Arch Microbiol (1983) 134:167–169

Archives of
Microbiology

Pathways and Regulation of N_2, Ammonium and Glutamate Assimilation by *Clostridium formicoaceticum*

M. Bogdahn[1], J. R. Andreesen[2], and D. Kleiner[1]

[1] Lehrstuhl für Mikrobiologie der Universität, D-8580 Bayreuth, Federal Republic of Germany
[2] Institut für Mikrobiologie der Universität, D-3400 Göttingen, Federal Republic of Germany

Abstract. *Clostridium formicoaceticum* possesses the following enzymes for the assimilation of N_2 and NH_4^+: nitrogenase, glutamine synthetase, NADH- and NADPH-dependent glutamate synthase, NADH- and NADPH-dependent glutamate dehydrogenase, NADPH-dependent alanine dehydrogenase, and NH_4^+-dependent asparagine synthetase. Nitrogenase and glutamine synthetase are repressed and alanine dehydrogenase is induced by NH_4^+, while the synthesis of the other enzymes is not influenced by the extracellular NH_4^+ level. Glutamate is degraded via glutamate mutase and β-methylaspartase.

Key words: Nitrogenase – Glutamine synthetase – Glutamate synthase – Glutamate dehydrogenase – Asparagine synthetase – Alanine dehydrogenase – β-Methylaspartase – *Clostridium formicoaceticum*

The genus *Clostridium* represents a group of heterogeneous species which are highly versatile with regard to their individual metabolism (Gottschalk et al. 1981). Referring to nitrogen metabolism, the degradation of amino acids, purines and pyrimidines by some members of this group has been studied in detail (Vogels and van der Drift 1976; Barker 1981), while the information about the metabolism of inorganic nitrogen compounds is sparse. Rosenblum and Wilson (1949) reported the ability of several clostridia to reduce N_2 to NH_4^+. The further assimilation of NH_4^+, however, has been only investigated in *Clostridium pasteurianum* (Nagatani et al. 1971; Dainty 1972; Kleiner 1979). We were interested whether the scheme of nitrogen assimilation by *C. pasteurianum* might be of general validity for clostridia.

Because of large differences in the physiology, we chose *Clostridium formicoaceticum* for comparison. This organism is quite versatile and can be grown in a defined medium (Leonhardt and Andreesen 1977). It differs from *C. pasteurianum* in its ability to reduce CO_2 to acetate (Andreesen et al. 1970). In addition, both organisms show no relationship with respect to their molar G + C content of the DNA (Gottschalk et al. 1981) and their 16 S rRNA nucleotide catalogue (Tanner et al. 1982).

Abbreviation. GOGAT, glutamine-oxoglutarate amidotransferase

Offprint requests to: D. Kleiner

Materials and Methods

The strains used in these studies were the type strain A1 (DSM 92; Andreesen et al. 1970) and a strain originally described as *Clostridium aceticum* (El Ghazzawi 1967), but renamed *C. formicoaceticum* (DSM 93; Andreesen et al. 1970). Strain DSM 93 was cultured in a medium containing potassium phosphate (20 mM), fructose (55 mM), $NaHCO_3$ (120 mM), $MgSO_4$ (0.4 mM), resazurin (1 mg/ml), 20 ml of both the mineral and the vitamine solution as given by Wolin et al. (1964), and varying amounts of yeast extract and NH_4Cl as will be indicated. Sodium thioglycollate (0.075%) or titanium-III-citrate (Zehnder and Wuhrmann, 1976) were used as reducing agents before inoculation of the medium. The organisms were cultivated at 37° C under N_2/CO_2 (4 : 1), the pH was kept between 8 and 8.5 by repeated titrations with anaerobic KOH.

Strain DSM 92 was either cultured with fructose as described by Andreesen et al. (1974) or with glutamate (68 mM) replacing fructose as the carbon source. The defined medium of Leonhardt and Andreesen (1977) was used to grow strain DSM 92 in the absence of NH_4^+ by sparging the culture with N_2/CO_2 (1 : 1).

