Mutagenized cell libraries of Corynebacterium glutamicum were screened for mutants that lost the ability to grow under low oxygen concentrations. The resulting high-oxygen-requiring mutants were used to clone wild-type DNA fragments that could complement the phenotype. Sequencing and subcloning analyses identified six genes, Cgl0807, Cgl1102, Cgl0600, Cgl1427, Cgl2857, and Cgl2859, as the genes responsible for complementation. Some of these genes showed cross-complementation of the mutants in oxygen-limiting static culture, suggesting the utility of these genes for improved growth and production under oxygen limitation.

Key words: Corynebacterium glutamicum; adaptation to oxygen limitation; amino acid production

For most aerobic industrial microorganisms, oxygen is a very important factor for growth and production. Especially, Corynebacterium glutamicum strains that produce amino acids require a large quantity of oxygen for efficient production. Under oxygen limitation, production strains usually accumulate undesirable organic acids, which results in damaged fermentation with decreased production yields. For this reason, considerable effort and expense have been devoted to the maintenance of a high oxygen supply. If we can develop so-called low-O₂ adapted strains that produce amino acids effectively even under oxygen limitation, a significant benefit will probably arise industrially, but there is no such technology at present. We have compared oxygen-requiring properties among typical aerobic bacteria, and we found that C. glutamicum grows under relatively low concentrations of oxygen.

On the basis of this finding, it is likely that C. glutamicum possesses some function for adaptation to limited oxygen availability. In this study, we attempted to screen for the relevant genes from the C. glutamicum genome by genetic complementation of mutants that lost the ability to grow under low oxygen concentrations.

Low oxygen conditions for growth on agar plates were established in a sealed 2.5-liter box using simple culture systems, Anaero Pack (Mitsubishi Gas Chemical, Tokyo), as described previously. Our preliminary studies revealed that the lower limit of oxygen concentration under which C. glutamicum can form colonies on BY agar plates was about 0.5% O₂. C. glutamicum wild-type strain WT-96, a single-colony derivative of C. glutamicum ATCC 31833, was mutagenized by N-methyl-N-nitro-N-nitrosoguanidine according to the standard protocol, and then we screened for mutants that lost the ability to grow at 0.5% O₂ by replica plating. These mutants formed colonies under atmospheric conditions (21% O₂), but most of them showed no growth at 0.5% O₂ or even at 6% O₂. Figure 1 showed the growth of five typical mutants, designated OX-3, OX-96, OX-109, OX-112, and OX-119, under different oxygen concentrations.

Using these five OX mutants as recipients, we attempted to clone wild-type DNA fragments that would complement the phenotype. For this purpose, chromosomal DNA from wild-type strain WT-96 was digested with BamHI, SalI, or EcoRI and ligated to BamHI- or SalI-digested pCS299 (or EcoRI-digested pCSEK20). The genomic library was used to transform the OX mutants. By this shotgun cloning method, we obtained seven plasmids that enabled those mutants to grow under 0.5% or 6% O₂. These plasmids included pEco1.9 carrying a 1.9-kb EcoRI DNA fragment that complemented mutant OX-3, pBam2.1 carrying a 2.1-kb BamHI DNA fragment that complemented mutant OX-96, pEco3.2 carrying a 3.2-kb EcoRI DNA fragment that complemented mutant OX-109, pEco1.1 carrying a 1.1-kb EcoRI DNA fragment that complemented mutant OX-112, and pBam1.8, pBam3.2, and pSal2.5 carrying a 1.8-kb BamHI DNA fragment, a 3.2-kb BamHI DNA fragment, and a 2.5-kb SalI DNA fragment respectively, that complemented mutant OX-119. As shown in Fig. 1, it is obvious that each plasmid substantially restored the growth of its corresponding mutant under low oxygen conditions (6% and/or 0.5% O₂), indicating that the cloned DNA fragments are involved in the phenotype. We also conducted similar shotgun cloning using 10 other high-oxygen-requiring mutants as recipients, but the newly cloned DNA fragments were identical to one or the other of the DNA fragments discussed above.

Sequencing and genome database analyses of the insert DNAs in the seven plasmids revealed the presence of one intact gene, Cgl0807, in plasmid pEco1.9, two intact genes, Cgl1101 and Cgl1102, in plasmid pBam2.1, two intact genes, Cgl2859 and Cgl2861, in plasmid pEco3.2, one intact gene, Cgl0600, in plasmid pEco1.1, one intact gene, Cgl1427, in plasmid pBam1.8.
two intact genes, Cgl2857 and Cgl2858, in plasmid pBam3.2, and two intact genes, Cgl2857 and Cgl2859, in plasmid pSal2.5 (Table 1). As for the three plasmids, pBam2.1, pEco3.2, and pBam3.2, that carried two genes on each insert, further subcloning experiments determined that Cgl1102, Cgl2859, and Cgl2857 respectively were responsible for the complementation. Taken together, six genes in total, Cgl0807, Cgl1102, Cgl0600, Cgl1427, Cgl2857, and Cgl2859, were identified as the genes involved in the high-oxygen-requiring phenotypes (Table 1). None of these is known for a relation to adaptability to low oxygen conditions in C. glutamicum, but hypotheses can be proposed, as below.

