FEBS Letters 496 (2001) 40–43 FEBS 24828

The 'strict' anaerobe *Desulfovibrio gigas* contains a membrane-bound oxygen-reducing respiratory chain

Rita S. Lemos[a], Cláudio M. Gomes[a], Margarida Santana[a], Jean LeGall[a,b], António V. Xavier[a], Miguel Teixeira[a,*]

[a] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, APT 127, 2780-156 Oeiras, Portugal*
[b] *Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30622, USA*

Received 27 February 2001; accepted 4 April 2001

First published online 18 April 2001

Edited by Richard Cogdell

Abstract Sulfate-reducing bacteria are considered as strict anaerobic microorganisms, in spite of the fact that some strains have been shown to tolerate the transient presence of dioxygen. This report shows that membranes from *Desulfovibrio gigas* grown in fumarate/sulfate contain a respiratory chain fully competent to reduce dioxygen to water. In particular, a membrane-bound terminal oxygen reductase, of the cytochrome *bd* family, was isolated, characterized, and shown to completely reduce oxygen to water. This oxidase has two subunits with apparent molecular masses of 40 and 29 kDa. Using NADH or succinate as electron donors, the oxygen respiratory rates of *D. gigas* membranes are comparable to those of aerobic organisms (3.2 and 29 nmol O_2 min^{-1} mg $protein^{-1}$, respectively). This 'strict anaerobic' bacterium contains all the necessary enzymatic complexes to live aerobically, showing that the relationships between oxygen and anaerobes are much more complex than originally thought.

Key words: Anaerobe; Aerobic respiratory chain; Cytochrome *bd*; *Desulfovibrio*

1. Introduction

Since the discovery of microbial sulfate respiration more than 100 years ago [1] sulfate-reducing bacteria were described as strict anaerobic organisms. However, in the last decade, several reports have shown that these bacteria, mainly from the *Desulfovibrio* genus, are quite resistant to oxygen [2,3]. In particular, sulfate-reducers were isolated from oxic environments close to anoxic or periodically anoxic zones (e.g. [4–6]). Also, these bacteria were shown to produce ATP in the presence of oxygen (e.g. [7,8]). The mechanisms of oxygen response by sulfate-reducing bacteria have been extensively studied in *Desulfovibrio gigas*. It was shown that in the presence of oxygen, this bacterium not only survives at the expense of its polyglucose carbon reserves, but also increases ATP production by substrate level phosphorylation [9,10]. In this process, NAD^+ is regenerated through a soluble redox chain coupling NADH oxidation to the complete reduction of oxygen to water. This chain, insensitive to cyanide, is composed of three cytoplasmic proteins: NADH:rubredoxin oxidoreductase, rubredoxin and rubredoxin:oxygen oxidoreductase [11–14]. However, the presence of a membrane-bound oxygen-reducing chain was not reported to date, and it was proposed that oxygen would be reduced by the same enzymes involved in the dissimilatory sulfate-reducing pathway (e.g. [3]).

The present work shows that membranes from *D. gigas*, grown in a fumarate/sulfate medium have a fully competent and canonical respiratory chain, allowing the reduction of dioxygen to water using reducing equivalents from NADH and succinate. In particular, one of the terminal components of this chain, a cytochrome *bd*, was isolated and is shown to be capable to completely reduce oxygen. Thus, *D. gigas* cells have the necessary enzymatic equipment to respire oxygen, a surprising feature in a supposedly strict anaerobe.

2. Materials and methods

2.1. Cell growth and cell extract preparation

D. gigas cells were grown in a fumarate/sulfate medium [15] in 10 l flasks at 37°C. Cells were also grown on the standard lactate/sulfate medium [16,17]. Unless otherwise stated, all experimental procedures were performed with the fumarate/sulfate grown cells. Cells were suspended in 10 mM Tris–HCl pH 7.6/1 mM PMSF buffer. After cell breakage the suspension was centrifuged at $12\,000\times g$ for 1 h. The supernatant (cell extract) was centrifuged at $150\,000\times g$ for 2 h and the pellet (membrane fraction) was resuspended in 10 mM Tris–HCl pH 7.6/1 mM PMSF buffer. The detergent *n*-dodecyl-β-D-maltoside (DM) was diluted in buffer and added to the membranes in a ratio of 2 g per g of protein. The suspension was stirred for 45 min at 4°C and centrifuged at $160\,000\times g$ for 1 h at 4°C.

