

TdaA Regulates Tropodithietic Acid Synthesis by Binding to the *tdaC* Promoter Region^{∇†}

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***Silicibacter* sp. TM1040, a member of the marine *Roseobacter* clade, produces the antibiotic and quorum signaling molecule tropodithietic acid (TDA), encoded by *tdaABCDEF*. Here, we showed that an LysR-type transcriptional regulator, TdaA, is a positive regulator of *tdaCDE* gene expression and binds to the *tdaC* promoter region.**

Silicibacter sp. TM1040 (here referred to as TM1040) is a member of the *Roseobacter* clade of the alphaproteobacteria that participates in a symbiosis with the marine dinoflagellate *Pfiesteria piscicida* (1, 2, 9, 10, 14–16). TM1040 produces an antibiotic called tropodithietic acid (TDA) (5, 17) that is an inducer of its own biosynthesis and a quorum signaling molecule (8).

Our previous genetic evidence suggests that TDA production requires six genes, *tdaA*, *-B*, *-C*, *-D*, *-E*, and *-F* (Fig. 1A), that are specifically involved in TDA biosynthesis (7, 8, 10, 20). Based on their organization, it is likely that *tdaAB* and *tdaCDE* constitute separate operons. To test this hypothesis, we used reverse transcription-PCR (RT-PCR) with pairs of *tdaA* and *tdaB*, *tdaB* and *tdaC*, and *tdaC* and *tdaE* oligonucleotides, as shown in Fig. 1A. The presence of amplified PCR products from *tdaA/tdaB* (Fig. 1B) indicates that *tdaA* and *tdaB* are transcribed on the same mRNA, while *tdaC*, *-D*, and *-E* are transcribed on a separate mRNA, indicating that *tdaAB* and *tdaCDE* are separate operons.

Based on the current data, the promoter of *tdaC* is located within the 363-bp intergenic region between *tdaB* and *tdaC*. To locate the promoter, we determined the transcription initiation site of *tdaC* by using rapid amplification of 5' cDNA ends (5'-RACE; GeneRacer kit; Invitrogen, Carlsbad, CA) and mapped the transcription initiation site to an A at –100 bp relative to the start codon of *tdaC* (Fig. 2A). A putative promoter sequence is present 33 bp 5' of the transcription initiation site, and its –10 (TATCCG) and –35 (TGAACA) sequences separated by 14 bp share similarities to *Escherichia coli* σ^{70} –10 (TATAAT) and –35 (TTGACA) consensus sequences (Fig. 2A). While the putative *tdaC* promoter is not a

perfect match to the *E. coli* σ^{70} consensus, it is not unusual to see this degree of mismatch to a promoter consensus sequence at both sites (4). The differences may suggest physiological requirements unique to *tda* gene expression.

Previously, we showed that transposon insertion in *tdaA*, a gene encoding an LysR-type transcriptional regulator (LTTR) (13), results in a loss of the ability to produce and respond to TDA (10). TdaA possesses a helix-turn-helix (HTH) DNA binding motif near its N-terminal end and an LysR substrate-binding domain at the C terminus, which is consistent with other LTTR family proteins (10). We searched for the LTTR consensus binding sequence, TN₁₁A, within an interrupted dyadic sequence (19), near the start of transcription initiation and the putative promoter of *tdaC*. The putative *tdaC* promoter contains two possible LTTR binding sites: site 1, TGTCN₆GATA (underlined characters indicate the conserved T/A sites in the LTTR binding sequence), and site 2, TCAGN₅CCGA (Fig. 2B), which is consistent with TdaA binding to the *tdaC* promoter region.

The databases contain homologs to *tdaC* and *tdaF* from other roseobacters, and the promoter regions of these genes are hypothesized to be similar to that of *tdaC* from TM1040 (8). An alignment of six of these sequences, including TM1040 *tdaC*, is shown in Fig. 2B and reveals that both sites 1 and 2 are conserved among these roseobacter promoters. Moreover, sites 1 and 2 share a consensus T(C/T)N₆GA motif in a dyadic region for all aligned DNA sequences except for *tdaF* of *Pseudovibrio* sp. JE062 (062C in Fig. 2B), suggesting that they share a mechanism to control transcription that involves TdaA or a homolog of the protein.

