AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Targeting the Nonmevalonate Pathway in *Burkholderia cenocepacia* Increases Susceptibility to Certain β-Lactam Antibiotics

Andrea Sass,[a] Annelien Everaert,[a] Heleen Van Acker,[a] Freija Van den Driessche,[a] Tom Coenye[a]

[a]Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium

ABSTRACT The nonmevalonate pathway is the sole pathway for isoprenoid biosynthesis in *Burkholderia cenocepacia* and is possibly a novel target for the development of antibacterial chemotherapy. The goals of the present study were to evaluate the essentiality of *dxr*, the second gene of the nonmevalonate pathway, in *B. cenocepacia* and to determine whether interfering with the nonmevalonate pathway increases susceptibility toward antibiotics. To this end, a rhamnose-inducible conditional *dxr* knockdown mutant of *B. cenocepacia* strain K56-2 (*B. cenocepacia* K56-2*dxr*) was constructed, using a plasmid which enables the delivery of a rhamnose-inducible promoter in the chromosome. Expression of *dxr* is essential for bacterial growth; the growth defect observed in the *dxr* mutant could be complemented by expressing *dxr in trans* under the control of a constitutive promoter, but not by providing 2-*C*-methyl-D-erythritol-4-phosphate, the reaction product of DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase). *B. cenocepacia* K56-2*dxr* showed markedly increased susceptibility to the β-lactam antibiotics aztreonam, ceftazidime, and cefotaxime, while susceptibility to other antibiotics was not (or was much less) affected; this increased susceptibility could also be complemented by *in trans* expression of *dxr*. A similarly increased susceptibility was observed when antibiotics were combined with FR900098, a known DXR inhibitor. Our data confirm that the nonmevalonate pathway is essential in *B. cenocepacia* and suggest that combining potent DXR inhibitors with selected β-lactam antibiotics is a useful strategy to combat *B. cenocepacia* infections.

KEYWORDS nonmevalonate pathway, *Burkholderia cenocepacia*, DXR, *Burkholderia cepacia* complex, beta-lactam antibiotics

Isoprenoids are a diverse class of naturally occurring compounds with widely varied roles in physiological processes in all living organisms. Animals (including humans), fungi, *Archaea*, and some bacteria synthesize isoprenoids using the mevalonate pathway (1). An alternative metabolic pathway for the biosynthesis of isoprenoids, the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway or nonmevalonate pathway, was discovered in the late 1980s (2). This pathway starts with the condensation of D-glyceraldehyde-3-phosphate and pyruvate to DOXP, catalyzed by the enzyme DOXP-synthase (DXS; in *Burkholderia cenocepacia* strain J2315, encoded by BCAM0911). Next, DOXP is converted to 2-*C*-methyl-D-erythritol-4-phosphate (MEP) by DOXP-reductoisomerase (DXR; encoded by BCAL2085). Subsequently, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) is formed by CDP-ME synthase (IspD; encoded by BCAL2016). CDP-ME is then phosphorylated by CDP-ME kinase (IspE; encoded by BCAL0802), forming CDP-ME-2-phosphate, which is subsequently converted to 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MER-cPP) by MER-cPP synthase (IspF, encoded by BCAL2015). This cyclic diphosphate molecule is the precursor of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (3, 4). The rate-limiting

Received 22 December 2017 **Returned for modification** 23 January 2018 **Accepted** 7 February 2018

Accepted manuscript posted online 12 February 2018

Citation Sass A, Everaert A, Van Acker H, Van den Driessche F, Coenye T. 2018. Targeting the nonmevalonate pathway in *Burkholderia cenocepacia* increases susceptibility to certain β-lactam antibiotics. Antimicrob Agents Chemother 62:e02607-17. https://doi.org/10.1128/AAC.02607-17.

Address correspondence to Tom Coenye, Tom.Coenye@Ugent.be.

step in the nonmevalonate pathway is the conversion of DOXP to MEP, which is catalyzed by DXR. Fosmidomycin, a natural phosphonic acid antibiotic, and FR900098, an *N*-acetyl derivative of fosmidomycin, are effective inhibitors of purified DXR from several bacterial species, but often have limited effects on whole bacterial cells (5, 6).

Burkholderia cepacia complex (Bcc) bacteria are highly resistant to most antibiotics and are most known for their ability to cause life-threatening respiratory tract infections in cystic fibrosis (CF) patients (7). Bcc bacteria, including *B. cenocepacia*, use the nonmevalonate pathway as sole pathway for isoprenoid biosynthesis. As this pathway is not present in human cells, it is a possible novel target for the development of antibacterial chemotherapy against Bcc infections (8). DXR (encoded by BCAL2085) was predicted to be essential in *B. cenocepacia* (9); a more recent study predicted that all five genes of the nonmevalonate pathway would be essential in *B. cenocepacia* J2315 (10). This makes the enzymes of the nonmevalonate pathway interesting candidates as drug targets. However, resistance against fosmidomycin, FR900098, and several other fosmidomycin derivatives was previously observed in a large collection of Bcc strains, and could partly be explained by the lack of a glycerol-3-phosphate transporter (GlpT) system, which is required for the import of fosmidomycin and FR900098 (8). However, Bcc species possess an alternative GlpT system, which can be induced by the addition of glucose-6-phosphate, leading to increased uptake of fosmidomycin. Unfortunately, a fosmidomycin resistance gene (*fsr*), which is involved in efflux of fosmidomycin and FR900098, is upregulated in treated cultures and is able to compensate for the increased fosmidomycin uptake in the presence of glucose-6-phosphate (8, 11). Isoprenoids are precursors for the biosynthesis of hopanoids, which are involved in regulation of membrane stability, permeability, and fluidity, and which contribute to the intrinsic resistance of Bcc bacteria against various antibiotics, including polymyxins, erythromycin, and chlorhexidine (11–13). Deletion of genes (BCAM2831 and BCAS0167) involved in hopanoid biosynthesis (squalene-hopene cyclases) resulted in the loss of swarming and swimming motility, suggesting that hopanoids play an important role in the physiology of *B. cenocepacia* (13). Several studies provide evidence that reducing the amount of hopanoids in the membrane of Bcc bacteria can increase susceptibility to antibiotics (11–15). For example, Malott et al. described resistance of *Burkholderia multivorans* isolates to fosmidomycin and colistin when used alone, but they observed antimicrobial synergy when both antibiotics were used in combination (11).

