Fermentation Enzymes in Strictly Aerobic Bacteria: Comparative Studies on Strains of the Genus *Alcaligenes* and on *Nocardia opaca* and *Xanthobacter autotrophicus*

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Nocardia opaca, *Xanthobacter autotrophicus* and 18 representative strains of the genus *Alcaligenes* were examined for the presence of NAD-dependent dehydrogenases for lactate, ethanol or 2,3-butanediol after the cells had been transiently cultivated under conditions of oxygen deficiency. Formation of these enzymes was derepressed in all strains except *Nocardia opaca* and *Alcaligenes latus*. The protein patterns of *Alcaligenes hydrogenophilus* and *Alcaligenes eutrophus* type strain and strains N9A, H16, B19, H1 and H20 obtained by PAGE were similar. The purified lactate dehydrogenases from these strains were strongly inhibited by 1 to 5 µM-oxaloacetate, had a broad substrate specificity and a high affinity for Matrex™ Gel Green A. *Alcaligenes faecalis* shared the properties of the lactate dehydrogenase but differed greatly with respect to its protein pattern. In *A. eutrophus* strain A7 a high activity of lactate dehydrogenase was detected, but the enzyme was not sensitive to oxaloacetate.

Alcohol dehydrogenase and 2,3-butanediol dehydrogenase were even more widely distributed than lactate dehydrogenase among the strains studied. In many cases the electrophoretic mobilities of both alcohol dehydrogenases were identical. The study results in the following taxonomical conclusions. *Alcaligenes eutrophus* type strain and strains N9A, H16, B19, H1 and H20 are almost identical with respect to protein and enzyme patterns as well as the presence of a derepressible L(+)–lactate dehydrogenase sensitive to oxaloacetate. The strains CH34, 707, A7 and JMP134 differ greatly from this core group and from each other and have to be considered as aberrant strains of *A. eutrophus*.

INTRODUCTION

The presence of NAD-linked dehydrogenases for lactate, 2,3-butanediol and ethanol in *Alcaligenes eutrophus* strain N9A had been demonstrated previously (Schlegel & Vollbrecht, 1980). These fermentation enzymes were not expected to be present in strictly respiratory bacteria and are only formed when *A. eutrophus* is cultivated under conditions of restricted oxygen supply (Schlegel & Steinbüchel, 1981). Recently, L(+)–lactate dehydrogenase (Steinbüchel & Schlegel, 1983*a, b*) and alcohol dehydrogenase (unpublished results) from *A. eutrophus* strain N9A have been purified to homogeneity and characterized. This organism served as a model for our studies on enzyme derepression in strictly respiratory bacteria under conditions of oxygen deficiency.

The lactate dehydrogenase from *A. eutrophus* is characterized by three major distinguishing properties. It can easily be purified several hundred-fold from crude extracts by chromatography on Matrex™ Gel Green A due to the remarkably high affinity of this enzyme for the carrier-linked triazine dye (Steinbüchel & Schlegel, 1983*a, c*). The purified lactate dehydrogenase exhibits a broad substrate spectrum allowing the reduction of 2-oxoacids other than pyruvate at

Abbreviations: CTAB, cetyltrimethylammonium bromide; PHB, poly-3-hydroxybutanoate.

a similar rate. Furthermore, enzyme activity is strongly inhibited by low concentrations of oxaloacetate (Steinbüchel & Schlegel, 1983*b*). The alcohol dehydrogenase from this bacterium exhibits 2,3-butanediol dehydrogenase activity, and both activities may be due to the same protein. Both activities were inhibited by 2-mercaptoethanol. The present study included *Xanthobacter autotrophicus* strain 7C, *Nocardia opaca* strain 1b and 18 different strains and species of *Alcaligenes*; the aim was to detect activities of lactate dehydrogenase, ethanol dehydrogenase and butanediol dehydrogenase after the incubation of these bacteria under conditions which resulted in derepression of these enzymes in strain N9A. Furthermore, the distribution of the unusual lactate and ethanol dehydrogenase among bacteria other than *A. eutrophus* was of interest. A further hint about taxonomical relationships was derived by comparing the electrophoresis patterns of cytoplasmic cell proteins.

METHODS

Bacterial strains. Sources and references of strains of the genus *Alcaligenes*, *N. opaca* and *X. autotrophicus* used in this study are listed in Table 1. Strain N9AF06 and strain TF97 are plasmid-free derivatives of strain N9A and the type strain, respectively, of *A. eutrophus*.

Growth of bacteria. Bacteria were grown heterotrophically in 4 l gluconate-mineral medium (Steinbüchel & Schlegel, 1983*a*) in glass fermenters (Schütt, Göttingen, F.R.G.). The cultures were aerated with 500 ml air min⁻¹ and agitated at 630 r.p.m. at 30 °C. After exponential growth with unrestricted oxygen supply to a density of approximately 2 g dry wt l⁻¹, 1.5% (w/v) sodium gluconate was added, and the aeration rate was decreased to 30 to 50 ml air min⁻¹ (Schlegel & Steinbüchel, 1981). The pH was continuously adjusted by a pH-stat to 7.0. After 15 to 20 h of cultivation under these conditions of restricted oxygen supply, the bacteria were harvested, washed with 100 mM-potassium phosphate buffer, pH 7.0, and frozen at -20 °C.

