Anaerobic growth and potential for amino acid production by nitrate respiration in *Corynebacterium glutamicum*

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Abstract Oxygen limitation is a crucial problem in amino acid fermentation by *Corynebacterium glutamicum*. Toward this subject, our study was initiated by analysis of the oxygen-requiring properties of *C. glutamicum*, generally regared as a strict aerobe. This organism formed colonies on agar plates up to relatively low oxygen concentrations (0.5% O_2), while no visible colonies were formed in the absence of O_2. However, in the presence of nitrate (NO_3^-), the organism exhibited limited growth anaerobically with production of nitrite (NO_2^-), indicating that *C. glutamicum* can use nitrate as a final electron acceptor. Assays of cell extracts from aerobic and hypoxic cultures yielded comparable nitrate reductase activities, irrespective of nitrate levels. Genome analysis revealed a *narK2GHJI* cluster potentially relevant to nitrate reductase and transport. Disruptions of *narG* and *narJ* abolished the nitrate-dependent anaerobic growth with the loss of nitrate reductase activity. Disruption of the putative nitrate:nitrite antiporter gene *narK2* did not affect the enzyme activity but impaired the anaerobic growth. These indicate that this locus is responsible for nitrate respiration. Agar piece assays using L-lysine- and L-arginine-producing strains showed that production of both amino acids occurred anaerobically by nitrate respiration, indicating the potential of *C. glutamicum* for anaerobic amino acid production.

Keywords *Corynebacterium glutamicum* · Oxygen limitation · Nitrate reductase · Nitrate respiration · Amino acid production

Introduction

Various L-amino acids including L-lysine, L-arginine, L-glutamine, and L-glutamate are widely manufactured by fermentation using regulatory mutants of *Corynebacterium glutamicum* (Kinoshita and Nakayama 1978). These fermentation processes require a large quantity of oxygen for efficient production (Leuchtenberger 1996). Under oxygen limitation, production strains usually accumulate undesirable organic acids, which results in damaged fermentation with decreased production yields. For this reason, it is necessary to keep dissolved oxygen above a certain level during fermentation. To this subject, considerable effort has been dedicated to investigation of aeration efficiency of fermentor tanks from the viewpoint of mechanical engineering (Oka 1999; Ikeda 2003). Actually, this subject is most

likely to be achieved by remodeling of fermentors. Nevertheless, capital investment is hampered by the recent situations where the world market is highly competitive and process economics are of primary importance. If we can develop strains that produce amino acids efficiently even under oxygen limitation, a significant benefit would likely arise industrially. However, there is no such technology at present.

Microorganisms that grow under anoxic conditions, such as facultative anaerobes and obligate anaerobes, possess a variety of anaerobic metabolism, which are divided into two opposing modes of metabolism: "fermentation" and "anaerobic respiration". In a typical "fermentation", most of carbon is excreted as partially reduced fermentation products such as lactate and ethanol to maintain redox balance. Due to this inevitable loss of carbon toward fermentation products, it is difficult to realize efficient amino acid production in parallel to fermentative metabolism. On the other hand, anaerobic respiration is one alternate process of respiration, where electron acceptors other than oxygen are used. For example, *Escherichia coli* possesses nitrate respiration systems and carries out the oxidation of NADH to NAD^+ and concomitant ATP synthesis using nitrate as an electron acceptor in environments where oxygen is absent (Gennis and Stewart 1996). Such metabolism of anaerobic respiration is not necessarily accompanied by undesirable fermentation products, unlike the metabolism of fermentation. Therefore, carbon in sugar might be effectively directed to amino acid biosynthesis, which is likely to realize amino acid production even under anaerobic conditions. Nevertheless, such electron transport systems supporting anaerobic respiration have not been known in *C. glutamicum*.

C. glutamicum is generally regarded as an aerobe (Abe et al. 1967; Sahm et al. 2000; Bott and Niebisch 2003; Mónica et al. 2006). However, no attention has so far been directed to the oxygen-requiring properties of this organism despite its industrial importance. For example, there is no information about the lower limit of oxygen concentrations where the organism can grow. Recently, the respiratory chain of *C. glutamicum* has been being elucidated by a few groups (Matsushita et al. 1998; Niebisch and Bott 2001; Sakamoto et al. 2001; Sone et al. 2001; Bott and Niebisch 2003; Nantapong et al. 2004). These studies have focused on the composition and the efficiency of the respiratory chain, and the oxygen usability of the organism remains to be elucidated. On the other hand, early taxonomic studies have shown that most wild-type strains belonging to the organism were positive toward nitrate reductivity (Abe et al. 1967), but there are no genetic and physiological evidences on the character. It also remains unclear whether the

organism grows by nitrate respiration using nitrate as an electron acceptor instead of oxygen.

The respiratory nitrate reductases have been extensively studied in several bacteria. In *E. coli*, *Bacillus subtilis*, and also *Mycobacterium tuberculosis*, an organism taxonomically related to *C. glutamicum* (Liebl 2005), respiratory nitrate reduction is known to be related to enzymes encoded by the *narGHJI* operon and the *narK* family (Hoffman et al. 1995; Wang et al. 1999; Sohaskey and Wayne 2003). NarG, H, and I are subunits of nitrate reductase, with NarG being the catalytic subunit, whereas NarJ functions in the assembly of the enzyme. Transport of nitrate and nitrite has been supposed to be due to *narK* and *narU* for *E. coli* (Noji et al. 1989; Rowe et al. 1994) and *narK2* for *M. tuberculosis* (Sohaskey and Wayne 2003). In the genome of *C. glutamicum* (BA000036), orthologs of the genes relevant to respiratory nitrate reductase and transport can be found and have been annotated as *narK2GHJI* (Cgl1186-1190), but there is no report about their functions (Bott and Niebisch 2003).

Toward exploitation of new strategies for cellular engineering to alleviate oxygen limitation during aerobic culture, we initiated our work by examining the oxygen-requiring properties of *C. glutamicum*, in connection with nitrate metabolism. As the result, it was shown that this organism can form colonies normally up to relatively low concentrations of oxygen whereas it does not grow at all under strict anaerobic conditions. In addition, the organism was found to show weak growth even under strict anaerobic conditions, provided that nitrate was supplied, revealing that this organism possesses a nitrate respiration system. Based on these findings, we show the possibility of anaerobic production of amino acids by *C. glutamicum*.

