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# A *dtsR* Gene-Disrupted Mutant of *Brevibacterium lactofermentum* Requires Fatty Acids for Growth and Efficiently Produces L-Glutamate in the Presence of an Excess of Biotin

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**A *dtsR* gene encoding a homolog of the $\beta$ subunit of some biotin-containing enzymes suppresses a detergent-sensitive mutation of *Brevibacterium lactofermentum* (E. Kimura *et al.*, 1996, *Biosci. Biotech. Biochem.* 60, 1565–1570), which has been used for the fermentative production of L-glutamate. When the *dtsR* gene was disrupted, the organism exhibited strict fatty acid auxotrophy; oleate or oleate ester, but not palmitate ester or stearate ester, supported the growth of the Δ*dtsR* mutant. Immunoblotting with an anti-DtsR antibody revealed that no intact DtsR was present in the cytosol of the Δ*dtsR* mutant. In the presence of an excess of biotin, the wild type strain did not produce L-glutamate whereas the Δ*dtsR* mutant efficiently produced it. The mechanism underlying the efficient production of L-glutamate by the Δ*dtsR* mutant is discussed as to the possible role of *dtsR* in fatty acid metabolism.** 

*Brevibacterium lactofermentum* is a Gram-positive, non-spore forming, non-pathogenic bacterium belonging to the Coryneform group, to which *Corynebacterium glutamicum* and *B. flavum* also belong. These bacteria are widely used for the fermentative production of amino acids and nucleotides. A significant amount of L-glutamate is produced by these bacteria in a biotin-limited medium (16). Although L-glutamate production is suppressed in the presence of an excess biotin, the addition of a detergent, polyoxyethylene sorbitan monopalmitate (Tween 40), or penicillin to excess biotin-containing medium induces L-glutamate production (11, 18). However, the mechanism underlying the induction of L-glutamate production has not been clarified.

We previously isolated a multicopy suppressor of a Tween 40-sensitive mutant of *B. lactofermentum* ATCC13869, and found that the suppressor carries a novel gene, *dtsR* (5). DtsR, the product of the *dtsR* gene, was suggested to be the target of Tween 40 (5). Thus, it seemed likely that inactivation of DtsR by Tween 40 induces L-glutamate production. As judged from the deduced amino acid sequence, DtsR is highly homologous to components of some biotin-containing enzyme complexes, such as the $\beta$ subunit of propionyl-CoA carboxylase of rat (6) or man (7), and the 12S subunit of methylmalonyl-CoA carboxyltransferase of *Propionibacterium freudenreichii* (20). Neither DtsR nor its homologs have a biotin-binding motif in their structures. On the other hand, a gene encoding a biotin-binding protein of *C. glutamicum* was recently cloned (2). This protein is highly homologous to an acyl-CoA carboxylase, which is assumed to be involved in fatty acid synthesis of *Mycobacterium leprae.*

To elucidate the in vivo role of DtsR with special regard to glutamate production, the *dtsR* gene was disrupted. We report here that the glutamate production by *B. lactofermentum* remarkably increased upon the disruption of *dtsR*.

## MATERIALS AND METHODS

*Materials.* Oleic acid and four kinds of polyoxyethylene sorbitan fatty acid ester, Tween 20 (monolaurate), Tween 40 (monopalmitate), Tween 60 (monostearate) and Tween 80 (monooleate), were purchased from Wako Chemicals Co. Restriction enzymes, T4 DNA polymerase, and bacterial alkaline phosphatase were obtained from Takara-shuzo Co.

*Bacterial strains and plasmids.* *B. lactofermentum* ATCC13869 was used. *Escherichia coli* JM 109 (14) was used for plasmid con-

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struction. Plasmid pDTR6 (5) carries *dtsR* and a replicon derived from pHM1519 (10).

*DNA manipulations.* Plasmid DNA was isolated by the method of Birnbaum (1). *B. lactofermentum* was transformed with a plasmid by means of electroporation as described (5). The transformant was selected on a CM2B plate (5) containing 4 $\mu$g/ml of chloramphenicol. For Southern blot analysis, genomic DNA (about 1 $\mu$g) of *B. lactofermentum* was restricted with *Eco*RI, *Bgl*II, *Sac*I or *Hin*dIII, size-fractionated on a 0.8% agarose gel, and then transferred to a Nytran filter (Hybond-N; Amersham). An about 2.3 kb *Pvu*II-*Kpn*I fragment of pDTR6 was labeled with digoxigenin-dUTP and used as a probe. Labeling, hybridization, washing and detection were performed using a "Nonradioactive DNA Labeling and Detection Kit" according to the manufacturer (Boehringer Mannheim).

