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Dual role of a single multienzyme complex in the oxidative decarboxylation of pyruvate and branched-chain 2-oxo acids in *Bacillus subtilis*

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The pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase activities of *Bacillus subtilis* were found to co-purify as a single multienzyme complex. Mutants of *B. subtilis* with defects in the pyruvate decarboxylase (E1) and dihydrolipoamide dehydrogenase (E3) components of the pyruvate dehydrogenase complex were correspondingly affected in branched-chain 2-oxo acid dehydrogenase complex activity. Selective inhibition of the E1 or lipoate acetyltransferase (E2) components *in vitro* led to parallel losses in pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex activity. The pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes of *B. subtilis* at the very least share many structural components, and are probably one and the same. The E3 component appeared to be identical for the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes in this organism and to be the product of a single structural gene. Long-chain branched fatty acids are thought to be essential for maintaining membrane fluidity in *B. subtilis*, and it was observed that the *ace* (pyruvate dehydrogenase complex) mutant 61142 was unable rapidly to take up acetoacetate, unlike the wild-type, indicative of a defect in membrane permeability. A single pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex can be seen as an economical means of supplying two different sets of essential metabolites.

The pyruvate dehydrogenase complex from *Bacillus stearothermophilus* (Henderson *et al.*, 1979; Henderson & Perham, 1980) and from *Bacillus subtilis* (Visser *et al.*, 1980; Hodgson *et al.*, 1983) comprises multiple copies of three different enzymes: pyruvate decarboxylase (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3) (EC 1.6.4.3). The E1 activity resides in E1α and E1β polypeptide chains and these, together with the E3 chains, are assembled round an E2 core of icosahedral symmetry (Henderson *et al.*, 1979; Hodgson *et al.*, 1983). The pyruvate dehydrogenase complex catalyses the oxidative decarboxylation of pyruvate to form acetyl-CoA, a key step in the oxidative degradation of glucose. Although vegetative growth of *B. subtilis* on glucose is almost entirely independent of the tricarboxylic acid cycle, the production of acetyl-CoA by the pyruvate dehydrogenase reaction is essential for normal growth (Klofat *et al.*, 1969; Fortnagel & Freese, 1968). Mutants defective in the enzyme cannot grow on glucose in the absence of supplementary acetate (Freese & Fortnagel, 1969; Hodgson *et al.*, 1983).

B. subtilis, unlike *Escherichia coli*, has a requirement for branched-chain fatty acids, such as isovalerate (3-methylbutyrate) and isobutyrate (2-methylpropionate), to act as starter units for the synthesis of branched-chain lipids for the cell membrane (Kaneda, 1973). The branched-chain 2-oxo acid dehydrogenase complex is therefore essential for normal growth, which is not so for *E. coli*, where the enzyme is absent. *E. coli* is unable to grow on branched-chain amino acids, e.g. leucine, since the transamination product 4-methyl-2-oxopentanoate cannot be oxidatively decarboxylated. In contrast, *B. subtilis* can grow on leucine as sole carbon and energy source, since the branched-chain catabolic

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pathway is present, and acetyl-CoA can be produced from it.

In the course of studies on *B. subtilis ace* mutant 61142, which is defective in the E1 subunits of the pyruvate dehydrogenase complex (Hodgson *et al.*, 1983), we found that it was unable to utilize leucine. In the present paper we pursue this observation and report evidence that a novel system is present in *B. subtilis* in which a single multienzyme complex is used oxidatively to decarboxylate both pyruvate and branched-chain 2-oxo acids.

Materials and methods

Reagents

Ethyl [3-^{14}C]acetoacetate (100 μCi, 4.69 Ci/mol), obtained from The Radiochemical Centre, Amersham, Bucks, U.K., was mixed with 0.2 ml of 0.33 M-NaOH and left at 22°C for 15 h. The mixture was adjusted to pH 7 by addition of about 20 μl of 2 M-H_2SO_4. The acetoacetate concentration was checked by assay with NADH and 3-hydroxybutyrate dehydrogenase (kindly given by Mrs. Denise Lowe). At least 75% of the radioactivity was in the form of acetoacetate. [U-^{14}C]Leucine (5 Ci/mol) was kindly given by Dr. P. J. F. Henderson (Department of Biochemistry, University of Cambridge). Sodium pyruvate and 2-oxoglutaric acid were from BDH. 2-Oxoisovalerate, 4-methyl-2-oxopentanoate and 3-methyl-2-oxopentanoate, all sodium salts, were purchased from Sigma.

