Anaerobic 2-ketogluconate metabolism of *Klebsiella pneumoniae* NCTC 418 grown in chemostat culture: involvement of the pentose phosphate pathway

JOOST A. SIMONS, JACKY L. SNOEP, SJOERD FEITZ, M. JOOST TEIXEIRA DE MATTOS and OENSE M. NEIJssel*

Department of Microbiology, Biotechnology Centre, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands

(Received 21 October 1991; accepted 25 November 1991)

Under anaerobic 2-ketogluconate-limited growth conditions \( (D = 0.1 \text{ h}^{-1}) \), *Klebsiella pneumoniae* NCTC 418 was found to convert this carbon source to biomass, acetate, formate, \( \text{CO}_2 \), ethanol and succinate. The observed fermentation pattern is in agreement with the simultaneous functioning of the pentose phosphate pathway and the Entner–Doudoroff pathway in 2-ketogluconate catabolism. When cultured at \( \text{pH} \ 8.0 \) apparent \( Y_{\text{ATP}} \) values were lower than those found at culture \( \text{pH} \ 6.5 \). This difference can be explained by assuming that at high culture \( \text{pH} \) values approximately 0.5 mol ATP was invested in the uptake of 1 mol 2-ketogluconate. Sudden relief of 2-ketogluconate-limited conditions led to lowering of the intracellular NADPH/NADP ratio and (possibly as a result of this) to inhibition of biosynthesis. Whereas production of ethanol stopped, lactate was produced at high rate. This product was formed, at least partly, via the methylglyoxal bypass.

**Introduction**

Organisms need to balance their NAD(P)H-producing and NAD(P)H-consuming reactions. In the absence of oxygen or other exogenous electron acceptors this can be achieved by internal redox reactions that result in the excretion of products of the same degree of reduction as the substrate (e.g. lactate production from glucose) or by producing, in a strict ratio, compounds that are more oxidized and compounds that are more reduced than the substrate. For example, under carbon-limited conditions fermentation of glucose by *Klebsiella pneumoniae* results in the production of acetate and ethanol in a ratio of 1:1 (Teixeira de Mattos & Tempest, 1983).

Under aerobic conditions, *K. pneumoniae* NCTC 418 is able to convert 2-ketogluconate intracellularly to 6-phosphogluconate by the combined action of an NADPH-dependent 2-ketogluconate reductase and gluconate kinase (Simons et al., 1991). 6-Phosphogluconate can be further catabolized via the Entner–Doudoroff pathway. In this way, 2-ketogluconate is converted to pyruvate without net NAD(P)H production (Fig. 1). However, when ammonia is used as nitrogen source, reducing power will be required for the production of biomass (approximate ‘molecular formula’ \( \text{C}_4\text{H}_7\text{O}_2\text{N} \): Herbert et al., 1971) from pyruvate:

\[
\frac{4}{3}\text{C}_3\text{H}_4\text{O}_3 + 1\text{NH}_3 + \frac{4}{3}[2\text{H}] \rightarrow \text{C}_4\text{H}_7\text{O}_2\text{N} + 2\text{H}_2\text{O} \ (1)
\]

Therefore, anaerobic growth on 2-ketogluconate will not be possible if the Entner–Doudoroff pathway is the sole catabolic route and yet we succeeded in growing *K. pneumoniae* anaerobically on this carbon source.

To investigate how this organism generates reducing equivalents necessary for biosynthesis, we grew *K. pneumoniae* NCTC 418 anaerobically in chemostat culture under 2-ketogluconate-limited growth conditions. The effect of the culture \( \text{pH} \) value on 2-ketogluconate metabolism and on the activities *in vitro* of several enzymes possibly involved in 2-ketogluconate metabolism was studied. Furthermore, we report on the effects of a sudden increase in the 2-ketogluconate concentration under anaerobic 2-ketogluconate-limited conditions.