2.2. Purification of the bd-type terminal oxidase

The cytochrome *bd* was purified from cells grown on the fumarate/sulfate medium. All purification steps were performed on a Pharmacia HiLoad system, at 4°C, and all buffers used contained 0.1% DM, 1 mM PMSF and were adjusted to pH 7.6 at 4°C. Purification steps were monitored by UV–vis spectroscopy and cytochrome *bd* containing fractions identified by the 650 nm absorption band. The DM extract was applied to a Q-Sepharose column equilibrated with 10 mM Tris–HCl buffer. The column was eluted from 0 to 1 M NaCl in the equilibrating buffer and cytochrome *bd* eluted at 80–140 mM NaCl. This fraction was diluted to lower the ionic strength, applied to a second Q-Sepharose column and eluted as in the first step. The cytochrome *bd* fraction, was collected at approximately 100 mM NaCl and loaded into a HTP ceramic column on 5 mM potassium phosphate buffer. This column was run from 5 to 1000 mM potassium

*Corresponding author. Fax: (35)-1-214428766.
E-mail: miguel@itqb.unl.pt

Abbreviations: DM, *n*-dodecyl-β-D-maltoside; DMN, dimethylnaphthoquinone; DQ, duroquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)02399-7

phosphate. The oxidase sample remained firmly attached to the resin and it was eluted only with 2 M of potassium phosphate. After elution the sample was immediately diluted to ~250 mM of potassium phosphate and concentrated. The final purification step consisted on a molecular filtration in a S-200 XK 26/60 column eluted with 10 mM Tris–HCl/150 mM NaCl.

2.3. Biochemical procedures

Protein concentration was determined by a modified microbiuret method for membrane proteins [18]. For heme extraction the protein was precipitated with acetone:HCl (19:1) and the heme extracted from the supernatant with ethyl acetate. The heme content was determined spectroscopically, by reduced minus oxidized difference of the visible spectra using the following extinction coefficients: 17.5 mM^{-1} cm^{-1} (562–575 nm) for heme *b* and 7.4 mM^{-1} cm^{-1} (628–652 nm) for heme *d*. Molecular masses of the subunits of the cytochrome *bd* were determined by 12% acrylamide SDS–PAGE [19]. Proteins were stained with Coomassie brilliant blue [20].

2.4. Spectroscopic techniques

UV–vis spectra were obtained on a Shimadzu UV-1630 spectrophotometer. EPR spectra were recorded on a Bruker ESP 380 spectrometer, equipped with an ESR 900 continuous-flow helium cryostat.

2.5. Enzymatic assays

Oxygen consumption was measured polarographically at 30°C using a Clark-type oxygen electrode (YSI Model 5300, Yellow Springs). Assays were carried out in a micro-chamber (600 μl) in 20 mM Tris–HCl, pH 7.6 buffer, unless otherwise stated. NADH (20 mM) or succinate (20 mM) were used as electron donors for the membrane suspension (13 and 0.5 mg of protein, respectively). For cytochrome *bd* a 40 nM solution (final concentration) was used. Quinones or *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) were added to a final concentration of 1 mM and kept reduced by 5 mM dithiothreitol or 5 mM ascorbate, respectively. The turnover number was calculated assuming that the concentration of O_2 in air-saturated buffer at 30°C is 223 μM. Phospholipids, when used, were prepared by a mild sonication treatment: 40 mg of asolectin (Merck) were sonicated in 3 ml of 20 mM Tris–HCl pH 7.5 buffer. For some assays, the substrate duroquinone (DQ, 0.7 mg) was sonicated together with the asolectin.

