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The Aerobic Nature of *Arcobacter nitrofigilis*

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The free-living nitrogen fixing bacterium, *Arcobacter nitrofigilis* which has been known to be a microaerophile, exhibited aerobic growth in brucella broth. In a level of oxygen equivalent to an air atmosphere (21% O_2), the maximum cell growth was observed in brucella broth. Low level of cell growth occurred in a level of low oxygen equivalent to lower than 2%, unless any other terminal electron acceptors other than oxygen were supplied in brucella broth. Membrane-bound cytochrome *b* and *c*, and soluble cytochrome *c* were found. The growth in an aerobic atmosphere, little growth at low oxygen level, and occurrence of cytochrome *c* mean that this species is an aerobe and obtains energy using energy-yielding respiration.

KEY WORDS □ *Arcobacter nitrofigilis*, aerobic, cytochrome

Some species of the genus *Campylobacter* have been recognized as important pathogens in human and animals (13, 15, 16). However, McClung and his colleagues (9, 10) isolated a free-living nitrogen-fixing *Campoylobacter nitrofigilis*, which is not associated with animals but with salt marsh grass, *Spartina alterniflora*. The cells are small, rigid, curved, motile, and rod shaped (0.2~0.9 μm wide and 1~3 μm long) and have a single pollar unsheathed flagellum (3, 10).

The species has been known to be microaerophilic, catalase- and oxidase-positive, and NaCl-requiring Gram-negative bacteria. Like other campylobacters, it can not utilize carbohydrates as a carbon and energy source. Instead, it utilizes amino acids or organic acids (tricarboxylic acid cycle intermediates). In nitrogen-deficient media, it can fix atmospheric nitrogen, but only under microaerobic conditions (9, 10).

Recently, Vandamme *et al.* (18) transferred *C. nitrofigilis* to a new genus, *Arcobacter*, as *Arcobacter nitrofigilis*. *A. nitrofigilis* is phylogenetically related to *Arcobacter cryaerophilus* which is aerobic, but not related to the genus *Campylobacter* which is microaerophilic (17, 18).

Characteristics differentiating *A. nitrofigilis* from other *Campylobacter* species include its nitrogenase activity and its requirement for at least 0.5% NaCl (10).

A. nitrofigilis and *A. cryaerophilus* are found in different habitats, i.e., *A. cryaerophilus* is associated with and causes diseases in animals, meanwhile *A. nitrofigilis* is found only in plant (9, 10, 11). Inclusion of the microaerophilic *A. nitrofigilis* with the aerobic *A. cryaerophilus* in a phylogenetic group shows the problem as mentioned in another group (4, 17).

No information has been available about the oxygen-dependent aerobic growth and the respiratory components of *A. nitrofigilis*. The present article describes the characteristics of aerobic growth and types of cytochromes in *A. nitrofigilis*.

MATERIALS AND METHODS

Bacterial strain

The type strain of *A. nitrofigilis* ATCC 33309 was obtained from the American Type Culture Collection. Stock cultures were grown at 30°C under an aerobic atmosphere in semisolid NaCl-supplemented brucella media (1.0% NaCl-added brucella broth with 0.15% agar) and transferred weekly.

Inocula and general cultivation condition

The top 1-cm of a 2-day-old culture grown in the semisolid brucella medium was removed to yield a homogeneous suspension. The suspension (0.05 m*l*) was inoculated into each 5-m*l* portion of test broth. For general tests, cultures were incubated statically in an aerobic atmosphere at 30°C except for the growth response at 6°C or 37°C. For testing growth response in the brucella broth, the organism was cultured in 5-m*l* volumes of media contained in 50-m*l* cotton-stoppered serum bottles. Cultures were incubated statically at 30°C for 48 hr in a sealed vessels (oxoid jar system) equipped with a vent to allow filling with various gas mixtures. Gas atmospheres were obtained manometrically by evacuating the Oxoid jar and refilling with various combinations of CO_2,

N_2, and O_2. For mass cultures of cytochrome scanning experiments, cells were grown in a biphasic culture system (50 m*l* NaCl-supplemented brucella broth overlaid onto 200 m*l* of NaCl-supplemented brucella agar containing 2.5% agar) at 30°C for 24 hr in an aerobic atmosphere.

