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Acetolactate Synthase from *Bacillus subtilis* Serves as a 2-Ketoisovalerate Decarboxylase for Isobutanol Biosynthesis in *Escherichia coli*[▽]

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A pathway toward isobutanol production previously constructed in *Escherichia coli* involves 2-ketoacid decarboxylase (Kdc) from *Lactococcus lactis* that decarboxylates 2-ketoisovalerate (KIV) to isobutyraldehyde. Here, we showed that a strain lacking Kdc is still capable of producing isobutanol. We found that acetolactate synthase from *Bacillus subtilis* (AlsS), which originally catalyzes the condensation of two molecules of pyruvate to form 2-acetolactate, is able to catalyze the decarboxylation of KIV like Kdc both in vivo and in vitro. Mutational studies revealed that the replacement of Q487 with amino acids with small side chains (Ala, Ser, and Gly) diminished only the decarboxylase activity but maintained the synthase activity.

We have previously shown that 2-keto acids generated from amino acid biosynthesis can serve as precursors for the Ehrlich degradation pathway (15) to higher alcohols (3). In order to produce isobutanol, the valine biosynthesis pathway was used to generate 2-ketoisovalerate (KIV), the precursor to valine, which was then converted to isobutanol via a decarboxylation and reduction step (Fig. 1A). The entire pathway to isobutanol from glucose is shown in Fig. 1A. To produce isobutanol, we overexpressed five genes, *alsS* (*Bacillus subtilis*), *ilvC* (*Escherichia coli*), *ilvD* (*E. coli*), *kdc* (*Lactococcus lactis*), and *ADH2* (*Saccharomyces cerevisiae*) (Fig. 1A). This *E. coli* strain produced 6.8 g/liter isobutanol in 24 h (Fig. 1B) and more than 20 g/liter in 112 h (3). More recently, we have found that an alcohol dehydrogenase (Adh) encoded by *yqhD* on the *E. coli* genome can convert isobutyraldehyde to isobutanol efficiently (5) (Fig. 1B).

One key reaction in the production of isobutanol is the conversion of KIV to isobutyraldehyde catalyzed by 2-ketoacid decarboxylase (Kdc) (Fig. 1C). Since *E. coli* does not have Kdc, *kdc* from *L. lactis* was overexpressed. Kdc is a nonoxidative thiamine PP_i (TPP)-dependent enzyme and is relatively rare in bacteria, being more frequently found in plants, yeasts, and fungi (8, 19). Several enzymes with Kdc activity have been found, including pyruvate decarboxylase, phenylpyruvate decarboxylase (18), branched-chain Kdc (8, 19), 2-ketoglutarate decarboxylase (10, 17, 20), and indole-3-pyruvate decarboxylase (13).

In this work, unexpectedly, we find that Kdc is nonessential for *E. coli* to produce isobutanol (Fig. 1). An *E. coli* strain overexpressing only *alsS* (from *B. subtilis*), *ilvC*, and *ilvD* (both from *E. coli*) is still able to produce isobutanol. Since *E. coli* is not a natural producer of isobutanol, it cannot be detected from the culture media in any unmodified strain. We identify that AlsS from *B. subtilis*, which was introduced in *E. coli* for acetolactate synthesis (Als), catalyzes the decarboxylation of 2-ketoisovalerate like Kdc both in vivo and in vitro. AlsS is part of the acetoin synthesis pathway and catalyzes the aldo condensation of two molecules of pyruvate to 2-acetolactate (Als activity) (Fig. 1C) (11). The overall reaction catalyzed by AlsS is irreversible because of CO_2 evolution. The first step in catalysis is the ionized thiazolium ring of TPP reacting with the first pyruvate, followed by decarboxylation. This intermediate then reacts with the second pyruvate. Deprotonation followed by C-C bond breakage produces 2-acetolactate. In this work, mutational approaches were used to assess the importance of Q487 in the Kdc activity of AlsS.

MATERIALS AND METHODS

Reagents. Restriction enzymes and Antarctic phosphatase were from New England Biolabs (Ipswich, MA). The rapid DNA ligation kit was from Roche (Mannheim, Germany). KOD DNA polymerase was from EMD Chemicals (San Diego, CA). Oligonucleotides were from Operon (Huntsville, AL).

