TdaA Regulates Tropodithietic Acid Synthesis by Binding to the tdaC Promoter Region^†

Haifeng Geng‡ and Robert Belas*

Department of Marine Biotechnology, University of Maryland Baltimore County, and Institute of Marine and Environmental Technology, 701 East Pratt Street, Baltimore, Maryland 21202

Received 7 March 2011/Accepted 18 May 2011

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 stop codon of tdaC (Fig. 2A). A putative promoter sequence is present 35 bp 5′ of the transcription initiation site, and its −10 (TATCCG) and −35 (TGAACA) sequences separated by 14 bp share similarities to Escherichia coli σ70 −10 (TATAAT) and −35 (TTGACA) consensus sequences (Fig. 2A). While the putative tdaC promoter is not a perfect match to the E. coli σ70 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, TN$_1$A, 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, TGTGCGAGATA (underlined characters indicate the conserved T/A sites in the LTTR binding sequence), and site 2, TCAAGGCAGA (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)N$_1$G$_1$A motif in a dyadic region for all aligned DNA sequences except for tdaF of Pseudoaltermonas 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 TdbA, a plasmid (pHG1014; lac::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 (lac::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

^ Corresponding author. Mailing address: Department of Marine Biotechnology, University of Maryland Baltimore County, and Institute of Marine and Environmental Technology, 701 East Pratt Street, Baltimore, MD 21202. Phone: (410) 234-8876. Fax: (410) 234-8896. E-mail: belas@umbc.edu.

‡ Present address: McKusick-Nathans Institute of Genetic Medicine, Department of Pediatrics, Johns Hopkins University School of Medicine, 733 N Broadway, Baltimore, MD 21205.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Published ahead of print on 27 May 2011.
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 µg/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::tdaC“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 expression (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 DH5α, with both pHG1014 (lacP::tdaAB+) and pHG1011 (tdaCp::lacZ). When incubated on Luria-Bertani agar containing X-Gal and the
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 promoter. 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.

FIG. 3. Expression of tdaC requires intact tdaA. (A) Upper three panels: a tdaCp::lacZ reporter plasmid was placed into HG1310 (tdaA::EZ-Tn5; TdaB). Into this strain were placed a plasmid control (pSRKGm; labeled Gm), a plasmid containing tdaA+tdaB' (pHG1012; lacC::tdaAB'; labeled tdaAB+), or a plasmid containing tdaA+tdaB' (pHG1014; lacC::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, lacC::tdaAB'; III, lacC::tdaA B'; and IV, no plasmid control.

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.
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 underscores 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.

We gratefully acknowledge the comments, advice, and encouragement given by the Belas Laboratory, as well as Harold Schreier, Kevin Sowers, and Rayford Payne.

This work was supported by a grant from the National Science Foundation (IOS-0842331).

REFERENCES

Downloaded from http://jb.asm.org on August 29, 2017 by guest