applied microbiology

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Complementation of an *Escherichia coli* K-12 Mutant Strain Deficient in KDO Synthesis by Forming D-Arabinose 5-Phosphate from Glycolaldehyde with Fructose 6-Phosphate Aldolase (FSA)

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Abstract: KDO (2-keto-3-deoxy-D-*manno*-octulosonate) is a landmark molecule of the Gram-negative outer membrane. Mutants without KDO formation are known to be barely viable. Arabinose 5-phosphate (A5P) is a precursor of KDO biosynthesis and is normally derived from ribulose 5-phosphate by A5P isomerases, encoded by *kdsD* and *gutQ* genes in *E. coli* K-12. We created a *kdsD gutQ*-deficient double mutant of strain BW25113 and confirmed that these cells are A5P auxotrophs. Fructose 6-phosphate aldolase (FSA) is known to utilize (among other donors such as dihydroxyacetone or hydroxyacetone) glycolaldehyde (GoA) as a donor compound and to provide A5P in vitro when glyceraldehyde 3-phosphate is the acceptor. We show here that this FSA function in vivo fully reverses the growth defect and the A5P deficiency in *kdsD gutQ* double mutants. Expression of both plasmid-encoded *fsaA*, *fsaA*^{A129S}, or *fsaB* genes as well as a chromosomally integrated form of *fsaA*^{A129S} led to maximal OD_{600} values of >2.2 when GoA was added exogenously (together with glucose as a C source) at a concentration of 100 μM (K_s values in the range of 4–10 μM). Thus, a novel bio-orthogonal bypass to overcome an A5P deficiency was opened. Lower GoA concentrations led to lower growth yields. Interestingly, mutant strains with recombinant *fsa* genes showed considerable growth yields even without exogenous GoA addition, pointing to yet unknown endogenous GoA sources in *E. coli* metabolism. This is a further example of the usefulness of FSA in rewiring central metabolic pathways in *E. coli*.

Keywords: *Escherichia coli*; KDO; arabinose 5-phosphate; glycolaldehyde; fructose 6-phosphate aldolase; FSA; auxotroph; metabolic bypass; lipopolysaccharide

Citation: Guitart Font, E.; Sprenger, G.A. Complementation of an *Escherichia coli* K-12 Mutant Strain Deficient in KDO Synthesis by Forming D-Arabinose 5-Phosphate from Glycolaldehyde with Fructose 6-Phosphate Aldolase (FSA). *Appl. Microbiol.* **2024**, *4*, 470–480. https://doi.org/10.3390/applmicrobiol4010032

Academic Editor: Ian Connerton

Received: 27 January 2024
Revised: 26 February 2024
Accepted: 1 March 2024
Published: 3 March 2024

1. Introduction

Cells of Gram-negative bacteria are typically surrounded by the outer membrane (OM) which is an asymmetric bilayer. The OM contributes to the viability of these bacteria. It functions as a permeability barrier as it keeps away various compounds such as antibiotics, enzymes, or detergent-like cholates. Gram-negative bacteria are, thus, less sensitive to a variety of antibiotics or bile acids [1]. Whereas the inner leaflet of the OM consists of glycerophospholipids, the outer leaflet is nearly exclusively composed of the glycolipid lipopolysaccharide (LPS) in *E. coli* with approximately t 2 × 10^6 LPS molecules per cell [2]. LPS is embedded in the OM via the so-called lipid A; lipid A is connected with the inner-core oligosaccharide of the LPS by KDO (2-keto-3-deoxy-D-*manno*-octulosonate) [3,4]. Lipid A and KDO constitute the most conserved part of LPS in Gram-negative bacteria and are essential for cell viability. In *E. coli* K-12, several different core oligosaccharide glycoforms exist in parallel, with an average of 2.1 molecules of KDO attached to lipid A (so-called KDO_2–lipid A) [5].

KDO biosynthesis starts with the enzyme D-arabinose 5-phosphate (A5P) isomerase (API) which converts the pentose phosphate pathway intermediate D-ribulose 5-phosphate

in a reversible reaction to A5P. *E. coli* K-12 hosts two API isoenzymes, encoded by the genes *kdsD* and *gutQ* [6,7]. API enzymes are highly conserved among most Gram-negative bacteria and appear to be the only de novo sources of A5P. If both API-encoding genes are knocked out in *E. coli*, this creates an auxotrophy for A5P even when cells are grown on rich media like LB or YT [3,7,8]. API-deficient mutant strains of *E. coli* K-12 are severely affected in their anabolism as they are unable to synthesize a proper LPS and, thus, are more sensitive to antibiotics or detergents [3].

