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Significance of pantothenate for glucose fermentation by *Oenococcus oeni* and for suppression of the erythritol and acetate production

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Abstract The heterofermentative lactic acid bacterium *Oenococcus oeni* requires pantothenic acid for growth. In the presence of sufficient pantothenic acid, glucose was converted by heterolactic fermentation stoichiometrically to lactate, ethanol and CO_2. Under pantothenic acid limitation, substantial amounts of erythritol, acetate and glycerol were produced by growing and resting bacteria. Production of erythritol and glycerol was required to compensate for the decreasing ethanol production and to enable the synthesis of acetate. In ribose fermentation, there were no shifts in the fermentation pattern in response to pantothenate supply. In the presence of pantothenate, growing *O. oeni* contained at least 10.2 μM HSCoA, whereas the HSCoA content was tenfold lower after growth in pantothenate-depleted media. HSCoA and acetyl-CoA are cosubstrates of phosphotransacetylase and acetaldehyde dehydrogenase from the ethanol pathway. Both enzymes were found with activities commensurate with their function in ethanol production during heterolactic fermentation. From the kinetic data of the enzymes and the HSCoA and acetyl-CoA contents, it can be calculated that, under pantothenate limitation, phosphotransacetylase, and in particular acetaldehyde dehydrogenase activities become limiting due to low levels of the cosubstrates. Thus HSCoA deficiency represents the major limiting factor in heterolactic fermentation of glucose under pantothenate deficiency and the reason for the shift to erythritol, acetate, and glycerol fermentation.

Keywords *Oenococcus oeni* · Lactic acid bacteria · Heterolactic fermentation · Erythritol · Acetate · Pantothenate · HSCoA

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Introduction

Oenococcus oeni (formerly *Leuconostoc oenos*) is a heterofermentative lactic acid bacterium used for malate degradation in wine fermentation (Radler 1963; Henick-Kling 1993; Dicks et al. 1995). Heterofermentative lactic acid bacteria ferment hexoses by the hexose-monophosphate pathway to equimolar amounts of lactate, ethanol and CO_2. In addition, *O. oeni* and some other lactic acid bacteria produce substantial amounts of erythritol and acetate and some glycerol (Veiga-Da-Cunha et al. 1992, 1993; Stolz et al. 1995). Erythritol is formed via fructose-6-P, which is cleaved by phosphoketolase to erythrose-4-P and acetyl-P. Erythrose-4-P is reduced to erythritol, and from acetyl-P acetate or ethanol are produced. Thus erythritol formation represents an alternative pathway for NAD(P)H reoxidation in addition to the typical ethanol pathway. As a consequence, part of the acetyl-P is excreted as acetate. The activity reported for acetaldehyde dehydrogenase from the ethanol pathway was much lower than required for ethanol production (Veiga-Da-Cunha et al. 1993), which could explain the need for the erythritol pathway. It was not clear, however, whether low acetaldehyde dehydrogenase activities represent the only limiting step or whether other limitations exist. Therefore the function of the enzymes within the ethanol pathway was studied quantitatively, in particular, the roles of acetaldehyde dehydrogenase, phosphotransacetylase and their cosubstrates HSCoA and acetyl-CoA.

Many lactic acid bacteria including *O. oeni* are auxotrophic for pantothenic acid, which is an intermediate of HSCoA synthesis (Garvie 1967; Dicks et al. 1995). HSCoA is required for fatty acid activation and is essential for reactions involving fatty acid CoA-esters. Within heterolactic glucose fermentation, HSCoA and acetyl-CoA are substrates of phosphotransacetylase and acetaldehyde dehydrogenase, respectively. Thus, HSCoA plays an important role in the ethanol pathway, but not in the pathways leading to erythritol, glycerol and lactate. Therefore glucose fermentation of *O. oeni* was analyzed to identify the ef-

fects of pantothenate (or HSCoA) deficiency in glucose fermentation and the shift to erythritol fermentation. By these experiments, it could be demonstrated that pantothenate deficiency leading to HSCoA deficiency is a major reason for the shift from heterolactic to erythritol/acetate fermentation.

