Evaluation of Heterologous Biosynthetic Pathways for Methanol-Based 5-Aminovalerate Production by Thermophilic Bacillus methanolicus

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The use of methanol as carbon source for biotechnological processes has recently attracted great interest due to its relatively low price, high abundance, high purity, and the fact that it is a non-food raw material. In this study, methanol-based production of 5-aminovalerate (5AVA) was established using recombinant *Bacillus methanolicus* strains. 5AVA is a building block of polyamides and a candidate to become the C5 platform chemical for the production of, among others, δ-valerolactam, 5-hydroxyvalerate, glutarate, and 1,5-pentanediol. In this study, we test five different 5AVA biosynthesis pathways, whereof two directly convert L-lysine to 5AVA and three use cadaverine as an intermediate. The conversion of L-lysine to 5AVA employs lysine 2-monooxygenase (DavB) and 5-aminovaleramidase (DavA), encoded by the well-known *Pseudomonas putida* cluster davBA, among others, or lysine α-oxidase (RaiP) in the presence of hydrogen peroxide. Cadaverine is converted either to γ-glutamine-cadaverine by glutamine synthetase (Spul) or to 5-aminopentanal through activity of putrescine oxidase (Puo) or putrescine transaminase (PatA). Our efforts resulted in proof-of-concept 5AVA production from methanol at 50°C, enabled by two pathways out of the five tested with the highest titer of 0.02 g l⁻¹. To our knowledge, this is the first report of 5AVA production from methanol in methylotrophic bacteria, and the recombinant strains and knowledge generated should represent a valuable basis for further improved 5AVA production from methanol.

**Keywords:** *Bacillus methanolicus*, thermophile, methanol, 5-aminovalerate, alternative feedstock

**INTRODUCTION**

The worldwide amino acid market is progressively growing at 5.6% annual rate and is estimated to reach US$25.6 billion by 2022, with amino acids used for animal feed production being its largest component (Wendisch, 2020). The growing demand for amino acid supply confronts the biotechnological industry with an unprecedented challenge of identifying suitable feedstocks,
especially in terms of replacing sugars and agricultural products, use whereof deteriorates food supply and threatens biodiversity (Cotton et al., 2020). Methanol, together with other one-carbon (C1) compounds, is considered a very promising substitute for feedstock that are conventionally used in biotechnological processes. The major advantages of using methanol as carbon source are its low production cost (e.g., methanol from steam reforming of methane), ease of transport and storage, and complete miscibility that bypasses the mass transfer barrier and potentially supports improvement in microbial productivities. However, what seems to cause a considerable difficulty in propagation of methanol as biotechnological feedstock is the limited selection of microorganisms capable to be used as their carbon and energy source. One of the compelling candidates to become a workhorse for the methanol-based production of amino acids is *Bacillus methanolicus*, a thermophilic methylotroph isolated from freshwater marsh soil by Schendel et al. (1990). The wild-type strain MGA3 naturally overproduces γ-aminobutyric acid and cadaverine (Nærdal et al., 2015; Irla et al., 2017; Brautaset et al., 2010; Table 1). MGA3 produces 0.4 g l\(^{-1}\) of L-lysine in high cell density fed-batch fermentations (Brautaset et al., 2010; Table 1); this titer was improved nearly 30-fold up to 11 g l\(^{-1}\) by plasmid-based overexpression of a gene coding for aspartokinase, a key enzyme controlling the synthesis of aspartate-derived amino acids (Jakobsen et al., 2009). Through application of a classical mutagenesis technique, a derivative of *B. methanolicus* MGA3 (M168-20) was constructed, which produces 11 g l\(^{-1}\) of L-lysine in high cell density methanol-controlled fed-batch fermentations (Brautaset et al., 2010); the L-lysine overproduction being caused among others by mutation in the *hom-1* gene coding for homoserine dehydrogenase (Hom) and in the putative lysine 2,3-aminomutase gene (locus tag BMMGA3_02505). The mutation in *hom-1* leads to the loss of catalytic activity of homoserine dehydrogenase and redirection of metabolic flux toward the L-lysine pathway and therefore its accumulation (Nærdal et al., 2011, 2017).

5-Aminovalerate (5AVA) is a product of L-lysine degradation, and it is mainly synthesized in a two-step process catalyzed by a lysine monooxygenase (DavB) and a δ-aminoovaleramidase amidohydrolase (DavA) (Revelles et al., 2005). 5AVA is a non-proteogenic five-carbon amino acid that could potentially be used as building block for producing biobased polymides (Adkins et al., 2013; Park et al., 2014; Wendisch et al., 2018). It is also a promising precursor for plasticizers and chemicals that are intermediates for bioplastic preparation: δ-valerolactam (Chae et al., 2017), 5-hydroxy-valerate (Sohn et al., 2021), glutarate (Adkins et al., 2013; Pérez-García et al., 2018), and 1,5-pentanediol (Cen et al., 2021). As summarized in Table 1, diverse approaches have been made at the establishment of microbial 5AVA production. *Pseudomonas putida* KT2440, which possesses davBA in its genome, can synthesize 20.8 g l\(^{-1}\) 5AVA from 30 g l\(^{-1}\) L-lysine in 12 h (Liu et al., 2014). Production of 5AVA was established in *Corynebacterium glutamicum* by heterologous overexpression of the DavB- and DavA-encoding genes (davBA) from *P. putida* with a final titer up to 39.9 g l\(^{-1}\) in a sugar-based fed-batch fermentation (Robles et al., 2016; Shin et al., 2016; Joo et al., 2017). 5AVA can be also produced in a process of bioconversion of L-lysine supplemented to the growth medium with molar yields of up to 0.942 achieved by *Escherichia coli* strains overproducing DavBA (Park et al., 2014; Wang et al., 2016). Moreover, when the recombinant *E. coli* strain expressing davAB genes was cultured in a medium containing 20 g l\(^{-1}\) glucose and 10 g l\(^{-1}\) L-lysine, 3.6 g l\(^{-1}\) 5AVA was produced, representing a molar yield of 0.45 (Park et al., 2013). Disruption of native lysine decarboxylase (CadA and LdcC) activity in *E. coli* strains overexpressing davBA limited cadaverine by-product formation, enabling increased accumulation of L-lysine following 5AVA production, with 5AVA yield of 0.86 g l\(^{-1}\) in glucose-based shaking flask fermentation (Adkins et al., 2013). Furthermore, Cheng et al. (2018) reported that the oxidative decarboxylation of L-lysine catalyzed by a L-lysine α-oxidase (RaiP) from *Scomber japonicus* led to 5AVA production. The production of RaiP was enhanced by the addition of 4% (v/v) ethanol and 10 mM H\(_2\)O\(_2\), which increased the 5AVA titer to 29.12 g l\(^{-1}\) by an *E. coli* host strain in a fed-batch fermentation (Cheng et al., 2018). Recently, in a similar L-lysine bioconversion strategy, an *E. coli* whole-cell catalyst producing RaiP was developed, converting 100 g l\(^{-1}\) of L-lysine hydrochloride to 50.62 g l\(^{-1}\) 5AVA representing a molar yield of 0.84 (Cheng et al., 2020).

Recent efforts have employed novel metabolic routes toward 5AVA. In *Pseudomonas aeruginosa* PAO1, the set of enzymes composed of glutamylpolyamine synthetase, polylamine:pyruvate transaminase, aldehyde dehydrogenase, and glutamine amidotransferase is essential for the degradation of diaminos through the γ-glutamylation pathway (Yao et al., 2011), which may lead to 5AVA production when cadaverine is degraded (Luengo and Oliva, 2020). Jorge et al. (2017) established a three-step 5AVA biosynthesis pathway consisting of the conversion of L-lysine to cadaverine by the activity of the enzyme LdcC, followed by cadaverine conversion to 5AVA through consecutive transamination, by a putrescine transaminase (PatA), and oxidation by a PatD. The heterologous overexpression of the genes ldcC, patA, and patD led to 5AVA production to a final titer of 5.1 g l\(^{-1}\) by an engineered *C. glutamicum* strain in a shake flask fermentation (Jorge et al., 2017). This pathway has served as basis for the establishment of a new three-step pathway toward 5AVA using the monoxygenase putrescine oxidase (Puo), which catalyzes the oxidative deamination of cadaverine, instead of PatA (Haupka et al., 2020).

