Uncoupler Resistance in *Escherichia coli*: the Role of Cellular Respiration

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Bioenergetic properties of a mutant strain of *Escherichia coli* K12 designated TUV, which is resistant to the protonophoric uncoupling agent 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) have been compared with those of its non-resistant parent, *E. coli* K12 Doc-S. Strain TUV grew and respired some 20–30% faster than strain Doc-S, and was cross-resistant to carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone and triphenyltin, but not to 2,4-dinitrophenol. Phosphorus nuclear magnetic resonance demonstrated the TTFB-mediated collapse of the transmembrane pH gradient at identical rates in starved cells of both strains, indicating that uncoupler access and function were unimpaired in the mutant under these conditions. Strain TUV displayed enhanced uncoupler resistance and maintained intracellular pH and ATP levels only when respiring. On the other hand, strain TUV also showed increased resistance to novobiocin, implying that its outer wall permeability had been lowered. We suggest that the active resistance of strain TUV results from the exclusion of uncoupler by the interaction of inner and outer membrane components in a manner modulated by the degree of cellular energization.

INTRODUCTION

It is generally agreed that a transmembrane proton electrochemical gradient plays an indispensable role in cellular energy transduction, linking the processes of respiration, ATP synthesis and active transport. One attractive feature of this chemiosmotic theory (Mitchell, 1961) is its ability to explain the mechanism of action of uncoupling agents, which are proposed to act as protonophores and dissipate the transmembrane protonmotive force (Δp). Excellent correlations have been observed between uncoupler potency *in vivo* and protonophoric activity *in vitro* (McLaughlin & Dilger, 1980), but several uncouplers have been reported to bind with high affinity to integral membrane proteins of both bacteria and mitochondria (Katre & Wilson, 1980; Partis *et al.*, 1984). It has been suggested that such interactions are important in uncoupling *in vivo*, and that uncoupler resistance arises from mutations affecting these binding proteins (Kell, 1982). The elucidation of the molecular mechanism of such resistance is thus important, both for our understanding of uncoupler action and for the information it may provide concerning the mechanism of cellular energy coupling.

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; FCCP, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone; MDP, methylenediphosphonic acid; MeP, methylphosphonate; TPP⁺, tetraphenylphosphonium; TPT, triphenyltin; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

Jones & Beechey (1987) isolated two mutant derivatives of *Escherichia coli* K12 Doc-S, designated strains TUV and CUV, which were resistant to the uncouplers 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) and carbonylcyanide *m*-chlorophenylhydrazine (CCCP). An enhanced resistance of growth, oxidative phosphorylation and proline transport was demonstrated. The parent strain, Doc-S, possesses a defective outer wall, which allows bulky hydrophobic species, such as deoxycholate, to penetrate to the cell membrane (Ahmed & Booth, 1983). Deoxycholate sensitivity was retained by strains TUV and CUV, and uncouplers were therefore believed to have equal access to their sites of action in all three strains, although this could not be proved directly.

Phosphorus nuclear magnetic resonance spectroscopy (^{31}P -NMR) provides an attractive means of investigating these cells. It allows the simultaneous non-invasive monitoring of many metabolically important compounds (inorganic phosphate, nucleotides, glycolytic intermediates), and the rapid estimation of intra- and extracellular pH from the corresponding phosphate chemical shifts. ^{31}P -NMR spectroscopy has been used extensively in studies of *E. coli* bioenergetics (e.g. Ogawa *et al.*, 1978; Ugurbil *et al.*, 1978; Slonczewski *et al.*, 1981), but has not been applied previously to the problem of uncoupler resistance.

In this paper, we extend our earlier work (Jones *et al.*, 1986; Jones & Beechey, 1987), making particular use of NMR spectroscopy to address the question of whether TTFB functions with equal efficacy in strains Doc-S and TUV. We present evidence indicating the active, energy-requiring nature of uncoupler resistance in strain TUV. Other data, however, suggest that some change in cell wall permeability is involved, a mutation which might be expected to produce passive uncoupler resistance (i.e. unaffected by the degree of cell energization). A possible scheme for rationalizing these apparently contradictory findings is proposed.

