Animal Cells and Systems 12: 41-46, 2008

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Isolation and Characterization of a Antimicrobial Compound from *Bacillus coagulans*

Emad Abd El-moniem Abada[1,2]

[1]*Laboratory of Developmental Genetics, Department of Life Science, Hanyang University, Seoul 133-791, Korea;* [2]*Botany and Microbiology Department, Faculty of Science, Helwan University, Ain Helwan, Cairo, Egypt*

Abstract: A bacterium strain called *Bacillus coagulans* was isolated from an industrial wastewater drainage and selected for its antimicrobial activities against bacteria and fungi. Characterization studies strongly suggested that this strain is *Bacillus coagulans*. Antimicrobial activity was found against gram-positive, gram-negative bacteria and yeast strain. Maximal activity was observed after 24 h when incubated at 30°C and pH 8. The activity was found to be stable at 75°C for 30 min and at pH range of 2-12. Analysis of the antimicrobial compound by SDS-PAGE suggested a molecular mass of approximately 7.5 KDa. The substance was characterized as a bacteriocin, because of its proteinaceous nature and low molecular weight. Our bacteriocin could potentially be used as a food preservative, because of its thermostable property and broad antimicrobial spectrum.

Key words: Antimicrobial activity, *Bacillus coagulans*, Bacteriocin, Food preservative

Formerly, secondary metabolites were defined as substances with a low molecular weight, which were not products of the primary metabolic pathway of the producing organism. In fact, it was thought that these products did not have a role in microbial primary functions or growth (Vining, 1992; Yarbrough et al., 1993).

Secondary metabolites are accepted to be essential for the producing cell as inhibitors of other organisms that compete for the same food supply or as regulators of cellular differentiation process. In addition, it was reported that they are indeed products of biosynthetic pathways, which have evolved to give these types of advantages (Demain, 1999; Demain and Fang, 2000). Indeed, these compounds are mostly biosynthesized by bacteria, fungi, algae, corals, sponges, plants and lower animals. About 100,000 secondary metabolites of a molecular weight below 2500 have been characterized, among them approximately 50,000 from microbial sources (Bezborodov, 1978). The microbial production of secondary metabolites is extremely sensitive to environmental factors and culture conditions (Bunch and Harris, 1986). Bacteria that show high production of natural products are those belonging to the genus *Bacillus* (Patel et al., 1995). Bacillus species are aerobic spore formers commonly found in soil and ground water. They are often encountered on plants and animals at the point of harvest or slaughter. Bacillus have been investigated for their ability to produce so called bacteriocin-like inhibitory substance (BLIS) (Motta et al., 2007). It has been reported that strains of *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. cereus* produce BLIS (Stein, 2005; Gray et al., 2006; He et al., 2006; Lisboa et al., 2006; Sharma et al., 2006). Among the Bacillus bacteriocins, a bacteriocin was found to be heat labile and not broadly effective for antimicrobial activity, although both cerein (Naclerio et al., 1993) and coagulin (Hyronimus et al., 1998) are not affected by several organic solvents. In the present study, we identified new bacteriocin isolated from *Bacillus coagulans* and its thermostablity property and broad antimicrobial spectrum were determined.

MATERIALS AND METHODS

Bacterial strains and fungi

Escherichia coli (NCTC-10418), *Pseudomonas aeruginosa* (NCIB-9016), *Klebsiella pneumoniae* (NCIB-9111), *Bacillus subtilis* (NCTC-6346), *Staphylococcus aureus* (NCTC-7447), *Candida albicans* (CBS-562), *Aspergillus Niger* (LTV-131).

Media

Nutrient agar medium (NA) was used as bacterial growth

Tel: +82-2-2220-0954; Fax: +82-2-2220-4474
E-mail: emadm_abada@yahoo.co.uk

medium for all experiments (3 g/l Beef extract, 5 g/l Peptone, 5 g/l NaCl, and 15 g/l Agar, pH 6.8). Dox's agar medium was used as fungi growth medium (20 g/l Sucrose, 2.0 g/l $NaNO_3$, 1 g/l Na_2HPO_4, 5 g/l $MgSO_4$-$7H_2O$, 5 g/l KCl, and 15 g/l Agar, pH 6.4).

