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Duplicate Isochorismate Synthase Genes of *Bacillus subtilis*: Regulation and Involvement in the Biosyntheses of Menaquinone and 2,3-Dihydroxybenzoate

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***Bacillus subtilis* has duplicate isochorismate synthase genes, *menF* and *dhbC*. Isochorismate synthase is involved in the biosynthesis of both the respiratory chain component menaquinone (MK) and the siderophore 2,3-dihydroxybenzoate (DHB). Several *menF* and *dhbC* deletion mutants were constructed to identify the contribution made by each gene product to MK and DHB biosynthesis. *menF* deletion mutants were able to produce wild-type levels of MK and DHB, suggesting that the *dhbC* gene product is able to compensate for the lack of MenF. However, a *dhbC* deletion mutant produced wild-type levels of MK but was DHB deficient, indicating that MenF is unable to compensate for the lack of DhbC. A *menF dhbC* double-deletion mutant was both MK and DHB deficient. Transcription analysis showed that expression of *dhbC*, but not of *menF*, is regulated by iron concentration. A *dhbA′*::*lacZ* fusion strain was constructed to examine the effects of mutations to the iron box sequence within the *dhb* promoter region. These mutations abolished the iron-regulated transcription of the *dhb* genes, suggesting that a Fur-like repressor protein exists in *B. subtilis*.**

Isochorismate synthase is responsible for converting chorismate to isochorismate and is necessary for the biosynthesis of both the respiratory chain component menaquinone (MK) and the *Bacillus subtilis* siderophore 2,3-dihydroxybenzoate (DHB) (Fig. 1). Although it is known that the carbon source, growth phase, and oxygen tension have regulatory effects, the actual signals that induce expression of respiratory chain components are unknown (37). However, the production of DHB is known to be regulated by iron concentration (1, 25, 26). Therefore, isochorismate is required by cells under very different environmental conditions.

B. subtilis has two distinct isochorismate synthases encoded by the *menF* (30) and *dhbC* (29) genes. MenF and DhbC are 47% identical at the DNA level and have 35% amino acid identity (29). *menF* is a promoter-proximal gene of the MK biosynthetic gene cluster located at 273° on the chromosome (30, 38). MK is a lipophilic, nonprotein redox component mediating electron transfer between dehydrogenases and cytochromes (37). *dhbC* is the second gene of the DHB biosynthetic gene cluster located at 291° on the chromosome (29). Under conditions of iron deprivation, *B. subtilis* synthesizes DHB, a component of the specific transport system for the uptake of extracellular iron (1, 25, 26).

This is not the only instance of gene duplication in *B. subtilis*. There are two thymidylate synthetases, TSaseA and TSaseB, that are encoded by the unlinked *thyA* and *thyB* genes, respectively (22). Both enzymes are functional in bacteria grown at 37°C or lower temperatures, but only TSaseA is active at 46°C (22). There are two different *a*-type terminal oxidases in *B. subtilis* that are encoded by separate loci (32). One of the oxidases is associated with a heme C-containing unit (32). The two membrane alkaline phosphatases of *B. subtilis* have substantial differences with respect to molecular weight, substrate specificity, and thermal stability (11, 36). The alkaline phosphatase genes have been cloned and, by sequence analysis, shown to have 63% amino acid identity (12). *B. subtilis* secretes at least seven proteases, four of which are serine proteases that have amino acid similarities (24). Also, *B. subtilis* has two citrate synthase genes, *citA* and *citZ* (13), whose expression is differentially regulated (14). The identification of two genes, *upp* and *pyrR*, in *B. subtilis* encoding uracil phosphoribosyltransferase has been recently reported (16).

This study was undertaken to determine what roles the duplicate isochorismate synthases play in the biosyntheses of MK and DHB. In addition, understanding the regulation of these two systems might suggest why these isozymes exist and provide insight into how *B. subtilis* responds to its environment.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* and *Escherichia coli* strains and plasmids are listed in Table 1. Media were from Difco Laboratories. Growth supplements and antibiotics were from Sigma Chemical Co. *B. subtilis* strains carrying integrative plasmids conferring resistance to either chloramphenicol or kanamycin were grown on Luria-Bertani (LB) agar plates containing 5 μg of chloramphenicol per ml or 10 μg of kanamycin per ml, respectively. *E. coli* strains carrying plasmids conferring ampicillin resistance were grown in Luria broth and on LB agar plates supplemented with 50 μg of ampicillin per ml. The *B. subtilis men* mutant strain RB1250 was maintained on tryptose-blood-agar-base plates containing 0.5% glucose and 18 μM menadione (TG_{18}); liquid media were supplemented with 5 μg of menatetrenone per ml. Iron starvation (IS) minimal medium was prepared by following the instructions of Chen et al. (3). MGT medium contained 1.5% agar, 1% Spizizen's solution, 0.5% glucose, and 0.05 mg of tryptophan per ml.

Genetic techniques and DNA manipulations. *B. subtilis* was transformed by the method of Piggot et al. (27). *E. coli* was transformed by the method of Hanahan (8). Plasmid DNA was prepared from *E. coli* by following a standard protocol (15). Restriction digestions, ligations, and subclonings were performed by following standard procedures (15). Enzymes were purchased from U.S. Biochemical Corp.

