 *m/z*. Unassigned, singly and >4 charged peptides were not selected for MS/MS and a 20 s dynamic exclusion was used.

The *Microbacterium* sp. C448 proteome used for protein identification was obtained from UniProt (*UP00002883, accessed Feb 2016*). The peak list files were analyzed using label-free quantitation in MaxQuant version 1.4.08 (Cox & Mann, 2008) with the default settings. The resulting LC-MS protein data were analyzed using the MaxQuant Software and then imported into Perseus version 1.5.1.6 (Tyanova *et al*, 2016) to perform statistical analyses. The data was first subjected to an initial round of processing where irrelevant protein identifications, such as those corresponding to commonly known protein contaminants, such as keratin, trypsin, and BSA powder, were removed from the dataset, and the data was linearized by a log transformation (base 2). To account for samples containing undetectable amounts of a given protein, all samples containing non-assigned number values (NaN) were filtered out using the default program settings. The quality of the replicates for each treatment was then assessed in two ways: first by comparing the expression patterns of the proteins within each sample to that of all the others, and then calculating the Pearson correlation of each comparison. The normality of the data was then assessed by generating histograms of the expression ratios of the samples. Finally, any residual missing signal intensity values were imputed using the default program settings.

Statistical analysis of the changes in protein expression for each treatment was carried out by performing a multiple sample, 2 sided T-test, with a maximum *p*-value of 0.01, and a false discovery rate (FDR) threshold value of 0.05.

3 RESULTS

3.1 Determination of substrate-induced sulfonamide biodegradation

To determine if the sulfonamide biodegradation pathway is upregulated in response to SMZ exposure, an induction assay was carried out using *Microbacterium* C448 cells previously grown in either the presence or absence of SMZ. This experiment was performed in the presence of CAM, an inhibitor of protein synthesis and it was found that after 8 hours of incubation, cells with previous SMZ exposure degraded 28.7% of the available SMZ compared to the 8.0% degraded by previously unexposed cells (Figure 2A). This resulted in an overall difference of 20.6% degradation between the two treatments (p = <0.001). In the absence of CAM, once again, previously exposed cells degraded a greater proportion of SMZ (67.4%) than previously unexposed cells (23.0%), with an overall difference of 44.4% between the two treatments (p = <0.001) (Figure 2B). Interestingly, the growth curves for previously unexposed cells remained consistent in both the presence and absence of CAM, while the slope of the growth curve for previously exposed cells was steeper when CAM was not present in the growth medium, indicating that CAM had a greater effect on previously unexposed cells (Figures 2C and D).

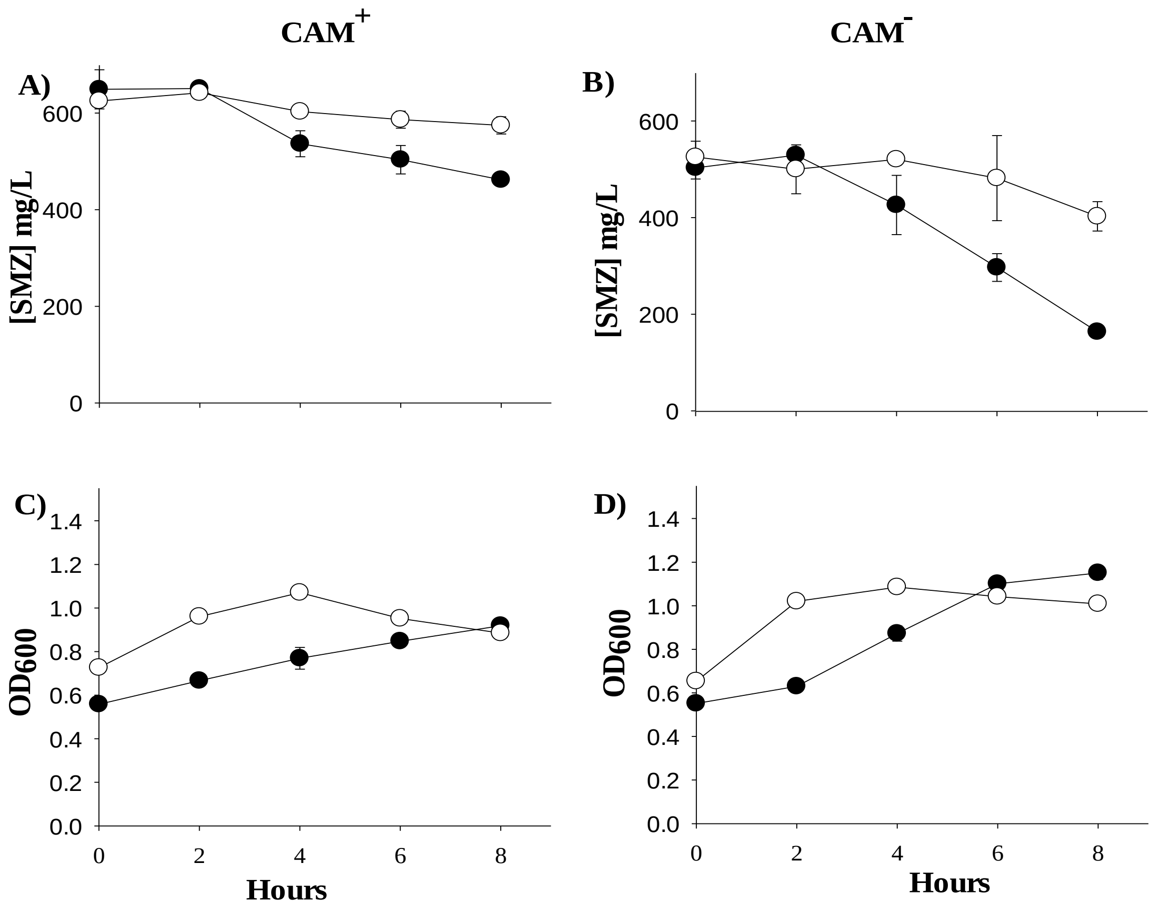


Figure 2. Inducibility of sulfamethazine biodegradation in *Microbacterium* C448. Sulfamethazine degradation (A and B) and bacterial growth (C and D) curves for cells that were either previously exposed or not previously exposed to SMZ prior to the start of the experiment. This assay was conducted in both the presence and absence of chloramphenicol (CAM). Data points represent the mean of three biological replicates; error bars indicate the standard deviation of these means.

