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NOTES

Presence of Cytochrome and Menaquinone in *Clostridium formicoaceticum* and *Clostridium thermoaceticum*

MECHTHILD GOTTWALD, JAN R. ANDREESEN,* JEAN LEGALL,[1] AND LARS G. LJUNGDAHL

Institut für Mikrobiologie der Universität Götingen, D-3400 Göttingen, and Department of Biochemistry, University of Georgia, Athens, Georgia 30602

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Cytochrome *b* and menaquinone have been demonstrated in the homoacetate-fermenting *Clostridium formicoaceticum* and *Clostridium thermoaceticum*.

In this report we would like to present evidence for the presence of heme in the homoacetate-fermenting *Clostridium formicoaceticum* and *Clostridium thermoaceticum*. Until now these microorganisms have been thought to contain only the corrinoid type of tetrapyrroles (7,13). In addition to heme the two bacteria also contain menaquinone.

C. formicoaceticum (DSM 92, ATCC 27076) was grown anaerobically in a medium containing fructose, 55 mM, or sodium-fumarate, 100 mM; $NaHCO_3$ or sodium-formate, 120 mM; peptone (Difco), 5 g/liter; yeast extract (Difco), 5 g/liter; K_2HPO_4, 58 mM; sodium-thioglycolate (Difco), 6.6 mM; pyridoxine, 5 μM; Fe-NH_4-citrate, 10^{-4} M; Na_2MoO_4, 10^{-4}M; Na_2SeO_3, 10^{-6}M. Ethylenediaminetetraacetate, $ZnSO_4$, $MnCl_2$, H_3BO_3, $CoCl_2$, $CuCl_2$, and $NiCl_2$ were added in concentrations as given in the SL 4 solution of Pfennig and Lippert (14).

C. thermoaceticum (DSM 521) was grown at 58 C under 100% CO_2 on glucose, 100 mM; $NaHCO_3$, 200 mM; tryptone (Difco), 5 g/liter; yeast extract (Difco), 5 g/liter; $(NH_4)_2SO_4$, 7.6 mM; sodium-thioglycolate (Difco), 4.4 mM; $MgSO_4$, 10^{-3} M; $Co(NO_3)_2$, 10^{-4} M; $Fe(NH_4)_2(SO_4)_2$, 10^{-4} M; Na_2WO_4, 10^{-5} M; and Na_2SeO_3, 10^{-6} M. Spores of *C. thermoaceticum* were activated by heating in a boiling water bath for 10 min. Cells of both bacteria were harvested at the end of the logarithmic growth phase or at the beginning of the stationary phase. Extracts were prepared using the French pressure cell (1,2). Acetone-hydrochloride extracts and pyridine hemochrome derivatives of the heme were prepared according to Jacobs and Wolin (8). For quantitative estimation of the cytochrome *a* $\Delta\epsilon$ (mM^{-1} cm^{-1}) of 20 (maximum of alpha-peak minus absorption at 568 nm) was used. Menaquinones were assayed as described by Kröger and Dadak (10) and fumarate reductase according to Bernath and Singer (4).

A cytochrome of *C. formicoaceticum* was detected in the pellet fraction of fumarate-grown cells obtained after 1 h of centrifugation of the crude extract at 48,000 × *g*. Difference spectra (reduced minus oxidized) of dithionite-reduced particles indicated a cytochrome and had alpha- and beta-peaks at 552 and 522 nm, respectively, and a gamma-peak at 424 nm (Fig. 1). Spectra obtained at low temperature (– 196 C) showed maxima at 548, 522, and 420 nm, respectively. The heme was completely extracted by acetone-hydrochloride. The pyridine-hemochrome derivative of the extracted heme gave a difference spectrum with maxima at 556, 526, and 426 nm (Fig. 2). These observations clearly indicated the presence of a *b*-type cytochrome. The cytochrome content in extracts of *C. formicoaceticum* cells grown on fumarate/bicarbonate was about 0.04 μmol per g of protein, and in extracts of cells grown on fumarate/formate, 0.12 μmole per g of protein. In cells grown on fructose the amount of cytochrome was too small to calculate.

Fumarate reductase activity in crude extracts of fructose-grown cells was 0.007 μmol/min per mg of protein and in fumarate grown cells, 0.19 μmol/min per mg of protein in both supernatant and pellet fraction.

