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The Lethal Phenotype Caused by Null Mutations in the *Escherichia coli htrB* Gene Is Suppressed by Mutations in the *accBC* Operon, Encoding Two Subunits of Acetyl Coenzyme A Carboxylase

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Insertion mutations in the *Escherichia coli htrB* gene result in the unique phenotype of not affecting growth at temperatures below 32.5°C but leading to a loss of viability at temperatures above this in rich media. When *htrB* bacteria growing in rich media were shifted to the nonpermissive temperature of 42°C, they continued to grow at a rate similar to that at 30°C but they produced phospholipids at the rate required for growth at 42°C. This led to the accumulation of more than twice as much phospholipid per milligram of protein compared with that in wild-type bacteria. Consistent with HtrB playing a role in phospholipid biosynthesis, one complementation group of spontaneously arising mutations that suppressed *htrB*-induced lethality were mapped to the *accBC* operon. This operon codes for the biotin carboxyl carrier protein and biotin carboxylase subunits of the acetyl coenzyme A carboxylase enzyme complex, which catalyzes the first step in fatty acid biosynthesis. Four suppressor mutations mapped to this operon. Two alleles were identified as mutations in the *accC* gene, the third allele was identified as a mutation in the *accB* gene, and the fourth allele was shown to be an insertion of an IS*1* transposable element in the promoter region of the operon, resulting in reduced transcription. The suppressor mutations caused a decrease in the rate of phospholipid biosynthesis, restoring the balance between the biosynthesis of phospholipids and growth rate, thus enabling *htrB* bacteria to grow at high temperatures.

During a screen for new *Escherichia coli* heat shock genes, two insertion mutations in the *htrB* gene were isolated. Bacteria carrying either of these two mutations grow in a completely wild-type manner at temperatures below 32.5°C but are inviable at higher temperatures in rich media (23). Although *htrB* was later shown not to be a heat shock gene (24), its unique temperature requirement is intriguing because, for the most part, *E. coli* cells growing between 21 and 37°C show very few temperature-dependent adaptive responses (20). One of the changes that occurs is an alteration in the composition of lipids that is required for the maintenance of membrane fluidity (30). Originally, we disregarded the possibility that HtrB may directly affect membrane structure because known mutants unable to correctly alter their lipid composition are viable at all temperatures (9, 40). Rather, on the basis of the similarity of the morphology of *htrB* bacteria grown at nonpermissive temperatures to that of cell wall biosynthesis mutants (5), we proposed that HtrB was involved in cell wall biosynthesis (23). However, further studies have led us to conclude that HtrB most likely does play a role in membrane structure.

This conclusion originated from the study of a multicopy suppressor of *htrB*, *msbB*. These multicopy suppressors are genes that when cloned onto multicopy plasmids rescue the Ts^- phenotype of *htrB*. The protein encoded by *msbB* may serve a role similar to that of HtrB, since the MsbB protein sequence is similar to that of HtrB (25). In addition, null mutations in either the *msbB* or *htrB* gene result in a similar and unique phenotype, namely, the ability to grow on fourfold higher concentrations of deoxycholate than can wild-type bacteria (25). The increased resistance to deoxycholate most likely indicates that *htrB* and *msbB* bacteria have alterations affecting membrane structure, possibly the lipopolysaccharide (LPS) layer.

In general, mutations that affect the LPS layer alter the resistance of bacteria to hydrophobic compounds. Although most known LPS mutants are hypersensitive to hydrophobic molecules (35), there are a few mutants which exhibit increased resistance to hydrophobic molecules. The best studied of these is a mutant of *Salmonella typhimurium*, a *pmrA* mutant (41). This mutant exhibits an increased resistance to the hydrophobic drug polymyxin B. The increase in resistance has been shown to be associated with a decrease in the positive charge of a portion of the LPS molecules, thus reducing the number of binding sites for the negatively charged polymyxin B (42). A similar type of change in LPS structure may lead to the increased resistance to deoxycholate of *htrB* and *msbB* bacteria. For example, a decrease in the amount of LPS molecules with negatively charged phosphoethanolamine residues could lead to fewer binding sites for the positively charged deoxycholate molecules.

Another indication that HtrB affects the membrane structure is that low levels of cationic detergents suppress its Ts^- phenotype (25). The cationic detergents may act by altering the interactions between the LPS molecules and divalent cations or polyamines. The addition of Ca^{2+} or Mg^{2+}

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TABLE 1. Strains

Strain	Relevant characteristic(s)	Reference or source
W3110	Wild type	Our collection
B178	W3110 *galE*	14
MLK53	W3110 *htrB1*::Tn*10*	23
MLK777	B178 *htrB1*::Tn*10* *zhb-43*::Tn*10*-Kan^r	This work
MLK993	MLK53 Ts$^+$1031 *zhb-43*::Tn*10*-Kan^r	This work
MLK1000	MLK53 Ts$^+$1123 *zhb-43*::Tn*10*-Kan^r	This work
MLK995	MLK53 Ts$^+$1043-1 *zhb-43*::Tn*10*-Kan^r	This work
MLK994	MLK53 Ts$^+$1043-6 *zhb-43*::Tn*10*-Kan^r	This work
MLK519	B178 *htrB1*::Tn*10* Ts$^+$1031(λ)	This work
MLK1067	W3110 *msbB*::Ωcam	25
MLK986	MLK53 *msbB*::Ωcam	25
MLK1086	MLK986 Ts$^+$1043-1 *zhb-43*::Tn*10*-Kan^r	This work
MLK1087	MLK986 Ts$^+$1043-6 *zhb-43*::Tn*10*-Kan^r	This work
DH5α	*recA1*	Bethesda Research Laboratories

reverses this rescue, possibly by competing for the same sites on the LPS molecules (25). These results have led us to propose that HtrB affects outer membrane structure and function (25).

In an attempt to further understand the role of HtrB in bacterial physiology, we have undertaken the study of a second type of *htrB* suppressor: single-copy, spontaneously arising, extragenic suppressor mutations. Presumably, these mutations directly or indirectly alter functions that are affected by the lack of HtrB. By mapping these suppressor mutations and identifying the genes that encode them, we hoped to clarify the role that HtrB plays in *E. coli* physiology. Consistent with the proposal that HtrB plays a role in membrane structure and function, we report here that one complementation group of such suppressors affects the biosynthesis of phospholipids and that HtrB may play a role in the coupling of phospholipid biosynthesis and growth rate.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are shown in Table 1. Early work with the suppressor mutations was done in the B178 background. B178 carries a *galE* mutation which blocks the mucoidy associated with wild-type bacteria (14). After the discovery that HtrB itself may affect membrane function, all mutations were moved into the wild-type W3110 background. All experiments presented here were performed using this background strain, unless otherwise indicated. Bacteria were grown in Luria-Bertani (LB) medium prepared as described previously (23). L agar is LB medium with 1% agar. Antibiotics were added when needed, at the following final concentrations: ampicillin, 50 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 10 μg/ml; chloramphenicol, 12 μg/ml; and kanamycin, 50 μg/ml.

Cell growth analysis. Bacterial growth, viability experiments, and photography were performed as described previously (23).

Plasmids. The pKS-1031 and pKS-1031-2 plasmids carry the 6-kb *Bam*HI fragment of λ transducing phage 6G3 (λ529) from the library made by Kohara et al. (26), cloned in both orientations into the *Bam*HI site of the pBluescript-KS plasmid (Stratagene). In pKS-1031, the fragment is oriented such that the T7 promoter on the plasmid is located 5′ to all of the open reading frames encoded on the fragment. The pBK2 plasmid was made by partially digesting pKS-1031 with *Kpn*I, digesting it to completion with *Bam*HI, isolating the 4-kb fragment from a low-melting-point agarose gel (FMC), and ligating the fragment to *Bam*HI-*Kpn*I-digested pBluescript-SK plasmid DNA. The p3/49 and p3/58 plasmids and the p2/53 plasmid are deletion derivatives of pKS-1031 and pKS-1031-2, respectively. These deletions were made according to the DNase I method of Hong (19). After partial DNase I digestion, the DNA was digested with *Eco*RI, ligated, and digested with *Pst*I to enrich for plasmids with deletions. The pE1 and pEK plasmids are the 900-bp *Eco*RV and *Eco*RV-*Kpn*I fragments of pKS-1031 cloned into the *Eco*RV and *Eco*RV-*Kpn*I sites of pBluescript-KS, respectively. The pGB-*accB* plasmid is the 2.3-kb *Eco*RV fragment from pKS-1031 cloned into the *Sma*I site of pGB2 (8). The pLac-*accC* plasmid was made by first digesting pKS-1031 with *Kpn*I and then partially digesting it with *Eco*RV. The 1.85-kb fragment was isolated from low-melting-point agarose and ligated with *Eco*RV- and *Kpn*I-digested pBluescript-KS plasmid DNA.

