TetR Is a Positive Regulator of the Tetanus Toxin Gene in Clostridium tetani and Is Homologous to BotR

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The TetR gene immediately upstream from the tetanus toxin (TeTXs) gene was characterized. It encodes a 21,562-Da protein which is related (50 to 65% identity) to the equivalent genes (botR) in Clostridium botulinum. TetR has the feature of a DNA binding protein with a basic pl (9.53). It contains a helix-turn-helix motif and shows 29% identity with other putative regulatory genes in Clostridium, i.e., uviA from C. perfringens and txeR from C. difficile. We report for the first time the characterization of C. tetani by electroformation, which permitted us to investigate the function of TetR. Overexpression of tetR in C. tetani induced an increase in TeTX production and in the level of the corresponding mRNA. This indicates that TetR is a transcriptional activator of the TeTX gene. Overexpression of botRA (60% identity with TetR at the amino acid level) in C. tetani induced an increase in TeTX production comparable to that for overexpression of tetR. However, botRC (50% identity with TetR at the amino acid level) was less efficient. This supports that TetR positively regulates the TeTX gene in C. tetani and that a conserved mechanism of regulation of the neurotoxin genes is involved in C. tetani and C. botulinum.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** C. tetani CN655 and recombinant strains were grown in broth containing tryptophane (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine-HCl (0.5 g/liter) (pH 7.2) under anaerobic conditions. Clostridium DNA was extracted and purified as previously described (18).

**DNA techniques.** Ligation, transformation, sequencing, and preparation of plasmid DNA from E. coli were conducted by standard procedures (22).

**Transformation of C. tetani by electroporation.** Competent cells from C. tetani CN655 were prepared in an anaerobic chamber. The bacteria of a Trypticase-yeast extract broth (10 ml) were recovered by centrifugation in the middle of the exponential growth phase, washed in distilled water, and suspended in 0.5 ml of 7 mM Na2HPO4 (pH 7.4) containing 1 mM MgCl2 and 270 mM sucrose. Plasmid DNA (1 to 5 μg) produced in E. coli HB101 was added to 50 μl of cell suspension. Electroporation was performed outside the anaerobic chamber. The bacteria were diluted in TGY, incubated for 3 h at 37°C, and plated onto TGY agar containing 5 μg of erythromycin per ml in an anaerobic chamber.

**Construction of plasmids for expression of tetR and botRC gene expression plasmids.** A DNA fragment containing the coding region of botR was amplified by PCR from C. tetani CN655 with primers introducing a NcoI site at the translational start codon and a PstI site immediately downstream of the stop codon. The amplification product cut by NcoI and PstI was cloned into the high-copy-number vector pAT19 downstream of the C. perfringens iota toxin gene promoter and upstream of the 3′ part of the iota toxin idp gene as previously described (pMRF306) (15). The resulting plasmid, pMRP365, was transferred into C. tetani CN655 yielding the CN655-OE strain.

A similar construction was done with botRC. The coding region of botRC was amplified by PCR from C. botulinum C 468 (11) by adding NcoI and PstI sites at...
the 5' and 3' parts, respectively, and was cloned into pMRP306 digested with NcoI-PstI. The resulting plasmid (pMRP319) was transferred into C. tetani CN655, yielding CN655-BotR/C.

RNA isolation and RNA dot blots. Total RNA was extracted from C. tetani cultures in the middle of the exponential growth phase by using Trizol (Gibco-BRL, Cergy Pontoise, France). The bacterial pellet from a 10 ml culture (optical density at 600 nm [OD600], 1.6 to 1.8) was washed twice in distilled water and suspended in 200 µl of 10 mM Tris-HCl (pH 7)-10 mM EDTA-20% sucrose containing 1 mg of lysozyme. The mixture was incubated for 30 min at 37°C and centrifuged. The pellet was suspended in 1 ml of Trizol, and the suspension was incubated for 5 min at room temperature. The subsequent steps were performed according to the manufacturer's recommendations.