Since *tdaAB* forms an operon, insertion of a transposon in *tdaA* (HG1310; *tdaA::EZ-Tn5*) is likely to have a polar effect on the transcription of *tdaB* (8). To determine the role of *tdaA* and separate TdaA activity from that of TdaB, a plasmid (pHG1014; *lacP::tdaA⁺B⁺*) bearing nucleotides from the start codon of *tdaA* to the stop codon of *tdaB* (*tdaA⁺B⁺*) was constructed such that the expression of these genes was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) (12). A second plasmid, pHG1012 (*lacP::tdaAB⁺*), was constructed that bore a truncated *tdaA*, resulting in a loss of 55 amino acids from the C terminus of TdaA and a wild-type copy of *tdaB*. These plasmids were individually moved into (i) a *tdaA* transposon

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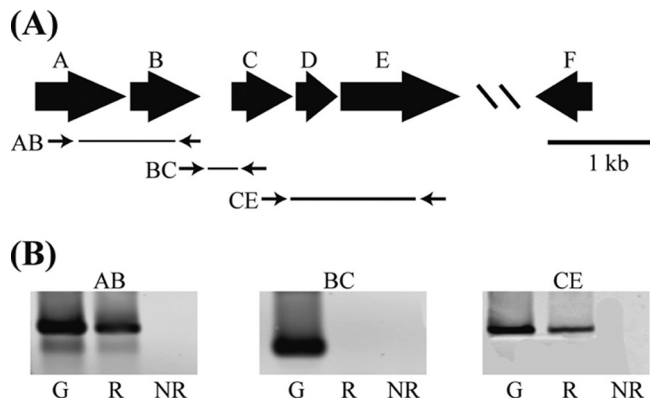


FIG. 1. (A) The *tdaA-tdaF* genes of *Silicibacter* sp. TM1040. Large black arrows indicate relative sizes and the directions of transcription of the genes, and the double hash mark indicates that an unknown amount of DNA separates *tdaE* from *tdaF*. Oligonucleotide primers (see Table S1 in the supplemental material) used in RT-PCR to determine transcriptional units are shown as thin arrows under the genes. (B) RT-PCR results showing that *tdaA* and *-B* are cotranscribed as an operon and *tdaC*, *-D*, and *-E* form a second operon. cDNA was reverse transcribed (R) from total RNA. Total genomic DNA (G) and no reverse transcriptase (NR) served as respective positive and negative controls.

insertion strain (HG1310; *tdaA::EZ-Tn5*) (10), (ii) a *tdaB* transposon insertion strain (HG1015; *tdaB::EZ-Tn5*) (10), and (iii) a *tdaC* transposon insertion strain (HG1080; *tdaC::EZ-Tn5*) (10), each of which also carried pHG1011 (*tdaCp::lacZ*) harboring a transcriptional fusion between the promoter region of *tdaC* and a promoterless *lacZ* (8). Figure 3

shows the results produced by each of these strains, where β -galactosidase activity was measured by its cleavage of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 60 μ g/ml of 2216 marine agar [Difco, BD Biosciences, Franklin Lakes, NJ]) after 3 days at 30°C. Pigment production, which coincides with TDA biosynthesis, was measured as previously described (15). As shown in Fig. 3, when either pHG1012 (*lacP::tdaAB*⁺) or pHG1014 (*lacP::tdaA*⁺*B*⁺) was placed in the *tdaC* mutant (HG1080; *tdaC::EZ-Tn5*), there was little or no detectable β -galactosidase activity, i.e., there was a dramatic loss of *tdaC* expression. This was expected, as previous studies have shown that transcription of *tdaC* is dependent on the presence of TDA, and defects in *tdaC* result in a loss of TDA synthesis (8). Strains with a defect either in *tdaB* (Fig. 3A; middle row) or in both *tdaA* and *tdaB* (Fig. 3A; top row) were complemented with plasmids bearing the respective wild-type genes upon IPTG addition. Complementation also resulted in the production of pigment (Fig. 3A; lower right image in each panel) and synthesis of TDA (data not shown). However, strains lacking a wild-type copy of *tdaA* failed to express *tdaC* and had a dramatic decrease in or loss of β -galactosidase activity, as illustrated in Fig. 3 (top row, middle). These results demonstrate the requirement for both TdaA and TDA in the transcription of *tdaC* and suggest that TdaA positively controls *tda* gene expression.

