Characterization of Hydrogen Photoevolution in *Oscillatoria chalybea* Detected by Means of Mass Spectrometry

Refat Abdel-Basset and Klaus P. Bader

Lehrstuhl für Zellphysiologie, Fakultät für Biologie, Universität Bielefeld, Postfach 10 01 31, D-33501 Bielefeld, Germany

Z. Naturforsch. **52c,** 775–781 (1997); received September 8/October 17, 1997

Photosynthesis, Mass Spectrometry, Hydrogen Photoevolution, Cyanobacterium

The filamentous non-heterocystous cyanobacterium *Oscillatoria chalybea* is capable to photoevolve molecular hydrogen when the cells are flushed to anaerobiosis with nitrogen or argon and exposed to short light flashes or continuous light. The light-induced hydrogen gas exchange of *Oscillatoria chalybea* has been investigated by direct determination of dynamic changes in the hydrogen partial pressure at *m/e*=2 in the H/D collector of a mass spectrometric set-up. By means of this technique also the time curves of the light-induced hydrogen gas exchange could be directly recorded. Depending on the chlorophyll concentration in the measuring cell we observed an increasing hydrogen content of the aqueous *Oscillatoria* suspension *i.e.* a dark evolution of molecular hydrogen. Upon the onset of light an initial rise of the H_2-signal was observed which was increasingly mixed or followed by a hydrogen uptake. The capability to photoevolve molecular hydrogen was maximal with young cultures and decreased with increasing age. The hydrogen evolution signals require relatively short dark adaptation to get pronounced; few seconds suffice for 2/3 of the hydrogen evolution amplitude. Prolonged dark adaptation maximizes the flash amplitudes. The hydrogen evolution signals do not deactivate by low flash frequency. *Oscillatoria chalybea* evolves molecular hydrogen following growth on nitrogen free or nitrate containing medium. Increase of the oxygen partial pressure of the assays completely abolishes the hydrogen evolution signals with an I_{50}-value of 6 μM.

Introduction

In recent years, biological hydrogen production regains scientific interest not least because of molecular hydrogen as being an extremely clean fuel under the perspective of industrial pollution in particular. Investigations have been reported on the light-induced production of molecular hydrogen *e.g.* in so-called „biophotoreactors" with more or less immobilized cells. However, much work still has to be done before real industrial and efficient production seems to be possible. Just a few specific problems shall be mentioned here: Depending on the organism, the reaction rates are far from being sufficiently high. Moreover, the generally high oxygen sensitivity of the enzyme(s) is problematic and consequently organisms with less oxygen sensitive enzyme complexes are desirable (for recent reviews and reports see Benemann, 1996; Boichenko and Hoffmann, 1994; Markov *et al.*, 1995; Oelze, 1994). Not too many cyanobacteria have been shown to photoevolve molecular hydrogen and among these were to our knowledge exclusively unicellular or heterocystous ones.

The filamentous non-heterocystous cyanobacterium *Oscillatoria chalybea* has been shown to possess the capability to fix atmospheric nitrogen in the absence of any combined nitrogen source in the culture medium. This reaction was detected and followed by the mass spectrometric measurement of $^{15}N_2$-uptake, hence by the direct registration of the real substrate of the nitrogenase complex *i.e.* molecular nitrogen. The described nitrogen fixation was strictly confined to nitrogen-free grown cultures; nitrate- or ammonium sulfate-grown cells showed no or only marginal rates of nitrogen fixation (Bader and Röben, 1995). An inherent property of the nitrogenase systems seems to be the concomitant (or even preponderant) reduction of protons leading to a simultaneous photoevolution of molecular hydrogen. Characteristics of the cyanobacterial nitrogenase have been described in detail by the groups of Böger, Bothe,

Reprint requests to Prof. Dr. Klaus P. Bader.
Fax: 05 21 / 106-6410.