Materials and methods

Bacterial strains and plasmids

The wild-type strains examined for oxygen-requiring properties were the four representative strains: *C. glutamicum* ATCC 13032, *C. glutamicum* subsp. *flavum* ATCC 14067, *C. glutamicum* subsp. *lactofermentum* ATCC 13869, and *C. glutamicum* subsp. *acetoacidophilum* ATCC 13870 (Abe et al. 1967; Kinoshita 1999; Ohnishi and Ikeda 2006). The aerobes, *Sinorhizobium meliloti* RU11/001 (Sourjik et al. 1998) and *Gluconobacter*

suboxidans IFO 12528 (Adachi et al. 2001), the facultative anaerobe *E. coli* W3110 (Bachmann 1972) and the aerotolerant anaerobe *Lactobacillus johnsonii* NCC 533 (Pridmore et al. 2004), were also used to examine for their oxygen-requiring properties for references. Strain ATCC 13032 was also used for construction of disrupted mutants of the chromosomal *narK2* (Cgl1190), *narG* (Cgl1189), and *narJ* (Cgl1187) genes. The wild-type ATCC 13032 is the type strain of *C. glutamicum*, the whole genome sequence of which has been determined (Ikeda and Nakagawa 2003). The other three strains, ATCC 14067, ATCC 13869, and ATCC 13870, were previously classified as *Brevibacterium flavum*, *Brevibacterium lactofermentum*, and *Corynebacterium acetoacidophilum*, respectively, but by recent molecular taxonomic studies, they are currently re-classified in the original species *C. glutamicum* (Liebl et al. 1991; Kinoshita 1999). The L-lysine- and L-arginine-producing strains used for agar piece assays were *C. glutamicum* AK-1 and *C. glutamicum* SU150, respectively, both of which are defined production strains developed by a genome-based approach from the wild-type ATCC 13032 (Ohnishi et al. 2002; Ikeda and Nakagawa 2003; Ikeda et al. 2006). L-Lysine producer AK-1carries only the *lysC311* mutation leading to desensitization of aspartokinase from synergistic inhibition by L-lysine plus L-threonine (Ohnishi et al. 2002; Ikeda et al. 2005). L-arginine producer SU150 carries two mutations that are essential for production of L-arginine: the *argB26* mutation leading to desensitization of the *argB* product from inhibition by L-arginine and a deletion mutation in the *argR* repressor gene (Ikeda et al. 2006). *C. glutamicum* SU62 and *C. glutamicum* SU101 used as indicator strains for agar piece assays were L-lysine- and L-arginine-auxotrophic mutants, respectively, derived by a round of mutagenesis with nitrosoguanidine from a wild-type strain *C. glutamicum* ATCC 31833. *E. coli* DH5α (Sambrook and Russell 2001) was used as a host for cloning of the PCR products.

Plasmid pESB30, which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum* (Mitsuhashi et al. 2004). It was constructed by ligating a 2.6 kb *Pst*I DNA fragment containing *sacB* (Schweizer 1992), the levansucrase gene of *Bacillus subtilis*, with *Pst*I-digested pHSG299 (Takeshita et al. 1987), a *E. coli* vector carrying the kanamycin gene. Plasmids pCnarK2d, pCnarGd, and pCnarJd that contained the internally deleted *narK2*, *narG*, and *narJ* genes, respectively, in vector pESB30 were used to replace the wild-type chromosomal genes by the deleted genes.

Media

Complete medium BY and minimal medium MM were used for investigation of oxygen-requiring properties of *C. glutamicum*. BY medium consisted of (per liter) 3 g of NaCl, 5g of yeast extract, 7g of meat extract, and 10g of peptone. MM medium consisted of (per liter) 10 g of glucose, 4 g of NH_4Cl, 1 g of KH_2PO_4, 3 g of K_2HPO_4, 2 g of urea, 0.4 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 1 mg of $MnSO_4 \cdot 5H_2O$, 5 mg of nicotinic acid, 5 mg of thiamine-HCl, and 0.1 mg of D-biotin (pH 7.2). Agar plates were made by the addition of Bacto-Agar (Difco) to 1.6%. MM agar medium was also used for investigation of amino acid production by the agar piece method. YPGD medium used for growth of *Gluconobacter suboxidans* contained 2% glycerol, 0.5% glucose, 0.5% peptone, and 1% yeast extract. When required, kanamycin was added at the final concentration of 20 μg/ml.

Culture conditions

For the growth test under several oxygen concentrations, cells were incubated at 30ºC under the indicated oxygen concentrations in a sealed 2.5-liter box, using simple culture systems, Anaero Pack (Mitsubishi Gas Chemical Inc., Tokyo, Japan). The anaerobic and microaerobic conditions during cultures were confirmed by the usage of the Anaerobic Indicator (Mitsubishi Gas Chemical) as recommended by the supplier.

For liquid culture in MM medium under the strict anaerobic conditions, 20 ml of medium supplemented with 1 mg/l of the oxygen indicator resazurin (7-hydroxy-3*H*-phenoxazin-3-one-10-oxide) was prepared in a stoppered 30-ml serum bottle, followed by flushing the medium with N_2 gas. A 0.2-ml amount of the seed culture grown aerobically at 30ºC in BY medium for 12 h was inoculated into the bottle filled with 20 ml of medium and incubated at 30ºC for 24 h. The strict anaerobic conditions during cultures were confirmed by the oxygen indicator resazurin.

For aerobic shaken culture in MM medium, A 0.5-ml amount of the seed culture grown aerobically at 30ºC in BY medium for 12 h was inoculated into 5 ml of medium in a L-type test tube and cultivated at 30ºC using a Monod shaker (Taitec Inc., Saitama, Japan) at 40 strokes/min.

Recombinant DNA techniques

Standard protocol (Sambrook and Russell 2001) was used for the construction, purification and analysis of plasmid DNA, and transformation of *E. coli*. Chromosomal DNA was extracted from protoplasts of *C. glutamicum* by the method of Saito and Miura (1963). The protoplasts were prepared by the method of Katsumata et al. (1984). Transformation of *C. glutamicum* by electroporation was carried out by the method of van der Rest et al. (1999), using Gene pulser and Pulse controller (BioRad, USA). PCR was performed with DNA Thermal Cycler GeneAmp 9600 (Perkin Elmer, USA), using Pyrobest DNA polymerase (Takara Bio Inc., Shiga, Japan), as follows: heating to 94°C for 5 min; for 25 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min, followed by extension for 3 min at 72°C. Fusion PCR for the construction of plasmids pCnarK2d, pCnarGd, and pCnarJd was performed in the modified conditions: heating to 94°C for 5 min; 10 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min; 72°C for 3 min, 20 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by extension for 3 min at 72°C. PCR products were purified using GENECLEAN III KIT (Qbiogene, CA, USA).

Generation of *nar*-disrupted mutants

Plasmids pCnarK2d, pCnarGd, and pCnarJd containing the internally deleted *narK2*, *narG*, and *narJ* genes, respectively, were constructed as follows and were used to replace the wild-type chromosomal genes with the deleted genes. Primers used in this study are listed in Table 1.

