Flux partitioning in the split pathway of lysine synthesis in *Corynebacterium glutamicum* 
Quantification by $^{13}$C- and $^1$H-NMR spectroscopy

Kirsten SONNTAG, Lothar EGGEILING, Albert A. DE GRAAF and Hermann SAHM
Institut für Biotechnologie, Forschungszentrum Jülich, Germany
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The Gram-positive *Corynebacterium glutamicum* has the potential to synthesize L-lysine via a split pathway, where amino-ketopimelate is converted to the ultimate lysine precursor diaminopimelate either by reactions involving succinylated intermediates, or by one single reaction catalysed by d-diaminopimelate dehydrogenase. To quantify the flux distribution via both pathways, $^{13}$C-enriched glucose was used and specific enrichments in lysine and in pyruvate-derived metabolites were determined by $^{13}$C- and $^1$H-NMR spectroscopy. Using a system of linear equations, the contribution of the d-diaminopimelate dehydrogenase pathway was determined to be about 30% for the total lysine synthesized. This was irrespective of whether lysine-accumulating mutants or the wild-type strain were analysed. However, when the distribution was determined at various cultivation times, the flux partitioning over the dehydrogenase pathway in a producing strain decreased from 72% at the beginning to 0% at the end of lysine accumulation. When ammonium sulphate was replaced by the organic nitrogen source glutamate, the ammonium-dependent d-diaminopimelate dehydrogenase pathway did not contribute to total lysine synthesis at all. Additional experiments with varying initial ammonium concentrations showed that in *Corynebacterium glutamicum* the flux distribution over the two pathways of lysine synthesis is governed by the ammonium availability. This is thus an example where an anabolic pathway is directly influenced by an extracellular medium component, probably via the kinetic characteristics of d-diaminopimelate dehydrogenase.

In *Corynebacterium glutamicum* lysine is synthesized either via the dehydrogenase variant or the succinylase variant of the diaminopimelate pathway (Fig. 1) [1, 2]. This is thus an unusual example of a split anabolic route. Split and convergent routes are usually reserved to catabolic processes where they occur to yield central metabolites like, for example, pyruvate or oxaloacetate [3]. Due to this peculiar pathway of lysine synthesis in *C. glutamicum* and also the fact that lysine is produced with this bacterium on an industrial scale [4], quantification of the use of both pathways has been attempted. With $^{13}$C-glucose as substrate, and several assumptions on total carbon flow in the cell, the use of the dehydrogenase variant was calculated to contribute 30–40% to total lysine synthesis [5]. In the second study devoted to this question, strains with altered metabolism were constructed and analysed [2], and it was concluded that the dehydrogenase variant contributes 55–75% to lysine synthesis. The two values are apparently in contradiction. They were calculated from indirect measurements and for different strains. Therefore, we here quantify the flux distribution by the use of $^{13}$C- and $^1$H-NMR spectroscopy. In similar studies with *C. glutamicum*, both on lysine and on glutamate production, $^{13}$C enrichments were determined only in the end products (lysine or glutamate) and then used for flux modelling [6, 7]. The essential element of the present analysis is the determination of $^{13}$C enrichments in metabolites before splitting of the diaminopimelate pathway, in addition to determination in the final product lysine. From these data and a generated simple linear equation, unequivocal flux distributions were determined.

**EXPERIMENTAL PROCEDURES**

**Strains and growth conditions**

The wild type of *Corynebacterium glutamicum* (ATCC 13032), its two lysine-producing mutants MH 20-22B [8] and DG 52-5 [9] and AS72 (derived from DG 52-5 lacking diaminopimelate dehydrogenase (Ddh) [2] were used. They were inoculated into 100 ml brain-heart infusion (Difco, Detroit, USA) as a preculture medium. Cells were grown for 16 h at 30°C on a reciprocal shaker (140 rpm), centrifuged (10 min, 4500 × g) and resuspended in 130 mM NaCl to the original volume; 1 ml of this suspension was inoculated into 60 ml minimal medium CgX [10]. Strain MH20-22B was grown on the same medium except that the ammonium sulfate concentration was increased to 40 g/l, and that 0.35 g/l L-leucine was included. The glucose used was [6-13C]glucose (99.9% atom enrichment) from Omicron, USA.
NMR spectroscopy

