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CHAPTER 3.1.9

The Genus *Zymomonas*

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Habitat

Zymomonas mobilis has been reported mainly from tropical and subtropical habitats, e.g., sugar-rich, plant saps from agave (Mexico; Lindner, 1928), sugar cane (Brazil and Fiji Islands; reviewed in Falcao de Morais et al., 1993), and palm wine from central Africa (Swings and De Ley, 1977). Other sources of this organism include fermenting sugarcane juice (Goncalves de Lima et al., 1970), fermenting cocoa beans (Ostovar and Keeney, 1973), and bees and ripening honey (Ruiz-Argueso and Rodriguez-Navarro, 1975). In Europe, *Z. mobilis* also appeared in spoiled beer and cider. One of the first written descriptions of "cider sickness" was presented by Lloyd (1903), in which he noted the presence of "sulphuretted hydrogen" in spoiled ciders. Barker and Hillier (1912) were the first to study cider sickness extensively and gave a description of the responsible bacterium. Cider sickness is recognized by frothing and abundant gas formation, a typical change in the aroma and flavor, reduction of sweetness, and development of a marked turbidity forming a heavy deposit. From the complex microflora of sick cider, Barker and Hillier isolated and purified a bacterium that caused the typical strong aroma and flavor in sterile cider upon reinfection; unfortunately, they did not give a Latin taxon to the new organism. In 1951, Millis isolated several bacteria from ciders and perries that resembled Barker's strains (Millis, 1951). It was Millis who demonstrated quite clearly that the cider sickness organism was a *Zymomonas*. Various strains formed approximately 1.9 mole of ethanol per 1 mole of glucose and about the same amount of CO_2. Furthermore, some H_2S, acetaldehyde and lactic acid could be detected. The optimal pH range for growth was 4.5 to 6.5 and the optimal temperature range was 25 to 31°C.

Pulque and Palm Wine

During his stay in Mexico in 1923 to 1924, Lindner studied the fermentation of agave sap to pulque, an alcoholic beverage containing approximately 4 to 6% ethanol (Lindner, 1928). Lindner discovered the causal organism of the fermentation, a bacterium which he called *Termobacterium mobile* (Lindner, 1931). Over the years this organism received many names. This very motile bacterium was able to ferment sucrose, fructose and glucose to ethanol and CO_2. Lindner suggested that these bacteria occur strictly in tropical regions, where they account for the alcoholic fermentations in palm wines, chica beer, etc., and where they are used for bread manufacture. *Zymomonas* strains are very well adapted to a great variety of plant juices in tropical areas around the world because in such environments sucrose, glucose, fructose, amino acids and growth factors are present. The bacterium is resistant to ethanol and grows at low pH values under anaerobic conditions. Kluyver and Hoppenbrouwers (1931) studied Lindner's isolate. Neither Lindner nor Kluyver and Hoppenbrouwers related the pulque bacteria to the cider sickness organisms described earlier by Barker and Hillier (1912).

Beer

Shimwell (1937) isolated a *Zymomonas* strain from beer, from the surface of brewery yards, and from the brushes of cask-washing machines. The cells were Gram-negative plump rods, 2 to 3 by 1 to 1.5 μm in young cultures and longer (without endospores) in old cultures. The organism was indifferent to hop antiseptic and capable of growing in a wide range of beers. Later, Ault (1965) recognized that *Zymomonas* is a serious contaminant in keg beers. These infections can occur because of anaerobiosis and presence of priming sugars. The bacteria produce a heavy turbidity, and the unpleasant odor of rotten apples is due to traces of acetaldehyde and H_2S. *Zymomonas* has not been reported in lager beers; the low temperature of the process, 8 to 12°C, is unfavorable for its growth.

Isolation

Several procedures are described in the literature for the isolation of *Zymomonas* strains

(Swings and De Ley, 1977). Barker and Hillier (1912) isolated the cider sickness organisms on beer wort gelatin as small colonies after 11 days incubation at 22°C. Swings (1974) described the isolation of *Zymomonas* from fresh palm wines in WL differential medium, which is designed for brewing and fermentation processes but contains a yeast and mold inhibitor (Difco). When the samples were streaked on the medium in Petri dishes and incubated at 30°C in the Gas Pak anaerobic system, colonies of 1 to 4 mm in diameter were obtained after 4 to 5 days. However, it was very important to use young wine, about 24-h old, because isolation from palm wines more than 48-h old was impossible; the viability of *Zymomonas* rapidly decreases in aged fermentation broths. Swings and De Ley (1977) described a selection medium containing 0.3% malt extract, 0.3% yeast extract, 2% glucose, 0.5% peptone and 0.002% cycloheximide. The medium is adjusted to pH 4.0, autoclaved, and then supplemented with ethanol to a final concentration of 3%.

For *Zymomonas* cultivation, Fein et al. (1983) described a defined medium consisting of (g per liter of distilled water): glucose, 20; KH_2PO_4, 3.5; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7\ H_2O$, 1.0; $FeSO_4 \cdot 7\ H_2O$, 0.01; and 2-[N-morpholino]ethanesulfonate (MES), 19.52. The medium is adjusted to pH 5.5 and autoclaved. Then, a sterile solution of biotin and Ca-pantothenate is added to final concentrations of 0.001 g/liter each. A complete medium was introduced by Bringer-Meyer et al. (1985; g per liter of distilled water): D-glucose, 20; yeast extract, 10; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 1.0; and $MgSO_4 \cdot 7\ H_2O$, 0.5.

Growth and Conservation Conditions

Zymomonas grows best between 25 and 30°C; 74% of the strains grow at 38°C, but rarely at 40°C (Swings and De Ley, 1977). Growth is slow at 15°C (Millis, 1951) and absent at 4°C. Members of the subspecies *pomaceae* do not grow at temperatures above 36°C; therefore a growth temperature test is a good method for distinguishing between the two subspecies.

Most *Zymomonas* strains are able to grow between pH 3.8 and 7.5. At pH 3.5, 40% of the strains develop, illustrating a good acid tolerance. This is not surprising because the natural niche of this organism is in acid palm wines, cider and beers at pH 4. We observed that death rapidly occurs in batch fermentation after all the sugar is fermented. The bacterial cells are kept alive on complex media at room temperature and transferred weekly. When lyophilized, the organism can be kept alive for several years.

Identification

In the 1970s, Swings and De Ley applied modern molecular biological techniques to compare approximately 40 different strains of *Zymomonas*. They concluded that all the strains of *Zymomonas* by then described belong to a single species, *Zymomonas mobilis*, with two subspecies: *Z. mobilis* subsp. *mobilis* (the organism isolated from pulque or palm wine and currently envisioned for industrial ethanol production) and *Z. mobilis* subsp. *pomaceae* (the organism responsible for beer and cider spoilage; Swings and De Ley, 1977). Growth at 36°C is the best phenotypic test to differentiate the subspecies *mobilis* from *pomaceae*; only the former grows.

Once *Zymomonas* has been isolated on an appropriate medium, its identification is a relatively simple matter. A listing of the phenotypic characteristics of *Zymomonas* is given in Table 1 (Montenecourt, 1985).

Inoculation of *Zymomonas* into media containing sugar will lead to growth and gas production only when glucose, fructose or possibly

Table 1. Phenotypic description of the genus *Zymomonas*.

1. Gram-negative rods, 2- to 6-µm length, 1- to 5-µm width
2. Either motile or nonmotile; motility can be easily lost; one to four lophotrichous flagella
3. Pleomorphic cell arrangement, rosettes, chains, filaments
4. Spores, capsules, intracellular storage compounds (lipids, glycogen and poly-β-hydroxybutyrate) absent
5. Catalase positive, oxidase negative
6. Anaerobic and microaeroduric
7. Ferments glucose and fructose producing ≥1.5 moles of ethanol and CO_2
8. Sucrose utilization inducible, may be accompanied by levan production
9. No other monosaccharides, disaccharides, polysaccharides or fatty acids metabolized
10. Contains pentacyclic triterpenoids (hopanoids), vaccenic acid, and sphingolipids in its cell membranes
11. Forms sorbitol and gluconic acid from sucrose or glucose fructose (presence of a periplasmic enzyme, glucose-fructose oxidoreductase)
12. G+C content, 47.5 to 49.5 mol %; genome size approximately 2,085 kb

Adapted from Montenecourt, 1985.

sucrose is present. The ratio (moles of ethanol produced to moles of glucose fermented) is at least 1.5, and only small amounts of lactic acid and traces of acetylmethylcarbinol (acetoin) are formed; thus, a pH indicator will not register any acidity. This feature makes *Zymomonas* a unique ethanol-producing bacterium, and as far as is known, no other bacteria behave in this manner. If a finer distinction is to be made, tests listed by Swings and De Ley (1977) may be applied. Recently, it was demonstrated that in comparison to other Gram-negative bacteria, *Zymomonas* strains contained a high level of cis-vaccenic acid (>60% of the total fatty acids; Tornabene et al., 1982). Furthermore, the lipopolysaccharide fraction from this organism is different from that of other Gram-negative species inasmuch as deoxyhexoses, pentoses, hexoses, aminopentose, uronic acid, phosphate and myristic acid are the principal constituents, whereas ketodeoxyoctulonic acid (KDO), heptoses or hydroxy fatty acids are not evident.

