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The protonophore resistance of *Bacillus megaterium* is correlated with elevated ratios of saturated to unsaturated fatty acids in membrane phospholipids

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Growth of the protonophore-resistant strain of *Bacillus megaterium*, strain C8, in the presence of oleic acid markedly reduced its resistance to low concentrations of carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Growth of the CCCP-sensitive wild-type strain in the presence of stearic acid increased the resistance of that strain to growth inhibition by protonophore. Studies of the membrane lipids indicated that in the absence of additions to the medium, membranes from C8 contained greatly reduced levels of monounsaturated fatty acids relative to the wild type; wild-type levels were restored by growth of C8 in the presence of oleic acid, concomitant with the loss of resistance. Conversely, growth of the wild type on stearic acid increased the ratio of saturated/unsaturated fatty acids in the membrane, concomitant with a modest increase in the resistance of the wild-type strain to CCCP. The exogenous oleic acid was preferentially incorporated into phosphatidylethanolamine, diphosphatidylglycerol, and 1,2-diacylglycerol, whereas stearic acid was incorporated preferentially into phosphatidylglycerol, and into the small component of free fatty acids. Depending upon the growth conditions, changes in membrane lipid-to-membrane protein ratio and in the ratios of polar lipid components were observed, but none of those changes correlated as did the changes in saturated fatty-acid-to-unsaturated fatty-acid ratio with protonophore resistance. This latter correlation was further suggested by experiments in which the protonophore resistance of wild type *B. megaterium* was shown to increase with increasing growth temperature without any temperature-dependent loss of protonophore efficacy. The experiments here support the hypothesis developed from work with *Bacillus subtilis* that changes in the fatty acid composition of the membrane phospholipids affect energy coupling, and make it clear that simple increases or decreases in the hydrolytic activity of ATPase in the uncoupler-resistant mutants of bacilli are not correlated with resistance in some direct way.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; P_i, inorganic phosphate.

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Introduction

Three recently isolated, protonophore-resistant mutants of *Bacillus subtilis* were shown to have pleiotropic, membrane-associated properties [1].

These included: elevated membrane ATPase activity; slightly elevated respiratory rates; the ability to synthesize ATP better than the wild type at submaximal levels of the electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) upon titration of the $\Delta\tilde{\mu}_{H^+}$ with any one of several agents; altered sensitivity to several membrane-active agents; and a few specific changes in the membrane-lipid composition, especially an increase in the ratio of $isoC_{15}/anteisoC_{15}$ fatty acids and a decrease in the monounsaturated $C_{16:1}$ fatty acids that are esterified in the phospholipids [1]. The decrease in the $C_{16:1}$ fatty acid (and, possibly, the ratio of $C_{16:0}/C_{16:1}$ fatty acids) was directly correlated with the protonophore resistance of both growth and ATP synthesis. This was most clearly demonstrated in experiments in which resistance to 5 μM CCCP was largely abolished by incorporation of exogenous plamitoleic acid into the phospholipids of the mutants during growth [2]. Other changes in membrane lipids did not show as close a correlation with resistance to protonophores [1,2].

The possibility that rather specific characteristics of the membrane phospholipids have an effect upon aspects of energy coupling is an intriguing challenge to the chemiosmotic formulation in which delocalized electrochemical gradients are the key force [3]. In that formulation, the only relevant property of the membrane would be an alteration in ion permeability such that the gradients were affected. This was not true of the *B. subtilis* mutants, since there were no changes in proton permeability or in the magnitude of the $\Delta\tilde{\mu}_{H^+}$ generated [1]. Thus, the interesting possibilities arise that the changes in the composition of the membrane may directly affect either the path of energy transduction or the H^+/ATP stoichiometry of proton-coupled ATP synthesis.