3.2 Sulfamethazine degradation in the presence of exogenous sulfur

3.2.1 Inorganic sulfur

The second objective of this study was to determine whether or not SMZ biodegradation was affected by the presence of exogenous sulfate (Na_2SO_4) in the growth medium. Initially, increasing amounts of Na_2SO_4 in the growth medium was associated with a decrease in the rate of SMZ degradation, with an impairment of degradation observed in the 50x treatment group (data not shown). However, it was hypothesized that the inhibitory effect could be due to the additional osmotic pressure conferred by the higher salt concentration. This hypothesis was tested by normalizing the molarity in all treatments to that conferred by the 50x Na_2SO_4, by addition of NaCl. When the molarity was normalized across the treatments there was no effect of increasing SO_4^{2-} concentrations on either sulfamethazine degradation or growth (Figure 3).

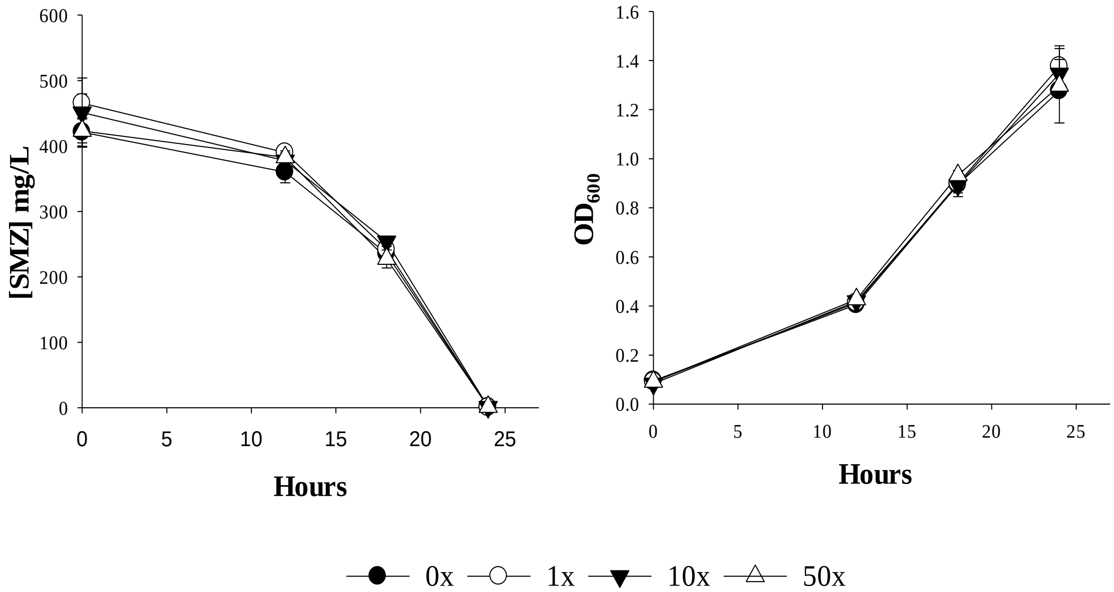


Figure 3. Effect of increasing exogenous sulfate on sulfamethazine biodegradation in *Microbacterium* C448. Degradation (A) and growth (B) curves for C448 isolates incubated in the presence of increasing amounts of exogenous Na_2SO_4 over the course of 24 hours. Na_2SO_4 was supplied at concentrations representative of 1x (1.7 mM), 10x (17.0 mM), and 50x (85 mM) the molar amount of sulfur present in the metabolized portion of SMZ (1.7 mM). NaCl was used to balance the molarity of Na^+. Data points represent the mean of three biological replicates; error bars indicate the standard deviation of these means.

3.2.2 Organic sulfur

A preliminary analysis was done to determine if *Microbacterium* C448 was able to utilize L-methionine as a source of sulfur in the absence of SMZ. Methionine did support growth, indicating that the cells were able to acquire sulfur from the amino acid with sucrose acting as a source of carbon (Figure 4). To determine whether or not organic sulfur had an effect on SMZ degradation rate, the sulfate inhibition assay was repeated using methionine as a source of organic sulfur. After 24 hours, cells grown on SMZ as the sole sulfur source (0x methionine) had degraded 93.0% of the available drug, while cultures in the 1x, 10x, and 50x methionine treatments had degraded 66.6% (p = 0.015), 35.6% (p = <0.001) and 6.6% (p = <0.001) respectively (Figure 5A). Additionally, there was no difference between the growth observed in the 0x and 1x treatments (OD_{600} = 0.94 vs. 0.99, p = 0.88), but further increasing the amount of methionine appeared to inhibit growth, with cultures being exposed to 10x and 50x methionine reaching a maximum OD_{600} of 0.79 (p = 0.006), and 0.45 (p = <0.001) respectively (Figure 5C). A similar trend was observed when the experiment was repeated using a SMZ concentration of 50 mg /L, with cultures in the 0x, 1x and 10x methionine treatments degrading significantly more of the drug than those containing 50x the methionine content within the first 12 hours (88.2%, 87.1% and 86.3% vs 70.7%, p = <0.001), however, this effect had diminished after 18 hours (Figure 5B). Furthermore, decreased cell growth was seen over the first 18 hours in the 50x treatment compared to the 0x, 1x and 10x treatments (p = <0.001) (Figure 5D).

