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Cloning and Sequencing of Some Genes Responsible for Porphyrin Biosynthesis from the Anaerobic Bacterium *Clostridium josui*

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The 6.2-kbp DNA fragment encoding the enzymes in the porphyrin synthesis pathway of a cellulolytic anaerobe, *Clostridium josui*, was cloned into *Escherichia coli* and sequenced. This fragment contained four *hem* genes, *hemA*, *hemC*, *hemD*, and *hemB*, in order, which were homologous to the corresponding genes from *E. coli* and *Bacillus subtilis*. A typical promoter sequence was found only upstream of *hemA*, suggesting that these four genes were under the control of this promoter as an operon. The *hemA* and *hemD* genes cloned from *C. josui* were able to complement the *hemA* and *hemD* mutations, respectively, of *E. coli*. The COOH-terminal region of *C. josui* HemA and the NH_2-terminal region of *C. josui* HemD were homologous to *E. coli* CysG (Met-1 to Leu-151) and to *E. coli* CysG (Asp-213 to Phe-454) and *Pseudomonas denitrificans* CobA, respectively. Furthermore, the cloned 6.2-kbp DNA fragment complemented *E. coli cysG* mutants. These results suggested that both *C. josui hemA* and *hemD* encode bifunctional enzymes.

Metal-chelating tetrapyrrole derivatives are contained in several essential components of most organisms, such as respiratory chain complexes, light-harvesting complexes, catalases, and peroxidases, and their biosynthesis routes have been studied in many organisms (3, 17, 24, 34, 47).

Recently, the genes involved in tetrapyrrole biosynthesis have been cloned by using *Escherichia coli* auxotrophs requiring some intermediates such as 5-aminolevulinic acid (ALA) and hemin for porphyrin synthesis from facultatively anaerobic bacteria such as *E. coli* (10, 11, 19, 23, 41, 50) and *Salmonella typhimurium* (12, 13) and from strict aerobes such as *Bacillus subtilis* (20, 36). Nothing is known, however, about the genes involved in porphyrin biosynthesis from strictly anaerobic bacteria. We have attempted to isolate interesting clones by a simple means: exposing *E. coli* transformants to long-wave UV light. Since porphyrins are excited by light of approximately 400 nm to exhibit pink fluorescence, organisms which overproduce porphyrins exhibit such fluorescence.

FIG. 1. Fluorescence of the transformants harboring pOR1 (1) and pBR322 (2). After overnight cultivation on an LB-ampicillin plate, cells were exposed to visible light (A) and UV light (B).

In this paper, we describe the cloning and nucleotide se-

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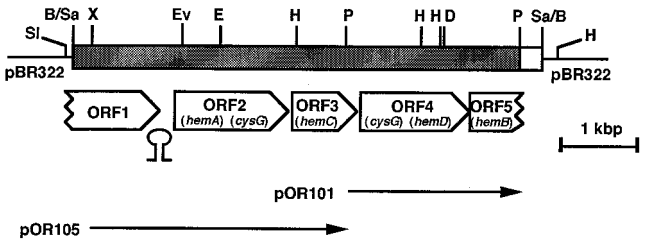

FIG. 2. Physical map of pOR1. Shaded and open bars show the cloned fragment. The shaded region was sequenced on both strands. Open arrows (ORF1 to ORF5) show the localization of each gene and the orientation of coding sequences. The regions encoding ORF1 and ORF5 are shown as rectangles with ragged left and right sides, respectively. The genes encoding homologous enzymes are indicated in parentheses. The symbol between ORF1 and ORF2 indicates the presence of a palindromic structure. pOR101 carries a 2.3-kbp *Pst*I-*Pst*I fragment at the *Pst*I site in pUC119. pOR105 carries a 3.4-kbp *Xba*I-*Pst*I fragment at the *Xba*I-*Pst*I site in pUC119. Arrows indicate the direction of *lacZ'* transcription. B, *Bam*HI; D, *Dra*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Sa, *Sau*3AI; Sl, *Sal*I; X, *Xba*I.

quence of the gene cluster responsible for porphyrin biosynthesis in a cellulolytic anaerobe, *Clostridium josui* (14, 15, 49).

