J Ind Microbiol Biotechnol (2015) 42:1547–1556
DOI 10.1007/s10295-015-1689-3

Control of carbon flux to glutamate excretion in *Klebsiella pneumoniae*: the role of the indigenous plasmid and its encoded isocitrate dehydrogenase

Mansi El-Mansi[1] · Francois Trappey[2] · Ewan Clark[3] · Malcolm Campbell[2,4]

Received: 29 April 2015 / Accepted: 14 September 2015 / Published online: 29 September 2015

Abstract *Klebsiella pneumoniae* (NCTC, CL687/80) harbors a large indigenous plasmid (p^{C3}), which in addition to encoding for citrate utilization, proline synthesis and glutamate excretion, it uniquely carries the structural gene (*icd*); encoding isocitrate dehydrogenase (ICDH). Flux analysis revealed that ICDH, despite its role in the generation of NADPH required for glutamate dehydrogenase, is not rate-limiting (controlling) in central metabolism as evidenced by a negative flux control coefficient and an adverse effect of overexpression (14-fold) on glutamate excretion. More significantly, however, this paper presents, for the first time, clear evidence that the accumulation of glutamate and its subsequent excretion is associated with the C3 plasmid-encoded regulatory elements, which trigger a shift-down in the activity of α-ketoglutarate dehydrogenase, both in the *K. pneumoniae* parental strain as well as in the *E. coli* exconjugants strains. This finding opens the door for the exploitation of regulatory elements as a tool for manipulating flux in microbial cell factories.

Keywords *Klebsiella pneumoniae* · Glutamate excretion · Isocitrate dehydrogenase · Glutamate dehydrogenase · α-Ketoglutarate dehydrogenase

✉ Mansi El-Mansi
mel.mansi@elizadeuniversity.edu.ng

[1] Centre for Research and Innovation, Faculty of Science, Elizade University, Ilara Mokin, Akure, Nigeria

[2] Department of Biology, Davidson College, Davidson, NC, USA

[3] Institute of Cell Biology, University of Edinburgh, Darwin Building, Mayfield Rd, Edinburgh EH9 3JR, Scotland, UK

[4] Genome Consortium for Active Teaching, Davidson, NC, USA

Introduction

Klebsiella pneumoniae possesses diverse metabolic capabilities and many strains have recently been used for the production of speciality fine chemical, e.g., *cis, cis*-muconic acid [19]. *K. pneumoniae* (NCTC, CL687/80) is a glutamate-producing organism that harbors a large indigenous plasmid (pC3), which, in addition to endowing the organism with the ability to utilize citrate as sole source of carbon, it also facilitates the excretion of glutamate and the biosynthesis of proline [28, 31]. The aforementioned phenotypes, which were lost following plasmid curing, were fully recovered by complementation suggesting that the three phenotypes are associated with the C3 plasmid rather than the chromosome [18, 31]. Further analysis [11, 18] revealed that the C3 plasmid carries the structural gene (*icd)* encoding the Krebs cycle enzyme isocitrate dehydrogenase (ICDH; EC 1.1.1.42); the enzyme catalyzing the oxidative decarboxylation of isocitrate to α-ketoglutarate; the immediate precursor for glutamate synthesis (Fig. 1). Understandably, the ability of this organism to excrete glutamate was equated with the plasmid-encoded ICDH [18, 31], especially because ICDH and glutamate dehydrogenase (GDH; EC 1.4.1.2) exhibit complementarities in co-factor requirements, thus forming a "moiety conserved cycle" [15] for $NDAP^+$/NADPH, H^+ (Fig. 1). It is noteworthy that under these circumstances, α-ketoglutarate forms a node (metabolic junction) at which GDH; for glutamate synthesis and α-ketoglutarate dehydrogenase (α-KGDH; EC 1.2.4.2) of the Krebs cycle are in direct competition for their common substrate [6, 25]. We have shown that this plasmid-encoded ICDH is an $NADP^+$-dependent enzyme [11], which is consistent with other members of *Enterobacteriaceae* such as *E. coli* [10] and other mesophiles, e.g., *Helicobacter pylori* [16] and *Mycobacterium tuberculosis* [13] as well as psychrophiles

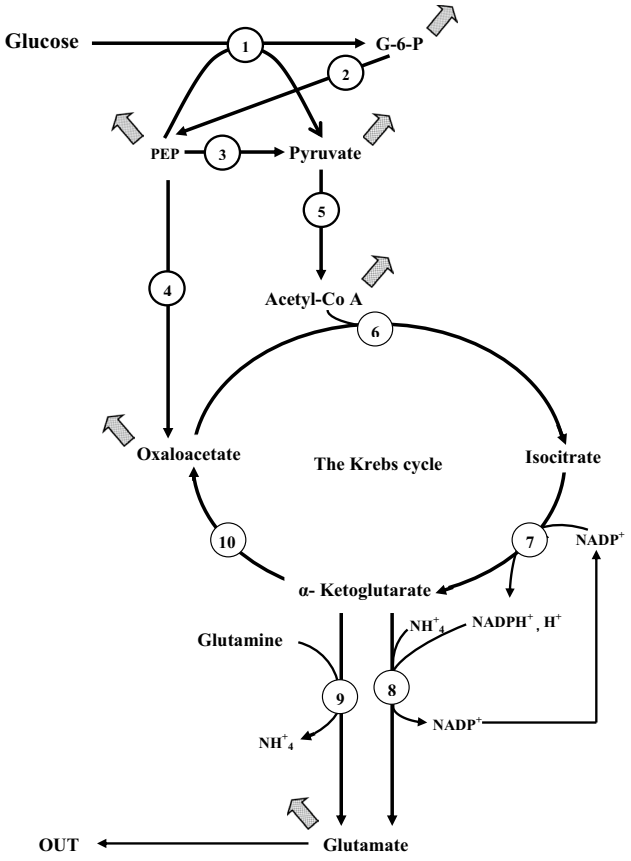

Fig. 1 Metabolic interrelations among various enzymes of central and intermediary metabolism *en route* to glutamate excretion in *K. pneumoniae* highlighting the competitions at the junction of α-ketoglutarate in the Krebs cycle and the "moiety conserved cycle" involving isocitrate dehydrogenase (ICDH) and glutamate dehydrogenase (GDH) for $NADP^+$/NADPH, H^+. Key: *1* PEP-glucose phosphotransferase system; *2* glycolysis; *3* pyruvate kinase; *4* PEP carboxylase; *5* pyruvate dehydrogenase; *6* citrate synthase; *7* isocitrate dehydrogenase; *8* glutamate dehydrogenase; *9* glutamine-oxoglutarate aminotransferase; *10* α-ketoglutarate dehydrogenase. *Large checkered arrows* indicate flux to intermediary metabolism for biosynthesis

[17]. This paper addresses the question of whether this plasmid-encoded ICDH is rate-limiting (controlling) with respect to flux to glutamate excretion and unravels the control mechanism that facilitates glutamate excretion in *K. pneumoniae* (NCTC, CL687/80). It is also hoped that DNA and deduced amino acids sequence comparisons will shed light on the evolutionary origin of this unique plasmid-encoded ICDH.

