Biofilm-Grown *Burkholderia cepacia* Complex Cells Survive Antibiotic Treatment by Avoiding Production of Reactive Oxygen Species

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Abstract

The presence of persister cells has been proposed as a factor in biofilm resilience. In the present study we investigated whether persister cells are present in *Burkholderia cepacia* complex (Bcc) biofilms, what the molecular basis of antimicrobial tolerance in Bcc persists is, and how persisters can be eradicated from Bcc biofilms. After treatment of Bcc biofilms with high concentrations of various antibiotics often a small subpopulation survived. To investigate the molecular mechanism of tolerance in this subpopulation, *Burkholderia cenocepacia* biofilms were treated with 1024 µg/ml of tobramycin. Using ROS-specific staining and flow cytometry, we showed that tobramycin increased ROS production in treated sessile cells. However, approximately 0.1% of all sessile cells survived the treatment. A transcriptome analysis showed that several genes from the tricarboxylic acid cycle and genes involved in the electron transport chain were downregulated. In contrast, genes from the glyoxylate shunt were upregulated. These data indicate that protection against ROS is important for the survival of persisters. To confirm this, we determined the number of persisters in biofilms formed by catalase mutants. The persister fraction in ΔkatA and ΔkatB biofilms was significantly reduced, confirming the role of ROS detoxification in persister survival. Pretreatment of *B. cenocepacia* biofilms with itaconate, an inhibitor of isocitrate lyase (ICL), the first enzyme in the glyoxylate shunt, reduced the persister fraction approx. 10-fold when the biofilms were subsequently treated with tobramycin. In conclusion, most Bcc biofilms contain a significant fraction of persisters that survive treatment with high doses of tobramycin. The surviving persister cells downregulate the TCA cycle to avoid production of ROS and at the same time activate an alternative pathway, the glyoxylate shunt. This pathway may present a novel target for combination therapy.

Introduction

*Burkholderia cenocepacia* is a member of the *Burkholderia cepacia* complex (Bce), a group of 17 closely related and phenotypically similar species [1]. These bacteria are opportunistic pathogens that can cause severe lung infections in immunocompromised people, including cystic fibrosis (CF) patients [2]. The prevalence and outcome of infections appear to be species dependent and infections with *Burkholderia multivorans* and *B. cenocepacia* are associated with high rates of transmission and high mortality [3]. *B. cenocepacia* J2315 belongs to the highly transmissible ET12 lineage which has infected many CF patients in Canada, the UK and various European countries [4]. Unfortunately, Bce organisms are difficult to eradicate because of their innate resistance to a wide range of antibiotics and their capacity to form biofilms [5]. Mechanisms of resistance include changes in lipopolysaccharide structure, the presence of several multidrug efflux pumps, inducible chromosomal β-lactamases and altered penicillin-bind-
lacking persister cell formation, suggesting that dormancy mechanisms are redundant [11]. Currently, it is assumed that toxin/antitoxin modules are involved in persister formation [12]. Toxins are proteins that inhibit important cellular functions such as translation or replication. This condition can be reversed by the expression of the corresponding antitoxin which can form an inactive complex with the toxin. By causing a reversible dormant state, toxins protect bacteria against antibiotics which require translation or replication. This condition can be reversed by the expression of the corresponding antitoxin which can form an inactive complex with the toxin. By causing a reversible dormant state, toxins protect bacteria against antibiotics which require active targets in order to be effective [11]. The phenomenon of persister formation in exponentially growing planktonic cultures has been demonstrated for several micro-organisms (including Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus) [13], but has so far not been investigated in the Bcc.

Recently, Kohanski et al. demonstrated that the production of reactive oxygen species (ROS) contributes to the antimicrobial activity of bactericidal antibiotics. The primary drug-target interactions stimulate the oxidation of NADH via the electron transport chain, which itself is dependent on the tricarboxylic acid cycle (TCA). A hyperactivation of the electron transport chain results in increased superoxide formation, leading to damage to iron-sulfur clusters in proteins with the release of ferrous iron. This ferrous iron can be oxidised in the Fenton reaction with the production of hydroxyl radicals capable of damaging proteins, DNA and lipids and ultimately leading to cell death [13].

The glyoxylate cycle is an anaplerotic pathway of the TCA cycle, which bypasses the decarboxylation steps in which NADH is produced. This pathway allows microorganisms to utilize simple carbon compounds as a carbon source [14]. The glyoxylate cycle is absent in humans, making it an interesting drug target [14]. For example, Van Schaik et al [15] found that inhibition of isocitrate lyase (ICL, the first enzyme of this shunt), by itaconate during experimental chronic *Burkholderia pseudomallei* lung infections forces the infection into an acute state, which can then be treated with antibiotics.

In the present study we wanted to investigate whether persister cells are present in *Bcc* biofilms, what the molecular basis of antimicrobial tolerance in *Bcc* persisters is, and how persisters can be eradicated from *Bcc* biofilms.

