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# An in vitro model for studies of attenuation of antibiotic-inhibited growth of *Aggregatibacter actinomycetemcomitans* Y4 by polyamines

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## Abstract

Polyamines are ubiquitous polycationic molecules that are present in all prokaryotic and eukaryotic cells, and they serve as important modulators of cell growth, stress, and cell proliferation. Polyamines are present at high concentrations in the periodontal pocket and could potentially affect the stress response of periodontal bacteria to antibiotics. The effects of polyamines on inhibition of growth by amoxicillin (AMX), azithromycin (AZM), and doxycycline (DOX) were investigated with the Y4 strain of *Aggregatibacter actinomycetemcomitans* (*Aa*). Bacteria were grown in brain heart infusion broth under the following conditions: (1) *Aa* only, (2) *Aa* + polyamine mix (1 mM putrescine, 0.4 mM spermidine, and 0.4 mM spermine), (3) *Aa* + antibiotic, and (4) *Aa* + antibiotic + polyamines. Growth curve analysis, minimal inhibitory concentration determination, and transcriptomic studies were conducted. The presence of exogenous polyamines produced a small, but significant increase in *Aa* growth, and polyamines attenuated the inhibitory effects of AMX, AZM, and DOX on growth. Transcriptomic analysis revealed that polyamines upregulate expression of ribosomal biogenesis proteins and small subunits, attenuate the bacterial stress response to antibiotics, and modulate bacterial nutritional pathways in a manner that could potentially increase the virulence of *Aa*. In summary, the polyamine-rich environment found in periodontal pockets appears to protect *Aa* and reduce its susceptibility to several antimicrobial agents in this in vitro model.

**KEYWORDS**
*Aggregatibacter actinomycetemcomitans*, amoxicillin, antimicrobial resistance, azithromycin, doxycycline, metatranscriptomic analysis

## 1 | INTRODUCTION

Although periodontitis affects nearly 65% of the adult population worldwide, one phenotype of the disease affects only children and circumpubertal adolescents. This form of the disease has a rapid rate of progression and can result in considerable loss of function at a very young age (Armitage, 2004). Therefore, a tremendous amount of research has been invested in elucidating the mechanisms that underlie this aggressive disease and identifying etiological factors.

Although the etiology of the disease is multifactorial, one organism, *Aggregatibacter actinomycetemcomitans* (*Aa*), a nonmotile, facultative gram-negative coccobacillus, has been consistently implicated in the etiopathogenesis of this phenotype of periodontitis. Studies have identified *Aa* in almost all individuals diagnosed with localized aggressive periodontitis (Zambon et al., 1983), and individuals who test positive for *Aa* in the oral cavity have demonstrated an increased risk of developing aggressive periodontitis (Fine et al., 2007). Importantly, successful therapeutic outcomes are associated with lower levels of *Aa*, whereas sites refractory to therapy contain a higher bioload of this species (Mandell et al., 1987). Numerous virulence factors have been identified that support its lifestyle in an anaerobic heme-rich, prooxidant environment and enable it to evade host defenses and invade epithelial and connective tissue (Christersson et al., 1985; Fives-Taylor et al., 1999; Taichman et al., 1980).

*Aa*'s ability to invade tissues makes it difficult to predictably eliminate from the periodontal pocket. The current approach for initial nonsurgical treatment of aggressive periodontitis involves use of systemic antibiotics in combination with scaling and root planing, which can induce a rapid and substantial reduction in bacterial levels and facilitate recolonization of the subgingival environment by host-compatible species (Feres et al., 2015).

The overall effectiveness of an antibiotic in treating periodontitis lies in its ability to alter the microbial ecology and promote a shift toward a nonpathogenic community. *Aa* has been shown to be susceptible to a variety of antibiotics in vitro, including amoxicillin (AMX), azithromycin (AZM), and doxycycline (DOX) (Veloo et al., 2012). Irrespective of their mechanism of action, most antibiotics (including gentamicin [Bustos et al., 2016], roxithromycin [Arslan et al., 2017], levofloxacin [Song et al., 2016], ceftazidime, piperacillin, and ciprofloxacin [Albesa et al., 2004]) have been shown to induce oxidative stress via generation of reactive oxygen species (ROS). Directly or indirectly, all bactericidal antibiotics promote the production of ROS (Kohanski et al., 2007).

When used to treat periodontitis, antibiotics have to function in an environment that is rich in polyamines (polycationic molecules that are breakdown products of amino acids). Polyamines, especially spermidine (SPD), spermine (SPM), and putrescine (PUT), have been associated with cell growth and are found at increased concentrations in areas with high cell proliferation and cell lysis (Miller-Fleming et al., 2015). It is not surprising, therefore, that they have been discovered to occur at higher concentrations in gingival crevicular fluid from sites with periodontitis and those with higher inflammatory burden when compared to healthy sites (Lamster et al., 1987). Polyamines have the potential to act as ROS scavengers and enhance the expression of stress response genes (Rhee et al., 2007). Therefore, we hypothesized that the high concentration of polyamines in the periodontal pocket could mitigate bacterial stress and decrease inhibition of *Aa* growth by antibiotics. In the present study, we tested this hypothesis by characterizing the effects of polyamines on the growth and stress responses of *Aa* cultured in the presence and absence of antibiotics used in periodontal antimicrobial chemotherapy.

## 2 | METHODS

### 2.1 Materials

DOX, PUT, SPD, and SPM were purchased from Sigma Chemical Company (St Louis, MO). AMX and AZM were purchased from US Pharmacopeia (Rockville, MD). Polyamine and AMX stock solutions were prepared in sterile water. AZM was prepared in ethanol and DOX was prepared in dimethyl sulfoxide.

