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Isolation and Characterization of Uncoupler-Resistant Mutants of *Bacillus subtilis*

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Three mutant strains of *Bacillus subtilis* were isolated on the basis of their ability to grow in the presence of 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The mutants (AG2A, AG1A3, and AG3A) were also resistant to 2,4-dinitrophenol, and AG2A exhibited resistance to tributyltin and neomycin. The mutants all exhibited (i) elevated levels of membrane ATPase activity relative to the wild type; (ii) slightly elevated respiratory rates, with the cytochrome contents of the membranes being the same as or slightly lower than those of the wild type; (3) a passive membrane permeability to protons that was indistinguishable from that of the wild type in the absence of CCCP and that was increased by addition of CCCP to the same extent as observed with the wild type; and (4) an enhanced sensitivity to valinomycin with respect to the ability of the ionophore to reduce the transmembrane electrical potential. Finally and importantly, starved whole cells of all the mutants synthesized more ATP than the wild type did upon energization in the presence of any one of several agents that lowered the proton motive force. Studies of revertants indicated that the phenotype resulted from a single mutation. Since a mutation in the coupling membrane might produce such pleiotropic effects, an analysis of the membrane lipids was undertaken with preparations made from cells grown in the absence of CCCP. The membrane lipids of the uncoupler-resistant strains differed from those of the wild type in having reduced amounts of monounsaturated C_{16} fatty acids and increased ratios of iso/anteiso branches on the C_{15} fatty acids. Correlations between protonophore resistance and the membrane lipid compositions of the wild type, mutants, and revertants were most consistent with the hypothesis that a reduction in the content of monounsaturated C_{16} fatty acids in the membrane phospholipids is related, perhaps causally, to the ability to synthesize ATP at low bulk transmembrane electrochemical gradients of protons.

During the past 15 years, the chemiosmotic model of energy coupling that had been advanced by Mitchell (28) almost 10 years earlier has achieved widespread confirmation and acceptance. According to this model, the energy made available by photosynthetic light capture, oxidation of substrates by the respiratory chain, or ATP hydrolysis by the membrane-associated F_1F_0-ATPase is transduced via an intermediary electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$). That is, these energy-producing processes are all associated with proton translocation across a relatively proton-impermeant coupling membrane such that a transmembrane pH gradient (ΔpH) and transmembrane electrical potential ($\Delta\psi$) are produced. In a bacterial cell or mitochondrion, the orientation of the $\Delta\bar{\mu}_{H^+}$ is outside positive and acid relative to the interior of the cell or organelle. Completing the energetic circuit are the energy-consuming processes that are catalyzed by integral membrane porters or enzymes. These processes, e.g., ATP synthesis and ion-coupled solute uptake, utilize and partially dissipate the $\Delta\bar{\mu}_{H^+}$. By contrast, lipid-soluble protonophores, by dissipating the $\Delta\bar{\mu}_{H^+}$ directly, may uncouple reactions that can generate a $\Delta\bar{\mu}_{H^+}$ from those that utilize this intermediate energy form (15, 27, 29).

Since, in this formulation, uncoupling by protonophores essentially renders the coupling membrane permeable to protons and incapable of sustaining a $\Delta\bar{\mu}_{H^+}$, the existence of uncoupler-resistant bacterial mutants that can grow oxidatively in the presence of protonophores is puzzling. Unless the mutation(s) simply involved an alteration in the outer surface layers, so that the protonophore could not reach the coupling membrane, or the acquisition of the ability to inactivate the protonophore, the existence of such mutants would seem to challenge the chemiosmotic view of protonophore action or proton flow during energy transduction (21). The characteristics of the mutants might support the view that protonophore action actually relates, at least in part, to an uncoupler-binding protein that may be associated with the ATPase (10, 11, 20). Alternatively, they might support one of the models of energy transduction in which the bulk $\Delta\bar{\mu}_{H^+}$ is less directly coupled to bioenergetic work than some more localized, and perhaps less protonophore-sensitive, pathway of proton flow (6, 8).

A small number of uncoupler-resistant mutant strains of *Escherichia coli* (16, 17, 37) and *Bacillus megaterium* (3, 4) have been isolated and described. These strains do not yet present a cohesive picture, the precise map positions of the mutations have not been determined, a possible change in passive proton permeability has not been excluded, and the primary biochemical change is unclear. In at least one of the *E. coli* mutants, complicating phenomena that may relate to proton movements through the periplasm obscure the bioenergetic problems of the strain (37). In the remaining reported mutants, both of *E. coli* and of *B. megaterium*, the F_1F_0-ATPase hydrolytic activity shows a decrease or temperature sensitivity whose basis has not yet been determined (3, 4, 17). Work in our laboratory on protonophore-resistant mutants (6, 8) previously focused on the C8 strain of *B. megaterium* studied by Decker and Lang (3, 4); efforts past

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the initial bioenergetic characterization, however, were frustrated by difficulties in purifying the ATPase from this strain. To retain the advantage of using an experimental organism which has only one unit cell membrane and a relatively uncomplicated cell surface structure, while increasing the opportunity for both genetic and biochemical characterization, we sought to isolate mutant strains of *Bacillus subtilis* that were resistant to the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In this paper we describe three mutant strains of *B. subtilis* that grow aerobically on malate in the presence of up to 5 μM CCCP. Unlike previously studied protonophore-resistant bacteria, these strains all exhibited increased ATPase activity. They also exhibited several other changes in membrane-associated functions and have an enhanced ability to synthesize ATP, relative to the wild type, when the $\Delta\bar{\mu}_{H^+}$ is lowered with one of several membrane-active agents. The analyses described here suggest that the mutation results from an alteration in membrane lipids that affects energy-coupling properties without changing the passive proton permeability of the membrane or the sensitivity of that permeability to CCCP.

MATERIALS AND METHODS

Growth of organisms and isolation of CCCP-resistant mutant strains. *B. subtilis* BD99 was grown at 30°C, with shaking, in Spizizen salts (38) supplemented with 0.1% (wt/vol) yeast extract and 50 μg each of L-histidine, L-threonine, and L-tryptophan per ml added from separate sterile solutions. The carbon source, also added from a separate sterile solution, was 50 mM DL-malate. CCCP-resistant mutants were isolated by a variation of the method described by Decker and Lang (3). Mid-logarithmic-phase cultures were mutagenized with ethyl methanesulfonate as described previously (41). The cells were washed free of ethyl methanesulfonate and allowed to grow for one generation; they were then plated at a high concentration (approximately 10^7 cells per plate) onto solid malate-containing medium to which 5 μM CCCP had been added. Colonies that arose after 48 to 72 h were picked and restreaked several times from single colonies on malate-CCCP plates. Three isolates, designated AG1A3, AG2A, and AG3A, grew well on such plates (with or without yeast extract), while the wild type did not. To verify that the mutants were derived from *B. subtilis* BD99, we confirmed the retention of the three auxotrophic markers in experiments involving the use of defined liquid media.

