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Differential permeability for lipophilic compounds in uncoupler-resistant cells of *Escherichia coli*

Edward G. Sedgwick and Philip D. Bragg

Department of Biochemistry, University of British Columbia, Vancouver (Canada)

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The *acrA* strain AS-1 of *Escherichia coli* is more sensitive than its parent W3110 to growth inhibition by Methylene blue, sodium dodecyl sulfate and novobiocin. UR-3 is an uncoupler-resistant strain isolated from AS-1 which is resistant to growth inhibition by carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 3,3',4',5-tetrachlorsalicylanilide (TCS) and tributyltin chloride, while remaining sensitive to the first group of compounds. A revertant of AS-1 acquired resistance to Methylene blue and sodium dodecyl sulfate but remained sensitive to uncouplers. In contrast to AS-1, proline uptake in UR-3 was resistant to uncouplers. Strain UR-3 grown in the presence of uncoupler incorporated elongation factor Tu to high levels in the outer membrane of the cell. A role for the outer membrane in the acquisition of uncoupler-resistance by UR-3 is suggested by the behaviour of the mutant to the fluorescence probe *N*-phenyl-1-naphthylamine. The fluorescence intensity of this probe was quenched by membrane energization in the wild-type strain W3110 but not in AS-1. UR-3 behaved like W3110, suggesting that an outer membrane barrier to neutral lipophilic compounds like *N*-phenyl-1-naphthylamine (NPN) and uncouplers had been restored in UR-3. By contrast, AS-1 and UR-3 both allowed energized uptake of the fluorescent lipophilic cation 2-(dimethylaminostyryl)-1-ethylpyridinium (DMP$^+$). It is concluded that lipophilic materials must permeate the outer membrane of *E. coli* by at least two different routes. However, uncoupler-resistance in UR-3 appears to be more complex than the provision of an outer membrane barrier to uncouplers. Thus, uncouplers readily discharged a pH gradient established in both AS-1 and UR-3 by addition of HCl to cell suspensions.

Introduction

According to the chemiosmotic hypothesis of Mitchell, membrane energization for ATP synthesis or solute transport involves the translocation of protons across the energy-transducing membrane [1]. ATP synthesis requires the use of the electrochemical potential gradient of protons by the ATP-synthase complex. Uncouplers dissipate the energized membrane state and so prevent ATP formation and inhibit the transport of those solutes which are energized by it. It has been proposed that uncouplers dissolve in the membrane lipid and act as mobile proton conductors to equilibrate the proton gradient. However, there is some evidence from the use of photoaffinity labelling uncouplers that uncouplers react with membrane proteins [2–6].

One possible approach to determining the mechanism of action of uncouplers, and so increasing our knowledge of the process of energy transduction, is to isolate bacterial mutants which are resistant to uncouplers.

This type of approach has been tried with *Bacillus* species and with *Escherichia coli* [7]. In previous studies [8–10] we have described the properties of a mutant, UV6, resistant to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Proline uptake and efflux, and the transmembrane pH gradient, were al-

Abbreviations: Bu_3SnCl, tri-*n*-butyltin chloride; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMP$^+$, 2-(dimethylaminostyryl)-1-ethylpyridinium; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; NPN, *N*-phenyl-1-naphthylamine; TCS, 3,3',4',5-tetrachlorosalicylanilide.

Correspondence: P.D. Bragg, Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, B.C., Canada, V6T 1Z3.

fected by CCCP in the parent strain but not in the mutant. However, equilibration by uncoupler of an artificially established proton gradient across the cell envelope was equally effective in both the parent and the mutant strains [8]. Growth of the mutant in the presence of CCCP caused the incorporation of the normally cytoplasmic protein elongation factor Tu into the outer membrane to become one of the three or four most abundant proteins in this membrane [10]. This suggested that CCCP had access to the cytosol to cause an alteration in the normal protein biosynthetic mechanisms. Furthermore it was observed that the mutant grown in the presence of CCCP incorporated significant amounts of the uncoupler into its inner and outer membranes [9].

