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Synthesis of Alternative Membrane-Bound Redox Carriers during Aerobic Growth of *Escherichia coli* in the Presence of Potassium Cyanide

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Aerobic growth of *Escherichia coli* with an oxidizable substrate as carbon source in the presence of low concentrations of KCN leads to the synthesis and integration into the membrane of menaquinone and cytochromes b_{558}, a_1 and *d* in addition to the redox carriers normally present under aerobic growth conditions, namely ubiquinone and cytochromes b_{562}, b_{556} and *o*. The results are discussed with reference to other phenotypic and genotypic modifications to the electron-transport chains of *E. coli*.

Escherichia coli can synthesize a variety of redox carriers depending on the conditions chosen for growth. Under conditions of vigorous aeration the membrane-bound carriers include ubiquinone and cytochromes b_{556}, b_{562} and *o*. Cytochrome *o* is a *b*-type cytochrome and appears as an additional cytochrome b_{556} component in low-temperature reduced minus oxidized difference spectra; cytochrome b_{562} is claimed to be a soluble component in *E coli* (Hager & Itagaki, 1967), yet a significant amount remains membrane-bound during particle preparation (see below), and a precise biochemical role remains to be established. Alternatively, under conditions of anaerobic growth, ubiquinone synthesis is decreased and replaced by menaquinone synthesis and, in addition to the cytochromes synthesized under aerobic conditions, cytochromes b_{558}, a_1, *d*, c_{549} and c_{552} are produced; the two *c*-type cytochromes are not apparently associated with the membrane-bound electron-transport system (for references see Haddock & Schairer, 1973). Although cytochromes b_{558}, a_1 and *d* are typical of anaerobic growth their concentrations in the cytoplasmic membrane of *E. coli* tend to increase under conditions in which aerobic growth is impaired, e.g.; (*a*) by poor aeration conditions or in the late exponential phase of growth (Shipp, 1972); (*b*) in respiratory-deficient mutants unable to synthesize components of the aerobic respiratory chain (Haddock & Schairer, 1973; Cox *et al.*, 1970); (*c*) during sulphate-limited aerobic growth conditions (Poole & Haddock, 1974*a*).

In an intensive study on the mechanism of adaptive cyanide resistance in *Achromobacter*, Arima and co-workers demonstrated that growth of this bacterium in the presence of cyanide resulted in the induced formation of cytochromes b_1 (specific *b*-type cytochromes were not specified), a_1 and *d* and insensitivity of the respiratory chain to cyanide (Mizushima & Arima, 1960*a*,*b*,*c*; Mizushima *et al.*, 1960; Arima & Oka, 1965; Oka & Arima, 1965). The major terminal oxidases of *E. coli*, as in *Achromobacter*, are cytochromes *o* and *d* (Castor & Chance, 1959). Pudek & Bragg (1974) reported spectral studies on the reaction of cyanide with cytochromes *o* and *d* in *E. coli*, and, from the kinetics of cyanide inhibition of NADH oxidation, concluded that the two cytochrome oxidases differed in their sensitivity to cyanide. The object of the present report was to test whether or not aerobic growth of *E. coli* in the presence of cyanide resulted in the synthesis of alternative electron-transport components.

Materials and methods

Bacterial strain, growth conditions and preparation of cells and particles. *E. coli* strain C-1 (*nal*^{R}; Poole & Haddock, 1974*b*), was grown aerobically at 37°C in the mineral salts medium described by Cohen & Rickenberg (1956) containing vitamin-free casamino acids (0.1%, w/v) and either sodium succinate (0.5%, w/v) or D-xylose (0.5%, w/v) in 2-litre baffled conical flasks each containing 625ml of growth medium agitated in a rotary shaker (L. H. Engineering Co., Stoke Poges, Bucks., U.K.) operating at about 200rev./min; the cultures were harvested in the early exponential phase of growth at E_{420} (10mm light-path) 0.5–0.7. Anaerobic growth was in the same medium containing D-xylose (0.5%, w/v) and vitamin-free casamino acids (0.1%, w/v) with or without KNO_3 (1%, w/v), K_2SeO_3 (1μM) and $(NH_4)_6Mo_7O_{24},4H_2O$ (1μM) as described previously (Haddock & Schairer, 1973). Cells were harvested and particles prepared as described by Haddock (1973).

Assay techniques. O_2 uptake and protein determinations were assayed as described previously (Haddock, 1973). Quinones were extracted, separated and determined as indicated by Haddock & Schairer (1973). The concentrations of the total *b*-type

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cytochromes and cytochrome *d* were calculated from room-temperature $Na_2S_2O_4$-reduced minus oxidized difference spectra (Haddock & Schairer, 1973) obtained with a wavelength-scanning spectrophotometer (Haddock & Garland, 1971). Since the spectral properties of cytochromes *o* and *d* cause mutual interference in $Na_2S_2O_4$-reduced +CO minus $Na_2S_2O_4$-reduced difference spectra measured in the region 400–460 nm, the concentration of cytochrome *o* was calculated from such spectra by using the decrease in extinction at 562nm relative to 575nm assuming that cytochrome *o* is the only *b*-type cytochrome reacting with CO (Castor & Chance, 1959) and the CO complex has a millimolar extinction coefficient of 42.2 litre·mmol^{-1}·cm^{-1} (R. K. Poole, personal communication). Difference spectra recorded at 77°K were obtained by using the same spectrophotometer with low-temperature accessories; the spectral band-width was 1 nm, the scanning speed was approx. 5 nm/s, the time-constant of the measuring circuit was 0.1 s and cuvettes of 0.2 cm light-path were used.

