Glycobiology and Extracellular Matrices: Benzothiazinones Mediate Killing of Corynebacterineae by Blocking Decaprenyl Phosphate Recycling Involved in Cell Wall Biosynthesis


doi: 10.1074/jbc.M113.522623 originally published online January 20, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M113.522623

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 22 of which can be accessed free at http://www.jbc.org/content/289/9/6177.full.html#ref-list-1
Benzothiazinones Mediate Killing of Corynebacterineae by Blocking Decaprenyl Phosphate Recycling Involved in Cell Wall Biosynthesis*

Received for publication, September 26, 2013, and in revised form, January 20, 2014  Published, JBC Papers in Press, January 20, 2014, DOI 10.1074/jbc.M113.522623


From the School of Biosciences, Institute of Microbiology and Infection, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom and the Institute for Bio and Geosciences Biotechnology (IBG-1), Research Centre Juelich, D-52425 Juelich, Germany

Background: Benzothiazinones inhibit cell wall arabinan biosynthesis, which is lethal for Corynebacterineae.

Results: Corynebacteria can evade the action of benzothiazinones in the absence of decaprenyl phosphorylribosine synthesis by increasing the intracellular decaprenyl phosphate pool.

Conclusion: Benzothiazinones induce synthetic lethality in Corynebacterineae by blocking decaprenyl phosphate recycling.

Significance: Increased production of decaprenyl phosphate is a new mechanism of resistance to benzothiazinones.

Benzothiazinones (BTZs) are a new class of sulfur containing heterocyclic compounds that target DprE1, an oxidoreductase involved in the epimerization of decaprenyl-phosphoribose (DPR) to decaprenyl-phosphoarabininose (DPA) in the Corynebacterineae, such as Corynebacterium glutamicum and Mycobacterium tuberculosis. As a result, BTZ inhibition leads to inhibition of cell wall arabinan biosynthesis. Previous studies have demonstrated the essentiality of dprE1. In contrast, Cg-UbiA a ribosyltransferase, which catalyzes the first step of DPR biosynthesis prior to DprE1, when genetically disrupted, produced a viable mutant, suggesting that although BTZ biochemically targets DprE1, killing also occurs through chemical synthetic lethality, presumably through the lack of decaprenyl phosphate recycling. To test this hypothesis, a derivative of BTZ, BTZ043, was examined in detail against C. glutamicum and C. glutamicum::ubiA. The wild type strain was sensitive to BTZ043; however, C. glutamicum::ubiA was found to be resistant, despite possessing a functional DprE1. When the gene encoding C. glutamicum Z-decaprenyl-diphosphate synthase (NCg02203) was overexpressed in wild type C. glutamicum, resistance to BTZ043 was further increased. This data demonstrates that in the presence of BTZ, the bacilli accumulate DPR and fail to recycle decaprenyl phosphate, which results in the depletion of decaprenyl phosphate and ultimately leads to cell death.

Tuberculosis (TB) remains the single most important bac-terial cause of mortality and morbidity globally, causing 8.7 mil-
biosynthesis, as illustrated through genetic experiments and inactivation of dprE1, is therefore essential for the formation of a complete M. tuberculosis and C. glutamicum cell wall (7).

The first step of the pathway for DPA biosynthesis is catalyzed by UbiA, a ribosyltransferase that generates decaprenyl-phosphoribose phosphate by condensation of phosphoribosyl pyrophosphate with decaprenyl phosphate, followed by dephosphorylation (Rv3807) affording DPR. However, unlike in M. tuberculosis, inactivation of ubiA in C. glutamicum produces a viable mutant that is unable to synthesize DPA and is devoid of cell wall arabinan, suggesting that synthesis of DPA and cell wall arabinan itself is not essential for survival in C. glutamicum (8, 9).

In this study, we examined the mode of action of BTZ inhibition on C. glutamicum and C. glutamicum:ubiA. The data shows that BTZ perturbs C. glutamicum growth but was ineffective against the Cg-ubiA mutant even though DprE1 was functionally intact. In addition, the inhibitory effect of BTZ on cell wall arabinan biosynthesis was examined when the functionally intact C. glutamicum Z cell wall arabinan biosynthesis was examined when the Cg-UbiA mutant even though DprE1 was functionally intact. In addition, the inhibitory effect of BTZ on cell wall arabinan biosynthesis was examined when the C. glutamicum Z-decaprenyl-diphosphate synthase, encoded by NCgl2203 (UppS), was overexpressed in C. glutamicum. The results demonstrate that overexpression of UppS was able to rescue the wild type strain (BTZ MIC 20 μg/ml) and shift the MIC of BTZ for this strain to >40 μg/ml. In conclusion, BTZ blocks the recycling of decaprenyl phosphate as in its presence, the cell continually synthesizes DPR, which is presumably toxic to the cells, and subsequently renders decaprenyl phosphate unavailable for the biosynthesis of other macromolecules, such as peptidoglycan, lipoparabinomannan, and arabinogalactan. However, the overexpression of a prenyl synthase supplements enough decaprenyl phosphate to aid in cell wall biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Reagents, and Enzymes**—All chemicals and solvents were from Sigma, Bio-Rad, and Fisher Chemicals (UK), unless otherwise stated, and were of AnaR grade or equivalent. Plasmids were propagated during cloning in Escherichia coli Top10 cells (Invitrogen). All restriction enzymes and Phusion DNA polymerase enzymes were sourced from New England Biolabs. A Bioline quick ligation kit was used to perform ligation reactions. Oligonucleotides were from MWG Biotech Ltd. and PCR fragments were purified using the QIAquick gel extraction kit (Qiagen). Plasmid DNA was purified using the QIAprep purification kit (Qiagen).