Growth experiments were conducted with 500-ml sidearm flasks containing 50 ml of culture medium and inoculated with late-logarithmic-phase cells so that the initial readings were 10 to 20 Klett units on a Klett-Summerson colorimeter (with a no. 42 filter). The sidearm flasks were incubated at 30°C, with shaking, and growth was monitored turbidimetrically. The effects of various inhibitors on growth were calculated by comparing the doubling times in the presence and absence of inhibitor.

Measurements of the $\Delta\bar{\mu}_{H^+}$. The magnitude of the $\Delta\bar{\mu}_{H^+}$ was studied by using cells that were harvested in the mid-logarithmic phase of growth and washed with and suspended in potassium phosphate buffer (pH 7.5). In other experiments, with starved cells in which ATP synthesis was monitored upon reenergization, measurements of the $\Delta\bar{\mu}_{H^+}$ were made on cells suspended in potassium phosphate buffer (pH 7.5 or 6.2). Measurements of the ΔpH were made by using the accumulation of weak acids or weak bases as described previously (6, 33). In most of the experiments shown in the tables, the ΔpH was measured by monitoring the distribution of 24 μM [^{14}C]benzoic acid. Although a measurable ΔpH was found at pH 6.2, this parameter was below detectable levels at pH 7.5. Thus at the latter pH, the $\Delta\bar{\mu}_{H^+}$ was essentially all in the form of a $\Delta\psi$. Nonetheless, 0.1 μM nigericin was routinely added to the suspensions, together with 10 mM malate, to ensure that a maximal $\Delta\bar{\mu}_{H^+}$, all in the form of a $\Delta\psi$, was generated. The $\Delta\psi$ was determined from the distribution of 4 μM [^{3}H]tetraphenylphosphonium (TPP^+) as described by others (36). 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. For calculations of the intracellular concentration of TPP^+, a water volume of 5 μl/mg of cell protein was determined from the distribution of ^3H_2O and [^{14}C]inulin (22).

Cellular ATP concentration. The intracellular ATP concentration was determined by the luciferin-luciferase assay as described previously (7), using an LKB 1251 luminometer. Cells were harvested by centrifugation for 10 min at 12,000 × *g* and washed and suspended in 50 mM potassium phosphate buffer (pH 7.5). Samples for ATP determinations were taken immediately and at various times after incubation at 30°C with shaking. The times by which 75 and 90% of the initial ATP had been depleted were calculated.

Preparation of membrane vesicles. Right-side-out membrane vesicles were used for the estimations of membrane cytochrome content via reduced-versus-oxidized difference spectroscopy. These vesicles were prepared in 50 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$ by the lysozyme method of Kaback (19). Everted membrane vesicles were used for assays of membrane-associated ATPase activity. These vesicles were prepared by passage of washed cells through a French pressure cell in the presence of protease inhibitor and DNase as described previously (14), with the following modifications. The cells were washed and broken in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5) containing 10 mM $MgCl_2$. The membranes were washed in 20 mM HEPES (pH 7.5) containing 100 mM NaCl and were subsequently suspended (to 5 to 20 mg of protein per ml) in 20 mM HEPES (pH 7.5) containing 10 mM NaCl and 0.4 M sucrose.

ATPase assays. The ATPase activity was measured by monitoring the release of P_i from ATP as described previously (14), except that the reaction mixtures contained 20 mM Tricine (pH 8; Sigma Chemical Co.), 10 mM ATP, and 2.5 mM $MgCl_2$.

Reduced-minus-oxidized difference spectra and determination of cytochrome content. Dithionite-reduced minus ferricyanide-oxidized difference spectra of membrane vesicles were recorded with a Perkin-Elmer 557 Dual Beam spectrophotometer as described previously (24). The concentrations of various cytochromes were estimated by using the following extinction coefficients and wavelength pairs: cytochrome aa_3, $\Delta A_{605-630}$, $\Delta\varepsilon = 20.5$/heme; cytochrome *b*, $\Delta A_{560-575}$, $\Delta\varepsilon = 17.5$; and cytochrome *c*, $\Delta A_{551-538}$, $\Delta\varepsilon = 17.3$.

Respiratory rates of whole cells. Mid-logarithmic-phase cultures grown in the presence or absence of 2 μM CCCP were harvested by centrifugation of 12,000 × *g* for 10 min and suspended in 50 mM potassium phosphate buffer (pH 7.5). Oxygen consumption by the suspended cells was measured at 30°C, in the presence of 10 mM malate, with a Yellow Springs Instruments model 53 Clark-type oxygen monitor (24).

Measurements of the passive proton permeability and buffering capacities of whole cells. Cell suspensions in 300 mM

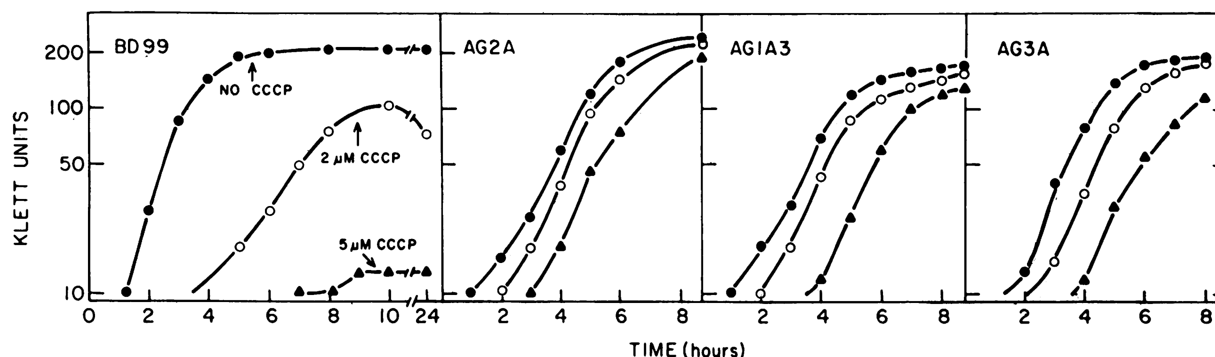

FIG. 1. Effect of CCCP on growth of wild-type *B. subtilis* BD99 and three CCCP-resistant strains in liquid medium. Liquid medium containing malate, yeast extract, and three essential amino acids was prepared at pH 7.0 as indicated in Materials and Methods. Either no CCCP (●), 2 μM CCCP (○), or 5 μM CCCP (▲) was added. The cultures were inoculated from late-logarithmic-phase cultures of the indicated strains, and growth was monitored as described in Materials and Methods.