Materials and methods

Bacterial strains and growth conditons

Oenococcus oeni strains B1 and B211 (Institut für Mikrobiologie und Weinforschung (IMW), Mainz), *Leuconostoc (L.) mesenteroides* (DSMZ 20240) and *Lactobacillus (Lb.) fermentum* (IMW) were used. The 16Sr RNA genes of the *O. oeni* strains were amplified by PCR with the Eubac5 and Eubac 3 oligonucleotide primers and showed more than 98% identity to *O. oeni* JCM6125 (Suzuki and Suzuki 1999). For maintenance, *O. oeni* was grown in modified tomato juice (TM) medium (DSMZ, Braunschweig), consisting of tryptone peptone (Difco, 20 g/l), proteose peptone (Difco, 5 g/l), yeast extract (GIBCO BRL, 5 g/l), supernatant of centrifuged tomato juice (250 ml/l), and 1 g Tween 80/l, at pH 6. For growth experiments, fermentation balances and enzyme assays, the bacteria were grown in modified MLD (Cavin et al. 1989) or B medium (Garvie 1967; Heerde and Radler 1978). Modified MLD medium contained casamino acids (Difco, vitamin free, 5 g/l), yeast extract (4 g/l), KH_2PO_4 (0.6 g/l), KCl (0.45 g/l), $MgSO_4 \cdot 7H_2O$ (0.13 g/l), $CaCl_2 \cdot 2H_2O$ (0.13 g/l), $MnSO_4 \cdot H_2O$ (0.003 g/l), and Tween 80 (2 ml/l) at pH 6. Medium B was prepared with the vitamin solution, tryptophan and cysteine as described by Heerde and Radler (1978) and Garvie (1967). Pantothenate was omitted from the vitamin solution and added where indicated. *O. oeni* was grown at 30 °C in 50-ml static cultures in rubber-stoppered bottles (100 ml) under a N_2 atmosphere. For anoxic conditions, the medium was degassed and kept under N_2. When appropriate, resazurin (200 µg/l) was included as redox indicator. Glucose or ribose (40 mM each) was added as indicated, and the medium was inoculated with 1 ml of a culture grown in the same medium.

Pantothenate-deficient bacteria (Tables 1, 3) for determining the fermentation products and HSCoA content in growing and resting bacteria were pregrown to an OD_{578} of 0.6 in MLD medium. The washed bacteria were diluted to an OD_{578} of 0.3 in medium B without pantothenate, grown to an OD_{578} of 0.6 and used for determination of HSCoA and fermentation products, or for suspensions of pantothenate-deficient bacteria.

Enzyme assays

Oenococcus oeni was grown in 50 ml modified MLD medium plus C-source to late exponential growth phase, collected by centrifugation, and resuspended in 0.1 M Tris, pH 7.4, and 5 mM dithiothreitol. The cell suspension was passed twice through a French press at maximal pressure and centrifuged at 10,000×*g* for 1 min; the supernatant (cell homogenate) was used. The homogenate was frozen in liquid N_2 and stored at –80 °C. Enzyme activities were measured photometrically at 30 °C and are given as µmol NAD(P)H or NAD(P) consumed, or as acetyl-CoA produced, per mg of protein (Bode et al. 1968) and min. Phosphotransacetylase activity was followed by acetyl-CoA formation from acetyl-P plus HSCoA (Klotzsch 1969) at 233 nm–260 nm, with $\Delta\varepsilon_{233-260nm}$=4.4 mM^{-1} cm^{-1}, in a Zeiss diode array photometer S10 with the WinAspect program. Alcohol dehydrogenase was measured at 365 nm by the NAD(P)-dependent oxidation of ethanol (DeMoss 1955). Acetaldehyde dehydrogenase was recorded photometrically (365 nm) under anoxic conditions by the acetyl-CoA (225 µM) dependent oxidation of NAD(P)H (0.5 mM) in buffer containing 0.1 M Tris/HCl, pH 7.4, and 5 mM dithiothreitol.