Bacterial strains

B. subtilis strain 168 *trp*C2 was kindly given by Dr. P. Oliver (Department of Genetics, University of Cambridge). *B. subtilis* mutants 61141R and 61142 (Freese & Fortnagel, 1969; Hodgson *et al.*, 1983), carrying auxotrophic markers *trp*C2 *met*C7 *ace*A, were kindly given by Dr. E. Freese. The *B. subtilis* mutants JH422 (markers *trp*C2 *cit*L22) (Rutberg & Hoch, 1970; Hoch & Coukoulis, 1978), 61484 (markers *trp*C2 *met*C7 *ace*A *bfm*A) and 61494 (markers *trp*C2 *met*C7 *ace*A *bfm*B) (Willecke & Pardee, 1971; Boudreaux *et al.*, 1981) were kindly supplied by the *Bacillus* Genetic Stock Center (Ohio State University, Columbus, OH 43210, U.S.A.). catalogue numbers 1A70, 1A470 and 1A471 respectively. Growth media were those described by Hodgson *et al.* (1983).

Purification and assay of enzymes

Pyruvate dehydrogenase complex was prepared from wild-type and mutant strains of *B. subtilis* as described by Hodgson *et al.* (1983). Pyruvate dehydrogenase complex, dihydrolipoamide dehydrogenase and pyruvate-driven E1 (reduction of 2,6-dichlorophenol-indophenol) activities were measured as described by Hodgson *et al.* (1983). 2-Oxoglutarate dehydrogenase complex and branched-chain 2-oxo acid dehydrogenase complex activities were assayed by replacing pyruvate with 2 mM-2-oxoglutarate or 2 mM-2-oxoisovalerate respectively. Branched-chain 2-oxo acid-driven E1 activity was measured by replacing pyruvate with 2 mM-2-oxoisovalerate in the assay mixture.

Uptake of leucine and acetoacetate by B. subtilis cells

Inocula of *ace* mutant 61142 and a complete *ace*$^+$ revertant of 61142 were added to separate 50 ml incubations containing L-broth and Spizizen salts supplemented with 10 mM-acetate, 10 mM-L-leucine and 20 mM-L-glutamate. The cells were allowed to grow at 37°C in an orbital shaker for 3 h to an A_{650} of 0.45 and were then harvested at 10000 rev./min in a Sorvall GSA rotor for 10 min. The cell pellets were resuspended in 25 ml of Spizizen M salts, containing in addition 20 mM-L-glutamate and 10 mM-acetate. The cells were washed with 25 ml of the same medium and were finally resuspended in 10 ml of the same medium. The two types of cell were adjusted to an A_{650} of 2.3, corresponding to 0.57 mg dry wt./ml, by addition of further medium. The cell suspension was left at 4°C with occasional aeration.

Samples (0.6 ml) were incubated with either 67 μM-L-[U-^{14}C]leucine (5 Ci/mol) or with 580 μM-[3-^{14}C]acetoacetate at 37°C with continuous agitation. Samples (0.1 ml) were removed at intervals, filtered on to 0.45 μm cellulose acetate filters (Millipore), washed with 5 ml of Spizizen M salts, containing in addition 20 mM-L-glutamate and 10 mM-acetate, and counted for radioactivity in a scintillation counter.

Other techniques

All other techniques, such as scintillation counting and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, were performed as described by Hodgson *et al.* (1983).

Results

A single mutation affects activities of both pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes

B. subtilis ace mutant 61142 requires a supplement of acetate to grow on glucose-based minimal medium (Freese & Fortnagel, 1969; Hodgson *et al.*, 1983). It was to be expected that a supplement of ketogenic amino acids might replace the acetate requirement for growth on glucose-based minimal medium. However, we found that a supplement of neither leucine alone nor any combination of leucine, valine or isoleucine could permit growth on glucose-based minimal medium. Moreover, wild-type *B. subtilis* strain *trp*C2 was able to grow, albeit slowly, on leucine as sole carbon and energy source,

whereas mutant 61142 was unable to do so. The partial *ace* revertant 61141R (Hodgson *et al.*, 1983) showed some growth on leucine, although it grew much more slowly than the wild-type.