Critical factors that can affect 5AVA accumulation in a production host are the presence of a native 5AVA degradation pathway in its genome and the end product-related inhibition. In some bacterial species, such as *P. putida* KT2440, *Pseudomonas syringae*, *Pseudomonas stutzeri*, and *C. glutamicum*, 5AVA is degraded by a GABAse (Figure 1), composed of two enzymes γ-aminobutyric acid aminotransferase (GabT) and succinic
TABLE 1 | Comparison of the 5AVA production by different engineered microbial strains and production of amino acids by B. methanolicus.

| Organism               | Approach                                                                 | 5AVA titer [g L⁻¹] | References          |
|------------------------|--------------------------------------------------------------------------|--------------------|---------------------|
| *Pseudomonas putida*   | KT2440 DavBA-based catalytic production of 5AVA from 30 g L⁻¹ L-lysine   | 20.80              | Liu et al., 2014    |
| *Corynebacterium glutamicum* | DavBA-based catalytic production of 5AVA from 30 g L⁻¹ L-lysine | 33.10              | Shin et al., 2016   |
|                        | Heterologous expression of davBA; sugar-based fed-batch fermentation    | 28.00              | Rohles et al., 2016 |
|                        | Heterologous expression of davBA; glucose-based fed-batch fermentation  | 39.93              | Joo et al., 2017    |
| *Escherichia coli*     | Heterologous expression of davBA; glucose-based fed-batch fermentation  | 5.10               | Jorge et al., 2017  |
|                        | Heterologous expression of davBA and deletion of cadA; glucose-based fermentation | 3.70               | Haupka et al., 2020 |
|                        | Heterologous expression of davBA; sugar-based fermentation; 10 g L⁻¹ L-lysine provided | 0.86               | Adkins et al., 2013 |
| *Bacillus methanolicus*| Heterologous expression of davBA; fed-batch whole-cell bioconversion of L-lysine maintained at 120 g L⁻¹ | 240.70             | Wang et al., 2016   |
|                        | Heterologous expression of davBA; glucose-based fed-batch fermentation; 120 g L⁻¹ L-lysine provided | 3.60               | Park et al., 2013   |
|                        | Heterologous expression of davBA; glucose-based fed-batch fermentation; 120 g L⁻¹ L-lysine provided | 0.50               | Park et al., 2013   |
|                        | Heterologous expression of davBA; glucose-based fed-batch fermentation; 120 g L⁻¹ L-lysine provided | 90.59              | Park et al., 2014   |
|                        | Heterologous expression of davBA; fed-batch whole-cell bioconversion of L-lysine maintained at 120 g L⁻¹ | 240.70             | Wang et al., 2016   |
|                        | Heterologous expression of davBA; sugar-based fermentation; 10 g L⁻¹ L-lysine provided | 3.60               | Park et al., 2013   |
|                        | Heterologous expression of davBA; glucose-based fed-batch fermentation; 120 g L⁻¹ L-lysine provided | 0.50               | Park et al., 2013   |
|                        | Heterologous expression of davBA; glucose-based fed-batch fermentation; 120 g L⁻¹ L-lysine provided | 90.59              | Park et al., 2014   |
|                        | Heterologous expression of davBA; fed-batch whole-cell bioconversion of L-lysine maintained at 120 g L⁻¹ | 240.70             | Wang et al., 2016   |

| Product in methanol-controlled fed-batch fermentation | Titer [g L⁻¹] | References |
|------------------------------------------------------|--------------|------------|
| *Bacillus methanolicus*                              | L-Glutamate  |
|                                                      | 60.00        | Heggeset et al., 2012 |
|                                                      | L-Lysine     |
|                                                      | 11.00        | Brautaset et al., 2010 |
|                                                      | γ-Aminobutyric acid |
|                                                      | 9.00         | Irla et al., 2017    |
|                                                      | Cadaverine   |
|                                                      | 11.30        | Nævdal et al., 2015  |

seomaldehyde dehydrogenase (GabD) (Park et al., 2013; Rohles et al., 2016; Pérez-Garcia et al., 2018); for example, GABAase from *Pseudomonas fluorescens* KCCM 12537 retains 47.7% activity when 5AVA is used as its substrate in comparison to when GABA is used (So et al., 2013). Based on the previous research, *B. methanolicus* seems a feasible candidate for 5AVA production because it does not possess the necessary genetic background for GABA-based 5AVA degradation, lacking the *gabT* gene in its genome (Irla et al., 2017). It was reported that 5AVA does not supports growth of *B. methanolicus* neither as sole carbon source nor as sole nitrogen source (Haupka et al., 2021). However, *B. methanolicus* displays low tolerance to 5AVA, with growth being impaired by addition of 1.17 g L⁻¹ 5AVA to the culture broth (Haupka et al., 2021).

Even though the application of diverse 5AVA biosynthetic pathways has led to significant improvement in titers and yields of 5AVA production in bacterial hosts, the most efficient processes rely on raw materials that contain sugar and/or agricultural products. Addressing shortages of global resources and food requires a replacement of the current mode of industrial biotechnology, which results in the need for novel biosynthetic pathways that utilize alternative raw materials such as methanol. Hence, in the present study we have selected five different pathways to establish methanol-based 5AVA production in the methylotrophic bacterium *B. methanolicus*. For two of the five pathways, proof-of-principle 5AVA production was achieved and our results should represent a valuable basis of knowledge and strains for further improved 5AVA production from methanol at 50°C.

**MATERIALS AND METHODS**

**Retrosynthesis Analysis**

Retrosynthesis analysis was conducted with RetroPath 2 (Delépine et al., 2018) (v6) and RetroRules (Duigou et al., 2019) (1.0.2, with hydrogen, in reversed direction) that translated reactions from MetaNetX (Moretti et al., 2016) into reaction rules, and KNIME (3.6.1). The “source” used in this analysis was 5AVA [InChI = 1S/C5H11NO2/c6-4-2-1-3-5(7)8/h1-4,6H2,(H,7,8)] and “sink” was the set of all metabolites from *E. coli* genome-scale model iJO1366 (Orth et al., 2011). We used at most four reaction steps and a diameter of eight chemical bonds around the reaction center. Those conservative parameters were used to limit the strength of the substrate promiscuity hypothesis and to limit our results to pathways most likely to compete with known pathways.