Fermentation reactions

For fermentation reactions, the bacteria were grown with glucose or ribose in modified MLD or B medium. For measurement in cell suspensions, bacteria in the late exponential growth phase were harvested, washed and resuspended at an OD_{578nm} of 6 in Mops buffer (Prohl et al. 1998) under anoxic conditions in rubber-stoppered bottles under N_2. After sugar addition, samples (0.5 ml) were withdrawn with syringes at various time intervals. The samples were centrifuged for 5 min at 10,000×*g*. In the supernatants, the substrates (glucose or ribose) and the products were determined by HPLC on an Aminex HPX87H column (300×7.8 mm, BioRad) (Prohl et al. 1998). Glucose, ribose, lactate, ethanol, acetate, mannitol, erythritol, glycerol, sorbitol, and alanine were identified by retention times and quantified with standard solutions by a refractive index and a UV light detector (215 nm). For the estimation of CO_2 production, it was assumed that 1 mol CO_2 is produced per mol of lactate or glycerol.

Measurement of HSCoA

For the measurement of HSCoA contents, *O. oeni* was grown with or without Ca-pantothenate. HSCoA was extracted and determined by a cyclic assay (Michal and Bergmeyer 1972). For HSCoA extraction, cell paste was incubated for 1 h in small volumes of buffer (50 mM K-phosphate, pH 7.5) with lysozyme (3 g/l). The suspension was then mixed and incubated for 10 min with three volumes of 1 M perchloric acid. HSCoA was determined from the supernatant after neutralization. The cellular HSCoA content was calculated from the HSCoA content (mg/g dry weight); 1 OD_{578nm} unit corresponds to 300 mg of dry mass/l, and 1 g dry weight to 2.5 ml cytoplasmic volume for *O. oeni*.

Results

Requirement for pantothenate for heterolactic glucose fermentation by *O. oeni*

Oenococcus oeni is known for pantothenate auxotrophy (Garvie 1967; Dicks et al. 1995), and in medium B, which is devoid of pantothenate, the bacteria showed only very weak growth with glucose or ribose. Low amounts of pantothenate (<1 mg/l) were sufficient for maximal stimulation of growth. Complex media such as tomato juice or MLD medium supported growth without pantothenate addition.

In the presence of pantothenate, glucose was nearly completely converted to the products of heterofermentative lactic fermentation, i.e. about 1 mol of lactate, ethanol, and CO_2 each (Table 1). These products contained 92% of the glucose carbon, and other products were found only in negligible amounts. In the absence of pantothenate, glucose fermentation was slow and substantial amounts of acetate and erythritol were produced (Table 1).

Cell suspensions of resting bacteria grown and incubated in the presence of pantothenate, produced nearly exclusively lactate, ethanol, and CO_2 (Table 1). When the bacteria were grown and incubated under pantothenate limitation, only 63% of the glucose carbon was regained in the products of heterolactic fermentation, and the amounts of acetate, erythritol, and glycerol increased, containing up to 31% of the glucose carbon (Table 1). The redox balance (O/R value) for the mixed heterolactic/eryth-

Table 1 Effect of pantothenate on glucose fermentation by growing and resting cells of *Oenococcus oeni*. The bacteria were grown in medium B without and with pantothenate, and the fermentation products were determined in the late exponential growth phase. For the cell suspension (resting cells), the washed bacteria were suspended in Mops incubation buffer without and with pantothenate as for growth (OD_{578nm}=4–6). The products are the average of two or three independent experiments. *%C* Carbon yield, *O/R* redox balance, NC not calculated

	Pantothenate (mg/l)	Products (mol/mol glucose)							
		Lactate	Ethanol	Acetate	Erythritol	Glycerol	CO_2	% C	O/R
Growing bacteria	1	0.90	0.95	0.08	0.04	≤0.01	0.91	99	0.94
	0	1.0	1.0	0.23	0.15	≤0.01	NC	NC	NC
Cell suspension	1	0.95	1.03	0.06	0.04	≤0.01	0.96	103	0.93
	0	0.65	0.56	0.39	0.21	0.07	0.72	94	1.03

