Cytochromes in *Streptococcus faecalis* var. *zymogenes* Grown in a Haematin-containing Medium

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SUMMARY

Functional cytochromes were found in the membrane fraction of *Streptococcus faecalis* var. *zymogenes* grown aerobically with haematin. Molar growth yields showed that the cytochrome system produced additional ATP. Inhibitors and uncouplers of oxidative phosphorylation verified the presence of cytochromes in the membrane fraction. Spectra indicated both *a* and *b* type cytochromes when cultures were grown with haematin. Without haematin, only a flavin system of electron transport developed without additional ATP generation. Bacteria grown anaerobically with haematin did not form cytochromes.

INTRODUCTION

The streptococci traditionally have been considered to be devoid of cytochromes; those species in which pathways to oxygen exist transfer electrons through flavin enzymes. Evidence is increasing, however, to show that certain strains of streptococci form cytochromes under appropriate growth conditions with a concomitant energy advantage.

Whittenbury (1964) reported the presence of bands corresponding to *a*₂ and *b* type cytochromes in *Streptococcus faecalis* (strain H69D5) when grown in medium supplemented with heated blood. He did not determine whether the cytochromes were functional, vestigial or artifacts resulting from cultural conditions. Bryan-Jones & Whittenbury (1969) reported the presence of a *b*₂ type cytochrome in the membrane fraction of *S. faecalis* (strain 581) when grown in a medium supplemented with haematin. This membrane fraction coupled the oxidation of NADH to the formation of ATP from ADP and inorganic phosphate; however, there was an endogenous ATP production of nearly 65 % due to adenylate kinase. The absorption bands did not appear in bacteria grown in a haematin medium under anaerobic conditions and it was concluded that oxygen was necessary for the appearance of cytochromes. Sijpesteijn (1970) reported that *S. lactis*, grown aerobically in a medium containing haemin, showed spectra of the *a*₂ and *b* types of cytochromes. Most recently, van der Wiel-Korstanje & de Vries (1973) described a strain of *Bifidobacterium*, isolated from faeces, which produced either cytochromes *b* and *a* or *b* and *d* when grown in a medium containing lysed red blood cells.

Gallin & VanDemark (1964) observed low levels of oxidative phosphorylation in *S. faecalis* (strain 10C1) during the oxidation of NADH by extracts of aerobically grown bacteria. They postulated that phosphorylation may have occurred at the 'NADH-flavin' level, or during the oxidation of naphthoquinone. Later, Smalley, Jahrling & VanDemark (1968) used molar growth yields as evidence for oxidative phosphorylation with the same organism, and obtained a P:O ratio approximating 0.6. Faust & VanDemark (1970) used fumarate as an electron acceptor for NADH oxidation in membrane fractions of *S. faecalis*

(strain 10C1) to demonstrate a P:O ratio of 0.19. In contrast to these findings Bryan-Jones & Whittenbury (1969) were unable to demonstrate oxidative phosphorylation in their strain of *S. faecalis* in the absence of haematin.

After a survey of several species of streptococci, a strain of *Streptococcus faecalis* var. *zymogenes* was selected for investigation of cytochromes. The selection was based on the fact that the organism grew vigorously in aerobic culture and on blood agar plates and was haemolytic.

