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L-Valine Production during Growth of Pyruvate Dehydrogenase Complex-Deficient *Corynebacterium glutamicum* in the Presence of Ethanol or by Inactivation of the Transcriptional Regulator SugR▽

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Pyruvate dehydrogenase complex-deficient strains of *Corynebacterium glutamicum* produce L-valine from glucose only after depletion of the acetate required for growth. Here we show that inactivation of the DeoR-type transcriptional regulator SugR or replacement of acetate by ethanol already in course of the growth phase results in efficient L-valine production.

Corynebacterium glutamicum is a gram-positive organism that grows on a variety of sugars, organic acids, and alcohols and is used for the production of amino acids, such as L-glutamate, L-lysine, and on a small scale also L-valine (6, 11, 12, 14, 16). L-Valine is essential for vertebrates and is used in infusion solutions and cosmetics and as a precursor for the chemical synthesis of herbicides (6, 10, 13). Recently, we engineered *C. glutamicum* for the production of L-valine by deletion of the gene *aceE* encoding the E1p enzyme of the pyruvate dehydrogenase complex (PDHC) (3). *C. glutamicum* Δ*aceE* produced significant amounts of pyruvate, L-alanine, and L-valine from glucose. Additional plasmid-bound overexpression of the L-valine biosynthetic genes *ilvBNCE* in *C. glutamicum* Δ*aceE* shifted the product spectrum from pyruvate and L-alanine toward L-valine. Deletion of the pyruvate: quinone oxidoreductase, phosphoglucose isomerase, and pyruvate carboxylase genes (*pqo*, *pgi*, and *pyc*, respectively) in *C. glutamicum* Δ*aceE*(pJC4ilvBNCE) further improved L-valine production (5). However, a common feature of the PDHC-deficient L-valine-producing strains was that L-valine production was decoupled from growth, i.e., took place only after the depletion of acetate, which is required for growth of all PDHC-deficient *C. glutamicum* strains. In this study, we succeeded in overcoming the nonproducing phenotype in the growth phase and present PDHC-deficient *C. glutamicum* strains producing L-valine in the presence of cellular growth.

The strains used in this study were *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) (5) and *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE). Deletion of *sugR* in *C. glutamicum* Δ*aceE* Δ*pqo* was performed as described previously for *C. glutamicum* Δ*sugR* (7), except that the selection agar plates additionally contained 85 mM potassium acetate. Deletion of *sugR* was verified by PCR using the primers sugRfow (5′-GTTCGTCGCGGCAATGATTGACG-3′) and sugRrev (5′-CTCACCACATCCACAAACCACGC-3′). DNA preparation, transformation, determination of amino acids, and preparation of media were performed as described previously (3). Shake flask fermentations were done aerobically at 30°C with 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker (diameter, 5 cm) at 120 rpm. The fed-batch fermentations were performed at 30°C as 200-ml cultures in a fed-batch-pro fermentation system from Dasgip. The pH was maintained at 7.0 by online measurement using a standard pH electrode (Mettler Toledo) and the addition of 2 M KOH and 2 M H_2SO_4. The dissolved oxygen was measured online by use of a polarimetric oxygen electrode (Mettler Toledo), which was adjusted to 30% saturation in a cascade by stirring at 100 to 1,200 rpm and aerating with 0.2 to 2 liters of air per minute. Foam development was prohibited by manual injection of small amounts of silicon antifoam (Roth). The fermentations were started with 4% (wt/vol) glucose, 0.5% (wt/vol) brain heart infusion (BHI) powder (Merck) plus 1% (wt/vol) acetate or plus 1% (vol/vol) ethanol. During the fed-batch processes, adequate amounts of 50% (wt/vol) glucose, 50% (wt/vol) acetate, or pure ethanol were injected. Glucose, acetate, lactate, and ethanol concentrations were determined by enzymatic tests from Roche Diagnostics.

Figure 1A shows the course of a shake flask fermentation of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE), representative of PDHC-deficient L-valine-overproducing *C. glutamicum* strains in minimal medium with glucose and acetate. Within 8 h, *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) grew to an optical density at 600 nm (OD_{600}) of about 26, consuming acetate completely and consuming a minor part of glucose. After depletion of acetate, the cells stopped growing, further consumed glucose, and produced more than 100 mM L-valine within 40 h. The cells secreted neither L-alanine nor L-lactate into the medium; however, they formed about 13 mM pyruvate. The growth and fermentation parameters for *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) are summarized in Table 1.