Reagents. NADH (disodium salt) was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Vitamin-free casamino acids were from Difco (Detroit, Mich., U.S.A.), D-xylose (grade II) was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and all other reagents were from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and were of the highest available purity.

Results

Preliminary experiments to determine the concentration of KCN necessary for growth inhibition indicated that with D-xylose as carbon source KCN had comparatively little effect on the mean generation time at concentrations below 1mM (1.5h in the absence of inhibitor, increasing to 2.3h in the presence of 1mM-KCN), but caused a lag in the onset of exponential growth after addition of inhibitor and decrease in the final cell yield, both effects becoming more apparent with increasing KCN concentrations. Nazar & Wong (1969) have reported that low concentrations of KCN (25μM) caused a transient inhibition of RNA synthesis, degradation of polyribosomes and inhibition of protein synthesis: all these effects were subsequently reversed with time and presumably account in part for the lag in growth that we observed. A possible explanation for the time-dependent reversal of these effects is the induction of an enzyme, such as rhodanese (EC 2.8.1.1), which could lower the intracellular cyanide concentration. Comparatively little is known about this enzyme in *E. coli* (references given in Westley, 1973) and this possibility was not explored further. With sodium succinate as carbon source no growth was observed in the presence of KCN concentrations greater than 250μM, and, at concentrations lower than this, KCN had a significant effect on the mean generation time (2.4h in the absence of inhibitor increasing to about 7h in the presence of 150μM-KCN).

The electron-transport chains of preparations from *E. coli* grown aerobically with sodium succinate as carbon source in the absence or in the presence of KCN (150μM) are compared in Table 1 and Fig. 1. Essentially similar results were obtained in a comparison of preparations from cells grown aerobically with a fermentative carbon source, D-xylose (which does not give catabolite repression), in the absence or in the presence of KCN (1mM). The data in Table 1 indicate that the presence of KCN in the growth medium: (*a*) has no effect on the NADH oxidase activity of particles (presumably any bound KCN from the growth medium would be washed free

Table 1. *Oxidase rates and concentrations of redox components in preparations from E. coli grown aerobically on sodium succinate with or without KCN*

Cells were grown, particles prepared and assays performed as indicated under 'Materials and methods'. Quinone concentrations were calculated after extraction of whole cells; other assays were performed with a particle preparation. The concentration of KCN resulting in 50% inhibition of NADH oxidase activity was calculated from a comparison of the rate of NADH oxidation by particles (0.5mg/ml) before and 2min after the addition of various concentrations of KCN.

Growth conditions ...	Aerobically	Aerobically+ KCN (150μM)
NADH oxidase rate (ng/stoma of O/min per mg of protein)	1146	1320
Concn. of KCN for 50% inhibition of NADH oxidase activity (μM)	75	500
Quinone concn. (nmol/g wet wt. of cells):		
Ubiquinone	88	91
Menaquinone	5	44
Cytochrome concn. (nmol/mg of protein):		
b-type cytochromes	0.082	0.102
Cytochrome *o*	0.033	0.021
Cytochrome *d*	0.005	0.026

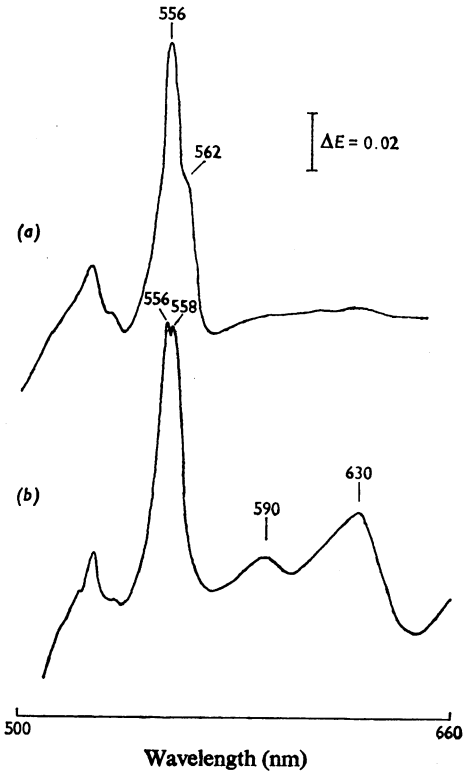

Fig. 1. *$Na_2S_2O_4$-reduced minus oxidized difference spectra recorded at 77°K obtained with particles from E. coli grown aerobically in the presence or in the absence of KCN*

