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# Bacterial Resistance to Uncouplers

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Uncoupler resistance presents a potential challenge to the conventional chemiosmotic coupling mechanism. In *E. coli*, an adaptive response to uncouplers was found in cell growing under conditions requiring oxidative phosphorylation. It is suggested that uncoupler-resistant mutants described in the earlier literature might represent a constitutive state of expression of this "low energy shock" adaptive response. In the environment, bacteria are confronted by nonclassical uncoupling factors such as organic solvents, heat, and extremes of pH. It is suggested that the low energy shock response will aid the cell in coping with the effects of natural uncoupling factors. The genetic analysis of uncoupler resistance has only recently began, and is yielding interesting and largely unexpected results. In *Bacillus subtilis*, a mutation in fatty acid desaturase causes an increased content of saturated fatty acids in the membrane and increased uncoupler resistance. The protonophoric efficiency of uncouplers remains unchanged in the mutants, inviting nonorthodox interpretations of the mechanism of resistance. In *E. coli*, two loci conferring resistance to CCCP and TSA were cloned and were found to encode multidrug resistance pumps. Resistance to one of the uncouplers, TTFB, remained unchanged in strains mutated for the MDRs, suggesting a resistance mechanism different from uncoupler extrusion.

**KEY WORDS:** Uncouplers; oxidative phosphorylation; ATP synthesis, $Na^+$ energetics, *E. coli*; *B. subtilis*; low-energy shock; stress response.

Uncouplers short-circuit the battery that is the cytoplasmic membrane of a bacterial cell, and it is not at all obvious that there should be a mechanism for uncoupler resistance. Yet mutants resistant to chemically unrelated uncouplers have been known for quite some time. The present review is a progress report, rather than a definitive description of the phenomenon, and will concentrate on recent developments. The comprehensive review of Krulwich and colleagues is an excellent guide to the often confusing literature on the various uncoupler-resistant mutants described over the years (Krulwich *et al.*, 1990).

## THE MECHANISM OF UNCOUPLING: CLASSICAL UNCOUPLERS

Our views on the mechanism of uncoupling have not changed since Mitchell's (1966) original model that envisioned an uncoupler as a proton shuttle (Fig. 1). Any weak acid that can permeate the membrane in the anionic form thus becomes an uncoupler. It is easy to see that a good uncoupler will be hydrophobic, with a delocalized negative charge to facilitate the permeation of the anionic species into the bilayer, and with a near neutral p$K$ to provide sufficient amounts of both the protonated and the anionic forms. It has been calculated that the shuttle mechanism will allow for about 1000 $H^+$ per uncoupler molecule to be carried across the membrane per second, the limit being set by the diffusion rate (Terada and van Dam, 1975; Terada, 1990). The best of the known uncouplers, SF 6847, is close to this limit, with 800 cycles/second. The properties of SF 6847 indeed closely fit the chemical portrait of an ideal uncoupler (Fig. 2). Perhaps not surprisingly, uncouplers tranport protons across the membrane significantly faster than the proton pumps of the

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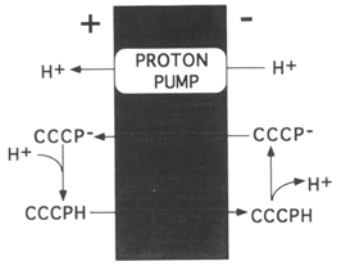


**Fig. 1.** A model for the mechanism of a classical uncoupler.

respiratory chain or the ATPsynthase, with SF 6847 uncoupling at a ratio of 1 molecule per 20 respiratory chains.

Of the very large number of known uncouplers, only FCCP, CCCP, TSA, PCP, and DNP are widely used in research. The reason for using these uncouplers is simply that they are readily available commercially (note that SF 6847 is now available from Wako Pure Chemical Industries, Ltd.). Using a large set of chemically unrelated uncouplers is very useful: indeed, if they all behave in the same manner, this is a strong indication of the effect being directed at the level of pmf.