Genetic manipulations. P1-mediated transductions were performed as described by Miller (31).

Isolation of mini-Tn*10*-Kan^r elements linked to the cold-sensitive (Cs$^-$) suppressor mutations was accomplished by P1 transduction of a library of mini-Tn*10*-Kan^r insertions (45) into the suppressor strains, selecting simultaneously for Kan^r and colony formation at 30°C. The Cs$^+$ Kan^r colonies were then restreaked at 30 and 42°C. Normal growth at 30°C but inviability at 42°C indicated that the wild-type copy of the suppressor mutation was cotransduced with the mini-Tn*10*-Kan^r marker.

To determine complementation of the Ts$^+$1031 Cs$^-$ phenotype with the Kohara et al. λ clones (26), an aliquot of each clone was used to infect a fresh culture of *htrB* Ts$^+$1031(λ) (MLK519) and the bacteria were plated at 30°C. Colonies that grew were restreaked at 30 and 42°C to identify which phage clones complemented the growth defect.

Cloning and mapping of the *zhb-43*::Tn*10*-Kan^r marker. The *zhb-43*::Tn*10*-Kan^r marker was transduced into strain CG1151 (MC1040-2 carrying the Cam^r vector Mu d5005 [15]). A library of mini-Mu clones was made as described by Groisman and Casadaban (15) and plated on W3110(Mu) (MLK47). Clones which carry the mini-Tn*10*-Kan^r marker were isolated by selecting simultaneously for Kan^r and Cam^r, and one of them was used to probe the Kohara library of λ clones (26) by the techniques previously described (23).

PCR. Polymerase chain reactions (PCRs) were carried out by the method of Innis and Gelfand (21). The two primers used to amplify the coding region of *accB* were 5′-GCAATC

TCGCCGCCGGTTGGC-3′ and 5′-GAACGGTCGCCGGA GCGGCT-3′. The primers used to amplify the promoter region of the *accBC* operon were 5′-CGACCTCGTCCTCCC TGACG-3′ and 5′-GAACGGTCGCCGGAGCGGCT-3′.

DNA sequencing. Sequencing was done with Sequenase (version 2.1) as described by the manufacturer (United States Biochemical). PCR products were sequenced by the snap-cooling method of Kusukawa et al. (28). The 5′-CGACCTCGTCCTCCCTGACG-3′ primer, used to make the PCR products, was also used to sequence across one IS*1* junction. The other junction was sequenced with a primer homologous to the IS*1* element, 5′-CCATCATACACT AAATCAG-3′.

Northern blot analysis. Isolation of RNA and Northern (RNA) blot analysis were performed as described previously (24). The *accBC* probe was the *Hin*dIII-*Pst*I DNA fragment internal to *accBC* and was labeled as described previously (24). To control for even loading of the RNA samples, the blot was stained with methylene blue after the hybridization procedure (18).

Western blot analysis. Western blots (immunoblots) were carried out as described by Ang and Georgopoulos (3). Streptavidin conjugated with alkaline phosphatase (Bethesda Research Laboratories) was used to detect the biotinylated BCCP with the chemiluminescence detection kit Western-light, from Tropix.

Fatty acid analysis. Bacterial cultures were first grown at their permissive temperatures to mid-log phase in LB medium, diluted into 50 or 100 ml of the same medium to an optical density at 595 nm (OD_{595}) of 0.05, and grown at 30 or 42°C to an OD_{595} of 0.4. The bacteria were harvested by centrifugation, and after the bacteria were washed twice with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9) or 10 mM $MgCl_2$, fatty acids were extracted by the method of Bligh and Dyer (6) as described by Ames (2). The phospholipid fraction was isolated by direct extraction of the washed bacteria. The LPS-enriched fraction was isolated from the monophase of this extraction, present in the insoluble pellet, as described by Galloway and Raetz (12). The pellet was hydrolyzed in 6 N HCl for 3 h at 100°C, and fatty acids were extracted by the method of Bligh and Dyer (6). Methyl esters of the fatty acids were made with BF_3 and analyzed by gas chromatography on Supelco SPB-1 fused silica (15 m by 0.25 mm with 1-μm film thickness), with helium as the carrier gas (10 lb/in^2) and a temperature program of 5°C/min, 150 to 250°C. Supelco bacterial fatty acid methyl ester CP mixture 4-7080 was used to determine retention times of the individual fatty acids.

Quantification of esterified fatty acids. Bacterial cultures were grown as described for fatty acid analysis. After pelleting by centrifugation, the bacteria were washed once with 10 mM $MgCl_2$, and the phospholipids were isolated by the method of Bligh and Dyer (6). Following lyophilization, the lipids were quantitated as hydroxamates by the method of Stern and Shapiro (38). Standard curves were obtained with L-α-phosphatidylcholine di-heptadecanoyl (Sigma). All phospholipid quantities are given using an average molecular weight of 700.

Quantification of 3-deoxy-D-manno-octulosonic acid. Bacterial cultures of 400 ml were grown as described for fatty acid analysis. After the bacteria were washed once with ice-cold 50 mM Tris (pH 7.9) and resuspended in the same buffer, they were passed twice through a French press. The suspension was centrifuged at 5,000 × *g* for 5 min to remove large debris, and the supernatant was centrifuged at 30,000 × *g* for 60 min at 4°C. The resulting pellet was used as the outer membrane fraction and was washed once with 50 mM Tris (pH 7.9) and once with 10 mM HEPES (pH 7.4) before use. The remaining supernatant was centrifuged for 60 min at 175,000 × *g* at 4°C. The resulting pellet was used as the inner membrane fraction and was washed as described above. The inner and outer membrane fractions were resuspended in 250 μl of distilled H_2O and assayed for protein as described below and for 3-deoxy-D-manno-octulosonic acid by the method of Weissbach and Hurwitz (46). LPS purchased from Sigma was used as the standard.

Protein determination. Following the washing procedures described above, a portion of each culture was lysed in 0.5% sodium dodecyl sulfate–10 mM EDTA–10 mM Tris (pH 7.9) at room temperature or at 55°C for highly concentrated suspensions of bacteria. Protein concentrations were determined with the bicinchoninic acid protein assay reagent purchased from Pierce, with bovine serum albumin as the standard.

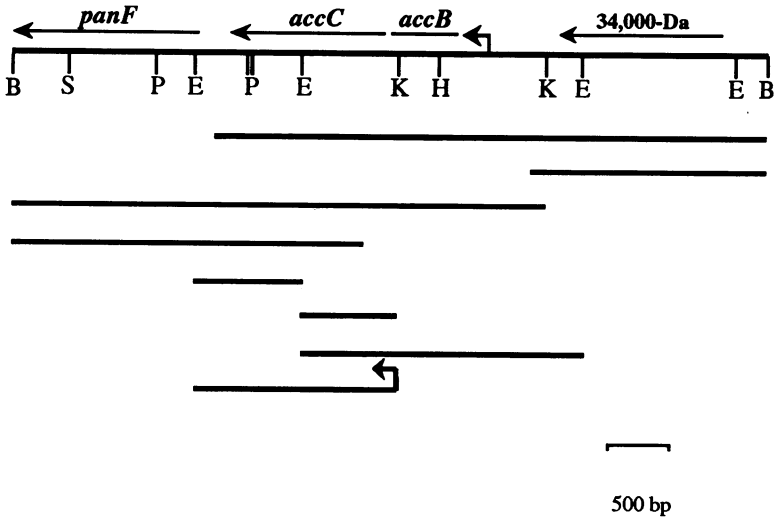
Determination of phospholipid biosynthesis rates. Bacteria were grown at their permissive temperatures to mid-log phase in LB medium and diluted to an OD_{595} equal to 0.05, 10 μCi of [1-^{14}C]acetate (NEN-Dupont) was added, and the 1-ml cultures were shifted to 42°C at time zero. Aliquots were taken at the appropriate times, and the phospholipids were extracted by the method of Bligh and Dyer (6). The chloroform-solubilized phospholipids were washed twice with 2 M KCl and once with distilled H_2O before scintillation counting.

