The Glycyl Radical Enzyme TdcE Can Replace Pyruvate Formate-Lyase in Glucose Fermentation

GARY SAWERS,†* CHRISTIAN HEßLINGER,‡ NATHALIE MULLER,* AND MANUELA KAISER‡

Nitrogen Fixation Laboratory, John Innes Centre, Norwich, United Kingdom, and Lehrstuhl für Mikrobiologie, Universität München, D-80638 Munich, Germany.

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Mutants of Escherichia coli unable to synthesize a functional pyruvate formate-lyase (PFL) are severely impaired in their capacity to grow by glucose fermentation. In a functional complementation assay designed to isolate the pfl gene from Clostridium butyricum, we fortuitously identified a gene that did not encode a PFL but nonetheless was able to complement the phenotypic defects caused by an E. coli pfl mutation. The clostridial gene encoded a basic 14.5-kDa protein (TcbC) which, based on amino acid similarity and analysis of immediately adjacent DNA sequences, was part of a transposon exhibiting extensive similarity to the product of the site-specific transposon Tn554 from Staphylococcus aureus. Our studies revealed that the clostridial TcbC protein activated the transcription of the E. coli tdcABCDEFG operon, which encodes an anaerobic L-threonine-degradative pathway. Normally, anaerobic synthesis of the pathway is optimal when E. coli grows in the absence of catabolite-repressing sugars and in the presence of L-threonine. Although anaerobic control of pathway synthesis was maintained, TcbC alleviated glucose repression. One of the products encoded by the tdc operon, TdcE, has recently been shown to be a 2-keto acid formate-lyase (C. Heßlinger, S. A. Fairhurst, and G. Sawers, Mol. Microbiol. 27:477–492, 1998) that can accept pyruvate as an enzyme substrate. Here we show that TdcE is directly responsible for the restoration of fermentative growth to pfl mutants.

Pyruvate formate-lyase (PFL) is a glycyl radical enzyme that catalyzes the nonoxidative dissimilation of pyruvate to acetyl coenzyme A (acytetyl-CoA) and formate when Escherichia coli grows anaerobically (for a review, see reference 15). The 170-kDa homodimeric PFL enzyme is interconverted between inactive and active forms. Activation of PFL to the radical-bearing species occurs only anaerobically and is catalyzed by an iron-sulfur protein called PFL-activating enzyme. Apart from inactive PFL, the other substrates in the reaction are S-adenosylmethionine and dihydrofolateoxidox.

The free radical in PFL is located directly on the polypeptide backbone at Gly-734 (37). Consequently, the active enzyme species is extremely susceptible to dioxygen (37). Exposure to dioxygen results in irreversible inactivation through specific cleavage of the polypeptide chain between Ser-733 and Gly-734 (37). This scission event results in the appearance of 82- and 3-kDa fragmentation products which can be readily identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Since only one radical is present per homodimer (15, 37), oxygenolytic cleavage of the polypeptide results in the appearance of a characteristic doublet that can be used as a diagnostic tool to identify the existence of active PFL molecules in the anaerobic cell. Only the full-length polypeptide is observable on Western blots of extracts derived from mutants unable to synthesize a functional PFL-activating enzyme (11, 15, 37).

To prevent irreversible damage to the enzyme when E. coli shifts from an anaerobic to an aerobic growth regimen, version of the active PFL enzyme back to the inactive, oxygen-stable form occurs. This reaction is catalyzed by the trifunctional AdhE enzyme (16).

A recent study has identified a second enzyme in E. coli with PFL activity (11). This enzyme, TdcE, is encoded by part of the multicistronic tdcABCDEFG operon, whose products form an anaerobic pathway that degrades L-threonine and L-serine to propionate and acetate, respectively, with concomitant generation of ATP (9, 11, 13). TdcE functions as a 2-keto acid formate-lyase, converting 2-keto-butyrate to propionyl-CoA and formate or pyruvate to acetyl-CoA and formate. Like PFL, TdcE is a glycyl radical enzyme, and the proteins have 82% amino identity (11). Moreover, introduction of the protein-based radical into TdcE is catalyzed by PFL-activating enzyme.