### 2.2 Bacterial culture

*Aa* strain Y4 (ATCC 43718; American Type Culture Collection, Manassas, VA) was grown in brain–heart infusion (BHI) broth (Difco, Becton, Dickinson and Co, Sparks, MD) at 37°C in a capnophilic (candle jar) environment containing 9% $O_2$ and 4% $CO_2$.

### 2.3 Growth curves

Fresh overnight cultures of *Aa* were diluted in BHI to 0.1 absorbance unit at 600 nm and added to sterile culture tubes (6 ml per tube). Stock solutions of antibiotics and polyamines were added to produce the following experimental conditions:

A. Control (*Aa* only, with no antibiotic or polyamine additions);
B. *Aa* + Polyamine (1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM)*;
C. Negative control (*Aa* +Antibiotic);
D. *Aa* + Antibiotic + Polyamine (1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM)*.

Antibiotic concentrations (4 $\mu$g/ml AMX, 0.5 $\mu$g/ml AZM, and 0.5 $\mu$g/ml DOX) were selected to provide moderate rather than complete inhibition of growth and are compatible with therapeutic concentrations observed in the gingival crevice (Walters & Lai, 2015). The polyamine concentrations designated by asterisks correspond to levels observed in gingival crevicular fluid samples obtained from patients with untreated periodontitis (Lamster et al., 1987). At 1.5-h intervals up to 9 h, a 1-ml aliquot was removed from each tube for spectrophotometric analysis at 600 nm. At least five replicates were obtained for each condition. Statistics were performed on final growth yield.

### 2.4 Minimal inhibitory concentration determinations

Fresh overnight cultures of *Aa* were diluted in BHI to 0.1 absorbance unit at 600 nm (approximately $10^6$ CFU/ml) and added to sterile snapped-cap Falcon culture tubes (6 ml per tube). Stock solutions of antibiotics and polyamines were added to create the following conditions:

1. Control (Antibiotic + *Aa*);
2. *Aa* + Antibiotic + Polyamine (mixture of 1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM);
3. *Aa* + Antibiotic + Polyamine (mixture of 1 mM SPD and 1 mM SPM).

Antibiotics were tested at concentrations of 0.125, 0.25, 0.5, 0.75, 1, and 2 $\mu$g/ml. The cultures were incubated overnight at 37°C in a candle jar environment containing 9% $O_2$ and 4% $CO_2$. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of each antibiotic that completely inhibited visible growth of the inoculum, as assessed by changes in turbidity. Seven replicate experiments were obtained for each condition.

## 2.5 Metatranscriptomic analysis

Fresh overnight cultures of *Aa* were diluted in BHI to 0.1 absorbance unit at 600 nm and added to sterile culture tubes (2 ml per tube). Stock solutions of antibiotics and polyamines were added to produce the following experimental conditions:

A. Control (*Aa* only, with no antibiotic or polyamine additions);
B. *Aa* + Polyamines (1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM);
$C_{AMX}$. *Aa* + AMX (4 $\mu$g/ml);
$D_{AMX}$. *Aa* + AMX (4 $\mu$g/ml) + Polyamines (1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM);
$C_{AZM}$. *Aa* + AZM (0.5 $\mu$g/ml);
$D_{AZM}$. *Aa* + AZM (0.5 $\mu$g/ml) + Polyamines (1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM).

Each sample was vortexed every 1.5 h to resuspend cultures. Cultures were removed from the incubator after 6 h of growth.

Total RNA was isolated from *Aa* cultures using the mirVana miRNA isolation kit (ThermoFisher Scientific). Bacteria were lysed and RNA was isolated according to the directions provided by the manufacturer. The concentration of total RNA was determined using the Qubit® RNA Assay Kit (Life Technologies). To evaluate the quality of the total RNA, RNA integrity value (RIN) was determined using the Agilent RNA 6000 Nano Reagents and RNA Nano Chips in an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (500 ng) was used to remove the DNA contamination using Baseline-ZERO™ DNase (Epicentre) according to the manufacturer's instructions. RNA was then purified using RNA Clean & Concentrator columns (Zymo Research). DNA-free RNA samples were used for rRNA removal by using Ribo-Zero™ Gold rRNA Removal Kit (Epidemiology; Illumina). Final purification was performed using RNA Clean & Concentrator columns (Zymo Research). rRNA-depleted samples were used for library preparation using the TruSeq™ RNA LT Sample Preparation Kit (Illumina) according to the manufacturer's instructions. Following library preparation, the final concentration of all the libraries was measured using the Qubit® dsDNA HS Assay Kit (Life Technologies), and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries were then pooled in equimolar ratios of 2 nM, and 4 pM of the library pool was clustered using the cBot (Illumina) and sequenced paired end for 300 cycles using the HiSeq 2500 system (Illumina) (2 × 150 bp PE).

Raw reads with >10% unknown nucleotides or with >50% low-quality nucleotides (quality value < 20) were discarded. Microbial transcripts were quality filtered using SolexaQA++, and aligned against the Human Oral Microbiome Database (HOMD) (Chen et al., 2010) using DIAMOND (Buchfink et al., 2015). Aligned sequences were annotated to the SEED database using Megan 6 (Huson et al., 2016) and metabolic pathways were visualized using KEGG. The metagenomic sequence classifier Kraken (Wood & Salzberg, 2014) was used along with our helper tool, kraken-biom (github.com/smdabdoub/kraken-biom), for taxonomic confirmation. A customized web application, VORTEX, was used for data visualization (Gupta et al., 2017).

