Occurrence of antimicrobial persistence in *Streptococcus pyogenes*

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Abstract

Streptococcus pyogenes (group A *Streptococcus*-GAS) is an important pathogen for humans. GAS has been associated with severe and invasive diseases. Despite the fact that these bacteria remain universally susceptible to penicillin, therapeutic failures have been reported in some GAS infections. Many hypotheses have been proposed to explain these antibiotic-unresponsive infections, however none of them have fully elucidated this phenomenon. In this study, we show that GAS strains have the ability to form antimicrobial-persistent cells when growing on abiotic surfaces in a biofilm-like environment. Our data suggest that efflux activity was possibly involved in this phenomenon. In fact, gene expression assays by real-time qRT-PCR showed upregulation of some genes associated with efflux pumps in persistent cells arising in the presence of penicillin. Phenotypic reversion assay and whole-genome sequence analysis indicated that this event was due to non-inherited resistance mechanisms. The small persistent colonies showed downregulation of genes associated with protein biosynthesis and cell growth, as demonstrated by gene expression assays. Moreover, the proteomic analysis showed that susceptible cells express higher levels of ribosome proteins. It is conceivable to suppose that the generation of persistent cells by *S. pyogenes* might be a mechanism of antimicrobial refractory during the course of real GAS invasive infections, which has been overlooked. Accordingly, the phenomenon described here might shed some light on the origin of therapeutic failures in *S. pyogenes* infections. Multifactorial mechanisms involving efflux pump activity, protein synthesis inhibition and cell-growth impairment seem to play roles in generation of GAS-persistent cells.

Introduction

Group A streptococci (GAS) has long been recognized as one of the most important disease-causing bacteria in humans. These bacteria are associated with different types of infections, including pharyngitis, impetigo, scarlet fever, cellulitis and abscesses (Ralph and Carapetis, 2012; Gerardi et al., 2015). GAS is also involved in severe invasive infections such as myositis and necrotizing fasciitis, and cases of toxic shock syndrome (Ralph and Carapetis, 2012; Gerardi et al., 2015). Additionally, some post infectious sequelae have been reported (Ralph and Carapetis, 2012; Gerardi et al., 2015).

GAS strains are typically susceptible to penicillin (Zacharioudaki and Galanakis, 2017). However, studies have reported treatment failures in 20–30% of patients receiving β -lactam therapy (Brook, 2007; Gidengil et al. 2013). Many explanations have been proposed to elucidate clinical failures of penicillin treatment, including protection of GAS-susceptible isolates by β -lactamase producers in the pharyngeal microbiota, penicillin tolerance, biofilm formation, bacterial internalization in host cells among others (Thulin et al., 2006; Walker et al., 2014; Fiedler et al., 2015). However, the contribution of each of these mechanisms for drug failures remains unclear (Walker et al., 2014).

Several mechanisms have also been described in other bacterial species to explain transient drug resistance including tolerance, small colony variants (SCVs), and persistence (Brauner et al., 2016). The phenomenon of tolerance is defined for bactericidal antibiotics when the minimum inhibitory

concentration (MIC) of the tolerant is equal to that of the susceptible strain, but the time required for bacterial death to occur is considerably longer. SCVs are characterized by their slow growth resulting in small colony sizes, which show mutations in genes often associated with auxotrophic phenotypes, electron transportation chain, and biosynthetic pathways (Proctor et al., 2006). Persistent bacteria occur in a subpopulation of cells capable of surviving in a phenotypic fashion at high concentrations of antimicrobials to which the bacteria are fully susceptible, with no change in the MIC (Huemer et al., 2020).

It is remarkable that high bacterial load was detected in tissue biopsy specimens from 17 patients presenting with GAS disseminated infections (necrotizing fasciitis or severe cellulitis) despite intravenous antibiotic therapy (clindamycin in combination with β -lactam antibiotic) for a prolonged time (Thulin et al. 2006). Those authors suggested that GAS survival inside macrophages could represent a mechanism preventing bacterial eradication. However, patterns of purely intracellular bacteria were observed in less than half of the biopsies analyzed (Thulin et al. 2006). Some in vitro studies have demonstrated the effect of biofilm and high cell density (HCD) in the failure of antibiotics to eliminate *E. coli*, mycobacteria, methicillin-resistant *Staphylococcus aureus* (MRS) and also in GAS (Nielsen et al., 2011; Ferro et al., 2015; Fiedler et al., 2015; Coates et al., 2018; Novais et al., 2020). Based on these studies, we hypothesized that persistent cells may also occur in GAS strains under these conditions. In fact, our findings demonstrated the generation of antimicrobial GAS-persistent cells in biofilm-like environments, which was associated with inhibition of both protein biosynthesis and cell growth, and possibly with an increased activity of intrinsic multidrug-resistant (MDR) efflux pumps.

