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NMR Metabolomic Analysis of Bacterial Resistance Pathways Using Multivalent Quaternary Ammonium Functionalized Macromolecules

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Abstract

Introduction Multivalent antimicrobial dendrimers are an exciting new system that is being developed to address the growing problem of drug resistant bacteria. Nuclear Magnetic Resonance (NMR) metabolomics is a quantitative and reproducible method for the determination of bacterial response to environmental stressors and for visualization of perturbations to biochemical pathways.
Objectives NMR metabolomics is used to elucidate metabolite differences between wild type and antimicrobially mutated *Escherichia coli* (*E. coli)* samples.
Methods Proton (^{1}H) NMR hydrophilic metabolite analysis was conducted on samples of *E. coli* after 33 growth cycles of a minimum inhibitory challenge to *E. coli* by poly(amidoamine) dendrimers functionalized with mannose and with C_{16}-DABCO quaternary ammonium endgroups and compared to the metabolic profile of wild type *E. coli*.
Results The wild type and mutated *E. coli* samples were separated into distinct sample sets by hierarchical clustering, principal component analysis (PCA) and sparse partial least squares discriminate analysis (sPLS-DA). Metabolite components of membrane fortification and energy related pathways had a significant p-value and fold change between the wild type and mutated *E. coli.* Amino acids commonly associated with membrane fortification from cationic antimicrobials, such as lysine, were found to have a higher concentration in the mutated *E. coli* than in the wild type *E. coli*. *N*-acetylglucosamine, a major component of peptidoglycan synthesis, was found to have a 25-fold higher concentration in the mid log phase of the mutated *E. coli* than in the mid log phase of the wild type.
Conclusion The metabolic profile suggests that *E. coli* change their peptidoglycan composition in order to garner protection from the highly positively charged and multivalent C_{16}-DABCO and mannose functionalized dendrimer.

Keywords Metabolomics · Quaternary ammonium compounds · Nuclear magnetic resonance · Dendrimers · DABCO · Antibiotic Resistance

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1 Introduction

New infectious diseases with novel drug targets emerge every year, and the occurrence of bacterial resistance to existing antibiotics is increasing at the same time that the number of new antibiotics produced per year is decreasing. This, in conjunction with a large quantity of bacteria in hospitals having at least one antibiotic that is ineffectual, is a critical global health concern (Mintzer et al. 2011; Alanis 2005; Martinez and Baquero 2000; Xue et al. 2013; Gonzáles-Bello 2017). Many of these multidrug resistant bacteria are gram negative (Hoerr et al. 2016; Gonzáles-Bello 2017), including strains of *Escherichia coli* (*E. coli*) (Gonzáles-Bello 2017).

E. coli is a great microbial on which to study resistance pathways for new antimicrobial agents due to the extensive genetic and metabolic studies that have been previously reported (Gonzáles-Bello 2017, Hoerr et al. 2016). In addition, *E. coli* has a short generation time, is easily mutable, is a commonly occurring infection and has a thin cell wall. The most common processes used by *E. coli* for antibiotic resistance include random beneficial genetic mutations, horizontal gene transfer, and strengthening of the cell wall and basic defense systems (Denamur and Matic 2006; Todar 2012; Gonzáles-Bello 2017; Anderson et al. 2012; Hoerr et al. 2016). *E. coli* have a beneficial mutation rate of 2 $x10^{-9}$ per genome per replication due to random genetic mutations (Denamur and Matic 2006; Martinez and Baquero 2000; Gonzáles-Bello 2017). Horizontal gene transfers occur due to the so far incurable habit of bacterial DNA uptake from the environment, swapping plasmids with other bacteria, and virus phage DNA injection. Bacterial communities that are antibiotic resistant can even shield antibiotic susceptible bacteria of different species from certain types of antibiotics (Perlin et al. 2009). Bacteria can also become antibiotic resistant by using efflux pumps, antibiotic degrading or altering enzymes, and alterations to their peptidoglycan layer composition (Martinez and Baquero 2000, Humann and Lenz 2009, Shepard and Ibba 2013, Todar 2012; Anderson et al. 2012).

Peptidoglycan, which is the main component in bacterial membranes, is composed of an alternating chain of *N*-acetylglucosamine and *N*-acetylmuramic acid interlinked with pentapeptide side chains. Changes to the peptidoglycan layer are known to change the permeability of the membrane and to alter the pattern of noncovalent interactions that are achieved by antibiotics interacting with the cell surface (Anderson et al. 2012; Shepard and Ibba 2013). For example, glycine is a common amino acid used to crosslink the peptidoglycan (Shepard and Ibba 2013; Hammes et al. 1973), and decreased amounts of crosslinking have been correlated with a decrease in bacterial survival rates (Loskill et al. 2014). Aminoacyl-tRNAs are redirected from protein synthesis to peptidoglycan synthesis in order to change the pentapeptide side chains, thereby protecting the bacteria against binding by environmental stressors such as antibiotics (Shepard and Ibba 2013). Generally, the pentapeptide side chain amino acids are switched out in order to change membrane fluidity, permeability, crosslinking, charge, and hydrophobicity to improve survivability (Loskill et al. 2014; Shepard and Ibba 2013; Humann and Lenz 2009; Anderson et al. 2012). Like genetic mutation, these defense mechanisms greatly increase the rate of antibiotic resistance in bacteria and decrease the number of currently effective antibiotics (Martinez and Baquero 2000; Gonzáles-Bello 2017; Anderson et al. 2012).