## 2.6 Bacterial viability determinations

Assays were conducted to confirm that control bacterial suspensions were viable and capable of transcription in the studies described above. An aliquot of overnight growth *Aa* culture was diluted to an absorbance at 670 nm of ~0.03. Half of the bacterial suspension was separated and sacrificed by exposure to heat (95°C for 10 min). The two suspensions were mixed to create five different proportions of live:dead cells (100:0, 90:10, 50:50, 10:90, and 0:100), which were used to standardize the bacterial viability assay. The assay (LIVE/DEAD BacLight assay kit L7012, ThermoFisher Scientific) was conducted by scanning fluorescence spectroscopy using the quantitative assay protocol provided by the manufacturer. The assay demonstrated that 90.7% of the bacteria were viable after 6 h of growth under experimental condition A (control).

# 3 | RESULTS

## 3.1 Effect of polyamines on inhibition of *Aa* growth by AMX, AZM, and DOX

Over the course of the growth period, AMX (4 $\mu$g/ml) inhibited bacterial growth yield to 26% of the untreated control (Figure 1). In the presence of a mixture of 1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM, AMX inhibited growth yield to 57% of control (difference statistically significant, $p < 0.05$). Addition of the polyamine mix to control cultures increased the growth yield slightly, but the effect was not statistically significant. Under similar experimental conditions (although the initial growth rate was lower because the initial bacterial density was slightly lower), AZM (0.5 $\mu$g/ml) decreased bacterial growth yield to 52% of control (Figure 2). In the presence of the polyamine mix, AZM inhibited growth yield to 84% of control (significant at $p < 0.05$). As previously noted, addition of polyamines to control cultures increased the growth yield, but the effect was not statistically significant. DOX (0.5 $\mu$g/ml) reduced bacterial growth yield to 56% of the untreated control (Figure 3). In the presence of the polyamine mix, DOX inhibited growth yield to 69% of control ($p < 0.05$). Again, addition of polyamines to control cultures increased growth yield, but the effect was not statistically significant. However, when data from all 17 replicates portrayed in Figures 1–3 were pooled, growth in the presence of polyamines was enhanced by 5% at 7.5 h (significant at $p = 0.003$).

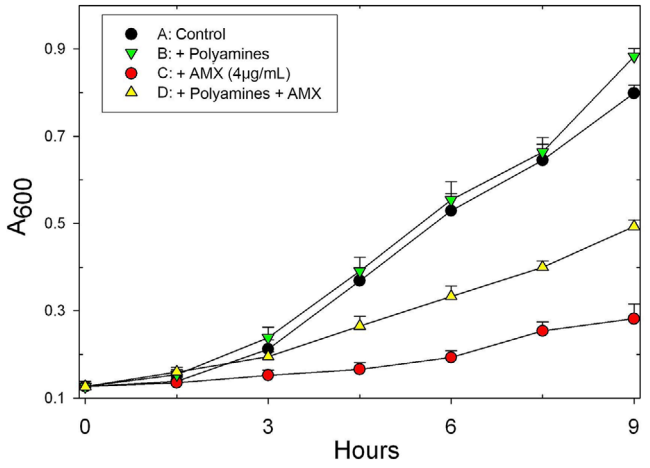


**FIGURE 1** Effect of polyamines on inhibition of *A. actinomycetemcomitans* growth by amoxicillin (AMX, 4 μg/ml). Data are represented as mean and SEM of five experiments. The polyamines included 1 mM putrescine, 0.4 mM spermidine, and 0.4 mM spermine, which approximate the mean concentrations found in gingival crevicular fluid at untreated periodontitis sites. Bacterial growth over the course of the experiment was enhanced in the presence of polyamines, but the effect was significant only in the presence of AMX ($p < 0.05$, repeated measures ANOVA with Holm–Sidak post hoc test)

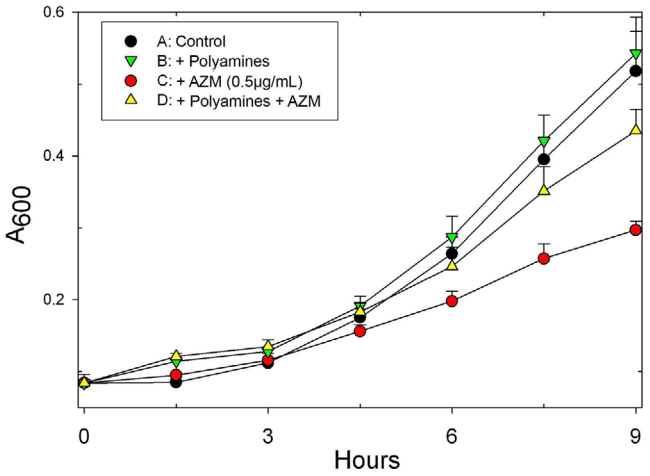


**FIGURE 2** Effect of polyamines (1 mM putrescine, 0.4 mM spermidine, and 0.4 mM spermine) on inhibition of *A. actinomycetemcomitans* growth by azithromycin (AZM, 0.5 μg/ml). The data are representative of five experiments. Bacterial growth over the course of the experiment was enhanced in the presence of polyamines, but the effect was statistically significant only in the presence of AZM ($p < 0.05$, repeated measures ANOVA with Holm–Sidak post hoc test)

## 3.2 Effect of polyamines on MIC for *Aa*

Polyamines had essentially no effect on the MIC for growth inhibition by AMX, regardless of whether the assays were conducted in the presence of a mix of 1 mM PUT, 0.4 mM SPD, and 0.4 mM SPM as well as a mix of 1 mM SPD and 1 mM SPM. However, the MIC for bacterial inhibition by AZM was significantly increased under both conditions. Similarly, the MIC for inhibition by DOX was increased in the presence of polyamines, but the difference was statistically significant only in the presence of a mixture of 1 mM SPD and 1 mM SPM (Table 1).

