Genomic Insights Into Antibiotic Resistance Pattern of Tetracycline Degrading Bacterium *Arthrobacter Nicotianae* OTC-16

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**Genomic insights into antibiotic resistance pattern of
tetracycline-degrading bacterium *Arthrobacter nicotianae* OTC-16**

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Abstract

Although many bacteria have the potential to remove antibiotic residues from environmental niches, the benefits of using antibiotic-degrading bacteria to manage antibiotic pollution should be assessed against the risk of the potential expansion of antimicrobial resistance. This study investigated the antibiotic resistance pattern of the bacterium *Arthrobacter nicotianae* OTC-16, which shows substantial biodegradation of oxytetracycline/tetracycline. The results showed that this strain could be resistant to at least seven categories, 15 antibiotics, based on antimicrobial susceptibility testing. The genome of *A. nicotianae* OTC-16 contains one chromosome(3,643,989 bp) and two plasmids (plasmid1,123,894 bp and plasmid2, 29,841bp). Of the 3,561 genes isolated, eight were related to antibiotic resistance. During oxytetracycline (OTC) degradation by the strain OTC-16, the expression of *ant2ia*, *sull1*, *tet33*, and *cml_e8* in the plasmid, and one gene(*tetV*) in the chromosome were tracked by real-time quantitative reverse transcription polymerase chain reaction(qRT-PCR). Only the plasmid-derived resistance genes were up-regulated under the pressure of OTC. The presence of OTC increased the tolerance of strain OTC-16 to streptomycin sulphate. This work deepens our understanding of the behavioural characteristics of resistance genes and adaptive evolution of resistant-drug bacteria under the pressure of antibiotics.

Keywords antibiotic-degrading bacteria; antibiotic resistance genes; Genome

Introduction

Antibiotics have received global attention for their wide application in human

therapy and livestock agriculture and their widely occurrence in terrestrial and aquatic environments^{1,2}, with the evidence leading to a measurable increase in antibiotic resistance genes (ARGs) and the potential to spread to the clinic. Environmental bacteria can utilize antibiotics as carbon or nitrogen sources. For example, there are specific antibiotic-degrading bacteria for β -lactams³, tetracyclines⁴ and sulphonamides⁵. These antibiotic-degrading bacteria have the potential to be developed for bioaugmentation/bioremediation of antibiotic-contaminated environments^{3,4} such as soils near pharmaceutical manufacturers, pharmaceutical wastewater, or livestock manure⁶. However, most of the bioaugmentation/bioremediation agents were applied to facilitate the removal of antibiotics at fixed-point sites, such as wastewater plants. One important reason for limiting the widespread use of biodegrading agents is that the strains are ecologically risky. Even after the death of the bioaugmentation agents, the naked DNA carrying the ARGs may exist for a long time, thus endangering human health through various pathways such as direct contact or pollution of the food chain⁷. In addition, the horizontal exchange of ARGs through mobile genetic elements such as integrons, plasmids, and transposons would increase resistance rapidly in many indigenous environmental bacteria as a result of exposure to resistant strains^{8,9}. Due to these high ecological risks, the World Health Organization has listed ARGs as one of the most important challenges threatening human health in the 21st century. Therefore, characterisation of the antibiotic resistance profile of these microbial inoculants is an essential step to assess the ecological risks before their application, which could guarantee their safe

67 use.

68 We previously isolated a tetracycline antibiotic-degrading bacterium
69 *Arthrobacter nicotianae* OTC-16, which shows striking biodegradation of
70 oxytetracycline/tetracycline in various matrices, for example, aqueous media under a
71 wide range of pH, temperature, and antibiotic concentration; manure samples from
72 different animals; and soils with different properties¹⁰. Preliminary analysis showed
73 that the application of strain OTC-16 did not increase some commonly studied
74 tetracycline-resistant genes in the soil such as *tetW*, *tetB*, *tetA*, and *tetG*, and could
75 effectively inhibit the proliferation of *tetW* and *tetB*. Nonetheless, these results are
76 insufficient to conclude that there is no risk in applying this degrading bacterium in
77 the environment. This study focused on the antibiotic resistance of *A. nicotianae*
78 OTC-16, with the aim of providing a theoretical basis for the safe use of a degrading
79 strain and further engineering modification to decrease its ecological risk. The
80 influence of the combined use of different drugs on the growth of the degrading strain
81 OTC-16, was also explored. The findings of this study will deepen our understanding
82 of the resistance pattern of the strain and elucidate the role of ARGs in antibiotic
83 resistance and adaptive evolution under the pressure of antibiotics, as well as provide
84 a theoretical basis for the safe use of degrading strains in the future.