For the construction of pCnarK2d, the 5'-region of the *narK2* gene was amplified by PCR using two primers narK2up800F and narK2FusR with the wild-type genomic DNA as a template. On the other hand, the 3'-region of the gene was also amplified with two primers narK2FusF and narK2down800R. Since the two primers narK2FusR and narK2FusF were complementary each other, fusion PCR was performed using the purified 5'-region-*narK2* and 3'-region-*narK2* fragments as templates and the primers narK2up800F and narK2down800R. The resulting 1.6-kb fragment contained the deleted *narK2* gene which was shortened from 1353 bp to 159 bp by in-frame deletion of its inner sequence. The fragment was digested with *Bgl*II and then ligated to *Bam*HI-digested pESB30 to yield pCnarK2d. Similarly, for the construction of pCnarGd, the 5'-region of the *narG* gene was amplified with two primers narGup800F and narGFusR, while the 3'-region of the gene was amplified with two primers narGFusF and narGdown800R. Both resultant fragments were

fused by PCR using the primers narGup800F and narGdown800R to generate a 1.6-kb fragment. It contained the deleted *narG* gene which was shortened from 3747 bp to 84 bp by in-frame deletion of its inner sequence. The fragment was digested with *Bgl*II and then ligated to the *Bam*HI-digetsed pESB30 to yield pCnarGd. For the construction of pCnarJd, the 5'-region of the *narJ* gene was amplified with two primers narJup800F and narJFusR, while the 3'-region of the gene was amplified with two primers narJFusF and narJdown800R. Both resultant fragments were fused by PCR using the primers narJup800F and narJdown800R. The resulting 1.6-kb fusion product, containing the deleted *narJ* gene shortened from 735 bp to 189 bp by in-frame deletion of its inner sequence, was digested with *Bgl*II and then ligated to the *Bam*HI-digested pESB30 to yield pCnarJd.

Defined chromosomal deletions of the *narK2*, *narG*, and *narJ* genes were constructed using pCnarK2d, pCnarGd, and pCnarJd, respectively, via two recombination events as described previously (Ohnishi et al. 2002). Strains carrying the *narK2*, *narG*, and *narJ* deletions in the wild-type background were designated strains ΔnarK2, ΔnarG, and ΔnarJ, respectively.

Nitrate reductase assay

C. glutamicum cells were grown on MM agar plates supplemented with and without 30 mM nitrate under the two oxygen conditions (microaerobic conditions 0.5% and atmospheric conditions 21%). Crude cell extracts were prepared by sonic disruption of the cells as described previously (Ozaki et al. 1985). Protein quantity was determined by the methods of Bradford (1976). Nitrate reductase activities in crude cell extracts were measured colorimetrically at 30°C by determining the amount of nitrite produced from nitrate by nitrate reductase, essentially by the method of MacGregor et al.(1974).

Measurement of nitrite in culture

The concentrations of nitrite in cultures were measured colorimetrically by the diazo coupling procedure (MacGregor et al. 1974) with slight modifications. Culture supernatants after removing cells by centrifugation were diluted to be appropriate concentrations for the determination. 900 μl of distilled water and 1 ml of a 1% solution of sulfanilic acid in 20% HCl were added to 100 μl of the diluted samples and were mixed thoroughly. Subsequently,

1 ml of 0.129% solution of *N*-1-naphthylethylenediamine diHCl was added. After 20 min, the absorbance was measured at 540 nm and compared with a known standard of nitrite.

Agar piece assays for amino acid production

The agar piece method developed for quantitative comparison of antibiotic production by *Streptomyces* (Ichikawa et al. 1971) was modified for assays for amino acid production under anaerobic conditions. First, MM agar media supplemented with and without 30 mM nitrate were cut out with a cork borer (6 mmΦ) to make agar pieces, followed by placing them separately in another Petri dish. Then, the L-lysine- and L-arginine-producing strains, AK-1 and SU150, respectively, grown for 24 h at 30ºC on BYG medium (containing 1.0% glucose in medium BY) were spread by toothpicks on the top of each of the agar pieces with and without nitrate and cultivated for 48 h at 30ºC under both atmospheric and anaerobic conditions. After cultivation, the agar pieces were sterilized by UV irradiation and then transferred onto bioassay plates at regular intervals. The bioassay plates consisted of two layers: (per plate) 15 ml of MM-bottom agar (2%) and 3 ml of MM-top agar (0.6%) supplemented with 0.1 ml of indicator-cell solution. The indicator-cell solution was prepared by cultivating the corresponding amino acid auxotroph (the L-lysine auxotroph SU62 or the L-arginine auxotroph SU101) overnight in 3 ml of BYG medium at 30ºC, washed with saline, and suspended in 3 ml of saline. The bioassay plates loaded with the sterilized agar pieces were cultured overnight at 30ºC and the resulting halos were compared.

Results

Oxygen-requiring properties on agar plates

To investigate the lower limit of oxygen concentrations where *C. glutamicum* can grow, we used simple anaerobic and microaerobic culture systems, Anaero Pack, as has often been reported (Yano et al. 2002; Osada et al. 2004; Hagiwara et al. 2006). By applying the systems, four representative wild-type strains of *C. glutamicum*, ATCC 13032, ATCC 14067, ATCC 13869, and ATCC 13870, were examined for their colony-forming abilities on BY agar plates under several oxygen concentrations (0%, 0.5%, 3%, 6%, and atmospheric conditions 21%) in a sealed 2.5-liter box. For references, the following four bacteria were also examined

for their growth: the facultative bacterium *E. coli* W3110, the aerotolerantly anaerobic lactic acid bacterium *Lactobacillus johnsonii* NCC 533, the aerobic nitrogen-fixing bacterium *Sinorhizobium meliloti* RU11/001, and the aerobic acetic acid bacterium *Gluconobacter suboxidans* IFO 12528. The *E. coli* and *L. johnsonii* strains grew at any oxygen conditions, but as expected, the former strain showed significantly better growth under aerobic conditions than under anaerobic conditions while the latter strain showed the opposite phenotype (Table 2). In contrast, all *C. glutamicum* strains examined could form colonies at and above 0.5% O_2 concentrations, but showed very weak growth at 0.5% O_2 and no growth in the absence of O_2 (Table 2), revealing that the limit of the colony-forming ability of *C. glutamicum* on BY agar plates was around 0.5% O_2. On the other hand, the aerobic *S. meliloti* and *G. suboxidans* strains required more or less higher oxygen concentrations for their normal growth than *C. glutamicum* (Table 2), suggesting that oxygen-requiring properties vary among aerobic bacteria and that *C. glutamicum* can grow up to relatively low concentrations of oxygen. When MM agar plates were used instead of BY agar plates, no significant differences in the growth dependency on oxygen were observed for the *C. glutamicum* strains except for the delay in the growth (data not shown).