**Methods**

*Micro-organism and growth conditions.* *Klebsiella pneumoniae* NCTC 418 was maintained by subculture on tryptic meat-digest agar slopes.
Organisms were cultured in a Porton-type chemostat with a working volume of approximately 500 ml, stirred at about 1000 r.p.m., or in a Multigen chemostat (New Brunswick Scientific) with a working volume of 300 ml (Herbert et al., 1965). In order to obtain 2-ketogluconate-limited growth conditions, simple salts medium was used as specified by Evans et al. (1970), but instead of citrate nitritriacetic acid (2 mM) was used as chelator. The medium contained approximately 50 mM-2-ketogluconate. This carbon source was sterilized by filtration (Seitz filter plates type EKS). The dilution rate \( D \) was set at 0-10 ± 0-01 h\(^{-1}\). The pH value of the culture was maintained automatically at a preset value ± 0.1 pH unit, using sterile 2 M-NaOH, and the temperature was maintained at 35 °C. To prevent excessive foaming silicone antifoaming agent (BDH; 1%, v/v) was added at a rate of approximately 0.5 ml h\(^{-1}\). Anaerobiosis was maintained by the method described by Teixeira de Mattos & Tempest (1983).

**Pulse experiments.** Pulse experiments were performed as described previously (Simons et al., 1991). The initial 2-ketogluconate concentration was approximately 15 mM.

**Preparation of 2-ketogluconate.** 2-Ketogluconate was prepared by a microbiological method in which sodium gluconate was oxidized by a strain of *Pseudomonas putida*, as described previously (Simons et al., 1991).

**Preparation of cell-free extracts.** Cell-free extracts were prepared according to Hommes et al. (1985). The buffer used throughout the preparation was 10 mM-sodium phosphate (pH 6-0) containing 5 mM-MgCl\(_2\), except in the case of preparing cell-free extracts for measuring methylglyoxal synthase activity. In the latter case, cells were washed with and resuspended in demineralized water.

**Enzyme assays.** Glucokinase was assayed according to Bergmeyer et al. (1974a), as modified by Leegwater (1983). 2-Ketoglucurate reductase was assayed as described previously (Simons et al., 1991). Isocitrate lyase activity was measured as described by Dixon & Kornberg (1959). 6-Phosphogluconate dehydrogenase was assayed according to Bergmeyer et al. (1974b) and glucose-6-phosphate dehydrogenase according to Lühr & Waller (1974). Transhydrogenase activities were determined according to Sweetman & Griffin (1971). D-Lactate dehydrogenase was determined as described by Streekstra et al. (1987b). Methylglyoxal synthase was measured as the rate of disappearance of dihydroxyacetone phosphate in the absence of nucleotide cofactors, according to Hopper & Cooper (1971) as modified by Streekstra (1990). Glyoxalase activity was measured in intact cells following the procedure of Cooper & Anderson (1970). The assays were performed at 35 °C with a Beckman DU 40 spectrophotometer. One Unit is defined as the amount of enzyme catalysing the conversion of 1 μmol substrate min\(^{-1}\).

**Measurement of NADPH and NADP levels.** NADPH was extracted from a culture sample with KOH (0-15–0-2 M; extraction pH approximately 12-5). NADP was extracted from a culture sample with HCl (0-15–0-2 M; extraction pH approximately 1-5). Samples were taken from these extraction solutions and kept at room temperature (NADPH) or boiled (NADP) for 10 min. Cell debris was removed by centrifugation (3000 g, 10 min). The acid extract was brought to pH 6-5 and the alkaline extract to pH 7-5. Determination of the nucleotides was based on the microcycling assay for NAD(H) of Bernofsky & Swan (1973) and was performed by adding 500 μl H\(_2\)O, 100 μl glycyglycine (1 M, pH 8-0), 50 μl 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (8-4 mM), 50 μl phenazine ethosulphate (33-2 mM) and 50 μl glucose 6-phosphate (20 mM) to 300 μl of the neutralized extraction solution. After 3 min, 50 μl glucose-6-phosphate dehydrogenase (70 U ml\(^{-1}\)) was added and the rate of MTT reduction was followed spectrophotometrically at 570 nm. Calibration was performed by adding 0, 0-2, 0-5 or 1-0 μM-NADPH or -NADP to the extraction solutions at the beginning of the extraction procedure. The assay was performed at 35 °C with a Beckman DU 40 spectrophotometer.