86 **Results and discussion**

87 **Antimicrobial susceptibility analysis**

Arthrobacter nicotianae OTC-16 was previously isolated for its efficient biodegradation of OTC and tetracycline, indicating that the strain was resistant to these two antibiotics. Antimicrobial susceptibility testing further confirmed that strain OTC-16 also exhibited obvious resistance to 13 other tested antibiotics, namely, sulfamethoxine, sulfamethoxazole, sulfamethazine, sulfamonomethoxine, cotrimoxazole, bacitracin, streptomycin, streptomycin sulphate, nalidixic acid, nystatin, furazolidone, isopropyl-b-D-thiogalactoside and metronidazole. In addition, strain OTC-16 showed low resistance to chloramphenicol, vancomycin, macrodantin, polymyxin, and kanamycin (Table 2). Overall, strain OTC-16 showed multiple resistance to antimicrobial agents, which might be related to its powerful enzyme systems for decomposing heterogeneous compounds¹¹⁻¹⁵.

Genomic features of *A. nicotianae* OTC-16

The whole genome of the newly isolated *A. nicotianae* OTC-16 was sequenced (accession number NZ_CP 033 081). Briefly, *A. nicotianae* OTC-16 contained one chromosome with a total length of 3,643,989 bp and two plasmids with lengths of 123,894 bp and 29,841bp, spectively (Figure 1); the GC-content of the genome was 61.71%. This genome contained 3,561 genes, including 67 tRNA, 19 rRNA, and 28 sRNA, with a total length of 379,7724 bp. **Figure S1** shows detailed GO functional information, from which three main functional domains were established: (i) cellular component, (ii) molecular function, and (iii)biological process. A total of 3,429 genes were annotated related to biological processes; 1,326 genes participated in metabolic processes, 992 genes functioned in cellular processes, and 132 genes responded to

stimuli. The statistics of KEGG annotation showed that genes of strain OTC-16 can be classified into six categories, with 30 genes related to biosynthesis of secondary metabolites, 12 genes participating in infectious diseases, 68 genes involved in xenobiotics biodegradation and metabolism, and 16 genes related to drug resistance (Figure 2). These drug resistance genes may contribute to the wide resistance of strain OTC-16 to antimicrobials.

The ARGs were obtained by CARD analysis, and detailed information is listed in Table 3. Briefly, eight antibiotic resistance types, a total of 23 copies of ARGs of strain OTC-16 were recognised and most of them, with four types (*sul1*, *tet33*, *ant2ia*, and *cml_e8*), 15 copies of ARGs located in plasmids. Cells of strain OTC-16 grew well when exposed to all the possible antibiotics encoded by the ARGs, as was observed in antimicrobial susceptibility testing. Tetracycline resistance involved *tet33* in both plasmid I and plasmid II, and *tetV* in the chromosome. Genes *tetV* and *tet33* encode efflux proteins, known as membrane-associated proteins, that export tetracyclines from the cell. The export of tetracyclines reduces intracellular drug concentration, and therefore, protects the ribosome within the cell. This may be the reason why strain OTC-16 grew well under the pressure of tetracycline and oxytetracycline. Since the *tet* genes were unable to decompose tetracycline antibiotics, the role of these genes in strain OTC-16 during tetracycline degradation should be considered in the future. The gene *ant2ia* encodes aminoglycoside transferases, which inactivate aminoglycosides and confer resistance to aminoglycoside antibiotics¹⁶. In antimicrobial susceptibility testing, strain OTC-16 showed high-resistance to

streptomycin and medium-resistance to tobramycin and kanamycin. The genes *sulI* and *cml_e8* provided strain resistance to sulfonamides and chloramphenicol, respectively. The antibiotic resistance spectrum experiment in this work also confirmed the existence and function of these ARGs. Strain OTC-16 grew well when facing sulfadimethazine, sulfamethoxazole, sulfamonomethoxine, and cotrimoxazole, and showed tolerance to chloramphenicol to some extent.