Isolation and identification of the bacterial strain with antimicrobial Activity

Bacterial samples were collected from industrial wastewater drainage (approximately 70 km south to Cairo). This drainage receives the wastewater from different factories of various industrial activities. The water samples were serially diluted and spread over nutrient agar plates. After incubation at 30°C for 24 h, an antagonistic strain was isolated and its antimicrobial activity was tested by the crowded plate technique (Hammond and Lambert, 1978). Purification of the antagonistic bacterial isolate was done by the streak plate method. On the basis of its morphological, physiological, and culture properties, the organism was identified as *Bacillus coagulans* according to Calus and Berkeley (1986) (Table 2).

Agar diffusion assay

Antibacterial activity was determined according to Gharieb and Abada (2005) with slight modifcation by using agar diffusion assay. Overnight broth cultures of bacteria were freshly prepared for each assay. Nutrient agar plates (15 ml) were prepared, allowed to set and then surface dried (37°C, 30 min). 500 µl of bacterial culture was spread over the surface of the dried agar plates using a sterile glass spreader and allowed to absorb in the agar for 10 min. The plates were dried, inverted, at 37°C for approximately 30 min until the bacterial overlay had dried. Ten µl of the culture supernatnt was pipetted onto a 6 mm sterile disc (Whattman filter paper), and the disc was placed onto the agar plate and incubated at 37°C for 24 h. For antifungal activity, 10 µl of the bacterial culture supernatnt was pipetted onto a 6 mm sterile disc (Whattman filter paper), which was then placed onto the Dox's medium plates opposite to the fungus disc (6 mm) and incubated at 30°C for a week. The diameters of the inhibition zone for each were recorded in mm.

Optimization of the culture conditions for the production of the antimicrobial compound

In order to determine the optimal medium composition for the production of antimicrobial compound, various physical and chemical conditions were used, including different incubation periods, carbon, nitrogen sources, temperature, and pH. Then, the antimicrobial activity of the supernatant was determined as mentioned above.

Effects of different treatments on antimicrobial activity

The effects of heat, pH, organic solvents, and proteolytic enzymes on antimicrobial activity were investigated. To investigate the effect of heat on antimicrobial compound activity, antimicrobial preparation was heated at 60, 75 and 90°C for 15 and 30 min, then immediately cooled in ice water. For pH stability, the pH was adjusted to levels between pH 2 and 12. Also, various organic solvents including acetone, acetonitrile, ethyl alcohol, and methyl alcohol at the final concentration of 10 % were added to the antimicrobial preparation and then incubated at 25°C for 1 h. The antimicrobial preparation was also treated with a final concentration of 1 mg/ml proteolytic enzymes (α-chymotrypsin, proteinase K, and pronase E; Sigma) in 50 mM phosphate buffer (pH 7.0) at 37°C for 1 h. The substance without any enzyme was used as negative control. After the incubation, the activities of treated samples were estimated by the agar diffusion assay.

Partial purification of the substance produced by *B. coagulans*

The substance was partially purified using by ammonium sulfate precipitation method. The culture broth was centrifuged at 708×g for 30 min at 4°C (Beckman Coulter, Inc., USA). The supernatant was adjusted to pH 8 and boiled for 10 min to inactivate proteases (Chintas et al., 1998; Ko et al., 2000). The cell-free supernatant was transferred to a beaker in an ice bath, and ammonium sulfate was added to 40% (final concentration). Subsequently, the mixture was centrifuged at 708×g for 45 min at 4°C and the pellet was then dissolved in 50 mM potassium phosphate buffer of pH 7.5. This solution was dialyzed against the same buffer overnight using a membrane with 3.5 KDa cut off (Spectrum Medical Inc., LA, USA). The dialyzed material (crude substance) was freeze-dried and then stored at –20°C until use.

