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Alkalophiles Have Much Higher Cytochrome Contents than Conventional Bacteria and than their own Non-Alkalophilic Mutant Derivatives

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SUMMARY: The membranes of two alkalophilic bacilli contain extraordinarily high cytochrome heme contents, at least 5.5 nmoles/mg membrane protein. Membranes from the non-alkalophilic derivatives of these bacterial strains contain much lower heme concentrations, especially lower b- and c-type cytochromes. The respiratory rates of whole cells of the non-alkalophiles were, nevertheless, equal to or slightly greater than those of the alkalophiles at their respective optimal pHs. Moreover, although alkalophiles expend energy to keep their cytoplasmic pH below the external pH, their growth yields on L-malate are comparable to conventional bacteria. Perhaps, among the special bioenergetic properties of alkalophilic bacteria, there are characteristics of the respiratory chain which facilitate particularly efficient energy transduction.

INTRODUCTION: During our studies of obligately alkalophilic bacilli, we observed that isolated membranes from Bacillus alcalophilus and Bacillus firmus RAB are rather intensely red in color, resembling the color of mitochondrial membranes. Non-alkalophilic mutant strains, B. alcalophilus KM23 and B. firmus RABN, derived from those species, were comparatively pale in color. The possession of a high level of respiratory chain components could be part of a constellation of special bioenergetic properties of alkalophiles. The non-alkalophilic strains had been characterized as lacking the electrogenic Na^+/H^+ antiporter which enables the alkalophilic strains to maintain a cytoplasmic pH that is lower than optimal external pHs (1-3). A markedly reduced level of cytochromes in the mutants might indicate an interesting regulatory

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effect of the antiporter deficiency upon respiratory properties. Therefore, we examined the cytochrome content of the membranes from B. alcalophilus, B. firmus RAB and their non-alkalophilic derivatives. An extraordinarily high level of cytochromes was found in the alkalophiles relative to the non-alkalophilic mutants and to other bacteria, without a correspondingly high rate of oxygen consumption by whole cells.

MATERIALS AND METHODS: B. alcalophilus (ATCC 27647), its non-alkalophilic derivative KM23, B. firmus RAB, and its non-alkalophilic derivative RABN were all grown with shaking at 30° C. in L-malate-containing medium, as described previously (1,2,4). The alkalophilic strains and non-alkalophilic strains were grown at pH 10.5 and 6.8, respectively.

Membrane vesicles were prepared from cells of each strain grown on L-malate to the late logarithmic phase, by a modification of Kaback's lysozyme method (5), as described elsewhere (3). The membranes were prepared in 100 mM potassium carbonate buffer, containing 10 mM $MgSO_4$, at pH 9.0. Dithionite-reduced minus air-oxidized difference spectra were recorded at room temperature with a Perkin Elmer 557 Dual Beam spectrophotometer to measure the cytochrome content of membrane vesicles. The following difference millimolar extinction coefficients and wavelength pairs were used: cytochrome (a+a_3), $\Delta A_{605-630}$, $\Delta\varepsilon$=20.5/heme (6); cytochrome b, $\Delta A_{560-575}$, $\Delta\varepsilon$=17.5 (7); and cytochrome c, $\Delta A_{551-538}$, $\Delta\varepsilon$=17.3 (8). Hemes could be extracted from the membrane vesicles of the two alkalophilic strains using acidified ethyl methyl ketone. Pyridine hemochromogens were then prepared according to the method of Falk (9). Reduced-minus-oxidized difference spectra were recorded for these compounds at room temperature. The following extinction coefficients were used to calculate heme concentrations: heme a, λ=587, ε=24 (10); protoheme IX, λ=557, $\Delta\varepsilon_{557-541}$=20.7 (9); and heme c, λ=551, $\Delta\varepsilon_{551-535}$=19.1 (10). The procedure gave very incomplete extraction of hemes from both non-alkalophilic strains; therefore the data derived from application of this method to those strains are not presented.