Methods

Bacterial isolates

Two hundred-eleven GAS strains were used to test the emergence of bacterial persistence to β -lactam antibiotics. These strains were obtained from infected patients and colonized individuals, in different regions of Brazil, from different clinical sites, the majority from symptomatic oropharyngeal infection cases. GAS was identified by routine methods and confirmed by latex agglutination tests (Streptococcal Grouping Kit; Oxoid, Basingstoke, Hampshire, United Kingdom). *Streptococcus pneumoniae* ATCC49619 was used as a control for minimal inhibitory concentration (MIC) and other antimicrobial susceptibility tests. To get some insights into the mechanisms associated with antimicrobial persistence in GAS cells, we randomly choose the GAS strain 37-97, since all 211 GAS analyzed were able to equally produce persistent cells under the experimental models used. This strain showed sequence type (ST) 62, *emm* 87 and was isolated from symptomatic oropharynx infection case.

MIC

MIC determination was done using the agar dilution method as recommended by the Clinical & Laboratory Standards Institute (CLSI) (Cockerill et al. 2011) with concentrations ranging from 0.0025–8 μ g/mL penicillin (Wyeth-Whitehall Ltda, Itapevi, SP, Brazil), 0.125–4 μ g/mL cephalexin (Sigma), 0.01–4 μ g/mL erythromycin (Sigma, St. Louis, Mo, USA), 0.06–4 μ g/mL azithromycin (Sigma; from), 0.005–1

µg/mL clindamycin (Sigma), 0.25–16 µg/mL chloramphenicol (Sigma), and 0.06–16 µg/mL tetracycline (Sigma). The MIC for ethidium bromide (EtBr; Sigma) was determined at concentrations of 0.015–4 µg/mL (Patel et al., 2010). Two biological experiments were performed (N=2).

Development of GAS persistent cells to β-lactams

The model used in this study to generate persistent cells was based on previous work done with *Staphylococcus aureus* strains (Novais et al., 2020). GAS strains (n=211) were grown in Todd Hewitt broth containing 0.5% (w/v) of yeast extract (THB-Y) at 37°C/6 h in order to reach the exponential phase. After centrifugation, the pellet was adjusted ($\sim 1-2 \times 10^{10}$ colony forming unit-CFU/mL) using the same broth. To produce a bacterial film, a 100-µL volume ($\sim 2-4 \times 10^7$ CFU/cm²) was spread onto the surface of THB-Y agar (covered with a cellophane membrane) containing 5% defibrinated sheep blood (BAB) and supplemented with 8 µg/mL penicillin (Pen; MIC=0.01 µg/mL) or 4 µg/mL cephalexin (Cep; MIC=0.5 µg/mL). Persistent cells grown (37°C/18 h) on cellophane membranes were removed for CFU determinations. To test whether defibrinated sheep blood interfered with the analysis, the experiments were also performed in the absence of blood. Antimicrobial susceptibility controls were performed exactly as described above but using inoculum size adjusted to concentrations recommended by CLSI ($\sim 10^6$ CFU/plate). Six biological experiments were performed with two technical replicates each. Two CFU determinations were carried out for each dilution (N=6).

Proteomic analysis

A proteomic analysis was done to assess differential protein expression between biofilm conditions (condition which allows persistence) and GAS susceptible cells grown on cellophane surfaces at low cell density (inoculum size adjusted to $\sim 10^6$ CFU/plate). Bacterial cells from strain 37-97 were collected from the cellophane membrane, suspended in phosphate buffered saline (PBS) (140 mM NaCl; 2.7 mM KCl; 8 mM Na₂HPO₄ and KH₂PO₄ 1.5 mM; pH 7.2) using vigorous shaking, and adjusted to OD_{600nm} = 0.4. Pellet was washed twice, resuspended in PBS and lysed with 106 µm beads (Sigma, USA) in a Bio101 Fast Prep system (BioSavant, Qbiogene, Carlsbad, CA) using six cycles (5 speeds/30 s pulse). After centrifugation, the protein concentration was estimated using a Qubit 2.0 (Invitrogen Life Technologies, CA, USA), and lysates diluted in sodium dodecyl sulfate polyacrylamide Gel (SDS-PAGE) sample buffer (1:1, v/v) (Laemmli, 1970). Proteins were separated using a 12.5% SDS-PAGE gel electrophoresis, and individual bands were isolated from the gels. All procedures used for the treatment of gel slices and trypsin digestion were performed as previously described (Shevchenko et al., 1996). The resulting peptides were desalted using an in-house reverse-phase microcolumn (POROS R2 resin, Applied Biosystems) and dried by vacuum centrifugation (Rodrigues et al., 2011). Peptides were solubilized in 20 µL of 0.1% (v/v) formic acid (FA), and 10 µL were injected into a trap column (Opti-Pak C18, Waters, Milford, MA). Liquid chromatography separation was performed using a reverse-phase capillary column (nanoEase C18, 100 mm × 100 µm, Waters) connected to a nano-HPLC system (Waters UPLC, Waters). The eluted peptides were introduced into an ESI-Q-TOF-MS/MS (Q-TOF Micro, Waters) controlled by MassLynx software (Version 4.1, Waters). Mass spectra (MS) were collected in the 50–2.000 m/z range, and the three most

abundant ions (charges +2, +3, and +4) were submitted for collision-induced dissociation (CID) using argon gas at 13 psi and 18–45 V. The raw data were converted to a peak list using the ProteinLynx Global software (version 4.0, Waters). Protein identification was considered valid if at least one peptide with minimum of 10 amino acids was observed with a maximum error tolerance of 50 ppm and Mascot score ≥ 46 ($p \leq 0.05$). The GenBank (Acc) access number, locus tag, and gene and protein names were determined using BLASTp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In addition, Uniprot BLAST analysis (www.uniprot.org/blast) was performed in order to identify homologs in *S. pyogenes* MGAS10750. Only e -values $\leq 1.0 \times 10^{-3}$ were considered in the database search.