### Materials and Methods

#### Strains and Culture Conditions

The strains used in the present study are shown in Tables 1 and 2 [16,17]. All strains were cultured at 37 °C on Luria-Bertani agar (LBA, Oxoid, Hampshire, UK) or on Mueller Hinton agar (MHA, Oxoid). Overnight cultures were diluted in Luria-Bertani broth supplemented with 800 μg/ml trimethoprim (Tp) (Ludeco, Brussels, Belgium) or in LBB supplemented with 800 μg/ml Tp and 0.2% rhamnose (Sigma Aldrich, Bornem, Belgium).

#### Minimal Inhibitory Concentration (MIC)

MICs were determined in duplicate according to the EUCAST broth microdilution protocol using flat-bottom 96-well microtiter plates (TPP, Trasadingen, Switzerland) [18]. Tobramycin and ciprofloxacin (Sigma Aldrich) concentrations tested ranged from 0.25 to 1024 μg/ml and from 0.25 to 128 μg/ml, respectively. Itaconate and 3-nitropropionate (3-NP) (Sigma Aldrich) concentrations ranged from 0.20 to 100 mM. 2-Thienylthiouroacetic acid (Sigma Aldrich) concentrations ranged from 5 to 625 nM. The MIC was defined as the lowest concentration for which no differences in absorbance were considered significant when the optical density (λ = 590 nm) was observed between the inoculated and blank wells after 24 h incubation. Differences in absorbance were considered significant when the 95%-confidence interval (calculated using Microsoft Excel) did not contain zero. All MIC determinations were performed in duplicate and replicates never differed more than two fold. When a two fold

### Table 1. Strains used in the present study.

| Strain     | LMG number | Strain information | Source (reference) |
|------------|------------|--------------------|--------------------|
| B. cenocepacia J2315 | LMG 16565 | CF patient, UK, ET12 strain | BCCM/LMG Bacteria Collection |
| B. cenocepacia K56-2 | LMG 18863 | CF patient, Canada | BCCM/LMG Bacteria Collection |
| B. cenocepacia CS424 | LMG 18827 | CF patient, Canada | Miguel Valvano [16] |
| B. cenocepacia MDL1 | CS424 ΔkatA mutant strain | Miguel Valvano [16] |
| B. cenocepacia MDL2 | CS424 ΔkatB mutant strain | Miguel Valvano [16] |
| B. cenocepacia D2 | ΔBCAL2118ΔBCAM1588 | This study |
| B. cenocepacia SDH7 | Overexpression of BCAM0967 | This study |
| B. cenocepacia SDH8 | Overexpression of BCAM0968 | This study |
| B. ambifaria AMMD | LMG 19182 | Pea rhizosphere, USA | BCCM/LMG Bacteria Collection |
| B. cenocepacia H2424 | LMG 24507 | Soil, USA, PHDC strain | John LiPuma [17] |
| B. lata ATCC 17769 | LMG 6992 | Soil, Trinidad and Tobago | BCCM/LMG Bacteria Collection |
| B. multivorans ATCC 17616 | LMG 17588 | Soil, USA | BCCM/LMG Bacteria Collection |
| B. vietnamensis ATCC 53617 | LMG 22486 | Wastewater, USA | BCCM/LMG Bacteria Collection |
| B. dolosa R-5670 | LMG 18943 | CF patient, US | BCCM/LMG Bacteria Collection |
| B. contaminans CCUG 34411 | LMG 16227 | CF patient, Sweden | BCCM/LMG Bacteria Collection |
| B. ubonensis NCTC 13147 | LMG 20538 | Surface soil, Thailand | BCCM/LMG Bacteria Collection |
| B. cepacia ATCC 25416 | LMG 1222 | Allium cepa, US | BCCM/LMG Bacteria Collection |
| B. pyrrocinia ATCC 15958 | LMG 14191 | Soil, US | BCCM/LMG Bacteria Collection |

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difference was observed between replicates, the lowest concentration was recorded as the MIC.

**Quantification of Persister Cells in Biofilms and Planktonic Cultures**

To determine the number of surviving cells, 24 h old biofilms or planktonic cultures were exposed to tobramycin, an aminoglycoside antibiotic, or ciprofloxacin, a fluoroquinolone, in concentrations ranging from 0.5 to 64× the MIC for 24 h. Biofilms were grown in the wells of a round-bottom 96-well microtiter plate (TPP), as described previously [19]. After 24 h of growth, the supernatant was removed and 120 μl of an antibiotic solution in physiological saline (PS) or 120 μl PS (= control) was added and the plates were further incubated at 37°C. Twelve wells were used for each condition. After 24 h of treatment, cells were harvested by vortexing and sonication (2×5 min) (Branson 3510, Branson Ultrasonics Corp, Danbury, CT) and quantified by plating on LB (n ≥2 for all experiments). For the planktonic experiments an overnight culture was diluted to an optical density of 0.1 (approximately 10⁷ cells/ml). After an additional 24 h growth in a shaking warm water bath, cell suspensions with an optical density of 1 (approximately 10⁹ cells/ml) were transferred to falcon tubes and centrifuged for 9 min at 5000 rpm. Cells were resuspended in an antibiotic solution in PS, or in PS and further incubated at 37°C. After 24 h of treatment the tubes were centrifuged, resuspended in PS and quantified by plating on LB (n ≥2 for all experiments).

