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The *tetAB* genes of the *Corynebacterium striatum* R-plasmid pTP10 encode an ABC transporter and confer tetracycline, oxytetracycline and oxacillin resistance in *Corynebacterium glutamicum*

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Abstract

The tetracycline resistance region of the 50-kb R-plasmid pTP10 from the clinical isolate *Corynebacterium striatum* M82B was analyzed in *Corynebacterium glutamicum* ATCC 13032 and confined to a 4.4-kb *Sph*I-*Sal*I DNA fragment. Nucleotide sequence analysis revealed two open reading frames, termed *tetA* and *tetB*, specifying proteins of 513 and 528 amino acids, respectively. The deduced amino acid sequences of *tetAB* displayed similarity to ATP-binding cassette transporters including StrV and StrW of *Streptomyces glaucescens* which are proposed to play a role in the export of streptomycin-like aminoglycosides. An antibiotic susceptibility screening in *C. glutamicum* showed that the *tetAB* genes confer resistance to tetracycline, oxytetracycline and to the structurally and functionally unrelated β-lactam antibiotic oxacillin.

Keywords: *Corynebacterium*; ABC transporter; Antibiotic export; Multidrug resistance

1. Introduction

Corynebacterium glutamicum is a Gram-positive soil bacterium which is used in a variety of biotechnological processes, especially in the fermentative production of L-amino acids. During the last decade, basic genetic tools for this organism have been developed and applied to improve the synthesis of selected amino acids by recombinant DNA techniques [1]. To obtain efficiently expressed antibiotic resistance genes for cloning purposes in *C. glutamicum*, we intended to characterize the 50-kb R-plasmid pTP10 which was identified in the human isolate *Corynebacterium striatum* M82B [2]. Curing experiments clearly demonstrated that pTP10 confers the resistance to the antibiotics kanamycin, erythromycin, chloramphenicol and tetracycline. A detailed restriction map of pTP10 was constructed by cloning and analyzing overlapping DNA fragments in *C. glutamicum* and *Escherichia coli* [3]. The restriction map of the

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kanamycin resistance region revealed striking similarities to the *aphA-1* gene specifying an aminoglycoside phosphotransferase enzyme. The erythromycin resistance gene *ermCX* encodes a 23S rRNA methyltransferase with the extended resistance profile to the antibiotic clindamycin [4]. In addition, the *cmx* gene of pTP10 mediates chloramphenicol resistance by an antibiotic export mechanism [5].

In the present study, we describe the characterization of the tetracycline resistance region of the R-plasmid pTP10 at the DNA sequence level. We identified two encoding regions whose deduced amino acid sequences showed a significant similarity to ATP-binding cassette (ABC) transporters. When expressed together in *C. glutamicum*, these tandem genes conferred resistance to the antibiotics tetracycline, oxytetracycline and oxacillin.

2. Materials and methods

2.1. Bacterial strains and media

The *C. glutamicum* wild-type strain ATCC 13032 (American Type Culture Collection, Manassas, VA, USA) was used in this study. *E. coli* DH5αMCR was used for all cloning procedures [6]. *C. glutamicum* and *E. coli* strains were routinely grown in LBG medium (10 g tryptone, 5 g yeast extract, 5 g NaCl and 2 g glucose per liter, pH 7.4) at 30 and 37°C, respectively. For *E. coli* strains carrying recombinant plasmids, LBG was supplemented with 50 μg ml^{-1} kanamycin.

2.2. DNA techniques and DNA sequence analysis

Plasmid DNA of *E. coli* was extracted by means of the QIAprep spin kit (Qiagen, Hilden, Germany). Recombinant plasmids were introduced into *E. coli* DH5αMCR by electroporation [6]. *C. glutamicum* cells were transformed by electroporation following the protocol described by Haynes and Britz [7]. Transformants were selected on LBG agar containing 25 μg ml^{-1} kanamycin.

Determination of the nucleotide sequence of the tetracycline resistance region was performed by the Institut für Innovationstransfer at the Universität Bielefeld (Bielefeld, Germany). The DNA sequence and protein data were analyzed as described previously [5]. The nucleotide sequence reported in this study has been deposited in the GenBank database under accession number U21300.

