Respiratory dependence of the CCCP-resistant luminescence of *Vibrio harveyi*

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The relation between respiration and luminescence of *Vibrio harveyi* was studied in the presence of the proton conductor CCCP (carbonyl cyanide *m*-chlorophenylhydrazine). CCCP affected both luminescence and oxygen uptake rate, but did not alter the cellular ATP level. In the absence of CCCP, luminescence and oxygen uptake rate were almost constant over the pH range 6.5–8.5. However, at 10 μ M-CCCP, both were apparently pH-dependent, with maxima observed at pH 8.5 and minima at pH 6.5. In the presence of CCCP (0.1–10 μ M), the changes in the luminescence were in close correlation with those in the oxygen uptake rate regardless of the pH. HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NaCN inhibited the CCCP-resistant luminescence. From these results, it is concluded that the CCCP-resistant luminescence is respiratory-dependent.

Introduction

In marine luminous bacteria, the relation between luminescence and the respiratory system has been extensively documented. Since the luciferase system can be viewed as an alternative electron transport pathway, competition between the two systems has been recognized, (Grogan, 1984; Hastings & Nealson, 1977; Nealson *et al.*, 1970; Ulitzur *et al.*, 1981; Watanabe *et al.*, 1975).

Carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), a proton-conducting uncoupler, has been used to study the luminescent pathway (Grogan, 1984; Guerrero & Makemson, 1989). CCCP is generally thought to increase proton permeability across the cell membrane, with consequent dissipation of the membrane potential and inhibition of ATP synthesis. Thus growth and the various cellular processes which depend on the proton-motive force are strongly inhibited in the presence of CCCP.

However, in our previous study, we found that *Vibrio harveyi* showed CCCP-resistant growth and luminescence at pH 8.5, but not at pH 6.5 (Wada *et al.*, 1992). A similar growth pattern has been found in other marine bacteria which possess a distinct respiratory chain involving a primary Na⁺ pump (Tokuda & Unemoto, 1983; Kogure & Tokuda, 1986). This pump translocates sodium ions from the inside to the outside of the cells at alkaline pH, as a result of electron transport. This suggested a close coupling between the luminescent system and the Na⁺-driven respiratory chain in *V. harveyi* at alkaline pH.

In this report we describe a close correlation between luminescence and respiratory activity in *V. harveyi* in the presence of CCCP.

Methods

Bacterial strain and preparation of cell suspension. *Vibrio harveyi* ATCC 14126 was incubated in PYG medium (pH 7.5) at 20 °C as described previously (Wada *et al.*, 1992). When OD₆₀₀ reached about 1.0, cells were harvested by centrifugation (10000 r.p.m., 1 min, 4 °C). After washing twice with 25 mM-Tris buffer containing 30 mM-MgSO₄·7H₂O, 10 mM-KCl and 0.3 M-NaCl, cells were resuspended in the same buffer. The pH (6.5–8.5) was adjusted with HCl. The cell suspension contained about 0.4 mg protein per ml, unless otherwise noted. When the effect of *n*-decanol on luminescence was studied, the suspension was diluted 10 times with the buffer. The suspension was kept on ice until use.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

Measurement of luminescence. Luminescence of the cell suspension was monitored with an ATP photometer (SAI Technology, Model 3000). Cell suspension (1 ml) was put in a glass vial and kept at 25 °C. After 5 min, luminescence was measured for 6 s and the peak value was monitored. Light intensity was expressed in count units of the photometer per mg cell protein.

Measurement of oxygen uptake rate. The oxygen consumption rate of the cell suspension was determined at 25 °C with an oxygen electrode (Rank). Measurement was started by the addition of 20 µl cell suspension to 2 ml of air-saturated buffer. The final cell concentration was the same as that for luminescent determination.

Measurement of ATP content. ATP was extracted from cells by the method of Goulbourne & Greenberg (1980). The cell suspension (0.2 ml) was mixed with 2 ml boiling Tris buffer (20 mM, pH 7.5 at 25 °C). After 10 min, samples were stored at -20 °C for later analysis. ATP content was measured by firefly luciferase-luciferin (Sigma).

Chemicals. CCCP and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were purchased from Sigma. *n*-Decanal was obtained from Nakarai Tesque (Kyoto, Japan). CCCP and *n*-decanal were dissolved in dimethyl sulphoxide (DMSO). HQNO was dissolved in methanol. In cell suspensions containing these chemicals, DMSO or methanol concentrations did not exceed 1% (v/v). This concentration of solvent did not affect luminescence or the oxygen uptake rate of *V. harveyi*. NaCN or KCN was added to the suspensions as aqueous solution.

Results

Effects of the proton-conductor CCCP on the luminescence of *V. harveyi* were determined at pH 6.5 and 8.5. Luminescence at pH 6.5 was immediately and almost completely inhibited by the addition of 1 µM-CCCP (Fig. 1 *a*), but at pH 8.5, considerable luminescence was observed even in the presence of 100 µM-CCCP (Fig. 1 *b*).

