



Efficient degradation of ivermectin by newly isolated *Aeromonas taiwanensis* ZJB-18,044

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Abstract Ivermectin (IVM) is a widely used antiparasitic agent and acaricide. Despite its high efficiency against nematodes and arthropods, IVM may pose a threat to the environment due to its ecotoxicity. In this study, degradation of IVM by a newly isolated bacterium *Aeromonas taiwanensis* ZJB-18,044 was investigated. Strain ZJB-18,044 can completely degrade 50 mg/L IVM in 5 d with a biodegradation ability of 0.42 mg/L/h. Meanwhile, it exhibited high tolerance (50 mg/L) to doramectin, emamectin,

rifampicin, and spiramycin. It can also efficiently degrade doramectin, emamectin, and spiramycin. The IVM degradation of strain ZJB-18,044 can be inhibited by erythromycin, azithromycin, spiramycin or rifampicin. However, supplement of carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation, can partially recover the IVM degradation. Moreover, strain ZJB-18,044 cells can pump out excess IVM to maintain a low intracellular IVM concentration. Therefore, the IVM tolerance of strain ZJB-18,044 may be due to the regulation of the intracellular IVM concentration by the activated macrolide efflux pump(s). With the high IVM degradation efficiency, *A. taiwanensis* ZJB-18,044 may

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serve as a bioremediation agent for IVM and other macrolides in the environment.

Keywords *Aeromonas taiwanensis* · Biodegradation · Efflux pump · Ivermectin · Tolerance

Abbreviations

IVM	Ivermectin
AVM	Avermectin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
CCCP	Carbonyl cyanide <i>m</i> -chlorophenylhydrazone
MSM	Mineral salts medium
LB medium	Luria–Bertani medium
MH medium	Mueller–Hinton medium
RNA	Ribonucleic acid
NCBI	The National Center for Biotechnology Information
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography mass spectrometry
DO	Dissolved oxygen
OD ₆₀₀	Optical density at 600 nm
MATE	Major facilitator superfamily
RND	Resistance-nodulation-division
ANOVA	Analysis of variance

Introduction

As a semi-synthetic insecticide, ivermectin (IVM) is a 22,23-dihydro derivative of avermectin (AVM) from *Streptomyces avermitilis* (Campbell et al. 1983; Chabala et al. 1980; Gonzalez Canga et al. 2009). IVM is widely used in agriculture, aquaculture, animal husbandry and pharmaceutical industries as an efficient broad-spectrum antiparasitic agent and acaricide

against nematodes and arthropods (Gonzalez Canga et al. 2009; Poul 1988). In addition to the treatment and control of parasitic infections of domestic animals (Uhlir and Volf 1992), IVM is also approved to treat human onchocerciasis (Aziz et al. 1982), lymphatic filariasis (Fischer et al. 1997), streptocerciasis (Omura 2008), scabies and pediculosis (Yates et al. 2003). Moreover, IVM can reduce malaria transmission by killing *Anopheles mosquitoes* (Chaccour et al. 2013; Foy et al. 2011). Therapeutic effect of IVM on trichinosis (Basyoni and El-Sabaa 2013), leishmaniasis and trypanosomiasis (Abe and Bignell 2000), piroplasmiasis (Batiha et al. 2019) were also reported. Furthermore, IVM was found to be capable of killing termites (Mo et al. 2005), body lice (Sangare et al. 2016) and *Strongyloides stercoralis* (Buonfrate et al. 2019). Currently, studies on antiviral (such as SARS-CoV-2) (Caly et al. 2020; Yang et al. 2020), anti-tuberculosis (Miró-Canturri et al. 2019) and anti-cancer effects of IVM are underway (Intuyod et al. 2019; Juarez et al. 2018; Omura and Crump 2014). These findings indicate the promising future of IVM in human health (Crump 2017).

Currently, increased attention has been paid to the toxicity and non-target effects of AVM and its derivatives such as IVM in terrestrial and aquatic environments (Lumaret et al. 2012; Sanderson et al. 2007). In contrast to low toxicity documented for mammals (Olsen and Snowman 1985), IVM has been shown to be extremely toxic to bees (Costa et al. 2014), silkworm and aquatic organisms, such as *Brachydanio rerio* (Hamilton–Buchanan), *Daphnia magna straus*, and *Gambusia affinis* (Davies et al. 1997; Katharios et al. 2002; Li 2010; Mladineo et al. 2006). Verdú et al. found that low doses of IVM can cause sensory and locomotor disorders in dung beetles (Ishikawa and Iwasa 2020; Verdú et al. 2015). It was also reported that IVM could pass through the blood-brain barrier of the turtle, and cause serious consequences (Panayotova-Pencheva 2016). Therefore, the released IVM may be a threat to the aquatic environments.

Pharmacokinetic studies indicated that most of the IVM is excreted from feces and urine in their nonmetabolized form by mammals (Sutherland and Campbell 1990). Residual IVM is mainly adsorbed into the soil. In addition to affect plant growth, IVM can also enter the plant through the plant roots, thereby endangering human health (Kumar et al.

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2005). Unlike most of other macrocyclic antibiotics (Masse et al. 2014), IVM is mainly degraded by photodegradation or microorganisms in terrestrial environments (Halley et al. 1993). However, adsorption of IVM to soil or feces significantly delayed its degradation (Davies et al. 1998; Floate et al. 2005; Wang et al. 2019; Wu et al. 2012). For example, over 70% of IVM remained after degradation for 70 d in the sediment of a simulated river way environment (Wu et al. 2012) and a half-life over 100 d of IVM was observed in marine sediment (Davies et al. 1998).

Currently, bioremediation has become one of the most promising tools to remove antibiotics and other pollutants in the environment (Alekseeva et al. 2011; He et al. 2014; Kumar et al. 2019; Pande et al. 2020). However, reports on biological degradation of IVM are rare (Hao 2009). In our previous research, a bacterium *Stenotrophomonas maltophilia* ZJB-14,120 was found to be capable of efficiently degrading abamectin, emamectin, erythromycin and spiramycin except IVM (Wang et al. 2015). Therefore, it is of interests to carry out researches on microbial degradation of IVM. Here we report the biological degradation of IVM by a macrolide-tolerant bacterium *Aeromonas taiwanensis* ZJB-18,044. *A. taiwanensis* ZJB-18,044 may serve as a biodegradation agent for IVM and other macrolides due to its high degradation efficiency.