RESULTS

Identification and cloning of the wild-type copies of the suppressor genes. Extragenic suppressors of the *htrB* insertion mutations arise spontaneously at a frequency of approximately 10^{-4} at the nonpermissive temperature of 42°C. Approximately one-third of these suppressor mutations show, to various degrees, a Cs$^-$ phenotype (i.e., slow or no growth at 30°C or below). Using this Cs$^-$ phenotype, complementation analysis with linked mini-Tn*10*-Kan^r markers (45) was performed to assign these suppressor mutations into several different complementation groups (data not shown). One of these classes consisted of two alleles, Ts$^+$1123 and Ts$^+$1031, that were extremely cold sensitive, being unable to form colonies at 30°C or below. Because of the tight Cs$^-$ phenotype of these two mutations, we were able to clone their corresponding wild-type genes by complementation.

The cloning of the wild-type genes in which the Ts$^+$1123 and Ts$^+$1031 mutations were located was done by first localizing a closely linked mini-Tn*10*-Kan^r element, *zhb-43*::Tn*10*-Kan^r. This was accomplished by cloning the *zhb-43*::Tn*10*-Kan^r marker into a mini-Mu plasmid (described in Materials and Methods) and using [α-^{32}P]dATP-labeled plasmid DNA to probe the overlapping λ clones of the *E. coli* genomic library made by Kohara et al. (26). The *zhb-43*::Tn*10*-Kan^r mini-Mu plasmid DNA hybridized to phage clones 6G3, 6G9, and 3C5 (λ529 to λ531), corresponding to the 71-min region on the *E. coli* chromosome. To identify the phage(s) that carries the intact wild-type gene, we infected *htrB* Ts$^+$1031(λ) (MLK519) bacteria with phage clones 3G10 to 4G11 (λ523 to λ535), covering a total of 70 kb of DNA on each side of the *zhb-43*::Tn*10*-Kan^r marker. Among those recombinant phages tested, only 21D3 and 6G3 (λ528 and λ529) complemented the Cs$^-$ phenotype of the Ts$^+$1031 mutation.

We further localized the complementation activity to a *Bam*HI fragment of approximately 6 kb that was isolated



	Complementation of suppressor mutations			
	Ts+ 1031	Ts+ 1123	Ts+ 1043-1	Ts+ 1043-6
pKS-1031	+	+	+	+
p3/58	+	+	+	+
p3/49	-	-	-	-
pBK2	+	+	+	+
p2/53	-	MR	-	-
pE1	-	MR	-	-
pEK	-	-	-	-
pGB-*accB*	MR	-	+	-
pLac-*accC*	-	+	-	+

FIG. 1. Restriction map of the pKS-1031 plasmid and complementation of the suppressor mutations. The directions of transcription and open reading frames of the four genes encoded on pKS-1031 are indicated above the restriction map. Restriction sites are marked with abbreviated forms of the names of the restriction endonucleases: B, *Bam*HI; S, *Sal*I; P, *Pst*I; E, *Eco*RV; K, *Kpn*I; H, *Hin*dIII. Deletion derivatives and subclones are shown below the restriction map, the bars indicate the portion of pKS-1031 that is cloned in each case, and the name of each clone is shown to the right along with its ability to complement. The arrow above the pLac-*accC* clone represents the *lacZ* promoter encoded on the vector. Complementation of the suppressor mutations is indicated with a plus symbol, marker rescue of the mutation is indicated as MR, and noncomplementing clones are indicated with a minus symbol.

from λ6G3 and cloned into pBluescript-KS (pKS-1031). A combination of methods, including partial DNA sequencing, analysis of the proteins encoded on this fragment with the T7 polymerase-promoter system of Tabor and Richardson (39), and comparison of the restriction map of pKS-1031 to the published restriction maps of this region (1, 22, 29, 34, 43) (data not shown), identified this 6-kb clone as carrying the genes coding for pantothenate permease (*panF*), BCCP (*accB*), biotin carboxylase (*accC*), and a 34,000-Da protein of unknown function. Figure 1 shows the arrangement of these genes on this 6-kb fragment.

A set of deletion derivatives and subclones of this fragment were made to determine which of these genes was required for complementation of the Cs^- phenotype of the Ts^+1031 and Ts^+1123 mutations. It was found that only deletion derivatives p3/58 and pBK2 were able to complement (Fig. 1). The *accBC* genes, coding for BCCP and biotin carboxylase, are the only genes common to both of these derivatives. These two genes have recently been shown to form an operon, with the promoter located 5′ to the *accB* gene (29) (Fig. 1).

Isolation of non-cold-sensitive suppressor alleles. To determine whether the extreme Cs^- phenotype was invariably linked with the ability of these mutations to suppress *htrB*, we isolated new alleles without prior screening for a Cs^- phenotype. To do this we transduced the *zhb-43*::Tn*10*-Kan^r marker into *htrB* mutant bacteria, and three independent isolates were grown overnight in liquid at 42°C to allow suppressor mutations to accumulate. The *zhb-43*::Tn*10*-Kan^r marker and any suppressor mutations linked to it were transduced back into *htrB* bacteria and identified by selecting simultaneously for Kan^r and colony formation at 42°C. Only one Ts^+ suppressor isolate from each of the three original cultures was characterized to ensure that each new suppressor was due to an independent mutational event. Two of three such suppressor mutations, Ts^+1043-1 and Ts^+1043-6, were shown to be linked to the *zhb-43*::Tn*10*-Kan^r marker by transduction, and both were mapped to the *accBC* operon by complementation studies (Fig. 1). Since both of these new suppressor strains formed colonies at 30°C, it appears that the extreme Cs^- phenotype is not a prerequisite for suppression. However, both mutations affected bacterial growth at 30°C (Fig. 2A); *htrB* Ts^+1043-6 bacteria grew slightly more slowly than the wild type, and *htrB* Ts^+1043-1 bacteria grew more slowly still, with a rate approaching that of *htrB* Ts^+1123 bacteria, which did not form colonies at 30°C.

Suppression of the HtrB phenotypes. All four of the suppressor mutations restored the ability of *htrB* bacteria to grow at 42°C, albeit at a lower growth rate than that of wild-type bacteria (Fig. 2B). The altered morphology that accompanies the loss of viability of *htrB* bacteria was also suppressed in these strains. Photographs of isogenic wild-type bacteria, *htrB* mutant bacteria, and *htrB* Ts^+1043-6 bacteria are shown in Fig. 2C. The *htrB* bacteria formed their characteristic bulges, whereas *htrB* bacteria with the Ts^+1043-6 suppressor mutation exhibited a wild-type morphology. Although the suppressor mutations suppressed the lethal phenotype caused by *htrB*, they did not suppress the increased deoxycholate resistance exhibited by *htrB* bacteria at 30°C. Whereas the MIC of deoxycholate for wild-type bacteria was 2.5%, both *htrB* and the suppressor strains grew on L agar supplemented with 10% deoxycholate (data not shown).

To further pursue the question of which of the HtrB phenotypes were reversed by the suppressor mutations, we checked their effects on *htrB msbB* double-mutant bacteria. The *msbB* gene was originally isolated as a multicopy suppressor of *htrB* and subsequently shown to code for a