A total of three *A. nicotianae* strains have been sequenced to date. Strain ZM05 (accession number [NZ_CP059853.1](#)) has only one plasmid (accession number [NZ_CP059854.1](#)) and strain NBRC14234 (accession number [NZ_BJNE00000000.1](#)) has none. To compare the differences in the resistance genes carried by the three strains, the ARGs listed in Table 3 were used as target genes to BLAST among the three strains with the NCBI software. BLAST results showed that the ARGs contained in the three strains differed. Although the ARGs were mostly located in plasmids in strain OTC-16, they all existed on chromosomes in strains ZM05 and NBRC14234. Strain ZM05 shared seven single-copy homologous resistance genes with strain OTC-16, the gene with the highest homology is *vanre*, with an identity value of 100%, followed by genes *vanrc* (99.41%) and *cara* (99.24%). While strain NBRC14234 had fewer ARGs, only the single copy genes, *bcra* and *ctab3*, were detected, and the homology was also lower than that of strain ZM05 (Table S1). Other detailed information can be found in the **Supplementary Information**. Comparison of the sequences of the three strains confirmed that the evolution of ARGs of the same bacterial species in different regions was different.

Common ARGs are more likely to evolve in similar and immediate surroundings. In this study, strains OTC-16 and ZM05 were both from China; they share 7 common ARGs. Meanwhile, strain NBRC14234, isolated from Japan, has only two low homologous common ARGs with strain OTC-16. Therefore, ARGs carried by strains reflected the evolution of the strain in the environment, and also possibly reflected the antibiotic pollution level where the strains survive.

Plasmids with ARGs replicated independently in cells, and can transfer to other bacteria through transformation and conjugation, leading to gene transport and diffusion. While in those strains with ARGs distributed on chromosomes, such as strains ZM05 and NBRC14234; the transmission of ARGs may be mainly achieved by transposition.

Expression of ARGs in OTC-16 under OTC stress

To study the expression characteristics of ARGs at different genetic loci, the behaviour of *sul1*, *tet33*, *ant2ia*, and *cml_e8*, located in plasmids and gene *tetV* carried by chromosomes, were tracked by qRT-PCR. The results showed that all five resistant genes could be expressed in strain OTC-16 with or without exposure to OTC. The expression levels of these genes increased at first and then decreased during incubation; however, the peak time was slightly different (Figure 3). Thus, the five tested ARGs were identified as constitutively expressed genes, which were automatically expressed with the growth of bacteria regardless of resistant stress. However, the expression levels of these genes were significantly different. Expression of gene *ant2ia* was dominant, and its relative expression value was approximately 10

times that of genes *tet33* and *sull*, 1,000 times that of *tetV*, and 10^5 times that of *cml_e8*. Overall, genes *ant2ia*, *tet33*, and *sull* were more active in strain OTC-16 than genes *tetV* and *cml_e8*. The antibiotic resistance spectrum experiment in this work confirmed the existence and expression of these ARGs. Strain OTC-16 could grow well when facing streptomycin, tetracycline, oxytetracycline, sulfadimethazine, and sulfamethoxazole. However, the appearance of chloramphenicol inhibited cell proliferation of this strain, possibly because of the low expression of gene *cml_e8*.

Figure 4 indicates that the presence of OTC generally suppressed the expression of genes *cml_e8* and *tetV*, but significantly promoted the relative expression of genes *ant2ia*, *sull*, and *tet33* during early culture (0 to 48 h). The maximum upregulated expression occurred at 24 h for gene *tet33*, and 48 h for genes *ant2ia* and *sull*; however, they all declined sharply 72 h later. The expression of genes *ant2ia* and *sull* at 168 h in OTC treatments was even lower than that in the controls. Overall, OTC evidently up-regulated the expression of these ARGs, but it is a short-term effect. The up-regulation of genes *sull* and *ant2ia* by OTC (Figure 4) implied that the presence of OTC improved the resistance of strain OTC-16 to antibiotics encoded by these genes.

