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THE INTERACTION OF MUCIDIN WITH ANAEROBICALLY GROWN CELLS OF *PARACOCCUS DENITRIFICANS*

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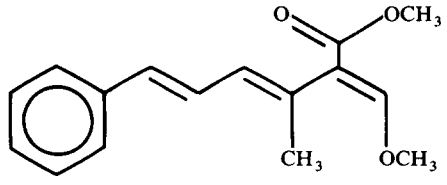
Mucidin similar to antimycin inhibits the electron flow to cytochrome *c* and the enzyme activities dependent on cytochrome *c* reduction in the cells of *Paracoccus denitrificans*, but it does not inhibit the electron flow to nitrate reductase and cytochrome *o*. Unlike antimycin mucidin does not permit a residual electron flow through the cytochrome bc_1 region. In the presence of antimycin the electron flow to nitrate is lower than in using mucidin in contrast with a higher extent of cytochrome *b* reduction. This result is in contradiction to the participation of the constitutive cytochrome *b* as an electron donor in the nitrate reduction.

Introduction

While the structure of the respiratory chain of mitochondria has essentially been clarified, there remains a variety of problems concerning the organization of the respiratory chain in bacteria. One of the frequently studied bacteria because of its similarity to the mitochondrial respiratory chain is the facultative anaerobic bacterium *Paracoccus denitrificans* (see Refs. 1–3 for a review). When grown under anaerobic conditions in the presence of nitrate as the terminal acceptor, the respiratory chain of *P. denitrificans* has a branched structure. experimental results in a number of papers have shown that the pathways to nitrate reductase and terminal oxidase (probably cytochrome *o*) branch off before the site of antimycin action [4–11], whereas pathways to nitrite reductase, N_2O reductase and cytochrome aa_3 diverge at the level of cytochrome *c* [4,12]. Recently the possible function of ubiquinol as a common electron donor for nitrate reductase and cytochrome *o* has been discussed [3,9,13]. In studying this region of the respiratory chain it proves advantageous to employ specific inhibitors of the mitochondrial complex bc_1 [4–11,14].



Scheme I. Structural formula of mucidin (strobilurin A).

Mucidin (Scheme I) is an antibiotic produced by the basidiomycete *Oudemansiella mucida* [15–18] coincident with strobilurin A isolated from *Strobilurus tenacellus* [18], whose synthesis was studied by means of isotopically labelled precursors [19]. Mucidin inhibits effectively mitochondrial respiration, the site of its inhibition being located between cytochrome *b* and c_1 [20–24]. In this paper the effect of mucidin on the rate of reduction of natural terminal acceptors (O_2, NO_3^-, NO_2^-) and/or ferricyanide and on the redox behaviour of cytochromes with anaerobically grown cells of *P. denitrificans* is described. A comparison

with a similar, but not identical effect of antimycin makes it possible to draw conclusions about the functional arrangement of redox components at the site of branching of the respiratory chain of this bacterium.

Material and Methods

Microorganism. P. denitrificans (NCIB 8944) was grown at 30°C in an anaerobic medium according to Tait [25] containing 50 mM succinate as the source of carbon and 10 mM nitrate as the terminal acceptor. The cells were harvested by centrifuging at an early stationary stage of growth, washed with 0.1 M sodium phosphate of pH 7.3; their dense suspension was stored in ice.

Measurement of enzyme activities. The rate of oxygen consumption was measured by means of the Clark electrode. The oxygraphic vessel was tempered to 25°C, it contained 3.5 ml of medium of the composition 0.1 M sodium phosphate (pH 7.3) and 10 mM sodium succinate. In following the reduction of nitrate and/or nitrite the same amount of medium contained additional components: 5 mM $NaNO_3$ or 1 mM $NaNO_2$. Aerobic conditions for measurements were obtained by shaking the samples in conical flasks; when working under anaerobic conditions closed test tubes were used, the samples being bubbled through with nitrogen. Nitrite concentration was determined colorimetrically [26] after the deproteinization of the samples with 1 ml saturated uranyl acetate solution. The consumption of ferricyanide was monitored by measuring the absorbance of the solution at 420 nm ($\epsilon_{420} = 1\ mM^{-1} \cdot cm^{-1}$ [27]).