METHODS

Molar growth yields. *Streptococcus faecalis* var. *zymogenes* strain TR was from the culture collection of the Laboratory of Microbiology, Cornell University. The partially defined medium of O'Kane & Gunsalus (1948) was used with various concentrations of substrates, and included/l: 100 mg cysteine, 0.02 mg lipoic acid and 0.5 g sodium acetate. Enzymically hydrolysed casein was substituted for acid-hydrolysed casein. The substrates, salts, and vitamins were filter-sterilized and added aseptically prior to inoculation of 50 ml volumes of medium. Haematin (Calbiochem) was added at 20 µg/ml unless otherwise noted. Haematin was prepared monthly by the filter-sterilization of 0.5 g haematin dissolved in 100 ml 0.2 M-KOH, and was stored at 5 °C. Anaerobic growth was obtained in 50 ml graduated flasks in an anaerobic incubator. The medium was rapidly cooled and inoculated (0.1 % from an 18 h culture) to avoid redissolving any oxygen. Aerobic growth was accomplished in 250 ml baffled flasks. After 11 to 15 h of growth at 37 °C the biomass became constant (determined for each substrate concentration by measurement at 650 nm with a Beckman DU spectrophotometer) and was immediately harvested. The spent medium was frozen, while the resulting bacterial pellet was washed twice with 35 ml 0.01 M-potassium phosphate buffer pH 7.2. The bacteria were dried in pre-heated weighing pans at 100 °C to a constant weight. Molar growth yields (Y), expressed as g dry wt/mol substrate, were calculated by the method of Bauchop & Elsden (1960), who defined the ATP yield as the number of moles of ATP produced per mole of substrate utilized.

Products of substrate fermentation. End-product analysis was carried out on the frozen spent medium with a High Efficiency Gas Chromatograph (Hewlett & Packard). The columns were packed with Polypack-2, a bead-like material made of ethylvinylbenzene cross-linked with divinylbenzene to form a polymer (not requiring a liquid phase). The carrier gas (helium) was adjusted to a flow rate of 15 ml/min, air at 280 ml/min, and hydrogen at 80 ml/min. The column oven temperature was 130 °C, while the injection portal and the flame detector were at 240 °C. Standards of lactate, pyruvate, formate, acetate, ethanol, acetylmethylcarbinol and 2,3-butanediol were used with a range of 10 and an attenuation of 8. The spent medium was injected directly into the portal.

Preparation of cell-free extracts. Bacteria were grown in a medium containing (g/l): tryptone, 10; yeast extract, 5; glucose, 5; K₂HPO₄, 2; at an initial pH of 7.2. When the pH reached 6.0 (1 % inoculum from an 18 h culture, 37 °C, shaking in baffled flasks for approx. 9 h) the growth was harvested at 10000 g for 15 min and washed twice in 0.01 M-N-tris-methyl-2-amino-ethane-sulfonate (TES; Calbiochem) pH 7.3, broken in an X-press (Biotech, New York, New York) at -25 °C and 20000 lb/in², then centrifuged at 48000 g for 25 min. The pellet was washed once in TES buffer, recentrifuged at 48000 g for 20 min, then resuspended in a few ml of TES and centrifuged at 3000 g for 10 min. The particulate fraction containing the high molecular weight membrane fraction was saved.

The original supernatant fraction containing the cytoplasm and very small particles (high-speed fraction) was centrifuged at 78000 g for 90 min and the resulting supernatant labelled

'diluted cytoplasm'. The pellet from this sedimentation was washed once in TES buffer and stabilized with 10 % sucrose. This fraction contained smaller particulate matter.

Measurement of NADH oxidation and coupled phosphorylation. The oxidation of NADH was followed spectrophotometrically at 340 nm on a Beckman DB-G grating spectrophotometer at 30 °C. Molar quantities of NADH were converted by the extinction at 340 nm using the molar extinction coefficient of Horecker & Kornberg (1948). The enzymic assay system to determine NADH oxidation contained: 15 μ mol TES buffer; 6 μ mol MgCl_2 ; 3 μ mol KF; 1.5 μ mol EDTA; 3 μ mol K_2HPO_4 ; 0.256 μ mol NADH; 0.05 to 1.0 mg protein of cell-free extracts; final pH 7.3, brought to a volume of 2.0 ml with distilled water. Protein was determined by the method of Kunitz (1952).