For many years, our group has been working with the *E. coli* enzyme fructose 6-phosphate aldolase (FSA) whose cognate physiological function is still enigmatic [9,10]. Its eponymous function is the reversible aldol formation of fructose 6-phosphate (F6P) from dihydroxyacetone (DHA) and glyceraldehyde 3-phosphate (G3P). *E. coli* K-12 hosts two genes (*fsaA* and *fsaB*) which encode isoenzymes of FSA [9]. FSA, in its recombinant form, has been used for a series of chemo-enzymatic syntheses in vitro as this aldolase is unusual in its broad donor and acceptor spectrum; for an overview and references, see [10]. For example, FSA utilizes other donor compounds (apart from DHA) such as hydroxyacetone [9] or glycolaldehyde (GoA) [11]. When GoA serves as a donor together with the acceptor G3P, A5P is formed in a reversible reaction in vitro [11–13]. We wanted to find out whether expression of *fsa* genes would allow a bypass in an *E. coli* mutant deficient in the API genes by providing A5P for anabolism. Therefore, we created a *kdsD* and *gutQ* double mutant of *E. coli* K-12. After proving that these cells were indeed A5P auxotrophs, we introduced and expressed *fsa* genes (either from plasmids or integrated into the chromosome). Thus, we show now that the A5P auxotrophy can be alleviated by adding GoA into the growth medium.

2. Materials and Methods

Bacterial strains of this study are listed in Table 1 and plasmids in Table 2; a list of PCR primers is given in Supplementary Table S1.

2.1. Growth Conditions

Bacteria were grown in Luria–Bertani (LB) medium [14] or MOPS minimal medium [15] with 28 mM glucose as the sole carbon source at 30 or 37 °C (determined by the plasmid used) and 180–200 rpm (INFORS HT, Bottmingen, Switzerland). If required, media were supplemented with antibiotics (100 µg/mL ampicillin, 50 µg/mL kanamycin, or 25 µg/mL chloramphenicol). In the cultures of the strains auxotrophic for A5P (Δ*gutQ* Δ*kdsD*), 50 µM A5P and 10 µM G6P were added to the media.

Table 1. *E. coli* strains. Methods employed to create the strains: (a) [16]; and (b) Δ*rbsK*::P_{tac}-$fsaA^{A129S}$:CRISPR/Cas [17].

Strain	Genotype	Reference
E. coli K-12		
BW25113	$lacI^q$ $rrnB_{T14}$ Δ$lacZ_{WJ16}$ *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$	[16]
BW25113 Δ*gutQ*	BW25113, Δ*gutQ*::FRT	[18]
BW25113 Δ*gutQ* Δ*kdsD*::*kan*	BW25113, Δ*gutQ*::FRT Δ*kdsD*::FRT-*kan*-FRT	This study [a]
BW25113 Δ*gutQ* Δ*kdsD*	BW25113, Δ*gutQ*::FRT Δ*kdsD*::FRT	This study [a]
BW25113 Δ*gutQ* Δ*kdsD* Δ*rbsK*::P_{tac}-$fsaA^{A129S}$	BW25113, Δ*gutQ*::FRT Δ*kdsD*::FRT Δ*rbsK*::P_{tac}-$fsaA^{A129S}$	This study [b]

2.2. Recombinant DNA Techniques

Standard molecular biological methods were followed [14]. For the chromosomal deletion of the *kdsD* gene, a recombineering method was carried out [16]. Yet, the integration of the $fsaA^{A129S}$ gene in the ribose operon was performed with the CRISPR–Cas9 method for *E. coli* [17]. DNA fragments were amplified by PCR from plasmids and chromosomal DNA. Plasmid constructs and chromosomal modifications were verified by colony PCR,

agarose gel electrophoresis (see Figure S1), and custom DNA sequencing (GATC Biotech AG, Konstanz, Germany).

Table 2. Plasmids used during this study.

Plasmid	Characteristics	Reference
pJF119EH	P_{tac}, *lacI*^{q}, RBS, Amp^{R}	[19]
pJF119*fsaA*	*fsaA* gene cloned into pJF119EH (Xba*I*/Hind*III*)	[20]
pJF119*fsaA*^{A129S}	*fsaA*^{A129S} gene cloned into pJF119EH (Xba*I*/Hind*III*)	[20]
pJF119*fsaB*	*fsaB* gene cloned into pJF119EH (Eco*RI*/Hind*III*)	This study
pKD46	*repA101* (Ts), *araC*, P_{araB}-ϒ-β-exo (red recombinase), Amp^{R}	[16]
pCO1-cat	P_{tac}-*aroC*-*aroA*, oriR6′ (first 36 bp), FRT-*cat*-FRT, *lacI*^{q}, Amp^{R}, Cm^{R}	[21]
pCP20	FLP^{+}, λ $cI857^{+}$, λ p_{R} Rep^{ts}, Amp^{R}, Cm^{R}	[22]
pCas	*repA101* (Ts), P_{cas}-*cas9*, P_{araB}-ϒ-β-exo (red recombinase), *lacI*^{q}, P_{trc}-sgRNA-*pMB1*, Km^{R}	[17]
pTargetF-Cm-*rbsK*	*pMB1*, sgRNA-*rbsK*, Cm^{R}	[23]

