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Full Length Research Paper

Screening of degradative plasmids from *Arthrobacter* sp. HW08 and their ability to degrade swainsonine

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***Arthrobacter* sp. HW08 is capable of swainsonine (SW) degradation with high efficiency. The aim of this study was to screen the degradative plasmids from *Arthrobacter* sp. HW08 and investigate their ability of degrading SW. A genomic library of *Arthrobacter* sp. HW08 was successfully constructed by *Bam*HI restriction digestion of genomic DNA and pUC19 vector, ligation and transformation into DH5α. Five plasmids of different sizes were screened from 100 genomic library strains, and were named as pUCSW-1, pUCSW-2, pUCSW-3, pUCSW-4 and pUCSW-5, and their insertion elements had sizes of 6 684, 5 093, 136, 1 452 and 2 082 bp, respectively. They contained 7 major open reading frames. SW (400 mg l^{-1}), as only carbon source, cultivated with the mixture of the five plasmid-transformants was degraded within 6 h. The degrading capability was equivalent to that of strain HW08. SW degradative ability of minimum combinations was pUCSW-2, 3, 5 > pUCSW-1, 2, 4 > pUCSW-1, 2, 5 > pUCSW-1, 3, 5, which were all slower than that of strain HW08. Any two or single transformant had no degradative ability. The results of this study support the search of the key genes of SW degradation and the investigating mechanism of SW biodegradation or exploration of its metabolic pathway, and even construction of transgene engineering bacteria to protect animals from lesion when locoweed is consumed.**

Key words: Swainsonine, biodegradation, degradative plasmids, *Arthrobacter* sp. HW08.

INTRODUCTION

Swainsonine (SW) is an indolizidine alkaloid, the main toxic component of the poisonous *Astragalus* and *Oxytropis* plants (generally referred to as locoweed) (Li, 2003; Molyneux and James, 1982; Ralphs et al., 2002b). Australian scholars used mannosidase to isolate SW from *Swainsona canescens* for the first time (Colegate et al., 1979), and this was followed by SW isolation from poisonous *Astragalus* and *Oxytropis* plants by many scholars around the world, including China (Cao et al., 1989). In addition, they proved that SW was the main toxin causing locoism in herbivores (Cook et al., 2009). In animals, SW can inhibit the activity of α–mannosidase in cells, affect the synthesis of cell glycoprotein (Elbein et al., 1981), cause oligosaccharide accumulation, depress cell function and result in a series of poisoning symptoms (Dorling et al., 1980; Hartley et al., 1989; James and Panter, 1989; James et al., 1970; Stegelmeier, 1999; Stegelmeier et al., 1999; Tulsiani et al., 1985).

It has been proved that SW is generated by locoweed endophytic fungi (Braun et al., 2003; Pryor et al., 2009; Ralphs et al., 2008; Yu et al., 2010). However, aside from grazing management strategies to reduce risk of poisoning (Ralphs et al., 2002a), traditional manual eradication to decrease the amount of locoweed (Li, 2003), supplementation with bentonite or mineral elements in daily ration binding to SW to reduce intoxication (Pulsipher et al., 1994), and immunology for SW-HSA immunoconjugate to prevent tissues containing blood urea nitrogen and serum enzymes of the experimental animals from *Oxytropis kansuensis* Bunge injuring (Tong et al., 2007, 2008), there are no other reports on effective methods to resolve locoweed intoxication.

Chinese scholars buried *O. kansuensis* Bunge in the

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soil, and six months later extracted and identified two strains of bacteria which were capable of degrading SW (Zhao, 2008; Zhao et al., 2009). These bacterial strains were further studied to optimize their conditions for degradation (Wang et al., 2010b), identify their SW degradation mechanism, and were hoped to be used in the elimination or reduction of SW concentration in animal bodies to reduce losses due to animal locoweed intoxication. Nevertheless, the degradation performance of the isolated strains was unsteady after passage. Our research team collected *Oxytropis ochrocephala* Bunge from Nanhua Mountain, Haiyuan County, Ningxia Hui Autonomous Region in October 2008 and buried them in the soil for six months. A strain of *Arthrobacter* sp. HW08 that was capable of SW degradation with high efficiency was obtained after enrichment culture and pure cultivation (Wang et al., 2010a). Based on this work, this study constructed a genomic library of strain HW08, screened for a degradative plasmid, and studied the effects of different transformant combinations on SW degradation. The findings of this study lay the foundation for research on SW degradation gene and degradation approaches.

