Journal of Agricultural Science and Technology B 1 (2011) 669-674
Earlier title: Journal of Agricultural Science and Technology, ISSN 1939-1250

General Evaluation of Biocide (Bt-ASF-1) Produced from Iraqi Isolate of *Bacillus thuringiensis*

A. S. Ahmed, S. Kadim, F. M. Sajet, J. A. Juwaed, A. Hamdy, I. Atyia and S. A. Alhamid
Department of Application Sciences, Division of Biotechnology, University of Technology, Baghdad 964, Iraq

Received: November 16, 2010 / Published: September 20, 2011.

Abstract: General evaluation of isolate *Bacillus thuringiensis* (Bt-ASF-1) used as biocide in meddle scale application was conducted. Some morphological and confirmation tests were achieved. The sensitivity tests had been accomplished by diffusion and dilution techniques to determine the response of isolate against the antibiotics. The results of diffusion tests showed to the sensitivity of bacteria to antibiotics of cefixime, erythromycin, gentamicin and tetracycline respectively. It was resistant to trimethoprim sulfonamide (TMP), bacitracin, penicillin and all its generations, and moderate resistance to nalidixic acid. Minimum Inhibitory Concentration (MIC) for amoxicillin was ranged between 30-40 μg/mL and these results are an approximation of the universal findings. Curing experiments showed the effective role of sodium dodecyl sulfate (SDS) (1.5%) comparing with temperature. The bacterial cells became sensitive to amoxicillin and TMP. The curing by temperature did not differ significantly from control treatment in plasmid pattern or antibiotics response. Plasmid profile referring that curing by SDS has been caused disturbance in beta -lactamase genes through the sensitivity to amoxicillin and remaining resistance to ampicillin. Curing isolate by SDS also became more sensitive to nalidixic acid, erythromycin and tetracycline respectively. It was found from the curing treatments the complexity distribution of r-genes between different plasmid size and chromosome but not effect on their insecticidal ability.

Key words: *Bacillus thuringiensis* (Bt-ASF-1), Iraqi isolate, general characteristics, antibiotic susceptibility tests, curing, plasmid profile.

1. Introduction

Bacillus thuringiensis was discovered in 1901 in Japan by Ishiwata and in 1911 and in Germany by Ernst Berliner, who discovered a disease called *Schlaffsucht* in flour moth caterpillars [1]. *B. thuringiensis* (or Bt) is a Gram-positive, soil-dwelling bacterium, spore-forming and commonly used as a pesticide. Additionally, *B. thuringiensis* also occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well as on the dark surface of plants.

This bacteria is return to Bacillaceae family and classified by Berliner in 1915 [2]. *B. thuringiensis* (B.t.) is a naturally-occurring soil bacterium that produces poisons which cause disease in insects. B.t. is considered ideal for pest management because of its specificity to pests and because of its lack of toxicity to humans or the natural enemies of many crop pests. To be effective, B.t. must be eaten by insects during their feeding stage of development, when they are larvae. B.t. forms asexual reproductive cells, called spores, which enable it to survive in adverse conditions. During the process of spore formation, B.t. also produces unique crystalline bodies [3].

B. thuringiensis regards as resistant to many antibiotics groups especially Beta-lactam and vary sensitivity of the Group aminoglycoside and tetracycline [4]. Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include diffusion (E-Test method, Kirby-Bauer method); dilution (Minimum Inhibitory Concentration (MIC) and diffusion &

Corresponding author: A. S. Ahmed, Ph.D., research fields: environmental and fermentation microbiology and technology. E-mail: plasmid20032003@yahoo.com.

Dilution (Stokes method) [5]. *B. thuringiensis* produce one or more potent broad-spectrum β-lactamase that affect all penicillins, cephalosporins, and carbapenems. However, *Bacillus* species may be susceptible to other drug classes, including vancomycin, macrolides, fluoroquinolones, or aminoglycosides, that could be used for therapy [6]. Minimum Inhibitory Concentration (MICs) for *B. thuringiensis* (in micrograms per milliliter and zone diameters in millimeters) showed resistance to penicillin G (MIC, 32; diameter, 8) and ampicillin (MIC, 16; diameter, 12) and a paradoxical susceptibility to piperacillin (MIC, 1; diameter, 27). The strain was also susceptible to 14 imipenem (MIC, 0.047; diameter, 34 mm), vancomycin (MIC, 2; diameter, 18), gentamicin (MIC, 0.5; diameter, 24) and ciprofloxacin (MIC, 0.25; diameter, 31) [7].