Boudreaux *et al.* (1981) found that mutant 61141 possessed less of the branched-chain 2-oxo acid dehydrogenase complex activity than did the parent strain 60015, and they concluded that it contained another mutation, *ace*B, distinct from *ace*A, the mutation originally described in the pyruvate dehydrogenase complex (Freese & Fortnagel, 1969). We therefore wondered whether the inability of the comparable mutant 61142 to grow on leucine-based minimal medium was due to a defect in the branched-chain 2-oxo acid dehydrogenase complex.

The cell-free extract of mutant 61142 contains no pyruvate dehydrogenase complex activity (Hodgson *et al.*, 1983). Neither could we detect any branched-chain 2-oxo acid dehydrogenase complex activity. This suggested that either two genetic lesions were present, as suggested by Boudreaux *et al.* (1981), or that a single lesion was affecting both enzyme activities. To distinguish between these alternatives, ten revertants were independently selected, five chosen for their ability to grow on leucine-based minimal medium and five for their ability to grow on glucose in the absence of acetate. It was found that all *ace*$^+$ revertants could grow on leucine as sole carbon and energy source and that all revertants selected for growth on leucine had simultaneously reverted to *ace*$^+$. The reversion frequency was about 1 in 10^7. This strongly suggests that a single lesion, presumably a point mutation (*ace*A), causes both defects. In other experiments (see below) we found that cell-free extracts of the partial *ace* mutant 61141R (Hodgson *et al.*, 1983) contained lowered activities for both pyruvate dehydrogenase and branched-chain 2-oxo-acid dehydrogenase complex, again suggesting that a single genetic lesion affected both enzyme complexes.

One explanation for this phenomenon might be that the mutations in mutants 61141R and 61142 lie in a shared E3 subunit [cf. Tu & Kaneda (1976)]. However, the *ace*A mutation of both mutants has been shown to reside in the E1 subunits (Hodgson *et al.*, 1983).

Co-purification of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex activities

Pyruvate dehydrogenase complex from the wild-type strain of *B. subtilis trp*C2 was purified to homogeneity as described by Hodgson *et al.* (1983). The 2-oxoglutarate dehydrogenase complex activity was cold-labile and easily separated from the pyruvate dehydrogenase complex. However, branched-chain 2-oxo acid dehydrogenase complex activity was found to co-purify with the pyruvate dehydrogenase complex, and the ratio between the two activities was constant over a 33-fold purification (Table 1). The purified enzyme contained only the four subunits of the pyruvate dehydrogenase complex as detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Hodgson *et al.*, 1983). The recovery of the two enzyme activities was similar and high, suggesting that a specific branched-chain 2-oxo acid dehydrogenase complex was not being selectively lost during the purification procedure. The purified enzyme catalysed the oxidative decarboxylation of the three branched-chain 2-oxo acids 2-oxoisovalerate, 4-methyl-2-oxopentanoate and 3-methyl-2-oxopentanoate in the proportions 1:0.07:0.09 (each substrate 2mM). Growth of strain *trp*C2 on 10mM-leucine as sole carbon source did not alter the ratios of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex activities in the cell-free extracts.

As mentioned above, *ace* mutant 61142 was found to contain no pyruvate dehydrogenase or

Table 1. *Purification of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes from wild-type B. subtilis*

The purification procedure is that described by Hodgson *et al.* (1983). Abbreviations used: PDHC, pyruvate dehydrogenase complex; OIVDHC, 2-oxoisovalerate dehydrogenase complex; OGDHC, 2-oxoglutarate dehydrogenase complex; Pyr-E1, activity of E1 component (reduction of 2,6-dichlorophenol-indophenol) in the presence of pyruvate; OIV-E1, activity of E1 component (reduction of 2,6-dichlorophenol-indophenol) in the presence of 2-oxoisovalerate.