PAGE and immunoblotting procedure. Proteins were precipitated from the supernatant of cultures (OD, 1.8) of wild-type and recombinant C. tetani strains with 10% trichloroacetic acid. The precipitate was collected by centrifugation and was washed with acetone. The solubilized proteins in 50 mM Tris-HCl (pH 7.5) and were then incubated by 0.1% SDS–10% PAGE were transferred electrophoretically to nitrocellulose sheets (Hybond C; Amersham). The nitrocellulose sheets were incubated for 1 h in phosphate-buffered saline containing 5% dried milk and were then incubated for 2 h in Rapid Hybridization Buffer (Amersham), with the PCR-amplified fragments corresponding to the TeTx gene which were 32P labeled with the Megaprime kit (Amersham). The membranes were washed in 0.1× SSC-0.1% sodium dodecyl sulfate at 60°C and exposed to X-ray films.

Immunoblotting procedure of Burnette (2) was used. Proteins separated by 0.1% SDS–10% PAGE were transferred electrophoretically to nitrocellulose sheets (Hybond C; Amersham). The nitrocellulose sheets were incubated for 1 h in phosphate-buffered saline containing 5% dried milk and were then incubated overnight at room temperature with a 1:4000 dilution of rabbit anti-TeTx antibodies. Bound antibodies were detected with peroxidase-labeled protein A and the chlomuniscence kit provided by Amersham.

Toxicity to mice. Serial twofold dilutions of samples (0.5 ml) in 50 mM sodium phosphate buffer (pH 6.3) containing 0.2% (wt/vol) gelatin were injected intraperitoneally into mice weighing 18 to 20 g. Four mice were used for each dilution.

RESULTS

Characterization of the tetR gene. Previous reports of the TeTx nucleotide sequence indicated the presence of tetR immediately upstream of the TeTx gene (7, 8). To obtain the complete sequence of tetR, we cloned a 2.4-kbp HindIII/EcoRI fragment and established the sequence of an area of 480 bp located upstream of the previously reported sequence (8). The nucleotide sequence and the deduced amino acid sequence of tetR are presented in Fig. 1. TetR has a calculated molecular mass of 21,562 Da and consists of 178 amino acids. It displays 50 to 65% identity with the corresponding proteins of C. botulinum strains. A sequence alignment is shown in Fig. 2. TetR exhibits the characteristic features of a DNA binding protein, i.e., a calculated basic pi (9.53) and a helix-turn-helix motif, and shows significant homology to UviA from C. perfringens and TtxR from C. difficile, two other putative Clostridium regulatory proteins (Fig. 2).

Overexpression of the tetR gene in C. tetani. To analyze the function of TetR, the tetR gene was overexpressed in C. tetani. A truncated promoter region of tetR was present in only the 2.4-kbp HindIII-EcoRI fragment and was too short to permit expression of the tetR gene. Thus, the coding region of tetR was amplified by PCR and cloned into the vector containing the promoter of the C. perfringens iap gene, which was used for Clostridium gene expression (15). The resulting plasmid, pMRP365, was transferred by electroporation into C. tetani CN655, yielding CN655-OE.

The production of the wild-type (CN655) and recombinant (CN655-OE) strains was monitored by measuring mouse lethal activity. As shown in Fig. 3, the lethal activity in the culture supernatant during the exponential growth phase was higher (six to eight times) in CN655-OE than that in the wild-type strain. The increase in TeTx production by CN655-OE was confirmed by Western blotting with specific antibodies against TeTx (Fig. 4). Transfection of pAT19 did not result in an increase in TeTx production compared with the wild type (data not shown). These data indicate that the tetR gene in high copy number and in trans position in C. tetani induced an increase in TeTx production. No other extracellular protein seemed to be overproduced, as shown in Fig. 5.

The specific mRNA of the TeTx gene was assayed by dot blot analysis in the wild-type and recombinant CN655-OE strains. The amounts of TeTx gene-specific mRNAs were approximately four times higher than those in the wild-type strain (Fig. 6). This was in agreement with the increase in the level of TeTx in culture supernatant quantified by mouse lethal activity and immunoblotting. These results show that TetR activates TeTx expression at the transcriptional level.

