Distribution of Cytochrome-like Respiration in Streptococci

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SUMMARY

The electron transport systems of 134 strains of streptococci were studied after aerobic growth on glucose in the presence of haematin, by examining the inhibition of electron transport as well as the cellular site of NADH oxidation. Each strain was placed into one of three possible groups: cytochrome-like NADH oxidase; flavin-like NADH oxidase; or no NADH oxidase. Most (88 %) of the strains of *Streptococcus faecalis* and its variants *liquefaciens* and *zymogenes* and a few strains of *S. lactis* and its variant *diacetylactis* contained cytochrome-like respiratory systems. Other streptococci including *S. faecium* fell into one of the other groups but did not contain cytochrome-like NADH oxidases.

INTRODUCTION

None of the members of the genus *Streptococcus* is known to be capable of haem synthesis. When grown in a glucose-yeast extract medium neither catalase nor cytochromes are produced. Whittenbury (1964), Bryan-Jones & Whittenbury (1969), Sijpsteijn (1970) and Ritchey & Seeley (1974*a*) have shown, however, that cytochromes are formed in some streptococci grown aerobically in media containing haem compounds. These organisms are blocked in the steps of haem synthesis but possess the genetic determinants to establish a membrane-bound cytochrome electron transport chain under appropriate conditions.

Ritchey & Seeley (1974*a*) demonstrated functional cytochromes in *Streptococcus faecalis* var. *zymogenes* by measuring molar growth yields and *in vitro* P:O ratios from membrane extracts. The cytochrome content of cells was also examined spectrophotometrically. Because of their complexities, however, these methods were unsatisfactory for a large-scale survey of a number of species. By investigations of the cellular site of NADH oxidase, and the selective inhibition of this enzyme pathway, the incidence of a cytochrome-like system in streptococci is demonstrated.

METHODS

Bacteria. The streptococci studied are listed in Table 1. The sources of strains are given where known. Strain identification was verified by a large number of physiological and biochemical tests.

Growth and harvesting of bacteria. Bacteria were grown in 2 l baffled flasks containing 450 ml medium of initial pH 7.6, containing (g/l): tryptone (Difco), 10; yeast extract (Difco), 5; glucose, 4; K₂HPO₄, 3. Haematin (0.5 g/100 ml of 0.2 M-KOH) was prepared monthly by filter sterilization and added to a final concentration of 20 µg/ml. The inoculum was 9 ml from a 10 to 20 h culture. The flasks were incubated at 30 °C with shaking until

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Table 1. Sources of strains and tests used to determine type of NADH oxidation

One unit was 0.178 of the change in E_{340} in 2 min in 0.1 ml extract from 1 g wet wt cells. For description of preparation of pellet and cytoplasm see Methods.

Organism	Strain no.	Received from*	Isolated from	Total units		NaN ₃ 0.1 M	Inhibition %		Type of NADH oxidation
				Pellet	Cytoplasm		KCN 0.5 mM	HOQNO 0.1 mM	
<i>Streptococcus pyogenes</i>	PAM	Authors	Human throat	0	190	59	21	6	F
	NEW	Authors	Human throat	0	77	72	32	5	F
	BIG	Authors	Human throat	0	0				N
	P-B	Authors	Human throat	0	0				N
	ST	Authors	Human throat	0	0				N
<i>S. equisimilis</i>	HS	Authors	Human throat	0	0				N
	208	CUC		0	130	46	20	9	F
	1AUG	N.L.N.	Bovine mouth	0	66	64	15	3	F
	T	CUC		0	81	52	27	2	F
	28AUG	J.F.T.	Equine lesion	0	115	70	40	0	F
<i>S. equi</i>	1AUG	N.L.N.	Bovine lesion	—	—				N
	10/1	J.F.T.		0	0				N
	6081	CUC		—	—				N
<i>S. dysgalactiae</i>	28AUG	J.F.T.	Bovine udder	—	—				N
	VT	J.F.T.	Bovine udder	—	—				N
<i>S. sanguis</i>	10556	ATCC		0	0				N
	10557	ATCC		0	0				N
	10558	ATCC		0	0				N
	WE4ERYR	J.M.R.		0	0				N
	Challis-6	J.M.R.		0	0				N
	FW35	J.M.R.		0	0				N
	Blackburn	J.M.R.		0	0				N
	84A	J.M.R.		0	0				N
	5042/48	J.M.R.		0	0				N
	RB742	J.M.R.		0	0				N
	K208	J.M.R.		0	0				N
	3437/48	J.M.R.		0	0				N
	B	CUC		0	107	47	22	8	F
	28AUG	J.F.T.		0	27	42	21	4	F
<i>S. agalactiae</i>	55617	N.L.N.	Bovine udder	0	35	38	16	3	F
	Ib†	N.L.N.	Bovine udder	0	75	63	31	5	F
	II†	N.L.N.	Bovine udder	0	96	39	17	7	F
	Ia†	N.L.N.	Bovine udder	0	187	30	10	10	F
	III†	N.L.N.	Bovine udder	0	43	56	11	5	F