2.3. Dose–Response Curves for A5P and GoA

Dose–response assays were performed using liquid MOPS-MM (containing 28 mM glucose) in shake flasks. The maximal OD_{600nm} achieved after 48 h of incubation at 37 °C (for A5P) or 30 °C (for GoA) was monitored. To assay A5P auxotrophy, an overnight culture of BW25113 Δ*gutQ*::FRT Δ*kdsD*::FRT-*kan*-FRT (10 mL MOPS-MM with 28 mM glucose, 10 µM G6P, 15 µM A5P, and 50 µg/mL Km) was washed twice with MOPS-MM containing 28 mM glucose. Then, cells were inoculated in 100 mL shake flasks containing 10 mL MOPS-MM with 28 mM glucose, 10 µM G6P, 50 µg/mL Km, and different A5P concentrations (0 µM; 5 µM; 10 µM; 25 µM; 40 µM; 50 µM; 60 µM; 70 µM; 100 µM) with an initial OD_{600nm} of 0.05. The shake flasks were incubated at 200 rpm and 37 °C for 48 h. The obtained OD_{600nm} values were plotted as a function of the A5P concentration; this resulted in a dose–response curve. Likewise, dose–response curves for glycolaldehyde were obtained with the A5P auxotroph strains grown on MOPS-MM containing 28 mM glucose, 100 µM IPTG, and different concentrations of GoA. A single colony, grown on MM-Agar with 28 mM glucose, 10 µM G6P, 50 µM A5P, and, if needed, the corresponding antibiotics (ampicillin 100 mg/L, kanamycin 50 mg/L), was inoculated in 10 mL MOPS-MM with 28 mM glucose, 10 µM G6P, 15 µM A5P, and the corresponding antibiotics in a 100 mL shake flask and incubated overnight at 200 rpm and 37 °C. An amount of 2 mL of the preculture was centrifuged at 16,500× *g* for 1 min and washed twice with 1 mL MOPS-MM with 28 mM glucose. The cell pellet was resuspended in 1 mL MOPS-MM with 28 mM glucose and inoculated with an initial OD_{600nm} of 0.05 in the main culture, which contained 10 mL MOPS-MM with 28 mM glucose, 100 µM IPTG, the corresponding antibiotics, and different GoA concentrations (0 µM; 10 µM; 25 µM; 40 µM; 50 µM; 90 µM; 100 µM; 125 µM). The cell culture was then incubated at 200 rpm and 30 °C for 48 h. The OD_{600nm} was measured when the incubation was finished. K_S and the maximal OD_{600nm} values were calculated with the software "GraphPad Prism" version 6.01 from GraphPad Software, Inc. (San Diego, CA, USA).

3. Results

3.1. Construction and Characterization of an E. coli K-12 Double Mutant Strain Deficient in API

E. coli K-12 strain BW25113 (see Table 1 for genotype) [16] was chosen as the parent strain. In order to block KDO formation, the two genes *gutQ* and *kdsD* for D-A5P synthesis were knocked out successively by a λ-red recombineering method [16]. Thus, the double mutant (strain BW25113 Δ*gutQ*::FRT Δ*kdsD*::FRT-*kan*-FRT) was obtained. We decided to keep the kanamycin resistance marker for easier detection of the mutant strain. During the last step of the double mutant generation, A5P (50 µM) was added to the cultures together with 10 µM of glucose 6-phosphate to induce the UhpT uptake system which transports A5P [24,25] (see Figure 1). The mutant strain was auxotrophic for supplementation of A5P as expected [7] and A5P had to be added even in complex media such as LB or 2YT, as yeast extract does not contain A5P [3]. We observed that cells of strain BW25113 Δ*gutQ* Δ*kdsD*::*kan* stopped growth after transfer into an A5P-free medium; they clumped together and eventually lysed. Microscopic analysis revealed that cells of this strain—in comparison with the wild-type BW25113—before lysis formed long chains indicating severe problems associated with division (see Figure S2). Therefore, growth curves in shake flasks with strain BW25113 Δ*gutQ* Δ*kdsD*::*kan* could not be obtained in the absence of A5P, a feature which has been reported for analogous *E. coli* mutants before [7].

A "bioassay" (auxanogram) was carried out with BW25113 Δ*gutQ* Δ*kdsD*::*kan* cells by adding varying concentrations of external A5P to assess the degree of this auxotrophy. Cells from an A5P-containing preculture were transferred (after washing) into in a MOPS-buffered mineral salt medium (MM) with glucose as the sole carbon source. A5P (together with G6P) was added at various concentrations.