Effect of nitrate on anaerobic growth

As described above, *C. glutamicum* is unable to grow anaerobically on both BY and MM agar plates. However, it was found that weak growth occurred even under strict anaerobic conditions on both agar plates supplemented with 30 mM nitrate (data not shown). To confirm this, we examined the ability of *C. glutamicum* ATCC 13032 cells to grow anaerobically in liquid medium with and without 30 mM nitrate. For this purpose, we used MM medium, which reflected the conditions for amino acid production processes. As shown in Fig. 1, the nitrate-dependent growth was observed in strictly anaerobic liquid cultures although cells grew slowly and to a low yield. Furthermore, we found that the anaerobic cultures in the presence of nitrate generated considerable amounts of nitrite in the medium as a result of nitrate reduction (Fig. 1). From these observations, we concluded that *C. glutamicum* is able to grow anaerobically by nitrate respiration. In the anaerobic cultures with nitrate, growth was gradually retarded after around 10 h (Fig. 1), probably due to accumulation of toxic nitrite. When the amount of nitrate added to the MM medium was varied between 1 mM and 100 mM, the dose dependence of the anaerobic growth and nitrite

production was observed up to 10 mM nitrate (data not shown). Above the concentration, no additional effects on both the anaerobic growth and nitrite production were observed although glucose was remaining in the cultures, suggesting that growth and nitrite production under anaerobic conditions would be limited by the capacity of this bacterium for reducing nitrate, not by the levels of nitrate or glucose.

When the effect of nitrate on anaerobic growth was examined for three other *C. glutamicum* strains, ATCC 14067, ATCC 13869, and ATCC 13870, we obtained the similar results, except for the last strain ATCC 13870, in which significantly positive effect of nitrate on anaerobic growth was not observed (data not shown). This result was reasonably accepted for us because the last strain ATCC 13870, previously classified as *Corynebacterium acetoacidophilum*, has been shown to be exceptionally negative toward nitrate reductivity in the early taxonomic studies (Abe et al. 1967).

We further examined whether growth occurred or not when nitrate was used as the sole nitrogen source in MM agar plates. However, no significant growth was observed under strict anaerobic conditions in any wild-type strains above (data not shown). This, along with the observation that anaerobic growth in the presence of nitrate was accompanied by nitrite production (Fig. 1), suggests that *C. glutamicum* could generally reduce nitrate to nitrite but not up to ammonia under the anaerobic conditions used. This consideration is supported by the absence of any obvious counterpart to *E. coli* nitrite reductase NrfA (Poock et al. 2002; Simon 2002) among protein sequences deduced from the *C. glutamicum* genome sequence (Ikeda and Nakagawa 2003).

Disruption of *narK2*, *narG*, and *narJ*

C. glutamicum has a set of genes, *narK2GHJI*, and this locus is likely responsible for the nitrate respiration. Based on the putative role of each gene product, we selected three key genes *narK2*, *narG*, and *narJ* to examine the effects of disruption of each gene on the anaerobic growth and nitrate reductase activities. For this purpose, we constructed three wild-type derivatives with in-frame deletions in the internal regions of the *narK2*, *narG*, and *narJ* genes (Fig. 2), and the resulting mutants were referred to as strains ΔnarK2, ΔnarG, and ΔnarJ, respectively. When these mutants were compared for their growth properties with the wild-type strain under atmospheric conditions, the similar growth profiles were observed (data not shown). However, as shown in Fig. 3, the three mutants

showed impaired growth in nitrate-supplemented MM liquid culture under strict anaerobic conditions. Especially, the growth of strains ΔnarG and ΔnarJ was thoroughly damaged, while strain ΔnarK2 showed partial growth relative to the wild-type strain.

We next assayed the nitrate reductase activities in the three mutants as well as the parental wild-type strain (Table 3). Each strain was grown with and without nitrate and harvested from atmospheric and microaerobic cultures. In the wild-type strain, there was no remarkable difference in the specific activity of cell-free extracts from cultures that had been grown with and without nitrate. Furthermore, the activities from cells of atmospheric and microaerobic cultures were almost equal. These results suggest that the *nar* genes in this organism are not regulated by either oxygen or nitrate levels, as in the case of the *narGHJI* operon of taxonomically related *M. tuberculosis* (Sohaskey and Wayne 2003). Minutely speaking, cell extracts of cultures with nitrate showed rather lower specific activities than extracts from nitrate-free cultures, especially in cell extracts from 0.5% O_2 cultures, but the reason for those minor differences in enzyme activities remains unclear. This could be due simply to increased damage of cells by accumulation of toxic nitrite.

Under these conditions, strains ΔnarG and ΔnarJ showed no nitrate reductase activities regardless of the culture conditions tested, while the wild-type levels of activities were detected in the cell-free extract of strain ΔnarK2 deficient in the putative nitrate:nitrite antiporter. These results indicate that nitrate reductase activity and also the phenotype of nitrate-dependent anaerobic growth in this organism are due to the *narK2GHJI* locus.

Nitrite production during aerobic shaken cultures

To examine how *C. glutamicum* ATCC 13032 behaves when oxygen and nitrate coexist, nitrite production was monitored during aerobic shaken cultures in MM medium supplemented with 30 mM nitrate (Fig. 4). Oxygen indicator resazurin was added to the medium to provide a visual indication of the presence of oxygen. Resazurin changes color from blue to pink in response to gradual oxygen depletion, and to colorless under strict anaerobic conditions (Guerin et al. 2001). Judged from the color of the culture medium (Fig. 4), the environment in our aerobic shaken cultures shifted from aerobic to anaerobic conditions during the exponential growth phase because respiring cells depleted oxygen faster than it could dissolve. Under the conditions, nitrite formation did not occur till

resazurin turned colorless, indicating that *C. glutamicum* cells dose not use nitrate as a final electron acceptor as long as oxygen is available.

Amino acid production under anaerobic conditions with nitrate

The occurrence of nitrate-dependent growth under anaerobic conditions might allow anaerobic production of amino acids by *C. glutamicum*. This was worth attempting because there was no report for anaerobic production of amino acids by this organism. To examine this possibility, we applied the agar piece method (Ichikawa et al. 1971) developed for antibiotic production by *Streptomyces*. In our modified agar piece assays, amino acid producers of *C. glutamicum* were cultivated on MM agar peaces supplemented with nitrate under strict anaerobic conditions, followed by bioassay of amino acids with the use of the corresponding amino acid auxotrophs. Two defined amino acid producers were used for this experiment: one was *C. glutamicum* AK-1, a producer of the aspartate family of amino acid L-lysine, and the other was *C. glutamicum* SU150, a producer of the glutamate family of amino acid L-arginine. As in the case of wild-type ATCC 13032, both strains, AK-1 and SU150, grew anaerobically on MM agar peaces supplemented with nitrate and showed nitrate-dependent production of L-lysine (Fig. 5A) and L-arginine (Fig. 5B), respectively, under strict anaerobic conditions. Based on the correlations between halo sizes and amino acid titers, the anaerobic production of L-lysine and L-arginine were calculated to be about 0.8 g/l and 0.4 g/l, respectively, while under ordinary atmospheric conditions L-lysine and L-arginine were both produced by about 2.0 g/l.