2.3. Antibiotic resistance assay

Determination of the minimal inhibitory concentration of antibiotics for *C. glutamicum* ATCC 13032 [pATTC4] was performed using liquid cultures supplemented with antimicrobial agents. For this purpose, 5×10^4 cells of an overnight culture grown in liquid LBG medium with 25 μg ml^{-1} kanamycin were washed twice with 1 ml PS buffer (8 g l^{-1} NaCl, pH 8.0). Subsequently, the cells were inoculated in 10 ml LBG medium supplemented with different concentrations of antimicrobial agents and incubated at 30°C for 20 h. The inhibitory concentration, specifying the antibiotic concentration at which no growth of the *C. glutamicum* strain occurs, was determined by measuring the optical density at 580 nm. *C. glutamicum* ATCC 13032 carrying the cloning vector pEBM2 was applied as a control strain.

3. Results and discussion

3.1. The tetracycline resistance region of the C. striatum R-plasmid pTP10 is located adjacent to the erythromycin resistance transposon Tn5432

In a previous study, the antibiotic resistance regions of pTP10 were identified by means of a plasmid library covering the complete R-plasmid [3]. Due to this functional analysis, the tetracycline resistance determinant and the erythromycin resistance gene *ermCX* were located on a 10.7-kb *Hin*dIII DNA fragment present in the *C. glutamicum* vector pCV200 (Fig. 1A). To identify a smaller DNA fragment able to mediate tetracycline resistance in *C. glutamicum*, subfragments of pCV200 [3] were cloned in *E. coli* DH5αMCR into compatible sites of the *E. coli-C. glutamicum* shuttle vector pEBM2 [8]. Subsequently, the deletion derivatives pATTC1–pATTC5 (Fig. 1B) were introduced into *C. glutamicum* ATCC 13032 by electroporation [7] using the pEBM2-encoded kanamycin resistance for primary selection.

