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The Hotdog Thioesterase EntH (YbdB) Plays a Role In Vivo in Optimal Enterobactin Biosynthesis by Interacting with the ArCP Domain of EntB▽†

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In response to iron limitation, the siderophore enterobactin is synthesized and secreted by *Escherichia coli*. Its biosynthesis is performed by a series of enzymes encoded by the Ent gene cluster. Among the genes of this cluster, *ybdB* has not been implicated in enterobactin production to date. We demonstrate here an in vivo role for the hotdog protein EntH (YbdB) in the optimal production of enterobactin. Indeed, we showed that EntH is a thioesterase specifically produced under iron limitation conditions. Furthermore, EntH interacts specifically with the aryl carrier protein (ArCP) domain of EntB, a crucial bifunctional enzyme of the enterobactin biosynthesis pathway and a potential target of EntH thioesterase activity. A strain devoid of EntH is impaired for growth under iron limitation associated with the presence of the salicylate inhibitor, correlating with the diminution of enterobactin production under these conditions. Normal growth and enterobactin production are restored upon expression of *entH* in *trans*. Inversely, unnecessary overproduction of EntH provokes a fall of the quantity of siderophore produced under iron starvation conditions. Our findings point to a proofreading role for EntH during biosynthesis of enterobactin in vivo. EntH thioesterase activity could be required for cleaving wrongly charged molecules on the carrier protein EntB. This is the first description of such a role in the optimization of a nonribosomal biosynthesis pathway for a protein of the hotdog superfamily.

Iron is an essential micronutrient for nearly all living systems. Under iron-limiting growth conditions, *Escherichia coli* produces enterobactin (also named enterochelin), a highly specific iron-chelating compound, which forms complexes with Fe^{3+} in the environment. The biosynthesis of this siderophore is one of the most thoroughly studied (7). The lack of iron results in the derepression by the Fur system of an entire collection of genes for the biosynthesis and transport of enterobactin. The 2,3-dihydroxybenzoate (DHB) enterobactin precursor is synthesized from chorismic acid by enzymes encoded by the *entC*, *entB*, and *entA* genes. In a second step, DHB and serine are polymerized and cyclized to form enterobactin by enzymes encoded by the *entE*, *entB*, and *entF* genes. The enzymatic steps are very well understood, leading to the possibility of producing enterobactin from an in vitro-reconstituted system (13).

Although many studies relating to the synthesis of enterobactin have been carried out for more than 30 years, no biological function has been allotted yet to *ybdB* (previously called *P15*), the last gene of the *entCEBA-ybdB* operon (28, 29) (Fig. 1A). All the genes involved in enterobactin synthesis, export, and import are clustered on the chromosome of *E. coli* and organized around three Fur-regulated bidirectional promoter-operator regions (Fig. 1A). Similarly to that of the *entCEBA* genes, *ybdB* expression depends on the *entCp* promoter, located upstream of *entC*, a promoter repressed by Fur when iron concentration is not limiting (5). This, together with the conservation of the *ybdB* gene at the end of the operon *entCEBA-ybdB* in many enterobacteriaceae, suggests that the YbdB protein could be involved in the synthesis of enterobactin. However, little information is available on *ybdB*: YbdB is not required for enterobactin formation in vitro (13), YbdB production from a plasmid carrying the *entCEBA-ybdB* operon has been observed in *E. coli* minicells (29), and YbdB has been shown to display an esterase activity in vitro on palmitoyl-coenzyme A (CoA) and *p*-nitrophenyl-butyrate (22). Finally, the structure obtained by crystallography shows that YbdB adopts a hotdog fold (3).

The hotdog fold was first observed in the structure of *E. coli* β-hydroxydecanoyl thiol ester dehydratase FabA, in which each subunit of this homodimeric enzyme contained a hotdog fold (24). A large superfamily of hotdog domains was defined using sequence analysis (10) and is described in the Pfam database under identification number CL0050. This family contains numerous prokaryotic, archaeal, and eukaryotic proteins displaying various catalytic activities (mainly thioesterases), involved in several metabolic activities, from lipid metabolism to detoxification and transcriptional regulation. Twelve proteins containing hotdog domains can be identified in *E. coli* (10): FabA, FabZ, MaoC, PaaI, TesB, YbaW, YbdB, YbgC, YciA, YdiI, YigI, and YiiD. Among them, seven have been crystallized: FabA (PDB no. 1MKA), PaaI (PDB no. 1P5U), TesB (PDB no. 1C8U), YbaW (PDB no. 1NJK), YbdB (PDB no. 1VH9), YbgC (PDB no. 1S5U), and YdiI (PDB no. 1VH5). Furthermore, four orthologs from other organisms have also been crystallized (FabZ [PDB no. 1U1Z] in *Pseudomonas aeruginosa*, MaoC [PDB no. 1Q6W] in *Archeoglobus*

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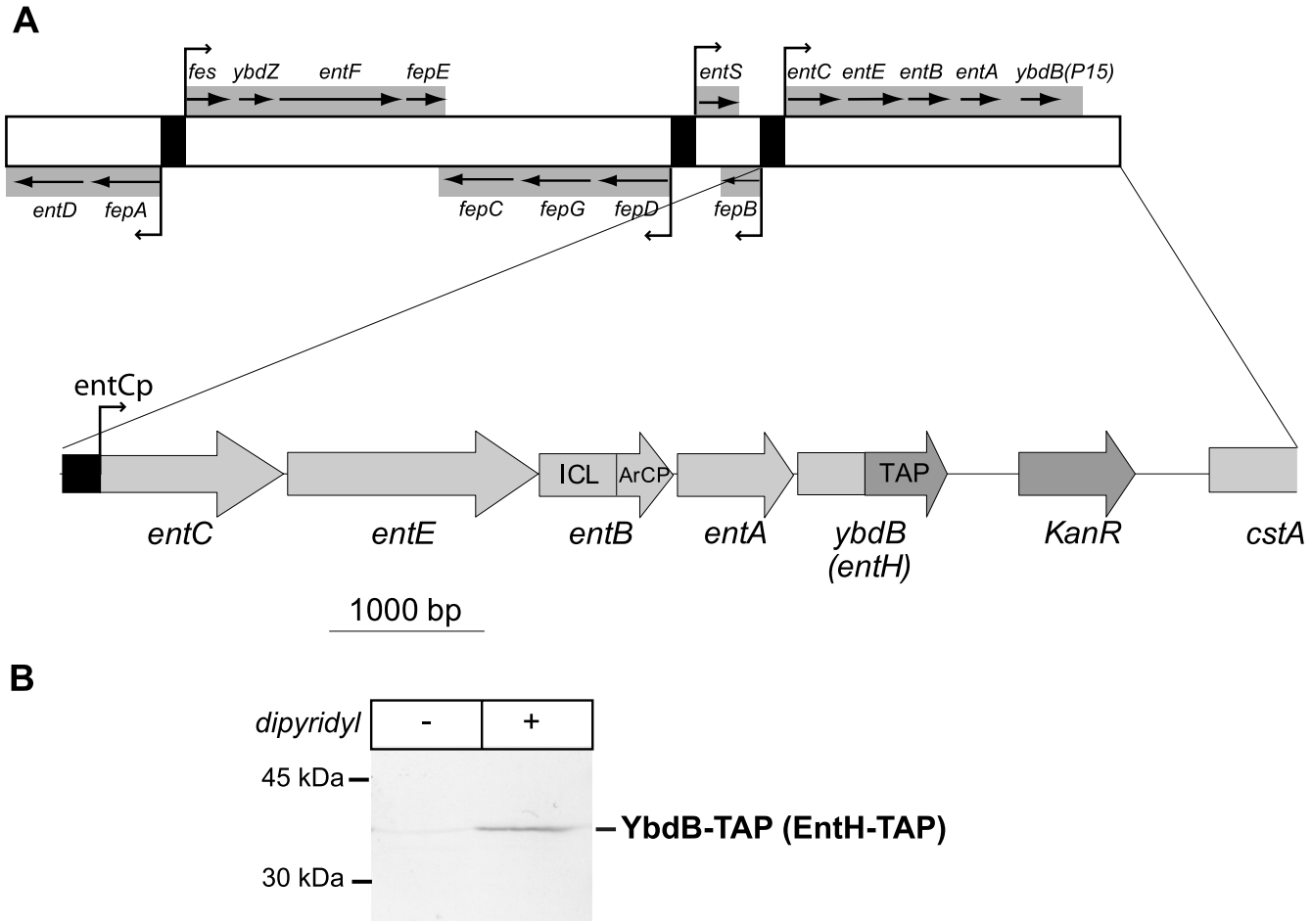


FIG. 1. (A) Genetic organization of the enterobactin biosynthesis locus in *E. coli* and organization of the *entCEBAH* operon after introduction of the TAP tag at the C terminus of the *entH* gene. The three Fur-regulated bidirectional promoter-operator regions are indicated by black squares, and the transcribed operons are highlighted in gray. (B) Expression of the *E. coli* EntH-TAP protein. Western blotting on whole-cell lysates of cell cultures in LB with (+) or without (−) 350 μM dipyridyl is shown. EntH-TAP was detected by a peroxidase anti-peroxidase antibody detecting the ProtA part of the TAP tag. References of molecular masses are indicated on the left of the membrane.

fulgidus, YciA [PDB no. 1YLI] in *Haemophilus influenzae*, and the YiiD [PDB no. 1T18] C-terminal domain in *Shewanella oneidensis*), experimentally confirming the hotdog fold prediction for all these proteins. Among the 12 *E. coli* proteins of the hotdog family, a physiological role is attributed only to the FabA and FabZ 3-hydroxydecanoyl-acyl carrier protein (ACP) dehydratases in fatty acid synthesis (19) and to the hypothetical PaaI and MaoC (PaaZ) thioesterases in phenylacetic acid degradation (9). TesB, an acyl-CoA thioesterase characterized in vitro, plays a role in lipid metabolism that is not clearly understood (27). Finally, an enzymatic genomic screen has evidenced in vitro thioesterase or esterase activities on CoA derivatives for YciA, YbgC, YdiI, and YbdB (22). Therefore, several hotdog proteins display esterase or thioesterase activities on acyl-ACP or acyl-CoA, and when they do not have a thioesterase activity, they often correspond to proteins able to interact with acyl-ACP or acyl-CoA derivatives, such as the two dehydratases FabA and FabZ or the regulator FapR in *Bacillus subtilis* (37). Recently, we found that the unknown-function hotdog protein YbgC, predicted to be a thioesterase, also interacts with ACP (15). ACP is a small acidic protein highly conserved in bacterial genomes and acting as a fatty acid carrier for fatty acid synthesis and delivery (34). Serine 36 of ACP is modified posttranslationally by a phosphopantetheine (Ppant) group providing the unique sulfhydryl of the protein.