Some strains required a modified treatment. *Alcaligenes faecalis* was cultivated at a temperature of 37 °C. *Alcaligenes latus* was grown on 1.5% (w/v) fructose at 35 °C. The gluconate medium for the cultivation of *Alcaligenes ruhlandii* was supplied with 1.5% (w/v) nutrient broth; the corresponding amount was also added when the aeration rate was decreased. The growth substrate for *Alcaligenes denitrificans* was glucose (1.5%, w/v). *Nocardia opaca* was grown in 1 litre Erlenmeyer flasks containing 300 ml gluconate-mineral medium. To induce oxygen deficiency, three exponentially grown cultures were combined in one 1 litre flask and then incubated without additional aeration. Samples for enzyme determination were taken throughout the incubation period.

Growth measurements. Optical densities were measured in 1 cm cuvettes at a wavelength of 436 or 546 nm in a Zeiss PL4 filter photometer. An absorbance of 10 corresponded to a bacterial density of about 2 mg dry wt ml⁻¹.

Preparation of protein extracts. Approximately 1 g wet wt of cells was resuspended in 4 ml 100 mM-potassium phosphate buffer, pH 7.0, and broken by ultrasonication (5 min) using an MSE (150-W) ultrasonic disintegrator with a probe of 9.5 mm diameter. The sonicate was then centrifuged for 1 h at 100000 *g* in an Omicron ultracentrifuge (Heraeus-Christ, Osterode, F.R.G.) to remove cell debris and particulate matter.

Determination of enzyme activities. The activities of lactate dehydrogenase (EC 1.1.1.27), alcohol dehydrogenase (EC 1.1.1.1) and 2,3-butanediol dehydrogenase (EC 1.1.1.4) were determined by measuring the absorbance changes at 366 nm accompanying NAD(H) reduction (oxidation) at 30 °C using a Zeiss PL4 filter photometer. Lactate dehydrogenase was assayed as described (Steinbüchel & Schlegel, 1983*a*). Assays of alcohol dehydrogenase (ethanol oxidation) and 2,3-butanediol dehydrogenase (butanediol oxidation) were performed in semicarbazide/pyrophosphate buffer, pH 8.9, and glycine/NaOH buffer, pH 9.2, containing 37 mM-2,3-butanediol as described by Bergmeyer (1974) and Höhn-Bentz & Radler (1978), respectively. In samples with no or low alcohol dehydrogenase and 2,3-butanediol dehydrogenase activities the reverse reactions of these enzymes were measured in 3.0 ml 100 mM-potassium phosphate buffer, pH 7.0, containing 0.24 mM-NADH and 2.0 mM-acetaldehyde or 2.0 mM-acetoin, respectively. One unit (U) of enzyme activity transformed 1 µmol substrate per minute.

Electrophoretic methods. PAGE was performed in a flat gel apparatus for vertical slab electrophoreses (Pantophor, Müller, Hann. Münden, F.R.G.) with 3 mm gels (7.5%, w/v, acrylamide) in Tris/borate buffer (30 mM-Tris, 71 mM-boric acid) at pH 8.9. Crude extracts were diluted with 100 mM-potassium phosphate buffer, pH 7.0, to give a protein concentration of about 10 mg ml⁻¹; 10% (w/v) sucrose and 0.005% amido black were added and 25 µl of this solution was applied to the gel. Electrophoresis was carried out at 400 V and 50 mA for about 1.5 h until the band of the tracking dye (amido black) had migrated 10.0 cm. Protein was stained with Coomassie brilliant blue (Weber & Osborn, 1969). For enzyme activity staining, gel strips were incubated at 30 °C in the dark in 100 mM-potassium phosphate buffer, pH 7.6, in the presence of (mg ml⁻¹): NAD, 0.7; 4-nitrobluetetrazolium chloride, 0.4; phenazine methosulphate, 0.025; and L(+)-lactate, 13 (for lactate dehydrogenase), or ethanol, 25 (for alcohol dehydrogenase), or 2,3-butanediol, 25 (for butanediol dehydrogenase), until bands of activity became visible (usually after 2 to 6 h). Relevant controls ensured that bands were specific

for the respective enzyme activity. The stain was discarded, and the gels were rinsed with water and then stored in 7% (v/v) acetic acid at 4 °C.

Preparation of Cibacron Blue F3G-A-Sepharose. Cibacron Blue F3G-A was coupled to Sepharose 6B-CL following the procedure of Atkinson *et al.* (1981).