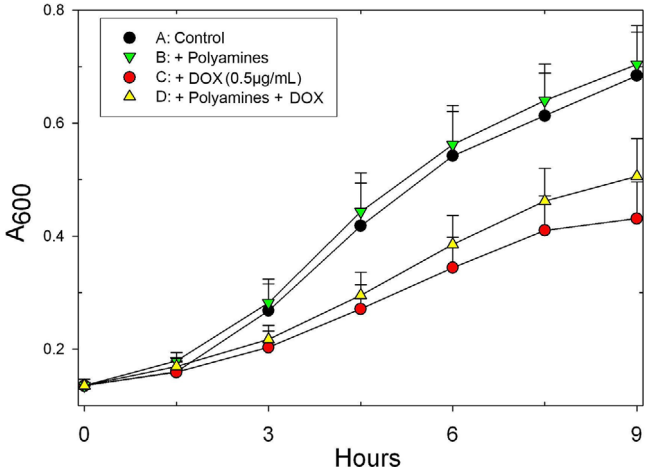


**FIGURE 3** Effect of polyamines (1 mM putrescine, 0.4 mM spermidine, and 0.4 mM spermine) on inhibition of *A. actinomycetemcomitans* growth by doxycycline (DOX, 0.5 μg/ml). The data are representative of seven experiments. Bacterial growth over the course of the experiment was enhanced in the presence of polyamines, but the effect was significant only in the presence of DOX ($p < 0.05$, repeated measures ANOVA with Holm–Sidak post hoc test)

## 3.3 Transcriptome analysis

The number of mapped transcripts, 9.0—12.4 million per sample, totaled 65,831,882 bases of sequenced cDNAs. A total of 840 functionally identifiable transcripts from the SEED database were found and matched belonging to 30 functional families.

### 3.3.1 Effect of AZM on *Aa*

We employed a pathway enrichment approach that relied on fold change in expression to examine expression of metabolic pathways. A total of 243 genes demonstrated upregulation and 149 were downregulated (Figure 4A and Table S1). A total of 249 of these genes could be mapped to unique Enzyme Commission (EC) identifiers. Not surprisingly, treatment with AZM led to a breakdown in the RNA machinery. For example, synthesis of ribosomes, RNA-associated proteins, RNA metabolism, and transcription factors were downregulated by 16%–143%, whereas transcription termination protein NusA was upregulated by 38%–110%. Downregulation of macrolide export system, especially the drug efflux system, was also observed. Additionally, downregulation of central metabolic pathways, including carbohydrates (e.g., the citrate cycle, pyruvate metabolism, and glycolysis/gluconeogenesis), lipid and protein and amino acid metabolism, and other membrane transport pathways, repression of stress response, ATP synthases, the CRISPR–Cas system, and lactate utilization path-

**TABLE 1** The effect of polyamines on the MIC of AMX, AZM, and DOX

| | MIC | | |
|---|---|---|---|
| **Antibiotic** | **1. Control** | **2. PUT + SPD + SPM** | **3. SPD + SPM** |
| AMX | 0.54 ± 0.06 | 0.50 ± 0.08 | 0.54 ± 0.07 |
| AZM | 0.54 ± 0.04 | 0.71 ± 0.04* | 0.75 ± 0.05* |
| DOX | 0.61 ± 0.11 | 0.79 ± 0.10 | 0.86 ± 0.09* |

*Note*: Data are represented as mean and SEM of seven experiments. PUT + SPD + SPM: 1 mM PUT + 0.4 mM SPD + 0.4 mM SPM. SPD + SPM: 1 mM SPD + 1 mM SPM. *Significantly different from control ($p < 0.05$, repeated measures ANOVA and Holm–Sidak post hoc test).

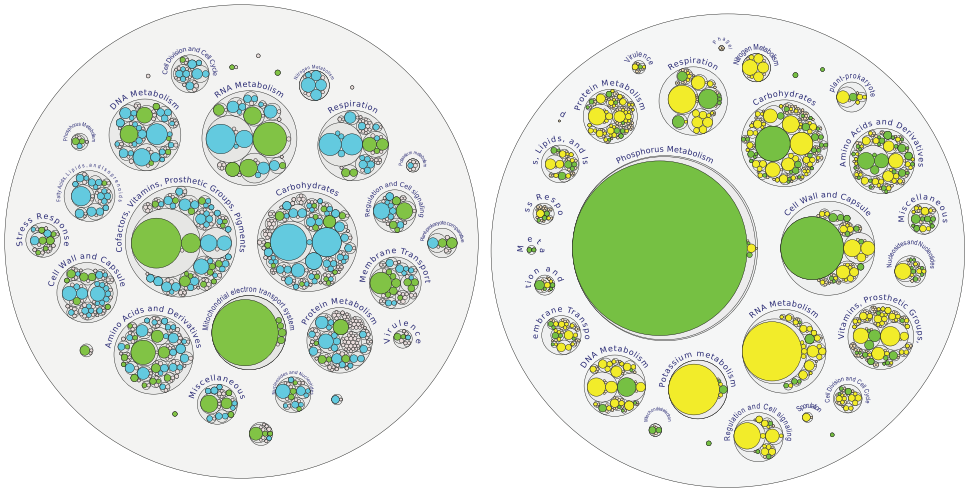


**FIGURE 4** Comparison of the effects of azithromycin (a) and amoxicillin (b) on transcriptional activity of *A. actinomycetemcomitans*. Data are portrayed in hierarchical circle packing plots of change in transcriptional activity following treatment of *A. actinomycetemcomitans* with azithromycin or amoxicillin. Each circle represents a gene and is sized by $\log_2$ fold change. Genes are grouped based on their functional role based on SEED classification. Genes that were significantly overexpressed ($\log_2$ fold change > 2) following antibiotic treatment when compared to controls are in aqua, whereas those that were underexpressed are shown in green. White circles indicate genes whose change in expression did not meet the above criteria. The data used in creating this figure are shown in Table S1