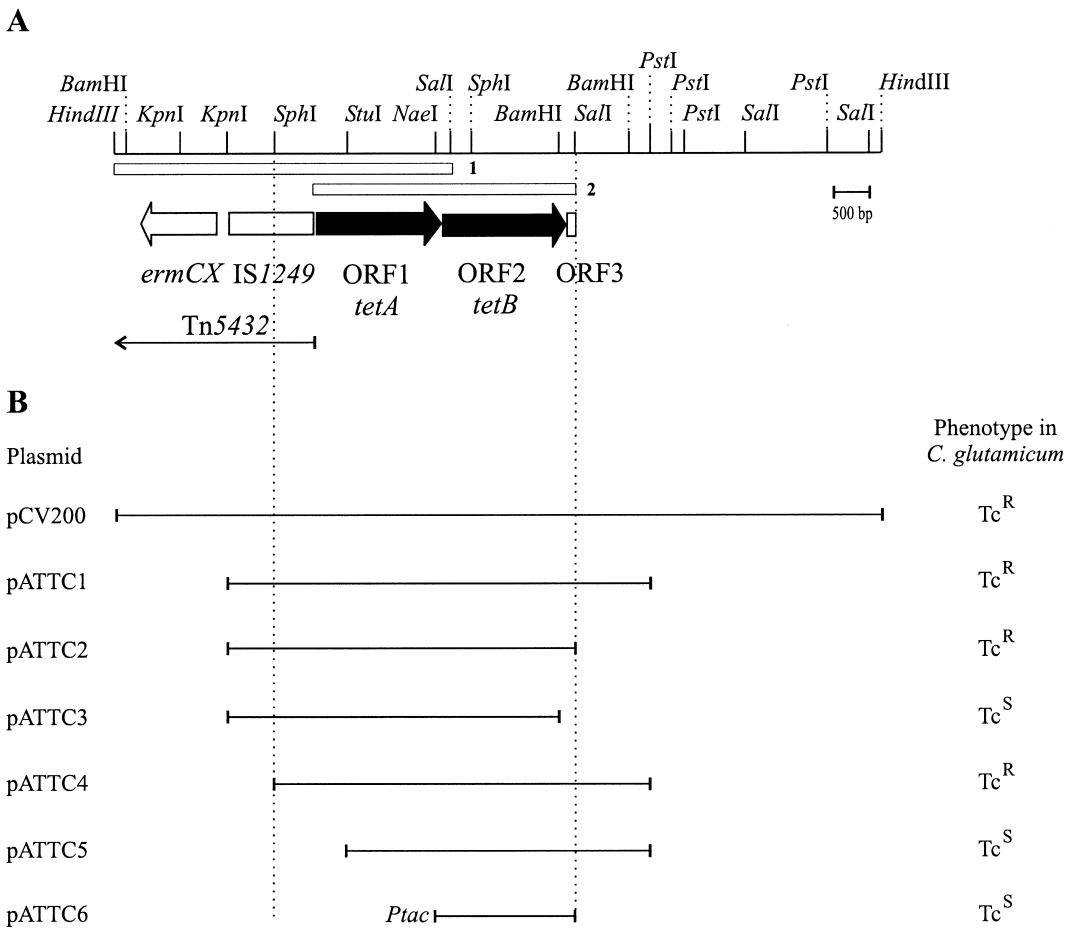

Fig. 1. Genetic and physical organization of the tetracycline resistance region of the *C. striatum* R-plasmid pTP10. (A) The restriction map of the 10.7-kb *Hin*dIII DNA fragment present in pCV200 is shown. The encoding regions located on the sequenced DNA fragment are marked by arrows indicating the direction of transcription. The 4.8-kb *Hin*dIII-*Sal*I fragment (box 1) comprising the erythromycin resistance gene *ermCX* and the insertion sequence IS*1249* of Tn*5432* was sequenced previously [4]. The nucleotide sequence of the overlapping 3.3-kb DNA fragment (box 2) was determined in the present study. (B) Plasmids and DNA fragments used for confining the tetracycline resistance region on the 10.7-kb *Hin*dIII fragment. The antibiotic resistance test was performed on LB agar containing 8 μg ml^{-1} tetracycline (Tc). In plasmid pATTC6, the ORF2 gene is under control of the *tac* promotor (P_{tac}) of the expression vector pZ8-1. The 4.4-kb *Sph*I-*Sal*I DNA fragment, sufficient to mediate tetracycline resistance in *C. glutamicum*, is indicated by dotted lines.

To determine the tetracycline resistance profile of the plasmids in *C. glutamicum*, the transformants were grown on LBG agar in the absence or presence of 8 μg ml^{-1} tetracycline. In control experiments, this tetracycline concentration completely inhibited the growth of the wild-type strain carrying the cloning vector pEBM2. In contrast, all pEBM2 derivatives containing a 4.4-kb *Sph*I-*Sal*I DNA fragment located adjacent to Tn*5432* were able to confer tetracycline resistance to *C. glutamicum* (Fig. 1B). Therefore, the entire tetracycline resistance determinant of pTP10 should be located on the DNA fragment present in plasmid pATTC4 (Fig. 1B).

3.2. The DNA fragment located adjacent to Tn5432 encodes two proteins showing homology to ABC transporters from Streptomyces glaucescens

The nucleotide sequence of a 3.3-kb DNA fragment of pATTC4 was determined and subsequently

Fig. 2. Global amino acid sequence alignments of the proteins deduced from *tetA* (A) and *tetB* (B) with the StrV and StrW proteins identified in *S. glaucescens* [13]. Asterisks denote identical amino acids, dots indicate similar amino acids. Gaps were introduced to optimize the protein alignment. Numbers on the right correspond to amino acid positions relative to the start of each protein sequence. The predicted transmembrane segments (I–V) of the TetAB proteins are indicated. The highly conserved Walker motifs A (GxSGxGKST) and B (KILmLDEAT) and the ABC signature (GxxGxxxSGGQxQR) which are involved in ATP-binding and hydrolysis are marked [11].

assembled with the previously sequenced portion of the cloned *Hin*dIII fragment of pCV200 (Fig. 1A). A computer-assisted search for encoding regions revealed the presence of two ORFs, designated ORF1 and ORF2, and the presence of a 5′ region of an incomplete ORF, termed ORF3 (Fig. 1A). Interestingly, the G+C content of the sequenced DNA fragment is 68% which is significantly higher than the mean G+C content of 57.6% reported for the *C. striatum* chromosome [9]. This may imply that the tetracycline resistance region was transferred to *C. striatum* by horizontal gene transfer from an unknown microorganism having a DNA with a high G+C content.