The hypothesis was tested by the experiment shown in Figure 5, in which the growth of *A. nicotianae* OTC-16 in different antibiotic treatments is shown. It was found that the cell growth of strain OTC-16 in the presence of streptomycin sulphate improved after the addition of OTC at a concentration of $100 \text{ mg} \cdot \text{L}^{-1}$. In contrast, the co-existence of sulfamethoxazole and OTC resulted in lower cell growth of strain OTC-16 than the individual antibiotic. However, the adverse effect of the co-existence

of sulfamethoxazole and OTC was alleviated at 36 h, which was supposed to be related to the up-regulation of *sulI* gene expression. Gao found that tetracycline stress can promote the transcriptional expression of tetracycline-related ARGs of *Shigella flexneri*¹⁷. Our study showed that the presence of OTC could also activate other ARGs of strain OTC-16 not related to OTC resistance, such as the sulphonamide resistance gene *sulI* and aminoglycoside resistance gene *ant2ia*. However, the resistance of the strain to these antibiotics is not completely improved, and depends on many factors, such as the type of antibiotics and duration.

The synchronised up-regulated expression of genes *tet33*, *sulI*, and *ant2ia* indicated that the three ARGs may have a synergistic regulation mechanism. Synergistic regulation refers to the process in which a series of transcription and translation systems of bacteria respond simultaneously to any kind of environmental pressure, such as antibiotics and heavy metals¹⁸. Through this mechanism, the addition of OTC can reduce the sensitivity of strain OTC-16 to other antibiotics. A similar phenomenon has been reported for heavy metals; for example, copper and zinc ions could coordinate the regulation of antibiotic resistance genes and enhance bacterial tolerance to chloramphenicol, tetracycline, thiomyacin, and other antibiotics¹⁹⁻²¹. In addition, similar fluctuation trends and the synchronous transcription phenomenon among the three ARGs suggested that they may have relatively close genetic loci or maybe regulated by the same or similar regulatory proteins. Analysing and mapping the gene drafts of strain OTC-16 showed that *ant2ia*, *sulI*, and *tet33* were all multiple copy genes, and these genes were closely connected in position and formed tandem

repeat *tet33-sulI- ant2ia* gene series located exclusively in plasmids 1 and 2. In addition, constant spacer regions of 4625 bp were observed between genes *tet33* and *sulI*, of 418 bp between genes *sulI* and *ant2ia*, and of 1492 bp between genes *ant2ia* and *tet33* (Figure 6). Based on the expression pattern and gene location, we speculated that these three genes may be in a resistance chain transmission vector, such as transposon and integron, and could be transferred horizontally²². Integrons are genetic entities that are defined by their ability to capture members of a large family of small mobile elements known as ‘gene cassettes’. Gene cassettes can be incorporated into a specific site of integrons by a site-specific recombinase encoded by the gene *intI*²³. To verify our speculation, the sequence of gene *intI* was examined in the plasmids of strain OTC-16. Finally, a total of 10 copies of gene *intI*, mostly with a constant sequence region (764 bp), were recognised. All genes were located adjacent to the ARGs in above mentioned cassettes; four were located in plasmid 1 and six in plasmid 2 (Figure 6). Integrons are a common genetic element involved in the development of multiple antibiotic resistance and play vital roles in improving the potential of bacteria to adapt to the surrounding environments^{24,25}. The survival of bacteria sometimes depends on their ability to capture integrons and transferability of the same plasmid²⁴. In this respect, integrons are products of the evolution of bacteria.