Expression of the tdc operon is very complex, being affected by at least five transcription factors (6, 8, 10, 41). Induction of operon expression occurs anaerobically and in the absence of catabolite-repressing sugars, such as glucose. The global transcription factor cyclic AMP (cAMP) receptor protein (CRP) provides the principal control of operon expression, with the LysR-like TdcA protein acting as an upstream regulator, possibly responding to L-threonine levels in the growth medium (8).

PFL enzyme activity has also been detected in a number of anaerobes, including Clostridium butyricum and Clostridium pasteurianum (35, 38). In contrast to the catabolic function PFL assumes in the enterobacteria, it has been proposed that PFL has an anabolic function in C. butyricum, providing formate for C1 metabolism (35). To understand the physiological function of the PFL protein of C. butyricum, we decided to attempt to isolate the corresponding pfl gene. During these studies, we serendipitously discovered a gene from C. butyricum whose product (TcbC) induced the synthesis of the TdcE protein in E. coli. This paper describes the identification and characterization of the TcbC protein.
Flasks filled to a maximum of 10% of their volumes, while anaerobic growth of anaerobic cultures. Aerobic cultures were grown in vigorously shaking conical flasks previously (11). Glucose was added to a final concentration of 20 mM in all media (TYEP contains, per liter, 10 g of Bacto Tryptone, 5 g of yeast extract, 1.5 g of NaCl, and 100 mM potassium phosphate [pH 6.5]), or the minimal medium described (27). Cell pellets were resuspended in 1 ml of Tris-EDTA buffer (29). Transformation of 2 μl (50 to 100 ng) of this plasmid DNA into strain RM202 yielded approximately 50,000 ampicillin-resistant colonies. Plasmid pT7-1011 was constructed by cloning the 900-bp EcoRI-HindIII fragment from pMU101 into EcoRI-HindIII-digested expression vector pTV7.5. The same insert was also cloned in the reverse orientation with respect to the T7Φ10 promoter of pTV7.5, yielding pT7-1014.

### MATERIALS AND METHODS

#### Strains and growth conditions

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<thead>
<tr>
<th>Strains or plasmid</th>
<th>Genotype or phenotype</th>
<th>Reference or source</th>
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<td>C. butyricum DSM-552</td>
<td>Type strain</td>
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Plasmids

- pT7-1011: Like pT7-5 but with insert in the opposite orientation relative to the T7Φ10 promoter.

#### Construction of a chromosomal DNA library

Chromosomal DNA was prepared as described by Ausubel et al. (3). Purified DNA (10 μg) was partially digested with Sau3AI, and DNA fragments between 3 and 8 kb in size were isolated after agarose gel electrophoresis and ligated into BamHI-digested pBR322 (29). The ligated DNA mixture was used to transform E. coli JM109. Approximately 10,000 ampicillin-resistant colonies were washed from the agar plates with 10 ml of LB medium and transferred to a sterile 250-ml flask. An additional 10 ml of LB medium was added, and the culture was incubated aerobically for 2 h at 37°C. Plasmid DNA was isolated and after treatment with RNase was resuspended in 1 ml of Tris-EDTA buffer (29). Transformation of 2 μl (50 to 100 ng) of this plasmid DNA into strain RM202 yielded approximately 50,000 ampicillin-resistant colonies.

#### Screening for clones capable of complementing an E. coli pfl mutant

Strain RM202 (Δ[pfl<sup>R</sup>]) was transformed with an aliquot of the C. butyricum chromosomal DNA gene library and plated on LB agar containing 50 μg of ampicillin ml<sup>-1</sup>. Plates were incubated aerobically at 37°C for 24 h. Each plate was overlaid with a mixture held at 45°C and containing 20 mM sodium pyruvate, 5 mg of benzyl viologen (BV) ml<sup>-1</sup>, and 25 mM potassium phosphate (pH 7.0). Molten agarose (0.4%, wt/vol) was included in the mixture to solidify the overlay (23). Colonies which were unable to synthesize active PFL were small and remained colorless, while the wild-type strain produced large colonies that became dark violet after being overlaid.

