RESEARCH ARTICLE

REVISED Crystal structure of *Pseudomonas aeruginosa* FabB C161A, a template for structure-based design for new antibiotics [version 2; peer review: 2 approved]

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v2 **First published:** 01 Nov 2021, **10**(Chem Inf Sci):1102
https://doi.org/10.12688/f1000research.74018.1
Latest published: 10 Jan 2022, **10**(Chem Inf Sci):1102
https://doi.org/10.12688/f1000research.74018.2

Abstract

Background: FabB (3-oxoacyl-[acyl-carrier-protein] synthase 1) is part of the fatty acid synthesis II pathway found in bacteria and a potential target for antibiotics. The enzyme catalyses the Claisen condensation of malonyl-ACP (acyl carrier protein) with acyl-ACP via an acyl-enzyme intermediate. Here, we report the crystal structure of the intermediate-mimicking *Pseudomonas aeruginosa* FabB (*Pa*FabB) C161A variant.
Methods: His-tagged *Pa*FabB C161A was expressed in *E. coli* Rosetta DE3 pLysS cells, cleaved by TEV protease and purified using affinity and size exclusion chromatography. Commercial screens were used to identify suitable crystallization conditions which were subsequently improved to obtain well diffracting crystals.
Results: We developed a robust and efficient system for recombinant expression of *Pa*FabB C161A. Conditions to obtain well diffracting crystals were established. The crystal structure of *Pa*FabB C161A was solved by molecular replacement at 1.3 Å resolution. Binding site comparison between *Pa*FabB and *Pa*FabF revealed a conserved malonyl binding site but differences in the fatty acid binding channel.
Conclusions: The *Pa*FabB C161A crystal structure can be used as a template to facilitate the design of FabB inhibitors.

Keywords

crystal structure, 3-oxoacyl-[acyl-carrier-protein] synthase 1, FabB, antibiotics

Open Peer Review

Approval Status ✓ ✓

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version 2 (revision) 10 Jan 2022	✓ view	✓ view
version 1 01 Nov 2021	? view	? view

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Competing interests: No competing interests were disclosed.

Grant information: The work was supported by the Research Council of Norway (grant number 273588). We made use of the Facility for Biophysics, Structural Biology and Screening at the University of Bergen (BiSS), which has received funding from the Research Council of Norway (RCN) through the NORCRYST (grant number 245828) and NOR-OPENSCREEN (grant number 245922) consortia.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Yadrykhins'ky V, Georgiou C and Brenk R. **Crystal structure of *Pseudomonas aeruginosa* FabB C161A, a template for structure-based design for new antibiotics [version 2; peer review: 2 approved]** F1000Research 2022, **10**(Chem Inf Sci):1102 https://doi.org/10.12688/f1000research.74018.2

First published: 01 Nov 2021, **10**(Chem Inf Sci):1102 https://doi.org/10.12688/f1000research.74018.1

REVISED **Amendments from Version 1**

A comparison of the fatty acid binding channel in *Pa*FabB and *Pa*FabF has been added. Some figures illustrating the structure of *Pa*FabB C161A have been combined and revised to make the article easier to comprehend and less repetitive. The role of an active site Phe in substrate and ligand binding and how active site mutations influence the conformation of this residue have been explained with more detail. In addition, we have made some minor changes to the text as suggested by the reviewers.

Any further responses from the reviewers can be found at the end of the article

Introduction

New antibiotics are urgently needed to maintain the high standard of living that we have got accustomed to as the antibiotics of today are losing effectiveness faster than they are being replaced by new treatment options.[1]

If no action is taken, by 2050 infections caused by drug-resistant pathogens will kill 10 million people a year worldwide, more than currently die from cancer.[2] A possible source for new targets for antibiotics is the fatty acid synthesis (FAS II) pathway (Figure 1A).[3] In this pathway, fatty acid synthesis is carried out by a series of monofunctional enzymes which are highly conserved among microbial pathogens. Genes coding for enzymes in the FAS II pathway have been found to be essential for *P. aeruginosa* in several genetic screens, including the gene for FabB (3-oxoacyl-[acyl-carrier-protein] synthase 1).[4–8]

Both, FabB and FabF (3-oxoacyl-[acyl-carrier-protein] synthase 2) catalyse the Claisen condensation of malonyl-ACP (acyl carrier protein) with acyl-ACP (Figure 1B), but differ in substrate specificity for the fatty acid chain.[3] Platensimycin

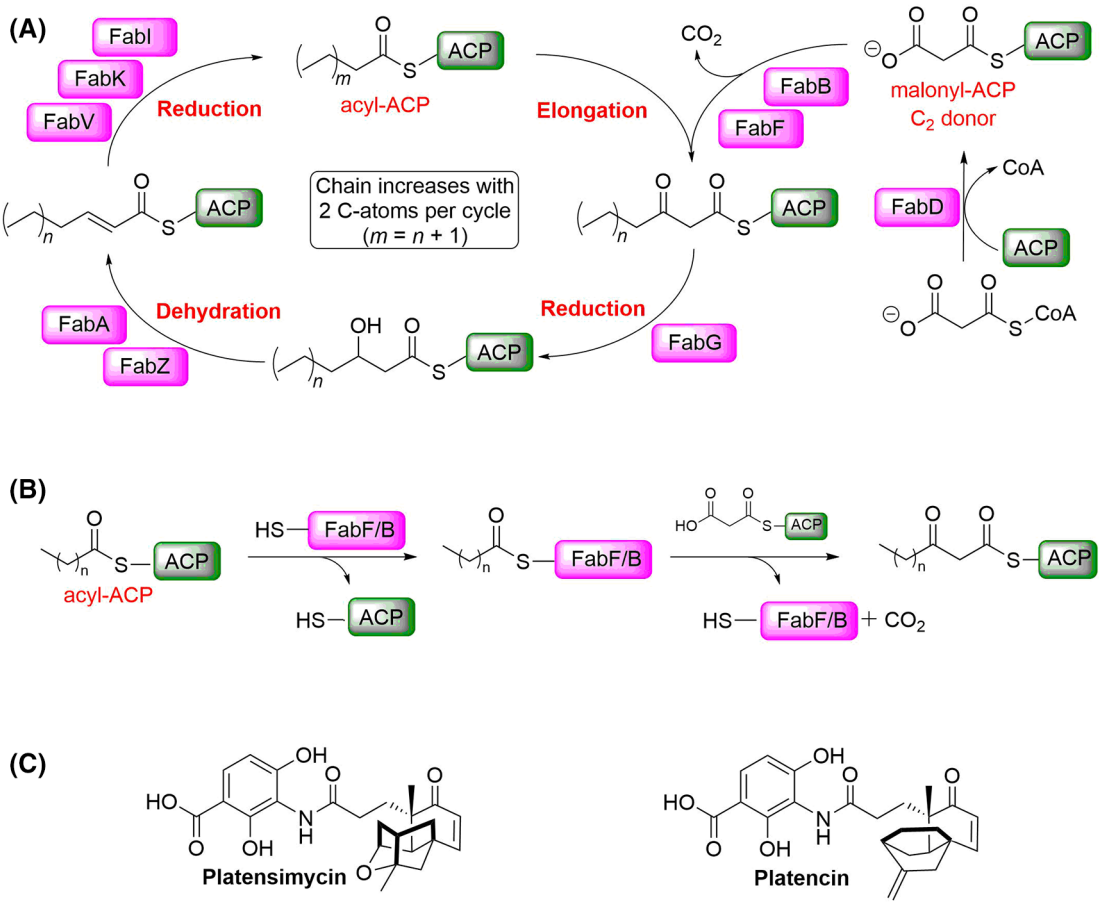

Figure 1. FAS II pathway and its inhibitors. A) Schematic overview of the elongation part of the FAS II pathway. B) Condensation reaction catalysed by FabF/B. (ACP: acyl carrier protein). C) Platensimycin and platencin have been reported as dual FabF/B inhibitors.

