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Uncoupler resistance in *E. coli* Tuv and Cuv is due to the exclusion of uncoupler by the outer membrane

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The uncoupler resistant bacterial strains *E. coli* Tuv and Cuv share the high deoxycholate sensitivity of the parent strain, Doc S. However, both Tuv and Cuv show greater resistance than Doc S to other detergents. Measurement of the periplasmic volume indicates that the outer membrane of Doc S is freely permeable to both TPP^+ and hydroxymethylinulin. Tuv and Cuv are able to exclude these compounds. EDTA treatment was necessary prior to measuring membrane potentials in Tuv and Cuv. Under conditions where $\Delta\psi$ could be measured, uncouplers acted to dissipate $\Delta\psi$ with equal potency in all strains. Uncoupler resistant proline uptake in Tuv and Cuv was abolished by EDTA treatment. Transduction experiments with phage P1 showed that uncoupler resistance could be transferred from Tuv to Doc S. Such transductants were no longer sensitive to novobiocin. The gene for uncoupler resistance cotransduced with the gene *pyrE* (82 min). Plating efficiency experiments with P1 suggests that detergent sensitivity in Doc S arises from an *rfa* (81 min) mutation. This mutation is no longer present in Tuv.

Introduction

Mitchell's chemiosmotic hypothesis requires that endergonic reactions in the energy-conserving membrane are driven by the bulk-phase protonmotive force (Δp) [1]. The hypothesis predicts that the collapse of Δp would lead to an inability to carry out energy-demanding reactions, such as ATP synthesis.

Uncouplers were envisaged to act as protonophores, dissipating Δp, and subsequently it has been demonstrated that uncouplers are capable of collapsing Δp [2]. On this basis, it would not be possible for organisms which synthesise ATP uniquely by aerobic electron transport-linked processes to grow in the presence of uncouplers. However, there have been many reports of uncoupler-resistant organisms [3–8]. Their existence requires explanation, since they challenge the concept that the bulk-phase Δp is essential for energy conservation.

Jones and Beechey [7] described the properties of two strains of *Escherichia coli* which were capable of growth in the presence of the uncouplers 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), and which showed differential sensitivities to uncouplers of different structures.

Using ^{31}P-NMR it was demonstrated that the ability of uncouplers to discharge the bulk-phase ΔpH was related to the level of energisation of the energy-conserving membrane [9]. Thus, in the absence of added and endogenous substrate, the effect of uncoupler on the bulk phase ΔpH of the resistant strain Tuv was the same as that on Doc S. However, when oxidisable substrates were present the pH gradient in strain Tuv showed a marked insensitivity to the presence of TTFB. Attention was drawn to the fact that the resistant strains showed greater resistance to the antibiotic novobiocin than Doc S [9].

Other workers have claimed that resistance to uncouplers found in other bacteria could be ascribed to changes in the fatty acid component of the phospholipid in the energy-conserving membrane [10,11].

In this paper we describe the lipid composition of the membranes of Doc S and the derived uncoupler-resistant strains, and have measured the effects of uncouplers on the membrane potential.

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; TPP^+, tetraphenylphosphonium cation; SDS, sodium dodecyl sulphate.

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Materials. CCCP was purchased from Sigma. All radiochemicals were obtained from Amersham International. Sodium deoxycholate was purchased from BDH. All other reagents were bought commercially.

Bacterial strains. E. coli K12 Doc S (*lacI pro trp his met*) is the parent of the uncoupler resistant strain Tuv. A second uncoupler resistant strain, Cuv, was derived from Tuv [7]. *E. coli* K12 J53 RP4 (*pro met* str^S tet^R kan^R $carb^R$), *E. coli* K12 CM2043 (*asnA31, asnB32, relA1, spoT1,* F^+) and *E. coli* K12 CM567 (*gltS7, gadS1, gadR2, argG6, pyrE41, thyA25, thi1, reaA1, rbsP1, rpsL8*) were used as control strains.

Growth of cells. Cells were maintained and grown under the conditions described by Jones and Beechey [7]. Cells were harvested at mid-logarithmic phase by centrifugation at $5000 \times g$ for 5 min at 4°C (Sorvall RC-5B) and washed once with buffer A. Buffer A contained choline chloride (150 mM), potassium chloride (5 mM) and Tris-HCl (30 mM, pH 7.0). Cells were resuspended to 10 mg dry weight/ml (4.2 mg protein/ml) in buffer A.