The *ent* genes encode two proteins that carry a Ppant group: EntB contains an aryl carrier protein (ArCP) domain, and EntF contains a peptidyl carrier protein domain on which elongation of enterobactin is taking place. Both domains are posttranslationally modified specifically by the Ppant transferase EntD. Furthermore, the structure of the ArCP domain of EntB is very similar to the structure of ACP (11). Therefore, by analogy with other hotdog proteins that target ACP (FabA, YbgC), we considered EntB and EntF two potential targets for the activity of the YbdB (thio) esterase.

In this study, we investigated the role of YbdB in enterobactin production in *E. coli*. First, we showed that the YbdB protein is indeed a thioesterase and that it is produced under iron starvation conditions. Second, we demonstrated a specific interaction between YbdB and the ArCP domain of EntB in vivo, dependent on the presence of the Ppant group on EntB. Third, although *ybdB* does not seem to be required for normal

TABLE 1. *E. coli* K-12 strains

Name	Relevant characteristics	Source or reference
C600	F$^-$ e14$^-$ (Mcr$^-$) or e14$^-$ (McrA$^+$) *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA25*	2
MC4100	*araD139* Δ(*argF-lac*)*205 flb-5301 pstF25 rpsL150 deoC1 relA1*	6
W3110	F$^-$ LAM-IN(*rrnD-rrnE*)1 *rph-1*	2
W3110Δ*entH*(*ybdB*)::Kan^r	Δ*ybdB*::Kan^r; P1 transduction from BW25113Δ*ybdB* to W3110	This work
W3110Δ*ydiI*::Kan^r	Δ*ydiI*::Kan^r; P1 transduction from BW25113Δ*ydiI* to W3110	This work
BTH101	F$^-$ *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	20
BW25113/pKD46	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*) Δ(*rhaBAD*)*568 rph-1* pKD46	8
BW25113/*entH-TAP*-Kan^r	*entH-TAP*-Kan^r; Ebm226/Ebm227 PCR on pJL72 into BW25113/pKD46	This work
W3110/*entH-TAP*-Kan^r	*entH-TAP*-Kan^r; P1 transduction from BW25113/*entH-TAP*::Kan^r to W3110	This work
BW25113Δ*entH*(*ybdB*)::Kan^r	Δ*ybdB*::Kan^r	1
BW25113Δ*ydiI*::Kan^r	Δ*ydiI*::Kan^r	1
BW25113Δ*entB*::Kan^r	Δ*entB*::Kan^r	1

production of enterobactin under iron starvation conditions, it becomes strikingly important if an inhibitory analogue of the enterobactin precursor is added. Finally, we showed that inversely the overproduction of YbdB inhibits the production of enterobactin. Because of this implication in enterobactin synthesis, we decided to rename YbdB EntH. We propose here a new type of physiological role for a hotdog protein: EntH (YbdB) could be involved in a proofreading function during enterobactin synthesis that involves a cleavage activity on the Ppant group of EntB carrying wrongly charged molecules. Finally, our study showed that hotdog proteins of *E. coli* may have evolved to accomplish diverse and moreover very specific functions. For example, the close EntH homologue YdiI had no effects like those of EntH on enterobactin synthesis.

MATERIALS AND METHODS

Bacterial strains. All strains used were derivatives of *E. coli* K-12 (Table 1). W3110Δ*entH*(*ybdB*)::Kan^r and W3110Δ*ydiI*::Kan^r were obtained by transduction of a P1 bacteriophage prepared on the BW25113Δ*entH*(*ybdB*)::Kan^r and BW25113Δ*ydiI*::Kan^r strains obtained from the Keio collection (1). Deletions were confirmed by PCR analyses. The BW25113*entH*-TAP strain was constructed with the technique of Datsenko and Wanner (8) to introduce the tandem affinity purification (TAP)-Kan^r cassette amplified with Ebm226 and Ebm227 oligonucleotides from the pJL72 plasmid (41). The construction was then transduced in the *E. coli* W3110 strain.

Plasmids. Plasmids (and their construction) and oligodeoxynucleotides are listed in Tables 2 and 3, respectively. For two-hybrid plasmid construction, gene sequences were amplified by PCR directly on W3110 colonies, using oligonucleotides introducing EcoRI and XhoI sites most of the time, XbaI and XhoI for *entC*, and PstI and XhoI for *entF*. The DNA fragments were then ligated into pT18 and pT25 plasmids using the same sites. Mutagenesis was performed using

TABLE 2. Plasmids[a]

Name	Relevant characteristics	Reference
pJL72 (pEB793)	*TAP*-Kan^r cassette; Amp^r; ColE1 ori	41
pBAD24 (pEB227)	Amp^r; pBR322 ori; P_{BAD} promoter	17
pBAD24-*entH* (pEB921)	Ebm196/Ebm197 *ybdB* PCR ligated in pBAD24 using EcoRI and HindIII	This work
pBAD24-*ydiI* (pEB967)	Ebm146/Ebm147 *ydiI* PCR ligated in pBAD24 using EcoRI and HindIII	This work
pBAD24-CBP (pEB602)	Amp^r; pBR322 ori; P_{BAD}-CBP	15
pBAD24-CBP-*entH* (pEB847)	Ebm196/Ebm197 *ybdB* PCR ligated in pBAD24 using EcoRI and HindIII	This work
pBAD24-*entH-TAP* (pEB883)	Ebm196/Ebm223 *ybdB* PCR ligated with *CtermTAP* in pBAD24	This work
pT25 (pEB354)	pKT25 derivative; Kan^r; p15A ori; P*lac* promoter T25 domain	15
pT18 (pEB355)	pUT18C derivative; Amp^r; ColE1 ori; P*lac* promoter; T18 domain	15
ybgC (pEB381-pEB380)	*ybgC5′-ybgC3′ ybgC* PCR in pT25 and pT18 using EcoRI and XhoI	15
yigI (pEB702-pEB715)	Ebm148/Ebm149 *yigI* PCR in pT25 and pT18 using EcoRI and XhoI	This work
yciA (pEB710-pEB713)	Ebm144/Ebm145 *yciA* PCR in pT25 and pT18 using EcoRI and XhoI	This work
ydiI (pEB654-pEB665)	Ebm146/Ebm147 *ydiI* PCR in pT25 and pT18 using EcoRI and XhoI	This work
ybaW (pEB580-pEB579)	Ebm106/Ebm107 *ybaW* PCR in pT25 and pT18 using EcoRI and XhoI	This work
fabA (pEB721-pEB720)	Ebm163/Ebm164 *fabA* PCR in pT25 and pT18 using EcoRI and XhoI	This work
paaI (pEB831-pEB720)	Ebm198/Ebm199 *paaI* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entH (pEB829-pEB830)	Ebm196/Ebm197 *entH* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entA (pEB977-pEB974)	Ebm308/Ebm309 *entA* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entB (pEB904-pEB902)	Ebm243/Ebm244 *entB* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entB(*S245A*) (pEB922-pEB919)	Mutagenesis Ebm251/Ebm252 on pEB904 and pEB902	This work
Flag-*entB* (pEB1018)	Hybridized Ebm363 and Ebm364 oligonucleotides inserted in pEB904 (PstI/XbaI)	This work
Flag-*entB*(*S245A*) (pEB1020)	Hybridized Ebm363 and Ebm364 oligonucleotides inserted in pEB922 (PstI/XbaI)	This work
entB ICL (pEB1002-pEB1000)	Ebm243/Ebm343 *entB* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entB ArCP (pEB1003-pEB1001)	Ebm342/Ebm244 *entB* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entE (pEB979-pEB976)	Ebm312/Ebm313 *entE* PCR in pT25 and pT18 using EcoRI and XhoI	This work
entC (pEB978-pEB975)	Ebm310/Ebm311 *entC* PCR in pT25 and pT18 using XbaI and XhoI	This work
entF (pEB916-pEB915)	Ebm250/Ebm262 *entF* PCR in pT25 and pT18 using PstI and XhoI	This work
acpP (pEB375-pEB379)	Ebm76/Ebm77 *acpP* PCR in pT25 and pT18 using EcoRI and XhoI	15

[a] Laboratory codes corresponding to our stock numbering are given in brackets. For the two-hybrid plasmids, both constructions in the pT25 and pT18 vectors are indicated on only one line per gene. Boldface type indicates the cloning vectors for each type of construct. ori, origin of replication.

TABLE 3. Oligodeoxynucleotides used in this study[a]

Oligodeoxynucleotide	Sequence	Sequence amplified
Ebm106	CACCGAATTCATGCAAACACAAATC	*ybaW*
Ebm107	ACCGCTCGAGAAGCTTACTTAACCATCTG	*ybaW*
Ebm144	CACCGAATTCATGTCTACAACACATAAC	*yciA*
Ebm145	ACCGCTCGAGAAGCTTACTCAACAGGTAAGGC	*yciA*
Ebm146	CACCGAATTCATGATATGGAAACGGAAAATC	*ydiI*
Ebm147	ACCGCTCGAGAAGCTTACAAAATGGCGGTCGTC	*ydiI*
Ebm148	CACCGAATTCATGAAGTGTAAGGATGATATG	*yigI*
Ebm149	ACCGCTCGAGAAGCTTAACCTACCATATAGGTG	*yigI*
Ebm163	CACCGAATTCATGGTAGATAAACGCGAATCC	*fabA*
Ebm164	ACCGCTCGAGAAGCTTAGAAGGCAGACGTATCCTG	*fabA*
Ebm196	AGAATTCATGATCTGGAAACGCCATTTAACGC	*entH*
Ebm197	ACTCGAGAAGCTTCATCCCAAAACTGC	*entH*
Ebm198	AGAATTCATGAGTCATAAGGCCTGGCAAAATG	*paaI*
Ebm199	ACTCGAGAAGCTTCAGGCTTCTCCTG	*paaI*
Ebm223	TTCCATGGATCCCAAAACTGCCGTACC	*entH*
Ebm226	GGGCGGCGTTGCTGCACTTGTCGGCTGGGTACGGCAGTTTTGGGATCCA TGGAAAAGAGAAG	TAP-Kan^r cassette
Ebm227	TCCGTTAACCGAGTTTTCCTGCAATCTCATCTAATTCTGTCGGGTCATAT GAATATCCTCCTTAG	TAP-Kan^r cassette
Ebm243	CACCGAATTCATGGCTATTCCAAAATTA	*entB*
Ebm244	ACTCGAGAAGCTTTATTTCACCTCGCG	*entB*
Ebm250	ACTCGAGAAGCTTTACCTGTTTAGCGTTG	*entF*
Ebm251	TCGACTACGGTCTGGATGCCGTACGCATGATGGCGCTGGC	*entB(S245A)*
Ebm252	GCCAGCGCCATCATGCGTACGGCATCCAGACCGTAGTCGA	*entB(S245A)*
Ebm262	CACCCTGCAGATGAGCCAGCATTTACC	*entF*
Ebm308	CACCGAATTCATGGATTTCAGCGGTAA	*entA*
Ebm309	ACTCGAGAAGCTTTTATGCCCCCAGCGTTG	*entA*
Ebm310	CACCTCTAGAAATGGATACGTCACTGGC	*entC*
Ebm311	ACTCGAGTTAATGCAATCCAAAAACGTT	*entC*
Ebm312	CACCGAATTCATGAGCATTCCATTCAC	*entE*
Ebm313	ACTCGAGAAGCTTCAGGCTGATGCGCGTG	*entE*
ybgC5′	CACCGAATTCGTGAATACAACGCTGTTTCG	*ybgC*
ybgC3′	ACCGCTCGAGTCACTGCTTAAACTCCGCG	*ybgC*
Ebm342	CACCGAATTCGTGGTGATGACTGAAGAA	*entB* ArCP
Ebm343	CACCCTCGAGAAGCTTTACAGTAATTCTTCAGT	*entB* ICL
Ebm363	GATTATAAAGATGACGATGACAAG	Flag up
Ebm364	CTAGCTTGTCATCGTCATCTTTATAATCTGCA	Flag down

[a] The oligodeoxynucleotides were provided by Eurogentec SA.

a QuikChange site-directed mutagenesis kit (Stratagene), using oligonucleotides Ebm251 and Ebm252 to obtain the pT25-*entB*(*S245A*) and pT18-*entB*(*S245A*) plasmids. The pT25-Flag-*entB* and pT25-Flag-*entB*(*S245A*) plasmids were obtained by introducing the Ebm363 and Ebm364 hybridized oligonucleotides encoding the Flag epitope in the corresponding two hybrid plasmids digested by PstI and XbaI.