Purification of lactate dehydrogenase from crude extracts. All steps were carried out at 0 to 4 °C in 100 mM-potassium phosphate buffer, pH 7.0, containing 4 mM-2-mercaptoethanol. Cells (6 g wet wt) were resuspended in 20 ml buffer and broken by ultrasonication (twice, 4 min) using an MSE (150-W) ultrasonic disintegrator with a probe of 19 mm diameter. The sonicate was centrifuged at 140000 g in a Beckmann L5-50B ultracentrifuge in a type 35 rotor (6 × 90 ml). To the resulting supernatant, which was referred to as crude extract, a solution containing 2.5% (w/v) cetyltrimethylammonium bromide (CTAB) was added dropwise to give a final concentration of 2.7 mg ml⁻¹. The precipitate was removed by centrifugation for 20 min at 40000 g. The supernatant (18 to 20 ml) was applied to a column (1.5 × 2.4 cm, 4 ml bed volume) of Matrex™ Gel Green A (Amicon Corp., U.S.A.). The column was washed with buffer which contained KCl in concentrations increasing stepwise. When lactate dehydrogenase activity began to appear in the eluate, 0.5 mM-NADH was added to the final elution buffer (containing 500 mM-potassium chloride in most cases). Fractions with high enzyme activity were combined, and salt and NADH were removed by using a diaflow chamber (Amicon Corp., U.S.A.) containing a PM10 filter. This Green A eluate was layered on top of a Cibacron Blue F3G-A-Sepharose column (1.5 × 2.4 cm, 4 ml bed volume). The column was washed successively with approximately 2 bed volumes buffer, 4 bed volumes buffer containing 100 mM-KCl and finally with buffer containing 500 mM-KCl. Lactate dehydrogenase was eluted from the column at the final step. Fractions with high enzyme activity were combined and referred to as Blue A eluate.

Protein determination. Soluble protein was routinely determined by the method of Bradford (1976).

Determination of poly-3-hydroxybutanoate. Poly-3-hydroxybutanoate (PHB) was determined by the infrared spectrometric method according to Jüttner *et al.* (1975).

Chemicals. Sepharose 6B-CL was obtained from Pharmacia. Matrex™ Gel Green A was purchased from Amicon (Lexington, U.S.A.) and phenazine methosulphate from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, Cibacron Blue F3G-A and 4-nitrobluetetrazolium chloride were from Serva (Heidelberg, F.R.G.).

RESULTS

Wide distribution of fermentation enzymes

All enzyme data refer to cells which, after growth, had been kept under conditions of restricted oxygen supply for about 15 to 20 h. The most remarkable result of this study pertains to the recognition of the wide distribution of the ability to form fermentation enzymes among strictly respiratory bacteria. Almost all strains studied proved to be able to synthesize NAD-dependent dehydrogenases for lactate, ethanol or 2,3-butanediol, if they were transiently cultivated under conditions of restricted oxygen supply. *Nocardia opaca* strain 1b and *A. latus* were the only strains which lacked these activities under the conditions described (Table 1, Fig. 1).

The formation of fermentation enzymes was accompanied by the synthesis of PHB in almost all those bacteria which are known to be able to form this storage material. *Alcaligenes hydrogenophilus*, for instance, accumulated PHB up to 45% of the bacterial cell mass. The role of PHB as an internal fermentation product (Senior & Dawes, 1973; Senior *et al.*, 1972; Schlegel & Steinbüchel, 1981) was herewith confirmed.

Formation and properties of NAD-dependent lactate dehydrogenase

Significant activities of NAD-dependent lactate dehydrogenases were detected in *A. faecalis*, *A. hydrogenophilus*, *X. autotrophicus* and *A. eutrophus* strains H16, N9A and derivatives, B19, TF97, H1, H20 and A7 following growth with restricted aeration. The presence of enzyme activity was demonstrated both by measuring the rate of pyruvate reduction and by activity staining after PAGE. The lactate dehydrogenase from each strain was purified several hundred-fold to approximately 95% homogeneity.

Lactate dehydrogenase from *X. autotrophicus* was highly sensitive to CTAB and was not further investigated due to the low recovery of activity after precipitation by CTAB. The lactate dehydrogenases of the other strains showed remarkably high affinities for Matrex™ Gel Green A;

Table 1. *Bacterial strains used and major properties of intact cells, enzyme activities, electrophoretic mobilities and PHB synthesis*

Enzyme activities were determined as described in Methods; the values represent the highest specific activities reached within 20 h after exposing the cells to restricted oxygen supply. The values in parentheses show activities for the reverse reaction, i.e. alcohol formation. The position of activity