3. Results

3.1. Oxygen uptake by D. gigas membranes

D. gigas membranes consume oxygen: upon addition of NADH or succinate, respiratory rates of 3.2 nmol O_2 min^{-1} mg^{-1} and 29 nmol O_2 min^{-1} mg^{-1}, respectively, were measured (Table 1). Full reduction of oxygen to water was confirmed by addition of catalase. The oxygen consumption rates are of the same order of magnitude of those found in the membranes of aerobic bacteria (e.g. *Rhodothermus marinus* [21]: 7.5 and 1.5 nmol O_2 min^{-1} mg^{-1}, at 37°C, for NADH and succinate, respectively (see Table 1)). Most interestingly, the respiratory rates of membranes from *D. gigas* cells grown in the standard lactate/sulfate medium were considerably lower than those obtained from the fumarate/sulfate growth: 0.25 and 0.37 nmol O_2 min^{-1} mg^{-1} for NADH and succinate, respectively (Table 1).

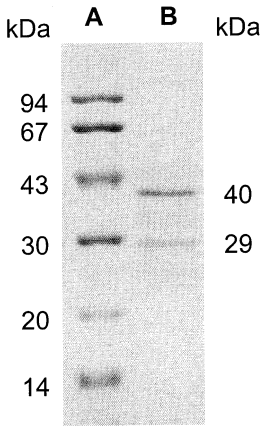

Fig. 1. SDS–PAGE of the isolated cytochrome *bd* oxidase from *D. gigas*. (lane A) Molecular mass markers and (lane B) cytochrome *bd*.

The effect of several inhibitors on the rate of oxygen consumption was tested. The membranes were incubated overnight, at 4°C, in the presence of 1 mM of each inhibitor in an ethanolic solution, for which an adequate controls using an equivalent amount of ethanol was performed (Table 2). 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) leads to a higher inhibition when succinate is the electron donor, which most probably reflects the inhibition of the succinate:quinone oxidoreductase activity. Rotenone, a specific inhibitor of type I NADH dehydrogenase inhibits approximately 20% of O_2 consumption, when NADH is used as a substrate, indicating the presence of this enzyme in *D. gigas* membranes (see below). Cyanide has a significant inhibitory effect (ca. 50%), indicating the presence of a cyanide-sensitive terminal oxidase. Antimycin A and DBMIB are inhibitors of Complex III (cytochrome bc_1). However, as they also inhibit cytochrome *bd* activity, it is not possible from these results to infer on the presence of such complex in *D. gigas* (see below).

Electron paramagnetic resonance spectra of *D. gigas* membranes are dominated by the features of fumarate:menaquinone oxidoreductase. Nevertheless, a detailed comparison of spectra obtained using NADH or succinate as reducing agents shows the presence of an axial component, at $g = 2.03$, 1.96. This is characteristic of reduced [2Fe–2S] centers, possibly associated with a type I NADH dehydrogenase (data not shown). Interestingly, these resonances were not observed in membranes from cells grown on sulfate. The canonical bc_1 complexes contain the Rieske iron–sulfur protein which, under reducing conditions, yield a characteristic EPR signature. However, no EPR spectra typical of Rieske centers were detected on reduced samples of *D. gigas* membranes from cells grown either on fumarate/sulfate or lactate/sulfate.

Table 1
Oxygen consumption rates (nmol O_2 min^{-1} mg $protein^{-1}$) measured on membranes from several organisms

	NADH-driven	Succinate-driven	Ref.
D. gigas F/S	3.2	29	This work
D. gigas L/S	0.25	0.37	This work
D. desulfuricans	4.5	n.d.	[35]
R. marinus	7.5	1.5	[21]

F/S denotes growth in a fumarate/sulfate medium and L/S in lactate/sulfate. In the case of *D. desulfuricans*, activities were measured in intact cells. n.d., not determined.