METHODS

Materials. Carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazine (FCCP) and triphenyltin (TPT) were purchased from Aldrich; methylphosphonate (MeP) from Alfa Produkte, Karlsruhe; 2,4-dinitrophenol (DNP) from BDH; and $^2\text{H}_2\text{O}$, amino acids, buffers, methylenediphosphonic acid (MDP) and novobiocin from Sigma. All chemicals were of at least analytical grade.

Organisms. *E. coli* K12 Doc-S (*lacI⁻z⁺y⁺a⁺ pro trp his met*), and its uncoupler-resistant derivative strains TUV and CUV have been described previously (Ahmed & Booth, 1983; Jones & Beechey, 1987). *E. coli* strains MRE600 and J53 RP4, both with wild-type cell walls, were also used.

Growth media. LB medium and succinate minimal medium were as given previously (Jones & Beechey, 1987). Glycerol minimal medium contained 5 ml glycerol l^{-1} , replacing succinate. Where appropriate, uncouplers were added aseptically, immediately before inoculation with cells.

Suspension media. These gave some osmolarity and buffering capacity but lacked respiratory substrates, which were added separately. Three media were employed, designed to provide optimal buffering capacity at different pH. All contained 40 mM-NaCl and 10 mM-KCl, supplemented by either 2-(*N*-morpholino)ethanesulphonic acid (MES; 150 mM), pH 6; *N*-(2-acetamido)iminodiacetic acid (ADA; 100 mM) plus piperazine-*N,N'*-bis-(2-ethanesulphonic acid) (PIPES; 40 mM), pH 6.1; or 3-(*N*-morpholino)propanesulphonic acid (MOPS; 150 mM), pH 7. These solutions are designated MES, ADA/PIPES and MOPS medium, respectively.

Growth. Cells were maintained and grown essentially as before (Jones & Beechey, 1987), except that growth was at 37 °C and was monitored spectrophotometrically at 600 nm.

Harvesting. Cells were harvested at mid-exponential phase (OD_{600} 0.8–1.2) by centrifugation at 17700 *g* for 10 min at 4 °C. They were washed once with the appropriate suspension medium, resuspended to 40–50 mg protein ml^{-1} , kept on ice and used within 14 h.

Assays. Protein was assayed by the Lowry method, using bovine serum albumin as standard. Cell samples were first lysed by vigorous mixing with an equal volume of 2 M-NaOH.

TTFB was assayed spectrophotometrically. Its absorption coefficient at 301 nm in water was measured as 6530 $\text{M}^{-1} \text{cm}^{-1}$.

The binding of TTFB to cells was assayed under conditions comparable to those of an NMR experiment: MOPS medium (pH 7), 25 mg protein ml^{-1} . Samples were incubated anaerobically for 10 min with TTFB (25–500 μM), then centrifuged for 1 min (Eppendorf 5412 microfuge). The supernatant was centrifuged again to ensure complete removal of cells, diluted 1:2 with water and its A_{301} determined, reading against a control sample to which only ethanol had been added. TTFB binding was calculated from the difference between the amount remaining in the supernatant and that originally added. To avoid introducing errors from Scatchard linearization,

K_d was estimated directly from the binding curve data, using the Marquardt algorithm for non-linear regression (Duggleby, 1984).

The procedure for monitoring deoxycholate sensitivity has been described previously (Jones & Beechey, 1987); tests of novobiocin sensitivity were conducted in an identical manner.

Respiratory studies. Harvested cells, grown in the absence of uncoupler, were studied using a Clark-type oxygen electrode (Yellow Springs YSI 5331) inserted into a water-jacketed, 3.3 ml reaction chamber, maintained at 25 °C. Cells were inoculated into the appropriate suspension medium to a final concentration of 0.3 mg protein ml⁻¹, and reoxygenated when necessary by bubbling for short periods with O₂/CO₂ (95:5, v/v).

