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Effect of R-Plasmid RP1 and Nutrient Depletion on the Gross Cellular Composition of *Escherichia coli* and Its Resistance to Some Uncoupling Phenols

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The resistance of *Escherichia coli* batch cultures depleted of carbon (C-dep), magnesium (Mg-dep), or phosphate (P-dep) against low concentrations of 3-chlorophenol, 4-chlorophenol, or 2-phenoxyethanol varied. C-dep cultures were always significantly more sensitive than Mg-dep or P-dep cultures. The presence of R-plasmid RP1 increased the sensitivity of C-dep cultures to 3- and 4-chlorophenol, yet had little effect on those cultures depleted in magnesium or phosphate ions. Cultures with R-plasmid RP1 had increased levels of β-polyhydroxybutyrate irrespective of the nature of the depleting nutrient. P-dep bacteria had less than one-third of the phospholipid of other cell types, this deficiency being compensated for by increases in fatty acid and neutral lipid content. The reduction in phospholipid content of P-dep cultures was entirely accounted for by decreased diphosphatidylglycerol and phosphatidylethanolamine levels in these cells.

The nature of a growth-limiting nutrient significantly influences the structure and composition of bacteria, especially the envelope (12, 15, 20, 30). Envelope changes have been associated with drug resistance by a mechanism of exclusion (2, 5, 6, 8, 9, 12, 16). With gram-negative bacteria such changes involve the outer membrane and may influence in vivo sensitivity to drugs (3).

There is now evidence that R-plasmid-mediated resistance sometimes involves drug exclusion as a result of envelope modification (17, 18, 29).

We have studied the interactions between R-plasmid RP1 and nutrient depletion of glycerol, magnesium, or phosphate, using *Escherichia coli* K-12. The effects of such interactions on gross chemical composition and on sensitivity to 3-chlorophenol, 4-chlorophenol, or 2-phenoxyethanol were measured. Low concentrations of these drugs uncouple oxidative phosphorylation from respiration (10, 22) by increasing the permeability of the cytoplasmic membrane to protons (14, 25). The uncoupling activity is related to the concentration of drug at the cytoplasmic membrane, and a linear relationship has been demonstrated between the initial rate of drug-induced proton translocation and drug concentration (10, 11). Variation in the drug concentrations iso-effective in proton translocation therefore probably reflects alteration in the ease of access of drug to its site of action through the wall (11).

MATERIALS AND METHODS

Organisms and chemicals. *E. coli* K-12 W3110, wild type and carrying R-plasmid RP1 (19), was used throughout. Stock cultures were maintained on solid minimal media (C-dep liquid media [below] plus 2% [wt/vol] agar) at 4°C after incubation for 16 h at 37°C.

2-Phenoxyethanol was obtained from Nipa Laboratories Ltd. (Pontypridd, U.K.), and its purity was checked by melting point, boiling point, and gas-liquid chromatography. Phospholipid standards were supplied by Lipid Products Ltd., South Nutfield, Redhill, Surrey, England. All other chemicals were of Analar grade (British Drug Houses Ltd., London, England).

Preparation of cell suspensions. Chemically defined liquid media were used in which growth of the cultures ultimately ceased at an optical density (E_{470}) of 2.0 as a result of depletion of glycerol (C-dep), magnesium (Mg-dep), or phosphate (P-dep), 1.52×10^{-2} M, 5.25×10^{-5} M, and 11.4×10^{-4} M, respectively. All other nutrients (NH_4FeSO_4, NH_4SO_4, NaCl, and KCl) were present in excess, and the media were buffered with 3-(*N*-morpholino)propane sulfonic acid (pH 7.5, 2.5×10^{-2} M).

Suspensions of bacteria were prepared from cultures grown for 48 h at 37°C in stirred 5-liter flat-bottomed flasks containing 2.5 liters of media. Harvesting was by centrifugation at 37°C (15,000 × *g*, 15 min), washing three times, and finally resuspending to an optical density (E_{470}) of 1.0 in glycyl-glycine buffer (pH 7.5, 2 mM, 37°C).

Drug sensitivity. To assess drug sensitivity, we measured rates of drug-induced proton translocation into bacteria by following the rate of change in pH of the extracellular phase, using a method based on that of Mitchell and Moyle (25). A 10-ml amount of a triple-washed cell suspension was transferred to a

jacketed vessel and well stirred at 37°C; the pH was measured with a glass-electrode, EIL 7050 pH meter and chart recorder. Amounts of HCl (0.005 M) sufficient to lower the pH to 3.8 were added to the suspension, and the subsequent rise in pH was monitored for approximately 10 min. Cell suspensions were preincubated for 5.0 min with various concentrations of the drugs prior to the addition of the acid. All determinations were performed twice in duplicate; initial rates of pH recovery were calculated and corrected for the rates of change of untreated cell suspensions. Results were finally expressed as rates of change of H^+ concentrations. Reproducibility was assessed by using means of five separate duplicate determinations with 0.21% (wt/vol) 2-phenoxyethanol for which χ^2 was 3.42 [χ^2 ($P = 0.05$, $\phi = 4$) = 9.49].