The outer membrane is known to act as a barrier to the permeation of lipophilic agents into cells of *E. coli* [11,12]. However, although our previous results suggested that uncoupler-resistance was not due to exclusion of the uncoupler at the outer membrane, it was decided to isolate uncoupler-resistant mutants from a strain, AS-1, of *E. coli* which showed significant permeability to lipophilic substances [13,14]. It was hoped in this way to avoid complications due to the outer membrane barrier. An analogous approach has been followed by Beechey and his colleagues [15–17].

This paper describes the properties of uncoupler-resistant strain UR-3 isolated from AS-1. Two pathways for the permeation of nonpolar substances through the cell envelope of *E. coli* were demonstrated by our experiments. The acquisition of uncoupler-resistance affected one of these pathways only. However, the generation of an outer membrane barrier to uncouplers does not appear to be the sole determinant of uncoupler-resistance in *E. coli*.

Materials and Methods

Bacterial strains

E. coli W3110, a wild-type K-12 strain, and AS-1, an *acrA* mutant derived from W3110 [13,14], were obtained from Dr. Yasuo Imae, Nagoya University, Japan. The CCCP-resistant strain UR-3 was isolated as a spontaneous mutant of AS-1 by streaking the parent organism on nutrient agar plates containing 150 μM CCCP.

Growth of cells

W3110, AS-1 and UR-3 were grown to stationary phase at 37°C with aeration from a 1% (v/v) inoculum on Penassay Broth (Difco) or on a minimal medium [18] using 0.4% (w/v) glucose as growth substrate. The cells were harvested by centrifugation and washed in the appropriate buffer as indicated in the legends to the table and figures. Cells were starved by incubation in buffer at 4°C for 24–48 h.

Determination of the sensitivity of growth to uncouplers and lipophilic compounds

The sensitivity of growth of W3110, AS-1 and UR-3 to sodium dodecyl sulfate, novobiocin and deoxycholate was determined by the method of Ahmed and Booth [19] using Penassay Broth agar medium. The sensitivity of growth to Methylene blue, CCCP, tri-n-butylin (Bu_3SnCl), and 3,3',4',5-tetrachlorosalicylanilide (TCS) was determined by plating cultures on the Penassay Broth agar medium containing these compounds at the concentrations indicated in Table I.

Proton uptake by intact cells

The cells sedimented from a 80 ml culture grown on minimal medium with glucose were washed twice by resedimentation (10000 × *g*; 15 min) from 50 mM Hepes-KOH buffer, pH 6.8, and resuspended in 1 ml 2 mM Tris-HCl buffer, pH 6.8, containing 0.3 M KCl. Proton translocation in response to addition of HCl was measured with a combination pH electrode as described before [8]. The assay system contained 0.2 ml cell suspension in 2 ml 2 mM Tris-HCl buffer, pH 6.8, containing 0.3 M KCl. 10 μl 10 mM HCl was added followed by 8 μl 5 mM CCCP (in ethanol). The changes in pH were recorded.

Fluorescence assays with NPN and DMP^+

The fluorescence intensity of *N*-phenyl-1-naphthylamine (NPN) was measured at 22°C with a Turner model 420 spectrofluorometer as previously described [20]. The reaction mixture (2 ml) in a cuvet of 1 cm light-path contained 50 mM Hepes-KOH buffer, (pH 7.4) and 2 mg of cell protein. The fluorescence of 2-(dimethylaminostyryl)-1-ethylpyridinium (DMP^+) was measured at 22°C with a SLM-Aminco SPF500C spectrofluorometer. The reaction buffer was 50 mM potassium phosphate (pH 7.5). The energy sources and

TABLE I

Sensitivity of growth of E. coli wild-type and CCCP-resistant isolates

The experiment was carried out as described in Materials and Methods. Revertants of AS-1 and UR-3 were isolated on the basis of their resistance to Methylene blue. R, resistance to the compound tested; S, sensitivity to the compound tested; VS, very sensitive to the compound tested; –, not tested.

