Insertional inactivation of a Tet(K)/Tet(L) like transporter does not eliminate tetracycline resistance in *Bacillus cereus*

Ole Andreas Økstad 1, Anne Grønstad 1,2, Toril Lindbäck, Anne-Britt Kolstø *

Biotechnology Centre of Oslo and School of Pharmacy, University of Oslo, P. O. Box 1125, Blindern, 0316 Oslo, Norway

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Abstract

*Bacillus cereus* ATCC 10987 and ATCC 14579 can be induced to high levels of resistance to tetracycline. The chromosomal *B. cereus* gene *bctl* encodes a transmembrane protein with homology to Gram-positive tetracycline efflux proteins and relation to other members of the major facilitator superfamily of transport proteins. A mutant strain containing an insertionally inactivated *bctl* gene did not show impaired tetracycline resistance. No additional altered phenotype was observed in the mutant. Accumulation studies suggested that the resistance mechanism involves a reduced sensitivity to intracellular tetracycline.

Keywords: Tetracycline resistance; Major facilitator superfamily; Active efflux; Transport; *Bacillus cereus*

1. Introduction

Bacterial resistance to tetracycline is mediated by two major mechanisms: ribosome protection and active efflux. *Bacillus* spp. are known to contain tetracycline efflux proteins from the related classes Tet(K) and Tet(L). The *tet(K)* and *tet(L)* genes are located on small and large plasmids or on the chromosome. A *tet(L)* gene has been sequenced from the *B. cereus* plasmid pBC16 [1]. Several *B. cereus* strains, however, among them *B. cereus* ATCC 10987 and ATCC 14579, can be induced to resistance to high concentrations of tetracycline (50 μg ml⁻¹) although lacking the pBC16 plasmid. Here we report a chromosomal gene from *B. cereus* ATCC 10987 and ATCC 14579 with homology to the Tet(K) and Tet(L) tetracycline transporters, as well as to QacA lipophilic organic cation transporters and TemA (tetracyclomycin A) and Mmr (methyleneomycin) resistance proteins. The Bctl protein possesses several features common to tetracycline efflux proteins, but does not confer tetracycline resistance in *B. cereus*.

2. Materials and methods

*B. cereus* strains were obtained from the American Type Culture Collection (ATCC). Bacteria were grown at 37°C in LB broth. Recombinant DNA ma-
Manipulations were performed according to standard protocols [2]. Plasmid vectors pUC18 and pUC19 were used for DNA subcloning, using 50 µg ml⁻¹ ampicillin for selection of transformants. E. coli BK2118 [3] was used for electroporation; exponentially growing cultures were harvested by centrifugation at 4°C, washed twice in ice-cold 1 mM HEPES buffer, pH 7.0 and once in ice-cold 10% glycerol and then resuspended in 1/500 volume of 10% glycerol. Aliquots of 40 µl were stored at -70°C and thawed on ice before use. DNA was added and electroporation performed in 2 mm electroporation cuvettes using the BioRad Gene Pulser (BioRad, Hercules, CA, USA). Electroporation conditions were as follows: capacitance 25 µF, 2.5 kV and 400 Ω 0.5 parallel resistance. Immediately after electroporation the suspension was diluted 25-fold with SOC medium and incubated at 37°C for 1 h before plating.

Sequencing was performed using an ALF automated sequencer (Pharmacia, Uppsala, Sweden) at the DNA Sequencing Laboratory, Biotechnology Centre of Oslo, or according to the Repeated Primer Extension Sequencing protocol (Amersham, UK). The GCG Sequence Analysis Software Program [4] was used to analyse the DNA sequence data.

Southern blotting was performed essentially as described by Kolstø et al. [5] using MagnaCharge nylon transfer membrane (MSI, Westboro, MA, USA). Immobilised DNA was hybridised overnight at 68°C [6].