Discussion

We have demonstrated in this study that *C. glutamicum*, generally regarded as an aerobe, is able to grow under strict anaerobic conditions in the presence of nitrate. Since the nitrate-dependent anaerobic growth was accompanied by nitrite production as a result of nitrate reduction, we concluded that this organism can grow anaerobically by nitrate respiration, albeit showly and to a low yield. This organism has a *narK2GHJI* locus. In this study, internal deletion of *narG* and *narJ* abolished the nitrate-dependent anaerobic growth, concomitantly with the loss of nitrate reductase activity. On the other hand, inactivation of *narK2* encoding the putative nitrate:nitrite antiporter resulted in reduced ability of

anaerobic growth without the loss of nitrate reductase activity. An explanation for this growth phenotype of strain ΔnarK2 can be that non-specific influx of nitrate into the cells might occur to some extent even without the putative specific transporter. These results are all consistent with the expected phenotypes inferred from the putative roles of those genes, thus showing that the *narK2GHJI* locus is responsible for anaerobic nitrate respiration in this organism.

In *E. coli*, there are two other nitrate reductase systems, in addition to the *narGHJI* operon. A second nitrate reductase encoded by *narZYWV* operon, which is structurally similar to the NarGHI enzyme, is constitutively expressed at low levels (González et al. 2006). A third nitrate reductase encoded by *napFDAGHBC* operon is expressed during anaerobic growth in the presence of low concentrations of nitrate (Wang et al. 1999). On the other hand, *M. tuberculosis* has two sets of genes, *narGHJI* and *narK2X* (Sohaskey and Wayne 2003). The *narGHJI* operon has been shown to be constitutively expressed and responsible for nitrate reducing activity, while the *narK2X* operon is induced under hypoxic conditions (Sohaskey and Wayne 2003). The *narX* gene encodes fused nitrate reductase with homology to parts of the NarG, NarJ and NarI proteins, but the gene product has been shown to have no detectable contribution to the respiration (Sohaskey and Wayne 2003). In the case of *C. glutamicum*, only the *narK2GHJI* locus could be found, and no other related genes have been identified. The putative nitrate:nitrite antiporter gene *narK2* constitutes the cluster with the *narGHJI* genes, which is a different point from other bacteria referred above. These findings suggest that the nitrate respiration system of *C. glutamicum* may be relatively simple, compared with those of other bacteria, and possibly due to so, this organism might have no outstanding ability of adaptation to oxygen limitation.

Interestingly, the nitrate reductase activity from cell-free extracts of *C. glutamicum* was independent of oxygen and nitrate levels. Nevertheless, the result is puzzling because nitrite production did not occur as long as oxygen was available in our aerobic shaken cultures (Fig. 4). Bacteria generally sense environmental changes such as the availability of oxygen and alternative electron acceptors and then respond by switching their regulatory mechanisms to ensure that the most energetically favorable route is active under a given environmental condition. Considering this common knowledge, our observation that oxygen respiration had priority over nitrate respiration (Fig. 4) is reasonable because the nitrate respiration gives rise to less ATP than oxygen respiration (Unden and Bongaerts 1997). Potentially, in the presence of oxygen, electrons could preferentially flow to the pathway to

oxygen probably due to higher electron-accepting affinity of cytochrome oxidases than nitrate reductase enzymes, thus preventing a less energy-efficient system of nitrate respiration. However, the actual mechanism(s) how oxygen is preferred to nitrate in this organism is unclear at present.

It is noteworthy that not only L-lysine but also L-arginine were produced under anaerobic conditions in our agar piece assays (Fig. 5AB). Biosynthesis of L-lysine does not necessarily require the TCA cycle flux because the amino acid is synthesized from glucose via pyruvate, oxaloacetate, and then L-aspartate. However, L-arginine is synthesized from the TCA cycle intermediate α-ketoglutarate, thus directly depending on the TCA cycle flux for its oversynthesis. Considering these, it is reasonable to consider that the TCA cycle is more or less operative during anaerobic nitrate respiration. This raises, in principal, the possibility of anaerobic production of any amino acids which can be aerobically produced by this organism, regardless of their biosynthetic pathways. In our agar piece assays, halo sizes of both L-lysine and L-arginine production were obviously small under anaerobic conditions with nitrate, compared with those under atmospheric conditions (Fig. 5AB). This is certainly due to the limited ability of *C. glutamicum* to grow anaerobically by nitrate respiration, suggesting the necessity for further improvement of anaerobic growth by nitrate respiration. In this organism, nitrate respiration brings about the production of toxic nitrite, which could be one of the reasons for the limited growth by nitrate respiration. In this connection, some other bacteria like *E. coli* and *B. subtilis* metabolize nitrate to nitrite and further to ammonia, thus can utilize nitrate as the nitrogen source (Cole 1996; Nakano et al. 1997; Simon 2002). If such the nitrite ammonification pathway could function in *C. glutamicum*, efficiency of anaerobic growth and production of amino acids by this organism might be improved.

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Fig. 1 Effect of nitrate on anaerobic growth (●) and nitrite production (*bars*) in *C. glutamicum* ATCC 13032. Cells were incubated in liquid MM medium in the presence (A) and absence (B) of 30 mM nitrate under strict anaerobic conditions. The cultures in the absence of nitrate (B) generated no detectable nitrite and thus no bar is shown. Data represent mean values from at least two independent cultures

Fig. 2 Schematic representation of *narJ*, *narG*, and *narK2* disruption in *C. glutamicum* ATCC 13032. Plasmids pCnarJd, pCnarGd, and pCnarK2d contain the corresponding sequences indicated by *thick arrows*, and the sequences contain the deleted *narJ*, *narG*, and *narK2* genes, respectively (*shadowed regions*). *Thin arrows* below the gene names indicate directions of transcription

Fig. 3 Growth of strain ΔnarK2 (◊), strain ΔnarG (▲), and strain ΔnarJ (■) in liquid MM medium supplemented with 30 mM nitrate under strict anaerobic conditions. For comparison, the profile of the wild-type strain ATCC 13032 (●) is shown as a control. Data represent mean values from three independent cultures