Species and strain	Reference or source*	Substrate	Specific activity† [mU (mg protein) ⁻¹]		
			LDH	ADH	BuDH
<i>A. eutrophus</i> H16	ATCC 17699, DSM 428 (Wilde, 1962)	Gluconate	353	15	235
<i>A. eutrophus</i> N9A	DSM 518 (Schuster, 1967)	Gluconate	489	26	285
<i>A. eutrophus</i> N9A-PHB ⁻ 02-HB ⁻ 1	DSM 1347	Gluconate	335	21	246
<i>A. eutrophus</i> N9AF06	Plasmid-free strain from DSM 518 (B. Friedrich, pers. comm.)	Gluconate	020	34	395
<i>A. eutrophus</i> B19	DSM 515 (Schuster, 1967)	Gluconate	435	12	327
<i>A. eutrophus</i> H1	ATCC 17698	Gluconate	401	15 (1433)	1391
<i>A. eutrophus</i> H20	Institut für Mikrobiologie, Göttingen, F.R.G.	Gluconate	296	18 (525)	795
<i>A. eutrophus</i> TF97	Plasmid-free strain from DSM 531 (Friedrich <i>et al.</i> , 1981 <i>a</i>)	Gluconate	082	28	512
<i>A. eutrophus</i> 707	ATCC 17707	Gluconate	10	60	417
<i>A. eutrophus</i> CH34	(Mergeay <i>et al.</i> , 1978)	Gluconate	15	11 (1034)	855
<i>A. eutrophus</i> JMP134	(Pemperton <i>et al.</i> , 1979)	Gluconate	10	87	26
<i>A. eutrophus</i> A7	(Schwien & Schmidt, 1982)	Gluconate	456	115	7 (1357)
<i>A. denitrificans</i>	CIP 21.75	Glucose	10	75	31
<i>A. faecalis</i>	CIP 62.31	Gluconate	548	0 (50)	73
<i>A. hydrogenophilus</i> 1978	(Ohi <i>et al.</i> , 1979)	Gluconate	68	21	813
<i>A. latus</i>	DSM 1123 (Palleroni & Palleroni, 1978)	Fructose	20	0 (0)	0 (0)
<i>A. paradoxus</i> 1360	ATCC 17722	Gluconate	0	57	0
<i>A. ruhlandii</i>	ATCC 15749, DSM 653	Gluconate + nutrient broth	20	123	39
<i>N. opaca</i> 1b	DSM 427	Gluconate	0	0 (0)	0 (0)
<i>X. autotrophicus</i> 7C	DSM 432	Gluconate	41	0 (326)	440

ND, not determined.

* ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; DSM, Deutsche Sammlung von Mikroorganismen.

† ADH, alcohol dehydrogenase; BuDH, 2,3-butanediol dehydrogenase; LDH, lactate dehydrogenase.

high activities were adsorbed by relatively small amounts of the affinity matrix, as has been described previously for the enzyme from *A. eutrophus* strain N9A-PHB⁻02-HB⁻1 (Steinbüchel & Schlegel, 1983*a*, *c*). Enzymes were eluted with 500 mM-potassium chloride and 0.5 mM-NADH in the final step. Only lactate dehydrogenase from *A. eutrophus* A7 was eluted by 150 mM-potassium chloride. The enzyme from *A. faecalis* did not elute as a sharp peak: activity was detected in every fraction tested including those to which no potassium chloride had been

Table 1.—*continued.*

bands in polyacrylamide gels is characterized by their relative mobility referred to amido black. For ADH and BuDH the pairs of values represent in each case two bands of activity. The values in parentheses represent relative mobilities of minor activity bands.

Relative mobilities of activity bands in PAGE†			PHB synthesis under restricted O ₂ ‡	Inhibition of BuDH by 2-mercaptoethanol§	Purified LDH
LDH	ADH	BuDH			
0.52	0.44, 0.46	0.44, 0.46	++	100.0	+
0.52	0.44, 0.46	0.44, 0.46	++	100.0	+
0.52	0.44, 0.46	0.44, 0.46	++	100.0	—
0.52	0.44, 0.46	0.44, 0.46	++	95.9	+
0.52	0.44, 0.46	0.44, 0.46	++	100.0	+
0.52	0.44, 0.46	0.44, 0.46	++	98.5	+
0.52	0.44, 0.46	0.44, 0.46	++	98.4	+
0.52	0.44, 0.46	0.44, 0.46	++	96.0	+
—	0.36, 0.39 (0.53, 0.56)	0.36, 0.39	++	100.0	—
(0.61)	0.39, 0.40	0.39, 0.40	+	96.8	—
—	0.32, 0.33 (0.42, 0.44)	0.32, 0.33	++	100.0	—
0.55	0.31 (0.42)	0.31	+	100.0	+
0.56	0.37, 0.39	0.26, 0.27	+	100.0	—
0.65 (0.76)	0.32 (0.50)	0.50	+	100.0	+
0.52	0.44, 0.46	0.44, 0.46	++	100.0	+
—	—	—	++	ND	—
—	0.56, 0.58 (0.26)	—	—	ND	—
0.56	0.39	0.26	—	76.0	—
—	—	—	—	ND	—
0.61 (0.68)	0.24	0.24	+	63.3	—

‡ The symbols for synthesis of PHB refer to the proportion of polymer relative to bacterial cell mass after exposing cells to restricted oxygen supply for 15–20 h: ++, 25–50%; +, up to 15%; —, PHB not detectable in significant amounts.

§ Inhibition of 2,3-butanediol dehydrogenase by 1 mM-2-mercaptoethanol was determined in the crude extract.

added. The broad elution profile may be due to the presence of two different enzymes as suggested by the results obtained by activity staining (Fig. 1*d*).