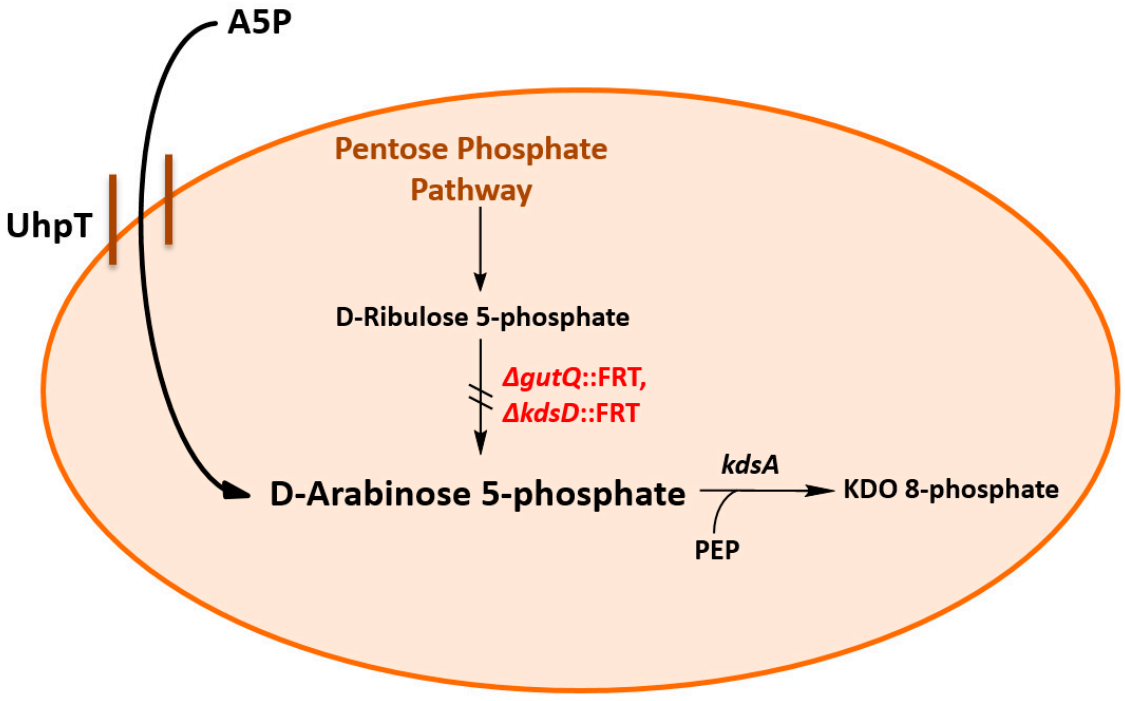

Figure 1. Scheme to highlight the role of A5P isomerases (encoded by *kdsD* and *gutQ*) and supplementation of an auxotroph for A5P (uptake via the UhpT transporter).

As can be seen from Figure 2 and Table S2, as little as 5 µM A5P gave a positive growth signal (increase in the final OD at 600 nm, OD_{600}) while no addition led to no OD increase, cell clumping, and formation of long chains (see Figure S2), and lysis was observed. A positive correlation was found with increasing A5P concentrations, final growth yields of up to OD ~3, with >60 µM of A5P. A K_S value of 17.7 ± 4.5 µM was, thus, determined. The wild-type strain BW25113 (without A5P supplementation) reached a maximal OD_{600} of ~3.

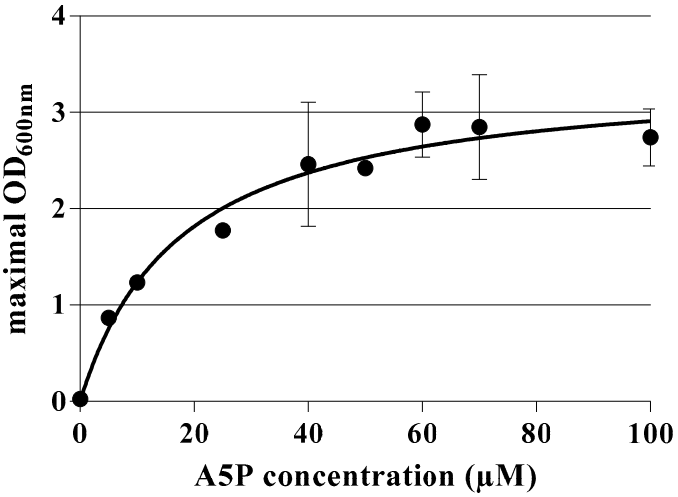

Figure 2. Dose–response curve with increasing A5P concentrations. Growth of double mutant BW25113 Δ*gutQ* Δ*kdsD*::*kan* on MOPS-MM with 28 mM of glucose as C source. Dependence of the maximal OD_{600nm} reached by BW25113 Δ*gutQ* Δ*kdsD*::*kan* on varying A5P concentrations. A maximal OD_{600nm} = 3.4 ± 0.3 determined with the software "GraphPad Prism" version 6.01 from GraphPad Software, Inc. (San Diego, CA, USA). For details, see Material and Methods. For uptake of A5P, 10 µM glucose 6-phosphate was added as inducer of UhpT [24]. Data are from two biological replicates with error bars.

3.2. Physiological Observations in kdsD gutQ Double Mutants with or without FSA Expression

GoA is a donor substrate for FSA [11,26] and A5P synthesis in vitro has been shown before by using FSA with the donor GoA and the acceptor G3P [12]. Further, a synthetic pathway ("glycoptimus", shown both in vitro and in vivo) from D-glucose, D-xylose, or L-arabinose leading to glycolic acid formation via GoA has been established in *E. coli* via plasmid-based expression of *kdsD* and *fsaA* genes [27].