Antibiotics designed using new scaffolds and directed at previously unaccessed bacterial metabolic pathways are needed because bacteria may be less likely to develop resistance to them. In addition, new antibiotics that allow for administration of a therapeutic cocktail that simultaneously targets multiple bacterial pathways could increase efficacy and decrease resistance. In particular, scaffolds that allow antibiotics to function multivalently are attractive because they can take advantage of multiple individual active units attached to a central core, thus concentrating several of the antibiotic moieties in close proximity. Dendrimers are an appealing multivalent scaffold because they are highly modular; the size of the dendrimer can be easily changed by changing dendrimer generation, and a variety of antibiotics in varying densities can be used (Mintzer et al. 2011; Paleos et al. 2010; Vembu et al. 2015; Chen et al. 2000; Lu and Pieters 2019). Poly(amidoamine) (PAMAM) dendrimers can be functionalized with carbohydrates, amino acids, antibiotics and other bioactive groups (Cloninger 2002; Andre et al. 2001; Roy 2003). Carbohydrate functionalized PAMAM dendrimers, i.e. glycodendrimers, have been shown to have higher activity than their carbohydrate monomer counterparts (Wolfenden et al 2015; VanKoten et al. 2016; Andre et al. 2001) and have been shown to disrupt bacterial activity (Roy 2003; Andre et al. 2001; Chabre and Roy 2008; Mintzer et al. 2011; VanKoten et al. 2016). Functionalization of the carbohydrate endgroups with an antibiotic has provided significantly

higher activity and a greater challenge for bacterial resistance than their monomer counterparts (García-Gallego et al. 2017; Wrońska et al. 2019; Mintzer et al. 2011; Chen et al 2000).

Quaternary ammonium compounds (QAC) are used as disinfectants and surface surfactants because of their antimicrobial properties (Engel et al. 2011; McDonnell and Russell 1999; Mintzer et al. 2011; Sreeperumbuduru et al. 2016; Jiao et al. 2017). QACs have a hydrophobic portion and a positively charged hydrophilic ammonium group. The large positive charge of the QACs disrupts and/or lyses the bacterial phospholipid membrane (McDonnell and Russell 1999; Mintzer et al. 2011; Sreeperumbuduru et al. 2016; Jiao et al. 2017). The QAC studied in this report is a 1,4-diazabicyclo-2,2,2-octane (DABCO) (Pokhrel et al. 2004) with a 16-carbon chain (C_{16}-DABCO). DABCO has been shown to be affective against both gram positive and negative microbes (Sreeperumbuduru et al. 2016). C_{16}-DABCO is attached to a mannose functionalized PAMAM dendrimer (Fig. 1). The C_{16}-DABCO and mannose functionalized dendrimer (DABCOMD) multivalently presents the positively charged ammonium units, and VanKoten *et al.* (2016) conducted a minimum inhibition study using DABCOMD to determine the minimum inhibitory concentration (MIC) against both positive and negative strains of bacteria. After challenging *E. coli* with DABCOMD for 33 growth cycles, DABCOMD showed 100-fold increased antimicrobial potency relative to the monomeric C_{16}-DABCO on a per active unit basis (VanKoten et al. 2016). The *E. coli* in the MIC study became very resistant to small molecule inhibitors including Ampicillin and monomeric DABCO, while the MIC remained almost unchanged for DABCOMD. Because of the effectiveness of DABCOMD in MIC assays coupled with a lack of development of resistance over many growth cycles, a comparison of the *E. coli* before and after exposure to DABCOMD for 33 growth cycles was performed using metabolomics.

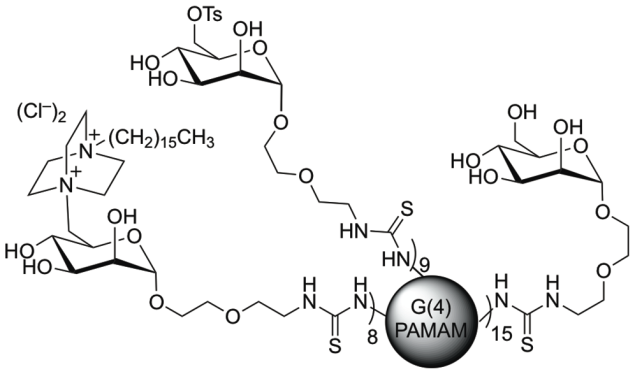

Fig. 1 Structure of the C_{16}-DABCO Mannose Functionalized Dendrimer (DABCOMD)

Metabolomics is the study of the small molecules (metabolites) produced in cellular metabolism. Metabolites are easily affected by the environment of the organism and can be used to distinguish phenotypes (Shulaev 2006; Barding et al. 2012; Hoerr et al. 2016). Metabolomics is a quantitative analysis which can be used to assess metabolite changes between wild type and mutants, making metabolomic profiling an ideal technique for studying the complex relationship that exists between an organism's metabolic pathways and its environment (Shulaev 2006; Barding et al. 2012; Dettmer et al. 2007; Hoerr et al. 2016; Lämmerhofer and Weckwerth 2013; Lindon et al. 2007). Since Nuclear Magnetic Resonance (NMR) metabolomics is a reliable and quantitative method, this technique is an important method for comparing the biochemical byproducts of mutated and wild type metabolism (Hoerr et al. 2016; Dettmer et al. 2005; Ammons et al. 2014). Thus, the goal for this research project was to determine how *E. coli* develop resistance to multivalent DABCOMD using proton (^{1}H) NMR hydrophilic metabolomics. The results reported herein were obtained using DABCOMD with *E. coli* in order to improve our understanding of the main resistance pathways and key mutations expected for bacterial systems upon exposure to multivalent antimicrobial compounds.

2 Methods

2.1 Samples

The FDA Strain Seattle 1946 *E. coli* (ATCC 25922) was used as the wild type, and the mutated samples were prepared as described in VanKoten et al. 2016. Four sample types were used: wild type mid log phase (WT ML), wild type stationary phase (WT S), mutant mid log phase (Mut ML) and mutant stationary phase (Mut S). Samples were grown, collected, and standardized as described in the Supplementary Material. A brief overview of procedures is show in Fig 2.

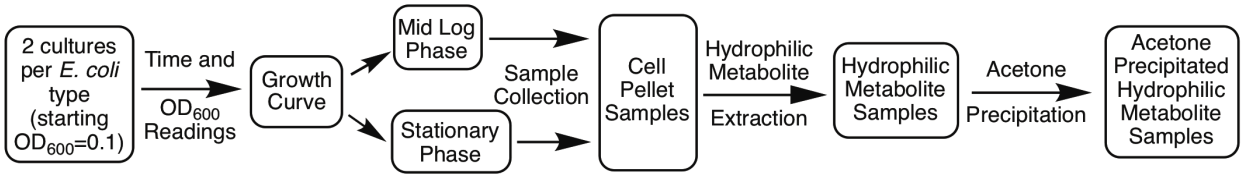

Fig. 2 Overview of procedures from culture to extracted hydrophilic metabolites ready for NMR buffer

2.2 Metabolite Extraction Procedures

Hydrophilic Extraction

The cell pellets were thawed on ice. Cold methanol (MeOH) (800 µL) and 170 µL of Millipore H_2O were added to each sample test tube and vortexed. The cells were sonicated for 5 min then vortexed. Cold chloroform (800 µL) and 400 µL of cold H_2O were added to each test tube. The samples were vortexed and incubated on ice for 15 min. The test tubes were centrifuged at 3,500 rpms for 15 min. The aqueous layer was transferred to a sterile Eppendorf tube and concentrated on a speed vacuum until dry. The samples were frozen at -80 ºC (Ammons et al. 2014 and 2015 and references therein).