Materials and methods

Materials

IVM (98%) was provided by Hebei Meihe Pharmaceutical Co., Ltd. (China). Avermectin (97%) and emamectin benzoate (73%) were gifts from Zhejiang Qianjiang Biochemical Co., Ltd. (China). Erythromycin (95%), spiramycin (90%) and rifampicin (98%) were purchased from J&K Chemical Ltd. (China). Doramectin (98%) and azithromycin dehydrate (98%) were purchased from Aladdin Chemical Reagent Co., Ltd. (China). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, reagent for qualitatively assaying the proton motive force-dependent pump) (Nagano and Nikaido 2009) was purchased from J&K Chemical Ltd. (China). Acetonitrile, methanol, dichloromethane and other chemicals were of analytical grade and commercially available.

Microbial enrichment, isolation and IVM degradation assays were performed with the mineral salts medium (MSM, pH 7.0–7.5) as described by Ali et al. (2010). The enrichment medium is MSM supplemented with 200 mg/L to 800 mg/L IVM. For cell cultivation, LB medium was used. *Aeromonas taiwanensis* was maintained on LB agar (2.5% w/v) plates. Antibiotic tolerance was assayed with Mueller–Hinton (MH) medium purchased from Aladdin Chemical Reagent Co., Ltd. (China). The media were autoclaved at 121 °C for 20 min.

Soil samples were collected from surface layer (2–10 cm) soils in flower beds in campus of Zhejiang University of Technology and the ground of IVM-producing workshop in Hebei Meihe Pharmaceutical Co., Ltd. (China). Pig feces samples were provided by the Institute of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences. Soil samples were treated as previous research (Nagano and Nikaido 2009; Wang et al. 2015). Pig feces samples were fresh pig manure after feeding different doses of IVM.

Isolation and identification of IVM-degrading microorganisms

The IVM-degrading microorganisms were enriched by introducing 1 g of soil or fresh pig feces to 250 mL flasks with 50 mL of MSM containing 200 mg/L IVM, and then incubated at 30 °C and 150 rpm on a rotary shaker in the dark for 2 d. The resulting culture (3 mL) were sequentially transferred to fresh MSM with 400 mg/L, 600 mg/L and 800 mg/L IVM, and then incubated as described above. The enrichment culture was diluted and plated on LB agar plates containing 400 mg/L IVM, and then incubated at 30 °C in dark for 36 h. After three cycles, the resulting colonies were picked up and introduced into 250 mL flasks with 50 mL LB medium and incubated as aforementioned. Then 2 mL of bacterial suspension was transferred into MSM containing 50 mg/L IVM in 180 mm × 18 mm test tube with silicone caps and subsequently incubated in dark at 30 °C, 150 rpm for 60 h. The reaction mixture was ultrasonicated and centrifuged at 10,000×*g* and 4 °C for 10 min. Then the resulting supernatants were extracted with dichloromethane. The extracts were dehydrated with anhydrous Na₂SO₄, supplemented with acetonitrile to the original volume and then examined by HPLC analysis

for IVM degradation ability. Strains capable of degrading over 65% of 50 mg/L IVM were primarily characterized with 16 S rRNA gene analysis. The 16S rRNA gene of the strains was amplified using the universal primer set 27f (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1492r (5'-TACGGY-TACCTTGTTACGACTT-3') (Lane 1991), and sequenced by TSINGKE Biotech (Hangzhou) Co., Ltd (China). Homology analysis was performed with 16S rRNA sequences in NCBI by Blast software. The multiple sequence alignment was analyzed by BioXM 2.6 (Thompson et al. 1994). With the highest degradation ability, strain ZJB-18,044 was selected for further study. Morphological characterization of strain ZJB-18,044 was performed after 24 h incubation on LB agar plate. Cell morphology was observed with Olympus CH20 light microscope (Olympus Microsystems, Japan). Physiological and biochemical characterization were carried out with a GEN III microplate and analyzed by a MicroStation (Biolog, USA). A phylogenetic tree based on the 16S rRNA gene sequences of strain ZJB-18,044 and closely related strains was constructed by the Neighbor-Joining method using MEGA7. The evolutionary distances were calculated with the Maximum Composite Likelihood method.

IVM biodegradation experiments

Unless otherwise mentioned, the biodegradation of IVM by strain ZJB-18,044 was carried out in 180 mm × 18 mm test tubes with silicone caps. To evaluate the effect of growing medium on IVM degradation, strain ZJB-18,044 was grown in 5 mL LB medium or 5 mL MSM containing 20 mg/L IVM for 12 h at 30 °C and 150 rpm. Then 2 mL of the resulting LB culture and MSM culture were introduced into 3 mL MSM and 3 mL LB medium (all supplemented with IVM to final concentration of 50 mg/L) respectively, and then cultivated for 72 h in the dark at 30 °C and 150 rpm. The culture broth was ultrasonicated and extracted as described above, then analyzed with HPLC.

Resting cells were prepared from the LB culture by centrifugation at 10,000 × *g* and 4 °C for 10 min. The resulting cells were washed once with an equal volume of sterile 0.2 M phosphate buffer (pH 7.5), and then resuspended in the same buffer. IVM tolerance and utilization of growing cells was carried out by

introducing 2 mL LB culture into 3 mL MSM supplemented with IVM to final concentration of 10 mg/L, 30 mg/L, 50 mg/L, 80 mg/L, 100 mg/L, 150 mg/L and 200 mg/L IVM. As for IVM tolerance and utilization of the resting cells, 2 mL resting cells suspension was used.