#### DNA manipulations

Work with recombinant DNA was carried out according to the methods of Sambrook et al. (29).

#### Analysis and subcloning of the DNA insert from pMU10

The 3,617-bp DNA insert of plasmid pMU10, which derived from the chromosome of C. butyricum, was sequenced completely on both strands (30). Appropriately designed oligonucleotides were used to complete the sequence. Plasmid pMU1011 was created by deleting the 3.09-kb EcoRI fragment from pMU10 and religating the remaining vector DNA. Plasmid pMU1012 was created in a similar manner by deleting the 3.74-kb HindIII fragment from pMU10 and religating the residual vector DNA. Plasmid pMU1013 was constructed by digesting pMU1012 with HindIII, filling in the protruding S<sup>5</sup>-ends with Klenow polymerase and deoxynucleoside triphosphates, and religating the vector DNA. Plasmid pT7-1011 was constructed by cloning the 900-bp EcoRI-BamHI fragment from pMU10 into EcoRI-BamHI-digested expression vector pTV7.5. The same insert was also cloned in the reverse orientation with respect to the T7Φ10 promoter of pTV7.5, yielding pT7-1014.

#### Preparation of cell extracts and determination of PFL enzyme activity

All steps were performed at 4°C unless otherwise indicated. Desalted crude extracts from approximately 0.5 to 1.0 g (wet weight) of cells were prepared as described by Kaiser and Sawyer (14). PFL enzyme activity was determined as described by Knappe and Blaschkowski (17) with [14C]formate (Amersham) (specific radioactivity, 1.48 to 2.22 GBq mmol<sup>-1</sup>). This assay measures the acetyl-CoA-dependent conversion of formate to pyruvate. Both the activating reaction and the conversion of formate to pyruvate were performed at 30°C in an anaerobic chamber. Assays were performed by mixing extracts (150 μg of protein) prepared from strains that were phenotypically PFL<sup>−</sup> ACT<sup>−</sup> and PFL<sup>−</sup> ACT<sup>−</sup> (ACT is the PFL-activating enzyme). Measurement of 2-ketobutyrate formate-lyase in these extract mixtures was performed according to the method of Heßlinger et al. (11). Assays were found to be reproducible to within 10% of the mean, and activity was linear with respect to time (up to 60 min) and protein concentration.

#### Other methods

Total RNA was isolated from cultures grown to mid-exponential phase, and primer extension analysis was carried out as described by Sawers and Böck (33). Analysis of the tcbC promoter was performed with oligonucleotide Tcb-1 (5'-CCTTTTGTTACTTCCAGCCACC-3'), and the sequence ladder was generated with pMU10 DNA. Analysis of the lac operon promoter was performed with oligonucleotide GS-1 (5'-GCGAGCGGAGCCGAATAAGGAC-3'), and the sequence ladder was generated with pD1 (11). The sequence ladder was generated with pD1 (11).
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C. butyricum dye BV (23). We used this system to identify plasmids from a store both anaerobic growth after 24 h on TYEP plates con-

E. coli tcpB antiserum was diluted 1,500-fold before use, and the antigen-antibody blotting was carried out according to the method of Towbin et al. (36). Anti-

SDS-PAGE of proteins was performed as described previously (19), and Western protein concentration was determined by the method of Lowry et al. (21).

