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Characterization of an *Escherichia coli* K12 Mutant that is Sensitive to Chlorate when Grown Aerobically

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Escherichia coli can normally grow aerobically in the presence of chlorate; however, mutants can be isolated that can no longer grow under these conditions. We present here the biochemical characterization of one such mutant and show that the primary genetic lesion occurs in the ubiquinone-8-biosynthetic pathway. As a consequence of this, under aerobic growth conditions the mutant is apparently unable to synthesize formate dehydrogenase, but can synthesize a Benzyl Viologen-dependent nitrate reductase activity. The nature of this activity is discussed.

Wild-type strains of *Escherichia coli* can grow in the presence of chlorate under aerobic conditions, but under anaerobic conditions growth is inhibited (Azoulay *et al.*, 1969*b*). This observation has been explained on the assumption that under anaerobic conditions chlorate, an analogue of nitrate, induces nitrate reductase (EC 1.7.99.4) and is converted into chlorite by the enzyme, and that chlorite is toxic to cells with the result that cell growth ceases: aerobic growth in the presence of chlorate can occur, since under these conditions nitrate reductase activity is repressed (Azoulay *et al.*, 1969*b*). By selecting colonies that were simultaneously neomycin-resistant and unable to grow on oxidizable substrates, mutants have been isolated that show a chlorate-sensitive phenotype when grown under aerobic conditions, in the presence of a fermentable carbon source (Giordano *et al.*, 1977*b*): some of these mutants exhibited a temperature-dependent phenotype, that is, under aerobic conditions in the presence of chlorate, they were able to grow at 22°C, but not at 32°C. A preliminary analysis indicated that the activity of the oxygen-dependent electron-transport chain in these mutants was decreased and, furthermore, that the synthesis of nitrate reductase was, in part-de-repressed under aerobic growth conditions.

The object of the present study was to characterize further one of these novel mutants with respect to the redox components synthesized and their functional activity in the cell.

Abbreviation used: Mops, 4-morpholinepropane-sulphonic acid.

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Material and Methods

Bacterial strains and growth conditions

The bacterial strains used and their relevant genotypic and phenotypic properties are listed in Table 1. Bacteria were grown in either a minimal medium (Davis & Mingioli, 1950; Azoulay *et al.*, 1969*a*) to which glucose (1 g/litre for aerobic growth or 2 g/litre for anaerobic growth) and other growth-factor supplements were added after sterilization or, alternatively, the complex L-medium of Lennox (1955). For anaerobic growth KNO_3 (1 g/litre) was also added.

Preparation of membrane particles

Membrane particles were prepared by the method of either Azoulay *et al.* (1969*a*) or Schairer *et al.* (1976) depending on the experiment to be performed.

Assay techniques

Formate dehydrogenase activity (EC 1.2.–.–) was assayed anaerobically in the presence of phenazine methosulphate as described by Pichinoty (1969).

Table 1. *Strains of E. coli K12 used*
The nomenclature of genes is that of Bachmann *et al.* (1976).

Strain	Relevant genetic loci	Resistance (R) or sensitivity (S) to aerobic growth in the presence of chlorate	Origin and other information
PA601	F^-, *thr*$^-$, *leu*$^-$, *his*$^-$, *pro*$^-$, *arg*$^-$, *thi*$^-$, *ade*$^-$, *gal*$^-$, *lacY*$^-$, *malE*$^-$, *xyl*$^-$, *ara*$^-$, *mtl*$^-$, *strR*, T_1R	R	Institut Pasteur
356-15	*chlA*$^-$ otherwise as PA601	R	Spontaneous mutation of PA601
356-24	*chlB*$^-$ otherwise as PA601	R	Spontaneous mutation of PA601
541	*pro*$^+$ otherwise as PA601	R	Derived from PA601 by P_1 transduction
72	as 541 with additional mutation	S	Giordano *et al.* (1977*b*)
AN59	*ubiB*$^-$	S	A. E. Lagarde (I. G. Young)

One unit was defined as 1 μmol of CO_2 formed/h per mg of protein.

Reduced Benzyl Viologen-dependent nitrate reductase activity (EC 1.7.99.4) was measured manometrically as described by Pichinoty (1963) and expressed as nmol of NO_2^- formed/h per mg of protein.

