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SHORT COMMUNICATION

Pleiotropic Properties of Mutations to Non-alkalophily in *Bacillus alcalophilus*

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Non-alkalophilic mutant strains of *Bacillus alcalophilus* exhibited a loss of Na^+/H^+ antiport activity and Na^+-coupling of solute transport, and decreased contents of membrane-bound cytochromes and of a membrane-bound chromophore that absorbs light at 526 nm. Evidence that these changes could result from a single mutation is presented.

INTRODUCTION

Obligately alkalophilic bacilli grow only in the extraordinary pH range of 9·0 to 12 (Guffanti *et al.*, 1978, 1980). Over this range of pH values for growth, the cytoplasmic pH is maintained at pH 9·5 or below (Guffanti *et al.*, 1978, 1980). Upon energization, isolated membrane vesicles can also produce a pH gradient, acid in, only if Na^+ is present (Mandel *et al.*, 1980). Studies of Na^+ and H^+ movements in cells (Guffanti *et al.*, 1980; Krulwich *et al.*, 1979), right-side-out vesicles and everted vesicles (Mandel *et al.*, 1980) indicate the presence of an electrogenic Na^+/H^+ antiporter which acidifies the cytoplasm or intravesicular space relative to the outside *milieu*. These observations led to the idea that the obligate nature of alkalophiles might be due to the antiporter activity, i.e. below pH 9·0 the cytoplasm might lack the capacity to buffer the protons taken up. Indeed, non-alkalophilic strains that could grow in the neutral pH range had lost the ability to grow above pH 9·0, and exhibited none of the Na^+ or H^+ movements attributed to the Na^+/H^+ antiporter (Guffanti *et al.*, 1980; Krulwich *et al.*, 1979; Mandel *et al.*, 1980). In *Bacillus alcalophilus*, loss of Na^+/H^+ antiporter activity was accompanied by a loss of the Na^+-dependence of solute transport systems; Na^+/solute symport is a common transport mechanism in this and other alkalophiles (Guffanti *et al.*, 1980; Kitada & Horikoshi, 1977, 1980). Several lines of evidence indicate that, in *B. alcalophilus*, both the loss of Na^+-coupling to solute transport and the loss of antiporter activity are primary effects of the mutation (Guffanti *et al.*, 1981). Similar findings have been reported in *Escherichia coli* (Zilberstein *et al.*, 1980). We have suggested that the mutation to non-alkalophily might affect a Na^+-translocating element that is common to Na^+-coupled symport and antiport systems in *B. alcalophilus* (Guffanti *et al.*, 1981). In the context of this suggestion and alternative hypotheses, we now consider evidence of further pleiotropic characteristics of non-alkalophilic mutants. We show that all the properties appear as an indivisible aggregate in many independent non-alkalophilic strains and are restored completely in revertants.

METHODS

Bacillus alcalophilus (ATCC 27647) was the wild-type strain used in these experiments. Strain KM23 and other KM strains were non-alkalophilic mutant derivatives of the wild-type; these were isolated, after mutagenesis with