C. josui FERM P-9684 (49), isolated from compost in Thailand, was cultivated in GS medium (16) with cellobiose as the sole carbon source, and its chromosomal DNA was isolated by the method of Saito and Miura (38). *C. josui* DNA was partially digested with *Sau*3AI, and 4- to 10-kbp fragments were fractionated by agarose gel electrophoresis. The *C. josui* gene bank was constructed by ligating the *Sau*3AI fragments with the vector pBR322 (39), which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase by using T4 DNA ligase. *E. coli* HB101 (39) was transformed with the chimera plasmids, plated onto Luria-Bertani (LB) agar medium containing ampicillin (100 μg/ml), and kept at 37°C overnight. One transformant fluorescing pink on UV irradiation at 375 nm was isolated (Fig. 1). It harbored a plasmid designated pOR1 with the 6.2-kbp *Sau*3AI fragment of *C. josui* at the *Bam*HI site in pBR322. The restriction map of the cloned fragment is shown in Fig. 2. Subcloning was performed with *E. coli* JM103 (58) and XL1-Blue (Stratagene, La Jolla, Calif.) as hosts and plasmids pUC118 and pUC119 as vectors. In Southern hybridization analysis, the 4.6-kbp *Xba*I-*Dra*I fragment hybridized with the *Xba*I-and-*Dra*I digest of chromosomal DNA of *C. josui* at the position corresponding to kbp 4.6 (data not shown), indicating that the cloned fragment originated from *C. josui* chromosomal DNA without any rearrangement.