0939–5075/97/1100–0775 $ 06.00

Gallon and others (Boison *et al.*, 1996; Brass *et al.*, 1992; Gallon, 1992; Villbrandt *et al.*, 1990). Recently, we were able to show that *Oscillatoria chalybea* evolves molecular hydrogen clearly distinct from proton reduction *via* nitrogenase. This photoevolution of hydrogen was observed with nitrate-grown cyanobacteria which, in turn, have easily been shown not to exhibit any $^{15}N_2$-uptake, hence, do not fix atmospheric nitrogen (Bader and Röben, 1995; Bader, 1996). Thus, *Oscillatoria chalybea*, contains one (or two) hydrogenase(s) leading to the flash-induced hydrogen evolution observed in this organism (Bader, 1996). The present paper is intended to describe physiological aspects of the light-induced hydrogen evolution in the filamentous non-heterocystous cyanobacterium *Oscillatoria chalybea* prior to biochemical and genetic approaches.

Materials and Methods

Oscillatoria chalybea from the Algal Collection in Göttingen (Germany) was cultivated in medium D (containing 1 g/l sodium nitrate) described by Kratz and Meyers (1955) on large clay plates as porous mechanical support in Petri dishes. The cultures were kept in a climatized room at 26 °C with a 14 h light/10 h dark cycle at a light intensity of approx. 12 μE x m^{-2} x s^{-1}. Protoplasts from *Oscillatoria chalybea* were isolated following enzymic digestion (glucuronidase, cellulase) essentially as previously described (Bader *et al.*, 1983).

Mass spectrometry was performed using a modified Stable Isotope Ratio Mass Spectrometer type 'Delta' from Finnigan MAT (Bremen, Germany). The modifications of the apparatus leading to a substantial increase in the sensitivity and the response time for dynamic measurements in particular, have been developed earlier and described in detail (Bader *et al.*, 1987). Further specifications and the calibration of the set-up for quantification of the respective signals have also been outlined (Bader *et al.*, 1992; compare also Bader and Röben, 1995). Hydrogen evolution and uptake was directly detected at m/e = 2 in a H/D collector and recorded on an SE 130–03 three-channel recorder from Goertz Metrawatt.

Oxygen flash measurements were performed amperometrically by means of the 'Three Electrode System' designed and described by Schmid and Thibault (1979). Flashes were supplied by the stroboslave 1539A from General Radio (Concord, Massachussetts, USA) and triggered by a laboratory-built pulse generator. At half intensity the flashes were 5 μs long and spaced 100 ms to 1000 ms apart as indicated in the figure. The amperometric oxygen signals were processed by using the calculation program developed by Schulder for an Atari Mega ST4 computer (Schulder *et al.*, 1992).

Results and Discussion

Under anaerobic conditions the filamentous non-heterocystous cyanobacterium *Oscillatoria chalybea* exhibits a strong hydrogen evolution signal upon illumination with short (5 μs) saturating flashes (Bader, 1996) or under continuous light (Fig. 1). No substantial differences are observed when the protoplasts are flushed to anaerobiosis with nitrogen or with argon, respectively. Maximal differences were in the region of about 15%. However, young cultures (1 to 2 weeks after inoculation) generally performed better than old cultures

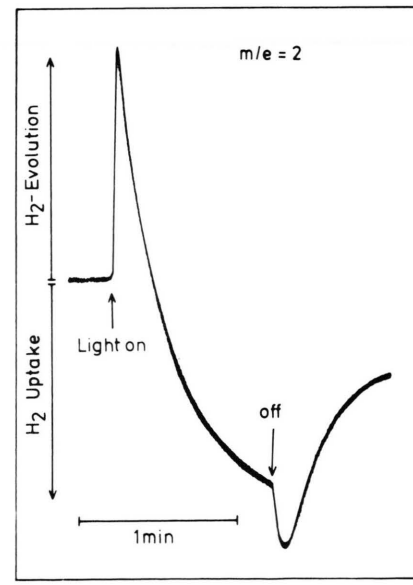


Fig. 1. Mass spectrometric recording of the hydrogen gas exchange in *Oscillatoria chalybea* upon illumination with 1 min red light after 30 min flushing with argon and 40 min dark adaptation. The signal was electronically detected *via* a H/D collector at *m/e* = 2. The evolution part of the composed signal corresponds to 11 pM molecular hydrogen. *Oscillatoria* preparations usually evolve hydrogen at a rate of 2–5 μM x mg Chl^{-1} x h^{-1}.

