Comparison of α-acetolactate synthase and α-acetolactate decarboxylase in Lactococcus spp. and Leuconostoc spp.

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COMPARISON OF α-ACETOLACTATE SYNTHASE AND α-ACETOLACTATE DECARBOXYLASE IN *LACTOCOCCUS* SPP. AND *LEUCONOSTOC* SPP.

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SUMMARY

Cell-free extracts of *Leuconostoc* and *Lactococcus* species were tested for their α-acetolactate synthase and α-acetolactate decarboxylase activities. In *Leuconostoc mesenteroides* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc lactis*, the Km of α-acetolactate synthase for pyruvate was close to 10 mM whereas it was 30 mM in *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. The Km of α-acetolactate decarboxylase for α-acetolactic acid was very low (0.3 mM) in *Leuconostoc* species in comparison to *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (60 mM). In the latter bacterium, α-acetolactate decarboxylase showed a sigmoidal dependance upon α-acetolactic acid and was activated by the three branched-chain amino acids: leucine, isoleucine and valine.

INTRODUCTION

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* spp. are used in the dairy industry for the production of diacetyl during the fermentation of milk. Citrate is the precursor of C_4 (acetoin, diacetyl and 2,3-butanediol) and C_5 (α-acetolactic acid (ALA)) compounds. ALA is produced by the condensation of active acetaldehyde with pyruvate by the α-acetolactate synthase (ALS). ALA is an unstable compound which can be decarboxylated chemically into acetoin and diacetyl or enzymatically into acetoin via the α-acetolactate decarboxylase (ALDC) (Hugenholz, 1993). ALS from *L. lactis* subsp. *lactis* biovar. *diacetylactis* has been purified: a very high Km value (50 mM) for pyruvate was found (Snoep *et al.*, 1992). ALDC from *Lactobacillus casei* has been purified (Rasmussen *et al.*, 1985) but no information is available on this enzyme in *Leuconostoc* and *Lactococcus* species. There is a large variation in diacetyl, acetoin and ALA production between *L. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* spp.: in *L. lactis* subsp. *lactis* biovar. *diacetylactis* acetoin, diacetyl, formate and acetate are the main products from citrate metabolism (Cogan, 1982; Schmitt *et al.*, 1988; Starrenburg and Hugenholz, 1991) whereas lactate and acetate are the main products in *Leuconostoc* spp. (Cogan, 1987; Schmitt and Diviès, 1992; Schmitt *et al.*, 1992). ALS and ALDC are the key enzymes involved in diacetyl, acetoin and ALA production: the aim of the present report is to achieve a comparative study of ALS and ALDC in *Lactococcus* and *Leuconostoc* species.

MATERIALS AND METHODS

Bacterial strains. The strains Z5, S3, F5, CD and F1 were obtained from SODIMA (Ivry sur Seine, France); 125, 20G, 18G, 25I, and 19D from INRA (Institut National de la Recherche Agronomique, CNRZ, Jouy en Josas, France); 20200 and 20186 from DSM (Deutsche Sammlung von Mikroorganismen, Göttingen, FRG); 195 from Boll (Arpajon, France); NCW1 from T.M. Cogan (National Dairy Products Research Centre, Fermoy, Co. Cork, Ireland). Strain D1 was isolated from the D1 starter obtained from the Chr. Hansen's Laboratorium (Hørsholm, Denmark) and the citrate-negative mutant 19D cit- was isolated from *Leuconostoc mesenteroides* subsp. *mesenteroides* 19D (Lin *et al.*, 1991).

