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Protonophore-resistance and cytochrome expression in mutant strains of the facultative alkaliphile *Bacillus firmus* OF4

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Two protonophore-resistant mutants, designated strains CC1 and CC2, of the facultative alkaliphile *Bacillus firmus* OF4 811M were isolated. The ability of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to collapse the protonmotive force ($\Delta\bar{\mu}_{H^+}$) was unimpaired in both mutants. Both resistant strains possessed elevated respiratory rates when grown at pH 7.5, in either the presence or absence of CCCP. Membrane cytochromes were also elevated: cytochrome *o* in particular in strain CC1, and cytochromes *aa*$_3$, *b*, *c* and *o* in strain CC2. Strain CC2 also maintained a higher $\Delta\bar{\mu}_{H^+}$ than the others when grown in the absence of CCCP. When grown in the presence of low concentrations of CCCP, strains CC1 and CC2 both maintained higher values of $\Delta\bar{\mu}_{H^+}$ than the wild-type parent and correspondingly higher capacities for ATP synthesis. In large-scale batch culture at pH 10.5, both mutant strains grew more slowly than the parent and contained significantly reduced levels of cytochrome *o*. Cells of strain CC1 also displayed a markedly altered membrane lipid composition when grown at pH 10.5. Unlike previously characterized protonophore-resistant strains of *B. subtilis* and *B. megaterium*, neither *B. firmus* mutant possessed any ability above that of the parent strain to synthesize ATP at given suboptimal values of $\Delta\bar{\mu}_{H^+}$. Instead, both resistant alkaliphile strains maintained a higher $\Delta\bar{\mu}_{H^+}$ and a correspondingly higher ΔG_p than the parent strain when growing in sublethal concentrations of CCCP, apparently as a result of mutational changes affecting respiratory chain composition. Also of note in both the mutant and the wild-type strains was a marked elevation in the level of one of the multiple terminal oxidases, an *aa*$_3$-type cytochrome, during growth at pH 7.5 in the presence of CCCP or during growth at pH 10.5, i.e. two conditions that reduce the bulk $\Delta\bar{\mu}_{H^+}$.

Introduction

Mutants of *Bacillus* species that are resistant to low concentrations of protonophoric uncouplers are of bioenergetic interest because they have been shown to manifest their resistance without excluding or inactivating the protonophore. Instead, the cells of uncoupler-resistant strains of *Bacillus subtilis* and *Bacillus megaterium* possess a greater capacity than their wild-type parents for performing oxidative phosphorylation at reduced values of the protonmotive force ($\Delta\bar{\mu}_{H^+}$) [1]; unless they can vary the H^+/ATP stoichiometry as a function of the bulk $\Delta\bar{\mu}_{H^+}$, these mutants stand in apparent contradiction of the chemiosmotic model of energy transduction [2]. Such a variation in H^+/ATP stoichiometry seems not to account for the phenomenon, since CCCP-resistance is not manifest in experiments in which imposed pH-gradients or diffusion potentials energize ATP synthesis, but only when respiration is the source of the $\Delta\bar{\mu}_{H^+}$ [1]. Thus far, these mutants all seem to arise from single mutations causing

Abbreviations: $\Delta\bar{\mu}_{H^+}$, protonmotive force; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential; ΔG_p, phosphorylation potential; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium; DMO, dimethyloxazolidine-2,4-dione; TCS, 3,3′,4′,5-tetrachlorosalicylanilide; EMS, ethyl methanesulfonate; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine.

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decreased desaturation of fatty acids; their membrane lipids show an increased ratio of saturated/unsaturated acyl side-chains [3,4]. Furthermore, their uncoupler-resistance can be modulated concomitantly with their lipid composition by the incorporation of saturated or unsaturated fatty acids in the growth medium [4,5]. We have interpreted these results in terms of a parallel coupling model [6], suggesting that the resulting changes in the physical properties of the membrane may enhance a direct pathway of proton movement between the proton-pumping complexes of the respiratory chain and the F_1F_0-ATP-synthase, for example by producing membrane domains with enhanced concentrations of these components [1].

With this hypothesis in mind, we attempted to isolate comparable uncoupler-resistant mutants of the alkaliphile *Bacillus firmus* OF4 [7]. This strain is naturally deficient in membrane fatty acid desaturase activity and its membrane lipids contain a very low percentage of unsaturated side-chains, thus precluding the type of mutation described above [8]. Any uncoupler-resistant strains were thus expected to arise by different means. Moreover, alkaliphiles growing at high pH have already adapted to the challenge of a low $\Delta\bar{\mu}_{H^+}$ [9]. Between pH 10 and pH 11, where aerobic growth on non-fermentable carbon sources is good, the $\Delta\bar{\mu}_{H^+}/F$ produced by respiration is only around -50 mV; a conventionally oriented $\Delta\psi$ of -180 mV is offset by a ΔpH, inside acid, of at least two pH units ($+120$ mV). Since the phosphorylation potential (ΔG_p) remains in the conventional range (10.5 kcal/mol, equivalent to -450 mV), energy coupling under these conditions cannot occur in a strict chemiosmotic manner by means of proton translocation with a normal H^+/ATP stoichiometry of 3–4. A variable H^+/ATP stoichiometry also appears unable to explain these findings [10]. Interestingly, these organisms possess very high concentrations of membrane cytochromes and have apparently fluid membranes with a very high content of bulky, *anteiso*, branched-chain lipid substituents. These properties might enhance the frequency of putative collisions between respiratory chain components and the proton-translocating F_1F_0-ATPase at high pH [9,10], allowing direct transfer of protons.