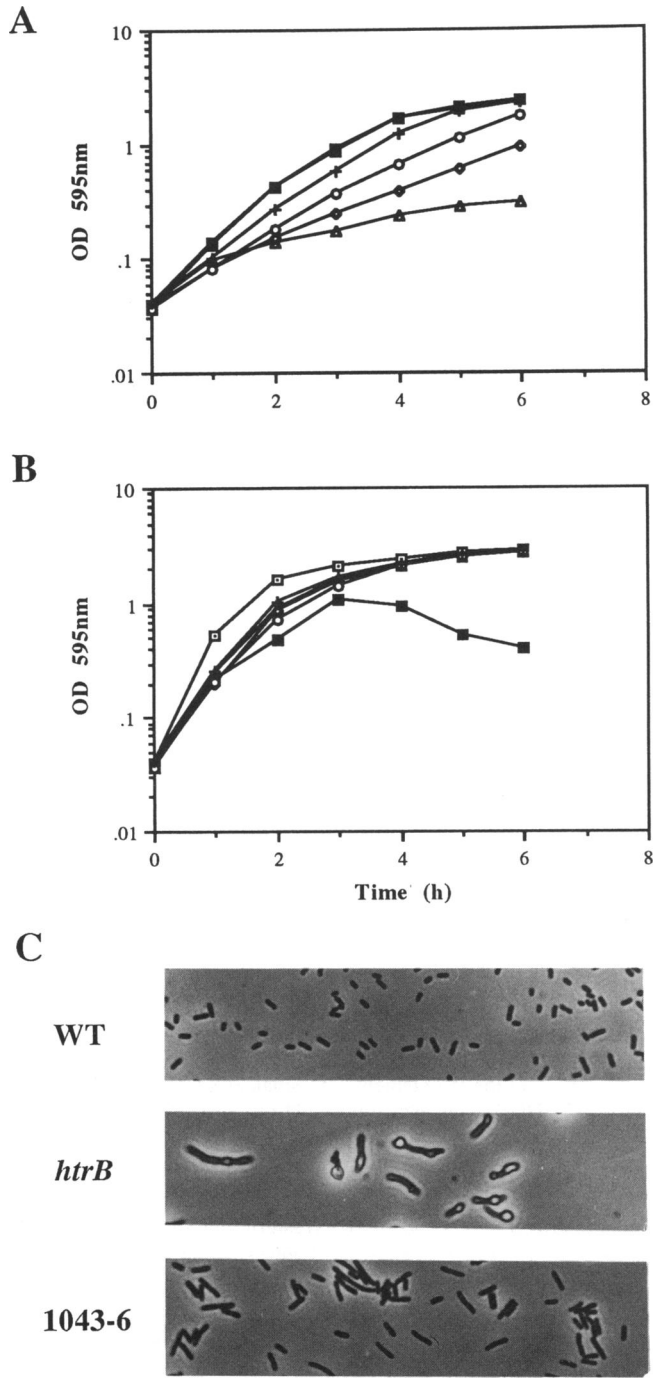


FIG. 2. Morphology and growth curves of wild-type, *htrB*, and various suppressor derivative bacteria. (A and B) Growth curves at 30°C (A) and 42°C (B) of isogenic wild-type (⊡), *htrB* (■), *htrB* Ts$^+$1031 (△), *htrB* Ts$^+$1123 (◇), *htrB* Ts$^+$1043-1 (○), and *htrB* Ts$^+$1043-6 (+) bacteria. (C) Photographs of wild-type (WT), *htrB*, and *htrB* Ts$^+$1043-6 (1043-6) bacteria grown at 42°C from an OD_{595} of 0.05 for 2.75 h.

protein similar to HtrB (25). We previously concluded that MsbB plays a role similar to and possibly redundant with that of HtrB because *htrB msbB* double-mutant bacteria exhibit phenotypes at 30°C that are not associated with either of the single mutations, including a heterogeneous alteration in morphology (i.e., filamentous and fat, misshapen cells) and a growth rate that is lower than that of wild-type bacteria (25). The presence of the Ts$^+$1043-1 or Ts$^+$1043-6 mutation suppressed the morphological alterations of the *htrB msbB* bacteria, but the slow-growth phenotype was actually accentuated in the triple mutants (data not shown).

Molecular analysis of the suppressor mutations. To identify which of the two genes in the *accBC* operon were mutated in these suppressor strains, we cloned the two wild-type genes individually on separate plasmids and used them to map the location of the four suppressor mutations. The *accB* gene, coding for BCCP, was subcloned into the low-copy-number vector pGB2 (8) on a 2.3-kb *Eco*RV fragment (pGB-*accB*). This fragment was cloned into a low-copy-number vector because its cloning, or the cloning of any fragment carrying the *accB* gene alone, onto higher-copy-number vectors has been unsuccessful thus far. The biotin carboxylase gene, *accC*, was cloned onto a 1.85-kb *Kpn*I-*Eco*RV fragment under the control of the *lac* promoter of pBluescript-KS (pLac-*accC*). Figure 1 shows the relationship of these subclones to the full-length pKS-1031 clone and the locations of the coding regions for BCCP and biotin carboxylase.

When either pLac-*accC* or pGB-*accB* was transformed into *htrB* bacteria, the bacteria formed colonies at 42°C. This finding was surprising, since the pKS-1031 plasmid containing the entire *accBC* operon did not rescue *htrB* bacterial growth at 42°C (data not shown). This ability to rescue appears to be the result of a stoichiometric imbalance of these two enzyme subunits, altering the activity of the acetyl coenzyme A (acetyl-CoA) carboxylase enzyme complex in a manner analogous to that caused by the suppressor mutations themselves (see below).

Complementation of the Cs$^-$ phenotype of the Ts$^+$1031 and Ts$^+$1123 mutations by pLac-*accC* and pGB-*accB* was determined by colony formation at 30°C. The pLac-*accC* plasmid complemented the Cs$^-$ phenotype of the Ts$^+$1123 mutation, indicating that this mutation mapped to the *accC* gene. We further localized Ts$^+$1123, by marker rescue, to the 950-bp *Eco*RV fragment (pE1) in the region encoding the carboxy terminus of the biotin carboxylase protein (Fig. 1). The Cs$^-$ phenotype of the Ts$^+$1031 mutation was not complemented by either of the subclones, despite the fact that it was complemented by the full-length pKS-1031 plasmid (Fig. 1). However, the pGB-*accB* plasmid rescued *htrB* Ts$^+$1031 mutant bacteria by recombination, indicating that the Ts$^+$1031 mutation was located within this fragment. The inability of both pLac-*accC* and pGB-*accB* to complement the Cs$^-$ phenotype of Ts$^+$1031 could indicate that this mutation exerts a polar effect on the expression of the *accB* gene or affects the promoter region of the *accBC* operon. To differentiate between these two possibilities, this mutation was characterized further, as described below.

Because the Ts$^+$1043-1 and Ts$^+$1043-6 suppressor mutations did not exhibit an extreme Cs$^-$ phenotype, one way to map them would have been to assess complementation by the reappearance of the Ts$^-$ phenotype of *htrB*. However, the above-mentioned ability of either pLac-*accC* or pGB-*accB* to rescue *htrB* at 42°C made this strategy impossible. Fortuitously, the presence of either pLac-*accC* or pGB-*accB* did not fully reverse the morphological alterations exhibited by *htrB msbB* double-mutant bacteria, so this double-mutant background was used to map the Ts$^+$1043-1 and Ts$^+$1043-6 mutations. When *htrB msbB* Ts$^+$1043-6 triple-mutant bacteria were transformed with the pLac-*accC* plasmid, the

resulting bacterial morphology was identical to that exhibited by the unsuppressed *htrB msbB* double mutant, indicating that Ts$^+$1043-6 most likely was a mutation in the *accC* gene (Fig. 1). The opposite result was obtained with the *htrB msbB* Ts$^+$1043-1 triple-mutant bacteria. The presence of the pGB-*accB* plasmid resulted in the appearance of filamentous cells, a phenotype exhibited by *htrB msbB* double-mutant bacteria carrying pGB-*accB*, indicating that Ts$^+$1043-1 was most likely a mutation in the *accB* gene (Fig. 1).

Analysis of the Ts$^+$1031 mutation. To determine whether the Ts$^+$1031 mutation was a polar mutation in the *accB* gene or a promoter mutation, we used PCR to amplify the *accB* gene from genomic DNA isolated either from bacteria carrying the Ts$^+$1031 mutation or from the isogenic wild-type strain. We first amplified and sequenced the coding region of the *accB* gene and found that there were no changes in the Ts$^+$1031 DNA sequence. We then amplified the promoter region of the operon and found that the PCR product made from the DNA of the Ts$^+$1031 mutant was approximately 750 bp longer than the corresponding PCR product made from wild-type DNA (data not shown). The sequencing of this PCR fragment showed that there was an IS*1* element inserted 215 bp upstream of the translational start codon for BCCP. Li and Cronan (29) have recently located the transcriptional start site of the *accBC* operon to 296 bp upstream of the *accB* coding region. Thus, the Ts$^+$1031 mutation was an IS*1* element inserted within this unusually long, 5′-untranslated leader region (Fig. 3A). The *accBC* promoter has previously been shown to be located in a region of bent DNA (27, 29, 32, 34); the IS*1* element has inserted at one end of this bent DNA region. Like most IS*1* insertion events (11), a 9-bp direct repeat was created in the *accBC* DNA (Fig. 3A).