Construction of deletion mutants. In-frame deletions of *menF* and *dhbC* were constructed in vitro. Plasmid pAI112 carries a 540-bp *Hin*dIII-*Pvu*II deletion within *menF* that was constructed as follows: plasmid pAI46 (21) was digested

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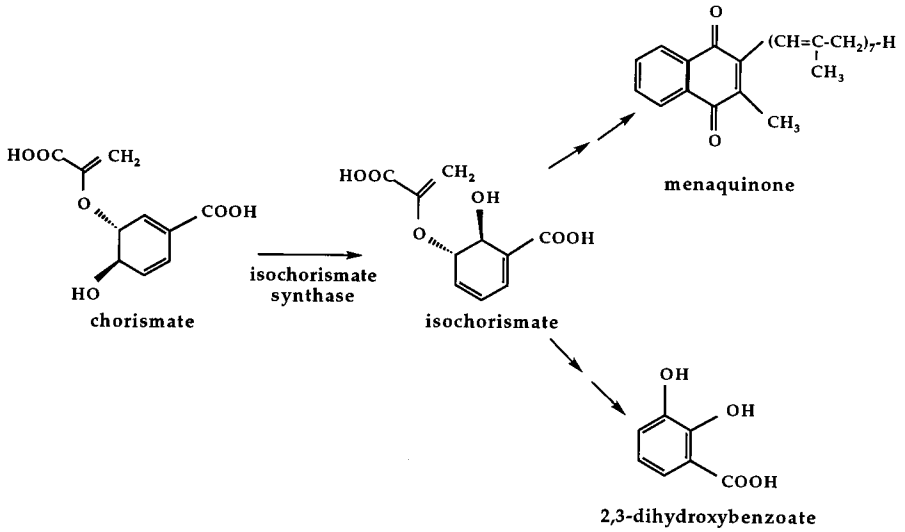

FIG. 1. Pathways for MK and DHB biosynthesis.

with *Sal*I, treated with Klenow, digested with *Sac*I, and ligated with the 1-kbp *Sac*I-*Pvu*II fragment of plasmid pAI79 (30). Plasmid pAI166 carries a fragment with a 980-bp deletion within *dhbC* that was generated by the technique of overlap extension PCR (10) with linear pENT4 as the template and the four synthetic oligonucleotides d1 (5′ GAAACAGACATGCAGTGG), d2 (5′ CCG CAGCATTGTCCGAAACTCTGCAATTCGGCCAGAAAGG), d3 (5′ CCTT TCTGGCCGAATTGCAGAGTTTCGGACAATGCTGCGG), and d4 (5′ AG GAAATGCGTCATCTGG). *B. subtilis* RB1 was transformed with pAI112 or pAI166 with selection for chloramphenicol resistance. Integrants that had undergone a double crossover event and lost the intact gene were identified by in situ hybridization with *menF*- or *dhbC*-specific oligonucleotide probes. Strain RB1120 (with a deletion in *menF*) was transformed with pAI166, and the process was repeated to obtain the double-deletion strain RB1250. Chloramphenicol-sensitive segregants were isolated and chromosomal deletions were confirmed by Southern hybridization. PCR-amplified fragments of chromosomal DNA served as templates for DNA sequencing and confirmed the sequences of the deletion junction regions.

MK determination. LB medium was pretreated with α,α-dipyridyl for 16 to 24 h at room temperature. Ten-milliliter aliquots of overnight cultures were used to inoculate 500 ml of LB medium with or without 210 μM α,α-dipyridyl in a 2.8-liter Fernbach flask. The culture was incubated at 37°C on a platform shaker at 250 rpm until 1 h after the transition point (T_0) between the exponential and stationary growth phases (the T_1 growth stage) was reached. Membranes were extracted in chloroform-methanol (2:1, vol/vol) and prepared by following the procedure of Meganathan and Cofell (17). Thin-layer chromatography was performed by following the procedure of Salton and Schmitt (31). A silica gel thin-layer plate (type SI250; Baker) was preactivated at 100°C for 30 min. The activated plate was spotted with 50.0 μl of each cell extract, menadione, and menatetrenone (Sigma) and placed into the chamber with iso-octane–diethyl ether (100:30, vol/vol). After the solvent front migrated 14 cm, quinones were visualized with long-wave (365 nm) UV light. To visualize by charring, a 5% sulfuric acid solution (in ethanol) was sprayed onto the plate, which was allowed to dry and baked at 100°C for 10 min.

DHB assay. The presence of DHB(G) in culture supernatants was determined by the method described by Peters and Warren (25). Briefly, *B. subtilis* strains were grown for 24 h in IS medium with and without 5 μM $FeCl_3$. Cell densities were determined by measuring optical densities at 600 nm (OD_{600}). The pH of a 1.0-ml sample of the culture was raised to 7.6 with 5 M NaOH after 0.45 ml of 10 mM $FeCl_3$ (in 100 mM HCl) was added. The A_{510} was determined, and A_{510}/OD_{600} was calculated.

β-Galactosidase assay. The desired *B. subtilis* strain was inoculated into 10.0 ml of LB in a 125-ml flask and incubated overnight at 28°C with shaking at 250 rpm. A 2.0-ml aliquot of the culture was used to inoculate 100 ml of the desired medium in a 1,000-ml flask. Cultures were grown in a 37°C Gyrotory shaker bath (New Brunswick Scientific), and cell samples were taken at half-hourly or hourly intervals. β-Galactosidase activity was determined by following the method of Zuber and Losick (40). Miller units (18) were calculated by the following equation: Miller units = $1{,}000/(\text{min})(\text{ml}) \times A_{420}/OD_{600}$, where min is the reaction time and ml is the volume of lysate used in the reaction mixture.

Primer extension analysis. The synthetic oligonucleotide PE4 (5′ TCCCTC TATACCCTTTGC 3′) was 5′ end labelled with [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont, New England Nuclear) with T4 polynucleotide kinase according to the supplier's protocol. Oligonucleotides were separated from unincorporated precursors on a Bio-Spin 6 chromatography column (Bio-Rad).

