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Carbonyl Cyanide-*m*-Chlorophenyl Hydrazone-Resistant *Escherichia coli* Mutant That Exhibits a Temperature-Sensitive Unc Phenotype

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Two spontaneous *Escherichia coli* mutant strains which are resistant to an oxidative phosphorylation uncoupler, carbonyl cyanide-*m*-chlorophenyl hydrazone, were isolated. Strain CM22 (*ccr-2*) was resistant to another uncoupler, pentachlorophenol, and to the inhibitors of proton-translocating ATPase, namely tributyltin and sodium azide. Carbonyl cyanide-*m*-chlorophenyl hydrazone or pentachlorophenol administered to cell suspensions of strain CM22 did not cause a pH change induced by H^+ influx, and a similar result was obtained with everted particles. The respiratory rate of strain CM22 with succinate was twice that of wild-type strain KH434. When carbonyl cyanide-*m*-chlorophenyl hydrazone was administered, a stimulation of O_2 uptake was observed in wild-type strain KH434 but not in the mutant strain CM22. Strain CM22 did not grow on succinate at 42°C. Isolation of a true revertant at a frequency of 10^{-8} demonstrated that the pleiotropic phenotype was induced by a single mutation. P1 transduction indicated that the mutant allele, *ccr-2*, was cotransduced with the *ilv* genes at a frequency of about 55%.

ATP formation by oxidative phosphorylation or photophosphorylation has been observed in bacterial, mitochondrial, and chloroplast membranes (3, 12, 16). According to the chemiosmotic hypothesis of Mitchell, the transmembrane gradient of the electrochemical potential of H^+ formed by the translocation of protons across the membrane is the motive force of ATP formation (15). The proton-translocating ATPase (H^+-ATPase) is thought to play the central role in ATP synthesis (11). The precise mechanism of uncouplers, which act to dissipate the proton motive force, is still obscure. One of the explanations on uncoupling action, currently supported by most investigators, is that uncouplers act as lipophilic proton conductors (15). However, some investigators insist that uncouplers bind to protein(s) for uncoupling action (4, 8, 9).

Ito and Ohnishi (10) have reported that mutant strains of *Escherichia coli* which are resistant to tributyltin, an inhibitor of H^+-ATPase, are also resistant to other inhibitors of H^+-ATPase, namely, sodium azide and *N,N'*-dicyclohexylcarbodiimide (DCCD), and to uncouplers, namely, carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) and pentachlorophenol (PCP). Some of the mutant strains exhibited a temperature-sensitive uncoupled (Unc) phenotype. The temperature-sensitive Unc phenotype and the resistance to inhibitors of H^+-ATPase and to uncouplers are induced by a single mutation. The mutant alleles were mapped in a region near the *unc* genes that code for protein subunits of H^+-ATPase (10). The results suggest that the uncouplers CCCP and PCP specifically interact with protein subunit(s) of H^+-ATPase.

Therefore, to confirm this suggestion we tried to isolate directly a mutant strain resistant to the uncoupler CCCP. In this paper, we report that a CCCP-resistant mutant strain, CM22 (*ccr-2*), is also resistant to another uncoupler, PCP, and to tributyltin and sodium azide, both inhibitors of H^+-ATPase. This strain exhibits the temperature-sensitive Unc phenotype, and the mutant allele *ccr-2* maps in a region near the *unc* genes that code protein subunits of H^+-ATPase.

MATERIALS AND METHODS

Bacteria. *E. coli* strain KH434 (*gal thy*), which was derived from strain W3350, was used as a wild-type strain. Strain IM20 (KH434 *ilv*) was isolated as an Ilv^- auxotroph by penicillin treatment after ethyl methane sulfonate mutagenesis (13). Strains IM20 and IM5 (HfrC *ilvA rel tna tonA22 bgl* $T2^r$) were used as recipient strains for the P1 transduction experiments.

Media. M63 medium (14) was used as the minimal medium with 40 mM sodium succinate or 0.4% (wt/vol) glucose as the carbon source. Thymine was added at a final concentration of 10 μg/ml when strain KH434 and its derivatives were grown. L broth supplemented with 10 μg of thymine per ml was used as

the rich medium. Cells were grown aerobically in all of the experiments.