Isolation of E. coli cell envelopes. The cell envelopes were prepared by sonicating 20 ml of cell suspension (6×15 s, MSE Soniprep 150, 15 μm amplitude). The sonicate was spun at $5000 \times g$ for 5 min. The supernatant was removed and the cell envelopes were sedimented by centrifugation at $120\,000 \times g$ for 60 min (MSE superspeed 65). The resulting pellet of cell envelopes was resuspended in buffer A (20 mg protein/ml). All procedures were carried out at 4°C.

Analysis of fatty acid content of membrane lipids. The fatty acid composition of the membrane fraction was investigated by gas chromatography of the fatty acid methyl esters [12]. The phospholipids were hydrolysed by refluxing in 10% (w/v) potassium hydroxide/ethanol. After acidification to pH 2, the liberated fatty acids were extracted with diethylether. The extract was washed twice with water and dried over anhydrous sodium sulphate. The ether was removed with nitrogen. The remaining fatty acids were esterified with 14% (v/v) borontrichloride/methanol. The samples were extracted into 40–60 petrol, and were again washed and dried. The petrol was removed with nitrogen, and the fatty acid methyl esters were dissolved in 'Spectro' hexane (BDH). Samples were run on a Carlo Erba Strumentazione HRGC 5300 Mega series gas-liquid chromatograph fitted with a Chrompack WCOT fused silica column (25 m × 0.32 mm; carrier gas, argon; flow rate, 60 ml/min). Data were collected by a Spectra-Physics SP 4270 integrator.

Effect of detergents on E. coli Doc S, Tuv and Cuv. Sensitivity to the detergents sodium deoxycholate, sodium dodecyl sulphate (SDS) and Triton X-100 was assayed by growth on solid medium in the presence of the test compound, using the method described by Booth et al. [13].

EDTA treatment of cells. EDTA (0.1 M) was added to a suspension of cells in buffer A to a final concentration of 1 mM. After 2 min at room temperature, the cells were sedimented by centrifugation and washed twice with buffer A. The cells were resuspended in buffer A to 10 mg dry weight/ml.

Cell compartment volume measurements. Cell compartment volumes were measured by the method of Stock et al. [14].

The following radioactive couples were used: hydroxy[^{14}C]methylinulin (50 nCi/ml)/^3H_2O (0.1 μCi/ml) (whole cell); [^{3}H]sucrose (0.2 μCi/ml)/hydroxy [^{14}C]methylinulin (50 nCi/ml) (periplasm); [^{14}C]sucrose (0.1 μCi/ml)/^3H_2O (0.1 μCi/ml) (cytoplasm).

Measurement of membrane potentials. The method of Ahmed and Booth was used to measure the bulk-phase membrane potential ($\Delta\psi$) [15]. Cells (1 mg dry weight/ml) were incubated for 20 min at 20°C in the presence of [^{14}C]TPP^+ (2 μM final concentration; 8.4 Ci/mol) and [^{3}H]sucrose (0.1 μCi/ml). Cells were sedimented by centrifugation, and samples of supernatant and pellet were assayed for radioactivity.

TTFB and CCCP were added in small ethanolic aliquots (10 μl) to give final concentrations up to 100 μM (in a total volume of 10 ml). The additions were made 5 min before sampling. The values for membrane potentials were calculated as described by Booth et al. [13]. All measurements were made in duplicate, and are the result of two or three experiments.

Proline uptake in E. coli cells. Cells (1.5 ml; 1 mg dry weight/ml) were incubated with ^3H_2O (0.1 μCi/ml) at 25°C for 30 min. [^{14}C]Proline (20 mM, 6.4 Ci/mol) was added to a final concentration of 45 μM, and 2×100 μl aliquots of the incubate were removed at 0, 0.5, 1.25, 2, 3 and 5 min. The cells were sedimented in a microfuge (30 s), and samples of supernatant and pellet assayed for radioactivity. Where required, TTFB or CCCP (10 nmol/mg dry weight) was added 5 min before the proline. The counts in the pellets were corrected for supernatant carryover.

Transduction of TTFB resistance from Tuv to Doc S. P1 phage was grown on Tuv in LB media, and the lysate was used to transduce Doc S [16]. After 15 min adsorption, LB medium was added. Samples were withdrawn at hourly intervals, washed with minimal medium and plated on minimal medium supplemented with succinate as the major carbon source, the required amino acids and 100 μM TTFB.