The goals of the present study were to determine whether *dxr* is truly essential in *B. cenocepacia* and to investigate whether interfering with the nonmevalonate pathway increased the susceptibility of *B. cenocepacia* to antibiotics.

RESULTS AND DISCUSSION

Construction of conditional mutants of *B. cenocepacia* K56-2. We constructed a *B. cenocepacia* K56-2 mutant (designated *B. cenocepacia* K56-2*dxr*), in which the expression of *dxr* (homolog of BCAL2085) is controlled by the rhamnose-inducible promoter P_{rha}. The correct alignment of P_{rha} with *dxr* was verified with Sanger sequencing (see additional data set 1 in the supplemental material). Quantitative real-time PCR (qPCR) was used to determine *dxr* expression in K56-2 and K56-2*dxr* under various conditions (Fig. 1). In the absence of rhamnose, *dxr* expression in K56-2*dxr* is very low (but not completely switched off), while in the presence of rhamnose, the mRNA expression level was similar to that observed in the wild-type strain.

Differences in planktonic growth between *B. cenocepacia* K56-2 and K56-2*dxr*. Growth curves of K56-2 and K56-2*dxr* obtained in a previously described basal salts medium (BSM-O) (16) in the presence and absence of rhamnose are shown in Fig. 2. K56-2*dxr* shows a growth defect, confirming that DXR is essential in K56-2, as previously suggested (9, 10). Growth was completely inhibited in K56-2*dxr* if the precultures used for the growth curves were grown in the absence of rhamnose, but growth resumed after a delay. Consecutive batch cultures in medium without rhamnose, starting from a preculture grown in the presence of rhamnose, show that growth is only inhibited after several generations. The growth rate then recovers to near wild-type levels upon

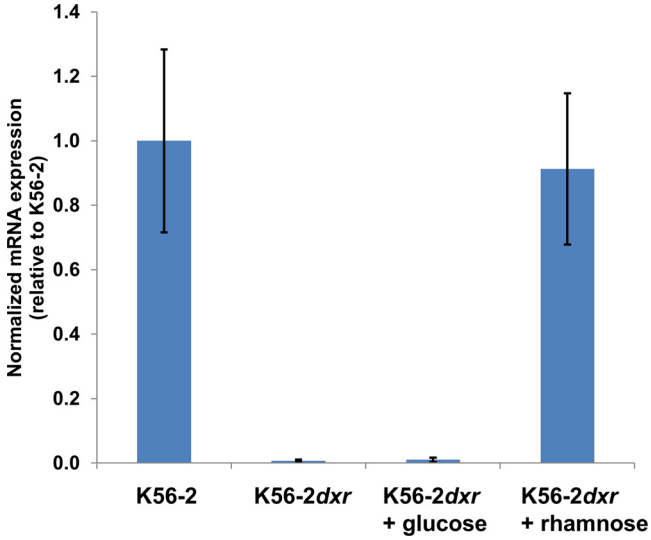

FIG 1 Relative mRNA expression of *dxr* measured by qPCR. Expression levels shown are relative to that of the expression observed in *B. cenocepacia* K56-2. Error bars represent range; $n = 2$.

further cultivation, and remains at this level (see Fig. S1 in the supplemental material). This indicates that, after withdrawal of rhamnose, levels of *dxr* mRNA and/or DXR protein initially remain sufficient to sustain growth. However, after several generations the protein is depleted, leading to complete growth inhibition. This is followed by selection of suppressor mutants, which grow at rates nearly identical to that of the wild type. The suppressor mutant(s) are most likely "false suppressor mutants," in which the rhamnose-inducible promoter and/or its regulators are mutated in such a way that the conditional *dxr* expression is converted to constitutive expression (17). Similar growth profiles were observed in other media (see Fig. S2 in the supplemental material), confirming that the phenotype is not specific for BSM-O medium. While we did not evaluate the effect of lack of *dxr* expression *in vivo*, it is worth mentioning that in a synthetic cystic fibrosis medium (18), a growth defect was also observed, suggesting that under the nutritional conditions encountered in the lungs of CF patients, *dxr* expression is important.

The growth defect in K56-2*dxr* could be complemented by expression of *dxr in trans* from a constitutive promoter (Fig. 3). However, growth could not be restored by adding MEP, the reaction product of DXR (highest concentration tested, 5 mM) (Fig. 4); this is likely due to a lack of uptake of MEP.