Compound tested	*E. coli* strain				
	W3110	AS-1	AS-1 Revertant	UR-3	UR-3 Revertant
2% SDS	R	S	R	S	R
50 μg/ml Methylene blue	R	S	R	S	R
4 mg/disc deoxycholate	R	S	–	S	–
1 mg/disc novobiocin	S	VS	–	VS	–
200 μM CCCP	S	S	S	R	R
75 μM Bu_3SnCl	R	S	–	R	–
150 μM TCS	S	S	–	R	–

inhibitors were used at the concentrations indicated in the legends. NPN fluorescence was excited at 340 nm and emission measured at 420 nm. For DMP$^+$ fluorescence the excitation and emission wavelengths utilized were 467 and 557 nm, respectively.

Preparation of cell envelopes and electrophoresis of proteins

Cell envelopes were prepared as previously described from cells grown in the absence or presence of 75 μM CCCP [8]. Sodium dodecyl sulfate gel electrophoresis was performed using the discontinuous buffer system of Laemmli [21]. The gels were stained with Coomassie blue.

Other assays

Proline uptake and the concentration of cellular inorganic phosphate were determined as described previously [8]. Protein concentration and ATPase activity were measured as described in Refs. 22 and 23.

Results

AS-1 is an *acrA* mutant derived from strain W3110. It was isolated as a strain with increased outer membrane permeability to nonpolar substances such as Methylene blue, mitomycin C, acridine orange and triphenyltetrazolium chloride [13]. It was found subsequently to be permeable to other lipophilic substances such as triphenylmethylphosphonium and tetraphenylphosphonium cations [14], and to sodium dodecyl sulfate. The nature of the *acrA* defect has not been characterized satisfactorily [12]. Strain UR-3 was isolated from AS-1 as a mutant spontaneously resistant to the uncoupler CCCP. W3110 and AS-1 showed similar growth sensitivity to the uncoupler. 20 μM CCCP caused 50% inhibition of growth in both strains. UR-3 retained outer membrane permeability to novobiocin, sodium dodecyl sulfate, deoxycholate, and Methylene blue, but was resistant to uncouplers CCCP, TCS and Bu_3SnCl (Table I). However, UR-3 was slightly more resistant to the detergents than the parent strain AS-1. On the basis of plate assays, UR-3 was 1.3-fold more resistant than AS-1 to sodium dodecyl sulfate, whereas with deoxycholate AS-1, but not UR-3, growth was inhibited at 2 mg detergent per disc. Significant growth inhibition occurred in both strains at 4 mg per disc. W3110 was insensitive to deoxycholate. A revertant of UR-3 acquired resistance to Methylene blue and remained resistant to CCCP (Table I). However, a similar revertant of AS-1 retained its sensitivity to CCCP.

Properties of UR-3

We have previously isolated a CCCP-resistant mutant (UV6) of *E. coli* [8]. This strain showed resistance of proline uptake to the presence of CCCP. Proline uptake in UR-3 was resistant to inhibition by 3.1 μM CCCP. This concentration of uncoupler inhibited uptake by 52%, 63% and 0% in strains W3110, AS-1 and UR-3, respectively.