(10 to 12 weeks) did. In this case we observed higher reaction rates of about 50 to 100% stimulation in the case of young cultures in relation to older ones (results not shown). Similar observations have been made by Luo and Mitsui (1994) who interpreted the substantial decrease of the hydrogen signals by the exhaustion of cellular glycogen content in the cells with increasing age of the cultures. The light-induced H_2-evolution was observed with nitrate- and nitrogen-free grown cells. As we have shown in a previous paper that *Oscillatoria chalybea* is capable to fix atmospheric nitrogen (Bader and Röben, 1995) but only in the case when the cultures have been grown on a nitrogen free medium, it can be concluded that the observed hydrogen evolution is largely (or exclusively) linked to the presence of a (nitrogenase independent) hydrogenase system in this organism. Under appropriate conditions the observed hydrogen signal at $m/e = 2$ is composed of an initial H_2-evolution followed (or mixed) with a H_2-uptake (Fig. 1). A similar transition has been tentatively ascribed to a non-active Calvin cycle in the dark which is initially activated during the first phases of an illumination thus funnelling electrons to an increasingly higher extent from proton reduction to CO_2-fixation *via* the Calvin cycle (Lee *et al.*, 1996; Greenbaum *et al.*, 1995). In the course of our experiments we shall investigate which type of hydrogenase(s) has to be considered for *Oscillatoria chalybea*. From the literature it can be expected that an uptake- as well as the reversible hydrogenase should operate, but also cases, where no bidirectional hydrogenase appears to be present or operational in a cyanobacterium have been reported (Tamagnini *et al.*, 1997). Moreover, we observed a dark evolution of molecular hydrogen in *Oscillatoria chalybea* which will be analyzed in a forthcoming paper.

Under our conditions *Oscillatoria* shows a dependence of the hydrogen evolution on the chlorophyll concentration which is overproportional in the sense that doubling the amount of chlorophyll increases the hydrogen photoevolution by a factor of about 5 (Fig. 2). (In this region of the chlorophyll concentration dependence measurements of the oxygen evolution yield a roughly proportional curve.) This observation might be correlated to the interpretation that in the case of light-induced hydrogen evolution reaction centres do not act independently one from the other but that it requires the cooperation of two (or more) reaction centres/charges by principle. A similar -although in the case of oxygen controversary- interpretation has been suggested by the group of Mauzerall in context with the question whether photosynthetic reaction centers act completely independently or in a type of concerted reaction (Mauzerall and Dubinsky, 1993). Also, a coordinated action of photosynthesis and respiration might be suggested as both processes appear to cooperate forming a common substrate pool for hydrogen metabolism. In contrast to O_2-evolution in the frame of the coherent Kok-model H_2-evolution as the consequence of 5 µs-flashes does not show any oscillation of the signal amplitudes but a virtually unchanged steady state signal starting from the very first flash of a sequence after extensive dark adaptation. This has been shown for *Oscillatoria chalybea* (Bader, 1996) and also in the early work with the green alga *Chlorella vulgaris* (Greenbaum, 1977). Accordingly, dark adaption between flash sequences and a low flash