B. firmus OF4 is a facultative alkaliphile and grows well at pH values as low as 7.5. Under these conditions, $\Delta\bar{\mu}_{H^+}/F$ is considerably greater (-140 mV) than at very alkaline pH, and energy coupling can be fitted by a chemiosmotic framework. It was thus of interest to determine whether energy-coupling could be altered at near neutral pH when cells were challenged by inclusion of protonophores in the growth medium. Here, we describe two protonophore-resistant mutants of *B. firmus* OF4 811M, whose detailed bioenergetic phenotypes are indeed different from those of the resistant, non-alkaliphilic *Bacillus* mutants described before.

Materials and Methods

Chemicals

[*phenyl*-^{3}H]Tetraphenylphosphonium (TPP^+) (35.5 Ci/mmol), [^{14}C]dimethyloxazolidine-2,4-dione (DMO) (50 mCi/mmol) and [^{14}C]methylamine (50 mCi/mmol) were from New England Nuclear. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was from Sigma, 3,3′,4′,5-tetrachlorosalicylanilide (TCS) from Eastman Kodak, and nigericin was a generous gift from Dr. R.L. Hamill, Lilly Research Laboratories, Indianapolis, IN. All other chemicals were of at least reagent grade.

Growth of organisms and isolation of protonophore-resistant strains

Bacillus firmus OF4 811M, a *met*$^-$, streptomycin-resistant derivative of *B. firmus* OF4 [7], was grown at 30°C with shaking. For growth at pH 7.5, the basal medium contained 94 mM Na_2HPO_4, 6 mM KH_2PO_4, 0.1% (w/v) $(NH_4)_2SO_4$ and 0.1 mM $MgSO_4$. For growth at pH 9, the basal medium contained 100 mM $NaHCO_3$, 0.1% (w/v) $(NH_4)_2SO_4$, 1 mM K_2HPO_4 and 0.1 mM $MgSO_4$; growth at pH 10.5 used the same medium, except that 100 mM Na_2CO_3 replaced the $NaHCO_3$. All media were supplemented with 0.1% (w/v) yeast extract, 1% (v/v) trace salts [11] and 50 mM sodium DL-malate (adjusted to pH 9 for the more highly alkaline media), which were added aseptically from separate sterile solutions. Growth was monitored spectrophotometrically from the absorbance at 600 nm, or, in a few experiments, using a Klett-Summerson colorimeter (No. 42 filter). Growth to an A_{600} of 1.0 (late logarithmic phase) did not result in any significant change in medium pH. For growth and bioenergetics studies, cells were grown in 50 ml medium in 250 ml Erlenmeyer flasks. Cells for cytochrome determinations were grown in 800 ml medium in 2-liter flasks, while those for lipid determinations were grown in 8 liters medium in 16-liter carboys, with vigorous aeration.

Growth of the parent strain at pH 7.5 was inhibited by 1 μM CCCP. Mutants were selected in pH 7.5 medium containing 2 μM CCCP, and screened for retention of the ability to grow at pH 10.5 and of methionine auxotrophy. Mutagenesis with ethyl methanesulfonate (EMS) was performed essentially as described previously [12].

Measurement of $\Delta\bar{\mu}_{H^+}$

Protonmotive force was measured in washed cells respiring on malate by the distribution of radioactive methylamine or DMO (ΔpH), and TPP^+ ($\Delta\psi$), essentially as described previously [13]. In cells grown at pH 7.5, the $\Delta\bar{\mu}_{H^+}$ was almost all in the form of a $\Delta\psi$, and ΔpH was below reliably detectable levels. Nigericin (0.1 μM) was therefore added to most suspensions to convert any residual ΔpH into a $\Delta\psi$ that could be mea-

sured as part of the whole. Corrections for non-specific binding of TPP^+ were calculated according to Rottenberg [14], and control measurements were also performed in the presence of butanol. An intracellular water volume of 10 μl/mg protein, measured for strain OF4 811M using [^{3}H]water and [^{14}C]inulin [15], was also used for strains CC1 and CC2, which exhibited the same cell dimensions as strain 811M and possessed identical amounts of cell protein per unit absorbance.

In some experiments, cells were starved overnight in 50 mM sodium phosphate buffer (pH 7.5), and then re-energized with 10 mM malate in the presence or absence of CCCP [16].

Phosphorylation potential

Cellular ATP, ADP and phosphate concentrations were assayed and ΔG_p calculated as described previously [16].

Respiratory rates

Mid-logarithmic phase cells were harvested and resuspended in basal medium (pH 7.5), or 50 mM sodium carbonate buffer (pH 10.5). Oxygen consumption was measured at 30°C, in the presence of 10 mM malate, using a Clark-type oxygen electrode (Yellow Springs). An oxygen content of 5 μl O_2/ml was taken for both buffers (manufacturer's literature).