Cell composition. Total readily extractable lipid (REL), phospholipids, fatty and neutral lipid, and β-polyhydroxybutyrate (PHB) were assayed gravimetrically in whole-cell preparations. Freeze-dried cells (approximately 0.5 g, accurately weighed) were suspended in 50 ml of chloroform-methanol (2:1) and extracted for 90 min at 37°C with shaking. Insoluble material was removed by rapid centrifugation (20,000 × *g*, 10 min) and reextracted with a further 50 ml of fresh solvent. The two extracts were bulked, filtered in a preweighed flask, and evaporated to dryness. REL was dried to constant weight over phosphorus pentoxide in vacuo. The weighed REL was taken up in 1 ml of solvent, and excess diethylether (30 ml) at −10°C was added to precipitate PHB (7). This was removed by centrifugation (20,000 × *g*, 15 min), and the ethereal extract was evaporated, dried, and weighed (as above). PHB was additionally characterized by infrared spectroscopy of itself and of its hydrolysis product and also by electron microscopy of the cells. Phospholipid content was similarly determined by precipitation of the ethereal extract with excess acetone (30 ml) at −10°C. The remaining acetone-soluble fraction contained fatty and neutral lipids. Determinations were performed in duplicate, and results are expressed as percent (dry weight). 2-Keto-3-deoxyoctonic acid (KDO), a marker for lipopolysaccharide, was assayed by the method of Weissbach and Hurrwitz (33). The relative amounts of each component phospholipid were determined by thin-layer chromatography, by use of the method and solvent system (A) of Minnikin and Abdulrahimzadeh (21). The developed plates were analyzed with a Chromoscan 200/201 densitometer (Joyce-Loebl, Gateshead, England).

RESULTS AND DISCUSSION

The data in Fig. 1 show plots of proton translocation rate against drug concentration for depleted cultures with and without R-plasmid RP1. C-dep suspensions containing the R plasmid were significantly more sensitive to 3-chlorophenol and 4-chlorophenol than the wild type. Sensitivity to 2-phenoxyethanol however did not alter significantly. P-dep and Mg-dep suspensions were markedly less sensitive to all three drugs than were C-dep ones. The presence of R-plasmid RP1 had little or no effect on the sensitivity of these suspensions.

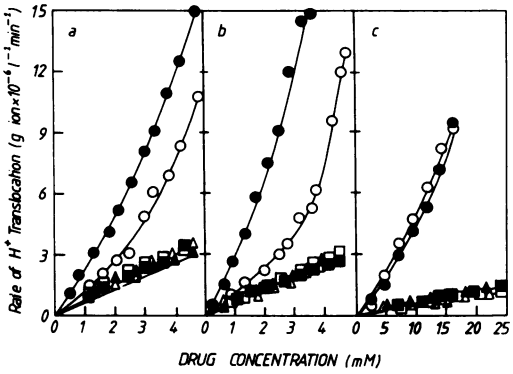

FIG. 1. *Effect of R-plasmid RP1 and nutrient depletion on the sensitivity of E. coli K-12 W3110 to (a) 4-chlorophenol, (b) 3-chlorophenol, and (c) 2-phenoxyethanol. Open symbols, Wild type; closed symbols, with R-plasmid RP1.* (○, ●) *Carbon-depleted;* (□, ■) *PO_4-depleted;* (△, ▲) *Mg-depleted.*

The level of uncoupling activity for a given drug, measured by proton translocation rate, is related to the concentration of drug at the cytoplasmic membrane. Variation in iso-effective concentrations for these drugs in cell suspensions depleted of different nutrients therefore probably reflects alterations in the ease of drug penetration through the outer envelope (11). Such mechanisms of resistance have been widely implicated for a number of gram-negative organisms, notably *Pseudomonas aeruginosa* where alteration in the composition of the cell envelope affects penetration by antimicrobial agents (2).

For many gram-negative organisms the nature of depleting nutrients can markedly affect cellular lipid composition (1, 13), lipopolysaccharide content (31), and drug sensitivity (4–6, 8, 9, 12). It seemed possible, therefore, that R-plasmid RP1 altered the envelope composition of *E. coli* cells under conditions of carbon starvation in such a way as to increase penetration by 3-chlorophenol and 4-chlorophenol but not 2-phenoxyethanol. This possibility was investigated further by examining the gross cellular composition of these cells.