	PDHC		OIVDHC		Ratio PDHC/	E3	OGDHC	Ratio Pyr-E1/
Step	(units)	(units/mg)	(units)	(units/mg)	OIVDHC	(units/mg)	(units)	OIV-E1
1. Cell-free extract	1220	0.30	77	0.019	15.9	0.80	57	1.41
2. Ultracentrifugation pellet	1240	1.50	99	0.12	12.5	3.2	52	1.28
3. Sepharose-2B pool	885	6.4	60	0.46	13.9	10.3	0	1.25
4. $(NH_4)_2SO_4$ precipitate	656	10.0	43	0.70	14.3	14.1	0	1.18
Yield	54%		56%					

branched-chain 2-oxo acid dehydrogenase complex activities and no pyruvate- or 2-oxoisovalerate-driven E1 activities during any step of the purification. Partial *ace* revertant 61141R, in contrast, contained pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex activities. When the pyruvate dehydrogenase complex was purified from this mutant at 22°C in the presence of Mg–thiamin pyrophosphate, as described by Hodgson *et al.* (1983), all four protein subunits were present and the ratio of 2-oxoisovalerate dehydrogenase complex to pyruvate dehydrogenase complex activity was similar to that in the wild type, although the K_m for both pyruvate (Hodgson *et al.*, 1983) and 2-oxoisovalerate was raised about 10-fold (Table 2). The enzyme isolated from mutant 61141R is cold-labile, the loss of activity being accompanied by dissociation of E1 subunits from the E2E3 subcomplex (Hodgson *et al.*, 1983). Incubation of the enzyme complex at 4°C led to loss of both branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase complex activities in parallel. This could be reversed by raising the temperature of the incubation to 24°C, which caused a simultaneous increase in both enzyme activities (Fig. 1). The ratio of the activities stayed approximately constant over 10-fold changes in them.

Purified pyruvate dehydrogenase complex from *B. stearothermophilus* (Henderson & Perham, 1980) also showed branched-chain 2-oxo acid dehydrogenase complex activity. The ratio of dehydrogenase complex activity with pyruvate as substrate to that with 2-oxoisovalerate as substrate was about 7.

Evidence that a single multienzyme complex is involved in the oxidative decarboxylation of pyruvate and branched-chain 2-oxo acids

We have shown above that pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes co-purify, that a single lesion in the E1 component of the pyruvate dehydrogenase complex in mutant 61142 causes loss of branched-chain 2-oxo acid dehydrogenase complex activity and that the lesion in the E1 subunits of mutant 61141R causes both enzyme activities to be affected similarly in terms of cold-lability and altered K_m. In line with these results, pyruvate was found to be a competitive substrate with 2-oxoisovalerate in the overall dehydrogenase complex reaction (Table 3).

At least part of the reaction catalysed by the E1 component must be in common for both substrates, since (1) titration of the enzyme complex with the E1 inhibitor thiamin thiothiazolone pyrophosphate (Gutowski & Lienhard, 1976) was observed to inhibit pyruvate dehydrogenase and 2-oxoisovalerate dehydrogenase complex activities to similar extents, (2) the K_i for acetyl phosphonate, an inhibitor competitive with pyruvate [R. A. Harrison, R. N. Perham & P. M. Slater, unpublished work, cited by Ambrose & Perham (1976)] was found to be the same (0.3 mM) for both pyruvate and 2-oxoisovalerate in the overall complex reaction, (3) incubation of the complex with 3-bromopyruvate inactivated both pyruvate- and 2-oxoisovalerate-driven E1 activities at the same rate, (4) pyruvate and 2-oxoisovalerate were competitive substrates for the E1-dependent reduction of 2,6-dichlorophenolindophenol (Table 3), (5) mixing purified E2E3 subcomplex from mutant 61142 (Hodgson *et al.*, 1983), which contained neither overall complex activity nor E1 activities for either substrate, with wild-type pyruvate dehydrogenase complex that had

Table 2. *Kinetic parameters of pyruvate dehydrogenase complex from B. subtilis wild-type and mutant 61141R*

Concentrations of other reactants were: 2.5 mM-NAD^+, 0.13 mM-CoA (for details see Hodgson *et al.*, 1983).

Bacterial strain ...	Wild-type		Mutant 61141R	
Substrate	$V_{max.}$ (units/mg)	K_m (mM)	$V_{max.}$ (units/mg)	K_m (mM)
Pyruvate	12	0.33	6	4
2-Oxoisovalerate	0.9	1.3	0.3	14

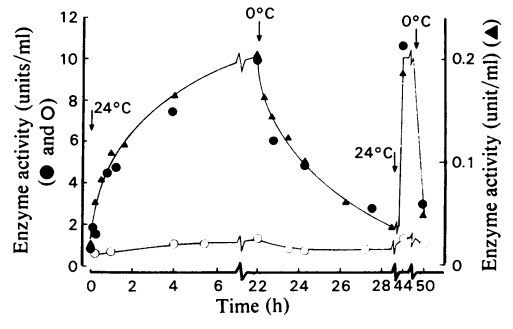

Fig. 1. *Cold-lability of pyruvate dehydrogenase and 2-oxoisovalerate dehydrogenase complex activities of B. subtilis mutant 61141R*