Main Features

Zymomonas mobilis is an aerotolerant, fermentative bacterium with a number of exceptional characteristics: over 95% of the glucose utilized by this organism is converted to an equimolar mixture of ethanol and CO_2, and only a small percentage (<3%) is incorporated into cell mass (Swings and De Ley, 1977). Catabolism of the only carbon sources, glucose and fructose, proceeds via the Entner-Doudoroff pathway and with a net production of a single mole of ATP per mole of glucose catabolized. *Zymomonas mobilis* lacks other pathways for glucose catabolism, is incapable of gluconeogenesis, and has an incomplete tricarboxylic acid cycle (Gibbs and De Moss, 1954; Dawes et al., 1970; Swings and De Ley, 1977). The presence of pyruvate decarboxylase and alcohol dehydrogenase enables the organism to perform a pure ethanol fermentation, i.e., to produce almost two moles of ethanol per mole of glucose. Because of its rapid growth, sugar catabolism (about 1 μmole glucose · min^{-1} · mg $^{-1}$ cell protein; Arfman et al., 1992), and tolerance towards high concentrations of substrate (up to 30% glucose; Kluyver and Hoppenbrouwers, 1931; Swings and De Ley, 1977; Loos et al., 1994) and product (up to 13% ethanol w/v; Rogers et al., 1982), *Z. mobilis* ferments ethanol very efficiently. *Zymomonas*, *Rhizomonas* and *Sphingomonas* belong to the same subclass (α-4) of the Proteobacteria (White et al., 1993). These genera contain sphinganine lipids in their membranes (Tahara and Kawazu, 1994). The physiological properties and biotechnological aspects of *Z. mobilis* have been reviewed (Swings and De Ley, 1977; Rogers et al., 1982; Baratti and Bu'Lock, 1986; Buchholz et al., 1987; Bringer-Meyer and Sahm, 1988; Viikari, 1988; Ingram et al., 1989; Sahm et al., 1992; Conway, 1992; Johns et al., 1992; Sprenger, 1993a; Sprenger et al., 1993b; Doelle et al., 1993; Yanase and Kato, 1994; Sprenger, 1996; Gunasekaran and Raj, 1999; Sprenger and Swings, 2000).

Metabolism: From Sugar Uptake to Ethanol Formation

Carbohydrate Transport

Zymomonas mobilis utilizes only three carbon sources: sucrose, glucose and fructose. Sucrose needs no uptake system because it is cleaved extracellularly and its moieties (glucose and fructose) are subsequently taken up into the cells by facilitated diffusion through a common transport protein (glucose facilitator GLF; DiMarco and Romano, 1985; Snoep et al., 1994; Weisser et al., 1996; Parker et al., 1997). *Zymomonas mobilis* appears to be the only known bacterium that relies solely on such an uniport type for sugar uptake, i.e., equilibration of external and internal sugar concentrations. No phosphoenolpyruvate (PEP)-dependent sugar uptake system has been detected yet (DiMarco and Romano, 1985). The uniporter needs no metabolic energy but cannot accumulate substances. Subsequent phosphorylation steps, however, distract free hexoses from the equilibrium so that effective sugar uptake and metabolism are warranted. Apart of glucose and fructose, various other hexoses and pentoses are GLF substrates (DiMarco and Romano, 1985; Schoberth and de Graaf, 1993; Parker et al., 1995; Parker et al., 1997; Weisser et al., 1995; Weisser et al., 1996), but D-mannose and D-xylose are no-growth substrates for wild-type *Z. mobilis* (but see Metabolic Engineering). Glucose is the preferred substrate (K_m ~4 mM) over fructose or xylose (K_m ~40 mM each). The GLF transports its substrates at high V_{max} (up to 1 μmole of substrate · mg^{-1} of cell protein · min^{-1}), with D-xylose being the best substrate, followed by glucose and fructose (Weisser et al., 1996; Parker et al., 1997). Growth on fructose leads to an increase in the transcription of *glf*, and the increased GLF may compensate for the lower affinity with fructose (Zembrzuski et al., 1992).

Owing to high glucose and fructose transport rates, equilibration between external and internal sugar concentrations can be rapid and may contribute to osmotic adjustment (Struch et al., 1991). Although not a growth substrate for *Z. mobilis*, the compatible solute sorbitol accumulates (up to 1 M, intracellular concentration) and further improves growth of *Z. mobilis* in high-

sugar environments (Loos et al., 1994). The sorbitol transporter is dependent on the proton motive force (Loos et al., 1994). Ethanol diffusion across the cell membrane has been determined using nuclear magnetic resonance (^{13}C-NMR) techniques (Schoberth et al., 1996).

Glycolytic Flux and Regulation of Metabolism

The glycolytic enzymes of the Entner-Doudoroff pathway and the fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase of *Z. mobilis* represent over half of the organism's soluble protein (Algar and Scopes, 1985; Osman et al., 1987; An et al., 1991). These enzymes (see Table 2 for details and references) are considered to operate at or near substrate saturation and do not seem to be subject to major regulations, e.g., by allosteric control (Algar and Scopes, 1985). The overall catabolic activity of *Z. mobilis* leads to a nearly unlimited flow of carbon through the glycolytic pathway. Thus each minute, *Z. mobilis* consumes an amount of glucose equal to one-third of its mass (Snoep et al., 1996; Parker et al., 1997). Regulation of enzyme activity has been shown for glucokinase through an absolute demand for inorganic phosphate, and to a lesser degree, by feedback inhibition through glucose-6-phosphate (Scopes and Bannon, 1995). Fructokinase is inhibited by glucose (K_i of 0.14 mM; Scopes et al., 1985b). Pyruvate kinase, which is allosterically regulated in most other organisms, appears not to be subject to any allosteric activator (Steiner et al., 1998). Growth on fructose induces about twofold the transcription of genes for peripheral sugar metabolism which lead to the introduction of fructose into glycolysis (i.e., by fructose uptake via GLF, fructose phosphorylation by fructokinase FRK, and

Table 2. Glycolytic enzymes and their genes.