Overexpression of botR/A and botR/C genes in C. tetani. Since TetR shows a high similarity to the equivalent regulatory genes (botRs) from C. botulinum, it was interesting to know if these genes of the same family can be functionally interchangeable. BotR/A shows an overall identity of 60% with TetR (Fig. 2). It was found that plasmid pMRP309 containing the botR/A gene under the control of its own promoter induced a significant increase in the production of BotNT/A and ANTPs and in the corresponding mRNAs in C. botulinum A (15). C. tetani CN655 was transfected by electroporation with pMRP309, yielding strain CN655-BotR/A. A comparable increase in the amount of TeTx assayed by the mouse test and immunoblotting was observed during the exponential growth phase of CN655-BotR/A and CN655-OE (Fig. 3). Mouse lethal activity in the culture supernatant of BotR/A was approximately eight times higher than that in the wild-type strain (Fig. 3). The overproduction of TeTx assayed by immunoblotting was increased by approximately 16 times in CN655-OE and 8 times in CN655-BotR/A compared to the wild-type strain (Fig. 4). Moreover, the amounts of mRNAs of the TeTx confirmed by Western blotting with specific antibodies against TeTx (Fig. 4). Transfection of pAT19 did not result in an increase in TeTx production compared with the wild type (data not shown). These data indicate that the tetR gene in high copy number and in trans position in C. tetani induced an increase in TeTx production. No other extracellular protein seemed to be overproduced, as shown in Fig. 5.

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gene were increased (approximately four times) in both strains (Fig. 6). This indicates that BotR/A was able to positively regulate the TeTx gene in C. tetani. The potential effect of BotR/C, which is less related to TetR at the amino acid level (50% identity) than BotR/A (60% identity), in C. tetani was investigated. Plasmid pMRP319, corresponding to the pAT19 vector containing the coding region of botR/C under the control of the iap gene promoter (15), was transferred into C. tetani CN655 by electroporation (CN655-BotR/C). The production of TeTx assayed by mouse lethal activity was three times higher in CN655-BotR/C than that in the wild-type strain and was eight times higher as determined by immunoblotting (Fig. 3 and 4). No significant increase in TeTx-specific mRNA was detected in CN655-BotR/C (Fig. 6). This shows that botR/C stimulated the expression of the TeTx gene, albeit at a lower extent than botR/A.

**DISCUSSION**

We report the complete sequence of the tetR gene from C. tetani, which is highly related to the botR genes from C. botulinum A, B, C, D, F, and G (1, 5, 6, 12, 13). TetR shows an overall level of identity of from 50% with BotR/C to 65% with BotR/F. This family of genes is related to other putative regulatory genes in Clostridium, such as uviA in C. perfringens and txeR in C. difficile (10, 16). TetR and the other related proteins possess the features of DNA binding proteins, i.e., high pI (pH 9.53) and the presence of a helix-turn-helix motif.

We succeeded in transforming C. tetani with pAT19, which is a shuttle vector between gram-positive and gram-negative bacteria and which contains a replication origin from Enterococcus fecalis (23). The electroporation conditions were similar to those used for the C. botulinum transformation (15). The present article is the first report of genetic transformation in C. te-
tani and construction of recombinant _C. tetani_ strains to investigate a gene function in this microorganism.

Overexpression of _tetR_ in trans position induces an increase in _TeTx_ production, as monitored by mouse lethality in culture supernatant and by Western blotting. A corresponding increase in the specific mRNA of the _TeTx_ gene indicates that _tetR_ positively regulates the transcriptional level of the toxin gene in _C. tetani_. It can not be ruled out that _tetR_ in cis position could be more efficient. _tetR_ seems to regulate specifically the _TeTx_ gene and to have no pleomorphic effect. We explored if the equivalent genes (_botR_ from _C. botulinum_) are functional in _C. tetani_. The high-copy-number vector pAT19 containing _botR/A_ or _botR/C_ was transferred by electroporation into _C. tetani_ CN655. BotR/A, which is more closely related to TetR than BotR/C, produced a higher increase in _TeTx_ production and in the specific mRNA, compared with BotR/C. This shows that BotR/A and BotR/C are functional in _C. tetani_. The different levels of effect between BotR/A and BotR/C could be due to a lower level of expression of _botR/C_, since _botR/C_ was under the control of the _iap_ promoter (pMRP365) and _botR/A_ was under the control of its own promoter (pMRP309). However, _tetR_ was constructed under the control of the _iap_ promoter and induced an equivalent activation of _TeTx_ gene expression equivalent to that of _botR/A_. The more distant relatedness of BotR/C to TetR than to BotR/A could explain the reduced efficiency of BotR/C in _C. tetani_. These data suggest a common mechanism of regulation of the neurotoxin genes in _C. tetani_ and _C. botulinum_.

