Energetics of Growth in a Tyrothricin-producing Strain of *Bacillus brevis*

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SUMMARY

The particulate NADH oxidase activity of *Bacillus brevis* is inhibited by tyrocidine and the kinetics of inhibition indicate that the NADH dehydrogenase region is the primary site of action. A decrease in NADH oxidase activity of electron transport particles and of NADH oxidase activity of whole organisms occurs in batch culture in the later stages of growth when production of tyrothricin increases. That respiration exhibited by growing cells takes place via the respiratory electron-transport system is indicated by the similarities in levels of activity and cyanide sensitivity of endogenous and NADH oxidation of growing cells. Exponential growth on glycerol asparagine media is diphasic, a slower growth rate commencing as the O_2 tension of the culture approaches zero and tyrothricin production increases. Molar growth yield data based on overall measurements of growth related to O_2 uptake in batch culture lead to low ATP/O ratios. In an attempt to determine Y_0 and ATP/O ratios under optimum conditions of growth a method is described which was based entirely on the growth and respiratory activity of exponentially growing cells. Such measurements led to higher values for Y_0 and to ATP/O ratios approaching 3, indicating the participation of three sites of phosphorylation during exponential growth. Tyrocidine and DNP inhibited growth, the tyrocidine at concentrations similar to those produced at the end of the exponential growth phase. Y_0/Y_{ATP} ratios (ATP/O ratios) on glycerol asparagine media were lower than on peptone yeast extract medium which yielded no tyrothricin, a possible indication that some form of uncoupling occurs in organisms grown on glycerol asparagine medium which does not yield tyrothricin.

INTRODUCTION

Previous reports (Fynn & Seddon, 1971; Seddon & Fynn, 1971; Fynn, Thomas & Seddon, 1972) have shown that *Bacillus brevis* (ATCC 10068) possesses an active respiratory electron-transport system as well as oxidative processes involved in the tricarboxylic acid (TCA) cycle. Studies with a different strain of *B. brevis* (*B. brevis* GB; producer of gramicidin S) have also shown the presence of a similar cytochrome system although cytochrome *o* was not mentioned (Vasil'eva, Groshev & Shestakov, 1970).

Tyrothricin produced by *Bacillus brevis* ATCC 10068 inhibits the particulate NADH oxidase system (Seddon & Fynn, 1971) whereas the NADH oxidase system found in the soluble fractions is unaffected (Fynn & Seddon, 1972). The relevance of these activities in the intact organism is unknown but it is known that these cyclic, decapeptide antibiotics are produced at the end of exponential growth when the organism experiences some limitation of nutrients necessary for growth (Mach, Reich & Tatum, 1963; Fujikawa, Suzuki & Kurahashi, 1968; Katz, 1968).

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This investigation was an attempt to reveal any correlation between the known effects of tyrothricin and tyrocidine on membrane systems and on the physiological aspects of growth and respiration of the intact, growing, tyrothricin-producing organism.

METHODS

Organism, growth and maintenance. *Bacillus brevis* ATCC 10068 was maintained and grown as described previously (Seddon & Fynn, 1971). The glycerol-asparagine (GA) medium was replaced by a peptone-yeast extract (PYE) medium (Fynn, Thomas & Seddon, 1972) for some studies. The dissolved O₂ concentration of the bacterial culture was measured by means of a submerged Clark-type O₂ electrode fitted into the incubation flask under aseptic conditions (oxygen content is quoted as percentage air-saturated media at 37 °C). The electrode and O₂ concentrations of the various gas mixtures used were calibrated as described by LeFevre (1969). Growth was measured by extinction at 600 nm in a Unicam SP 600 spectrophotometer calibrated for dry weight, which was determined after centrifuging and washing the bacteria with basal salts medium (Seddon & Fynn, 1970) and by drying to constant weight in a vacuum oven at 80 °C. Growth on predetermined amounts of oxygen was made by first equilibrating the culture with the gas mixture required (equilibrium was followed by measuring the O₂ content using the submerged oxygen electrode) and then sealing the flask with a sterile rubber bung. Growth, as dry weight, on 100% oxygen was three times that on 20.9% oxygen (air), indicating that in the latter experiments growth was not limited by the GA nutrients but by the amount of O₂ available for their oxidation (Table 2).