Gene	Function/enzyme	Protein characterization	Sequence reference
glf	Glucose/fructose transporter	DiMarco and Romano, 1985 Parker et al., 1995, 1997 Weisser et al., 1995, 1996	Barnell et al., 1990
glk	Glucokinase	Scopes et al., 1985	Barnell et al., 1990
frk	Fructokinase	Doelle, 1982 Scopes et al., 1985 Weisser et al., 1996	Zembrzuski et al., 1992
pgi	Glucose 6-P isomerase	Hesman et al., 1991	Hesman et al., 1991
zwf	Glucose 6-P dehydrogenase	Scopes et al., 1985 Scopes, 1997	Barnell et al., 1990
pgl	6-Phosphogluconolactonase	Scopes, 1985	n.a.
edd	6-Phosphogluconate dehydratase	Scopes and Griffiths-Smith, 1984	Barnell et al., 1990
eda	KDPG aldolase	Scopes, 1984	Conway et al., 1991
gap	Ga 3-P dehydrogenase	Pawluk et al., 1986	Conway et al., 1987d
pgk	Phosphoglycerate kinase	Pawluk et al., 1986	Conway and Ingram, 1988
pgm	Phosphoglycerate mutase	Pawluk et al., 1986	Yomano et al., 1993
eno	Enolase	Pawluk et al., 1986	Burnett et al., 1992
pyk	Pyruvate kinase	Pawluk et al., 1986 Steiner et al., 1998	Steiner et al., 1998
pdc	Pyruvate decarboxylase	Bringer-Meyer et al., 1986 Neale et al., 1987	Brau and Sahm, 1986 Conway et al., 1987b Neale et al., 1987 Reynen and Sahm, 1988
adhA	Alcohol dehydrogenase I (zinc-dependent)	Wills et al., 1981 Neale et al., 1986	Keshav et al., 1990
adhB	Alcohol dehydrogenase II (iron-dependent)	Wills et al., 1981 Neale et al., 1986	Conway et al., 1987c
pdhA-D	Pyruvate dehydrogenase complex		Neveling et al., 1998
gfo	Glucose-fructose oxidoreductase	Zachariou and Scopes, 1986 Hardman and Scopes, 1988 Wiegert et al., 1997	Kanagasundaram and Scopes, 1992a Wiegert et al., 1997
gnl	Gluconolactonase		Kanagasundaram and Scopes, 1992b
sacA	Sucrase or invertase (intracellular)	Gunasekaran et al., 1990 Yanase et al., 1991	
sacB	Sucrase or invertase B (extracellular)	O'Mullan et al., 1992 Yanase et al., 1995	Kyono et al., 1995
levU	Levansucrase	Yanase et al., 1992 Yanase et al., 1991	Song et al., 1993 Kyono et al., 1995

isomerization to glucose-6-phosphate by phosphoglucose isomerase PGI; Scopes et al., 1985b; Hesman et al., 1991; Barnell et al., 1992; Zembrzuski et al., 1992).

Control on glycolytic flux is mainly exerted by glucose-6-phosphate dehydrogenase as shown by overexpression studies of several glycolytic enzymes of *Z. mobilis* (Snoep et al., 1995; Snoep et al., 1996). When overexpressing each of the first genes (*glf*, *zwf*, *edd*, *glk*) of the Entner-Doudoroff pathway (see Fig. 1), only recombinants with elevated glucose-6-phosphate dehydrogenase had a 10–13% higher glycolytic flux than that of the native organism, whereas increasing the expression of various other glycolytic operons caused a significant decrease in the glycolytic flux and growth rate. The latter was attributed to a protein burden effect (Snoep et al., 1995). Additional evidence for a major role of glucose-6-phosphate dehydrogenase in flux control was given by kinetic investigations which showed that the enzyme is allosterically inhibited by phosphoenolpyuvate (Scopes, 1997). Furthermore it was proposed that differences in transcript stability of the glycolytic enzymes of Z. mobilis represent a mechanism to balance the high levels of the ethanologenic enzymes (Meija et al., 1992). Hence, expression of glycolytic and

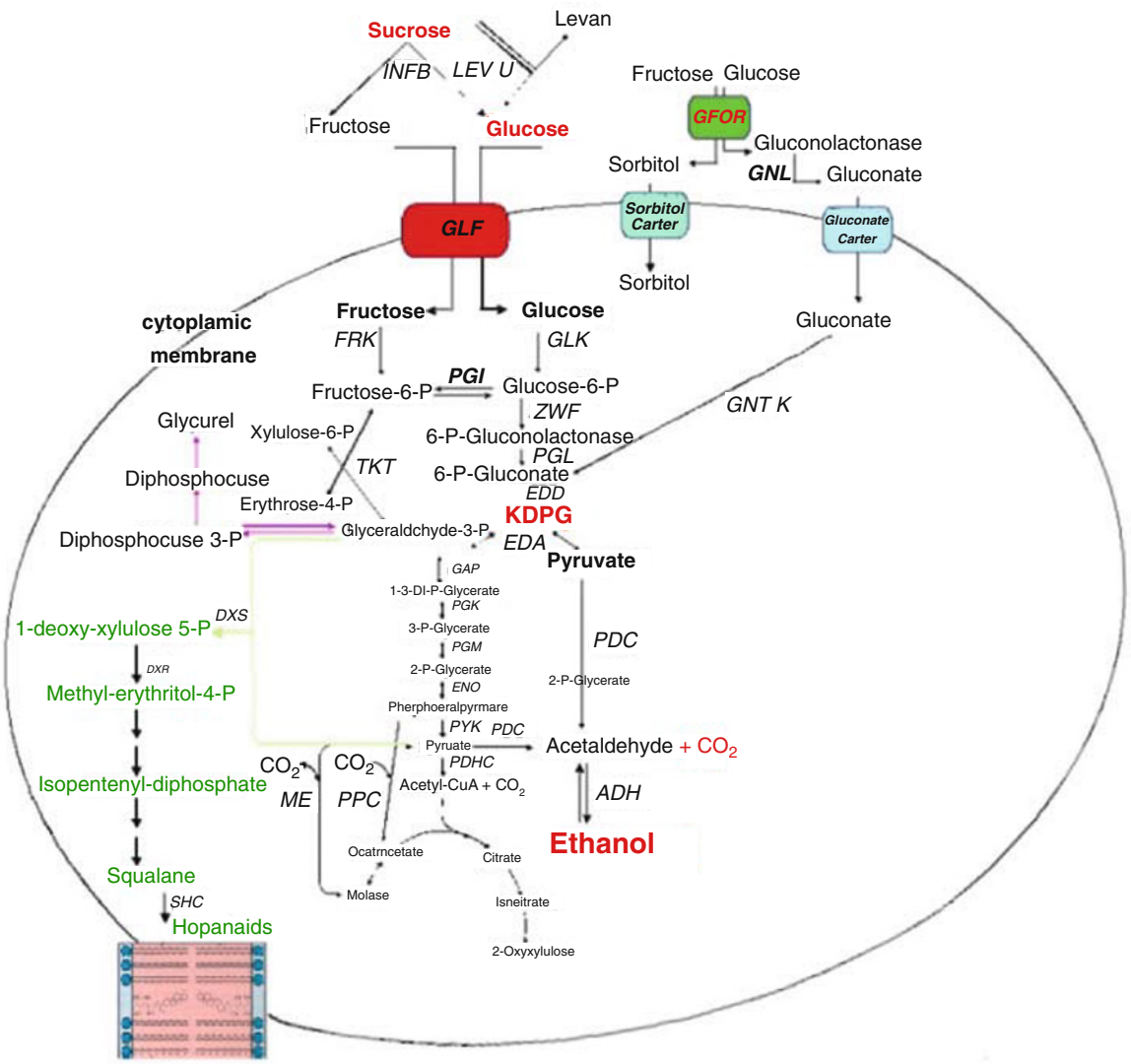

Fig. 1. Overview of main metabolic features in a schematic *Zymomonas mobilis* cell. Steps in carbohydrate metabolism (uptake and metabolism of sucrose, glucose, fructose, gluconate, sorbitol) are given together with anabolic reactions (incomplete tricarboxylic acid (TCA) pathway, formation of by-products, formation of hopanoids). Abbreviations: DXR = deoxyxylulose 5-phosphate reductoisomerase; DXS = 1-deoxy-xylulose 5-phosphate synthase; GNT K = gluconate kinase; ME = malic enzyme; PGL = phosphogluconolactonase; PPC = phosphoenol-pyruvate carboxylase; TKT = transketolase. For other abbreviations of enzymes see Table 2.

fermentative enzymes relies mainly on mRNA stability (the half-lives of transcripts for the glycolytic enzymes were in the range of 8–18 minutes; Meija et al., 1992). This relative mRNA stability may distinguish highly expressed glycolytic genes from biosynthetic genes. Differential expression of the *gap* and *pgk* genes, which form an operon, also has been attributed to differences in mRNA stability (Eddy et al., 1989).

When used in in vitro fermentations, the Entner-Doudoroff enzymes were remarkably resistant to ethanol inactivation and produced up to 16.5% (w/v) ethanol (Scopes and Griffiths-Smith, 1986). In living cells, 3-phosphoglycerate accumulated in the presence of 10% (w/v) ethanol, as detected by ^{31}P NMR spectroscopy. Enzyme assays confirmed that phosphoglycerate mutase and enolase were inhibited 31% and 40%, respectively, in the presence of 10% (w/v) ethanol in the test system (Strohhäcker et al., 1993).