Total cellular RNA was isolated from strain RB1 grown to T_0 in IS medium with and without 5 μM $FeCl_3$ as described for the RNase protection assay. Ten micrograms of RNA from *B. subtilis* RB1 or *Saccharomyces cerevisiae* was coprecipitated with 4×10^6 cpm of radiolabelled oligonucleotide and resuspended in 20.0 μl of hybridization buffer (RPA II kit; Ambion). Samples were heated at 90°C for 3 min and incubated at 42°C for 16 to 20 h. The nucleic acids were precipitated and resuspended in 11.0 μl of distilled water. Primer extension was performed with the First-Strand cDNA synthesis kit (Pharmacia Biotech) according to the supplier's protocol, except that the 37°C incubation was increased to 2 h. The extension reaction was stopped by the addition of 89.0 μl of STE buffer (150 mM NaCl, 150 mM EDTA, 10 mM Tris-HCl [pH 7.5]) and extracted once with 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acids in the aqueous layer were precipitated and resuspended in 3.0 μl of distilled water and 2.0 μl of loading buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 2 mM EDTA). Primer extension products were resolved on 5% acrylamide–7 M urea gels (National Diagnostics). To serve as size comparison markers, DNA sequencing products derived from pAI169 with PE4 as the primer were included on the gel. Dried gels were exposed to Biomax MR film (Kodak) at −80°C for 12 to 20 h.

RNase protection assay. The [^{32}P]UTP-labelled cRNA probe was prepared with the MAXIscript in vitro transcription kit (Ambion). Template DNA was prepared by incorporating the T7 promoter elements into the target sequence by PCR with the GeneAmp PCR reagent kit (Perkin-Elmer Cetus) and a Perkin-Elmer DNA thermal cycler 480. A 323-bp fragment containing portions of *dhbA* and *dhbC*, including intergenic sequences (see Fig. 5), was amplified from linearized pENTB2 template with oligonucleotides 5′ TTGAGAATTCAGCCT TCGGATATTGCGG and 5′ GGATCCTAATACGACTCACTCACTATAGG GAGGCAATTCGGCCAGAAAGG. The core T7 promoter sequences (underlined) were appended to the 5′ end of the *dhbA*-specific primer. Transcription was carried out in the presence of [α-^{32}P]UTP (Amersham) with the purified PCR product as the template according to the manufacturer's instructions. Reaction mixtures were loaded onto 5% acrylamide–8 M urea gels. Following electrophoresis at 200 V for 1 h, the gel was exposed to a X-Omat AR film (Kodak) for 1 to 5 min to visualize the transcription products. The full-length transcript was eluted overnight from the gel slice at 37°C.

Total cellular RNA was prepared from *B. subtilis* RB1 grown in IS medium with and without 5 μM $FeCl_3$ at growth stages T_{-2}, T_{-1}, T_0, T_1, T_2, and T_3 with the RNaid PLUS kit (Bio 101). RNA integrity was confirmed on ethidium bromide-stained agarose gels. RNase protection was performed with an RPA II kit (Ambion). To hybridize cellular RNA to the cRNA probe, 5.0 μg of *B. subtilis* total cellular RNA was mixed with the cRNA probe (3×10^4 cpm), heated to 90°C, and incubated overnight at 42°C. Unprotected RNA was digested at 37°C for 30 min in RNase digestion buffer (Ambion) containing 0.5 U of RNase A and 20.0 U of RNase T_1. Protected RNA fragments were resolved on 5% acrylamide–7 M urea gels at 60 W (1,200 to 2,000 V). Dried gels were exposed to X-Omat AR film (Kodak) at −80°C for 12 to 18 h.

Site-directed mutagenesis of the iron box sequence. The technique of overlap extension PCR (10) was used to make the nucleotide sequence changes in the iron box with linear pENT4 as the template and the four synthetic oligonucleotides FeA (5′ ttgacagagctgAGACATACTCAGCCTTGCC), FeB (5′ TTGAT

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
B. subtilis		
RB1	Marburg strain 168, *trpC2*	Our laboratory
RB941	RB1ΩpAI200, Cam^r *menF′*::*lacZ*	9
RB1076	RB1ΩpAI60*cat*, Cam^r Men$^{+/-}$, *menF* insertion disruption	20
RB1120	RB1Δ*menF*, MK$^+$ DHB$^+$, 540-bp *Hin*dIII-*Pvu*II deleted	This study
RB1209	RB1Δ*menF*, Men$^+$ DHB$^+$, 160-bp *Rsa*I-*Sau*3A deleted	X. Qin
RB1210	RB1Δ*menF*, Men$^+$ DHB$^+$, 300-bp *Rsa*I-*Pst*I deleted	X. Qin
RB1238	RB1Δ*menF*, Men$^+$, DHB$^+$, 160-bp *Hae*III-*Hin*cII deleted	X. Qin
RB1250	RB1120Δ*menF*, Δ*dhbC*, MK$^-$ DHB$^-$, 980 bp of *dhbC* and 540 bp of *menF* deleted	This study
RB1255	RB1Δ*dhbC*, MK$^+$ DHB$^-$, 980 bp of *dhbC* deleted	This study
RB1273	RB1ΩpAI173, *dhbA′*::*lacZ* Amy$^-$ Kan^r Lac$^+$, mutated *dhb* promoter iron box	This study
RB1274	RB1ΩpAI174, *dhbA′*::*lacZ* Amy$^-$ Kan^r Lac$^+$	This study
E. coli		
JM107	e14, (*mcrA*) Δ(*lac-proAB*) *thi-1 gyrA96 endA1 hsdR17 relA1 supE44*, [F′ *traD36 proAB lacI*^{q}ZΔM15]	Our laboratory
JM109	e14, (*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	L. Flaherty
DH5α	F$^-$ ϕ 80d*lac*ZΔM15 Δ(*lacZYA-argF*) *deoR recA1 endA1 hsdR17 supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco, BRL
Plasmids		
pSGMU2	*E. coli* replicon, Amp^r Cam^r, cloning vector	J. Errington
pENTB2	pBR322 with ~4.5 kbp of *B. subtilis* DNA, carries *dhbC*, *dhbE*, and part of *dhbB*	6
pENT4	pBR322 with ~6 kbp of *B. subtilis* DNA, carries *dhbA*, *dhbC*, *dhbE*, *dhbB*, and part of *dhbF*	6
pMD433	*E. coli* replicon, Amp^r Kan^r, *amyE* integration, *lacZ* fusion vector	4
pAI46	700-bp *Hin*dIII fragment cloned into pSGMU2, *men* clone	21
pAI79	1.6-kbp *Hin*dIII-*Sac*I fragment cloned into pBS$^-$, *men* clone	30
pAI112	*menF* deletion plasmid, 1.0-kbp *Pvu*II-*Sac*I fragment of pAI79 cloned into *Sac*I and Klenow-filled *Sal*I sites of pAI46	This study
pAI166	*dhbC* deletion plasmid, 1.3-kbp *Bam*HI-*Sac*I fragment of overlap extension PCR product cloned into *Bam*HI-*Sac*I sites of pSGMU2	This study
pAI173	*dhbA′*::*lacZ* translational fusion in pMD433, mutated iron box	This study
pAI174	*dhbA′*::*lacZ* translational fusion in pMD433	This study