KCl containing KSCN, valinomycin, and carbonic anhydrase were prepared as described by Maloney (26). Volumes (2 ml) of the cell suspensions were incubated at 30°C in a water-jacketed chamber in which a combination electrode was placed. The suspensions were vigorously stirred throughout the experiment. The suspensions were allowed to equilibrate before measurements of proton permeability were made; the length of this equilibration was the minimal time required to achieve a steady base-line pH measurement. Longer equilibration times, e.g., periods in the range of hours as used by Maloney (26), resulted in lysis of some of the suspensions, especially of mutant strain AG2A. After base-line drift had ceased, an acid pulse of 3 to 5 μl of 0.1 N HCl was added with a Hamilton syringe. The values for outer buffering (B_o) and total buffering (B_t) were determined as described by Maloney (26) and by Scholes and Mitchell (35). The internal buffering (B_i) was calculated from the formula $B_i = B_t - B_o$. Using the buffering capacity measurements and the rate at which pH approached final equilibrium, the passive proton permeability ($C_m{}^{H^+}$) was calculated as described by Scholes and Mitchell (35). The proton conductance was expressed as micromoles of H^+ per second per pH unit per gram of protein, where protein was determined by the method of Lowry et al. (25) with egg-white lysozyme as a standard. Estimates of the conductance (in microsiemens per square centimeter of membrane surface) were made by using the calculations described by Mitchell and Moyle (29) and assuming that the cells had the dimensions of a cylinder 0.7 μm in diameter by 2.5 μm long.

Determinations of ATP synthesis and the phosphorylation potential (ΔG_p). For the determination of L-malate-dependent ATP synthesis, cells of wild-type BD99 and its three CCCP-resistant derivatives were depleted of ATP by starvation in a variation of the method described previously (6). Mid-logarithmic-phase cultures were harvested and washed by centrifugation at 12,000 × *g* for 10 min with 50 mM potassium phosphate buffer (pH 7.5 or 6.2). The washed cells were suspended to 100 Klett units. The suspensions were shaken at 30°C, and samples were taken periodically until the ATP content had been depleted by at least 80 to 90%. At these times, which varied from strain to strain, 10 mM malate was added to initiate ATP synthesis. When inhibitors were included, cell suspensions were pretreated with ethanolic solutions of the inhibitors for 5 min at 30°C. Ethanol was kept at or below 0.1% (vol/vol), which did not affect the experimental parameters. As indicated in the descriptions of specific experiments, samples were taken before and at various times after energization for the determination of ATP, ADP, or P_i, as described previously (9). The phosphorylation potential was calculated from the equation

$$\Delta G_p = \Delta G^0 + RT2.3\log([ATP]/[ADP][P_i])$$

where brackets denote concentrations. The value used for ΔG^0 was 7.3 kcal/mol (30.54 kJ/mol), taken from Rosing and Slater (32).

Preparation, fractionation, and identification of membrane lipids. Total lipids were extracted from right-side-out membrane vesicles by the method of Bligh and Dyer (1). For separation of neutral and polar lipid fractions, the lipids were fractionated on a silicic acid column and then purified to single lipid species by silica gel thin-layer chromatography, as described previously (2). The methods then used for identification and quantitation of individual lipids have recently been outlined in detail (2). For analysis of the fatty acids, fatty acid methyl esters were prepared by the method of Morrison and Smith (30) with boron trichloride-methanol instead of boron trifluoride-methanol reagent. The methyl esters were extracted and examined by gas-liquid chromatography and mass spectrometry as described previously (2).

Chemicals. Valinomycin, gramicidin, carbonic anhydrase, luciferin, luciferase, and CCCP were purchased from Sigma. [*phenyl*-^{3}H]TPP$^+$ (35.5 Ci/mmol) was a product of New England Nuclear. Tri-*n*-butyltin chloride was purchased from Aldrich Chemical Co. Authentic lipids were purchased from Serdary Research Laboratories, Inc., Sigma, or Alltech Associates, Inc., Applied Science Division. Fatty acid methyl esters were from Supelco, Inc., and Alltech. All other chemicals were obtained commercially at the highest purity available.

RESULTS

Growth characteristics. The growth of the wild type, BD99, and three mutant strains in liquid medium containing one of several low concentrations of CCCP is shown in Fig. 1. Although growth of the wild type was appreciably inhibited by 2 μM CCCP and almost completely inhibited by 5 μM CCCP, all three of the mutant strains grew quite well, after a lag, in the presence of 5 μM CCCP. All three mutant strains were also more resistant than the wild type to 2 mM 2,4-dinitrophenol (data not shown). Strains AG1A3 and AG3A showed sensitivities to gramicidin (1 μM), neomycin (5 μg/ml), and tributyltin (0.5 μg/ml) that were the same as

TABLE 1. Bioenergetic properties of the CCCP-resistant strains

Strain	ATPase activity (mean ± SD)[a]	Cellular ATP (mM)[b]	Time of starvation (min)[b] to reduce ATP by: 75%	90%	Respiratory rate (natom of O/min/mg)[b]	Total cytochromes (nmol/mg of membrane protein)[b]
Wild type	0.122 ± 0.0013	3.09	14	35	723	0.65
AG2A	0.213 ± 0.014	3.29	8	21	856	0.66
AG1A3	0.190 ± 0.022	3.02	12	20	882	0.65
AG3A	0.187 ± 0.022	3.71	5	17	825	0.48

[a] Average of at least four separate determinations. Activity is expressed as micromoles of P_i per minute per milligram of membrane protein.
[b] Average of at least three independent determinations, with standard deviations within 10% of the values.

that of the wild type, whereas AG2A appeared to be more sensitive than all of the other strains to gramicidin and somewhat less sensitive to either neomycin or tributyltin.

Bioenergetic properties of the mutant strains. A comparative study of several bioenergetic properties was undertaken. The most impressive finding was that all three mutant strains, and especially AG2A, exhibited higher ATPase activity than the wild type as measured by the hydrolysis of ATP by membrane vesicle preparations (Table 1). The increased ATPase activity probably accounts for the observation (Table 1) that the cellular ATP levels of the mutants declined more rapidly upon starvation than did the level of ATP in the wild type. The respiratory rates of the three mutant strains were comparable to one another and were all slightly higher than that of the wild type. On the other hand, the total membrane cytochrome content, calculated from reduced-minus-oxidized difference spectra, was the same in the mutants as in the wild type, except that AG3A had slightly lower total values; the spectral patterns were the same in all the strains examined (data not shown).