MATERIALS AND METHODS

Culture medium

Luria-Bertani medium (LB) for enrichment culture contained (g l^{-1}) 5 g yeast extract, 10 g peptone and 10 g NaCl (pH 7.2). The mineral salts medium (MSM) used in the degradation tests comprised (g l^{-1}) 5.0 g NH_4NO_3, 1.5 g $MgSO_4$, 5.0 g $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, 5.0 g NaCl and 1.5 g K_2HPO_4 (pH 7.2); SW was added to the medium after autoclaving. Media were solidified, if necessary, by the addition of 15 g agar per liter.

Strain and plasmids

Arthrobacter sp. HW08 was isolated from locoweed-contaminated soil in our laboratory, and kept in China General Microbiological Culture Collection Center (CGMCC NO: 3313) with the Patent No. 200910218983.5 (Wang et al., 2010-06-09). Its 16S rDNA sequence has been included in the GenBank under the Registry No. GQ921838. *Escherichia coli* DH5α [*supE44, ΔlacU169 (Φ80 lacZΔM15), hsdR17, recA1*, *endA1*, *gyrA96*, *thi-1* and *relA1*] was kept in the laboratory. The pUC19 plasmid was purchased from TaKaRa.

Enzymes and reagent kits

Restriction enzyme *Bam*HI, dephosphorylating enzymes, DNA marker, X-Gal and IPTG were all purchased from TaKaRa. Bacteria genome extraction reagent kit, plasmid DNA fast extraction kit and DNA segments (gel) fast recovery purification kit were purchased from Tiangen Biotech (Beijing) Co., Ltd. SW standard was provided by Biotoxin & Molecular Toxiology Laboratory, College of Veterinary Medicine, Northwest A&F University (Wang et al., 2011). Methyl-α-D-mannopyranoside (me-Gal), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) were purchased from SIGMA. Other chemicals used in this study were of analytical grade and were obtained from commercial sources.

Construction of genomic library from *Arthrobacter* sp. HW08

The genomic DNA of *Arthrobacter* sp. HW08 was extracted and purified using the kit and treated with *Bam*HI partial digestion. Purified digestion segments were quickly recovered. *Bam*HI complete digestion was performed on pUC19 plasmids and segments were quickly recovered for dephosphorylation. Genome digestion segments were combined with dephosphorylated cloning vehicle at a proportion of 5:1, placed at 16°C for one night, and transferred into *E. coli* DH5α using the calcium chloride method (Sambrook et al., 2001). Recombinant strains were screened by blue-white screening. All white colonies made up the genomic library. They were inoculated into LB liquid medium for cultivation and kept as glycerol stock. At the same time, 50 µl of genomic library bacteria was inoculated into a 2 ml MSM containing 100 mg l^{-1} SW and incubated at 30°C and 180 r min^{-1} for 72 h. Then, 200 µl sample was collected and SW content was detected by thin layer chromatography (TLC) (Molyneux et al., 1991) before and after cultivation.

Screening and sequencing analysis of SW degradative plasmids

One percent of genomic library MSM was reinoculated into a 5 ml fresh MSM containing 100 mg l^{-1} SW for cultivation for 72 h. About 50 µl of the forementioned culture medium was put into LB solid medium containing ampicillin in spread plate method and incubated at 30°C for 16 h. 100 single colonies were selected, and were used in plasmid extraction and restriction enzyme identification. Plasmids of different sizes were transferred into *E. coli* DH5α for LB slant stroke generation cultivation. Bacteria was washed off with MSM, mixed at a certain proportion, and incubated with 100 mg l^{-1} SW at 30°C and 180 r min^{-1} for 12 h. Then, 200 µl samples were collected and SW content was detected by gas chromatography (GC) using the internal standard method (Zhao et al., 2007). *E. coli* DH5α (pUC19) MSM with 100 mg l^{-1} SW was used as the control.