Cytochrome content

Everted vesicles were prepared by passage of washed cells through a French pressure cell at 18000 psi in the presence of proteinase inhibitors (5 mM *p*-aminobenzamidine and 0.2 mM phenylmethylsulfonyl fluoride) and DNAse. The suspension buffer contained (mM): 250 sucrose, 50 KCl, 50 Tricine-KOH, 10 $MgCl_2$ (pH 8). After low-speed centrifigation (12000 × g_{max}, 10 min) to remove unbroken cells, the vesicles were pelleted (203000 × g_{max}, 90 min), then resuspended in 1–2 ml buffer containing 50 mM Tricine-KOH, 5 mM $MgCl_2$ (pH 8). Cytochromes were assayed with a Perkin-Elmer 557 dual-beam spectrophotometer. Ascorbate/TMPD-reduced minus air-oxidized spectra were used to quantitate cytochromes *c* and aa_3; dithionite was then added to the reduced samples to measure *b*-type cytochromes. Cytochrome *o* was quantitated from carbon monoxide/dithionite minus dithionite spectra. The following wavelength pairs and millimolar extinction coefficients were used: $(a + a_3)$, $\Delta A_{600-620}$, $\Delta\epsilon = 20.5\ mM^{-1}$ [17]; *b*, $\Delta A_{560-575}$, $\Delta\epsilon = 17.5$ [18]; *c*, $\Delta A_{551-538}$, $\Delta\epsilon = 17.3$ [19]; *o*, $\Delta A_{416-430}$, $\Delta\epsilon = 145.0$ [20]. Total protein was assayed by the Lowry method [21], with bovine serum albumin as standard.

Membrane lipid composition

Right-side-out membrane vesicles were prepared from whole cells by lysozyme treatment [22,23]. Total lipids were extracted from the vesicles by the method of Bligh and Dyer [24]. The chromatographic methods used for the identification and quantitation of individual lipid components have been described previously [8].

Results

Growth properties

Two CCCP-resistant strains, designated CC1 and CC2, were isolated. Strain CC2 was obtained following EMS mutagenesis, while strain CC1 arose spontaneously. As shown in Fig. 1, growth of both mutants in liquid medium at pH 7.5 was only slightly inhibited by 2 μM CCCP, in contrast to that of the parent strain, which was inhibited almost completely. Under these conditions, both mutants could grow in the presence of up to 3 μM CCCP. All three strains were less sensitive to CCCP at pH 9, where the uncoupler is a less potent protonophore, and grew in the presence of up to 10 μM CCCP. At pH 9, strain CC2 was no more resistant than its parent, but CC1 retained a slightly enhanced resistance, growing slowly but reproducibly with 15 μM CCCP in three separate experiments. Resistance to no more than 2 μM CCCP could also be demonstrated in completely minimal medium (pH 7.5), lacking the yeast extract but with added methionine (100 μg/ml). It proved difficult to demonstrate the CCCP-resistant phenotype reproducibly on plates, but when mid-logarithmic phase cells were streaked onto plates (same com-

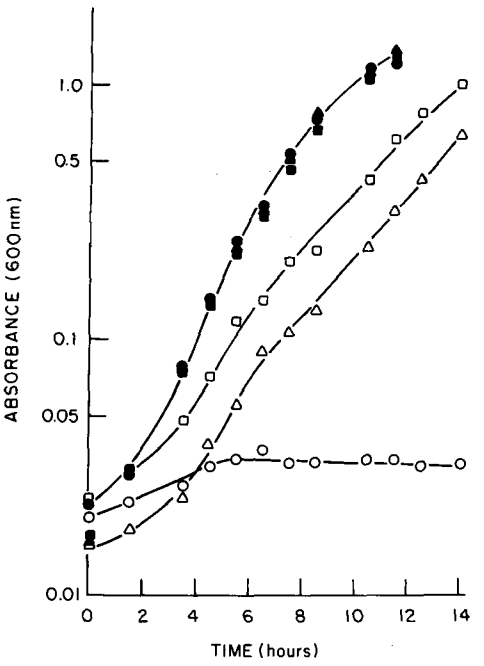

Fig. 1. Growth of *B. firmus* OF4 811M and its protonophore-resistant derivative strains CC1 and CC2. Cells were grown aerobically at 30°C and pH 7.5 with 50 mM sodium malate as carbon source, in the absence (closed symbols) or presence (open symbols) of 2 μM CCCP. Key: ○, ●, 811M; □, ■, CC1; △, ▼, CC2.

position as pH 7.5 medium plus 15 g/l agar) prewarmed to 30°C and containing various concentrations of CCCP, strains CC1 and CC2 grew well in the presence of 3 μM CCCP, whereas strain 811M did not grow.

Growth of strains CC1 and CC2 at pH 7.5 was cross-resistant to the protonophore TCS (0.1 μM), but not to tributyltin chloride (all strains grew with 0.1 μM, none with 0.3 μM). The latter compound can uncouple by functioning as a Cl^-/OH^- exchanger, but may also inhibit the F_1F_0-ATP synthase directly. In all cases, the pH of the medium did not change significantly during growth, ruling out any cell-induced reduction in uncoupler potency by alkalinization of the medium. Uncoupler degradation was also discounted; filtered medium containing 2.5 μM CCCP in which strain CC2 had grown would not support the growth of the parent strain.

To investigate any possible consequences of the uncoupler-resistant phenotype on alkaliphily, the growth of parent and mutants in the absence of uncoupler was compared over a range of pH values. The upper and lower limits of growth, approx. pH 11.0 and 7.0, were the same in all strains. Strain CC2 grew consistently more slowly than strain 811M at pH values above 10 (20–25% slower at pH 10.5 and up to 40% slower at pH 10.8; three separate experiments). Growth of 50 ml batch cultures of strain CC1 was indistinguishable from that of strain 811M, but 8 liter cultures of CC1 in carboys grew up to 10% more slowly than those of 811M at pH values above 10. Again, the pH of the medium did not change by more than 0.3 pH units during growth to the late logarithmic phase.