All spectra were run on a Bruker AMX-400 WB spectrometer operating at a frequency of 100.6 MHz for $^{13}$C, using a 10-mm broadband probe head for $^{13}$C-NMR and a 5-mm inverse probe head for $^1$H-NMR. For $^{13}$C-NMR spectroscopy, 3 ml culture supernatant or extract was mixed with 0.45 ml D$_2$O. Sorbitol (50 mM) was used as an internal standard. Proton-decoupled $^{13}$C-NMR spectra were obtained with the following spectral parameters: 90° pulses, 19.5 kHz spectral width, 12.6 s repetition time, 4000 accumulations and 16 K data points. Field stabilization was achieved by locking on D$_2$O frequency.

For $^1$H-NMR. 2 mM sodium 3-trimethylsilyl-(2,2,3,3-$^3$H$_4$)propionate was added to each sample as an internal standard. Spectral parameters were as follows: 90° pulses, 6 kHz spectral width, 16 K data points, 30 s repetition time, 2 s presaturation time of the water signal and 40 accumulations. For each sample two $^1$H-NMR spectra were run: one without and a second with broad band $^{13}$C-decoupling using the GARP composite pulse decoupling scheme.

For the determination of $^{13}$C enrichments from $^{13}$C spectra, signal areas of each carbon atom were determined by integrating the corresponding peak. Afterwards lysine, alanine or valine (50 mM, with 1.1% natural-abundance $^{13}$C) was added to the sample, a second spectrum was run and signal areas were again integrated. Calculation of the $^{13}$C enrichments followed Eqn (1):

$$^{13}\text{C atom } \% = \frac{A_i}{A_i - A_1} \times \frac{N}{c_{as}}$$ (1)

where $A_i$ is the peak area of carbon atom in the first spectrum, $A_1$ the peak area of carbon atom in the second spectrum, $N$ the natural-abundance $^{13}$C of added standard (0.55 mM), and $c_{as}$ the millimolar concentration of the amino acid under consideration in the sample, as determined by HPLC.

When using $^1$H-NMR spectroscopy, $^{13}$C enrichments were calculated from the ratio of the satellite/centre peak area of each proton [7]. In the non-decoupled $^1$H spectrum, signals of the $^{13}$C-bound protons appear symmetrically as satellite signals around the signal of $^{13}$C-bound protons. In the $^{13}$C-decoupled $^1$H spectrum, signals of $^{13}$C protons appear at the same position as signals of $^{12}$C-bound protons: thus no $^{13}$C satellites are present. Therefore, subtracting the $^{13}$C-decoupled spectrum from the non-decoupled spectrum allowed us to quantify the $^{13}$C satellite signal areas very accurately without any interference from the base line or background signals from $^{12}$C-bound protons (Fig. 2). The enrichments were calculated according to Eqn (2):

$$^{13}\text{C atom } \% = \frac{\text{signal area }^{13}\text{C-H}}{\text{signal area }^{13}\text{C-H} + \text{signal area }^{13}\text{C-H}}$$ (2)

RESULTS

Development of flux distribution model

Fig. 3 shows a scheme of the carbon distribution of the lysine building blocks aspartate and pyruvate within the dehydrogenase and succinylase variant of the diaminopimelate pathway. The succinylase variant has L,L-diaminopimelate as intermediate with a C-2 rotational axis of symmetry. Therefore, two physically different D,L-diaminopimelate species arise in the following epimerase reaction if specifically lab-
Fig. 2. Proton NMR spectra showing the signals of H6 of lysine derived from C. glutamicum strain MH20-22B after incubation with [6-13C]glucose. (A) Non-13C-decoupled; (B) with broadband 13C decoupling; (C) difference spectrum obtained after subtracting B from A. Asterisks indicate the positions of the 13C satellite signals. Signals marked X stem from impurities in the lysine sample and partly overlap with one of the 13C satellites of H6. In the difference spectrum C, the signals X are removed except for small dispersion-like residuals due to different lineshapes in A and B that integrate to zero. The baseline offset, clearly visible in A and B, is also completely removed in C.