Formate-dependent nitrate reductase activity (EC 1.2.2.1 with EC 1.7.99.4) was measured in membrane particles or whole resting cells, in the presence of either O_2 or N_2, in reaction mixtures containing 0.25 M-KNO_3, 10 mM-sodium formate and 100 mM-phosphate buffer, pH 7.0. Glucose-dependent nitrate reductase activity was measured in whole resting cells in the same way as the formate-dependent activity, formate being replaced by 20 mM-glucose. Nitrite was determined by the method of Rider & Mellon (1946). One unit was defined as 1 nmol of NO_2^- formed/h per mg of protein or mg dry wt. of cells.

NADH–$NADP^+$ transhydrogenase activities were determined by the method of Schairer *et al.* (1976). Membrane particles (30–50 μg of protein/ml) were added to 50 mM-Tris/acetate buffer, pH 7.5, containing 5 mM-$MgCl_2$, 6 mM-hydrazinium dichloride, 4 μM-NADH, 130 mM-ethanol, 4 units of alcohol dehydrogenase (EC 1.1.1.2) and 2 mM-KCN. The energy-independent activity was measured at 25°C by following NADPH formation at 334 nm after addition of 6 μM-$NADP^+$. The increase in the reaction rate after addition of 5 mM-ATP reflects an ATP-dependent activity. For the assay of the respiration-dependent activity, KCN was omitted, and 2.5 mM-D-lactate was substituted for ATP. One unit was expressed as 1 nmol of NADPH formed/min per mg of protein. Quenching by acridine dye was measured in a 2 ml reaction mixture containing 50 mM-KCl, 10 mM-$MgCl_2$, 50 mM-Mops/KOH buffer, pH 6.5, 2.5 mM-9-amino-6-chloro-2-methoxyacridine and membrane particles (about 600 μg of protein). Fluorescence was measured at 25°C with a Fica spectrofluorimeter connected to a chart recorder. Fluorescence was excited by light at 430 nm and emission was measured at 500 nm. The respiration, dependent quenching coupled to oxygen and nitrate was determined after the addition of 5 mM-formate, 2.5 mM-D-lactate or 2.5 mM-NADH. For anaerobic assays carried out in the presence of 20 mM-nitrate, all the solutions were strongly de-aerated by bubbling with N_2. The ATP-dependent quenching was determined after the addition of 2.5 mM-ATP.

O_2 uptake in whole resting cells, previously grown either aerobically or anaerobically with nitrate, was measured with the Clark electrode in the presence of 20 mM-glucose, D-lactate, succinate or formate. Respiratory activity was expressed as nmol of O_2 consumed/min per mg of cell dry wt. (Giordano *et al.*, 1977*a*).

Cytochrome contents were calculated from reduced-minus-oxidized difference spectra recorded at room temperature (20°C) in a Beckman ACTA III double-beam spectrophotometer. Samples were reduced with either 0.1 mM-sodium dithionite or 1 mM-sodium formate and oxidized with either 1 mM-H_2O_2 or 1 mM-KNO_3. The following millimolar extinction coefficients were used (expressed as litre·$mmol^{-1}$·cm^{-1}): for cytochrome *b* at $A_{560}-A_{575}$ a value of 17.5 (Jones & Redfearn, 1966); for cytochrome *a* at A_{594}–A_{610} a value of 8.5, as suggested by Meyer & Jones (1973); for cytochrome *d* at $A_{630}-A_{610}$ a value of 8.5 (Jones & Redfearn, 1966).

Reconstitution of nitrate reductase activity in vitro

This was carried out by the 'complementation technique' of Azoulay *et al.* (1969*a*) in reaction mixtures containing supernatant extracts (17 mg/ml) of the *chlA* mutant, anaerobically grown with nitrate, and of other strains grown under various conditions. Reconstituted reduced Benzyl Viologen-dependent nitrate reductase activity was assayed as described (Azoulay *et al.*, 1969*a*).

Extraction and chromatography of quinones

This was carried out as described by Cox & Gibson (1966). Aerobically grown bacteria (20 g dry wt.) were harvested by centrifugation, transferred to a round-bottomed flask, and suspended in 300ml of acetone. The acetone was removed by rotary evaporation for 2h. After the addition of 300ml of diethyl ether, evaporation was continued for a further 2h until a volume of 60ml was obtained. The residue was resuspended in 150ml of light petroleum (b.p. 65–95°C), and the volume was decreased to approx. 15ml by rotary evaporation.

