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OXYGEN RESISTANT STRAIN OF N_2-FIXING *ESCHERICHIA COLI*

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Oxygen resistant N_2-fixing *Escherichia coli*, which can grow at a high oxygen concentration in a nitrogen-free medium, were produced by several times of cultivation under a condition of 0.03% oxygen. Six isolated resistant strains could grow at an oxygen concentration ten times that of the parent strain. The *nif*-genes of these six strains were integrated into a chromosome. At low oxygen, they showed one third the nitrogenase activity to the parent strain, but more so at a high oxygen concentration. It is thus evident that the amount of nitrogenase protein in a cell is a factor determining the oxygen resistance of nitrogenase.

Biological N_2-fixation, the reduction of dinitrogen to ammonia, is a prokaryotic function catalyzed by the oxygen labile enzyme nitrogenase. In *Klebsiella pneumonia*, functions required for the expression of nitrogenase are encoded within the *nif* (nitrogen fixation) gene cluster(1). This gene cluster was cloned to the plasmid pRD1 by Dixon et al(2) using *in vivo* genetic engineering techniques. Pühler et al(3) and Klipp(4) reported the cloning of the entire *nif*-genes of the plasmid pRD1 on a multicopy plasmid vehicle(pWK120 or pWK220) in *Escherichia coli*. Elmerich et al. also constructed the plasmid pCE1 carrying the entire *nif*-genes(5). Using the plasmids pWK220 and pCE1, much significant information regarding matters such as construction(4), regulation(4), and sequence(6) has been obtained. *E. coli*, carrying the above plasmid, can grow under anaerobic or microaerobic conditions in a nitrogen-free medium. Little information is available, however, on the oxygen resistance in nitrogen-free medium other than reports on the regulation of nitrogenase under microaerobic conditions(7).

Abbreviations: SM, streptomycin; CM, chloramphenicol.

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The present study was conducted to examine oxygen resistant N_2-fixing *E. coli* originally carrying the plasmid pWK220. Oxygen resistant strains were isolated from cultured broth previously cultured several times at a 0.03% oxygen concentration. In these strains, *nif*-genes were integrated into a chromosome, pWK220 was lost, and nitrogenase content decreased.

Materials and Methods

Bacterial Strains, Phages and plasmids. These are listed in Table 1. *E. coli* HIF0001 was a streptomycin(SM) resistant derivative of *E. coli* W3110(8), obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis described previously(9). HIF1000 was a transformant of HIF0001 with the plasmid pWK220. T4 and T7 phage lysate preparation and phage check were conducted according to Nashimoto and Fujisawa(10).

Medium. The nitrogen-free medium was prepared according to Cannon et al. (11), and to which 0.01 % resazurin and 25 μg/ml chloramphenicol(CM) were added. To remove oxygen from the medium, platinum asbestos in a visking tube was added, followed by stirring for 2-3 days in an anaerobic chamber(10% H_2 and 90% N_2). Anaerobic conditions were monitored by the disappearance of pink color due to resazurin.

Growth characterization. The pre-culture was shaken at 30° C overnight using Luria broth(12). To starve the nitrogen source, 0.3ml of pre-culture was added to 10ml of the nitrogen-free medium and the system was shaken for 12h in the air. The starved culture(0.6ml) was inoculated to the nitrogen-free medium(6.0ml) in a high pressure tube(38ml). A slight pink color appeared by inoculation, but with 1-2h incubation, it disappeared by respiration. When pink could no longer be seen, various amount of oxygen were injected at 24h intervals. The oxygen concentration specified in the results and discussion was the initial concentration at 24h intervals. Growth was monitored as absorbance at 660nm.

DNA techniques. Plasmid content was checked by using crude lysate and separating the DNA by agarose gel electrophoresis, as described by Kado and Liu(13). Southern transfer and hybridization were carried out as follows. Bands of plasmid and chromosomal DNA, visualized in agarose gels after electrophoresis, were transferred onto nitrocellulose filters and then hybridized(68° C) with digoxigenin-labelled *nifKDH*(EcoRI-HindIII fragment from pHI001) as a probe for homologies(14). The digoxigenin labelled method was carried out exactly according to the manual of Boehringer Mannheim.

Nitrogenase activity. Nitrogenase activity was monitored as the reduction of acetylene to ethylene. Nitrogenase reaction was made to occur as follows. 2ml of pre-culture were added to 300ml of nitrogen-free medium and incubated at 30° C for 12h in the anaerobic chamber, causing nitrogenase to be induced and oxygen removed. After incubation, 6ml of cultured broth, 0.06ml of 5.0mg/ml tetracycline and 0.24ml of 50% glucose were introduced in to a high pressure tube along 3.2ml of acetylene and various amounts of oxygen. The oxygen concentration in this system was the initial concentration. Tetracycline was added as an inhibitor of cell growth and protein synthesis. After shaking at 30 ° C for 12h, ethylene formation was measured by gas chromatogroph using a porapak R column(2mm x 3m) at 50° C, according to Takeda(15). Protein content of the entire cell was measured by the method of Lowry(16), using bovine serum albumin the standard.