**Strains, Genomic DNA, Plasmids, and Primers**

Bacterial strains and plasmids used in this study are listed in Table 2. The *E. coli* strain DH5α was used as general cloning host, and *B. methanolicus* strains MGA3 and M168-20 were used as expression hosts. The following strains were the source of genetic material for cloning of the 5AVA synthesis pathways: *E. coli* MG1655, *Rhodococcus qinghongii* DSM45257, *Paeonarthrobacter aurescens* DSM420116, *Kocuria rosea* DSM20447, *Peribacillus simplex* DSM1321, and *P. putida* KT2440. The L-lysine-α-oxidase-coding regions from *Trichoderma viride* (GenBank
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FIGURE 1 | Schematic view of five 5AVA biosynthesis pathways and a 5AVA degradation pathway. Five different pathways for potential 5AVA production in Bacillus methanolicus were tested; two pathways have L-lysine as precursor, and three pathways have cadaverine as an intermediate metabolite, obtained by conversion of L-lysine by a lysine decarboxylase (CadA). (A) DavBA pathway: L-lysine conversion to 5AVA by lysine 2-monoxygenase (DavB) and 5-aminovaleramidase (DavA). (B) RaiP pathway: conversion of L-lysine to α-ketolysine by a L-lysine α-oxidase (RaiP) and spontaneous decarboxylation of α-ketolysine in the presence of hydrogen peroxide. (C) SpuI pathway: cadaverine to γ-glutamine-cadaverine (γ-glut-cadaverine) by glutamylpolyamine synthetase (SpuI), with subsequent activity of polyamine:pyruvate transaminase (SpuC), aldehyde dehydrogenase (KauB), and glutamine amidotransferase class I (PauD2); γ-glutamine-aminopentanal: γ-glutamine-aminopentanal, γ-glutamine-aminovalerate: γ-glutamine-aminovalerate. (D) PatA pathway: cadaverine to 5-aminopentanal through activity of putrescine aminase (PatA) and 5-aminopentanal conversion to 5AVA by 5-aminopentanal dehydrogenase (PatD). (E) Puo pathway: cadaverine to 5-aminopentanal through activity of putrescine oxidase (Puo), followed by 5AVA formation by PatD. 5AVA is degraded to glutarate by GABAse activity, a combination of γ-aminobutyrate aminotransferase (GabT) and succinate semialdehyde dehydrogenase (GabD), although this activity was not found in B. methanolicus (Irla et al., 2017).

AB937978.1) and S. japonicus (GenBank AB970726.1) were codon-optimized for B. methanolicus MGA3 expression and synthesized by Twist Biosciences (Supplementary Table S1 and Supplementary Material). The davBA operons from alternative hosts Williamsia sterculiae CPCC 203464, Roseobacter denitrificans OCh 114 strain DSM 7001, and Parageobacillus caldoxylosilyticus B4119 (davA only) were codon-optimized for expression in B. methanolicus, synthesized and provided in the pUC57 plasmid from GenScript (Supplementary Table S1 and Supplementary Material). Isolated genomic DNA of Bacillus megaterium DSM32 was purchased from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). All primers (Sigma-Aldrich) used in this research are listed in Table 2.

Molecular Cloning
The E. coli DH5α competent cells were prepared according to the calcium chloride protocol as described in Green and Rogers (2013) or purchased as chemically competent NEB 5-α E. coli cells (New England Biolabs). All standard molecular cloning procedures were carried out as described in Sambrook and Russell (2001) or according to manuals provided by producers. Chromosomal DNA was isolated as described in Eikmanns et al. (1994). PCR products were amplified using CloneAmp HiFi PCR Premix (Takara) and purified using a QIAquick PCR Purification Kit from Qiagen. DNA fragments were separated using 8 g l⁻¹ SeaKem LE Agarose gels (Lonza) and isolated using a QIAquick Gel Extraction Kit (Qiagen). The colony PCR was performed using GoTaq DNA Polymerase (Promega). The sequences of cloned DNA fragments were confirmed by Sanger sequencing (Eurofins). B. methanolicus MGA3 was made electrocompetent and transformed by electroporation as described previously (Jakobsen et al., 2006). Recombinant DNA was assembled in vitro by means of the isothermal DNA assembly method (Gibson et al., 2009), employing the NEBuilder HiFi DNA Assembly Kit or ligation with T4 DNA ligase.
### TABLE 2 | Bacterial strains, plasmids, and primers used in this study.

| Strain name | Relevant characteristics | References |
|-------------|--------------------------|------------|
| *Escherichia coli* DH5α | General cloning host, F− thi-1 endA1 hsdR17 (r− m−) supE44 lacU169 (ΔlacZΔM15) recA1 gyrA96 relA1 | StrataGene |
| *E. coli* MG1655 | Wild-type strain | ATCC 47076 |
| *Bacillus methanolicus* MGA3 | Wild-type strain | ATCC 53907 |
| *Bacillus methanolicus* M160-20 | 1<sup>st</sup>-generation S−(2-aminoethyl)cysteine-resistant mutant of MGA3; L-lysine overproducer | Brautaset et al., 2010 |
| *Rhodococcus qingshengii* DSM32 | Wild-type strain | DSM45257 |
| *Paenarthrobacter aurescens* DSM20116 | Wild-type strain | DSM20116 |
| *Kocuria rosea* DSM20447 | Wild-type strain | DSM20447 |
| *Penibacillus simplex* DSM1321 | Wild-type strain | DSM1321 |
| *Pseudomonas putida* KT2440 | Wild-type strain | DSM6125 |

**Genomic DNA**

| Relevant characteristics | References |
|--------------------------|------------|

**Plasmid**

| Relevant characteristics | References |
|--------------------------|------------|

(Continued)
### TABLE 2 | Continued

| Primer | Sequence 5' → 3' | Characteristics |
|--------|------------------|-----------------|
| davBA_Pp_F1 | atagttgatggataaacttgttcacttaaggaggtagtacatatgaaagaagaaccgcc | davBA from P. putida; fw |
| davBA_Pp_R1 | aacgacggccagtgaattcgagctcatggtacggatcttaaattttaacttgatattcttttgg | raip from P. simplex; fw |
| davB_Pp_F1 | gatggataaacttgttcacttaaggaggtagtacatatgagagttacaacatcagttgg | dava from P. putida; pr; rv |
| davB_Pp_R1 | aacgacggccagtgaattcgagctcatggtacggatcttaaattttaacttgatattcttttgg | raip from P. simplex; rv |
| davA_Pc_F1 | ccaagattagcatttaaactagttttgttaaacaattacataaataggggtagtagctatagtgaaacrataatagtgacactagcc | dava from P. putida; pm; pm-
| davA_Pc_R1 | tctagacctatggcgggtaccttaataaacatctgttcttctttcattcatc | patD from P. putida; pm; pm-
| davB_Ws_F1 | ggataaacttgttcacttaaggaggtagtacatatgagagttacaacatcagttgg | dava from P. putida; pm; pm-
| davB_Ws_R1 | acggccagtgaattcgagctcactagttatcagcctttacgcaggtgc | raip from S. japonicus; rv |
| davA_Pp_F1 | ccaagattagcatttaaactagttttgttaaacaattacataaataggggtagtagctatagtgacactagcc | dava from P. putida; pm; pm-
| davA_Pp_R1 | tctagacctatggcgggtaccttaataaacatctgttcttctttcattcatc | raip from S. japonicus; rv |
| raiptfw | ctgcttacctagggggaatggcatcttcaacgcggctt | raip from T. viride; rv |
| Katafw | gtaaacaattacataaataggggtagtagctatagcacaacaaatagaagaaaattactaacaagc | kata from B. halodenitrificans; fw |
| katafw | gtaaacaattacataaataggggtagtagctatagcacaacaaatagaagaaaattactaacaagc | kata from B. halodenitrificans; rv |
| AVA1 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; fw |
| AVA2 | aacgacggccagtgaattcgagctcatggtacggatcttaaattttaacttgatattcttttgg | patD from P. putida; fw |
| AVA3 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; rv |
| AVA4 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; pr |
| AVA5 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; pr |
| AVA6 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; pr |
| AVA7 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; pr |
| AVA8 | ttcactaagggggaatggcatcttcaacgcggctt | patD from P. putida; pr |
| AVA9 | cgaacgtgtatcgccttaactaataaataggggtagtagctatagc | pavo from P. putida; fw |
| AVA10 | cgaacgtgtatcgccttaactaataaataggggtagtagctatagc | pavo from P. putida; rv |
| AVA11 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; fw |
| AVA12 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA13 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA14 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA23 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA27 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA25 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA32 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| AVA33 | ttcactaagggggaatggcatcttcaacgcggctt | patD from E. coli; rv |
| MI09 | gtaaacaattacataaataggggtagtagctatagc | pavo from Kocuria rosea; fw |
| MI10 | gtaaacaattacataaataggggtagtagctatagc | pavo from Kocuria rosea; rv |

CmrR, chloramphenicol resistance; KanR, kanamycin resistance.
pMI2mp plasmid was obtained via site-directed mutagenesis (SDM) of pTH1mp performed as previously described with CloneAmp HiFi PCR Premix (Liu and Naismith, 2008). The detailed description of plasmid creation is presented in Supplementary Material.