Pyruvate dehydrogenase complex from mutant 61141R was purified up to the final stage of precipitation with $(NH_4)_2SO_4$ (Hodgson *et al.*, 1983) and kept at 4°C for 24h. The enzyme was then diluted 10-fold in 50 mM-potassium phosphate/1 mM-$MgCl_2$/0.2 mM-thiamin pyrophosphate, pH 7, to give a protein concentration of 2.3 mg/ml, and incubated at 24°C for 24h. This sequence of temperature changes was repeated again during the next 50h. At intervals the pyruvate dehydrogenase (●), 2-oxoglutarate dehydrogenase (O) and 2-oxoisovalerate dehydrogenase (▲) complex activities were measured.

Table 3. *Substrate competition between pyruvate and 2-oxoisovalerate for B. subtilis pyruvate dehydrogenase complex*

Theoretical enzyme activity for one enzyme capable of utilizing two substrates competitively is based on

$$V_{total} = \frac{V_{Pyr}\left(1+\frac{[Pyr]}{K_m^{Pyr}}\right)+V_{OIV}\left(1+\frac{[OIV]}{K_m^{OIV}}\right)}{1+\frac{[Pyr]}{K_m^{Pyr}}+\frac{[OIV]}{K_m^{OIV}}}$$

where V_{Pyr} and V_{OIV} are enzyme velocities in presence of pyruvate or 2-oxoisovalerate alone (Segel, 1975), and the following kinetic constants apply: dehydrogenase complex activity, K_m^{Pyr} 0.33 mM, K_m^{OIV} 1.4 mM; catalytic activity of E1 measured by the rate of reduction of 2,6-dichlorophenol-indophenol in the presence of pyruvate or 2-oxoisovalerate (DCPIP-E1 activity), K_m^{Pyr} 0.023 mM, K_m^{OIV} 3.3 mM.

Substrate	Dehydrogenase complex activity (units/ml)	Theoretical enzyme activity (units/ml): Two separate enzymes	Theoretical enzyme activity (units/ml): One enzyme
2 mM-Pyruvate	7.60		
4 mM-2-Oxoisovalerate	0.52		
2 mM-Pyruvate plus 4 mM-2-oxoisovalerate	5.82	8.12	5.46

Substrate	DCPIP-E1 activity	Theoretical enzyme activity (units/ml): Two separate enzymes	Theoretical enzyme activity (units/ml): One enzyme
2 mM-Pyruvate	2.13		
4 mM-2-Oxoisovalerate	2.84		
2 mM-Pyruvate plus 4 mM-2-oxoisovalerate	2.14	4.97	2.17

been inactivated by treatment with *N*-ethylmaleimide in the presence of pyruvate, led to a simultaneous appearance of branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase complex activities (Table 4) caused by migration of E1 subunits from the inactivated complex to the potentially active subcomplex (Hodgson *et al.*, 1983), and (6) limited proteolysis of the E1 subunits with trypsin or chymotrypsin (P. N. Lowe, J. A. Hodgson & R. N. Perham, unpublished work) led to simultaneous falls in pyruvate dehydrogenase and 2-oxoisovalerate dehydrogenase complex and E1 activities.

Direct evidence that a single E2 core is involved comes from the following experiment. Incubation of *N*-ethyl[2,3-^{14}C]maleimide with *B. subtilis* pyruvate dehydrogenase complex in the presence of pyruvate led to modification of the lipoic acid residues in the E2 component and consequently to inactivation of pyruvate dehydrogenase complex activity, as described by Hodgson *et al.* (1983). The inactivation was pseudo-first-order (half-life, $t_{\frac{1}{2}}$ = 15 min), and the incorporation of radiolabelled inhibitor into the E2 subunits was 5.1 ± 0.2 nmol/mg of complex for complete inactivation. The 2-oxoisovalerate dehydrogenase complex activity was destroyed simultaneously, but slightly more quickly ($t_{\frac{1}{2}}$ = 8.5 min). An identical incubation of the enzyme with *N*-ethyl[2,3-^{14}C]maleimide, but in the presence of 2-oxoisovalerate, not pyruvate, led to a final incorporation of 4.9 ± 0.4 nmol/mg of complex and a simultaneous loss of 2-oxoisovalerate dehydrogenase complex ($t_{\frac{1}{2}}$ = 8.5 min) and pyruvate dehydrogenase complex ($t_{\frac{1}{2}}$ = 15.5 min) activities. Similar results were obtained with the enzyme from *B. stearothermophilus*, except that both enzyme activities were lost at exactly the same rate in the presence of *N*-ethylmaleimide and pyruvate or