ways were noted. This was accompanied by a two- to eightfold upregulation in synthesis of critical cell wall and cell membrane components including Lipid A, lipopolysaccharide, peptidoglycan, sialic acid, and cell cytoskeleton and in quorum sensing. One interesting finding was the respiratory response of these bacteria to treatment with AZM. A large-scale downregulation of anaerobic respiratory pathways with a concomitant upregulation of aerobic mechanisms was observed. Of particular importance was the upregulation of cytochrome pathways, which are normally not dominant in an obligate anaerobe. Also upregulated was the chorismate synthesis pathway.

### 3.3.2 Effect of AMX on *Aa*

Treatment with AMX led to repression of 355 genes (Figure 4B and Table S1). Not surprisingly, many of these genes encoded cell wall and cell membrane components, including peptidoglycan. As with AZM, a massive downregulation occurred in genes encoding most central metabolic pathways and mitochondrial electron transport. Notably, downregulation of glycine, serine, and threonine metabolism was observed in response to AMX. The respiratory response of *Aa* to AMX was similar to AZM, with downregulation of anaerobic respiratory pathways and upregulation of aerobic systems.

### 3.3.3 Effect of a polyamine-rich environment on response to antibiotics

To understand the impact of polyamines on antibiotic therapy, we first examined the behavior of *Aa* in a polyamine-rich environment. A large-scale downregulation of several functions was observed under these conditions, with repression of 481 genes (Figure 5 and Table S1). A striking effect was observed in upregulation of respiratory pathways, including ATP synthases, and in ribosomal activity. We then interrogated the transcriptome for differences in response to antibiotics in a polyamine-rich environment. Interestingly, although the two different antibiotics affected different functional pathways in *Aa*, a polyamine-rich environment attenuated the effects of both medications. In the presence of polyamines, the ability of AMX to repress cell wall transcription and the ability of AZM to stall ribosomes were diminished or reversed. However, the presence of polyamines did not alter antibiotic-induced stress response in *Aa*.

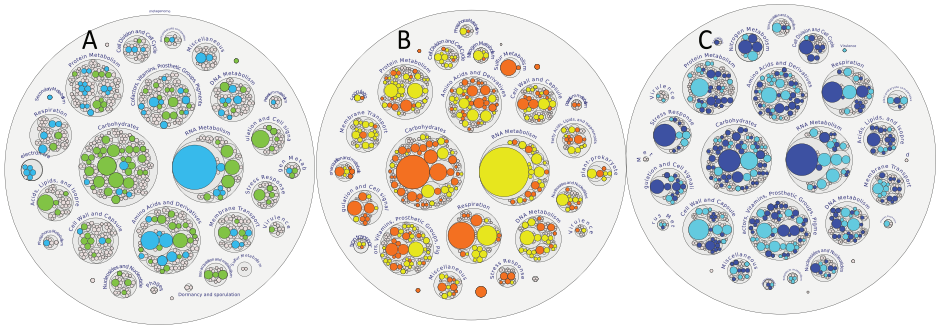


**FIGURE 5** Effect of a polyamine-rich environment (mixture of 1 mm PUT, 0.4 mm SPD, and 0.4 mm SPM) on *A. actinomycetemcomitans* and its response to antibiotics. Hierarchical circle packing plot of change in transcriptional activity of *Aa*. Each circle represents a gene and is sized by $\log_2$ fold change. Genes are grouped based on their functional role based on SEED classification. In panel (a), genes that were significantly overexpressed ($\log_2$ fold change > 2) in a polyamine-rich environment when compared to controls are in aqua, whereas those that were underexpressed are shown in green. White circles indicate genes whose change in expression did not meet the above criteria. Panel (b) shows the effects of polyamines on gene transcription in *Aa* treated with 4 $\mu$g/ml amoxicillin. Genes that were significantly overexpressed ($\log_2$ fold change > 2) in polyamine-rich environment are shown in yellow, whereas those downregulated are in orange. White circles indicate genes whose change in expression did not meet the above criteria. Panel (c) shows the effects of polyamines on gene transcription in *Aa* treated with 0.5 $\mu$g/ml azithromycin. Genes that were significantly overexpressed ($\log_2$ fold change > 2) in a polyamine-rich environment are shown in dark blue, whereas those downregulated are in aqua. White circles indicate genes whose change in expression did not meet the above criteria. The data used in creating this figure are shown in Table S1

## 4 | DISCUSSION

Ornithine decarboxylase and SPD synthase are important enzymes in the polyamine synthesis pathway. *Aa* lacks these enzymes and is unable to produce its own supply of polyamines in the usual way, but is capable of utilizing exogenous PUT, SPD, and SPM. For this reason, exogenous polyamines may play a role in modulating antibiotic resistance, stress, and cell growth. Our findings indicate that polyamines have a multitude of effects on planktonic *Aa* in vitro.