Table 1. (*continued*)

Organism	Strain no.	Received from*	Isolated from	Total units		Inhibition %			Type of NADH oxidation
				Pellet	Cytoplasm	NaN ₃ 0.1 M	KCN 0.5 mM	HOQNO 0.1 mM	
<i>Streptococcus agalactiae</i>	II†	N.L.N.	Bovine udder	0	65	51	15	6	F
	970	N.L.N.		0	143	43	24	11	F
	971	N.L.N.		0	89	61	13	2	F
<i>S. salivarius</i>	TR	Authors	Human throat	0	0				N
	129	CUC		0	0				N
<i>S. bovis</i>	37124	CUC		0	0				N
	37134	CUC		0	0				N
	37132	CUC		0	0				N
	37130	CUC		0	0				N
<i>S. equinus</i>	7A	CUC		0	49	44	10	2	F
	19B	CUC		0	16	57	10	8	F
	12A	CUC		0	52	56	18	1	F
	19A	CUC		0	31	48	5	11	F
	13A	CUC		0	83	42	23	0	F
	11B	CUC		0	18	61	24	2	F
	B	H.B.N.		0	0				N
	C	H.B.N.		0	0				N
<i>S. uberis</i>	MAR	H.B.N.		0	0				N
	VET	N.L.N.	Bovine udder	0	46	46	8	8	F
	28VET	J.F.T.		0	90	54	13	5	F
<i>S. mutans</i>	10449	CUC		0	0				N
	6715	CUC		0	0				N
<i>S. sp. group G</i>	TR	CUC		0	181	62	21	6	F
<i>S. sp. group E</i>	I†	J.F.T.	Swine	0	37	55	17	10	F
	II†	J.F.T.		0	102	60	28	12	F
	III†	J.F.T.		0	18	64	25	7	F
<i>S. faecium (durans)</i>	VET	J.F.T.	Bovine faeces	0	81	40	4	7	F
	530ED	T.M.C.	Food	0	619	75	18	15	F
	8989	T.M.C.	Food	0	281	55	11	6	F
	8017.5D	T.M.C.	Food	0	195	67	25	10	F
	9217	T.M.C.	Human faeces	0	318	41	11	0	F
	9136	T.M.C.	Human faeces	0	131	54	22	12	F
	731P1	T.M.C.	Human faeces	0	225	61	23	3	F
	731P3	T.M.C.	Human faeces	0	283	80	16	5	F
	725P1	T.M.C.	Human faeces	0	229	49	6	4	F
	25307	CUC	Human faeces	0	110	65	7	12	F

Table 1. (*continued*)

Organism	Strain no.	Received from*	Isolated from	Total units		Inhibition %			Type of NADH oxidation
				Pellet	Cytoplasm	NaN ₃ 0.1 M	KCN 0.5 mM	HOQNO 0.1 mM	
<i>Streptococcus faecalis</i> var. <i>zymogenes</i>	27C1	CUC		335	190	0	89	46	Cy
	C	CUC		633	334	2	90	61	Cy
	31	CUC		0	70	52	19	10	F
	36C3	L.N.Z.		738	418	3	94	71	Cy
	26C1	L.N.Z.		477	201	9	92	64	Cy
	SP4	L.N.Z.		372	131	7	92	55	Cy
	SP1	L.N.Z.		572	207	0	89	70	Cy
	x-14	L.N.Z.		629	298	0	90	49	Cy
	84A	L.N.Z.		654	260	1	89	67	Cy
	458G	T.M.C.	Human faeces	0	410	40	30	10	F
<i>S. lactis</i>	TR	Authors	Human faeces	394	182	11	92	72	Cy
	I3	Authors	Raw milk	0	25	40	18	15	F
	14	Authors	Raw milk	0	0				N
	18	Authors	Raw milk	0	18	7	29	8	F
	TR	CUC		317	113	18	90	12	Cy
	7962	CUC		0	89	0	15	9	F
	10-19	J.F.T.	Milk	0	210	18	25	1	F
	C	H.B.N.		444	86	22	62	8	Cy
	21	H.B.N.		583	69	20	75	21	Cy
	L21	H.B.N.		0	42	43	31	0	F
<i>S. lactis</i> var. <i>diacetylactis</i>	MAR9	H.B.N.		0	130	36	15	5	F
	Chi	H.B.N.		298	128	21	79	15	Cy
<i>S. cremoris</i>	PL	H.B.N.		0	56	25	30	11	F
	BM	H.B.N.		0	39	24	19	7	F
	MAR9	H.B.N.		0	0				N
	chi	H.B.N.		0	30	21	19	9	F