Acetone Precipitation

The samples were thawed on ice. The pellets were suspended in 250 µL D_2O and 1,250 µL Acetone. The samples were frozen at – 80 ºC overnight. The samples were thawed on ice. They were cloudy with precipitated proteins once thawed. The samples were centrifuged at 2,000 rpms for 30 min. The protein pellet was discarded. The speed vacuum was used to dry the metabolite samples. The metabolite pellet was frozen at –80 ºC (Ammons et al. 2014 and 2015 and references therein).

2.3 NMR Sample Preparation and Data Processing

Note: All D_2O used was NMR grade.

NMR Buffer Preparation

An imidazole stock solution was made by adding 27.230 g of imidazole to 4.0 mL of D_2O. 3-(Trimethylsilyl)propane-1-sulfonate sodium salt (DSS) (21.23 mg) was added to 4.0 mL of D_2O to make a DSS stock solution. In 5.0 mL of D_2O, 0.345 g $NaH_2PO_4 \cdot H_2O$, 0.355 g Na_2HPO_4 (anhydrous) and 0.040 g NaN_3 were dissolved to create a $NaPO_4$ buffer stock solution. A 30 mL D_2O buffer solution was made by combining 300 µL of the DSS stock solution, 60 µL of the imidazole stock solution, 1,500 µL of the $NaPO_4$ buffer stock solution, and 28,140 µL of D_2O (Ammons et al. 2014 and 2015 and references therein).

Sample Preparation

The metabolite samples were placed on ice, and 700 µL of the NMR buffer was added. Three of the same metabolite pellet types were combined into one 700 µL NMR buffer solution. The samples were gently vortexed then transferred to a capped NMR Tube.

Data Acquisition

The samples were acquired using a Bruker DRX 600 MHz NMR. ^{1}H NMR spectra were acquired using Topspin as the data acquisition program. The solvent was set as D_2O, and the number of scans per sample was set to 64. The receiver gain and shimming were checked to ensure they were correct. The spectra were Fourier transformed and phased using the H_2O and DSS resonances.

Data Analysis

The spectra were processed and profiled using Chenomx NMR Suite 8.2 software (Chenomx 2016). Later, the same data was chosen at random to profile again to check for errors. The concentrations of the identified metabolites in each sample were standardized to the concentration of the starting sample. XLSTAT (Addinsoft 2019) was used for hierarchical clustering and for principal component analysis (PCA) for each sample type and corresponding standardized metabolite concentrations. The metabolites and their concentration data were transformed into comma delineated form (CSV). The CSV data were uploaded to the MetaboAnalyst website version 4.0 for statistical analysis (Chong et al 2019 and references therein). A volcano plot of p-value versus fold change and sparse partial least squares discriminate analysis (sPLS-DA), Pattern Hunter and the very important features (VIP) data were used to determine relationships among statistically significant metabolite changes. sPLS-DA was used to eliminate outliers in each sample type. The list of metabolites generated for each sample type was used in the MetaboAnalyst Pathway Analysis tool and using KEGG pathways (Kanehisa and Goto 2000 and references therein) in order to identify altered metabolomic pathways in the mutants in response to the DABCOMD.

3 Results

E. coli (ATCC 25922, denoted WT for wild type) were mutated in the presence of C_{16}-DABCO and mannose functionalized dendrimers (DABCOMD) for 33-growth cycles (denoted Mut for mutant) as previously described by VanKoten *et al.* 2016. The hydrophilic metabolites were extracted from the mid log and stationary phases for both the wild type and mutant samples as summarized in Fig. 2 (Ammons et al. 2014 and 2015 and references therein). ^{1}H NMR spectra of the hydrophilic metabolites for the mid log and stationary phases of both the wild type and mutant samples were obtained. Fig. 3a displays an example spectrum for each sample type. Chenomx NMR Suite 8.2 software was used to identify and quantify the hydrophilic metabolites present in each sample. These metabolite identities and their concentrations for each sample type were entered into MetaboAnalyst 4.0 for sPLS-DA analysis; sPLS-DA was used to eliminate outliers in each sample type. Wild type stationary and mutant stationary phases each contained eight nonoutlier samples. Wild type mid log phase contained eleven nonoutlier samples, whereas the mutant mid log phase contained nine nonoutlier samples. The metabolite identities and concentrations were analyzed using XLSTAT Hierarchical Clustering, from which the number of unique sample types was determined and the data were classified accordingly. XLSTAT Hierarchical Clustering grouped the four sample types into their own classes as shown in Fig. 3b. None of the samples from the mid log or stationary phases had overlapping data sets, and the mutant and wild type sample sets were also separated into their own nonoverlapping classes. This demonstrates that the wild type mid log samples, the wild type stationary samples, the DABCOMD mutated mid log samples, and the DABCOMD mutated stationary samples all form unique sample sets.