Efflux pump activity

Efflux pump activity was initially assessed using the pump substrate ethidium bromide (EtBr) as suggested previously (Patel et al., 2010). First, the EtBr MIC for the strain 37-97 was determined using concentrations ranging from 0.015–4.0 $\mu\text{g/mL}$. Additionally, bacterial cells were grown on biofilm environment formed on the surface of cellophane membranes placed on BAB plates containing 4.0 $\mu\text{g/mL}$ of EtBr (MIC=0.06 $\mu\text{g/mL}$). Cells were removed from the cellophane membranes for CFU determinations. Controls were performed exactly as described above but using inoculum size adjusted to low cell density ($\sim 10^6$ CFU/plate). Six biological experiments were performed with two technical replicates each. Two CFU determinations were done for each dilution (N=6).

GAS persistence to other antimicrobial drugs

The same tests were performed to assess if the persistence mechanisms observed for GAS cells were associate to β -lactams only, or to universal mechanisms as those involving efflux pumps. The ability of GAS strain 37-97 to become persistent to different antimicrobials was tested in high concentrations of erythromycin (4 $\mu\text{g/mL}$; MIC= 0.12 $\mu\text{g/mL}$), azithromycin (4 $\mu\text{g/mL}$; MIC= 0.12 $\mu\text{g/mL}$), clindamycin (1 $\mu\text{g/mL}$; MIC= 0.01 $\mu\text{g/mL}$), chloramphenicol (16 $\mu\text{g/mL}$; MIC= 1.0 $\mu\text{g/mL}$), or tetracycline (16 $\mu\text{g/mL}$; MIC= 0.12 $\mu\text{g/mL}$). Plates were examined after incubation (37°C/18 h) and the CFU determined. Controls were performed exactly as described above but using inoculum size adjusted to $\sim 10^6$ CFU/plate, as recommended by CLSI. For each antimicrobial tested, three biological experiments were performed with two replicates each. Two CFU determinations were carried out for each dilution (N=3).

Effect of cyanide 3-chlorophenylhydrazone (CCCP) efflux pump inhibitor in antimicrobial refractory

Because CCCP inhibits proton motive force and also the transcription of some transport associated genes (Baron and Rolain, 2018), the ability of this compound to inhibit the formation of persistent cells was tested. The CCCP (Sigma) at 100 μM final concentration (Clancy et al., 1996) was incorporated to BAB (covered with a cellophane membrane) supplemented with erythromycin (4 $\mu\text{g/mL}$; MIC=0.12 $\mu\text{g/mL}$), azithromycin (4 $\mu\text{g/mL}$; MIC=0.12 $\mu\text{g/mL}$), clindamycin (1 $\mu\text{g/mL}$; MIC=0.01 $\mu\text{g/mL}$), chloramphenicol (16 $\mu\text{g/mL}$ MIC=1.0 $\mu\text{g/mL}$), tetracycline (16 $\mu\text{g/mL}$; MIC=0.12 $\mu\text{g/mL}$), or EtBr (4 $\mu\text{g/mL}$; MIC=0.06 $\mu\text{g/mL}$). BAB plates without CCCP were used to control bacterial growth. In addition, controls were performed exactly as above but using inoculum size adjusted to $\sim 10^6$ CFU/plate. Three

biological experiments were performed with two technical replicates each. Two CFU determinations were carried out for each dilution (N=3).

Phenotypic switching test

Persistent GAS cells of strain 37-97 grown on BAB plates containing 8 µg/mL penicillin were subjected to successive passages (up to 500 generations) on BAB without antibiotics. After passaging, bacterial growth was adjusted to concentrations recommended by CLSI (~10⁶ CFU/plate), and the penicillin MIC was determined using the agar dilution method (CLSI; Cockerill et al., 2011). Two biological experiments were performed (N=2).

Whole-genome sequencing

Total DNA from penicillin-persistent and -susceptible cells of the strain 37-97 was obtained using the Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA). Genomic libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina HiSeq (125 pb reads). The genome assembly was carried out using Newbler v3.0 (Margulies et al., 2005). Scaffolds were aligned against a reference genome (*S. pyogenes* strain NGAS743; Acc: CP007560) using cross match (version 0.990329; www.phrap.org/phrap.docs/phrap.htm). Intra-scaffold and inter-scaffold gaps resulting from repetitive sequences were resolved by in silico gap filling. Any remaining gaps in the genomic sequence from penicillin-persistent cells of the 37-97 strains (37-97P) were filled with "N" with estimated sizes based on the complete sequence of strain 37-97 (37-97S). The sequenced genomes were annotated using RAST 2.0v (Overbeek et al., 2014). Differences in single nucleotide polymorphisms (SNPs) between samples 37-97S and 37-97P were evaluated using cross match with parameter discrepancy lists. The generated list was compared to the Newbler assembly ace file and genome annotation. SNPs were verified by resequencing on an ABI 3730 DNA Analyzer (Life Technologies – Applied Biosystem; Carlsbad, CA, USA). Reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit in 36-cm capillaries with POP7 polymer according to the manufacturer's instructions.