Membrane potential. Membrane potential ($\Delta\psi$) was measured according to the method of Ahmed & Booth (1983), using [¹⁴C]tetraphenylphosphonium (TPP⁺).

NMR studies. The spectrometer was constructed around an Oxford Instruments 8.5 T superconducting wide-bore magnet, interfaced to Nicolet computing equipment. Standard samples [15 ml bacterial suspension, 1 ml ²H₂O (field/frequency lock), 0.2 ml silicone antifoam, 0.2 ml 200 mM-MeP (internal reference) and 0.1 ml 100 mM-NaH₂PO₄; final cell concentration 25 mg protein ml⁻¹] were placed in a 20 mm diameter, flat-bottomed NMR tube, and maintained at 21 °C. A capillary containing MDP served as an external reference. The antifoam was tested for uncoupling activity, but showed none.

Samples were oxygenated continuously by two flows of O₂/CO₂ (95:5, v/v): a flow of 130 ml min⁻¹ through four capillaries inserted to the bottom of the sample, and a surface-directed flow of 1 l min⁻¹ (Slonczewski *et al.*, 1981). In a bench test of a sample of strain Doc-S respiring on succinate, an oxygen tension of 250 μ M-O₂ was maintained, well above the K_m of *E. coli* MRE600 for O₂ (0.7 μ M) (Ogawa *et al.*, 1978).

Fourier transform ³¹P-NMR spectra were acquired at 145.8 MHz with broad-band proton decoupling, and using a single 40° pulse in order to allow a rapid recycle time (405 ms, including a relaxation delay of 200 ms). Each transient comprised 4096 data points and the sweep width was $\pm$ 5000 Hz. Spectra were accumulated from blocks of between 128 and 1480 transients (total acquisition times 0.9–10.4 min). A 10 s delay was programmed between the acquisition of each spectrum, during which time substrates and reagents could be added via an injection line. Line-broadening of 20 Hz was imposed on the transformed spectra, which were assigned from the literature (Ugurbil *et al.*, 1978; Slonczewski *et al.*, 1981).

Titration curves of chemical shift against pH were constructed for phosphate and MeP by treating samples with small volumes of 1 M-NaOH and 1 M-HCl; the compounds behaved identically in all three suspension media. The chemical shifts of intracellular phosphate and MeP were assumed to follow the same curves as in the media. This assumption was verified directly for pH values above 6.3 by titrating samples of anaerobic *E. coli* Doc-S following 40 min incubation with 200 μ M-TTFB. Below pH 6.3, two phosphate resonances were resolved, probably due to the presence of a residual transmembrane pH gradient, opposing a Donnan membrane potential (Ogawa *et al.*, 1981). Such a Δ pH (around 0.2 pH units) would not be competent to drive ATP synthesis, as there would be no net protonmotive force.

RESULTS

Binding of TTFB to cells

The binding properties of TTFB appeared virtually identical in strains Doc-S, TUV and MRE600, being monophasic with a K_d in strain TUV of $217 \pm 3 \mu$ M (two determinations). This probably represents non-specific binding, as small amounts of tight, specific binding ($K_d < 10 \mu$ M; e.g. Partis *et al.*, 1984) would be impossible to detect with this assay. Under typical conditions employed for NMR studies (100 μ M-TTFB), around 75% of the uncoupler is bound to the cells.

Growth and cross-resistance properties

At 37 °C in the absence of uncoupler, the growth rate of strain TUV on succinate minimal medium, pH 7, was up to 25 % higher than that of strain Doc-S (fastest doubling times: TUV, 90 min; Doc-S, 120 min), consistent with previous observations at 30 °C (Jones & Beechey, 1987). Under these conditions, the respiratory rate of strain TUV was $17 \pm 5\%$ greater than that of strain Doc-S (three separate determinations on cultures growing simultaneously in the same incubator), although the actual rates varied somewhat [strain Doc-S, 800 ng-atom O min⁻¹ (mg protein)⁻¹, $\pm 10\%$].