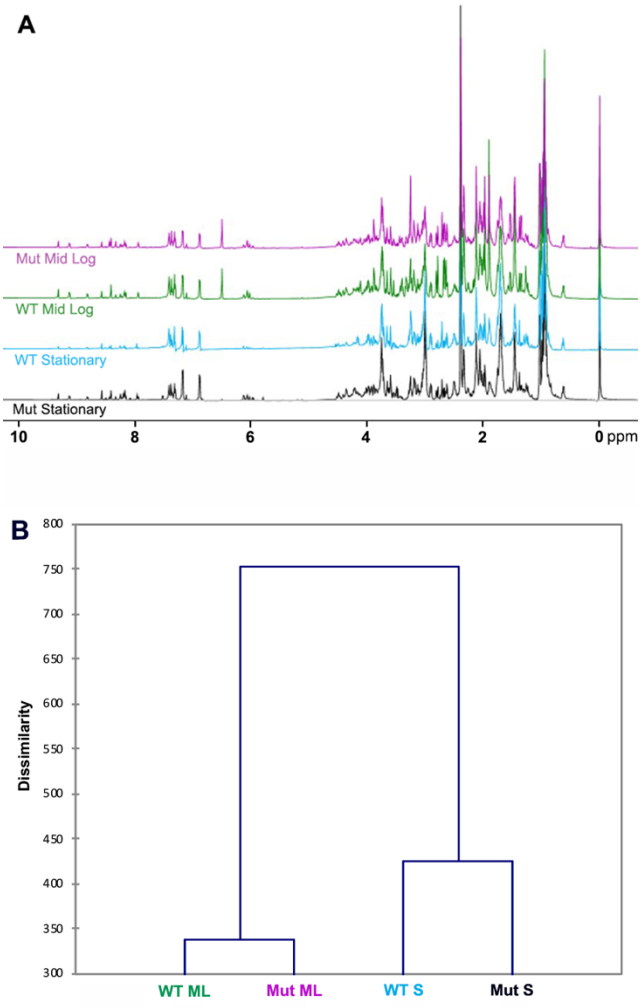

Fig. 3 A An example ^{1}H NMR spectrum for each sample type. **B** Hierarchical Clustering indicating that there are 4 unique sample types. No overlap between sample types was observed

The name of the metabolite and its concentration for each sample were entered into XLSTAT and MetaboAnalyst 4.0 to determine sample separation, metabolite-to-sample correlation, and the statistical significance for each metabolite. To determine the separation of sample types and the metabolite indicators for each sample type, multivariate statistical analyses were performed using sPLS-DA 2D and 3D plots and a PCA biplot (Fig. 4). Fig. 4a and 4b show that each sample type has significant separation on each axis, accounting for 54.2% and 67.4% of the variation. This indicated that only the first two components needed to be considered for separation and analysis. The PCA biplot where 56.0% of the variation was accounted for in the first two factors as shown in Fig. 4c, also indicated that consideration of the first two components was sufficient. Factor 1 separated the wild type from the mutant and factor 2 separated the mid log from the stationary phase. Fig. 4c shows which metabolites are indicative of each sample type. *N*-acetylglucosamine, for example, was indicative of the DABCOMD mutated samples due to its significantly higher concentration in the samples from mutated *E. coli* than in the samples from wild type *E. coli*.

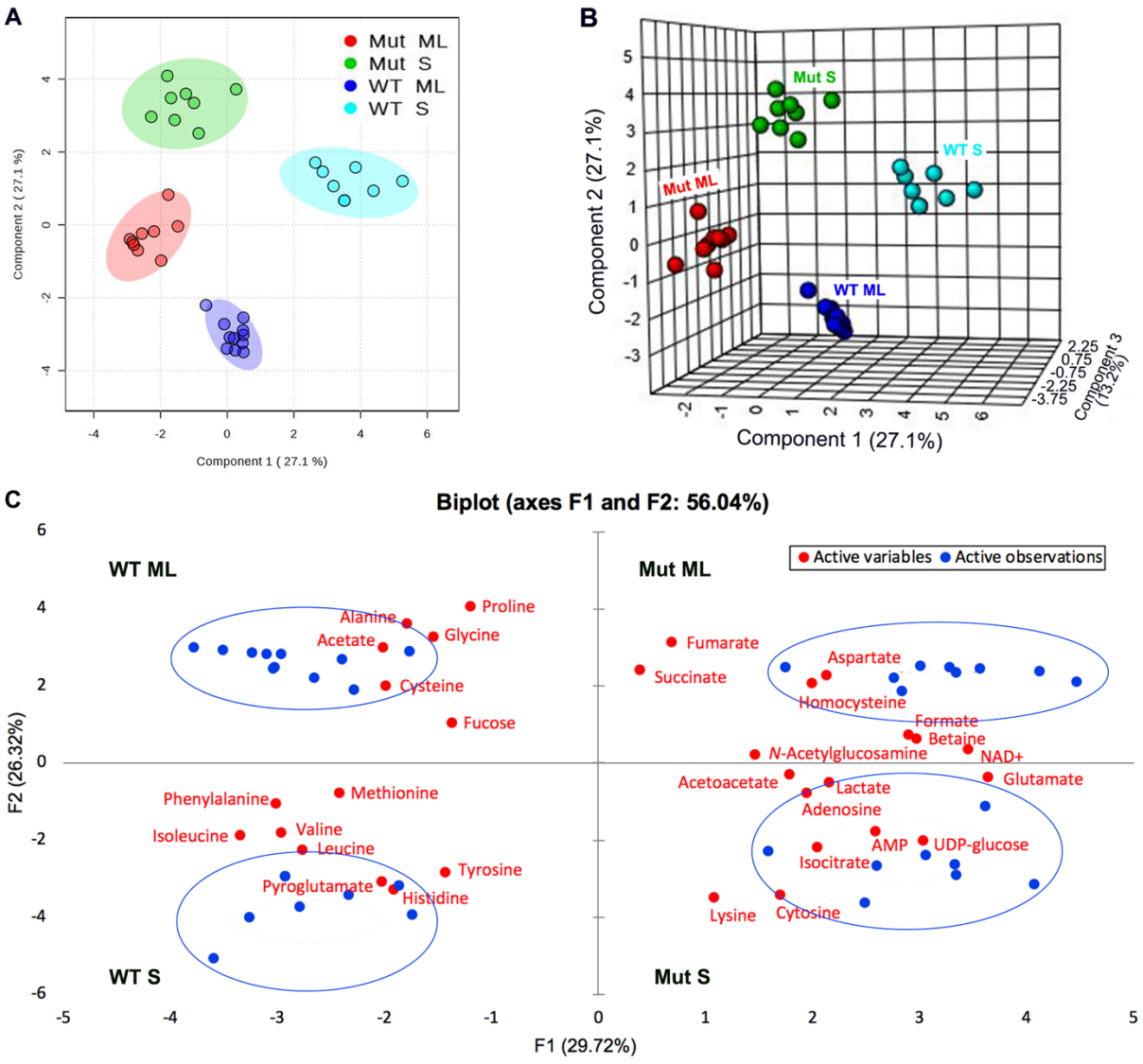

Fig. 4 A 2D sPLS-DA and **B** 3D sPSL-DA plots demonstrate that each sample type can be separated into a distinct group without overlap. **C** PCA biplot shows the sample type separation (blue) and which metabolites are the most indicative of each sample type (red)