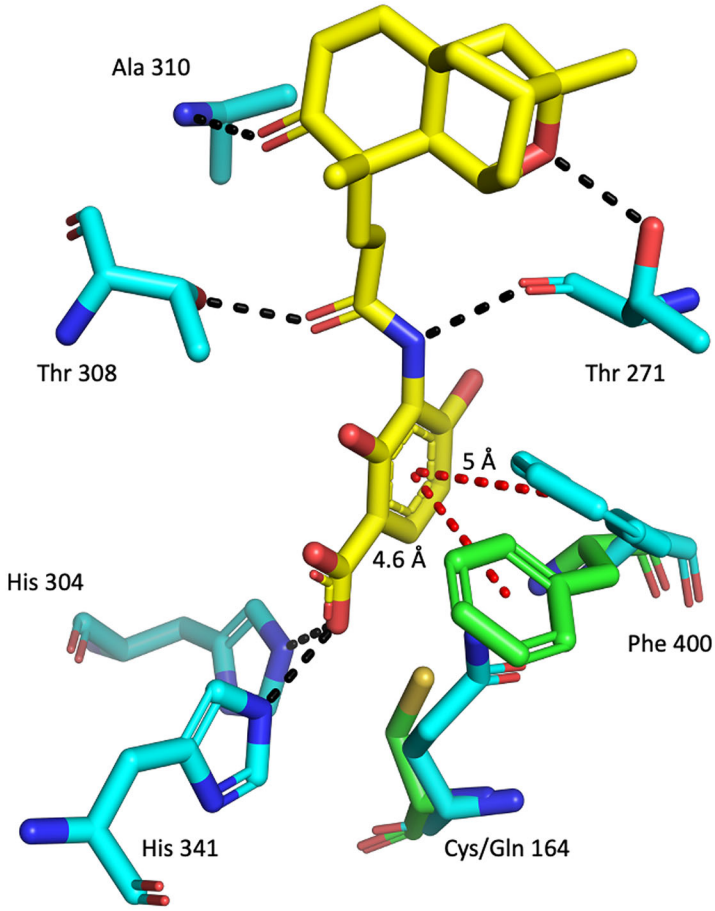

Figure 2. The malonyl binding site of FabF. Alignment of apo w. t. *Pa*FabF (green sticks – PDB ID: 4JPF , for clarity only Phe400 and Cys164 is shown) and *Pa*FabF C164Q (cyan sticks – PDB ID: 7OC1) in complex with platensimycin (yellow sticks). Hydrogen bonds are indicated as black dashed lines and aromatic interactions as red dashed lines. Compared to the apo structure, Phe400 is rotated in the holo structure to create space for the ligand to bind.

and platencin (Figure 1C) have been reported as FabF and FabB inhibitors binding into the malonyl binding site.[9,10] However, it has been shown that these compounds do not bind potently to the apo-enzyme, but only to the lauryl-FabF/B intermediate (Figure 1B) and to intermediate-mimicking variants. In these variants, the active site Cys is replaced with either Gln or Ala (Figure 2).[9,11] In the Ala variant, the presumably negatively charged Cys in the w. t. form is replaced with a neutral residue, thus mimicking more closely the charge of the lauryl intermediate. In the Gln variant, the amide group in the side chain in addition mimics the acyl group of the intermediate (Figure 1B). Both variants have been used to study binding of malonyl-competitive inhibitors to FabF.

To facilitate structure-based design of FAS II inhibitors, knowledge of the structures in this pathway is essential. Recently, we have reported the crystal structure of *Pa*FabF and the reaction intermediate-mimicking variant *Pa*FabF C164Q.[12] Here, we report the crystal structure of an intermediate-mimicking *Pa*FabB variant at 1.3 Å resolution. As in our hands *Pa*FabF C164A was more stable than *Pa*FabF C164Q and thus better suited for biophysical studies, we focused our efforts on *Pa*FabB C161A.[13]

Results and discussion

Protein expression and purification

The gene coding for *P. aeruginosa* PA14 FabB C161A was synthesised and cloned in a bacterial plasmid pET-28a (+)-TEV vector after a DNA sequence coding for a 6-His-tag followed by a TEV protease cleavage site. To find good expression conditions, seven widely used *E. coli* strains were transformed with the plasmid (BL21 (DE3), BL21 (DE3) pLysS, C41 (DE3), C41 (DE3) pLysS, C43 (DE3), C43 (DE3) pLysS and Rosetta (DE3) pLysS) and screened for protein expression. The best results were obtained with Rosetta (DE3) pLysS cells (data not shown). Therefore, this cell line was used for all subsequent protein expression experiments.

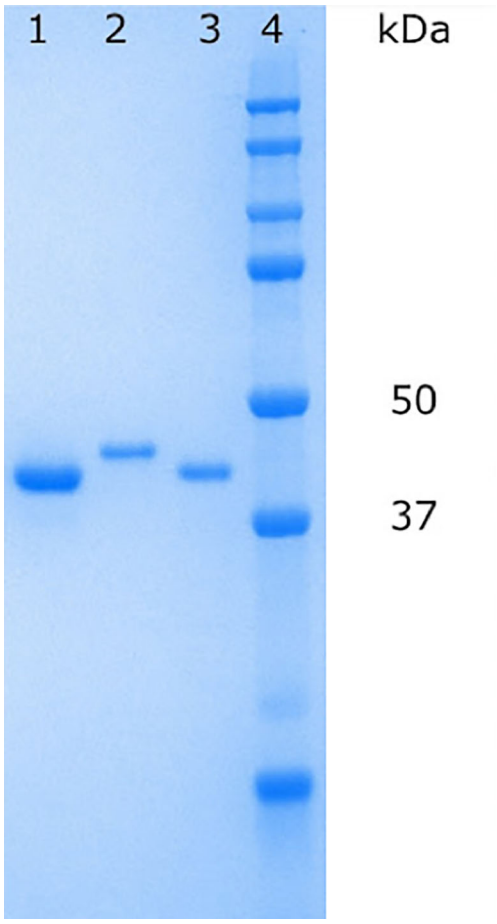

Figure 3. Protein samples on an SDS–PAGE gel. Lane 1: *Pa*FabB C161A (without His-tag) after inverse affinity chromatography, lane 2: 6-His-tagged *Pa*FabB C161A after SEC purification, lane 3: *Pa*FabB C161A (without His-tag) after SEC purification, lane 4: protein ladder.

His-tagged *Pa*FabB C161A was purified using affinity chromatography with a Ni column followed by size exclusion chromatography (SEC). To obtain FabB lacking the His-tag, the protein obtained after affinity chromatography was cleaved with TEV protease. The cleaved protein was separated from the protease and the tag by inverse affinity chromatography followed by SEC. In both cases, pure protein was obtained as judged by SDS-PAGE gel electrophoresis (Figure 3). Typical yields for His-tagged *Pa*FabB C161A were 26 mg/L and for cleaved *Pa*FabB C161A 7 mg/L.