Strains and plasmids. A list of many of the strains, plasmids, and oligonucleotides used is given in Table 1. JCL16 (2) is BW25113 (*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$) (7) with F′ transduced from XL-1 Blue to supply *lacI*^{q}. JCL260 is JCL16 Δ*adhE* Δ*fnr-ldhA* Δ*frdBC* Δ*pflB* Δ*pta*. The *ilvC* gene was inactivated by P1 transduction with JW3747 (6).

To clone *alsS*, pSA69 (3) was digested with AatII and SalI. A shorter fragment was purified and cloned into plasmid pCS27 (16) cut with the same enzymes, creating pZL8. Both *alsS* single-site mutations (Q487N and Q487A) were introduced using PCR-directed mutagenesis. To introduce the mutation into *alsS*, pSA69 was used as a PCR template with A306 and A124 (Q487N) and A307 and A124 (Q487A). The beginning of the *alsS* gene located on pSA69 was also amplified from the AatII site upstream of the ribosome binding site to the 1,458th base in the *alsS* gene, using primers A300 and A305. These two fragments were then joined by splice overlap extension. The products were digested with AatII and SalI and cloned into pSA69 cut with the same enzyme, creating pSA163 and pSA164.

For protein overexpression and purification, the wild type and *alsS* variants were amplified with primers A297 and A298. PCR products were digested with BamHI and SalI and cloned into pETDuet-1 (Novagen, Madison, WI) cut with the same enzymes (Table 2).

Medium and culture conditions for isobutanol production. M9 medium (64 g $Na_2HPO_4 \cdot 7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mg thiamine per liter water) containing 36 g/liter glucose, 5 g/liter yeast extract, 30 μg/ml kanamycin, and 1 ml/liter of trace metal mix A5 [2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 49.4 mg $Co(NO_3)_2 \cdot 6H_2O$ per liter water] was used for cell growth. Preculture in test tubes containing 3 ml of medium was performed at 37°C overnight on a rotary shaker (250 rpm). Overnight culture was diluted 1:100 into 20 ml of fresh medium in a 250-ml screw cap conical flask. Cells were grown

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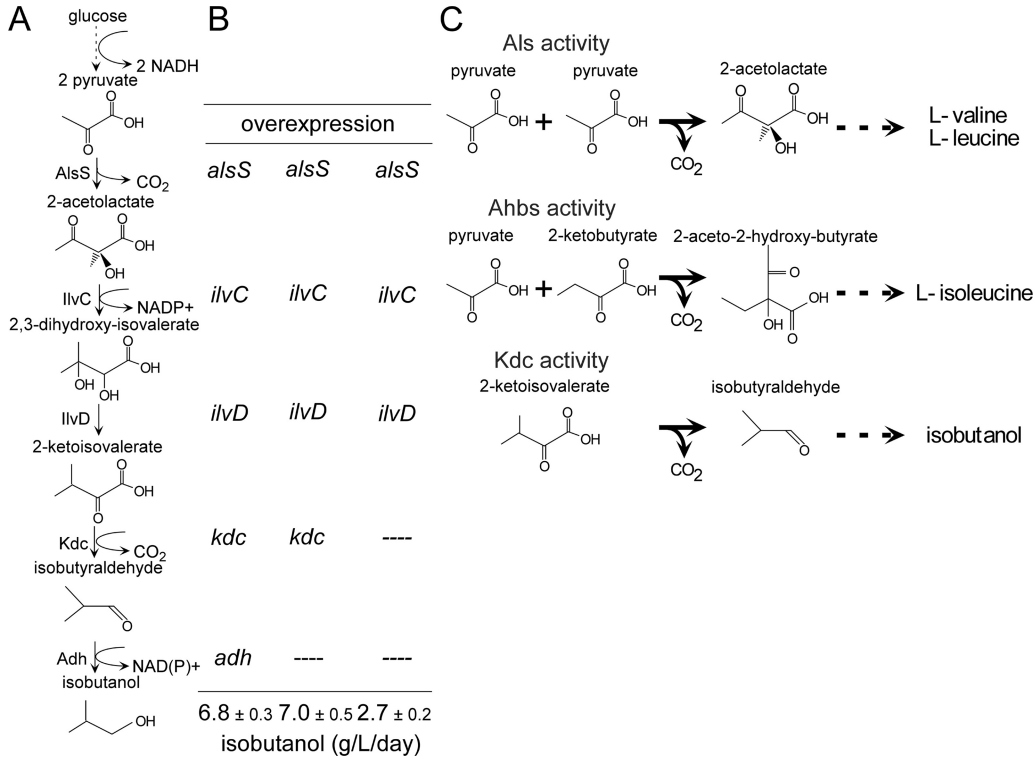