Materials and methods

Maintenance and propagations of microorganisms

A freeze-dried culture of *Klebsiella pneumoniae* (NCTC, CL687/80) was obtained from the National Collection of Type Culture, NCTC; Colindale Avenue, London. All other organisms were from the culture collection of the corresponding author.

Culture preparation and growth conditions

Lyophilized cultures of *K. pneumoniae* and *E. coli* parental strains were first resuscitated in LB containing 0.5 % glucose and incubated over night with shaking at 30 (*K. pneumoniae*) or 37 °C (*E. coli*). Once the wild types (parental strains) were resuscitated, they were checked for purity and identity. Both cultures were found to be pure and conform to the phenotypic and genotypic description listed in Bergey's Manual of Systematic Bacteriology [21]. Once the phenotypic and genotypic trait of the organisms were determined and found to be satisfactory, samples of each culture were stored at −80 °C in LB containing 30 % glycerol. Cured strains of *K. pneumoniae* and exconjugants strains of *E. coli* were grown in LB.

For growth on minimal media, the organisms were grown aerobically in batch cultures at 30–37℃ for *K. pneumoniae* and *E. coli* strains, respectively in simple defined medium as previously described [3].

Rich and minimal media

Luria Broth (LB) and LB Agar were made as previously described [23], while glucose minimal medium was reconstituted as previously described [11].

Conjugal transfer of the C3 plasmid of *K. pneumoniae*

Conjugal transfer of the C3 plasmid from *K. pneumoniae* (NCTC, CL687/80) into *E. coli* and thence into another recipient strain of *E. coli* were carried out as previously described [31].

Agarose gel electrophoresis

Electrophoresis of restriction fragments were electrophoresed using TBE buffer at 6.0 V per cm for 4 h as previously described [23]. PCR fragments and cloned PCR inserts were analyzed in 0.6 % TBE Agarose gels.

Measurements of enzymic activities

Isocitrate dehydrogenase (ICDH) assay

Isocitrate dehydrogenase (ICDH) activity was measured spectrophotometrically following the increase in absorbance at 340 nm due to the formation of NADPH, as previously described [9].

α-Ketoglutarate dehydrogenase (α-KGDH) assay

Measuring the activity of this enzyme through the reduction of NAD^+ to NADH, H^+ was not possible, due to high NADH oxidase activity in the crude extract. It follows; we employed the ferricyanide reduction assay as previously described [22], which is a measure of the activity of the α-KGDH component (E_1). In this assay, the formation of ferrocyanide, a consequence of oxidative decarboxylation of potassium α-ketoglutarate in the presence of ferricyanide as electron acceptor, was followed colourimetrically. One unit of enzymic activity was defined as the amount of enzyme required to produce one nmol of ferrocyanide per min at 30 °C. Specific activity is expressed as units per milligram of protein, which was determined using the Biuret method, with crystalline bovine serum albumin (BSA) as the standard.

Determination of glutamate and acetate

Glutamate concentration in the culture filtrate was determined enzymically following the increase in absorbance at 450 nm using the Sigma-Aldrich kit. On the other hand, acetate concentration was determined using HPLC (organic acid column) and confirmed enzymically as previously described [7, 8].

Plasmid DNA extraction and isolation

The indigenous plasmid of the organism under investigations, *K. pneumoniae*, was isolated and its molecular mass was determined as previously described [11]. Preparation of recombinant plasmids was carried as previously described [23].

Amplification and cloning of the plasmid-encoded *icd* cistron from *K. pneumoniae* NCTC, CL687/80

PCR primers, synthesized by Sigma-Proligo, for amplifying the *K. pneumoniae* (NCTC, CL687/80) *icd* gene from isolated plasmid DNA were designed using the sequence of MGH78578 (WashU, http://genome.wustl.edu/genome.cgi?GENOME=Klebsiella%20pneumoniae&SECTION=assemblies). The sequences of the primers were as follows:

Forward: (22-mer) 5′-ATGGAAAGCAAAGTAGTAG TTC

Reverse: (22-mer) 5′-TTACATGTTCGCGATAATCGCG

Plasmid DNA PCR

Amplifications of the plasmid-encoded *icd* cistron of *K. pneumoniae (*NCTC, CL687/80) were carried out using the following sequence of cycles: 95 °C, 5 min once; 95 °C, 1 min; 45 °C, 1 min; 72 °C, 1 min; 30 cycles' total. The reaction contained 1.5 mM $MgCl_2$ and the amplified amplicon was cloned into the Invitrogen pCR®2.1 TA cloning vector (part #K2020-20) using T4 DNA ligase (included in kit) and Promega's rapid ligation buffer (part #M8221). Chemically competent JM109 *E. coli* cells (Promega part #L2001) were then transformed and plated on LB-Amp agar containing X-gal and IPTG for blue-white screening. White colonies were picked up and cultured overnight for plasmid isolation using Promega's procedure for the Wizard plus SV mini-prep Kit (part #A1330).

Sequencing of *icd*

DNA sequencing was carried out by Retrogen, Inc (San Diego, CA 92121) and the results obtained (chromatogram and text) were checked and verified against each other. In the protocol, the T7 promoter was used for forward priming while M13 was used for reverse priming.