Media. Cells were grown at 37°C in Luria-Bertani (LB) medium (26) or in minimal medium buffered with Tris (MM9Tris; 1× M9 salts, 10 mM Tris-HCl [pH 6.8], 0.3% Casamino Acids, 0.5 μg/ml thiamine, 0.2% glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$). Iron limitation was induced by adding 250 μM 2,2′-dipyridyl to the liquid or agar media in LB or 100 μM 2,2′-dipyridyl to MM9Tris. When indicated, salicylate was also added at 500 μM in the liquid or agar media. Both products were purchased from Sigma. Plasmids were maintained with ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

Purification of CBP-EntH and continuous spectrophotometric assay of EntH thioesterase activity. Calmodulin binding peptide (CBP)-EntH protein was purified from a 500-ml culture of MC4100/pBAD-CBP-*entH* in LB media. At an optical density at 600 nm (OD_{600}) of 0.8, production of CBP-EntH was induced with 0.1% arabinose for 2 h. The cells were broken by sonication in 10 ml of buffer A (50 mM Tris-HCl [pH 8], 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$), and the extract was then centrifuged for 30 min at 27,000 × *g* before incubation on 600 μl of calmodulin beads (Stratagene) on a wheel for 2 h at 4°C. Then, four successive 10-ml washes were performed with buffer A, buffer A containing 0.5 M NaCl, buffer A containing 0.1% Triton X-100, and buffer A. CBP-EntH was eluted from the beads in 3 ml of buffer B (50 mM Tris-HCl [pH 8], 150 mM NaCl, 10 mM β-mercaptoethanol, 2 mM EGTA) and then concentrated to 2.5 mg/ml.

Thioesterase assays were performed as previously described for YbgC (43). Acyl-CoA substrates were purchased from Sigma. Hydrolysis reactions of the acyl-thioester substrates were monitored at 25°C by measuring the absorbance of 5-thio-2-nitrobenzoate at 412 nm (extinction coefficient = 13.6 mM^{-1} cm^{-1}), which was formed by reacting DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)] with the CoASH liberated from the acyl-CoA substrates. To allow high substrate concentrations to be used in the assays, reactions were carried out in a quartz cuvette with a 3-mm light path. Each assay reaction mixture (100 μl) contained EntH protein (6.5 μM for *n*-propionyl-CoA and palmitoyl-CoA and 0.13 μM for benzoyl-CoA), acyl-CoA substrate (0.025 to 4 mM), DTNB (2 mM), NaCl (150 mM), and 50 mM Tris-HCl (pH 8). The kinetic parameters of maximum velocity (V_{max}) and K_m were determined from initial velocity data, measured as a function of substrate concentration, using the equation $V = V_{max}[A]/([A] + K_m)$, where $[A]$ is the substrate concentration, V is the initial velocity, and K_m is the Michaelis constant. The k_{cat} value was calculated from the ratio of V_{max} to the total enzyme concentration.

Siderophore assay. Chrome azurol S (CAS) agar plates were prepared as previously described (39). Their exact final composition is the following: 1× M9 salts (19.5 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl), 6.05% CAS, 5 μM $FeCl_3$, 7.29% hexadecyltrimethylammonium bromide, 3% 1,4-piperazinediethanesulfonic acid (PIPES), 0.3% Casamino Acids, 0.2% glucose, 0.5 μg/ml thiamine, and 1.5% agar. When needed, ampicillin (100 μg/ml) was added. As indicated in the figure legends, the plates could also contain 0.01% arabinose or 500 μM salicylate. On these plates, enterobactin production is induced because of the precise balance of the $FeCl_3$ and hexadecyltrimethylammonium bromide concentrations, without addition of dipyridyl. Production of siderophores is detected by the appearance of an orange halo. Cultures in LB of the different strains were grown at 37°C for 24 h. One microliter of a normalized dilution of cells was spotted on the CAS plates. Plates were then incubated at

30°C. Measurements of the halos correspond to the difference (in millimeters) between the diameter of the halo and the diameter of the disc formed by the cells (data in Fig. 6A and C are averages for seven measurements).

A siderophore assay on culture supernatants was also performed. The CAS assay solution was prepared exactly as previously described (39). Overnight precultures at 37°C were done in MM9Tris with antibiotics if required. Four-milliliter cultures in MM9Tris with or without 500 μM salicylate were then inoculated in triplicate at initial OD_{600}s of 0.05. When the cultures reached OD_{600}s of 1.5 to 2.0, the CAS assay was performed as described in reference 39 on 500 μl of supernatant. The measure corresponds to the percentage of absorbance reduction at 630 nm compared to that for the reference assay performed on the uninoculated culture medium.

Bacterial two-hybrid technique. We used the adenylate cyclase-based two-hybrid technique (20). Pairs of proteins to be tested were fused to the two catalytic domains T18 and T25 of adenylate cyclase, using plasmids pT18 and pT25, respectively. After cotransformation of the BTH101 strain with the two plasmids expressing the fusions, selection plates were incubated at 30°C for 48 h. Three milliliters of LB medium supplemented with ampicillin, kanamycin, and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was inoculated and grown at 30°C for 18 h. Then, 2-μl drops were spotted on MacConkey plates supplemented with 1% maltose and incubated for 24 h at 30°C.

Copurification on calmodulin beads. The C600 strain was cotransformed with the pBAD24-CBP or pBAD24-CBP-*entH* and pT25-Flag-*entB* or pT25-Flag-*entB*(*S245A*) plasmids. A 200-ml culture was prepared at 37°C in LB with ampicillin and kanamycin and induced for 90 min with 0.05% arabinose and 0.5 mM IPTG to a final OD_{600} of approximately 2. The cells were pelleted, and an extract was prepared by sonication in 10 ml calmodulin binding buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$) and centrifugation for 30 min at 27,000 × *g*. For each copurification assay, 1.4 ml of extract was incubated on 40 μl of calmodulin beads washed in calmodulin binding buffer. After 90 min of incubation at 4°C on a wheel, the beads were washed five times in 1 ml of calmodulin binding buffer, resuspended in 40 μl of Laemmli loading buffer, and heated for 10 min at 96°C. Twenty-five microliters of each sample was analyzed by Western blotting.

Protein electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransfer onto nitrocellulose membranes, and Western blot analysis were performed as previously described (16). The peroxidase anti-peroxidase soluble complex and monoclonal anti-Flag M2 were purchased from Sigma.

Bioinformatic analyses. Alignment was performed with the TCOFFEE program with default settings (31), followed by manual modifications. In addition to the 11 sequences of hotdog proteins from *E. coli*, 3 sequences of hotdog proteins from other organisms were used to add structural information. The corresponding unrooted tree was constructed by a maximum likelihood method using PHYLM with the Jones-Taylor-Thornton model (14). Drawing was realized using the Treeview program (30). Bootstrap values were computed by PHYLM for 100 replicates of the original data set.

RESULTS

YbdB protein is produced under iron limitation conditions. Among the genes coding for unknown-function hotdog proteins, *ybdB* is the only one to be localized in an operon of a very well-known function, i.e., enterobactin synthesis (28, 29). Surprisingly, even if there is no doubt that *ybdB* belongs to the *ent* operon, the production of the YbdB protein has never been followed during iron starvation.

In order to detect this protein in vivo and to look for potential protein partners, we constructed a recombinant *E. coli* strain in which the *ybdB* gene is tagged at its 3′ end with the TAP tag sequence. In this strain, the only YbdB protein produced is tagged at its C terminus by the TAP tag (Fig. 1A). This strain grew as well as the wild-type strain in iron-limiting medium, showing that production of enterobactin was not affected by the chromosomal insertion of the cassette (data not shown). Using this strain, we followed the production of YbdB-TAP under iron-rich or iron starvation conditions caused by dipyridyl addition and analyzed the proteins by SDS-PAGE and Western blotting to detect the TAP tag. YbdB-TAP levels significantly increased under conditions of iron starvation (Fig. 1B). This demonstrated that *ybdB* is regulated like the other genes of the *entCEBA* operon. For this reason, we renamed this gene *entH*, the names of enzymes EntA, -B, -C, -D, -E, -F, and -G being already used (EntG has been used for the enterobactin synthetase component of the bifunctional enzyme EntB).