### Media and Conditions for Shake Flask Cultivations

*E. coli* and *P. putida* strains were cultivated at 37°C in Lysogeny Broth (LB) or on LB agar plates supplemented with antibiotics when necessary. *P. aerogenes* DSM2011 and *K. rosea* DSM20447 were cultivated at 30°C and 225 rpm in medium 53 (casein peptone, tryptic digest, 10.0 g l\(^{-1}\), yeast extract, 5.0 g l\(^{-1}\), glucose, 5.0 g l\(^{-1}\), NaCl, 5.0 g l\(^{-1}\); pH adjusted to 7.2–7.4); *R. qingshengii* DSM45257 was grown at 28°C and 225 rpm in medium 65 (glucose, 4.0 g l\(^{-1}\), yeast extract, 4.0 g l\(^{-1}\), malt extract, 10.0 g l\(^{-1}\); adjusted to pH to 7.2); and *P. simplex* DSM1321 was cultivated in nutrient medium (peptone 5 g l\(^{-1}\) and meat extract 3 g l\(^{-1}\); pH adjusted to 7.0) at 30°C and 200 rpm. For preparation of crude extracts, electrophoretic cells and transformation *B. methanolicus* strains were cultured at 50°C in SOB medium (Difco) supplemented with antibiotics when necessary. For 5AVA production experiments, recombinant *B. methanolicus* strains were cultivated in 250-ml baffled shake flasks at 50°C and 200 rpm in 40 or 50 ml MVcM medium containing 200 mM methanol. The MVcM medium contained the following, in 1 l of distilled water: K2HPO4, 4.09 g; Na2HPO4*H2O, 1.49 g; (NH4)2SO4, 2.11 g; it was adjusted to pH 7.2 before autoclaving. The MVcM medium was supplemented with 1 ml 1 M MgSO4*7H2O solution, 1 ml trace element solution, and 1 ml vitamin solution (Schedel et al., 1990). One mole of MgSO4*7H2O solution contained 246.47 g of MgSO4*7H2O in 1 l of distilled water. The trace element solution contained the following, in 1 l of distilled water: FeSO4*7H2O, 5.56 g; CuSO4*2H2O, 27.28 mg; CaCl2*2H2O, 7.35 g; CoCl2*6H2O, 40.50 mg; MnCl2*4H2O, 9.90 g; ZnSO4*7H2O, 287.54 mg; Na2MoO4*2H2O, 48.40 mg; H3BO3, 30.92 mg; and HCl, 80 ml. The vitamin solution contained the following, in 1 l of distilled water: biotin, thiamine hydrochloride, riboflavin, d-calcium pantothenate, pyridoxine hydrochloride, nicotinamide, 0.1 g each; p-aminobenzoic acid, 0.02 g; folic acid, vitamin B12 and lipico acid, 0.01 g each (Schedel et al., 1990). When needed, 10 g l\(^{-1}\) xylose (v/v) was added for induction. For precultures, a minimal medium supplemented with 0.25 g l\(^{-1}\) yeast extract, designated MVcMY, was used. Antibiotics (chloramphenicol, 5 µg ml\(^{-1}\) and/or kanamycin, 25 µg ml\(^{-1}\)) were supplemented as necessary. Cultivations were performed in triplicates with start OD600 of 0.1–0.2. Growth was monitored by measuring OD600 with a cell density meter (WPA CO 8000 Biowave).

### Determination of Amino Acid Concentration

For the analysis of amino acid concentrations, 1 ml of the culture sample was taken from the bacterial cultures and centrifuged for 10 min at 11,000 rpm. Extracellular amino acids were quantified by means of high-pressure liquid chromatography (HPLC, Waters Alliance e2695 Separations Module). The samples underwent FMOC-Cl (fluorenylmethyloxycarbonyl chloride) derivatization before the analysis, according to the protocol described before (Haas et al., 2014), and were separated on a column (Symmetry C18 Column, 100 Å, 3.5 µm, 4.6 mm × 75 mm, Waters) according to the gradient flow presented in Table 3, where A is an elution buffer 50 mM Na-acetate pH = 4.2 and B is an organic solvent, acetonitrile. The detection was performed with a Waters 2475 HPLC Multi Fluorescence Detector (Waters), with excitation at 265 nm and emission at 315 nm.

#### Enzyme Assays

In order to determine enzymatic activity, crude extracts of recombinant *B. methanolicus* cells were prepared according to Dreier et al. (2020). *B. methanolicus* strains were inoculated in SOB medium and grown to exponential phase (OD600 = 0.8). Recombinant expression was induced by addition of 10 g l\(^{-1}\) xylose 2 h after inoculation. A total amount of 50 ml culture broth was harvested by centrifugation at 7,500 rpm and 4°C for 15 min and washed twice in ice-cold buffer used for specific enzyme assay before storing at −80°C. The cells were thawed in ice and disrupted by sonication using a Fisherbrand Sonic Dismembrator (FB-505) with 40% amplitude with 2 s on and 1 s off-pulse cycles for 7 min. Cell debris was then removed by centrifugation (at 14, 000 rpm and 4°C for 1 h). Protein concentrations were determined by Bradford assay (Bradford, 1976), using bovine albumin serum (Sigma) as standard.

L-Lysine α-oxidase activity was assayed by measuring the rate of hydrogen peroxide formation, as described elsewhere (Tani et al., 2015a). The reaction was initiated by adding crude extracts from *B. methanolicus* strains to the reaction media (50°C) consisting of 100 mM L-lysine and 50 mM pH 7 phosphate buffer, resulting in a total volume of 1 ml. Next, the sample was quenched by addition of 50 µl 2 M HCl. After neutralization with 50 µl 2 M NaOH, 200 µl of the mixture was withdrawn and transferred to 800 µl of a second reaction mixture containing 50 mM pH 6 phosphate buffer, 30 mM phenol, 2 units ml\(^{-1}\) peroxidase from horseradish (Sigma) and 0.5 mM 4-aminotyrosine. Formation of quinonemine dye from oxidative coupling of phenol and 4-aminotyrosine (Job et al., 2002) was determined by measuring absorbance at 505 nm using a Cary 100 Bio UV-visible spectrophotometer (Varian). One unit (U) of RaiP activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol hydrogen peroxide per minute.

Catalytic activities of PatA and PatD or putrescine oxidase and PatD were measured by using a coupled reaction, and...
cadaverine was used as substrate instead of putrescine, as previously described elsewhere, with modifications (Jorge et al., 2017). The 1-mL assay mix contained 0.1 M Tris–HCl pH 8.0, 1.5 mM α-ketoglutarate, 2.5 mM cadaverine, 0.1 mM pyridoxal-5’-phosphate, and 0.3 mM NAD. In this coupled reaction, cadaverine was converted to SAVA via 5-aminopentanal and one unit of coupled enzyme activity was defined as the amount of the enzyme that formed 1 μmol of NADH (ε340 nm = 6.22 mM−1 cm−1) per minute at 50°C.

The coupled DavAB assay was performed as described in Liu et al. (2014) with some modifications. Five hundred microliters of crude extract was added into 50-mL Falcon tubes filled with 4 mL 100 mM phosphate buffer pH 7.0 supplemented with 10 g l−1 l-lysine. The tubes were incubated for 40 h at 30 or 50°C with stirring at 200 rpm. The samples for quantification of SAVA concentration through HPLC (see section “Determination of Amino Acid Concentration”) were taken at the beginning of incubation, after 16 h and after 40 h.