Spectra. Difference spectra of cytochromes were examined in 3 ml closed cuvettes by means of spectrophotometer Cary 118 C equipped with a scattered transmission accessory. The content of the reference cuvette was oxidized with solid potassium ferricyanide.

Chemicals. The mucidin used was prepared in the laboratory of Dr. Musílek (Institute of Microbiology, Czechoslovak Academy of Sciences, Praha, Czechoslovakia) and was obtained as a gift from Dr. Šubík (Food Research Institute, Bratislava, Czechoslovakia) and from the above institute; antimycin was from Sigma (München, F.R.G.). The concentrations of the stock solutions of inhibitors in ethanol were verified by spectrophotometry in the ultraviolet region [23]. The other chemicals used were products of Lachema (Brno, C.S.S.R.) and all were of analytical grade of purity.

Results

It was found that mucidin decreases oxygen reduction in cells of *P. denitrificans*. The dependence of the rate of respiration on mucidin concentration is given in Fig. 1. From this it is evident that at the titre of about 1 nmol mucidin per 1 mg dry weight of cells the rate of respiration was reduced to 40% of the original value and remained constant at further increase in mucidin concentration. This result is in keeping with earlier information on the branched structure of the terminal respiratory chain of *P. denitrificans* with the presence of at least two different terminal oxidases (cytochrome *o* and/or aa_3) one of which (cytochrome *o*) is insensitive to antimycin [3]. As documented in Fig. 2 with the same mucidin titre nitrite reductase activity of cells is blocked completely and the formation of nitrite from nitrate under anaerobic conditions reaches its maximal stimulation. Since similar results to those given in Figs. 1 and 2 were obtained with the use of antimycin [6,7], it is probably that the site of

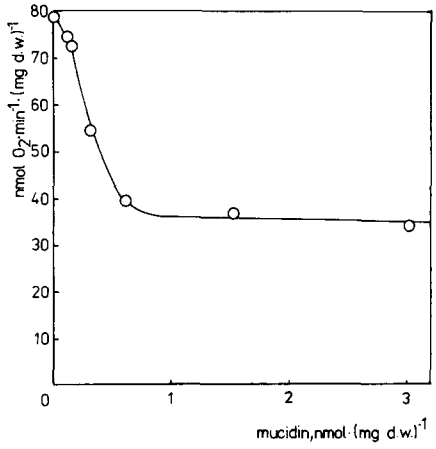


Fig. 1. The inhibitory action of mucidin on the oxygen consumption in the cells of *P. denitrificans*. Conditions of measuring see Material and Methods; 2 mg dry weight of cells were used.

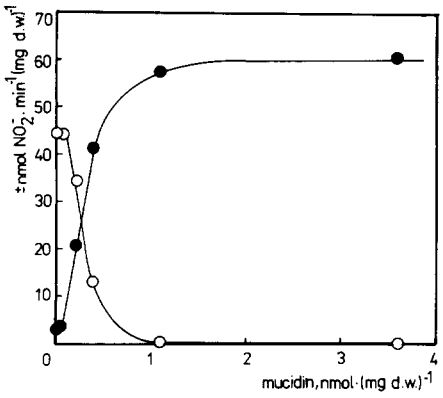


Fig. 2. The effect of mucidin on the reduction of nitrite or nitrate by the cells of *P. denitrificans*. (○) Specific activity of nitrite consumption; the reaction mixture was anaerobic and contained 1 mM sodium nitrite. (●) Specific activity of nitrite production; the aerobic reaction mixture contained 5 mM sodium nitrate. 1.7 mg dry weight of cells was used in the experiments.

mucidin inhibitory action in respiratory chain of *P. denitrificans* is also located in the region of cytochrome *b* and c_1. The finding that mucidin concentrations employed inhibit neither the terminal oxidase – cytochrome *o* – nor the nitrate reductase is in agreement with the fact that the branching of the respiratory chain of *P. denitrificans* takes place before the site of mucidin inhibition.