**RNA Extraction and Microarray Analysis**

Biofilms were grown as described above and exposed to an antibiotic (concentration of 4×MIC) or a 0.9% NaCl solution (untreated controls) for 24 h. Treated and untreated *B. cenocepacia* J2315 sessile cells were harvested by vortexing (5 min) and sonication (5 min) and transferred to sterile tubes. RNA was extracted immediately following harvesting of the cells, using the Ambion RiboPure Bacteria Kit (Ambion, Austin, TX) according to the manufacturer’s instructions and the procedure included a DNase I treatment for 1 h at 37°C. After extraction, RNA of both treated and untreated samples was concentrated using Microcon YM-50 filter devices (Milipore, Billerica, MA) and linearly

| Table 2. The MIC and fraction of persisters for early and late Bcc clonal isolates obtained from infected CF patients. |
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| **Species** | **Strain** | **Time interval (years)** | **MIC** | **Average % persisters (n = 3)** | **SD** |
| **Clonal isolates from Canada** |  |  |  |  |  |
| *B. multivorans* | C8298 | 8 | Early | 64 | 0.1551 | 0.2352 |
|  | D2156 |  | Late | 64 | 0.0068 | 0.0076 |
| *B. multivorans* | C8814 | 5 | Early | 32 | 0.3411 | 0.3080 |
|  | D0999 |  | Late | 128 | 0.0012 | 0.0008 |
| *B. multivorans* | C6396 | 9 | Early | 64 | 0.0031 | 0.0004 |
|  | D0913 |  | Late | 128 | 0.0005 | 0.0003 |
| *B. cenocepacia* | C4053 | 6 | Early | 128 | 0.0004 | 0.0004 |
|  | C6121 |  | Late | 64 | 0.0019 | 0.0009 |
| *B. cenocepacia* | C3921 | 10 | Early | 1024 | 0.0249 | 0.0270 |
|  | C9343 |  | Late | 1024 | 0.0051 | 0.0044 |
| *B. cenocepacia* | C6483 | 4 | Early | 256 | 0.0019 | 0.0031 |
|  | C8474 |  | Late | 256 | 0.0214 | 0.0363 |
| *B. cenocepacia* | C4629 | 7 | Early | 128 | 0.0226 | 0.0326 |
|  | C8482 |  | Late | 128 | 0.0036 | 0.0007 |
| *B. cenocepacia* | C5876 | 8 | Early | 128 | 0.0020 | 0.0004 |
|  | D0465 |  | Late | 256 | 0.0000 | 0.0001 |
| *B. cenocepacia* | C5424 | 3 | Early | 256 | 0.0527 | 0.0753 |
|  | C7376 |  | Late | 128 | 0.0114 | 0.0152 |
| **Clonal isolates from the US** |  |  |  |  |  |
| *B. cenocepacia* | AU0326 | 11 | Early | 256 | 0.0066 | 0.0095 |
|  | AU18962 |  | Late | 512 | 0.0276 | 0.0441 |
| *B. cenocepacia* | AU0734 | 13 | Early | 256 | 0.0010 | 0.0007 |
|  | AU21801 |  | Late | 256 | 0.0001 | 0.0001 |
| *B. vietnamiensis* | AU0808 | 12 | Early | 16 | 0.0019 | 0.0025 |
|  | AU21645 |  | Late | 8 | 0.0092 | 0.0116 |
| *B. dolosa* | AU3503 | 10 | Early | 32 | 0.0040 | 0.0044 |
|  | AU21993 |  | Late | 32 | 0.0821 | 0.0702 |
| *B. dolosa* | AU0265 | 14 | Early | 128 | 0.9246 | 0.7618 |
|  | AU21961 |  | Late | 128 | 0.1230 | 0.1847 |

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amplified using the MessageAmp II-Bacteria Kit (Ambion) prior to the microarray analysis. The method was first optimised with the use of standard *Escherichia coli* RNA. Assessment of RNA yield and quality, cDNA synthesis and the hybridization and washing of the custom made 4 × 644K arrays (Agilent, Santa Clara, CA) was performed as described previously [20]. The gene expression analysis was performed using GeneSpring GX 7.3 (Agilent) and data were normalized using the procedure recommended for two-colour Agilent microarrays. Only features dedicated to *B. cenocepacia* J2315 that were labelled “present” or “marginal” were included in the analysis. After this initial filtering, a student’s T-test analysis was performed (p, 0.05). The experimental protocols and the raw microarray data can be found in ArrayExpress under the accession number E-MEXP-3532.

### Quantitative RT-PCR

In order to validate the microarray results, the expression of 11 selected genes was examined using RT-qPCR. Biofilms were treated and RNA was extracted as described above. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Forward and reverse primers were developed using tools available on the NCBI website and they were compared with the *B. cenocepacia* J2315 genome sequence using BLAST to determine their specificity (Table 3). The primer concentration used was 600 nM (300 nM for BCAM1588). All qPCR experiments were performed on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler as described previously [20]. Each sample was spotted in duplicate and control samples without added cDNA were included in each experiment. The initial 3 min denaturation step at 95°C was followed by 40 amplification cycles, consisting of 15 s at 95°C and 60 s at 60°C. A melting curve analysis was included at the end of each run. To allow accurate normalization of our data, we also included three reference genes (BCAL2694, BCAS0175, BCAM2784) for which we confirmed expression stability using GeNorm [21] prior to the actual analyses (Figure S1).