We found that BotR/A stimulates expression of both the BoNT and ANTP genes (15). The −10 and −35 regions of the neurotoxin and ANTP gene promoters in _C. botulinum_ A, B, C, D, F, and G and _C. tetani_ contain conserved sequences (1, 12). Moreover, BotR/A seems to interact directly with the promoter region and the conserved motifs could represent binding sites for the regulatory proteins (15). TetR could be also a regulatory protein which binds the promoter region of the _TeTx_ gene. Whether TetR and BotR are involved in a cascade of regulatory proteins is unknown. It has been found that short peptides from casein hydrolysates are important for toxigenic-

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**FIG. 3.** Mouse lethal activity in culture supernatants of wild-type _C. tetani_ CN655 (C) and recombinant strains overexpressing the _tetR_ (A), _botR/A_ (B), and _botR/C_ (C) genes. The mouse lethal activity (LD_50_) is plotted against the OD_600_ for each culture. The means and standard deviations of the values from two experiments are indicated.

**FIG. 4.** Production of _TeTx_ assayed by Western blotting with anti-TeTx antibodies in wild-type _C. tetani_ CN655 (A) and in recombinant strains overexpressing the _tetR_ (CN655-OE) (B), _botR/A_ (CN655-BotR/A) (C), and _botR/C_ (CN655-BotR/C) (D) genes. Supernatants of each culture (OD_600_) were concentrated by trichloroacetic acid precipitation, 20 μg of protein was loaded on lane 1, and serial twofold dilutions were loaded in the subsequent lanes. In panels B and D, the upper bands correspond to the whole _TeTx_ and the lower bands correspond to the H chain.

**FIG. 5.** PAGE of extracellular proteins (50 μg) of recombinant strain overexpressing _tetR_ (CN655-OE) (lane 1) and of _C. tetani_ wild-type CN655 (lane 2). H and L, heavy and light chains of _TeTx_, respectively.
esis in C. tetani (19), but the environmental signals which trigger neurotoxin production remain to be determined.

The presence of highly conserved genes in the close vicinity of the clorastidial neurotoxin genes, which are functionally interchangeable, constitutes additional evidence that the locus of clorastidial neurotoxin genes derived from a common ancestor. However, the NTNH and HA genes, which lie upstream from the BoNT genes in the different C. botulinum toxinotypes, are missing in C. tetani. The tetR gene is the only ANTP gene which was found in C. tetani.

Vaccination against tetanus is extremely effective in preventing this disease, and widespread vaccination has almost eradicated tetanus from developed countries. Current tetanus vaccines are produced by formaldehyde treatment of TeTx produced by wild-type C. tetani to yield the immunogenic toxoid. A novel generation of tetanus vaccines involves production of the C-terminal part (fragment C) of TeTx, which is nontoxic and is able to induce neutralizing antibodies. The production of large quantities of recombinant fragment C in various organisms such as E. coli, Lactococcus lactis, Bacillus, and Pichia pastoris (3, 4, 9, 14, 24) was attempted. Our findings on the genetic transformation of C. tetani and on the identification of TetR as a positive regulator open the possibility of using C. tetani as an engineering system for vaccine production. It may be possible to construct C. tetani strains which produce large amounts of TeTx or fragment C. C. tetani has the advantage of secreting a soluble form of TeTx, and this organism is already used in industrial fermentation.

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