We wondered whether the findings with respect to protonophore resistance and membrane lipids was unique to *B. subtilis* or might represent a more general phenomenon. Therefore, we undertook a re-examination of *Bacillus megaterium* and an uncoupler-resistant mutant thereof that had been isolated by Decker and Lang [4,5] some time ago. Previous studies by Decker and Lang [4,5] and by our laboratory [6,7] had indicated that the resistant strain, C8, synthesized more ATP than its wild-type parent strain when the $\Delta\tilde{\mu}_{H^+}$ had been reduced by protonophore treatment to comparable levels in the two strains. This relative ability to synthesize more ATP at submaximal $\Delta\tilde{\mu}_{H^+}$ values was exhibited by C8 cells energized by either malate or ascorbate [6], and could be demonstrated in isolated membrane vesicles that had been loaded with ADP and P_i and were energized with ascorbate [7]. Of particular note in the current connection was that, unlike the similar mutants of *B. subtilis*, protonophore-resistant strain C8 from *B. megaterium* had reduced rather than increased membrane ATPase activity [4,6]. It therefore offered an opportunity to explore further the hypothesis that specific changes in the membrane lipids rather than other changes, such as that in the activity of the ATPase, are directly related to the protonophore-resistance of growth and oxidative phosphorylation. Indeed, the studies reported here support that hypothesis.

Materials and Methods

Organisms and growth conditions. The wild-type *B. megaterium* used in all the studies was ATCC19213, and the uncoupler-resistant strain was the C8 derivative of that wild type, originally isolated by Decker and Lang [4,5]. The bacteria were routinely grown at 30 °C on Spizizen salts [8] containing DL-malate (50 mM) and 0.1% yeast extract. When cells were grown in the presence of fatty acid supplements, those supplements, like the carbon source, were added from separate sterile solutions. In experiments in which the fatty acid supplement was radioactive, either ^{14}C-stearic acid, 2.15 GBq/mmol, or ^{14}C-oleic acid, 2.97 GBq/mmol was employed.

Growth experiments were conducted using 500 ml sidearm flasks containing 50 ml of culture medium, and inoculated with late logarithmic phase cells so that the initial readings were 10–20 Klett units on a Klett–Summerson colorimeter (No. 42 filter). The sidearm flasks were incubated at the temperatures indicated for individual experiments and monitored by turbidimetric readings.

Isolation and characterization of membrane lipids. Lipids were extracted from right-side-out membrane vesicles that were prepared from late logarithmic phase cells by the lysozyme method of

Kaback [9]. Cells were routinely washed before the lysozyme treatment, with cells that had been grown in the presence of fatty acid supplements being washed with buffered bovine serum albumin as described previously [2]. The lipid extraction was carried out by the method of Bligh and Dyer [10]. Details of the methods for separation of neutral and polar lipids and for identification of the individual lipids have recently been described [11]. Methylesters of fatty acids used in the fatty acid analyses were prepared by the procedure of Morrison and Smith [12] using boron-trichloride-methanol instead of boron trifluoride-methanol reagent. The methylesters were extracted and examined by gas-liquid chromatography and by mass spectrometry as described earlier [11].

Measurements of the transmembrane electrical potential, $\Delta\psi$. The $\Delta\psi$ was determined from the distribution of 4 μM [^{3}H]tetraphenylphosphonium as described by others [13]. Corrections for probe binding were made by subtracting background values obtained in the presence of 10 μM gramicidin or 5% butanol, both of which gave similar results. A cell volume of 10 μl/mg cell protein was used in the calculations [6]. Protein was determined by the method of Lowry et al. [14] using bovine serum albumin as a standard.

Chemicals. Non-radioactive fatty acids were purchased from Sigma Chemical Company, as was gramicidin. Radioactive fatty acids were purchased from Amersham Corporation. Radioactive tetraphenylphosphonium was from New England Nuclear, and the non-radioactive probe was from ICN K & K. All other chemicals were purchased commercially at the highest available purity.

Results

After a distinct lag, wild-type *B. megaterium* grew only slightly more slowly in the presence of 2 μM CCCP than it did without the protonophore (Fig. 1), but growth in the presence of CCCP stopped below the final level of growth in its absence (not shown). No growth at all, even after overnight incubation, occurred in unsupplemented or oleic acid-supplemented cultures of the wild type in the presence of 3 μM CCCP (Fig. 1). On the other hand, addition of stearic acid to the growth medium facilitated growth of the wild type