A global regulatory protein (Grp) with high sequence similarity to the *Escherichia coli* global regulator Lrp has been discovered and can complement a glutamate-uptake mutant of *E. coli* (Peekhaus et al., 1995). The *grp* gene lies adjacent to an operon for a high-affinity glutamate carrier; its true function, however, remains to be elucidated (Peekhaus and Krämer, 1996).

Pyruvate Decarboxylase

Pyruvate decarboxylase (PDC, EC 4.1.1.1), a key enzyme in ethanol fermentation, catalyzes the conversion of pyruvate to acetaldehyde and carbon dioxide and depends on thiamine diphosphate and Mg(II) ions for its catalytic activity. Though PDC is widely distributed in fungi and higher plants, it is rare in prokaryotes and unknown in animals (Candy and Duggleby, 1998). In addition to being in *Z. mobilis* (Hoppner and Doelle, 1983; Bringer-Meyer et al., 1986; Neale et al., 1987; Diefenbach and Duggleby, 1991), bacterial PDCs have been found in *Sarcina ventriculi* (Stephenson and Dawes, 1971; Lowe and Zeikus, 1992), *Acetobacter* species (King and Cheldelin, 1954; De Ley and Schell, 1959), and *Erwinia amylovora* (Haq and Dawes, 1971; Haq, 1984). In *Z. mobilis*, PDC is one of the most abundant proteins, contributing 4–6% to the soluble cell protein content (Bringer-Meyer et al., 1986; An et al., 1991). The enzyme is a homotetramer with a subunit molecular mass of 60.79 kDa calculated from the DNA sequence (Neale et al., 1987; Conway et al., 1987b; Reynen et al., 1988). In contrast to genes in *Saccharomyces cerevisiae*, only one gene seems to code for PDC in *Z. mobilis* (Neale et al., 1987; König, 1998). *Zymomonas mobilis* PDC exhibits normal Michaelis-Menten kinetics with a K_m for pyruvate of 0.3–0.4 mM (Bringer-Meyer et al., 1986; Neale et al., 1987). This is exceptional because all other PDCs that have been characterized up to now are allosterically regulated by the substrate or other activator molecules such as pyruvamide (König, 1998). Recently, determination of the 3D crystal structure of the PDC from *Z. mobilis* revealed that the interface area between the dimers of the enzyme is much larger than in the yeast PDC. In addition, the dimers are more tightly packed in the PDC from *Z. mobilis*, thus preventing large rearrangements in the quarternary structure and locking the enzyme in an activated conformation (Dobritzsch et al., 1998). The critical amino acid residues involved in cofactor binding (Asp^{440}, Asn^{467}, Gly^{469}), substrate binding (Asp^{27}, His^{113}, His^{114}, Tyr^{290}, Thr^{388}, Glu^{473}) and catalysis (Glu^{473}) of the *Z. mobilis* PDC have been identified by site-directed mutagenesis (Sun et al., 1995; Pohl, 1997; Candy and Duggleby, 1998; Chang et al., 1999). A side reaction catalyzed by pyruvate decarboxylase is the carboligase activity, where the activated acetaldehyde bound to the thiamine diphosphate cofactor is condensed to a second aldehyde molecule (Bringer-Meyer and Sahm, 1988; Bornemann et al., 1995; Bruhn et al., 1995). This acyloin condensation reaction is used industrially for the production of phenylacetylcarbinol ([R]-1-hydroxy-1-phenylpropan-2-one), an intermediate in the synthesis of L-ephedrine. In *Z. mobilis* PDC, alanine replacement of the tryptophan (Trp^{392}) located near the active center enhanced the carboligase activity of the enzyme, increasing phenylacetylcarbinol formation by a factor of four (Bruhn et al., 1995).

Alcohol Dehydrogenase Isoenzymes

Zymomonas mobilis possesses two isoenzymes of fermentative alcohol dehydrogenase (ADH, EC 1.1.1.1): ADH I (a zinc-containing enzyme) and ADH II (an iron-containing enzyme; Wills et al., 1981; Hoppner and Doelle, 1983; Kinoshita et al., 1985; Neale et al., 1986). ADH activity is essential for the obligatory fermentative metabolism of *Z. mobilis*; therefore it is not surprising that the two ADH isoenzymes are abundant proteins in *Z. mobilis*, representing 2–5% of the soluble cell protein (Mackenzie et al., 1989). The ADH I, encoded by *adhA*, is a homotetramer with a subunit molecular mass of 36 kDa, and it contains one zinc atom per subunit (Wills et al., 1981; Neale et al., 1986; Keshav et al., 1990). The protein level of ADH I, which is more active at the early stages of growth, was found to decline in stationary-phase cells (Viikari, 1988; Keshav et al., 1990; An et al., 1991). The gene *adhB* encodes ADH II, also a homotetrameric enzyme with a subunit molecular mass of 40 kDa and one

iron atom per subunit. This enzyme catalyzes half the acetaldehyde reduction in *Z. mobilis* (Wills et al., 1981; Neale et al., 1986; Conway et al., 1987d). Under aerobic conditions, ADH I activity is fully conserved, but ADH II activity decays as the enzyme-bound Fe^{2+} atoms are oxidized (Tamarit et al., 1997). Whereas ADH I also oxidizes butanol, ADH II has almost no activity towards this substrate (Kinoshita et al., 1985; Neale et al., 1986). The enzyme has been found to transfer the pro-*R* hydrogen of NADH onto acetaldehyde (Glasfield and Brenner, 1989). Together with ADH IV of *Saccharomyces cerevisiae* and propanediol oxidoreductase of *E. coli*, ADH II of *Z. mobilis* forms a group of structurally related enzymes belonging to the iron-activated group III of dehydrogenases (Conway and Ingram, 1989; Reid and Fewson, 1994). These proteins share no homology with zinc-containing ADH enzymes (Cabriscol et al., 1994). The ADH II of *Z. mobilis*, identified as a major stress protein, was induced both by exposure to ethanol and by elevated temperature (Michel, 1993). The enzyme is expressed from tandem promoters which share partial sequence identity with the *E. coli* consensus sequence for heat shock proteins (An et al., 1991; Mackenzie et al., 1989). By a polymerase chain reaction (PCR)-mediated random mutagenesis, ADH II of *Z. mobilis* has been altered to produce more thermally stable variants. The same in vitro random mutagenesis technique enabled isolation of variant enzymes that had substrate specificities different from that of the wild-type enzyme, e.g., mutant enzymes active with butanol or with NADP (Rellos et al., 1997).

Ethanolic Fermentation

Zymomonas mobilis performs a highly productive ethanol fermentation and offers a number of advantages over the traditional yeast fermentation, i.e., higher sugar uptake and ethanol yield, lower biomass production, and oxygen independence (Rogers et al., 1982). Fermentations with *Z. mobilis* compared to those with yeast have a limited substrate range, which is restricted to glucose, fructose and sucrose (Swings and De Ley, 1977). Furthermore, the low salt tolerance of *Z. mobilis* poses problems for the fermentation of molasses, which usually have a high salt content (Montenecourt, 1985; Skotnicki et al., 1982). Nevertheless, it has been shown that glucose as well as unsterile hydrolyzed B-starch can be converted efficiently to ethanol in a continuous process employing a fluidized bed reactor (Bringer et al., 1984; Weuster-Botz, 1993a; Weuster-Botz et al., 1993b). In addition, processes for simultaneous saccharification and ethanol fermentation of starch or sugar cane have been developed (Kim and Rhee, 1993; Krishna et al., 1998). When glucose is fermented by nonaerated cultures, only insignificant amounts of by-products are formed. However, during growth of *Z. mobilis* in fructose-containing media the formation of acetoin, acetic acid, acetaldehyde, glycerol and dihydroxyacetone was more pronounced, and the cell yield was lower than when grown in glucose (Viikari, 1988; Johns et al., 1992; Horbach et al., 1994). Various genes encoding enzymes required for utilization of other carbon sources, e.g., starch, cellulose, raffinose, lactose, xylose or mannose, have been transferred into *Z. mobilis*; for details, the reader is referred to the chapter on metabolic engineering and to recent reviews (Doelle et al., 1993; Sprenger, 1993a; Gunasekaran and Raj, 1999). Another strategy for an efficient ethanol fermentation of such carbon sources is the transfer into *E. coli*, other enteric bacteria and also Gram-positive bacteria of the *Z. mobilis* genes *pdc* and *adhB* encoding pyruvate decarboxylase and alcohol dehydrogenase II, respectively (for a review see Ingram et al., 1998; Ingram et al., 1999).