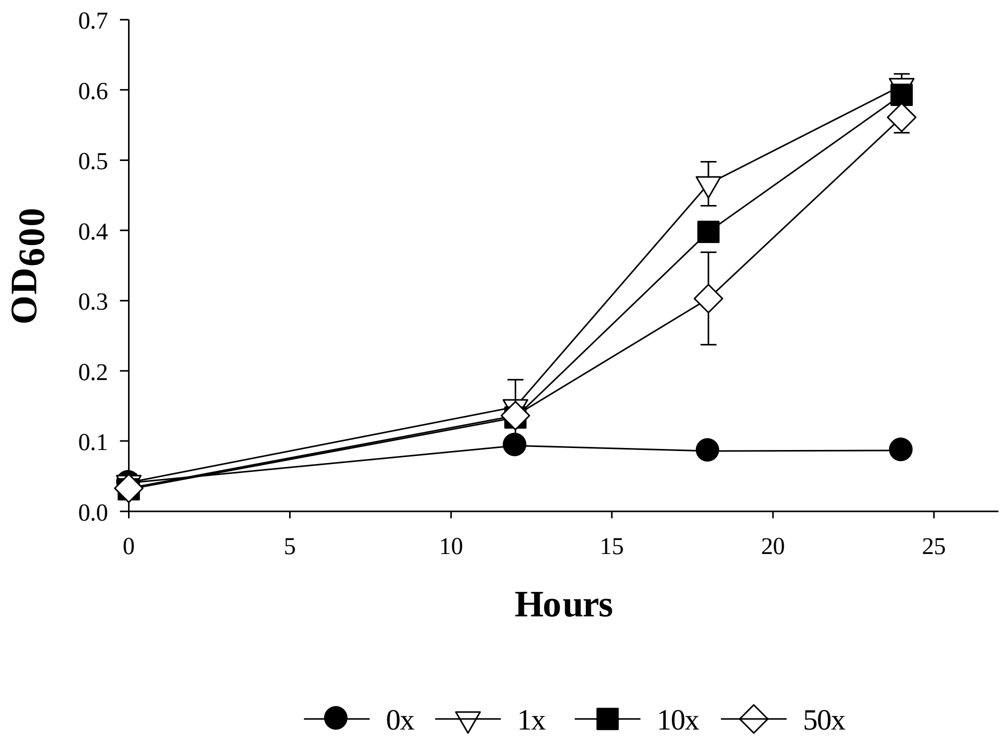


Figure 4. Effect of exogenous methionine on growth of *Microbacterium* C448. Growth curve for C448 isolates grown in MS medium with methionine as the sole sulfur source over 24 hours. Methionine was supplied at concentrations representative of 1x (1.7 mM), 10x (17.0 mM), and 50x (85 mM) the molar amount of sulfur present in the metabolized portion of SMZ (1.7 mM). This experiment was performed in the presence of sucrose. Data points represent the mean of three biological replicates; error bars indicate the standard deviation of these means.

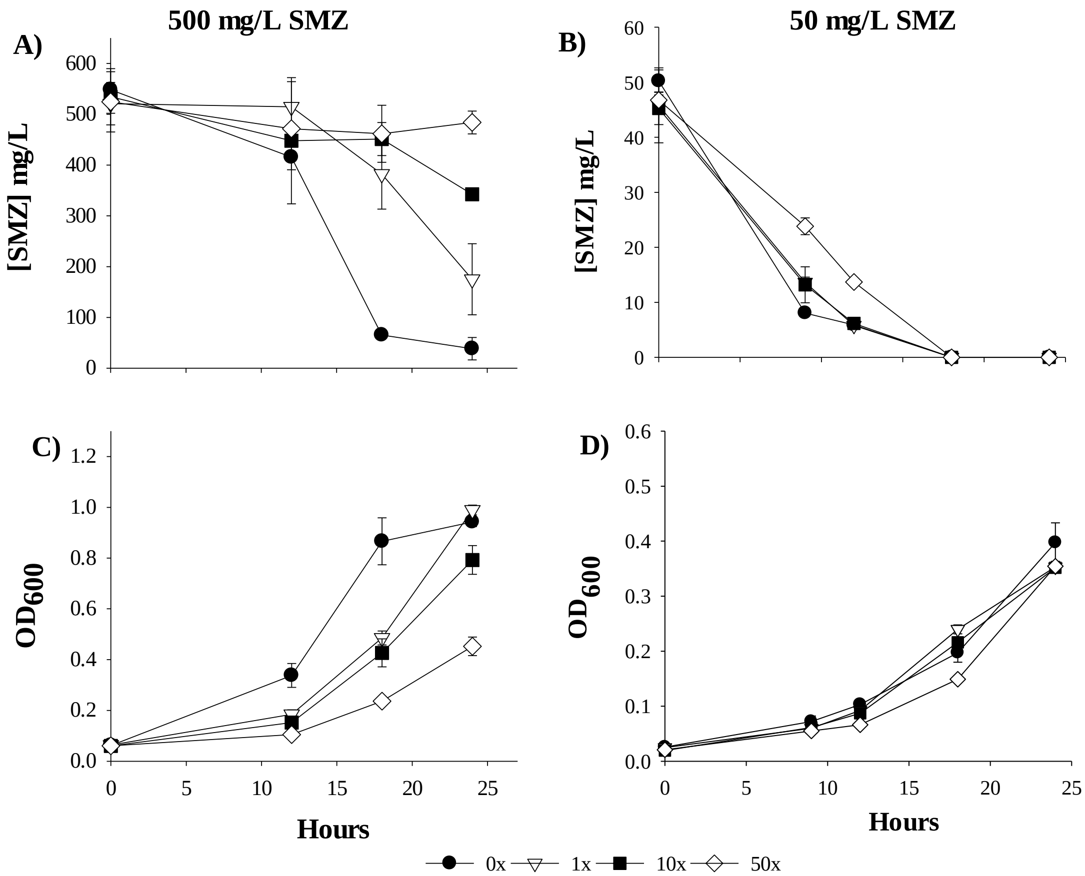


Figure 5. Effect of exogenous methionine on sulfamethazine biodegradation by *Microbacterium* C448. Degradation (A and B) and growth (C and D) curves for C448 isolates incubated in the presence of increasing amounts of exogenous methionine over the course of 24 hours. Methionine was supplied at concentrations representative of 1x (1.7 mM), 10x (17.0 mM), and 50x (85 mM) the molar amount of sulfur present in the metabolized portion of SMZ. Data points represent the mean of three biological replicates; error bars indicate the standard deviation of these means.

3.3 Sulfamethazine degradation in the presence of exogenous nitrogen

Since methionine is composed of carbon, sulfur and nitrogen, a second set of experiments were performed where the total nitrogen content varied, but sulfur content of each treatment was kept constant for each treatment. In these experiments, nitrogen was supplied in inorganic (NH_4Cl) or organic carbon-containing (glutamate) forms. In both cases, the presence of exogenous nitrogen stimulated both cellular growth and SMZ degradation in C448 (Figure 6). A distinct decrease in the rate of SMZ degradation was observed in the absence of NH_4Cl, compared to cultures with 1x, 10x, and 50x NH_4Cl over the first 72 hours of incubation (49.5% vs 74.9%, 77.6%, and 80.1% respectively, p = <0.01) (Figure 6A). Additionally, increasing amounts of NH_4Cl in the culture medium profoundly increased microbial growth with maximum OD_{600} values of 0.90, 1.69, 3.14 and 3.94 for the 0x, 1x, 10x, and 50x treatments respectively (Figure 6C).

When glutamate was used as a source of nitrogen, cultures containing SMZ as the sole source of nitrogen (0x glutamate) degraded 7.6% of the available SMZ, while cultures with 1x, 10x, and 50x glutamate degraded 85.1%, 100% and 100% respectively within the first 24 hours of incubation (Figure 6B). Once again, increasing amounts of glutamate in the growth medium accelerated cell growth, with cultures containing 10x and 50x glutamate reaching a final OD_{600} of 1.19 and 1.16 respectively (Figure 6D). These values were significantly higher than those seen when glutamate was either absent (0.74; p = 0.005) or supplied at the 1x concentration (0.92; p = <0.001) (Figure 6B).