RESULTS AND DISCUSSION

Selection, Design, and Construction of Heterologous Biosynthetic Pathways for SAVA Biosynthesis in B. methanolicus

Due to the fact that B. methanolicus is a thermophile, a typical issue concerning implementation of biosynthetic pathways from heterologous hosts is the lack of thermostability of the transferred enzymes. It was shown before that a screening of diverse donor organisms allows to identify pathways active at 50°C. It was shown before that a screening of diverse donor organisms allows to identify pathways active at 50°C. We could not identify a complete davBA operon from a thermophilic host; however, thermophilic P. caldoxylosilyticus possesses a putative davA gene and was also included in this study. All selected davBA operons were co-transfected and cloned into the pBV2xp vector under control of the xylose-inducible promoter as described in the Supplementary Material. The finished vectors were used to create the following B. methanolicus strains: MGA3_DavBA(2p), MGA3_DavBA(Ws), MGA3_DavBA(Rd), MGA3_DavBA(Bd), and MGA3_DavBA(2p) (Table 4). Furthermore, selected davBA operons were expressed as single genes using compatible pBV2xp and pM12mp plasmids for gene expression (Supplementary Material). The davB genes from P. putida and W. sterculiae were cloned under control of the xylose-inducible promoter in plasmid pBV2xp, while the davA gene from P. caldoxylosilyticus was cloned into the pM12mp plasmid under control of the mdh promoter constitutively active in methylotrrophic conditions. The combination of two plasmids (2p) expressing single genes resulted in creation of the following B. methanolicus strains: MGA3_DavB(2p)Pa and MGA3_DavB(2p) (Table 4).

For expression of the RaiP pathway, the B. methanolicus strains MGA3_RaiP(2p), MGA3_RaiP(2p), and MGA3_RaiP(2p) (Table 4) carried heterologous raiP gene sequences from the prokaryote P. simplex and from the eukaryotic genetic donors.

TABLE 4 | List of B. methanolicus strains used in this study with abbreviated strain names.

| Abbreviated strain name | Recombinant B. methanolicus strains created in this study |
|-------------------------|----------------------------------------------------------|
| MGA3_EV                 | MGA3(pBV2xp)                                             |
| MGA3_DavBA(2p)          | MGA3(pBV2xp-davBA(2p))                                   |
| MGA3_DavBA(Ws)          | MGA3(pBV2xp-davBA(Ws))                                   |
| MGA3_DavBA(Rd)          | MGA3(pBV2xp-davBA(Rd))                                   |
| MGA3_DavBA(Bd)          | MGA3(pBV2xp-davBA(Bd))                                   |
| MGA3_DavA(2p)           | MGA3(pBV2xp-davA(2p))                                    |
| MGA3_DavA(Ws)           | MGA3(pBV2xp-davA(Ws))                                    |
| M168-20_EV              | M168-20(pBV2xp)                                          |
| M168-20_DavBA(2p)       | M168-20(pBV2xp-davBA(2p))                                |
| M168-20_DavA(2p)        | M168-20(pBV2xp-davA(2p))                                 |
| M168-20_RaiP(2p)        | M168-20(pBV2xp-raiP(2p))                                 |
| M168-20_RaiP(2p)        | M168-20(pBV2xp-raiP(2p))                                 |
| M168-20_RaiP(Tv)        | M168-20(pBV2xp-raiP(Tv))                                 |
| M168-20_RaiP(2p)        | M168-20(pBV2xp-raiP(2p))                                 |
| M168-20_RaiP(2p)        | M168-20(pBV2xp-raiP(2p))                                 |
| M168-20_RaiP(2p)        | M168-20(pBV2xp-raiP(2p))                                 |
| MGA3_Cad                | MGA3(pTH1mp-cadA(pBV2xp))                                |
| MGA3_PatA(2p)           | MGA3(pTH1mp-cadA(pBV2xp)-AVA(2p))                        |
| MGA3_Spul               | MGA3(pTH1mp-cadA(pBV2xp)-AVA(2p))                        |
| MGA3_Kat                | MGA3(pTH1mp-katA(pBV2xp))                                |
| MGA3_Pud(2p)            | MGA3(pTH1mp-katA(pBV2xp)-AVA(2p))                        |
| MGA3_Pud(2p)            | MGA3(pTH1mp-katA(pBV2xp)-AVA(2p))                        |
S. japonicus and T. viride, respectively, the two latter with characterized RaIP activity (Arinbasarova et al., 2012; Tani et al., 2015a). The full length of codon-optimized sequences derived from S. japonicus and T. viride is present in the Supplementary Table S1. The original S. japonicus sequence encodes a protein with 617 amino acids and has a 52.2% GC content, while the sequence codon optimized for B. methanolicus has a GC content of 29%. The T. viride-derivative sequence was adjusted from the GC content of 42.5 to 28.6%. The substitution of nucleotides did not alter their coding amino acid sequences.

Among the pathways used for cadaverine formation from L-lysine through activity of E. coli-derived lysine decarboxylase CadA (EC 4.1.1.18, encoded by cadA) as an intermediate, we considered a multistep diamine catabolic pathway of P. aeruginosa PAO1 (Spul pathway, Figure 1C) (Yao et al., 2011). In order to test this pathway for methanol-based 5AVA production, the MGA3_Spul strain was constructed through transformation of B. methanolicus wild type with two vectors pTH1mp-cadA and pBV2xp-AVA \textit{Pp}, the first one carrying the cadA gene and the latter the genes encoding the Spul pathway (Table 4 and Supplementary Material). The Spul pathway that converts cadaverine to 5AVA is composed of the following enzymes: glutamylpolyamine synthetase (EC 6.3.1.2, Spul), polyamine:pyruvate transaminase (EC 2.6.1.113, SpuC), aldehyde dehydrogenase (EC 1.2.1.3, KauB), and glutamine amidotransferase class I (EC 6.3.5.2, PauD2) (Yao et al., 2011).

Another pathway, also predicted by our retrosynthesis analysis, potentially leading to production of 5AVA from L-lysine is a three-step pathway composed of CadA, PatA (EC 2.6.1.82, PatA), and 5-aminopentanal dehydrogenase (EC 2.6.1.19, PatD) (PatA pathway, Figure 1D). In order to test this pathway, two strains were constructed, MGA3_PatA\textit{Ec} and MGA3_PatA\textit{Bm}, through transformation of B. methanolicus with pTH1mp-cadA plasmid, and pBV2xp-AVA\textit{Ec} or pBV2xp-AVA\textit{Bm}, respectively (Table 4). As described in the Supplementary Material, the lysine decarboxylase-encoding gene (cadA) was placed under control of the \textit{mdh} promoter in a rolling circle vector pTH1mp. The \textit{E. coli}-derived patAD operon encoding previously characterized enzymes was placed under control of the xylose-inducible promoter in pBV2xp, resulting in pBV2xp-AVA\textit{Ec} (Samsonova et al., 2003). The genes of the patAD operon in \textit{B. megaterium} were identified based on a BLAST search of its genome and were cloned into pBV2xp, yielding pBV2xp-AVA\textit{Bm} (Altschul et al., 1990). While the existence of prior art makes it a solid candidate, we knew that its second step catalyzed by PatA may suffer from an unfavorable thermodynamic (predicted close to 0 kJ mol$^{-1}$) (Noor et al., 2012).