During fermentation of sucrose by *Z. mobilis*, the formation of by-products such as levan and sorbitol decreases the ethanol yield (Viikari, 1988). Three types of transfructosylation occur in the presence of sucrose, resulting in the formation of free fructose, oligosaccharides and levan (Viikari, 1988). *Zymomonas mobilis* possesses three different sucrases: an intracellular sucrase (SacA or syn. InvA), an extracellular levansucrase (SacB or syn. LevU or syn. SucZE2), and an extracellular sucrase (SacC or syn. InvB or syn. SucZE3) (Kannan et al., 1997; Yanase et al., 1998; Song et al., 1999). Extracellular levansucrase and extracellular sucrase are involved in the sucrose metabolism of *Z. mobilis*, whereas the function of the intracellular sucrase is not understood (Kannan et al., 1997). The genes encoding for the extracellular levansucrase and sucrase are clustered on the chromosome (Gunasekaran et al., 1995), and the transcription of both genes was induced significantly when sucrose was added to the medium (Song et al., 1999). Extracellular levansucrase and sucrase do not carry an amino-terminal signal peptide usually found in proteins translocated across the cytoplasmic membrane (Gunasekaran et al., 1995; Kyono et al., 1995). In *E. coli* that carry the *Z. mobilis sucZE2* gene, a part of the expressed levansucrase was translocated across the inner membrane (Yanase et al., 1998), whereas the *Z. mobilis* gene for the extracellular sucrase expressed in *E. coli* did not lead to the enzyme's secretion (Kannan et al., 1995; Yanase et al., 1998). Levansucrase (EC 2.4.1.10) hydrolyses β-fructosides but not α-glucosides and catalyzes

levan formation from sucrose as well as from raffinose (Sangiliyandi et al., 1999). The optimum temperature for the polymerase activity (30°C) was lower than that for the hydrolase activity (50°C; Yanase et al., 1992; Sangiliyandi et al., 1999).

Sugar and Ethanol Tolerance

GLUCOSE-FRUCTOSE OXIDOREDUCTASE (GFOR) AND THE FORMATION OF SORBITOL The formation of sorbitol and gluconic acid from sucrose (or mixtures of glucose and fructose) by *Z. mobilis* has attracted much interest in the past, as yields of up to 90% for gluconic acid and up to 92% of sorbitol were reported for mixtures of glucose and fructose of up to 750 g/liter (Rehr et al., 1991; Silveira et al., 1999). A novel enzyme, glucose-fructose oxidoreductase (GFOR), with tightly bound NADP(H) as cofactor, has been detected (Zachariou and Scopes, 1986). The 3D crystal structure of the homotetramer GFOR enzyme has been elucidated recently (Kingston et al., 1996). So far, this enzyme is unique to *Zymomonas mobilis*, where it occurs in both subspecies, *mobilis* and *anaerobia* (Sprenger and Swings, 2000). The physiological function of this periplasmic enzyme (Loos et al., 1991; Aldrich et al., 1992) apparently lies in the formation of the compatible solute sorbitol. Sorbitol is accumulated in the cell (up to 1 M; Loos et al., 1994) and helps to counteract the detrimental effects of high sugar concentrations (Loos et al., 1994). A GFOR-deficient mutant, unable to form sorbitol (ACM3963; Kirk and Doelle, 1993), failed to grow in 1 M sucrose solutions, but after introduction of the wild-type *gfo* gene, it regained sorbitol forming ability and subsequently grew in high sucrose media (Wiegert et al., 1996). The periplasmic location of GFOR takes advantage of the concomitant presence of both substrates, glucose and fructose, at saturating concentrations (Sprenger, 1996), as the glucose facilitator prefers glucose over fructose (Weißer et al., 1995). The GFOR is formed as a preprotein with an unusually long N-terminal extension that serves as a signal sequence for the protein's export to the periplasm (Wiegert et al., 1996; Halbig et al., 1999). This signal sequence contains a so-called twin-arginine motif, which is a hallmark of a new class of Sec-independent protein export, the TAT or twin-arginine translocation in bacteria (Berks, 1996; Sargent et al., 1998; Berks et al., 2000). The TAT pathway transports folded proteins together with their redox cofactors to the bacterial periplasm.

HOPANOIDS IN *Z. MOBILIS*: MEDIATORS OF ETHANOL TOLERANCE AND FIRST INDICATORS OF THE EXISTENCE OF A NOVEL BIOSYNTHETIC PATHWAY FOR THE ISOPRENIC C_5 UNIT *Zymomonas mobilis* is capable of tolerating ethanol concentrations up to 13% (w/v). This ethanol tolerance is remarkably high and comparable to that of *Saccharomyces cerevisiae* (Rogers et al., 1982). The *Z. mobilis* cytoplasmic membrane contains a number of different hopanoids (Fig. 2): these membrane-stabilizing, pentacyclic

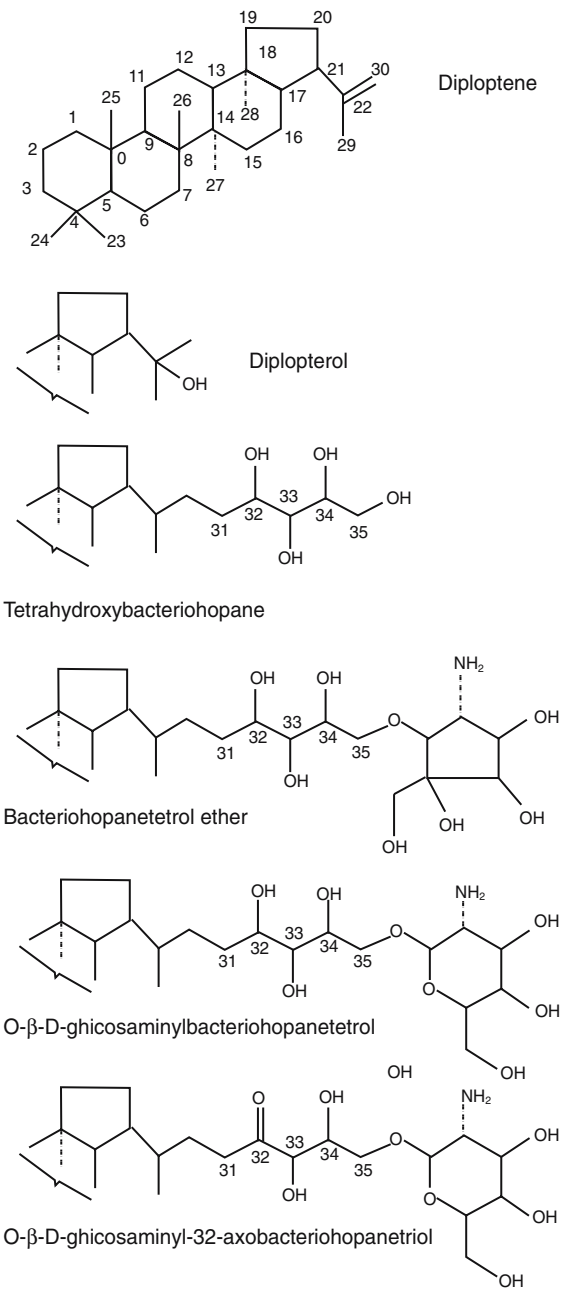

Fig. 2. Hopanoids from *Zymomonas mobilis*.

triterpenoids are present at concentrations of 30 mg/g cell dry weight (i.e., they contribute 40–50% to the organism's total lipid content; Hermans et al., 1991; Sahm et al., 1993; Moreau et al., 1997; Kannenberg and Poralla, 1999). Hopanoids play an important role in the ethanol tolerance of *Z. mobilis* by reinforcing the cytoplasmic membrane against the fluidizing effects of high concentrations of ethanol (Horbach et al., 1991; Sahm et al., 1993). Exposure to high levels of ethanol leads to an induction of several stress proteins (An et al., 1991) and their genes *adhB*, *dnaK*, and *groESL* (encoding for alcohol dehydrogenase II and the heat shock proteins DnaK, GroES and GroEL) have been cloned and characterized (Conway et al., 1987c; Michel, 1993; Barbosa et al., 1994). In continuous cultures at high ethanol concentrations and low dilution rates, sustained oscillations of biomass occur due to fluctuations of cell viability (Ghommidh et al., 1989; Vaija et al., 1993; Daugulis et al., 1997).