Fig. 4 Nitrite production by *C. glutamicum* ATCC 13032 during aerobic shaken cultures in MM medium supplemented with 30 mM nitrate. The amount of nitrite in the medium (*bars*) was monitored with cell growth (●). The photographs above show the colors of oxygen indicator resazurin in the cultures. Resazurin changes color from blue to pink in response to gradual oxygen depletion, and to colorless under strict anaerobic conditions. Data represent mean values from at least two independent cultures

Fig. 5 Production of L-lysine (A) and L-arginine (B) by nitrate respiration under strict anaerobic conditions. The L-lysine- and L-arginine-producing strains, *C. glutamicum* AK-1 and SU150, respectively, were tested for production ability on MM agar pieces with and without 30 mM nitrate under both atmospheric and anaerobic conditions. After cultivation, the agar pieces were sterilized by UV irradiation and then transferred onto bioassay plates containing L-lysine or L-arginine auxotrophs as indicator strains. The plates were incubated overnight at 30ºC

Table 1 Oligonucleotide primers. The *Bgl*II recognition sites are *underlined*

Primer	Sequence (5'→3')
narK2up800F	CAAAGATCTAACCGAAAAGCGGGCA
narK2FusR	AAAGCGGTGGCGATGATGAAGAATTTCGAGTCCCAATGTTCAGGATCTT
narK2FusF	AAGATCCTGAACATTGGGACTCGAAATTCTTCATCATCGCCACCGCTTT
narK2down800R	TCGAGATCTTCCGGAACGTCAGT
narGup800F	ACCAGATCTGGCACCACGGTGTTT
narGFusR	AATTCTGGTGACCTCATCGCGGTTAGACTTCCCAGAAGAAGTAGTTGTAGT
narGFusF	ACTACAACTACTTCTTCTGGGAAGTCTAACCGCGATGAGGTCACCAGAATT
narGdown800R	GATAGATCTGCGTACACTTTTCGG
narJup800F	TTTAGATCTACGACGCCGACCGT
narJFusR	CGGATGAGCTCCATGTAGCTGTTAGGCACAAAGTGATCCGGAATTTTG
narJFusF	CAAAATTCCGGATCACTTTGTGCCTAACAGCTACATGGAGCTCATCCG
narJdown800R	GTGAGATCTCGAGTCATTGTCGAG

Table 2 Growth on agar plates under various oxygen concentrations. Culture was carried out on BY agar plates at 30ºC for three days. Only for *G. suboxidans* IFO 12528, YPGD agar plates were used. Colonies on agar plates visible after 24 h (++), 48 h (+), and 72 h (+/–); –, no growth observed

	Oxygen concentrations (%)				
Strain	21	6	3	0.5	0
Corynebacterium glutamicum					
ATCC 13032	++	++	++	+	–
ATCC 14067	++	++	++	+	–
ATCC 13869	++	++	++	+	–
ATCC 13870	++	++	++	+	–
Lactobacillus johnsonii NCC 533	+	+	+	+	++
Escherichia coli W3110	++	++	++	++	+
Sinorhizobium meliloti RU11/001	++	+/–	–	–	–
Gluconobacter suboxidans IFO 12528	++	+	+	+/–	–

Table 3 Nitrate reductase activity in cell-free extracts. Results represent the mean values (nmol of NO_2^-/min/mg of protein) ± standard deviations. Cells were grown under atmospheric conditions and under 0.5% O_2 conditions for 24 h and 48 h, respectively. *ND* Not detected

Strain	Nitrate reductase activity			
	Air		0.5% O_2	
	−Nitrate	+Nitrate	−Nitrate	+Nitrate
Wild type	20.2 ± 5.2	18.7 ± 3.9	24.9 ± 4.5	11.3 ± 3.2
ΔnarK2	24.5 ± 4.3	14.1 ± 1.0	24.9 ± 1.5	22.1 ± 2.7
ΔnarG	ND	ND	ND	ND
ΔnarJ	ND	ND	ND	ND

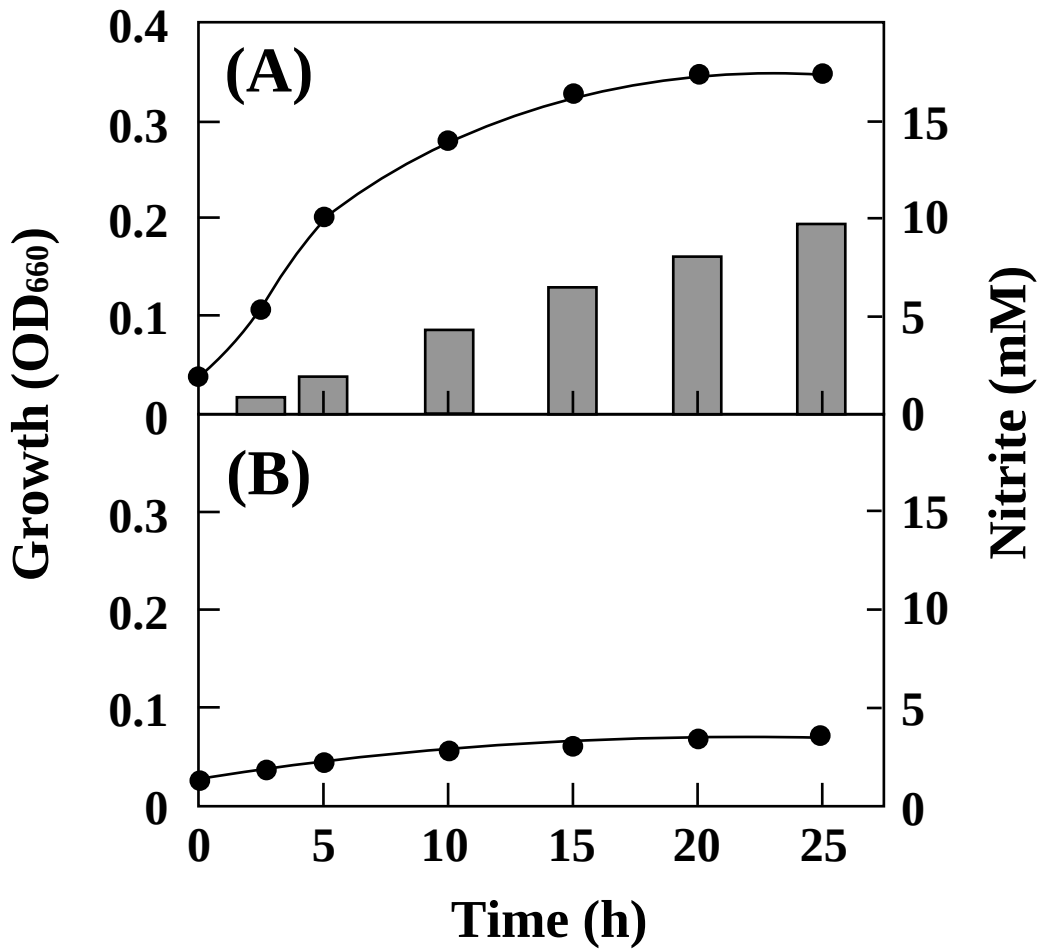
(A)
(B)
Growth (OD660)
Nitrite (mM)
Time (h)
0.4
0.3
0.2
0.1
0
15
10
5
0
0
5
10
15
20
25