General tests

The type strain of *A. nitrofigilis* was checked for purity by streaking. In order to identify the type strain of *A. nitrofigilis* used in this study the following physiological and biochemical characteristics were determined by the procedures that have been described in the previous report (10) with exception that the brucella media were used in this study: Gram staining, growth at 6°C or 37°C, rapid coccal transformation, activity of catalase, oxidase, urease or phosphatase, nitrate or nitrite reduction, hydrolysis of hippurate, esculin, or starch, growth in the presence of 1% bile, 1% glycine or 3.5% NaCl, and growth in anaerobic growth with 0.1% fumarate. Tests were performed in triplicate.

Measurement for aerobic growth

Growth reponses in the brucella broth were estimated turbidometrically at 660 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer using an 1-cm cuvette.

Cytochrome analyses

Cell suspensions were obtained by harvesting the cells from three to five Roux bottles, centrifuged at 10,000×*g* for 10 min, and suspending the cell pellets in 40 mM Tris-HCl buffer (pH 7.5). The cells were disrupted by using a model 300 sonic dismembrator (Fisher Scientific Co.) at full power (five 30 sec bursts). The preparation was centrifuged at 10,000×*g* for 10 min to remove whole cells and large debris. The supernatant (crude extract) was then centrifuged at 100,000×*g* for 90 min to obtain the soluble and membrane fractions. The membrane fraction was suspended in 40 mM Tris-HCl buffer (pH 7.5).

Cytochrome scans were performed at room temperature by using a model 2000 spectrophotometer (Bausch & Lomb) with a light path of 1 cm, band path of 1 nm, and scan speed of 50 nm per min. The membrane and soluble fractions were used to determine dithionite-reduced *minus* air-oxidized difference spectra. The types and concentrations of cytochromes were estimated by using extinction coefficients and wavelength pairs reported by Jones and Redfearn (6), Cross and Anthony (2), and O'Keeffe and Anthony (12). Protein concentrations were determined by the method of Bradford (1).

RESULTS AND DISCUSSION

Identification of the type strain of *A. nitrofigilis*

Morphological, physiological, and biochemical tests were performed to identify the type strain of *A. nitorfigilis* used in this study.

Microscopic observation of the strain showed that the cells were small, rigid, curved, motile, and rod-shaped and had a single pollar flagellum. The test results for the strain were positive for the following traits: activity of catalase, oxidase, and urease, growth in the presence of 3.5% NaCl, and growth at 6°C, and nitrate reduction. The strain showed negative reactions for the following traits: Gram reaction, growth at 37°C, rapid coccal transformation, phosphatase activity, nitrite reduction, hydrolysis of hippurate, esculin, and starch, growth in the presence of 1% bile and 1% glycine, and growth in anaerobic growth with 0.1% fumarate.

The characteristics of the strain used in this study corresponded to those of the type strain of *A. nitrofigilis* indicated in the previous report (10).

Oxygen-dependent aerobic growth

The strain of *A. nitrofigilis* used in this study showed the typical characteristics of aerobic growth in the brucella broth (Table 1). Unlike microaerophilic organisms, the growth of the strain was not inhibited, instead it was maximum, in an aerobic atmosphere (21% O_2). This suggests this strain is aerobic, not microaerophilic. In a low level of air atmosphere (lower than 2% O_2), this species exhibited the low level cell growth in the brucella broth, when any other electron acceptors other than oxygen were added.

As defined by Krieg and Hoffman (8), the characteristics of an aerobic organim is similar to those of an microaerophilic organism in that both kinds of organisms exhibit oxygen-dependent growth, have the strict respiratory type of energy metabolism, and are capable of respiring with oxygen as a terminal electron acceptor (positive reaction to the Kovacs oxidase test) (7, 19). An aerobe can grow, however, a micro-

Table 1. *Effect of O_2 concentrations on the growth of A. nitrofigilis*[a].