Differences in *in vitro* antimicrobial susceptibility. MICs of fosmidomycin and FR900098 were >500 μg/ml for *B. cenocepacia* K56-2 and K56-2*dxr*, irrespective of whether rhamnose was present. This is in agreement with concentrations previously reported for *B. cenocepacia* strain LMG 16656 (8). Subsequently, we determined the MIC for a range of antibiotics, for *B. cenocepacia* K56-2, *B. cenocepacia* K56-2 in the presence of subinhibitory concentration of FR900098 (250 μM), *B. cenocepacia* K56-2*dxr*, *B. cenocepacia* K56-2*dxr* in the presence of rhamnose, and for the complemented strain (Table 1). Deletion of *dxr* resulted in an increased susceptibility toward all β-lactam antibiotics tested, with the most pronounced differences for aztreonam (16-fold difference), the cephalosporin ceftazidime (16-fold), and cefotaxime (32-fold). Although differences in MIC were also observed for the carbapenems tested, these were lower (2-fold for meropenem and 4-fold for imipenem). For the other antibiotics, difference in MIC between the wild type and the *dxr* mutant were either small (bacitracin, chloramphenicol, ciprofloxacin, tetracycline, and rifampin) or no difference was observed (erythromycin and tobramycin). For the wild-type strain, the addition of rhamnose did not change the MIC of any of the antibiotics tested; the addition of rhamnose to the *dxr* mutant returned all MICs to wild-type levels, indicating that the reduced expression of

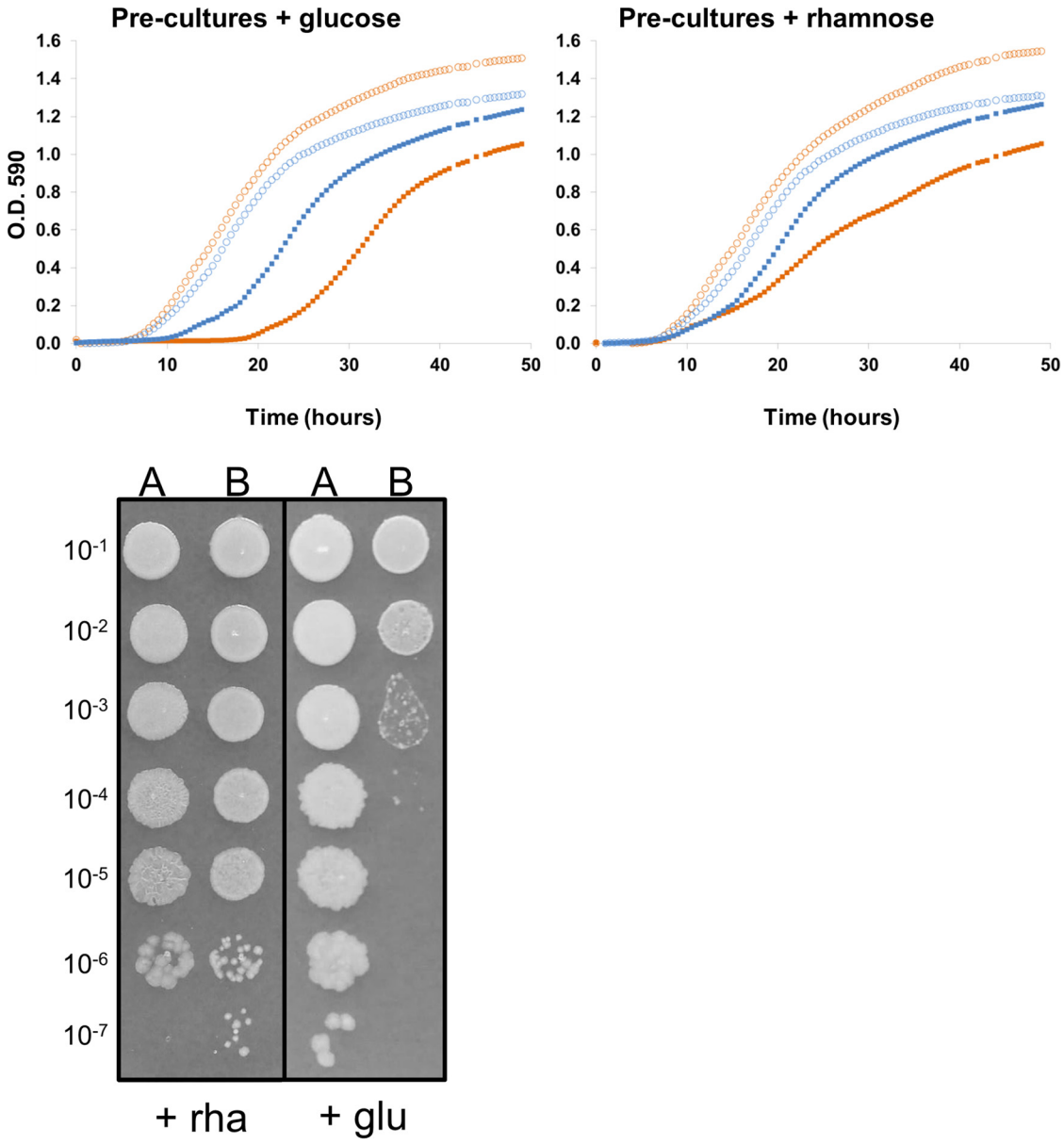

FIG 2 Growth of *B. cenocepacia* K56-2 wild type and the K562*dxr* conditional mutant in permissive and nonpermissive conditions. Top panels: planktonic growth. Solid squares, K56-2*dxr*; open circles, K56-2 wild type; orange, BSM-O with 0.5% glucose; blue, BSM-O with 0.5% rhamnose. Absence of rhamnose inhibits growth of K56-2*dxr*, but growth resumes after a delay (left). Addition of rhamnose enables growth of the conditional mutant to near wild-type levels. Growth inhibition in the presence of glucose is less pronounced if precultures are grown in the presence of rhamnose (right). Growth curves are representative of three biological replicates, performed in 96-well round bottom microtiter plates. Bottom panel: colony formation on agar plates. A, K56-2 + pSCrhaB2; B, K56-2*dxr*. Cultures grown in BSM-O with 200 mg/liter trimethoprim without addition of sugars were normalized to an optical density at 590 nm (OD_{590}) of 1.0, and then a 1:10 dilution series was performed. Aliquots of 15 μl of the diluted cultures were spotted onto BSM-O (with 200 mg/liter trimethoprim) with 0.5% rhamnose (left) or 0.5% glucose (right).

dxr was the cause for the MIC changes (Table 1). Expressing *dxr in trans* under the control of a constitutive promoter returned MIC levels of antibiotics (with the exception of bacitracin) for the *dxr* mutant back to wild-type levels. Interestingly, when the DXR inhibitor FR900098 was added along with the antibiotics, MICs for the β-lactam antibiotics were (much) lower and in the same range as that observed for the *dxr* mutant (Table 1). Compared to FR900098, the potentiating effect of fosmidomycin was less pronounced (see Fig. S3 in the suplemental material), which is in line with the previously observed lower antimicrobial activity of the latter (8).

