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NITRITE REDUCTION WITH FORMATE IN *PSEUDOMONAS DENITRIFICANS* ATCC 13867

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SUMMARY

Formate served as an electron donor in dissimilatory nitrite reduction in resting cells of a denitrifier, *Pseudomonas denitrificans*. Pyruvate also donated electrons to nitrite. Pyruvate reduced nitrite at the same rate as formate after some lag period. Citrate, the carbon source for cultivation medium employed in this study, was less effective for nitrite reduction. Two distinct cytochromes were shown to be involved in the electron transfer from formate to nitrite.

Denitrification is a type of respiration using nitrogenous oxides as electron acceptors in place of oxygen and has attracted attention not only as a tool of studying the regulation of energy metabolism but as a microbiological system applicable to waste water treatment. Dissimilatory nitrate reductase and nitrite reductase catalyze earlier steps of denitrification from nitrate. In the case of nitrate reductase, formate has been considered generally as the major electron donor in microorganisms such as *Escherichia coli* [1, 2] and *Pseudomonas denitrificans* [3]. However, for nitrite reductase, the role of formate remains unknown though there have been several reports on the electron carriers participating in the reduction of nitrite. In this communication, we describe that formate serves as an effective electron donor for nitrite reduction in resting cells of *P. denitrificans*. Participation of cytochromes in the formate-dependent reduction of nitrite is also reported.

MATERIALS AND METHODS

Pseudomonas denitrificans ATCC 13867 was grown anaerobically at 30° C in a synthetic medium containing (per 1): KNO_3 5 g, NH_4Cl 1 g, Na_3-citrate 6 g,

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KH_2PO_4 0.5 g, K_2HPO_4 1 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 10 mg, $CaCl_2$ 20 mg, $CuSO_4 \cdot 5H_2O$ 1 mg, $ZnSO_4 \cdot 7H_2O$ 1 mg, $MnCl_2 \cdot 6H_2O$ 0.1 mg, $CoCl_2$ 2 mg, $Na_2MoO_4 \cdot 2H_2O$ 2.5 mg and $Na_2SeO_4 \cdot nH_2O$ 0.2 mg. Cell growth was monitored turbidimetrically at 580 nm. Cells harvested at the late logarithmic growth phase were washed three times with 33 mM sodium potassium phosphate buffer (pH 7.0), and suspended in the same buffer.

Nitrite reductase activity was assayed by measuring decrease of nitrite at 30° C. The reaction mixture contained 1.5 mmol of sodium potassium phosphate buffer (pH 7.0), 30 μmol of sodium nitrite, 150 μmol of sodium formate, sodium pyruvate or sodium citrate, and appropriate amounts of cells (wet weight 150 mg) in a total volume of 15 ml. The reaction was initiated by the addition of nitrite after incubation for 5 min. Aliquots of the mixture were withdrown with appropriate intervals and the concentration of nitrite remained was assayed spectrophotometrically by a diazo-coupling method according to Nicholas and Nason [4]. Ammonia formed was determined by the indophenol method [5].

The difference spectra of cells were obtained in Thumberg-type cuvettes (1 cm path length) under helium with a double-beam spectrophotometer equipped with double end-on type photomultipliers, Shimadzu MPS-5000 (Shimadzu Seisakusho Co., Kyoto, Japan). Cells incubated with formate and nitrite were referred to as "formate-reduced cells" and "nitrite-oxidized cells", respectively.

RESULTS

Time-course changes in nitrite reduction with various electron donors in resting cells of *P. denitrificans* are shown in Figure 1. Reduction of nitrite occurred linearly when formate was used as an electron donor (specific activity, 6.8 nmol/min per mg-wet cells). Pyruvate served as an effective electron donor (6.0 nmol/min per mg-wet cells) comparable to formate except that some lag period was required for the initiation of nitrite reduction. Citrate, the carbon source in growth medium, reduced nitrite at a lower rate by three fold (1.9 nmol/min per mg-wet cells) after a lag period under the same conditions. No detectable nitrite reduction occurred when lactate was used (data not shown). Low amounts of ammonia (a twentieth or lower of the amounts of nitrite disappeared) were produced. Most of nitrite disappeared would be reduced to compounds other than ammonia.