IS*1* elements have been shown to exert polar effects on transcription, as well as create new promoters at their site of insertion (11). These promoters are created by fusing a preexisting −35 promoter recognition sequence, within the IS*1* element, to potential −10 promoter recognition sequences in the genome. In this case it is likely that transcription from the *accBC* promoter terminated within the element and that the small quantity of residual transcription seen was due to initiation at a newly created promoter, much weaker than the *accBC* promoter, as illustrated in Fig. 3A. The activity of this promoter was low probably because the spacing between the putative −10 and −35 regions is 6 bp shorter than the average spacing between −10 and −35 regions (33).

Northern blot analysis was performed to determine the effect of the IS*1* element insertion on the transcription of the operon. To determine whether any of the other suppressor mutations affect the expression of the operon, we included RNA from the other suppressor strains, as well as *htrB* and wild-type bacteria, grown at either 30 or 42°C. As shown in Fig. 3B, only the Ts$^+$1031 mutation had a substantial effect on *accBC* expression; the insertion of the IS*1* element was found to greatly reduce the transcription of this operon at both 30 and 42°C.

One would expect that such a large decrease in the amount of mRNA would be reflected by the amount of BCCP and biotin carboxylase protein present in the cell. Using streptavidin conjugated to alkaline phosphatase and a chemiluminescent substrate, biotinylated BCCP was detected on Western blots. As shown in Fig. 3C, the quantity of biotinylated BCCP was indeed reduced at 30°C, but surprisingly, at 42°C the reduction was not as much as would be expected considering the results obtained from the Northern blot experiment.

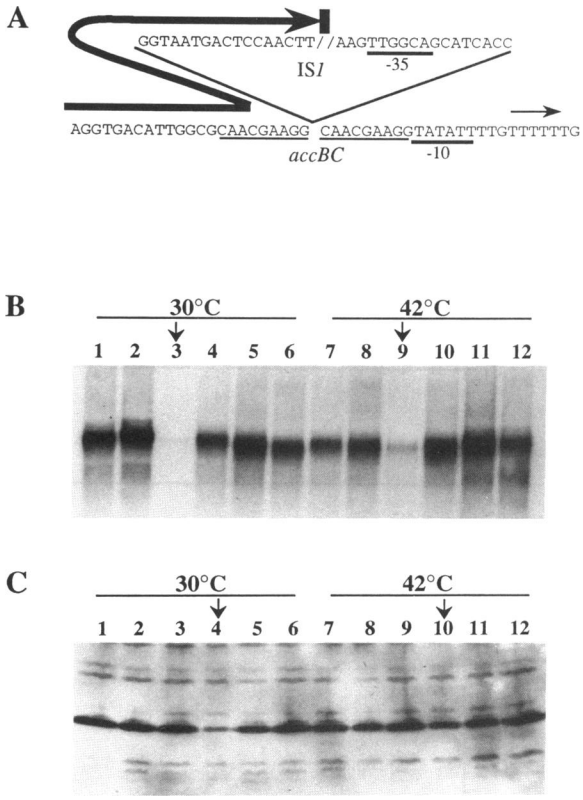


FIG. 3. Effects of the IS*1* transposable element insertion mutation, Ts$^+$1031, on the expression of the *accBC* operon. (A) Model of the mechanism by which the insertion of the IS*1* element decreases the transcription of the *accBC* operon. The IS*1* element is indicated above the *accBC* DNA sequence, and the exact point of insertion in the *accBC* DNA is indicated by a gap in the sequence. The boldface arrow, initiating from the left, represents transcription from the *accBC* promoter. The bar at which this arrow ends indicates transcriptional termination within the IS*1* element. Potential −35 and −10 RNA polymerase recognition signals are marked. Thin lines below the *accBC* sequence indicate the 9-bp repeat created by the insertion of the IS*1* element. The small arrow to the right, above the *accBC* DNA sequence, represents the lower level of transcription initiated at this putative new promoter. (B) Northern blot of RNA isolated from wild-type (lanes 1 and 7), *htrB* (lanes 2 and 8), *htrB* Ts$^+$1031 (lanes 3 and 9), *htrB* Ts$^+$1123 (lanes 4 and 10), *htrB* Ts$^+$1043-1 (lanes 5 and 11), and *htrB* Ts$^+$1043-6 (lanes 6 and 12) bacteria grown at 30 or 42°C. (C) Western blot analysis of biotinylated BCCP protein from wild-type (lanes 1 and 7), *htrB* (lanes 2 and 8), *htrB* Ts$^+$1123 (lanes 3 and 9), *htrB* Ts$^+$1031 (lanes 4 and 10), *htrB* Ts$^+$1043-1 (lanes 5 and 11), and *htrB* Ts$^+$1043-6 (lanes 6 and 12) bacteria grown at 30 or 42°C. The arrows above the lanes serve to highlight the *htrB* Ts$^+$1031 results. The bar to the right of the Western blot indicates the position of the BCCP protein.

Fatty acid analysis of *htrB* and the suppressor mutations. The only other known mutation of the *accBC* operon is *fabE* (16). This mutation results in a Ts$^-$ phenotype and has recently been shown to be a point mutation in the *accB* gene near the region encoding the biotin attachment site of BCCP (29). When *fabE* or *fabD* Ts$^-$ mutants (FabD catalyzes the second step in fatty acid biosynthesis) are grown at semipermissive temperatures, their fatty acid compositions are altered (16, 17). This alteration reflects the use of most of the