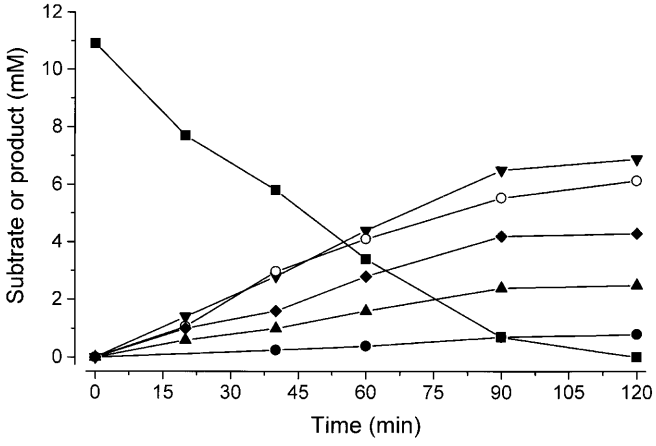

Fig. 1 Kinetics of glucose degradation and product formation by resting cells of *Oenococcus oeni* in Mops buffer. The cell suspensions ($OD_{578\,nm}$=5) of bacteria grown under pantothenate limitation with glucose were incubated under anoxic conditions. Glucose (■), acetate (◆), erythritol (▲), lactate (▼), ethanol (❍), glycerol (●)

ritol fermentation was close to 1, demonstrating that erythritol production is a consequence of the increased acetate formation. The kinetics of glucose fermentation were followed for pantothenate-limited resting cells (Fig. 1). Erythritol and acetate as well as lactate and ethanol were excreted from the beginning, and the relative amounts of the products were constant throughout. Therefore, heterolactic fermentation and the erythritol pathway were used simultaneously in the bacteria.

Fermentation of ribose is not affected by pantothenate limitation

The effects of pantothenate limitation on the fermentation of pentoses were tested in growing and resting bacteria. In the presence of pantothenate, ribose was fermented to acetate and lactate in equimolar concentrations. In the absence of pantothenate, the growth rate on ribose was poor, but the fermentation products of growing and resting cells were the same as with pantothenate (not shown).

Enzyme activities in the ethanol branch

The enzyme activities of the ethanol branch were measured in cell homogenates of bacteria grown with pantothenate, and are compared to the rate of ethanol production (Table 2). The ethanol production rate was calculated from the growth rate and the molar growth yield of the bacteria on glucose. The activities of phosphotransacetylase and ethanol dehydrogenase were high and greatly exceeded the rate of ethanol formation. Acetaldeyde dehydrogenase activity was much lower and comparable to the

Table 2 Activities of the enzymes of the ethanol branch in *O. oeni*, *Leuconostoc mesenteroides* and *Lactobacillus fermentum*. The bacteria were grown on glucose in MLD medium. *PTA* Phosphotransacetylase, *ALDH* acetaldehyde dehydrogenase, *ADH* alcohol dehydrogenase. Ethanol production rate (*a*) was calculated from the growth yields (Y_{Gluc}) and the growth rates (μ) according to $a=\mu/Y_{Gluc}$ [µmol ethanol (g protein)$^{-1}$ min^{-1}], assuming that 1 ethanol is produced per glucose. Y_{Gluc} (9.2, 9.7, and 10.5 g dry weight/mol glucose for *O. oeni*, *Lc. mesenteroides*, and *Lb. fermentum*, respectively) and μ (0.023, 0.049, and 0.065 h^{-1}, respectively, for *O. oeni*, *L. mesenteroides*, and *Lb. fermentum*) were determined for the bacteria growing in MLD medium. *ND* Not determined