Sub-cloning into expression vector

New PCR primers, synthesized by Sigma-Proligo for amplifying the *icd* gene from pFT01, were designed with restriction sites for ligation into the expression vectors pTYB 2 and pTYB 12 of the IMPACT-CN system (New England Bio Labs E6900S). PCR conditions for both reactions were as follows: 95 °C 5 min once; 95 °C 1 min; 62 °C 1 min; 72 °C 1 min; 30 cycles total. Both reactions contained 1.5 mM $MgCl_2$. The C-terminal fusion vector pTYB 2 and the N-terminal fusion vector pTYB 12 were chosen for expression and they, along with the PCR amplicons were double digested with *Nde*I (New England Bio Labs R0111S) and *Sma*I (New England Bio Labs R0141S) in buffer 4 (New England Bio Labs B7004S). The digested vectors were purified and treated with shrimp alkaline phosphatase (SAP; Promega M8201). Vectors and amplicons were then loaded onto a 0.8 % Agarose gel for purification by electroelution. Purified fragments were then ligated into pTYB 2 and 12 using Invitrogen's T4 DNA ligase (15224-017) and Promega's rapid ligation buffer (M8221). Competent cells of *E. coli* JM109 (Promega) were transformed and after 1 hour of incubation at 37 °C, the transformation mix was plated onto LB-amp agar.

Nucleotide and amino acid sequence analysis

BLAST was used to detect regions of similarity/identity between the query, the plasmid-born *icd* sequence, and data banks. Sequences used for comparison were:

Escherichia coli AAC74220.1; *K. pneumoniae* ORF (from Washington University Genome Sequencing Center, (http://genome.wustl.edu/genome.cgi?GENOME=Klebsiella%20

pneumoniae); *Salmonella enterica* and *Citrobacter diversus* CIT42 AAC45920

Results

Control of carbon flux to glutamate excretion in *K. pneumoniae*

The data shown in Fig. (2) highlight the pattern of growth (OD_{420nm}) and output to glutamate excretion during growth of *K. pneumoniae* (NCTC, CL687/80) on glucose (55.6 mM) as sole source of carbon in batch culture under aerobic conditions at 30 °C. Under these conditions, a specific growth rate (μ) of 0.73 was observed. As can be seen from Fig. (2), glutamate excretion mirrored microbial growth in a manner that is typical of primary metabolites and although no glutamate could be detected in the first 5 h of growth, throughput to glutamate excretion thereafter increased linearly as a function of biomass (Fig. 2 inset). Once glucose was fully consumed, no more biomass or glutamate was made.

In another set of experiments aimed at assessing the question of whether the plasmid-encoded ICDH is subject to reversible inactivation at the end of growth on glucose; in preparation to adaptation and assimilation of the excreted acetate, the organism was grown on glucose (0.6 mM) minimal medium and the catalytic activities of ICDH and isocitrate lyase (ICL) were monitored (Fig. 3a, b). While, ICDH activity increased in an autocatalytic manner as a function of time (Fig. 3a), it increased linearly as function of biomass formation (Fig. 3b). Once glucose is fully exhausted (Fig. 3a), the organism reversibly inactivated ICDH and induced the glyoxylate bypass enzyme ICL in preparation for adaptation and assimilation of the excreted acetate. As can be seen from Fig. (3a), following the exhaustion

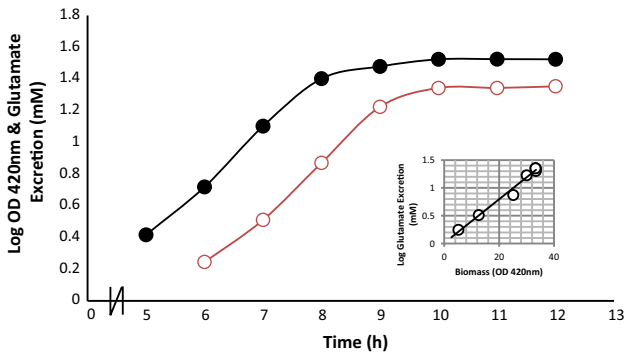

Fig. 2 Pattern of growth (OD_{420nm}) and glutamate excretion in *K. pneumoniae* during growth on glucose minimal medium under aerobic condition at 30 °C The *inset* shows the pattern of glutamate excretion as a function of biomass formation (OD_{420nm}). Key: Biomass, *filled circle*; Glutamate, *circle*

of glucose, the organism simultaneously inactivates ICDH and induces ICL. Once the excreted acetate is fully consumed, the organism reversibly activates ICDH. Reversible inactivation of ICDH is catalysed through the activities of the bi-functional regulatory enzyme ICDH kinase/phosphate; *ace*K, a member of the glyoxylate bypass operon (*Ace* operon) [3, 5, 7, 9].

Effect of the C3 indigenous plasmid on the specific activity of α-ketoglutarate dehydrogenase (α-KDGH) and flux to glutamate excretion

The output (throughput; mmol g^{-1}-dry weight) and flux (mmol g^{-1}-dry weight h^{-1}) to glutamate excretion following growth of *K. pneumoniae* and its cured derivative as well as *E. coli* and *E. coli* exconjugants bearing the C3 plasmid on glucose as sole source of carbon were determined (Table 1). In addition, the individual impact of the C3 indigenous plasmid and the recombinant plasmid pCR2.1 encoding the *K. pneumoniae* ICDH on the specific activities of α-KDGH and ICDH in *K. pneumoniae* and *E. coli* strains has been assessed and the results are shown in Tables (2) and (3), respectively.

Cloning of the *K. pneumoniae* plasmid-encoded *icd*

Primer design for the cloning of the plasmid-encoded ICDH

Using the *Klebsiella pneumoniae* reference genome ((Washington University, http://genomeold.wustl.edu/projects/bacterial/kpneumoniae/), PCR primers were designed (see "Materials and Methods") for the amplification of the *icd* cistron from the purified indigenous plasmid DNA. The *icd* cistron was amplified using PCR and the resulting amplicon (Fig. 4a) was cloned into pTYB2 and pTYB12 to give pCR 2.1. Each ligation mixture was then used to transform competent cells of *E. coli* JM109. Following overnight incubation at 37 °C, the recombinant (white) clones (Ampicillin resistant, β-galactosidase-deficient) were selected for further analysis. A recombinant plasmid carrying the *icd* amplicon in the forward (p^{icd}) and another carrying the reverse ($p^{icd\text{-}rev}$) orientations were successfully isolated (Fig. 4b). Confirmation of orientation was established through *PstI* restriction endonuclease mapping and gel electrophoresis (Fig. 4c).