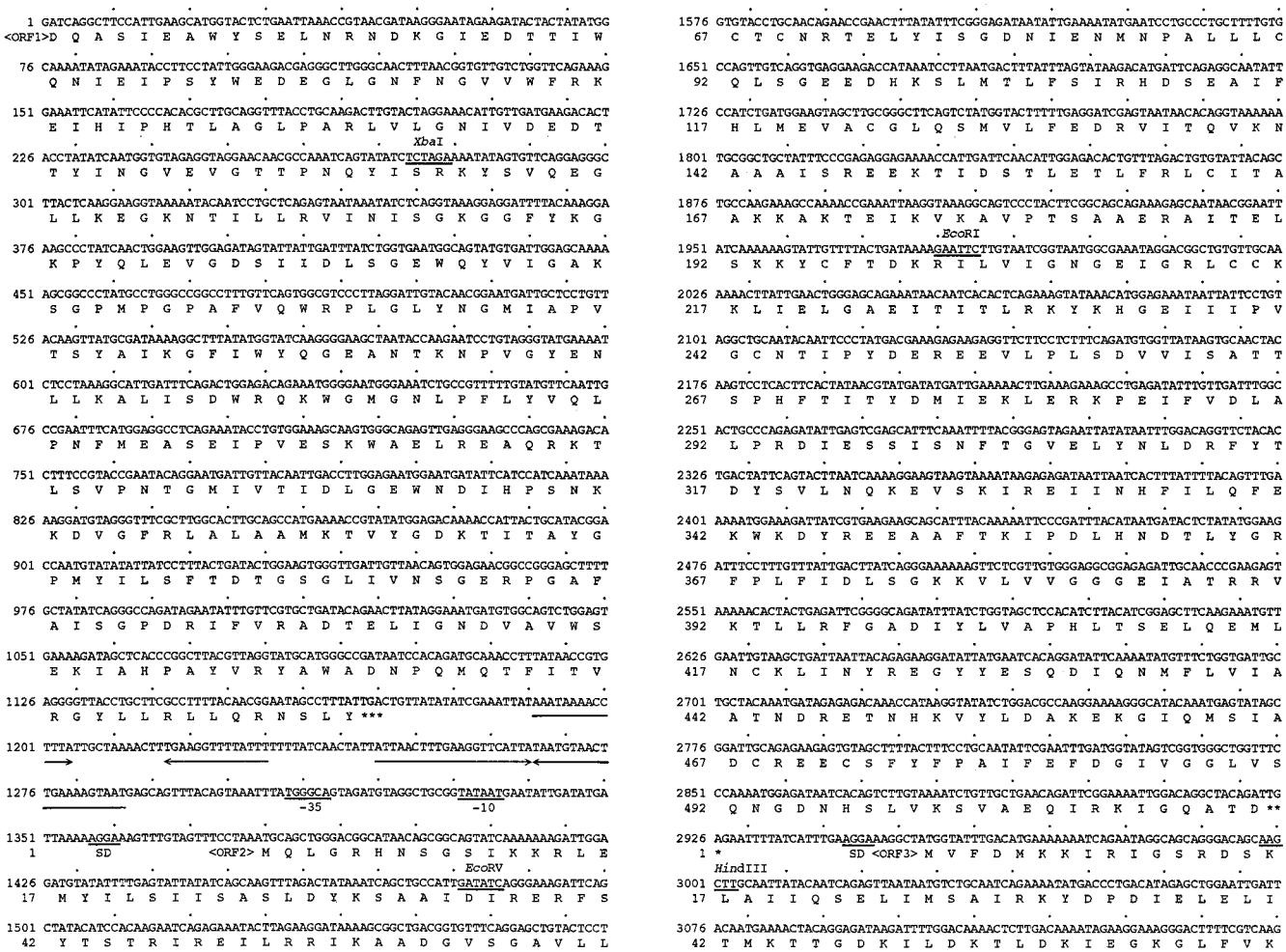

FIG. 3. Nucleotide and deduced amino acid sequences of the 6.0-kbp fragment of the *C. josui* chromosome. The underlined nucleotide sequences marked −35 and −10 refer to the sites for recognition and binding of RNA polymerase. SD indicates a possible ribosome-binding site. The stop codons are indicated by three asterisks. Palindromic sequences between ORF1 and ORF2 are shown by horizontal arrows.

The DNA sequence of the 6.0-kbp *Sau*3AI-*Pst*I fragment of pOR1 from *C. josui* was determined by the dideoxy-chain termination method (40) by using single-stranded DNA templates and a Sequenase DNA sequencing kit (United States Biochemical Co., Cleveland, Ohio) according to the supplier's protocol (Fig. 3). The deletion-bearing plasmids for DNA sequencing determination were constructed by exonuclease III and mung bean nuclease digestion as described by Henikoff (22), with some modifications, and single-stranded DNAs were prepared by infecting *E. coli* MV1184 harboring pUC118 or pUC119 derivatives with M13KO7 (53). Sequence data were analyzed by using the program GENETYX-MAC, version 5.0 (Software Development Co., Ltd., Tokyo, Japan).

As a result, five open reading frames (ORFs) (ORF1 to ORF5) were found in the 6.0-kbp fragment (Fig. 3). ORF1 (Fig. 2 and 3), encoding 389 amino acid residues, was incomplete, i.e., the initiation codon was not contained in this fragment. Immediately downstream of ORF1, two palindromic structures, which were followed by a putative promoter sequence and four ORFs (ORF2 to ORF5) of 1,545, 885, 1,512, and 616 bp, were detected. ORF5, the last ORF, did not contain any stop codon (Fig. 2 and 3) in the 6.0-kb fragment, indicating that ORF5 was also incomplete. Each ORF was preceded by a typical ribosome-binding site upstream of its ATG initiation codon. Only one putative promoter sequence, ^{1308}TGGGCA^{1313} as the −35 region (consensus for *E. coli*, TTGACA) and ^{1331}TATAAT^{1336} as the −10 region (consensus for *E. coli*, TATAAT), was found with the consensus distance of 17 bp upstream of ORF2, and no other promoter sequence was identified in the nucleotide sequence, suggesting that ORF2 to ORF5 are transcribed from this promoter in a polycistronic mRNA, i.e., the genes form an operon.

Amino acid sequences deduced from ORF2, ORF3, ORF4, and ORF5 were homologous to those of HemA, HemC, HemD, and HemB, respectively, of *E. coli* and *B. subtilis*, as described below (Fig. 2).

The NH_2-terminal region of the ORF2 protein (Leu-20 to Trp-343) was highly homologous to HemA proteins of *B. subtilis* (identity, 29%) (36), *E. coli* (33%) (10, 29, 52), and *S. typhimurium* (31%) (12) (Fig. 4A), which synthesize ALA via the C_5 pathway, but not homologous to HemA proteins of *Rhizobium meliloti* (27), *Agrobacterium radiobacter* (9), *Bradyrhizobium japonicum* (32), *Saccharomyces cerevisiae* (51), chickens (7), rats (48), mice (43), and humans (4), which synthesize ALA via the C_4 pathway. The plasmid pOR105 (Fig. 2), containing ORF2, complemented *E. coli hemA* mutants AN344 (provided by Y. Murooka) and SASX41B (provided by B. Bachmann; CGSC4806). These results indicate that ORF2 encodes HemA protein, NAD(P)H-dependent glutamyl-tRNA reductase (20), which is involved in ALA synthesis via the C_5