Preparation of cell-free extracts. *Lactococcus* strains were grown at 30°C in modified M17 (Terzaghi and Sandine, 1975) medium (10 mM citrate was added) and *Leuconostoc* strains in modified MRS (de Man *et al.*, 1960) medium (Tween 80 and acetate were omitted). Cells were harvested at the end of the exponential growth phase, washed in 50 mM phosphate buffer (pH 6.0) and resuspended in 10 ml of the same buffer. The suspension was sonicated at 0°C for 5 min (Vibra Cell Sonics Materials, Danbury, Conn., USA). The debris were removed by centrifugation at 25,000 g, 4°C for 20 min. The protein concentration was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of ALA. The ester of ALA (α-methyl-α-acetoxy ethyl acetoacetate) was obtained from Oxford Chemicals Ltd. (Brackley, Northants, UK). It was transformed to ALA, ethanol and acetate by adding two equivalents of NaOH. The saponification reaction was carried out at room temperature for 30 min.

Enzyme assays. ALS activity was assayed at 45°C; the reaction mixture contained 70 mM sodium acetate buffer (pH 5.3), 0.17 mM thiamine pyrophosphate, enzyme solution and the reaction was started by addition of pyruvate (Cogan *et al.*, 1984). The ALA produced was transformed into acetoin by addition of 65 mM HCl and assayed by the method of Westerfeld (1945). ALDC activity was assayed at 30°C; the reaction mixture contained 200 mM potassium phosphate (pH 6.0), enzyme solution and the reaction was started by addition of ALA. Acetoin production was measured by the method of Westerfeld and corrected for the chemical decarboxylation of ALA. ALA obtained from saponification is a racemic mixture of D-and L-ALA, but the ALDC decarboxylates only the D isomer (Rasmussen *et al.*, 1985). The ALA concentrations given in tables and figures represent the concentration of one isomer. One unit of ALS (ALDC) activity represented the formation of 1 µmol ALA (acetoin) per min. The apparent Km for allosteric enzymes was defined as substrate concentration at which the velocity is half of the maximum velocity. For Michaelian enzymes, double reciproced plot was used.

RESULTS

Screening of ALS and ALDC activities. Six *Lactococcus* and ten *Leuconostoc* strains were tested for ALS and ALDC activity (Table 1). ALS was present in all the strains, a great variation in the ALS activity, ranging from 0.018 to 0.380 U/mg was observed. *Lactococcus* strains showed globally higher activities than *Leuconostoc* strains. ALDC activity was tested at two ALA concentrations: 3.6 and 48.4 mM. The activity of ALDC from the *Lactococcus* strains (except of strain D1) was much (> 10-fold) lower at 3.6 mM than at 48.4 mM, suggesting that the Km value for ALA of ALDC was higher than 3.6 mM. No ALDC activity was found in *L. lactis* subsp. *lactis* biovar. *diacetylactis* D1. In the *Leuconostoc* strains, ALDC activities were similar at both ALA concentrations and ranged from 0.109 to 1.142 U/mg.

Saturation kinetics. The effect of increasing substrate concentration on the ALS and ALDC activity was studied in *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5, *Leuc. mesenteroides* subsp. *cremoris* 195, *Leuc. mesenteroides* subsp. *mesenteroides* 19D and *Leuc. lactis* NCW1 (Fig.1). ALS showed a sigmoidal dependance upon pyruvate concentration in *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5, *Leuc. mesenteroides* subsp. *mesenteroides* 19D and *Leuc. lactis* NCW1 contrarily to *Leuc. mesenteroides* subsp. *cremoris* 195. Furthermore, Km and Hill coefficient values were calculated from data presented in Fig. 1. The Hill coefficients were 2.59, 0.78, 1.72 and

Table 1. α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALDC) activities in *Lactococcus* and *Leuconostoc* species.