Oxygen concentration (%)	Turbidity[b]
<2[c]	0.041 ± 0.016
4	0.205 ± 0.008
8	0.238 ± 0.007
12	0.241 ± 0.032
16	0.240 ± 0.028
21	0.254 ± 0.017

[a] Cells were grown statically in 5-m*l* brucella broth at 30°C for 48 hr under different levels of oxygen concentration.

[b] Turbidities were measured at 660 nm. The values are the averages ± standard deviations for four to six replicate cultures.

[c] The condition represents the oxygen concentration lower than 2%.

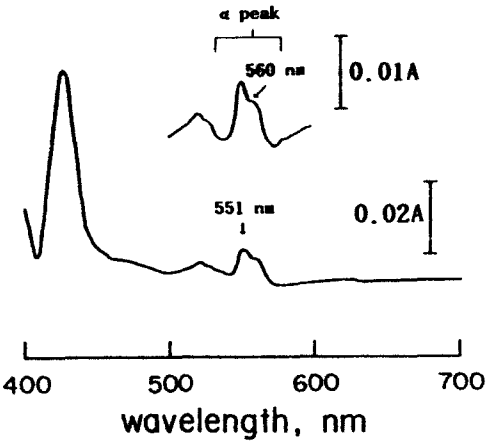

Fig. 1. *Dithionite-reduced minus air-oxidized spectrum of the membrane fraction of A. nitrofigilis.* Protein concentration was 0.27 mg/ml. The spectrum was measured at room temperature at a scan speed of 50 nm·min^{-1}. The light path was 1 cm.

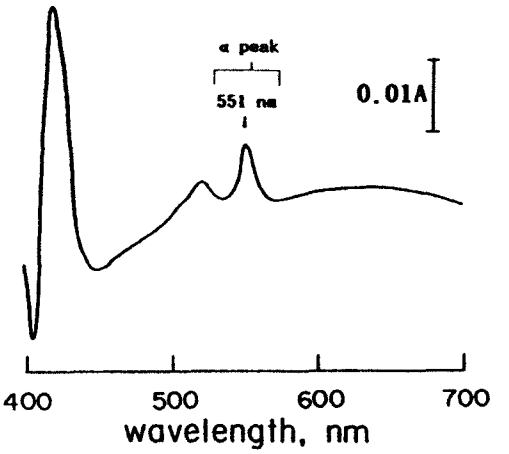

Fig. 2. *Dithionite-reduced minus air-oxidized spectrum of the soluble fraction of A. nitrofigilis.* Protein concentration was 0.34 mg/ml. The spectrum was measured at room temperature at a scan speed of 50 nm·min^{-1}. The light path was 1 cm.

aerophile fails to grow or grow poorly in a level of oxygen equivalent to or higher than that present in an aerobic atmosphere (i.e., oxygen is beneficial and harmful to microaerophilic microorganisms).

McClung *et al.* (10) have reported that *A. nitrofigilis* can fix atmospheric nitrogen only under a microaerobic condition, when any nitrogen sources are not given in media, and this species is obligately microaerophilic. However, no detailed information about the microaerophily and little information about the nitrogen fixation of this species in complicated media in an aerobic atmosphere have been available. As shown in the Table 1, the strain of *A. nitrofigilis* used in this study grew very well in the brucella broth in an aerobic atmosphere. Regardless of the oxygen-labile nitrogenase complex activity in an aerobic atmosphere, the strain might utilize amino acids, as a nitrogen source, contained in the brucella media. If in an aerobic atmosphere, the strain can grow in nitrogen-deficient media, it has to fix atmospheric nitrogen. In this case, the oxygen-labile nitrogenase complex should be protected against the oxygen toxicity. The unidentified slimy compound produced by this strain (unpublished data) may play an important role in respiratory protection. If oxygen is not used up rapidly at the cell surface, it might reach oxygen-labile enzymes within the cell. The importance of respiratory protection is emphasized by the discovery of certain microaerophilic mutants of the normally aerobic nitrogen-fixing bacterium, *Azotobacter chroococcum* (14). Whether the oxygen-labile nitrogenase complex from the strain cultured in an aerobic atmosphere is still biochemically active or not should be further studied.