Total RNA was prepared essentially as described by Igo and Losick [7] from 10 ml cultures with and without tetracycline added. Northern blotting was done using Hybond N⁺ nylon membranes (Amersham, UK). RNA slot blots were prepared using vacuum blotting (Minifold II, Schleicher and Schuell, Germany). 100 µg DNase I-treated RNA was loaded in each slot, transferred to a nylon membrane, fixed for 2 h at 80°C and hybridised [6]. RNA was quantified by phosphorimaging (Storm 840, Molecular Dynamics, USA). 23S rDNA was used as a control probe for normalising the total amounts of RNA loaded on the filters. For RT-PCR, reverse transcription was performed using 5 µg Dnase I-treated RNA, 20 U AMV-Reverse Transcriptase (AMV-RT, Promega, Madison, WI, USA), 100 ng oligonucleotide primer, 80 U RNasin (Promega), and 1 mM each dNTP in 1× reaction buffer at 42°C for 1.5 h. For the PCR reaction 5 µl reverse transcriptase reaction product was amplified using 2.5 U Taq DNA polymerase³ (Promega), 0.4 µM of each primer, and 2 mM each dNTP in 1× Thermo DNA polymerase reaction buffer with 1.5 mM MgCl₂ added. RNA not treated with AMV-RT was included as a negative control. PCR was run for 30 cycles, each consisting of 1 min denaturation (94°C), 1 min annealing (62°C), and 2 min polymerisation (72°C). Oligonucleotide primers were made at the DNA Synthesis Laboratory, Biotechnology Centre of Oslo.

B. cereus ATCC 10987 was adapted to growth on tetracycline by cultivation for 2 days on 0.5 µg ml⁻¹ and subsequently gradually increasing the drug concentration to 5 µg ml⁻¹. Tetracycline accumulation studies were performed by inoculating 1:100 volume of an overnight culture in LB broth into fresh medium. Exponentially growing cultures were washed in 10 mM Tris-HCl, pH 8.0, and uptake of [³H]tetracycline (0.89 Ci mmol⁻¹, DuPont-New England Nuclear) was assayed in 50 mM potassium phosphate/10 mM MgSO₄, pH 6.6 in the presence or absence of 2,4-dinitrophenol (DNP) by a filtration method [8].

3. Results and discussion

A 4.7 kb HindIII clone (Bc69) of B. cereus ATCC 10987 genomic DNA was originally used as an anonymous probe in the construction of physical maps of Bacillus thuringiensis and B. cereus chromosomes [6]. Bc69 was mapped to the 410 kb NotI fragment of the NotI physical map of B. cereus ATCC 10987 [5], and was shown by sequencing to contain a part of the bcll gene. The 5' end of the gene was acquired by construction and screening of two additional genomic libraries, prepared using AccI and EcoRI for digestion. Two clones overlapping Bc69 were isolated and sequenced. The sequences were assembled into a contig of 2475 bp containing two open reading frames (ORFs): bcll of 1323 nucleotides (441 amino acids) and bgclr of 807 nucleotides (269 amino acids).
The ORFs were in the same orientation, separated by a 213 bp intergenic region (Fig. 1).

The deduced amino acid sequences were compared to protein sequences from SwissProt, release 34.0 (11/96) and Trembl, release 3.0 (05/97) by FastA searches [9]. Bcll displayed similarity to several drug resistance efflux proteins, with the strongest homology to members of the Tet(K) and Tet(L) classes of tetracycline resistance determinants from Gram-positive bacteria (26–29% identity, 57–59% similarity in 393 aa). Bcgll showed 29–59% identity (51–75% similarity in 220–266 aa) to glutamate racemases, with the highest identity to the homologue from Bacillus subtilis.