A hemoprotein was found also in particles obtained from extracts of *C. thermoaceticum* by centrifugation at 100,000 × *g* for 1 h. The heme was extracted with acetone-hydrochloride and the pyridine hemochrome was prepared. It had a difference spectrum (reduced minus oxidized) with maxima at 556, 523, and 420 nm as shown in Fig. 3. The results also indicate a *b*-type

[1] Present address: Laboratoire de Chimie Bactérienne, C.N.R.S.31, Chemin Joseph Aiguier, 13274-Marseille Cedex 2, France.

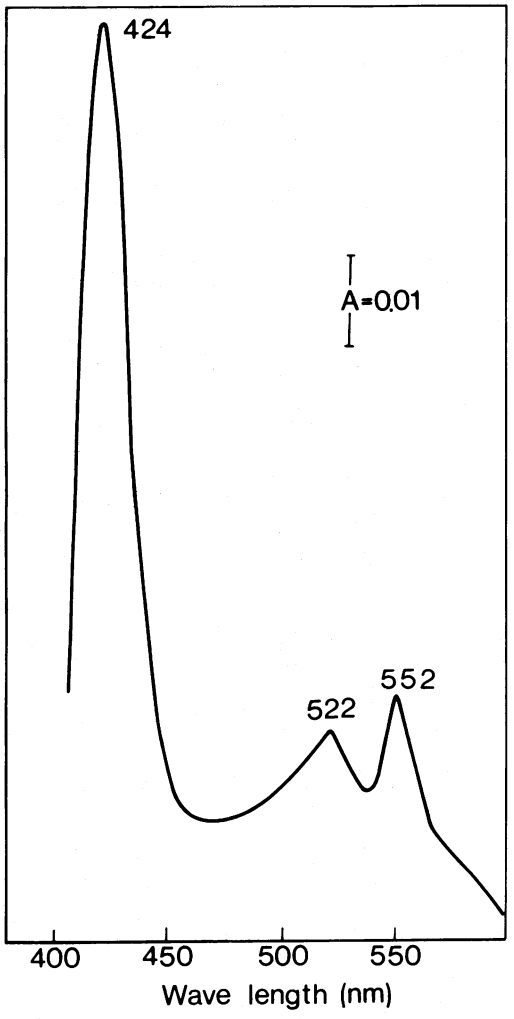

FIG. 1. *Dithionite-reduced minus air-oxidized difference spectrum of a crude particle preparation (5 mg of protein/ml) of C. formicoaceticum.*

cytochrome. The amount of the heme was about 0.19 μmol per g of particle protein.

Menaquinones were assayed in cells of both *C. formicoaceticum* and *C. thermoaceticum*. The amount in the former was estimated to be 0.03 μmol per *g* of protein in fumarate/bicarbonate-grown cells, and in *C. thermoacetium*, 0.3 μmol per g of protein.

The positions of the alpha- and beta-peaks of the spectra of the reduced cytochrome and of the pyridine derivate of *C. formicoaceticum* are of special interest since they indicate the possible presence of a *c*-type cytochrome. However, since the acetone-hydrochloride extraction leads to the complete removal of the heme the cytochrome must be considered a *b*-type according to the recommendations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (1973) which state that *b*-type cytochromes contain hemes that are not covalently bound to the protein.

Special attention was drawn to the possibility that a contamination was responsible for the cytochrome content. Therefore *C. formicoaceticum* was grown from a colony obtained after repeated application of the dilution agar shake culture method (12). A pasteurization step was included before each transfer. Cytochrome was consistently found in the fumarate grown cultures. Furthermore, the strain of *C. formicoaceticum* isolated by El Ghazzawi (5)(DSM 93; ATCC23439) also contained cytochromes. Cells of *C. thermoaceticum* were grown from three different cultures kept separately for many years, (i) from a broth culture maintained in liquid medium for 7 years; (ii) from an agar stab culture transferred yearly since 1958; (iii) from a spore culture untouched