Fig. 1

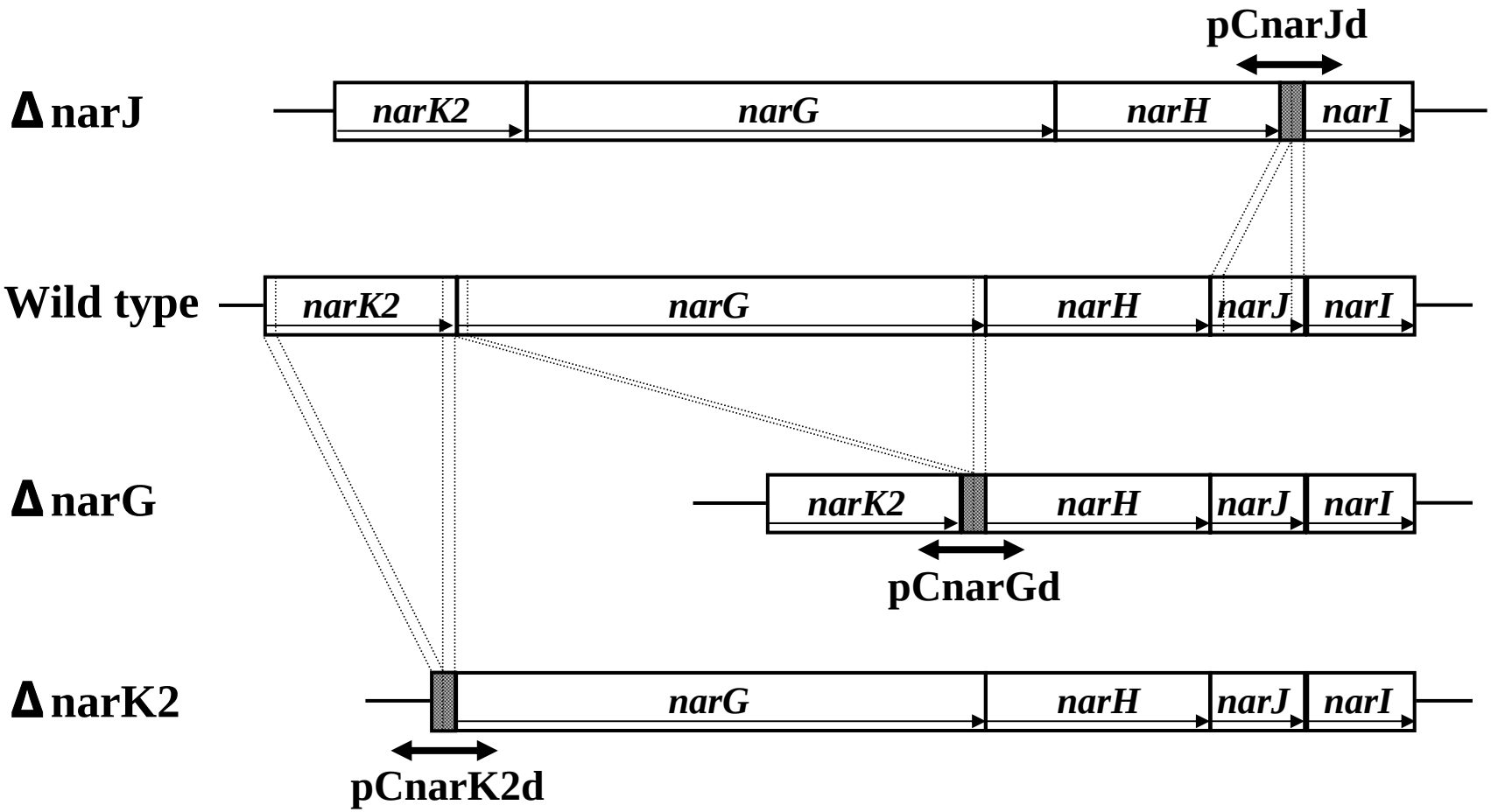
pCnarJd
ΔnarJ
narK2
narG
narH
narI
Wild type
narK2
narG
narH
narJ
narI
ΔnarG
narK2
narH
narJ
narI
pCnarGd
ΔnarK2
narG
narH
narJ
narI
pCnarK2d