The eluates from the Green A column, which contained about 50% of the lactate dehydrogenase activity, were applied to Cibacron Blue F3G-A-Sepharose. Lactate dehydrogenase activities appeared in the eluate at 500 mM-potassium chloride in all cases. Only lactate dehydrogenase from *A. eutrophus* A7 was eluted from the column by 100 mM-potassium chloride.

The similarity of the lactate dehydrogenases from *A. eutrophus* strains H16, N9A, B19, TF97, H1 and H20 or from *A. hydrogenophilus* was confirmed by the almost coincident migration behaviour during gel electrophoresis (Fig. 1d) and by the substrate specificity of the purified enzymes (Table 2). In addition, all enzymes showed the characteristic sensitivity to 1 μ M-oxaloacetate as was described for the extensively characterized lactate dehydrogenase from *A. eutrophus* strain N9A-PHB-02-HB-1 (Steinbüchel & Schlegel, 1983b). The properties of the lactate dehydrogenase from *A. faecalis* were similar, but 5 μ M-oxaloacetate was required to reach inhibitory effects of more than 95%, whereas 1 μ M caused almost no inhibition. The enzyme from *A. eutrophus* strain A7 differed with respect to its lower affinity for the triazine-dye affinity matrices, in reducing oxaloacetate at a significant rate and in its insensitivity to inhibition by oxaloacetate.

Only low lactate dehydrogenase activities [20 mU (mg protein)⁻¹] were detected in *A. eutrophus* strain CH34. No increase of enzyme activity was achieved when the incubation conditions were varied or when lower aeration rates (resulting in 0, 6 and 36% of the maximum respiration rates) were employed. Furthermore, neither variation of the buffer systems nor desalting of crude extract on Sephadex G-25 resulted in increased enzyme activities. None of the

Fig. 1. (Continued on facing page). Electropherograms of cytoplasmic fractions of various strains of the genus *Alcaligenes* and of *N. opaca* and *X. autotrophicus*. About 200 to 250 μ g protein of the crude extracts of cells, which had been incubated for 15 to 20 h under conditions of restricted oxygen supply, were separated in Tris/borate buffer, pH 8.9, as described in Methods. After electrophoresis, the gels were stained either for protein (a) or for activity of alcohol dehydrogenase (b), 2,3-butanediol dehydrogenase (c) and L(+)-lactate dehydrogenase (d). The arrows in (a) indicate the position of membrane-bound hydrogenase (MH), soluble hydrogenase (SH), alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) in the core strains of *A. eutrophus*, and of amido black (AB). In (b), (c) and (d) only those bands are shown which were specific for the respective enzyme activity. Bands which appeared independently from NAD or substrate are not shown.

Fig. 1 (continued)

Table 2. *Properties of lactate dehydrogenases from different strains of the genus Alcaligenes*

Assay samples for the determination of substrate specificity and oxaloacetate inhibition contained in a final volume of 3.0 ml: 95 mM-potassium phosphate buffer, pH 7.0; 0.24 mM-NADH; 3.0 mM-substrate; about 0.15 to 0.30 µg purified enzyme; and $\pm$ 1 or 5 µM-oxaloacetate. Reaction was started by adding the enzyme. Results are given as the percentage of the activity obtained with pyruvate. Affinity towards triazine-dye affinity media was derived from the conditions leading to the elution of the enzyme from the respective medium: + + + +, 500 mM-KCl + 0.5 mM-NADH; + + +, 500 mM-KCl; + +, 150 mM-KCl; +, 100 mM-KCl.

Substrate specificity	<i>A. eutrophus</i> N9A	<i>A. eutrophus</i> N9AF06	<i>A. eutrophus</i> H16	<i>A. eutrophus</i> B19	<i>A. eutrophus</i> TF97	<i>A. eutrophus</i> H1	<i>A. eutrophus</i> H20	<i>A. hydrogenophilus</i> 1978	<i>A. faecalis</i> A7
Glyoxylate	3.3	7.7	9.1	3.4	8.6	9.1	8.3	5.7	4.8
Pyruvate	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
2-Oxobutyrate	135.4	116.0	136.5	121.9	131.5	161.7	111.3	97.5	171.2
2-Oxovalerate	136.9	113.4	136.5	98.1	117.8	147.0	131.1	82.0	102.2
3-Methyl-2-oxobutyrate	42.0	37.0	29.8	31.7	40.1	45.7	37.5	24.9	14.3
3-Methyl-2-oxovalerate	6.2	5.1	5.2	3.4	6.2	5.3	5.0	3.8	2.4
4-Methyl-2-oxopentanoate	41.2	31.4	31.0	23.4	35.6	38.2	29.3	27.9	11.4
Oxaloacetate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Oxoglutarate	88.7	85.7	115.5	89.9	102.7	114.7	66.5	126.8	223.5
Affinity to Matrex™	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + + *
Gel Green A									
Affinity to Cibacron Blue F3G-A Sepharose	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Oxaloacetate concn (µM) resulting in $\geq$ 95% inhibition	1	1	1	1	1	1	1	1	5
									ND

ND, Not determined.