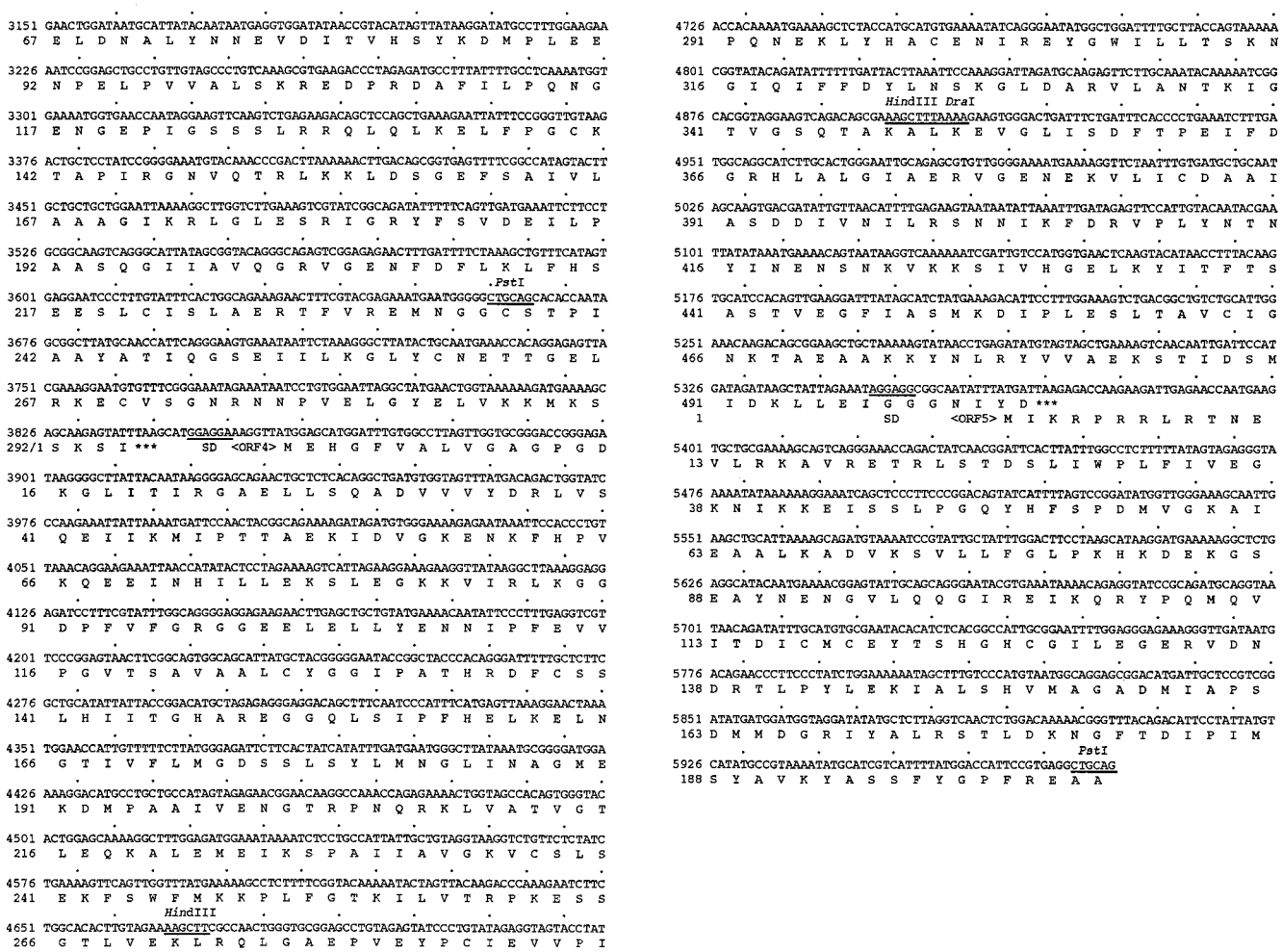

FIG. 3—*Continued.*

FIG. 4. Homology analysis of the predicted amino acid sequence from ORF2 (*hemA*) from *C. josui*. (A) Alignment of the predicted amino acid sequences in the NH_2-terminal region in HemA of *C. josui* (C.j) and NAD(P)H-dependent glutamyl-tRNA reductases (HemA) of *B. subtilis* (B.s), *E. coli* (E.c), and *S. typhimurium* (S.t). (B) Alignment of the predicted amino acid sequences in the COOH-terminal region in HemA of *C. josui* (C.j) and in the NH_2-terminal region of CysG of *E. coli* (E.c). The putative $NADP^+$-binding site is underlined. Shaded residues represent amino acids which are identical to those in *C. josui* HemA.