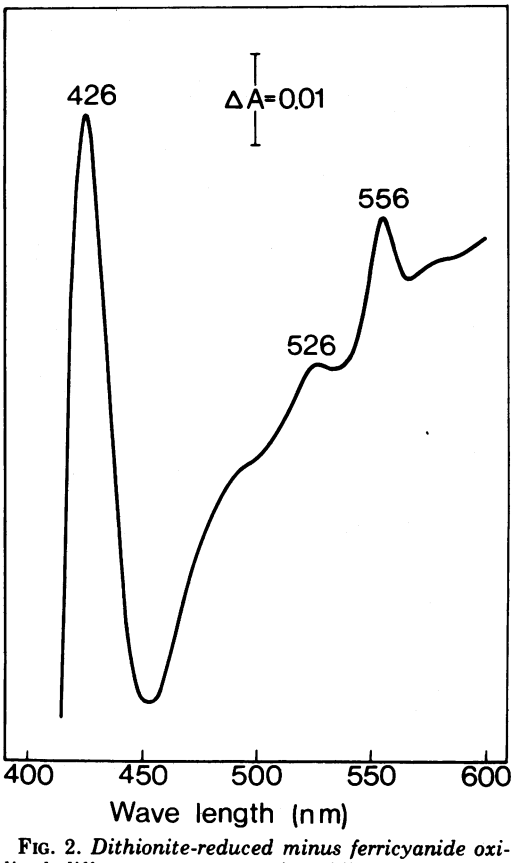

FIG. 2. *Dithionite-reduced minus ferricyanide oxidized difference spectrum of pyridine hemochrome from 0.12 g of particle protein from C. formicoaceticum extracted with acetone-hydrochloride. Final volume was 2 ml.*

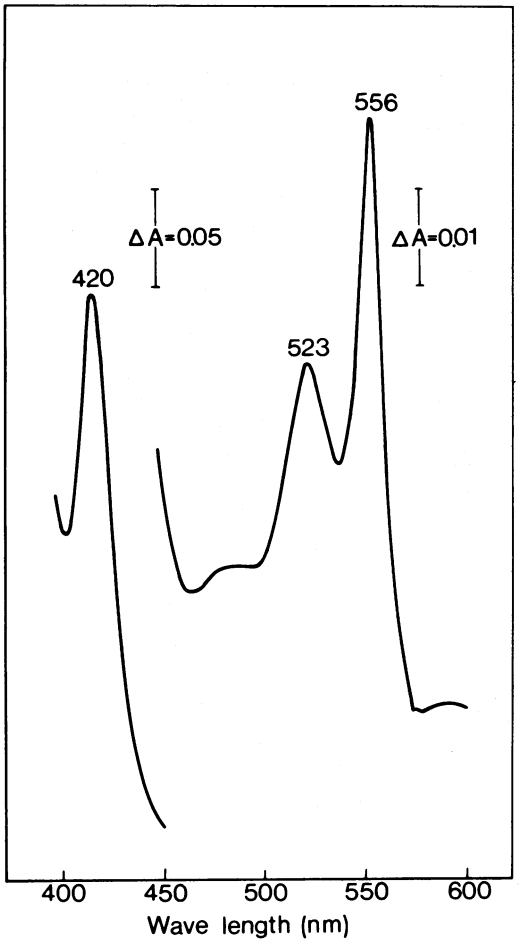

FIG. 3. *Dithionite-reduced minus air-oxidized difference spectrum of pyridine-hemochrome from 0.19 g of particles from C. thermoaceticum extracted with acetone-hydrochloride. Final volume was 2.5 ml.*

since 1948. In all three cultures grown on glucose, cytochromes were found in about equal amounts.

The presence of cytochrome *b* and of menaquinone in *C. formicoaceticum* may be rationalized by its ability to use fumarate as an electron acceptor. It is well known that these two electron carriers are involved in the fumarate reductase system (6, 8, 9, 16—19).

The existence of the two electron carriers in *C. thermoaceticum* is more difficult to explain. This bacterium does not grow on fumarate (3) and a fumarate reductase has not been observed. However, the growth yield of *C. thermoaceticum* is higher than expected when grown on sugars and it has been suggested that this bacterium may perform an electron transport phosphorylation in the course of CO_2 reduction to acetate (3). The presence of the two electron carriers is compatible with this suggestion.

Although A. J. D'Eustachio in a preliminary communication (Bacteriol. Proc. p. 71, 1966) and Newton and Kamen in their review (11) claim the presence of a heme compound in *C. pasteurianum*, to our knowledge this is the first report of the presence of cytochromes in clostridia. This finding may raise the question if the two homoacetate-fermenting clostridia ought to be reclassified. However, this should await analysis of deoxyribonucleic acid nucleotide ratios and an investigation whether a plasmid is responsible for the synthesis of the heme or if its synthesis is directed by the bacterial genome.

