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Anaerovibrio glycerini sp. nov., an anaerobic bacterium fermenting glycerol to propionate, cell matter, and hydrogen

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Abstract. A strictly anaerobic, Gram-negative bacterium was isolated in continuous culture from black freshwater sediment with glycerol as sole source of carbon and energy. It was present in such sediments at 10^8 cells per ml. The isolate was highly specialized and used only glycerol and the glycerol residue of diolein as substrate, and fermented it quantitatively to propionate. During growth in mineral medium, small amounts of hydrogen were produced which corresponded exactly to the calculated amount of electrons released in cell matter formation from glycerol. Yeast extract enhanced cell yields with glycerol, but did not support growth itself. In cell-free extracts, benzylviologen-dependent hydrogenase activity as well as a b-type cytochrome and some of the enzymes of the methylmalonylCoA pathway were found. The guanine-plus-cytosine content of the DNA was 34.3 ± 1.0 mol% and corresponded well with that of *Anaerovibrio lipolytica* which was found to be 31.4 mol%. The consequences of the electron balance of this glycerol fermentation are discussed with respect to glycerol fermentation by other propionic acid-forming bacteria.

Key words: *Anaerovibrio glycerini* sp. nov. – Glycerol fermentation – Propionate formation – Hydrogen metabolism – Hydrogenase – Cytochrome b

Glycerol is an important constituent of lipids in biomass. It is also present at high concentrations as an osmoregulant in fungi (Jennings 1984), yeasts (Larsson and Gustafsson 1987; André et al. 1988), green algae (Brown 1978), and cyanobacteria (Reed et al. 1984; Hagemann et al. 1987), in the latter case as glycosyl-glycerol. Since glycerol can easily enter the glycolytic pathway via oxidation and phosphorylation, aerobic or nitrate-dependent degradation by eukaryotic or prokaryotic cells does not cause any major problems (Lin 1976, 1977). Sulfate-dependent glycerol oxidation to acetate and CO_2 has been proven unequivocally only recently (Stams et al. 1985).

For fermenting bacteria, glycerol degradation poses a problem by its redox state which is by 2 electrons more reduced than sugars. Therefore, many fermenting bacteria can degrade glycerol only if fumarate is present as an external electron acceptor, e.g. *Escherichia coli* (Quastel et al. 1925; Miki and Lin 1975) or *Streptococcus faecalis* (Gunsalus 1947). Homoacetogenic bacteria use CO_2 as electron acceptor in glycerol oxidation to acetate (Eichler and Schink 1984). Fermentation to butyrate requires acetate (Bhat and Barker 1947) or pyruvate (Azova 1967) as cosubstrate. Several clostridia also form from glycerol 1,3-propanediol (Nakas et al. 1983; Forsberg 1987) which is the reduced end product of glycerol fermentation also in lactic acid bacteria (Sobolov and Smiley 1960; Schütz and Radler 1984), *Klebsiella pneumoniae* (Abeles et al. 1960; Streekstra et al. 1987) and "*Bacillus amaracrylus*" (Voisenet 1918). 1,3-Propanediol was described already in 1881 by Freund as a product of glycerol fermentation (Braak 1928). Several strict anaerobes dismutate glycerol to 1,3-propanediol and 3-hydroxypropionate (Schink and Stieb 1983; Stieb and Schink 1984). *Citrobacter freundii* ferments glycerol to mainly formate, acetate, and ethanol (Magasanik et al. 1953). Fermentation of glycerol to propionate is at first sight a simple reaction which is carried out by several classical propionic acid bacteria (Stjernholm and Wood 1960), as well as by *Selenomonas ruminantium* and *Anaerovibrio lipolytica* (Hobson and Mann 1961).

Thus various pathways have evolved in anaerobic bacteria to solve the problem of excess electron release during glycerol degradation. The present study was initiated to examine which one of these fermentative pathways predominates in an anoxic environment such as a freshwater creek sediment. Fermentative glycerol degradation by rumen contents leads to acetate, propionate, butyrate, and lactate (Wright 1969). Direct enrichment from freshwater creek sediments with glycerol in strictly anaerobic batch cultures always led to acetate- and 1,3-propanediol-forming facultative anaerobes (unpublished results from our laboratory). In order to better mimic natural conditions, enrichment cultures were also started in continuous culture. From these enrichments, a new propionate-forming bacterium was isolated which is described here in detail. It proved to be the predominant glycerol degrader in these sediments as evidenced by direct dilutions of sediment material.