Nitrogenase was measured in vivo by the acetylene reduction method as outlined (Kleiner 1979). Cell extracts were prepared by anaerobic passage through a French press (Leonhardt and Andreesen 1977). Glutamine synthetase was measured by the ATP dependent formation of γ-glutamyl-hydroxamate from glutamate and hydroxylamine (Kleiner 1979), glutamate dehydrogenase, glutamate synthase, asparagine synthetase and ^{14}C-methylammonium uptake were assayed as described (Kleiner 1979, 1982), α-ketoglutarate reductase was measured according to Lerud and Whiteley (1971), β-methylaspartase as described by Barker et al. (1959). β-Methylaspartate was prepared according to Barker and Smyth (1961). One unit of enzyme activity was defined as 1 μmol product formed per min. Protein was determined by the microbiuret method (Goa 1953), and ammonium according to Fawcett and Scott (1960).

Results

A summary of enzyme activities is given in Table 1. Both strains showed essentially the same activities within the statistical errors. Nitrogenase could be detected in vivo only when the bacteria were grown in media without NH_4Cl and

Table 1. Activities of the enzymes involved in the assimilation of inorganic nitrogen compounds by *Clostridium formicoaceticum*. All enzymes except nitrogenase were tested in vitro. Activities are expressed in U per mg protein except for nitrogenase, the activities of which are expressed in $\mu mol \cdot h^{-1} \cdot OD^{-1} \cdot ml^{-1}$. The NH_4^+-free medium contained 0.2% yeast extract, except for nitrogenase measurements, where 0.05% yeast extract was used

Enzyme	Growth conditions	
	with NH_4^+ (18 mM)	without NH_4^+ [a]
Nitrogenase	< 0.01	0 − 0.38
Glutamine synthetase	0.04 ± 0.02	0.3 ± 0.15
GOGAT (NADPH)[a]	0.02	0.04
GOGAT (NADH)	0.07 ± 0.03	0.07 ± 0.03
Glutamate dehydrogenase (NADPH)	2.5 ± 1.0	2.5 ± 1.0
Glutamate dehydrogenase (NADH)	0.6 ± 0.2	0.6 ± 0.2
Alanine dehydrogenase (NADH)	< 0.0002	< 0.0002
Alanine dehydrogenase (NADPH)	0.004 ± 0.001	< 0.0002
Asparagine synthetase	0.06 ± 0.02	0.06 ± 0.02

[a] Highest activities observed (rapidly decreasing)

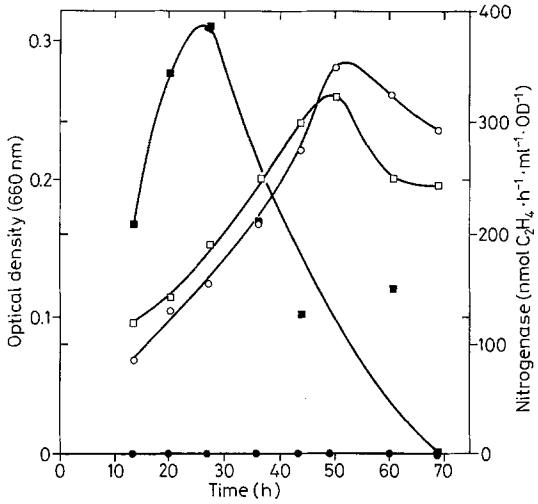

Fig. 1. Growth and nitrogenase activity of *Clostridium formicoaceticum* DSM 93 with N_2 plus 0.05% yeast extract or with 18 mM NH_4Cl plus 0.2% yeast extract as nitrogen sources. Symbols: □ growth with N_2; ○ growth with NH_4Cl; ■ nitrogenase activity with N_2; ● nitrogenase activity with NH_4Cl in the medium

containing less than 0.2% yeast extract. The highest activity was detected in cells of the exponential growth phase (Fig. 1). The decline in activity during the late exponential and the stationary phase might be due to a reduced metabolic activity, resulting in either a decreased ATP level or a decreased flux of reducing equivalents to the nitrogenase. The presence of NH_4Cl in the medium completely suppressed nitrogenase activity. An equimolar presence of tungstate (10^{-7} M) besides molybdate proved not to be inhibitory for strain DSM 92. The organisms could be grown in the defined medium for an extended period with N_2 as nitrogen source.

