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Elucidation of Genes Relevant to Microaerobic Growth of *Corynebacterium glutamicum*

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Research aims

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 cost have been devoted to maintenance of high oxygen supply. If we can develop so-called 'low-O_2 adapted strains' that produce amino acids effectively even under oxygen limitation, a significant benefit would likely arise industrially. However, there is no such technology at present. We previously compared the oxygen-requiring properties among typical aerobic bacteria and found that *C. glutamicum* can grow up to relatively low concentrations of oxygen.[1)] 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.

Methods

The wild-type strain of *C. glutamicum* used in this study is strain WT-96, a single-colony derivative of *C. glutamicum* ATCC 31833. Complete medium BY and minimal medium MM were used for investigation of growth dependency on different oxygen concentrations in *C. glutamicum*. For the growth test on BY agar plates under different 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). For static culture in MM liquid medium, a 0.05-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 test tube and cultivated at 30ºC. Mutagenized cell libraries of strain WT-96 was prepared by incubating cells at 30ºC for 30 min in 50 mM Tris-maleate buffer (pH 6.0) containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (400 μg/ml). The genomic library of strain WT-96 was constructed by ligating its chromosomal DNA digested with BamHI, SalI, or EcoRI to BamHI- or SalI-digested pCS299P or EcoRI-digested pCSEK20.

Results

Screening for oxygen-high-requiring mutants. Our preliminary studies revealed that the lower limit of oxygen concentrations where *C. glutamicum* can form colonies on BY agar plates was around 0.5% O_2.[1)] Mutagenized cell libraries of *C. glutamicum* wild-type strain WT-96 were screened for mutants that lost the ability to grow at 0.5% O_2 by replica plating. These mutants could form colonies under atmospheric conditions (21% O_2), but most of them showed no growth at 0.5% O_2 and even at 6% O_2. 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.

Cloning of DNA fragments that complement oxygen-high-requiring phenotype. By the shotgun cloning method using those five mutants as recipients, we obtained seven plasmids that enabled those mutants to grow at 0.5% or 6% O_2. These plasmids include 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 growth of its corresponding mutant under low oxygen conditions (6% and/or 0.5% O_2), indicating that the cloned DNA fragments are involved in the phenotype. We also conducted similar shotgun cloning using other ten oxygen-high-requiring mutants as recipients. However, newly cloned DNA fragments were identical to either of the DNA fragments above.

Identification of the genes responsible for the complementation. Sequencing and genome database analyses[2)] 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