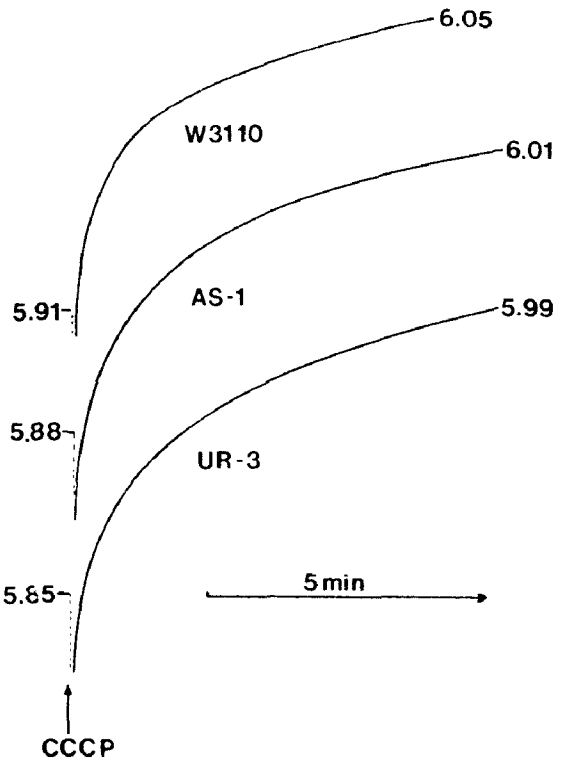

Fig. 1. Effect of CCCP on the uptake of protons by starved cells of W3110, AS-1 and UR-3. The experiment was carried out as described in Materials and Methods. The pH values of the reaction mixtures after addition of 10 μl 10 mM HCl and following addition of 12.5 μM CCCP are indicated on the traces.

The mechanism of uncoupling according to the Chemiosmotic hypothesis involves dissipation of the transmembrane pH gradient/membrane potential [1]. The ability of CCCP to discharge a pH gradient established by addition of HCl to a cell suspension was examined using W3110, AS-1 and UR-3. Addition of acid to the lightly-buffered suspensions of cells which had been starved overnight at 4°C lowered the external pH's of the suspensions to about 5.9 from the initial pH of 6.8. CCCP (12.5 μM) catalyzed rapid uptake of protons in all cases (Fig. 1).

Examination of cell envelopes of AS-1 and UR-3 by SDS-gel electrophoresis revealed no evident differences in protein composition between the strains. As in the case of uncoupler-resistant strain UV6 [9,10], growth of UR-3 in the presence of 7.5 μM CCCP resulted in uptake of CCCP (or a derivative of it), the envelopes becoming bright yellow in colour, and in the incorporation of a high level of elongation factor Tu into the cell envelope fraction (Fig. 2). The elongation factor was associated primarily with the outer membrane of the envelope (also see Ref. 10).

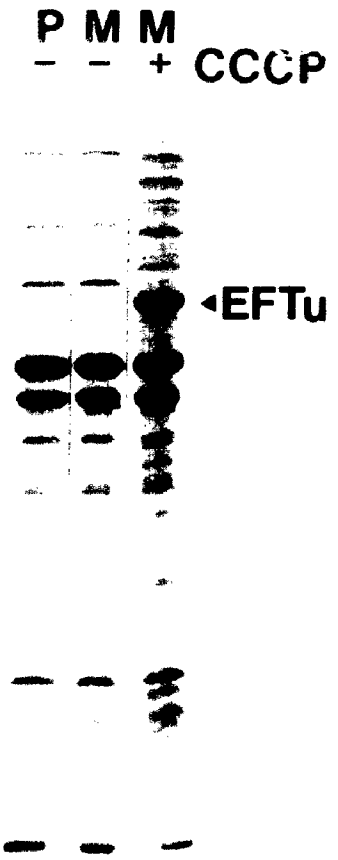

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cell envelopes of parent strain AS-1 (P) and CCCP-resistant mutant strain UR-3 grown in the absence (M–) and presence (M+) of 75 μM CCCP. EFTu, elongation factor Tu. Fifty μg protein was loaded in each lane.

ATPase activities of membranes of W3110, AS-1 and UR-3 were all in the range of 105–112 nmol ATP hydrolyzed/min/mg protein. ATPase activities were unaffected by the absence or presence of Na^+ suggesting that uncoupler-resistance in UR-3 was not due to the possession of a Na^+-ATPase [24]. In contrast with the high levels of cytosolic phosphate found previously in the uncoupler-resistant strain UV6 growing on succinate [8], the phosphate concentrations of UR-3, AS-1 and W3110 were all in the range of 27–46 mM.