The light-petroleum extracts of strains 541 and 72, and solutions of ubiquinone-10 (0.5mg/l) and menaquinone-K_3 (0.5mg/ml), were chromatographed on silica-gel plates with chloroform/light petroleum (3:1, v/v) as solvent. After exposure of part of the plate to I_2 vapour or diazotized *p*-nitroaniline, the compounds were eluted with diethyl ether and further purified by a second chromatography on the same plates. The compounds were eluted with carbon tetrachloride for n.m.r. analysis and mass-spectroscopy studies, or with ethanol for spectroscopic analysis, then evaporated (Gibson, 1973).

Spectroscopy

N.m.r. spectra were obtained with a Cameca 250Mhz apparatus. Mass spectra were obtained with a DS 50 mass spectrometer. In both cases, ubiquinone-10 and ubiquinone-8 were used as standards. Concentrations of ubiquinone-8 and menaquinone were determined by difference u.v. spectroscopy between the oxidized form in ethanol and the $NaBH_4$-reduced form, with a Beckman ACTA III spectrophotometer, and the appropriate millimolar extinction coefficients (expressed as litre·mmol^{-1}·cm^{-1}): for ubiquinone-8 a value of 12.7 at 275nm (Crane & Barr, 1971) and for menaquinone-9 a value of 18.3 at 245nm (Dunphy & Brodie, 1971).

Results

Respiratory activity of strain 72

This mutant is unable to grow with succinate or D-lactate as sole carbon source, and exhibits a respiratory activity with glucose that is some 40% lower than that of the parental strain 541 (Giordano *et al.*, 1977*b*). These initial observations are confirmed and extended by the results in Table 2, which demonstrate that strain 72 exhibits a much decreased respiratory activity towards a variety of different substrates when grown either aerobically or anaerobically in the presence of NO_3^- as compared with strain 541.

Nitrate reductase activity of strain 72

When assayed anaerobically under an atmosphere of N_2, whole resting cells of strains 541 and 72, previously grown anaerobically with nitrate, couple the reduction of nitrate to the oxidation of formate or glucose (Table 3). When assayed in the presence of O_2, the formate-dependent nitrate reductase activities are lowered by 75% for strain 541 and 32% for strain 72, whereas the glucose-dependent nitrate reductase activity is completely inhibited and lowered by 60% for strain 72 compared with the determinations performed under N_2.

Membrane particles derived from strain 72, grown aerobically with or without nitrate, have only a very low formate-dependent nitrate reductase activity (Table 4); these particles do, however, possess a reduced Benzyl Viologen-dependent nitrate reductase activity (Giordano *et al.*, 1977*b*). This latter activity is completely inhibited by heating membrane particles at 100°C for 5min and is 90% inhibited on the addition of 1mM-NaN_3, indicating the enzymic nature of the reaction. This conclusion was confirmed by similar results obtained with resting cells (results not shown).

Table 4 also shows that membrane particles derived from strains 541 and 72, previously grown anaerobically in the presence of nitrate, exhibit a formate-dependent nitrate reductase activity that is lowered by 50% when assayed in the presence of O_2.

Formate oxidation by membrane particles derived from strain 72

Membrane particles from strain 72, previously grown aerobically on complex medium with added

Table 2. *Effects of growth conditions on the respiratory activities of whole cells of strains* 541 *and* 72 *towards different substrates*

O_2-uptake studies were performed as indicated in the Materials and Methods section on cells grown either aerobically or anaerobically with NO_3^-. Substrates were added at a concentration of 15mM. The indicated values were corrected for the endogenous activity obtained when no substrate was added. These endogenous values represent about 2% of the value obtained with the substrate.

	Respiratory activity (nmol of O_2 consumed/min per mg dry wt. of cells)			
Growth conditions ...	Aerobically		Anaerobically+NO_3^-	
Substrate Strain ...	541	72	541	72
Glucose	94	51	48	21
Succinate	15	<1	10	<1
D-Lactate	40	5	51	25
Formate	33	<1	100	50

glucose, have a very low formate dehydrogenase activity when compared with the parent strain 541 (Table 4). However, membrane particles from both strains, prepared from cells grown anaerobically in the presence of nitrate, exhibit the same high formate dehydrogenase activity, an activity that is not shown by the *chlB* mutant 356-24. Clearly then, the mutation in strain 72 does not result primarily in the loss of formate dehydrogenase activity, but as a secondary consequence of the mutation formate dehydrogenase is not expressed under all growth conditions.