RESULTS

Functional complementation of an E. coli pfl deletion mutant. Mutants unable to synthesize an active PFL grow very poorly in rich medium under fermentative growth conditions (14). Moreover, since these strains do not produce formate, they are incapable of inducing the synthesis of the formate-

hydrogen lyase pathway unless formate is supplied exogenously (28). Strains that synthesize a functional formate-hydrogen lyase pathway can be easily and rapidly identified by overlaying colonies of anaerobically cultivated E. coli strains with a solution containing pyruvate or formate and the redox indicator dye BV (23). We used this system to identify plasmids from a C. butyricum chromosomal DNA gene library that could re-

store both anaerobic growth after 24 h on TYEP plates contain-

ing 0.4% (wt/vol) glucose (TGYEP) and a BV phenotype to the E. coli Δpfl mutant RM202 (32). Approximately 10,000 transformants were screened, and five dark violet colonies were isolated. However, only one of these clones had the additional desired phenotype of restoring appreciable growth to the pfl mutant under fermentative conditions. Significantly, no complementation was observed in a pfl act double null mutant (RM221), indicating that a PFL-like enzyme was responsible for the phenotype. In liquid minimal medium, MC4100 had a specific growth rate of 1.02 h⁻¹ when grown anaerobically, while the pfl mutant RM202 showed no discernible growth and the complemented mutant had a specific growth rate of 0.18 h⁻¹. The plasmid isolated from the complemented mutant was termed pMU10.

Nucleotide sequence analysis of plasmid pMU10. The complete 3,617-bp nucleotide sequence of the pMU10 insert was determined on both strands. The G+C content of the DNA was approximately 28%, which is characteristic for C. butyri-

cum. Analysis of the nucleotide sequence did not reveal an open reading frame with similarity to the sequence encoding PFL. Instead, two complete open reading frames (tcpB and tcpC; transposon from C. butyricum) and one incomplete open reading frame (tcpA') were identified (Fig. 1). The stop codon of the tcpA gene and the initiation codon of the tcpB gene overlap, while three base pairs separate the stop codon of the tcpB gene from the initiation codon of the tcpC gene. The products of all three genes show high similarity to polypeptides encoded by the Staphylococcus aureus transposon Tn554 (Fig. 1) (24). Southern blotting experiments with the insert from pMU10 revealed a single copy of the tcb genes on the chromo-

some of C. butyricum (data not shown).

Restoration of a PFL⁺ phenotype by pMU10 results from induction of the anaerobic synthesis of the E. coli TdcE en-

zyme. The fact that a BV⁺ phenotype was conferred by pMU10 indicates that the transformants must have recovered the ability to make endogenous formate and strongly suggests that a PFL or PFL-like enzyme is responsible. Furthermore, no complementation by pMU10 was observed in strain RM221 with a deletion of the act gene, confirming this supposition.

Since none of the three putative tcp gene products encoded by pMU10 is likely to function as a PFL enzyme, the possibility that one or more of the gene products encoded by pMU10 may induce the synthesis of an E. coli enzyme which can functionally replace PFL was considered. To test this hypothesis, we performed PFL enzyme assays in which [¹⁴C]formate was converted to [¹⁴C]pyruvate with acetyl-CoA as a substrate. Due to the extreme sensitivity of the radical-bearing species of PFL to oxygen, the activation and assay of PFL and, by implication, TdcE were performed anaerobically by mixing crude extracts of appropriate strains as described in Materials and Methods and by Kaiser and Sawers (14). This test demonstrated that mixing an extract of an act mutant with an extract from a pfl mutant restored a PFL enzyme activity which defined the wild-

type level of PFL. No enzyme activity was detectable when the mixture lacked a functional PFL-activating enzyme. Mixing an extract from a pfl mutant and an extract from a pfl act double null mutant carrying pMU10 resulted in a PFL activity that was approximately 8% of that observed in a PFL⁺ strain. Again, this activity was completely dependent on the presence of a functional PFL-activating enzyme. This low activity is in accord with the partial restoration of anaerobic growth in minimal medium (see above). Specifically, the levels of enzyme activity (in nanomoles of [¹⁴C]formate converted per minute per milligram of protein) of extracts of several strains transformed with pMU10 were as follows (PFL and ACT phenotypes are shown in parentheses): 234M11 (PFL⁺ ACT⁺) plus RM202 (PFL⁻ ACT⁻), 610; 234M11 (PFL⁺ ACT⁻) plus RM221 (PFL⁻ ACT⁻), <1; RM202 (PFL⁻ ACT⁻) plus RM221/ pMU10, 42; and RM221 (PFL⁻ ACT⁻) plus RM221/pMU10, <1.