SW degradative plasmid DNA sequence was determined by automated sequencing at Nanjing Genescript Biotech. Co., Ltd. Both strands of the DNA fragment were cloned in pUC19 vector and sequenced using M13-47 and RV-M universal primers. Major open reading frames were found by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and their homologies were analyzed with Blast software.

Effect of different combinations of 5 transformants on SW degradation ability

With 400 mg l^{-1} SW as the sole carbon source, the MSM containing one or combinations of several transformants was incubated at 30°C and 180 r min^{-1} for 12 h. 200 µl of samples were collected every 3 h for bacterial density measurement using spectrophotometer (Spectronic Instruments, Rochester, NY) and SW content detection using GC internal standard method (Zhao et al., 2007). The MSM of *Arthrobacter* sp. HW08 containing 400 mg l^{-1} SW was used as the control.

Detection of SW

TLC was applied for qualitative and half-quantitative SW detection (Molyneux et al., 1988, 1991; Molyneux and Roitman, 1980) and GC internal standard method was used for quantitative detection (Zhao et al., 2007). The latter was performed in our laboratory. Briefly, the culture supernatant was lyophilized after centrifugation at 10,000 ×*g* for 5 min, and the lyophilized powder was used for derivatization with BSTFA + TMCS. SW concentration was

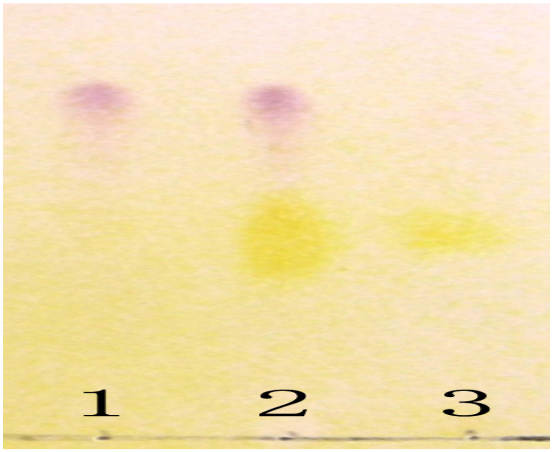

Figure 1. Thin-layer chromatogram of SW degradation by genomic library bacteria. 1, 100 mg l^{-1} SW standard substance in methanol; 2, genomic library bacteria with 100 mg l^{-1} SW in MSM, 0 h; 3, genomic library bacteria with 100 mg l^{-1} SW in MSM, 72 h.

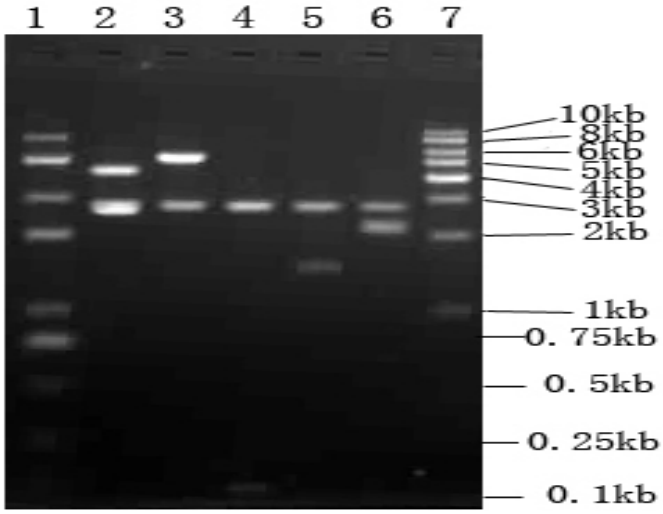

Figure 2. Agarose gel electrophotogram of degradative plasmid digestion with *Bam*HI. 1, Trans2K plus II DNA Marker; 2, plasmid pUCSW-1 (about 4.2, 2.7 and 2.5 kb); 3, plasmid pUCSW-2 (about 5.1 and 2.7 kb); 4, plasmid pUCSW-3 (about 2.7 and 0.1 kb); 5, plasmid pUCSW-4 (about 2.7 and 1.5 kb); 6, plasmid pUCSW-5 (about 2.7 and 2.1 kb); 7, 1 kb DNA ladder.