A putative bcll ATG start codon was located 11 bp downstream of a putative ribosome binding site, 5' -AGGAGGAGG-3', with a calculated minimal free energy of −13.6 kcal mol⁻¹ [10]. A dyad symmetry recognised as a probable Rho-independent terminator structure was located 23 bp downstream of the bcll stop codon. The 27 nt symmetric sequence was predicted to form an 11 bp stem-5 nt loop structure with a minimal free energy of −12.4 kcal mol⁻¹ [10], followed by a 5 nt poly(U) tail known to be an additional stabilising feature. The level of expression of tet(L) and tet(K) genes is presumed usually to be regulated by an mRNA attenuation mechanism involving formation of alternative stem-loop structures in the 5' end of the molecule [11]. No sequence-forming pairs of alternative inverted repeats nor any ORFs showing amino acid sequence identity to the previously known highly conserved Tet(K) or Tet(L) leader peptides were identified in the 118 bp non-coding region upstream of bcll. In Clostridium perfringens tet(A)P, a 12 bp palindromic sequence identified upstream of the coding region may represent part of a binding site for a regulatory protein [12]. No equivalent palindromic sequence was found upstream of bcll.

Griffith et al. [13] suggested the presence of a major facilitator superfamily (MFS) of related proteins including transporters for antibiotics, antiseptics, quinolones, amino acids, and sugars. The MFS is divided into five superfamilies, family (III) consisting of several drug transport proteins including the Tet(K) and Tet(L) tetracycline transporters and the QacA and Mmr proteins. Using the PileUp [14] and ClusTree programs [15] a phylogenetic tree of Bcll and selected MFS-related proteins was constructed (Fig. 2). The tree indicated, as was also suggested by FastA searches and multiple sequence alignments, that Bcll is evolutionarily connected to the MFS subfamily (III), showing the closest relation to Tet(K) and Tet(L) members. The B. cereus protein seems to form an independent branch separate from these subclasses.

Hydropathy analysis of Bcll using PepWindow [16] with a window size of 14 residues suggested 14 hydrophobic regions of sufficient length to form plasma membrane-spanning domains. Accordingly,
by membrane topological analysis using alkaline phosphatase and β-galactosidase fusions, Tet(K) and QacA from *Staphylococcus aureus* have been shown to contain 14 transmembrane segments [17,18]. Analysis by the PhdTopology program of the PredictProtein server at EMBL, Heidelberg [19] indicated that *B. cereus* Bct1 contains 12 transmembrane helices, as is suggested for the Gram-negative Tet transporters. According to both models, two Cys residues are located in transmembrane segments, representing potential sites for redox regulation of Bct1 structure or function. Two positively charged residues (Lys-126 and Arg-237 in the 14-helix model, Arg-257 and Arg-412 in the 12-helix model) were likewise predicted to be embedded in the hydrophobic membrane environment. No acidic membrane-localised residues were found, a feature thought to be necessary for efflux of metal-tetracycline [20].

To test whether bct1 was induced in the presence of tetracycline, total RNA was prepared from cultures grown in LB in the presence or absence of tetracycline. When the RNA was examined by Northern analysis using a radiolabelled 0.7 kb *PacI/MscI* fragment internal to the bct1 gene as a probe (Fig. 1), no bct1 transcript could be detected in any of the samples.

As the Northern analysis indicated low levels of
bct1 transcription, a more sensitive assay was used: RNA was subjected to analysis by RT-PCR, employing two oligonucleotide primers specific for the bct1 gene. A 0.7 kb DNA fragment was amplified from RNA isolated from cultures grown in LB as well as in LB with tetracycline added, showing that bct1 is transcribed both in the presence and in the absence of the antibiotic. The bct1 gene probe was used for hybridising slot-blot of *B. cereus* ATCC 10987 RNA isolated from exponentially growing cultures in LB with or without 10 μg ml⁻¹ of tetracycline. No induction of bct1 transcription was detected in cells grown in the presence of the antibiotic.