In order to obtain some information on the electron carriers participating in the nitrite reduction with formate, difference spectra of cells were taken under several conditions. Figure 2-A shows formate-reduced minus nitrite-oxidized difference spectrum. A peak of a cytochrome (α-band) was detected at 553 nm, with a shoulder at 559 nm. Spectrum obtained with formate-reduced cells against cells with no addition had no detectable peak (Fig. 2-B),

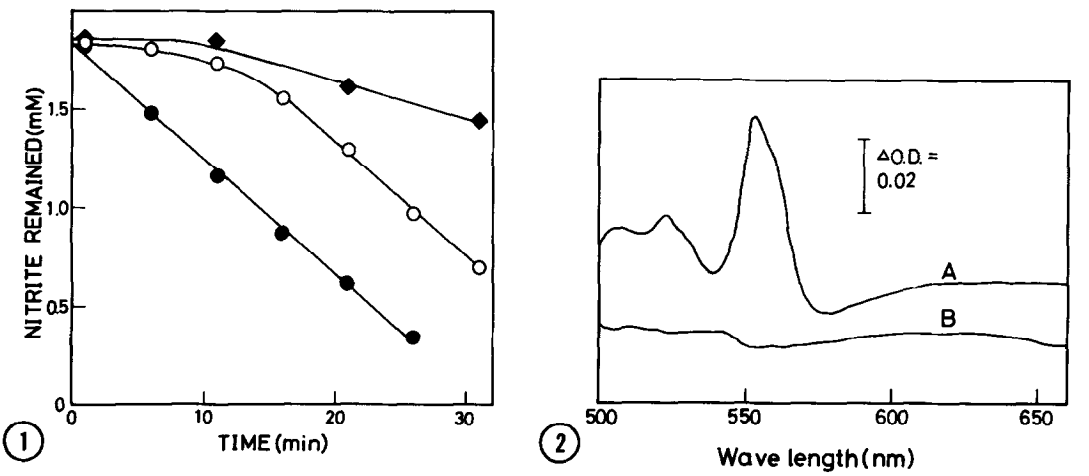

Fig. 1 Reduction of nitrite by resting cells of *P. denitrificans* with sodium formate (●), sodium pyruvate (○) and sodium citrate (◆). Each electron donor was added at a final concentration of 10 mM.

Fig. 2 A:Formate-reduced minus nitrite-oxidized difference spectrum of resting cells grown anaerobically with nitrate. Spectrum B was obtained with formate-reduced cells against cells with no addition. The reaction mixture in Thumberg-type cuvettes contained 150 μmol of sodium potassium phosphate buffer (pH 7.0), cells (wet weight 45 mg) and 30 μmol of sodium formate or 30 μmol of sodium nitrite in 3 ml. Base lines were obtained with the cell suspension under helium. The reductant, formate, and the oxidant, nitrite, were then added to each cuvette from side arms and the spectra were taken after incubation for 5 min.

indicating that these two cytochromes were in reduced state in the absence of nitrite even without exogenously added formate. Cells grown aerobically without nitrate exhibited neither of these peaks (data not shown).

Further, we took difference spectra of steady state cells reducing nitrite with formate (Fig. 3). Spectrum A—difference spectrum:(nitrite-oxidized + formate) minus nitrite-oxidized—had a peak at 553 nm. The shoulder at 559 nm shown in Figure 2-A was not observed. Spectrum B in Figure 3—formate-reduced minus (formate-reduced + nitrite)—had a broad peak from 553 nm to 559 nm. Comparing with Figure 2-A, this peak would consist of two α-bands of cytochromes mentioned above with nearly equal heights. These results show that cytochrome 559 was nearly completely oxidized and some portion of cytochrome 553 was in reduced state in steady state cells reducing nitrite with formate.