The results of a series of growth studies are summarized in Table 1. Compared with strain Doc-S, strain TUV displayed markedly increased resistance to TTFB, FCCP and TPT, but was not cross-resistant to DNP. Strain CUV was three times more resistant than strain TUV to

Table 1. *Uncoupler-resistant growth of E. coli strains*

Figures indicate the concentration (μM) of uncoupler causing a 50% reduction in growth rate, relative to control flasks, under the specified conditions. The same amount of ethanol (solvent for the uncouplers) was present in all flasks and had negligible effects on growth. ND, Not determined.

Carbon source	pH	Uncoupler	Strain...	Uncoupler I_{50} (μM)			
				Doc-S	TUV	CUV	MRE600
Succinate	7	TTFB		12	120	120	25
Succinate	6	TTFB		3	35	35	ND
Glycerol	7	TTFB		10	150	ND	ND
Succinate	7	FCCP		2	25	80	<5
Succinate	7	DNP		80	70	150	150
Succinate	7	TPT		<0.5	4	3	ND

FCCP, consistent with results obtained with the structurally similar CCCP (Jones & Beechey, 1987), and was weakly cross-resistant to DNP, but its resistance to TTFB and TPT was not further enhanced. Resistance to TTFB did not require any specific carbon source, that of strain TUV being unaffected by the substitution of glycerol (Table 1) or acetate (Jones & Beechey, 1987) for succinate. These respiratory substrates are all non-fermentable. In the three strains tested, TTFB was three to four times more potent at pH 6 than at pH 7. The TTFB-induced conductance of phospholipid bilayers shows a similar pH dependence, being 3.5 times higher at pH 6 than at pH 7 (Dilger & McLaughlin, 1979).

TPT may uncouple cells by catalysing transmembrane Cl^-/OH^- exchange (Selwyn *et al.*, 1970), but can also inhibit the F_0F_1 ATP synthase directly (Kagawa *et al.*, 1979). The relative importance of these processes is unclear, but tributyltin requires at least 40 mM extracellular Cl^- to uncouple *E. coli* effectively (Sedgwick *et al.*, 1984). It thus seems unlikely that TPT would uncouple cells in succinate minimal medium, containing less than 2 mM Cl^- (from histidine.HCl), and more likely that the effects of the compound arise from inhibition of the ATP synthase.

Sensitivity to novobiocin

In order to compare the cell wall permeabilities of the strains, their sensitivity to novobiocin, an inhibitor of DNA gyrase (Cozzarelli, 1980), was investigated (Fig. 1). Strain Doc-S was clearly the most sensitive, while the resistance of strains TUV and CUV appeared similar to that of the wild-type J53 RP4.

Deprivation of respiratory substrate

Strain TUV was grown overnight in LB medium, then transferred to incomplete minimal medium, pH 7, containing 100 μM -TTFB but lacking both succinate and citrate. After various preincubation periods, succinate and citrate were provided and subsequent growth was monitored. The rationale was that only active, energy-requiring resistance would be impaired by this deprivation of respiratory substrate in the presence of uncoupler, with passive resistance remaining unaffected.

In control experiments done in the absence of uncoupler, the lag time of strain TUV following the reintroduction of the carbon sources was under 30 min, even after 120 min preincubation in incomplete medium. Preincubation for up to 5 min in incomplete medium containing 100 μM -TTFB did not affect this lag time, but 10 min preincubation increased it to 10–12 h (three experiments). Similar results were obtained when FCCP (15 μM) was substituted for TTFB.

Respiratory studies

The results of a typical experiment comparing harvested cells of strains Doc-S and TUV are shown in Fig. 2. Cells were introduced into the reaction chamber and incubated in the absence of respiratory substrate or uncoupler. Following an initial short burst of respiration, oxygen consumption in both strains fell to a constant low rate. After 15 min incubation, 100 μM -TTFB

Fig. 1. Sensitivity of *E. coli* strains to novobiocin. The filter discs on each plate were treated with (reading anticlockwise from the top) 0, 35, 180, 350, 530 and 700 μg novobiocin, respectively. The enhanced resistance of strains TUV and CUV, compared with strain Doc-S, is readily apparent.

