Identification and Phylogenetic Characterization of Cobalamin Biosynthetic Genes of Ensifer adhaerens

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Ensifer adhaerens CSBa was screened as a cobalamin producer. The draft genome sequence revealed that the strain possesses 22 cobalamin biosynthetic genes (cob genes). The cob gene arrangement on the genome of E. adhaerens CSBa was similar to that of other Ensifer species, and most similar to that of Pseudomonas denitrificans SC510. The cobN sequence phylogeny was generally congruent with that of the 16S rRNA gene, and it is suggested that E. adhaerens CSBa might have inherited the cob genes from common ancestors of the Ensifer species. It was also suggested that the cob genes can be laterally transferred.

Key words: cobalamin biosynthetic genes, cob, Ensifer, Sinorhizobium, phylogeny

Cobalamin (vitamin B₁₂) is one of the most structurally complex, nonpolymeric biomolecules biosynthesized only by bacteria and archaea (2, 12, 15). Today, cobalamin is industrially produced exclusively by biosynthesis, and more than 30 genes are involved in the biosynthesis through oxygen-dependent (aerobic) and oxygen-independent (anaerobic) pathways (9). The aerobic pathway of Pseudomonas denitrificans has been studied intensively, and the anaerobic pathway has been studied thoroughly in several bacteria, such as Salmonella typhimurium and Bacillus megaterium (9). These studies have targeted mainly the identification of cobalamin biosynthetic genes and the functions of their encoded enzymes. Genetic engineering techniques involving these genes have also been employed to produce cobalamin (9); however, there has been little discussion about the diversity and phylogeny of cobalamin biosynthetic genes among bacteria, although functional gene diversity is one of the main current issues in microbial ecology (2, 4, 6, 11, 17). In the present study, we tried (i) to screen for cobalamin-producing bacteria, (ii) to reveal the composition of cobalamin biosynthetic genes (cob genes) of one of the obtained cobalamin producers, and (iii) to compare the gene arrangement and sequences with other bacteria, so that light could be shed on the phylogeny of the cob genes and their relationship with the evolutionary phyllogenetic background.

Cobalamin is an essential growth factor for most marine eukaryotic algae, and Croft et al. (3) isolated a cobalamin-producing bacterium, Halomonas sp., from a nonaxenic culture of the marine microalgae Amphidinium oculatum (Dinophyta). On the other hand, we previously isolated bacteria from nonaxenic cultures of freshwater Chlorella (Chlorophyta) (14). We expected cobalamin producers to be included within the isolates, taking into account the report by Croft et al. (3). In order to screen for cobalamin-producing bacteria from the isolates, we first screened for cobalamin auxotrophic freshwater algae, and revealed that Monomastix minuta NIES-255 and NIES-256 (Chlorophyta) are cobalamin auxotrophs (see Supplemental material). Using the former strain, we successfully obtained two potential cobalamin producers, B. megaterium CSBa and Ensifer adhaerens (= Sinorhizobium morelense) CSBa (see Supplemental material). The cob genes of the former has already been reported (9) but those of the latter have not yet been examined; therefore, we selected E. adhaerens CSBa as the material in the subsequent experiments. Additionally, the production of cobalamin by E. adhaerens CSBa was confirmed by a brief test (see Supplemental material).

The cob genes of E. adhaerens CSBa were analyzed as follows. E. adhaerens CSBa was cultivated on 1.5% agar medium of 10-fold diluted Bacto Nutrient Broth (Difco, Detroit, MI, USA) at 26°C for 1 week, and the colonies were collected from the medium and suspended in a 1.5-mL microtube containing 1 mL sterile distilled water. The cell suspension was centrifuged at 10,000 rpm for 10 min and the supernatant was removed. The pellet of bacterial cells was then subjected to DNA extraction and purification using ISoil (Nippon Gene, Tokyo, Japan). Thirty micrometres of the obtained DNA was subjected to pyrosequencing commercially carried out by Hokkaido System Science (Sapporo, Hokkaido, Japan) with a 454 GS-FLX (Roche) by which 575,172 sequences (225,813,225 bp nucleotides) were generated. Each sequence was assembled using GS De novo Assembler software, and a draft genome containing 76 contigs was produced. cob Genes were searched from the obtained sequences by BLAST (1), which revealed that three regions contain sequences similar to those of cob genes. After start/stop codons were detected on the nucleotide sequences of the three regions using the gene prediction software...
MetaGene Annotater, the open reading frames (ORFs) highly homologous to cob genes were specified. The nucleotide sequences of the ORFs were translated to amino acid sequences with BioRuby (5). The obtained amino acid sequences were subjected to BLAST and Conserved Domain Search (8) using the database in GenBank, and the cob genes of E. adhaerens CSBa were identified. In addition, a neighbor-joining (NJ) tree was constructed based on the cobN sequence by the method described previously (16).