In the presence of polyamines, the inhibitory effects of AMX, AZM, and DOX on *Aa* growth were significantly attenuated. Although a statistically significant increase in MIC was observed for bacteriostatic agents AZM and DOX in a polyamine-rich environment, a similar effect was not evident for AMX. Low concentrations of bactericidal agents such as AMX can produce bacteriostatic effects. Because the MIC of a bactericidal agent tends to be relatively close to its minimum bactericidal concentration, however, it may be inherently more difficult to demonstrate a polyamine-induced increase in MIC with AMX than with a bacteriostatic agent. It is possible that polyamines transiently attenuate inhibition of *Aa* growth by AMX, and that this effect is most obvious during the first few hours (during the lag and log phases) of bacterial growth. This is plausible when examined in the context of AMX transport into the cell. Small hydrophilic molecules (e.g., $\beta$-lactam antibiotics) enter the cell through porin channels in the cell membrane, whereas macrolides are hydrophobic and are able to diffuse through the cell membrane (Delcour, 2009). Polyamines have been shown to decrease outer membrane permeability by inhibiting porin-mediated influx in *Escherichia coli* (Vega & Delcour, 1996). Polyamines likely bind to an internal pore-exposed site and trigger channel closures (Delcour, 2009). By impairing AMX entry into the cell periplasm, polyamines could reduce its bactericidal activity. A wild-type strain of *P. aeruginosa* showed increased resistance to imipenem in the presence of polyamines, but a mutant strain devoid of outer membrane porin OprD was susceptible to the antibiotic (Kwon & Lu, 2007). Polyamine interaction with outer membrane porins could play a role in blocking influx of certain antibiotics into the intracellular space.

To help explain the mechanisms by which polyamines mitigate *Aa* inhibition by antibiotics, transcriptomic studies were conducted on bacterial responses to AMX and AZM, because polyamines had a more profound effect on these two agents than DOX. RNA was isolated from samples obtained after 6 h of growth. Under these conditions, the bacteria were still in log phase and their viability was ~90%, suggesting that most bacteria were actively producing mRNA.

We discovered potential mechanisms by which polyamines support and enhance virulence mechanisms in *Aa*. For instance, a substantial upregulation of ribosomal biogenesis proteins and small subunits was observed in a polyamine-rich environment. This is in line with previous reports that polyamines stimulate assembly of 30S ribosomal subunits (Igarashi et al., 1974) and bind to ribosomes to increase codon accuracy during protein synthesis (Ito & Igarashi, 1986). Due to their polycationic nature, polyamines can interact with and act as electrostatic bridges for negatively charged molecules including DNA, RNA, proteins, and ROS.

We also found evidence that polyamines attenuate the bacterial stress response to antibiotics. For instance, we discovered a four- to 11-fold upregulation of catalase, and a two- to fourfold upregulation in superoxide dismutase, suggesting that polyamines can modulate oxidative stress. Polyamines also modulated genes in the glutathione redox pathway, which plays a role in oxidative stress reduction. Glutathione is oxidized by hydrogen peroxide to form water, whereas glutathione reductase (which underwent a 4.5-fold increase) reduces the oxidized glutathione molecule (Birben et al., 2012). A 2.18-fold increase in fructose-1,6-bisphosphatase transcription suggests that the pentose phosphate pathway may also be affected. This pathway is involved

in cellular metabolism and the production of NADPH, an important reducing agent in the glutathione redox pathway (Birben et al., 2012). Upregulation of DNA-binding proteins dps and HU-$\alpha$, which have a protective role in environmental stress by wrapping DNA and stabilizing it, provides further support of this hypothesis. Moreover, multiple components responsible for cytochrome c maturation were downregulated significantly. This downregulation may play an important role in cell survival. The presence of antibiotics leads to the production of ROS via the electron transport chain (Kohanski et al., 2007), in which cytochromes play a prominent role (Reguera, 2018). Reducing levels of cytochrome c could potentially reduce overall levels of ROS in the cell.

Polyamines also appear to confer protection against antibiotics by modulating bacterial nutrition. For instance, although both AMX and AZM downregulated lactate utilization and upregulated sialic acid metabolism, we observed an 18- to 25-fold upregulation of lactate utilization and a two- to fivefold downregulation in sialic acid catabolic pathways in a polyamine-rich environment. *Aa* is unique among oral pathogens in that it is capnophilic, preferring to use lactate from the extracellular matrix as a carbon source instead of simple sugars (Brown & Whiteley, 2009). It is thought that this preference for this growth limiting carbon source is an evolutionary trait that supports life in the periodontal pocket. Sialic acid is another potential carbon source, and one that is host derived. Our data suggest that in conditions of antimicrobial-induced stress, *Aa* switches from a slow-growth carbon source to a more readily available carbon source for its nutrition. It has been demonstrated that oral and gut pathogens conscript sialic acid for nutrition and niche colonization (MacDonald et al., 2016). Downregulation of lysine and threonine metabolism was also observed in response to treatment with both AMX and AZM and restored in the presence of polyamines. *Aa* poses a lysine riboswitch that regulates transcription of lysine transporters. This mode of regulation allows transcription to be linked to intracellular lysine concentrations, thus allowing bacteria to fine-tune expression in real time in response to changing lysine levels (Jorth & Whiteley, 2010). Lysine is important in the activation of leukotoxin from its protoxin form, and therefore, the present study suggests that polyamines might play a role in increasing the virulence of *Aa*.