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ethyl methanesulphonate or spontaneous mutation, by growth at pH 7·0, as described previously (Krulwich *et al.*, 1979). KMR strains were revertants of strain KM23 that had regained the ability to grow at pH 10·5 after mutagenesis. The wild-type and KMR strains were grown at pH 10·5 and KM strains at pH 7·0 in the L-malate-containing media described elsewhere (Guffanti *et al.*, 1978; Krulwich *et al.*, 1979). For growth in complete darkness, growth vessels were wrapped with light-proof foil. For growth in 'low iron' medium, cells were grown for four passages in media from which iron was omitted from the trace salts. Membrane vesicles were prepared essentially by the method of Kaback (1971), with slight modifications (Mandel *et al.*, 1980). All vesicle preparations were done at pH 9·0 in 100 mM-potassium carbonate buffer containing 10 mM-$MgSO_4$. Production of a pH gradient, acid inside, by the vesicles was assayed by accumulation of methylamine as described previously (Mandel *et al.*, 1980). The energy source was 20 mM-ascorbate plus 2 mM-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). Uptake of α-aminoisobutyric acid (AIB) by vesicles was determined by a filtration assay using oxygenated reaction mixtures containing 1 mg vesicle protein ml^{-1}, 40 μM-AIB, 20 mM-ascorbate and 2 mM-TMPD. When present, Na^+ was added to a final concentration of 10 mM. Initial rates were determined and corrected for AIB binding by unenergized vesicles. Cytochrome contents were determined from difference spectra of reduced versus oxidized vesicles as described previously (Lewis *et al.*, 1980). For visualization of the chromophore that absorbs light at 526 nm, absolute absorbance spectra were recorded against a water reference in a Perkin Elmer 557 spectrophotometer operated in the dual-beam mode. The amount of chromophore was estimated by $\Delta A_{526-505}$.

RESULTS AND DISCUSSION

In addition to the loss of Na^+-coupling, recent studies have shown that non-alkalophilic mutants have much lower membrane cytochrome contents than their wild-type parents and that the decreases are especially pronounced in *b*- and *c*-type cytochromes (Lewis *et al.*, 1980). During spectrophotometric studies of the membranes it was also observed that vesicles from wild-type *B. alcalophilus* contained a chromophore that exhibits an absorption band at 526 nm (Fig. 1). Absorption at this wavelength was unchanged by reduction with dithionite or oxidation by ferricyanide (results not shown). Non-alkalophilic strains such as KM23 showed little or no light absorption at 526 nm (Fig. 1). While perhaps unrelated, it was notable that after several days of growth of non-alkalophilic mutants, a brown pigment appeared in the culture medium; no such pigment was found in cultures of the wild-type.

Dozens of independently isolated non-alkalophilic strains (KM strains) and independently isolated revertants of KM23 (KMR strains) have been studied. As shown in Table 1 for three alkalophilic strains (wild-type *B. alcalophilus* and two KMR strains) and three non-alkalophilic strains, the pleiotropic properties are expressed in each strain. That is, Na^+/H^+ antiport activity (as assayed by energy- and Na^+-dependent acidification of the intravesicular space), Na^+-dependent uptake of AIB, cytochrome contents above 5 nmol (mg membrane protein)$^{-1}$ and high levels of the chromophore that absorbs light at 526 nm were found in all wild-type and revertant strains. By contrast, all KM strains exhibited no Na^+/H^+ antiporter activity, no Na^+-dependent AIB uptake, and much lower cytochrome and chromophore contents (Table 1). AIB uptake by vesicles of the KM strains was assayed at pH 7·0 as well as pH 9·0. At the lower pH, appreciable uptake was observed with all three mutants, but this uptake was Na^+-independent, as previously reported for whole cells of KM23 (Krulwich *et al.*, 1979).

The frequencies of spontaneous mutation of the wild-type to non-alkalophily (1 in $1·6 \times 10^9$) and reversion of non-alkalophilic strain KM23 to alkalophily (1 in $0·62 \times 10^9$) are consistent with a single mutation. Again, the distinction between strains was growth of non-alkalophiles at pH 7·0 (but not at pH 10·5) and growth of alkalophiles at pH 10·5 (but not at pH 7·0).