CCCP is neither inactivated by nor ineffective in reducing the $\Delta\bar{\mu}_{H^+}$ of the resistant strains. The $\Delta\psi$ values of the wild-type and mutant strains were comparable when measured in the absence of CCCP under conditions in which cells were energized at pH 7.5, with nigericin added to prevent the development of a ΔpH (Table 2). Addition of 2 μM CCCP to such cells reduced the $\Delta\psi$ by about 50%. Addition of 10 μM CCCP abolished the $\Delta\psi$ entirely. Thus CCCP was as effective in reducing the $\Delta\psi$ of the resistant strains as that of the wild type in cell suspension.

Two different experiments were conducted to explore whether the resistant strains were able to inactivate CCCP in the growth medium. First, mutant cells were grown to the mid-logarithmic phase in the presence of 2 μM CCCP. The cells were aseptically removed from the cultures by centrifugation, and the supernatant fluids were tested for their ability to inhibit growth of the wild-type strain. Controls included supernatant from cultures of mutant cells grown in the absence of CCCP as well as authentic CCCP at 2 μM. The inhibition of growth by CCCP-containing supernatant was at least as good as that by fresh CCCP. A second series of experiments established that similar supernatants affected ATP synthesis and generation of a $\Delta\psi$ by wild-type cells in a manner identical to that for inhibition by fresh CCCP (data not shown).

Passive proton permeability and buffering capacities of the mutant strains and effect of CCCP. The patterns of decay of an acid pulse administered to suspensions of the wild-type and mutant strains under deenergized conditions at pH 6.7 are shown in Fig. 2A. The patterns appear quite similar, and, indeed, calculated values for buffering capacity and membrane proton conductance (Table 3) do not differ very much among the strains. The value of $C_m{}^{H^+}$ was approximately 2 μmol of H^+/s per pH unit per g of protein. With the assumptions noted in Materials and Methods, this value

TABLE 2. Effect of the addition of CCCP on the $\Delta\psi$ of washed cells of wild-type *B. subtilis* and CCCP-resistant strains in buffered suspensions[a]

Strain	$\Delta\psi$ (mV) after addition of CCCP at: 0 μM	2 μM	10 μM
Wild type	−162	−87	0
AG2A	−157	−82	0
AG1A3	−162	−80	0
AG3A	−168	−80	0

[a] Cells were washed with 50 mM potassium phosphate buffer (pH 7.5) and suspended in the same buffer containing 10 mM malate and 0.1 μM nigericin. CCCP was added at the indicated concentrations. After 15 min, the $\Delta\psi$ values were determined as described in Materials and Methods.

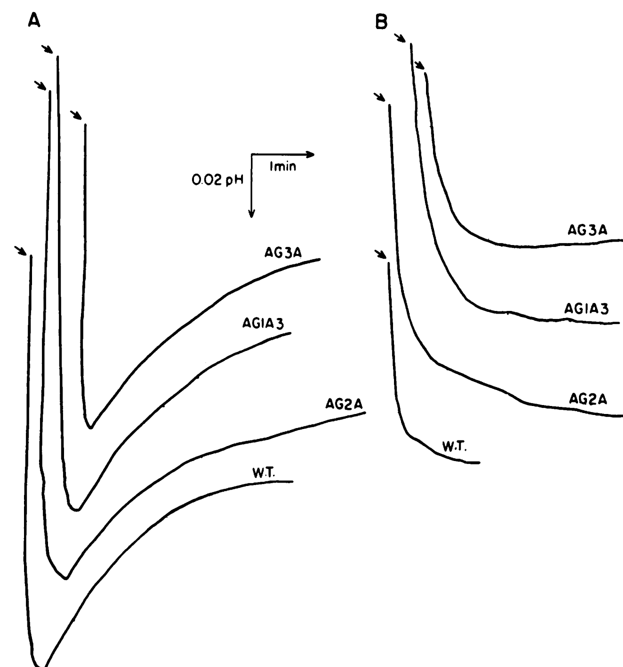

FIG. 2. Changes in the external pH of suspensions of the wild-type and CCCP-resistant strains after an acid pulse. Cells of the wild-type and three mutant strains were suspended to 4.0 mg of protein per ml at pH 6.7 in KCl containing valinomycin and KSCN as described in Materials and Methods. Where indicated by the arrows, 5 μl of 0.1 N HCl was added. (A) No other additions. (B) 2 μM CCCP was added 5 min before the acid pulse.

TABLE 3. Proton conductance of the membranes and the buffering capacities of the cytoplasm and outer cell surface of wild-type *B. subtilis* and CCCP-resistant strains at pH 6.7[a]

Strain	Mean buffering capacity (μmol of H^+/pH unit per mg of protein) ± SD			Mean $C_m^{H^+}$ (μmol of H^+/s per pH unit per g protein) ± SD
	B_i	B_o	B_t	
Wild type	0.51 ± 0.08	0.43 ± 0.06	0.94 ± 0.3	2.02 ± 0.45
AG2A	0.67 ± 0.04	0.39 ± 0.09	1.06 ± 0.11	2.08 ± 0.21
AG1A3	0.47 ± 0.08	0.53 ± 0.14	1.00 ± 0.2	2.03 ± 0.21
AG3A	0.54 ± 0.16	0.52 ± 0.1	1.06 ± 0.1	2.02 ± 0.71

[a] Washed cells were suspended in a water-jacketed chamber at 30°C in 300 mM KCl at pH 6.7 in the presence of 10 μM valinomycin, 30 mM KSCN, and 1 mg of carbonic anhydrase per ml. Values for cytoplasmic buffering capacity (B_i), external buffering capacity (B_o), total buffering capacity (B_t), and membrane proton conductance ($C_m^{H^+}$) were calculated as described in Materials and Methods after a small amount of 0.1 N HCl had been added.

gives an electrical conductance of 1.37 μS/cm^2 of membrane surface. Importantly, addition of 2 μM CCCP prior to the acid pulse renders the entire internal buffering capacity immediately accessible to titration in the wild type and all three of the mutant strains (Fig. 2B).

Inhibition of ATP synthesis by CCCP in wild-type and mutant cells. Starved whole cells were energized with malate as described in Materials and Methods. Such energization resulted in similar amounts of ATP synthesis by the wild-type and mutant strains over a 6-min time course, with the shapes of the curves showing some small differences (Fig. 3). This synthesis, by all strains, was inhibited by 75 to 90% upon treating the cells for 30 min at pH 6.7 with 100 μM dicyclohexylcarbodiimide (DCCD) before initiating ATP synthesis under the usual experimental conditions. Thus the synthesis observed is attributable to a DCCD-sensitive ATP synthase.