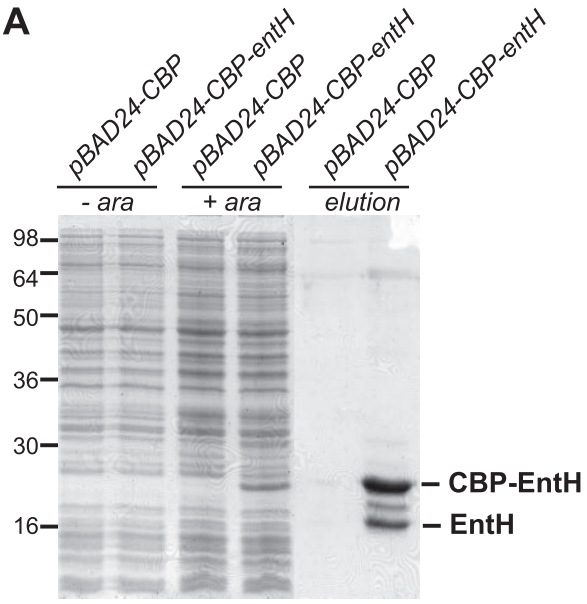
EntH displays a thioesterase activity. We then confirmed the thioesterase activity of EntH in vitro. In order to purify EntH, we used the MC4100 strain transformed by the pBAD24-CBP-*entH* plasmid, permitting the overproduction of a tagged CBP-EntH protein. We purified this recombinant protein on calmodulin beads as described in Materials and Methods. We obtained a mixture of two proteins corresponding to the tagged CBP-EntH and the copurified wild-type EntH proteins (Fig. 2A). We then tested the thioesterase activity on diverse acyl-CoA thioesters (*n*-propionyl-CoA, palmitoyl-CoA, and benzoyl-CoA) with the DTNB assay (see Materials and Methods), using high concentrations of both protein and substrate to follow the approach described for the study of YbgC (43). A specific thioesterase activity was detected on the three substrates tested (Fig. 2B). Interestingly, the k_{cat}/K_m value was at least 100-fold higher for benzoyl-CoA than for *n*-propionyl-CoA or palmitoyl-CoA, indicating that the physiological substrate of EntH should be chemically related to the aromatic benzoyl-CoA substrate. To ascertain that the thioesterase activity detected was really due to EntH and not to trace amounts of contaminating proteins, we performed the exact same purification and concentration procedures on a mock culture of MC4100/pBAD24-CBP (Fig. 2A). No thioesterase activity was detected on this preparation on any of the three substrates (data not shown).

EntH interacts in vivo with the ACP domain of EntB. EntH (YbdB) has never been implicated in enterobactin biosynthesis. Furthermore, in vitro synthesis reconstitution has been performed successfully without EntH, indicating that it is not required for enterobactin synthesis (13). In order to understand the role of EntH in enterobactin biosynthesis, we first looked for protein partners by using the TAP method (32) on cell lysates of the EntH-TAP-expressing strain grown under iron-limiting conditions. Unfortunately, no specific interacting protein was obtained, and in particular, no enzyme of enterobactin biosynthesis was isolated in complex with EntH (data not shown).

We then made assumptions for potential partners, based on the known partners of other hotdog proteins. As explained in the introduction, since several hotdog proteins are known to interact with the ACP (15, 24), we hypothesized that EntH might interact with the ArCP domain of EntB. We decided to test this interaction using the in vivo two-hybrid technique with *E. coli*. In parallel, we tested the interaction with EntF, a protein of the pathway containing a peptidyl carrier peptide domain, also modified by a Ppant group. Finally, we decided to test systematically the interactions between all the proteins involved in enterobactin synthesis encoded by the *entCEBAH* operon and *entF*. Informative results are summarized in Fig. 3A.

EntH interacted with EntB in the two possible plasmid combinations (Fig. 3B). No interaction was detected between EntH

A



Coomassie Blue

B

Acyl-CoA substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
n-Propionyl-CoA	1.0	0.029	$2.7x10^1$
Palmitoyl-CoA	0.18	0.076	$4.2x10^2$
Benzoyl-CoA	0.082	7.9	$9.6X10^4$

FIG. 2. (A) Purification of CBP-EntH. The purification was performed on 500-ml cultures of MC4100/pBAD24-CBP (control) and of MC4100/pBAD24-CBP-*entH* as described in Materials and Methods. Cell extracts before induction and after induction with 0.5% arabinose and 30 μl of the eluates (2 ml in total) from the calmodulin beads before concentration were loaded on an SDS-12% PAGE gel. The proteins were detected by Coomassie blue staining. References of molecular masses are indicated in kDa on the left of the gel. (B) Steady-state kinetic constants for EntH thioesterase-catalyzed hydrolysis of acyl-CoA thioesters at pH 8 and 25°C determined by a DTNB assay as described in Materials and Methods.

and the other proteins encoded by the operon or *entF* (Fig. 3B and data not shown). EntH oligomerized strongly, which fits with a general property of hotdog proteins (Fig. 3B). EntC, EntA, and EntB were also able to oligomerize, whereas EntE and EntF did not (Fig. 3A and data not shown). This may be consistent with the report that EntF functions as a monomer (13). Finally, we did not find other interactions between proteins encoded by this operon (data not shown). This is consistent with a report failing to isolate an enterobactin synthesis complex by immunoprecipitation (18).

The detection of an interaction between EntH and EntB by the two-hybrid technique provides evidence for an interaction occurring in vivo. However, good practice prescribes that a new interaction detected by any type of two-hybrid technique should be confirmed by using another method and by showing its specificity (4). First, we tested the specificity toward EntH by testing the interaction of EntB with seven other hotdog proteins of *E. coli* (FabA, PaaI, YbaW, YbgC, YciA, YdiI, and YigI). EntB did not interact with any of these other hotdog proteins (data not shown). We then tested the specificity toward EntB: we constructed a point mutation in EntB, replacing serine 245 with alanine, preventing the modification of EntB on this residue by the Ppant transferase EntD. This mutant protein, named EntB(S245A), was correctly produced, as demonstrated by its ability to interact with itself and with the wild-type version of the protein (Fig. 3A). The interaction between the mutant EntB(S245A) protein and EntH was significantly lowered, with an almost total inhibition in the case of the T18-EntH/T25-EntB(S245A) combination (Fig. 3B). Therefore, the interaction between EntH and EntB is specific and should reflect a related function of these two enzymes.

To verify our first assumption, that EntH interacted with EntB because it contained an ArCP domain, we tried to identify the domain of EntB interacting with EntH. We constructed two-hybrid plasmids expressing the isochorismate lyase (ICL) domain of EntB involved in DHB synthesis (residues 1 to 207) and the ArCP domain of EntB involved in polymerization of the precursors (residues 200 to 285). An interaction was detected between EntH and the ArCP domain of EntB in one of the two combinations of plasmids, T25-EntH/T18-EntB-ArCP (Fig. 3B). The detection of an interaction in only one combination of plasmids can result from different factors, including

A

		T25							
		EntH	EntB	EntB S245A	EntB ICL	EntB ArCP	EntF	ACP	other Hotdog proteins
T18	EntH	++	++	-/+	-	-	-	-	ND
	EntB	++	+	+	-	-	-	ND	-
	EntB S245A	+	+	+	-	-	ND		
	EntB ICL	-	-	-	-	-	ND		
	EntB ArCP	+	-	-	-	-	ND		
	EntF	-	-	ND	ND	ND	-		
	ACP	-	ND						
	other Hotdog proteins	ND	-						

B

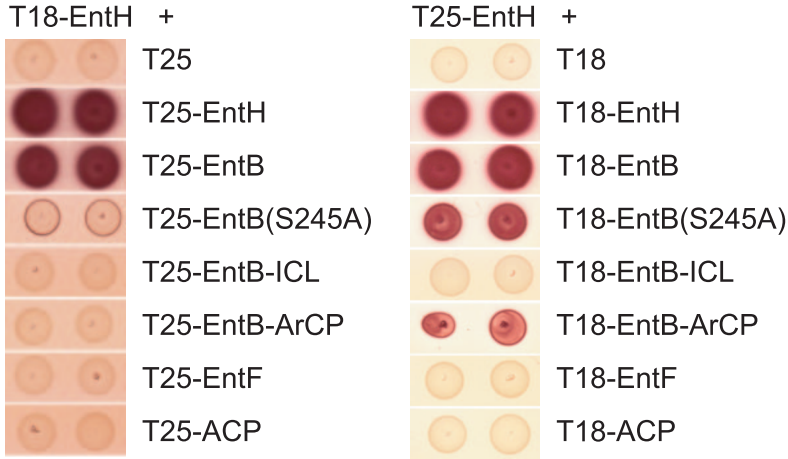


FIG. 3. EntH interacts specifically with the ArCP domain of EntB. The BTH101 strain was cotransformed with two compatible plasmids derived from the pT18 and pT25 plasmids. Positive interactions were detected on MacConkey plates containing 1% maltose by apparition of a red coloration after 24 h at 30°C. The other hotdog proteins tested against EntB were FabA, PaaI, YbaW, YbgC, YciA, YdiI, and YigI. ND, not determined. (A) The table summarizes the results for all the combinations tested. (B) The pictures below illustrate the coloration obtained on MacConkey plates for the most informative parts of the table concerning EntH interactions.

the correct folding of the fusion proteins, the ratio of the two proteins (one vector is high copy and the other low copy), and the way the proteins interact in space to put T18 and T25 domains at a distance enabling reconstitution of adenylate cyclase activity. No interaction with the ICL domain of EntB was detected in either combination (Fig. 3B). To verify even further the specificity of the recognition, we tested the interaction of EntH with the ACP protein, homologous to the ArCP domain of EntB. No interaction with this protein was detected (Fig. 3B).

Finally, as a second method for confirming the interaction between EntH and EntB, we chose a copurification technique. We used the CBP tag, which enables purification on calmodulin beads in the presence of calcium. We first tagged EntH at its N terminus with CBP (pBAD24-CBP-*entH* plasmid) and EntB with the Flag epitope, using the compatible pT25 plasmid (pT25-Flag-*entB*) in order to be able to detect EntB. The C600 strain was cotransformed by both plasmids, and purification on calmodulin beads was performed as described in Materials and Methods. T25-Flag-EntB was specifically copurified with CBP-EntH on calmodulin beads (Fig. 4, compare lane 2 to lane 1). The experiment was repeated with a pT25-Flag-*entB*(*S245A*) plasmid. The T25-Flag-EntB(S245A) protein was produced in quantities similar to those of T25-Flag-EntB (data not shown). However, the T25-Flag-EntB(S245A) protein was not retained with CBP-EntH on calmodulin beads (Fig. 4, compare lane 3 to lane 4). Some unspecific binding of the T25-Flag-EntB proteins on the calmodulin beads occurred (Fig. 4, lanes 1 and 4). However, upon CBP-EntH binding and saturation of the calmodulin beads, this unspecific binding was drastically reduced, as can be observed in lane 3, where there is no more T25-Flag-EntB(S245A) binding. This demonstrates that the binding of T25-Flag-EntB observed in lane 2 is highly specific.