Wendisch et al. (17) previously showed that glucose consumption of wild-type *C. glutamicum* (ATCC 13032) was reduced during growth on glucose plus acetate compared to growth on glucose as the sole carbon source. In accordance, several authors (7, 8, 15) recently identified the DeoR-type

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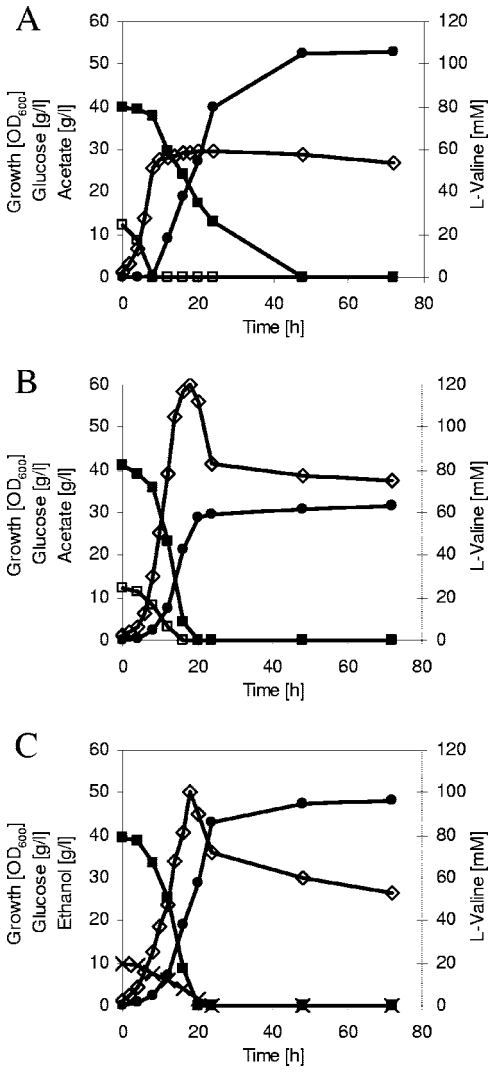

FIG. 1. L-Valine accumulation during representative shake flask batch cultivations of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) (A), of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE) in minimal medium initially containing glucose (4%), potassium acetate (1%), and BHI powder (0.5%) (B), and of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilv BNCE) in minimal medium initially containing glucose (4%), ethanol (1%), and BHI powder (0.5%) (C). ◇, growth; ■, glucose; □, acetate; ×, ethanol; ●, L-valine. Three independent fermentations were performed, and all three showed comparable results.

regulator SugR to be responsible for acetate-mediated repression of *ptsG*, *ptsI*, and *ptsH*, encoding the glucose-specific enzyme II and the general components enzyme I and Hpr of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). To test whether the repressive effect of SugR on the PTS genes and, thus, a decreased glucose consumption in the presence of acetate are the reasons for the nonproducing phenotype of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) in the growth phase, we deleted the *sugR* gene in *C. glutamicum* Δ*aceE* Δ*pqo*, transformed the resulting strain with plasmid pJC4ilvBNCE, and performed shake flask fermentations. In minimal medium with glucose and acetate, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR* (pJC4ilvBNCE) grew within 18 h to an OD_{600} of about 60 and in fact produced about 60 mM L-valine in the growth phase ($t = 0$ to 20 h) (Fig. 1B). After depletion of both substrates, the OD_{600} dropped to about 40. In addition to L-valine, the strain secreted small amounts of L-alanine and pyruvate and about 50 mM L-lactate (Table 1). The glucose consumption rate of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE) in the first 8 h of fermentation was about five times higher than that of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE), whereas the acetate consumption rate was significantly lower (Table 1). These results indicate an intracellular surplus of the precursor pyruvate in the presence of acetate in the SugR-deficient mutant. In addition, these findings suggest that the repressive effect of SugR on the PTS genes and, in consequence, the reduced glucose consumption rate in the presence of acetate might be responsible for the nonproducing phenotype of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) during growth.

Although *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE) showed L-valine production in the growth phase, the overall L-valine formation was about 40% lower than that of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE). Therefore, we tested for an alternative possibility to overcome the nonproduction phenotype of this strain during growth. Since *C. glutamicum* possesses ethanol and acetaldehyde dehydrogenase activities (1, 2, 9) and since glucose consumption seemed not to be affected by the presence of ethanol in the growth medium (1), we characterized the growth and (by-)product formation of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) in minimal medium with glucose plus ethanol instead of glucose plus acetate. The cells grew within 18 h to an OD_{600} of about 50, and within 24 h they produced about 90 mM L-valine and small amounts of L-ala-

TABLE 1. Growth rates, substrate consumption rates, $Y_{X/S}$, final L-valine concentrations, and by-product concentrations in shake flask fermentations of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) and *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE)[a]

Strain	Substrates	Growth rate (h^{-1})	Substrate consumption rate [mmol/(g CDW × h)][b]		$Y_{X/S}$ (g CDW/mol C)[c]	Final L-valine concn (mM)	By-product (mM)		
			Acetate or ethanol	Glucose			L-Alanine	Pyruvate	L-Lactate
C. glutamicum Δ*aceE* Δ*pqo*(pJC4ilvBNCE)	Glucose + acetate	0.38 ± 0.02	3.30 ± 0.30	0.18 ± 0.04	19 ± 3	106 ± 9	<1	13 ± 5	<1
C. glutamicum Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE)	Glucose + acetate	0.31 ± 0.02	1.90 ± 0.10	0.80 ± 0.05	38 ± 7	63 ± 4	5 ± 2	14 ± 6	47 ± 10
C. glutamicum Δ*aceE* Δ*pqo*(pJC4ilvBNCE)	Glucose + ethanol	0.31 ± 0.02	1.80 ± 0.12	1.05 ± 0.08	36 ± 7	93 ± 6	5 ± 2	3 ± 1	<1

