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THE ELECTRON-TRANSPORT SYSTEM OF *MICROCOCCUS LUTEA* (*SARCINA LUTEA*)

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SUMMARY

1. The membrane-bound electron-transport system of *Micrococcus luteus* was investigated. It contained cytochromes a_{598}, b_{562}, b_{557}, c_{554}, c_{549}, a menaquinone (MK-8), carotenoids, and NADH, malate, and succinate dehydrogenases. Sensitivities to inhibitors indicate the difference between this system and the mammalian one. The *M. luteus* system was insensitive to antimycin A, and showed only low sensitivity to rotenone. It was effectively inhibited by cyanide, piericidin A, dicoumarol, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, and ultraviolet irradiation at 360 mμ.

2. A carotenoidless mutant was isolated which showed properties similar to the parent organism except that there was no detectable succinate oxidase activity, and the malate oxidase system activity increased 8-fold in electron-transport particles. Similar sensitivities to inhibitors were found.

3. A scheme for electron transport in *M. luteus* is suggested.

INTRODUCTION

As a continuation of a comparative survey of electron-transport systems of bacteria in progress in this laboratory, we have been engaged in a study of the respiratory system of *Micrococcus luteus* (*Sarcina lutea*), a Gram-positive aerobic bacterium. *M. luteus* is non-photosynthetic and contains carotenoids of the β-carotene and xanthophyll types[1,2]. The functions of these carotenoids in the cell are not well understood. They are reported to be intimately associated with the cell membrane, probably as a pigment–protein complex[3]. MATHEWS AND SISTROM[4,5] suggest that the carotenoids may serve a protective function against lethal photodynamic reactions. It has been suggested that the carotenoids in Mycoplasma serve a structural function and may provide transport mechanisms for fatty acids and sugars[6].

M. luteus contains a menaquinone (MK-8) as its sole quinone[7]. It also contains a mitochondrial type cytochrome sequence[8]. The membrane fraction is reported to contain NADH oxidase, succinate oxidase and cytochrome *c* oxidase activities[3,9].

However, little is known of the actual pathways of electron transport or the relationships between the respiratory system and carotenoids in this bacterium. Therefore, we felt a more detailed investigation of the electron-transport system of *M. luteus* utilizing inhibition studies, dual-wavelength techniques and comparison with a carotenoidless mutant, would be of value.

MATERIALS AND METHODS

Chemicals. NADH was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Piericidin A was a gift from Prof. N. Takahashi, University of Tokyo, Tokyo, Japan. *N*-Methyl-*N*-nitroso-*N*-nitroguanidine was purchased from Koch-Light Laboratories, Colnbrook, Great Britain. Nutrient broth and nutrient agar were obtained from Oxoid, London, Great Britain. All other chemicals were obtained from British Drug Houses, Poole, Great Britain, and were the finest grade obtainable. Glass double-distilled water was used throughout.

Culture and maintenance. M. luteus (*Sarcina lutea*, NCIB 8553) was maintained on nutrient agar slopes at 5°. A slope was used to inoculate 150 ml of medium (13 g/l nutrient broth) in a 500-ml flask; the bacteria were grown with shaking (200 rev./min) on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 30° to late log phase. 5 ml of this was used to inoculate 600 ml of medium in 2-l flasks and was grown to late log phase at 30° as above. The cells were harvested on a Sharples continuous-flow refrigerated centrifuge and were washed twice with distilled water. The cells were suspended in 0.05 M sodium–potassium phosphate buffer (pH 7.4) and sonicated at full output on a Branson sonicator for 15 min at 30-sec intervals with cooling in ice. The suspension was centrifuged for 10 min at $12\,500 \times g$ at 5° in an MSE-18 centrifuge. The supernatant was carefully removed and centrifuged 1.5 h at $105\,000 \times g$ in an MSE-50 centrifuge at 5°. The electron-transport particles were prepared freshly for each experiment. They were washed and recentrifuged before use. Protein was assayed by the modified biuret mothed of GORNALL *et al.*[10].

Measurement of oxidase activities. O_2 uptake was measured at 30° using a Clark electrode. The reaction mixture contained 500 μmoles KH_2PO_4–Na_2HPO_4 buffer (pH 7.4), 1.5–3.0 mg particle protein, 15 μmoles substrate and glass-distilled water to a final volume of 2.2 ml.

Determination of quinone and carotenoid levels. Menaquinone was assayed by a modification of the semi-micro method for quinone determination of PUMPHREY AND REDFEARN[11]. The quinone was not reduced with $NaBH_4$. $\Delta A_{249\,m\mu}$, with a molecular absorbance coefficient of $\varepsilon = 19\,000$ (ref. 12), was used to determine the concentration. Carotenoid levels were determined as described by MATHEWS AND SISTROM[3].

