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Analysis of a Prodigiosin Biosynthetic Gene Cluster from the Marine Bacterium *Hahella chejuensis* KCTC 2396

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Abstract Marine bacterium *Hahella chejuensis* KCTC 2396 simultaneously produced red antibiotic prodigiosin and undecylprodiginine. A complete set of the prodigiosin biosynthetic gene cluster has been cloned, sequenced, and successfully expressed in a heterologous host. Sequence analysis of the gene cluster revealed 14 ORFs showing high similarity to *pig* and *red* genes from *Serratia* spp. and *Streptomyces coelicolor* A3(2), respectively, and the gene organization was almost similar to that of *pig* genes. These genes were named *hap* for *Hahella* prodigiosin, and determined to be transcribed as a single operon, by RT-PCR experiment. Based on the *hap* gene mutagenesis experiments and comparative analysis with *pig* and *red* genes, we propose a prodigiosin-biosynthetic pathway in KCTC 2396.

Key words: Marine bacterium, algicidal agent, prodigiosin, *hap*

Prodiginines are a large family of pigmented tripyrrole antibiotics, with medical potential as immunosuppressants and antitumour agents [4, 5, 11, 12], which are produced by actinomycete and other eubacterial strains [3]. The red-colored linear tripyrrolyl prodiginine produced by *Serratia marcescens* is called prodigiosin, which has a series of close relatives bearing the same prodigiosene core with different substituents. A red antibiotic produced by actinomycete *Streptomyces coelicolor* A3(2) was found to be the mixture of linear undecylprodiginine and its isomeric, cyclic derivative, butyl-*meta*-cycloheptylprodiginine, in the ratio of 2:1 [15].

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Following intensive studying on the undecylprodiginine biosynthesis, at the biochemical and molecular levels [1, 14], the organization of the undecylprodiginine biosynthetic gene cluster was well examined, and the biosynthetic pathway was subsequently proposed, which was based on functional and comparative analyses on several key enzymes. These studies demonstrated that undecylprodiginine is derived from one unit of proline, one unit of glycine, one unit of serine, and several units of acetate, via a convergent pathway involving condensation of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC) and 2-undecylpyrrole at a late stage, with butyl-*meta*-cycloheptylprodiginine possibly being derived from undecylprodiginine by oxidative cyclization.

In contrast, a pathway for prodigiosin biosynthesis in *Ser. marcescens* has been recently proposed, which showed that the terminal step in the biosynthesis involves the enzymatic coupling of a stable bipyrrolyl MBC with a volatile monopyrrolyl 2-methyl-3-amylpyrrole (MAP) to form prodigiosin [6]. In a genetic work, two cosmid clones, containing the entire gene clusters (≈21 kb) for prodigiosin from two *Serratia* spp. chromosomes, were cloned, sequenced, and analyzed comparatively [6]. Recently, a more valid prodigiosin pathway was proposed, and some experimental data for the genes encoding the pathway-specific key enzymes have been available [16].

Marine bacterium *Hahella chejuensis* KCTC 2396 produced two prodiginines; one red antibiotic prodigiosin (major secondary metabolite) with algicidal activity [8] and another minor undecylprodiginine [10]. In this paper, we describe the organization and function of the prodigiosin biosynthetic gene cluster in this bacterium, and propose the prodigiosin biosynthetic pathway.

MATERIALS AND METHODS

Genomic Library Construction and Sequence Analysis

Two kinds of *H. chejuensis* KCTC 2396 genomic libraries were constructed; one with randomly sheared genomic fragments (2 kb and 5 kb) into a pUC-derived high-copy plasmid vector, and another the fosmid library harboring ca. 40 kb inserts using the CopyControl pCC1FOS Fosmid Library Production Kit (Epicentre, U.S.A.). About 8x sequencing coverage was obtained with RISA 384 [13] and ABI Prism 3700/3730 XL (Applied Biosystems, U.S.A.) using standard dye-terminator chemistry. Base-calling, fragment assembly, and partial finishing were carried out by using the PHRED/PHRAP/CONSED software package (http://www.phrap.org/). Putative protein-coding sequences (CDSs) from the contig sequences were predicted by GLIMMER [2] and were subjected to BLAST search against public protein sequence databases.