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Materials and methods

Sources of bacterial strains

Cells of *Anaerovibrio lipolytica* ATCC 33276, DMS 3074 were kindly provided by Dr. H. Hippe, Deutsche Sammlung von Mikroorganismen GmbH, Braunschweig, FRG.

Enrichment cultures for new glycerol degraders were inoculated with black sediments from two freshwater creeks near Konstanz, FRG, and with anoxic sewage sludge from the municipal sewage plant at Konstanz, FRG.

Media and growth conditions

All cultivation procedures were essentially as previously described (Widdel and Pfennig 1981; Schink and Pfennig 1982). The mineral medium for enrichment and further cultivation was carbonate-buffered and sulfide-reduced, and contained the trace element solution SL 10 (Widdel et al. 1983). The pH was 7.2–7.4. Strain LGS 4 was enriched in a continuous culture with the same medium containing 10 mM glycerol and 10 mM sodium sulfate. The culture vessel (500 ml, with about 30 ml N_2/CO_2 (90%/10%) headspace) was inoculated with 20 ml of anoxic mud. After 3 days of batch growth, a dilution rate of 1 d^{-1} was adjusted, and samples were taken daily for microscopical and end product analysis. Pure cultures were isolated in agar shake dilutions according to Pfennig (1978). Growth experiments were carried out at 28° C unless stated otherwise. Growth yields and substrate conversion stoichiometries were determined either in 23 ml screw cap tubes or in half-filled 60 ml serum bottles under N_2/CO_2 (90%/10%) atmosphere.

Calibration of culture turbidity to cell dry matter content was based on gravimetric determinations in 500 ml cultures. Determination of pH-dependence of growth was carried out in the basal mineral medium which contained in addition trishydroxymethyl aminomethane, potassium phosphate, and acetate, each at 10 mM concentration.

Cytological characterization

The Gram type was determined according to Magee et al. (1975). *Escherichia coli* and *Acetobacterium woodii* were used as controls. The guanine-plus-cytosine content of the DNA was determined by thermal denaturation (DeLey 1970) after extraction of the DNA (Marmur 1961) using *Escherichia coli* strain K 12 as reference. Cytochromes were identified taking redox difference spectra of dithionite-reduced minus air-oxidized cell extracts or membrane preparations with a Shimadzu UV-300 spectrophotometer.

Enzyme assays

Cell-free extracts were prepared by lysozyme treatment and by French press cell disruption under a nitrogen atmosphere strictly avoiding contact with air. Conversion of glycerol to glyceraldehyde phosphate was followed as glycerol- and ATP-dependent reduction of dichlorophenol indophenol (De Vries et al. 1973). Pyruvate oxidation was assayed with benzyl viologen (Odom and Peck 1981) and with NAD, hydrogenase activity with benzyl viologen according to Schink (1985) and with NAD as well. Phosphotransacetylase, malate dehydrogenase, and acetate kinase were determined by standard procedures (Bergmeyer 1974), malate dehydrogenase also with dichlorophenol indophenol as acceptor (Stams et al. 1984).

Chemical determinations

Fatty acids were assayed by gas chromatography (Schink and Pfennig 1982), glycerol enzymatically (Bergmeyer 1974). Hydrogen was quantified with a Hewlett-Packard 5880 A-series gas chromatograph with a molecular sieve column (5 Å) and a thermal conductivity detector. Protein was determined by a microbiuret method (Kuenen and Veldkamp 1972).

Chemicals

All chemicals used were of analytical or reagent grade quality and obtained from Merck, Darmstadt, or Fluka, Neu-Ulm, FRG. Biochemicals and enzymes were purchased from Boehringer, Mannheim, FRG, and Sigma Chemical Co., München, FRG.