Test of minimal inhibitory concentration. Cells were grown to log phase in the rich medium and were washed with M63 medium. About 10^6 cells per plate were put on plates containing succinate, thymine, and an inhibitor. The concentrations of the inhibitors were: CCCP, 25, 30, 40, 50, and 60 μM; PCP, 75, 100, 150, 200, and 300 μM; sodium azide, 50, 100, 200, and 300 μM; DCCD, 1.5, 3, and 5 mM; and tributyltin, 10^{-3}, 2×10^{-3}, 3×10^{-3}, and 4×10^{-3}% (vol/vol).

Chemicals. CCCP was purchased from Sigma Chemical Co. Tributyltin, sodium azide, and DCCD were purchased from Wako Chemical Co. PCP was provided by T. Higuti (Tokushima University).

Isolation of mutant strains. Strain KH434 was grown in the rich medium overnight and was washed twice in M63 medium. Cells (about 5×10^8 per plate) were spread on plates which contained M63 medium supplemented with succinate, thymine, and 50 μM CCCP. The cells were incubated at 30°C for 3 days.

Measurement of H^+ influx. Cells were grown in the rich medium at 30°C to log phase. Cells were washed twice and suspended in 0.2 M KCl to give a concentration of 250 μg of protein per ml. After the addition of uncoupler to the cell suspension, the pH change was measured at 30°C by a pH meter (Hitachi-Horiba F-7ss) equipped with a recorder.

Preparation of membrane particles. Cells were grown in the rich medium at 30°C to log phase. Cells were washed twice, suspended in 0.2 M KCl, and sonicated at 19.5 kHz for 10 min with a sonicator (Kaijo Denki TA-4201). After low-speed centrifugation at 2,000 × *g* for 20 min, the supernatant fraction was used for membrane particles. The protein concentration was adjusted to 1.5 mg/ml in 0.2 M KCl.

Measurement of O_2 uptake. Cells were grown in the rich medium at 30°C to log phase. Cells were washed twice and were suspended in M63 medium to give a concentration of 350 μg of protein per ml. The O_2 uptake was measured at 30°C by the Clark-type oxygen electrode after succinate (at a final concentration of 25 mM) and an uncoupler were added.

RESULTS

Isolation of CCCP-resistant mutant strains. At a concentration of 50 μM CCCP, the wild-type strain KH434 could grow with glucose as the carbon source but not with succinate (data not shown). This observation suggests that CCCP inhibits the ATP formation coupled to the oxidative metabolism of tricarboxylic acid cycle intermediates. To isolate CCCP-resistant strains, 5×10^8 cells of the wild-type strain per plate were spread on plates containing succinate, thymine, and CCCP and were incubated at 30°C for 3 days. Colonies which appeared were purified twice on selective plates. Two spontaneous mutant strains, CM21 (*ccr-1*) and CM22 (*ccr-2*), were isolated. Mutant strains CM21 and CM22 were examined to determine whether they were resistant to another uncoupler, PCP, and to the inhibitors of H^+-ATPase, namely, tributyltin, sodium azide, and DCCD. Strain CM21 was resistant to the uncouplers CCCP and PCP and also to tributyltin. Strain CM22 was resistant to the uncouplers and also to tributyltin and sodium azide (Table 1).

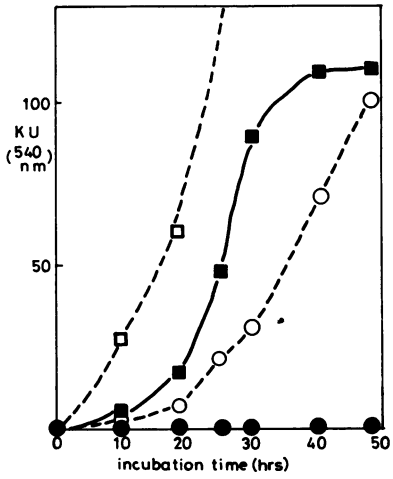

FIG. 1. Resistance of strain CM22 to CCCP. Cells of strains CM22 and KH434 were grown to 6×10^8 cells per ml in rich medium, washed, and diluted to 6×10^6 cells per ml in M63 medium containing 30 μM CCCP supplemented with glucose or succinate. Aerobic incubations were performed at 30°C with a shaker (Yamato BT-22, 180 shakes per min), and turbidity was measured in Klett units (540 nm). Symbols: ○, KH434, glucose; ●, KH434, succinate; □, CM22, glucose; ■, CM22, succinate.