Degradation of IVM by strain ZJB-18,044 was further examined in 5 L fermenter (BIOTECH, China). LB culture of strain ZJB-18,044 cultivated in 500 mL flasks containing 100 mL LB at 30 °C and 150 rpm for 12 h were pooled and introduced into the 5 L fermenter (with 2.7 L MSM supplemented with IVM to final concentration of 50 mg/L or 100 mg/L) at a 10% (V/V) inoculation size. The initial incubation conditions were 30 °C, 200 rpm, aeration rate 2.0 L/(L min), and pH 7.5. The dissolved oxygen (DO) was maintained above 30% throughout the fermentation and samples were withdrawn every 12 h.

The utilization and tolerance of strain ZJB-18,044 towards avermectin (16-membered-ring macrolide), doramectin (16-membered-ring macrolide), emamectin (16-membered-ring macrolide), erythromycin (14-membered-ring macrolide), azithromycin (15-membered-ring macrolide), spiramycin (16-membered-ring macrolide) or rifampicin (ansa macrolide) were investigated by incubating the strain in MSM or MH medium separately containing the macrolides in the dark at 30 °C and 150 rpm for 72 h. Then the biomass and macrolides concentrations were determined.

To determine whether the aforementioned macrolides can induce the IVM degradation, IVM (final concentration 50 mg/L) was introduced into 48 h cultures of strain ZJB-18,044 in LB or LB separately supplemented with the macrolides, and then further incubated for 72 h.

The IVM degradation metabolites of strain ZJB-18,044 was determined by HPLC-MS analysis of the IVM degradation products of growing cells. The 4 h to 32 h culture broth of strain ZJB-18,044 in MSM with 50 mg/L IVM was ultrasonicated and centrifuged at 10,000 × *g* and 4 °C for 10 min. Then the resulting supernatants were extracted with dichloromethane for IVM and corresponding metabolites. The HPLC-MS was performed with a Thermo Scientific LTQ XL mass analyzer attached to a Dionex UltiMate 3000 system equipped with a Hypersil gold C18 column (100 mm × 2.1 mm × 3 μm). Sample (20 μL) was loaded. The elution method is 8 min 2%–100% acetonitrile, 5 min 100%–2% acetonitrile, 2 min 2%

acetonitrile in total 15 min. The flow rate of the mobile phase was 0.6 mL/min.

The IVM-tolerant mechanism of strain ZJB-18,044 was investigated in 3 L MSM medium supplemented with 50 mg/L, 100 mg/L and 200 mg/L IVM within 5 L fermenter and incubated as described above. Samples were withdrawn at 0, 6, 12, 24, 36, 48, 60, 72, 96 and 120 h, and then centrifuged at $10,000\times g$ and 4 °C for 10 min. The harvested cells were resuspended and disrupted for determination of intracellular IVM as previous research (Wang et al. 2015).

Analytical methods

Biomass was assayed by measuring the optical density at 600 nm (OD_{600}). Concentration of AVM and its derivatives (IVM, doramectin and emamectin) and other macrolide antibiotics was determined by Agilent HPLC-1260 Infinity (Agilent, USA) using a Unitary C18 column (250 mm \times 4.6 mm \times 5 μ m) (Acchrom, China) as previous research (Wang et al. 2015). Briefly, AVM, IVM, doramectin and emamectin were monitored at 245 nm with acetonitrile-water (90:10, v/v) at 2.0 mL/min. Other macrolides were monitored at 215 nm with K_2HPO_4 buffer (0.05 M, pH 8.2)-acetonitrile (50:50, V/V) at 1.0 mL/min. The data was analyzed with analysis of variance (ANOVA) statistics with SPSS (IBM, USA).

Results

Isolation and identification of IVM-degrading microorganisms

Among 157 different single colonies obtained from enrichment and streaking, 12 bacterial strains capable of efficiently degrading IVM (over 65% degradation) were isolated and primarily identified based on 16 S rRNA gene analysis (Table 1). Strain ZJB-18,044, a bacterium with the highest IVM degradation (75.3%), was isolated from a pig feces sample. The strain was a Gram negative, motile and rod-shaped bacterium. The morphologies of the colonies were round, raised, pale yellow and translucent on LB agar plates. The physical and biochemical characteristics of strain ZJB-18,044 (Table 2) suggested that the isolate was *Aeromonas caviae* (identity 0.52). Homology analysis of the partial nucleotide sequence of 16S rRNA gene

(1413 bp, GenBank accession number: MK359642) showed that strain ZJB-18,044 had similarity above 99% with *Aeromonas taiwanensis* strain A2-50 (GenBank accession number: NR116585, 99.72%) and *Aeromonas caviae* strain W20 (GenBank accession number: KC840846, 99.65%). The phylogenetic tree constructed based on the 16S rRNA gene sequences of strain ZJB-18,044 and closely related strains is shown in Fig. 1. According to the phylogenetic tree, strain ZJB-18,044 is closest to *Aeromonas taiwanensis* strain A2-50, which is different with physiological data from the Biolog system. However, there is no data of *Aeromonas taiwanensis* in the Database of Biolog system (<https://www.biolog.com/wp-content/uploads/2020/04/00A-005rC-Biolog-Database-Book.pdf>). Therefore, strain ZJB-18,044 was identified as *Aeromonas taiwanensis*.

Effects of temperature (20–35 °C) and initial pH (pH 4–10) on IVM degradation by *A. taiwanensis* strain ZJB-18,044 were investigated. As depicted in Fig. 2, from 28 to 35 °C, IVM degradation reached above 65%. And as depicted in Fig. 3, from pH 7 to 9, IVM degradation reached above 69%. Therefore, the optimum temperature and initial pH were 28 °C and 7.5, respectively (Figs. 2 and 3). The results showed that ZJB-18,044 could degrade IVM in a temperature range of 28 °C to 35 °C and an alkaline environment.