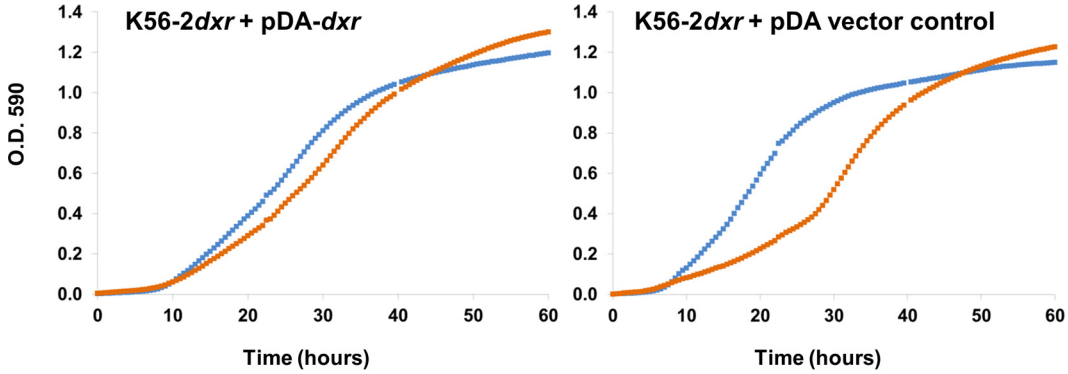

FIG 3 Expression of *dxr in trans* complements the growth defect observed in *B. cenocepacia* K56-2*dxr*. Growth medium, LBB with 200 mg/liter trimethoprim. Orange, 0.5% glucose; blue, 0.5% rhamnose. pDA-*dxr* expresses *dxr* constitutively. Growth curves represent the average of 4 biological replicates, performed in 96-well round bottom microtiter plates.

The effect of reduced *dxr* expression or of adding FR900098 was most pronounced for aztreonam, ceftazidime, and cefotaxime. Aztreonam and cefotaxime both target penicillin-binding protein (PBP) 3 in *Escherichia coli* (19, 20). In *Burkholderia pseudomallei*, the deletion of BSSP1219, the gene encoding a PBP3, results in ceftazidime resistance, suggesting that ceftazidime targets PBP3; a homolog of BSSP1219 is present in *B. cenocepacia* (21). PBP3 is involved in peptidoglycan synthesis, as it is part of the peptidoglycan synthesis core of the divisome, and it modulates the interaction of FtsW with lipid II (22). Lipid II [undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc] is the precursor for peptidoglycan synthesis, and the lipid undecaprenyl phosphate (C_{55}-P) is synthesized from C_5 isopentenyl units (23), which are in turn produced by the nonmevalonate pathway (24). Interestingly, reduced *dxr* expression and the addition of the DXR inhibitor FR900098 also reduced the MIC for bacitracin (by 2- and 4-fold, respectively). Bacitracin inhibits cell wall formation by binding to C_{55}-isoprenyl pyrophosphate (C_{55}-IPP), which is responsible for transporting peptidoglycan precursors across the bacterial cell membrane (25). Our data suggest that reducing *dxr* expression in *B. cenocepacia* K56-2 or adding FR900098 (partially) inhibits cytoplasmic steps of peptidoglycan biosynthesis, leading to lower levels of C_{55}-P and C_{55}-IPP and increased susceptibility to antibiotics that target interaction partners of C_{55}-P and C_{55}-IPP. Carbapenems, such as meropenem and imipenem, target PBP2 (26). In contrast to PBP3 (which is part of the divisome), PBP2 is part of the elongasome (responsible for

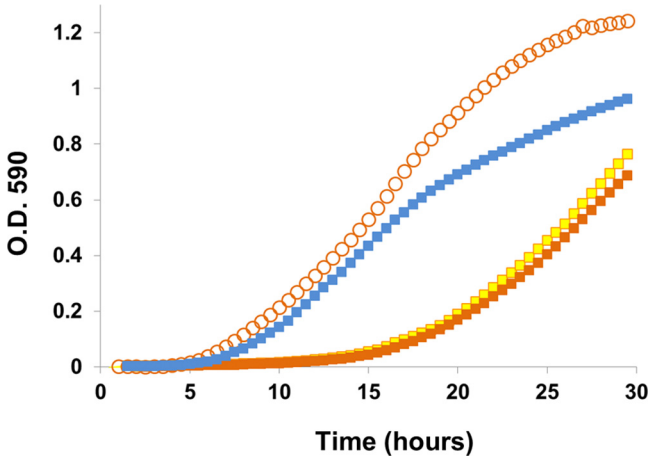

FIG 4 Growth of *B. cenocepacia* K56-2*dxr* in the presence of 2-*C*-methyl-D-erythritol 4-phosphate (MEP). Growth medium, BSM-O. Solid squares, K56-2*dxr*; open circles, K56-2 wild type; orange, 0.5% glucose; blue, 0.5% rhamnose; yellow, 5 mM MEP.