Sequence similarity and identity of the plasmid-born *icd* with other *icd* orthologs

The plasmid-born *icd* was sequenced; GenBank accession number: BankIt1420989, seq1 HQ822274, and its deduced amino acid sequence was determined and compared with *K. pneumoniae* reference strain (MGH78578—Washington University), as well other organisms (data not shown). A sequence identity value of 96 % was established.

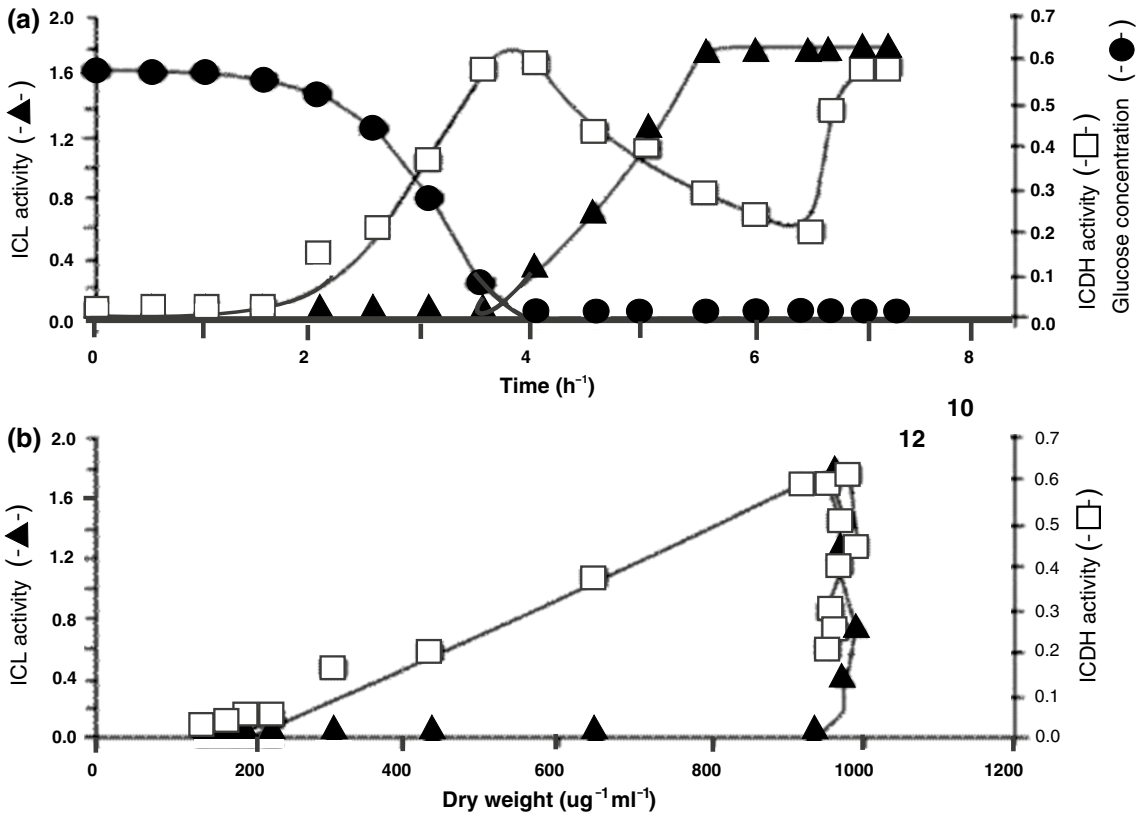

Fig. 3 A composite graph displaying: **a** the patterns of enzymatic activities of ICDH, *square*; isocitrate lyase (ICL), *filled triangle* and glucose utilization; *filled circle* as a function of time, and **b** the patterns of enzymatic activities of ICDH, *square* and ICL, *filled triangle* as a function of biomass

Table 1 Throughput (mmol g^{-1} dry weight) and flux (mmol g^{-1} dry weight h^{-1}) to glutamate excretion following growth of different strains of *K. pneumoniae* and *E. coli* on glucose minimal medium

Organisms/parameter	*K. pneumoniae* (NCTC, CL687/80)	*K. pneumoniae*/pCR2.1 recombinant	*E. coli* K12	*E. coli/pC3 exconjugant strain*	*E. coli* EB106/pCR2.1 recombinant
Throughput	3.45 mM ± 0.06; N4	2.81 mM ± 0.08; N4	0.03 mM ± 0.04; N4	3.08 mM ± 0.06; N5	0.09 mM ± 0.05; N3
Specific growth rate (μ)	0.73 ± 0.04; N4	0.65 ± 0.05; N4	0.68 ± 0.04; N4	0.62 ± 0.03; N5	0.61 ± 0.04; N3
Flux	2.52	1.83	0.02	1.91	0.055

Glutamate concentrations in the culture filtrates was determined spectrophotometrically as described in the section of "Materials and Methods". *K. pneumoniae* cured (plasmid-less) strain did not grow on glucose minimal medium because it lacks ICDH activity, which renders the organism glutamate auxotroph

Table 2 Effect of the C3 indigenous plasmid and the recombinant plasmid pCR2.1-encoding ICDH on the specific activity of α-ketoglutarate dehydrogenase (α-KGDH; nmol ferrocyanide mg^{-1} protein at 30 °C) and isocitrate dehydrogenase (ICDH; nmol NADPH μg^{-1} dry weight at 30 °C)

Organisms/medium	*K. pneumoniae* (parental strain)	*K. pneumoniae* cured strain (plasmid-less)	*E. coli* K12	*E. coli/pC3 Exconjugants*	*E. coli* EB106/pCR2.1 recombinant
Lauria Broth	0.15 ± 0.06; N5	0.21 ± 0.06; N4	0.49 ± 0.06; N4	0.21 ± 0.08; N5	0.56 ± 0.05; N4
Glucose minimal medium	0.21 ± 0.06; N4	No growth	0.62 ± 0.06; N5	0.23 ± 0.06; N4	0.66 ± 0.08; N5

The activities and specific activities of ICDH and α-KGDH were determined as described in the section of "Materials and Methods"

Table 3 Effect of the C3 indigenous plasmid on the specific activity of isocitrate dehydrogenase (ICDH; nmol NADPH μg^{-1} dry weight at 30 °C) carried on the recombinant plasmid pCR2.1