**Analyses.** Bacterial dry weight was measured by the method of Herbert et al. (1971). Protein was determined according to Gornall et al. (1949). 2-Ketogluconate, acetate, lactate, succinate, formate and ethanol concentrations were determined by HPLC (LKB) with an Aminex HPX 87H organic acids column (Bio-Rad) at a temperature of 65 °C with 5 mM-H\(_2\)SO\(_4\) as eluent, using a 2142 refractive index detector (LKB) and an SP 4270 integrator (Spectra Physics). CO\(_2\) produced by the cultures was determined by passing the gas from the fermenter through a CO\(_2\) analyser (Servomex IR gas analyser PA 404).

**Calculations.** The specific rate of NAD(P)H oxidation was calculated, using equation (1) (see Introduction) and the fermentation scheme given in Fig. 1a, b:

\[
\frac{q_{NADPH}}{q_{NADP}} = \frac{q_{2-ketogluconate} + 4/3q_{biomass} + 2q_{glutamate} + 2q_{ethanol}}{q_{NADP} + q_{2-ketogluconate} + 5/3q_{CO_2} (PP)}
\]

(2)

Assuming that the flux through the pentose phosphate pathway \([\frac{q_{CO_2} (PP)}{q_{NAD(P)}}]\) equals the rate of CO\(_2\) production that cannot be explained by cleavage of formate and production of succinate \([q_{CO_2}(PP) = q_{CO_2} (\text{observed}) + q_{formate} + q_{2-ketogluconate} - q_{ethanol} - q_{NADP} - q_{glucose} \text{ glyceraldehyde 3-phosphate by the gluconeogenic pathway and glucose-6-phosphate dehydrogenase}.\) When lactate is produced via the methylglyoxal bypass, \(q_{NADPH}\) can be calculated by subtracting \(q_{lactate}\) from equation (3). Redox balances (%H) were calculated as \(q_{NADPH}/q_{NADP} \times 100\).

For the calculation of specific rates of ATP production \(q_{ATP}\) it was assumed that an ATP-dependent transhydrogenase, consuming 1 ATP/NADP formed, served to generate the NADPH (necessary for 2-ketogluconate reduction and biomass formation) that was not produced by the action of the pentose phosphate pathway, that is:

\[
\frac{q_{2-ketogluconate} + 4/3q_{biomass} - q_{CO_2} (PP)}{q_{2-ketogluconate} + 4/3q_{biomass} - q_{CO_2} (PP)}
\]

(3)

Thus (see Fig. 1a, b, \(q_{ATP} = \frac{q_{ethanol} - q_{2-ketogluconate} (ED) + 5/3q_{CO_2} (PP) - q_{2-ketogluconate} + 4/3q_{biomass} - q_{CO_2} (PP)}{q_{ethanol} - q_{2-ketogluconate} (ED) - q_{2-ketogluconate} - q_{CO_2} (PP)}\) is the flux through the Entner–Doudoroff pathway. It follows that:

\[
q_{ATP} = \frac{q_{ethanol} - q_{2-ketogluconate} (ED) + 5/3q_{CO_2} (PP) - q_{2-ketogluconate} + 4/3q_{biomass} - q_{CO_2} (PP)}{q_{ethanol} - q_{2-ketogluconate} (ED) - q_{2-ketogluconate} - q_{CO_2} (PP)}
\]

(4)

When lactate is formed via the methylglyoxal bypass \(q_{ATP}\) can be calculated by subtracting \(2q_{lactate}\) from equation (4).