The data from each phase was entered into MetaboAnalyst 4.0 to generate a volcano plot as shown in Fig. 5. The volcano plot marks as significant every metabolite with a p-value of 0.05 or less and a fold change of 1.5 or higher. Fig. 5a shows that cysteine, glycine and histidine are higher in concentration in the wild type mid log phase than in the mutant mid log phase, whereas all other statistically significant metabolites were in higher concentrations in the mutant mid log phase than in the wild type mid log phase. *N*-acetylglucosamine had a 25-fold increase in the samples from the mutant mid log phase as compared to the wild type mid log samples. The statistically significant variations in metabolite concentrations were more evenly spread between the stationary phases of the wild type and mutant samples, as shown in Fig 5b. Fig. 5c and 5d display the very important features plot (VIP scores) determined by the PSL-DA. Metabolites with high VIP scores are highly correlated to other metabolites. Interestingly, lysine appeared in the VIP scores but not in the volcano plot because the volcano plot had a cut off at 1.5-fold change whereas lysine only had a 1.3-fold change. By using the VIP scores in conjunction with the other statistical analyzes, important metabolites that have been shown in previous work with antimicrobials were revealed.

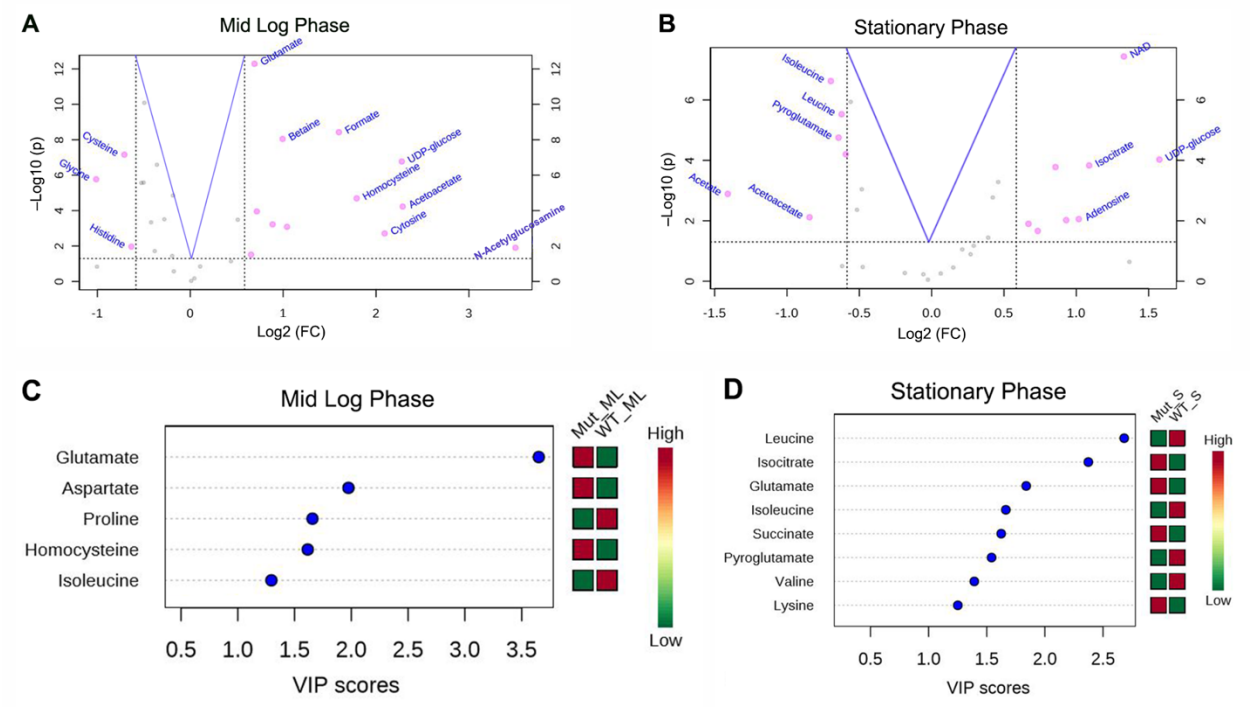

Fig. 5 Volcano plots displaying metabolites with a significant p-values and fold changes for **a** the mid log phase and **b** the stationary phase. Important features (VIP) scores from the PLS-DA for important metabolites and their corresponding concentrations in **c** the mid log phase and **d** the stationary phase

The pathways that are likely involved with the statistically significant metabolites during their mid log and stationary phases are listed in Table 1. Pattern Hunter and Pathway Analysis on MetaboAnalyst 4.0 aided in determining the most likely pathways involved with several of the statistically significant metabolites. Pattern Hunter was used to ensure significance and correct correlation among the metabolites in a given pathway. For example, histidine was found to be a statistically significant metabolite in our studies. Histidine has an important role in aminoacyl-tRNA biosynthesis, yet it did not correlate to the other metabolites in that pathway and was therefore omitted from Table 1. Further studies are needed to determine the pathways involved in histidine's fold change. Lysine, on the other hand, showed only a 1.3-fold increase in the mutant stationary phase relative to the wild type stationary phase, and Pattern Hunter showed a positive correlation with aspartate, glutamate, and AMP and a negative correlation with Cysteine, glycine, isoleucine and proline. Thus, the direction of fold change and corresponding metabolites for lysine correctly correlated with additional metabolite data provided in Table 1.

Table 1 Fold change of statistically significant metabolites in their corresponding metabolic pathway for the mid log and the stationary phase.