Results

Enrichment, isolation, and cellular characterization

An anaerobic enrichment culture vessel containing 500 ml mineral medium with 10 mM glycerol as sole organic carbon source was inoculated with 20 ml of freshwater creek sediment and incubated as a batch culture for 3 days at 25° C. During this time, a motile straight rod grew up which fermented glycerol to 1,3-propanediol and acetate. After starting the medium flow at a dilution rate of 1 d^{-1}, the sediment was slowly washed out of the vessel, but some sandy sediment particles still remained for the following 6 days. During this time, a very motile curved rod established itself, and the product pattern changed to propionate only.

The predominant bacterium, strain LGS 4, could easily be isolated in agar dilution series. It formed small, cone-shaped yellow to orange colonies with a slightly rough surface in the agar. Cells were curved rods, $0.5 \times 2-10$ µm in size, with slightly pointed ends (Fig. 1). They were highly motile at the beginning, but motility was lost after about 1 year of cultivation. To the end of log phase growth, dark granular inclusions of probably reserve material were visible. If higher substrate concentrations were used (50 mM), cells formed long chains of up to 10 "waves". Cells stained Gram-negative, and no spores were formed. The guanine-plus-cytosine content of the DNA was 34.4 ± 1.0 mol%.

The same type of propionate-forming highly motile curved rods was isolated by direct dilution of sediment material from the same site as well as from other freshwater creek and ditch sediments at about 10^8 cells per ml of sediment sample. These strains were not characterized any further. In most-probable-number dilution experiments, propionate was the main fermentation product in the last positive dilution tubes.

Physiology

Strain LGS 4 was strictly anaerobic. Although selected and isolated at 25° C, the temperature optimum of growth was 37° C; the temperature limits were 10 and 42° C. The growth

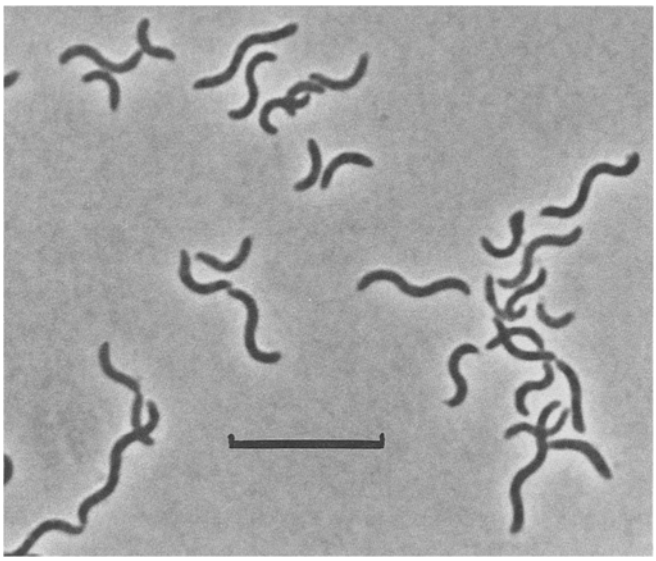

Fig. 1. Phase contrast photomicrograph of *Anaerovibrio glycerini* strain LGS 4 in the late log phase of growth. Bar equals 10 µm

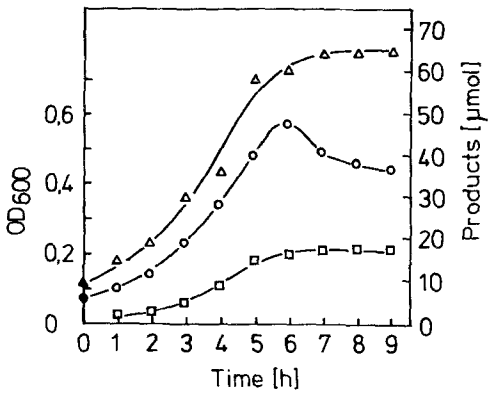

Fig. 2. Growth and product formation by *Anaerovibrio glycerini* strain LGS 4 at 37° C with 85 µmol glycerol. (○) Optical density at 600 nm wavelength; (△) propionate formed; (□) hydrogen formed

rate at 25°C was 0.12 h^{-1} (t_d = 5.8 h), at 37°C 0.43 h^{-1} (t_d = 1.6 h).