Luminescence and respiratory activity were determined at pH 6.5 and 8.5 as a function of CCCP concentration (Fig. 2). Oxygen uptake rate at pH 6.5 was slightly enhanced at 0.1 µM-CCCP, but markedly reduced at more than 1 µM. At pH 8.5, however, oxygen uptake was stimulated throughout the concentration range (0.1–10 µM) tested. Although changes in the luminescence and the oxygen uptake rate with respect to the CCCP concentration were more significant at pH 6.5 than at 8.5, the changes in luminescence and oxygen uptake closely paralleled each other. This correlation implied a respiratory-dependence of the luminescence in the presence of CCCP.

The effects of pH on the CCCP-resistant luminescence and the oxygen uptake rate are shown in Fig. 3. In the absence of CCCP, the luminescence is almost constant over the pH range tested (6.5–8.5). However, in the presence of 10 µM-CCCP, the luminescence became pH-dependent, with the maximum luminescence at pH 8.5 and the minimum at pH 6.5. The response of the oxygen

Fig. 1. Effects of CCCP on the luminescence of *V. harveyi* at (a) pH 6.5 and (b) pH 8.5. After determination of luminescence at zero time, CCCP was added to the suspension. (a) ○, 0; ●, 0.1; ■, 1.0; □, 10 µM-CCCP (final concentrations). (b) ○, 0; ●, 1.0; ■, 100 µM-CCCP (final concentrations).

uptake rate to the external pH in the presence and absence of CCCP gave a profile similar to the luminescence data (Fig. 3).

To ensure that the CCCP-resistant luminescence was respiratory-dependent, cells were treated with respiratory inhibitors. Luminescence in the presence of 10 µM-CCCP at pH 8.5 was inhibited by 2 mM-NaCN and 100 µM-HQNO (Fig. 4). Under these conditions, the oxygen uptake rates with CCCP, with CCCP + NaCN and with CCCP + HQNO were 96.0 ± 7.6 , 18.4 ± 1.0 and 23.7 ± 2.2 nmol oxygen min⁻¹ (mg protein)⁻¹, respec-

Fig. 2. Effects of CCCP on the luminescence and oxygen uptake rate of *V. harveyi* at (a) pH 6.5 and (b) pH 8.5. Luminescence (●) and oxygen uptake rate (○) were determined 5 min after CCCP addition. Bars represent errors of the duplicate assay.

tively. These results indicate that the CCCP-resistant luminescence at pH 8.5 was respiratory-dependent. It seems most likely that inhibitor blocking of electron flow through the respiratory chain and a resultant inhibition of functioning of the Na⁺ pump lead to a decrease in the electron flow rate through the luminescent system in the presence of CCCP (see Discussion).

Since aldehyde synthesis from fatty acids requires both NADPH and ATP, this reaction can be a limiting step in bacterial luminescent systems. Addition of an aldehyde (tetradecanal) causes a stimulation of *in vivo* luminescence, both in the absence and the presence of respiratory inhibitors (Grogan, 1984; Guerrero & Makemson, 1989; Makemson, 1986), and had a similar effect on luminescence in the presence of CCCP in this study. Inhibition of luminescence caused by 10 μM -CCCP at pH 6.5 and that of CCCP-resistant luminescence by 2 mM-KCN at pH 8.5 could be partly restored by 50 μM -decanal (Fig. 5). However, neither CCCP nor KCN affected intracellular ATP levels (Table 1). Thus, a decrease in the cellular ATP pool does not seem to be a

Fig. 3. Effects of pH on the luminescence (a) and oxygen consumption (b) of *V. harveyi* in the presence of CCCP. CCCP was added to a final concentration of 10 μM (○). DMSO was used as a control (●). Luminescence was determined 5 min after CCCP or DMSO addition. Bars represent errors of the duplicate assay.

Fig. 4. Inhibition of the CCCP-resistant luminescence of *V. harveyi* by respiratory inhibitors at pH 8.5. CCCP was added to give a final concentration of 10 μM . Just after the determination of the luminescence at zero time, NaCN and HQNO were added to the suspension to give final concentrations of 2 mM and 100 μM , respectively. ●, CCCP; ○, CCCP + HQNO; ■, CCCP + NaCN. Data represent mean values of duplicate assay (deviations are too small to show in this figure).

cause of the inhibition of luminescence, although deficiency in a long-chain aldehyde may be partly involved.

Fig. 5. Effect of decanal addition on the luminescence of *V. harveyi* in the presence of 10 μ M-CCCP at (a) pH 6.5 and (b) pH 8.5. The cell suspension contained about 0.04 mg protein ml⁻¹. Decanal was added to a final concentration of 100 μ M. KCN was added to a final concentration of 2 mM. Each arrow represents the addition of the reagent. DMSO was added to 1% (v/v) as a control for decanal addition.