We next asked if TdaA in the absence of TDA affects *tdaC* transcription by transforming a heterologous (*LacZ*⁻, non-TDA-producing) bacterium, *E. coli* DH5 α , with both pHG1014 (*lacP::tdaA*⁺*B*⁺) and pHG1011 (*tdaCp::lacZ*). When incubated on Luria-Bertani agar containing X-Gal and the

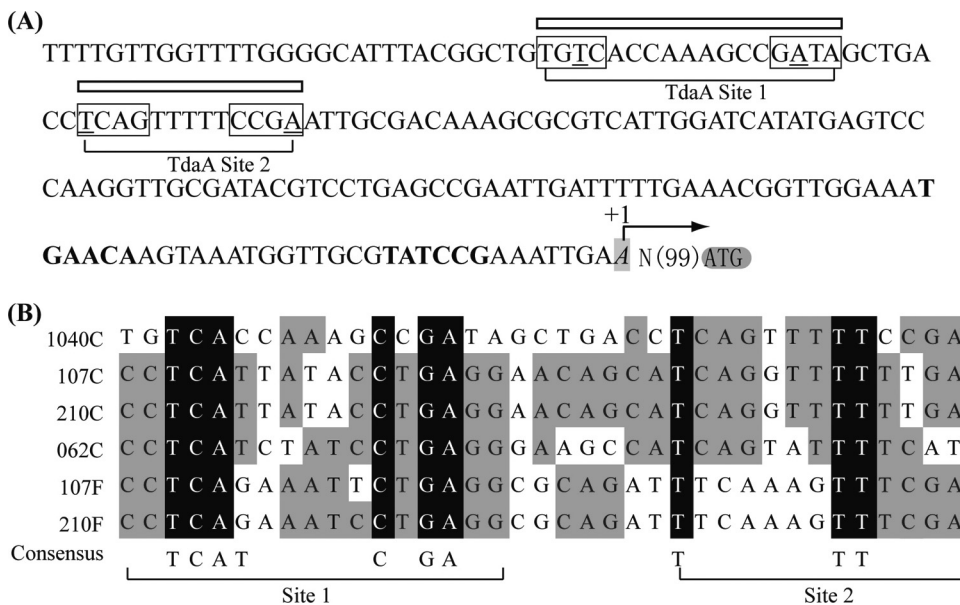


FIG. 2. The 363-bp intergenic region between *tdaB* and *tdaC*. (A) The intergenic region contains two LysR binding sites, TN₁₁A, within an area of interrupted dyad symmetry (rectangles). The transcription initiation site (+1) as determined by 5'-RACE, the *tdaC* start codon (ATG), and the putative *tdaC* promoter -10 and -35 sequences (bold letters) are indicated. N(99) represents 99 bp in this region that are not shown. (B) TdaA binding sites 1 and 2 are conserved in the regulatory regions of genes involved in TDA biosynthesis. Clustal W alignment of TdaA nucleotide binding sites 1 and 2 in the upstream regulatory region of *tdaC* from TM1040 (1040C), *Phaeobacter gallaeciensis* BS107 *tdaC* (107C), *P. gallaeciensis* 2.10 *tdaC* (210C), *Pseudovibrio* sp. JE062 *tdaC* (062C), TM1040 *tdaF* (1040F), *P. gallaeciensis* BS107 *tdaF* (107F), and *P. gallaeciensis* 2.10 *tdaF* (210F) are shown from top to bottom in the alignment. The resulting consensus sequence is highlighted in black, whereas gray letters denote partial matches.