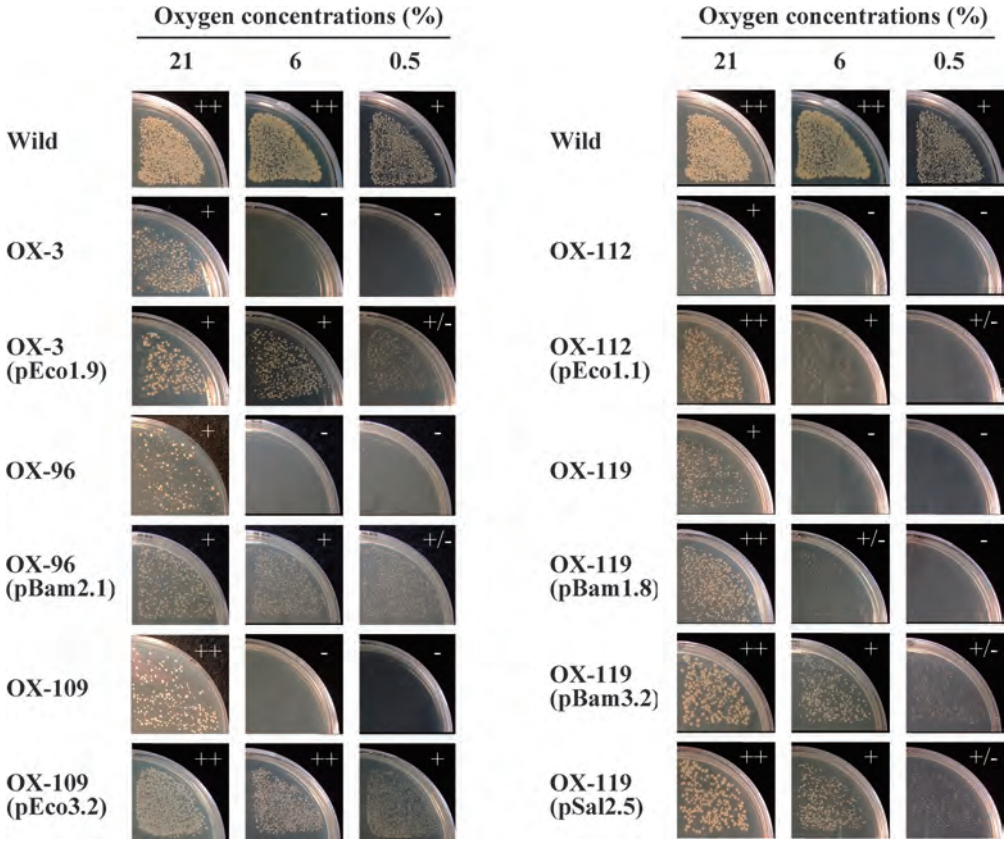

Fig. 1. Growth on agar plates under different oxygen concentrations. Appropriate dilutions (ca. 10^3/ml) of cultures of *C. glutamicum* wild-type strain WT-96, its oxygen-high-requiring mutants, and their transformants were spread onto BY agar plates and cultured at 30ºC for two days under indicated oxygen concentrations. Growth levels are signified as follows: ++, good growth; +, weak growth; +/−, marginal growth; -, no growth.

genes Cgl2857 and Cgl2859 in plasmid pSal2.5. As for the three plasmids, pBam2.1, pEco3.2, and pBam3.2, which carried two genes on each insert, further subcloning experiments identified that Cgl1102, Cgl2859, and Cgl2857, respectively, were responsible for the complementation. Taken together, six genes in total were identified as the genes involved in the oxygen-high-requiring phenotypes: (i) Cgl0600 encoding a putative RNA polymerase σ^{24} subunit, (ii) Cgl0807 encoding a putative siderophore-interacting protein involved in iron transport, (iii) Cgl1102 encoding a putative ferredoxin involved in electron transfer, (iv) Cgl1427 encoding a putative cytidylate kinase involved in nucleic-acid biosynthesis, and (v) Cgl2857 and (vi) Cgl2859 both encoding putative membrane proteins.[3] None of those are known for their relations to adaptability to low oxygen conditions in *C. glutamicum*. In addition, putative membrane proteins encoded by Cgl2857 and Cgl2859 remain to be assigned even a tentative function. Since orthologs of the two genes could not be found in other microorganisms except *C. glutamicum* R and *Corynebacterium efficiens*, the function might be unique to so-called 'glutamic-acid bacteria'.

Cross-complementation of different mutants with each gene. The fact that the two separate genes Cgl1427 and Cgl2857 were obtained by functional complementation of a single mutant OX-119 (Fig. 1) has suggested the cross-complementation ability of the gene(s). To verify this possibility for the six genes all, we introduced five plasmids into the five different mutants and examined their cross-complementation abilities in oxygen-limiting static culture with MM medium. As for plasmids to introduce the four genes Cgl0807, Cgl1102, Cgl0600, and Cgl1427, we used pEco1.9, pBam2.1d, pEco1.1, and pBam1.8, respectively, all carrying single genes alone. As for the other two genes Cgl2857 and Cgl2859 locating next to each other on the genome, we used plasmid pSal2.5 carrying the gene cluster, because the two gene products, putative membrane proteins, likely play a single role by forming a complex. The three plasmids pEco1.1, pBam1.8, and pSal2.5 could more or less restore the growth defects of not only the corresponding mutants but any other mutants, indicating cross-complementation abilities of the four genes (Cgl0600, Cgl1427, Cgl2857, and Cgl2859).[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 other three mutants, indicating the limited cross-complementation abilities of the two genes (Cgl0807 and Cgl1102).[3] The similar results were obtained on solid plates under different oxygen concentrations.

Conclusion

Mutagenized cell libraries of *Corynebacterium glutamicum* were screened for mutants that lost the ability to grow under low oxygen concentrations. The resulting oxygen-high-requiring mutants were used to clone wild-type DNA

fragments that could complement the phenotype. Sequencing and subcloning analyses showed that the following six genes were responsible for the complementation: (i) Cgl0600 encoding a putative RNA polymerase σ^{24} subunit, (ii) Cgl0807 encoding a putative siderophore-interacting protein involved in iron transport, (iii) Cgl1102 encoding a putative ferredoxin involved in electron transfer, (iv) Cgl1427 encoding a putative cytidylate kinase involved in nucleic-acid biosynthesis, and (v) Cgl2857 and (vi) Cgl2859 both encoding putative membrane proteins. Some of these genes showed cross-complementation of the mutants in oxygen-limiting static culture, suggesting applicability of those genes to improved growth and production under oxygen limitation.

References

1) Takeno, S., Ohnishi, J., Komatsu, T., Masaki, T., Sen, K., and Ikeda, M. Anaerobic growth and potential for amino acid production by nitrate respiration in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **75**, 1173–1182 (2007)

2) Ikeda, M., and Nakagawa, S. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological process. *Appl. Microbiol. Biotechnol.* **62**, 99–109 (2003)

3) Ikeda, M., Baba, M., Tsukamoto, N., Komatsu, T., Mitsuhashi, S., and Takeno, S. Elucidation of genes relevant to the microaerobic growth of *Corynebacterium glutamicum*. *Biosci. Biotechnol. Biochem.* **73**, 2806–2808 (2009)