Crystallization of *Pa*FabB C161A

Crystallization trials of His-tagged *Pa*FabB C161A and FabB C161A lacking the His-tag were attempted using the JCSG+, PACT premier, HELIX (only His-tagged *Pa*FabB C161A) and LFS screens. No promising crystallization conditions for His-tagged *Pa*FabB C161A were found using these screens. In contrast, 11 different conditions resulted in crystals of *Pa*FabB C161A lacking the His-tag (Table 1, Figure 4). All of these conditions contained PEG 3350 between 20 and 25% and a number of conditions contained ethylene glycol. Further, the majority of the conditions contained 0.1 M Bis-Tris propane, and 0.2 M sodium iodide. Therefore, these components were kept for further optimization trials. The pH of the initial conditions varied from 5.5 to 8.5. As crystals grown in a buffer of pH 7.5 were visually judged to be more regular (e. g. the crystal shown in Figure 4B), this pH was fixed during optimization. These considerations resulted in an optimization matrix where the concentration of PEG 3350 was varied between 5 and 30% and the protein concentration between 9 and 23 mg/mL. Ethylene glycol was added to all conditions at either 10 or 20% while 0.2 M sodium iodide and 0.1 M Bis-Tris propane were fixed (Figure 5). Under 32 conditions, crystals were obtained. These were mounted and used for diffraction experiments.

Six different conditions led to well-diffracting crystals (Figure 5). For these, data sets with resolutions between 2 and 1.3 Å could be collected. For the best diffracting crystal, the resolution was set limited based on the distance of the

Table 1. Conditions in which crystals of PaFabB C161A (without His-tag) were formed. (PEG-polyethylene glycol; EG-ethylene glycol.)

Well screen	Buffer	Salt	Precipitant 1	Precipitant 2
F2 LFS	0.1 M Bis Tris Propane pH 6.5	0.2 M Sodium bromide	20% *w/v* PEG 3350	10% *v/v* EG
F3 LFS	0.1 M Bis Tris Propane pH 6.5	0.2 M Sodium iodide	20% *w/v* PEG 3350	10% *v/v* EG
F4 LFS	0.1 M Bis Tris Propane pH 6.5	0.2 M Potassium thiocyanate	20% *w/v* PEG 3350	10% *v/v* EG
G3 LFS	0.1 M Bis Tris Propane pH 7.5	0.2 M Sodium iodide	20% *w/v* PEG 3350	10% *v/v* EG
E3 PACT premier		0.2 M Sodium iodide	20% *w/v* PEG 3350	
F2 PACT premier	0.1 M Bis-Tris propane pH 6.5	0.2 M Sodium bromide	20% *w/v* PEG 3350	
F3 PACT premier	0.1 M Bis-Tris propane pH 6.5	0.2 M Sodium iodide	20% *w/v* PEG 3350	
G2 PACT premier	0.1 M Bis-Tris propane pH 7.5	0.2 M Sodium bromide	20% *w/v* PEG 3350	
G3 PACT premier	0.1 M Bis-Tris propane pH 7.5	0.2 M Sodium iodide	20% *w/v* PEG 3350	
B2 JCSG+		0.2 M Sodium thiocyanate	20% *w/v* PEG 3350	
D6 JCSG+	0.1 M Tris pH 8.5	0.2 M Magnesium chloride hexahydrate	20% *w/v* PEG 8000	
H10 JCSG+	0.1 M BIS-Tris pH 5.5	0.2 M Ammonium acetate	25% *w/v* PEG 3350	

detector from the crystal and no data were discarded. Based on the CC1/2 and I/sigI values (Table 2) it is likely this FabB crystal diffracted to an even higher resolution than 1.3 Å. The crystal structure was determined using a homology model created based on *Vibrio cholerae* FabB (*Vc*FabB, PDB Id 4XOX) as search model. The crystal was in the space group C 2 2 21 and contained 2 protein molecules in the asymmetric unit.

Crystal structure of *Pa*FabB C161A

*P*aFabB C161A crystallized as a dimer and has the same overall fold as observed before for FabB and FabF from other organisms (Figure 6). The rmsd between *Pa*FabB C161A and *Vc*FabB (the protein with the highest sequence identity in the PDB (72%), PDB Id 4XOX) is 0.42 Å while the rmsd to w. t. *Pa*FabF is 0.84 Å (sequence identity 41%, PDB Id 4JPF). The two catalytic histidines, His296 and His331, are highly conserved and well aligned with the catalytic histidines from both *Vc*FabB and *Pa*FabF (Figure 6B).

Due to the high concentration of ethylene glycol (20% *v/v*) and salt in the well and protein buffers, respectively (150 mM NaCl and 200 mM NaI), 18 ethylene glycol molecules and 11 ions (Cl^- and I^-) were identified and placed in the crystal structure of *Pa*FabB C161A during refinement (Figure 7A). Some of these molecules were found to bind in the active site of the protein (Figure 7B). The chloride ion Cl 1 binds tightly (B factor for Cl 1 is 18 $Å^2$, average B-factor for protein atoms is 16.8 $Å^2$, average ions B factors is 30.7 $Å^2$) in the active site of chain B, in close proximity to the catalytic residues His296 (3.3 Å) and His331 (3. 2Å). Moreover, Cl 1 forms two additional interactions with an ethylene glycol (EDO511, average B factor 18 $Å^2$) and a water molecule (HOH227) in the active site.

Comparison of malonyl binding site in *Pa*FabB and *Pa*FabF

Although, the overall sequence identity between *Pa*FabB and *Pa*FabF is only 41%, the conservation in the malonyl binding site is much higher. Apart from Thr271 in FabF that is replaced by Val268 in FabB, all active site residues