NADH–NADP⁺ transhydrogenase activities

The NADH oxidase activity of membrane particles prepared from aerobically grown cells of strain 72 is one-tenth that shown by a similar preparation prepared from strain 541. In accordance with this, Table 4 indicates that the ATP-dependent transhydrogenase activity is the same for both strains 541 and 72, but the respiration-dependent transhydrogenase activity of strain 72 is only 39% of that shown by strain 541. These results can best be ascribed to a generalized defect in the functional activity of the aerobic electron-transport chain(s) in strain 72, as discussed below.

Table 3. *Effects of aerobic and anaerobic assay conditions on the formate- and glucose-dependent nitrate reductase activities of resting cells of strains* 541 *and* 72

Cells were grown anaerobically in the presence of nitrate. Assays were performed as indicated in the Materials and Methods section and are expressed as nmol of NO_2^- formed per mg dry wt. of cells. The indicated values were corrected for the endogenous activity obtained when no substrate was added. These endogenous values represent about 2% of the value obtained with the substrate.

	Formate-dependent nitrate reductase activity		Glucose-dependent nitrate reductase activity	
	N_2	O_2	N_2	O_2
Strain 541	6000	1500	1700	0
Strain 72	7000	4800	2300	870

Energy-dependent quenching of 9-amino-6-chloro-2-methoxyacridine by membrane particles of strains 541 and 72

In membrane particles from strain 541, the respiratory- and ATP-driven quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence resembles results presented for atebrin quenching in an *E. coli* prototroph (Haddock & Downie, 1974; Haddock & Kendall-Tobias, 1975). Thus respiratory-driven acridine-dye quenching with NADH, D-lactate and formate as electron donors and either O_2 or NO_3^- as terminal electron acceptors can be demonstrated in membrane particles derived from strain 541 grown aerobically or anaerobically with O_2 or NO_3^- respectively. In addition membrane particles from strain 541 exhibited a similar ATP-dependent acridine-dye quenching as did equivalent particles prepared from strain 72 (Figs. 1*a* and 1*c*).

Strain 72, however, when grown aerobically with or without NO_3^- gave membrane particles that did not show any quenching with acridine dye with formate as substrate and O_2 as electron acceptor (Fig. 1*a*); with NADH or D-lactate as respiratory substrate, quenching with acridine dye was observed,

Table 4. *Enzymic activities of membrane particles from strains* 541, 72 *and* 356-24, *grown in different conditions*

Cells grown under a variety of conditions; membrane particles were prepared and assayed as described in the Materials and Method section. The formate-dependent nitrate reductase was measured under N_2 or O_2 and is expressed in nmol of NO_2^- formed/h per mg of protein. The formate dehydrogenase activity was determined as indicated in the Materials and Method section and is expressed in μmol of CO_2 formed/h per mg of protein. The NADH–NADP⁺ transhydrogenase activity is expressed in nmol of NADPH formed/min per mg of protein. Abbreviation: N.D., not determined.

Strain	Growth conditions	Formate-dependent nitrate reductase activity		Formate dehydrogenase activity	NADH–NADP⁺ transhydrogenase activities		
		N_2	O_2		Energy-independent	Respiration-dependent	ATP-dependent
541	Aerobically	0	0	11.6	2.8	13.6	19
	Aerobically+NO_3^-	0	0	11.6	N.D.	N.D.	N.D.
	Anaerobically+NO_3^-	2000	700	20.0	N.D.	N.D.	N.D.
72	Aerobically	25	20	0.2	3.3	5.3	21.3
	Aerobically+NO_3^-	20	20	0.3	N.D.	N.D.	N.D.
	Anaerobically+NO_3^-	5000	2400	21.0	N.D.	N.D.	N.D.
356-24	Aerobically	0	0	0	N.D.	N.D.	N.D.
	Aerobically+NO_3^-	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Anaerobically+NO_3^-	0	0	0	N.D.	N.D.	N.D.