RNA Preparation and RT-PCR

KCTC 2396 grown on Difco Marine Broth 2216 (MB) agar plate was inoculated in 50 ml of MB liquid for 48 h at 30°C with vigorous shaking. The cells were then harvested by centrifuging at 10,000 ×*g* for 20 min and suspended in 1 ml of chloroform and 5 ml of Trizol reagent (Invitrogen, U.S.A.), and mixed thoroughly for 1 min by vortexing. The mixture was incubated at 37°C for 10 min, followed by centrifugation (10,000 ×*g* for 20 min). The supernatant solution was washed with an equal volume of chloroform. The RNA was then precipitated with 2 volumes of absolute isopropanol at −70°C for 1 h, and centrifuged (20,000 ×*g* for 30 min). The resultant RNA pellet was washed twice with 70% ethanol, and dissolved in RNase-free water [7]. The extracted total RNA was further purified by spin column and DNase I treatment according to the manufacturer instructions (Qiagen, Germany).

RT-PCR was carried out using the OneStep RT-PCR Kit with primers, which were designed based on the sequences of RT-PCR target genes. RT-PCR reactions were performed in 50 μl with 1.3 μg of total RNA and 50 pmol of each primer with OneStep RT-PCR Enzyme Mix (Qiagen). The thermocycler program used for the RT-PCR reactions was as follows: 50°C for 30 min, 95°C for 15 min, 35× (94°C for 30 s, 50–59°C for 30 s, 72°C for 1 min), and 72°C for 10 min. A description of the primers (annealing temperature) to amplify the two adjacent *hap* genes is shown in Table 1.

Mutagenesis on *hap* Genes

Transposon random mutagensis on recombinant HC81006F09-R4 fosmid in the EPI300 host that was used in the KCTC 2396 fosmid library construction was carried out using an EZ::TN <KAN-2> Insertion Kit (Epicentre). For a gene-targeted disruption, isolated HC81006F09-R4 was transferred to BL21(DE3) host, and the mutagenesis was carried out according to the TargeTron Gene Knockout System (Sigma-Aldrich, U.S.A.) user guide.

Table 1. RT-PCR primers and conditions for PCR.

Primer[a]	Target gene	Sequence	T_a[b] (°C)	Product size (bp)
hapAorf5-F	*hapA-orf5*	5'-tgttcgcctgcgcagttcctatct-3'	58	1,512
hapAorf5-R		5'-gtggggcagggcgtacagagattt-3'		
orf5hapB-F	*orf5-hapB*	5'-tctctgtacgccctgccccactat-3'	55	910
orf5hapB-R		5'-tttccacggcgtctttcactaatg-3'		
hapBC-F	*hapB-C*	5'-ggacagcgtcggcatgggca-3'	50	1,005
hapBC-R		5'-gcccgccagggccagagct-3'		
hapCD-F	*hapC-D*	5'-gccgcatccgcaccggaga-3'	50	1,004
hapCD-R		5'-gttggagaccagcgagcc-3'		
hapDE-F	*hapD-E*	5'-ctgcggcatttcagccgt-3'	50	1,003
hapDE-R		5'-cccgcgcttgccgataatcga-3'		
hapEF-F	*hapE-F*	5'-ccaacccccgctgatcatcag-3'	55	1,133
hapEF-R		5'-ccgacgaataacgtgcgcg-3'		
hapFGH-F	*hapF-G-H*	5'-cggtgcgcgacgacaaag-3'	55	1,049
hapFGH-R		5'-ggcttccgcctgttgcga-3'		
hapHI-F	*hapH-I*	5'-gaggacctggatatggcggcgca-3'	55	997
hapHI-R		5'-ccatcagggacaggatggaggg-3'		
hapIJ-F	*hapI-J*	5'-gctggtgcgcagactggacg-3'	58	1,041
hapIJ-R		5'-cgccgcgaatgacgctgtag-3'		
hapJKLM-F	*hapJ-K-L-M*	5'-ccgtaaccgctacccatgcgg-3'	55	1,500
hapJKLM-R		5'-ccagtcatggccgcgaacc-3'		
hapMN-F	*hapM-N*	5'-cactcaccgcggccctttaac-3'	58	961
hapMN-R		5'-cttacgcgccaggtccgcgtc-3'		

[a]Forward (-F) and reverse (-R) primers are indicated.
[b]T_a, the annealing temperature used during PCR.