Bioenergetic properties

In the absence of CCCP, the only detectable difference between the protonmotive forces of the three strains was the higher $\Delta\psi$ of strain CC2 grown at pH 7.5 (Table I). This difference became more apparent when the membrane potentials were measured in the presence of nigericin, to convert any small undetected

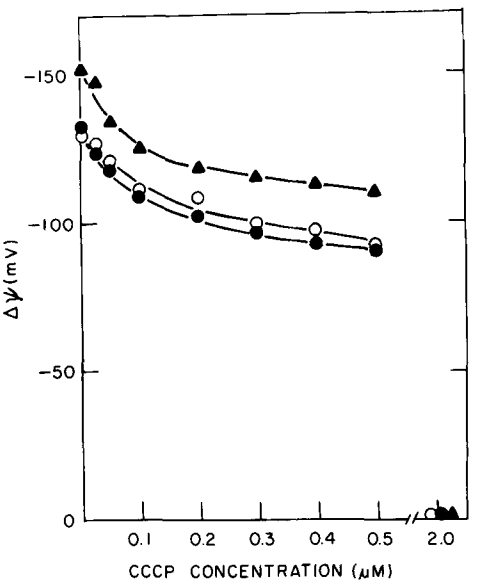

Fig. 2. Effect of CCCP on $\Delta\psi$ of *B. firmus* OF4 strains. Cells were grown at pH 7.5 in the absence of CCCP, washed and resuspended in basal salts. Respiration was initiated by the addition of 10 mM sodium malate. Membrane potentials were measured by the distribution of [*phenyl*-^{3}H]TPP$^+$, in the presence of 0.1 μM nigericin. At least five determinations were made in each case; errors did not exceed 10%. Key: ●, 811M; ○, CC1; ▲, CC2.

ΔpH into a $\Delta\psi$. When cells suspended in basal salts (pH 7.5) were assayed in the presence of various concentrations of CCCP, the $\Delta\psi$ of strain CC2 was consistently 15–20 mV larger than those of the other two strains, which were indistinguishable from each other (Fig. 2). CCCP at 2 μM collapsed the $\Delta\psi$ of all three strains; under these conditions, the protonophore is probably more effective than in the slightly richer growth medium, as indicated earlier. A similar titration of $\Delta\psi$ with valinomycin revealed that this ionophore decreased the $\Delta\psi$ of the two mutants more effectively than that of the parent strain (Fig. 3). The extra 15–20 mV of $\Delta\psi$ in strain CC2 was particularly susceptible to collapse by as little as 25 nM valinomycin. These findings provide

TABLE I

Protonmotive force components of B. firmus OF4 strains

Cells were grown to mid-logarithmic phase at pH 7.5 or pH 10.5, in the absence of CCCP. After washing, they were resuspended in basal salts, pH 7.5 or pH 10.5 as appropriate, plus 10 mM sodium malate. ΔpH and $\Delta\psi$ were assayed as described in the Materials and Methods section. Numbers (mV) represent mean ± S.D. At least five independent determinations were averaged for each estimate of $\Delta\psi$. For statistical analysis, data from strains 811M and CC2 were compared using a one-tailed Student's *t*-test: *, significant at $P = 0.01$.

Bioenergetic parameter	Assay conditions	Growth conditions					
		pH 7.5			pH 10.5		
		811M	CC1	CC2	811M	CC1	CC2
ΔpH (mV)	no additions	≤ −15	≤ −15	≤ −15	+135	+129	+135
$\Delta\psi$ (mV)	no additions	−126 ± 6	−121 ± 9	−138 ± 7	−181 ± 3	−179 ± 4	−181 ± 4
$\Delta\psi$ (mV)	+0.1 μM nigericin	−133 ± 9	−130 ± 5	−152 ± 6 *	−183 ± 4	−181 ± 3	−182 ± 4

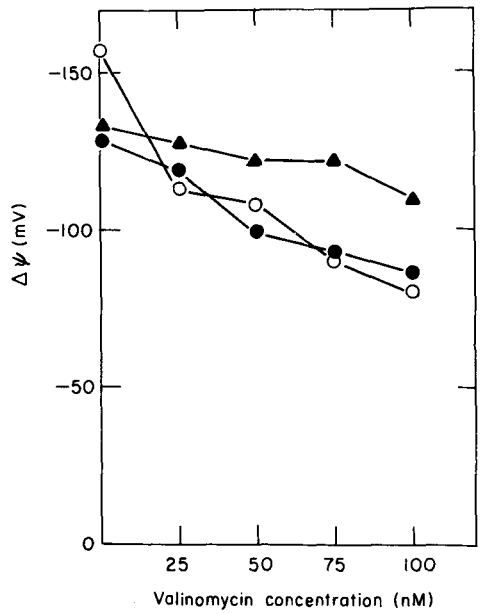

Fig. 3. Effect of valinomycin on $\Delta\psi$ of *B. firmus* OF4 strains. Washed cells, grown at pH 7.5 without CCCP, were resuspended in 100 mM potassium phosphate (pH 7.5) and energized with 10 mM sodium malate. $\Delta\psi$ was measured without addition of nigericin. At least four determinations were made in each case, and errors did not exceed 10%. Key: ▲, 811M; ●, CC1; ○, CC2.

further evidence that the general susceptibility of the mutant strains to hydrophobic ionophores was not diminished. A similar hypersensitivity to valinomycin was noted in the protonophore-resistant *B. subtilis* strains [3].