* Broad elution profile.

lactate dehydrogenase activities were activated by fructose 1,6-bisphosphate and MgCl_2 , which are known to stimulate the activities of other lactate dehydrogenases (Garvie, 1980).

Formation of NAD-dependent alcohol and butanediol dehydrogenases

Almost all of the strains studied contained high activities of alcohol and butanediol dehydrogenases which were separable by electrophoresis into two close but distinct bands in some strains. In those strains in which the properties of lactate dehydrogenase were similar, the electrophoretic mobilities of alcohol and butanediol dehydrogenase activities in polyacrylamide gels were coincident. The alcohol dehydrogenase from *A. eutrophus* N9A was purified to homogeneity (not shown here in detail). The purified enzyme had ethanol dehydrogenase activity as well as 2,3-butanediol dehydrogenase activity. The two activities could not be separated by a variety of methods. Extensive kinetic studies, which included the examination of inhibitory effects of various physiological metabolites and of 2-mercaptoethanol, strengthened the conclusion that in *A. eutrophus* both activities are due to the same enzyme protein. Furthermore, detailed studies of the purified alcohol dehydrogenase confirmed that the lower of the two close bands detected by electrophoresis represents a modified form of the native enzyme.

The electrophoretic mobilities of the alcohol dehydrogenase and butanediol dehydrogenase from *X. autotrophicus*, *A. faecalis*, *A. paradoxus*, *A. denitrificans*, *A. ruhlandii* and *A. eutrophus* strains CH34, A7, JMP134 and 707 were different from each other (Table 1 and Fig. 1). The discrete bands corresponding to each activity were close together in samples from a wide range of strains. The coincidence of the ethanol and butanediol dehydrogenase activity bands detectable in the electropherograms of some species indicates that both enzyme activities are probably due to the same enzyme protein. Distinct bands for alcohol dehydrogenase activity without butanediol dehydrogenase activity were found only in the gel tracks of *A. eutrophus* strains 707, JMP134 and A7. *Alcaligenes paradoxus* was the only strain in which alcohol dehydrogenase activity but no butanediol dehydrogenase activity was detected.

The butanediol dehydrogenases of most strains were almost completely inhibited by 1 mM-2-mercaptoethanol, as was shown previously for the purified enzyme from *A. eutrophus* strain N9A. Only the butanediol dehydrogenases from *A. ruhlandii* and *X. autotrophicus* were inhibited to a minor extent (Table 1).

Protein pattern

The protein patterns obtained by electrophoretic separation of the cytoplasmic proteins of *A. hydrogenophilus* and *A. eutrophus* strains H16, N9A, B19, TF97, H1, and H20 were almost identical (Fig. 1a). The protein pattern of all other strains deviated from this type to various extents. The highest degree of similarity between strains that differed from *A. eutrophus* existed between *A. denitrificans* and *A. ruhlandii*; this similarity was reflected not only by the protein pattern (Fig. 1a) but also by the pattern resulting from activity staining (Fig. 1b-d).

Plasmid-free strains

Identical activities of the fermentation enzymes were detected in the wild-type strain N9A of *A. eutrophus* and in its plasmid-free derivative N9AF06 as well as in the wild-type strain of the type strain of *A. eutrophus* and its plasmid-free derivative strain TF97. This indicates that these plasmids, which appear to be identical with the megaplasmid pHG1 isolated from *A. eutrophus* H16 (Friedrich *et al.*, 1981a), are not involved in the synthesis of fermentation enzymes.

However, the absence of the plasmid was reflected by the electrophoretic protein patterns. Compared to other strains the protein patterns of strains N9AF06 and TF97 lacked at least one major band (relative mobility of about 0.08, Fig. 1a).

Hydrogenase activities are derepressed in *A. eutrophus* under various growth conditions, those of restricted oxygen supply included (Friedrich *et al.*, 1981b), and can contribute 1 to 2% of the total cell protein (K. Schneider, personal communication). The protein band absent from N9AF06 and TF97 was not one of the hydrogenases; activity staining of the gels indicated that the relative mobility of soluble hydrogenase was 0.35 and of the membrane-bound enzyme, 0.24. Furthermore, the electrophoretic mobilities of the soluble and membrane-bound hydrogenase

purified from strain H16, which were kindly provided by Dr K. Schneider, were also not identical to the plasmid-encoded protein with a relative mobility of 0.08, the major protein band.