## UNCOUPLER RESISTANCE IN *E. coli*

### Resistant Mutants

Uncoupler-resistant mutants of *E. coli* were isolated in several laboratories by plating cells on media containing fairly high levels of uncouplers: up to 250 $\mu$M CCCP (Sedgwick *et al.*, 1984). Individual mutants had varying spectra of resistance to different uncouplers (for a review, see Krulwich *et al.*, 1990). Physiological changes accompanying uncoupler resistance appear to be pleiotropic. For example, the following set of traits has been reported for a particular mutant resistant to CCCP and TSA: (1) protonophoric activity of uncouplers in mutant cells is comparable to the wild type; (2) proline transport is resistant to uncouplers; (3) mutant cells become apparently impermeable to a fluorescent probe *N*-phenyl-1-naphthylamine (Sedgwick and Bragg, 1992).

So far, it looks like the resistant strains of *E. coli* described in the literature are likely to possess more than one mutation, affecting permeability to uncoupler and perhaps changing the bioenergetic properties of the cell. The apparent low frequency of

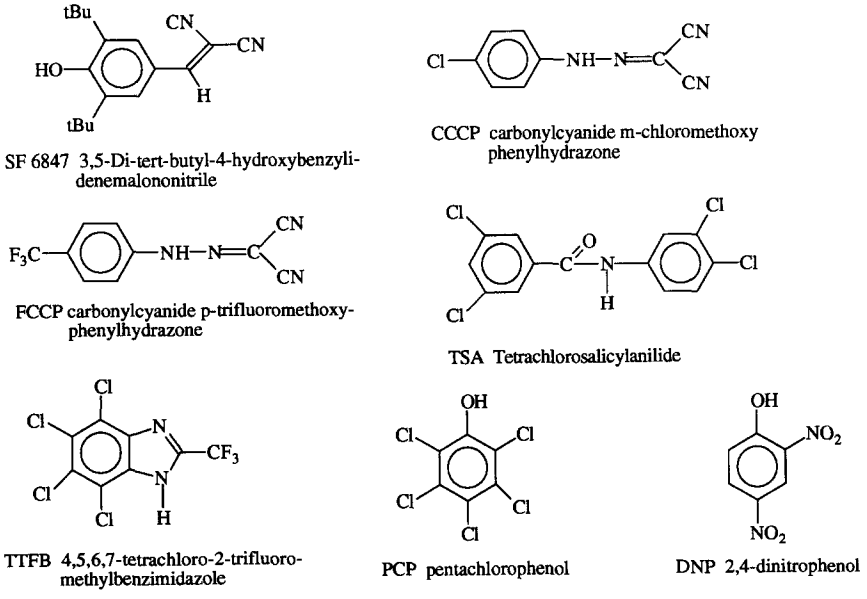


**Fig. 2.** Examples of commonly used uncouplers. The protonophoric activity decreases in the order of SF 8647 > FCCP > TTFB > CCCP > TSA > PCP > DNP, as determined in experiments with artificial membranes and mitochondria. The same sequence should be expected for bacterial membrane vesicles, but the action on whole cells will strongly depend on the outer membrane permeability which is lower for larger molecules. According to our observations, CCCP is more active than SF 6847 in wild type *E. coli* cells.

**Table I.** Resistance Properties of a Collection of *E. coli* CCCP-Resistant Mutants

| Strains | Compounds tested | | | |
|---|---|---|---|---|
| | CCCP | TSA | Ampicillin | Nalidixic acid |
| K12 wild type | – | – | – | – |
| KLE 701 | + | + | + | + |
| KLE 702-710 | + | + | – | – |

a mutational event, and even the reversion to the "wild type," is not necessarily good evidence for a single locus change. Indeed, mutants can form microcolonies in the presence of a deleterious factor, from which additional mutants can spring up, giving the impression of single-mutation events. The "reversion" to wild phenotype can in fact be caused by a suppressor mutation in an unrelated locus. It is also possible that the complex phenotype is due to a mutation in a regulatory locus that in turn affects a whole set of genes. A pertinent example is a mutation activating the *mar* locus which in turn controls a large number of genes conferring resistance to "multiple antibiotics" in *E. coli* (Cohen *et al.*, 1993).