	Mid Log Phase		Stationary Phase	
Indicated Pathway	**Metabolite (CAS #)**	**Fold Change[a,b]**	**Metabolite (CAS #)**	**Fold Change**
Citric Acid Cycle	Fumarate (142-42-7)	+1.5	Succinate (110-15-6)	+1.9
	NAD^+ (53-84-9)	+1.6	Isocitrate (320-77-4)	+2.1
			NAD^+ (53-84-9)	+2.4
Pyruvate Metabolism	Acetate (71-50-1)	-2.7	Acetate (71-50-1)	-2.7
Aminoacyl-tRNA Biosynthesis	Lysine (56-87-1)	+1.3	Lysine (56-87-1)	+1.3 (VIP)
	Aspartate (56-84-8)	+1.3 (VIP)	Glutamate (56-86-0)	+1.3 (VIP)
	Glutamate (56-86-0)	+1.6 (VIP)	Glycine (56-40-6)	+1.8
	AMP (61-19-8)	+1.8	AMP (61-19-8)	+1.6
	Cysteine (54-90-4)	-1.5	Leucine (61-90-5)	-1.6 (VIP)
	Glycine (56-40-6)	-2.0	Isoleucine (73-32-5)	-1.6 (VIP)
	Isoleucine (73-32-5)	-1.4 (VIP)	Valine (72-18-4)	-1.4 (VIP)
	Proline (609-36-9)	-1.5 (VIP)		
Peptidoglycan Synthesis	*N*-acetyl (7512-17-6) glucosamine	+25		
	Lactate (50-21-5)	+2.4		
	Glutamate (56-86-0)	+1.6		

[a]A positive fold change is indicative of a higher concentration in the mutant. [b]The metabolites with VIP next to them were determined to be very important features by the PSL-DA.

4 Discussion

Multiple metabolites that are likely to be involved in energy related pathways and cell membrane composition had both a significant p-value and fold change. The most significant concentration changes between the wild type and the mutant were common components of peptidoglycan synthesis, which is a critical component of the cell membrane. *N*-acetylglucosamine had the largest observed fold change, which was a 25-fold increase in concentration in samples from the mutant compared to those from the wild type during the mid log phase. Many likely components of aminoacyl-tRNA biosynthesis significantly changed in concentration when comparing mutant to wild type in both the mid log and the stationary phases. One likely explanation for the observed concentration changes is a diversion from aminoacyl-tRNA biosynthesis to peptidoglycan synthesis. Common amino acid substitutes found in the peptidoglycan pentapeptide side chain, such as lactate, glutamate and lysine had significantly higher concentrations in the mutant than the wild type. Lysine has been shown to lower the permeability of the cell membrane to cationic molecules (Shepard and Ibba 2013) because this amino acid inserts positive charge on the membrane to reduce the attraction of the cationic antimicrobial to the membrane. Since the polyalanine tail of the peptidoglycan pentapeptide side chain is a common target for antibiotics, bacterial mutants commonly switch the last alanine amino acid of the pentapeptide side chain for a different amino acid such as lactate (Mengin-Lecreulx et al. 1994; Humann and Lenz 2009; Anderson et al. 2012).

The mutant samples revealed a 2-fold decrease in glycine for the mid log phase and a 1.5-fold increase in glycine in the stationary phase when compared to wild type. Glycine is involved in crosslinking of the peptidoglycan, and crosslinking increases the rigidity of the membrane structure (Loskill et al. 2014). A possible explanation for the significant fold change reversal for glycine is due to cells rapidly dividing in the mid log phase and peptidoglycan crosslinking occurring to a lesser extent. When bacteria are in the stationary phase, they are expected to settle into a configuration that provides higher survivability, and the extra rigidity of the cell membrane could be less of a hindrance to normal functioning (Humann and Lenz 2009; Mengin-Lecreulx et al. 1994). Homocysteine levels were 4-fold higher and cysteine levels were 1.5 lower in the mutant than the wild type. Their intermediate, cystathionine,

can be utilized in peptidoglycan synthesis (Mengin-Lecreulx et al. 1994). When cystathionine is added to media, bacteria uptake and incorporate it into their peptidoglycan layer (Mengin-Lecreulx et al. 1994). This truncates the pentapeptide side chain by excluding the D-ala-D-ala tail. Lacking a polyalanine tail can provide protection against some antimicrobials (Loskill et al. 2014; Sheppard and Ibba 2013; Anderson et al. 2012). Thus, the *E. coli* grown in the presence of DABCOMD most likely changed their peptidoglycan composition to aid in repelling and protecting against this cationic antimicrobial, and this explains the shifting levels of cysteine and homocysteine.

The synthesis of extra components for the peptidoglycan layer in the mutant would be expected to be energetically costly for the mutant bacteria. Acetate, an energy-related metabolite associated with pyruvate and the citric acid cycle, had a lower concentration in the mutant than the wild type. Spent energy-related metabolites such as NAD^+ and AMP, on the other hand, were present in higher concentrations in the mutant than in the wild type. Likely components of both the citric acid cycle and aminoacyl-tRNA biosynthesis showed statistically significant changes in the concentrations of metabolites in both the mid log and stationary phases. The mutant *E. coli* most likely required more energy-related metabolites, yet they were still able to compensate for their mutations and obtain the same growth density and comparable growth curves as the wild type.

Quaternary ammonium antimicrobial compounds induce the formation of a hole in the bacterial membrane (Sreeperumbuduru et al. 2016; McDonnell and Russell 1999; Jiao et al. 2017). The DABCOMD studied here, which bears multiple quaternary ammonium groups per macromolecule, is a particularly effective quaternary ammonium antibacterial agent because the dendrimer framework brings multiple cationic groups into close proximity and thereby enforces multiple simultaneous cation/membrane interactions at the cell surface. The results of this ^{1}H NMR metabolomics study reveal that *E. coli* grown in the presence of DABCOMD show a significant increase in metabolites most likely associated with peptidoglycan component synthesis, which is critically important for strengthening the bacterial membrane. Changes in energy related pathways were also observed, indicating that the mutated bacteria were required to work harder in order to maintain the same level of function as wild type *E. coli*.