elld substrates are used. In contrast, in the dehydrogenase variant only one of these species arises due to label retention by D-diaminopimelate dehydrogenase activity [12]. On the premises of epimerization of either chiral centre of L,L-diaminopimelate to the D-configuration, the 13C enrichment in each carbon atom of lysine resulting from the labelling in the precursors aspartate and pyruvate can be modeled as a function of the flux partitioning f of the dehydrogenase pathway.

\[ L_2 = \frac{1}{2} (1 + f) A_2 + \frac{1}{2} (1 - f) P_2 \]  
\[ L_6 = \frac{1}{2} (1 + f) P_2 + \frac{1}{2} (1 - f) A_2 \]

where \( L_2 \) and \( L_6 \) are the enrichments in C-2 and C-6 of lysine, \( A_2 \) the enrichment in C-2 of aspartate and \( P_2 \) the enrichment in C-2 of pyruvate. Solution of Eqs (3) and (4) yields

\[ f = \frac{L_6 - L_2}{2 \times P_2 - L_6 - L_2} \]

It is thus possible to calculate directly the contribution of the dehydrogenase variant from the measured parameters \( P_2 \), \( L_2 \), and \( L_6 \).

Fig. 3. Scheme of carbon atom distribution from aspartate and pyruvate in lysine produced by both variants. \( A_1 \) are the 13C enrichments in aspartate carbons \( A_1 \) and \( P_1 \) those in pyruvate. The lysine species \( A \) is formed when only the dehydrogenase variant operates, whereas when only the succinylase variant operates an equimolar mixture of species \( A \) and \( B \) is formed.

Assessment of the validity of the flux distribution model

To assess whether total epimerization of either chiral centre of L,L-diaminopimelate to the D-configuration actually occurs, we took advantage of mutants of C. glutamicum with inactivated D-diaminopimelate dehydrogenase [2]. Strain 52-5 Ddh+ (AS72) was grown with 200 mM 50% enriched [6-13C]glucose and, after 48 h of incubation, the specific enrichment in carbon atoms of lysine accumulated was determined using 13C-NMR. The resulting 13C enrichments are shown in Table 1 (first line). Values are accurate to about 5%. Within this experimental error, C-2 and C-6 have identical labelling, as have C-3 and C-5. This symmetrical labelling around C-4 is proof that indeed an equal mixture of the two possible lysine species within the succinylase variant was
Table 1. Contribution of the dehydrogenase variant for the total lysine synthesized with different strains of *C. glutamicum*. Determination of the $^{13}$C enrichment in C-5 of lysine ($L_5$) and alanine ($A_2$) was achieved by $^1$H and $^{13}$C-NMR spectroscopy. The partitioning coefficient $f$ was calculated from $L_2$, $L_6$, and $A_2$. n.d., not determined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$L_2$</th>
<th>$L_5$</th>
<th>$L_6$</th>
<th>$L_7$</th>
<th>$L_8$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>$f$</th>
</tr>
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<tr>
<td>DG 52-5 Ddh$^+$</td>
<td>3.8</td>
<td>7.6</td>
<td>29.7</td>
<td>9.8</td>
<td>31.7</td>
<td>7.9</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>MH 20-22B</td>
<td>4.9</td>
<td>7.2</td>
<td>23.7</td>
<td>8.4</td>
<td>24.9</td>
<td>4.9</td>
<td>2.7</td>
<td>25.3</td>
</tr>
<tr>
<td>DG 52-5</td>
<td>4.0</td>
<td>5.4</td>
<td>19.4</td>
<td>4.2</td>
<td>19.3</td>
<td>3.6</td>
<td>1.7</td>
<td>20.2</td>
</tr>
<tr>
<td>ATCC 13032</td>
<td>n.d.</td>
<td>10.3</td>
<td>22.8</td>
<td>5.8</td>
<td>23.9</td>
<td>8.0</td>
<td>4.2</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Fig. 4. Non-$^{13}$C-decoupled proton NMR spectrum of lysine derived with $[6^{-13}$C]glucose. Assignments were confirmed with H, H COSY 2D NMR spectroscopy (not shown). Asterisks indicate the positions of the $^{13}$C satellite signals.