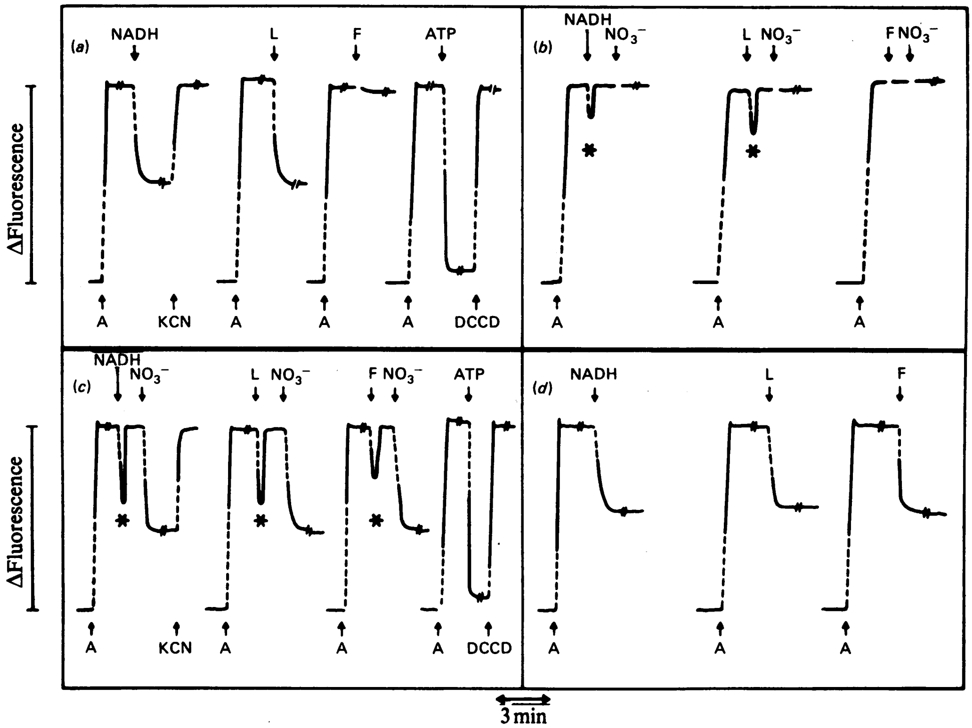

Fig. 1. *Quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence in membrane particles from strain* 72
Cells were grown aerobically with (*a*) or without (*b*) nitrate or anaerobically in the presence of nitrate (*c*, *d*). Particles were prepared and the quenching of acridine-dye fluorescence was measured as indicated in the Materials and Methods section. Although Helma cuvettes specially adapted for anaerobiosis, and N60 nitrogen (Air-Liquide France, Marseille, France) containing less than 0.1 p.p.m. of O_2 were used, and despite strong de-aeration of solutions used for the anaerobic assays (*b* and *c*), the remaining traces were sufficient to support a brief burst of respiratory-driven dye quenching on the addition of substrate, as indicated by *. Abbreviations: A, 9-amino-6-chloro-2-methoxyacridine; F, formate; L, D-lactate; DCCD, dicyclohexylcarbodi-imide.

but only to about 50% of the extent obtained with membrane particles from strain 541. The lack of formate-dependent acridine-dye quenching in membranes from aerobically grown strain 72 confirms other results described in this paper, e.g. lack of formate dehydrogenase activity and the inability of formate to reduce the cytochrome components of the electron-transport chain.

Significantly, with NO_3^- as the terminal electron acceptor, no respiratory-driven acridine-dye quenching was observed with particles prepared from cells of both strains grown anaerobically in the presence of NO_3^- (Fig. 1*b*): this indicates that the nitrate reductase activity observed with strain 72 under these growth conditions is either kinetically incompetent to serve as a terminal electron reaction centre to support respiratory-driven acridine-dye quenching or is functionally different from the nitrate reductase activity synthesized under anaerobic growth conditions.

With membrane particles from strain 72 grown anaerobically in the presence of nitrate, a decreased fluorescence quenching (70% of the extent observed with particles from strain 541) was seen with NO_3^- as electron acceptor and NADH, D-lactate or formate as substrate (Fig. 1*c*). The addition of the same substrates to particles prepared from a pleiotropic mutant defective in formate dehydrogenase and nitrate reductase (ChlA⁻, strain 356-15) did not lead to any quenching with nitrate as electron acceptor, no quenching with acridine dye was observed with formate, but a normal extent of quenching was recorded with NADH or D-lactate as the respiratory substrate (results not shown).

Complementation between soluble extracts of strains 72 and 356-15

To determine whether strain 72 accumulates some of the constituents of the nitrate reductase complex [peptides A, B and C described by Enoch & Lester

(1974)] in its cytoplasm, we used the complementation technique *in vitro*, described by Azoulay *et al.* (1969*a*), which allows the reconstitution of the nitrate reductase activity.