AGTGGATTGATAATTCAATTATAAAAATAATCATATCC), FeC (5′ TT GAATTATCAATCCACTATCAATAGATTGCGTTTTTCAAGG), and FeD (5′ ttgacaggatccAACAGCTTCGCCTATTCC). Lowercase letters indicate non-*dhb*-specific sequences appended to the oligonucleotide primers to facilitate cloning. The overlap extension product and the wild-type PCR product were digested with *Pvu*II and *Bam*HI and cloned into the *Sma*I-*Bam*HI sites of pMD433 (4). pMD433 is a member of a set of *lacZ* translational fusion vectors that are capable of integrating into the *amyE* locus of the *B. subtilis* chromosome. The DNA sequences of the PCR-amplified fragments of the mutated iron box construct (pAI173) and the wild-type promoter construct (pAI174) were confirmed. Linearized plasmids were transformed into RB1 with selection on LB agar plates containing 10 μg of kanamycin per ml. All of the transformants screened were unable to degrade starch, suggesting that the *lacZ* fusion plasmids had integrated into *amyE*. Genetic linkage experiments confirmed that *amyE* was 100% linked by transformation to the kanamycin resistance marker of plasmid pAI173 or pAI174. DNA sequence analysis of PCR-amplified fragments of the chromosome confirmed the sequence of the cloned material carried by the integrated *lacZ* fusion plasmids.

RESULTS

To determine what role *menF* and *dhbC* gene products play in MK and DHB biosyntheses, strains carrying in-frame deletion mutations were created. The *menF* deletion and insertion strains are shown in Fig. 2. Strains RB1209 and RB1210 have deletions encompassing 11 and 22% of the *menF* open reading frame, respectively. Strain RB1238 carries a 160-bp deletion encompassing the putative *menF* ribosome binding site, initiation codon, and first 23 codons. Strain RB1120 has 38% of the *menF* open reading frame deleted. The insertion strain RB1076 has the *cat* gene inserted into the *Hin*cII site within *menF* (20). Over 80% of *dhbC* has been deleted in strain

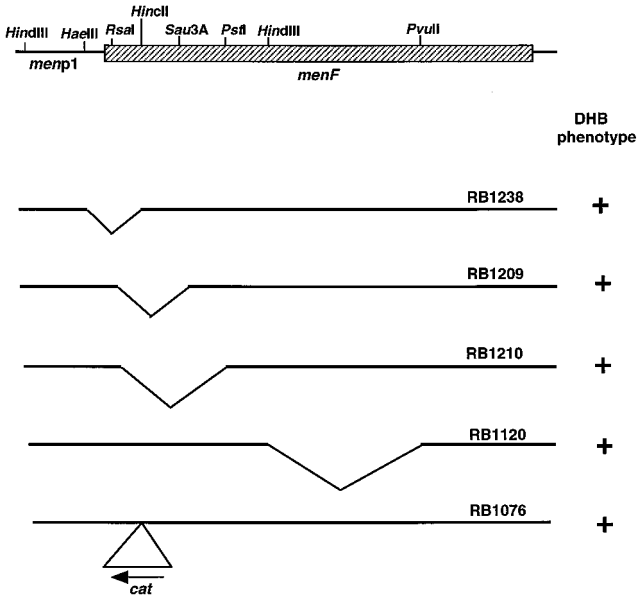

FIG. 2. *menF* deletion and insertion strains. Relevant restriction sites of the *menF* region are shown. Fragments deleted: RB1238, the 160-bp *Hae*III-*Hin*cII fragment; RB1209, the 160-bp *Rsa*I-*Sau*3A fragment; RB1210, the 300-bp *Rsa*I-*Pst*I fragment; RB1120, the 540-bp *Hin*dIII-*Pvu*II fragment. RB1076 has the *cat* gene inserted into the *Hin*cII site.