Detection of Prodigiosin

E. coli hosts containing recombinant fosmid were inoculated in 3 ml of LB liquid containing chloramphenicol (25 μg/ml) at 37°C overnight. A part of the culture (0.2 ml) was transferred to 100 ml of fresh LB liquid containing chloramphenicol. To induce the prodigiosin production, 1× CopyControl Induction Solution (Epicentre) and 0.1 mM IPTG was added to the host strain suspensions EPI300 and BL21(DE3), respectively, and further cultivation was carried out for 24 h at 30°C with vigorous shaking. The pigment from the cell pellet was extracted with 10 ml of 100% acetone, the extract was centrifuged at 10,000 ×*g* for 10 min, and the white pellet was discarded. The acetone extract was dehydrated with sodium sulfate and evaporated. The dried extract was dissolved in 50% methanol (a final concentration of 5 mg/ml), and 10 μl of that was analyzed by LC-ESI-MS technique.

LC-ESI-MS Analysis

Liquid chromatography mass spectrometry (LC-MS) was performed using a Finnigan LCQ Advantage MAX ion-trap mass spectrometer (Thermo Electron Co., U.S.A.) equipped with an electrospray ionization (ESI) source. HPLC separations was performed on a Finnigan Surveyor Modular HPLC System (Thermo Electron Co.), using a XTerra MS C18 (5 μm, 2.1×150 mm, Waters, Ireland). Mobile phase A was water and mobile phase B was acetonitrile-both contained 0.1% formic acid. Gradient elution at a flow rate of 0.2 ml/min was performed as follows: 0–30 min 10–100% B (linear gradient) and 30–50 min 100% B (isocratic). Full-scan mass spectra were obtained in the positive ion mode and range *m/z* 50–700. Data-dependent tandem mass spectrometry (MS/MS) experiments were controlled by the menu-driven software provided with the Xcalibur system.

Nucleotide Sequence Accession Number

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession number DQ266254.

RESULTS

Analysis of Prodigiosin Gene Cluster

In the course of the genome sequencing project for *H. chejuensis* KCTC 2396, one scaffold containing genes homologous to those involved in the biosynthesis of prodigiosin from *Ser. marsescens* and undecylprodiginine from *S. coelicolor* A3(2) was selected. Six contigs in this scaffold were joined into one high-quality sequence (59.4 kb) by primer walks on plasmid templates before the completion of entire genome sequencing. The sequence analysis has revealed the identification of a ca. 40-kb

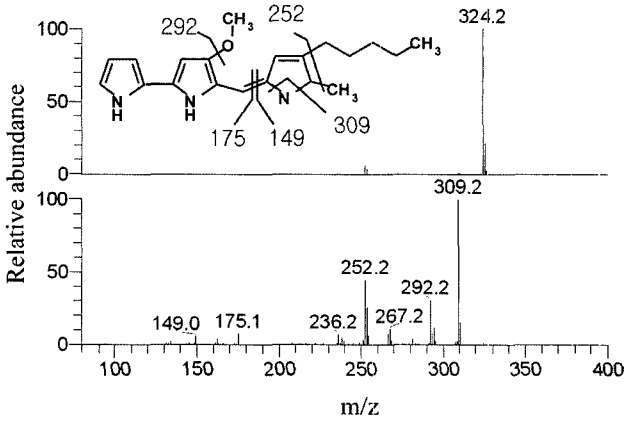

Fig. 1. Full MS and MS/MS spectral analysis of the red pigment (prodigiosin) extracted from the HC81006F09-R4 clone.

region containing the pigment gene cluster. Among fosmid clones encompassing this gene cluster, HC81006F09-R4 producing a red pigment around *E. coli* host cells was detected.