**Bacterial Strains and Growth Conditions**—E. coli Top 10 cells were routinely grown in Luria-Bertani broth (LB, Difco) at 37 °C. C. glutamicum ATCC 13032, C. glutamicum-pVWEx2, and C. glutamicum-pVWE2-uppS (10 μg/ml tetracycline) were grown on rich brain heart infusion medium (BHI, Difco) and subsequently induced with 0.5 mM isopropyl β-d-thiogalactopyranoside at an A500 0.6 and incubated at 30 °C for 24 h. Cells were harvested (15 min, 7,000 × g), washed twice with phosphate-buffered saline, and resuspended in 50 mM MOPS (pH 7.9) containing lysozyme (0.2 mg/ml). The resuspended cell pellets were incubated at 37 °C for 1 h to allow cell lysis. The suspension was centrifuged (20 min, 27,000 × g, 20 min) and the supernatant checked for the presence of the Cg-UppS protein on SDS-PAGE gels.

**Effect of BTZ on C. glutamicum Strains**—To determine the C. glutamicum and C. glutamicum:pVWE2-MIC of BTZ, ~10^8 cells were used to inoculate 2 ml of BHI broth containing 0 to 20 μg/ml of BTZ43 in a stepwise gradient and the A500 measured up to 48 h. To determine the effect of increasing concentrations of BTZ043 on C. glutamicum:ubiA and C. glutamicum-pVWE2-uppS, ~10^8 cells were used (30 °C, BHI, 200 rpm) to inoculate 2 ml of BHI containing 0 and 20 μg/ml of BTZ043 and the A500 measured up to 48 h.

**Construction of Plasmids and Strains**—C. glutamicum-pppS (NCgl2203) was cloned in pVWE2 under the isopropyl β-d-thiogalactopyranoside-inducible Ptap promoter and was amplified from genomic DNA using the primers: 2203vwx, forward, 5’-GGATGGTCTGAGGAAATATAGTGTAGTGGAT- TCAAGTA-3’ and 2203vwx, reverse, 5’-ATTAGGATCTTCCGCTATTCG-3’ (restriction sites underlined). The PCR product was ligated into plasmid pVWEx2 using XbaI and BamHI restriction sites, yielding C. glutamicum-pVWE2-pppS. The empty pVWE2 and pVWE2-pppS plasmids were electroporated into C. glutamicum using a standard protocol (10). The pVWE2-pppS plasmid was subsequently sequenced by Eurofins MWG Operon. The C. glutamicum:ubiA strain was obtained as described previously (8).

**Expression of uppS in C. glutamicum**—C. glutamicum-pVWE2-pppS cultures were grown overnight and then used to inoculate 50 ml of BHI (30 °C, 180 rpm, 10 μg/ml of tetracycline) and subsequently induced with 0.5 mM isopropyl β-d-thiogalactopyranoside at an A500 0.6 and incubated at 30 °C for 24 h. Cells were harvested (15 min, 7,000 × g), washed twice with phosphate-buffered saline, and resuspended in 50 mM MOPS (pH 7.9) containing lysozyme (0.2 mg/ml). The resuspended cell pellets were incubated at 37 °C for 1 h to allow cell lysis. The suspension was centrifuged (20 min, 27,000 × g, 20 min) and the supernatant checked for the presence of the Cg-UppS protein on SDS-PAGE gels.

**Effect of BTZ043 on C. glutamicum**—To determine the C. glutamicum and C. glutamicum:pVWE2-MIC of BTZ043, ~10^8 cells were used to inoculate 2 ml of BHI broth containing 0 to 20 μg/ml of BTZ043 in a stepwise gradient and the A500 measured up to 48 h. To determine the effect of increasing concentrations of BTZ043 on C. glutamicum:ubiA and C. glutamicum-pVWE2-pppS, ~10^8 cells were used (30 °C, BHI, 200 rpm) to inoculate 2 ml of BHI containing 0 and 20 μg/ml of BTZ043 and the A500 measured up to 48 h. The cultures were also grown for 48 h as above and the viability count was determined by spotting 10 μl of serially diluted cultures (up to dilution 10^-6) on BHI plates for C. glutamicum:ubiA and C. glutamicum-pVWE2-pppS. The MIC was defined as the minimal concentration required to completely inhibit 99% of the growth.