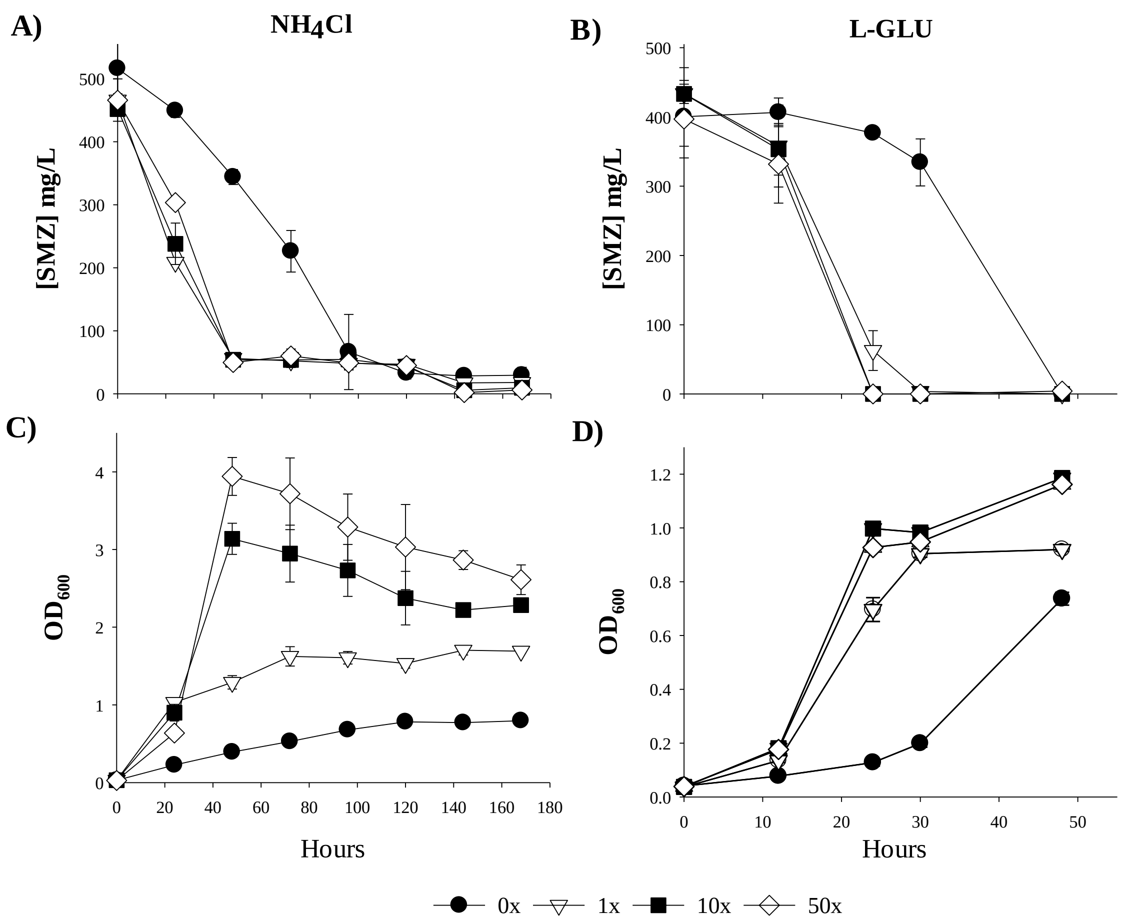


Figure 6. Effect of exogenous sources of nitrogen on sulfamethazine biodegradation by *Microbacterium* C448. Degradation (A and B) and growth (C and D) curves for C448 cultures incubated in the presence of increasing amounts of exogenous NH_4Cl (A and C) or glutamate (B and D). The concentrations of NH_4Cl and glutamate used represent 1x (1.7 mM), 10x (17.0 mM) and 50x (85 mM) the molar amount of nitrogen present in the metabolized portion of SMZ (1.7 mM). Data points represent the mean of three biological replicates; error bars indicate the standard deviation of these means.

3.4 Sulfamethazine degradation in the presence of exogenous carbon

Finally, to distinguish between the effects of exogenous nitrogen and carbon on SMZ degradation, increasing amounts of sucrose were added to the growth medium and SMZ degradation monitored over one week's incubation. Levels of the other nutrients were held constant. Increasing amounts of sucrose in the growth medium accelerated both growth and SMZ degradation with complete degradation being observed in cultures treated with 1x, 10x, and 50x during the first 24 hours of the assay (Figures 7A and B). On the other hand, cultures containing SMZ as the sole carbon source (0x sucrose) took 120 hours for complete SMZ degradation, with only 12.4% of the drug being metabolized within the first 24 hours (Figure 7A). Similar to the nitrogen experiments, cultures treated with 50x sucrose had the most overall growth, with a maximum OD_{600} of 0.98, while cultures lacking exogenous carbon reached a peak OD_{600} of 0.18 (p=<0.001) (Figure 7B).

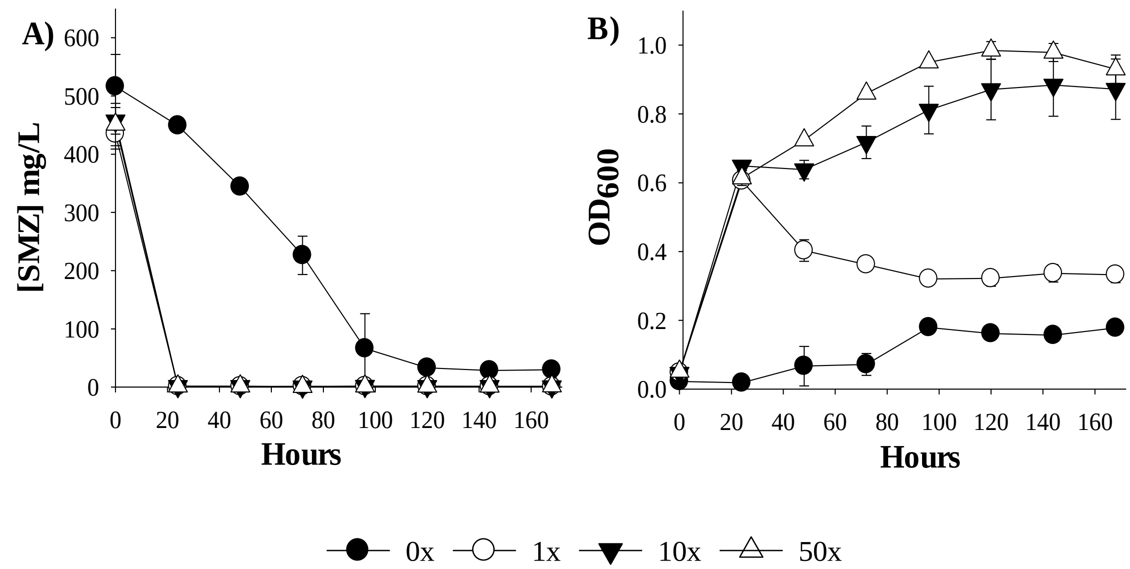


Figure 7. Effect of increasing amounts of exogenous sucrose on sulfamethazine biodegradation in *Microbacterium* C448. Degradation (A) and growth (B) curves for C448 incubated in the presence of increasing amounts of exogenous sucrose over the course of one week. The concentrations of sucrose used represent 1x (10.8 mM), 10x (108 mM) and 50x (540 mM) the molar amount of carbon present in the metabolized portion of SMZ (10.8 mM). Data points represent the mean of three biological replicates; error bars indicate the standard deviation of these means.