Measurement of growth yields and calculation of ATP/O ratio with whole organisms. Maximum growth on known amounts of O₂ was measured as the extinction at 600 nm. Knowing the amount of O₂ consumed and the dry wt of bacteria formed the Y_0 (g dry wt bacteria formed/atom of oxygen consumed) value was calculated. Using the Elsdén constant (Y_{ATP} ; g dry wt bacteria formed/mol of ATP generated by catabolism of the energy source) of 10.5 (Kormancikova, Kovac & Vidova, 1969) a Y_0/Y_{ATP} ratio (which is equivalent to the ATP/O ratio) was obtained.

A second method of calculating the ATP/O ratio of intact, growing organisms was also used. As molar growth yield estimations depend upon the fact that anabolic and catabolic processes are coupled throughout growth it was decided to use only data obtained from bacteria in the exponential phase of growth since, under such conditions, anabolic and catabolic processes would be expected to be favourably coupled.

For bacteria in the exponential phase of growth the total number of organisms increases with time according to the equation

$$n = n_0 e^{0.693t/T}, \quad (1)$$

where n_0 is the original number and n the number at time t ; T being the mean generation time (Dean & Hinshelwood, 1966).

The amount of oxygen consumed in a given time during the exponential growth phase will be given by the relationship

$$d[O]/dt = rn, \quad (2)$$

where $d[O]/dt$ is the amount of oxygen consumed in a given time and r is a constant and is the amount of oxygen consumed/unit no. of bacteria/unit time interval.

Substituting equation (1) in equation (2) we obtain

$$\frac{d[O]}{dt} = rn_0 e^{0.693t/T}, \quad (3)$$

which gives

$$d[O] = rn_0 e^{0.693t/T} dt.$$

Integration between the limits 0 and t gives

$$\begin{aligned} [O] &= \int_0^t rn_0 e^{0.693t/T} dt \\ &= rn_0 T/0.693 [e^{0.693t/T}]_0^t, \end{aligned} \quad (4)$$

where $[O]$ is the amount of oxygen consumed.

If the time interval is equal to the mean generation time then $t = T$ and the equation becomes

$$[O] = rn_0 T/0.693. \quad (5)$$

In the exponential phase the number of bacteria is directly proportional to their dry weight. Thus equation (1) can be written

$$d = d_0 e^{0.693t/T}, \quad (6)$$

where d_0 is the original dry weight and d is the dry weight at time t .

Similarly equation (5) becomes

$$[O] = rd_0 T/0.693 \quad (7)$$

for the amount of oxygen consumed in time T .

The increase in bacterial dry weight in time T will be, from equation (6),

$$2d_0 - d_0 = d_0. \quad (8)$$

Therefore in one generation time the increase in g dry weight of organisms/g atom oxygen consumed (Y_0) will be (8) divided by (7)

$$\begin{aligned} &= d_0 / rd_0 (T/0.693) \\ Y_0 &= 0.693/rT, \end{aligned}$$

where r is the units of g atoms oxygen min^{-1}/g dry wt bacteria. Hence

$$Y_0/Y_{\text{ATP}} \equiv \text{ATP/O} = 0.693/10.5rT.$$

Knowing the mean generation time (T) and the g atoms oxygen consumed min^{-1}/g , dry wt bacteria (r) then Y_0 and the Y_0/Y_{ATP} ($= \text{ATP/O}$) ratio can be calculated.

