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学位論文の内容の要旨

A well known microorganism, Gram-positive coryne-form bacterium, *Corynebacterium glutamicum*, has been industrially used for the production of the important amino acids, such as L-glutamate and L-lysine. Thus, this bacterium, nowadays, is one of the most popular topics in applied microbiology. In order to increase the production of amino acids in this bacterium, most of the published data mainly reported on the metabolic engineering for the enzymes of which the function is directly involved to the metabolic regulation for the production of a distinct amino acid. To date, little has been reported on its respiratory chain though the relationship between aerobic energy metabolism and amino acid production has been wildly well known to be important. The productivity of several amino acids by amino acid producing organisms has been reported to be depended on the respiratory proton pumps, without understanding of its respiratory chain thoroughly. Since the respiratory chain of *C. glutamicum* remains unclear, therefore, it is essential to understand a full detail of its respiratory chain, which is important to expand scientific view as well as providing the other clues for amino acid production improvement. Based on this aspect, therefore, the objective of this study is to reveal the components involved in *C. glutamicum* respiratory chain, such as NADH dehydrogenase.

The first part of this work is on the study of type II NADH gehydrogenase (NDH-2),

coded by *ndh* gene, of which the function is closely related to the respiratory pathway as being the main enzyme for transferring electron to menaquinone-pool in *C. glutamicum*. The study was performed by constructing NDH-2 inactivated and over-expressed strains derived from lysozyme sensitive strain, KY9714. When compared with KY9714, the strain with disrupted *ndh* showed no growth defect on glucose minimum medium, while over-expressed strain displayed the delay of growth rate. On the other hand, clear difference was observed on lactate minimum medium where the disruptant showed better growth than its parental strain, and the severe growth defect was observed in the over-expressed one. This is corresponded to the finding that the disrupted strain contains higher L-lactate dehydrogenase activity (L-LDH) than wild type, whereas, this enzyme including D-lactate dehydrogenase (D-LDH) of the over-expressed strain is much decreased. The strain with low L-LDH and D-LDH activities, would reduce an ability to catabolize lactate in such an environment, with only lactate being a carbon source. Therefore, bacterial cell could not produce much metabolites and energy leading to the serious defect on the growth. Other enzymes that are also affected by NDH-2 disruption or over-expression are cytoplasmic lactate dehygrogenase (LDH) and cytoplasmic malate dehydrogenase (MDH), in which the *ndh* disruptant shows relatively high activities of these enzymes, but the contrary in the over-expressed enzyme. It has been reported that MDH-MQO (malate:quinone oxidoreductase) are functioning together for re-oxidation of NADH in the strain lacking NDH-2 (Molenaar et al., 2000). This study indicated that not only MDH-MQO system but also LDH-L-LDH system function as NADH oxidation system. The activity was reproduced with L-lactate as a substrate in the presence of both membrane and cytoplasmic fractions isolated from disruptant strain. Whereas, in the over-expressed strain, the oxidation of NADH is mainly done by NDH-2.

To further understand the function of NDH-2, this enzyme was purified from the over-expressed strain and characterized as described in Chapter 2. UV-visible and fluorescence spectra showed that NDH-2 of *C. glutamicum* contained non-covalently bound FAD. The purified enzyme has an ability to oxidize NADPH apart from NADH, both of which are able to donate electron to oxygen and various artificial quinone analogs at acidic and neutral pH, respectively. However, the reduction of native quinone, menaquinone-2, was observed only with NADH, while the transfer of electron to oxygen was observed more intensively with NADPH. This study revealed that NDH-2 of *C. glutamicum* is a source of reactive oxygen species production of superoxide and hydrogen peroxide concomitant with the oxidation of NADH and NADPH most of which are mainly produced from the oxidation of NADPH. Such a unique character of NADPH oxidation that mostly found in eukaryotic

enzyme suggests that NDH-2 of *C. glutamicum* in more alike to eukaryotes than prokaryotes and thus might be evolved from the same ancestor. This theory has been supported by the construction of phylogenetic three where the results showed that *C. glutamicum* NDH-2 is more related to the group of yeast and fungi than bacteria.

The last section of this work is the characterization of *cydAB* gene, which seems to code a cytochrome *bd*-type qinol terminal oxidase. According to the previous reports, this enzyme also exists in some specific growth condition of *C. glutamicum*. In this study, we expected that it might be work as a CN-resistant bypass oxidase system, and thus we tried to construct the disruption of *cydB* gene. The disruptant strain showed only slight growth defect on either rich or glucose minimum media without causing any serious effects. NADH and TMPD oxidase activities were somewhat different between wild-type and disruptant strains. Low temperature-reduced minus oxidized difference spectra of the membranes purified from these strains displayed the same spectral feature of heme *a*, heme *b* and heme *c*, but with decreased of hemes *a* and *c* level in the *cydB* disruptant strain. However, the cyanide sensitivity of either NADH or TMPD oxidase activities was not much changed between wild-type and disruptant. Thus, these results suggested that *cydAB* genes of *C. glutamicum* might not encode a cytochrome *bd* oxidase or a cyanide-resistant bypass oxidase.