FIG. 1. Schematic representation of the pathway for isobutanol production. (A) The Kdc-dependent synthetic pathway for isobutanol production. (B) Isobutanol production with the Kdc-dependent and -independent synthetic pathways. IlvC, acetohydroxy acid isomeroreductase; IlvD, dihydroxy acid dehydratase. (C) Enzymatic reaction of Als, Ahbs, and Kdc activities.

at 37°C for 3 h, followed by adding 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Production was performed under microaerobic conditions at 30°C on a rotary shaker (250 rpm) for 24 h. Isobutanol was quantified by a gas chromatography-flame ionization detector as previously described (3). Secreted pyruvate was quantified by a high-performance liquid chromatography as previously described (2).

Protein purification. The wild type and AlsS variants were synthesized from a His-tag plasmid in *E. coli* strain BL21 Star (DE3) (Invitrogen, Carlsbad, CA) followed by purification with Ni-nitrilotriacetic acid (NTA) spin columns (Qiagen, Valencia, CA). Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA).

Als activity assay. An enzyme assay for Als activity of AlsS was carried out in 1 ml of morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0) containing 80 nM AlsS, 100 mM MOPS (pH 7.0), 1 mM $MgCl_2$, 0.1 mM TPP, 10 mM acetate, and various concentrations of pyruvate at 37°C for 10 min. The reaction was terminated by acidification of the solution with 0.1 ml of 50% H_2SO_4. The mixture was incubated for an additional 25 min at 37°C to allow for the acid hydrolysis of the acetolactate to acetoin. Acetoin formation was measured as described previously (11). One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate into product in 1 minute under these conditions. The K_m values for pyruvate and the V_{max} were extrapolated after nonlinear regression of the experimental points with the Gauss-Newton method using Matlab.

Kdc activity assay. The enzyme assay for Kdc activity of AlsS was carried out in reaction mixtures containing 80 nM AlsS, 100 mM MOPS (pH 7.0), 1 mM $MgCl_2$, 0.1 mM TPP, 10 mM acetate, and various concentrations of KIV at 37°C for 1 h. The production of isobutyraldehyde was confirmed to be linear over 1 h. Isobutyraldehyde was measured by a gas chromatography-flame ionization detector as previously described (3). One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate into product in 1 minute under these conditions. The K_m values for KIV and the V_{max} were extrapolated after nonlinear regression of the experimental points with the Gauss-Newton method using Matlab.

RESULTS AND DISCUSSION

Analysis of the Kdc-independent isobutanol production. Since *E. coli* does not have any Kdc, we first hypothesized that pyruvate dehydrogenase (PDH) or 2-ketoglutarate dehydrogenase (KGDH) of *E. coli* could catalyze the conversion of KIV to isobutyryl coenzyme A, which is followed by the conversion of isobutyryl coenzyme A to isobutyraldehyde and then isobutanol by aldehyde and alcohol dehydrogenases. To test these possibilities, we deleted *aceE* and *sucA*, which encode subunits of the PDH and KGDH complexes, respectively. However, this double knockout strain with overexpression of *alsS*, *ilvC*, and *ilvD* was still capable of producing isobutanol (data not shown), indicating that neither PDH nor KGDH catalyzes the reaction in the Kdc-independent isobutanol production.