This study has limitations related to oversimplification and sample size. *Aa* Y4 is a laboratory strain that has been studied extensively, but it has characteristics that differ from many clinical isolates. Y4 is not highly leukotoxic and does not carry the JP2 genotype (Yoshida et al., 2012). In comparison to many clinical strains, Y4 has lost some of its "attachment or surface-related" features (Fine et al., 1999) and does not produce poly-*N*-acetylglucosamine, a surface polysaccharide that helps some clinical strains of *Aa* resist phagocytic sacrificing (Venketaraman et al., 2008). Changes in gene presence and expression in Y4 and other laboratory strains of *Aa* cannot be accounted for in this in vitro model. Moreover, this single strain of planktonic *Aa* may not be representative of a naturally occurring biofilm, and it is possible that polyamines might affect antibiotic effects on *Aa* in a biofilm environment differently. The dose dependence of the effects of polyamines was not characterized. Instead, a mixture of polyamines was used at concentrations similar to those occurring in the gingival crevicular fluid of untreated periodontitis patients. Similarly, the experimental antibiotic concentrations were in the therapeutic range observed in the gingival crevicular fluid of patients undergoing systemic antimicrobial chemotherapy. The experimental conditions used in the present study were focused, but are representative of conditions that occur in vivo. Moreover, although the open-ended transcriptomic approach gives us a global view of gene alterations in response to AMX or AZM, these changes only represent a single time point. However, we believe that within the limitations of an in vitro planktonic model and small number of replicates, there is evidence to suggest that polyamines play very important roles in attenuating the effects of antimicrobials. Further investigation into the effects of polyamines is required in order to determine important pathways and mechanisms that may prevent or inhibit antibiotic effectiveness.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that supports the findings of this study is available in the supplementary material for this article (Table S1).

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/omi.12353.

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## REFERENCES

Albesa, I., Becerra, M. C., Battán, P. C., & Páez, P. L. (2004). Oxidative stress involved in the antibacterial action of different antibiotics. *Biochemical and Biophysical Research Communications*, *317*, 605–609. https://doi.org/10.1016/j.bbrc.2004.03.085

Armitage, G. C. (2004). Periodontal diagnoses and classification of periodontal diseases. *Periodontology 2000*, *34*, 9–21. https://doi.org/10.1046/j.0906-6713.2002.003421.x

Arslan, M., Timocin, T., & Ila, H. B. (2017). In vitro potential cytogenetic and oxidative stress effects of roxithromycin. *Drug and Chemical Toxicology*, *40*, 463–469. https://doi.org/10.1080/01480545.2016.1264410

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal*, *5*, 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Brown, S. A., & Whiteley, M. (2009). Characterization of the L-lactate dehydrogenase from *Aggregatibacter actinomycetemcomitans*. *PLoS ONE*, *4*, e7864. https://doi.org/10.1371/journal.pone.0007864

Buchfink, B., Xie, C., & Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nature Methods*, *12*, 59–60. https://doi.org/10.1038/nmeth.3176

Bustos, P. S., Deza-Ponzio, R., Páez, P. L., Albesa, I., Cabrera, J. L., Virgolini, M. B., & Ortega, M. G. (2016). Protective effect of quercetin in gentamicin-induced oxidative stress in vitro and in vivo in blood cells. Effect on gentamicin antimicrobial activity. *Environmental Toxicology and Pharmacology*, *48*, 253–264. https://doi.org/10.1016/j.etap.2016.11.004

Chen, T., Yu, W. H., Izard, J., Baranova, O. V., Lakshmanan, A., & Dewhirst, F. E. (2010). The Human Oral Microbiome Database: A web accessible

resource for investigating oral microbe taxonomic and genomic information. *Database*, *2010*, baq013. https://doi.org/10.1093/database/baq013

Christersson, L. A., Slots, J., Rosling, B. G., & Genco, R. J. (1985). Microbiological and clinical effects of surgical treatment of localized juvenile periodontitis. *Journal of Clinical Periodontology*, *12*, 465–476. https://doi.org/10.1111/j.1600-051x.1985.tb01382.x

Delcour, A. H. (2009) Outer membrane permeability and antibiotic resistance. *Biochimica and Biophysic Acta*, *1794*, 808–816. https://doi.org/10.1016/j.bbapap.2008.11.005

Feres, M., Figueiredo, L. C., Soares, G. M. S., & Faveri, M. (2015). Systemic antibiotics in the treatment of periodontitis. *Periodontology 2000*, *67*, 131–186. https://doi.org/10.1111/prd.12075

Fine, D. H., Furgang, D., Schreiner, H. C., Goncharoff, P., Charlesworth, J., Ghazwan, G., Fitzgerald-Bocarsly, P., & Figurski, D. H. (1999). Phenotypic variation in *Actinobacillus actinomycetemcomitans* during laboratory growth: Implications for virulence. *Microbiology*, *145*, 1335–1347. https://doi.org/10.1099/13500872-145-6-1335

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., McKiernan, M., & Gunsolley, J. (2007). *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: Longitudinal cohort study of initially healthy adolescents. *Journal of Clinical Microbiology*, *45*, 3859–3869. https://doi.org/10.1128/JCM.00653-07

Fives-Taylor, P. M., Meyer, D. H., Mintz, K. P., & Brissette, C. (1999). Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontology 2000*, *20*, 136–167. https://doi.org/10.1111/j.1600-0757.1999.tb00161.x

Gupta, N., Dabdoub, S., & Kumar, P. (2017). VORTEX- Visualizations for omics related technology. *Journal of Dental Research*, *96*(Special Issue A), 2533. https://iadr.abstractarchives.com

Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S. Ruscheweyh, H. J., & Tappu, R. (2016). MEGAN community edition-interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS Computational Biology*, *12*, e1004957. https://doi.org/10.1371/journal.pcbi.1004957