Gene expression analysis

Total RNA from penicillin-persistent (8 µg/mL penicillin plates) and -susceptible GAS was prepared from cells (strain 37-97) grown on cellophane membranes placed on BAB plates using the RNeasy Mini kit (Qiagen; Maryland, USA) and quantified by a Qubit 2.0 Fluorometer (ThermoFisher Scientific Brasil; São Paulo, Brazil). RNA quality was analyzed by gel electrophoresis. For some experiments, gene expression was also performed in presence of CCCP 100 µM. To test the effect of clindamycin in the expression of the efflux-associated locus *MGAS10750_Spy1819*, total RNA was prepared from GAS persistent cells grown on cellophane membranes on BAB plates containing 1 µg/mL clindamycin (MIC=0.01 µg/mL). The real-time quantitative reverse transcriptase PCR (real-time RT-qPCR) was performed using Power SYBR Green RNA-to-CT™ 1-Step Kit (Applied Biosystems; Foster City, CA, USA) as recommended ("Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR"; Applied Biosystems). The rRNA 16S gene was used as an endogenous control. The calibrator sample was total

RNA from susceptible cells of strain 37-97. The reaction was performed in a Step One™ Real Time PCR System (Applied Biosystems). Data were analyzed using Step One Software 2.2 (Applied Biosystems). All primers were validated as recommended in the cited guide and listed in **Supplementary Table S1**. Three biological replicates were performed with three technical replicates each (N=3).

Statistical tests

Two-tailed unpaired Student's t test was used to analyze most the binary experiments. To analyze the hypothesis that the expression of efflux pump-associated genes increases in the persistent GAS cells, one-tailed unpaired t test was calculated using GraphPad Prism version 9.00 for windows (GraphPad Software, La Jolla, CA, USA). In addition, to confront the null hypothesis, Scaled Jeffreys–Zellner–Siow (JZS) Bayes Factor for two-samples t test was calculated to test the alternative hypothesis for $r=0.707$ (Rouder et al., 2009).

Results

Persistence to β -lactam antibiotics

As expected, all GAS strains tested ($n=211$) for MIC determinations were susceptible to penicillin, and MIC values varied from 0.0025–0.02 $\mu\text{g/mL}$. Both MIC50 and MIC90 were 0.01 $\mu\text{g/mL}$. However, persistent cells (tiny hemolytic colonies) were formed for the 211 GAS in all penicillin concentrations higher than the MIC tested (**Figure 1A and 1B**). To observe a possible influence of defibrinated sheep blood on persistence formation, GAS strain 37-97 was grown at biofilm conditions on BAB plates or on BAB plates without blood supplementation, both containing 8 $\mu\text{g/mL}$ penicillin, covered or not with cellophane membranes. Persistent cells were formed in all conditions. Similar to the results obtained for penicillin, persistent cells could also arise on BAB plates containing 4 $\mu\text{g/mL}$ cephalixin (**Figure 1A**). The average of persistent cells corresponded to 2.7% of the total cell population grown in the absence of penicillin ($6.0 \pm 2.4 \times 10^{10}$ CFU/mL). Likewise, the mean percentage of persistent cells on 4 $\mu\text{g/mL}$ cephalixin was 1.8 % of the total cell population grown in the absence of drugs ($6.0 \pm 2.4 \times 10^{10}$ CFU/mL) (**Figure 1B**).

Drug susceptibility could be reverted when persistent cells were submitted to serial passaging on BAB plates without antibiotics, with previously persistent cells returning to their original state of drug susceptibility (MIC=0.01 $\mu\text{g/mL}$). Additionally, DNA from penicillin-persistent and susceptible cells of strain 37-97 underwent whole-genome sequencing (WGS). Both sequences were identified as *S. pyogenes* ST62. WGS alignments generated in MAUVE showed high identity and perfect synteny of collinear blocks (**Figure 2A**). There was also no difference in the absence or presence of mobile genetic elements, genomic islands, or unique genes in the persistent cells (Acc: CP041615) compared with that of susceptible ones (Acc: CP041408). Despite some differences in SNPs observed in the WGS, these could not be confirmed by Sanger resequencing of these regions, thus mutations were not associated with the emergence of persistent cells (**Figure 2B**) ruling out the phenomenon known as SCVs