We obtained a collection of uncoupler-resistant mutants by selecting a minimal medium with succinate and using a moderate concentration of CCCP that is only 2-fold higher than the minimal inhibitory concentration for the wild type. Ten colonies were tested for resistance to a number of compounds to get a general idea of the possible nature of resistance (Table I). The compounds chosen for testing would allow a general discrimination between a decrease in permeability versus an uncoupler-specific mechanism of resistance. Of the ten mutants tested, one was resistant to ampicillin. The target for ampicillin is in the peptidoglycan layer, so that resistance to this antibiotic indicates that the mechanism is probably a decrease in the permeability of the outer membrane. The same mutant was resistant to nalidixic acid, whose target is the cytoplasmic enzyme DNA-gyrase. All other mutants were resistant to CCCP and a chemically unrelated uncoupler TSA, but were sensitive to ampicillin and nalidixic acid. The latter mutants are unlikely to be affected in the general permeability properties of the cell envelope.

In order to get a better understanding of the mechanism of uncoupler resistance, we attempted to clone a CCCP-resistant mutation from *E. coli*. The locus cloned into the wild type conferred resistance to a lower level of uncoupler than the mutation. This suggested that a different locus, and not the mutation, had been cloned. Our attempts to clone an uncoupler-resistant mutation have so far failed, indicating that this might indeed be a multilocus trait.

### Cloning Uncoupler Resistance Genes from *E. coli*

The rationale for cloning uncoupler resistance was fairly straightforward: a chromosomal library in a high-copy vector pUC18 was prepared from DNA of a CCCP-resistant mutant, and wild type cells transformed with this library were selected on plates with CCCP. This selection yielded two clones that appeared to carry an overlapping insert. Deletion analysis established a minimal functional fragment conferring uncoupler resistance, and sequencing of the locus suggested two open reading frames, *emrAB* (Lomovskaya and Lewis, 1992). The sequence was used to amplify this locus by PCR from the wild type, and sequencing showed that it was identical to the genes cloned from the mutant. Thus, some wild type locus was clonsed from the CCCP-resistant mutant, and the elevated resistance of the recombinant cells was apparently due to the overexpression of the proteins from the multicopy plasmid.

A homology search indicated interesting similarities between the new genes and known proteins in the database. EmrB encodes a 51-kD peptide that has a typical structure of an integral membrane protein, with 14 putative $\alpha$-helix domains. It shares significant homology with export pumps for specific antibiotics from producing *Streptomyces* species, as well as homology to multidry resistance pumps (MDRs) that were found in gram-positive species: QacA of *Staphylococcus aureus* (Rouch *et al.*, 1990) and Bmr of *B. subtilis* (Neyfakh *et al.*, 1991). The best studied efflux pump from this family is TetA that has 12 transmembrane domains and acts as a [tetracycline $\cdot$ $Mg^{2+}$]/$H^+$-antiporter (Levy, 1992). The export pumps of this family [the major facilitator family, named after its simplest member, the glucose facilitator of eukaryotic cell (Marger and Saier, 1993)] share a "drug extrusion" consensus **G***PIL***GP***VL***GG**6 (Tennent *et al.*, 1989) that is also present in EmrB.

Homology with pumps that extrude fairly hydrophobic antibiotics clearly pointed to the possibility of EmrB acting as an extrusion pump for uncouplers. However, the relation of EmrB to translocases seemed puzzling, since it would make little sense for a cell to spend energy trying to extrude the uncoupler

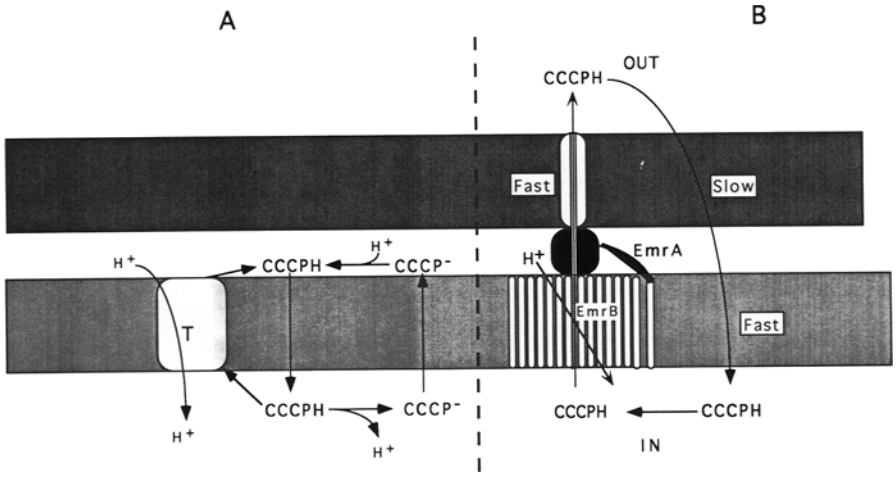


**Fig. 3.** Models of an uncoupler extrusion pump. A translocase (T) extruding uncouplers across the cytoplasmic membrane will only increase the efficiency of uncoupling. The model proposes that the EmrAB translocase extrudes uncoupler across both membranes, taking the advantage of the low permeability of the outer membrane to large hydrophobic molecules.