A typical growth curve at 37°C in mineral medium is shown in Fig. 2. The cell density increased within 6 h to its maximum and decreased again shortly afterwards by about 20%, in hand with disappearance of the inclusion bodies (see above). Glycerol was quantitatively converted to propionate, cell matter, and hydrogen which appeared in screw cap bottles as small bubbles on the wall. The stoichiometry of substrate conversion was reexamined several times with various volumes of culture fluid. As shown in Table 1, hydrogen was always formed together with propionate. The amount of hydrogen released corresponded to the amount of cell material formed, according to the equation:

$$8\,C_3H_8O_3 \rightarrow 6\,(C_4H_7O_3) + 5H_2 + 6H_2O.$$

($C_4H_7O_3$) stands for cell material (Pfennig and Biebl 1976). The amount of hydrogen formed did not change no matter if acetate, fumarate, or yeast extract was added to the medium. Yeast extract increased the cell yield during growth with glycerol by about 38%. No growth occurred with yeast extract alone. Growth yields were proportional to glycerol supplied up to 25 mM glycerol in the medium.

Of a broad variety of sugars, organic acids, alcohols, lipids, and lipid analogues (see listing at the end of the discussion), only glycerol and diolein (glycerol dioleic ester) were used as substrates. From diolein, about equimolar amounts of propionate were formed indicating that only the glycerol residue was fermented (Table 1). Exact determinations of cell yields etc. were hampered in this case by the low solubility of diolein in water.

Strain LGS 4 grew well in freshwater medium as well as in saltwater medium in the presence of 20 g NaCl and 3 g $MgCl_2 \times 6\ H_2O$ per liter. Phosphate concentrations up to 50 mM did not inhibit growth significantly. The optimal pH for growth was 6.5 – 7.5, with limits at pH 5.0 and 8.5.

Sulfate, sulfite, thiosulfate, sulfur, and nitrate were not reduced. Molecular nitrogen was not fixed. Vitamins were not required for growth.

Biochemistry

Enzymes were studied in cell-free extracts of strain LGS 4 as well as in cell suspensions in the presence of cetyl trimethyl ammonium bromide. No dihydroxyacetone kinase, acetate kinase, or phosphotransacetylase activity could be detected, but a small activity of benzylviologen-dependent hydrogenase (32 mU per mg protein) was found which did not react with NAD. NAD could not be reduced with glycerol or glycerophosphate. However, reduction of dichlorophenol indophenol was achieved with either glycerophosphate (160 – 210 mU per mg protein) or with glycerol in the presence of ATP. Small activites of NAD-dependent malate dehydrogenase (38 mU per mg protein) and of fumarase (40 mU per mg protein) were detected. Succinate was converted to propionate by cell-free extracts at 0.6 mU per mg protein.

Membrane preparations contained a b-type cytochrome at 24.5 mg or 380 nmol per g protein as shown in redox difference spectra (Fig. 3; Deeb and Hager 1964). Reduction of this cytochrome was possible with glycerol phosphate or with glycerol in the presence of ATP and small amounts of the cytoplasmic fraction; fumarate reoxidized the cytochrome. Thus, electrons from glycerol phosphate oxidation can be channeled via the cytochrome to fumarate in the cytoplasmic membrane.

Discussion

Physiology and biochemistry

The new type of Gram-negative anaerobic bacteria described in this study is highly specialized on glycerol utilization, and forms propionate as sole organic fermentation product. The high degree of specialization can be due to the fact that this bacterium lacks acetate kinase and phosphotransacetylase. It cannot form acetate from acetyl-CoA, therefore, and this may be the reason why it is restricted to substrates that can be fermented exclusively to propionate. With this, it differs from all other propionate-forming bacteria described so far. Glycerol fermentation to propionate releases a considerable amount of energy (calculation after Thauer et al. 1977):

$$C_3H_8O_3 \rightarrow C_3H_5O_2^- + H^+ + H_2O$$
$$\Delta G'_0 = -150\ \text{kJ per mol.}$$

Table 1. Stoichiometry of substrate degradation and product and cell matter formation by *Anaerovibrio glycerini* strain LGS 4

Substrate (amount) (μmol)	Net OD_{600}	Dry matter (mg)[a]	Substrate assimilated (μmol)[b]	Products formed (μmol)		Electron recovery (%)	Yield (g/mol)
				Propionate	H_2		
Glycerol							
100	0.45	1.2	15.5	87	14	103	12.0
200	0.44	2.5	32.2	178	20	105	12.6
Diolein							
~200	n.d.	n.d.	n.d.	212	n.d.	>100	n.d.