ORF1 of the tetracycline resistance region most probably starts at the ATG codon located 42 bp downstream of IS*1249* and encodes a protein of 513 amino acids with a deduced molecular mass of 53.7 kDa (Fig. 2A). ORF2 initiates immediately downstream of the ORF1 stop codon and specifies

Table 1
Minimal inhibitory concentration for *C. glutamicum* (*C.g.*) strains carrying the plasmid pEBM2 and its derivative pATTC4 with the cloned *tetAB* genes

Antimicrobial agents	Minimal inhibitory concentration (μg ml^{-1})	
	C.g. [pEBM2]	*C.g.* [pATTC4]
Amikacin	0.1	0.1
Amoxicillin	0.1	0.1
Ampicillin	0.1	0.1
Bacitracin	0.25	0.25
Cefotaxim	0.25	0.25
Ceftriaxone	0.75	0.75
Cefuroxime	0.5	0.5
Cephalothin	0.1	0.1
Chlortetracycline	0.25	0.25
Doxycycline	0.1	0.1
Ethidium bromide	0.75	0.75
Fusidic acid	0.25	0.25
Minocycline	0.1	0.1
Novobiocin	5	5
Oxacillin	0.5	4
Oxytetracycline	0.25	10
Penicillin G	0.25	0.25
Sodium dodecycl sulfate	21250	250
Tetracycline	0.75	12
Vancomycin	0.1	0.1

a protein of 528 amino acids with a calculated molecular mass of 55.9 kDa (Fig. 2B). A putative promoter region resembling the −35 and −10 consensus promoter sequences of *C. glutamicum* is located upstream of the assigned start codon of ORF1 and thus within the inverted repeat of IS*1249*. Therefore, the expression of ORF1 and possibly ORF2 might be mediated by a hybrid promoter of the −35 region which is provided by IS*1249*.

Analysis of the deduced amino acid composition of ORF1 and ORF2 revealed that the proteins are composed of a predominantly hydrophobic N-terminal domain and a hydrophilic C-terminal domain. In the N-terminal portion of each protein, the neural network system Predict Protein [10] indicates the presence of five hydrophobic segments with a minimum length of 18 amino acids which are predicted to be putative transmembrane α-helices (Fig. 2). In addition, the C-terminal end of the ORF1 protein contains the Walker motif B and an ABC signature whereas Walker motifs A and B could be identified in the C-terminal part of the ORF2 protein (Fig. 2). These highly conserved motifs are typical features of ATP-binding domains and involved in the binding and hydrolysis of ATP [11].

Database searches with the deduced amino acid sequences of ORF1 and ORF2 revealed significant similarity to several members of the ABC transporter superfamily that are implicated in membrane transport of a large variety of substrates [11]. A global protein alignment calculated 28% identity and 46% similarity between the ORF1 protein and the ABC transporter StrV from *S. glaucescens* (Fig. 2A). A comparison of the ORF2 protein with the ABC transporter StrW from *S. glaucescens* revealed 31% identity and 48% similarity (Fig. 2B). The amino acid sequence similarity between the ORF2-encoded protein and StrW was strongest in the C-terminal domain comprising 36% identical amino acids with an additional 21% conservative substitutions (Fig. 2B). This is in accordance with the observation of Higgins [12] that the transmembrane domain of ABC transporters is less well conserved than the ATP-binding domain. Moreover, the ORF1 and ORF2 proteins revealed significant similarity to the ABC transporters FxtA (43%) and FxtB (53%) which are

part of the exochelin locus in *Mycobacterium smegmatis* (GenBank AF027770).