F, flavin-like; N, no NADH oxidase; Cy, cytochrome-like; —, did not grow aerobically in a haematin-containing medium.

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† Strain number also designates sero-type.

the late-exponential phase of growth was reached. Since generation times varied for different species the time of incubation was altered to give a constant final growth state. Bacteria were harvested by centrifuging at 15000 *g* for 15 min (21500 *g* for 15 min was used where cell packing was insufficient), then washed twice in 0.015 M-2-*n*-morpholino-ethanesulphonic acid (MES) at pH 7.3.

Bacterial extracts. Bacteria (1 g wet wt) were resuspended in 25 ml MES and then broken in an X-Press (BBL, Rockville, Maryland, U.S.A.) at -25°C and 20000 lb in $^{-2}$ (138 MPa). The resulting suspension was centrifuged at 40000 *g* for 15 min and the pellet discarded. The supernatant fluid was recentrifuged at 78000 *g* for 90 min. The supernatant fluid from this centrifugation contained the cytoplasm. The pellet (resuspended in 5 ml MES with 10 % sucrose) contained the vesicles and small membrane fragments. Certain strains and particular species were harder to break than others, and this introduced an inaccuracy in that a preparation with high breakage liberated more units of NADH oxidase into the cell-free fraction. Between 50 and 80 % of the bacteria from any species were broken by the X-Press. Thus, if a strain was reported to have 400 units of NADH oxidase/g wet wt the maximum error was ± 120 units. The variation did not affect the inhibitor data or data on the site of cellular NADH oxidase. Duplicate breakage of a single strain indicated that no more than ± 10 % variation would result.

NADH oxidase activity. The assay for NADH oxidase activity was performed as previously described (Ritchey & Seeley, 1974*a*). If the primary assay of 0.1 ml of the membrane fraction showed very high levels of activity, the 5 ml membrane fraction was diluted. Inhibitors of NADH oxidation [0.20 ml of 1.0 M-NaN₃ to give a final concentration of 0.1 M, 0.05 ml of 0.02 M-KCN to give a concentration of 0.5 mM, or 5.0 μl of 2-*n*-heptyl-4-hydroxyquinoline-*n*-oxide (HOQNO) to give a final concentration of 0.1 mM] were added in three subsequent assays.

The 25 ml fraction containing the cytoplasm was then assayed for enzymic activity. Normally 0.2 ml of the 25 ml fraction contained the optimum concentration of activity for these assays. Since inhibitors were used to confirm the predominant type of NADH oxidase, their effect on the cytoplasmic NADH oxidase was not examined if the membrane fraction contained a cytochrome-linked NADH oxidase. The term 'NADH oxidase' is used in a very broad sense and refers to the primary pathway from NADH to oxygen, which is either flavin-linked or cytochrome-linked. Descriptive terms used here to designate these pathways are 'flavin-like' or 'cytochrome-like'. These terms are used to recognize the limitations of classifying NADH oxidases in specific categories where complete individual electron carrier systems were not isolated and characterized. If the cytochrome-like pathway was not present in the 5 ml membrane fraction, inhibitors were used to confirm the presence of any flavin-like NADH oxidase.

RESULTS

The NADH oxidase activity was measured in the membrane fraction and cytoplasm of the 134 strains of streptococci (Table 1). A few strains did not grow aerobically under these conditions, although they grew anaerobically in the same medium. In some instances neither the particulate nor the soluble fraction of aerobically haematin-grown cells contained any NADH oxidase activity. These strains were regrown and harvested in the mid-exponential phase of growth. An organism was classified as not containing NADH oxidase (group 3) if activity could not be found under any of these conditions.