Organisms/medium	*K. pneumoniae* (parental strain)	*K. pneumoniae* cured strain (plasmid-less)	*K. pneumoniae*/ pCR2.1 recombinant	*E. coli* K12	*E. coli exconjugants*	*E. coli* EB106/ pCR2.1 recombinant
Luria broth	0.55 ± 0.06; N5	Not Detectable; N4	7.85 ± 0.06; N4	0.48 ± 0.06; N5	1.14 ± 0.08; N3	8.08 ± 0.05; N4
Glucose minimal medium	0.65 ± 0.06; N4	No growth	8.42 ± 0.08; N5	0.58 ± 0.05; N5	1.18 ± 0.05; N4	8.28 ± 0.06; N5

Microorganisms and growth conditions were as described previously

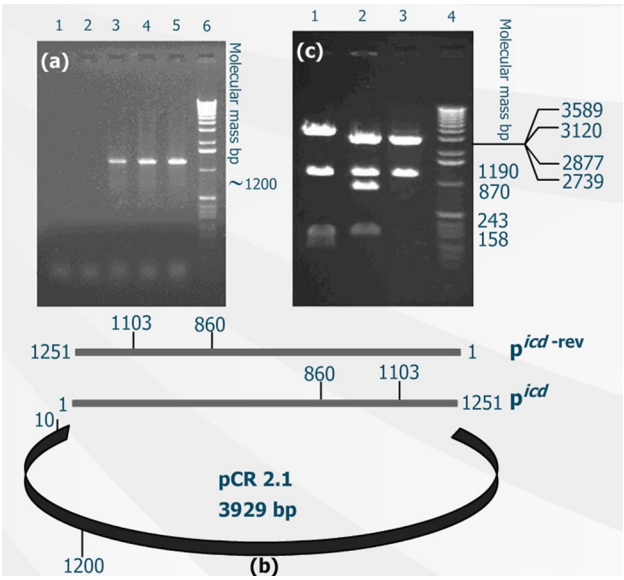

Fig. 4 Amplification of the *icd* cistron and its subsequent cloning into the plasmid vector pCR 2.1. The digits 10 and 1200, on the plasmid vector as well as 860 and 1103 on the *icd* amplicon denote restriction sites for the enzyme *Pst*1. Electrophoresis was performed as described in "Materials and Methods". Figure's subsets are as follows: **a** amplification of the *icd* cistron. The contents in each lane are as follows: *lanes 1* and *2*, represent negative controls; *lanes 3, 4* and *5* represent different quantities of the PCR product; amplicons of *icd*-cistron. *Lane 6* represents molecular mass ladder of linear DNA fragments, **b** ligation of the *icd* amplicon in the forward and reverse orientations into the plasmid vector pCR 2.1 to give p^{icd} and $p^{icd\text{-rev}}$, respectively, and **c** a restriction endonuclease digests of the recombinant plasmids and the plasmid vector pCR2.1. (*Lane 1*): *Pst*I digest of $p^{icd\text{-}rev}$ cloned 3′ to 5′ orientation; (*Lane 2*): *Pst*I digest of p^{icd} cloned 5′ to 3′ orientation; (*Lane 3*): *Pst*I digest of the plasmid vector, pCR 2.1, and (*Lane 4*): molecular mass markers

Discussions

Control of carbon flux to glutamate excretion in *K. pneumoniae* and the pattern of ICDH and isocitrate lyase (ICL) activities

During growth on glucose, as sole source of carbon, *K. pneumoniae* (NCTC, CL687/80) diverts large fraction of the primary carbon source to glutamate excretion (Fig. 2). Glutamate plays a central role as an acceptor of inorganic nitrogen (ammonia) as well as a donor of its amino group to various metabolites of intermediary metabolism for the biosynthesis of nitrogen-containing monomers, e.g., purines, pyrimidines and, NAD^+ [6]. Although glutamate can be generated through the activities of glutamate dehydrogenase (GDH), glutamine-oxoglutarate aminotransferase (GOGAT) and other α-ketoglutarate-α-amino acids aminotransferases, the GOGAT is particularly important under conditions of nitrogen limitations due to the low affinity of GDH for ammonia [14]. The patterns of ICDH and ICL activities during growth of the organism under investigation on glucose (Fig. 3a, b) showed that ICDH activity increased autocatalytically as a function of time and linearly as a function of biomass; at a rate (*P* value) of 2.3 (±0, 03; $n = 4$) units (μmol^{-1} NADPH mg^{-1} dry $weight^{-1}$ min^{-1}, at 30 °C). On the other hand, ICL activity was not detected until after glucose was fully exhausted and growth ceased (Fig. 3b). Once glucose was fully utilized, the organism reversibly inactivated ICDH and induced the glyoxylate bypass enzyme ICL (Fig. 3b) in preparation for adaptation and assimilation of the excreted acetate. This finding lends further support to the original observations reported for *E. coli* [3, 7]. It should be noted, however, that assimilation of the excreted acetate following the exhaustion of glucose yielded no new biomass, which is in contrast to earlier observation made on *E. coli* following growth on pyruvate [9, 10]. Quantitative analysis revealed that pyruvate sustained a much higher flux to acetate excretion (16.81 $mml^{-1}g^{-1}$ dry weight) than does glucose (4.89 $mml^{-1}g^{-1}$ dry weight), which in turn accounts for this discrepancy, i.e., formation of new biomass from the excreted acetate following growth on pyruvate, but not glucose. The synthesis of no biomass from the excreted acetate suggests that the excreted acetate following growth on glucose was a perfect match for maintenance requirements, which under these circumstances include the synthesis of isocitrate lyase (ICL) and other members of the glyoxylate bypass operon. Once the excreted acetate was fully utilized, ICDH was reversibly activated and, no further ICL was made (Fig. 3b). The composite graph (Fig. 3a, b)

demonstrates that, upon exhaustion of glucose, the organism simultaneously turns on the "acetate-switch" [5, 7, 12, 32], which in turn brings about the expression of the glyoxylate bypass enzymes ICL, malate synthase (MS) and isocitrate dehydrogenase kinase/phosphatase (ICDH K/P). The latter bi-functional regulatory enzyme catalyzes the reversible inactivation of a large fraction of ICDH, thus enabling the organism to successfully adapt and assimilate the excreted acetate as sole source of carbon, which is consistent with earlier observations [5, 7, 12].