Methylammonium transport: An active uptake of ^{14}C-methylammonium as an indicator for ammonium uptake (see Kleiner 1981) could not be detected. However, the repression of nitrogenase by NH_4Cl indicated metabolization and thus uptake of ammonium. The decrease of extracellular ammonium concentrations to values below 10 µM (data not shown) during growth on fructose suggests active uptake.

Glutamine synthetase (EC 6.3.1.2): This enzyme was detected in both strains; its level decreased in the presence of NH_4Cl (Table 1). Glycine (1 mM), alanine (10 mM) or serine (3 mM) did not inhibit the glutamine synthetase activity in contrast to the *Escherichia coli* enzyme (Stadtman and Ginsburg, 1974), but ADP (2 mM) slightly lowered the activity to 78%. No change in activity was observed in extracts of ammonium grown cells after incubation with phosphodiesterase (0.1 U per ml extract) in order to split off potentially modifying AMP-groups as observed for *E. coli* (Stadtman and Ginsburg 1974).

Glutamate synthase (GOGAT, glutamine-oxoglutarate amido transferase, EC 1.4.1.13): Both NADH and NADPH activities were detected, the NADH dependent activity being considerably higher. Although strict anaerobic conditions were maintained during extract preparation and assay, the NADPH dependent activity proved extremely unstable. Generally a decrease of over 90% was found within 2 h, leading to a wide variation of the results. The NADH dependent activity was more stable; a decline to 77% was observed within 24 h. These observations suggest the existence of two different enzymes. This is supported by the difference in inhibition with 6-diazo-5-oxo-norleucine, a potent inhibitor of GOGAT (Nagatani et al. 1971). While 50% inhibition of the NADH dependent activity was observed at 2 µM, roughly 50 − 100 µM were required for 50% inhibition of the NADPH dependent activity. No variation in response to the nitrogen source was found.

Glutamate dehydrogenase (EC 1.4.1.2 NAD, EC 1.4.1.3 NAD(P), EC 1.4.1.4 NADP): High activities were found in both strains with NADPH and NADH as cofactors, the K_m for NH_4^+ being 7.6 and 10 mM for the NADH and NADPH dependent activities. No dependence on the ammonium level in the medium could be detected.

Alanine dehydrogenase (EC 1.4.1.1): Like *Clostridium pasteurianum* (Mortenson, 1978) both *C. formicoaceticum* strains contained a NADPH dependent alanine dehydrogenase, but only if ammonium was present in the medium.

Asparagine synthetase (EC 6.3.1.1): *C. formicoaceticum* contained an ammonium dependent asparagine synthetase activity, which seemed to be independent of the extracellular NH_4Cl.

Glutamate degradation: Glutamate is a good substrate for growth of *C. formicoaceticum* in a defined medium (Andreesen et al. 1970; Leonhardt and Andreesen 1977). Two different pathways of glutamate degradation by anaerobic bacteria are known (Buckel and Barker 1974): i) via glutamate dehydrogenase and α-ketoglutarate reductase leading to 2-hydroxyglutarate, and ii) via a B_{12} dependent glutamate mutase and β-methylaspartase. Pathway i) was first suggested by the high glutamate dehydrogenase activities present in extracts of fructose grown cells (Table 1) and of glutamate grown cells (1.8 and 0.06 U per mg with NADH and NADPH as cofactors, respectively), which also worked in the direction of α-ketoglutarate formation (less efficient). However, operation of pathway ii) is likely to occur in *C. formicoaceticum*, since no α-ketoglutarate reductase but a very high β-methylaspartase activity could be detected in glutamate grown cells (10 U per mg protein), which was absent in fructose grown cells.