Formed, and a prerequisite for calculating the partition coefficient $f$ (Fig. 3) when both variants operate.

Another prerequisite is the determination of $^{13}$C enrichments in the carbon atoms of either aspartate or pyruvate. Since these intermediates are only found in limited amounts in culture fluids [9], we undertook label determination in alanine derived directly from pyruvate. In a culture of strain DG 52-5, 12 mM alanine was found, with 3.0% $^{13}$C enrichment at C-2 and 24.6% at C-3 determined. In addition, excreted valine arising from the condensation of two pyruvate molecules was also analysed in this culture. The specific enrichments determined were 3.2% at C-2 and C-3 (both equivalent to C-2 of alanine) and 24.3% at C-4 and C-5 (equivalent to C-3 of alanine). Similar determinations were made for other cultivations (not shown), justifying the use of either of these metabolites for specific label determination in pyruvate.

Flux partitioning in lysine producers

The lysine-producing strain *C. glutamicum* MH20-22B was grown with 525 mM $[13$C$]$glucose (50% enriched) for 72 h. It accumulated 210 mM lysine and 1 mM alanine. The $^{13}$C spectrum was recorded directly from the culture fluid. In addition, the $^1$H-NMR spectrum (with and without decoupling) of the isolated lysine and alanine was recorded (Fig. 2). Quantification by both $^1$H- and $^{13}$C-NMR gave identical values, and the enrichments determined are given in Table 1. Using Eqn (5), $f$ was calculated as 0.33. This means that the dehydrogenase variant contributes only 33% to the formation of total lysine accumulated, and the succinylase variant the remaining 67%. In addition to the *C. glutamicum* strain MH20-22B, strain DG 52-5 was also analysed, which has a lower capacity to accumulate lysine [2, 9]. When grown with 200 mM $[13$C$]$glucose (50% enriched) it accumulated 29.2 mM lysine and 9.8 mM alanine. The quantitative analysis of the $^{13}$C enrichments showed that, in this strain, 32% of the accumulated lysine was synthesized over the dehydrogenase variant.

In previous experiments we obtained a reduction of the lysine yield by 55–75% with Ddh$^-$ mutants [2], and concluded that it is this part of lysine which is synthesized over the dehydrogenase variant in the corresponding Ddh$^+$ strains. We therefore determined the flux partitioning and lysine yield in strain DG 52-5 using NMR under conditions as used by Schrumpf et al. [2]. The flux partitioning was 33% for both conditions with comparable lysine yields (not shown). The Ddh$^-$ mutant (AS72) was grown in parallel and 5.1 mM lysine found for the conditions given by Schrumpf et al., but 19.7 mM under present conditions. This shows that cultivation conditions have a great influence on final lysine accumulation with the Ddh$^-$ mutant, which therefore cannot be used to reach any conclusions concerning flux partitioning in the parent strain.

Flux partitioning in the wild type

Since the wild type of *C. glutamicum* excretes only very limited amounts of lysine (0.02 mM), it was necessary to use cytosolic amino acids for specific enrichment determinations. *C. glutamicum* ATCC 13032 was grown identically as de-
Table 3. Nitrogen-dependent flux partitioning in C. glutamicum. Strain DG 52-5 was grown on minimal medium where ammonium was replaced by 140 mM glutamate, strain MH20-22B at varying concentrations of ammonium [NH₄] sulphate. Aₓₓ is the total absorbance at 600 nm to measure growth at the time of harvest; all other abbreviations as for Table 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>[NH₄]</th>
<th>[Lysine]</th>
<th>Growth</th>
<th>L₂</th>
<th>L₆</th>
<th>Aₓₓ</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>DG 52-5</td>
<td>(140 Glu)</td>
<td>4.6</td>
<td>22.0</td>
<td>6.5</td>
<td>6.0</td>
<td>n.d.</td>
<td>0</td>
</tr>
<tr>
<td>MH 20-22B</td>
<td>600</td>
<td>4.3</td>
<td>11.4</td>
<td>13.4</td>
<td>7.1</td>
<td>4.0</td>
<td>51 ± 3</td>
</tr>
<tr>
<td></td>
<td>150</td>
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<td>14.1</td>
<td>11.1</td>
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<tr>
<td></td>
<td>38</td>
<td>3.7</td>
<td>14.2</td>
<td>10.8</td>
<td>10.0</td>
<td>3.6</td>
<td>6 ± 3</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>1.9</td>
<td>11.6</td>
<td>11.7</td>
<td>11.9</td>
<td>n.d.</td>
<td>0</td>
</tr>
</tbody>
</table>