**BTZ043 Inhibits DPA Synthesis in C. glutamicum Strains**—To characterize the effect of BTZ043 on DprE1, p-[14C]Rpp was synthesized as described previously (11) and supplemented as a substrate for the in vitro synthesis of DPA. Accumulation of DPR in the presence of BTZ043 was chosen as a parameter to monitor the effect of BTZ043. Cell membranes from C. glutamicum and C. glutamicum:ubiA were prepared as described previously and assayed for DPA biosynthesis (12). Decaprenyl phosphate (50 μg, 5 mg/ml stored in ethanol, 1 μl) was dried under nitrogen and resuspended in buffer A (50 mM MOPS, 10 mM MgCl₂, pH 8.0) and sonicated. The basic assay mixture consisted of 400 μg of membranes and a P60 fraction (13), 25 mM ATP, 25 mM FAD, 25 mM NAD, 25 mM NADP, and BTZ043 (20 μg/ml in DMSO) in a final volume of 80 μl of buffer A and initiated by the addition of 65,000 cpm of p-[14C]Rpp. Reactions were incubated at 30 °C for 1 h and quenched by the addition of 4 ml of CHCl₃/CH₃OH/H₂O (10:10:3, v/v) and mixed for 15 min. The assay mixture was combined with 1.75 ml of CHCl₃ and 0.75 ml of H₂O, mixed for 15 min, and centrifuged at 3000 × g for 10 min. The lower organic phase was removed and washed twice with 2 ml of CHCl₃/CH₃OH/H₂O (3:47:48, v/v), centrifuged at 3000 × g for 15 min, recovered, and dried under nitrogen. The resulting products were resuspended in 20 μl of TATGCCTTCCAGATCTCG-3’.
To characterize the effect of BTZ043 on DprE1, DP-[14C]A was screen (Kodak) for 24 h.

DPA formation, BTZ043 (20 $\mu$g-[14C]pp as described previously (11). Briefly, to prevent (14). The resulting assay products were resuspended in 20 $\mu$L of CHCl$_3$/CH$_3$OH (2:1, v/v) and equal counts (10,000 cpm) were extracted as described previously and dried under nitrogen.

::

CH$_3$OH (2:1, v/v) and an aliquot was subjected to TLC analysis using silica gel plates (5735 Silica Gel 60F254, Merck) developed in CHCl$_3$/CH$_3$OH/H$_2$O/NH$_4$OH/CH$_3$COONH$_4$ (180:140:23:9:9, v/v), and visualized by phosphorimaging by exposing the TLCs to a phosphorimaging screen (Kodak) for 24 h.

DprE1 Is Functional in C. glutamicum and C. glutamicum::ubiA—To characterize the effect of BTZ043 on DprE1, DP-[14C]R was supplemented as a substrate for the in vitro synthesis of DP[14C]A (11). Cell membranes from C. glutamicum and C. glutamicum::ubiA were assayed for DPA biosynthesis activity as described previously. DP[14C]R was prepared using $p$-[14C]pp as described previously (11). Briefly, to prevent DPA formation, BTZ043 (20 $\mu$g/ml in DMSO) was added to the assay in a final volume of 80 $\mu$L (including buffer A, co-factor mixture, membranes, and P60). The resulting DP[14C]R was extracted as described previously and dried under nitrogen (14).

The resulting assay products were resuspended in 20 $\mu$L of CHCl$_3$/CH$_3$OH (2:1, v/v) and an aliquot was subjected to TLC analysis using silica gel plates (5735 Silica Gel 60F254, Merck) developed in CHCl$_3$/CH$_3$OH/H$_2$O/NH$_4$OH/CH$_3$COONH$_4$ (180:140:23:9:9, v/v) and visualized by autoradiography by exposure of TLCs to x-ray film (Kodak X-Omat). The DP[14C]R thus prepared was used as a substrate for determining the functionality of DprE1 in C. glutamicum and C. glutamicum::ubiA.

The basic assay mixture consisted of DP[14C]R (20,000 cpm) dried and resuspended in buffer A, 500 $\mu$g of membranes, and P60 fraction, co-factor mixture and BTZ043 (20 $\mu$g/ml in DMSO) in a final volume of 80 $\mu$L of buffer A. Reactions were incubated at 30 °C for 1 h and quenched by the addition of 4 ml of CHCl$_3$/CH$_3$OH/H$_2$O (10:10:3, v/v), and mixed for 15 min. The assay mixture was combined with 1.75 ml of CHCl$_3$ and 0.75 ml of H$_2$O, mixed for 15 min, and centrifuged at 3,000 × g for 10 min. The lower organic phase was removed and washed twice with 2 ml of CHCl$_3$/CH$_3$OH/H$_2$O (3:47:48, v/v), centrifuged at 3,000 × g for 15 min, recovered, and dried under nitrogen.