We wanted to know whether *E. coli* FSAA might open a bypass reaction in the API-deficient strain BW25113 Δ*gutQ* Δ*kdsD*::*kan* by condensing G3P and GoA to A5P. Therefore, the genes encoding the native FSAA or the variant $FSAA^{A129S}$ (which has an improved catalytic efficiency towards F6P [23,26,28]) were expressed in the *E. coli* double mutant from the plasmids pJF119*fsaA* and pJF119$fsaA^{A129S}$ [20,23], respectively. These expression vectors contained an ampicillin resistance marker and the *fsa* genes were under the control of the P_{tac} promoter (IPTG-inducible). Thus, IPTG was added for induction (final concentration of 100 µM) and external GoA was added to provide a donor compound for FSA activity. We assumed that the cells would supply the acceptor G3P from glycolysis. As controls, *E. coli* K-12 wild-type (wt) strain BW25113, BW25113 Δ*gutQ* Δ*kdsD*::*kan*, BW25113 Δ*gutQ* Δ*kdsD*, and BW25113 Δ*gutQ* Δ*kdsD*::*kan* pJF119EH (for genotypes, see Table 1) were used.

While the wild-type BW25113 showed, as expected, normal growth, none of the double mutant strains could grow without *fsa*-bearing plasmids (less than one doubling in OD). Cells of strain BW25113 Δ*gutQ* Δ*kdsD*::*kan* (or its pJF119EH-bearing derivative) clumped and lysed when incubated without the presence of external A5P on MOPS-MM (see Supplementary Figure S2). Addition of various concentrations of GoA did not prevent this. However, cells of BW25113 Δ*gutQ* Δ*kdsD*::*kan* carrying either pJF119*fsaA* or pJF119$fsaA^{A129S}$ were able to grow in the presence of different GoA concentrations (see Figure 3a,b). Also, pJF119 carrying the other *E. coli* FSA-encoding gene, *fsaB* (see Table 2), was able to complement the API deficiency in BW25113 Δ*gutQ* Δ*kdsD* (Figure 3c). For data sets, see Table S3.

Furthermore, we wondered if a single chromosomally integrated copy of $fsaA^{A129S}$ might also be sufficient to restore growth. We, therefore, integrated a P_{tac}-controlled copy of the gene into the *rbsK* locus. This strain, BW25113 Δ*gutQ* Δ*kdsD* Δ*rbsK*::P_{tac}-$fsaA^{A129S}$, also grew without external addition of A5P if GoA was provided (Figure 3d).