Western blot analysis with anti-PFL antiserum and the crude extracts used in determining PFL enzyme activity revealed that pfl deletion mutants transformed with pMU10 exhibit two strong cross-reacting polypeptides (Fig. 2A, lane 6). Examination of lane 4 in Fig. 2A reveals that cross-reacting polypep-
E. coli membranes, the polypeptides were challenged with anti-PFL antibodies, according to the method of Laemmli (19), and after transfer to nitrocellulose membranes, the polypeptides were challenged with anti-PFL antibodies. Crude extracts from various E. coli strains were separated in 7.5% (wt/vol) polyacrylamide gels according to the method of Laemmli (19), and after transfer to nitrocellulose membranes, the polypeptides were challenged with anti-PFL antibodies.

The positions of the intact PFL and its specific oxygenolytic fragmentation product were shown. Lane 1, MC4100 (wild type) grown anaerobically (100 μg of protein); lane 2, MC4100 grown anaerobically (7.5 μg of protein); lane 3, 234M11 grown anaerobically (7.5 μg of protein); lane 4, RM202 (pfl act) grown anaerobically (150 μg of protein); lane 5, RM221 (pfl act) grown anaerobically (150 μg of protein); lane 6, RM202/pMU10 grown an aerobically (150 μg of protein); lane 7, RM221/pMU10 grown anaerobically (150 μg of protein); lane 8, RM226 (pfl act) grown anaerobically (150 μg of protein). (B) Western blot demonstrating that induction of TdcE synthesis by pMU10 occurs anaerobically. Lane 1, MC4100 grown anaerobically (7.5 μg of protein); lane 2, MC4100 grown anaerobically (7.5 μg of protein); lane 3, RM202 (pfl act) grown anaerobically (100 μg of protein); lane 4, RM202/pBR322 grown anaerobically (100 μg of protein); lane 5, RM202/pMU10 grown anaerobically (100 μg of protein); lane 6, RM202/pMU10 grown anaerobically (100 μg of protein).

The TcbC protein encoded by pMU10 is necessary and sufficient to induce E. coli TdC enzyme synthesis. Various subclones of the DNA insert of pMU10 were constructed to determine which of the three tcb genes is responsible for anaerobic induction of the E. coli TdC enzyme synthesis (Fig. 4A). Plasmid pMU1011 has an 897-bp DNA insert that includes a small portion of the tcbB operon (4, 12). The results clearly show that no polypeptide was induced by pMU10 in W3110/pfl, confirming that the PFL-like protein induced by pMU10 was the glycyl radical enzyme TdC.

Finally, we determined 2-ketobutyrate formate-lyase activity (11) in the same mixture of extracts used to determine PFL activity. The activity determined for the extract mixture containing RM202 plus RM221/pMU10 was 51 nmol of NADH formed min⁻¹ mg of protein⁻¹, while no activity was detectable in the absence of PFL-activating enzyme.

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whether the gene is transcribed from its own promoter and whether transcription is affected by aerobic or anaerobic growth of *E. coli*. Total RNA was isolated from RM221/pMU10 grown aerobically and from RM221/pMU10, RM221/pMU1011, and RM221/pMU1012 grown anaerobically, and the 5' end of the *tcbC* transcript was determined with oligonucleotide Tcb-1, as described in Materials and Methods. Transcription initiated from an adenosine residue 184 bp upstream of the presumptive GTG translation start site of the *tcbC* gene (Fig. 6). The levels of transcription were similar under aerobic and anaerobic growth conditions. Moreover, while a transcript of the same length as and an intensity similar to those of pMU10 was also observed for pMU1011, no transcript for pMU1012 was observed (Fig. 6A). This result is consistent with these DNA sequences being absent in pMU1012. Analysis of transcription from pMU1013, which carries the frameshift mutation in the *tcbC* gene, gave the same result as that observed for pMU10 and pMU1011 (Fig. 6A).