E. coli strain C1 was grown aerobically with sodium succinate as carbon source in the absence (trace *a*) or in the presence of 150 μM-KCN (trace *b*). Cells were harvested, particles prepared and low-temperature difference spectra recorded as indicated under 'Materials and methods'. The final protein concentrations in the cuvettes were 12.0 mg/ml (trace *a*) and 12.2 mg/ml (trace *b*).

during the preparation of the particles), but that this activity shows a significant difference in sensitivity to cyanide inhibition, and, in the absence of added thiosulphate, this difference in sensitivity is unlikely to be due to the presence of rhodanese activity in the particles; (*b*) results in a small increase in the total concentration of *b*-type cytochromes in the membrane (but with little effect on the cytochrome *o* content) and a much larger increase in the concentration of cytochrome *d*; (*c*) although having no effect on the ubiquinone content, results in a large increase in the menaquinone content of cells. From the low-temperature reduced minus oxidized difference spectra shown in Fig. 1 it can be seen that the presence of KCN in the growth medium leads to an increased synthesis of a specific *b*-type cytochrome, cytochrome b_{558}, as well as cytochrome *d*; an increase in the concentration of cytochrome a_1 is also noticeable at 77°K but is more difficult to detect in room-temperature difference spectra.

Since the presence of KCN in the medium during aerobic growth of *E. coli* resulted in the synthesis of redox carriers typically present in anaerobically grown cells, the effects of KCN on the cytochrome content of *E. coli* grown under anaerobic conditions, with D-xylose as carbon source either fermentatively (with no added terminal electron acceptor) or 'oxidatively' (with KNO_3 as terminal electron acceptor), were investigated. Low-temperature reduced minus oxidized difference spectra of whole cells grown under these two different conditions were similar to those described previously (Haddock & Schairer, 1973; Ruiz-Herrera & De Moss, 1969), and the additional presence of KCN (1 mM) in the growth medium had no effect on the observed cytochrome pattern in either case. From these preliminary studies therefore it appears that the presence of KCN in the growth medium has a specific effect on the cytochromes synthesized under aerobic conditions with no detectable effect on the cytochrome composition of anaerobic cells; no attempt was made to estimate changes in quinone concentrations under these last-mentioned conditions.

Discussion

The results presented here can be interpreted by assuming that during aerobic growth in the presence of cyanide the normal aerobic respiratory chain of *E. coli*, involving ubiquinone and cytochromes b_{556} and *o*, and possibly cytochrome b_{562}, is inhibited because of the high affinity of cytochrome *o* for KCN (Pudek & Bragg, 1974), and, as a result of this inhibition, alternative redox carriers, menaquinone and cytochromes b_{558}, a_1 and *d*, are synthesized. Kinetic evidence suggests that cytochrome a_1 does not function as a terminal oxidase in *E. coli* and its role remains obscure (Downie, 1974), but cytochrome *d* does have an oxidase function (Castor & Chance, 1959; Downie, 1974) and is less sensitive to cyanide inhibition than is cytochrome *o* (Pudek & Bragg, 1974). Cytochrome *d* is, however, sensitive to higher concentrations of KCN, since no growth occurs with sodium succinate in the presence of KCN at concentrations above 250 μM, but with a fermentative carbon source, xylose, growth occurs at KCN concentrations of at least 1 mM.

Attempts to correlate observed changes in redox components, after alterations to the growth conditions of the cell, with their functional organization and metabolic significance are dubious in the absence of more detailed knowledge. The following information is so far available for *E. coli*: (*a*) the synthesis of

cytochromes b_{556}, *o* and possibly cytochrome b_{562} appears to be constitutive; (*b*) cytochromes b_{558}, a_1 *d* appear to show co-ordinate synthesis under certain growth conditions; (*c*) ubiquinone synthesis requires aerobic conditions; (*d*) menaquinone is synthesized both under anaerobic growth conditions, with a postulated role in anaerobic electron transport with fumarate as terminal acceptor (Newton *et al.*, 1971), and under aerobic growth conditions (Cox *et al.*, 1970; Haddock & Schairer, 1973) where its role is uncertain; (*e*) the presence of cytochromes b_{558}, a_1 and *d* in the membranes results in electron transport, with O_2 as terminal acceptor, coupled to one potential energy-conservation site (Brice *et al.*, 1974; Downie, 1974; Poole & Haddock, 1974*a*) compared with two such sites in respiratory chains containing only cytochromes b_{556}, b_{562} and *o* (Lawford & Haddock, 1973). This last-mentioned observation could explain the large increase caused by KCN in the mean generation time with sodium succinate as the growth carbon source (all cellular ATP synthesized via oxidative phosphorylation) but the relatively small effect of KCN on the mean generation time with xylose as carbon source (ATP synthesized from both glycolysis and oxidative phosphorylation).

From this brief survey it is apparent that the mechanisms that operate to control the synthesis of the various redox components of the cytoplasmic membrane are complex, and presumably quinone synthesis and cytochrome synthesis respond to different signals. Previous suggestions that the availability of O_2 in the growth medium is the controlling parameter are obviously too simple, and it is more likely that control is exerted by variations in the concentrations of cellular components that reflect the internal redox potential of the cells, as discussed by Wimpenny (1969) and Harrison (1972).

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