TABLE 1 MIC values of antibiotics belonging to different classes for *B. cenocepacia* K56-2, *B. cenocepacia* K562*dxr*, and the complemented mutant[a]

Antibiotic	MICs (μg/ml)[b] against:					
	Wild type	*dxr* mutant	Wild type + rhamnose	*dxr* mutant + rhamnose	*dxr* mutant + pDA-*dxr*	Wild type + FR900098
Aztreonam	>2,048	128	>2,048	>2,048	>2,048	64
Ceftazidime	128	8	128	128	256	8
Cefotaxime	2,048	64	2,048	2,048	1,024	16
Meropenem	16	8	16	16	32	8
Imipenem	128	32	128	128	128	32
Bacitracin	32,000	16,000	32,000	32,000	8,000	8,000
Chloramphenicol	32	16	32	32	16	32
Ciprofloxacin	4	2	4	4	4	4
Erythromycin	512	512	512	512	512	512
Rifampin	16	32	32	32	32	16
Tetracycline[c]	8	4	8	8		2
Tobramycin	256	256	256	256	512	256

[a]Determined in the presence and absence of rhamnose (0.5%) or FR900098 (250 μM).
[b]Determined in LBB.
[c]The MIC for tetracycline could not be measured in the *dxr* mutant expressing *dxr in trans*, as this plasmid contains a tetracycline resistance marker.

elongation of rod-shaped cells) and interacts with several other proteins in the Rod system (27). The different targets of the β-lactam antibiotics tested (PBP2 versus PBP3) could explain the different effects of reduced *dxr* expression and the potentiating activity of FR900098. The genome of *B. cenocepacia* strain J2315 (very similar to that of strain K56-2) encodes two PBP3s (BCAM1362 and BCAL3468 genes, 49% similarity); this likely explains the strain's extreme resistance to aztreonam (MIC, >2,048 μg/ml), ceftazidime (MIC, 128 μg/ml), and cefotaxime (MIC, 2,048 μg/ml) observed in *B. cenocepacia* (in contrast, for *Pseudomonas aeruginosa*, EUCAST clinical breakpoints for aztreonam and ceftazidime are 16 and 8 μg/ml, respectively).

Conclusions. Our data confirm that *dxr*, and by extension the nonmevalonate pathway, is essential in *B. cenocepacia* and is a potentially interesting drug target. Interfering with this pathway, either by reducing the expression of *dxr* (coding for the key enzyme DXR) or by inhibiting DXR activity using the acetylated fosmidomycin derivative FR900098, drastically reduced the MIC of certain β-lactam antibiotics. This was most obvious for β-lactam antibiotics that target interaction partners of C_{55}-P and C_{55}-IPP, explaining the link between reduced isoprenoid synthesis and resistance to these antibiotics. High concentrations of aztreonam (up to 985 mg/kg sputum) can be obtained after inhalation therapy (28) and these concentrations are above the MIC that is obtained in the wild type in combination with FR900098. It will be interesting to explore whether the combination of carefully selected β-lactam antibiotics and DXR inhibitors could provide an alternative strategy to combat *B. cenocepacia* infections.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* and *B. cenocepacia* strains were grown in Luria-Bertani agar or broth (LBA or LBB) or in more defined and phosphate-buffered media, BSM-O or M9-O (16). BSM-O is based on a basal salt medium (18.6 mM K_2HPO_4, 7.2 mM NaH_2PO_4, 37.4 mM NH_4Cl, 0.1 g/liter nitrilotriacetic acid, 0.2 g/liter $MgSO_4{\cdot}7\ H_2O$, 0.012 g/liter $FeSO_4{\cdot}7\ H_2O$, 0.003 g/liter $MnSO_4{\cdot}H_2O$, 0.003 g/liter $ZnSO_4{\cdot}7\ H_2O$, and 0.001 g/liter $CoSO_4{\cdot}7\ H_2O$) (29) and supplemented with yeast extract (5 g/liter), Casamino Acids (2 g/liter), and 0.5% (wt/vol) glycerol (16). M9-O is based on M9 (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8 mM NaCl, and 10 mM NH_4Cl) and was supplemented with the same organic substrates as those for BSM-O. Media were supplemented with 0.5% (wt/vol) rhamnose or glucose, 50 μg/ml gentamicin (Gen), 40 μg/ml kanamycin (Kan), trimethoprim (Tmp, 50 or 200 μg/ml), or tetracycline (Tet; 20 or 200 μg/ml) (all from Sigma-Aldrich) as required. Liquid cultures were grown in a shaking incubator at 37°C.

Construction of conditional *dxr* mutant. A conditional knockdown mutant was constructed by replacing the native promoter of the target gene *dxr* (BCAL2085 in *B. cenocepacia* J2315) with the plasmid-borne rhamnose-inducible promoter P_{rha} (16, 30), using homologous recombination. Primers are listed in Table 3. The genome contigs of *B. cenocepacia* K56-2 were obtained from the *Burkholderia* Genome Database (http://www.burkholderia.com/; whole genome shotgun sequence of *Burkholderia cenocepacia* K56-2Valvano, RefSeq NZ_ALJA02000015). Primers DXR F1/DXR R2 were designed to amplify

TABLE 2 Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference or source
B. cenocepacia strain		
K56-2	Wild type, ET-12 lineage, CF isolate	Lab collection
K56-2*dxr*	K56-2 P_{rha}::*dxr*	This study
K56-2*dxr* + pDA-	K56-2*dxr* vector control for complementation experiments	This study
K56-2*dxr* + pDA*dxr*	K56-2*dxr*, complemented with constitutively expressed *dxr*	This study
K56-2 + pSCrhaB2	Wild type, transformed with a plasmid conveying Tmp resistance	This study
E. coli strains		
DH5α	For propagation of replicative plasmids pDA- and pDA-*dxr*	Lab collection
DH5α λpir	λpir, for propagation of suicide plasmid pSC200 and its derivatives	Biomedal
HB101	pRK2013; helper strain for triparental matings	Lab collection
Plasmids		
pRK2013	ori_{colE1}, RK2 derivative, Kan^r, *mob*$^+$, *tra*$^+$	35
pSC200	pGpΩTp derivative, ori_{R6K}, *rhaR*, *rhaS*, P_{rha}, *e-gfp*, *dhfr*, Tmp^r	16
pSC200*dxr*	pSC200 carrying fragment of *dxr*	This study
pDA$^-$	Derivative of pDAI-SceI, ori_{pBBR1}, Tet^r, I-SceI gene removed and self-ligated	This study
pDA-*dxr*	pDA carrying the *dxr* gene under a constitutive *dhfr* promoter	This study
pSCrhaB2	ori^{pBBR1}, *rhaR*, *rhaS*, P_{rhaB}, *Tmp*^{r}, *mob*$^+$	31