The resulting residue was resuspended in 20 $\mu$L of CHCl$_3$/CH$_3$OH (2:1, v/v) and equal counts (10,000 cpm) were subjected to TLC analysis using silica gel plates (5735 Silica Gel 60F254, Merck) developed in CHCl$_3$/CH$_3$OH/H$_2$O/NH$_4$OH/CH$_3$COONH$_4$ (180:140:23:9:9, v/v) and visualized by phosphorimaging by exposing the TLCs to a phosphorimaging screen (Kodak) for 24 h.

Increased Synthesis of Decaprenyl Phosphate Occurs in C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-ppS Treated with BTZ043—To analyze the effect of overexpression of decaprenyl-phosphate synthase on the cell wall component AG in C. glutamicum, overnight cultures of C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-ppS were used to subculture 20 ml of BHI media and grown to A$_{600}$ 0.4–0.6. The cultures were induced with 0.5 mM isopropyl $\beta$-D-thiogalactopyranoside and half of the culture was treated with BTZ043 at 0.75× MIC (15 $\mu$g/ml) for 2 h. Both treated and untreated cultures were labeled with 1.0 $\mu$Ci/ml of [14C]glucose (250–360 mCi/mm, PerkinElmer Life Sciences) and grown for 48 h (30 °C, 200 rpm). The mycolylarabinogalactan complex was isolated and sugar hydrolysis was performed on all the samples as previously described (15). The radioactive sugar samples were analyzed by loading equal counts (20,000 cpm) on HPTLC-cellulose plates developed three times in formic acid/water/tertiary butanol/methylethyl ketone (3:3:8:6) and visualized by autoradiography by exposing the TLC to x-ray film (Kodak X-Omat) for 4 days.

To analyze the effect of overexpression of decaprenyl-phosphate synthase on the cell wall component LAM/LM in C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-ppS were used to subculture 40 ml of BHI media and grown to A$_{600}$ 0.4–0.6. The cultures were induced with 0.5 mM isopropyl $\beta$-D-thiogalactopyranoside and labeled with 1.0 $\mu$Ci/ml of [14C]glucose (250–360 mCi/mm, PerkinElmer Life Sciences) and grown for 1 h (30 °C, 200 rpm). At time 0 h, the culture was split into 5-ml aliquots and BTZ043 (20 $\mu$g/ml) was introduced into half of the aliquots to afford treated and untreated cultures. The cultures were then sampled every 2 h by using 5-ml aliquots, centrifuged, and snap frozen using liquid nitrogen. LAM/LM analysis was performed on all the samples as previously described (8). The [14C]LAM/LM samples were analyzed by loading equal counts (3000 cpm) on SDS-PAGE gel and visualized by autoradiography by exposing the gels to x-ray film (Kodak X-Omat) for 14 days.

RESULTS

Effect of BTZ043 on C. glutamicum Wild Type and C. glutamicum::ubiA—The BTZ analog BTZ043 targets DprE1, which is a FAD-containing oxidoreductase conserved across
the Corynebacterineae (14). Sequence alignment analysis of
Rv3790 and NCg0187 (the open reading frames encoding
DprE1 from M. tuberculosis and C. glutamicum, respectively)
shows that the amino acid sequences of these two orthologs are
65% identical (5). Furthermore, Cg-DprE1 also contains all the
conserved active site residues, including Cys-414, which corre-
sponds to Cys-387 in Mt-DprE1 that forms a covalent semi-
mercaptal linkage with the activated nitroso group of BTZ043
(Fig. 1B). C. glutamicum and M. tuberculosis share very similar
cell wall architecture and due to its genetically tractable
genome, we have used C. glutamicum extensively as an excel-
lent model organism to study the molecular genetics of myco-
bacterial cell wall biosynthesis (8, 16, 17). C. glutamicum::ubiA
is a particularly interesting genetically modified strain, in as
much as it is completely devoid of arabinan in its cell wall. UbiA
encodes for a decaprenylphosphoryl-5-phospho-D-ribose
synthase and is non-essential in C. glutamicum. Because the
primary action of BTZ043 is reportedly due to the blockage of
cell wall arabinan biosynthesis, we cultured C. glutamicum
(wild type) and C. glutamicum::ubiA in liquid media over a 48-h
period and examined their susceptibility to BTZ043 at a range
of concentrations (Fig. 2, A and B). We determined that the
MIC of BTZ043 against wild type C. glutamicum is 20 μg/ml
(Fig. 2A). Although C. glutamicum::ubiA is inherently slow
growing, when we repeated the experiment with this mutant,
we observed that BTZ043 failed to inhibit the growth of this
arabinan-deficient mutant at a concentration corresponding to
the MIC of the wild type strain (Fig. 2B). Furthermore, we con-
ducted cell viability assays against C. glutamicum and C.
glutamicum::ubiA against increasing concentrations of BTZ043;
our data demonstrates that BTZ043 elicits a 1000-fold decrease
in cell viability against C. glutamicum in comparison to C.
glutamicum::ubiA in which BTZ043 failed to inhibit its growth
with no observable effect on cell viability (Fig. 2C). Therefore, C.
glutamicum::ubiA was determined to be resistant to BTZ043, in
comparison with the sensitive wild type C. glutamicum strain.