Igarashi, K., Sugawara, K., Izumi, I., Nagayama, C., & Hirose, S. (1974). Effect of polyamines on polyphenylalanine synthesis by Escherichia coli and rat-liver ribosomes. *European Journal of Biochemistry*, *48*, 495–502. https://doi.org/10.1111/j.1432-1033.1974.tb03790.x

Ito, K., & Igarashi, K. (1986). The increase by spermidine of fidelity of protamine synthesis in a wheat-germ cell-free system. *European Journal of Biochemistry*, *156*, 505–510. https://doi.org/10.1111/j.1432-1033.1986.tb09609.x

Jorth, P., & Whiteley, M. (2010). Characterization of a novel riboswitch-regulated lysine transporter in *Aggregatibacter actinomycetemcomitans*. *Journal of Bacteriology*, *192*, 6240–6250. https://doi.org/10.1128/JB.00935-10

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, *130*, 797–810. https://doi.org/10.1016/j.cell.2007.06.049

Kwon, D. H., & Lu, C. D. (2007). Polyamine effects on antibiotic susceptibility in bacteria. *Antimicrobial Agents and Chemotherapy*, *51*, 2070–2077. https://doi.org/10.1128/AAC.01472-06

Lamster, I. B., Mandella, R. D., Zove, S. M., & Harper, D. S. (1987). The polyamines putrescine, spermidine and spermine in human gingival crevicular fluid. *Archives of Oral Biology*, *32*, 329–333. https://doi.org/10.1016/0003-9969(87)90087-2

MacDonald, N. D., Lubin, J.-B., Chowdhury, N., & Boyd, E. F. (2016) Host-derived sialic acids are an important nutrient source required for optimal bacterial fitness in vivo. *mBio*, *7*, e02237-15. https://doi.org/10.1128/mBio.02237-15

Mandell, R. L., Ebersole, J. L., & Socransky, S. S. (1987). Clinical immunologic and microbiologic features of active disease sites in juvenile periodontitis. *Journal of Clinical Periodontology*, *14*, 534–540. https://doi.org/10.1111/j.1600-051x.1987.tb00996.x

Miller-Fleming, L., Olin-Sandoval, V., Campbell, K., & Ralser, M. (2015). Remaining mysteries of molecular biology: The role of polyamines in the cell. *Journal of Molecular Biology*, *421*, 3389–3406. https://doi.org/10.1016/j.jmb.2015.06.020

Reguera, G. (2018). Biological electron transport goes the extra mile. *Proceedings of the National Academy of Sciences*, *115*, 5632–5634. https://doi.org/10.1073/pnas.1806580115

Rhee, H. J., Kim, E.-J., & Lee, J. K. (2007). Physiological polyamines: Simple primordial stress molecules. *Journal of Cellular and Molecular Medicine*, *11*, 685–703. https://doi.org/10.1111/j.1582-4934.2007.00077.x

Song, M., Wu, H., Wu, S., Ge, T., Wang, G., Zhou, Y., Sheng, S., & Jiang, J. (2016). Antibiotic drug levofloxacin inhibits proliferation and induces apoptosis of lung cancer cells through inducing mitochondrial dysfunction and oxidative damage. *Biomedicine & Pharmacotherapy*, *84*, 1137–1143. https://doi.org/10.1016/j.biopha.2016.10.034

Taichman, N. S., Dean, R. T., & Sanderson, C. J. (1980). Biochemical and morphological characterization of the killing of human monocytes by a leukotoxin derived from *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, *28*, 258–268. https://doi.org/10.1128/IAI.28.1.258-268.1980

Della Vega, A. H., & Delcour, A. H. (1996). Polyamines decrease *Escherichia coli* outer membrane permeability. *Journal of Bacteriology*, *178*, 3715–3721. https://doi.org/10.1128/jb.178.13.3715-3721.1996

Veloo, A. C. M., Seme, K., Raangs, E., Rurenga, P., Singadji, Z., Wekema-Mulder, G., & van Winkelhoff, A. J. (2012). Antibiotic susceptibility profiles of oral pathogens. *International Journal of Antimicrobial Agents*, *40*, 450–454. https://doi.org/10.1016/j.ijantimicag.2012.07.004

Venketaraman, V., Lin, A. K., Le, A., Kachlany, S. C., Connell, N. D., & Kaplan, J. B. (2008). Both leukotoxin and poly-*N*-acetylglucosamine surface polysaccharide protect *Aggregatibacter actinomycetemcomitans* cells from macrophage killing. *Microbial Pathogenesis*, *45*, 173–180. https://doi.org/10.1016/j.micpath.2008.05.007

Walters, J., & Lai, P.-C. (2015). Should antibiotics be prescribed to treat chronic periodontitis? *Dental Clinics of North America*, *59*, 919–933. https://doi.org/10.1016/j.cden.2015.06.011

Wood, D. E., & Salzberg, S. L. (2014). Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biology*, *15*, R46. https://doi.org/10.1186/gb-2014-15-3-r46

Yoshida, A., Ennibi, O.-K., Miyazaki, H., Hoshino, T., Hayashida, H., Nishihara, T., Awano, S., & Ansai, T. (2012). Quantitative discrimination of *Aggregatibacter actinomycetemcomitans* highly leukotoxic JP2 clone from non-JP2 clones in diagnosis of aggressive periodontitis. *BMC Infectious Diseases*, *12*, 253. https://doi.org/10.1186/1471-2334-12-253

Zambon, J. J., Christersson, L. A., & Slots, J. (1983). *Actinobacillus actinomycetemcomitans* in human periodontal disease: Prevalence in patient groups and distribution of biotypes and serotypes within families. *Journal of Periodontology*, *54*, 707–711. https://doi.org/10.1902/jop.1983.54.12.707

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