The objective of further work was a more detailed comparison of the inhibitory action of mucidin and antimycin and the application of possible differences in their action to a better recognition of the branching site. In one of the experiments the rates of nitrite accumulation in nitrate reduction under anaerobic conditions in the presence of the saturating concentrations of inhibitors were compared (see Table I). It should be pointed out that nitrite does not normally accumulate during anaerobic reduction of nitrate when the inhibitor is not present (see line 1 in the table). It became evident that in using mucidin the established rate of NO_2^- accumulation was significantly higher than in using antimycin (in the experiment described in the table by 45%, in other experiments not quoted here by 18 and 33%). Further it can be seen from Table I that the residual activity of nitrite reductase in the inhibition of the respiratory chain with antimycin is higher in comparison with mucidin, when the electron flow to nitrite is near zero (cf. Fig. 2). The results of the experiment in Table I suggest that in the cells in the presence of saturating concentration of antimycin the residual flow of electrons passes through the terminal branch of the respiratory chain containing cytochromes *c*. This conclusion is confirmed by measuring the rate of reduction of ferricyanide, an artificial acceptor which also in *P. denitrificans* abstracts electrons mainly from the cytochrome *c* region [6,7,13,28]. From the time-courses of ferricyanide consumption in Fig. 3 it follows that in the presence of mucidin the rate of reduction of the artificial acceptor was lower by 8% than in the presence of antimycin. The effect of the two inhib-

TABLE I

THE REDUCTION OF NITRATE AND NITRITE IN THE PRESENCE OF RESPIRATORY INHIBITORS

The cells of *P. denitrificans* were kept anaerobic in a reaction mixture containing 5 mM $NaNO_3$ (A) or 1 mM $NaNO_2$ (B) as described in the Material and Methods section.

Inhibitor	A Increment of NO_2^- (nmol·min^{-1}·mg^{-1})	B Decrement of NO_2^- (nmol·min^{-1}·mg^{-1})
–	0	38.5
Antimycin	50.5	6
Mucidin	73.3	1

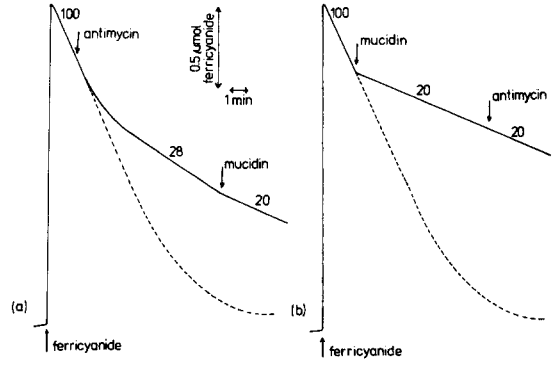


Fig. 3. Influence of mucidin and antimycin on the rate of ferricyanide reduction by the cells of *P. denitrificans*. 3 ml of reaction medium contained 0.5 mg dry weight of cells. Indicated additions: potassium ferricyanide 2 µmol, mucidin 0.75 µg (2.9 nmol), antimycin 0.5 µg (0.9 nmol).

itors did not have additive character. Whereas the reduction rate of ferricyanide in the presence of the saturating amount of antimycin was further lowered by the addition of mucidin (record a), the addition of antimycin to cells inhibited with mucidin had no effect (record b). The use of mucidin which probably completely stops the electron flow through the bc_1 segment of the respiratory chain of *P. denitrificans* (cf. Fig. 2 and Table I) thus make it possible to distinguish residual antimycin-insensitive ferricyanide reductase activity of *P. denitrificans* cells described in an earlier paper [13] into a part corresponding to electron flow through the bc_1 segment in the presence of antimycin and a part which might be due to ferricyanide interaction with a component of the respiratory chain different from cytochrome *c* (cf. also Ref. 13).