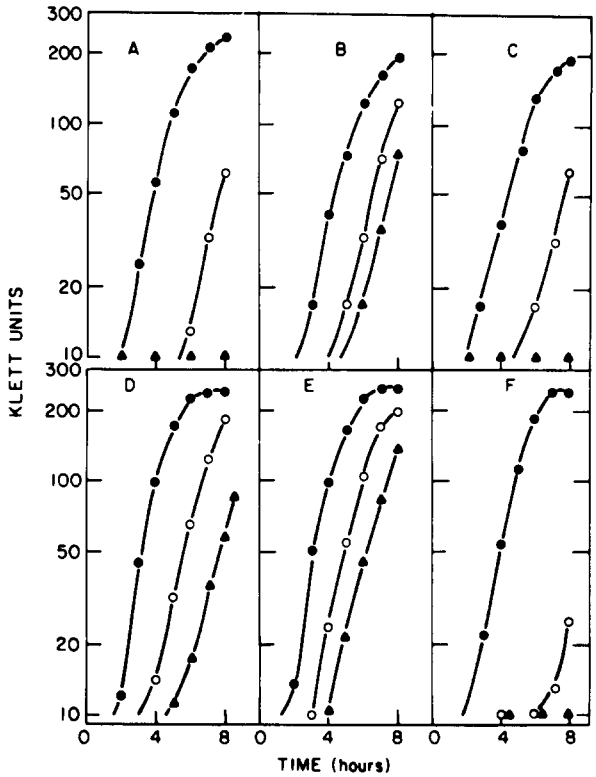

Fig. 1. The effect of CCCP of the growth of wild-type *Bacillus megaterium* and mutant C8 in the presence and absence of stearic or oleic acid. Cells of the wild type (A, B, C) or C8 (D, E, F) were grown on malate medium at 32° C without CCCP (●), with 2 μM CCCP (○), or with 3 μM CCCP (▲). The growth medium contained no fatty acid (A, D), 10 μM stearic acid (B, E), or 5 μM oleic acid (C, F).

in the presence of 3 μM CCCP and enhanced the rate of growth in the presence of 2 μM protonophore (Fig. 1). The CCCP-resistant strain, C8, grew on both 2 and 3 μM CCCP in the absence of fatty-acid additions to the medium (Fig. 1). Addition of stearic acid to the growth medium had a very slightly enhancing effect on growth in the presence of CCCP. By contrast, supplementation of the medium with oleic acid markedly reduced the growth of C8 in the presence of CCCP, such that its sensitivity was even greater than unsupplemented wild-type cells. In these experiments, we utilized the highest concentrations of the fatty acids that did not appreciably inhibit growth, therefore using a higher concentration of stearic than oleic acid. Although not shown, identical experiments with palmitic acid and palmitoleic

acid gave precisely the same pattern of results although the effect of these shorter chain fatty acids was less pronounced.

The observations above suggested that in *B. megaterium,* as in *B. subtilis,* relative resistance to protonophores might correlate with an increase in the ratio of saturated/unsaturated fatty acids in the membrane phospholipids. The fatty acid composition of the membrane lipids of the wild type and C8 strains, grown under various conditions, is shown in Table I. The wild-type strain, grown without any additions, contained four different unsaturated fatty acids, $isoC_{16:1}$, $nC_{16:1}$, $isoC_{17:1}$, and $nC_{18:1}$, together representing about 18% of the total fatty-acid content of the membrane. Strain C8 lacked all but $nC_{16:1}$ which, in this mutant, represented only about 3% of the total fatty acid of the membrane; the $nC_{16:0}$ content of C8 was significantly elevated relative to the wild type. The other major difference between the wild type and mutant strains grown without additions was the elevation in C8 of the level of $isoC_{15:0}$ fatty acid, with some reduction in the other branched form of the same chain length, the ante-$isoC_{15:0}$ fatty acid.