Discussion

The observations on nitrogen metabolism by *Clostridium formicoaceticum* showed several similarities with other clostridia, but also remarkable differences. Like *C. pasteurianum*, it fixed N_2 as other saccharolytic clostridia (Rosenblum and Wilson 1949), and contained the enzymes of the glutamate cycle (glutamine synthetase and GOGAT; Nagatani et al. 1971), an NH_4^+ dependent asparagine synthetase (Kleiner 1979) and an NADPH dependent alanine dehydrogenase (Mortenson 1978). In contrast to *C. pasteurianum*, where no glutamate dehydrogenase was found (Mortenson 1978; Kleiner 1979), *C. formicoaceticum* contained both the NADH and the NADPH dependent activities like several proteolytic clostridia (Buckel and Barker, 1974). The apparent constitutive nature of both glutamate dehydrogenase activities is somewhat puzzling, since due to the high K_m values of 7.6 and 10 mM for NH_4^+ their function can only be marginal under N_2 fixing conditions, if we assume an intracellular NH_4^+ concentration of around 0.5 mM as in other N_2 fixing species (Kleiner et al. 1981). On the other hand, no major catabolic role can be assigned to these enzyme(s), since we have shown (this paper), that glutamate degradation occurs via methylaspartate (Buckel and Barker 1974).

Another remarkable feature is the occurrence of both an NADH and an NADPH dependent GOGAT activity in *C. formicoaceticum*. In contrast, *C. pasteurianum* contained only the NADH dependent enzyme (Nagatani et al. 1971; Kleiner 1979). So far the occurrence of both activities has only been detected in *Selenomonas ruminantium* (Smith et al. 1980). Both activities may be located on different proteins, as indicated by the different stabilities and sensitivities towards 6-diazo-5-oxo-norleucine.

Nitrogenase and glutamine synthetase are fully and partially repressed, respectively, while alanine dehydrogenase is induced in the presence of ammonium. This suggests the operation of a nitrogen control circuit in *C. formicoaceticum*.

Acknowledgements. This work was supported by "Forschungsmittel des Landes Niedersachsen" (to J.R.A.) and by a grant from the Deutsche Forschungsgemeinschaft (to D.K.).

References

Andreesen JR, El Ghazzawi E, Gottschalk G (1974) The effect of ferrous ions, tungstate and selenite on the level of formate dehydrogenase in *Clostridium formicoaceticum* and formate synthesis from CO_2 during pyruvate fermentation. Arch Microbiol 96:103–118

Andreesen JR, Gottschalk G, Schlegel HG (1970) *Clostridium formicoaceticum* nov. spec. Isolation, description and distinction from *Clostridium aceticum* and *Clostridium thermoaceticum*. Arch Mikrobiol 72:154–174

Barker HA (1981) Amino acid degradation by anaerobic bacteria. Ann Biochem 50:23–40

Barker HA, Smyth RD (1961) β-Methyl-L-aspartatic acid. Biochem Prep 8:89–92

Barker HA, Smyth RD, Wilson RM, Weissbach H (1959) The purification and properties of β-methylaspartase. J Biol Chem 134: 320–328

Buckel W, Barker HA (1974) Two pathways of glutamate fermentation by anaerobic bacteria. J Bacteriol 117:1248–1260

Dainty RH (1972) Glutamate biosynthesis in *Clostridium pasteurianum* and its significance in nitrogen metabolism. Biochem J 126:1055–1056

El Ghazzawi E (1967) Neuisolierung von *Clostridium aceticum* Wieringa und stoffwechselphysiologische Untersuchungen. Arch Mikrobiol 57:1–19