Further information on the mechanism of the action of the above inhibitors can be obtained by examining the redox state of cytochromes *b* and *c* with the aid of difference spectroscopy. Since in the branched respiratory chain of *P. denitrificans* oxygen takes electrons simultaneously both from the site before and after the region of antimycin or mucidin attack, it is more advantageous to work under anaerobic conditions, using nitrogen terminal acceptors NO_2^- or NO_3^- which oxidize the bc_1 complex of the respiratory chain from those different sides. The difference spectra obtained are given in Fig. 4. It follows from them that the addition of inhibitors to cells respiring nitrite resulted in lowering the degree of reduction of cytochrome *c* (maximum at 550 nm) and caused the appearance of the peak attributable to reduced cytochrome *b*. A subsequent addition of nitrate resulted in a partial cytochrome *b* reoxidation. Fig. 4 also shows that the degrees of reduction of cytochromes *b* and *c* utilizing NO_2^- or a mixture of NO_2^- and NO_3^- are different, depending on the inhibitor employed. Whereas the higher degree of cytochrome *c* reduction in the presence of antimycin is evidently the consequence of the residual electron flow through segment bc_1 (see above), the higher degree of reduction of cytochrome *b* in the presence of antimycin evidently follows from a different site of inhibitory action of the two inhibitors in the bc_1 region. The differences in the action of the two inhibitors might be related to the functional organization of the redox components of segment bc_1 as schematically shown in Fig. 5 which should be considered as a working model.

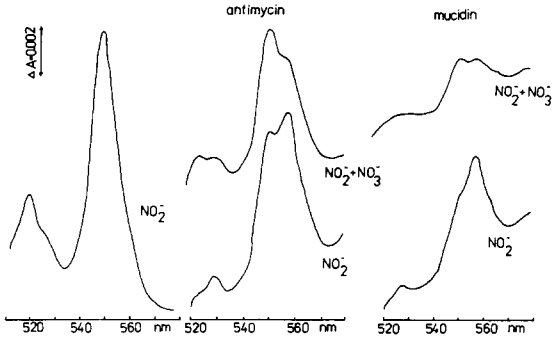


Fig. 4. Difference absorbance spectra of *P. denitrificans* cells while utilizing nitrite or a mixture of nitrite and nitrate in the presence of antimycin or mucidin. 4.7 mg dry weight of cells were suspended in 3 ml of medium. After exhaustion of oxygen 20 μmol of the terminal acceptor nitrite or nitrate were added. This was incubated for 2 min and the spectra registered. The addition were: 5 μg (9.1 nmol) antimycin or 7.5 μg (29.1 nmol) mucidin.

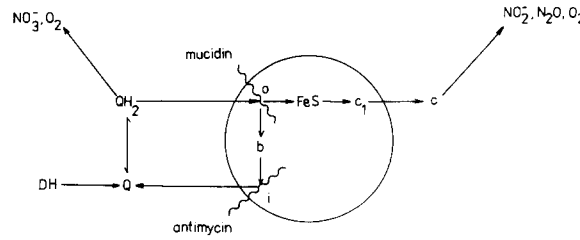


Fig. 5. Preliminary localization of the intervention sites of mucidin and antimycin into the respiratory chain of *P. denitrificans*. Explanation of symbols: b means cytochromes *b* in complex bc_1; c_1 and c mean cytochromes c_1 and cytochrome *c*; Q and QH_2 designate ubiquinone and ubiquinol; DH is dehydrogenase; o and i represent centre *o* and centre *i*; ∿∿ refers to the intervention site of the inhibitor.