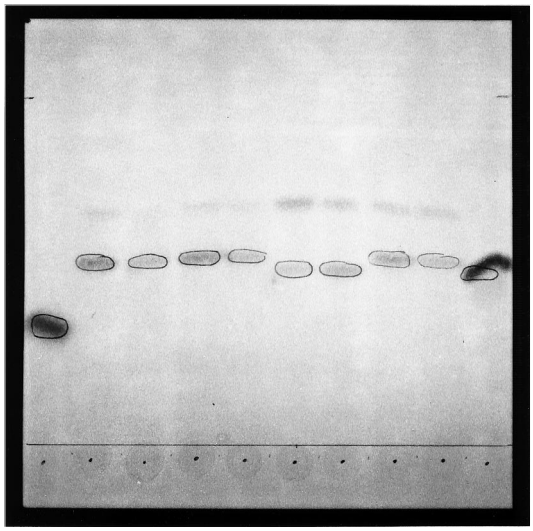

FIG. 3. Thin-layer chromatogram of *B. subtilis* membrane preparations. The membranes were extracted with chloroform-methanol (2:1, vol/vol) (17). Fifty-microliter aliquots of the membrane extracts were spotted onto a silica gel thin-layer plate and developed with iso-octane–diethyl ether (100:30, vol/vol) (31). Lanes 2, 4, 6, and 8, membranes extracted from bacterial cells grown in LB medium; lanes 3, 5, 7, and 9, membranes extracted from bacterial cells grown in LB medium with 210 μM α,α-dipyridyl. Lane 1, purified menadione; lanes 2 and 3, RB1; lanes 4 and 5, RB1120; lanes 6 and 7, RB1250; lanes 8 and 9, RB1255; lane 10, purified menatetrenone. Long-wave (365 nm) UV light-absorbing spots corresponding to quinones are circled. Sulfuric acid charring was performed by spraying the silica gel plate with a 5% solution of sulfuric acid (in ethanol) and baking the plate at 100°C for 10 min.

RB1255, and the double-deletion strain RB1250 has 38% of *menF* and 80% of *dhbC* deleted.

Effect of deletion mutations on MK biosynthesis. MK-deficient *B. subtilis* strains are unable to grow on minimal medium plates without supplementation with a MK homolog. However, MK-deficient mutants can grow on complex medium without supplementation, albeit very poorly. The *menF* deletion strains RB1120, RB1209, RB1210, and RB1238 were all Men^+ and grew as well as wild-type strain RB1 on tryptose-blood-agar-base, LB, TG_{18}, and unsupplemented MGT agar media. Strain RB1076 had a partial Men^- phenotype, presumably because of polar effects on downstream *men* genes caused by the *cat* gene insertion. Growth phenotypes indicated that *dhbC* deletion strain RB1255 is MK sufficient. The double-deletion mutant strain RB1250 grew very poorly on tryptose-blood-agar-base and LB agar media but formed larger colonies on TG_{18} agar medium. These data suggest that strain RB1250 has a Men^- phenotype.

To test by direct chemical measurement whether strains RB1120, RB1250, and RB1255 were capable of MK synthesis, thin-layer chromatography of *B. subtilis* membranes was performed (Fig. 3). Strains RB1, RB1120, RB1250, and RB1255 were grown in LB medium with and without the iron chelator α,α-dipyridyl. The bacterial membranes were extracted with chloroform-methanol (17), spotted onto a thin-layer plate, and developed with iso-octane–diethyl ether (31). Menadione, which lacks an isoprenoid side group, and menatetrenone (MK4), which has four isoprenoid units in the side chain compared with seven isoprenoid units in MK7 (the isoprenolog formed by *B. subtilis*), were included as controls. UV light-absorbing spots corresponding to MK7, which migrated slightly faster than the MK4 control, were present in membrane extracts of strains RB1, RB1120, and RB1255. The MK4 control ran somewhat aberrantly because of its position on the chromatographic plate; however, it was routinely seen to migrate more slowly than MK7. There was no detectable spot corresponding to MK7 in the membrane extract of strain RB1250; only a spot corresponding to MK4, the quinone added to the culture medium of this strain to support growth, was found. Sulfuric acid charring was used to identify UV light-absorbing chromatogram components as carbon-containing compounds. The UV light-absorbing spots were visualized by charring (Fig. 3), confirming the presence of naphthoquinones in this region of the chromatogram. Although this procedure was not quantitative, visual estimates of MK7 concentrations showed no major differences in the membranes of cells grown in LB medium or in LB medium with 210 μM α,α-dipyridyl (Fig. 3), suggesting that MK biosynthesis is not regulated by iron.

Effect of deletion mutations on DHB biosynthesis. Extracellular DHB levels were measured to determine what effect the *menF* and *dhbC* deletions had on DHB biosynthesis. The four *menF* deletion strains and the *cat* gene insertion strain were positive for DHB production compared with strain RB1. The A_{510}/OD_{600} values (see Materials and Methods) for strain RB1 and all test strains were similar but varied between 0.15 and 0.3 in different experiments. A_{510}/OD_{600} values of 0.02 to 0.04 were observed when 5 μM $FeCl_3$ was added to the cultures. These data indicate that DHB production is inhibited by iron and does not rely upon the *menF* gene product. Average A_{510}/OD_{600} values for the *dhbC* deletion strain RB1255 and the double-deletion strain RB1250 were 0.025 and 0.005, respectively, which confirms that *dhbC* is required for DHB biosynthesis. In addition, MenF is unable to compensate for the loss of DhbC because the *dhbC* deletion strain RB1255 does not produce DHB.

Effect of iron concentration on *menF* expression. Strain RB941 has, integrated at the *men* locus, the first 32 codons of