### Flow Cytometry

The induction of ROS by tobramycin was confirmed by staining of treated and untreated biofilms with the ROS-specific fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA, Sigma Aldrich), followed by quantification of labeled cells by flow cytometry. After 24 h of treatment, as described above, 22 µl DCFHDA (0.1 mM) was added to each well. After a 30 min incubation at 37°C in the dark, the wells were rinsed and 100 µl PS was added. Tobramycin-treated and untreated sessile cells were harvested by scraping and then transferred to sterile tubes. The tubes were centrifuged for...

### Table 3. Primer sequences.

| Gene        | Annotation                  | FW primer                          | RV primer                         |
|-------------|-----------------------------|------------------------------------|-----------------------------------|
| Glyoxylate shunt | Malate synthase | GCCCGGACCGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG              |
| BCAM2118    | Malate synthase            | GACCGTGGTCGGCTCCTCGG               | GCCCGGACCGCTGAAAGATCA             |
| BCAM1588    | Isocitrate lyase           | TCCCTGCGCGTCGTCCTCTC               | ATCCCGGAGGAGGAGTGTGA             |
| Tricarboxylic acid cycle | Aconitate hydratase | GTCCGCGGCTGAAAGATCA                | CACCGTGGTCGGCTCCTCGG             |
| BCAM0961    | Aconitate hydratase        | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM0211    | Dihydrolipoamide dehydrogenase | GTTCCGCGGCTGAAAGATCA            | CACCGTGGTCGGCTCCTCGG             |
| BCAM0970    | Succinate dehydrogenase     | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| Other genes | Malate dehydrogenase       | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM2318    | Putative ferredoxin oxidoreductase | GTTCCGCGGCTGAAAGATCA            | CACCGTGGTCGGCTCCTCGG             |
| Reference genes | ATP synthase beta chain | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM0289    | Glutamate synthase         | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM0421    | DNA gyrase B subunit       | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM1459    | Calcineurin-like phosphoesterase | GTTCCGCGGCTGAAAGATCA          | CACCGTGGTCGGCTCCTCGG             |
| BCAM1659    | Ribose transport permease  | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM1861    | Acetoactetyl-CoA reductase | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM2694    | Dehydrogenase              | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM3991    | Tryptophane synthase beta chain | GTTCCGCGGCTGAAAGATCA         | CACCGTGGTCGGCTCCTCGG             |
| BCAM2784    | Aspartate transaminase      | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| BCAM0175    | Hydrolase                  | GTTCCGCGGCTGAAAGATCA               | CACCGTGGTCGGCTCCTCGG             |
| doi:10.1371/journal.pone.0058943.t003
6 min, at 9000 rpm), the cells were resuspended and 100 fold diluted in PS. All solutions were prepared using MQ water (Millipore, Billerica, MA) and were filter-sterilized before use (Puradisk FP30; Whatman, Middlesex, UK). Labeled cells were quantified using a Cyan ADP flow cytometer (Beckman Coulter, Suarlèce, Belgium) with a 488 nm argon laser and a 530–540 nm emission filter. Data of at least 50000 cells were collected for each sample.

**Effect of Superoxide Dismutase (SOD), Isocitrate Lyase (ICL) or Succinate Dehydrogenase (SDH) Inhibition on Survival of Bcc Persisters**

To confirm the importance of protection against ROS for the survival of persister cells, the number of surviving cells was quantified in biofilms treated with tobramycin (4 × MIC, 24 h) in combination with the SOD inhibitor diethyldithiocarbamate (0.05 mM) (Sigma Aldrich). To confirm the importance of ICL for persistence, the number of surviving cells was quantified in treated (4 × MIC, 24 h) and untreated biofilms grown in LBB supplemented with 50 mM itaconate or 10 mM 3-NP, two ICL inhibitors. These concentrations were below the MIC of these inhibitors for the strains tested (data not shown). Itaconate and 3-NP solutions were neutralised with NaOH in LBB and sterilized by filtration. To evaluate the role of SDH in persistence, the number of surviving cells was determined in biofilms grown in LBB supplemented with 250 nM 2-thenoyltrifluoroacetone, a concentration well below the MIC (data not shown).

**Construction of ICL Mutant**

The genome of *B. cenocepacia* J2315 contains two ICL encoding genes, BCAL2118 and BCAM1588. To confirm their importance, we constructed a double ICL mutant. All *B. cenocepacia* mutant strains were constructed following the protocol described by Hamad et al., which allows for the creation of unmarked nonpolar gene deletions [22]. Briefly, this mutagenesis procedure requires the upstream and downstream regions flanking the target gene to be cloned into pGPI-SceI-XCm plasmid. The PCR amplifications of these regions (about 500 bp each) were performed with the

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**Figure 1.** The percentage surviving cells after treatment of *B. cenocepacia* J2315 with tobramycin or ciprofloxacin. The results from the biofilm experiments are indicated with open circles and those from the planktonic cultures with triangles. Error bars represent min-max (n = 2).