Table 1. ATP content of *V. harveyi* in the presence of CCCP

Cellular ATP levels were determined 5 min after CCCP addition at pH 6.5 and 8 min after CCCP addition at pH 8.5. Data are means from a duplicate assay.

pH	ATP content [μ g ATP (mg cell protein) ⁻¹]
6.5 DMSO	1.10 $\pm$ 0.10
10 μ M-CCCP	1.22 $\pm$ 0.12
8.5 10 μ M-CCCP	0.40 $\pm$ 0.08
10 μ M-CCCP + 2 mM-KCN	0.58 $\pm$ 0.03

Discussion

Both the luminescence and the oxygen uptake rate of *V. harveyi* were sensitive to the proton-conductor CCCP. These processes were more resistant to CCCP at alkaline

than at acidic pHs (Fig. 2 and 3). Although Guerrero & Makemson (1989) reported only the inhibitory effects of CCCP on bacterial luminescence and respiration, our results show more detailed responses of these processes to CCCP in *V. harveyi*. Stimulation of both luminescence and oxygen uptake by CCCP was observed at pH 6.5 and 8.5, although the range of CCCP concentration was different (Fig. 2); less than 0.1 μ M at pH 6.5, and more than 0.1 μ M at pH 8.5.

As CCCP increases proton permeability across the cell membrane, the following changes will generally occur: changes in the cytoplasmic cation environment, dissipation of membrane potential and a consequent increase in respiration. If the perturbation of the cytoplasmic cation environment by CCCP is extensive, respiration and any other cellular metabolism will cease. In fact, Tokuda & Unemoto (1983) showed that, at pH 6.5, CCCP caused leakage of K⁺ and inhibition of growth in *Vibrio alginolyticus*. However, at pH 8.5, the cytoplasmic cation environment was almost conserved and substantial growth was observed even in the presence of CCCP. These results can be explained by a switching of respiratory chain system from H⁺-dependence (H⁺ pump) to Na⁺-dependence (Na⁺ pump) (Tokuda & Unemoto, 1983).

Our previous study (Wada *et al.*, 1992) found that *V. harveyi* possessed a Na⁺-dependent NADH oxidase, and was able to grow in the presence of CCCP at pH 8.5, but not at pH 6.5. From these results, *V. harveyi* was considered to possess a respiratory-dependent primary Na⁺ pump similar to that of *V. alginolyticus*. Although cytoplasmic cation environments were not checked, the responses to CCCP in *V. harveyi* presented in this report would be explained by analogy as follows: at acidic pH, maintenance of the cytoplasmic cation environment depends on H⁺ circulation driven by H⁺ pumps, and addition of CCCP would result in severe perturbation of cytoplasmic pH and the cation environment. On the other hand, under alkaline conditions, when the Na⁺ pump is functional, *V. harveyi* is able to maintain a normal cytoplasmic cation environment even in the presence of CCCP. Thus, normal cellular works including light production proceed at pH 8.5. In other words, high sensitivity at acidic pH and insensitivity at alkaline pH to CCCP would be attributed to the H⁺ and Na⁺ pumps, respectively.

The inhibition of bacterial luminescence by CCCP has been considered a consequence of a decrease in intracellular ATP, which is required for aldehyde recycling (Guerrero & Makemson, 1989). Restoration of the CCCP-induced inhibition by *n*-decanal shown in Fig. 5 seems to be consistent with this idea. However we observed that CCCP had no apparent effect on intracellular ATP level (Table 1). Thus, although lack of

aldehyde itself may be partly involved in the inhibition, it seems unlikely that the energy from ATP hydrolysis is directly coupled to the luminescence.

It is more reasonable to explain the results in the context of the respiratory electron transport activity or the rate of electron flow. The close correlation between luminescence and oxygen uptake rate (Fig. 2) strongly supports this idea. The oxygen uptake rate corresponds closely to the respiratory electron transport activity, indicating that the luminescence is linked to or depends on the respiratory electron transfer. This linkage was further supported by the inhibition of CCCP-resistant luminescence by NaCN and HQNO (Fig. 4). As stated above, in the presence of CCCP at pH 8.5, maintenance of the cellular cation environment is presumed to be fastidious and strictly dependent on the Na⁺ pump. Inhibition of the Na⁺ pump by blocking electron flow through the respiratory chain system could result in a perturbation of the cellular ionic environment. This, in turn, would lead to a decrease in respiratory electron transport activity and hence the luminescence, although NaCN and HQNO both enhance luminescence in the absence of CCCP (data not shown).

We previously concluded (Wada *et al.*, 1992) that luminescence was respiratory-dependent at pH 8.5. In this study, we further observed that luminescence in the presence of CCCP was closely associated with respiratory activity over the pH range 6.5–8.5.

We conclude that the luminescence of *V. harveyi* is respiratory-dependent in the presence of CCCP. This conclusion is consistent with our hypothesis that luminescence in *V. harveyi* primarily depends on the respiratory activity.

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