Discussion

In recent years a number of experimental facts has been accumulated witnessing an almost general distribution of enzymes quinol-cytochrome *c* (and/or plastocyanine) oxidoreductases in nature and their analogical structure and mechanism of action (for survey see Ref. 29). The employment of specific inhibitors is of great importance for iden-

tifying those enzymes in electron-transfer chains and for studying their function. Mostly in this way evidence has been obtained witnessing the participation of the bc_1 complex in the aerobic respiratory chain of *P. denitrificans* [1,14,30,31].

This paper deals with the investigation of the attack of β-methoxy acrylate ('moa') antibiotic-mucidin (strobilurin A) into a branched respiratory chain of the anaerobically grown bacterium *P. denitrificans*. The results of measuring of enzyme activities (Figs. 1 and 2) and of the redox state of cytochromes (Fig. 4) have made it possible to localize the sites of inhibition of mucidin into the bc_1 segment similarly as is the case in mitochondria [20–24]. It has also appeared (Fig. 2) that, besides antimycin [6,7] and hydroxylamine [7], mucidin is another inhibitor abolishing the inhibitory effect of oxygen on the reduction of nitrate in *P. denitrificans* cells [32].

In Fig. 5 there is a schematic representation of the possible functional arrangement of the Q-bc_1 region in the anaerobic respiratory chain of *P. denitrificans*. The indicated cytochrome *b* in the picture means the components with E_{m7} equalling −40 mV and +110 mV found by a potentiometric analysis of the membranes [33]. A preliminary location of the intervention sites of the inhibitors and the inclusion of the fundamental features of Mitchell's Q-cycle [34] have been motivated by an experimentally found higher degree of cytochrome *b* reduction in the presence of nitrite and antimycin than in the presence of nitrite and mucidin (Fig. 4). Similarly John and Papa [14] have observed an increase in the reduction of cytochromes *b*-566 and *b*-560 in membranes from cells of *P. denitrificans* after an oxygen pulse in the presence of antimycin. In a scheme depicted in a recently published paper [11] it is assumed that in *P. denitrificans* antimycin blocks the electron flow from QH_2 to FeS protein. In this way of inhibition it would, however, be difficult to explain the described redox behaviour of cytochromes *b*.

It is characteristic of the sketch in Fig. 5 that ubiquinol is considered to be the common donor of electrons for the bc_1 segment, nitrate reductase and cytochrome *o*. This function of QH_2 was postulated earlier [3,9] and it was also shown that the redox behaviour of ubiquinone was consistent with this assumption [13]. The possibility that some of the constitutive cytochromes *b* in the bc_1 segment might serve as a donor for nitrate reduction, often encountered in earlier reviews (e.g., see Refs. 1, 2 and 35), is rendered doubtful by our finding (cf. Table I and Fig. 4) that in the presence of antimycin the electron flow to nitrate is lower than in using mucidin in contrast with a considerably higher degree of cytochrome *b* reduction. According to Fig. 5 the oxidation of cytochromes *b* observed on adding nitrate to membranes [4,5], to membranes in the presence of antimycin or mucidin (Ref. 13 and Fig. 4) can be interpreted as a consequence of the change in the degree of ubiquinone pool reduction (cf. also Ref. 12) due to the flow of electrons from QH_2 to the nitrate reductase complex. This interpretation does not exclude a possible contribution of the redox change of further, perhaps minor, cytochrome *b* components to the observed effect brought by NO_3^- addition. Such a *b* type component could be a part of nitrate reductase complex (e.g., see Refs. 3 and 36, but see Ref. 33).

Experimental results in this paper do not make it possible to determine the nature of the observed electron flow insensitive to antimycin but inhibited with mucidin through the bc_1 segment (Table I and Fig. 3). It is possible to speculate that the rate of this action equals the dismutation of radical $QH^{\cdot}$ generated in the centre *o* by the transfer of one electron from QH_2 to FeS protein and cytochrome c_1, which does not take place after blocking centre *o* with mucidin.

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