Bacteria	PTA	ALDH		ADH		Ethanol production rate
		NADPH[a]	NADH[a]	NADP[a]	NAD[a]	
	µmol substrate (g protein)$^{-1}$ min^{-1}					
O. oeni	4,600	2	80	2,400	150	83
L. mesenteroides	15,500	8	150	970	90	168
Lb. fermentum	ND	28	270	ND	ND	206

Table 3 HSCoA contents and production of ethanol, acetate and erythritol from glucose by growing and resting cells of *O. oeni*. HSCoA contents and the products were determined in the late logarithmic growth phase (growing cells) or after preparation of cell suspensions in Mops buffer from bacteria grown under the same conditions. For the resting cells, the pantothenate content was the same during growth and in the cell suspension. *E. coli* were grown aerobically on glucose. *ND* Not determined

Growth condition	HSCoA		Ethanol	Acetate	Erythritol
	mg/g dw	μM	(mol/mol glucose)		
O. oeni (growing cells)					
Medium B, no pantothenate	<0.002	<1.0	0.9	0.23	0.18
Medium B+1 mg pantothenate/l	0.02	10.0	0.95	0.01	0.01
MLD medium	0.101	51.4	0.95	0.02	0.03
O. oeni (resting cells)					
No pantothenate	<0.004	<2.0	0.48	0.37	0.17
1 mg pantothenate/l	0.024	12.0	0.81	0.02	0.05
E. coli AN387 (wild-type)					
M9 medium+glucose	1.81	460	ND	ND	ND

ethanol production rate. The acetaldehyde dehydrogenase activity might be underestimated, since the enzyme from anaerobic bacteria presumably requires additional constituents for full activity (Nair et al. 1994; Lorenz 1997). Acetaldehyde dehydrogenase used preferentially NADH as electron donor, whereas ethanol dehydrogenase was specific for NADP(H). The enzyme activities in the closely related *L. mesenteroides* and in *Lb. fermentum*, which also grow by heterolactic fermentation, were comparable to those from *O. oeni*. Again, the aldehyde dehydrogenases showed the lowest activities, similar to the ethanol production rates (Table 2), and used preferably NADH. In summary, all enzymes of the ethanol branch were present in activities required for the production of 1 mol ethanol per mol glucose under the respective growth rates. The low activities for acetaldehyde dehydrogenase were observed for all strains irrespective of whether these strains tend to excrete erythritol (*O. oeni*) or not (*Lb. fermentum*).

Bacterial HSCoA contents and relation to enzyme activities

The HSCoA contents in the bacteria were determined after growth in medium B with and without pantothenate supplementation (Table 3). The HSCoA measured includes HSCoA, acetyl-CoA and other fatty acid-CoA esters. After growth without pantothenate, the HSCoA contents were below the detection limit. In bacteria grown in pantothenate-supplemented medium B, significant amounts of HSCoA were found, and these amounts were even higher after growth in the MLD and TM media. In *Escherichia coli* wild-type, significantly higher contents were found, in agreement with published values (Jackowski 1996). Thus, *O. oeni* contains HSCoA only after growth in the presence of pantothenate, and the contents are generally lower than in *E. coli*. The HSCoA contents did not decrease significantly in resting *O. oeni* incubated with or without pantothenate for ≥26 h.

The K_m value of phosphotransacetylase for HSCoA was determined from cell homogenates. In the presence of saturating acetyl-phosphate, the enzyme activity responded in a hyperbolic curve to increasing HSCoA concentrations (not shown). The double-reciprocal plot of $1/v$ against $1/[HSCoA]$ according to Lineweaver-Burk gave a linear relation that is characteristic for Michaelis-Menten type kinetics and an apparent K_m for HSCoA of 40 μM and V_{max} of 4600 U/g protein. For acetaldehyde dehydrogenases from various sources, apparent K_m values for acetyl-CoA are 15 μM or higher (Kazahaya et al. 1972; Bruchhaus and Tannich 1994; Yan and Chen 1990), and the K_m for *O. oeni* might be similar. The K_m values suggest that phospotransacetylase and acetaldehyde dehydrogenase have to operate in *O. oeni* at non-saturating HSCoA and actyl-CoA levels, in particular under pantothenate limitation.