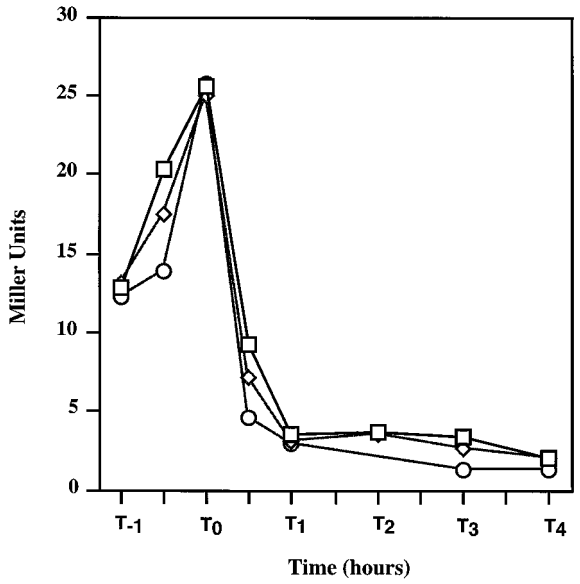

FIG. 4. Expression of *menF'*::*lacZ* in LB medium with and without 200 μM α,α-dipyridyl or 20 μM $FeSO_4$. Strain RB941 was grown in LB medium (□), LB medium with 200 μM α,α-dipyridyl (○), and LB medium with 20 μM $FeSO_4$ (◇). Cell samples were taken at half-hourly or hourly intervals, and β-galactosidase activity was determined as described in Materials and Methods. Timepoints: T_0 indicates the transition point between the exponential and stationary growth phases; T_{-1}, 1 h before T_0; T_1 to T_4, 1 to 4 h after T_0, respectively.

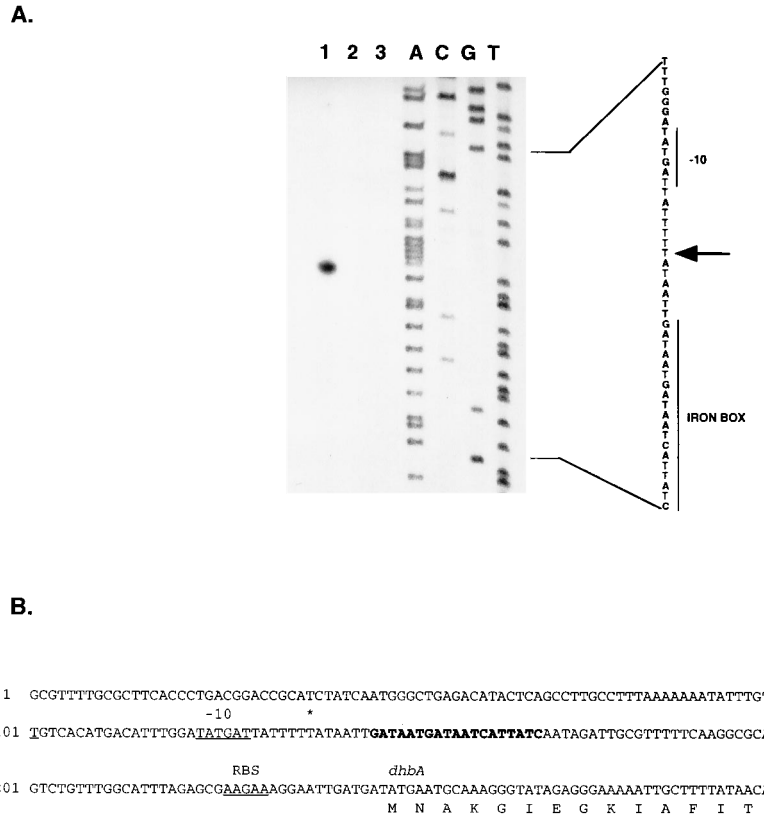

FIG. 5. Locations of the 5′ termini of *dhb* transcripts by primer extension. (A) Primer extension. Lane 1, extension product obtained by hybridizing 4×10^6 cpm of ^{32}P-labelled oligonucleotide PE4 to 10.0 μg of RNA isolated from strain RB1 grown in IS medium and treated with reverse transcriptase; lane 2, 4×10^6 cpm of oligonucleotide PE4 hybridized to 10.0 μg of RNA isolated from strain RB1 grown in IS medium plus 5 μM $FeCl_3$ and treated with reverse transcriptase; Lane 3, 4×10^6 cpm of oligonucleotide PE4 hybridized to 10.0 μg of yeast RNA and treated with reverse transcriptase; lanes A, C, G, and T, nucleotide sequence derived from pAI169 with PE4 as the primer. The DNA sequence of the sense strand is shown to indicate the locations of the −10 promoter element and the iron box with respect to the transcriptional start site (arrow). (B) Nucleotide sequence of the first 300 bp of the *B. subtilis dhb* operon (29; GenBank accession number U26444). A portion of the *dhbA* gene is indicated. Abbreviations and notations: RBS, ribosome binding site; *, an RNA 5′ terminus maps to this nucleotide; underlined nucleotides, promoter sequences; nucleotides in boldface type, sequence identical to the *E. coli* Fur operator consensus sequence.

menF fused to the ninth codon of *E. coli lacZ*, thereby creating a MenF′::LacZ fusion protein (9). To study the expression of *menF*, strain RB941 was grown in LB medium, LB medium plus 200 μM α,α-dipyridyl, and LB medium plus 20 μM $FeSO_4$. The results of the β-galactosidase assay indicate that the $menp_1$ promoter, which controls the expression of *menF*, is unaffected by iron concentration (Fig. 4). Maximal promoter activity occurred at the end of the exponential growth phase and declined rapidly thereafter, a finding which is in close agreement with the previous studies of *menp*1 (9, 19). The kinetics of *menp*1 activity in strain RB941 grown in IS medium or IS medium plus 5 μM $FeCl_3$ were similar (data not shown), confirming that *menF* expression is not regulated by iron.

Identification of the *dhb* promoter region and 5′ termini of transcripts. Plasmid integration studies suggested that transcription of *dhbC* is dependent upon a promoter upstream from the adjacent *dhbA* gene (29). Primer extension analysis was performed to identify the 5′ termini of transcripts initiating upstream of *dhbA*. A reverse transcription product was obtained with primer PE4 as shown in Fig. 5A, lane 1. The extension product was 133 nucleotides in length and localized the apparent 5′ terminus of *dhb* mRNA to nucleotide number 131. An extension product was also obtained with a primer which annealed several bp upstream of the PE4 primer site, confirming the 5′ terminus of mRNA (data not shown). The deduced transcription start site is consistent with the locations of promoter elements ^{96}TTGACTN$_{17}$TATGAT (Fig. 5B) likely to be recognized by the Eσ^A form of RNA polymerase.