BTZ043 Inhibits DPA Synthesis in C. glutamicum and C.
glutamicum::ubiA with a Functional DprE1—We prepared mem-
bane preparations from C. glutamicum and C. glutamicum::ubiA,
which were subsequently examined for their ability to synthesize
DPA in the absence and presence of BTZ043. Initial experi-
ments used p-[14C]Rpp as an exogenous substrate with extracts
to monitor the conversion of p-[14C]Rpp to DP[14C]R and
DP[14C]A, which were evaluated using thin-layer chromatog-
raphy (TLC) followed by phosphorimaging (Fig. 3A). Analysis
of the 14C-labeled products by TLC clearly demonstrates that
C. glutamicum is able to utilize p-[14C]Rpp as a substrate and
synthesize DP[14C]A (Fig. 3A, lane 3). However, this conversion
to DP[14C]A is inhibited when reaction mixtures are incubated
with BTZ043, affording only DP[14C]R (Fig. 3A, lane 2). We

FIGURE 1. Structure and mode of inhibition of BTZ043. A, structure of the antitubercular compound BTZ043 that targets DprE1. B, BTZ043 is reduced into a
nitroso-derivative by the flavin cofactor of DprE1 (36). This electrophilic nitroso-derivative irreversibly binds Cys-387 in the active site of DprE1 (14, 35, 36) and
forms a semi-mercaptal adduct that renders the enzyme inactive.
also performed control experiments that included known standards (DPA, Fig. 3A, lane 1) and an enzyme blank (Fig. 3A, lane 6). However, no $^{14}$C-labeled products were seen when reactions were carried out using membranes prepared from C. glutamicum:ubiA either in the absence or presence of BTZ043 (Fig. 3A, lanes 4 and 5). The inability of C. glutamicum::ubiA to synthesize DPR/DPA is due to the absence of UbiA, which is a decaprenylphosphoryl-5-phospho-β-o-ribose synthase and is required for transfer of ribose 5-phosphate from $p$-$^{14}$C|Rpp to the lipid carrier decaprenyl monophosphate, and represents the first committed step in the biosynthetic pathway leading toward DPA formation.

To examine endogenous DprE1 activity directly in C. glutamicum and C. glutamicum::ubiA, C. glutamicum was examined for its susceptibility to BTZ043 in liquid media at a range of concentrations (0, 1, 4, 10, and 20 µg/ml). C. glutamicum::ubiA was examined for its susceptibility to BTZ043 in liquid media at a final concentration of 20 µg/ml (MIC for C. glutamicum), C. the cell viability of C. glutamicum and C. glutamicum::ubiA was tested at a range of BTZ043 concentrations (0, 1, 5, 10, 15, and 20 µg/ml) by spotting ~10$^6$-10$^8$ cells on BHISagar plates incubated at 30 °C for 48 h after treatment with 20 µg/ml of BTZ043.

Furthermore, the synthesis of DPA is blocked in the presence of BTZ043, thus indicating that both C. glutamicum and C. glutamicum::ubiA express a functional copy of DprE1 and is targeted effectively by BTZ043 (Fig. 3B, lanes 2 and 4). The apparent lack of BTZ043 inhibition of C. glutamicum::ubiA grown in culture is in stark contrast to the effect of BTZ043 on wild type C. glutamicum, and suggests that its mode of action is not due solely to DprE1 inhibition. This synthetic viable phenotype is manifested by the interruption of ubiA in C. glutamicum, which serves to alleviate sensitivity toward BTZ043. Because UbiA signifies the first committed step in the biosynthetic pathway leading toward DPA formation, we hypothesized that the primary effect of BTZ043 was to create a situation whereby exposure of C. glutamicum to the drug causes an accumulation of DPR, which is presumably toxic to the cells. Subsequently, this results in a failure by the cell to recycle decaprenyl phosphate and its limited availability causes a severe growth phenotype in Corynebacterineae. This BTZ043-induced chemical synthetic lethality is removed in C. glutamicum::ubiA because decaprenylphosphoribose phosphate is not being produced as a precursor to DPR formation, thus it allows the recycling of decaprenyl phosphate to occur unhindered, which is required for cell wall peptidoglycan biosynthesis.