Response of fluorescence probes in UR-3

We have used NPN previously to probe membrane energization in *E. coli* [20,25,26]. Two types of behaviour were identified. Quenching of NPN fluorescence was induced by substrate oxidation or ATP hydrolysis. The change in fluorescence intensity was attributed to an alteration of the binding of NPN to the outer membrane or to an alteration in its environment in the outer membrane on inner membrane energization. The probe also responded in the inner membrane to changes in the redox state of the respiratory chain, particularly of cytochromes *o* and *d* [26,27]. Fig. 3 shows changes in the fluorescence intensity of NPN in cell suspensions of W3110, AS-1 and UR-3 on addition of the respiratory chain substrate D-lactate. (Addition of glucose gave similar results.) Oxidation of substrate by W3110 resulted in fluorescence quenching as the inner membrane was energized. Quenching was reversed rapidly when the dissolved oxygen in the cuvet had been depleted and the redox state of the respiratory chain altered. The cells of AS-1 showed only the second phase, that is, the response of the probe to the redox state of the cytochromes. This was not due to impaired oxidation of D-lactate. By contrast, the initial quenching phase was restored in UR-3. DMP^+ cation was introduced by Bereiter-Hahn as a probe of the metabolic state of mitochondria. Changes in fluorescence intensity of this probe were attributed to changes in the environment of the probe and not to alterations in ion gradients or to pH changes [28]. By contrast, Mewes and Rafael [29] suggested that in mitochondria DMP^+ responds to membrane potential. However, Midgley and his coworkers [30,31] have shown that fluorescence intensity changes of DMP^+ in *E. coli* are associated with uptake and efflux of the dye. Fig. 4 shows the response of the fluorescence intensity of DMP^+ to alterations in the metabolic state of strains W3110, AS-1 and UR-3. Addition of glucose to a cell suspension of W3110 caused fluorescence quenching which partially reversed when the system became

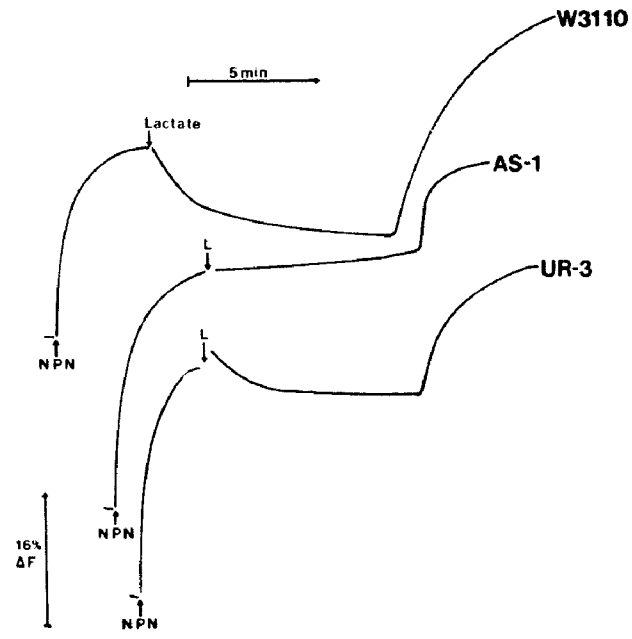

Fig. 3. Changes in the fluorescence intensity of NPN in the presence of intact cell suspensions of W3110, AS-1 and UR-3. The procedure is described in Materials and Methods. L, D-lactate added to a concentration of 10 mM. The time (8–10 min following lactate addition) at which the reaction solution became anaerobic is indicated by a rapid increase in fluorescence intensity.