3.5 Free amino acid content of dairy and swine manure

In order to determine if concentrations of sulfur-containing amino acids present in agricultural manures were sufficient to have an effect on the SMZ biodegradation pathway, concentrations of cysteine and methionine were quantified using free amino acid extracts taken from the supernatants of four independent manure samples (one dairy and three swine samples). Only the dairy sample contained detectable amounts of methionine (68.2 μM) (Table 6), which was present at a molarity 25 times lower than that which resulted in an inhibition of SMZ degradation (1.7 mM) (Figure 5A). Additionally, the molar amounts of glutamate and asparagine were determined. Only the dairy manure sample contained detectable amounts of asparagine (2.09 μM), which was present at a concentration that was approximately 3250 times lower than the minimum concentration of glutamate that was shown to accelerate SMZ biodegradation in C448 (1.7 mM) (Figure 6A) (Table 6).

Table 6. Concentrations of select free amino acids in dairy and swine manures

Manure type	**[Met] μM**	**[Cys] μM**	**[Glu] μM**	**[Asp] μM**
Dairy	68.2± 3.3	ND	ND	2.09 ± 0.2*
Swine 1	ND	ND	ND	ND
Swine 2	ND	ND	ND	ND
Swine 3	ND	ND	ND	ND

Limit of detection: 2 pM
* = was only detected in 2 of the three samples
ND= not detected

3.6 Comparative proteomic analysis

In order to identify proteins that were differentially regulated in response to SMZ exposure, a comparative proteomic analysis was carried out where cells were grown in either MS supplimented with sucrose only, SMZ only, or both sucrose and SMZ. Of the 1,544 proteins identified by LC-MS, 36 were found to be significantly upregulated, and 92 proteins downregulated, by a factor of 2 or more, in response to SMZ (p = <0.01) (Table 7). Four of the genes encoding five proteins that showed the greatest increase in abuncance in response to SMZ, appeared to be located within the same genomic region of *Microbacterium* C448 (Figure 8). These proteins include two dehydrogenases, an oxidoreductase and a transcriptional regulator belonging to the GntR family (Table 7). Interestingly, there was no change in the abundance of the top five proteins in degradation deficient mutant C544, with the exception of 2-oxoisovalerate dehydrogenase subunit beta (UniProt ID: W0ZAF5), in which only a 2.6 fold increase was seen (Table 7), despite sharing 100% sequence similarity.

Table 7. Differentially expressed proteins in response to SMZ in *Microbacterium* C448 and C544

UniProt ID	Gene[c]	Protein Annotation	Fold-Change[a]		
			C448 SMZ	**C448 SMZ/SUCROSE**	**C544[b] SMZ/SUCROSE**
W0ZC29	*gabD*	Succinate-semialdehyde dehydrogenase (NADP(+)) 1	+224.7[d]	+175.0	--[e]
W0ZAF5	*MIC448_2420014*	2-oxoisovalerate dehydrogenase subunit beta	+163.1	+146.8	+2.6
W0Z942	*pps*	Phosphoenolpyruvate synthase	+147.9	+117.1	--
W0Z8E4	*MIC448_2420013*	Glyoxalase/bleomycin resistance protein/dioxygenase	+57.7	+45.9	--
W0ZB11	*MIC448_2420012*	Transcriptional regulator, GntR family	+28.0	+13.3	--
W0Z8S2	*MIC448_2580005*	Uncharacterized protein	+13.8	--	--
W0Z5N9	*aceB*	Malate synthase	+10.4	--	--
W0ZEQ2	*MIC448_930014*	Putative Extracellular ligand-binding receptor	+9.6	+3.7	--
W0Z7P4	*glpX*	Fructose-1,6-bisphosphatase	+9.5	+2.9	--
W0Z7Z0	*ydiA*	Putative phosphoenolpyruvate synthase regulatory protein	+9.3	+7.5	--
W0ZBY2	*MIC448_300004*	Putative sugar transport permease	+8.6	--	+6.2
W0ZD06	*xylG*	Fused D-xylose transporter subunits of ABC superfamily ATP-binding	+8.1	+4.5	+7.4

UniProt ID	Gene	Protein Annotation	Fold-Change[a]		
			C448 SMZ	C448 SMZ/SUCROSE	C544[b] SMZ/SUCROSE
W0ZC75	*MIC448_410004*	Flavin-dependent oxidoreductase, F420-dependent methylene-tetrahydromethanopterin reductase	+4.4	+9.8	+5.3
W0ZDB5	*poxB*	Pyruvate dehydrogenase (Pyruvate oxidase),thiamin-dependent, FAD-binding	+4.1	+9.6	+7.4
W0Z8X7	*MIC448_2660004*	Universal stress protein UspA	+4.1	+4.6	--
W0Z6T2	*aceA*	Isocitrate lyase	+4.0	--	--
W0ZCM3	*gabT*	4-aminobutyrate aminotransferase,PLP-dependent	+3.9	+4.3	--
W0Z7L4	*treZ*	Malto-oligosyltrehalose trehalohydrolase	+3.6	+2.4	+3.3
W0Z818	*bglB*	Thermostable beta-glucosidase B	+3.4	+48.7	+45.5
W0Z5L8	*MIC448_1310006*	Acyl-CoA dehydrogenase, C-terminal domain protein	+3.3	--	-365.9[f]
W0Z9V3	*gabD*	Succinate-semialdehyde dehydrogenase I, NADP-dependent	+3.2	+3.1	+1.5
W0ZD15	*gap*	Glyceraldehyde-3-phosphate dehydrogenase	+3.2	--	+1.4

UniProt ID	Gene	Protein Annotation	Fold-Change[a]		
			C448 SMZ	C448 SMZ/SUCROSE	C544[b] SMZ/SUCROSE
W0Z9W7	*MIC448_1620010*	Uncharacterized protein	+3.0	--	--
W0Z6P3	*lpdC*	Dihydrolipoyl dehydrogenase	+2.9	+2.5	--
W0Z746	*MIC448_1100021*	Putative enoyl-CoA hydratase 1	+2.7	--	--
W0Z8F2	*MIC448_2040025*	Putative transport protein (ABC superfamily,atp_bind)	+2.6	--	--
W0Z555	*ilvE*	Branched-chain-amino-acid aminotransferase	+2.6	--	+2.0
W0Z991	*MIC448_1680006*	Putative RutC family protein YjgH	+2.5	+2.0	-1.1
W0Z7H9	*MIC448_1310007*	YceI family protein	+2.3	--	--
W0ZDL6	*MIC448_920051*	NhaP-type Na+/H+ and K+/H+ antiporters	+2.2	--	--
W0Z8I2	*gluC*	Glutamate transport system permease protein GluC	+2.2	--	--
W0Z8R3	*acs*	Acetyl-coenzyme A synthetase	+2.2	--	+1.5