To determine essential components for the Kdc-independent isobutanol production, we measured isobutanol production from the strain overexpressing different combinations of *alsS*, *ilvC*, and *ilvD* (Fig. 2). The strain overexpressing *alsS* alone produced isobutanol, but the strain overexpressing *ilvC* and *ilvD* did not (Fig. 2A). When *ilvC* and *ilvD* were overexpressed with *alsS*, isobutanol production increased nearly ninefold (Fig. 2A). Because the only known activity of AlsS is acetolactate synthase, it is unclear how the strain could produce isobutanol only with *alsS* overexpression. As a control experiment, *ilvI* and *ilvH* (*E. coli*), which encodes an acetohydroxy acid synthase (Ahas) instead of AlsS (*B. subtilis*), were overexpressed. The strain overexpressing *ilvI* and *ilvH* (*E. coli*)

TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description[a]	Reference
E. coli strains		
BW25113	*rrnB*$_{T14}$ Δ*lacZWJ16 hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	7
JCL16	BW25113 F′ [*traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15]	2
JCL260	Same as JCL16 but Δ*adhE* Δ*fnr-ldhA* Δ*frdBC* Δ*pflB* Δ*pta*	3
SA296	Same as JCL260 but Δ*ilvC*	This work
KS145	Same as JCL16 but Δ*ilvI* Δ*ilvB*	4
Plasmids		
pSA55	ColE1 *ori*; Amp^r; $P_L$lacO$_1$::*kivd-ADH2*	3
pSA69	p15A *ori*; Kan^r; $P_L$lacO$_1$::*alsS-ilvC-ilvD*	3
pCS27	p15A *ori*; Kan^r; $P_L$lacO1::MCS1	16
pZL8	p15A *ori*; Kan^r; $P_L$lacO$_1$::*alsS*	This work
pSA159	Derivative of pPETDuet-1 with *alsS*	This work
pSA166	Derivative of pPETDuet-1 with *alsS*(*Q487A*)	This work
pSA187	Derivative of pPETDuet-1 with *alsS*(*Q487I*)	This work
pSA188	Derivative of pPETDuet-1 with *alsS*(*Q487S*)	This work
pSA205	Derivative of pPETDuet-1 with *alsS*(*Q487G*)	This work
pSA206	Derivative of pPETDuet-1 with *alsS*(*Q487L*)	This work
Oligonucleotides		
A124	ACGCAGTCGACCTAGAGAGCTTTCGTTTTCATGAGT	3
A297	CGGGATCCGTTGACAAAAGCAACAAAAGAACAAA	This work
A298	ACGCAGTCGACCTAGAGAGCTTTCGTTTTCATGAGT	This work
A300	AATAAGACGTCTAAGAAACCATTATTATCATG	This work
A305	GAATGCAACCATGTCATATGTGCTG	This work
A306	ATGGAACGACAGCACATATGACATGGTTGCATTCAACCAATTGAAAA AATATAACCGTAC	This work
A307	ATGGAACGACAGCACATATGACATGGTTGCATTCGCCCAATTGAAAA AATATAACCGTAC	This work

[a] For oligonucleotides, the sequences are shown (5′→3′).

did not produce isobutanol. Increasing AlsS levels in *E. coli* led to a parallel increase in the formation of acetoin, which is the product of spontaneous decarboxylation of 2-acetolactate (1). To test whether some enzymes in *E. coli* could utilize acetoin as a substrate for isobutanol production, acetoin was fed to the *E. coli* culture. Neither isobutyraldehyde nor isobutanol was detected from this culture, indicating that acetoin was not a precursor of isobutanol in this pathway (data not shown). To confirm that the Kdc-independent pathway used the same route as the Kdc-dependent pathway, the *ilvC* gene on the genome was deleted (Fig. 2A). The deletion of *ilvC* abolished isobutanol production, indicating that this Kdc-independent pathway utilized KIV as a precursor (Fig. 2A).

We then supplied KIV to the medium to assess the capability to utilize KIV for isobutanol production (Fig. 2B). The strain without AlsS did not produce isobutanol in the presence of 6 g/liter of KIV (Fig. 2B), but addition of KIV to strains overexpressing *alsS* led to isobutanol production in the wild-type and Δ*ilvC* backgrounds (Fig. 2B). These tests revealed that Kdc-independent isobutanol production requires overexpression of *alsS* and a high concentration of KIV. Feeding of KIV to the strain overexpressing *alsS*, *ilvC*, and *ilvD* did not change the production of isobutanol (Fig. 2C), presumably because the concentration of KIV may already saturate the enzyme which utilizes KIV for isobutanol production or the efficiency of KIV uptake may decrease (Fig. 2C).