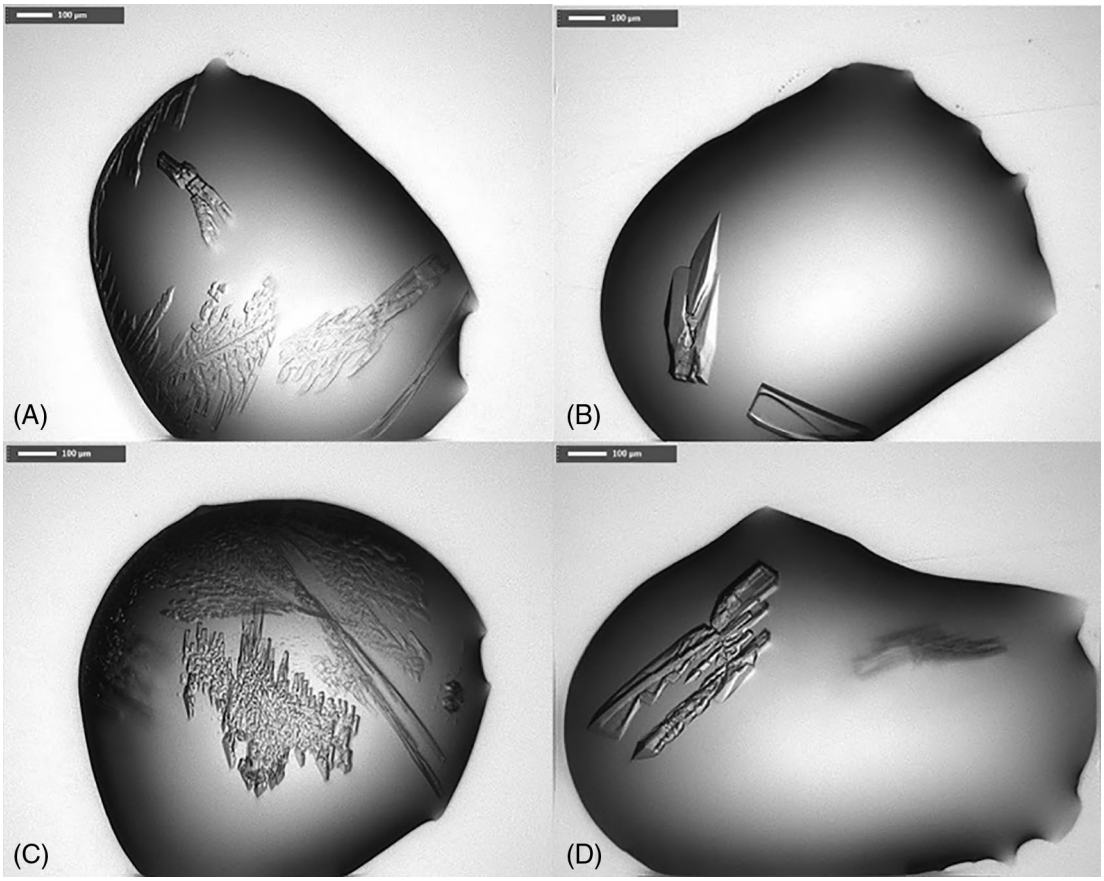

Figure 4. Selected *Pa*FabB C161A crystals obtained from various screens. A) condition F3 from LFS, B) G3 from LFS, C) F3 from PACT premier, D) G3 from PACT premier (for composition of crystallization buffer see Table 1).

PEG 3350 %

	5	5	10	10	15	15	20	20	25	25	30	30	
	1	2	3	4	5	6	7	8	9	10	11	12	*Pa*FabB C161A mg/mL
A					3					3		3	23
B					1		3			2,3		3	21
C					1,2		3			3		3	19
D					1,2		3						17
E					2		1,3					3	15
F					1,2		1,3						13
G					1,2		1,3			3			11
H							3			3		3	9
	10	20	10	20	10	20	10	20	10	20	10	20	

Ethylene glycol %

Figure 5. Plate layout for optimization of crystallization conditions. The numbers in the cells indicate the ratio between protein solution and crystallization buffer in the drops (drop 1-1:1 ratio, drop 2-1:2 ratio, drop 3-2:1 ratio). Coloured cells indicate conditions from which crystal were harvested and mounted for diffraction experiments. Green cells indicate conditions under which diffracting crystals were obtained.

Table 2. Data-collection and refinement statistics of *Pa*FabB C161A. Values in parentheses are for the highest resolution shell.

Data collection and processing	
Space group	C 2 2 21
a, b, c (Å)	74.23, 102.30, 188.77
α, β, γ (°)	90.00 90.00 90.00
Solvent content (%)	40
Diffraction data	
Resolution range (Å)	47.2-1.3 (1.38-1.30)
Unique reflections	339190 (54366)
Multiplicity	13.4 (12.3)
R merge (%)	5.8 (49.4)
Completeness (%)	99.7 (97.3)
I/sigI	22.4 (4.6)
CC (1/2)	99.9 (92.8)
Refinement	
R work/R free	0.114/0.137
Quaternary structure	dimer
Protein residues (in a dimer)	808
Water molecules (in a dimer)	492
Ions (in a dimer)	Iodide (11), Chloride (6)
Ligands (in a dimer)	1,2-ETHANEDIOL (18)
R.m.s.d.s	
Bonds (Å)	0.013
Angles (Å)	1.75
Ramachandran plot,residues in (%)	
Favoured regions	790 (96%)
Allowed regions	33 (4%)
Outlier regions	0 (0%)
Average B factors (Å^2)	
Protein atoms	16.8
Ions, Ligands, Waters	30.7, 27.3, 30.0
PDB code	7PPS

involved in hydrogen-bond interactions with platensimycin are conserved between the two enzymes (Figure 8A). That makes it highly likely that ligands binding into this pocket in FabF may also bind to FabB with a similar affinity, and thus opens up the possibility for the designing of dual inhibitors for both FabF and FabB that will lead to a complete inhibition of the last step of the fatty acid elongation cycle.

The highly conserved Phe400/391 (numbering based on *Pa*FabF/*Pa*FabB) in the malonyl binding site was previously identified to play a pivotal role in substrate specificity and ligand binding, as this residue adopts different conformations in the apo and the intermediate-binding state (Figure 2).[9,12] In w. t. apo *Pa*FabF (PDB Id 4JPF, Figure 8A and B), Phe400 is in a 'closed' conformation (dihedral angle C-CA-CB-CG = -177.1 °). Upon the mutation of the catalytic residue Cys164 to Gln (PDB Id 7OC1 – Figure 8A and C) or Ala, the enzyme has been shown to mimic the intermediate-binding state and to trap the Phe400 into the 'open' conformation (dihedral angle C-CA-CB-CG = 168.8 °) as also found when a fatty acid is bound (e.g. PDB ID 2GFY). The reason for this is likely that the closed conformation is stabilized by a sulphur-pi interaction between the catalytic Cys and the Phe.[14] Once this interaction is disturbed through either binding the fatty acid or a point mutation of Cys, Phe adopts the then energetically more favourable open conformation. Here, the

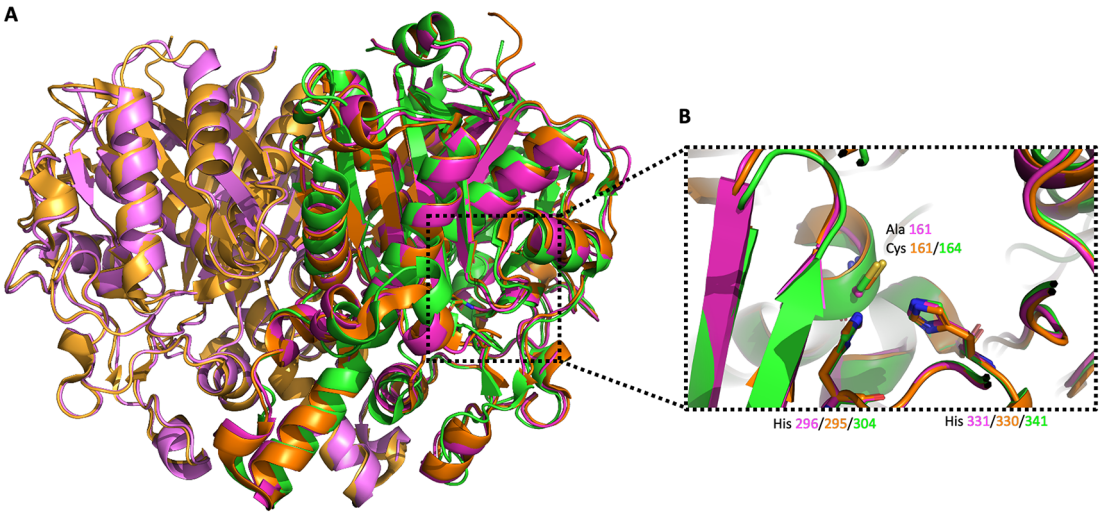

Figure 6. Alignment of *Vc*FabB (PDB Id 4XOX) dimer, *Pa*FabF (4JPF) monomer and *Pa*FabB C161A (7PPS) dimer. A) The three different enzymes are shown in orange/light orange, green and magenta/light magenta cartoon style, respectively. B) Alignment of the active site catalytic triad of *Vc*FabB, w. t. *Pa*FabF and *Pa*FabB C161A.