DISCUSSION

Since the first report of the presence of NAD-dependent dehydrogenases for lactate, ethanol and butanediol in *A. eutrophus* N9A (Schlegel & Vollbrecht, 1980), evidence has accumulated that many obligately aerobic bacteria, which rely only on a strictly respiratory metabolism, can form fermentation enzymes. This indicates that the corresponding genetic information is present. Typical fermentation products were detected in cultures of various *Pseudomonas* strains and *Paracoccus denitrificans* (Vollbrecht, 1980; Vollbrecht & El Nawawy, 1980), and alcohol and 2,3-butanediol dehydrogenases were detected in strains of *Paracoccus denitrificans* (Nokhal, 1981) that had been cultivated under conditions of restricted oxygen supply. Recently, heterofermentative lactic acid fermentation was demonstrated in *Thiobacillus neapolitanus* (Beudeker *et al.*, 1981). The present study expanded the group of strictly aerobic bacteria known to carry genetic information for fermentation enzymes. Among 20 strains investigated, only in *A. latus* and *N. opaca* were none of these enzyme activities detected. In view of the wide distribution of derepressible fermentation enzymes the question arises whether these 'silent genes' (Riley & Anilionis, 1978) are redundant or may be profitable for this group of strictly aerobic bacteria.

A good correlation exists between the occurrence and properties of the three fermentation enzymes and the taxonomical assignment of the strains and species studied, with few exceptions. PAGE of the cytoplasmic proteins provided further characters for strain differentiation. The electrophoretic mobilities of the three enzymes as well as the banding patterns of the total soluble proteins reflected the differences and similarities detected by enzyme measurements and characterization. The suitability of protein electropherograms for differentiating various species of the genus *Alcaligenes* had been demonstrated before (Kerstens & De Ley, 1980).

Alcaligenes eutrophus strains H16, N9A and derivatives, B19, H1, H20 and TF97 were very similar to each other and indistinguishable with respect to both the electropherograms of cytoplasmic proteins and various properties of the three fermentation enzymes. *Alcaligenes hydrogenophilus* strain 1978 isolated in Japan (Ohi *et al.*, 1979) was almost indistinguishable from most of the autotrophic *A. eutrophus* strains. As the similarity pertains to substrate specificity and even includes the presence of two hydrogenases (Schlegel, 1983), this strain should probably be considered to be a subspecies of *A. eutrophus* rather than a separate species.

Apart from the core of *A. eutrophus* and *A. hydrogenophilus*, *A. faecalis* CIP 62.31 was the only organism from which the lactate dehydrogenase was highly sensitive to oxaloacetate. However, the protein patterns after electrophoresis as well as the enzyme activity patterns differed significantly from the organisms mentioned above. On the basis of other data, *A. faecalis* CIP 62.31 is phenotypically and genotypically an aberrant strain, not belonging to *A. faecalis*, but belonging to the core group of *A. eutrophus* (K. Kersters, personal communication). By extending the investigations on the distribution of this remarkable lactate dehydrogenase the relationships between the species and strains of *Alcaligenes*, which is a taxonomically and physiologically heterogeneous group of bacteria, may be clarified.

This study has revealed that all other strains investigated differed widely from the core of *A. eutrophus* strains. Consequently, there are several reasons for removing *A. eutrophus* strain 707, which is considered as a slightly aberrant *A. eutrophus* strain (K. Kersters, personal communication) and *A. eutrophus* strain JMP134, A7 and CH34 from the core of *A. eutrophus*. *Alcaligenes ruhlandii* and *A. denitrificans* showed some similarities to each other with respect to the protein pattern as well as to the enzyme pattern. This is in agreement with the results from Kersters *et al.* (1981).