The generation of a $\Delta\psi$ upon energization was also very similar in all the strains, as measured by TPP^+ uptake. The generation of comparable $\Delta\psi$ values by all the strains is consistent with their identical proton permeabilities and makes it more unlikely that the mutants are less (or more) permeable to some other ion than is the wild type. Both malate-dependent ATP synthesis and TPP^+ uptake were completely abolished by the addition of 1 μM valinomycin before energization, whether or not CCCP was also added. The addition of 2 μM CCCP alone reduced TPP^+ uptake by the wild type and the three mutant strains to a very comparable extent. In contrast, the effect of 2 μM CCCP on ATP synthesis was considerably more pronounced in the wild type. Two of the resistant strains, AG1A3 and AG3A, exhibited a consistent sigmoidicity in the time course for ATP synthesis in the presence of 2 μM CCCP. Elimination of nigericin had no effect on the pattern and almost no effect upon the magnitudes of the parameters measured (data not shown).

In Table 4, the calculated values for $\Delta\bar{\mu}_{H^+}$ and ΔG_p from the experiments of the type depicted in Fig. 3 are shown. Steady-state levels of ATP synthesized upon energization of wild-type and mutant cells are very similar in the absence of CCCP and yield $\Delta G_p/\Delta\bar{\mu}_{H^+}$ values that are all approximately 3. On the other hand, in the presence of 2 μM CCCP the levels of ATP synthesized by the three mutant strains were less affected than that synthesized by the wild type, whereas all the strains showed similar reductions in the $\Delta\bar{\mu}_{H^+}$, thus resulting in a $\Delta G_p/\Delta\bar{\mu}_{H^+}$ that was significantly higher for the mutants (at about 5.3) than for the wild type (at 4.3).

The apparent ability of the mutants to synthesize more ATP than the wild type at reduced $\Delta\bar{\mu}_{H^+}$ levels could result from some bioenergetically relevant mutational change. It was important, however, to examine the possibility that the mutants were exhibiting some adaptation to CCCP and that even wild-type cells might adapt, albeit not as well, to the presence of a protonophore. Wild-type cells and cells of mutant strain AG1A3 were grown to the mid-logarithmic phase on malate medium containing 2 μM CCCP. The cells were harvested and compared with cells of the same strains grown in the absence of CCCP with respect to malate-dependent ATP synthesis and generation of a $\Delta\psi$, precisely as described above. The results with cells that had been grown in the presence of CCCP were identical to those with control cells; i.e., no enhanced resistance to CCCP was observed with either wild-type or mutant cells. In a different experiment, late-logarithmic-phase cells of the wild type (only) that had been grown in the presence of 2 μM CCCP were found to produce a gummy layer that rendered them difficult to filter and wash and that also conferred some CCCP resistance. Such cells formed chains and appeared compromised when studied with a microscope. Importantly, they excluded CCCP, showing a reduced effect of CCCP both on the $\Delta\psi$ and on ATP synthesis. This was unlike the resistance exhibited by mutant cells throughout their growth cycle.

Effects of other inhibitors on ATP synthesis. It was of interest to determine whether the apparent ability of the mutant strains to synthesize more ATP at a given, suboptimal, $\Delta\bar{\mu}_{H^+}$ was restricted to a condition in which the $\Delta\bar{\mu}_{H^+}$ was titrated with protonophores or in which the $\Delta\psi$ was the sole component of the $\Delta\bar{\mu}_{H^+}$. First, we examined the effect of various concentrations of valinomycin on the $\Delta\psi$ and ATP synthesis, again at pH 7.5. Two differences between the wild-type and mutant strains were discernible in these experiments (Fig. 4). The $\Delta\psi$ of all three mutant strains was more sensitive to reduction by valinomycin than that of the wild type (Fig. 4A). For example, at 0.05 μM valinomycin, the wild type retained a $\Delta\psi$ of about −135 mV, whereas the mutants exhibited $\Delta\psi$ values between −70 and −90 mV. Despite this greater sensitivity of the $\Delta\psi$ to valinomycin, the mutants synthesized somewhat more ATP at given $\Delta\psi$ levels achieved by valinomycin treatment than did the wild type; this is illustrated by a plot of steady-state values for ATP synthesized versus the $\Delta\psi$ (Fig. 4B).

A second set of experiments was done to explore the effect of various concentrations of nigericin on ATP synthesis and the $\Delta\bar{\mu}_{H^+}$ at pH 6.2. At this pH, starved cells of the wild-type and mutant strains all generated a $\Delta\psi$ between −115 and −139 mV and a ΔpH of −72 to −88 mV upon energization with malate (Table 5). The total $\Delta\bar{\mu}_{H^+}$ of all the strains was thus close to −200 mV at pH 6.2. Nigericin at 0.015 μM reduced both the $\Delta\psi$ and the ΔpH produced upon energization, so that the total $\Delta\bar{\mu}_{H^+}$ was now between −101 and −128 mV. We were surprised at first by the finding that nigericin affected both the $\Delta\psi$ and the ΔpH at pH 6.2, whereas at pH 7.5 it could be used as an electroneutral agent, affecting only