(Fig. 3A). The apparent key to this puzzle comes from the second peptide EmrA, which shows homologies with auxiliary proteins of peptide exporters such as the hemolysin pump of *E. coli*. There is good evidence that the function of HlyD is to join the inner and outer membrane and organize the transport of hemolysin across the cell envelope in a single step (Schulein *et al.*, 1992). By analogy, EmrA could organize such a single-step extrusion route for uncouplers and other hydrophobic substances (Lomovskaya and Lewis, 1992). The outer membrane is a fairly good barrier for uncouplers and other bulky hydrophobic substances (Nikaido, 1994). A rapid export of uncouplers across the cell envelope and a slow leaking back across the outer membrane would then make pumping uncouplers a feasible mechanism for resistance. Our results show that overexpression of EmrAB slows penetration of TSA into the cell.

The function of EmrAB is hardly to export CCCP, but rather, hydrophobic, potentially toxic substances in general. This type of mechanism is restricted to gram-negative species that have an outer membrane. It is noteworthy that the MDRs of gram-positive species only protect cells from fairly hydrophilic substances such as $TPP^+$ that do not penetrate that easily into the cytoplasmic membrane. (See Lewis, 1994 for a review of bacterial MDRs.)

Having failed to clone uncoupler resistance mutations, it seemed prudent to generate a collection of uncoupler-sensitive strains and use these for cloning. The straightforward selection for a negative trait, ampicillin enrichment in the presence of uncoupler, only produced the wild type, apparently due to adaptation to uncoupling. In a modification of this approach, an uncoupler-resistant mutant was used as starting material for ampicillin enrichment in the presence of uncoupler. The resistant strain was also mutagenized with *mini*-Tn10, *lacZ*, *kan*, to simplify subsequent cloning. This approach yielded a good enrichment for uncoupler-sensitive mutants. The transposon carries kanamycin resistance, and this trait was used to $P_1$-transduce the insertional mutations into the wild type. A collection of 20 independent uncoupler-sensitive strains was obtained.

The difference in uncoupler sensitivity between the mutants and the wild type was not large, about 1.5-fold. The transposon creates translational *lacZ* fusions with downstream genes, allowing for a convenient measurement of promoter activity. The $\beta$-galactosidase activity in one of the strains, KLE309, was induced by uncouplers. The insertion was cloned, and a partial sequence of the coding region of the fused gene showed that it is identical to an open reading frame identified from the *E. coli* genome sequencing project (Burland *et al.*, 1994). This gene, *emrD*, is apparently another MDR: it is homologous to EmrB and has the drug extrusion consensus. Unlike *emrAB*, this is a single-gene locus, and placing *emrD* on a multicopy plasmid did not increase the resistance level of the wild type. It is likely that there might be an EmrA-like protein acting in conjunction

with EmrD that is coded by a gene from a different locus, so that overexpressing EmrD alone will have no effect on resistance.

