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Uptake of Branched-Chain α-Keto Acids in *Bacillus subtilis*

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Bacillus subtilis has a constitutive system for the uptake of α-keto-β-methylvalerate, α-ketoisovalerate, and (probably) α-ketoisocaproate. A mutation, *kauA1*, which blocks the uptake of α-keto-β-methylvalerate and α-ketoisovalerate, is located between *metB* and *citK* on the *B. subtilis* chromosome.

Bacillus subtilis strain CU906 is a derivative of strain 168I⁻ which carries an *ilvB* deletion mutation and a *leuB* mutation and, therefore, requires the branched-chain amino acids isoleucine, valine, and leucine. It can also satisfy these growth requirements with the corresponding α-keto acids: α-keto-β-methylvalerate (KMV), α-ketoisovalerate (KIV), and α-ketoisocaproate (KIC). We mutagenized strain CU906 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and imposed a penicillin selection (4) for mutants unable to use the α-keto acids for growth. A mutant strain, CU961, was isolated that could not use 0.3 mM DL-KMV or KIV to satisfy its isoleucine and valine requirements, although it could still use 0.3 mM KIC to satisfy its leucine auxotrophy. We have named the mutation responsible for this phenotype *kauA1;* its genetic map position is in the *metB-citK* region (Fig. 1), unlinked to the *ilvB* and *leuB* mutations. We have isolated two other mutants with similar defects; their mutations lie very close to *kauA1* and are probably in the same gene. The *kauA* mutations are silent in strains that do not carry *ilv* mutations; i.e., such

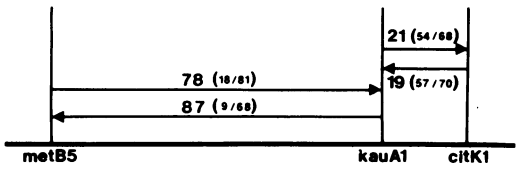

FIG. 1. *Genetic map of the metB-citK region of the chromosome of B. subtilis showing the position of the kauA1 marker. A three-point cross was carried out between two strains isogenic for an ilvB deletion (necessary for scoring kauA1, which is silent in the absence of a requirement for isoleucine or valine). The donor in the phage PBS1 transduction was strain CU450 (trpC2, ilvBΔ1, citK1). The recipient was strain CU968 (trpC2, ilvBΔ1, metB5, kauA1). Selection was for Met⁺ or for Kau⁺. Of 81 Met⁺ transductants tested, none were Kau⁻ and CitK⁻; all other recombinant classes were found. This indicates the order of genes shown. Data from a two-point cross linking citK1 and kauA1 are included in the figure. Map distances are given as phage PBS1 transduction units, calculated as (100 − percentage of co-transfer of the two markers). The arrows point from the selected to the unselected markers. The numbers in parentheses indicate the actual number of co-transductants found among the total colonies tested in each cross. Genetic techniques used were previously reported (4).*

TABLE 1. *Rate of KIV uptake in the presence of various inhibitors*[a]

Inhibitor[b]	Rate of uptake (%)	
	5 μM inhibitor concn	100 μM inhibitor concn
L-Leucine	–[c]	53
L-Isoleucine	–	63
L-Valine	–	74
KIC	31	3.7
DL-KMV	48	2.5
KIV	60[d]	–
Pyruvate	–	96
α-Ketoglutarate	–	85
α-Ketobutyrate	–	53

[a] The rate of KIV uptake is given as a percentage of the rate of uptake seen in this experiment with no inhibitor added. The [KIV] was 1.6 μM and the rate of uptake with no inhibitor present was observed to be 1.7 nmol/min per mg of protein. Uptake rates were measured as described in Fig. 3A.

[b] Inhibitors were adjusted to pH 7 before use.

[c] A dash indicates that the assay was not performed.

[d] In this assay, enough unlabeled KIV was added to bring the final [KIV] to 6.6 μM. This point may thus be directly compared to the other values in this column.

strains are prototrophic. The aminotransferase activities of the mutant strain CU961 for isoleucine and valine, measured in both the forward and reverse directions with α-ketoglutarate and L-glutamate by the method of Taylor and Jenkins (3), did not differ significantly from those of the parent strain CU906.

Strain CU992 (*trpC2 kauA1*) and the isogenic strain CU120 (*trpC2*) were tested for uptake of KIV; the results are shown in Fig. 2. The uptake of KIV was virtually absent in the strain carrying the *kauA1* marker, whereas essentially linear uptake was observed for at least 5 min in the Kau$^+$ strain (Fig. 2, inset). Since similar activity was seen whether or not the glucose-salts growth medium contained KIV, the uptake system for KIV appears to be constitutive.