Determination of cytochrome levels. The concentration of cytochrome in the electron-transport particles was calculated routinely from the dithionite-reduced *minus* oxidized difference spectra. The presence of cytochrome a_3 was determined by the CO–dithionite-reduced *minus* reduced spectra. NADH-reduced *minus* oxidized difference spectra were also done. On the assumption that the molecular absorbance coefficients for *M. luteus* cytochromes are similar to those for the mammalian ones, the following values were used: cytochrome *a*, for $A_{598\,m\mu} - A_{623\,m\mu}$ $\varepsilon = 24\,000$ (ref. 13); for cytochrome *b*, for $A_{560\,m\mu} - A_{570\,m\mu}$ $\varepsilon = 20\,000$ (ref. 14); for cytochrome *c*, for $A_{550\,m\mu} - A_{540\,m\mu}$ $\varepsilon = 21\,000$ (ref. 15). A low temperature spectrum (77° K) (NADH-reduced *minus* oxidized) was done through the kind courtesy of Dr. Derek Bendall, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

Dual-wavelength spectrophotometry. The reduction states of the individual cytochrome components of *M. luteus* particles were determined using an Aminco–Chance dual-wavelength spectrophotometer (Aminco Instrument Co., Silver Spring, Md.,

U.S.A.). All measurements were carried out at 25°. The system contained 500 μmoles sodium–potassium phosphate buffer (pH 7.4), 1.0–3.0 mg particle protein and glass-distilled water to a final volume of 3.0 ml. The reaction was initiated by the addition of 9 μmoles of substrate. The following wavelength pairs were used: cytochrome *a* 598 and 610 mμ, cytochrome *b* 562 and 570 mμ, cytochrome *c* 550 and 540 mμ.

Mutagenesis of M. luteus. Cells in early log phase were treated with the mutagen, *N*-methyl-*N*-nitroso-*N*-nitroguanidine (50 μg/ml), for 15 min at room temperature. The cells were washed once with sterile 0.05 M sodium–potassium phosphate buffer (pH 7.4), resuspended in buffer to give a cell concentration of 10^5/ml, and plated on nutrient agar plates. Colourless colonies were replated and characterized. A carotenoidless mutant, M-7, was obtained which was positively identified by Dr. A. G. Baird-Parker, Unilever Research Laboratory, Sharnbrook, Great Britain, as a member of the *M. luteus* family. It had similar biochemical properties as regards the electron-transport particles, but contained no carotenoids, showed no detectable succinate oxidase activity and had a more active membrane-bound malate oxidase system than the parent.

RESULTS

The low temperature (77°K) NADH-reduced *minus* oxidized difference spectrum of *M. luteus* electron-transport particles is shown in Fig. 1. This spectrum suggests the presence of cytochromes a_{598}, b_{562}, b_{557}, c_{554} and c_{549}. Room temperature spectra did not differentiate between the two cytochrome *B* and *C* types. A room temperature CO–dithionite-reduced *minus* reduced difference spectrum indicated the presence of a cytochrome a_3. There was no evidence for a cytochrome *o* which is in line with the finding of CASTOR AND CHANCE[16].

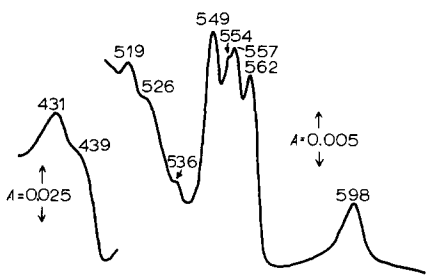

Fig. 1. Low temperature (77°K) difference spectra of *M. luteus* electron-transport particles, NADH-reduced *minus* oxidized. Protein concn., 5.3 mg/ml.

The concentrations of components of the respiratory chain in the particles are given in Table I. For comparison the cytochrome levels of the carotenoidless mutant are given, as well as cytochrome and quinone levels of electron-transport particles from beef heart[17] and from *Mycobacterium phlei*[18].

Oxidase-system activities are also given in Table I for particles from *M. luteus* and its mutant. Although the NADH oxidase activities are very similar, the malate oxidase activity in the mutant is about 8 times higher. The addition of supernatant, FAD, or NAD^+ did not affect oxidase activities in either organism.

TABLE I

CYTOCHROME, MENAQUINONE, CAROTENOID CONTENT AND OXIDASE SYSTEM ACTIVITIES OF *M. luteus* AND M-7 ELECTRON-TRANSPORT PARTICLES

All concentrations are expressed in μmoles/g protein. Oxidase activities are expressed as μgatoms O_2/min per mg protein. Values for *M. luteus* and M-7 were obtained as described in the text, and are representative of at least 10 preparations.