		Specific activity (U/mg)		
			ALDC	
	Strain	ALS with 80 mM pyruvate	with 3.6 mM α-acetolactic acid	with 48.4 mM α-acetolactic acid
Lactococcus lactis subsp. *cremoris*	Z5	0.160	0.002	0.018
Lactococcus lactis subsp. *lactis*	S3	0.058	0.002	0.040
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*	125	0.250	0.004	0.120
	F5	0.380	0.006	0.200
	D1	0.100	0	0
	CD	0.190	0.003	0.058
Leuconostoc mesenteroides subsp. *cremoris*	F1	0.031	0.143	0.162
	20200	0.018	0.113	0.130
	195	0.055	0.113	0.130
Leuconostoc mesenteroides subsp. *mesenteroides*	20G	0.048	0.152	0.141
	19D	0.120	0.130	0.137
	19D cit-	0.029	0.149	0.179
Leuconostoc mesenteroides subsp. *dextranicum*	18G	0.043	0.109	0.117
Leuconostoc paramesenteroides	20186	0.036	0.950	1.142
Leuconostoc lactis	251	0.219	0.461	0.517
	NCW1	0.050	0.188	0.212

1.62 for strains F5, 195, 19D and NCW1, respectively. The Km value for pyruvate was higher in *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 (30 mM) than in the *Leuconostoc* strains (11, 9, and 9 mM for 195, 19D and NCW1, respectively). ALDC showed a sigmoidal dependance upon ALA concentration in *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 (Hill coefficient = 1.89) and obeyed Michaelis-Menten kinetics in the *Leuconostoc* strains. The Hill coefficients were 0.93, 0.95 and 0.89 for strains 195, 19D and NCW1, respectively. Furthermore, the Km value for ALA was 60 mM in *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 whereas it was close to 0.3 mM for the *Leuconostoc* strains.

Effect of pH on ALS and ALDC activity. The effect of pH on ALS and ALDC activity from *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5, *Leuc. mesenteroides* subsp. *cremoris* 195, *Leuc. mesenteroides* subsp. *mesenteroides* 19D and *Leuc. lactis* NCW1 is shown in Fig. 2. ALS from *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 and *Leuc. lactis* NCW1 showed an optimum activity at pH 5.5, which dropped rapidly below pH 5.5 whereas a slower decrease was observed above this pH. About 60% of the maximum activity was conserved at pH 6.5. For *Leuc. mesenteroides* subsp. *cremoris* 195 and *Leuc. mesenteroides* subsp. *mesenteroides* 19D, the optimum pH was 5.1 and the activity decreased rapidly for higher and lower pH values. Similar results were obtained for ALDC, except for the strain NCW1 in which the optimum pH was 6.0.

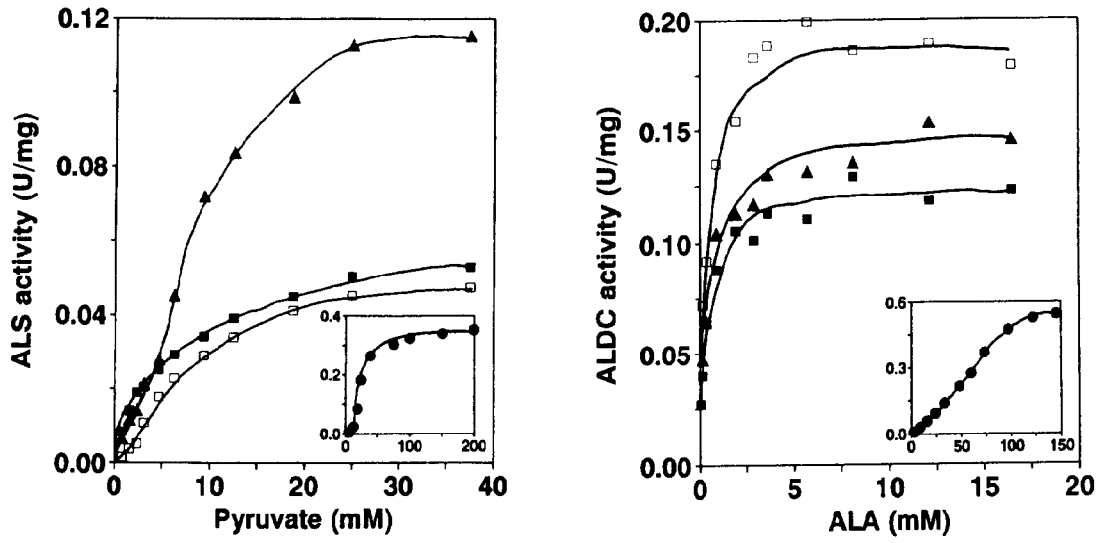

Fig. 1. Effect of substrate concentration on the activity of α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALDC). Symbols: ■ , *Leuc. mesenteroides* subsp. *cremoris* 195; ▲ , *Leuc. mesenteroides* subsp. *mesenteroides* 19D; □ , *Leuc. lactis* NCW1. Insert: ● , *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5.