Although PE4 gave a very strong reverse transcription product with RNA isolated from cells grown in IS medium, no primer extension product was obtained with RNA isolated from *B. subtilis* grown in IS medium with 5 μM $FeCl_3$ (Fig. 5A, lane 2). Thus, expression of the *dhb* genes appears to be regulated by iron.

Effect of iron concentration on transcription of *dhbC*. RNase protection experiments were performed to confirm that transcription of *dhbC* is dependent upon an upstream promoter and regulated by iron concentration. A 323-bp DNA fragment with *dhbA-dhbC*-specific sequences (Fig. 6) was amplified by PCR to serve as the template for cRNA synthesis. Total cellular RNA was isolated from RB1 cells grown in IS medium with and without 5 μM $FeCl_3$ at the growth stages T_{-2}, T_{-1}, T_0, T_1, T_2, and T_3. The [^{32}P]UTP-labelled cRNA was hybridized to *B. subtilis* RNA, and unprotected RNA was digested with RNase A and RNase T_1. The major protected RNA species corresponds to the full-length transcript probe (Fig. 6, lanes 1 through 6). The undigested cRNA probe is slightly larger than the *B. subtilis* RNA protected fragment because the probe contains 15 nucleotides that are not *dhb*-specific sequences. Less-well-defined, smaller fragments are presumably probe degradation products, because corresponding bands are visible in a lighter exposure of the undigested probe lane (Fig.

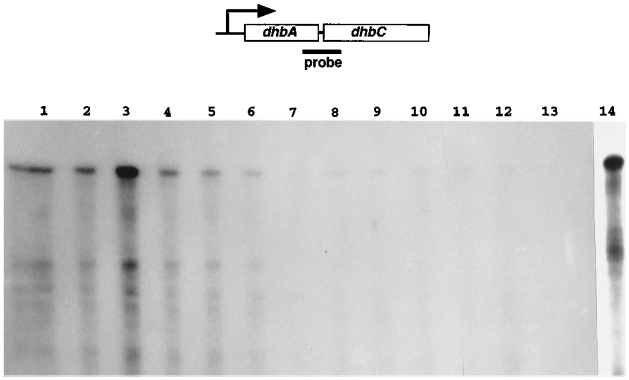

FIG. 6. RNase protection with a *dhbA-dhbC*-specific probe. RNase protection assays were performed with a 323-nucleotide cRNA probe; 308 nucleotides were specific to *dhbA-dhbC* sequences as shown, and 15 nucleotides were nonspecific. The ^{32}P-labelled probe (3×10^4 cpm) was hybridized with the segment of RNA indicated by the line above "probe" and digested with a combination of RNases A and T_1. Protected fragments were separated by electrophoresis on a 5% acrylamide gel containing 8 M urea. An autoradiograph of the dried gel is shown; to show probe degradation products, a gel subjected to shorter exposure was substituted in lane 14. Lanes 1 through 12, 3×10^4 cpm of the probe and 5.0 μg of *B. subtilis* RB1 RNA treated with RNase: lane 13, 3×10^4 cpm of the probe and 5.0 μg of yeast RNA treated with RNase; lane 14, 3×10^4 cpm of the probe not treated with RNase; lanes 1 to 6, RNA isolated from strain RB1 grown in IS medium at T_{-2}, T_{-1}, T_0, T_1, T_2, and T_3, respectively; lanes 7 to 12, RNA isolated from strain RB1 grown in IS medium with 5 μM $FeCl_3$ at T_{-2}, T_{-1}, T_0, T_1, T_2, and T_3, respectively.

6, lane 14). This confirms that no transcripts initiate in the *dhbA-dhbC* intercistronic space. Transcription was drastically reduced by the addition of 5 μM $FeCl_3$ to IS medium (Fig. 6, lanes 7 through 12), confirming that expression of *dhbC* is regulated by iron. Maximal transcription of the *dhb* genes occurs at the T_0 growth stage. These data agree with the fact that DHB production reaches a plateau shortly after the culture enters stationary phase (19).

Effect of iron box mutagenesis on iron regulation of *dhb* gene transcription. Immediately downstream from the apparent transcription start point is the palindromic sequence ^{138}GATA ATGATAATCATTATC (Fig. 5B). This sequence is identical to the consensus sequence for the *E. coli* Fur binding site (5) called an iron box. In *E. coli*, the Fur protein acts as a repressor in the presence of iron by binding to operator sites of iron-regulated genes and blocking transcription. Iron boxes are always located within the promoter or transcription initiation regions of iron-regulated genes.

Site-directed mutagenesis was performed to determine whether the iron box located upstream of *dhbA* is involved in the iron regulation of *dhb* gene expression. Eight of the 9 bases mutated (Fig. 7A) were among the 10 most highly conserved bases identified by sequence comparisons of all *E. coli* Fur binding sites (5). Fusions were constructed so that the wild-type or mutated promoter regions controlled the expression of DhbA′::LacZ fusion proteins. The fusion constructs were incorporated into the *amyE* locus of the *B. subtilis* chromosome. Resulting fusion proteins have the first 24 amino acids of DhbA fused to the sixth amino acid of LacZ.

Strain RB1274, with the wild-type *dhb* promoter, was grown in IS medium with and without 5 μM $FeCl_3$. As shown in Fig. 7B, the expression of the DhbA′::LacZ fusion protein was regulated by iron concentration. Maximal *dhb* promoter activity occurred at growth stage $T_{0.5}$ when the cells were grown in IS medium. The addition of 5 μM $FeCl_3$ completely repressed transcription of the *dhbA′::lacZ* fusion gene.