It was revealed that E. adhaerens CSBa has 22 cob genes: cobA, cobB, cobC, cobD, cobE, cobF, cobG, cobH, cobI, cobJ, cobK, cobL, cobM, cobN, cobO, cobP, cobQ, cobS, cobT, cobU, cobV, and cobW (accession numbers, AB705623 to AB705625); this composition of cob genes is the same as that of P. denitrificans SC510 (9). Recently, the increasing availability of whole genome sequences for a considerable number of bacteria is revealing that many bacteria possess at least some of these 22 genes in the aerobic pathway, regardless of whether they are known as cobalamin biosynthesizing bacteria (cf. Table S1). Other members of the genus Ensifer, such as Ensifer melloti (= Sinorhizobium melloti) 1021, Ensifer medicae (= Sinorhizobium medicae) WSM419, and Ensifer fredii (= Sinorhizobium fredii) NGR234, are examples that have the same 22 genes (cf. Fig. S1). Ensifer species are potential nodule-forming bacteria, and it was demonstrated that E. melloti requires cobalamin-dependent ribonucleotide reductase for symbiosis with its plant host (12). Some other nodule-forming Rhizobiales bacteria, such as Rhizobium leguminosarum WSM2304, also have these 22 genes (cf. Fig. S1). It is therefore possible to speculate that nodule-forming Rhizobiales bacteria generally, if not always, have cobalamin biosynthesis ability. Many bacteria possess both cobalamin-dependent and -independent isozymes. Campbell et al. (2) suggested that cobalamin-dependent isozymes likely confer some advantage under specific environmental conditions, and that these isozymes may be important for the survival of E. melloti within a plant host. Not only E. adhaerens CSBa but also other members belonging to nodule-forming Rhizobiales bacteria have been detected in algal-bacterial consortia (13). It is possible that cobalamin biosynthesis ability might be a key to the symbiotic associations between them. In addition to the above cob genes, it is known that some other genes are necessary for the synthesis of cobalamin: bluB is one of the genes required for cobalamin biosynthesis in E. melloti (10). The present study revealed that E. adhaerens CSBa also has this gene (accession number, AB705624).

The 22 cob genes of E. adhaerens CSBa are located in three regions (regions 1 to 3), one of which is separated into three subregions (3a to 3c) by multiple ORFs (Fig. 1), forming a tight cluster (operon) within each region or subregion. The above four reference nodule-forming bacteria, i.e., E. melloti 1021, E. medicae WSM419, E. fredii NGR234, and R. leguminosarum WSM2304, have similar cob gene operons to those of E. adhaerens CSBa (Fig. 1, S1). These bacteria show a high degree of similarity: every pair of two adjacent genes of cobH, cobI, cobJ, and cobK, and that of cobD, cobB, cobC, overlaps, except that E. medicae WSM419 and E. fredii NGR234 have no overlapping between cobB and cobC.
Ecological studies. The above congruency presented here described based on the NJ-tree topology, it is fair to assume that the cobN of E. adhaerens CSBA might be inherited from common ancestors of the four Ensifer members; however, cobN of P. denitrificans SC510 is distant from those of other Pseudomonas members but within the Ensifer cluster close to E. adhaerens CSBA. The other 21 cob genes of P. denitrificans SC510 also showed the same phylogenetic relationship to E. adhaerens CSBA (data not shown). The above NJ-tree topology, as well as the high degree of similarity in the cob gene arrangement described above, suggests that an ancestor of P. denitrificans SC510 obtained the 22 cob genes from an Ensifer bacterium phylogenetically closely related to E. adhaerens CSBA, perhaps by lateral gene transfer. Krishnapillai (7) named the cob operon as one of the prokaryotic DNA segments postulated to be involved in lateral gene transfer, since the cob operons in Escherichia coli and Synechocystis sp. had a higher GC content (59%) than the genome average of 52%. The average GC content of the 22 cob genes of E. adhaerens CSBA and P. denitrificans SC510 is approximately 66%, and that of the genomes of Ensifer species and P. denitrificans is approximately 62–63% each, which unfortunately does not help to elucidate the validity of the above suggestion in the present study. P. denitrificans SC510 and its mutants have been well studied and used for the commercial production of cobalamin for decades. It was somewhat surprising that we stumbled on the possible origin of the cob genes of P. denitrificans SC510 in Rhizobiales bacterium. In the present study, we revealed that E. adhaerens CSBA is a cobalamin producer and has 22 cob genes. It was suggested that cob gene phylogeny is generally congruent with that of the 16S rRNA gene, but the genes can be laterally transferred. Bertrand et al. (1) suggested that a previously undescribed group of bacteria could dominate the B12 biosynthesizing community in a certain environment, indicating the potential benefits of cob genes as a tool for ecological studies. The above congruency presented here would be valuable as a guide to the further study of the diversity of cobalamin producers and the distribution of cobalamin-dependent events in microbial ecology.

The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank and assigned accession numbers AB705623–AB705625.

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