Sequence analysis and comparisons of the plasmid-encoded *icd* with other chromosomally encoded orthologs from other organisms and evolutionary origin

The plasmid-born *icd* was sequenced (GenBank accession number: BankIt1420989 seq1 HQ822264) and its deduced amino acid sequence was determined and compared with its counterpart of *K. pneumoniae* reference strain (MGH78578—Washington University), as well other organisms. Sequence comparisons revealed that the differences between the plasmid-born *icd* sequence and its chromosomally encoded orthologs in the *K. pneumoniae* reference strain and *E. coli* K12 were negligible. Further sequence analysis identified the presence of the glyoxylate bypass operon (*aceBAK*) enzymes within the *K. pneumoniae* genome, thus lending further support to our findings (Fig. 3b.) that the plasmid-encoded ICDH of *K. pneumoniae* is subject to reversible inactivation following the exhaustion of glucose.

Flux analysis of central and intermediary metabolism *en route* to glutamate excretion in *K. pneumoniae*

Glutamate has recently been shown to be a very dominant intracellular metabolite during growth of *E. coli* on glucose, glycerol or acetate as sole carbon source [4]. To assess whether ICDH activity is rate-limiting with respect to glutamate excretion in *K. pneumoniae,* we modeled fluxes through central and intermediary metabolism using Gepasi, version 3.30 and found that the flux control coefficient of ICDH is negative with respect to flux through central and intermediary metabolism, thus ruling out ICDH as a rate-limiting (controlling) during growth on glucose (data not shown).

From the glucose input (27.80 mmol g^{-1} dry weight), and outputs (throughputs) to glutamate (5.51 mmol g^{-1} dry weight) as well as precursors throughputs to biosynthesis [26], a chart for metabolic throughputs through various enzymes of central and intermediary metabolism *en route* to glutamate excretion by *K. pneumoniae* was constructed (Fig. 5). When fructose was used as sole source of carbon instead of glucose, no acetate was excreted due to the suppressive effect of Cra (*Fru*R) on phosphotransacetylase, which is required for acetate excretion. Under these circumstances, neither ICDH inactivation nor expression of ICL was observed.

From the specific growth rate (μ) of the organism on glucose (0.76) and throughputs through various enzymes (Fig. 5), fluxes through ICDH, GDH and succinyl CoA synthetase were calculated. Analysis of fluxes at the junction of α-ketoglutarate revealed that the presence of the C3 plasmid brought about a dramatic 2.68-fold (268 %) increase in the flux through GDH relative to succinyl CoA synthetase of the Krebs cycle, which in turn enables the diversion of α-ketoglutarate from the Krebs cycle to glutamate excretion. Furthermore, the C3 plasmid had no effect on ICDH in the *E. coli* exconjugant strains. The presence or absence of α-KGDH activity in glutamic acid-producing Corynebacteria has been the subject of much debate. However, such a debate has finally been resolved not only by the detection of the enzymatic acclivity, but also by the presence of a fully operational Krebs cycle during the course of glutamate production in coryneform bacteria [2, 24, 25, 29]. The ability of the C3 plasmid to affect a shift-down in the expression of α-KGDH is reminiscent with the impact of the switch from biotin-rich to biotin-limiting conditions during growth of coryneform bacteria [24–26]. The finding that mutant strains deficient in α-KGDH and/or succinyl CoA synthetase excrete large quantities of glutamate [1, 25] lends further support to the data presented in this paper. The shift-down in the specific activity of α-KGDH observed in the parental strain of *K. pneumoniae* as well as the *E. coli* exconjugants strongly suggests that the pC3 plasmid carries regulatory elements that are capable of suppressing α-KGDH activity. This is reminiscent with suppressive effect observed in *Corynebacterium glutamicum* for DtR1 (DRP); a novel global regulatory protein, on the expression of DtSR1 [20], which in turn leads to glutamate overproduction. The question of whether the C3 plasmid elements down regulates the activity of α-KGDH through protein phosphorylation [24] or by impacting acetyl CoA carboxylase proteins (dstR1, Dstr2 and AccBC) [20, 31], though not pertinent to the argument of this paper, is nevertheless interesting and awaits further investigations.

Phylogeny and evolutionary origin of the plasmid-encoded ICDH of *K. pneumoniae*

An extensive phylogenetic analysis of all published amino acids sequences of ICDH revealed that the ICDH family is composed of three distinct subfamilies [27]. While subfamily I comprises prokaryal $NADP^+$-dependent ICDH enzymes, subfamily II includes eukaryal $NADP^+$-dependent, homodimeric ICDH enzymes, together with the prokaryal enzyme from *Sphingomonas yanoikuyae*. It