The functional subcloning of the tetracycline resistance determinant in *C. glutamicum* indicated that ORF1 and ORF2 might be necessary to mediate tetracycline resistance (Fig. 1). Since a putative promoter region was only identified upstream of ORF1, the ORF2 gene located on a 1.9-kb *Nae*I-*Sal*I DNA fragment (Fig. 1A) was cloned under the control of the *tac* promoter of plasmid pZ8-1 (Degussa AG, Germany) to ensure its expression in *C. glutamicum*. The antibiotic resistance assay clearly demonstrated that neither ORF1 (pATTC3) nor ORF2 (pATTC6) alone were able to confer tetracycline resistance in *C. glutamicum* (Fig. 1B). Although the deduced proteins of ORF1 and ORF2 share 39% similarity, they differ in the highly conserved Walker motifs and in the ABC signature (Fig. 2). This might indicate that the ORF1 and ORF2 proteins function as a heterodimer similar to the homologous proteins StrV and StrW which are proposed to be involved in the export of streptomycin-like compounds in *S. glaucescens* [13]. Beyer and co-workers suggested that, for instance, the StrV protein could take part in the substrate recognition while the StrW protein may catalyse the hydrolysis of ATP during the export process. Due to these genetic data and the structural similarities, we conclude that ORF1 and ORF2 encode an ABC transporter which is involved in the export of the antibiotic tetracycline from the bacterial cell. Therefore, the corresponding genes of the R-plasmid pTP10 were termed *tetA* and *tetB*, respectively.

3.3. The *tetAB* genes conferred resistance to the antibiotics tetracycline, oxytetracycline and oxacillin in *C. glutamicum* ATCC 13032

As described above, the deduced amino acid sequence of the *tetAB* genes revealed homology to members of the ABC transporter superfamily, most of which confer a so-called 'specific drug resistance' phenotype in bacteria [11]. Currently, the only known bacterial multidrug resistance ABC transporter is the LmrA protein of *Lactococcus lactis* conferring resistance to a number of structurally and functionally unrelated toxic compounds [11]. To identify an extended antibiotic resistance profile mediated by the *tetAB* gene products, we performed a functional analysis of the *tetAB* genes in *C. glutamicum*. For this purpose, the tetracycline sensitive control strain *C. glutamicum* ATCC 13032 [pEBM2] and *C. glutamicum* ATCC 13032 [pATTC4], carrying the *tetAB* genes cloned on pEBM2, were tested for growth in the presence of various antibiotics and antimicrobial agents. The minimal inhibitory concentration for both strains was determined by monitoring the growth in liquid LBG medium supplemented with different concentrations of antimicrobial substances. The results of the susceptibility screening are summarized in Table 1.

As expected, the *tetAB* genes of pTP10 increased the minimal inhibitory concentration of tetracycline for *C. glutamicum* by a factor of 16 when present in the cloning vector pEBM2 (Table 1). Most interestingly, the extended susceptibility screening in *C. glutamicum* revealed that the *tetAB* genes also mediate resistance to the tetracycline derivative oxytetracycline (a 40-fold increase of the inhibitory concentration) and to the semisynthetic β-lactam antibiotic oxacillin (an 8-fold increase of the inhibitory concentration). Furthermore, no significant effect on the resistance to other antibiotics, especially to other tetracycline derivatives (chlorotetracycline, minocycline, doxycycline) and to other β-lactam antibiotics (penicillin G, ampicillin, amoxicillin) was observed. Nevertheless, the action of the *tetAB* genes against the structurally and functionally unrelated antibiotics tetracycline/oxytetracycline and oxacillin suggests that the gene products might resemble a multidrug transport system.

The characterization of the *tetAB* genes is also of medical importance since antibiotic resistance may play a crucial role in the failure of antibiotic treatment of *C. striatum*-mediated human infections. Interestingly, Martinez-Martinez et al. [14] reported that 97% of the clinical samples of *C. striatum* were resistant to tetracycline. In addition, an antibiotic susceptibility screening showed that oxacillin exhibited a reduced activity against *C. striatum* strains [15]. In an early study, Roberts et al. [16] identified a *tetM* homologous gene in *C. striatum* strains by heterologous hybridization experiments suggesting a resistance mechanism that is based on *tetM*-mediated ribosomal protection. Our study at

the DNA sequence level expanded the known mechanisms for the occurrence of tetracycline (and oxacillin) resistance in *C. striatum* strains from clinical samples. According to the presented data, the resistance profile of clinical strains might alternatively be mediated by an antibiotic efflux mechanism of an ABC transporter.

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