Degradation and tolerance towards IVM of *A. taiwanensis* strain ZJB-18,044

Effects of IVM concentrations on the growth and IVM degradation of strain ZJB-18,044 were investigated by incubating the growing cells (Fig. 4a) and resting cells (Fig. 4b) in MSM supplemented with 10–200 mg/L IVM for 3 d. The data showed that the strain could grow under all tested IVM concentrations. The biomass increased from 10 mg/L IVM to 50 mg/L IVM, and then dropped at higher IVM concentrations. No significant changes in the biomass were observed in the resting cell treatments. As for IVM degradation, the IVM degradation of the resting cells (the highest IVM degradation (11.3 mg/L) was obtained under 100 mg/L IVM) was much lower than that of the growing cells (over 35 mg/L IVM was degraded under 50–200 mg/L IVM) (Fig. 4). This may due to the requirement of coenzymes for the biodegradation,

Table 1 Microorganisms with IVM degradation ability and their IVM degradation rate

Serial number	Identification	Degradation rate (%) ^a
H15	<i>Bacillus cereus</i>	66.1 ± 3.5
H36	<i>Bacillus subtilis</i>	72.1 ± 5.2
I13	<i>Comamonas testosteroni</i>	70.1 ± 4.6
M19	<i>Sphingobacterium multivorum</i>	69.2 ± 3.2
S22	<i>Acinetobacter bouvetii</i>	72.2 ± 6.1
S31	<i>Spingobacterium psychroaquaticum</i>	68.9 ± 5.5
U25	<i>Xanthomonas</i> sp.	65.9 ± 4.7
S32	<i>Acinetobacter calcoaceticus</i>	71.2 ± 4.6
S34	<i>Pseudomonas straminea</i>	70.1 ± 5.4
U11	<i>Stenotrophomonas maltophilia</i>	68.6 ± 6.2
S27	<i>Escherichia coli</i>	67.9 ± 3.7
M6	<i>Aeromonas taiwanensis</i>	75.3 ± 4.3

^aDegradation rate: IVM degradation of microorganisms grown in MSM medium containing 50 mg/L IVM was incubated at 30 °C and 150 rpm in the dark for 3d. The value is the mean ± SD of triplicates

which can be produced by the growing cells, whereas the coenzymes are limited in the resting cells. Thus, the growing cells were used for subsequent experiments.

In the treatments of pre-culturing in 50 mg/L IVM containing LB and MSM, the OD₆₀₀ was 1.28 and 1.02, respectively. Regardless of the difference in biomass, there was no significant difference in IVM degradation in the two treatments (75.3% in the treatment of IVM containing LB and 75.2% in the treatment of IVM containing MSM, respectively).

Large scale degradation of IVM by *A. taiwanensis* strain ZJB-18,044 was further investigated in a 5 L fermenter (Fig. 5). Strain ZJB-18,044 can completely degrade 50 mg/L IVM in 5 d with OD₆₀₀ value 1.93 (Fig. 5a). The strain also can degrade 98.92% of 100 mg/L IVM in 10 d with OD₆₀₀ value 2.61 (Fig. 5b).

Degradation of other macrolides by *A. taiwanensis* strain ZJB-18,044

Among the tested macrolides, strain ZJB-18,044 can grow well in MSM containing emamectin, doramectin or spiramycin. It grew poorly in MSM containing AVM and could not grow in MSM containing azithromycin, erythromycin or rifampicin. This was consistent with its degradation towards all tested macrolides (Fig. 6). Furthermore, it could grow in MH medium supplemented with the tested macrolides except for azithromycin and erythromycin (Table 3).

Effect of other macrolides on IVM degradation by *A. taiwanensis* strain ZJB-18,044

Effect of other tested macrolides on IVM degradation by the strain was investigated by supplementing IVM (final concentration of 50 mg/L) to the 48 h culture grown in LB or LB separately containing the antibiotics described. As depicted in Fig. 6, strain ZJB-18,044 almost completely degraded 50 mg/L IVM in 5 d in LB medium and LB medium pre-cultured with IVM, AVM, doramectin and emamectin. No significant IVM degradation was observed when the strain was pre-cultured in LB medium with erythromycin, azithromycin, spiramycin and rifampicin (Fig. 7). However, the IVM degradation in these treatments can be recovered with the addition of CCCP (Fig. 7).

Identification of IVM degradation metabolites

HPLC-MS analysis revealed that there were two main metabolites with retention times at 3.96 and 4.82 min among the IVM degradation metabolites. By comparison, two IVM degradation metabolites can be presumed from the mass spectrum corresponding to the retention time at 3.96 min (*m/z* 750.0, Metabolite A) and 4.82 min (*m/z* 150.0, Metabolite B) (Fig. S1). The IVM degradation metabolites of strain ZJB-18,044 were proposed in Fig. S2.

Table 2 Physiological and biochemical characteristics of strain ZJB18044

Characteristic	Results	Characteristic	Results
Lincomycin	+	Gelatin	B
Guanidine HCl	+	Nalidixic acid	–
Dextrin	+	Glycyl-L-prolin	+
D-Maltose	+	L-Alanine	+
D-Trehalose	+	1% NaCl	+
D-Cellobiose	+	L-Glutamic acid	+
Gentiobiose	–	L-Histidine	+
Sucrose	+	L-Pyrogutamic acid	–
D-Turanose	–	Lithium chloride	+
Stachyose	–	Pectin	–
D-Raffinose	–	D-Galacturonic acid	–
α -D-Lactose	–	L-Galactonic acid lactone	–
D-Melibiose	–	D-Gluconic acid	+
β -Methyl-D-glucoside	+	Troleandomycin	+
D-Salicin	+	Glucuronamide	–
<i>N</i> -Acetyl-D-glucosamine	+	Mucic acid	–
<i>N</i> -Acetyl-D-galactosamine	–	Quinic acid	–
<i>N</i> -Acetyl neuraminic acid	–	D-Saccharica acid	–
α -D-Glucose	–	<i>p</i> -Hydroxy-phenylacetic acid	–
D-Mannose	–	Methyl pyruvate	–
D-Fructose	–	D-Lactic acid methyl ester	–
D-Galactose	+	L-Lactic acid	+
3-Methyl glucose	–	Citric acid	–
D-Fucose	–	α -Keto-glutaric acid	–
1% Sodium lactate	+	D-Malic acid	–
L-Rhamnose	–	L-Malic acid	+
Inosine	+	Bromo-succinic acid	+
D-Sorbitol	–	Tween 40	–
D-Mannitol	–	γ -Amino-butryric acid	–
D-Arabitol	–	α -Hydroxy-butryric acid	+
myo-Inositol	–	β -Hydroxy-D,L butyric acid	–
Glycerol	+	α -Keto-butryric acid	+
D-Glucose-6-PO ₄	–	Acetoacetic acid	+
D-Fructose-6-PO ₄	+	Propionic acid	+
D-Aspartic acid	–	Sodium bromate	–
Minocycline	–	Rifamycin SV	+
D-Serine	+	Formic Acid	+