Pinchot's (1953) method was used to measure ATP formation (coupled oxidative phosphorylation). In addition to the reaction mixture used for the enzymic assay of NADH oxidase the following reagents were used: 4.0 μ mol AMP; 0.225 μ mol ADP; 10 μ mol glucose 0.06 mg bovine serum albumin; 15 units hexokinase (lyophilized, yeast, salt free; Calbiochem); 30 units glucose-6-phosphate dehydrogenase (Torula yeast; Calbiochem); with or without 0.22 μ mol NADP. In this reaction NADH is oxidized, decreasing extinction at 340 nm. The ATP formed is converted to glucose-6-phosphate by hexokinase and subsequently oxidized by glucose-6-phosphate dehydrogenase with a simultaneous reduction of NADP. NADP reduction is measured as the difference in the rate of extinction decrease at 340 nm between reactions with and without NADP. Consequently, if the membrane fractions couple the oxidation of one mole of NADH to the reduction of one mole of NADPH, the slope (μ mol NADH oxidized; $E_{340}/\text{min}/\text{mg}$ protein) of the whole reaction is one, thus a P:O ratio of one. If coupling does not occur the reaction with NADP has the same slope as the reaction without NADP, due solely to NADH oxidation (a P:O of zero).

In a separate measurement ATP production due to adenylate kinase activity was subtracted from the total ATP formed, leaving only ATP formed from oxidative phosphorylation. This control was used separately without NADH as substrate, thus permitting observation of the endogenous ATP formed by adenylate kinase.

Concentrated stock solutions of inhibitors and uncouplers were dissolved in ethanol or distilled water and used in 0.2 to 0.05 ml and 2 μ l portions, respectively. When examining their effect upon the P:O ratio of the control the difference was taken as the P:O ratio of the whole reaction with effector, and the reaction without NADP with effector.

Spectrophotometric absorption spectra. Difference absorption spectra were obtained from membrane suspensions of bacteria grown aerobically with and without haematin. The growth was thoroughly washed in phosphate buffer (0.15 M) to eliminate any excess haematin. Since the bacteria were harvested in exponential phase at a relatively high pH, most of the haematin remained suspended. Bacteria were broken in an X-press and, after centrifuging, the low-speed fraction containing the particulate material was used. Spectra were obtained using a Cary 14 Recording Spectrophotometer employing a cell pathlength of 0.2 cm. Air was the oxidant and a few crystals of sodium dithionite the reductant. The reduced-cyanide minus reduced-spectrum was the difference between the dithionite reference and a cuvette with the same dithionite-reduced sample plus a few crystals of KCN. A reduced-carbon monoxide minus reduced-spectrum was the difference between the dithionite-reference and the same dithionite-reduced sample bubbled for a few minutes with CO.

Fig. 1. Molar growth yields with (a) glucose and (b) mannitol. See text for methods and discussion.

RESULTS AND DISCUSSION

A preliminary report (Ritchey, VanWie & Seeley, 1972) presented evidence for the development of a cyanide-sensitive oxidative pathway in hematin-grown *Streptococcus faecalis* var. *zymogenes*. Manometric techniques also revealed that resting suspensions of haematin-grown bacteria had an increased rate of oxygen uptake, and that haematin could not be used as a substrate. In this communication, functional cytochrome(s) have been identified and probably account for greater ATP production when there is a source of haematin.

Changes in ATP production were monitored either by molar growth yields or in cell-free extracts as P:O ratios *in vitro*.

End-product analysis of the spent medium from the molar growth yields (Y) on glucose showed that the primary product during aerobic growth was acetate (92 %), with small amounts of lactate, acetylmethylcarbinol and ethanol. During anaerobic growth lactate was the primary product (89 %) with small amounts of acetate and only traces of ethanol and acetylmethylcarbinol or 2,3-butanediol. The aerobic Y_{glucose} of 40.6 (Fig. 1a) can be expected to be the product of slightly less than 4 mol ATP/mol substrate. The anaerobic Y_{glucose} of 20.5 should be slightly more than 2 mol ATP. From these data the Y_{ATP} can be considered to be 10.2 (g dry wt/mol ATP). Bauchop & Elsdén (1960) observed an anaerobic Y_{glucose} of 22 with *S. faecalis* and a Y_{ATP} of 10.5. Since these organisms closely resemble each other, the molar growth yields seem in close enough agreement to substantiate this method.