### Low-energy Shock

Evidently, studies of uncoupler resistance have not been initiated with the aim of uncovering some peculiar change in the cell envelope permeability, or even to find MDR's, for that matter. Rather, uncoupler resistance has been used as a tool to probe the paradigm of classical chemiosmosis. The alternative to the classical view is the "localized" model according to which protons travel directly from a generator such as a respiratory chain component into the ATP-synthase (Westerhoff *et al.*, 1988; Rottenberg, 1990). Realistically, it does not seem that the localized model is viable as an alternative mechanism; what remains to be seen is whether the localized mode exists under some special conditions as an additional mechanism. One interesting possibility would be for a cell to express a localized mechanism under conditions where the permeability of the membrane is compromised. We sought to find whether *E. coli* might have an adaptive response to uncouplers, as they do to heat, oxidants, mutagens, and some other factors. Cells were grown in minimal medium with succinate, where oxidative phosphorylation is the only mechanism of ATP synthesis (Rosen, 1986). Addition of large amounts of uncoupler completely inhibited growth, but moderate amounts of uncoupler that also completely arrested growth allowed for an eventual recovery to fairly high growth rates (Naroditskaya *et al.*, 1993; Fig. 4). A similar result was observed with a number of different uncouplers. Recovery of growth from uncouplers (Kinoshita *et al.*, 1984; Avetisyan *et al.*, 1989; Nakano and Onoda, 1989) or an eventual acceleration of growth in the presence of uncoupler (Gage and Neidhardt, 1993) have also been observed in a medium with fermentable substrates, but this is less relevant to the present discussion. The presence of a "low energy shock" adaptive response suggests that if localized coupling exists, it should be induced as part of the adaptive mechanism. The existence of an adaptive response also increases the validity of studying uncoupler-resistant mutants, which can now be envisioned as constitutively expressing low energy shock response, rather than being some monsters created in a test tube.

Could the adaptive response be satisfactorily explained by an induction of MDRs that extrude

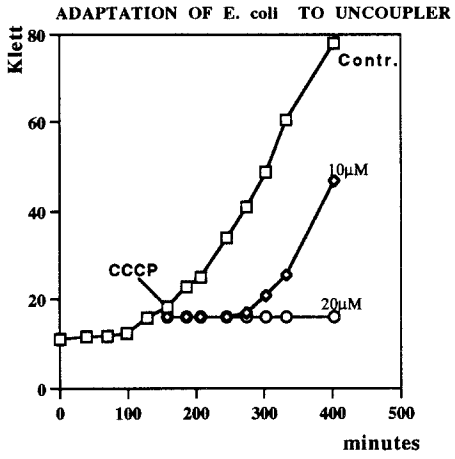


**Fig. 4.** Cells were grown at 37°C with aeration in a minimal medium with succinate. Uncoupler was added at the time indicated.

uncoupler from the cell? Cells mutated in either *emrB* or *emrD* were more sensitive to CCCP and TSA, but an adaptive response was still observed, indicating the presence of an additional resistance mechanism. Wild type cells adapted to all uncouplers that were tested, but recovery from TTFB was sluggish. Moreover, mutations in *emrB* or *emrD* did not affect the sensitivity to TTFB, suggesting that resistance to this uncoupler might be due to a mechanism different from extrusion. A mutant specifically affected in TTFB resistance was identified from the uncoupler-sensitive collection. This strain, KLE610, was also sensitive to all other uncouplers we tested.

The transposon insertion from KLE610 was cloned and sequenced. The insert appeared to be in the *dsd* locus (McFall, 1987) coding for the D-serine deaminase. The insertion was upstream the coding region, adjacent to a gene that had been implicated in the regulation of *dsdA*. The insertion appears to be in an open reading frame that could code for a membrane protein with significant homology to the gluconate translocase from *B. subtilis* (Reizer *et al.*, 1991). This is a puzzling finding indeed, since a locus involved in D-serine deamination is hardly an obvious candidate for participating in uncoupler resistance. However, there is an interesting connection between the product of D-serine deamination, $NH_3$, and uncoupling. Neijssel and co-authors (1990) have described an "ammonia uncoupling cycle" in *E. coli* (Fig. 5) which is observed when cells are transferred from a low- to a high-ammonia medium. At low ammonia, an uptake translocase for $NH_4^+$ is expressed, which will lead to a typical uncoupling

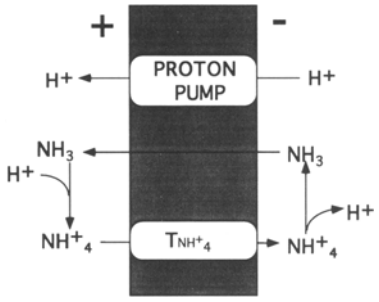


**Fig. 5.** The ammonium uncoupling cycle.

cycle in the presence of high $NH_3$ concentrations. The D-serine deaminase is a massively produced enzyme and will generate considerable amounts of $NH_3$ in the cytoplasm that can leak out of the cell and trigger an uncoupling cycle. Thus, having a gene protecting the cell from uncoupling within a locus that makes an uncoupler might not be accidental. Our current efforts are directed at clarifying the role of the *dsd* locus in uncoupler resistance, as well as cloning other genes that might be involved in low energy shock/uncoupler resistance.