a 283-nucleotide (nt) fragment spanning the 5′ region of *dxr*. The fragment was digested with restriction enzymes XbaI and NdeI (Promega) and ligated into digested pSC200 downstream of P_{rha}, resulting in plasmid pSC200*dxr*. The recombinant plasmid was transformed into *E. coli* DH5α λpir with the heat shock method using LBA supplemented with 50 μg/ml Tmp and 0.5% glucose as selective agar. Exconjugants were screened for presence of insert by PCR using primers rhaF/200-MCS-R. The recombinant plasmid was transferred into *B. cenocepacia* K56-2 by triparental mating (16). Overnight liquid cultures of *B. cenocepacia* K56-2, *E. coli* HB101 (pRK2013), and *E. coli* donor strain were mixed in equal volumes, and 300 μl of the mix was spotted onto LBA with 0.5% rhamnose and incubated at 37°C for 6 h. Cells were washed off the agar with physiological saline and plated on LBA containing 50 μg/ml Gen (to select against *E. coli*), 200 μg/ml Tmp and 0.5% rhamnose. Plates were incubated at 37°C for 48 h, and exconjugants were screened for correct insertion of the plasmid by PCR using primers rhaF/DXR R4. A successful exconjugant (here referred to as K56-*2dxr*) was subcultured on LBA plus 200 μg/ml Tmp and 0.5% rhamnose, and the respective PCR product was Sanger sequenced (GATC Biotech, Additional data set 1).

To allow growth experiments on LBB with Tmp, *B. cenocepacia* K56-2 wild type was transformed with a replicative plasmid (pSCrhaB2) carrying the *dhfr* gene (31).

Construction of *B. cenocepacia* K56-2*dxr* expressing *dxr in trans*. To complement the *dxr* mutant, a modified version of pDAI-SceI, a plasmid used for constitutive I-SceI (homing endonuclease) expression from the constitutive P1 promoter for *dhfrIIb* (32), was used. I-SceI was excised using NdeI and XbaI and the plasmid was gel cleaned. The linear plasmid was then either blunted and self-ligated, giving rise to the vector control plasmid pDA$^-$, or ligated with the *dxr* gene, amplified from the *B. cenocepacia* K56-2 genome using primers dxr-exprF and dxr-exprR and restricted with NdeI and XbaI, giving rise to pDA-*dxr*. Correct insertion of *dxr* was screened by PCR using primers pDA-F and rha1R. Plasmids pDA$^-$ and pDA-*dxr* were transformed into K56-*2dxr* by triparental mating, using LBA with 200 Tet, 200 Tmp, 50 Gen, and 0.5% rhamnose as selective medium. *dxr* on pDA-*dxr* of a successful exconjugant of K56-*2dxr* was Sanger-sequenced (GATC Biotech, Additional data set 1).

RNA extraction and cDNA synthesis. Cells of wild-type and mutant *B. cenocepacia* K56-2 were cultured in either M9-O or LBB. Both media were supplemented with 0.5% rhamnose or glucose as required. After 24 h incubation at 37°C, cells were collected by centrifugation and RNA was extracted using the RiboPure bacteria kit (Ambion) according to the manufacturer's instructions. RNA concentra-

TABLE 3 Primers

Name	Sequence[a]	Purpose
DXR F1	TTATTAATACATATGCAAAAACGTCTGACATTGCTC	Amplification of *dxr* fragment
DXR R2	TATCTAGAAGCCGTCGCTCTTCGACAC	Amplification of *dxr* fragment
rhaF	CACGTTCATCTTTCCCTGGT	Confirmation of fragment insertion and plasmid recombination
200-MCS-R	AACACTTAACGGCTGACATGG	Confirmation of insertion of fragment in plasmid
DXR R4	AGGCCCTTGTTCATCATCG	Confirmation plasmid recombination in K56-2*dxr*
dxr-exprF	AATAATAATCATATGCAAAAACGTCTGACATTGCTC	Amplification of *dxr* for complementation
dxr-exprR	AATTCTAGATTCATGAGCACCCCATTCAGAC	Amplification of *dxr* for complementation
pDA-F	GCAACTGGTCCAGAACCTTG	Confirmation of *dxr* insertion into pDA$^-$
rha1R	CTCTCATCCGCCAAAACAGC	Confirmation of *dxr* insertion into pDA$^-$

[a]Restriction sites are underlined.

tions were determined using a BioDrop μlite (Isogen Life Science), and 500 ng of each RNA sample was used to synthesize cDNA using the qScript cDNA synthesis kit (QuantaBio).

Quantitative real-time PCR (qPCR). qPCR experiments were performed on a Bio-Rad CFX96 real-time PCR detection system using the Perfecta SYBR green Fastmix (QuantaBio), according to the manufacturer's instructions. The PCR cycling protocol included an initial denaturation at 95°C for 3 min, followed by 40 amplification cycles consisting of 15 s at 95°C and 1 min at 60°C; a melting curve analysis was performed at the end of each run. All PCR data were normalized to the mean expression value of three reference genes (BCAS0175, BCAM2784, and BCAL2694) that were stably expressed under all conditions (33).