The red pigment was extracted from the HC81006F09-R4 clone using 100% acetone, and analyzed by LC-MS/MS spectrometry. The pigment having a molecular weight of *m/z* 324.2 ($[M+H]^+$) showed an identical fragmentation pattern and retention time with that of authentic prodigiosin from KCTC 2396 [8] (Fig. 1), which represents the successful heterologous expression of this prodigiosin gene cluster. Consequently, HC81006F09-R4 insert DNAs were completely sequenced, and the functional prodigiosin gene cluster was analyzed.

Extensive genetic analysis of the 35,739-bp sequenced region of HC81006F09-R4 revealed that it contains a total of 26 ORFs, which are supposed to be, either directly or indirectly, involved in prodigiosin production. Homology searches against the database with the deduced amino acid sequences of the ORFs identified fourteen as putative genes for prodigiosin biosynthesis, and named as for *Hahella* pigment (prodigiosin). Similarities between the *hap* genes and the representative homologues are summarized in Table 2. Interestingly, all the *hap* genes, except *hapL*, from KCTC 2396 showed high similarities with the prodigiosin-structural genes (*pig*) from *Serratia* sp. ATCC 39006 or *Ser. marcescens* ATCC 274, and also exhibited similarities with the undecylprodiginine genes (*red*) from *S. coelicolor* A3(2).

Computer analysis of the 3-terminal region of the insert DNA sequence revealed the presence of two ORFs encoding a two-component signal transduction system. The ORFs (designated *orf9* and *orf10*), which are transcribed in the opposite direction to the *hap* genes (*hapA-hapN*), are 38% and 59% identical to the deduced amino acids of structural genes for signal transduction histidine kinase and response regulator from *Vibrio vulnificus*, respectively. Thus, it is

Table 2. Comparison of Hap proteins with homologs.

Protein	Identity (%)*			Putative function	Representative gene
	Pig [39006]	Pig [274]	Red [A3(2)]		
HapA	50	48	48	L-Prolyl-PCP dehydrogenase	*pigA*
HapB	41	43	-	H_2MAP oxidase/dehydrogenase	*pigB*
HapC	53	55	43	Condensation enzyme	*pigC*
HapD	51	51	-	3-Acetyloctanal synthase	*pigD*
HapE	58	61	38	3-Acetyloctanal aminotransferase	*pigE*
HapF	58	61	22	HBC *O*-methyl transferase	*pigF*
HapG	46	52	48	Peptidyl carrier protein	*pigG*
HapH	56	60	52	HBM synthase/aminotransferase	*pigH*
HapI	42	41	47	L-Prolyl-AMP ligase	*pigI*
HapJ	37	37	39	Pyrrolyl-β-ketoacyl ACP synthase	*pigJ*
HapK	46	43	52	Hypothetical protein	*pigK*
HapL	-	-	29	4'-Phosphopantetheinyl transferase	*redU*
HapM	31	32	30	HBM oxidase/dehydrogenase	*pigM*
HapN	56	58	28	Oxidoreductase	*pigN*

*Percentage of identity was obtained by aligning the deduced amino acid sequences using BlastP. Pig [39006], Pig protein from *Serratia* sp. ATCC 39006; Pig [274], Pig protein from *Ser. marcescens* ATCC 274; Red [A3(2)], Red protein from *S. coelicolor* A3(2).

assumed that *orf9* and *orf10* should regulate the prodigiosin production in KCTC 2396. In addition to the prodigiosin biosynthetic genes and regulatory genes, a putative membrane-associated protein gene (*orf5*) and three putative microcystin-dependent protein genes (*orf11*, *orf12*, and *orf13*) are also located near to the gene cluster. Thus, based on homology analysis using BLAST searches, this cluster was apparently divided into three functional categories: genes for MBC