Measurements of oxygen uptake. Oxidative activity of growing organisms was assayed with an oxygen electrode at 37 °C. Samples of growing culture were rapidly transferred into the reaction vessel (vol. 3 ml) and the rate of oxygen uptake immediately measured. Samples were diluted, where necessary, with basal salts medium (Seddon & Fynn, 1970) to give measurable rates of oxidation. The conditions of assay were such that at no time did the concentration of oxygen become limiting nor did the diffusion of oxygen into the assay solution ($\leq 0.05\%$ $\text{O}_2 \text{ min}^{-1}$) lead to false low values of oxygen uptake. NADH oxidase activity of growing culture samples was measured with the system described but with the addition of lysozyme and NADH (Fynn & Seddon, 1971). Preparation of electron-transport particles (ETP) and measurement of ETP NADH oxidase activity was described by Seddon & Fynn (1970). The effects of various components on oxidase activities were assayed by

addition of such components (0.05 ml and less) to the reaction mixture at the concentrations shown.

Electron-transport activity. NADH-cytochrome *c* reductase, NADH dehydrogenase and cytochrome *c* oxidase activities were all measured on the ETP preparation as previously described (Fynn & Seddon, 1972).

Measurement of phosphorylation coupled to NADH oxidation in cell-free extracts. ATP formation was measured using the glucose-hexokinase trapping system described by Pinchot (1967). The disappearance of P_i was measured in the same system by the method of Fiske & Subba Row (1925).

Tyrosine determination. The content of tyrosine in whole cells was assayed as described by Seddon & Fynn (1971). The purified preparation, after passage through an acid alumina column (Mach *et al.* 1963), was measured for tyrosine content.

Protein determination. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine serum albumin (Fraction V) as standard.

Materials. The chemicals used throughout this work were of A.R. quality when available. More specialized chemicals were obtained from Sigma Chemical Co, St Louis, Missouri, U.S.A., except tyrocidine and bovine serum albumin which were obtained from the Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A., and the Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex, respectively.

RESULTS

The NADH oxidase activity of an ETP preparation from bacteria grown on GA media was 100% inhibited by tyrocidine at a final concentration of $0.2 \mu\text{mol/mg}$ protein. Table 1 indicates that the site of inhibition is at the NADH dehydrogenase region of the electron-transport chain. Cytochrome *c* oxidase was little affected by the concentrations of tyrocidine used. The ETP NADH oxidase from bacteria grown on PYE media required much higher concentrations of tyrocidine for 100% inhibition of NADH oxidase activity. No tyrosine was detected from growth on PYE media. NADH oxidase activity of the ETP preparation decreased with growth of the culture whereas tyrosine production increased with growth (Fig. 1*c*, *d*). Also shown (Fig. 1*e*) is a decrease in NADH oxidase activity of whole bacteria with growth of the culture. Measurements of NADH oxidase activity of whole organisms was made after lysozyme treatment had (i) resulted in loss of endogenous oxidation and (ii) rendered the bacteria permeable to NADH such that measurement of the full NADH oxidase could be obtained in the whole organism. Fig. 2 shows that activity measured within the initial 2 min of lysozyme treatment most probably represents that possessed by the intact organism since lower values were obtained after prolonged incubation with lysozyme or passage through a French pressure cell. (Again (Fig. 1*e*) whole cell NADH oxidase activity is much higher in organisms from the first exponential phase of growth than from the second (post-exponential) phase.) The values given in Fig. 1(*e*) are those measured within 2 min of lysozyme treatment.

Both endogenous and NADH oxidase activities of exponentially growing cells were inhibited 100% by 5 mM-KCN. Since Seddon & Fynn (1971) showed that ETP NADH oxidase is sensitive to KCN inhibition, it may be inferred that endogenous oxidation of growing cells is via the cyanide-sensitive respiratory electron-transport chain.

The measured oxidative activity of cells during the exponential phase of growth on GA media was $232 \pm 5.4 \text{ ng atoms } 0 \text{ min}^{-1}/\text{mg dry wt}$ of organisms (Table 3). Fig. 1(*a*) shows a typical growth curve of *Bacillus brevis*. At a cell density of $290 \mu\text{g dry wt cells ml}^{-1}$ (E_{600} of 0.65) there was a break in the exponential growth curve, the generation time of 115.4 ± 1.4

Table 1. *The activities of various segments of the ETP NADH oxidase system and their inhibition by tyrocidine*

Enzyme activities were measured as described in the Methods section.