UniProt ID	Gene	Protein Annotation	Fold-Change[a]		
			C448 SMZ	C448 SMZ/SUCROSE	C544[b] SMZ/SUCROSE
W0ZCM8	*ssdA*	Succinate-semialdehyde dehydrogenase (NADP(+))	+2.1	+3.1	--
W0ZAT0	*ssdA*	Alkyl hydroperoxide reductase AhpD	+2.1	--	--
W0ZA54	*MIC448_1650011*	Aminomethyltransferase	+2.0	--	+2.4
W0ZAL3	*MIC448_250019*	Uncharacterized protein	+2.0	--	+3.1

[a]Fold changes reported relative to protein abundance detected in C448 sucrose treatment group with a *p*-value of <0.01
[b]*Microbacterium* sp. Strain C544 is the degradation deficient mutant that was generated by UV mutagenesis.
[c] Gene names taken from the UniProt database (The UniProt Consortium 2017).
[d] + signifies an increase in protein abundance in response to SMZ
[e] -- signifies no significant change in protein abundance in response to SMZ
[f] - signifies a decrease in protein abundance in response to SMZ

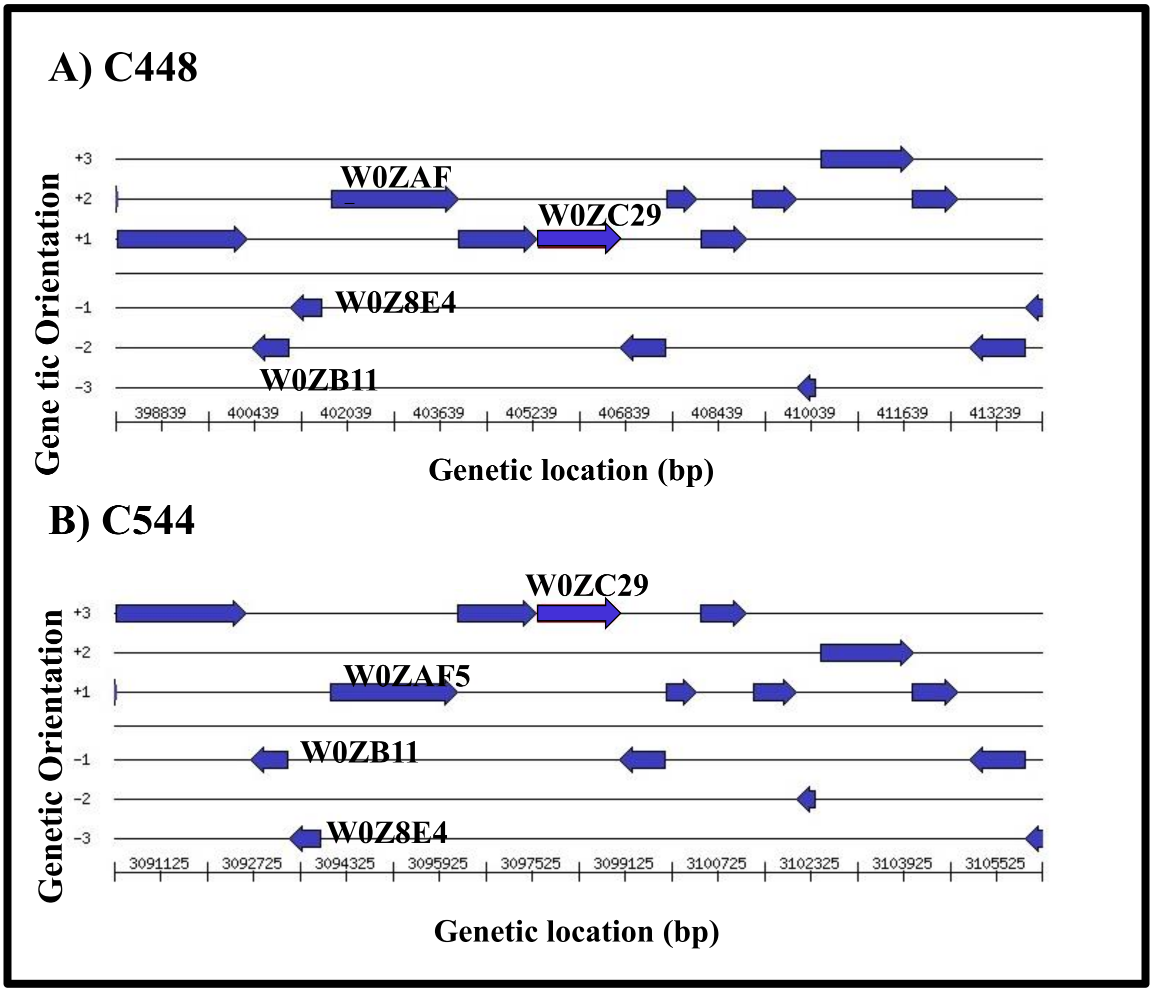


Figure 8. Genomic locations the genes encoding for 4 of the 5 proteins that showed the greatest increase in expression following sulfamethazine exposure in *Microbacterium* sp. Strain C448 and C544. W0ZAF5: 2-oxoisovalerate dehydrogenase subunit beta; W0ZC29: Succinate-semialdehyde dehydrogenase (NADP(+)) 1; W0Z8E4 : Glyoxalase/bleomycin resistance protein/dioxygenase; W0ZB11: Transcriptional regulator, GntR family. Genomic construct modified from RAST SEED viewer (Overbeek *et al*, 2005).

4 DISCUSSION

4.1 Sulfonamide biodegradation is induced by SMZ

The impact of previous exposure to SMZ on ability of C448 to biodegrade the antibiotic was analyzed. The results were interpreted by comparing the amount of degradation observed in cells previously exposed to SMZ to that of cells that had no previous exposure to the drug. This experiment was run in the presence of chloramphenicol (CAM), an inhibitor of protein synthesis, with the prediction that, if the pathway has to be induced, previously unexposed cells would not degrade SMZ. We observed a more efficient SMZ degradation by cultures previously exposed to SMZ compared to those that were previously unexposed (Figure 2A). One reason for this would be that the previously exposed cells were already actively degrading SMZ prior to the beginning of the assay, and would already have all of the necessary proteins required for SMZ degradation, allowing for degradation to occur in the presence of CAM. On the other hand, cells grown in the absence of SMZ would need to synthesize these proteins in order to degrade SMZ. When the experiments were repeated in the absence of CAM, there was an overall increase in the degradation rates of both previously exposed and unexposed cells, however previously exposed cells still degraded a greater proportion of SMZ over time than previously unexposed cells (Figure 2B).