scribed for strain DG 52-5 but, at the end of the exponential growth phase, lysine and alanine were isolated from a cyto-
solic preparation. The ¹³C enrichments determined are given in Table 1, showing a 24% contribution of the dehydrogenase variant for lysine synthesis at the time of harvesting.

Flux partitioning as a function of culture time

During batch cultivation, cells pass various growth phases and physiological stages, as is especially evident when metabolites are excreted [8]. We therefore analysed whether the flux distribution changed with cultivation time. For this purpose C. glutamicum strain MH20-22B was grown with 550 mM [¹³C]glucose (50% enriched), and samples were taken to determine the specific enrichments at five time points (and for amino acid determination as well). Fig. 4 shows an illustrative example of an H-NMR spectrum of lysine obtained after 48 h of cultivation. The collected data are given in Table 2. Apparently, the calculated partition coefficients f (in this case f₂) for the total lysine present at the various time points decreased from 72% to 33%. Although these values indicate considerable changes in the use of both variants of lysine synthesis with cultivation time, they reflect the cumulative changes of both variants in accumulating lysine at each time point. Therefore, the instantaneous partition coefficients f were estimated from graphs constructed (not shown) for the relative rates of formation of lysine synthesized via the dehydrogenase variant and lysine synthesized via the succinylase variant. As can be seen in Table 2 (last column), the use of the dehydrogenase variant f changed dramatically from 72% at the beginning to zero at the final stage of lysine formation, with the largest part being synthesized via the succinylase variant.

Flux partitioning as a function of ammonium availability

The unexpected finding of dynamic changes in the flux distribution led to the question of whether ammonium availability might govern the in vivo flux distribution. The reasons are that, in the culture medium, ammonium is finally almost quantitatively fixed in cells and lysine [8], and that it is also a direct substrate of the d-diaminopimelate dehydrogenase with low affinity towards ammonium [12]. Consequently, strain DG 52-5 was grown with an organic nitrogen source, namely L-glutamate, where it can be expected that no free (or only limited) ammonium is available within the cell. The resulting ¹³C enrichments at C-2 and C-6 of the lysine produced were identical (Table 3), as was the case for lysine synthesized by the Ddh⁻ strain of this strain (Table 1). Therefore, under these conditions the dehydrogenase variant did not contribute to lysine synthesis. In a second experiment the ammonium concentration was directly varied and four different initial concentrations assayed for their effect on f in strain MH20-22B. In this experiment cultures contained only 74 mM 99% enriched [¹³C]glucose. Samples were taken after a short cultivation time of 7 h to reduce the cumulative changes and this explains the very low lysine concentrations. Table 3 shows the ¹³C enrichments determined and the calculated partition coefficients. Use of the dehydrogenase variant was clearly related to the external ammonium concentration, with very limited or even no use below the initial concentration of 38 mM.

DISCUSSION

Coryneform bacteria such as Corynebacterium glutamicum have the potential to produce large quantities of amino acids. These bacteria have therefore been attractive objects for metabolite flux analysis by NMR spectroscopy. Using [¹³C]-NMR, intracellular and extracellular metabolites were quantified in C. glutamicum ssp. flavum producing lysine from unlabelled as well as [¹³C]-enriched glucose [7, 13]. Enriched [¹³C]glucose was also used in a study of glutamate formation with the coryneform Microbacterium ammoniaphilum [6]. In the latter study specific ¹³C enrichments were determined in excreted glutamate, which enabled these authors to calculate that the major fraction of glutamate was formed via glycolysis, phosphoenolpyruvate carboxylase and the first third of a turn of the Krebs cycle.