The data in Table 1 show the total REL, PHB, phospholipid, fatty and neutral lipid, and KDO content for P-dep, Mg-dep, and C-dep cultures with and without the R-plasmid RP1. Bacteria containing the R plasmid possessed higher amounts of PHB than their corresponding wild types. This difference was most marked under carbon-depleted conditions where PHB constituted 5.8% of the cells' dry weight. This probably accounted for the increased REL for these cells, there being no significant variation in REL for the remainder. Although this observation is interesting per se, it is difficult to

TABLE 1. *Chemical analysis of whole-cell preparations of E. coli K-12 W3110 under conditions of different nutrient depletions and carrying R-plasmid RP1*[a]

Nutrient depletion	R-plasmid RP1	Total REL (%, dry wt)	PHB (%, dry wt)	Phospholipid (%, dry wt)	FAN (%, dry wt)	KDO (μg/mg, dry wt)
Carbon	−	11.03 ± 2.17	0.223 ± 0.45	4.61 ± 0.39	6.20 ± 0.39	11.16 ± 0.24
Carbon	+	14.80 ± 0.23	5.831 ± 0.48	4.43 ± 0.21	4.53 ± 0.21	9.74 ± 1.35
Magnesium	−	10.68 ± 1.82	0.672 ± 0.29	4.69 ± 0.75	5.30 ± 0.75	9.11 ± 0.19
Magnesium	+	10.72 ± 0.96	1.281 ± 0.07	4.69 ± 0.81	4.74 ± 0.81	10.63 ± 1.92
Phosphate	−	11.78 ± 1.37	1.226 ± 0.21	1.36 ± 0.23	9.20 ± 0.23	8.44 ± 0.42
Phosphate	+	11.54 ± 0.25	1.728 ± 0.12	1.31 ± 0.06	8.51 ± 0.06	9.16 ± 0.18

[a] REL, Readily extractable lipid; PHB, β-polyhydroxybutyrate; FAN, fatty and neutral lipid; KDO, 2-keto-3-deoxyoctonic acid.

TABLE 2. *Phospholipid composition of whole-cell preparations of E. coli K-12 W3110 under conditions of different nutrient depletions and carrying R-plasmid RP1*

Nutrient depletion	R-plasmid RP1	Composition (%, dry wt)[a]			
		DPG	PE	PG	PC
Carbon	−	1.634 ± 0.06	2.090 ± 0.19	0.583 ± 0.06	0.184 ± 0.03
Carbon	+	1.701 ± 0.11	1.943 ± 0.17	0.602 ± 0.005	0.184 ± 0.02
Magnesium	−	1.773 ± 0.19	2.302 ± 0.04	0.397 ± 0.12	0.226 ± 0.04
Magnesium	+	2.025 ± 0.06	1.960 ± 0.08	0.511 ± 0.14	0.198 ± 0.01
Phosphate	−	0.600 ± 0.09	0.367 ± 0.04	0.342 ± 0.06	0.160 ± 0.02
Phosphate	+	0.586 ± 0.06	0.301 ± 0.03	0.372 ± 0.02	0.178 ± 0.01

[a] DPG, Diphosphatidyl glycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine.

interpret it in terms of the observed alterations in drug sensitivity, especially since PHB is an intracellular inclusion and unlikely to affect penetration of the outer envelope by these drugs. Under certain conditions it might, however, act as a sink for high intracellular drug uptake, reducing the available drug concentration at the site of action and thereby increasing resistance; such a mechanism of resistance has been suggested for "fattened" *Staphylococcus aureus* cells towards phenol (16). The increased production of PHB in $RP1^+$ bacteria may prove of survival advantage in some circumstances, especially in an environment depleted of an energy source. Of the remaining cellular components studied, none varied significantly with the presence or absence of the R plasmid. The mechanism of the varying drug sensitivity remains unknown. However, we suggest that increased sensitivity might possibly result from a more subtle perturbation of the envelope such as might be caused by a change in lipopolysaccharide not affecting the KDO (27) assay or by variation in envelope proteins.

Phospholipid content was markedly reduced under P-dep conditions to 1.3% (dry weight), from approximately 4.5% (dry weight) for C-dep and Mg-dep cells. The phospholipid loss was compensated for by a corresponding increase in fatty and neutral lipid content. This situation is analogous to the findings with *Bacillus subtilis*, where phosphate depletion leads to a decreased cell phospholipid content and increased phosphate-free polar lipid content (24), and suggests some duality of function between the two (22, 23). The decrease in phospholipid content was found to originate from reduction in diphosphatidylglycerol and phosphatidylethanolamine, whereas phosphatidylglycerol and phosphatidylcholine content remained relatively stable (Table 2). Phospholipids have been implicated as ionophores for a number of metal cations (31) and amino acids (26). Variation in the phospholipid composition of bacterial cells, such as this, would therefore profoundly affect their ability to transport and assimilate a number of nutrients. Changes in relative proportions of neutral and phosphatide lipids have been implicated in gentamicin resistance (28). In this study no variation in drug sensitivity was observed between Mg-dep and P-dep cultures, despite their fatty and neutral lipid-to-phospholipid ratios being markedly different.

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