論文審査の結果の要旨

アミノ酸生産菌として有名な *Corynebacterium glutamicum* のアミノ酸生産性は培養における通気条件に強く依存しており、より高いアミノ酸生産性を得るためには、酸素と密接に関係するエネルギー生成能の解析が必要である。*C. glutamicum* の呼吸鎖電子伝達系には、エネルギー生成能の高いシトクロム酸化酵素系の呼吸鎖ルートに加え、エネルギー生成能の低いバイパス・オキシダーゼ系が機能している。このバイパス呼吸鎖によるエネルギー生成能の調節は、アミノ酸生産を含む本菌の物質代謝と密接に関係していると考えられ、生理学的にも実用上でも重要な意味を持つと考えられる。

本研究では、*C. glutamicum* のエネルギー代謝系の中心を成す電子伝達系の構造と機能を解明し、その知識を利用した電子伝達系の改変によるエネルギー代謝系の操作を目指している。そのため、特に本菌のエネルギー代謝系、特に中央代謝経路、と密接に結びついて機能しているNADH脱水素酵素およびシアン耐性バイパス・オキシダーゼの本体と考えられるシトクロムdに注目して研究を行った。

まづ、*C. glutamicum* の呼吸鎖で機能し、NADHの酸化にともなうエネルギー生成に関与

しているタイプII・NADH脱水素酵素（Ndh）のエネルギー代謝における役割を、その欠損株および過剰発現株をもちいることで解析した。Ndhの欠損は、その生育に影響を及ぼさなかったが、過剰発現株の生育は親株と比較して大幅に低下した。このNdhの欠損および過剰発現は、呼吸鎖シトクロム成分の組成やエネルギー生成能に大幅な影響を与えることはなかった。しかし、Ndhの欠損は、呼吸鎖のL-乳酸酸化活性の増加を導き、逆にNdhの過剰発現は、呼吸鎖のL-乳酸およびL-リンゴ酸酸化活性の減少を導いた。また、L-乳酸およびL-リンゴ酸に依存したNADH酸化活性をNdh欠損株の細胞膜および細胞質から再構成できることを明らかにした。このようにして、本菌においては、呼吸鎖で機能するNdhに加え、呼吸鎖L-乳酸酸化系と細胞質のNAD依存性乳酸脱水素酵素がカップルしてNADHの再酸化能を行うことができることを明らかにした。同時に、本研究で、Ndhの欠損、つまり乳酸酸化系の増加、は本菌のグルタミン酸生産を低下させ、逆にその過剰発現、つまり乳酸酸化系の減少、はグルタミン酸生産を増加させることを明らかにした。

次に、NADH 脱水素酵素（Ndh）をその過剰発現株から精製し,その性質について解析した。本酵素はその細胞膜から高い NADH:ubiquinone-1 酸化還元活性を示す酵素として精製され,その分光および蛍光分析から非共有結合型 FAD を補欠分子族とするフラビン酵素であることを示すとともに、NADH と NADPH を、それぞれ中性および微酸性領域で、種々の人工色素やキノン化合物を電子受容体として酸化できることを明らかにした。特に、NADH の酸化は、*C. glutamicum* の本来のキノンである menaquinone-2 を最も良い電子受容体としたが、NADPH の酸化においては酸素への電子伝達活性が強く見られた。また、本酵素は、特に NADPH を酸化する際、高い活性酸素種の生成能があり、分子系統学的にも酵母やカビの NADH 脱水素酵素に類似していると考えられた。さらに、この酸素への直接の電子伝達能が本菌呼吸鎖のバイパス・オキシダーゼ活性の本体である可能性が示された。

これらの研究に加えて、本菌のシアン耐性バイパス・オキシダーゼの本体と考えられるシトクロム *d*（Cyd）の遺伝子破壊株を作成し、そのバイパス・オキシダーゼ活性への関与を検討した。しかしながら、得られた Cyd 欠損株はその生育、呼吸鎖成分、エネルギー生成能に本質的な影響をあたえることがなく、Cyd は少なくとも通常の培養条件下では発現しておらず、そのため、バイパス・オキシダーゼの機能を有する本体と考えることはできないことが明らかにされた。

これらの研究成果は、*C. glutamicum* の Ndh の特異な機能および位置づけを明らかにするにとどまらず、その欠損・過剰発現がエネルギー代謝系を改変することによって発酵生産能を制御することが可能であることを示す画期的なものであると評価された。