Cgl0600 encodes a putative RNA polymerase sigma factor SigD, one of seven sigma factors identified in the C. glutamicum genome.9) This sigma factor belongs to the group of environmentally responsive transcriptional regulators, although it has not yet been studied with respect to its function.9) Thus the Cgl0600 gene product probably plays an important role in the transcription of the genes involved in adaptation to microaerobic conditions. Cgl0807 and Cgl1102 encode a putative siderophore-interacting protein and a putative ferredoxin respectively. Siderophores, small iron chelators, facilitate cellular iron transport to help the delivery of iron to heme proteins such as cytochromes, while ferredoxins, small proteins containing iron and sulfur atoms organized as iron-sulfur clusters, mediate electron transfer in a range of metabolic reactions, including respiration. Enhancement of these functions might improve cellular respiratory activity, thereby contributing to increased adaptability to microaerobic conditions. Cgl1427 encodes a putative cytidylate kinase catalyzing the phosphorylation of cytidine 5-monophosphate to cytidine 5-diphosphate in the presence of ATP. There appears to be no relation of this enzyme to microaerobic growth, but the increased activity of the enzyme might improve cellular ability in nucleic-acid biosynthesis under oxygen-limited stress conditions, enhancing adaptability to microaerobic conditions. Cgl2857 and Cgl2859 both encode putative membrane proteins. These gene products remain to be assigned even a tentative function at present. Since orthologs of the two genes could not be found in other microorganisms, except for C. glutamicum R and Corynebacterium efficiens, the function might be unique to so-called glutamic-acid bacteria.

C. glutamicum is aerobic, the cells would have to have sensory systems to gauge oxygen availability in...
their environments or mechanisms to facilitate oxygen availability to the respiratory components of the cells. The membrane proteins might be involved in such oxygen sensing or delivering.

The fact that two separate genes, Cgl1427 and Cgl2857, were obtained by functional complementation of a single mutant, OX-119 (Fig. 1, Table 1), suggests the cross-complementation ability of the genes. To verify this possibility for six genes, we introduced five plasmids into the five different mutants and examined their cross-complementation abilities in oxygen-limiting static culture with MM medium (Fig. 2). As for plasmids to introduce the four genes Cgl0807, Cgl1102, Cgl0600, and Cgl1427, and pSal2.5, we used pEco1.9, pBam2.1d, pEco1.1, and pBam1.8 respectively, all carrying single genes (Table 1). Plasmid pBam2.1d was constructed as follows: The region containing the Cgl1102 gene was amplified from plasmid pBam2.1 by PCR using primers pr1 (5’-GGATCCGCACTCAGTCG-3’) and pr2 (5’-TACTACGGATCCATCCTGGTACAG-3’), which introduced BamHI sites (underline), digested with BamHI, and then ligated to the BamHI-digested pCS299P to yield pBam2.1d. As for the other two genes, Cgl2857 and Cgl2859, located next to each other on the genome, we used plasmid pSal2.5 carrying the gene cluster (Table 1), because the two gene products, putative membrane proteins, would play a single role by forming a complex. As shown in Fig. 2, the three plasmids pEco1.1, pBam1.8, and pSal2.5 more or less restored the growth defects not only of the corresponding mutants but also of any other mutants, indicating cross-complementation abilities of the four genes (Cgl0807, Cgl1427, Cgl2857, and Cgl2859). Plasmid pBam1.8, containing the Cgl1427 gene, exhibited acceleratory effects on the growth of every mutant, except for OX-3. The other two plasmids, pEco1.9 and pBam2.1d, showed cross-complementation with respect to the two corresponding mutants, OX-3 and OX-96, but showed no substantial cross-complementation toward the other three mutants, indicating the limited cross-complementation abilities of the two genes (Cgl0807 and Cgl1102). Similar results were obtained on solid plates under different oxygen concentrations (data not shown).

Industrial strains that have traditionally been constructed by multiple rounds of mutagenesis generally have weak constitutions, showing sensitivities to various stresses, including limited oxygen availability, as compared to the ancestral wild-type strains. Although the genetic backgrounds of such traits remain undefined, the genes identified in this study are promising targets for cellular engineering of the industrial strains to improve their performance under oxygen limitation.

References