SmithKline Beecham (1981) patented Amoxicillin or amoxicillin/clavulanate potassium tablets, and first sold the antibiotic in 1998 under the tradenames of Amoxicillin, Amoxil, and Trimox. Amoxicillin is a semisynthetic antibiotic [8]. *B. thuringiensis* is closely related to *B. cereus* , a soil bacterium, and *B. anthracis*, the cause of anthrax: the three organisms differ mainly in their plasmids [1].

Interest in *B. thuringiensis* plasmids started at the end of the 1970s when a correlation was established between the formation of crystals and the presence of certain plasmids [9, 10].

Plasmid patterns were always underestimated, mostly due to the possibility that *B. thuringiensis* strains may spontaneously lose some of their plasmids and to the unreliability of the plasmid extraction techniques [11]. In the other hand these Cry proteins coded by genes (*cry* genes) harbored in megaplasmids although it has also been suggested that they are present in the chromosome [12]. The number of plasmids in different strains of BT varies from 2 to 12 with sizes ranging from *ca.* 1.5 to *ca.* 150 Md. The crystal protein genes are mostly located in large plasmids (15-120 Md) [13]. Curing plasmid by using SDS 0.02%, the results indicated that in each strain of *Bacillus thuringiensis* only a single plasmid involved in bioactivity, although the size of the implicated plasmid varies from one strain to another [14]. Derivatives cured of one or more plasmids were often detected as colonies having an unusual morphology such as in the size (big, small), compared with that of the parental strain [15]. Our study aimed to identify the sensitivity of the local isolate of *B. thuringiensis* against the antibiotics by diffusion and determine the MIC towered one type and finally the plasmid profile of isolate and the effect of curing agents on the stability the plasmids with relation to antibiotic resistance.

2. Materials and Methods

2.1 Microorganism

Bacillus thuringiensis bacterium was supplied by the research projects laboratory (Dr. A. S. Ahmed) of biotechnology division/application sciences dep./Technology university. This isolate was identified by the mentioned laboratory. The isolate was activated and re-purified on nutrient agar and stored at 4 °C in refrigerator. Several morphological tests were achieved to confirm this isolate.

2.2 Antibiotic Discs

Many groups of antibiotic disc were used in susceptibility tests which supplied from Indian company (Himedia, exp., 2011). These antibiotic discs are Vancomycin (30 µg), Methicllin (5 µg), Cefixime (5 µg), Gentamicin (10 µg), Amikacin (10 µg), Nalidixic acid (30 µg), Tetracycline (30 µg), Bacitracin (10 µg), Trimethoprim Sulfonamide TMP (10 µg), Erythromycin (15 µg), Amoxicillin (30 µg), Ampicillin, Streptomycin (10 µg).

2.3 Media, Solutions and Reagents

Nutrient agar, Tryptic Soya Broth (biolife company); Standard Amoxicillin supplied by Kemadia; SDS supplied by Pharmacia company.

2.4 Antibiotic Susceptibility Tests Methods

Diffusion and dilution methods were used to determine the general susceptibility tests against the range of antibiotic discs and minimal inhibitory concentration (MIC) for one selected antibiotic respectively. Serial concentrations of antibiotic in sterile distil water were prepared in stock solutions separately (5, 10, 20, 30, 40, 60 and 80 μg/mL) and sterilized through sterile membrane filter (0.45 micron). After preparation nutrient agar and poured in the plates, pure active colony of *B. thuringiensis* isolate was spreading. Wells were made on the solid agar medium by special empty sterile tube to give final 0.1 mL volume for each well. The final concentrations of antibiotic putted in the wells were 0.5, 1, 2, 3, 4, 6 and 8 μg respectively. The cultural plates were incubated at 37 °C for 24 hr. The diameter of zone formation was measured.

2.5 Curing Experiments

Temperature treatment (exposure the liquid culture to 55 °C/10 minute and test with the selected antibiotic discs) and SDS (by inoculated the active culture of bacteria in nutrient broth supplemented with 1.5% of SDS and incubated for 24 hr) were used. Then the loopful of culture was spreading on the same medium with agar and incubated for 24 hr. The growth colonies were selected and used in sensitivity tests by diffusion technique. Plasmid profile was achieved by using the standard extraction kit and ladder supplied from Hi-Pure company (India).