Growth of the C8 strain in the presence of stearic acid did not affect the fatty acid content except to increase the content of $C_{18:0}$ itself. On the other hand, growth of the wild type in the presence of stearic acid, a condition that somewhat enhanced the protonophore resistance of this strain (Fig. 1), markedly decreased the level and kinds of unsaturated fatty acids in the membrane in addition to increasing the content of $C_{18:0}$. The only unsaturated fatty acid found in wild-type cells that were supplemented with stearic acid was $nC_{16:1}$; this one unsaturated fatty acid was present at the same levels as in unsupplemented wild-type membranes (7–8%) and hence represented a total unsaturated fatty acid content above that of C8 (3%) but well below that of unsupplemented wild type (18%). Stearic acid supplementation did not alter the ratio of $isoC_{15:0}$/anteiso$C_{15:0}$. As further shown in Table I, supplementation of C8 with oleic acid, a condition associated with greatly increased sensitivity of this strain to CCCP (Fig. 1), raised the percentage of unsaturated fatty acid back up to the 18% level found in the wild type. Interestingly, although the supplement was not converted to any significant extent to other fatty acids found in the membrane (see footnote to Table III), the increase in unsaturated fatty acids involved not only an expected elevation of $C_{18:1}$, but also of other unsaturated fatty acids. Moreover, the ratio of $isoC_{15:0}$/anteiso$C_{15:0}$ fatty acids in membranes from oleic acid-supplemented C8 was in between that of unsupplemented C8 and wild-type membranes; the decrease in this ratio upon supplementation must be secondary to the incorporation

TABLE I

FATTY ACID COMPOSITION OF THE MEMBRANE LIPIDS OF *BACILLUS MEGATERIUM* AND CCCP-RESISTANT STRAIN C8

Fatty acid	*Bacillus megaterium,* wild type, grown with		C8, growth with			
	no additions	10 μM stearic acid	no additions	2 μM CCCP	10 μM stearic acid	5 μM oleic acid
$isoC_{15:0}$	3 [a]	2	21	23	20	10
anteiso$C_{15:0}$	58	63	44	45	47	54
$isoC_{16:0}$	4	4	4	4	4	4
$isoC_{16:1}$	3	0	0	0	0	3
$nC_{16:0}$	2	7	13	11	9	3
$nC_{16:1}$	7	8	3	4	3	7
$isoC_{17:0}$	10	5	7	7	7	6
$isoC_{17:1}$	3	0	0	0	0	1
$nC_{18:0}$	2	12	2	3	7	1
$nC_{18:1}$	5	0	0	0	0	7

[a] Values are the % of total fatty acid. They are the means of 3–4 determinations of each of at least two independent preparations. The deviations were no more than 15% of the values shown.

TABLE II

MEMBRANE LIPID-TO-MEMBRANE PROTEIN RATIOS AND POLAR LIPID COMPOSITION OF MEMBRANES FROM WILD-TYPE *BACILLUS MEGATERIUM* AND STRAIN C8

	Bacillus megaterium, wild type, grown with		C_8, grown with			
	No additions	10 μM stearic acid	no additions	2 μM CCCP	10 μM stearic acid	5 μM oleic acid
Membrane lipid-to-membrane protein (mg/mg)	0.57 [a]	0.33	0.49	0.52	0.47	0.66
% Polar lipid represented by:						
phosphatidylglycerol + acyl-lysophosphatidylglycerol	65	72	70	68	68	57
phosphatidylethanolamine	15	10	13	12	14	24
glycolipid and phosphoglycolipid	14	12	7	12	12	4
diphosphatidylglycerol	6	6	10	8	6	15

[a] Values are the means of 3–4 determinations on each of at least two independent preparations. The deviations were within 15% of the vlaues shown.

of exogenous unsaturated fatty acids into the phospholipids, since, again, there was essentially no conversion of the supplement to other fatty acids. Finally, with respect to fatty acid content, cells of strain C8 that had been grown in the presence of 2 μM CCCP had the same fatty acid content as cells of C8 grown without protonophore (Table I).

It was of interest to determine whether other changes in the membrane lipids accompanied the changes in fatty acids and to assess where the incorporation of the exogenous fatty acids had occurred. As shown in Table II, strain C8 had a slightly lower membrane lipid-to-membrane protein ratio than the wild type, on the borderline of significance. Neither the presence of CCCP nor the presence of stearic acid in the growth medium of the mutant altered that ratio. The presence of oleic acid, however, did result in a significant change, i.e., an increase in the lipid-to-protein ratio of the membrane. Supplementation of the wild type with stearic acid resulted in a significant change in the opposite direction, i.e., a reduction in the membrane lipid-to-membrane protein ratio (Table II). Although not shown, the percentage of the total membrane lipid that was neutral lipid was between 20 and 30% under all conditions examined, generally closer to 30%. Also, and importantly, the composition of the neutral lipid fraction was essentially unchanged by any of the conditions employed, being over 90% 1,2-diacylglycerol, with free fatty acids accounting for the remainder.