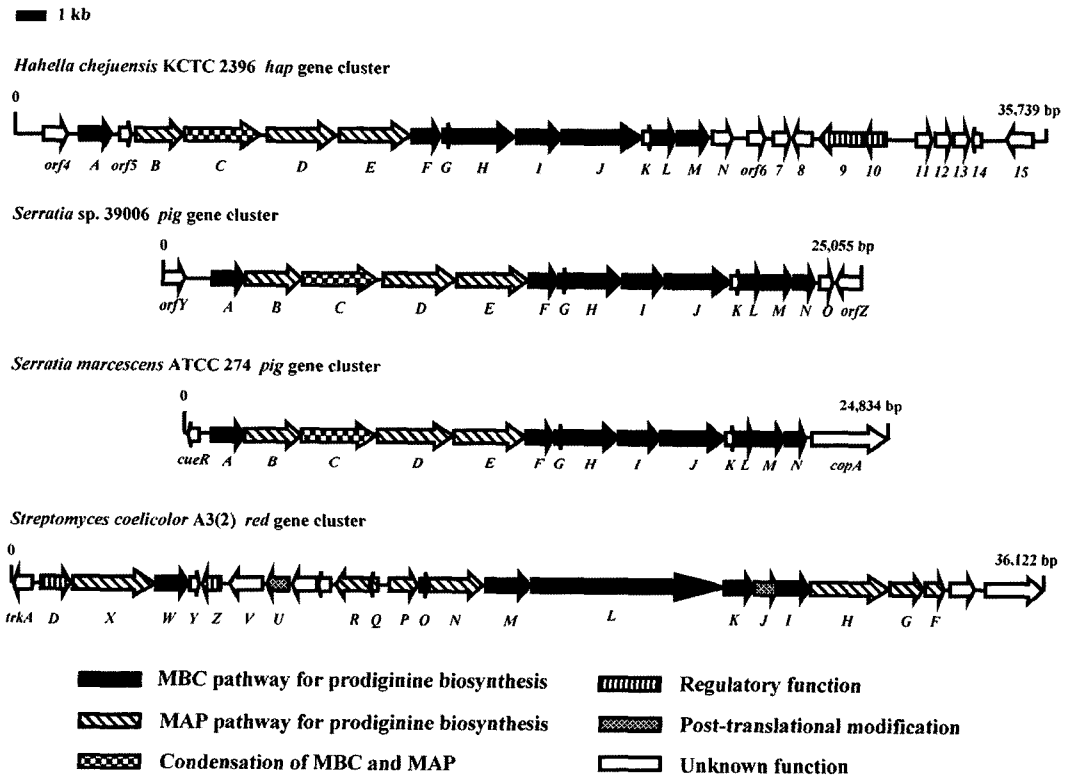

Fig. 2. Comparison of the prodiginine biosynthetic clusters.
The *pig* [16] and *red* [1] genes are divided by their functions, which were described by the previously published data. The directions of transcription are indicated by arrowheads.

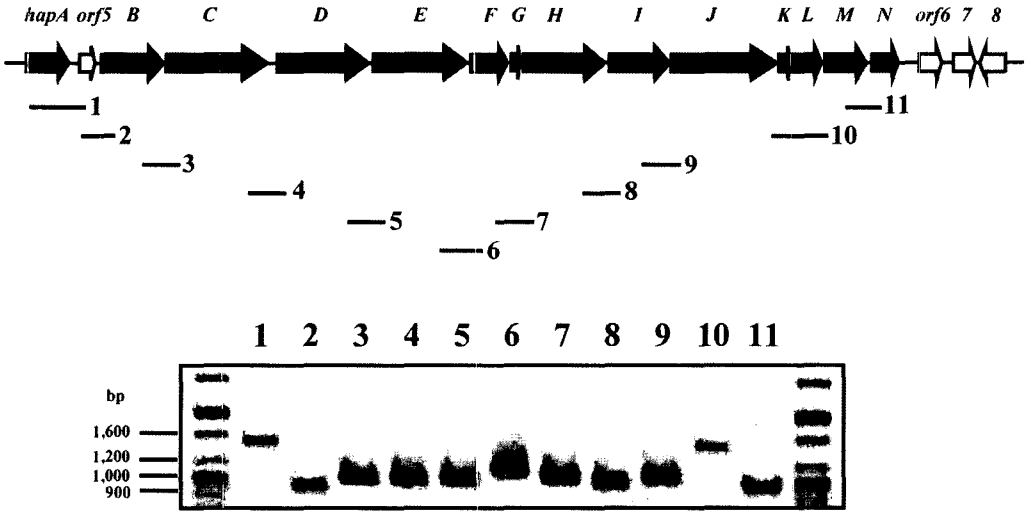

Fig. 3. Gene organization of the prodigiosin biosynthetic operon from *Hahella chejuenesis* KCTC 2396.
Black arrows indicate structural genes for the prodigiosin biosynthesis. Open reading frames are shown as white arrows. The direction of transcription is indicated by arrowheads. The nucleotide numbers, marked on the end of the lines, indicate relative positions in the nucleotide sequence of GenBank accession number DQ266254. The lower panel shows agarose gel electrophoresis of RT-PCR products. The expected PCR products for each well are indicated in the gene map. The first lane was loaded with a molecular weight marker.

and MAP production, regulatory functions, and unknown functions (Fig. 2).