Respiratory rates of whole cells were assayed by oxygen uptake, using a Yellow Springs Instrument Model 53 Clark-type oxygen monitor. Cells in the late logarithmic phase were harvested, and resuspended in 25 mM potassium phosphate buffers at pH 6.8, or 25 mM potassium carbonate buffer at either pH 8.5 or 10.5. The cell protein concentrations were in a range from 0.03 to 0.1 mg cell protein/ml. Experiments were conducted at cell protein concentrations within that range such that the rate of oxygen uptake increased linearly with increasing protein concentration. Samples (3.0 ml) were allowed to become air saturated in the monitor chamber and were equilibrated to 30°C. for 3 min. The rate of oxygen uptake was determined both before and after the addition of 3.3 mM L-malate. The L-malate concentration used allowed the maximal rate of oxygen uptake. The respiratory rates were unaffected by the addition of 10 μM gramicidin. Protein concentra-

tions were determined by the method of Lowry et al. (11), using egg white lysozyme as a standard.

The growth yield (Y) of the two alkalophilic strains on L-malate was determined by the method of Stouthamer (12) and is expressed as mg dry weight per mmole substrate. For cultures which required yeast extract for growth, one culture with yeast extract only was used as a control.

RESULTS AND DISCUSSION: As shown in Table I, membranes from B. alcalophilus and B. firmus RAB contain at least 5.5 nmoles of cytochrome heme/mg protein. Assays of copper content should clarify the estimates of cytochrome a, which are affected in each of the techniques by assumption of an extinction coefficient. However, even if each value represents only a reasonable estimate, the cytochrome contents of the alkalophiles are extraordinarily high. Values reported by other investigators for conventional non-alkalophilic bacteria include 0.72 and 0.76 nmoles heme/mg membrane protein for Escherichia coli (13) and Bacillus cereus (14), respectively. Even unusually high levels found previously, e.g., 4.66 nmoles heme/mg membrane protein in Nitrosomonas (15) and 3.88 nmoles heme/mg membrane protein in particles from Azobacter vinelandii (8), are exceeded in the alkalophiles. As an additional frame of reference, typical heme contents of whole beef heart mitochondria and rat liver mitochondria are 2.6 and 0.94 nmoles/mg protein (16,17).

Estimations of the cytochrome contents from difference spectra indicated a markedly lower level in the two non-alkalophilic strains than in the parent strains (Table I); the b- and c-type cytochromes were decreased relatively more than a-type cytochromes. The difference between the membranes from the alkalophilic and non-alkalophilic strains with respect to the extractability of hemes was interesting, and could relate to changes in various membrane properties.

TABLE I

CYTOCHROME CONTENTS OF MEMBRANES FROM ALKALOPHILIC BACTERIA AND THEIR NON-ALKALOPHILIC MUTANT DERIVATIVES

Cytochrome contents were assayed in wild type strains by analysis of difference spectra and by extraction of hemes followed by pyridine hemochromogen preparation, as described in Materials and Methods. For the non-alkalophilic strains only the former method was employed, because acidified ethyl methyl ketone failed to give quantitative extraction from their membranes.

	Concentration of cytochrome (nmoles/mg membrane protein)					
	a-type		b-type		c-type	
Bacterial Strain	Difference Spectra	Heme Extraction	Difference Spectra	Heme Extraction	Difference Spectra	Heme Extraction
Bacillus alcalophilus	0.51	1.3	2.2	1.7	2.6	2.5
Bacillus alcalophilus KM23	0.23	-	0.54	-	0.50	-
Bacillus firmus RAB	0.57	1.7	2.7	1.1	3.8	2.7
Bacillus firmus RABN	0.43	-	0.50	-	0.53	-

TABLE II

RESPIRATORY RATES OF WHOLE CELLS OF ALKALOPHILIC BACTERIA AND THEIR NON-ALKALOPHILIC MUTANT DERIVATIVES

Washed L-malate-grown cells of each strain were resuspended in 25 mM buffer at the indicated pH. Oxygen uptake, in the presence of 3.3 mM L-malate, was measured as described under Materials and Methods.