Whenever haematin was added to the growth medium the products of aerobic growth were nearly the same, but the Y_{glucose} was increased to 50.8. Haematin added to the medium of anaerobically grown bacteria changed neither the products nor the Y_{glucose} . When growth is aerobic on glucose with haematin, 5 mol ATP are generated per mol substrate as compared with 4 due entirely to substrate level phosphorylation in non-haematin grown cultures.

Smalley *et al.* (1968) calculated a P:O ratio in the absence of haematin from the difference in the aerobic Y_{mannitol} and the aerobic Y_{glucose} to indicate oxidative phosphorylation of 0.6 to 0.7 in *S. faecalis* 10C1. Product analysis of mannitol grown *S. faecalis* var. *zymogenes* indicated production of about 4 mol ATP/mol substrate. Aerobically Y_{mannitol} was 41.8 but with haematin, while the same products were formed, Y_{mannitol} was 58.4 (Fig. 1b and Table 1). We present molar growth yields as buttressing evidence for oxidative phosphorylation. Whenever Y_{mannitol} and Y_{glucose} were compared in bacteria grown aerobically with haematin,

Table 1. *Molar growth yields (Y) for S. faecalis var. zymogenes*

Substrate	Anaerobic		Aerobic					
			No addition		Haematin 20 µg/ml		Haematin 40 µg/ml	
	Y	ATP*	Y	ATP*	Y	ATP*	Y	ATP*
Glucose	20.5	2.01	40.6	3.98	50.8	4.98	50.8	4.98
Mannitol	—	—	41.8	4.19	58.4	5.73	—	—

See text for methods.

* ATP was measured in mol ATP/mol substrate.

a P:O ratio of 0.75 was obtained. Extra ATP production can be realized by the utilization of the extra pair of electrons on mannitol. Evidently the inclusion of haematin in the growth medium, with subsequent cytochrome induction, enables this bacterium to couple the oxidation of NADH to the formation of ATP. Haematin was not used as a substrate to provide energy because the addition of 40 µg haematin/ml, instead of the normal 20 µg/ml, did not increase the aerobic Y_{glucose} ; the same y-axis intercept indicated that haematin was not used as a source of energy.

In the absence of haematin ATP production from oxidative phosphorylation, as suggested by molar growth yields, did not occur in *S. faecalis* var. *zymogenes*. This differs from the findings of Smalley *et al.* (1968) with *S. faecalis* 10C1, but is similar to the findings of Bryan-Jones & Whittenbury (1969) with *S. faecalis* strain 581. In an unpublished survey we have found variation in the mechanism of aerobic ATP generation not only between *Streptococcus* spp., but also between strains of a given species (e.g. *S. faecalis*).

Table 2 shows the effect of several inhibitors upon the oxidation of NADH by the high-speed membrane fraction from bacteria grown without haematin. These membranes contained little cytoplasm as the NADH oxidase activity had been purified 25-fold from the original broken preparation. The concentrations of NaN_3 used inhibited the activity in membranes from growth with haematin less than those from growth without haematin. Potassium cyanide blocked NADH oxidation more efficiently in the haematin-induced pathway, implying blockage of a cytochrome system. An inhibitor, 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO), reported by Jackson & Lightbown (1956) to be more effective with bacterial preparations than antimycin, displayed discriminatory inhibition of the cytochrome system. Antimycin A also partially inhibited NADH oxidation in this system, supporting the evidence of a Site II level of oxidative phosphorylation. Very low concentrations of both rotenone and amytal showed inhibition at the Site I level of a cytochrome respiratory chain.