Table 4. *Reconstitution of pyruvate dehydrogenase complex activity by mixing E2E3 subcomplex with N-ethylmaleimide-inactivated enzyme complex*

The 100% value is the 2-oxo acid dehydrogenase complex activity of the wild-type enzyme before its inactivation with *N*-ethylmaleimide. For abbreviations see Table 1.

Time (min)	Pyruvate dehydrogenase complex activity (%)	Pyruvate dehydrogenase complex activity (units/ml)	Activity ratio PDHC/OIVDHC
Before treatment with *N*-ethylmaleimide	100	5.4	16.7 ± 2.8 (S.D.)
0	1.5		
3	3		14.5
31	5		15.2
300	30		15.9

2-oxoisovalerate. Assuming a stoicheiometry of the subunits of 1:1:1:0.5 (E1α:E1β:E2:E3) and one acetylatable lipoic acid residue per E2 polypeptide chain (Henderson & Perham, 1980; Stanley *et al.*, 1981), about 6nmol of inhibitor per mg of complex should be incorporated for complete inhibition. Thus the same E2 core is acylated when either pyruvate or 2-oxoisovalerate is used as substrate.

There is already evidence that the E3 subunit is shared by the branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase complexes (Tu & Kaneda, 1976). We extended this as follows. *B. subtilis* mutant JH422 contains very low dihydrolipoamide dehydrogenase, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complex activities, less than 1% those of the wild-type strain (Hoch & Coukoulis, 1978). We confirmed this and in addition found that the branched-chain 2-oxo acid dehydrogenase complex activity was similarly low. Pyruvate-, 2-oxoglutarate- and 2-oxoisovalerate-driven E1 activities (dichlorophenol-indophenol reduction) were present in amounts similar to those measured in the wild-type. All three 2-oxo acid dehydrogenase complex activities were restored by addition of purified E3 isolated from *B. subtilis* mutant 61141R (Hodgson *et al.*, 1983). This strongly suggests that a single type of E3 subunit, coded for by a single gene, is shared by all three dehydrogenase complexes.

Other B. subtilis mutants

B. subtilis mutants 61484 and 61494 are derived from mutant 61141 and should contain the same mutation, *ace*A (Willecke & Pardee, 1971; Boudreaux *et al.*, 1981), whereas mutant 61494 contains the marker *bfm*B, which does not affect the branched-chain 2-oxo acid dehydrogenase complex (Boudreaux *et al.*, 1981). We found that cell-free extracts of both mutants 61484 and 61494 contained normal or elevated 2-oxoglutarate dehydrogenase complex activity, elevated E3 activity and lowered pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex activities. The last two activities were cold-labile, and the K_m values for pyruvate and 2-oxoisovalerate in both mutants were raised relative to those in the wild-type. In all these respects, mutants 61484 and 61494 resembled the mutant that we have designated 61141R (Hodgson *et al.*, 1983). A further resemblance was that all three mutants showed a partial independence of acetate for growth on glucose-based minimal medium (Hodgson *et al.*, 1983).

Growth studies on wild-type and mutant strains of B. subtilis

The experiments presented above suggest that the inability of *ace* mutant 61142 to grow on leucine is due to the defect in its branched-chain 2-oxo acid dehydrogenase complex. If this were the sole reason, it might be expected that the organism would grow on compounds that yield acetate through pathways that do not involve the pyruvate dehydrogenase or branched-chain 2-oxo acid dehydrogenase complex reactions. However, we found that mutant 61142 was unable to grow on acetoacetate, isovalerate, palmitate, stearate, leucine, valine or isoleucine (all at 10mM) either as sole carbon source or added as supplement to glucose. The wild-type strain *trp*C2