In a further series of experiments, the $\Delta\bar{\mu}_{H^+}$ and ΔG_p values of cells growing logarithmically at pH 7.5 were assayed following a sudden challenge with 1 μM CCCP. The uncoupler caused an immediate, substantial lowering of both $\Delta\psi$ and ΔG_p in all three strains (Table II), with no indication of resistance in strains CC1 and CC2 at 15 min after CCCP addition. Over the following 5–7 h, however, strains CC1 and CC2 resumed logarithmic growth (at a lower rate than in the absence of CCCP), while 811M grew only slightly. Subsequent assays revealed that both resistant strains, in contrast to strain 811M, had significantly increased their $\Delta\psi$ and ΔG_p (Table II). Furthermore, the values of these parameters attained by strain CC2 were significantly higher than those of strain CC1 ($P = 0.05$, one-tailed Student's *t*-test). The behavior of the cells was unaffected by the growth conditions (±CCCP) of the initial inocula.

We conducted further experiments to study ATP synthesis by starved and re-energized cells immediately after a challenge with CCCP. Washed cells were starved overnight and re-energized with malate in the presence or absence of various concentrations of CCCP. The data of Table III confirm the significantly elevated $\Delta\psi$ of strain CC2 (Fig. 2, Table I), and ATP synthesis at a given concentration of CCCP was significantly higher in CC2 than in the other strains. However, at any given $\Delta\psi$ between −125 and −75 mV, the amount of ATP synthesized was very similar in all three strains.

Respiratory properties

The respiratory rates of all three strains were identical at pH 10.5 (Table IV). At pH 7.5, however, strains CC1 and CC2 both respired 40–50% faster than the parent. Respiratory rates were not significantly altered by growth in the presence of 1 μM CCCP. Addition of 1 μM CCCP to the test samples caused no increase in respiration, indicating a lack of measurable respiratory control. The respiration of all three strains was affected similarly by 20 μM antimycin A (50–60% inhibition) or by 50 μM HQNO (45–55% inhibition), inhibitors of the cytochrome bc_1 complex.

TABLE II

The effects of CCCP on ΔG_p and $\Delta\psi$ of growing cells of B. firmus OF4 strains

Strain	Inoculum grown in presence of 1 μM CCCP	Sample taken 15 min after addition of 1 μM CCCP to log-phase cells [a]			Samples taken from cells grown on 1 μM CCCP for 5–7 h [b]		
		ΔG_p (kcal/mol)	ΔG_p (mV)	$\Delta\psi$ (mV)	ΔG_p (kcal/mol)	ΔG_p (mV)	$\Delta\psi$ (mV)
811M	no	9.0±0.2	−390±8	−53±2	9.2±0.1	−398±4	−50±2
CC1	no	9.0±0.2	−390±8	−46±3	9.8±0.1	−424±4 **	−81±1 **
	yes	9.1±0.2	−394±8	−59±3	9.6±0.2	−416±8 **	−77±5 **
CC2	no	9.1±0.2	−394±8	−56±2	10.0±0.1	−433±4 **	−95±2 **
	yes	9.1±0.2	−394±8	−62±3	10.0±0.2	−433±8 **	−98±2 **

[a] Cells of each strain were grown at pH 7.5 (1 ml inoculum used for 50 ml culture) until early logarithmic phase (100 Klett units). CCCP was added to 1 μM and incubation was continued for 15 min before the determinations were made. The values are the mean±S.D. of five independent experiments. All $\Delta\psi$ values presented in this table were measured in the presence of nigericin. Values of ΔG_p (kcal/mol) and $\Delta\psi$ in control cells (not treated with CCCP) were: 811M, 10.7±0.2, −132±11; CC1, 10.6±0.2, −131±9; CC2, 10.9±0.3, −155±12.

[b] Cells were grown at pH 7.5 in the presence of 1 μM CCCP. Measurements were made after 5–7 h of growth when the cultures of CC1 and CC2 were growing logarithmically and were at a turbidity of about 150 Klett units, whereas 811M had grown slightly to a turbidity of about 50 Klett units. The values are the mean±S.D. of five independent experiments. Data from strains CC1 and CC2 were compared with those from 811M using a one-tailed Student's *t*-test: **, significant at $P = 0.01$.

TABLE III

Effects of CCCP on ATP synthesis and $\Delta\psi$ in starved and re-energized cells of B. firmus OF4 strains

Cells were grown to mid-logarithmic phase at pH 7.5 in the absence of CCCP, harvested, resuspended in 50 mM sodium phosphate, 5 mM $MgSO_4$ (pH 7.5) and starved overnight. Following preincubation for 5 min with various concentrations of CCCP, sodium malate (10 mM) was added, and the cells were aerated at 30°C. ATP synthesis was followed over a 10 min time-course. In all cases, a steady-state, maximal level of ATP was reached within 5 min. This maximum amount of ATP synthesized is presented in each case, along with the corresponding $\Delta\psi$, measured in the presence of nigericin. At least three determinations were made in each case. For statistical analysis, data from strains 811M and CC2 were compared using a one-tailed Student's *t*-test: *, significant at $P = 0.05$; **, significant at $P = 0.01$.

[CCCP] (μM)	811M		CC1		CC2	
	ATP synthesized (mM)	$\Delta\psi$ (mV)	ATP synthesized (mM)	$\Delta\psi$ (mV)	ATP synthesized (mM)	$\Delta\psi$ (mV)
0	2.5 ±0.4	−124± 6	2.75±0.7	−125± 7	3.3 ±0.6	−139± 8 *
0.05	1.35±0.3	−103± 8	1.5 ±0.4	−99±10	2.6 ±0.3 **	−124± 7 *
0.1	0.5 ±0.2	−76±10	0.4 ±0.2	−79± 9	1.3 ±0.3 **	−112± 3 *
0.2	0	−51± 7	0	−58± 8	0.35±0.2	−87±10 **

Cytochrome content

Four types of cytochrome could be distinguished in membrane vesicles of the *B. firmus* OF4 strains harvested at the late logarithmic stage of growth: types aa_3, *b*, *c* and *o*. An additional *d*-type cytochrome, with an absorbance peak at 620 nm in dithionite-reduced minus oxidized spectra, appeared in cells grown to stationary phase (not shown). The cytochromes from comparable cells grown in the presence of CCCP were also assayed. Results are presented in Table V.