+ positive, – negative, B borderline

IVM tolerance mechanism of strain ZJB-18,044

To investigate the IVM tolerance mechanism of strain ZJB-18,044, extracellular and intracellular IVM concentration (C_e and C_i , respectively) of samples from the culture under different IVM concentrations in 5 L fermenter were analyzed. As depicted in Fig. 7, the

IVM degradation process included 3 steps. In Step one (0–6 h), C_e in all treatments decreased rapidly with a simultaneously increase in C_i (Fig. 8a–d). In Step two (6–12 h), C_i in treatment A, B and C all decreased while C_e increased, which may be due to the expel out of IVM by the active efflux (Fig. 8a–c). As to treatment D, C_e and C_i only changed slightly

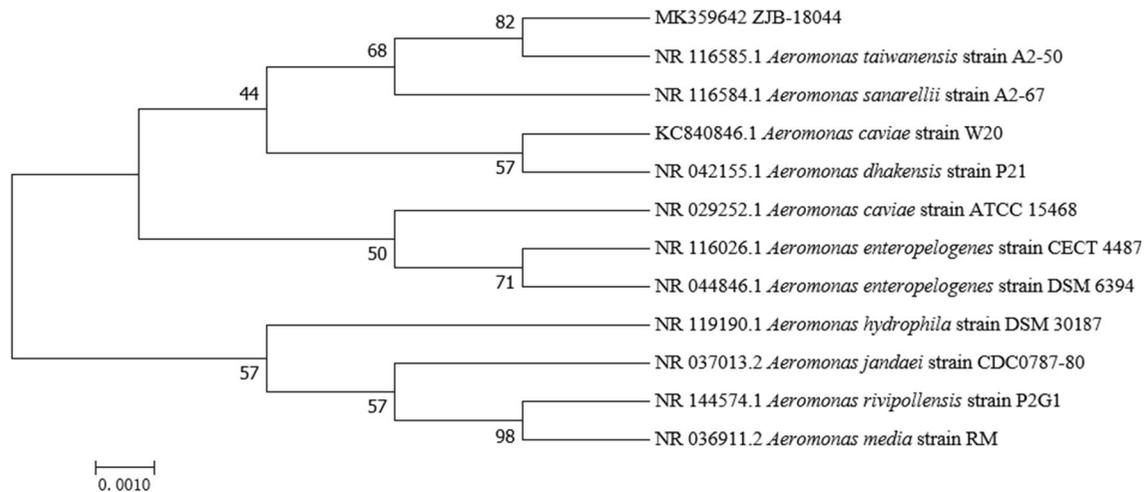


Fig. 1 Phylogenetic tree constructed with the Neighbor-Joining method based on the 16S rRNA gene sequences of strain ZJB-18,044 and closely related strains. Numbers presented in front of the strains are GenBank accession numbers of the sequences.

Evolutionary distances were computed with Maximum Composite Likelihood method. The scale bar represents 0.001 substitutions per nucleotide position. Evolutionary analysis was conducted with MEGA7

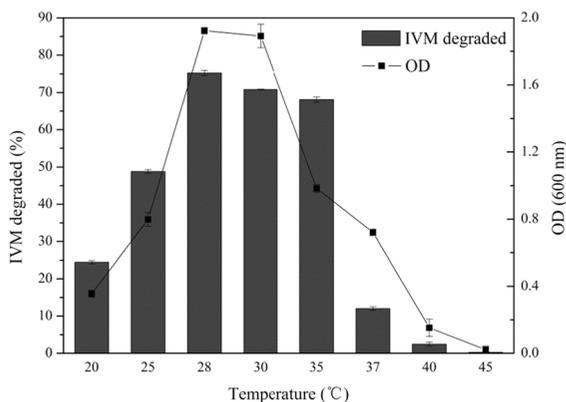


Fig. 2 Effect of temperature on growth and IVM degradation of *A. taiwanensis* strain ZJB-18,044 grown in MSM medium. The strain was incubated at initial pH 7.0 and 150 rpm in the dark for 3 d. Error bars: mean \pm SD of triplicates

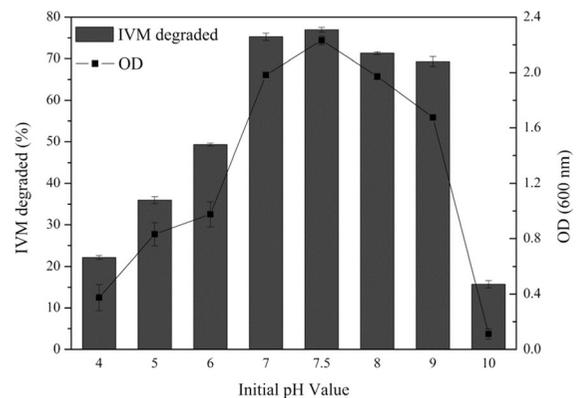


Fig. 3 Effect of initial pH value on growth and IVM degradation of *A. taiwanensis* strain ZJB-18,044 grown in MSM medium. The strain was incubated at 28 °C and 150 rpm in the dark for 3 d. Error bars: mean \pm SD of triplicates

(Fig. 8d), which may result from the inactivation of the active efflux by CCCP which caused de-energization of the cytoplasmic membrane. The total IVM concentrations in all treatments remained stable in these two steps. In the last step (12 h-120 h), no significant changes in C_i in all treatments was observed (Fig. 8a–d). However, C_i in treatment D was much higher than those in treatment A, B and C. Except for treatment D, C_e and total IVM in other treatments all decreased.