Enzyme system	Activity (nmol substrate oxidized or reduced min ⁻¹ mg protein 25 °C)	Inhibition by tyrocidine	
		Concentration (μmol/mg protein)	Inhibition (%)
NADH oxidase	90	0.2	100
NADH oxidase*	205	0.2	15
NADH oxidase*	205	1.5	100
NADH-cytochrome <i>c</i> reductase	44	0.2	100
NADH dehydrogenase	440	0.2	100
Cytochrome <i>c</i> oxidase	570	0.2	18

* ETP NADH oxidase activity from organisms grown on PYE media. All other values are for organisms grown on GA media.

min increasing to 491.6 ± 6.3 min. From Fig. 1(b) it can be seen that this change in growth rate coincided with a limitation in the oxygen supply, the O₂ concentration in the liquid phase having decreased to zero. A comparison of Fig. 1(a), (b) and (c) indicates that the onset of oxygen starvation is followed by a slower growth rate and an increased production of tyrothricin.

All attempts to measure phosphorylation coupled to NADH oxidation in subcellular fractions (ETP ± supernatant, supernatant ± ETP) were unsuccessful. Attempts to determine ATP/O ratios (Y_0/Y_{ATP}) using molar growth yields related to the consumption of a known amount of O₂ yielded values substantially less than 3 (values approximating to 1 were obtained) (Table 2). ATP/O ratios, however, determined using molar growth yield data from exponential phase organisms gave much higher ATP/O ratios (2.49 ± 0.06) for organisms growing on GA media (Table 3). Exponential growth on PYE media, where tyrothricin production was undetected, resulted in even higher values for Y_0 and Y_0/Y_{ATP} ratios consistent with phosphorylation coupled to three sites of phosphorylation. If oxidative phosphorylation is the major contributory system to ATP formation, one might expect uncouplers of oxidative phosphorylation to have inhibitory effects on growth. Fig. 3 shows that 2,4-dinitrophenol (DNP) addition, either to the initial GA growth media or to cells in the exponential phase of growth, resulted in complete cessation of growth; tyrocidine had a similar effect to DNP in indicating that perhaps free tyrocidine leads to uncoupling of oxidative phosphorylation (Fig. 3).

DISCUSSION

Respiratory metabolism of *Bacillus brevis* has been investigated to see if ATP synthesis resulted from oxidative processes under conditions of antibiotic production. Glazer, Silaeva & Shestakov (1966), investigating the phosphate metabolism of *B. brevis* GB, suggested it was not. *Bacillus brevis* GB produces gramicidin S (a tyrocidine molecule), and because these cyclic decapeptides uncoupled oxidative phosphorylation (Neubert & Lehninger, 1962; Hunter & Schwartz, 1967) it was argued that oxidative phosphorylation was absent under conditions of antibiotic production. Previously Seddon & Fynn (1971) showed that the tyrocidine produced by *B. brevis* ATCC 10068 strongly inhibited the ETP NADH oxidase system; present results indicate that this inhibition occurs at the NADH dehydrogenase region of the electron-transport chain. Correlated with these findings is the observation that

Fig. 1. (a) Diphasic growth curve of *Bacillus brevis* grown on GA media. (b) Oxygen content of culture media during growth of *B. brevis* on GA media. Percentage O_2 content is quoted in relation to air-saturated sterile GA media at 37°C as 100%. (c) Tyrothricin production in relation to growth on GA media. (d) ETP NADH oxidase activity in relation to growth on GA media. (e) Whole cell NADH oxidase activity in relation to growth on GA media. NADH oxidase activities were assayed in all cases at 37°C .

as tyrothricin production increases with continued growth so the ETP NADH oxidase activity falls (Fig. 1 c, d). It would appear from the results that tyrothricin at a concentration as low as 5 mg/l (20 nmol tyrothricin/mg dry wt bacteria) already shows inhibitory effects on the ETP NADH oxidase (Fig. 1 d).