In vitro studies with cell-free extracts of *Z. mobilis* revealed that biosynthesis of the triterpenic moiety of the hopanoids proceeded from isopentenyl diphosphate via the intermediates farnesyl diphosphate and squalene (Shigeri et al., 1991); two enzymes involved in this part of isoprenoid biosynthesis in *Z. mobilis*, squalene synthase and squalene cyclase, were studied in detail and the corresponding genes were cloned and characterized (Reipen et al., 1995; Koukkou et al., 1996; Perzl et al., 1998).

However, the results of feeding studies with ^{13}C-labeled glucose and NMR spectroscopic analysis of the resulting labeling pattern of the triterpenoid moiety of the hopanoids clearly contradicted the classical mevalonate pathway for C_5 isoprenoid building-block isopentenyl diphosphate (IPP) biosynthesis (Flesch and Rohmer, 1989; Rohmer et al., 1989; Rohmer et al., 1993; Rohmer et al., 1996; Horbach et al., 1993; Sahm et al., 1993). Only recently was it evident that the unique characteristics of *Z. mobilis* could be of substantial help for the discovery of a novel, mevalonate-independent pathway for IPP biosynthesis, with glyceraldehyde-3-phosphate and pyruvate as precursors of isoprenic units (Rohmer et al., 1996). In this pathway, 1-deoxyxylulose 5-phosphate is formed as the first intermediate (Broers, 1994; Sprenger et al., 1997; Lois et al., 1998). Four further reactions of the pathway have been identified (Fig. 3; Takahashi et al., 1998; Rohdich et al., 1999; Lüttgen et al., 2000; Herz et al., 2000). The novel pathway is present in bacteria, green algae and the chloroplasts of higher plants (methylerythritol-4-phosphate pathway; Rohmer, 1999).

Special Features: Aerobic Metabolism, Pyruvate Dehydrogenase Multienzyme Complex

Aerobic Metabolism

Although strictly fermentative, *Z. mobilis* is an aerotolerant bacterium owing to the presence of a respiratory chain and the antioxidant enzymes catalase and superoxide dismutase (Belaich and Senez, 1965; Bringer et al., 1984; Pankova et al., 1985). However, at glucose concentrations >100 mM, oxygen inhibits growth due to an accumulation of acetaldehyde (Bringer et al., 1984; Pankova et al., 1985; Ishikawa et al., 1990). In the presence of oxygen, less NADH is available for the reduction of acetaldehyde to ethanol and therefore acetaldehyde accumulates to growth-inhibiting concentrations (Bringer et al., 1984; Pankova et al., 1985). Recently, continuous chemostat cultures of *Z. mobilis* growing aerobically with low glucose concentrations (<100 mM) exhibited a 2–2.5-fold increase in molar growth yields (Toh and Doelle, 1997; Zikmanis et al., 1997; Zikmanis et al., 1999). It was suggested that an inhibition of the proton pumping membrane ATPase by acetaldehyde in the presence of oxygen is the main cause for this increase. In this situation, membrane energization would be brought about by the respiratory activity (Toh and Doelle, 1997; Zikmanis et al., 1999).

The composition and function of the *Z. mobilis* electron transport chain is poorly understood, although the presence of a constitutive respiratory chain, containing cytochromes b, c and d, has been reported long ago (Belaich and Senez, 1965). Further studies have shown a structure with several membrane oxidoreductases and with branched electron pathways to oxygen. A hypothetical scheme is shown in Fig. 4. Cytoplasmic membrane vesicles oxidize NADH with a high specific rate, reaching 0.2-0.3 U · mg protein^{-1} (Bringer et al., 1984; Strohdeicher et al., 1990; Kalnenieks et al., 1995). Apart from the NADH oxidase, *Zymomonas* contains a membrane-linked NADPH oxidase with a similar activity (Bringer et al., 1984; Pankova et al., 1985). Two minor membrane oxidase activities have been reported: glucose oxidase, corresponding to a pyrroloquinoline quinone (PQQ)-containing glucose: ubiquinone oxidoreductase (Strohdeicher et al., 1988; Strohdeicher et al., 1990), and D-lactate oxidase (Kalnenieks et al., 1998). It has been demonstrated that electrons from both NADH and glucose are transported to oxygen via the quinone pool (Strohdeicher et al., 1990). Coenzyme Q_{10} (ubiquinone) has been found to

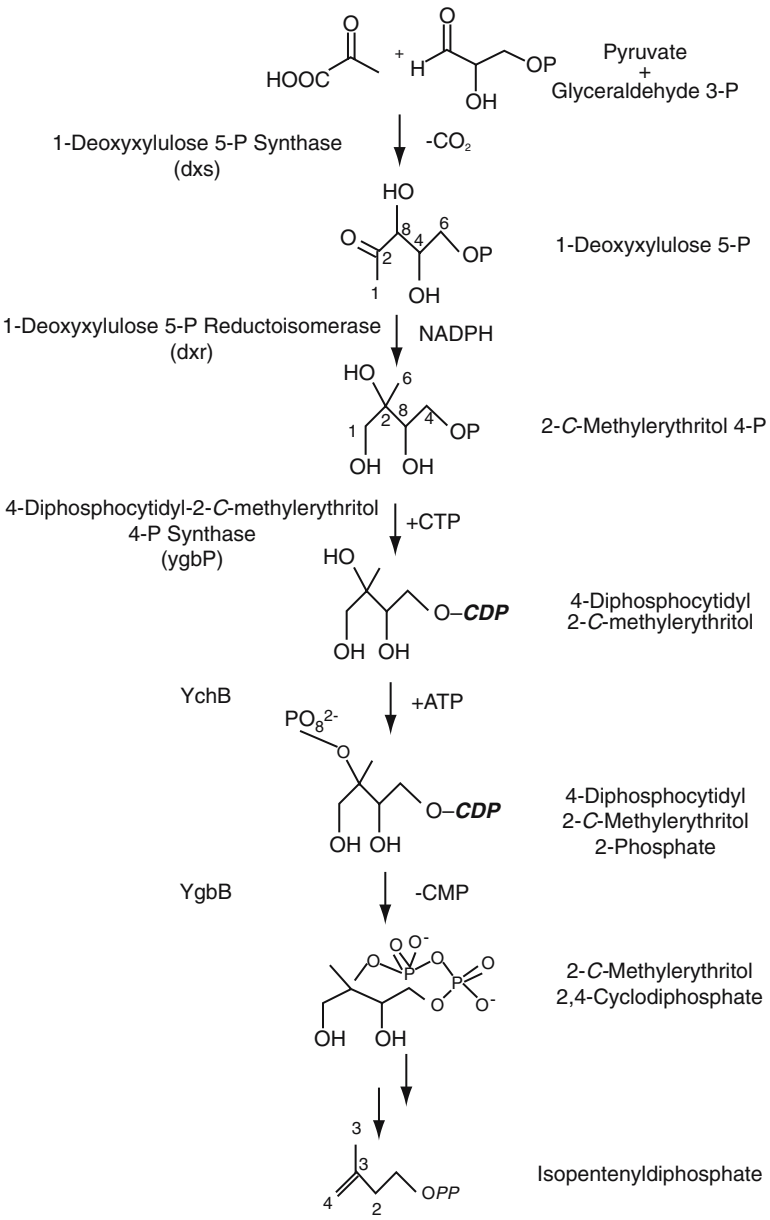

Fig. 3. The 2-methylerythritol 4-phosphate (MEP) pathway to isopentenyl diphosphate.

be the only quinone species in *Zymomonas* membranes (Strohdeicher et al., 1990).