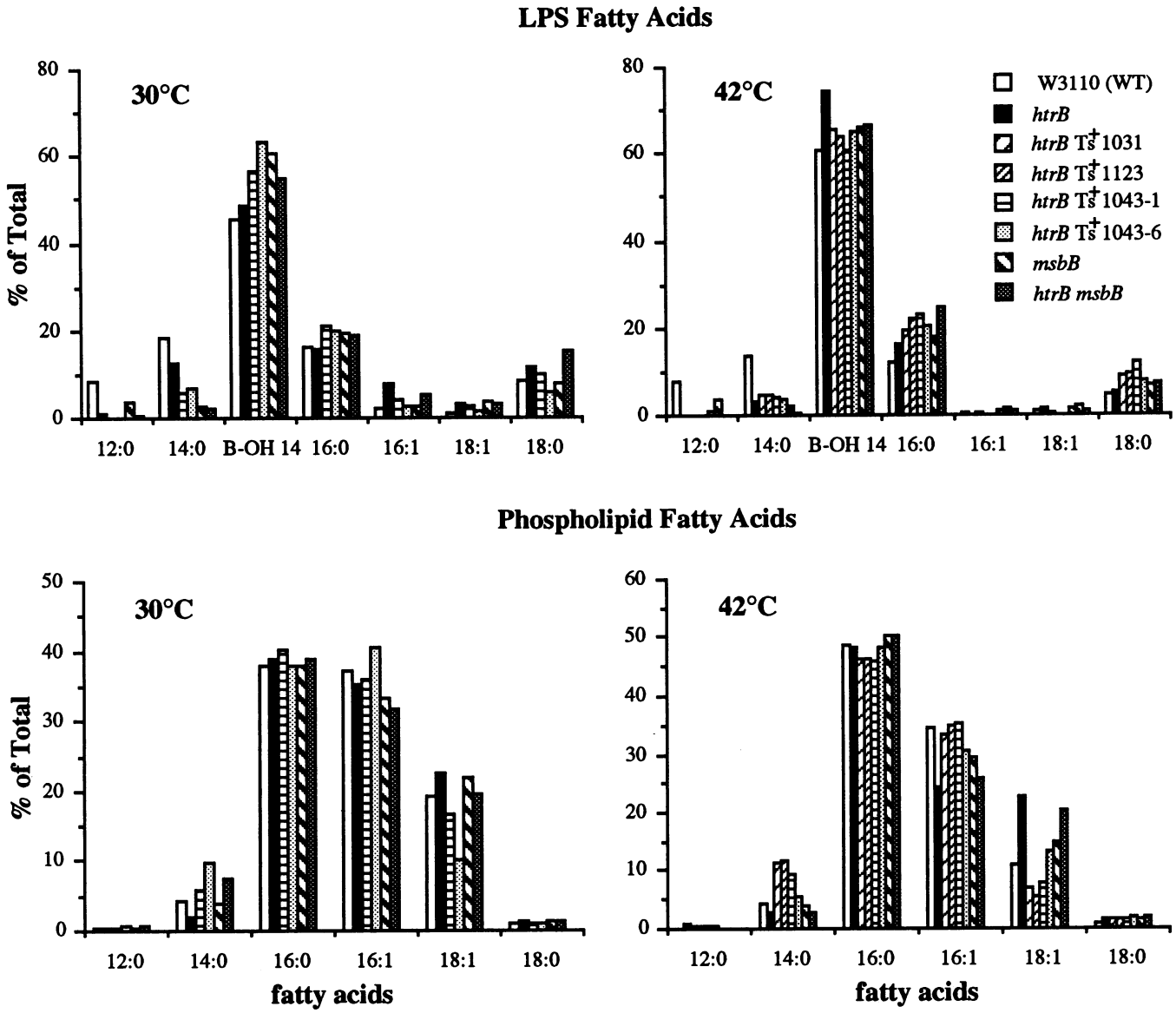


FIG. 4. Fatty acid compositions. Shown are graphical representations of the percent fatty acid composition of phospholipid and LPS fractions from wild-type (WT), *htrB*, *htrB* suppressor strains, *msbB*, and *msbB htrB* bacteria. β-Hydroxymyristic acid is abbreviated as β-OH 14. The phospholipid and LPS fatty acid percentages at 30 and 42°C for wild-type, *htrB*, *msbB*, and *htrB msbB* bacteria are the averages from four independent experiments. The fatty acid percentages for phospholipid and LPS fractions at 42°C for the suppressor strains are the averages from two independent experiments. The fatty acid percentages for phospholipid and LPS fractions at 30°C for the suppressor strains are from a single experiment.

residual enzymatic activity to form β-hydroxymyristic acid, the major fatty acid of LPS.

Because of the observed effects of the *fabE* and *fabD* mutations, we reasoned that the *accBC* suppressor mutations may also alter fatty acid composition and that this might compensate for the effects caused by the lack of HtrB. To determine whether this was the case, we analyzed the fatty acid compositions of both phospholipid- and LPS-enriched fractions isolated from *htrB* bacteria, the suppressor strains, and isogenic wild-type bacteria. It was found that both the LPS and phospholipid fatty acid compositions of *htrB* bacteria were altered (Fig. 4). The LPS fatty acids from *htrB* bacteria grown at either 30 or 42°C exhibited reproducible reductions in lauric acid (12:0) and myristic acid (14:0). At 30°C, there was a slight increase in palmitoleic acid (16:1), and at 42°C, there was an increase in palmitic acid (16:0) and β-hydroxymyristic acid (compare the open and black bars in Fig. 4). Such increases may compensate for the lack of the smaller fatty acids. Rather than reversing these changes, the suppressor mutations actually accentuated the observed decreases in myristic acid (14:0) at 30°C, suggesting that these changes were probably not the cause of *htrB* lethality.

Although the *htrB* mutation had only a slight effect on phospholipid fatty acid composition at 30°C, at 42°C the ratio of the two unsaturated fatty acids, palmitoleic acid (16:1) and *cis*-vaccenic acid (18:1), was considerably altered (compare open and black bars in Fig. 4). The 18:1/16:1 ratio for *htrB* bacteria was 0.93, whereas wild-type bacteria had a ratio of

0.32. However, the total percentage of unsaturated fatty acids remained similar to that for the wild type, 45.8% for wild-type bacteria and 47.3% for *htrB* bacteria. All four suppressors reversed the effect on the 18:1/16:1 ratio, and in the case of the Ts$^+$1031, Ts$^+$1123, and Ts$^+$1043-1 suppressor mutations, there was a slight overcompensation, resulting in 18:1/16:1 ratios between 0.16 and 0.22. Although the ability of the suppressor mutations to reverse the alterations in fatty acid composition indicates that these changes were linked to *htrB* lethality, it is unlikely that these changes were the direct cause of *htrB* lethality, since they are similar to and not as extreme as those changes caused by the Vtr mutation of the *fabF* gene, which has no effect on bacterial viability (9).

In an attempt to further define which of the fatty acid composition changes were associated with *htrB* lethality, we also determined the effects of a null mutation in the *msbB* gene and the effects of an *htrB msbB* double mutation. Because HtrB and MsbB appear to share similar functions but, unlike HtrB, MsbB is not required for growth under any condition tested (25), we reasoned that by comparing changes caused by the *msbB* null mutation with those caused by the *htrB* mutation we could determine which changes were associated with the nonlethal membrane alterations and which were associated with *htrB* lethality.

We found that the *msbB* mutation caused a qualitative alteration in LPS fatty acids similar to that seen with *htrB*. Thus, these changes were most likely associated with nonlethal changes in membrane structure. The *msbB* mutation resulted in a slight change in the phospholipid 18:1/16:1 ratio but not as much as that caused by the *htrB* mutation, a result consistent with the phospholipid fatty acid changes being associated with *htrB* lethality. The *htrB* and *msbB* changes in LPS fatty acids appear to be additive, since the *htrB msbB* double mutation resulted in an effect that was greater than that seen with either single mutation (Fig. 4). At 42°C the double mutation had an effect similar to that of *htrB* alone. This was an expected result, since in all other respects *htrB* has been shown to be epistatic to *msbB* at 42°C (25).

The quantity of phospholipids per milligram of protein. Although the results from the fatty acid analysis suggested that the changes in phospholipid fatty acid composition were associated with *htrB* lethality, no clear relationship between these changes and lethality could be discerned. However, during this analysis we noted an overall increase in the amount of fatty acids present in the phospholipid fraction per milligram of protein from *htrB* bacteria grown at 42°C. To determine whether *htrB* bacteria indeed had increased quantities of phospholipids, we used the hydroxamic quantification method of Stern and Shapiro (38). We standardized the amount of phospholipid to total cellular protein since the quantity of protein per OD_{595} unit of bacteria was not affected by the presence of the *htrB* mutation (data not shown). As shown in Table 2, at 42°C, *htrB* bacteria accumulate more than twice as much phospholipid per milligram of protein as wild-type bacteria do. In each case, the presence of the suppressor mutations inhibited this overproduction, leading to a phospholipid-to-protein ratio that was 94 to 123% of that seen with wild-type bacteria.

The ability of the Ts$^+$1043-1 and Ts$^+$1043-6 mutations to suppress the morphological phenotypes of the *htrB msbB* double mutant at 30°C suggests that this phenotype may also be caused by an increase in phospholipids. However, at 30°C the phospholipid levels for the *htrB msbB* double mutant and both of the single mutants were similar to that of wild-type bacteria (Table 2). Therefore, the morphological changes must be associated with another aspect of membrane biosynthesis that can also be suppressed by the Ts$^+$1043-1 and Ts$^+$1043-6 mutations. Consistent with the *msbB* mutation having no deleterious effects on bacterial growth, no increase in phospholipids at 30 or 42°C was observed (Table 2). Like all other phenotypes tested, *htrB msbB* double-mutant bacteria exhibited the same phenotypes at 42°C as *htrB* mutant bacteria, including the twofold overproduction of phospholipids (Table 2).

TABLE 2. Phospholipid levels for *htrB* and related bacteria at various temperatures

Growth temp (°C)[a]	Strain or relevant genotype	μg of phospholipid/ mg of protein	% of wild-type level
42	W3110 (wild type)	139 ± 10	100
	htrB	326 ± 27	235
	htrB Ts$^+$1031	171 ± 2	123
	htrB Ts$^+$1123	153 ± 8	110
	htrB Ts$^+$1043-1	166 ± 10	119
	htrB Ts$^+$1043-6	130 ± 19	94
	msbB	138 ± 30	99
	htrB msbB	290 ± 30	209
30	W3110 (wild type)	163 ± 14	100
	htrB	146 ± 20	90
	msbB	127 ± 5	78
	htrB msbB	136 ± 9	83

[a] Bacteria were grown at the indicated temperature from an OD_{595} of 0.05 to an OD_{595} of 0.4. For more details, see Materials and Methods.