Using strain CU120, the kinetics of the uptake system for KIV was studied. The results of a substrate saturation experiment are shown in Fig. 3A; a double-reciprocal plot of these

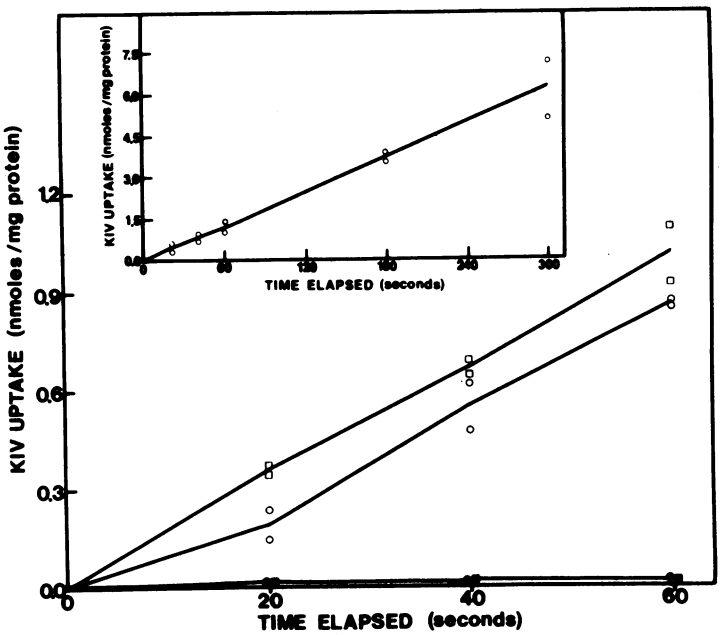

FIG. 2. *Uptake of KIV versus time with strains CU992 (trpC2, kauA1) and CU120 (trpC2). Symbols:* ○, *CU120 grown in minimal medium;* □, *CU120 grown with KIV;* ●, *CU992 grown in minimal medium;* ■, *CU992 grown with KIV. For each time point, two separate determinations were made; the line is drawn through their mean. Cells were grown for 5 h at 37 C with aeration in 70 ml of medium containing (in micrograms per milliliter): L-glutamate, 100; L-tryptophan, 50; D-glucose, 5,000; and, where applicable, KIV, 40; in BS. BS is a salts solution containing (in milligrams per milliliter): $(NH_4)_2SO_4$, 2; K_2HPO_4, 14; KH_2PO_4, 6; sodium citrate·$2H_2O$, 1; and $MgSO_4 \cdot 7H_2O$, 0.2. After harvesting, cells were washed twice with BS, resuspended in about 5 ml of BS, and kept on ice prior to their immediate use. Samples were removed, and total cell protein (usually about 300 μg/ml) was estimated by the method of Lowry et al. (1), using bovine serum albumin as standard. For the uptake assay, 0.2 ml of cell suspension was equilibrated in 0.4 ml of 0.2% D-glucose in BS at 30 C for 5 min. Then, 5 μl of [^{14}C]KIV (49 μCi/μmol; 0.2 μmol/ml) (prepared by a modification of the method of Meister [2] and adjusted to pH 7) was added and the tube was reincubated at 30 C. The final concentration of KIV in this assay was thus 1.7 μM. Samples of 100 μl were removed at appropriate times and filtered through 0.45-μm pore size nitrocellulose filters (Matheson-Higgins Co.) presoaked with 0.15 M NaCl and immediately washed with 8 ml of 0.15 M NaCl. Filters were dried for 12 min at 165 C and counted in a fluid containing 4.02 g of 2,5-diphenyloxazole (PPO) and 0.10 g of 1,4-bis[2]-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP) per liter of toluene in a Beckman LS-230 scintillation counter for 10 min. Background counts for the assay were determined by using tubes equilibrated at 30 C for 5 min and then chilled to 0 C; the assay was run as above but at 0 C. These counts were subtracted from the experimental values obtained at 30 C to calculate the counts taken up by the cells after various elapsed times. The number of moles of KIV taken up was estimated from these data by counting a known quantity of [^{14}C]KIV solution absorbed into filters, which were subsequently dried and counted as above. Data for the inset were obtained in a separate experiment from that shown in the figure.*