3. Result and Discussion

3.1 Bacterial Isolate

In Fig. 1, pure colonies of local isolate of *Bacillus thuirngiensis* were grew on the surface of nutrient agar. The active and pure colony of the isolate was confirmed by several tests (Table 1). Colony and cells shape, motility, gram stain, blood hemolysis, lecithin hydrolyses and spores production. All these tests were

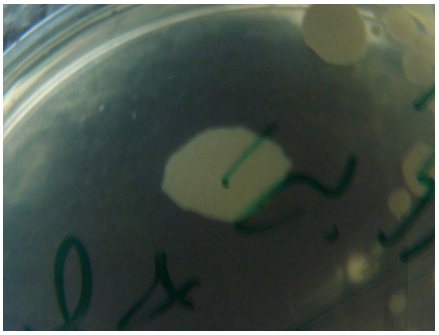


Fig. 1 Colony of *Bacillus thuringiensis*.

Table 1 General properties of local isolate *Bacillus thuringiesis.*

Test	Result
Gram stain	+
Colony shape on nutrient agar	Large, sandy cream, irregular edges, small convex.
Lecithinase production on egg yolk agar medium	+
Mortality (wet film)	+ (Mid-fast) single, double not more than 3 cells.
Spore forming and location	+ , sub terminal limited swelling
Blood hemolysis	Alpha and few beta hemolysis

corresponding to the standard morphological and confirmation tests of *B. thuringeinsis*.

3.2 Antimicrobial Susceptibility Tests

The results of *in vitro* susceptibility test for local isolate of *B. thuringiensis* are as fallow.

3.2.1 Diffusion Test

Table 2 is illustrated the sensitivity test of the local isolate *B. thuringiensis* toward many groups of antimicrobial agents of selected types like β-lactam (Ampicillin, Amoxicillin, Bacitracin, Amikacin, Methicillin); Aminoglycoside (Streptomycin, Gentamicin); antimetabolites (Sulfa drugs like TMS); Quinolones (Nalidixic acid); Tetracyclines; Macrolides (Erythromycin) and glycopeptides (Vancomycin); Chloramphenicol. The results indicated that the isolate was resistant to all β-lactam Methicillin, Amikacin, Ampicillin and Amoxicillin in this study. This result considers analogue to many studies [4]. The isolate was resistant to antimetabolites (TMS) and sensitive to remaining antibiotics types but in different ranges. The most effective antibiotic was the Cefixime (50 mm) and erythromycin (30 mm)

then Tetracycline, Gentamicin and chloramphenicol. Vancomycin and Nalidixic acid were the moderate in their effects. In the line of standard reference (Table 2), the values are represent the standard inhibition zones which derived by finding the MIC values and zone diameters for many different microbial strains. The susceptibility tests for the local isolate of *B. thuringiensis* was not similar to the standard or international strains and that is a normal result as mentioned in many references [16]. The resistance of this isolate to the many generations and derivatives of penicillin returns to the production of several modified types of beta-lactamase [17].

3.2.2 Dilution Tests

In order to determine the minimum inhibitory concentration of one of the antibiotic that resisted by *B. thuringiensis*, we selected amoxicillin because it was concedered the modern type of beta-lactam group. The technique followed in the determination of the MIC was the wells made on agar medium and these wells are same in the geometric scales. The final size of each well was 0.1 mL, so the volume of antibiotic titer solution calculated according to this size. In final we obtained serial concentrations of antibiotic (amoxicillin). The MIC is flagged between 3-4 µg/0.1 mL or 30-40 µg/mL). These results do not differ significantly with Refs. [18, 19].

Table 3 and Fig. 2 illustrated the MIC determination of amoxicillin against the local isolate of *B. thuringiensis*.

3.3 Curing of Plasmids

Fig. 3 and Table 4 showed the results of the effect the curing agents on plasmid pattern and susceptibility against selected antibiotic discs respectively. The curing by temperature had no effects on plasmid or sensitivity phenomenon compared with parent isolate. The isolate has tow maga-plasmids (large and small size) and tow flexible plasmids (10-20 Kbp). The curing by SDS (1.5%) was clearly effect on plasmid and sensitivity against antibiotics. The curing isolate

Table 2 Antibiotics susceptibility tests of *Bacillus thuringiensis*.

Antibiotic disc	Zone diameter (mm)	Bacterial sensitivity (*)	Standard reference (**)
Amikacin (10 µg)	0.0	R	
Ampicillin (10 µg)	0.0	R	
Amoxicillin (30 µg)	0.0	R	
Bacitracin (10 µg)	0.0	R	
Cefixime (5 µg)	50	S	(≥ 17 mm)
Erythromycin (15 µg)	30	S	(≥ 18 mm)
Gentamicin (10 µg)	20	S	(≥ 17 mm)
Methicillin (5 µg)	0.0	R	
Nalidixic acid (10 µg)	20	S	
Tetracycline (30 µg)	18	S	
Vancomycin (0 µg)	10	M	(< 12 mm)
Streptomycin (10 µg)	20	S	(> 15 mm)
Trimeth.-Sulfa. (10 µg)	0.0	R	
Chloramphenicol (30 µg)	18	S	(≥ 18 mm)

*: R = Resistance; S = Sensitive; M = Intermediate, **: Derived by finding the MIC values and zone diameters for many different microbial strains [16].