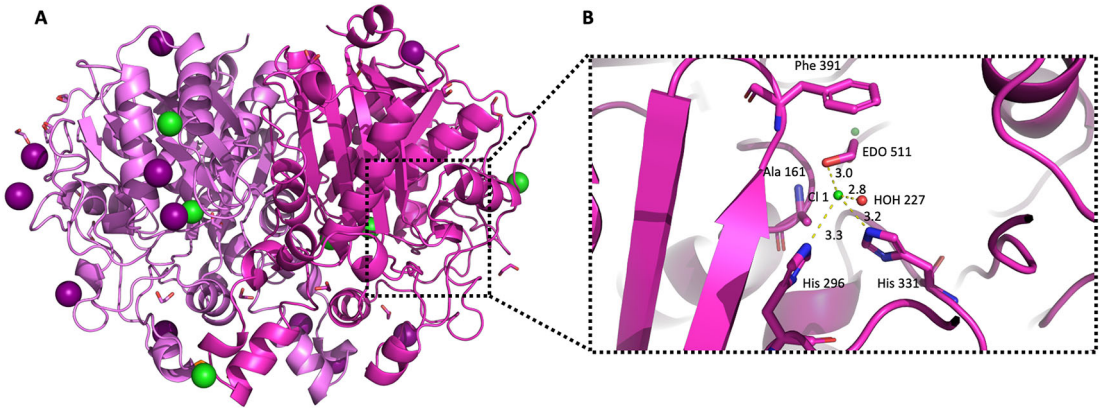

Figure 7. Crystal structure of *Pa*FabB C161A highlighting bound buffer components. A) The structure the of *Pa*FabB C161A homodimer is shown in carton style coloured in magenta/light magenta. Iodine and chloride ions are shown with deep purple and green colour, respectively. B) Active site residues are shown as magenta sticks, water molecules and chloride ions are shown as red and green spheres, respectively, while the distances between the chloride ion Cl1 and the neighbouring molecules are shown as yellow dashed lines.

catalytic residue Cys161 of *Pa*FabB was mutated to Ala161. As can be seen from the crystal structure (Figure 8A and D), Phe391 adopts the 'open' conformation as expected for an intermediate-mimicking FabB variant (dihedral angle C-CA-CB-CG = 170.2 °).

Comparison of the fatty acid chain binding channel in and in *Pa*FabB and *Pa*FabF

FabB and FabF catalyse the condensation of C4-C14 saturated fatty acids, but they show different levels of acceptance of unsaturated fatty acids.[3,15,16] FabB is able to catalyse the elongation of cis-3-decenoyl-ACP up to three times, and synthesise cis-5-dodecenoyl-ACP, cis-7-tetradodecenoyl-ACP and cis-9-hexadodecenoyl-ACP. Cells lacking FabB are auxotroph for unsaturated fatty acids, making FabB an essential gene for the bacteria.[4,17,18] In contrast, FabF but and not FabB was shown to be responsible for the condensation of cis-9-hexadodecenoyl-ACP to cis-11-octadodecenoyl-ACP, the last step for the synthesis of vaccenic acid.[19]

Despite the fact that the crystal structures of both FabF and FabB have been published some time ago, it is still not clear what the molecular reasons for the observed fatty acid selectivity are. In a recent publication, a gating mechanism was proposed that regulates access to the fatty acid binding sites of FabB and FabF through a significant conformational change of two active site loops. However, based on the presented data no conclusions about the observed substrate selectivity can be drawn.[20]

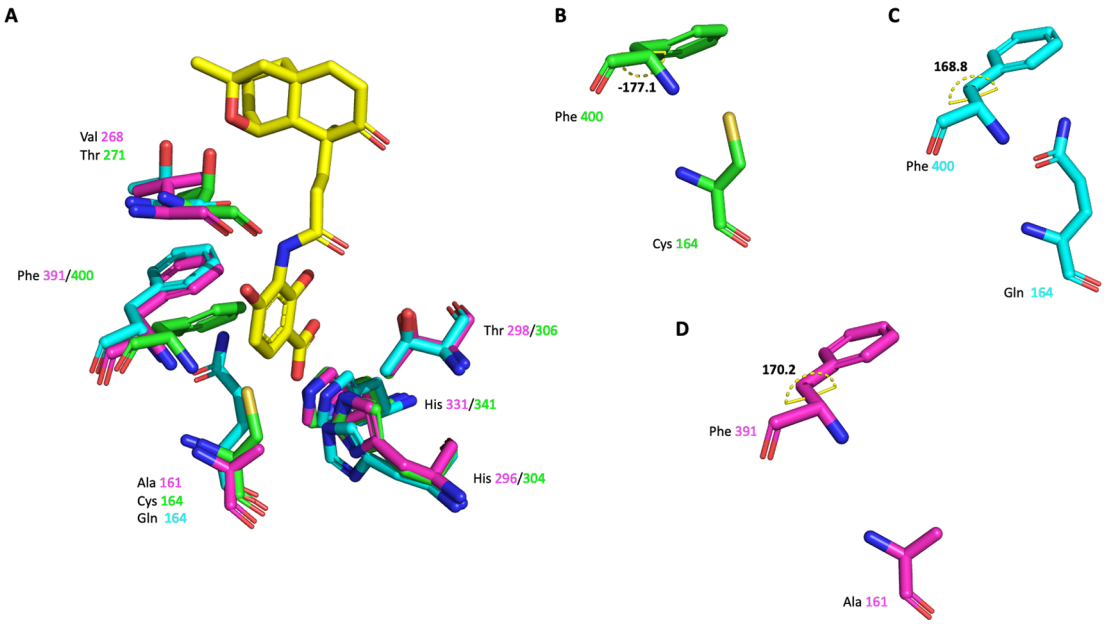

Figure 8. Alignment of the malonyl binding site of *Pa*FabF (PDB Id 4JPF), *Pa*FabF C164Q (7OC1) and *Pa*FabB C161A (7PPS). A) The active site residues of the three different enzymes are shown as sticks. Platensimycin binding to *Pa*FabF C164Q is shown as yellow sticks. Side chain conformation and dihedral angle C-CA-CB-CG of Phe391/400 is shown in B) for *Pa*FabF C) for *Pa*FabF C164Q and D) for *Pa*FabB C161A.