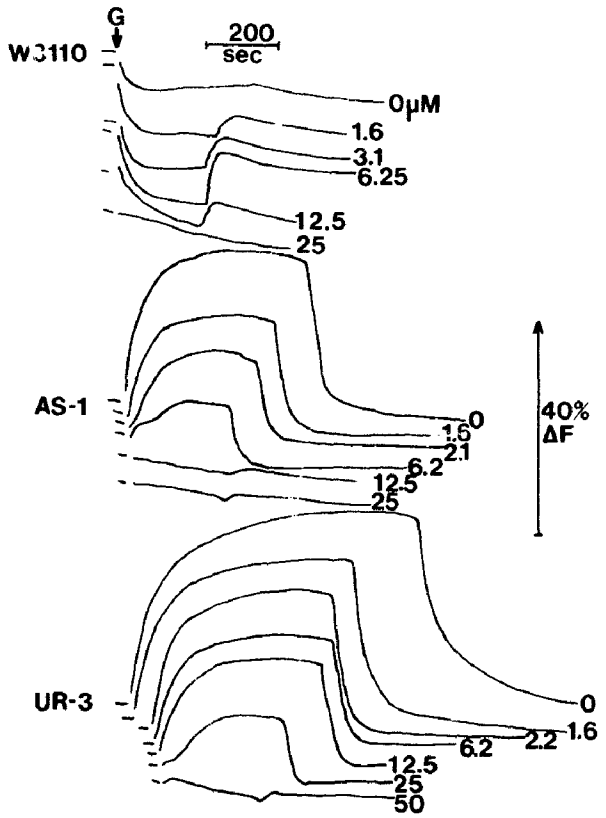

Fig. 4. Effect of uncoupler CCCP on the fluorescence of DMP^+ with intact cells of W3110, AS-1 and UR-3. The procedure is described in Materials and Methods. G, D-glucose added to 10 mM concentration. The numbers indicate the μM concentration of CCCP preincubated with the cell suspension for 5 min prior to the addition of DMP^+ at zero time. The initial fluorescence increments obtained on addition of DMP^+ to the cell suspensions have been omitted from these traces.

anaerobic on depletion of the dissolved oxygen in the medium. The extent of the initial quenching was enhanced by low concentrations of CCCP and the recovery of fluoresence on anaerobiosis was more dramatic. Fluorescence quenching was absent in the presence of 25 μM CCCP. By contrast, fluorescence intensity was enhanced by metabolism of glucose in strains AS-1 and UR-3 (Fig. 4). Quenching occurred at anaerobiosis. CCCP abolished the glucose-induced enhancement of fluorescence. The fluorescence response was more resistant to quenching by CCCP in UR-3 compared with AS-1.

Discussion

The uncoupler-resistant mutant UR-3 retains the sensitivity of its parent strain AS-1 to sodium dodecyl sulfate and Methylene blue, substances unable to permeate envelopes of wild-type strains like W3110, and yet is resistant to uncouplers such as CCCP, TCS and Bu_3SnCl. That the resistance is due to a change in the composition and/or structure of the outer membrane is supported by the slightly greater resistance to detergents of UR-3 compared with AS-1, and by the behaviour of UR-3 with the fluorescent probe NPN. The initial energy-dependent phase of quenching of fluorescence, absent in AS-1, is restored in UR-3. The initial phase has been attributed to a change in the binding of NPN to the outer membrane or to an alteration in its environment [20]. The latter might involve movement through the outer membrane energized by interactions with the inner membrane. The simplest explanation of the loss of the first phase is that NPN can permeate the outer membrane in AS-1 and that this pathway is blocked in UR-3, at the same time that resistance to uncouplers is acquired. However, this hypothesis does not explain all observations. Thus, CCCP, TCS and Bu_3SnCl rapidly catalyze the uptake of protons from the external medium into both AS-1 and UR-3 (Fig. 1). In a previous paper [8], we have suggested that uptake of protons occurred across the outer membrane into the periplasmic space. This is inconsistent with the hypothesis that the barrier to uncoupler is due solely to alterations in the outer membrane. Since the initial phase of quenching of NPN is energy-dependent, it likely involves inner/outer membrane interactions. That is, energization of the inner membrane may effect the behaviour of NPN at the outer membrane. A possible hypothesis is that this linkage is affected in AS-1 and restored in UR-3.