Taken together, the data obtained in vivo by the two-hybrid technique and the copurification experiment demonstrate that EntH interacts specifically with EntB and that this interaction

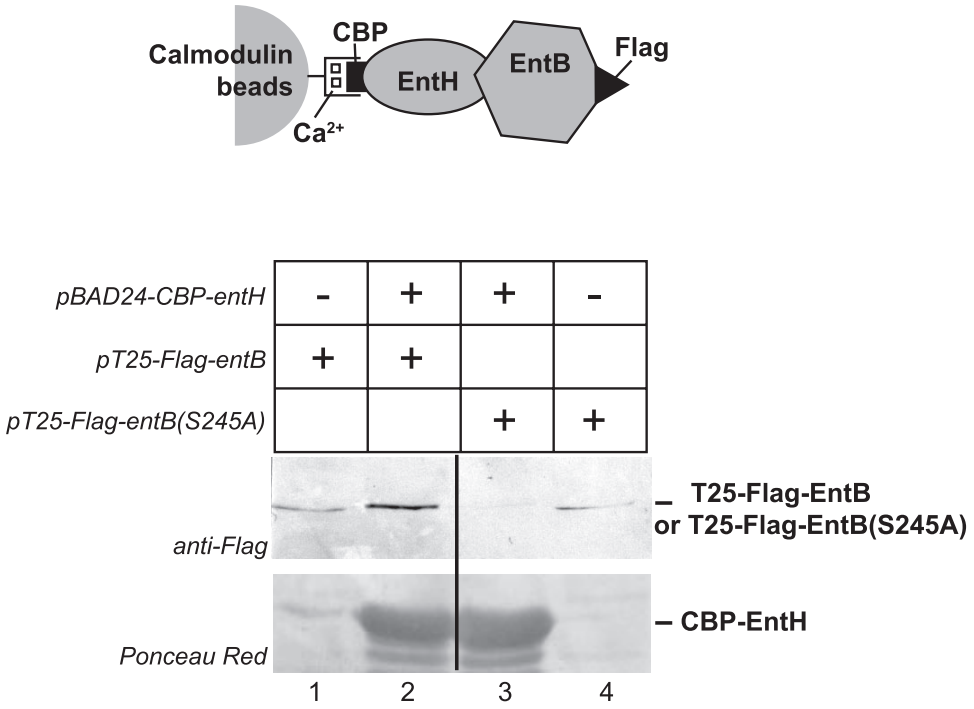


FIG. 4. EntB is copurified with EntH. The C600 strain was cotransformed with two compatible plasmids encoding T25-Flag-EntB [or T25-Flag-EntB(S245A)] and pBAD24-CBP-*entH* (or pBAD24-CBP). After extract preparation, samples were purified on calmodulin beads as indicated in Materials and Methods. Western blot analysis was then performed using anti-Flag monoclonal antibodies. The upper scheme depicts the principle of the experiment. 1, pBAD24-CBP/pT25-Flag-*entB*; 2, pBAD24-CBP-*entH*/pT25-Flag-*entB*; 3, pBAD24-CBP-*entH*/pT25-Flag-*entB*(*S245A*); 4, pBAD24-CBP/pT25-Flag-*entB*(*S245A*).

is impaired when EntB cannot be modified by the Ppant group. Therefore, EntH is involved in the polymerization step of enterobactin biosynthesis.

***entH* is required for optimal production of enterobactin in vivo.** The *entH* gene belongs to the *entCEBAH* operon and is regulated by iron limitation. Furthermore, we have demonstrated a specific physical interaction in vivo between EntH and the ArCP domain of EntB. Therefore, both the genetic and physical associations of EntH with the Ent proteins led us to test whether EntH was involved in enterobactin biosynthesis in vivo.

We first checked the phenotype of a Δ*entH* mutant. We used the BW25113Δ*entH*::Kan^r strain of the Keio collection (1) and transduced the mutation into a W3110 background. We followed growth in iron-limiting conditions induced by addition of dipyridyl to MM9Tris minimal medium. The Δ*entH* mutation did not exhibit any growth phenotype compared to the wild type under this condition (Fig. 5, left-hand plate and growth curve). Therefore, EntH did not seem necessary for enterobactin production. However, we then tested the growth of the Δ*entH* mutant in the presence of 500 μM salicylate. This compound is an analogue of the DHB precursor of enterobactin synthesis and can be loaded similarly on EntB and EntF in the second step but then would block the following processing of the molecule (13). Upon addition of dipyridyl and salicylate, the mutant W3110Δ*entH* displayed a significant reduction in growth compared to wild-type W3110 (Fig. 5, right-hand plate and growth curve). We scanned this growth inhibition on plates at different salicylate concentrations. The phenotype could be detected when salicylate concentration was above 50 μM and increased with salicylate concentration (data not shown). This effect was complemented by the production of EntH in *trans* using the pBAD24-*entH* plasmid (Fig. 5, right-hand plate and growth curve). This result showed that EntH was required for correct growth in the strong inhibitory condition caused by iron limitation and salicylate and hence suggested that EntH was required for optimal enterobactin production.

To confirm this, we analyzed the enterobactin production of the different strains. As analyzed on CAS reporter plates containing 500 μM salicylate, the Δ*entH* mutant produced one-third the amount of enterobactin produced by the wild-type strain (Fig. 6A). This inhibition was partially complemented by the production of EntH in *trans* using the pBAD24-*entH* plasmid (Fig. 6A). Enterobactin production was also assayed in culture supernatants containing 500 μM salicylate, using the CAS liquid assay as described in Materials and Methods. In this experiment, the Δ*entH* mutant behaved like the Δ*entB* mutant negative control, confirming the strong defect in enterobactin production (Fig. 6B). Enterobactin production was restored at the wild-type level upon production of EntH in *trans* using the pBAD24-*entH* plasmid, even without addition of arabinose (Fig. 6B).

Therefore, we conclude that EntH is required for optimal enterobactin production upon iron limitation and the presence of salicylate. EntH could prevent the blockage of enterobactin synthesis provoked by incorporation of salicylate, a precursor analogue, at the beginning of the polymerization step.

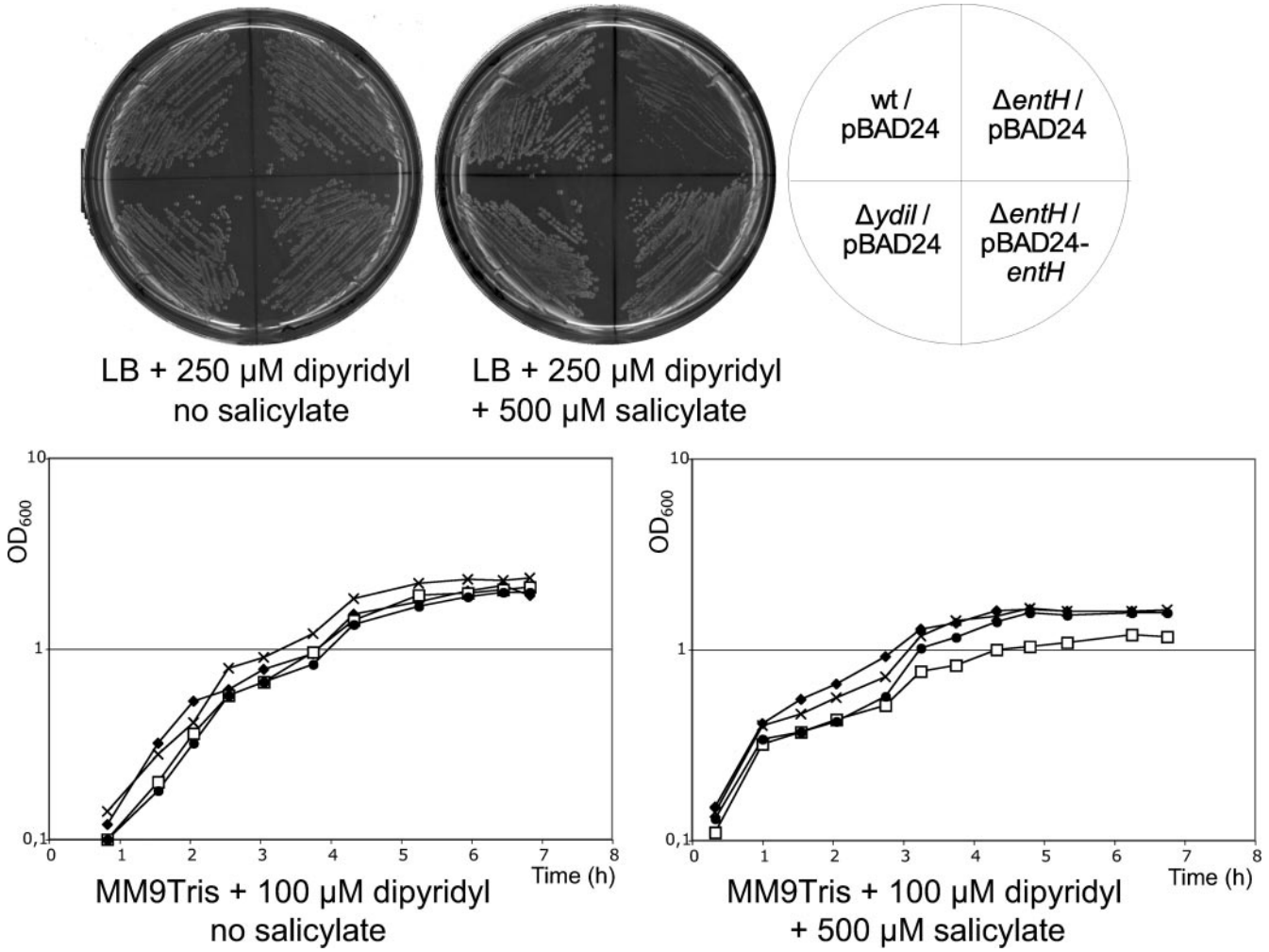


FIG. 5. Growth of the Δ*entH* mutant is affected in the presence of salicylate. Strains W3110/pBAD24, W3110Δ*entH*/pBAD24, W3110Δ*ydiI*/pBAD24, and W3110Δ*entH*/pBAD24-*entH* were plated on LB plates containing 250 μM dipyridyl, 0.01% arabinose, and ampicillin, as indicated on the right-hand scheme. The left-hand plate was incubated for 24 h at 30°C, and the right-hand plate, containing 500 μM salicylate, was incubated for 36 h at 30°C. The corresponding growth curves were obtained following cultures in MM9Tris liquid medium containing 100 μM dipyridyl and ampicillin at 37°C, without arabinose. wt, wild type; ◆, W3110/pBAD24; □, W3110Δ*entH*/pBAD24; ●, W3110Δ*ydiI*/pBAD24; and ×, 5W3110Δ*entH*/pBAD24-*entH*.

Overexpression of EntH inhibits enterobactin production. Taking the problem in reverse, we decided to test the effect of overproducing EntH when not needed, i.e., without salicylate addition. We again used the pBAD plasmid permitting the overproduction of proteins, using arabinose as an inducer. The mutant W3110Δ*entH* produced as much enterobactin as the wild-type W3110 strain, as detected on CAS plates (Fig. 6C), consistent with the fact that the mutant W3110Δ*entH* showed no growth phenotype during iron limitation produced by dipyridyl addition (Fig. 5, left-hand plate and growth curve). Under the same conditions, overexpression of EntH with 0.01% arabinose produced a significant decrease in siderophore production: the strain W3110/pBAD24-*entH* produced two-thirds the amount of enterobactin produced by the control strain W3110/pBAD24, whereas no effect was visible when another hotdog protein, such as YdiI, was overproduced (Fig. 6C and see below). This inhibitory effect of EntH overproduction in the Δ*entH* or wild-type strains was not observed when salicylate was present (Fig. 6A and B and data not shown). To understand better these inverse effects, we compared EntH protein levels under the different conditions using TAP-tagged EntH proteins. Without arabinose induction, quantities of EntH-TAP protein produced in the W3110/EntH-TAP recombinant strain under iron starvation or from a pBAD24-*entH*-TAP plasmid are comparable (see Fig. S1 in the supplemental material). Upon 0.01% arabinose induction, amounts of EntH-TAP are 50 times greater with the plasmid than from chromosome expression (see Fig. S1 in the supplemental material).