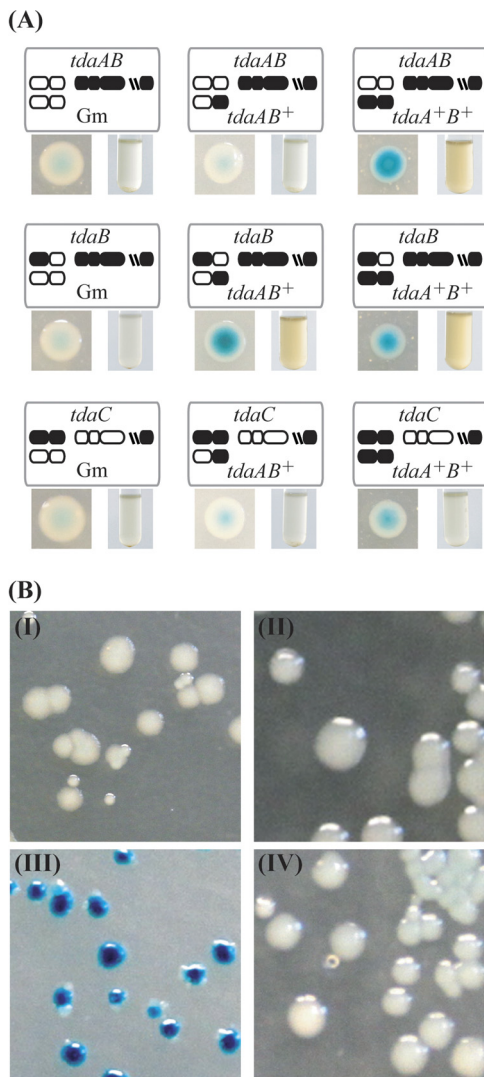


FIG. 3. Expression of *tdaC* requires intact *tdaA*. (A) Upper three panels: a *tdaCp::lacZ* reporter plasmid was placed into HG1310 (*tdaA::EZ-Tn5*; TdaA⁻ TdaB⁻). Into this strain were placed a plasmid control (pSRKGM; labeled Gm), a plasmid containing *tdaA*⁻ *tdaB*⁺ (pHG1012; *lacP::tdaAB*⁺; labeled *tdaAB*⁺), or a plasmid containing *tdaA*⁺ *tdaB*⁺ (pHG1014; *lacP::tdaA*⁺*B*⁺; labeled *tdaA*⁺*B*⁺). Expression of *tdaC* is indicated by a blue colony resulting from the cleavage of X-Gal by β-galactosidase (lower left image in each panel). TDA production was assessed by measuring the amount of brown pigment produced by the culture (lower right image in each panel) whose synthesis is correlated with TDA production (3, 6, 10). Controls: middle three panels are the same set of plasmids in a *tdaB* background, while the lower three panels are the same set of plasmids in a *tdaC* background. (B) Expression of *tdaC* in *E. coli* is *tdaA* dependent. The same three plasmids as described for panel A were placed in an *E. coli* DH5α background. Panels: I, pSRKGM; II, *lacP::tdaAB*⁺; III, *lacP::tdaA*⁺*B*⁺; and IV, no plasmid control.

appropriate antibiotics, *E. coli* harboring both plasmids synthesized β-galactosidase, indicating expression of *tdaC* (Fig. 3B, III). These results confirm that TdaA is required for transcription of *tdaC* and surprisingly demonstrate that transcription of *tdaC* in *E. coli* does not require TDA.

The results thus far suggest that TdaA upregulates *tdaC* expression by binding to DNA near the putative *tdaC* pro-