Plasmid-derived ARGs (*ant2ia*, *sulI*, and *tet33*) were greatly induced by external addition of OTC and had obviously higher expression activity compared with genes carried by chromosomes (*tetV*). The results are consistent with the report by Qin¹⁶, who claimed that genes associated with mobile genetic elements generally showed

higher levels of expression under antibiotic-treated conditions than those in chromosomes, based on analysing the transcriptomics of 12 multidrug-resistant *Acinetobacter baumannii*. The proposition was further verified by Liu²⁶, who conducted metagenome and macro-transcriptome analysis on sludge and found that ARGs with high transcription abundance are mainly related to plasmid-derived resistance genes. We proposed that the tetracycline-specific efflux pump gene *tet33* of the major facilitator super family (MFS) on the plasmids maybe an important genetic element for strain OTC-16 to resist OTC; however, its role in the degradation process of OTC still needs to be further verified. The inherent ARGs improve the resistance of the degrading strain to various antibiotics and enhance its adaptability to the environment when the host bacterium was applied as bioaugmentation/biodegradation agent. However, the presence of these genes also increases the risk of the expansion of antimicrobial resistance, especially those located in mobile genetic elements such as plasmids and integrons, just like genes *ant2ia*, *sull*, and *tet33* in this work. Actually, plasmid conjugation is one of the dominant mechanisms of horizontal gene transfer^{27,28}, contributing to the increasing resistance of indigenous bacteria. A recent study showed that a conjugative antibiotic resistance plasmid, RP4, harboured by the donor strain *Pseudomonas putida* KT2442 could be disseminated to bacteria affiliated with more than 15 phyla²⁹. Therefore, from this point of view, strain OTC-16 may currently pose a high ecological threat, mainly due to the resistance plasmids. However, despite the ecological risks, one cannot deny the significant application prospects of the strain. In contrast, the expression behaviour of the resistant genes in

this study provides some inspiration for genetic modification of the strain. Since most resistance genes are located on plasmids, a strategy capable of bypassing the development of resistance by shearing redundant resistance genes on plasmids and even directly eliminating plasmids could be adopted in the future to reduce the proliferation and spread of resistance genes in the environment.

Conclusions

Many ARGs unrelated to tetracycline degradation were identified in an efficient oxytetracycline/tetracycline-degrading bacterium *A. nicotianae* OTC-16, which make the strain resistant to at least seven categories, 15 antibiotics. Actively expressed genes, such as *ant2ia*, *sull*, and *tet33*, are mostly located in plasmids and are incorporated into integrons as ‘gene cassettes’ with obvious mobile characteristics. This study provides valuable information for improved and safe application of this strain and insights to reducing its ecological risk by genetic modification. These findings deepen our understanding of the ecological role and adaptive evolution of antibiotic-degrading microorganisms in the environment.

Materials and methods

Strain and medium

The OTC-degrading strain *A. nicotianae* OTC-16, used in this study, was previously isolated from active sludge around a pharmaceutical factory in Taizhou, Zhejiang province, China. Luria-Bertani (LB) medium (pH 7.2) containing 10 g · L⁻¹ tryptone, 5 g · L⁻¹ yeast extract, and 10 g · L⁻¹ NaCl, was used for incubation of the strain. The kits used in this study included the Axygen® AxyPrep Plasmid Miniprep

Kit, RNAiso plus Kit (Takara®Biochemicals, Hangzhou, China), TRANS DNA extraction kit (TransGen Biotech, Beijing, China), PrimeScript™ RT reagent Kit with gDNAEraser (Perfect Real Time, Takara Biochemicals, Hangzhou, China), MightyAmp for Real Time (TB Green Plus; Takara Biochemicals, Hangzhou, China), and Omega EZNATM soil DNA Kit (Omega Bio-Tek, Inc, USA).

Antimicrobial susceptibility testing

Susceptibility of the OTC-16 strain to drugs was determined using antibiotic discs (µg per disc, unless indicated). A single colony of strain OTC-16 was inoculated into LB liquid medium, and 80 µL of bacterial suspension was spread evenly on LB solid medium in petri dishes. Discs soaked with antibiotics were then placed in these plates. The inhibitory effects of antibiotics on strain OTC-16 were assessed after 24 h by measuring the diameter of the bacteriostatic circle of each disc. Absence of a bacteriostatic circle indicated that the strain was highly resistant to the target drug; appearance of an obvious bacteriostatic circle(diameter≥1.3 cm) indicated that the strain was sensitive to the antibiotic; and when the diameter of bacteriostatic circle was less than 1.3 cm, the strain was deemed to have medium-resistance to the drug. The experiment was performed in triplicate for each drug, with a total of 52 commonly used antibiotics tested. The antibiotics tested in this experiment were purchased from Solarbio (Beijing, China) with a purity≥99%.