Discussion

As a potent and broad-spectrum antiparasitic agent and acaricide, IVM has been widely applied in various fields such as aquaculture and husbandry. However, in the husbandry most of the administered IVM was released in feces or urine to environment with its unmetabolized form. Either in water or firmly bound to the soil, IVM has a long elimination half-life (Floate et al. 2005; Wang et al. 2019; Wu et al. 2012). IVM has adverse effects not only on non-target

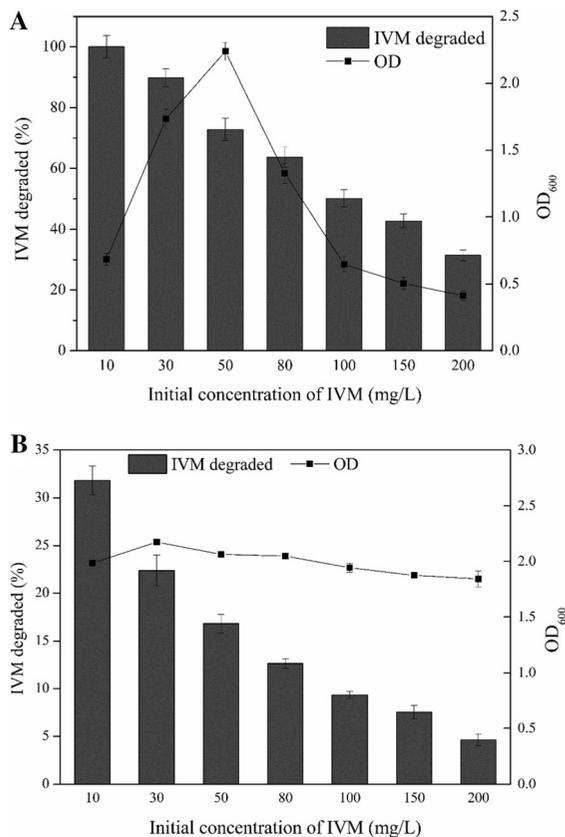


Fig. 4 Effect of IVM concentrations on growth and IVM degradation of *A. taiwanensis* strain ZJB-18,044 grown in MSM medium with growing cells (a) and resting cells (b). The strain was incubated at 28 °C and 150 rpm in the dark for 3 d. Error bars: mean \pm SD of triplicates

organisms such as terrestrial microbes and aquatic organisms, but also on other organisms through the food chain. That is, the releasing IVM may cause ecological problems (Floate et al. 2005; Lumaret and Errouissi 2002; Wang et al. 2019). Therefore, investigation on bio-degradation of IVM is of great importance.

In the present research, *A. taiwanensis* strain ZJB-18,044, a bacterium with potent IVM degradation, was isolated and characterized. The data revealed that this strain is a macrolides tolerant bacterium and capable of degrading some macrolides such as IVM, doramectin and emamectin. The strain can efficiently degrade IVM at a temperature from 28 to 35 °C and initial pH (pH 7–9). Furthermore, growing cells of strain ZJB-18,044 exhibited more potent IVM degradation ability than resting cells.

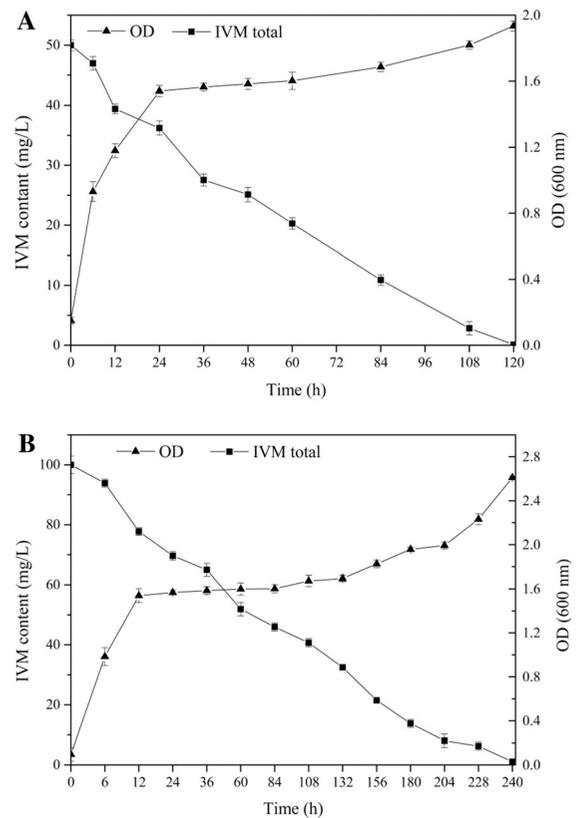


Fig. 5 Degradation of 50 mg/L (a) and 100 mg/L (b) IVM by growing cells of *A. taiwanensis* strain ZJB-18,044 in 5L fermenter. The reaction mixture was incubated at 30 °C and 150 rpm for 5 d or 10 d in MSM medium. Error bars: mean \pm SD of triplicates

Up to date, there is only one available report on microbial degradation of IVM (Hao 2009). Hao isolated a bacterial strain *Pseudomonas* sp. strain K1 from pig feces, which can degrade 15.7% of 40 mg/L IVM in 30 d at 30 °C and 150 rpm. Its average IVM degradation rate (8.72 μ g/L/h under 40 mg/L IVM) is 47.7 times lower than that of the strain ZJB-18,044 (0.416 mg/L/h under 50 mg/L IVM). Thus, strain ZJB-18,044 can serve as an efficient IVM degrading agent.