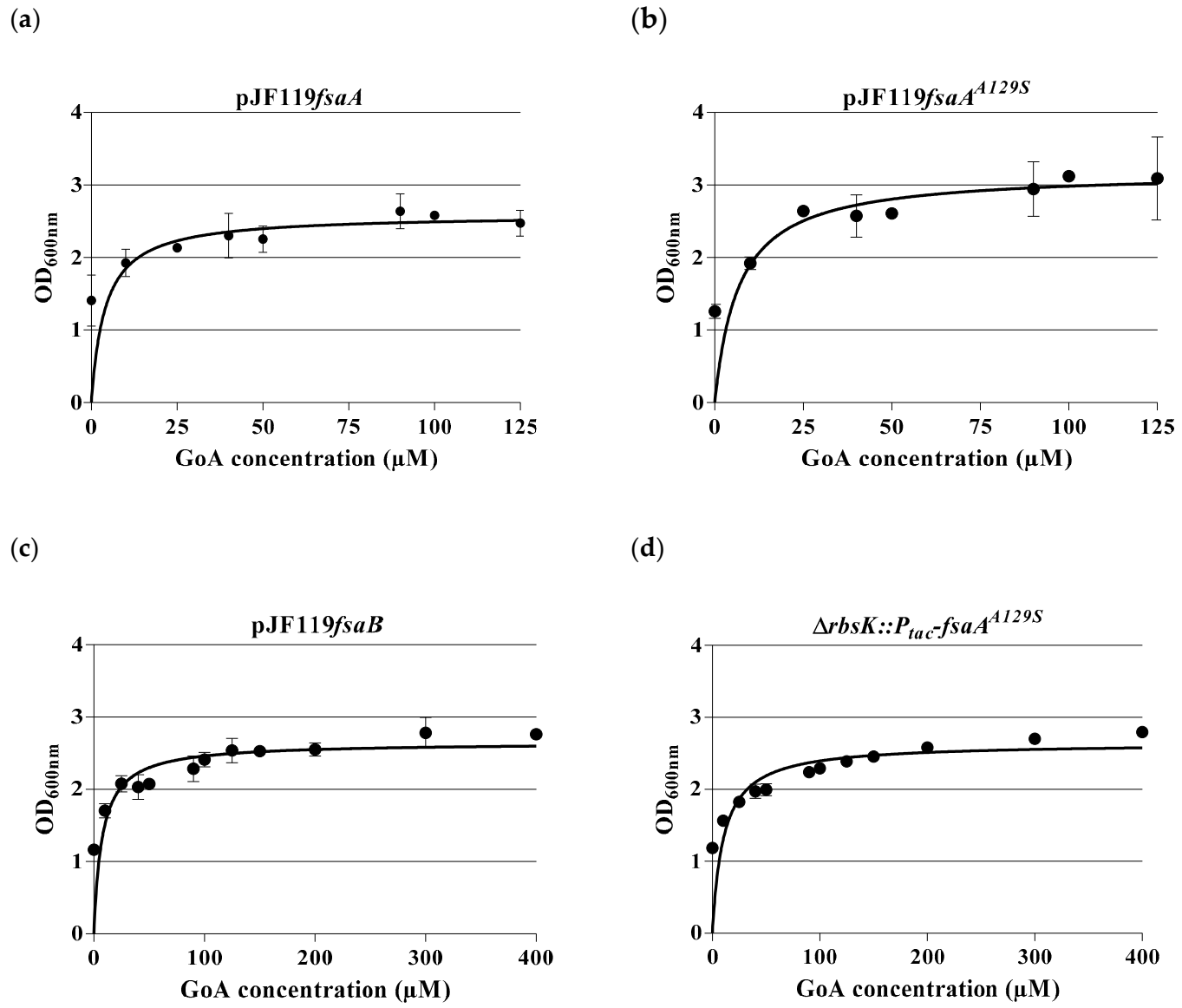

Figure 3. Dose–response curves for glycolaldehyde in double mutants deficient for API with various *fsa*-bearing plasmids (**a**–**c**) or a chromosomal integration (**d**). Growth yields were determined as final OD_{600nm} values after 48 h of incubation on MOPS-MM containing 28 mM glucose and 100 µM IPTG. (**a**) BW25113 Δ*gutQ* Δ*kdsD*::*kan* pJF119*fsaA* (K_S = 4.1 ± 3.6; $OD_{600nm\ max.}$ = 2.6 ± 0.3). (**b**) BW25113 Δ*gutQ* Δ*kdsD*::*kan* pJF119*fsaA*^{A129S} (K_S = 6.8 ± 3.8; $OD_{600nm\ max.}$ = 3.2 ± 0.3). (**c**) BW25113 Δ*gutQ* Δ*kdsD* pJF119*fsaB* (K_S = 7.5 ± 3.0; $OD_{600nm\ max.}$ = 2.6 ± 0.1). (**d**) BW25113 Δ*gutQ* Δ*kdsD* Δ*rbsK*::P_{tac}-*fsaA*^{A129S} (K_S = 10.4 ± 3.8; $OD_{600nm\ max,}$ = 2.6 ± 0.1). Average values of two independent biological replicates with error bars are shown. Note that in Figure 3c,d, the range of GoA is different.

The four different strains showed a similar reaction versus GoA addition: the higher the GoA concentration present in the medium, the higher the maximal OD_{600nm} reached by these strains. GoA concentrations above 100 µM, however, did not lead to significant growth yield improvement. It is worth noting that even without GoA supplementation, a maximal $OD_{600nm} \geq 1$ was reached by all double mutant strains carrying *fsa* plasmids. This we interpret as a hint that *E. coli* metabolism provides yet unknown sources of GoA (see Section 4).

4. Discussion

KDO is a landmark molecule of the Gram-negative outer membrane. Mutants without KDO formation are barely viable [3]. We created *kdsD* and *gutQ* double mutants of *E. coli* K-12 strain BW25113 and confirmed, as expected from the work of Woodard's group, that these cells are A5P auxotrophs. If A5P was removed from the growth media by centrifugation and washing, cells ceased to grow and most of them eventually lysed or formed long chains of cells which ceased to divide (see Supplementary Figure S2); this is also in accordance with earlier reports [7]. Gain-of-function suppressor mutants have been reported to arise from *kdsD gutQ* double mutants when cultured for prolonged times in liquid media. These mutants grew with a prolonged generation time and showed a growth defect above 42 °C; the LPS layer consisted of lipid IV_A free of KDO and the cells were super-susceptible to a variety of antibiotics, SDS, or bile salts. The compensatory suppressor mutants were also considered as endotoxin-free [3]. We did not look for such suppressor mutants, however, as this was not within the scope of the present study.

Our idea was to assay whether expression of *fsa* genes would complement the growth defects of API-lacking cells. This was performed in the presence or absence of glycolaldehyde (GoA). It turned out that only those double mutant strains with ectopic expression of *fsa* genes (*fsaA*, *fsaB*, or $fsaA^{A129S}$ either from plasmids or inserted under P_{tac}-control in a chromosomal locus) were able to regain growth in the absence of externally added A5P. Externally added GoA improved growth yields but, remarkably, even without GoA addition, cell densities (measured as OD_{600}) of >1 were obtained. This was not observed with double mutants that did not carry recombinant *fsa* genes.

FSAA and its variant FSAA A129S have been shown in the past to allow bypass reactions in the central metabolism of *E. coli*. The group of Stephanopoulos showed that a block in the DXS reaction (providing 1-deoxyxylulose 5-phosphate from pyruvate and G3P for the methylerythritol biosynthesis pathway of isoprenoids in *E. coli*) could be overcome by FSA by adding exogenous hydroxyacetone with GoA (from an engineered D-arabinose catabolic pathway) to 1-deoxyxylulose [29]; this reaction has been previously shown in vitro [30]. Recently, we reported that FSAA A129S was able to bypass a block by phosphofructokinase in *E. coli* mutants (Δ*pfkA* Δ*pfkB* Δ*zwf*), again following the knowledge that F6P can be cleaved to G3P and DHA. This opened a pathway for the production of DHA and glycerol from glucose [23]. The opposite reaction, e.g., growth on glycerol via DHA to F6P in specific mutants, was achieved as well by the group of the late Arren Bar-Even [31]. While the present manuscript was in preparation, the Walther's group proved that FSAA can be used to implement a novel in vivo metabolic pathway to yield the product acetyl-CoA. Their starting point was ethylene glycol, which had to be oxidized to GoA with the help of an oxygen-resistant variant of FucO; GoA was then condensed with G3P to A5P by FSAA [32].