Although aerobically growing cultures apparently do not produce extra ATP for biomass synthesis, oxidative phosphorylation can be measured in nongrowing cells and membrane vesicles (Kalnenieks et al., 1993). The energy coupling sites of the electron transport chain vary depending on the aeration of the culture. In the membranes of anaerobically cultivated cells, ATP synthesis is linked mainly to the site I (i.e., NADH dehydogenase I; Kalnenieks et al., 1995). On the other hand, for aerobically cultivated cells the buildup of membrane potential (Kim et al., 1995) and oxidative phosphorylation (Kalnenieks et al., 1996) takes place in the respiratory chain region downstream from site I. There are two kinetically distinguishable NADH oxidase activities in *Z. mobilis* membranes: one with a low K_m for NADH (around 7 μM) and another one with a higher K_m (around 60 mM; Kim et al., 1995; Kalnenieks et al., 1996). This points to the presence of two different membrane-bound NADH dehydrogenases, as was also found for other bacterial respiratory chains (e.g., for *E. coli*; Calhoun et al., 1993). It has been suggested that the NADH oxidase with the low K_m corresponds to the energy-coupling NADH: ubiquinone oxidoreductase of type I, and that the oxidase with the high Km represents the energy non-generating, type II NADH: ubiquinone oxidoreductase (Kalnenieks et al.,

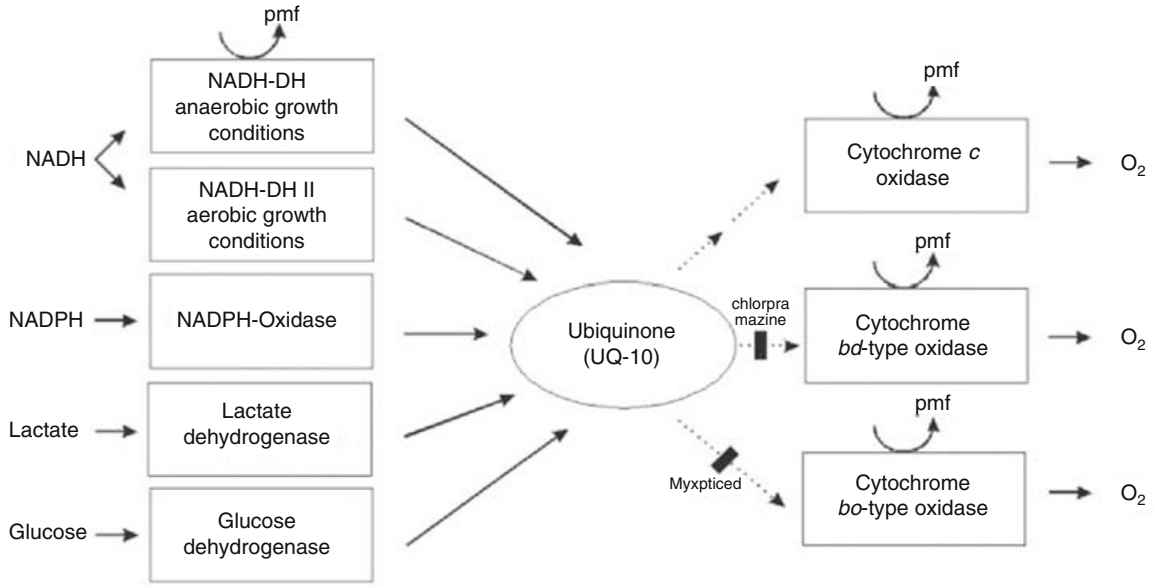

Fig. 4. Electron transport chain of *Zymomonas mobilis*; pmf, proton motive force.

1996). Spectral features of several types of cytochromes (b, c and d) have been observed in membrane preparations (Belaich and Senez, 1965; Pankova et al., 1985; Kalnenieks et al., 1998). In spite of high rates of respiration, the cytochrome content in *Z. mobilis* is comparatively low, being approx. 2–3 times lower than that in *E. coli* (Kalnenieks et al., 1998). From spectroscopic data it has been postulated that electrons, coming from the quinone pool, are distributed between three branches, terminated by 1) cytochrome *bd*, 2) a cytochrome *bo*-like component and 3) a cytochrome *a*-type terminal oxidase (Kalnenieks et al., 1998). At present it can be outlined that the respiratory chain of *Z. mobilis* is composed of several membrane oxidoreductases and of branched electron pathways to oxygen (Fig. 4). The presence of a respiratory chain in *Z. mobilis* supports the suggestion made by Swings and De Ley (1977) that this organism may have originated from aerobic ancestors.

Pyruvate Dehydrogenase Multienzyme Complex

In *Z. mobilis*, the formation of acetyl-CoA from pyruvate is catalyzed by a pyruvate dehydrogenase (PDH) complex (Bringer-Meyer and Sahm, 1993). The occurrence of this enzyme complex in *Z. mobilis* is surprising because anaerobically growing bacteria usually employ pyruvate formate lyase (EC 2.3.1.54) or pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) for acetyl-CoA synthesis (Sawers and Watson, 1998; Chabriere et al., 1999). The formation of acetyl-CoA from pyruvate represents a main junction of catabolic and anabolic pathways. Up to 98% of the pyruvate is converted to the fermentation end products ethanol and CO_2, whereas only a small part of the pyruvate is oxidatively decarboxylated by the reaction of the PDH complex to acetyl coenzyme A, CO_2 and NADH (Sahm et al., 1992). Inasmuch as *Z. mobilis* lacks the 2-oxoglutarate dehydrogenase complex and other enzymes of the tricarboxylic acid cycle, the PDH complex plays an exclusively anabolic role in this organism (Bringer-Meyer and Sahm, 1993). Bacterial pyruvate dehydrogenase complexes are composed of multiple copies of three different enzymes: pyruvate dehydrogenase (E1p; EC 1.2.4.1), dihydrolipoamide transacetylase (E2p; EC 2.3.1.12) and lipoamide dehydrogenase (E3; EC 1.8.1.4; Reed and Hackert, 1990). In *Z. mobilis*, the PDH complex consists of four polypeptides, similar to the situation found in Gram-positive bacteria, with an E1α subunit of 38.6 kDa, an E1β subunit of 49.8 kDa, an E2 subunit of 48 kDa and an E3 subunit of 50 kDa. The E2 core of the complex is arranged to form a pentagonal dodecahedron, again resembling the quaternary structures of PDH complexes of Gram-positive bacteria and eukaryotes (Neveling et al., 1998a; Neveling et al., 1998b). The structural genes (*pdhA*αβ, *pdhB*, *lpd*) encoding the PDH complex of *Z. mobilis* are located in two distinct gene clusters, *pdhA*αβ and *pdhB*-orf2-*lpd* (Neveling et al., 1998b). Like the dihydrolipoamide acetyltransferases of *S. cerevisiae* and numerous other organisms, the product of the *pdhB* gene of *Z. mobilis* contains a single

lipoyl domain. In addition, the E1β subunit was found to contain an amino-terminal lipoyl domain, a property that is exceptional among PDH complexes (Neveling et al., 1999).

Genetics and Metabolic Engineering

Plasmids, Gene Transfer, Mutagenesis and Mutant Isolation

Most strains of *Z. mobilis*, including the type strains (ATCC10988 = ZM1; ATCC29191 = ZM6; ATCC31821 = CP4), contain several natural plasmids (in a size range from 1.6 kb to >40 kb; Rogers et al., 1982; Tonomura et al., 1982; Scordaki and Drainas, 1987). Plasmid profiling has been used to clarify uncertain strain status (Yablonsky et al., 1988; Degli-Innocenti et al., 1990). For the most part, the *Z. mobilis* plasmids remain cryptic, although some functions as antibiotic or heavy metal resistances have been assigned to them (Tonomura et al., 1982; Walia et al., 1984; Scordaki and Drainas, 1987; Ogale and Deobagkar, 1988). For genetic engineering, various broad host range plasmids (from several incompatibility groups), shuttle and/or expression vectors have been constructed (Byun et al., 1986; Brestic-Goachet et al., 1987; Conway et al., 1987a; Cho et al., 1989; Reynen et al., 1990)—some of them with inducible promoter features (e.g., *lacI*q/P*tac* vectors; Arfman et al., 1992; Reipen et al., 1995). Using plasmid R68.45, chromosome transfer between donor and recipient strains of *Z. mobilis* was reported (Skotnicki et al., 1982; Stokes et al., 1983). No bacteriophage has been reported for *Z. mobilis* yet. This, however, need not mean that no phages exist for *Zymomonas* but rather indicates the lack of in-depth investigations. A physical map of ZM4 strain has been constructed recently which allowed to estimate the genome size to about 2,085 kb (Kang and Kang, 1998) and the full genomic sequence of this strain is currently underway.