Whenever haematin was incorporated in the growth medium, cell-free extracts of purified membranes exhibited an NADH oxidation which was sensitive to many inhibitors of a classical cytochrome-mediated electron transport system. Extracts from cultures grown without haematin were less sensitive to the inhibitors rotenone, amytal, HOQNO, antimycin A, and KCN. The NADH oxidation system under these conditions is flavin in nature and is only sensitive to high concentrations of these inhibitors (Faust & VanDemark, 1970). Our results concur with those of Bryan-Jones & Whittenbury (1969), since antimycin A and KCN were found to be potent inhibitors (NaN_3 was a weaker inhibitor) of NADH oxidation in membrane preparations from haematin-grown bacteria. The reason that the haematin-induced pathway is less sensitive to NaN_3 could be due to the nature of the haem group as it appears in the apoenzyme of the cytochrome. Since NaN_3 will bind thioester bonds,

Table 2. *Effect of inhibitors on NADH oxidation and oxidative phosphorylation*

Fractions were prepared and tested as described in Methods.

Inhibitor	Concn (mm)	Inhibition of NADH oxidation* (%)	
		Haematin-grown membranes	Non-haematin grown membranes
NaN ₃	10.0	20	38
	50.0	62	100
KCN	0.6	43	12
	1.0	100	20
HOQNO	0.1	72	15
Antimycin A	0.008	44	6
Amytal	0.01	15	4
Rotenone	0.04	21	7
Batho. S.	0.1	12	N.D.

Uncoupler	Concn (mm)	Uncoupling of oxidative phosphorylation	
		Haematin-grown membranes P:O	Non-haematin-grown membranes P:O
Complete	0	0.28	0.00
KCN	0.6	0.30	N.D.
Batho. S.	0.1	0.04	N.D.
Antimycin A	0.008	0.08	N.D.
HOQNO	0.1	0.00	N.D.

N.D., not tested; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; and Batho. S., bathophenanthroline disulphonate.

* The absolute rate of NADH oxidation corresponds to 0 % inhibition.

one could expect to see greater inhibition of a flavin oxidase system where the riboflavin moiety is bound to the apo-enzyme by a thioester bond.

No ATP generation from NADH oxidation was found with non-haematin extracts when up to 1.0 mg (protein) of the high-speed fraction or the cytoplasm was used. The high-speed fraction (0.05 mg protein) from haematin-grown cells coupled the oxidation of NADH to the formation of ATP (Table 2). In several experiments P:O ratios from 0.14 to 0.36 were found, depending upon the speed and temperature of the isolation. The ATP formed by adenylate kinase was subtracted from the ATP formed in the whole reaction and was never more than 10 %. Adenylate kinase activity was inhibited by AMP, EDTA, and KF and was partially eliminated by washing the high-speed fraction. Antimycin A and HOQNO gave 44 % and 72 % inhibition of NADH oxidation and uncoupled the ATP formation (Table 2). Significant results were obtained with bathophenanthroline disulphonate, since this compound decreased the P:O ratio by 85 %, but inhibited the NADH oxidation by only 12 %. KCN did not decrease the P:O ratio, but inhibited the NADH oxidation by 43 %.

The uncoupler, bathophenanthroline disulfonate, supports the present evidence that ATP production can be separated from the electron-respiratory chain without inhibition of electron transport. Similarly, when KCN was used only the rate of electron transport was affected, not ATP production. Such findings can be attributed to the purity of the high-speed fraction of membranes and the successful inhibition of the adenylate kinase. The data suggest inhibition of the rate of electron transport in a cytochrome system, which can be independent of any ATP formation, and also suggest uncoupling of phosphorylation resulting from cytochrome-mediated electron flow.

Table 3. *Sites of NADH oxidase in S. faecalis var. zymogenes*

Fractions were prepared as described in the text.