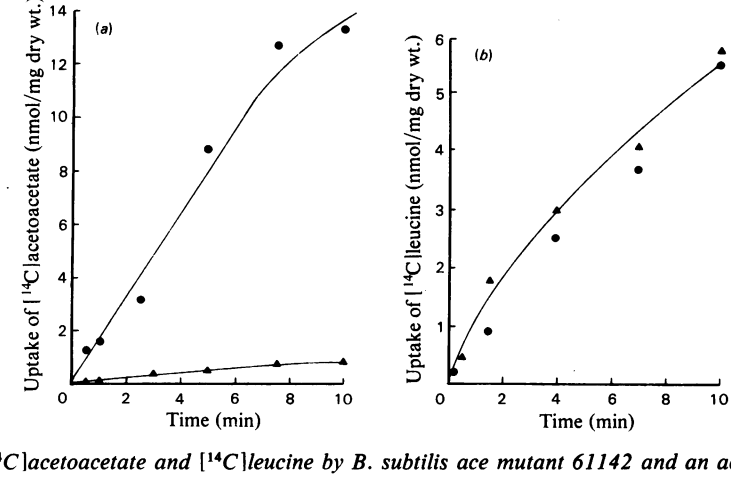

Fig. 2. *Uptake of [^{14}C]acetoacetate and [^{14}C]leucine by B. subtilis ace mutant 61142 and an ace$^+$ revertant of mutant 61142*

B. subtilis mutant 61142 or an *ace*$^+$ revertant of mutant 61142 were incubated with either 580μM-[3-^{14}C]acetoacetate (*a*) or 67μM-[U-^{14}C]leucine (*b*). Cells were prepared and uptake of radiolabel was measured as described in the Materials and methods section. Uptake with *ace* mutant 61142 (▲) and *ace*$^+$ revertant (●) is expressed as nmol of radiolabelled substrate per mg dry wt. of bacterial cells.

and the *ace*$^+$ revertants of mutant 61142 grew on these media. That the mutant 61142 did not grow on any of the branched-chain amino acids is not surprising, since a single branched-chain 2-oxo acid dehydrogenase complex is thought to be involved in the oxidative decarboxylation.

We also noticed that much lower concentrations of acetate as sole source were needed to sustain growth of *ace*$^+$ revertants of mutant 61142 compared with growth of *ace* mutant 61142. This suggested that the inability of *ace* mutant 61142 to grow on long- and short-chain fatty acids might be due to some problem in transport. Direct evidence for that explanation is shown in Fig. 2. An *ace*$^+$ revertant of mutant 61142 could rapidly take up externally added [^{14}C]acetoacetate, whereas *ace* mutant 61142 could not (Fig. 2*a*). However, the uptake of [^{14}C]leucine, an active transport process, was very similar in both *ace* mutant 61142 and the *ace*$^+$ revertant (Fig. 2*b*).

Discussion

We have been unable to distinguish the pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes of *B. subtilis* by genetic or chemical techniques, and the simplest explanation is that the complexes are one and the same, although it should be recognized that our results do not exclude the possibility that specialized E1 subunits of apparently identical M_r are responsible for the initial binding of each substrate. A few preliminary experiments suggested that the complexes are also identical in *B. stearothermophilus*. Namba *et al.* (1969) described a partial purification of a branched-chain 2-oxo acid dehydrogenase complex from *B. subtilis* and distinguished the enzyme from the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities of *B. subtilis*. All our results indicate that the pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes at the very least share many structural components. It remains possible that Namba *et al.* (1969) were examining a quite distinct enzyme system, since the activities that they describe, even after a 40-fold purification, were lower than those that we found in the cell-free extract of *B. subtilis trp*C2. We think it unlikely that we have missed another branched-chain 2-oxo acid dehydrogenase complex activity in our cells. In the cell-free extracts of mutant 61142 we could detect no branched-chain 2-oxo acid dehydrogenase complex activity in the total absence of pyruvate dehydrogenase complex activity. On the other hand, we do agree with Namba *et al.* (1969) that the branched-chain 2-oxo acid dehydrogenase and 2-oxoglutarate dehydrogenase complexes are distinct, since only the latter is cold-labile (Table 1).