Determination of molecular weight of the antimicrobial substance by SDS-PAGE

The molecular size of the crude substance was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% acrylamide gel. For estimation of the size, the low molecular weight marker was used (Biorad). Twenty µl of samples was mixed with five µl of 5× sample buffer and boiled for 10 min. After electrophoresis at constant current of 20 mA for 3 h, the gel was removed and cut into two vertical parts. One part of the gel, containing the sample and the molecular weight standard, was stained with Coomassie brilliant blue R250, and the other, containing only the sample, was tested for antimicrobial activity as previously described (Kim et al., 2000; Matsusaki et al., 1996) with some modification. The gel was immediately fixed in 20% isopropanol and 10% acetic acid for 2 h and washed in sterilized distilled water for 4 h. The gel was then placed onto a Petri dish and overlaid with 5 ml of 0.7% agar

Table 1. Inhibitory spectrum of the antimicrobial substance produced by *Bacillus coagulans*

Indicator	Antimicrobial activity in agar diffusion test
Gram-positive	
Bacillus subtilis	+
Staphylococcus aureus	+
Gram-negative	
Escherichia coli	+
Klebsiella pneumoniae	+
Salmonella typhimurium	+
Fungi	
Aspergillus niger	-
Candida albicans	+

+ indicate control diameter over 6 mm.
- no activity

containing the indicator strains. The plate was incubated for 24 h at 37°C and analyzed for clear zones.

RESULTS AND DISCUSSION

Isolation and identification of the strain producing an antimicrobial substances

Of the 30 bacterial strains isolated from wastewater drainage, one strain showed high antimicrobial activity against various indicator organisms (Table 1). The isolate showed antimicrobial activity against not only various gram-positive, but also gram-negative, bacteria. Furthermore, fungi such as *Candida albicans* was also inhibited. Thus, it showed a very broad spectrum of inhibition ranging from prokaryotes to some eukaryotes. The isolate was identified as an endospore-forming, gram-positive, catalase-positive, and motile rod. The strain was identified as *Bacillus coagulans*, based on morphological and biochemical properties defined by Bergey's Manual (Claus and Berkeley, 1986; Holt et al., 1994).

Effect of culture conditions and supplements on the production of antimicrobial substance

The effect of incubation periods, temperature, pH, carbon sources and nitrogen sources on the antimicrobial compound production were determined.

Effect of incubation periods and pH on the antimicrobial activity

In order to investigate the effect of different incubation periods and pH on the production of the antimicrobial, *B. coagulans* was cultivated in nutrient broth (NB) medium with different pHs (2-12) in flask cultures with shaking. The optimal pH for was determined to be 8 after 24 h incubation period (Fig. 1, 2).

Table 2. Identification of the strain *Bacillus coagulans*

Characters	Results
Gram reaction	+
Shape	Rod
Arrangement of cells	Regular
Spore formation	+
Catalase	+
Oxidase	-
H_2S production	-
Growth on NaCl (2-7%)	+
Growth at pH 5	+
Citrate utilization	-
Nitrate reduction	-
Methyl red	-
VP	+
Indole	-
Levan	+
Amylase	-
Lipase	+
Cellulase	-
Pectinase	-
Gelatinase	+
Raffinose	-
Melibiose	-
Cellobiose	+
L-Rhaminose	-
L-Arabinose	+
D-Glucose	+
L-Tyrosine	-
DL-Tryptophane	-
L-Alanine	-
L-Lysine	-
DL-nor-Valine	-
DL-Alanine	-
L-Methionine	-

Effect of temperature on the antimicrobial activity

To find the optimal temperature for the production of the antimicrobial compound, *Bacillus coagulans* was cultivated at various temperatures. The optimum temperature was found to be 30°C (Fig. 3). When the temperature during the incubation process was below 30°C or an initial pH of the medium below 8, the antimicrobial product was decreased. In addition, the amount of antimicrobial product in the medium was dependent upon the phase of bacterial growth (data not shown).