5 Conclusion

Novel antimicrobials are needed to combat the increasing occurrence of antibiotic resistant bacteria, and multivalency has been shown to improve the effectiveness of antimicrobial compounds. NMR metabolomics is a reliable and quantitative method to study the effect of antimicrobials on bacterial biochemical pathways. Here, NMR hydrophilic metabolomics was used to study the metabolic changes associated with mutation of *E. coli* by DABCOMD, a multivalent quaternary ammonium compound. Mutated and wild type samples were easily separated by hierarchical clustering, PCA and sPLS-DA analyses. Common components of energy pathways and peptidoglycan synthesis were found to have significant p-values and fold changes when comparing the wild type *E. coli* to the mutated *E. coli* in both the mid log and the stationary phases. Lysine, an amino acid commonly associated with decreasing the effectiveness of cationic antimicrobials, was found to have a higher concentration in the mutant than in the wild type. The mutated *E. coli* also had a 25-fold increase in *N*-acetylglucosamine (a major component of the peptidoglycan) during the mid log phase. Overall, the NMR hydrophilic metabolite data indicates that *E. coli* are most likely altering their peptidoglycan composition to protect their membranes from the large positive charge found on the multivalent C_{16}-DABCO and mannose functionalized PAMAM dendrimers.

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Author Contributions MLA grew and collected bacterial culture samples, extracted the metabolites, prepared the samples for NMR spectral acquisition, processed the NMR data, performed data analysis and interpretation, and wrote the manuscript. MJC oversaw all aspects of the research project and co-wrote the manuscript.

Compliance with Ethical Standards

Conflict of interest Both authors declare that they have no conflicts of interest.
Research involving human and/or animal participants This article does not contain any studies with human and/or animal participants performed by either of the authors.

References

Addinsoft (2019) XLSTAT 2019: Data Analysis and Statistical Solution for Microsoft Excel. Paris, France.

Alanis, A. J. (2005) Review Article: Resistance to Antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research*, 36, 697-705.

Ammons, MC. B., Tripet, B. P., Carlson, R. P., Kirker, K. R., Gross, M. A., Stanisich, J. J., Copié, V. (2014) Quantitative NMR Metabolite Profiling of Methicillin-Resistant and Methicillin-Susceptible Staphylococcus aureus Discriminates between Biofilm and Planktonic Phenotypes. *Journal of Proteome Research*, 13, 2973-2985.

Ammons, MC. B., Morrissey, K., Tripet, B. P., Van Leuven, J. T., Han, A., Lazarus, G. S., Zenilman, J. M., Stewart, P. S., James, G. A., Copié, V. (2015) Biochemical Association of Metabolic Profile and Microbiome in Chronic Pressure Ulcer Wounds. *PLOS one*, 10(5), 1-22. doi:10.1371/journal.pone.0126735.

Anderson, R. J., Groundwater, P. W., Todd, A., Worsley, A. J. (2012). Microorganisms: In Antibacterial Agents: Chemistry, Mode of Action, Mechanisms of Resistance and Clinical Applications. (pp. 3-32). West Sussex, UK: Wiley.

Anderson, R. J., Groundwater, P. W., Todd, A., Worsley, A. J. (2012). Glycopeptide Antibiotics: In Antibacterial Agents: Chemistry, Mode of Action, Mechanisms of Resistance and Clinical Applications. (pp. 305-318). West Sussex, UK: Wiley.

Andre, S., Pieters R. J., Vrsaidas, I., Kaltner, H., Kuwabara, I., Liu, F-T., Liskamp, R. M. J., Gabius, H-J. (2001) Wedgelike Glycodendrimers as Inhibitors of Binding of Mammalian Galectins to Glycoproteins, Lactose Maxiclusters, and Cell Surface Glyconjugates. *ChemBioChem*, 2, 822-830.

Barding, G. A., Salditos, R., Larive, C.K. (2002) Quantitative NMR for Bioanalysis and Metabolomics. *Analytical Bioanalytical Chemistry*, 404, 1165-1179.

Chabre, Y., Roy, R. (2008) Recent Trends in Glycodendrimer Syntheses and Applications. *Current Topics in Medicinal Chemistry*, 8(14), 1237-1285.

Chen, C. Z., Beck-Tan, N. C., Dhurjati, P., Van Dyk, T. K., LaRossa, R. A., Cooper, S. L. (2000) Quaternary Ammonium Functionalized Poly(propylene imine) Dendrimers as Effective Antimicrobials: Structure-Activity Studies, *Biomacromolecules*, 1, 473-480.

Chenomx (2016) N.M.R. Suite 8.2. Edmonton: Chenomx Inc.

Chong, J., Wishart, D. S., Xia, J. (2019) Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocols in Bioinformatics*, 68, e86.

Cloninger, M. J. (2002) Biological Applications of Dendrimers. *Current Opinions in Chemical Biology*, 6, 742-748.

Denamur, E., Matic, I. (2006) Evolution of Mutation Rates in Bacteria. *Molecular Microbiology*, 60 (4), 820-827.

Dettmer, K., Aronov, P. A., Hammock, B. D. (2007) Mass Spectrometry-Based Metabolomics. *Mass Spectrometry Review*, 26, 51-78.

Engel, R., Ghani, I., Montenegro, D., Thomas, M., Klaritch-Vrana, B., Castaño, A., Friedman, L., Leb, J., Rothman, L., Lee, H., Capodiferro, C., Ambinder, D., Cere, E., Awad, C., Sheikh, F., Rizzo, JL., Nisbett, L-M., Testani, E., Melkonian, K. (2011) Polycationic Glycosides. *Molecules*, 16, 1508-1518.

García-Gallego, S., Franci, G., Falanga, A., Gómez, R., Folliero, V., Galdiero, S., Javier de la Mata, F., Galdiero, M. (2017) Function Oriented Molecular Design: Dendrimers as Novel Antimicrobials. *Molecules*, 22, 1581-1610.

Gonzáles-Bello, C. (2017) Antibiotic Adjuvants- A Strategy to Unlock Bacterial Resistance to Antibiotics. *Bioorganic and Medicinal Chemistry Letters*, 27, 4221-4228.

Hammes, W., Schleifer, K. H., Kandler, O. (1973) Mode of Action of Glycine on the Biosynthesis of Peptidoglycan. *Journal of Bacteriology*, 116(2), 1029-1053.