**Results and Discussion**

*Klebsiella pneumoniae* NCTC 418 was found to be able to use 2-ketogluconate as the sole carbon and energy source under anaerobic conditions. Just as under aerobic conditions, cells could convert this carbon source, after it had been taken up, to 6-phosphogluconate by means of an NADPH-dependent 2-ketogluconate reductase and gluconate kinase (Table 1). Since catabolism via the Entner–Doudoroff pathway does not deliver the reducing power necessary for production of biomass from 2-ketogluconate, other pathways must (also) be involved in the anaerobic catabolism of this carbon source.
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Fig. 1. ATP- and NAD(P)H-consuming and producing reactions in the conversion of 2-ketogluconate to pyruvate via the Entner–Doudoroff pathway or the pentose phosphate pathway (a) and in the formation of fermentation products (b).

Table 1. Specific enzyme activities in *K. pneumoniae* NCTC 418, grown anaerobically in chemostat culture under 2-ketogluconate limitation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity*</th>
<th>pH 6.5</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Ketogluconate reductase</td>
<td>3</td>
<td>0.59 ± 0.04</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>Gluconate kinase</td>
<td>3</td>
<td>0.02 ± 0.00</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>3</td>
<td>0.18 ± 0.03</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>3</td>
<td>0.14 ± 0.03</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Transhydrogenase (ATP-dependent)</td>
<td>3</td>
<td>0.01 ± 0.01</td>
<td>&lt;0.01 ± 0.00</td>
</tr>
<tr>
<td>Transhydrogenase (ATP-independent)</td>
<td>3</td>
<td>0.01 ± 0.01</td>
<td>&lt;0.01 ± 0.00</td>
</tr>
<tr>
<td>α-Lactate dehydrogenase</td>
<td>2</td>
<td>0.51 ± 0.03</td>
<td>1.29 ± 0.07</td>
</tr>
<tr>
<td>Methylglyoxal synthase</td>
<td>2</td>
<td>0.18 ± 0.02</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Glyoxalase</td>
<td>2</td>
<td>3.2 ± 0.2</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>

To investigate the anaerobic metabolism of 2-ketogluconate by *K. pneumoniae* NCTC 418, we grew this organism in chemostat culture under 2-ketogluconate-limited conditions. It was found that 2-ketogluconate was converted to biomass, acetate, formate, CO₂, ethanol and succinate (Table 2). This latter product was not formed via the glyoxylate shunt, since no isocitrate lyase activity was detectable in these cells. So, not only was reducing power invested in the production of...
biomass, but also in the formation of ethanol and succinate. If the Entner–Doudoroff pathway was the sole catabolic route, the rate of NAD(P) reduction would equal the rate of 2-ketogluconate consumption (see Fig. 1a). It can be calculated that the actual rate of NAD(P)H oxidation was about twice the rate at which NAD(P) could be reduced by the sole functioning of this pathway in 2-ketogluconate catabolism.

It has been observed for growth of *K. pneumoniae* on a variety of carbon sources that the measured rate of CO₂ production closely agrees with the rate that can be calculated from the fermentation pathways (Teixeira de Mattos & Tempest, 1983; Streekstra et al., 1987a), indicating that the net effect on CO₂ production of CO₂-fixing and -producing steps in biomass formation by this organism is negligible. However, the observed CO₂ production rate for growth on 2-ketogluconate was significantly higher than would be the case if cleavage of formate and production of succinate, respectively, were the only CO₂-producing and -consuming reactions.

An explanation for the apparent discrepancy between the rate of NAD(P) reduction and NAD(P)H oxidation would be that NAD is reduced by anaerobic activity of the pyruvate dehydrogenase complex. Recently, this enzyme system was found to be active under anaerobic conditions in *Enterococcus faecalis* when this organism was grown on pyruvate (Snoep et al., 1990). However, functioning of this enzyme complex in 2-ketogluconate catabolism does not explain the discrepancy between observed and calculated CO₂ production rates, since the CO₂ production accompanying pyruvate oxidation would still equal \( q_{\text{acetate}} + q_{\text{ethanol}} - q_{\text{formate}} \).