pathway. ORF2 was termed *hemA*. In addition to having similarity with other HemA proteins, *C. josui* HemA had similarity (23%) in its COOH-terminal region (Asp-361 to Asp-515) with the NH_2-terminal region of CysG protein of *E. coli* (35, 55, 56) (Fig. 4B). Recently, M. J. Warren et al. have reported that the NH_2 terminus of *E. coli* CysG was involved in the dehydrogenation of dihydrosirohydrochlorin (precorrin-2) and ferrochelation, which convert precorrin-2 into siroheme (54). The $NADP^+$-binding site (21, 45) identified as Asp-14 to Asn-41 in *E. coli* CysG (54) was conserved in *C. josui* HemA as Lys-377 to Val-404 (Fig. 4B). These results suggest that the *hemA* gene of *C. josui* is responsible for two different steps in porphyrin biosynthesis, i.e., the synthesis of ALA from glutamate and siroheme from precorrin-2.

The amino acid sequence predicted from ORF3 displayed a high degree of homology with sequences of porphobilinogen deaminases (PBG-Ds) (hydroxymethylbilane synthase [HMB-S]; EC 4.3.1.8) which are encoded by the *hemC* genes of *B. subtilis* (36), *E. coli* (1, 50), humans (37), *S. cerevisiae* (25), and *Euglena gracilis* (46) (Fig. 5). An extract from *E. coli* BL21(DE3) cells (Novagen, Madison, Wis.) harboring pER1 (constructed by inserting PCR products containing the *hemC* region into pET-16b vector purchased from Novagen) had PBG-D activity (data not shown). These results indicate that ORF3 corresponds to the *hemC* gene. The cysteine residue in the dipyrromethane cofactor-binding site which was identified as Cys-242 in *E. coli* PBG-D (44) was conserved in *C. josui* HemC as Cys-237 and is present in all other PBG-Ds reported so far (Fig. 5). One Asp and six Arg residues which were identified as catalytic sites for tetrapyrrole synthesis in PBG-D from *E. coli* (30) are conserved in *C. josui* HemC as Asp-86, Arg-13, Arg-128, Arg-129, Arg-146, Arg-152, and Arg-173 and are also conserved in the other PBG-Ds (Fig. 5).

ORF4 encodes a polypeptide of 504 amino acids, and its COOH-terminal region downstream of Met-247 revealed 24% identity with the HemD protein, uroporphyrinogen III (UroIII) synthase (EC 4.2.1.75), from *B. subtilis* (20) (Fig. 6A). pOR101 (Fig. 2) complemented *E. coli hemD* mutant SASZ31 (provided by B. Bachmann; CGSC7153). On the basis of these results, ORF4 was identified as *hemD*. The NH_2-terminal region (Met-1 to Phe-246) of *C. josui* HemD revealed 49 and 39% identities with the COOH-terminal region (Asp-213 to Phe-454) of the *E. coli* CysG protein (35, 55, 56) and with the whole of the *Pseudomonas denitrificans* CobA protein (8) (Fig. 6B), respectively. Both proteins are *S*-adenosylmethionine-dependent UroIII methylases. Therefore, HemD of *C. josui* might catalyze sequential reactions to synthesize UroIII from HMB and then precorrin-2, which are intermediate compounds in both vitamin B_{12} and siroheme biosyntheses.