Cytochrome analyses

The dithionite-reduced *minus* air-oxidized spectra of the membrane fraction and of the soluble fraction are shown in Fig. 1 and 2, respectively. The wavelengths of α peak, which is important to identify the types of cytochromes, of cytochrome *b* and of cytochrome *c* were 560 nm and 551 nm, respectively.

The concentrations of the both cytochromes are summarized in Table 2. The concentration of cytochrome *c* in the membrane fraction was 1.4-fold and 1.3-fold higher than that of cytochrome *b* in the membrane fraction and that of the cytochrome *c* in the soluble fraction, respectively. Cytochrome *b* was not detected in the soluble fraction. So far, any other cytochrome *a*, *o*, and *d* as a terminal oxidase have not yet been determined. However, any types of terminal oxidase might be present, because *A. nitrofigilis* is positive to the Kovacs oxidase reaction. Usually, a positive Kovacs oxidase organism is usually associated with organisms that possess cytochrome *c* and are of considerable importance to frequently exhibit higher H^+/O ratios (7, 19). The composition of cytochromes found in this species resembled that reported for other aerobic or microaerophilic microorganisms in that they possess membrane-bound cytochromes *b* and *c*, and soluble cytochrome *c* (5, 19)

The study should be further carried out to know the composition and structure of respiratory system of this species. It might be interested in

Table 2. *Wavelengths of peaks and concentrations of cytochromes in A. nitrofigilis*[a].

Fraction	Membrane fraction		Soluble fraction
Cytochromes	Cytochrome *b*	Cytochrome *c*	Cytochrome *c*
Peak of wavelength (nm)	560	551	551
Concentration[b] (nanomoles per mg protein)	1.26	1.79	1.36

[a]Cells were grown in the brucella biphasic media at 30°C for 24 hr in an aerobic atmosphere.
[b]Concentrations were determined by using the following extinction coefficients: $\triangle\varepsilon$ of cytochrome $b = 17.5\ mM^{-1}\ cm^{-1}$ at 560 nm; $\Delta\varepsilon$ of cytochrome $c = 17.3\ mM^{-1}cm^{-1}$ at 551 nm in dithionite-reduced *minus* air-oxidized specta.

whether the compositions and concentrations of respiratory components including cytochromes associate with the shifts in the level of oxygen concentration from a microaerobic to an aerobic condition, and *vice versa*.

So far, there has been little information about the differentiation of an aerobe from a microaerophile. Although there have been known to be many similar characteristics such as oxygen-dependent growth, energy metabolism, and occurrence of cytochrome *c* between an aerobe and a microaerophile, a major difference is that an aerobe can grow in the presence of a level of oxygen equivalent to or higher than that present in an air atmosphere (21% O_2), however, a microaerophile can not due to lack of any defence mechanisms against the toxic forms of oxygen. Some facultatives are capable of growing aerobically by respiring with oxygen. However, a major difference between aerobes and facultatives is that in an anaerobic atmosphere, facultatives obtain energy using fermentation and grow, meanwhile aerobes do using anaerobic respiration, if other electron acceptors other than oxygen are supplied in media.

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초 록: *Arcobacter nitrofigilis*의 호기적 특성

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미산소성 세균으로 알려진 질소고정균 *Arcobacter nitrofigilis*는 brucella 액체배지에서 호기적 생장의 특성을 나타내었다. 일반대기의 산소조건(21% O_2)하에서 최대생장의 특성을 보여주었다. 2% 이하의 산소조건하에서 산소 이외의 다른 최종산소수용체를 첨가하지 않은 액체배지에서 이 세균은 적은 정도의 생장을 나타내었다. 이 세균은 전자전달계 구성성분인 세포막에 함유되어 있는 cytochrome *b* 및 *c*, 그리고 용해성 cytochrome *c*를 가지고 있었다. 일반대기의 산소조건에서의 최대생장, 낮은 산소 농도하에서의 적은 정도의 생장 및 cytochrome *c*의 존재로 볼 때 이 세균은 미산소성 세균이나 통성혐기성 세균이 아니고, 호기성 세균임을 나타내주며, 또한 이 세균은 산소를 이용한 호흡에 의해 에너지를 얻음을 나타내준다.