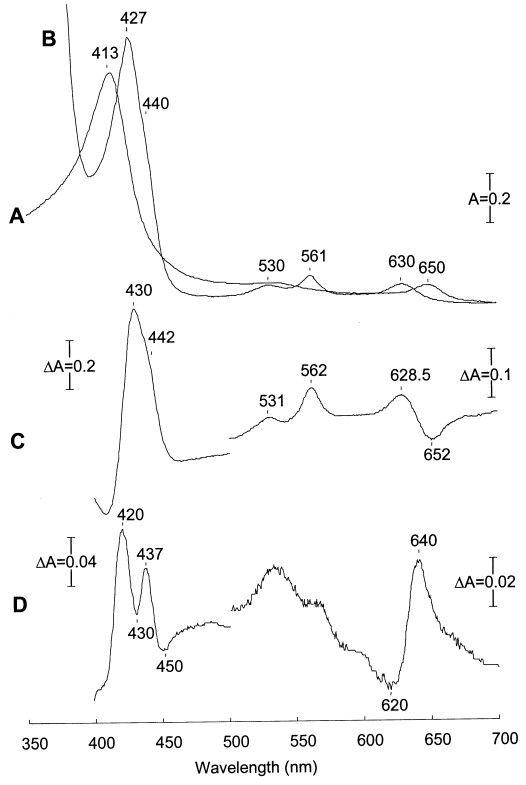

Fig. 2. Visible spectra of the isolated cytochrome *bd* oxidase from *D. gigas*. (A) Oxidized absorption spectra; (B) reduced absorption spectra; (C) reduced minus oxidized difference spectrum and (D) reduced plus CO minus reduced spectrum.

3.2. The bd-type oxidase

The full reduction of dioxygen to water by *D. gigas* membranes indicates the presence of at least one terminal oxygen reductase. Accordingly, a canonical cytochrome *bd* was purified from *D. gigas* membranes, grown on fumarate/sulfate, consisting of two subunits with apparent molecular masses of 40 and 29 kDa (Fig. 1). The electronic absorption spectrum of the oxidized and reduced forms of the purified cytochrome *bd* oxidase (Fig. 2, trace A and B) is characteristic of this class of enzymes: the spectrum of the oxidized form is dominated by an intense absorption peak at 650 nm due to heme *d*. A small broad band at 600 nm, only observed in a more concentrated preparation (data not shown), is due to the α-band of heme *b*-600 (analogous to *b*-595 in *Escherichia coli* [22]). Upon reduction the 650 nm band shifts to 630 nm. Cytochrome *b*-561 (analogous to *b*-558 in *E. coli* cytochrome *bd*) gives rise to an α-band at 561 nm in the reduced spectrum and at 562 nm in the reduced minus oxidized difference spectrum (Fig. 2, trace C), with a β-band at 530 and 531 nm, respectively. Binding of CO to the reduced form of the enzyme induces a red shift of the 630 nm band: the CO-reduced minus reduced spectrum shows a through at 620 nm and a peak at 640 nm (Fig. 2, trace D); the trough at 450 nm is also attributed to heme *d* [23].

As cytochromes *bd* are quinol oxidases, several quinone analogues were tested for catalytic activity: ubiquinone-1, plumbagin, dimethylnaphthoquinone (DMN), and DQ. The activity with the first three quinols was negligible, as well as with the ascorbate/TMPD pair. Only with DQ a significant activity was obtained. When phospholipids were added to the enzyme the oxidase activity was enhanced about 13 fold; if DQ was sonicated together with the lipids, instead of being added to the reaction mixture, the activity increases ca. 50 fold. The product of oxygen reduction was tested by the addition of catalase and proved to be H_2O. When DQ was sonicated together with lipids, the quinol oxidase activity has a turnover number of 3.5 s^{-1}, a value within the expected range for *bd*-type oxidases (10 s^{-1} and 1.3 s^{-1} for the *Bacillus firmus* [24] and *Bacillus stearothermophilus* [25] enzymes, respectively).

D. gigas cytochrome *bd* is relatively insensitive to cyanide, a characteristic of this type of oxidases. However, HQNO, a quinone analogue that competes for quinone binding sites, inhibited approximately 60% of the oxidase activity. Antimicin A and DBMIB were also proven to be highly efficient inhibitors of the *bd* oxidase, while rotenone had no effect (Table 2).