Effect of carbon sources on the antimicrobial activity

When compared with the controls, among the wide variety

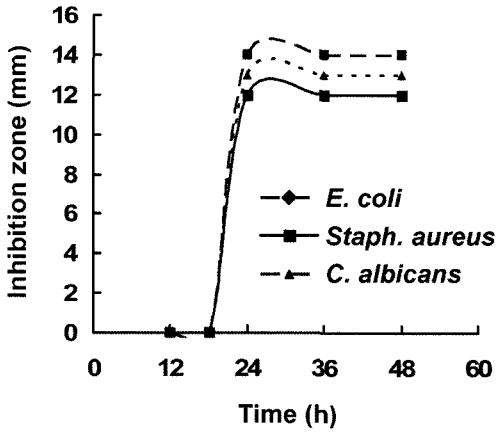

Fig. 1. Effect of incubation period on the production of bacteriocin.

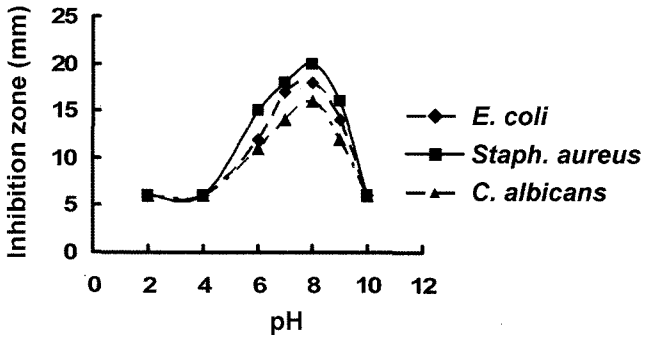

Fig. 2. Effect of different pHs on the production of bacteriocin.

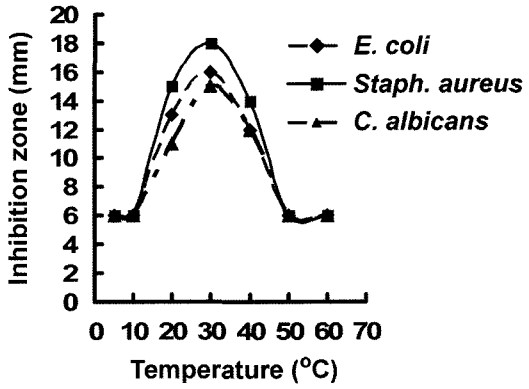

Fig. 3 Effect of different temperatures on the production of bacteriocin.

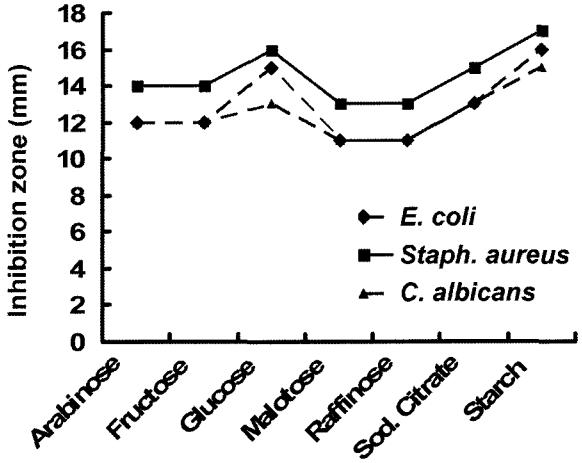

Fig. 4. Effect of different carbon sources on the production of bacteriocin.

of carbon sources tested, NB medium amended with arabinose, fructose, glucose, maltose, raffinose, sodium citrate and sucrose. Our data showed that glucose enhanced the antimicrobial activity (Fig. 4).

Effect of nitrogen sources on the antimicrobial activity

Nitrogen sources (alanine, arginin, asparagine, lysine, serine, methionine, phenyl-alanine, tryptophan, tyrosine and valine) at concentrations of 2 g/l were added to NB medium supplemented with glucose as best carbon source. Higher antimicrobial activity was achieved when peptone used as a nitrogen source (data not shown).