Characterization of wild type and AlsS variants. After exhausting all other possibilities, we hypothesized that AlsS could catalyze the decarboxylation of KIV and give isobutyraldehyde without the nucleophilic attack of the second pyruvate in the presence of a high concentration of KIV and a low concentration of pyruvate. Because the overexpression of *alsS*, *ilvC*, and

TABLE 2. Kinetic parameters of the wild-type AlsS (*B. subtilis*) and the variants for acetolactate synthase and decarboxylase activity[a]

Amino acid at residue 487	Pyruvate			KIV		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ratio	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ratio
Q	13.6 ± 0.8	121 ± 13	8.9 ± 1.1	300 ± 35	8.9 ± 1.2	0.03 ± 0.005
A	8.7 ± 0.5	58 ± 8	6.7 ± 1.0	186 ± 27	1.1 ± 0.5	0.006 ± 0.003
G	1.6 ± 0.4	11 ± 5	6.9 ± 3.6	175 ± 18	0.8 ± 0.2	0.005 ± 0.001
S	1.1 ± 0.6	11 ± 6	10 ± 7.7	154 ± 21	0.8 ± 0.3	0.005 ± 0.002
L	ND	ND	ND	342 ± 45	5.4 ± 0.9	0.02 ± 0.003
I	ND	ND	ND	323 ± 26	4.8 ± 0.5	0.01 ± 0.002

[a] The values shown after the ± signs are standard deviations. ND, not detectable.

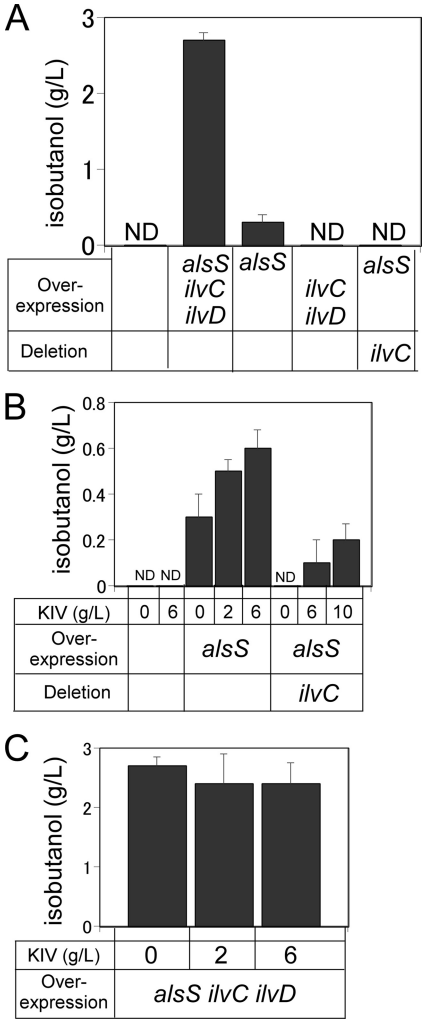

FIG. 2. Summary of results for isobutanol production without Kdc in *E. coli*. The cells were grown in M9 medium containing 5 g/liter yeast extract and 36 g/liter glucose in shake flasks at 30°C with 0.1 mM IPTG for 24 h. Overexpressed and deleted genes and KIV supplementation are indicated below the graphs. (A) Isobutanol production using various enzymes. (B and C) Isobutanol production with the supply of KIV. ND, not detectable.

ilvD significantly decreases the secretion of pyruvate to below a detection limit of 0.1 mM (the host strain without these plasmids secretes ~7 mM pyruvate), it is possible that under this condition, KIV reacts with TPP and undergoes decarboxylation, and then escapes by giving isobutyraldehyde before undergoing the carboligation.

Using the crystal structure of *Klebsiella pneumoniae* AlsS, Pang et al. (14) showed that the extended side chain of Gln483 causes steric hindrance with the larger substrate, 2-ketobutyrate, which explains why AlsS reacts very poorly with a larger 2-keto acid as the second substrate (9, 14). Gln483 in *K. pneumoniae* AlsS has been shown to be in close proximity to the first pyruvate and also involved in second-substrate specificity (14), suggesting that Gln483 may play a role in the release of aldehydes. The residue corresponding to Gln483 in *K. pneumoniae* AlsS is Gln487 in *B. subtilis* AlsS. To test whether Gln487 could play a role in decarboxylation, we replaced Gln487 with various other amino acids.