Overexpression of Cg-uppS, a Prenylphosphate Synthase Protects C. glutamicum from Inhibition by BTZ043—The above data suggests that a fine balance exists in terms of the supply of decaprenyl phosphate for the biosynthetic pathways leading to DPA and lipid II formation, which ultimately result in the for-
Benzothiazinone-induced Synthetic Lethality

FIGURE 4. Effect of BTZ043 treatment on C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS. The growth and viability of C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS was examined in the presence of BTZ043. A, the growth rates of both C. glutamicum and C. glutamicum-pVWEx2-uppS were monitored in the absence of BTZ043 in liquid media for 48 h to check the effect of overexpression. B, the growth of C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS was measured in the presence of BTZ043 at 1× MIC (20 μg/ml) and 2× MIC (40 μg/ml). The optical density (A562) obtained for C. glutamicum-pVWEx2-uppS was higher than C. glutamicum-pVWEx2 at both 1× MIC and 2× MIC, indicating increased tolerance to BTZ043. C, cell viability counts were determined for C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS treated for 48 h at 1× and 2× MIC of BTZ043 after plating on BHI-agar plates.

FIGURE 5. Overexpression of uppS in C. glutamicum elicits an increase in decaprenyl phosphate synthesis. Cell-free extracts prepared from C. glutamicum-pVWEx2 (Cg-pVWEx2) and C. glutamicum-pVWEx2-uppS (Cg-pVWEx2-uppS) were examined for any increased biosynthesis of decaprenyl phosphate in the presence and absence of BTZ043 (20 μg/ml), by labeling the assay mixtures with exogenous [14C]IPP. Assays were quenched after a 1-h incubation at 30 °C and reaction products were extracted with organic solvent. Equal counts (20,000 cpm) were loaded onto a reverse phase silica gel plates (Silica Gel 60 RP-18 F254S, Merck) developed in methanol/acetic acid (82:18, v/v) and visualized by phosphorimaging.

UppS Overexpression Results in an Increased Synthesis of Decaprenyl Phosphate in C. glutamicum—We prepared membrane extracts from C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS, which were subsequently examined for their ability to synthesize decaprenyl phosphate in the absence and presence of BTZ043. We made use of [14C]IPP as an exogenous substrate supplied with membrane and cytosolic extracts to monitor the conversion of [14C]IPP to [14C]decaprenyl phosphate, which was evaluated using TLC followed by phosphorimaging (Fig. 5). Analysis of the 14C-labeled products by TLC clearly demonstrates that C. glutamicum is able to utilize [14C]IPP as a substrate and synthesize [14C]decaprenyl phosphate (Fig. 5). We conducted a densitometric analysis of the bands migrating on the TLC that correspond to decaprenyl phosphate and then calculated the apparent and actual cpm incorporation into [14C]decaprenyl phosphate from a total of 20,000 cpm loaded per reaction. C. glutamicum produced 481 ± 72 cpm/μg of protein, whereas overexpression of uppS in C. glutamicum increases the amount of [14C]decaprenyl phosphate by 77% (853 ± 16 cpm/μg of protein) and is illustrated by the increase in density of the band migrating on a TLC, corresponding to a standard decaprenyl phosphate (Fig. 5). When assays were supplemented with 20 μg/ml of BTZ043, synthesis of the decaprenyl phosphate pool appears to reduce in wild type C. glutamicum-pVWEx2 and remain relatively unchanged in C. glutamicum-pVWEx2-uppS, when compared with assays con-
Benzothiazinone-induced Synthetic Lethality

Overexpression of UppS Restores LAM Biosynthesis in C. glutamicum Treated with BTZ043—C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS were examined for their ability to synthesize LAM when treated with BTZ043 (20 μg/ml) (Fig. 7). Exponentially growing cultures of C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS were treated with BTZ043 and samples were removed every 2 h from BTZ043-treated cultures (as well as non-treated cultures). Following lipoglycan extraction, the presence of [14C]LAM was detected in SDS-PAGE gels by autoradiography (Fig. 7). The SDS-PAGE analysis showed that in C. glutamicum-pVWEx2 cultures treated with BTZ043 (20 μg/ml), production of LAM and LM is significantly reduced over time when compared with untreated cultures of C. glutamicum-pVWEx2 (Fig. 7, A and B). However, modest levels of LAM and LM are still being produced in C. glutamicum-pVWEx2 and C. glutamicum-pVWEx2-uppS treated with BTZ043 (Fig. 7, C and D). Thus, C. glutamicum-pVWEx2-uppS is able to continually synthesize LAM in the presence of BTZ043 at 2× MIC.