The polar lipid content of C8 membranes differed from that of the wild type in having slightly more diphosphatidylglycerol and having less glycolipid and phosphoglycolipid (Table II). Supplementation of the wild type with stearic acid resulted in a small increase in the phosphatidylglycerol component, already the major polar lipid, and in a small decrease in the amount of phosphatidylethanolamine. Growth of C8 in the presence of CCCP or stearic acid affected the minor polar lipid components, appearing to increase the content of glycolipid and phosphoglycolipid in that strain; growth of C8 in the presence of stearic acid also lowered the content of diphosphatidylglycerol. Growth of the mutant strain in the presence of oleic acid had more pronounced effects, increasing the phosphatidylethanolamine and diphosphatidylglycerol appreciably, and lowering the relative amount of phosphatidylglycerol (Table II). The increase in phosphatidylethanolamine and diphosphatidylglycerol during growth on an unsaturated fatty acid supplement had been seen in the protonophore resistant mutants *B. subtilis* [2]. As in those mutants, these increases in strain C8 correlated with preferential incorporation of labelled oleic acid into these two polar lipids (as well as the 1,2-diacylglycerol fraction of the neutral lipids) relative to the incorporation pattern of stearic acid in the same strain (Table III). By

TABLE III

DISTRIBUTION OF LABEL FROM RADIOACTIVE FATTY-ACID GROWTH SUPPLEMENTS AMONG MAJOR MEMBRANE LIPID FRACTIONS FROM *BACILLUS MEGATERIUM* STRAINS

Cells of the indicated strains were grown in the presence of either 10 μM [^{14}C]stearic acid or 5 μM [^{14}C]oleic acid. The cells were harvested in the late logarithmic phase and lipids were extracted as described under Materials and Methods. At least 98% of the labelled fatty acid supplement was recovered from extracted lipids in its original form, i.e., unchanged in chainlength or degree of saturation.

Strain	Radioactive fatty acid growth supplement	Radioactive fatty acid incorporation in major fractions (nmol labelled substrate incorporated per mg total membrane lipid)					
		1,2-diacyl-glycerol	free fatty acids	phosphatidyl-ethanolamine	phosphatidyl-glycerol	diphosphatidyl-glycerol	glycolipid and phosphoglycolipid
Wild type	stearic acid	3.6 [a]	0.9	2.5	9.7	0.5	3.0
C8	stearic acid	3.5	0.8	2.3	9.9	0.4	2.9
	oleic acid	9.9	0.3	3.8	3.9	1.4	2.3

[a] The values are the means of 3–4 determinations on each of at least two independent preparations. The deviations were no more than 20%.

contrast, stearic acid showed relatively greater incorporation into phosphatidylglycerol and into the small free fatty component in C8 and in wild type type (Table III).

As in *B. subtilis* [2], none of the changes in polar lipid composition that were observed in *B. megaterium* cells grown in the presence of exogenous fatty acids correlated with protonophore resistance. Rather, protonophore resistance was correlated with the saturated fatty acid/unsaturated fatty acid ratio. It was of interest, then, to see whether manipulation of the saturated fatty acid/unsaturated fatty acid content without additions to the medium would also affect protonophore resistance. Since Fulco [15] and colleagues have demonstrated that this ratio increases dramatically in *B. megaterium* as the growth temperature is increased, the CCCP resistance of growth was examined as a function of growth temperature of wild type *B. megaterium*. As shown in Fig. 2, *B. megaterium* was more sensitive to CCCP during growth at 24°C than at the usual growth temperature of 30°C, and was much less sensitive at the higher growth temperature of 35°C. Control experiments were conducted to ascertain whether these changes in CCCP resistance might reflect greater or lesser efficacy of the protonophore at different temperatures. Measurements of $\Delta\psi$ were made in the presence and absence of 2 μM CCCP in cells grown and incubated at 24, 30 or 35°C. Control values for the $\Delta\psi$ were −138, −139, and −141 mV (with standard deviations of 1.4 mV), respectively, at the three temperatures. In the presence of CCCP, the values at 24, 30, and 35°C were −65.5, −68.5 and −57 mV, respectively, with somewhat higher standard deviations in a range from 1.4 to 9 mV. Thus, the enhanced resistance to protonophore as temperature was increased did not relate to a diminished capacity of the protonophore to reduce $\Delta\psi$.