Relation of intracellular HSCoA contents to acetate/erythritol formation

The relation of the HSCoA contents to the function of the heterolactic fermentation was studied by measuring the HSCoA contents and the fermentation products (Table 3). Only the most significant products for the ethanol branch and the acetate/erythritol pathways are shown in Table 3. In growing as in resting cells, bacteria with a high HSCoA content produced ethanol in amounts typical for heterolactic fermentation, but no acetate or erythritol. Low HSCoA contents, however, correlated with high amounts of acetate and erythritol, and decreased amounts of ethanol.

Discussion

Erythritol fermentation as a consequence of pantothenate or HSCoA limitation

In addition to its requirement as a general growth factor (Garvie 1967), pantothenate is needed for heterolactic glucose fermentation by *O. oeni*. In the presence of pantothenate, the bacteria show typical heterolactic fermentation, whereas under pantothenate limitation considerable amounts of erythritol, acetate and glycerol are produced. As shown in this report, the redox balances for the mixed

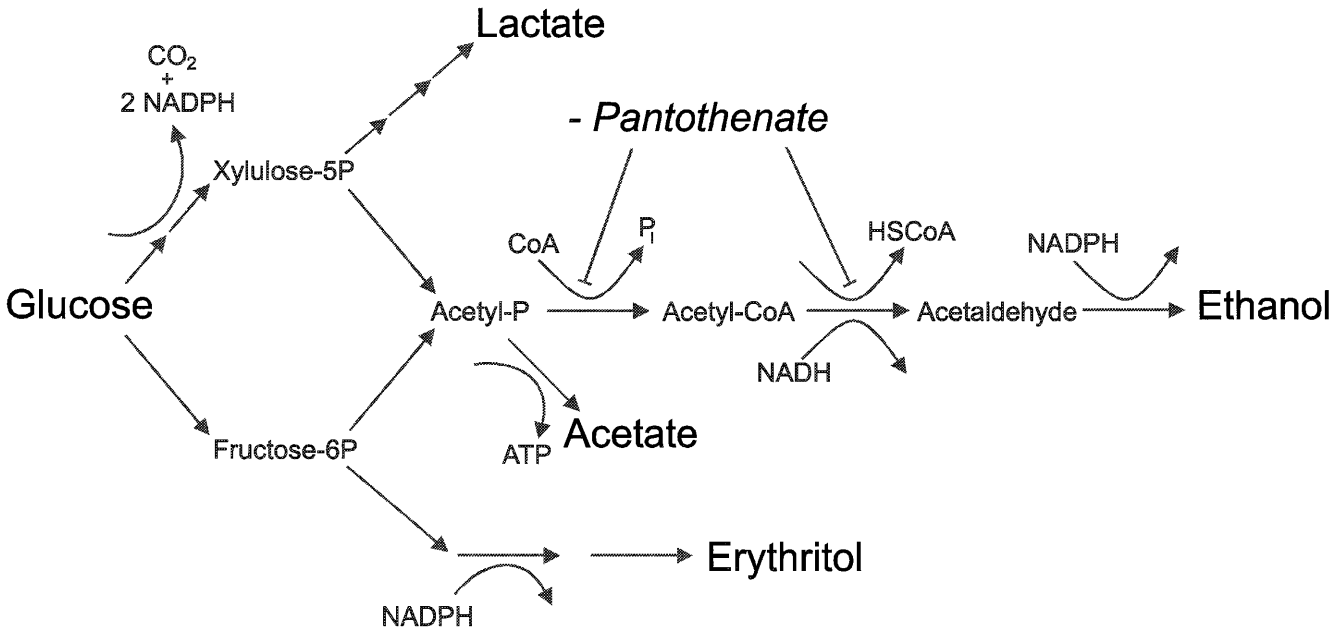

Fig. 2 Glucose fermentation by *O. oeni*: the major routes resulting in the production of lactate, ethanol and CO_2 (heterolactic fermentation using phosphoketolase reaction). The alternative pathway resulting in acetic acid and erythritol (modified from Veiga-Da-Cunha et al. 1993) is also shown. Only for the ethanol branch, catalyzing the conversion of acetylphosphate to ethanol by phosphotransacetylase (PTA), acetaldehyde dehydrogenase (ALDH) and ethanol dehydrogenase (ADH), are all reaction steps and cosubstrates shown. The reactions (PTA, ALDH) limited by low HSCoA or acetyl-CoA contents are indicated