Preparation	*Cytochromes*			*Quinone*	*Carotenoid*	*Oxidase activities*		
	a	*b*	*c*			*Succinate*	*Malate*	*NADH*
M. luteus	0.15	0.91 0.51*	0.59	6.3	6.1	0.013	0.036	0.162
M-7	0.13	1.12	0.90	—	0	0	0.233	0.175
Beef heart[17]	1.62	0.85	0.63	4.8	—	—	—	—
M. phlei[18]	0.27	0.65 0.18*	0.62	12.0	—	—	—	—

* Substrate reduced: NADH in *M. luteus*; NAD^++β-hydroxybutyrate in *M. phlei*.

TABLE II

EFFECT OF INHIBITORS ON OXIDASE SYSTEM ACTIVITIES IN *M. luteus* ELECTRON-TRANSPORT PARTICLES

Oxidase activities were measured as described in the text. All values represent at least 5 preparations.

Inhibitor	*Concn. and time of exposure*	*Inhibition of oxidase system* (%)		
		Malate	*NADH*	*Ascorbate+ubiquinone*
KCN	10 μM	60	60	72
	0.1 mM	80	82	100
Ultraviolet	10 min	40	50	—
irradiation at 360 mμ	15 min	100	82	0
Piericidin A	5 mμmoles/mg protein	—	80	—
Rotenone	140 mμmoles/mg protein	30	40	—
Antimycin A	1.0 μmole/mg protein	8	25	—
2-*n*-Heptyl-4-hydroxy-quinoline-*N*-oxide	0.5 μmole/mg protein	54	80	—
Dicoumarol	10 mμmoles/mg protein	83	80	—
o-Phenanthroline	0.5 mM	15	22	—

TABLE III

REDUCTION LEVELS OF CYTOCHROMES IN *M. luteus* ELECTRON-TRANSPORT PARTICLES

All values are representative of at least 5 preparations. NADH and malate were used as substrates. Reactions were carried out as described in the text.

Cytochrome	(%) *Aerobic steady state*			(%) *Anaerobic state*		
	NADH	*Malate*	*Ascorbate+ubiquinone*	*NADH*	*Malate*	*Ascorbate+ubiquinone*
a	6	—	—	95	—	—
b	25	31	2	56	64	7
c	24	27	29	83	85	63

The results of studies with respiratory inhibitors are shown in Table II. The sensitivity of M-7 to inhibitors is similar to that of the parent. M-7 seems to be slightly more sensitive to ultraviolet irradiation and to *o*-phenanthroline.

Table III shows the aerobic steady-state and reduced-state levels of cytochromes in the electron-transport particles. Preliminary studies with M-7 particles gave similar results.

Studies with inhibitors using dual-wavelength spectroscopy indicated the following apparent sites of action: piericidin A inhibiting at low concentrations at the flavoprotein level, and at higher concentration, after cytochrome *c*; KCN inhibiting after cytochrome *c*; ultraviolet irradiation inhibiting before or at the cytochrome *b* region; 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide inhibiting before the cytochrome *b* region.

DISCUSSION

Although it has been reported in the literature[9,19,20] that *M. luteus* is susceptible to lysozyme treatment, our strain under the growth conditions employed showed only low susceptibility. Sonication was therefore used as a routine method of obtaining electron-transport particles.

The presence of a mammalian type cytochrome system, menaquinone, and carotenoids of the β-carotene and xanthophyll types in the membrane-bound electron-transport system was confirmed. A low temperature (77°K) difference spectrum (substrate-reduced *minus* oxidized) indicated the presence of a mammalian type cytochrome *a*, one mammalian type cytochrome *b*, one with its maximum at 557 mμ, and two mammalian type cytochrome *c*'s. Room temperature difference spectra did not differentiate between the two *B* and *C* types. Room temperature CO–dithionite-reduced *minus* reduced spectra indicated the presence of a cytochrome a_3 type.

As seen from Table I, the cytochrome and quinone levels in *M. luteus* particles are similar to those of beef heart with the exception of cytochrome *a*. This is more comparable with cytochrome *a* levels in *M. phlei*. As with *M. phlei* particles, only a portion of the cytochrome *b* reducible chemically can be reduced by substrate. The substrate reduction levels of cytochromes *a* and *c* are similar to dithionite levels.

The carotenoids appear very tightly bound to the membrane fraction and are apparently intimately associated with the respiratory components although no evidence has been obtained for their participation in electron transport. Preliminary experiments, in which particles were treated with 1% Nonidet p. 40 or 1% sodium deoxycholate, did not completely separate the carotenoids from the cytochromes in our strain. SALTON *et al.*[21], however, have reported the separation of carotenoids and cytochromes in *M. luteus*. A possible close association of carotenoid with cytochrome *b* in *Micrococcus lysodeikticus* in which cytochrome *b* could not be obtained free of carotenoid has been reported[22]. Our strain of *M. luteus* seems to show a similar association.