The mechanism by which the *acrA* mutation causes an increase in permeation of nonpolar and amphiphilic compounds is unknown [12]. The increased permeation of such contrasting agents as the negatively charged sodium dodecyl sulfate anion and the tetraphenylphosphonium cation suggest that a large disordering of the outer membrane may have occurred. This does not seem likely since resistance to one class of non-polar compounds, the uncouplers, is manifested in UR-3 with retention of some of the permeability characteristics of AS-1. Furthermore, reversion to an $acrA^+$ phenotype in AS-1 does not give uncoupler-resistance. Revertants to an $acrA^+$ phenotype in UR-3 retain resistance to uncouplers. At least two distinct pathways for permeation of lipophilic compounds through the outer membrane of *E. coli* must exist. Non-polar compounds, such as the uncouplers tested in our experiments, must penetrate via a pathway which is blocked in UR-3. Amphiphilic compounds such as sodium dodecyl sulphate, Methylene blue, mitomycin, acridine dyes, and lipophilic cations must permeate through the pathway opened by the *acrA* mutation. Comparison of AS-1 and UR-3 with respect to their behaviour with the fluorescent probes NPN and DMP^+ illustrates this difference. UR-3 recovers the permeability characteristics of the wild-type strain W3110 to NPN in contrast to AS-1. UR-3 retains the behaviour of AS-1 with

respect to amphiphilic compounds such as DMP^+. Moreover, both strains are equally permeable to tetraphenyl phosphonium (unpublished data). However, uptake of DMP^+ by UR-3 is more resistant to inhibition by uncouplers. This suggests that some overlap or interaction occurs between these two pathways.

It is interesting to compare the behaviour of UR-3 with uncoupler-resistant strains of *E. coli* isolated by Beechey and his coworkers [15–17]. As with the work described in the present paper, Beechey's group isolated uncoupler-resistant mutants from a strain of *E. coli* Doc-S unusually sensitive to the detergent deoxycholate and to ionophores [15]. Strain TUV showed resistance of growth, proline transport and oxidative phosphorylation to the uncouplers FCCP, TTFB and Bu_3SnCl, while retaining sensitivity to deoxycholate [15,16]. This behaviour is analogous to that observed with UR-3. A significant difference between TUV and UR-3 is that the former has lost the permeability of its parent strains to lipophilic cations like tetraphenylphosphonium [17]. Furthermore, TUV is resistant to novobiocin in contrast with Doc-S [16]. Both AS-1 and UR-3 are very sensitive to novobiocin. Beechey and his colleagues have shown recently that the detergent and novobiocin sensitivity of Doc-S arises from a mutation in the *rfa* gene (81 min). This mutation is absent in TUV [17]. By contrast the mutation in AS-1 is in the *acrA* gene (10 min) and appears to be retained in UR-3. UR-3, compared with TUV, is novobiocin-sensitive. Thus, it is unlikely that the uncoupler resistance of UR-3 is associated with the *rfa* gene. Beechey and his co-workers [17] have concluded that resistance to uncouplers in TUV is due to exclusion of the uncoupler by the outer membrane. As with our studies described here, anomalies still exist. The uncoupler TTFB collapsed the transmembrane pH gradient of resting cells, measured by phosphorus nuclear magnetic resonance, to a like degree in both Doc-S and TUV implying that the uncoupler could permeate the cell envelope in TUV [16]. This behaviour resembles that of UR-3 in the proton-equilibration experiments described above. Furthermore, these workers found that the transmembrane pH gradient in TUV was more resistant to collapse by uncoupler when there was active cellular respiration [16]. It is difficult to rationalize these effects with a mechanism of resistance which relies solely on the passive exclusion of uncoupler by the outer membrane. A complex interaction between inner and outer membrane permeation and energization is implied by Beechey's and our studies.

Acknowledgements

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