*Media and culture conditions.* *E. coli* and *B. lactofermentum* were grown on LB medium (9) at 37°C, and CM2B medium (5) at 30°C, respectively, unless otherwise specified. Where specified, chloramphenicol (40 $\mu$g/ml for *E. coli* and 4 $\mu$g/ml for *B. lactofermentum*) was added to the medium. The glutamate production medium contained, per liter, 80 g glucose, 1.5 g $KH_2PO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 0.45 mg thiamine HCl, 40 ml soybean protein hydrolysate, and 0.45 mg *d*-biotin. The medium pH was adjusted to 7.2 with $NH_3$ gas. A jar fermenter (0.5 l) was used for the glutamate production culture under pH control with $NH_3$ gas.

*Construction of pKCTX-KdE.* Plasmid pKCTX-KdE used for the disruption of *dtsR* was constructed as follows. A *Xba*I-*Kpn*I fragment (about 3 kbp) of pDTR6 carrying *dtsR* was cloned into the *Xba*I-*Kpn*I site of pHSG399 (17), which carries a chloramphenicol resistance gene, to construct pHSGX-K. pHSGX-KdE carrying $\Delta$*dtsR* was constructed by digestion of pHSGX-K with *Eco52*I and self-ligation. A temperature-sensitive replication origin derived from pHSC4 (US Patent No. 08/408.188) was introduced into the *Kpn*I site of pHSGX-KdE using a *Kpn*I linker to construct pKCTX-KdE (Fig. 1).

*Antibody and Western blotting.* Immunoblotting was carried out with an anti-DtsR antibody, which was raised in rabbits against a synthetic peptide corresponding to Leu272-Asp290 of DtsR.

*Glutamate production.* The glutamate content of a culture was determined with a glutamate-sensitive electrode (Asahi Chemical Industry Co. Ltd.).

## RESULTS

*Isolation of a $\Delta$dtsR mutant.* To elucidate the role of DtsR in vivo, a chromosomal deletion of *dtsR* was constructed. A null allele of *dtsR* was constructed on a plasmid by deleting 600 bp of the *Eco52*I fragment of *dtsR* (Fig. 1). Plasmid pKTCX-KdE, which carries a gene conferring chloramphenicol resistance, a temperature-sensitive replicon derived from pHSC4, and the null allele of *dtsR*, was introduced into *B. lactofermentum* ATCC13869. The transformant was selected at a permissive temperature (25°C) on a CM2B agar plate supplemented with chloramphenicol. Derivatives of the transformant, which could grow on CM2B agar plates containing chloramphenicol at a non-permissive temperature (34°C), were then selected. These derivatives should have pKCTX-KdE integrated into the chromosomal *dtsR* region through homologous recombination between the plasmid-borne *dtsR* gene fragment and chromosomal *dtsR*. The chromosomal integration was confirmed with one of the derivatives by Southern blot