Table 3 Minimum inhibitary concentration (MIC) of amoxicillin on *Bacillus thuringiensis*.

No.	Concentration (µg/0.1 mL)	Zone diameter (mm)
1	0.5	0.0
2	1	0.0
3	2	0.0
4	3	20
5	4	28
6	6	33
7	8	40

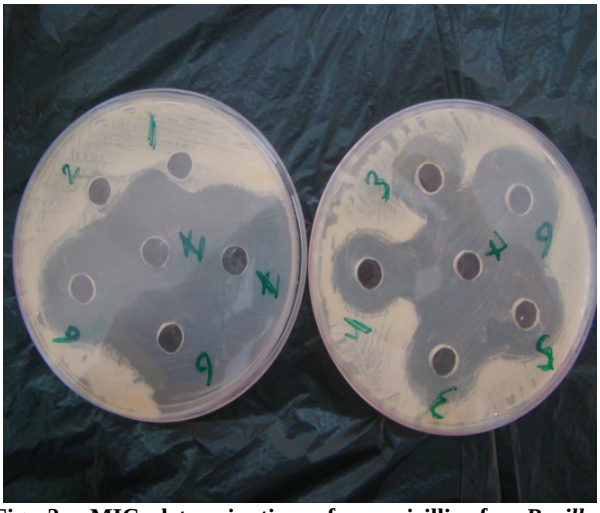
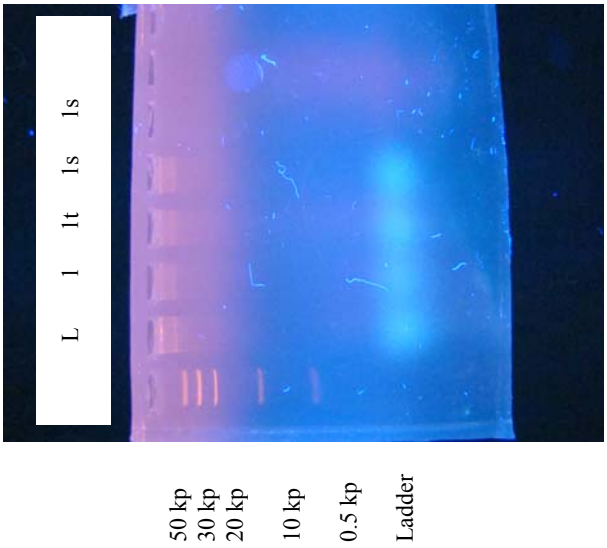


Fig. 2 MIC determination of amoxicillin for *Bacillus thuringiensis*.



L: Ladder DNA,
1: *B. thuringiensis* (control references),
1t: Temperature treatment (55 °C for 10 min.),
1s: SDS (1.5% treatment).

Fig. 3 Plasmid profile pattern of Iraqi Bt-ASF -1(*Bacillus thuringiensis*).

Table 4 Effect of curing agents on antibiotic susceptibility of *B. thuringiensis*.

Antibiotic disc	Zone diameter (mm)		
	Control	Curing agents	
		Temp.	SDS (1.5%)
Ampicillin (10 µg)	0.0	0.0	0.0
Amoxicillin (30 µg)	0.0	0.0	28
Erythromycin (15 µg)	30	34	42
Tetracycline (30 µg)	18	17	22
Nalidixic acid (10 µg)	16	21	32
Trimeth.-Sulfa. (10 µg)	0.0	0.0	42

seemed loss the megaplasmid. The chromosomal bands not present according to the specify of the extraction kits and the ladder indicator. The results of the effect the curing agents on antibiotic susceptibility tests were illustrated in Table 4. Curing by temperature had no effect on the sensitivity of isolate against the antibiotics because there was no change on the pattern of the chromosome and large plasmids as compare with the control. The effect SDS was clear in change the sensitivity of isolate. It became sensitive against TMP and amoxicillin and more sensitive agaist nalidixic acid and streptomycin and no change towered ampicillin and tetracycline. These change return to cure the plasmid that harbors the resistance against TMP and amoxicillin and allows more sensitive against nalidixic acid and streptomycin. The remaining the same resistance against ampicillin may return to stability this trait as chromosomal gene. This change in antibiotic response by SDS was remarked but in use the 0.02% through the curing of different sizes of plasmids [14].