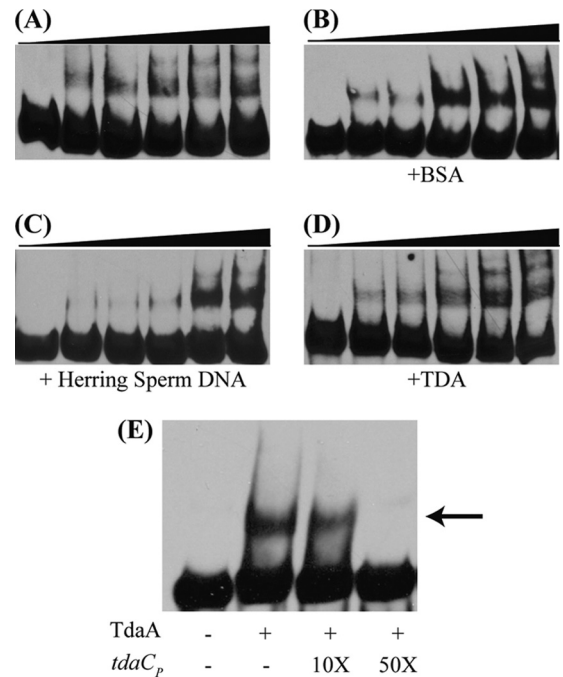


FIG. 4. Electrophoretic mobility shift assays of TdaA binding to *tdaC* promoter DNA. (A) Increasing concentration of TdaA (0, 10, 25, 50, 100, and 200 nM, respectively, from the left to right in each gel) causes a shift in multiple *tdaC* DNA bands. The addition of BSA (B), nonspecific competitor DNA (herring sperm DNA) (C), or 500 nM high-performance liquid chromatography (HPLC)-purified TDA (D) does not affect the shift in *tdaC* DNA. (E) The addition of cold, unlabeled *tdaC* promoter DNA results in a reduction in the amount of shifted, labeled *tdaC* DNA.

moter. This hypothesis was tested using *in vitro* electrophoretic mobility shift assays (EMSA) with purified TdaA protein and a DNA fragment bearing the *tdaC* promoter region. A C-terminal six-histidine-tagged TdaA was expressed from plasmid pET-21a(+) (Novagen, Madison, WI) and purified by nickel affinity chromatography (His-Trap column, GE Healthcare, Piscataway, NJ). A 225-bp segment of DNA from -182 to +43 relative to the transcriptional initiation site of *tdaC*, including both putative TdaA binding sites, was amplified by PCR with 5' biotin-labeled primers (see Table S1 in the supplemental material). DNA bound to TdaA was separated from unbound DNA by electrophoresis with a 5% nondenaturing polyacrylamide gel, and the resulting DNA bands were detected by chemiluminescence (LightShift EMSA kit; Pierce Thermo Fisher Scientific, Rockford, IL).

Increasing amounts of TdaA reduced the mobility of *tdaC* DNA in a concentration-dependent manner (Fig. 4A). The shift in *tdaC* mobility was unaffected by the addition of either a nonspecific protein (bovine serum albumin [BSA]; Fig. 4B) or purified TDA (Fig. 4D). The addition of nonspecific DNA (herring sperm DNA; Fig. 4C) slightly enhanced the shifted bands but did not significantly change the resulting pattern. However, an excess of unlabeled *tdaC* DNA added to the reaction abolished the observed shifts (Fig. 4E).

The EMSAs do not support a hypothesis that TDA is necessary for binding of TdaA to the *tdaC* promoter DNA. Yet, TDA acts as an autoinducer (8), and the results shown in Fig.

3 indicate that β -galactosidase activity from a strain containing wild-type *tdaC*⁺ is enhanced compared to that of a strain lacking TDA (*tdaC*) and underscore that TDA enhances *tdaC* expression. How can this contradiction be resolved?

The transcriptional initiation of *tdaC* is 100 bp upstream of the start of translation. Why does *tdaC* have such a lengthy leader? One possibility, currently under investigation, is that the 100-bp leader contains a binding site for a repressor whose function may be TDA dependent. This hypothesis adequately explains the EMSA results and expression of *tdaC* in *E. coli* (Fig. 3B). Other possibilities may also explain the contradiction. A simple one is that IPTG induction of *tdaA*⁺ results in a physiologically excessive amount of TdaA, and at such high levels, TdaA activates *tdaC* expression in the absence of TDA, similar to what has been reported in *Neisseria meningitidis* CrgA (11) and *E. coli* NhaR (18). It is also possible that *E. coli* produces a TDA-like molecule that may compensate for the lack of TDA; however, we have not detected such a molecule or activity. Last, TDA activation of *tdaC* expression may occur through a mechanism that does not directly involve TdaA, perhaps via the activities of TdaI, a hybrid histidine kinase (8), or another regulator, such as FlaC (2).

In conclusion, the results underscore the importance of TdaA as a regulator and activator of *tda* genes, whose activity requires binding to *tda* promoter DNA. We speculate that TdaA is relatively low in the hierarchy of proteins controlling *tda* gene expression, with other regulatory proteins, such as FlaC and CtrA (2), situated above TdaA and perhaps controlling *tdaA* expression.

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