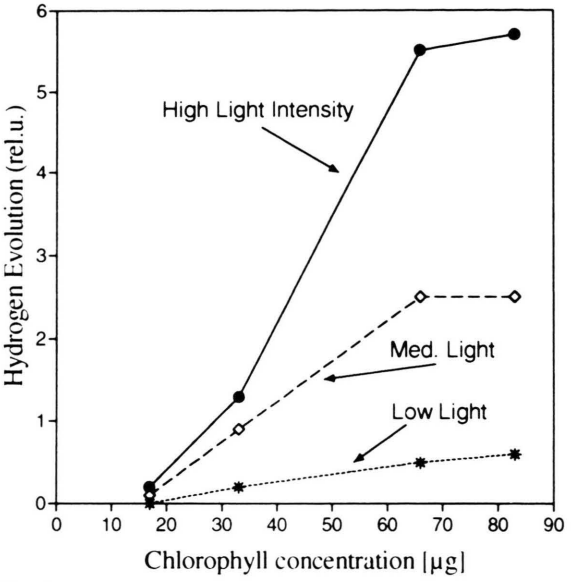
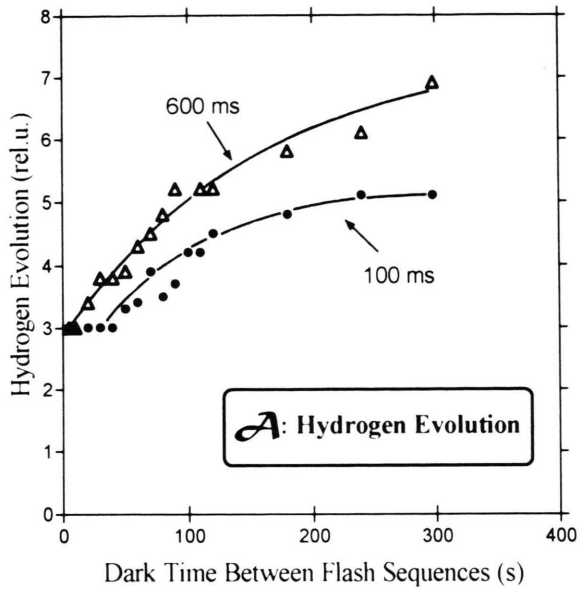
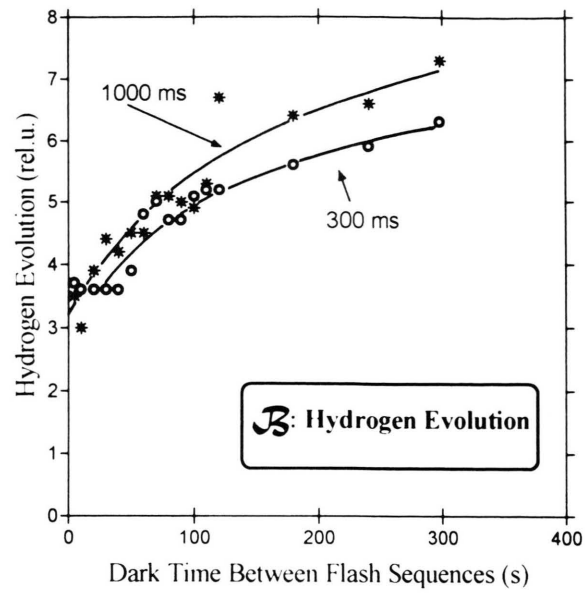


Fig. 2. Dependence on the light-induced hydrogen evolution in *Oscillatoria chalybea* on the chlorophyll concentration in the mass spectrometric measuring cell. The chlorophyll content (µg as indicated) was contained in the total volume of 3 ml reaction assay. The cumulated signals were induced by a sequence of 10 Xenon flashes which were 5 µs long and spaced 300 ms apart (strobos-lave 1539A from General Radio at high, medium and low light intensity).





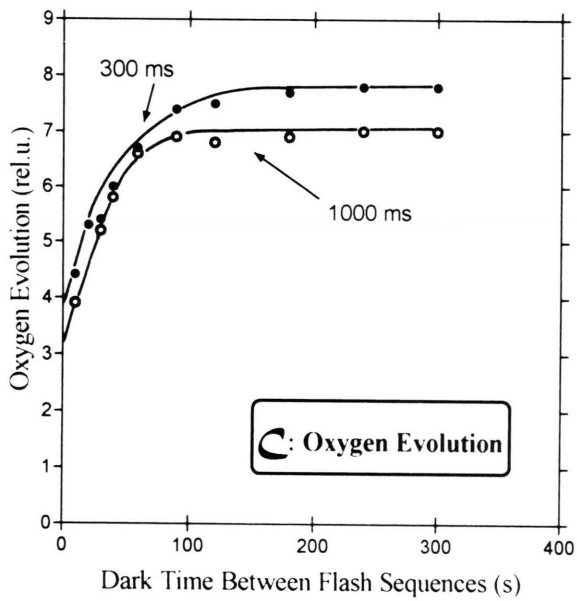


Fig. 3. Dependence of the light-induced hydrogen (a & b) and oxygen (c) on the flash frequency – 1.66 and 10 Hz (a), 3.33 and 1 Hz (b & c) and on the dark time between the flash series. Gas exchange was recorded as the cumulated signals at $m/e = 2$ and at $m/e = 32$, respectively, induced by 10 flashes (5 μs).