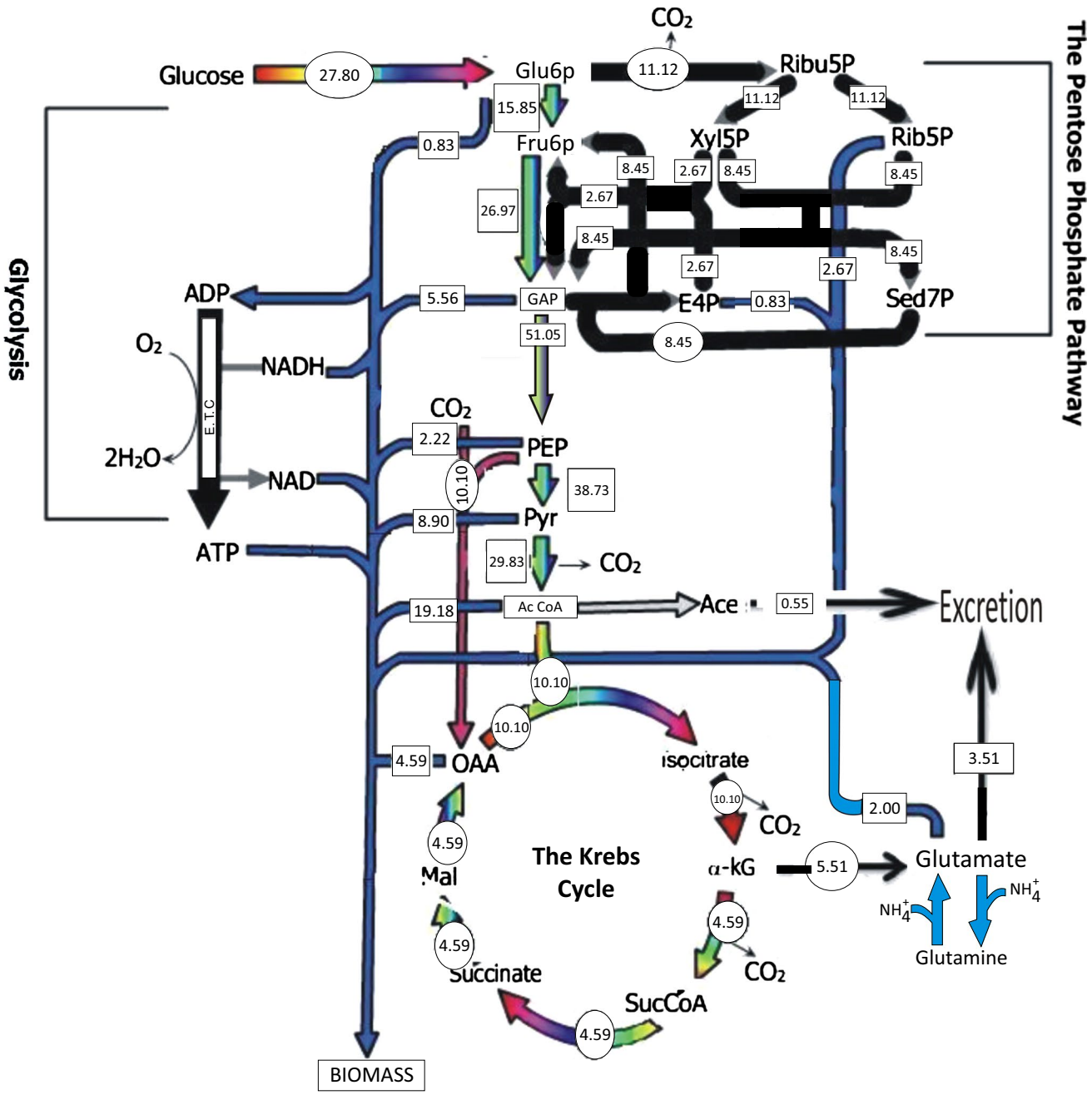

Fig. 5 *Metabolic chart* highlighting the throughputs through various enzymes of central and intermediary metabolism *en route* to glutamate excretion during unlimited growth of *K. pneumoniae* on glucose, under aerobic conditions, in batch cultures at 30 °C. The differing affinities between the pentose phosphate pathway on one hand and glycolysis on the other for their common substrate (glucose 6 phosphate) as well as biosynthesis of 40:57:3, respectively, were taken into consideration when calculating the throughputs at this junction as demonstrated by ^{13}C-radiolabelled distribution in other glutamate-producing bacteria (Shirai et al. [26]). It is noteworthy that the metabolic chart shown, a modification of Varela et al. [30] represents throughputs to glutamate excretion and biosynthesis after 11 h of growth on glucose (CF, Fig. 2); at which point the majority of excreted acetate was assimilated

has also been shown that subfamilies I and II are monophyletic, composed entirely of homodimers with sequence identities ranging from 48–74 and 61–100 %, respectively. Subfamily III, on the other hand, contains all NAD^+-dependent multimeric mitochondrial ICDH enzymes as well as the prokaryal homodimeric $NADP^+$-dependent enzyme from *T. thermophilus*. According to this phylogenetic study [27], the plasmid-encoded ICDH of *K. pneumoniae* can be grouped with other $NADP^+$-dependent ICDH orthologs from *Archaeoglobus fulgidus, Bacillus subtilis,*

Caldococcus noboribetus, Escherichia coli, Sphingomonas yanoikuyae, Streptococcus salivarius, Thermus aquaticus and Thermus thermophilus (subfamily I). Compared with the thermophilic ICDH from *A. Fulgidus,* the plasmid-born ICDH contains six cysteine residues, as opposed to only one in the thermophilic enzyme. These differences may suggest that the abundance of cysteine residues adversely affect the thermostability of the enzyme. The demonstration of high sequence identity among ICDH enzymes across the domain of prokarya [16, 17] strongly suggests that prokaryotic organisms must have acquired ICDH from a primordial archaeon through lateral gene transfer.

Conclusions

This paper concludes that the plasmid-encoded ICDH is not rate-limiting (controlling) with respect to flux to glutamate excretion in *K. pneumoniae* (NCTC, CL687/80) as evidenced by a negative flux control coefficient and the diminution of carbon flux to glutamate excretion following ICDH overexpression (14-fold). The paper further concludes that the control of glutamate excretion rests with the ability of the C3 plasmid to affect a shift-down in the activity of α-KGDH by some 60 %, thus rendering it rate-limiting (controlling) and, in turn, a legitimate target for metabolic engineering. Moreover, DNA- and deduced amino sequence analysis concluded that the differences between this plasmid-encoded ICDH and other chromosomally encoded orthologs from "glutamate non-producing" organisms are negligible and not directly correlated with any of the motifs associated with the catalytic or regulatory properties of the enzyme. The high degree of sequence identity among prokaryal ICDH, including the plasmid-encoded enzyme of the organism under investigation, strongly suggests that prokaryotic organisms must have acquired the structural gene *icd* from a primordial archaeon, presumably through lateral gene transfer.

Acknowledgments MEM wishes to thank Chief Michael Ade. Ojo for his entrepreneurial vision and moral values and Professor Valentine A. Aletor, VC, Elizade University, for constant interest in research and encouragements. The authors wish to thank Adrian Agho and Adesanya Adetayo for skilled assistance in the preparation of the graphs. FT and AMC wish to acknowledge the James G. Martin Genomics programme for technical and logistical support to FT.