Humann, J., Lenz, L. L. (2009) Bacterial Peptidoglycan Degrading Enzymes and their Impact on Host Muropeptide Detection. *Journal of Innate Immunity*, 1, 88-97.

Hoerr, V., Duggan, G. E., Zbytnuik, L., Poon, K. K. H., Große, C., Neugebauer, U., Methling, K., Löffler, B., Vogel, H. J. (2016) Characterization and Prediction of the Mechanism of action of antibiotics through NMR Metabolomics. *BMC Microbiology*, 16(82), 1-14. doi:10.1186/s12866-016-0696-5

Jiao, Y., Niu, L-n., Ma, S., Li, J., Tay, F. R., Chen, J-h. (2017) Quaternary Ammonium-Based Biomedical Materials: State-of-the-Art, Toxicological Aspects and Antimicrobial Resistance. *Progress in Polymer Science*, 71, 53-90.

Kanehisa, M., Goto, S. (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acid Research*, 28, 27-30.

Lämmerhofer, M., Weckwerth, W., Eds, (2013) NMR-Based Metabolomic Analysis: In Metabolomics in Practice: Successful Strategies to Generate and Analyze Metabolic Data. (pp. 209-233). Weinheim, Germany: Wiley-VCH.

Lindon, J. C., Nicholson. J. K., Holmes, E. (2007). NMR Spectroscopic Techniques for Application to Metabonomics: In the Handbook of Metabolomics and Metabonomics. (pp. 55-112). Oxford, UK: Elsevier.

Loskill et al. (2014) Reduction of the Peptidoglycan Crosslinking Causes a Decrease in the Stiffness of the *Staphylococcus aures* Cell Envelope. *Biophysical Journal*, 107, 1082-1089.

Lu, W., Pieters, R. J. (2019) Carbohydrate-Protein Interactions and Multivalency: Implications for the Inhibition of Influenza A Virus Infections. *Expert Opinion on Drug Discovery*, 14(4), 387-395.

Martinez, J. L., Baquero, F. (2000) Minireveiw: Mutation Frequency and Antibiotic Resistance. *Antimicrobial Agents and Chemotherapy*, 44(7), 1771-1777.

McDonnell, G., Russell, A. D. (1999) Antiseptics and Disinfectants: Activity, Action and Resistance. *Clinical Microbiology Review*, 12(1), 147-226.

Mengin-Lecreulx, D., Blanot, D., VanHeijenoort, J. (1994) Replacement of Diaminopimelic acid by Cystathionine or Lanthionine in the Peptidoglycan of *Escherichia coli*. Journal of Bacteriology, 176(14), 434-437.

Mintzer, M. A., Dane, E. L., O'Toole, G. A., Grinstaff, M. W. (2011) Exploiting Dendrimer Multivalency to Combat Emerging and Reemerging Infectious Diseases. *Molecular Pharmaceutics*, 9, 342-354.

Paleos, C. M., Tsiourvas, D., Sideratou Z., Tziveleka, L-A. (2010) Drug Delivery using Multifunctional Dendrimers and Hyperbranched Polymers. *Expert Opinion on Drug Delivery*, 7, 1387-1398.

Perlin, M. H., Clark, D. R., McKenzie, C., Patel, H., Jackson, N., Kormanik, C., Powell, C., Bajorek, A., Myers, D. A., Dugatkin, L.A., Atlas, M. R. (2009) Protection of Salmonella by Ampicillin-Resistant Escherichia coli in the presence of otherwise Lethal Drug Concentrations. *Proceedings of the Royal Society B*, 276, 3759-3768.

Pokhrel, S., Nagaraja, K. S., Varghese, B. (2004) Preparation, Characterization and X-ray Structure Analysis of 1,4-diazabicyclo-2,2,2-octane (DABCO) and Ammonium Cation with Tetrathiomolybdate Anion. *Journal of Structural Chemistry*, 45(5), 900-905.

Roy, R. (2003) A Decade of Glycodendrimer Chemistry. *Trends in Glycoscience and Glycotechnology*, 15(85), 291-310.

Sreeperumbuduru, R. S., Abid, Z. M., Claunch, K. M., Chen, H. H., McGillivray, S. M., Simanek, E. E. (2016) Synthesis and Antimicrobial Activity of Triazine Dendrimers with DABCO Groups. *Royal Society of Chemistry Advances*, 6, 8806-8810.

Shepard, J., Ibba, M. (2013) Direction of Aminoacylated transfer RNAs into Antibiotic Synthesis and Peptidoglycan-Mediated Antibiotic Resistance. *FEBS Letters*, 587(18), 2895-2904.

Shulaev, V. (2006) Metabolomics technology and bioinformatics. *Briefings in Bioinformatics*, 7(2), 128-139.

Todar, K., (2012). Textbook of Bacteriology (Online). University of Wisconsin, http://textbookofbacteriology.net. Accessed 9 April 2015

VanKoten, H.W., Dlakic, W. M., Engel, R., Cloninger, M. J. (2016) Synthesis and Biological Activity of Highly Cationic Dendrimer Antibiotics. *Molecular Pharmaceutics* 13, 3827-3834.

Vembu, S., Pazhamalai, S., Gopalakrishnan, M. (2015) Potential Antibacterial Activity of Triazine Dendrimer: Synthesis and Controllable Drug Release Properties. *Bioorganic and Medicinal Chemistry*, 23, 4561-4566.

Wolfenden, M., Cousin, J., Nangia-Makker, P., Raz, A., Cloninger, M. (2015) Glycodendrimers and Modified ELISAs: Tools to Elucidate Multivalent Interactions of Galectins 1 and 3. *Molecules*, 20, 7059-7096.

Wrońska, N., Majoral, J. P., Appelhans, D., Bryszewska, M., Lisowska, K. (2019) Synergistic Effects of Anionic/Cationic Dendrimers and Levofloxacin on Antibacterial Activities. *Molecules*, 24, 2894-2905.

Xue, X., Chen, X., Mao, X., Hou, Z., Zhou, Y., Bai, H., Meng, J., Da, F., Sang, G., Wang, Y., Lou, X. (2013) Amino-Terminated Generation 2 Poly(amidoamine) Dendrimer as a Potential Broad-Spectrum, Nonresistance-Inducing Antibacterial Agent. *AAPS Journal*, 15, 132-142.