Fawcett JW, Scott JE (1960) A rapid and precise method for the determination of urea. J Clin Path 13:156–159

Goa J (1953) A microbiuret method for protein determination. Scand Clin Lab Invest 5:218–222

Gottschalk G, Andreesen JR, Hippe H (1981) The genus *Clostridium*. Nonmedical aspects. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The procaryotes. A handbook on habitats, isolation and identification of bacteria. Springer, Berlin Heidelberg New York, pp 1767–1803

Kleiner D (1979) Regulation of ammonium uptake and metabolism by nitrogen fixing bacteria. III. *Clostridium pasteurianum*. Arch Microbiol 120:263–270

Kleiner D (1981) The transport of NH_3 and NH_4^+ across biological membranes. Biochim Biophys Acta 639:41–52

Kleiner D (1982) Ammonium (methylammonium) transport by *Klebsiella pneumoniae*. Biochim Biophys Acta 688:702–708

Kleiner D, Phillips S, Fitzke E (1981) Pathways and regulatory aspects of N_2 and NH_4^+ assimilation in N_2 fixing bacteria. In: Bothe H, Trebst A (eds) Biology of inorganic nitrogen and sulfur. Springer, Berlin Heidelberg New York, pp 131–139

Leonhardt U, Andreesen JR (1977) Some properties of formate dehydrogenase accumulation and incorporation of ^{185}W-tungsten into proteins of *Clostridium formiaceticum*. Arch Microbiol 115:227–284

Lerud RF, Whiteley HR (1971) Purification and properties of α-ketoglutarate reductase from *Micrococcus aerogenes*. J Bacteriol 106:571–577

Mortenson LE (1978) Regulation of nitrogen fixation. In: Horecker BL, Stadtman ER (eds) Curr top cell regul, vol 13. Academic Press, New York, pp 179–232

Nagatani H, Shimizu M, Valentine RC (1971) The mechanism of ammonia assimilation in nitrogen fixing bacteria. Arch Mikrobiol 79:164–175

Rosenblum ED, Wilson PW (1949) Fixation of isotopic nitrogen by *Clostridium*. J Bacteriol 57:413–414

Smith JC, Hespell RB, Bryant MP (1980) Ammonia assimilation and glutamate formation in the anaerobe *Selenomonas ruminantium*. J Bacteriol 141:593–602

Stadtman ER, Ginsburg A (1974) The glutamine synthetase of *Escherichia coli* structure and control. In: Boyer PD (ed) The enzymes, vol X. Academic Press, New York, pp 755–807

Tanner RS, Stackebrandt E, Fox GE, Gupta R, Magnum LJ, Woese CR (1982) A phylogenetic analysis of anaerobic eubacteria capable of synthesizing acetate from carbon dioxide. Curr Microbiol 7:127–132

Vogels GD, Van der Drift C (1976) Degradation of purines and pyrimidines by microorganisms. Bacteriol Rev 40:403–468

Wolin EA, Wolfe RS, Wolin MJ (1964) Viologen dye inhibition of methane formation by *Methanobacillus omelianskii*. J Bacteriol 87:993–998

Zehnder AJB, Wuhrmann K (1976) Titanium-III-citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science 194:1165–1166

Received January 5, 1983/Accepted February 2, 1983

Note Added in Proof

Recently evidence for an ammonium transport system in *C. formicoaceticum* was obtained when the cells were treated as follows: The organisms were grown under N_2 fixing conditions, harvested anaerobically in the logarithmic phase, and incubated anaerobically for 3–5 min in a medium containing 1% fructose, 1% $NaHCO_3$ and 20 mM potassium phosphate pH 7.6. Methylammonium uptake then occurred at a rate of about 0.1 nmol per min per mg protein. After 20 min more than 70% of the intracellular label could be chased from the cells by the addition of 50 mM NH_4^+. Methylammonium uptake was repressed to more than 90% if 18 mM NH_4Cl was present in the growth medium.