As API are also found in several Gram-negative pathogenic bacteria, they are considered as interesting targets for the development of antibacterials [33,34]. With our approach of using *gutQ* and *kdsD* mutants, A5P is channeled to KDO8P synthesis starting from glucose (via G3P) and the simple compound GoA. This prevents its mixing with compounds of the PPP (summarized in Figure 4). A5P is, thus, delivered by an orthogonal biosynthetic pathway. This could be used to label KDO8P specifically by using labeled GoA. This could also help in studies where the interaction of KDO_2–lipid A with the innate immune system is analyzed. KDO_2–lipid A is the conserved structure in LPS of most Gram-negative bacteria. LPS notoriously acts as endotoxin, leading to fever ("pyrogen") and toxic shock in mammals as it stimulates the innate immune system via the TRL-4 receptor on macrophages. Purified KDO_2–lipid A is sufficient to evoke this endotoxic effect [35]. Specific *E. coli* mutants (with defects in the *rfaD* gene) have been obtained with improved production of KDO_2–lipid A to develop lipid A adjuvants as part of bacterial vaccines [36]. Our findings in A5P formation from inexpensive precursors could help to further improve this production.

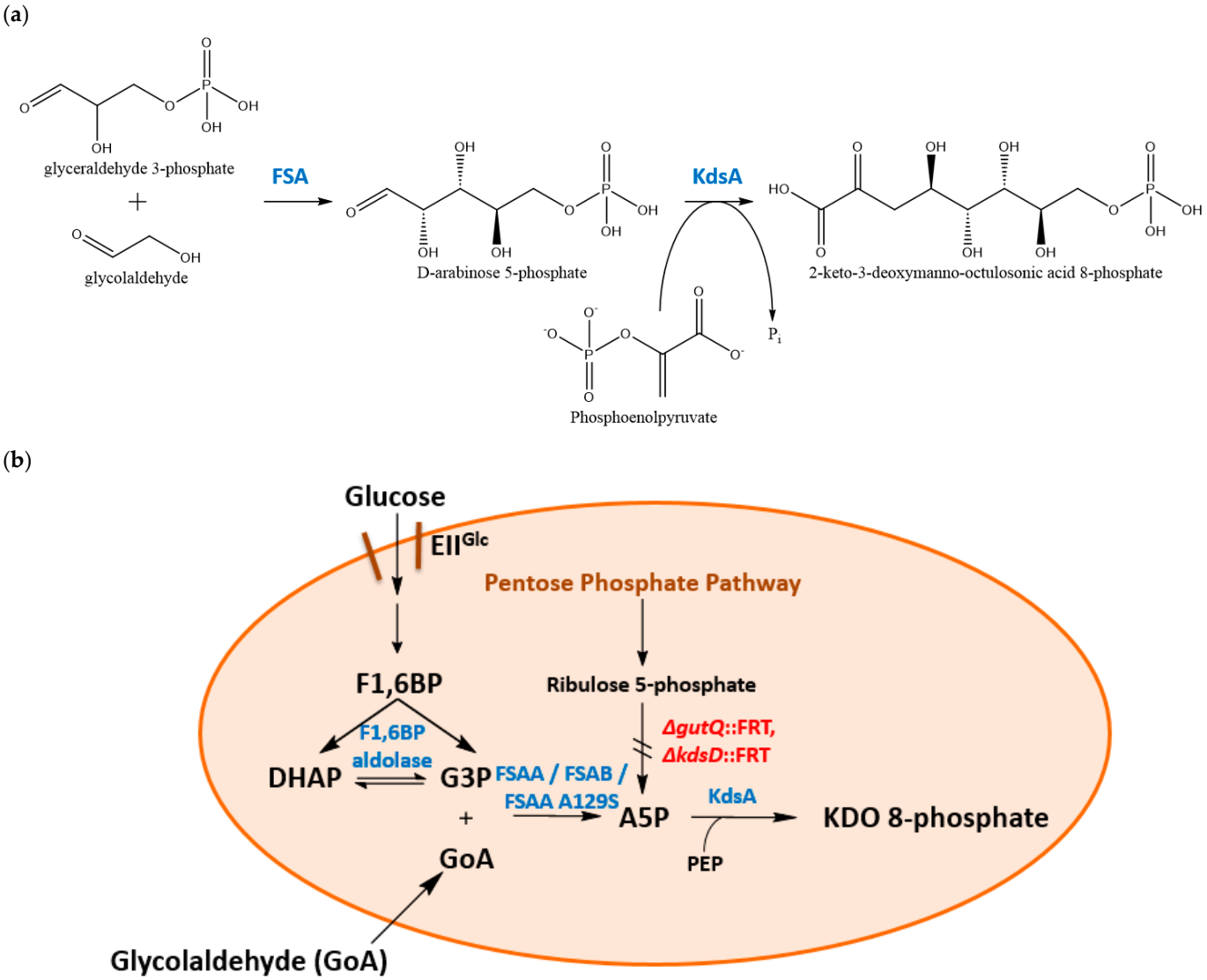

Figure 4. (**a**) Addition of glycolaldehyde (GoA) to glyceraldehyde 3-phosphate (G3P) by FSA enzymes to arabinose 5-phosphate (A5P), and condensation of A5P with phosphoenolpyruvate (PEP) to form 2-keto-3-deoxymanno-octulosonic acid 8-phosphate (KDO 8-phosphate) by KDO 8-phosphate synthetase (KdsA). (**b**) Scheme of complementation by FSA enzymes in *E. coli* K-12 *kdsD gutQ* double mutant by adding external GoA to G3P, thereby providing the cells with A5P, a precursor of KDO.

Residual growth (after removal of A5P) was found in the control *kdsD gutQ* double mutants to reach OD values of ~0.3 with the onset of clumping and eventual lysis. The observation that *kdsD gutQ* double mutants with the expression of recombinant *fsa* genes could grow up to an OD_{600} of ~1.2 (starting OD ~0.05) (see Figure 3) in the absence of both external compounds, e.g., A5P or GoA, deserves to be discussed in more detail. This growth feature points to hidden intracellular sources of GoA when cells grow on glucose as a sole carbon source. The literature on in vivo formation of free GoA, however, is scarce or based only on in vitro measurements [37]. GoA is a highly reactive Maillard agent which leads to non-enzymatic post-translational protein modifications in vivo, N^6-carboxymethyllysine being the predominant glycated end product [37]. From biochemical studies, a one-substrate reaction of bacterial transketolase on ß-hydroxypyruvate (HPA) is known which could release free GoA; HPA in turn could be formed by action of YeaB from 3-phosphohydroxypyruvate in the L-serine biosynthesis pathway [38].