analyzed by injecting 2 µl of derivatization sample into GC-14C gas chromatography spectrometer (Shimadzu, Japan) with a data handing system and FID, using high purity nitrogen (99.999%) as the mobile phase at a rate of 2 ml min^{-1}. The split ratio was adjusted to 30:1. Column, injector, and FID temperatures were 210, 280 and 300°C, respectively.

Nucleotide sequence accession number

The nucleotide sequences of SW degradation-related genes determined in this study and named from *SWDR1* to *SWDR7* have been annotated and deposited in the GenBank database with the accession number HQ132735, HQ132736, HQ132737, HQ132738, HQ132739, HQ132740 and HQ132741, respectively.

RESULTS

Genomic library of *Arthrobacter* sp. HW08

After extracting and digesting of HW08 genomic DNA, ligating with pUC19 vector and transfer into *E. coli* DH5α, about 30, 000 white colonies were obtained, indicating that the HW08 genomic library was constructed successfully. According to TLC detection, purple spots in the sample disappeared after cultivation for 72 h (Figure 1), indicating that 100 mg l^{-1} SW was degraded and the library strain had utilized SW as the sole carbon source.

Screening and sequencing analysis of SW degradative plasmids

After the streak dilution plate technique on the earlier-mentioned 72 h culture solution, 100 colonies were selected for minipreparation of plasmids by alkaline lysis method. Five plasmids of different sizes were obtained after digestion with *Bam*HI and agarose electrophoresis identification (Figure 2). They were labeled as pUCSW-1, pUCSW-2, pUCSW-3, pUCSW-4 and pUCSW-5, and had sizes of about 9.4, 7.8, 2.8, 4.2 and 4.8 kb, respectively. The mixture of transformants were transferred into *E. coli* DH5α, respectively, was capable of degrading 100 mg l^{-1} SW within 12 h by GC detection (Figures 3 and 4). On the other hand, *E. coli* DH5α (pUC19) had no capability for SW degradation.

Sequences of the five plasmid DNAs were analyzed by ORF Finder, Blast and DNAMAN software. Table 1 shows the distance matrix and homology matrix of five sequences, indicating that the coherency with each other was probably low. They contained seven major functional genes which were related to SW degradation, and named as *SWDR1* to *SWDR7*, respectively. In comparison with tat pathway signal sequence domain-containing protein, glutathionylspermidine synthase, riboflavin biosynthesis protein RibD and GDSL-like lipase/acylhydrolase domain protein of *Arthrobacter aurescens* TC1, the encoding amino acid identities of *SWDR1*, *SWDR2*, *SWDR5* and *SWDR6* exhibited 84, 97, 85 and 94%, respectively. While *SWDR3* and *SWDR4* were 47 and 40% in contrast with glycosyltransferase of *Sanguibacter keddieii* DSM 10542, *SWDR7* was 53% for two component transcriptional regulator of *Mycobacterium vanbaalenii* PYR-1.

Effect of degradative plasmid-transformants on combined degradation

Table 2 shows the bacterial density and the ability of the five transformants to degrade 400 mg l^{-1} SW in MSM