Purification and characterization of wild type and AlsS variants. To assay the Kdc activity (Fig. 1C, bottom) of AlsS, His-tagged wild-type AlsS was expressed from a His-tag plasmid and purified as described in Materials and Methods. The Kdc activity of the His-tagged wild-type AlsS was 5.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, while isobutyraldehyde production was not detected from a negative control experiment without AlsS. Although the activity was weak, AlsS surely showed the decarboxylase activity toward KIV in vitro.

The kinetic parameters were measured for AlsS variants (Table 2). The k_{cat}/K_m values for pyruvate of Q487 variants with small residues (Q487A, Q487G, and Q487S) were similar to that of the wild type, while the k_{cat}/K_m values for KIV of these variants decreased dramatically (Table 2). Q487L and Q487I replacements impaired Als activity (Table 2). However, the k_{cat}/K_m values for KIV of these variants were similar to that of the wild type. The wild type and all variants showed extremely high K_m values for KIV (Table 2), which may explain why an increase of the flux toward KIV is required for the decarboxylase activity of AlsS. Further analysis is required to explain the relationship between Q487 and decarboxylase activity.

Effects of Q487 replacements for isobutanol production. To test these AlsS variants' capability to produce isobutanol, which requires both Als (Fig. 1C, top) and Kdc (Fig. 1C, bottom) activities, these *alsS* variants were overexpressed with *ilvC* and *ilvD*. Isobutanol production with AlsS (Q487N) was similar to the production achieved using wild-type AlsS (Fig. 3A), presumably because the side chain of Asn has an amine group, like Gln. However, the replacement of Q487 with valine, alanine, glycine, serine, leucine, and isoleucine nearly abolished isobutanol production (Fig. 3A). According to the results of the performed enzyme assays (Table 2), the replacement of Q487 with glycine and serine maintained Als activity and decreased Kdc activity. The ratios of K_m for KIV to K_m for pyruvate of Q487G and Q487S were 109 ± 30 and 140 ± 79, respectively, while the ratio for the wild-type enzyme was 20 ± 2.9. The strains with either Q487G or Q487S cannot produce isobutanol, presumably because the Kdc activity of Q487G and Q487S could not compete with the Als activity. Enzyme assays showed that Q487L and Q487I replacements impaired Als activity. We showed that increased flux toward KIV was important for isobutanol production when using AlsS for Kdc activity (Fig. 2A and B), but the K_m values for KIV of Q487L and Q487I were 342 ± 45 mM and 323 ± 26 mM, respectively (Table 2), which were extremely high. Because these K_m values are extremely high, the strains with these replacements could not produce isobutanol, presumably because the intracellular concentration of KIV would not be high enough for the Kdc activity. No replacements were found that could increase isobutanol production.

Effects of Q487 replacements for Als activity. To distinguish between Als and Kdc activities in these variants, we tested the growth rate of an *E. coli* strain KS145 (Δ*ilvI* Δ*ilvB*) expressing various Q487 variants in a minimum glucose medium supplemented with L-isoleucine. AlsS is a distant homologue of Ahas, which is responsible for both Als (Fig. 1C, top) and 2-aceto-2-hydroxy butyrate synthase (Ahbs) (Fig. 1C, middle) activities