Whole-genome sequencing

Arthrobacter nicotianae OTC-16 was cultivated aerobically at 30°C for 24 h in LB medium. DNA extraction was conducted using the TRANS DNA extraction kit

according to the manufacturer's protocol. The genome of *A. nicotianae* OTC-16 was sequenced using a PacBio RS II platform and Illumina HiSeq 4000 platform at the Beijing Genomics Institute (BGI, Shenzhen, China). The tRNAs and rRNAs were identified using tRNAscan-SE³⁰, RNAmmer³¹ and Rfam databases. The best hit was abstracted using the BLAST alignment tool for function annotation. Seven databases, including KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Cluster of Orthologous Groups of proteins), NR(Non-Redundant Protein Database), Swiss-Prot, GO (Gene Ontology), TrEMBL (Translation of European Molecular Biology Laboratory) and EggNOG (Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups) were used for general function annotation. Virulence factors and resistance genes were identified based on the core dataset in VFDB (the Virulence Factors of Pathogenic Bacteria) and CARD (the Comprehensive Antibiotic Research Database).

Quantitative polymerase chain reaction

Freshly cultured bacterial suspensions with OD_{600nm}= 1.8 were inoculated into treatment (LB broth spiked with 100 mg · L⁻¹ OTC, hereafter referred to as LB^R broth) and control (LB broth only) at a dose of 3 % (v/v) and then incubated at 30 °C for 7 d. The cultures were performed in triplicate, and the bacterial suspensions were sampled every 24 h. Total RNA was extracted using the RNAiso plus Kit. The obtained RNA was dissolved in ribonuclease-free water, and high-quality RNA with purity and concentration which met the PCR standard was saved and used in the subsequent experiments. Approximately 1µg of RNA was used for real-time PCR in

each sample. Complementary DNA (cDNA) was synthesised using a PrimeScript™ RT reagent Kit with a gDNAEraser, according to the manufacturer's instructions. Expression of five antibiotic resistance genes (*tetV*, *sul1*, *tet33*, *ant2ia*, and *cml_e8*) was tracked by real-time qRT-PCR with reference to the whole genome sequence of *A. nicotianae* strain OTC-16 (NZ_CP 033 081). The primers for the candidate genes were designed using the primer analysis software Primer 5.0, and the primer specific verification was performed on the National Center for Biotechnology Information (NCBI). The 16S rRNA V3 region (simply referred to as 16S rRNA) was used in the study as the reference gene for quantitative analysis. Detailed information on the primers is listed in Table 1.

Real-time qRT-PCR was performed with Mighty Amp™ for Realtime PCR Kit and conducted on an ABI StepOnePlus Real-Time PCR System (PerkinElmer Applied Biosystems, Foster City, CA, USA). The specificity of the amplified product was confirmed by the melting temperature. With 16S rRNA as the internal reference gene, the results of the qRT-PCR were normalised to obtain the relative value of resistance gene expression. The relative value of gene expression and the relative expression of resistance genes were calculated using the following two formulas:

$$\text{Relative value of gene expression} = \frac{N1}{N2} \quad (1)$$

$$\text{Relative expression of resistance gene} = \frac{VT}{VCK} \quad (2)$$

Where *N1* denotes the copy number of target gene, *N2* denotes the copy number of the 16S rRNA gene, *VT* denotes the gene expression value of treatment group genes, and *VCT* denotes the gene expression value of control group genes.

Response of cell growth to co-existence of multiple antibiotics

Freshly cultured bacterial suspensions with $OD_{600nm}=1.8$ were inoculated into 5 mL of LB^R broth at a 3% (v/v) dose. Streptomycin sulphate and sulfamethoxazole were selected as target antibiotics in this experiment at a final concentration of 100 mg · L⁻¹. Four groups were designed for each target antibiotic (TA): (1) TA + strain OTC-16; (2) TA +OTC +strain OTC-16; (3) OTC +strain OTC-16; and (4) strain OTC-16 only. The cultures were performed in triplicate, and the OD_{600nm} values of the bacterial suspension were recorded every 12 h.