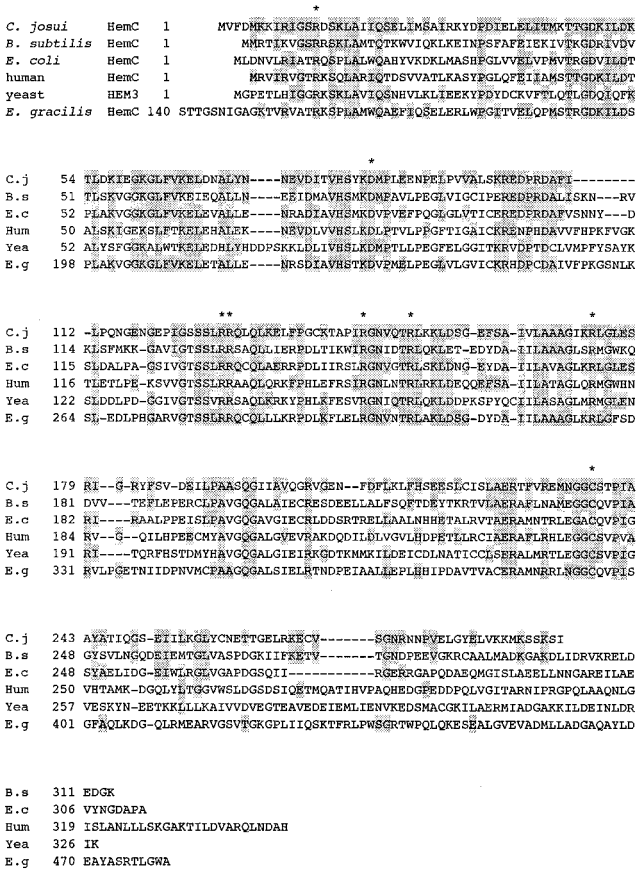

FIG. 5. Alignment of the predicted amino acid sequence from ORF3 (*hemC*) of *C. josui* (C.j) and the amino acid sequences of PBG-Ds (HMB-S) (HemC) of *B. subtilis* (B.s), *E. coli* (E.c), humans (Hum), *S. cerevisiae* (Yea), and *E. gracilis* (E.g). Shaded residues represent amino acids which are identical to those in *C. josui* HemC. Conserved amino acids which are candidates for the catalytic sites discussed in a previous paper (30) are marked by asterisks.

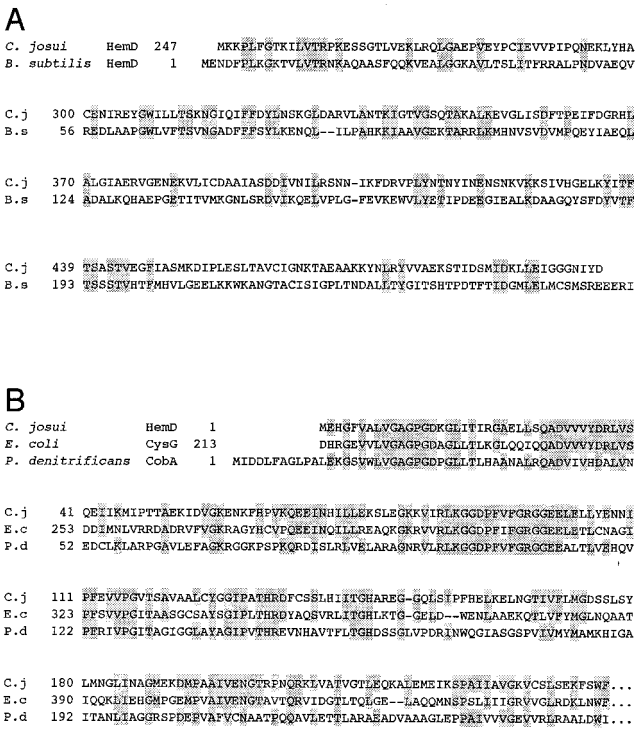

FIG. 6. Homology analysis of the predicted amino acid sequence from ORF4 (*hemD*) from *C. josui*. (A) Alignment of the predicted amino acid sequences in the COOH-terminal region in HemD of *C. josui* (C.j) and UroIII synthase (HemD) of *B. subtilis* (B.s). (B) Alignment of the predicted amino acid sequences in the NH_2-terminal region in HemD of *C. josui* (C.j) and *S*-adenosylmethionine-dependent UroIII methylases (CysG and CobA) of *E. coli* (E.c) and *P. denitrificans* (P.d). Shaded residues represent amino acids which are identical to those in *C. josui* HemD.

The NH_2-terminal region of the amino acid sequence predicted from ORF5 (205 residues) showed a high degree of similarity with PBG synthases (ALA dehydratase) (EC 4.2.1.24) of *B. subtilis* (20), *E. coli* (11, 28), *S. cerevisiae* (31), humans (57), and rats (6) (Fig. 7), whereas the 6.0-kbp fragment sequenced in this study did not contain the region encoding the COOH-terminal moiety. The amino acid sequence of *C. josui* HemB contained a short motif (Cys-117 to Cys-127) similar to a zinc-binding domain, including two cysteines and two histidines in a zinc finger (5, 26), and this motif was highly conserved in all PBG synthases (Fig. 7). This incomplete gene, however, was not sufficient for complementing *E. coli hemB* mutant RP523 (provided by B. Bachmann; CGSC7199), probably because of the defectiveness of the *C. josui hemB* gene.