For these experiments, strain 356-15 (*chlA*) was grown anaerobically with nitrate and strain 72 aerobically. Washed cells were disrupted in the French Press and crude extracts were centrifuged twice at 200000*g* for 90 min to obtain supernatants cleared of all sedimentable material (Azoulay *et al.*, 1969*a*). Incubation at 32°C for 2 h of reaction mixtures containing equivalent amounts of extracts from strains 72 and 356-15 results in the reconstitution of a nitrate reductase activity, but the separate incubation of the two extracts does not give any reconstitution (Table 5). The amount of reconstituted activity is 37 % of that obtained between the extracts of strains 356-15 and 356-24 (mutant *chlB*) both grown anaerobically in the presence of nitrate. We must stress that the parental strain 541 grown in aerobic conditions gives extracts with no Benzyl Viologen-dependent nitrate reductase and which cannot complement with the extracts of the strain 356-15. The ability of soluble extract 72 to complement means that it contains the product of gene *chlA* which accumulates, possibly together with other components of the nitrate reductase complex, in the cytoplasm of strain 72, because of an inability to integrate within the cytoplasmic membrane.

Cytochrome content of membranes from aerobically grown strain 72

During anaerobic growth, the addition of nitrate induces the synthesis of a specific *b*-type cytochrome,

Table 5. *Effects of growth conditions on the reconstitution of nitrate reductase by complementation in vitro of the soluble extracts from various strains with the supernatant extract from mutant* 356-15 (*chlA*) *grown anaerobically in the presence of nitrate*

Reduced Benzyl Viologen-dependent nitrate reductase was reconstituted (Azoulay *et al.*, 1969*a*) in a reaction mixture containing 17 mg of protein/ml. All the supernatant extracts used to make these different complementations do not have any reduced Benzyl Viologen-dependent nitrate reductase activity and a separate incubation of the supernatants does not result in any reconstitution of this activity.

Complementation of strain 356-15 with:	Growth conditions	Reconstituted reduced Benzyl Viologen-dependent nitrate reductase
Strain 356-24	Anaerobiosis+nitrate	11 000
Strain 356-24	Anaerobiosis	5000
Strain 72	Aerobiosis	4000
Strain 72	Aerobiosis+nitrate	5000
Strain 541	Aerobiosis	0
Strain 541	Anaerobiosis+nitrate	0

cytochrome $b_{556}^{NO_3^-}$, which is kinetically (Haddock *et al.*, 1976) and genetically (Ruiz-Herrera & De Moss, 1969) distinct from other *b*-type cytochromes synthesized by *E. coli* (Azoulay *et al.*, 1977). The presence of cytochrome $b_{556}^{NO_3^-}$ is required for the expression of the membrane-bound formate-dependent nitrate reductase activity (Enoch & Lester, 1972, 1974).

The maximum absorption of membrane-bound *b*-type cytochromes of strain 72 grown aerobically with or without nitrate was shifted 2 nm toward lower wavelengths compared with similar data obtained with aerobically grown cells from strain 541. This shift can best be explained by assuming that the relative contents of the different *b*-type cytochromes (b_{558}, b_{556}, b_{562} and *o*) are not the same in membranes from the two different strains. Nevertheless, the total content of the *b*-type cytochromes (0.40 and 0.50 nmol/mg of protein), cytochrome a_1 (0.09 and 0.06 nmol/mg of protein) and cytochrome *d* (0.34 and 0.36 nmol/mg of protein) were similar for strains 541 and 72 respectively. The *b*-type cytochromes of strain 72, grown aerobically in the presence of nitrate, cannot be reduced by sodium formate; in addition, if reduced with low concentrations of sodium dithionite (0.1 mM), they cannot subsequently be reoxidized by nitrate even at high concentration (10 mM) and after extensive incubation (30 min). The complete opposite was observed when strains 541 and 72 were grown anaerobically with nitrate; the membrane-bound *b*-type cytochromes were reduced quickly and extensively by formate and partially reoxidized by nitrate.

Analysis of the quinone content of strain 72

Aerobically grown cells of strain 541 contained ubiquinone (80 nmol/mg dry wt. of cells) and menaquinone (8.5 nmol/mg dry wt. of cells) at concentrations similar to those found in other strains (Haddock & Schairer, 1973).