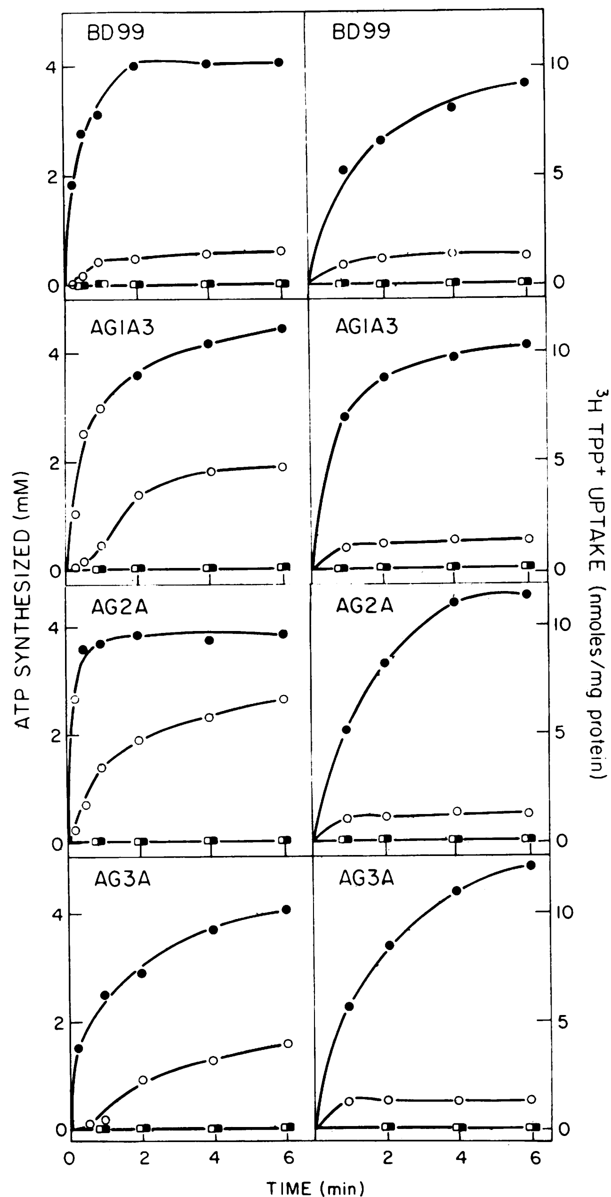

FIG. 3. Effect of CCCP on ATP synthesis and the generation of a $\Delta\psi$ by wild-type strain BD99 and the uncoupler-resistant mutants at pH 7.5. The cellular content of ATP was lowered by starvation in 50 mM potassium phosphate buffer (pH 7.5) as described in Materials and Methods. After starvation, the cell suspensions were preincubated with 0.1 μM nigericin for 5 min. Some cells were also preincubated with 1 μM valinomycin (■), 2 μM CCCP (○), or both valinomycin and CCCP (□) for 5 min. Malate, to 10 mM, was added at the point indicated as zero time on the graphs. Control cell suspensions (●) contained only malate. After malate addition, samples were removed at intervals to measure ATP content and the uptake of [^{3}H]TPP$^+$. The left-hand axis shows values of ATP content at a given time point minus the content at zero time; the zero-time values were approximately 10% of the steady-state contents of cells energized in the absence of inhibitors.

TABLE 4. Relationship between the $\Delta\tilde{\mu}_{H^+}$ and the ΔG_p in the presence and absence of 2 μM CCCP at pH 7.5[a]

Strain	No addition			2 μM CCCP added		
	ΔG_p (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	$\Delta G_p/\Delta\tilde{\mu}_{H^+}$	ΔG_p (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	$\Delta G_p/\Delta\tilde{\mu}_{H^+}$
Wild type	−485	−162	3.0	−374	−87	4.3
AG2A	−480	−157	3.1	−431	−82	5.3
AG1A3	−500	−162	3.1	−427	−80	5.3
AG3A	−485	−168	2.9	−417	−80	5.2

[a] Steady-state values for the ΔG_p and $\Delta\tilde{\mu}_{H^+}$ were calculated from the experiments depicted in Fig. 3.

the ΔpH. However, previous observations of nigericin effects upon the $\Delta\psi$ have apparently been made by other investigators (12, 18) and stand as a caveat against assumptions about ionophore actions in new experimental situations. The sensitivity of the mutants to nigericin was completely comparable to that of the wild type with respect to reductions in the $\Delta\tilde{\mu}_{H^+}$. In contrast, ATP synthesis was again less affected in the mutants than in the wild type.

Pleiotropic restoration of wild-type properties in revertants, consistent with single mutations. Spontaneous revertants of each of the mutant strains were selected by plating cells of each strain on malate-containing plates and then replicating onto plates containing the same medium plus 5 μM CCCP. Approximately 200,000 colonies of each strain were thus screened. Two or three colonies of each strain that appeared CCCP sensitive subsequently proved to be sensitive in growth experiments in liquid medium. The CCCP-sensitive revertants also exhibited sensitivity to valinomycin that was comparable to that of the wild-type rather than the mutant parent and no longer had elevated levels of membrane-associated ATPase (Table 6). Indeed, one revertant exhibited levels of ATPase activity that were even significantly lower than those of the wild type.

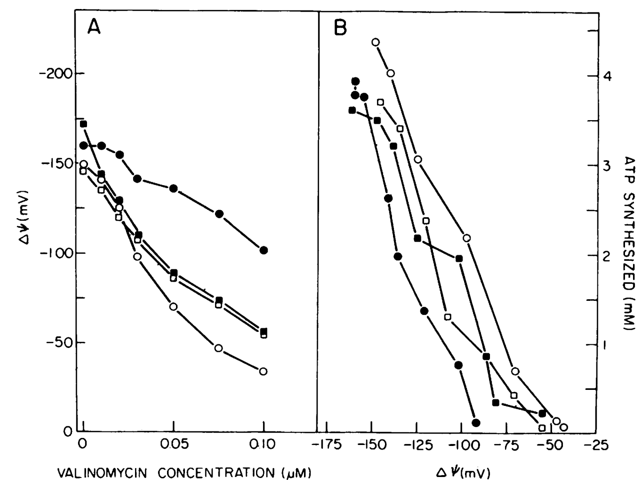

FIG. 4. Effect of various valinomycin concentrations on the generation of a $\Delta\psi$ and ATP synthesis at pH 7.5 in the wild-type and mutant strains. Cells of each strain, starved at pH 7.5 as described in Materials and Methods, were pretreated for 5 min with various concentrations of valinomycin, after which 10 mM malate was added to initiate the generation of a $\Delta\psi$ and ATP synthesis. (A) The magnitude of the $\Delta\psi$ was calculated from the steady-state uptake of [^{3}H]TPP$^+$ in wild-type (●), AG1A3 (○), AG2A (■), and AG3A (□) cells. (B) The amount of ATP synthesized is shown, with the same symbols for the four strains.

TABLE 5. Effect of nigericin on the proton motive force and ATP synthesis at pH 6.2[a]

Strain	Nigericin	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	ATP concn (mM)
Wild type	–	−115 ± 10	−76 ± 10	−191 ± 20	4.68 ± 0.9
	+	−90 ± 14	−14 ± 5	−104 ± 19	0.1 ± 0.1
AG1A3	–	−116 ± 5	−85 ± 13	−201 ± 18	3.9 ± 0.6
	+	−92 ± 14	−36 ± 15	−128 ± 29	0.94 ± 0.5
AG2A	–	−139 ± 3	−88 ± 9	−227 ± 12	4.81 ± 0.8
	+	−63 ± 13	−42 ± 16	−105 ± 29	0.91 ± 0.7
AG3A	–	−123 ± 14	−72 ± 8	−195 ± 22	4.73 ± 0.8
	+	−67 ± 14	−34 ± 11	−101 ± 25	2.41 ± 1.0

[a] Starved cells were energized with malate at pH 6.2. When present (+), 0.015 μM nigericin was added to cells 5 mins before the addition of 10 mM malate. Results are given as the mean plus or minus the standard deviation.