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REFERENCES

- ATKINSON, T., HAMMOND, P. M., HARTWELL, R. D., HUGHES, P., SCAWEN, M. D., SHERWOOD, R. F., SMALL, D. A. P., BRUTON, C. J., HARVEY, M. J. & LOWE, C. R. (1981). Triazine-dye affinity chromatography. *Biochemical Society Transactions* **9**, 290–293.
- BERGMAYER, H. U. (1974). *Methoden der Enzymatischen Analyse*, vol. 1, 3rd edn. Weinheim: Verlag Chemie.
- BEUDEKER, R. F., DE BOER, W. & KUENEN, J. G. (1981). Heterolactic fermentation of intracellular polyglucose by the obligate chemolithotroph *Thiobacillus neapolitanus* under anaerobic conditions. *FEMS Microbiology Letters* **12**, 337–342.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.
- FRIEDRICH, B., HOGREFE, C. & SCHLEGEL, H. G. (1981a). Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. *Journal of Bacteriology* **147**, 198–205.
- FRIEDRICH, C. G., FRIEDRICH, B. & BOWIEN, B. (1981b). Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*. *Journal of General Microbiology* **122**, 69–78.
- GARVIE, E. L. (1980). Bacterial lactate dehydrogenases. *Microbiological Reviews* **44**, 83–105.
- HÖHN-BENTZ, H. & RADLER, F. (1978). Bacterial 2,3-butanediol dehydrogenases. *Archives of Microbiology* **116**, 197–203.
- JÜTTNER, R. R., LAFFERTY, R. M. & KNACKMUSS, H. J. (1975). A simple method for the determination of poly- β -hydroxybutyric acid in microbial biomass. *European Journal of Applied Microbiology* **1**, 233–237.
- KERSTERS, K. & DE LEY, J. (1980). Classification and identification of bacteria by electrophoresis of their proteins. In *Microbiological Classification and Identification*, pp. 273–297. Edited by M. Goodfellow & R. G. Board. London: Academic Press.
- KERSTERS, K., SEGERS, P., GAYRAL, J. P. & DE LEY, J. (1981). The taxonomy of the genus *Alcaligenes* studied by auxanography and DNA:rRNA hybridizations. In *Abstracts of the IV International Conference on Culture Collections*, p. 34. Brno.
- MERGEAY, M., HOUBA, C. & GERITS, J. (1978). Extrachromosomal inheritance controlling resistance to cadmium, cobalt and zinc ions: evidence from curing in a *Pseudomonas*. *Archives internationales de physiologie et de biochimie* **86**, 440–441.
- NOKHAL, T.-H. A. A. (1981). *Paracoccus denitrificans: Neuisolation und Untersuchungen zur Taxonomie und Stoffwechselphysiologie*, pp. 117–151. Dissertation, Universität Göttingen, F.R.G.
- OHI, K., TAKADA, N., KOMEMUSHI, S., OKAZAKI, M. & MIURA, Y. (1979). A new species of hydrogen-utilizing bacterium. *Journal of General and Applied Microbiology* **25**, 53–58.
- PALLERONI, N. J. & PALLERONI, A. V. (1978). *Alcaligenes latus*, a new species of hydrogen-utilizing bacteria. *International Journal of Systematic Bacteriology* **28**, 416–426.
- PEMPERTON, J. M., CORNEY, B. & DON, R. H. (1979). Evolution and spread of pesticide degrading ability among soil microorganisms. In *Plasmids of Medical, Environmental and Commercial Importance*, pp. 287–299. Edited by K. N. Timmis & A. Pühler. Amsterdam: Elsevier/North Holland Biomedical Press.
- RILEY, M. & ANILIONIS, A. (1978). Evolution of the bacterial genome. *Annual Review of Microbiology* **32**, 519–560.
- SCHLEGEL, H. G. (1983). Studies on the regulation and genetics of enzymes of *Alcaligenes eutrophus*. In *Microbial Ecology and Metabolism*. Edited by G. A. Codd & W. D. P. Stewart. London, New York: Academic Press (in the press).
- SCHLEGEL, H. G. & STEINBÜCHEL, A. (1981). Die relative Respirationsrate (RRR), ein neuer Belüftungsparameter. In *Fermentation*, pp. 11–26. Edited by R. M. Lafferty. Wien, New York: Springer Verlag.
- SCHLEGEL, H. G. & VOLLBRECHT, D. (1980). Formation of the dehydrogenases for lactate, ethanol and butanediol in the strictly aerobic bacterium *Alcaligenes eutrophus*. *Journal of General Microbiology* **117**, 475–481.
- SCHUSTER, E. (1967). *Chemolithotrophes Wachstum von Hydrogenomonas H16 im Chemostaten mit elektrolitischer Knallgaszerzeugung*. Dissertation, Universität Göttingen, F.R.G.
- SCHWIEN, U. & SCHMIDT, E. (1982). Improved degradation of monochlorophenols by a constructed strain. *Applied and Environmental Microbiology* **44**, 33–39.
- SENIOR, P. J. & DAWES, E. A. (1973). The regulation of poly- β -hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Biochemical Journal* **134**, 225–238.
- SENIOR, P. J., BEECH, G. A., RITCHIE, G. F. & DAWES, E. A. (1972). The role of oxygen limitation in the formation of poly- β -hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. *Biochemical Journal* **128**, 1193–1201.
- STEINBÜCHEL, A. & SCHLEGEL, H. G. (1983a). NAD-linked L(+)-lactate dehydrogenase from the strict aerobe *Alcaligenes eutrophus*. 1. Purification and properties. *European Journal of Biochemistry* **130**, 321–328.
- STEINBÜCHEL, A. & SCHLEGEL, H. G. (1983b). NAD-linked L(+)-lactate dehydrogenase from the strict aerobe *Alcaligenes eutrophus*. 2. Kinetic properties and inhibition by oxaloacetate. *European Journal of Biochemistry* **130**, 329–334.
- STEINBÜCHEL, A. & SCHLEGEL, H. G. (1983c). The rapid purification of lactate dehydrogenase from *Alcaligenes eutrophus* in a two-step procedure. *European Journal of Applied Microbiology and Biotechnology* **17**, 163–167.
- VOLLBRECHT, D. (1980). Oxygen deficiency and excretion of metabolites by strictly aerobic bacteria. *Biotechnology Letters* **2**, 49–54.
- VOLLBRECHT, D. & EL NAWAWY, M. A. (1980). Restricted oxygen supply and excretion of metabolites. I. *Pseudomonas* spec. and *Paracoccus denitrificans*. *European Journal of Applied Microbiology and Biotechnology* **8**, 135–143.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.
- WILDE, E. (1962). Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas*. *Archiv für Mikrobiologie* **43**, 109–137.