In liquid medium, strain CM22 showed good growth with 30 μM CCCP, which completely inhibited the growth of the wild-type strain KH434 with succinate as the carbon source (Fig. 1). A similar observation was made with 40 μM CCCP (data not shown). Strain CM22 showed

TABLE 1. Minimal inhibitory concentrations for uncouplers and inhibitors of H^+-ATPase[a]

Strain	Mutation allele	Minimal inhibitory concn				
		CCCP (μM)	PCP (μM)	Tributyltin ($\times 10^{-3}$%)	Sodium azide (μM)	DCCD (mM)
KH434	None	25	75	1	50	1.5
CM21	*ccr-1*	60	150	3	50	1.5
CM22	*ccr-2*	>60	300	3	300	1.5

[a] Each mutant strain was grown on plates containing succinate, thymine, and an inhibitor at 30°C for 2 days.

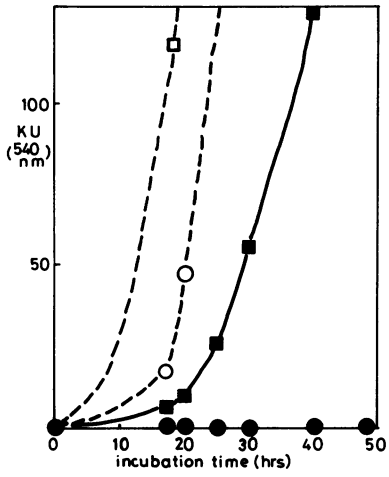

FIG. 2. Resistance of strain CM22 to tributyltin. Cells of strains CM22 and KH434 were grown to 6 × 10^8 cells per ml in rich medium, washed, and diluted to 6 × 10^6 cells per ml in M63 medium containing 2.5 × 10^{-4}% (vol/vol) tributyltin supplemented with glucose or succinate. Aerobic incubations were performed at 30°C with a shaker (Yamato BT-22, 180 shakes per min), and turbidity was measured in Klett units (540 nm). Symbols: ○, KH434, glucose; ●, KH434, succinate; □, CM22, glucose; ■, CM22, succinate.

good growth with 2.5 × 10^{-4}% (vol/vol) tributyltin, which completely inhibited the growth of the wild-type strain KH434 with succinate as the carbon source (Fig. 2). A similar observation was obtained with 5 × 10^{-4}% (vol/vol) tributyltin (data not shown). At these concentrations of CCCP and tributyltin, ATP formation by oxidative phosphorylation in the wild-type strain KH434 was probably blocked since growth occurred with glucose as the carbon source (Fig. 1 and 2).

Lack of uncoupler-induced pH change in strain CM22. According to the chemiosmotic hypothesis of Mitchell (15), uncouplers act as they do mainly because of their behavior as lipophilic proton conductors across the membrane. However, we cannot rule out a possible interaction between uncouplers and other membrane components during the dissipation of the proton motive force. To determine whether a membrane component is involved in uncoupling action, we investigated the uncoupler-induced H^+ influx in cells where the inside negative electrochemical potential difference of H^+ exists. When CCCP or PCP was administered in cell suspensions of the wild-type strain KH434, the pH change induced by H^+ influx was observed (Fig. 3). The H^+ influx values were 27.3 nmol of H^+ per mg of protein per min at 2 μM CCCP, 35.7 nmol of H^+ per mg of protein per min at 4 μM CCCP, 11.4 nmol of H^+ per mg of protein per min at 30 μM PCP, and 16.4 nmol of H^+ per mg of protein per min at 45 μM PCP. On the other hand, in the mutant strain CM22, H^+ influx was not observed when these uncouplers were added (a small H^+ influx of 1.6 nmol of H^+ per mg of protein per min was observed at 45 μM PCP) (Fig. 3).