Fig. 2. Respiratory properties of *E. coli* Doc-S and TUV. Cells were introduced into the reaction chamber (containing MOPS medium) at time zero. TTFB (100 μM) was added after 15 min, and succinate (5 mM) after a further 10 min. Respiratory rates [$\text{ng-atom O min}^{-1} (\text{mg protein}^{-1})$] are indicated on the curves. —, Doc-S; ---, TUV.

was added, a concentration which prevents the growth of strain Doc-S, but not that of strain TUV (Table 1). With strain Doc-S, a further burst of respiration ensued, believed to represent the rapid oxidation of endogenous substrate reserves. A similar loss of respiratory control has been noted in another strain of *E. coli* K12 (Burstein *et al.*, 1979). The respiratory rate of strain Doc-S gradually declined towards its previous level, but the addition of succinate 10 min later failed to elicit any respiratory response. Respiration of strain TUV, in contrast, was little affected by 10 min incubation with TTFB and cells continued to respond to added succinate, even though similar incubations with uncoupler caused large increases in culture lag times (see previous section). The reason for this discrepancy is unknown.

Fig. 3. Effects of TTFB on Δ pH of starved cells of *E. coli* Doc-S and TUV. Results are representative of several experiments (Doc-S, $n = 2$; TUV, $n = 3$). ■, Intracellular pH; □, extracellular pH. (a) Sample: 16.5 ml strain Doc-S, MOPS medium. Oxygenation commenced at time zero; TTFB (100 μ M) was added 60 min later. (b) Identical sample and protocol to (a), but using strain TUV.

These effects of TTFB on strain Doc-S could be explained in two ways: uncoupler-binding to membrane proteins causing a direct inhibition of succinate transport and/or respiration; or the dissipation of Δp removing the driving force for transport (Gutowski & Rosenberg, 1975). The first alternative is considered unlikely. If metabolite transport were inhibited directly in strain Doc-S, then the transporters for succinate, glycerol and acetate must all have become resistant in strain TUV, since TTFB-resistant growth occurs on all three substrates. Furthermore, succinate respiration by strain Doc-S was not inhibited immediately upon addition of TTFB, but continued unaffected for a further 60 min (data not shown), presumably the time required for the oxidation of all endogenous substrate. Thus TTFB does not inhibit respiration or transport directly, and strain TUV must either maintain Δp in the presence of TTFB or transport succinate at values of Δp below those required by strain Doc-S.

Membrane potential

A membrane potential of 130 mV was measured with strain Doc-S suspended at pH 7, in good agreement with a previous study (Ahmed & Booth, 1983). In contrast, strain TUV took up the probe only after pretreatment with EDTA, when a $\Delta\psi$ of 135 mV was obtained. Addition of TTFB [4 nmol (mg dry weight)⁻¹] caused complete dissipation of $\Delta\psi$ in both strains. These data provide further evidence for a change in envelope permeability in strain TUV.

³¹P-NMR spectroscopy

Effects of TTFB on cells lacking respiratory substrate. Cell samples were oxygenated *in situ* for 60 min in the absence of added substrate in order to deplete them of endogenous reserves. During this starvation the β -NTP resonance declined by up to 80%. The starved cells were then treated with TTFB and subsequent changes in cytoplasmic and extracellular pH were monitored. TTFB dissipated Δ pH equally rapidly in starved cells of both strains (Fig. 3).

Effects of TTFB on succinate-fed cells. Cell samples (in MES or ADA/PIPES medium) were incubated with succinate, then challenged with TTFB. Preliminary work, done in the absence of uncoupler, revealed the expected extracellular alkalinization as succinate was transported into the cells via the succinate/H⁺ symport (Gutowski & Rosenberg, 1975), but intracellular pH was maintained at between 7.3 and 7.5. In these experiments, Δ pH could be reduced by both succinate transport and uncoupler action, but the two processes were distinguishable by the direction of net proton movement: succinate uptake causes extracellular alkalinization, intracellular pH remaining constant, whereas uncoupling is associated with intracellular acidification, extracellular pH remaining constant.