While a single mutation in a Na^+-translocating element could account for simultaneous loss of Na^+-dependent antiport and symport activities, it was less clear how such a mutation could result in the changes in cytochrome content. Since one of the roles of the Na^+/H^+ antiporter is regulation of the cytoplasmic pH, we considered the possibility that perturbations of cytoplasmic pH, upon loss of the antiporter, might mediate changes in

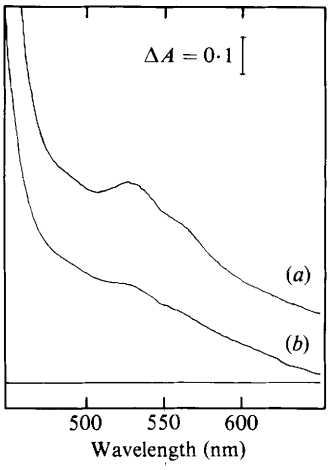

Fig. 1. Spectra showing the chromophore that absorbs at 526 nm. Absolute absorbance spectra were recorded as described in Methods. A few grains of potassium ferricyanide were added to prevent reduction of cytochromes. Trace (*a*) is of vesicles prepared from wild-type *B. alcalophilus* and trace (*b*) is of vesicles prepared from strain KM23. Protein concentrations for both preparations were 3·0 mg ml^{-1}.

Table 1. *Bioenergetic characteristics of membrane vesicles from non-alkalophilic strains of B. alcalophilus and revertants thereof*

Strain	Production of pH gradient $[H^+]_{in} > [H^+]_{out}$ upon energization	AIB uptake at pH 9·0 [nmol min^{-1} (mg protein)$^{-1}$]		Total membrane cytochrome content [nmol (mg membrane protein)$^{-1}$]	Absorption band at 526 nm [$\Delta A_{526-505}$ (g membrane protein)$^{-1}$]
		$-Na^+$	$+Na^+$		
Wild-type	Yes	0·3	1·4	5·31	11·0
KMR4	Yes	0·5	2·5	5·22	12·2
KMR101	Yes	0·1	1·5	6·50	18·0
KM23	No	0·2	0·2	1·27	<1·0
KM41	No	0·2	0·2	1·66	2·1
KM104	No	0·12	0·12	0·80	<1·0

cytochrome contents. In enteric bacteria, changes in cytoplasmic pH have recently been posited to initiate certain behavioural responses (MacNab & Kihara, 1981; Repaske & Adler, 1981). Growth of wild-type *B. alcalophilus* at pH 9·0 rather than pH 10·5 resulted in an appreciable decrease in the contents of cytochromes (especially *c*-type), to 3·5 nmol (mg protein)$^{-1}$. Conversely, growth of strain KM23 at pH 8·5 instead of pH 7·0 resulted in a significant increase in the membrane content of cytochromes, to 2·0 nmol (mg protein)$^{-1}$, as compared to the values in Table 1. Thus, imposed changes in external pH, that presumably lead to some modulation of intracellular pH, can have an impact upon cytochrome content. Perhaps mutational loss of the antiporter changes the typical cytoplasmic pH such that cytochrome contents are then altered as a consequence of changes in gene expression or membrane assembly. Importantly, while the cytochrome content was decreased upon growth of the wild-type at pH 9·0, Na^+-coupling was still observed (data not shown); the loss of Na^+-coupling is not, then, a secondary consequence of cytochrome reduction.

It was notable that the content of the chromophore was generally higher at high pH. The possibility that this material was a photoreceptor (for an ion pump?) of the type described in

halobacteria (Lanyi, 1981; Stoeckenius *et al.*, 1979) was considered. However, there was no diminution in chromophore content after growth of the wild-type in total darkness; moreover, light conditions had no perceptible effect on any of the determinations made. It was also possible that the chromophore was related to the obviously high iron demand for alkalophilic growth. If so, strain KM23 might be induced to produce more of the chromophore under conditions in which iron was growth-limiting. However, growth of strain KM23 in 'low iron' medium failed to cause an increase in this material (results not shown). In the wild-type, growth on 'low iron' medium caused a decrease in chromophore content as well as in the contents of cytochromes *b* and *c*. The nature of the chromophore is still unclear. Preliminary experiments indicate that it is associated with a protein whose integrity is necessary for the absorption at 526 nm. It will be of special interest to determine the nature of the chromophore, and whether it is related to Na^+-coupling.

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