Therefore, we concluded that strong overproduction of EntH had an inhibitory effect on enterobactin production under iron starvation conditions, yet this overproduction is still beneficial to bacteria when salicylate is present.

Characterization of the hotdog proteins of *E. coli*. In this study, we show a new type of physiological role for a protein of the hotdog superfamily: a role in nonribosomal biosynthesis

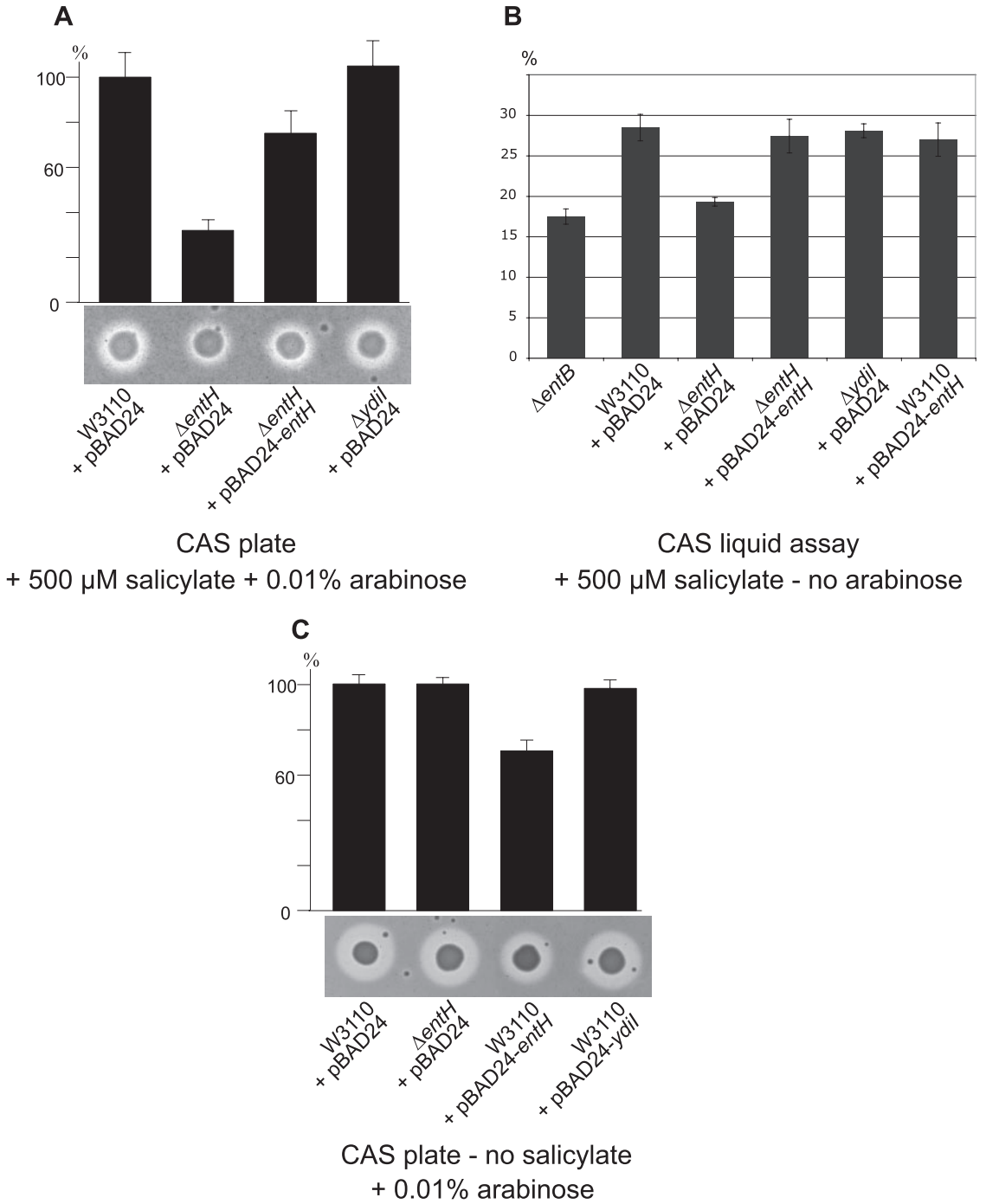


FIG. 6. (A) Enterobactin production in the presence of salicylate for the strains W3110/pBAD24, W3110Δ*entH*/pBAD24, W3110Δ*entH*/pBAD24-*entH*, and W3110Δ*ydiI*/pBAD24. Enterobactin production was assayed on CAS plates containing 0.01% arabinose, ampicillin, and 500 μM salicylate at 30°C as described in Materials and Methods. The level of enterobactin production by the W3110/pBAD24 strain is arbitrarily set to 100%. Seven measurements were taken for each strain. Photographs of representative plates are shown under the graph. (B) Enterobactin production in the presence of salicylate for the strains BW25113Δ*entB*, W3110/pBAD24, W3110Δ*entH*/pBAD24, W3110Δ*entH*/pBAD24-*entH*, W3110Δ*ydiI*/pBAD24, and W3110/pBAD24-*entH*. Enterobactin level in culture supernatants was assayed by the CAS method as described in Materials and Methods. Cultures were performed in triplicate in minimal medium containing ampicillin and 500 μM salicylate at 37°C. The values represent the percentages of reduction in absorbance at 630 nm compared to the level for the reference assay performed on uninoculated medium. (C) Enterobactin production without salicylate for the strains W3110/pBAD24, W3110Δ*entH*/pBAD24, W3110Δ*entH*/pBAD24-*entH*, and W3110Δ*entH*/pBAD24-*ydiI*. Enterobactin production was assayed on CAS plates containing 0.01% arabinose and ampicillin at 30°C as described in Materials and Methods. The level of enterobactin production by the W3110/pBAD24 strain is arbitrarily set to 100%. Seven measurements were taken for each strain. Photographs of representative plates are shown under the graph.

A

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                    10        20        30        40        50        60        70        80        90       100
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PaaI           1    ----------MSHKAWQNAHAMYENDACAKAIGIDIISMDEG--------FAVVTMTVT----AQMLNGHQS-GHGGQLFSLADTAFAYACNS- 70
YigI           1    ---MKCKDDMSAVLTAEQALKLVGEMFVYHMPFNRALGMELERYEKE--------FAQLAFKNQ---PMMVGNWAQSILHGGVIASALDVAAGLVCVGS 85
YbaW           1    ------------------------------------------------------MQTQIKVR-----GYHLDVYQHVNNARYLEFLEEARWDGLENS 38
YbgC           1    ------------------------------------------------MNTTILFRWPVEVY---YEDTDAGGVVYHASYVAFYERARTEMLRHH 43
YciA Haein     1    ---------------------------------MSANFTDKNGRQS---KGVLLLRTLAM-----PSDTNANGDIFGGWIMSQMDMGGAILAKEI 54
YciA           1    ------------------------------------------MSTTHNVP---QGDLVLRTLAM-----PADTNANGDIFGGWLMSQMDIGGAILAKEI 49
YdiI           1    ----------MIWKRKITLEALNAM-GEGNMVGFLDIRFEHIGDD---------TLEATMPVD-----SRTKQPFGLLHGGASVVLAESIGSVAGYIC 73
EntH           1    ----------MIWKRHLTLDELNAT-SDNTMVAHLGIVYTRLGDD---------VLEAEMPVD-----TRTHQPFGLLHGGASAALAETLGSMAGFMM 73
FabA           1    MVDKRESYTKEDLLASGRGELFGAKGPQLPAPNMLMMDRVVKMTETGGNFD---KGVVEAELDINPDLWFFGCHFIGDPVMPGCLGLDAMWQLVGFYLGW 97
FabZ           1    ----------MTTNTHTLQIEEILELLPHRFPFLLVDRVLDFE-EG--------RFLRAVKNVSVNEPFFQGHFPGKPIFPGVLIIEAMAQATGILAFK 80
FabZ Pseud     1    ----------------MMDTNETREYLPHRYPFLLVDRVVELDIEG--------KRIRAYKNVSINEPFFNGHFPEHPIMPGVLIIEAMAQAAGILGFK 75
TesB-I         1    ----------MSQALKNLLTLL----------NLEKIEEG-----------LFRGQSE--------DLGLRQVFGGQVVGQALYAAKETV--- 51
TesB-II      133    ----------ETQIAQSLAHLLPPVLKDKFICDRFLEVRPVEFHNPLKGHVAEPHRQVWIRA---NGSVPDDLRVHQYLLGYASDLNFLPVALQP 214
Q8E989 Sheon   1    ----------DELLNRLRQTWHSTIPVSEFMQIAPLSFTDG--------ELSVSAPLA------PNINLHHTMFAGSIYTIMTLTGWGMVWLQ 69
YiiD         155    FLMIKPVATLDDILHRGDWCAQLQQAWYEHIPLSEKMGVRIQQYTGQ--------KFITTMPET------GNQNPHHTLFAGSLFSLATLTGWGLIWLM 239
                                                                                  ---------  ----------------------