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**Table 4.** The average percentage surviving cells for different Bcc strains after treatment of biofilms with high concentration of tobramycin or ciprofloxacin (n ≥3).

| Strain                  | Tobramycin (4 × MIC) | Ciprofloxacin (4 × MIC) |
|-------------------------|----------------------|-------------------------|
|                         | % surviving cells    | SD                      | % surviving cells | SD          |
| *B. cenocepacia* J2315  | 0.3409               | 0.1877                  | 10.2425           | 1.4721      |
| *B. cenocepacia* K56-2  | 0.0064               | 0.0072                  | 3.7081            | 3.5486      |
| *B. cenocepacia* C5424  | 0.0319               | 0.0038                  | 37.9861           | 15.6789     |
| *B. cenocepacia* H12424 | 0.0007               | 0.0007                  | 3.6601            | 0.6902      |
| *B. ambifaria* ANMD     | 0.0096               | 0.01424                 | 14.6604           | 11.3466     |
| *B. lata* ATCC 17769    | 0.8581               | 1.5047                  | 4.6846            | 2.3581      |
| *B. multivorans* ATCC17616 | 0.0011            | 0.0012                  | 26.1484           | 21.3644     |
| *B. ubonensis* LMG 20358 | 0.0548              | 0.0867                  | 0.6486            | 1.1232      |
| *B. contaminans* LMG 16227 | 0.0183             | 0.0268                  | 10.5396           | 9.1080      |
| *B. dolosa* LMG 18943   | 0.1165               | 0.0839                  | 18.6143           | 6.3400      |
| *B. cepacia* LMG 1222   | 0.2369               | 0.4611                  | 4.3806            | 5.0242      |
| *B. vietnamiensis* LMG 22486 | 0.8721            | 1.1513                  | 1.8217            | 1.8081      |
| *B. pyrrocinia* LMG 14191 | 2.4287             | 3.4043                  | 6.7670            | 6.6825      |

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Exconjugants were selected in the presence of Tp (200 \mu g/ml) (24 h). The mean percentage of cells with a high fluorescent signal (>30) is indicated (n = 7). Differences between treated and untreated biofilms are statistically significant (p<0.05). doi:10.1371/journal.pone.0058943.g002

**Results and Discussion**

**Presence of Persister Cells**

The presence of persister cells has been proposed as an important factor in biofilm resilience [6]. To investigate the presence of these dormant, multidrug-tolerant phenotypic variants in Bcc biofilms, mature biofilms were treated with tobramycin, an aminoglycoside antibiotic, frequently used in the treatment of
Table 5. Differences in gene expression expressed as fold changes in treated vs untreated biofilms.

| Gene number | Annotation                                           | Microarray | qPCR   |
|-------------|------------------------------------------------------|------------|--------|
| Glyoxylate shunt                                    |            |           |        |
| BCAL2122    | Malate synthase                                      | 1.4*       | −3.3*  |
| BCAL2118    | Isocitrate lyase AceA                                 | 2.3*       | 1.9    |
| BCAM1588    | Isocitrate lyase                                      | 3.1*       | 1.6    |
| BCAL0813    | RNA polymerase factor sigma 54                       | −1.5*      | −      |
| BCAL1949    | Glyoxylate carboligase                                | −1.5       | −      |
| Tricarboxylic acid cycle                            |            |           |        |
| BCAM0961    | Aconitate hydratase                                   | −1.1       | −      |
| BCAM2701    | Aconitate hydratase                                   | −1.3*      | −1.3   |
| BCAM1833    | Aconitate hydratase/methylisocitrate dehydratase     | 1.5*       | 1.1    |
| BCAL2735    | Isocitrate dehydrogenase                              | 1.3*       | −2.5*  |
| BCAL2736    | Isocitrate dehydrogenase                              | 1.4        | 1.5    |
| BCAL1515    | α-ketoglutarate dehydrogenase E1                      | −2.0*      | −      |
| BCAL1516    | Dihydrolipoamide succinyltransferase                 | −3.3*      | −      |
| BCAL1517    | Dihydroxyoamide dehydrogenase                        | −2.5*      | −      |
| BCAL2207    | Putative dihydrolipoamide dehydrogenase              | −1.3*      | −      |
| BCAL1215    | Dihydroxyoamide dehydrogenase                        | −1.4*      | −      |
| BCAL0956    | Succinyl-CoA synthetase beta chain                    | −2.0*      | −      |
| BCAL0957    | Succinyl-CoA synthetase subunit alpha                 | −3.3*      | −10.0* |
| BCAM0967    | Putative succinate dehydrogenase                     | −1.7*      | −      |
| BCAM0968    | Putative succinate dehydrogenase                     | −2.5*      | −      |
| BCAM0969    | Succinate dehydrogenase flavoprotein                 | −2.5*      | −      |
| BCAM0970    | Succinate dehydrogenase iron-sulfur subunit          | −5.0*      | −25.0* |
| BCAL2908    | Fumarate hydratase                                   | −1.3*      | 1.9*   |
| BCAL2287    | Putative fumarate dehydrogenase                      | 1.0        | −      |
| BCAM0965    | Malate dehydrogenase                                 | 1.0        | −2.0*  |
| BCAL2746    | Putative citrate synthase                            | −1.3       | −      |
| BCAM0964    | Putative lyase                                       | −1.4*      | −      |
| BCAS0023    | Hpc/HpaI aldolase/citrate lyase family               | −2.5*      | −      |
| BCAM0972    | Type II citrate synthase                             | −5.0*      | −      |
| Oxidative phosphorylation                           |            |           |        |
| BCAL2142    | Cytochrome o ubiquinol oxidase subunit III            | −2.0*      | −      |
| BCAL2143    | Ubiquinol oxidase polypeptide I                       | −2.0*      | −      |
| BCAL0750    | Cytochrome c oxidase polypeptide I                    | −1.7*      | −      |
| BCAL0752    | Cytochrome c oxidase assembly protein                 | −2.5*      | −      |
| BCAL0753    | Hypothetical protein                                 | −2.5*      | −      |
| BCAL0754    | Putative cytochrome c oxidase subunit III            | −2.0*      | −      |
| BCAL0030-0037| F$_{o}$F$_{o}$ ATP synthase subunit A-c            | −1.7*~−2.5* | −      |
| BCAL2331-2343| NADH dehydrogenase subunit B-N                    | −2.5~−10.0* | −      |
| NAD(P)H production                                   |            |           |        |
| BCAL3276    | NAD-kinase                                           | 1.4*       | −      |
| BCAL0672    | Isocitrate dehydrogenase kinase/phosphatase         | 1.3*       | −      |
| BCAL3359    | Glutamate dehydrogenase                              | 4.2*       | −      |
| BCAL3395    | Malic enzyme                                         | 1.7*       | −      |
| Response to oxidative stress                         |            |           |        |
| BCAL1250    | Putative glutathione 5-transferase                   | 1.6*       | −      |
| BCAL3331    | Putative glutathione 5-transferase                   | 3.4*       | −      |
| BCAL0463    | Putative thioredoxin                                 | 1.6*       | −      |
| BCAL2013    | AhpC/TSA family protein                              | 1.9*       | −      |
infected CF patients. The experiments were carried out using *B. cenocepacia* J2315, a member of the epidemic ET12 lineage of which the genome has been sequenced. The MIC was previously determined to be 256 \( \mu \text{g/ml} \) [18]. Biofilms were treated with various concentrations of tobramycin, ranging from 0.5 to 64 \( \times \text{MIC} \) for 24 h.