Because we have previously proposed that HtrB plays a role in outer membrane function (25), possibly affecting the LPS layer, we also determined the amount of LPS present in *htrB* bacteria. This determination was made by two methods. We first determined the amount of LPS fatty acids per milligram of protein by gas chromatography and found that there was no increase compared with the amount in wild-type bacteria. We also used the thiobarbituric acid method (46) to quantify the amount of 3-deoxy-D-manno-octulosonic acid residues present on LPS and found that there was no increase; wild-type bacteria had 546 ± 47 μg of LPS per mg of protein, and *htrB* bacteria had 591 ± 30 μg of LPS per mg of protein. However, we did find that there was an increase in the amount of LPS in the inner membrane fraction, accompanying a decrease in the amount of LPS in the outer membrane fraction. Whereas 83% of the LPS from wild-type bacteria sedimented with the outer membrane fraction, only 48% of the LPS from *htrB* bacteria sedimented with the outer membrane fraction. Determination of the amount of phospholipid in the two fractions indicated that both the inner and outer membranes contain increased quantities of phospholipids (data not shown). The shift of LPS to the inner membrane fraction was most likely a consequence of increased amounts of phospholipids in the outer membrane, thus decreasing its overall buoyant density, so that it fortuitously sedimented with the inner membrane fraction.

Determination of the rate of phospholipid biosynthesis. To establish the nature of the overproduction of phospholipids in *htrB* bacteria and the means by which the *accBC* mutations suppressed *htrB* lethality, we determined the rate of [1-^{14}C]acetate incorporation into phospholipids. As shown in Fig. 5, the rate of phospholipid biosynthesis was reduced by approximately 30 to 40% in all four suppressor strains compared with that of the wild type. This was an expected result, since all of the suppressor strains exhibited reduced

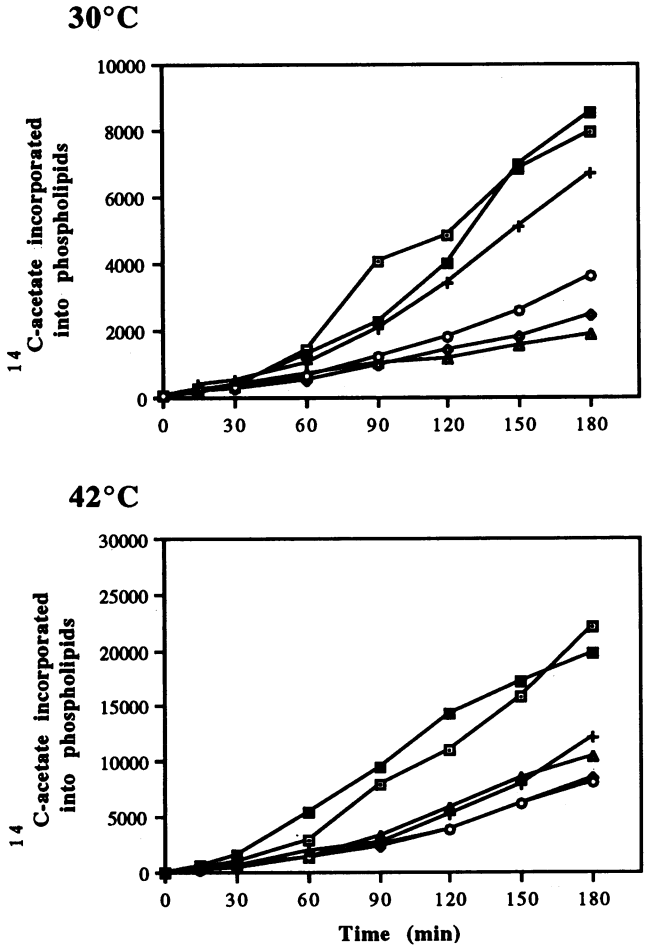


FIG. 5. Rate of phospholipid biosynthesis at 30 and 42°C. Shown is a graph of the [1-^{14}C]acetate incorporated into phospholipids as a function of time for wild-type (⊡) and *htrB* (■) bacteria and the Ts$^+$1031 (△), Ts$^+$1123 (◇), Ts$^+$1043-1 (○), and Ts$^+$1043-6 (+) suppressor strains.

rates of growth and the Ts$^+$1031 mutation caused a decrease in the transcription of the *accBC* operon.

As mentioned previously, Ts$^+$1031 and Ts$^+$1123 bacteria exhibit a Cs$^-$ phenotype and are unable to form colonies at 30°C. Both the Cs$^-$ and the slow-growth phenotypes are caused by the suppressor mutations and are not affected by the presence of the *htrB* mutation (data not shown). To determine whether the inability of Ts$^+$1031 and Ts$^+$1123 bacteria to form colonies at 30°C was due to a failure to synthesize phospholipids, we also measured the rate of phospholipid biosynthesis at 30°C. As shown in Fig. 5, both of these strains continued to synthesize phospholipids at a rate that was approximately 20 to 30% of that of wild type. Since these strains continued to synthesize phospholipids at 30°C, it is not obvious why Ts$^+$1031 and Ts$^+$1123 bacteria do not form colonies on L-agar plates even after prolonged incubation. The fact that Ts$^+$1043-1 bacteria exhibited a slightly a higher rate of fatty acid biosynthesis at 30°C than the two Cs$^-$ suppressor strains and formed small colonies at 30°C suggests that colony formation may require a threshold amount of phospholipid biosynthesis and that Ts$^+$1031 and Ts$^+$1123 bacteria do not exceed this threshold, but Ts$^+$1043-1 bacteria do.

Because the presence of either the pLac-*accC* or the pGB-*accB* plasmid allowed *htrB* bacteria to grow at 42°C, we also measured the rate of phospholipid biosynthesis for wild-type and *htrB* strains carrying these plasmids. The presence of either of these plasmids led to a decrease in the rate of fatty acid biosynthesis in both wild-type and *htrB* bacteria. The presence of pLac-*accC* reduced the rate of fatty acid biosynthesis by approximately 65%, whereas pGB-*accB* reduced the rate by approximately 30%. The amount of reduction caused by these two plasmids directly reflected their abilities to allow *htrB* bacteria to grow at 42°C. Mutant *htrB* bacteria carrying pLac-*accC* formed almost wild-type-size colonies at 42°C, whereas those carrying pGB-*accB* formed only small colonies. The reduction in fatty acid biosynthesis caused by pGB-*accB* may be enough to permit colony formation of *htrB* bacteria at 42°C but not enough for rapid growth. As proposed above, the presence of either of these plasmids probably disrupts the stoichiometric balance of the subunits composing the acetyl-CoA carboxylase complex. This disruption and consequent reduction in fatty acid biosynthesis may also explain why the *accB* gene cannot be cloned alone on higher-copy-number plasmids. The increased amounts of BCCP may disrupt the complex to such a degree that fatty acid biosynthesis is dramatically affected.

At both 30 and 42°C, *htrB* bacteria exhibited wild-type rates of phospholipid biosynthesis. This indicates that the overproduction of phospholipids may not be the result of an increased rate of phospholipid biosynthesis, but rather it may reflect the uncoupling of the rate of phospholipid biosynthesis from the rate of growth. Consistent with this, when *htrB* bacteria were shifted to 42°C, they continued to grow at a rate similar to that at 30°C, as judged by OD_{595} (9) (Fig. 2). However, *htrB* bacteria synthesized phospholipids at the rate required for wild-type bacteria to grow at 42°C. Unlike the rate of phospholipid biosynthesis, the rate of protein synthesis remained coupled to the rate of growth at high temperatures (data not shown). Thus, the increase in phospholipid levels per milligram of protein was actually the consequence of protein biosynthesis remaining coupled to the reduced rate of growth and phospholipid biosynthetic rates increasing with temperature, independently of growth rate.

The uncoupling between growth and phospholipid biosynthesis rates is best exemplified by the ratio of incorporated [1-^{14}C]acetate counts into phospholipid per milligram of protein. For wild-type bacteria this ratio was 2,500 cpm/mg of protein. In contrast, the ratio for *htrB* bacteria was 16,400 cpm/mg of protein. The *accBC* suppressor mutations appear to reduce phospholipid biosynthesis so that growth and phospholipid biosynthesis are once again coupled. The corresponding ratios for Ts$^+$1031 and Ts$^+$1041-6 bacteria were 3,600 and 3,100 cpm/mg of protein, respectively, much reduced compared with that for *htrB* bacteria and similar to those for the wild type.