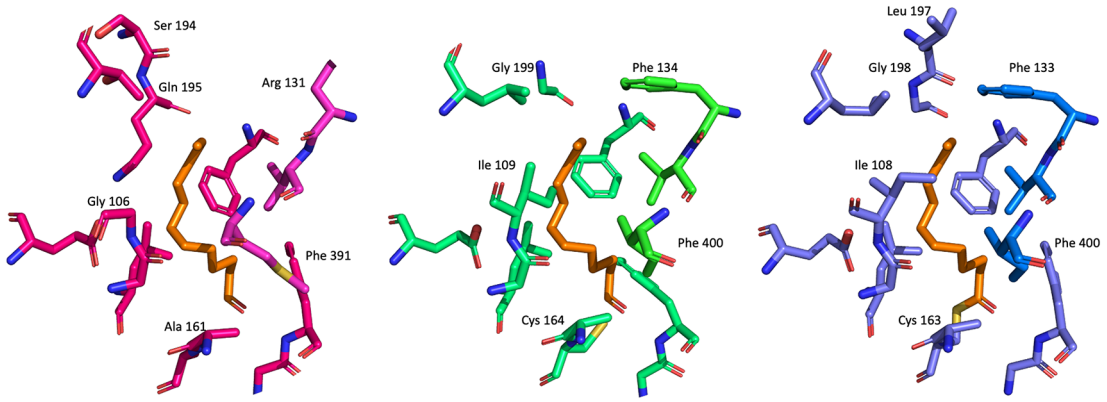

Figure 9. Comparison of the fatty acid binding channel residues of the homodimers of *Pa*FabF (PDB Id 4JPF), *Pa*FabB C161A (7PPS) and *Ec*FabF (2GFY). The fatty acid binding channel site residues of the three different enzymes are shown as blue/light blue, green/light green and magenta/light magenta sticks, respectively. C12 fatty acid binding to *Ec*FabF is superimposed with the remaining structures and shown as orange sticks.

The fatty acid binding channel is located at the interface of the homodimer (Figure 9). No crystal structures of FabF/B with unsaturated acids for which the enzymes appear to be selective have been published so far. The entrance to the fatty acid channel is lined with identical residues in FabF and FabB. However, further into the channel, FabF has a Ile (108/109 *Ec*FabF/*Pa*FabF numbering). This residue has been shown to rotate to allow the binding of fatty acids longer than C6.[15] The equivalent residues in *Pa*FabF is Gly106 making the channel wider in this part of the pocket and potentially also more flexible. This might explain the substrate promiscuity of FabB, but more detailed studies are needed to confirm this. A better understanding of the driving forces for the observed selectivity will also help with the design of selective of dual FabF/B inhibitors.

Conclusions

In this study, the first high-resolution crystal structure of *Pa*FabB C161A is reported. This structure can now serve as a template for the structure-based design of FabB inhibitors. The C161A mutation of FabB in this crystal structure causes Phe 391 to be in the 'open' conformation (Figure 8) and allows targeting of the intermediate-acylated state of FabB; in a similar manner to the natural antibiotic platensimycin. Furthermore, due to the high conservation of the overall fold and the high sequence identity in the malonyl binding site between the structure reported here with *Pa*FabF, the structures can be used as a template for the design of novel dual FabF/B inhibitors. In contrast, compounds extending deep into the fatty acid channel are likely to be selective, but more work is needed to get a better understanding of what drives the substrate selectivity in this channel.

Methods

Recombinant protein production and purification

The gene coding for *P. aeruginosa* PA14 FabB (ORF number (open reading frame): PA14_43690), with the single point mutation C161A was synthesised and cloned in a bacterial plasmid pET-28a(+)-TEV vector using the cloning sites NdeI/BamHI by Genscript. The plasmid had a DNA sequence coding for a 6-His-tag followed by a TEV protease cleavage site before *Pa*FabB. Seven different *E. coli* strains (OverExpress C41(DE3) SOLOs and C43(DE3) SOLOs from Biosearch technologies; BL-21(DE3), BL-21(DE3) pLysS, C41(DE3) pLysS and C43(DE3) pLysS from Lucigen, and Rosetta (DE3) pLysS from Merck) were heat-shock transformed with the synthesised plasmid. Expression of *Pa*FabB in each transformed cell line was tested as per manufacturer protocol.

E. coli Rosetta (DE3) pLysS competent cells yielded the highest protein expression, based on SDS-PAGE analysis, and were used as expression system for large-scale protein production and purification. Transformed cells were inoculated in 50 mL of LB medium supplemented with kanamycin (30 μg/mL) and chloramphenicol (50 μg/mL) overnight at 310 K. Pre-culture stocks were prepared by mixing the overnight culture with glycerol (final concentration 40% *v/v*), aliquoted and kept in –80 °C until use. For large-scale expression, 0.1 mL of pre-culture stock was inoculated in 100 mL of LB medium supplemented with kanamycin (30 μg/mL) and chloramphenicol (50 μg/mL) overnight at 310 K. The entire volume was then transferred into 900 mL of LB-medium containing antibiotics and the cell growth continued until OD_{600} reached 0.7. Protein expression was then induced by adding IPTG to a final concentration of 1 mM and the expression continued for another 3-3.5 hours.

Cells were harvested by centrifugation (15 minutes, 5000 *g*, 277 K), resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1 mM DTT, 10% glycerol (*v/v*), pH 7.4) with addition of one tablet of Complete EDTA-free protease inhibitor cocktail (Roche) and incubated with magnet stirring for 60 minutes at 277 K. 20 U (units) of DNAse I (Sigma Aldrich) was added per cell pellet, before the mixture was sonicated on ice by an ultrasonic processor (Sonics, Vibra-Cell VC130) for a total of two minutes with 10 seconds pulses with amplitude 70%. The debris and insoluble protein were pelleted by centrifugation at 15000 rpm, 277 K, for 30 minutes. The supernatant was collected and filtered with Whatman filter units 0.2 μM (GE healthcare) using a syringe. The protein was then purified using a Ni^{2+} Sepharose High Performance HisTrap HP 5 mL column (GE Healthcare) with an increasing imidazole gradient from 0 to 500 mM. The fractions containing *Pa*FabB C161A were pooled and TEV protease was added to remove the affinity tag. The mixture was dialyzed with buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl) overnight at 277 K and the cleaved protein was purified by passage through a Ni^{2+} HisTrap column. SEC was then performed on a HiLoad 26/600 Superdex 75 pg column (Cytiva) with equilibration buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 7.4). Purity was confirmed by SDS–PAGE (Mini-PROTEAN TGX Stain-Free Precast Gel; Bio-Rad) and the final concentration of *Pa*FabB C161A was determined using a NanoDrop ND-1000 (Thermo Fisher Scientific). The extinction coefficient used was 0.666 $(mg/mL)^{-1}$ cm^{-1} (calculated using the final protein sequence).

Crystallization and X-ray data collection

For crystallization trials JCSG+ (MD1-37), PACT premier (MD1-29) and LFS (Ligand Friendly Screen, MD1-122) crystallization screens from Molecular Dimensions were used. *Pa*FabB C161A lacking the His-tag (23 mg/mL) in 20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 7.4, was mixed with well buffer in different ratios (2:1, 1:1 and 1:2) on a Triple Sitting Drop 96-well plate (TTP Labtech) using a crystallography Mosquito LCP (TTP LabTech). The plates were incubated at 20°C. Optimization (Figure 5) of the initial hit conditions (Table 1) was achieved by varying the precipitants and protein concentrations while keeping the salt and buffer concentration constant. Optimisation led to rod-shaped crystals (250 × 100 × 10 μm) in multiple drops (Figure 4).

Crystals with a final concentration of precipitant lower than 25% (*w/v*) were cryoprotected with a mixture consisting of the crystallization buffer and Cryomix 9 from CryoSol MD1-90 (Molecular Dimensions) (final composition of the cryo-mixture: 0.2 M NaI, 0.1 M Bis-Tris propane pH 7.5, 5% (*w/v*) PEG 3350, 10% (*v/v*) EG 5% (*v/v*), diethylene glycol,

5% (*v/v*) 1,2-propanediol, 5% (*v/v*) dimethyl sulfoxide, 5% (*v/v*) glycerol, 5 mM NDSB 201 (3-(1-Pyridinio)-1-propanesulfonate), 5% (*v/v*) 1,4-dioxane) prior to flash-cooling in liquid nitrogen.