We found that most cells were killed when J2315 biofilms were treated with tobramycin in concentrations ranging from 1 to 4 \( \times \text{MIC} \), but even at higher concentrations (up to 64 \( \times \text{MIC} \)), some of the cells survived, indicating the presence of persisters (Fig. 1). Similar results were obtained for other *Bcc* strains (Table 4).

Treatment with ciprofloxacin, a fluoroquinolone, resulted in an even higher fraction of surviving cells (Fig. 1). The presence of persisters is not unique to biofilms as we found similarly-shaped survival curves in planktonic cultures, although the fraction of surviving cells was considerably lower (Fig. 1).

Mulcahy et al. [28] analyzed clonal pairs of early and late *P. aeruginosa* isolates from single CF patients and found that in the majority of these patients, cultures of late isolates contained increased numbers of drug-tolerant persister cells. We analyzed clonal pairs of early and late *Bcc* isolates, obtained from 14 CF patients. The MICs and the fraction of persister cells are shown in Table 2. In general, the MICs were very similar (i.e. within a two-fold dilution), indicating that the strains did not acquire resistance during the course of the infection. Only in four patients the persister fraction was higher in the late isolates. In three patients there was no difference between the early and the late isolates and in eight patients the number of surviving cells was lower in the late than in the early isolate. Our observations suggest that, unlike in *P. aeruginosa*, the fraction of persisters does not increase with the duration of colonisation.

### Table 5. Cont.

| Gene number | Annotation                      | Microarray | qPCR  |
|-------------|--------------------------------|------------|-------|
| BCAL2106    | Glutathion peroxidase           | 1.6*       | –     |
| BCM2318     | Putative ferredoxin oxidoreductase | –10.0*    | –33.3*|
| Fe-storage  |                                |            |       |
| BCAM2627    | Putative hemin ABC transporter protein | 5.2*       | –     |
| BCAM2630    | Hemin importer ATP binding subunit | 2.8*       | –     |
| BCAM2224    | Putative pyochelin receptor protein FptA | 2.7*       | –     |
| BCAL1790    | Putative iron-transport protein | 2.5*       | –     |
| BCAL1347    | Putative Fe uptake system extracellular binding protein | 2.5*       | –     |
| BCAM2228    | Putative pyochelin synthetase PchF | 2.1*       | –     |
| BCAL1789    | Putative iron-transport protein | 2.0*       | –     |
| BCAL1371    | Putative TonB-dependent siderophore receptor | 2.0*       | –     |
| BCAL1702    | Putative ornibactin biosynthesis protein | –2.2*     | –     |

*: no qPCR experiments were performed. #: significant change in expression between the treated and the untreated biofilms (n = 3, \( p<0.05 \)). \$: range of fold changes for the various subunits.