When strain RB1273 (having the mutated iron box) was

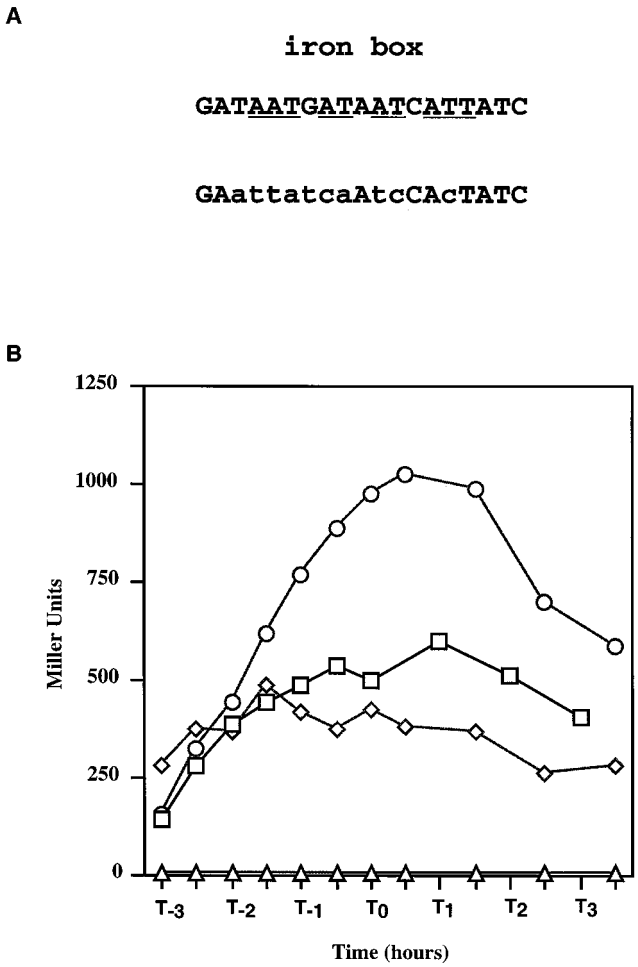

FIG. 7. Effects of the iron box mutation on the expression of *dhbA′::lacZ*. (A) The 19-bp palindromic sequence within the promoter region of the *dhb* gene cluster that is identical to the consensus *E. coli* Fur binding site is shown (iron box). Underlined nucleotides identify the 10 most highly conserved bases of Fur operator sequences (4). Lowercase letters indicate the nucleotide changes created to mutate the iron box. (B) β-Galactosidase activity levels of strains RB1273 (□) and RB1274 (○) grown in IS medium and strains RB1273 (◇) and RB1274 (△) grown in IS medium with 5 μM $FeCl_3$. Cell samples were taken at half-hourly or hourly intervals, and levels of β-galactosidase activity were determined as described in Materials and Methods. Timepoints are as described in the legend to Fig. 3.

grown under these same conditions, expression of the DhbA′::LacZ fusion protein was not regulated by iron (Fig. 7B). The nucleotide changes also caused a modest decrease in the level of β-galactosidase expression.

DISCUSSION

Isochorismate is required by *B. subtilis* under two different environmental conditions: for MK biosynthesis under varying conditions of carbon source availability and for DHB biosynthesis when the extracellular iron concentration is low. It seems likely that the intracellular competition for isochorismate is strong, and that genes have evolved to encode two enzymes for the synthesis of this intermediate. *B. subtilis* thus has two isochorismate synthase genes, one located within the MK biosynthetic gene cluster (*menF*) and the other located within the DHB biosynthetic gene cluster (*dhbC*).

Strains carrying deletions of *menF* produced wild-type levels of MK and DHB, indicating that DhbC is able to provide sufficient amounts of isochorismate for MK biosynthesis. However, *dhbC* deletion strains did not produce detectable levels of DHB, suggesting that MenF is unable to provide sufficient isochorismate for DHB biosynthesis. One possibility is that the cell makes insufficient MenF to synthesize the level of isochorismate needed for DHB biosynthesis.

Expression of *dhbC*, but not *menF*, is controlled by iron concentration. The *menF* promoter, *menp*1, appears to be responsive to the carbon source and the growth phase (28). Transcription of *dhbC* is controlled by a promoter upstream of *dhbA*. RNase protection experiments showed that expression of *dhbC* was maximal at the end of exponential growth and declined in stationary phase.

Mutations within the iron-box sequence of the *dhb* promoter abolished iron regulation of transcription, while causing a modest overall effect on transcription. These results suggest that a Fur-like repressor protein exists in *B. subtilis*. Chen et al. identified two iron-regulated promoters of *B. subtilis* that have sites with 57 to 73% identity to the *E. coli* Fur operator sequence (3). Mutations in the putative iron box of one of these promoters partially alleviated iron repression. Schneider and Hantke identified two iron-box sequences with 78% identity to the *E. coli* Fur box upstream of the *B. subtilis* iron-hydroxamate-uptake gene *fhuD* (34); these sequences are recognized in vivo by *E. coli* Fur (35). Although Fur has not been isolated from *B. subtilis*, a Fur-like regulatory protein, DtxR, has been identified in two other gram-positive species, *Corynebacterium diphtheriae* (1) and *Brevibacterium lactofermentum* (23). The *C. diphtheriae* DtxR operator site is a 19-bp palindromic sequence with little similarity to the Fur operator sequence (39). A sequence similar to that of the DtxR operator site was found within an iron-regulated promoter of *Streptomyces pilosus* (7). These studies suggest that gram-positive bacteria possess mechanisms for iron-concentration-dependent regulation of gene expression that are generally similar to the Fur-iron box paradigm of *E. coli*. However, the structure of the Fur-like regulatory protein and corresponding operator sequences may vary considerably from species to species.

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