The arrangement of the gene cluster responsible for porphyrin biosynthesis in *C. josui* (Fig. 2 and 8) was similar to that of the gene cluster in *B. subtilis*, although a gene corresponding to *hemX* was not found between *hemA* and *hemC*. Homology analysis of HemAs suggested that in *C. josui*, ALA was possibly synthesized via the C_5 pathway, which was also found to be the case in *Clostridium thermoaceticum* (33). Therefore, some clostridia seem to use the C_5 pathway for ALA synthesis. The *hemL* gene, encoding glutamate-1-semialdehyde-2,1-aminotransferase (EC 5.4.3.8), which is involved in ALA synthesis via the C_5 pathway, was not included in the fragment cloned from *C. josui* in this study, although the *hemL* genes of several organisms, such as *S. typhimurium*, *E. coli*, *B. subtilis*, and plants, have been cloned and sequenced (13, 18, 19, 20). Since the *hemL* gene is located downstream of the *hemB* gene in *B. subtilis*, the *hemL* gene of *C. josui* might also occur downstream of *hemB* (ORF5).

In addition, HemA and HemD might be involved in the biosynthesis of vitamin B_{12} or siroheme (Fig. 8). We examined the vitamin B_{12} productivity of *C. josui* by performing a microbiological assay with vitamin B_{12} auxotrophic *E. coli* 215 (42) according to the method of the Association of Official Analytical Chemists (2). When *C. josui* was cultivated at 45°C for 4 days in 20 ml of GS medium (16) containing biotin (0.2 mg/liter), *p*-aminobenzoic acid (0.4 mg/liter), and $CoCl_2 \cdot 6H_2O$ (20 mg/liter) instead of yeast extract, it accumulated 30 ng of CN-vitamin B_{12} in total. Furthermore, pOR1 (Fig. 2) was able to complement *E. coli cysG* mutants AT718 and AT2455 (provided by A. Nishimura; ME5358 and ME5461). Homology analysis and complementation experiments indicated that the HemA and HemD proteins of *C. josui* each contained two putative catalytic domains with different functions and therefore may be bifunctional enzymes (Fig. 2, 4, and 6).

Our results showed that these genes responsible for porphyrin synthesis were arranged in a more compact organization in *C. josui* than in the other bacteria and suggested that the gene cluster might be involved in the synthesis of vitamin B_{12} and siroheme. To our knowledge, this is the first report describing the genes responsible for porphyrin biosynthesis from a strictly anaerobic bacterium.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB,

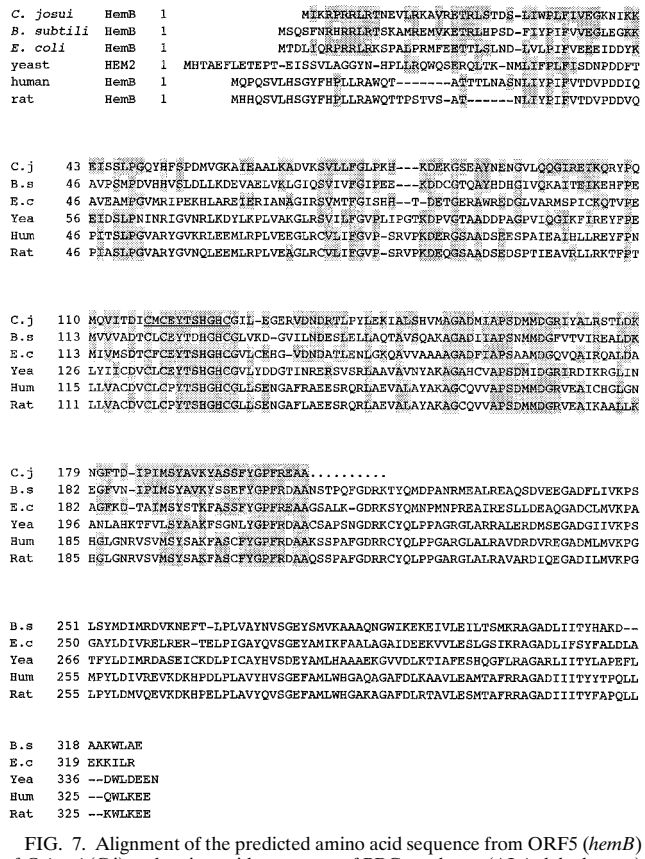

FIG. 7. Alignment of the predicted amino acid sequence from ORF5 (*hemB*) of *C. josui* (C.j) and amino acid sequences of PBG synthases (ALA dehydratase) (HemB) of *B. subtilis* (B.s), *E. coli* (E.c), *S. cerevisiae* (Yea), humans (Hum), and rats (Rat). Shaded residues represent amino acids which are identical to those in *C. josui* HemB. A short motif similar to a zinc-binding domain is underlined.