To see if these findings reflected the situation in the intact, growing organism NADH oxidation in such organisms was measured. Although higher values were obtained for NADH oxidation by whole organisms, the pattern of activity remained the same (Fig. 1 e) – high

Fig. 2. NADH oxidase activity of *Bacillus brevis* and the effects of lysozyme, time and disruptive treatment on such activity. (a) Organisms harvested from the exponential phase of growth on GA media. (b) Organisms harvested from the postexponential phase of growth on GA media. 1, Whole organisms; 2, whole organisms immediately after lysozyme treatment (within the initial 2 min); 3, whole organisms 60 min after lysozyme treatment; 4, whole organisms 120 min after lysozyme treatment; 5, whole organisms after passage ($3\times$) through the French pressure cell at 10000 to 16000 lb/in² and 0 to 5 °C. All assays were at 37 °C.

Table 2. Molar growth yields of *Bacillus brevis* grown on defined amounts of oxygen

Percentage oxygen content is based on pure oxygen as 100%.

Oxygen content of flask (%)	Total growth (mg dry wt/l)	Y_0	Y_0/Y_{ATP}
1.1	43	10.8	1.03
4.0	165	11.3	1.08
20.9	470	6.4	0.62
100.0	1570	4.4	0.42

Table 3. ATP/O ratios calculated from the mean generation time and oxidative activity of *Bacillus brevis* during exponential growth on GA and PYE media.

All values are quoted as mean $\pm$ S.E.M. (number of experiments).

	Mean generation time (min)	Oxidative activity (ng atoms 0 min^{-1} /mg dry wt)	Y_0	Y_0/Y_{ATP}
GA media	115.4 ± 1.4 (19)	232.1 ± 5.4 (19)	26.1 ± 0.6	2.49 ± 0.06
PYE media	59.1 ± 0.3 (17)	359.5 ± 8.7 (17)	33.3 ± 0.8	3.17 ± 0.08

$P < 0.001$

Fig. 3. The effects of uncouplers of oxidative phosphorylation on the growth of *Bacillus brevis*. ●, Normal GA growth conditions; ■, GA media containing 5×10^{-5} M-tyrocidine; □, GA media containing 10^{-4} M DNP; ▲, normal GA growth conditions with DNP, 10^{-4} M final concentration, added at ↑; ○, normal GA growth conditions with tyrocidine, 5×10^{-5} M final concentration, added at ↑.

activity during rapid exponential growth and a gradual decrease during later stages of growth. The high NADH oxidase activity immediately after lysozyme treatment compared with such activity after passage through a French pressure cell (Fig. 2*a, b*) implies that if tyrothricin inhibits ETP NADH oxidase, then full activity of the antibiotic is not observed in the intact organism (possibly due to compartmentalization). Results with exponentially growing bacteria (Fig. 1*e*, Fig. 2) indicate that, at least with organisms producing little or no tyrothricin, NADH oxidase activity values after lysozyme treatment are equal to or greater than the whole organism respiratory activity (values ≤ 340 ng atoms $\text{o min}^{-1}/\text{mg dry wt cells}$ compared with values ≤ 285 ng atoms $\text{o min}^{-1}/\text{mg dry wt cells}$ respectively). There is therefore sufficient electron-transport respiratory activity after lysozyme treatment to account for the respiratory rates of whole organisms growing on GA media. The fact that values for NADH oxidase activity are even greater than the whole organism respiratory activity may reflect some uncoupling of respiration rates.