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Author contributions

X.Z. experimental design, data analysis and wrote original draft. R.Z. wrote the manuscript, performed the majority of the experiments, and ran data analysis. H.L. conceived of the idea, experimental design and co-wrote the paper. W.L. assisted with antimicrobial susceptibility testing and interpreting the result. J.M. writing-review and editing.

Competing interests

The authors declare no competing interests.

Figure Legends

Fig. 1 Circular map of the chromosome (a), plasmid1 (b) and plasmid2 (c) of strain OTC-16. From inner to outer: Genome size; Forward Strand Gene(colored by COG classification); Reverse Strand Gene(colored by COG classification); Forward Strand ncRNA; Reverse Strand ncRNA; Repeat gene; G+C content; GC-SKEW(calculated as $(G-C)/(G+C)$); green/purple peaks out/inside the circle indicate values higher or lower than 1, respectively.

Fig. 2 KEGG function annotation distribution map of strain OTC-16.

Fig. 3 Relative abundance of resistance gene expression at 24-168 h.

Fig. 4 The expression levels of resistance genes *tet33*, *sul1*, and *ant2ia* changed at 24-168 h.

Fig. 5 Effect of streptomycin sulfate(a) and sulfamethoxazole(b) on the growth of strain OTC-16.

Fig. 6 The sites of gene *sul1*, *tet33* and *ant2ia* on plasmid 1 and plasmid 2.

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Table 1. Primers and annealing temperatures for qPCR assays

Primer	primer sequence (5'-3')	T _m (°C)
<i>tet33-F</i>	TCCCGCACTGCTACACGA	55
<i>tet33-R</i>	GCGAGATGGCACCGAACA	
<i>cml_e8-F</i>	CGTGCCAGCGAACCAGAAG	55
<i>cml_e8-R</i>	TGGGTGCCAGGAAGGTGAAT	
<i>tetV-F</i>	GCACTCGTCTTCTCCACCTT	55
<i>tetV-R</i>	GGTTCCTCCAGCCCATTAG	
<i>ant2ia-F</i>	CATCCCGTGGCGTTATCC	55
<i>ant2ia-R</i>	CTGGGCAGGTAGGCGTTT	
<i>sul1-F</i>	CGCACCGGAAACATCGCTGCAC	65
<i>sul1-R</i>	TGAAGTTCCGCCGCAAGGCTCG	
<i>16S rRNA-F</i>	CCTACGGGAGGCAGCAG	62
<i>16S rRNA-R</i>	ATTACCGCGGCTGCTGG	

Table 2. Inhibitory effect of tested drugs on growth of strain OTC-16

Antibiotic categories	Antibiotics (µg per disc)	Test effect	Antibiotic categories	Antibiotics (µg per disc)	Test effect
Tetracyclines	Chlorotetracycline(30)	+ -	Polypeptide	Polymyxin(300)	+-
	Doxycycline(30)	+		Bacitracin(0.04)	-
	Oxytetracycline(30)	-		Vancomycin(30)	+-
	Minocycline(30)	+	Polyene		
	Tetracycline(30)	-		Nystatin(100)	-
Sulfonamides	Sulfadimethoxine(100)	-	Quinolones	Ciprofloxacin(5)	+
	Sulfamethoxazole(30)	-		Ciprofloxacin(5)	+
	Sulfadimethazine(300)	-		Ofloxacin(5)	+
	Sulfamonomethoxine (30)	-		Enoxacin(10)	+
	Cotrimoxazole(23.75)	-		Enrofloxacin(10)	+
Macrolides	Erythromycin(15)	+	Lincosamides	Norfloxacin(10)	+
	Midecamycin(30)	+		Nalidixic acid(30)	-
Aminoglycosides	Gentamicin(10)	+	Penicillins	Clindamycin(2)	+
	Amikacin(30)	+		Lincomycin(20)	+-
	Tobramycin(10)	+-		Piperacillin(100)	+
	Neomycin(30)	+		Ampicillin(20)	+
	Kanamycin(30)	+-	Chloramphenicols	Amoxicillin(20)	+
	Kanamycin sulfate(30)	+-		Penicillin(20)	+
	Streptomycin(10)	-	Nitrofurans	Chloramphenicol(30)	+-
	Streptomycin sulphate(10)	-		Macroclantin(300)	+-
			Glycopeptides	Furazolidone(300)	-
Cephalosporin	Cefotaxime(30)	+		Vancomycin(30)	+
	Aventiamycin IV(30)	+	Others	Rifampicin(5)	+
	Cefalexin(30)	+			
	Cefazolin(30)	+		Isopropyl-b-D-thiogalactoside(0.01g/ml)	-
	Ceftriaxone(30)	+		Metronidazole(5)	-
	Mefoxin(30)	+			
	Cefoperazone(75)	+			