In our study, we have also included a pathway confirmed through retrosynthesis analysis where the step of cadaverine transamination (PatA pathway, Figure 1D) is replaced by its oxidative deamination (Puo pathway, Figure 1E) because this reaction displays a more favorable thermodynamic (predicted close to $-100$ kJ mol$^{-1}$ in cell conditions) in comparison to PatA. While a cadaverine oxidase has not been identified before, it was shown that putrescine oxidase encoded by \textit{puo}

retrains up to 14% of its maximal activity when cadaverine is used as a substrate (Okada et al., 1979; Ishizuka et al., 1993; van Hellemond et al., 2008; Lee and Kim, 2013). We have therefore decided to express three different versions of the \textit{puo} gene derived from \textit{K. rosea}, \textit{P. aurescens}, and \textit{R. qingshengii}, together with the \textit{E. coli}-derived \textit{patD} gene from the pBV2xp plasmid (for details see Supplementary Material), which led to creation of the following strains: MGA3_Puo\textit{Kr}, MGA3_Puo\textit{Pa}, and MGA3_Puo\textit{Rq}, respectively (Table 4). In order to prevent oxidative stress caused by H$_2$O$_2$ formation, a native gene encoding catalase was homologously expressed from pTH1mp plasmid in all constructed strains.

**Testing Recombinant \textit{B. methanolicus} Strains for 5AVA Production From Methanol**

The plasmids designed and built as described in the above Section were used for transformation of wild-type \textit{B. methanolicus} cells and resulted in the creation of 26 different strains (Table 4) which were then tested for their ability to synthesize 5AVA. All strains were cultivated in minimal medium supplemented with methanol as the sole carbon and energy source, and the 5AVA titer was evaluated after the strains had reached the stationary growth phase as described in the following sections.

**Expression of the DavAB-Encoding Genes Resulted in no 5AVA Biosynthesis in \textit{B. methanolicus}**

In the first attempt, we heterologously expressed genes encoding the DavBA pathway in \textit{B. methanolicus} MGA3 (Figure 1A). In addition to the well-known \textit{davba} operon from \textit{P. putida} (gamma-proteobacteria), the alternative \textit{davb} operon from \textit{W. sterculiae} (actinobacteria) and \textit{davab} from \textit{R. dentificicans} (alpha-proteobacteria) were tested for 5AVA formation in \textit{B. methanolicus} MGA3. Moreover, the only enzyme identified from a thermophilic host, DavA from \textit{P. caldoxylosilyticus} (bacilli), was combined with the before-mentioned lysine 2-monooxygenases (DavB). \textit{P. caldoxylosilyticus} has a reported optimum growth temperature from 50 to 65$^\circ$C (Fortina et al., 2001).

Several considerations were made with regard to strain design, namely, adjusting the GC content and the types of codons present in the open reading frames in the genomic DNA of a donor and designing suitable expression cassettes. In total, seven different \textit{B. methanolicus} strains were constructed: MGA3(pBV2xp-davb\textit{Pa}) named MGA3_DavB\textit{Pa}, MGA3(pBV2xp-davb\textit{Wr}) named MGA3_DavB\textit{Wr}, MGA3(pBV2xp-davba\textit{Rd}) named MGA3_DavB\textit{Rd}, MGA3(pBV2xp-davba\textit{Tc}) named MGA3_DavB\textit{Tc}, MGA3(pBV2xp-davb\textit{Kr}) named MGA3_DavB\textit{Kr}, MGA3(pBV2xp-davb\textit{Pq}) named MGA3_DavB\textit{Pq}, and MGA3(pBV2xp-davb\textit{Rq}) named MGA3_DavB\textit{Rq} (2p) (Table 4). However, in none of the tested strains (MGA3_DavB\textit{Pa}, MGA3_DavB\textit{Wr}, MGA3_DavB\textit{Rd}, MGA3_DavB\textit{Tc}, MGA3_DavB\textit{Kr}, MGA3_DavB\textit{Pq}, MGA3_DavB\textit{Rq}, MGA3_DavB\textit{Rd}), the active pathway was expressed; and following no 5AVA accumulation was observed during shake flask cultivations in any constructed strain (data not shown).
The first reaction step from l-lysine to 5-aminopentanamide requires O2 (Figure 1A), and due to the high O2 demand to facilitate the assimilation of methanol, we also tested 5AVA formation from the alternative carbon source mannitol. Neither was this strategy successful. Furthermore, the DavAB pathway was also tested in the genetic background of l-lysine-overproducing B. methanolicus strain M160-20. Specifically, the following strains were constructed: M168-20_DavBAp, M168-20_DavAwpBwp(2p), and M168-20_DavAwpBwp(2p); however, none of them produced any detectable 5AVA (data not shown). Taken together, the DavBA pathway did not enable 5AVA formation. It is not clear whether this was caused by low enzymatic stability at 50°C (only P. caldoxyslylisiticus is known to be thermophilic among the organisms found to be source organisms for the two genes). In order to exclude the effect of elevated temperature on the DavAB activity, we tested enzymatic activity at 30°C for selected strains (MGA3_DavBAp, MGA3_DavAwp, MGA3_DavAwpBwp, and MGA3_DavAwpBwp); however, no DavAB activity was detected (data not shown). The reason why the functional DavAB pathway was not expressed in B. methanolicus remains unknown.

**RaiP Pathway Is Functional in B. methanolicus and Supports 5AVA Production**

Methanol-based 5AVA biosynthesis was attempted via heterologous expression of RaiP encoding gene rap in MGA3. The strains MGA3(pBV2xp-riap) named MGA3_RaiPp, MGA3(pBV2xp-riap) named MGA3_RaiPp, and MGA3(pBV2xp-riap) named MGA3_RaiPp (Table 4) carry the rap gene from the bacterium P. simplex and rap genes with codon-optimized sequences from the eukaryotic donors S. japonicus and T. viride, respectively. The T. viride-derived RaiP was shown to be stable at temperatures up to 50°C (Arinbasarova et al., 2012). It is reported that the RaiP protein from S. japonicus is thermally stable for at least 1 h in temperatures up to 60°C, with its highest activity registered at 70°C (Tani et al., 2015b). Moreover, although there is no kinetic characterization of RaiP from P. simplex available, this bacterium is classified as mesophilic, with growth optimum at 30°C (Yumoto et al., 2004). To examine the activity of RaiP in the constructed B. methanolicus strains, l-lysine α-oxidase activity was measured at 50°C. While the empty vector control strain has shown no RaiP activity, the highest RaiP specific activity was observed in crude extracts from strain MGA3_RaiPp, being 62.1 ± 1.4 mU mg⁻¹ (Figure 2A). The values of RaiP activity for strains MGA3_RaiPp and MGA3_RaiPp were 1.4 ± 0.3 mU mg⁻¹ and 12.0 ± 4.4 mU mg⁻¹, respectively (Figure 2A). It is not clear if the poor activity of heterologous RaiP from genetic donors S. japonicus and P. simplex was caused by low enzymatic stability at 50°C, and the reason for that remains to be investigated.

HPLC analysis of supernatant from MGA3_RaiPp strain cultivated in minimal medium revealed 16.15 ± 1.62 mg L⁻¹ 5AVA and 0.27 ± 0.04 mg L⁻¹ l-lysine. In contrast, the l-lysine level in the MGA3 strain harboring the empty vector plasmid pBV2xp (MGA3_EV) was 37.8 ± 7.2 mg L⁻¹ (Figure 2B). Even though a slight RaiP activity was observed in crude extract of the strains MGA3_RaiPp and MGA3_RaiPp, no 5AVA production was observed for those strains (data not shown). Let us note here that the 5AVA titer in the methanol-based shaking flask fermentation of strain MGA3_RaiPp was significantly inferior to that in previously reported glucose-based fermentations in E. coli (Cheng et al., 2018).