The results at later stages of growth, where tyrothricin is produced, are more difficult to interpret. Fig. 1(*e*) and 2 indicate that initial values for NADH oxidase activity, even within 2 min of lysozyme treatment, are low. One cannot decide whether this is a result of lysozyme treatment allowing tyrothricin to interact with the respiratory system or whether it is a true reflection of the level of NADH oxidase in the intact organism (due to tyrothricin having already interacted with the respiratory electron-transport chain). Since, in bacterial extracts, tyrothricin interacts with the respiratory electron-transport chain, then it is not at all surprising that cell-free extracts of tyrothricin producing organisms have no observable oxidative phosphorylation. Phosphorylation coupled to oxidation has been shown (Hunter

& Schwartz, 1967) to be more potently inhibited by the peptide antibiotics than the oxidative processes themselves. Thus, even though small levels of tyrothricin could result, on fractionation, in uncoupling and failure to observe oxidative phosphorylation in bacterial extracts, this does not necessarily preclude such processes in the intact organism.

Studies with whole organisms should clarify this problem and indeed the results using Y_0 values suggest that a considerable amount of ATP formation is via oxidative phosphorylation. The discrepancy between the two different approaches has been interpreted as indicating the different conditions prevailing in each case. As observed by Hadjipetrou, Gerrits, Teulings & Stouthamer (1964), values obtained from growth yields by measuring total amount of oxygen consumed led to ATP/O ratios less than 3; values approximating 1 being obtained here with *Bacillus brevis* on GA media. They suggested that such low values may result from a considerable amount of oxygen being consumed towards the end of growth with no resultant net synthesis in cellular material. The situation is probably even more complicated in the present investigation in that low O_2 concentrations in the growing culture may lead to a slower growth rate and increased tyrothricin production as suggested in Fig. 1(a)–(c). Tyrothricin itself may then lead to inefficiency in coupling of phosphorylation to oxidation.

The alternative method used here, i.e. measuring the mean generation time and the oxidative activity of exponentially growing cells, gave higher values for the ATP/O ratios. This approach, however, is not without its pitfalls. All the assumptions made in the derivation of this method must be met if accurate values are to be obtained. Oxygen consumption (catabolism) must be completely coupled to synthesis of cellular material (anabolism); oxygen consumption not connected with the respiratory electron-transport chain (for example, hydroxylation reactions resulting in the fixation of molecular oxygen) will lead to erroneously low ATP/O ratios. One must determine, therefore, the amount of oxygen consumed via the electron-transport chain. Since whole organism respiration and NADH oxidation are completely inhibited by cyanide (inhibitor of cytochrome oxidase) it is inferred that negligible amounts of oxygen consumption take place by such side-reactions in *Bacillus brevis*. There is also the possibility, albeit unlikely, that the substrates are metabolized by energy-producing pathways (as yet unknown) other than oxidative phosphorylation and the accompanying oxygen uptake is a (constant) side-reaction. Such a case would also require the cyanide-sensitive respiratory process not being coupled to oxidative phosphorylation, a rather remote possibility. The finding that DNP, an uncoupler of oxidative phosphorylation, completely inhibits further growth, strongly implies that oxidative phosphorylation is necessary for growth.

There remains the problem of whether tyrothricin production leads to uncoupling of oxidative phosphorylation. The ATP/O ratio for cells grown on GA media, which in tyrothricin is produced, is 2.49 ± 0.06 compared with 3.17 ± 0.08 for cells grown on PYE media, where tyrothricin production is undetected; for these two sets of results $P < 0.001$, showing them to be different. It is tempting to suggest the lower value obtained with GA-grown cells is a result of slight uncoupling due to traces of tyrothricin produced during the exponential growth phase (one must also concede that the two sets could fall into different groups for a variety of reasons). The results imply that oxidative phosphorylation, with three sites of phosphorylation, is operative in *Bacillus brevis*.

Finally the physiological aspects of growth presented here show that the onset of a slower growth rate and of tyrothricin production is not a result of the lack of nutrients but of a lack in the supply of oxygen, necessary for oxidative metabolism. This condition occurs when the oxygen requirement of the bacterial culture outstrips the supply of oxygen, by diffusion, to the growth media.

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