                         110       120       130       140       150       160       170       180       190       200
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PaaI           70    ----------QGLAAVASACTIDFLRPGFAGD--TLTATAQVRHQGKQ------TGVYDIEIVNQ-------QQKTVALFRGKSHRIGGTITGE 139
YigI           86    TLTRHETISEDELRQRLSRMGTIDLRVDYLRPG-RGE--RFTATSSLLRAGNK-------VAVARVELHNE-------EQLYIASATATYMVG------- 161
YbaW           38    -DSFQWMTA------HNIAFVVVNININYRRPAVLSD--LLTITSQLQQLNGK-------SGILSQVITLE------PEGQVVADALITFVCIDLKTQKA 116
YbgC           44    HFSQQALMA------ERVAFVVRKMTVEYYAPARLDD--MLEIQTEITSMRGT-------SLVFTQRIVNA-------ENTLLNEAEVLVVCVDPLKMKP 121
YciA Haein     55    AH----------GRVVTVAVES-MNFIKPISVGD--VVCCYGQCLKVGRS-------SIKIKVEVWVKKVASEPIGEKYCVTDAVFTFVAVDNNGRS 131
YciA           50    AH----------GRVVTVRVEG-MTFLRPVAVGD--VVCCYARCVQKGTT-------SVSINIEVWVKKVASEPIGQRYKATEALFKYVAVDPEGKP 126
YdiI           74    T----------EGEQKVVGLEINANHVRSAREGRVRGVCKPLHLGSR---------HQVWQIEIFDE-------KGRLCCSSRLTTAIL------- 136
EntH           74    T----------RDGQCVVGTELNATHHRPVSEGKVRGVCQPLHLGRQ---------NQSWEIVVFDE-------QGRRCCTCRLGTAVLG------ 137
FabA           98    LGG----------EGKGRALGVGEVKFTGQVLPTAK-KVTYRIHFKRIVNRR-----LIMGLADGEVLV-------DGRLIYTASDLKVGLFQDTSA 171
FabZ           81    SVG--------KLEPGELYYFAGIDEARFKRPVVPGD---QMIMEVTFEKTRR------GLTRFKGVALV--------DGKVVCEATMMCARSREA---- 151
FabZ Pseud     76    MLDV-------KPADGTLYYFVGSDKLRFRQPVLPGD---QLQLHAKFISVKR------SIWKFDCHATV--------DDKPVCSAEIICAEKKL------ 146
TesB-I         51    ----------------PEERLVHSFHSYFLRP---GD--SKKPTIYDVETLRD-----GNSTSARRVAAIG-------NGKPIFYMTASFQAPEAGFEHQ 118
TesB-II      215    HGIGFL--------EPGIQIATIDHSMWFHRP---FN--LNEWLLYSVESTSAS-----SARGFVRGEFYT------QDGVLVASTVQEGVMRNHN---- 286
Q8E989 Sheon  70    QQLL----------NVDGDIVLADAHIRYLAPVTSAPSVKVEWPDTN---LSPLQRGRKAKVKLEVQLFC--------DGKLCAQFDGLYVSVPKM---- 144
YiiD         240    LRER----------HLGGTIILADAHIRYSKPI-SGKPHAVADLGALSGDLDRLARGRKARVQMQVEIFG--------DETPGAVFEGTYIVLPAKPFGP 320
                                  ------------------        -------------      ------------      ---------------

                         210       220
                         ....|....|....|....|...
PaaI           140   A---------------------  140
YigI           161   ---------------------  161
YbaW           117   LALEGELREKLEQMVK------  132
YbgC           122   RALPKSIVAEFKQ---------  134
YciA Haein     132   RTIPRENNQELEKALALISEQPL 154
YciA           127   RALPVE----------------- 132
YdiI           136   ---------------------  136
EntH           137   ---------------------  137
FabA           172   F--------------------- 172
FabZ           151   ---------------------  151
FabZ Pseud     146   ---------------------  146
TesB-I         119   KTMPSAPAPDGLPS--------  131
TesB-II        286   ---------------------  286
Q8E989 Sheon   144   ---------------------  144
YiiD           321   YEEGGNEEE------------  329
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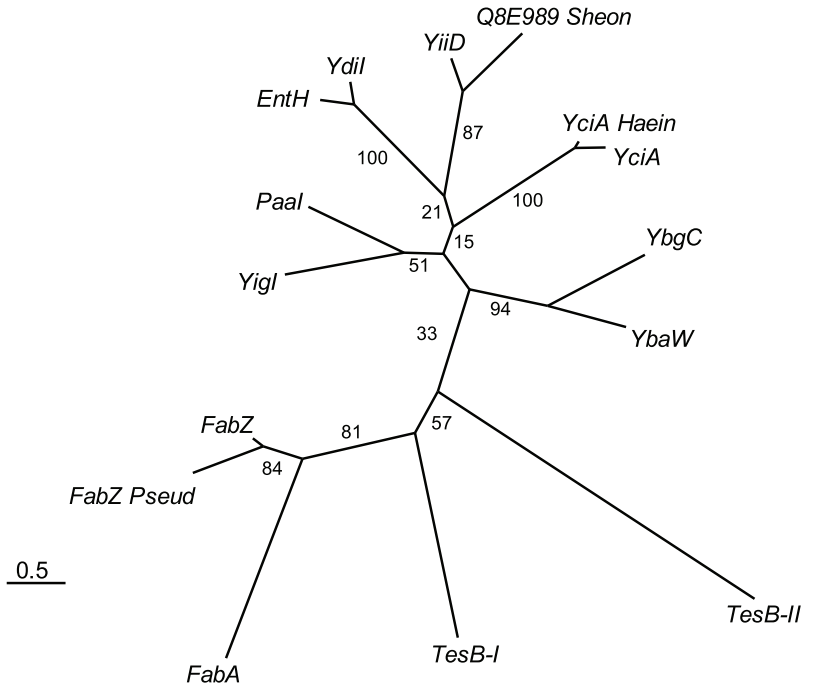
B



pathways, such as the enterobactin siderophore biosynthesis pathway. To place EntH into an evolutive point of view in the hotdog protein family and to try to get information about the functions of other proteins of this family, we constructed an alignment of 11 of the hotdog proteins of *E. coli* based on secondary structures of *E. coli* or other organism proteins (Fig. 7A). TesB shows an internal repeat (TesB-I and TesB-II) described as a double hotdog fold (25). The more distant MaoC hotdog protein was excluded from this study because it significantly differs from the other hotdog proteins and it generates problems of legibility for alignments. We then constructed the corresponding unrooted tree based on the most conserved regions of the proteins (Fig. 7B). Several of the branches have low bootstrap values, showing the high divergence of these proteins despite very similar structures. Similarly, the active sites are not conserved or located at the same residue positions in the structure (Fig. 7A). On the other hand, some of the proteins may have diverged more recently, such as YbgC and YbaW (49% similarity) or PaaI and YigI (38% similarity). It is especially true for the YdiI and EntH proteins, which are twins in the bootstrap 100 tree (Fig. 7B). This is in accordance with the high similarity of these two proteins (59% identity, 75% similarity; see also alignment in Fig. 7A). Because of their high similarity, it was tempting to think that these two proteins could have similar functions.

YdiI is not involved in enterobactin synthesis. Because of the high sequence similarity between EntH and YdiI, we hypothesized that YdiI may also be involved in enterobactin synthesis. We first checked the phenotype of a Δ*ydiI* mutant. We used the BW25113Δ*ydiI*::Kan^r strain of the Keio collection (1) and transduced the mutation in a W3110 background. Similarly to the Δ*entH* deletion, the Δ*ydiI* mutation did not exhibit any growth phenotype compared to the wild-type strain on dipyridyl (Fig. 5, left-hand plate and growth curve). However, contrary to the Δ*entH* deletion, the Δ*ydiI* mutation did not exhibit any growth phenotype on a dipyridyl-plus-salicylate plate (Fig. 5, right-hand late and growth curve). This is consistent with the fact that the Δ*ydiI* mutant was not affected either in enterobactin production, as shown by the CAS plate assay (Fig. 6A and B). We then constructed the double mutant Δ*entH* Δ*ydiI* and tested its growth phenotype on dipyridyl with or without salicylate. The double mutant behaved exactly like the simple Δ*entH* mutant (data not shown).

We also compared the effect of overexpression of YdiI to the effect obtained with EntH. The expression of YdiI had no effect on enterobactin production (Fig. 6C). This last finding highlights the very specific effect of the overproduction of EntH on enterobactin production and rules out the possibility of an indirect energetic effect coming solely from the production of a protein. Therefore, all these results showed that despite high similarities, YdiI and EntH display different functions in vivo.

DISCUSSION

Previous studies on the response of *E. coli* to iron limitation failed to define a function for the YbdB protein encoded by the last gene of the operon *entCEBA-ybdB* (*P15*). In this work, we have shown that the hotdog protein YbdB is involved in vivo in enterobactin siderophore production.

We showed that EntH is a thioesterase, which is physiologically produced under iron starvation conditions like the other Ent proteins. We then showed in vivo a specific interaction between EntH and the ArCP domain of EntB. This domain is homologous to the ACP protein that plays a role as cofactor in fatty acid synthesis, and it is modified posttranslationally in a similar way on its serine 245 residue by a Ppant group (Fig. 8). Then, it plays a role as carrier for the precursors of enterobactin in synthesis to be delivered to EntF for the oligomerization step (Fig. 8). Importantly, EntH interaction with an EntB protein mutated at the serine 245 residue was nearly abolished, demonstrating that the interaction between EntH and the ArCP domain of EntB required a posttranslationally modified Ppant-EntB protein.

There was no evident effect of the deletion of the *entH* gene on enterobactin production or on growth under iron starvation conditions. However, we found that EntH is required for optimal growth and enterobactin production when we added to the growth media salicylate, an analogue of DHB and an inhibitor of the pathway. In reverse, the overproduction of EntH can inhibit enterobactin production in vivo. It has to be stressed that, considering either the deletion mutant phenotype or the inhibitory overproduction phenotype, the effect of EntH was specific. Indeed, there were no similar effects of the *ydiI* deletion or of the YdiI overexpression despite the high sequence similarities between the EntH and YdiI proteins (Fig. 5 and 6). From these results, we concluded that EntH is implicated in vivo in optimal enterobactin production.

We were able to detect an interaction in vivo between EntB and EntH by using the two-hybrid technique, and this was confirmed by copurification of the two proteins. Surprisingly, we did not detect any other interactions between the Ent proteins by using the two-hybrid technique or TAP on EntH-TAP (Fig. 3 and data not shown), despite the facts that these enzymes are likely to function as a protein complex and that many interactions have been studied indirectly. More particularly, the ArCP domain of EntB should interact with EntE and EntF, as it was studied by combinatorial mutagenesis (23). In

FIG. 7. Sequence analysis of 11 *E. coli* hotdog proteins. (A) Sequence alignment. The alignment shown is based on the sequence of 11 hotdog proteins from *E. coli*, with 3 additional sequences of hotdog proteins from other organisms. Only the hotdog domain sequence of YiiD is presented, and the two hotdog domains of TesB (TesB-I and TesB-II) are displayed as two separate entities. The secondary structure elements (green, α-helices; red, β-strands) of PaaI (PDB no. 1psuA), YbaW (PDB no. 1njkA), YbgC (PDB no. 1s5uA), YciA (*Haemophilus influenzae*; PDB no. 1yliA), YdiI (PDB no. 1vh5A), EntH (PDB no. 1vh9A), FabA (PDB no. 1mkbA), FabZ (*Pseudomonas aeruginosa*; PDB no. 1u1za), TesB (parts I and II of the double hotdog fold; PDB no. 1c8u), and Q8F989_Sheon (PDB no. 1T82; a YiiD partial homolog from *Shewanella oneidensis*) are indicated on the alignment. Residues identified previously to have crucial roles in catalysis are indicated in bold and underlined (21, 24, 25, 40). The black dotted line indicates regions used for the construction of the tree. (B) Unrooted tree. Numbers at nodes indicate the bootstrap values. The scale bar represents the average number of substitutions per site.