Proteomic analysis

A total of 61 proteins were only detected in the biofilm growth of the strain 37-97, a condition that led to antimicrobial persistence formation (**Supplementary Table S2**). The most remarkable feature was the low frequency of L ribosomal proteins (LRP) in these GAS cells (3.3%). Seventy-nine proteins were only detected when GAS strain 37-97 was grown using an inoculum size recommended for MIC determination (**Supplementary Table S3**), a condition for susceptibility. However, the most frequently detected proteins under this condition were the LRP proteins (31.6%), which play essential roles in ribosome assembly and are crucial for protein synthesis and cell growth (**Figure 3**). These data suggest a decrease in growth activity under biofilm condition, which is consistent with the tiny colonies formed by persistent cells. Some multidrug resistance (MDR) efflux pump components were only detected under condition of cell agglomeration, including a protein associated with the periplasmic component of the efflux system that belongs to the resistance-nodulation-cell-division (RND) family (Uniprot access: Q1J790). Multiple sugar transport ATP-binding protein MsmK (Uniprot access: Q1J4L0) and the multidrug resistant ABC transporter ATP-binding and permease protein (Uniprot access: Q1J8L9) were also observed under this condition (**Supplementary Table S2**). A total of 128 proteins were found in both conditions (**Supplementary Table S4**).

Efflux pump activity

Efflux pump substrates (EtBr and different classes of antimicrobials) were used to assess the role of efflux pump activity in the formation of persistent cells. The MIC value of strain 37-97 for EtBr was 0.06 µg/mL. However, in the condition used to allow persistent formation, growth was observed at concentrations of EtBr as high as 4 µg/mL (**Figure 4A**), possibly indicating intense efflux activity. The percentage of EtBr-refractory cells recovered at concentration of 4 µg/mL was about 6% of the GAS cell population grown at biofilm condition in the absence of EtBr ($5.5 \pm 2.0 \times 10^{10}$ CFU/mL) (**Figure 4B**).

The data showed that persistent cells were formed for all antimicrobials tested independent on the class of the drug analyzed. Persistent cells grew at MIC levels and at concentrations as high as 4 µg/mL erythromycin, 4 µg/mL azithromycin, 1 µg/mL clindamycin, 16 µg/mL chloramphenicol, and 16 µg/mL tetracycline (**Figure 4A**). The percentage of persistent cells recovered, considering all antimicrobials tested, ranged from 0.32% to 5.0% of the cell population ($5.5 \pm 2.0 \times 10^{10}$ CFU/mL) grown in the absence of antimicrobials (**Figure 4B**).

Since the resistance-nodulation-division (RND) family of efflux pumps was one of the drug/proton antiporters detected in the proteome performed with cell grown under agglomeration condition, we used the pump inhibitor CCCP to dissipate the proton-motive force. Control plates with CCCP (100 µM) without antibiotic caused no effect on bacterial growth. Despite the inhibition by CCCP of persistent cells to clindamycin and chloramphenicol (**Figure 4C**), this compound did not inhibit persistence to β-lactams or other antimicrobials tested.

Gene expression analysis

Of the 15 genes analyzed that were associated with the efflux pumps, seven showed some levels of upregulation in penicillin-persistent cells compared with those of susceptible GAS cells (**Figure 5A**). Among these, genes of an operon associated with efflux pumps of the RND family showed increases of ≥ 4 -fold, which included *MGAS10750_Spy1817* (gene product: ABC transporter ATP binding protein; $p=0.0156$), *MGAS10750_Spy1818* (gene product: ABC transporter permease protein; $p=0.0088$), and *MGAS10750_Spy1819* (gene product: periplasmic component of efflux system, $p<0.001$). An increase in transcripts of >4 -fold was also observed for a gene product annotated as belonging to a major facilitator superfamily, the multidrug resistance protein B (*MGAS10750_Spy0495*) ($p=0.04$). Another gene upregulated was a homolog of the multiple sugar transport ATP-binding protein MsmK (*MGAS10750_Spy1776*), which displayed a 2.2-fold increase in expression levels. Despite this difference was not very expressive ($p=0.1490$), protein of this same family was detected only on the proteome of cells grown in the biofilm environment. The loci *MGAS10750_Spy0043* and *MGAS10750_Spy1633* (*norA* homologue) showed about 2-fold increase ($p<0.001$ and $p=0.0031$, respectively; **Figure 5A**).

Because clindamycin was one of the antibiotics completely inhibited by CCCP, we also investigated the effect of this antibiotic in the overexpression of *MGAS10750_Spy1819*, which is part of the ABC transport operon. Our data showed an overexpression of about 9-fold in these gene transcripts. It was observed that CCCP had simultaneously affected the transcript levels of this ABC operon and two genes homologs to *ihk* (*MGAS10750_Spy1815*) and *irr* (*MGAS10750_Spy1816*) encoding the two-component regulatory system (TCS) *Ihk/Irr*, which are adjacent to and upstream this operon. Our data also demonstrated that both the operon and TCS were downregulated in the presence of CCCP (ABC operon: $p=0.03$, $p=0.021$, $p<0.001$ and *ihk/irr*: $p=0.0057$, $p=0.0389$, respectively; **Figure 5B**). Similar to the downstream genes belonging to the ABC operon, *ihk/irr* homologs also displayed increased levels of transcripts (4-fold) for persistent cells grown in the absence of CCCP ($p<0.001$ and $p=0.01$), compared with the susceptible GAS cells (**Figure 5B**). These data suggest that the *Ihk/Irr* system could be acting as a regulator of this operon. Indeed, consistent with an increase in pump activity, genes (*MGAS10750_Spy1765* and *MGAS10750_Spy1120*) annotated as belonging to the MarR and GntR families (pump negative transcriptional regulators) were downregulated in the persistent cells ($p=0.0032$ and $p=0.0137$; **Figure 5C**).