Plating efficiency of P1 on Doc S and Tuv. The plating efficiency of P1 on cells of Doc S, Tuv, CM2043 and a TTFB-resistant transductant of Doc S was determined as in Ref. 16.

Transduction of E. coli CM567 (pyrE$^-$) with P1 lysate from Doc S and Tuv (pyrE$^+$). Transduction of *E. coli* CM567 (*pyrE*$^-$) was performed with P1 lysates from Doc S and Tuv. P1 phage could not be grown on Doc S

in the normal way, and so lysates from Doc S were prepared by temperature induction of Doc S lysogenised with P1 c1.100 cm (a strain of P1 containing the c1.100 temperature-sensitive control gene). It has been shown previously that sensitivity to uncouplers correlates well with sensitivity to novobiocin [9]. For technical reasons it was easier to measure the sensitivity of the transductants to novobiocin as described in Ref. 9.

Results and Discussion

Fatty acid composition of the membranes of E. coli Doc S and Tuv

The fatty acid methyl esters were analysed by gas chromatography. Ten residues were resolved (Table I). Not all of these were identified, but the resolution was sufficient to compare samples. No significant differences were observed in samples from Doc S and Tuv. The presence of TTFB in the growth medium had no noticeable effect on the fatty acid composition of the membranes of Tuv.

Thus, the fatty acid composition of the membranes from Tuv appears to have no relation to the degree of uncoupler resistance exhibited by the cell. This contrasts with the findings of Krulwhich and co-workers [10,11], who showed that there is a constitutive change in the fatty acid composition of uncoupler resistant strains of *Bacillus megaterium* and *B. subtilis*. Herring et al. [6] showed that in *E. coli* UV6 there is an adaptive change in fatty acid composition when grown in the presence of uncoupler. Again, this is not the case with Tuv, suggesting that the mechanism of resistance in this strain is different to that found in other strains.

Detergent sensitivities of E. coli Doc S, Tuv and Cuv

Doc S, Tuv and Cuv have been tested routinely for deoxycholate sensitivity, and have invariably shown much greater sensitivity to this detergent than a control strain, J53 RP4, which has a wild-type cell envelope [7]. This was originally interpreted as an indication of the extremely permeable nature of the outer membrane of Doc S and its derivatives, Tuv and Cuv, to organic anions up to a molecular mass of 400 Da. This would include both of the uncoupler molecules used in this study.

However, with SDS and Triton X-100, the results were very different. J53 RP4 was able to grow normally on LB agar plates supplemented with 2% (w/v) SDS or 2% (w/v) Triton X-100. Doc S was unable to tolerate the presence of these detergents at 1% (w/v). In marked contrast, both Tuv and Cuv showed a resistance to 2% (w/v) SDS or Triton X-100, similar to that exhibited by J53 RP4.

There is a major change in the permeability of the outer membrane towards SDS (M_r 288) and Triton X-100 (M_r 646) in the resistant strains, although it is difficult to explain the sensitivity to deoxycholate (M_r 415) when the other detergents, which have similar molecular weights, have no discernible effects.

TABLE I

Gas-liquid chromatography of fatty acid methyl esters prepared from E. coli

Cells were grown to late log phase in * minimal medium and + minimal medium supplemented with 100 μM TTFB. Lipids were extracted, hydrolysed and the methyl esters prepared as described in Materials and Methods. ?, unidentified.

Retention time (min)	Putative identity of fatty acid	Strain and conditions		
		* Doc S (% of total)	* Tuv (% of total)	+ Tuv + TTFB (% of total)
1.0	C_{12}	1.1	1.1	1.0
1.7	C_{14}	3.0	2.9	3.1
2.3	?	2.2	2.4	2.4
3.0	C_{16}	32.8	33.1	33.4
4.2	$C_{16.1}$	16.9	17.0	16.6
5.0	$C_{\Delta 17}$	15.3	15.8	15.9
5.6	C_{18}	7.1	6.8	6.9
6.8	$C_{18.1}$	17.4	17.6	17.8
8.2	?	4.2	3.3	2.9

TABLE II

Volumes of cell compartments in E. coli

Values given are in μl/mg dry wt., an average of eight experiments ± S.D.