Examination of the DNA sequence upstream of the transcription initiation site of the *tcbC* gene revealed sequences which match four of the six consensus nucleotides of the *E. coli* 210 and 235 RNA polymerase recognition sequences (Fig. 6B). TcbC activates transcription of the *tdc* operon promoter. Recent studies have shown that the *tdcE* gene is cotranscribed with the *tdcA, -B, -C, -D, -F, and -G* genes (11). Transcript mapping studies were therefore undertaken to determine whether the clostridial TcbC protein activated transcription of the *tdc* promoter. Expression of the *tdc* operon is induced anaerobically and is catabolite repressed (41). Primer extension analysis of the transcript generated by wild-type strain FM420 grown in the absence or presence of glucose revealed that transcription levels were lower in the presence of glucose (Fig. 7; compare lanes 1 and 2). The 5' end of the transcript was three nucleotides downstream of that identified in a previous study (9). It should also be noted that the absence of glucose is not the condition for anaerobic growth under which *tdc* operon expression is optimal (41).

Introduction of pMU10 into FM420 resulted in high levels of transcription even in the presence of glucose (Fig. 7, lane 3).

FIG. 4. The *tcbC* gene of pMU10 induces TdcE synthesis. (A) The subclones derived from pMU10 are shown. The ability and inability of the constructs to induce TdcE enzyme synthesis and to complement an *E. coli* pfl mutant are indicated by plus and minus signs, respectively. The white box at the end of the *tcbC* gene in pMU1013 indicates that the gene sequence is out of frame. (B) Western blot showing the ability of the various pMU10 plasmid derivatives to induce anaerobic synthesis of TdcE. Lane 1, MC4100 (10 μg of protein); lane 2, RM221 (Δpfl Δacr) pMU10; lane 3, RM221/pMU1011; lane 4, RM221/pMU1012; lane 5, RM221/pMU1013; lane 6, RM221/pBR322.
Analysis of the pfl mutant RM202 transformed with pBR322 demonstrated that glucose strongly repressed tdc transcription, whereas in the presence of pMU10, transcription from the tdc promoter was no longer repressed by glucose (Fig. 7, lanes 4 and 5). The lower transcript seen in Fig. 7 does not originate from the tdc operon but results from fortuitous hybridization of the oligonucleotide with a distinct mRNA species that is unaffected by the presence of the TcbC protein.

Measurement of the activity of the L-threonine deaminase enzyme, encoded by the tdcB gene (7), revealed that the activity in extracts derived from RM202/pBR322 grown anaerobically in the presence of glucose was 0.06 \( \mu \text{mol of NADH oxidized min}^{-1} \mu \text{g of protein}^{-1} \), while the activity in RM202/pMU10 grown under the same conditions was 0.64 \( \mu \text{mol of NADH oxidized min}^{-1} \mu \text{g of protein}^{-1} \). The latter activity is in a range similar to that observed previously in crude extracts of E. coli K-12 strains grown anaerobically in the presence of cAMP (27). These results correlate well with the observed induction of tdc expression by pMU10 (compare lanes 4 and 5 in Fig. 7). The activity of TdcB in RM202/pMU10 grown aerobically was 0.08 \( \mu \text{mol of NADH oxidized min}^{-1} \mu \text{g of protein}^{-1} \), which confirms that pMU10 induces expression of tdc only anaerobically.

**DISCUSSION**

The data presented here corroborate and provide independent support for the findings of Heßlinger et al. (11) that TdcE has a substrate spectrum similar to that of PFL and can partially substitute for PFL in anaerobic catabolism. Indeed, PFL and TdcE have the same activating enzyme. Anaerobic TdcE synthesis was not induced by the TcbC protein (encoded by pMU10) to levels equivalent to those observed for PFL. Our estimates based on densitometry of Western blots indicate that TdcE attained levels of 5 to 10% of those seen for PFL. This is in good agreement with the PFL enzyme activity determined for TdcE, making the assumption that PFL and TdcE have similar \( K_m \) values for formate in this enzyme assay, and with the fact that complementation of the pfl mutation was only partial. It will be of interest to determine whether, when over-
produced to levels similar to those observed with PFL, TdcE can replace PFL completely.