Cells of the non-resistant strain 811M grown at pH 10.5 contained significantly higher levels of cytochromes, particularly types aa_3 and *c*, than those grown at pH 7.5, in agreement with an earlier study [7]. The previous study reported higher concentrations, but these may have been overestimated due to overlap in the absorbance peaks of the dithionite-reduced minus ferricyanide-oxidized spectra used for quantitation. By contrast, the assays employed in the present study may produce somewhat underestimated values due to incomplete reduction or oxidation by the reagents. Both resistant strains also contained high levels of cytochromes aa_3 and *c* when grown at pH 10.5, although their cytochrome *o* levels were both some 40% lower than those of strain 811M.

At pH 7.5, strain CC1 contained higher levels of cytochrome *o* than the parent strain, while strain CC2 contained elevated levels of four cytochrome types: aa_3, *b*, *c* and *o* in a pattern somewhat akin to that of pH-10.5-grown cells of the parent. These changes may account at least in part for the higher respiratory rates of strains CC1 and CC2. Interestingly, growth of strains 811M and CC1 in the presence of CCCP at pH 7.5 caused cytochrome aa_3 to be elevated to the level found in strain CC2 grown without the uncoupler; growth with CCCP caused little further elevation in the cytochromes of strain CC2.

TABLE IV

Respiratory properties of B. firmus OF4 strains

Cells were grown to mid-logarithmic phase in the presence or absence of CCCP and assayed at the same pH as grown, as described in the Materials and Methods section. Respiratory rates are presented as mean ± S.D., with the number of independent determinations in parentheses. * Significant versus 811M at $P = 0.01$ (one-tailed Student's *t*-test).

Growth conditions			Respiratory rate (ngatom O/min per mg protein)
pH	strain	[CCCP] (μM)	
7.5	811M	0	751± 47 (8)
		1	723± 25 (2)
7.5	CC1	0	1075± 85 (7) *
		1	1225±136 (2)
7.5	CC2	0	1112±133 (5) *
		1	1249±124 (2)
10.5	811M	0	725± 51 (3)
10.5	CC1	0	721± 59 (3)
10.5	CC2	0	672± 45 (3)

Membrane lipid composition

The polar lipid composition, neutral lipid composition and distribution of acyl side-chains for the three strains are presented in Tables VI–VIII. The neutral/polar lipid ratio of pH-7.5-grown cells was 25 : 75 in all three strains. No significant differences were apparent among any of the strains grown at pH 7.5 in the absence of CCCP. Instead, marked differences in lipid composition were observed between strain CC1 and the others upon comparison of pH-10.5-grown cells. The neutral/polar lipid ratios were 35 : 65 for strains 811M and CC2, and 40 : 60 for strain CC1. Phosphatidylethanolamine and cardiolipin were elevated in strain CC1 at the expense of phosphatidylglycerol (Table VI). Changes in neutral components were less marked (Table

TABLE V

Cytochrome content of B. firmus OF4 strains

Cells were grown, harvested, and assayed as described in Materials and Methods. The values are mean ± S.D., with the number of independent determinations in brackets. Statistical analysis was performed using a one-tailed Student's *t*-test (*, **) significant versus 811M grown under the same conditions at $P = 0.05$ (*) or $P = 0.01$ (**). †, Significant versus the same strain growth without CCCP at $P = 0.05$.

Growth conditions			Cytochrome content (nmol/mg protein)			
pH	Strain	[CCCP] (μM)	aa_3	*b*	*c*	*o*
7.5	811M	0	0.07 ± 0.02 (4)	0.54 ± 0.10 (4)	0.68 ± 0.10 (4)	0.25 ± 0.04 (4)
		1	0.12 ± 0.03 (2) †	0.56 ± 0.20 (2)	0.79 ± 0.18 (2)	0.40 ± 0.22 (2)
7.5	CC1	0	0.05 ± 0.03 (4)	0.64 ± 0.13 (4)	0.66 ± 0.18 (4)	0.35 ± 0.05 (3) *
		1	0.12 ± 0.02 (2) †	0.67 ± 0.16 (2)	0.82 ± 0.15 (2)	0.31 ± 0.14 (2)
		2	0.13 ± 0.01 (2) †	0.74 ± 0.10 (2)	1.04 ± 0.21 (2)	0.31 ± 0.07 (2)
7.5	CC2	0	0.12 ± 0.01 (5) **	0.87 ± 0.16 (5) **	0.98 ± 0.16 (5) **	0.38 ± 0.08 (4) *
		1	0.15 ± 0.03 (2)	0.74 ± 0.08 (2)	0.98 ± 0.20 (2)	0.23 ± 0.04 (2)
		2	0.13 ± 0.01 (3)	0.76 ± 0.10 (3)	1.09 ± 0.16 (3)	0.27 ± 0.13 (3)
10.5	811M	0	0.18 ± 0.03 (4)	0.62 ± 0.05 (4)	1.19 ± 0.08 (4)	0.35 ± 0.04 (3)
10.5	CC1	0	0.18 ± 0.04 (4)	0.74 ± 0.20 (4)	1.04 ± 0.24 (4)	0.22 ± 0.01 (3) **
10.5	CC2	0	0.18 ± 0.03 (4)	0.59 ± 0.16 (4)	0.92 ± 0.23 (4)	0.19 ± 0.03 (3) **

TABLE VI

Polar lipid composition of B. firmus OF4 strains

Cells were grown in the absence of CCCP, at pH 7.5 or pH 10.5, as indicated. At least two independent determinations of lipid composition were made for each strain; individual components did not vary by more than 2% between preparations.