## GRAM-POSITIVE BACTERIA

An obvious advantage in studying uncoupler resistance in gram-positive species is the presence of only one membrane, providing a reasonable expectation for resistance to affect the mechanism of energy transduction, rather than uncoupler permeation. The protonophoric activity of uncouplers in resistant mutants from *Bacillus megaterium* and *B. subtilis* was comparable to that of wild type cells (Decker and Lang, 1978; Guffanti *et al.*, 1981). This important finding shows that resistance has to do with the results of uncoupling, rather than with the uncouplers *per se*. The resistant *B. subtilis* strain has a mutation in a gene coding for the fatty acid desaturase, leading to a higher level of saturated fatty acids, and a more solid-state membrane (Dunkley *et al.*, 1991). Similar changes in fatty acid composition were observed in the *B. megaterium* mutant (Clejan *et al.*, 1988). It has been suggested that a more solid membrane could be more conducive to complex formation between redox chain components and the ATPsynthase, leading to localized coupling (Dunkley *et al.*, 1991). An important unanswered question regarding these mutants is whether their phenotype is indeed representative of some *in vivo* situation. It seem that *B. subtilis* has an adaptive response to uncouplers (see Fig. 5A of Alper *et al.*, 1994), and it would be interesting to learn if it leads to an increase in the content of the membrane fatty acids. According to our findings, the fatty acid composition of *E. coli* adapted to CCCP remains unchanged; of course, *B. subtilis* might be different in this regard.

A fair amount of interesting functional data has been reported on *B. megaterium* and *B. subtilis* mutants from two laboratories, with a general agreement of results (see Krulwich *et al.*, 1990). The basic observation is that ATP is made in mutants in the presence of uncouplers at pmf levels when ATP synthesis is not observed in the wild type (Decker and Lang, 1978; Guffanti *et a.*, 1981, 1983, 1987).

Interestingly, results reported for the uncoupler-resistant *Bacillus* mutants are very similar to the findings of Krulwich and co-authors with alkaliphilic *Bacilli* living at pH 9–10. The cytoplasmic pH is kept at around 8 in these cells, creating an inverse pH gradient. The total measured pmf is around 50 mV, yet ATP synthesis is observed at a high rate. The same cells grown at pH 7 do not have ATP synthesis at 50 mV pmf (Guffanti and Krulwich, 1992; Krulwich *et al.*, 1990). Taken together, the results from uncoupler-resistant *Bacilli* and alkaliphilic bacteria present a challenge to the classical chemiosmotic theory. At the same time, measurements of pmf are by necessity indirect and thus prone to artifacts (like $TPP^+$ extrusion by MDRs; see Neyfakh *et al.*, 1991). It is not likely that the local coupling controversy will be settled on the merits of measurements of the energetics of ATP synthesis alone. The utility of these measurements is mainly in identifying a convenient system in which to search for a possible functional complex of ATPsynthease and a redox chain component performing localized coupling.

## NATURAL UNCOUPLERS

What are the natural conditions of uncoupling? Numerous deleterious factors will affect the membrane permeability and lead to uncoupling. A list of such likely factors will include futile cycles (such as the $NH_3$ cycle), oxidants, detergents (such as bile acids in the gut), heavy metals, organic solvents, fatty acids (Wojtczak and Schonfeld, 1993; but see Skulachev, 1991); pore-forming antimicrobial peptides such as animal defensins (Lehrer *et al.*, 1991) and plant thionins (Bohlmann and Apel, 1991), possibly

extremes of pH, and heat. What is conspicuously absent from this list is an antimicrobial substance that is a classical uncoupler. Most of the factors on the list indeed lack a specific protonophoric activity, but an increased proton permeability is the first and predominant consequence of nonspecific membrane damage.