Synthesis of TdcE normally is induced in the absence of both oxygen and catabolite-repressing sugars. *E. coli* strains harboring TcbC synthesize TdcE even in the presence of glucose, but only under anaerobic conditions. Our studies have shown that TcbC functions at the transcriptional level, inducing expression of the *tdcABCDEF* operon without altering the site of transcription initiation.

How is TcbC able to abrogate glucose repression and activate *tdc* transcription? Although TcbC is encoded by part of a presumptive transposon from *C. butyricum*, activation of *E. coli* *tdc* transcription is unlikely to be the result of a transposition event, since by analogy with Tn554 of *S. aureus*, transposition of the *C. butyricum* transposon is expected to require the TcbA and TcbB proteins (24, 25). Neither of these proteins is required for complementation of the *pfl* mutant. Also, a transposition event would not be expected to affect *tdc* transcription only anaerobically. Furthermore, our transcript mapping data did not indicate any alteration in the location of the start site of the *tdc* transcript, which might be anticipated if a transposon were to insert in the neighborhood of the promoter. Rather, we believe that TcbC may be functioning directly by interacting with the *tdc* transcription machinery. As mentioned above, the deduced amino acid sequences of the tcbA, tcbB, and tcbC products are highly similar to the corresponding TnpA, TnpB, and TnpC proteins (24, 25). Insertion of Tn554 occurs with high frequency, is site specific, and is always in one orientation. Mutational analyses have demonstrated that TnpC determines the orientation specificity of the element (3). Although *tnpc* mutants are still capable of transposition, the frequency is reduced by approximately 2 orders of magnitude compared with the wild-type element. Insertion of Tn554 at its unique chromosomal site, *att554*, requires a core hexanucleotide recognition sequence for the transposase complex (26). Thus, in *S. aureus*, it is likely that TnpC may be involved in binding the transposase complex to the *att554* recognition sequence. By analogy, TcbC may be a DNA binding protein that fortuitously recognizes a sequence in the *tdcABCDEF* operon promoter region with similarity to the *C. butyricum* transposase *att* site. This, however, does not provide an explanation of how TcbC may activate transcription. Assuming that TcbC functions in a direct manner (rather than indirectly, for example, by affecting cAMP levels in glucose-grown cells), it is improbable that TcbC simply overrides transcriptional control of the *tdc* promoter, since anaerobic transcriptional regulation is maintained. Hence, it is anticipated that TcbC-induced transcriptional activation still depends on Fnr and ArcA (6). Since anaerobic regulation of *tdc* expression is strongly dependent on the cAMP-CRP complex (41), it is conceivable that TcbC is capable of enhancing *tdc* expression in the presence of glucose either by substituting for cAMP-CRP in transcriptional activation or by improving interaction of the complex with the CRP binding site at the *tdc* promoter at lower cAMP levels. The former is unlikely, since TcbC has no similarity to CRP at the primary sequence level. However, TcbC is a very basic protein (pI = 10.32), and although it has no obvious DNA binding domain, it nevertheless has some features of histone-like proteins. Expression of *tdc* has been shown to be influenced both by the topological constraint of the DNA (40) and by integration host factor, which functions in concert with cAMP-CRP (39, 41). It is therefore possible that TcbC facilitates the binding of CRP to the *tdc* promoter by influencing DNA topology. The consequences of this could be either that the cAMP-CRP complex has a higher affinity for the CRP binding site in the *tdc* promoter or that the efficiency of transcriptional activation through improved CRP-RNA polymerase interaction is increased. We are currently conducting in vitro studies with purified TcbC to address this possibility.

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