After extraction of lipids, separation by t.l.c. and detection by exposure to I_2 vapour, cells of aerobically grown strain 72 showed three characteristic components (Fig. 2). The first of these (S1) migrated as menaquinone (S7) and was shown to be menaquinone after purification and assay (16 nmol/mg dry wt. of cells). Spot 3 (S3) had the same R_F value (0.53) as 2-octaprenylphenol (S4), which is also present in the lipid extract of the ubiB$^-$ mutant (strain AN59) (Cox *et al.*, 1969). Clearly compound S3 does not have the same u.v.-absorption spectrum or R_F values as ubiquinone-8 (S6; $R_F = 0.44$) or ubiquinone-10 (S9), and cannot be reduced by $NaBH_4$ (Fig. 3). Compound 2 (S2), which had the same u.v. spectrum as component S3 but a different R_F value (0.68), must be regarded as a new unidentified compound. It should be noted that both compounds 2 and 3 can be detected after spraying with diazotized *p*-nitroaniline, a reagent specific for phenolic compounds (Gibson, 1973).

Identification of compounds S2 and S3

N.m.r. spectrometry. With compound S3, the various proton signals obtained indicated that it was

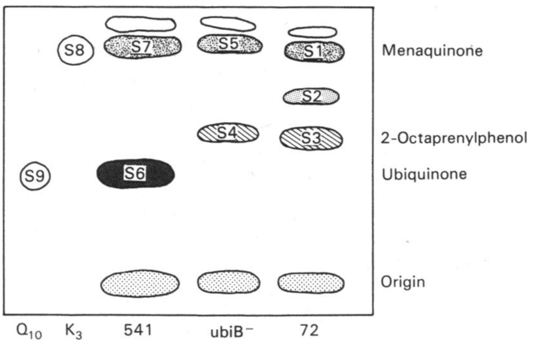

Fig. 2. *T.l.c. of lipid extracts from strains* 541, 72 *and AN59 (ubiB⁻)*
Separation was performed on silica-gel plates, and the various components (S1–S9) were detected after exposure to I_2 vapour. Further details are given in the Materials and Methods section.

definitely 2-octaprenylphenol. However, compound S2, isolated by t.l.c. in the same experiment as compound S3, could not be analysed with sufficient precision by n.m.r. The concentration of compound S2 was very low and spectra were difficult to interpret, owing to the presence of several impurities introduced with the solvents used in the extraction procedure. Nevertheless, it was possible to conclude that compound S2 is a phenolic compound, probably containing more than one hydroxy group.

Mass spectrometry. The mass spectrum of 2-octaprenylphenol is described by Cox *et al.* (1969). Our mass spectrum for compound S3 showed the same fragmentation with a weak molecular peak at 638 with the more important peak at 690. For compound S2, the presence of impurities was too high and the spectrum was not analysed in detail; however, fragments were present in the 640–650 mass range.

Discussion

From these studies, it appears that the primary genetic lesion in strain 72 results in its inability to synthesize ubiquinone-8. On the observations reported by Gibson and co-workers (Cox & Gibson, 1966, 1974; Cox *et al.*, 1969; Young *et al.*, 1971; Gibson, 1973) this pattern of accumulation of

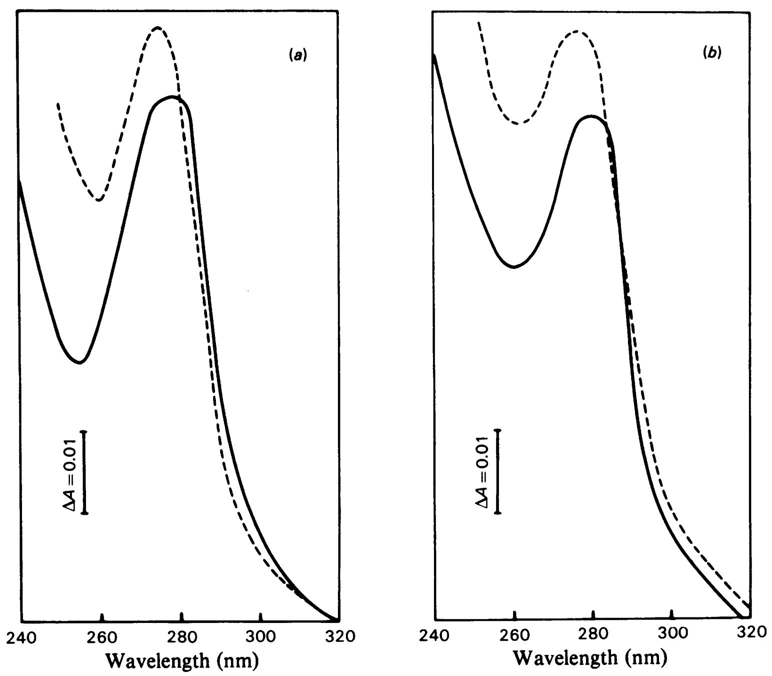

Fig. 3. *U.v.-absorption spectra of compounds S2 and S3*
The spectra of compounds S2 (*a*) and S3 (*b*) were obtained in ethanolic solution before (——) and after (----) reduction with $NaBH_4$.