frequency as such are required to maximize the flash-induced H_2-evolution (Fig. 3a/b). Even at 1000 ms dark intervals between the flashes no deactivation of the excited states seems to exist. Under identical conditions photosynthetic oxygen evolution which requires the cooperation of 4 successively accumulated charges within one and the same reaction center is reduced already (Fig. 3c). When complete flash patterns of oxygen evolution are recorded, the requirement of a high flash frequency for the successive accumulation of oxidizing equivalents without any loss by intermediate deactivations is most clearly seen in the region of Y_3 and Y_4 with the steady state evolution level being much less inferred (Fig. 4). It should be noted, however, that the requirement of a prolonged dark adaptation in order to get high hydrogen amplitudes is only needed to really maximize the signals in the sense that short dark intervals (5 s) on the other hand are sufficient for

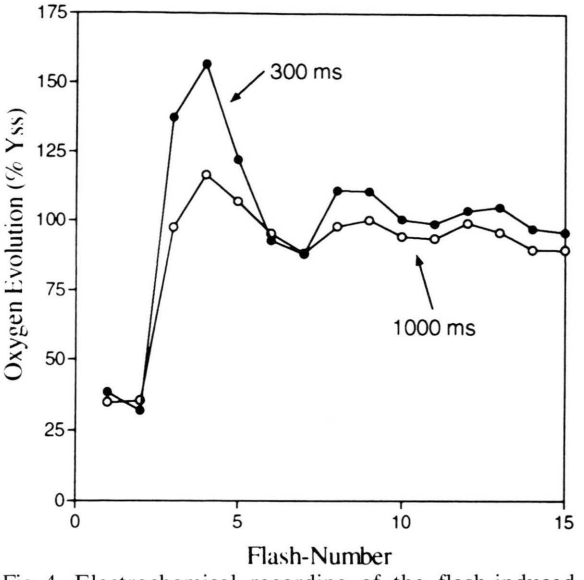


Fig. 4. Electrochemical recording of the flash-induced oxygen evolution pattern in *Oscillatoria chalybea* on a flash electrode. The Xenon-flashes were 5 μs long and triggered at a frequency of 3.33 and 1 Hz, respectively.

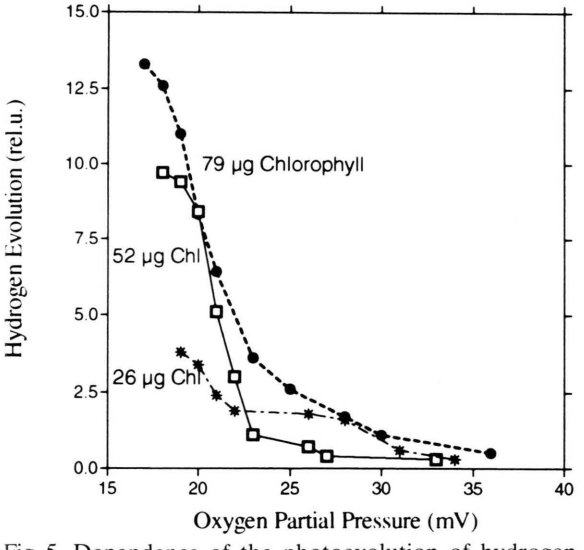


Fig. 5. Dependence of the photoevolution of hydrogen in *Oscillatoria chalybea* on the oxygen partial pressure of the mass spectrometric assay. Under these conditions an oxygen partial pressure of 20 mV corresponds to 5.6 μM O_2.

about 2/3 of the maximum hydrogen flash yield (Bader and Abdel-Basset, 1997). Fig. 5 shows the dependence of the light-induced hydrogen evolution on the oxygen partial pressure of the measuring assay. At an oxygen concentration of 6 μM the hydrogen evolution is inhibited by 50%. This is in accordance with the literature; it should be noted, however, that recent reports describe hydrogenase systems even under very high oxygen partial pressures (Yih *et al.*, 1996).