	Percentage of total polar lipid content					
	811M		CC1		CC2	
	7.5	10.5	7.5	10.5	7.5	10.5
Phosphatidylglycerol	57	53	50	35	56.5	51.5
Phosphatidylethanolamine	21	20	20	30	21.5	19
Cardiolipin	14.5	16.5	15	25	14	18.5
Phosphatidic acid	4.5	2.5	4	2	5	2.5
Other	3	8	3	8	3	7.5

TABLE VII

Neutral lipid composition of B. firmus OF4 strains

Neutral lipids were assayed in the same cell extracts used for Table VI. At least two independent determinations of lipid composition were made for each strain; individual components did not vary by more than 2% between preparations.

	Percentage of total neutral lipid content					
	811M		CC1		CC2	
	7.5	10.5	7.5	10.5	7.5	10.5
Free fatty acids	4.5	4	4	12.5	3.5	3.5
1,3-Diacylglycerol	20	22.5	20	24	20	21.5
1,2-Diacylglycerol	24.5	33.5	26	28	26.5	27
Squalene	10	13.5	10	8	10	13
Dehydro- or tetrahydro-squalene	6	12	6	5	6	12
C_{40} isoprenoids	30	13.5	29	18	29	19
C_{50} isoprenoids	5	1	5	4.5	5	4

TABLE VIII

Fatty acid composition of B. firmus OF4 lipids

The same cell samples were used as for Tables VI and VII. At least two independent determinations of lipid composition were made for each strain; individual components did not vary by more than 4% between preparations.

Fatty acid	Percentage of total fatty acid content					
	811M		CC1		CC2	
	7.5	10.5	7.5	10.5	7.5	10.5
iso-12:0	5.5	1.5	5	1	4	2
n-13:0	5.5	1.5	5	1	6	0
iso-14:0	8	9	10	5.5	8	10.5
n-14:0	10	8.5	9	7.5	10	8.5
iso-15:0	29	27.5	28	10.5	30	26.5
anteiso-15:0	15	25	16	48.5	15	25.5
iso-16:0	1.5	1.5	1	2	2	1
n-16:0	6.5	8	6	7.5	6	8
iso-17:0	5	6.5	6	2.5	6	6.5
anteiso-17:0	0	1	0	2	0	0.5
n-17:0	13.5	9	14	10	13	10.5
n-18:0	0.5	1	0	0	0.5	0.5
n-16:1	0	0	0	1	0	0
iso-17:1	0	0	0	1	0	0
anteiso-17:1	0	0	0	1	0	0
n-18:1	0	0	0	1	0	0

VII), but squalene derivatives were lowered and free fatty acids raised 3-fold. Compared with strain 811M, isoprenoids were somewhat elevated in both strains CC1 and CC2 grown at pH 10.5. An additional feature of pH 10.5-grown strain CC1 was a 5-fold increased ratio of 15:0 *anteiso/iso* side-chains (Table VIII). To investigate the possible relevance of the altered lipid composition of strain CC1 to its uncoupler-resistance at pH 7.5, lipids were also analyzed from strains CC1 and CC2 grown at pH 7.5 in the presence of 2 μM CCCP. This treatment, however, did not affect the composition of polar or neutral lipids in either strain, which remained indistinguishable from that of strain 811M grown without CCCP at the same pH (not shown).

Discussion

In quantitative terms, the CCCP-resistance of *B. firmus* OF4 strains CC1 and CC2 is similar to that of previously characterized mutants of *B. subtilis* and *B. megaterium*, but is an order of magnitude below the resistance reported in *E. coli* strains, where outer membrane involvement has been implicated in at least some of the mutants [1,25]. Indeed, the *B. firmus* strains were particularly sensitive to the protonophores and inhibitors employed in this study.

In previous studies, it was shown that the ability of CCCP to dissipate $\Delta\bar{\mu}_{H^+}$ in protonophore-resistant strains of both *B. megaterium* and *B. subtilis* was unimpaired, but that the bacteria displayed an enhanced ability to synthesize ATP at suboptimal values of $\Delta\bar{\mu}_{H^+}$ [1,3]. The uncoupling potency of CCCP also appeared unimpaired in *B. firmus* strains CC1 and CC2. In contrast to the resistant strains of *B. subtilis* and *B. megaterium*, however, neither CC1 nor CC2 synthesized more ATP than their wild-type parent at equivalent, submaximal values of $\Delta\bar{\mu}_{H^+}$ (Tables II, III), suggesting that a different mechanism of resistance operates in the alkaliphile strains.