### Organic Solvent Resistance

Organic solvents partition into the membrane and cause a proton leak leading to uncoupling. Bacteria do confront organic solvents in nature, and not necessarily as a result of an oil spill, since soil microorganisms harbor plasmids carrying genes for catabolism of hydrocarbons. For example, the Tol plasmid allows *Pseudomonas putida* to use toluene, or crude oil as the sole source of carbon. Bacteria are highly sensitive to organic solvents, and toluene is administered to *P. putida* cultures in the form of a vapor. At the same time, mutants of both *Pseudomonas* and *E. coli* highly resistant to organic solvents have been isolated (Inoue and Horikoshi, 1989; Aono *et al.*, 1991). Colonies of these mutants grow on plates covered with organic solvents. One would expect that these mutants are similar to mutants resistant to classical uncouplers. It seemed useful to learn if a mutant resistant to CCCP will also be resistant to a substance whose uncoupling effect is based on a very different mechanism. We tested spontaneous mutants resistant to CCCP for resistance to tetralin and cyclohexane, and found that all were sensitive. Similarly, mutants resistant to tetralin and cyclohexane were sensitive to CCCP. The genes conferring resistance to tetralin and cyclohexane were cloned and sequenced (Ferrante *et al.*, in preparation). The main mechanism of resistance to these organic solvents appears to be unrelated to uncoupling, even though tetralin also acts as an uncoupler (Sikkema *et al.*, 1992; 1994). An additional mutation was required to impart resistance to a more polar solvent, xylene, to a tetralin-resistant mutant. This additional mutation might in fact be related to uncoupler-resistance mutations.

## LOW pmf and $Na^+$ ENERGETICS

Apart from a possible localized coupling, a good way for bacteria to respond to conditions of increased membrane conductivity would be to switch to $Na^+$-based energetics (Skulachev, 1989). The nonspecific permeability of a lipid bilayer to $Na^+$ is about 6 orders of magnitude lower than it is to protons. This means that when the conductivity of the membrane increases, for example with increasing of temperature, proton leakage becomes a real problem, while $Na^+$ flux remains negligible. The thermophilic bacterium *Clostridium fervidus* lives at 70°C and couples all its active transports to $Na^+$ (Speelmans *et al.*, 1993). The $Na^+$ gradient is generated by a primary V-type $Na^+$ ATPase. The bacterium is an anaerobe and gets its ATP from fermentation. Thermophilic aerobes face a more severe problem: ATP synthesis, unlike active transport, requires a high level of pmf. However, aerobic thermophiles use a $H^+$-ATPsynthase to make ATP. Marine bacteria, such as *Vibrio alginolyticus*, have a primary $Na^+$-pump as one of the elements of the respiratory chain that also pumps out protons. The pump is induced at alkaline pH when the pmf is low and the $Na^+$-motive force is used for active transport and motility (Tokuda, 1992). However, the most critical energy consumer, ATPsynthase, requires a pmf. Finally, in the most extreme case of living at low pmf, alkaliphilic *Bacilli* have an electrogenic $Na^+/H^+$ antiporter that makes a $Na^+$ gradient that fuels all active transport (Krulwich and Guffanti, 1989), yet again oxidative phosphorylation relies on a $H^+$-based respiratory chain and ATPsynthase. The clearly documented case of $Na^+$-based membrane energetics is that of the marine anaerobe *Propionigenum modestum* that employs an unusual primary pump: a decarboxylase coupled with $Na^+$ extrusion, and an $F_1F_0$-type ATPsynthase that has a preference for $Na^+$ (Dimroth, 1992). A $Na^+$-based oxidative phosphorylation has been reported for *E. coli* growing in alkali medium (Dibrov, 1991; Avetisyan *et al.*, 1993), but a $Na^+$-stimulated ATPase is yet to be identified.

The above examination shows that $Na^+$-based energetics is not the universal answer to the problem of making ATP under conditions of low pmf. It is indeed surprising that *Vibrio alginolyticus*, when confronted with a mild challenge of living at pH 8.5, will switch most of its energetics, but *not* ATP synthesis, to a $Na^+$-cycle. The fact that bacteria switch to a $Na^+$-cycle at low pmf to solve some of their problems, yet do not have a $Na^+$-ATPsynthase, suggests a different solution for the most important process in energy transformation (but see Skulachev, 1994). Whether this solution requires a modification of the mechanism of

oxidative phosphorylation as we know it remains an intriguing open question.

## ACKNOWLEDGMENTS

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