Results and Discussion

In assessing the oxygen sensitivity of E. coli carrying pWK220 in the nitrogen-free medium, the growth rate and final cell density were found to progressively increase with continued cultivation at a 0.03% initial oxygen concentration at 24h intervals(Fig.1A). In the first cultivation, the growth rate of HIF1000 was quite slow. However, after several cultivations (0.6ml of cultured broth was inoculated to the next 6ml of fresh medium), the growth rate and final cell density were becoming greater(Fig.1A). HIF1000 was slightly inhibited by oxygen at the 0.03% initial oxygen concentration(Fig.2A), and consequently, the above results indicate possibly the accumulation of the oxygen resistant strain. This accumulation is reported here for the first time and an attempt was made to isolate the oxygen resistant strains and characterize them.

From the independent cultured broth, we selected 6 strains as high growth rate strains, and designated them as HIF2001-2006 (Table1). The oxygen resistant strains may possibly not have been derived from HIF1000 but from some other N_2-fixing bacteria, and we tried to confirmed the derivation form HIF1000 by phage sensitivity and antibiotic resistance. All 6 strains were T4 and T7 phage sensitive, and SM(100 μg/ml) and CM(25 μg/ml, originally due to pWK220) resistance. All 6 strains are thus *E. coli* and the derivative of HIF1000. The growth curves of HIF2001-2006 at 0.3% initial oxygen concentration are shown in

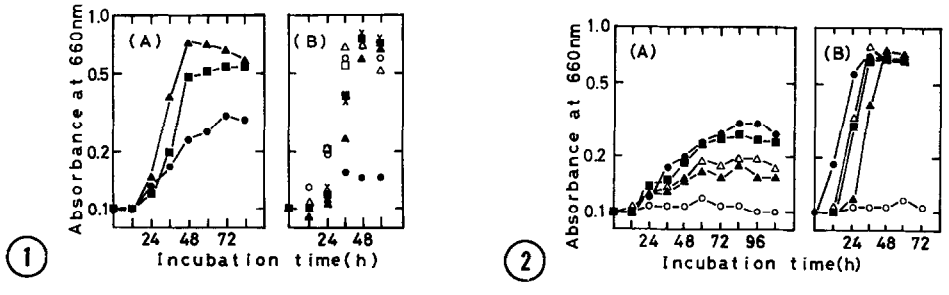


Fig.1 Growth curves of parent strain and oxygen resistant strains.
(A),Accumulation of oxygen resistant strain at the 0.03% initial oxygen concentration. The first(●), the 5th(■), and the 7th(▲) cultivation.
(B),Growth curves of oxygen resistant strains at the 0.3% initial oxygen concentration. HIF1000(●), HIF2001(■), HIF2002(▲), HIF2003(○), HIF2004(△), HIF2005(□), and HIF2006(×).

Fig.2 Comparison of oxygen resistance between (A),parent strain(HIF1000) and (B),oxygen resistant strain(HIF2001).
0.01%(●), 0.03%(■), 0.1%(△), 0.3%(▲), and 1.0%(○) of initial oxygen.

Table 1. Bacterial strains, phages, and plasmids

	Marker	Source or references
Escherichia coli		
W3110		(8)
HIF0001	Sm^r	This work
HIF1000	Sm^r Cm^r *Nif*$^+$	This work
HIF2001-HIF2006	Sm^r Cm^r *Nif*$^+$	This work
Plasmid		
pWK220	Cm^r *Nif*$^+$	(3)(4)
pHI001	Ap^r *nifKDH*	This work
Bacteriophage		
T4 phage		(10)
T7 phage		(10)

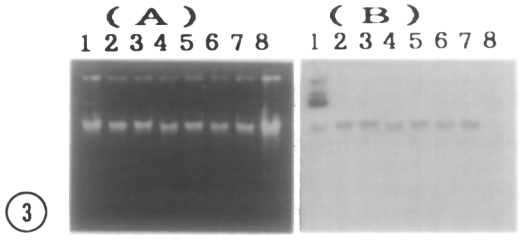
Fig.1B. HIF1000 showed little growth while considerable growth was noted for HIF2001-2006. The growth characterization of these 6 strains was virtually the same except lag time(Fig.1B).