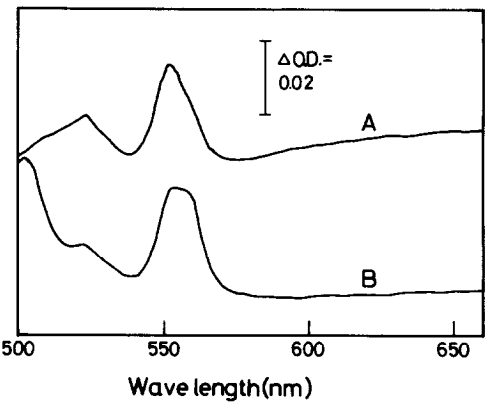

Fig. 3 Difference spectra of steady state cells reducing nitrite with formate. Spectrum A:(nitrite-oxidized + formate) minus nitrite-oxidized. Spectrum B:Formate-reduced minus (formate-reduced + nitrite). The reaction mixture in cuvettes was same that in Figure 2. For spectrum A, a pair of cuvettes containing cells and nitrite were incubated anaerobically for 3 min at 30° C. Base line was obtained with the cuvettes. Formate was then added to one cuvette from the side arm and incubated for another 3 min for spectrum measurement. Formate and nitrite were added by reverse order for sepctrum B.

DISCUSSION

The results obtained here clearly demonstrated that formate acts as an effective electron donor for dissimilatory nitrite reduction which leads to produce gaseous substance(s) in *P. denitrificans* ATCC 13867. It would be the first case to show the participation of formate in nitrite reduction in a denitrifying organism. In many microorganisms, various carbon compounds have been employed to support nitrite reduction, but their fate in cells has been remained unknown. In *P. denitrificans*, formate exhibited higher ability of nitrite reduction than citrate, the carbon source used in this study. This strongly suggests that formate has an important role in physiology of dissimilatory nitrite reduction.

Two cytochromes were found to participate in electron flow from formate to nitrite. Recently, Walter *et al.* reported that cytochrome c-553 and cytochrome b-559 had a role as electron transport mediators in nitrite reduction with glutamate in the same strain that was employed in the present study [6]. The cytochromes participating in nitrite reduction with formate would be iden-

tical with those reported by them. The proportion of cytochrome c-553 to cytochrome b-559 in formate-reduced minus nitrite-oxidized difference spectrum (Fig. 2) was similar to that in glutamate-reduced minus nitrite-oxidized difference spectrum.

The difference spectrum of steady state cells reducing nitrite with formate was, however, different from that with glutamate. We obtained similar results to those reported by Walter *et al.* [6], in which the spectrum of the steady state cells with glutamate—glutamate-reduced minus (glutamate-reduced + nitrite)—was identical with glutamate-reduced minus nitrite-oxidized difference spectrum (data not shown). This indicates that the steady state cells with glutamate had both of the cytochromes in nearly completely oxidized state. On the other hand, nitrite-reducing cells with formate contained cytochrome b-559 in nearly completely oxidized state and c-553 some portion of which remained in reduced state (Fig. 3).

A peak at 503 nm was also observed as illustrated in Figures 2 and 3. The appearance of this peak requiring some lag period was accompanied by the reduction of nitrite with formate. The same peak was also detected in the difference spectra of glutamate-nitrite system, but was not mentioned by Walter *et al.* [6]. The precise nature of this peak is now under study.

Electron carriers participating in dissimilatory nitrite reduction have been studied intensively in a certain bacterium which was tentatively called *P. denitrificans* (Iwasaki strain) [7]. However, it was shown that the bacterium belongs to *Alcaligenes* species [7,8].

Recently, Abou-Jaudé *et al.* reported that formate acts as an electron donor for nitrite reduction in *E. coli* [9]. The reduction product from nitrite is ammonia in *E. coli* and is different from that in denitrifiers. It would be doubtful whether formate plays an important role in nitrite reduction in *E. coli* because of its low efficiency compared with glucose, the carbon source employed in the culture.

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