A route that results in the generation of both NAD(P)H and CO₂ is the pentose phosphate pathway. Activities *in vitro* of the (NADP-dependent) key enzymes 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase were detectable in cell-free extracts from *K. pneumoniae* that had been grown anaerobically on 2-ketogluconate (Table 1) and were significantly higher than those found under (anaerobic) glucose limitation [pH 6.5, \( D = 0.1 \text{ h}^{-1} \): 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase activities were 0.08 and 0.07 U (mg protein)⁻¹ respectively]. If 2-ketogluconate were catabolized partly by means of the pentose phosphate pathway the ratio of 'extra' NAD(P)H/CO₂ produced would be 5/3 (Fig. 1a). Indeed, at both culture pH values tested this ratio was found to be 1-7 and when it was assumed that the CO₂ production that cannot be explained by cleavage of formate and production of succinate resulted from activity of the pentose phosphate pathway the redox balances were found to be 97% (pH 6.5) and 99% (pH 8.0), respectively (Table 2). Therefore, we conclude that the Entner–Doudoroff pathway and the pentose phosphate pathway function simultaneously in the anaerobic catabolism of 2-ketogluconate. The calculated flux through the latter pathway was, respectively, 42% (pH 6.5) and 66% (pH 8.0) of the total flux of 2-ketogluconate catabolism.

The reason for the importance of this route in the (anaerobic) catabolism of 2-ketogluconate may reside in the fact that *K. pneumoniae* first has to reduce this carbon source in an NADPH-dependent reaction. So, NADPH must be produced at high specific rates to sustain both anabolism and catabolism of 2-ketogluconate, whereas only low specific transhydrogenase activities *in vitro* could be detected in these cells, especially at a high culture pH value (Table 1). Thus, the probable function of this route is to provide NADPH. A consequence of the functioning of the pentose phosphate pathway is that the conversion of 2-ketogluconate to pyruvate is accompanied by net NADH production. Therefore, to maintain redox neutrality, reduced compounds like ethanol and succinate have to be produced. An interesting observation is that the yield on 2-ketogluconate was higher at pH 6.5 than at pH 8.0. This difference cannot be explained by a shift in fermentation pattern, leading to a decrease in the amount of ATP produced per 2-ketogluconate catabolized. On the contrary, it can be calculated that more ATP per 2-ketogluconate was synthesized at high culture pH values (1.9 ATP/KGA at pH 8.0 and 1.7 ATP/KGA at pH 6.5). Thus, as can be seen in Table 2, the apparent \( Y_{\text{ATP}} \) values for cells growing under 2-ketogluconate limitation were lower at high culture pH values. With anaerobic gluconate-limited cultures of *K. pneumoniae* similar observations were made and these were explained by postulating that at high culture pH values 0.5 mol ATP was invested in the uptake of 1 mol gluconate (Streekstra et al., 1987a). This is in agreement with the suggestion that, in *Escherichia coli*, at low pH (5-5) one proton is taken up per mol of (undissociated) organic acid transported, whereas at high pH (7-5) two protons are taken up per mol of acid, one in association with the substrate itself and one in association with the carrier molecule (Ramos & Kaback, 1977). The observed pH dependence of apparent \( Y_{\text{ATP}} \) values for growth of *K. pneumoniae* on 2-ketogluconate is in agreement with this hypothesis. Indeed, in order to obtain equal \( Y_{\text{ATP}} \) values for both acid and alkaline growth conditions 0.55 mol ATP has to be taken into account for the uptake of 1 mol 2-ketogluconate under the latter conditions. In conclusion, the data presented provide evidence that at high culture pH values approximately 0.5 mol ATP is invested in the uptake of 1 mol 2-ketogluconate.