We next examined H^+ influx in membrane particles. Everted particles were prepared by sonication. The inside of each particle was positive (acid), and the outside was negative. Therefore, 3 μmol of H^+ was added in the particle suspension (3.5 ml) to make the outside positive (acid). The measurement of the external pH required higher concentrations of uncoupler because the internal spaces of the everted particles

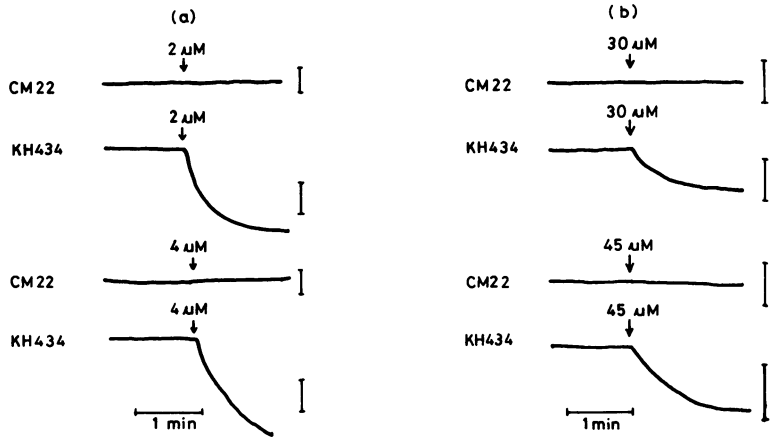

FIG. 3. pH change by uncouplers. Cells of the indicated strains were suspended in 0.2 M KCl to give a concentration of 250 μg of protein per ml. Each vertical line indicates 10 nmol of H^+. (a) CCCP was administered at a concentration of 2 or 4 μM. (b) PCP was administered at a concentration of 30 or 45 μM.

were smaller than those of the intact cells. When CCCP was administered to the particles from strain KH434, a pH change induced by H^+ influx was observed (Fig. 4). The rates of H^+ influx were 1.11 nmol of H^+ per mg of protein per min at 20 μM CCCP and 1.20 nmol of H^+ per mg of protein per min at 30 μM CCCP. On the other hand, H^+ influx was not observed at these concentrations of CCCP with particles from strain CM22 (Fig. 4).

The compatible observations that uncouplers do not induce H^+ influx in intact cells and everted particles of strain CM22 suggest that uncouplers interact with a membrane component for the uncoupling action.

Measurement of respiratory rates. It is also known that uncouplers stimulate O_2 uptake at low concentrations (2). When 4 μM CCCP was administered in a cell suspension of strain KH434 with 25 mM succinate as substrate, stimulation of O_2 uptake was observed (Fig. 5). The respiratory rates were 21.7 nmol of O_2 per mg of protein per min with 25 mM succinate and 39.0 nmol of O_2 per mg of protein per min with the addition of CCCP. When strain CM22 was examined, no stimulation of O_2 uptake was observed at the same concentration of CCCP (Fig. 5). The respiratory rate was 43.0 nmol of O_2 per mg of protein per min throughout the experiments with the addition of CCCP. A significant difference in the respiratory rates of the two strains with succinate should be noted. The respiratory rate of strain CM22 was twice that of strain KH434. The increased respiratory rate suggests that strain CM22 carries a mutation in a component for oxidative phosphorylation.

Temperature-sensitive Unc phenotype of the mutant strain CM22. The CCCP-resistant mutant strain CM22 was also resistant to tributyltin and sodium azide, both inhibitors of H^+-ATPase (Table 1). This observation suggests that strain CM22 carries a mutation in a protein subunit of H^+-ATPase. Thus, it should exhibit an Unc phenotype, which means that the cells should grow in the presence of glucose as the carbon source but not in the presence of succinate; these are characteristics of strains carrying mutations in protein subunits of H^+-ATPase (7). In fact, strain CM22 had temperature-sensitive growth on succinate. When the experiment was performed at 42°C in the absence of the inhibitors, the mutant strain CM22 grew in the presence of glucose as the carbon source but not in the presence of succinate. On the other hand, the wild-type strain KH434 grew in the presence of either of these carbon sources (Fig. 6). This lack of growth on succinate at 42°C indicates that strain CM22 carries a mutation in a protein subunit of H^+-ATPase.

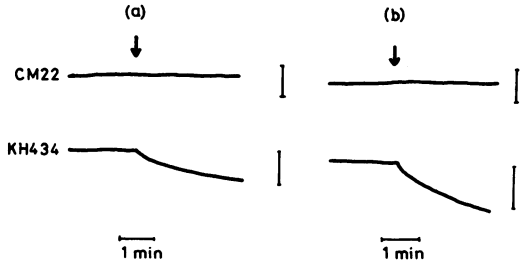

FIG. 4. pH change of everted particles by CCCP. Cells of the indicated strains were suspended in 0.2 M KCl and sonicated. After low-speed centrifugation, the supernatant fraction was used for the everted particles. Protein concentration was adjusted to 1.5 mg/ml in 0.2 M KCl. Experimental procedures were similar to those used for Fig. 3. CCCP was administered at concentrations of 20 (a) or 30 (b) μM. Each vertical line indicates 5 nmol of H^+.