Strain ZJB-18,044 can efficiently degrade IVM but not AVM. This is contrary to *Stenotrophomonas maltophilia* ZJB-14,120 (Wang et al. 2015) and *Bacillus cereus* strain AVM1 (Li 2010), which can degrade AVM but not IVM. Among doramectin, emamectin and spiramycin, strain ZJB-18,044 displayed the highest degradation towards emamectin. Except *S. maltophilia* ZJB-14,120, reports on

microbial degradation of emamectin are rare (Wang et al. 2015). As to spiramycin, it is reported that *Aspergillus sclerotiorum* and *Microcystis aeruginosa* can degrade spiramycin (Liu et al. 2012a, b). Due to the lack of researches on doramectin, emamectin or spiramycin degradation by *A. taiwanensis*, it is important to elucidate macrolides-degrading enzymes/genes in *A. taiwanensis* strain ZJB-18,044.

HPLC-MS analysis of the IVM degradation products revealed that there are two metabolites (Fig. S1).

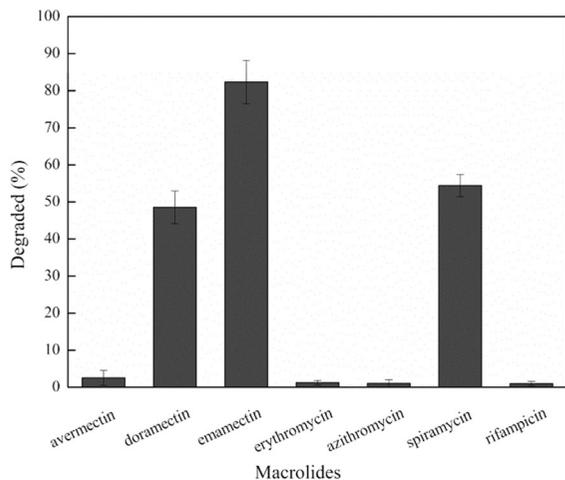


Fig. 6 Degradation of macrolides by growing cells of *A. taiwanensis* strain ZJB-18,044. The macrolides were separately introduced into the growing cell suspension to 50 mg/L, incubated at 28 °C and 150 rpm in the dark for 3 d. Error bars: mean \pm SD of triplicates

A primary biodegradation pathway for IVM of *A. taiwanensis* strain ZJB-18,044 was proposed (Fig. S2). As depicted in Fig. S2, the IVM biodegradation begins with the breakage of the C–O bond between C4 and O11, and forms metabolite A (the ion at m/z 750.0) and metabolite B (m/z 150.0). Up to date, the information on the mechanism of microbial degradation of IVM is

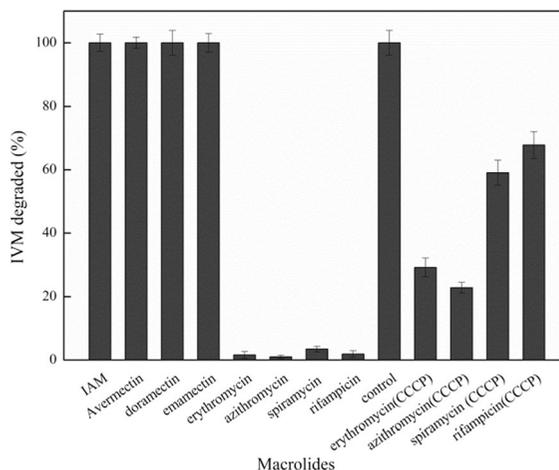


Fig. 7 Degradation of IVM by growing cells of *A. taiwanensis* strain ZJB-18,044 grown in LB medium supplemented with IVM, avermectin, doramectin, emamectin, erythromycin, azithromycin, spiramycin and rifampicin. The last 4 bars stand for the reaction mixture additionally supplemented with 8 mg/L CCCP. Cells cultivated in pure LB medium were set as controls. The reaction mixture containing 50 mg/L IVM was incubated at 28 °C and 150 rpm in the dark for 5 d. Error bars: mean \pm SD of triplicates

Table 3 Growth of *A. taiwanensis* Strain ZJB-18,044 in MSM and MH with different kinds of macrolides

Substrates	MSM ^a (OD _{600 nm})	MH ^b (OD _{600 nm})
Avermectin	0.0812 \pm 0.0015	1.2423 \pm 0.0312*
Doramectin	0.7285 \pm 0.0032**	1.0412 \pm 0.0507
Emamectin benzoate	0.7418 \pm 0.0011**	1.1466 \pm 0.0231*
Erythromycin	0.0429 \pm 0.0015	0.0483 \pm 0.0042
Azithromycin	0.0373 \pm 0.0047	0.0557 \pm 0.0095
Spiramycin	0.4973 \pm 0.0011*	1.1133 \pm 0.0523
Rifampicin	0.0302 \pm 0.0018	1.0289 \pm 0.0132*
Control	0.0461 \pm 0.0054	0.0492 \pm 0.0016

^a50 mg/L different macrolides were respectively added into MSM as sole carbon source, incubated at 30 °C and 150 rpm in the dark for 3 d. The value is the mean \pm SD of three replicates

^b100 mg/L different macrolides were respectively added into MH as antibiotic stress, incubated at 30 °C and 150 rpm in the dark for 3 d. The value is the mean \pm SD of triplicates