References

1. Asakura Y, Kimura E, Usuda Y, Kawahara Y, Matsui K, Osumui T, Nakamatsu T (2007) Altered metabolic flux due to deletion of *odhA* causes L-Glutamate overproduction in *Corynebacterium glutamicum*. Appl Environ Microbiol 73(4):1308–1319
2. Becker J, Klopprogge C, Schroder H, Wittman C (2009) Metabolic engineering of the tricarboxylic acid cycle for improved lysine production by *Corynebacterium glutamicum*. Appl Environ Microbiolol 24:7866–7869
3. Bennett PM, Holms WH (1975) Reversible inactivation of the isocitrate dehydrogenase of *Escherichia coli* ML 308 during growth on acetate. J Gen Microbiol 87:37–51
4. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. Nat Chem Biol 5:593–599
5. Cozzone AJ, El-Mansi EMT (2005) Control of isocitrate dehydrogenase activity by protein phosphorylation in *Escherichia coli*. J Mol microbiol Biotechnol 9:132–146
6. Demain A, Sanchez S (2012) Microbial synthesis of primary metabolites: current advances and future prospects; in Fermentation Microbiology and Biotechnology, 2nd edn. CRC Taylor and Francis, London. ISBN 0-8493-5334-3
7. El-Mansi EMT (1998) Control of metabolic interconversion of isocitrate dehydrogenase between the catalytically active and inactive forms in *Escherichia coli*. FEMS Microbiol Letts 166(2):333–339
8. El-Mansi EMT (2005) Free-CoA mediated regulation of intermediary and central metabolism: an hypothesis, which accounts for the excretion of α-ketoglutarate during aerobic growth of *E. coli* on acetate. Res Microbiol 156:874–879
9. El-Mansi EMT, Nimmo HG, Holms WH (1986) Pyruvate metabolism and the phosphorylation state of isocitrate dehydrogenase of *Escherichia.coli*. J Gen Microbiol 132:797–806
10. El-Mansi EMT, Holms WH (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J Gen Microbiol 135:2875–2883
11. El-Mansi EMT, Anderson KJ, Inche CA, Knowles LK, Platt DJ (2000) Isolation and curing of the *Klebsiella pneumoniae* large indigenous plasmid using sodium dodecyle sulphate. Res Microbiol 151(3):201–209
12. El-Mansi EMT, Cozzone AJ, Shiloach J, Eikmanns B (2006) Control of carbon flux among various enzymes of central metabolism during growth of *Escherichia coli* on acetate. Curr Opin Microbiol 9:173–179
13. Hatzopoulos GN, Kefala G, Mueller-Dieckmann J (2008) Cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of isocitrate dehydrogenase 2 (Rv0066c) from *Mycobacterium tuberculosis*. Acta Crystallogr Sect F Struct Biol Cryst Commun 64(Pt 12):1139–1142
14. Hirasawa T, Kim J, Shirai T, Furusawa C, Shimizu H (2012) Molecular mechanisms and metabolic engineering of glutamate overproduction in *Corynebacterium glutamicum*. Subcell Biochem 64:261–281
15. Hofmeyr JHS, Kaeser H, van der Merwe KJ (1986) Metabolic control analysis of moiety-conserved cycles. Eur J Biochem 155:631–641
16. Huang D, Liu J, Shen G (2010) Cloning, expression, and enzymatic characterization of isocitrate dehydrogenase from *Helicobacter pylori*. Protein J 28:443–447
17. Ishii A, Suzuki M, Sahara T, Takada Y, Sasaki S, Fukunaga N (1993) Genes encoding two isocitrate dehydrogenase isoenzymes of a psychrophilic bacterium, *Vibrio sp* strain ABE-1. J Bacteriol 175(21):6873–6880
18. Juerez A, Pares R, Vives-Rego J (1982) Effect of the carbon source and cyclic AMP on isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase in *Klebsiella pneumoniae* C3. Can J Microbiol 28(10):1101–1106
19. Jung HM, Jung MY, Oh MK (2015) Metabolic engineering of *Klebsiella pneumoniae* for the production of cis, cis-muconic

acid. Appl Microbiol Biotechnol. 99(12):5217–25. doi:10.1007/s00253-015-6442-3
20. Kimura E (2002) Triggering mechanism of L-glutamate overproduction by *dstR1* In Coryneform bacteria. J Biosci Bioeng 94:545–551
21. Orskov I (1984) Genus v. Klebsiella. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Philadelphia, pp 461–465
22. Pettit FH, Hamilton L, Munk P, Namihira G, Eley MH, Willmiams CR, Reed LJ (1973) α-keto acid dehydrogenase complexes XIX. Subunit structure of the *Escherichia coli* alpha-ketoglutarate dehydrogenase complex. J Biol Chem 10(15):5282–5290
23. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York
24. Schultz C, Niebisch A, Gebel L, Bott M (2007) Glutamate production by *Corynebacterium glutamicum*: dependence on the oxoglutarate dehydrogenase inhibitor protein OdhI and protein kinase PknG. Appl Microbiol Biotechnol 76(3):691–700
25. Shimizu H, Tanaka H, Nakato A, Nagahisa K, Kimura E, Shioya S (2009) Effects of changes in enzyme activities on metabolic flux redistribution around the 2-oxoglutarate branch in glutamate production by Corynebacterium glutamicum. Bioprocess Biosyst Eng 25:291–298
26. Shirai T, Fugimura K, Furusawa C, Nagashisa K, Shioya S, Shimizu H (2007) Study on roles of anaplerotic pathways in glutamate overproduction of *Corynebacterium glutamicum* by metabolic flux analysis. Microb Cell Fact 6(1):19–27
27. Steen I-H, Torleiv L, Birkeland N-K (1997) Biochemical and phylogenetic characterization of isocitrate dehydrogenase from a hyperthermophilic archaeon, *Archaeoglobus fulgidus*. Arch Microbiol 168:412–420
28. Tomas J, Rague M, Pares R, Jofer J (1982) Citrate utilizing ability linked to glutamic acid excretion governing conjugative plasmid in *Klebsiella pneumoniae* C3. Zentralblatt für Bakteriologie Mikrobiologie und Hygiene: I. Abt. Originale C: Allgemeine, angewandte und ökologische Mikrobiologie 3(4):457–465
29. Uy D, Delaunay S, Georgen JL, Engaser JM (2005) Dynamics of glutamate synthesis and excretion fluxes in batch and continuous cultures of temperature-triggered *Corynebacterium glutamicum*. Bioprocess Biosyst Eng 27:153–162
30. Varela CE, Agosin M, Baez M, Klapa G, Stephanopoulos G (2003) Metabolic flux distribution in *Corynebacterium glutamicum* in response to osmotic stress. Appl Microbiol Biotechnol 60:547–555
31. Vives-Rego J, Jofre J, Imperial J, Pares R (1979) The effect of 2-oxoglutarate and biotin in the release of amino acids by *Citrobacter intermedius* C3. Rev Esp Fisiol 35(4):475–480
32. Wolfe AJ (2005) The acetate switch. Microbiol Mol Biol Rev 69(1):12–50