4. Discussion

Altogether, the data presented in this report show that a fully operative oxygen-reducing and membrane-bound respiratory chain is present in *D. gigas* grown in a fumarate/sulfate medium. Besides the fumarate/succinate oxidoreductase, which is induced under these conditions [26], these membranes also have a significant NADH dehydrogenase activity. The inhibition by rotenone, together with the EPR data, suggest the presence of a canonical Complex I. Most importantly, a membrane-bound terminal oxygen reductase was isolated and shown to be a member of the cytochrome *bd* family. Since this enzyme is quite insensitive to cyanide, while the oxygen uptake by *D. gigas* membranes is significantly inhibited by this anion, it may be hypothesized that there is a second oxygen reductase, of the heme-copper superfamily. Indeed, pyridine hemochrome spectrum of the extractable hemes from *D. gigas* membranes suggests the presence of A-type hemes (data not shown), and the closely related species, *Desulfovibrio vulgaris* Myazaki has genes encoding such an enzyme [27]. The expression of a canonical Complex III (quinol:cytochrome *c* oxidoreductase) could not be established, as the typical inhibitors of

Table 2
Inhibition of oxygen consumption in the membranes from the fumarate/sulfate grown cells and in the isolated *bd* cytochrome

Inhibitor (1 mM)	Inhibition in O_2 consumption (%)		
	membranes		cytochrome *bd*
	NADH-driven	succinate-driven	duroquinol-driven
HQNO	18	59	60
Rotenone	21	n.d.	0
Antimycin A	38	44	50
DBMIB	58	84	58
KCN	63	43	9

this complex, DBMIB and antimycin also inhibit the cytochrome *bd*, and the EPR resonances characteristic of the Rieske protein could not be detected. Nevertheless, there are previous examples of aerobic respiratory chains, involving cytochrome *c*:oxygen oxidoreductases, where such a complex is absent and substituted by a multiheme cytochrome complex [21]. On the other hand, if only the cytochrome *bd* is present in *D. gigas*, since it is a quinol oxidase, a Complex III is not necessary, as it happens, for example, in *E. coli* [28] and most probably in *Haemophilus influenzae* [29].

A careful analysis of the unfinished genome of another sulfate-reducing bacterium, *D. vulgaris* Hildenborough [30], shows that this organism contains putative genes encoding cytochrome *bd* oxidase as well as for a heme-copper terminal oxidase. In fact, the presence of D-type hemes in a sulfate-reducing bacterium, *Desulfovibrio africanus* was reported several years ago [31]. One of the most conserved subunits of type-I NADH dehydrogenase, the TYKY or NUOI subunit, is also present in the genome. Interestingly, putative genes encoding regulatory proteins that control aerobic/anaerobic gene expression (*Fnr* and *ArcAB*) involved in the regulation of cytochrome *bd* expression (e.g. [32]) are also present in *D. vulgaris*. Thus, it appears that the presence of a canonical oxygen respiratory chain may be a general feature of sulfate-reducing bacteria, an observation with a significant implication for the understanding of the physiology, ecology, and possible pathogenicity (e.g. [33]) of this important group of microorganisms.

Finding the enzymatic machinery adequate for aerobic respiration on an organism classified as strict anaerobe is surprising but agrees with several recent reports. It also supports the proposals for an urgent revision of the classification of microorganisms in terms of their response to oxygen [2,34]. The sensitivity to oxygen is most probably related to the type of terminal acceptor used, i.e. sulfate-reducing bacteria may be strict anaerobes while respiring sulfate, but not when using a different electron acceptor. Furthermore, it illustrates the plasticity of the microbial world and points out the importance of further studies on the relationships between anaerobes and oxygen, relations which are much more complex than what has been thought for more than a century.

Acknowledgements: This work was supported by FCT-Portugal Grants 36560/99 and 36558/99 to M.T. M.S. and R.S.L. are recipients of PRAXIS XXI Grants BPD/3382/96 and BD/19867/99.