Strain	Whole cell	Cytoplasm	Periplasm
J53 RP4	2.2 ± 0.1	1.3 ± 0.11	0.8 ± 0.11
Doc S	1.7 ± 0.12	1.6 ± 0.12	0.1 ± 0.02
Tuv	2.7 ± 0.14	1.7 ± 0.09	1.05 ± 0.04
Cuv	2.6 ± 0.06	1.6 ± 0.11	1.1 ± 0.08

Cell compartment volume measurements

The cytoplasmic volumes of Doc S, Tuv and Cuv were determined under the same conditions as were used to measure membrane potentials. Whole cell and periplasmic volumes were measured at the same time (Table II).

The volumes of the whole cell (1.7 μl/mg dry wt.), cytoplasm (1.6 μl/mg dry wt.) and periplasm (0.1 μl/mg dry wt.) for Doc S agree closely with those published by Ahmed and Booth [15]. It is, however, misleading to conclude that Doc S has no periplasmic space. The treatment of Doc S with EDTA/lysozyme results in the rapid release of alkaline phosphatase, a periplasmic enzyme (data not shown). It would appear that the defect in the cell wall of Doc S results in permeability to molecules up to at least 5 kDa (hydroxymethylinulin), but less than 80 kDa (alkaline phosphatase).

Tuv and Cuv do not share this characteristic with Doc S. These strains appear to have a normal permeability barrier towards hydroxymethylinulin, effectively excluding it.

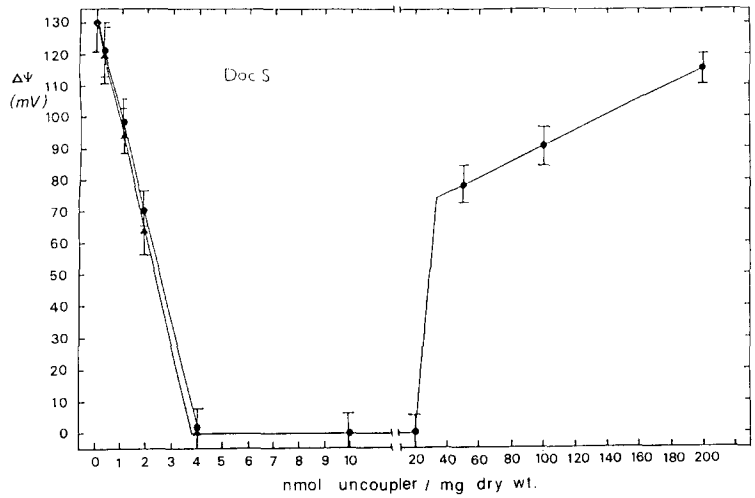

Fig. 1. The effect of uncouplers on the membrane potential of Doc S. Membrane potentials were determined as in methods. Values given are an average of four experiments. ●, TTFB; ▲, CCCP.

Effect of uncouplers on the membrane potential of E. coli

Doc S. Whole cells without prior treatment with EDTA were used. Fig. 1 shows that the resting membrane potential prior to uncoupler addition was 130 ± 10 mV. This is the mean ± S.D. for four experiments.

When the cells were titrated with uncoupler, the membrane potential decreased linearly, as seen in Fig. 1, reaching zero at 4 nmol uncoupler/mg dry weight. At concentrations of uncoupler greater than 20 nmol/mg dry weight, significant amounts of TPP^+ accumulated in the cells, giving an apparent enhancement of the membrane potential (Fig. 1). This is thought to be due to ion-pairing between the anionic uncoupler and the cationic probe, the complex then dissolving in the hydrophobic regions of the cell.

Tuv and Cuv. No TPP^+ uptake was observed in Tuv and Cuv. This is typical of wild-type strains of *E. coli*, where the cell wall excludes organic cations [17]. Thus, it was necessary to pretreat cells with 1 mM EDTA. Such treatment had no effect on the magnitude of the cytoplasmic volume in these cells (data not shown).