Additionally, the expression of genes associated with protein biosynthesis and cell growth/division were evaluated. For all these genes, the transcript levels decreased, but for *bcaT* homolog (gene product: branched-chain-amino acid aminotransferase) this decrease was less than 2-fold. The *guaA* homolog (gene product: GMP synthesis [glutamine hydrolyzing]), which is involved in the GTP pathway, was 2-fold down-regulated ($p=0.016$). Decreased expression was also observed for *relA* (gene product: GTP pyrophosphokinase; $p=0.003$) and *typA* (gene product: GTP-binding protein *TypA/BipA*; $p=0.0314$). Finally, the *ftsA* homolog, which is essential for cell division, was reduced 3-fold ($p=0.0187$) (**Figure 5D**).

Among the genes associated with the stress conditions studied, which includes some genes related to oxidative stress, the majority was down-regulated in penicillin-persistent GAS cells. An increase was only observed for a *dpr* homolog (gene product: hydrogen peroxide resistance regulator), which was about 2-

fold ($p=0.03$) more expressed compared with the susceptible GAS cells (**Figure 5E**). Finally, we examined the expression of three genes homologous to toxin-antitoxin (TA) systems found in the genome of *S. pyogenes* strain 37-97. Increased expression was observed for the *hicA/B* homologs (2.8-fold and 3.5-fold increase, respectively; $p=0.007$ and $p=0.017$, respectively) for persistent cells (**Figure 5F**).

Discussion

The influence of biofilm development and high bacterial density on antimicrobial resistance in vitro and in vivo has been described by others (Thulin et al., 2006; Rio-Marques et al., 2014; Karlake et al., 2016; Li et al. 2017; Novais et al., 2020). Despite the fact that it might be a reason for failures in the drug therapy, antimicrobial persistence remains unexplored in *Streptococcus pyogenes*. Here, we showed that GAS cells in agglomerated environments, such those encountered in a biofilm growth, persist to various classes of antimicrobials, corresponding to about 0.3 to 6.0% of the total bacterial population. It is important to emphasize that drug persistence was not a particular characteristic of only one or a few representatives of GAS because all 211 isolates from our collection presented the same phenomenon. Phenotypic reversion was observed, indicating the involvement of non-inherited antimicrobial resistance mechanisms. Also, no mutation was detected in the persistent tiny colonies discarding the phenomenon of SVCs (Proctor, 2019; Huemer et al. 2020). There is no question that bacterial resistance acquired through genetic mechanisms is the major reason for clinical failures during antimicrobial therapy for many other pathogens. However, the importance of non-inherited resistance should not be disregarded, mainly concerning infections affecting immunocompromised patients, those associated with biofilm production, or severe and invasive infections where a high bacterial load can accumulate at the site of infection (Thulin et al., 2006).

It was observed that CCCP fully restored the susceptibility to clindamycin and chloramphenicol, suggesting the involvement of proton efflux pumps in GAS persistence/refractory to these drugs. In fact, a gene of the ABC operon of the RND family (that uses proton gradient force across inner membrane to exclude drugs; Eicher et al., 2014) was almost 9-fold overexpressed in GAS-persistent cells induced by clindamycin, and was detected only in the proteome of cells grown under biofilm conditions. It is possible that additional efflux pumps, not importantly affected by CCCP, may be involved in the extrusion of the other antimicrobials tested. This assumption is supported by the fact that CCCP did not recover GAS susceptibility to the pump substrate EtBr. Indeed, about 50% of the efflux-associated genes analyzed were upregulated in penicillin-persistent cells.

Typically, overexpression of efflux pumps confers resistance to different classes of antimicrobial agents and some dyes, such as EtBr, in other bacterial species (DeMarco et al., 2007; Martins et al., 2011; Sun et al., 2014; Wang et al., 2019). The involvement of conserved RND proteins in reducing *S. aureus* persistence to β -lactams and glycopeptides has also been demonstrated (Quiblier et al., 2011). Similar to our findings, Poudyal and Sauer found increased expression of genes associated with an ABC transporter and other transport systems in *Pseudomonas aeruginosa* grown in biofilm conditions, suggesting that these mechanisms contributed to the persistence phenotype of *P. aeruginosa* to tobramycin (Poudyal and

Sauer, 2017). In fact, in our study, a homolog of *marR*, a negative pump regulator, was down-regulated in persistent cells. In line with these data, increased resistance in *Burkholderia thailandensis* was attributed to enhanced efflux pump activity and was detected after repression of a *marR* homolog (Sabrin et al., 2019). Additionally, we found that a gene in the GntR family of regulators was also down-regulated in penicillin-persistent GAS cells. It is remarkable that a *norG* knockout in *S. aureus* (a member of the GntR family) led to a 3-fold increase in the expression of an *abcA* gene encoding a protein of the ABC transport system with a concomitant increase in resistance to β -lactams (Truong-Bolduc and Hooper, 2007).