References

[1] Beijerinck, W.M. (1895) Centralbl. Bakt. Abt. 1, 1–9, 49–59, 104–114.
[2] LeGall, J. and Xavier, A.V. (1996) Anaerobe 2, 1–9.
[3] Cypionka, H. (2000) Annu. Rev. Microbiol. 54, 827–848.
[4] Bade, K., Manz, W. and Szewzyk, U. (2000) FEMS Microbiol. Ecol. 32, 215–223.
[5] Battersby, N.S., Malcolm, S.J., Brown, C.M. and Stanley, S.O. (1985) FEMS Microbiol. Ecol. 31, 225–228.
[6] Krekeler, D., Tseke, A. and Cypionka, H. (1998) FEMS Microbiol. Ecol. 25, 89–96.
[7] Dilling, W. and Cypionka, H. (1990) FEMS Microbiol. Lett. 71, 123–128.
[8] Dannenberg, S., Kroder, M., Dilling, W. and Cypionka, H. (1992) Arch. Microbiol. 158, 93–99.
[9] Santos, H., Fareleira, P., Xavier, A.V., Chen, L., Liu, M.Y. and LeGall, J. (1993) Biochem. Biophys. Res. Commun. 195, 551–557.
[10] Fareleira, P., LeGall, J., Xavier, A.V. and Santos, H. (1997) J. Bacteriol. 179, 3972–3980.
[11] Chen, L., Liu, M.Y., LeGall, J., Fareleira, P., Santos, H. and Xavier, A.V. (1993) Eur. J. Biochem. 216, 443–448.
[12] Chen, L., Liu, M.Y, LeGall, J., Fareleira, P., Santos, H. and Xavier, A.V. (1993) Biochem. Biophys. Res. Commun. 193, 100–105.
[13] Gomes, C.M., Silva, G., Oliveira, S., LeGall, J., Liu, M.Y., Xavier, A.V., Rodrigues-Pousada, C. and Teixeira, M. (1997) J. Biol. Chem. 272, 22502–22508.
[14] Frazão, C., Silva, G., Gomes, C.M., Matias, P., Coelho, R., Sieker, L., Macedo, S., Liu, M.Y., Oliveira, S., Teixeira, M., Xavier, A.V., Rodrigues-Pousada, C., Carrondo, M.A. and LeGall, J. (2000) Nat. Struct. Biol. 7, 1041–1045.
[15] Miller, J.D.A. and Wakerley, D.S. (1966) J. Gen. Microbiol. 43, 101–107.
[16] LeGall, J., Mazza, G. and Dragoni, N. (1965) Biochim. Biophys. Acta 99, 385–390.
[17] Peck, H.D. (1966) Biochem. Biophys. Res. Commun. 22, 108–112.
[18] Waters, C. (1978) Anal. Biochem. 70, 241–243.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Diezel, W., Kopperschläger, G. and Hoffmenn, E. (1972) Anal. Biochem. 48, 621–623.
[21] Pereira, M.M., Carita, J.N. and Teixeira, M. (1999) Biochemistry 38, 1276–1283.
[22] Miller, M.J. and Gennis, R.B. (1983) J. Biol. Chem. 258, 9159–9165.
[23] Jünemann, S. (1997) Biochim. Biophys. Acta 1321, 107–127.
[24] Gilmour, R. and Krulwich, T.A. (1997) J. Bacteriol. 179, 863–870.
[25] Sakamoto, J., Koga, E., Mizuta, T., Sato, C., Noguchi, S. and Sone, N. (1999) Biochim. Biophys. Acta. 1411, 147–158.
[26] Hatchikian, E.C. and LeGall, J. (1972) Biochim. Biophys. Acta. 267, 479–484.
[27] Kitamura, M., Mizugai, K., Taniguchi, M., Akutsu, H., Kumagai, I. and Nakaya, T. (1995) Microbiol. Immunol. 39, 75–80.
[28] Ingledew, W.J. and Poole, R.K. (1984) Microbiol. Rev. 48, 222–271.
[29] Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A. and Merrick, J.M. et al. (1995) Science 269, 496–512.
[30] Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org.
[31] Jones, H.E. (1971) Arch. Mikrobiol. 80, 78–86.
[32] Govantes, F., Orjalo, A.V. and Gunsalus, R.P. (2000) Mol. Microbiol. 38, 1061–1073.
[33] Loubinoux, J., Mory, F., Pereira, I.A.C. and Le Faou, A. (2000) J. Clin. Microbiol. 38, 931–934.
[34] Mortelmans, K. (1998) Soc. Industr. Microbiol. News 48, 261–265.
[35] Abbollahi, H. and Wimpenny, J.W.T. (1990) J. Gen. Microbiol. 136, 1025–1030.