*Significant, ** very significant

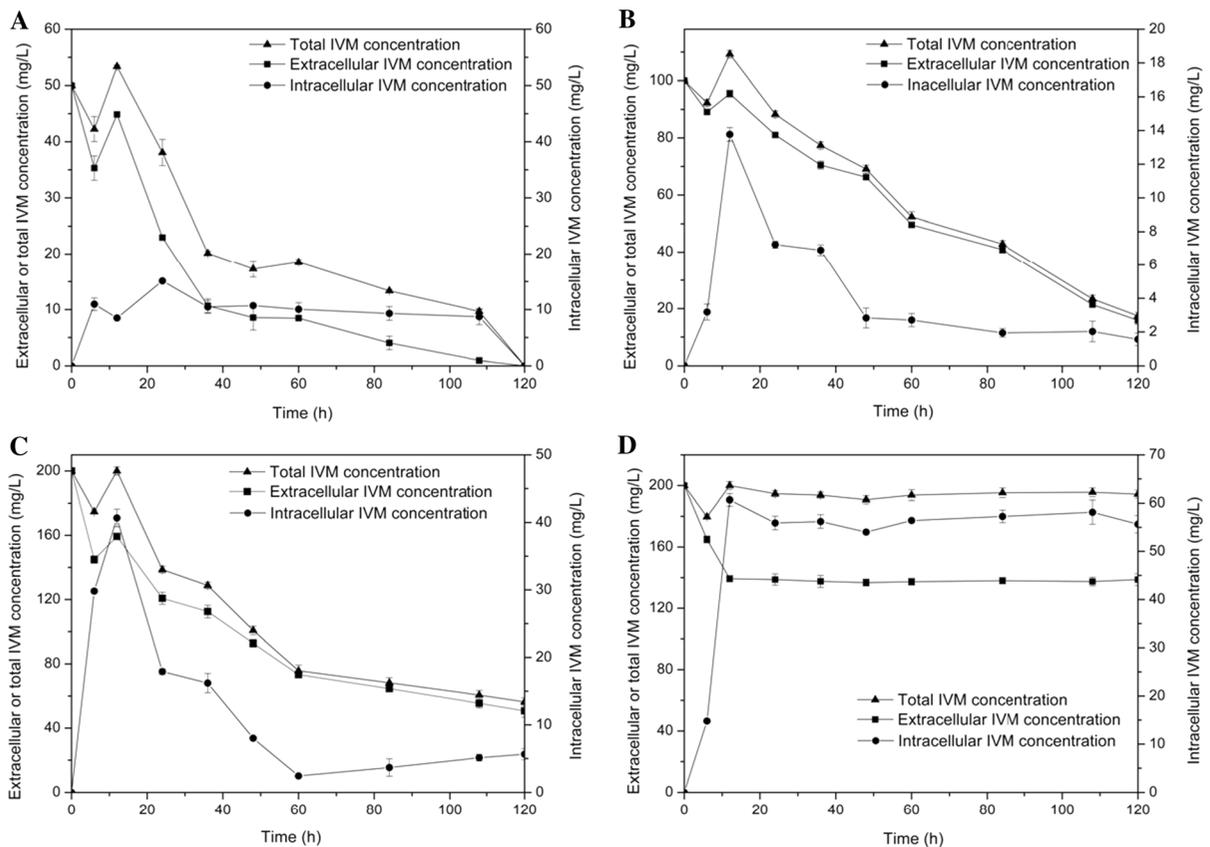


Fig. 8 Profiles of extracellular, intracellular IVM concentration (ν), total IVM concentration (π) and intracellular IVM content (λ) in *A. taiwanensis* strain ZJB-18,044 growing cells containing 50 mg/L IVM (a), 100 mg/L IVM (b), 200 mg/L IVM (c) and

200 mg/L IVM with 8 mg/L CCCP (d). The reaction mixture was incubated at 30 °C and 150 rpm in 5 L fermenter for 5 d. Error bars: mean \pm SD of triplicates

quite rare. These findings provided a primary understanding on microbial degradation of IVM.

IVM can be degraded by strain ZJB-18,044 pre-cultured with or without IVM in LB medium, which indicates that IVM degradation of this strain is an inherent metabolism. Furthermore, IVM could not be degraded by strain ZJB-18,044 on LB with erythromycin, azithromycin or rifampicin, and the mechanism of the inhibition still remains uncovered. However, addition of CCCP can partially recover the IVM degradation ability of ZJB-18,044 inhibited by erythromycin, azithromycin or rifampicin. Since CCCP is an uncoupler of oxidative phosphorylation, this demonstrated that the efflux pumps on the cytoplasmic membrane might affect the intracellular macrolides concentration (Pages and Amaral 2009). Reports on overexpression of such efflux pumps indicated that a decrease in the concentration of

intracellular antibiotics could lead to a decrease in microbial drug sensitivity (Nikaido and Pages 2012). Macrolide efflux pumps systems are also found in *Aeromonas* sp. including *Aeromonas salmonicida* (containing efflux pumps to multi-class antibiotics, including macrolide) (Valdes et al. 2015) and *Aeromonas hydrophila* (with efflux pump to intrinsic multidrug resistance) (Hernould et al. 2008). Genome analysis of *Aeromonas taiwanensis* revealed the possible presence of efflux pumps such as major facilitator superfamily (MFS), MacB family and resistance-nodulation-division (RND) (Wang et al. 2014). The MFS, MacB and RND efflux pump are closely related to the bacterial drug resistance (Colclough et al. 2020; Greene et al. 2018; Kumar et al. 2020). This may provide the basis for the difference in intracellular and extracellular IVM concentrations.

The IVM tolerance mechanism of *A. taiwanensis* strain ZJB-18,044 was described in Fig. 8. The mechanism is similar with the AVM tolerance mechanism of *S. maltophilia* strain ZJB-14,120 (Wang et al. 2015). According to previous hypothesis, there were significant IVM adsorption and discharge processes in step 1 and step 2. IVM degradation mainly took place in step 3. Compared to the extracellular IVM, the intracellular IVM only accounts for a small portion of total IVM during the degradation (Fig. 8a–c) due to the pump out of excess IVM by efflux pumps. The low IVM concentration inside the cells can avoid the harmful effects of excess IVM. The role of efflux pumps in the regulation of intracellular IVM concentration is further evidenced in Fig. 8c and d.

In summary, the IVM degradation of *A. taiwanensis* strain ZJB-18,044 and its IVM tolerance mechanism were primarily explored in this study. The strain ZJB-18,044 can degrade and tolerate IVM, emamectin, doramectin and spiramycin. Due to its degradation ability, *A. taiwanensis* strain ZJB-18,044 can be applied in the treatment of water and soils contaminated by IVM, doramectin, emamectin and spiramycin. Due to the lack of deep insight into the IVM degradation mechanism, low IVM tolerance and long degradation time required (5 d for completely degradation of 50 mg/L IVM), future work will focus on the elucidation of the IVM degradation pathway, improvement IVM tolerance and degradation efficiency (reduce the time needed) of *A. taiwanensis* strain ZJB-18,044.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

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