	Extracts*						Total Units† (TU)
	Low-speed membranes		High-speed membranes		Cytoplasm		
	TU†	SA‡	TU†	SA‡	TU†	SA‡	
Haematin-grown	369	22.0	394	36.0	182	1.7	945
Non-haematin-grown	26.0	1.6	31.0	3.3	340	2.5	397

* Total extracts being 1 g wet wt bacteria.

† Total units = $0.178 \Delta E_{340}/2 \text{ min}/0.1 \text{ ml extract}$.

‡ SA = specific activity (units/mg protein).

Fig. 2. Reduced-oxidized and reduced cyanide-reduced spectra of *S. faecalis* var. *zymogenes* grown in the presence of haematin and a haematin control. See text for preparation and results. - - -, Haematin control; —, haematin-grown membranes.

The sites of NADH oxidation were explored (Table 3). Cultures grown with haematin: (1) have more than twice the total NADH oxidizing power of non-haematin-grown cultures; and (2) have the site of NADH oxidation primarily in the membranes, thus agreeing with the findings of Bryan-Jones & Whittenbury (1969). The high-speed fractions from haematin-grown cultures had a deep brown colour with a red tint, while those from non-haematin-grown cultures were white with a yellow tint. Thus another outstanding difference between the haematin-induced and the flavin pathways is that bacteria grown with haematin have a membrane-bound NADH oxidase system, while those grown without haematin have a soluble NADH oxidase. The usual bacterial cytochrome systems are obligately membrane-bound. Henderson (1971) reported that there has been only one preliminary description of a 'soluble' system which was capable of oxidative phosphorylation. Thus, knowledge of the location of the NADH oxidase system can contribute to the identification of a cytochrome system.

Spectral data support the theory of cytochrome induction (Fig. 2). A notable difference is

seen between the peaks of the haematin (568, 538 and 425 nm) and those of the membrane preparation of haematin-grown cells (559, 531 and 429 nm). When properly washed, little or no absorption was seen at 425 nm in cells to which haematin was added after growth. Membranes of anaerobically grown bacteria never exhibited spectra around 425 to 430 nm. The effect of KCN upon the reduced spectra of the same membrane suspensions (aerobically haematin-grown) is shown in the upper curves of Fig. 2. In the reduced-cyanide minus reduced-spectrum a marked difference is evident between the absorption maximum of the haematin control at 425 nm and that at 435 nm for the membranes of cells grown with haematin. When NADH (1 μ mol NADH or lactic acid plus lactate dehydrogenase) was used to obtain a reduced- minus oxidized-spectrum with HOQNO preventing reoxidation the results were almost identical with those in the lower portion of Fig. 2; however, the intensity of the absorption was less. The advantage of this procedure was that the haematin control was not reduced. In contrast, when NADH reduced the preparation in the presence of KCN, the same shift in the solet region was seen as that appearing in the upper left of Fig. 2. If neither HOQNO nor KCN were used, only slight reduction could be seen due to the reoxidation of the cytochrome system. In the reduced-carbon monoxide minus reduced-spectrum a cytochrome of the *b* type (559) did not appear. A cytochrome of the *a* type did appear in the lower solet region and was significantly different than the haematin control.

The spectral data revealed a membrane-bound cytochrome of the *b* type which may be the same as that found by Bryan-Jones & Whittenbury (1969) since both had peak at 559 nm. The exact identity of the cytochrome oxidase reported in this communication has not been determined.

The data presented lead to the conclusion that the membranes of haematin-grown cells contained: (1) the components giving cytochrome spectra; (2) the major portion of the NADH oxidase; and (3) the mechanism for cell-free oxidative phosphorylation. Molar growth yields gave evidence for oxidative phosphorylation in growing cultures which contained haematin, thus evidence for a functional cytochrome system. When bacteria were grown without haematin: (1) a soluble flavin system developed, and (2) oxidative phosphorylation did not occur.

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