[a] Values are means ± standard deviations.
[b] Cell dry weight (CDW) was calculated from the OD_{600} (after 8 h) using a ratio of 0.3 g CDW $liters^{-1}$ per OD_{600} (4). Substrate consumption rates were calculated for acetate, ethanol, and glucose for the first 8 h of fermentation.
[c] $Y_{X/S}$ is given as g CDW per mol C from either acetate or ethanol.

TABLE 2. $Y_{X/S}$, final L-valine concentrations, overall $Y_{P/S}$, productivity, and by-product concentrations in fed-batch fermentations of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) and *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE)[a]

Strain	Substrates	Total C consumed (mol C)	Fermentation time (h)	$Y_{X/S}$ [g CDW/mol C][b]	L-Valine concn (mM)	$Y_{P/S}$ (mol-C/mol-C)[c]	Productivity [mmol/(g CDW × h)]	By-product (mM)		
								L-Alanine	Pyruvate	L-Lactate
C. glutamicum Δ*aceE* Δ*pqo*(pJC4ilvBNCE)	Glucose + acetate	4.57 ± 0.24	47 ± 4	11 ± 5	210 ± 22	0.23 ± 0.02	0.25 ± 0.03	<1	7 ± 3	<1
C. glutamicum Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE)	Glucose + acetate	4.70 ± 0.34	41 ± 4	24 ± 7	160 ± 11	0.17 ± 0.05	0.23 ± 0.01	17 ± 3	14 ± 6	25 ± 16
C. glutamicum Δ*aceE* Δ*pqo*(pJC4ilvBNCE)	Glucose + ethanol	7.53 ± 0.41	66 ± 5	19 ± 5	301 ± 27	0.20 ± 0.02	0.30 ± 0.05	26 ± 4	20 ± 3	<1

[a] Values are means ± standard deviations.
[b] Cell dry weight (CDW) was calculated from the OD_{600} (after 8 h) using a ratio of 0.3 g CDW liters^{-1} per OD_{600} (4). $Y_{X/S}$ is given as g CDW per mol C from either acetate or ethanol.
[c] The overall $Y_{P/S}$ were calculated with the total carbon from either acetate plus glucose or ethanol plus glucose.

nine and pyruvate (Fig. 1C) (Table 1). The glucose consumption rate of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) in medium containing glucose plus ethanol was about sixfold higher than that in medium containing glucose plus acetate, and the ethanol consumption rate was lower than that of acetate (Table 1). Replacement of acetate in the fermentation medium by ethanol thus represents an elegant way to achieve L-valine production in the growth phase of PDHC-deficient *C. glutamicum* strains.

Although *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE) on glucose plus acetate and *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilv BNCE) on glucose plus ethanol produced L-valine already in the course of growth, the overall L-valine formation was lower than that with *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) on glucose plus acetate as substrates (Fig. 1A, B, and C). Comparing the data shown in the figures, we speculated that the lower overall L-valine production is due to the higher biomass formation. To further study L-valine production, we carried out comparative fed-batch fermentations with both strains and determined L-valine accumulation and other fermentation parameters (Table 2). As in the shake flask fermentations, we observed a strict separation into growth and production phases with *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4ilvBNCE) on glucose plus acetate. In contrast, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE) on glucose plus acetate and *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) on glucose plus ethanol formed L-valine throughout the cultivations (data not shown). The biomass-specific yield ($Y_{X/S}$) of *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR*(pJC4ilvBNCE) was higher, and L-valine yield ($Y_{P/S}$) and productivity were about the same; however, the final L-valine concentration was significantly lower than that of *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE) (Table 2). In addition, the cells formed significant amounts of L-alanine, pyruvate, and L-lactate (Table 2). When *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4ilvBNCE) was cultivated in the presence of ethanol instead of acetate, the overall L-valine accumulation and also $Y_{X/S}$ were higher, whereas the $Y_{P/S}$ and productivity were about the same under both conditions (Table 2). However, the cells cultivated in the presence of ethanol secreted significant amounts of L-alanine and pyruvate (Table 2), indicating an intracellular accumulation of the L-valine precursor pyruvate under these conditions and thus suggesting a further potential to increase L-valine production with *C. glutamicum* Δ*aceE* Δ*pqo*(pJC4ilvBNCE).

Taken together, our data show that the nonproduction phenotype in the growth phase of PDHC-deficient *C. glutamicum* L-valine producers can be overcome either by inactivating of SugR or by replacing acetate by ethanol in the growth medium. However, further studies are necessary to improve the overall L-valine yields and productivity.

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