The oxygen sensitivities of HIF1000 and of HIF2001 were compared at various oxygen concentrations(Fig.2). HIF2001 was about ten times more oxygen resistance than HIF1000. Fig.2. also shows HIF2001 to be not only oxygen resistant but also to have a higher growth yield.

HIF2001-2006 may be oxygen resistance as a result of some mutation with pWK220. The plasmid pWK220 of 6 strains were thus checked by agarose gel-electrophoresis(Fig.3A). Lane 1(HIF1000) showed two DNA bands, one chromosomal and the other pWK220. But Lane2-7 (HIF2001-2006) only showed a chromosomal band. We could not detect pWK220 from HIF2001-2006. These 6 strains may lose pWK220 but retain the ability to fix nitrogen. We looked for the genes encoding N_2-fixation by Southern blotting using *nifKDH* as a probe. Fig.3 shows the results of Southern blotting. Lanes2-7 (HIF2001-2006) showed high affinity of the probe to the chromosomal band. Southern blotting suggested the *nif*-genes to be integrated to the chromosome, pWK220 to be lost, and all 6 strains to consequently be oxygen resistant. Lane 1(HIF1000) showed affinity to the chromosomal and pWK220 bands. This affinity may be due to integration of pWK220 to chromosome and/or aggregation between chromosome and broken pWK220. The plasmid pWK220 had a high molecular weight(about 50kb)(4) and could be easily broken by the DNA extraction. No clarification was made of the mechanisms of the integration of pWK220 into the chromosome. However, there are several reports on the integration of N_2-fixing genes into

chromosomes. Cannon et al.(11) showed the spontenious integration and Klingmuller et al.(17,18), the selection of an integrated strain, using cholate resistance or Mu phage. They consider nitrogen-fixing genes to be integrated by homology of his-region or homology of the genes responsible for nitrate reductase production or functions. HIF2001-2006 may also be integrated in the same manner. The parent strain W3110 may reasonably be considered to be *recA+*.

By the integration of *nif*-genes into the chromosome and loss of pWK220, the host strain can grow at higher oxygen concentration, but physiologically, it is not clear why these strains became oxygen resistant. For clarification of this point, nitrogenase activity (acetylene reducing activity) was measured at several oxygen concentrations using whole cells of HIF1000 and HIF2001. Acetylene reducing activity was not quantitatively reflect N_2-fixing activity, but was qualitatively enough to monitor N_2-fixing activity. By the addition of tetracycline, the cells would not grow nor would nitrogenase be further induced. At low oxygen concentration, the parent strain HIF1000 showed about three times more nitrogenase activity than HIF2001(Fig.4). However, at higher oxygen condition, HIF2001 showed about two times(3% of initial oxygen concentration) and about four times(10%) more nitrogenase activity than HIF1000. It would thus appear that the amount of the nitrogenase protein in a cell influences to the oxygen sensitivity of nitrogenase activity *in vivo*. The difference between the amounts of nitrogenase in HIF1000 and HIF2001 was



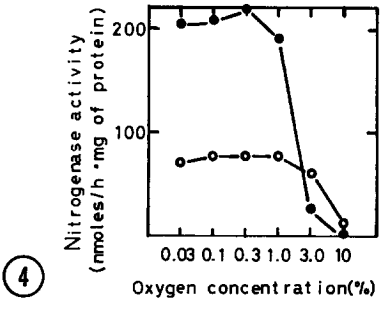


Fig.3 Agarose gel-electrophoresis of crude lysate(A) and Southern hybridization(B).
Lane 1, HIF1000;lane 2, HIF2001; lane 3, HIF2002; lane 4, HIF2003;lane 5, HIF2004; lane 6, HIF2005; lane 7, HIF2006;lane 8, HIF0001.

Fig.4 Comparison of nitrogenase activity between HIF1000(●) and HIF2001(○) at various initial oxygen concentration.

reasonable since pWK220 in HIF1000 was a high copy number plasmid and *nif* genes in HIF2001 were integrated in chromosome. A possible explanation for the above influence is as follows; By a decrease in nitrogenase protein, the efficiency of reducing power from pyruvate or of ATP to nitrogenase(19) increases. This efficiency may be important to oxygen sensitivity. The growth curves of HIF1000 and HIF2001 at the same oxygen concentration, show the higher growth yield of HIF2001(Fig.2.). This would indicate that a energy efficiency of HIF2001 is higher than that of HIF1000. Oelze et al.(20,21), using continuous culture of *Azotobacter vinelandii*, showed that the high respiration activity of *A. vinelandii* was not for decreasing oxygen in a cell, but for supplying high reducing power for nitrogenase to protect nitrogenase from oxygen. Their results agree with the above possibility.

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