The value of the Michaelis–Menten constant for T. viride-derived RaiP for l-lysine has been estimated (Km = 5.85 mg L⁻¹) (Kusakabe et al., 1980). Therefore, the precursor levels in the B. methanolicus strains should not be a limiting factor for production of 5AVA. The RaiP-mediated production is mainly utilized in the l-lysine biocconversion approach, utilizing E. coli strains as whole-cell biocatalysts (Cheng et al., 2018, 2020, 2021) where high concentrations of the precursor were used; for example, the molar yield of 0.942 was obtained from 120 g l⁻¹ l-lysine (Park et al., 2014). However, construction and testing of the B. methanolicus strains M168-20_RaiPp, M168-20_RaiPp, and M168-20_RaiPp (Table 4), based on the l-lysine-over-producing mutant M168-20 (Brautaset et al., 2010), did not result in any improved 5AVA production (data not shown).

The lack of 5AVA production in MGA3_RaiPp and MGA3_RaiPp, as well as low 5AVA titer produced by strain MGA3_RaiPp, might be related to the spontaneous conversion step that follows RaiP activity. This could be a limiting factor for the RaiP-mediated production of 5AVA. Three compounds are produced in a reaction catalyzed by RaiP: α-ketolysine, NH₃, and H₂O₂ (Mai-Prochnow et al., 2008; Cheng et al., 2018). In a second spontaneous step of 5AVA synthesis, the intermediate α-ketolysine is oxidatively decarboxylated to form 5AVA in the presence of H₂O₂ as an oxidizing agent. It was shown that the addition of H₂O₂ into the culture broth has led to an 18-fold increase of 5AVA titers in comparison with the control condition without H₂O₂ (final titer 29.12 g l⁻¹) in a 5-1 fermenter (Cheng et al., 2018). The RaiP-mediated 5AVA production may be increased by enzymatic conversion of α-ketolysine in an approach different to ours, where spontaneous reaction of oxidative decarboxylation occurs. Recently, an artificial synthetic pathway for the biosynthesis of 5AVA in E. coli was developed, consisting of three steps: conversion of l-lysine to α-ketolysine via RaiP, decarboxylation of α-ketolysine to produce 5-aminopentanal via α-ketoacid decarboxylase, and oxidation of 5-aminopentanal to 5AVA via aldehyde dehydrogenase. The expression of the artificial pathway resulted in a yield increase of 774% compared to the single gene pathway (Cheng et al., 2021). This approach is potentially a feasible strategy we have shown in our study that E. coli-derived PatD is active as a 5-aminopentanal dehydrogenase in B. methanolicus and participates in 5AVA biosynthesis (see Section “The PatA Pathway Supports 5AVA Accumulation in B. methanolicus”).

**Use of the SpuI Pathway Does Not Lead to 5AVA Production in B. methanolicus**

Three different pathways that use cadaverine as an intermediate product have been tested for their feasibility for production of 5AVA in B. methanolicus. Cadaverine biosynthesis in B. methanolicus cells was enabled through the activity of lysine decarboxylase encoded by a heterologously expressed cadA...
FIGURE 2 | Evaluation of RaiP enzyme activity (A) and amino acids production (B) in recombinant B. methanolicus strains. B. methanolicus strains MGA3_EV, MGA3_RaiP\textsubscript{St}, MGA3_RaiP\textsubscript{Ps}, or MGA3_RaiP\textsubscript{Tv} were cultivated in a shaking flask culture. The grown cells were harvested, washed twice with 50 mM phosphate buffer (pH 7.0), and disrupted by sonication. After centrifugation, the crude extracts were directly used for the RaiP assay. MGA3_EV and MGA3_RaiP\textsubscript{Tv} were cultivated for 27 h, and supernatants were obtained by centrifugation for HPLC analysis. The error bars represent standard deviation of technical triplicates.

(Nærdal et al., 2015). Cadaverine can be converted to 5AVA through activity of a multistep diamine catabolic pathway derived from P. aeruginosa PAOI (Spul pathway, Figure 1C) (Yao et al., 2011). The MGA3(pTH1mp-cadA)(pBV2xp-AVA\textsubscript{Pp}) strain called MGA3_SpuI (Table 4) did not accumulate any 5AVA during methanol-based growth in minimal medium, despite the accumulation of the precursor, cadaverine, at the level of 118.8 ± 5.1 mg l\textsuperscript{-1} similar to the empty vector control strain (130.0 ± 5.3 mg l\textsuperscript{-1}) (Table 5). The cadaverine titers of 130.0 ± 5.3 mg l\textsuperscript{-1} achieved by MGA3_Cad are higher than the L-lysine titer of 37.8 ± 7.2 mg L\textsuperscript{-1} achieved by MGA3_EV in this study (Figure 2B). This is in accordance with previous findings of Nærdal et al. (2011, 2015) who attributed high cadaverine titers for production strain in relation to L-lysine titer in empty vector

| Strain     | Growth rate [h\textsuperscript{-1}] | Coupled activity of PatAD or Puo-PatD [mU mg\textsuperscript{-1}] | Lysine [mg l\textsuperscript{-1}] | Cadaverine [mg l\textsuperscript{-1}] | 5AVA [mg l\textsuperscript{-1}] |
|------------|------------------------------------|---------------------------------------------------------------|----------------------------------|-------------------------------------|-------------------------------|
| MGA3_Cad   | 0.37 ± 0.01                         | 0 ± 0                                                          | Not detected                     | 123.0 ± 5.3                        | 0.0 ± 0.0                     |
| MGA3_SpuI  | 0.33 ± 0.01                         | N.A.                                                          | Not detected                     | 118.8 ± 5.1                        | 0.0 ± 0.0                     |
| MGA3_PatA\textsubscript{Ec} | 0.12 ± 0.02                         | 7 ± 4                                                          | Not detected                     | 1.47 ± 0.17                        | 23.7 ± 2.7                   |
| MGA3_PatA\textsubscript{Bm} | 0.15 ± 0.03                         | 170 ± 37                                                       | Not detected                     | 0.71 ± 0.11                        | 8.3 ± 4.1                    |
| MGA3_Kat   | 0.35 ± 0.01                         | 0 ± 0                                                          | Not detected                     | Supplemented (500 mg L\textsuperscript{-1}) | 0.0 ± 0.0                     |
| MGA3_SpuI  | 0.32 ± 0.00                         | N.A.                                                          | Not detected                     | Supplemented (500 mg L\textsuperscript{-1}) | 0.0 ± 0.0                     |
| MGA3_PatA\textsubscript{Ec} | 0.14 ± 0.02                         | 7 ± 4                                                          | Not detected                     | Supplemented (500 mg L\textsuperscript{-1}) | 31.8 ± 2.3                   |
| MGA3_PatA\textsubscript{Bm} | 0.17 ± 0.04                         | 170 ± 37                                                       | Not detected                     | Supplemented (500 mg L\textsuperscript{-1}) | 77.7 ± 5.5                   |
| MGA3_Kat   | 0.32 ± 0.00                         | 0 ± 0                                                          | 3.1 ± 0.5                        | Supplemented (500 mg L\textsuperscript{-1}) | 0.0 ± 0.0                     |
| MGA3_Puo\textsubscript{Ec} | 0.28 ± 0.01                         | 0 ± 0                                                          | 5.0 ± 0.7                        | Supplemented (500 mg L\textsuperscript{-1}) | 0.0 ± 0.0                     |
| MGA3_Puo\textsubscript{Ps} | 0.29 ± 0.01                         | 0 ± 0                                                          | 4.9 ± 0.9                        | Supplemented (500 mg L\textsuperscript{-1}) | 0.0 ± 0.0                     |
| MGA3_Puo\textsubscript{Rq} | 0.29 ± 0.00                         | 0 ± 0                                                          | 3.7 ± 0.2                        | Supplemented (500 mg L\textsuperscript{-1}) | 0.0 ± 0.0                     |

The B. methanolicus strains expressing pathways that use cadaverine as an intermediate (Spul, PatA, or Puo pathways) were cultivated for 24 h, and supernatants were obtained by centrifugation for HPLC analysis. Catalytic activities of PatA and PatD or Puo and PatD were measured by using a coupled reaction, and cadaverine was used as substrate (see Section “Enzyme Assays”). The standard deviation of technical triplicates is shown. NB: RaiP activity and 5AVA production for the RaiP pathway is shown Figure 2.
control strain to a metabolic pull which deregulated flux through the L-lysine biosynthesis pathway.