The slope of the growth curve for previously exposed cells was steeper when CAM was not present in the growth medium, while the growth rate of previously unexposed cells remained consistent throughout, indicating that CAM had a greater inhibitory effect on preciously exposed cells (Figures 2C and 2D). Preliminary growth experiments revealed a slower growth rate in C448 cells exposed to SMZ than cells that were just grown on sucrose (data not shown). One possible explanation for this could be that bacteriostatic antibiotics, such as SMZ and CAM lower bacterial cellular respiration (Lobritz *et al.*, 2014). It is possible that cellular respiration was already reduced in cells previously exposed to SMZ relative to unexposed cells, and the subsequent addition of CAM resulted in a further reduction of cellular respiration and a more pronounced inhibition of growth. One way to clarify these results would be to either pre-incubate the cells for 30 minutes with CAM prior to the addition of SMZ to allow the drug to affect protein synthesis before beginning the assay. Also getting a measurement of the total protein content at each time point and reporting the values as a function of the concentration of SMZ degraded over time per ng protein would normalize the results.

4.2 Influence of exogenous nutrients on SMZ biodegradation

Sulfamethazine degradation was inhibited by the presence of exogenous methionine (Figure 5) with an inverse relationship between the concentration of methionine and SMZ degradation. However, it is unlikely that this suppression of SMZ degradation would be observed in manures due to the fact that the methionine content of the dairy (68.2 μM) and swine (not detected) manures were well below that which was found to suppress pathway activity (1.70 mM) (Table 6; Figure 4). One explanation for this relationship would be that SMZ degradation is at least in part associated with the process of sulfur assimilation in C448. While bacterial sulfur assimilation generally begins with the acquisition of sulfate from the surrounding environment, many soil-dwelling bacteria are also able to assimilate sulfur from a variety of organic compounds such as alkanesulfonates, dibenzothiopene, taurine, and methionine, when sulfate is limiting (Vermeij and Kertesz 1999; Ohshiro and Izumi 1999; Mirleau *et al.*, 2005; Hullo *et al.*, 2007; Kertesz 2000). Once sulfate enters the cell, it is converted into sulfite, sequentially reduced and eventually incorporated into methionine and cysteine (Keretesz 2000; Fuchs *et al*, 2011). The fact that C448 was able to grow with either SMZ or methionine as the sole sulfur source suggests that it is able to assimilate sulfur from both of these organic compounds (Figures 4 and 5). Furthermore, pathways such as the S-adenosyl-methionine (SAM) recycling pathway have been characterized in bacteria such as *Bacillis subtillis* (Hullo *et al.*, 2007; Rodinov *et al.*, 2007), *Klebsiella aerogenes* (Seiflein and Lawrence 2006), and *Pseudomonas aeruginosa* (Kertesz 2000) that facilitate the conversion of methionine to cysteine. Therefore, it is possible that increased concentrations of methionine in the culture medium inhibited SMZ degradation due to the fact that methionine is both one of the main products of sulfur assimilation and can be converted into cysteine – the other main product of sulfur metabolism. This would also explain why increasing amounts of exogenous Na_2SO_4 had no effect on SMZ degradation (Figure 3), because both forms of sulfur (SMZ and SO_4^{2-}) would have to undergo a series of transformations, once taken up by bacteria, in order to synthesize methionine and cysteine.

Increasing amounts of methionine were associated with a substantial inhibition of cell growth with SMZ was present at an initial concentration of 500 mg/L (Figure 5C). However, this effect was diminished when the starting concentration of SMZ was reduced to 50 mg/L (Figure 5D), suggesting that the growth inhibition was a result of the higher SMZ concentrations, rather than that of methionine itself. This is further illustrated by the fact that increasing concentrations of methionine had little effect on bacterial growth when C448 was grown in the absence of SMZ (Figure 4).

In contrast to what was seen in the methionine experiment, the addition of sucrose, ammonium, or glutamate to the MS medium stimulated both proliferation and SMZ degradation in C448 (Figures 6 and 7). This is consistent with the fact increasing the carbon and nitrogen content in soils stimulates both microbial growth and metabolic activity (Peacock *et al.,* 2001; Larkin *et al.,* 2006), and the addition of ammonium nitrate to soils lead to an increase in proportion of gram-positive bacterial species compared to untreated soils (Peacock *et al.,* 2001). While the concentration of amino acid asparagine, which has a nitrogen-containing side chain, in the dairy manure sample was well below what was found to have a stimulatory effect on the pathway (2.09 μM vs 1.70 mM) (Table 6; Figure 6), it is likely that SMZ degradation would still be stimulated due to the high carbon content of manures (Larkin *et al.*, 2006). Carbon contents of solid dairy and swine manures were found to be 0.19 mol kg^{-1} and 0.06 mol kg^{-1} respectively, which were both well above the lowest concentration (540 mM) shown to have a stimulatory effect on degradation (Figure 7) (Larkin *et al.*, 2006).

Based on these results, it can be concluded that SMZ biodegradation is stimulated by sulfur limitation or by elevated levels of carbon/ nitrogen nutrient sources. When either carbon or nitrogen are present at non-limiting amounts, not only is bacterial growth stimulated, but sulfur becomes the limiting nutrient. Given that 1) bacteria in high carbon conditions are more efficient at consuming organic substrates (Peacock *et al.,* 2001), and 2) SMZ is a source of organic sulfur, it can be hypothesized that SMZ degradation is stimulated in C448 as a means of satisfying its sulfur requirements.

4.3 Comparative proteomic analysis of SMZ biodegradation in C448

Once SMZ is taken up by *Microbacterium* C448, it is perhaps split into two aromatic moieties: ADMP, which is excreted out of the cell, and the hypothesized sulfanilic acid intermediate which is metabolized by the bacterium (Figure 1; Topp *et al.*, 2013). In general, bacterial degradation of aromatic compounds is a specialized process that occurs in two main steps: the hydroxylation-mediated destabilization of the aromatic ring and the cleavage of hydroxylated intermediate (McLeod and Eltis, 2008; Fuchs *et al.*, 2011). Both steps require a substantial amount of oxidative power and rely on the activities of oxidoreductases, such as oxidases and dehydrogenases to facilitate degradation (McLeod and Eltis, 2008; Gan, 2011). This process occurs via a variety of peripheral metabolic pathways, eventually generating substrates that will be used for central metabolism (Fuchs *et al.*, 2011).