Operon Structure and Function of the Prodigiosin Biosynthetic Genes

Comparison of the *red* with *pig* gene clusters revealed that they are dissimilar in the gene organization and operonic structure, in spite of the genes' functional identity among them. The *red* cluster consists of 23 genes organized into four transcriptional units, and is larger than the *pig* clusters (14–15 genes in a single operon). Considered from the prodigiosin production in *Serratia* spp. and KCTC 2396, the gene organization and operon structure of the *hap* cluster was expected to be more similar to those of *pig* clusters from the two *Serratia* spp. than the *red* cluster from *S. coelicolor* A3(2) (Fig. 2).

To determine the operon structure of the *hapA-hapN* gene cluster, RT-PCR analysis was performed with the total RNA extracted from the KCTC 2396 cells producing prodigiosin. As shown in Fig. 3, the RT-PCR products with the expected sizes (Table 1) were amplified for all the samples, whereas no PCR products were obtained in the control using only PCR reaction. The results imply that the *hapA-hapN* genes are transcribed as the same transcription unit in the KCTC 2396 cells.

Several transposon insertions in *hap* genes (*hapA*, *B*, *C*, *D*, *E*, *H*, and *J*) and an additive *hapD*-targeted disruption resulted in white colonies, in all cases, compared with the HC81006F09-R4 fosmid clone constitutively producing prodigiosin. The total metabolites from the white mutants were extracted as described in Materials and Methods for identification of prodigiosin, and analyzed by LC-ESI-MS spectrometry. As shown in Table 3, the white mutants were not able to produce prodigiosin at all, whereas red wild-type HC81006F09-R4 produced prodigiosin. Interestingly, a disruption of *orf7* (51% identity with an uncharacterized conserved bacterial protein, *Ralstonia metallidurans* CH34), located at 1.4 kb downstream from the 3'-end of the *hap* operon, caused complete inhibition of prodigiosin production. The results imply that the mutated *hap* genes and *orf7* are functionally involved in prodigiosin production in KCTC 2396 cells.

Proposed Prodigiosin Biosynthetic Pathway

The biosynthetic pathways for undecylprodiginine and its structural analog prodigiosin were proposed in which

Table 3. Detection of prodigiosin from the *hap* gene-insertional mutants of the HC81006F09-R4 fosmid.

Compound	HC81006F09-R4	Transposon insertion-mutated gene							
		hapA	*hapB*	*hapC*	*hapD*[c]	*hapE*	*hapH*	*hapJ*	*orf7*
Prodigiosin	+[a]	−[b]	−	−	−	−	−	−	−

[a]Detected by LC-ESI-MS technique, but not determined quantitatively.
[b]Not detected at all.
[c]Also disrupted by the gene-targeted mutagenesis.