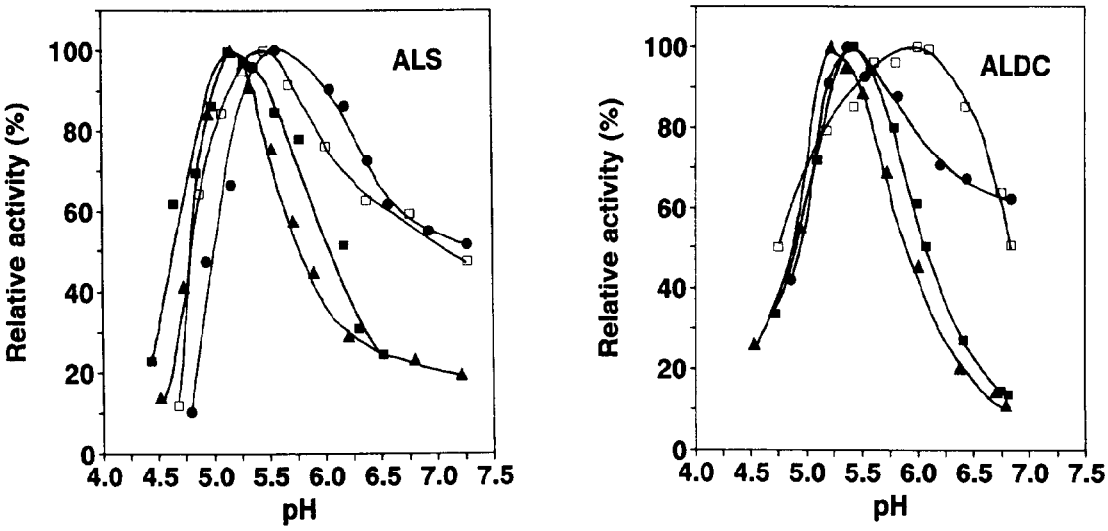

Fig. 2. Effect of pH on the activity of α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALDC). The concentration of pyruvate for ALS measurement was 80 mM, the concentration of α-acetolactic acid for ALDC measurement was 3.6 mM for 195, 19D and NCW1 and 60 mM for F5. ALS and ALDC activities were expressed as the percentage of maximum activity. Symbols: ● , *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5; ■ , *Leuc. mesenteroides* subsp. *cremoris* 195; ▲ , *Leuc. mesenteroides* subsp. *mesenteroides* 19D; □ , *Leuc. lactis* NCW1.

Effect of the branched-chain amino acids. The Km value of ALDC for ALA was surprisingly high (60 mM) in *L. lactis* subsp. *lactis* biovar. *diacetylactis*. Also, several compounds were tested as potential effectors for ALDC. ALDC activity was assayed with 3.6 mM ALA. No effect was observed with NAD(P), NAD(P)H, ATP and ADP. The 3 branched-chain amino acids (leucine, isoleucine and valine) were found to be activators for *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 (Table 2). Addition of 10 mM isoleucine, valine and leucine led to a 3-, 6- and 12-fold increase in ALDC activity, respectively (the seventeen other amino-acids had no effect on ALDC (data not shown)). Leucine, isoleucine and valine had no effect on the ALDC of *Leuconostoc* strains and on the ALS activity for both *Leuconostoc* and *Lactococcus* strains (data not shown).

Table 2. Effect of addition of leucine, isoleucine and valine on α-acetolactate decarboxylase (ALDC) activity[a].