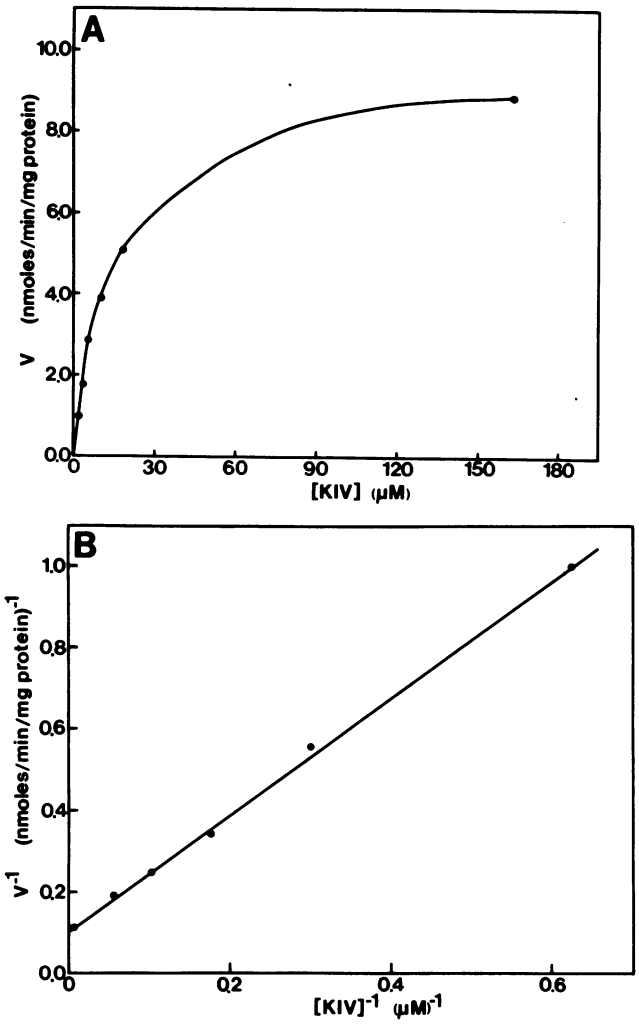

FIG. 3. *(A) Rate of KIV uptake versus external concentration of KIV. The uptake assay was performed as described in Fig. 2. Cells were grown without KIV. Unlabeled KIV (adjusted to pH 7) was added to assay tubes to increase the external concentration of KIV; the quantity of [^{14}C]KIV present was the same as described in Fig. 2. The rate of uptake was calculated by measuring KIV uptake in triplicate assay samples at 1 and 3 min elapsed time and finding the KIV uptake per minute. (B) Double-reciprocal plot of the data presented in (A).*

data appears in Fig. 3B. The measured K_m for KIV in this experiment was 14 μM; the V_{max} of uptake was 9.6 nmol/min per mg of protein.

To investigate the specificity of the KIV uptake system, the rate of KIV uptake was measured for strain CU120, with various potential inhibitors present at either 5 or 100 μM in the usual uptake assay (Table 1). Strong inhibition was seen with KMV and KIC. A lower but significant amount of inhibition occurred with the branched-chain amino acids. It was not due to contamination of the amino acid solutions by α-keto acids.

We examined the inhibition of KIV uptake by KMV and KIC in strain CU120 (Fig. 4). The inhibition due to KMV and to KIC was found to be competitive; the measured K_i values for uptake were 3.5 and 4.1 μM for racemic KMV and for KIC, respectively.

The system affected by the *kauA1* mutation appears to function in the uptake of the three branched-chain α-keto acids (KIV, KMV, and

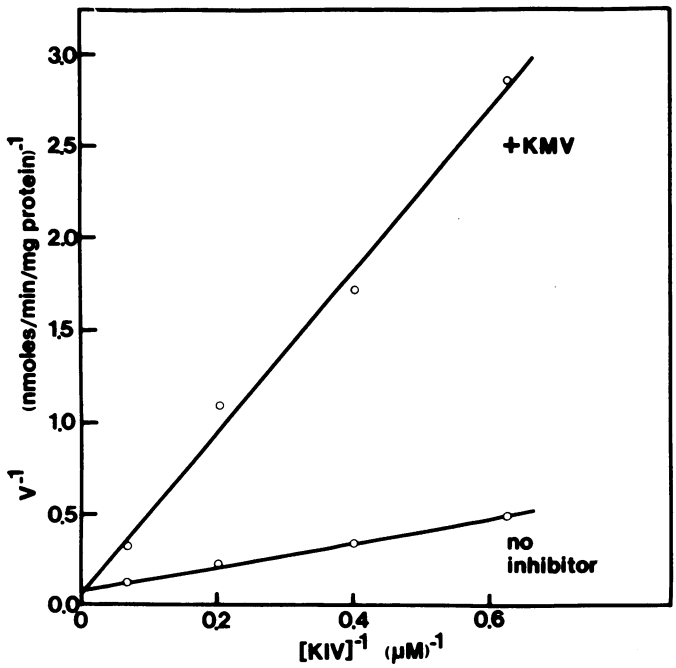

FIG. 4. *Double-reciprocal plots of rate of KIV uptake versus external concentration of KIV in the absence and presence of 20 μM* DL-*KMV. This experiment was performed as described in Fig. 3A. For each data point, the average of two separate determinations is shown. In this experiment, the K_m for KIV was 9.7 μM and the V_{max} for uptake was 15 nmol/min per mg of protein. An analogous experiment was performed with 20 μM KIC, and similar results were obtained. In this latter experiment, the K_m for KIV was 20 μM and the V_{max} was 24 nmol/min per mg of protein.*

KIC) in *B. subtilis*. Since the mutant strain, CU961, originally isolated from the penicillin selection was able to utilize externally supplied KIC, there must be an alternate system for the uptake of KIC in *B. subtilis* that does not function in the uptake of KIV or KMV.

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