Exogenously added GoA was shown previously to complement a pyridoxine auxotrophy in an *E.coli* B mutant WG3. In this strain, the authors found a defect in a glycolaldehyde

dehydrogenase (forming GoA by reduction of glycolate) and proposed a possible origin of GoA from glyoxylate via glycolate [39]. More recent work in the literature discusses GoA as precursor of L-4-hydroxythreonine synthesis (aldol reaction of LtaE on glycine and GoA) and, thus, as a precursor of a minor pathway (not the main reaction) to pyridoxal phosphate in *E. coli*. Here, the endogenous origin of GoA could be 3-phosphohydroxypyruvate (see above) [38]. In the folate biosynthesis pathway, dihydroneopterin aldolase FolB liberates GoA [40]. GoA could also be formed by reduction of the dialdehyde glyoxal via YqhD, a metabolite which is formed by degradation of autoxidized compounds in many organisms [41]. An unspecific monoamine oxidase (MaoA) from *E. coli* has been shown to provide GoA from ethanolamine in vitro [42]. Further work is necessary to elucidate the internal source(s) of GoA which leads to formation of A5P and complementation of the *kdsD* and *gutQ* defects.

5. Conclusions

We have shown that the expression of multifunctional enzyme FSA allows a bypass in an API-deficient mutant strain of *E. coli* BW25113 by using G3P (from glycolysis) and GoA to form A5P, a precursor of the outer membrane compound KDO. This helped to reinstall full growth and to overcome the detrimental effects on viability caused by KDO deficiency. Full complementation in terms of growth yields took place with exogenously added GoA, while lower growth yields were achieved even without GoA addition, pointing to hidden endogenous sources of GoA. This constitutes another example for the establishment of bio-orthogonal metabolic pathways (bypass reactions) in *E. coli* with the help of FSA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4010032/s1, Figure S1: Agarose gel electrophoresis, Figure S2: Light microscopy photos of cells Table S1: PCR primers, Table S2: Dose-response curve for various A5P concentrations, shake flask cultures, $OD_{600\ nm}$ measured, Table S3: Dose-response curve for various exogenous GoA concentrations, shake flask cultures, maximal $OD_{600\ nm}$ measured.

Author Contributions: Conceptualization, E.G.F. and G.A.S.; methodology, E.G.F.; software, E.G.F.; validation, E.G.F. and G.A.S.; formal analysis, E.G.F.; investigation, E.G.F.; resources, G.A.S.; data curation, E.G.F.; writing—original draft preparation, E.G.F. and G.A.S.; writing—review and editing, E.G.F. and G.A.S.; visualization, E.G.F.; supervision, G.A.S.; project administration, G.A.S.; funding acquisition, none. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data are available within this paper and the supporting information.

Acknowledgments: We thank members of our institute for help with cloning (Natalie Trachtmann, Katrin Gottlieb).

Conflicts of Interest: The authors declare no conflicts of interest.

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