	Oxygen Uptake (n-atoms O/min/mg cell protein)		
Bacterium	pH 6.8	pH 8.5	pH 10.5
B. alcalophilus	-	996	987
B. alcalophilus KM23	1351	176	-
B. firmus RAB	-	983	979
B. firmus RABN	1404	328	-

As shown in Table II, the rate of oxygen uptake by L-malate-grown whole cells of the alkalophiles, in the presence of L-malate, was almost 1,000 n-atoms O/min/mg cell protein. This is very similar to rates reported for E. coli (18) in the presence of Krebs cycle intermediates. Moreover, non-alkalophilic B. alcalophilus KM23 and B. firmus RABN exhibited slightly higher rates of oxygen uptake at their optimal pH for growth, pH 6.8, than did the alkalophilic parent strains at their optimum of pH 10.5. At the intermediate pH of 8.5, at which both strains grew suboptimally, the alkalophilic strains retained a greater rate of respiration.

The lack of correlation between whole cell respiratory rates and cytochrome heme contents is notable. Moreover, the growth yields of B. alcalophilus and B. firmus RAB on L-malate were found to be 37 and 31 mg dry weight/mmole of L-malate, respectively. These yields were completely comparable to those obtained for Bacillus megaterium and Arthrobacter pyridinolis in parallel determinations (data not shown). Thus, neither substrate nor O_2

consumption is elevated in the alkalophiles, whereas the cytochrome content is unusually high. It will be of particular interest to examine the proton translocation properties (H^+/O ratios) and other characteristics of respiratory chain structure and function in the alkalophiles. Jones et al. (19), have suggested that bacteria vary greatly in H^+/O ratios, depending upon the specific array of cytochromes and other respiratory chain components. Were the alkalophiles to possess an unusual ability to translocate protons, this ability could relate importantly to the problem of ATP synthesis in these bacteria (1).

The cytochrome contents are markedly reduced in non-alkalophilic strains whose primary defect is in the Na^+/H^+ antiporter (2-4). Revertants are found to regain both antiporter activity and wild type levels of cytochromes (data not shown). The basis for these observations will be studied further. Among other approaches, attempts will be made to isolate non-alkalophilic (Na^+/H^+ antiporter-deficient) strains with high cytochrome contents.

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REFERENCES:

1. Guffanti, A.A., Susman, P., Blanco, R., and Krulwich, T.A. (1978) J.Biol.Chem. 253, 708-715.
2. Guffanti, A.A., Blanco, R., Benenson, R.A., and Krulwich, T.A. (1980) J.Gen.Microbiol., in press.
3. Mandel, K.G., Guffanti, A.A., and Krulwich, T.A. (1980) J.Biol. Chem., in press.
4. Krulwich, T.A., Mandel, K.G., Bornstein, R.F., and Guffanti, A.A. (1979) Biochem.Biophys.Res.Commun. 91, 58-62.
5. Kaback, H.R. (1971) in Methods in Enzymology (Jakoby, W.B., ed.) Vol. 22, pp. 99-120, Academic Press, New York.
6. Van Gelder, B.F. (1966) Biochim.Biophys.Acta 118, 36-46.
7. Deeb, S.S., and Hager, L.P. (1964) J.Biol.Chem. 239, 1024-1031.
8. Jones, C.W., and Redfearn, E.R. (1966) Biochim.Biophys.Acta 113, 467-481.
9. Falk, J.E. (1964) Porphyrins and Metalloporphyrins, Elsevier, Amsterdam.

10. Rieske, J.S. (1967) in Methods in Enzymology (Estabrook, R.W., and Pullman, M.E., eds.) Vol. 10, pp. 488-493, Academic Press, New York.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275.
12. Stouthamer, A.H. (1969) in Methods in Microbiology (Norris, J.R., and Ribbons, D.W., eds.) Vol. 1, pp. 629-663, Academic Press, New York.
13. Itagaki, E. (1964) J.Biochem. 55, 432-445.
14. Doi, R.H., and Halvorson, H. (1961) J.Bacteriol. 81, 51-58.
15. Falcone, A.B., Shug, A.L., and Nicholas, D.J.D. (1962) Biochem. Biophys.Res.Commun. 9, 126-131.
16. Green, D.E., and Wharton, D.C. (1963) Biochem.Zeitschr. 338, 335-348.
17. Chance, B., and Hess, B. (1962) J.Biol.Chem. 234, 2404-2412.
18. Lawford, H.G., and Haddock, B.A. (1973) Biochem.J. 136, 217-220.
19. Jones, C.W., Brice, J.M., Downs, A.J., and Drozd, J.W. (1975) Eur.J.Biochem. 52, 265-271.