In contrast to many investigations dealing with hydrogen photoevolution we failed to observe any substrate dependent signal increase. Neither glucose nor fructose nor sucrose nor pyridine nucleotides induced a significant improvement of the proton reduction rate in *Oscillatoria*. In the case of extracts from *Anacystis nidulans* NAD(P) / NAD(P)H have been shown to be efficient electron donors/acceptors for the reversible hydrogenase (Schmitz and Bothe, 1996). Carbohydrates, organic acids and alcohols have been screened and in part described to be potent electron donors for hydrogen production in the case of *Synechococcus* sp. strain Miami BG 043511. In this case, however, the hydrogen evolution is supposed to be based on nitrogenase activity (Luo and Mitsui, 1994). However, also stimulatory effects of *e.g.* fructose on the hydrogen uptake have been described (Troshina *et al.*, 1996). In the case of *Oscillatoria chalybea,* with none of the investigated cultures an increase in the hydrogen evolution signal could be observed following the addition of one of the possible substrates. Thus, it looks as if *Oscillatoria* does not suffer from any substrate limitation as far as the electron transfer to protons is concerned. This might be related to an interrelationship between various electron transport chains in this organism. As *Oscillatoria chalybea* (like the other cyanobacteria) is a prokaryote and lacks cell organells all electron transport dependent redox reactions have to be carried out in the same thylakoid membrane system. Thus, the above mentioned relations between electron transport chains have to be assumed and have been shown and described in several aspects already. As an example, only the investigations on the phenomenon of chlororespiration shall be mentioned here (Bennoun, 1982; Gruszecki *et al.*, 1994; Maione and Gibbs, 1986). If, however, we assume that the substrates which supply the electrons necessary for proton reduction are funnelled into photosystem I *e.g.* from the respiratory electron transport chain, it should be possible to somehow deprive this electron source. As a consequence the light-induced hydrogen evo-

Table I. Effect of exogenous substrates (carbohydrates/pyridin nucleotides) and CO_2 on the mass spectrometrically measured hydrogen photoevolution in *Oscillatoria chalybea.*

Oscillatoria chalybea	Addition					
Normal cells	Fructose	Glucose	Sucrose	NADH	NADPH	CO_2
25 ± 7.7pM H_2	−6.5%	−11.7%	−20.8%	−19.5%	−3.5%	–
Depleted cells						
10.8 ± 2.9pM H_2	+41%	+46%	+62%	+4%	+46%	+67%

Oscillatoria protoplasts were depleted by preillumination for 2 h in weak red light (725.8 nm). Controls were incubated for the same time but without illumination prior to the measurements. Signals were obtained with a series of 10 flashes spaced 300 ms apart and detected at m/e = 2. All chemicals were added reaching a final concentration of 0.1 mM.

lution should be inhibited. Table I shows that in fact *Oscillatoria chalybea* progressively looses its capacity to reduce protons when the cells are exposed to a far red preillumination before the analyzing light is given. This means that only under such conditions the endogenous substrates can be exhausted thus leaving not enough reductants for hydrogen evolution. The depleted cells can then be re-supplemented by electron donors by adding exogenous substrates like the above mentioned glucose, fructose and sucrose. Under these conditions also alcohols are effective to some extent (results not shown). Under the conditions of red light depletion the inhibition of light-induced hydrogen evolution can be restored by approx 50%. The function of the respiratory electron transport chain as being a source of electrons for proton reduction can be further substantiated by analysis of the effect of typical respiration inhibitors like cyanide, SHAM and Antimycin A (Table II). The latter which is known to inhibit respiration in the region of the cytochrome b/c_1 (complex III) decreases the hydrogen evolution signals by about 70%. Most interestingly, the classical respiration inhibitor cyanide (effect on the cytochrome a/a_3 complex) impairs the reduction of protons to about the same extent as does salicylhydroxamic acid, an inhibitor which is known to affect the alternate respiratory system and the effect of the two inhibitors is additive. This suggests that not only the cytochrome pathway but also the alternate respiration feed in electrons into the proton reduction system. Our observations and interpretations fit earlier observations that also the alternate respiration is operational in *Oscillatoria chalybea* (Bader and Schmid, 1989).

Acknowledgement

The financial support from the 'Alexander von Humboldt-Stiftung' (R. A.-B.) and from the 'Deutsche Forschungsgemeinschaft' (K. P. B.) is gratefully acknowledged.

Table II. Effect of various respiration inhibitors on the light-induced hydrogen evolution in *Oscillatoria chalybea* measured by mass spectrometry.

Control	Antimycin A	Cyanide	SHAM	Cyanide + SHAM
43.8 ± 24.7 pM H_2	11.8 ± 3.4	13.9 ± 7.9	12.0 ± 4.5	4.2 ± 1.9
(100%)	(26.9%)	(31.7%)	(27.4%)	(9.5%)

Signals were obtained with a series of 10 flashes spaced 300 ms apart and detected at m/e = 2. Inhibitors were applied at a final concentration of 1 mM except Antimycin A which was added to give a final concentration of 0.2 mM.

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