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Figure 3. Effect of knocking out catalase function on survival of persisters. The number of surviving cells in biofilms formed by *B. cenocepacia* C5424 and two catalase mutants after treatment with tobramycin in a concentration of 4 \( \times \text{MIC} \) (512 \( \mu \text{g/ml} \)). Error bars represent standard deviation (n = 3).

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Figure 4. Effect of superoxide dismutase inhibition on survival of persisters. The number of surviving cells in *B. cenocepacia* J2315 biofilms after treatment with tobramycin alone (4 \( \times \text{MIC}, 24 \text{h} \)) or in combination with diethyldithiocarbamate (DETC 0.05 mM). Error bars represent standard deviation (n = 3, \( p<0.05 \)).

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Induction of ROS by Tobramycin

Recently, it was shown that bactericidal antibiotics induce the production of reactive oxygen species (ROS) [13]. Tobramycin is known to corrupt protein synthesis and by using ROS-specific staining and flow cytometry, we confirmed that tobramycin also drastically increased ROS production in treated sessile cells. Most of the cells in treated, unlike untreated biofilms showed a high fluorescent signal (Fig. 2).

Figure 5. Effect of isocitrate lyase inhibition on B. cenocepacia J2315. The number of surviving cells in B. cenocepacia J2315 biofilms after treatment with tobramycin (4×MIC, 24 h) or ciprofloxacin (4×MIC, 24 h) for biofilms grown in LB or LB supplemented with 50 mM itaconate (ita) or 10 mM 3-nitropropionate (3-NP). Error bars represent standard deviation. Statistically significant differences are indicated by an asterix (p<0.05, n ≥3). doi:10.1371/journal.pone.0058943.g005

Gene Expression in Cells Treated with High Doses of Tobramycin

A transcriptome analysis showed a considerable change in gene expression in sessile cells treated with high doses of tobramycin relative to untreated sessile cells. Of the 8729 B. cenocepacia J2315 sequences represented on the array, 2688 (30.8%) were significantly (p<0.05) upregulated, while the expression of 2413 sequences (27.6%) was significantly (p<0.05) downregulated. 669 (9.4%) protein-coding genes were significantly (p<0.05) upregu-

Figure 6. Effect of isocitrate lyase inhibition on different Bcc strains. The number of surviving cells in different Bcc biofilms after treatment with tobramycin (4×MIC, 24 h) for biofilms grown in LB or LB supplemented with 50 mM itaconate. Statistically significant differences are indicated by an asterix (p<0.05) (n≥3). doi:10.1371/journal.pone.0058943.g006
Glutamate dehydrogenase (BCAL3359) was upregulated and findings, BCAL0813, encoding a RNA polymerase factor sigma 54 encoding genes were also found to be upregulated. In line with these rate to the glyoxylate shunt, as well as both isocitrate lyase (ICL) can inactivate isocitrate dehydrogenase, thereby directing isocitrate dehydrogenase kinase-phosphatase, a bifunctional enzyme that the NADPH producing enzymes, was also upregulated. Isocitrate BCAL3395, the gene coding for malic enzyme, which is one of cellular metabolism towards the synthesis of NADPH and away from the formation of NADH) was upregulated. Similarly, BCAL3276, the gene coding for NAD-kinase, an enzyme that mediates the formation of NADP (a key coenzym known to tilt cellular metabolism towards the synthesis of NADPH and away from the formation of NADH) was upregulated. Similarly, BCAL3395, the gene coding for malic enzyme, which is one of the NADPH producing enzymes, was also upregulated. Isocitrate dehydrogenase kinase-phosphatase, a bifunctional enzyme that can inactivate isocitrate dehydrogenase, thereby directing isocitrate to the glyoxylate shunt, as well as both isocitrate lyase (ICL) encoding genes were also found to be upregulated. In line with these findings, BCAL0813, encoding a RNA polymerase factor sigma 54 which represses the glyoxylate pathway [32] was downregulated. Glutamate dehydrogenase (BCAL3359) was upregulated and α-ketoglutarate dehydrogenase (BCAL1515) was downregulated indicating an increased production of α-ketoglutarate, a ROS scavenger. Other proteins involved in response to oxidative stress which were significantly upregulated include proteins involved in glutathione biosynthesis, thioredoxin, peroxiredoxine and glutathione peroxidase. The expression levels of selected genes were confirmed by qPCR (Table 5).

Eradication of Persister Cells

Our transcriptome analysis suggested that protection against ROS is important in survival of persister cells. To confirm this we determined the number of persisters in biofilms formed by catalase mutants. B. cenocepacia contains two catalases of which one (KatA) is a specialized catalase/peroxidase which helps maintaining the normal activity of the TCA cycle, while KatB is a classical catalase/peroxidase which plays a global role in cellular protection against oxidative stress by converting toxic H₂O₂ into H₂O and O₂ [20]. We found the persister fraction to be slightly reduced in biofilms formed by the ΔkatA mutant but almost 40-fold reduced in biofilms formed by the ΔkatB mutant (Fig. 5). Furthermore, the number of surviving cells was almost 100 times lower after addition of the superoxide dismutase inhibitor diethyldithiocarbamate (DETC) (Fig. 4). Superoxide dismutases are antioxidant enzymes that detoxify O₂⁻ by a dismutation reaction generating H₂O₂ and O₂ [16]. The inhibitor had no effect on untreated biofilms in the concentrations tested, but reduced the number of surviving cells in a concentration dependent manner when combined with tobramycin. Together, these results indicate that protection against ROS is indeed important for the survival of persister cells.