Growth curves. Bacterial cultures were grown overnight in LBB supplemented with 200 μg/ml Tmp and 0.5% rhamnose as required and subsequently diluted to a final inoculum of 5×10^5 CFU/ml in various growth media, including LBB, BSM-O, M9-O, M9 with 0.5% glucose, tryptic soy broth (TSB), Mueller-Hinton broth (MHB), and synthetic cystic fibrosis medium (SCFM; [18]). 2-*C*-Methyl-D-erythritol 4-phosphate lithium salt (MEP) was purchased from Sigma-Aldrich and added to a final concentration of 5 mM. Glucose and rhamnose were added to a final concentration of 0.5% as required. A total of 200 μl of each bacterial suspension was added in duplicate to a flat-bottomed 96-well microtiter plate (SPL Life Sciences), and bacterial growth was monitored by measuring absorbance at 590 nm in an Envision multilabel plate reader (PerkinElmer, Waltham, MA), every 30 min for 48 to 72 h at 37°C, until the stationary phase was reached. Growth curves for consecutive reinoculated batch cultures were performed in a Cell Growth Quantifier (Aquila Biolabs).

Evaluation of *in vitro* antimicrobial susceptibility. Antimicrobial susceptibility was investigated by determination of the MIC according to the EUCAST broth microdilution guidelines (34). Antibiotics tested included bacitracin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, imipenem, rifampin, tetracycline, and FR900098 (Sigma-Aldrich), meropenem (Hospira Benelux), tobramycin, aztreonam (TCI Europe), and fosmidomycin (Invitrogen). MICs were determined in triplicate using flat-bottomed 96-well microtiter plates, with concentrations adapted to the high antimicrobial resistance of *B. cenocepacia*. Planktonic bacterial cultures were grown overnight in the appropriate medium and added to the 96-well plates at a final inoculum of 5×10^5 CFU/ml. Plates were incubated for 24 h at 37°C, and absorbance was measured at 590 nm using an Envision plate reader. Initial tests for the potentiating effect of fosmidomycin and FR900098 were performed in MHB, with concentrations of potentiator ranging from 4 to 500 μM. Further MIC analyses were then conducted with FR900098 at a concentration of 250 μM, because this showed the highest effect on MIC values, and in LBB, because the complemented mutants did not grow in MHB. Neither Tmp nor Tet was added to the MIC tests.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02607-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENT

The authors acknowledge funding support by the Research Foundation—Flanders (FWO). The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. Haemers T, Wiesner J, Van Poecke S, Goeman J, Henschker D, Beck E, Jomaa H, Van Calenbergh S. 2006. Synthesis of alpha-substituted fosmidomycin analogues as highly potent *Plasmodium falciparum* growth inhibitors. Bioorg Med Chem Lett 16:1888–1891. https://doi.org/10.1016/j.bmcl.2005.12.082.
2. Lange BM, Rujan T, Martin W, Croteau R. 2000. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97:13172–13177. https://doi.org/10.1073/pnas.240454797.
3. Odom AR. 2011. Five questions about non-mevalonate isoprenoid biosynthesis. PLoS Pathog 7:e1002323. https://doi.org/10.1371/journal.ppat.1002323.
4. Eisenreich W, Bacher A, Arigoni D, Rohdich F. 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell Mol Life Sci 61:1401–1426. https://doi.org/10.1007/s00018-004-3381-z.
5. Kuzuyama T, Shimizu T, Takahashi S, Seto H. 1998. Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. Tetrahedron Lett 39:7913–7916. https://doi.org/10.1016/S0040-4039(98)01755-9.
6. Chofor R, Sooriyaarachchi S, Risseeuw MD, Bergfors T, Pouyez J, Johny C, Haymond A, Everaert A, Dowd CS, Maes L, Coenye T, Alex A, Couch RD, Jones TA, Wouters J, Mowbray SL, Van Calenbergh S. 2015. Synthesis and bioactivity of beta-substituted fosmidomycin analogues targeting 1-deoxy-D-xylulose-5-phosphate reductoisomerase. J Med Chem 58:2988–3001. https://doi.org/10.1021/jm5014264.
7. Scoffone VC, Chiarelli LR, Trespidi G, Mentasti M, Riccardi G, Buroni S. 2017. *Burkholderia cenocepacia* infections in cystic fibrosis patients: drug resistance and therapeutic approaches. Front Microbiol 8:1592. https://doi.org/10.3389/fmicb.2017.01592.
8. Messiaen AS, Verbrugghen T, Declerck C, Ortmann R, Schlitzer M, Nelis H, Van Calenbergh S, Coenye T. 2011. Resistance of the *Burkholderia cepacia* complex to fosmidomycin and fosmidomycin derivatives. Int J Antimicrob Agents 38:261–264. https://doi.org/10.1016/j.ijantimicag.2011.04.020.
9. Juhas M, Stark M, von Mering C, Lumjiaktase P, Crook DW, Valvano MA, Eberl L. 2012. High confidence prediction of essential genes in *Burkholderia cenocepacia*. PLoS One 7:e40064. https://doi.org/10.1371/journal.pone.0040064.
10. Wong YC, Abd El Ghany M, Naeem R, Lee KW, Tan YC, Pain A, Nathan S. 2016. Candidate essential genes in *Burkholderia cenocepacia* J2315 identified by genome-wide TraDIS. Front Microbiol 7:1288. https://doi.org/10.3389/fmicb.2016.01288.
11. Malott RJ, Wu CH, Lee TD, Hird TJ, Dalleska NF, Zlosnik JE, Newman DK, Speert DP. 2014. Fosmidomycin decreases membrane hopanoids and