An organism was classified as flavin-like (group 2) if fewer than 10 units of enzyme activity were present in the 5 ml of the particulate fraction (this represented the lower limit

of assay sensitivity) and there was detectable soluble NADH oxidase. The criteria used to classify either pathway were arbitrary since a precedent for such a scheme has not yet been established. The limits for assigning either units of activity or percentage inhibition were determined by careful examination of the data. The presence of a flavin-like system was confirmed by measuring inhibition of the rate of NADH oxidation. With 0.1 M-NaN₃, inhibition was greater than 35 %, with 0.5 mM-KCN it was less than 35 %, and with 0.1 mM-HOQNO less than 25 %. The species which were difficult to fit into this classification were *Streptococcus lactis*, *S. lactis* var. *diacetylactis* and *S. cremoris*. These species (the lactic streptococci) may have a different type of flavin-linked NADH oxidase, in that their soluble NADH oxidase was relatively insensitive to NaN₃.

An organism was labelled 'cytochrome-like' (group 1) if there were more than 250 units of enzymic activity present in the 5 ml of the particulate fraction. The presence of a cytochrome system was confirmed by the degree of inhibition of the rate of NADH oxidation. With 0.1 M-NaN₃, inhibition was less than 20 %, with 0.5 mM KCN it was greater than 75 %, and with 0.1 mM HOQNO greater than 35 %. Again the lactic streptococci presented the exceptions: those with a cytochrome-like NADH oxidase system were not very sensitive to HOQNO.

Streptococci were divided into six general groups, depending upon the type of NADH oxidase found. (i) The species which failed to reveal any type of NADH oxidase: *S. equi*, *S. dysgalactiae*, *S. sanguis*, *S. salivarius*, *S. bovis*, *S. thermophilus* and *S. mutans*. (ii) The species containing strains, some of which were devoid of NADH oxidase and others with a flavin-type NADH oxidase: *S. pyogenes*, *S. zooepidemicus* and *S. cremoris*. (iii) The species with strains which contained the flavin-type NADH oxidase alone: *S. equisimilis*, *S. agalactiae*, *S. equinus*, *S. uberis*, *Streptococcus* group G, *Streptococcus* group E, and *S. faecium* (including strains formerly known as *S. durans*). (iv) *S. faecalis* var. *liquefaciens*, the only species which contained strains with a cytochrome-like NADH oxidase alone. (v) Strains of *S. faecalis*, *S. faecalis* var. *zymogenes* and *S. lactis* var. *diacetylactis* which produced either the cytochrome-like or the flavin-like NADH oxidase systems. The strains of *S. faecalis* and *S. faecalis* var. *zymogenes* which produced the flavin-like system were old laboratory cultures, by which is meant a strain which had been isolated more than one year prior to this study and which had usually been carried in laboratory media for many sub-transfers. The term old laboratory culture is used to call attention to the fact that mutations might have occurred during the time of sub-culturing, thus affecting components necessary for the cytochrome pathway. (vi) *S. lactis* the only species which contained strains which fell into the main groups i, ii, and iv.

DISCUSSION

The ability of certain streptococci to metabolize haem compounds has remained obscure until recently because good growth occurred in many media without haematin and where cytochromes did appear they were inducible. In the present study all strains which produced cytochromes in haematin-containing medium also produced a flavin system when grown in a medium devoid of haematin, and were thus haematin-dependent for cytochrome formation but not for respiration.

The object of this investigation was to identify species of the genus *Streptococcus* able to form cytochromes when grown under certain defined conditions. Using cell-free extracts of newly isolated strains, the distinction between the cytochrome and flavin systems was easily made. Cytochrome-like enzymic activity, when it occurred, was present in the washed membranes at levels of at least 290 units as seen in *S. faecalis* var. *liquefaciens* strain T91

(Table 1). This activity was significantly more sensitive to KCN and HOQNO but less sensitive to NaN_3 than was the activity of the flavin system.

Under our cultural conditions, several strains produced less than 50 units of flavin-linked NADH oxidase/g wet wt bacteria. These strains were only weakly oxidative, and would have been classified as lacking NADH oxidase if particular attention had not been given to cultivation of batches. Several species were found to grow aerobically only if haematin were present. This effect was not due to the induction of cytochromes, however, since NADH oxidase was not produced. Ritchey & Seeley (1974*b*) have reported that 13 strains of *S. sanguis* were protected from hydrogen peroxide in the presence of haematin under aerobic conditions. These strains gave increased molar growth yields but did not produce cytochromes.