intermediary biosynthetic compounds is consistent with the idea that the mutation in strain 72 resembles the *ubiB* or possibly the *ubiH* mutations previously described. However, the accumulation of a new phenolic compound with an isoprenoid chain and a mobility different from other known intermediates of the ubiquinone-8-biosynthetic pathway suggests that the mutation in strain 72 is not identical with the *ubiB* and *ubiH* mutations. There is a possibility that this new compound is 6-hydroxy-2-octaprenylphenol, an intermediate postulated by Gibson (1973), but this so far has not been demonstrated. Clearly the chemical identification of compound 2 and the genetic characterization of strain 72 will resolve this problem.

As a secondary consequence of the inability of strain 72 to synthesize ubiquinone-8, two further phenotypic changes occur. First, strain 72 appears unable to synthesize formate dehydrogenase under aerobic growth conditions and yet is clearly genetically competent to do so, since formate dehydrogenase activity is present in anaerobically grown cells. Secondly, strain 72 synthesizes a reduced Benzyl Viologen-dependent nitrate reductase when grown under aerobic conditions. There are several explanations for this latter observation, including: (*a*) the possibility that the anaerobic nitrate reductase is de-repressed and synthesized under aerobic growth conditions as a result of this mutation (the difference in specific activity between the aerobically grown and anaerobically grown preparations might, in part, be ascribed to the known oxygen-lability of the enzyme); (*b*) as a result of this mutation, another (unspecified) reductase may be induced, which primarily reduces some compound other than nitrate, but which can catalyse a Benzyl Viologen-dependent nitrate reductase, albeit at a very low rate. At this time we are unable to differentiate between the possibilities.

The presence of some peptides of nitrate reductase in the soluble extracts of aerobically grown strain 72 would explain their ability to reconstitute nitrate reductase by complementation *in vitro* with extracts of mutant 356-15 (*chlA*) grown anaerobically in the presence of nitrate. The cytoplasmic accumulation of these peptides in the extracts of aerobic strain 72 would be due to the inability to bind to the membrane; this inability is supposed to result from the inhibition by oxygen of the association mechanism of these peptides, as shown by Azoulay *et al.* (1969*a*) in their studies of nitrate reductase reconstitution.

The absence of formate oxidase activity from, and the decreased rates of oxidation of other substrates in, strain 72 when grown aerobically but in the presence of fully active formate-dependent nitrate reductase activity in preparations from cells grown anaerobically in the presence of nitrate is clearly in accordance with the results of Wallace & Young (1977). These latter authors, using ubiquinone-deficient (ubi$^-$), menaquinone-deficient (men$^-$) and double mutants of the ubi$^-$men$^-$ type, showed that there is an obligatory requirement for ubiquinone for the functional activity of the aerobic electron-transport chain(s) in *E. coli* and that menaquinone cannot replace ubiquinone. Conversely, nitrate-dependent electron transport in *E. coli* requires a quinone of some sort, but either menaquinone or ubiquinone could serve for functional activity. The formate dehydrogenase has been considered either as a single metalloenzyme (Enoch & Lester, 1972) or as two different proteins with a common subunit containing molybdenum and selenium (Ruiz-Herrera & DeMoss, 1969); it would moreover be associated with a peptide specific for ubiquinone-8 and also menaquinone.

The reason why the mutant selection procedure, based on the isolation of strains unable to grow aerobically in the presence of chlorate (Giordano *et al.*, 1977*b*), should result in the appearance of mutants deficient in the ability to synthesize ubiquinone is obscure. However, this can be rationalized if, as a result of such a mutation, a reductase is synthesized under aerobic growth conditions that is capable of reducing nitrate and, more significantly, chlorate to the toxic chlorite. It is significant that the ubiB$^-$ mutant (AN59) is also sensitive to chlorate when grown aerobically (B. A. Haddock, unpublished work), though further work is required to confirm that this is a generalized property of respiratory-deficient mutants.

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