potentiates the effects of colistin on *Burkholderia multivorans* clinical isolates. Antimicrob Agents Chemother 58:5211–5219. https://doi.org/10.1128/AAC.02705-14.
12. Schmerk CL, Welander PV, Hamad MA, Bain KL, Bernards MA, Summons RE, Valvano MA. 2015. Elucidation of the *Burkholderia cenocepacia* hopanoid biosynthesis pathway uncovers functions for conserved proteins in hopanoid-producing bacteria. Environ Microbiol 17:735–750. https://doi.org/10.1111/1462-2920.12509.
13. Schmerk CL, Bernards MA, Valvano MA. 2011. Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in *Burkholderia cenocepacia*. J Bacteriol 193:6712–6723. https://doi.org/10.1128/JB.05979-11.
14. Malott RJ, Steen-Kinnaird BR, Lee TD, Speert DP. 2012. Identification of hopanoid biosynthesis genes involved in polymyxin resistance in *Burkholderia multivorans*. Antimicrob Agents Chemother 56:464–471. https://doi.org/10.1128/AAC.00602-11.
15. Loutet SA, Valvano MA. 2011. Extreme antimicrobial peptide and polymyxin B resistance in the genus *Burkholderia*. Front Microbiol 2:159.
16. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, Campopiano DJ, Govan JR, Valvano MA. 2007. A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. J Bacteriol 189:3639–3644. https://doi.org/10.1128/JB.00153-07.
17. Hamad MA, Di Lorenzo F, Molinaro A, Valvano MA. 2012. Aminoarabinose is essential for lipopolysaccharide export and intrinsic antimicrobial peptide resistance in *Burkholderia cenocepacia*. Mol Microbiol 85:962–974. https://doi.org/10.1111/j.1365-2958.2012.08154.x.
18. Palmer KL, Aye LM, Whiteley M. 2007. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J Bacteriol 189:8079–8087. https://doi.org/10.1128/JB.01138-07.
19. Sykes RB, Bonner DP, Bush K, Georgopapadakou NH. 1982. Azthreonam (SQ 26,776), a synthetic monobactam specifically active against aerobic gram-negative bacteria. Antimicrob Agents Chemother 21:85–92. https://doi.org/10.1128/AAC.21.1.85.
20. Kocaoglu O, Carlson EE. 2015. Profiling of beta-lactam selectivity for penicillin-binding proteins in *Escherichia coli* strain DC2. Antimicrob Agents Chemother 59:2785–2790. https://doi.org/10.1128/AAC.04552-14.
21. Chantratita N, Rholl DA, Sim B, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Thanwisai A, Chua HH, Ooi WF, Holden MT, Day NP, Tan P, Schweizer HP, Peacock SJ. 2011. Antimicrobial resistance to ceftazidime involving loss of penicillin-binding protein 3 in *Burkholderia pseudomallei*. Proc Natl Acad Sci U S A 108:17165–17170. https://doi.org/10.1073/pnas.1111020108.
22. Leclercq S, Derouaux A, Olatunji S, Fraipont C, Egan AJ, Vollmer W, Breukink E, Terrak M. 2017. Interplay between penicillin-binding proteins and SEDS proteins promotes bacterial cell wall synthesis. Sci Rep 7:43306. https://doi.org/10.1038/srep43306.
23. Bouhss A, Trunkfield AE, Bugg TD, Mengin-Lecreulx D. 2008. The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol Rev 32:208–233. https://doi.org/10.1111/j.1574-6976.2007.00089.x.
24. Tatar LD, Marolda CL, Polischuk AN, van Leeuwen D, Valvano MA. 2007. An *Escherichia coli* undecaprenyl-pyrophosphate phosphatase implicated in undecaprenyl phosphate recycling. Microbiology 153:2518–2529. https://doi.org/10.1099/mic.0.2007/006312-0.
25. Williamson DA, Carter GP, Howden BP. 2017. Current and emerging topical antibacterials and antiseptics: agents, action, and resistance patterns. Clin Microbiol Rev 30:827–860. https://doi.org/10.1128/CMR.00112-16.
26. Zhanel GG, Simor AE, Vercaigne L, Mandell L, Canadian Carbapenem Discussion G. 1998. Imipenem and meropenem: comparison of in vitro activity, pharmacokinetics, clinical trials and adverse effects. Can J Infect Dis 9:215–228. https://doi.org/10.1155/1998/831425.
27. Cho H, Wivagg CN, Kapoor M, Barry Z, Rohs PD, Suh H, Marto JA, Garner EC, Bernhardt TG. 2016. Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously. Nat Microbiol 1:16172. https://doi.org/10.1038/nmicrobiol.2016.172.
28. Dalhoff A. 2014. Pharmacokinetics and pharmacodynamics of aerosolized antibacterial agents in chronically infected cystic fibrosis patients. Clin Microbiol Rev 27:753–782. https://doi.org/10.1128/CMR.00022-14.
29. Hareland WA, Crawford RL, Chapman PJ, Dagley S. 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J Bacteriol 121:272–285.
30. Cardona ST, Mueller CL, Valvano MA. 2006. Identification of essential operons with a rhamnose-inducible promoter in *Burkholderia cenocepacia*. Appl Environ Microbiol 72:2547–2555. https://doi.org/10.1128/AEM.72.4.2547-2555.2006.
31. Cardona ST, Valvano MA. 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. Plasmid 54:219–228. https://doi.org/10.1016/j.plasmid.2005.03.004.
32. Flannagan RS, Linn T, Valvano MA. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. Environ Microbiol 10:1652–1660. https://doi.org/10.1111/j.1462-2920.2008.01576.x.
33. Van Acker H, Sass A, Bazzini S, De Roy K, Udine C, Messiaen T, Riccardi G, Boon N, Nelis HJ, Mahenthiralingam E, Coenye T. 2013. Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. PLoS One 8:e58943. https://doi.org/10.1371/journal.pone.0058943.
34. Hasselmann C, Microbiology ESC. 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infect 9:ix–xv. https://doi.org/10.1046/j.1469-0691.2003.00790.x.
35. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76:1648–1652.