Effect of different treatments on antimicrobial activity

The antimicrobial substance was stable under 60°C and the activity remained after 30 min at 75°C. The residual activity was still detected in the range of pH 2-11 (Table 3). The antimicrobial activity was not affected by treatments with organic solvents (data not shown). However, the antimicrobial activity was completely lost by treatments with proteinase K and α-chymotrypsin, and partially inactivated by pronase E (data not sown). This indicated that the antimicrobial substance produced by *B. coagulans* was of proteinaceous nature that could be classified as a bacteriocin.

Table 3. Effect of temperature and pH on the stability of the antimicrobial substance

Treatment	Inhibition zone (mm[1])		
	Staph. aureus	*E. coli*	*C. albicans*
Control[2]	18.2	16	15
Heat treatment			
60°C, 15 min	18	16	14.8
75°C, 15 min	18	15.5	14.6
90°C, 15 min	13	10	9
60°C, 30 min	18	16.2	15.3
75°C, 30 min	17.8	15.8	15
90°C, 30 min	10.3	9.5	9
pH treatment			
pH 2	16.3	13.3	12.5
pH 4	16.4	13.7	12.5
pH 6	16.9	14	13
pH 8	17.8	15.5	13.9
pH 10	17.6	14.7	13.5
pH 12	14.1	12.3	11

[1]diameter (mm) of clear zone included disk (6 mm)
[2]Control was made from NB that was treated the same as the antimicrobial preparation.

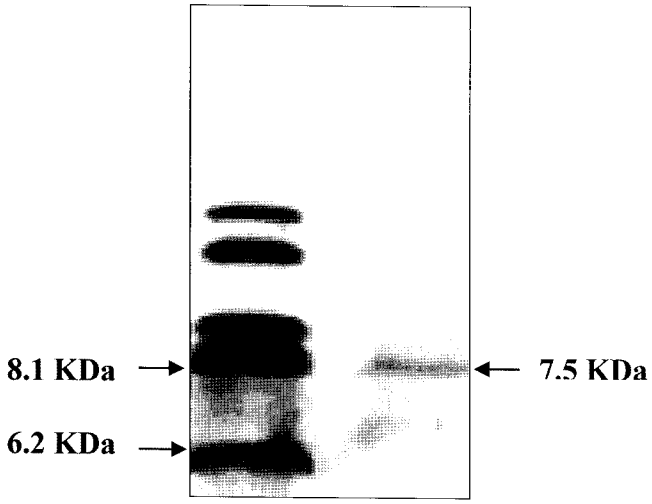

Fig. 5. Tricine/SDS-PAGE of the cell-free supernatant from the overnight culture of *Bacillus coagulans* (lane 2), Lane 1, MW standards.

Determination of molecular weight of the bacteriocin by SDS-PAGE

The apparent molecular weight of the bacteriocin was determined by SDS-PAGE. The molecular mass of the bacteriocin was approximately 7.5 KDa, and a clear band of the antimicrobial substance was observed (Fig. 5). The antimicrobial substance was identified as bacteriocin due to its low molecular weight.

Most of the bacteriocins show a narrow spectrum of antimicrobial action, and they inhibit strains closely related to the producers. Only a few bacteriocins from gram-positive bacteria inhibit diverse groups of gram-positive bacteria, and very few inhibit gram-negative bacteria (Klaenhammer, 1988; De Vuyst and Vandamme, 1994; Zheng et al., 1999). It has been reported that coagulin isolated from *B. coagulans* strain exhibited a bactericidal and a bacteriolytic mode of action against indicator cells (Hyronimus et al., 1998). The apparent molecular mass was estimated to be about 3-4 KDa by SDS-PAGE. The antimicrobial substance of our bacteriocin was stable at 75°C for 30 min, at pH ranging from 2-12. The high stability of the antimicrobial substance from *B. coagulans* at various temperature, pHs, and organic solvents are advantageous. Like nisin, bacteriocins and bacteriocin-like substances produced by lactic acid bacteria are only stable at acidic and/or neutral condition and are inactivated at pH above 8.0 (De Vuyst and Vandamme, 1994). Considering these points, the antimicrobial substance identified in this study has a good potential for preservation of non-acidic fermented foods.