X-ray data were collected from single crystals at the DESY synchrotron (Hamburg, Germany) at the P11 high-throughput MX beamline. In each case, crystals were maintained at 100 K and the X-ray wavelength was 0.976246 Å. Data were processed with the automatic data processing pipeline of P11 beamline, using XDS.[21]

Structure solution and refinement

The structure was solved by molecular replacement using Dimple[22] from the CCP4i2 suite.[23] As search model, a homology model generated from wt. *Vc*FabB (PDB Id 4XOX) with 72% sequence identity was used. Refinement was performed using REFMAC5[24] while inspection of electron-density and difference density maps and model manipulation was achieved using *Coot*.[25] During refinement, water molecules, ions and side-chain conformers were included. The model geometry was assessed using *MolProbity*,[26] the PDB redo server[27] and the PDB validation tools. The crystallographic data and refinement statistics are listed in Table 2. The figures were generated with *PyMOL* v.2.4.1 (Schrödinger, LLC) and VMD v.1.9.3.[28]

Data availability

Protein Data Bank: The crystal structure of *Pa*FabB C161A with the PDB Id 7PPS, https://doi.org/10.2210/pdb7PPS/pdb.

Acknowledgements

We acknowledge DESY (Hamburg, Germany), a member of the Helmholtz Association HGF, for the provision of experimental facilities. Parts of this research were carried out at PETRA III and we would like to thank Johanna Hakanpää and Sofiane Saouane for assistance in using the P11 beamline. Beamtime was allocated for the proposal via the BAG-20190768 EC. We thank Khan Kim Dao for excellent support with protein purification and Ludvik Olai Espeland for help with preparing Figure 1.

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Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 31 January 2022

https://doi.org/10.5256/f1000research.119244.r119242

✔ **Bjarte A. Lund**
Hylleraas Centre for Quantum Molecular Sciences, Department of Chemistry, UiT The Arctic University of Norway, Tromsø, Norway

The article has been revised to my satisfaction. No further comments

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, structural biology, enzymology,

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 12 January 2022

https://doi.org/10.5256/f1000research.119244.r119243

✔ **Klaus Reuter**
Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Marburg, Germany

All mistakes have been corrected and all concerns have been addressed. The paper may now be indexed as it is, apart from one further correction that has to be done:

- page 10, penultimate line: replace "... selective of dual ..." with "... selective or dual ...".

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: protein crystallography; enzymology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 06 December 2021

https://doi.org/10.5256/f1000research.77724.r101462

?

Klaus Reuter
Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Marburg, Germany

First of all: Apart from the fact that I do not insist on CC* or CC1/2 values in Table 2, I do absolutely agree with every single concern of the 1st reviewer making it unnecessary to restate all these points.

Further (minor) issues:

- Page 3, Introduction, 1st paragraph, line 3: Replace “drug-resistant infections” with “infections caused by drug-resistant pathogens”.
- Page 3, Introduction, 2nd paragraph, line 4: Replace “w.t. enzyme” with “apo-enzyme”.
- Page 3, Figure 1 (B): In the first partial reaction of the FabF/B-catalysed reaction, the authors indicate the incoming HS-FabF/B (above the reaction arrow) but forget to indicate the leaving HS-ACP.
- Page 4, Results/Protein expression and purification, 2nd paragraph, line 2: Replace “… FabB with a cleaved His-tag …” with“… FabB lacking the His-tag …”.
- Page 4, Results/Crystallization of *Pa*FabB C161A, 1st paragraph, line 2: Replace “cleaved FabB” with “FabB lacking the His-tag”.
- Page 5, Results/Crystal structure of *Pa*FabB C161A, 1st paragraph, line 3: rmsd values for superimposed structures are given with three significant digits, respectively, which suggests inappropriately high accuracy. 0.424 Å should be replaced by 0.42 Å and 0.843 Å by 0.84 Å.
- Page 5, Results/Crystal structure of *Pa*FabB C161A, 1st paragraph, line 5: Replace “… from both *Vc*FabB as well as the ones from *Pa*FabB …” with“… from both *Vc*FabB and *Pa*FabB …”.

- Page 6, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 1: The 1st sentence of this paragraph has to be reformulated. It does not make sense in its present form.

- Page 7, Table 2: Again, several values are given with inappropriately high accuracy. Multiplicity: 6.84 (6.56) should be replaced by 6.8 (6.6); I/sig(I): 19.11 (3.45) should be replaced by 19.1 (3.5); Ramachandran plot, residues in (%): (96.02%) should be replaced by (96.0%) and (3.98%) by (4.0%).

- Page 8, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 1: Replace "w.t." with "apo-".

- Page 8, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 2: Replace "When mutating ..." with "Upon the mutation of ...".

- Page 8, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 8: Replace "... is mutated to Val268 ..." with "... is replaced by Val268 ..." or "... corresponds to Val268 ...".

- Figure 5: It is not immediately obvious if the figure shows the homodimer or a single subunit only. This information should be given. If the complete dimer is shown (which I presume) it would make sense to present its subunits in different colours or shades of colour. The same applies to subsequent figures.

Major concern:

- The many figures showing FabB or the superpositions of different FabB/F enzymes are not very meaningful. FabB and FabF differ in substrate specificity for the fatty acid chain. The authors should definitely discuss the reasons for the respective substrate specificity by means of available structures.

- I wish the authors had explained why the C161A mutation leads to the open conformation of the active site (see reviewer 1).

In summary, the manuscript is scientifically sound and significant, although somewhat greater effort should have been put into its presentation.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: protein crystallography; enzymology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Dec 2021

Ruth Brenk, University of Bergen, Bergen, Norway

Thank you Klaus for reviewing our paper and for very constructive feedback!

Below follows a point-to-point reply to all issues that you have raised.

- First of all: Apart from the fact that I do not insist on CC* or CC1/2 values in Table 2, I do absolutely agree with every single concern of the 1st reviewer making it unnecessary to restate all these points.

We have addressed all points that Bjarte Lund has raised. Please refer to the reply to his comments to see what we have changed.

- Page 3, Introduction, 1st paragraph, line 3: Replace “drug-resistant infections” with “infections caused by drug-resistant pathogens”.

Done

- Page 3, Introduction, 2nd paragraph, line 4: Replace “w.t. enzyme” with “apo-enzyme”.

Done

- Page 3, Figure 1 (B): In the first partial reaction of the FabF/B-catalysed reaction, the authors indicate the incoming HS-FabF/B (above the reaction arrow) but forget to indicate the leaving HS-ACP.

Corrected as suggested

- Page 4, Results/Protein expression and purification, 2nd paragraph, line 2: Replace “… FabB with a cleaved His-tag …” with“… FabB lacking the His-tag …”.

Done

- Page 4, Results/Crystallization of *Pa*FabB C161A, 1st paragraph, line 2: Replace “cleaved FabB” with “FabB lacking the His-tag”.

Done

- Page 5, Results/Crystal structure of *Pa*FabB C161A, 1st paragraph, line 3: rmsd values for superimposed structures are given with three significant digits, respectively, which suggests inappropriately high accuracy. 0.424 Å should be replaced by 0.42 Å and 0.843 Å by 0.84 Å.