Strain	Specific activity (U/mg)			
	No amino acid	Leucine	Isoleucine	Valine
L. lactis subsp. *lactis* biovar. *diacetylactis* F5	0.008	0.102	0.022	0.051
Leuc. mesenteroides subsp. *cremoris* 195	0.106	0.101	0.103	0.106
Leuc. mesenteroides subsp. *mesenteroides* 19D	0.127	0.122	0.118	0.124
Leuc. lactis NCW1	0.190	0.189	0.165	0.167

[a] α-acetolactic acid (ALA) concentration was 3.6 mM, 10 mM amino acids were added in the reaction mixture

DISCUSSION

Leuconostoc and *Lactococcus* species show differences in the characteristics of ALS and ALDC. Among the six *Lactococcus* strains and the ten *Leuconostoc* strains tested, ALS activity was globally higher in *Lactococcus* species but this difference was not so high as found (> 40-fold less activity in *Leuconostoc* strains) by Hugenholz and Starrenburg (1992). In *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5, ALS was found allosteric and had a high Km value for pyruvate (30 mM), confirming the results obtained by Snoep *et al.* (1992). Also, a high Km value for pyruvate permitted the removal of the toxic pyruvate without competition between the ALS and enzymes such as lactate dehydrogenase and pyruvate dehydrogenase. ALS from *Leuc. mesenteroides* subsp. *mesenteroides* 19D and *Leuc. lactis* NCW1 was allosteric whereas it was Michaelian in *Leuc. mesenteroides* subsp. *cremoris* 195. In both cases, the Km for pyruvate was near 10 mM.

During growth in milk, *Leuconostoc* strains produce acetoin only when the pH decreases below 5.0 whereas *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains produce this compound even at the beginning of the fermentation, when the pH is near 6.5 (Cogan, 1975). One hypothesis of this behaviour could be higher ALS and ALDC activities in *L. lactis* subsp. *lactis* biovar. *diacetylactis* at the pH range 6.0-7.0. The present work shows that in *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5, ALS and ALDC activities were near of 60% of their maximum activity at pH 6.5. *Leuc. mesenteroides* subsp. *cremoris* 195 and *Leuc. mesenteroides* subsp. *mesenteroides* 19D had lower activities at this pH (about 20%), but this was not the case for *Leuc. lactis* NCW1 which had high levels of ALS and ALDC activities at pH 6.5.

Km of ALDC for ALA in *Leuconostoc* strains (0.3 mM) was much lower than in *Lactobacillus casei* (4.8 mM)

(Rasmussen *et al.*, 1985) . In contrast, ALDC from *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 was allosteric and had a high Km for ALA (60 mM). The activation of ALDC by leucine, isoleucine and valine in *L. lactis* subsp. *lactis* biovar. *diacetylactis* could constitute a regulation mechanism for the synthesis of the branched-chain amino acids. However, *L. lactis* subsp. *lactis* biovar. *diacetylactis* F5 was auxotrophic for leucine, isoleucine and valine which implied the absence of regulation of branched-chain amino acid synthesis by ALDC. The gene encoding ALDC has been recently identified in a *Lactococcus lactis* strain which did not require leucine, isoleucine and valine for growth (Chopin, 1993). This gene is downstream of the *leu-ilv* cluster (containing the structural genes necessary for the synthesis of the branched-chain amino acids) and is cotranscribed with the *leu-ilv* genes. This suggests the importance of ALDC in the regulation of the synthesis of leucine, valine and isoleucine. *Lactococcus* strains isolated from dairy products are auxotrophic for branched-chain amino acids, this auxotrophy resulted from accumulated mutations and deletions within the amino acid biosynthetic genes (Chopin, 1993) which might be a consequence of the adaptation of dairy lactococci to milk or dairy products. In spite of the incapacity of the dairy *Lactococcus* strains to synthetize the branched-chain amino acids, the ALDC enzyme was however activated by these amino acids.

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