4. Conclusions

The Iraqi isolate of *B. thuringienis* was characterized by their resistant against most beta lactam and TMP antibiotic discs and variable sensitivity toward other groups of antibiotics. It has high sensitivity against the cefixime and erythromycin and mild against vancomycin. These results are not different widely in comparision with the universal strains. The result of MIC against amoxicillin does not differ significantly to the many references. SDS 1.5% was more effectiveness as a curing agents comparing with temperature on antibiotic susceptibility. It became sensitive against TMP and amoxicillin and more sensitive agaist nalidixic acid and streptomycin and no change to ampicillin and tetracycline. These variable results refer to the new distribution of R-genes between low and high copy number plasmids and chromosomes of our isolate which not found in other references. Results indicated to a noticeable change in plasmid pattern by using the curing agents (SDS or heat treatment) compared to control, although for a clear change in sensitivity to antibiotics. In the next study, we will go to link between curing treatments and biocide activity of our strain.

References

[1] C. Clayton, T. Yamamoto, Invitation paper (C.P. Alexander Fund): history of *Bacillus thuringiensis* berliner research and development, Canadian Entomologist 124 (1992) 587-616.
[2] M. Madigan, J. Martinko, Brock Biology of Microorganisms, 11th ed., Prentice Hall, 2005.
[3] D.T. John, H.J. James, Antimicrobial susceptibility testing: general considerations, in: P.R. Murray, E.J. Baron, M.A.

Pfaller, F.C. Tenover, R. Yolken (Eds.), Manual of Clinical Microbiology, 7th ed., American Society for Microbiology, Washington DC, 1999, pp. 1469-1473.

[4] B. Myer, History of Antibiotics, 2008, available online at: www.About.com.

[5] NCCLS Performance Standards for Antimicrobial Susceptibility Testing, National Committee for Clinical Laboratory Standards, Wayne, Pa, 2003.

[6] D.J. Weber, S.M. Saviteer, W.A. Rutalla, *In vitro* susceptibility of *Bacillus* spp. to selected antimicrobial agents, Antimicrob Agents Chemother 32 (1988) 642-645.

[7] E. Hernandez, F. Ramisse, J. Ducoureau, T. Cruel, J. Cavallo, *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice, J. Clin. Microbiol. 36 (1998) 2138-2139.

[8] M. Bellis, History of antibiotics, in: Antibiotic Timeline, available online at: www.about.com.

[9] B.C. Carlton, Patterns of plasmid DNA in crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*, Plasmid 3 (2009) 92-98.

[10] J.M. Gonza'lez, Jr., B.J. Brown, B.C. Carlton, Transfer of *Bacillus thuringiensis* plasmids coding for endotoxin among strains of *B. thuringiensis*, Proc. Natl. Acad. Sci. USA 79 (1982) 6951-6955.

[11] A. Reyes-Ramı'rez, J. Barra, Plasmid patterns of *Bacillus thuringiensis* type strains, Appl. Environm. Microb. 74 (2008) 125-129.

[12] J. Kronstad, W.H.E. Schnepf, H.R. Whiteley, Diversity of locations for *Bacillus thuringiensis* crystal protein genes, J. Bacteriol. 154 (1983) 419-428.

[13] A.I. Aronson, W. Beckman, P. Dunn, *Bacillus thuringiensis* and related insect pathogens, in: J.L. Ingraham (Ed.), Microbiol. Rev., American Society for Microbiology, Washington, D.C., 1986, pp. 1-24.

[14] M. González, H. Dulmage, B. Carlton, Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*, Plasmid 5 (1981) 351-365.

[15] A. Sivropoulou, L. Haritidou, S. Aptosoglou, S. Koliais, Correlation of the insecticidal activity of the *Bacillus thuringiensis* A4 strain against *Bactrocera oleae* (Diptera) with the 140-kDa crystal polypeptide, Current Microbiology 41 (2000) 262-266.

[16] L. Priscott, P. Harley, D. Klien, Antimicrobial chemotherapy, in: Microbiology, 6th ed., McGraw Hill, 2005, p. 779.

[17] A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol. Apr. 45 (1966) 493-496.

[18] I. Materon, A. Queenan, M. Koehler, K. Bush, T. Palzkill, Biochemical characterization of beta-lactamases Bla1 and Bla2 from *Bacillus anthracis*, Antimicrob. Agents Chemother. 47 (2003) 2040-2042.

[19] J.M. Andrewsand, R. Wise, Susceptibility testing of *Bacillus* species, J. Antimicrob Chemother 49 (2002) 1039-1046.