Done

- Page 5, Results/Crystal structure of *Pa*FabB C161A, 1st paragraph, line 5: Replace "… from both *Vc*FabB as well as the ones from *Pa*FabB …" with"… from both *Vc*FabB and *Pa*FabB …".

Done

- Page 6, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 1: The 1st sentence of this paragraph has to be reformulated. It does not make sense in its present form.

This sentence reads now: "*The highly conserved Phe400/391 (numbering based on PaFabF/PaFabB) in the malonyl-CoA binding site was previously identified to play a pivotal role in substrate specificity and ligand binding, as this residue adopts different conformations in the apo and the intermediate-binding state*."

- Page 7, Table 2: Again, several values are given with inappropriately high accuracy. Multiplicity: 6.84 (6.56) should be replaced by 6.8 (6.6); I/sig(I): 19.11 (3.45) should be replaced by 19.1 (3.5); Ramachandran plot, residues in (%): (96.02%) should be replaced by (96.0%) and (3.98%) by (4.0%).

Done

- Page 8, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 1: Replace "w.t." with "apo-".

Done

- Page 8, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 2: Replace "When mutating …" with "Upon the mutation of …".

Done

- Page 8, Results/Active site and differences to *Pa*FabF, 1st paragraph, line 8: Replace "… is mutated to Val268 …" with "… is replaced by Val268 …" or "… corresponds to Val268 …".

Done

- Figure 5: It is not immediately obvious if the figure shows the homodimer or a single subunit only. This information should be given. If the complete dimer is shown (which I presume) it would make sense to present its subunits in different colours or shades of colour. The same applies to subsequent figures.

We have added the information to the relevant figures (now 6 and 7) and colored the subunits as suggested.

- The many figures showing FabB or the superpositions of different FabB/F enzymes are not very meaningful. FabB and FabF differ in substrate specificity for the fatty acid chain. The authors should definitely discuss the reasons for the respective substrate specificity by means of available structures.

We have inserted a new section to discuss the substrate selectivity with respect to fatty acids "Comparison of fatty acid chain binding channel in and in *Pa*FabB and *Pa*FabF". In addition, we have deleted the old Figure 5.

- I wish the authors had explained why the C161A mutation leads to the open conformation of the active site (see reviewer 1).

An explanation has been added to what is now the second paragraph in the section "Comparison of malonyl-CoA binding sites in *Pa*FabB and *Pa*FabF".

We hope that we have answered all issues that you have raised to your full satisfaction. If not, please let us know what else we should modify.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 November 2021

https://doi.org/10.5256/f1000research.77724.r98455

?

Bjarte A. Lund
Hylleraas Centre for Quantum Molecular Sciences, Department of Chemistry, UiT The Arctic University of Norway, Tromsø, Norway

Yadrykhins'ky *et al.* present an interesting story on the exploration of a new system for the development of new antibiotics. The authors make a convincing case for why fatty acid synthesis is a good target. Furthermore, the authors convey a clear technical step towards the goal by engineering a mutant variant of one enzyme in the pathway which mimics the intermediate step of the enzymatic reaction. An intermediate state that has been found to be susceptible to other inhibitors.

My objections to this paper are not on the technical and scientific soundness of the work but on its presentation.

- The introduction is short and does not clearly convey to me as a non-expert how the mutation of a Cys to Gln/Ala would make the enzyme resemble the intermediate state. I need to read all the way until Figure 9 and the Discussion to be informed about the Phe-residue that flips between open/closed conformations.

- After reading the paper, it is still not clear to me whether Gln was tried, or whether other amino acids could have had the same effect. Perhaps it is not important since Ala apparently worked.

- In the Abstract, acyl-intermediate could more clearly be written as acyl-enzyme intermediate.

- Results, Protein expression, and purification, TEV site would more precisely be termed TEV protease cleavage site.

- Results, Crystallization (Figure 2) **C164A/C161A is mixed non-consistently.** I can only assume this is a mix-up with the group's other work with FabF. But it is very confusing to the reader.

- Clearly out of scope to the paper, but it would have been interesting to know if iodine-phasing was tested.

- It is unclear how the resolution cutoff was decided. I/sig(I) is still very high at the highest resolution shell. It would also have been useful if CC* or CC1/2 was presented in Table 2.

- The very short Discussion-segment could have been merged with the Results-section. The discussion does raise the very interesting possibility of dual-target inhibitors that would presumably be of high interest with regards to the emergence of resistance. This could have been emphasized earlier as well.

- Methods, The sonication amplitude appears to lack a unit, presumably %. For the protein concentration determination, the extinction coefficient used should be given.

- Reference 21 appears to be a mistake.

All in all, I did appreciate the work and look forward to the continuation of the story.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, structural biology, enzymology,

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Dec 2021

Ruth Brenk, University of Bergen, Bergen, Norway

Thank you Bjarte for reviewing our article!

Here follows a point-to-point reply to the issues that you have raised.

- The introduction is short and does not clearly convey to me as a non-expert how the mutation of a Cys to Gln/Ala would make the enzyme resemble the intermediate state. I need to read all the way until Figure 9 and the Discussion to be informed about the Phe-residue that flips between open/closed conformations.

We have added a new Figure (now Figure 2) together with some explanatory text in the introduction to make this clearer.

- After reading the paper, it is still not clear to me whether Gln was tried, or whether other amino acids could have had the same effect. Perhaps it is not important since Ala apparently worked.

We have initially tried the Gln variant, but because this was less stable, we decided to work with the Ala variant instead. We have added this explanation to the end of the introduction.

- In the Abstract, acyl-intermediate could more clearly be written as acyl-enzyme intermediate.

Done.

- Results, Protein expression, and purification, TEV site would more precisely be termed TEV protease cleavage site.

Done.

- Results, Crystallization (Figure 2) **C164A/C161A is mixed non-consistently.** I can only assume this is a mix-up with the group's other work with FabF. But it is very confusing to the reader.

This was indeed a mix-up with the number from FabF which has now been corrected (figure legend for what is now Figure 3).

- Clearly out of scope to the paper, but it would have been interesting to know if iodine-phasing was tested.

We agree that this would be interesting, but we have not yet tested this. We might try it in the future.

- It is unclear how the resolution cutoff was decided. I/sig(I) is still very high at the highest resolution shell. It would also have been useful if CC* or CC1/2 was presented in Table 2

The resolution was limited by the distance of the detector. Unfortunately, we had major problems with shipping crystals to the synchrotron this spring and lost two consecutive shipments due to shipping/custom problems. As the work presented in this paper is from a master thesis, there was no time to repeat the measurements, but it looks like the crystals might diffract even beyond 1.3 Å. We have added CC1/2 to Table 2.

- The very short Discussion-segment could have been merged with the Results-section. The discussion does raise the very interesting possibility of dual-target inhibitors that would presumably be of high interest with regards to the emergence of resistance. This could have been emphasized earlier as well.

We have renamed the Results section to Results and Discussion and the old Discussion section to Conclusions. The discussion about dual-target inhibitors for both, the malonyl-

CoA binding site and the fatty acid channel (see comments from Klaus Reuter) has been added to what is now the Results and Discussion section.

- Methods, The sonication amplitude appears to lack a unit, presumably %. For the protein concentration determination, the extinction coefficient used should be given.

Done

- Reference 21 appears to be a mistake.

Deleted.

We hope that we have answered to all issues that you have raised to your full satisfaction. If not, please let us know what else we should address.

Competing Interests: No competing interests were disclosed.