Discussion

The current study, together with the recent work on protonophore resistance in *B. subtilis*, suggests that a change in the fatty acids of the membrane phospholipids, specifically an increase in the saturated fatty acid/unsaturated fatty acid ratio, is a general basis for protonophore resistance in bacilli. In *B. subtilis*, the reduction in unsaturated fatty acids in protonophore-resistant mutants was exclusively in $nC_{16:1}$ [1], whereas in *B. megaterium* strain C8, all four of the unsaturated fatty-acid species of the membrane were reduced, with the $nC_{16:1}$ being the only one that was found at all in the mutant membranes. Indeed, the patterns of unsaturated fatty-acid content in mutant and wild type under various conditions lead to the suggestion that the desaturase is a likely site of mutation, and that either there is more than one desaturase system or desaturation

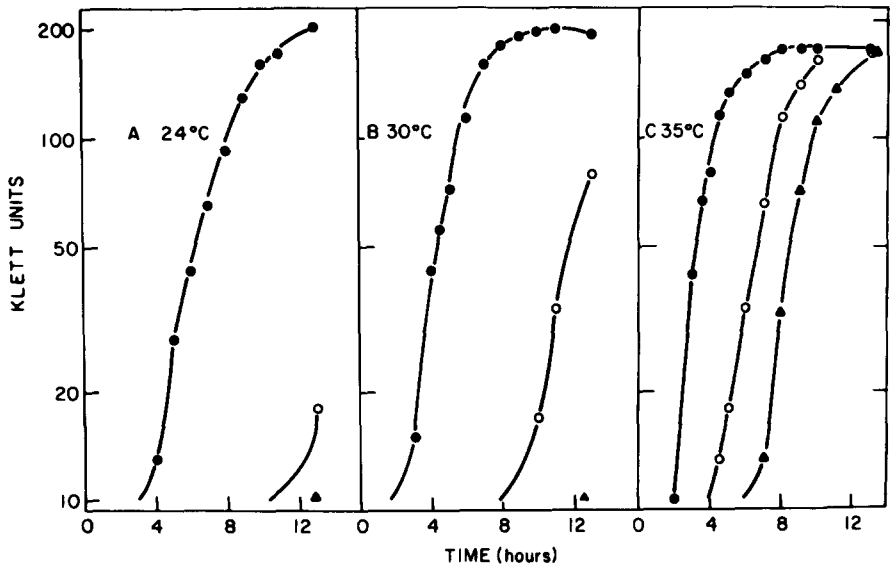

Fig. 2. The effect of temperature upon the sensitivity of the growth of wild-type *Bacillus megaterium* to inhibition by CCCP. Cells were grown at 24° C (A), 30° C (B), or 35° C (C) without CCCP (▲), or with the addition of 2 μM CCCP (○), or 3 μM CCCP (▲).

of $nC_{16:0}$ fatty acids persists relative to other substrates upon various alterations of a single system. We hypothesize that increases in the saturated fatty acid-to-unsaturated fatty-acid ratio of the membrane lipids are translated into an ability of mutants or conditionally altered wild-type strains to synthesize more ATP than controls at a given, sub-maximal level of the $\Delta\tilde{\mu}_{H^+}$. The mechanism(s) of that translation is (are) yet to be elucidated and may offer a new avenue for approaching some of the elusive issues vis-à-vis energy coupling.