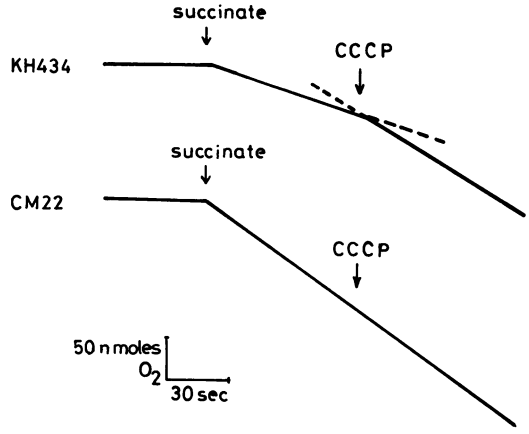

FIG. 5. Effect of CCCP on O_2 uptake. Cells of the indicated strains were suspended in M63 medium to give a concentration of 350 μg of protein per ml. Respiration started with 25 mM succinate. CCCP was administered at a concentration of 4 μM.

Reversion test of strain CM22. Twenty spontaneous Unc^+ revertants were isolated on succinate plates at 42°C and were tested to determine whether the resistances to CCCP, PCP, tributyltin, and sodium azide had reverted to the same levels as in the wild-type strain KH434. Out of 20 revertants, 1 reverted simultaneously to normal sensitivities to CCCP, PCP, tributyltin, and sodium azide. Another 13 of the revertants remained resistant to the four inhibitors. The remaining six strains reverted to normal sensitivities to some of the four inhibitors. The details are shown in Table 2. Furthermore, when the stimulation of respiratory rate and H^+ influx induced by an uncoupler were examined, the true revertant behaved similarly to the wild-type strain KH434 (data not shown).

The true revertant was isolated at the frequen-

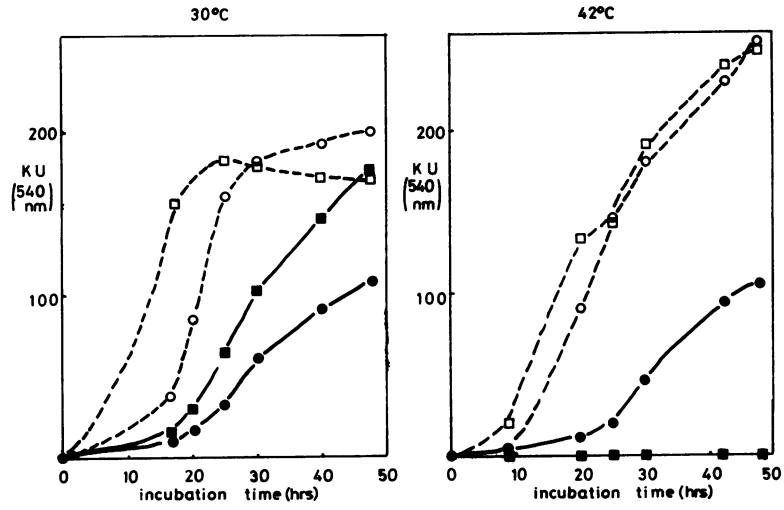

FIG. 6. Temperature-sensitive Unc phenotype of strain CM22. Cells of strains CM22 and KH434 were grown to 6×10^8 cells per ml in rich medium, washed, and diluted to 6×10^6 cells per ml in M63 medium. Glucose or succinate was used as the carbon source. Aerobic incubations were performed at 30 or 42°C with a shaker (Yamato BT-22, 180 shakes per min), and turbidity was measured in Klett units (540 nm). Symbols: ○, KH434, glucose; ●, KH434, succinate; □, CM22, glucose; ■, CM22, succinate. The approximate numbers of viable cells per milliliter after 30 h at 42°C were: KH434, glucose, 10^9; KH434, succinate, 5.8×10^8; CM22, glucose, 10^9; CM22, succinate, 4.0×10^6.

cy of 10^{-8}. The isolation of a true revertant indicates that the pleiotropic phenotype was induced by a single mutation.