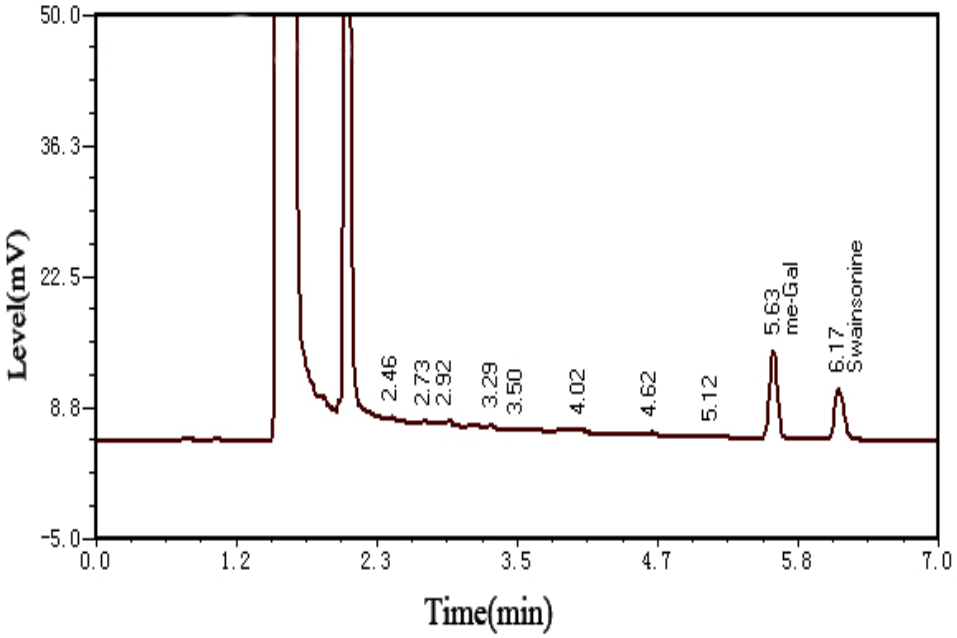

Figure 3. Gas chromatogram of MSM of transformants containing 100 mg l^{-1} SW. The result shows the gas chromatogram of the mixture of five transformants containing 100 mg l^{-1} SW incubated for 0 h and determined by GC internal standard method. Appearance time of me-Gal (internal standard substance) and 100 mg l^{-1} SW was 5.63 and 6.17 min, respectively.

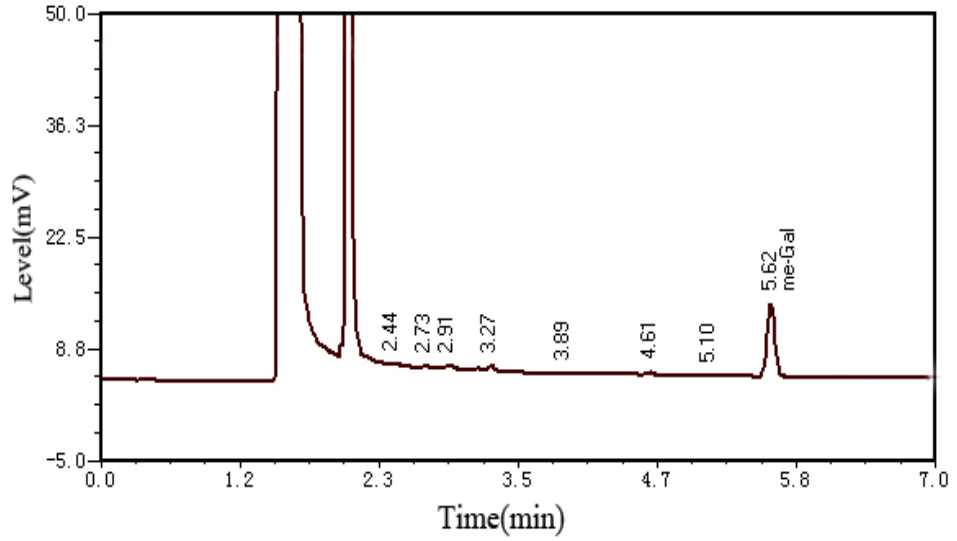

Figure 4. Gas chromatogram of transformants degrading SW incubated for 12 h. The result shows the SW degradation of the mixture of five transformants containing 100 mg l^{-1} SW incubated for 12 h and determined by GC internal standard method. Appearance time of me-Gal (internal standard substance) was 5.62 min. The peak of SW disappeared in contrast with Figure 3.

within 12 h. When several transformants combined at a certain proportion in MSM with SW as the sole carbon source, the combined effect of 5 transformants showed complete degradation of 400 mg l^{-1} SW within 6 h, which had no significant difference when compared with that of *Arthrobacter* sp. HW08 ($P>0.05$). The SW degradation capability of other minimum combinations was pUCSW-2, 3, 5 > pUCSW-1, 2, 4 > pUCSW-1, 2, 5 > pUCSW-1, 3, 5, which were entirely slower than that of *Arthrobacter* sp. HW08. Furthermore, single transformant or the combination of two and other combinations of three could not degrade SW.