Fig. 2

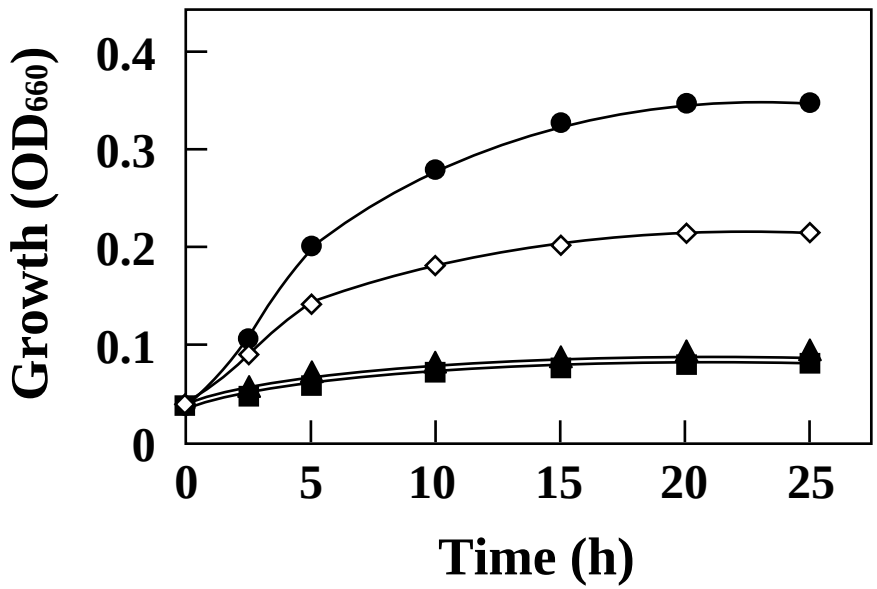

Fig. 3

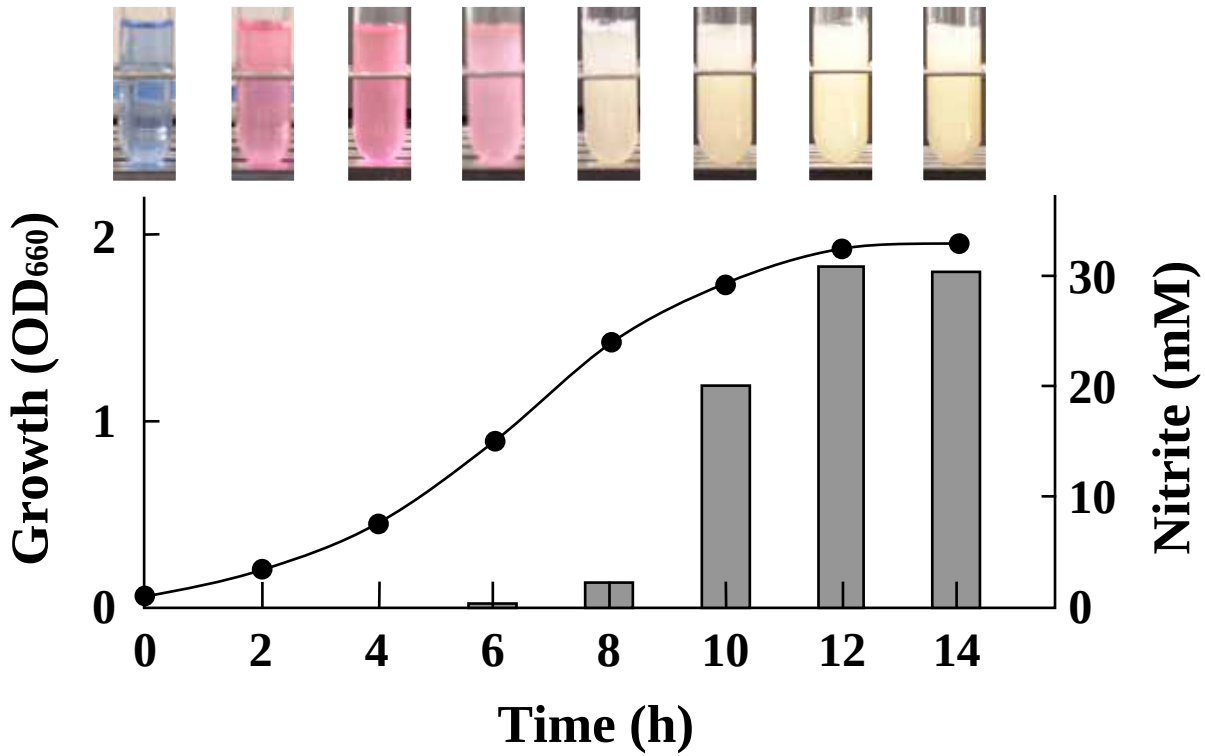

Fig. 4

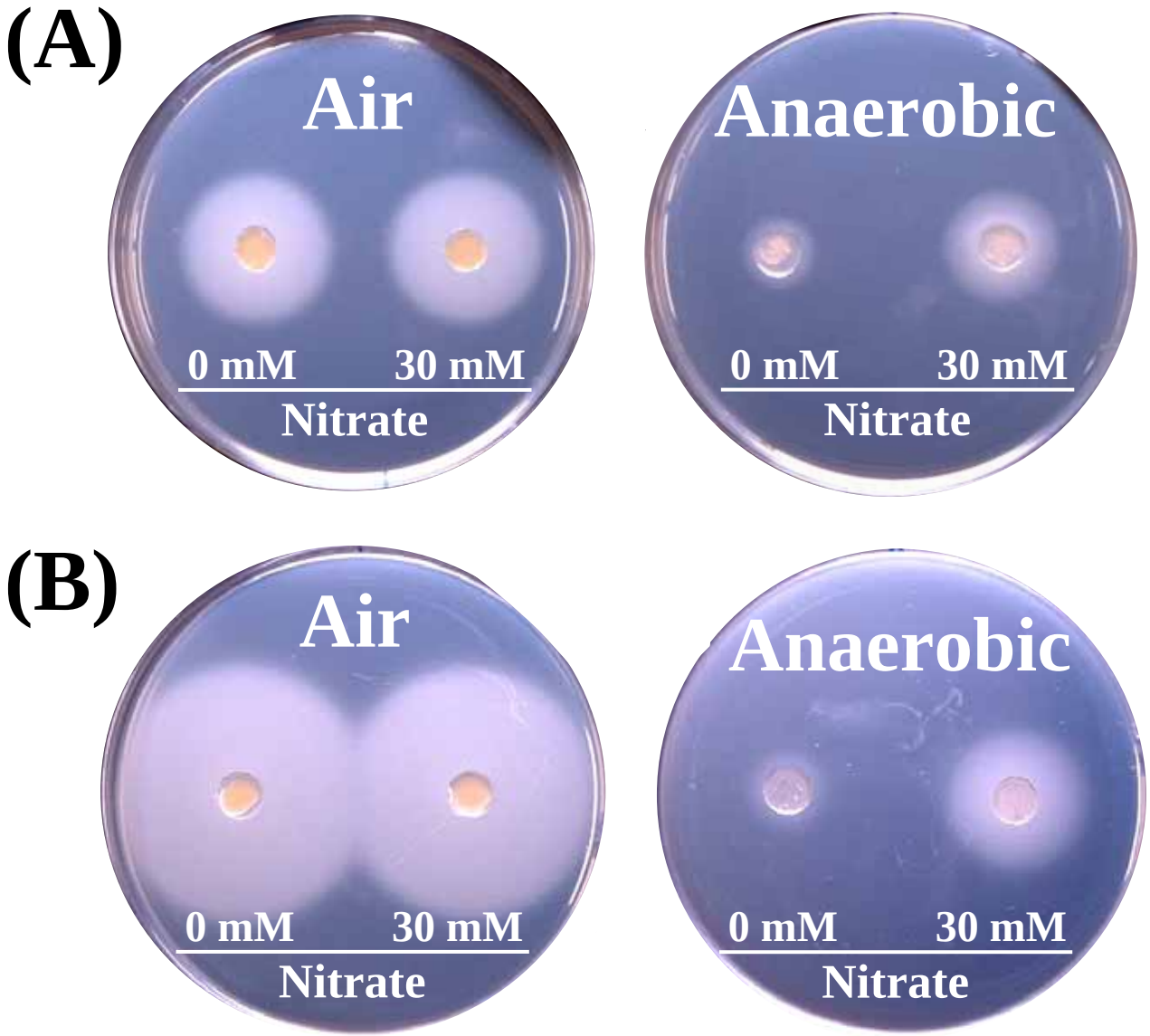
(A)
Air
0 mM
30 mM
Nitrate
Anaerobic
0 mM
30 mM
Nitrate
(B)
Air
0 mM
30 mM
Nitrate
Anaerobic
0 mM
30 mM
Nitrate

Fig. 5