Our results with *B. subtilis* mutant JH422 confirm the suggestion of Tu & Kaneda (1976) that the E3 subunits of the branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase complexes are common. However, Tu & Kaneda (1976) also reported that the mutation in the E3 subunits that they found caused no loss of 2-oxoglutarate dehydrogenase complex activity. In contrast, Hoch & Coukoulis (1978), using mutant JH422, showed that this more extreme lesion in E3 abolished pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complex activities. Our own results support those of Hoch & Coukoulis (1978) and suggest that the incomplete inhibition of E3 in the mutation studied by Tu & Kaneda (1976) was responsible in some way for the retention of 2-oxoglutarate dehydrogenase activity.

Our conclusion is that a single E3 gene codes for the dihydrolipoamide dehydrogenase needed in the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase activities of *B. subtilis*. The dihydrolipoamide dehydrogenase of *E. coli* pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (there is no branched-chain 2-oxo acid dehydrogenase complex) is coded for by a single structural gene (Guest & Creaghan, 1973; Perham *et al.*, 1978). Similarly, there appears to be only one dihydrolipoamide dehydrogenase associated with the distinct pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex activities of mammalian mitochondria (Pettit *et al.*, 1978; Danner *et al.*, 1979), although conformational isomers of the enzymes may exist (Williams, 1976). On the other hand, Sokatch *et al.* (1981) have reported that in *Pseudomonas putida* there are two different dihydrolipoamide dehydrogenases, one for the inducible branched-chain 2-oxo acid dehydrogenase complex and the other for the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes. This appears to be the only instance of functionally different dihydrolipoamide dehydrogenases occurring in the same organism. Whether there are two *lpd* genes remains to be determined.

B. subtilis mutant 61484 (genotype *ace*A, *bfm*A, *met*C7, *trp*C2) was first isolated by Willecke & Pardee (1971) and was obtained by mutagenesis of mutant 61141 (genotype *ace*A, *met*C7, *trp*C2) described by Freese & Fortnagel (1969). It was shown to be deficient in branched-chain 2-oxo acid dehydrogenase (*bfm*A) and pyruvate dehydrogenase (*ace*A) complex activities (Willecke & Pardee, 1971), and there was a later report by Boudreaux *et al.* (1981) that they were unable to separate the *bfm*A and *ace*A mutations by transduction. The mutant 61141 that we obtained from the *Bacillus* Genetic Stock Center and separately as a kind gift

from Dr. Freese in fact elaborates a functional pyruvate dehydrogenase complex, although the E1 component exhibits cold-lability of binding to E2E3 and has a K_m for pyruvate raised from 0.4mM to 4.3mM (Hodgson *et al.*, 1983). We believe it to represent a partial revertant of mutant 61141 as originally described (Freese & Fortnagel, 1969), and we designated it 61141R [Hodgson *et al.* (1983), and see above]. In the light of the experiments reported in the present paper, the inability to resolve the *bfm*A and *ace*A mutations is readily explained by the overlap of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase activities in a single complex. The symbols *bfm*A, *ace*B and *ace*A may very well be synonymous.

Some years ago, one of us (Perham, 1975) coined the term 'principle of parsimony' to express the multiple use of common subunits in different structures. Many examples can now be cited (Perham *et al.*, 1978), including the use of a common polypeptide chain in the various 2-oxo acid dehydrogenase complexes. The pyruvate dehydrogenase complex of *B. subtilis* exemplifies this principle to its limit, since it doubles up as the branched-chain 2-oxo acid dehydrogenase complex of the organism. In other genera, these complexes appear to be distinct, presumably so that they can be separately controlled. There may be a rationalizing explanation in the fact that, in *Bacillus*, terminally methyl-branched fatty acids with 14 to 18 carbon atoms in the chain provide the major fatty acid component (60–90%) of the cell membranes (Kaneda, 1973). These fatty acids are synthesized from branched short-chain fatty-acyl-CoA molecules, which are in turn produced by oxidative decarboxylation of the 2-oxo acids obtained by transamination of leucine, isoleucine and valine (Kaneda, 1973; Namba *et al.*, 1969). It has been suggested that the long-chain branched fatty acids are essential for the maintenance of the fluidity of the cell membrane at low temperatures and are therefore important for survival (Willecke & Pardee, 1971). A direct effect on the *B. subtilis* cell membrane of a deficiency in the branched-chain 2-oxo acid dehydrogenase complex activity dehydrogenase complex activity is illustrated in Fig. 2, where the *ace* mutant 61142 cannot rapidly take up acetoacetate. In these terms, a bifunctional pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complex can be seen as an economical means of supplying two different sets of metabolites that are always essential to the cell.

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