Of the enterococci (all serological group D) only *S. faecalis* and its two varieties produced the cytochrome-like system. All 18 strains of *S. faecium* failed to form cytochromes. Of the 40 strains of the *S. faecalis* group only five did not form cytochromes. These five strains were old laboratory cultures which may have been genetically altered. A block at any site of the cytochrome pathway would result in the establishment of a mutant strain. The number of loci needed to code for an entire cytochrome system must be large, thus increasing the chances for the occurrence and persistence of mutants incapable of cytochrome induction. This possibility is increased since the maintenance of a culture in the laboratory is likely to have been in haematin-free media. It should be noted, however, that several old laboratory cultures produced the cytochrome system.

Although it was not our purpose to examine the types of flavin-like electron transport systems in the streptococci, sufficient evidence was gathered to conclude that the lactic streptococci may have a flavin system differing from those of other streptococci since it was less sensitive to NaN_3 . Of 15 strains of the lactic streptococci (all serological group N), four formed cytochromes. The other strains contained either a flavin NADH oxidase or no NADH oxidase.

London & Kline (1973), upon examining the aldolases of several species of the genera *Streptococcus*, *Lactobacillus* and *Pediococcus*, found that the electrophoretic mobility rates of the fructose diphosphate aldolases of *S. faecalis* strain MR and *S. cremoris* strain ATCC19259 were somewhat similar but they differed equally from *S. durans* (*S. faecium*). This would seem to relate to our finding that *S. faecalis* and the lactic streptococci form cytochromes but *S. faecium* does not. London & Kline (1973) presented a phylogenetic map which placed the enterococci and the group N streptococci near the genera *Lactobacillus* and *Pediococcus*, in a linkage closer than to any other member of the genus *Streptococcus*. These findings are in general agreement with those of Whittenbury (1965) which demonstrated a close linkage between *Pediococcus* and *enterococcus*. Species of the genus *Pediococcus*, however, appeared to be physiologically and biochemically more like *S. faecium* than *S. faecalis*. Furthermore, *S. faecalis* but not *S. faecium* produces cytochromes when grown in a haematin-containing medium. Additional work may be needed to reclassify *S. faecalis* at the genus level. The ability of some strains of *S. lactis* to form cytochromes suggests ancestral linkages not too remote from *S. faecalis*. Although *Bergey's Manual of Determinative Bacteriology* (1974) states that members of the genus *Streptococcus* do not contain haem compounds, this view must be modified to recognize that *S. faecalis* and *S. lactis* may contain cytochromes in appropriate conditions. The extent to which cytochrome formation occurs under natural conditions, e.g. during infections where suitable haem precursors for cytochrome formation are available, remains to be determined.

Cytochrome induction in streptococci has been shown to occur exclusively under aerobic

conditions and is dependent upon the environment for exogenous haem. Van der Wiel-Korstanje & de Vries (1973), using another faecal inhabitant, *Bifidobacterium longum*, found that lysed red blood cells induced cytochromes *b* and *a* aerobically or cytochromes *b* and *d* anaerobically during nitrate reduction. These findings parallel the data on cytochrome induction in streptococci to the extent that in both cases a haem compound was necessary for aerobic cytochrome formation. If an anaerobic cytochrome system exists in *S. faecalis*, it has not yet been demonstrated; however, the conditions of the intestine have not been simulated, since an alternate electron acceptor such as nitrate has never been provided. Langston & Williams (1962) reported that some group D streptococci were capable of nitrate reduction.

Another faecal inhabitant, *Escherichia coli*, contains aerobic cytochromes as well as anaerobic cytochromes for nitrate reduction. The aerobic cytochromes are induced by exposure to highly oxidative conditions and produce additional energy for growth. Cytochromes may confer a similar advantage upon *S. faecalis*. *Streptococcus faecalis* var. *zymogenes* produces 4 mol ATP/mol glucose aerobically with the flavin electron transport system (Ritchey & Seeley, 1974*a*). With the cytochrome system, 5 mol ATP were produced. Since the flavin system may be non-functional in an anaerobic environment, only 2 mol ATP would be formed from fermentation. If these organisms had an anaerobic cytochrome system, however, the additional energy yield, possibly 5 instead of 2 mol ATP, would be of enormous advantage.

A significant finding (Smalley, Jahrling & VanDemark, 1968) was that *S. faecalis* strain 10C1 was capable of oxidative phosphorylation without haematin. We found that cytochromes were induced when this organism was grown with haematin but that the energy yield was only slightly increased. The aerobic molar growth yield of 53.5 with the flavin system increased only to 56.2 with the cytochrome system. It appears that the cytochrome system which developed in the presence of haematin was favoured over the flavin system possibly because the former has a higher capacity for binding most terminal electron acceptors (Niederpruem & Hackett, 1958).

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