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LITERATURE CITED

1. Andreesen, J. R., E. El Ghazzawi, and G. Gottschalk. 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.
2. Andreesen, J. R., and L. G. Ljungdahl. 1973. Formate dehydrogenase of *Clostridium thermoaceticum*. Incorporation of selenium-75, and the effects of selenite, molybdate, and tungstate on the enzyme. J. Bacteriol. **116**:867-873.
3. Andreesen, J. R., A. Schaupp, C. Neurauter, A. Brown, and L. G. Ljungdahl. 1973. Fermentation of glucose, fructose, and xylose by *Clostridium thermoaceticum*: effect of metals on growth yield, enzymes, and the synthesis of acetate from CO_2. J. Bacteriol. **114**:743-751.
4. Bernath, P., and T. P. Singer. 1962. Succinic dehydrogenase, p. 597-614. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
5. El Ghazzawi, E. 1967. Neuisolierung von *Clostridium aceticum* und stoffwechselphysiologische Untersuchungen. Arch. Mikrobiol. **57**:1-19.
6. Hatchikian, E. C. 1974. On the role of menaquinone-6 in the electron transport of hydrogen: fumarate system in the strict anaerobe *Desulfovibrio gigas*. J. Gen. Microbiol. **81**:261-266.
7. Irion, E., and L. G. Ljungdahl. 1968. Isolation of Co-5'-deoxyadenosylcobinamide guanosine diphosphate, α-(2-methyladenyl)-Co-5'-deoxyadenosylcobamide, and Co-methylcorrinoids from *Clostridium thermoaceticum*. Biochemistry **7**:2350-2355.
8. Jacobs, N. S., and M. S. Wolin. 1963. Electron-transport system of *Vibrio succinogenes*. I. Enzymes and cyto-

chromes of the electron-transport system. Biochim. Biophys. Acta **69**:18-28.
9. Kröger, A. 1974. Electron-transport phosphorylation coupled to fumarate reduction in anaerobically grown *Proteus rettgeri*. Biochim. Biophys. Acta **347**:273-289.
10. Kröger, A., and V. Dadak. 1969. On the role of quinones in bacterial transport: the respiratory system of *Bacillus megaterium*. Eur. J. Biochem. **11**:328-340.
11. Newton, J. W., and M. D. Kamen. 1961. Cytochrome systems in anaerobic electron transport, p. 397-423. *In* I. C. Gunsalus and R. Y. Stainer (ed.), The bacteria. II. Metabolism. Academic Press Inc., New York.
12. van Niel, C. B. 1971. Techniques for the enrichment, isolation and maintenance of the photosynthetic bacteria, p. 3-28. *In* A. San Pietro (ed.), Methods in enzymology, vol. 23, part A. Academic Press Inc., New York.
13. O'Brien, W. E., and L. G. Ljungdahl. 1972. Fermentation of fructose and synthesis of acetate from carbon dioxide by *Clostridium formicoaceticum*. J. Bacteriol. **109**:626-632.
14. Pfennig, N., and K. D. Lippert. 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55**:245-256.
15. Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. 1973. Cytochromes, p. 34-38. *In* Enzyme nomenclature. Elsevier Scientific Publishing Company, Amsterdam.
16. Sone, N. 1974. The redox reactions in propionic acid fermentation. IV. Participation of menaquinone in the electron transfer system in *Propionibacterium arabinosum*. J. Biochem. **76**:137-145.
17. deVries, W., W. M. C. vanWyck-Kapteyn, and S. K. H. Oosterhuis. 1974. The presence and function of cytochromes in *Selenomonas ruminantium*, *Anaerovibrio lipolytica* and *Veillonella alcalescens*. J. Gen. Microbiol. **81**:69-78.
18. deVries, W., W. M. C. vanWyck-Kapteyn, and A. H. Stouthamer. 1973. Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. J. Gen. Microbiol. **76**:31-41.
19. White, D. C., M. P. Bryant, and D. R. Caldwell. 1962. Cytochrome-linked fermentation in *Bacteroides ruminicola*. J. Bacteriol. **84**:822-828.