To gain information about the function of the Bct1 protein we performed insertional inactivation of bct1 in *B. cereus*. A 2.6 kb region of genomic DNA from the *B. cereus* type strain ATCC 14579 containing bct1 and bcglr genes was sequenced. The predicted Bct1 and Bcg1r proteins were highly conserved, showing 86% and 97% sequence identity respectively to the homologues from the ATCC 10987 strain. A 1.5 kb EcoRI-ClaI fragment containing the bct1 gene from ATCC 14579 was subcloned in pMal-c2, and a 0.4 kb 5' region of the gene was substituted with a spectinomycin resistance cassette. The choice of a different strain for this experiment was due to a low efficiency of both protoplast transformation, electroporation and conjugation of *B. cereus* ATCC 10987. The EcoRI-ClaI fragment containing the spectinomycin resistance cassette was subcloned in the conjugal suicide vector pAT113 [21] and used in transconjugal transfer from *E. coli* JM83 (pRK24) to the *B. cereus* type strain ATCC 14579 as described by Pezard et al. [22]. Replacement of the bct1 gene by the recombinant construct was verified by hybridisation, and the mutant strain was shown by RT-PCR analysis not to transcribe a functional bct1 gene. The mutant strain did not show impaired resistance to tetracycline, exhibiting an ability comparable to the wild-type strain to grow on concentrations up to 50 μg ml⁻¹ tetracycline in LB. No indication of tetracycline transport was found when Bct1 was expressed in inverted vesicles of *E. coli* NM81 (T.A. Krulwich, personal communication). We therefore conclude that Bct1 does not perform tetracycline efflux in *B. cereus*. The mutant did not show any changes in growth rate, temperature tolerance, sugar metabolism characteristics (API CH50 test kit, bioMérieux, France), sporulation or haemolytic activity compared to the wild-type. The two strains also exhibited an equal resistance pattern to 13 additional antibiotics. Expression of Bct1 was not found to complement Na⁺/H⁺ antiporter-deficient *E. coli* NM81 [23] or K⁺-uptake-deficient *E. coli* TK2420 [24], indicating the inability of Bct1 to transport Na⁺ and K⁺ (T.A. Krulwich, personal communication).

To study the nature of the tetracycline resistance we assayed the accumulation of tetracycline in *B. cereus* ATCC 10987 adapted to growth on 5 μg ml⁻¹ tetracycline and in cells not previously exposed to the drug, thus unable to grow above 1 μg ml⁻¹. Both the adapted and the non-adapted strains were cultivated for 6 days in LB broth without tetracycline prior to the measurements, with daily re-inoculation in fresh medium. To measure accumulation, [³H]tetracycline was added to a final concentration of 5.6 μM (2.7 μg ml⁻¹, 5 μCi ml⁻¹) and samples were analysed during 5-30 min of incubation. The adapted *B. cereus* ATCC 10987 accumulated as much tetracycline as the non-adapted strain, however only the adapted strain was able to grow in the presence of tetracycline (data not shown). Thus after adaptation, *B. cereus* ATCC 10987 can endure higher intracellular tetracycline concentrations, showing that a mechanism different from an efflux pump is involved in the antibiotic resistance. To support this conclusion DNP was added to an aliquot of the culture in each experiment to collapse the proton gradient across the plasma membrane, thereby inhibiting the active efflux of tetracycline via proton/tetracycline antiporters. As described for tetracycline-sensitive strains of *E. coli* [8], we observed that tetracycline accumulation decreased by more than 50% in the presence of DNP, again suggesting the absence of a functional tetracycline efflux protein and indicating that an energy-dependent and an energy-independent component are involved in uptake of the drug. The decrease occurred in all *Bacillus* strains examined, including a non-adaptable tetracycline-sensitive *B. cereus* strain. Considering that the adapted strain was still resistant after cultivation for more than 6 days without exposure to tetracycline, it is likely that a genetic alteration has occurred during adaptation by either amplification or mutational selection, or both. This could involve a mutation in a
ribosomal target molecule or a change in a gene conferring resistance by another non-efflux type mechanism.

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References