The Ihk/Irr two-component system is involved in the regulation of various streptococcal processes, including virulence (Han et al. 2012; Kachroo et al., 2020). It is notable that *ihk/irr* were overexpressed in a non-human primate model of GAS necrotizing myositis, and these genes were implicated in GAS resistance to polymorphonuclear phagocytosis (Kachroo et al., 2020). The fact that CCCP inhibited *ihk/irr* gene regulators and the RND family operon concomitantly, and that both the operon and *cis* regulators displayed increased expression in GAS-persistent cells, raises an interesting hypothesis about another possible role for the Ihk/Irr system beyond virulence regulation. However, despite the genes co-localization and the concomitant regulation of *ihk/irr* and the genes of the ABC transport operon by CCCP, molecular cloning strategies are needed to validate the hypothesis that *ihk/irr* may not only regulate GAS virulence but also this transport system in 37-97 strain. Corroborating this hypothesis, microarrays data from Voyich et al (2004) obtained from an *irr* mutant of the GAS strain, JRS500, revealed that a number of ABC transport genes were downregulated in this mutant, as well as the *msmK* gene.

Oxidative stress has also been associated with antimicrobial persistence in *E. coli* (Wu et al., 2012). In that study, a *dpr* homolog was upregulated in penicillin-resistant GAS cells. The *dpr* gene encodes the non-specific DNA binding protein Dps (peroxide resistance protein, Dpr), and homologs have been identified in different bacterial species associated with protection against multiple stressors (Leszczynska et al., 2013). Dps protein forms self-aggregates and an insoluble complex with DNA. In *E. coli*, the aggregates formed in the stationary growth phase correlated with increased persistence (Leszczynska et al., 2013). Also, the induction of *dps* in *E. coli* resulted in overexpression of the toxin/antitoxin (TA) system MqsR/MqaA (Kim et al., 2010). In fact, in our study, the TA system of the HicAB family was upregulated in GAS-persistent cells. However, the contribution of (p)ppGpp and of TA system in *E. coli* persistence remains controversial (Goormaghtigh et al., 2018).

In addition to efflux pumps, stress conditions, and TA systems, slow-growing cells and stringent responses have also been implicated in antimicrobial persistence (Goormaghtigh et al., 2018). The persistent GAS cells produced tiny colonies, indicating a condition of slow growth. Actually, genes associated with protein biosynthesis were downregulated in the penicillin-persistent GAS cells, including homologs of *typA/bipA* (important in ribosome assembly) and *guaA* (essential in GTP synthesis). The inhibition of *guaBA* operon by (p)ppGpp in *E. coli* led to low levels GTP and increased bacterial survival during amino acid starvation (Hauryliuk et al., 2015). These results agree with the proteomic data that showed increased expression of ribosome protein L in GAS cells grown at low density population compared with cells grown on biofilm condition. Indeed, the expression of the *ftsA* gene, which is

essential for cell growth, was reduced in penicillin-persistent GAS cells. It is notable that a substantial reduction in transcription and translation was previously observed for antimicrobial-persistent *E. coli*, which was associated with increased expression of different RNases (including RNase E, which is involved in the specific degradation of *ftsA-ftsZ* transcripts) (Radzikowski et al., 2016).

In conclusion, we showed that GAS cells can become persistent to high concentrations of β -lactams and other antimicrobials when cells are agglomerated in conditions such as those observed in biofilm formation in biotic or abiotic surfaces. Our data suggest that increased efflux pump activity and growth arrest are mechanisms associated with this phenotypic resistance in GAS cells, which have also been observed for other persistent bacteria. It is possible that this phenomenon might have some implications for failures in antimicrobial therapy that have been reported for some GAS clinical infections (Brook, 2007; Gidengil et al. 2013), including those severe and sometimes lethal invasive diseases, for which high bacterial load ($\sim 10^7$ CFU/cm²) can be achieved in the infected tissues, despite the use of effective antimicrobial therapy (Thulin et al., 2006), and thus it should not be overlooked. Finally, further studies with *ihk/irr* are required to clarify the role of this TC system in antimicrobial clinical failures due to a possible upregulation in both intracellular persistence of GAS in macrophages and appearance of antimicrobial persistent cells in biofilm like-environments.

Declarations

AUTHORS' CONTRIBUTIONS

Conception and design of the work: AMSF, BTFC and RBZ. The experiments were carried out by AZC, CLM, CNG, MCM, MCNM, TTA and USL. Data were analyzed and interpreted by AMSF, AZC, BTFC, CLM, and RBZ. The whole genome sequencing and analysis were performed by AMNB, ATRV, LGPA and PJP. The manuscript was written and revised by AMSF, BTFC and CLM. All authors read and approved the final manuscript.