CCCP-resistant strains exhibit alterations in membrane lipids that are restored to a wild-type pattern in revertant strains. A single mutation apparently resulted in pleiotropic changes, such as altered sensitivities to membrane-active agents, altered levels of membrane-associated activities, and altered properties of energy coupling. This focused our attention on possible compositional changes in the coupling membrane of the mutants that might lead to the complex phenotype. We analyzed the membrane lipids in cells of the wild type, mutant, and revertant strains grown in the absence of CCCP. CCCP was omitted to allow a direct comparison between the wild type (which cannot be grown in the presence of protonophore) and mutants and thus clarify possible effects of the mutation on membrane lipids. All the strains exhibited a majority of properties in common, including membrane lipid/membrane protein ratios (by weight) in a range from 0.65 to 0.73 ± 0.1 or less; neutral lipid/polar lipid ratios of either 25%:75% or 30%:70%; and a simple neutral lipid composition of 1,2-diacylglycerol and free fatty acids that were present in the neutral lipid fraction, respectively, at 90 to 98% and 2 to 10% with a 4 to 8% standard deviation in determinations on two to four independent samples.

The polar lipid composition was more complex. For most of the strains examined, the wild-type composition of approximately 12% phosphatidylethanolamine, 70% phosphatidylglycerol, and small percentages of at least six other components was observed. Only strain AG3A had a significant alteration in membrane polar lipid composition, having approximately double the wild-type content of phosphatidylethanolamine and a lower content of phosphatidylglycerol than the other strains (Table 7). The wild-type pattern was restored in the revertant of AG3A.

The fatty acid fraction, derived almost entirely from fatty acids esterified in phospholipids, showed two interesting differences between the wild type and the uncoupler-resistant strains (Table 8). All the uncoupler-resistant strains exhibited a significant increase in the ratio of iso-C_{15}/anteiso-C_{15} fatty acids; this increase was dramatic in AG1A3 and AG2A and was somewhat less dramatic in AG3A. The revertant of AG1A3 showed partial restoration, and revertants of the other two mutant strains showed complete restoration, of the wild-type ratio. The second change observed in the uncoupler-resistant mutants was a reduction, by approximately 50%, in the relative content of monounsaturated n-C_{16} fatty acids. This was accompanied in AG1A3 and AG2A, but not in AG3A, by an increase in the saturated C_{16} fatty acid content. All of the revertant strains exhibited restoration of the wild-type levels of monounsaturated C_{16} fatty acids.

The membrane lipids of one of the protonophore-resistant strains, AG1A3, were analyzed by using lipids from cells that had been grown in the presence of 5 μM CCCP. The only difference observed between these preparations and the others from this strain was a slight increase in the lipid/membrane protein ratio (data not shown).

DISCUSSION

The three newly isolated mutant strains of *B. subtilis* exhibited resistance to low levels of CCCP and 2,4-dinitrophenol during aerobic growth on malate. The mutants neither inactivated nor excluded protonophores from access to the coupling membrane. Importantly, there was no detectable change in the one membrane-associated property that would allow a simple chemiosmotic interpretation of the mutant phenotype. That is, the coupling membrane was not altered with respect to passive proton permeability or the effect of CCCP on that permeability. The value for $C_m{}^{H^+}$ found in all four strains, approximately 1.37 $\mu S/cm^2$ of membrane surface, was in the range of values reported for other bacteria (26, 35). Similarly, the buffering capacities of the surface and cytoplasm of the three mutant strains were comparable to those properties of the wild-type strain; the values calculated from the acid-pulse experiments were, moreover, within the same range as those determined in earlier titrations of untreated and detergent-solubilized cells of *B. subtilis* (22). The similar $\Delta\tilde{\mu}_{H^+}$ values found in mutant and wild-type strains, under various conditions, make it unlikely that a change in the permeability to some ion other than protons underlies the phenotype.

The complex phenotype of the mutants, which is somewhat different among the three strains, suggested that an alteration of the membrane had occurred. Respiratory rates and ATPase activity were enhanced, there was an increased sensitivity to valinomycin, and all the strains showed at least some altered resistance to more than one chemically distinct membrane-active agent. The possibility that these various characteristics resulted from multiple mutations is unlikely, since spontaneous revertants of each of the mutant strains were readily isolated from a screen of no more than a few hundred thousand colonies and each of the revertants appeared to have lost a full array of mutant properties. On the other hand, both the reversion frequencies and the differences between the mutant strains may reflect a mutational site that is complex (e.g., the fatty acid desaturase complex).

Analyses of the membrane lipids indicate that the mutation in each of the resistant strains results in an altered

TABLE 6. Properties of spontaneously occurring revertants of each of the CCCP-resistant mutant strains

Strain	Growth on malate medium + 5 μM CCCP	ATPase activity of everted vesicles (μmol/min per mg of protein)[a]	% Abolition of $\Delta\psi$ by 0.03 μM valinomycin at pH 7.5
Wild type	–	0.122 ± 0.013	9
AG2A	+	0.213 ± 0.014	40
Revertant AG2AR35-1	–	0.118 ± 0.001	5
AG1A3	+	0.190 ± 0.022	35
Revertant AG1A3R7	–	0.077 ± 0.002	11
AG3A	+	0.187 ± 0.022	40
Revertant AG3AR40-1	–	0.114 ± 0.001	14

[a] Activity is the average of at least four separate determinations plus or minus the standard deviation.