Table 1. Distance matrix and homology matrix of 5 sequences by DNAMAN software analysis.

Sequence name	Distance matrix					Homology matrix (%)				
pUCSW-1	0					100				
pUCSW-2	0.634	0				36.6	100			
pUCSW-3	0.691	0.544	0			30.9	45.6	100		
pUCSW-4	0.649	0.646	0.699	0		35.1	35.4	30.1	100	
pUCSW-5	0.658	0.689	0.654	0.659	0	34.2	31.1	34.6	34.1	100

Table 2. SW degradation effects on combinations of five kinds of degradative plasmid-transformants.

Group	0 h	3 h		6 h		9 h		12 h	
	OD_{600}	OD_{600}	Degradation ratio (%)	OD_{600}	Degradation ratio (%)	OD_{600}	Degradation ratio (%)	OD_{600}	Degradation ratio (%)
12345	0.658	0.681	0	0.692	100	0.639	100	0.627	100
124	0.669	0.688	0	0.779	88.43	0.866	100	0.822	100
125	0.598	0.600	0	0.667	46.93	0.658	100	0.628	100
135	0.622	0.643	0	0.697	38.66	0.757	94.59	0.728	100
235	0.639	0.655	0	0.703	95.08	0.698	100	0.671	100
HW08	0.646	0.677	0	0.792	100	0.720	100	0.704	100

DISCUSSION

Arthrobacter sp. strains are the third most abundant in catalogued biotransformation reactions, only after *Pseudomonas* and *Rhodococcus* sp. strains. *Arthrobacter* strains are metabolically diverse and are capable of catabolizing a variety of chemical and environmentally relevant compounds because they contain genes or pathways for the catabolism of any of these compounds (Mongodin et al., 2006). The experiment revealed that *Arthrobacter* sp. HW08 was particularly well-endowed to metabolize SW genetically (Wang et al., 2010a).

Microbial degradation genes can be located in the plasmid and/or chromosome (Takahashi et al., 2009). Plasmids can independently exist outside the chromosome, can be passed on to the next generation, and can not be generally combined with host chromosome. Degradative plasmids might not be steady in structure, and are apt to cause disappearance or reduction of the function to degrade organic matter. In contrast, when the degradation genes are in the chromosome, the degradative function of the organic matter can be passed on to the filial generation quite steadily. By continuous streak cultivation on LB slant for 100 times at 30 ℃ for 2 days, it was found that the degradation effect of the 100th generation has no significant difference with the original parent bacteria after further investigation. This proves that SW degradation capability of *Arthrobacter* sp. HW08 is relatively steady and its functional gene is probably located in the chromosome.

Then, we found that there were no catabolic plasmids present in the *Arthrobacter* sp. HW08 (unpublished). The construction of a genomic library of strain HW08 and SW degradation assay resulted in the isolation of five recombinant plasmids with SW degradation capabilities. Distance matrices have been used for a long time in structural bioinformatics, mostly for protein struc-ture comparison and alignment and for inferring protein-protein interactions (Kloczkowski et al., 2009). The distance matrix of the five degradative plasmid DNA sequences had bad correlated with each other badly. Different combinations of five kinds of plasmid-transformants demonstrated different SW degradation capability. SW content had no change in one or any of the two combinations of plasmids, but some of the three urgent combination resulted in degradation. Subsequently, we cultured individual cells carrying plasmids no.1 to 5 in the presence of SW and collected the media to mix them with various combinations. The degradation capability was similar to the reports described earlier, which indicating that the three plasmids, probably provided a subunit of an enzyme complex which could degrade the SW.

Further studies are needed to clarify swainsonine degradation mechanism of combination of plasmid-transformants and seek for key genes of SW degradation so as to construct transgene engineering bacteria and solve the problem of locoweed poisoning.

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