Fig. 4. Effects of TTFB on Δ pH and intracellular ATP of cells respiring on succinate. Results are representative of several experiments (Doc-S, $n = 3$; TUV, $n = 4$). ■, Intracellular pH; □, extracellular pH. (a) Sample: 12.8 ml strain Doc-S, ADA/PIPES medium. Oxygenation commenced at time zero, with succinate (78 mM) present; TTFB (150 μ M) was added 33 min later. (b) ^{31} P-NMR spectrum (1480 transients) of strain Doc-S acquired 87.0–98.6 min after time zero, as indicated in (a). Assignments: 1, phosphomonoesters; 2, intracellular P_i ; 3, extracellular P_i ; 4, γ -NTP + β -NDP; 5, α -NTP + α -NDP + NAD(P)(H); 6, NDP-glucose; 7, β -NTP. Sample and initial treatment identical to (a), but using strain TUV. Oxygenation interrupted after 118 min and more succinate (72 mM) added. Oxygenation resumed 21 min later. (d) Spectrum (1480 transients) of strain TUV acquired 118.8–129.4 min after time zero, as indicated in (c), and plotted with a vertical scale expansion twice that of (b).

The addition of 150 μ M-TTFB to strain Doc-S (Fig. 4a) caused a gradual intracellular acidification, although succinate uptake continued and the medium continued to alkalinize. Intracellular acidification was significantly slower than that observed with starved cells (Fig. 3a). After the dissipation of Δ pH was complete, the indicated pH was 6.95 ± 0.1 in all three experiments. A slow alkalinization followed, but the phosphate signal did not split, indicating that intra- and extracellular pH rose together. A similar pattern of events occurred with strain TUV (Fig. 4c) but intracellular pH in all four cases was maintained at 7.4 ± 0.1 , despite the presence of the uncoupler.

Even after the dissipation of Δ pH, both strains retained a large β -NTP resonance (Fig. 4b). Interruption of the oxygen supply caused a rapid decline in this resonance (Fig. 4d), indicating that oxidative phosphorylation had continued to operate while oxygen was available. The availability of substrate thus allows even strain Doc-S to resist uncoupling to some extent. Higher concentrations of TTFB (200–750 μ M) caused significant intracellular acidification and the disappearance of the β -NTP resonance in both strains (data not shown).

Fig. 5. Effects of TTFB on Δ pH and intracellular ATP of cells respiring on glycerol. Results are representative of several experiments (Doc-S, $n = 2$; TUV, $n = 5$). ■, Intracellular pH; □, extracellular pH. Assignments as for Fig. 4, plus: 8, extracellular methylphosphonate (MeP); 9, intracellular MeP; 10, methylenediphosphonic acid (MDP). (a) Sample: 16.5 ml strain Doc-S, MOPS medium. Oxygenation commenced at time zero. Glycerol (79 mM) was added after 46 min, followed 14 min later by TTFB (100 μ M). (b) Spectrum (512 transients) acquired 105.3–108.9 min after time zero, as indicated in (a). Oxygenation was interrupted during this period, in order to improve the signal/noise ratio. (c) Identical protocol to (a), but using strain TUV. (d) Spectrum (512 transients) acquired without oxygenation 105.3–108.9 min after time zero, as indicated in (c).

Effects of TTFB on glycerol-fed cells. Cells were grown in glycerol minimal medium (pH 7), harvested and resuspended in MOPS medium. In control experiments without uncoupler, glycerol metabolism, presumably phosphorylation, caused the depletion of intracellular NTP and phosphate (which sometimes became difficult to detect), and a corresponding increase in the phosphomonoester resonance. Extracellular pH gradually fell, possibly due to the excretion of acidic products of incomplete glycerol oxidation. Both strains accumulated MeP, as shown by the splitting of its NMR resonance (Slonczewski *et al.*, 1981).