heterolactic/erythritol fermentation are close to 1 in resting bacteria, proving that the two pathways depend on each other. In *Saccharomyces cerevisiae*, pantothenate deficiency affects acetate production and consumption, but the reason for this is different (Taherzadeh et al. 1996).

HSCoA as a major limiting factor for the ethanol pathway

Phosphotransacetylase and acetaldehyde dehydrogenase require HSCoA and acetyl-CoA, respectively, as substrates and thus must be affected by the available HSCoA (Fig. 2). V_{max} of phosphotransacetylase is very high, but with the cellular HSCoA contents the actual activities can be estimated, by using the Michaelis-Menten equation, to be much lower (Table 4). Under pantothenate limitation, phosphotransacetylase activity decreases to values (<112 U/g protein) close to the minimal activity required for ethanol production (83 U/g protein).

The same arguments apply to acetaldehyde dehydrogenase (Table 4). Here it is assumed that the acetyl-CoA content corresponds maximally to the total HSCoA content. At a high HSCoA content, the actual activities of acetaldehyde dehydrogenase amount to maximally 50% of the ethanol production rate. Presumably the actual activities of the enzyme are higher than measured due to the lability of the enzyme (Lorenz 1977), in agreement with the functional pathway present under these conditions. In bacteria with low HSCoA contents, the actual activities drop to <11% of the required activities, a level that apparently is no longer sufficient to maintain operation of the pathway. Therefore the enzymes of the ethanol pathway, including acetaldehyde dehydrogenase, are of sufficient activity to support the heterolactic pathway and ethanol production if enough HSCoA is present in the bacteria. Only under pantothenate and HSCoA deprivation does erythritol fermentation takes place.

Previously, substantially lower activities for acetaldehyde dehydrogenase were found, and it was concluded that low activities of this enzyme cause the limitation in the ethanol pathway (Veiga-Da-Cunha et al. 1993). However,

Table 4 Effect of various cellular HSCoA contents on the activity of phosphotransacetylase and of acetaldehyde dehydrogenase of *O. oeni*. The activities of phosphotransacetylase (*PTA*) and acetaldehyde dehydrogenase (*ALDH*) were calculated for particular HSCoA contents of the bacteria (compare Table 3) using the Michaelis-Menten relation $v=(V_{max}\cdot[HSCoA])/(K_m+[HSCoA])$. For simplification, it is assumed that [HSCoA] and [acetyl-CoA], as the substrates of PTA and ALDH, respectively, correspond to the total HSCoA contents from Table 3. V_{max} for PTA and ALDH are taken from Table 2. K_m of PTA for HSCoA was 40 μM, K_m of ALDH for acetyl-CoA was taken as 15 μM (Kazahaya et al. 1972; Bruchhaus and Tannich 1994: Yan and Chen 1990)

Condition	HSCoA (total) (μM)	PTA	ALDH
		U/g protein	
HSCoA saturation (V_{max})	–	4600	80
+Pantothenate (growing bacteria, medium B)	10.0	934	32
+Pantothenate (cell suspension)	14.8	1240	40
–Pantothenate (growing bacteria)	<1.0	<112	<5
–Pantothenate (cell suspension)	<2.0	<219	<9

using NADH instead of NADPH as the donor, substantially higher activities are found, which fit the role of the enzyme in the ethanol pathway. Accordingly, acetaldehyde dehydrogenase from the closely related *L. mesenteroides* also shows highest activities with NADH (Kazahaya et al. 1972).

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