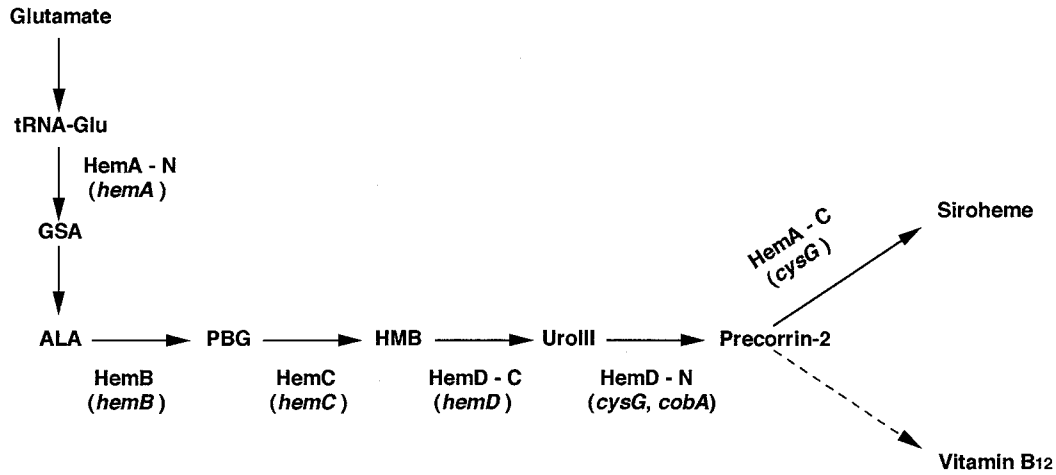

FIG. 8. Proposed pathway of porphyrin biosynthesis in *C. josui*. -N and -C show the NH_2-terminal region and the COOH-terminal region, respectively. The genes encoding homologous enzymes are given in parentheses. The *C. josui* proteins correspond to the following enzymes: HemA-N, NAD(P)H-dependent glutamyl-tRNA reductase; HemB, PBG synthase; HemC, HMB-S; HemD-C, UroIII synthase; HemD-N, *S*-adenosylmethionine-dependent UroIII methylase; HemA-C, siroheme synthase. The broken arrow shows that vitamin B_{12} is synthesized in several steps from precorrin-2. tRNA-Glu, glutamyl-tRNA; GSA, glutamate-1-semialdehyde..

DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D28503.

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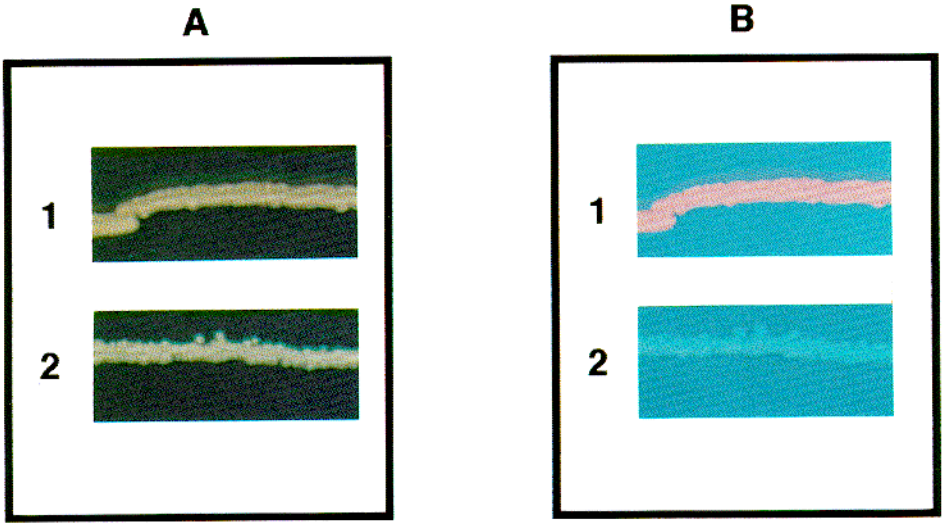
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