Persisters are typically considered as “dormant” (i.e. metabolically inactive) cells [11] but our data challenge that paradigm. While it is true that some pathways (i.e. TCA cycle) were found to be downregulated, other pathways were upregulated, and these may be a novel therapeutic target for combination therapy. The importance of the upregulation of the glyoxylate shunt for the survival of persister cells was confirmed by inhibition
of ICL, the first enzyme in the shunt. Because persisters are thought to be pre-existent, biofilms were grown in the presence of these inhibitors. During treatment the inhibitors were removed because of pH incompatibility with the antibiotics used. Pre-treatment of B. cenocepacia biofilms with the ICL inhibitor itaconate in a concentration of 50 mM reduced the persister fraction approximately 10 fold when the biofilms were subsequently treated with tobramycin. Pre-treatment with 10 mM 3-NP, a more potent ICL inhibitor resulted in similar reductions (Fig. 5). The concentrations of the ICL inhibitor used were below the MIC and they did not affect growth in the untreated biofilms. In addition, we did not observe an effect of either inhibitor on ROS production (data not shown). The additional killing observed after combined treatment with tobramycin and an ICL inhibitor was not observed with ciprofloxacin (Fig. 5). This may suggest that this effect is related to the type of antibiotic and/or the magnitude of its effect on biofilms. However, this remains to be investigated. Similar results were obtained in other Bcc bacteria (Fig. 6).

To confirm the results obtained by chemically inhibiting ICL, we constructed a B. cenocepacia J2315 mutant in which both ICL genes were inactivated. Surprisingly, there was no significant decrease in the number of surviving cells after treatment with tobramycin in the D2 mutant compared to wild type (Fig. 7A), but we did find an additional effect after adding 3-NP to the mutant. Iaconate and 3-NP are succinate analogues and besides inhibiting ICL, they also inhibit succinate dehydrogenase (SDH) [33].

We hypothesized that an upregulation of ICL in persisters not only limits the production of NADH (and thus limits ROS production) but also leads to an increased intracellular succinate concentration. Succinate can be oxidised by SDH, thereby generating FADH2 which can lead to basal production of ATP in persister cells in which other energy-generating systems are downregulated. Iaconate and 3-NP likely kill persisters by shutting down the remaining ATP production by inhibiting both ICL and SDH. To test this hypothesis we constructed a SDH antisense overexpression mutant in the D2 background, in which SDH is inactivated if rhamnose is present. However, we noticed that Tp (required to maintain the plasmid) and/or rhamnose (required to induce antisense RNA expression) had a mild influence of biofilm formation such as, and on the number of persisters in biofilms (data not shown), which made interpretation of these data impossible. However, addition of an ubiquinone type SDH inhibitor, 2-thenoyltrifluoroacetone, to B. cenocepacia D2 biofilms significantly (p<0.05) reduced the number of surviving persisters (Fig. 7B), while not affecting the MIC for tobramycin (data not shown).

The reaction catalysed by ICL also leads to the production of glyoxylate. In B. cenocepacia glyoxylate can be used to form malate (through the activity of malate synthase) or tartronate semialdehyde (through the activity of glyoxylate carboligase). There are two malate synthases of which only one (BCAL2122) was slightly upregulated and glyoxylate carboligase (BCAL1949) was slightly downregulated (Table 5).

**Conclusion**

Our results contribute to a better understanding of the molecular mechanisms responsible for the antimicrobial tolerance of Bcc biofilms by demonstrating that these biofilms contain tolerant persister cells. In these surviving persister cells, the TCA cycle was downregulated and the expression of genes involved in the electron transport chain was also downregulated. This way the cells avoid the production of ROS. At the same time, persister cells activate an alternative pathway, i.e. the glyoxylate shunt. When biofilms were grown in the presence of an inhibitor of ICL and SDH, less persisters survived. Similar results were obtained in different species, indicating that this mechanism is widespread within the Bcc. So far, most anti-persister strategies focused on “reawakening” persisters for efficient killing [34], but we found that inhibiting a cellular target can also reduce the number of surviving persisters. This finding may provide novel approaches for treatment of persister-related infections.

**Supporting Information**

**Figure S1 Average expression stability values (M) of remaining control genes during stepwise exclusion of the least stable control gene (between brackets).** Genes are ranked from left to right in order of increasing expression stability (decreasing M value). Genes labeled with an asterix were used for normalization. (TIF)

**Figure S2 Differentially expressed protein-coding genes ordered by functional category.** (DOCX)

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**Author Contributions**

Contributed to writing the paper: AS NB KDR EM. Conceived and designed the experiments: TC HVA HM NB. Performed the experiments: HVA AS TM KDR SB CU. Analyzed the data: HVA TC. Contributed reagents/materials/analysis tools: EM AS KDR NB GR. Wrote the paper: HVA HM GR TC.

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