When the cells had been treated with EDTA, the resting membrane potentials of Tuv and Cuv were determined at 135 ± 6 mV and 140 ± 8 mV, respectively, the results being an average (± S.D.) from six experiments (Fig. 2). These values are not significantly differ-

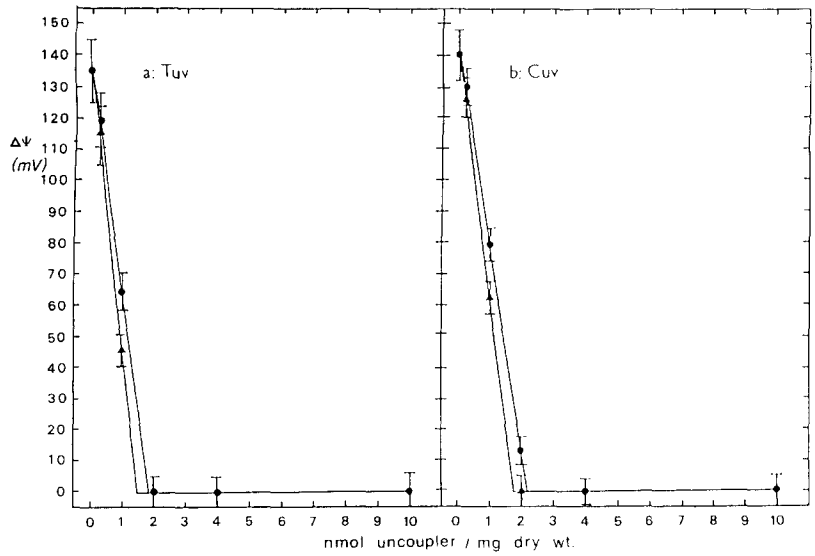

Fig. 2. The effect of uncouplers on the membrane potential of EDTA treated cells of Tuv (a) and Cuv (b). Values given are an average of six experiments. ●, TTFB; ▲, CCCP.

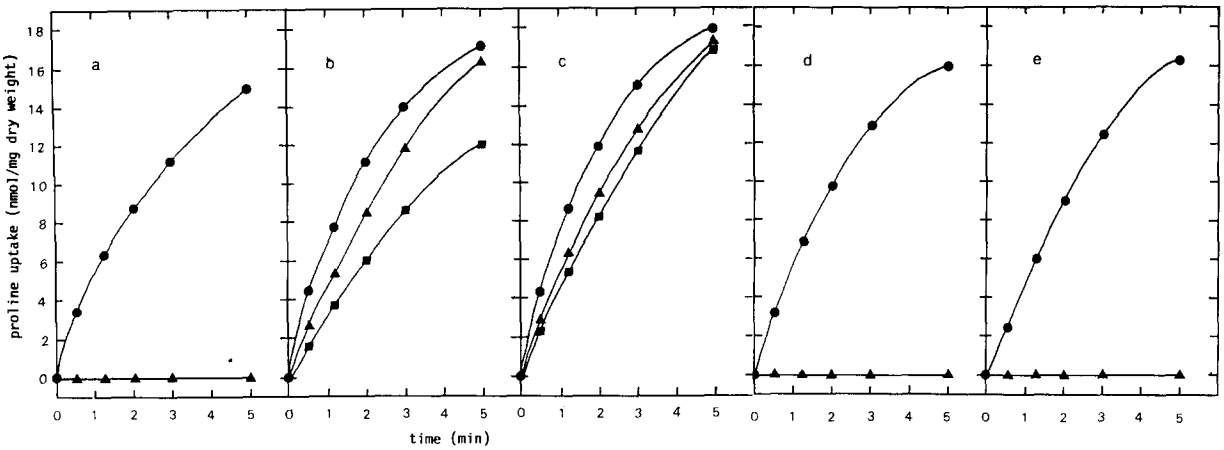

Fig. 3. The effect of uncouplers on proline uptake in untreated cells of Doc S (a), Tuv (b) and Cuv (c), and in EDTA-treated cells of Tuv (d) and Cuv (e). Cells were incubated in the absence of uncoupler (●), or in the presence of 10 nmol TTFB/mg dry weight (▲) or 10 nmol CCCP/mg dry weight (■).

ent to those obtained with Doc S. The effect of TTFB on the membrane potentials of these cells is shown in Fig. 2. There is a linear decline in the membrane potential, reaching zero in both cases at approx. 2 nmol TTFB/mg dry weight. As with Doc S, higher concentrations of TTFB gave an apparent enhancement of the membrane potential. CCCP had similar effects on the membrane potentials of strains Tuv and Cuv.

Thus, under conditions where it is feasible to measure the membrane potential, it appears that uncouplers are able to discharge it completely, regardless of the phenotype of the cell involved.