Note: +: Strain OTC-16 is high sensitive to the drugs (Diameter of bacteriostatic circle ≥ 1.3 cm); -: strain OTC-16 is high resistant to the drugs (No bacteriostatic circles were observed); +-: strain OTC-16 is medium resistant to the drugs (Diameter of bacteriostatic circle < 1.3 cm).

515 **Table 3.** ARGs information of strain OTC-16

Location	Gene_id	Subject_id	Gene_type	Antibiotic_resistance	Original_Resistance_Type
Chromosome	ZAF-05GL000241	ardb_2199	<i>tetv</i>	tetracycline	tet_efflux
Chromosome	ZAF-05GL000615	ardb_115	<i>vanre</i>	vancomycin	vane
Chromosome	ZAF-05GL000913	ardb_1951	<i>cml_e6</i>	chloramphenicol	cml
Chromosome	ZAF-05GL001175	ardb_2604	<i>cara</i>	lincosamide,streptogram macrolide	mls_abc
Chromosome	ZAF-05GL001392	ardb_2322	<i>bcra</i>	bacitracin	bcr
Chromosome	ZAF-05GL001549	ardb_2778	<i>catb3</i>	chloramphenicol	catb
Chromosome	ZAF-05GL002124	ardb_2395	<i>vanrc</i>	vancomycin	vanc
Chromosome	ZAF-05GL003407	ardb_2606	<i>bcra</i>	bacitracin	bcr
Plasmid1	ZAF-05GL003435	ardb_598	<i>tet33</i>	tetracycline	tet_efflux
Plasmid1	ZAF-05GL003441	ardb_571	<i>sul1</i>	sulfonamide	sul
Plasmid1	ZAF-05GL003442	ardb_2203	<i>ant2ia</i>	tobramycin,kanamycin, sisomicin,dibekacin, gentamicin	ant
Plasmid1	ZAF-05GL003445	ardb_598	<i>tet33</i>	tetracycline	tet_efflux
Plasmid1	ZAF-05GL003450	ardb_571	<i>sul1</i>	sulfonamide	sul
Plasmid1	ZAF-05GL003451	ardb_2203	<i>ant2ia</i>	tobramycin,kanamycin, sisomicin,dibekacin, gentamicin	ant
Plasmid1	ZAF-05GL003527	ardb_1888	<i>cml_e8</i>	chloramphenicol	cml
Plasmid2	ZAF-05GL003540	ardb_598	<i>tet33</i>	tetracycline	tet_efflux
Plasmid2	ZAF-05GL003544	ardb_571	<i>sul1</i>	sulfonamide	sul
Plasmid2	ZAF-05GL003545	ardb_2200	<i>ant2ia</i>	tobramycin,kanamycin, sisomicin,dibekacin, gentamicin	ant
Plasmid2	ZAF-05GL003548	ardb_598	<i>tet33</i>	tetracycline	tet_efflux
Plasmid2	ZAF-05GL003552	ardb_571	<i>sul1</i>	sulfonamide	sul
Plasmid2	ZAF-05GL003553	ardb_2200	<i>ant2ia</i>	tobramycin,kanamycin, sisomicin,dibekacin, gentamicin	ant
Plasmid2	ZAF-05GL003556	ardb_598	<i>tet33</i>	tetracycline	tet_efflux
Plasmid2	ZAF-05GL003560	ardb_571	<i>sul1</i>	sulfonamide	sul

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Figures

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KEGG Pathway Classification

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