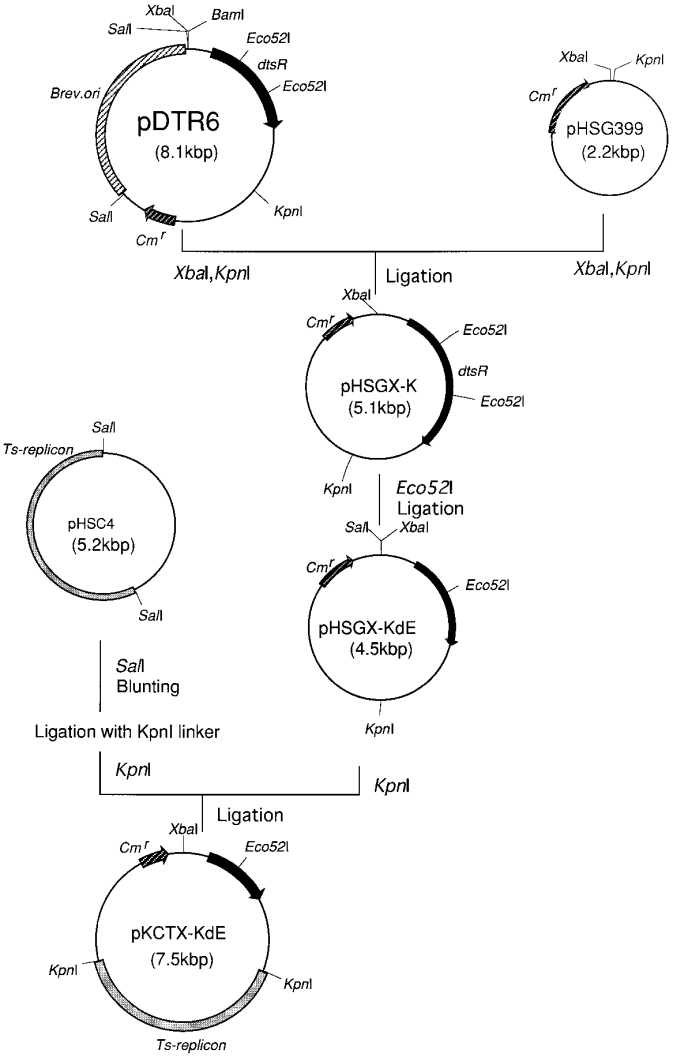


**FIG. 1.** Construction of plasmid pKCTX-KdE used for the disruption of *dtsR*. The construction of plasmids was carried out in *E. coli* JM109. Cm$^r$, a gene conferring chloramphenicol-resistance; Ts-replicon, a temperature-sensitive replicon. For details, see the text.

analysis of the *dtsR* region using linearized pHSG399 as a probe. Chloramphenicol-sensitive mutants of this derivative were next selected by means of replica-plating onto CM2B agar plates containing 1 mg per ml of Tween 80 at 34°C. As described in a later section, the addition of Tween 80 was crucial for successful isolation of $\Delta$*dtsR*. These mutants should have lost the gene conferring the resistance to chloramphenicol through the second homologous recombination between the wild type and mutant *dtsR* genes, and thus carry either the wild type or mutant *dtsR* on the chromosome. Chromosomal DNAs of five chloramphenicol-sensitive mutants were restricted and analysed by Southern hybridization with a 2.3 kbp *Pvu*II-*Kpn*I fragment of pDTR6 containing *dtsR* as a probe, and one of the mutants,