DISCUSSION

Four single-copy extragenic suppressors of *htrB* have been isolated and mapped to the *accBC* operon, which codes for BCCP and biotin carboxylase. These two proteins associate with a heterodimer of carboxyltransferase to form the acetyl-CoA carboxylase enzyme complex. This complex catalyzes the first step in fatty acid biosynthesis, namely, the carboxylation of acetyl-CoA to form malonyl-CoA. Two of the four suppressor mutations, Ts$^+$1123 and Ts$^+$1043-6,

were mapped to the *accC* gene, encoding biotin carboxylase, whereas the Ts$^+$1043-1 allele was mapped to the *accB* gene, encoding BCCP. The fourth mutation, Ts$^+$1031, was identified as an insertion of an IS*1* transposable element in the promoter region of the operon.

The effect of this IS*1* element was a large reduction of *accBC* operon transcription. We were surprised to find that such a large change in mRNA levels had only a small effect on the biotinylated BCCP levels at 42°C. One possible explanation for this is that the *accB* gene is under translational regulation and, hence, a low intracellular level of mRNA may have little effect on BCCP levels. The unusually long, 5′-untranslated region of this mRNA (29) could serve such a function. Alternatively, if a constant level of biotinylated BCCP is maintained in the cell (irrespective of the quantity of unbiotinylated BCCP), changes in mRNA levels may have little effect on the levels of biotinylated BCCP. One argument against this latter suggestion is that Fall and Vagelos (10) showed that most of the BCCP isolated from *E. coli* is in the biotinylated form. However, if unbiotinylated BCCP is unstable, either in vivo or during the isolation procedure, it would appear that a majority of the BCCP in the cell is biotinylated.

Whether the effects of the Ts$^+$1031 mutation were mediated through a small decrease in both BCCP and biotin carboxylase levels or through a larger decrease in biotin carboxylase levels alone is not known at this time. At 30°C, the Ts$^+$1031 mutation must affect the levels of both biotin carboxylase and BCCP, since neither pGB-*accB* nor pLac-*accC* complemented the Cs$^-$ phenotype of Ts$^+$1031; only the presence of the pKS-1031 plasmid, which carries both genes, resulted in growth at 30°C. It appears that suppression can be mediated through either of these gene products, since the Ts$^+$1123 and Ts$^+$1043-6 alleles were identified as mutations in the *accC* gene and Ts$^+$1043-1 was identified as a mutation in *accB*. These results also suggest that suppression was mediated through the activity of acetyl-CoA carboxylase enzyme complex as a whole, rather than through any one of its individual components.

Because these suppressor mutations mapped to an operon whose products are involved in phospholipid biosynthesis, we studied the effects of the *htrB* null mutation on this process. When *htrB* bacteria are grown at temperatures above 33°C in rich media, they lose viability rapidly (23). This loss of viability is associated with a twofold increase in the amount of phospholipid per milligram of protein. The overproduction of phospholipids was the consequence of synthesizing phospholipids at a rate that appears to be in excess of that required to accommodate the reduced growth rate of *htrB* bacteria at 42°C. This uncoupling of phospholipid biosynthesis and growth rates appears to be integral part of *htrB* lethality at high temperatures, since the suppressor mutations most likely rescue by reducing the rate of phospholipid biosynthesis, thus matching the reduced rate of growth. The ability of either the pGB-*accB* or pLac-*accC* plasmid alone to rescue the lethal phenotype of *htrB* bacteria also appears to be the result of a decrease in the rate of phospholipid biosynthesis, presumably caused by an imbalance in the levels of the individual subunits of the complex.

The *htrB* mutation also affected the fatty acid composition of both LPS and phospholipids. At both 30 and 42°C, the LPS fatty acids from *htrB* bacteria were relatively depleted in lauric and myristic acid residues but relatively enriched in the larger fatty acid residues compared with those from the wild-type bacteria. The absence of MsbB, a protein with a sequence similar to that of HtrB, had a similar effect on LPS fatty acids. These changes in LPS fatty acids may be the cause of or reflect other changes in the LPS molecule that result in the increased deoxycholate resistance exhibited by *htrB* and *msbB* bacteria (25). Consistent with this, the suppressor mutations did not reverse the increased deoxycholate resistance, nor did they reverse the observed changes to LPS fatty acid composition. The increased deoxycholate resistance and alterations in LPS fatty acid composition are the only phenotypes of *htrB* that we have been able to identify at permissive temperatures. These results suggest that LPS synthesis may be the primary target of the *htrB* mutation. Unlike the changes in phospholipid composition, the changes to LPS fatty acid composition were not accompanied by changes in the quantity of LPS. This could indicate that whereas phospholipid biosynthesis is limited by the rate of fatty acid biosynthesis, LPS biosynthesis is controlled at some other step in its biosynthesis.

In contrast to the changes to LPS, the phospholipid fatty acid composition changes exhibited by *htrB* bacteria at 42°C were reversed by the presence of the suppressor mutations. As mentioned previously, we do not believe that these changes cause *htrB* lethality, because the observed changes in fatty acid composition are reminiscent of those exhibited by bacteria with the Vtr mutation in the *fabF* gene, encoding β-ketoacyl-acyl carrier protein synthase II, which are able to grow at all temperatures (9). This enzyme elongates palmitoleic acid, forming *cis*-vaccenic acid. The activity of the wild-type enzyme itself is altered by temperature, such that as the temperature rises the activity of the enzyme decreases, leading to a relative decrease in *cis*-vaccenic acid levels at higher temperatures (13). The Vtr mutation results in an increase in the activity of this enzyme at all temperatures such that high levels of *cis*-vaccenic acid are synthesized independently of growth temperature (9). Similar to the Vtr mutation, the imbalance in phospholipid biosynthesis and growth rates caused by the lack of HtrB may somehow increase the activity of β-ketoacyl-acyl carrier protein synthase II such that *cis*-vaccenic acid levels are increased at the expense of palmitoleic acid levels. The decrease in smaller fatty acids in the LPS fractions from the suppressor strains could be the result of the decrease in fatty acid biosynthesis altering the balance between the utilization of the smaller fatty acids for elongation and their acylation to the lipid A portion of LPS.

Originally, we interpreted the formation of the bulges and filaments by *htrB* bacteria at the nonpermissive temperatures to be the consequence of changes in cell wall structure (23). Although the *htrB* mutant may have an altered cell wall structure, because of our finding of an excess of phospholipids in *htrB* bacteria we now suggest that the formation of the bulges may be more analogous to the formation of bulges caused by an overproduction of poly-β-hydroxybutyrate (37). Poly-β-hydroxybutyrate is a homopolymer of D-(−)-3-hydroxybutyrate produced as a storage molecule by a wide variety of bacteria. *E. coli* does not normally produce this polymer. However, when the genes encoding the biosynthetic enzymes for the polymer are expressed in *E. coli*, large quantities of it are produced, constituting up to 80% of its dry weight (37). Such high levels of poly-β-hydroxybutyrate can lead to altered morphologies, including the formation of bulges and filaments (37). *E. coli* may respond to the presence of excess phospholipids in *htrB* bacteria in the same manner in which it deals with the large quantities of this polymer, in both cases leading to the formation of bulges and filaments.

Taking all of our data together, it appears that the lethal

Ts^- phenotype of *htrB* is caused by the combination of an overall reduced growth rate and its uncoupling from the rate of phospholipid biosynthesis at high temperatures. Because *htrB* bacteria can grow at high temperatures under slow-growth conditions (23), such as in minimal media, we believe that the Ts^- phenotype exhibited by these bacteria is not wholly caused by the growth temperature but instead is a consequence of the increased growth rate at higher temperatures. When *htrB* bacteria are grown in rich media at temperatures above 33°C, they continue to grow at a rate that is similar to the rate at which they were growing at the permissive temperature of 30°C. The inability to adjust their growth rate in rich media at high temperatures suggests that in the absence of HtrB, the rate of some essential process is limited.

It is not clear why phospholipid biosynthesis does not remain coupled to the rate of growth in *htrB* bacteria. Growth rate limitation in itself does not lead to uncoupling, since a variety of mutant strains exhibit slow-growth phenotypes without associated lethality (4, 7, 36, 44, 47). It appears that HtrB is uniquely involved in the coupling of phospholipid biosynthesis and growth rate under conditions of rapid growth. If the *htrB* mutation primarily affects the LPS layer of the outer membrane, as we have previously proposed (25), the intriguing possibility exists that HtrB provides a link between the regulation of phospholipid biosynthesis, LPS biosynthesis, and bacterial growth.

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