The addition of TTFB to strain Doc-S (Fig. 5a) initiated an intracellular acidification, and Δ pH fell, even though acidification of the medium continued. In contrast, strain TUV (Fig. 5c) maintained its intracellular pH above 7.3, and Δ pH gradually increased. The continued resolution of intra- and extracellular phosphate and MeP resonances illustrates the maintenance of a Δ pH by strain TUV, but not by strain Doc-S (Fig. 5b,d). At a higher concentration of TTFB (200 μ M), Δ pH collapsed rapidly in both strains (data not shown).

DISCUSSION

Uncoupler resistance is a complex phenomenon, with resistant cell lines often displaying many phenotypic alterations. In order to better comprehend the nature of resistance in *E. coli* TUV and CUV, we have drawn a distinction between active and passive mechanisms, and attempted to establish their relative importance *in vivo*. This discussion will focus mainly on the better characterized strain TUV.

Previous studies of uncoupler resistant *E. coli* have provided some indications of active resistance. In the first-reported strain, SWL14 (Date *et al.*, 1980), proline transport was resistant in both cells and right-side-out vesicles. An enhanced resistance to uncoupler-mediated dissipation of ΔpH was reported for vesicles of a further strain, CM22 (Ito *et al.*, 1983). In *E. coli* UV6, the best-characterized mutant to date, ΔpH of whole cells was also reported as being less susceptible to collapse by uncoupler, but vesicles were not examined (Sedgwick *et al.*, 1984). Unfortunately, interpretation of results with this strain was complicated by the occurrence of ill-defined H^+ movements, possibly across the outer membrane; it was hoped that the use of derivatives of strain Doc-S would avoid such problems. Strains CM22 and UV6 both showed multiple cross-resistances, similar to those of strains TUV and CUV.

Two lines of evidence indicate the importance of active resistance in strain TUV. Firstly, the large increases in lag times when uncoupler-treated cultures are temporarily deprived of substrate point to some protective effect of respiratory metabolism. Secondly, NMR samples of strain TUV show superior resistance only when respiratory substrate is available. The ability of strain Doc-S to maintain intracellular NTP in the presence of TTFB is also enhanced by the presence of substrate, provided that the intracellular pH has not fallen too low. The role of respiratory substrate in uncoupler resistance appears not to have been examined previously in this manner, but has proved informative in this study.

The immediate, uncoupler-mediated dissipation of ΔpH in starved cells of strains Doc-S and TUV, as monitored by NMR spectroscopy (Fig. 3), provides direct evidence that TTFB has equal and facile access to the cytoplasmic membranes of both, and argues against any reduction in the envelope permeability of strain TUV under these conditions. The fact that ΔpH is dissipated at similar rates in both strains also argues against any decrease in proton transport rates within the cytoplasmic membrane of strain TUV, implying that uncoupler function within this membrane is unimpaired. Furthermore, the pH dependence of TTFB's uncoupling activity in strains Doc-S and TUV is identical to that of its protonophoric activity in phospholipid bilayers (Dilger & McLaughlin, 1979), arguing against the involvement of bacterial membrane proteins in the uncoupling process. Dissipation of ΔpH , however, was always slow (≤ 0.05 pH units min^{-1}), raising the possibility that proton translocation is not rate-limiting in the uncoupling process. For example, FCCP dissipates the mitochondrial Δp within seconds (Bakker *et al.*, 1973), but ΔpH collapses slowly, possibly because of slow counterion movements (Ogawa *et al.*, 1981). If similar processes limit the rate of collapse of ΔpH in *E. coli*, then any impairment of TTFB's protonophoric activity in strain TUV might remain undetected.