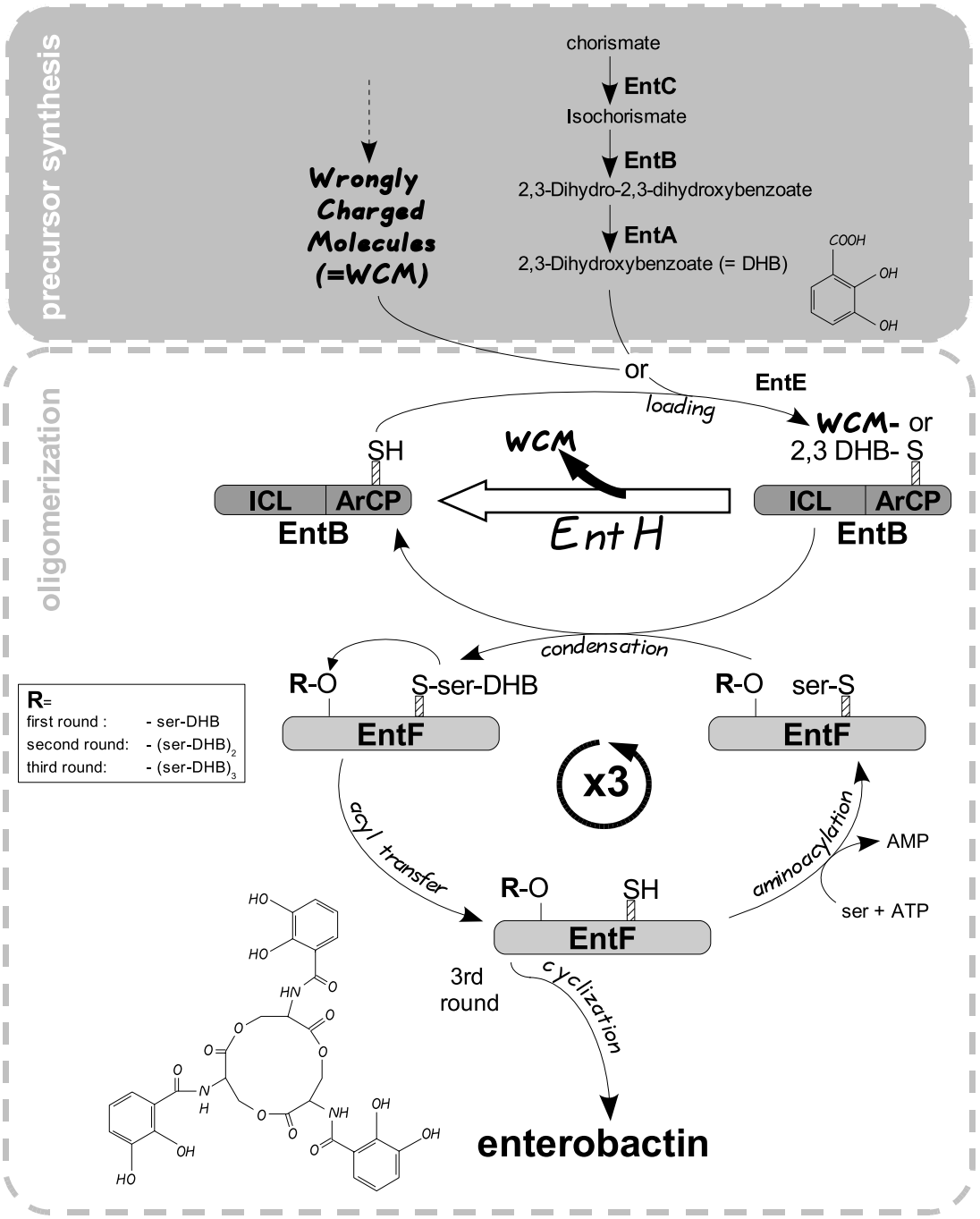


FIG. 8. Scheme of the enterobactin biosynthesis pathway based on previous work (7, 12, 13) with the proposed role played by EntH: EntH removes wrongly charged molecules from the ArCP domain of EntB, thus preventing the blocking of the biosynthesis pathway. ser, L-serine. R represents the molecule attached to the Ppant group on EntF during the three cycles required for the full synthesis of enterobactin. The hatched box represents the Ppant group.

addition to the EntB/EntH interaction, we were able to detect dimerization only for EntC, EntB, EntA, and EntH. However, it has never been possible to isolate a protein complex corresponding to the enterobactin synthase, either by immunoprecipitation (18) or by gel filtration chromatography (13). Therefore, as suggested in previous studies, the complex is likely to be transient and loosely associated. This suggests a stronger interaction between EntB and EntH. The enterobactin synthetase has also been suggested to be associated with the cytoplasmic membrane, but this association is not required for activity (18). However, the Ent proteins display a characteristic behavior upon osmotic shock, suggesting a loose association with the cytoplasmic membrane that might correspond to a specific localization or compartmentalization (18). Using the

EntH-TAP strain for spheroplast production and extract preparation, we observed that EntH-TAP was mainly cytoplasmic (data not shown).

We have shown that EntH displays a thioesterase activity on acyl-CoA substrates in vitro, like most hotdog proteins (22, 40, 43). The low k_{cat}/K_m values for *n*-propionyl-CoA and palmitoyl-CoA substrates reflect that these acyl-CoA substrates do not correspond to the physiological substrate of EntH. However, the k_{cat}/K_m value for benzoyl-CoA is 100-fold higher (9.6×10^4 M^{-1} s^{-1}). This may be related to the chemical nature of the real substrates of EntH, which we propose to be related to DHB, such as salicylate for example. Intermediates of enterobactin biosynthesis (DHB usually) are bound to the ArCP domain of EntB via a thioester linkage to the Ppant posttranslational modification (Fig. 8). Because EntH binding to EntB is dependent on the presence of the Ppant group on EntB, as demonstrated by two-hybrid and copurification experiments (Fig. 3 and 4), it is likely that EntH displays a thioesterase activity on EntB.

Under iron-limiting conditions provoked by dipyridyl addition, EntH is not required for optimal growth or for enterobactin production. However, it becomes necessary when an analogue of DHB, such as salicylate, is added to the medium (Fig. 5 and 6A and B). Therefore, we hypothesized that EntH may be involved in a quality control or proofreading role for the synthesis of enterobactin precursors taking place on EntB. It would cleave the thioester bound existing between the Ppant group of EntB and wrongly charged molecules (such as salicylate) by a thioesterase activity. In this regard, it has to be stressed that the EntB/EntH interaction depends on the presence of the Ppant group on EntB. This hypothesis is strengthened by the fact that on the contrary, artificial overproduction of EntH can be deleterious to enterobactin production (Fig. 6C). This effect is compatible with a thioesterase activity on the thioester bound linking correct enterobactin precursors to the Ppant group of EntB. This inhibition could be due to an equilibrium displacement resulting from EntH overproduction, leading to cleavage of correctly charged EntB-Ppant-DHB. Surprisingly, the same expression plasmid has been used to produce EntH under the two conditions, in one case for restoring correct proofreading in the presence of salicylate (Fig. 5 and 6A and B) and in another case to produce an inhibitory effect without salicylate (Fig. 6C). In fact, complementation by the pBAD24-*entH* plasmid in the presence of salicylate was total without addition of arabinose and intermediate with 0.01% arabinose, suggesting that a mild inhibitory effect might already occur in the last case (compare Δ*entH* plus pBAD24-*entH* in Fig. 6A and B). Inverse effects of *entH* production depending on the conditions highlight the importance of the precise amount of EntH in the cell and of its stoichiometry with the other Ent proteins.

The role of EntH in removing wrong intermediates during enterobactin synthesis is similar to features described before for other nonribosomal biosynthesis pathways. In these pathways, whereas the final product is released by a well-studied thioesterase domain, a second independent thioesterase of type II has been often shown to be important for efficient product formation (36). For example, in pyochelin synthesis, a siderophore produced by *Pseudomonas aeruginosa*, a thioesterase of type II has been proposed to be involved in proofreading by removing misloaded amino acids (33). Concerning antibiotic synthesis pathways, it was suggested that the role of this thioesterase is to regenerate misacylated synthases, which result from the apo to holo conversion of the enzymes by Ppant transferases that use not only free CoA but also acyl-CoAs as Ppant donors (38). In the Ent system, this would mean that EntD may add the Ppant group to EntB already in an acylated form inherited from the acyl-CoA precursor. However, we found that EntH is not required under simple iron starvation conditions. Therefore, it does not seem that EntH is necessary for the regeneration of EntB at the beginning of the pathway and the hypothesis is not adequate. It was also proposed before that a proofreading mechanism for the molecules charged on the peptidyl carrier peptides is necessary if wrong molecules can be incorporated by mistake (33). Indeed, in the Ent system, it has been shown that EntE can incorporate incorrect molecules on EntB, such as salicylate, and that salicylate can be transferred to EntF but then blocks the synthesis pathway (12, 13). Because the growth of the *entH* mutant is impaired when salicylate is added in parallel with a decrease in enterobactin production, this second hypothesis is in accordance with our results, and in the Ent system, the external EntH thioesterase could be involved in proofreading and removal of wrongly charged molecules on EntB ArCP domain as described on our model presented in Fig. 8. *entH* is present in the other enterobactin-producing pathogenic strains of *E. coli* and in species of *Shigella* and *Salmonella*. Such a conservation is suggestive of an essential function for EntH that is not apparent under standard laboratory conditions. Salicylate is produced under conditions of iron limitation by many bacterial species, where it acts as a siderophore precursor. Enterobacterial EntH may prevent enterobactin blockage by salicylate-producing organisms that compete for the same iron-poor ecological niche.

EntH is the first hotdog protein described to date to play such a proofreading role in nonribosomal peptide syntheses. This adds a new entry to the list of cellular functions performed by the proteins of this family. These functions can be very diverse and furthermore remain unknown for most of the hotdog proteins, but common features can be extracted. It appears that they all have the ability to interact with an ACP-like domain or with CoA (references 10 and 15 and this work). Most of them are thioesterases, and when this is not the case, they still interact with thioesters of ACP or CoA. Furthermore, for most of the known functions, they participate in metabolic pathways, such as phenolic compound degradation or fatty acid or siderophore synthesis pathways. For example, PaaI and MaoC are involved in phenylacetic acid degradation (9), TesB may be used to enhance 3-hydroxydecanoic acid production from polyhydroxyalkanoates in *E. coli* (42), and a *tesB*-like gene has also been involved in polyhydroxyalkanoate metabolism in *Alcanivorax borkumensis* (35). These enzymatic activities are of great interest for biotechnological applications, which is certainly why the unknown-function proteins of the hotdog family have been all targeted in structural genomic projects. Nonribosomal synthesis pathways acting on phenolic compounds, such as the enterobactin synthesis pathway, are also involved in the synthesis of many valuable chemicals, such as antibiotics and vitamins, which shows the importance of better understanding the role that the hotdog proteins play in these pathways.

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