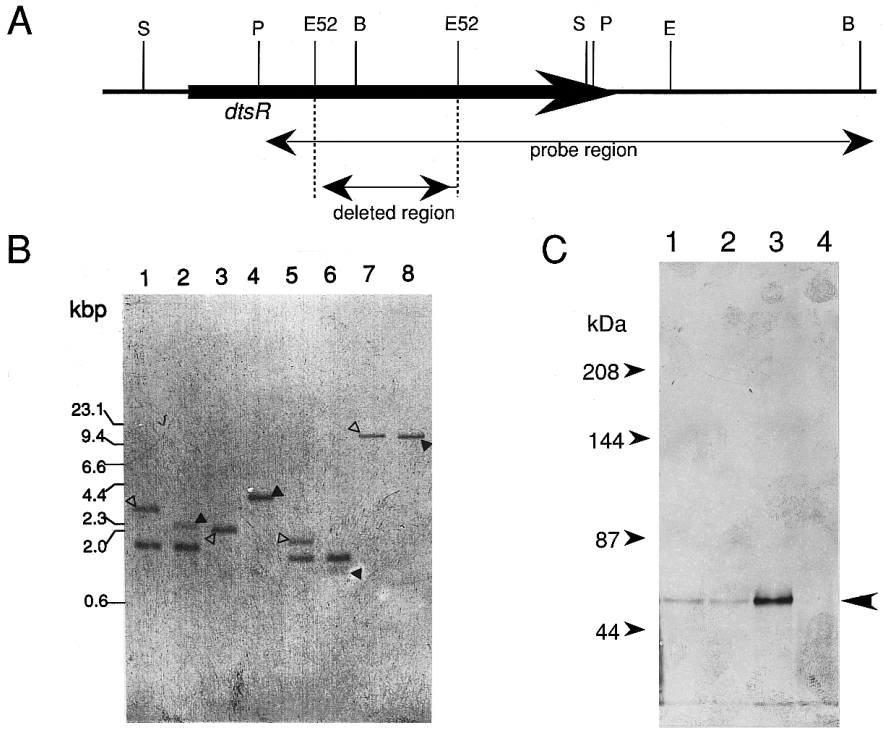


**FIG. 2.** Disruption of the *dtsR* gene. (A) A restriction map of the chromosomal *dtsR* locus. S, *Sac*I; P, *Pvu*II; E52, *E52*I; B, *Bgl*II; E, *Eco*RI. (B) Southern blot analysis of genomic DNA from the wild type *B. lactofermentum* (lanes 1, 3, 5 and 7) and its Δ*dtsR* mutant, AJ13132 (lanes 2, 4, 6 and 8). Chromosomal DNA was digested with *Eco*RI (lanes 1 and 2), *Bgl*II (lanes 3 and 4), *Sac*I (lanes 5 and 6), or *Hin*dIII (lanes 7 and 8), and then analysed on an agarose gel, followed by Southern hybridization with a 2.3 kb *Pvu*II-*Kpn*I fragment carrying *dtsR* as a probe, as described under MATERIALS AND METHODS. Open arrowheads indicate the wild type restriction fragment, which became shorter (*Eco*RI, *Sac*I and *Hin*dIII) by 600 bp or longer (*Bgl*II) upon the Δ*dtsR* mutation. The corresponding fragments of the Δ*dtsR* mutant are indicated by closed arrowheads. Some of them hybridized with the probe less efficiently due to the deletion. (C) Immunoblotting of the DtsR protein. Cell-free extracts (10 μg protein) of wild type *B. lactofermentum* ATCC13869 (lane 1), ATCC13869 harboring pSAC4 (lane 2), ATCC13869 harboring pDTR6 (lane 3), and Δ*dtsR* strain AJ13132 (lane 4) were analysed by SDS-PAGE, followed by immunoblotting with the anti-DtsR antibody. The position of DtsR is indicated by an arrowhead.

AJ13132, was found to be the *dtsR* null mutant, which lacks the internal *Eco*52I fragment (Figs. 2A and B). Furthermore, the deletion was confirmed by sequencing of the Δ*dtsR* region (data not shown).

*Immunoblotting of the DtsR protein.* To confirm that the Δ*dtsR* mutant expresses no intact DtsR protein, cell free extracts were analysed by SDS-PAGE, followed by immunoblotting with the anti-DtsR antibody raised against the Leu272-Asp290 region of DtsR (Fig. 2C). When cell-free extracts of the wild type was examined, a single band corresponding to a molecular mass of about 68 kDa was detected (lane 1). The amount of this band did not change when the wild type cells harbored pSAC4 (5), the vector used for the *dtsR* cloning (lane 2). On the other hand, when the wild type cells harbored pDTR6 carrying *dtsR*, the amount of the band markedly increased (lane 3). Furthermore, this band was not observed for the cell-free extract of the Δ*dtsR mutant* (lane 4). The molecular mass of this band material coincided with the calculated molecular mass (68 kDa) of DtsR. Taken together, these results indicate that the 68 kDa band represents DtsR, which is absent in the Δ*dtsR* mutant.

*Fatty acid auxothrophy of the* Δ*dtsR strain.* During the course of isolation of the Δ*dtsR* mutant, we found that the mutant required fatty acids for its growth. When a CM2B agar plate was used without any supplement for isolation of the Δ*dtsR* mutant, only the wild type strain was isolated. It seemed possible that a certain nutrient became essential upon the disruption of *dtsR*. Since the involvement of *dtsR* in fatty acid synthesis was anticipated, we attempted to isolate Δ*dtsR* in the presence of Tween 80 (polyoxyethylene sorbitan monooleate) and obtained the Δ*dtsR* mutant, AJ13132. Then, growth of the Δ*dtsR* mutant on CM2B liquid medium supplemented with oleic acid or various fatty acid esters (Tweens) was examined (Fig. 3). Oleate, Tween 80 and Tween 20 (laurate ester) supported growth of the Δ*dtsR* strain although Tween 20 was less effective than others. On the other hand, neither Tween 40 (palmitate ester) nor Tween 60 (stearate ester) was effective. These results suggest that DtsR is

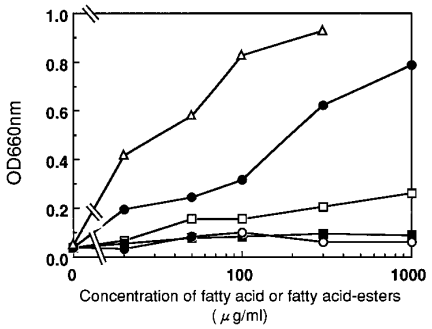


**FIG. 3.** Effects of fatty acid and fatty acid esters on growth of the Δ*dtsR* mutant. The Δ*dtsR* mutant, AJ13132, was grown on CM2B liquid medium containing the specified amounts of fatty acid or fatty acid esters at 30 °C for 30 hrs. The cell density was measured at 660 nm. □, Tween 20; ○, Tween 40; ■, Tween 60; ●, Tween 80; △, oleate.

involved in the synthesis of some fatty acids although the requirement for a definite length of the alkyl chain or the existence of an unsaturated bond for the mutant's growth is not known at present. It may be worthy of note that Tween 40 and Tween 60, which did not support the mutant growth, have been reported to induce L-glutamate production in excess biotin (19). In contrast, neither Tween 80 nor Tween 20 induces the production (19).