The evidence discussed so far may be interpreted in favour of a model in which uncoupler resistance is maintained by active, substrate-dependent processes, with retention of ΔpH (in agreement with Ito *et al.*, 1983; Sedgwick *et al.*, 1984). A possible origin of the active resistance of strain TUV lies in its 25%-enhanced respiratory rate, which could promote faster proton translocation and greater protonophore resistance. The cytochromes of strains Doc-S and TUV appear qualitatively and quantitatively indistinguishable (data not shown), suggesting an unchanged $\text{H}^+/\text{2e}^-$ stoichiometry; thus proton translocation in strain TUV is unlikely to increase by more than 25%. Such a modest increase seems insufficient to explain the strain's 10-fold enhanced TTFB resistance (Table 1), unless other factors are also involved.

Evidence that the situation is more complicated than was at first thought comes from the finding of novobiocin resistance in strains TUV and CUV, suggesting a reduced envelope permeability in these strains. Preliminary genetic analysis (by O. Michelsen, University of Odense, Denmark) indicates that strain Doc-S carries a mutation conferring resistance to phage P1 and cotransducible with the *pyrE* locus, which has reverted to wild-type in strain TUV. A likely location of the change is the *rfa* operon. This operon encodes several enzymes concerned with lipopolysaccharide synthesis (Nikaido & Vaara, 1985), and *rfa* mutant strains can be extremely sensitive to novobiocin. (This cannot be the only lesion affecting the envelope of strain Doc-S and its derivatives, since strains TUV and CUV retain abnormal sensitivity to deoxycholate.) It is not yet known whether strain TUV carries further mutations from its parent (strain CUV, presumably, must), but a *rfa* back-mutation could account for the reduction in novobiocin sensitivity, and might lead to a more effective exclusion of TTFB and FCCP. Such a

mutation could also account for the cross-resistance pattern of strain TUV, including the retention of sensitivity to DNP, a hydrophilic uncoupler which could still reach the cell membrane through the pores of the outer wall. Interestingly, strain UV6 also retained sensitivity to DNP (Sedgwick *et al.*, 1984). Furthermore, the *rfa* operon lies at 81 min on the chromosome, quite close to the *unc* operon (83.5 min), raising the possibility that some of the mutations documented by Ito and co-workers (Ito & Ohnishi, 1981, 1982; Ito *et al.*, 1983) originated in the *rfa* region. The reduced permeability of strain TUV to TPP⁺ is further evidence of a cell envelope change.

Although the above findings might appear to lead to an essentially trivial explanation of uncoupler resistance in these strains, two interesting questions remain. Firstly, one would expect resistance arising from a cell envelope mutation to be passive, whereas the resistance of strain TUV contains an essential active component. Secondly, the uncoupler resistance of strains TUV and CUV is much greater than that of strains possessing wild-type envelopes, even though the former strains retain abnormal sensitivity to deoxycholate. A possible solution to the first problem comes from a report that cellular energization may render the wild-type *E. coli* envelope less permeable to uncouplers (Helgersson & Cramer, 1977). The nature of cell membrane-cell wall interactions in *E. coli* remains obscure, but proteins spanning the periplasm have recently been described, which may be involved in the energy-dependent opening and closing of cell wall pores (Higgins *et al.*, 1988). Thus, the access of uncouplers to the cell membrane of strain TUV may become impaired by changes in cell wall permeability resulting from cellular energization. A partial recovery of envelope integrity might also explain the enhanced growth and respiratory rates of the resistant strains.

In conclusion, it appears that complex cell membrane-cell wall interactions may occur even in deoxycholate-sensitive strains of *E. coli*, and that these might be modulated by the degree of cellular energization. Our results are generally compatible with previous studies of uncoupler resistance in *E. coli*, but the diverse properties of the various mutant strains may not all be explicable in terms of a single underlying mechanism. We would, however, draw a distinction between results obtained with *E. coli* and those obtained with resistant strains of the Gram-positive organisms *Bacillus megaterium* and *B. subtilis*. In the latter species, ATP synthesis was maintained despite the unimpaired dissipation of Δp by uncouplers, and resistance correlated with changes in membrane lipids (Krulwich *et al.*, 1987; Clejan *et al.*, 1988). While the biochemistry of uncoupler resistance in *E. coli* remains intriguing, the type of mutation exemplified by the *Bacillus* species may be more challenging from a bioenergetic standpoint.

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