Experiments were carried out in 17 ml Hungate tubes containing 10 ml liquid medium under a N_2/CO_2 (90%/10%) atmosphere

[a] Cell dry matter formation was calculated after a formula determined experimentally as described in Materials and methods (0.1 OD_{600} = 26.8 mg dry matter/l)

[b] Conversion of substrate to dry matter was calculated using the formula: 8 $C_3H_8O_3 \rightarrow 6(C_4H_7O_3) + 5\ H_2 + 6\ H_2O$. According to this formula, 12.9 μmol glycerol is assimilated to form 1 mg of cell dry matter and 8.1 μmol H_2

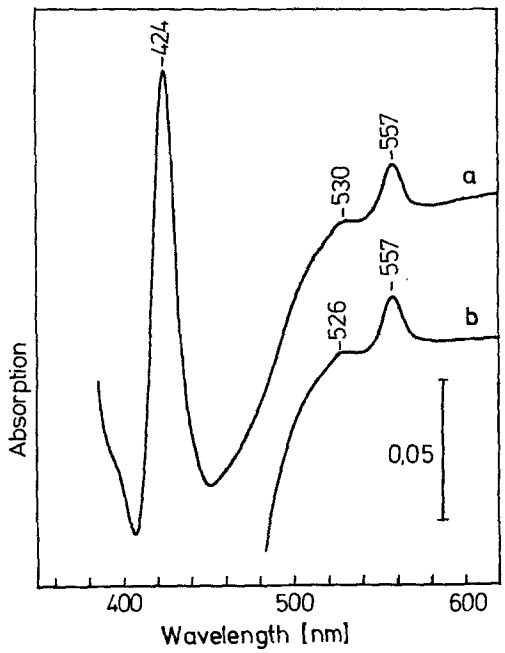

Fig. 3. Redox difference spectrum of a crude cell extract of *Anaerovibrio glycerini* strain LGS 4. The extract contained 3.1 mg protein/ml. **a** Dithionite-reduced minus air-oxidized extract; **b** dithionite-reduced minus $K_3Fe(CN)_6$-oxidized extract

Since about 70–75 kJ are needed for irreversible synthesis of 1 mol of ATP (Thauer et al. 1977), about 2 ATP could be formed per glycerol fermented. Activation of glycerol to glycerophosphate requires 1 ATP, and 2 ATP are gained in the glyceraldehydephosphate dehydrogenase and pyruvate kinase reaction. Assuming that propionate is formed via the methylmalonyl CoA pathway, further 2/3 ATP could be synthesized by electron transport phosphorylation in fumarate reduction with electrons derived from glycerophosphate dehydrogenation (Schink 1988). Our enzyme studies showed only poor activities of the enzymes involved, but they basically support the assumption of this pathway. This is further corroborated by the presence of a cytochrome b in the membranes which is reduced by glycerophosphate and oxidized by fumarate. The growth yield determined (12.0 g per mol glycerol in mineral medium; 17.8 g in the presence of 0.1% yeast extract) allows the calculation of a Y_{ATP} of 7.8 or 10.8 g cell matter under the respective growth conditions, which is well in the range of those calculated for other anaerobic bacteria growing under similar conditions (Stouthamer 1979). The energy metabolism of this new isolate appears to be basically understood, therefore.

Of special interest was the formation of small amounts of molecular hydrogen which could not be attributed to energy metabolism. Since glycerol is more reduced than cell material the bacterium has to release the excess electrons via the benzylviologen-dependent hydrogenase which in vivo is probably coupled to a ferredoxin-like electron carrier. In classical *Propionibacterium* sp., hydrogenases have never been reported. Thus, these bacteria would have difficulties growing with a substrate as reduced as glycerol. However, since they are usually grown in the presence of high amounts of yeast extract (De Vries et al. 1977; Stouthamer 1980) the problem of electron release during glycerol utilization has never been realized. That complex medium additions such as peptone could act as electron acceptor in glycerol fermentation was already suggested by Braak (1928). To our knowledge, this is the first reported case of a hydrogenase in a fermenting bacterium which acts only in balancing the assimilatory electron metabolism.