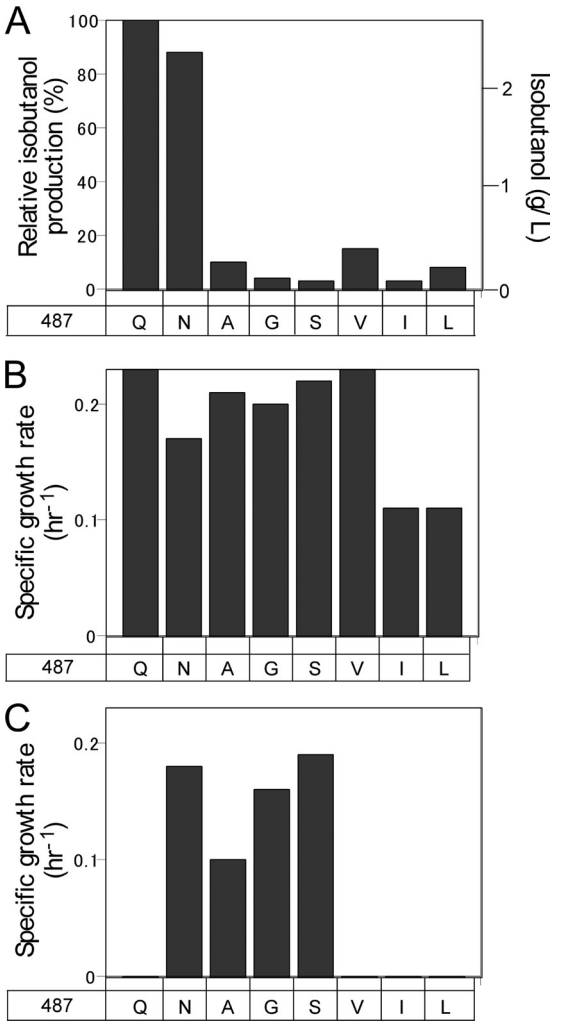

FIG. 3. Effect of Q487 on the decarboxylase activity of AlsS. (A) Isobutanol production with the AlsS variants. The cells were grown in M9 medium containing 5 g/liter yeast extract and 36 g/liter glucose in shake flasks at 30°C with 0.1 mM IPTG for 24 h. (B and C) Specific growth rate of *E. coli* strain KS145 (Δ*ilvI* Δ*ilvB*) with the AlsS variants in M9 medium containing 1% glucose and 39.5 μg · ml^{-1} L-isoleucine (B) and 35 μg · ml^{-1} L-valine and 39.5 μg · ml^{-1} L-leucine (C). The *ilvC* and *ilvD* genes were overexpressed with the *alsS* gene.

in the branched chain amino acid biosynthesis. The KS145 strain does not have Als and Ahbs activities (Fig. 1C); thus, the specific growth rate in the minimal medium with L-isoleucine reflects the Als activity. Figure 3B shows that all of the Q487 variants retain significant Als activity. Considering that most of the Q487 variants did not generate isobutanol (Fig. 3A), we conclude that AlsS is indeed responsible for the Kdc activity observed in isobutanol synthesis, and that Q487 is important for this activity. KS145 cells expressing Q487L or Q487I showed slow growth with the L-isoleucine supplement (Fig. 3B), indicating that these replacements would reduce Als activity. These results were consistent with the results of enzyme assays (Table 2). In the structure model of *K. pneumoniae* AlsS, the C-1 carbonyl oxygen of the modeled second pyruvate is hydrogen-bonded to the side chain of Gln483 (14). Thus, the nonpolar side chain of isoleucine and leucine in the 487th residue would reduce the binding affinity of the second pyruvate to the site. No growth phenotype was observed in any strain while grown on minimum glucose medium supplemented with L-valine, L-leucine, and L-isoleucine (data not shown).

Effects of Q487 replacements for Ahbs activity. AlsS reacts very poorly with the larger substrate, 2-ketobutyrate (9). If these replacements change the second substrate specificity by removing steric hindrance, the AlsS variants would gain the Ahbs activity so that the KS145 cells expressing the variants could grow in minimal medium with L-valine and L-leucine supplementation. As predicted, KS145 cells expressing the wild-type AlsS were unable to grow without L-isoleucine (Fig. 3C), indicating that the wild-type AlsS was not capable of catalyzing Ahbs reaction, which is consistent with previous studies (12). Interestingly, the AlsS variants which contain small residues (Ala, Gly, and Ser) at the 487th residue rescued the growth of KS145 under the same conditions (Fig. 3C). This result suggests that the replacement of Q487 with small side chain amino acids would make the substrate binding site larger so that the variants are able to react with 2-ketobutyrate as the second substrate (Ahbs activity) (Fig. 1C, middle).

Our evidence shows that Kdc is not essential for isobutanol production and that AlsS, previously known only to have Als activity, can catalyze the decarboxylation of KIV. The use of mutational studies allowed us to identify that Q487 is important for Kdc activity. For further analysis, mutational studies can be expanded to other residues in the catalytic center of AlsS. These studies will give us implications to distinguish important residues for Als and Kdc activities. Protein structural analysis, such as X-ray crystallography, can also be applied to increase our understanding of the catalytic mechanism of Kdc activity of AlsS.

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