A comparative proteomic analysis of C448 cells grown in the presence and absence of SMZ identified two oxidoreductases — NAD dependent succinate semialdehyde dehydrogenase (UniProt ID: W0ZC29), and glyoxylase/bleomycin resistance protein/ dioxygenase (UniProt ID: W0Z8E4) among the top proteins to have the greatest increase in expression in response to SMZ (Table 7). Previously, succinate semialdehyde dehydrogenases have been found to be involved in the catabolism of aromatic compounds in *Escherichia coli* (Skinner and Cooper 1982; Diaz 2001), *Klebsiella pneumoniae* (Sanchez *et al.*, 1982) and *Arthrobacter nicotinovorans* pAO1 (Chiribau *et al.*, 2006). This protein was also found to have 56% sequence similarity with the succinate semialdehyde dehydrogenase protein (UniProt ID: A0A1E5KA91) in sulfadiazine degrading bacterium *Arthrobacter* sp. D4 (Deng *et al.,* 2016). Additionally, several proteins belonging to the glyoxalase/bleomycin resistance protein/ dioxygenase superfamily have also been shown to be involved in the metabolism of aromatic compounds in *Bacillis subtilis* (Tam *et al.*, 2006; Duy *et al.*, 2007). Interestingly, the genes encoding for these three proteins appear to be located within the same genomic region of *Microbacterium* C448 (Figure 8). Also within this region is the gene encoding for a transcriptional regulator belonging to the GntR family (UniProt ID: W0ZB11) (Table 7; Figure 8). GntR regulators are commonly involved in the regulation of bacterial aromatic degradation pathways in a wide variety of bacteria (Gerischer 2002; Tropel and van der Meer, 2004). The close proximity of these genes could suggest the presence an operon that is involved in the degradation of SMZ and other sulfonamide antibiotics.

Another protein of interest identified in this analysis is fructose, 1-6-bisphosphatase (UniProt ID: W0Z7P4), which showed a 9-fold increase in expression in response to SMZ (Table 7). Previous work done by Qi *et al.* (2007) demonstrated that fructose-1,6-bisphosphatase (Fbp) was necessary for the degradation of aromatic compounds in gram positive bacteria *Corynebacterium glutamicum.* In the Qi et al. study, the ability of *C. glutamicum* to degrade aromatic compounds was lost in Fbp knockout strains, but restored when a construct containing the Fbp gene was reintroduced into the bacterium (Qi *et al.*, 2007). They were also able to identify this protein as a bridge between peripheral and central metabolism (Qi *et al.*, 2007). A BLAST comparison of the C448 Fbp enzyme against that of *C. glutamicum* revealed that there is a 59% sequence similarity between the two enzymes. Furthermore, this enzyme was also found to have

60% sequence similarity with the Fpb enzyme in the sulfadiazine degrading bacterium *Arthrobacter* sp. D4 (Deng *et al*., 2016).

The C-terminal domain of the acyl-coA dehydrogenase (UniProt ID: W0Z5L8) protein was also found to be upregulated in response to SMZ in C448 (Table 7). This protein was previously identified in a pull through assay where C448 lysates were passed over a SMZ bound column and acyl-coA dehydrogenase was the only protein that bound to the SMZ molecule (R. Marti, unpublished data). Furthermore, the gene for this protein appears to be disrupted by an insertion in the degradation deficient mutant *Microbacterium* C544. Additionally, this protein shares 96% and 78% sequence similarity to hypothetical proteins in sulfonamide degrading *Arthrobacter* species D2 and D4 respectively (Deng *et al.*, 2016).

Additionally, the increase in abundance of the universal stress protein A (Uniprot code: W0Z8X7) suggests that SMZ exposure promotes a bacterial stress response in C448 (Table 7). Proteins belonging to the universal stress protein A (UspA) family are usually upregulated in response to different types of stressors including high temperatures, cell cycle arrest, oxidative stress or cellular starvation (Dowds, 1994; Kvint *et al.,* 2003; Nanchin *et al.,* 2005; Jenkins *et al.,* 2011). These UspA proteins also protect against oxidative damage in *Listeria monocytogenes* (Gomes *et al.,* 2011) and *Bacillis subtilis* (Dowds, 1994).

5 Conclusions and future perspectives

Overall this study was able to shed some light on the sulfonamide degradation pathway in C448. The pathway was shown to be induced in response to SMZ, and was influenced by the concentration of different nutrient sources in the culture environment. SMZ degradation was inhibited by methionine, but stimulated in the presence of sucrose, NH_4Cl, and glutamate. From a potential bioremediation perspective, understanding how the presence of these nutrients in manures and soils influence the amount of SMZ degradation would be useful for determining what combinations of nutrient sources in manures, agricultural soils and surface waters would maximize SMZ degradation in these environments. Based on the results presented here, an environment that is either low in methionine or high in sucrose, ammonium or glutamate would increase SMZ degradation. While the concentrations of methionine in the dairy and swine manures used in this study was drastically lower than the concentrations that were found to have an effect on the pathway (Table 6), other organic sulfur compounds in the form of sulfonates, sulfate esters, and are commonly found in the environment (Kertesz & Mirleau, 2004) and it would be worthwhile to look into how SMZ degradation would be influenced by the presence of these compounds.

One question raised by these results would be how a combination of exogenous carbon, nitrogen and sulfur sources nutrients would influence the activity of the degradation pathway. This study only looked at how the concentrations of each nutrient source individually influenced SMZ degradation, but this would not be the case in a real world setting where bacteria have access to a combination of various carbon, nitrogen and sulfur sources. A study by Farrell *et al.*, found that high concentrations of organic carbon was associated with a decrease in the rate of organic nitrogen uptake in bacteria (2014). Given the fact that SMZ is an organic source of nitrogen, it would be interesting to explore the relationship between carbon content and SMZ uptake in C448.

The comparative proteomic analysis done in this study identified several potential candidate proteins for the SMZ degradation pathway (Table 7). Moving forward, the next logical step would be to conduct a function analysis in order to confirm their involvement in the pathway. One way this could be done is by generating gene knockout strains of C448 for each of the selected protein genes, and looking at how the degradation phenotype is affected would be affected.

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7 CURRICULIM VITAE

Post-secondary Education

Graduate Degree MSc in Biology (2015 – Present)
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Undergraduate Degree BSc, Hon. Spec. in Genetics and Biochemistry (2010 – 2015)
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Student Research Assistant (2013-2015)
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Scholarships and Awards

Queen Elizabeth Aiming for the Top Scholarship (2010, 2011, 2012, 2014)
CIS Academic All-Canadian (2011, 2012)
Leib Pillersdorf Award for Academic Excellence (2010, 2011)
Western Scholarship of Distinction (2010)

Conference Presentations

Malcolm, T,* Renaud, J. and Topp, E. (2017). Regulation of sulfonamide antibiotic biodegradation by *Microbacterium* sp. Strain C448. Poster presented at the Canadian Society of Microbiologists 68th Annual Conference, Waterloo, ON