The protonophore-resistant phenotype of *B. firmus* strain CC2 at pH 7.5 may be explained in terms of a model in which the elevated cytochromes allow faster respiration and the generation of a larger $\Delta\bar{\mu}_{H^+}$. All aerobic extreme alkaliphiles possess extremely high concentrations of membrane cytochromes [9]. A multiplicity of species has been identified by redox potentiometry [26,27], and a caa_3-type terminal oxidase has been purified from *B. firmus* RAB [28]. The respiratory chain of these organisms is proton pumping; there are no primary, respiration-coupled Na^+ pumps [29]. The facultative *B. firmus* OF4 grown at pH 7.5 possesses lower concentrations of membrane cytochromes than when grown at pH 10.5 [7]. The CC2 mutation appears to alter this pattern, such that cells grown at pH 7.5 contain cytochrome concentrations more typical of wild-type cells grown at pH 10.5. On the other hand, the poorer growth of strain CC2 above pH 10 correlates with the reduced levels of cytochromes *b* and *o* noted in pH-10.5-grown cells. The possession of an *o*-type terminal oxidase, in addition to the caa_3-type, was suggested in earlier studies of alkaliphilic bacilli [26] (Plass, R.J., Hicks, D.B. and Krulwich, T.A. (1990) Biophys. J. 57, 557a [Abstr.]). Although the $\Delta\bar{\mu}_{H^+}$ of strain CC2 at pH 10.5 was no lower than that of the parent strain, this

bulk phase parameter may be a less direct and complete determinant of the energetic status of the cells at high pH [9,10]. The alkaliphile at pH 10.5 may require higher concentrations of specific respiratory chain complexes to facilitate their interaction with the ATP-synthase, for example, by collisions allowing direct proton transfer. Altered cytochrome patterns leading to the generation of an equal bulk $\Delta\bar{\mu}_{H^+}$, but changing the proportion of a component involved in direct coupling, might affect this coupling mode adversely.

Strain CC2 may carry a mutation in a gene concerned with cytochrome expression and/or organization. The resulting phenotype is strongly pH-dependent, with cytochromes generally elevated in pH-7.5-grown cells but with some types depleted in pH 10.5-grown cells. Multiple changes in cytochrome levels resulting from single transposon Tn5 insertions have been documented for *Rhizobium phaseoli* [30] and *Bradyrhizobium japonicum* [31]. Similarly, a cytochrome aa_3-deficient mutant of *B. subtilis* contained reduced amounts of cytochrome *c* and elevated cytochrome *o* [32]. In *E. coli*, expression of the two terminal oxidases is controlled by the two-component *arc* system, such that cytochrome *o* levels are decreased by anaerobic growth while cytochrome *d* levels are elevated [33]. A similar type of control system could be envisaged for cytochrome aa_3 in alkaliphilic *B. firmus*, since the level of this oxidase correlates inversely with $\Delta\bar{\mu}_{H^+}$; cytochrome aa_3 increases when $\Delta\bar{\mu}_{H^+}$ is reduced by CCCP at pH 7.5, or when cells are grown at a more alkaline pH at which $\Delta\bar{\mu}_{H^+}$ is lower (Table V). Strain CC2 may have lost some element of this control system, as its expression of cytochrome aa_3 is high under all conditions of $\Delta\bar{\mu}_{H^+}$.

Strain CC1 also appears to possess a mutation affecting the respiratory chain composition and function, but the relationship between the observed changes and the protonphore-resistant phenotype is less clear. While its raised respiratory rate at pH 7.5 may be linked to its elevated cytochrome *o*, there was no significant difference between the cytochromes of strains 811M and CC1 when both were grown with 1 μM CCCP, although the respiratory and growth rates of CC1 were far superior. In the absence of CCCP, the increased respiratory rate of strain CC1 was not reflected in a higher $\Delta\bar{\mu}_{H^+}$, in contrast to strain CC2. When grown in the presence of 1 μM CCCP, however, strain CC1 behaved similarly to strain CC2, sustaining higher values of $\Delta\bar{\mu}_{H^+}$ and ΔG_p than the wild-type parent. Surprisingly, marked lipid changes were observed when strain CC1 was grown at pH 10.5, but not at pH 7.5 where resistance is manifest. One possibility is that the membrane lipid changes have no relevance to the uncoupler-resistance, and that strain CC1 carries more than one mutation. The original CCCP-resistant isolate from pH 7.5 medium was plated at pH 10.5 before selection of a single colony, and a secondary mutation could have occurred at this stage. Alternatively, there may be some subtle, so far undetermined change at pH 7.5 (e.g., in quinones), which allows enhanced function of the rate-limiting energetic step at this pH when $\Delta\bar{\mu}_{H^+}$ is lowered, but which would not permit growth at pH 10.5 without concomitant alterations in lipid composition. Strain CC1 retains more nearly wild-type growth and respiratory rates at pH 10.5, even though its cytochrome composition, with reduced cytochrome *o*, is very similar to that of CC2, whose growth is slower at this pH; possibly the lipid changes are responsible for this difference.

Thus, in both resistant strains, protonophore-resistance is associated with an elevated respiratory rate, alterations in the respiratory chain composition, and the generation of an elevated $\Delta\bar{\mu}_{H^+}$, either constitutively (strain CC2) or when grown in the presence of CCCP (strain CC1). A good correspondence between $\Delta\bar{\mu}_{H^+}$ and ΔG_p was shown for both mutants growing at pH 7.5 in the presence of CCCP, consistent with the operation of a chemiosmotic type of coupling mechanism at this pH. Further clarification of the current strains may be possible if more and genetically accessible mutants can be produced. We have recently developed a system for the introduction of the transposon Tn917 into *B. firmus* OF4 [34]. A library of insertional mutants, as employed in the generation of cytochrome mutants of *Rhizobium* species [30,31], offers a promising approach to the selection of demonstrably single insertions resulting in CCCP-resistance. Of particular interest would be any protonophore-resistant mutants unable to grow at high pH; such strains might show more clear-cut changes than those of CC1 and CC2.

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