A study of substrate reduction of cytochromes using dual-wavelength spectroscopy indicated the sequence: cytochrome $b \rightarrow$ cytochrome $c \rightarrow$ cytochrome $a \rightarrow O_2$.

The response of the electron-transport particles to inhibitors indicated that this system possesses distinct differences from a mammalian system. The ubiquinone + ascorbate oxidase, entering at the cytochrome *c* level (as shown by dual-wavelength studies) is completely inhibited by 0.1 mM cyanide. The malate and NADH oxidase

systems are inhibited 80%. Studies with *M. lysodeikticus*[23], which possesses a mammalian type cytochrome system, indicate the presence of a relatively cyanide-insensitive pathway to O_2. It has been suggested that either an auto-oxidizable *b*-type cytochrome or a flavoprotein may be involved. Since there is evidence for two *b*-type cytochromes in *M. luteus* particles, one of these may be involved in terminal oxidation. There is no evidence that one of them is cytochrome *o*. We found a fast, followed by a slow rate of reduction by substrate of cytochrome *b* in both *M. luteus* and M-7 particles. We are not able to say whether this indicates that two types or pools of *b* are involved or whether it is only the result of some dislocation of cytochrome *b* from the membrane during preparation of the particles.

Ultraviolet irradiation is an effective inhibitor of electron transport in *M. luteus* and M-7 particles. It destroys or alters the menaquinone in such manner that it can no longer participate in electron transport. Determinations of menaquinone in particles before and after irradiation showed that over 30% of the quinone originally present is destroyed. There were spectral alterations in the quinone remaining. Ultraviolet irradiation can also alter protein structure and has been reported to break metal–protein bonds[14]. Therefore, a part of the observed inhibition may be due to this effect rather than solely to destruction of the quinone.

Dicoumarol was an effective inhibitor in this system. This inhibitor has been commonly assumed to be a menaquinone antagonist, but Cox *et al.*[25], in studies with ubiquinone- and menaquinone-deficient mutants of *Escherichia coli*, found inhibition in the absence of menaquinone. They suggest that another site must be operating in this inhibition. Dicourmarol has been reported to inhibit a flavoprotein NADH dehydrogenase[26].

Inhibition of electron transport in *M. luteus* by rotenone and piericidin A has been previously reported[27]. Piericidin A is much more effective than rotenone, as is found with most bacterial systems. SNOSWELL AND COX[28] found that piericidin A affects ubiquinone-containing oxidase systems probably at or near the quinone level in *E. coli*. However, they also found inhibition of the nonubiquinone-containing systems for α-glycerophosphate and dihydroorotate oxidation. The site for this latter piericidin A inhibition is still unknown. It is interesting that it should be effective in a nonubiquinone-containing organism, *M. luteus*. As stated above, in this organism, piericidin A appears to act at or near the flavoprotein level at low concentrations, and after cytochrome *c* at higher concentrations. Since it has been suggested that piericidin A inhibits oxidative-phosphorylation capacity in mammalian submitochondrial particles[29], it would be of interest to investigate more thoroughly its inhibition sites in bacteria.

The response to antimycin A is comparable with that found in most other bacterial systems (see GELMAN *et al.*[30] for summary). Antimycin A is not effective except at very high concentrations. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide is a more effective inhibitor. Its site of action has been described by KOGUT AND LIGHTBOWN[31]. These authors also indicate that it can affect flavins. ALEEM[32] reported that it inhibits NADH-linked oxidative phosphorylation in *Nitrobacter agilis* between the flavoprotein and cytochrome levels. Our preliminary studies show it may have its main site of action before cytochrome *b* in *M. luteus*. Dual-wavelength experiments showed that the level of aerobic steady-state reduction achieved by cytochrome *b* in the presence of substrate and this inhibitor is the same as or less than that in the absence of inhibitor. Cytochrome *c* aerobic steady-state levels showed similar results, indicating the

presence of a possible site before *c* but not necessarily between *b* and *c*. Flavoprotein aerobic steady-state levels increase markedly on the addition of inhibitor. Work is in progress further to define the inhibition site in this bacterium.

The low inhibition observed with *o*-phenanthroline indicates that non-haem iron may play a role in electron transport in this system.

From the above results, we suggest the following possible scheme for the electron-transport system of *M. luteus*:

$$\text{Substrate} \rightarrow \text{flavoprotein} \rightarrow [\text{MK-8, cyt. } b_{557}, \text{ cyt. } b_{562}] - ? \rightarrow O_2$$
$$\downarrow$$
$$[\text{cyt. } c_{554}, \text{ cyt. } c_{549}] \rightarrow [\text{cyt. } a_{598}, \text{ cyt } a_3] \rightarrow O_2$$

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