Ecology

Most of the known glycerol-fermenting bacteria form 1,3-propanediol as reduced product, together with acetate. The theoretical ATP yield of such a fermentation is, depending on whether hydrogen or formate can be formed in addition, in the range of 0.5–0.67 ATP per glycerol fermented. This is a very low yield of utilizable energy compared to that of our strain LGS 4 (1.67 ATP per glycerol). Nonetheless, in batch culture enrichments 1,3-propanediol-forming facultative aerobes always outcompeted other types of fermenting bacteria, because they grow considerably faster. Contrary to a batch culture, a continuous culture at low dilution rates enriches for bacteria with high substrate affinities (Veldkamp 1977), and it was this method which led to isolation of strain LGS 4. Its metabolism appears to be the most efficient one for fermentative degradation of glycerol, and it is not astonishing that this bacterium proved to be the most abundant glycerol fermenter in the low-sulfate freshwater environments studied. Only sulfate-dependent glycerol oxidation (Stams et al. 1985) could possibly compete with this high energy efficiency.

Taxonomy

Strain LGS 4 represents a new type of anaerobic bacterium that cannot be affiliated with any of the existing species. As a Gram-negative strictly anaerobic, non-sporing propionate former, it belongs to the family Bacteroidaceae and has to be compared with the genera *Selenomonas, Propionispira, Pectinatus*, and *Anaerovibrio*. All these known genera ferment numerous substrates including sugars, organic acids etc. to mainly propionate and acetate. *Selenomonas* has a guanine-plus-cytosine content of the DNA at 53–61 mol%, and *Propionispira* and *Pectinatus* are at 36.7 and 39.8 mol% (Krieg and Holt 1984). For *Anaerovibrio*, the guanine-plus-cytosine content was never determined. Therefore, this value was determined in the present study, and turned out to be 31.5 ± 1.2 mol%, close to that of strain LGS 4 (34.3 ± 1.0 mol%). Since strain LGS 4 appears most related to *Anaerovibrio* also with respect to its temperature optimum of growth and its slight lipolytic activity (degradation of diolein), it is suggested to affiliate strain LGS 4 with this genus as a new species, *Anaerovibrio glycerini*.

A. gly.ce.ri'ni sp. nov. glycerinum. M. L. n. glycerol, glycerini, of glycerol, referring to utilization of glycerol as sole substrate.

Curved, spiral-shaped cells, 0.5–2–10 µm in size, with slightly pointed ends, occasionally forming dark inclusion bodies. Motile in young cultures, Gram-negative, non-sporeforming. Strictly anaerobic chemoorganotroph, glycerol and diolein, perhaps other glycerolesters used as sole source of carbon and energy. Propionate only organic fermentation product. In mineral media, small amounts of hydrogen are formed. Grows in freshwater medium as well as in the presence of up to 2% (w/v) sodium chloride. Sulfate, sulfite, thiosulfate, sulfur, or nitrate not reduced. Indole not formed, gelatine or urea not hydrolyzed.

Cytochrome b present, no catalase activity.

Substrates that did not support growth included glucose, fructose, mannose, lactose, sucrose, maltose, salicin, mannitol, melibiose, melezitose, raffinose, sorbose, rhamnose, trehalose, arabinose, xylose, ribose, lactate, pyruvate, tartrate, malate, fumarate, succinate, aspartate, glutamate, citrate, formate, acetate, propionate, butyrate, methanol, ethanol, propanol, ethylene glycol, 1,2-propanediol, 1,3-propanediol, 2,3-butanediol, acetoin, diacetyl, glycolate, glyoxylate, glycolaldehyde, oxalate, malonate, betaine, yeast extract, peptone, casamino acids, H_2/CO_2 (80%/20%).

All substrates were given at 10 mM or 0.1% concentration. Enrichment from anoxic sediments in mineral medium either in continuous culture or isolation by direct dilution of sediment or sludge material.

pH range: 5.0–8.5; Temperature range 10–42° C, optimum at 37° C.

DNA base ratio: 34.4 ± 1.0 mol% G + C (thermal denaturation). Habitats: anoxic muds of freshwater origin, sewage sludge. Type strain: LGS 4, DSM 5192, deposited with the Deutsche Sammlung von Mikroorganismen GmbH, Braunschweig, FRG.

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