When, under aerobic conditions, a 2-ketogluconate-limited culture of *K. pneumoniae* was suddenly relieved of
its limitation (a so-called pulse experiment) cells reacted by catabolizing this carbon source at a highly elevated rate, whereas growth came to a complete standstill. It was suggested that this growth inhibition was caused by a depletion of the NADPH pool, due to the high rate at which NADPH was now oxidized by 2-ketogluconate reductase (Simons et al., 1991). Addition of excess 2-ketogluconate to a 2-ketogluconate-limited culture of \textit{K. pneumoniae} under anaerobic conditions had the same inhibitory effect on biosynthesis (Table 2). Again, after a pulse the specific rate of 2-ketogluconate catabolism (and thus the rate of NADPH oxidation) was increased. Measurements of intracellular levels of NADPH and NADP revealed that addition of excess 2-ketogluconate led to a decrease in the NADPH level and (thus) a concomitant increase in the NADP level (Table 3). As a result of the 2-ketogluconate pulse, the intracellular NADPH/NADP ratio was lowered by a factor of 2.4. Thus the growth inhibiting effect of a sudden excess of 2-ketogluconate could indeed be mediated by a decrease in the NADPH level or the NADPH/NADP ratio in the cell.

The calculated flux through the pentose phosphate pathway increased when 2-ketogluconate was pulsed, but not to the same extent as the rate of 2-ketogluconate catabolism. Thus, the relative contribution of the pentose phosphate pathway to 2-ketogluconate catabolism was lowered. Whereas specific production rates of CO$_2$, acetate and succinate were increased, production of ethanol stopped (Table 2). The generated reducing power was now disposed of by production of lactate, a product also found under aerobic conditions after 2-ketogluconate addition (Simons et al., 1991). Lactate could be formed via two distinct pathways (see Fig. 1b); from pyruvate by the soluble (NAD-linked) d-lactate dehydrogenase (pyruvate reductase; Garvie, 1980) or via the so-called methylglyoxal bypass. This latter pathway, consisting of the enzymes methylglyoxal synthase and glyoxalase (see Cooper, 1984), can convert dihydroxyacetone phosphate into d-lactate without accompanying ATP production, thereby enabling cells to uncouple ATP generation from catabolism. The production of lactate by \textit{K. pneumoniae} after relief of carbon limitation under anaerobic conditions is well-known and has been ascribed to activity of the latter pathway (Teixeira de Mattos et al., 1984). The relevant enzymes of both lactate-producing pathways were found to be present in cell-free extracts from \textit{K. pneumoniae} that had been grown anaerobically on 2-ketogluconate (Table 1). However, since growth of \textit{K. pneumoniae} stopped when 2-ketogluconate was pulsed to a 2-ketogluconate-limited culture, one would expect the energy requirement of the cells to decrease. If it is assumed that all of the lactate produced after a 2-ketogluconate pulse was formed via the methylglyoxal bypass the calculated specific rate of ATP production was indeed lowered (Table 2). However, if one assumes lactate production by means of lactate dehydrogenase this would lead to an almost doubling of the specific ATP production rate as compared to steady-state conditions. These calculations strongly suggest that lactate production by \textit{K. pneumoniae} in response to a 2-ketogluconate pulse is the result of methylglyoxal bypass activity.

In conclusion, the data presented in this work provide evidence that in \textit{K. pneumoniae} the pentose phosphate pathway plays an important role in the anaerobic catabolism of 2-ketogluconate. Sudden relief of 2-ketogluconate-limited conditions leads to an increase in the rate of 2-ketogluconate catabolism and (possibly as a result of the accompanying decrease in the intracellular NADPH/NADP ratio) to inhibition of biosynthesis. Since anabolism and catabolism are now totally uncoupled, some form of energy dissipation has to take place. Most likely, this is effected by production of lactate via the methylglyoxal bypass.

\begin{table}
\centering
\caption{Effect of pulsing 2-ketogluconate on levels of NADPH and NADP in an anaerobic 2-ketogluconate-limited chemostat culture of \textit{K. pneumoniae} NCTC 418}
\begin{tabular}{llll}
\hline
 & & [\mu mol (g dry wt)$^{-1}$] & & \\
\hline
Steady state & 0.36 ± 0.03 & 0.46 ± 0.00 & 0.79 ± 0.05 \\
t = 10 min & 0.21 ± 0.02 & 0.63 ± 0.01 & 0.33 ± 0.02 \\
t = 30 min & 0.26 & 0.60 & 0.43 \\
\hline
\end{tabular}
\end{table}

\textbf{References}