TABLE 7. Membrane polar lipid composition of wild-type, CCCP-resistant, and revertant *B. subtilis* strains[a]

Polar lipid	% Composition of strain						
	BD99	AG1A3	AG1A3R7	AG2A	AG2AR35-1	AG3A	AG3AR40-1
Phosphatidylethanolamine	12 ± 4	15 ± 5	10 ± 4	10 ± 4	12 ± 2	24 ± 3	16 ± 2
Phosphatidylglycerol	70 ± 4	65 ± 8	70 ± 6	70 ± 6	70 ± 4	58 ± 5	66 ± 4
Diphosphatidylglycerol	4 ± 2	6 ± 2	5 ± 2	7 ± 3	7 ± 3	4 ± 2	5 ± 1
Monoglycosyl diacylglycerol	2 ± 2	6 ± 1	5 ± 1	5 ± 2	6 ± 2	3 ± 1	4 ± 2
Diglycosyl diacylglycerol	4 ± 2	4 ± 1	5 ± 2	4 ± 0	2 ± 0	5 ± 2	4 ± 1
Aminoacyl phosphatidylglycerol	2 ± 1	0	1 ± 0	0	0	2 ± 0	0
Phosphoglycolipid	5 ± 2	3 ± 1	3 ± 1	2 ± 1	0	4 ± 1	5 ± 1
Unidentified	1 ± 1	1 ± 1	1 ± 0	2 ± 0	3 ± 1	0	0

[a] Membrane lipids were prepared and fractionated as described in Materials and Methods. The values shown are the averages of four determinations on each of two to four independent preparations and are shown with the standard error of the mean.

membrane composition, with some variability in the details among the three uncoupler-resistant strains. Taken together, the data support the hypothesis that a reduction in the monounsaturated C_{16} fatty acids in the phospholipids is correlated, perhaps causally, with protonophore resistance. Although the increase in the iso-C_{15}/anteiso-C_{15} fatty acid ratio is striking, there is at least one uncoupler-resistant mutant (AG3A) in which the ratio is indistinguishable from that of one of the uncoupler-sensitive revertant strains (AG1A3R7). It is interesting that the one revertant, AG1A3R7, whose iso-C_{15}/anteiso-C_{15} fatty acid pattern was not completely restored to that of the wild type also exhibited particularly low ATPase values; it may well be that one change in fatty acids is associated with protonophore resistance, while secondary lipid changes contribute to other components of the mutant and revertant phenotypes. The conclusion that the iso-C_{15}/anteiso-C_{15} ratio is not directly correlated with protonophore resistance is supported by experiments with mutant strains of *B. subtilis* isolated previously (40), whose iso-C_{15}/anteiso-C_{15} content can be varied by appropriate manipulation of the growth conditions. Increasing the iso-C_{15}/anteiso-C_{15} ratio of such strains did not confer protonophore resistance (data not shown). By contrast, and as reported in the companion paper (23), we have demonstrated that manipulation of the $C_{16:1}$ content of the membrane phospholipids by adding exogenous fatty acids to the growth medium results in a modification of protonophore resistance in accord with the hypothesis developed here. Moreover, those studies support the view that changes in the fatty acid branching pattern are secondary to the change in the concentration of monounsaturated fatty acids.

It is notable that both the change in the degree of fatty acid saturation and the change in fatty acid branching patterns of the mutant strains would, a priori, be expected to result in a less fluid membrane. Herring et al. (13) have reported that a CCCP-resistant strain of *E. coli* produces a less fluid membrane, when grown in the presence of CCCP, than that of either the mutant or the wild type grown in the absence of protonophore; the change in fluidity in that system was attributed to a change in the membrane protein/membrane lipid ratio.

Of greatest interest to us is the possible mechanistic relationship between a change in the fatty acid content of membrane phospholipids and the energy-coupling properties of the cell in the absence of a change in proton permeability. The most impressive property from the bioenergetic point of view, and perhaps the property that relates most directly to CCCP resistance, is the apparent ability of the mutants to synthesize ATP more effectively than the wild-type parent when the $\Delta\bar{\mu}_{H^+}$ has been reduced by CCCP, valinomycin (pH 7.5), or nigericin (pH 6.2). This capacity (albeit subtle) was observed in experiments with different inhibitors and at different pH values. It is therefore unlikely that a change in the surface charge of the membrane confers the resistance by a bioenergetically trivial mechanism in which a particular charged species of ionophore, e.g., protonophore anion form or positively charged valinomycin-potassium complex, would be inhibited in its penetration of or cycling in the membrane. That possibility is, in any event, essentially precluded by the finding that both valinomycin and CCCP are efficacious in abolishing the $\Delta\psi$.

We are inclined toward the hypothesis that a mutational change that alters the membrane fatty acid composition leads to pleiotropic effects on such properties as the activity or integration of some integral membrane proteins (e.g., ATPase), the sensitivities to various inhibitors, and the ability to synthesize ATP at reduced levels of the $\Delta\bar{\mu}_{H^+}$. With respect to the last property, it is pertinant to note proposals considered by many investigators (e.g., 5, 31, 39) and recently formulated by Rottenberg and Hashimoto (34)

TABLE 8. Fatty acid composition of membrane lipids from wild type, CCCP-resistant, and revertant strains of *B. subtilis*

Strain	Growth on 5 μM CCCP	Fatty acid (% of total membrane fatty acids)[a]					
		iso-$C_{15:0}$	anteiso-$C_{15:0}$	iso-$C_{16:0}$	n-$C_{16:0}$	n-$C_{16:1}$	iso-$C_{17:0}$
Wild type	−	16 ± 4	36 ± 6	5 ± 1	7 ± 1	16 ± 2	18 ± 0
AG1A3	+	32 ± 7	19 ± 4	4 ± 1	14 ± 2	7 ± 2	18 ± 0
AG1A3R7	−	26 ± 5	28 ± 6	5 ± 0	10 ± 2	13 ± 1	18 ± 1
AG2A	+	34 ± 4	21 ± 4	3 ± 0	15 ± 3	7 ± 1	14 ± 0
AG2AR35-1	−	19 ± 4	30 ± 5	4 ± 1	6 ± 1	14 ± 2	16 ± 1
AG3A	+	22 ± 3	29 ± 5	7 ± 1	9 ± 2	6 ± 2	19 ± 0
AG3AR40-1	−	18 ± 2	36 ± 6	4 ± 2	6 ± 1	15 ± 3	18 ± 0

[a] Fatty acids making up 3% or less of the total, with no differences between strains, are omitted. Values shown are the means of four determinations on each of two to four independent preparations, given with standard errors of the mean.

with special emphasis on a possible role of membrane lipids. That is, if energy-coupling can occur by either a bulk chemiosmotic mechanism or a more localized pathway of proton translocation that involves the membrane more actively, one could envision a mutational change in the membrane such that the latter pathway was now favored. This could be achieved either by a global change in some property such as fluidity or by some far more specific interaction of membrane lipids and proteins. If the membrane-associated pathway for proton translocation were less sensitive to uncoupling than energy transduction through the bulk, then the results reported here would be obtained. Another basis for the findings that must also be considered is that the mutational changes in the phospholipids lead to a change in the H^+/ATP stoichiometry of the ATPase at relatively low levels of the $\Delta\bar{\mu}_{H^+}$. Once manipulation of the membrane lipids and its effect upon energy-coupling are further documented, it may be possible to design experiments involving the use of protonophore resistance that will test models of parallel coupling or altered mechanistic stoichiometries in a more direct way than has generally been feasible in other experimental systems.

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