It is not obvious that the mutations to protonophore resistance in *Escherichia coli* involve changes in the membrane lipids that approximate those found in the bacilli. Bragg and colleagues [16–18] have described the properties of an uncoupler-resistant strain of *E. coli* that, unlike the bacilli, shows profound changes in membrane lipids during growth on CCCP relative to growth in its absence [17]. There are at least indications, assessed by the finding of abnormal membrane localization of the elongation factor *Tu*, that there are also gross changes in membrane-associated functions in the presence, but not in the absence of CCCP [18]. These findings differ from those in bacilli, in which the presence of CCCP in the growth medium did not affect the fatty acid composition or membrane lipid-to-protein ratio [1]. Comparisons between the lipids of wild type *E. coli* and its protonophore-resistant mutant, both grown in the absence of CCCP, did not clearly show changes in the saturated/unsaturated fatty acids of the inner membrane [17]. Nonetheless, the complex phenotype of the *E. coli* mutant, with many membrane-associated alterations, leaves open the possibility that its mutational basis is a variation on the theme elaborated here.

Taken together, the studies of protonophore-resistant bacilli fail to support the possibility that a particular alteration, i.e., increase or decrease, in the ATPase activity is necessarily associated with resistance. Since the earliest mutants that were isolated on the basis of protonophore-resistant growth, both in *E. coli* [19,20] and *B. megaterium* [4,5], had reduced levels of membrane ATPase activity, it was attractive to hypothesize that the primary mutation in such strains might be in the F_1F_0 ATPase such that both the energy-coupling properties and the hydrolytic activity were altered. However, the uncoupler-resistant strains of *B. subtilis* exhibited somewhat elevated rather than reduced ATPase activity, and addition of palmitoleic acid to those mutants dramatically lowered their protonophore resistance without altering their ATPase activity [1,2].

Apart from the support that the current study lends to the view that specific changes in membrane lipids may alter the energy-coupling properties of the cells, there are observations with respect to the membrane lipids themselves that are of intrinsic interest. Firstly, it is notable that alterations in the concentration of unsaturated fatty acids in the phospholipids tend to be accompanied by an inverse change in the ratio of $isoC_{15:0}$/ $anteisoC_{15:0}$ fatty acids. Similar observations were made with *B. subtilis* [1,2] and, in a very different kind of experiment, with *Bacillus stearothermophilus* [21]. It is likely that the bulkier anteiso-branched fatty acid may be more easily accommodated, from some packing point of view, when a double bond is present in the other fatty acid on the phospholipid, Secondly, there are several interesting features in the patterns of incorporation of exogenous fatty acids and in the effects of exogenous fatty acids on membrane composition. As in *B. subtilis* [2], exogenous unsaturated fatty acid is incorporated preferentially into phosphatidylethanolamine and diphosphatidylglycerol, among the polar lipids, whereas exogenous saturated fatty acid is incorporated preferentially into phosphatidylglycerol. During growth of mutant C8 in the presence of oleic acid there was an elevation of the content of phosphatidylethanolamine and diphosphatidylglycerol complement of the total polar lipid; this had also been observed during growth of protonophore-resistant mutants of *B. subtilis* in the presence of palmitoleic acid [2]. The exogenous saturated and unsaturated fatty acids also differ with respect to a preferential distribution in free fatty acids vs. 1,2-diacylglycerol, respectively, among the neutral lipids of *B. megaterium*. The bases for these discrete and apparently general distribution preferences is not yet clear.

It is also interesting and entirely unclear how the incorporation of exogenous $nC_{18:1}$ leads to the reappearance of other species of unsaturated fatty acids in the C8 membrane without direct conversion of the supplement to these other species; similarly, the unsaturated fatty acid supplement leads to secondary changes in the relative content of the two types of branched chain fatty acids. These effects may reflect regulatory phenomena at the level of synthesis and/or constraints related to packing.

Finally, growth of *B. megaterium* wild type (but, interestingly, not C8) on stearic acid led to a significant decrease in the membrane lipid-to-protein ratio, whereas growth of the mutant on oleic acid led to an increase in that ratio. It is possible that the details of the phospholipid milieu influence protein incorporation into the membrane in some specific general direction. Such increases or decreases in membrane protein incorporation might even account for the secondary effects of mutations to protonophore resistance on the activities of some membrane-associated activities, i.e., some apparent changes in activity may turn out to be changes in the actual levels of enzyme protein.

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