DISCUSSION

In 2009, it was reported that a new class of compounds called benzothiazinones exhibit extremely potent bactericidal activity against M. tuberculosis, as well as against drug-resistant clinical isolates, such as MDR-TB and XDR-TB strains (1). The most active compound, BTZ043, was shown by both biochemical and genetic experiments to target DprE1, a FAD-containing oxidoreductase responsible for the conversion of DPR to DPX (Fig. 8). Recently, the x-ray crystal structure of DprE1 in complex with BTZ043 was solved, highlighting the importance of a reactive Cys-387 residue in the active site, which forms a covalent semi-mercaptal linkage with the nitroso group of the molecule (Fig. 1B), thus providing the structural basis for suicide inhibition of DprE1 by BTZ (14, 19). DprE2 is a NADH-dependent reductase, which partners DprE1 as an “epimerization pair” that serves to reduce DPX to DPA (3). DPA is the sole substrate that is utilized by an array of membrane-embedded arabinofuranosyltransferases that are responsible for assembling the d-arabinan, which is an essential domain that is covalently attached to both LM and linear galactan, ultimately forming LAM and AG, respectively. EmbA, EmbB, and EmbC are three such arabinofuranosyltransferases that are responsible, in part, for the biosynthesis of d-arabinan; each of these enzymes are targeted by ethambutol, a front-line drug currently in clinical use as part of the directly observed treatment, short course regimen (20, 21). However, due to the potency of BTZ043, DprE1 has been lauded by many within the mycobacterial research community as a “magic drug target.” Targeting d-arabinan biosynthesis ultimately results in the removal of covalent linkage between peptidoglycan and the outer mycolate layers, and is a salutary approach to development of new antimycobacterial agents. However, this is insufficient evidence to explain the vulnerable nature of DprE1 as a magic drug target, sitting at a critical intersection of cell wall biosynthesis. We sought to investigate the vulnerability of DprE1 as a drug target and the results from this study serve to deconvolute the obser-
vation that implicates DprE1 as being a particularly susceptible target within cell wall biosynthesis.

Because *M. tuberculosis* and *C. glutamicum* share a remarkably similar cell wall and many of the genes involved in cell wall biosynthesis are syntenic, *C. glutamicum* represents an excellent model organism for studying the molecular genetics associated with cell wall biosynthesis in *M. tuberculosis* (22–26). In this regard, we have successfully generated a panel of *C. glutamicum* mutant strains that incur lesions in cell wall arabinan due to the loss of genes that encode specific arabinofuranosyltransferases or proteins involved in the processing of DPA. *C. glutamicum*::ubiA is a mutant strain that exhibits an extremely interesting phenotype, which is a complete loss of cell wall arabinan and covalently attached corynomycolates (8, 9, 27). This mutant becomes even more pertinent when we consider the fact that *C. glutamicum* is susceptible to the bactericidal activity of BTZ043, displaying an MIC of 20 μg/ml. The question must be asked, “why is *C. glutamicum*, a seemingly non-arabinan requiring member or the Corynebacterineae, susceptible to BTZ043, which has a reported mode of action as the blockage of D-arabinan formation by virtue of DprE1 inhibition?”

The data in this study does not exclude the possibility that killing results directly from toxicity associated with the accumulation of the DprE1 substrate DPR. Overproduction of Cg-UbiA could be toxic, either because of DP sequestration as we propose or through DPR accumulation. However, simultaneous overproduction of Cg-UppS and Cg-UbiA should either be lethal, if DPR accumulation is toxic, or rescue growth, if DP sequestration is actually killing the bacilli. We attempted to clarify this by amplifying Cg-ubiA from genomic DNA and cloning it into the inducible vector pEKEx2, which we have used successfully in previous studies on a number of membrane-embedded arabinosyltransferases (26, 28). We obtained a number of clones with the appropriate length, using a variety of PCR conditions and at least three different polymerases. However, upon sequencing different small mutations were present resulting in at least one non-acceptable amino acid change in our experiments. We then changed the cloning strategy by using another inducible vector pCLTON2, but again experienced similar non-acceptable mutations in Cg-ubiA. We therefore suggest that a weak expression even under non-inducing conditions is not tolerated when using an *E. coli* cloning host. We then attempted direct cloning in *C. glutamicum* but were again not successful in obtaining a correctly validated gene sequence. Altogether, this points to a delicate and tight control of Cg-ubiA expression, which is in line with the physiological data obtained in our studies.