*L-Glutamate production by the Δ*dtsR* mutant.* It is known that an excess biotin in the medium prevents the L-glutamate production by the wild type *B. lactofermentum* (16). The growth of and glutamate production by the wild type and Δ*dtsR* mutant were examined in the presence of an excess biotin and 1 mg/ml of Tween 80 (Fig. 4). The mutant grew much more slowly than the wild type did. The glutamate production by the wild type was inefficient in the presence of excess biotin. In marked contrast, the production of L-glutamate by the Δ*dtsR* mutant took place efficiently even in the presence of excess biotin, indicating that the glutamate production by this organism becomes insensitive to excess biotin upon the disruption of *dtsR*.

## DISCUSSION

Successful isolation of the Δ*dtsR* mutant of *B. lactofermentum* required fatty acid supplementation to the selection medium. The isolated Δ*dtsR* strain, AJ13132, was indeed found to be a fatty acid auxotroph. DtsR, presumably a cytosolic protein because of its hydrophilicity (5), exhibits significant homology to some subunits which comprise biotin enzyme complexes, such as the β subunit of propionyl-CoA carboxylase (6, 7) or the 12S subunit of methylmalonyl-CoA carboxyltransferase (20). Since DtsR does not seem to contain biotin, as its homologs do not, it is likely that DtsR forms a complex with another subunit, which contains biotin. A gene encoding a protein with a biotin-binding motif was recently cloned from *Corynebacterium glutamicum* (2), which is taxonomically close to *B. lactofermentum* (8). The *C. glutamicum* protein exhibits more than 70 % identity to the acyl-CoA carboxylase catalyzing the committed step in fatty acid synthesis of *Mycobacterium leprae* (2). This protein may be the biotin-containing counterpart of DtsR.

The glutamate production by the wild type of *B. lactofermentum* does not take place in the presence of an excess amount of biotin. On the other hand, glutamate production by the Δ*dtsR* mutant efficiently took place even in the presence of excess biotin. Both biotin-limitation and Tween 40 supplementation to media are known to induce glutamate production by the wild type. We speculate that the functional level of the complex containing DtsR decreases under these conditions, which leads to glutamate production, as is the case for the Δ*dtsR* mutation. The functional level of the complex comprising DtsR and the putative biotin subunit should be limited under biotin-limiting conditions. The Tween 40-sensitive mutation of *B. lactofermentum* was suppressed on the overexpression of DtsR (5), suggesting that DtsR is a target of the action of Tween 40, a fatty acid analogue. Tween 40 presumably inhibits DtsR and thus decreases the functional level of DtsR molecules. It has been reported that an oleate auxotrophic mutant of a Coryneform bacterium efficiently produces glutamate (3, 4, 12, 13). This auxotroph may also have a mutation causing a decrease in the functional level of the DtsR-containing complex.

Oleate constitutes about 50% of the total cellular fatty acids content of Coryneform bacteria (15). When *Microbacterium ammoniaphilum*, a glutamate producer, is grown in the presence of Tween 40, the oleate

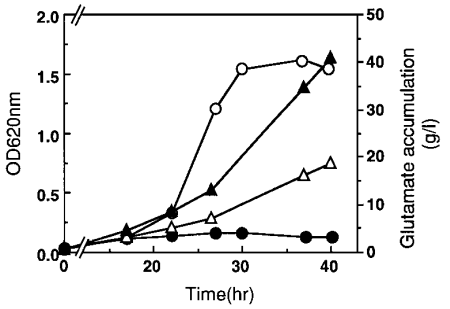


**FIG. 4.** Growth of and glutamate production by the wild type *B. lactofermentum* ATCC13869 (○,●) and its Δ*dtsR* (△,▲) mutant, AJ13132. The strains were grown on CM2B medium containing excess biotin (0.3 μg/ml) and Tween 80 (1 mg/ml) in a jar fermenter. Glutamate production (●,▲) was examined as described under Materials and Methods. The cell density (○,△) was measured at 620 nm after 51-fold dilution. Under the biotin-limiting condition, the wild type strain usually produces 30 to 40 g of L-glutamate per liter (16).

content was reported to decrease with a compensatory increase in the stearate content (15), indicating that the detergent causes an alteration in fatty acid synthesis. DtsR, a target of the detergent, may be involved in this metabolic change.

These observations and our results, taken together, indicate that DtsR plays a role in fatty acid synthesis, and thus affects glutamate production through the metabolic coupling between fatty acid and glutamate syntheses, although the mechanism underlying this coupling remains largely unknown.

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