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ETHICAL CONCERNS

This work was submitted to the Ethics Committee of the Hospital Universitario Clementino Fraga Filho, UFRJ/RJ under the # 4-485-002.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no competing interest.

DATA AVAILABILITY STATEMENTS

All data generated or analyzed during this study are contained within the article and in the supplementary material.

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Figures

Figure 1

Emergence of GAS persistent cells for penicillin and cephalexin. (A) Persistent cells of the strain 37-97 grown at different concentrations of penicillin above MIC. The arrow indicates the range of drug

concentrations used. The tip of arrow indicates the highest concentration tested. (B) Persistent cells recovered from biofilm environments at concentrations of 8 $\mu\text{g/mL}$ penicillin (MIC=0.01 $\mu\text{g/mL}$) or 4 $\mu\text{g/mL}$ cephalalexin (MIC=0.5 $\mu\text{g/mL}$).

Figure 2

Analysis of synteny and circular comparison of the chromosomes. (A) Genome alignments were performed using MAUVE. 37-97P: DNA sequence of the penicillin-persistent cells of strain 37-97 grown at 8 $\mu\text{g/mL}$ penicillin (MIC=0.01 $\mu\text{g/mL}$), and 37-97S: DNA sequence of the susceptible cells of strain 37-97. (B). Circular comparison of the chromosomes was generated by Blast Ring Image Generator (BRIG) using genome sequences obtained from 37-97P (red) and 37-97S (blue).

Figure 3

Proteomic analysis of GAS strain 37-97 grown in biofilm environments (BC; biofilm condition) and at condition of low cell density (LCD). Color gradient indicates the frequency of proteins involved in a biological process according to InterPro (www.ebi.ac.uk/interpro). More intense color: Highest number of proteins. Lightest color: Smallest number of proteins. White color: Absence of proteins.

Figure 4

GAS cells grown in biofilm environments are persistent to ethidium bromide and to a number of non- β -lactam antimicrobials. (A) Persistent cells of the strain 37-97 grown in biofilm environments in different drug concentrations. The arrow indicates the range of drug concentrations used in these experiments. The tip of arrow indicates the highest concentration. The drugs tested were ethidium bromide (EtBr), erythromycin (Ery), azithromycin (Azi), clindamycin (Cli), chloramphenicol (Chl) and tetracycline (Tet). The MICs for the drugs tested were 0.06 $\mu\text{g/mL}$ (EtBr), 0.12 $\mu\text{g/mL}$ (Ery), 0.12 $\mu\text{g/mL}$ (Azi), 0.01 $\mu\text{g/mL}$ (Cli), 1

μg/mL (Chl), or 0,12 μg/mL (Tet). (B) Persistent cells recovered from biofilm at concentrations containing 4 μg/mL EtBr, 4 μg/mL Ery, 4 μg/mL Azi, 1 μg/mL Cli, 16 μg/mL Chl, or 16 μg/mL Tet. (C) Inhibition of antimicrobial persistence by the efflux pump inhibitor, cyanide 3-chlorophenylhydrazine (CCCP). Top panel: Left plate was supplemented with 1 μg/mL clindamycin, and right plate with 1 μg/mL clindamycin and 100 μM/mL CCCP. Bottom panel: Left plate was supplemented with 16 μg/mL chloramphenicol, and right plate with 16 μg/mL chloramphenicol and 100 μM/mL CCCP. Note the hemolysis produced by tiny (almost invisible) colonies for GAS cells grown in biofilm environments. Persistence to these antibiotics was completely inhibited in the presence of CCCP.

Figure 5

Analysis of the gene expression in penicillin-persistent GAS cells, strain 37-97. (A) Transcript levels of genes associated with efflux pumps in penicillin-persistent cells of GAS. (B) Influence of CCCP in the expression of genes associated with ABC-type efflux pump (Locus_tag: MGAS10750_Spy1817, MGAS10750_Spy1818, and MGAS10750_Spy1819) and *ihk/irr* regulators in penicillin-persistent GAS cells; (C) Transcript levels of genes associated with negative transcriptional regulators of efflux pump in penicillin-persistent GAS cells. (D) Transcript levels of genes associated with protein biosynthesis and cell growth in penicillin-persistent GAS cells; (E) Transcript levels of genes associated with stress conditions in penicillin-persistent GAS cells; (F) Transcripts levels of genes associated with toxin-antitoxin (TA) systems in penicillin-persistent GAS cells. Genes locus tag annotated in the genome of the strain MGAS10750 (Acc: NC_008024) were used as a reference for primer design. a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$. For all gene expression assays, the respective calibrator sample (susceptible cells) was assigned relative quantification equal 1. For all tests, JZS Bayes factor agrees with t test, except for the gene locus MGAS10750_Spy1019 for which the Bayes Factor (BF=1.042144) was in favor of the alternative hypothesis; *bsaA* (BF=1.289759; in favor of alternative hypothesis); *sodA* (BF=1.213086; in favor of the alternative hypothesis); *relB* (BF= 1.349216; in favor of the alternative hypothesis). RQ: Relative quantification.

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