REFERENCES

Bezborodov AM (1978) On secondary metabolities: their functions and biogenesis. *Folia Microbiol* (Praha) 23: 509-510.

Bunch AW and Harris RE (1986) The manipulation of micro-organisms for the production of secondary metabolites. *Biotechnol Genet Eng Rev* 4: 117-144.

Claus D and Berkeley RCW (1986) Genus *Bacillus* Cohn 1872. In: Sneath PHA, Mair NS, Sharpe ME, and Holt JG (eds), *Bergey's Manual of Systematic Bacteriology*, vol. 2 Williams & Wilkins, Baltimore. pp.1105-1139.

Demain AL (1999) Pharmaceutically active secondary metabolites of microorganisms. *Appl Microbiol Biotechnol* 52: 455-463.

Demain AL and Fang A (2000) The natural functions of secondary metabolites. Adv *Biochem Eng Biotechnol* 69: 1-39.

De Vuyst L and Vandamme EJ (1994) Antimicrobial potential of lactic acid bacteria, In: De Vuyst L and Vandamme EJ (eds), Bacteriocins of Lactic Acid Bacteria. Microbiology, Genetics and Applications. Blackie Academic & Professional, Glasgow, Scotland. pp.91-142.

Gray Ej, Lee KD, Souleimanov AM, Di Falco MR, Zhou X, Ly A, Charles Tc, Driscoll BT, and Smith DL (2006) A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain Bacillus thuringiensis NEB17: Isolation and classification. *J Appl Microbiol* 100: 545-554.

Klaenhammer TR (1988) Bacteriocins of lactic acid bacteria. *Biochimie* 70: 337-349.

Hammond SM and Lambert PA (1978) Antimicrobial Actions, Edward Arnold Ltd, London. pp.8-9.

He L, Chen W, and Liu Y (2006) Production and partial characterization of bacteriocin like peptides by bacillus licheniformis ZJU12. *Microbiol Res* 161: 321-326.

Holt GJ, Krieg NR, Sneath PHA, Staley JT, and Williams ST (1994) Genus *Bacillus*, *in Bergey's Manual of Determinative Bacteriology*, 9th ed. Williams & Wilkins, Baltimore, U.S.A, pp 559-562.

Hyronimus B, Le Marrec C, and Urdaci MC (1998) Coagulin, a bacteriocin-like inhibitory substance produced by bacillus coagulans. *J Appl Microbiol* 85: 42-50.

Lisboa MP, Bonatto D, Bizani D, Henriques JA, and Brandelli A (2006) Characterization of a bacteriocin-like substance produced by Bacillus amyloliquefaciens isolated from Brazillin Atlantic forest. *Int Microbiol* 9: 111-118.

Mearns-Spragg A, Bregu M, Boyd KG, and Burgess JG (1998) Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett Appl Microbiol* 27: 142-146.

John GH, Kreig NR, Smeath HAP, Staley JT, and Williams ST (1994) Bergay's Manual of Determinative of Bacteriology, 9th edition, pp 605-702

Motta AS, Cannavan FS, Tsai S-M, and Brandelli A (2007) Characterization of a broad range antibacterial substance from a new Bacillus species isolated from Amazon basin. *Arch Microbiol* 188: 367-375.

Sharma N, Kapoor G, and Neopaney B (2006) Characterization of a new bacteriocin produced from a novel isolated strain of Bacillus lentus NG121. *Antonie Van Leeuwenhoek* 89: 337-343.

Stein T (2005) Bacillus subtillis antibiotics: structures, syntheses and specific functions. *Mol Microbiol* 56: 845-857.

Vining LC (1992) Secondary metabolism, inventive evolution and biochemical diversity *a review Gene* 115: 135-140.

Yarbrough GG, Taylor DP, Rowlands RT, Crawford MS, and Lasure LL (1993) Screening microbial metabolites for new drugs-theoretical and practical issues. *J Antibiot* (Tokyo) 46: 535-544.

Zheng G and Slavik MF (1999) Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. *Lett Appl Microbiol* 28: 263-367.

[Received January 3, 2008; accepted February 18, 2008]