Effect of uncouplers on energy-dependent proline uptake

Proline uptake was measured in both untreated and EDTA treated cells, in the presence and absence of uncouplers. In the absence of uncoupler, Doc S maintained the uptake of proline over 5 min (Fig. 3a). The addition of 10 nmol TTFB/mg dry weight (sufficient to reduce the membrane potential to zero; see Fig. 1) completely inhibited proline uptake. With cells of Tuv and Cuv which had not been treated with EDTA, uncoupler-resistant proline uptake was observed (see Fig. 3b and c), as reported by Jones and Beechey [7].

However, Figs. 3d and e show that when Tuv and Cuv were treated with EDTA, uncoupler-resistant proline uptake was completely abolished.

Hence, it appears that uncoupler-resistant proline uptake in Tuv and Cuv is due to the exclusion of the uncoupler at the outer membrane of the cell. It is only when the lipopolysaccharide coat of the cell is disrupted with EDTA that uncouplers are able to pass freely across the outer membrane.

Transduction of TTFB resistance from Tuv to Doc S

P1 phage grown on Tuv was used to transduce Doc S. The transductants were then selected for TTFB resistance. No resistance was seen within 3 h of transduction. However, after that time the transduced Doc S did grow in the presence of TTFB. Since the P1 phage takes approx. 2% of the host genome during transduction, the observation of transduction of TTFB resistance from Tuv to Doc S suggests that few genes are involved. The time taken to acquire resistance in Doc S indicates that a component of the cell has to be replaced with the product of the uncoupler resistance gene(s) before uncoupler tolerance is exhibited.

Parallel experiments with the control strain CM2043 revealed that it is not possible to increase the natural resistance of this strain with P1 phage grown on Tuv.

Plating efficiency of P1 on E. coli

Phage P1 recognises and binds to the lipopolysaccharide coat of *E. coli* during infection [18]. Plating efficiency gives an estimate of the degree of binding of P1 to the bacterial cell wall. The efficiency of adsorption of phage P1 to Doc S was approx. 1% that of Tuv, CM2043, and a TTFB-resistant transductant of Doc S. These observations suggest that in Doc S there is a mutation in the lipopolysaccharide biosynthetic genes (*rfa*), which are located at 81 min on the *E. coli* chromosome. The absence of this defect in the uncoupler resistant strain is indicative of a reversion mutation in the *rfa* genes.

Transduction of E coli CM567 (pyrE$^-$) with P1 lysate from Doc S and Tuv (pyrE$^+$)

The *rfa* genes are located at 81 min on the *E. coli* chromosome, approx. 0.5 min from the *pyrE* genes [19]. The predicted cotransduction frequency of these genes by P1 is 50% [20]. *E. coli* CM567 (*pyrE*$^-$) was transduced to *pyrE*$^+$ with P1 grown on Doc S. Half of the transductants (9/16) became sensitive to novobiocin, confirming that the novobiocin/uncoupler sensitivity gene is located within 0.5 min of the *pyrE* gene. When

this experiment was repeated with Tuv as the donor, no novobiocin-sensitive transductants were observed among the *pyrE* transductants. Thus, it was possible to cotransduce the *rfa* mutation with the *pyrE* gene from strain Doc S, but not from strain Tuv. This suggests that uncoupler sensitivity in Doc S is related to a mutation in the *rfa* genes, and that a reversion in this mutation is responsible for uncoupler resistance in Tuv and Cuv. However, this change is not sufficient to account fully for the properties of Tuv and Cuv, since these strains remain very sensitive to deoxycholate. It is not clear how this is achieved, although it probably involves a further mutation in the outer membrane. Similarly, such a permeability change does not form a rational basis for explaining the differential sensitivity of Tuv and Cuv to different uncouplers [7].

In summary, the results presented in this paper suggest that Doc S contains a mutation in the *rfa* genes which causes the cell wall of this strain to be unusually permeable to lipophilic molecules, such as detergents, uncouplers, triphenyltins and TPP^+. A reversion of this mutation in Tuv and Cuv leads to a multiple resistance phenotype, such that cells of these strains are able to grow in the presence of titres of detergent (this paper), uncoupler [7], and trialkyltin [9] which inhibit the growth of Doc S.

Although Tuv and Cuv share many of the phenotypic characteristics of other uncoupler-resistant bacteria, sufficient evidence has been presented to show that resistance in some of the latter strains is not due to permeability changes in the outer membrane [3,11]. Elucidation of the mechanism of resistance in such strains is more challenging from a bioenergetic standpoint.

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