The present work is restricted to the relatively short reaction sequence of lysine formation in C. glutamicum from aspartate plus pyruvate. Originally it was assumed that these reactions only proceed via succinylated intermediates of the diaminopimelate pathway [14] which, together with the non-symmetrical ¹³C-labelling found in lysine [15], led to the suggestion that channelling of intermediates might occur. However, the present experiment with the C. glutamicum Ddh⁻ strain shows that, if the succinylase variant is exclusively used, the expected symmetrical labelling pattern in lysine results, indicating that no channelling occurs. This was the key prerequisite for our flux model to be used for unequivocal determinations of the flux distribution. With C. glutamicum strain DG 52-5 and MH20-22B, 32% of the total accumulated lysine was found to be synthesized over the dehydrogenase variant. This compares well with the value of 30–40% calculated for a different lysine overproducer [5]. Therefore, most of the lysine excreted by the different strains is synthesized via the succinylase variant. One could thus argue that, in the strains that synthesize lysine for overpro-
duction plus lysine for cellular demands, use of the dehydrogenase variant is a result of increased total flux. However, the analysis of the C. glutamicum wild type (with f = 24%) conclusively shows that this is not the case. Moreover, due to the fact that we determined the $^{13}$C enrichments in lysine and alanine in the cytosolic fraction at one time point during cultivation, we determined the instantaneous use of the dehydrogenase variant. This proves that, even in the wild type, which is not as severely influenced in total metabolite flow as overproducers (which have mutations in feedback-regulated enzymes [10, 16]) or altered export mechanisms [8, 17], both the succinylase variant and the dehydrogenase variant operate jointly.

We previously found that the Ddh$^{-}$ strains of C. glutamicum DG 52-5 and MH20-22B accumulated less lysine than their parent strains [2]. It was assumed that the reduced amount of lysine reflects that part which is synthesized via the dehydrogenase variant. However, the present NMR analysis, which is not dependent on specifically constructed strains and indirect conclusions, shows that this assumption is invalid. The reduced amount obtained with the Ddh$^{-}$ strains must reflect a secondary effect of the Ddh$^{-}$ mutation, which only becomes apparent under specific cultivation conditions. This has to be further investigated.

Most interesting is the result that f decreased with increasing cultivation time. We confirmed by two independent experiments that ammonium is related to this changing of flux distribution. In the experiment where ammonium was replaced by glutamate, the free ammonium concentration in the cell can be assumed to be extremely low, since the amino group of glutamate is transferred via transamination to other substrates [3]. In addition, it has been shown for C. glutamicum ssp. flavum that, during growth with glutamate as nitrogen source, the activities of the nitrogen-scavenging system, consisting of glutamine synthetase and glutamate synthase, are increased more than tenfold [18]. The two activities of the scavenging system are also increased in C. glutamicum [18] and Escherichia coli [19] at extracellular concentrations of ammonium sulfate below 1 mM. In the second experiment, where we used initial ammonium concentrations over an extreme range of 600–38 mM, the dehydrogenase variant was preferably used for lysine synthesis at the higher concentrations. Since it is assumed that the non-protonated form of ammonium ($NH_3$) diffuses rapidly through the membrane into the cell, whereas the protonated form ($NH_4^+$) prevails [20], it can be assumed that the intracellular ammonium concentration is also high. Therefore, it is most likely that the ammonium concentration directly influences the flux distribution between the succinylase and dehydrogenase variants by increasing the in vivo activity of the diaminopimelate dehydrogenase. This enzyme has a low affinity ($K_m = 36$ mM) for ammonium [21], which explains the sensitivity of f over the large range of ammonium concentrations assayed. Of course, a prerequisite for these considerations is that the enzyme activities of the succinylase and dehydrogenase variant are not regulated. A regulation in catalytic activity or expression is not known for any of the five respective enzymes. Even during growth on different media or at different cultivation times no substantial change in enzyme activities has been found [8, 22]. Taken together, all data are consistent with the flux distribution over both variants being principally governed by the ammonium availability for the dehydrogenase reaction. In contrast to a genetic regulation of nitrogen-metabolizing systems [18, 19], it is thus a kinetic effect in response to the ammonium availability. Whether one of the variants represents a specific need for the cell cannot be answered, but, as is known [2], at least the dehydrogenase variant is in principle dispensable for the cell, though its absence has unknown consequences.

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REFERENCES