Linkage of the *ccr-2* allele to *ilv* genes. It is probable that strain CM22 carries a mutation in a protein subunit of H^+-ATPase since the strain is resistant to the inhibitors of H^+-ATPase (tributyltin and sodium azide) and since the strain exhibits the Unc phenotype at 42°C. It is known that *unc* genes coding for protein subunits of H^+-ATPase are mapped at 83.5 min on the chromosome of *E. coli* and that they could be linked to *ilv* genes by P1 transduction (7). Therefore, experiments in P1 transduction were carried out to learn where the mutant allele *ccr-2* is mapped on the chromosome.

Strain IM20, which was an *ilv* strain derived from KH434, was used as a recipient strain. Strain IM20 was infected with P1 *vir* phage grown in strain CM22. A total of 50 ilv^+ transductants were scored. Of these transductants, 29 coinherited the resistances to the four inhibitors and the lack of growth on succinate at 42°C. This observation indicates that the mutant allele *ccr-2* is linked to the *ilv* marker at a frequency of 58%. Then, when strain IM5 carrying a mutation in the *ilvA* gene was used as the recipient strain, the mutant allele *ccr-2* was linked to the *ilvA* gene at a frequency of 50% (20 out of 40 tested). From the linkages to the *ilv* genes, it is reasonable that the mutant allele *ccr-2* lies in the *unc* genes coding for protein subunits of H^+-ATPase.

TABLE 2. Reversion test of strain CM22

No. of revertants	Effect of inhibitor[a]			
	CCCP	PCP	Tributyltin	Sodium azide
1	S	S	S	S
13	R	R	R	R
1	R	R	R	S
1	R	R	S	R
2	R	R	S	S
1	S	R	S	R
1	S	S	R	R

[a] S means that the growth of the given revertant strain was inhibited on a plate containing 25 μM CCCP, 75 μM PCP, 10^{-3}% tributyltin, or 50 μM sodium azide—concentrations which inhibited the growth of wild-type strain KH434. R means that the revertant strain was not inhibited under those conditions. A total of 20 Unc^+ revertants picked up on succinate plates at 42°C were tested.

DISCUSSION

We isolated spontaneous mutant strains which were resistant to an uncoupler, CCCP. Mutant strain CM22 (*ccr-2*) was examined more extensively, and the following observations were made. (i) The mutant strain CM22 (*ccr-2*) is resistant not only to the uncouplers CCCP and PCP but also to inhibitors of H^+-ATPase, namely, tributyltin and sodium azide. (ii) The mutant carries a mutation in a membrane component since the H^+ influx was not observed with the administration of an uncoupler, CCCP or PCP.

(iii) The respiratory rate of strain CM22 is twice that of the wild-type strain KH434. (iv) Strain CM22 exhibits the Unc phenotype at 42°C. (v) The pleiotropic phenotype of strain CM22 results from a single mutation because a true revertant was isolated at a frequency of 10^{-8}. (vi) The mutant allele *ccr-2* is mapped in a region close to the *unc* genes. These observations indicate that strain CM22 carries a mutation in a protein subunit of H^+-ATPase and that the uncouplers CCCP and PCP interact with H^+-ATPase.

It is possible that a mutation in H^+-ATPase affects the accessibility of inhibitors to membrane sites. However, this is not likely since partial revertants, which restore the sensitivities to some of the four inhibitors, are isolated by a single-step reversion (Table 2). It is likely that the substitution of a different amino acid at the mutated site or of an amino acid at a different site in protein subunit(s) of H^+-ATPase induces an alteration on the specific interactions between H^+-ATPase and the inhibitors.

Strain CM22 cannot grow on malate or fumarate, as well as succinate, at 42°C (data not shown). This phenotype is reasonable for a mutant strain carrying a mutation in a protein subunit of H^+-ATPase. However, more analysis is needed to determine on which side of the *ilv* genes the mutant allele *ccr-2* lies.

The conclusion drawn here is compatible with the observations made with tributyltin-resistant mutant strains of *E. coli* (10), PCP-resistant mutant strains of *E. coli* (manuscript in preparation), and a CCCP-resistant mutant strain of *Bacillus megaterium* (5, 6). Extensive study of the CCCP-resistant mutant strain CM22 will provide more information on ATP formation by oxidative phosphorylation.

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