**The PatA Pathway Supports 5AVA Accumulation in *B. methanolicus***

In the next step, two versions of the PatA pathway (Figure 1D) derived from either *E. coli* or *B. megaterium* were tested in strains MGA3(pTH1mp-cadA)(pBV2xp-AVAEc) named MGA3_PatAEc and MGA3(pTH1mp-cadA)(pBV2xp-AVAEm) named MGA3PatAEm (Table 4), respectively. The optimal temperature of PatA derived from *E. coli* is 60°C, which means that it is a thermostable enzyme that should be active at 50°C, which is a temperature used for the production experiment. PatA was shown to have a broad substrate range including cadaverine and, in lower extent, spermidine, but not ornithine (Samsonova et al., 2003). This property was used by Jorge et al. (2017) who have shown in their study that it is possible to use PatA and PatD derived from *E. coli* to establish conversion of cadaverine to 5AVA, confirming experimentally the broad substrate range of those two enzymes. The *B. megaterium*-derived PatA was characterized only superficially with regard to its substrate spectrum and not optimal temperature or thermostability (Slabu et al., 2016); however, its host organism is known to have a wide temperature range for growth up to 45°C (Vary et al., 2007). The multiple-sequence alignment with *E. coli*-derived enzymes showed identity of 63 and 38% for PatA and PatD, respectively (Okada et al., 1979). Both *E. coli* and *B. megaterium*-derived versions of the pathway are functional in *B. methanolicus*, with the combined PatAD activity of 7 ± 4 mU and 170 ± 37 mU mg⁻¹ (Table 5). Final 5AVA titers of 23.7 ± 2.7 and 8.3 ± 4.1 mg L⁻¹ (Table 5) were achieved, which is considerably lower than 5AVA titers of 0.9 g L⁻¹ obtained by wild-type *C. glutamicum* strain transformed with plasmids for expression of *ldcC* (coding for lysine decarboxylase) and *patDA* (Jorge et al., 2017). For both producer strains, the concentration of unconverted cadaverine is similar: 1.7 ± 0.1 mg L⁻¹ and 1.5 ± 0.2 mg L⁻¹ for MGA3_PatAEm and MGA3_PatAEm, respectively (Table 5). While *Kₘ* for cadaverine has not been assessed, it has been shown to be 811 mg L⁻¹ for putrescine for *E. coli*-derived PatA; assuming similar *Kₘ* for cadaverine, it may explain why full conversion of cadaverine has not occurred (Samsonova et al., 2003). Due to relatively high *Kₘ* for putrescine of PatA, we decided to test how supplementation with external cadaverine affects 5AVA accumulation. In fact, for both MGA3_PatAEm and MGA3_PatAEm, 5AVA titers increased to 31.8 ± 2.3 and 77.7 ± 5.5, respectively, when the growth medium was supplemented with 500 mg L⁻¹ cadaverine (Table 5). These results indicate that the enhancement of precursor supply is one potential target for subsequent metabolic engineering efforts to increase 5AVA titers. Another important consideration for activity of transaminase is availability of keto acid that acts as amino group acceptor. It was shown that *E. coli* and *B. megaterium*-derived PatA can use either pyruvate or 2-oxoglutarate as amino group acceptors (Slabu et al., 2016); the intracellular concentrations of those compounds in *B. methanolicus* MGA3 cells are 3.2 and 2.7 mM, respectively (Brautaset et al., 2003). Knowing that *Kₘ* for 2-oxoglutarate for *E. coli*-derived PatA is 19.0 mM (Samsonova et al., 2003), recovery of the keto acids may be a limitation for 5AVA accumulation. This issue could be potentially solved by heterologous production of alanine dehydrogenase or L-glutamate oxidase which catalyzes reactions where pyruvate or 2-oxoglutarate is produced (Böhmer et al., 1989; Sakamoto et al., 1990; Slabu et al., 2016).

**Use of the Puo Pathway Leads to 5AVA Production in *B. methanolicus***

Lastly, a pathway that relies on an activity of the monoxygenase putrescine oxidase (Puo, EC 1.4.3.10) was tested (Figure 1E). Puo catalyzes the oxidative deamination of cadaverine in lieu of cadaverine transamination catalyzed by PatA. It was shown that different putrescine oxidases can use cadaverine as their substrate with 9–14% of their maximal activity shown when putrescine is a substrate (Desa, 1972; Okada et al., 1979; van Hellemond et al., 2008; Lee et al., 2013). Moreover, putrescine oxidases derived from *K. rosea* (*Micrococcus rubens*) and *Rhodococcus* are thermostable and optimal activity of *P. aurescens*-derived Puo is at 50°C (Desa, 1972; van Hellemond et al., 2008; Lee et al., 2013). The disadvantage of this pathway is that it requires O₂, the supply of which may be difficult to control. Furthermore, due to formation of hydrogen peroxide in the reaction catalyzed by Puo, the oxidative stress may increase when this pathway is active. In order to avoid detrimental effect of hydrogen peroxide accumulation, catalase was overproduced in the recombinant strains containing the Puo pathway: MGA3(pTH1mp-katA)(pBV2xp-AVAEC) named MGA3_PuoEC, MGA3(pTH1mp-katA)(pBV2xp AVAEm) named MGA3_PuoEm, and MGA3(pTH1mp-katA)(pBV2xp-AVAEm) named MGA3_PuoEm (Table 4). To achieve sufficient levels of the pathway precursor, cadaverine, we have decided not to rely on plasmid-based production of lysine decarboxylase and to add cadaverine to the growth medium, instead. The tested recombinant strains with the Puo pathway did not produce 5AVA, which is consistent with no Puo-PatD activity detected in crude extracts (Table 5). The Puo pathway was shown to be active in *C. glutamicum* where titer of 0.1 ± 0.0–0.4 ± 0.0 g L⁻¹ 5AVA was achieved (Haupka et al., 2020).

**CONCLUSION**

In the search for 5AVA production from the sustainable feedstock methanol, we have screened five pathways toward 5AVA biosynthesis in *B. methanolicus*. No 5AVA production was observed for DavBA, Puo, and Spu pathways. However, the pathways relying on RaiP and PatA activities were functional in shake flask cultures of *B. methanolicus*, which led to 5AVA production from methanol for the first time, respectively, up to 16.15 ± 1.62 mg L⁻¹ or 23.7 ± 2.7. RaiP and PatA pathways are targets for further optimizations which could increase the 5AVA titers in the constructed strains. For instance, the improvement of substrate utilization and H₂O₂ availability or decomposition efficiency might contribute to the increase in the yield of 5AVA. Moreover, our study shows that the availability of supplemented
cadaverine has high impact on 5AVA titer when the PatA pathway is employed. Another factor that needs to be considered is tolerance to 5AVA, which was shown to be low (Haupka et al., 2021). Recently, adaptive laboratory evolution experiments resulted in the selection of a mutant strain of *B. methanolicus* that displays tolerance to approximately 46 g l\(^{-1}\) 5AVA (Haupka et al., 2021), which could be employed as a platform to develop high-titer 5AVA production strains. This shows that methanol has the potential to become a sustainable feedstock for the production of 5AVA.

## DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

LB, MI, IN, and SL: study design and experimental work. BD: bioinformatic analysis. LB and MI: writing—original draft preparation. TB: writing—review and editing and project administration. SH, TB, MI, and IN: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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## SUPPLEMENTARY MATERIAL

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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