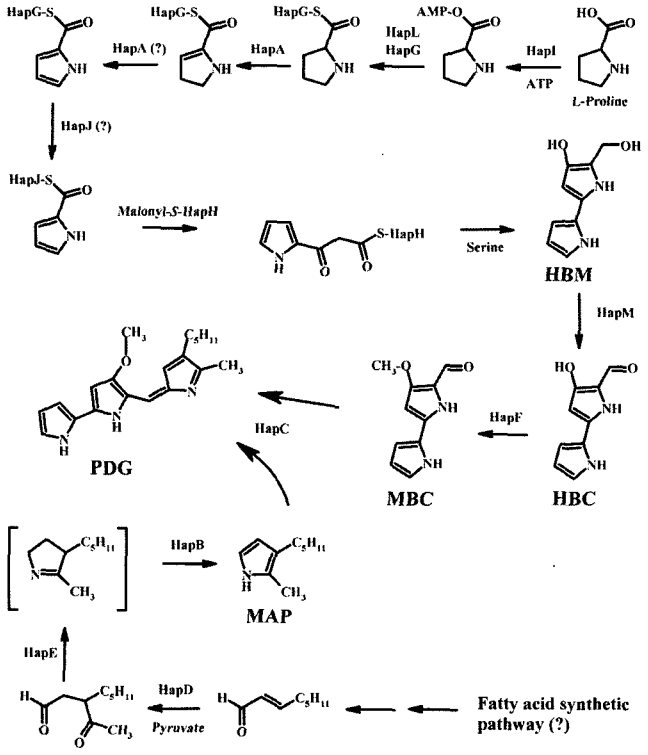

Fig. 4. Proposed pathway for the bifurcated biosynthetic routes in prodigiosin production in *Hahella chejuenesis* KCTC 2396.
The gene designations for each step are shown. The names for the key intermediates and final products in this pathway are indicated as follows: HBM, 4-hydroxy-2,2'-bipyrrole-5-methylalcohol; HBC, 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde; MBC, 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde; PDG, prodigiosin; MAP, 2-methyl-3-amylpyrrole.

bipyrrole MBC is a common building block of them. Although MBC was supposed to share generally a similar biosynthetic route, some catalytic reactions and corresponding enzymes have been proposed and described differently between *Serratia* sp. ATCC 39006 and *S. coelicolor* A3(2) [1, 6, 16]. This discrepancy appears to be originated from insufficient functional characterization for the putative catalytic genes in the two strains, which also makes it difficult to assign the roles of *hap* genes in the MBC pathway. Overall, the MBC pathway in KCTC 2396 has been proposed as shown in Fig. 4 (see Table 2 for the assigned function of each *hap* gene), based on the biosynthetic pathway of prodigiosin from *Serratia* sp. ATCC 39006 [6, 16].

Although MBC was supposed to share similar biosynthetic steps among the microorganisms, two monopyrroles, MAP and 2-undecylpyrrole (a monopyrrolyl counterpart for MAP), were reported to be synthesized through different steps from each other. In general concept, the monopyrrolyl biosynthetic pathway appears to follow the fatty acid biosynthesis, the initial steps for which would produce various monopyrrolyl building blocks with different carbon chains in length. As in the case of MBC, the proposed MAP biosynthetic pathway in KCTC 2396 would be generally similar to that in *Serratia* sp. ATCC 39006, although additive experimental data are required to confirm the KCTC 2396 MAP pathway. Finally, synthesized MBC and MAP intermediates should be condensed to generate the final product prodigiosin (Table 2 and Fig. 4).

DISCUSSION

In the course of the genome sequencing project for *H. chejuensis* KCTC 2396, one red fosmid clone (HC81006F09-R4) from the KCTC 2396 library pool was selected, inserts of which were homologous to several undecylprodiginine *red* genes of *S. coelicolor* A3(2) and prodigiosin *pig* genes of *Serratia* spp. It was shown that HC81006F09-R4 contained the entire gene cluster (*hapA-hapN)* needed for prodiginine production. Additionally, the successful sequencing and exhaustive sequence analysis of the KCTC 2396 genome (7.2 Mb) show us no more gene clusters related to the prodiginine production on the KCTC 2396 genome, except the *hap* gene cluster cloned in HC81006F09-R4. From the above descriptions, it was assumed that prodigiosin and undecylprodiginine could be synthesized by *E. coli* containing HC81006F09-R4. However, we could not detect undecylprodiginine at all, although prodigiosin was constitutively produced by the heterologous expression in *E. coli*.

The usefulness of prodiginines as future prospective pharmaceutical agents [9, 17] promoted researchers to study the biosynthesis of prodigiosin and its analogs from a viewpoint of the biosynthetic step and catalytic enzymes. KCTC 2396 is able to produce a mixture of at least two prodiginines, which contain the common linear tripyrrolyl structure with cytotoxic C-6 methoxy substituent. Therefore, KCTC 2396 could provide several new strategies, both *in vivo* and *in vitro*, to develop novel prodigiosin derivatives with an improved function and activity.

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