However, our study does highlight the role of UppS, a decaprenyl-phosphate synthase, in rescuing *C. glutamicum* from the bactericidal effect of BTZ043 (Fig. 8). The implication here being that inhibition of DprE1 becomes acutely important when DPR begins accumulating in the cell membrane, and represents a metabolic “dead end” that halts the recycling of decaprenyl phosphate back into cell wall biosynthetic processes (Fig. 8). When UppS is overexpressed in *C. glutamicum*, the resultant phenotype is an increased tolerance to BTZ043 (Fig. 8). This increased tolerance can be attributed to the increased biosynthesis of decaprenyl phosphate, which is required primarily for the production of lipid II, an essential intermediate involved in peptidoglycan biosynthesis. This data is in accordance with our previous observations that although *C. glutamicum* has a cell wall with enough plasticity to dispense with D-arabinan, it still requires peptidoglycan to protect against the external osmotic stress across the cytoplasmic membrane (15, 29). In this regard, a decrease in the expression of uppS from *Bacillus subtilis* has been shown to confer increased suscepti-
bility to many late-acting cell wall antibiotics, such as β-lactams (30). However, this effect was significantly more pronounced when experiments were repeated using fosfomycin and D-cycloserine, which interfere with the early stages of peptidoglycan biosynthesis (30). In Gram-positive organisms, such as *B. subtilis*, the undecaprenyl phosphate (C55-P) is the major polyprenyl carrier used in the synthesis of peptidoglycan as well as other cell wall components such as wall teichoic acids (31, 32). Furthermore, similar effects have been demonstrated in *Staphylococcus aureus*, whereby Targocil, an inhibitor of late-stage cell wall teichoic acid biosynthesis, causes an accumulation of undecaprenyl-linked intermediates, thus preventing recycling of the undecaprenyl-phosphate lipid carrier (33). Whereas lipid II is important for peptidoglycan biosynthesis, *Corynebacteri- neae*, such as *M. tuberculosis* and *C. glutamicum*, also use decaprenyl phosphate in the production of DPA and decaprenyl phosphomannose, which is directed toward the formation of β-arabinan and lipomannan (which is the precursor to LAM), respectively. We sought to investigate the effects of *uppS* overexpression on the biosynthesis of these cell wall polysaccharides when exposed to BTZ043. Our analysis of the total sugar content from mAGP isolated from *C. glutamicum*-pVWEx2 revealed an overall decrease in arabinose upon treatment with BTZ043. This result was expected because BTZ inhibits the production of the sole decaprenyl phosphate-based arabinose donor, DPA, which is required for biosynthesis of β-arabinan. However, we were able to counter this effect by overexpressing *uppS*, which resulted in a continued incorporation of [14C]arabinose into mAGP even up to BTZ043 concentrations that were 0.75 × MIC (Fig. 6). Similarly, examination of the LAM pool from the BTZ043-treated *C. glutamicum*-pVWEx2 strain demonstrates a decrease in both LM and LAM, because both molecules employ decaprenyl phosphate-based sugar donors, in the form of decaprenyl phosphomannose and DPA. Because we

---

**FIGURE 8.** A schematic representation of the proposed mechanism of action of BTZ on DprE1 inhibition and decaprenyl phosphate recycling. DPA synthesis is crucial for biosynthesis of arabinan components of LAM and AG and begins with transfer of ribose phosphate from phosphoribosyl pyrophosphate (pRPP) to decaprenyl phosphate and follows the pathway where DprE1/E2 generates DPA from DPR. Treatment with BTZ043 (star) results in accumulation of DPR (highlighted in a broken circle) stalling LAM and AG biosynthesis and a block in decaprenyl phosphate recycling. Overexpression of UppS, the prenyl synthase (highlighted in a broken circle) causes restoration of the recycling pathway by supplementing enough decaprenyl phosphate for synthesis of cell wall components.
observe a commensurate decrease in the formation of LM and LAM upon exposure to BTZ043, the notion that BTZ043 acts by inhibiting DPA formation is a false dichotomy. Indeed, over-expression of upPS restores the synthesis of both LM and LAM lipoglycan pools in the presence of BTZ043 (Fig. 7).

Mycobacteria can develop natural resistance to BTZs due to the occurrence of a single mutation in the operon coding for the Cys-387 residue in the active site of DprE1. This reaction is catalyzed either by DprE1 itself (36) or by other oxygen-insensitive nitroreductases (34). Bacterial nitroreductases, such as NfnB are capable of rendering the drug inactive by reducing the nitro group to the corre-

Bacterial nitroreductases, such as NfnB are capable of rendering the nitroso-derivative to react with the Cys-387 residue in the active site of DprE1. This reaction is catalyzed either by DprE1 itself (36) or by other oxygen-insensitive nitroreductases (34). Bacterial nitroreductases, such as NfnB are capable of rendering the drug inactive by reducing the nitro group to the corresponding amine as observed in case of M. smegmatis (34). In this regard, the results obtained from this study imply that increased expression of decaprenyl phosphate synthase (upPS) might provide an alternative mechanism for Corynebacteri-

aureum, respectively (1). BTZ043 functions as a pro-drug (34, 35) that requires reduction of the nitro group to an electrophilic nitroso-derivative to react with the Cys-387 residue in the active site of DprE1. This reaction is catalyzed either by DprE1 itself (36) or by other oxygen-insensitive nitroreductases (34). Bacterial nitroreductases, such as NfnB are capable of rendering the drug inactive by reducing the nitro group to the corresponding amine as observed in case of M. smegmatis (34). In this regard, the results obtained from this study imply that increased expression of decaprenyl phosphate synthase (upPS) might provide an alternative mechanism for Corynebacteri-

**REFERENCES**


20. Belanger, A. E., Besra, G. S., Ford, M. E., Mikusová, K., Belisle, J. T., Bren-