Mutant isolation and enrichment still poses remarkable problems (Sprenger et al., 1993b; Pencreac'h et al., 1996; Pappas et al., 1997) and up to now, no working method for gene disruption or even gene replacement has been reported, although an integrative shuttle vector has been reported recently (Delgado et al., 1995). Auxotrophic mutants have been won spontaneously, or after induction by UV light, by chemical mutagens (alkylating agents such as ethyl methane sulfonate [EMS] or nitrosoguanidine; Typas and Galani, 1992), or following transposon mutagenesis (Pappas et al., 1997). Mutants range from auxotrophs for amino acids or vitamins (Goodman et al., 1982; Eddy et al., 1988; Karunakaran and Gunasekaran, 1989; Pencreac'h et al., 1996; Estevez et al., 1997) to mutants with altered metabolic markers (fructose utilization; Bringer-Meyer et al., 1985; Suntinanalert et al., 1986; glucokinase; DiMarco and Romano, 1985; Aitabdelkader et al., 1996; alcohol dehydrogenases; Wecker and Zall; 1987; mannitol degradation; Buchholz et al., 1988; sorbitol formation; Kirk and Doelle, 1993). Furthermore, various strains with tolerance versus elevated temperatures (e.g., 42°C), molasses, salts or ethanol have been isolated as well as flocculent strains (see references in Ingram et al., 1989). Mutants resistant to allyl alcohol were found to be deficient in both alcohol dehydrogenases (Wecker and Zall, 1987; O'Mullan et al., 1995). These strong acetaldehyde-forming mutant strains grow and ferment poorly and are dependent on the presence of oxygen (Wecker and Zall, 1987).

Methods for introduction of foreign genes mainly rely on conjugation from *E. coli* donor cells (see references in Ingram et al., 1989; Sprenger, 1993a) or on electroporation (Okamoto and Nakamura, 1992; Lam et al., 1993; Liang et al., 1998), the latter at still low frequencies. Successful chemical transformation using spheroplasts (Yanase et al., 1986) or a chemical ($CaCl_2$) method with whole cells (Browne et al., 1984; Su and Goodman, 1987), yielding up to $1.8 \cdot 10^5$ transformants/μg of plasmid DNA, have been reported, but reproduction of these results has been difficult in other laboratories. *Zymomonas mobilis* is inherently resistant to nalidixic acid, which kills *E. coli* cells. In conjugations from *E. coli* donors to *Z. mobilis* recipients, nalidixic acid is therefore employed to discriminate against the donor cells (Uhlenbusch et al., 1991; Arfman et al., 1992). The other inherent antibiotic resistances limit the marker genes mainly to chloramphenicol and tetracycline. However, spontaneous resisters against both antibiotics also are found. The strain ZM6 (ATCC29191) and its derivatives are more sensitive to ampicillin than are the strains ZM1 (ATCC10988) and CP4; therefore replicons with Amp^R-genes may be utilized in ZM6 and its derivatives.

Metabolic Engineering

Nearly as soon as the paramount large-scale, ethanol-producing capabilities of *Z. mobilis* became widely known (Rogers et al., 1982), attempts to broaden the limited substrate and product range of *Z. mobilis* were started. Genetic and metabolic engineering of *Z. mobilis* have attracted many groups to study the unusual efficiency of sugar metabolism and ethanol produc-

tion and to improve the limited substrate and product range of the organism. Several reviews during the last years have dealt with these issues in depth and are recommended for further reading (Ingram et al., 1989; Conway, 1992; Johns et al., 1992; Sprenger, 1993a; Doelle et al., 1993; Sprenger et al., 1993b; Sprenger, 1996). Successful examples are summarized in Table 3. The main goals were to enlarge the substrate spectrum to the utilization of constituents of abundantly available and renewable carbon sources from wood and straw (e.g., hemicelluloses with D-xylose, L-arabinose and D-mannose as main monosaccharide constituents), whey (lactose), starch (maltose) and cellulose (cellobiose).

Many groups have worked especially on lactose as a carbon source, however, only with limited success. Main problems were that this disaccharide needs to be taken up by an energy-consuming step (proton-symporting lactose permease LacY), genes for galactose catabolism have to be co-introduced, and finally inhibitory by-products (such as galactonic acid or galactitol) may limit the growth and ethanol formation from lactose (literature reviewed in Sprenger, 1993a). More recently, catabolism of D-xylose, L-arabinose and D-mannose has been achieved with good ethanol yields (Feldmann et al., 1992; Zhang et al., 1995; Deanda et al., 1996; Weisser et al., 1996; de Graaf et al., 1999). Sugar uptake of D-xylose and D-mannose is gratuitous as the GLF transporter takes both sugars at good rates (Weisser et al., 1996; Parker et al., 1997). Introduction of a single phosphomannose-isomerase gene (*pmi*) from *E. coli* resulted in a mannose-positive phenotype of *Z. mobilis*, as another gratuitous reaction (involving fructokinase) took care of the mannose phosphorylation step (Weisser et al., 1996). Successful transfer of the plasmid-borne *pmi* gene could be monitored by growth of the *Z. mobilis* exconjugants on mannose as sole C-source; this can now be used as an alternative to the antibiotic-resistance markers, which are limited (Weisser et al., 1996). For D-xylose and L-arabinose catabolism, the peripheral enzymes have been introduced from various heterologous hosts into recombinant strains of *Z. mobilis* (Liu et al., 1988; Feldmann et al., 1992; Zhang et al., 1995; Deanda et al., 1996). After this was achieved it became evident that additional activities of the central pentose-phosphate metabolism are required (Feldmann et al., 1992; Zhang et al., 1995). Whereas transketolase is present at very low activity, transaldolase appears to be absent from *Z. mobilis* (Feldmann et al., 1992; Zhang et al., 1995). Introduction and expression of transketolase and transaldolase from *E. coli* were necessary to isolate xylose-positive clones of *Z. mobilis* (Zhang et al., 1995; de Graaf et al., 1999). In the same line, L-arabinose-fermenting clones have been isolated (Deanda et al., 1996). The path of ^{13}C-labeled xylose in recombinant *Z. mobilis* cells has recently been followed by NMR techniques (de Graaf et al., 1999; Kim et al., 2000).

To enlarge the product spectrum of *Z. mobilis*, an L-alanine dehydrogenase gene from *Bacillus sphaericus* has been introduced and was expressed from the strong *pdc* promoter. This led to a portion of pyruvate being diverted from the normal ethanologenic route to a fermentative L-alanine route. To reduce the overwhelming PDC activity, a thiamine-auxotroph strain was used which was then starved for thiamine in the

Table 3. Examples of successful metabolic pathway engineering in *Zymomonas mobilis*.

Heterologous gene(s)	Donor	Enzymes expressed	Pathway	Refs.
lacZY, galEKT	*E. coli*	lactose permease, β-galactosidase, galaktokinase, UDP-Gal-Epimerase, UDP-Glc-Transferase	lactose degradation galactose degradation	Buchholz et al. 1989
araBAD, tktA, talB	*E. coli*	L-arabinose isomerase, L-ribulokinase, L-ribulose 5-phosphate 4-epimerase, transketolase, transaldolase	L-arabinose degradation	Deanda et al. 1996
xylAB, tktA, talB	*E. coli*	xylose isomerase, xylulokinase transketolase, transaldolase	D-xylose degradation	Zhang et al. 1995
xylAB, tktA, talB	*Klebsiella pneumoniae*	xylose isomerase, xylulokinase	D-xylose degradation	Feldmann et al. 1992 de Graaf et al. 1999
	E. coli	transketolase, transaldolase		
pmi (manA)	*E. coli*	phosphomannose isomerase	D-mannose degradation	Weisser et al. 1996
alaD	*B. sphaericus*	L-alanine dehydrogenase	L-alanine formation	Uhlenbusch et al. 1991
?	*B. brevis*	α-glucosidase	ethanol formation from maltose (no growth)	Strzelecki et al. 1993
crtBEIY	*Erwinia uredovora*	carotene biosynthetic enzymes	β-carotene production	Misawa et al. 1991

medium to reduce the activity of thiamine-diphosphate-requiring PDC. This resulted in a major rerouting of pyruvate to L-alanine (Table 3; Uhlenbusch et al., 1991). The strong isoprenoid-forming pathway of *Z. mobilis* was successfully altered into a carotenoid pathway by introduction of *crt* genes from *Erwinia* species (Table 3; Misawa et al., 1991).

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